PERSPECTIVES OF BIOCHEMICAL PLANT PATHOLOGY

Saul Rich, Editor

Perspectives of Biochemical Plant Pathology

Perspectives of Biochemical Plant Pathology

The Symposia and Lectures
Commemorating the 75th Anniversary
of the
Department of Plant Pathology and Botany

May 7 and 8, 1963
New Haven, Connecticut

Edited by Saul Rich

The Connecticut Agricultural Experiment Station
Bulletin 663 **December 1963**

The symposia and lectures commemorating the 75th Anniversary of the Department of Plant Pathology and Botany, and their publication, were made possible by the Lookwood Trust, an endowment of the Station.

Preface

This publication reports the lectures and discussions that commemorated the 75th Anniversary of the Department of Plant Pathology and Botany of The Connecticut Agricultural Experiment Station. The sessions were held in New Haven on May 7 and 8, 1963. The symposia represented various aspects of biochemical plant pathology. This theme ties together the major interests of the current staff of the Department. It also represents a major, contemporary line of research in plant pathology. The currency of its interest can be affirmed by the fact that the symposia were attended by 200 scientists representing laboratories all over the world. The discussions following each paper are faithfully reported here, but the cold print cannot convey the enthusiasm and vigor with which these discussions were pursued. The vitality of the discussions was a tribute to the caliber of papers presented, and to the skill of the discussion leaders.

Dr. McNew's analysis of ever-expanding plant pathology, and Dr. Horsfall's nostalgic Thaxteriana were the highlights of the banquet held the evening of May 7. That evening, the 165 people who attended the banquet were entertained also by the darting witticisms of E. M. Stoddard who acted as Master of Ceremonies. From his 52 years as a professional plant pathologist, Mr. Stoddard was able to see things in their proper perspective. He made it quite plain that our present, intense scientific involvements, as always are precursors for things to come.

Although the official commemoration began at 1:30 P.M. on May 7 and ended at 5:00 P.M. May 8, the success of this relatively short affair was assured by the conscientious and enthusiastic efforts of many people. My own thanks go to the speakers and discussion leaders; to P. M. Miller and the other members of the Department of Plant Pathology and Botany who worked so hard to make our guests welcome; to all the members of the Station staff who helped us enthusiastically whenever they were asked; and to Miss Lois Pierson who made sure that all loose ends were properly knotted. I am particularly grateful to L. V. Edgington, R. J. Lukens, and M. L. Zucker who not only served as chairmen, but also did the initial editing of the manuscripts for their respective symposia. The whole affair would not have been possible without the support of our Board of Control and the Lockwood Trust Fund.

Saul Rich, *Chairman*
75th Anniversary Committee
DEPARTMENT OF PLANT PATHOLOGY
AND BOTANY

Contents

Welcome

James G. Horsfall

Director, The Connecticut Agricultural Experiment Station

Ladies and Gentlemen: On behalf of my colleagues at the Experiment Station, may I welcome you to New Haven, to The Connecticut Agricultural Experiment Station, and to these Lockwood Symposia. I would particularly like to bid welcome to our colleagues from other countries—from Argentina, Britain, Australia, Canada, Holland, Germany, and Japan.

To my friend, Dr. Uritani from Japan, I would like to use the phrase I learned in Japan to welcome the audiences that came to hear my lectures, "Yoku. Irasshaimashita."

We are proud to see so many of you here. We are flattered and highly pleased. When we were planning this affair, we hardly knew how best to announce it. One must not appear too forward in saying: "Look here, come and help us celebrate our Diamond Jubilee," but obviously many of you found out about it anyway.

Well, what was the world like in 1888, when Thaxter came here? There were no typewriters or carbon paper, so that one did not have to prepare his reports in triplicate and quadruplicate. Our letterheads, Thaxter's letterheads, in 1888 said that the horse-cars left the New Haven Green every half-hour on the half-hour. The telephone was just 10 years old. The first telephone exchange in America was here in New Haven, and we were one of its earliest customers. Thaxter's letterhead said that we could "be spoken" from the Railroad Station and from a feed store down on Chapel Street.

In March of the year occurred the famous blizzard of 1888. The horse-cars on Whitney Avenue were snowed in up to their roofs, I am told. The Johnstown flood was to occur the next year, and—on a happier note—the Gibson girl was to appear about 5 years later. Now 1888 was only 25 years after de Bary's classic paper of 1853, which many people consider to mark the birth of plant pathology; it was 6 years after the birth of Bordeaux mixture, and about 7 years before the first use of formaldehyde in plant disease control.

There are some exhibits here in the auditorium which you can examine if you find time. One of them comprises a few samples of some of the classic, very old books of plant pathology. Being an early institution, we have many old books in plant pathology and botany. Another of our exhibits is labelled "Colorful Thaxter." Besides some of Thaxter's

choicer quotations, it contains his original wash-boiler sprayer, the use of which caused him to coin the phrase, "squirt-gun botany." Exhibited there, too, is the famous potato tuber on which Thaxter etched his monogram with *Streptomyces* in 1890 to demonstrate the cause of potato scab. You will find an exhibit featuring a monograph on peach yellows written by the Mayor of New Haven in 1844. In this monograph, His Honor the Mayor talks about insect transmission, transmission by budding, the effect of the disease on photosynthesis, and he even proposes chemotherapy for control. The article was published in the ALBANY CULTIVATOR, the precursor of the COUNTRY GENTLEMEN, which is now also extinct. We have a complete file of the early editions.

Well, enough of history. May I say again how delighted we are to see you. I hope you make good talk together during your visit here.

I. Natural Biochemical Resistance to Disease in Plants

Introduction

Milton L. Zucker

The Connecticut Agricultural Experiment Station

The fact that many plants show a natural resistance to disease has long been used by plant geneticists in their breeding programs. Rapid advances in modern biochemistry have now made it possible for plant pathologists to investigate the biochemical bases of this resistance. The aim of these investigations is to obtain knowledge that can be used to induce biochemical resistance to disease in plants. This symposium session should illustrate progress which has been made in this field, and it will emphasize a number of the problems which remain to be solved.

It is becoming increasingly clear that many types of resistance involve biochemical interactions between host tissue and invading pathogen. Thus, the investigator has a doubly difficult task, for he must not only understand pathology but must also be competent to study the biochemistry of plants. Some of the discussion in the symposium will be concerned with biochemical pitfalls, rather than progress, which may be encountered. If these difficulties can be avoided, then work on the biochemistry of resistance to disease in plants will contribute much to plant biochemistry, as well as to plant pathology.

The Biochemical Basis of Disease Resistance Induced By Infection

Ikuzo Uritani

Laboratory of Biochemistry, Faculty of Agriculture
Nagoya University, Japan

In recent years many biochemical studies have examined mechanisms of disease resistance of higher plants. However, we have not progressed very far in our understanding of the biochemical basis of resistance. In this paper biochemical work on the relationship between resistance and metabolic change induced by infection will be reviewed, and general principles will be discussed from a biochemical view point.

Our principle studies concern sweet potato roots infected by the black rot fungus, *Ceratocystis fimbriata,* and I should like to bring this specific disease into focus. I will discuss our results in the following order: 1) biochemical changes in the roots after infection, and 2) the relationship between biochemical changes and resistance.

First, I will describe briefly the methods of inoculation and sampling. Asexual spores of the fungus are produced by shake culture, and the spores are inoculated on the surface of slices of sweet potato tissue 2 to 3 cm thick. The inoculated tissue is incubated at 28 to 30 C for a period of time, and then the infected tissue is separated from the adjacent, uninjured tissue.

Necrotic tissue resulting from infection we will call infected tissue, and the tissue adjacent to it we will call diseased tissue. Tissue incubated under the same conditions except for inoculation is called cut tissue. Fresh tissue of sweet potato roots served as another control. These four kinds of tissue were analyzed for certain chemical substances and enzyme activities.

Biochemical changes in the roots after infection: Many researchers have demonstrated an increase in oxygen uptake of the host when it is infected by the pathogen (6, 37). In the black rot of sweet potato, the tissue also shows an increment in respiratory rate after infection (5, 33). A steady increase in rate occurs over a period of 1 to 2 days. Usually the tissue located nearer the infected tissue shows the greater change in respiratory rate. The cutting treatment itself induces a comparatively small increase in oxygen uptake which attains a constant level within 1 day after cutting.

As a general rule, the respiratory rate is controlled by the ADP* level in the tissue (6, 21, 25, 37). We can describe two possible ways in which the ADP concentration can be increased. The one may be the acceleration of ATP-utilizing systems, such as those involved in protein synthesis, production of organic phosphorus compounds, and protoplasmic work. The other may result from the uncoupling of oxidative phosphorylation, or the induction of respiratory enzyme systems not involving phosphorylation. Many workers have attempted to learn whether or not the respiratory increase in diseased tissue results from the acceleration of ATP-utilizing systems (6, 7, 37). In our experiments the protein content of the tissue increases while the amino acid content decreases (1). There is also an increase in organic phosphate at the expense of inorganic phosphate. These data suggest that ATP-utilizing systems are being activated, and they agree with results of others who have studied different host-parasite systems (28, 29, 30, 31). However, not all of our results or those of others are consistent with this idea (5, 20). Although it was expected that ion absorption would increase more in diseased tissue, we found the opposite effect. For example, the ability to absorb radioactive phosphate from solution is greater in cut than in diseased tissue.

The increase in ADP concentration may abolish the Pasteur effect; thus, intermediates of carbohydrate breakdown may accumulate (37, 41). These intermediates can be used effectively for the formation of phenolic compounds which are usually produced in diseased plants. In the case of the black rot disease, chlorogenic acid and isochlorogenic acid are the main phenolic compounds found in diseased tissue (36). Polyphenols are produced during the 72-hour period of incubation, the greatest amount being synthesized in the second layer of diseased tissue. The amount of phenolics formed decreases in the inner layers. In the first layer of tissue which gradually becomes infected during incubation, the concentration of polyphenols is substantially reduced because of the oxidation by polyphenol oxidase which is active in the injured tissue penetrated by the fungus. Cutting treatment also shows some increment in the phenolic compounds. However, the levels are much lower than those in diseased tissue.

According to the work of Sprinson and his collaborators with bacteria (27), erythrose-4-phosphate is condensed with phosphoenolpyruvate to form precursors of phenolic compounds. These two compounds may also be intermediates of phenolic synthesis in higher plants. Erythrose-4-phosphate, a 4 carbon compound, can be produced from glucose via the pentose phosphate pathway. If this occurs in the tissue, then the ratio of

Abbreviations used in this paper:
* ADP and ATP are adenosine di and triphosphate; CoA is coenzyme A; DEAE-cellulose is diethylaminoethylcellulose.

CO_2 released from the C_6 position of glucose compared with that from the C_1 position should be low during the active synthesis of phenolic compounds. Our experimental results show that an increase in the C_6/C_1 ratio does occur at the 72-hour stage following the initial period of active phenolic production in diseased tissue (3).

We should like to present a scheme for the mechanism of respiratory increase in connection with polyphenol formation (Fig. 1). In response to infection, ATP-utilizing systems are accelerated, increasing the ADP concentration. As a result, C_3, C_4, C_5, and C_6 intermediates accumulate; thus, synthesis of polyphenolic compounds are induced by the increased supply of these precursor molecules.

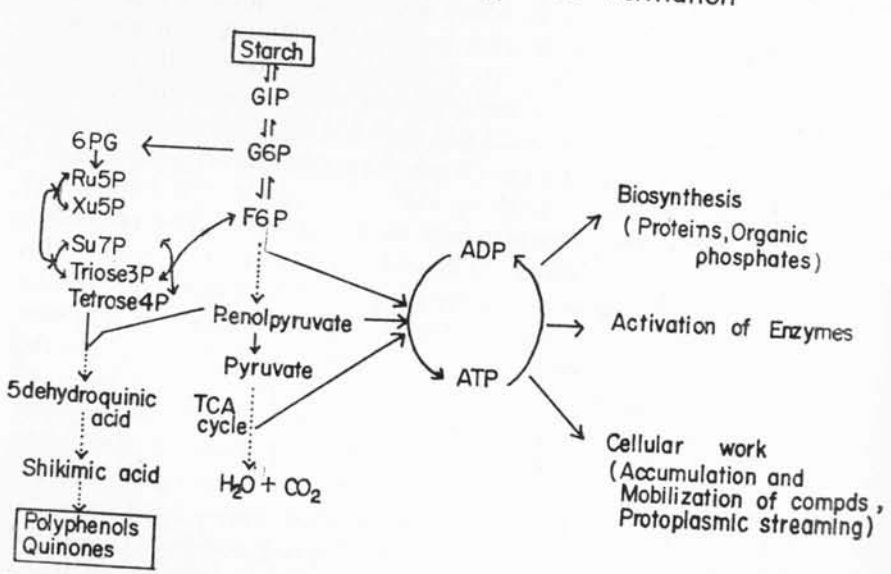


Figure 1. A scheme proposed to explain how polyphenol formation can cause increased respiration. Abbreviations: G1P, glucose-1-phosphate; G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; Su7P, sedoheptulose-7-phosphate; TCA cycle, tricarboxylic acid cycle; 6PG, 6-phosphogluconate; Ru5P, ribulose-5-phosphate; Xu5P, xylulose-5-phosphate.

Among the ATP-utilizing systems, the most important one should be protein synthesis. What are the changes in protein metabolism of diseased tissue? Stahmann and his associates have presented the first evidence that specific antigenic compounds are induced by infection (12). Diseased tissue of sweet potato synthesizes antigenic compounds which are absent or barely detectable in fresh or cut tissue (39). Some of these components were detected as immunological lines in an agar gel system

containing diseased sweet potato proteins and the antiserum. A specific band associated with diseased tissue was found by starch-gel electrophoresis. Starch-gel immunoelectrophoresis also revealed the presence of antigenic compounds not detected in cut tissue. Four components, labeled A, B, C, and D, showed precipitation lines on agar gel. Components A and C are found in every tissue and do not change on infection. Components B and D are specific to, or increase greatly in disease tissue. Component C exhibits beta-amylase activity. Its identity was confirmed by the continuous arc line produced between the tissue extract and pure beta-amylase. Component D is a peroxidase. No enzymatic activities have been associated with component A or B. In our laboratory Hyodo repeated the immunological work using antisera taken from Japanese rabbits (14). In addition to the components found with the antisera prepared in Dr. Stahmann's laboratory, another precipitation line was observed. The new antigen is similar to but not identical with component B, and is called B′.

We subjected diseased sweet potato proteins to DEAE-cellulose column chromatography. Every third tube of effluent was concentrated with Carbowax and was assayed for immunological reaction in agar gel. More than 20 antigenic components were detected in this experiment by investigating the precipitation lines and by examining their enzymatic properties (17). Some components were detected only in diseased tissue while others were found in cut tissue as well, but not in fresh tissue.

Since component D is a peroxidase, Kawashima carried out some biochemical experiments on peroxidase activity of the tissue (18). He found that little change occurred in the peroxidase level during the first day following infection. Then a rapid increase in activity begins and continues until approximately the 3rd day after infection. The first layer of diseased tissue (1 mm thick) shows less activity than the 2nd and 3rd layers, because it is injured by infection. Cut tissue also increases in activity, but to a much smaller extent than diseased tissue.

Protein extracts of diseased, cut, and fresh tissue were subjected to starch-gel electrophoresis. Four peroxidases were detected as the main components. Each of the tissue extracts was also chromatographed on columns of DEAE-cellulose. Recovery of total peroxidase activity from the columns is quite good. Component D, mentioned above is found in fraction IV of the column eluates. The level of activity of the individual peroxidases present varies greatly between the fresh, cut, and diseased tissues. Antigenicity is associated with the fraction I peroxidase as well as with fraction IV.

Polyphenol oxidase increases in the black rot infected sweet potato, as well as in other diseased plants (10, 14). Therefore, changes in polyphenol oxidase activity were also investigated by column chromatography and electrophoresis. We compared extracts of fresh, cut, and

diseased tissue. Three polyphenol oxidase fractions were separated on the columns and labeled I, II, and III in order of elution. Fraction II is common to every tissue. Fraction III is produced in response to both cutting and infection. Only diseased tissue produces a fraction I polyphenol oxidase with antigenic property. Upon electrophoresis, fraction II and III each yield two to three sub-components which show polyphenol oxidase activity after reacting immunologically with antiserum. The lines can be detected by staining reactions after addition of chlorogenic acid and alanine.

In addition to peroxidase and polyphenol oxidase activity, cytochrome oxidase increases in diseased tissue (35). Ascorbic acid, assumed to be associated with the respiratory system, also increases in diseased tissue (34). The ratio of the oxidized form to the reduced form is lower in diseased tissue than it is in cut tissue.

In connection with the formation of polyphenols, I would like to mention the change in coumarin compounds in diseased plants. Umbelliferone, scopoletin, and esculetin, as well as the glycosides of umbelliferone and scopoletin, skimmin, and scopolin are found in black rot infected sweet potato (22, 32).

Umbelliferone accumulates to a greater extent in the first layer of diseased tissue than in the second and third layers, in contrast to polyphenols as chlorogenic acid which are destroyed by oxidation in the first layer. The total amount of polyphenols synthesized in diseased tissue is about 1000 times greater than that of umbelliferone.

Next, I would like to discuss some remarkable changes in lipid metabolism and relate them to the alterations in carbohydrate and respiratory metabolism already described. The formation of the sesquiterpene, ipomeamarone in diseased tissue of the sweet potato was first reported by Hiura (13), and the chemical structure was determined by Kubota and his associates (19). This compound is distinctive in that it possesses a furan ring. They found other compounds of similar structure, ipomeanine and ipomeanic acid. Akazawa separated more than 10 furanoterpenes in the crude oil of the diseased plants (2). He used thin-layer chromatography on silica for this study.

Ipomeamarone increases substantially in infected tissue and in diseased tissue very closely adjacent to infected tissue (Fig. 2). In considering the relation of ipomeamarone synthesis to disease resistance, it is necessary to take another fact into account. It has been found that such terpenes are also produced by sweet potato slices in response to treatment with chemical agents such as mercuric chloride and monoiodoacetate, as well as by infection with other pathogens (28, 38).

The biosynthetic pathway of furanoterpene formation is not functional in fresh tissue. However, during incubation of slices which have been inoculated or treated chemically, the pathway is completed or at least

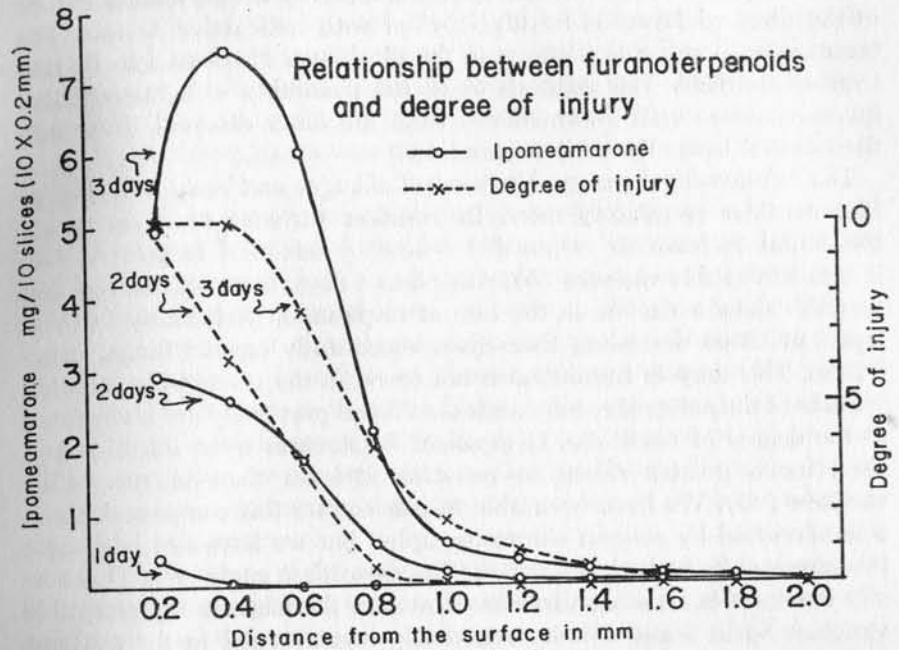


Figure 2. Relation between degree of injury to sweet potato and the production of ipomeamarone by the injured tissue.

activated. Consequently, when mercuric chloride is supplied to tissue previously treated with other chemicals, or pre-inoculated, the synthesis of furanoterpenes is more rapid than in tissue without any pretreatment.

Ipomeamarone is a typical terpene, and its biosynthesis is assumed to involve acetyl-CoA, mevalonate, geranyl-pyrophosphate, and farnesyl-pyrophosphate as intermediates. This assumption has been partially confirmed by Akazawa who has showed that radioactive acetate and mevalonate are incorporated into ipomeamarone and other furanoterpenes (4).

The lipid content of fresh sweet potato tissue is very low. In diseased tissue the phospholipids increase twice as much as in cut tissue, while the sterols increase only slightly, and the neutral lipids do not change (15). Imaseki has found that the incorporation of C^{14}-acetate into the neutral lipid fraction containing terpenes, is greater in diseased than in cut tissue (16). Silica gel column chromatography of this radioactive fraction revealed the presence of several components in diseased tissue which are not found in cut tissue. Furthermore, the distribution of radioactivity among the components common to both types of tissue differs between the two.

Although the concentration of furanoterpene is very low in diseased

tissue compared to that in the adjacent infected tissue, ipomeamarone in the diseased layers is readily labelled with radioactive acetate. The most active incorporation occurs in the third layer of tissue, 1 to 1.5 mm from the surface. This suggests to us the possibility of a migration of ipomeamarone or its intermediates from the inner diseased tissue into the infected tissue where it accumulates.

The relationship between biochemical changes and resistance: Let us first consider respiratory rates. In resistant varieties of sweet potato the initial increase in respiration following infection is greater than it is in susceptible varieties (5). After 3 to 4 days, however, the resistant varieties show a decline in the rate of respiration, particularly in those layers of tissue defending themselves successfully against fungal penetration. This drop in respiration is not found in the susceptible varieties.

Some of the antigenic compounds mentioned previously are also related to the degree of resistance. Component B increases upon infection and to a much greater extent in resistant varieties than in susceptible varieties (40). We have been able to concentrate this component about a hundredfold by column chromatography, but we have not been able to determine its biological function. The peroxidase component D generally increases in resistant varieties. However, the increase in susceptible varieties Norin 4 and 5 is approximately that attained in the resistant Norin 1.

Several investigations have been made of the relation between peroxidase formation and resistance (10, 41). Some workers claim that there is a positive correlation. As I have demonstrated here, peroxidase produced during infection is composed of multi-components. Therefore, we should like to emphasize that it is necessary to determine the concentration of each component produced during infection. Preliminary experiments suggest that the change in fraction I and III peroxidases may parallel the magnitude of resistance of the tissue.

The extent of polyphenol ovidase activity in diseased tissue has also been investigated in correlation with the magnitude of resistance (10). However, as I have just pointed out in relation to peroxidases, it is necessary to determine the change in activity shown by each kind of polyphenol oxidase produced before we can obtain a clear view. Our preliminary evidence indicates that the polyphenol oxidase fraction III may be related to the defense reaction.

A number of workers have found that the amount or rate of production of phenolic compounds themselves parallels the magnitude of resistance to disease (11, 41). However, in the black rot disease of sweet potato, we have not obtained such a clear relation. The amount of phenolic compounds in Norin 10, a resistant variety, is about that found in the susceptible Norin 4. Several workers have focused their attention on the relationship between coumarin formation and resistance. Both

umbelliferone and scopoletin are produced in larger amounts in the resistant Norin 10 sweet potato inoculated with the black rot organism than in the similarly treated susceptible Norin 4 (23). Umbelliferone formation responds faster to the infection than does scopoletin formation. Under similar conditions of inoculation the total amount of furanoterpenes, including ipomeamarone, is also much larger in Norin 10 than in Norin 4 (37, 41).

As a basis for our experimental studies on the production of phenols, coumarins, and terpenes during infection, we have assumed that the chemical factors related to the defense reaction are accumulated in infected tissue and in diseased tissue adjacent to the site of infection.

The concentrations of material near the site of infection provide a basis for comparing the effects of phenols and coumarins on the growth of the black rot fungus in vitro. Chlorogenic acid and isochlorogenic acid show little inhibitory effect, even at a concentration of 0.5 per cent which approaches the concentration in diseased tissue. When the fungus is grown on agar containing these two compounds, it produces a browning of the substrates within 2 days after inoculation. This browning is due to the oxidation of the polyphenols by the mycelial phenol oxidase. Similar results are obtained using liquid media. On the other hand, umbelliferone and scopoletin inhibit fungal growth considerably at a concentration of 0.1 per cent (32). However, this level is much greater than that found in diseased tissue. The inhibitory effect of ipomeamarone is the most striking one. A marked inhibitory activity is obtained at a concentration of 0.1 per cent (41). It should be pointed out that the concentration of ipomeamarone in the infected tissue often exceeds 1.0 per cent. Both ipomeamarone and umbelliferone inhibit the respiration of *Ceratocystis fimbriata,* although quinone was without effect over the brief experimental period examined. Perhaps the formation of physical barricades should be considered as a possible explanation for the resistance associated with some plant phenols.

The data from our experiments and the results from others (8, 9, 24, 26) suggest that phytoalexin-like compounds accumulate in the infected tissue and in the adjacent non-infected tissue. The production of such compounds may be related to the operation of a lipid pathway which involves acetyl-CoA.

We have shown that several biochemical changes as well as the production of a number of metabolites during infection can be related to the defense reaction. It should be emphasized though, that they may be a reflection of the changes in protein metabolism induced by infection.

We may wonder how such remarkable biochemical changes are produced in diseased tissue, especially in resistant varieties. I can give no explanation of the mechanism of this phenomenon of so-called hypersensitivity. However, hypersensitivity may be a reflection of non-com-

patability between the protoplasm of the host and that of the pathogen. An investigation at the level of cell proteins and nucleic acids might be the proper approach to solving the mechanism of the hypersensitive reaction.

I am deeply indebted to Dr. Milton Zucker at The Connecticut Agricultural Experiment Station for his valuable advice and help in the preparation of the manuscript. The biochemical studies on black rot of sweet potato which I have shown have been done in collaboration with Dr. T. Akazawa, Dr. H. Imaseki, Mr. N. Kawashima, Mr. T. Minamikawa, and Mr. H. Hyodo. I am grateful to them for their helpful discussions during the preparation of this paper.

LITERATURE CITED

1. Akazawa, T., and I. Uritani. 1955. Respiratory increase and phosphorus and nitrogen metabolism in sweet potato infected with black rot. Nature 176:1071-1072.
2. Akazawa, T. 1960. Chromatographic isolation of pure ipomeamarone and re-investigation of its chemical properties. Arch. Biochem. Biophys. 90:82-89.
3. Akazawa, T., and I. Uritani. 1962. Pattern of carbohydrate breakdown in sweet potato roots infected with Ceratocystis fimbriata. Plant Physiol. 37:662–670.
4. Akazawa, I., Uritani, I., and Y. Akazawa. 1962. Biosynthesis of ipomeamarone. I. The incorporation of acetate-2-C^{14} and mevalonate-2-C^{14} into ipomeamarone. Arch. Biochem. Biophys. 99:52-59.
5. Akutsu, K. 1962. A biochemical study of respiration and ion absorption in sweet potato with black rot. M.S. Thesis. Nagoya Univ.
6. Allen, P. J. 1953. Toxins and tissue respiration. Phytopathology 43:221-229.
7. Allen, P. J. 1954. Physiological aspects of fungus diseases of plants. Ann. Rev. Plant Physiol. 5:225-248.
8. Condon, P., and J. Kuć. 1960. Isolation of a fungitoxic compound from carrot root tissue inoculated with Ceratocystis fimbriata. Phytopathology 50:267-270.
9. Cruickshank, I. A. M., and D. R. Perrin. 1963. Studies on phytoalexins. VI Pisatin: the effect of some factors on its formation in Pisum sativum L., and the significance of pisatin in disease resistance. Australian J. Biol. Sci. 16:111-128.
10. Farkas, G. L., and Z. Király. 1958. Enzymological aspects of plant diseases. I. Oxidative enzymes. Phytopathol. Z. 31:251-272.
11. Farkas, G. L., and Z. Király. 1962. Role of phenolic compounds in the physiology of plant disease and disease resistance. Phytopathol. Z. 44:105-150.
12. Heitefuss, R., D. J. Buchanan-Davidson, M. A. Stahmann, and J. C. Walker. 1959. Electrophoretic and immunochemical studies of proteins in cabbage infected with Fusarium oxysporum f. conglutinans. Phytopathology 50:198-205.
13. Hiura, M. 1941. Studies in storage and rot of sweet potato (2). Gifu Norin Semmon Gakko Gakujutsu Hokoku 50:1-5.
14. Hyodo, H., T. Asahi, and I. Uritani. 1963. Polyphenol oxidases in diseased and cut sweet potato. Abstracts of the Annual Meeting of Agr. Chem. Soc. Japan at Tokyo, April. 1-3.
15. Imaseki, H., S. Takei, and I. Uritani. 1961. Lipid metabolism in sweet potato with black rot. J. Japanese Biochem. Soc. 31:637.
16. Imaseki, H., and I. Uritani. 1963. A biochemical study of the mechanism of ipomeamarone synthesis in sweet potato with black rot. Ann. Phytopathol. Soc. Japan 28:81.

17. Kawashima, N., H. Hyodo, and I. Uritani. 1962. An immunological study of protein metabolism in sweet potato with black rot. J. Japanese Biochem. Soc. 34:463-464.
18. Kawashima, N., and I. Uritani. 1963. Occurrence of peroxidases in sweet potato infected by the black rot fungus. Agr. Biol. Chem. 27:409-417.
19. Kubota, T., and T. Matsuura. 1953. Chemical studies on the black rot disease of sweet potato. J. Chem. Soc. Japan 74:101-109, 197-199, 248-251, 668-670.
20. Kuroda, S. 1961. Pathological physiology of sweet potato with black rot. Gifu Univ. Press, Japan. 91 p.
21. Lardy, H. A., and H. Wellmann. 1952. Oxidative phosphorylations: role of inorganic phosphate and accepter systems in control of metabolic rates. J. Biol. Chem. 195:215-224.
22. Minamikawa, T., T. Akazawa, and I. Uritani. 1962. Isolation of esculetin from sweet potato roots with black rot. Nature 195:726.
23. Minamikawa, T., T. Akazawa, and I. Uritani. 1963. Analytical study of umbelliferone and scopoletin synthesis in sweet potato roots infected by Ceratocystis fimbriata. Plant Physiol. 38:493-497.
24. Perrin, D. R., and W. Bottomley. 1962. Studies on phytoalexins. V. The structure of pisatin from Pisum sativum L. J. Am. Chem. Soc. 84:1919-1922.
25. Siekevitz, P., and Van R. Potter. 1953. Intramitochondrial regulation of oxidative rate. J. Biol. Chem. 201:1-13.
26. Sondheimer, E. 1961. Possible identity of a fungitoxic compound from carrot roots. Phytopathology 51:71-72.
27. Srinivasan, P. R., M. Katagiri, and D. B. Sprinson. 1959. The conversion of phosphoenolpyruvic acid and D-erythrose-4-phosphate to 5-dehydroquinic acid. J. Biol. Chem. 254:713-715.
28. Suzuki, N., K. Kasai, Y. Yamazaki, T. Araki, S. Toyoda, and T. Takahashi. 1957. Studies on the violet root rot of sweet potatoes. Bull. Natl. Inst. Agr. Sci. (Japan). Ser. C. No. 8:1-173.
29. Tomiyama, K. 1955. Cytological studies on resistance of potato plants to Phytophthora infestans. II. The death of the intracellular hyphae in the hypersensitive cell. Ann. Phytopathol. Soc. Japan 19:149-154.
30. Tomiyama, K. 1955. Cytological studies on resistance of potato plants to Phytophthora infestans. III. The time required for the browning of midrib cell of potato plants infected by Phytophthora infestans. Ann. Phytopathol. Soc. Japan 19:165-169.
31. Tomiyama, K., and N. Takase. 1956. Physiological studies on the defense reaction of potato plant to the infection of Phytophthora infestans. I. Changes in the physiology of potato tuber induced by the infection of Phytophthora infestans and their varietal differences. Hokkaido Natl. Agr. Expt. Sta., Research Bull. 71:32-50.
32. Uritani, I., and I. Hoshiya. 1953. Isolation of coumarin substances from black-rotted sweet potato and their physiology. J. Agr. Chem. Soc. Japan 27:161-164.
33. Uritani, I., and S. Takita. 1953. Abnormal increase of the respiration of black-rotted sweet potato. J. Agr. Chem. Soc. Japan 27:168-174.
34. Uritani, I., and K. Iechika. 1953. Some knowledge concerning ascorbic acid in black-rotted sweet potato. J. Agr. Chem. Soc. Japan 27:688-692.
35. Uritani, I., and T. Akazawa. 1953. Activation of the respiratory enzyme systems of black-rotted sweet potato. J. Agr. Chem. Soc. Japan 27:789-795.
36. Uritani, I., and M. Miyano. 1955. Derivatives of caffeic acid in sweet potato attacked by black rot. Nature 175:812.
37. Uritani, I., and T. Akazawa. 1959. Alteration of respiratory pattern in infected plants, p. 349-390. *In* J. G. Horsfall and A. E. Dimond, [ed.], Plant Pathology. Vol. 1, Academic Press, New York.
38. Uritani, I., M. Uritani, and H. Yamada. 1960. Similar metabolic alterations induced in sweet potato by poisonous chemicals and by Ceratocystis fimbriata. Phytopathology 50:30-34.

39. Uritani, I., M. A. Stahmann. 1961. Changes in nitrogen metabolism in sweet potato with black rot. Plant Physiol. 36:770-782.
40. Uritani, I., and M. A. Stahmann. 1961. The relationship between antigenic compounds produced by sweet potato in response to black rot infection and the magnitude of disease resistance. Agr. Biol. Chem. 25:479-486.
41. Uritani, I. 1961. The role of plant phenolics in disease resistance and immunity, p. 98-124. *In* G. Johnson and T. A. Geissman, [ed.], Symposium of Plant Phenolic Substances. Colorado State Univ., Fort Collins.

Discussion

Stahmann: In his letter inviting me to discuss this paper, Dr. Dimond indicated that I should evaluate the significant points and introduce new aspects before calling for discussions from the floor.

In my estimation, the great significance of Professor Uritani's paper is indicated in its title. That is, *that the very act of infection induces disease resistance with a biochemical basis.* This biochemical basis for resistance is reflected in changes in the proteins, probably enzymes, and products of their action in cells adjacent to the infected site. It was the use of infection in a plane, on the surface of a cut potato, that made this demonstration possible. It is significant that every method for protein analysis, immunochemical, chromatographic, or electrophoretic, indicated changes in the proteins of cells adjacent to the infection plane. Most significant is the fact that these changes appeared to be greatest in varieties that carry genes for resistance.

To me, this is a particularly important conclusion, because when I started studies on the biochemistry of plant diseases and disease resistance as a graduate student, I began with a hypothesis suggested by my professors, that since the colored outer scales of onions contained toxic phenolic compounds that were correlated with resistance and that inhibited fungal growth, resistance in other plants might also be of a similar passive character. However, it was only after years of unsuccessful search for other passive biochemical agents associated with resistance that I was forced to change my point of view and to consider that resistance may be an active process in plants, triggered off by the infection. As you know, this is the case in animals. However, resistance in plants differs from that in animals in that the resistant character need only be manifest in the few cells surrounding the infection site. This work suggests that such is the case in sweet potatoes.

A second significant conclusion is that *there is a profound interaction between the parasite and the host which causes changes in protein synthesis of the host cells immediately around the infection site.* Our new data extend this conclusion to other diseases. At the same time that Professor Uritani was working in my laboratory, Professor Tomiyama

was also studying *Phytophthora infestans* on white potato tubers. Using gel electrophoresis, Tomiyama detected changes in proteins of cells adjacent to the infection plane. Of particular significance was his demonstration that some changes are race specific. An example is the relative production of peroxidase isoenzymes in the following: a control uncut tuber; a tuber cut but uninfected; a tuber cut and infected by a compatible race (Race 1) of *P. infestans* to which the tissue is susceptible; and a tuber cut and infected by the incompatible race (Race 0) to which the tissue is resistant. He found that the greatest change in peroxidase activity was induced by the race to which the host is resistant. The race to which the host is susceptible caused little change. Thus of these two races of *P. infestans* which differ in their ability to infect the tuber and to induce changes in the peroxidase of the potato, only the race to which the tuber is resistant increases peroxidase activity.

Figure 1 from Dr. Tomiyama's work shows the distribution of polyphenol oxidase and peroxidase in the tuber. The solid line shows activities in tissue whose cut surface had been infected by the incompatible race to which the tissue is resistant. The dashed line represents tissue infected by the compatible race to which it is susceptible. The dotted line shows the response in cut but uninfected tissue. Note that the resistant race caused the increase in polyphenol oxidase to extend much further into the underlying tissue. A second significant finding of Dr. Tomiyama was that treatment with a pectinase preparation, shown at B, also induced an increase in polyphenol oxidase and peroxidase.

Dr. Richard C. Staples of the Boyce Thompson Institute has spent his sabbatical year with us. He has shown that rust infection by *Uromyces phaseoli* in bean leaves also induces marked changes in the proteins of the host. He has very cleverly combined electrophoresis in polyacrylamide gels and enzymatic specificity to focus onto the specific isozyme patterns of several enzymes in healthy, rust infected, and uredospore extracts. With this technique he was able to show that rust growth is accompanied by the appearance of one new malate dehydrogenase isozyme and the continuation of one otherwise lost during development of the healthy leaf. One new isozyme is contributed by the fungus, the other by the leaf. Both enzymes are cytoplasmic proteins.

Table 1 summarizes data obtained by Dr. Staples in his survey of changes in the isozyme pattern of enzymes induced by rust. Many enzymes showed no change following rust infection, but with some there was an increase in the number of isozyme bands in the supernatants from the infected bean leaves. The total enzymatic activity varied; in some cases it increased, in others it decreased or showed no change. From a study of substrate specificity and susceptibility to heat, Dr. Staples has concluded that rust infection may induce the formation of new host isozymes or allow the continued synthesis of an isozyme that

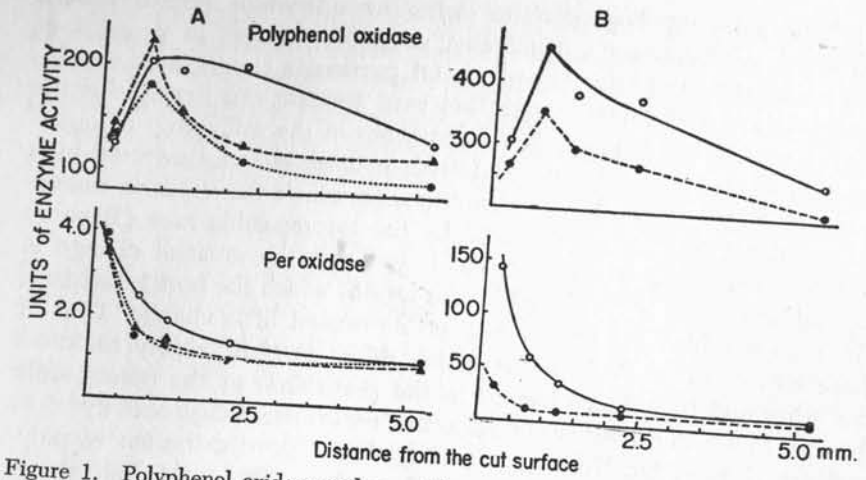


Figure 1. Polyphenol oxidase and peroxidase activity curves in the tissue adjacent to the cut surface of potato tubers. In A, the solid line represents the activity in the tissue whose cut surface had been infected by the incompatible race (race 0) of *Phytophthora infestans* to which the tissue is resistant. The dashed line represents the tissue infected by the compatible race (race 1) to which the tissue is susceptible, and the dotted line represents cut but uninfected tissues. In B, the solid line represents the tissue whose cut surface had been treated with pectinase; the dashed line represents cut but infected tissue.

normally disappears as the leaf matures. I think it is significant that he has shown alterations in the isozyme pattern of several, but not all enzymes that he has investigated.

Dr. Klaus Rudolph, who is now with us from Gottingen, is extending these investigations to a bacterial disease, halo blight of beans caused by *Pseudomonas phaseolicola*. As illustrated in Fig. 2, he has shown that this bacterial infection caused changes in profiles that can be detected by chromatography on diethylaminoethylcellulose. Here are the profiles from resistant and susceptible bean leaves before and after

Table 1. Changes in Enzymes Induced by Rust (*Uromyces phaseoli*) (With Dr. R. C. Staples)

Enzyme	Isozyme in supernatant from bean leaves		Total enzyme activity
	Healthy	Infected*	
Malate Dehydrogenase	2	4	No Change
Succinate Dehydrogenase	0	4	Increase
Acid Phosphatase	2	4	Decrease
Alkaline Phosphatase	2	5	No Change
Cytochrome Oxidase	3	5	Increase
Peroxidase	7	7	Increase

* 12 days after infection

infection. Note that infection of the susceptible variety reduced the size of the peaks shown at A as compared to B. In contrast, infection of a resistant variety caused an increase in these same components eluting at C and D on the right. Thus, bacterial infection also induces changes in the host. Following Dr. Staples' success with gel electrophoresis, he has applied this technique to halo blight and shown changes in the isozyme pattern of three enzymes.

Thus, the work of my two Japanese associates, Drs. Uritani and Tomiyama, has clearly shown that fungus infection on the surface of the cut, sweet or white potatoes induced changes in the protein synthesis of only those host cells located within a few millimeters of the infection plane. Furthermore, the extent of these changes in host proteins were greatest when the host was resistant or when the fungus race was unable to cause disease in the hypersensitive or resistant host. The work of Dr. Staples and Dr. Rudolph has extended these studies to a rust and bacterial infection. Here too, changes in protein synthesis in host cells of the bean leaf have been demonstrated. It is more difficult to show the location of the change in the leaf, however, recent experiments involving

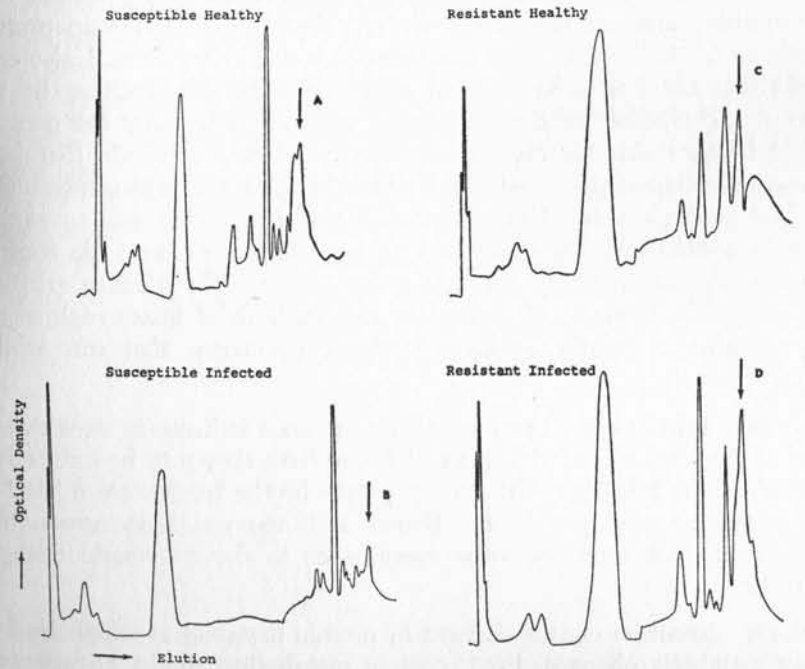


Figure 2. Profiles obtained by chromatography of an extract containing supernatant proteins from resistant and susceptible bean leaves before and after infection with *Pseudomonas phaseolicola*. The optical density at 220 mμ is plotted against the volume required for elution.

strip inoculations suggest that some changes in the acid phosphatase isozymes occur throughout the plant. Thus, we conclude that there is a profound interaction of the parasite with the host resulting in alterations in protein biosynthesis by the host.

We have also obtained evidence that *the protein metabolism of the parasite may be altered by the host.* This was studied by my student, Dr. Dawson Deese, who investigated pectinase synthesis by *Fusarium* and *Verticillium* in resistant and susceptible hosts. He was able to show that those varieties of tomato, potato, or banana which have high resistance to these pathogens, are also capable of suppressing the synthesis of fungal pectin hydrolyzing enzymes. Dr. Staples has shown that when the rust fungus infects bean leaves, there is a suppression of two rust isozymes of malate dehydrogenase and one of acid phosphatase of the germinated uredospore. The heat stability and substrate specificity of some fungal enzymes were changed by an interaction with the host.

From all of these studies, I conclude that there is a profound interaction of these various plant pathogens and the host tissues that they invade. As a result of this interaction, there are alterations in the protein synthesis of the host cells. It seems highly significant that whenever the extent of this alteration has been compared between resistant and susceptible varieties, we have observed that the alteration in protein synthesis of the host appears greater with resistant varieties. I also conclude that the host cells have an effect upon protein synthesis by the parasite. This effect of the host upon protein synthesis by the parasite could be the basis for race specialization. Thus, I conclude that both disease development or resistance depends upon a complex interaction of host and parasite. The effect that this interaction has upon the protein synthesizing systems of either pathogen or host determines whether the parasite will produce a disease, or if the plant is resistant or susceptible. It may well determine the synthesis of toxins such as the phytoalexins as pisatin, ipomeamarone or coumarins that can inhibit fungal growth.

Galston: I'd like to ask you whether you could indicate to us which, if any, of the biochemical changes which you have shown to be induced as a result of the infection of the sweet potato by the fungus are related to the resistance displayed by the tissue? Is it ipomeamarone production only, or do you attribute some significance to the enzymatic changes as well?

Uritani: Infection causes changes in protein metabolism which lead to other metabolic changes. First, protein metabolism might be affected. Other types of metabolism might subsequently be induced or increased. There are some cases in which the change in ATP metabolism is of prime

importance. However, I think that this may also be induced by a changing protein metabolism.

Zucker: I wonder if you have investigated the effects of light on the changes in protein metabolism of the sweet potato. We have found that light stimulates protein synthesis in slices of potato tubers.

Uritani: In my case I haven't investigated the effects of light. I am not sure of the effect. Do you mean effects on photosynthesis?

Zucker: Yes, in part. I am more interested in the induction of chloroplast development by light.

Uritani: I am not sure about this. You work with stem tissue, while we work with roots. Perhaps someone else might be able to answer this question.

Cruickshank: I have listened to Dr. Uritani's presentation with considerable interest and I wish to know 1) whether the synthesis of ipomeamarone, an end product of an obviously complicated reaction involving many intermediates, is induced in sweet potato by a number of fungal species and 2) if this compound shows any selective toxicity toward the pathogens of sweet potato as exemplified by the action of pisatin toward the pathogens and nonpathogens of peas?

Uritani: Ipomeamarone is toxic to both pathogenic and nonpathogenic fungi and some bacteria as well. So far as we have investigated, there is no clear-cut relation between its inhibitory effect and pathogenicity. This is different from your results with peas.

The Role of Phenolic Compounds in Disease Resistance

Joseph Kuć

Department of Biochemistry, Purdue University
Lafayette, Indiana

Why phenolics? Of the countless compounds found in plants the phenolics have persistently been assigned a role, though often vague, in disease resistance mechanisms in plants. Is this role justified, or are researchers guilty of wishful thinking in attempting to assign a metabolic role to a class of substances whose existence or production is at times difficult to justify within our present sphere of knowledge?

Before starting into a defense of the role of phenolics it is imperative that the term is defined. Phenolics comprise a vast class of compounds including: anthocyanins, leucoanthocyanins, anthoxanthins, hydroxybenzoic acids, glycosides, sugar esters of phenolic acids, esters of hydroxycinnamic acids, and coumarin derivates. In the past the term "tannin," and more recently the term "polyphenol," have been used to refer to any or a collection of these compounds. Reference in the literature to either of these terms, therefore, can only be relied upon to imply the presence of a compound with one or more phenolic hydroxyl groups.

Phenolics are synthesized in plants via either the shikimic acid pathway (11, 13) the acetate-malonate pathway (1, 2) or modifications of both pathways (16). Some plants accumulate high concentrations of phenols especially in external tissue, and investigators have at times correlated this accumulation with resistance to various pathogens and non-pathogens (3, 10, 12). A great deal of evidence has appeared indicating the production, speed of production, or liberation of phenolics by plant tissue after infection is important in disease resistance (4, 5, 9, 10, 15). The latter approach, dependent on a specific host-pathogen interaction, offers an element of specificity difficult to realize when considering only pre-formed phenolics. The accumulation of phenolics has been detected in the infected tissue as well as in healthy tissue in advance of the microorganism. Coupled with the increase in phenolic content, an increase in phenoloxidase and peroxidase, in or adjacent to infected tissues, has also often been reported (6, 14). When one considers the antibiotic properties of many plant phenolics, the increased antibiotic activity reported for their quinones (7, 8) the localization of these compounds around points of infection, and finally the universal occurrence of plant

phenolics, it is evident why researchers have often implicated these compounds with disease resistance mechanisms in plants. One other factor should be considered, if I may be allowed a teleological digression. Phenolics represent a vast array of compounds, and though, of course, far simpler in structure than proteins, they do offer a variety of structure which is not approached by any other class of naturally-occurring compounds, certainly a wider variety than the common amino acids, sugars, or fatty acids found in nature. I could even be nasty and include proteins as phenolics because of the tyrosine component. This variety of structure does provide a basis for a degree of specificity as antimicrobial agents and suggests a partial explanation for the specificity of host-pathogen interaction.

Disease resistance mechanisms in animals start with the skin and include the rather nonspecific mobilization of white blood cells and the highly specific antibody-antigen response. All three mechanisms are based upon preventing the organism from getting established in the animal. We accept these mechanisms and are not bewildered by the fact that we have included in the defense both specific and non-specific weapons. On the other hand, among plant pathologists I detect a degree of "resistance" to the concept of general mechanisms for disease resistance in plants. They are skeptical because such mechanisms may not explain the high specificity of certain pathogens for certain hosts and, therefore, the specificity of disease resistance. Undoubtedly, highly specific substances, mechanisms or interactions are involved with resistance, but what about the over-all general resistance of plants to the multitude of microorganisms in the environment? How do we explain the nonpathogenicity of some microorganisms or the pathogenicity of some on a very limited host range? Actually, in nature, resistance is the rule and susceptibility the exception. Perhaps the general mechanisms take care of 99.9 per cent of disease resistance, and the other 0.1 per cent (where general resistance fails or where the pathogen overcomes general resistance) is accounted for by highly specific mechanisms. Perhaps our approach to resistance should be to find why resistance fails in the relatively few cases of susceptibility in nature. After all, there is nothing sacred about a specific response mechanism for resistance. I believe there is much to be said for the concept of a rather general response, but a specific mechanism "triggering" this response. Certainly the nature of mechanisms "triggering" metabolic reactions leading to resistance warrants more study.

The basic tenet of disease resistance in animals is the prevention of growth and reproduction of the pathogen by a resistant host. I believe the basic role of phenolics in disease resistance of plants is to stop growth of the pathogen. The one observation common to all resistance mechanisms observed in plants is limited growth of the pathogen. The

strategy (and this strategy has its political and military merits) appears to be "stop the bug before it gets started and before it starts producing toxins or spreads to other tissues." There are countless microorganisms capable of producing extracellular hydrolytic enzymes and phytotoxic substances, and yet there are relatively few plant pathogens. I emphasize again the fact that resistance is the rule and not the exception. A microorganism penetrating into a plant encounters cell wall components including protein, pectin, cellulose, hemicellulose, and lignin. The substrates for adaptive enzyme formation are there, and many microorganisms produce extracellular, hydrolytic enzymes capable of degrading these substrates. Yet, not all microorganisms capable of producing these enzymes attack all plants they penetrate. It is difficult for me to explain the specificity of host-pathogen interaction solely on the basis of extracellular enzyme production. I definitely, however, do not minimize the important role that may be played by extracellular microbial enzymes and toxins in the over all disease syndrome—in actually causing what we call the susceptible reaction or symptoms. If the organism grows, it can produce extracellular products, hence the appearance of characteristic symptoms. I have yet to find, however, conclusive evidence that after penetration into a plant a microorganism cannot obtain ample nutrition for survival without first producing hydrolytic enzymes or toxins. Nevertheless, it cannot be denied that some pathogens are intercellular, some intracellular, and some just rot all tissue in their path.

The inactivation of extracellular microbial enzymes by phenols or quinones present in plants or produced by plants after penetration is certainly a defense mechanism; it curtails symptom expression. However, this inactivation of enzymes is often thought of as a means of inhibiting the organism by limiting its source of nutrients. I suggest the possibility that the phenomenon is primarily concerned with limiting the spread of the pathogen and thereby curtailing the appearance of a susceptible reaction. I can't help coming back to the age-old controversy of "what came first – the chicken or the egg?"

In other words, is the pathogen inhibited in the resistant host because its extracellular enzymes are inactivated, or is the lack of extracellular enzymes due to the fact that growth of the pathogen is curtailed?

The only answer that appears to fit is that many disease resistance mechanisms are operative in plants. Some function as barriers, others inactivate extracellular toxins and enzymes, and still others act as metabolic inhibitors of the pathogen itself. This is consistent with observations in animals – skin as a barrier, white blood cells to destroy pathogens, antibodies to destroy or agglutinate pathogens and to detoxify microbial toxins. In any one host-pathogen interaction several of these mechanisms may function to impart resistance, and therefore resistance

based on one mechanism and one mechanism alone is a dangerous assumption, even with single gene resistance. A correlation of a metabolic function with resistance is one thing, but to limit resistance to a single mechanism is unfounded.

I would like to introduce some aspects of recent work in our laboratories concerning a nonspecific host response and a "triggering" mechanism. We studied the resistance of certain apple trees to the incitant of apple scab, *Venturia inaequalis*, as well as to organisms never known to attack apple foliage. The disease reactions of apples to races of *V. inaequalis* are of three kinds. The first is a highly resistant reaction (some people would call this a hypersensitive reaction or immunity) which is usually characteristic of apple varieties that are resistant to all known races of the pathogen. Symptoms appear on the leaves as minute pinpoint pits 36 to 48 hours after inoculation. No sporulation is evident. The second resistant reaction appears on leaves approximately 7 to 10 days after inoculation as restricted, necrotic, regular, irregular, or stellate lesions with no sporulation. The third reaction, or susceptible reaction, appears 10 to 14 days after inoculation and is characterized by large sporulating lesions. In severe cases leaves may abscise. In all cases the fungus spore germinates on the surface, forms first a germ tube, and then an appressorium. Penetration through the cuticle commences immediately following appressorium formation, and here is where differences start. In the hypersensitive-type of resistance, cells immediately around the point of penetration collapse, and a minute red-brown pit forms. The fungus makes little or no further growth. In the second resistant reaction, the organism penetrates and makes growth beneath the cuticle. Then after 7 to 10 days, cells beneath the organism collapse, further growth of the fungus is arrested, and non-sporulating lesions form. In the susceptible reaction, the fungus makes abundant growth beneath the cuticle, and after 10 to 14 days the fungus sporulates and breaks through the cuticle. Only some time after sporulation do host cells collapse. Certainly, if growth of the fungus itself was causing collapse of host cells, the most rapid collapse would occur with the susceptible host-pathogen combination, but this is not the case. When leaves showing either of the resistant reactions are washed with water, the concentrated washings are inhibitory to spore germination of *V. inaequalis*. Washings of uninoculated leaves, or leaves with freshly sporulating lesions, do not inhibit germination. Alcoholic extracts of uninoculated and inoculated leaves behave the same way. Examination of mechanically injured leaf tissue indicated a rapid hydrolysis of phloridzin by a β-glycosidase to glucose and phloretin. Phloretin then appeared to be oxidized by a phenol oxidase to produce a dihydroxy intermediate which was converted to a transient inhibitor, probably a toxic quinone, followed by polymerization. Inhibition of spore germination increased as phloridzin

was converted to phloretin, and the phloretin was oxidized. Inhibition decreased as polymerization progressed. Injury of leaf tissue in the presence of sodium metabisulfite or sodium bisulfite did not prevent β-glycosidase action and phloretin accumulated, but under these conditions oxidation did not occur and inhibition of spore germination was not evident. Inhibiting the β-glycosidase activity with a gluconolactone resulted in oxidation of phloridzin but with little hydrolysis and low inhibitory activity. Thus, the liberation of phloretin via β-glycosidase hydrolysis of phloridzin, followed by oxidation of phloretin, produced a compound highly fungitoxic to *V. inaequalis*. The potential for producing this series of reactions which produces the inhibitor was demonstrated to exist in resistant as well as susceptible hosts, and this series of reactions certainly is not complex or specific. The question arises why was this reaction "triggered" so quickly in the most highly resistant host-pathogen combination, and at best very slowly in the susceptible host-pathogen combination.

Recently we have obtained symptoms identical to the hypersensitive-type resistance by inoculating leaves with completely incompatible organisms such as *Helminthosporium carbonum, H. maydis,* and *H. turcicum*. In all cases spores germinate, form germ tubes and appressoria, followed by rapid collapse of host tissue with the liberation of fungitoxic products. In summary, it is important that the nonspecific defense mechanism based on host response should not be ignored. This type of mechanism, also genetically controlled, may help to explain the observation that disease resistance is the rule and not the exception in nature.

LITERATURE CITED

1. Bentley, R., and J. G. Keil. 1961. The role of acetate and malonate in the biosynthesis of penicillic acid. Proc. Chem. Soc., March:111-112.
2. Bu'Lock, J. D., and H. M. Smalley. 1961. Biosynthesis of aromatic substances from acetyl and malonyl-coenzyme A. Proc. Chem. Soc., June:209-211.
3. Clauss, E. 1961. Die phenolischen Inhaltsstaffe der Sammenschatem von Pisum sativum L. und ihre Bedeutung fur die Resistenzgegen die Erreger der Fusskrankheit. Naturwissenschaften 48:106.
4. Cruickshank, I. A. M., and D. R. Perrin. 1960. Isolation of a phytoalexin from Pisum sativum L. Nature (London) 187:799-800.
5. Davis, D., P. E. Waggoner, and A. E. Dimond. 1953. Conjugated phenols in the fusarium wilt syndrome. Nature (London) 172:959-961.
6. Farkas, G. L., Z. Király, and F. Solymosy. 1959. The biochemical mechanism of hypersensitive reaction in rust and virus infected plants. 9th Intern. Botan. Congr., Montreal 2:111.
7. Farkas, G. L., and G. A. Ledingham. 1959. Studies on the polyphenol-polyphenoloxidase system of wheat stem rust uredospores. Can. J. Microbiol. 5:37-46.
8. Kaul, R., and M. Shaw. 1960. The physiology of host-parasite relations. VI. Oxidation-reduction changes in wheat leaf sap caused by rust infection. Can. J. Botany 38:399-407.

9. Király, Z., and G. L. Farkas. 1962. Relation between phenol metabolism and stem rust resistance in wheat. Phytopathology 52:657-664.
10. Kuć, J., R. E. Henze, A. J. Ullstrup, and F. W. Quackenbush. 1956. Chlorogenic and caffeic acids as fungistatic agents produced by potatoes in response to inoculation with Helminthosporium carbonum. J. Am. Chem. Soc. 78:3123-3125.
11. Levy, C. C., and M. Zucker. 1960. Cinnamyl and *p*-coumaryl esters as intermediates in the biosynthesis of chlorogenic acid. J. Biol. Chem. 235:2418-2425.
12. Link, K. P., and J. C. Walker. 1933. The isolation of catechol from pigmented onion scales and its significance in relation to disease resistance in onions. J. Biol. Chem. 100: 379-383.
13. McCalla, D. R., and A. C. Neish. 1959. Metabolism of phenyl propanoid compounds in salvia. II. Biosynthesis of phenolic cinnamic acids. Can. J. Biochem. and Physiol. 37:537-547.
14. Maine, E. C., and A. Kelman. 1960. Changes in the oxidation rates of polyphenols and ascorbic acid in tobacco stem tissue invaded by Pseudomonas solanacearum. Phytopathology 50:645.
15. Müller, K. O. 1958. Studies in phytoalexins I. The formation and the immunological significance of phytoalexin produced by Phaseolus vulgaris in response to infection with Sclerotinia fructicola and Phytophthora infestans. Australian J. Biol. Sci. 11:275-300.
16. Underhill, E. W., J. E. Watkin, and A. C. Neish. 1957. Biosynthesis of quercetin in buckwheat. Part I. Can. J. Biochem. and Physiol. 35:219-228.

Discussion

Sondheimer: It is a very great pleasure for me to participate in this symposium and to serve as discussion leader for Dr. Kuć's excellent paper. Before asking for questions and comments from the floor, I would like to go on a little bit with one of the topics already mentioned by Dr. Kuć, namely the dangers that are inherent in the words "plant phenolics" and "polyphenolics." In many papers one sees these terms applied with experimental connotations. That is, if for example an investigator observes an increase in the rate of oxygen uptake on infection of a plant then he is apt to attribute this to the presence of "polyphenolics" on the basis of such tests as an increase in the fluorescence, or the Folin-Denis test, and several other equally non-specific tests. In a few cases some attempts have been made to distinguish between the various phenolics present by means of qualitative paper chromatography. However, it seems to me that all of these procedures are apt to lead to ambiguous interpretation. The major reason for making this statement is the evidence that has been accumulating recently on the effects of different phenols on enzymic activities. Possibly one of the most dramatic illustrations of this type of behavior is the study by Gamborg, Wetter, and Neish (Can. J. Biochem. and Physiol. 39:113, 1961) on the effect of phenols in the oxidation of reduced diphosphopyridine nucleotide by peroxidase. Experiments were carried out with dialyzed extracts from pea epicotyls, spruce shoots, and crystalline horse-radish peroxidase.

These authors found that for maximum activity, the system required Mn^{++}, hydrogen peroxide, and a phenol. *p*-Coumaric acid was the most potent activator, but *p*-hydroxyphenylpropionic acid, *p*-hydroxyphenylpyruvic acid, or resorcinol could replace *p*-coumaric acid, but were less effective. On the other hand, ferulic, sinapic, chlorogenic, caffeic acids, and hydroquinone were potent inhibitors. I would like to draw particular attention to the results obtained with *p*-hydroxyphenylpyruvic acid. When this compound was used in the absence of any other added phenol at a concentration of 5×10^{-4} M it gave weak activation effects. If on the other hand the system also contained 5×10^{-4} M *p*-coumaric acid the above concentration of *p*-hydroxyphenylpyruvic acid caused a 50 per cent inhibition with pea epicotyl peroxidase. Another example that I can cite is the results we obtained with IAA oxidase from etiolated peas (Sondheimer and Griffin, Science 131:672, 1960). With an enzyme preparation obtained from an acetone powder it was found that the rate of IAA oxidation could be very nicely regulated by the ratio of *p*-coumaric acid to chlorogenic acid that we used. Thus, under standardized conditions the following data were obtained.

p-Coumaric acid added (μg/ml)	Chlorogenic acid added (μg/ml)	Destruction of IAA after 20 min. at 30°C (%)
0	0	37
1	0	73
0	1	0
1	1	65
1	2	0

Although many additional examples could be listed, these two suffice to show how futile it is to attempt to attribute changes in enzymic activities to variations in the concentration of "polyphenolics." Now it is true that all of these results were obtained with cell-free extracts, and it is not justifiable to extrapolate from these data to the behavior expected from intact cell systems. However, many of the characteristic effects caused by fungal or bacterial infection of plants are accompanied by the loss of some cellular integrity. Therefore, many of the biochemical effects of infection, such as a change in the rate of oxygen uptake, a change in the C_6/C_1 ratio in glucose oxidation, or a change in the IAA levels may be correlated with the change of one given phenolic constituent, rather than with the total "polyphenolics."

Since the techniques being developed at the present time are sufficiently sensitive to permit quantitative detection and separation of a large number of different phenolics, I would like to make a most urgent plea for the more frequent use of such procedures. I feel very strongly that the progress that can be made in clearing up the function of

phenolics in the biochemistry of plant diseases is very closely linked to the role of the individual compounds as activators and inhibitors of enzymes.

And now I would like to ask for comments or questions from the floor on Dr. Kuć's paper.

Sondheimer: I would like to begin by asking Dr. Kuć whether he has located the difference in oxidation products of phloridzin before and after removal of the glucose?

Kuć: In both cases we are forming a dihydroxy compound prior to oxidation to a quinone. In the oxidation process we can account for the uptake of a mole of oxygen per mole of substrate lost. We can then pick up the introduction of a hydroxyl group by various chemical reactions. We haven't as yet had enough of the dihydroxy compound to conduct long experiments as to the inhibitory properties of it versus the quinone, but we hope we can get enough of the dihydroxy compound to do this.

Sondheimer: But most of all you get changes in the phenolic group previously protected by that glucose.

Kuć: No, I feel that oxidation of phloridzin and phloretin occurs on the ring outside. I think it involves actually the introduction of a hydroxyl group at the 2-position of the *p*-hydroxyphenyl ring.

Sondheimer: But the inhibitory properties of the two compounds you tried were different.

Kuć: That's right.

Sondheimer: That's why I wondered about the glucose bond.

Kuć: It is possible that the glucose bond has some influence on the toxicity of the resultant dihydroxy compound or its quinone.

Sproston: Dr. Kuć, you touched on the problem. How important do you think polymerization of phenols is in lowering toxicity? How general do you think it is?

Kuć: The polymerization of quinones resulting from the oxidation of phenols is very important. First of all, you can't have an aqueous solution of an ortho quinone such as that from catechol or chlorogenic acid for any length of time. This is very reactive. It polymerizes. I think a great deal of difficulty has arisen because people have oxidized phenols with polyphenol oxidase, and to get the reaction going at full strength and for a period of time, they take a known amount of phenol and polyphenol oxidase and incubate them for an hour. When they test the solution, they find there is no inhibitory activity. The fact remains that

the phenols have been oxidized to quinones, the quinones have polymerized, things come out of solution, you get melanin-like material and you've lost your activity. However, I have often thought of another process which this type of reaction suggests. That is, the oxidation of phenols to quinones and their addition to substrates in the host thereby making the substrates unavailable for the microorganism, tying up perhaps pectin, protein-cellulose complexes, and cell wall materials. Thereby the pectin is prevented from acting as an adaptive substrate for the production of some of the pectin splitting enzymes. Or the quinone complexes may be merely masking some of these substances, acting as a barrier of polymerized phenols. I think the active agent as an inhibitor of the organism in many of these cases is the quinone, and this quinone is a transient thing especially at the test tube level. Once it forms, unless you have some means of reversing the situation, it polymerizes and leaves the solution.

Kelman: Do the resistant and susceptible apple varieties differ with respect to the rate at which phloretin is formed following infection by the apple scab fungus?

Kuć: One of the great problems in plant pathology is that plants are not blessed with kidneys, urine, blood, and other convenient sites for detecting metabolic changes. We are faced with the problem of inoculating a leaf, and studying metabolic changes before we actually see symptoms. We take it on good faith that since we've inoculated hundreds of leaves before and have gotten symptoms that we will get symptoms on this leaf. If you examine the susceptible reaction after the organism has penetrated into the host, but before you observe the collapse of cells, and if you take care to inactivate the β-glycosidase by putting the tissue into liquid nitrogen or extracting in boiling alcohol, you can see that there isn't any phloretin, or very little phloretin, produced in the infected, susceptible leaf. Phloretin is produced within 24 to 36 hours after inoculation in the hypersensitive, resistant reaction. But if you just injure that host, you can start off the whole series of reactions. The difference between susceptible and resistant hosts is not in the content of phloridzin or enzymes affecting its hydrolysis and oxidation, but rather in the interaction initiating these changes.

Kelman: Is there any fundamental difference between resistant and susceptible varieties with respect to the rate of phloretin formation when you inoculate with *Helminthosporium* sp.?

Kuć: The reaction in response to *Helminthosporium carbonum, H. maydis, H. turcicum* and some of the other organisms we've worked with, in regard to phloridzin, phloretin, the action of the β-glycosidase and phenol oxidase, as far as we can say, is not different from the situation

with the hypersensitive reaction of the very resistant host to the organism. The lines which exhibit this hypersensitive reaction are resistant to all known races of the apple scab fungus. We have worked a good deal with *Malus astrosanguinia* which shows this type of resistance.

Kelman: Is it correct that the resistant and susceptible apple scab lines cannot be differentiated on the basis of their reaction to an organism such as *Helminthosporium*?

Kuć: That is correct for all of the lines that we have tested, and we have tested about 10 commercial varieties including McIntosh, Jonathan, Red Delicious, Yellow Delicious, some exotic lines, certain Russian varieties, and *M. atrosanguinia.* We've also tested Geneva and other varieties that are differential hosts. They all are resistant to and apparently respond in the same way to *Helminthosporium carbonum.*

Kelman: Do you have any idea what the triggering mechanism is?

Kuć: I was waiting for someone to ask me that. No, I don't have any idea what the triggering mechanism is. We are working on this, and we've got some very confusing leads indicating that there is a material produced by germinating spores which can cause the collapse of host cells, if you first get an appressorium and get this mysterious thing called an infection peg formed. But strangely, and I don't want to commit myself to the truth of this yet, you can apparently get this toxin if you have apple scab spores germinating on a membrane. If appressoria form, you can get the toxin, but as far as we can tell, for some reason, you may not get this toxin produced on a susceptible leaf. But if you take the toxin and put it on a susceptible leaf, you can get the collapse of cells.

Sussman: It seems to me that there is a fundamental question in common between the talks that were presented in series here today. The question really could be phrased in these terms: What is the primary effect of the manifold changes demonstrated by Dr. Uritani and Dr. Stahmann in enzyme complement, and what are the effects of the secondary products such as phenols etc. on these enzymes? This is a two-fold question, therefore, and one to which I have not yet heard a satisfactory answer. I think its importance resides perhaps in another question. What is the physiological role *in vivo* of enzymes? I'm struck by the fact that peroxidase, which has been shown by these speakers to exist as isozymes, also has another role than its peroxidative one, in which it serves as a DPNH-oxidase. My question, therefore, concerns the relationship between the phenols and the oxidases. Is it possible that resistant creatures appear to produce more of the DPNH-oxidase, that is, the actual peroxidases, by virtue of the fact that the phenols which are also produced cause an increase in their activity leading to deleterious effects on the pathogen?

Kuć: Yes, the production of phenols would markedly influence some of these enzymes. But we also have a difference in the substrate specificity with peroxidases and polyphenol oxidases. This may account for the difference in oxidation products that are formed. I do agree with you that the phenols could influence the activity and production of the phenolases and peroxidases, and that the linkage with the DPN-DPNH system would influence the oxidation or stability of any quinones that might be formed.

Sondheimer: May I add a comment? I think you can go a step further. The complete activity or lack of activity of the DPNH-oxidases or the peroxidases such as these which attack indole acetic acid can be completely modified by the type of phenolics produced in the tissue and by the concentration of phenolics. This may not be their actual function *in vivo*, but in test tube experiments it's no trick at all to change an inert protein into a very highly active one by addition of very low concentrations of specific phenolics. Possibly this does have a direct bearing on plant disease resistance because in many instances you are dealing with cell macerates and this would approach conditions as they exist in test tube experiments.

Natural Inhibitors of Fungal Enzymes and Toxins In Disease Resistance

R. J. W. Byrde

Long Ashton Research Station, University of Bristol, England

Resistance of plants to fungal disease has often been ascribed in the past to the production of physical barriers or the presence of toxic substances. These substances were generally considered to be present in higher or lower concentrations in the healthy plant, and to act by directly preventing growth of fungi; in other words, they were fungistatic materials both *in vitro* and *in vivo*. Recently, it has become clear that specific antifungal compounds may be formed as a direct response to fungal invasion, and this aspect of disease resistance has been discussed in a preceding paper. There also seems to be some evidence to support the concept of resistance due to the presence or appearance of compounds not necessarily directly fungitoxic, but which have the ability to inactivate the extracellular enzymes of a fungus attempting to invade the plant. This paper attempts to summarise the scattered evidence which is available on this topic, and also to speculate on the possibility that the inhibition of fungal enzyme biosynthesis (as distinct from activity), by naturally-occurring compounds, may also be a factor in disease resistance.

Enzymes and toxins involved in pathogenicity of fungi

The basic assumption is being made that secretion of extracellular enzymes and toxins plays an important role in the pathogenicity of fungi towards higher plants. First put forward by de Bary (1886), this hypothesis is now supported by a wealth of evidence, although much of the detail is not fully understood.

Among the extracellular enzymes involved, the pectolytic enzymes have assumed particular importance. Attention has been directed to them largely because of the fact that many fungi penetrate via the middle lamella, which is known to be predominantly pectic in nature. The role of the pectolytic enzymes has been the subject of recent exhaustive reviews by Husain and Kelman (1959) and by Wood (1960), and will not be considered here in more detail. Cellulolytic enzymes have also been implicated in many host-parasite relationships (Husain and Kelman, 1959; Wood, 1960). The association of arabans and galactans with uronides in pectic materials has led to the suggestion that hemicel-

lulases may be important in plant tissue maceration (van Sumere et al., 1957; Wood, 1960), while recent evidence of a protein component in the middle lamella (Ginzburg, 1958) has prompted the suggestion that proteolytic enzymes could also be involved in tissue disintegration (Wood, 1960; Kuć, 1962).

There is evidence of the importance of fungal polyphenol oxidase in some disease syndromes (Husain and Kelman, 1959). An extracellular glucosidase may be responsible for the pathogenicity to oats of the variety *avenae* of *Ophiobolus graminis*. The enzyme can break down the glucoside avenacin, a component of the host toxic to the parent species *O. graminis*, which lacks the enzyme and is unable to attack oats (Turner, 1961).

The status of toxins in the production of disease symptoms has been reviewed by Brian (1955) and by Ludwig (1960).

Effect of classical enzyme inhibitors and of fungicides on some extracellular enzymes

(a) *Effects on pectolytic enzymes*

The pectolytic enzymes are known to be in general resistant to inactivation by classical enzyme inhibitors (McColloch and Kertesz, 1947; Rahman and Joslyn, 1953), although van Sumere et al. (1957) found them partially inactivated by silver nitrate, sodium sulfide, cysteine and iodoacetate, and Rahman and Joslyn by arsenate and sodium azide. Furthermore, of a range of 117 fungicides and chemotherapeutants tested at a final concentration of 0.016 per cent by Husain and Dimond (1958) against the polygalacturonase (PG) of *Ceratostomella ulmi* only N-*t*-tridecylazomethine and postassium benzothiazolyl thioglycollate showed an inhibitory effect greater than 40 per cent and the latter is in any event not a very effective fungicide *in vitro*. Husain and Rich (1958) reported partial inactivation of the PG of *Cladosporium cucumerinum* by ferbam, nabam, ziram, dichlone, and pyridine-thione (sodium salt). 1, 4-Dioxyanthraquinone-2-sulfonic acid was shown by Grossmann (1962) to be effective in inactivating a pectinase at a concentration of 10^{-3} M, and to act as a systemic chemotherapeutant against tomato wilt.

(b) *Effects on cellulolytic enzymes*

Horsfall (1956) cited the effect of a long-chain aliphatic amine which was active against *Lentinus lepideus* when the fungus was growing with cellulose as carbon source, but inactive when glucose was substituted (Finholt, Weeks and Hathway, 1952); it seemed likely that the effect arose from the inhibition of a cellulolytic enzyme system. Reese and Mandels (1957) tested 175 compounds, including classical inhibitors, against the cellulases of three nonpathogenic fungi. The relatively few

compounds showing inhibitory properties include Ag^{+}, Hg^{++}, Mn^{++}, some phenolic compounds, nabam, zineb and several halogenating agents. Husain and Dimond (1958) also found Ag^{+} and Hg^{++} active against the cellulase of *C. ulmi*, but nabam and 8-hydroxyquinoline sulfate were found to be ineffective, although both had been reported active against other cellulases.

Naturally occurring inhibitors of fungal enzymes and toxins

(a) *Plant extracts*

The effect of a plant extract on the activity of a fungal pectolytic enzyme was apparently first noted by Chona (1932) who showed that the pectinases of several plant-pathogenic fungi were inhibited by potato and apple juices. He considered that the effect was attributable to the presence of Mg^{++} or potassium phosphate in potato juice and of malic acid in apple juice. This and further instances of the inhibition of fungal pectolytic or macerating enzymes by plant extracts are summarized for convenience in Table 1, which also indicates where the inhibition pattern suggested a basis for disease resistance.

There are also reports of the inactivation of other extracellular enzymes by plant extracts – thus Kneen and Sandstedt (1946) and Miller and Kneen (1947) noted the presence of amylase inhibitors in cereals and sorghum respectively, and Laskowski and Laskowski (1954) described trypsin inhibitors in soy and lima beans. Natural cellulase inhibitors were reported by Mandels, Howlett and Reese (1961) and by Bell, Aurand and Etchells (1960). An interesting example of the inactivation of a fungal toxin implicated in a disease syndrome has been quoted by Romanko (1959) who found that intact tissue of varieties of oat resistant to Victoria blight (caused by *Helminthosporium victoriae*) was able to incapacitate the toxin Victorin.

(b) *Phenolic compounds*

In several instances of fungal enzyme inactivation, evidence has been obtained that the inhibitors are of phenolic origin. These are summarized in Table 2, which also shows those examples where varietal resistance appeared to be associated with enzyme inhibition effects. Unambiguous proof of a direct relationship is not easy to establish, but it is at least desirable that possible growth-inhibiting effects of the polyphenols at relevant concentrations should be discounted experimentally, as in the instance of *Sclerotinia fructigena* on apple. Polyphenols have also been implicated in the ability of raspberry leaf extracts to inactivate a number of plant viruses (Cadman, 1958).

These phenolic substances probably owe their effect on extracellular enzymes to their ability to precipitate proteins, and it is probably

Table 1. Inhibition of extracellular fungal enzymes by crude plant extracts

Plant tissue	Enzyme inhibited*	Origin	Possible role in resistance indicated	Reference
Apple fruit, potato tuber	ME	5 plant-parasitic fungi	Yes	Chona (1932)
Apple fruit	PG	Commercial pectinases and *Penicillium brevicompactum*	No	Kieser et al. (1950)
Pear sap	Pectinase (PMG?)	Commercial pectinase	No	Weurman (1953)
Apple fruit, broad bean leaf (oxidised extracts)	PG, ME	*Botrytis cinerea* and *Sclerotinia fructigena*	No	Cole (1956)
Various vegetable storage organs	ME	*Sclerotinia sclerotiorum*	Yes	Echandi and Walker (1957
Grape (and other plants') leaves	PG	Purified commercial enzyme, or mouldy cucumber flowers	Yes	Bell and Etchells (1958) Etchells et al. (1958) Bell et al. (1962)
Grape (and other plants') leaves	Cellulase (Cx)	Mouldy cucumber flowers or commercial enzyme	Yes	Etchells et al. (1958) Bell et al. (1960) Bell et al. (1962)
Broad bean leaf (oxidised)	PG	*Botrytis cinerea* and *B. fabae*	Yes	Deverall and Wood (1961b)

* Symbols: PG = Polygalacturonase; PMG = Polymethylgalacturonase; ME = Macerating enzyme.

Table 2. Inhibition of fungal and plant enzymes by polyphenolic substances

Polyphenolic substance	Enzyme inhibited*	Origin of enzyme	Possible role in resistance indicated	Reference
Tannic acid	PG	Commercial pectinase, and *Penicillium brevi-compactum*	No	Kieser et al. (1950)
Oxidised polyphenols (apple fruits)	PG, ME	*Sclerotinia fructigena*	Yes	Byrde (1956, 1957)
Tannin, chebulinic acid	PMG, ME	*Fusarium oxysporum* f. *lycopersici*	No	Grossmann (1958)
Tannin (Myrobalans)	PG, PMG	Commercial fungal pectinases	No	Hathway and Seakins (1958)
Polyphenols (apple fruits), chlorogenic acid, *d*-catechin	PME	Apple	No	Pollard et al. (1958)
Leuco-anthocyanins (pear)	PG	Yeast, and commercial pectinases	No	Pollard et al. (1958)
Catechins (tea)	PME	Tea	No	Ramaswamy and Lamb (1958)
Oxidised polyphenols (apple)	PG	*S. fructigena, Botrytis cinerea, Penicillium expansum*	No	Cole (1958) Wood and Cole (1958) Cole and Wood (1961)
Oxidised leuco-anthocyanins (apple)	PG	*S. fructigena*	No	Cole (1958) Cole and Wood (1961)
Polymerised polyphenols (persimmon)	PG	*Gloeosporium kaki*	Yes	Tani (1959)
Oxidised *d*-catechin, *l*-epicatechin and leuco-cyanidin†	PG, ME	*S. fructigena*	Yes	Byrde et al. (1960)
Oxidised dihydroxyphenylalanine	PG	*B. fabae*	Yes	Deverall and Wood (1961b)
Oxidised catechol	PG	*B. cinerea*	Yes	Deverall and Wood (1961b)
Tannin or tannin-like substance (grape leaf)	PG	Commercial enzyme	Yes	Porter et al. (1961)
Oxidised polyphenols (cocoa bean)	PG, ME	*Phytophthora palmivora*	Yes	Spence (1961a, b)

*Symbols: PG = Polygalacturonase; PME = Pectinmethylesterase; PMG = Polymethylgalacturonase; ME = Plant macerating enzyme.
† Forsyth & Roberts (1958)

significant that many of the instances cited refer to oxidised and polymerised forms of the phenolics, while Noveroske, Williams and Kuć (1962) showed that host resistance to *Venturia inaequalis* could be "broken" by a polyphenol oxidase inhibitor, 4-chlororesorcinol. There is evidence that molecular size and shape have an effect in the inactivating properties of the phenolic compounds, in that those based on a C_{15} monomer (such as *d*-catechin, *l*-epicatechin and leuco-anthocyanins) have been found more effective than those based on a C_9 structure (such as chlorogenic acid) (Byrde, Fielding and Williams, 1960; Cole and Wood, 1961).

To what extent enzyme inactivation is specific with respect to enzyme is a matter for speculation. It seems unlikely that the phenomenon is anything more than a protein precipitation: the ability of the inhibitory preparations to precipitate gelatin has been noted (Byrde, 1957; Pollard et al., 1958). Also, oxidized phenolic compounds are known to inactivate polyphenol oxidase itself in the assay system for this enzyme, and to inactivate hyaluronidase (Vincent and Segonzac, 1953). Moreover, Hathway and Seakins (1958) were able to reactivate a fungal PG by treatment of the tannin-enzyme complex with 80 per cent acetone and Mejbaum-Katzenellenbogen et al. (1959) claimed that precipitates of tannin with another enzyme could be decomposed by treatment with caffeine solution, liberating free active enzymes. Nevertheless, minor differences in inactivating different pectolytic enzymes have been reported by Pollard et al. (1958) and by Hathway and Seakins (1958).

By contrast, the inhibitor of PG and cellulase present in grape leaves was reported to inhibit PG by a competitive mechanism (Bell and Etchells, 1958).

(c) *Effect of divalent metals*

Another example of pectolytic enzyme inhibition by characterized substances is the effect of divalent metal ions, and particularly calcium, on the susceptibility of pectic acid to hydrolytic attack. Kertesz (1951) cited four instances where calcium and magnesium were inhibitory, and mention has already been made of the suggestion by Chona (1932) that such salts were responsible for the inhibitory effect of potato juice. Edgington and Dimond (1959) and Edgington, Corden and Dimond (1961) demonstrated that the pectic substances of calcium-deficient tomatoes, which were more susceptible to *Fusarium oxysporum* f. *lycopersici*, were more easily released by pectic enzymes than those in normal tissue. These and other effects were attributed to differences in calcium binding of the pectic substances. Deverall and Wood (1961a) also noted the effect of calcium deficiency in increasing the susceptibility of *Vicia faba* to *Botrytis cinerea* and *B. fabae*, while Wallace

et al. (1962) attributed resistance of apples to rots partly on the basis of polyvalent ions in protopectin linkages.

(d) *Allicin*

There is at least one other report of a naturally-occurring compound active as an enzyme inhibitor, although in this case the enzymes studied were mostly neither extracellular nor were they of fungal origin. Wills (1956) showed that the inhibitory effect of extracts of garlic towards a number of enzymes might be explained by the presence of allicin, $CH_2{:}CHCH_2.SO.S.CH_2CH{:}CH_2$. At a concentration of 5×10^{-4}M, this compound inhibited nearly all the sulfhydryl enzymes tested and its effects could be reversed with cysteine or glutathione. In view of its effect being primarily against –SH enzymes, it may be that pectolytic enzymes would be unaffected.

Inhibition of enzyme biosynthesis as a factor in disease resistance

It is clear that, while disease resistance may arise from the inactivation of extracellular fungal enzymes of vital importance to the pathogen, a similar effect would operate if the *formation* of the enzyme were inhibited. The work of Deese and Stahmann (1962a,b) for example, has shown that levels of pectolytic and cellulolytic enzymes were lower in infected resistant hosts than in infected susceptible plants. This section draws attention to two instances in recent work where inhibition of biosynthesis has been unambiguously demonstrated.

The amino acid L-canavanine:

$$(NH_2C(=NH)NHO(CH_2)_2CHNH_2COOH)$$

occurs in a number of leguminous plants and may be present in their seeds in concentrations as high as 2 to 3 per cent (Bell, 1958). It is also known to act as an antimetabolite for the amino acid L-arginine. There are several examples of its effect in inhibiting biosynthesis of microbial enzymes (e.g. Richmond, 1959). It has also been shown to inhibit leaf and stem rusts of wheat (Samborski and Forsyth, 1960).

In recent work* the compound has also been found to be effective in inhibiting the induced biosynthesis of PG by *Sclerotinia* (*Monilinia*) *fructigena*. In passing, it may be noted that no evidence has been obtained in this work to suggest that D-isomers of amino acids active as chemotherapeutants (e.g. Kuć, Williams and Shay, 1957; van Andel, 1958) owe their effect to this mode of action.

An effect of more general occurrence is the inhibition of inducible enzyme synthesis by high concentrations of glucose. The so-called "glucose effect" (e.g. Mandelstam, 1961) has also been demonstrated

* To be submitted for publication in the Biochemical Journal.

in studies* with *S. fructigena* on the induction of PG biosynthesis by D-galacturonic acid. It is interesting to speculate to what extent similar phenomena (Page, 1961) may partly account for the differential effect of high and low sugar levels on disease predisposition first noted by Horsfall and Dimond (1957).

To sum up, there is clear evidence of the importance of extracellular enzymes in the pathogenicity of fungi to plants. These enzymes, although on the whole resistant to classical inhibitors, may be inactivated by some plant extracts. In particular, oxidised phenolic compounds have been implicated in this effect, which probably reflects a nonspecific precipitation of proteins. Unambiguous evidence that these inactivation phenomena are directly responsible for disease resistance *in vivo* is however scanty. Resistance may also be due to inhibition of enzyme biosynthesis by naturally-occurring compounds.

LITERATURE CITED

Andel, O. M. van. 1958. Investigations of plant chemotherapy. II. Influence of amino acids on the relation of the plant to the pathogen. Tijdschr. Plantenziekten 64:307-327.

Bary, A. de. 1886. Ueber einige Sclerotinien und Sclerotienkrankheiten. Botan. Ztg. 44:377-474.

Bell, E. A. 1958. Canavanine and related compounds in Leguminosae. Biochem. J. 70:617-619.

Bell, T. A., L. W. Aurand, and J. L. Etchells. 1960. Cellulase inhibitor in grape leaves. Bot. Gaz. 122:143-148.

Bell, T. A., and J. L. Etchells. 1958. Pectinase inhibitor in grape leaves. Bot. Gaz. 119:192-196.

Bell, T. A., J. L. Etchells, C. F. Williams, and W. L. Porter. 1962. Inhibition of pectinase and cellulase by certain plants. Bot. Gaz. 123:220-223.

Brian, P. W. 1955. The role of toxins in the etiology of plant diseases caused by fungi and bacteria, p. 294-319. *In* J. W. Howie and A. H. O'Hea, [ed.], Mechanisms of microbial pathogenicity. Cambridge University Press, Cambridge.

Byrde, R. J. W. 1956. The varietal resistance of fruits to brown rot. I. Infection experiments with *Sclerotinia fructigena* Aderh. and Ruhl. on certain dessert, culinary and cider varieties of apple. J. Hort. Sci. 31:188-195.

Byrde, R. J. W. 1957. The varietal resistance of fruits to brown rot. II. The nature of resistance in some varieties of cider apple. J. Hort. Sci. 32:227-238.

Bryde, R. J. W., A. H. Fielding, and A. H. Williams. 1960. The role of oxidized polyphenols in the varietal resistance of apples to brown rot, p. 95-99. *In* J. B. Pridham, [ed.], Phenolics in plants in health and disease. Pergamon Press, Oxford.

Cadman, C. H. 1958. Some properties of an inhibitor of virus infection from leaves of raspberry. J. Gen. Microbiol. 20:113-128.

Chona, B. L. 1932. Studies in the physiology of parasitism XIII. An analysis of the factors underlying specialization of parasitism with special reference to certain fungi parasitic on apple and potato. Ann. Botany (London) 46:1033-1050.

Cole, J. S. 1956. Studies in the physiology of parasitism. XX. The pathogenicity of

* To be submitted for publication in the Biochemical Journal.

Botrytis cinerea, Sclerotinia fructigena and Sclerotinia laxa, with special reference to the part played by pectolytic enzymes. Ann. Botany (London) 20:15-38.

Cole, M. 1958. Oxidation products of leuco-anthocyanins as inhibitors of fungal polygalacturonase in rotting apple fruit. Nature (London) 181:1596-1597.

Cole, M., and R. K. S. Wood. 1961. Pectic enzymes and phenolic substances in apples rotted by fungi. Ann. Botany (London) 25:435-452.

Deese, D. C., and M. A. Stahmann. 1962a. Pectic enzymes and cellulase formation by Fusarium oxysporum f. cubense on stem tissues from resistant and susceptible banana plants. Phytopathology 52:247-255.

Deese, D. C., and M. A. Stahmann. 1962b. Pectic enzymes in Fusarium-infected susceptible and resistant tomato plants. Phytopathology 52:255-260.

Deverall, B. J., and R. K. S. Wood. 1961a. Infection of bean plants (*Vicia faba* L.) with *Botrytis cinerea* and *B. fabae*. Ann. Appl. Biol. 49:461-472.

Deverall, B. J., and R. K. S. Wood. 1961b. Chocolate spot of beans (*Vicia faba* L.)-interactions between phenolase of host and pectic enzymes of the pathogen. Ann. Appl. Biol. 49:473-487.

Echandi, E., and J. C. Walker. 1957. Pectolytic enzymes produced by Sclerotinia sclerotiorum. Phytopathology 47:303-306.

Edgington, L. V., M. E. Corden, and A. E. Dimond. 1961. The role of pectic substances in chemically induced resistance to Fusarium wilt of tomato. Phytopathology 51:179-182.

Edgington, L. V., and A. E. Dimond. 1959. Correlation between the wilt resistance of plants and the nature of their pectic substances. Phytopathology 49:538.

Etchells, J. L., T. A. Bell, and C. F. Williams. 1958. Inhibition of pectinolytic and cellulolytic enzymes in cucumber fermentations by Scuppernong grape leaves. Food Technology 12:204-208.

Finholt, R. W., M. Weeks, and C. Hathaway. 1952. New theory on wood preservation. Industr. Engng. Chem. 44:101-105.

Forsyth, W. G. C., and J. B. Roberts. 1958. Cacao "leucocyanindin". Chem. Ind: 755.

Ginzburg, B. Z. 1958. Evidence for a protein component in the middle lamella of plant tissue: a possible site for indolylacetic acid action. Nature (London) 181:398-400.

Grossmann, F. 1958. Uber die Hemmung pektolytischer Enzyme von *Fusarium oxysporum* f. *lycopersici* durch Gerbstoffe. Naturwissenschaften 45:113-114.

Grossmann, F. 1962. Therapeutische Wirkung von Pektinase-Hemmstoffen gegen die *Fusarium*-welke der Tomato. Naturwissenchaften 49:138-139.

Hathway, D. E., and J. W. T. Seakins. 1958. The influence of tannins on the degradation of pectin by pectinase enzymes. Biochem. J. 70:158-163.

Horsfall, J. G. 1956. Principles of fungicidal action. Chronica Botanica, Waltham, Mass. 274 p.

Horsfall, J. G., and A. E. Dimond. 1957. Interactions of tissue sugar, growth substances, and disease susceptibility. Z. Pflanzenkrankh. U. Pflanzenschutz 64:415-421.

Husain, A., and A. E. Dimond. 1958. The function of extracellular enzymes of Dutch elm disease pathogen. Proc. Nat. Acad. Sci. U. S. 44:594-601.

Husain, A., and A. Kelman. 1959. Tissue is disintegrated, p. 143-188. *In* J. G. Horsfall and A. E. Dimond, [ed.] Plant Pathology, Vol. 1. Academic Press, New York.

Husain, A., and S. Rich. 1958. Extracellular pectic and cellulolytic enzymes of Cladosporium cucumerinum. Phytopathology 48:316-320.

Kertesz, Z. I. 1941. The pectic substances. Interscience Publishers, Inc., New York. 628 p.

Kieser, M. E., A. Pollard, and A. M. Stone. 1950. The clarification of apple juice. II. The estimation of pectic enzyme activity. Ann. Rep. Agr. Hort. Res. Sta. Bristol, 1949: 153-159.

Kneen, E., and R. M. Sandstedt. 1946. Distribution and general properties of an amylase inhibitor in cereals. Arch. Biochem. Biophys. 9:235-249.

Kuć, J. 1962. Production of extracellular enzymes by Cladosporium cucumerinum. Phytopathology 52:961-963.

Kuć, J., E. B. Williams, and J. R. Shay. 1957. Increase of resistance to apple scab following infection of host with phenylthiourea and D-phenylalanine. Phytopathology 47:21-22.
Laskowski, M., and M. Laskowski, Jr. 1954. Naturally occurring trypsin inhibitors. Advances Protein Chem. 9:203-242.
Ludwig, R. A. 1960. Toxins, p. 315-357. *In* J. G. Horsfall and A. E. Dimond, [ed.], Plant Pathology, Vol. 2. Academic Press, New York.
McColloch, R. J., and Z. I. Kertesz. 1947. Pectic enzymes VIII. A comparison of fungal pectin-methylesterase with that of higher plants, especially tomatoes. Arch. Biochem. 13:217-229.
Mandels, M., W. Howlett, and E. T. Reese. 1961. Natural inhibitors of cellulase. Can. J. Microbiol. 7:957-959.
Mandelstam, J. 1961. Induction and repression of β-galactosidase in non-growing Escherichia coli. Biochem. J. 79:489-496.
Mejbaum-Katzenellenbogen, W., W. Dobryszycka, J. Bogusawska-Jaworska, and B. Morawiecka. 1959. Regeneration of protein from insoluble protein-tannin compounds. Nature (London) 184:1799-1800.
Miller, B. S., and E. Kneen. 1947. The amylase inhibitor of Leoti sorghum. Arch. Biochem. 15:251-264.
Noveroske, R. L., E. B. Williams, and J. Kuć. 1962. Breakdown of host resistance to Venturia inaequalis by a polyphenol-oxidase inhibitor. Phytopathology 52:23.
Page, O. T. 1961. Induced variation in Fusarium oxysporum. Can. J. Botany. 39:1509-1519.
Pollard, A., M. E. Kieser, and D. J. Sissons. 1958. Inactivation of pectic enzymes by fruit phenolics. Chem. Ind: 952.
Porter, W. L., J. H. Schwartz, T. A. Bell, and J. L. Etchells. 1961. Probable identity of the pectinase inhibitor in grape leaves. J. Food Sci. 26:600-605.
Rahman, M. B., and M. A. Joslyn. 1953. Properties of purified fungal polygalacturonase. Food Research 18:301-304.
Ramaswamy, M. S., and J. Lamb. 1958. Studies on the "fermentation" of Ceylon tea. X. Pectic enzymes in tea leaf. J. Sci. Food Agric. 9:46-51.
Reese, E. T., and M. Mandels. 1957. Chemical inhibition of cellulases and β-glucosidases. Research Report No. 17 (Microbiology Series) Quartermaster Research and Development Center, Natick, Mass.
Richmond, M. R. 1959. The differential effect of arginine and canavanine on growth and enzyme formation in *Staphlococcus auteus* 524 SC. Biochem. J. 73:155-167.
Romanko, R. R. 1959. A physiological basis for resistance of oats to Victoria blight. Phytopathology 49:32-36.
Samborski, D. J., and F. R. Forsyth. 1960. Inhibition of rust development on detached wheat leaves by metabolites, antimetabolites and enzyme poisons. Can. J. Botany. 38:467-476.
Spence, J. A. 1961a. Probable mechanism of resistance of varieties of cocoa to black pod disease caused by *Phytophthora palmivora* (Butl.) Nature. (London) 192:278.
Spence, J. A. 1961b. Black-pod disease of cocoa II. A study of host-parasite relations. Ann. Appl. Biol. 49:723-734.
Sumere, G. F. van, C. van-Sumere-de Preter, and G. A. Ledingham. 1957. Cell-wall splitting enzymes of *Puccinia graminis* var. *tritici*. Can. J. Microbiol. 3:761-770.
Tani, T. 1959. Enzymic degradation of kaki fruits' polysaccharides by *Gloeosporium kaki* (In Japanese, English summary). Tech. Bull. Fac. Agric. Kagawa Univ. 10:29-35 (Biol. Abs. 36: 2561. 1961).
Turner, E. M. C. 1961. An enzymic basis for pathogenic specificity in *Ophiobolus graminis*. J. Exp. Botany 12:169-175.
Vincent, D., and G. Ségonzac. 1953. Action du tanin sur l'hyaluroanidase. C. R. Soc. Biol. 147:1776-1779.
Wallace, J., J. Kuć, and H. N. Draudt. 1962. Biochemical changes in the water-insoluble material of maturing apple fruit and their possible relationship to disease resistance. Phytopathology 52:1023-1027.

Weurman, C. 1953. Pectinase inhibitors in pears. Act. Bot. Neerl. 2:107-121.
Willis, E. D. 1956. Enzyme inhibition by allicin, the active principle of garlic. Biochem. J. 63:514-520.
Wood, R. K. S. 1960. Pectic and cellulolytic enzymes in plant disease. Ann. Rev. Plant Physiol. 6:299-322.
Wood, R. K. S., and M. Cole. 1958. The degradation of pectic substances by plant pathogens. Chem. Ind: 533.

Discussion

Whitaker: Pectinases are one offensive weapon of plant pathogens, cellulases are another and I should like to open this discussion by contrasting briefly some general problems facing these two groups of enzymes. At the molecular level, cellulose presents fewer problems to an enzyme than does pectin. Firstly, cellulose appears to be basically a uniform linear polymer. This allows cellulase to approach its glycosidic linkages without having to contend with side chains of other sugars, such as those which now appear to be important features of the structure of many pectins. Secondly, carboxyl groups are not basic elements of the structure of cellulose. They may arise from oxidation after the cellulose has been laid down, but unless the cellulose has been very badly mistreated, they are usually minor features. The presence of carboxyl groups in the substrate can have several important consequences to an enzyme, and I'd like to consider just two of them. In the case of pectin, the presence of free or esterified carboxyl groups at carbon 6 opens up another reaction path for cleavage of the polymer: the route exploited by the "transeliminase" group of pectinases. Cleavage of glycosidic linkages by these enzymes is accompanied by removal of hydrogen, leaving a double bond between carbon atoms 4 and 5. Furthermore, carboxyl groups give a substrate additional means of forming complexes with proteins. At higher pH's where carboxyl groups are ionized, the complex may arise by purely electrostatic interactions with the basic groups on proteins. At lower pH's the complexes may arise by coupling through hydrogen bonds. A long polysaccharide chain with many carboxyl groups may bind several protein molecules simultaneously, and this sort of multiple binding can so cross-link an enzyme that it is effectively immobilized. Thus, if the carboxyl groups of a particular pectin are not esterified or are not already complexed, then, particularly at lower pH's, I think an enzyme should approach that substrate with caution. It may have designs on the array of glycosidic linkages before it, but the array of carboxyl groups in the background may have designs on the enzyme. In this sense, pectin has a potential of its own as a natural enzyme inhibitor.

These considerations are at a molecular level. Beyond that level, cellulases encounter problems of their own. Cellulose is laid down in

fine threads which may contain up to several thousand individual chains. Within those threads, the chains tend to be so closely packed together that even water molecules have difficulty moving between them. Thus, it is out of the question for a molecule as large as an enzyme molecule to do so. In fact, many enzyme molecules might have trouble moving freely between the capillary spaces separating many microfibrils. One problem of cellulases, then, is that at a given location, at a given time, in a given mass of pure cellulose, only a very small fraction of the linkages present is accessible to the enzyme. This is one reason why the enzymic attack on cellulose tends to be a rather slow and highly localized process. This physical structure may not come under the heading of a natural inhibitor, but I should think it has discouraged many microorganisms from careers in cellulose chemistry.

These are some general problems of cellulases and pectinases. A problem of more immediate interest to a plant pathologist is control of their activities. There have been several *in vitro* studies of potential inhibitors of cellulases; most of the effective ones are broad spectrum enzyme poisons and at present it would appear that the parent microorganism offers an easier target than its extracellular enzymes.

Another matter, which may be a necessary antecedent of effective control is a thorough understanding of the action of these enzymes in plant tissues. An enzymologist has many legitimate interests for which *in vitro* studies are perfectly adequate, but the conditions under which he studies these reactions are often a far cry from the reaction conditions in nature. To begin with, if the enzymologist is dealing with an extracellular enzyme such as a cellulase, the enzyme preparation was probably obtained from a culture filtrate after growing the organism in a semi-synthetic liquid medium. Enzymes aren't the only secretions of microorganisms into such media, and among these other secretions may be products such as polysaccharides or peptides which readily complex with enzymes. The complexing agents present may vary from one set of cultural conditions to another and thus the choice of cultural conditions may have an important bearing on several properties of the enzyme, e.g. its apparent thermostability or its electric charge distribution. Furthermore, when the enzymologist measures activity toward insoluble substrates, he usually adds a dilute solution of this somewhat battered enzyme to a suspension of a substrate which has been thoroughly beaten, extracted, and swollen to increase its accessibility to large molecules. Before the enzyme can get to work, it must diffuse from this dilute solution through the thin stationary film that surrounds any insoluble particle in water, then diffuse, if it can, into the mass of the substrate. Soluble hydrolysis products released by the enzyme must retrace these diffusion steps. It seems to me that these aren't the operating conditions for microorganisms. They tend to maintain their surface in intimate contact with tissues that they are attacking and these surfaces can secrete fresh, concentrated enzymes directly into the site and absorb hydrolysis products from it.

This question of the relevance of *in vitro* to *in vivo* effects is a fundamental one. I wonder, Dr. Byrde, if you would care to comment on it and its relevance to pectinases.

Byrde: I think we would all accept how difficult it is to interpret from *in vitro* to *in vivo*. It's equally difficult, I think, to find out what is happening *in vivo*. As soon as you start extracting plant tissue or macerating it, you probably release inhibitors at once which might inactivate the enzyme. In fact, what you get out of the tissue may not be a reflection of what is happening *in vivo* at all.

Edgington: Are the adaptive cellulolytic and pectolytic enzymes of vascular pathogens inhibited *in vivo* by glucose, sucrose and other substrates encountered outside of the vascular system, and could such an inhibition of adaptive enzyme formation confine the pathogen to the vascular system?

Byrde: I'm not very familiar with the position in vascular diseases, as I haven't worked with them, but it seems to me that your suggestion is plausible insofar as it fits the facts as I know them. Sucrose has been shown by other workers to inhibit adaptive enzyme formation. We've found that glucose will do it for our system and I have no reason to believe that sucrose wouldn't be equally effective.

Bateman: I would like to raise a point concerning *in vitro* and *in vivo* production of pectic enzymes by plant-pathogenic fungi. Our studies with *Rhizoctonia solani* indicate that the enzymes which the organism is able to elaborate in culture differ, both with respect to properties and reaction products released in reaction mixtures, when compared to those we can obtain from disease tissue. These are quite reproducible differences from experiment to experiment. In other words, the properties of the enzymes themselves, their physical properties and their reaction products, are distinctly different. Whether or not the information we get from *in vivo* systems gives us a true picture of what happens in the plant or not, it does present a picture different from that obtained in studies with culture filtrates.

Byrde: This question of complex formation which you raised, Dr. Whitaker, might be involved. The different enzyme activities that one gets might reflect the same enzyme complexed in different ways.

Whitaker: Are the enzymic properties the same?

Bateman: So far as heat stability, stability at different pH levels, these properties are different.

Whitaker: Complexing could definitely modify these properties.

Bateman: So far as the reaction products are concerned, we get galacturonic acid as the end product of hydrolysis by the culture filtrate enzymes. The reaction products from diseased tissue extracts are di-, tri-, and tetragalacturonic acids. Using a common substrate such as sodium polypectate, we get complete hydrolysis in the former case and incomplete hydrolysis in the latter. This may indicate that a component of the system is lacking in extracts of diseased tissue. I think this illustrates the difference one can obtain by working with two different systems of this type.

Mandels: Would Dr. Byrde be willing to speculate on the nature of the products obtained from the autooxidation of catechin and epicatechin?

Byrde: I think you should ask a better chemist than myself, but I'll speculate on that. My colleague Mr. A. H. Williams and I have tested a dimer from tea leaves, this dimer had a molecular weight of the order of 700, based on epigallo catechin gallate. We had evidence that the dimer gave us about 40 per cent inhibition of extra-cellular pectolytic enzymes. Of course polymers can be inhibitory. The few experiments we have carried out suggest that a molecular weight of about 700 is a minimum requirement. Even phenolic chemists would be reluctant to postulate structures for some of these polymers.

Mandels: We've obtained natural inhibitors of cellulase from a number of plants. The inhibitors appear to be polymeric phenols. However, they lost their inhibitory activity when they were maintained at pH 8 for one-half hour.

Byrde: I'm not very certain, but I think that the products you would get by enzymic oxidation with polyphenol oxidase are the same as those obtained by autooxidation at pH 8.

Rudolph: Dr. Grossman from the department of Plant Pathology at Göttingen found that unoxidized apple juice was much more inhibitory to pectolytic enzymes of *Fusarium oxysporum* than was oxidized juice (Phytopathol. Z. 45:1, 1960). For instance, acetone extracts of certain apple varieties inhibited the pectic enzymes more than water extracts which became oxidized. He concluded that the apple contained inhibitory polymerized tannins that lost their activity upon oxidation.

Byrde: Thank you for your comment on Dr. Grossman's interesting work. I think I perhaps over-simplified the picture. I've given the impression that oxidized, polymerized forms of phenolics are always more toxic, but that is not true. Perhaps I've had this outlook because in my particular work that has been the case. However, Dr. Pollard and Miss Kieser, colleagues of mine at Long Ashton working on fruit juices, have found that leuco-anthocyanins present in pear juice are themselves, without any further oxidation, highly inhibitory to pectolytic enzymes. They also found in one case that chlorogenic acid inhibited some of these enzymes, but became less effective when it was oxidized.

Byrde

Rantela: You have shown that the macerating activity of enzymes in culture filtrates can be inhibited by polyphenols. That is, the enzyme activity is inhibited after the enzyme is produced. Is there any indication that the biosynthesis of the enzymes can be inhibited by such compounds?

Byrde: It is difficult to show that the biosynthesis of the enzymes is inhibited if, at the same time as it is produced, its activity is inhibited. You would have difficulty in distinguishing between inhibition of biosynthesis and inhibition of activity if you had the phenols present all the time. You wouldn't know whether the phenols prevented the enzyme from being produced or were inactivating it after it had been produced. If you got less enzyme you couldn't be sure whether 80 per cent less enzyme had been formed or whether there was an 80 per cent inhibition of the enzyme produced.

Stahmann: In our studies with Deese on the relationship between pectolytic enzyme activity and resistance when *Verticillium* or *Fusarium* was grown on tissue from resistant and susceptible potatoes, tomatoes, and banana tissue, we found a correlation between resistance and a reduction in enzyme activity or the amount of pectin hydrolyzing enzymes produced by the parasite. We also asked the question as to whether or not this was due to a reduction in the production of pectin-hydrolyzing enzymes or an inhibition of their activity. It's a difficult question to answer. We tried to answer it by mixing extracts from the resistant varieties of potatoes that had shown a reduced activity, with enzyme preparations containing a known amount of activity. If there had been an inhibition of activity, we hoped to get an inhibition of the control preparation when the two were mixed. However, as I remember the results, we did not get any inhibition, which I think would tend to indicate that there was a supression of enzyme synthesis by potatoes in these resistant varieties.

I think that disease is, in a sense, a biochemical battle between the parasite and the host. Among the offensive weapons that the parasite uses are the extra-cellular hydrolytic enzymes such as pectinases, cellulases, and other hydrolytic enzymes. In response to the presence of the parasite, I think that the host is also capable of mobilizing biochemical defensive weapons. Among these we may have enzyme in-

hibitors and inhibitors of protein biosynthesis which block or reduce the synthesis of hydrolytic enzymes by the parasite. Obviously the situation is extremely complex. I believe that we should study plant diseases on the biochemical battle field where the host and parasite are in intimate contact. Dr. Whitaker and others have pointed out that there is a difference in the biosynthesis and activity that the parasite carries out in synthetic media compared to what it does on its natural substrate, the host. Here is the area where I think more work is needed in order to see clearly the interrelationships between the host and parasite when they are in intimate contact as disease symptoms are being formed.

Cowling: I was much interested in your statement that cellulolytic enzymes act primarily at points of contact with the microorganism and, by virtue of their large particle weight, that the enzymes would be unable to permeate and act within the fine structure of plant cell walls. With my electron microscope and light microscope, we have obtained evidence that the cellulolytic enzymes of many wood-destroying fungi cause dissolution of cell wall material not only at point of contact between fungal hyphae and the wood cell wall but also at considerable distances (in terms of hyphal diameters) from the hyphae. Is it your view that dissolution of the cellulose in plant cell walls is accomplished primarily in close proximity to the fungi? At how great a distance from the hyphae do you believe extracellular cellulolytic enzymes will act?

I certainly agree with your suggestion that it is highly speculative to extrapolate from studies of isolated enzyme systems developed in semisynthetic media to the far more complex situation existing *in vivo*, particularly during pathogenesis. To avoid this kind of error, we have attempted to characterize the chemical transformations that occur in wood in progressive stages of decomposition by pure cultures of known fungi, and to develop from such evidence hypotheses regarding the mechanism of enzyme action involved in the presence of the microorganism. Degree of polymerization (DP) measurements on holocellulose prepared from decayed wood samples have shown, for example, that brown-rot fungi preferentially attack amorphous cellulose in the early stages of decay, leading to a very rapid drop in average DP. This suggests that the enzymes in fact are capable of action within the fine structure of wood cell walls.

Do you have experimental evidence from other enzyme-cellulosic substrate systems that show that the enzymes are not able to permeate the fine capillaries that exist between microfibrils and between cellulose molecules in the amorphous regions of the cellulose? In this connection you may be aware of the relatively low molecular weight cellulase which Petterson, Porath, and I described recently (Biochim. Biophys. Acta 67:1, 1963). An enzyme with a particle weight of 11,000 is still a very

large molecule. But we also have obtained evidence from measurements of the anti-shrink efficiency of polyethylene glycols of even 20,000 in average molecular weight that suggest that such molecules may find access to a portion of the cell wall structure of spruce and maple sapwood.

Whitaker: The yardstick for close proximity which I had in mind was the diffusion path into the substrate from the surface of the organism (as opposed to the diffusion + convection path in the substrate + liquid medium which prevails in *in vitro* assays). The maximum effective length of this path would depend, I should think, on the size, shape, stability and concentration gradient of the enzyme, the prevailing temperature, the permeability of the substrate and on the enzyme-synthesizing capacity and patience of the organism. Most microfibrils appear to have diameters of the order of a few hundred Ångstrom units and, neglecting subsequent widening by enzymic attack, the channels between them would be fine enough, I should think, to block the passage of very large proteins and to slow down the diffusion rate of small ones. However, given enough time, a molecule which can navigate through a channel will diffuse right through it. Craig's data on the escape rates of proteins through cellophane membranes gives an indication of the advantages of low molecular weight for diffusion through amorphous cellulose.

II. Plant Chemotherapy

Introduction

Lloyd V. Edgington

The Connecticut Agricultural Experiment Station

Interest in chemotherapy of diseases has expanded rapidly. However, we have few instances of successful commercial acceptance of chemotherapeutants. It appears to me that we are limited in success by our inability to select chemicals which will move within the plant to the locale of the diseased tissue, and will be selective, inhibiting the microplant, or fungus, but not inhibiting the macroplant or host.

The largest, relevant body of work on the movement of exogenous compounds in plants has been done by plant physiologists concerned with the translocation of growth regulators. Our first speaker, Dr. Mitchell, is one of the leaders in this field. To discuss Dr. Mitchell's paper, we have invited Dr. Davis, a plant pathologist who has studied the merits of growth regulators as chemotherapeutants.

The *modus operandi* of chemotherapeutants will be presented by Dr. Dimond, whose accomplishments in this area of research are well known to all of you. Because chemotherapy is widely regarded as the most likely control measure for systemic infections of trees, we have asked Dr. Cowling to lead the discussion of Dr. Dimond's presentation. Dr. Cowling is a forest pathologist vitally concerned with the degradation of woody tissues by fungal enzymes.

The chemotherapy of rusts has also received much attention. Because rusts are obligate parasites, their chemotherapy presents special problems worthy of separate consideration. This subject will be covered by Dr. Samborski, whose research on the physiology of the cereal rusts has led him into studies of their chemotherapy. Dr. Daly, an equally keen student of the host-parasite relations of rusts, will lead the discussion of Dr. Samborski's paper.

Progress in Research on Absorption, Translocation, and Exudation of Biologically Active Compounds in Plants

John W. Mitchell

Crops Research Division, Agricultural Research Service
U. S. Department of Agriculture, Beltsville, Maryland

About 25 years ago, it was learned that some exogenous substances induced plants to respond systemically when placed on a single leaf. Interest then developed in studying absorption and translocation of these chemicals and much research effort was soon aimed in this direction. Now, however, effort to learn how plants translocate exogenous substances has decreased, except perhaps in the field of weed control. But there are many questions yet to be answered, if we are to utilize fungicides, bactericides, and other pest-control chemicals most effectively and safely. The questions that still confront us are fundamental ones which are really difficult to answer with our present methods and store of knowledge.

Use of the Water Stream for Transport

We all know of the excellent effort that has been made, particularly here at The Connecticut Experiment Station, toward learning how water involved mainly in transpiration can be utilized to carry and distribute chemotherapeutants within plants (2, 3). To be sure, the water stream that moves up through plants represents a remarkable distribution system capable of rapidly transporting required minerals from below ground level 300 feet upward through a redwood tree, where these substances are distributed among cells in the uppermost branches. In studying this water-transport system of plants, we clocked the speed of molecules moving up with the water stream through the stems of young bean plants and found the rate to be a foot a minute, 60 feet an hour (5). Similar plants carried southern bean mosaic virus particles, which are thousands of times larger than many ordinary molecules, at about the same rate of a foot a minute. With this efficient translocation system at hand, there is no question but that we should attempt to utilize it fully as a means of distributing chemotherapeutants within plants.

But what about the other no less remarkable translocation system, the phloem? This second system translocates carbon compounds required for the growth and maturation of every cell in crop and ornamental

plants, including the giant *Sequoia*, mentioned earlier. This amounts to a lot of carbon in the form of sugar and other compounds. Of importance in chemotherapy is the fact that this phloem translocation system can carry molecules uncommon to the plant. It can also move particles that vary widely in size, such as viruses and chemotherapeutants.

The question is: Can we discover a way to utilize this phloem translocation system effectively in the chemotherapy of plants? Our first reaction is that this is too difficult, that not enough chemical can be moved in the phloem, or that the phloem is too sensitive to environmental changes to be used in this way.

But the fact is that herbicide people are now using the phloem translocation system very successfully and safely in killing weeds, and saving American farmers millions of dollars. Entomologists also are using this system to some extent to control insects. Can pathologists use this downward translocation system to control disease organisms effectively? With the right chemicals, this may be just as feasible as it is when chemicals are used to control weeds.

Use of the Phloem for Transport

Using growth regulators, scientists recently learned some things about phloem transport which might be of interest to pathologists studying chemotherapeutants. We know that transport in the phloem involves a vital process. The phloem must therefore be continuous and uninjured in order for plants to translocate molecules effectively downward from the leaves. Many kinds of regulating chemicals are readily absorbed by leaves and translocated down petioles into stems of plants. If, however, the petiole is severed and the cut end placed in a small amount of water, compounds absorbed by the leaf blade will not move from the blade through the petiole and out into the water, possibly because of callose accumulation at the cut surface of the petiole (1). Nor do translocatable exogenous substances move out of the cut surfaces of roots (7). From the standpoint of systemic disease control, this suggests that injury to the phloem by pathogens would interfere with the downward translocation of a therapeutant.

Assuming, however, that the phloem is uninjured, it would not be expected that a chemotherapeutant would be translocated from a leaf to the stem and roots unless photosynthate were also being translocated from the leaf to the stem and root. This assumption is based on the fact that leaves effectively translocate regulating chemicals, herbicides, and viruses only when photosynthates are being translocated over the same pathway to other parts of the plant.

Environmental conditions adverse to photosynthate transport are, therefore, also adverse to downward movement of chemicals through the

phloem. As one would expect, young rapidly expanding leaves and leaves growing in deep shade translocate growth-regulating chemicals and herbicides less readily than do more mature leaves and leaves growing in sunlight (6). The same would no doubt be true concerning translocation of chemotherapeutants.

Effect of Metabolism on Transport

Another important reason why many substances are not translocated readily through the phloem is that plants are equipped with enzymes which can effectively change or degrade many kinds of exogenous substances after such a compound enters the plant through the leaves. As a result, a substance which enters a leaf may be degraded and parts of it synthesized into another or several other compounds. It is known that these degradation products often do not possess biological activity equal to that of the original substance.

Some recent experiments clearly illustrate the ability of leaves to degrade or alter growth substances, and no doubt leaves may just as readily degrade some of the compounds pathologists attempt to use as chemotherapeutants. In these experiments, radioactive naphthalenelactamide, a compound that can induce marked growth responses, was applied quantitatively along the outer edge of bean leaves. Two days later this chemical could not be detected as such in the main veins of these leaves, in the petioles, or in the stems. The main veins of the treated leaves did contain, however, one easily detectable reaction product which gave an R_F of 0.67 compared with 0.92 for the lactamide applied to the leaves. This same initial reaction product was found in the petioles and stems. However, two additional metabolites were also detected in the petioles and these gave widely different R_F values. The stems contained still a fourth compound that differed from the parent lactamide and all the other reaction products detected (8). It is apparent from this study that different parts of the plant contained different enzymes which limited translocation of the original intact molecule. To be effective, a therapeutant active in *in vitro* tests would, of course, have to resist any reactions within the plant that reduced its activity.

One can, in some cases a least, partially block the ability of a plant to degrade a biologically active compound and in this way increase the chances of the compound being systemically effective. This has been done by altering the structure of the chemical slightly, and in such a way that the chemical retains the desired biological activity.

This was illustrated recently by Leafe (4). In his country, a weed known as cleavers failed to respond to a commonly used herbicide, 4-chloro-2-methylphenoxyacetic acid. Cleavers changed this chemical almost as soon as it was absorbed by the leaves and converted it into in-

active degradation products. To prevent this, a single alkyl group was introduced in place of a hydrogen associated with the alpha carbon. This change blocked the ability of the leaves to degrade the molecule, and at the same time the necessary herbicidal properties were retained. These results point to the possibility of using structural changes to control degradation in the case of chemotherapeutants.

Along the same line of thought, Linder and I are now studying the metabolism of some phthalamic acids. One compound in this family of regulators is apparently changed by bean plants into degradation products that do not possess growth-regulating activity. This degradation occurs within 2 or 3 days after measured amounts are applied. In contrast, another closely related phthalamic regulator apparently remains intact and active for more than a week when an equivalent amount is applied. These results also illustrate the possibility of controlling degradation rates by selecting compounds that have appropriate molecular configurations.

Also of interest is the fact that some regulating substances are metabolized at vastly different rates depending upon where the chemical is applied to the plant. For example, Linder found that 2,3,6,-trichlorophenylacetic acid was a very effective regulator when 130 μg was applied to the stem of a bean plant. However, when the same amount was applied to the leaves, very little growth response developed (10). Using the phthalamic acids mentioned earlier, the difference was even more striking. Applied to stems of bean plants, many of these phthalamic compounds are very active growth regulators. Applied to the leaves, they are ineffective. We are attempting to learn whether this difference in activity is due to rapid degradation of these compounds in the leaves and less rapid degradation in the stems.

Exudation

There is another relatively new and unexplored area of interest in connection with chemotherapy. This involves exudation of compounds from roots in biologically effective amounts.

Plants are capable of absorbing many kinds of exogenous substances. Once inside the plants, some of these substances are moved downward into the roots. Of importance here is the fact that roots are capable of exuding some of these substances into surrounding soil. The question is: Can pathologists utilize root exudation in developing methods for controlling some soil-inhabiting pathogens?

For a long time it has been generally known that plants exude endogenous substances from their roots. Microbiologists have carried out intensive research in this field (11). On the other hand, it was less than 10 years ago when it was first learned plants can absorb, translocate,

and exude exogenous substances from their roots. We also learned that the amounts exuded can greatly alter the growth and behavior of other plants nearby (9). So far, root exudation of exogenous substances has received little attention.

Proof of root exudation was obtained by using a very simple experiment with the plant regulator alpha-methoxyphenylacetic acid. Two bean plants were grown with their roots in soil contained in one pot. Another pair of comparable plants was grown, each plant with its roots in a separate pot. Alpha-methoxyphenylacetic acid was applied to only one plant of each pair.

Within a few days both plants with their roots in the same pot developed growth responses to the chemical. It should be emphasized that only one plant of the pair had received the chemical, but both plants responded.

In contrast, considering the pair of plants with their roots in separate pots, only the plant to which the chemical was applied developed a growth response.

The proximity of aerial parts of all treated and untreated plants was the same, thus, proving that this chemical did not move through the air to bring about these results. The only other pathway possible was from the roots of the treated plant, through the soil, and to the roots of the untreated one. This pathway was, of course, possible only when the plants had their roots in the same pot.

Subsequently, more elaborate methods and apparatus were developed for studying root exudation in detail. By placing roots of plants in aerated water, it was possible to recover a number of growth-regulating substances exuded following application of these chemicals to the leaves. Through the use of C^{14}-tagged compounds, it was possible to identify the compounds exuded.

Mechanism of Root Exudation

With these current methods, we have learned that many kinds of plants, including monocotyledonous and dicotyledonous ones, are capable of absorbing and translocating certain exogenous substances to their roots, and then exuding these compounds into the surrounding soil. The exuded substance can be absorbed by the roots of nearby plants. The receiver plant need not be of the same species as the donor. For example, a compound can be exuded from the roots of sunflower, and then absorbed by the roots of cotton in amounts that greatly alter growth of the cotton plant.

Exudation is fairly rapid. When a compound was applied to the leaves of the plants at Beltsville, a detectable amount exuded from the roots in only 8 hours. The amount exuded increased with time for about

72 hours and then the rate of exudation decreased. We believe that this variation in the rate of exudation was probably proportional to and limited by the amount of the compound absorbed by the leaves and translocated to the roots. This assumption is based on the fact that we were unable to overload the absorption-translocation system of our experimental plants.

To learn more about this rather unexpected observation, we attempted to overload the translocation system of bean plants in two ways. First, we determined the minimum leaf dosage which resulted in a barely detectable amount of root exudation. We then increased the dosage level until it reached 80 times that required to give a threshold response. This amount of the chemical proved to be toxic to the leaves and we were, therefore, unable to increase the dosage further. At all times during the 80-fold increase we observed that the rate of exudation was proportional to the amount of the chemical applied to leaves. Over the range used, the more chemical we applied, the more of the compound exuded from the roots. These results suggest that the exudation system functioned in a passive way since, under the conditions of the experiment, the rate of exudation paralleled in a linear fashion the wide range of dosages applied to the leaves.

Linder attempted to overload the translocation system in another way. He placed a stable form of a growth regulator in water surrounding the roots and allowed the plant to absorb the chemical, to translocate it upward, and to develop growth responses. It was then assumed that the translocation systems of the plant contained many molecules of the compound. At this time a radioactively tagged form of the regulator was applied to the leaves. For comparison, an equal amount of the tagged compound was also applied to leaves of other plants which had not previously received the root treatment. Exudation of the tagged compound was the same regardless of whether the plants had been pretreated with the untagged form of the same compound or not.

We also learned that exudation depends on phloem transport. To play its part and make root exudation possible, the phloem must function efficiently. This was demonstrated by the fact that removal of about one-third of the root decreased exudation by approximately one-third. Removal of two-thirds of the root decreased exudation by approximately two-thirds, and removal of all the root resulted in no detectable exudation from the cut surface of the stem. Exudation from injured phloem did not occur (7).

Killing different amounts of the roots with steam also decreased exudation by amounts proportional to the amount of root killed. From this, it appears that interference with phloem translocation is reflected proportionally in the amount of the compound that exudes from the roots.

There are now eight exogenous compounds that are known to exude from roots in readily detectable amounts following application of these substances to the leaves of plants. So far we have not found a common structural characteristic associated with the ability of plants to absorb, translocate, and exude these substances.

The most effective compound, as far as exudation is concerned, is 2-methoxy-3,6-dichlorobenzoic acid. Linder collected this compound, as it exuded from roots of six bean plants during a 24-hour period. He then applied this exuded regulator to another, previously untreated, bean plant. The amount of the benzoic acid or a growth-regulating metabolite of it exuded from roots of the original six plants was sufficient to kill the plant to which it was applied.

In conclusion, it should be emphasized that so far, in studying exudation, we have used only substances that are growth regulators. We have limited our studies to these compounds because it is easy to detect exudation with these substances. It is difficult to detect exudation of compounds that are not growth regulators only because we have not yet developed methods for this purpose. However, by using organisms other than plants to detect exudation, for example, fungi, nematodes, or bacteria, it will be possible to find exogenous, nongrowth-regulating chemicals that exude from roots after application to leaves. There are, no doubt, many compounds other than growth regulators which plants can absorb into their leaves and stems, translocate, and exude from their roots in biologically effective amounts. Might there not be some exogenous compounds that exude in amounts sufficient to protect plants against certain nematodes, fungi, bacteria, and parasitic weeds? We may some day control certain kinds of weeds by means of exuded herbicides, and prevent attacks by some kinds of nematodes, fungi, and soil-borne bacteria with these chemicals. To attain these goals, we must understand more fully absorption, translocation, exudation, and the fate of the compounds we apply.

LITERATURE CITED

1. Currier, H. B. 1957. Callose substance in plant cells. Am. J. Botany 44:478-488.
2. Dimond, A. E. 1962. Surface factors affecting the penetration of compounds into plants, p. 368-382. *In* H. F. Linskens and M. V. Tracey, [ed.], Modern methods of plant analysis, Vol. 5. Springer-Verlag, Berlin.
3. Horsfall, J. G. 1956. Principles of fungicidal action. Chronica Botanica, Waltham, Mass. 279 p.
4. Leafe, E. L. 1962. Metabolism and selectivity of plant-growth regulator herbicides. Nature (London) 193:485-486.
5. Linder, P. J., and J. W. Mitchell. 1960. Rapid transport of alpha methoxyphenylacetic acid introduced directly into the water stream of bean plants. Botan. Gaz. 121:139-142.
6. Mitchell, J. W., and J. W. Brown. 1946. Movement of 2,4-dichlorophenoxyacetic acid stimulus and its relation to the translocation of organic food materials in plants. Botan. Gaz. 107:393-407.

7. Mitchell, J. W., P. J. Linder, and M. B. Robinson. 1961. Mechanism of root exudation of α-methoxyphenylacetic acid in the bean plant. Botan. Gaz. 123:134-137.
8. Mitchell, J. W., P. J. Linder, and B. C. Smale. 1960. Growth regulators and therapeutants–their absorption, translocation, and metabolism in plants. The Nature and Fate of Chemicals Applied to Soils, Plants, and Animals. ARS Sp. Rpt. 20-9, Agricultural Research Service, U.S.D.A.
9. Preston, W. H., Jr., J. W. Mitchell, and Wilkins Reeve. 1954. Movement of alpha-methoxyphenylacetic acid from one plant to another through their root systems. Science 119:437-438.
10. Unpublished data.
11. Woods, F. W. 1960. Biological antagonisms due to phytotoxic root exudates. Botan. Rev. 26:546-569.

Discussion

Davis: My remarks will be limited to two questions and then I will open discussion on the floor. Considering yesterday's discussion regarding the role of natural products such as phenolics in disease and evidence that certain of these phenolics seem to be concentrated to a greater extent in areas of infection in resistant plants than in susceptible plants, I would like to ask Dr. Mitchell what he thinks about the possibility of mobilization and redistribution of natural inhibitors such as phenolics towards areas of infection? Secondly, what do you think of the possibility of artificially loading plants with natural inhibitors as a mode of chemotherapy? This question is based on the fact that the plants which contain these inhibitors have a way of detoxifying them by storing them in a combined form which could be released upon contact with an invading organism.

Mitchell: We do not have any means of mobilizing phenolic compounds that are produced by plants. But we are aware of the fact that plants are capable of mobilizing compounds very effectively to points of high metabolic activity. I think, in every case, induced mobilization has been associated with induction of metabolic activity. For example, the herbicide people have great difficulty in killing the resting buds of mesquite. They can get 2,4,5-trichlorophenoxyacetic acid into the top of the mesquite plant. The plant will translocate the chemical downward, bypassing these resting buds and having no effect on them. As a result, these buds may eventually grow. On the other hand, if the buds are growing at the time the plant is treated, the chemical is mobilized to the buds and subsequent growth is prevented. This is a good example of how mobilization is associated with metabolic activity and growth. We have also demonstrated this by chemically stimulating meristematic growth. For example, if you apply a growth regulator, 2,4-D, to the leaf of a plant, you can then measure the amount that is mobilized in the stem. If you apply the same amount of 2,4-D to the leaf and at the same time

treat the stem with a different growth accelerator such as indoleacetic acid, you might find that the amount of 2,4-D mobilized in the stem is three times as much as that mobilized without the indoleacetic acid. We have done this with many compounds, again illustrating that mobilization, as we understand it with growth regulators, is always associated with increased metabolic activity. As far as I know, we cannot separate these two phenomena and thus induce a chemical to move to one specific part of a plant without at the same time inducing meristematic activity in that part of the plant. It may be that since some of your pathogens may increase metabolic activity, they may influence, to some extent, mobililization of some compounds and perhaps some of the phenol compounds would be mobilized as a result.

Now as to the second question; "Can we apply naturally occurring substances that might be beneficial in disease control to the outside of the plant and get better results than if we used synthetically prepared compounds?" Again, we have had no experience in the field of pathology in this respect, but we have had a little experience with growth regulators. The naturally occurring compound, gibberellic acid, as far as I know, is the most readily absorbed and translocated growth regulator known. We have been unable to induce stem curvature with gibberellic acid apparently because the compound is moved so rapidly across the stem when applied unilaterally that the stem grows more or less uniformly and the plant grows straight up. This lack of curvature is apparently caused by a very rapid movement of a chemical from one side of the stem to the other. I do not know of any synthetically prepared compounds which are moved this rapidly across the stem. Also, some years ago, we obtained a growth regulator from the endosperm of immature bean seeds. This regulator behaves in a similar way to gibberellic acid. It too appears to move so readily across stems that it fails to induce stem curvature. Whether this means that some of your naturally occurring compounds would be translocated more readily than synthetic ones, I cannot say, but we have found some very effectively translocated natural compounds in the field of growth regulators.

Galston: I would like to ask Dr. Mitchell and also Dr. Davis, since this pertains to a question he raised, whether one should not now consider the naturally occurring kinins as important in the mobilization of substances in plants? The work of Mothes, of Osborne, and of others has shown that a local application of the synthetic material, kinetin, and more recently of naturally occurring kinins which can be extracted from various plant sources, will cause a rapid migration of labelled amino acids to the locale of application. I would like to ask specifically whether Dr. Mitchell knows of any instances in which labelled synthetic or natural auxins have been caused to migrate to the locale of kinin application?

As a corollary to this question I would also like to ask about the role of natural and synthetic auxins in also causing a mobilization of other materials. The work of Osborne with senescent bean leaves has shown that local application of 2,4-D will create an island of green tissue in an otherwise yellowing leaf, and will act like kinetin in causing a channeling of exogenously labelled amino acids to that site. Do you have any comments on this problem in general?

Mitchell: I think that is a very good suggestion: the use of kinetin in attempting to mobilize various compounds. I do not know of any experiment where this has been done to mobilize plant hormones and naturally occurring auxins but it would be very interesting to try with some of the compounds that you have been interested in, especially in connection with disease control.

Now as to the mobilizing effect of some of the synthetically prepared compounds on naturally occurring auxins, we all know that when a synthetically prepared growth regulator is applied, a series of events is set in progress when high concentrations are present. This ultimately leads to the production of new cells. I assume that in this process many regulators are involved. No doubt some may be moved in from other plant parts. Perhaps in this series of processes some are actually manufactured, but we visualize this as starting with an initial simple response, the nature of which we do not know at present. If sufficient stimulator is there, the response develops to where we have this very complex situation, the production of more cells. Many hormones are involved in these processes and no doubt some are mobilized and some produced at the site.

Dimond: Dr. Mitchell, your discussion of the excretion from the roots of compounds applied to leaves in relation to the control of soil-borne pathogens is a most intriguing possibility. Have your studies indicated that excretion occurs under normal conditions of growth or are special environmental conditions necessary?

Mitchell: No special environmental conditions are necessary. We have obtained exudation from many kinds of plants, in fact, all kinds that we have tested with the exception of corn plants. This is a very interesting situation. Why do corn plants not exude chemicals readily exuded by other plants? No special conditions are required to obtain exudation. I should point out, however, that the amount of chemical exuded is not very great. The amount exuded is in terms of micrograms. We are dealing here with exceedingly active compounds so that only a few micrograms are necessary, but, from the standpoint of pathology, one wonders about the concentration at the root surface. Even though very minute amounts

are involved, the concentration at the root surface may be relatively high and, therefore, biologically effective.

Palmer: Dr. Mitchell, you talked about the degradation of certain of these agents by enzymes after they entered the plant. Have any agents which are initially ineffective as herbicides been added to plants, and then the enzymes made them effective?

Mitchell: In the case of napthalenelactamide, the compound was apparently changed into degradation product A, a very effective growth regulator. In fact, it caused seven times as much growth regulation at a distance from the point of application as did the parent acid, naphthaleneacetic acid. Whether this is due to inherent activity in the molecule, brought about by enzymatic activity, or to some effect on translocatability, we do not know. But there was an increase in the activity of this compound once it was changed into a degradation product. I think the entomologists have noticed this also in some of their systemic compounds: an increase in activity following absorption of the insecticide and action of enzymes.

Horsfall: Dr. Mitchell, you mentioned at least two compounds which you found excreted into the soil, the alpha-methoxyphenylacetic acid and I think you mentioned a trichlorobenzoic acid. How much substitution can you do on the rings of these compounds without influencing their translocatability?

Mitchell: You can't do very much. I might read you a list of the compounds which we now know to exude and you can see how closely related they are: DL-alpha-methoxyphenylacetic acid, meta-chloro-alpha-methoxyphenylacetic acid, meta-fluoro-alpha-methoxyphenylacetic acid, para-fluoro-alpha-methoxyphenylacetic acid, 2,3,6-trichlorobenzoic acid, 2,3,5,6-tetrachlorobenzoic acid, 2, 3, 6-trichlorophenylacetic acid, and 2-methoxy-3,6-dichlorobenzoic acid. The substituted benzoics were tried but failed to exude.

Horsfall: You didn't put any hydroxyls on those rings?

Mitchell: We have had no hydroxylated benzoic acids that exuded so far.

Horsfall: You tested them however?

Mitchell: Yes, we checked them in a limited way.

Zentmeyer: I would like to make a comment or two and then ask Dr. Mitchell a question. I have been working considerably on the role of root exudates in relation to pathogenesis of some root fungi, especially

Phytophthora. We have some very interesting evidence that where we have striking chemotaxy of zoospores of *Phytophthora cinnamoni* to the roots of avocado, some material is being exuded, primarily from the region of elongation. One of my questions is: Do you have any evidence from your work as to what region of the root is involved in exudation?

Mitchell: With growth regulators we can see no difference. When we cut off the elongating portion of the root we get a proportional decrease in exudation. If cut further back towards the stem, the proportionate decrease remains the same, i.e., equal to the amount of root removed. And finally, if all the root is removed, we have been unable to get exudation from the cut surface of the stem.

Zentmeyer: The other comment is: We also have some evidence that ethionine, 4-ethylthio-2-aminobutyric acid, is translocated downward. This movement, however, has been detected only biologically so far. We have indications that you can apply this material to the foliage and obtain reduction of size of stem cankers. We've analyzed this in relation to chemotaxy to see if this material would also reduce the attraction of the roots for the zoospores, and so far, unfortunately, I have to report there is no correlation between the application of ethionine to the foliage and the reduction of chemotaxy of zoospores to the exudate. I wondered also if you have any indications of translocation of this type of material in a downward direction?

Mitchell: We have experiments in progress now on this very point, but I hesitate to report on them now. They are preliminary. There is some evidence that we have an effect.

Stahmann: I would like to ask Dr. Mitchell a question about the efficiency of this translocation of exudates. What percentage of compounds applied to leaves is excreted from the roots?

Mitchell: When we applied 20 micrograms of acid to the total surface of the primary leaf of the bean plant, we found approximately 15 per cent exuded in 24 hours. This is the only case where we have measured the actual amount. But it will give you some idea of the amount placed on the surface of the leaf in comparison with the amount that came out of the root.

Howard: Dr. Mitchell, am I to assume that you have only done this with annual plants? For instance, with the chlorinated benzoic acids in trees, we get variable results. We have used them for several years as chemotherapeutants. One year we get excellent results; the next year we will get no results. That is why we're noncommittal sometimes. Have you anything on higher plants where the growth of the plant may effect the translocation?

Mitchell: With respect to trees, no. We need to study the translocation in trees the year round. I don't believe anyone has done this yet and it seems a very good thing to do, if we are to try to utilize the translocation systems of trees. It would be an easy matter to start January first and study during the entire year the ability of the tree to translocate substances through its water translocation systems, and also through its phloem. I think you would get a very interesting annual picture of the behavior of these two translocation systems. No, we haven't experimented with trees but we certainly would like to do so.

The Modes of Action of Chemotherapeutic Agents in Plants

A. E. Dimond

The Connecticut Agricultural Experiment Station

Chemotherapy is the control of disease by compounds that act from within the plant. The most obvious type of chemotherapy, and the best known, is the direct action of a compound on the pathogen. Many studies have taken this direct approach and sought compounds that are toxic to the pathogen *in vitro,* low in phytotoxicity, and sufficiently systemic and stable in the host to be useful.

During the course of such studies, a number of compounds have been found that alleviate disease under conditions that preclude the possibility of direct toxicity to the pathogen. These are now known to modify the host or to react in an important way with enzymes or phytotoxic products of the pathogen.

As our knowledge of the biochemistry of pathogenesis and of natural disease resistance have improved, plant pathologists have begun to use this information in developing chemotherapeutic agents. The parallel between natural biochemical resistance and plant chemotherapy is clear. Yesterday's discussions on biochemical resistance have provided an excellent background for considering the nature of chemotherapeutic action. In addition, the general aspects of chemotherapy have already been thoroughly reviewed (10, 13, 26, 33, 35, 37).

The known chemotherapeutic agents may be classed into three general categories, according to their mode of action. The first contains the systemic fungicides and bactericides, which act directly upon the pathogen. The second consists of compounds that act upon the host itself, causing it to become more resistant to disease. The third group of chemotherapeutic agents is the least well-known. These interact with toxic compounds or enzymes produced by the pathogen that have an important role in the induction of disease. Our discussion will be confined to a consideration of the first two groups only.

Systemic fungicides and bactericides. The systemic fungicides and bactericides are toxic to the pathogen directly or become toxic after modification in the plant. To determine that a compound is a systemic toxicant is more difficult than it seems. A compound that is toxic to the pathogen *in vitro* and active in chemotherapy assays may or may not act as a systemic toxicant. A compound must be shown to be unmodified

in the plant and to be present in toxic concentrations if its acts directly. As an illustration, Crowdy, Grove and Pramer (7) identified streptomycin chromatographically in treated plants in concentrations toxic to the pathogen. Later Pramer, Robinson, and Starkey (36) used two strains of *Erwinia chrysanthemi,* one susceptible and the other resistant to streptomycin, and by this means demonstrated that the control of bacterial infection in streptomycin-treated chrysanthemum cuttings results from systemic bactericidal action.

By contrast, the control of crown gall with antibiotics had been assumed to result from systemic bactericidal action until Klemmer, Riker, and Allen (27), in comparable experiments, showed that an antibiotic-resistant strain of *Agrobacterium tumefaciens* failed to induce galls when inoculated into antibiotic-treated plants. This led them to conclude that the transformed gall cells of the host were susceptible to chlortetracycline, oxytetracycline, and chloramphenicol, whereas normal host cells were unaffected by these antibiotics. Such studies provide elegant examples of the need for critically designed experiments to determine if a chemotherapeutic agent acts directly upon the pathogen or not.

The action of nickel compounds in combating the cereal rusts is an example of direct action. Forsyth (20) has shown that both nickelous ion and sulfadiazine inhibit the respiration and development of *Puccinia recondita* in wheat. However, the effect of sulfadiazine is temporary. Many years ago Gassner and Hassebrauk (21) reported the chemotherapeutic effect of sulfa compounds against wheat rust in greenhouse tests, but were unable to reproduce the effect in field experiments. Forsyth has at last provided the explanation: the effect is too short-lived to be useful. In the field, nickel sulfate sprays have given almost perfect control of stripe and leaf rust in bluegrass (23).

A chemotherapeutic agent may be modified in the plant to a form that is fungitoxic. In this case *in vitro* toxicity of the chemotherapeutic agent may be low, but fungitoxic materials can be isolated from the treated plant. The derivatives of cycloheximide provide an illustration of such behavior. The oxime and acetate of cycloheximide are absorbed and translocated in plants more efficiently than is cycloheximide itself (22). Lemin and Magee (31) noted that cycloheximide acetate is not fungitoxic, but fungitoxic materials are found in plants treated with this antibiotic. They conclude that the acetate is hydrolyzed in plant tissues, freeing cycloheximide.

A fungitoxic grouping must of necessity be biologically reactive, and plant tissues frequently respond to the presence of biologically active molecules by detoxifying them. To circumvent this, active chemotherapeutic agents have sometimes been modified so that the toxophore is masked, a method that has been discussed by Sijpesteijn (37) and Oort and van Andel (35). The essential concept can be illustrated by the

studies of Byrde and Woodcock (4). In a series of di-esters derived from 2,3-dichloro-1,4-napthahydroquinone, the rates of alkaline hydrolysis under standard conditions were compared with their fungitoxicity and found well correlated. Such masking of fungitoxic groupings is potentially useful in chemotherapy. When masking prevents liberation of the fungitoxic grouping in host tissue except in the presence of the pathogen, the chemotherapeutic agent will have a longer useful life in the plant and be selectively toxic.

Systemic fungicides present an unusual problem in the combat of the vascular wilt diseases. Here, the pathogen is restricted to vascular tissue of the roots and stem, and the chemotherapeutic agent must be distributed similarly. Freely translocated compounds move to leaf tissue and are ineffective. Quaternary ammonium compounds have been tried for this purpose because they are adsorbed to xylem tissue and protect it against fungal attack (17). However, these compounds do not distribute in stems but remain adsorbed where they are introduced. Polyvalent cations, introduced prior to the chemotherapeutant, improve its transport in the stem, but the effect is inadequate and the introduced ions are somewhat phytotoxic (18). Edgington (14) has investigated the relation between molecular structure, fungitoxicity, and mobility in plant stems of quaternary ammonium compounds. He finds that fungitoxicity decreases as the aliphatic chain length decreases, and that mobility of these compounds increases simultaneously. The best compromise between mobility and fungitoxicity for therapy of wilt diseases apparently consists of a quaternary ammonium compound with a side chain of 6 to 8 carbon atoms.

An alternate approach to this distributional problem involves suppressing the ionization of the quaternary ammonium ion in a nonphytotoxic organic solvent that will penetrate through overlying tissues and carry the chemotherapeutic agent into xylem. Then, in the presence of the transpiration stream, the agent regains its ionic charge and is adsorbed to xylem tissue. This approach is also an exploitable one, if a suitable solvent system can be developed.

Compounds that modify the host. (*a*) *Agents that modify carbohydrate levels in plant tissues.* Quite apart from the effect of nutrient elements in affecting resistance to disease, resistance and susceptibility have been related by Horsfall and Dimond (25) to the sugar content of plant tissues. Light, boron deficiency, and the action of growth regulators, such as 2,4-D and maleic hydrazide, and of fungicides, such as nabam and captan, all affect the sugar levels in plant tissues. In turn, these treatments cause plants to become more susceptible to some pathogens and more resistant to others. On this basis, the rusts, powdery mildews, and chocolate spot of beans are considered as "high sugar diseases," i.e., tissues high in sugar are susceptible; and *Alternaria* on tomato, *Helminthosporium* on cereals, and Dutch elm disease are "low sugar diseases," i.e., tissues low in sugar are attacked.

Sijpesteijn (37) has given a clear illustration of how this idea may be used in chemotherapy. To improve the translocation of sodium dimethyldithiocarbamate as a fungicide in plant tissues, the corresponding S-carboxymethyl-N,N-dimethyldithiocarbamate was prepared. This proved to be readily translocated in plant tissue, and to be active as a chemotherapeutic agent against cucumber scab. Also, the compound produced formative effects in plants, resembling those produced by 2,4-D. Cucumber seedlings treated with this material were frequently low in soluble carbohydrates. This suggested cucumber scab is a high sugar disease, and when the chemotherapeutic agent reduces sugar levels in leaves, they become resistant. When cucumber seedlings were fed on glucose solutions, they were more severely attacked by *Cladosporium* than corresponding control plants. Moreover, when seedlings absorbed both glucose and the chemotherapeutic agent, sugar levels in tissues were high and the disease was severe.

We do not as yet fully understand the basis of the relation between carbohydrate levels of tissue and resistance or susceptibility to disease. For the "low sugar diseases," Dr. Byrde discussed yesterday one way in which the relation might well be explained. Doubtless different mechanisms are involved in other cases. The pectolytic and cellulolytic enzymes are frequently important in pathogenesis and some pathogens produce them as adaptive enzymes. The presence of sugars in host tissues suppresses the adaptive formation of these enzymes. Consequently, tissues high in sugars will resist invasion by such pathogens. When such fungal enzymes are constitutive, the presence of sugar in host tissue will have no effect.

The sugar content of tissues is a useful index, whether or not it determines resistance or susceptibility as such. This relation offers a useful approach to plant chemotherapy. Also, it may yield important information on the biochemistry of pathogenesis. This is a fertile field for further exploration.

(*b*) *Agents that modify amino acid metabolism of the host.* Studies in The Netherlands had shown that sodium dimethyldithiocarbamate is poorly translocated in plants. Van Andel (40) explored the effect of amino acids in improving the transport of this fungicide. She soon observed that some amino acids improved the control of cucumber scab when sodium dimethyldithiocarbamate was present, whereas other amino acids exerted a chemotherapeutic effect in their own right. Glycine and DL-α-alanine improved the action of dithio- or thiocarbamates. DL-serine, DL-threonine, L-threo-ß-phenyl serine or DL-histidine, applied alone to cucumber seedlings, reduced the severity of attack by *Cladosporium cucumerinum*. DL-serine also reduced the severity of

chocolate spot on broad bean and of late blight on tomato. This finding calls to mind the earlier study of Kuć, Williams, and Shay (30) in which D-phenylalanine was reported to increase the resistance of apple leaves to scab. Except for L-threo-ß-phenyl serine (which is unnatural), the D forms of the amino acids were more active than the DL mixtures and the L forms were inactive. Sap from treated plants was mildly fungitoxic but not invariably so. Subsequently, van Andel (41) has studied the auxin activity of the amino acids having a chemotherapeutic effect and finds that they predominantly act as anti-auxins. They promote root elongation, even when auxin is present. The single exception, DL-ß-phenyl-ß-alanine, was not examined in this regard, but has auxin activity in the epinasty test on tomato, whereas the others do not. It may prove significant that many of the amino acids having chemotherapeutic activity are both unnatural in occurrence and affect auxin action.

(*c*) *Agents that may influence the phenolic composition of plants.* In this category fall two types of compounds: those that may influence the oxidases in plant tissues, and those that may serve as precursors in the synthesis of phenolic compounds.

The report by Kuć, Williams, and Shay (30) that phenylthiourea increased the resistance of apple leaves to scab, reminds us that this compound is an inhibitor of polyphenol oxidase. Recently, Sijpesteijn and Pluijgers (38) examined a series of phenylthioureas in respect to their potency as inhibitors of polyphenol oxidase, uptake by cucumber seedlings, and their efficacy in control of cucumber scab in chemotherapy assays. Their data suggest a good correlation between inhibition and control of scab. This is a stimulating matter, in light of the known inhibition of pectolytic activity by polyphenols and their oxidation products (9).

The role of phenolic compounds in natural biochemical resistance has been well brought together by our speakers of yesterday afternoon. An intriguing aspect of plant chemotherapy is the possibility of supplying existing biochemical systems in plants with precursors of compounds that function in resistance. Chlorogenic acid has already been correlated with resistance of potatoes to *Verticillium* wilt (34). Levy and Zucker (32) have determined the biosynthetic pathway of chlorogenic acid in the potato tuber, and Zucker and Levy (42) have studied the factors affecting its synthesis. It now becomes possible to supply plant tissues with precursors, phenylalanine, for example, so that the titer of chlorogenic acid may be increased beyond the normal content. Kuć (28) and Kuć et al. (29) have measured the production of chlorogenic acid and its precursors in response to infection and stressed that the situation is a dynamic and not a static one. These situations are not unique in plant tissues as infection occurs, and to exploit them in plant chemotherapy is an intriguing possibility.

(*d*) *Agents that modify the pectins in host tissues.* One of the first promising compounds in our studies on the chemotherapy of *Fusarium* wilt of tomato was 4-chloro-3,5-dimethyl phenoxyethanol (12). This compound causes formative effects on tomato suggestive of the action of 2,4-D. Soon thereafter, Davis and Dimond (8) reported the action of standard growth regulators, such as indole acetic acid and naphthalene acetic acid, in reducing symptoms of *Fusarium* wilt. When the fungicide 2-mercaptobenzothiazole was modified by the addition of a carboxymethyl group, the resulting compound, sodium 2-benzothiazolyl mercaptoacetate also reduced the severity of disease and caused formative symptoms in the plant (11). These compounds could not be tolerated by plants in concentrations that are fungitoxic, and sap of treated plants showed no fungitoxicity. Evidently the chemotherapeutic action is indirect.

Compounds that affect the growth of plants vary widely in their specific growth regulating properties. Corden and Dimond (5) examined a related series of naphthalene-substituted aliphatic acids and esters, measuring their effectiveness as chemotherapeutic agents, their fungitoxicity, and assaying their action to prevent leaf abscission, to stimulate root initiation, and to inhibit root elongation. Within this series of compounds, the chemotherapeutic activity was poorly correlated with the ability of the compounds to prevent leaf abscission and to stimulate initiation of roots, but chemotherapeutic activity was well correlated with the action of compounds to prevent elongation of roots. These results suggested a modification of the pectic compounds of the plant by growth regulators. Edgington and Walker (19) had already shown a progressive increase in severity of *Fusarium* wilt with a decrease in calcium nutrition of the plant.

Corden and Edgington (6) then studied the interaction between calcium deficiency in tomato plants and treatment with naphthalene acetic acid in relation to their resistance to *Fusarium* wilt. Plants receiving a normal supply of calcium are, of course, moderately susceptible to wilt. Plants deficient in calcium are highly susceptible. Plants treated with naphthalene acetic acid are resistant. But calcium-deficient plants treated with naphthalene acetic acid proved to be highly susceptible to wilt. This study demonstrated a calcium requirement for the resistance to wilt induced by growth regulators. It suggested the cause as well.

Pectic enzymes macerate plant tissues slowly when they contain a normal amount of calcium but do so rapidly when calcium is not present (24). Evidently the cross-linking by calcium between pectic acid chains reduces their solubility, makes them more rigid, and less available to the chain-splitting pectic enzymes. In calcium-deficient plants, pectolytic enzymes produced by *Fusarium* can readily attack the pectins exposed at pits in vessels and cause plugging readily. Naphthalene acetic acid,

apparently, increases the amount of free carboxyl groups in pectic compounds in plant tissues. When calcium is in normal supply, it forms salt bridges between pectic chains and thus reduces their availability to fungal enzymes.

Edgington, Corden, and Dimond (16) have analyzed the nature of pectic materials in tomato plants under the influence of varying calcium supply and presence or absence of naphthalene acetic acid, administered at concentrations that are inhibitory to growth. Calcium-deficient plants, whether treated with naphthalene acetic acid or not, contained more water-soluble pectin than normal plants, whereas those treated with growth regulator only contained a lower than normal amount of water-soluble pectin. Treatments leading to a high content of water-soluble pectin produced plants that are highly susceptible to wilt and pectins that are readily hydrolyzed by enzymes. Treatments resulting in a low content of water-soluble pectins produced plants resistant to disease and pectic substances that are hydrolyzed slowly by fungal enzymes. Resistance of plants to disease and of their pectic substances to attack by enzymes is also related to the calcium content of the pectic substances.

(*e*) *Agents that modify the morphology of woody tissues.* In woody plants, resistance to vascular wilt diseases may be modified through morphological changes that restrict the invasion of the pathogen. No conifer suffers from a vascular wilt disease. The reason, perhaps, is merely the anatomy of the wood, which lacks vessels and contains a higher proportion of wall substance in relation to void space of conductive tissue than vessel-containing wood. Also, the tracheids are much shorter than are the functional vessels in angiosperms. A number of years ago, Banfield (1) noted the poor ability of the pathogen causing Dutch elm disease to penetrate cell walls. Young elms with thick annual rings frequently recover from the disease by outgrowing the fungus, whereas old trees with thin annual rings seldom do. When a tree grows rapidly, it merely leaves the pathogen behind, and newly developed tissues are healthy.

This principle has been considered in chemotherapy of wilt diseases. The critical consideration is to increase the amount of wall substance that the pathogen must penetrate to keep pace with the growth of the tree. In principle, resistance can result from increasing the diametric rate of growth, as happens naturally in young trees as compared with old ones, or it can result from altering the nature of the growth so that fewer vessels are formed, especially the large, early season vessels. Beckman (2) has shown that sodium 2-benzothiazolylmercaptoacetate (11) and the related compound sodium 4,5-dimethyl-2-thiazolylmercaptoacetate, both active in suppressing symptoms of Dutch elm disease, inhibit the rate of growth of tissues in the presence of physiologically normal amounts of auxin. He correlated this chemically-induced inhibition in development of spring wood with the reduced expression of symptoms in elms affected with Dutch elm disease. These two chemotherapeutic agents have proven erratically phytotoxic in practice, and alternative growth regulating materials have been sought that are less phytotoxic.

Recently, two new active materials have been found. Edgington (15) has markedly reduced the symptoms of Dutch elm disease in inoculated trees with mixed isomers of aminotrichlorophenylacetic acid. When applied in early spring, this preparation modifies the structure of the woody tissues that develop subsequently in a striking way. A layer of dense, starch-filled cells interrupt the normal continuity of the annual ring. This layer apparently acts as a barricade which the pathogen does not penetrate readily.

Smalley (39), working with the related, but somewhat more phytotoxic, 2,3,6-trichlorophenylacetic acid has reported its efficacy in preventing Dutch elm disease. This compound also modifies growth of the plant and is also more effective when applied early in the growing season. Smalley has noted the ready development of tyloses in vessels of treated trees, and has called attention to the possibility of their acting as an internal barricade in a vessel. The tyloses may function in preventing longitudinal invasion of the tree by the pathogen. That tylose and gum formation can act as an effective barrier to invasion of vascular pathogens has been effectively demonstrated by Beckman, Halmos, and Mace (3).

Summary

The presently promising chemotherapeutic agents may be classed as systemic toxicants and compounds that modify the resistance of the plant to infection or invasion. Some systemic bactericides and fungicides are presently in use. Some act directly upon the pathogen. Others are moved in the plant and converted to a toxic form. The use of groups to mask the toxophore so that the compound is selectively toxic to the pathogen has been attempted, and translocation of the introduced compound has been concomitantly improved in some instances. In the wilt diseases, distribution of the chemotherapeutic agent must be restricted to tissues subject to invasion. Cationic toxicants are adsorbed to xylem tissues and sometimes protect these tissues against attack. Adsorption may be controllable so that proper distribution of the chemotherapeutic agent is achieved.

Carbohydrate levels of plant tissues provide a useful index of susceptibility to attack by some pathogens and resistance to attack by others. Some chemotherapeutic agents effectively modify the carbohydrate level in tissues and their resistance to disease. When pectolytic enzymes are adaptive, their formation is usually suppressed by the presence of sugars. In instances where pectolytic enzymes are both adaptive and essential

in pathogenesis, high carbohydrate levels in the host will cause it to be resistant. This is one but probably not the only mechanism by which high carbohydrate levels induce resistance in the host toward low sugar diseases.

Certain unnaturally occurring amino acids increase the resistance of plants to disease. Amino acids that act in this way affect auxin action. The fundamental basis of their activity as chemotherapeutic agents is unknown as yet.

Inhibitors of polyphenol oxidase and precursors of phenolic compounds that function in natural biochemical resistance have exerted moderate activity in preventing *Venturia* infection on apple leaves and *Cladosporium* infection on cucumber seedlings. This approach to chemotherapy is promising.

A variety of compounds that have growth regulating activity increases the resistance of plants to disease. These compounds apparently affect the nature of the pectins in plant tissues, so that they are more resistant to attack by pectolytic fungal enzymes.

These and related compounds also modify the morphology of woody tissues so that vascular wilt fungi do not so readily invade them. Chemotherapy of Dutch elm disease by this means apparently involves barricade formation, either through production of small, dense cells, or tylose formation in vessels, or both.

LITERATURE CITED

1. Banfield, W. M. 1941. Distribution by the sap stream of spores of three fungi that induce vascular wilt diseases of elm. J. Agr. Research 62:637-681.
2. Beckman, C. H. 1958. Growth inhibition as a mechanism in Dutch elm disease therapy. Phytopathology 48:172-176.
3. Beckman, C. H., S. Halmos, and M. E. Mace. 1962. The interaction of host, pathogen, and soil temperature in relation to susceptibility to Fusarium wilt of bananas. Phytopathology 52:134-140.
4. Byrde, R. J. W., and D. Woodcock. 1953. Fungicidal activity and chemical constitution. II. Compounds related to 2,3-dichloro-1,4-naphthaquinone. Ann. Appl. Biol. 40:675-687.
5. Corden, M. E., and A. E. Dimond. 1959. The effect of growth regulating substances on disease resistance and plant growth. Phytopathology 49:68-72.
6. Corden, M. E., and L. V. Edgington. 1960. A calcium requirement for growth regulator induced resistance to Fusarium wilt of tomato. Phytopathology 50:625-626.
7. Crowdy, S. H., J. F. Grove, and D. Pramer. 1954. Systemic distribution of antibiotics and the control of plant disease. Proc. 8th Intern. Botan. Congr., Paris. Sec. 24:88-90.
8. Davis, D., and A. E. Dimond. 1956. Site of disease resistance by plant growth regulators in tomato. Phytopathology 46:551-552.
9. Deverall, B. J., and R. K. S. Wood. 1961. Chocolate spot of beans (Vicia faba L.)–interactions between phenolase of host and pectic enzymes of the pathogen. Ann. Appl. Biol. 49:473-487.

10. Dimond, A. E. 1959. Plant chemotherapy, p. 221-228. *In* C. S. Holton, [ed.], Plant pathology, problems and progress, 1908-1958. Univ. Wisconsin Press, Madison.
11. Dimond, A. E., and D. Davis. 1953. The chemotherapeutic activity of benzothiazole and related compounds for Fusarium wilt of tomato. Phytopathology 43:43-44.
12. Dimond, A. E., D. Davis, R. A. Chapman, and E. M. Stoddard. 1952. Plant chemotherapy as evaluated by the Fusarium wilt assay on tomatoes. Conn. Agr. Expt. Sta. (New Haven), Bull. 557, 82 p.
13. Dimond, A. E., and J. G. Horsfall. 1959. Plant chemotherapy. Ann. Rev. Plant Physiol. 10:257-276.
14. Edgington, L. V. 1962. The relation of fungitoxicity to translocation of N-alkyl quaternary ammonium compounds. Phytopathology 52:923.
15. Edgington, L. V. 1963. A chemical that retards development of Dutch elm disease. Phytopathology 53:349.
16. Edgington, L. V., M. E. Corden, and A. E. Dimond. 1961. The role of pectic substances in chemically induced resistance to Fusarium wilt of tomato. Phytopathology 51:179-182.
17. Edgington, L. V., and A. E. Dimond. 1960. Effect of charge of ionized chemotherapeutants on their translocation through xylem. Phytopathology 50:239-240.
18. Edgington, L. V., and A. E. Dimond. 1960. Controlling translocation of quaternary ammonium chemotherapeutants with cations and solvents. Phytopathology 50:634.
19. Edgington, L. V., and J. C. Walker. 1958. Influence of calcium and boron nutrition on development of Fusarium wilt of tomato. Phytopathology 48:324-326.
20. Forsyth, F. R. 1962. Inhibition by nickel of the respiration and development of established infections on Thatcher wheat caused by Puccinia recondita Rob. ex Dism. Can. J. Botany 40:415-423.
21. Gassner, G., and K. Hassebrauk. 1936. Untersuchungen zur Frage der Getriederostbekämpfung mit chemischen Mitteln. Phytopathol. Z. 9:427-454.
22. Hamilton, J. M., M. Szkolnik, and E. Sondheimer. 1956. Systemic control of cherry leaf spot fungus by foliar sprays of acti-dione derivatives. Science 123:1175-1176.
23. Hardison, J. R. 1963. Commercial control of Puccinia striiformis and other rusts in seed crops of Poa pratensis by nickel fungicides. Phytopathology 53:209-216.
24. Holden, Margaret. 1950. A study of enzymes that can break down tobacco leaf components. 3. Fungal polygalacturonase on leaf fiber. Biochem. J. 47:415-420.
25. Horsfall, J. G., and A. E. Dimond. 1957. Interactions of tissue sugar, growth substances, and disease susceptibility. Z. Pflanzenkrankh. U. Pflanzenschutz. 64:415-421.
26. Horsfall, J. G., and F. L. Howard. 1959. Therapy, p. 563-604. *In* J. G. Horsfall and A. E. Dimond, [ed.], Plant pathology, Vol. 1. Academic Press, New York.
27. Klemmer, H. W., A. J. Riker, and O. N. Allen. 1955. Inhibition of crown gall by selected antibiotics. Phytopathology 45:618-625.
28. Kuć, J. 1957. A biochemical study of the resistance of potato tuber tissue to attack by various fungi. Phytopathology 47:676-680.
29. Kuć, J., R. E. Henze, A. J. Ullstrup, and F. W. Quackenbush. 1956. Chlorogenic and caffeic acids as fungistatic agents produced by potatoes in response to Helminthosporium carbonum. J. Amer. Chem. Soc. 78:3123-3125.
30. Kuć, J., E. B. Williams, and J. R. Shay. 1957. Increase of resistance to apple scab following injection of host with phenylthiourea and D-phenylalanine. Phytopathology 47:21-22.
31. Lemin, A. J., and W. E. Magee. 1957. Degradation of cycloheximide derivatives in plants. Plant Disease Reptr. 41:447-448.
32. Levy, C. C., and M. Zucker. 1960. Cinnamyl and *p*-coumaryl esters as intermediates in the biosynthesis of chlorogenic acid. J. Biol. Chem. 235:2418-2425.

33. Livingston, J. E., and M. T. Hilborn. 1959. The control of plant diseases by chemotherapy. Econ. Botany 13:3-29.
34. McLean, J. G., D. J. LeTourneau, and J. W. Guthrie. 1961. Relation of histochemical tests for phenols to Verticillium wilt resistance of potatoes. Phytopathology 51:84-89.
35. Oort, A. J. P., and O. M. van Andel. 1960. Aspects of chemotherapy. Mededel. Landbouwhogeschool En Opzoekingsstas. Staat Gent 25:981-992.
36. Pramer, D., R. S. Robinson, and R. L. Starkey. 1956. The mode of action of antibiotics in the control of plant disease. Phytopathology 46:341-342.
37. Sijpesteijn, A. Kaars. 1961. New developments in the systemic combat of fungal diseases of plants. Tijdschr. Plantenziekten 67:11-20.
38. Sijpesteijn, A. Kaars, and C. W. Pluijgers. 1962. On the action of phenylthioureas as systemic compounds against fungal diseases of plants. Mededel. Landbouwhogeschool En Opzoekingsstas. Staat Gent 27:1199-1203.
39. Smalley, E. B. 1962. Prevention of Dutch elm disease by treatments with 2,3,6-trichlorophenyl acetic acid. Phytopathology 52:1090-1091.
40. Van Andel, O. M. 1958. Investigations on plant chemotherapy. II. Influence of amino acids on the relation plant-pathogen. Tijdschr. Plantenziekten 64:307-327.
41. Van Andel, O. M. 1962. Growth regulating effects of amino acids and dithiocarbamic acid derivatives and their possible relation with chemotherapeutic activity. Phytopathol. Z. 45:66-80.
42. Zucker, M., and C. C. Levy. 1959. Some factors which affect the synthesis of chlorogenic acid in disks of potato tuber. Plant Physiol. 34:108-112.

Discussion

Cowling: Dr. Dimond has very ably reviewed the three principle modes of action of chemotherapeutants for the control of plant disease. He has indicated the general mechanism of direct action on the pathogen and emphasized several specific methods of enhancing the resistance of the host plant. As discussion leader, I should like to extend briefly our consideration of chemotherapy by direct action by calling attention to specific theoretical mechanisms which may be derived from some of the essential differences between pathogens and higher plants. We will then open the topic for discussion from the floor.

Both in medical and plant chemotherapy, the development of successful therapeutic chemicals has been largely a product of empirical rather than synthetic research approaches. Hopefully, however, our knowledge of the essential metabolic, physiological, and structural differences between pathogens and hosts will enlarge sufficiently to permit a more rational approach in the future.

As Dr. Dimond has indicated, action on a pathogen already well-established within a living host requires a highly selective mode of action. The compound must either be more toxic to the pathogen than to the host or else it must be selectively accumulated to lethal levels in the pathogen. Such selective toxicity or accumulation is made possible by differences in the essential metabolism, structure, or physiological functions of the host and pathogen.

Unfortunately for our purposes in chemotherapy, the broad current of comparative biochemical evidence points to similarity rather than heterogeneity of metabolism in living organisms, particularly in energy-yielding reactions. Among the synthetic activities of host and pathogen, however, there are a variety of unique attributes of pathogens which may provide the key to direct action by chemotherapeutic materials. The proper question to raise here is: What is it that phytopathogenic fungi and bacteria have or do, that higher plants do not have or do? Our list is not long although it surely is incomplete.

Let us first examine the cell walls of pathogens and hosts. The cell walls of fungi characteristically contain chitin. This substance does not occur in higher plants. Thus, chemical agents that degrade chitin or inhibit its synthesis would tend to act specifically on the pathogen. The effect of a chitinase on the chitin of fungi would be analogous to the action of lysozyme on the capsular polysaccharides of bacteria infections in man. The effect of chitinase inhibitors may be analogous with the action of penicillin on bacterial cell-wall synthesis. Actually, the fungitoxicity of griseofulvin has been suggested by Brian (Ann. Botany, 13:59, 1949) to be the result of its interference with chitin synthesis, either by itself acting as a growth regulator, or by interfering with a regulator of chitin synthesis. Stunting, branching, distortion, and other abnormalities of hyphal growth provide evidence of interference with cell-wall synthesis of fungi by griseofulvin. This antibiotic has demonstrated some chemotherapeutic activity (Nature, London, 165:347, 1951).

Many of the polysaccharides in fungi are 1-2, 1-3, and 1-6 linked, whereas those in higher plants, particularly woody plants, usually are 1-4 linked. Selective interference with the synthesis or degradation of these materials also might be helpful. Another difference between the cell walls of higher plants and those of fungi and bacteria is the large amount of proteinaceous material which is associated with the cell wall polysaccharides of the microorganisms. If we knew more about the biochemical mechanisms of synthesis of these cell wall proteins, we might have another potential principle of chemotherapeutic action.

Second, the enzymes polygalacturonase and cellulase are produced in abundance by many phytopathogenic fungi and bacteria. Although they have been reported in higher plants, particularly in germinating seedlings, enzyme levels in higher plants usually are very low. Yesterday, Drs. Byrde and Whitaker discussed the occurrence of natural inhibitors to enzymes of these two types which are produced primarily by pathogenic organisms.

Third, another difference between pathogens and hosts is the fact that a larger proportion of the cells of the pathogen than of the host are undergoing cell division during pathogenesis. Thus, a variety of anti-

mitotic agents and inhibitory analogs of purines and pyrimidines may be applied to infected plants and become involved in so-called lethal syntheses that lead to the production of "ineffective" genetic material in the pathogen. Antimitotic agents that have found use in medical chemotherapy include fluoro- and bromuracil, and folic acid antagonists such as aminopterin. Other antimitotic agents of potential utility are the nitrogen mustards that function as alkylating and mutagenic agents, the lignan podophyllin, chloral hydrate, and mercaptoethanol.

Fourth, the sterol ergosterol is the principal and most abundant compound of its type in fungi; ergosterol is known to occur in only a few green plants (mostly algae) (Ann. Rev. Plant Physiol. 4:383, 1953). Interference with sterol metabolism in fungi holds promise of being an effective basis for chemotherapy.

Fifth, differences in cell membrane permeability between fungus and host cells is well-established, although poorly understood. Spores of many organisms thus selectively accumulate heavy metals, for example. This phenomenon will be discussed by Drs. McCallan, Miller, and Sussman later this afternoon.

The possibility that the membranes of the pathogen can be selectively disrupted and cause injury to fungus pathogens is suggested by the deleterious action of polymyxins and other peptide antibiotics on the permeability of bacterial cell membranes. Comparative studies of the permeability of fungi and host cells may lead to the development of chemotherapeutants based on differential permeability effects.

Sixth, plants have been shown to produce a host of antimicrobial substances (Econ. Botany 13:281, 1959). Such natural products which already exist in plants may in themselves provide nonphytotoxic but antimicrobial substances useful in chemotherapy. Dr. Dimond has already called attention to the possibility of encouraging the synthesis of naturally inhibitory phenolic products in host plants. Another example of this kind, for which preliminary evidence has recently been obtained in our laboratory at Yale, is the increase in amount of pinosylvin which develops in pine stumps treated with borax following thinning of pine plantations. The pinosylvins apparently make the stump a less suitable court of infection for the root decay fungus, *Fomes annosus*.

Seventh, vitamin deficiencies are almost unknown in higher plants but are well established in many phytopathogenic fungi. Advantage might be taken of this difference in development of chemotherapeutants.

Eighth, although they have not yet been characterized structurally, specific reproductive hormones have been demonstrated in some fungi. Identical substances are very likely not to occur in higher plants.

Ninth, differences in later stages of nitrogen metabolism suggest that competitive analog inhibition of vital transformation of nitrogenous materials may offer a key to chemotherapy of fungus diseases.

In concluding our discussion I should like to thank Dr. Dimond for his very fine presentation and to call attention to a rather clever play on words which Dr. Horsfall has used to indicate something of the unique character of The Connecticut Agricultural Experiment Station. "We here are not so much concerned about how to grow plants as we are to learn more about how plants grow." This statement of emphasis on fundamental research applies equally well to the Department of Plant Pathology here at the Station and to the Station as a whole. It would be my hope, and I'm sure that of Dr. Dimond and his department, that this tradition will be maintained and that future progress in chemotherapy will evolve from continuing attention to the fundamental principles of selective action.

Kuć: I am very intrigued by the high and low sugar level relationship to disease resistance. I would like to have both of your comments, if you care to, on the possibility of phenolic compounds (of course, being a phenol man) influencing the availability of sugars. In other words, the sugar level itself may not be the determining factor, but rather a sugar level in relation to the phenolic compounds which may be influencing the availability of the sugar would be important. The reason I bring this to your attention, is the role of phloridzin in inducing artificial diabetes in animals, where we prevent the reabsorption of glucose in the kidney. Consequently, the glucose appears in the urine. Apparently phloridzin has this effect on the absorption of the glucose by living cells. Therefore, phloridzin, and other phenols in plants, may have a similar effect. The balance of phenol and sugar would have a profound influence on the availability of sugar. Therefore, we have high and low sugar diseases.

Dimond: This is, indeed, an intriguing possibility. I think there is very little that I could add to that at the present time. I have already said that I don't believe the simple generalization about sugar levels is fully understood, and to approach the problem in the manner you have suggested may give us a better insight into it.

I would like to comment on a related aspect of this which bears upon a question that Dr. Davis posed to Dr. Mitchell about the possibility of phenolic compounds being introduced into plants to increase their resistance and their translocatability. As we know, the things that are characteristically phloem-transported are frequently related to sugar molecules and their esters. One of the many ways that occurs to me of utilizing the phenolic compounds in a translocatable form would be to introduce a glycoside in the hope that the pathogen might hydrolyze it selectively. This would involve some rather pious hopes that the type of compound could be found which would be unlocked only by the

pathogen and not by the host, but I think it does remain as a possibility to be explored for selective toxicity.

Kelman: I would like to pose a problem for Dr. Dimond. A student who worked with us investigated translocation of cycloheximide in slash pine in relation to control of fusiform rust, one of the major diseases affecting southern pines. This rust is a stem-invading fungus causing a gall to form on the stem of affected trees. Cycloheximide prevented germination of the spore stages of the fusiform rust fungus at very low concentrations. Furthermore, cycloheximide was translocated in slash pine seedlings at concentrations high enough to inhibit a test-assay organism. However, it had no apparent effect on the fungus in the tissue of the infected host. I wonder if Dr. Dimond would like to suggest a possible explanation for this type of situation?

Dimond: Of course I cannot explain the situation, but the possibility that occurs to me is that while the compound may be transported readily in host tissue through the arteries, as you might think of them, the dispersal from the artery to the site of infection may be a very slow process. This is more likely to involve diffusion types of transport than main stream flow, and it is entirely conceivable to me that this would limit the concentration available to the pathogen so that it would always be below a toxic level. Is this a possibility?

Kelman: Yes, although in the tissue infected by the pathogen there is apparently not much interference with downward translocation of food. The cycloheximide can be detected in the phloem and xylem tissue.

Dimond: I am not questioning this. These I consider highways.

Kelman: I think it is true. It is very difficult to detect the actual level of cycloheximide in infected tissue.

Dimond: I can illustrate this in a very homely way. I used to live in the Town of Cheshire, a few miles north of here. I had to buy bottled gas, although a few hundred yards away from me ran a high pressure gas line all the way from Texas. This was a matter of the inefficiency of diffusion across a few hundred feet, and so I had to buy my gas in bottles.

Kelman: I think that is a very good simile.

Zentmyer: I would like to make a comment on ethionine in relation to Dr. Dimond's reference to the work in Holland, I believe, on the greater toxicity of the D-forms of various amino acids as compared to the L-forms. In our work (with W. Moje and J. B. Kendrick, Jr.) ethionine, as a systemic material and also as a fungicide, has shown much more effectiveness in the L-form than in the D-form. For instance, the ED_{50} is 25 ppm for the L-form in PDA agar as contrasted to over 1000 ppm

of the D-form. For the DL-form the ED_{50} was approximately 50 ppm. This material has shown some quite interesting reactions in relation to the structure of the L and D forms, and I wonder if perhaps the differences here might not be related to the differences in mechanisms of action, either on the fungus or the host? For instance, we have good evidence now that ethionine is acting as an antimetabolite of methionine, which has a very similar structure with a methyl group instead of an ethyl group. Would you care to make any comment on that?

Dimond: I do not know that my comment will be helpful, but let us assume that *Phytophthora* predominantly depends for its pathogenesis on pectic enzymes. Then apart from the fungitoxicity of ethionine, one of its main effects would be upon affecting the composition of the pectins of the host. Methionine is a methyl donor and, if ethionine acts as a competitive inhibitor for methionine, then the pectin composition of the host might be considerably modified. In this reaction, one would expect L-ethionine to be more reactive than D-ethionine.

Cruickshank: I have been interested in Dr. Cowling's comments in connection with the change of disease reaction by the unnatural isomers of amino acids; nickel chloride, and borax. We have found in Canberra that the application of $HgCl_2$ and $CuCl_2$ at physiologic concentrations to the endocarp of pea pods induces the formation of pisatin at concentrations adequate to inhibit some pathogens of peas *in vitro*. It appears that the introduction of such compounds as heavy metal ions induces phytoalexin formation and that this might be the reason for change in disease reaction. I would be pleased to have your comments.

Cowling: It is difficult to envision exactly what might be the relationship between something like borax and pinosylvin synthesis or something like the metal ions you spoke of, and other organic substances which are toxic. The same sort of effect which I spoke about has been reported by Jorgensen to result from the addition of substrate amounts of acetate and ethanol, also in nontoxic concentrations.

Cruickshank: In this regard we find that we can induce the formation of pisatin in pea pods by a large number of chemicals, too, although I mentioned the materials which gave us the most spectacular results. It does appear that by inducing what very well may be called a servo function, one triggers off a mechanism which is not very specific as far as the final part is concerned.

Chemically Induced Rust Resistance

B. J. Samborski

Canada Department of Agriculture Research Station
Winnipeg, Manitoba

Rust diseases of field crops are a major problem and control measures are still almost entirely dependent on the use of resistant varieties. This dependence on breeding for resistance stimulated genetic studies on host plants and pathogens, and very substantial progress has been made. The ultimate objective of studies on the physiology and biochemistry of parasitism is surely to determine the chemical nature of the genetic interaction which leads to resistance or susceptibility. Some progress has been made but the most optimistic assessment must be that a background of necessary information is being obtained which will lead to definitive experiments in the future (2, 53).

The majority of workers have studied the physiology of disease development. Although comparisons may be drawn between diseased, resistant, and susceptible hosts, it is not certain whether such data will permit extrapolation to the initial interactions leading to resistance or susceptibility. Müller's (35) direct approach has not been used by rust workers but appears to warrant serious consideration. This paper will deal largely with physical and chemical treatments which have been found to alter rust resistance. Before proceeding with this analysis, some genetic characteristics of host-pathogen interactions will be considered since biochemical interpretations must correspond to the observed genetic phenomena.

The terms pathogenicity and virulence will be used according to the definitions proposed by Miles (33) and subsequently adopted by Day (10). Pathogenicity is the general attribute of a pathogenic organism while virulence is the pathogenicity of a given stable, homogeneous strain of a species in relation to a particular host genotype. A similar usage of the terms has been described by Loegering and Powers (31). This is a useful distinction since it immediately implies that resistance to an avirulent strain of a pathogen is associated with the action of specific genes acting in a background which is favorable for rust development. It also implies that pathogenicity and virulence may be determined by quite different biochemical mechanisms.

With respect to rust diseases, resistance is not necessarily synonymous with the absence of disease and a resistant plant may suffer considerable damage if inoculum is plentiful. Resistance is obtained when reproduction of the pathogen is curtailed and all host genes which reduce the rate of reproduction of the pathogen should be considered as genes for resistance.

Flor's (14) formulation of the gene-for-gene hypothesis and his emphasis on studying the pathogen with host material containing single genes for rust resistance stimulated similar studies by other workers. Back-cross lines, differing essentially in single genes for rust resistance, are now being used extensively to study host-pathogen interactions (5, 19 24, 31). These studies suggest that the resistant reaction type is a function of the host gene. Different strains of rust may be avirulent or virulent. If the strain is avirulent, then the interaction with a host gene for resistance results in the reaction type which is characteristic of that gene for resistance. If it is virulent, a susceptible reaction results. It is on this precise gene-for-gene relationship that biochemical considerations should be centered.

While it is true that any one gene for resistance conditions a characteristic reaction type, it must be emphasized that the corresponding gene for avirulence is always involved in the interaction. Therefore, it is just as valid to state that interaction of a resistant host with an avirulent pathogen results in the reaction type which is characteristic of that gene for avirulence. At present it seems necessary to fall back on the rather meaningless statement that reaction type results from the interaction of a host and a pathogen. This is perfectly true but it is not particularly helpful in analyzing disease symptoms in terms of the relative contribution of each component of the interaction.

Genes for resistance, or for virulence or avirulence, are not always completely dominant or recessive, and interactions where the host or pathogen are heterozygous with respect to corresponding genes may produce an atypical reaction type. This is probably a quantitative effect rather than a qualitative difference stemming from formation of hybrid protein. Studies on enzyme-activity levels in inbred lines of corn and hybrids of such lines gave no indication of different or more efficient enzyme systems in the hybrid (21). Similarly, it appears that hybrid protein does not occur in microorganisms (56). This could be expected from current concepts of protein synthesis in which the template is a short-lived messenger RNA produced under genetic control as required for synthesis of specific proteins (38). Since messenger RNA is a copy of a gene, that is, of a DNA chain coding for a given protein, it is reasonable to expect that two messenger RNA's and consequently two independent proteins would be produced in heterozygotes of diploid organisms.

A hypothetical model of host-parasite interactions could assist in the assessment of the data on induced changes in rust resistance. However, a variety of models can be readily devised and the purely speculative nature of any one model should be clearly recognized. The possible

chemical nature of the interactions will not be considered, but it can be assumed that resistance results from the conversion of an innocuous compound to one which is toxic, to one or both members of the host-pathogen complex. Since virulence is usually a recessive character, it can also be assumed that the innocuous compound (precursor) is secreted by avirulent strains and not by virulent strains of the pathogen. This model can be simply illustrated for a single gene interaction.

Pathogen genotype	Host genotype rr	Host genotype RR
VV	Susceptible. Precursor secreted by pathogen. No conversion to toxin by host gene.	Resistant. Precursor secreted by pathogen and converted to toxin by host gene.
vv	Susceptible. No precursor secreted by the pathogen.	Susceptible. No precursor secreted by the pathogen.

However, a system which invokes a direct precursor to toxin conversion would require a different toxin for each distinct gene interaction. While this is possible, it makes the model distinctly unattractive. If a toxin is implicated it seems necessary to postulate that it arises as a side reaction following a metabolic derangement in the incompatable host-parasite interaction. The same toxic compound could then result from a variety of biochemical lesions. It is obvious that the identification of such a toxin would provide little, if any, information on the action of the specific genes which determine the interaction. The evidence for induced toxins as a result of incompatable host-pathogen interactions has been recently reviewed by Müller (36) but little is known about their origin. In wheat, it has been shown that hydrolysis of the glucoside 4-0-glucosyl-2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one yields a phytotoxic aglucone, 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (12). However, there is no convincing evidence that rust resistance is directly related to this reaction.

It seems best to assume that the genes for resistance and virulence or avirulence control the production or activity of particular enzymes. These enzymes could be preformed or induced. Indeed, Lindegren (30) has proposed that all gene-systems are exclusively adaptive.

A model of host-pathogen interactions should also account for the different reaction types which are obtained. Such reaction types may merely reflect different rates of interlocking enzyme systems or the host gene conditioning a particular reaction type may be controlled by a suppressor gene. Following infection with an avirulent strain of the pathogen, the interaction would initially be of a compatable type since the R (resistance gene) gene would not function. However, profound

metabolic changes occur in rust-infected cells (1, 27, 52). At some level of altered host metabolism, the suppressor gene may no longer function; the R gene would then become active and would prevent further development of the pathogen. This unmasking of an R gene at a certain level of metabolic change, and consequently at a certain stage of development of the pathogen, could explain the consistency of the observed reaction types. The R gene conditioning an immune reaction would be immediately operative, or if a suppressor gene was present, its controlling action could be removed by much smaller changes in host metabolism.

It is of course necessary to postulate that such suppressors are present in all varieties of the host and to give them a pleiotropic action extending beyond their effect on R genes. Furthermore, the occurrence of galls and witches' brooms indicates the extent to which the regulatory mechanisms of host cells can be changed by disease-inciting organisms.

The occurrence of genes for mature plant resistance suggests that such suppressor or regulatory effects may be operative in host plants. Seedling plants of some wheat varieties may be completely susceptible while mature plants are resistant. Since the R genes are obviously present in seedlings, it is possible that their activity is suppressed until the plants reach a certain stage of development.

Finally, it is possible that any massive change in the internal environment wherein the genes operate may circumvent or render them inactive. Such massive changes are usually associated with treatments which alter a resistant interaction to one which is susceptible.

A good example of induced susceptibility to rust is obtained when infected wheat leaves are detached and kept on water or sugar solutions (42). Marked proteolysis occurs and soluble nitrogenous compounds and carbohydrates accumulate in these leaves. The effect of detachment on rust reaction is quite unspecific and only certain immune reactions remain unchanged (17). Flangas and Dickson (13) suggested that the effect of detachment on resistance might stem from the inability of such leaves to form induced enzymes which are necessary for rust resistance. Detached resistant wheat leaves do not become susceptible if they are maintained on solutions of benzimidazole or kinetin at concentrations which have no effect on spore germination or on development of rust on detached leaves of susceptible wheats (17, 41, 42, 54). This type of induced resistance apparently operates by permitting the normal activities of resistance genes. The leaves appear to maintain their normal metabolic state and presumably synthesis of an induced enzyme could occur (41, 42, 55). The effect of benzimidazole or kinetin can be nullified by an exogenous supply of glucose (42, 54) or various analogues of benzimidazole (54, 55). These latter effects can in turn be nullified by concentrations of cobalt which alone do not affect rust development. It

has been suggested that benzimidazole, or a derivative of it, and cobalt may form a vitamin B_{12}-like factor which is required by the detached leaf to maintain the normal metabolic activities (54). However, the mode of action of benzimidazole in detached leaves has not been determined.

An interaction between kinetin and indole acetic acid (IAA) has been demonstrated in other systems (34), but its significance if any for rust development is unknown. Susceptible wheat plants fed continuously with IAA remained susceptible although characteristic morphological changes occurred in the treated plants (46). Similar treatment of moderately resistant plants induced a higher level of resistance. This was probably an indirect effect, since the IAA-treated plants were pale green and reduced carbohydrate levels would limit rust development. In contrast, resistant wheat plants become highly susceptible after treatment with maleic hydrazide, while the reaction of susceptible plants remained unchanged (46). As with detached leaves, profound metabolic changes occur in maleic hydrazide-treated leaves, characterized by marked increases in amino acids and carbohydrates (47). The effect of maleic hydrazide on rust resistance is also quite unspecific with respect to different host-pathogen combinations. It is probable that induced susceptibility in detached leaves and maleic hydrazide-treated plants results from similar biochemical changes in host metabolism.

Induced rust resistance has been observed with a number of compounds classed as metabolites and antimetabolities. Susceptible host-pathogen interactions were used in all these studies, and it is not known whether there is any relationship between these observations and gene-conditioned rust resistance. A treatment may mimic the chemical interactions which occur between a resistant host and an avirulent pathogen. Information about the nature of this gene conditioned chemical interaction would greatly facilitate the assessment of studies on induced rust resistance. However, the information derived from studies on chemically induced resistance may indicate metabolic areas in the host-pathogen complex which are vulnerable to chemical attack.

Hassebrauk (22) and Hotson (25) showed that rust development was inhibited by sulphonamides; this inhibition could be reversed by para-aminobenzoic acid and folic acid. Oxythiamine, an antithiamine, effectively controlled rust on detached wheat leaves and its action was reversed by thiamine (43). No particular evaluation of these observations is possible apart from the obvious one that the availability of the co-enzymes concerned is essential for rust development.

A number of workers have observed a marked effect of exogenously supplied amino acids on disease susceptibility of host plants. Van Andel (3) found that amino acids active against *Cladosporium cucumerinum* Ell. et Artl. were compounds that do not occur normally in plants.

D-isomers of serine, threonine, and histidine were also effective while the L-forms were inactive. Comparable concentrations of DL-serine, DL-threonine, and DL-histidine prevent the development of leaf rust and stem rust on detached wheat leaves (43). Van Andel (3) suggested that all active compounds might act as antimetabolites in her system. Similarly, the D-isomer of phenylalanine reduced the susceptibility of apple leaves to *Venturia inaequalis* (Cke.) Wint., while the L-isomer was ineffective (28). A number of amino acids which controlled *Aphanomyces* root rot of peas could also act as antimetabolites (39, 40). However, wheat leaves supplied with L-methionine were resistant to rust (43) and L-methionine was more effective than the D-form in reducing pathogenesis of *Aphanomyces euteiches* Drechs, on peas (40).

The majority of the compounds tested were nonfungitoxic and presumably act by modifying host metabolism. Many of the compounds tested by Van Andel interfered with auxin action (4). Papavizas and Davey emphasized the importance of the presence of methyl groups in conjunction with amino groups in compounds which exert an effect on symptom development (40). They suggested that the ability of compounds to enter into transmethylation reactions leading to pectin synthesis might be related to their effects on the *Aphanomyces* root rot disease of peas. Corden and Dimond (6) have attributed the induction of wilt resistance by growth regulators to demethylation of plant pectins and formation of rigid cross-linkages, principally as calcium salts. The salt bridges thus formed would render the pectic substances resistant to attack by fungal polygalacturonase.

It is not known whether altering the chemical constitution of plant cell walls would influence rust development, but cell wall composition cannot be the determining factor in rust resistance to specific strains of the pathogen. The majority of amino compounds active in inducing resistance to rust probably act as amino acid analogues. It is not certain whether their effect comes from a direct action on the pathogen or by altering host metabolism. Inhibition by the amino acid analogues, canavanine, para-fluorophenylalanine, and ethionine was reversed by the corresponding amino acids and, less effectively, by glycine. Phenylsydnones may act in a similar manner since they are structural analogues of phenylalanine and glycine (8). Such analogues have diverse effects in living systems and critical studies on their action in the rust-host system have not been carried out.

The simplest explanation would assume inactivation of enzyme activity following incorporation into host, or pathogen protein, or both. However, enzyme activity might only be affected if the analogue was incorporated into an active site. To elaborate, incorporation of canavanine could be deleterious to those enzymes in which arginine participates in the active site.

An interesting action of analogues have been described by Stekol et al. (51). S-adenosyl derivatives of ethionine and methionine are formed by the methionine-activating enzyme of rat liver and yeast to approximately the same extent. The methionine derivative is utlilized more rapidly than the ethionine derivative. They suggested that the formation of S-adenosyl-L-ethionine may be considered as an ATP-trapping action that prevents the reutilization of the adenosine moiety. Such ATP-trapping actions could have profound effects on rust development since synthetic reactions would be depressed.

A number of carbohydrates, including xylose, sorbose, and sugar alcohols, inhibit rust development on detached leaves (43, 48, 50), presumably through an effect on the metabolism of endogenous carbohydrate. Although the precise mode of action of these compounds on the host-pathogen system is not known, dulcitol inhibits the metabolism of glucose in wheat leaves (45) and L-sorbose-1-phosphate is a strong, noncompetitive inhibitor of brain and tumor hexokinase (29). Daley et al. (7) observed sugar alcohol formation in rust-infected leaves and suggested that formation of sugar alcohols would act as a metabolic sink to maintain low TPNH/TPN ratios during high rates of respiration via the pentose pathway. An exogenous supply of sugar alcohol would act reversibly to inhibit oxidative pentose metabolism and inhibit rust development.

Fuchs and his school at Göttingen have studied the effect of semicarbazide and thiosemicarbazide on stem rust of wheat (18, 49). Treated leaves were resistant to rust and were characterized by increased levels of amino acids and decreased levels of sugars. Similar results were obtained with the *Brassica-Peronospora* system (23, 37). The metabolic changes in treated plants could result, at least in part, from the effects of these carbonyl reagents on pyridoxal phosphate. Semicarbazide and thiosemicarbazide did not inhibit spore germination at concentrations that induced resistance to stem rust. It would be desirable to have further information on their ability to enter rust spores and their effect on spore metabolism.

A few antibiotics have been studied as rust-control chemicals, but have so far contributed little to our understanding of host-parasite interactions (11). Recent reports on a crude preparation from *Streptomyces* sp. are interesting but the active compound has not been isolated and its chemical nature and mode of action are not known (9, 20). A similar paucity of information exists regarding the activity of formulations which contain nickel ions. Nickel is a potent eradicant of rust infections (15, 16, 26) and the organism appears to be particularly susceptible to nickel poisoning. Fundamental studies on the action of nickel would be most desirable.

To conclude, it is obvious that only broad generalizations can be deduced from the studies on chemically induced rust resistance. However, it is worth noting that a number of compounds that induce resistance to rust probably act by limiting the availability of essential coenzymes. Whether such effects are related to genetically conditioned resistance must await the biochemical determination of gene action in interactions leading to resistance or susceptibility. There is an urgent need for such studies. Elucidation of the biochemistry involved in this interaction should provide a powerful stimulus for devising a rational chemotherapy of rust diseases. However, it should be noted that resistance to rust may involve the death of host tissue. If this means that a phytotoxic compound is formed, this principle would be useful in chemotherapy only if it is the pathogen that either converts an innocuous compound to its toxic form or induces the host plant to carry out this reaction.

LITERATURE CITED

1. Allen, P. J. 1959. Metabolic considerations of obligate parasitism, p. 119-129. *In* C. S. Holton, [ed.], Plant pathology, problems and progress. 1908-1958. Univ. of Wisconsin Press, Madison.
2. Allen, P. J. 1959. Physiology and biochemistry of defense, p. 435-467. *In* J. G. Horsfall and A. E. Dimond, [ed.], Plant pathology Vol. 1. Academic Press, New York.
3. Andel, O. M. van. 1958. Investigations on plant chemotherapy. II. Influence of amino acids on the relation plant-pathogen. Tijdschr. Plantenziekten 64:307-327.
4. Andel, O. M. van. 1962. Growth regulating effects of amino acids and dithiocarbamic acid derivatives and their possible relation with chemotherapeutic activity. Phytopathol. Z. 45:66-80.
5. Anderson, R. G. 1961. The inheritance of leaf rust resistance in seven varieties of common wheat. Can. J. Plant Sci. 41:342-359.
6. Corden, M. E., and A. E. Dimond. 1959. The effect of growth-regulating substances on disease resistance and plant growth. Phytopathology 49:68-72.
7. Daly, J. M., R. E. Inman, and A. Livne. 1962. Carbohydrate metabolism in higher plant tissues infected with obligate parasites. Plant Physiol. 37:531-538.
8. Davis, D., H. J. Becker, and E. F. Rogers. 1959. The chemotherapy of wheat and bean rust diseases with sydnones. Phytopathology 49:821-823.
9. Davis, D., L. Chaiet, J. W. Rothrock, J. Deak, S. Halmos, and J. D. Garber. 1960. Chemotherapy of cereal rust with a new antibiotic. Phytopathology 50:841-843.
10. Day, P. R. 1960. Variation in phytopathogenic fungi. Ann. Rev. Microbiol. 14:1-16.
11. Dickson, J. G. 1959. Chemical control of cereal rusts. Botan. Rev. 25:486-513.
12. Elnaghy, M. A., and P. Linko. 1962 The role of 4-0-glucosyl-2, 4-dihydroxy-7-methoxy-1, 4-benzoxazin-3-one in resistance of wheat to stem rust. Physiol. Plantarum 15:764-771.
13. Flangas, A. L., and J. G. Dickson. 1961. Complementary genetic control of differential compatability in rusts. Quart. Rev. Biol. 36:254-272.
14. Flor, H. H. 1956. The complementary genic systems in flax and flax rust. Advances in Genet. 8:29-54.
15. Forsyth, F. R., and B. Peturson. 1959. Chemical control of cereal rusts. IV.

The influence of nickel compounds on wheat, oat and sunflower rusts in the greenhouse. Phytopathology 49:1-3.
16. Forsyth, F. R., and B. Peturson. 1960. Control of leaf and stem rust of wheat by zineb and inorganic nickel salts. Plant Disease Reptr. 44:208-211.
17. Forsyth, F. R., and D. J. Samborski. 1958. The effect of various methods of breaking resistance on stem rust reaction and content of soluble carbohydrate and nitrogen in wheat leaves. Can. J. Botany. 36:717-723.
18. Fuchs, W. H., and R. Bauermeister. 1958. Uber die Wirkung von Thiosemikarbazid auf die Entwicklung von *Puccinia graminis tritici* auf Weizen. Naturwissenschaften 14:343-344.
19. Green, G. J., D. R. Knott, I. A. Watson, and A. T. Pugsley. Seedling reactions to stem rust of lines of Marquis wheat with substituted genes for rust resistance. Can. J. Plant Sci. 40:524-538.
20. Hagborg, W. A. F., J. F. T. Spencer, and W. S. Chelack. 1961. Antibiotic P-9 in the control of cereal rusts. Can. J. Botany 39:1725-1732.
21. Hageman, R. H., J. F. Zieserl, and E. R. Leng. 1963. Levels of nitrate reductase activity in inbred lines and F_1 hybrids in maize. Nature (London) 197:263-265.
22. Hassebrauk, K. 1952. Untersuchungen uber die Einwirkung von sulfonamiden und sulfonen auf Getreideroste. III. Untersuchungen uber den Wirkungsmechanisms von Sulfonamiden und sulfonen. Phytopathol. Z. 19:56-78.
23. Heitefuss, R., and W. H. Fuchs. 1960. Untersuchungen zur pathologischen Physiologie von *Peronospora brassicae* (Gaum.) auf *Brassica cleracea*. Phytopathol. Z. 37:348-378.
24. Hooker, A. L., and W. A. Russell. 1962. Inheritance of resistance to Puccinia sorghi in six corn inbred lines. Phytopathology 52:122-128.
25. Hotson, H. H. 1952. The inhibition of wheat stem rust by sulfadiazine and the reversal of this effect by para-amino benzoic and folic acids. Phytopathology 42:11.
26. Keil, H. L., H. P. Frohlich, and J. O. Van Hook. 1958. Chemical control of cereal rusts. I. Protective and eradicative control of rye leaf rust in the greenhouse with various chemical compounds. Phytopathology 48:652-655.
27. Király, Z., and G. L. Farkas. 1959. Biochemical trends in plant pathology. Phytopathol. Z. 34:341-364.
28. Kuć, J., E. Barnes, A. Daftsios, and E. B. Williams. 1959. The effect of amino acids on susceptibility of apple varieties to scab. Phytopathology 49:313-315.
29. Lardy, H. A., V. D. Wiebelhaus, and K. M. Mann. 1950. The mechanism by which glyceraldehyde inhibits glycolysis. J. Biol. Chem. 187:325-337.
30. Lindegren, C. C. 1963. Viruses, genes and cistrons. Nature (London) 197:566-568.
31. Loegering, W. Q., and H. R. Powers, Jr. 1962. Inheritance of pathogenicity in a cross of physiological races 111 and 36 of Puccinia graminis f. sp. tritici. Phytopathology 52:547-554.
32. Matthews, R. E. F. 1960. Virus inactivation in vitro and in vivo, p. 461-506. *In* J. G. Horsfall and A. E. Dimond, [ed.], Plant pathology Vol. 2. Academic Press, New York.
33. Miles, A. A. 1955. The meaning of pathogenicity, p. 1-16. *In* J. W. Howie and A. T. O'Hea, [ed.], Mechanisns of microbial pathogenicity. Cambridge University Press, Cambridge.
34. Miller, C. O. 1961. Kinetin and related compounds in plant growth. Ann. Rev. Plant Physiol. 12:395-408.
35. Müller, K. O. 1958. Studies in Phytoalexins. I. The formation and the immunological significance of Phytoalexin produced by *Phaseolus vulgaris* in response to infections with *Sclerotinia fructicola* and *Phytophthora infestans*. Australian J. Biol. Sci. 11:275-300.
36. Müller, K. O. 1959. Hypersensitivity, p. 469-519. *In* J. G. Horsfall and A. E. Dimond, [ed.], Plant pathology Vol. 1. Academic Press, New York.
37. Nielsen, J. 1960. Weitere Untersuchungen zur Pathologischen Physiologie von *Peronospora parasitica* auf *Brassica oleracea*. Phytopathol. Z. 40:117-142.

38. Ochoa, S. 1962. Enzymatic mechanisms in the transmission of genetic information, p. 153-166. *In* M. Kasha and B. Pullman, [ed.], Horizons in biochemistry. Academic Press, New York.
39. Papavizas, G. C., and C. B. Davey. 1963. Effect of sulfur-containing amino compounds and related substances on Aphanomyces root rot of peas. Phytopathology 53:109-115.
40. Papavizas, G. C., and C. B. Davey. 1963. Effect of amino compounds and related substances lacking sulfur on Aphanomyces root rot of peas. Phytopathology 53:116-122.
41. Person, C., D. J. Samborski, and F. R. Forsyth. 1958. Effect of benzimidazole on detached wheat leaves. Nature (London) 180:1294-1295.
42. Samborski, D. J., F. R. Forsyth, and C. Person. 1958. Metabolic changes in detached wheat leaves floated on benzimidazole and the effect of these changes on rust reaction. Can. J. Botany 36:591-601.
43. Samborski, D. J., and F. R. Forsyth. 1960. Inhibition of rust development on detached wheat leaves by metabolites, antimetabolites, and enzyme poisons. Can. J. Botany 38:467-476.
44. Samborski, D. J., C. Person, and F. R. Forsyth. 1960. Differential effects of maleic hydrazide on the growth of leaf and stem rusts of wheat. Can. J. Botany 38:1-7.
45. Samborski, D. J., R. Rohringer, and C. Person. 1961. Effect of rust-inhibiting compounds on the metabolism of wheat leaves. Can. J. Botany 39:1019-1027.
46. Samborski, D. J., and M. Shaw. 1957. The physiology of host-parasite relations. IV. The effect of maleic hydrazide and indoleacetic acid on the rust resistance of Khapli and Little Club Wheats. Can. J. Botany 35:449-455.
47. Samborski, D. J., and M. Shaw. 1957. The physiology of host-parasite relations. IVa. The effect of maleic hydrazide on the carbohydrate, nitrogen and free amino acid content of the first leaf of Khapli wheat. Can. J. Botany 35: 457-461.
48. Sempio, C. 1942. Influenza di alcuni glucidi isomeri sullo suiluppo della ruggine del fagiola di altre malattie fungine. Riv. Biol. (Perugia) 34:52-56.
49. Siebert, R. 1961. Biochemische Untersuchungen zum Wirt-Parasit-Verhaltnis am Biespiel von *Puccinia graminis tritici*. Phytopathol. Z. 40:221-244.
50. Silverman, W. 1960. The development of stem rust on wheat leaves treated with some sugars and sugar alcohols. Phytopathology 50:114-119.
51. Stekol, J. A., E. Bedrak, V. Mody, N. Burnette, and C. Somerville. Inhibition of the synthesis of diphosphopyridine nucleotide by ethionine administration in the liver of rats and mice. J. Biol. Chem. 238:469-473.
52. Uritani, I., and T. Akazawa. 1959. Alteration of the respiratory pattern in infected plants, p. 349-390. *In* J. G. Horsfall and A. E. Dimond, [ed.], Plant pathology Vol. 1. Academic Press, New York.
53. Walker, J. C., and M. A. Stahmann. 1955. Chemical nature of disease resistance in plants. Ann. Rev. Plant Physiol. 6:351-366.
54. Wang, D. 1959. Effect of benzimidazole, dimethylbenzimidazole, glucose and metal ions on the development of *Puccinia graminis tritici* on detached leaves of Khapli wheat. Can. J. Botany 37:239-244.
55. Wang, D., M.S.H. Hao, and E. R. Waygood. 1961. Effect of benzimidazole analogues on stem rust and chlorophyll metabolism. Can. J. Botany 39:1029-1036.
56. Yanofsky, C., and P. St. Laurence. 1960. Gene action. Ann. Rev. Microbiol. 14:311-340.

Discussion

Daly: I first would like to acknowledge, for everyone present, the excellent summation that Dr. Samborski has made. In a research area that frequently is overly optimistic, he has given us a critical evaluation of the most important aspects. In reviewing his major arguments, there are two features that emerge. First, the major emphasis on rust control by chemicals has centered on the limited part of the life cycle of rust fungi which is concerned chiefly with mycelial growth. Second, at present there are few principles upon which we can hang our chemotherapeutic hats.

Although it is probable that the latter failure will be rectified with continued research, it also is possible that effective chemical control may be quite difficult if it is sought only at this particular stage in the life cycle of rust fungi. Control at this stage may require a degree of selective toxicity not easily obtained. All available evidence suggests an extremely close association between host and parasite. As Dr. Samborski suggests, there is a real possibility that effective chemical treatments damaging to the rust may be sufficiently harmful to the host so that, although economic control is possible (half a loaf is better than none), full yield potentials may not be realized.

If we examine the entire life cycle of rusts, particularly those that attack cereals, there are several soft spots that as yet have not been exploited. Some of these soft spots represent stages for which there is no ready analogy in the host. They appear to be unique to the parasite, and perhaps are more amenable to control by chemicals that may have no comparable effects on the biochemistry or physiology of the host.

The initial events of penetration consist of an orderly sequence of organelle differentiation: appressorium, penetration peg through stomates, substomatal vesicle, and finally infection hyphae which establish haustoria. Until recently, attempts to reproduce the sequence, even on surfaces which simulated the leaf surface, met with only rare success. Consequently, it was thought that differentiation was a specific response to the physical contours and convolutions of the host surface. Paul Allan of Wisconsin made the significant finding that all these structures could be induced in sequence on liquid surfaces by addition of small amounts of lipid-like chemicals extracted from several natural products such as cotton. If differentiation is under chemical control, there is a reasonable expectation that chemicals can be found which abort or repress any or all of the organelles which appear essential to infection. This approach is different in principle to inhibition of spore germination *per se*, which until now has been the chief means of rapid screening. Further, the fact that chemicals inducing normal organelle differentiation on the host may be part of the cuticular layer opens the way to investigations directed

toward incorporation of anti-differentiation agents into the cuticle. Another possible use of such information is the application of natural or synthetic chemicals that induce differentiation of appressoria or penetration pegs before the stomate is reached by the fungus. In either event (inhibition or premature formation) penetration may be substantially reduced or abolished.

Even after penetration there are possibilities as yet unexplored. This morning we had discussion centered about the role of translocation in the effective distribution of chemotherapeutants. I would like to show some results obtained by Mr. Avinoam Livne in his recent Ph. D. thesis. His results suggest that translocation patterns are disturbed markedly by disease, and that perhaps the economic losses accompanying infection might be reduced by application of chemicals that maintain normal translocation patterns in diseased plants.

Mr. Livne was concerned primarily with the balance of carbon in diseased tissue. Infection by rust fungi increases the total dry weight even though loss of carbon by respiration is enhanced. He carefully examined fixation of $C^{14}0_2$ by photosynthesis of both healthy and infected primary leaves of bean, and found that there was a reduction in rate of fixation once symptoms were evident. During periods of maximum dry weight gains and respiration rates, photosynthesis declined to rates one-third those of healthy tissue. Suspecting that there might be a compensation for the reduction, he found that uninfected trifoliate tissues of diseased bean plants had rates of photosynthesis approximately twice as high as corresponding leaves from healthy plants.

Mr. Livne then checked translocation patterns in order to determine if the stimulated rates of photosynthesis might be functionally significant. In brief, he was able to demonstrate quite conclusively that infected leaves not only reduce the rate of photosynthesis, but that over 97 per cent of the $C^{14}O_2$ fixed remained in the leaf and was not exported to growing tissues. In contrast, comparable leaves from healthy plants furnished considerable materials for growth and development of other organs. Healthy leaves on infected plants were not only stimulated to fix more CO_2 but exported much more. Instead of export to new growth, however, photosynthate is translocated to a considerable extent to infected primary leaves, which on healthy plants receive practically no support from photosynthesis in other organs.

In discussing this phenomenon, Mr. Livne has raised the question as to whether *tolerance* to disease might involve mechanisms whereby infected plants are better able to continue more normal patterns of translocation than are varieties which suffered substantial economic loss even though the infection levels are the same. If such is the situation, can chemicals be applied which assist normal translocation when applied at the critical stage when grain or fruit are developing? Some recent work on kinetin

and benzimidazole by Mothes which was mentioned earlier suggest this might be possible. In this situation, altered translocation is not only a means of distributing chemotherapeutants but a process that may be altered by chemotherapeutants leading to induced tolerance to disease.

In the course of this work, it was found that carbohydrates, previously detected by feeding radioactive glucose, were formed from products of photosynthesis in diseased tissue. In normal leaves, approximately 80 per cent of the radioactivity in the carbohydrate fraction was found in glucose, fructose, and sucrose. In diseased tissue, nearly 70 per cent was found in carbohydrates which appear to be trehalose, a hexitol or octitol, and a pentitol. We have been able to demonstrate an enzyme system in diseased, but not healthy, tissue which will form the latter material from ribose-5-phosphate. Perhaps this type of system will be amenable to selective inhibition of fungal development although at present the functional significance of pentitols is not known.

Finally, little attention has been paid to the inhibition or alteration of spore production. In addition to antisporulants there is the possibility of using self-inhibitors which have been demonstrated in rusts. One highly effective way might be to induce chemically the transformation from uredia to telia in the life cycle of rust. In the Great Plains, several generations of the repeating uredial stage are required in order to create severe epidemics. During final maturation of cereals, there is a conversion to the overwintering telial stage. The factors responsible for the conversion are not known but certainly deserve our attention. If the non-repeating telial conditions could be induced by chemicals, we would have a highly effective method of rapidly chopping down the spread and continued development of rust.

All of the foregoing is speculation. The feasibility of chemical control by methods such as those outlined by Dr. Samborski, or by those given just now, depends on much additional information. In throwing the subject open to discussion, both Dr. Samborski and myself would like to emphasize how little is known with respect to basic biochemistry and physiology of rust diseases, especially information of potential use in control by chemicals.

Zelitch: It seems to me, as something of an outsider, that there is another possible method of controlling organisms like the rusts which has not been discussed today. This bears on the problem that was raised of the nature of the penetration of the pathogen into the leaf tissue. Rusts and many other pathogens penetrate leaf tissue through the stomata of the leaves. In our laboratory we have recently demonstrated the preventing of stomatal opening by a large number of common enzyme inhibitors including alpha-hydroxysulfonates which inhibit glycolic acid oxidase.

A few years ago, before we were aware of this property of these inhibitors in closing stomata, Dr. M. E. Corden was carrying out bean rust assays and noted that these alpha-hydroxysulfonates were also quite effective in preventing infection by the bean rust fungus. We have been studying the effects of stomatal control on transpiration and photosynthesis in leaves, and have not yet investigated the effect on pathogenesis. I would suggest however, that many of the observations that have been reported here today could very well be accounted for by the effect of the compounds used on stomata opening. In this connection I would like to ask Dr. Samborski whether he has any evidence on the effect of these genes that produce rust resistance on either the stomatal numbers or stomatal movement, and I would like to ask Dr. Daly if he knows whether or not his compounds affect stomatal opening?

Samborski: I won't answer both questions because I know that Daly has some information which is pertinent, unpublished information. But he is very willing to discuss his unpublished information. That is why I have so many publications.

My answer has to be a rather indirect one. There are certain varieties which, as far as we know, are completely susceptible to the prevalent cultures of rust, and if you just look at it in terms of virulence or avirulence they should be very badly damaged in the field but they are not. At the end of the season they will carry a relatively small percentage of rust. It may be 40 per cent instead of 100 per cent. These varieties are of a completely susceptible type, and so I have always felt that they have an exclusion mechanism of some sort. While this mechanism may be stomatal closing, one must realize that with the leaf rust the stomate closes immediately when the fungus forms an appressorium over them, so that penetration is always through closed stomata. Whether there are different types of closures, I don't know.

Barratt: I have two points I would like to make about Dr. Samborski's talk. The first concerns the inhibitory effect of sorbose in *Neurospora* and it is, as in many fungi, strongly inhibitory. The inhibition is reversed by glucose competitively. We know that there are morphological changes in the fungus as a result of sorbose inhibition, and that sorbose is not actually built into the hyphae. This is now getting into the area raised by Dr. Cowling when he suggested that one of the things that fungi have that the host cells don't have is a chitin wall. It may be that the sorbose is acting by affecting the synthesis of chitin in the fungus cell wall. It should not then be toxic to the host. Another comment along this same line is that I agree that mannitol is probably a "sink" because we know that *Neurospora* accumulates about 20 per cent dry weight as mannitol when grown on high levels of glucose or sucrose. The other point, concerning the first part of Dr. Samborski's talk, is that I would be

a bit hesitant about stating there are not hybrid proteins produced in heterozygous conditions. I have been working for some time on this question, generally using *Neurospora* as the test system, and working with a series of mutants which are all blocked at the same step – the synthesis of the enzyme glutamic dehydrogenase. One can take these mutants pair by pair and put them on a medium on which neither will grow individually, and they will grow together. This phenomenon is called complementation. But one can isolate the protein from the individual complementing strains. There is indeed a protein produced here, and one can demonstrate clearly that complementation occurs only in strains that produce a non-enzymatic, inactive but nevertheless cross-reacting protein (immunologically). This has been demonstrated in other systems. Protein-protein or polypeptide-polypeptide chain interactions do occur. Hybrid protein formation has been suggested as a very important mechanism in parasitism, particularly in the fungi because of the heterokaryon (dikaryon) formation in *Basidiomycetes.*

Samborski: I skipped part of my manuscript with respect to your first comment. Certainly sorbose-1-phosphate has been demonstrated as a strong inhibitor of hexokinase in certain systems. I did not go into that. Your second comment is very interesting. I was not aware of that. Has it been published anywhere?

Barratt: Yes, there have been several papers published on this. It has been demonstrated by Woodward with adenylsuccinase; in fact the work was done at Yale and later in Texas. It has now been shown by Fincham at John Innes, and we have been doing the same line of work. I think that it is perfectly clear cut that polypeptide-polypeptide chain interaction does occur. Indeed you are right that each ribosome is coded with one bit of genetic information from one of the alleles. Interaction is not at the ribosomal level, but apparently after the abnormal proteins are produced. These are sufficiently similar so that the polypeptide chains can fold and complement one another making a functional protein.

Daly: I would like to add just one comment to the reference on mannitol. We have been very careful to indicate that this is a hexitol. We do not have very good chemical proof yet that it is mannitol.

Heggestad: I was very much interested in the things Dr. Samborski had to say including the statement that the biochemical explanation must ultimately fit the observed genetic phenomena. There was another statement made later that I would like to have him comment on. You stated that there were marked changes in the amino acids and carbohydrates brought on by maleic hydrazide. Please comment on the nature of these major changes.

Samborski: I didn't elaborate on the changes that occur as a time sequence. Initially there is an increase in the fructose, glucose, and sucrose and with time, at the concentrations of maleic hydrazide under the conditions that we worked with, the leaves would start yellowing, and it was pretty obvious that proteolysis was going on. A wide variety of amino acids would accumulate including all the normal amino acids, and very high levels of glutamine. There were high levels of sucrose in these leaves.

Uritani: May I ask you a question which is not directly related to chemotherapy of rusts? When the resistant variety of the host is attacked by the rust fungus, we can observe hypersensitivity to lead to the death of the host cells. Do you think that this phenomenon may be related to the production of a protein to supress the particular host RNA or DNA metabolism, under the control of the fungus gene?

Samborski: I do not know. Maybe someone would like to comment.

Uritani: I will ask the question regarding the system between the rust fungus and the host. Is there a similarity to the bacteria-bacteriophage system?

Samborski: There is a similarity in the specificity of each host-parasite interaction, but I would not like to extend the analogy to the biochemical interaction which occurs in each system. The rust fungus is a much more complex organism than a phage.

Uritani: The T-even bacteriophage can duplicate in the normal strain of *Escherichia coli,* but the amber mutants of bacteriophage T-4 cannot grow in the normal strains, though several new enzyme activities appear in the host at an early stage. On the other hand, the host itself is killed by the attack of the mutants. This is the work of Dr. J. S. Wiberg et al. of the Massachusetts Institute of Technology. I think that the basis of this phenomenon might be similar to that of hypersensitivity in plant disease.

Samborski: Yes it may definitely be of some useful value and we should watch the phage work very carefully.

III. Fungicidal Action

Introduction

Raymond J. Lukens

The Connecticut Agricultural Experiment Station

Today, the agricultural scientist is faced with the dilemma of choosing useful compounds from a titanic array of materials. How can we pick successful fungicides without discarding something of value? Here at The Connecticut Agricultural Experiment Station, Thaxter and his successors have played an active role in this search for better fungicides. Their interest in the development of more efficient testing and screening procedures has been accompanied by a continuing search for the principles of fungicidal action.

The preceding symposia on biochemical resistance and chemotherapy examined the interactions of host, pathogen, and fungitoxicant. Now we will restrict ourselves to the interaction between pathogen and fungitoxicant. It will soon become clear that even this simplified relation is extremely complex.

First we will discuss structure-activity relations, or fungitoxicity from the chemist's viewpoint. Most fitting, this aspect of the problem will be presented by E. Y. Spencer. Dr. Spencer, trained as an organic chemist, is Director of the Science Service Laboratory at London, Ontario. This laboratory was established by the Canadian Department of Agriculture to explore the fate of agricultural chemicals in the environment. The discussion of Dr. Spencer's paper will be led by S. S. Block, a chemist who has made substantial contributions to our understanding of the relation of chemical structure to fungitoxicity.

Next, we will examine fungitoxicity from the viewpoint of the fungus. What happens to the vital processes of fungi treated with fungitoxicants will be described by H. D. Sisler. Dr. Sisler and R. G. Owens, who will discuss his paper, are leaders in this area of research.

Finally, the important and controversial problem of the uptake of fungitoxicants by fungi will be explored for us by S. E. A. McCallan and L. P. Miller. This team of plant pathologist and chemist has thrown new light on pathogen-fungitoxicant relations, and revised our thinking about the fungitoxic dose. The discussion of their paper will be led by A. S. Sussman, whose extensive studies on the physiology of *Neurospora* ascospores includes the relation between spore dormancy and the uptake of fungitoxicants.

Structure and Activity Relations Among Fungicides

E. Y. Spencer

Research Institute, Canada Department of Agriculture
London, Ontario

Many compounds are toxic to a broad spectrum of organisms without showing any desirable selectivity. Thus, many a potential fungicide is discarded due to its phytocidal activity. The goal, therefore, amongst fungicidal chemicals, is to find those that exhibit as great a difference in toxicity as possible between the host and parasite.

A rational approach to this search would be ideal. However, an examination of the fungicides now in use will reveal that most, if not all, have been selected empirically. Granted, after the initial selection, some "rational" modifications may have been made in some cases. One logical development would seem to emerge from utilizing a difference in some aspect of the physiology of the host and parasite. Having found, for example, some critical enzyme difference, a chemical might be found which would capitalize on this for selective toxicity. Unfortunately, studies to date indicate a remarkable similarity in the physiology and metabolism of plant pathogens and hosts. It is therefore not surprising to find most fungicides are selective not by the inhibition of a single enzyme. The margin of selectivity between a higher and lower plant is considerably less than between insects and mammals. This is indicated in Table 1.

It is of interest to note that one of the earlier organophosphorus insecticides, parathion, exhibits little selective toxicity. However, a later development, and an empirical one, malathion, marked an era in the production of a synthetic biocide with remarkable selectivity. A few years lapsed before an explanation for this specificity of action was found. With this also came an explanation for potentiation in activity by certain chemicals which were causing concern over the loss in toxicity specificity. As an outgrowth of this work some selective organophosphorus insecticides were developed on "rational" principles with low mammalian toxicity (O'Brien et al., 1958). However, as is so often the case, the theory was pushed "too far," as indicated in the loss of selectivity for the cis isomer of thiono Phosdrin (Spencer, 1961). Much still remains to be done in providing explanations for the selective action of other types of organophosphorus insecticides. However, the problem has been somewhat simplified by the more specific action of these compounds on certain enzymes, by contrast with most fungicides.

In studying the toxicity of chemicals as fungicides or fungistats, using the concentration of the toxicant in the external solution can be quite misleading. The selective uptake by the spore is often remarkable so that a far more valid estimation of toxicity is on a weight of uptake per unit weight of organism (McCallan, 1957; Miller et al., 1953). By use of labelling with radioactive tracers, more precise data of actual uptake by spores have become available (Somers, 1961). Thus, with a standard unit for comparison, the relatively low and nonspecific toxicity of fungicides by comparison with insecticides is indicated in Table 1.

Table 1. Approximate LD_{50} values ($\mu g/g$) for some toxicants

Toxicant	Subject	Toxicity	Reference
Malathion	Mouse	1590.0	Seume and O'Brien,
	Housefly	13.0	1960
Phosdrin, *cis* isomer	Mouse	2.0	Spencer, 1961
	Housefly	0.3	
Thiono Phosdrin,	Mouse	17.0	ibid.
cis isomer	Housefly	3.2	
Phosdrin, *trans* isomer	Mouse	14.5	ibid.
	Housefly	45.0	
Thiono Phosdrin,	Mouse	700.0	ibid.
trans isomer	Housefly	3.5	
Sulfur	*Neurospora sitophila*	11,500	McCallan, 1957
Mercury	*N. sitophila*	5,030	ibid.
Silver	*N. sitophila*	165	ibid.
Glyodin	*N. sitophila*	5,800	Miller et al., 1953
Dichlone	*N. sitophila*	560	ibid.
Captan	*N. crassa*	6,900	Somers, 1961

Selective toxicity requires that the chemical be preferentially taken up by the spores and then that it be inherently toxic to critical enzymes of the spore. Included in these two aspects is the possibility that the spore membrane permeability may be adversely altered. A potential fungicide might have the required toxicity for the critical internal constituents of a spore but not have the required structure to permeate the spore membrane. Therefore, considerable work has been done to find the structure for optimum penetration of the toxophore. In many instances, however, there are not two discreet elements in the fungicide structure, but rather either the whole molecule is involved or a number of substituents add to the over-all effect. Fungicidal action has been differentiated by Ferguson into two classes, namely chemical reaction and physical mechanism. The latter is nonspecific and usually reversible. Since the toxicant is considered to be in equilibrium between the external phase and biophase a measure of the "activity" can be obtained by

determining the degree of saturation in the external phase. High values indicate physical toxicity, while low ones suggest a chemical toxicity mechanism. However, caution has to be used in interpreting some high values. These may be due to a high capacity for a spore to take up a toxicant with an almost equal ability for its subsequent detoxification (Somers, 1961).

From these general remarks it can be concluded that fungicides are in general of relatively low toxicity and specificity by comparison with many toxins. Their selectivity has to depend on the preferential uptake by the spores with the subsequent often rather indiscriminate reaction with cellular components. Further knowledge concerning the detoxification mechanisms and selective permeability will assist in the development of more specific fungicides on rational principles. Meantime, in view of the state of knowledge of structure and reactivity among fungicides, and since fungitoxic mechanisms are to be discussed by Dr. Sisler and permeation into cells by Drs. McCallan and Miller, I feel the topic assigned to me of structure and activity relations among fungicides can best be discussed by examining the change in reactivity of several fungicides as members of a homologous series, as well as examining the varied structures of a number of fungicides.

Inorganic fungitoxicants have been in use for a considerable time. The most common one is copper, as in Bordeaux mixture, which has been well reviewed by Martin (1959). On a basis of minimum toxic dosage the inorganic cationic compounds are relatively low in toxicity by contrast with most organic or organo-metal complex toxicants. Their fungitoxicity was correlated with their ability to form chelate compounds by Horsfall (1956). Somers (1959) suggested activity decreases in order of decreasing electrode potential from copper, silver, mercury at one extreme to zinc, iron, and calcium at the other. Although there has been some disagreement over the mechanism and correlation by Miller (1960), Somers (1960) re-confirmed his suggestion "that there is a common, non-specific, toxic action for most metal cations which can be related to their strength of covalent binding to the surface inorganic groups present outside the cell protoplast." Somers' (1961) results supports the proposal of Danielli and Davies (1951) of a correlation between the electronegativity value of the cation and the logarithm of its ion concentration at the ED_{50} value.

Inorganic mercury fungicides have been largely replaced by the more selective and less mammalian-toxic organo-mercury compounds. From a survey of material of type formula RHgX where R is alkyl or aryl and X an anion, the latter was insignificant in fungicidal efficiency against the test organism employed (Gassner, 1950). The effectiveness varied with the nature of the R group. However, effective seed treatment materials are found with a wide variation in structure from 2-methoxy-

ethyl mercuric chloride and methylmercuric dicyandiamide on the one hand to *N*-(ethylmercuri)-p-toluene sulfonanilide on the other (Martin, 1961). The history and activity of organomercury compounds is reviewed by Martin (1959).

Having mentioned the use of inorganic copper, some reference should also be made to organo-copper compounds. The most common is copper 8-hydroxyquinolinolate or oxinate. Much has been written in an attempt to explain its toxicity (Martin, 1959). Was the toxicity due to the copper or the oxine or both? From the suggestion by Albert (1951) and substantiated by Block (1955, 1957) the 2:1-oxine:Cu chelate complex has the required lipid solubility to penetrate the spore while the ionized non-lipoidal 1:1-oxine:Cu is the toxicant. He showed the importance of chelation by comparing the activity of oxine, 5,7-dihalogeno, and 5,7-dinitro-oxine over a pH range. Oxine which loses its ability to chelate as the pH is lowered, also loses its fungitoxicity (Zentmyer, 1943, 1944). The other two, where their chelating property is not affected over this pH range, retained their toxicity. In addition, the methyl ether of 5,7-dichloro-oxine, which cannot chelate, is inactive.

Durkee (1958) in our laboratory showed from examination of a number of hydroxyquinolines and pyridines, chelation was necessary for lipoid solubility and resultant spore penetration. However, the 8-hydroxy group of quinoline was important for toxicity, not just chelation ability, since other quinoline and pyridine carboxylic acid derivatives which formed copper chelates had low fungitoxicity.

Byrde et al. (1958, 1961) examined the effect of 5-alkyl substitution on oxine activity in the absence of copper and found it maximum with the pentyl and hexyl derivative. However, in order to further substantiate the significance of chelation, 5-n-pentyl-6-hydroxyquinoline (which cannot chelate) was synthesized and found to have an ED_{50} against *Aspergillus niger* 80 times greater than the corresponding 5-n-pentyl oxine (Woodcock, 1961). Therefore, although an alkyl substituent enhances activity, maximum fungitoxicity occurs where chelation is possible. In the 6-n-alkyloxine series, reactivity increased to a maximum for the n-hexyl and n-heptyl derivative, the former being twice as active as the corresponding 5-alkyloxine (Rich, 1960). Since solubilities are probably not very different, possibly a steric factor is significant.

Another attempt to incorporate lipophilic character for penetration, as well as ionic character for ultimate toxicity, was attempted by Grier and Ramp (1961). They synthesized a series of salts of salicylic acid with the benzoic acid ester of oxine. The most active compounds were the oxine ester salts with no additional substitution in the quinoline ring with the exception of 5,7-dibromo-8-quinolinyl benzoate, 3,5-diiodo-salicylic acid salt.

By contrast with many other metals, such as mercury and copper, tin

and its inorganic salts possess low fungicidal activity. However, an investigation of a series of organic derivatives of tetravalent tin indicated a number of effective fungicides (van der Kerk and Luijten, 1954). Of mono, di, tri and tetra-alkyl tin compounds the trialkyl compound was the most effective by far. Variation in the X group of R_3SnX indicated little effect on toxicity while varying the R group, an optimum activity was reached with tributyl derivatives with triethyl and triphenyl derivatives next. Mixed alkyl aryl substituents showed intermediate activity while substitution in the phenyl substituents reduced activity. All the trialkyl tin compounds were found to be phytotoxic (Hartel, 1958, 1962) while triphenyl tin salts had low phytotoxicity with the hydroxide being less than the acetate (Pieters, 1962). It is of interest to note that phytotoxicity can be reduced while maintaining fungitoxic properties through formulation (Pieters, 1962). In a study of derivatives of germanium and lead (elements either side of tin, in group IV of the periodic table), lead compounds exhibited antifungal activity comparable with those of tin while the activity of germanium compounds was much lower (Kaars Sijpesteijn et al., 1962).

Returning to a purely inorganic fungicide, sulfur is one of the oldest and still important. Although its efficiency is low – up to 50,000 ppm by weight of spores (McCallan, 1954) – it is influenced to some extent by the particle size and form. In spite of its simplicity and selectivity there is much speculation as to its mode of action as indicated in reviews (McCallan, 1957; Martin, 1959; Rich, 1960).

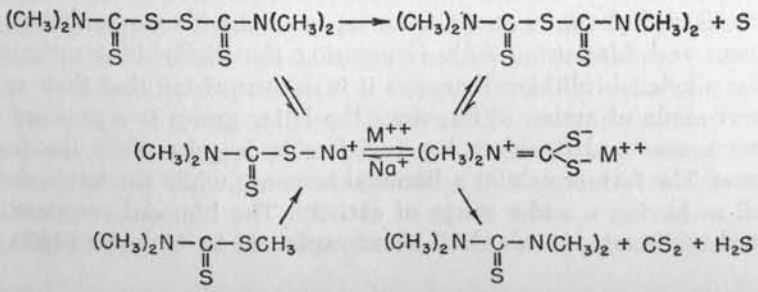
With the granting of a patent to Tisdale and Williams in 1934 for derivatives of dithiocarbamates, an era dawned for organo-sulfur fungicides to be followed by Dimond et al. (1943) reporting on the properties of alkylenebisdithiocarbamates. A great deal has since been published on the properties of derivatives of these two groups. It has been well summarized in the recent monograph by Thorn and Ludwig (1962) and by Rich and Horsfall (1961), so that only some salient points will be mentioned here.

The monoalkyldithiocarbamates, due to their instability, have not been studied to any extent. However, recently a patent has been issued for a form stabilized in a concentrated solution with the addition of a primary or secondary aliphatic or aromatic amine to react with any isothiocyanate (Dorman and Linguist, 1957). Comparing the dialkyldithiocarbamates and the alkylenebisdithiocarbamates it is not surprising that their reactivity and mode of action differ, since the latter group is a product of a primary amine and therefore has two free hydrogens while the former has none. The former exhibit a bimodal response while the latter do not, as well as having a wider range of activity. The bimodal response first reported by Dimond et al. (1941) and explained by Goksøyr (1955) re-

calls the activity of copper oxinate to the extent that it is the half chelate which is much more toxic than the full chelate.

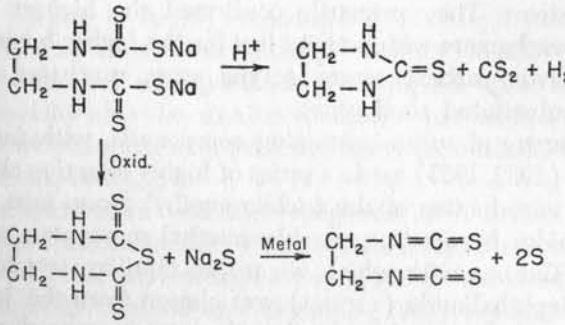
Included in the dithiocarbamates are the metallic dithiocarbamates, the thiuram sulfides, and the esters. Systematic studies of the fungicidal properties of dialkyldithiocarbamates have indicated a decrease with increasing chain length of the dialkyl groups with the dimethyl derivative being the most active (Goldsworthy et al., 1943; Parker-Rhodes, 1943; Barratt and Horsfall, 1947; van der Kerk and Klöpping, 1952). Aromatic derivatives are inactive. This reactivity is similar with the corresponding thiuram derivatives. The alkyl esters of dimethyldithiocarbamate are much less toxic, the methyl ester being the least active of a series. The canonical form $R_2N^+ = C\langle^{S^-}_{S^-}\rangle M$ was suggested as an essential structure in which the dialkyldithiocarbamates must ultimately exist to exert fungicidal activity (van der Kerk and Klöpping, 1952). Presumably they penetrate the spore in a nonionized form and are reduced or hydrolyzed as the case may be to the above canonical form. Closely related compounds not able to form the "thiouride" ion are much less active. This structure was substantiated by Chatt et al. (1956) for a homologous series which decreased in activity with increasing chain-length of alkyl groups. Thus, other properties as relative lipoid-water solubility are factors contributing to changing fungitoxicity in this homologous series. However, it appears difficult to reconcile the low toxicity of the methyl ester of dimethyldithiocarbamate with the high value for the relatively insoluble zinc salt.

The analogous structure $RO^+ = C\langle^{S^-}_{S^-}$ contributes little to the structure of xanthates (Chatt et al., 1956) which have low fungitoxicity. The anomalous behavior of "tetramethylthiuram oxide" which is highly fungicidal (Klöpping and van der Kerk, 1951b) and which theoretically would be unable to produce a thiouride ion similar to thiuram was later explained by White (1954) of our laboratory. He showed that the compound was incorrectly named and should be designated dimethylthiocarbamyldimethylcarbamyl sulfide and therefore contains the desired grouping $-Me_2NC(S).S.C(O)NMe_2$. Some of the overall reactions can be summed up:



A study of some bisdithiocarbamates indicated that the fungicidal activity decreased with increasing length of the carbon chain (Davies and Sexton, 1946). Part of this may be due to solubility limitations. Replacement of the active hydrogens on the nitrogen atoms greatly reduced activity as well as specificity greatly below that of the corresponding dimethyldithiocarbamate salt. It is not surprising that the spectrum of activity and specificity as well as mode of action (as indicated by toxicity response curves) differ between the dialkyldithiocarbamates and the ethylenebisdithiocarbamates. The latter are able to be converted to isothiocyanates. Examination of the monoisothiocyanate showed low activity while the diisothiocyanate was even more active than nabam with reduction in activity as the homologous series was ascended (Klöpping and van der Kerk, 1957).

It has been observed that nabam, the water-soluble disodium salt of ethylenebisdithiocarbamate, on exposure to air formed an insoluble precipitate. Meantime, Thorn and Ludwig (1954) and Ludwig et al. (1954) from our laboratory showed that nabam was converted to a mixture from which besides ethylenethiourea, CS_2 and H_2S, the toxic ethylenethiuram monosulfide and its polymer were identified. This was confirmed by Kaars Sijpesteijn and van der Kerk (1954) and reconciled with the diisothiocyanate. The catalysis of the interchange by metallic ions was then reported by Ludwig et al. (1955) so that production of the final toxicant, the diisothiocyanate and byproducts may be outlined as follows:



Although the dithiocarbamates dominate the organo-sulfur fungicide field, others have been found that are fungitoxic. Ethylenethiourea, a byproduct of ethylenebisdithiocarbamate decomposition, although of low toxicity itself, reaches a maximum value with an 8 carbon N-alkyl chain (Rich and Horsfall, 1954; Ross and Ludwig, 1957). Since the ultimate toxicity appears to be due to a physical effect, an increase in lipoid solubility was more important than in the case of the bisisothiocyanates, where, because of chemical toxicity, penetration probably is of less importance.

Rhodanine (2-mercaptothiazolidone-4) has some fungicidal activity and contains the >N-C(S)S- group common to the salts of dimethyldithiocarbamates as well as thiram which are very effective. It was logical, therefore, to add substituents, particularly at the 3 and 5 position, in an attempt to increase the fungitoxicity. Maximum activity was found with 3 carbons in the chain (Bradsher et al., 1954). Examination of substituted phenyl and benzyl derivatives in the 3 position gave no consistent pattern for fungitoxicity. While many electron attracting substituents in the para position were effective, one of opposite polarity, namely 3-(p-methoxyphenyl)-rhodanine was one of the two most active. Also substitution with chlorine or bromine in the ortho or meta position, as well as methyl and carboxyl in the meta position, increases the activity over para substitution. In addition, the 3-phenyl derivative was more active than the 3-benzyl derivative to *Aspergillus niger* while the reverse was found for *Bacillus subtilis* and *Escherichia coli* (Brown et al., 1956). Among a series of substituted rhodanines, van der Kerk (1956) found 3-(p-chlorophenyl)-5-methyl rhodanine highly fungicidal while the N-alkyl derivatives have little activity. He found that the active materials were sufficiently reactive to undergo formation of an isothiocyanate. Thus the alkylene bisdithiocarbamates and the appropriate rhodanine derivatives may both act through a reactive isothiocyanate intermediate.

Carter et al. (1963) have recently reported the fungitoxic properties of an extended series of dithiocarbamate and hydroxydithioformic acid (xanthate) derivatives. They primarily confirmed the highest toxicity for dimethyldithiocarbamate with activity less for the higher homologues, monoalkyl derivatives, carboxy esters, acrylic ester, xanthates, isothiocyanates and N-substituted rhodanines.

Continuing a survey of sulfur-containing compounds, with fungicidal activity, Kittleson (1952, 1953) made a series of highly effective chemicals by the fortuitous introduction of the trichloromethyl group into a series of amides and imides by reacting perchloromethyl mercaptan with the appropriate salt. The one with which we are all familiar, tetrahydro-N-trichloromethylthiophthalimide (captan) was chosen from the 16 active compounds produced. It was thought that the activity was due to the $>NSCCl_3$ group. This was tested to some extent by incorporation of this group into some sulfonamides (Waeffler et al., 1955). The most promising was N-methyl-sulfonyl-N-trichloromethyl-thio-4-chloroaniline. However, another test of the significance of $>NSCCl_3$ was made by replacing the N by O. It was found that even as simple a compound as trichloromethanesulfenyl chloride ($MeOS.CCl_3$) was active against two fungi (Sosnovsky, 1956). Since nitrogen did not seem essential Uhlenbroek et al. (1957a, b) studied the significance of the trichloromethylthiol group by incorporation into a number of thiolsulfonic esters and disulfides. Trichloromethyl methyldisulfide is nonfungitoxic while there is some activity with aromatic derivatives, the nitrophenyl compound being highly fungitoxic. It will be noted that activity increases with greater number of electronegative substituents. On the other hand, the trichloromethylthiolsulfonates (RSO_2SCCl_3) are quite fungitoxic irrespective of the electronegativity of the R group. As aryl sulfonates are strongly electronegative, substituents would not be expected to have much effect in changing the activity of the adjacent sulfur. Johnson et al. (1957) similarly confirmed the low activity of aliphatic trichloromethylthiolsulfonates while the fungitoxicity of the aryl derivatives was not changed significantly by substituents. However, toxicity was greatly reduced by replacing the trichloromethyl by the nitrophenyl group.

Horsfall and Rich (1954) had earlier postulated that -CO-NR-CO was the toxic group of the captan series by analogy of the similar dosage response curves with the antibiotic cycloheximide which contains the same grouping. However, in view of subsequent results already mentioned, it appears to be untenable. The toxophore cannot be attributed to one particular part of the molecule.

As mentioned earlier, maximum fungitoxicity is often reached in a homologous series when there is an appropriate lipoid-water partition coefficient. However, Richmond and Somers (1962a, b) have found no such correlation in studying the toxicity of a series of captan-related compounds to spores of *Neurospora crassa*. In addition, the lack of specificity, as indicated by the high ED_{50} level of 6900μg captan per g of dry spores, is largely explained by the high rate of detoxification by the spores. Thus the net amount reaching the active sites is much less. Possibly, the lack of importance of the lipoid/water coefficient for optimum toxicity may be due to evidence that captan and related compounds are absorbed by a process requiring metabolic energy (Richmond and Somers, 1962a, b).

Of interest as a potential fungicide is tetrachloroethylmercaptocyclohexene dicarboximide (Thomas et al., 1962) recently reported. Here the familiar trichloromethylmercapto group has been modified without losing activity. Lukens and Horsfall (1962) replaced the $SCCl_3$ group entirely by a series from phenyl to phenylbutyl and found the phenyl derivative inactive while fungitoxicity rose as the carbon chain increased to four. They found the same for similar derivatives of *o*-benzendisulfonimide and suggested that low activity of the phenyl derivative was due to steric hindrance. However, even the optimum toxicity was 1/100 that of captan so that the trichloromethyl mercaptan group plays an added role beyond just an aid in penetration.

The quinones, like the dithiocarbamates, were established chemicals of the rubber industry long before their investigation as potential fungicides. It was not until 1940 that the first one was investigated, namely

tetrachloro-*p*-benzoquinone (Cunningham and Sharvelle, 1940). Although very effective, it was also phytotoxic and light-sensitive. However, these defects were somewhat overcome with 2,4-dichloronaphthoquinone. Effects of modification on the benzo- and naphthoquinones were reviewed by McNew and Burchfield (1951). They listed the quinones in the following increasing order of activity: anthraquinone, benzoquinone, phenanthraquinone, and naphthoquinone. Substituents modify effectiveness and so cause overlapping in the above groups. Halogenation improves activity, decreases phytotoxicity and water solubility with the order decreasing from Cl>Br>I. Hydroxyl or methoxyl groups ortho to the carbonyl increase water solubility and phytotoxicity while alkyl groups reduce fungitoxicity. In naphthoquinones where substitution can be other than in the ortho position to the carbonyl, activity is not destroyed. Ortho and para quinones differ only slightly in their fungitoxicity. Replacing fluorine by chlorine increased effectiveness (Tehon, 1951). Byrde and Woodcock (1953) found that replacing vicinal chlorine atoms by groups of equal size such as methyl or methoxyl destroyed fungitoxicity. Acetylation of the corresponding hydroquinone reduced phytotoxicity only, evidently due to their stability on the leaf while being absorbed by the spore and hydrolyzed by a fungal esterase.

Turning to nitrogen heterocyclic systems, Wellman and McCallan (1946) discovered the activity of the glyoxalidine or 2-imidazoline nucleus. In order to have it reach its target or penetrate the spore, the nucleus required a hydrophobic chain. This also increased phytotoxicity as would be expected. However, examination of a homologous series with substitution at position 2 indicated maximum fungitoxicity with a side chain of 13-17 carbon atoms whereas phytotoxicity was greatest with a 11-13 carbon chain. Variation in the substituent in the 1 position had little effect on the fungistatic activity on the 2-heptadecyl derivative. Horsfall and Rich (1951) in an extensive survey of heterocyclic nitrogen compounds found a number that evidently were potentially fungitoxic but required a lipophilic substituent to enable the toxophore to penetrate the spore. One of the most active was an oxygen analogue of imidazoline, namely the 2-hendecyl derivative of 2-oxazoline. Rader et al. (1952) further substantiated this by finding the most active compound in the 2-alkyl 4,4,6-trimethyl tetrahydropyrimidine series was the 17 carbon derivative. An earlier paper of McNew and Sundholm (1949) reported on the effectiveness of substituted pyrazoles. Pyrazole was unreactive, while the 3,5-dimethyl derivative showed weak fungitoxicity but this was considerably improved with the 4-nitroso derivative. Other derivatives as nitro, amino, or phenylazo were ineffective. Maximum activity was found with the 1-phenyl derivative. It would be of interest to study the effect of the 1-alkyl compounds on fungitoxicity for a more direct comparison

with the imidazolines and other N-heterocyclic compounds already mentioned.

The effect of increasing the lipophilic property on the fungitoxicity of pyridine by quaterization was shown to increase to a maximum with the tetradecyl derivative (LoCicero et al., 1948). Subsequent substitution in the 2 or 4 position of N-dodecylpyridinium bromide showed little change in activity.

A recent addition to the surface active fungicides was found in the n-alkyl guanidine series. Brown and Sisler (1960) found maximum fungitoxicity to *Monilinia fructicola* with the guanidine acetate containing a 13-14 carbon alkyl group while maximum phytotoxicity was shown by the 10 carbon alkyl group. This relationship is very similar to that of the imidazolines. Byrde et al. (1962) confirmed the chain length for optimum activity and compared this with the corresponding alkyl amines. The weaker activity of the latter may be due to weaker basicity, unless the guanidine group itself is critical, in addition to its basicity.

Another group of effective fungicides are based on derivatives of S-triazine (Wolf et al., 1955). Activity was narrowed down to various substituents of 2,4-dichloro-s-triazine, in the 6 position. The 6-arylamino derivatives were more stable and effective than the aryloxy analogues. Chloro, bromo, or methyl substitution in the ortho position of the 6-phenylamino group gave better foliage protection than the *para* isomer and this in turn better than the *meta*.

During a study of potential organophosphorus toxicants, van den Bos et al. (1960) synthesized a number of substituted bis (dimethylamido) phosphoryl derivatives, including the following heterocyclic systems: pyrrole, indazole, imidazole, 1,2,3 and 1,2,4-triazole. Of these, the most promising was 5-amino-3-phenyl-1-bis (dimethylamido)-1,2,4-triazole (Elings, 1962). Besides being effective in the control of powdery mildew, it exhibits systemic activity on application to roots, and as might have been predicted, it has cholinesterase-inhibiting properties. Thus, it is toxic to mammals, as well as being an effective aphicide. Replacement of phenyl by alkyl groups up to C_5H_{11} has no effect on fungitoxicity, while increasing slightly phytotoxicity. However, substitution of the phenyl by parachloro or methoxy reduces activity while replacement of aryl by benzyl, phenylethylene, carboxyethyl ester destroys fungitoxicity. Similarly, replacement of one or both dimethylamido substituents by ethoxy groups or 'O' by thiono 'S' cancels out any fungitoxicity activity. Therefore, in this phosphorylbisdimethylamido triazine, originally examined for possible insecticidal and acaricidal activity, a potential systemic fungicide was found which is effective against powdery mildew and whose activity is greatly modified by slight variations in substituents in different parts of the molecule.

Electrophilic substituents such as the nitro group in the ortho or para position activate others in aromatic compounds. Thus, 6-(2-octyl)-phenyl-2,4-dinitrocrotonate (Karathane) originally designed as an acaricide is also effective against powdery mildew. A closely related one is 2-(2-butyl)-4,6-dinitrophenyl 3,3-dimethylacrylate. Bates et al. (1962) found that 2-methyl-4,6-dinitrophenol and its aliphatic esters were similar in *in vitro* fungitoxicity while aromatic esters were less active. However, the benzoic ester was found to be a very effective protectant fungicide. This was shown to be due to enzymic hydrolysis of the ester by the plant and not the spore, so that ultimate toxicity is due to the substituted free phenol.

Nitrated chlorobenzenes probably owe their activity to the labile halogen caused by the introduction of electronegative groups. In the study of 2,4-dichloro-3,5-dinitrobenzoates, however, the free acid was found to be the least reactive, while the esters increased from isopropyl to ethyl with the methyl ester the most reactive (Summers and Turner, 1963). Since there was low phytotoxicity, presumably the methyl ester gave optimum selective penetration of the spore. An examination of a number of chlorinated nitrobenzenes indicated increased activity and decreased water solubility with increased substitution (Eckert, 1962). Phytotoxicity diminished with high chlorination. Solubility-toxicity data indicated structurally nonspecific inhibitors from the application of Ferguson's thermodynamic activity principle.

In the course of routine screening against spores of *Botrytis cinerea*, 3-methyl-2,4-dichloro-6-nitroaniline showed high activity in the impregnation test (Clark and Hams, 1961). From this lead a great many related compounds were examined. Omission of the methyl group had no effect as did monomethylation of the nitrogen, while dimethylation reduced activity. Increasing chlorination as in 2,4,6-trichloro-3-nitroaniline had no effect, while a fourth chlorine reduced activity greatly. Some halogen is necessary for activity as replacement by methyl or methoxy groups lowered fungitoxicity. Replacing the amino group by a phenolic hydroxyl reduced activity somewhat, as did acetylation. The naphthalene series showed no promise. Of significance was the fact that all except the phenolic derivatives were inactive in the spore germination test. We therefore see that suitable biological activity can be found with established compounds by trial and error and that the choice of the bioassay can be critical. The ultimate activity of the basic molecule modified by slight changes in substituent groups cannot as yet be predicted.

The discovery of 6-methyl-quinoxaline-2,3-dithiolcarbonate as being effective against the resistant mildew fungi is, I feel, another example of serendipity in the field of fungicidal development (Sasse, 1960). The initial research followed the line of looking for potentially suitable chelating agents. One of these, 2,3-dimercapto-quinoxaline, exhibited no outstanding biological properties. However, blocking the SH group such as forming carbonic acid derivatives surprisingly resulted in compounds with effective acaricidal and fungicidal properties. With this lead, numerous blocking agents and substituents about the quinoxaline nucleus were investigated resulting in the ultimate selection of the above-mentioned dithiolcarbonate derivative as the most promising.

In spite of considerable searching for effective systemic fungicides, no outstanding ones seem to have been found. Sulfonamides and certain phenoxyacetic acids have exhibited this property (Cremlyn, 1961). Recently a series of phenylthioureas were examined for systemic fungitoxicity and the unsubstituted phenyl derivative was found to be the most active by comparison with the *p*-methoxy, *p*-nitro, *p*-chloro and 3,4-dichloro compounds (Kaars Sijpesteijn and Pluijers, 1962). Systemic control of powdery mildew has been found with such antimetabolites as the 8-aza derivatives of three bases, adenine, guanine, and uracil, occurring in RNA (Dekker, 1962). Another antimetabolite, fluorophenylalanine, has also exhibited a systemic effect against two fungi tested (van Andel, 1962).

Among the numerous antibiotics listed (Sharvelle, 1961), only two have achieved commercial level of productivity, namely streptomycin and cycloheximide (Goodman, 1962). The former is a glycoside of 1,3-diguanidino-2,4,5,6-tetrahydroxy cyclohexane streptose and 2(methylamino)glucose while the latter is 3-[2-(3,5-dimethyl-2-oxocyclohexyl)-2-hydroxyethyl] glutarimide. Griseofulvin is another antibiotic which has been examined extensively as a systemic fungicide, isolated in 1939 (Oxford, et al., 1939). It is 7-chloro-4,6-dimethoxycoumaran-3-one-2-spiro-1′-(2′-methoxy-6′-methyl-cyclohex-2′-en-4′-one) (Grove et al., 1952). Chemical modifications (Crowdy et al., 1959) resulted in four types of compounds:

(a) replacement of methyl ether by ethyl ether or dechlorination did not affect the systemic activity.
(b) isomerization and alkoxylation of the resulting enol retained *in vitro* activity but no systemic properties.
(c) the trione showed weak systemic activity but inactive *in vitro*.
(d) replacing the methoxy of the enol by amino or making the DL-diastereoisomer inactivated the product.

From such different and in some cases relatively complex compounds, it is difficult to correlate any particular structure with activity, although attempts have been made.

In addition to "foreign" antibiotics, examples can be cited of internally-produced antifungal agents. Virtanen and Hietala (1955) isolated an antifusarium factor from rye seedlings and identified as benzoxazolinone. Later, Virtanen et al. (1957) and Smissman et al. (1957) identified

6-methoxy-2(3)-benzoxazolinone as an antifungal and insecticidal factor from young corn and wheat plants. The methoxyl group seemed to have little effect on antifungal properties. We have shown the presence of an antifungal agent in young barley plants quite unrelated to that in corn, rye or wheat (Ludwig et al., 1960). Following further purification we have shown that the material is a strong base, contains a guanidine nucleus and is light-sensitive (Koshimizu et al., in press). One product of acid hydrolysis has been identified (Stoessl, unpublished). There are many more examples of naturally-occurring fungitoxic substances both in plants and the soil (Cremlyn, 1961). It is obvious from the diverse structural nature of both synthetic and naturally-occurring fungitoxic substances that there is no set pattern as a guide. Discovery is in large measure as yet fortuitous. As indicated initially, relative toxicity is much lower than for most insecticides. There is a narrow margin of selectivity between the spore and the host plant so that much toxicity is based on the selective accumulation of the toxicant by the spore. This in turn kills the spore by the nonselective inhibition of a number of enzymes. Therefore, at present, it is difficult to design for high specificity. As more information is obtained from fungitoxic mechanisms and factors effecting permeability, the design of more selective fungicides will be possible.

LITERATURE CITED

Albert, A. 1951. Selective toxicity with special reference to chemotherapy. Methuen and Co., London. 228 p.

Andel, O. M. van. 1962. Fluorophenylalanine as a systemic fungicide. Nature (London) 194:790.

Barratt, R. W., and J. G. Horsfall. 1947. Fungicidal action of metallic alkyl bisdithiocarbonates. Conn. Agr. Expt. Sta. (New Haven) Bull. 508:1-51.

Bates, A. N., D. M. Spencer, and R. L. Wain. 1962. Investigations on fungicides. V. The fungicidal properties of 2-methyl-4,6-dinitrophenol (DNC) and some of its esters. Ann. Appl. Biol. 50:21-32.

Block, S. S. 1955. Fungitoxicity of the 8-quinolinols. J. Agr. Food Chem. 3:229-234.

Block, S. S. 1957. Reversal of fungitoxicity of copper-8-quinolinolate. J. Agr. Food Chem. 4:1042-1046.

Bos, B. G. van den, M. J. Koopmans, and H. O. Huisman. 1960. Investigations on pesticidal phosphorous compounds. I. Fungicides, insecticides and acaricides derived from 3-amino-1,2,4-triazole. Rec. Trav. Chim. 79:807-822.

Bradsher, C. K., F. C. Brown, and R. J. Grantham. 1954. Synthesis and fungistatic activity of some 5-(1-methyl-2-thioalkylethylidene)-rhodanines. J. Am. Chem. Soc. 76:114-115.

Brown, F. C., C. K. Bradsher, E. C. Morgan, M. Tetenbaum, and P. Wilder, Jr. 1956. Some 3-substituted rhodanines. J. Am. Chem. Soc. 78:384-388.

Brown, I. F., and H. D. Sisler. 1960. Mechanisms of fungitoxic action of *n*-dodecylguanidine acetate. Phytopathology 50:830-839.

Byrde, R. J. W., D. R. Clifford, and D. Woodcock. 1958. Fungicidal activity and chemical constitution. VI. The activity of some substituted 8-hydroxyquinolines (oxines). Ann. Appl. Biol. 46:167-177.

Byrde, R. J. W., D. R. Clifford and D. Woodcock. 1961. Fungicidal activity and chemical constitution. IX. The activity of 6-*n*-alkyl-8-hydroxyquinolines. Ann. Appl. Biol. 49:225-232.

Byrde, R. J. W., D. R. Clifford, and D. Woodcock. 1962. Fungicidal activity and chemical constitution. XI. The activity of *n*-alkyl guanidine acetates. Ann. Appl. Biol. 50:291-298.

Byrde, R. J. W., and D. Woodcock. 1953. Fungicidal activity and chemical constitution. II. Compounds related to 2,3-dichloro-1,4-napthaquinone. Ann. Appl. Biol. 40:675-687.

Carter, G. A., J. L. Garraway, D. M. Spencer, and R. L. Wain. 1963. Investigations on fungicides. VI. The antifungal activity of certain dithiocarbamic and hydroxydithioformic acid derivatives. Ann. Appl. Biol. 51:135-151.

Chatt, J., C. A. Duncanson, and L. M. Venanzi, 1956. Electronic structure of dithiocarbamates and xanthates. Nature (London) 177:1042-1043.

Clark, N. G., and A. F. Hams. 1961. Antifungal activity of substituted nitroanilines and related compounds. J. Sci. Food and Agr. 12:751-757.

Cremlyn, F. J. W. 1961. Systemic fungicides. J. Sci. Food and Agr. 12:805-812.

Crowdy, S. H., J. F. Grove, and P. McCloskey. 1959. The translocation of antibiotics in higher plants. 4. Systemic fungicidal activity and chemical structure in griseofulvin relatives. Biochem. J. 72:241-249.

Cunningham, H. S., and E. G. Sharvelle. 1940. Organic seed protectants for lima beans. Phytopathology 30:4.

Danielli, J. F., and J. T. Davies. 1951. Reactions at interfaces in relation to biological problems. Advances in Enzymol. 11:35-39.

Davies, W. H., and W. A. Sexton. 1946. Chemical constitution and fungistatic action of organic sulfur compounds. Biochem. J. 40:331-334.

Dekker, J. 1962. Systemic control of powdery mildew by 6-azauracil and some other purine and pyrimidine derivatives. Mededel. Landbouwhogeschool En Opzoekingsstas. Staat Gent 27:1214-1221.

Dimond, A. E., J. W. Heuberger, and J. G. Horsfall. 1943. A water soluble protectant fungicide with tenacity. Phytopathology 33:1095-1097.

Dimond, A. E., J. W. Heuberger, and E. M. Stoddard. 1941. Role of the dosage response curve in the evaluation of fungicides. Conn. Agr. Expt. Station (New Haven) Bull. 451:635-667.

Dorman, S. C., and A. B. Linguist, Jr. 1957. Stabilization of salts of monoalkyl dithiocarbamic acids. U. S. Pat. 2,791,605.

Durkee, A. B. 1958. Fungitoxicity of some substituted pyridines and quinolines related to 8-quinolinol (oxine). J. Agr. Food Chem. 6:194-196.

Eckert, J. W. 1962. Fungistatic and phytotoxic properties of some derivatives of nitrobenzene. Phytopathology 52:642-649.

Elings, H. 1962. Experiences with 5-amino-3-phenyl-1-bis (dimethyl amido) phosphoryl-1,2,4-triazole, a new fungicide. Proc. Brit. Insecticide Fungicide Conf., Brighton, England 1961:451-459.

Gassner, G. 1950. Die chemotherapeutische Bewertung von Quecksilberverbindungen in den verschiedenen Beizverfahren. Phytopathol Z 17:1-35.

Goksøyr, J. 1955. The effect of some dithiocarbamyl compounds on the metabolism of fungi. Physiol. Plantarum 8:719-835.

Goldsworthy, M. C., E. L. Green, and M. A. Smith. 1943. Fungicidal and phytocidal properties of some metal dialkyl dithiocarbamates. J. Agr. Research 66:277-291.

Goodman, R. N. 1962. The impact of antibiotics upon plant disease control. Advances in Pest Control 5:1-46.

Grier, N., and J. A. Ramp. 1961. Oxine ester salicylates as antifungal agents. J. Org. Chem. 26:4032-4037.

Grove, J. F., J. MacMillan, T. C. P. Mulholland, and M. A. T. Rogers. 1952. Griseofulvin. IV. Structure. J. Chem. Soc. 1952:3977-3987.

Hartel, K. 1958. Organic tin compounds as a crop fungicide. Quart. J. Tin. Research Inst. 43:9-14.

Hartel, K. 1962. Triphenyltin compounds. Agr. Vet. Chem. 3:19-24.

Horsfall, J. G. 1956. Principles of fungicidal action. Chronica Botanica Co., Waltham, Mass. 279 p.
Horsfall, J. G., and S. Rich. 1951. Fungitoxicity of heterocylic nitrogen compounds. Contrib. Boyce Thomp. Inst. 16:313-347.
Horsfall, J. G., and S. Rich. 1954. Fungitoxicity of sulfur-bridged compounds. Science 119:582.
Johnston, T. P., H. C. Rueggeberg, and S. S. Block. 1957. Fungicidal activity of trichloromethyl thiolsulfonates. J. Agr. Food Chem. 5:672-674.
Kaars Sijpesteijn, A., and G. J. M. van der Kerk. 1954. Investigations on organic fungicides. VII. The biochemical mode of action of bisdithiocarbamates and diisothiocyanates. Biochim. Biophys. Acta 13:545-552.
Kaars Sijpesteijn, A., and C. W. Pluijers. 1962. On the action of phenylthioureas as systemic compounds against fungal diseases of plants. Mededel. Landbouwhogeschool En Opzoekingsstas. Staat Gent 27:1199-1203.
Kaars Sijpesteijn, A., F. Rijkens, J. G. A. Luijten, and L. C. Willemsens. 1962. On the antifungal and antibacterial activity of some trisubstituted organogermanium, organotin, and organolead compounds. Antonie van Leeuwenhoek 28:346-356.
Kerk, G. J. M. van der. 1956. The present state of fungicide research. Mededel. Landbouwhogeschool En Opzoekingsstas. Staat Gent 21:305-339.
Kerk, G. J. M. van der, H. L. Klöpping. 1952. Investigations on organic fungicides. VII. Further considerations regarding the relations between chemical structure and antifungal action of dithiocarbamate and bisdithiocarbamate derivatives. Rec. Trav. Chim. 71:1179-1197.
Kerk, G. J. M. van der, and J. G. A. Luijten. 1954. Organotin compounds III. Biocidal properties of organotin compounds. J. Appl. Chem. (London) 4:314-319.
Kittleson, A. R. 1952. A new class of organic fungicides. Science 115:84-86.
Kittleson, A. R. 1953. Preparation and some properties of *N*-trichloromethylthiotetrahydrophthalimide. J. Agr. Food Chem. 1:677-679.
Klöpping, H. L., and G. J. M. van der Kerk. 1951a. Investigations on fungicides. IV. Chemical constitution and fungistatic activity of dithiocarbamates, thiuram sulphides and structurally related compounds. Rec. Trav. Chim. 70:917-939.
Klöpping, H. L., and G. J. M. van der Kerk. 1951b. Investigations on organic fungicides. V. Chemical constitution and fungistatic activity of aliphatic bisdithiocarbamates and isothiocyanates. Rec. Trav. Chim. 70:949-961.
Koshimizu, K., E. Y. Spencer, and A. Stoessl. Can. J. Botany (in press).
LoCicero, J. C., D. E. H. Frear, and H. J. Miller. 1948. The relation between chemical structure and fungicidal action in a series of substituted and unsubstituted pyridinium halides. J. Biol. Chem. 172:689-693.
Ludwig, R. A., E. Y. Spencer, and C. H. Unwin. 1960. An antifungal factor from barley of possible significance in disease resistance. Can. J. Botany 38:21-29.
Ludwig, R. A., G. D. Thorn, and D. M. Miller. 1954. Studies on the mechanism of fungicidal action of disodium ethylene bisdithiocarbamate (nabam). Can. J. Botany 32:48-54.
Ludwig, R. A., G. D. Thorn, and C. H. Unwin. 1955. Studies on the mechanism of fungicidal action of metallic ethylenebisdithiocarbamates. Can. J. Botany 33:42-59.
Lukens, R. J., and J. G. Horsfall. 1962. Fungitoxicity of *N*-substituted phthalimides. Phytopathology 52:925.
McCallan, S. E. A. 1957. Mechanism of toxicity with special reference to fungicides. p. 77-95. *In* Plant Protection Conf. 1956. Academic Press, New York.
McNew, G. L., and H. P. Burchfield. 1951. Fungitoxicity and biological activity of quinones. Contrib. Boyce Thomp. Inst. 16:357-374.
McNew, G. L., and N. K. Sundholm. 1949. The fungicidal activity of substituted pyrazoles and related compounds. Phytopathology 34:721-751.
Martin, H. 1959. Scientific principles of crop protection. 4th ed. Arnold, London. 359 p.
Martin, H. 1961. Guide to chemicals used in crop protection. 4th ed. Canada Dept. Agr., London, Ontario. 387 p.

Miller, L. P. 1960. Fungitoxicity of metal ions. Nature, (London) 185:545-546.
Miller, L. P., S. E. A. McCallan, and R. M. Weed. 1953. Rate of uptake and toxic dose on a spore weight basis of various fungicides. Contrib. Boyce Thomp. Inst. 17:173-195.
O'Brien, R. D., G. D. Thorn, and R. W. Fisher. 1958. New organophosphate insecticides developed on rational principles. J. Econ. Ent. 51:714-718.
Oxford, A. E., H. Raistrick, and P. Simonart. 1939. Studies in the biochemistry of micro-organisms. LX. Griseofulvin, $C_{17}H_{17}O_6Cl$, a metabolic product of *Penicillium griseo-fulvum* Dierckx. Biochem. J. 33:240-248.
Parker-Rhodes, A. F. 1943. Studies in the mechanism of fungicidal action. V. Nonmetallic and sodium dithiocarbamic acid derivatives. Ann. Appl. Biol. 30:170-179.
Pieters, A. J. 1962. Triphenyltin hydroxide, a fungicide for the control of *Phytophthora infestans* on potatoes, and some other fungus diseases. Proc. Brit. Insecticide Fungicide Conf. Brighton, England. 1961:145-50.
Rader, W. E., C. M. Monroe, and R. R. Whetstone. 1952. Tetrahydropyrimidine derivatives as potential foliage fungicides. Science 115:124-125.
Rich, S. 1960. Fungicidal chemistry, p. 553-602. *In* J. G. Horsfall and A. E. Dimond, [ed.], Plant pathology Vol. 2. Academic Press, New York.
Rich, S., and J. G. Horsfall. 1954. Fungitoxicity of ethylenethiourea derivatives. Science 120:122-123.
Rich, S., and J. G. Horsfall. 1961. Fungitoxicity of carbamic and thiocarbamic acid esters. Conn. Agr. Expt. Sta. (New Haven) Bull. 639:1-95.
Richmond, D. V., and E. Somers. 1962. Studies on the fungitoxicity of captan. I. The structural specificity of captan and six *n*-trichloromethylthio analogues. Ann. Appl. Biol. 50:33-43.
Richmond, D. V., and E. Somers. 1962. Studies on the fungitoxicity of captan. II. The uptake of captan by conidia of *Neurospora crassa*. Ann. Appl. Biol. 50:45-56.
Ross, R. G., and R. A. Ludwig. 1957. A comparative study of fungitoxicity and phytotoxicity in a homologous series of *N*-*n*-alkyl ethylenethioureas. Can. J. Botany 35:65-95.
Sasse, K. 1960. A new group of non-phosphorus acaricides. Hofchen-Briefe Wiss. U. Praxis 13:197-207.
Seume, F. W., and R. D. O'Brien. 1960. Potentiation of the toxicity to insects and mice of phosphorothionates containing carboxy ester and carboxy amide groups. Toxicol. Appl. Pharmacol. 2:495-503.
Sharvelle, E. G. 1961. Nature and use of modern fungicides. Burgess Publishing Co., Minneapolis, Minn. 308 p.
Smissman, E. E., J. B. LaPidus, and S. D. Beck. 1957. Isolation and synthesis of an insect resistance factor from corn plants. J. Am. Chem. Soc. 79:4697-4698.
Somers, E. 1959. Fungitoxicity of metal ions. Nature (London) 184:475-476.
Somers, E. 1960. Fungitoxicity of metal ions. Nature (London) 187:427-428.
Somers, E. 1961. The fungitoxicity of metal ions. Ann. Appl. Biol. 49:246-253.
Sosnovsky, G. 1956. Trichloromethylthio- derivatives of biological interest. J. Chem. Soc. 1956:3139-3141.
Spencer, E. Y. 1961. The dissimilar selective toxicity of two vinyl phosphorothionate isomers (thiono Phosdrin). Can. J. Biochem. and Physiol. 39:1790-1792.
Stoessl, A. (unpublished results).
Summers, L. A., and J. A. W. Turner. 1963. Fungitoxicity of 2,4-dichloro-3,5-dinitrobenzoates. Nature. (London) 197:495-496.
Tehon, L. R. 1951. Fungistatic potencies of some fluorinated *p*-benzoquinones. Science 114:663-664.
Thomas, W. D., Jr., P. H. Eastburg, and M. D. Bankuti. 1962. The comparative fungicidal properties of acidic nitrogen derivatives of poly-alkyl sulfenyl chlorides. Phytopathology 52:754.
Thorn, G. D., and R. A. Ludwig. 1954. The aeration products of disodium ethylene bisdithiocarbamate. Can. J. Chem. 32:872-879.
Thorn, G. D., and R. A. Ludwig. 1962. The dithiocarbamates and related compounds. Elsevier Monographs, New York. 298 p.
Tisdale, W. H., and I. Williams. 1934. Disinfectant. U. S. Patent No. 1,972,961.

Uhlenbroek, J. H., and M. J. Koopmans. 1957. Investigations on agricultural fungicides. II. Compounds structurally related to trichloromethyl thiolsulphonates. Rec. Trav. Chim. 76:657-665.
Uhlenbroek, J. H., M. J. Koopmans, and H. O. Huisman. 1957. Investigations on agricultural fungicides. I. Trichloromethyl thiolsulphonates. Rec. Trav. Chim. 76:129-146.
Virtanen, A. I., and P. K. Hietala. 1955. 2(3)-Benzoxazolinone, an anti-Fusarium factor in rye seedlings. Acta Chem. Scand. 9:1543-1544.
Virtanen, A. I., P. K. Hietala, and O. Wahlroos. 1957. Antimicrobial substances in cereals and fodder plants. Arch. Biochem. Biophys. 69:486-500.
Waeffler, R., R. Gasser, A. Margot, and H. Gysin. 1955. Über organische Fungizide (Sulfonamid-Derivate). Experientia 11:265-266.
Wellman, R. H., and S. E. A. McCallan. 1946. Glyoxalidine derivatives as foliage fungicides. I. Laboratory studies. Contrib. Boyce Thomp. Inst. 14:151-160.
White, R. W. 1954. The structure of the thiuram oxides. Can. J. Chem. 32:867-871.
Wolf, C. N., P. H. Schuldt, and M. M. Baldwin. 1955. *s*-Triazine derivatives- A new class of fungicides. Science 121:61-62.
Woodcock, D. 1961. Structure-activity correlations of some antifungal compounds. Soc. Chem. Ind. (London), Monograph 1961 (15):92-105.
Zentmyer, G. A. 1943. Mechanism of action of 8-hydroxyquinoline. Phytopathology 33:1121.
Zentmyer, G. A. 1944. Inhibition of metal catalysis as a fungistatic mechanism. Science 100:294-295.

Discussion

Block: Let me say first how honored I am to be invited here and that, on my part, I have just returned the courtesy by joining the American Phytopathological Society. This may be hard to explain at home, since I am in chemical engineering—imagine a chemist working in a department of chemical engineering joining the American Phytopathological Society. But really, the reason for my joining the phytopathologists is, as you see here today, that this discipline provides the best forum I know for people interested in fungicides and their action.

In 35 minutes Dr. Spencer covered many things. Let me comment on a few to help provoke discussion. Dr. Spencer began with the subject of selectivity and pointed to the fact that, based upon the amount of toxicant on the organism rather than the amount merely in solution, fungicides are not nearly as toxic as insecticides. Many insecticides are toxic in amounts of just a few ppm whereas the ED_{50} for most fungicides is greater than 1000 ppm. Thus, fungicides are both nonselective and low in toxicity.

This brings to mind the work of Cavallito (J. Biol. Chem. 164:29, 1946). He was interested in the selectivity of penicillin as compared with certain other antibiotics. He added cysteine, thioglycolate, and other thiols to solutions of these antibiotics and measured the rates at which the antibiotics were inactivated. He found that cysteine rapidly inactivated all of the antibiotics but that the other materials operated more

slowly or not at all. He correlated the selectivity of the antibiotic with its inactivation. The antibiotics which were more generally reactive, that is, which were inactivated rapidly by most compounds, were nonselective whereas penicillin which was selective was inactivated rapidly only by cysteine.

Living matter is abundant in proteins and enzymes containing the very reactive thiol group. These groups may be found in constitutional protein, not as vital to the cell function as the functional thiol groups of vital enzymes. But they are present and capture the toxicant molecules randomly, preventing most of them from reaching their target. It is like shooting an elephant with buckshot. With enough buckshot you can kill the elephant, but one well-directed "BB" in the eye of the animal will do the job.

Perhaps we ought to look not only for activity but for selectivity as well. For example, we might screen our fungicides against thiol compounds and select those that are not tied up. These materials should be able to push through and reach their sites of action rather than become bound by nonvital cellular components. Statistically, those compounds that are easily bound by cellular thiols do not have much chance to get far. Look at our fungicides. All that I know about are reversed by thiols, and they are nonselective and low in activity as indicated by the true ED_{50}.

All through Dr. Spencer's paper, he referred to so many carbons on the side chain to get the greatest activity. This is a big numbers game we all play. Some years ago, Dr. Ferguson, as Dr. Spencer has mentioned, gave us an explanation for this phenomenon and Ross and Ludwig (Can. J. Botany 35:65, 1957) used it in their study of the alkyl ethylenethiourea series.

As we go up a homologous series, we find the toxic concentration decreases up to a point after which the toxicity drops off to zero. Now, let us assume that the inherent toxicity goes on increasing but the solubility becomes too little. If this is true, we could take an alkyl phenol molecule and extend it with methylenes all the way to Los Angeles and we should have potentially a very toxic material. The trouble, of course, would be that it would not exert its toxicity because it would be too insoluble. So we would hire a fellow with a chemical tack hammer and send him off to Pittsburgh to tack on a few sulfonic acid groups and to Chicago to stick on a few carboxyls, and then to Denver for some hydroxyls and we should end up with the most toxic fungicide in the western world. You will note that I did not include Russia. This is because Russia has a larger land mass, extending from Leningrad to Vladivostok.

Seriously though, Ferguson's Principle based upon thermodynamic activities states that for compounds manifesting "physical" toxicity,

the "true" toxicity decreases as the homologous series is ascended so our cross-country fungicide would unfortunately be a flop, though you must admit a flop of heroic proportions. But, in his work, Ferguson considered only toxicity in water solution or in the vapor phase. What would be the result if we were to use an oil system? This is not just a question of academic interest. Our friends who run jet aircraft are worried by microorganisms that grow in the fuel, plugging things up and causing corrosion. In an oil system, saturation should be reached with the least amount of compound in the lowest member of the series, and the compounds are more soluble as the carbon chain is increased. This is opposite to the case in water and should reverse the toxicity series; that is, the first member should give the greatest inhibition with the least concentration, according to Ferguson's Principle.

Of interest to this discussion is a news item that appeared last week. The Phillips Petroleum Company claim that one of their additives for jet fuel, which is used as a deicing agent, is very effective as a bactericide-fungicide. It is supposed to be more effective than many of the potent compounds we are familiar with. The composition of this material was not revealed, but my information is that it is simply the monomethyl ether of ethylene glycol plus a little glycerine. So you see, in such a system Coca Cola might prove to be a powerful toxicant, which, of course, would make Rachel Carson very happy.

I want to correct one error in Dr. Spencer's paper. Let me forward this comment, however, with the fact that the error is not Dr. Spencer's: it is mine. I can correct it, therefore, with impunity. Dr. Spencer stated that $CH_3SO_2SCCl_3$, according to Johnston et al., is nontoxic whereas the aromatic trichloromethyl thiolsulfonates are toxic. This is incorrect. I know, because I was that "et al." The compound mentioned is highly toxic. In our reported work we were looking for foliage fungicides and our technique missed compounds that were volatile, as is this one. Dr. Spencer also mentioned the work of Uhlenbroek and Koopmans who reported CH_3SSCl_3 to be nontoxic. I cannot prove this one way or the other, but I do not believe it. I would think that this would be a very toxic compound, but it is even more volatile than $CH_3SO_2SCCl_3$, and this could account for it being found nontoxic. My reason for believing it to be toxic is that we made CCl_3SSCCl_3 which proved to be a highly active compound. Dimethyl disulfide is weakly antifungal; thus CH_3SSCCl_3 might be expected to be in-between.

This gets to the question that Dr. Spencer raised as to why the zinc salt of dimethyl dithiocarbamate was toxic whereas the methyl ester was not. Might I suggest that this may be due to the fact that the zinc salt ionizes while the ester does not. We made $(CH_3)_2NCS_2SCCl_3$ and tested it. In this case the dithiocarbamate ion can be released and the compound is as toxic as the dithiocarbamate itself.

I think I have raised a few questions. Some of you may wish to pick up some of the points from Dr. Spencer's discussion, or that I have raised.

Horsfall: With all due respect to my colleagues Dr. McCallan, Dr. Spencer, and others, who say that fungicides are weak because their activity is of the order of several thousand ppm, whereas that of DDT, let us say, is 1 ppm, I would like to remind them of Seymour Block's analogy that 15,000 buckshot are needed to kill one elephant, but if put in his eye, only one buckshot would be sufficient. DDT goes into the nerve of an insect. If one calculated the amount of nerve in an organism affected by DDT, DDT would not be effective at 1 ppm. It would probably be 1000 ppm. Since an insect is a much more complex organism than a fungus cell, you have to inhibit only a very minor portion of his anatomy until he cannot navigate anymore, and the elephant analogy is a good one. When we say that fungicides are inefficient we are only saying that we do not know what the local killing system is.

Howard: I would disagree with Dr. Spencer. I do not know what test he used, but at Rhode Island in a series of growth tests, fungi are killed 100 per cent at levels as low as 0.03 ppm with certain fungicides. I think that he is way off on killing power of some chemicals.

Miller: I would like to make a comment on what Dr. Horsfall has just mentioned. Bacteria are also very small, but there are antibiotics which will inactivate bacteria at very low concentrations on the basis of the amount of penicillin that is required per unit weight of bacteria. With penicillin this is in the order of about 1μg per g, while we do not have any fungicide that even approaches this in innate toxicity. I think Dr. Howard was talking about the concentration in the applied solution, and results can be very misleading when toxicity is expressed on this basis.

Kuć: Would either the discussion leader or speaker care to comment on the mechanism by which tetrachlorobenzoquinone is decomposed by light and thereby loses its fungitoxicity?

Spencer: All I will say in that connection is that one of our fellows is irradiating maleic hydrazide and getting some very interesting degradation products, but he assures me that it is very rash for anyone to postulate a mechanism. You can postulate almost anything, but you cannot prove it.

Burchfield: It has been shown that tetrachlorobenzoquinone decomposes on exposure to light, but the products are unknown. However, the photolysis of benzoquinone itself has been studied and the products formed are reported in the literature. These include hydroquinone and a dimer.

Fungitoxic Mechanisms

Hugh D. Sisler

Department of Botany, University of Maryland, College Park

I. Introduction

Chemical control of fungal diseases of plants is at present primarily a matter of protection. Various toxicants, ranging from inorganic compounds to antibiotics, are used for this purpose. The outstanding features of these compounds are their high toxicity to fungi and low toxicity to higher plants and animals. It is the purpose of this discussion to examine aspects of the toxic mechanisms of some of these compounds and to consider factors which relate to their selective action.

II. Inhibition of Cell Wall Synthesis

Selective toxicity of penicillin and certain other antibacterial agents is related to inhibition of synthesis of polymers unique to the bacterial cell walls (Salton, 1960; Strominger, 1962). The action of these compounds leads to the production of osmotically-fragile forms and ultimately to death of cells by lysis.

Presence of chitin in cell walls of many fungi and its absence in higher plants suggests the possibility that interference with chitin synthesis might provide a basis for selective toxicity simlar to that of pencillin. In fact, there is evidence that toxicity of the antibiotic, griseofulvin, may be associated with inhibition of chitin synthesis. Brian (1949, 1960) noted a strong general correlation between antibiotic-induced hyphal distortion and the presence of chitin in fungal walls.

The idea that inhibition of chitin synthesis should produce lethal osmotic fragility in fungi could be misleading, however, because this polymer may not be primarily responsible for cell wall strength in many species. In *Neurospora sitophila*, for instance, Owens et al. (1958) showed that only about 5 per cent of the wall material is chitin, whereas hemicelluloses and cellulose account for 53 and 32 per cent, respectively. Cell walls of *Saccharomyces cereviseae* contain only about 3 per cent chitin and are composed mainly of glucan, mannan, and protein (Falcone and Nickerson, 1956). Blumenthal and Roseman (1957) showed that the chitin content of fungi varies widely. In some *Aspergillus* spp., 25 per cent of the dry weight of the cells is chitin, but in *Glomerella cingulata* chitin accounts for only 3.8-6.3 per cent of the dry weight.

Thus in *N. sitophila, G. cingulata, S. cereviseae,* and other fungi where chitin content is low, polymers other than chitin may be mainly responsible for stabilizing the protoplasts.

The possibility that certain antifungal antibiotics affect wall synthesis in *S. cereviseae* was investigated by Schockman and Lampen (1962). No appreciable difference was found in the sensitivity of walled cells or protoplasts to nystatin, filipin, pimaricin, candicidin, cycloheximide, or streptimidone. This indicates that interference with cell wall synthesis is not their primary mode of action.

There is likewise no evidence that any of the fungicides in common use interefere specifically with cell wall synthesis. Whether chitin synthesis or synthesis of other cell wall components will provide a basis for selective control of plant pathogenic fungi remains to be determined. Success should be most readily achieved in species where a single wall component is critical in stabilizing the protoplast. Clearly, additional knowledge of fungal walls is needed for a rational approach to this problem.

III. Effects on Permeability

Several types of fungicidal compounds cause leakage of metabolites from cells. This phenomenon is usually attributed to changes in membrane permeability and is frequently encountered in the toxic action of ionic surface-active agents, but it has also been noted to occur with Ag^{+} (Miller and McCallan, 1957; Tröger, 1958), Cu^{++} (Tröger, 1958; Richardson and Thorne, 1962), polyene antibiotics (Scholz et al., 1959; Kinsky, 1961), sodium-N-methyldithiocarbamate (Wedding and Kendrick, 1959) and maneb (Morehart and Crossan, 1962). A direct action of the inhibitors on the cell mebranes is probably responsible for these changes in permeability, although osmotic damage may result from activation or release of lytic enzymes or a weakening of the cell wall structure. In fact, leakage of metabolites, which are capable of passing freely through the cytoplasmic membrane, could result from damage to internal binding sites.

In addition to the fact that they cause leakage of metabolites, there is other evidence which indicates that certain of these toxicants cause membrane damage. Protoplasts of bacteria prepared from cells pretreated with a bactericidal concentration of a detergent are not sensitive to osmotic shock as are those prepared from untreated cells (Newton, 1960). This indicates that marked changes in permeability of the membrane are produced by the toxicant. Some compounds which cause leakage of metabolites also render cells capable of metabolizing exogenous substrates which are not metabolized by untreated cells because of permeability restrictions. The pyruvate ion, for instance, is not decarboxylated by untreated yeast cells, but it is readily decarboxylated by

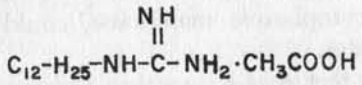
cells treated with a toxic dose of dodine (Brown and Sisler, 1960), nystatin (Sutton et al., 1961; Kottke and Sisler, 1962) or glyodin (Kottke and Sisler, 1962). Pyruvate decarboxylase apparently becomes accessible to the pyruvate ion after permeability of cells is altered by the toxicants.

Loss of ability to maintain metabolites concentrated within the cell is sufficient to cause death. Drastic metabolic changes undoubtedly result when critical metabolites are lost. Therefore, it should be established early in studies of fungitoxic mechanism whether a compound alters permeability. Otherwise, metabolic alterations, in cases where membrane damage occurs, may be erroneously attributed to a direct action of the toxicants on metabolic reactions in the cells.

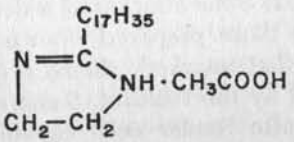
It seems appropriate to mention at this point that leakage of metabolites is not a general response to all types of toxicants. Cycloheximide, iodoacetamide, or sodium azide, for instance, do not cause weight losses in *Neurospora* (Kinsky, 1961) nor does toxic concentration of captan (Montie and Sisler, 1962) cause yeast cells to leak phosphorus compounds.

Dodine and glyodin (Fig. 1) are cationic surface active agents which are used successfully in plant protection. Compounds of this type are toxic to a wide spectrum of bacteria and fungi and would probably injure protoplasts of higher plants if they came in contact with them in sufficient quantity. Apparently the cuticle and cell wall exclude the toxicants, and thus prevent injury to the plant cell. Adsorption of the toxicants by ionized galacturonic acid groups of pectin in the cell walls may play a significant role in protecting the higher plant protoplasts.

More structural specificity than is found in the synthetic surface active agents would contribute to greater selectivity. The antibiotic, nystatin, undoubtedly possesses considerable structural specificity, and it is more selective than ordinary ionic surfactants. Lampen et al. (1962)



Dodine



Glyodin

Figure 1. Structures of dodine and glyodin.

present evidence that sterols in the yeast cell membrane may be the critical binding sites for this antibiotic. Bacterial cells which contain little or no sterol do not bind the antibiotic and thus are not injured by it.

IV. Mechanism of Action of Selected Fungicides

Research on the mechanism of action of protective fungicides has shown that the effect of a toxicant is usually not limited to a specific reaction or even to a single phase of metabolism. Therefore, the toxic mechanisms of these compounds cannot be readily classified on the basis of the phase of cell metabolism which is affected. In the remaining discussion the problem will be approached from the chemical point of view and the manner in which individual compounds or classes of compounds affect various cell processes will be considered.

A. *Sulfur*

The mechanism of action of elemental sulfur has been a subject of interest for many years. It is reduced to H_2S by fungal spores but the H_2S does not account for toxicity (Miller et al., 1953). These authors suggest that sulfur acts as a hydrogen acceptor in metabolic systems and, in doing so, disturbs the normal hydrogenation and dehydrogenation reactions in the cell. Owens (1960) proposed an interesting theory that polysulfide free radicals, produced in metabolic breakdown of 8 membered rings of elemental sulfur to H_2S, may be responsible for toxicity. He suggested that the reactive polysulfide free radicals may form stable cross linkages with protein and other cellular components.

The basis of toxic action of sulfur in the cell is still not clear, but it presents one of the most intriguing unsolved problems in fungicidal mechanisms.

B. *Dithiocarbamates*

Much has been written about the mode of action of dithiocarbamates, but space permits only brief comments to be made about them here. Thorough discussion of these compounds can be found in the excellent monograph by Thorne and Ludwig (1962) and in recent articles by Rich and Horsfall (1961) and Kaars Sijpesteijn and Janssen (1959).

Dithiocarbamates are usually divided into two groups primarily on the basis of evidence for 2 different mechanisms of action. The first group, the dialkyldithiocarbamates, includes compounds like sodium dimethyldithiocarbamate and tetramethylthiuram disulfide (thiram) which are derived from dialkyl amines, whereas the second group, the monoalkyl dithiocarbamates, include compounds like sodium-N-methyldithiocarbamate (Vapam) and sodium ethylenebisdithiocarbamate (nabam)

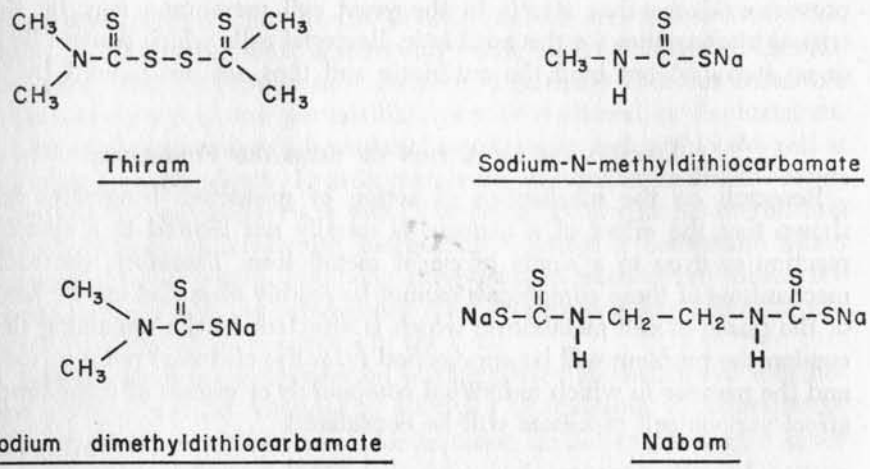


Figure 2. Structures of thiram, sodium dimethyldithiocarbamate, sodium-N-methyldithiocarbamate, and nabam.

which are derived from monoalkyl amines. Structures of these compounds are illustrated in Fig. 2.

1. *Dialkyl dithiocarbamates*

Dialkyl dithiocarbamates are strong chelating agents and it was the general thought for a while that they acted by depriving the cell of needed metals. More recent data, however, indicate that a heavy metal ion is required for high toxicity of these fungicides (Goksøyr, 1955; Kaars Sijpesteijn et al., 1957; Smale, 1957; Richardson and Thorne, 1961). According to Goksøyr (1955), a 1:1 complex of Cu^{++} and dithiocarbamate ion (D^-) is responsible for toxicity of dialkyl dithiocarbamates at low concentrations. This complex (DCu^+) presumably attaches to vital components of the cell and thus prevents growth.

It remains to be determined whether the 1:1 complex is the ultimate toxicant. Owens (1960) has discussed the possibility that free radicals rather than dithiocarbamate ions may be the active forms of thiram and ferbam.

Lowe and Phillips (1962) observed that sodium diethyl dithiocarbamate and other toxic chelating agents catalyze incorporation of copper into the diethyl ester of mesoporphyrin, and they suggested that the mode of action of these compounds might be associated with the incorporation of copper into a porphyrin precursor of an essential haem-type pigment. Toxicity of this type might be appropriately termed "lethal catalysis."

The mechanism of action of dialkyldithiocarbamates in fungal cells remains obscure, even though much research has been devoted to the

problem. Literature summarized by Thorne and Ludwig (1962) show that more than 25 enzymes of various types are inhibited by these compounds. Certainly not all of the enzymes are equally sensitive, but the fact that so many are inhibited suggests that the fungicides are not very specific in their action at the biochemical level. It would seem to be a safe assumption that a limited number of reactions are clearly more sensitive than others and that high potency of the toxicants is related to their inhibition of these reactions. Sensitive sites probably occur in resistant as well as susceptible cells. Thus specificity would be conditioned more by uptake and detoxification than by presence or absence of susceptible sites.

In the cell, toxicity of these compounds might involve one or more of the following:

(a) Chelation of required heavy metals.
(b) Attachment of a 1:1 complex of metal and dithiocarbamate ions to enzymes.
(c) Attachment of dithiocarbamate ions to metals bound to proteins.
(d) Reaction of free radical intermediates with cellular components.
(e) Lethal catalysis.

However, the chemical basis of toxic action cannot be settled until the sites of action in the cell are established.

2. *Monoalkyldithiocarbamates*

According to van der Kerk and Klöpping (1952) monoalkyldithiocarbamates (e.g., nabam and sodium-N-methyldithiocarbamate) have a mode of action which differs from that of the dialkyldithiocarbamates. The evidence for different modes of action has been summarized by Thorne and Ludwig (1962). By virtue of a reactive hydrogen on the nitrogen atom, compounds in the former group tend to undergo conversion to isothiocyanates as follows (Kaars Sijpesteijn and van der Kerk, 1954):

$$R-\underset{H}{N}-\overset{S}{\overset{\|}{C}}-S^{(-)} \longrightarrow SH^{(-)} + R-N=C=S$$

According to Kaars Sijpesteijn and van der Kerk (1954), toxicity of these dithiocarbamates is considered to be mediated via an isothiocyanate which reacts with SH groups in the following manner:

$$R-N=C=S + RSH \longrightarrow R-\underset{H}{N}-\overset{S}{\overset{\|}{C}}-S-R$$

Ludwig and Thorne (1953) found ethylenethiuram monosulfide among the decomposition products of nabam and considered it a candidate with properties which might account for the toxicity of nabam solutions. Kaars

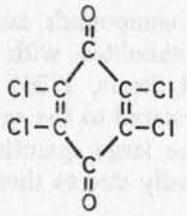
Sijpesteijn and van der Kerk (1954), however, postulated that this compound is formed via an isothiocyanate intermediate and that the two compounds are in equilibrium. The latter authors, of course, considered the isothiocyanate to be responsible for toxicity.

The fact that sodium-N-methyldithiocarbamate is a successful soil fungicide and nematocide suggests that it produces a potent volatile toxicant such as methylisothiocyanate. Formation of methylisothiocyanate from this compound in the soil has in fact been demonstrated by Gray (1962), Turner et al. (1962) and Munnecke et al. (1962). Considering all the evidence favoring the isothiocyanate hypothesis for toxicity of monoalkyldithiocarbamates, the case seems very strong. On the other hand, it is rather difficult to believe that compounds differing so slightly as some of the mono and dialkyl dithiocarbamates should be successful fungicides and yet act by entirely different mechanisms. There is evidence that isothiocyanates may not be entirely responsible for toxicity of monoalkyldithiocarbamates. Rich and Horsfall (1961) discuss this point in detail and review the evidence in opposition to the isothiocyanate theory. Nabam or its breakdown product, ethylenethiuram monosulfide, have not been shown to produce an isothiocyanate except in anhydrous chloroform in the presence of zinc sulfate or iron sulfate (Ludwig et al., 1955; Ludwig and Thorne, 1960). The biochemical data of Owens (1960) show differences in the effects of methylisothiocyanate and ethylenebisdithiocarbamates. Similarly, Wedding and Kendrick (1959) noted differences in the metabolic effects of sodium-N-methyldithiocarbamate and methylisothiocyanate. The former compound, but not the latter, caused alteration in the permeability of the mycelium of *Rhizoctonia solani*. Moorehart and Crossan (1962) demonstrated that maneb alters permeability of *Colletotrichum capsici*, which suggests that it and sodium-N-methyldithiocarbamate act by similar mechanisms.

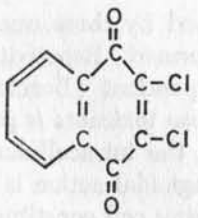
It appears that nabam, sodium-N-methyldithiocarbamate, and other dithiocarbamates of this type may, by virtue of their potential to form isothiocyanate, have an inhibitory mechanism not found in dialkyldithiocarbamates. However, a mechanism of action identical with that of dialkyldithiocarbamates is also possible.

C. *Quinones*

The quinones, dichlone and chloranil (Fig. 3), are active fungicides which have been used for several years in the control of fungal diseases. The chemical basis for the fungitoxicity of quinones has been considered in detail by McNew and Burchfield (1951) and Owens (1953). Two mechanisms of toxicity appear most likely for these compounds. They are: (a) binding of the quinone nucleus to SH and NH_2 groups in the fungus cell, and (b) disturbance of electron transport systems.



Chloranil



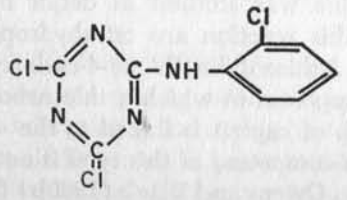
Dichlone

Figure 3. Structures of chloranil and dichlone.

Owens and Novotny (1958) showed that dichlone affects the metabolism of *Neurospora sitophila* at many sites. The fungicide inhibits phosphorylation, certain dehydrogenases and carboxylases, and inactivates coenzyme A. Both mechanisms, (a) and (b), may be involved in the toxic action of dichlone in *N. sitophila*. Coenzyme A inactivation is irreversible, which indicates that the coenzyme is bound to the dichlone nucleus by replacement of a chlorine atom (Owens and Novotny, 1958). Endogenous respiration is stimulated and oxidation is apparently uncoupled from phosphorylation. It is possible that dichlone prevents phosphorylation in the normal electron transport system or fits into an oxidation-reduction system and diverts electron transport along an abnormal pathway in which oxidation is not coupled to phosphorylation. From the standpoint of economy of the toxicant, the latter mechanism would be very efficient and might operate even when the quinone is bound to sulfhydryl or amino groups.

D. *s-Triazines*

Fungitoxicity of *s*-triazines, like dichlone, is dependent on the presence of active halogen atoms in the molecule. Burchfield and Storrs (1956) showed that Dyrene, which is 2-4-dichloro-6-(*o*-chloroanaline)-*s*-triazine (Fig. 4), will react with many metabolites including glutathione, para-aminobenzoic acid, pyridoxine, and proteins. The halogens of the *s*-tria-



Dyrene

Figure 4. Dyrene.

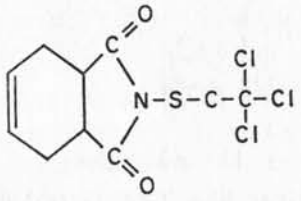
zine are displaced by these nucleophilic compounds and alkylated derivatives are formed. Reactivity of metabolites with s-triazines are strongly pH dependent (Burchfield and Storrs, 1956) and, therefore, selectivity of these toxicants is probably related to the quantity taken up by cells and to the intracellular pH. The large quantity of *s*-triazines required for fungicidal action is undoubtedly due to their indiscriminate reaction with many cell constituents.

E. *Captan and -SCCl$_3$ Compounds*

A class of fungitoxic compounds, which is of considerable interest to plant pathologists, contains the -SCCl$_3$ group. Much of what is known about the mechanism of action of this group of compounds has been derived from studies of the tetrahydrophthalimide derivative, captan (Fig. 5).

1. *Reaction with sulfhydryl compounds*

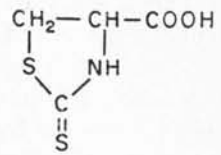
Captan is highly reactive with the sulfhydryl compounds, cysteine and glutathione (Lukens and Sisler, 1958). When these compounds are present in the medium, cells are protected from the toxic effects of captan, but toxic effects are not reversed in cells previously exposed to the inhibitor.



Captan

Figure 5. Captan.

In an attempt to establish a basis for toxic action in cells, the reaction of captan with cysteine was studied in detail by Lukens and Sisler (1958). Products of this reaction are tetrahydrophthalimide, thiophosgene, CS_2, H_2S, HCl, 2-thiazolidinethione-4-carboxylic acid (TTC) and cysteine. TTC is a compound in which a thiocarbonyl fragment derived from the -SCCl$_3$ group of captan is linked to the -SH and amino group of cysteine (Fig. 6). A compound of this type is not formed when captan reacts with glutathione. Owens and Blaak (1960b) found that glutathione yielded only one detectable product, oxidized glutathione, in reacting with captan. There is evidence that unstable trithiocarbonates are formed when captan reacts with -SH compounds (Lukens, 1959; Owens and



2–Thiazolidinethione–4–carboxylic Acid

Figure 6. 2-Thiazolidinethione-4-carboxylic acid.

Blaak, 1960b). In the breakdown of trithiocarbonates, some CS_2 is formed which contains one sulfur atom originating from captan and one originating from the reacting sulfhydryl compound (Lukens, 1959). Captan does not react directly with serine, but when serine is included in a reaction mixture with captan and cysteine, the thiophosgene released by cysteine will react with serine (Lukens and Sisler, 1958). Thus, SH compounds will release thiophosgene from the -SCCl$_3$ group of captan and it will react with more SH groups and with other types of groups as well.

These studies of the reactivity of captan suggest that one or more of the following reactions may account for toxicity:

(a) Oxidation of SH groups or formation of trithiocarbonates with cellular -SH compounds.
(b) Stripping of sulfur from SH groups.
(c) Reaction of thiophosgene, a breakdown product of captan, with cell metabolites to form derivatives of the type illustrated by TTC.

The role of these reactions in toxicity will be discussed in the following section. It should be noted at this point that CS_2 is evolved when captan is added to cultures of yeast cells (Lukens and Sisler, 1958) and the odor of thiophosgene is also evident, suggesting that reactions similar to those just discussed occur in cells treated with captan.

2. *Effects on cell growth and metabolism*

Growth-inhibitory doses of captan produce strong inhibition of respiration in cells of *Fusarium roseum* (Hochstein and Cox, 1956), *N. sitophila* (Owens and Novotny, 1959), and *S. pastorianus* (Montie and Sisler, 1962). These observations indicate that enzymes in the respiratory system of fungal cells are as sensitive to captan as spore germination or growth. Subsequent investigations of the effect of captan on metabolic reactions in this system reveal this to be the case. Owens and Novotny (1959)

showed that captan interferes with many metabolic reactions in *Neurospora,* including keto acid utilization and the sythesis of citrate from acetate. A detailed investigation of citrate synthesis (Owens and Blaak, 1960a) showed that inhibition resulted from inactivation of coenzyme A. This inhibition, however, could be reversed by mild reducing agents, such as glutathione, which suggests that a simple oxidation or trithiocarbonate formation is involved in the inactivation of coenzyme A.

The effect of captan on metabolism of S. *pastorianus* has also been investigated (Montie and Sisler, 1962). Fermentation of glucose, by intact cells or by extracts from cells pretreated with toxic doses of captan, was affected by the same magnitude as growth. Inhibition of triosephosphate dehydrogenase, a SH-dependent enzyme, apparently accounts for inhibition of fermentation.

There is little doubt that captan inhibits many vital reactions in cells where sulfhydryl groups are involved, and this inhibition is of sufficient magnitude in short-term experiments to account for inhibition of growth. However, metabolism and viability of spores of *N. sitophila* tend to recover from toxic effects of low doses of captan (Owens and Novotny, 1959). A similar recovery of cells of S. *pastorianus* has also been noted (Montie and Sisler, 1962). These fungistatic effects most likely result from oxidation of SH groups, and subsequent recovery probably results from reduction of these groups by residual metabolic activity. Thus the reversible reactions, oxidation of SH groups or trithiocarbonate formation, seem to be responsible for the fungistatic action of low doses of captan. Can an excess of reactions of this type account for the fungicidal action of higher concentrations of the toxicant? It is possible that they can, but other mechanisms must be considered in light of the fact that added SH compounds fail to reverse toxicity of higher doses of the fungicide (Lukens and Sisler, 1958; Richmond and Somers, 1962). This suggests that simple oxidation may not account for the fungicidal action. However, permeability restrictions may prevent these SH compounds from entering the cells in sufficient quantity to restore the inactivated SH groups.

The significance of thiophosgene in the toxic action of captan has not been determined, but its reactivity with compounds other than those containing SH groups suggest that it may be responsible for fungicidal effects.

Stripping of sulfur from SH groups in the breakdown of trithiocarbonates may also contribute to the fungicidal action of captan. Lukens (1963) showed that about 14 per cent of the CS_2 released when cells of S. *pastorianus* are treated with captan contains sulfur originating from cellular sources. The significance of this stripping of sulfur in the toxic action of captan is not clear, but the irreversible nature of the phenomenon indicates that it could serve as a basis for fungicidal action.

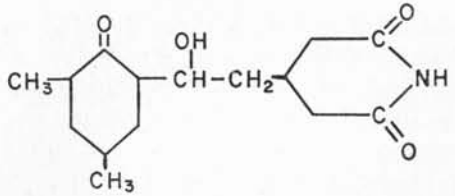
According to Richmond and Somers (1962), the large uptake of captan by spores of *Neurospora crassa* is dependent on reaction with SH groups. However, they consider the reactions with sulfhydryl groups to be detoxification rather than the essential toxic mechanism. These conclusions are based on the observations that subtoxic doses of sulfhydryl reagents, especially iodoacetate, markedly reduce the uptake of captan, but do not change its toxicity. This phenomenon is difficult to visualize, but it might be explained by the fact that sulfhydryl compounds not only decompose the $-SCCl_3$ group of captan, but also react with the decomposition products. Reaction with sulfhydryl groups may be necessary to activate captan, but further reaction of the products, thiophosgene for instance, with more sulfhydryl compounds may be in competition with fungicidal reactions and lead to detoxification. Since some SH groups are tied up by iodoacetate, there would be less uptake and activation, but the captan taken up would be relatively more effective because of less detoxification. In a system where unreacted captan was not removed during the germination period, as it was in the experiments of Richmond and Somers, sulfhydryl reagents should contribute to increased toxicity.

It would be difficult to accept a simple detoxification role for the reactions of SH groups with captan, although considerable detoxification probably occurs by this mechanism. The fact, that SH compounds react so readily with captan, suggests that they play a role in the activation of fungicide or that it is their inactivation which accounts for toxicity.

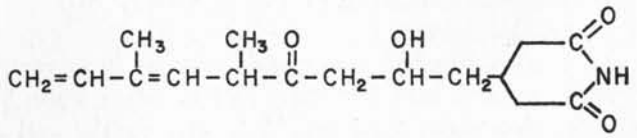
A problem in need of further investigation is the role of the R group to which the $-SCCl_3$ group is attached. It has been suggested that the R group stabilizes and regulates the reactivity of the $-SCCl_3$ group (Lukens and Sisler, 1958). However, this group may be directly involved in the final toxic action as suggested by Horsfall and Rich (1953). A common mechanism of action for the various types of R groups of $-SCCl_3$ compounds might be through reactive free radical intermediates formed when the $-SCCl_3$ group is removed in reactions with -SH compounds.

F. *Cycloheximide*

Cycloheximide is a highly potent antifungal antibiotic which is used to control certain plant diseases. The structure of this compound (Kornfield et al., 1949) and that of a closely related antibiotic, streptimidone (van Tamelen and Haarstad, 1960), are shown in Fig. 7. Both antibiotics contain a glutarimide ring, but the group attached to this structure differs somewhat in the 2 molecules. However, it can be seen that the two groups are probably synthesized from the same or similar precursors. The molecules are identical through the glutarimide ring to the 2-hydroxyethyl portion of the attached group, which suggests that this configuration may be critical for fungitoxic action.



Cycloheximide



Streptimidone

Figure 7. Structures of cycloheximide and streptimidone.

Several investigators have sought to explain the mechanism of action of cycloheximide. Effects on respiration have been studied in several organisms and various investigators have reported essentially no effect (McCallan et al., 1954; Kerridge, 1958) or varying degrees of inhibition of this process by the antibiotic (Walker and Smith, 1952; Sisler and Marshall, 1957; Tsukada et al., 1962). Reports of the effect of heavy metals on toxicity of cycloheximide indicate that they increase toxicity (Greig et al., 1958), have no effect (Gundersen, 1961), or decrease toxicity (Blumaverová and Starka, 1959). The role of heavy metals, if any, in toxicity remains to be determined, but it is possible that the metals may be involved in the attachment of the antibiotic to its site of action in the cell. Latuasan and Berends (1958) reported that cycloheximide and other imides inhibit alcohol dehydrogenase in yeast, but this could not be confirmed by Westcott (1962).

Coursen and Sisler (1960) observed that vitamin A alcohol and certain methyl ketones reverse the toxicity of low concentrations of cycloheximide to *S. pastorianus*. The significance of this antagonism is not understood, but it is interesting that part of the structure of both cycloheximide and streptimidone are apparently derived from isoprene units, which are intermediates in the synthesis of vitamin A.

Coursen and Sisler (1960) investigated the effect of the antibiotic on metabolism of *S. pastorianus* using C^{14}-labeled glucose and P^{32}-labeled phosphate. Most amino acids, several organic acids of the citric acid cycle, and a number of organic phosphorus compounds were synthesized from a C^{14} glucose substrate in cells treated with toxic doses of cycloheximide. Label in glutamic acid was sharply reduced and labeled glutamine,

which was high in untreated cells, was not detectable in treated cells. It was suggested that cycloheximide may interfere with synthesis of glutamine—a compound resembling the glutarimide portion of the antibiotic. Glutamine did not reverse the toxicity of cycloheximide and, therefore, it appears that the effects of the antibiotic are not limited to glutamine synthesis or possibly the effects on glutamine synthesis result from inhibition at other sites. The effect of the antibiotic on glutamine synthetase has not yet been determined.

Synthesis of glutamine from glutamic acid resembles protein synthesis in some respects. There is evidence from several sources which indicates that cycloheximide inhibits protein synthesis in fungi. Kerridge (1958) showed that cycloheximide causes a marked inhibition of protein and deoxyribonucleic acid synthesis in *Saccharomyces carlsbergenesis* at minimum growth-inhibiting concentrations without appreciably affecting respiration or glycolysis. Ribonucleic acid synthesis continued for a while in treated cells at a normal or moderately-inhibited rate. Similar effects of the antibiotic on protein and nucleic acid synthesis in *Aspergillus nidulans* were demonstrated by Shepherd (1958).

Siegel (1962) made a detailed study of the effect of the antibiotic on protein synthesis and other aspects of metabolism in *S. pastorianus*. The observations of Coursen and Sisler (1960), relating to the effect of the antibiotic on synthesis of amino acids, organic acids, and phosphorus compounds, were confirmed and extended. Using C^{14} glucose or P^{32} phosphate in 40-minute incubation periods, it was shown that free nucleotides and ribonucleic acid reach higher levels in treated than in control cells. Inhibition of protein synthesis was nearly complete, which is in agreement with the results of Kerridge (1958) and of Shepherd (1958). DNA synthesis was also inhibited, but to a lesser extent than protein synthesis.

The effect of the antibiotic on protein synthesis *in vitro* was determined in a system prepared from the cells of *S. pastorianus* (Siegel and Sisler, 1963). Protein synthesis in this system was remarkably sensitive to cycloheximide, being inhibited 50% by 0.2 μg/ml of the antibiotic.

Further research is needed to define the nature of the inhibition more precisely. The fact that the effects of cycloheximide on protein synthesis in cells are immediate, and the demonstration of a direct effect on protein synthesis *in vitro*, suggest that inhibitory effects on DNA synthesis are possibly secondary to those on protein synthesis. Changes noted in levels of organic acids, free amino acids, and free nucleotides are probably a reflection of the effects of the antibiotic on protein synthesis.

An outstanding feature of cycloheximide is its high potency on a μg/g basis. The 50% inhibitory dose for cells of *S. pastorianus* was determined by Westcott (1962) to be 0.38 μg/g fresh weight. These observations for high potency are borne out in the biochemical studies of its effects on

protein synthesis *in vitro*. The antibiotic apparently attacks only a limited number of sites in sensitive cells, and therefore it is not consumed in indiscriminate reactions with nonvital cellular components as is the case with organic fungicides.

Cycloheximide provides an example of what can be achieved in potency and specificity at the biochemical level. Unfortunately the sites attacked are not peculiar to fungi, and thus it falls short of being an ideal toxicant. In any case, the direction of progress in fungitoxicity will probably be toward specific metabolic inhibitors like cycloheximide. These must differ from cycloheximide, however, in that the sites which they attack should be characteristic of the pathogen, but not of the host.

One evident weakness of specific metabolic inhibitors is the ease with which organisms may develop resistance to them by a metabolic bypass. This factor may prove to be the chief obstacle to their practical success.

A number of organic fungicides have been in use for more than 10 years without notable loss in effectiveness. These compounds, being somewhat indiscriminate in their action, affect metabolism at several sites making it difficult for organisms to develop resistance to them except through impermeability or detoxification mechanisms. In this respect, these compounds are superior to more specific types of inhibitors and for this reason they may continue for some time in the future to be our best means of chemical control of plant diseases.

LITERATURE CITED

Blumaverová, M., and J. Starka. 1959. Reversal of antibiotic action of cycloheximide (Actidione) by bivalent metal ions. Nature (London) 183:261.

Blumenthal, H. J., and S. Roseman. 1957. Quantitative estimation of chitin in fungi. Bacteriol. 74:222-224.

Brian, P. W. 1949. Studies on the biological activity of griseofulvin. Ann. Botany (London) 13:59-77.

Brian, P. W. 1960. Griseofulvin. Trans. Brit. Mycol. Soc. 43:1-13.

Brown, I. F., and H. D. Sisler. 1960. Mechanisms of fungitoxic action of n-dodecylguanidine acetate. Phytopathology 50:830-839.

Burchfield, H. P., and E. E. Storrs. 1956. Chemical structures and dissociation constants of amino acids, peptides and proteins in relation to their reaction rates with 2,4-dichloro-6-(*o*-chloroanalino)-*s*-triazine. Contrib. Boyce Thompson Inst. 18:395-418.

Coursen, B. W., and H. D. Sisler. 1960. Effect of the antibiotic cycloheximide on the metabolism and growth of *Saccharomyces pastorianus*. Am. J. Botany 47:541-549.

Falcone, G., and W. J. Nickerson. 1956. Cell-wall-mannan-protein of baker's yeast. Science 124:272-273.

Goksøyr, J. 1955. The effect of some dithiocarbamyl compounds on the metabolism of fungi. Physiol. Plantarum 8:719-835.

Gray, R. A. 1962. Rate of Vapam decomposition in different soils and other media. Phytopathology 52:734.

Grieg, M. A., R. A. Nalk and A. J. Gibbons. 1958. Effect of actidione on yeast fermentation. J. Bacteriol. 75:489-491.

Gundersen, K. 1961. Cycloheximide, the active substance in *Streptomyces griseus* antagonism against *Fomes annosus*. Acta Horti Gotoburgensis XXIV:1-26.

Hochstein, P. E., and C. E. Cox. 1956. Studies on the fungicidal action of N-(trichloromethylthio)-4-cyclohexene-1,2-dicarboximide (Captan). Am. J. Botany 43:437-441.

Horsfall, J. G., and S. Rich. 1953. Fungus killers. Yale Sci. Mag. 27:12-13.

Kaars Sijpesteijn, A., and G. J. M. van der Kerk. 1954. Investigations on organic fungicides. VIII. The biochemical mode of action of bisdithiocarbamates and diisothiocyanates. Biochim. Biophys. Acta 13:545-552.

Kaars Sijpesteijn, A., and M. J. Janssen. 1959. On the mode of action of dialkyldithiocarbamates on moulds and bacteria. Antonie van Leeuwenhoek 25:422-438.

Kaars Sijpesteijn, A., M. J. Janssen, and G. J. M. van der Kerk. 1957. Investigations on organic fungicides. XI. The role of metals and chelating agents in the fungitoxic action of sodium dimethyldithiocarbamate (NaDDC). Biochem. Biophys. Acta 23:550-557.

Kerk, G. J. M. van der, and H. L. Klöpping. 1952. Investigations on organic fungicides. VII. Further considerations regarding the relations between chemical structure and antifungal action of dithiocarbamate and bisdithiocarbamate derivatives. Rec. Trav. Chim. 71:1179-1197.

Kerridge, D. 1958. The effect of Actidione and other antifungal agents on nucleic acid and protein synthesis in *Saccharomyces carlsbergensis*. J. Gen. Microbiol. 19:497-506.

Kinsky, S. C. 1961. Alterations in the permeability of *Neurospora crassa* due to polyene antibiotics. J. Bacteriol. 82:889-897.

Kornfield, E. C., R. G. Jones and T. V. Park. 1949. Structure and chemistry of actidione, an antibiotic from *Streptomyces griseus*. J. Amer. Chem. Soc. 71:150-159.

Kottke, M., and H. D. Sisler. 1962. Effect of fungicides on permeability of yeast cells to the pyruvate ion. Phytopathology 52:959-961.

Lampen, J. O., P. M. Arnow, Z. Borowska, and A. I. Laskin. 1962. Location and role of sterol at nystatin binding sites. J. Bacteriol. 84:1152-1160.

Latuasan, H. E., and W. Berends. 1958. The action mechanism of Actidione. Rec. Trav. Chim. 77:416-422.

Lowe, M. B., and J. N. Phillips. 1962. A possible mode of action of some antifungal and anti-bacterial chelating agents. Nature (London) 194:1058-1059.

Ludwig, R. A., and G. D. Thorne. 1953. Studies of the breakdown of disodium ethylene bisdithiocarbamate (Nabam). Plant Disease Reptr. 37:127-129.

Ludwig, R. A., and G. D. Thorne. 1960. Chemistry and mode of action of dithiocarbamate fungicides. Advances Pest Control Research. III. 219-252.

Ludwig, R. A., G. D. Thorne, and C. H. Unwin. 1955. Studies on the mechanism of fungicidal action of metallic ethylenebisdithiocarbamates. Can. J. Botany 33:42-59.

Lukens, R. J. 1959. Chemical and biological studies on a reaction between captan and dialkyldithiocarbamates. Phytopathology 49:339-343.

Lukens, R. J. 1963. Personal communication.

Lukens, R. J., and H. D. Sisler. 1958. Chemical reactions involved in the fungitoxicity of captan. Phytopathology 48:235-244.

McCallan, S. E. A., L. P. Miller, and R. M. Weed. 1954. Comparative effect of fungicides on oxygen uptake and germination of spores. Contrib. Boyce Thompson Inst. 18:39-68.

McNew, G. L., and H. P. Burchfield. 1951. Fungitoxicity and biological activity of quinones. Contrib. Boyce Thompson Inst. 16:357-374.

Miller, L. P., and S. E. A. McCallan. 1957. Toxic action of metal ions to fungus spores. J. Agr. and Food Chem. 5:116-122.

Miller, L. P., S. E. A. McCallan, and R. M. Weed. 1953. Quantitative studies on the role of hydrogen sulfide formation in the toxic action of sulfur to fungus spores. Contrib. Boyce Thompson Inst. 17:151-171.

Montie, T. C., and H. D. Sisler. 1962. Effects of captan on glucose metabolism and growth of *Saccharomyces pastorianus*. Phytopathology 52:94-102.

Morehart, A. L., and D. F. Crossan. 1962. The effect of manganese ethylene bisdithiocarbamate (maneb) on some chemical constituents of *Colletotrichum capsici*. Toxicol. Appl. Pharmacol. 4:720-729.
Munnecke, D. E., K. H. Domsch, and J. W. Eckert. 1962. Fungicidal activity of air passed through columns of soil treated with fungicides. Phytopathology 52:1298-1306.
Newton, B. A. 1960. The mechanism of the bactericidal action of surface active compounds: A Summary. J. Appl. Bacteriol. 23:345-349.
Owens, R. G. 1953. Studies on the nature of fungicidal action I. Inhibition of sulfhydryl-, amino-, iron-, and copper-dependent enzymes *in vitro* by fungicides and related compounds. Contrib. Boyce Thompson Inst. 17:221-242.
Owens, R. G. 1960. Effects of elemental sulfur, dithiocarbamates, and related fungicides on organic acid metabolism of fungus spores. Developments Indust. Microbiol. 1:187-205.
Owens, R. G., and H. M. Novotny. 1958. Mechanism of action of the fungicide dichlone (2,3-dichloro-1,4-naphthoquinone). Contrib. Boyce Thompson Inst. 19:463-482.
Owens, R. G., and H. M. Novotny. 1959. Mechanism of action of the fungicide captan [*N*-(trichloromethylthio)-4-cyclohexene-1,2-dicarboximide]. Contrib. Boyce Thompson Inst. 20:171-190.
Owens, R. G., H. M. Novotny, and M. Michels. 1958. Composition of conidia of *Neurospora sitophila*. Contrib. Boyce Thompson Inst. 19:355-374.
Owens, R. G., and G. Blaak. 1960a. Site of action of captan and dichlone in the pathway between acetate and citrate in fungus spores. Contrib. Boyce Thompson Inst. 20:459-474.
Owens, R. G., and G. Blaak. 1960b. Chemistry of the reactions of dichlone and captan with thiols. Contrib. Boyce Thompson Inst. 20:475-498.
Rich, S., and J. G. Horsfall. 1961. Fungitoxicity of carbamic and thiocarbamic acid esters. Conn. Agr. Expt. Sta. (New Haven) Bull. 639, 95 p.
Richardson, L. T., and G. D. Thorne. 1961. The interaction of thiram and spores of *Glomerella cingulata* Spald. & Schrenk. Can. J. Botany 39:532-540.
Richardson, L. T., and G. D. Thorne. 1962. Stimulation of spore germination and growth of *Glomerella cingulata* by copper and other heavy metal ions. Phytopathology 52:865-869.
Richmond, D. V., and E. Somers. 1962. Studies of the fungitoxicity of Captan. II. The uptake of captan by conidia of *Neurospora crassa*. Ann. Appl. Biol. 50:45-56.
Salton, M. R. J. 1960. Microbial cell walls. Wiley, New York. 94 p.
Scholz, R., H. Schmitz, Th. Bücher, and J. O. Lampen. 1959. Über die Wirkung von Nystatin auf Bäckerhefe. Biochem. Z. 331:71-86.
Shepherd, C. J. 1958. Inhibition of protein and nucleic acid synthesis in *Aspergillus nidulans*. J. Gen. Microbiol. 18: iv-v.
Shockman, G. D., and J. O. Lampen. 1962. Inhibition by antibiotics of the growth of bacterial and yeast protoplasts. J. Bacteriol. 84:508-512.
Siegel, M. R. 1962. Effect of cycloheximide on the biochemical activity of *Saccharomyces pastorianus*. Ph.D. Thesis, University of Maryland.
Siegel, M. R., and H. D. Sisler. 1963. Inhibition of protein synthesis *in vitro* by cycloheximide. Manuscript in preparation.
Sisler, H. D., and N. L. Marshall. 1957. Physiological effects of certain fungitoxic compounds on fungus cells. J. Wash. Acad. Sciences 47:321-329.
Smale, B. C. 1957. Effects of certain trace elements on the fungitoxicity of sodium dimethyldithiocarbamate. Ph.D. Thesis, University of Maryland.
Strominger, J. L. 1962. Biosynthesis of bacterial cell walls. Federation Proc. 21:134-143.
Sutton, D. D., P. M. Arnow, and J. O. Lampen. 1961. Effect of high concentrations of nystatin upon glycolysis and cellular permeability in yeast. Proc. Soc. Exptl. Biol. Med. 108:170-175.
Tamelin, E. E. van, and V. Haarstad. 1960. Structure of the antibiotic streptimidone. J. Amer. Chem. Soc. 82:2974-2975.

Thorne, G. D., and R. A. Ludwig. 1962. The dithiocarbamates and related compounds. Elsevier, New York. 298 p.
Tröger, R. 1958. Studien zur Fungicedwirkung der Schwermetallsalze. Arch. Mikrobiol. 29:430-437.
Tsukada, Y., T. Sugimori, K. Imai, and H. Katagiri. 1962. Action of cycloheximide on *Zygosaccharomyces soja*. J. Bacteriol. 83:70-75.
Turner, N. J., M. E. Corden, and R. A. Young. 1962. Decomposition of Vapam in the soil. Phytopathology 52:756.
Walker, A. T., and F. G. Smith. 1952. Effect of actidione on growth and respiration of *Myrothecium verrucaria*. Proc. Soc. Exptl. Biol. Med. 81:556-559.
Wedding, R. T., and J. B. Kendrick, Jr. 1959. Toxicity of *N*-methyldithiocarbamate and methylisothiocyanate to *Rhizoctonia solani*. Phytopathology 49:557-561.
Wescott, E. W. 1962. Uptake of cycloheximide by sensitive and resistant yeast and effect of the antibiotic on alcohol dehydrogenase. Ph.D. Thesis, University of Maryland.

Discussion

Owens: From Dr. Sisler's summary of the status of our knowledge of fungicidal mechanisms, I believe it is apparent that some progress has been made toward an understanding of biochemical mechanisms. It is equally apparent that there is much more to be learned. We do not know yet the chemical basis for observed differences in absorption of the same fungicide by different species of fungi, nor do we know the chemical basis for differences in susceptibility of various fungi to the same chemical.

The work of Burchfield and Storrs on the *s*-triazines has indicated to us the importance of pH and of the pK values of cell constituents in toxicant interactions with cytoplasmic constituents. These factors have been shown repeatedly to determine the rates of reactions between enzymes and inhibitors and it is possible that pH along with other factors, as yet unknown, play a role in species specificity.

Little is known yet about the detoxication of fungicides. Dr. Sisler pointed out the work of Richmond and Somers on captan. They consider the reaction between captan and thiols to be a detoxication reaction rather than the reaction responsible for toxicity. Reactions with excess thiols and amines certainly are detoxication reactions. This has been recognized for a long time, but I know of few studies on detoxication *per se*. What knowledge we have has been obtained incidentally from studies on reactions that have to do with toxicity.

Richmond and Somer's objections to the hypothesis that thiols are involved in captan toxicity are hardly tenable in the face of the facts. They base their objection on the finding that treatment of spores of *Neurospora crassa* with sublethal amounts of iodoacetate or iodoacetamide reduced the uptake, but not the toxicity of captan upon subsequent treatment with the latter. Our interpretation of their finding is that this

strongly supports the hypothesis because it illustrates joint action of two sulfhydryl reactants. Partial reaction of the sulfhydryl components of the spores with one reagent should result in a reduced requirement of the second if they react with the same groups. Apparently, they assumed that the iodo reagents had reacted with all available sulfhydryl groups, but had this been the case, the spores would have been killed before treatment with captan*.

Barratt: I have been away from this field of the mode of action of dithiocarbamates for nearly 15 years now, and it is interesting to see that while some progress has been made there is an element of confusion. I am wondering if anyone approached this problem with the use of isotopes? Have any studies been made using labelled dithiocarbamate derivatives? I ask this question particularly because it seems this might be a way of telling where the toxicants are accumulating. Are they mitochondrial bound or do they undergo inhibitory reactions in the soluble portions of the cell? If they are inhibiting the aconitase then one should be able to demonstrate that these compounds are indeed being bound onto the mitochondria and the mitochrondrial fractions should, therefore, be radioactive. Has anyone done any experiments along this line?

Owens: There has been a little work along this line, not specifically in the sense that you mentioned, but to find out where ferbam and thiram accumulated in higher plants. I do not know of any work specifically on the intercellular distribution of dithiocarbamates in spores.

Barratt: I just want to comment that we should be very careful not to consider the fungal cell as a bag of enzymes.

Owens: I agree with you. The fact that they inhibit enzymes which are associated with mitochondria would suggest that they do get to the mitochondria.

Barratt: The fact is that activity of aconitase in an active cell system is not the same as the activity of aconitase from the mitochondrial fraction.

Owens: We were working with intact spores. We incubated intact spores with acetate and then measured intermediates formed from acetate in the intact spores. We used different levels of toxicants and correlated inhibition at this particular site with toxicity.

Zucker: Dr. Sisler mentioned that a leakage of metabolites from fungal cells occurs in treatment with some fungicides, and he postulated a

* Dr. Owens also discussed the dithiocarbamates. This portion of the discussion is not included here because it has appeared since as a part of *Owens, R. G. 1963. Chemistry and physiology of fungicidal action. Ann. Rev. Phytopathology 1: 77-100.*

damage to cell permeability. Perhaps other mechanisms might be involved. In bacteria, excretion of metabolites can be an active process, just as uptake is active. If the fungicide inhibited uptake but not excretion then a "leakage of metabolites" would result. Was pyruvate penetration into damaged cells active or passive?

Sisler: We enter into the dispute of active vs. passive uptake here. I have a recent paper on my desk by Leggett, Olson and Spangler (Proc. Nat. Acad. Sci. U.S. 48: 1949-1962) which indicates that ion uptake (cation) by yeast is a passive process. I do not believe pyruvate entrance into treated cells is anything but a passive process. These toxicants also cause cells to leak metabolites like pyruvate. Thus a permeability barrier must have been altered.

Burchfield: I would like to make one remark on the subject of attack on the membrane. It may not always be the case that reactions which involve the cell membrane are lethal. In studies with dyrene, a biological alkylating agent, we observed that uptake by spores was very rapid. The curve soon reached a maximum after which part of the fungicide was released back into the solution. This was accompanied by the release of endogenous radioactive phosphate. This indicates that the permeability of the membrane was altered; yet this phenomenon occurred at concentrations lower than those required to prevent spore germination.

Sisler: Well, we had this in mind. We were hoping to find compounds which would alter permeability without producing lethal effects. When you get alteration of permeability, which we presume occurs when pyruvate is metabolized, the effects are lethal. In the particular case of the pyruvate ion, we could detect no metabolism unless toxic levels of the fungicides were used.

Kuć: The question I have is directed to one or both of you. Do you have direct evidence supporting the inactivation of CoA as the limiting factor in the TCA inhibition?

Owens: Are you speaking now of the inhibition of citrate synthesis by thiram?

Kuć: I am speaking about the inhibition of the oxidation of the CoA as the limiting factor in the citrate synthesis.

Owens: Yes, we have indirect evidence which I pointed out to you. First, thiram reacts *in vitro* with coenzyme A very readily just as it reacts with other thiols. We know that coenzyme A functions in this pathway because we have demonstrated it in these particular spores. We have found nothing else in the pathway between acetate and citrate which is susceptible to inhibition by mild oxidizing agents. This is the extent of our evidence.

Kuć: The point I was making is would you have to have this block at this point between pyruvate acetyl CoA to get a block in citrate synthesis?

Owens: We used acetate, not pyruvate.

Kuć: Yes, but in the normal metabolism couldn't you have a block at the glyceraldehyde dehydrogenase step which also is SH-dependent and, therefore, not have the material ever getting down to the CoA step?

Owens: We use sodium acetate so that glyceraldehyde dehydrogenase was not in the pathway we examined. You are thinking, I think, of using glucose as a substrate.

Kuć: That is right.

Owens: We were using acetate as the substrate. There are only three steps involved between converting acetate to citrate—reaction of acetate with ATP to form acetyladenylate, reaction of the latter with CoA to form acetyl CoA, and then addition of the acetyl moiety to oxalacetate to yield citrates.

Kuć: In the normal metabolism of the cell you would not just suddenly find acetate.

Owens: We provide acetate in the incubation system. This is used as a substrate to induce increases in citrate. With our treatment in this system we are able to determine whether or not the acetate is being incorporated into citrate in a normal way, and we find that it is inhibited by some dithiocarbamates and not by others.

Sussman: In view of the ubiquity of CoA, I would be interested in Dr. Owen's views of how specificity is to be invoked for the action of the fungicides with which he works.

Owens: I have not invoked specificity. As a matter of fact, I do not think that they are specific. We postulate that they will react with sulfhydryl groups wherever they occur. Coenzyme A, as you say, is ubiquitous. I think that probably every site in the cell or in metabolism where coenzyme A occurs will be inhibited. We have used this system only as a model system because we happen to know some of the components in it.

Sussman: I am referring to the cells of host plants, for example, which would be expected to have CoA, as do the microorganisms.

Owens: Yes, if we could work with bare plant cells I am sure that we would find they would be killed also. Fortunately, they are protected by coats of suberin or wax which prevent the fungicides from coming into contact with the cytoplasm in large amounts.

Uptake of Fungitoxicants By Spores

S. E. A. McCallan and Lawrence P. Miller

Boyce Thompson Institute for Plant Research, Inc., Yonkers, N.Y.

We are met here to honor and celebrate the 75th anniversary of the founding of the Department of Plant Pathology and Botany at The Connecticut Agricultural Experiment Station with the appointment of Ronald Thaxter as mycologist. It is an interesting coincidence to note that at almost the same time as this founding Wüthrich in Germany started the first studies on the uptake of copper and mercury by the spores of several species of fungi of agricultural interest. Thus, this field of study is not as new as is commonly thought, nor did it have to await the developments of the refined techniques of the mid-20th century.

Since the objective of most fungicides is to kill or to inhibit the germination of fungus spores or to a lesser extent to kill or inhibit the growth of mycelial cells, we are faced with the question of uptake of the fungitoxicant. The contact between toxicant and spore may be merely a surface one, i.e. adsorption, or the toxicant may penetrate within the spore, i.e. absorption, or probably both processes are involved. Admittedly the distinction between these actions often cannot be clearly differentiated. A survey of the now relatively extensive literature reveals increasing support for the concept that reactions take place within the spore rather than on the surface as was the more popular concept. In fact there is much evidence indicating the ready movement of materials both into and out of spores.

In general in toxicological studies it is customary to express toxicity in terms of the amount of toxicant required per unit weight of the organism. However, in dealing with such small organisms as bacteria or spores this concept of the true dose is usually ignored. It has become customary to speak in terms of the concentration of the ambient solution and more or less to ignore the quantity of spores. As will be shown, there is a very definite relation between the quantity of toxicant taken up by spores and the resulting response. When the actual amounts of toxicant taken up are known it becomes possible to plot a true dosage-response curve in contrast to the apparent dosage-response curve which is based on external concentrations.

Early Studies on the Uptake of Copper, Mercury, and Other Toxicants

As befits an historical occasion it is interesting to note the extent of the early work on the uptake of toxicants. In 1892 Wüthrich (59) demonstrated that spores of *Phytophthora infestans* and *Peronospora*

viticola readily took up copper from dilute solutions of copper sulfate. Conidia of *Claviceps purpurea* and uredospores of *Puccinia graminis* took up lesser amounts. When large amounts of copper were taken up germination was inhibited, but with only small amounts the spores were still viable. Other workers in continental Europe, notably Herzberg (14), Tubeuf (55), Hecke (13), and Volkart (56) subsequently showed that copper is not fungicidal to smut spores, but merely fungistatic. Hecke, for example, found that the copper taken up by spores of *Ustilago crameri* could not be removed by washing with water. However, when he used 0.5 per cent hydrochloric acid the copper was removed and germination proceeded similarly to that of the untreated spores. Hecke's work was quantitative and uptake values as high as 1 per cent of the spore weight were obtained within a few minutes.

In 1922 Pichler and Wöber (39) presented the results of uptake of copper, mercury, silver, and cerium by corn smut spores. Uptake was very rapid, half of the total being taken up within a minute and the total amount taken up was as high as 5 per cent of the spore weight. Silver and mercury were taken up in greater quantities than copper. Autoclaved spores took up more copper than living spores presumably because of the greater accessibility of the spore constituents. The uptake did not follow the adsorption curve.

Beginning in 1927, Bodnár and Terényi in a series of papers (2, 3, 4, 51) confirmed much of the earlier work on the uptake of copper and mercury by smut spores. They were unable to demonstrate the uptake of sulfate, chloride, nitrate, or acetate when in association with copper. The copper was found to exchange partially with hydrogen ions and with ions of alkalies such as calcium and magnesium. Uptake followed the adsorption equation. The effect of copper ammonium sulfate, unlike other forms of copper, was not reversible and hence the action was fungicidal rather than fungistatic. The uptake and toxicity of mercury was much influenced by the particular mercuric salt used. The uptake was greatest with mercuric acetate but mercuric bromide was the most toxic. In contrast to salts of copper, mercuric chloride and bromide were believed to be taken up in molecular form.

Thus it will be seen that nearly 40 years ago there was already an appreciable background of information on the uptake of fungitoxicants by fungus spores. Further details on these and later studies are given in the review by McCallan and Miller (22).

Determination of Uptake Using Labeled Fungitoxicants

By the use of modern radioisotope techniques it is possible to determine more readily very small quantities of the ions or compounds used as fungitoxicants. Also it is possible to label spores so that the effects of

the toxicants on the outward movement of cellular constituents can be measured. Samples of the spores being treated can be removed at appropriate intervals during the uptake studies so that the effects on germination can be directly related to the quantity of toxicants removed from solution by the spores. In 1950, Miller and McCallan initiated a series of studies (18, 20, 21, 22, 24, 25, 26, 29, 30, 31, 33, 34, 35, 57) on the uptake of toxicants employing radioisotope techniques. Spores of a large number of different species were used ranging in mean size from 5 to over 8,000 cubic microns (19, 32). The toxicants investigated were silver, mercury, zinc, cadmium, copper, cerium, 2-heptadecyl-2-imidazoline (glyodin base),2,3-dichloro-1,4-naphthoquinone (dichlone), *n*-dodecylguanidine acetate (dodine), and ferric dimethyldithiocarbamate (ferbam).

In general for most toxicants and spores of most species of fungi studied the uptake was rapid and the quantities taken up large in terms of spore weight. For example when spores of *Neurospora sitophila* were exposed to a dose of 2,160 μg of glyodin base per gram of spores, 1,525 μg were taken up within the first half minute. At the end of 15 minutes, 1,740 μg had been taken up. When the amount of glyodin base available was in large excess the amount taken up relative to the total was naturally less. When 34,000 μg per gram were available, 13,600 μg were taken up in the first 0.5 minute and 26,300 at the end of 15 minutes exposure (33). Similar results have been obtained with the uptake of silver (33), mercury, and other toxicants (35). The uptake of cadmium and of zinc by the species studied was generally slower and the quantities taken up relatively small. However, prolonged exposure increased uptake and toxicity (30).

Spores of different species vary widely as to the quantities of toxicant they will take up. The relative quantities taken up are not consistent from toxicant to toxicant nor is there a relationship between the degree of toxicity and ease of uptake. There is specificity between species as to uptake as well as to toxic effects. Spores of a given species may take up very large amounts without effect on germination, while those of another species may take up less with pronounced toxic effects. For example, spores of *Neurospora sitophila* take up large amounts of glyodin base while those of *Aspergillus niger* take up much less, while with cerium the reverse holds.

Tomizawa et al. (52) have recently demonstrated that Hg^{203}-labeled phenylmercuric acetate, and S^{35}- and Zn^{65}-labeled zinc ethylene bisdithiocarbamate (zineb) are rapidly taken up from dilute solution by spores of *Pirucularia oryzae, Cochlioblus miyabeanus,* and *Colletotrichum lagenarium.* Similarly Brown and Sisler (5) found that dodine was rapidly taken up by yeast cells, and by spores of *Monilinia fructicola.*

Ross and Ludwig (43) found that among the alkylethylenethioureas

the octyl compound was taken up by spores of *Monilinia fructicola* in amounts equivalent to 2,300 μg per gram without preventing germination. By employing spectrophotometric methods Somers (46) has shown the rapid uptake of a homologous series of alkyldimercaptathiazoles by spores of several different species of fungi. Maximum uptake took place with the amyl homologue. Quantities as high as 67,000 μg per gram of *Aspergillus niger* spores were reported.

Uptake of Sulfur

Sulfur appears to be unique among fungitoxicants in that while it is apparently readily taken up by spores it is not accumulated but is reduced and given off as hydrogen sulfide. Spores of all species appear to have this property (23) and the most active, such as *Cephalosporium acremonium*, can produce as much as 6,400 μg of hydrogen sulfide per gram of spores (32) per hour for the first two hours of exposure. This reduction occurs only with viable spores. The reaction is not a simple one between sulfur and SH compounds since carbon dioxide in equimolar quantities is one of the products (21). The formation of hydrogen sulfide from sulfur may actually be a detoxification process. Probably toxicity results from an interference with the normal hydrogenation and dehydrogenation process of the spore (32).

Dosage-response Curves and ED_{50} Values

When the quantities of toxicant taken up are plotted against the germination response on log-probability paper, straight lines usually result as shown, for example, for glyodin base against *N. sitophila* and *Venturia pyrina*, for silver against *M. fructicola, Alternaria oleracea, Aspergillus niger* and *V. pyrina* (33), and for sulfur and *N. sitophila* as measured by H_2S production (32). From such dosage-response curves the ED_{50} values may be obtained, several series of which have been published (18, 24, 28, 35). The values in μg per gram of spores are in general very high, for example, 11,500 for the effect of sulfur on *N. sitophila*, 5,800 to 9,300 for glyodin base against several fungi, 2,830 to 5,030 for mercury, and 385 to over 1,400 for dichlone. The most toxic material found to date is silver with ED_{50} values from 85 to 360 micrograms per gram of spore weight. Studies by Burchfield and Storrs (6), Owens and Novotny (38), Miller (26), and Richmond and Somers (42) have shown ED_{50} values in the range of 1,500 to 2,500 or more μg per gram of spores of various fungi for *s*-triazines, dodine, and captan.

A comparison of the efficacy of some of the fungitoxicants with various drugs, toxins, and biocides has been made by Miller and McCallan (18, 26, 29). While herbicides and insecticides are known

which give ED_{50} values of around 1 μg/g of weed or insect weight with the fungitoxicants, quantities from 85 to 10,000 or more μg/g are required. As progress continues this gap between the effectiveness of the fungicides and other biocides should gradually be eliminated.*

Uptake by Mycelial Pellets

Studies by McCallan and Miller (20) on uptake by mycelial pellets of *Aspergillus niger* and *Alternaria oleracea* showed that the rate was much slower for mycelium than for spores and uptake continued for many hours. When the pellets were comminuted to some 50 μ in size the rate of uptake of silver was increased 2- to 3-fold by *A. niger*, but comminution had no effect on uptake by *A. oleracea*. Despite the slow rate of uptake the ED_{50} values for silver and dichlone were more or less comparable for pellets and conidia.

Barnes (1) working with 8-quinolinol has likewise reported that the spores of *A. niger* are much more active in uptake than the mycelium. More recently various workers have reported on the uptake of fungitoxicants by yeast cells.

Effect of Toxicants and Ions on the Uptake of Other Toxicants

The uptake of toxicants may be markedly affected by the presence of other toxicants or ions especially when presented to the spores in simultaneous treatments as shown by Miller and McCallan (30, 31, 34, 35). Similar materials tend to interfere with one another more than unrelated ions or compounds presumably because of the competition for the same receptor sites. Thus with dissimilar compounds such as glyodin base, silver, and cerium the spores will take up these toxicants at about the same rate and same extent regardless of whether they are treated singly, simultaneously, or consecutively. On the other hand, spores treated with cerium will release some of the cerium when exposed to neodymium or other rare earths (24, 35). Tomizawa et al. (52) found that the uptake of phenylmercuric acetate was but little affected by the presence of dissimilar compounds such as zineb, copper sulfate, or dichlone.

Mercury exhibits a marked effect in reducing the uptake of silver, but the opposite is not the case since pretreatments with sliver increase

* In the report by Sisler presented at this symposium, reference was made to the work of Westcott (58) who showed that the ED_{50} value for cycloheximide was 0.38 μg/g of wet weight of cells of *Saccharomyces pastorianus*. This represents an important break-through with respect to the innate toxicity of fungitoxicants as compared to other biocides. Other toxicants of comparable effectiveness will no doubt be found in the future.

the uptake of mercury (30). When mercury is supplied as mercuric acetate the addition of bromide or iodide ions, especially the latter, greatly increases the toxicity of mercury (31). Recently Richmond and Somers (41, 42) have studied the effect of pretreating spores of *Neurospora crassa* with various metabolic inhibitors on the subsequent uptake of captan. In general there was little or no effect on germination but the uptake of captan was markedly reduced, especially by iodoacetic acid and *p*-chloromercuri benzoate. This is believed to be evidence that the high uptake of captan is a result in large part of the decomposition of captan by cell thiols before the captan reaches its site of action.

Permeation

In the whole phenomenon of toxicity of fungicides there is probably no more intriguing question than that of permeation. Do toxicants permeate into spores or are they largely adsorbed on the spore surface? For some reason there appears to be a considerable body of opinion which contends that many toxicants do not permeate the spore walls. In order to understand the nature of permeation it is first necessary to know more about the physical structure of the cell as well as its chemical nature. With the advent of the electron microscope a considerable advance has been made; unfortunately, however, the sum total of knowledge is still meager for fungus spores. Sisler and Cox (45) have reviewed this question and point out that the cell walls of fungi are complex and variable structures. Among the substances reported are chitin, cellulose, and lipids. However, the generally accepted view is that the cell wall plays a relatively unimportant role in limiting penetration of compounds of low molecular weight. The important barrier is generally believed to be the semi-permeable cytoplasmic membrane. According to the classical views of Davson and Danielli (7) the membrane is lipoprotein in nature and consists of a layer of lipids two molecules thick with a monomolecular layer of protein on the inner and outer surfaces. Hence the property of lipid solubility has been considered paramount by many in consideration of the ability of fungitoxicants to permeate the spore (40, 48).

The idea of a more or less simple membrane lying inside the cell wall and following its general shape is now largely abandoned. Newer concepts of the membranes are presented in a recent review by Miller (27). It appears that the membrane may show small penetrating invaginations or pinocytes. There are also believed to be deep folds of the membrane penetrating into the cytoplasm. It has been suggested that the membrane may actually be in motion and not a fixed system. The membrane contains many enzymes and thus reactions may occur near the cell surface. The main obstacle to free diffusion is still believed to be the thin hydrophobic lipid layer; however, the configuration of the

molecule is important in penetration and factors other than lipid solubility must be concerned.

There is also believed to be a network of membrane-bound cavities, the endoplasmic reticulum, permeating the entire cytoplasm from cell membrane to nucleus. Physiological studies indicate that not all of the cell is behind an osmotic barrier, but that certain parts are freely available to externally applied electrolytes or non-electrolytes. This part is termed the apparent free space or outer space, while the volume not so available is the apparent osmotic volume. The membranous surface of the cell is increased many times by the formation of vesicles which are thought to carry fluids and move into the innermost parts of the cytoplasm where they are broken down and the contents released. Thus with such a complex cell structure and network of membranes, emphasis on cell surface and attempted distinctions between adsorption and absorbtion become somewhat meaningless.

There is an increasing volume of direct evidence that many toxicants do penetrate within the spore or cell rather than accumulate on the outside. Thus in the first studies on uptake by spores, Wüthrich apparently demonstrated the presence of copper in the protoplasm of the spores by the ferrocyanide reaction. Goldsworthy and Green (10, 11), employing reagents that form colored precipitates with copper, showed the presence of copper within spores of *Monilinia fructicola* and *Glomerella cingulata* which had been exposed to Bordeaux mixture. The copper appeared to be distributed uniformly throughout the protoplasm and not fixed on the cell walls. Germinating spores took up more copper than dormant spores. Copper taken up by living mycelium was more firmly fixed than that taken up by mycelium previously killed. Earlier, Goldsworthy (9) had also been able to demonstrate particles of sulfur in the spores and germ tubes of uredospores of *Tranzschelia punctata* exposed to the action of lime sulfur.

Tröger has carried out extensive studies on the interaction between copper compounds and conidia of *Fusarium decemcellulare* (53, 54). He has demonstrated the presence of copper within the conidia and also has shown that conidia which have taken up copper excrete amino acid complexes of this toxicant. Mudd and Anderson (36) have given a very clear demonstration of the distribution of some metals in the cells of bacteria. Cells of *Fusabacterium, Eberthella typhosa, Shigella dysenteriae,* and *Vibrio commance* were exposed to dilute solutions of silver, lead, mercury, and nickel for 30 seconds. By means of the electron microscope the inner protoplasm was shown to be selectively darkened, but not the cell walls.

Rowley and associates (44) have disintegrated bacteria which were exposed to penicillin and have concluded that the antibiotic was not associated with the cell wall but was fixed in the interior of the cells.

Likewise Miller and McCallan (30) disintegrated by grinding, spores of *N. sitophila* which had been exposed to silver. The silver remained with the protoplasmic contents in the supernatant after the cell wall fragments had settled to the bottom. An effective demonstration of the distribution of toxicants within the cell was provided by Owens and Miller (37). They disintegrated sonically spores of *N. sitophila* and *A. niger* which had taken up C^{14}-labeled dichlone and glyodin base, and Ag^{110}, Zn^{65}, Cd^{115}, Hg^{203}, and Ce^{144}. It was concluded that little or none of the toxicant is associated with the cell walls which accounted for 35 per cent of the total weight, but instead was associated with the protoplasmic contents.

Goksøyr (8) has concluded that the uptake of dithiocarbamate compounds by yeast cells results in a very rapid equilibrium and hence surface adsorption is indicated rather than permeation. However the uptake of glyodin base, an active surface reactant, is not correlated with the surface area of spores of different species (32).

Sussman and associates in a series of papers (16, 17, 49, 50) have studied the physiology of the cell surface of ascospores of *Neurospora tetrasperma* which unlike most spores used in toxicity studies are dormant until subjected to a heat treatment. Viable spores, as well as killed spores, possess binding sites of strongly negative character to which cations are readily adsorbed. When dormancy is broken the presence of such adsorbed toxic cations prevents germination presumably because they are then able to penetrate. However, prior adsorption on the surface did not appear to result in faster penetration into the interior; nor did the blocking of the adsorbing sites cause any decrease in the uptake of silver ions. Thus it was concluded that adsorption on the surface of the cell is not a necessary prelude to penetration.

Recently Kottke and Sisler (15) have proposed that since yeast cells do not ferment the pyruvate ion under anaerobic conditions this system may be used to measure the effect of toxicants in changing the permeability of the cell by allowing the penetration of pyruvate.

Somers (47) working with Cu^{64} has recently substantiated much of the earlier work on the uptake of copper. Spores killed by heat took up more copper than viable ones and washing with dilute HCl removes much of the copper. With three species there was little copper in the cell wall fragments, but with *Alternaria tenuis* 42 per cent of the copper was distributed in the cell walls.

As indicated earlier, the uptake of silver by spores (29, 30) markedly changes the permeability and there is a loss of cellular components such as phosphorus compounds. Brown and Sisler (5) demonstrated that toxic doses of dodine notably altered the permeability of yeast cells, and to a lesser extent spores of *Monilinia fructicola*, with a resulting loss of phosphorus compounds and ninhydrin-positive substances. Recently

Gottlieb et al. (12) have reported that the antibiotic filipin enters both cell wall and cytoplasmic fractions of exposed cells of *S. cerevisiae* and causes a loss in dry weight as well as leakage of nitrogen, phosphorus, and nucleic acid compounds.

Actually there appears to be not only a ready inward movement of toxicants into spores, but also a more or less free outward movement of some of the cellular components. Spores labeled with P^{32} or S^{35} show a release of phosphorus or sulfur compounds into the ambient solution (31) when first suspended in water. Such outward movement can also be demonstrated by noting the loss of dry weight on suspension in water. This amounted to over 38 per cent in the case of *M. fructicola* as shown in Table 1 for the mean of a number of experiments. Loss is also evident for spores of *N. sitophila* and *A. niger*. This loss of cellular constituents has little or no effect on germinability. This procedure is in fact more or less routine, in obtaining spores for germination tests of fungicides.

Table 1. Percentage of dry weights of spores leached out on suspension in distilled water

Species	Per cent dry weight removed	Standard deviation
Monilinia fructicola	38.6	18.2
Neurospora sitophila	25.0	12.6
Aspergillus niger	14.4	5.4

Conclusions

The now fairly extensive data on uptake of fungicides by spores and other cells seem to warrant the following general conclusions:

1. The important criterion in studying toxicity of fungicides is to determine the uptake on a spore weight basis. Expressions of concentration of toxicant in the ambient solution without reference to the quantity of spores are somewhat meaningless.
2. In general most fungitoxicants have been shown to be taken up rapidly and in relatively large amounts.
3. There is a marked difference between fungus species in the amounts of the various toxicants taken up.
4. Mycelium and in some instances yeast cells appear to take up fungitoxicants more slowly than spores.
5. Fungitoxicants are notably less effective than are other active biocides indicating nonspecific and general enzyme poisoning.
6. There is a release of cellular contents from normal untreated spores into the aqueous medium without viability being affected.
7. Spores in general appear to be readily permeated by fungitoxicants and most of the toxic reactions probably take place within the spores.

LITERATURE CITED

1. Barnes, D. E. 1955. Mechanism of action of the fungicide copper oxinate. 116 p. Ph.D. Thesis, Univ. Florida.
2. Bodnár, J., and A. Terényi. 1930. Biochemie der Brandkrankheiten der Getreidearten. II. Biophysikalische und biochemische Untersuchungen über die Kupferadsorption der Weizensteinbrandsporen (Tilletia tritici [Bjerk.] Winter). Z. Physiol. Chem. 186:157-182.
3. ———— 1932. Biochemie der Brandkrankheiten der Getreidearten. IV. Wirkungsmechanismus der Quecksilbersalze auf die Weizensteinbrandsporen [Tilletia tritici (Bjerk.) Winter]. Z. physiol. Chem. 207:78-92.
4. Bodnár, J., Villányi, and A. Terényi. 1927. Biochemie der Brandkrankheiten der Getreidearten. I. Die Kupferadsorption der Weizensteinbrandsporen (Tilletia Tritici [Bjerk.]) aus Kupferverbindungen. Z. physiol. Chem. 163:73-93.
5. Brown, I. F., and H. D. Sisler. 1960. Mechanisms of fungitoxic action of *n*-dodecylguanidine acetate. Phytopathology 50:830-839.
6. Burchfield, H. P., and E. E. Storrs. 1957. Effects of chlorine substitution and isomerism on the interactions of *s*-triazine derivatives with conidia of *Neurospora sitophila*. Contrib. Boyce Thompson Inst. 18:429-452.
7. Davson, H., and J. F. Danielli. 1943. The permeability of natural membranes. Cambridge Univ. Press, Cambridge. 361 p.
8. Goksøyr, J. 1955. The effect of some dithiocarbamyl compounds on the metabolism of fungi. Physiol. Plantarum 8:719-835.
9. Goldsworthy, M. C. 1928. The fungicidal action of liquid lime sulfur. Phytopathology 18:355-360.
10. Goldsworthy, M. C., and E. L. Green. 1936. Availability of the copper of Bordeaux mixture residues and its absorption by the conidia of *Sclerotinia fructicola*. J. Agr. Research 52:517-533.
11. ———— 1938. Effect of low concentrations of copper on germination and growth of conidia of *Sclerotinia fructicola* and *Glomerella cingulata*. J. Agr. Research 56:489-505.
12. Gottlieb, D., H. E. Carter, J. H. Sloneker, Ling Chi Wu, and E. Gaudy. 1961. Mechanisms of inhibition of fungi by filipin. Phytopathology 51:321-330.
13. Hecke, L. Z. landwirtsch. Versuch. Oesterr. 5:22, 933. 1902; 6:765. 1903. Cited by Bodnár et al. (4).
14. Herzburg, P. Vergleichende Untersuchungen über landwirtschaftlich wichtige Flugbrandarten. Dissertation. Halle 1895. Cited by Bodnár et al. (4).
15. Kottke, M. and H. D. Sisler. 1962. Effect of fungicides on permeability of yeast cells to the pyruvate ion. Phytopathology 52:959-961.
16. Lowry, R. J., and A. S. Sussman. 1956. Physiology of the cell surface of *Neurospora* ascospores. II. Interference with dye adsorption by polymyxin. Arch. Biochem. Biophys. 62:113-124.
17. Lowry, R. J., A. S. Sussman, and B. von Böventer. 1957. Physiology of the cell surface of *Neurospora* ascospores III. Distinction between the adsorption and entrance phases of cation uptake. Mycologia 49:609-622.
18. McCallan, S. E. A. 1957. Mechanisms of toxicity with special reference to fungicides. Proc. Intern. Plant Protect. Conf., 2nd, Fernhurst Research Sta., Eng., 1956; 77-95.
19. ———— 1958. Determination of individual fungus spore volumes and their size distribution. Contrib. Boyce Thompson Inst. 19:303-320.
20. McCallan, S. E. A., and L. P. Miller. 1957. Effect of fungicides on oxygen consumption and viability of mycelial pellets. Contrib. Boyce Thompson Inst. 18:483-495.
21. ———— 1957. Equimolar formation of carbon dioxide and hydrogen sulfide when fungus tissue reduces sulfur. Contrib. Boyce Thompson Inst. 18:497-506.
22. ———— Innate toxicity of fungicides. 1958. Advances Pest Control Research 2:107-134.

23. McCallan, S. E. A., and F. Wilcoxon. 1931. The fungicidal action of sulphur. II. The production of hydrogen sulphide by sulphured leaves and spores and its toxicity to spores. Contrib. Boyce Thompson Inst. 3:13-38.
24. Miller, L. P. 1956. Use of radioactive tracers in studying fungicidal action. Conf. Radioactive Isotopes in Agriculture. East Lansing, Mich. 223-233.
25. ———— 1960. Uptake and innate toxicity of dodine (*n*-dodecylguanidine acetate) to fungus conidia. Phytopathology 50:646.
26. ———— 1961. Use of radioisotopes in plant pathological research. Proc. Southeastern Seminar on Atomic Progress in Agr. Clemson Coll., N. C. 180-194.
27. ———— 1962. Natural membranes, p. 345-367. *In* S. B. Tuwiner, [ed.], Diffusion and membrane technology. Reinhold Publishing Corp., New York.
28. Miller, L. P., and S. E. A. McCallan. 1955. Some examples of the use of radioisotopes in studies of pesticide action. Rept. Intern. Hort. Congr., 14th, Netherlands. 546-555.
29. ———— 1956. Use of radioisotopes in tracing fungicidal action. Proc. Intern. Conf. on Peaceful Uses Atomic Energy, Geneva, 1955. 12:170-176.
30. ———— 1957. Toxic action of metal ions to fungus spores. J. Agr. Food Chem. 5:116-122.
31. ———— 1960. Mechanism of action studies on the effect of fungitoxicants in destroying fungus conidia. Proc. Intern. Congr. Crop Protect., 4th, Hamburg, 1957. 2:1379-1384.
32. Miller, L. P., S. E. A. McCallan, and R. M. Weed. 1953. Quantitative studies on the role of hydrogen sulfide formation in the toxic action of sulfur to fungus spores. Contrib. Boyce Thompson Inst. 17:151-171.
33. ———— 1953. Rate of uptake and toxic dose on a spore weight basis of various fungicides. Contrib. Boyce Thompson Inst. 17:173-195.
34. ———— 1953. Accumulation of 2-heptadecyl-2-imidazoline, silver, and cerium by fungus spores in mixed and consecutive treatments. Contrib. Boyce Thompson Inst. 17:283-298.
35. ———— 1954. The use of radioisotopes in studying the affinity of various toxicants for fungus spores. Proc. Radioisotope Conf., 2nd, Oxford, Engl. 1:381-390.
36. Mudd, S., and T. F. Anderson. 1942. Selective "staining" for electron micrography. The effects of heavy metal salts on individual bacterial cells. J. Exptl. Med. 76:103-108.
37. Owens, R. G., and L. P. Miller. 1957. Intracellular distribution of metal ions and organic fungicides in fungus spores. Contrib. Boyce Thompson Inst. 19: 177-188.
38. Owens, R. G., and H. M. Novotny. 1959. Mechanism of action of the fungicide captan [*N*-(trichloromethylthio)-4-cyclohexene-1,2-dicarboximide]. Contrib. Boyce Thompson Inst. 20:171-190.
39. Pichler, F., and A. Wöber. 1922. Biologische Studien über die Adsorption aus verschiedenen Metallsalzlösugen. Biochem. Z. 132:420-438.
40. Rich, S., and J. G. Horsfall. 1952. The relation between fungitoxicity, permeation, and lipid solubility. Phytopathology 42:457-460.
41. Richmond, D. V., and E. Somers. 1962. Studies on the fungitoxicity of captan. I. The structural specificity of captan and six *n*-trichloromethylthio analogues. Ann. Appl. Biol. 50:33-43.
42. ———— 1962. Studies of the fungitoxicity of captan. II. The uptake of captan by conidia of *Neurospora crassa*. Ann. Appl. Biol. 50:45-56.
43. Ross, R. G., and R. A. Ludwig. 1957. A comparative study of fungitoxicity and phytotoxicity in an homologous series of *N*-*n*-alkylethylenethioureas. Can. J. Botany 35:65-95.
44. Rowley, D., P. D. Cooper, P. W. Roberts, and E. Lester Smith. 1960. The site of action of penicillin. I. Uptake of penicillin on bacteria. Biochem. J. 46: 157-161.
45. Sisler, H. D., and C. E. Cox. 1960. Physiology of fungitoxicity, p. 507-552. *In* J. G. Horsfall and A. E. Dimond, [ed.], Plant pathology Vol. 2. Academic Press, New York.

46. Somers, E. 1958. The uptake of a homologous series of fungicides by plant tissue and fungal spores. Can. J. Botany 36:997-1009.
47. ———— 1961. Uptake of fungicides by fungal cells. Soc. Chem. Ind. (London) Monograph. 15:54-63.
48. ———— 1962. Mechanisms of toxicity of agricultural fungicides. Sci. Prog. (London) 50:218-234.
49. Sussman, A. S., and R. J. Lowry. 1955. Physiology of the cell surface of *Neurospora* ascospores. I. Cation binding properities of the cell surface. J. Bacteriol. 70:675-685.
50. Sussman, A. S., B. von Böventer-Heidenhain, and R. J. Lowry. 1957. Physiology of the cell surface of *Neurospora* ascospores. IV. The functions of surface binding sites. Plant Physiol. 32:586-590.
51. Terényi, A. 1930. Biochemie der Brandkrankheiten der Getreidearten. III. Über die Kupferadsorption der Haferflugbrandsporen (*Ustilago avenae* [Pers.] Jans). Z. physiol. Chem. 192:274-280.
52. Tomizawa, C., T. Sato, and H. Yoshii. 1960. Intake of fungicides by fungus spores. Bull. Natl. Inst. Agr. Sci. Tokyo Ser. C, No. 12:171-180.
53. Tröger, R. 1956. Studien über die fungicide Kupferwirkung bei *Fusarium decemcellulare*. Arch. Mikrobiol. 25:166-192.
54. ———— 1961. Untersuchungen über die Wirkung von Kalzium Kupfervirbindungen auf die Oxydationsfermente und oxydablen Substanzen bei *Fusarium decemcellulare* (Brick). Zentr. Bakterol. Parasitenk. Abt. II 114:725-744.
55. Tubeuf, C. von. 1901. Studien über die Brandkrankheiten des Getreides und ihre Bekämpfung. Arb. Biol. Abt. Land-U. Forstwirtsch. Kaiserl. Gesundh. 2:179-349.
56. Volkart, A. Landwirtsch. Jahrb. Schweiz 20:443. 1906. Cited by Bodnár *et al.* (4).
57. Weed, R. M., S. E. A. McCallan, and L. P. Miller. 1953. Factors associated with the fungitoxicity of ferbam and nabam. Contrib. Boyce Thompson Inst. 17:299-315.
58. Westcott, E. W. 1962. Uptake of cycloheximide by sensitive and resistant yeast, and effect of the antibiotic on alcohol dehydrogenase. Ph.D. Thesis. University of Maryland. 27 p.
59. Wüthrich, E. 1892. Ueber die Einwirkung von Metallsalzen und Säuren auf die Keimfähigkeit der Sporen einiger der verbreitetsten parasitischen Pilze unserer Kulturpflanzen. Z. Pflanzenkrankh. 2:16-31, 81-94.

Discussion

Sussman: It is perhaps not inappropriate at this gathering to mention a saying of one of the former associates of Professor Thaxter's at Harvard. I think Dr. Horsfall will appreciate this. This is something that was given to me as a piece of advice before I went into my doctoral exam by Cap Weston, whom many of you will remember. I was worried, as most of us were at such a time, about not knowing various things, and he said, "Al, it is all very simple. If they ask you about hemoglobin in a horse, say you don't know anything about that, but in a mold. . . ." Well in any event, I introduce my discussion in this way because an ascomyceteologist, like myself, is somewhat removed from the kind of research that has been performed so elegantly and extensively by Drs. McCallan and Miller. That is, as they point out, there are considerable

differences between the spores formed even within a single species of fungus. In the case of *Neurospora,* conidia and ascospores differ in ways which are obvious morphologically. For example, in the conidium the electron micrographs that I have seen by Dr. Alex Miller of Cal Tech and Aaron Sharpkin show that there is really one single wall which perhaps although lamellate is still a single component. By contrast the ascospore is a dormant cell in *Neurospora,* which many of you will remember requires a heat shock before it will germinate. This dormant cell has a complex three-layered wall. I should like to remind you of a few of the differences, which Dr. McCallan presented accurately, from which it is readily apparent that kinetics can be very misleading. Now the kinetics he displayed for the uptake of toxicants in the conidia showed the kind of hyperbolic curve that one would assign perhaps to adsorption isotherm, the kinetics of which are almost identical to those one obtains with the ascospores. But there is a major difference and that difference resides in the possession by the ascospores of an outer coat consisting almost completely of uronic acid residues which can be stripped off. This coat, therefore, provides the ascospore with an acid character which results in its ability to accumulate bases of various kinds in rather large amounts. Therefore, all one has to do is to steep the cells in a substance like methylene blue and immediately the solution is cleared. Even killed cells, and living cells kept at 0° C, will adsorb in this way. Consequently it appears as though this is a rather nonspecific kind of adsorptive uptake which is characteristic of this cell. There are other differences in the nonspecificity attached to the uptake and so on, but I won't dwell on these. I would simply like to note that it seems to me important, in view of the differences between spores formed by the same organism, to note the diversity of mechanisms of uptake and its meaning in nature. I should like to point out that higher plants have recently been shown to possess uronic acid coats which, too, appear to function as almost ion exchange absorbers in the sense that I have suggested for ascospores. In addition I should like to point out that bacterial spores in the work of Britt and Gerhardt (J. Bact. 76:288, 1958), have been shown to accumulate basic antibiotics by much the same mechanism.

This brings me to the next subject, as a prelude to further discussion by Dr. Miller and Dr. McCallan. This concerns some of the remarks made earlier about the use of polymyxin and its effect upon cell membranes. Polymyxin, being strongly basic, was used by us very early in the game. We were interested in seeing how this naturally occurring material would affect the spores. In fact polymyxin is adsorbed by ascospores and, if left on the cell surface, it will inhibit germination. On the other hand, dormant cells are not at all affected by this antibiotic in that respiration is not impaired and polymyxin can be eluted, after which germination can proceed normally.

Now in connection with the question that Dr. Zucker asked after Dr. Sisler's paper, it is clear there is no continuing release of material by secretory mechanisms which he would like to invoke, such as might occur in other cells. You can study the release of materials, and there is very little from the germinating ascospores. On the other hand, in the presence of polymyxin they spew their guts out in the sense that many materials can be shown to be released by the cell in response to this antibiotic. This observation is not original with us, but has been shown by Few and Shulman (J. Gen. Microbiol. 9:454, 1953) and others in England for bacteria, and by Norman (Arch. Bioch. Biophys. 58:461, 1955), in higher plant roots as well.

Another thing I should like to mention, which I don't think was mentioned in the extensive review of Dr. McCallan, concerns the use of enzymes as micro-surgical devices in the study of the complexity of fungus spores and spore walls. It has been mentioned previously that spheroplasts and protoplasts have been obtained in bacteria, but to my knowledge there has been very little work in the fungi. There has been some recent work in which snail juice, the favorite material for this kind of endeavor, has been applied. I should like to note that lysozyme, which to my knowledge has been used successfully only with the bacteria and some *Actinomycetes*, has been found to be effective on a fungal system which is related to that discussed by Dr. Sisler, namely, pyruvate oxidation. In the case of lysozyme and *Neurospora* ascospores, treatment with this enzyme which is supposed to work on mucopolysaccharides is able to induce the oxidation of exogenously added pyruvate whereas cells without such treatment will not do so. In addition, these cells which respond by germinating in the presence of furfural will now be poisoned by furfural after lysozyme treatment. Whatever effect the lysozyme has appears to be a grievous one which can be reversed by the addition of the proper osmoticum. Therefore, I should like to point out that there are some new techniques which are available to the mycologists and the plant pathologists which can aid in the study of uptake. Furthermore, the diversity of creatures must be taken in account before generalizations can be made. Now I would like to turn the floor open to discussions on Dr. McCallan's paper.

Miller: I should like to mention that Dr. Sussman's suggestion about the use of enzymes is a very good one. We have done some preliminary experiments with the lysozyme and snail gut enzyme. They do have an effect and we have some indication that we can prevent the germination of the spores by the use of these enzymes. This work has only been preliminary and we are not in a position to make any report of our findings. It is evident that it is a fruitful mode of attack. We would very much like to be able to do what you mentioned has been done

with bacteria, so that we could isolate the membranes but we do not see our way clear as to how this can be done.

Chapman: I would like to ask Dr. McCallan, does the difference in the rate of uptake of silver between spores and mycelial pellets indicate a qualitative difference between the mechanism of action of this element on these two parts of the fungus, or does it indicate a quantitative difference in the same mechanism?

McCallan: I think it is probably only a difference in degree. We also, of course, have studied respiration of fungus spores and of pellets and we find that the species that we worked with the spores respire much more than does the mycelium. We think the spores are physiologically much more active than mycelium.

Sisler: I agree with Dr. McCallan that these toxicants probably go readily into the cell. I would like to know, however, how you justify your description of the cell membrane in view of the fact that fungus protoplast must be stabilized with substances like sucrose. That is, there must be a stabilizing substance like sucrose or else they will burst. Does this not imply that the membrane is continuous?

McCallan: I don't know.

Sisler: I believe Dr. McCallan said the membrane was not continuous.

Sussman: I believe Dr. Sisler is referring to the question whether pinocytosis in fact occurs? The question is really, how much evidence is there for the participation of a mechanism involving the invagination of a membrane, so that materials could be taken up by engulfment, or pinocytosis.

Miller: I think as you pointed out in your discussion, Dr. Sussman, there isn't any evidence that this is true in the case of fungus spores. There is much evidence, of course, that in other cells you have the endoplasmic reticulum in particular and various structures which indicate that the cell is far different from a sphere with a membrane around it. There isn't much information on spores, and what information there is does not indicate so far that there is an endoplasmic reticulum.

Owens: Dr. Miller may not be aware of this, I am not sure, but there are some recent papers by McAlear and Moore on electron photomicrographs showing endoplasmic reticulum in the developing ascospores of several fungi, showing the continuum between the cytoplasmic membrane of the spore and the endoplasmic reticulum just as in other cells.

Sussman: May I answer part of that Dr. Miller? Whereas this is true and it has been shown that infoldings in the membrane occur, it is necessary to prove that uptake can occur through this means. I think

Dr. Sisler's question was a functional question directed not so much to the presence of these things but to their functionality. Have they done anything on that? I suspect not—they are electron microscopists, aren't they?

Owens: I am not sure what Dr. Sisler is referring to. I think it is the bursting of cells in pure water and I believe he referred to the use of sucrose to maintain osmotic equilibrium. In fungal spores, I have had the experience that no matter what kind of solution I put them in they would remain visible; they do not burst. Other cells might, but we often suspend spores in pure water with no ill effect.

Sussman: That is certainly the case for ascospores, but we can do even better than that, I suspect. We can suspend them in concentrated sulfuric acid for a day and they will survive.

If there are no other questions, I would like to direct one which I think bears on a subject raised previously, and that concerns the question of the effect of cations as fungitoxins. There are a couple of theories which are current now, one of them being stressed by a previous speaker. This is the suggestion by Dr. Spencer that electronegativity could be correlated with the effectiveness of metals on cells of fungi. I would like to suggest another possibility which bears in turn upon something said by another speaker about sulfhydryl groups. Shaw, as some of you will probably remember, has suggested that the solubility products constant for the mercaptides of the various metals can be correlated very frequently with the toxicity. I wonder whether Dr. Miller, Dr. McCallan, or Dr. Spencer would care to comment on this aspect of fungitoxicity.

Miller: Somers wrote an article which was published in Nature in which he showed a correlation between the toxic effects of various metal ions and their electronegativity, but in an article that was published in a subsequent issue, we took the data which he presented and eliminated those cations which are not really toxic to fungus spores and showed that for the rest of the curve the correlation was not significant. He did not really accept our conclusions, and I think Dr. Spencer could tell you a little more about what he did after that. Do you wish to comment?

Spencer: You pointed that out. Somers disagreed with the suggestion that metals less toxic than zinc be disregarded since they have a measurable ED_{50}. He extended his study to more metal ions and confirmed his hypothesis of a correlation of electronegativity with ED_{50} values using a spore germination test.

Cowling: Dr. Sisler has called attention to the fact that chitin is not present in all fungi. Have studies been made to determine the composition of the cell walls of phytopathogenic fungi?

Miller: I do not know that I can really answer this, but I think as somebody mentioned earlier, Dr. Owens worked on the cell wall constituents of *Neurospora sitophila* which of course is not a phytopathogenic fungus. I think there isn't too much known of the constituents of the cell wall of the phytopathogenic fungi or others for that matter.

Sussman: Perhaps I can supplement that just a bit, Dr. Miller. There has been increasing numbers of studies on the subject for some of the fungi and very interestingly it has been revealed that even within the single group of *Phycomycetes,* there is a diversity of wall types and I should like to report on just two of these. The difference in walls is associated with the presence or absence of chitin and its concomitant occurrence with cellulose. Two groups of the *Phycomycetes* have been separated on the basis of a comparison in other ways with well-known plants like spinach, or well-known animals like a horse by the use of enzymatic studies as well.

For example, Vogel has shown that lysine metabolism of a well-known animal variety is associated with the presence of chitin, and the plant-type enzyme is associated with the presence of cellulose. Some of these studies were performed by Frey, a Swiss who used X-ray diffraction techniques which are susceptible, I think, to re-investigation now because of the need for the removal of lipids which obscure some of the patterns. The work of Aronson on *Allomyces* has revealed the presence of glucans and other materials. A student of mine is working on the characterization of these walls and has shown other sugars to be present, as well as some chitin. The work of Bartnicki-Solomon, and Walter Nickerson has shown protein as well as sugar components, so that there is some evidence accumulating to show first there is chitin in many species; second that it may or may not be associated with cellulose, glucans, and proteins. As you know it is very important in these studies to show the nature of the sugars which in fact may lead to the kind of diversity that you suggest as distinguishing the fungi from all groups of creatures. I might also add that even when one does characterize, as Dr. Owens has done, the chemicals in the conidial wall, there is another dimension that is needed, especially in the case of complex walls. This is the chemical nature of each of the components of this very complex association of polymeric materials, so that the proper morphological orientation is provided as well as its chemical nature. This we are a long way from accomplishing.

A Vignette On Roland Thaxter

James G. Horsfall

The Connecticut Agricultural Experiment Station

Seventy-five years ago on the morning of July 1, 1888, Roland Thaxter got off the horse car on Whitney Avenue in New Haven, Connecticut, walked up the Suburban (now Huntington) Street hill, and presented himself to Dr. Samuel W. Johnson as the first plant pathologist of The Connecticut Agricultural Experiment Station and, perhaps, the tenth plant pathologist in the nation.

Thus was set in motion the train of events that eventuated in this Diamond Jubilee Celebration of plant pathology at the Station.

Thaxter was convinced that *mycology* sounded more intellectual than *plant pathology,* and so he called himself mycologist. He called his new laboratory a "mycotheca" from the two Greek words "*mikos*" for fungus and "*theka*" for box. He grew his plants in what he called a "sass" garden.

Objective of This Paper

This paper comprises no biography of Thaxter. Clinton (1936) and Weston (1933) have published good biographies. This is a story of Thaxter's three rough years thrashing in the boneyard of primitive plant pathology. This is a story of his struggle to balance the science and the art of plant pathology. We modern plant pathologists still struggle with the conflict between the art and the science of plant pathology (Horsfall, 1959). At least we have had the benefit of 75 intervening years of experience and precedents. At least we have the extension plant pathologist on the one hand to represent the art, and the research worker on the other hand to represent the science. But we still have a great gray limbo in between. This was not so clear to Thaxter. He knew only the great gray area. His was the struggle to illuminate it.

His struggles did much to elevate the scientific end of plant pathology.

Thaxter's Letters

I shall attempt to tell Thaxter's tale through his letters, chiefly through his letters to his old professor, W. G. Farlow at Harvard. Methodical Farlow collected them all and bound them. They were graciously made available to me for the first time many years ago by

Professor W. H. Weston and again recently by Professor I. Mackenzie Lamb.

Thaxter had the intriguing habit of setting up bogeymen in his letters. Then he would stand back, take aim, and fire his trusty old blunderbuss at them. This propensity makes his letters a joy to read. Like the McCoys and the Hatfields, he enjoyed feuding.

The three most interesting bogeymen were (1) J. C. Arthur, his distinguished mycological contemporary, (2) the farmers of Connecticut, and (3) his employer, The Connecticut Agricultural Experiment Station. We shall quote choice bits of his battles with each. Items 2 and 3 are the ones describing his struggles to advance the science of plant pathology in the face of terrific pressure to satisfy the practical pressing problems of the day—to get on with the science while running a clinic withal.

Bogeyman, J. C. Arthur

The feud with J. C. Arthur begins in a letter to Farlow dated November 18, 1889, when Thaxter had been 17 months in his new post. He described the convention of the newly formed Association of American Agricultural Colleges and Experiment Stations.

> I found the proceedings were largely jabbering . . . I wish that the restraining hand had been behind me when I was called upon for "remarks." I ventured to suggest that I was skeptical about the value of copper compounds as fungicides *per se* and instanced the fact that various molds grew readily upon them as a possible indication in this respect. The laughter which greeted this suggestion was so derisive that I wished devoutly that I was on another planet. . . .
>
> Arthur struck me as most unpleasant not merely because he laughed at me the loudest either.

Isn't this delightful? As far as I know, Thaxter never forgave Arthur's guffaw.

By the time we next hear of Arthur, Thaxter has long been back at Harvard. His student, G. P. Clinton, by then at The Connecticut Agricultural Experiment Station, wrote Thaxter on September 8, 1902:

> I have been invited to present my smut [taxonomy] story to the North American Flora [a publication] of the New York Botanical Garden. . . . Earle . . . said Arthur was to monograph the Uredineae and contemplated a number of changes among the genera of this group. What do you and Professor Farlow honestly think about my trying anything on this after the thesis is out of the way?

Whereupon Thaxter took aim at the Botanical Garden and Arthur together:

> I have no sympathy for Bronx ways. If you get out your monograph, it will make no difference whether some ignoramous gets at it or not as far as

> your own work is concerned, which will indeed be an authoritative deliverance on the subject. Heaven deliver us from J. C. Arthur.

A year or so later on January 7, 1903, Thaxter wrote further to Clinton.

> I hoped I might see you at Washington where I was last week at the grand combination meeting of all creation [A.A.A.S.], but think on the whole you missed nothing. The various botanical meetings killed one another off to a large extent. I for one shall never go to another and to make matters worse, I got stuck in for President of the morphologists and only discovered it to my horror when it was too late to retreat. I saw a lot of people for a few minutes, Tracy, the New York gang, and the Washington gang—Spaulding, Duggar, McMillan, etc., etc., but had little satisfaction out of anybody. I told Arthur that he ought to be burned at the stake with Kunze and made myself disagreeable to Earle and others. Moral: keep away from such things.

And then on December 21, 1906, Thaxter writes to Clinton:

> . . . I dread the advent of the succeeding part [of the North American Flora] which as Arthur told me two years ago, is going "to surprise people." Personally I shall not be surprised at anything even if he abandons his aecial system and substitutes some other equally unnecessary.

The last entry I have found in the Arthur feud appears in Thaxter's letter to Clinton on April 9, 1907:

> No, I have not yet seen Smith's Uredineae (I call him Smith because I feel sure there must be something wrong about his own nomenclature) but I suppose I must come to it.

His Constituents as Bogeymen

That Thaxter considered Connecticut farmers as bogeymen shows only in his letters to his old professor Farlow at Harvard. One finds no hint of it in his files of letters to farmers, and no hint of it in his printed reports. Clearly, this was a straw man that he delighted to use for target practice.

Thaxter's feelings did not derive from embarrassment, as in the Arthur feud, but from the frustration for his basic research that derived from the pressure to solve the immediate clinical problems of the plant pathology of the day. There were no extension men to take the pressure off.

He first states his case in a long letter to Farlow on October 27, 1888, four months after he had marched up the Suburban Street hill into the pages of plant pathological history. He writes:

> I am favored with rotten potatoes and decaying apples occasionally and the prospect is that mycological department will interest the rural constituency. There was a grand pow-wow of assembled Grangers at the Station the other day, and you may imagine my sensations when I was called on for prandial remarks. There was something delightfully humorous in the whole thing, horrible as was my whole predicament. But the worst came later. A farmer writes Dr. [Director] Jenkins, having described the thoughts that had been bourne in on him "while digging some rotten potatoes" suggests that that thing would be a field for "Prof. Thaxter" and closes with this humiliating slander. "His style, judging from his remarks at the Exp. Sta. meeting will be popular with the farmer," which is sad if true.

A year later on November 30, 1889, he writes Farlow again in the same vein.

> I have half expected a letter from you for the last week to cheer me up during my second attack of malarial chills which I have just begun to leave behind. There is only one redeeming point about it which was that I was too sick last Tuesday to go to the field meeting of the Board of Agriculture which was held at the Station; at which I was expected to make some "remarks—after dinner remarks on rot by Roland Thaxter." Imagine it. I really believe there may be such a thing as a special Providence after all.

And then comes a particularly juicy exchange of letters anent Thaxter's now famous classic bulletin on "Fungicides." Farlow writes to Thaxter on April 12, 1890: "Your paper on washes, lotions, and other remedies was received this morning. I always thought I should prefer Eau Celeste. Is that the same as Eau Bénite?" And Thaxter replies by return mail:

> I am glad you appreciate the beauties of my lotion bulletin . . . Bordeaux mixture is the vilest compound imaginable, but it would give me intense satisfaction to spray a select committee of Connecticut farmers with it until they could not see of their eyes and the moss started from their backs, then to soar away to lands where farmers are unknown and the blight, rust, mildew, mold, rot, rusts, scab, scald, smut, and spot are known by bitter tasting names.

And then years later from the warm and comfortable security of Harvard to which "he soared away" he wrote Clinton on October 26, 1902: "I remember old [blank] well; and recollect, too, that after I had spent half a day perspiring over his grapes, which I personally squirted, he charged me full retail price for a couple of boxes of strawberries, but he let me spray, and at that time I considered it . . . the mark of an unusually enlightened mind."

The Station as a Bogeyman

To Thaxter, the Station was a bogeyman too, but he was a little bit confused about it. As his letters will show, he sometimes thought the Station was pretty good, sometimes pretty bad. He confused his

frustrations with his institution. His job was difficult; his research did suffer; he was indeed frustrated. His position was almost analogous to the frustrated businessman who has a hard day at the office and berates his wife when he goes home.

The job of the Station was difficult, too; its research suffered, too; and it was also frustrated. In that sense Thaxter's problems were equated to those of the Station. It is understandable that he criticized the Station for problems beyond itself.

Atwater had said in his very first report as director of the Station dated May 1, 1876 (page v): "It has been felt from the first that the more abstract scientific investigations would afford not only the proper, but also the most widely and permanently useful field of labor. But the need of a fertilizer control system was so pressing . . . that it seemed absolutely necessary to turn the first efforts in this direction." Atwater was a realist and Thaxter was, too.

The immediate problems press as hard in 1963 as in 1876, but by now there are more of us and we can now do both the abstract and the practical research. Thaxter was only one and he was dreadfully stressed. Even he did both the abstract and practical research and, thus he helped to set the pattern which has enabled the Station to thrive for these 88 years.

Thaxter's feud with the Station began early. He wrote to Farlow on the 30th day after his arrival. He says:

> Your letter of the 20th reached me safely and was most welcome particularly as Mrs. T. and I have both been dismally homesick here. . . . The fact is that just what to do here is a rather ugly question. There is no farm connected with the Station, only a small sass garden, and no adjacent farms, so that experiments with fungicides and what not must be carried on at arms length in whatever corners of the state a "mildew" or "rust" or "blight" is accommodating enough to appear. I wish you would give me a little fatherly advice.

On August 13, 1888, Farlow gave him "fatherly advice." He said: "I am sorry you feel so strongly about Connecticut. You will be more forgiving as you grow older." And on October 3, 1888, he wrote Thaxter again: "Mr. Eliot [i.e. President Charles Eliot of Harvard] told me today that you were troubled with malaria and wished to leave New Haven. Is that so?" And Thaxter replied by return mail: "I suppose that what Mr. Eliot said rests on the fact that when I was sick last summer, I wrote to Charles that if I were going to be subject to malaria here, I was afraid it might end in forcing me to leave New Haven. I am now feeling uncomfortably well and hope I may escape future trouble." "Uncomfortably well", he says!

Thaxter wrote to Farlow again early in February, 1889:

> I think there is going to be much for me to do here of one kind or another but there will never be any chance for any work outside of the "practical investigation" of diseases. This Station is nothing if not practical and lives subject to the gaze of an incredulous and exacting bucolic eye. The work of this Station is really first rate of its kind and of much value to the farming community but the attitude seems to be one of defense against adverse criticisms. . . . I do not believe that my work here will ever amount to a row of pins. Do you suppose the College will ever give you an assistant . . . and that in such case would you be satisfied with me for such a place? Barkis was never readier than I shall be to take it. The situation here is ideal of its kind and everyone is kindness itself. But I am afraid that I have more ambition to do good work than I thought and impressed with the fact that life is short and uncertain. . . . I may tell you that we are laying by in the family stocking the whole of my salary here. Perhaps some day this stocking will get full enough to warrant my giving the whole bucolic constituency a sound ducking in sulphate of copper and running away where I can be absolutely impractical or as impractical as I choose.

And Farlow gave him some more fatherly advice on February 26, 1889:

> I have been very busy and yet even if I had had time I should not have answered your last letter at once for the following reason—I could not make up my mind as to whether you were in earnest about your wish to return to Cambridge or whether you were suffering from a temporary attack of the blues. Why this desire to leave an "ideal" place?
>
> Cambridge is far from being an ideal place [Harvardmen, take notice!] . . . you were thinking of Cambridge as it seemed to you when a student. If you come back at some future time, as I hope you will, and shall try to bring about if circumstances are favorable, you will be in a very different condition than what you were before. You will certainly have to teach, and lecturing will become more and more laborious and you will have to keep the run of all sorts of things which will use up your time quite as much as the work of a practical mind now does in New Haven. You see how much my time and energy now have to be spent on what seems to be purely mechanical and clerical . . . dirty work must be done [here, too.] Do not be too discouraged where you are, but wait long enough to be able to settle in your own mind whether you really do not think the practical agricultural work is as good as unpractical teaching and herbarium work. That is one evil instead of another, and you ought not to make up your mind at once.

Even in 1889 college professors thought that teaching is a load to be borne! Farlow clearly saw that college teaching is the analogue of Station service to its constituents. Both students and farmers must be served.

On March 4, 1889 Thaxter replied:

> As concerns the matter of my going back to Cambridge, what I wrote you was not born out of blues but is what I felt before I came here and have felt ever since. . . . Here I see no future that can amount to anything. The object of the institution being primarily a rot, smut, and scab elixir, to

> speak metaphorically, for which I care as little as possible. I thought I might feel differently after being here for awhile, and it is possible that I might, but all idea of serious work must be given up if I do, not only because evenings and Sundays are not sufficient for it, but because there is only a handful of books in town of which I have the greater part. . . . As I said, this is an ideal place *of its kind* and I think I should be worse off rather than better if I left it for anywhere but Cambridge. As you say, Cambridge is not ideal by any means, but as a scientific center it is nearer to this than one can find in America. I should be sorry to be precipitate in my flight in decency to them here.

He signs it "Sincerely, Barkis."

On March 30, 1889, Farlow sent a clear, curt, and complete answer: "My life is spent in mechanical drudgery. WGF"

On November 3, 1889 Thaxter wrote to Farlow:

> They are going to send me to Washington to the medicine talk of the Experiment Stations. [Land Grant College meeting, that is. See also another note earlier.] I never went to one, but I shrink from that sort of thing. I think they ought to have a pitched battle over the potato rot to decide who shall have it for his private and particular property and fix a fine for mentioning it in anybody's bulletin. I shudder when I think what a predicament the profession would be in had not the all wise and far seeing creator *not* created the potato rot. Yet I foresee the time when the bryophytic back itself will crack under it. The cedar apple and the barberry rust will not become wholly unpresentable quite so soon. But, alas, when they are gone what form of natural or artificial selection can get up a new rust, rot, smut, scab, or maggot on which we can all comfortably lean. But pray forgive my biliousness which lies at the door of the malarial germ.

Farlow could be as pithy as Thaxter. I suspect that Thaxter imitated Farlow. Farlow replied on November 4, 1889:

> I never knew before that there was an advantage of having malaria, but there is only one thing worse than "a rot conference," smallpox, perhaps not even that for one does not recover from a "rot conference" without having his mind "pitted."
>
> I must try by my lectures to get some money to fit up my new laboratory and museum. This is a desperate world. My whole life will of necessity be spent in trying to get together the means for allowing others to work. I never hope to have any time for real work. Perhaps the next generation will be in a better position for work in consequence but they will never know it.

Thaxter could have said the same.

More pithiness. Farlow wrote Thaxter on November 12, 1889:

> I am much obliged for the *Phytophthora* paper [*Phytophthora phaseoli*, that is]. I suppose that I am a nag, but I am exceedingly glad to have two copies of your papers for one copy as you know goes to feed the little pigs in the laboratory [graduate students, that is] and the other I keep for the repacious devouring of the old hog himself.

On December 31, 1889, Thaxter wrote a long letter to Farlow about an article on original research in the Botanical Gazette. He says,

> Original research is a precious slow coach and is not wanted by the constituency for which the Stations are created. . . . They do not want pure science, they want mud pies, the sloppier the better, and as ninety-nine out of a hundred of them have idea on the subject prevalent B.C. it's quite natural. But I must stop this dreadful habit of growling.

Farlow continued to bombard Thaxter. He wrote on February 6, 1890: "A Cambridge professor is only a drudge. Real work must be done elsewhere," and Thaxter replied with some asperity in February 17, 1890: "You are ungrateful in what you say of the professors' lot at Harvard with practically five clear months for working unhampered by class exercises." No teaching load, long vacations, he says. The grass is greener beyond the fence!

Thaxter to Farlow on March 13, 1890 says: "I mail you with this a copy of my 'report' on the noxious farmers of Connecticut. I send you but one copy because the piggy-wiggies in the laboratory have not your digestive process and might get malignant dyspepsia of the mind at that." Apparently they had been discussing a successor here for Thaxter when he would arrange to go to Cambridge and Setchell was mentioned.

> In regard to his taking this position in case I should go back to Cambridge, they might be glad to have him come here provided that he would agree to stay some destined time, but I hardly think they would care to have him for a year or two with the definite prospect of his leaving at the termination of this time since it takes about a year to learn the ropes and to get on the right side of the more formal aspects, and above all to become adept in the use of the Thaxter squirt-gun. . . . The only criticism of my last year's report that has reached me from the constituents in the state, is that it is too scientific to be comprehensive to the ordinary mortal. So you can imagine what encouragement this is to do anything scientific.

The last word that I know of from Thaxter about his old Experiment Station was in a letter dated December 17, 1919 to Clinton about his recent visit there. This was 13 years before he died. He must have gone soft in his old age because he said: "I am sorry to make such a flying visit to my old and happy hunting ground; but I enjoyed myself greatly."

I suspect that Thaxter would be secretly pleased but publicly offended to learn that recently the U.S. Department of Agriculture has named a new lima bean, Thaxter, a new bean resistant to Thaxter's *Phytophthora* mentioned in his letter to Farlow on November 12, 1889.

Thaxter was a mycologist whose ideal in life was to work on such problems as interested him while enfolded in the warm and comforting cloak of his alma mater. He had to make a tremendous adjustment to

fit the new environment here. Had he been a graduate of an agricultural college there would have been nothing novel in farmers' meetings.

I think the evidence that he wrote with tongue in cheek is that he *did* make a sprayer, he *did* write a bulletin on fungicides, and he *did* think of a farmer who allowed him to do experimental spraying as having "an unusually enlightened mind."

Similarly the correspondence about the Station represents an attempt at adjustment rather than a real feud. What was more natural than for Thaxter to bare his soul to his idol at his alma mater? And Farlow's replies probably had much to do with Thaxter sticking to the job as long as he did.

Thaxter was obviously not at home here. That is not necessarily to his discredit or to the Station's work. Both did pioneer; he did make both practical and scientific contributions; and he did demonstrate that mycological science could be put to work for agriculture, and that was one of the things he was hired for.

As we said at the outset, Thaxter had no experimental farm for his research, only a small "sass garden." Sass, or garden sauce, is said to be of two kinds, long sass (as beets and carrots) and short sass (as onions, potatoes, and pumpkins). Since Thaxter worked on onions and potatoes, we may assume that Thaxter was professionally a short-sass man.

LITERATURE CITED

Clinton, G. P. 1936. Biographical memoir of Roland Thaxter 1858-1932. Nat. Acad. Sci. U.S. Biographical Memoirs 17:55-68.

Horsfall, J. G. 1959. A look to the future—The status of plant pathology in biology and agriculture, p. 63-70. *In* C. S. Holton [ed.], Plant pathology, problems and progress 1908-1958. University of Wisconsin Press, Madison.

Weston, W. H., Jr. 1933. Roland Thaxter (1858-1932), his influence on plant pathology. Phytopathology 23:565-571.

The Ever-Expanding Concepts Behind 75 Years of Plant Pathology

George L. McNew

Managing Director, Boyce Thompson Institute for Plant Research, Inc., Yonkers, N. Y.

Plant pathology has been a schizophrenic profession ever since Julius Kühn tried to assemble its body of knowledge into the first textbook on the subject (Die Krankheiten der Kulturgewächse, ihre Ursachen und ihre Verhütung) in 1858. It is neither an art nor a science but it dabbles freely in both realms.

The confusion in the collective minds of phytopathologists is well reflected in the action of their associates. The mycologists, virologists, bacteriologists, and parasitologists frequently look upon them as interlopers in their realms of pure science. The administrators of agricultural science have long been confused as to what to do with them. This is reflected by their being bound administratively at one time or another to plant physiology as at Texas and North Carolina, botany as at Purdue University and Iowa State University, agronomy at the University of Illinois, given their freedom as vegetable pathologists at the University of Wisconsin and Cornell University, or made into the handmaidens of crop specialists in the U.S.D.A. The agronomists and horticulturists have ever stood ready to annex their functions as a part of plant culture and have on occasion, as at the University of Illinois, achieved their ambitions, and retarded the normal development of a crucially needed science for almost a full half century before seeing the error of their ways. Yes, plant pathology has been a headache to the administrators.

Today, the profession has achieved a degree of autonomy, proved its right to stand on its own two feet with one planted in the realm of science and the other in the art of crop culture; but plant pathologists are not quite certain where the next step should be taken in exercising this new freedom. In the United States, some 2,200 strong, they represent a force for progress and economic security in meeting the material needs of a nation, but they look for leadership in exercising the birthright given them by society. Where shall they turn for an inspiration? Back to their antecedents or to new arenas of endeavor?

Origin and Basic Need of the Science

The science of plant pathology was spawned in the backyard of mycology. It suffered the growing pains and rebellion of youth with

bacteriology as the bacteriologists released themselves from the bonds of their mycological parent and then rushed back later to claim their birthrights as microbiologists. Plant pathology proved in its young manhood to be the pioneer in the wildernesses of virology and nematology where it cleared the forest and sowed the seeds to support the many who were to follow from the realms of bacteriology, nematology, biochemistry, and biophysics. In its seniority, it often has chosen physiology as an avocation and a hobby for its relaxed moments, and some of the more adventuresome young progeny have dared to cast an eye toward biochemistry and the realm of molecular behavior. The question the sages of the profession have been asking themselves is, "Should we turn back to the wells of these professions for strength and inspiration for the next century of progress in man's enduring and growing battle with disease in plants?" A philosophy is needed.

Unfortunately, plant pathologists' obsession with the art of plant disease regulation has prevented them from reaching the pinnacle of either science or philosophy. Some unkind wag, with a discerning mind, has observed that a scientist is a person who learns more and more about less and less in nature until eventually he knows everything about nothing. The philosopher, on the other hand, takes a broader viewpoint and learns less and less about more and more until he knows nothing about everything.

Since the end result from either approach to knowledge finds the practitioners in the same pasture of learning, it is perfectly reasonable to expect scientists to become doctors of philosophy. The plant pathologists have readily accepted the title but have rarely achieved the perfection demanded of either science or philosophy. The plant pathologists of this generation and their predecessors have learned something about a few things in phytopathology but they have escaped ultimate perfection and self satisfaction. The fact they appreciate how little they know may bid well for the future. They do, however, need a philosophy conceived from their present state of ignorance but based on the soundness of the past.

When one looks around for a model to guide the profession out of the wilderness, perhaps it is not to be found in the largest of our great universities but in one of the smaller institutions which has experienced all the pangs of growth and uncertainty but by progressive foresight and determination has always moved ahead in the forward march of plant pathology.

The Connecticut Agricultural Experiment Station offers such a case study, and we would like to return to it from time-to-time in our discussion this evening. Surely its plant pathology has been subject to schizophrenia. What could be more schizophrenic than a precise man

of Harvard such as Dr. Thaxter drawing gorgeous, beautiful illustrations of *Urocystis cepulae* by night and inventing a wash boiler sprayer for applying Bordeaux mixture by day?

Connecticut's plant pathology was started in 1888 with an invitation from the Board of Control to all agriculturists to "send in all plants suffering maladies such as. . . ." For the past 75 years, everything that is putrid, mildewed, blighted, or rotten in the State of Connecticut has been finding its way into the hands of the plant pathologists but they have been able to keep their hands clean and their minds clear to attack the big problems of disease. From the second years of Roland Thaxter to the present incumbent in the chair of plant pathology, they have been testing fungicides but have always claimed it was to learn about how chemicals behave rather than to help others control plant disease. Surely it takes a philosopher to maintain such a position for 75 years while doing a terrific practical service to the people of their state.

Plant pathology has gone through four distinct stages in developing a science and is well into its fifth phase at present. In their order of development, these were: the mystical period, when neither cause nor logic of disease induction was understood; the predisposition phase, when the agents of disease were being identified but cause and result of diseases were confounded; the etiological period, when identification of causes was predominant; the ecological period, when the influence of environment occupied a predominant place; and the physiological era that we have been gradually approaching for the past two decades. After we have discussed each of these in more detail, we may well ask ourselves, "Where next?" What are the big problems that must be solved, if the science of plant pathology must not stagnate, and its art is to be pushed on to perfection?

The Mystical Period to the End of the Dark Ages

The ancients were well aware of the significance of plant diseases: the rusts, blasts, and mildews. In an era of ignorance, the only rational idea that could be offered was that disease was a means of venting on mankind the wrath of the gods. Records may be found in the Old Testament of the Bible in Genesis 41:23; I Kings 8:37; Deuteronomy 28:22; Amos 4:9; Haggai 2:16-17; and II Chron. 6:28.

If a grandfather of plant pathology must be named, he must be Cleidemus whose observations on diseases of fig, olive, and grape were so freely quoted by Aristotle. Theophrastus elaborated on these teachings of his master. By the time of the Roman era, the stars had come to have a share in the cause of plant disease, but Pliny graciously excused the sun from responsibility. In the Roman culture, there developed

a pair of rust-gods known as Rubigus and Rubigo and an annual festival was developed to placate them. Never has plant pathology risen to such social heights since.

During the dark ages, from the fall of the Roman Empire (A.D. 476) until the seventeenth century, there were few bright spots. It may come to you as a shock to learn that the most conspicuous was an Arabian country gentleman who turned encyclopedist—Ibn-el-Awam. He wrote and lived in Seville, Spain, in the tenth century where he accurately catalogued the diseases of trees and grapes.

The mystical period in plant pathology continued through the Renaissance period. By the eighteenth century the horticulturists were attempting to name and classify plant diseases much as their hosts were being named by Linnaeus and others. This preoccupation with classifying diseases on a superficial or artificial basis is not too important except it set the psychological stage all too well for plant pathologists to be oriented toward classification of causes for the next hundred years. It was during this period that the masterly treatises of Joseph Pitton de Tournefort in France, Christian Sigismund Eysfarth in Germany, Johann Christian Fabricius the great Danish entomologist, Michael Adamson in France, Stephen Hales in England, were written; and many other observers were cataloguing diseases and gradually convincing themselves that the fungous growth associated with so many of the diseases was not a plant excrescence but were a distinct body in themselves.

The Predisposition Period

Once the idea was established that fungi were bodies in their own right and not part of a disease syndrome, the only problem remaining was to decide whether they were the cause or result of the disease condition. Beginning about the time of Franz Unger, or roughly from 1800 to 1853, there was a strong conviction that the fungi in morbid tissue were there because disease had predisposed the tissues to their invasion.

We must remember that during this period botanical science was overwhelmed with fascinating new theories and investigations on plant reaction to environment, plant physiology, sexual behavior, plant nutrition, etc. These new aspects of botany were replacing taxonomy as the intellectual star in man's research destiny, but the idea of diseases had to await the refutation of the theory of spontaneous generation by Pasteur in 1860.

The Austrian, Franz Unger, gave close attention to plant diseases in his lectures on vegetable physiology at the University of Vienna from 1830 to 1870. He classified the fungi as entophytes, i.e. disease organisms, but not as parasites. He was firmly convinced that they originated from

disease tissues even though they were distinct organisms worthy of their own names. He emphasized that internal disorganization of nutrition processes in tissues was responsible for disease. Looking back from the vantage point of 130 years, what a shame his ideas were not oriented just a shade further in the proper direction! Had his concepts been based on the real facts that disease was a new physiological state in plants born of mutual interplay or symbiosis of plant and pathogen, plant pathology might be one hundred years further ahead today than it is. Unfortunately man is not inspired or psychic and has to move tediously step by step, so this masterly movement was not to come until the concept of predisposition had been killed and true etiology of disease had been firmly established.

Those living in Unger's era were dominated by him and helped bolster his predisposition concepts in the era 1833 to 1853. In their books on plant pathology Wiegmann and Meyers expanded his concepts as they brought together knowledge on the horticultural aspects of disease.

During this era the mycologists came into their own and it was only inevitable that their great interest in taxonomy would direct their attention to the study of fungi in plant tissues. It was during this period that such classical scholars as Bulliard, DeCandolle, Tulasne brothers, Lévielle, and others did such a beautiful job of cataloguing and illustrating plant pathogens without quite revealing what their real import was. A plant pathologist needs only thumb through the brilliant and beautifully illustrated studies of the Tulasne brothers on the *Ustilaginales, Erysiphales, Urediniales,* and *Claviceps* to realize how much a man can learn while still missing the main point. However, if people such as they had done their job less well in working out the life cycles, studying the polymorphism of different species of plant fungi, and describing the general morphology of the fungi, plant pathology would have been retarded another 50 years. It is well to remember that such an eminent person as the Reverend Berkeley was drawing beautiful pictures of *Phytophthora infestans* in 1846 while people were starving to death from the loss of their potato crop. There was a crying need for a fresh viewpoint, but it was not to come until 7 years later when the cause of disease was firmly established.

The Etiological Period

One may question the exact date at which the fundamental nature of plant disease was established. Certainly Benedict Prevost in 1807 proved that bunt of wheat was caused by a fungus, but his ideas were never accepted. Modern plant pathology had to await the arrival on the scene of Henrich Anton de Bary of the Universities of Tübingen, Halle, and Strassburg. He never professed to be a plant pathologist, but he was its

father, regardless of whether he recognized his offspring or not. His doctoral dissertation on "Die Brand Pilz" in 1853 established beyond doubt that smut diseases were caused by fungi. If we do not accept this date as the origin of phytopathology, we would choose 1861 when he proved *Phytophthora infestans* caused late blight of potatoes, or 1865 when he demonstrated experimentally the long-accepted suspicion that the aecial stage of *Puccinia graminis* on barberry was responsible for starting rust outbreaks. With de Bary, plant pathology was born.

Anton de Bary may have been a mycologist who merely studied the substrate of his fungi, but surely his contemporary Julius Kühn was a real plant pathologist. His textbook on "Die Krankheit der Kulturgewächse, ihre Ussachen und ihre Verhütung" in 1858 was the first real textbook in plant pathology based on causal relations. It marked the beginning of a long period extending into the modern era.

A new breed of people sprang up to serve as disciples of de Bary and Kühn. They sometimes like to trace their ancestry back to the master of all pathology, Louis Pasteur, who in 1860 laid the theory of spontaneous generation of microorganisms to rest forever in its grave of irrefutable facts long after the Italian masters had refuted it. However, the alter ego of this new profession soon came to reside in Robert Koch who laid down a few logical rules for the proof of pathogenesis known as Koch's postulates. In proving a bacillus was the cause of anthrax in cattle he developed the four key requirements for proof of pathogenicity in 1876.

For the next 80 years, plant pathologists were obsessed with the idea that Koch's postulates were the heart and soul of their science. They became wedded to the microscope, the Petri dish, and the hypodermic syringe. We regret to say these essential tools too often become a substitute for intellectual curiosity. Plant pathologists concentrated their attention in isolating, identifying, and inoculating disease agents to such an extent they almost forgot to ask themselves the fundamental questions of why diseases were induced by pathogens and why the host became susceptible or resistant.

Regardless of this shortcoming, they entered a robust period of growth as truth after truth began to reveal the diversity of attack on plants and the enormity of the depradations. It was during this era that Hartig brought out his marvelous treatise on tree diseases in 1874. Burrill proved that bacteria could cause plant diseases in 1879. Viruses were proved to be infectious by Adolph Mayer in 1886, and to be filterable by Iwanowski in 1892. The concept of a *contagium vivum fluidum* was advanced by Beyerinck in 1896.

It was during this era of etiology and in this ferment of ideas that plant pathology was born at the Connecticut Agricultural Experiment Station in 1888. The establishment of the Hatch act provided Connecticut

with $15,000 of federal funds and half of this was allocated to the tiny station at New Haven. With this new federal support the Board of Control then had a total income of $18,606.15 to expend—and they spent it down to the last 15 cents. This $7,500 windfall caused the Board of Control no small concern. They held meetings on February 17 and April 2 to debate the several proposals for new investigations. Eventually they decided in favor of plant pathology over the runner-up, entomology, but history does not reveal the name of the master strategist and politician who maneuvered this wise decision. The record only shows that a mycological lab was established and the State spent $348.13 exclusive of salaries on it that first year.

The Board of Control announced in its report for 1888 that: "The station was fortunate in securing the services of Dr. Roland Thaxter, a graduate of Harvard University. Books and apparatus including a Zeiss microscope of approved construction have been provided and as soon as heating arrangements, now making, are finished, Dr. Thaxter will begin to culture various injurious fungi. . . ."

This momentous event in Connecticut occurred exactly three years after the Section of Mycology was established in the U. S. Dept. of Agr. with F. Samson Scribner as its head, one year before the U.S.D.A. changed this section's name to Vegetable Pathology, four years after J. C. Arthur went to the New York Experiment Station in Geneva to work on fireblight, and in the same year that plant pathology was born in Delaware, North Dakota, and New Jersey. The profession was yet to wait 17 years until the first chair of plant pathology apart from mycology was to be established at Cornell, 19 years before L. R. Jones went to the University of Wisconsin, and 20 years for the birth of the American Phytopathological Society.

Thus, the new program was launched in July 1888 under the most favorable auspices with a highly creative man who was to earn an undying place in the sphere of mycology, and a general concept that he was to investigate disease in its broadest aspects. We will leave the discussion of this man to more capable hands but the speaker would be remiss in his duties tonight if he did not point out that the events of the next two years were to exert a tremendous influence on the course of activity and psychology of the department that was to be developed. Since we are certain the other speaker tonight will be hesitant to give you the real interpretation of Thaxter's significance, let us give you the real facts.

In keeping with his fundamental interests, a mycologist is always a mycologist. Some of Dr. Thaxter's beautiful drawings of *Urocystis cepulae* appear in the annual report of 1889. We doubt that a single member of today's department could duplicate the feat. The same mycologist justified his existence by combating the onion smut caused

by this *U. cepulae* in the field. He claimed "5 grammes of sodium sulphide" in a 10-foot row worked wonders. This study was made the same year Bolley introduced formaldehyde and may very well be the first soil treatment introduced to control a specific plant pathogen. It cast a shadow of impending events because after this, chemotherapy by Stoddard and Dimond was inevitable.

What causes common scab of potatoes? Thaxter was most certain it was a species of *Oospora* that could be cultured and inoculated. And the young man forever linked his name indelibly with scab by inoculating a tuber with a pure culture of *Actinomyces scabies* so it was monographed with his initials.

By October 31, 1889 he was busy photographing trees sprayed with Bordeaux mixture. He ignored a few niceties of statistics and randomization of plots but in a straightforward manner he arrived at the right conclusion. His heirs often have done as well, but you know they have made very extensive improvements in methodology. They went around in circles on vegetables instead of straight rows of quince when Jim Horsfall applied his agile mind to the subject. By the time Saul Rich had improved on the experimentation with fungicides, he made an innovation by substituting McIntosh apples for quince. Apparently his director insisted on efficiency, so he used one-half of a tree instead of whole rows of them, or else he had to improve the efficiency of his testing because of the multitude of compounds that were born in the fertile imagination of Thaxter's successors. In any event this Station's pathologists developed a control for apple scab that saved the McIntosh apple as a crop for this State.

Thaxter had his own opinion of "squirt-gun botanists," as he was prone to refer to plant pathologists somewhat contemptuously, but it was not beneath his dignity to invent a wash-boiler device for applying spray materials. It was not so intricate as the power sprayers on the ingenious spiral spray plots of a later date, but it was quite advanced for its day and age.

Great was the progress along these lines as the years rolled around. By 1890, this great applied mycologist could advertise the use of double acting pumps and the Vermorel nozzles. By 1891, Thaxter had taught plant pathologists how to convince everyone of the importance of plant diseases by the use of devastating photographs of plants "before and after." Could anyone look at his photographs of poor quince trees in the 1891 annual report and feel that there was any reason why a sane man should not practice chemical therapy?

By 1893, the creative individuals at Connecticut had developed a barrel pump which they recommended, without in any way weakening their promotion of their own knapsack sprayer. Their ever-growing search for efficiency had gotten them to the stage where they had a

one-horse and two-man "squirt-gun botany" department. By 1895, according to the photographs in their annual report, they had progressed to being two-horse squirt-gun botanists. You will also note in photographs in the annual report of that date they had begun to take on the air of a high-class experiment station with straw hats, jackets, and neckties on their spray operators. This, however, we hasten to explain occurred only after Thaxter had deserted his duties in the service of man's welfare in favor of the *Laboulbeniales*, sick bugs, and Harvard University. His successor, William Sturgis, had taken over the position as mycologist.

In this same year the Experiment Station's facilities were fortified by a new installation. It gained a small greenhouse. Here was the opening wedge needed to start the studies on epiphytology of plant diseases which was to become a function in the research of G. P. Clinton, Florence A. McCormick, and P. J. Anderson of the Windsor Substation.

This short digression illustrates how men have accepted the responsibility of battling plant diseases on behalf of the farmers of this state and nation without losing sight of the fact that there is a greater responsibility to understand why disease and disease-control practices operate as they do.

Thaxter insisted that Bordeaux was being studied to develop an understanding of how chemicals perform. Dimond and Horsfall 60 years later were helping chemists design new types of organic molecules so they could develop theories that would lead to still more effective molecules. The applied research did not become an end in itself—it was merely the stepping stone and the motivation behind the search for ever-broadening concepts. This probably is why the small body of men at Connecticut has exerted such a strong influence on plant pathology.

Research is the art of asking one's self the proper questions. If questions are properly framed, nature always gives sound, intelligible answers. This group here in Connecticut has been asking itself the proper questions for many years—not how many wheels on the spray cart or how many horses drew the rig, but why chemicals succeed or fail, why fungi run rampant then desist, why disease follows parasitism, etc. Because these questions have been properly framed, this small body of men —so few in number—has contributed, on the average, one member to the National Academy of Sciences each 20 years: Roland Thaxter, 1912; Clinton, 1930; and Horsfall, 1953. It is doubtful whether any other department of plant pathology in the United States can boast an equal record for the manpower available in plant pathology.

How well these honors are deserved may be clearly discerned from examining the multitude of firsts and near misses in science here at New Haven: observation of the race of *Phytophthora infestans* on tomatoes in 1889 (but the great problem of biotypes awaited definition by Eriksson in 1896), one of the first uses of soil fungicides in 1889

(sodium sulfides), identification and naming of downy mildew of lima beans and potato scab in 1890, Clinton's monograph on the *Ustilaginales* in 1905, detection of crystals of tobacco mosaic virus in plant tissues in 1914 (but the key fact of infectivity escaped proof), studies on X-disease of peaches in relation to wild hosts (1933), development of modern concepts of chemotherapy, development of the concept of metal chelation as an explanation of fungitoxicity (1943), studies on physiology of wilt (1955), development of antiozonants to prevent flecking of tobacco, Horsfall's two books on fungicides (1945, 1955), and Horsfall and Dimond's editing of Plant Pathology Treatise (1960), to name only a few of the many contributions.

The Connecticut Agricultural Experiment Station had the foresight to launch its program long before many states had seen the need of scientific investigation of plant diseases. The first appointee was a fit disciple of the etiological period. He dived into the problems of identifying the fungous parasites responsible for disease and in perfecting methods of avoiding their ravages. Following his brief tenure, the same line of research was continued and expanded by his successors, William C. Sturgis and G. P. Clinton. Theirs were masterly contributions of the type needed for their day and age.

The incipient research in virology was not pursued so skillfully as mycology. The modern field of virology really should have been born in New Haven. As early as 1845 one Darling Noyes, who served as mayor of the city, described peace yellows in the Philadelphia area and reported its successful transmission by budding. He concluded it was some sort of fluid infectious agent that spread throughout the plant and that it might very well be associated with an insect. Such brilliance over 40 years before peach yellows defeated Erwin F. Smith of the U.S.D.A. and drove him into the more rational field of bacterial diseases, all went to naught. Sturgis did not pick up these ideas when he began to examine the yellows disease and calico of tobacco. Clinton and Stoddard returned to making bud experiments almost 90 years later. Detailed studies on tobacco mosaic by Clinton did not develop any revolutionary concepts since he concluded it was a disease of the chlorophyll caused by an enzyme.

The Connecticut plant pathologists have given little attention to the bacterial diseases over the years beyond the study of potato scab.

The Ecological Period

As plant pathologists catalogued the various causes of plant diseases in the latter half of the nineteenth century, it became obvious that environment exercised a vital role in regulating the severity of diseases. As the mode of dissemination was identified attention was naturally

focused on survival and potency of inoculum as well as predisposition of plants to attack and prolongation of the susceptible period. Obviously, the climatic and edaphic factors were second only to the presence of the plant pathogen in determining how severe plant diseases would become.

Plant disease forecasting services were needed to determine when and where disease control measures would have to be applied. The effects of humidity and temperature on infection and duration of favorable combinations of the two on successful parasitic establishment soon came to be a prevailing obsession at the major graduate training centers such as Cornell Unversity, the University of Wisconsin, and Iowa State University. Epidemiology began to take its place alongside Koch's postulates as a subject worthy of investigation. Soon a plant pathologist began to consider himself underprivileged if he did not have a battery of Wisconsin soil tanks to regulate soil temperature and humidity-temperature control cabinets to carry on inoculation tests. The very basic observation of J. C. Walker and his associates at Wisconsin, that the inherited resistance of some varieties of cabbage could be modified by soil temperature, served only to emphasize the need of control over the environment in breeding for disease resistance and the use of controlled inoculations.

The effect of light intensity and air currents on dissemination and survival of fungous spores and bacterial inoculum soon became a major concern to the epidemiologist. They had to know how far inoculum could be carried in a viable condition and how long it would withstand desiccation or exposure to different types of irradiation regardless of whether it was in the air or on and in the seed. Again the plant pathologists became involved in a job of cataloguing—this time the conditions of weather. The great graduate school at Wisconsin led in this era of plant pathology but others eagerly followed its lead.

The effect of weather conditions on inoculum survival was studied by many persons but probably no research gave more decisive results than the observations of Neil E. Stevens on bacterial wilt of sweet corn. He concluded that severity of this disease could be predicted by setting up a scale of degree-days of temperature required in late winter to permit survival of the vector.

As a result of such research on epidemiology, the U.S.D.A. was able to establish a division on disease forecasting to determine when crops should be treated. This is a necessary service to serve as a partner to basic research on design of protective chemicals and methods of their deposition and retention.

The study of the effect of soil conditions on inoculum survival was long delayed apparently because the plant pathologists were confused by the complexity of the situation. Everyone knows that ordinary garden

soil may have only one or two pathogens among the couple of billion other microorganisms per gram of soil and plant debris. They were competing for food substances, both the minerals from the soil and decaying organic matter. However, the forces of antibiosis latent in this situation were largely ignored until the first third of the twentieth century had passed. Probably the shock of Fawcett's retiring address as President of the American Phytopathological Society, or his student Richard Weindling's discovery that glyotoxin from the fungus he assumed to be *Trichoderma viride* would prevent damping-off of citrus seedlings, oriented the plant pathologists to look back over this area of investigation. Gradually a volume of knowledge on antibiosis began to be accumulated by plant pathologists long before Flemming's discovery of penicillin or Waksman's development of streptomycin. It remained for Garrett, however, to become the apostle of exploiting the forces of antibiosis in the soil in the control of plant diseases.

Early in the etiological period, plant pathologists became aware that soil fertility could not be ignored as a force in predisposition of plants to parasitic invasion. A voluminous literature has grown up but is not fully appreciated because no disease can be controlled completely by fertilizing the soil. It is admitted that diseases may be alleviated by liming the soil for club root of crucifers, increasing the hydrogen ion concentration by sulfur for potato scab and bacterial wilt of solanaceous plants, use of nitrates judiciously on crops exposed to bacterial pathogens or obligate parasites, addition of adequate potassium in presence of foliage blights, and restriction of this element in certain overgrowth and gall diseases. In general, an imbalance of fertilizer that promotes excessive growth and succulence favors the highly specialized parasites such as the rusts, mildews, and bacteria, while any condition that suppresses strong vegetative growth or retards normal development favors the facultative saprophytes, which are fundamentally poor parasites and attack only weakened or injured plants.

It is interesting to note that the Connecticut Agricultural Experiment Station is one of the very few that has established a climatology department. Interestingly enough, Paul Waggoner, a plant pathologist trained in this field, has been placed in charge, so we can hope to see new developments coming from this organization.

The subject of environmental effects is too intricate to be developed further here but suffice it to point out that the study of ecology during the period 1880 to 1960 has been extensive. Much is known about the effect of edaphic and climatic factors on the pathogen but much less is understood about the effect in predisposing the plant to attack and to recovery.

The study of environmental influences made it obvious that plant disease agents lead a most tenuous existence in spite of their ingenious

adaptation to plant life. If plants could be protected for a crucial period of a week or even a few days or hours, the crop ordinarily would escape severe damage. Since it was impossible to regulate the weather during these crucial periods, other means were sought. Starting with Millardet's development of Bordeaux mixture on foliage in October 1882, Thaxter's sodium sulfide treatment of soil for onion smut, and Bolley's formaldehyde treatment for seed grains in 1890, a body of knowledge on use of chemicals grew up. The copper ion and the sulfur atom were the mainstays for many years. The mercuric ion joined the arsenal in minor roles as a disinfectant and seed treatment. The introduction of the organic mercurials by Riehm in 1912 was a tremendous advance in this chemical warfare. Intensive studies of different copper compounds came in the next two decades but the development of cuprous oxide and tribasic copper sulfate merely revealed that regulation of water solubility was all that could be achieved with inorganic ions.

The possibilities of organic compounds as fungicides were clearly established by S. E. A. McCallan and Frank Wilcoxon, but this great, promising new era was not opened until W. H. Tisdale and I. Williams' announcement of the alkyl dithiocarbamate fungicides in 1934. This was more than a new form of sulfur, it was a designed organic molecule which could be modified and adjusted to meet many special demands. This discovery served to emphasize that formaldehyde was but the forerunner of a whole class of new organic fungicides, bactericides, and nematocides that could be used in plant protection. The U. S. Rubber Co. sensed this possibility and appropriated $5,000 to explore the possibilities. Of the first 23 compounds chosen by W. P. ter Horst on an absolutely fallacious theory of oxidation-reduction potential, two proved to be outstanding fungicides in the hands of Eric Sharvelle and J. G. Horsfall. Even after their discovery these compounds were relegated to a back shelf because they could not compete with cheap cuprous oxide, until it was demonstrated that the true measure of cost of Spergon was to be appraised by benefits received and safety in use rather than cost per pound.

The response of industry was most gratifying. Carbide and Carbon, Dow, Rohm & Haas, du Pont, U. S. Rubber, and many others entered the field. Since there were no sound theories to guide them, they depended strictly upon empirical testing. Thousands of compounds were synthesized and discarded to find the one promising material that would destroy one form of plant life without injuring its host. Gradually a volume of knowledge built up around this empirical approach so certain relationships of chemical structure to activity were conceived.

Among the significant discoveries during this period, the Connecticut Experiment Station was to contribute a substantial share. It cooperated with Dr. Hester and Rohm and Haas in discovering the alkylene bis-

dithiocarbamates, the most widely used fungicides of this era. It is interesting to note that Dimond, Heuberger, and Horsfall characterized this compound as a water soluble, protectant fungicide with tenacity. Later studies were to show the material was neither fungicidal nor persistent. As a matter of fact, they had made a novel approach to plant disease control by applying a bland, inactive substance that would generate an ephemeral fungitoxicant *in situ* so as to control plant pathogens without building up heavy deposits of toxicants that would be potentially capable of exerting an adverse effect on the crop plant. This group was well in the forefront in studying potential relations of chemical structure to activity, the role of chelation processes in fungitoxicity, the relation of lipid solubility to cell permeation, the use of dosage-response curves in assaying toxicity and studying potential differences in mode of action, and the relationship of particle size to protective ability.

By 1945, no serious new principles had evolved because plant pathologists had been so loathe to enter into physiological research, much less attempt to understand the vagaries of the organic molecule in a living system. The chemists synthesized while the plant pathologist evaluated performance but the blind were leading the blind.

The lack of perception in fungicide research was not unique. A similar hesitancy to plunge into the physiology of parasitism, the biochemistry of pathogenesis, the chemical forces involved in antibiosis, the effect of mineral nutrient balance on cellular response to pathogens and comparable areas prevailed during this period when ecology ruled supreme.

The Physiological Era

Following World War II the plant pathologists began to awaken to the fact that their research was leading into too many blind alleys. There is only so much any one can do by isolating and inoculating pathogens, determining their nutrient requirements and cultural characteristics, defining their cardinal temperature requirements for growth and parasitism, measuring their response to toxicants, breeding crop plants for resistance, and studying survival of pathogens in different environments.

Something was missing when men who were supposed to be scientists were operating in the realm of the horticultural and mycological arts. Too many people were doing the same things over and over, merely substituting the name of one organism for another, or of one crop for the next one in a rotation. New principles were not emerging to guide the footsteps to a higher plateau of achievement. The science of plant pathology was rapidly reaching a state of art where it could be practiced by an agronomist, or horticulturist, without devising new principles. If plant pathologists were to provide leadership they would have to find more

penetrating concepts as to the nature of plant diseases, plant resistance to attack, and response of parasites to toxicants in the environment.

Eyes were turned backward to see if something vitally important had not been missed. Perhaps there was a message for the 1940's, 1950's, and 1960's in previous observations. After all, plant pathologists had provided far more data on the physiology of fungi than either the mycologists or physiologists, but they had to wait for bacteriologist J. W. Foster [Chemical Activities of Fungi. Academic Press (1949)] to assimilate and interpret the data on physiology of fungi, and eventually for Vincent W. Cochrane to give it a mycological interpretation in his book on physiology of fungi (Wiley, 1958).

L. R. Jones did classical pioneering work on the pectic enzymes secreted by the soft rot bacteria in 1909, but he moved to Wisconsin and established the stronghold of phytopathological ecology and others never picked up his idea. However, the enzymes secreted by a parasite must become a part of the host physiology and hence be fundamental to pathogenesis. In Wisconsin, J. C. Walker, H. R. Angell, and K. P. Link had demonstrated that phenolic substances in onion scales were responsible for resistance of purple onions to smudge, and G. Greathouse correlated alkaloid content of roots with resistance to the Texas root rot fungus, but the biochemistry of plant resistance did not flourish in the barren soil of phytopathology. The phytopathologists looked upon a variety of abnormal growth and flowering responses without concerning themselves with such frivolous things as auxins. It remained for the Japanese chemists and physiologists to call to the world's attention the potent attributes of gibberellic acid and its relatives. The pathologists had helped design hundreds of biologically active molecules and develop the most staggering, superficial concepts as to the relationship of chemical structure to fungitoxicity. They discussed molecules as though they could be broken into fragments and digested part by part without considering the whole. Action was interpreted strictly by groups without concern for the physical as well as the chemical properties of the total molecule.

All of this began to change about 1948 after some of the leaders began to complain about the artificiality of the research. Answers began to be found in the reaction of living cells rather than in theory. Theories were good only to the extent that they encouraged penetrating, decisive experimentation. Ernst Gäumann directed his tremendous encyclopedic abilities to interpreting the principles of plant infection and the nature of chemical agents—the so-called toxins—in pathogenesis.

The Connecticut plant pathologists dove into this problem of disease-induction with the penetrating research of A. E. Dimond and others. New ideas and new concepts on how wilt diseases result from parasitism of the *Fusaria* were developed.

The growing field of fungicide research was given greater depth and perspective as a new breed of plant pathologists and biochemists joined the research endeavor. At Connecticut, Boyce Thompson Institute, Maryland, London (Canada), Rothamsted, and in Utrecht, Holland, the scientists began to probe into the response of living cells to toxicants. Theory after theory has fallen by the wayside and the supposedly marvelous new innovations were found to be relatively poor performers in one or more aspects. The ideas on permeation of spores and changes in the plasma membrane by L. P. Miller and his colleagues opened new vistas. Pinning down the biochemical mechanisms of fungitoxicants proved much more complicated than R. G. Owens, H. D. Sisler, C. E. Cox, R. A. Ludwig, and R. J. Lukens had assumed it would be. Molecules change in the living cell and the cell changes in the presence of the toxicant so the cutest of preconceived ideas must be modified or abandoned.

In spite of the difficulties encountered, a set of new principles has begun to emerge on the deposition of spray materials as defined by H. P. Burchfield, and on selective permeation by the spore by S. Rich and J. G. Horsfall, and by L. P. Miller and S. E. A. McCallan. R. G. Owens has been able to tie down the chemical mechanism of some of the quinones and dithiocarbamates and G. Zentmyer's concept on chelation of essential metals by 8-hydroxyquinoline has been revised so it seems to have some reasonable validity.

This is an exciting era we are now in. Plant pathologists are following the pathways of the plant physiologists toward biochemistry. As they journey into this new area they find much more than an opportunity for mimicry. They are finding substances new to science such as the α-amino acid that acts as Armin Braun's tabtoxine responsible for the chlorotic halo in wildfire of tobacco. A dearth of fundamental information, both on plants and their pathogens, lies revealed before their penetrating scrutiny.

Gradually there is evolving a philosophy that there is something known as the disease state which has a biochemistry all of its own. For example, a nematode in the root of a plant induces root knot not only by secreting substances to dissolve the cell walls, but also by stimulating nucleic acid metabolism, according to R. G. Owens. Nuclei grow relentlessly but forget to divide. The wound tumor virus changes *Rumex* tissue so it has a prodigious requirement for phosphates but its phosphatases are so active it cannot build up a normal reserve, according to C. A. Porter and L. H. Weinstein. The rust fungus carries its own inhibitor in the uredospore but when removed from its influence will germinate. According to R. C. Staples it has an essentially normal Krebs cycle for releasing energy and it produces new amino acids, but he and Burchfield found it incapable of synthesizing these into proteins. A marigold secretes a material to repel nematodes, but the exact nature of the substance remains to be defined.

Every observation such as these poses more questions than it solves. In the search for answers, the plant pathologist will inevitably not only procure new knowledge to direct his efforts in devising new methods of controlling parasites or antidoting their effects, but will also make material contributions to plant physiology, veterinary medicine, and human medicine.

The new concepts on the physiology of parasitism and pathogenesis open many exciting new pathways for attacking old problems. As we learn more about the chemistry of the pathogen's attack and the host's response to invasion, a number of new lines of research must open for us.

The Problems Ahead

The fundamental nature of resistance and parasitism is so imperfectly understood that much remains to be done on the biochemistry of the relationship. There are undoubtedly many natural chemicals that impart immunity or otherwise retard the pathogen. These must be isolated and purified so their use in chemotherapy can be studied. There must also be methods of altering the host's physiology so as to impart temporary resistance either by disturbance of the normal hormonal balance or development of metabolites that are abnormal for that period of the plant's history. The Dutch elm disease may eventually be controlled by accentuating differentiation of tracheal tubes during the early season period of susceptibility. The natural process of walling off infection in the sapwood might be controlled once we know more about lignin deposition via the quinic acid pathway of metabolism.

The biochemistry of soil antagonisms holds the future to exploitation of antibiosis. The types of biologically active molecules in the soil will offer leads as to how we can shift the microbial balance in favor of the beneficial and neutral microorganisms and place the pathogens at increasing disadvantage. We must recognize that there is much validity to Garrett's classification of soil pathogens as normal inhabitants and temporary soil colonizers that temporarily find circumstances favorable to them. These relationships are dependent upon the balance of nutrient supplies and release of metabolites that are selectively toxic. In either case, the problem becomes one of identifying, separating, purifying, and studying the biological effects of each material in the substrate.

The proliferation of abnormal growth must be reduced to its simplest physical and chemical terms. The autocatalytic nature of crown gall tissue once cells are preconditioned and incited to gall development, the apparent permanent changes in host tissues induced by the wound tumor virus, and many similar changes must be investigated because

there is suggestion that the acquired trait imparted by pathogenesis becomes self-perpetuating beyond the influence of the pathogen. If this is so, either the DNA of the host nucleus has been changed, a new RNA has been added in the cytoplasm where it becomes self-duplicating, or there has been an irrevocable change in the host proteins. The more probable answer lies in the second possibility, but a full investigation is warranted.

The mystery of obligate parasitism must be solved. There is sufficient evidence accumulated to make it obvious that this is not a simple case of inadequate sugars, amino acids, or growth accessory factors. As one studies the problem, he becomes increasingly aware that all obligate parasites are either intracellular or have haustoria which penetrate the cell. The strong propensity for the haustorium of a rust or powdery mildew, for example, to come into close contact with the host nucleus has long attracted our attention. Since the fungus alone does not seem to be deficient in its energy conversion system or in its capacity to synthesize amino acids, attention is focused on the inability to convert amino acids into proteins. This suggests that either messenger RNA or transfer RNA may be missing in the parasite and it must procure these vital substances from the host nucleus as they are released into its cytoplasm. If so, then the proteins of the fungus would have the same sequential arrangement of amino acids as the hosts. This in due time would indicate a community of capabilities in producing enzymes and would provide a logical explanation to H. H. Flor's theory of matching genes in host and parasite. In substance, the whole problem may eventually prove to be closely allied to that of virus physiology and multiplication if it does revolve around competition and supplementary action of RNA's from the host and pathogen.

The effect of mineral balance must be investigated on a much more comprehensive scale than heretofore. The studies must embrace prevalence of the parasite, the aggressiveness of the attack, the predisposition of the host to attack, and the capacity of the host to recover from invasion or neutralize its effects. The increasing use of balanced fertilizers, the heavier fertilizer applications to stimulate crop productivity, and the decline in organic matter all serve to bring about drastic dislocation in both the balance and quantity of mineral nutrients available to the plant and pathogen. The decrease in microbial populations in many soils contributes to drastic fluctuations in nutrient supply as well as prevalence of parasites. The trends of soils to be almost depleted of nutrients by heavy cropping and to be restored by heavy applications of fertilizers are certain to increase in the years ahead. Plant pathologists must know not only what is likely to happen to different classes of parasites but why the nutrient supply has a particular effect.

The mechanism of fungitoxicity must be further explored to determine

what happens to these and other chemical protectants inside the fungous cell. The toxicants now in use are quite inadequate and must be improved upon both as to innate toxicity for the pathogen and selective action against the pest. There are unlimited possibilities for designing inert molecules such as nabam that will generate evanescent fungicides *in situ* in such limited quantities that they will be of little hazard to the host for the consumer of the crop. It is possible to develop systemic materials which will either change the host's basic metabolism so it will become resistant or will disinfest its tissue. The chemical control of plant diseases is very much in its infancy because so little is known about the mechanisms of plant protection or disinfestation.

The fate of protective chemicals in the environment must be investigated much more thoroughly not only to improve their effectiveness but also to make certain that they are safe in use. The studies on formulation, deposition, depletion of spray deposits and the products of decomposition must be pursued aggressively. If plant pathologists are to use fungicides, they must know what is to be expected of the molecules and particles after they are deposited. Repetition of applications should be based on residual deposits rather than on some arbitrary time scale that ignores chemical stability, response to environment, type of deposit, or growth rate of the host.

Knowledge must be obtained on the fate of the chemical that reaches the soil and whether it becomes bound to soil colloids by base exchange or is decomposed by soil microorganisms. The truth of the matter is that the chemist has been able to synthesize molecules which far outrun the present capacity of the soil microorganisms to digest them, be they fungicides, herbicides, insecticides, or detergents. The time may come when we will have to train microorganisms to digest and dispose of our residual sprays in the soil before they reach the surface or underground water supplies. Although Rachael Carson's "Silent Spring" is a grossly exaggerated and distorted view of the hazards from using chemicals on crops, it is a voice that should not be ignored. It is everyone's responsibility to see that these chemicals are used wisely even though none of the fungicides have been condemned or even criticized severely in the frantic hysteria of the misinformed.

The gene control over parasite and host must be explored from a fresh viewpoint. Of course, the search for natural sources of resistance must go on and genes for resistance must be incorporated into as many crops as possible. This is an unending service, just as the search for better chemicals will ever expand as long as our agriculture and industry remain progressive. However, what is needed are some new genes not to be matched by the parasites. The idea of a synthetic gene is not too far away. Good, sound knowledge on the nucleotides involved in gene action is available and within the present decade many of the

codes will have been ascertained. When, and if, it becomes possible to synthesize a new combination of nucleotides and get it to operate in a living cell unlimited possibilities will be opened in plant disease control.

At the moment, the plant pathologist is confronted with a matter of prime concern in saving the diversity of germplasm inherent in our cultivated crops. The plant breeder by inbreeding and hybridization is discarding genes left and right which we may sorely need in the future to breed resistant varieties for an unheralded parasitic race of pathogens. How are we to save these "worthless" genes until we need them? One possibility would be to set aside a reasonable acreage for open-pollinated crops as has been done for corn in South Dakota. However, this is totally inadequate because some genes are inevitably lost in choosing seed stock. This problem could be solved once and for all if someone would develop a method of culturing the haploid tissue from pollen grains in culture media. If each pollen member of a tetrad was placed in culture every gene of the plant would be preserved *ad infinitum*. However, someone would have to develop a method of using these cultures as sperms or else convert them to autodiploids that could produce only one genetic kind of egg cell or sperm cell. Louis Nickell working with bean tissue and F. C. Steward with carrot tissue have shown that new plants can be developed from single diploid cells, so this is not an unreasonable dream. Methods of inducing diploidy and tetraploidy are available so all we need is someone to cultivate the pollen tube of angiosperms in synthetic media. Dr. W. Tulecke of Boyce Thompson Institute has succeeded in cultivating pollen of certain gymnosperms but the angiosperms have resisted his greatest efforts. The plant pathologists have such a great stake in this problem, they have every right to enter the field.

We visualize the time when germplasm banks will be maintained in tremendous protected laboratories safe from random selection of breeding materials, and from catastrophies such as nuclear energy from an atomic explosion. Not only would this serve as a storehouse of registered germplasm, it would make available to the geneticist, for the first time, true autodiploids where the individual cells would have gene purity, i.e. every gene matched with its own kind of allele and no heterozygosity.

Summary

The plant pathologists have learned much in the past 75 years on the cause of disease, the influence of environment, and the physiological forces behind disease. However, there is no shortage of problems today. All we need is the vision and determination to attack them and new techniques and skills to solve them.

The past 75 years have merely served to define the problems before us. The intellectual aspects are more challenging than at any time in the past, and the opportunities to serve our fellow men were never brighter. The challenge of this moment could never have been visualized when Roland Thaxter came to New Haven—or, for that matter, when our beloved associates of today began to converge here some 24 years ago. Today we understand only enough to know where to attack our problem.

We have attained neither the perfection of science which would permit us to be self-satisfied with what we know, nor a philosophy strong enough to lure us away from continuing the search for a deeper knowledge. We know only that we are dedicated to a search for the ultimate truths about disease and its control.

Conclusion

Saul Rich

The Connecticut Agricultural Experiment Station

These symposia and the banquet speeches were of great interest to all who heard them, particularly to those of us here at The Connecticut Agricultural Experiment Station. But the formal discussions were only part of the intellectual harvest. Between the sessions, scientists gathered in small groups to exchange ideas, to query and expostulate. This "quarrelsome interest" is the very ferment of scientific progress. New ideas came from across the country and from other lands. New ideas returned with homeward bound travelers to be tested in the laboratory and in the field.

What each scientist retained was important in his own research. The importance of the fundamental studies discussed, however, is broader than the narrow interests of the specialists. This can best be illustrated by the following quotation from the Pesticides Report of the President's Scientific Advisory Committee released on May 16, 1963. "The goal of the development of safer, more specific and less persistant chemicals is not unreasonable. But the attainment of this goal will require extending the research efforts away from empirical approaches to more fundamental studies in such aspects as mode of action of pesticides, comparative toxicology, the metabolism of compounds in insects and in higher plant forms, and the processes of chemical degradation and inactivation in nature." In these symposia we were helping to implement the recommendations of the Pesticides Report. We explored how plants protect themselves, and what they can do to degrade and inactivate foreign molecules, whether these foreign compounds come from a pathogen or from man. In addition, we discussed the current thinking about the mode of action of fungicides. These descriptions of fundamental studies illustrate how we are projecting our research away from empiricism.

Here were representatives of related and overlapping disciplines, examining the evidence from their own viewpoints. Plant pathologists, biochemists, plant physiologists, and many other specialists blended their knowledge in a single cauldron. Throughout ran a dominant theme: The enzymology of the diseased plant and the poisoned pathogen.

The first symposium described biochemical defense mechanisms in plants.

Dr. Uritani introduced us to studies on phytoalexins now progressing in his own laboratory and at other laboratories throughout the world. Dr. Stahmann further illustrated how a pathogen intrudes its own biochemistry on that of the host, and how in the process both are altered. Still unknown are the variables which decide the ultimate victory of host or pathogen.

Dr. Kuć spoke convincingly of his belief that phenolic compounds are the most important phytoalexins. Dr. Sondheimer pointed out the technical difficulties of interpreting experiments with phenolic compounds. Still, the phenolic compounds have been invoked repeatedly in plant disease resistance. Are we being misled by ubiquity, or are we justified in accepting the seemingly obvious?

Dr. Byrde reported naturally occurring compounds which can inhibit the pectinases and cellulases used by fungi to attack their hosts. Dr. Whitaker related the structure of pectins and celluloses to their resistance to attack by fungal enzymes. He questioned the relevance of *in vitro* studies on isolated, extracellular enzymes.

The second symposium concerned the introduction of compounds into plants to increase disease resistance.

Dr. Mitchell described how foreign molecules move in plants, and the factors influencing their movement. Dr. Davis raised the possibility of whether we could use such information to control the movement and concentration of phytoalexins in plants.

Dr. Dimond analyzed our present knowledge about plant chemotherapy. Dr. Cowling listed differences between host and pathogen that could be useful in chosing or designing chemotherapeutants.

Dr. Samborski spoke of chemically induced rust resistance, and hoped that these studies would illuminate genetic resistance in plants. Dr. Daly pointed out weaknesses in the biology and physiology of rusts that should be considered in our control attempts.

The third symposium discussed the poisoning of fungi.

From Dr. Spencer's report of the multitudinous studies relating chemical structure to fungitoxicity, it appears that we are not ready to abandon empiricism completely. Although we would have it otherwise, empirical explorations are an essential beginning for our biological studies. Before we can find out how Nature operates, we must have well-defined biological responses to examine. Before we can study fungitoxicity, we must have chemically defined fungitoxicants. Biologically active compounds, most of them found empirically, are our only keys to biological activity. Dr. Spencer and Dr. Block followed the fundamental currents through the empirical swamp.

Dr. Sisler examined the various biochemical mechanisms by which fungicides can harm fungi. His discussion was extended by Dr. Owens, who enlarged on the fungitoxic action of the dithiocarbamates. It is

apparent that the successful fungicides are those with multiple mechanisms of action.

Dr. McCallan and Dr. Miller described how fungi absorb fungitoxicants at remarkable rates and in surprising quantities. Whether fungi really have any strong barriers to permeability was discussed by Dr. Sussman.

At our banquet, we were caught up in history, and became a part of the eternal parade of men and events.

As Dr. Horsfall spoke, Roland Thaxter came alive for us. Thaxter's irascible, pungent views of plant pathology and of bucolic Connecticut are now hilariously piercing. But as we laughed with Thaxter, so we identified with him. Had he not been so eager to do fundamental research, he would not have felt so frustrated. The struggles of yesterday continue today. Yet in spite of his frustration, Thaxter succeeded at Connecticut and afterwards. Later he could look back at his callow years with a tolerance that included The Connecticut Agricultural Experiment Station.

Dr. McNew was our historian, giving us an account of plant pathology, and his view of the role played by the Connecticut Station's Department of Plant Pathology and Botany. He concluded with a fascinating analysis of the important areas in plant pathology that need to be explored.

The banquet speeches, befitting the occasion, reminded us that "What is past, is prologue." We were further reminded, however, that what is present is also prologue, and much remains to be done.

List of Speakers

S. S. Block
Department of Chemical Engineering
University of Florida
Gainesville, Florida

R. J. W. Byrde
Department of Plant Pathology
Long Ashton Research Station
University of Bristol
Bristol, England

E. B. Cowling
Forestry School
Yale University
New Haven,Connecticut

J. M. Daly
Department of Plant Pathology
University of Nebraska
Lincoln, Nebraska

David Davis
New York Botanical Garden
Bronx Park
New York, New York

A. E. Dimond
Department of Plant Pathology and Botany
The Connecticut Agricultural Experiment Station
New Haven, Connecticut

L. V. Edgington
Department of Plant Pathology and Botany
The Connecticut Agricultural Experiment Station
New Haven, Connecticut

J. G. Horsfall
The Connecticut Agricultural Experiment Station
New Haven, Connecticut

Joseph Kuć
Department of Biochemistry
Purdue University
Lafayette, Indiana

R. J. Lukens

Department of Plant Pathology and Botany
The Connecticut Agricultural Experiment Station
New Haven, Connecticut

S. E. A. McCallan

Boyce Thompson Institute for Plant Research, Inc.
Yonkers, New York

G. L. McNew

Boyce Thompson Institute for Plant Research, Inc.
Yonkers, New York

L. P. Miller

Boyce Thompson Institute for Plant Research, Inc.
Yonkers, New York

J. W. Mitchell

Crops Research Branch
U. S. D. A.
Beltsville, Maryland

R. G. Owens

Boyce Thompson Institute for Plant Research, Inc.
Yonkers, New York

Saul Rich

Department of Plant Pathology and Botany
The Connecticut Agricultural Experiment Station
New Haven, Connecticut

B. J. Samborski

Research Branch
Canada Department of Agriculture
Winnipeg, Manitoba
Canada

H. D. Sisler

Botany Department
University of Maryland
College Park, Maryland

Ernest Sondheimer

Chemistry Department
State University College of Forestry
Syracuse, New York

E. Y. Spencer

Research Branch
Canada Department of Agriculture
London, Ontario
Canada

M. A. Stahmann

Department of Agricultural Biochemistry
University of Wisconsin
Madison, Wisconsin

E. M. Stoddard

Department of Plant Pathology and Botany
The Connecticut Agricultural Experiment Station
New Haven, Connecticut

A. S. Sussman

Department of Botany
University of Michigan
Ann Arbor, Michigan

Ikuzo Uritani

Laboratory of Biochemistry
Nagoya University
Anjo, Aichi
Japan

D. R. Whitaker

Division of Applied Biology
National Research Council
Ottawa, Ontario
Canada

M. L. Zucker

Department of Plant Pathology and Botany
The Connecticut Agricultural Experiment Station
New Haven, Connecticut

Additional Participants

R. W. Barratt

Department of Biological Sciences
Dartmouth College
Hanover, New Hampshire

D. F. Bateman

Department of Plant Pathology
Cornell University
Ithaca, New York

H. P. Burchfield

Southwest Research Institute
8500 Culebra Road
San Antonio, Texas

R. A. Chapman

Department of Plant Pathology
University of Kentucky
Lexington, Kentucky

I. A. M. Cruickshank

Division of Plant Industry
C. S. I. R. O.
Canberra, Australia

A. W. Galston

Biology Department
Yale University
New Haven, Connecticut

H. E. Heggestad

Agricultural Research Service
U. S. D. A.
Beltsville, Maryland

F. L. Howard

Department of Plant Pathology and Entomology
Rhode Island University
Kingston, Rhode Island

Arthur Kelman

Department of Plant Pathology
North Carolina State College
Raleigh, North Carolina

G. R. Mandels

Pioneering Research Division
U. S. Army Natick Laboratories
Natick, Massachusetts

J. K. Palmer

Central Research Laboratory
United Fruit Company
Norwood, Massachusetts

G. S. Rantela

Forestry School
Yale University
New Haven, Connecticut

Klaus Rudolph

Department of Biochemistry
University of Wisconsin
Madison, Wisconsin

Thomas Sproston, Jr.

Department of Botany
University of Vermont
Burlington, Vermont

Israel Zelitch

Department of Biochemistry
The Connecticut Agricultural Experiment Station
New Haven, Connecticut

G. A. Zentmyer

Department of Plant Pathology
University of California
Riverside, California

Bulletin 663

December 1963

The Connecticut Agricultural Experiment Station

New Haven