PREVENTION OF MOLD ON HIGH MOISTURE HAY WITH EMPHASIS ON THE FATTY ACIDS AS FUNGICIDAL AGENTS

Thesis for the Degree of M. S.

MICHIGAN STATE COLLEGE

Bernard David Lewis

1951

This is to certify that the

thesis entitled

Prevention of Mold on High Moisture Hay With Emphasis on the Fatty Acids as Fungicidal Agents

presented by

Bernard D. Lewis

has been accepted towards fulfillment of the requirements for

M.S. degree in Bacteriology

W. L. Mallmann
Major professor

Date March 15, 1951

O-169

PREVENTION OF MOLD ON HIGH MOISTURE HAY WITH EMPHASIS ON THE FATTY ACIDS AS FUNGICIDAL AGENTS

by

Bernard David Lewis

A THESIS

Submitted to the School of Graduate Studies of Michigan State College of Agriculture and Applied Science in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Department of Bacteriology and Public Health

1951

6/29/51
Gift

TABLE OF CONTENTS

LIST OF TABLES

LIST OF GRAPHS

ACKNOWLEDGEMENTS

The author wishes to express his sincere thanks to Dr. Walter L. Mallmann, under whose constant supervision, personal guidance, and unfailing interest this investigation was undertaken.

Grateful acknowledgement is also due to Mr. Elbert S. Churchill for his helpful suggestions and assistance in one way or another.

The investigator also extends his sincere thanks to Mr. Robert Kleis and other members of the Agricultural Engineering Department at Michigan State College for their assistance and the supply of materials used in this study.

The writer appreciates the financial support of the J. I. Case Company for the fellowship which they provided for this study.

INTRODUCTION

The methods used today in the curing of hay result in considerable loss of nutritive value while the hay is being reduced in moisture content to a point which will prevent heating and molding during storage.

Several qualifications must be met in the production of quality hay. These qualifications are: (a) purity of the hay, (b) retention of a high percentage of leaves, (c) pliability of stems, (d) color, (e) absence of moisture and moldiness.

In the late eighteen hundreds, it was found that 25 to 50 percent of the dry matter in hay would be lost by exposing the hay to rain. This represents a considerable loss of the most digestible portions of hay. When weather conditions are unfavorable during the haying season, the longer the hay is exposed the greater the loss encountered. Besides loss due to weather conditions, the present methods of moisture reduction cause the fragile leaves to become dry and they are easily lost. This results in a heavy loss of protein. For these reasons, a way for improving the curing process has long been sought.

The specific purpose of this study was to explore the possibilities of using chemical agents to inhibit the development of microorganisms responsible for the spoilage of most baled hay, and the possible application of the agents in the preservation of baled hay with moisture contents in excess of 20 percent by weight. Particular attention is paid to possible use of the short chain fatty acids. This is an entirely new approach to the prevention of mold development in hay.

HISTORICAL REVIEW

A. Methods of Preservation

Most of the literature dealing with the prevention of mold development in stored hay attacks the problem from a physical point of view. Most workers have used methods such as drying and heating, while little experimentation seems to have been carried out with chemical treatment.

It has long been recognized that high relative humidity and moisture content are the primary factors in the control of mold growth in stored products. McHargue (17) found that molds were able to develop on corn having 15 percent moisture, if no ventilation was provided. Thom and LaFevre (29) gave a critical value of 13 percent moisture for mold growth on corn. Wright (36) stated that "the relative humidity, rather than the moisture content of the material, is the primary factor affecting mold growth". He found that samples of artificially dried grass, stored at 13 percent moisture content, could be held for periods greater than one year without mold development.

Based on moisture contents, methods have been developed to prevent molding by reducing the moisture. For centuries, hay has been field dried before being placed in storage. Methods of speeding the drying process, such as the use of forced air, have been employed in the hay mow.[1]

Heat, as a drying agent, has been employed to produce quality hay. Hendrix (8) pointed to the fact that the heat due to respiration and bacterial action in hay can drive off considerable amounts of moisture. Terry (26) suggested that low temperature (cold storage) could be tried to prevent spoilage in hay. He also indicated that a hay pasteurizer, having a temperature above 125° F. might be used. The latter idea actually has been applied by Cheveley (3) in the use of hot air dryers.

The use of chemical methods to prevent molding in hay has not been widely explored. Henry (9) stated, "salt and lime scattered over damp hay (in the mow) tends to prevent fermentation and checks the growth of molds". In storage problems, similar to the one afforded by hay, chemical means of preservation have been used. Rigler and Greathouse (22) used the fatty acids in the prevention of root rots. Humphrey and Fleming (12) listed several oils and salts which have been used to preserve wood against fungi. Mallmann and Michael (18) showed the value of chlorophenols, and related compounds, in preventing mold development in paper. Horsefall (11) described the action and uses of most of the modern fungicides.

---

1. The workers here are too numerous to mention. Some references are Journal Agricultural Engineering 1947, 28: 141-144, 257-258, 289-290, 294.

Shepherd et al. (25) used sulfur dioxide and other gases for the preservation of forage products. Similar work was carried out previously by Klotz (14) using nitrogen trichloride, and other gases, as fungicides in the storage of fruit.

B. Fungi Involved in Hay

Examination of the literature on hay and other grasses revealed no complete studies of fungi which cause moldiness on hay.

Thom and Raper (30) discuss members of the aspergilli that would appear in this problem. Almost all the aspergilli are well adapted for growth on a medium such as moist hay. Their frequency in stored hay led Cohn (4) to believe that they were the cause of "heating" in damp hay.

Thom (28) reports the presence of penicillia in almost every type of substratum. Many studies (17) of molds as a cause of deterioration in corn have shown members of the Penicillium group as the destructive agents. Ruschman (23) found that the destruction of the flax fibers was due to penicillia. Thom identified Penicillium roqueforti and its related strains as the characteristic molds of silage.

Hay contains an abundance of carbohydrate material. A great deal of work has been done on the decomposition of carbohydrates by fungi, including celluloses, hemicellulose and pectins. Ward (32) studied the pathogenicity of penicillia as wood-destroying fungi. Dox and Neidig (6) showed that several of the aspergilli and penicillia were able to destroy the pentosans found in large quantities in soil and manure.

Many species of soil inhabiting molds are capable of transferring cellulose and lignin portions of plant material. A comprehensive idea of these activities, and of their importance in the economy of nature, is summarized by Thaysen and Bunker (27).

EXPERIMENTAL PROCEDURE

A. Isolation and Identification of Fungi on Hay

1. Laboratory Procedure (Hay Fungi)

Isolation and identification of the molds on hay were carried out so that an over-all picture could be formed as to the types of fungi involved in this problem.

The bales of hay used for the isolation were obtained from farms in the vicinity of Lansing, Michigan. The bales, from which isolations were made, all showed visible moldiness. This hay was the first cutting of the spring.

The isolations of the molds were made by suspending small samples of the bale in sterile water. Various dilutions of these samples were plated in potato dextrose agar, pH 4.5, or Sabarouds maltose agar, pH 4.5. The plates were incubated for four days to two weeks at room temperature. In this way adequate time was allowed for the development of slow growing species.

The penicillia isolated in this study were identified by the aid of Thom's "The Penicillia" (28). The aspergilli were identified from descriptions in Thom and Raper's "Manual of the Aspergilli" (30). The rest of the cultures were identified only as to genera, by use of the key in Gilman's "Soil Fungi" (7).

TABLE I

THE TEN COMMONEST FUNGI ISOLATIONS FROM HAY
IN LANSING MICHIGAN AREA

Organism	No. Isolations from 30-40% Moisture Hay	No. Isolations from 10-20% Moisture Hay
Mucor sp.	18	11
Aspergillus niger	11	10
Aspergillus fumigatus	9	4
Penicillium expansum	11	2
Rhizopus sp.	6	9
Alternia sp.	5	8
Fusarium sp.	9	1
Aspergillus glaucus	3	6
Aspergillus repens	4	2
Trichoderma sp.	3	0

Note: 18 samples of 30-40% moisture hay used for isolations.
12 samples of 10-20% moisture hay used for isolations.

2. Results and Discussion

In Table I are presented the ten commonest molds isolated from 30 bales of hay. These molds represent over 300 isolations. The most frequently isolated fungi were found to be members of three groups: Mucor, Aspergillus and Penicillium. An attempt was made to differentiate between the fungus flora of high moisture hay and hay having a low moisture content. The number of samples was not sufficient to show any definite trend. However, the results obtained indicate that the same molds were predominant in both types of samples.

Thom and Raper (30) point out that the Aspergillus glaucus group is most conspicuous upon concentrated material, such as drying plant products. The Aspergillus niger group, which is probably the most common of the aspergilli, is a predominant cause of mildew. Aspergillus fumigatus is an extremely cosmopolitan mold and occurs with particular frequency on vegetable material, undergoing slow decomposition, and upon imperfectly stored grains.

The presence of the penicillia is to be expected. Cereal grains and their products are a favorable host material for this group of organisms. Their presence is directly connected with the presence of moisture. It has been found that penicillia are unable to grow at the low percentage of moisture at which members of the A. glaucus group are still active.

Mucors are found in greater abundance in the soil than any other group of soil fungi. Compared with other fungi, isolated in this study, mucors were found to grow most rapidly on the media used.

TABLE II

FUNGI ISOLATED FROM HAY AS COMPARED WITH GENERA REPORTED PRESENT IN SOIL*

Hay Fungi		Soil Fungi	
1.	Mucor	1.	Absidia
2.	Rhizopus	2.	Mucor
3.	Aspergillus	3.	Rhizopus
4.	Rhodatorula and other yeasts	4.	Zygorhynchus
		5.	Saccharomyces
5.	Penicillium	6.	Chaetomium
6.	Scopulariopsis	7.	Monilia
7.	Cephalosporium	8.	Oidium
8.	Trichoderma	9.	Sporotrichum
9.	Alternaria	10.	Botrytis
10.	Cladosporium	11.	Aspergillus
11.	Sporotrichum	12.	Penicillium
12.	Verticillium	13.	Scopulariopsis
13.	Fusarium	14.	Verticillium
14.	Helminthosporium	15.	Cephalosporium
15.	Chaetomium	16.	Trichoderma
16.	Oidium	17.	Acrostalagmus
17.	Sterile white mycelium	18.	Zygodesmus
		19.	Dicoccum
18.	Pullularia	20.	Cephalothecium
		21.	Basisporium
		22.	Dematium
		23.	Acremoniella
		24.	Cladosporium
		25.	Alternaria
		26.	Fusarium
		27.	Melanconium
		28.	Coniothyrium
		29.	Sclerotium
		30.	Sterile red and white mycelium

* The soil genera are from a list compiled by Waksman, S. A., Soil Fungi and Their Activities. Soil Science. 2:139. 1916.

Their extreme proliferation proved to be a handicap, in that they masked slower growing organisms.

Workers in the field of soil fungi have found results similar to those in this paper. Waksman (33) lists the most widely spread groups of soil fungi as the Mucor, Aspergillus, Penicillium, Trichoderma and Rhizopus. Table II shows the main groups of fungi found in hay as compared to the main groups found in soil by Waksman (33). All genera isolated from hay are seemingly represented in soil. In both cases, the predominant groups are the mucors, the aspergilli and the penicillia.

Werkenthin (34) concluded that there is a rather constant and characteristic fungus flora in the soil. Aspergilli seemed to be the predominant fungi in southern soils, while penicillia and mucors are found more extensively in northern soils, and occur only occasionally in southern soils. Jensen (13) showed four typical groups present in the soil of Denmark. These are Mucor, Penicillium, Aspergillus, and Trichoderma. In addition, he found Fusarium and Alternaria present frequently in field soils. Other fungi found occasionally in field soils are Hormodendrum, Cephalosporium and Botrytis.

The reports, of the above mentioned workers, show that a parallel may be drawn between the groups found predominantly in soil and those present in hay, Table II. It is reasonable to assume from these data that the types of fungi causing mold on hay are the predominating fungi in the soil upon which the hay is growing.

3. Laboratory Procedure (Soil Fungi)

Isolation of soil fungi was attemped to establish whether there actually is a correlation between the fungus flora found in hay and that found in the soil. For these tests, soil samples were collected from the fields in which the hay, previously used in isolation, had been grown. The soil samples were collected in sterile flasks, under aseptic conditions, and were brought immediately to the laboratory. The soil samples were then placed in sterile water blanks and plated by the dilution plate method. Sabarouds dextrose agar, pH 4.5, was used for plating. All plates were incubated at room temperature and held for three weeks observation. The resulting colonies were then picked and purified by the dilution plate method.

Thirty soil samples were collected and tested. It was not anticipated that the results would include all genera or species that were present in the soil from which the samples had been taken. However, it was hoped that these random samples would indicate the most common fungi to be found in these particular soils.

4. Results and Discussion

The results obtained showed three main groups to be present in an overwhelming majority. As in the case of hay, these three groups proved to be the mucors, penicillia and aspergilli. Of the thirty samples investigated, thirty contained aspergilli (thirteen of which were *Aspergillus niger*), and ten contained penicillia.

The result of this study indicates, within certain limitations, that three predominant types of fungi grow in soil and in hay. The predominance of these three main groups does not rule out the implication of other groups as causative agents in the molding of hay. The area of sampling in this experiment was too limited to make a blanket statement to the effect that the elimination of any specific groups would solve the entire mold problem in hay. However, the results of the data compiled show a more or less constant flora is present in hay and in the soil upon which members of this flora are growing. The large variety of fungi found points to the need of controlling all mold growth on hay, if the problem is to be solved.

B. A Study of Mold-inhibiting Agents

Inherent characteristics in mold-inhibiting materials made most compounds unsuitable for use in this problem. In selecting the material, the main facts to be taken into consideration were: (1) toxicity; (2) solubility; (3) methods of application; (4) cost of material to the farmer.

The inhibiting agent had to be toxic to fungi, yet be totally non-toxic to animals. This fact ruled out a great many of the fungicides known today, especially such as the heavy metals, copper, mercury, arsenic, etc. Because of this fact, attention was turned to the use of the fatty acids which are naturally occurring in many substances and have been used as mycostatic preservatives for a long time.

Other reasons for experimentation with the fatty acids can also be enumerated. They are relatively soluble and, therefore, could be easily applied in a solution. Their cost is comparatively low. This is an essential factor for so extensive a problem as the one encountered in the protection of the hay. The fatty acids are also easily produced on a commercial basis and thus readily obtained.

1. A Discussion of the Fatty Acids

The disinfectant value of strongly dissociated mineral acids, such as sulfuric and hydrochloric, depends upon the number of free hydrogen ions present. The weak organic acids, however, exert a more toxic effect than would be indicated by the degree of dissociation.

In determining the mode of action of the fatty acids as disinfecting agents, various theories have been presented. Many workers have attributed the action to a particular entity on the basis of their experimental work. However, the effect of an acid seems to be a collective one in which several attributing factors must be taken into consideration.

The factors which must be considered when discussing the effect of organic acids upon microorganisms can be enumerated under the following headings:

1. The hydrogen ion concentration.
2. The characteristic anion.
3. The undissociated molecule.
4. The surface tension.
5. The molecular weight and size of the molecule.
6. Specificity of certain acids for certain microorganisms.

Acetic acid in vinegar is one of the most ancient preservatives. Wolf and Shunk (35) found that it was more toxic to the six species of bacteria investigated than was hydrochloric, sulfuric, citric, tartaric, malic, or formic acids. In the experiments of Bitting (1), acetic acid was as toxic to fungi as benzoic, boric, or salicylic acids. It killed Penicillium expansum, Alternaria soloni, and Oidium lactis under conditions in which citric, lactic, malic or tartaric acids only slightly retarded their growth.

The toxicity of acetic acid to fungi has been discussed by many workers. Doran (5) indicates that the toxicity to fungi by acetic acid is due largely to the undissociated molecule and partially to the hydrogen concentration. Kahlenberg and True (15) found that undissociated acetic acid is toxic to molds. According to True (31), the $C_2H_3O_2$ ion is not toxic. However, Wolf and Shunk (35) found that the limit for the growth of Bacterium tabacum was pH 5.9, when the medium was adjusted with acetic acid, and pH 4.6 when the medium was adjusted with malic acid. They concluded that the hydrogen ion alone is not responsible for the toxicity of acetic acid.

Kahlenberg and True (15) found that formic acid was the most toxic to fungi of the fatty acids, being strongly dissociated. Propionic, butyric and valeric acid exhibited a similar inhibitory activity and were slightly more active than acetic, although the latter is more strongly dissociated.

The relationship of molecular structure to mycostatic power of the fatty acids was demonstrated by Hoffman, Schweitzer and Gaston (10). By using substituted acids, they showed that, by altering the position of the substituted group, the fungicidal power would be altered. Monochloroacetic, alpha-chloropropionic and di-chloropropionic acids showed less disinfecting power than normal propionic acid. The introduction of chlorine on the alpha carbon decreased the mycostatic power of the acid with the same effect on mono-chloroacetic acid. A chlorine atom on the carbon next to the carboxyl group affects the mycostatic properties of the acid, whereas the same substituent on the beta

TABLE III

PHYSICAL PROPERTIES OF ORGANIC ACIDS

	Formula	Molecular Weight	pH of IN Solution	Surface Tension IN Solution
Monobasic				
Acetic	$CH3COOH$	60.04	2.36	61.85
Propionic	C_2H_5COOH	74.06	2.40	50.36
Butyric	C_3H_7COOH	88.08	2.36	36.16
Valeric	C_4H_9COOH	102.11	2.40	32.44
Dibasic				
Oxalic	$(COOH)_2$	126.06	.90	75.03
Malonic	$CH_2(COOH)_2$	104.05	1.55	70.98
Succinic	$(CH_2)_2(COOH)_2$	118.07	2.20	66.58

carbon atom has no effect.

Langmuir (16) pointed out that halogen substitution had an appreciable effect on the dissociation constant of a fatty acid. The theory is rather widely held that the biological activity of an acid is dependent on the dissociation constant; that is, the less ionized the acid, the more powerful its biological effect. The result of Langmuir's work showed that the relative mycostatic power of acetic, monochloroacetic, propionic, alpha-chloropropionic acids, when compared with their respective dissociation constants, are in agreement with this theory.

Bruenn (2), by adding varying amounts of a salt containing a common anion to acetic and lactic acid, determined that a decrease in the number of free hydrogen ions resulted in a decrease of disinfectant power. He concluded that the disinfectant power of the organic acids depends upon the hydrogen ion concentration only. Norton and Hsu (19) agree that the disinfectant action of acids is due to their hydrogen ion. Decreasing the number of free hydrogen ions in formic acid, and increasing the undissociated molecule, by the addition of a common ion, decreases the toxic action of the acid for bacteria. These workers increased the number of free anions without changing the hydrogen ion concentration or the number of undissociated molecules. They determined that the acid anions act upon the hydrogen ions as positive catalyzers, increasing their germicidal powers.

Paul, Birstein and Reusz (20) reported that the organic acids, acetic and butyric, were more toxic for bacteria than hydrochloric, when equal isohydric solutions were considered. This they explained on the basis of the additive toxic effect which the anion and undissociated molecule might exert.

Reid (21) indicated that there is a relationship between the surface tension of the acids and their disinfecting powers. He demonstrated that the lowering of surface tension is inversely proportional to the length of the carbon chain. Therefore, as the antiseptic properties of an acid increases, the surface tension of the acid decreases.

The dibasic acids, oxalic, malonic and succinic, decrease in toxicity to fungi as the series is ascended. Paus, mentioned by Reid (21), noted that, with the exception of oxalic and malonic, all dibasic acids were less active against Escherichia coli and Salmonella typhosa than were the normal fatty acids. He concluded that there was little relationship between the hydrogen ion concentration and growth, but that the kind of acid, as well as the acidity, was responsible for their germicidal action.

All the literature discussed in the preceeding paragraphs emphasizes the fact that the exact mode of action of the fatty acids is undecided. Too many of these workers have disregarded the fact that the mode of action of these acids is a combination of the various factors presented, rather than the result of a single factor.

2. Laboratory Experiments on the Effect of Some Fatty Acids on Fungi

The fungi selected for these tests were obtained from isolations previously mentioned. They were chosen as examples of the predominant groups which were found by the author, in hay from the Lansing, Michigan area.

An agar plate method was used for testing the effect of the various acids on the organisms. In all experiments carried out, tryptose glucose extract agar (T.G.E.) was used. The pH of the media, before the addition of the acids, was 6.0-6.2. The acids were diluted in sterile water, using the medium as the last diluent. The final volume of all plates was ten ml.

Suspensions of conidia of the test fungi were made by washing the cultures with sterile water. One-tenth ml. of the suspensions was then transferred, by pipette, to the plate containing acid concentration ranging from 0.1-1.0 percent. The plates were incubated at room temperature, and results were recorded every day for three weeks (Table IV). Plates on which well developed colonies were observed were recorded as positive.(x) Where only a slight growth or dwarf colonies could be observed with the naked eye, plus-minus (±) was indicated. Plates showing no growth at the end of three weeks were considered to be negative (-).

3. Results and Discussion

The data in Table IV show that as the carbon chain of the monobasic acids was lengthened, there was an increase in the mold inhibiting effect. There is one noticeable exception in the results for valeric acid. The inability of this acid to show increased inhibition to fungi at higher concentrations can be explained by its insolubility in water. Table IV also illustrates the specificities that the acids

TABLE IV

INHIBITION OF MONOBASIC AND DIBASIC ACIDS ON FUNGI

	Non-buffered Acetic					Propionic					Butyric					Valeric				
% Con.	.1	.3	.5	.7	1	.1	.3	.5	.7	1	.1	.3	.5	.7	1	.1	.3	.5	.7	1
A	x	x/-	-	-	-	x	x/-	x/-	-	-	x	-	-	-	-	x	x/-	-	-	-
B	x	x	-	-	-	x	-	-	-	-	x	-	-	-	-	x	-	-	-	-
C	x	-	-	-	-	-	-	-	-	-	-	-	-	-	-	x	-	-	-	-
D	x	$\underline{x}$	-	-	-	x	$\underline{x}$	-	-	-	-	-	-	-	-	x	-	-	-	-
Buffered pH 3.5																				
A	x	x	-	-	-	x	x	-	-	-	x	-	-	-	-	x	x	-	-	-
B	x	x	x	-	-	x	-	-	-	-	x	-	-	-	-	x	x	-	-	-
C	x	x	-	-	-	x	-	-	-	-	-	-	-	-	-	x	x	-	-	-
D	x	x	-	-	-	x	x	-	-	-	x	-	-	-	-	x	x	-	-	-
	Non-buffered Oxalic					Malonic					Succinic									
A	x	$\underline{x}$	-	-	-	x	x	$\underline{x}$	-	-	x	x	x	x	x					
B	x	x	-	-	-	x	x	x	-	-	x	x	x	x	x					
C	x	$\underline{x}$	-	-	-	$\underline{x}$	$\underline{x}$	-	-	-	x	x	x	x	x					
D	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x					

Key
A. . Aspergillus fumigatus B . . Rhizopus nigricans C . . Mucor sp. D . . Penicillium frequetans

have for certain fungi. The data in this table show that an acid concentration greater than 0.5 percent would be required to assure inhibition of the most common molds.

In discussing the results recorded in Table IV, it is interesting to note that at the end of the three week observation period, the ($\underline{X}$) colonies were no larger than they were when growth was first observed at four to six days. Upon transplanting portions of these colonies into nutrient broth or on Sabarouds agar, there was observed a natural abundant growth in three days. All controls in the above experiments were positive in 72 to 96 hours.

When valeric, butyric, propionic and acetic acids were buffered (using a KH_2PO_4 and NaOH system) to a pH of 3.5, the inhibiting concentrations of the acids were almost the same as in those of a non-buffered solution (Table IV). This indicates that in the monobasic acids, the undissociated molecule is more important than the hydrogen ion as a cause of toxicity towards the fungi tested here. These data show a relationship between inhibiting power and surface tension. Table III indicates that the surface tension of the acids decreases as the carbon chain becomes longer. If two acids are compared and one is more active as a surface tension depressant than the other, the one which induces the lower surface tension will be present in the greater concentration at the interface between the fungus and the medium and therefore will have the greater effect.

Graph I shows the necessary pH and concentrations of propionic acid which were mycostatic to Aspergillus niger, Penicillium frequetans and Rhizopus nigricans. These results were obtained by buffering the acid to various pH levels and determining the molar concentration required to inhibit the fungi at that pH. The points on the graph represent the lowest concentration of acid which inhibited at the particular pH. These results are similar to those of other workers.[1]

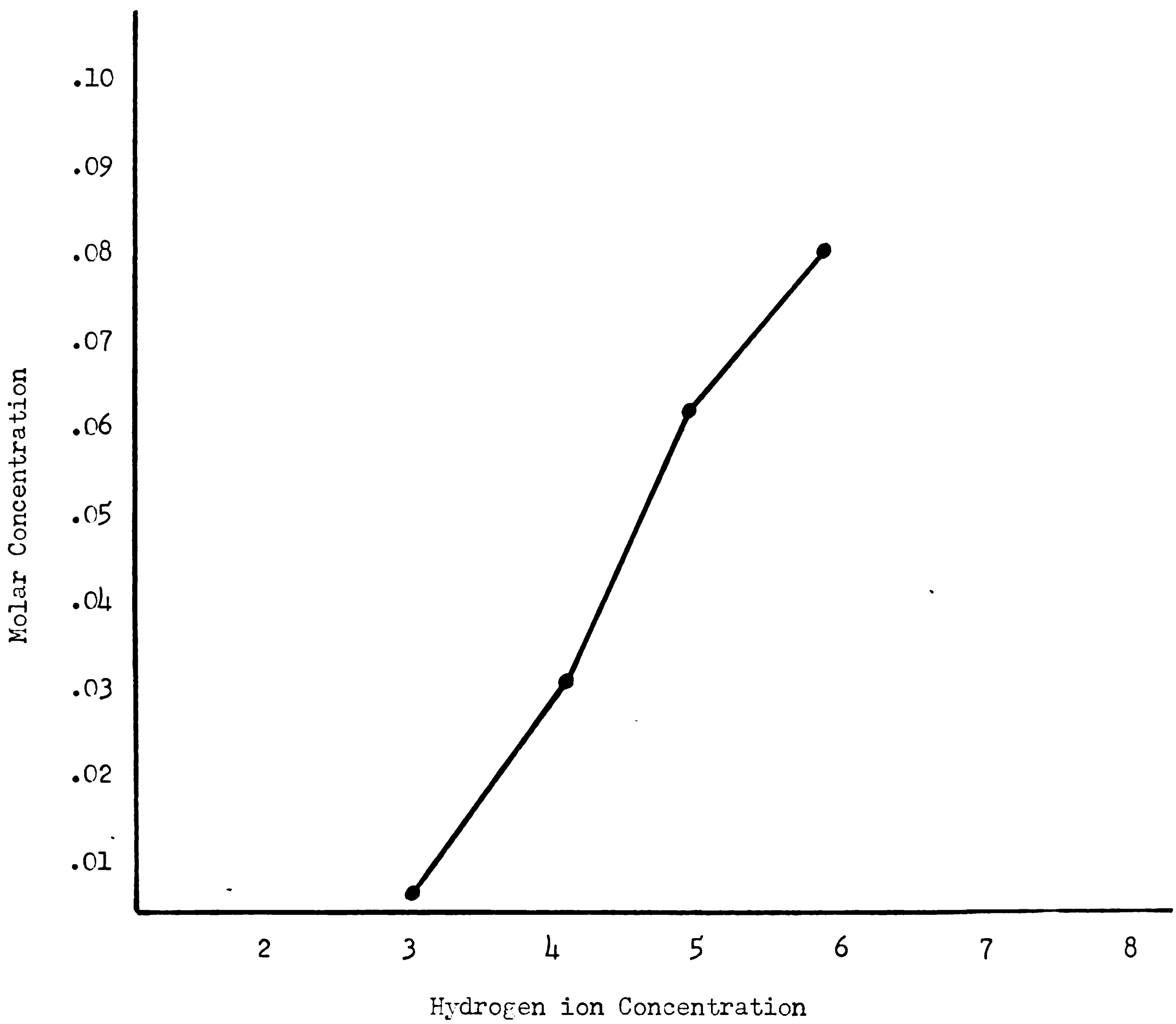
When an acid was added to the medium for testing its fungicidal properties, a change in the pH of the medium occurred as recorded in Table V. All pH readings were measured by a Beckman line pH meter. An examination of the data presented in Tables IV and V clearly indicated that inhibition of the fungi, by fatty acids, varies inversely with the dissociation constants of the acids. In contrast, it should be noted that the dibasic show the opposite results. This could be explained by the extremely low pH of these acids, making their action more like that of the mineral acids, or by the theory that branched carbon chains will not have the inhibiting properties of straight carbon chains.

The sodium and calcium salts of propionic acid have had use in industry as mycostatic agents.[2] Using the plate method previously mentioned, results were obtained which showed that the pH of a medium must be well below 4.0 before any inhibition could be detected. The organisms used in this test were: Aspergillus niger, Penicillium frequetans and Rhizopus nigricans. It would appear from the results

---

1. Hoffman, C., Schweitzer, T. R., Gaston, D. Fungistatic properties of the fatty acids and possible biochemical significance. Food Res. 6:539. 1939.
2. Macy, H., J. C. Olson. Preliminary observations on the treatment of parchment paper with sodium or calcium propionate. Jour. Dairy Sci. 22:527. 1939.

GRAPH I



Concentrations of propionic acid mycostatic to Aspergillus niger, Penicillium frequetans and Rhizopus nigricans.

obtained, that the propionic ion was the inhibiting factor, and the presence of the calcium and sodium ions was insignificant. It can be seen that nothing would be gained by using a salt, at a low pH, to obtain results that would be more efficiently produced by using the corresponding acid.

After observing the effect of the fatty acids on fungi, it was decided to apply these acids to the problem of moldiness of hay. From the laboratory results, there was definite indications that the acids might inhibit mold formation during the storage period in the curing of hay.

For ease of application, an aqueous solution of the acids was desirable. This somewhat limited the acids which would be used. As was previously pointed out, increasing the length of the carbon chains increased the inhibiting powers of these acids. However, the problem of solubility limited the length of the carbon chains which could be used. Acids having carbon chains longer than valeric are too insoluble in water to give concentrations which would act as inhibiting agents. For this reason, only the acids from acetic through valeric were used for treating hay.

TABLE V

VARIATION IN HYDROGEN ION CONCENTRATION OF TRYPTOSE GLUCOSE AGAR AFTER ADDITION OF ORGANIC ACIDS

% Composition	1	.7	.5	.3	.1	Dissociation Constant
Monobasic Acids						
Acetic	3.10	3.29	3.32	3.41	3.63	1.86×10^{-5}
Propionic	3.20	3.30	3.40	3.50	3.70	1.4×10^{-5}
Butyric	3.33	3.40	3.48	3.50	3.79	1.48×10^{-5}
Valeric	3.36	3.41	3.50	3.57	3.85	1.60×10^{-5}
Dibasic Acids						
Oxalic	1.60	1.79	1.80	2.13	2.50	4.90×10^{-5}
Malonic	2.22	2.50	2.50	2.60	2.72	1.61×10^{-3}
Succinic	3.10	3.18	3.19	3.30	3.48	6.60×10^{-5}

4. Laboratory Experiments Using Fatty Acids as Mold-inhibitors on Hay

The hay used for these experiments was obtained from various fields in the vicinty of Lansing, Michigan. The hay was mowed and field dried in windrows, until a moisture content, ranging from 30 to 40 percent, was reached. When the proper moisture content was reached, the hay was divided into 300 gram portions. Both chopped hay and regular hay were used.

The test solution was applied to the hay by spraying with a paint type, spray gun, using air pressure of 30 pounds. The spray gun was held approximately two feet from the hay. Known volumes and concentrations of the test materials were used. The hay was then placed in two quart, wide mouth jars. It was found that 300 grams of hay (35 percent moisture) would solidly fill one of these jars, hence simulating as closely as possible the condition of a bale. Some jars were covered with paper, allowing an air passage, while others were covered with glass lids. The jars were incubated, both at room temperature and at 37° C. Daily checks were made for the appearance of visible mold or deterioration of the hay.

a. <u>Control test 1</u>. Hay samples, ranging from 35 to 40 percent moisture, were placed in jars and incubated at 37° C., while other samples were placed in jars and allowed to stand at room temperature. None of this hay was treated in any way. The results obtained showed that hay with 35 percent moisture possessed visible mold at the end of 48 hours incubation. The jars covered with paper showed the same amount of mold as did the jars covered with a glass cap. Therefore, it can be assumed that the passage of air had little effect on the development of mold in this test. Within one week, all jars were completely filled with mycelia and beads of moisture, due to respiratory activity of both mold and hay. A putrid odor was also observed.

b. Control test 2. Tests were made in which the moisture content of the hay was reduced below 30 percent. It was found that hay containing less than 20 percent moisture would occasionally produce visible mold spoilage. Below 15 percent, all samples showed negative mold development.

The results of these control tests are similar to work reported by Terry (26), who found that hay in which the moisture content was reduced below 20 percent, in not more than three-and-one-half days, was free of mold. Terry also stated, "When the temperature is eighty to ninety degrees Fahrenheit, the drying process must be completed in less than two days, if mold is to be avoided".

Some known commercial fungicides were tested by the method described above. These compounds have been used in mold problems similar to that encountered in hay. The compounds used were: D.H.A.S.[1], Thiourea, and a commercial compound, V.H.C.9[2], specifically sold for the prevention of mold on high moisture hay. Aqueous solutions of D.H.A.S. and Thiourea, ranging from one-tenth to one percent, were sprayed on 300 grams of hay. V.H.C.9 was applied in powder form to another 300 grams of hay, according to the manufacturer's instruction. After treating the hay with these chemicals, all samples were placed in jars and incubated in a manner similar to that of the previously mentioned controls.

The results of the above experiment are presented in Table VI. These data show that the concentrations of the test materials were too low to be effective in the prevention of mold development on hay. Since Thiourea and D.H.A.S. produce a toxic effect in animals, when used in higher concentrations, and since these concentrations would be very costly to use in large quantities, it seemed inadvisable to attempt any further work with these compounds.

Table VI shows that V.H.C.9 was ineffective in the concentrations used. In these tests, as much as 50 grams of compound was applied to 300 grams of hay. These quantities were far in excess of those deemed necessary, by the manufacturer, to prevent mold development.

---

1. Dow Chemical Company, Midland, Michigan.
2. VHC9 H. D. Campbell Co., Rochelle, Illinois - Advertising pamphlet - Hudson Chemical Co. New Crop Preserving VHC9.

In succeeding experiments, using the spray method on 300 grams samples of hay, the effect of the fatty acids on hay fungi was tested. Hay with 35 to 40 percent moisture by weight, was used in all experiments. The data in Table VII show that a definite inhibiting force is present in these acids which indicates that they could be used in this particular problem. Butyric acid would be the best acid because of its inhibiting properties, according to these results, since necessary concentration of this acid, in aqueous solution, would be lower than any of the other acids. However, the odor of both butyric and valeric acid would be a detriment which would eliminate any wide use by farmers in practical application. Propionic acid, in concentration over two percent, showed results equal to those of the other acids. The odor of this acid would not minimze its use; in fact, four days after spraying there was a lack of any residual odor on the hay.

In comparing the data of Table IV with those of Table VII, there is a direct correlation between the inhibiting qualities of the acids on test organisms in plate media, and on the organisms in their normal environmental conditions. The only deviation was in the effect of valeric acid upon the fungi in the hay. The inability of valeric acid to inhibit fungi at higher concentrations can be explained by its lower solubility in aqueous solutions; this prevented an even distribution of the acid on the hay with the spray method of application.

In making up the solution to be sprayed, it was found that 150 to 200 ml. of each solution was needed to give satisfactory results on 300 to 400 grams of hay. The amount did not vary with the

TABLE VI

COMMERCIAL FUNGICIDES TESTED ON HIGH MOISTURE HAY

Hours	THIOUREA			DHAS			VHC9	CONTROLS	
	.1%	.5%	1%	.1%	.5%	1%	50 gms.	37C	25C
24 Hrs.	-	-	-	-	-	-	-	-	-
48 Hrs.	-	-	-	-	-	-	-	-	$\underline{x}$
96 Hrs.	x	x	x	x	-	-	x	x	x
120 Hrs.	x	x	x	x	-	-	x	x	x
144 Hrs.	x	x	x	x	$\underline{x}$	-	x	x	x
168 Hrs.	x	x	x	x	x	x	x	x	x

x . . . Heavy mycelial development

$\underline{x}$ . . . Small patches of mycelia

\- . . . No visible mycelia

concentrations, indicating that there must be a total coverage of the hay surface to prevent molding.

Graph 2 shows the relationship of percent of concentration of the acids to the length of time in which visible moldiness would be observed. As the length of the carbon chain of the fatty acids increased, the concentration required for the inhibition of mycelial development decreased. The one exception, as was previously pointed out, was valeric acid; only the lowest concentration of this acid acted in accordance with the carbon chain theory. This, again, due to the insolubility of the acid.

The addition of aqueous solutions raised the moisture content of the hay up to 70 to 80 percent by weight. The addition of great amounts of moisture would rule out the practicability of this operation in the field. However, if hay with a moisture content of 70 to 80 percent can be prevented from molding, then by some other method of application, such as vaporization, the acid could be applied without adding to the moisture content of the hay.

At the end of three months, the moisture content of the hay in the glass jars was still approximately 35 percent. At this time, the hay began to develop visible mycelial patches. When this was observed, the total contents would become moldy within three to four days. This indicated that the acids were not fungicidal, but rather, mycostatic in nature. Under natural conditions, it is probable that a bale of hay would dry much faster. The static period would be long enough so that, if the acid were applied, and the hay allowed to dry to a moisture

TABLE VII

CONCENTRATIONS OF ACIDS AND DAYS REQUIRING MOLD DEVELOPMENT ON HAY

	0.5%	1%	2%	3%	4%	5%	Control
3 days							
Acetic	x	x	x	x	x	$\underline{x}$	x
Propionic	$\underline{x}$	$\underline{x}$	-	-	-	-	x
Butyric	-	-	-	-	-	-	x
Valeric	-	-	-	-	-	-	x
6 days							
Acetic	x	x	x	x	x	x	x
Propionic	x	x	$\underline{x}$	-	-	-	x
Butyric	$\underline{x}$	-	-	-	-	-	x
Valeric	$\underline{x}$	-	-	-	-	-	x
9 days							
Acetic	x	x	x	x	x	x	x
Propionic	x	x	x	-	-	-	x
Butyric	x	-	-	-	-	-	x
Valeric	x	x	x	$\underline{x}$	-	-	x
15 days							
Acetic	x	x	x	x	x	x	x
Propionic	x	x	x	-	-	-	x
Butyric	x	$\underline{x}$	-	-	-	-	x
Valeric	x	x	x	$\underline{x}$	-	-	x

x . . . Heavy mycelial development
$\underline{x}$ . . . Scattered mycelial development
- . . . No visible mycelia

content below 20 percent in three to four weeks, there would be no visible moldiness.

5. Field Tests Using Fatty Acids as Mold Inhibitors

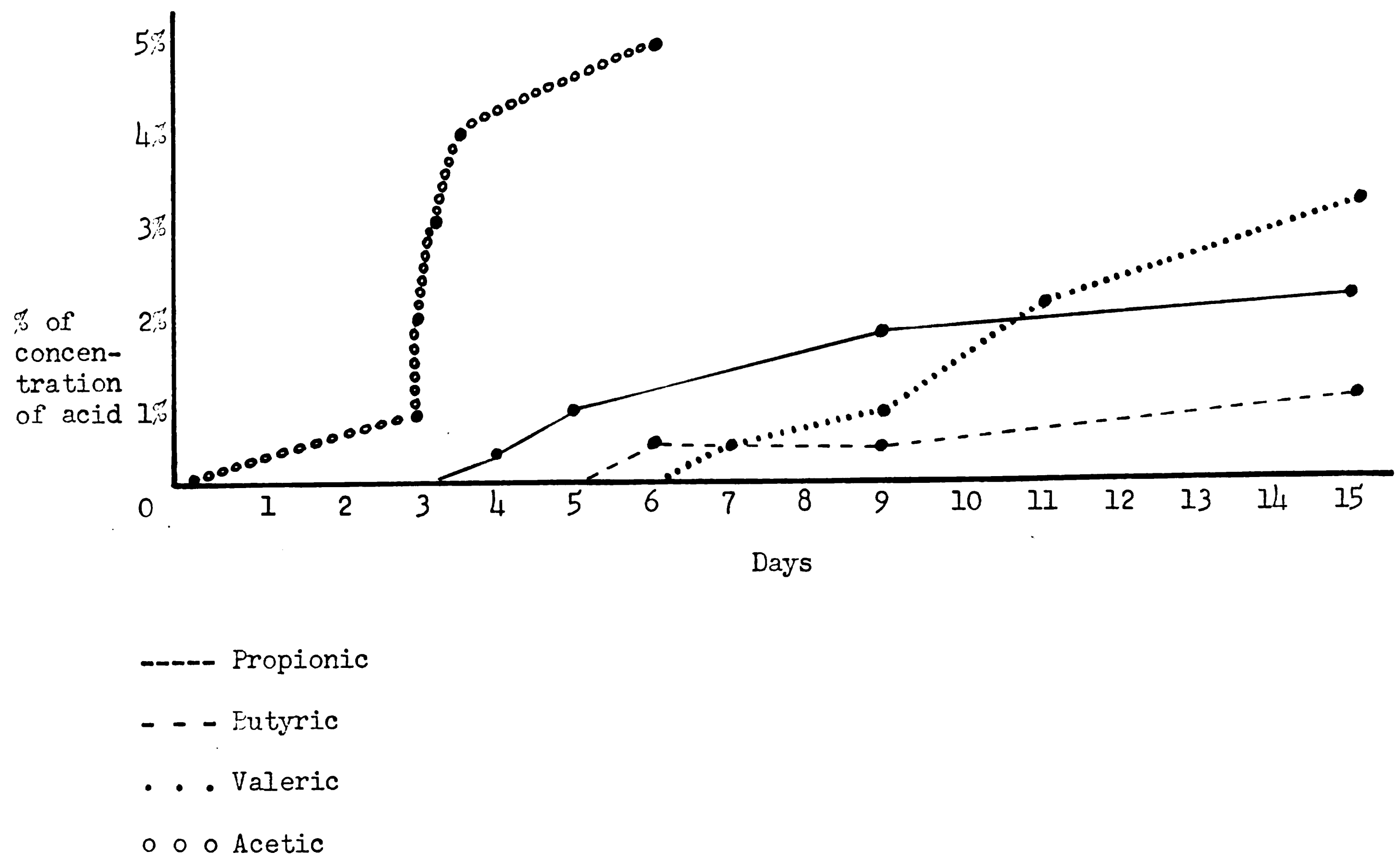
The procedure, used in the laboratory tests, was followed on a larger scale in the field. However, tests which were carried out under natural conditions introduced problems that could not be entirely foreseen during laboratory work.

In the first test, the application of the acid was made with the spray gun used in the laboratory procedure. The acid was applied to windrows of hay which had a 40 percent moisture content. This hay was then baled immediately. The coverage and penetration of the acid in this method was far too inadequate, as shown by the fact that all bales molded considerably within one week.

To overcome the problem of coverage, a new means of application was devised. Attachments were made on the mowing apparatus, and the acid was applied as the hay was being cut. The new procedure in the field was as follows: A five percent solution of propionic acid was sprayed from a series of jets on a pipe, which extended parallel to the cutter bar, three feet above the ground and eight feet ahead of the bar. A fine fog of the acid was sprayed ahead of the cutter bar, giving good coverage of the hay. The hay was windrowed and cured, then baled at 35 percent moisture content.

The results of this experiment showed no inhibition to

Relationship of days to % concentration of acid in showing visible mycelia



mold formation in the test bales. Within a few minutes after spraying, there appeared to be no residual acid on the hay. The acid seemed to volatilize rapidly during the earliest stages of curing, thus affording no protection against mold development in the bale. Since the hay was high in moisture content, there was considerable heating in these experimental bales.

It was impossible to carry out further field studies after this attempt, due to the fact that the end of the haying season had arrived. The weather conditions became unfavorable, and there was a lack of standing hay for any more tests.

CONCLUSION

The problem of mold development in hay, as pointed out in this paper, is a complex one, both from the standpoint of contributing factors and methods of control. In high moisture content hay, the host material is extremely compact, the amount of fungi very high and the moisture and temperature conditions exceedingly beneficial to the growth of these fungi.

The mold population of hay has been shown to be wide and varying, containing, in greatest numbers, the typical cosmopolitan fungi found in the soil upon which the hay is growing. Any chemical method of treatment, to prevent mold development, must eliminate all the various types of molds present on the hay; chemicals, which show a specificity for only certain groups of these fungi, will be of little value in solving this problem.

In the chemical treatment of hay, the action of the chemical need only be mycostatic in nature. If the mold present at the time of baling could be held in a state of inanimation, until a critical moisture content of the hay could be reached, the problem of mold in hay would be eliminated.

The laboratory experiments in this thesis indicated that the short chain fatty acids have sufficient mycostatic powers to be useful in preventing the development of mold in high moisture hay. By use of the fatty acids, high moisture content hay could be baled and placed in the barn, without fear of mold deterioration in the hay.

Propionic acid proved to be the most practical of the fatty acids for actual use. This acid is low in cost, free from unpleasant odor, easily obtained, readily soluble and proved effective in laboratory tests for the preservation of hay. It is necessary, however, to develop a practical method of application of the acid to the hay before baling, without materially changing the moisture content of the hay. The method developed must insure that volatilization of the acid should not take place before the bale is formed.

LITERATURE CITED

1. Bitting, K. G. The effect of certain agents on the development of some molds. U. S. Dept. Ag. Washington, D. C. Bull. 381, p. 176. 1920.

2. Burenn, W. Über das Desinfektionsvermögen der Säuren Inaug Dissert. (May) 1913.

3. Cheveley, S. W. Grass drying. Ivor Nicholson & Watson Lmtd. London. 1-137. 1937.

4. Cohn of Breslau. Spontaneous combustion of hay. Vet. Jour. 15: 310. 1890.

5. Doran, W. L. Acetic acids and pyroligneous acid in comparison with formaldehyde as soil disinfectants. Jour. Agr. Res. 44:57. 1932.

6. Dox, A. W., R. E. Neidig. Pentozans in lower fungi. Jour. Bio. Chem. 19:267-269. 1911.

7. Gilman, J. C. Soil fungi. The Collegiate Press Inc. Ames, Iowa. 1945.

8. Hendrix, A. T. Heat generated in chopped hay and its relationship to the drying effect. Jour. Ag. Eng. 28:286. 1947.

9. Henry, W. A., F. B. Morrison. Feeds and feeding. The Henry Morrison Co. 19th Ed. 1927.

10. Hoffman, C., T. R. Schweitzer, D. Gaston. The effect of chlorine substitution on the fungistatic properties of acetic and propionic acids. Jour. Am. Chem. Soc. 62:988-989. 1940.

11. Horsefall, J. G. Fungicides and their action. Chronica Botanica Co. Waltham, Mass. 1945.

12. Humphrey, C. J., R. M. Fleming. The toxicity to fungi of various oils and salts, particularly those used in wood preservation. U. S. Dept. Ag. Washington, D. C. Bull. 227. 1915.

13. Jensen, H. L. Fungus flora of the soil. Soil Sci. 31:123-158. 1931.

14. Klotz, L. J. Nitrogen trichloride and other gases as fungicides. Hilgardia. 10:1-52. 1936.

15. Kahlenberg, L., R. H. True. On the toxic action of dissolved salts and their electrolytic dissociation. Bot. Gaz. 22:81-124. 1896.

16. Langmuir, I. Forces near the surface of molecules. Chem. Rev. 6:465. 1929.

17. McHargue, J. S. The cause of deterioration and spoiling of corn and corn meal. Jour. Ind. Eng. Chem. 12:257. 1920.

18. Mallmann, W. L., C. E. Michael. The development of mold on cold storage eggs and methods of control. Mich. State College Ag. Exp. Station. Tech. Bull. 174. 1940.

19. Norton, J. F., P. H. Hsu. The physical chemistry of disinfection. Jour. Inf. Dis. 18:180-194. 1916.

20. Paul, Birstein and Reusz. Biochem. Ztschr. 29:202. 1910.

21. Reid, J. D. The disinfectant action of certain organic acids. Amer. Jour. Hyg. 16:540. 1932.

22. Rigler, N. E., G. A. Greathouse. The chemistry of resistance of plants to Phymatotrichium. Amer. Jour. Bot. 27: 701-704. 1940.

23. Ruschmann, G. An abstract in Review of Applied Mycology. 3:87. 1924.

24. Snow, D. Mould deterioration of feeding stuffs in relation to humidity of storage. Ann. App. Bio. 31:102-116. 1944.

25. Shepherd, J. B., H. G. Wiseman, R. E. Ely, C. G. Melin, C. H. Gordon. Tests with sulfur dioxide for forage preservation. U. S. Dept. Ag. Washington, D. C. Dairy Ind., B.D.I.M.-Inf. 106. 1950.

26. Terry, C. W. Relation of time and operating schedule to hay quality, mold development and economy of operation. Jour. Ag. Eng. 28:141-144. 1947.

27. Thaysen, A. C., H. J. Bunker. The microbiology of cellulose, hemicellulose, pectins and gums. Oxford Univ. Press. London. 1927.

28. Thom, C. The Penicillia. Williams & Wilkins Co. Baltimore. 1930.

29. Thom, E., E. LeFevre. Flora of corn meal. Jour. Ag. Res. 22:179. 1921.

30. Thom, C.,K. B. Raper. Manual of the Aspergilli. Williams & Wilkins Co. Baltimore. 1945.

31. True, R. H. The toxic action of a series of acids and of their sodium salts on Lupinus albus. Amer. Jour. Sci. 9:183-192. 1900.

32. Ward, H. M. Penicillium as a wood destroying fungus. Ann. Bot. 12:565-566. 1898.

33. Waksman, S. Fungus flora of the soil. Soil Sci. 3:565-589. 1917.

34. Werkenthin, F. C. Fungus flora of Texas soils. Phytopathology. 6:241-253. 1916.

35. Wolf, F. A., I. V. Shunk. Tolerance to acids of certain bacterial plant pathogens. Phytopathology. 11:244-250. 1921.

36. Wright, N. C. The storage of artificially dried grass. Jour. Ag. Sci. 31:194-197. 1940.