

1940

Relationship of the lipolytic and proteolytic activities of various penicillia to the ripening of blue cheese

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RELATIONSHIP OF THE LIPOLYTIC AND PROTEOLYTIC ACTIVITIES
OF VARIOUS PENICILLIA TO THE RIPENING OF BLUE CHEESE

by

Christian Jensen

A Thesis Submitted to the Graduate Faculty
for the Degree of

DOCTOR OF PHILOSOPHY

Major Subject Dairy Bacteriology

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GENERAL INTRODUCTION

The ripening of blue cheese involves the breakdown of fats and proteins through the activities of the respective enzymes, lipases and proteases. These enzymes are elaborated in large quantities by the penicillia employed in the ripening and are also present in small amounts in raw milk.

Flavor development in blue cheese is generally believed to be largely the result of fat hydrolysis. Some penicillia are capable of hydrolyzing fat with an accumulation of caproic, caprylic and capric acids and their readily hydrolyzable salts; these constituents are believed to be responsible for the peppery flavor of blue cheese. When grown under unfavorable conditions the penicillia are thought to decompose the fatty acids to methyl ketones through a beta-oxidation; the very important methyl-n-amyl ketone is formed from caprylic acid. The methyl ketones contribute to the typical flavor of blue cheese.

The proteolytic enzymes decompose milk proteins to their various degradation products, such as peptones, peptids, amino acids and ammonia. Although these enzymes supposedly do not function in flavor development, they are of importance in breaking down the curd to the proper consistency. Absence of proteolytic enzymes probably would result in cheese fully developed in flavor but possessing a hard brittle body.

The time required for ripening of blue cheese is undoubtedly responsible for much uncertainty concerning the quality of cheese and is

also of economic importance from the standpoint of capital turnover. Hence, the possibility of employing cultural methods in selecting strains of penicillia for the purpose of standardizing the quality of the cheese, as well as to reduce the time of ripening, is of importance to the cheese industry.

GENERAL STATEMENT OF PROBLEM

The object of the work reported herein was to determine the:

1. Lipolytic and proteolytic activities of various penicillia employed in the ripening of blue cheese.
2. Relationship between the lipolytic activities of penicillia and the ripening of blue cheese, from the standpoint of (a) time of ripening and (b) quality of the cheese.
3. Relationship between the proteolytic activities of penicillia and the ripening of blue cheese, with respect to (a) time of ripening and (b) quality of the cheese.
4. Feasibility of employing cultural methods in the selection of strains of penicillia suitable for the ripening of blue cheese.

GENERAL REVIEW OF LITERATURE

Manufacture of Blue Veined Cheeses

Various procedures have been proposed for the manufacture of roquefort and related types of cheese. Marre (51) published a text in which a method is described for the manufacture of roquefort cheese from sheep's milk. Procedures for the manufacture of stilton and wensleydale cheeses were outlined by Benson (5). Golding (23) proposed a method of making wensleydale cheese under Canadian conditions. Funder (22) reported a modified procedure for producing roquefort cheese in Norway.

A method for manufacturing goats' milk roquefort cheese in California was suggested by Hall and Phillips (32). The product was reported to be equal in quality to imported roquefort cheese made from sheep's milk.

In the United States various attempts have been made to manufacture a modified roquefort type cheese from cows' milk. One such method was published by Thom, Matheson and Currie (76), who concluded that the flavor and texture of cows' milk cheese can approximate that of sheep's milk cheese but cannot duplicate it. The investigation was continued by Matheson (52) and, after extensive research, he outlined a detailed procedure for the manufacture of cows' milk roquefort cheese, which he believed could be made successfully and profitably. Goss, Nielson and Mortensen (31) proposed a modified method for the manufacture of blue

cheese from cows' milk. Lane and Hammer (46) suggested a method for the manufacture of blue cheese from homogenized cows' milk. This involved modifications in the manufacturing procedure other than the homogenization. After an extensive study of various factors involved in the ripening of blue cheese, Lane and Hammer (47) reported that cheese could be rather consistently prepared from homogenized milk which was lighter in color, softer in body, possessed a more peppery flavor and ripened faster than cheese made from unhomogenized milk. These workers also found homogenized cows' milk to be generally more satisfactory than unhomogenized goats' milk for the manufacture of blue cheese. Homogenization pressures of 3000 plus 500 pounds ordinarily produced most satisfactory results, although 2000 plus 500 pounds also gave good results.

Microorganisms of Importance in Ripening of Blue Veined Cheeses

The literature dealing with the general types of microorganisms associated with ripening of blue veined cheeses reveals a close agreement in results obtained by various investigators. Thom (71) concluded that lactic acid bacteria and Penicillium roqueforti are the only organisms necessary in the ripening of roquefort and related types of cheese; this mold was also found in stilton, gorgonzola and brinse cheeses. From stilton cheese Percival and Mason (59) isolated various organisms, including Streptococcus acidilactici, a species of Thyrothrix, Penicillium glaucum and a round form of Torula; Penicillium glaucum was reported to be checked in growth by the Thyrothrix. Lactic acid bacteria

were predominant in the early stages of ripening; there was then a gradual decline in the number of these organisms and in the later stages of ripening Penicillium glaucum and a form of Torula were the most abundant. Thom and Matheson (75) reported the presence in roquefort cheese of various types of organisms consisting of Penicillium roqueforti, the common lactic acid bacteria, including both the Bacillus lactis acidi and the Bacillus bulgaricus groups, some liquefying organisms, a small number of yeasts and the varied flora of the surface slime. The slime on the surface of cheese was not believed to function in the development of cheese flavor but to serve only as an index of humidity and temperature conditions in cheese ripening. According to Evans (21), the essential organisms in the ripening of roquefort cheese are Streptococcus lacticus and Penicillium roqueforti. The surface slime was found to consist of micrococci, rod forms and yeasts. Evans also concluded that enzymes contained in the slime are unnecessary for the ripening of cheese.

Penicillia Associated with Ripening of Blue Veined Cheeses

The mold associated with the ripening of blue veined cheeses was referred to by many of the earlier workers as Penicillium glaucum. Thom (71), in studying fungi employed in cheese ripening, isolated and described the mold in roquefort cheese and named it Penicillium roqueforti.

Attempts have been made to differentiate the various strains of penicillia concerned with blue veined cheese ripening. Of twelve cheeses examined by Steuart (68), including roquefort, gorgonzola, stilton,

wensleydale and blue caerphilly, Penicillium roqueforti was predominant in eight but completely absent in three. Golding (24) reported that the dominant mold in wensleydale cheese differed from Penicillium roqueforti, since the strain from wensleydale cheese produced a slight amount of acid in whey-peptone-litmus gelatin, whereas Penicillium roqueforti produced none. He also found that, when compared with the wensleydale mold, Penicillium roqueforti digested casein four times as rapidly. Similarly, Arnaudi (2) found comparatively large variations in morphologic and cultural characteristics of fifteen strains of penicillia isolated from an equal number of gorgonzola cheese. This investigator concluded that, by the isolation and selection of cultures, strains could be obtained which had a more beneficial effect on cheese than others. According to Thom (73), different strains of Penicillium roqueforti show such large variations in their biochemical characteristics that their practical usefulness is affected, and differences in the strains were found to influence the quality of the cheese. Eight cultures of penicillia associated with the ripening of blue cheese were studied by Lane (45). He found they varied considerably. Certain mold strains regularly produced cheese with a desirable flavor, whereas others were associated with cheese lacking in flavor or having off flavors.

General Action of the Penicillia Associated with
Ripening of Blue Veined Cheeses

Various investigators have noted that penicillia employed in blue veined cheese ripening have the ability to utilize acids for their

development. Thom, Matheson and Currie (76) reported that in the ripening of roquefort cheese Penicillium roqueforti plays a major role. They believed that ripening is brought about by the mold through enzyme production, which causes reduction of acidity and partial decomposition of the fat and protein. From a study of the volatile acidity of stilton cheese, Hiscox (37) concluded that volatile acids produced by bacteria during the early stages of ripening are utilized by the molds, so that there is a decrease in the amount of volatile acids after extended ripening. Laxa (49) found that certain volatile acids studied by him were utilized by Penicillium glaucum. Rahn (61) reported that Penicillium glaucum showed a preference for the lower fatty acids and that they were completely decomposed by this mold. Similarly, Bryant (8) found that the lower fatty acids were utilized by Penicillium roqueforti when this mold was inoculated into sterile skim milk containing these acids and also that more of the acids of lower molecular weight were utilized than of higher molecular weight.

Factors Affecting Growth of Various Penicillia

From the work of different investigators, it is evident that the growth of Penicillium roqueforti is influenced by variations in environmental conditions. Golding (26) reported that the growth of Penicillium roqueforti in milk was increased by the addition of small amounts of citric acid and suggested that the type of starter might influence mold growth. In a synthetic medium acetic acid increased mold growth, whereas citric acid had an inhibiting effect. Laxa (49)

determined the effect of acetic, butyric and caprylic acids on the growth of Penicillium roqueforti and Oidium lactis by growing the molds on media containing them. He reported that the toxicity of these fatty acids increased with the increase in molecular weight.

According to Bryant (7), more rapid mold growth was obtained when blue cheese were inoculated with new spores than when either mycelium or old spores were used.

Naylor, Smith and Collins (55) obtained the heaviest growth of Penicillium roqueforti on a synthetic medium adjusted to pH 4.5 and containing a small amount of ammonium chloride. In an endeavor to produce a strong enzyme extract from Penicillium roqueforti, Golding (28) obtained excellent mold growth by using a modified Dox salt solution with increased proportions of casein and dextrose. Maximum mold felts were obtained when the medium contained 5 per cent casein and 20 per cent dextrose.

Golding (27) found that the addition of ammonium chloride to curd did not increase the growth of Penicillium roqueforti but had a slight detrimental effect.

Golding (29) reported that the introduction of air and oxygen, as well as the removal of carbon dioxide, favored the growth of mold in cheese; skewering cheese was the most effective method of increasing mold growth. Later he subjected wensleydale cheese in steel cylinders to continuous and also to intermittent air pressures; although both types of pressure increased mold growth in the cheese, the intermittent method gave the better results. Thom and Currie (74) found that, among several species of penicillia and aspergilli, only Penicillium roqueforti produced

colonies showing fairly good development in an atmosphere of 75 per cent carbon dioxide, which was the equivalent of 5 per cent free oxygen in the mixture. In a recent report, Golding (30) concluded that the growth of Penicillium roqueforti in cheese is not limited as much by the absence of oxygen as by the presence of carbon dioxide and that the purpose of skewering or punching a cheese is to permit the carbon dioxide to escape rather than to admit oxygen.

In a study of salt as a factor in mold ripened cheese, Thom (72) found that in the presence of a high concentration of salt (about 4.0 per cent) in roquefort cheese Penicillium roqueforti developed normally. There occurred only a negligible growth of the usual contaminating types of microorganisms, and the growth of Oidium lactis was completely stopped. Golding (25) investigated the salt tolerance of Penicillium roqueforti and reported that the salt concentrations normally found in roquefort cheese may slow up but not stop mold growth. According to Bryant (7), the concentration of salt found in blue cheese inhibits the growth of bacteria normally found in this type of cheese.

Lane and Hammer (48) reported that when 2 per cent salt was added to the curd before hooping better mold growth sometimes resulted; the cheese quite regularly were lighter in color and certain flavor defects were usually controlled.

Lipase in Milk

The early investigations by Thatcher and Dahlberg (70) and by Palmer (58) failed to demonstrate the presence of lipase in milk, but

more recent findings of Nair (54), Henneberg (35), Kay, Mattick and Tolley (41), Mattick and Kay (53) and Hilsman and Courtaoy (36) have definitely established it as a normal constituent of raw milk. Pfeffer, Jackson and Weckel (60) reported that lipase is present in the skim milk fraction and that it is found in a more concentrated form in the slime of the separator bowl. Krukovsky and Herrington (42) studied the activity of lipase in milk and found an increase in free fatty acids between the time of milking and the time of delivery to the pasteurizing plant. When raw milk was warmed to about 30° C. and cooled, the subsequent rate of lipolysis was greatly increased. They believed that two types of lipase are present in milk, as evidenced by different reactions toward formaldehyde. Krukovsky and Sharp (43) induced lipolysis of fat by shaking raw cows' milk while the fat was in a liquid or partially liquid state, and the lipolysis was continued after the milk was cooled and held at 2.0° C. They attributed the effect of shaking to an alteration in the surface character of the fat globules which created more favorable conditions for lipolysis. In a study of the effects of homogenization upon milk, Sharp and de Tomasi (64) reported increased development of rancidity with increasing homogenizing pressure and a rise in temperature up to 36.7° C. However, rancidity development was retarded at temperatures ranging from 37.8° to 46.1° C. Dorner and Widmer (18) found a much increased activity of lipase in milk as a result of homogenization and believed that the more extensive lipolysis was due to the greatly increased surface area of the fat globules. They found that lipase was inactivated by the following exposures: 54.4° C. for 20 minutes, 58.9° C. for 10 minutes, 62.8° C. for

5 minutes and 70° C. instantaneous exposure. They also believed that lipase is present in the milk serum.

In a preliminary paper dealing with homogenization of raw milk for blue cheese, Lane and Hammer (46) reported that the curd was more flaky, lighter in color and possessed a more typical flavor and aroma than that from unhomogenized milk. The rancid flavor and odor which characterized the curd from homogenized milk were attributed to the action of lipase upon the finely divided fat. This observation was substantiated by more extensive studies conducted by Lane and Hammer (47), who concluded that milk lipase definitely aids in cheese ripening since cheese made from raw skim milk plus fat constituents and subjected to homogenization quickly developed rancidity and the flavor and aroma of methyl-n-amyl ketone, whereas no early rancidity or ketone flavor and aroma developed in the cheese made from pasteurized skim milk plus the fat substances. Pfeffer, Jackson and Weckel (60) suggested that the increased activity of lipase in homogenized raw milk is not due entirely to decrease in the size of the fat globules.

Flavor Production in Blue Veined Cheeses

Flavor production in blue veined cheeses and identification of the specific substances responsible for the flavor and aroma of cheeses belonging to this group have received considerable attention. Orla-Jensen (57) concluded that the chief aroma constituent of roquefort cheese was the very sharp-tasting ethyl butyrate. No data were offered in support of this conclusion, the substance apparently being identified by smell.

Weigmann (80) assigned the rancid sharp flavor of French roquefort, English stilton and Italian gorgonzola cheeses to the activity of the green penicillia, since the characteristic flavor was observed after spore formation. Flavor production in roquefort cheese was studied in detail by Currie (15), who concluded that the peppery taste or burning effect produced on the palate and tongue by this type of cheese is due to caproic, caprylic and capric acids, together with their easily hydrolyzable salts, which are formed as a result of hydrolysis of milk fat. Currie (16) also reported that roquefort cheese from sheep's milk is far more peppery than cheese of equal ripeness from cows' milk. This, Currie believed, was probably due to the relatively high percentage of sharp tasting acids obtained on hydrolysis of sheep's milk. Corminhauf (13) found a direct relationship between the amount of volatile acids and intensity of flavor in roquefort cheese. Lane (45) compared eight strains of penicillia which were found to produce variable amounts of volatile acids in blue cheese, although approximately the same acids were produced by the different strains. He reported that one mold in particular regularly was capable of producing very fine cheese, whereas others were associated with cheese lacking in flavor. As a rule cheese containing relatively large amounts of these volatile acids possessed more of the characteristic peppery flavor and aroma of blue cheese than the product containing small amounts of acids. Subsequent investigations by Lane and Hamner (47) substantiated these results. It was pointed out by them that the volatile acidity values for cheese made from homogenized milk were roughly two to four times the values for cheese made from

unhomogenized milk. They also found that cheese made from pasteurized homogenized milk did not ripen as rapidly as cheese made from raw homogenized milk. However, when compared with cheese made from raw unhomogenized milk, the pasteurized homogenized product showed a more rapid development of volatile acid, higher acid values on fat and a more characteristic flavor.

Coulter and Combs (14) accelerated blue cheese ripening materially by addition of steapsin to the milk or curd. Although generally acceptable to the trade, the cheese was regarded as inferior by competent judges who criticized it as possessing a bitter flavor. Cheese ripened without mold but with steapsin alone developed rancid and atypical flavors.

In a study dealing with methyl ketone formation in the decomposition of coconut fat, Starkle (67) discussed the possibility of these compounds being of importance in the rancidity of butter and cheese. This investigator concluded that the characteristic aroma substances formed in mold ripened roquefort cheese are methyl ketones, instead of butyric acid esters. In a steam distillate from roquefort cheese, he obtained about 2 drops of material that had intensive odors of methyl-amyl and methyl-heptyl ketones. The mixed semi-carbazone had a crude melting point of 105° to 107° C. The amount recovered was too small to permit separation and identification of the components. It was saponified with sulfuric acid and the odors of methyl-amyl and methyl-heptyl ketones were noted.

Aeklin (1) studied the formation of methyl ketones by Penicillium glaucum from the ammonium salts of normal monocarboxylic fatty acids (from butyric acid up to and including myristic acid).

Although no ketone was formed from butyric or valeric acids, methyl ketone production from the remainder of the series was demonstrated. He concluded that the reaction occurs as a beta-oxidation, thus resulting in the formation of a keto acid, which is then further oxidized to the ketone and carbon dioxide, acetic acid and possibly some secondary alcohols. Acklin also investigated the formation of methyl-propyl ketone from caproic acid by Penicillium glaucum and found that the percentage yield was dependent upon the concentration of the acid in the substrate. Tricaproin produced greater yields of the ketone than the corresponding acid. Unbuffered solutions of this ester, with an initial pH of 7.6, gave 48 per cent of the ketone, whereas solutions buffered to pH 7.6 and 4.2 gave percentage yields of the ketone of 35 and 30 per cent, respectively.

Stokes (69) studied the formation of methyl ketones from fatty acids by the action of molds on coconut oil. Presumably the beta carbon atom is attacked, resulting in the formation of a keto acid. In a normal decomposition this is further broken down, resulting in the formation of a fatty acid containing two less carbon atoms and acetic acid. He believed that methyl ketone is formed because the adsorption of toxic fatty acid by the molds impedes normal respiration, resulting in a condition wherein the keto acid is decomposed to methyl ketone and carbon dioxide. Stokes also was of the opinion that only the acids up to lauric acid are absorbed and consequently ketones of higher molecular weight than methyl-nonyl ketone are not formed.

In an investigation of fat as a nutrient medium for Penicillium glaucum, Küssner (14) found that the decomposition resulted in

formation of fatty acids of lower molecular weight. The double bonds of the unsaturated fatty acids were believed to play an important part in the fat hydrolysis, since analysis of the decomposition products revealed a lowering in iodine value, which was accompanied by an increase in the acetyl value. The formation of hydroxyl groups at the double bonds was believed to be a step in the breakdown of the unsaturated fatty acids. Glycerol was quickly utilized by the molds.

Hammer and Bryant (31) studied the flavor constituents of blue cheese and concluded that methyl-n-amyl ketone is an important flavor contributant. They believed that this compound is formed from caprylic acid through the action of Penicillium roqueforti under unfavorable conditions of growth.

GENERAL METHODS

Sources of Cultures

The strains of penicillia studied were from various sources. Most of them were from culture collections, whereas others were isolated from various samples of blue veined cheeses. The sources and identifications of the organisms are presented in the following tabulation.

Culture no.	Source	Identification
Ia. St. Col. collections:		
1	Mold No. 1	<u>P. roqueforti</u>
3	Mold No. 3, isolation from Danish blue cheese	<u>P. oxalicum</u>
4	Mold No. 4	Unidentified <u>Penicillium</u>
5	Danish A mold	<u>P. roqueforti</u>
6	Danish B mold	<u>P. roqueforti</u>
7	Mold No. 834, J. M. Rosell	<u>P. roqueforti</u>
8	Mold No. 835, J. M. Rosell	<u>P. roqueforti</u>
9	Mold No. P6	<u>P. roqueforti</u>
10	Mold No. 33, N. S. Golding	<u>P. roqueforti</u>
11	Mold No. 16, N. S. Golding	<u>P. gorgonzola</u>
12	P. Simonart	<u>P. gorgonzola</u> , Biourge
13	P. Simonart	<u>P. gorgonzola</u> type
14	P. Simonart	<u>P. stilton</u> , Biourge
15	P. Simonart	<u>P. roqueforti</u> , Thom
16	C. B. Lane, isolation from Butterfly brand cheese	<u>P. gorgonzola</u>
17	C. B. Lane, isolation from Neptune brand cheese	Unidentified <u>Penicillium</u>
18	C. B. Lane, isolation from European brand cheese	<u>P. chrysogenum</u>
20	C. B. Lane	Unidentified <u>Penicillium</u>
21	C. B. Lane, isolation from Grove City cheese	<u>P. roqueforti</u>
22	C. B. Lane, obtained from C. Thom culture collection	<u>P. roqueforti</u>

23	C. B. Lane, isolation from Zenith brand cheese	Unidentified <u>Penicillium</u>
24	C. B. Lane, source not given	<u>P. roqueforti</u>
	Misc. collections:	
25	Isolation from air	<u>P. roqueforti</u>
26	Isolation from imported French Roquefort cheese	<u>P. roqueforti</u>
26b	Isolation from imported French Roquefort cheese	<u>P. roqueforti</u>
27	Isolation from imported French Roquefort cheese	<u>P. roqueforti</u>
41	Univ. of Minn. collection, H. C. Macy	<u>P. roqueforti</u>
42	Isolation from blue cheese	Unidentified <u>Penicillium</u>

Basic Medium

Czapek's solution agar, originally recommended by Czapek and later modified by Dox (19) and by Thom (73), was used as the basic substrate; it had the following composition.

Water	1000.0 ml.
Sodium nitrate	2.0 gm.
Mono potassium phosphate	1.0 gm.
Magnesium sulfate	0.5 gm.
Potassium chloride	0.5 gm.
Ferrous sulfate	0.01 gm.
Sucrose	150.0 gm.
Agar	15.0 to 20.0 gm.

Organic nutrients and inorganic constituents were added to the medium according to the special type of decomposition being studied.

Methods for Determination of Lipolytic Activities of *Penicillia*

Media for determination of lipolysis

For the lipolytic studies the media were prepared according to

methods suggested by Long and Hammer (50), with the exception that Czapek's solution agar was used as the basic substrate instead of beef infusion agar. The media employed were prepared as follows.

a. Natural fat technique: The medium consisted of Czapek's solution agar containing dispersed natural fat, either butterfat or cottonseed oil. Proper distribution of the fat was obtained by preparing an emulsion consisting of 3.0 ml. of fat, 0.5 gm. of agar and 100 ml. of water; the mixture was sterilized in a 4 ounce screw cap bottle and, after it had cooled, the fat was dispersed as minute globules by vigorous agitation. Approximately 0.5 ml. of an emulsion was generally used per plate and was added immediately before pouring with agar.

b. Nile blue sulfate technique: The medium consisted of Czapek's agar containing dispersed fat (butterfat or cottonseed oil) and Nile blue sulfate. The emulsion was prepared as outlined under (a). Approximately 0.5 ml. of an emulsion was added to each plate before pouring with agar. Fifteen ml. of the agar containing Nile blue sulfate, usually in the proportion of 1 part to 15,000 parts of agar although in some instances in the proportion of 1 part to 10,000 parts of agar, was used per plate.

c. Simple triglyceride technique: The medium consisted of Czapek's agar containing various dispersed simple triglycerides. Emulsions were prepared as outlined under (a) with the exception of the amounts of materials used in the mixtures. From 5 to 6 per cent of tripropionin, tributyrin or trivalerin, from 3 to 4.75 per cent of tricaproin, triheptylin, tricapyrin, tricaprillin or triolein and from 2 to

2.75 per cent trilaurin, trimyristin, tripalmitin or tristearin were used in the respective emulsions; all of the triglycerides were of the normal acids. The usual amount of an emulsion (0.5 ml.) was added to each plate before pouring. When simple triglycerides of relatively high melting points* were used, it was necessary to heat the mixtures to temperatures above their melting points and, after vigorous agitation, 0.5 ml. of an emulsion was quickly transferred to a plate.

When the Nile blue sulfate technique was employed with simple triglyceride studies, it was applied as outlined under (b).

Inoculation of plates for determination of lipolysis

A loop of spores was transferred from an 8 day Penicillium culture growing on an agar slant to a screw cap bottle containing 100 ml. of sterile water. After the bottle had been shaken vigorously, a loop of the suspension of spores was used to inoculate the center of each plate.

Incubation of plates for determination of lipolysis

The plates were usually incubated at 21° C. for periods ranging from 6 to 13 days, although in certain instances higher or lower temperatures were used. Ordinarily the plates were incubated in an unlimited supply of air, but in some trials a restricted air supply was used; details of the method are given later.

*Those triglycerides which are solid at ordinary room temperature, including tricaprin, trilaurin, trimyristin, tripalmitin and tristearin.

Examination of plates for determination of lipolysis

a. Natural fat technique: Hydrolysis of butterfat or cottonseed oil was detected by the appearance of the dispersed fat; unhydrolyzed globules were translucent and upon hydrolysis became opaque.

b. Nile blue sulfate technique: Lipolysis of butterfat and cottonseed oil was detected by the color of the dispersed fat; the unhydrolyzed globules were pink and upon hydrolysis became blue.

c. Simple triglyceride technique: Hydrolysis of tripropionin, tributyrin and trivalerin was evidenced by the formation of a clear zone in the area immediately surrounding the colony, whereas with tricaproin, triheptylin, tricapyrylin, tricaprin and trilaurin hydrolysis was detected by the appearance of the dispersed fat; unhydrolyzed fat was pink and hydrolyzed globules blue.

d. Modified Nile blue sulfate technique: This method was applied only to butterfat and cottonseed oil and lipolysis was observed by the change of color of the dispersed fat; the unhydrolyzed fat was pink and the hydrolyzed product was blue.

A wide field binocular with 7, 20 and 40 magnifications was used in the examination of the plates for lipolysis. Usually a source of substage light was employed in the examination of fat dispersed in the medium underneath the conidial growth. Occasionally a light source above the stage was used to advantage in the inspection of fat globules at the surface of the medium. For detailed observation of individual globules a microscope was helpful.

Evaluation of lipolytic activities of various penicillia

The lipolytic activity of a mold culture was evaluated by measuring and recording the diameter (in centimeters) of the growth and of the corresponding hydrolyzed area. When most of the fat globules in the measured area were hydrolyzed lipolysis was regarded as uniform, when only a portion of the globules were hydrolyzed it was considered nonuniform and when no lipolysis was noted it was considered negative.

Methods for Determination of Proteolytic Activities of Penicillia

Media for determination of proteolysis

The medium employed most commonly in studies on proteolysis consisted of Czapek's solution agar containing sterilized skim milk in the proportion of 1 part of milk to 10 parts of agar; 15 ml. of the mixture was used per plate.

In some of the trials a modified milk agar method, suggested by Thom (71), was used. The medium was prepared by mixing together hot agar and hot acidified milk in equal portions; a flocculent precipitate of curd formed in the agar.

Inoculation of plates for determination of proteolysis

The plates were inoculated as described under the method for inoculation of the plates for determination of lipolysis.

Incubation of plates for determination of proteolysis

The plates were usually incubated at 21° C. for periods ranging from 6 to 14 days; however, variations in the time and temperature were used occasionally. The method most often used consisted of holding the plates for 4 days in air and thereafter in an atmosphere of carbon dioxide gas. The carbon dioxide atmosphere was obtained by placing the plates inside of glass bell jars, properly sealed so as to prevent the escape of gas. Carbon dioxide gas was introduced slowly into the bottom of a jar, while air was permitted to escape through a vent at the top. After the air had been replaced by carbon dioxide, the vent was closed. No attempt was made to maintain the carbon dioxide at a constant level, since the only purpose in using the gas was to retard growth of the colonies and permit diffusion of protease to become more conspicuous.

Examination of plates for determination of proteolysis

a. Acidified milk agar technique: Proteolysis was detected by the appearance of a clear zone underneath and in the area surrounding the colony.

b. Carbon dioxide technique: Proteolysis was also determined by the appearance of a clear zone in the agar around the colony.

Evaluation of proteolytic activities of various penicillia

The proteolytic activity of a mold culture was evaluated by measuring and recording the diameter (in centimeters) of the growth

and of the corresponding hydrolyzed area.

Manufacture of Cheese

Milk used in making the cheese came from the regular milk supply of Iowa State College market milk laboratory. The cheese was made according to the method suggested by Lane and Hammer (47) which is as follows.

Fresh, whole milk, testing 3.4 to 3.7 per cent butterfat, was heated to 32.2° C. and homogenized at 2500 + 500 pounds pressure. After the milk was cooled to 31.7° C. it was placed in a cheese vat, and 2 per cent of lactic acid culture was added. When the acidity reached 0.19 to 0.20 per cent, calculated as lactic acid, the milk was set at 31.1° C. with commercial rennet at the rate of 3 ounces per 1000 pounds of milk. After a holding period of 60 to 80 minutes, the curd was cut with 0.5 inch curd knives. Immediately after cutting, the whey acidity was 0.13 to 0.14 per cent. The curd was stirred occasionally during the following 90 to 120 minutes, or until the whey had attained an acidity of 0.17 to 0.19 per cent. At the end of this period the curd was ordinarily ready for dipping; in rare instances, when it was unusually soft, a slight toughening was produced by removal of a small portion of whey, heating it with steam and then returning it to the cheese vat. At no time was the temperature of the curd permitted to exceed 33.3° C.

The curd was dipped by first draining a portion of the whey from the vat and then transferring the curd with a scoop to an adjacent draining vat covered with a large single layer of cloth of the type

commonly employed by swiss cheese manufacturers. Drainage of the curd was facilitated by raising and lowering the corners of the cloth at frequent intervals. Sufficient curd was dipped on the draining cloth each time to make two cheese. One or 2 minutes after draining was begun, the mold was mixed with the curd, and then after 2 or 3 minutes the curd was quickly placed in the hoops.

When a draining cloth was used for more than one dipping, it was thoroughly washed and scalded so as to prevent contamination of subsequent lots of curd. In a number of trials an individual sterilized cloth was used for each lot of curd dipped.

Since the curd obtained from homogenized milk was comparatively soft, the hoops were turned frequently after filling to prevent the cheese from sticking to the hoops and to insure a closed surface. Ordinarily the hoops were turned every 15 minutes for the first 2 hours and then each hour for 6 to 8 hours.

The dry salting method was used exclusively with the cheese. Usually salt equal to 5.5 per cent of the total weight of the cheese was incorporated into a lot of cheese during three successive saltings over an 8 to 10 day period.

After the salting was finished the cheese were rinsed and skewered; ordinarily 50 holes were punched in each of the flat sides of a cheese with a needle about 0.12 inch in diameter. The cheese were placed on edge on special racks in a curing room in which a temperature of about 8.3° C. and a humidity of 90 per cent or above were maintained. Two times during the curing period the cheese were temporarily removed from

the curing room, soaked in cold water for several minutes and brushed with a stiff bristled brush to remove surface slime. After about 3 months in the curing room the cheese were wrapped in tinfoil and placed in a cold room having a temperature of 1.7° to 4.4° C.

Preparation of Mold Powder

Mold powder was prepared according to the method suggested by Hussong and Hammer (39). Whole wheat bread, cut into small cubes and sterilized in a bottle, was inoculated with a mold culture. After about 10 days incubation at 21.1° C., the spore covered bread was dried, pulverized and stored in sterile glass containers.

Inoculation of Curd

The curd usually was inoculated with mold by dusting about 2 gm. of the prepared powder on 10 pounds of curd; this was enough curd for 2 cheese. In some of the early trials the curd was inoculated with aqueous spore suspensions obtained by flooding 10 to 12 day old Czapek's agar slopes of penicillia with sterile water and scraping the spores from the surface of the growth with an inoculating needle. In some of the later trials a similar method of inoculation was employed with the exception that the cultures were grown on Czapek's agar plates. Both methods were unsatisfactory because the number of spores introduced into the curd from a culture was usually insufficient to establish it in the cheese.

Examination and Scoring of Cheese

Each lot of cheese was examined twice for flavor and mold growth. In both examinations a small wedge shaped portion was removed from each cheese in order to compare the mold strains present with those used in inoculating the curd. After the first examination the exposed surface of the cheese was sealed with paraffin and placed in the curing room and ripening continued. In the second examination each cheese was cut into halves, so that the entire cross section of a cheese was exposed. Small portions of cheese were removed from the surface of the freshly cut area and scored for flavor, while mold development was observed by noting the character and extent of growth in the exposed area.

EXPERIMENTAL

Comparison of Methods Employed in Determination of Lipolytic
and Proteolytic Activities of Various Penicillia

In order to evaluate certain cultural methods for determining the lipolytic and proteolytic activities of various penicillia, comparisons were made of different procedures.

Review of literature

Sommaruga (66) studied the lipolytic activities of microorganisms by growing them on solid media in which was dispersed 2.0 per cent olive oil or other fat. Disappearance of fat globules from the zone surrounding bacterial colonies was evidence of hydrolysis. Eijkman (20) poured melted inoculated agar over a thin layer of fat in a petri dish, and lipolysis was indicated by clear areas forming beneath the colonies of bacteria.

In a study of the action of Nile blue sulfate as a fat stain, Thorpe (77) reported that the dye is a sulfate of diethylaminophenanthoxazine. The red stain, which is not present in the original dye but is formed in the aqueous solution, is the corresponding oxazone. The amount of red stain could be increased by heating Nile blue sulfate with dilute sulfuric acid. Smith (65) showed that fat globules in tissues could be stained by Nile blue sulfate and that the dye colored olein and other

neutral fats red, whereas oleic acid was colored blue, this color resulting from the formation of a blue soap between the oxazine base and the fatty acid. The ability of Nile blue sulfate to color various triglycerides and fatty acids was investigated by Boeminghaus (6). He concluded that the dye is a specific for olein and its fatty acid, since these were colored much more intensely than were palmitin and stearin and their corresponding acids. Kaufman and Lehman (40) found that the unsaturated triglycerides and their fatty acids were well stained with Nile blue sulfate. However, certain other saturated compounds also absorbed the stain. Results obtained by Rettie (62) supported the use of Nile blue sulfate as a fat stain. He showed that the oxazone of the dye is pink and, since it is soluble in fat and fat solvents, it is responsible for the color in the stained fat; this constituent Rettie called Nile pink.

Turner (78) used Nile blue sulfate in the detection of lipolytic bacteria. The dye was added to the inoculated medium, in which was dispersed 10 per cent of a sterile fat emulsion, and a study was made of the changes occurring in the fat globules during hydrolysis by the bacteria. He concluded that unsaturated fatty acids are deeply stained with Nile blue sulfate. Later, Turner (79) substantiated the use of Nile blue sulfate in detecting bacterial lipolysis. However, the dye was inhibitory for some organisms. Hussong (38) modified Turner's technique in a study of bacterial rancidity in butter. Nile blue sulfate was used in the proportion of 1:10,000 and fat in the ratio of 1:2000. The fat was emulsified in 0.5 per cent agar, and the emulsion was sterilized and then added to beef infusion agar at the time of pouring the plates.

Streak cultures were made over the agar surface; lipolysis was indicated by a change in the color of the dispersed fat and by deep blue areas around the growth. Collins and Hammer (12) and Hammer and Collins (34) determined lipolysis of simple triglycerides and natural fats by a method similar to that employed by Hussong. These investigators reported that tributyrin, tricaproin, tricaprylin and triolein were colored bright red by the dye; tricaprin, trilaurin, trimyristin, tripalmitin and tristearin were colored red to a degree which decreased rapidly with the increased melting points of the triglycerides. The natural fats studied also were colored with different intensities of red. Hydrolysis of tricaproin and tricaprylin was more easily identified by a disappearance of the globules than by color changes with Nile blue sulfate; however, with tricaprin the reverse was true. The manner of dispersing fats and triglycerides seemed to have no effect upon hydrolysis. These investigators also found a variation in the colors of fatty acids. Caproic, caprylic and oleic acids were intensely blue; capric and lauric acids varied in their intensities of blue, whereas only slight amounts of the blue dye were absorbed by myristic, palmitic and stearic acids. In a study of various methods of detecting lipolysis by microorganisms, Long and Hammer (50) concluded that the Nile blue sulfate technique gave clear cut results but, because of the toxicity of the dye, was limited in its usefulness. The natural fat technique, in which no dye was used, gave good results, but it was difficult to detect weakly lipolytic organisms by this method. In a recent investigation of the action of microorganisms on fats, Castell and Bryant (10) pointed out certain errors in previous interpretations of

color changes occurring in fat globules stained with Nile blue sulfate. They concluded that the change in color could not be used as a specific indication of hydrolysis of fat, since besides extraction of the blue oxazine form of the dye from the aqueous solution by fatty acids or other substances within the globules, similar color changes resulted by the reduction of oxazone (from pink to blue).

Ayres (3) employed casein agar for identification of proteolytic bacteria by noting the clear areas formed around the colonies. Later, Ayres and Mudge (4) suggested the use of various dry milk agar media for detection of acid forming and proteolyzing bacteria by observing the precipitation and digestion of casein in areas surrounding the colonies.

Comparison of methods for determining lipolysis

Comparison of natural fat technique with Nile blue sulfate technique. Comparison of the natural fat technique with the Nile blue sulfate technique for determination of the lipolytic activities of certain penicillia was made with six mold strains on butterfat and on cottonseed oil; the cultures were incubated 8 days at 21° C. The Nile blue sulfate medium contained 1 part of the dye in 15,000 parts of agar. The results are presented in table I.

a. Results with butterfat: The natural fat technique resulted in greater growth of the colonies, when compared with the Nile blue sulfate method, in all six comparisons. The maximum diameter of the colonies with the natural fat technique was 9.0 cm., the minimum was

TABLE I

COMPARISON OF NATURAL FAT TECHNIQUE WITH NILE BLUE SULFATE TECHNIQUE
FOR DETERMINATION OF LIPOLYTIC ACTIVITIES
OF CERTAIN PENICILLIA

Incubation 8 days at 21° C.

a. Lipolysis of butterfat				
Culture no.	Technique	Diameter of		Lipolysis*
		colony cm.	lipolysis cm.	
5	Natural fat	7.5	7.7	Slight
	Nile blue sulfate	4.2	5.2	Good
7	Natural fat	6.3	0.0	Imperceptible
	Nile blue sulfate	6.0	5.6	Slight
8	Natural fat	8.5	0.0	Imperceptible
	Nile blue sulfate	5.6	5.5	Moderate
9	Natural fat	9.0	9.0	Good
	Nile blue sulfate	5.9	5.9	Very good
15	Natural fat	6.8	6.7	Good
	Nile blue sulfate	4.0	3.5	Good
25	Natural fat	7.7	7.5	Good
	Nile blue sulfate	4.0	3.5	Good

b. Lipolysis of cottonseed oil				
Culture no.	Technique	Diameter of		Lipolysis*
		colony cm.	lipolysis cm.	
5	Natural fat	8.9	8.9	Slight
	Nile blue sulfate	4.0	3.8	Good
7	Natural fat	8.0	0.0	Imperceptible
	Nile blue sulfate	4.3	4.3	Moderate
8	Natural fat	8.4	0.0	Imperceptible
	Nile blue sulfate	4.3	4.3	Moderate
9	Natural fat	9.0	9.0	Good
	Nile blue sulfate	4.0	4.0	Very good
15	Natural fat	7.3	7.5	Slight
	Nile blue sulfate	5.3	5.3	Good
25	Natural fat	8.0	6.7	Slight
	Nile blue sulfate	4.0	4.0	Good

*Lipolysis refers to the perceptibility of fat hydrolysis.

6.3 cm. and the average was 7.6 cm.; corresponding diameters with the Nile blue sulfate method were 6.0, 4.0 and 5.0 cm.

In the six comparisons the natural fat technique showed the more extensive lipolysis in four instances, while the Nile blue sulfate exhibited the more in two comparisons. The maximum, minimum and average diameters of lipolysis with the natural fat technique were 9.0, 0.0 and 5.2 cm., respectively, as compared with corresponding values of 5.9, 3.8 and 4.3 cm. with Nile blue sulfate.

Lipolysis was more readily detected with the Nile blue sulfate technique than with the natural fat technique. With the natural fat technique lipolysis was good in three comparisons, slight in one and imperceptible in two; with the Nile blue sulfate method it was very good in one, good in three, moderate in one and slight in one.

b. Results with cottonseed oil: In general, the results with cottonseed oil were practically the same as those with butterfat. Greater mold development occurred on the natural fat medium than on Nile blue sulfate agar. The maximum diameter of the colonies on the natural fat medium was 9.0 cm., the minimum was 7.3 cm. and the average was 8.3 cm., as compared with corresponding diameters on the Nile blue sulfate medium of 5.3, 4.0 and 4.3 cm.

In six comparisons the natural fat technique showed the more extensive lipolysis in four instances, while Nile blue sulfate exhibited the more in two comparisons. The maximum, minimum and average diameters of lipolysis with the natural fat technique were 9.0, 0.0 and 5.3 cm., respectively, as compared with corresponding values of 5.3, 3.8 and

4.3 cm. with Nile blue sulfate.

Lipolysis was more readily detected with the Nile blue sulfate than with the natural fat technique. Lipolysis with the natural fat technique was good in one instance, slight in three and imperceptible in two, whereas with the Nile blue sulfate technique it was very good in one, good in three and moderate in two.

Eight additional trials were made with butterfat; the cultures were incubated 8 days at 21° C. The Nile blue sulfate medium contained 1 part of the dye to 10,000 parts of agar. The growth and lipolysis curves of the cultures were plotted from values obtained by calculating the square of the radius of each colony and of the hydrolyzed area; the radius squared (r^2) was regarded as a function of the area. The results are given in figure 1.

In all cases the inhibitive action of Nile blue sulfate with respect to mold growth and lipase development was evident from the relative slopes of the growth and lipolysis curves with the natural fat, as compared with the Nile blue sulfate technique. While colony growth progressed more rapidly with the natural fat technique, lipolysis was generally more perceptible with the Nile blue sulfate technique. Cultures 1 and 7 showed relatively rapid rates of growth on the natural fat medium when compared with the Nile blue sulfate medium; however, no lipolysis was evident with the natural fat technique while it was noticeable with the Nile blue sulfate technique. Culture 14 showed relatively slow growth and no evident lipolysis with either technique, but colony growth proceeded at a greater rate with the natural fat technique than with the Nile

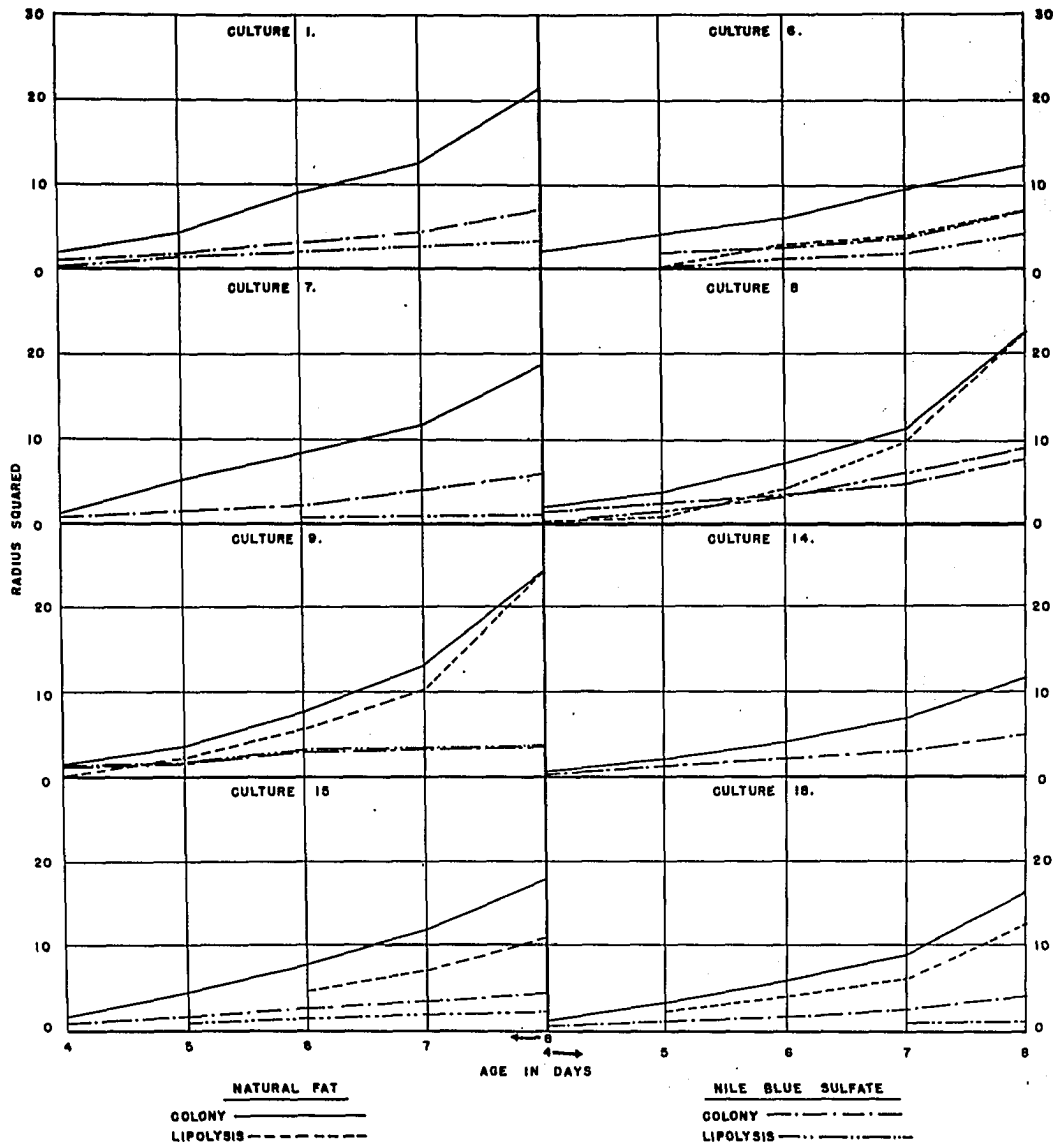


FIGURE 1. COMPARISON OF NATURAL FAT TECHNIQUE WITH NILE BLUE SULFATE TECHNIQUE FOR DETERMINATION OF LIPOLYTIC ACTIVITIES OF CERTAIN PENICILLIA ON BUTTERFAT

blue sulfate technique. With cultures 6, 8, 9, 15 and 18 growth and lipolysis progressed more rapidly with the natural fat than with the Nile blue sulfate technique. However, lipolysis was in all cases more evident with the Nile blue sulfate technique.

Some of the cultures (7, 8, 9 and 15) included in figure 1 were also considered in table I. With a few exceptions there was a general agreement in the results obtained by any one of the cultures.

Eight additional trials were made with cottonseed oil; the cultures were incubated 8 days at 21° C. The Nile blue sulfate medium contained 1 part of the dye to 10,000 parts of agar. Figure 2 presents the results.

The results were generally similar to those obtained with butterfat (figure 1). Cultures 1, 6, 7, 8, 10 and 15 showed more growth and lipolysis with the natural fat than with the Nile blue sulfate technique; however, lipolysis was more evident with the Nile blue sulfate technique. An exception was noted with culture 13; it showed fairly rapid growth and lipolysis with the natural fat technique, while growth was somewhat retarded and no lipolysis was noted with the Nile blue sulfate technique. Culture 14 showed slow growth and no evidence of lipolysis with both techniques, although the growth was somewhat more rapid on the natural fat than on the Nile blue sulfate medium.

A number of the cultures (7, 8 and 15) included in figure 2 were also considered in table I. With a few exceptions the results obtained with any one of the cultures were in general agreement.

From the data presented in table I and figures 1 and 2, it is

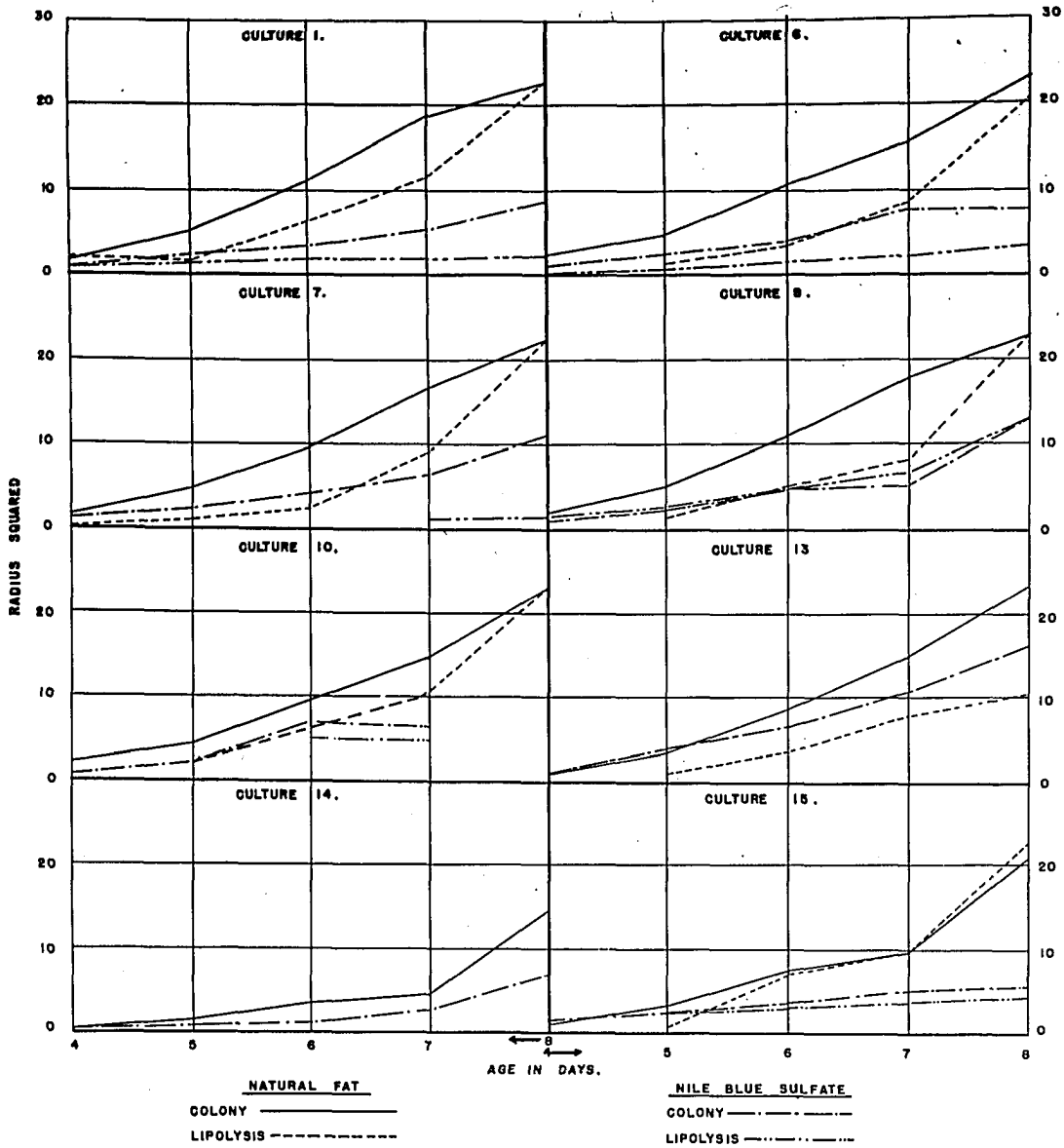


FIGURE 2. COMPARISON OF NATURAL FAT TECHNIQUE WITH NILE BLUE SULFATE TECHNIQUE FOR DETERMINATION OF LIPOLYTIC ACTIVITIES OF CERTAIN PENICILLIA ON COTTONSEED OIL

evident that when the natural fat technique and the Nile blue sulfate technique were compared for the determination of lipolytic activities of certain penicillia on butterfat and cottonseed oil, the greatest growth occurred with the natural fat technique. However, lipolysis of the fats was more readily detected with the Nile blue sulfate than with the natural fat technique, as evidenced by the perceptibility of lipolysis. In most instances the Nile blue sulfate technique gave the more distinct differentiation between the unhydrolyzed and the hydrolyzed fat.

Comparison of natural fat technique with modified Nile blue sulfate technique. In order to compare the natural fat with the modified Nile blue sulfate technique for determining the lipolytic activities of certain penicillia on butterfat and cottonseed oil, the natural fat plates referred to in table I were flooded after incubation with a solution of Nile blue sulfate consisting of 1 part of the dye to 1000 parts of 50 per cent alcohol. After the plates had been exposed to the Nile blue sulfate about 10 minutes, they were rinsed free of the dye with water. The alcoholic solution caused a collapse of the conidial growth so that it flattened against the surface of the agar exposing underlying dispersed fat globules for examination. The results with six cultures are presented in table II.

a. Results with butterfat: The modified Nile blue sulfate technique gave a much more clear cut differentiation of the penicillia cultures than the natural fat procedure; it was especially useful in distinguishing differences between slightly lipolytic cultures. The maximum, minimum and average diameters of lipolysis with the natural

TABLE II

COMPARISON OF NATURAL FAT TECHNIQUE WITH MODIFIED NILE BLUE SULFATE
TECHNIQUE FOR DETERMINATION OF LIPOLYTIC ACTIVITIES
OF CERTAIN PENICILLIA

Incubation 8 days at 21° C.

a. Lipolysis of butterfat				
Culture no.	Technique	Diameter of		Lipolysis
		colony cm.	lipolysis cm.	
5	Natural fat		7.7	Slight
	Modified n. b. s.*	7.5	7.7	Very good
7	Natural fat	6.3	0.0	Imperceptible
	Modified n. b. s.		**	Good
8	Natural fat		0.0	Imperceptible
	Modified n. b. s.	8.5	6.4	Good
9	Natural fat		9.0	Good
	Modified n. b. s.	9.0	9.0	Very good
15	Natural fat		6.7	Good
	Modified n. b. s.	6.8	6.7	Good
25	Natural fat		7.5	Good
	Modified n. b. s.	7.7	7.5	Good

b. Lipolysis of cottonseed oil				
5	Natural fat		9.0	Slight
	Modified n. b. s.	8.9	9.0	Very good
7	Natural fat		0.0	Imperceptible
	Modified n. b. s.	8.0	8.0	Good
8	Natural fat		7.3	Slight
	Modified n. b. s.	8.4	8.4	Very good
9	Natural fat		9.0	Good
	Modified n. b. s.	9.0	9.0	Very good
15	Natural fat		7.5	Slight
	Modified n. b. s.	7.3	7.5	Good
25	Natural fat		6.7	Slight
	Modified n. b. s.	8.0	8.0	Good

*n. b. s. refers to Nile blue sulfate.

**Although the fat globules had not become definitely blue most of them were colored peach red and each was surrounded by a tenacious pellicle, on puncturing an oily fluid escaped.

fat technique were 9.0, 0.0 and 5.3 cm., respectively, as compared with corresponding values of 9.0, ** and 6.2 cm. with the modified procedure.

Lipolysis was much more readily detected with the modified Nile blue sulfate technique than with the natural fat procedure. With the natural fat technique lipolysis was good in three comparisons, slight in one and imperceptible in two; with the modified Nile blue sulfate procedure it was very good in two and good in four.

An additional advantage of the modified Nile blue sulfate procedure, as compared with the natural fat technique, was its adaptability to the microscopic examination of fat globules within the heavy sporulating areas of the cultures. The disadvantage with the modified technique lay in the impossibility of studying the rates of growth and of lipolysis, since the flooding procedure prevented obtaining more than one reading from a set of plates.

b. Results with cottonseed oil: The results with cottonseed oil were similar to those obtained with butterfat. The maximum, minimum and average diameters of lipolysis with the natural fat technique were 9.0, 0.0 and 6.6 cm., respectively, as compared with corresponding values of 9.0, 7.5 and 8.3 cm. with the modified procedure.

Lipolysis was much more readily detected with the modified Nile blue sulfate technique than with the natural fat procedure. With the latter technique lipolysis was good in one comparison, slight in four

**Although the fat globules had not become definitely blue most of them were colored peach red and each was surrounded by a tenacious pellicle, on puncturing an oily fluid escaped.

and imperceptible in one, whereas with the former procedure it was very good in three and good in three.

The data presented in table II show that for the determination of the lipolytic activities of certain penicillia on butterfat and cottonseed oil the modified Nile blue sulfate technique regularly gave better results than the natural fat technique. The superiority of the modified technique was most noticeable in the perceptibility of fat hydrolysis, the lipolysis rating from good to very good in all instances, whereas it rated from imperceptible to good with the natural fat technique.

Effect of certain factors on detection of lipolysis

Concentration of fat in medium. The effect of the concentration of fat in the medium on the detection of lipolysis, as determined by Nile blue sulfate, was studied with two penicillia cultures on butterfat and on cottonseed oil. The fat emulsions consisted of 97 ml. of 0.5 per cent agar solution plus 3 ml. melted butterfat or cottonseed oil. The cultures were incubated 6 days at 21° C. and the results are presented in table III.

a. Results with butterfat: The concentration of butterfat emulsion in the medium had no appreciable effect on mold growth or on lipolysis, but with increased concentrations of fat there were more globules within a given area and lipolysis seemed more noticeable than with lower concentrations. This advantage was offset by nonuniform lipolysis when 1.5 ml. of butterfat emulsion was employed.

TABLE III
EFFECT OF CONCENTRATION OF FAT IN MEDIUM ON
DETECTION OF LIPOLYSIS

(Nile blue sulfate technique)

Incubation 6 days at 21° C.

a. Lipolysis of butterfat				
Culture no.	Medium	Diameter of		Lipolysis
		colony cm.	lipolysis cm.	
	15 ml. Czapek's agar + butterfat emulsion:			
5	0.5 ml.	4.7	3.8	Good*
	1.0 ml.	4.8	3.7	Good*
	1.5 ml.	5.0	3.9	Good**
9	0.5 ml.	4.8	4.6	Good*
	1.0 ml.	4.8	4.6	Very good*
	1.5 ml.	4.8	4.6	Very good**
b. Lipolysis of cottonseed oil				
	15 ml. Czapek's agar + cottonseed oil emulsion:			
5	0.5 ml.	5.1	5.0	Good*
	1.0 ml.	4.8	4.6	Good*
	1.5 ml.	4.8	4.6	Very good**
9	0.5 ml.	4.9	4.9	Good*
	1.0 ml.	5.0	5.0	Very good*
	1.5 ml.	5.0	5.0	Very good**

*Uniform lipolysis

**Nonuniform lipolysis

b. Results with cottonseed oil: The data agree with the results on butterfat. The concentration of cottonseed oil in the medium had no material effect on the growth of the molds or on lipolysis. Increased fat concentration in the medium made lipolysis seem more noticeable than with lower concentrations but this advantage was offset by non-uniform lipolysis when 1.5 ml. of cottonseed oil emulsion was employed.

From the data presented in table III it is evident that the concentration of butterfat or cottonseed oil in the medium had no appreciable effect on the detection of lipolysis when the amount of fat emulsion varied from 0.5 ml. to 1.0 ml. per plate (15 ml. agar). However, when 1.5 ml. fat emulsion was added, the uniformity of lipolysis was appreciably affected; lipolysis appeared to be more noticeable, but it was non-uniform.

Concentration of Nile blue sulfate in medium. In studying the effect of the concentration of Nile blue sulfate in the medium on the detection of lipolysis of butterfat, two strains of penicillia were used; they were selected because of their known behavior toward the concentrations of dye ordinarily employed. Culture 7 represented about the average of the strains of penicillia with respect to its reaction toward Nile blue sulfate, whereas culture 9 was regularly inhibited by the dye. The results are presented in table IV.

One part of Nile blue sulfate to 10,000 parts of agar showed the most pronounced inhibitive action toward the mold growth. After 12 days the colonies formed under-developed, irregular growths, indicating marked inhibition. The intense pink color of the fat globules made the

TABLE IV

EFFECT OF CONCENTRATION OF NILE BLUE SULFATE IN MEDIUM
ON DETECTION OF LIPOLYSIS OF BUTTERFAT

Incubation 12 days at 21° C.

Culture no.	Concentration of nile blue sulfate in medium	Diameter of colony cm.	Lipolysis
	1 part of nile blue sulfate in:		
7	10,000 parts of agar	4.6	Moderate
	15,000 parts of agar	6.7	Moderate
	20,000 parts of agar	6.7	Moderate
	30,000 parts of agar	7.5	Slight*
9	10,000 parts of agar	6.9	Good
	15,000 parts of agar	8.6	Good
	20,000 parts of agar	8.8	Good
	30,000 parts of agar	8.9	Slight*

*Fat globules pale in color

change from red to blue upon hydrolysis of the fat readily noticeable. The 1 to 30,000 dye-agar ratio showed the least toxicity of the concentrations studied; however, the fat globules were pale in color, due to the low concentration of nile blue sulfate, and as a consequence gave poor differentiation between the neutral and the hydrolyzed fat globules. When the medium contained 1 part of nile blue sulfate to 15,000 or 20,000 parts of agar, the most satisfactory results were obtained, since the colony growth appeared quite normal and the fat was colored sufficiently to give clear differentiation between the unhydrolyzed and the hydrolyzed globules.

The data presented in table IV show that with the nile blue sulfate technique for determining the lipolytic activities of certain penicillia on butterfat, the best results were obtained when the medium contained 1 part of the dye to 15,000 and 20,000 parts of agar. The fat was colored sufficiently to give a clear differentiation between the unhydrolyzed and hydrolyzed fat and the colony growth was normal. With higher concentrations of nile blue sulfate in the medium, mold growth was considerably inhibited, whereas, with lower concentrations, the fat was inadequately colored to give clear cut results.

Effect of certain factors on detection of proteolysis

In the preliminary trials dealing with the proteolytic action of various penicillia on Czapek's solution skim milk agar, inconsistent results were obtained. At times certain molds produced conspicuous proteolysis, whereas in other trials the proteolytic action of the

cultures was not evident. This suggested the existence in the technique of significant factors which hitherto had been considered unimportant and, accordingly, various factors affecting detection of proteolysis by various penicillia were investigated.

Temperature of incubation. The effect of temperature of incubation on the detection of proteolysis was studied with six strains of penicillia on Czapek's solution skim milk agar. Three sets of plates were inoculated; one was incubated at 12° C., one at 20° C. and one at 28° C. The results after 9 days incubation are presented in table V.

The penicillia grew more rapidly at 20° C. than at 28° or 12° C., as indicated by the sizes of the colonies. The maximum, minimum and average diameters of the cultures incubated at 12° C. were 5.0, 3.6 and 4.3 cm., respectively, as compared with corresponding values at 20° C. of 8.4, 5.9 and 7.1 cm. and with similar values at 28° C. of 5.1, 3.1 and 4.4 cm.

Proteolysis was more readily detected with the cultures incubated at 28° C. than with those incubated at 20° or 12° C. With the exception of culture 18 proteolysis with the cultures incubated at 20° or 12° C. was imperceptible in all instances, whereas at 28° C. it was very good in three comparisons, good in one and imperceptible in two.

From the data given in table V it is apparent that proteolysis by certain penicillia on Czapek's solution skim milk agar was more readily detected when the cultures were incubated at 28° C. than at 20° or 12° C. When the cultures were incubated at 12° or 20° C., proteolysis was perceptible in one instance, while at 28° C. it was perceptible in four out

TABLE V

EFFECT OF TEMPERATURE OF INCUBATION ON DETECTION OF PROTEOLYSIS

Cul- ture no.	Proteolysis after 9 days on Czapek's solution skim milk agar at								
	12° C.			20° C.			28° C.		
	Diameter of colony		Prote- olysis	Diameter of colony		Prote- olysis	Diameter of colony		Prote- olysis
cm.	cm.	cm.		cm.	cm.		cm.		
14	3.6	0.0	Imper- ceptible	5.9	0.0	Imper- ceptible	3.1	0.0	Imper- ceptible
15	4.6	0.0	Imper- ceptible	8.3	0.0	Imper- ceptible	4.3	4.8	Very good
16	4.7	0.0	Imper- ceptible	6.1	0.0	Imper- ceptible	4.9	4.9	Good
18	4.7	4.7	Good	7.6	7.6	Good	4.5	4.8	Very good
20	5.0	0.0	Imper- ceptible	8.4	0.0	Imper- ceptible	5.1	0.0	Imper- ceptible
25	4.0	0.0	Imper- ceptible	6.3	0.0	Imper- ceptible	4.6	5.1	Very good

of six cases.

Carbon dioxide. The effect of carbon dioxide on the detection of proteolysis was studied with three cultures of penicillia on Czapek's solution skim milk agar and on beef infusion skim milk agar. Three sets of conditions were compared; (a) incubation of the cultures 8 days in the air, (b) incubation of the cultures 3 days in the air followed by 5 days in an atmosphere where most of the air had been replaced by carbon dioxide and (c) incubation of the cultures in an atmosphere where the air had been largely replaced by carbon dioxide. No attempt was made to control the exact amount of carbon dioxide, since its only purpose was to retard mold growth. The cultures were all incubated at 21° C. The plates were examined for evidence of proteolysis after 4, 6 and 8 days. Table VI gives the results.

a. Results with Czapek's solution skim milk agar: Proteolysis was less conspicuous when the cultures were incubated in air than in an atmosphere composed mainly of carbon dioxide. Proteolysis was most conspicuous when the cultures were incubated in air 3 days followed by 5 days in carbon dioxide, whereas it was practically imperceptible with cultures incubated in the air.

The inhibitive effect of carbon dioxide on the growth of the penicillia was apparent from the comparative sizes of the colonies incubated under the different conditions. The cultures grown in air developed normally, those incubated 3 days in air followed by 5 days in carbon dioxide were definitely retarded in growth, while those incubated 8 days in carbon dioxide showed only a small amount of development.

TABLE VI
EFFECT OF CARBON DIOXIDE ON DETECTION OF PROTEOLYSIS

Incubation at 21° C.

Proteolysis on Czapek's solution skim milk agar								
Cul- ture in no. days	Age in days	In air 8 days		In air 3 days followed by 5 days in carbon dioxide		In carbon dioxide 8 days		
		colony cm.	proteolysis cm.	colony cm.	proteolysis cm.	colony cm.	proteolysis cm.	
		Diameter of						
		colony	proteolysis	colony	proteolysis	colony	proteolysis	
		cm.	cm.	cm.	cm.	cm.	cm.	
	4	4.4	0.0	2.9	0.0	1.6	1.1	
9	6	7.4	0.0*	5.0	5.1	3.4	5.4	
	8	8.7	0.0	6.4	6.5	5.3	5.4	
	4	3.4	0.0	4.4	0.0	2.9	2.4	
25	6	6.0	0.0*	7.7	6.8	5.3	5.2	
	8	8.0	0.0	8.6	8.5	7.8	7.4	
	4	4.5	0.0	4.1	0.0	3.1	1.7	
41	6	7.4	0.0	7.3	6.0	5.7	5.2	
	8	8.6	0.0	8.5	8.3	8.1	8.7	
Proteolysis on beef infusion skim milk agar								
	4	2.8	0.0	2.9	0.0	3.3	0.0	
9	6	4.0	0.0	5.1	0.0	5.4	3.8**	
	8	4.7	0.0	6.2	5.2**	7.4	0.0	
	4	2.0	0.0	2.2	0.0	2.6	0.0	
25	6	3.0	0.0	4.2	0.0	4.9	3.5**	
	8	3.8	0.0	5.7	4.6**	0.0	0.0	
	4	2.3	0.0	2.8	0.0	3.0	0.0	
41	6	3.2	0.0	5.2	0.0	4.4	0.0	
	8	3.7	0.0	6.5	0.0	6.3	0.0	

*Suggestion of proteolysis, not positive

**Faintly proteolytic

b. Results with beef infusion skim milk agar: Proteolysis was not evident when the cultures were grown in air on beef infusion milk agar. Slight proteolysis was noted with cultures 9 and 25 after 3 days in air and 5 days in carbon dioxide, while culture 41 showed no evidence of proteolysis. After 6 days in carbon dioxide, proteolysis was slight with cultures 9 and 25, while it was absent with culture 41. After 8 days proteolysis was absent with all of the cultures incubated under carbon dioxide.

From the data presented in table VI it is evident that proteolysis was less conspicuous when the cultures were incubated in air than when they were incubated in an atmosphere composed mainly of carbon dioxide. The data also show that beef infusion skim milk agar was unsatisfactory for the detection of proteolysis by the penicillia, whether the cultures were incubated in air or in an atmosphere composed mainly of carbon dioxide.

Comparison of acidified milk agar and carbon dioxide techniques for determination of proteolytic activities of certain penicillia

The acidified milk agar technique, suggested by Thom (71), consisted of growing the penicillia on a medium prepared by pouring together hot agar and hot acidified skim milk in equal proportions. The milk was acidified by the addition of 2.0 ml. of a 5 per cent acetic acid solution to 98 ml. skim milk. A flocculent precipitate of curd formed in the agar. The plates were incubated in air at 21° C. and examined after 4, 6 and 10 days; proteolysis was evidenced by the formation of a

clear zone in the medium under and around the colony. With the carbon dioxide technique the cultures were grown on Czapek's solution milk agar in the air 4 days and then in an atmosphere in which the air had been almost entirely replaced by carbon dioxide. The temperature of incubation was 21° C. and the plates were examined after 4 and 10 days. The results of nine comparisons of the acidified skim milk agar technique with the carbon dioxide technique for determining the proteolytic activities of certain penicillia are presented in table VII.

The data show that more clear cut results were obtained with the carbon dioxide technique than with the acidified milk agar technique. The diameters of the proteolyzed areas were somewhat smaller with the acidified milk agar technique, the maximum diameter being 7.5 cm., the minimum 3.9 cm. and the average 5.1 cm., as compared with maximum, minimum and average diameters with the carbon dioxide technique of 8.3, 3.5 and 5.4 cm., respectively.

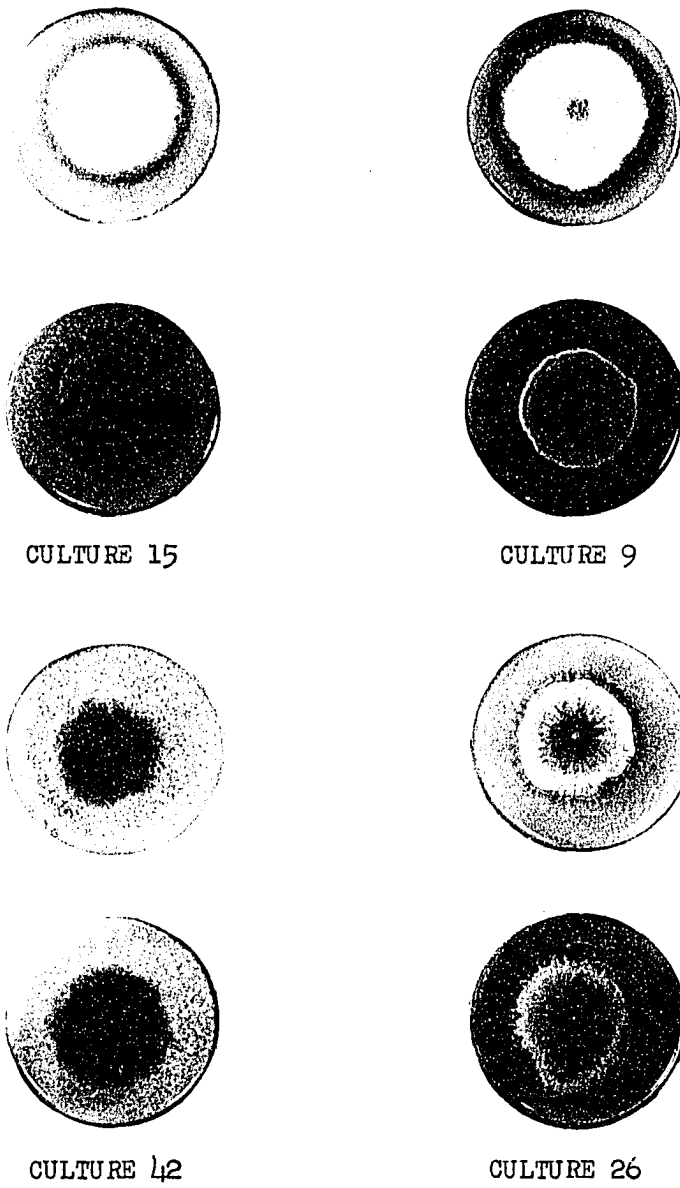
The two methods compared favorably with respect to proteolysis; however, with the acidified milk agar technique the lines of demarcation between the unhydrolyzed and hydrolyzed areas were not as sharply defined as with the carbon dioxide technique. Proteolysis with the acidified milk agar technique was very good in two comparisons, good in two, moderate in four and slight in one, compared with four very good, one good, three moderate and one slight with the carbon dioxide procedure.

Figure 3 presents the results of four comparisons of the carbon dioxide and acidified milk agar techniques for the detection of

TABLE VII

COMPARISON OF ACIDIFIED MILK AGAR TECHNIQUE WITH CARBON
DIOXIDE TECHNIQUE FOR DETERMINATION OF PROTEOLYTIC
ACTIVITIES OF CERTAIN PENICILLIA

Results							
Culture no.	Age in days	Acidified milk agar technique (Incubated in air at 21° C.)			Carbon dioxide technique (Incubated in air 4 days + 6 days in carbon dioxide at 21° C.)		
		Diameter of		Proteolysis	Diameter of		Proteolysis
		colony	proteolysis		colony	proteolysis	
		cm.	cm.		cm.	cm.	
6	4	1.4	1.4	Slight	3.5	0.0	Imperceptible
	10	7.7	6.9	Moderate	5.2	5.4	Moderate
7	4	1.1	0.6	Slight	2.7	0.0	Imperceptible
	10	3.7	3.9	Slight	4.6	4.8	Moderate
8	4	1.1	1.0	Good	2.5	2.5	Moderate
	10	4.5	4.0	Good	5.4	5.5	Very good
9	4	1.2	1.2	Good	4.6	0.0	Imperceptible
	10	6.2	5.7	Good	8.2	8.3	Very good
14	4	2.0	2.0	Slight	3.3	0.0	Imperceptible
	10	7.2	7.5	Moderate	5.1	3.5	Slight
15	4	1.6	1.0	Good	3.1	0.0	Imperceptible
	10	5.5	4.5	Very good	5.2	5.5	Very good
25	4	1.5	1.2	Moderate	3.2	0.0	Imperceptible
	10	4.2	4.2	Very good	5.3	5.7	Very good
27	4	2.0	1.4	Slight	3.8	0.0	Imperceptible
	10	5.7	4.8	Moderate	5.3	5.7	Moderate
41	4	1.0	0.0	Imperceptible	2.6	0.0	Imperceptible
	10	4.8	4.4	Moderate	5.1	4.7	Good



ACIDIFIED MILK AGAR TECHNIQUE CARBON DIOXIDE TECHNIQUE

FIGURE 3. COMPARISON OF ACIDIFIED MILK AGAR TECHNIQUE WITH CARBON DIOXIDE TECHNIQUE FOR DETECTION OF PROTEOLYTIC ACTIVITIES OF CERTAIN PENICILLIA

proteolysis by various penicillia. Although proteolysis was readily detected with both techniques, the results obtained with the carbon dioxide technique were more evident and the boundaries of the proteolyzed areas were more clearly defined than with the acidified milk agar technique.

From the data presented in table VII and figure 3, it is apparent that somewhat more clear cut results were usually obtained with the carbon dioxide technique in the detection of proteolysis by certain penicillia as compared with the acidified milk agar technique, when the cultures were incubated at 21° C. Although the two methods compared favorably with respect to perceptibility of proteolysis, the lines of demarcation between the unhydrolyzed and hydrolyzed areas were not as sharply defined with the acidified milk agar as with the carbon dioxide technique.

Lipolytic and Proteolytic Activities of Various Penicillia

Various cultures of penicillia employed in the ripening of blue veined cheeses differ in their morphologic and physiologic characteristics. It is, therefore, to be expected that variations in results will occur in cheese ripening. Lipolysis and proteolysis are believed to be the principal functions of the penicillia in cheese ripening. Accordingly, the lipolytic and proteolytic activities of the penicillia associated with the ripening of blue veined cheeses were investigated.

Review of literature

The ability of molds employed in the ripening of blue veined cheeses to elaborate enzymes capable of bringing about decomposition of fats and proteins has been reported by various investigators.

The production of lipase by Penicillium glaucum was observed by Camus (9), who found that a variation in the rate of inoculation into a solution of monobutyrim caused a variation in the production of lipase. In a study of the lipase production of Penicillium glaucum and other microorganisms, Laxa (49) noted two types of hydrolysis. Some bacteria and molds were capable of decomposing all of the triglycerides in a fat, whereas others could hydrolyze only those of lower molecular weights. The two strains of Penicillium glaucum studied by Laxa were capable of hydrolyzing fat. The lipolytic properties of Penicillium glaucum were also observed by Orla-Jensen (56) and by Rahn (61), who noted that the mold has powerful fat hydrolyzing properties. From an extensive study of the fat hydrolysis occurring during the ripening of roquefort cheese, Currie (15) concluded that the decomposition is due to a water soluble lipase produced by Penicillium roqueforti and results in an accumulation of free and combined forms of fatty acids and their hydrolyzable salts. This investigator grew Penicillium roqueforti on Czapek's solution in which cane sugar had been replaced by pure butterfat, tributyrin, ethyl butyrate, glycerol, butyric acid or ammonium butyrate. The mold was capable not only of growing these substances but also of hydrolyzing them and utilizing their constituents as a source of carbon. Currie also noted lipolysis of ethyl acetate, ethyl butyrate, triacetin and

tributylin with a material prepared by triturating air dried mold mycelium with powdered glass.

That proteolysis occurs during the ripening of blue cheese was pointed out by Currie (15), who cited some unpublished data from Dox showing the percentage distribution of nitrogen in a prime, well ripened cheese to be as follows: caseoses 10.7, peptones 8.6, amino acids 29.1, ammonia 6.1 and insoluble nitrogen 45.5 per cent; small amounts of tyrosin were also noted. According to Collins (11) and to Naylor, Smith and Collins (55), the esterase and protease activities of Penicillium roqueforti are proportional to the mold growth. Golding (26) concluded that low concentrations of acetic acid in milk reduced digestion of casein by Penicillium roqueforti, whereas the addition of citric acid resulted in increased digestion.

Lipolytic activities of various penicillia

The lipolytic activities of various penicillia on butterfat and on cottonseed oil were studied both with the natural fat and the Nile blue sulfate techniques, while their actions on different triglycerides were investigated only with the Nile blue sulfate procedure. Since none of the penicillia studied were capable of hydrolyzing trimyristin, tripalmitin or tristearin, the data obtained with these triglycerides were omitted.

The lipolytic activities of the cultures were rated by determining the ratio of colony growth to the extent of lipolysis, as expressed by $\frac{L}{C}$; L represented the diameter in cm. of the hydrolyzed

area and C the diameter of the colony. When the $\frac{L}{C}$ value was 1.00 the diameters of the colony and the hydrolyzed area were the same, and when it was less than 1.00 the area lipolyzed was smaller than that of the colony.

Lipolytic activities of certain penicillia on butterfat.

Table VIII gives the results obtained with 19 cultures in determining the lipolytic activities on butterfat, after an incubation of 8 days at 21° C.

a. Results with natural fat technique: The data show wide differences between the lipolytic activities of the cultures, as indicated by the comparative $\frac{L}{C}$ values and the uniformity and intensity of lipolysis. The pronounced lipolytic cultures showed a maximum $\frac{L}{C}$ value of 1.00, a minimum of 0.75 and an average of 0.91, while one moderately lipolytic culture exhibited a value of 0.65 and the slightly lipolytic cultures showed maximum, minimum and average values of 0.44, 0.18 and 0.34, respectively. Cultures with $\frac{L}{C}$ values of 0.00 were considered nonlipolytic on butterfat, according to the natural fat technique. With respect to uniformity and intensity of lipolysis, cultures 9 and 21 were uniform and very pronounced; cultures 5, 6, 15 and 25 were uniform and pronounced; cultures 8, 18 and 20 were nonuniform and pronounced; culture 10 was uniform and moderate; cultures 23, 24 and 26 were nonuniform and slight; and cultures 1, 7, 13, 14, 16 and 22 were nonlipolytic.

b. Results with Nile blue sulfate technique: With a few exceptions the results were in general agreement with those obtained by

TABLE VIII
LIPOLYTIC ACTIVITIES OF CERTAIN PENICILLIA
ON BUTTERFAT

Incubation 8 days at 21° C.

a. Results with natural fat technique				
Culture no.	Diameter of colony cm.	$\frac{L^*}{C}$ value	Uniformity of lipolysis	Lipolysis
1	9.0	0.00		
5	8.4	0.95	Uniform	Pronounced
6	6.9	0.75	Uniform	Pronounced
7	8.6	0.00		
8	9.0	0.86	Nonuniform	Pronounced
9	9.0	1.00	Uniform	Very pronounced
10	8.0	0.65	Uniform	Moderate
13	9.0	0.00		
14	6.8	0.00		
15	8.5	0.80	Uniform	Pronounced
16	5.1	0.00		
18	8.0	0.87	Nonuniform	Pronounced
20	9.0	1.00	Nonuniform	Pronounced
21	9.0	1.00	Uniform	Very pronounced
22	9.0	0.00		
23	9.0	0.44	Nonuniform	Slight
24	9.0	0.18	Nonuniform	Slight
25	7.7	0.97	Uniform	Pronounced
26	9.0	0.39	Nonuniform	Slight

Table continued on page 65.

Table VIII continued.

b. Results with Nile blue sulfate technique				
1	5.2	0.61	Nonuniform	Moderate
5	5.2	0.80	Uniform	Pronounced
6	5.2	0.80	Uniform	Pronounced
7	5.0	0.44	Nonuniform	Slight
8	5.4	0.94	Nonuniform	Pronounced
9	3.7	1.00	Uniform	Very pronounced
10	3.8	0.52	Uniform	Moderate
13	4.3	0.00		
14	4.4	0.00		
15	4.0	0.67	Uniform	Pronounced
16	2.1	0.00		
18	4.0	0.55	Nonuniform	Moderate
20	5.7	0.66	Uniform	Pronounced
21	5.8	0.93	Uniform	Very pronounced
22	6.0	0.00		
23	4.6	0.67	Nonuniform	Moderate
24	4.2	0.26	Nonuniform	Slight
25	3.3	0.97	Nonuniform	Pronounced
26	5.7	0.37	Nonuniform	Slight

* $\frac{L}{C}$ value = $\frac{\text{Diameter in cm. of area containing hydrolyzed fat}}{\text{Diameter in cm. of colony area}}$

means of the natural fat technique. The pronounced lipolytic cultures showed maximum, minimum and average $\frac{L}{C}$ values of 1.00, 0.66 and 0.85, respectively, as compared with corresponding values of the moderately lipolytic penicillia of 0.67, 0.52 and 0.59 and of the slightly lipolytic cultures of 0.44, 0.26 and 0.37. In regard to uniformity and intensity of lipolysis, cultures 9 and 21 were uniform and very pronounced; cultures 5, 6, 15 and 20 were uniform and pronounced; cultures 8 and 25 were nonuniform and pronounced; culture 10 was uniform and moderate; cultures 1, 18 and 23 were nonuniform and moderate; cultures 7, 24 and 26 were nonuniform and slight, whereas cultures 13, 14, 16 and 22 were nonlipolytic according to the technique employed.

Lipolytic activities of certain penicillia on cottonseed oil. The lipolytic activities on cottonseed oil were studied with 18 cultures. Table IX gives the results obtained after 7 days at 21° C.

a. Results with natural fat technique: The lipolytic activities on cottonseed oil varied considerably, as indicated by the comparative $\frac{L}{C}$ values and the uniformity and intensity of lipolysis. The pronounced lipolytic cultures showed a maximum $\frac{L}{C}$ value of 1.00, a minimum of 0.72 and an average of 0.83, while the moderately lipolytic cultures showed maximum, minimum and average values of 0.83, 0.61 and 0.79, respectively; one slightly lipolytic culture showed an $\frac{L}{C}$ value of 0.74. Five cultures with $\frac{L}{C}$ values of 0.00 were regarded as nonlipolytic according to the natural fat technique. In regard to uniformity and intensity of lipolysis, culture 9 was uniform and very pronounced; cultures 6, 8, 10, 15, 21 and 25 were uniform and pronounced; cultures 1

TABLE IX
LIPOLYTIC ACTIVITIES OF CERTAIN PENICILLIA
ON COTTONSEED OIL

Incubation 7 days at 21° C.

a. Results with natural fat technique				
Culture no.	Diameter of colony cm.	$\frac{L}{C}$ value	Uniformity of lipolysis	Lipolysis
1	8.7	0.78	Nonuniform	Pronounced
5	6.6	0.61	Uniform	Moderate
6	7.8	0.74	Uniform	Pronounced
7	8.2	0.74	Nonuniform	Slight
8	8.1	0.72	Uniform	Pronounced
9	8.3	1.00	Uniform	Very pronounced
10	7.7	0.84	Uniform	Pronounced
13	7.7	0.72	Nonuniform	Moderate
14	4.2	0.00		
15	7.3	0.85	Uniform	Pronounced
16	5.2	0.00		
18	7.5	0.93	Nonuniform	Pronounced
20	6.9	0.00		
21	6.9	0.76	Uniform	Pronounced
22	6.0	0.83	Nonuniform	Moderate
23	7.6	0.00		
24	8.0	0.00		
25	8.0	0.83	Uniform	Pronounced

Table continued on page 68.

Table IX continued.

b. Results with Nile blue sulfate technique				
1	4.5	0.62	Nonuniform	Moderate
5	4.0	0.80	Uniform	Pronounced
6	5.5	0.58	Uniform	Moderate
7	5.0	0.40	Nonuniform	Slight
8	5.7	0.93	Nonuniform	Pronounced
9	4.8	1.00	Uniform	Very pronounced
10	5.4	0.82	Uniform	Pronounced
13	6.5	0.00		
14	3.2	0.00		
15	4.3	0.88	Uniform	Pronounced
16	2.8	0.00		
18	3.6	0.80	Nonuniform	Moderate
20	5.2	0.00		
21	5.1	1.00	Uniform	Very pronounced
22	5.8	0.00		
23	5.6	0.00		
24	6.0	0.41	Nonuniform	Slight
25	4.2	0.96	Uniform	Pronounced

and 18 were nonuniform and pronounced; culture 5 was uniform and moderate; cultures 13 and 22 were nonuniform and moderate; culture 7 was nonuniform and slight; while cultures 14, 16, 20, 23 and 24 were nonlipolytic.

b. Results with Nile blue sulfate technique: Except in a few instances the results were in general agreement with those obtained with the natural fat technique. The pronounced lipolytic penicillia showed maximum, minimum and average $\frac{L}{C}$ values of 1.00, 0.80 and 0.91, respectively, as compared with corresponding values of the moderately lipolytic cultures of 0.80, 0.58 and 0.67 and of the slightly lipolytic molds of 0.41, 0.40 and 0.415. Some of the cultures were considered nonlipolytic since they gave $\frac{L}{C}$ values of 0.00. With respect to uniformity and intensity of lipolysis cultures 9 and 21 were uniform and very pronounced; cultures 5, 10, 15 and 25 were uniform and pronounced; culture 8 was nonuniform and pronounced; culture 6 was uniform and moderate; cultures 1 and 18 were nonuniform and moderate; cultures 7 and 24 were nonuniform and slight; whereas cultures 13, 14, 16, 20, 22 and 23 were nonlipolytic.

From the data presented in tables VIII and IX it is evident that there was considerable variation in the lipolytic activities of various penicillia on butterfat and cottonseed oil, as determined by the natural fat and the Nile blue sulfate techniques. This variation is evident in the $\frac{L}{C}$ values and in the uniformity and the intensity of lipolysis of the cultures.

With a few exceptions there was a general agreement between

the natural fat technique and the Nile blue sulfate procedure with respect to the lipolytic activities of the penicillia.

Lipolytic activities of certain penicillia on some simple triglycerides. The lipolytic activities of eight penicillia on some simple triglycerides were determined by means of the Nile blue sulfate technique, or by the disappearance of the triglycerides. The dye was added to the medium in the proportion of 1 part to 10,000 parts of agar. The results after 9 days at 21° C. are presented in table X.

The data show considerable variation in the lipolytic activities of the penicillia on the simple triglycerides; this was indicated by the relationship of the diameter of the colony to the area which showed lipolysis, as represented by the expression $\frac{L}{C}$.

Tripropionin exhibited a high degree of toxicity toward the penicillia, as shown by the sizes of the colonies; the maximum colony diameter on the medium containing tripropionin was 4.5 cm., the minimum was 0.0 cm. and the average was 2.3 cm. None of the cultures showed hydrolysis of tripropionin.

Tributyryn also inhibited growth of the penicillia but the inhibition was not as marked as with tripropionin. The maximum, minimum and average diameters of the colonies were 6.0, 2.0 and 3.6 cm., respectively. The penicillia caused hydrolysis of tributyrin quite readily, as evidenced by maximum, minimum and average $\frac{L}{C}$ values of 1.75, 1.00 and 1.27, respectively. The highest values were shown by cultures 12, 13, 18 and 41, while relatively low values were shown by cultures 5, 9, 15 and 25.

TABLE X

LIPOLYTIC ACTIVITIES OF CERTAIN PENICILLIA ON SOME

Incubation 9 days at 21° C.

Culture no.	Tripropionin		Tributyryn		Trivalerin		Tricaproin		Triheptylin	
	Diameter of colony cm.	$\frac{L}{C}$ value	Diameter of colony cm.	$\frac{L}{C}$ value	Diameter of colony cm.	$\frac{L}{C}$ value	Diameter of colony cm.	$\frac{L}{C}$ value	Diameter of colony cm.	$\frac{L}{C}$ value
5	3.7	0.00	4.8	1.02	4.4	1.01	2.6	0.60	3.4	0.76
9	3.2	0.00	6.0	1.00	5.7	1.01	2.6	1.00	2.3	1.00
12	0.8	0.00	2.0	1.75	1.6	1.00	1.0	0.00	1.2	0.00
13	0.0	0.00	2.0	1.40	5.1	1.02	0.6	+ *	3.5	+ *
15	3.4	0.00	3.4	1.06	3.2	0.70	2.9	0.97	2.4	0.88
18	0.0	0.00	2.4	1.29	2.6	1.19	3.6	+ *	4.8	0.75
25	2.9	0.00	4.2	1.04	4.6	0.78	2.0	0.54	3.3	1.00
41	4.5	0.00	3.7	1.60	3.0	1.23	3.6	0.94	4.6	0.91

*Lipolysis positive, but too poorly defined for accurate measurement.

TABLE X

GROWTH OF CERTAIN PENICILLIA ON SOME SIMPLE TRIGLYCERIDES

Incubation 9 days at 21° C.

Tricaproin		Triheptylin		Tricaprylin		Tricaprin		Trilaurin		Triolein	
Diameter of colony	$\frac{L}{C}$ value	Diameter of colony	$\frac{L}{C}$ value	Diameter of colony	$\frac{L}{C}$ value	Diameter of colony	$\frac{L}{C}$ value	Diameter of colony	$\frac{L}{C}$ value	Diameter of colony	$\frac{L}{C}$ value
cm.		cm.		cm.		cm.		cm.		cm.	
2.6	0.60	3.4	0.76	3.4	0.93	3.7	0.88	4.1	+ *	3.0	1.00
2.6	1.00	2.3	1.00	2.4	1.00	6.0	1.00	2.9	+ *	2.9	1.00
1.0	0.00	1.2	0.00	9.0	0.00	9.0	0.00	8.0	0.00	9.0	0.51
0.6	+ *	3.5	+ *	7.8	0.00	0.7	0.00	6.3	0.00	9.0	+ *
2.9	0.97	2.4	0.88	3.3	0.87	3.2	0.93	2.9	+ *	3.5	1.00
3.6	+ *	4.8	0.75	3.0	+ *	9.0	+ *	6.2	0.00	0.5	0.00
2.0	0.54	3.3	1.00	3.0	0.66	1.8	1.20	2.3	+ *	2.2	1.00
3.6	0.94	4.6	0.91	1.8	1.00	1.7	0.63	3.2	+ *	4.7	1.00

ate measurement.

The maximum diameter of the colonies on trivalerin was 5.7 cm., the minimum was 1.6 cm. and the average 3.8 cm. This triglyceride was readily hydrolyzed by all eight cultures, as indicated by maximum, minimum and average $\frac{L}{C}$ values of 1.23, 0.70 and 0.99, respectively. Cultures 5, 9, 12, 13, 18 and 41 showed high $\frac{L}{C}$ values, while cultures 15 and 25 exhibited low values.

Tricaproin also retarded the growth of some cultures. The maximum diameter of the colonies was 3.6 cm., the minimum was 0.6 cm. and the average was 2.3 cm. Marked differences were noted in the lipolytic activities of the cultures on tricaproin. The maximum $\frac{L}{C}$ value was 1.00, the minimum was 0.00 and the average was 0.51. The highest $\frac{L}{C}$ values were shown by cultures 9, 15 and 41, intermediate by cultures 5 and 25 and low values by cultures 12, 13 and 18.

When the cultures were grown on the medium containing triheptylin, the maximum colony diameter was 4.8 cm., the minimum was 1.2 cm. and the average was 3.2 cm. The maximum, minimum and average $\frac{L}{C}$ values were 1.00, 0.00 and 0.66, respectively. The values for cultures 9, 15, 25 and 41 were relatively high, those for cultures 5 and 18 were medium and the minimum values were shown by cultures 12 and 13.

The growth of the penicillia on the medium containing tricapyrin showed relatively large differences, as indicated by maximum, minimum and average colony diameters of 9.0, 1.8 and 4.2 cm., respectively. There were also definite differences in the lipolytic activities of the cultures, the maximum $\frac{L}{C}$ value being 1.00 and the average value 0.56. The minimum value was expressed as plus; this indicated positive

lipolysis which was too indefinite for accurate measurement. The highest $\frac{L}{C}$ values were shown with cultures 5, 9, 15 and 41, while culture 25 showed an intermediate value and culture 18 a low value; cultures 12 and 13 were nonlipolytic.

The maximum diameter of a colony grown on the medium containing tricaprln was 9.0 cm., the minimum was 0.7 cm. and the average was 4.3 cm. There was considerable variation in the lipolytic activities of the penicillia, as shown by maximum, minimum and average $\frac{L}{C}$ values of 1.2, 0.00 and 0.58, respectively. The highest values were shown by cultures 5, 9, 15 and 25, an intermediate value by culture 41 and low values by cultures 12, 13 and 18.

On the medium which contained dispersed trilaurin, the maximum, minimum and average diameters of the colonies were 8.0, 2.3 and 4.5 cm., respectively. Cultures 5, 9, 15, 25 and 41 showed slight but positive lipolysis of trilaurin, as indicated by the plus $\frac{L}{C}$ values, while negative results were obtained with cultures 12, 13 and 18.

The colonies on the medium containing triolein showed a maximum diameter of 9.0 cm., a minimum of 0.5 cm. and an average of 4.4 cm. The lipolytic activities of the cultures on triolein showed wide differences, as indicated by maximum, minimum and average $\frac{L}{C}$ values of 1.00, 0.00 and 0.69, respectively. High $\frac{L}{C}$ values were shown by cultures 5, 6, 15, 25 and 41, an intermediate value by culture 12 and low values by cultures 13 and 18.

Additional trials were also made comparing the lipolytic

activities of certain penicillia on some simple triglycerides on a medium containing Nile blue sulfate in the proportion of 1 part of the dye to 15,000 parts agar. The plates were inspected twice for lipolysis and colony growth. The first examination was made after an incubation period of 4 days in air plus 6 days in an atmosphere consisting mostly of carbon dioxide at 21° C. The plates were then incubated 8 additional days in the carbon dioxide atmosphere, after which they were again examined. The purpose of incubating the plates in carbon dioxide was to retard growth and thus prevent a heavy growth, which rendered the examination for lipolysis underneath the colonies difficult. The results are presented in table XI.

a. Results with cultures incubated 4 days in air plus 6 days in carbon dioxide at 21° C.: The data show significant differences in the lipolytic activities of certain penicillia on some triglycerides, as evidenced by their $\frac{L}{C}$ values.

The toxicity of tripropionin toward the penicillia was evident from the sizes of the colonies. The maximum diameter of a colony was 2.0 cm., the minimum was 0.5 cm. and the average was 1.4 cm. With culture 14 no growth was evident. No evidence of lipolysis of tripropionin was noted with any of the cultures and the $\frac{L}{C}$ values were 0.00.

Tributyryn also inhibited mold growth, although the inhibition was not as great as with tripropionin. The maximum diameter of the colonies was 3.9 cm., the minimum was 2.2 cm. and the average was 2.9 cm. Tributyrin was readily hydrolyzed by all of the cultures, as evidenced by relatively high $\frac{L}{C}$ values. The maximum, minimum and average $\frac{L}{C}$ values

TABLE XI

LIPOLYTIC ACTIVITIES OF CERTAIN PENICILLIA ON SOME

Culture no.	Tripropionin		Tributyryn		Trivalerin		Tricaproin		Triheptylin	
	Diameter of colony cm.	$\frac{L}{C}$ value	Diameter of colony cm.	$\frac{L}{C}$ value	Diameter of colony cm.	$\frac{L}{C}$ value	Diameter of colony cm.	$\frac{L}{C}$ value	Diameter of colony cm.	$\frac{L}{C}$ value
a. Cultures incubated 4 days in air + 6 days in										
6	2.0	0.00	2.2	1.09	3.4	1.00	4.5	0.60	3.9	0.59
7	1.0	0.00	3.9	0.85	4.4	0.77	4.9	0.39	4.8	0.48
14	0.0	0.00	2.5	1.12	1.6	1.00	4.2	0.45	3.0	0.63
25	0.5	0.00	2.3	1.13	2.9	1.03	3.0	0.57	3.4	0.44
27	2.0	0.00	3.8	1.05	3.8	0.84	3.8	0.55	4.1	0.56
b. Cultures incubated 4 days in air + 14 days in										
6	4.0	0.00	3.7	1.14	4.9	0.98	5.1	0.78	3.9	0.59
7	2.0	0.00	4.7	1.23	5.5	0.96	6.5	0.77	6.1	0.85
14	0.5	0.00	3.6	1.14	3.1	0.90	5.2	0.67	4.2	0.29
25	0.9	0.00	4.4	1.37	4.2	1.10	4.4	0.82	4.1	0.88
27	3.8	0.00	5.7	1.07	6.0	1.02	5.0	0.56	5.8	0.71

*Lipolysis positive, but too poorly defined for accurate measurement.

TABLE XI

OF CERTAIN PENICILLIA ON SOME SIMPLE TRIGLYCERIDES

Tricaproin	Triheptylin		Tricaprylin		Tricaprin		Trilaurin		Triolein		
	Diameter of colony cm.	$\frac{L}{C}$ value	Diameter of colony cm.	$\frac{L}{C}$ value	Diameter of colony cm.	$\frac{L}{C}$ value	Diameter of colony cm.	$\frac{L}{C}$ value	Diameter of colony cm.	$\frac{L}{C}$ value	
4 days in air + 6 days in carbon dioxide at 21° C.											
4.5	0.60	3.9	0.59	3.9	0.64	3.7	0.70	3.3	*	3.8	0.58
4.9	0.39	4.8	0.48	4.4	0.39	4.1	0.41	4.4	0.00	4.6	0.00
4.2	0.45	3.0	0.63	3.8	0.39	4.1	0.49	4.1	0.00	4.1	0.00
5.0	0.57	3.4	0.44	2.6	0.54	3.0	0.87	2.8	0.00	3.6	0.69
4.8	0.55	4.1	0.56	3.5	0.51	3.5	0.60	3.7	0.00	3.8	*
4 days in air + 14 days in carbon dioxide at 21° C.											
5.1	0.78	3.9	0.59	4.4	0.84	3.8	0.87	3.5	*	4.2	0.95
5.5	0.77	6.1	0.85	5.8	0.59	4.5	0.84	5.2	0.00	5.5	0.73
5.2	0.67	4.2	0.29	4.1	0.68	5.0	0.84	4.5	*	4.5	*
4.4	0.82	4.1	0.88	4.2	0.62	4.6	0.91	4.0	*	5.0	0.52
5.0	0.56	5.8	0.71	4.7	0.68	4.9	0.92	5.1	0.00	6.0	*

measurement.

were 1.13, 0.85 and 1.05, respectively. With the exception of culture 7 which showed the lowest $\frac{L}{C}$ value, the molds showed relatively uniform lipolysis on tributyrin.

The maximum colony diameter on the medium containing trivalerin was 4.4 cm., the minimum was 1.6 cm. and the average was 3.2 cm. Although trivalerin showed less inhibitory effect than tributyrin toward certain penicillia, it was more toxic toward culture 14, which developed a colony measuring 1.6 cm. in diameter compared to 2.5 cm. on tributyrin. The five cultures produced pronounced lipolysis on trivalerin, as shown by maximum, minimum and average $\frac{L}{C}$ values of 1.03, 0.77 and 0.92, respectively. The highest values were shown by cultures 6, 14 and 25 and the lowest by cultures 7 and 27.

The restraining influence of tricaproin on mold growth was less marked than with the triglycerides of lower molecular weights. The maximum, minimum and average diameters of the colonies were 4.9, 3.0 and 4.1 cm., respectively. The maximum $\frac{L}{C}$ value was 0.60, the minimum was 0.39 and the average was 0.51. The highest values were shown by cultures 6, 25 and 27 and the lowest by cultures 7 and 14.

The maximum, minimum and average diameters of the colonies on triheptylin were 4.8, 3.0 and 3.8 cm., respectively. Less variation was noted in the lipolytic activities of the cultures than with the lower triglycerides, as indicated by a maximum $\frac{L}{C}$ value of 0.63, a minimum of 0.44 and an average of 0.54. The highest values were noted with cultures 6, 14 and 27 and the lowest with cultures 7 and 25.

Colonies on the medium which contained dispersed tricaprylin

showed fairly normal development; the maximum colony diameter was 4.4 cm., the minimum was 2.6 cm. and the average diameter was 3.6 cm. The cultures were moderately lipolytic on tricaprylin, as indicated by a maximum $\frac{L}{C}$ value of 0.64, a minimum of 0.39 and an average value of 0.49. The highest $\frac{L}{C}$ values were shown by cultures 6, 25 and 27 and the lowest by 7 and 14.

The growth of the cultures on tricaprin showed marked similarity to the corresponding cultures on tricaprylin. The maximum, minimum and average diameters of the colonies were 4.1, 3.0 and 3.7 cm., respectively. All of the cultures caused hydrolysis of tricaprin. The maximum, minimum and average $\frac{L}{C}$ values were 0.87, 0.41 and 0.61, respectively. The highest values were shown by cultures 6, 25 and 27 and the lowest by 7 and 14.

Although trilaurin showed some restraining action with respect to the growth of certain of the molds, fairly normal development of the cultures occurred in all cases. The maximum colony diameter was 4.4, the minimum was 2.8 and the average was 3.7 cm. Culture 6 was the only mold in the group which showed definite lipolysis of trilaurin; this action was so slight and indefinite that no accurate measurement of the hydrolyzed area could be made, and consequently the lipolytic action was indicated by a plus sign.

Triolein seemed to have no restraining effect on the growth of the penicillia. The maximum, minimum and the average sizes of the colonies were 4.6, 3.6 and 3.9 cm., respectively. The $\frac{L}{C}$ values showed moderate lipolysis with cultures 6 and 25, there was slight but positive

lipolytic action with culture 27, while negative activity was indicated with cultures 7 and 14.

b. Results with cultures incubated 4 days in air plus 14 days in carbon dioxide at 21° C.: The data show results similar to those obtained on shorter incubation with the exception of a few instances where changes in the $\frac{L}{C}$ values had occurred. Tripropionin again showed marked toxicity toward the penicillia, as evidenced by the diameters of the colonies. The maximum diameter of the colonies was 4.0 cm., the minimum was 0.5 cm. and the average 2.2 cm. Cultures 7, 14 and 25 showed the greatest inhibition. None of the cultures caused lipolysis on tripropionin.

The maximum diameter of the colonies on tributyrin was 5.7 cm., the minimum was 3.6 cm. and the average was 4.4 cm. The $\frac{L}{C}$ values indicated pronounced lipolysis with all of the cultures on tributyrin; the maximum, minimum and average values were 1.37, 1.07 and 1.19, respectively. The highest values were shown by cultures 7 and 25 and the lowest by cultures 6, 14 and 27.

The cultures on trivalerin showed maximum, minimum and average diameters of 6.0, 3.1 and 4.7 cm. The $\frac{L}{C}$ values of the cultures showed pronounced lipolysis on the triglyceride, as indicated by a maximum value of 1.10, a minimum of 0.90 and an average of 0.99. Cultures 25 and 27 showed the highest and cultures 6, 7 and 14 the lowest $\frac{L}{C}$ values.

Tricaproin had relatively little inhibitive influence on the growth of the penicillia studied. The maximum, minimum and average

diameters of the colonies were 6.5, 4.4 and 5.2 cm. The cultures were moderately lipolytic on tricaproin, as indicated by the $\frac{L}{C}$ values; the maximum value was 0.82, the minimum was 0.56 and the average was 0.72. Cultures 6, 7 and 25 had the highest values and cultures 14 and 27 the lowest.

The cultures showed comparatively good growth on triheptylin; the maximum colony diameter was 6.1 cm., the minimum was 3.9 cm. and the average 4.8 cm. The cultures varied rather widely in their abilities to cause lipolysis of triheptylin, as evidenced by maximum, minimum and average $\frac{L}{C}$ values of 0.88, 0.29 and 0.66, respectively. The highest values were noted with cultures 7, 25 and 27 and the lowest with cultures 6 and 14.

The maximum size of the colonies on tricaprylin was 5.8 cm., the minimum was 4.1 cm. and the average was 4.6 cm. in diameter. The cultures did not show as wide differences in their lipolytic activities on tricaprylin as on the lower triglycerides. The maximum $\frac{L}{C}$ value of a culture was 0.84, the minimum was 0.59 and the average was 0.68. The highest values were shown by cultures 6, 14 and 27 and the lowest by cultures 7 and 25.

Tricaprin seemed to exhibit a slight inhibitive effect on the growth of some of the cultures while others were not retarded in growth. The colonies showed a maximum diameter of 5.0 cm., a minimum of 3.8 cm. and an average of 4.5 cm. in diameter. The cultures did not show large differences in their $\frac{L}{C}$ values, as indicated by maximum, minimum and average values of 0.92, 0.84 and 0.88, respectively.

The maximum diameter of a colony on trilaurin was 5.2 cm., the minimum was 3.5 cm. and the average was 4.5 cm. Cultures 6, 14 and 25 showed indication of lipolytic activity on trilaurin; however, the lipolysis was slight and too indefinite to be measured, consequently a plus sign was used to designate positive hydrolysis of the triglyceride. Negative results with respect to lipolysis were obtained with cultures 7 and 27 and indicated by $\frac{L}{C}$ values of 0.00.

The cultures showed normal growth on triolein. The maximum, minimum and average diameters were 6.0, 4.2 and 5.0 cm., respectively. Large variations occurred in the $\frac{L}{C}$ values. Cultures 6, 7 and 25 showed the corresponding values of 0.95, 0.73 and 0.52, while cultures 14 and 27 showed slight but definite lipolysis as indicated by plus values.

The lipolytic activities of certain penicillia on some triglycerides were again compared and the results are presented in figures 4 and 5. The results are similar to those given in tables X and XI with the following exceptions; cultures 5 and 15 showed active lipolysis of tripropionin; culture 15 exhibited pronounced lipolysis of trivalerin; culture 15 showed no lipolysis of trilaurin; none of the cultures showed lipolysis of trimyristin or tripalmitin, two triglycerides were not included in the data reported in the tables.

From the data presented in tables X and XI and figures 4 and 5, it is evident that there was considerable variation in the lipolytic activities of various penicillia on different triglycerides. In only a few instances was the lipolysis of tripropionin noted. However,

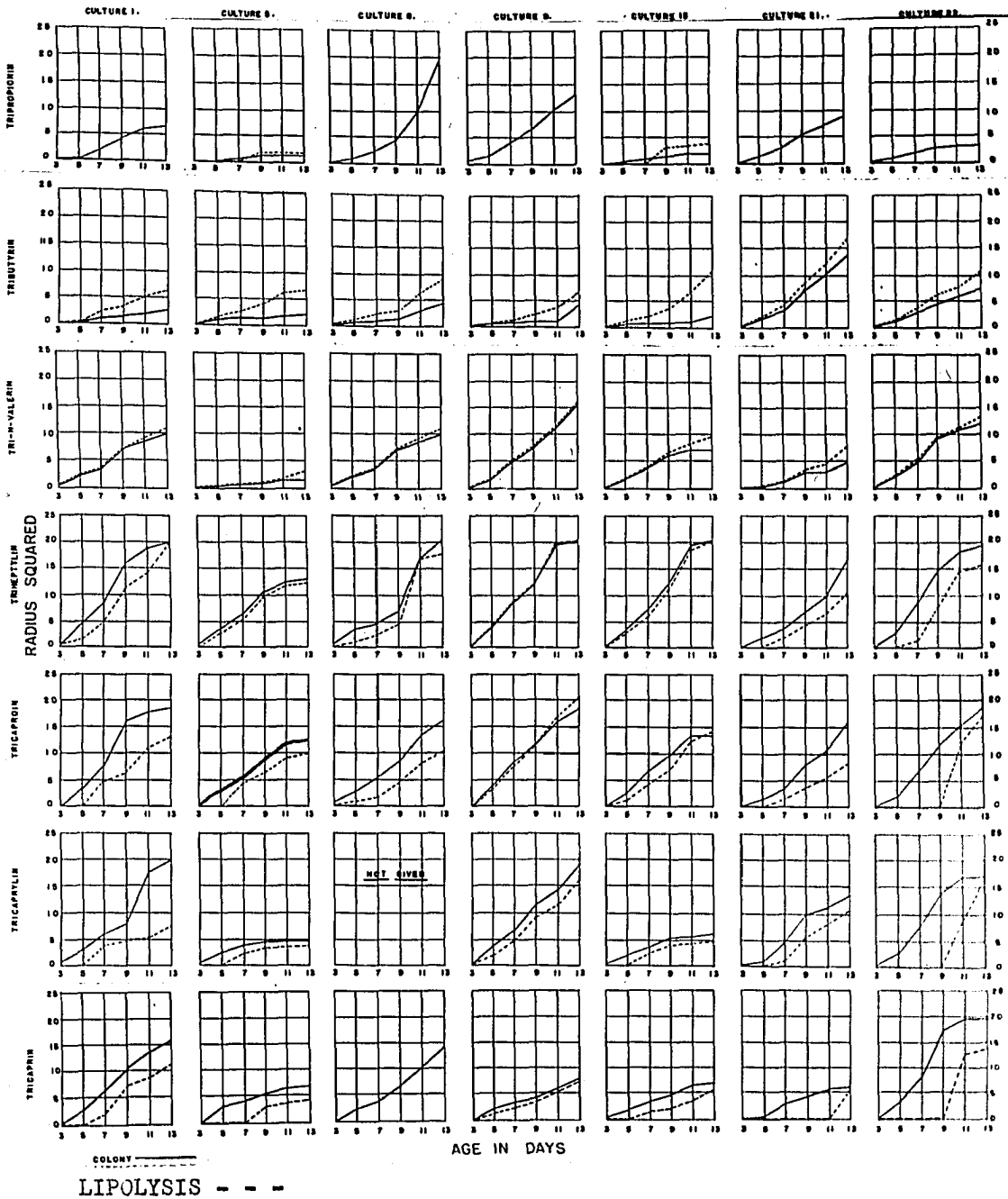


FIGURE 4. LIPOLYTIC ACTIVITIES OF CERTAIN PENICILLIA ON SOME SIMPLE TRIGLYCERIDES

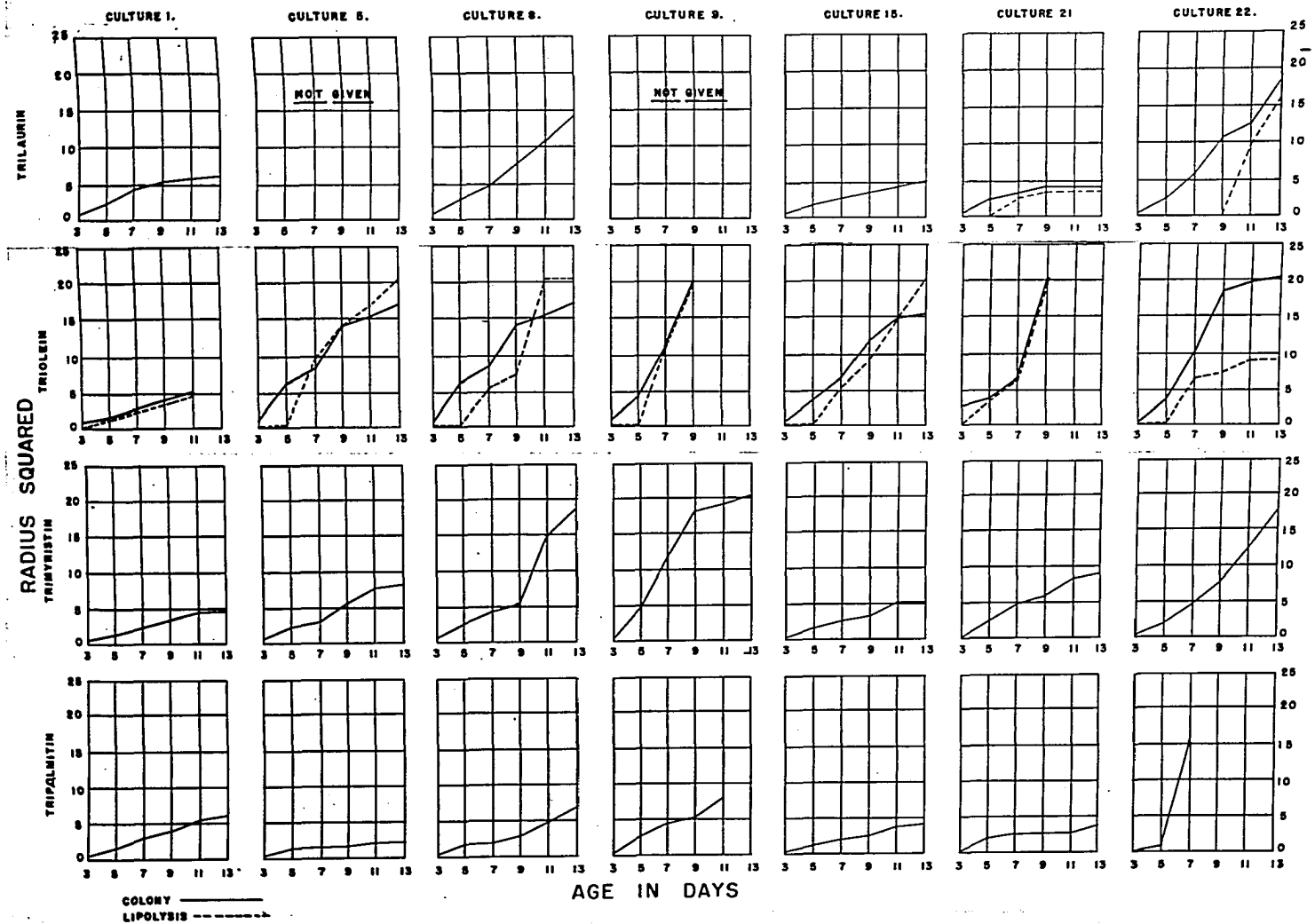


FIGURE 5. LIPOLYTIC ACTIVITIES OF CERTAIN PENICILLIA ON SOME SIMPLE TRIGLYCERIDES

tributylin and trivalerin was readily hydrolyzed by all of the cultures. As the molecular weights of the triglycerides increased, variations in the lipolytic activities of the cultures became more conspicuous. Some cultures showed relatively gradual declines in their lipolytic activities while others declined sharply on the lower triglycerides, beginning with tricaproin. While most of the cultures were actively lipolytic on the triglycerides from tributyrin to and including tricaproin, several showed negative results with trilaurin.

It is also evident that some of the triglycerides were toxic with respect to the penicillia; this was evidenced by retarded culture growth. The cultures were variously affected by the different triglycerides, some of them showing relatively high sensitivity while others were more resistant. In general the triglycerides that exhibited the most pronounced toxicity toward the penicillia were tripropionin, tributyrin, trivalerin, tricaproin and trilaurin. According to the data from figure 5, it is also evident that trimyristin and tripalmitin were usually toxic toward the molds. The least toxicity was evident with triheptylin, tricaproin, tricaprylin and triolein.

Factors Affecting Lipolysis of Fats

Effect of carbon dioxide and nitrogen on lipolytic activities of certain penicillia on butterfat

In studying the effect of certain gases on the lipolytic activities of the penicillia, the inoculated plates were placed under a

bell jar, after which the bottom of the jar was sealed with stopcock grease to a glass plate to prevent leakage of the gas mixture. A definite fraction of air was then evacuated from the jar, as determined with a manometer; this was followed by introduction of the gas into the jar until normal atmospheric pressure was obtained. When the gas mixture consisted of carbon dioxide and nitrogen, the air was first replaced by carbon dioxide by permitting a stream of the gas to enter the jar slowly through a tube which extended almost to the bottom of the container. The incoming carbon dioxide gradually replaced the air which was emitted through a vent at the top of the jar. A definite fraction of carbon dioxide was then evacuated and nitrogen was introduced until normal atmospheric pressure was regained.

The effect of carbon dioxide and nitrogen on the lipolytic activities of certain penicillia was studied with five cultures on butterfat dispersed in Czapek's solution agar, using the Nile blue sulfate technique (1 part dye in 15,000 parts agar). The results after 8 days at 21° C. are presented in table XII.

The data show marked differences in the lipolytic activities of the penicillia when grown under the influence of different gas mixtures. When the cultures were grown in air, the maximum colony diameter was 6.3 cm., the minimum was 4.5 cm. and the average was 5.3 cm. The maximum, minimum and average $\frac{L}{C}$ values were 0.89, 0.17 and 0.58, respectively. Cultures 5, 15 and 25 showed uniform and pronounced lipolysis, while cultures 8 and 26 exhibited nonuniform and slight lipolytic action.

TABLE XII

EFFECT OF CARBON DIOXIDE AND NITROGEN ON I
OF CERTAIN PENICILLIA ON BUTYR

Incubation 8 days at 21°

Culture no.	Czapek's solution agar + Nile blue sulfate (1 part 3 parts air + 1 part carbon dioxide)									
	Normal air supply					3 parts air + 1 part carbon dioxide				
	Diameter of colony cm.	$\frac{L}{C}$ value	Uniformity of lipolysis	Lipolysis	Diameter of colony cm.	$\frac{L}{C}$ value	Uniformity of lipolysis	Lipolysis	Diameter of colony cm.	v
5	4.5	0.84	Uniform	Pronounced	5.8	0.74	Nonuniform	Slight	7.3	0.
8	5.1	0.18	Nonuniform	Slight	4.9	0.83	Nonuniform	Slight	7.6	0.
15	6.3	0.81	Uniform	Pronounced	5.0	0.35	Nonuniform	Slight	6.9	0.
25	4.5	0.89	Uniform	Pronounced	3.8	0.76	Nonuniform	Slight	6.3	1.
26	6.0	0.17	Nonuniform	Slight	5.3	0.59	Nonuniform	Slight	7.3	0.

TABLE XII

AND NITROGEN ON LIPOLYTIC ACTIVITIES
 PENICILLIA ON BUTTERFAT

on 8 days at 21° C.

sulfate (1 part of dye + 15,000 parts agar):									
oxide 3 parts air + 1 part nitrogen					3 parts carbon dioxide + 1 part nitrogen				
Lipolysis	Diameter	$\frac{L}{C}$	Uniformity	Lipolysis	Diameter	$\frac{L}{C}$	Uniformity	Lipolysis	
	of colony	cm.	value			of	of colony		
Light	7.3	0.85	Uniform	Pronounced	5.9	0.93	Uniform	Pronounced	
Light	7.6	0.71	Uniform	Moderate	7.2	0.76	Nonuniform	Moderate	
Light	6.9	0.74	Uniform	Pronounced	3.9	0.79	Uniform	Pronounced	
Light	6.3	1.00	Uniform	Very pronounced	4.9	0.89	Uniform	Pronounced	
Light	7.3	0.73	Uniform	Moderate	6.3	0.74	Nonuniform	Moderate	

The cultures incubated in 3 parts air and 1 part carbon dioxide showed respective maximum, minimum and average diameters of 5.8, 3.8 and 5.0 cm. The maximum $\frac{L}{C}$ value was 0.83, the minimum was 0.35 and the average was 0.65. All five cultures showed nonuniform lipolysis and were considered only slightly lipolytic.

Incubation of the penicillia in an atmosphere consisting of 3 parts air and 1 part nitrogen resulted in colonies which showed denser conidial growth and more pronounced lipolysis, when compared with the same cultures grown in air. The maximum colony diameter was 7.6 cm., the minimum was 6.3 cm. and the average was 7.5 cm. The maximum, minimum and average $\frac{L}{C}$ values were, respectively, 1.00, 0.71 and 0.81. Cultures 5 and 15 showed uniform and pronounced lipolysis, cultures 8 and 26 showed nonuniform and moderate lipolysis, while culture 25 exhibited uniform and very pronounced lipolytic action.

In an atmosphere consisting of 3 parts carbon dioxide and 1 part nitrogen, the growth of the penicillia was slightly greater and the lipolytic activities were more pronounced than with the same cultures incubated in a normal air supply. The maximum, minimum and average colony diameters were 7.2, 3.9 and 5.6 cm., respectively; the maximum, minimum and average $\frac{L}{C}$ values were 0.93, 0.74 and 0.82, respectively. Cultures 5, 15 and 25 showed uniform and pronounced lipolysis and cultures 8 and 26 exhibited nonuniform and moderate hydrolysis of butter-fat.

From the data presented in table XII it is evident that there were marked variations in the lipolytic activities of the penicillia on

butterfat when grown under the influence of different gas mixtures. This variation is evident in the $\frac{L}{C}$ values and in the uniformity and perceptibility of lipolysis. The lipolytic activities of the penicillia were retarded when the cultures were grown in an atmosphere consisting of 3 parts air and 1 part carbon dioxide, while their activities were considerably accelerated in 3 parts air and 1 part nitrogen and slightly accelerated in 3 parts carbon dioxide and 1 part nitrogen.

Effect of 4 per cent sodium chloride on lipolytic activities of certain penicillia on cottonseed oil

The effect of sodium chloride on the lipolytic activities of certain penicillia on cottonseed oil was investigated with 12 cultures by means of the natural fat technique. Table XIII gives the results after 8 days at 25° C.

The data show that 4.0 per cent sodium chloride in the medium resulted in a slight to pronounced decrease in colony growth, when compared with growth on the medium to which no salt had been added. The maximum diameter of colonies grown on medium which contained no salt was 9.3, the minimum was 4.5 and the average was 7.1 cm., as compared with the respective maximum, minimum and average diameters of 8.9, 1.5 and 5.2 cm. for the colonies grown on agar to which 4 per cent sodium chloride had been added.

The medium which contained no salt showed maximum, minimum and average $\frac{L}{C}$ values of 1.00, 0.00 and 0.52, while the medium to which 4.0 per cent sodium chloride had been added showed corresponding values

TABLE XIII

EFFECT OF 4 PER CENT SODIUM CHLORIDE ON LIPOLYTIC ACTIVITIES
OF CERTAIN PENICILLIA ON COTTONSEED OIL

(Natural fat technique)

Incubation 8 days at 25° C.

Culture no.	Per cent sodium chloride added per 100 grams medium							
	0.0 per cent				4.0 per cent			
	Diameter of colony cm.	$\frac{L}{C}$ value	Uniformity of lipolysis	Lipolysis	Diameter of colony cm.	$\frac{L}{C}$ value	Uniformity of lipolysis	Lipolysis
1	8.4	0.70	Nonuniform	Moderate	7.2	0.55	Nonuniform	Slight
5	8.7	0.70	Uniform	Pronounced	5.2	0.38	Uniform	Moderate
6	5.5	0.91	Uniform	Pronounced	5.2	0.77	Uniform	Moderate
7	8.8	0.34	Nonuniform	Slight	6.3	0.63	Nonuniform	Slight
8	6.2	0.81	Nonuniform	Moderate	8.9	0.56	Nonuniform	Slight
9	4.5	1.00	Uniform	Pronounced	3.2	0.94	Uniform	Pronounced
10	9.3	0.43	Uniform	Moderate	7.0	0.57	Nonuniform	Moderate
11	5.8	0.00			5.0	0.40	Nonuniform	Slight
12	9.0	0.00			3.4	0.88	Nonuniform	Slight
13	8.8	0.45	Nonuniform	Slight	4.5	0.44	Nonuniform	Slight
14	6.5	0.00			1.5	0.00		
15	4.5	0.95	Uniform	Pronounced	4.4	0.91	Nonuniform	Moderate

of 0.94, 0.00 and 0.58, respectively. Although there was no appreciable decrease in the $\frac{L}{C}$ values, lipolysis was less intense in the presence of salt.

The results given in table XIII indicate a slight decrease in the lipolytic activities of certain penicillia on cottonseed oil when the medium contained 4 per cent sodium chloride.

Effect of temperature on lipolytic activities of certain penicillia on cottonseed oil

The effect of temperature on the lipolytic activities of certain penicillia on cottonseed oil was studied with 11 cultures by means of the natural fat technique. One set of cultures was incubated at 25° C., while another group was grown at 9° to 10° C. The results after 7 days are presented in table XIV.

The data show that greater mold growth resulted when the cultures were incubated at the higher than at the lower temperature. When the cultures were incubated at 25° C., the maximum colony diameter was 9.0 cm., the minimum was 4.5 cm. and the average 7.5 cm., as compared with corresponding measurements of 5.5, 2.3 and 4.2 cm. at 9° to 10° C.

The penicillia showed greater lipolytic action at 25° C. than at 9° to 10° C., as indicated by the comparative $\frac{L}{C}$ values. The maximum $\frac{L}{C}$ value of colonies grown at 25° C. was 0.91, the minimum was 0.00 and the average was 0.48, as compared with similar values of 0.63, 0.00 and 0.31, respectively, when the cultures were grown at 9° to 10° C.

TABLE XIV

EFFECT OF TEMPERATURE ON LIPOLYTIC ACTIVITIES
OF VARIOUS PENICILLIA ON COTTONSEED OIL

(Natural fat technique)

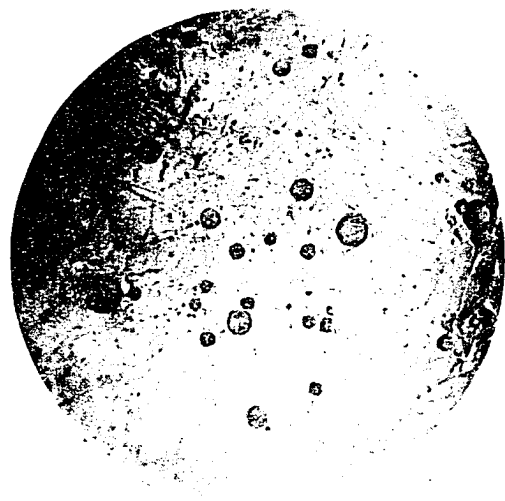
Cultures incubated 7 days at:				
25° C.			9° to 10° C.	
Culture no.	Diameter of colony cm.	$\frac{L}{C}$ value	Diameter of colony cm.	$\frac{L}{C}$ value
1	8.4	0.59	4.1	0.48
5	8.7	0.69	4.5	0.55
6	5.5	0.91	4.1	0.61
7	8.8	0.34	5.1	0.29
8	8.0	0.62	4.5	0.41
10	9.0	0.78	5.0	0.63
11	5.8	0.00	4.1	0.00
12	9.0	0.00	5.5	0.00
13	8.8	0.45	3.8	0.00
14	6.5	0.00	2.3	0.00
15	4.5	0.88	2.8	0.50

From the data presented in table XIV it is apparent that the penicillia showed greater lipolytic action on cottonseed oil when the cultures were grown at 25° C. than at 9° to 10° C., as indicated by their comparative $\frac{L}{C}$ values. It is also evident that considerably greater growth of the cultures occurred at the higher than at the lower temperatures; this was evidenced by the relative diameters of the colonies.

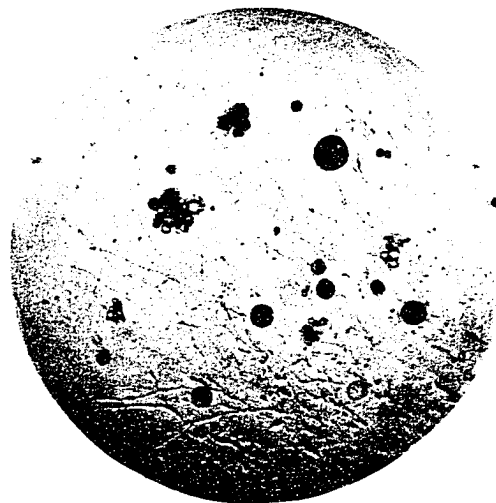
General Observations of Changes Occurring in Lipolysis
of Certain Fats and Triglycerides with
Nile Blue Sulfate Technique

In the study of the lipolytic activities of various penicillia with the Nile blue sulfate technique, the changes which occurred in the physical appearance of globules during hydrolysis were noted. The observations were usually made with a wide field binocular; however, a microscope with a magnification of 100 diameters was frequently employed for closer scrutiny of the globules. In some instances photomicrographic reproductions were made to illustrate changes which occurred in the fats and triglycerides. The photomicrographs represented a magnification of 100 diameters and were obtained on cultures incubated for a period of 8 days at 21° C. The colors were reproduced from the original plates by tinting.

Lipolysis of butterfat and cottonseed oil: Figure 6 shows the lipolysis of butterfat and cottonseed oil by typical Penicillium cultures. Nile blue sulfate was added to the medium in the proportion of 1 part of dye to 15,000 parts agar.



BUTTERFAT



COTTONSEED

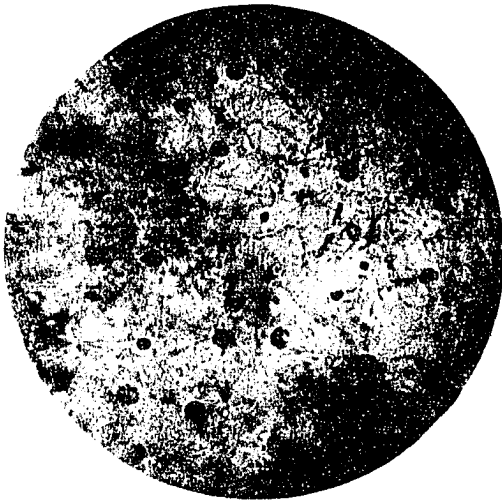
FIGURE 6. LIPOLYSIS OF BUTTERFAT AND COTTONSEED OIL BY A TYPICAL PENICILLIUM CULTURE

The unhydrolyzed butterfat was represented by the pink globules, while the blue globules were representative of the hydrolyzed fat. Intermediate stages of hydrolysis were indicated by various shades of the globules from peach red to the violet colors. Inspection of individual globules revealed the oily character of the pink unhydrolyzed fat as contrasted with the tenacious pellicle surrounding the globules which had undergone changes in color. When the pellicles were punctured an oil like fluid escaped from the globules. Examination of the medium, containing Nile blue sulfate, underneath the colonies revealed that it was practically devoid of color. The dissipation of color from the medium became more pronounced with the age of the cultures.

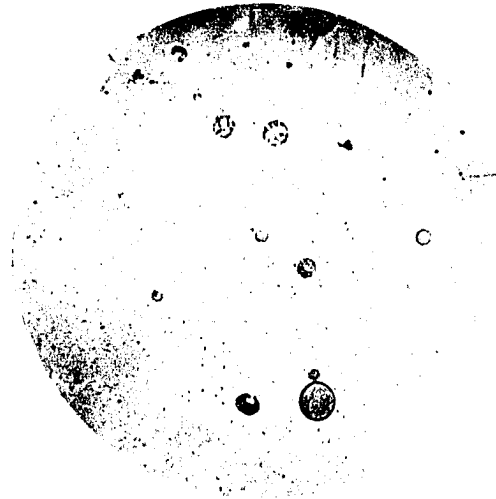
Cottonseed oil exhibited changes on hydrolysis quite similar to those observed with butterfat. The globules underwent a series of color changes from pink or red through various shades of purple and finally to blue. A tenacious pellicle was also observed with the hydrolyzed cottonseed oil globules.

Lipolysis of triglycerides: In the lipolysis of tripalmitin, tributyrin and triolein, hydrolysis was evidenced by the disappearance of the triglyceride globules; when this extended beyond the area of the mold colonies, it was evident by the formation of a clear area in the medium. Nile blue pink dye was readily absorbed by the lower triglycerides.

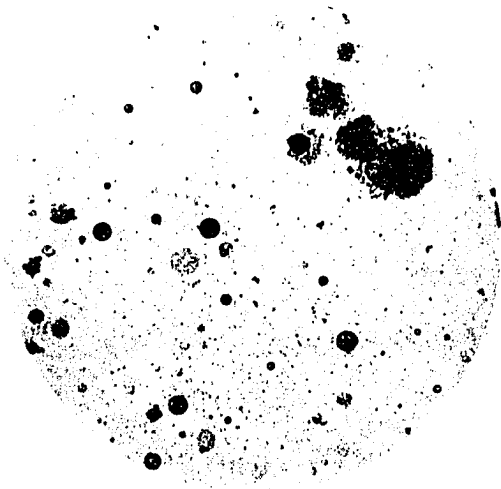
Figure 7 shows the lipolysis of tripalmitin, tripalmitin, tripalmitin and triolein by typical cultures with the Nile blue sulfate



TRICAPROIN



TRICAPRYLIN



TRICAPRIN



TRIOLEIN

FIGURE 7. LIPOLYSIS OF DIFFERENT TRIGLYCERIDES BY A TYPICAL PENICILLIUM CULTURE

technique.

Tricaproin showed unhydrolyzed globules of an intense pink hue; as hydrolysis progressed they changed to peach red, later to reddish purple, then to purple and to blue. The color changes were accompanied by the disintegration or solution of the globules, as indicated by their gradual disappearance.

With tricaprylin various stages of lipolysis were noted. The globules ranged in color from pink to shades of blue. Some of the blue globules were of concave and others of crater like appearance, indicative of the gradual disappearance of the products of hydrolysis.

In the lipolysis of tricaprin there was no indication of the disappearance of the products of hydrolysis. The blue spherical globules appeared to be characteristic of the early stages of hydrolysis, while later the material constituting the hydrolyzed globules assumed the form of plaques, as shown by the large masses of blue material.

The illustration showing lipolysis of triolein under the margin of a colony is typical with respect to the hydrolysis of this triglyceride. The unhydrolyzed globules were vividly red, while upon hydrolysis they became intensely blue.

Proteolytic Activities of Various Penicillia

In the study of the proteolytic activities of various penicillia, the extent of proteolysis was determined by measuring the diameter of the clear area formed in milk agar under and around a mold

colony. The relationship between the diameter of a colony and of its hydrolyzed area was designated by the mathematical expression $\frac{P}{C}$; P represents the diameter in centimeters of the hydrolyzed area and C the diameter of the colony. When the $\frac{P}{C}$ value was 1.00 the diameters of the colony and the hydrolyzed area were the same, and when it was less than 1.00 the area of the proteolyzed area was smaller than that of the colony. The intensity of proteolysis was estimated by observing the degree of transparency of the hydrolyzed area and was designated accordingly as negative, slight, moderate, pronounced or very pronounced.

The proteolytic activities of certain penicillia were studied with 14 cultures by means of the acidified milk agar and the carbon dioxide techniques. The results after 8 days at 21° C. are presented in table XV.

a. Results with acidified milk agar technique: The data show wide differences in proteolytic activities of the penicillia, as indicated by the relative $\frac{P}{C}$ values and the observations on the intensity of proteolysis. The maximum, minimum and average $\frac{P}{C}$ values were, respectively, 1.12, 0.00 and 0.65, while the proteolysis ranged from negative to very pronounced. Culture 6 showed very pronounced proteolysis and it had a $\frac{P}{C}$ value of 0.97; cultures 1, 5, 9, 15 and 25 were regarded as pronounced proteolytic and their $\frac{P}{C}$ values ranged from 0.78 to 1.12; cultures 10 and 41 were moderately proteolytic and their $\frac{P}{C}$ values were 0.79 and 0.77, respectively; cultures 7, 8, 14 and 27 were slightly proteolytic and their $\frac{P}{C}$ values varied from 0.11 to 0.89; while cultures 26 and 42 showed no evidence of proteolysis and

TABLE XV

PROTEOLYTIC ACTIVITIES OF CERTAIN PENICILLIA

Incubation 8 days at 21° C.

a. Results with acidified milk agar technique			
Culture no.	Diameter of colony cm.	$\frac{P}{G}$ value	Proteolysis
1	4.9	1.00	Pronounced
5	5.0	1.12	Pronounced
6	4.5	0.97	Very pronounced
7	4.2	0.41	Slight
8	4.3	0.21	Slight
9	5.1	1.11	Pronounced
10	4.7	0.79	Moderate
14	4.6	0.89	Slight
15	4.9	0.90	Pronounced
25	4.2	0.78	Pronounced
26	4.4	0.00	Negative
27	4.1	0.11	Slight
41	4.8	0.77	Moderate
42	4.0	0.00	Negative

b. Results with carbon dioxide technique			
Culture no.	Diameter of colony cm.	$\frac{P}{G}$ value	Proteolysis
1	4.9	1.00	Pronounced
5	5.0	1.11	Very pronounced
6	4.5	0.98	Moderate
7	4.2	1.02	Very pronounced
8	4.2	1.02	Pronounced
9	5.0	1.11	Very pronounced
10	4.5	0.94	Moderate
14	3.5	0.17	Slight
15	4.8	0.87	Pronounced
25	4.7	1.10	Pronounced
26	4.9	0.00	Negative
27	4.1	0.90	Moderate
41	4.8	0.81	Slight
42	4.5	0.91	Slight

their $\frac{P}{C}$ values were 0.00. Although culture 14 showed a relatively high $\frac{P}{C}$ value of 0.89, it was regarded as slightly proteolytic since the milk contained in the agar was not digested to the point where the medium was transparent but exhibited a cloudy effect suggestive of slight proteolytic activity.

b. Results with carbon dioxide technique: The data indicate wide differences in proteolytic activities of the penicillia. The maximum, minimum and average $\frac{P}{C}$ values were 1.11, 0.00 and 0.78, respectively and the intensity of proteolysis ranged from negative to very pronounced. Cultures 5, 7 and 9 showed very pronounced proteolysis and their $\frac{P}{C}$ values ranged from 1.02 to 1.11; cultures 1, 8, 15 and 25 showed pronounced proteolysis and their $\frac{P}{C}$ values ranged from 0.87 to 1.10; cultures 6, 7 and 27 were moderately proteolytic and their $\frac{P}{C}$ values varied from 0.90 to 0.98; cultures 14, 41 and 42 exhibited slight proteolytic activities with $\frac{P}{C}$ values ranging from 0.17 to 0.91; while culture 26 showed negative proteolysis.

Additional trials were made with 16 cultures to determine the proteolytic activities of the penicillia with the carbon dioxide technique. The results after 11 days at 21° C. are presented in table XVI.

There was marked variation in the proteolytic activities of the penicillia. Cultures 1, 5, 6, 7, 16, 18 and 21 were very pronounced proteolytic with $\frac{P}{C}$ values ranging from 1.01 to 1.20; culture 20 showed pronounced proteolysis with a $\frac{P}{C}$ value of 1.00; cultures 4, 8 and 10 showed moderate proteolysis and had $\frac{P}{C}$ values of 0.89,

TABLE XVI

THE PROTEOLYTIC ACTIVITIES OF CERTAIN PENICILLIA

(Carbon dioxide technique)

Incubation 11 days at 21° C.

Culture no.	Diameter of colony cm.	$\frac{P}{C}$ value	Proteolysis
1	6.3	1.13	Very pronounced
4	5.4	0.89	Moderate
5	4.4	1.20	Very pronounced
6	4.5	1.10	Very pronounced
7	5.3	1.02	Very pronounced
8	5.9	0.93	Moderate
10	4.7	1.10	Moderate
11	4.5	0.93	Slight
12	7.5	0.00	
15	7.4	1.00	Slight
16	5.4	1.01	Very pronounced
18	5.1	1.30	Very pronounced
20	5.6	1.00	Pronounced
21	6.9	1.04	Pronounced
22	6.7	1.00	Slight
24	4.5	0.88	Slight

0.93 and 1.10, respectively; while with culture 12 negative results were obtained, as indicated by a $\frac{P}{C}$ value of 0.00.

Some of the cultures studied were also considered in table XV. There was a general agreement between the results obtained with a given culture.

From the data presented in tables XV and XVI it is evident that there was considerable variation in the proteolytic activities of various penicillia on milk agar, as determined by the acidified milk agar and carbon dioxide techniques. Evidence of this is apparent in the $\frac{P}{C}$ values and intensity of proteolysis of the cultures. There was a general agreement between the results obtained with the two techniques; however, in a few instances they failed to concur. With both techniques the activities of the cultures ranged from nonproteolytic to very pronounced proteolysis.

Effect of Various Factors on Proteolytic Activities of Certain Penicillia

The effect of temperature, of carbon dioxide and of nitrogen on the proteolytic activities of certain penicillia was studied with seven cultures on Czapek's solution skim milk agar. The rates of growth and of proteolysis were investigated under the following growth conditions: (1) air at 28° C.; (2) air plus 10 per cent carbon dioxide at 28° C.; (3) carbon dioxide at 28° C.; (4) nitrogen at 28° C.; (5) air at 19° C. and at 12° C. The growth and proteolysis curves were plotted in a manner similar to that employed in plotting

the lipolysis curves in figures 1 and 2. The results are presented in figure 8.

Since no growth occurred with culture 25 when grown in air at 28° C., no data were obtained for plotting. The remaining cultures showed slow growth in air at 28° C., as indicated by the gradual increase in the slopes of the curves. Cultures 12, 15 and 18 showed proteolysis curves which preceded but were almost parallel to the growth curves, while no proteolysis was noticeable with the remaining cultures.

When the cultures were incubated in an atmosphere which consisted of air plus 10 per cent carbon dioxide gas at 28° C., growth occurred slowly, as shown by the slight rise of the curves. Marked differences were noted in rates of proteolysis with the seven cultures, as indicated by the relative slopes of the curves. Cultures 12, 15 and 18 showed pronounced proteolysis, cultures 16 and 25 were slowly and slightly proteolytic, whereas cultures 14 and 20 showed no indication of proteolysis.

When the penicillia were grown in an atmosphere of carbon dioxide at 28° C., only a negligible amount of growth occurred with cultures 12, 14, 15, 18, 20 and 25, while no growth was noticeable with culture 16.

The growth of the penicillia incubated in an atmosphere of nitrogen at 28° C. proceeded slowly, as indicated by the slowly ascending growth curves. Proteolysis was pronounced with cultures 16 and 18 while none was evident with 12, 20 and 25. Cultures 12, 14 and 20 were

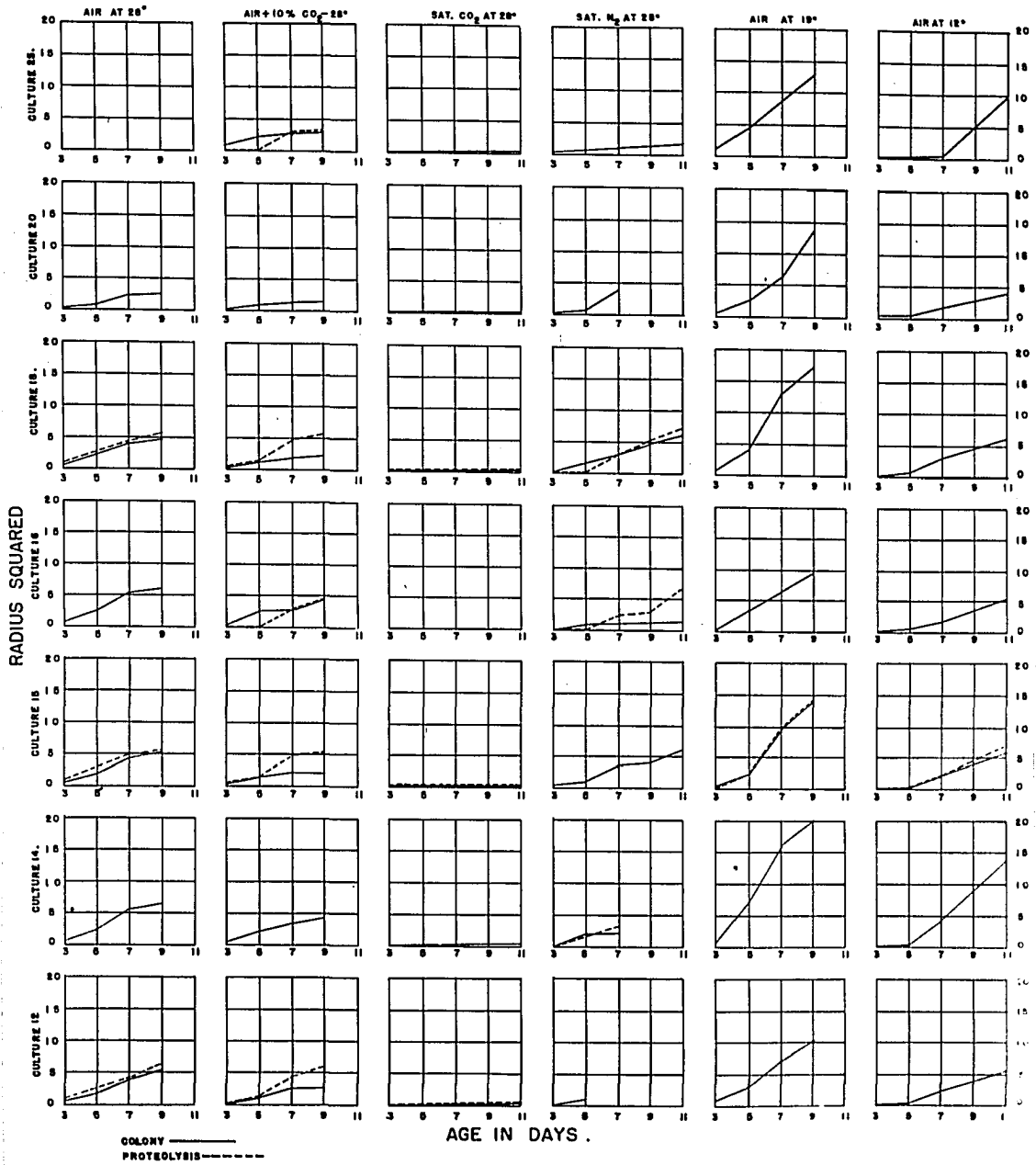


FIGURE 8. EFFECT OF VARIOUS FACTORS ON PROTEOLYTIC ACTIVITIES OF CERTAIN PENICILLIA

contaminated with other molds after 5 and 7 days and consequently the tests were discontinued.

When the cultures were incubated in air at 19° C., the growth was comparatively rapid as shown by the corresponding curves. However, proteolysis was noticeable to a slight extent with only one culture.

The growth of penicillia in air at 12° C. was somewhat retarded, when compared with the development of the same cultures at 19° C. With exception of culture 15, none of the seven cultures showed evidence of proteolytic activity at 12° C.

From the data presented in figure 8 it is apparent that the rates of growth and of proteolysis of various penicillia are affected by different growth conditions. The cultures grew more slowly but showed greater proteolytic activities in air at 28° C. than at 19° or 12° C.; they were somewhat retarded in growth but proteolysis was unaffected when grown at 28° C. in an atmosphere in which 10 per cent of the air had been replaced by carbon dioxide; culture growth and proteolysis at 28° C. were almost stopped in an atmosphere which consisted almost entirely of carbon dioxide; growth was usually unaffected but proteolysis was slightly accelerated at 28° C. in an atmosphere which consisted almost completely of nitrogen.

Effect of Type of Inoculating Material on Mold Growth in Cheese

In the studies on the effect of the type of inoculating

material on the mold growth in ripened cheese, agar slope, agar plate and dry bread cultures of the molds were employed.

Inoculation of curd with agar slope cultures

The effect of inoculation of curd with the spores from agar slopes on the mold development in blue cheese was studied with 17 Penicillium. Cultures were grown on Czapek's solution agar slopes in large test tubes (25 mm. in diameter x 200 mm. in length). After 10 days at 21° C., aqueous suspensions of the mold spores were prepared by flooding the slopes with sterile water and scraping the spores from the surfaces by means of an inoculating needle. When the curd had drained sufficiently on the draining cloth, it was sprinkled with a freshly prepared spore suspension; distribution of the spores was attained by thoroughly mixing the mass while the mold suspension was being added. After 8 weeks ripening each cheese was examined with respect to predominant mold types. A sample for plating was obtained from a wedge cut from the cheese. The cut surface was scraped with a sterile spatula and from different parts of the new surface small portions of cheese containing mold growth were collected and placed in a sterile water blank which contained 0.1 per cent sodium citrate. After the cheese had been thoroughly emulsified by triturating with a sterile glass rod, a loopful of the emulsion was transferred to a test tube which contained approximately 15 ml. melted agar at 45° C. The tube was rolled between the hands to obtain uniform distribution of the inoculant throughout the medium, after which the mixture was poured

into a petri dish. The plates were incubated at room temperature. When the newly formed molds became visible, representative colonies were picked from different parts of the plate to agar slopes. Each culture was subsequently reisolated twice to insure its purity. A wide field, low power binocular facilitated the selection of individual mold colonies for successive isolations. The isolated cultures were compared with those used in the original cheese inoculations. When the cultures isolated from the cheese were not identical with the inoculated cultures but still were recognized as typical strains of P. roqueforti, they were regarded as contaminants. The results are given in table XVII.

The inoculation of curd with spores from agar slopes was unreliable in establishing the cultures in cheese. In a total of 46 cheese the inoculated molds were predominant in 15, present in 2 and absent in 29 after a ripening period of 8 weeks. Cultures 1, 6, 11, 13 and 15 were predominant in two of three inoculations; culture 14 was predominant in the one cheese in which it was used; cultures 12, 21 and 23 were present in one of three inoculations; culture 18 was present in one of two inoculations; and cultures 3, 8, 9, 10, 16 and 20 were absent in all trials (according to the technique used).

Although no attempt was made to identify the contaminating mold cultures isolated from the cheese, observations indicated striking similarity of some of the cultures to P. roqueforti, whereas others did not belong to this group of penicillia, as indicated by their morphological characteristics. Various types of contaminating molds were

TABLE XVII

EFFECT OF INOCULATION OF CURD WITH AGAR SLOPE CULTURES
ON MOLD DEVELOPMENT IN BLUE CHEESE

Ripened 8 weeks

Culture no.	Types of molds developed in ripened cheese			Remarks on contaminating molds in cheese
	Trial no.	Inoculated molds were	Contaminating molds were	
1	1	Absent	Pred.*	Appeared like typical <u>P. roqueforti</u>
	4	Pred.	Absent	
	6	Pred.	Absent	
3	1	Absent	Pred.	Appeared like typical <u>P. roqueforti</u> Appeared like typical <u>P. roqueforti</u> Two types; one typical <u>P. roqueforti</u> ; one strong moldy odor, restricted growth, drops on surface
	4	Absent	Pred.	
	1	Absent	Pred.	
5	4	Present	Pred.	Strong moldy odor, restricted growth, drops on surface
	6	Pred.	Absent	
6	1	Absent	Pred.	Two types; one typical <u>P. roqueforti</u> ; one strong moldy odor, restricted growth, drops on surface Strong moldy odor, restricted growth, drops on surface
	4	Pred.	Present	
	6	Pred.	Absent	
8	1	Absent	Pred.	Two types; one typical <u>P. roqueforti</u> ; one strong moldy odor, restricted growth, drops on surface Typical <u>P. roqueforti</u> type Strong moldy odor, restricted growth, drops on surface
	4	Absent	Pred.	
	6	Absent	Pred.	
9	1	Absent	Pred.	Strong moldy odor, restricted growth, drops on surface Strong moldy odor, restricted growth, drops on surface
	4	Absent	Pred.	
	6	Absent	Pred.	Strong moldy odor, restricted growth, drops on surface

Table continued on page 107.

Table XVII continued.

	1	Absent	Pred.	Strong moldy odor, restricted growth, drops on surface
10	4	Absent	Pred.	Strong moldy odor, restricted growth, drops on surface
	6	Absent	Pred.	Strong moldy odor, restricted growth, drops on surface
	1	Absent	Pred.	Strong moldy odor, restricted growth, drops on surface
11	4	Pred.	Present	Two types; one typical <u>P. roqueforti</u> ; one strong moldy odor
	6	Pred.	Absent	
	1	Absent	Pred.	Typical <u>P. roqueforti</u>
12	3	Absent	Pred.	Typical <u>P. roqueforti</u>
	5	Pred.	Present	Two types; one typical <u>P. roqueforti</u> ; one strong moldy odor
	2	Absent	Pred.	Typical <u>P. roqueforti</u>
13	3	Pred.	Absent	
	5	Pred.	Absent	
14	5	Pred.	Absent	
	2	Absent	Pred.	Typical <u>P. roqueforti</u>
15	3	Pred.	Absent	
	5	Pred.	Absent	
	2	Absent	Pred.	Two types; one typical <u>P. roqueforti</u> ; one strong moldy odor
16	3	Absent	Pred.	Two types; one typical <u>P. roqueforti</u> ; one strong moldy odor
	3	Absent	Pred.	Two types; one typical <u>P. roqueforti</u> ; one strong moldy odor
18	3	Absent	Pred.	Two types; one typical <u>P. roqueforti</u> ; one strong moldy odor
	5	Pred.	Absent	
	2	Absent	Pred.	Typical <u>P. roqueforti</u>
20	3	Absent	Pred.	Two types; one typical <u>P. roqueforti</u> ; one strong moldy odor
	5	Absent	Pred.	Two types; one typical <u>P. roqueforti</u> ; one strong moldy odor
	2	Absent	Pred.	Typical <u>P. roqueforti</u>
21	3	Absent	Pred.	Strong moldy odor, restricted growth, drops on surface
	5	Pred.	Absent	
	2	Absent	Pred.	Typical <u>P. roqueforti</u>
23	3	Present	Pred.	Strong moldy odor, restricted growth, drops on surface
	5	Absent	Pred.	Strong moldy odor, restricted growth, drops on surface

*Pred. means predominant.

noted but the most prevalent was a Penicillium which formed a colony with a compact base, restricted margin, and possessed a strong moldy odor; this, or apparently related types, had drops of transpired fluid on the surface of the colonies, which ranged from colorless to faint violet shades.

From the data presented in table XVII it is evident that the inoculation of curd with spores from agar slopes was unreliable in establishing the cultures in blue cheese. The inoculated cultures were absent according to the technique used in a majority of the cheese after 8 weeks ripening.

Inoculation of curd with agar plate cultures

The effect of inoculation of the curd with spore suspensions from agar plate cultures of certain penicillia on the molds developing in blue cheese was studied with 19 cultures. With the exception that the cultures were grown on agar plates, the general method was similar to that used with the agar slope cultures already discussed. The results after 16 weeks are presented in table XVIII.

The data show that the inoculation of curd with spores from agar plate cultures of penicillia was unreliable in the establishment of inoculated mold in the cheese. From a total of 31 cheese, the inoculated penicillia were predominant in seven, present in five and absent in 19 after a ripening period of 16 weeks. Cultures 1 and 10 were predominant in all cheese inoculated with them; culture 6 was predominant in only one of three inoculations; cultures 7, 8 and 15 were each predominant in one and absent in one cheese; culture 25

TABLE XVIII

EFFECT OF INOCULATION OF CURD WITH SPORES FROM AGAR PLATE CULTURES
ON MOLD DEVELOPMENT IN BLUE CHEESE

Ripened 16 weeks

Types of molds developed in ripened cheese				
Culture no.	Trial no.	Inoculated molds were	Contaminating molds were	Remarks on contaminating molds in cheese
1	7	Pred.	Absent	
	10	Pred.	Absent	
5	7	Absent	Pred.	Appeared like typical <u>P. roqueforti</u>
	8	Absent	Pred.	Two types; one typical <u>P. roqueforti</u> ; one atypical <u>Penicillium</u>
6	7	Absent	Pred.	Appeared like typical <u>P. roqueforti</u>
	8	Pred.	Present	Appeared like typical <u>P. roqueforti</u>
	10	Absent	Pred.	Appeared like typical <u>P. roqueforti</u>
7	7	Absent	Pred.	Appeared like typical <u>P. roqueforti</u>
	8	Pred.	Present	Appeared like typical <u>P. roqueforti</u>
8	8	Pred.	Absent	
8	10	Absent	Pred.	Appeared like typical <u>P. roqueforti</u>
9	10	Absent	Pred.	Strong, moldy odor, colony restricted in growth, drops on surface
10	10	Pred.	Present	On Czapek's solution agar, white, broadly spreading <u>Penicillium</u>
11	10	Absent	Pred.	Appeared like typical <u>P. roqueforti</u>
12	7	Absent	Pred.	Appeared like typical <u>P. roqueforti</u>
13	9	Absent	Pred.	Strong, moldy odor, colony restricted in growth, drops on surface
14	7	Absent	Pred.	Appeared like typical <u>P. roqueforti</u>
	8	Absent	Pred.	Appeared like typical <u>P. roqueforti</u>
15	7	Absent	Pred.	Appeared like typical <u>P. roqueforti</u>
	9	Pred.	Absent	
16	7	Absent	Pred.	Appeared like typical <u>P. roqueforti</u>
	9	Absent	Pred.	Appeared like typical <u>P. roqueforti</u>

Table continued on page 110.

Table XVIII continued.

18	9	Absent	Pred.	Appeared like typical <u>P. roqueforti</u>
20	9	Absent	Pred.	Appeared like typical <u>P. roqueforti</u>
21	8	Present	Present	Appeared like typical <u>P. roqueforti</u>
22	9	Present	Present	Three types of penicillia isolated
	8	Present	Present	Appeared like typical <u>P. roqueforti</u>
24	9	Absent	Pred.	Two distinct species of penicillia isolated
	8	Present	Present	Appeared like typical <u>P. roqueforti</u>
25	9	Present	Present	Appeared like typical <u>P. roqueforti</u>
	10	Absent	Pred.	Appeared like typical <u>P. roqueforti</u>

was present in two cheese and absent in one; cultures 21 and 22 were present in all cheese inoculated with them; and cultures 5, 9, 11, 12, 13, 14, 16, 18 and 20 were absent according to the technique used in all cheese inoculated with them.

From the data presented in table XVIII it is evident that the inoculation of curd with spores from agar plate cultures of penicillia was unreliable in establishing the inoculated mold in cheese. The inoculated cultures were absent in most of the cheese after 16 weeks ripening.

Inoculation of curd with dry bread cultures

The effect of inoculation of curd with dry bread cultures of certain penicillia on the molds developing in blue cheese was studied with 12 cultures in 24 trials. The mold powder was prepared and inoculations were made in accordance with the procedure described under general methods. Table XIX presents the results.

Inoculation of the curd with bread cultures of certain penicillia was effective in implantation of the molds in blue cheese, as shown by the types of molds present in the cheese after a ripening period of 8 weeks. In the 24 cheese the inoculated cultures were predominant in all instances. In 17 cheese only the predominant penicillia were found, while in 7 contaminating types of penicillia were noted. The contaminating molds produced strong moldy odors and the colonies on Czapek's solution agar were restricted in growth, zonate and drops of transpired fluid formed on their surfaces.

TABLE XIX

EFFECT OF INOCULATION OF CURD WITH BREAD MOLD CULTURES
ON MOLD DEVELOPMENT IN BLUE CHEESE

Ripened 8 weeks

Types of molds developed in ripened cheese				
Culture no.	Trial no.	Inoculated molds were	Contaminating molds were	Remarks on contaminating molds in cheese
5	11	Pred.	Absent	
	12	Pred.	Absent	
6	11	Pred.	Absent	
	12	Pred.	Absent	
8	11	Pred.	Absent	
	12	Pred.	Absent	
9	11	Pred.	Present	Atypical <u>Penicillium</u> , colony on Czapek's solution agar, white, broadly spreading
	12	Pred.	Absent	
	11	Pred.	Absent	
14	12	Pred.	Present	Strong, moldy odor, colony restricted, zonate, drops abundant
	11	Pred.	Absent	
15	12	Pred.	Present	Strong, moldy odor, colony restricted, zonate drops abundant
	11	Pred.	Absent	
16	12	Pred.	Present	Strong, moldy odor, colony restricted, zonate, drops abundant
	11	Pred.	Present	
25	12	Pred.	Present	Strong, moldy odor, colony restricted, zonate, drops abundant
	11	Pred.	Absent	
26	12	Pred.	Present	Strong, moldy odor, colony restricted, zonate, drops abundant
	11	Pred.	Absent	
26b	12	Pred.	Absent	
	11	Pred.	Absent	
27	12	Pred.	Absent	
	11	Pred.	Absent	
41	11	Pred.	Absent	
	12	Pred.	Absent	

The data given in table XIX show that when the curd was inoculated with bread mold cultures, the penicillia were successfully established in the cheese, the inoculated molds being predominant in the ripened cheese in all instances.

Growth of Certain Penicillia in Cheese During Ripening

When the cheese considered under table XIX had been scored the second time, freshly cut surfaces of a number of them were photographed to demonstrate the extent of mold growth with certain inoculated strains of penicillia. The results are presented in figures 9 to 16.

Effect of Lipolytic and Proteolytic Activities of Various Penicillia on Ripening of Blue Cheese

In studying the effect of the lipolytic and proteolytic activities on the ripening of blue cheese, only those cheese were considered in which the inoculated molds predominated after ripening. The penicillia were classified on the basis of their general lipolytic activities into slightly, moderately and pronounced lipolytic molds, as tested on agar plates which contained dispersed natural fats or simple triglycerides. A similar classification of the penicillia was made on the basis of their proteolytic activities into slightly, moderately and pronounced proteolytic molds, the proteolytic activities of the cultures being determined with the carbon dioxide technique.



FIGURE 9. BLUE CHEESE RIPENED BY CULTURE 5 SHOWING GOOD MOLD GROWTH

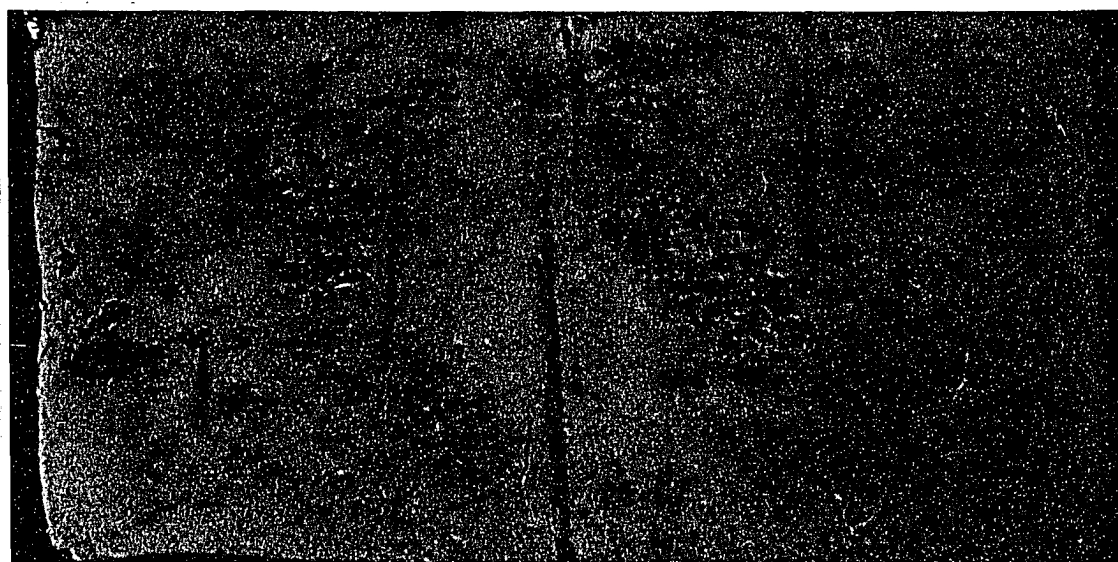


FIGURE 10. BLUE CHEESE RIPENED BY CULTURE 6 SHOWING GOOD MOLD GROWTH

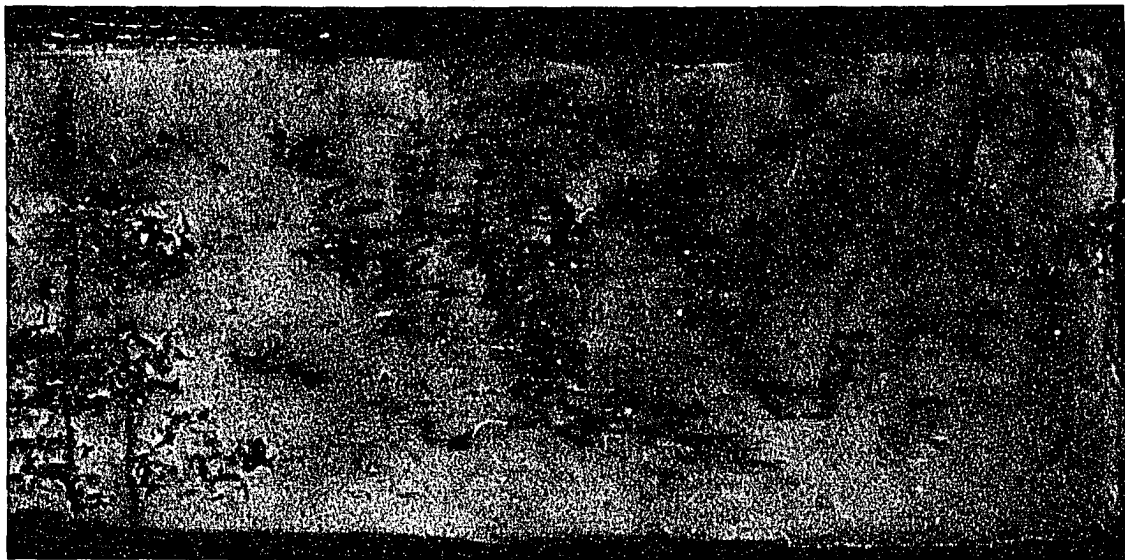


FIGURE 11. BLUE CHEESE RIPENED WITH CULTURE 9 SHOWING GOOD MOLD GROWTH



FIGURE 12. BLUE CHEESE RIPENED WITH CULTURE 14 SHOWING GOOD MOLD GROWTH



FIGURE 13. BLUE CHEESE RIPENED BY CULTURE 15 SHOWING GOOD MOLD GROWTH



FIGURE 14. BLUE CHEESE RIPENED BY CULTURE 16 SHOWING POOR MOLD GROWTH

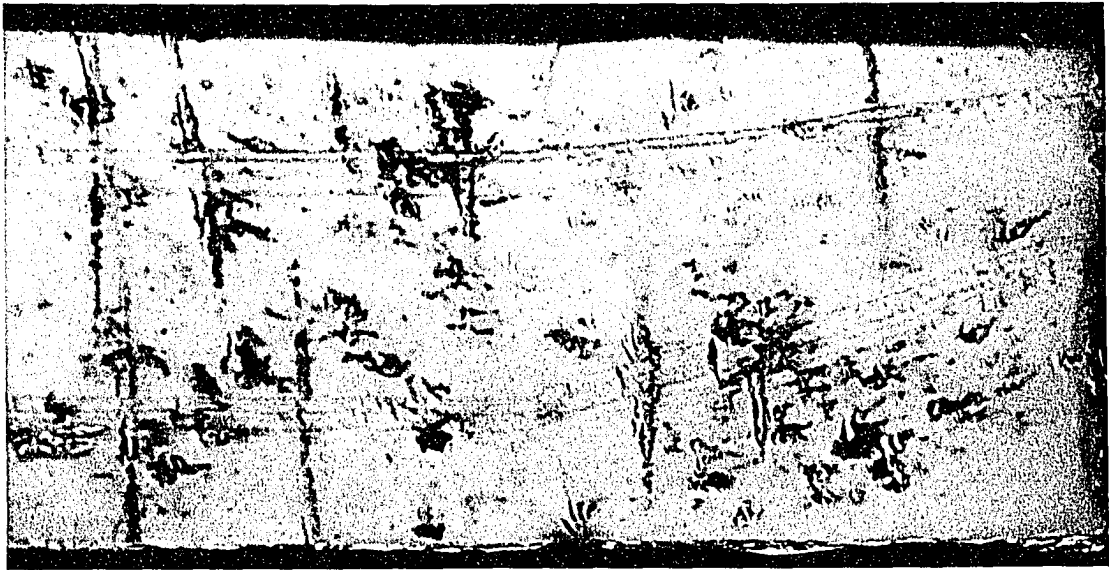


FIGURE 15. BLUE CHEESE RIPENED BY CULTURE 25 SHOWING GOOD MOLD GROWTH



FIGURE 16. BLUE CHEESE RIPENED BY CULTURE 27 SHOWING FAIR MOLD GROWTH

In some trials no moderately proteolytic cultures were used in preparing the cheese.

The effect of the lipolytic and proteolytic activities of various penicillia on the ripening of blue cheese was studied in three groups of trials. In the first group, 18 cheese inoculated with the spores of 11 cultures from agar slopes were examined after 8 and 12 weeks; in the second group 16 cheese inoculated with the spores of 11 cultures from agar plates were inspected after 16 and 48 weeks; while in the third group 24 cheese inoculated with 12 bread cultures were inspected after 8 and 18 weeks. The results are presented in table XX.

Cheese inoculated with agar slope cultures and inspected after 8 and 12 weeks

After 8 weeks the eight cheese inoculated with penicillia which were slightly lipolytic and proteolytic showed good mold growth in three instances, fair in three and poor in two; three cheese inoculated with cultures that were moderately lipolytic and pronounced proteolytic showed good growth in two instances, fair in none and poor in one; while seven cheese inoculated with pronounced lipolytic and proteolytic molds exhibited good growth in five instances, fair in two and poor in none. Cheese ripened with slightly lipolytic and proteolytic penicillia showed better flavor development than those ripened by the more active molds. Flavor development in eight cheese ripened with the former penicillia was good in three cases, fair in one and poor in four; three cheese ripened by molds which were moderately lipolytic

TABLE XI

EFFECT OF LIPOLYTIC AND PROTEOLYTIC ACTIVITIES OF VARIOUS
ON THE RIPENING OF BLUE CHEESE

Culture no.	Identification of culture	Lipolytic activity of culture	Proteolytic activity of culture	Cheese lot no.	Mold growth in cheese	Remarks on ripening of cheese
a. Results, cheese inoculated with agar slope culture to 8 weeks						
1	<u>P. roqueforti</u>	Moderate	Pronounced	4a	Good	Fair, bitter
				6a	Good	Poor, bitter
5	<u>P. roqueforti</u>	Pronounced	Pronounced	4c	Good	Fair, very bitter
				6c	Good	Poor, very bitter
6	<u>P. roqueforti</u>	Pronounced	Pronounced	4d	Good	Poor, bitter, unclean
				6d	Good	Fair, bitter
11	<u>P. gorgonzola</u>	Slight	Slight	4h	Good	Fair, musty
				6h	Fair	Poor, fermented
12	<u>P. gorgonzola</u> , Biourge	Slight	Slight	3a	Poor	Good, slightly unclean
				5a	Poor	Poor, musty
13	<u>P. gorgonzola</u> type	Slight	Slight	3b	Fair	Poor, very unclean
14	<u>P. stilton</u> , Biourge	Slight	Slight	5b	Good	Poor, very unclean
15	<u>P. roqueforti</u> , Thom	Pronounced	Pronounced	3c	Fair	Fair, unclean, h
				5d	Fair	Poor, very unclean
18	<u>P. chrysogenum</u>	Moderate	Pronounced	5e	Poor	Poor, bitter, unclean
21	<u>P. roqueforti</u>	Pronounced	Pronounced	5g	Good	Poor, fermented, unclean
23	Unidentified <u>Penicillium</u>	Slight	Slight	3a	Fair	Good, slightly musty
b. Results, cheese inoculated with agar plate culture to 16 weeks						
1	<u>P. roqueforti</u>	Moderate	Pronounced	7a	Good	Fair, green, bitter
				10a	Fair	Fair, green
5	<u>P. roqueforti</u>	Pronounced	Pronounced	7b	Good	Fair, green, bitter
				8a	Poor	Fair
6	<u>P. roqueforti</u>	Pronounced	Pronounced	7c	Poor	Fair
				8b	Poor	Fair, green
7	<u>P. roqueforti</u>	Slight	Pronounced	7d	Poor	Poor, fruity, unclean
				8c	Fair	Fair
8	<u>P. roqueforti</u>	Pronounced	Pronounced	8d	Fair	Good
10	<u>P. roqueforti</u>	Moderate	Moderate	10c	Fair	Fair, green
15	<u>P. roqueforti</u> , Thom	Pronounced	Pronounced	9b	Poor	Poor, green, bitter
21	<u>P. roqueforti</u>	Pronounced	Pronounced	8f	Fair	Poor, unclean, bitter
22	<u>P. roqueforti</u>	Slight	Slight	9f	Poor	Fair, green
24	<u>P. roqueforti</u>	Slight	Slight	8g	Fair	Fair
				8h	Good	Good
25	<u>P. roqueforti</u>	Pronounced	Pronounced	9h	Good	Fair, green

Table continued on page 120.

TABLE XI

AND PROTEOLYTIC ACTIVITIES OF VARIOUS PENICILLIA
IN THE RIPENING OF BLUE CHEESE

Culture	Cheese lot no.	Mold growth in cheese	Remarks on flavor of cheese	Mold growth in cheese	Remarks on flavor of cheese
Inoculated with agar slope culture technique after:					
			8 weeks	12 weeks	
iced	4a	Good	Fair, bitter	Good	Fair, butyric
	6a	Good	Poor, bitter	Good	Fair, green
iced	4c	Good	Fair, very bitter	Good	Good
	6c	Good	Poor, very bitter	Good	Fair, unclean
iced	4d	Good	Poor, bitter, unnatural	Good	Good
	6d	Good	Fair, bitter	Fair	Fair, green
	4h	Good	Fair, musty	Good	Fair, musty
	6h	Fair	Poor, fermented	Fair	Poor, very musty
	3a	Poor	Good, slightly unclean	Poor	Poor, musty
	5a	Poor	Poor, musty	Poor	Poor, musty
	3b	Fair	Poor, very unclean, unnatural	Good	Good
	5b	Good	Poor, very unclean	Fair	Fair
	5c	Good	Good	Good	Good, butyric
iced	3c	Fair	Fair, unclean, high flavor	Fair	Good
iced	5d	Fair	Poor, very unclean	Fair	Poor
iced	5e	Poor	Poor, bitter, unnatural	Poor	Fair
iced	5g	Good	Poor, fermented, bitter	Good	Good
	3a	Fair	Good, slightly musty	Fair	Fair, slightly unclean
Inoculated with agar plate culture technique after:					
			16 weeks	48 weeks	
iced	7a	Good	Fair, green, bitter	Good	Fair, musty, raw
	10a	Fair	Fair, green	Good	Very good
iced	7b	Good	Fair, green, bitter	Good	Good, suggestion of butyric
	8a	Poor	Fair	Good	Good, slightly fruity
iced	7c	Poor	Fair	Good	Good
	8b	Poor	Fair, green	Fair	Good
iced	7d	Poor	Poor, fruity, unclean	Good	Good
	8c	Fair	Fair	Good	Fair, fruity
iced	8d	Fair	Good	Good	Fair
te	10c	Fair	Fair, green	Good	Good
iced	9b	Poor	Poor, green, bitter	Good	Fair, raw, bitter, musty
iced	8f	Fair	Poor, unclean, bitter	Good	Very poor, raw, butyric
	9f	Poor	Fair, green	Good	Fair
	8g	Fair	Fair	Good	Poor, fruity
iced	8h	Good	Good	Good	Good
	9h	Good	Fair, green	Good	Very good

Table XX continued.

		c. Results, cheese inoculated with bread culture technique					
						8 weeks	
5	<u>P. roqueforti</u>	Pronounced	Pronounced	1-1	Good	Good	
				1-2	Good	Good	
6	<u>P. roqueforti</u>	Pronounced	Pronounced	2-1	Good	Good	
				2-2	Good	Fair,	bitter, butyri
8	<u>P. roqueforti</u>	Pronounced	Pronounced	3-1	Good	Good	
				3-2	Good	Fair	
9	<u>P. roqueforti</u>	Pronounced	Pronounced	4-1	Good	Very	good
				4-2	Good	Fair,	musty, butyri
14	<u>P. stilton</u> , Biourge	Slight	Slight	5-1	Good	Fair	
				5-2	Good	Fair,	musty
15	<u>P. roqueforti</u> , Thom	Pronounced	Pronounced	6-1	Good	Good	
				6-2	Good	Fair,	musty
16	<u>P. gorgonzola</u>	Slight	Slight	7-1	Poor	Very	poor
				7-2	Poor	Fair,	slightly mu
25	<u>P. roqueforti</u>	Pronounced	Pronounced	8-1	Fair	Good	
				8-2	Good	Fair,	musty, green
26	<u>P. roqueforti</u>	Slight	Slight	9-1	Fair	Fair,	musty, slight
				9-2	Good	Good	
26b	<u>P. roqueforti</u>	Slight	Slight	10-1	Poor	Poor,	very butyri
				10-2	Good	Good	
27	<u>P. roqueforti</u>	Slight	Slight	11-1	Poor	Poor,	very butyri
				11-2	Good	Good	
41	<u>P. roqueforti</u>	Pronounced	Pronounced	12-1	Fair	Good	
				12-2	Good	Good	

se inoculated with bread culture technique after:

		8 weeks		18 weeks	
	1-1	Good	Good	Good	Fair, slightly butyric
ced	1-2	Good	Good	Good	Good
	2-1	Good	Good	Good	Good, slightly butyric
ced	2-2	Good	Fair, bitter, butyric	Good	Good
	3-1	Good	Good	Good	Fair, musty
ced	3-2	Good	Fair	Good	Fair
	4-1	Good	Very good	Good	Fair, slightly butyric
ced	4-2	Good	Fair, musty, butyric	Good	Fair
	5-1	Good	Fair	Good	Good
	5-2	Good	Fair, musty	Poor	Fair
	6-1	Good	Good	Good	Poor, butyric
ced	6-2	Good	Fair, musty	Good	Poor, butyric, musty
	7-1	Poor	Very poor	Poor	Fair
	7-2	Poor	Fair, slightly musty	Good	Fair
	8-1	Fair	Good	Good	Good
ced	8-2	Good	Fair, musty, green, bitter	Good	Good
	9-1	Fair	Fair, musty, slightly bitter	Good	Fair, slightly butyric
	9-2	Good	Good	Good	Fair
	10-1	Poor	Poor, very butyric	Good	Fair
	10-2	Good	Good	Good	Fair, musty
	11-1	Poor	Poor, very butyric	Good	Fair
	11-2	Good	Good	Fair	Fair, slightly musty
	12-1	Fair	Good	Good	Good
ced	12-2	Good	Good	Good	Good

and pronounced proteolytic exhibited good flavor development in none of the trials; while the flavor was fair in one and poor in two; seven cheese ripened by pronounced lipolytic and proteolytic molds showed good flavor development in none of the trials, fair in three and poor in four.

Nine cheese in the group were inoculated with five cultures of P. roqueforti and these showed good mold development in seven instances, fair in two and poor in none; one cheese inoculated with P. chrysogenum showed poor growth; six cheese inoculated with three cultures of P. gorgonzola exhibited good growth in two instances, fair in two and poor in two instances; one cheese ripened by P. stilton showed good growth; and one cheese inoculated with an unidentified Penicillium showed fair mold development. The cheese ripened by P. roqueforti showed good flavor development in none, fair in five and poor in five; one cheese ripened by P. chrysogenum showed poor flavor development; six cheese ripened by P. gorgonzola possessed good flavor in one, fair in one and poor in four; one cheese ripened by P. stilton showed good flavor development; and one cheese ripened with an unidentified Penicillium showed good flavor development.

After a ripening period of 12 weeks the mold growth in eight cheese inoculated with slightly lipolytic and proteolytic penicillia was good in three instances, fair in one and poor in four; three cheese inoculated with cultures which were moderately lipolytic and pronounced proteolytic exhibited good mold growth in two instances, fair in none and poor in one; when pronounced lipolytic and proteolytic

penicillia were inoculated, the cheese showed good mold growth in four instances, fair in three and poor in none. Flavor development in the cheese ripened by the pronounced lipolytic and proteolytic and the moderately lipolytic and pronounced proteolytic penicillia was better than in cheese ripened with slightly lipolytic and proteolytic penicillia. The flavor development in eight cheese ripened by slightly lipolytic and proteolytic penicillia was good in two instances, fair in three and poor in three; all three cheese ripened by cultures which were moderately lipolytic and pronounced proteolytic showed fair flavor development; while cheese ripened with pronounced lipolytic and proteolytic molds were good in four cases, fair in two and poor in one instance.

Of the nine cheese inoculated with P. roqueforti, six showed good growth and three exhibited fair growth; one cheese inoculated with P. chrysogenum showed poor growth; two of the six cheese inoculated with P. gorgonzola showed good growth, two exhibited fair growth and two showed poor growth; one cheese inoculated with P. stilton showed good growth; and one cheese inoculated with an unidentified Penicillium exhibited fair growth. The cheese ripened with P. roqueforti showed good flavor development in four instances, fair in four and poor in one; one cheese ripened with P. chrysogenum showed fair flavor; six cheese ripened by P. gorgonzola exhibited good flavor development in one, fair in two and poor in three; one cheese ripened by a culture of P. stilton showed good flavor; while one cheese ripened by an unidentified Penicillium showed fair flavor development.

Cheese inoculated with agar plate cultures and inspected after 16 and
13 weeks

After 16 weeks ripening the mold growth was fair in one and poor in one of the two cheese ripened by slightly lipolytic and proteolytic penicillia; one cheese inoculated with a moderately lipolytic and proteolytic culture showed fair growth; two cheese inoculated with a culture which was slightly lipolytic and proteolytic exhibited fair growth in one and poor in one; three cheese inoculated with a culture that was moderately lipolytic and pronounced proteolytic showed good growth in two and poor in one; while nine cheese inoculated with pronounced lipolytic and proteolytic penicillia exhibited good growth in three, fair in two and poor in four. Cheese ripened with pronounced lipolytic and proteolytic penicillia showed somewhat better flavor development after 16 weeks ripening than those ripened with the less active molds. Two cheese ripened with slightly lipolytic and proteolytic penicillia showed fair flavor development; one cheese ripened with a moderately lipolytic and proteolytic mold had a fair flavor; of two cheese ripened by slightly lipolytic and proteolytic cultures one showed fair and one poor flavor development; three cheese ripened by a moderately lipolytic and pronounced proteolytic mold showed fair flavor development in one and poor in two; while nine cheese ripened with pronounced lipolytic and proteolytic cultures exhibited good flavor development in two, fair in five and poor in two instances.

The cheese were all inoculated with cultures of

P. roqueforti. Of the 16 cheese in the group, four showed good mold growth, six fair and six poor. The flavor of the cheese was good in two instances, fair in 11 and in three it was poor.

After 48 weeks ripening two cheese inoculated with slightly lipolytic and proteolytic penicillia showed good mold growth; one cheese inoculated with a moderately lipolytic and proteolytic culture showed good growth; two cheese inoculated with a slightly lipolytic and pronounced proteolytic mold exhibited good growth; three cheese inoculated with molds which were moderately lipolytic and pronounced proteolytic showed good growth; and nine cheese inoculated with pronounced lipolytic and proteolytic penicillia showed good growth in eight, fair in one and poor in none.

The flavor of cheese ripened with pronounced lipolytic and proteolytic penicillia was usually better than that of cheese ripened with the less active molds. Two cheese ripened by slightly lipolytic and proteolytic penicillia showed good flavor development in none, fair in one and poor in one; one cheese ripened by a moderately lipolytic and proteolytic culture possessed a good flavor; two cheese ripened with a slightly lipolytic and pronounced proteolytic mold showed good flavor in one and fair in one; two cheese inoculated with a moderately lipolytic and pronounced proteolytic culture showed good flavor in one and fair in one; while nine cheese ripened by pronounced lipolytic and proteolytic penicillia exhibited good flavor development in six cases, fair in two and poor in one instance.

Of the 16 cheese inoculated with 11 cultures of

P. roqueforti, 15 showed good and one fair growth. The flavor was good in nine, fair in five and poor in two cheese.

Cheese inoculated with dry bread mold cultures and inspected after 8 and 18 weeks

After 8 weeks cheese inoculated with slightly lipolytic and proteolytic penicillia usually showed poorer mold growth than those inoculated with pronounced lipolytic and proteolytic molds. Mold growth in 10 cheese ripened with slightly lipolytic and proteolytic penicillia was good in five instances, fair in one and poor in four cases; 14 cheese inoculated with pronounced lipolytic and proteolytic penicillia showed good growth in 12 cases, fair in two and poor in none. Cheese ripened by slightly lipolytic and proteolytic penicillia showed less satisfactory flavor development than those ripened with pronounced lipolytic and proteolytic molds. Flavor development in cheese ripened by slightly lipolytic and proteolytic penicillia was good in three cases, fair in four and poor in three; cheese ripened by pronounced lipolytic and proteolytic penicillia showed good flavor development in nine instances, fair in five and poor in none.

Twenty cheese in the group were inoculated with 10 cultures of P. roqueforti; of these 16 showed good, two fair and two poor mold growth; two cheese inoculated with P. gorgonzola showed poor growth; while two cheese inoculated with P. stilton exhibited fair growth. The flavor development in cheese ripened with P. roqueforti was good in 17 instances, fair in six and poor in two; two cheese ripened with

P. gorgonzola showed fair flavor in one and poor in one instance; while two cheese ripened with P. stilton exhibited fair flavor development.

After 18 weeks ripening cheese inoculated with pronounced lipolytic and proteolytic penicillia usually showed better mold growth than those ripened with slightly lipolytic and proteolytic molds. The 14 cheese inoculated with pronounced lipolytic and proteolytic penicillia showed good mold growth in 13 instances, fair in one and poor in none; whereas 10 cheese inoculated with slightly lipolytic and proteolytic cultures exhibited good growth in seven instances, fair in one and poor in two. Cheese ripened with pronounced lipolytic and proteolytic penicillia generally showed better flavor development than those ripened by the less active molds. The flavor of cheese ripened by the pronounced lipolytic and pronounced proteolytic penicillia was good in seven cases, fair in five and poor in two; while the flavor of cheese ripened by slightly lipolytic cultures was good in one and fair in nine instances. Culture 15, a pronounced lipolytic and proteolytic culture, was used in both instances when the ripened cheese possessed a poor flavor, which was described as being butyric and musty.

The 20 cheese inoculated with 10 cultures of P. roqueforti showed good mold development in 19 instances and fair growth in one; two cheese inoculated with a culture of P. gorgonzola exhibited good mold growth; while two cheese inoculated with P. stilton showed good growth in one and poor in one case. Flavor development in cheese ripened with P. roqueforti was good in seven, fair in 11 and poor in two instances; the flavor of two cheese ripened by P. gorgonzola was

fair; while the flavor of two cheese ripened with P. stilton was good in one instance and fair in one.

From the data presented in table XX it is evident that the lipolytic and proteolytic activities of various penicillia had a general effect on the ripening of blue cheese, as shown by the flavor developed in the cheese during ripening. Cheese ripened with pronounced lipolytic and proteolytic penicillia generally showed better mold growth and ripened earlier with the development of a good flavor than those ripened with the less active molds. During the early stages the cheese ripened by the pronounced lipolytic and proteolytic penicillia usually possessed bitter and green flavors, but these tended to disappear and good flavor usually resulted. Cheese ripened with the slightly lipolytic and proteolytic penicillia frequently showed poor mold growth and flavor development. However, the results were not always consistent since in a few instances certain pronounced lipolytic and proteolytic penicillia resulted in cheese with poor flavor.

It is also evident that there was marked variation in the flavor of cheese ripened by different strains of penicillia. Cheese ripened by cultures of P. roqueforti usually showed good mold growth but not always good flavor development, some cheese were fair and others were of poor flavor. Cheese ripened by a culture of P. chrysogenum resulted in poor growth and flavor; cheese ripened with P. gorgonzola showed from good to poor growth and usually from fair to poor flavor; one culture of P. stilton usually produced good growth and from good to fair flavor in the cheese; and one culture of an

unidentified Penicillium resulted in fair growth and flavor.

DISCUSSION

The greater perceptibility of lipolysis of butterfat and cottonseed oil by certain penicillia with the Nile blue sulfate than with the natural fat technique was apparently due to the intense color imparted to the fat by the Nile blue sulfate which gave a sharp contrast between the unhydrolyzed and the hydrolyzed fat. Although the natural fat technique resulted in larger mold colonies, due to the toxic action of Nile blue sulfate, the uncolored fat globules (unhydrolyzed and hydrolyzed) were relatively inconspicuous against the variously colored background of the culture growth. Only the pronounced lipolytic molds gave results which were sufficiently conspicuous so that definite measurement of the extent of lipolysis could be made, whereas some of the moderately and all of the slightly lipolytic cultures showed negative lipolytic activities with the natural fat technique. Culture 1 was typical with regard to this characteristic; it was moderately lipolytic with Nile blue sulfate but nonlipolytic with the natural fat technique.

The modified Nile blue sulfate technique would be expected to give more conspicuous results than the natural fat technique in detecting lipolytic activities of the penicillia. The flooding procedure facilitated the examination of fat globules under the central part of the colony. The dye (1 part Nile blue sulfate to 1000 parts 50 per cent alcoholic solution) also stained the fat globules

intensely; this facilitated the examination of them for evidence of lipolysis.

Although varied concentrations of butterfat or cottonseed emulsions in the media had no appreciable effect on the lipolytic activities of various penicillia, uniform dispersions were essential for evaluating the different cultures; cultures which showed uniform lipolysis with the usual fat dispersions gave nonuniform lipolysis when excessive concentrations of fat were employed in the media.

The relatively conspicuous proteolysis obtained with certain penicillia cultures on Czapek's solution milk agar at 28° C., as compared with inconspicuous results at lower temperatures, was due primarily to greater enzyme activity at the higher temperature. While active proteolysis likely occurred at 20° C., it did not progress as rapidly as the culture growth and the proteolyzed area was obscured by the colonies. The slow growth of the molds at 28° C., as compared with 20° C., showed that the optimum growth temperature had been exceeded.

The carbon dioxide technique for determining the proteolytic activities of certain penicillia consistently gave the more satisfactory results when compared with the acidified milk agar procedure. In some instances certain cultures showed only slight proteolysis of skim milk with the acidified milk agar technique, while they were pronounced proteolytic with the carbon dioxide procedure.

The failure to obtain evidence of proteolysis on beef infusion milk agar suggests that the good supply of nitrogenous nutrients with beef infusion makes the hydrolysis of casein less necessary.

Whether the cultures were incubated in the air or in an atmosphere consisting almost entirely of carbon dioxide, unsatisfactory results were obtained. Since the cultures did not grow as rapidly on beef infusion as on Czapek's agar, the difficulty apparently can not be attributed to the colony growth obscuring the proteolyzed area.

It is evident that relatively wide differences existed in the lipolytic activities of various cultures. Since the penicillia have been classified mainly on the basis of their morphological characteristics, physiological variations among different cultures of a species would be expected. Consequently, it was not surprising that cultures of P. roqueforti obtained from different sources varied with respect to their lipolytic activities. Although striking similarities in the lipolytic activities were exhibited by some cultures, none showed identical reactions. In general, cultures which were moderately and pronounced lipolytic on butterfat and cottonseed oil showed similar reactions on the different triglycerides. Certain penicillia which were negatively lipolytic on butterfat and cottonseed oil were pronounced lipolytic on some of the lower, readily hydrolyzable triglycerides. However, their lipolytic activities decreased rapidly as the molecular weights of the triglycerides increased.

It was of interest to note the variation in the apparent toxicity of different triglycerides toward various penicillia. It seemed probable that this effect was not due to the triglycerides but rather to the acids set free by them. In general the lower triglycerides exhibited the greatest toxic effect on the molds but in

certain instances some of the higher triglycerides exerted marked toxic influence. However, all of the molds did not react similarly toward the triglycerides. While the growth of some cultures was markedly inhibited, others showed normal development.

Inspection of individual butterfat and cottonseed oil globules suggested that many (especially with certain cultures) had undergone some lipolysis without the accompanying change in color from pink to blue with the Nile blue sulfate technique. The globules were surrounded by a tenacious pellicle and they ranged in color from peach red to purple. The failure of fat globules to show the characteristic color change may have been due to a dissipation of the oxazine form of the Nile blue sulfate by the mold mycelium since examination of the medium underneath the colonies showed it was practically devoid of color. The dissipation of color from the medium became more pronounced as the cultures aged. In view of these observations it seemed advisable, in the study of the lipolytic activities of various penicillia on butterfat and cottonseed oil, to carefully inspect individual fat globules for minor color changes and also for the presence of a pellicle.

The disappearance of some of the lower triglycerides upon lipolysis by the penicillia was primarily due to the solubility of the lower fatty acids and glycerol in the medium. The solubility of the fatty acids decreases with increased molecular weight and this is reflected in the comparatively slow disappearance of some of the globules.

It is evident that the lipolytic activities of various penicillia are affected to a marked degree by certain gases. The decreased lipolytic activities of the cultures incubated in an atmosphere which consisted of 3 parts of air and 1 part of carbon dioxide, as compared with the cultures grown in a normal air supply, was to be expected since carbon dioxide retarded development of the molds. The effect of carbon dioxide on lipolysis by the penicillia is probably of importance with respect to cheese ripening since an accumulation of the gas in cheese during ripening would be expected to retard mold growth, with consequent diminished rate of fat decomposition. However, nitrogen accelerated the growth and lipolytic activities of various penicillia; this was most pronounced when cultures were incubated in an atmosphere which consisted of 3 parts of air and 1 part nitrogen and less pronounced in a mixture of 3 parts carbon dioxide and 1 part nitrogen. It did not seem possible that the penicillia which are known to be aerobic would be able to grow in the absence of oxygen. It seemed probable that with the methods employed in removing the air from the bell jars, in which the cultures were grown, some air may have remained in the petri dishes; this in addition to the oxygen which may have been contained in the medium was possibly sufficient to support the mold growth.

Apparently wide differences existed in the proteolytic activities of various penicillia. Since the cultures ranged from slightly to pronounced proteolytic, the relative activities of various penicillia would be expected to play an important role with respect to

the texture of the ripened cheese.

It is evident that the dry bread culture method was the most effective in establishing the molds in blue cheese, when this procedure was compared with the agar slope and the agar plate culture methods. The failure of the agar slope and agar plate culture methods of inoculation to establish the inoculated molds in cheese was due to the relatively small number of spores in comparison with the quantity of curd inoculated. Some cultures which showed heavy spore formation became established in the cheese in some instances when inoculated from agar slope or plate cultures. The penicillia were effectively implanted when cheese were inoculated with dry bread cultures, since these contained relatively large numbers of spores.

Irrespective of the method of inoculation, certain cheese contained contaminating types of penicillia. In some cheese these were predominant, while in others they constituted a small minority. Frequently, more than one type of contaminating mold was found in the ripened cheese. It is not surprising that various contaminating molds should be present in the ripened cheese, since they were made from raw milk which undoubtedly contained various types of molds. From the standpoint of cheese ripening, it is apparently essential to employ heavy inoculations of mold culture in the curd in order to obtain early predominance of the inoculated molds in the cheese and to prevent the development of objectionable flavors from the contaminating molds present in the curd prior to the inoculation.

Variations in response to the growth environment with

different cultures are to be expected, since it is quite possible that some cultures are more sensitive than others to the concentrations of carbon dioxide and sodium chloride in cheese. The cultures were not consistent with respect to the growth in cheese. A culture exhibited good growth in one cheese while in another cheese, usually from a different trial, mold development was poor. Such variation in growth by the same culture may have been due to differences in the texture of the cheese. Poor growth was frequently noted in close textured cheese. Although poor mold growth was occasionally found with cultures of P. roqueforti, it was much more frequent with P. gorgonzola; this may have been due to definite differences in the sensitivity of the two species to the environmental conditions in cheese. One culture, P. chrysogenum, grew well in cheese but the growth was atypical with respect to blue cheese and, therefore, the culture was regarded as showing poor growth.

That the lipolytic and proteolytic activities of various penicillia should affect the ripening of blue cheese is to be expected, since flavor development is believed to be associated with the decomposition of fats and proteins through the activities of lipase and protease, respectively. The bitter and green flavor sometimes noted during ripening in cheese inoculated with pronounced lipolytic and proteolytic penicillia is probably an essential characteristic of such cultures and indicates rapid decomposition of fat and protein accompanied by an accumulation of the decomposition products in the cheese. Such flavor suggests a rapid accumulation of fatty acids and

as conditions became less favorable for mold growth, the acids were oxidized to the respective methyl ketones, thus resulting in early ripening.

Variation in the ripening of blue cheese with different strains of P. roqueforti was primarily due to the marked variations in their lipolytic and proteolytic activities. However, the results were not always consistent, since in a few instances certain pronounced lipolytic cultures frequently resulted in poor cheese. Evidence of this was noted with cultures 15 and 25, these were strikingly similar with respect to their colony characteristics and their lipolytic and proteolytic activities, yet they exhibited marked variation in regard to cheese ripening; culture 15 usually resulted in poor flavor, while culture 25 quite consistently produced good flavor in the ripened cheese. Cultures of P. gorgonzola seemed to be relatively slow in ripening the blue cheese; this was probably associated with the slight lipolytic activities of these molds. P. stilton usually resulted in good cheese; although this mold showed no evidence of causing hydrolysis of butterfat and tricaproin, it was moderately lipolytic on the different triglycerides. P. stilton also showed good mold growth in the cheese. In view of these observations it would seem that the selection of mold cultures for blue cheese ripening should be made on the basis of strain rather than species.

SUMMARY AND CONCLUSIONS

1. The lipolysis of butterfat and cottonseed oil by various penicillia was more readily detected with the Nile blue sulfate technique than with the natural fat technique. Although the natural fat technique resulted in the greater colony growth, the Nile blue sulfate technique usually gave the more distinct differentiation between the unhydrolyzed and the hydrolyzed fat.

2. The modified Nile blue sulfate technique for determining the lipolytic activities of various penicillia on butterfat and cottonseed oil gave a more distinct differentiation between the unhydrolyzed and the hydrolyzed fat than the natural fat technique.

3. No appreciable difference in effect on the detection of lipolysis by various penicillia was noted when 0.5 ml. or 1.0 ml. of a 3 per cent fat emulsion was used per plate (15 ml. agar). However, when 1.5 ml. fat emulsion was used the uniformity of lipolysis was affected.

4. With the Nile blue sulfate technique for determining lipolysis of butterfat by certain penicillia, the best results were obtained when the media contained 1 part of dye in 15,000 or 20,000 parts of agar. Higher concentrations of the dye caused marked inhibition of mold growth, while lower concentrations failed to color the fat properly.

5. Proteolysis was more readily detected on Czapek's solution milk agar when the cultures were incubated:

- a. At 28° C. rather than at 21° or 12° C.
- b. At 21° C. in the air 4 days followed by 4 days in an atmosphere charged with carbon dioxide rather than at 21° C. in air for 6 days or 8 days.

6. At 21° C. proteolysis was more readily detected on Czapek's solution milk agar than on beef infusion milk agar when the cultures were incubated 4 days in the air followed by 4 days in carbon dioxide, or when they were grown for 8 days in carbon dioxide.

7. Somewhat more clear cut results were obtained with the carbon dioxide technique (incubation on Czapek's solution milk agar with incubation at 21° C. for 4 days followed by 4 days in carbon dioxide) in the detection of proteolysis than with the acidified milk agar technique.

8. There was considerable variation in the lipolytic activities of various penicillia on butterfat and cottonseed oil, as determined by the natural fat and the Nile blue sulfate techniques. According to the ratio of the colony size to the extent of lipolysis ($\frac{L}{C}$ value) and the intensity and uniformity of lipolysis, the cultures ranged from nonlipolytic to very pronounced lipolytic.

9. There was considerable variation in the lipolytic activities of various penicillia on different triglycerides, according to the Nile blue sulfate technique. Only a few cultures showed

lipolysis of tripropionin, while all readily hydrolyzed tributyrin and trivalerin. As the molecular weights of the triglycerides increased, variations in lipolytic activities of the cultures became more conspicuous. Some cultures showed gradual declines in their lipolytic activities, whereas others declined sharply on the triglycerides beginning with tricaproin. While most cultures were actively lipolytic on the triglycerides from tributyrin up to and including tricaproin, several showed no lipolysis with trilaurin.

10. There was considerable variation in the toxic effect of different triglycerides on various penicillia. In general, the triglycerides that exhibited the most pronounced toxicity, in the order of their effect, were tripropionin, tributyrin, trivalerin, tricaproin, trilaurin, trimyristin and tripalmitin. The least toxic were triheptylin, tricaproin, tricapyrylin and triolein.

11. The lipolytic activities of various penicillia on butterfat were retarded when the cultures were grown in an atmosphere consisting of 3 parts air and 1 part carbon dioxide, while their activities were markedly accelerated in 3 parts air and 1 part nitrogen and slightly accelerated in 3 parts carbon dioxide and 1 part nitrogen.

12. The lipolytic activities of various penicillia on butterfat declined slightly when 4 per cent sodium chloride was added to the medium.

13. Certain penicillia showed greater lipolytic action on cottonseed oil when the cultures were incubated at 25° C. than at 9° to 10° C.; more rapid mold growth also occurred at the higher temperature.

14. There was considerable variation in the proteolytic activities of various penicillia, as determined by the acidified milk agar and the carbon dioxide techniques. There was a general agreement between the results obtained with the two techniques.

15. The rates of growth and of proteolysis of certain penicillia were affected by different growth conditions. The cultures grew more slowly but showed greater proteolytic activities in air at 28° C. than at 19° or 12° C.; the cultures were somewhat retarded in growth but proteolysis was unaffected when grown at 28° C. in an atmosphere in which 10 per cent of the air had been replaced by carbon dioxide; culture growth and proteolysis at 28° C. were almost stopped in an atmosphere which consisted principally of carbon dioxide; growth was usually unaffected but proteolysis was slightly accelerated at 28° C. in an atmosphere consisting for the most part of nitrogen.

16. The inoculation of cheese curd with dry bread cultures of penicillia was the only satisfactory method of establishing the penicillia in cheese. Suspensions of spores from agar slope or plate cultures failed to introduce a sufficient number of mold spores for establishment of the inoculated cultures.

17. Blue cheese ripened with pronounced lipolytic and proteolytic penicillia usually showed better mold growth and earlier development of a good flavor than those ripened by less active molds. The bitter and green flavors often found, during the early period of ripening, in cheese ripened with the pronounced lipolytic and proteolytic penicillia gradually disappeared, and good flavors usually

resulted. When the cheese were ripened with slightly lipolytic and proteolytic penicillia, they frequently showed poor mold growth and flavor development. However, the results were not always consistent since in a few instances certain pronounced lipolytic and proteolytic penicillia resulted in cheese with poor flavor.

18. There were marked variations in the flavors of cheese ripened with different strains of penicillia. Cheese ripened with P. roqueforti usually showed good mold growth but not always good flavor. Cheese ripened with a culture of P. chrysogenum showed poor growth and flavor; cheese ripened with P. gorgonzola showed from good to poor growth and usually from fair to poor flavor; one culture of P. stilton usually produced good growth and from good to fair flavor in the cheese; while one culture of an unidentified Penicillium gave fair growth and flavor.

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