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SOME OF THE FACTORS INFLUENCING THE GROWTH OF MOLDS IN BUTTER

By

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INTRODUCTION

The market value of butter is determined, in a large measure, by flavor and aroma, but the general appearance and physical structure also have considerable influence upon a critical and discriminating buyer.

One of the most disturbing defects of market butter is due to the growth of molds which produce discolored areas on the surface of the butter, wrapper or packing, often sufficiently serious to cause the rejection of such butter by the dealers. On the other hand, the mold growth may be such that there are no marked, visible signs of molding to mar the appearance of the butter but quite enough to bring about decided, deleterious changes in the flavor and aroma. The discoloration of the butter due to the growth of molds is always a serious matter in itself, but when the flavor and aroma may be affected simultaneously, or even independently, as they are by the development of certain molds, the situation assumes very serious proportions. As a rule the market has been thinking of the molding of butter, principally, as a defect in appearance because this is the most striking manifestation of the development of these microörganisms.

Generally, moldy butter is encountered as an isolated case,

altho at times an outbreak of moldiness in butter from certain creameries may assume the proportions of an epidemic. When such a situation arises, losses to the creameries manufacturing such butter are often heavy, involving hundreds or thousands of dollars, quite sufficient to cause a minor financial crisis for large creameries, and a major crisis for smaller establishments. In recent years, moldy butter has attracted much more attention than it did at one time. The reason for this is not entirely clear altho changes in the manufacturing methods and a more discriminating market may be factors which have had a part in this increased interest.

The appearance of mold in butter has been more frequent in unsalted butter, and occurs most commonly during the early spring and summer months. However, isolated cases may be encountered at any season of the year and in various grades of butter and margarine.

The mold problem is really serious for the creamery industry, considering the damage done by the mold in producing the typical surface discoloration and deterioration of the product. A number of investigators have interested themselves in a study of some of the factors which may influence the growth of the mold in the butter, but there are a number of factors which have not been studied carefully, and which must be understood clearly before the industry may assume control of the situation. For instance, it has been agreed, quite generally, that salt has a preservative action in butter and tends to prevent moldiness. Nevertheless, many cases of mold growth in quite highly salted butter

continue to occur. The problem is not simple, nor will it be easy to solve. There are so many possible factors which may influence the growth of molds in butter that each of them must be studied thoroly before a proper evaluation of each of them can be made for individual species of molds and mixtures of species which may occur in a given lot of butter. The relationships which these factors have, one to the other, must be established before the problem can be approached with a reasonable degree of assurance that control can be exercised.

The studies reported here have been undertaken in an attempt to provide further data from which future research may be directed toward a more complete solution of the problem.

REVIEW OF LITERATURE

There is no indication in the literature as to the original observation of the molding of butter. Undoubtedly, mold growth occurred on butter made under primitive conditions and stored where there was every opportunity for growth. For many years articles on dairying have made reference to the appearance of discolored areas on butter, but during the period before mycological laboratory methods were perfected little definite information was available. Saccardo (111) in 1886 stated that Oospora ruberrima had been reported by Trabut as occurring on butter in Algiers. The same year, Duclaux (34) mentioned the fact that molds were present in butter. In 1892, Siedel (124) found that granular butter, stored for several months in brine, changed in flavor and developed roquefort, turnipy or beet flavors which he attributed to molds. McWeeney (80) in 1894 described and illustrated a fungus causing dark brown spots on butter exhibited at Cambridge which he considered to be like Dematium pullulans DeBary but which was identified as Cladosporium herbarum Link. Reference to the presence of mold in butter was made in 1896, from Sweden anonymously (4) and by Wågepetersen (141). Shaw (125) reported in 1896 that several samples of butter in Portland, Oregon

developed bluish-black isolated spots on the surface, which eventually became covered with the growth of a fungus which was identified as Stemphylium butyri. He also stated that a similar case had been noted in North Carolina. In an extended report upon experimental exports of unsalted butter to the London market, published in 1898 by the U. S. Department of Agriculture, Alvord (2) stated that the results were unsatisfactory because a portion of the butter became moldy. In a monograph which has been quoted widely, Gripenberg (53) discussed the investigations carried on during the years 1896-1898 in Finland to determine the causes of moldy butter. The molding of butter had become a serious problem for the exporters of Finnish butter and demanded some study. During the last thirty years there have been increasing numbers of references to the appearance of mold in butter and results of research upon sources, species of molds encountered, factors influencing growth, chemical activity and methods for control have been published. Many of these will be discussed in the subsequent pages.

Factors Influencing the Growth of Molds in Butter

There are many factors which may be considered as affecting the growth of molds, among which the food supply, moisture, atmospheric conditions, temperature and miscellaneous physical and chemical environment are the most noteworthy. Furthermore, the species

of mold undoubtedly is an important factor. These factors may be considered separately.

A. Food Supply

Butter is a substance which carries a variety of food elements such as fats, proteins, carbohydrates, and mineral salts, which should provide nutriment for the molds that may be present in the butter.

1. Fats and Related Substances. Inasmuch as fat is the principal constituent of butter it is well to consider the part which fat may play in the nutrition of some of the common fungi. In an investigation of the use of fats by certain fungi, Schmidt (117) employed cultures of Penicillium sp., Aspergillus sp., Mucor racemosus and Phycomyces nitens but inasmuch as only slight differences in the action of these species were noted, Aspergillus niger was used principally. Various oils were added to a mineral solution and it was found that the molds grew and utilized the fats, especially the neutral fats. Schmidt pointed out that these may be factors influencing the growth. The decomposition of a glyceride was considered as beginning with a splitting into glycerol and the fatty acids, which in turn might be attacked. If the oil covered the surface of the nutrient solution, the molds were shut off from oxygen and did not grow. Duclaux (35) melted a sample of butter and de-

canted a portion of the fat. The remainder of the sample was emulsified and inoculated with a species of Penicillium. Changes took place in the fats and there were variations in the proportion of fatty acids released. Butyrin was saponified more easily by the culture than caproin but both of these were more readily attacked than the other glycerides. Later, Duclaux (36) found that if an oil was impure, and carried a substance which imbibed water, the surface of the substance might be covered by an abundant vegetation after a few days. In this Penicillium glaucum predominated. Water played an important role in this growth of mold especially since it may carry various nutrients and furnish moisture for germination. In a study of rancidity of butter, Hanus (56) reported that the glycerides of the saturated fatty acids were decomposed by molds but that those of the unsaturated acids were not. He pointed out later (57) that the molds first attacked the lactose and proteins and later decomposed the fats. Eichholz (39) (40) made the statement that microörganisms could not grow in pure butterfat but that Penicillium glaucum could show an especially good development and cause an intense roquefort flavor in butter rich in casein. Crampton (25) (26) concluded from his experiments with moldy oleomargarine that where nitrogenous or other non-fatty material afforded a nutriture medium for the growth of molds, edible fats could be hydrolyzed so that fatty acids and

glycerol were liberated. Laxa (72) found that molds brought about an important decomposition of butterfat; but that the splitting of all glycerides did not take place in the same degree. The glycerides of the insoluble fatty acids which have the higher molecular weights were found to be hydrolyzed by the molds more easily than those of lower molecular weights and the toxicity of the freed, soluble fatty acids increased with the increasing molecular magnitude. Employing cultures of Mucor mucedo, Oidium albicans, Penicillium glaucum and an unidentified mold, Schrieber (118) found that pure fat by itself was not a foodstuff for microörganisms but where other nutrients were available the molds were able to split the fat. As a result of his experiments Bahn (99) concluded that the decomposition of fat took place only in the presence of organic nitrogen. Penicillium glaucum and several other species of molds were able to decompose fat in a nutrient medium but not under anaerobic conditions. Kühl (70) (71) reported that molds isolated from butter were able to attack fat but this process took place after the molds had made their original development on the curd of butter. Boussy (109) investigated the value of fatty substances as food for Phycomyces nitans, Rhizopus nigricans and Sterigmatocystis nigra and stated that fat was just as good food for these molds as carbohydrates. As a substrate he made use of Raulin's liquid which furnished sources of

nutriment other than fat. His studies (110) were continued with similar results when a number of other species were used. Söhngen (125) found that a variety of molds were capable of producing lipase which split the fat in butter and margarine. In extensive investigations Spieckermann (126) (127) studied the effect on fats of cultures of Penicillium glaucum, as well as Aspergillus glaucus, Aspergillus flavus and several species of Monilia. The fats were split in a variety of ways when some nitrogen compounds were present in the substrate. Batten and Bywaters (9) in experiments with sterile cacao butter observed that molds would not grow on it at any temperature unless water was present. Colonies of mold did appear on a solidified emulsion of cacao butter and water (about 30 per cent) after 3 months at room temperature. When small quantities of sterile prune juice agar were mixed with the cacao butter vigorous growth was observed in less than a week. Bywaters (21) also stated that pure fats were very resistant to the attack of fungi, probably because of the lack of nitrogenous and other elements required for protoplasmic growth. As a result of researches on the rancidity of vegetable margarine, Jacobson (62) decided that the influence of molds on absolutely dry cocoanut oil was of no significance whatever but if only traces of water were present rancidity might occur. Stokoe (129) found that Penicillium glaucum when inoculated into pure fats pro-

duced little change but when the fats were emulsified with nutrient media, the mold developed within a few days. He (130) stated that the development of rancidity in water-free fats was not due to the activity of microörganisms. The products formed as a result of the action of Penicillium sp. were discussed in a later publication by the same author (131). Flieg (49) studied the action of Aspergillus niger upon a variety of fats in a synthetic medium which encouraged the early growth of the fungus and found that the different fats were attacked in different ways. After reviewing the literature extensively, Stärkle (128) stated that the presence of water and nitrogenous substances was necessary for the growth of molds in cocoa fat. Zikes (147) likewise made the statement that pure fats and waxes did not support the growth of fungi and that positive results in the past were probably due to adherent traces of proteins and carbohydrates. In a recent publication, Patil and Hammer (92) reported that butterfat in a purified condition seemed quite resistant to the growth of microörganisms. Strained fat and ghee kept much better than butter or ghee containing added water.

Schmidt (117) found that when a mineral nutrient was provided, oleic and palmitic acids were more or less satisfactory sources of carbon for Aspergillus niger, after glycerides were

broken down into the component acids and glycerol. According to Bokorny (14) butyric acid may be used as food for molds, but Bitting (11) found that a 0.2 per cent solution retarded the growth of Penicillium, while Oidium lactis did not develop in more than 0.1 per cent. Crampton (26) found that Coniothecium sp. attacked the fatty acids freed in oleomargarine, with a preference for those of lower molecular weights. Rahn (99) agreed that fungi show a preference for the lower acids. Laxa (72) reported the utilization of volatile fatty acids released in the splitting of butterfat by molds. Spieckermann (126) found that certain molds were able to use fatty acids especially if they were finely divided. Boussy (110) was able to obtain good growth of a number of species of mold on oleic, palmitic and stearic acids, when they were used in connection with Raulin's medium. Tausson (132) reported that oleic, stearic and palmitic acids were utilized by A. flavus altho the saturated acids were more suitable than the oleic acid. Kiesel (66) was of the opinion that fatty acids show considerable toxicity towards Aspergillus niger, especially those containing the most carbon. In this respect the molecular structure also appeared to be significant. The differences in toxicity were explained on the basis of the differences in penetrability of the protoplasmic layer of the cell for various substances.

The glycerol released by the splitting of fats can be utilized quite readily by fungi according to Burr (19) who found that Penicillium glaucum and Aspergillus niger would grow in 43 per cent glycerol solutions. Ehrlich (38) demonstrated that Oidium lactis utilized glycerol as a food. Roussy (110) came to the conclusion that the several species of molds which he was studying developed much better on the fatty acids than on glycerol with the exception of species of Penicillium and Aspergillus. Glycerol did not serve particularly well as a source of carbon according to Schmidt (117).

When ivory-nut oil and lecithin were added to a nutrient medium, von Euler (145) found that the growth of Penicillium glaucum and Rhizopus chinensis was extensive after the medium was irradiated by ultra-violet light.

It may be seen by a perusal of the literature upon the value of fat, fatty acids, glycerol and related substances, that pure fat is not available for direct use by molds, and that sufficient quantities of water and nutrient substances appear to be necessary before the splitting and utilization of the fat can be accomplished. There seems to be some question as to the action of molds upon the free fatty acids. A difference appears to exist between the value of the different acids, either because of their composition, struc-

ture or some other factor. Large quantities might be toxic and small amounts stimulating. Apparently glycerol and lecithin are able to furnish a satisfactory food supply for many molds.

2. Proteins and Related Substances. The literature abounds with references to experiments undertaken to determine the types of nitrogen compounds most useful for various species of mold. It is so extensive that no attempt will be made to review it at this time. Since normal butter contains a considerable quantity of rich nitrogenous materials in the form of the droplets of buttermilk it may be assumed that the molds will find a variety of proteins and related compounds quite sufficient for growth, providing the molds are in such a position that they are able to obtain the substances directly.

The question may arise as to the possibility of molds fixing nitrogen when they are attempting to grow in such a substance as pure butter-fat. Dox (32) (33) reported that he found no evidence that Aspergillus fumigatus could fix nitrogen. The literature upon the subject of nitrogen fixation has been reviewed very thoroly by Duggar (37) who came to the conclusion that nitrogen fixation could not be established for Aspergillus niger, Penicillium digitatum, Penicillium expansum and some other forms while Phoma betae showed signs of fixation when growing on mangel and sugar

beet decoction with sugar.

As far as the utilization of the protein fraction of butter is concerned, Batten and Bywaters (9), Burr and Wolff (19), Crampton (26), Eichholz (40), and Laxa (73), all expressed the opinion that a high curd content favored the growth of molds in butter. Boekhout and deVries (12) held that the percentage of curd in itself was not a factor because the merest traces of curd were sufficient to encourage the growth of mold. This observation seems to be more in accordance with the facts relating to the quantities of nitrogen material necessary for the development of fungi.

3. Carbohydrates and Mineral Salts. The value of various carbohydrate and mineral elements or salts as food for molds has been studied by many workers. It would be impractical to present a review of the voluminous literature upon this subject. In connection with studies on butter, it was reported anonymously (5) that lactose was not a very food source of food for Oidium lactis or Mucor mucedo, but fairly satisfactory for Penicillium glaucum. In solutions containing sugar a marked submerged mycelial development was noted. This observation is of interest in light of the findings presented later in this thesis. Boekhout and deVries (12) likewise reported that lactose was not the best type of sugar for

Hormodendrum cladosporioides. In his studies on the influence of lactose on the decomposition of casein by microörganisms, Laxa (73) found that proteolysis was favored by the presence of lactose. According to Ehrlich (38) Oidium lactis was able to utilize lactic acid which might be formed by the hydrolysis of lactose.

The fact, that for years the majority of studies upon molds have been made upon synthetic media containing a variety of inorganic salts, is evidence enough to indicate that mineral elements are satisfactory and necessary food sources, providing they are present in the proper compounds and in the proper quantities. Normal buttermilk carries a variable quantity yet more or less satisfactory variety of most of the inorganic elements essential for the development of many fungi.

Summary. As a whole, butter contains a variety of food elements some of which are readily available for use. A great deal would depend upon the molds being properly oriented so that they were in intimate contact with the most easily utilizable compounds. In butter the protein, carbohydrate and mineral salts are present in the buttermilk which is finely dispersed thruout the mass of butterfat. As pointed out by Rahn and Boysen (102) these particles may be extremely small and so numerous that the majority of the droplets of buttermilk are sterile. Further than this, some

of the droplets may be largely water with the slightest traces of food-stuffs, if any, unless the spores of the molds have germinated and produced sufficient mycelium and enzymes to attack the abundant supply of adjacent fat.

B. Moisture

It is generally recognized that moisture is necessary for the germination of the spores of molds and that the development of hyphae is accellerated when sufficient moisture is available. In order to utilize any foodstuff, water in sufficient quantities must be present. This has been indicated in connection with the availability of fat as a nutrient for fungi.

1. Moisture in Substrate. Batten and Bywaters (9) prepared a series of blocks of cacao butter containing from 0 to 20 per cent of water in an emulsified state and inoculated mold cultures into the center of the blocks. Under these conditions, the growth of the mold was slow, no matter how high the percentage of water present. This may, of course, have been explained by other factors, such as a lack of suitable food or oxygen. Mold did develop on solidified emulsions of cacao butter and water (about 30 per cent) when spores were inoculated on the surface. Burr and Wolff (19) found that when butter contained water in large droplets

instead of being in the normal, finely-divided state described by Boysen (15) and Rahn and Boysen (102), mold development was favored. They pointed out that nutrients were not available unless sufficient moisture was present. Combs and Eckles (23) made the statement that the moisture content is a factor governing the development of molds in butter. As pointed out elsewhere, Duclaux (36) found that water was required for the germination of mold spores. According to Gripenberg (53), wood and paper used in butter packages sustained the growth of molds when sufficient amounts of moisture were present. Hood and White (60) were of the opinion that normal butter contained sufficient moisture for mold development. This seems to be certain since the moisture in butter is present largely in connection with the principal food constituents such as proteins, carbohydrates and mineral matter. In his investigations on the causes of rancidity of vegetable margarine, Jacobsen (62) found that oils became rancid thru mold action when small quantities of water (0.2 to 0.5 per cent) were present. König, Spieckermann and Bremer (68) reported that a multiplication of molds occurred in three sorts of cottonseed meal only when the water content was higher than 14 per cent. McWeeney (80) warned against superfluous moisture in butter if molding were to be prevented. As pointed out elsewhere, Patil and Hammer (92) observed the development of

microörganisms in butterfat and ghee only when water was present with these substances. Thom and Shaw (139) reported the presence of mold in butter with a water content ranging from 7.38 to 18 per cent. According to Welte (145), Penicillium glaucum made good growth after four days on bread containing 33 per cent of water but less satisfactory development in six days when the water content was 20 to 25 per cent. With Aspergillus nidulans, no growth was obtained, even after six days, when 25 per cent or less of moisture was present. He quoted Flügge as authority for the statement that mold growth was completely hindered when the water content was as low as 10 to 12 per cent.

It is evident from a review of this literature that water in the substrate is essential for the development of molds. Just what the minimum moisture requirement may be under different conditions is not so clear. The fact remains, however, that normal butter contains a high percentage of moisture in proportion to the most easily utilized food constituents and should provide a satisfactory substrate for mold growth from the standpoint of moisture.

2. Moisture in Atmosphere. The matter of humidity is another consideration of some importance. Combs and Eckles (23) held that the moisture content of the atmosphere in which butter was stored had a considerable influence upon the development of

molds. This is in agreement with the observations of Orla Jensen (63) that Cladosporium butyri, Oidium lactis and Penicillium glaucum grew best when butter was kept in a moist room. According to König, Spieckermann and Bremer (68), molding was increasingly abundant with an increase in humidity. Macy and Pulkrabek (81) stored samples of unsalted butter wrapped in mold-contaminated parchment under conditions of varying humidity. Nineteen samples kept at 1° C. for 50 to 60 days at approximately 70 per cent relative humidity showed no mold. The same was found to be true of ten samples stored at 12° C. for 30 days at about this humidity while two other samples at the same temperature and humidity after 50 to 80 days were moldy. A high relative humidity (90 to 100 per cent) favored the growth of molds upon comparable samples kept at the same temperatures. Thom and Shaw (139) found that samples of butter inoculated with various molds and kept at room temperature, for several days showed no growth of mold at ordinary humidities but when the samples were placed in a moist chamber the growth was active. At the low humidities none of the cultures appeared to develop, regardless of the moisture content of the butter. The failure of mold to grow on butters with high protein content when they were kept at low humidities indicated that the moisture in the atmosphere was the essential factor since the cultures developed in the moist chamber in butters

with a low protein content. Studies were also reported where definite humidities, namely, 100, 90, 79.6, and 69.6 per cent were employed. The growth of molds was found to be greatest at 100 per cent, good at 90 per cent, considerable at 79.6 per cent and little or none at 69.6 per cent relative humidity.

Unquestionably humidity is a very important factor in the growth of molds in butter.

C. Atmosphere

The importance of a sufficient supply of oxygen for the development of molds in butter has been suggested by a number of investigators, among which are, Boekhout and deVries (12), Burr (18), Burr and Wolff (19), Duclaux (36), Gripenberg (53), Hood and White (60), and Rogers (108).

Lopriore (78) observed that the germination of spores of Mucor mucedo was slowed by the presence of 10 per cent carbon dioxide in the atmosphere. Undiluted CO_2, altho producing total inhibition, did not kill the spores even after an exposure of three months. The formation of sporangia was more readily suppressed than spore germination. Brown (17) reported that within quite wide limits, the oxygen pressure had very little effect upon the germination and growth of certain molds such as Botrytis, Fusarium,

and Alternaria. The germination and growth of these organisms was retarded by CO_2, especially at lower temperatures with a scanty food supply.

According to Porodko (95) it did not appear that aerobic organisms such as A. niger, P. glaucum and M. stolonifer were particularly sensitive to changes in oxygen pressure. Sevenster (120) (121) (122) stated that butter stored under vacuum was not in a favorable environment for the growth of molds.

Karsner and Saphir (65) studied the effect of high partial pressures of oxygen upon a number of species of molds grown on Sabourand's agar in petri dishes kept in glass jars. They found that concentrations of oxygen of 76 per cent or more exercised a definite inhibitory effect upon some molds.

Rippel and Bortels (106) observed that the development of Aspergillus niger from spores was hampered by removing the CO_2 from the atmosphere. They held that CO_2 was necessary for the functioning of plant cells.

According to Rockwell and Highberger (107) a species of Mucor was found to be inhibited in growth when incubated at 37° C. in a jar containing 4 per cent NaOH to remove some of the CO_2 from the atmosphere.

The notion that molds are strict aerobes seems to be faulty but it is evident that they do require at least some oxygen

for their normal development, altho excessive quantities are inhibitory. How important these facts may be in connection with the molding of butter is not fully explained. One must bear in mind that a certain amount of air is entrapped in ordinary butter, the amount and distribution varying at different times as shown by Rahn (101). The extent of the surface of butter exposed to the air also may be a factor. Reisz (104) has shown that a cylinder ($r^2\pi h$) of butter exposes 14.5 per cent and a cube (r^3) 24.1 per cent more surface than an equal quantity of butter in the form of a sphere ($^4/_3\ r^3\pi$). If air is essential for the development of mold in butter, the shape of the package may be worthy of consideration.

D. Temperature

Temperature is a factor which influences profoundly, the growth of microörganisms. Molds are no exception to this.

Welte (145) reported that Penicillium glaucum and Mucor stolonifer showed the best growth at room temperature while Aspergillus nidulans was able to grow well at 36 to 37° C. In his studies on Aspergillus niger and Penicillium glaucum, Thiele (135) observed that the maximum temperature for growth was not constant and depended upon the medium. The minimum for P. glaucum was 1.5 to 2.0° C.

and for A. niger, 6 to 8° C. Later, he reported (136) that P. glaucum made only scanty growth at 30 to 40° C. Thom (137) found that few Penicillium species grew normally at 37° C. but nearly all showed rapid growth at 12 to 30° C. The development was progressively reduced by lower temperatures. At 10 to 20° C. it was slow but good, while at temperatures approaching 0° C. the growth was very much slower. Ames (3), in studying the minimum temperature for germination of spores of Monilia fructigena, Penicillium digitatum, Rhizopus nigricans and other storage-rot fungi, found that the spores of these species had a minimum germination point at 1° C., 1° C. and 3° C. while their optimim temperatures for growth were 25° C., 25° C. and 36° C. respectively. He pointed out that the minimum temperature for fructification in all cases was several degrees higher than that for growth. Brooks and Cooley (16) determined that the spores of Alternaria sp., Botrytis cinerea, and Penicillium expansum germinated slowly at 0° C. on cornmeal agar. Aspergillus niger failed to germinate at 10° C. Meyer (84) advanced the opinion that it was not permissible to speak of an optimum temperature for the growth of fungi except in the sense that it was the temperature which permitted the greatest rate of growth under strictly specified conditions other than temperature.

These reports are representative of many others which

indicate that each species shows different responses to different temperatures for germination, growth or fructification.

In respect to the growth of molds in butter at different temperatures, the reports are largely in generalities, altho Boekhout and deVries (12) reported that moldiness had appeared in samples of butter stored at -3 to -6° C. for four weeks to four and one-half months. A report from the U. S. Department of Agriculture (6) recommended that butter be stored below 2° F. (-16.67° C.) to prevent mold development. Hood and White (60) pointed out that molds grew in butter over a wide range of temperatures but were checked at temperatures approaching the freezing point of water. This was in agreement with the views of Rogers (108) expressed several years before. Macy and Pulkrabek (81) observed that temperature was a factor which influenced the development of mold on experimental butters. Stokoe (129) remarked that temperature entered into the factors favoring the production of rancidity of oleomargarine by molds, on the basis that this defect was more prevalent in the summer months.

In a general way it appears evident that low temperatures impede the development of molds in butter but experimental evidence for different species and specific temperatures is lacking. Butter has been found to be moldy even when stored at relatively low temperatures for sufficient periods of time.

E. Miscellaneous Chemical and Physical Environment.

There are many factors which may be considered under this category, but only a few will be considered here.

1. Salt. A number of investigations have been undertaken regarding the effect of salt (sodium chloride) upon the growth of molds. Eschenhagen (43) reported that Aspergillus niger would grow in a 17 per cent solution of sodium chloride in an inorganic medium, Penicillium at 18 per cent and Botrytis cinerea at 12 per cent. Kosinski (69) was of the opinion that a 0.26 molar solution (1.523 per cent) of sodium chloride did not effect the respiration of Aspergillus niger. According to Gustafson (54), spores of Aspergillus niger failed to germinate in 0.5 molar solution of sodium chloride (about 3 per cent). Lindet (76) explained the antiseptic action of salt upon the basis of its depriving microörganisms of a portion of their elementary structure through plasmolysis. Wöltje (146) studied the effect of various concentrations of sodium chloride in different media upon the growth of eighteen species of Penicillium. The killing concentration of salt at 15 to 17° C. for conidia was found to be from 6 to 26 per cent and for mycelium from 8 to 27 per cent, depending upon the species. Extensive microscopic studies of the effect of salt upon the growth of Penicillium expansum, Alternaria solani, and Oidium lactis were reported by

Bitting (11). She found that salt retarded growth and produced stunted development. It took longer for the conidia to germinate in higher salt concentrations. Oidium lactis was most susceptible and did not grow in 15 per cent salt. Holliard (85) found that sodium chloride at concentrations up to 10 per cent lowered the activity of Sterigmatocytis nigra. Golding (51) studied four species of Penicillium, isolated from blue-veined cheese and inoculated into sweet skimmilk and Czapek's medium containing 4, 8, 12, and 16 per cent of salt. The salt decreased the power to digest casein as well as the growth of these organisms.

Observations have been made in a general way upon the effect of salt on the appearance of mold in butter. Alvord (2) reported that unsalted butter exported to England from the United States became moldy. Dean and Harcourt (29) found that salt was more effective in preventing mold than other preservatives. Barr and Wolff (19) (20) reported that salted butter with a normal curd content was not an exceptionally good substrate for molds and that mold spores could not germinate well when 2 per cent salt was present in the butter. Species of Mucor did not develop in salted butter and the growth of Penicillium was retarded. Rahn, Brown and Smith (100) found that Oidium lactis increased in unsalted but not in salted butter. The keeping quality of butter and margarine was

improved when they contained about 3 per cent of salt according to Fischer and Gruenert (48). Hastings (59) held the opinion that mold spores could not germinate in salted butter. Combs and Eckles (23) also concluded that the salt content of butter had a considerable influence upon the development of mold. Jacobsen (62) reported that vegetable margarine was well preserved only when salt or other preservatives were dissolved in the water droplets so that the molds were restrained. Abbott and Ashenfelter (1) made the observation that mold counts increased more rapidly in unsalted than in salted butter. The idea that heavy salting of liners, wrappers, tubs, etc. as well as the soaking of parchment paper in brine, decreased the possibilities of the development of mold upon the surface of butter or package, has been upheld by anonymous writers (4), (6) (7), and by Bŏggild (13), Burr (18), Davis (27), Dean (28), Ibsen (61), Rogers (108), and Zoffman (148).

More detailed studies of salt effects with especial reference to the butter industry have been undertaken. As reported elsewhere, Siedel (124) attributed to the action of molds, the roquefort, turnipy or beet flavors which developed in granular butter immersed in 10 per cent and saturated brine. While he did not record actual visible growth, he implied that some development had occurred. Gripenberg (53) made use of a butter "serum" which

consisted of the portion of butter removable after butter was melted and most of the fat decanted. When this serum contained 18 and 20 per cent of salt, molds (Penicillium sp. and Trichosporium sp.) were found growing in it after five to six months. When this material was diluted, the fungi grew much better. Hanging drops of butter serum containing 0, 10, and 25 per cent of salt were inoculated with Penicillium crustaceum and Trichosporium collae. In the unsalted serum growth was active after nine days for both types, with 10 per cent salt the growth was slight, while with 25 per cent no signs of growth were evident. Parchment paper dipped in salted butter serum showed no molding in the 24 per cent salt serum but increasingly heavy growth occurred in serum when the salt was present in decreasing amounts. McKay and Larsen (79) studied the growth of Penicillium glaucum in media containing various quantities of salt. The growth after two days was luxuriant when less than 9 per cent salt was present, noticeable with 9 per cent but only a trace of growth with 10 per cent salt. Fettick (45) followed the development of Oidium lactis and Penicillium glaucum in unsalted butter as well as butter containing 3 per cent of salt by plating samples at the beginning of the storage period and after seven weeks, two months, and four months. In the salted butter the number of molds decreased immediately and disappeared within the two

months while they increased steadily in the unsalted sample. He also prepared sterilized butter containing 0, 0.5, 1, 2, 3, 4, 5, and 6 per cent of salt. These samples were inoculated with Oidium lactis, Mucor macedo and Penicillium glaucum and stored for one week in a dark place at 17° C. Platings were made at the end of this period. All three species decreased in proportion to the increased percentage of salt. No growth was obtained in samples containing 4 per cent or more of salt. The effect of various percentages of salt in Czapek's medium was studied by Thom (138). Petri plate cultures of 21 species of Penicillium and 10 species of Aspergillus were prepared, using Czapek's solution with and without agar, and containing 10 per cent of sodium chloride. After nineteen days all of the Penicillium and Aspergillus cultures had grown on the solid medium, but only sixteen of the Penicillium and nine of the Aspergillus cultures in the liquid. The extent of growth varied with the different species. Growth of P. pinophilum, P. lilacinum, P. luteum, P. digitatum, P. purpurogenum, P. roseum, P. duclauxii, A. nidulans, A. fumigatus, and a check culture of O. lactis was stopped or reduced to a negligible amount in the liquid medium. Further, twelve species of Penicillium were inoculated on Czapek's medium containing 0, 5, 10, and 15 per cent of sodium chloride. After thirty-four days growth had occurred in

all samples reported. There was, relatively, less development, however, with increasing percentages of salt. It was determined that Oidium lactis was reduced to negligible growth when the amount of salt exceeded 6 per cent.

Thom and Shaw (139) investigated the growth of three cultures of Alternaria, four of Mucor, two of P. roqueforti, and individual cultures of the following, Cunninghamiella sp., Fusarium sp., O. lactis, Penicillium sp., P. expansum, P. stoloniferum, P. chrysogenum, P. purpurogenum, Rhizopus nigricans, Trichoderma sp. and a red mold on Czapek's agar containing 6.5 per cent and 14.4 per cent of salt. The plates were kept in a moist chamber. All grew in 6.5 per cent of salt with Oidium least and Penicillium best. In 14.4 per cent of salt, Alternaria and Penicillium alone developed, with the latter showing better growth. In an 18 per cent salt Czapek's agar, three species of Penicillium and Aspergillus repens grew but in 21 per cent the spores of P. chrysogenum were the only ones to germinate. Thom and Shaw noted that the growth on salted agar was better than on salted butter of the same per cent of salt. Denning (31) found butter in good condition after eight months storage at 50° F. in a 20 per cent solution of sodium chloride. Later experiments (32) indicated that butter kept in 30 per cent of salt had been preserved in good condition. Hormodendrum cladosporioides cultures were seeded into a medium

containing peptone, levulose and nutrient salts and varying quantities of sodium chloride by Boekhout and deVries (12). Five cultures grew in the medium containing 13.4 per cent salt, four in 14.5 per cent, two in 16 per cent, two in 17 per cent, and none in 18, 19, or 20 per cent. Samples of butter containing 0, 2, 2.5, and 3 per cent of salt were placed in Erlenmeyer flasks at 21° C. and held for one month after inoculating with two Hormodendrum cultures. Growth occurred only on the unsalted butter. It is evident that Boekhout and deVries obtained better growth on the salted, synthetic medium.

Paraschtschuk (90) (91) studied the growth of several species of mold on malt agar containing varying percentages of salt. His results are shown in the following table:

Culture	Percent of salt			
	5%	10%	18%	25%
Mucor sylvaticus	***	***	-	-
Mucor racemosus	***	**	-	-
Mucor hiemalis	***	***	-	-
Mucor roseum	***	**	-	-
Rhizopus arirus	***	**	-	-
Cladosporium herbarum nigr.	***	***	**	-
Stilbaciae graphium	***	*	-	-
Oidium lactis	**	-	-	-

	5%	10%	18%	25%
Penicillium glaucum	***	***	***	-

Legend: ***= abundant; ** = medium; * = scanty; - = none.

Unquestionably salt has a marked effect upon the growth of the molds. According to the foregoing evidence the influence varies, however, depending upon the species and the substrate. Altho unsalted butter is most commonly affected, the fact remains that mold appears often on salted butter. This occurrence demands explanation.

2. Degree of Acidity. Schaffer (115) stated that the growth of molds was rather favored by the low acidity of butter made from pasteurized cream. According to Laxa (73) the presence of lactic acid did not hinder markedly the peptonization of casein by molds. In studies carried on by Boekhout and deVries (12) two cultures of Hormodendrum cladosporioides were grown on a base medium containing lactic acid. One culture was checked by 0.75 per cent lactic acid, while the other was not restrained until the percentage of acid reached 1.0 per cent.

None of these investigations was based upon carefully controlled conditions in butter so give no satisfactory idea of the effect of acidity in butter upon the growth of molds therein.

A large number of papers dealing with the effect of

hydrogen-ion concentration upon the development of molds have been presented in recent years. Only two of these are mentioned here. Gustafson (55) studied the effect of various hydrogen-ion concentrations upon the respiration of Penicillium chrysogenum. He found that the rate of respiration was not effected within the range of pH 4.0 and pH 8.0. Johnson (64) at the Iowa station reviewed previous investigations and reported that the acid and alkaline reactions inhibiting the growth of seven molds were as follows:

	acid	alkaline
Mucor glomerula	pH 3.2 - 3.4	pH 8.7 - 9.2
Fusarium bullatum	pH 2.0 - 2.2	pH 9.2 - 11.2
Aspergillus oryzae	pH 1.6 - 1.8	pH 9.0 - 9.3
Fusarium oxysporum	pH 1.8 - 2.0	pH 9.2 - 11.1
Penicillium variable	pH 1.6 - 1.8	pH 10.1 - 11.1
Aspergillus terricola	pH 1.6 - 1.8	pH 9.0 - 9.3
Penicillium italicum	pH 1.9 - 2.2	pH 9.1 - 9.3

These investigations clearly indicate that most molds grow over a wide range of hydrogen-ion concentrations. The majority of species are favored by an acid medium and the limits of acidity reached in ordinary butter are such that the hydrogen-ion concentrations, as such, should not be much of a factor in influencing mold development. The nature of the substances bringing about the

changes in pH appears to be of much greater significance.

3. Light. Altho the general opinion, expressed by Rogers (108) that molds developed much better in the dark than in the light, has been held by most investigators, Kolkwitz (67) and Maximow (82) suggested that at times light accellerated the metabolism of fungi. In practise however, butter is kept in packages and storage rooms where light does not gain direct access to it. Consequently, one would not expect light to be much of a factor in the growth of molds on butter.

4. Vitamines. There have been some investigations into the effect of growth-stimulating substances such as vitamines upon the development of molds. Linossier (77) (78) pointed out that Oidium lactis, Aspergillus niger, and Penicillium glaucum were able to grow in pure culture in media lacking vitamines but containing necessary nutrients. When the nutrients were reduced greatly, the addition of vitamine-containing substances such as orange juice sometimes stimulated growth. Lepeschkin (75) reviewed the literature upon the subject and concluded that vitamines were not of great importance in the nutrition of molds.

At any rate, butter ordinarily contains vitamines and in sufficient quantities and variety in most cases to satisfy the slight demands of the fungi.

Species of Molds Isolated from Butter

Many species of molds have been isolated from butter but they have not always been shown to be the specific causes of defects in appearance, flavor or aroma of such butter. The species of molds are important factors inasmuch as one must determine what types actually grow in butter even under the most favorable conditions. Many forms encountered when butter is plated may exist in butter only in the form of spores which do not germinate under the conditions existing in the product. Every species encountered should be studied to determine whether or not it can develop in ordinary butter.

It is not necessary to present at this time anything more than a catalog of some of the species that have been isolated from butter, giving the species name applied by the investigator who reported it, and the key number to locate the authority in the bibliography. In addition attention may be called to the fact that See (119) described a number of species isolated from paper. It is possible that some of these might be encountered on parchment paper used for wrapping butter.

Alternaria sp. (22) (139)

Aspergillus sp. (18) (26) (90) (91) (128) (129)

Aspergillus flavus (9)

Aspergillus glaucus (113) (129)

Aspergillus niger (19) (62)

Aspergillus oryzae (9)

Botricanus scostan (90) (91)

Botrytis sp. (58)

Chaetomium sp. (36)

Cladosporium sp. (90) (91) (129) (139)

Cladosporium butyri (62) (63)

Cladosporium herbarum (80) (90) (91)

Coniosporium sp. (26)

Coniothecium sp. (26)

Dematium sp. (70)

Dematium pullulans (80)

Epicoccum sp. (22)

Eurotium repens (68)

Eurotium rubrum (68)

Hormodendrum cladosporioides (12)

Monilia sp. (83) (90) (91) (96)

Monilia alba (90) (91)

Monilia candida (90) (91)

Monilia roseum (90) (91)

Mucor sp. (18) (22) (53) (56) (58) (90) (91) (105) (139)

Mucor hiemalis (90) (91)

Mucor mucedo (5) (19) (46) (57) (133)

Mucor petrinsularis (36)

Mucor racemosus (90) (91)

Mucor spinosus (56)

Mucor sylvaticus (90) (91)

Oidium lactis (5) (8) (22) (39) (45) (58) (63) (83) (86) (87) (88) (89) (90) (91) (96) (100) (105) (113) (133) (139)

Oidium variicolor (97)

Oospora ruberrima (111)

Penicillium sp. (18) (19) (26) (35) (53) (58) (90) (91) (105) (128) (131)

Penicillium brevicaule (142) (143)

Penicillium chrysogenum (139)

Penicillium crustaceum (53)

Penicillium expansum (139)

Penicillium glaucum (5) (9) (19) (36) (39) (40) (45) (46) (62) (63) (70) (90) (91) (113) (129) (130) (133)

Penicillium olivaceum (?) (80)

Penicillium roqueforti (22) (70) (139)

Rhizopus arirus (90) (91)

Stemphylium butyri (93) (123) (139)

Sterigmatocystis sp. (36)

Stilbaciae graphium (90) (91)

Trichosporium collae (53)

Verticillium sp. (36)

Molds isolated from paper. See (119).

PURPOSE OF STUDY

The purpose of the studies reported on subsequent pages has been to determine the influence of certain factors upon the growth of molds with special reference to their development in butter. It has been the intention of the writer to investigate some of the basic considerations with a hope that the results might offer opportunities for their interpretation in terms of the complex interrelationships of the various factors which appear to be operating in such a valuable and concentrated food product as butter. It was anticipated that many phenomena would appear, to throw light on some of the perplexing problems at present recognized, and to serve as bases upon which further studies might rest.

METHOD OF PROCEDURE

In the beginning, it was necessary to decide what factors appeared to be most important in their influence upon the growth of molds, particularly in butter. It was thought that these were as follows: (1) Food supply, (2) Moisture, (3) Temperature, (4) Atmosphere, and (5) Miscellaneous chemical and physical factors, especially the effect of various concentrations of sodium chloride.

Species of Molds Used in Experiments

In order to have one fixed condition thruout the experiments, ten species of molds were selected to be used in the studies to be undertaken. These cultures were representative of types of molds which have been found commonly in butter. As a matter of convenience in the presentation of the experimental data, reference will be made to them by number. These cultures and their corresponding key numbers are as follows:

1. Alternaria humicola Oudemans
2. Aspergillus flavus Link
3. Aspergillus niger Van Tieghem
4. Hormodendrum cladosporioides (Fresenius) Saccardo
5. Mucor sylvaticus Hagem
6. Oospora lactis var. A. (Fresenius) Lindan

7. Oospora lactis var. B. (Fresenius) Lindau

8. Penicillium biforme Thom

9. Penicillium expansum Link

10. Rhizopus nigricans Ehrenberg

The Mucor and Rhizopus stock cultures were carried on potato dextrose agar, the Oospora cultures on whey agar and the remainder on Czapek's agar. These media gave the most luxuriant growth for the particular species. Inoculations in the various experiments were made directly from cultures ten days to two weeks in age, particular care being taken to transfer none of the culture medium.

Methods Employed in Experiments

The methods employed for preparing the different substrata, for maintaining the desired temperature, humidity, etc., will be discussed under the respective subdivisions of the material presented.

Manner of Recording Results

The extent of growth made by the ten species on different substrata and under other varying conditions will be recorded uniformly by the following symbols:

- \- No visible growth
- ± Questionable visible growth
- \+ Slight, visible growth
- ++ Moderate growth
- +++ Abundant growth
- ++++ Normal growth

Note: Wherever these symbols are arranged in parentheses, they indicate subsurface growth.

In a similar manner, the color produced by the cultures under the various conditions will be registered according to the following symbols:

- W - White
- Y - Yellow
- G - Green
- B - Black
- Br - Brown
- C - Cream

EXPERIMENTAL

The experimental data will be presented under the following subdivisions:

A. Food supply
    1. Fats and related substances
    2. Proteins and related substances
    3. Carbohydrates and related substances
    4. Mineral constituents
    5. Combinations of (1), (2), (3), and (4).

B. Moisture
    1. Moisture in substrate
    2. Moisture in atmosphere

C. Temperature

D. Atmosphere
    1. Ordinary
    2. Reduced CO_2
    3. Vacuum
    4. Oxygen-free.

E. Miscellaneous chemical and physical conditions
    1. Salt content.

A. Food Supply

The primary requirement for the growth of microorganisms is a satisfactory food supply. Butter furnishes a variety of foodstuffs, principally fat but also proteins, carbohydrates and mineral salts in varying amounts. Each of these constituents has been studied as a source of food for the ten species of molds previously selected.

1. Fats and Related Substances.

a. Fresh Butterfat.

Methods. Fresh unsalted butter was melted at a temperature of 50 to 55° C. and placed in a warm, separatory funnel. The curd and water which settled out were drawn off. The fat was washed five times in the funnel by shaking with it equal quantities of water at 60 to 65° C. After each washing, the mixture was allowed to stand until the layers of fat and water were distinct, whereupon the water was drawn from the funnel. The washed fat was tempered to 55° C. and left at this temperature for 24 hours during which time it was filtered thru filter paper to remove traces of water. The filtered fat was tubed in ten cubic centimeter amounts and autoclaved. When ready to use, the fat was melted and poured into sterile petri plates and solidified quickly. Analyses of the autoclaved fat showed it to be water-free. The cultures were streaked across the surface

of the fat as uniformly as possible. The plates were placed in piles on a rack in a humidor consisting of a ten gallon, covered, earthenware crock in the bottom of which was a one inch layer of water containing sufficient bichloride of mercury to maintain it as a sterile fluid. The relative humidity, determined by a wet-dry bulb hygrometer, was maintained at 100 per cent. The temperature of incubation ranged from 20 to 25° C.

Results. The extent of growth in the fresh butterfat is indicated in Table I. After one week the only visible growth was noted with Culture 5 * and it was evident that the development was restricted to the point of inoculation where sufficient residual nutriment may have been present in the mycelium inoculated. At the end of six weeks, Culture 5 * had produced a few black sporangia, while Culture 5 had shown no further increase, rather a slight diminution in the mass of growth. A microscopic examination of the plates after six weeks incubation revealed signs of germination of the conidia in the case of Cultures 3, 4, 5, 7, 8, and 9, but very little hyphal development except in Culture 5 where the mycelium had developed extensively.

b. Old Butterfat.

Methods. The method for preparing the old butterfat was the same as that previously described for fresh butter-

* See key on page

TABLE I

GROWTH OF MOLD CULTURES ON FRESH BUTTERFAT

Culture No.	Extent of growth at 20-25°C., high humidity, after						
	4 days	1 week	2 weeks	3 weeks	4 weeks	6 weeks	6 weeks Micro-scopic
1	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-
3	-	-	-	-	-	+B	+
4	-	-	-	-	-	-	+
5	-	+	+	+	+	+	+
6	-	-	-	-	-	-	-
7	-	-	-	-	-	-	+
8	-	-	-	-	-	-	+
9	-	-	-	-	-	-	+
10	-	-	-	-	-	-	-
Check	-	-	-	-	-	-	-

fat. The butter used was unsalted, one month old, and decidedly cheesy in flavor. The surface was discolored with spots of mold. The conditions for inoculation and incubation were precisely the same as those for the fresh butterfat.

Results. As shown in Table II growth was evident after four days in the case of Cultures 3 and 8 but again this development was at the point of inoculation. Culture 5 produced a few black sporangia while Culture 8 formed a light green spot. At the end of one week, Culture 10 showed slight aerial mycelium which disappeared within the following week. Culture I began to produce a green spot after three weeks. This became deeper in color during the subsequent period. Culture 9 developed a small green spot after the third week. A dark green streak appeared along the line of inoculation in the case of Culture 4 at the expiration of the six weeks incubation period. The microscopic examination indicated that Cultures 1, 2, 3, 4, 8, and 9 had made slight growth during the trial. In general, the growth on this type of butterfat was somewhat better than that on the fresh butterfat.

c. Butterfat from Washed Cream.

Methods. Four pounds of 30 per cent sweet cream were diluted to three gallons with ordinary tap water and heated to 35° C. The diluted cream was then separated in a centrifugal separator. The resultant cream was diluted again in the same

TABLE II

GROWTH OF MOLD CULTURES ON OLD BUTTERFAT

Culture No.	Extent of growth at 20-25°C., high humidity, after 4 days	1 week	2 weeks	3 weeks	4 weeks	6 weeks	6 weeks Microscopic
1	-	-	-	+G	+	+G	+
2	-	-	-	-	-	-	+
3	+B	+	+	+	+	+	+
4	-	-	-	-	-	+G	+
5	-	-	-	-	-	-	-
6	-	-	-	-	-	-	-
7	-	-	-	-	-	-	-
8	+G	+	+	+	+	+	+
9	-	-	-	-	+G	+	+
10	-	+W	-	-	-	-	-
Check	-	-	-	-	-	-	-

way, heated, and separated as before. The process was repeated until the cream had been diluted and separated ten times. The cream that was finally obtained was placed at 12° C. and allowed to stand overnight. During this period the fat had formed a solid mass floating above a slightly cloudy serum. The mixture was heated to 50° C. and placed in a warm separatory funnel. In this way, it was possible to remove the serum. The fat was washed five times with water at 60 to 65° C. Thereafter, it was placed at 55° C. for 24 hours during which time it was allowed to filter thru ordinary filter paper. An analysis showed that all water had been removed. The fat was placed in test tubes and autoclaved. When desired for use the fat was melted, poured into petri plates and solidified promptly. The conditions of inoculation and incubation were the same as those described in the preceding experiments.

Results. As in the previous experiment, Cultures 3 and 8 gave evidence of growth after four days, according to the data presented in Table III. Culture 10 had developed a noticeable aerial mycelium during this period but as happened before, this mycelium gradually disappeared. A scanty aerial mycelium was sent up by Culture 5 after one week but this likewise disappeared after five weeks. A green streak following the line of inoculation of Culture 4, appeared at the end of four weeks, while Culture 9 had

T A B L E III

GROWTH OF MOLD CULTURES ON BUTTERFAT FROM WASHED CREAM

Culture No.	Extent of growth at 20-25°C., high humidity, after 4 days	1 week	2 weeks	3 weeks	4 weeks	6 weeks	6 weeks Microscopic
1	-	-	-	-	-	-	-
2	-	-	-	-	-	-	+
3	+B	+	+	+	+	+	+
4	-	-	±	±	+ G	+	+
5	-	+W	+	+	+	-	+
6	-	-	-	-	-	-	-
7	-	-	-	-	-	-	-
8	+G	+	+	+	+	+	+
9	-	-	-	±	+G	+	+
10	+W	+	-	-	-	-	+
Check	-	-	-	-	-	-	-

produced a slight green spot during this same period. The microscopic examination made after six weeks showed that Cultures 2, 3, 4, 5, 8, 9, and 10 had been able to develop slightly near the point of inoculation.

d. Butterfat Washed with Alcohol.

Methods. A portion of the fresh butterfat used in the experiment reported in Table I was mixed with equal parts of 95 per cent ethyl alcohol and shaken thoroly. The fat was drawn off by means of a separatory funnel, placed at 55° C. and filtered thru paper, after which it was placed on a waterbath to drive off any remaining alcohol. The final analysis showed no traces of water in the fat. The fat was tubed and autoclaved. When desired, it was melted and poured into petri dishes where it was solidified. The fat was inoculated and incubated in the same manner as described in the preceding experiments.

Results. Table IV gives the results of the experiment. Culture 4 as before produced a few black sporangia after four days. At the end of one week, Culture 5 had sent forth scanty aerial mycelium but as had happened in the previous experiments, this disappeared after six weeks. Culture 2 appeared to grow on this preparation of butterfat and after a period of one week, a few yellowish-green sporangia were visible. After three

TABLE IV

GROWTH OF MOLD CULTURES ON BUTTERFAT WHICH HAS BEEN WASHED WITH ALCOHOL

Culture No.	Extent of growth at 20-25°C., high humidity, after 4 days	1 week	2 weeks	3 weeks	4 weeks	6 weeks	6 weeks Micro-scopic
1	-	-	-	-	-	+G	+
2	-	+G	+	+	+	+	+
3	+G	+	+	+	+	+	+
4	-	-	-	+G	+	+	++
5	-	+W	+	+	+	-	-
6	-	-	-	-	-	-	-
7	-	-	-	-	-	-	-
8	-	-	-	-	+G	+	+
9	-	-	-	-	+G	+	+
10	-	-	-	-	-	-	-
Check	-	-	-	-	-	-	-

weeks, Culture 4 developed to such an extent that green streaks were evident and green spots were produced by Cultures 8 and 9, Finally, at the end of six weeks, Culture 1 was able to produce a small, green spot. The microscope revealed rather extensive development of Culture 4 and slight mycelial growth of Cultures 1, 2, 3, 8, and 9. In general, the growth in the fat washed with alcohol was slightly better than that observed on the other preparations.

e. Fresh Butterfat plus Water.

Method. The fresh butterfat, as prepared in the experiment (a) described previously, was used in the trial. In order to provide a substrate in which the fat and water might be more intimately associated and in a more finely-divided state, a medium containing 1.5 per cent of washed agar was prepared. Fifteen grams of Bacto agar were placed in a cloth bag which was suspended in running water overnight in order to remove some of the soluble and finely divided impurities. After the washed agar had been dried at 55° C. it was weighed and sufficient distilled water added to make a 1.5 per cent concentration of the agar. This became a substrate which supplied sufficient water but very little nutriment. Similar batches of this same medium were prepared at various times, sometimes containing 1.0 per cent and sometimes 1.5 per cent of agar depending upon circumstances. This medium was placed in

measured amounts in test tubes and autoclaved. When ready for use in combination with the fat, the agar and fat were melted and the butterfat added to the agar in the proportions of four parts of agar to one part of fat. The mixture was shaken thoroly until the fat was in a finely-divided state and then poured into petri plates and cooled on a cold surface as quickly as possible. The droplets of fat were evenly dispersed thruout the solidified agar. The fat-agar mixtures were inoculated by streaking the cultures across the surface. The plates were stored at room temperature (20 to 25° C.) for a period of six weeks, at a relative humidity varying from 60 to 65 per cent.

Results. The fact that molds made very meagre growth on the washed agar substrate is indicated in Table V. The results reported are representative of other lots of this same medium used in later experiments. Very slight growth was obtained at any time, and in most cases it was barely visible, especially on the surface altho a slight penetration of the mycelium below the surface was noted in some instances. As shown in Table VI, the presence of water led to much more extensive growth of all the cultures with the exception of Cultures 6 and 7. Cultures 1, 3, and 4 grew especially well. It is significant to note that Cultures 8 and 9 were able to produce a more or less typical odor of

TABLE V

GROWTH OF MOLD ON 1.5% WASHED AGAR

Culture No.	Extent of growth at 20-25° C., ordinary humidity, after					
	4 days	1 week	2 weeks	3 weeks	4 weeks	4 weeks Micro-scopic
1	+W	+	+	+	+	+
2	+	+	+	+	+	+
3	+	+	+	+	+	+
4	+	+	+	+	+	+
5	+W	+	+	+	+	+
6	-	-	-	-	-	-
7	-	-	-	-	-	-
8	±	+	+	+	+	+
9	±	±	±	±	±	+
10	+W	+	+	+	+	+
Check	-	-	-	-	-	-

TABLE VI

GROWTH OF MOLD ON 1.5% WASHED AGAR PLATES WITH FRESH BUTTERFAT ADDED

(4 parts of agar medium: 1 part of butterfat)

Culture No.	Extent of growth at 20-25°C., ordinary humidity, after					
	4 days	1 week	2 weeks	3 weeks	4 weeks	4 weeks Microscopic
1	+W	+W	++G	++	++	+
2	-	+Y	+Y-G	+	+	+
3	+B	++B	++	++	++	+
4	±	++G	++	++	++	+
5	+W	+	+	+	+	+
6	-	-	-	-	-	-
7	-	-	-	-	-	-
8	- (*)	+G	+	+	+	+
9	- (*)	+G	+	+	+	+
10	+W	+	+	+	+	+
Check	-	-	-	-	-	-

(*) Roquefort odor.

Roquefort cheese before there were any signs of visible growth.

f. Old Butterfat plus Water.

Methods. The proceedure was the same as that just described under (e) with the exception that the old butterfat prepared for experiment (b) was employed.

Results. The growth of the molds on old butterfat was increased when water was present, as the data in Table VII indicate. The development of Cultures 1, 3, and 4 was particularly good. Cultures 6 and 7 failed to grow. It may be noted that the Roquefort cheese odor which appeared in the fresh butterfat with water in the case of Cultures 8 and 9 was not evident in the old butterfat-water mixture.

g. Alcohol-extracted Butterfat plus Water.

Methods. The method of preparing the substrate was the same as that described previously in (e) except that the butterfat washed with alcohol (see experiment (d) preceding) was used. The methods of inoculation and incubation were the same as those followed in experiment (e).

Results. The growth of Cultures 1, 2, 3, and 4 was quite extensive as shown in Table VIII. As before, cultures 6 and 7 did not show any signs of development. The Roquefort cheese odor was apparent in the case of Culture 8 at the end of four days.

TABLE VII

GROWTH OF MOLD ON 1.5% WASHED AGAR PLATES

WITH OLD BUTTERFAT ADDED

(4 parts of agar medium: 1 part of butterfat)

Culture No.	Extent of growth at 20-25°C., ordinary humidity, after					
	4 days	1 week	2 weeks	3 weeks	4 weeks	4 weeks Microscopic
1	+W	+	++G	++	++	+
2	-	-	+Y-G	+	+	+
3	+B	++	++	++	++	+
4	±	++G	++	++	++	+
5	+W	+	+	+	+	+
6	-	-	-	-	-	-
7	-	-	-	-	-	-
8	-	-	+G	+	+	+
9	-	-	+G	+	+	+
10	+W	+	+	+	+	+
Check	-	-	-	-	-	+

T A B L E VIII

GROWTH OF MOLD ON 1.5% WASHED AGAR PLATES WITH ALCOHOL EXTRACTED, BUTTERFAT ADDED

(4 parts of agar medium: 1 part of butterfat)

Culture No.	Extent of growth at 20-25°C., ordinary humidity, after 4 days	1 week	2 weeks	3 weeks	4 weeks	4 weeks Micro-scopic
1	+W	+	++G	++	++	+
2	+Y	++Y-G	++	++	++	+
3	+B	++	++	++	++	+
4	±	++G	++	++	++	+
5	+W	+	+	+	+	+
6	-	-	-	-	-	-
7	-	-	-	-	-	-
8	- (*)	+G	+	+	+	+
9	-	+G	+	+	+	+
10	+W	+	+	+	+	+
Check	-	-	-	-	-	-

(*) Roquefort odor.

In general, the growth was practically the same as when water was added to the fresh and old butterfats.

h. 0.5 per cent Aqueous Emulsion of Milk Lecithin.

Methods. A highly purified lecithin obtained from milk by Dr. W. E. Petersen and Dr. L. M. Thurston of the Minnesota Agricultural Experiment Station was emulsified by thoro shaking in water. It produced a stable, milky emulsion which was tubed and autoclaved. After sterilization, a flocculent precipitate was formed but it remained uniformly suspended after cooling. The emulsion was inoculated from the various cultures and left at room temperature (20 to 25° C. for a period of three weeks, at a relative humidity of 60 to 65 per cent.

Results. Table IX gives the results obtained with this emulsion of lecithin. With Culture 3, a white mycelium and a few black sporangia appeared on the walls of the tube just above the surface of the liquid after three days. Culture 8 produced a good, green surface growth at the end of one week. Cultures 1, 2, and 9 produced white rings near the surface of the solution after two weeks but at the end of three weeks were growing reasonably well on the surface. All of the cultures showed subsurface mycelial development, which was especially good in the case of Culture 10. Unquestionably lecithin was a fairly good source of food for these species.

TABLE IX

GROWTH OF MOLDS IN 0.5% AQUEOUS SOLUTION OF MILK LECITHIN

Culture No.	Extent of growth at 20-25°C., ordinary humidity, after 3 days	1 week	2 weeks	3 weeks
1	-	-	+W (++)	++G (++)
2	-	-	+W (+)	++Y G (+)
3	+ W B	+	+ (+)	+B (+)
4	-	-	+G (+)	+G (+)
5	-	-	- (+)	- (+)
6	-	-	- (+)	- (+)
7	-	-	- (+)	- (+)
8	-	++G	++ (+)	++ (+)
9	-	-	+W (+)	+ (+)
10	-	-	- (+)	- (+)
Check	-	-	-	-

i. 0.5 per cent Aqueous Emulsion of Milk Lecithin in 1.5 per cent Washed Agar.

Methods. A portion of the sterile lecithin emulsion, prepared according to the methods described in the preceding trial, was added to an equal amount of melted sterile, 1.5 per cent washed agar made up in five cubic centimeter amounts in test tests. After thoro mixing, the medium was slanted. The agar slants were inoculated over the surface in the usual manner. The tubes were incubated at room temperature (20 to 25° C.) for three weeks at a relative humidity of 60 to 65 per cent.

Results. It will be noted in Table X that growth began promptly in all cases, and became practically normal after three weeks with the exception of Cultures 6 and 7 which already have been shown to do very poorly on fat or fat-like substrata. The development of the cultures was much better upon the solid medium than upon the fluid preparation.

j. 1 per cent Glycerol in 1.5 per cent Washed Agar.

Methods. Chemically pure glycerol was added to melted, sterile, 1.5 per cent washed agar in amounts sufficient to make a 1 per cent solution. This mixture was tubed, autoclaved and slanted. The usual agar stroke was made with each culture. The cultures were then placed in a humidor which consisted of a

T A B L E X

GROWTH OF MOLDS ON 1.0% WASHED AGAR SLANTS WITH 0.5% AQUEOUS SOLUTION OF MILK LECITHIN ADDED

(Equal parts of agar medium and lecithin solution)

Culture No.	Extent of growth at 20-25° C., ordinary humidity after 3 days	1 week	2 weeks	3 weeks
1	++W	+++G	+++G	+++
2	+W	++Y	++Y G	++
3	+W	+W	+B	++
4	+W	++G	+++G	+++
5	++W	+++	+++W-B	+++
6	+W	+	+	+
7	+W	+	+	+
8	+W	+	+G	++
9	+W	+	+++G	+++
10	++W	+++	+++W-B	+++
Check	-	-	-	-

covered ten gallon metal churn, equiped with a false, perforated bottom, under which was placed a quantity of water to which bichloride of mercury had been added to maintain the sterility of the water. The relative humidity was maintained at 100 per cent. The temperature of incubation was 20 to 25° C. and the storage period three weeks.

Results. Table XI gives the results of this experiment. It will be observed that Cultures 2, 6, 7, and 10 showed some visible growth after three days. With the remainder, growth became noticeable after one week. However, none of the cultures exhibited any further surface growth, with the exception of Culture 2 which had developed quite extensively after three weeks. The significant result was the extensive subsurface growth of nearly all cultures. This subsurface development in most cases was much more marked than the surface growth. Cultures 1 and 4 produced remarkable dark green subsurface mycelium. This same phenomenon appeared in aqueous solutions of glycerol, studied in preliminary unreported trials.

k. 1 per cent Butyric Acid in 1.5 per cent Washed Agar.

Methods. Purified butyric acid was added to measured amounts of tubed, melted, sterile, 1.5 per cent washed

TABLE XI

GROWTH OF MOLDS ON 1.5% WASHED AGAR CONTAINING 1% GLYCEROL

Culture No.	Extent of growth at 20-25° C., high humidity, after 3 days	1 week	2 weeks	3 weeks
1	±	+W (++)	+W (+++G)	+G (+++G)
2	+W (+)	+G (++)	+G (++)	++G (+++)
3	±	+B (+)	+B (+)	+B (++)
4	± (+G)	+G (++G)	+G (+++G)	+G (+++G)
5	± (++)	+W (++)	+W (++)	+W (++)
6	+W (++)	+W (++)	+W (+++)	+W (+++)
7	+W (++)	+W (++)	+W (+++)	+W (+++)
8	±	+G (+)	+G (+)	+G (+)
9	± (+)	+G (++)	+G (+++)	+G (+++)
10	+W (+)	+W (+)	+W (+)	+W-B (+)
Check	-	-	-	-

agar medium in quantities sufficient to make a 1 per cent solution. These tubes were shaken thoroly and slanted. After inoculation the cultures were placed in a metal humidor (described previously) at 20 to 25° C. for a period of three weeks, and at a relative humidity of 100 per cent.

Results. Table XII reveals the fact that the cultures under investigation were unable to develop in this concentration of butyric acid. This is in agreement with the results of preliminary studies.

1. 1 per cent Palmitic Acid in 1.5 per cent Washed Agar.

Methods. Purified palmitic acid was added in a sufficient quantity to a sterile, melted 1.5 per cent washed agar medium to make a 1 per cent concentration. After warming the mixture in a steam bath until the palmitic acid melted, it was shaken thoroly and placed as quickly as possible in sterile tubes. The tubes were rotated thoroly so that the acid was fairly well dispersed and the medium solidified as quickly as possible in a slanted position. The acid hardened on the surface in areas ranging from 0.1 mm. to 5.0 mm. in diameter with equal areas of clear agar between them, while the bulk of the medium was clear. The inoculations were made over the surface in such a way that the cultures covered

TABLE XII

GROWTH OF MOLDS ON 1.5% WASHED AGAR CONTAINING 1% BUTYRIC ACID

Culture No.	Extent of growth, at 20-25° C., high humidity, after 3 days	1 week	2 weeks	3 weeks
1	-	-	-	-
2	-	-	-	-
3	-	-	-	-
4	-	-	-	-
5	-	-	-	-
6	-	-	-	-
7	-	-	-	-
8	-	-	-	-
9	-	-	-	-
10	-	-	-	-
Check	-	-	-	-

representative areas of the solidified acid. The tubes were incubated under the same conditions prevailing in the previous experiment.

Results. The growth on the medium containing palmitic acid as the source of nutriment was fairly good, as shown in Table XIII. All cultures exhibited some surface growth which was much better than that obtained on the agar checks. The remarkable feature of this series, however, was the extensive development of Cultures 1, 2, 4, 5, 6, and 10 below the surface. The mycelium grew directly away from the surface of the slants and in the case of Cultures 1 and 4, the hyphae were dark green in color.

m. 1 per cent Stearic Acid in 1.5 per cent Washed Agar.

Methods. The 1 per cent concentration of stearic acid in 1.5 per cent washed agar substrate was prepared in the same manner as the preceding mixture of palmitic acid and agar. The emulsion obtained in this case, however, was more uniform. The medium became very cloudy. The inoculation and incubation conditions were identical with the foregoing (k) and (l).

Results. Table XIV gives the results of this experiment. The only surface development observed in this medium occurred with Culture 1 where a small area of growth appeared about the point of inoculation. The medium was so opaque that it was

T A B L E XIII

GROWTH OF MOLDS ON 1.5% WASHED AGAR CONTAINING 1% PALMITIC ACID

Culture No.	Extent of growth, at 20-25° C., high humidity, after 3 days	1 week	2 weeks	3 weeks
1	+W(++G)	+G(++G)	+G(++G)	+G(++G)
2	+W(++)	+Y-G(++)	+Y-G(++)	+Y-G(++)
3	+W(+)	+B(+)	+B(+)	+B(+)
4	+W(+)	+G(++G)	+G(++G)	+G(++G)
5	+W(++)	+W(++)	++W(++)	++W(++)
6	-(+)	+W(+)	+W(++)	+W(++)
7	-(+)	+W(+)	+W(+)	+W(+)
8	-	+G(+)	+G(+)	+G(+)
9	-	+G(+)	+G(+)	+G(+)
10	+W(++)	++W(++)	++W-B(++)	++W-B(++)
Check	-	-	-	-

T A B L E XIV

GROWTH OF MOLDS ON 1.5% WASHED AGAR CONTAINING 1% STEARIC ACID

Culture No.	Extent of growth, at 20-25° C., high humidity, after 3 days	1 week	2 weeks	3 weeks
1	+F	+F	+F-G	+G
2	-	-	-	-
3	-	-	-	-
4	-	-	-	-
5	-	-	-	-
6	-	-	-	-
7	-	-	-	-
8	-	-	-	-
9	-	-	-	-
10	-	-	-	-
Check	-	-	-	-

impossible to determine whether any subsurface growth had taken place.

n. 1 per cent Oleic Acid in 1.5 per cent Washed Agar.

Methods. Purified oleic acid was measured into definite amounts of sterile melted 1.5 per cent washed agar medium in tubes so that the concentration of acid was 1 per cent. The mixture was shaken thoroly and solidified as quickly as possible in a slanting position. The oleic acid appeared on the surface in clear droplets, approximately 1 mm. in diameter and close together. The conditions of inoculation and incubation were exactly the same as those used in the three preceding trials.

Results. As indicated in Table XV, the growth on this substrate was better than that obtained on media containing any of the other acids commonly found in butterfat. Surface development was quite good in all excepting Cultures 3, 6, 7, and 8. The subsurface mycelium was remarkably heavy, especially in Cultures 2, 3, 4, 7, and 9. Cultures 1 and 4 produced their characteristic dark green hyphae in the depth of the medium.

2. Proteins and Related Substances.

a. 1 per cent Aqueous Solution of Peptone

Methods. A 1 per cent solution of Bacto peptone

T A B L E XV

GROWTH OF MOLDS ON 1.5% WASHED AGAR CONTAINING 1% OLEIC ACID

Culture No.	Extent of growth, at 20-25° C., high humidity, after 3 days	1 week	2 weeks	3 weeks
1	+W(++)	++W-G(++G)	++G(++G)	++G(++G)
2	+G(++)	++G(+++)	++G(+++)	++G(+++)
3	- (+)	+B(++)	+B(++)	+B(+++)
4	+G(++)	++G(++G)	++G(+++G)	++G(+++G)
5	+W(++)	++W(++)	++W(++)	++W(++)
6	- (+)	+W(++)	+W(++)	+W(++)
7	- (+)	+W(++)	+W(+++)	+W(+++)
8	- (+)	+G(++)	+G(++)	+G(++)
9	- (+)	+G(++)	++G(+++)	++G(+++)
10	+W(++)	++W(++)	++W-B(++)	++W-B(++)
Check	-	-	-	-

was prepared in distilled water, tubed and autoclaved. The peptone solution was inoculated in the usual manner with each of the cultures. The tubes were incubated under conditions identical with those employed in the preceding experiment.

Results. The growth of the cultures began promptly in this solution as indicated in Table XVI. At the end of one week, the development of all cultures was good, and in some cases perfectly normal. While some subsurface development was noted, it was not nearly as extensive as that upon the surface.

b. Curd Portion of Butter.

Methods. Fresh, unsalted butter was melted at 60 to 65° C., and placed in a warm separatory funnel from which the so-called curd was withdrawn with ease, as it settled below the melted fat. This curd, which consisted principally, of protein, was tubed and autoclaved. The water content was 86.3 per cent. The curd was inoculated with the test cultures. The tubes were placed in a humidor (as described under the preparation of fresh butterfat), where the relative humidity remained at 100 per cent during the storage period. The temperature of incubation was 20 to 25° C.

Results. The growth of all cultures, excepting Cultures 1 and 8 became visible within three days, as is shown in Table XVII. At the end of one week, the development was practi-

TABLE XVI

GROWTH OF MOLDS IN 1% AQUEOUS SOLUTION OF PEPTONE

Culture No.	Extent of growth, at 20-25° C., high humidity, after 3 days	1 week	2 weeks	3 weeks
1	+W (++)	++W-G (+)	++W-G	++G
2	++W	++W-Y	+++Y-Br	++++Br
3	++W (+)	+++B (+)	++++B (+)	++++B (+)
4	++G (+)	+++G (+)	++++G (+)	++++G (+)
5	++W (++)	+++W (+)	+++W (+)	++++W (+)
6	+++W (++)	++++W (++)	++++W (++)	++++W (++)
7	++W (++)	+++W (++)	++++W (++)	++++W (++)
8	++G (+)	+++G (+)	++++G (+)	++++G (+)
9	++W (+)	++++W	++++W	++++W
10	+++W (++)	++++W (+)	++++W (+)	++++W-B (+)
Check	-	-	-	-

TABLE XVII

GROWTH OF MOLDS ON CURED PORTION OF BUTTER

Culture No.	Extent of growth at 20-25°C., high humidity, after 2 days	4 days	1 week	2 weeks
1	-	++++G	++++	++++
2	++W	+++Y G	++++	++++
3	+B	+++B	++++	++++
4	++G	++++G	++++	++++
5	+++W B	++++	++++	++++
6	++W	++	+++	++++
7	+W	+++C	++++	++++
8	-	++++G	++++	++++
9	+G	++++G	++++	++++
10	+++W B	++++	++++	++++
Check	-	-	-	-

cally normal. The growth reached a maximum after two weeks and further incubation resulted in no noticeable changes. It will be noted that growth was both rapid and luxuriant.

c. Dialyzed Curd from Butter.

Methods. A portion of the curd obtained by the method outlined in the previous experiment was placed in a collodion sac and dialyzed in distilled water for 24 hours. It was then tubed and autoclaved. The final preparation contained 89.3 per cent water. The conditions of inoculation and incubation were identical with those employed in the previous trial.

Results. The growth of the molds did not commence as promptly as it did on normal curd preparations, as indicated in Table XVIII. After four days, however, the growth was marked and it reached a maximum after one week. In general, the development was not quite as vigorous as that observed on untreated curd.

d. Washings from Cream.

Methods. The sera (obtained from the successive washings of cream as explained in a previous experiment) were collected after each separation, tubed and autoclaved. The first, third, sixth and tenth rinsings were selected for this purpose. The

T A B L E XVIII

GROWTH OF MOLDS ON DIALYZED CURD FROM BUTTER

Culture No.	Extent of growth at 20-25°C., high humidity, after 2 days	4 days	1 week	2 weeks
1	-	+++G	++++	++++
2	+W	+++Y G	+++	+++
3	+W	++++B	++++	++++
4	+++G	++++G	++++	++++
5	++W	++++W B	++++	++++
6	+W	++	+++	+++
7	+W	++	+++	+++
8	-	+++G	++++	++++
9	-	+++G	+++	+++
10	+W	+++W B	++++	++++
Check	-	-	-	-

first washing appeared quite milky, resembling diluted skimmilk. The tenth washing was practically clear. The washings were inoculated in the usual manner with the various cultures. The tubes were kept at 20 to 25° C. for one week, at a relative humidity of 60 to 65 per cent.

Results. Table XIX gives the results obtained when the washings were inoculated with the test cultures. Growth was practically normal in the serum from the first washing after one week with the exception of Cultures 6 and 7. In the third washings, Cultures 1, 8, and 9 were the only ones to show any development and this was rather scanty. Culture 1 was able to grow on the sixth and tenth washings while none of the others produced any visible growth with the exception of a slight development of Culture 8 on the tenth washing. It was very apparent that most of the nutrients must have been removed from the cream by the early washings.

3. Carbohydrates and Related Substances

a. 1 per cent Aqueous Solution of Lactose.

Methods. A 1 per cent aqueous solution of chemically pure lactose was prepared in distilled water, tubed and autoclaved. The tubes were inoculated in the usual manner and kept for three weeks at a temperature of 20 to 25° C. and a relative

TABLE XIX

GROWTH OF MOLDS ON WASHINGS FROM CREAM

Culture No.	Extent of growth at 20-25°C., ordinary humidity							
	1st washing		3rd washing		6th washing		10th washing	
	3 days	1 week	3 days	1 week	3 days	1 week	3 days	1 week
1	++++G	++++	+	++	+	+	+	+
2	++Y	++++	-	-	-	-	-	-
3	-	++B	-	-	-	-	-	-
4	++++G	++++	-	-	-	-	-	-
5	+++W	+++	-	-	-	-	-	-
6	-	+W	-	-	-	-	-	-
7	+W	+	-	-	-	-	-	-
8	+++G	++++	+G	+G	-	-	-	+G
9	+G	+++	+G	+G	-	-	-	-
10	++W	++++	-	-	-	-	-	-
Check	-	-	-	-	-	-	-	-

NOTE: All cultures on 3rd, 6th and 10th washings showed slight sub-surface growths.

humidity of 100 per cent, in the metal humidor previously described.

Results. Table XX indicates that the only signs of growth after four days incubation took place in the depth of the liquid or downward from the surface where the conidia floated. At the end of one week, feeble surface growth was noted with the exception of Cultures 6 and 7. The subsurface development was somewhat more extensive. After three weeks, Cultures 1 and 8 had sent up considerable mycelium from the surface, but at the same time the subsurface growth was equally good. In general, the development in the lactose solution was not marked at any time.

b. 1 per cent Solution of Lactose in 1.5 per cent Washed Agar.

Methods. One gram of chemically pure lactose was added to 100 cubic centimeters of 1.5 per cent washed agar, and the mixture tubed autoclaved and slanted. The usual inoculations were made and the tubes incubated under the conditions described in the previous trial.

Results. The surface growth was very slight even after three weeks incubation as shown in Table XXI. Subsurface development in most cases was quite marked. Cultures 1 and 4 produced dark green mycelium which penetrated deeply into the solid

TABLE XX

GROWTH OF MOLDS IN 1% AQUEOUS SOLUTION OF LACTOSE

Culture No.	Extent of growth at 20-25° C., high humidity, after 5 days	1 week	2 weeks	3 weeks
1	- (+)	+W (+)	++G (++G)	++G (++G)
2	- (+)	+W (+)	+W (+)	+W (+)
3	- (+)	+W (+)	+W (+)	+W (+)
4	-	-	+G	+G (+)
5	- (+)	+W (+)	+W (+)	+W (+)
6	- (+)	- (+)	- (+)	+W (+)
7	-	- (+)	- (+)	+W (+)
8	- (+)	+W (+)	+W (+)	++W-G (++)
9	- (+)	+W (+)	+W (+)	+W (+)
10	- (+)	+W (+)	+W (++)	+W (++)
Check	-	-	-	-

TABLE XXI

GROWTH OF MOLDS ON 1.5% WASHED AGAR CONTAINING 1% LACTOSE

Culture No.	Extent of growth at 20-25° C., high humidity, after 3 days	1 week	2 weeks	3 weeks
1	- (+)	+W (++)	+W (+++G)	+W (+++G)
2	+W (+)	+Y-G (++)	+Y-G (++)	+ (+++)
3	+W (+)	+B (+)	+B (++)	+ (++)
4	+G (++G)	+G (++G)	+G (+++G)	+ (+++G)
5	+W (++)	+W (++)	+W (++)	+ (++)
6	+W (++)	+W (++)	+W (++)	+ (++)
7	+W (++)	+W (++)	+W (++)	+ (++)
8	+G (++)	+G (++)	+G (+++)	+ (+++)
9	+G (+)	+G (++)	+G (+++)	+ (+++)
10	+ (+)	+W (+)	+W (+)	+ (+)
Check	-	-	-	-

medium, at right angles with the side walls of the tube. This is illustrated plainly in Plates I and II. The growth resembled that obtained on solid media containing glycerol.

c. 1 per cent Lactic Acid in 1.5 per cent Washed Agar.

Methods. Purified lactic acid was added to measured quantities of sterile, melted 1.5 per cent washed agar in tubes, in amounts sufficient to make a 1 per cent solution. After thoro mixing the tubes were slanted. The slants were inoculated in the usual manner and incubated at 20 to 25° C. for three weeks in a metal humidor (described elsewhere) where the relative humidity was maintained at 100 per cent.

Results. The results of this trial are given in Table XXII. It will be noted that Cultures 3 and 4 made moderate, Cultures 1, 2, 8, 9, and 10 slight, and Cultures 5, 6, and 7 no growth in this medium. The subsurface development was particularly good in the case of Cultures 3 and 8. Culture 4 sent dark green hyphae into the depth of the medium. Where growth appeared, it was better than that obtained on the pure agar substrate.

d. Diffusate from "Curd" of Butter.

Methods. The diffusate obtained in the dialysis

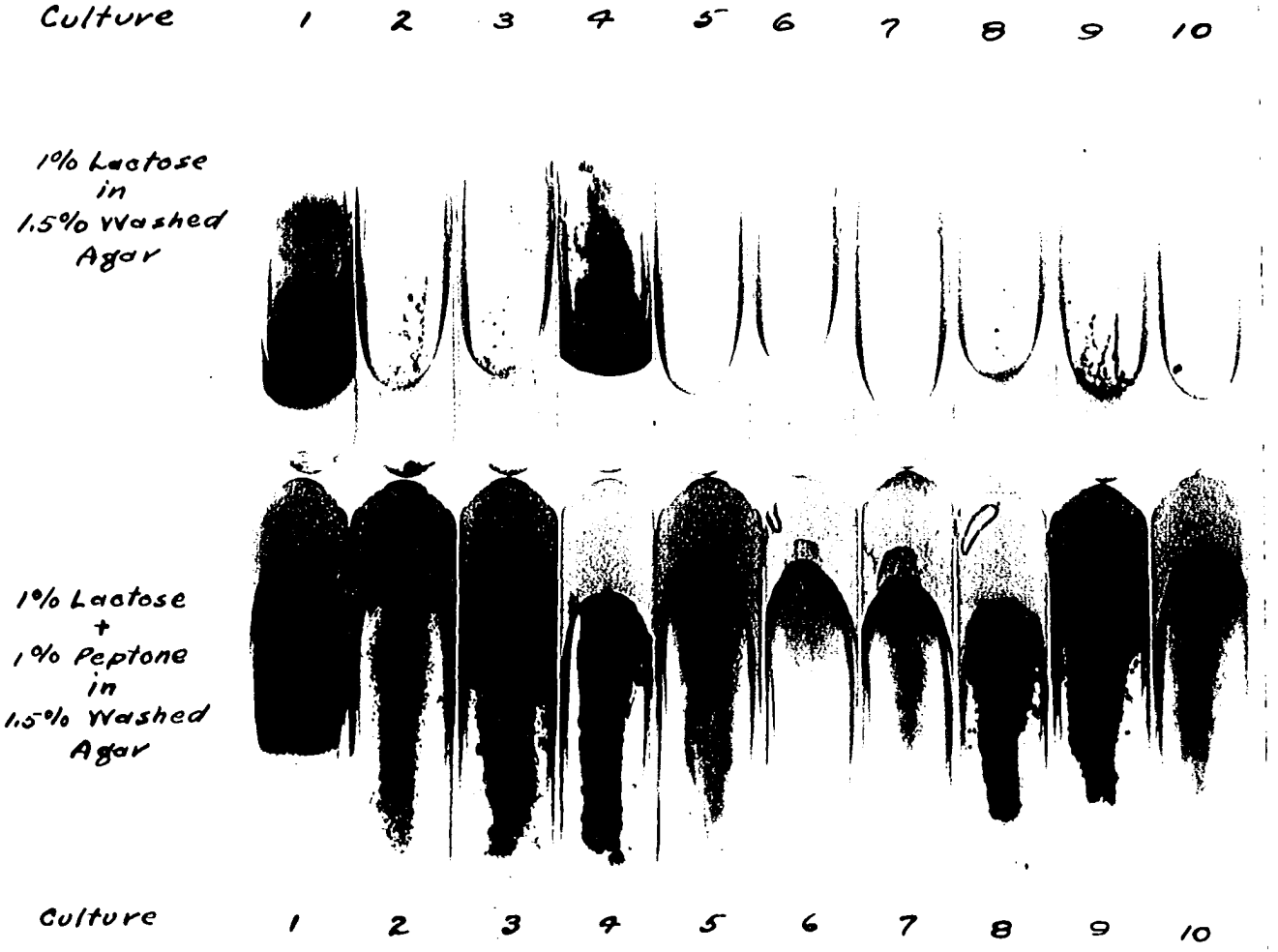

Plate I

Comparative Growth of Test Cultures on 1% Lactose in 1.5% Washed Agar and on 1% Lactose + 1% Peptone in 1.5% Washed Agar.

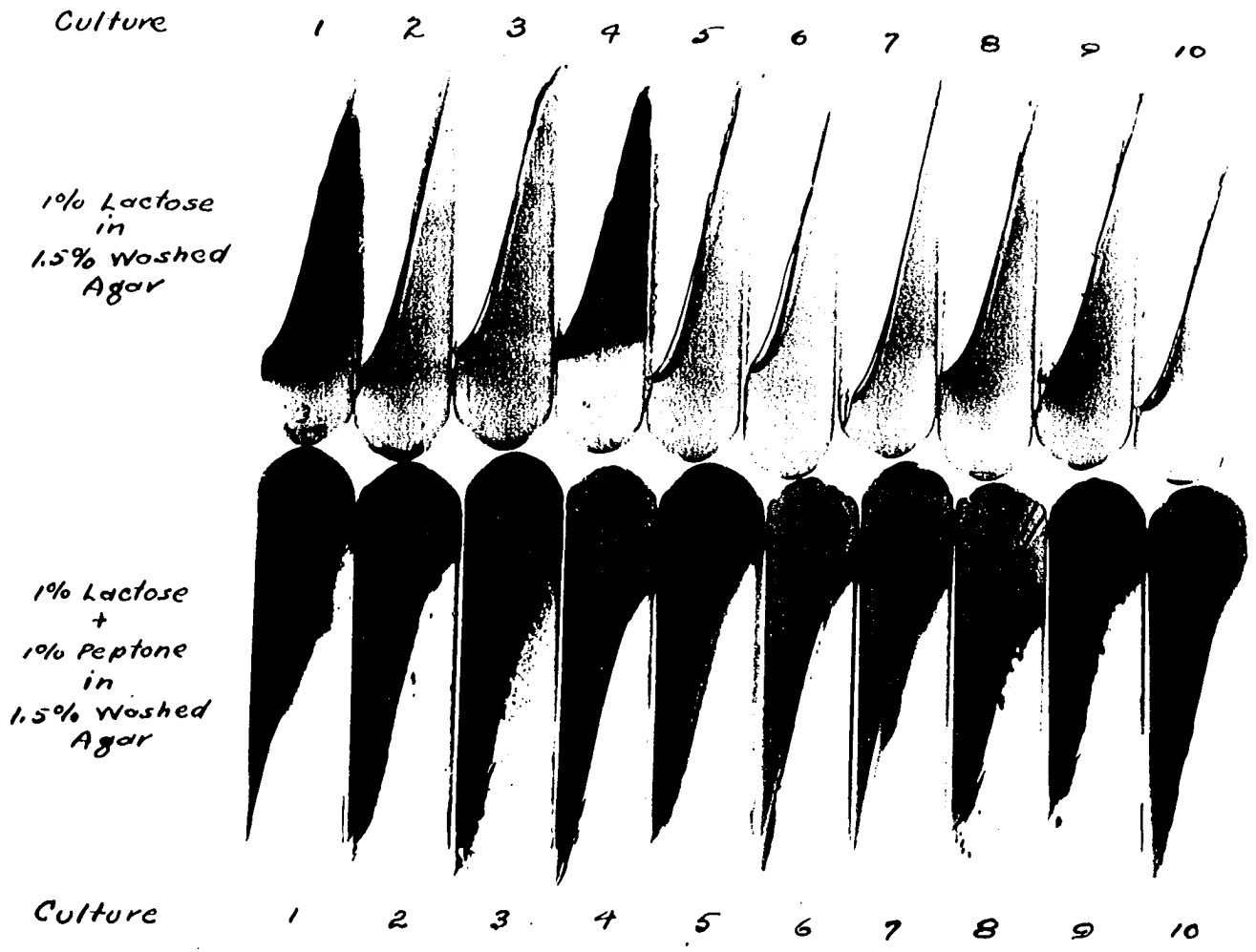

Plate II

Photograph Showing Penetration of Mycelium into Substrate.

(Note: The apparent clouding in the medium containing lactose and peptone together is due largely to shadows cast from the surface mycelium.)

T A B L E XXII

GROWTH OF MOLDS ON 1.5% WASHED AGAR CONTAINING 1% LACTIC ACID

Culture No.	Extent of growth, at 20-25° C., high humidity, after 3 days	1 week	2 weeks	3 weeks
1	-	-	+G	+G
2	$+W^{(+)}$	$+W^{(+)}$	$+Y^{(+)}$	$+Y-G^{(+)}$
3	$+T^{(+)}$	$+B^{(++)}$	$++B^{(++)}$	$++B^{(++)}$
4	-	+G	$++G^{(+)}$	$++G^{(+G)}$
5	-	-	-	-
6	-	-	-	-
7	-	-	-	-
8	$+G^{(+)}$	$+G^{(++)}$	$+G^{(++)}$	$+G^{(++)}$
9	-	$+W^{(+)}$	$+G^{(+)}$	$+G^{(+)}$
10	-	+W	+W	+W
Check	-	-	-	-

of the curd from butter, as explained in previous pages, was tubed and autoclaved. The diffusate contained 99.7 per cent of water and 0.00238 per cent of nitrogen, at the time of inoculation. It was inoculated and kept at 20 to 25° C. for three weeks in a humidor at a relative humidity of 100 per cent.

Results. Table XXIII gives the results of this experiment. It will be noted that growth commenced promptly except with Culture 7 which did not produce visible mycelium until the second week. Cultures 2, 3, 5, 6, 8, and 9 reached the point of maximum development after four days. The growth of Cultures 1, 4, and 10 became especially luxuriant. Apparently, the food materials were readily available as evidenced by the prompt growth but were so rapidly utilized by certain of the cultures that the development reached an early maximum.

4. Mineral Salts

a. Milk Ash in 0.75 per cent Aqueous Solution.

Methods. One pint of fresh skimmilk was evaporated to dryness over a water bath, the residue incinerated in a muffle furnace and the ash recovered. A 0.75 per cent aqueous solution was prepared, tubed and autoclaved. The inoculated tubes were incubated at 20 to 25° C. for two weeks, at a relative humidity of

T A B L E XXIII

GROWTH OF MOLDS ON DIFFUSATE FROM CURD OF BUTTER

Culture No.	Extent of growth at 20-25° C., high humidity, after 2 days	4 days	1 week	2 weeks	3 weeks
1	++W	+++W	+++	+++	++++G
2	++Y	++Y G	++	++	++
3	+B	+	+	+	+
4	++C	++	++	++	+++G
5	+W	+	+	+	+
6	+W	+	+	+	+
7	-	-	-	+W	+
8	++G	++	++	++	++
9	+G	++	++	++	++
10	+W	+	+	+	+++
Check	-	-	-	-	-

60 to 65 per cent. At the end of two weeks, a cubic centimeter of sterile skimmilk was added to each tube to determine whether the cultures were in a viable condition.

Results. As shown in Table XXIV, no growth occurred with most of the cultures. There was a slight subsurface mycelium in the case of Culture 10, and a barely visible development of Culture 2. Three days after the sterile milk was added, excellent growth was obtained from all except Cultures 2 and 7.

b. Milk Ash in 0.75 per cent Aqueous Solution Neutralized with Hydrochloric Acid.

Methods. The solution of milk ash prepared according to the method outlined in the foregoing experiment was neutralized with hydrochloric acid. The methods of tubing, sterilizing and inoculating and the conditions of incubation were identical with those followed in the previous trial.

Results. It will be noted from Table XXV that fairly good growth took place after four days in the case of Cultures 1, 8, and 9, while Cultures 2 and 3 showed scanty mycelial development. At the end of two weeks all cultures exhibited some degree of growth, excepting Cultures 6 and 7. Cultures 5 and 10 however, produced only subsurface mycelium. The addition of sterile milk showed that none of the cultures had been killed; all grew well in

T A B L E XXIV

GROWTH OF MOLDS ON 0.75% AQUEOUS SOLUTION OF MILK ASH

Culture No.	Extent of growth, at 20-25°C., ordinary humidity, after 4 days	1 week	2 weeks	Growth after adding 1 cc. sterile milk 3 days
1	-	-	-	+++G
2	+	+	+	++Y G
3	-	-	-	+B
4	-	-	-	+++G
5	-	-	-	++W
6	-	-	-	++W
7	-	-	-	+W
8	-	-	-	+++G
9	-	-	-	+++G
10	- (+)	- (+)	- (+)	+++W
Check	-	-	-	-

TABLE XXV

GROWTH OF MOLDS ON 0.75% AQUEOUS SOLUTION OF MILK ASH MADE NEUTRAL WITH HCl.

Culture No.	Extent of growth at 20-25°C., ordinary humidity, after 4 days	1 week	2 weeks	Growth after adding 1 cc. sterile milk 3 days
1	++W	++	++	+++W G
2	+W	+	+	++W Y
3	+W B	+	+	++B
4	-	+W	+	++G
5	-	- (+)	- (+)	++W
6	-	-	-	+++W
7	-	-	-	+W
8	++W	++	++	+++G
9	++W	++	++	+++G
10	- (+)	- (+)	- (+)	+++W
Check	-	-	-	-

three days, with Culture 7 again the least active.

5. Combinations of Various Foodstuffs.

a. Emulsion of Butterfat, Milk Lecithin and Water.

Methods. Five parts of old, sterile butterfat, prepared according to the methods described in earlier experiments, were mixed thoroly with one part of a 1 per cent sterile, aqueous emulsion of lecithin, and the mixture poured into petri plates. These plates were inoculated by streaking the cultures across the surface and were incubated at 20 to 25° C. for three weeks, at a relative humidity of 100 per cent in the earthenware humidor previously described.

Results. The growth on the mixture of butterfat, lecithin and water was not much better than that obtained on fat alone, as is clearly demonstrated in Table XXVI. Culture 1 began its development promptly and showed the best growth. Cultures 3 and 4 produced a scanty but visible surface growth.

b. Butterfat plus Dry Milk Ash.

Methods. Some of the old butterfat used in previous experiments was melted, poured into petri plates and solidified promptly. Dry milk ash was sprinkled freely over the surface. The cultures were streaked across the surface in such a way that

TABLE XXVI

GROWTH OF MOLDS ON EMULSION OF BUTTERFAT IN PETRI PLATES WITH 1% AQUEOUS SOLUTION OF MILK LECITHIN (5 parts of butterfat: 1 part of lecithin solution)

Culture No.	Extent of growth at 20-25°C., high humidity, after 3 days	1 week	2 weeks	3 weeks
1	+W	+	+	++
2	-	-	-	-
3	-	-	-	+B
4	-	-	-	+G
5	-	-	-	-
6	-	-	-	-
7	-	-	-	-
8	-	-	-	-
9	-	-	-	-
10	-	-	-	-
Check	-	-	-	-

they came into contact both with the ash and the fat. The plates were incubated for three weeks in the earthenware humidor at a temperature of 20 to 25 ° C. and relative humidity of 100 per cent.

Results. Table XXVII gives the results obtained. The growth was somewhat better than that secured on pure butterfat. Visible mycelium was observed in the case of Cultures 1, 2, 3, 4, 8, and 9 after three weeks incubation. Cultures 5, 6, and 7 showed no signs of development when the plates were examined under the microscope, while culture 10 gave evidence of the germination of some of the conidia and abortive mycelium.

c. Mixtures of Butterfat, Milk Ash and Water.

Methods. Two parts of sterile fresh butterfat and one part of milk ash (prepared in accordance with methods previously described) were mixed thoroly and added to four parts of a sterile melted, 1.5 per cent washed agar medium. The mixture was shaken thoroly and poured into petri plates. After it had become hard, the cultures were streaked across the surface. The plates were incubated for four weeks, at a temperature of 20 to 25° C. and a relative humidity of 60 to 65 per cent.

Results. Table XXVIII indicates that growth began within a few days and increased slightly during the period of four weeks, with the exception of Cultures 6 and 7. Cultures 1,

T A B L E XXVII

GROWTH OF MOLDS ON BUTTERFAT IN PETRI PLATES WITH MILK ASH SPRINKLED OVER SURFACE

Culture No.	Extent of growth at 20-25°C., high humidity, after 1 week	2 weeks	3 weeks	3 weeks Micro-scopic
1	+W	+G	++G	+
2	-	+Y G	+	+
3	++B	++	++	+
4	-	-	++G	+
5	-	-	-	-
6	-	-	-	-
7	-	-	-	-
8	-	-	+G	+
9	-	-	+G	+
10	-	-	-	-
Check	-	-	-	-

T A B L E XXVIII

GROWTH OF MOLDS ON 1.5% WASHED AGAR PLATES WITH FRESH BUTTERFAT, AND SATURATED SOLUTION OF MILK ASH ADDED

(4 parts of agar medium: 2 parts of butterfat: 1 part of ash solution)

Culture No.	Extent of growth at 20-25°C., ordinary humidity, after					
	4 days	1 week	2 weeks	3 weeks	4 weeks	4 weeks Microscopic
1	+W	+G	+G	++	++	+
2	+Y	++	++ Y G	++	++	+
3	+B	++	++	++	++	+
4	+G	++G	++	++	++	+
5	+W	+	+	+	+	+
6	-	-	-	-	-	-
7	-	-	-	-	-	-
8	+W	+W	+G	+	+	+
9	+W	+G	+	+	+	+
10	+W	+	+	+	+	+
Check	-	-	-	-	-	-

2, 3, and 4 made the most noticeable progress in this medium. The microscopic examination failed to show any signs of germination of the conidia from Cultures 6 or 7; the failure of these cultures to grow on fats, minerals or combinations of the two substances has been a regular phenomenon.

d. Aqueous Solution of Lactose and Peptone.

Methods. A solution containing 1 per cent Bacto peptone and 1 per cent chemically pure lactose was prepared in distilled water, tubed and autoclaved. After inoculation the samples were incubated at 20 to 25° C. for three weeks in the metal humidor previously described, and where the humidity was maintained at 100 per cent.

Results. Table XXIX shows that all the cultures grew rapidly in the medium and soon reached a normal state of development as was the case with the peptone alone. The only effect which the lactose seemed to exert was an increase in the amount of subsurface mycelium. The protein appeared to be the more readily utilized substance.

e. Mixture of Lactose and Peptone in 1.5 per cent Washed Agar.

Methods. Sufficient lactose and peptone were added to a 1.5 per cent washed agar medium to give a 1 per cent concentration of each of them. This mixture was tubed, autoclaved

TABLE XXIX

GROWTH OF MOLDS IN AQUEOUS SOLUTION CONTAINING 1% LACTOSE AND 1% PEPTONE

Culture No.	Extent of growth, at 20-25° C., high humidity, after 3 days	1 week	2 weeks	3 weeks
1	+W (+)	+++G (++)	+++G (++)	+++++G (++)
2	++W (+)	++W	+++Y-Br	++++Y-Br
3	++W-B (+)	++B (+)	++++B (+)	++++B (+)
4	++G (+)	++++G (+)	++++G (+)	++++G (+)
5	++W (++)	+++W (++)	+++W (+)	+++++W (+)
6	++W (++)	++++W (++)	++++W (++)	++++W (++++)
7	++W (++)	++++W (++)	++++W (++)	++++W (++)
8	++W (+)	++++G (+)	++++G (+)	++++G (++)
9	++W (+)	++++W (+)	++++W (+)	++++W (+)
10	+++W (++)	++++W-B (++)	++++W-B (+)	++++W-B (+)
Check	-	-	-	-

and slanted. The slants were inoculated on the surface in the usual manner. The tubes were incubated under the same conditions as those described in the previous experiment.

Results. It will be observed in Table XXX that excellent growth was obtained in this medium. In contrast with the lactose-washed agar medium, the surface growth was predominant altho some subsurface mycelium was produced, the contrast between the growth upon the lactose-peptone-washed agar and the lactose-washed agar is shown clearly in Plates I and II. When lactose was the main source of food, the mycelium of the molds studied seemed preferably to penetrate into the depth of the medium, but when a nitrogen compound, such as peptone, was available at the same time the growth became characteristically concentrated on the surface.

f. Sterile, Unsalted Butter.

Methods. A sample of fresh 30 per cent cream was autoclaved and churned in a sterile Dazey churn, washed with sterile water and worked with sterile paddles. Small pieces of this butter were transferred to sterile test tubes and allowed to harden overnight in the cooler. After inoculation by streaking the cultures along the surface, the tubes were placed in a humidor at a temperature of 20 to 25° C. and a relative humidity of 100 per cent, for three weeks.

TABLE XXX

GROWTH OF MOLDS ON 1.5% WASHED AGAR CONTAINING 1% LACTOSE AND 1% PEPTONE

Culture No.	Extent of growth at 20-25° C., high humidity, after 3 days		1 week		2 weeks		3 weeks	
1	+W	(+)	++W-G	(+)	+++W-G	(+)	++++G	(+)
2	++W	(+)	+++W-Y-G	(+)	+++Y-G	(+)	++++Y-G	(++)
3	++B	(+)	++++B	(+)	++++B	(+)	++++B	(+)
4	+++G	(+Br)	++++G	(+Br)	++++G	(+Br)	++++G	(+Br)
5	+++W	(+)	+++W	(+)	+++W	(++)	+++W	(+++)
6	++W	(+)	++W	(+)	++W	(++)	+++W	(++)
7	++W	(+)	+++W	(+)	+++W	(++)	+++W	(++)
8	++++G	(+)	++++G	(+)	++++G	(+)	++++G	(++)
9	+++W	(+)	++++	(+)	++++G	(++)	++++G	(++)
10	+++W	(+)	+++W	(++)	+++W	(++)	+++W	(++)
Check	-		-		-		-	

Results. The growth of the cultures upon this combination of all of the foodstuffs previously studied is described in Table XXXI and illustrated in Plate III. The most extensive development was noted in the case of Cultures 1, 3, 4, 5, 8, 9, and 10. The butter was discolored to the greatest extent with Cultures 1 and 4. Culture 2 showed slight visible mycelium and a few yellowish-green sporangia. No visible growth of Cultures 6 or 7 was observed. The unsalted butter appeared to furnish ample food for the growth of the majority of the cultures studied.

B. Moisture.

The influence which moisture has upon the growth of molds may be considered from two standpoints, namely, the moisture contained in the substrate itself and the moisture carried by the atmosphere.

1. Moisture in Substrate.

It has been shown in experiments reported in the previous pages, that the presence of water facilitates the growth of molds on butterfat. Inasmuch as butter contains a considerable percentage of water, especially in intimate association with the most useful foodstuffs contained in the butter in the form of droplets of buttermilk, it was not considered essential to investigate the influence of water of constitution upon the growth of molds, further

TABLE XXXI

GROWTH OF MOLDS ON STERILE, UNSALTED BUTTER

Culture No.	Extent of growth at 20-25°C., high humidity, after 4 days	1 week	2 weeks	3 weeks
1	-	+G	+++G	++++
2	-	-	+Y G	+
3	+B	++B	++	+++
4	+G	+++G	+++	+++
5	++W	+++W B	+++	+++
6	-	-	-	-
7	-	-	-	-
8	-	++G	++	+++
9	+G	+++G	+++	+++
10	-	+G	+++W B	+++
Check	-	-	-	-

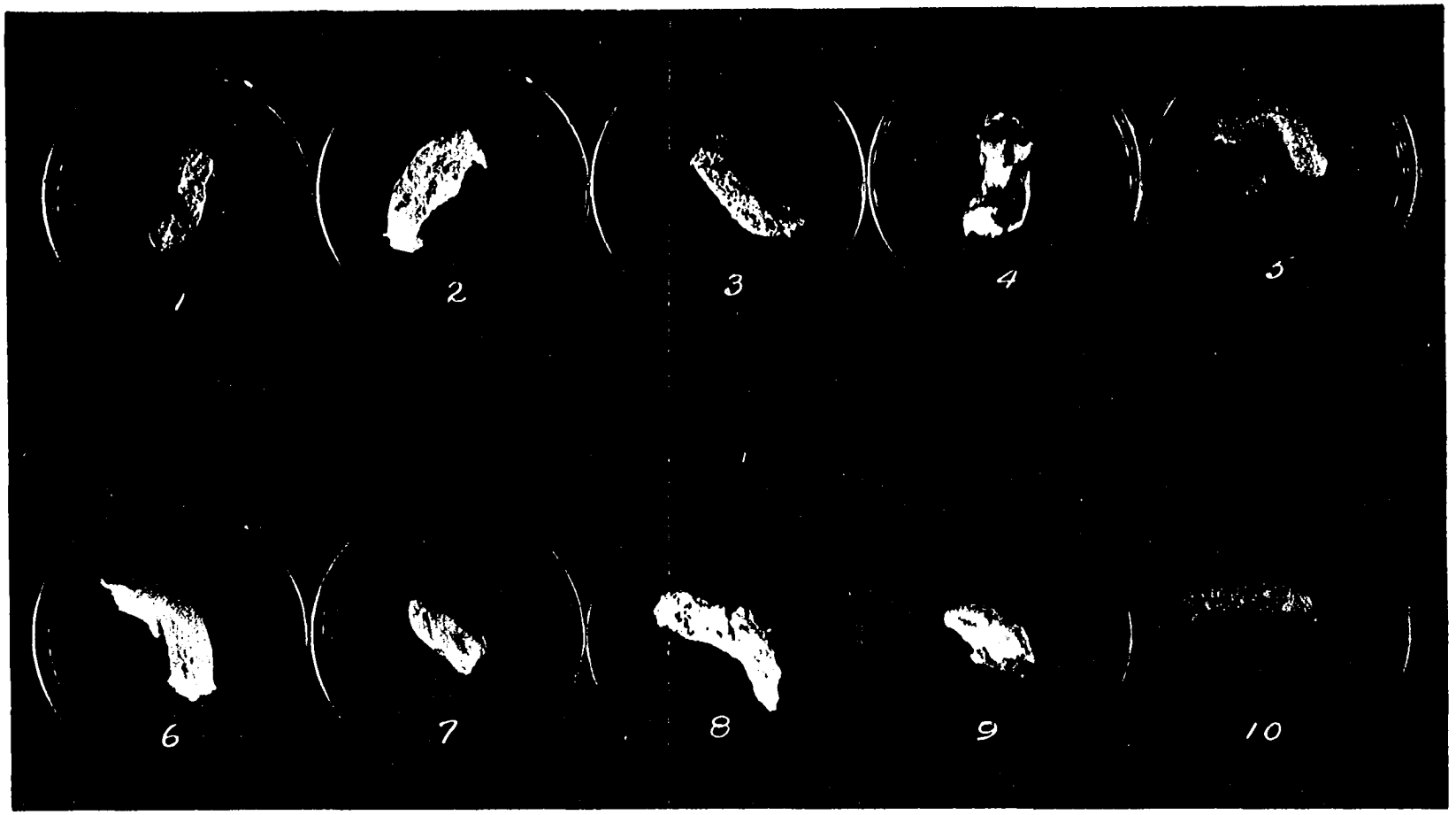

Plate III

Typical Appearance of Growth of Test Cultures on Unsalted Butter.

than that reported in previous or subsequent pages in connection with other factors.

2. Moisture in Atmosphere.

a. Effect of Humidity upon Growth of Molds on Unsalted Butter.

Methods. Sweet cream, testing 35 per cent fat was autoclaved, cooled and churned in a sterile Dazey churn. The butter was washed with sterile water, worked with sterile paddles, due precautions being taken to prevent contamination. Small blocks of the finished butter were placed in sterile petri plates and held overnight at 0° C. in order to harden the butter. The following day the cultures were streaked over the surfaces of the samples. The plates were kept at 10 to 12° C. for six weeks, one set in a metal humidor (previously described) at a relative humidity of 100 per cent, the other set in the laboratory at a relative humidity of 70 to 75 per cent.

Results. The comparison between the growth of the molds on the samples of butter kept at different humidities is shown in Table XXXII. At the end of six weeks, none of the butters kept at the lower humidity gave any evidence of growth. In the samples kept in an atmosphere saturated with moisture, Cultures 1 and 4 produced dark green smudgy areas which spread some distance

T A B L E XXXII

EFFECT OF HUMIDITY UPON GROWTH OF MOLDS INOCULATED ON SURFACE OF STERILE UNSALTED BUTTER

Culture No.	Extent of growth at 10° C., after periods of 1 week		2 weeks		3 weeks		4 weeks		6 weeks	
	Dry	Humid	Dry	Humid	Dry	Humid	Dry	Humid	Dry	Humid
1	-	-	-	-	-	+G	-	+++G	-	++++
2	-	-	-	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-	-	-	-
4	-	-	-	-	-	+G	-	+++	-	+++
5	-	-	-	-	-	-	-	+W-B	-	+
6	-	-	-	-	-	-	-	+W	-	+
7	-	-	-	-	-	-	-	-	-	-
8	-	+W	-	+W	-	++W	-	++	-	++++W-G
9	-	+W	-	+W	-	++W	-	++W-G	-	+++
10	-	-	-	-	-	-	-	+W	-	+
Check	-	-	-	-	-	-	-	-	-	-

from the line of inoculation; Culture 5 showed delicate, white, aerial threads of mycelium, bearing smoky-grey and black sporangia; Culture 6 produced a slight, white felt-like mass; Cultures 8 and 9 formed white and green spots scattered over the surface and also gave to the butter a distinctly Roquefort cheese odor; while Culture 10 developed a scanty, white aerial mycelium. There were no signs of growth with Cultures 2 and 3. It will be pointed out later that these cultures do not grow well at 10° C. and for this reason did not develop on the samples studied in this trial.

b. Effect of Humidity upon the Growth of Molds on Unsalted Butter Churned from Inoculated Cream.

Methods. Eleven lots of sweet, 40 per cent cream were placed in Erlenmeyer flasks and autoclaved. When they were cooled they were inoculated with the cultures being studied, except one lot which was held as a check. The inoculated creams were allowed to stand at room temperature (20 to 25° C.) for four days after which they were placed at 0° C. until cooled sufficiently to churn. During the four days, all of the cultures had grown extremely well and covered the surface of the cream with a deep layer of mycelium bearing the characteristic fruiting bodies. Each lot of cream was churned in a sterile Dazey churn, washed with sterile water and worked with sterile paddles under careful condi-

tions. Pieces of the felt-like mass formed by the mycelium were removed as far as possible from the butter. Small blocks of the butter were placed in petri plates. One cubic centimeter of water was placed in each of the plates of one set of samples and replenished daily while the other set was left without the addition of water. Both lots were incubated at 20 to 25° C. for six weeks.

Results. It may be noted from Table XXXIII that some of the samples maintained at low humidities showed the growth of mold after one or two weeks but in most cases the development upon the samples at higher humidities gave evidence of more favorable growing conditions. Culture 4 became clearly visible after one week under both conditions and produced a very dark green smudge and surface spots. A delicate, white web of mycelium was formed by Culture 2 on the dry samples, but bright yellowish-green sporangia on the butter well supplied with moisture. Culture 8 produced white spots under both conditions. There were no visible signs of growth in the case of Cultures 1, 6, 7, and 10.

C. Temperature.

The fact that temperature has a profound effect upon the growth of microörganisms is generally appreciated. Each species has its minimum, optimum and maximum temperatures which vary with the

T A B L E XXXIII

EFFECT OF HUMIDITY UPON GROWTH OF MOLDS ON UNSALTED BUTTER CHURNED FROM INOCULATED STERILE CREAM

Culture No.	Extent of growth at 20-25° C., after 1 week		2 weeks		3 weeks		4 weeks		5 weeks		6 weeks	
	Dry	Humid	Dry	Humid	Dry	Humid	Dry	Humid	Dry	Humid	Dry	Humid
1	-	-	-	-	-	-	-	-	-	-	-	-
2	-	+W-Y	+W	+W-Y	+	++Y-G	+	++	+	++	+	++
3	-	+B	-	+B	-	+B	-	+	-	+	-	+
4	++G	++G	+++G	+++G	+++	+++	+++	+++	+++	+++	+++	+++
5	-	+W	-	++	-	++	-	++	-	++	-	++
6	-	-	-	-	-	-	-	-	-	-	-	-
7	-	-	-	-	-	-	-	-	-	-	-	-
8	-	+W	+W	+W	+W	+W	+	+	+	+	+	+
9	-	+G	-	++G	-	++G	-	++	-	++	-	++
10	-	-	-	-	-	-	-	-	-	-	-	-
Check	-		-		-		-		-		-	

conditions under which the organisms are existing. Butter is kept at a wide range of storage temperatures but these are, in most cases, near 0° C. or below it. Occasionally, lots of butter will be exposed to higher temperatures for considerable periods of time. The ten species of molds used in these experiments were seeded on various substrata in order to observe the effect of different temperatures upon their growth. The temperatures used did not go below 0° C. but subsequent studies should be pursued at lower temperatures for extended periods.

1. Growth on Whey Broth.

Methods. Fresh skimmilk was heated to 35° C. and maintained at that temperature while dilute hydrochloric acid was added slowly, with constant stirring. The milk was brought to the isoelectric point of casein, pH 4.6 to 4.7 by comparison with methyl red standards. The finely-granulated casein was removed by filtering thru cheesecloth. The resultant whey was autoclaved for fifteen minutes to coagulate the heat-labile proteins and filtered thru cotton. The whey was neutralized with N/1 sodium hydroxide to pH 6.8 and 0.5 per cent Bacto peptone added. The mixture was autoclaved for 15 minutes to precipitate the acid-soluble substances and filtered thru paper after which it was tubed and sterilized. Three sets of inoculated samples were prepared and one set stored at 20 to 25° C. and 40 to 50 per cent relative humidity, another

set at 10 to 12 ^{o} C. and 70 to 75 per cent humidity and the other at 0^{o} to 2^{o} C. and 70 to 75 per cent humidity.

Results. Table XXXIV gives the results of this experiment. It is evident that all of the cultures found the whey broth to be an excellent source of food as shown by the active growth at 20^{o} C. after one week. A certain amount of subsurface mycelium was present in most cases, especially after three weeks at this temperature. Culture 2 was very slow to develop at 10^{o} C. and when it did the mycelium was scanty. Culture 3 likewise made slight progress at this temperature. The growth of all cultures was retarded when the whey broth was kept at 0^{o} C. Cultures 2, 3, and 10 gave no evidence of growth within three weeks time. Cultures 1 and 5 produced traces of visible mycelium in the depths of the liquids. Time was an apparent factor at the lower temperatures altho certain of the species did not show any growth after longer periods of incubation.

2. Growth on Whey Agar Slants.

Methods. A solid medium containing 1.2 per cent Bacto agar was prepared from whey broth made according to the method outline in the previous experiment. This medium was tubed, autoclaved and slanted. After inoculation, the three sets of samples were stored under the conditions described in the foregoing trial.

T A B L E XXXIV

EFFECT OF TEMPERATURE UPON THE GROWTH OF MOLDS IN WHEY BROTH

Culture No.	Extent of growth at ordinary humidity after 1 week			2 weeks			3 weeks		
	20°C.	10°C.	0°C.	20°C.	10°C.	0°C.	20°C.	10°C.	0°C
1	++++(++)	+(++)	-(+)	++++(++)	+(++)	-(+)	++++(++)	++(++)	-(+)
2	++++(++)	-	-	++++(++)	-	-	++++(++)	+	-
3	++++	+	-	++++	+(+)	-	++++(++)	+(+)	-
4	++++	+++(+)	+	++++	+++(+)	++(+)	++++	+++(+)	++(+)
5	+++(++)	++(++)	-	+++(++)	++(++)	-(+)	+++(++)	++(++)	-(++)
6	++++	+++(+)	+(+)	++++	+++(+)	+(+)	++++	+++(+)	+(+)
7	+++(++)	+++(++)	-(+)	++++(++)	+++(++)	-(+)	++++(+)	++(+)	+(+)
8	++++(+)	+++(+)	+	++++(+)	+++(+)	+(+)	++++(+)	++++(+)	+(+)
9	+++(+)	+++(+)	-	++++(+)	+++(+)	-(+)	++++(+)	+++(+)	+(+)
10	++++(++)	++(++)	-	++++(++)	+++(+)	-	++++(++)	+++(+)	-
Check	-	-	-	-	-	-	-	-	-

Results. All of the cultures grew luxuriantly after one week at 20° C. as indicated in Table XXXV. With the exception of Culture 2 which failed to grow and Culture 3 which developed poorly, all the cultures showed practically normal growth at 10°C. At 0° C. the development of those cultures which grew at all was less luxuriant, while Cultures 2, 3, and 10 failed to produce any visible mycelium. Low temperatures exhibited a marked effect upon the growth especially with Cultures 2 and 3.

3. Growth in Sweet Buttermilk.

Methods. Buttermilk was obtained from a churning of fresh, sweet cream, tubed and autoclaved. The inoculated samples were divided into three lots and incubated under the conditions explained in the last two experiments.

Results. Table XXXVI shows that the cultures kept at 20° C. did not develop quite as promptly in the buttermilk as they did in whey broth or whey agar. Growth at the end of three weeks was reasonably good in all cases at this temperature. The development at 10° C. was somewhat less in most instances; no visible growth was obtained with Culture 2 while Culture 3 gave barely visible growth after three weeks. Cultures 2, 3, and 10 again failed to grow at 0° C. during the observation period. In general, the surface growth on buttermilk was somewhat less than it had been on

T A B L E XXXV

EFFECT OF TEMPERATURE UPON THE GROWTH OF MOLDS ON WHEY AGAR SLANTS

Culture No.	Extent of growth at ordinary humidity, after 1 week			2 weeks			3 weeks		
	20°C.	10°C.	0°C.	20°C.	10°C.	0°C.	20°C.	10°C.	0°C.
1	++++	+++	+	++++	++++	+	++++	++++	++
2	++++	-	-	++++	-	-	++++	-	-
3	+++	+	-	+++	+	-	+++	+	-
4	++++	++++	+	++++	++++	++	++++	++++	++
5	+++	++	+	++++	++	+	++++	++	++
6	+++	+++	+	++++	+++	+	++++	+++	++
7	+++	+++	+	++++	+++	+	++++	+++	+
8	++++	++++	++	++++	++++	++	++++	++++	+++
9	++++	+++	+	++++	+++	+	++++	+++	+
10	++++	++++	-	++++	++++	-	++++	++++	-
Check	-	-	-	-	-	-	-	-	-

TABLE XXXVI

EFFECT OF TEMPERATURE UPON THE GROWTH OF MOLDS IN SWEET BUTTERMILK

Culture	Extent of growth at ordinary humidity, after								
	1 week			2 weeks			3 weeks		
No.	20°C.	10°C.	0°C.	20°C.	10°C.	0°C.	20°C.	10°C.	0°C.
1	+	++	-	++	++	-	+++	+++	+
2	+++	-	-	+++	-	-	++++	-	-
3	++	-	-	+++	-	-	++++	+	-
4	++++	++	-	++++	++	-	++++	+++	++
5	+++	+++	++	+++	+++	++	++++	+++	++
6	++++	++	++	++++	++	++	++++	++	++
7	++++	++	++	++++	++	++	++++	++	++
8	+++	+++	+++	+++	+++	++	+++	+++	+++
9	+++	++	++	+++	++	++	+++	+++	++
10	++++	+	-	++++	++	-	++++	++	-
Check	-	-	-	-	-	-	-	-	-

whey broth and whey agar.

4. Growth on Sterile Butter.

Methods. A batch of sweet cream was autoclaved and, after cooling, churned in a sterile Dazey churn, washed with sterile water and worked with sterile paddles. The finished butter was divided into four portions, one of which was left unsalted. Sterile salt was worked into the other lots of butter in varying amounts, giving a final salt content (by analysis) of 1.2 per cent, 2.6 per cent, and 2.9 per cent respectively. Blocks of each lot of butter were placed in petri plates and allowed to harden over night at 0° C. The samples were inoculated by streaking the cultures across the surfaces. One set of samples was incubated at 0° C. and the other at 10° C. in metal humidors (previously described), where the relative humidity was maintained at 100 per cent.

Results. The observations made upon the samples over a period of six weeks are reported in Table XXXVII. At 0° C., Culture 9 showed growth after two weeks, Culture 1 after four weeks, and Culture 8 after six weeks in the unsalted butter, while none of the others showed any apparent development even after six weeks incubation. Cultures 1 and 8 produced small white spots at this temperature in the butter containing 1.2 per cent salt. Otherwise, all cultures were retarded by the combined influence of low temper-

T A B L E XXXVII

EFFECT OF TEMPERATURE UPON THE GROWTH OF MOLDS ON STERILE BUTT

	Extent of growth, at 0°C. and 10°C., high humidity, after 1 Week								after 2 Weeks				
Culture	Unsalted		1.2% Salt		2.5% Salt		2.9% Salt		Unsalted		1.2% Salt		2.6%
	°C.		°C.		°C.		°C.		°C.		°C.		
No.	0	10	0	10	0	10	0	10	0	10	0	10	0
1	-	-	-	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-	-	-	-	-	-
5	-	-	-	-	-	-	-	-	-	-	-	-	-
6	-	-	-	-	-	-	-	-	-	-	-	-	-
7	-	-	-	-	-	-	-	-	-	-	-	-	-
8	-	+W	-	-	-	-	-	-	-	+W	-	-	-
9	-	+W	-	-	-	-	-	-	+W	+W	-	-	-
10	-	-	-	-	-	-	-	-	-	-	-	-	-
Check	-	-	-	-	-	-	-	-	-	-	-	-	-

NOTE: The same series of samples, maintained at the same temperatures but under conditions of low humidity, failed to show any evidence of development of molds.

VII

THE GROWTH OF MOLDS ON STERILE BUTTER

). and 10°C., high humidity, after periods of

					2 Weeks								5 Weeks		
% Salt	Unsalted		1.2% Salt		2.6% Salt		2.9% Salt		Unsalted		1.2% Salt		2.6% Sa	
°C.	°C.		°C.		°C.		°C.		°C.		°C.		°C.	
10	0	10	0	10	0	10	0	10	0	10	0	10	0	10
-	-	-	-	-	-	-	-	-	-	+C	-	-	-	-
-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-	-	+C	-	-	-	-
-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
-	-	+W	-	-	-	-	-	-	-	++W	-	+W	-	-
-	+W	+W	-	-	-	-	-	-	+W	++W	-	+W	-	-
-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

maintained at the same temperatures,
humidity, failed to show any evidence

5 Weeks								4 Weeks							
Unsalted		1.2% Salt		2.6% Salt		2.9% Salt		Unsalted		1.2% Salt		2.6% Salt		2.9% Salt	
°C.		°C.		°C.		°C.		°C.		°C.		°C.		°C.	
0	10	0	10	0	10	0	10	0	10	0	10	0	10	0	10
-	+G	-	-	-	-	-	-	+G	+++G	-	+G	-	+G	-	-
-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
-	+G	-	-	-	-	-	-	-	+++G	-	+G	-	-	-	-
-	-	-	-	-	-	-	-	-	+W	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-	+W	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
-	++W	-	+W	-	-	-	-	-	++W-G	-	+W	-	+G	-	+W
+W	++W	-	+W	-	-	-	-	+W	+++W-G	-	+W	-	+W-G	-	+W
-	-	-	-	-	-	-	-	-	+W	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

4 Weeks							6 Weeks					
% Salt	2.6% Salt		2.9% Salt		Unsalted		1.2% Salt		2.6% Salt		2.9% Salt	
°C.	°C.		°C.		°C.		°C.		°C.		°C.	
10	0	10	0	10	0	10	0	10	0	10	0	10
+G	-	+G	-	-	+W	++++G	+W	++G	-	+G	-	+W-G
-	-	-	-	-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-	-	-	-	-
+G	-	-	-	-	-	++G	-	++G	-	+G	-	+G
-	-	-	-	-	-	+W	-	+W	-	-	-	-
-	-	-	-	-	-	+W	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-	-	-	-	-
+W	-	+G	-	+W	+W	++++W-G	+G	++W-G	-	++G	-	++W-G
+W	-	+W-G	-	+W	+G	++++W-G	-	++G	-	++G	-	++G
-	-	-	-	-	-	+W	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-	-	-	-	-

ature and salt. At 10° C. growth in the unsalted butter was extensive in most instances and after a period of six weeks, Cultures 1 and 4 produced dark green smudges, Cultures 5 and 10 developed slight aerial mycelia, Culture 6 showed a small amount of white mycelium, and Cultures 8 and 9 formed white and green spots, while Cultures 2 and 3 were not able to grow. In the butter containing 1.2 per cent of salt, and kept at 10° C., Culture 5 produced a few aerial hyphae, while Cultures 1, 4, 8, and 9 grew quite well and produced distinct areas of moldiness. The butters containing 2.6 per cent, and 2.9 per cent salt showed slight growth of Cultures 1, 4, 8, and 9. The low temperatures clearly restrained the growth of most of the species studied, and the effect became more noticeable as the percentage of salt increased.

5. Growth on Butter Made from Inoculated Cream.

Methods. Eleven batches of fresh, sweet cream in flasks were autoclaved and cooled, after which ten lots were inoculated with the experimental cultures and the other left as a check. The inoculated cream samples were kept at 20 to 25° C. for four days. During this time, all cultures grew exceedingly well and produced a surface felt with normal fructification. These samples were churned in individual, sterile Dazey churns, washed with sterile water and worked with sterile paddles. Visible pieces of the felt were removed

as far as possible. Each lot of butter was divided into four parts, one remained unsalted, while the others were worked with different amounts of sterile salt in order to obtain varying concentrations. The actual salt content in the various lots of butter varied as indicated in Table XXXVIII. It was very difficult to obtain exactly the same results in each case, because of the difficulty of working the butter under aseptic conditions. Blocks of the butters were placed in petri plates and hardened at 0° C. One set of plates was placed at 20 to 25° C., another at 10 to 12° C. and another at 0 to 2° C. in metal humidors (previously described), at a relative humidity of 100 per cent and observations made during a period of six weeks.

Results. The combined effect of temperature and salt concentration on the growth of the various species is shown in Table XXXVIII. It will be noted that Cultures 1, 6, 7, and 10 failed to make any visible growth under any of the conditions. Cultures 2 and 3 grew reasonably well at 20° C. in all salt concentrations but apparently could not develop at 10° C. or 0° C. Culture 4 grew at all three temperatures but was affected somewhat by increased percentages of salt, especially at 0° C. The only visible growth secured with Culture 5 was on the unsalted sample at 20° C. The results with Culture 8 were somewhat irregular but it grew at all temperatures. With the higher salt concentrations and lower temperatures its development was retarded or checked. Culture 9 was not

TABLE XXXVIII

EFFECT OF TEMPERATURE UPON GROWTH OF MOLDS IN BUTTER MADE FROM INOCULATED STERILE CREAM

Culture No.	Per Cent of Salt	Extent of growth, high humidity, after 2 weeks			4 weeks			6 weeks		
		20°C.	10°C.	0°C.	20°C.	10°C.	0°C.	20°C.	10°C.	0°C.
1	0.	-	-	-	-	-	-	-	-	-
	1.5	-	-	-	-	-	-	-	-	-
	2.7	-	-	-	-	-	-	-	-	-
	4.2	-	-	-	-	-	-	-	-	-
2	0.	+	-	-	++	-	-	++	-	-
	1.4	++	-	-	+++	-	-	+++	-	-
	2.2	+++	-	-	+++	-	-	+++	-	-
	4.0	++	-	-	++	-	-	++	-	-
3	0.	+	-	-	+	-	-	+	-	-
	1.5	++	-	-	+++	-	-	+++	-	-
	2.3	++	-	-	++	-	-	++	-	-
	4.5	++	-	-	++	-	-	++	-	-
4	0.	+++	++	+	+++	+++	++	+++	++++	++
	1.6	++	++	-	++	+	+	++	+	+
	2.7	++	+	-	+	+	-	+	+	-
	3.3	+	+	-	+	+	-	+	+	-
5	0.	++	-	-	++	-	-	++	-	-
	1.1	-	-	-	-	-	-	-	-	-
	2.3	-	-	-	-	-	-	-	-	-
	3.5	-	-	-	-	-	-	-	-	-
6	0.	-	-	-	-	-	-	-	-	-
	1.4	-	-	-	-	-	-	-	-	-
	1.6	-	-	-	-	-	-	-	-	-
	3.0	-	-	-	-	-	-	-	-	-

T A B L E XXXVIII (Continued)

EFFECT OF TEMPERATURE UPON GROWTH OF MOLDS IN BUTTER MADE FROM INOCULATED STERILE CREAM

Culture No.	Per Cent of Salt	Extent of growth, high humidity, after 2 weeks			4 weeks			6 weeks		
		20°C.	10°C.	0°C.	20°C.	10°C.	0°C.	20°C.	10°C.	0°C.
7	0.	-	-	-	-	-	-	-	-	-
	0.9	-	-	-	-	-	-	-	-	-
	2.1	-	-	-	-	-	-	-	-	-
	3.1	-	-	-	-	-	-	-	-	-
8	0.	+	+	+	+	++	++	+	++	++
	0.7	-	+	-	-	++	-	-	++	+
	2.3	+	-	-	+	++	-	+	++	-
	3.1	+	-	-	+	-	-	+	-	-
9	0.	++	+	-	++	++	-	++	++	-
	1.3	++	+	-	++	++	-	++	++	-
	2.2	++	+	-	++	++	-	++	++	-
	3.0	+	+	-	+	++	-	+	++	-
10	0.	-	-	-	-	-	-	-	-	-
	1.3	-	-	-	-	-	-	-	-	-
	2.1	-	-	-	-	-	-	-	-	-
	4.0	-	-	-	-	-	-	-	-	-
Checks	0-4.5	-	-	-	-	-	-	-	-	-

effected particularly by the salt content of the butter but failed to appear at 0° C. Evidently, the effect of temperature varied with the species of mold and the composition of the substrate as influenced by the salt content.

D. Atmosphere

Most species of molds are considered aerobic, or at best facultative, and this would indicate that a satisfactory supply of oxygen was a factor in their growth. Experiments were undertaken to determine how the mold growth on butter might be affected by changes in atmospheric conditions.

1. Ordinary Air Supply.

In the experiments previously reported, and in which a plentiful supply of oxygen was provided, the species of molds making up the experimental group, were able to grow satisfactorily when the food supply, moisture, temperature and other conditions were favorable. These results may be considered as checks, demonstrating that the molds grew well when an abundant supply of air was present.

2. Reduced Air Supply--Partial Vacuum.

Methods. Sterile cream was churned in a sterile Dazey churn, washed with sterile water and worked with sterile paddles. Blocks of butter were placed in petri plates (in which the covers

were kept slightly raised by the use of small wire staples) and hardened at 0° C. The surface of the butter was inoculated with the molds being studied. The plates were then placed in an ordinary sterile, glass desiccator. After a liter of water was placed in the bottom of the jar to provide adequate humidity the lid was put in place and sealed with vaseline. A vacuum of 25 inches was drawn and maintained during the period of study. The temperature of incubation was 20 to 25° C.

Results. The results of the trial are given in Table XXXIX. Growth was not very active during the first week but increased somewhat during the subsequent three weeks. The development was never very great, however, and not comparable with that obtained under ordinary atmospheric conditions. The reduction of the amount of available oxygen appeared to retard the development of these species.

3. Partial Removal of Carbon Dioxide.

Methods. The butter used was prepared in the same manner as that for (2) above. The plates were held in a sterile desiccator in the bottom of which was placed a liter of a 10 per cent aqueous solution of sodium hydroxide. This was added to absorb some of the carbon dioxide, while at the same time it furnished a source of moisture. The lid of the desiccator was sealed with

TABLE XXXIX

EFFECT OF REDUCED AIR SUPPLY UPON GROWTH OF MOLDS ON STERILE, UNSALTED BUTTER

Extent of growth, at 20°-25°C., high humidity, under 25" vacuum, after	Culture No.										
	1	2	3	4	5	6	7	8	9	10	Check
1 week	+	+	+	+	-	-	-	+	-	-	-
4 weeks	+	++	++	++	+	-	+	++	+++	+	-

vaseline. The temperature of incubation was 20 to 25° C.

Results. Altho the growth of the cultures was not extensive during the first week, Table XL shows that excellent development of all except Cultures 6 and 7 occurred after four weeks. However, these last named cultures actually showed more visible growth under these conditions than they had shown at any other time when seeded on butter. Apparently, a reduction in the amount of carbon dioxide does not seriously deter the growth of the species under observation.

4. Removal of Oxygen.

Methods. The butter used was prepared in the manner described in (2) and (3) above. The plates were placed in a sterile desiccator and the lid sealed with vaseline, after 100 grams of pyrogallic acid had been placed in the bottom. A funnel was introduced thru an opening in the side of the desiccator and 500 cubic centimeters of a 10 per cent aqueous solution of sodium hydroxide added to the pyrogallic acid. The funnel was removed quickly and the opening closed and sealed. The temperature of incubation was 20 to 25° C.

Results. Table XLI points out clearly that no growth appeared in any of the samples even after a period of four weeks. The exhaustion of oxygen apparently made conditions unfavor-

T A B L E XL

EFFECT OF PARTIAL REMOVAL OF CARBON DIOXIDE FROM ATMOSPHERE UPON GROWTH OF MOLDS ON STERILE, UNSALTED BUTTER

Extent of growth at 20°-25°C., high humidity (10% solu. of NaOH) in desiccator, after	Culture No.										
	1	2	3	4	5	6	7	8	9	10	Check
1 week	+	+	+	+	+	+	+	++	++	++	-
4 weeks	+++	++	+++	+++	++++	+	+	+++	+++	++++	-

T A B L E XLI

EFFECT OF REMOVAL OF OXYGEN FROM THE ATMOSPHERE

UPON THE GROWTH OF MOLDS ON STERILE, UNSALTED BUTTER

(Oxygen exhausted by NaOH- pyrogallic acid mixture in sealed desiccator.)

Extent of growth at 20°-25° C., high humidity, after	Culture No.										
	1	2	3	4	5	6	7	8	9	10	Check
1 week	-	-	-	-	-	-	-	-	-	-	-
4 weeks	-	-	-	-	-	-	-	-	-	-	-

able for the development of the molds being studied.

E. Miscellaneous Chemical and Physical Factors.

1. Salt.

Sodium chloride has been considered a food preservative for many years. Inasmuch as most butter contains some salt, and since unsalted butter has appeared to be more susceptible to molding than the salted type, experiments were conducted to determine the effect of various concentrations of sodium chloride upon the growth of the species of molds selected for this study.

a. Whey Broth plus Salt.

Methods. Whey broth prepared in the manner described in previous experiments was used as a substrate. Sodium chloride was added in different amounts to various portions so that the final salt contents (by weight) in the different lots of broth were 10 per cent, 15 per cent, and 20 per cent. An unsalted portion was left as a check. The solutions were tubed and autoclaved. After inoculation the samples were placed at 20 to 25° C. in a room with a relative humidity of 40 to 50 per cent, and observed for three weeks.

Results. The record of the observations is given in Table XLII. The growth of all the cultures was excellent

TABLE XLII

EFFECT OF SALT UPON THE GROWTH OF MOLDS IN WHEY BROTH

Extent of growth at 20-25° C., ordinary humidity, after 1 week

Culture No.	Salt content 0 %	10 %	15 %	20 %
1	++++(++)	-(+)	-	-
2	++++(++)	+++	++	-
3	++++	+++(+)	-	-
4	++++	+++(+)	-	-
5	+++(++)	-(+)	-	-
6	++++	-	-	-
7	+++(++)	-	-	-
8	++++(+)	+++(+)	++	-
9	+++(+)	+++(+)	++	+
10	++++(++)	-	-	-
Check	-	-	-	-

2 weeks				3 weeks			
Salt content				Salt content			
0 %	10 %	15 %	20 %	0 %	10 %	15 %	20 %
++++(++)	-(++)	-	-	++++(++)	-(++)	-(+)	-
++++(++)	+++(+)	++(+)	-	++++	+++(+)	++(+)	-
++++	+++(+)	-(++)	-	++++(++)	+++(+)	-(++)	-
++++	+++(+)	-	-	++++	+++(+)	-(+)	-
+++(++)	-(+)	-	-	+++(++)	-(+)	-	-
++++	-	-	-	++++	-	-	-
++++(++)	-	-	-	++++(++)	-	-	-
++++(+)	+++(+)	+++(+)	+	++++(++)	+++(+)	+++(+)	++
++++(+)	++++(+)	++(+)	++	++++(+)	+++(+)	++(+)	++
++++(++)	-(+)	-	-	++++(++)	-(+)	-	-
-	-	-	-	-	-	-	-

in the unsalted broth, especially on the surface. In the broth containing 10 per cent of salt Cultures 2, 3, 4, 8, and 9 had produced abundant mycelium and fructification at the end of three weeks. Cultures 1, 5, and 10 produced only subsurface growth while Cultures 6 and 7 were checked entirely. In the 15 per cent salt broth, Cultures 2, 8, and 9 grew fairly well on the surface while Cultures 1, 3, and 4 showed some subsurface mycelium. The remaining cultures gave no growth with 20 per cent salt. The only species to show any development in the broth were Cultures 8 and 9 and they grew quite well. It is evident that salt exercised a restraining effect upon the growth of molds but it varied considerably with the different species.

b. Whey Agar plus Salt.

Methods. The whey agar which was used in this experiment was similar to that described on previous pages. Sodium chloride was added to portions of the medium so that the final concentrations by weight were 10 per cent, 15 per cent, and 20 per cent. These and the unsalted check were tubed, autoclaved, and slanted. After inoculation they were incubated under conditions similar to those in the preceding experiment.

Results. All cultures developed luxuriantly on the unsalted slants as reported in Table XLIII. In the 10 per cent

TABLE XLIII

EFFECT OF SALT UPON THE GROWTH OF MOLDS ON WHEY AGAR SLANTS

Extent of growth at 20-25° C., ordinary humidity, after

Culture No.	1 week				2 weeks				3 weeks			
	salt content				salt content				salt content			
	0%	10%	15%	20%	0%	10%	15%	20%	0%	10%	15%	20%
1	++++	++	-	-	++++	+++	+	-	++++	+++	+	-
2	++++	+++	++	-	++++	+++	++	-	++++	+++	++	-
3	+++	+++	+	-	+++	++	+	-	+++	++	+	-
4	++++	+++	-	-	++++	+++	+	-	++++	+++	++	-
5	+++	+	-	-	+++	+	-	-	+++	+	-	-
6	+++	-	-	-	+++	-	-	-	+++	-	-	-
7	+++	-	-	-	+++	-	-	-	+++	-	-	-
8	++++	++++	++	+	++++	+++	++	+	++++	+++	++	+
9	++++	++	++	+	++++	++	++	+	++++	++	++	+
10	++++	+	-	-	++++	+	-	-	++++	+	-	-
Check		-		-		-		-		-		-

salt agar, Cultures 6 and 7 were the only ones that failed to grow altho Cultures 5 and 10 showed very meagre growth. When the salt content reached 15 per cent, Cultures 2, 4, 8, and 9 showed moderate growth and Cultures 1 and 4 slight growth. The others remained dormant. As found in the previous experiment, in the 20 per cent concentration of salt Cultures 8 and 9 were the only ones to grow and their growth was very scanty. The growth on the solid medium was somewhat better than it was on the whey broth, but the influence of the salt was demonstrated to be in the same general direction.

b. Sterile, Sweet Buttermilk plus Salt.

Methods. Buttermilk was obtained directly from a churning of sweet cream. The necessary salt was added to yield solutions containing 5 per cent, 10 per cent, 15 per cent, and 20 per cent. An unsalted portion was kept as a check. All lots were tubed and autoclaved. After inoculation they were incubated under the conditions described under (a) and (b) above.

Results. The growth of every culture was luxuriant in the unsalted buttermilk after one week as is shown in Table XLIV. In the 5 per cent salt buttermilk, development was excellent after two weeks in the case of Cultures 1, 2, 3, 4, 8, and 9, moderately good with Cultures 5, and 10, but only slight with Cultures 6 and 7. The 10 per cent salt concentration complete-

TABLE XLIV

EFFECT OF SALT UPON GROWTH OF MOLDS IN STERILE, SWEET BUTTERMILK

Culture No.	Extent of growth at 20-25° C., ordinary humidity after 4 days					Extent of gr humidi	
	Salt content of Buttermilk					Salt cont	
	0 %	5 %	10 %	15 %	20 %	0 %	5
1	+++W	+W	-	-	-	+++W-G	++
2	++W-Y	++W-Y	++Y	-	-	+++Y-G	++
3	++W-Br	+W-Br	+Br	-	-	+++Br	++
4	+++G	++G	+G	-	-	+++G	++
5	++W	+W	-	-	-	+++W-B	+
6	++W	-	-	-	-	+++W	
7	+++W	-	-	-	-	+++W	
8	++W	+W	+W	-	-	+++W-G	
9	+++G	++G	+W-G	+W-G	-	+++G	++
10	+++W-B	+W	-	-	-	+++W-B	+
Check	-	-	-	-	-	-	

MILK

ent of growth at 20–25° C., ordinary humidity after 1 week

Salt content of Buttermilk 0 %	5 %	10 %	15 %	20 %
+W-G	+++W-G	-	-	-
+Y-G	+++Y-G	++Y-G	+W	-
+Br	+++Br	+Br	-	-
+G	+++G	+G	-	-
+W-B	++W-B	-	-	-
+W	-	-	-	-
+W	-	-	-	-
+W-G	+W	+W	-	-
+G	+++G	+++W-G	+W-G	-
+W-B	++W-B	-	-	-
-	-	-	-	-

Extent of growth at 20–25° C., ordinary humidity after 2 weeks

Salt content of Buttermilk 0 %	5 %	10 %	15 %	20 %
+++G	+++W-G	++G	-	-
+++Y-G	+++Y-G	+++Y-G	+W	-
+++Br	+++Br	++Br	+W	-
+++G	+++G	++G	-	-
+++W-B	++W-B	-	-	-
+++W	+W	-	-	-
+++W	+W	-	-	-
+++G	+++G	++W	+W	-
+++G	+++G	+++G	++W-G	+W-G
+++W-B	++W-B	-	-	-
-	-	-	-	-

ly inhibited Cultures 5, 6, 7, and 10 but the remainder were able to grow quite well. When the percentage of salt was increased to 15 per cent, only Cultures 2, 3, 8, and 9 were active. With 20 per cent salt, Culture 9 alone produced visible growth in the buttermilk. It will be noted that the growth of certain species is retarded more in the salted buttermilk than it was in the whey broth or agar.

d. Sterile, Sour Buttermilk plus Salt.

Methods. A sample of buttermilk was obtained from a churning of sour cream butter. Salt was added to portions of this buttermilk so as to give concentrations of 5, 10, 15, 20, and 25 per cent and an unsalted check was also retained. These solutions were tubed and autoclaved. After inoculation, they were incubated at 20 to 25° C. for two weeks at a relative humidity of 60 to 65 per cent.

Results. Table XLV presents the results obtained in this experiment. In the unsalted buttermilk the growth after two weeks was quite luxuriant but it had been slower than that obtained on the sweet buttermilk. The development of the cultures in the 5 per cent salt solution was practically the same as that obtained in the unsalted samples, in some cases less and in other cases greater. In the buttermilk containing 10 per cent of salt, Cultures

T A B L E XLV

EFFECT OF SALT ON GROWTH OF MOLDS IN STERILE, SOUR BUTTERMILK

Culture No.	Extent of growth at 20-25° C., ordinary humidity after 3 days. Salt content of Buttermilk 0 %	5 %	10 %	15 %	20 %	25 %	Extent of humidity Salt 0 %	5 %
1	++W	+W	+W	-	-	-	+++W-G	++G
2	++Y-G	+Y	+Y	-	-	-	++Y-G	+++Y
3	+W-B	+B	+B	-	-	-	++B	++B
4	+G	+G	-	-	-	-	++G	+++G
5	++W	+W	-	-	-	-	++W	++W-G
6	++W	+W	-	-	-	-	+++W	++W
7	++W	+W	-	-	-	-	+++W	++W
8	+W	+W	+W	+W	-	-	++G	++G
9	++W-G	+W-G	+W	-	-	-	++G	+++G
10	++W	+W	-	-	-	-	++W	++W-B
Check	-	-	-	-	-	-	-	-

growth at 20–25° C., ordinary after 1 week.				Extent of growth at 20–25° C., ordinary humidity after 2 weeks.					
content of Buttermilk				Salt content of Buttermilk					
10 %	15 %	20 %	25 %	0 %	5 %	10 %	15 %	20 %	25 %
+W	-	-	-	+++W-G	++G	+W	-	-	-
++Y	-	-	-	+++Y-G	++Y-G	+++Y-G	+Y-G	-	-
+++B	-	-	-	++B	+++B	+++B	+Br	-	-
-	-	-	-	+++G	+++G	++G	-	-	-
-	-	-	-	+++W-B	++W-B	-	-	-	-
-	-	-	-	+++W-Y	++W-Y	-	-	-	-
-	-	-	-	+++W-Y	++W-Y	-	-	-	-
+W	+W	-	-	+++G	++G	+++W-G	+G	-	-
+++G	+W	-	-	+++G	+++G	+++G	++G	+W	-
-	-	-	-	+++W-B	++W-B	-	-	-	-
-	-	-	-	-	-	-	-	-	-

1, 2, 3, 4, 8, and 9 were able to grow quite well. When the concentration of salt was increased to 15 per cent Cultures 2, 3, 8, and 9 were the only ones to produce visible mycelium and fruiting bodies. The 20 per cent solution retarded all the cultures except Culture 9 and in a 25 per cent concentration of salt none were able to grow during the two weeks incubation period. In order to determine whether the cultures that had failed to grow on the buttermilk containing 15 per cent of salt were simply retarded or actually killed, a portion of each sample was plated on whey agar. Normal colonies were obtained from all such platings, proving that the spores were still viable but unable to grow in the strong brine. In a similar way, when samples were taken from the 25 per cent solution, it was found that Cultures 2, 3, 5, 8, 9, and 10 had been unharmed by the salt while Cultures 1, 4, 6, and 7 apparently had been destroyed since no colonies were obtained. Accordingly, it would appear that moderately strong brines simply retard the growth of certain species while higher concentrations may actually destroy some species.

e. Sterile Butter plus Salt.

Methods. A batch of sweet cream was autoclaved, cooled, churned in a sterile Dazey churn, and washed with sterile water. The butter was divided into five lots, one of which was retained as a check without salt. Sterilized salt was added in

varying amounts to the other four lots. All were worked as thoroly and carefully as possible with sterile paddles. The salt contents of the four lots of salted butter, as determined by analysis, were 0.5, 0.8, 1.4, and 1.7 per cent, respectively. Small blocks of each were placed in petri plates and hardened at 0° C. overnight. The next day they were inoculated by streaking the cultures across the surface. The intention was to incubate these samples at 5 to 6° C. at a high humidity for six weeks, but circumstances made it necessary to remove the plates from the humidor after one week. During the next two weeks they were kept at 5 to 6° C. but at a lower humidity. At the end of three weeks it was necessary to remove the samples from the cooler. For the following three weeks they were kept at 20 to 25° C. at a high humidity. Consequently, the conditions are so variable that the results are not very satisfactory. Analyses of the butters showed the following compositions:

% salt	0.0	0.5	0.8	1.4	1.7
% water	14.0	14.4	14.2	14.2	14.8
% salt in brine	0.0	3.4	5.3	8.9	10.5

This makes it possible to interpret the results in terms of those obtained with buttermilk, whey broth etc.

Results. The results of this trial are recorded in Table XLVI. No growth was visible in any of the samples held at

TABLE XLVI

EFFECT OF SALT UPON GROWTH OF MOLDS ON STERILE BUTTER

Extent of growth - 1st week at 6°C., high humidity; 2d and 3d weeks at 6

Culture No.	4 days Salt Content					1 weeks Salt Content					2 we Salt Cont		
	0%	0.5%	0.8%	1.4%	1.7%	0%	0.5%	0.8%	1.4%	1.7%	0%	0.5%	0.
1	-	-	-	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	—	-	-	-	-	-	-
3	-	-	-	-	-	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-	-	-	-	-	-
5	-	-	-	-	-	-	-	-	-	-	-	-	-
6	-	-	-	-	-	-	-	-	-	-	-	-	-
7	-	-	-	-	-	-	-	-	-	-	-	-	-
8	-	-	-	-	-	-	-	-	-	-	-	-	-
9	-	-	-	-	-	-	-	-	-	-	+T	-	-
10	-	-	-	-	-	-	-	-	-	-	-	-	-
Check	-	-	-	-	-	-	-	-	-	-	-	-	-

d weeks at 6°C., ordinary humidity; 4th, 5th and 6th week at 20-25°C. high humidity

2 weeks Salt Content					3 weeks Salt Content					4 weeks Salt Content			
0%	0.5%	0.8%	1.4%	1.7%	0%	0.5%	0.8%	1.4%	1.7%	0%	0.5%	0.8%	1.4%
-	-	-	-	-	+W-G	-	-	-	-	+W-G	-	-	-
-	-	-	-	-	-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-	-	+W-G	-	-	-
-	-	-	-	-	+G	-	-	-	-	+G	-	-	-
-	-	-	-	-	-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-	-	-	-	-	-
+W	-	-	-	-	+W	-	-	-	-	+G	-	-	-
-	-	-	-	-	-	-	-	-	-	+W	-	-	-
-	-	-	-	-	-	-	-	-	-	-	-	-	-

humidity.

5 weeks Salt Content					6 weeks Salt Content						
1.4%	1.7%	0.%	0.5%	0.8%	1.4%	1.7%	0%	0.5%	0.8%	1.4%	1.7%
-	-	++++W-G	++G	+G	+G	+G	+++	++	++	++	+
-	-	+W	+I	+I	+I	++I-G	+	++	+	+	+++
-	-	+++B	+++B	++B	++B	+B	+++	+++	++	++	++
-	-	+++G	++G	+G	+G	+G	+++	+++	++	++	++
-	-	++W-B	+W	-	-	-	++	++	-	-	-
-	-	-	-	-	-	-	-	—	-	-	-
-	-	-	-	-	-	-	-	-	-	-	-
-	-	+W	+G	+W	+W	+W	+	+	+	+	+
-	-	+++G	+++G	++G	++G	++G	+++	+++	+++	+++	+++
-	-	++W	-	-	-	-	++	++	-	-	-
-	-	-	-	-	-	-	-	-	-	-	-

6° C. during the period of two weeks, with the exception of Culture 9 which had produced a small white spot in the unsalted butter. After 3 weeks, cultures 1, 4, and 9 had developed visible colored areas in the unsalted butter. The following week witnessed the growth of Cultures 3 and 10 in addition to those mentioned. After two weeks at room temperature active development was observed for all cultures, with the exception of Cultures 6 and 7. At the end of the six week period, Cultures 1, 2, 3, 4, 8, and 9 had shown some growth in all samples, salted and unsalted, with the extent of this development greatest in the latter. Cultures 5 and 10 were checked by the salt in the butter containing 0.8 per cent or more. Cultures 6 and 7 did not grow at all. When it is realized that the highest salt concentration on the basis of brine was 10.3 per cent in the case of the butter containing 1.7 per cent of salt, it is not surprising that a number of species were able to grow as the same cultures had demonstrated their ability to do so in buttermilk containing an equivalent amount of salt. The checking of the growth of Cultures 5 and 10 in butter with a salt-in-brine percentage of over 5.3 per cent, also is in accordance with the results obtained in buttermilk. These facts emphasize the importance of considering the salt content of butter in terms of the salt-in-brine percentage when it is to be taken into account as a preservative or deterrent of mold growth.

f. Sterile Butter plus Salt.

Methods. Sweet cream was autoclaved, cooled, churned in a Dazey churn, and washed with sterile water. The butter was divided into four lots, one of which remained unsalted to serve as a check, and sterile salt added to the three portions in amounts sufficient to give final salt contents of 1.2, 2.6, and 2.9 per cent respectively. Each lot of butter was worked separately with sterile paddles and under aseptic conditions. Blocks of each butter were placed in petri plates and held at 0° C. overnight for hardening. The samples were inoculated on the surface and placed in a metal humidor (described previously) and stored at 10° C. for six weeks at a relative humidity of 100 per cent.

The compositions of these four lots of butter as determined by analysis were as follows:

% Salt	0.0	1.2	2.6	2.9
% Water	15.9	15.6	16.0	16.0
% Salt-in-Brine	0.0	7.1	13.9	15.3

Results. The data obtained in this experiment are reported in Table XLVII. Cultures 8 and 9 were the only ones to show growth during the first two weeks and then only on the unsalted butter. After three weeks, Cultures 1 and 4 grew on the unsalted

T A B L E XLVII

EFFECT OF SALT UPON THE GROWTH OF MOLDS ON STERILE BUTTER

Extent of growth at 10° C., high humidity, after

Culture No.	1 week Salt content 0%	1.2%	2.6%	2.9%	2 weeks Salt content 0%	1.2%	2.6%	2.9%
1	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-
5	-	-	-	-	-	-	-	-
6	-	-	-	-	-	-	-	-
7	-	-	-	-	-	-	-	-
8	+W	-	-	-	+	-	-	-
9	+W	-	-	-	+	-	-	-
10	-	-	-	-	-	-	-	-
Check	-	-	-	-	-	-	-	-

3 weeks Salt content				4 weeks Salt content				6 weeks Salt content			
0%	1.2%	2.6%	2.9%	0%	1.2%	2.6%	2.9%	0%	1.2%	2.6%	2.9%
+G	-	-	-	+++G	+G	+G	-	++++	++G	+G	+W-G
-	-	-	-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-	-	-	-
+G	-	-	-	+++G	+G	-	-	+++	++G	+G	+G
-	-	-	-	+W	-	-	-	+	+W	-	-
-	-	-	-	+W	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-	-	-	-
++	+W	-	-	++	+	+G	+W	++++WG	++WG	++G	++W-G
++	+W	-	-	+++WG	+	+W-G	+W	+++	++G	++G	++G
-	-	-	-	+W	-	-	-	+	-	-	-
-	-	-	-	-	-	-	-	-	-	-	-

Note: The same series of samples, maintained at the same temperature, but under conditions of low humidity, failed to show any evidence of development of the molds.

butter, while Cultures 8 and 9 also developed on the butter containing 1.2 per cent salt. Cultures 1, 4, 8, and 9 developed extensively in the unsalted butter after four weeks and Cultures 5, 6, and 10 became barely visible. In the 1.2 per cent salt butter, Cultures 1, 4, 8, and 9 grew slightly. Culture 1 produced a small dark green spot on the butter with 2.6 per cent salt, while Cultures 8 and 9 grew both on the 2.6 per cent and 2.9 per cent salt samples. During the six weeks period, Cultures 1, 4, 8, and 9 developed exceedingly well, but Cultures 5 and 10 set up scanty aerial mycelium, in the unsalted butter. In the butter containing 1.2 per cent salt, Cultures 1, 4, 8, and 9 grew quite extensively, and Culture 5 very sparsely. On the butters with the higher salt concentrations Cultures 1, 4, 8, and 9 produced fairly distinct discolorations. These results are more or less in accordance with previous observations of the growth of these molds on media containing salt, when the salt content is considered from the standpoint of its concentration in the brine. The failure of Cultures 6 and 7 to show visible growth even in unsalted butter has been the rule altho there is evidence to lead to the belief that they actually may be growing even tho a visible mycelium does not appear. The explanation of the failure of Cultures 2 and 3 to develop may be found in the temperature of incubation. Previous observations demonstrated that these species did not grow

well in the most favorable substrate at temperatures as low as 10° C. It may also be stated that a set of samples similar to those considered in Table XLVII was kept at the same temperature but at a relative humidity of 70 to 75 per cent. These samples gave no signs of growth in any instance after a period of three weeks.

g. Butter (Made from Inoculated Cream) plus Salt.

Methods. The butter was made from sterile cream inoculated and handled according to the proceedure outlined in previous experiments. The butter in each case was divided into four lots and worked with varying percentages of salt. The amount of salt in each lot of butter is indicated in Table XLVIII. Attention should be called to the fact that considerable difficulty was encountered in churning the cream inoculated with Culture 10. It took over an hour while ordinarily the churning period was ten or fifteen minutes. At the other extreme, it required only two minutes to churn the creams containing Cultures 2 and 3. In these three samples the water content was exceedingly high and the butter little more than a paste. The per cent of water in the various samples was as follows:

Culture	% Water
1.	Range 16.8 to 19.4
2.	" 46.2 to 50.8
3.	" 35.0 to 36.8

TABLE XLVIII

EFFECT OF SALT UPON GROWTH OF MOLDS IN BUTTER MADE FROM INOCULATED STERILE CREAM

Culture No.	Per Cent of Salt	Extent of growth at 20-25°C., high humidity, after 1 week	2 weeks	3 weeks	4 weeks	5 weeks	6 weeks
1	0.	-	-	-	-	-	-
	1.5	-	-	-	-	-	-
	2.7	-	-	-	-	-	-
	4.2	-	-	-	-	-	-
2	0.	+W-Y	+W-Y	++Y-G	++	++	++
	1.4	++Y-G	++Y-G	+++	+++	+++	+++
	2.2	++Y-G	+++Y-G	+++	+++	+++	+++
	4.0	+Y-G	++Y-G	++	++	++	++
3	0.	+B	+B	+	+	+	+
	1.5	++B	++B	+++	+++	+++	+++
	2.3	++B	++B	++	++	++	++
	4.5	+B	++B	++	++	++	++
4.	0.	++G	+++G	+++	+++	+++	+++
	1.6	+G	++G	++	++	++	++
	2.7	+G	++G	++	++	++	++
	3.3	+G	+G	+	+	+	+
5	0.	+W	++W	++	++	++	++
	1.1	-	-	-	-	-	-
	2.3	-	-	-	-	-	-
	3.5	-	-	-	-	-	-
6	0.	-	-	-	-	-	-
	1.4	-	-	-	-	-	-
	1.6	-	-	-	-	-	-
	3.0	-	-	-	-	-	-

T A B L E XLVIII (Concluded)

EFFECT OF SALT UPON GROWTH OF MOLDS IN BUTTER MADE FROM INOCULATED STERILE CREAM

Culture No.	Per Cent of Salt	Extent of growth at 20-25°C., high humidity, after 1 week	2 weeks	3 weeks	4 weeks	5 weeks	6 weeks
7	0.	-	-	-	-	-	-
	0.9	-	-	-	-	-	-
	2.1	-	-	-	-	-	-
	3.1	-	-	-	-	-	-
8	0.	+W	+W	+	+	+	+
	0.7	-	-	-	-	-	-
	2.3	+W-G	+G	+	+	+	+
	3.1	-	+G	+	+	+	+
9	0.	+G	++G	++	++	++	++
	1.3	+G	++G	++	++	++	++
	2.2	+G	++G	++	++	++	++
	3.0	+G	+	+	+	+	+
10	0.	-	-	-	-	-	-
	1.3	-	-	-	-	-	-
	2.1	-	-	-	-	-	-
	4.0	-	-	-	-	-	-
Checks	0-4.5	-	-	-	-	-	-

(Continued)

Culture		% Water
4.	Range	18.4 to 20.0
5.	"	15.0 to 19.4
6.	"	21.0 to 21.8
7.	"	21.6 to 23.4
8.	"	15.0 to 19.5
9.	"	19.2 to 20.2
10.	"	47.4 to 51.2

The percentage of salt-in-brine under these conditions is as follows:

Culture		% Salt in Brine		
1.	0	7.2	13.8	18.4
2.	0	2.9	4.5	7.3
3.	0	3.9	5.9	11.4
4.	0	7.4	12.4	15.2
5.	0	9.3	12.7	15.3
6.	0	6.0	6.9	12.5
7.	0	4.0	8.2	12.1
8.	0	4.5	11.3	13.3
9.	0	6.2	10.3	12.9
10.	0	2.5	4.2	7.8

The butter samples were stored for six weeks at a temperature of 20 to 25° C. Each plate contained one cubic centimeter of added water which was replenished at intervals to maintain a high humidity.

Results. The results of this experiment are arranged in Table XLVIII. It will be seen that Cultures 1, 6, 7, and 10 failed to give any visible evidence of growth altho alterations in the aroma of the butter were quite marked. Culture 5 showed some development of aerial mycelium in the unsalted but was not able to grow in the salted butter. The other cultures gave irregular results which are not easy to explain but Cultures 2, 3, 4, 8, and 9 showed a tendency to grow at all salt concentrations used; the trend being toward less growth in the most highly salted sample. Plate IV illustrates the character of the growth of Culture 4 on butters containing various amounts of salt. The unsalted sample would scarcely be recognized as butter.

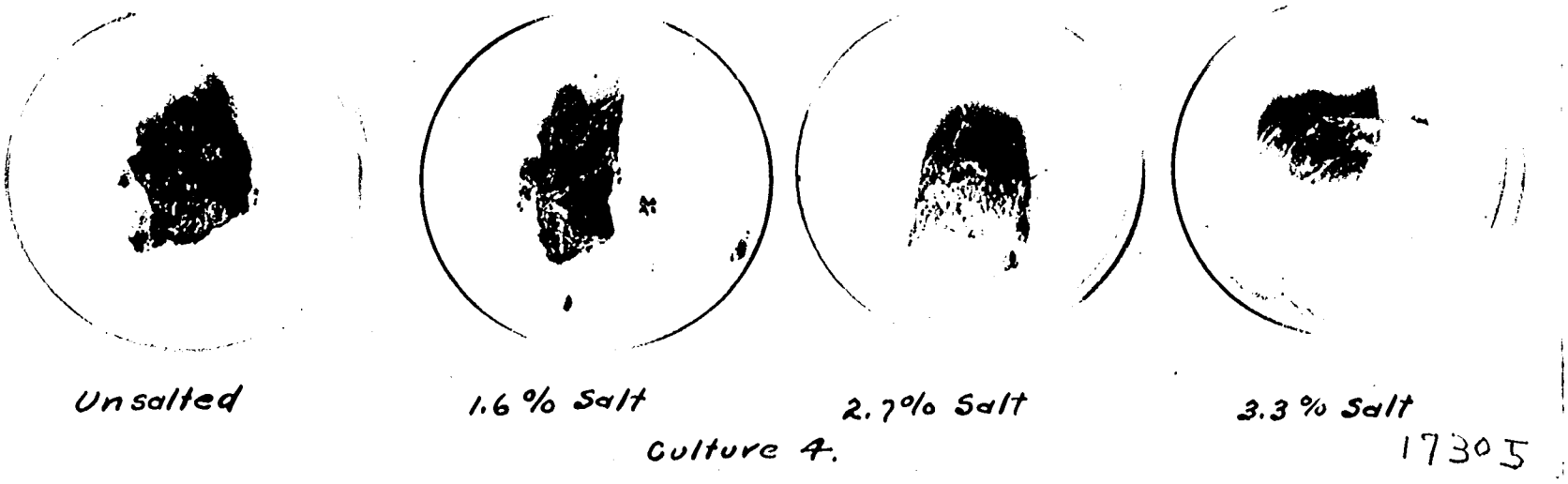

Plate IV

Eff ct of Salt Concentration upon the Growth of Hormodendrum cladosporioides in Butter

DISCUSSION OF RESULTS

The data obtained in the experiments reported in the preceding pages have given clues to some of the more important factors influencing the growth of molds in butter. For convenience, these factors will be discussed in the order in which they were studied.

Food Supply. As pointed out elsewhere, microorganisms must have sufficient and satisfactory nutrients if they are to develop and carry on the metabolic processes of a growing cell. While certain types of organisms are able to subsist on compounds which are practically pure inorganic substances, the majority of the familiar forms require a diversity of foodstuffs. It is generally agreed that fungi seek especially compounds containing carbon, oxygen, hydrogen and nitrogen, particularly in the form of organic substances. In nature, most of the materials carrying these four elements also contain others such as phosphorus, sulphur and a great variety of mineral elements, either intimately as a part of such compounds, or as impurities. The familiar substances, commonly classed as fats, proteins, carbohydrates and mineral salts and the materials related to them furnish the great variety of essential elements for the purposes of microbiological activity. Butter is

a substance consisting principally of a mixture of fats which carry a rich supply of carbon, oxygen, and hydrogen. Held within this mass of fat are droplets of material consisting of such substances as the proteins, casein and albumin, the carbohydrate, lactose, a variety of mineral salts and by-products of the decomposition of any of these compounds, all in association with a relatively large amount of water.

It seemed desirable that a study should be made of the relative value of the various constituents of butter as foods for molds, specifically for those cultures which were selected as representative of the species commonly found in butter. Inasmuch as fat is the principal constituent of butter, it was made the subject of the first trials. It is quite generally agreed that fat is not the most desirable food for microorganisms. This does not mean however that all forms fail to utilize the various glycerides. While it is not easy to obtain fat in an absolutely pure state, it is possible to purify it with reasonable success without altering it character appreciably. As reported in previous pages, the species of molds studied did not make significant growth on purified butterfat unless some water were present. This is quite reasonable since a certain amount of water is necessary for any biological process involving the metabolism of living protoplasm. When growth

was obtained on the butterfat to which water had been added, there was a possibility that it was due to other substances present as impurities in the fat. At any rate, development was not extensive under these conditions and althe germination of conidia occurred in some cases, the amount of mycelium formed was seldom great. Microscopic studies showed that in some instances, initial development occurred but was soon checked and followed by a disintegration of the hyphae. The slight development on purified fats might well be attributed to a supply of nutrients within the inoculum, and present in such minute quantities that growth was soon impossible. The trials with butterfat obtained from various sources and treated in different ways, did not show any particular advantage of one type of butterfat over another. None of them were to be considered as very satisfactory direct sources of food for the early development of the molds being studied, even when water was present to improve the opportunities for growth. These results are in line with previous investigations and in accordance with the observation that butter oils seldom are affected with mold growth.

Lecithin, which contains nitrogen and phosphorus in addition to carbon, hydrogen and oxygen, is known to exist in butter. When this substance, obtained from dried buttermilk and in a highly purified condition, was emulsified in water and inoculated with the

experimental cultures, growth was observed in every case. In this preparation some of the cultures developed only in the depth of the liquid, but the species which have been observed to produce the most extensive growth on butter developed reasonably well upon the surface. When a solid substrate was afforded the cultures, the growth was much more marked.

Everyone familiar with the butter industry appreciates that molding most often occurs after butter has been stored for a period of time. During this time the fats may be hydrolyzed so that glycerol and the constituent acids are released. In this connection, it was desirable to consider the value of glycerol and some of the organic acids commonly present in the mixed glycerides known as fat. It was found that glycerol did not serve as a particularly favorable source of food for the cultures used, at least in the concentration employed. The subsurface development in almost every instance was much better than that upon the surface of the medium, whether the glycerol was in a liquid or solid medium. The only water-soluble fatty acid studied, was butyric. In the 1 per cent aqueous solution of this common hydrolytic product of butterfat, growth was not observed in any instance. Apparently it was toxic for the cultures studied. The growth in the unsaturated oleic acid was much better than that obtained on the saturated palmitic acid which in turn appeared to be more nearly suited to the demands of the molds

studied than the other typical saturated fatty acid, stearic. Since the oleic acid predominates in butterfat there may be occasions where it is called upon to furnish the food supply of molds.

In a general way, butterfat, lecithin, and related substances, were not particularly satisfactory sources of food for the early development of the conidia of the mold cultures used. Normal growth was not obtained in any of these substances, even where sufficient quantities of water were present. It is conceivable, however, that these compounds may be utilized readily by molds which have been able to make their early development of mycelium upon substrata furnishing more suitable food supplies, and after the enzymes necessary to split the fats into readily assimilable compounds have been formed. Many of the typical flavors and odors produced in butter, cheese, etc., such as those characteristic of Roquefort cheese, are associated with fat hydrolysis by molds. It is only necessary to call attention to the fact that such a flavor and aroma was noticeable in some of the fat, water and agar combinations, before there were any visible signs of mycelium. Of course, this hydrolysis of the fat may have been the result of enzymes carried by the inoculum but it is possible that the germinated hyphae produced enzymes when they were called upon to supply nourishment with fat as the only available source of food. Fat and its related

compounds must be considered as potential sources of food for mold growth.

Nitrogen is one of the most important of the elements for the metabolism of the living cell. Many nitrogen-bearing compounds are suitable for this purpose but such substances as natural proteins, their split products, such as peptone, amino acids, etc., and related compounds are generally considered to be the most satisfactory sources of nitrogen for microorganisms. Organisms which can obtain their nitrogen directly from the atmosphere, as elementary nitrogen, are known but the species under investigation have not been shown to be capable of fixing nitrogen in this manner. Rather, they must use other sources of supply. The portion of butter which furnishes nitrogen compounds in considerable amounts is known as the curd. This is really a misnomer as the casein, albumin, and similar nitrogenous complexes normally found in milk are carried over into the butter in much the same form as they exist in milk, or more correctly, in buttermilk. This buttermilk is held in butter principally in the form of tiny, individual droplets, with a full quota of the water which makes up the bulk of buttermilk. As a starting point in the investigation of the value of nitrogenous substances as a source of food for molds, solutions of peptone were used. The germination and hyphal development of the cultures

upon this substrate were both rapid and luxuriant and growth was as extensive as one might hope to see. In contrast with the tendency toward subsurface development in the fatty substances, the growth on peptone was predominately on the surface. The fact that the cultures made more noticeable surface development upon lecithin preparations than upon fats, or fatty acids, may be due to the presence of a nitrogen-bearing radical in the lecithin molecule.

In order to obtain an idea of the usefulness of the actual curd portion of butter for the growth of molds, the fat was removed from a sample of typical butter so that the curd and water portion became available. This substance consisted of a mixture of casein, albumin, lactose, mineral salts etc. with about 85 per cent of water. The growth of the test cultures upon this substrate was prompt and luxuriant. When this material was dialyzed to remove some of the soluble constituents such as lactose, mineral salts, and other diffusable compounds, the residue was found to serve adequately as a source of food for the molds being studied. While growth was not quite as rapid as upon the original mixture it became equally extensive in a short time. This demonstrated that the important constituents were not diffused thru a collodion membrane and indicated that the principal sources of subsistence were the colloidal compounds of nitrogen.

As a further test of the non-fatty portions of butter, which were to be considered as largely nitrogenous, from the theoretical standpoint at least, the sera obtained from the successive washings and separations of cream were employed as substrata for the cultures. It was found that the first washings were quite satisfactory sources of nutrients but each successive washing left less and less material for the needs of a growing cell. This may be considered to indicate that a rich source of food is incorporated in butter in the form of the solids-not-fat in cream and that the purification of fat by successive washings and separations may remove so much of the food for fungi that growth in the finished product consisting largely of fat would be materially retarded. Preliminary studies now underway would lead the writer to believe that this is actually the case.

Inasmuch as lactose is a constituent of butter, an investigation to determine its suitability as a food for the experimental cultures was undertaken. In an aqueous solution of lactose, growth was very meagre, especially on the surface. Some subsurface mycelial development was noted, especially in the bottom of the liquid. When the lactose was held in a washed agar medium, the growth was somewhat better, especially in the depth of the agar. A penetration of the substrate occurred with all cultures. The species of Hormodendrum and Alternaria grew luxuriantly below the surface and

carried a deep green and almost black color in their mycelia while very scanty growth appeared on the surface.

Lactic acid which is so commonly formed by the fermentation of lactose did not prove to be a satisfactory food source and in general, growth of the molds studied was poor at the concentration used.

The diffusate obtained by the dialysis of the curd of butter served as a much more satisfactory substrate for the cultures than pure lactose solutions. This may be attributed to the presence of soluble nitrogen compounds and mineral salts in addition to lactose. At any rate with the diffusate the growth was limited almost entirely to the surface of the liquid which is suggestive of the results obtained with nitrogenous substances. Development was most active within a few days and continued incubation did not lead to any significant increase in the visible mycelium. This would tend to indicate an early exhaustion of essential food elements. The belief that nitrogen compounds were the principal sources of nourishment was substantiated in part by an observation upon the pH of the substrate in different cultures. The initial pH of the diffusate was pH 6.4. After three weeks the hydrogen ion concentration of the medium seeded with Cultures 6 and 7, which developed the least, was pH 6.2 in each case. In the other cultures,

the reaction was altered to points from pH 7.0 to 7.6 which was suggestive of alkaline by-products of protein hydrolysis.

At any rate, it is certain that lactose did not serve as satisfactory material for encouraging the growth of the test cultures. The more abundant growth in the depth of the medium was in accordance with a similar phenomenon in the case of substrata containing glycerol, fatty acids and fats, all of which are compounds lacking nitrogen. The fact, that subsurface growth was also noticeable in the non-nutritive washed agar medium, which served as a check, might be taken to indicate that where the food supply was not especially favorable the tendency was toward deeper penetration of the substrate. This does not appear to be an entirely satisfactory explanation for this phenomenon, however. The species of Hormodendrum and Alternaria showed a tendency to spread into the depth of butter, much as they did on the above-mentioned media, and produced similar dark-colored mycelium even when an adequate and desirable food supply was available. Some reason other than the lack of suitable food must be sought to explain this situation.

Most everyone will concede than mineral elements of various sorts are essential for the growth of microorganisms. Fortunately, minute traces are ordinarily sufficient to meet the needs of the living cell. Usually, in a natural product like milk, they

exist in combination with other constituents or as impurities, even after attempted purifications. One would not expect molds to grow in such a highly alkaline preparation as a solution of milk ash. The results presented demonstrate this. However, a concentration such as a 0.75 per cent solution was not sufficient to destroy the conidia as evidenced by the fact that they were capable of normal growth when sources of nitrogen, carbon, etc. were supplied. It is quite surprising that growth occurred in such an ash solution even when it was neutralized since theoretically no nitrogen or carbon were present. These elements might have been supplied from some other source when molds were able to grow in a neutralized ash solution.

Growth of the cultures in mixtures of butterfat, lecithin and water was not conspicuous. The surface tension may be a factor in this since both of the former substances depress the surface tension, the combination perhaps more than either alone. The presence of particles of milk ash on butterfat, in an environment well supplied with water, appeared to encourage the development of certain species but could not be considered as giving a good growth. When the solutions of ash were mixed more intimately with the fat, the growth was much better which probably means that the mineral salts added a stimulus for the attack upon fat.

The results with a combination of peptone and lactose in liquid or solid media indicated how important a nitrogenous compound really is for the development of fungi. While the proportion of growth upon the surface of the medium was much greater than upon simple lactose substrata, there was more of a tendency for subsurface mycelium to occur than was the case in pure peptone solutions. This may mean that there is something fundamental about the mode of development of molds upon nitrogenous and non-nitrogenous media. The disturbance in the normal surface development of molds in the presence of such substances as lactose, glycerol, fatty acids and fat is striking.

Altogether, butter provides a wide variety of foodstuffs, quite sufficient to sustain the growth of molds. The important consideration is not the amount or quality of the food, however, but its availability. The physical structure of butter must be taken into account, since the most desirable nutrients are dispersed in minute droplets thruout the mass of fat. If the molds happen to be situated where this food is accessible, and if other factors are favorable, growth should take place freely.

Moisture. The effect which moisture has upon the growth of molds is quite clear. One appreciates that all living protoplasm has a relatively high moisture content and that the normal

nutrition of the cell takes place from a food supply dispersed in an aqueous dispersion medium. It has been demonstrated that moisture-free fat is not a good substrate from growth. Butter regularly carries a reasonable amount of moisture even upon a basis of percentage total composition. When it is borne in mind that butter is more than 80 per cent fat, and that the water, constituting from 12 to 16 per cent of the total weight, is largely in association with the nitrogenous compounds as well as the lactose, mineral salts and various traces of other compounds in the form of buttermilk droplets, it may be appreciated that there should be a plentiful supply of moisture for the growth of molds. It has already been pointed out that by actual analysis the curd portion of butter was about 85 per cent water. Therefore, it may be assumed with reasonable assurance of correctness that the moisture conditions in normal butter are perfectly satisfactory.

The humidity of the atmosphere, however, is not such a constant factor, especially in relation to the outer surface of butter. Sometimes butter is stored in a dry, well-ventilated refrigerator or again in a damp place where the air is stagnant and evaporation slight so that the moisture on the surface of the butter is retained. The results of the experiments reported in preceding pages indicate that humidity may play a considerable part in the

development of molds on butter. There is a significant fact which stands out in this connection however. It appeared that sterile butter which was inoculated upon the surface with mold spores failed to show any mold growth when kept in a storage room at a low humidity but when the atmosphere was saturated with moisture extensive growth took place. On the other hand, butters made from cream in which molds had been growing for several days developed moldy areas even under conditions of low humidity. Apparently there is a fundamental difference involved. In the case of the first lot of butter the conidia found it difficult or impossible to germinate because of the lack of moisture brought about by evaporation on the surface, while in those butters where the molds were growing actively, the process was checked only temporarily during churning and continued afterwards because of the abundant supply of moisture obtained thru the mycelium from the reservoirs of water in the droplets of buttermilk. This feature may be of some practical significance. If the infection of the cream takes place before churning and the molds are able to establish a mycelial development before the butter is made, growth may continue and the surface of the butter marred in appearance, even tho the product be stored in an atmosphere low in moisture and conducive to rapid evaporation. On the other hand, if contamination takes place after the butter

is made, and the finished product kept at low humidities, mold growth may not appear. Undoubtedly, a dry, well-ventilated storage room may be an important factor in the prevention of the molding of butter.

Temperature. The temperature at which mold growth occurs varies decidedly with the individual species of molds and the nature of the substrate. The results of the experiments reported in previous pages demonstrate clearly that Aspergillus niger and Aspergillus flavus were checked when the temperature of incubation was below 10° C. This would indicate that these two species need not be considered as responsible for the discoloration of butter as long as the product is kept at low temperatures. The growth of Rhizopus nigricans was inhibited at a temperature of 0° C. Species of Penicillium, Alternaria and Hormodendrum were not as sensitive to low temperatures. This is significant since these species have been shown to be among the most common causes of molding in butter, even when it is kept at low storage temperatures. The amount of salt present in the butter also affected the growth of the various species at different temperatures. The amount of salt present had a more noticeable effect on the growth of the molds at low than at high temperatures. Time is a very important factor in the growth

at lower temperatures. As might be expected, species which are able to grow at low temperatures may require a considerable period before they can produce noticeable growth. It is interesting to note also that the species which are least inhibited by salt are the ones which grow at the lower temperatures. Since the amount of salt in the butter has a marked effect upon the freezing point of the water droplets containing the food materials, with a high salt content the nutrients may remain in a liquid substrate at temperatures much below the ordinary freezing point of water. Consequently, those species able to grow at low temperatures and at high salt concentrations may bring about the molding of butter in storage if sufficient time is allowed for their growth. The temperatures used in these studies were not especially low so it would be desirable to continue the investigations at much lower temperatures with species known to be common causes of molding in butter.

Atmosphere. The effect of atmospheric conditions, aside from humidity, upon the growth of molds is worthy of consideration. The results of the experiments reported herein indicate that a sufficient supply of oxygen is essential for the development of the ordinary molds. When the amount of available oxygen was reduced by a partial evacuation of the air within a sealed container in which

infected butter was stored, the growth was retarded but, in time, development began. It is possible that oxygen was released from the food constituents after the atmospheric oxygen was consumed and eventually permitted satisfactory growth of most of the species studied. Where the oxygen was removed so that anaerobic conditions existed no growth of molds occurred. Butter is seldom stored under conditions where oxygen is completely excluded. In some instances it is placed in containers upon which a partial vacuum is drawn. Consequently, under ordinary commercial conditions, the supply of oxygen may be considered as reasonably satisfactory. In the sealed containers, mold growth may appear as it does in certain instances in canned condensed milk. This condition was studied in the experiment where butter was kept in a partial vacuum. It is not economically feasible however, to handle butter under conditions where all oxygen is removed. It may of course be possible to replace the oxygen by some inert gas but experiments along this line are necessary to demonstrate the practicability of such a plan. The partial removal of carbon dioxide from the atmosphere in which butter was stored did not lead to a retardation of mold development in the experimental samples. How essential carbon dioxide is for mold growth has not been determined but further studies should be made to ascertain this point. Without doubt, atmospheric conditions are

factors which influence mold development but to what extent, is not yet evident. The fact that certain species were able to grow so extensively in the depth of media containing carbohydrates, fatty acids, glycerol etc. raises a question as to these relationships.

Salt. The amount of salt which butter contains is unquestionably an important factor influencing the growth of molds in this product. The results presented in the foregoing pages demonstrate that the effect of salt upon growth depends especially upon the individual species of molds, the amount of moisture present and the temperature. In accordance with many previous investigations, Oospora lactis was checked by a slight concentration of sodium chloride in the substrate. The disappearance of this fungus in salted butter may be explained on such a basis. The species of Mucor and Rhizopus studied were only slightly more resistant to salt and consequently, one would not expect them to be important in the molding of salted butter. Aspergillus niger and Aspergillus flavus were found to be quite resistant to high salt concentrations but when butter was kept at temperatures below 10° C. growth was impeded. For this reason, their importance as causes of molding in butter stored at the usual commercial temperatures is slight. The remainder of the molds studied, namely Alt-

ernaria, Hormodendrum and Penicillium were found to be capable of growing in quite high salt concentrations and at the lower temperatures. Inasmuch as these species produce the most marked changes in the appearance, flavor and aroma of butter when they are able to develop, they are of major importance. The species of Alternaria and Hormodendrum studied produced dark green almost black, smudgy areas on butter on which they grew. Their mycelia spread considerable distances from the point of infection, both along the surface and to considerable depths in the butter. The species of Penicillium which were used did not mar the appearance of the butter particularly, altho in some cases sufficient green fruiting bodies were formed to give a slight, dusty discoloration of the surfaces. The effect of the cultures of Penicillium was largely upon the flavor and aroma. Butters in which these species were growing, became distinctly cheesy in flavor and odor, and in these characters suggested Roquefort cheese. In any consideration of the effect which salt may have upon the growth of mold in butter, the concentration of the salt in each of the droplets of water within the butter must be taken into account. Even though a gravimetric analysis of butter may indicate a high salt content, it is important to know how much water there is present to carry this salt. Some of the experiments reported in the preceding pages illustrate this point.

If the water and salt percentages are both high, the concentration of salt in the water may not be any higher than when the water and salt contents are both low. Equally, if the water content is low and the salt content moderately high, the brine may be quite highly concentrated. Then, also, the salt may be unevenly distributed in the minute droplets of moisture within the butter. Some may represent a strong, and others a weak brine. The number of conidia present in butter, even under extreme conditions, seldom will be large, and never considerable in proportion to the number of water droplets. It is perfectly conceivable that an individual conidium may be allocated to a droplet of water containing little or no salt and an abundant supply of food, and accordingly be able to germinate and develop without restraint. It would only take one such instance to lead to serious consequences as far as molding is concerned. Thus the effect which salt may have upon the growth of mold on butter will depend entirely upon the relative concentration of the salt in the droplets in which the mold spores or mycelium may be located. The molding of salted butter may have a partial explanation in this condition and even though the composition of the butter and type of mold may at first thought have been considered as a sufficient reason for expecting protection against such a contingency.

Species of Molds. It is clearly evident that the species of molds must be considered as factors in the molding of butter. A wide variety of species have been isolated from butter. Among these, the ten species selected for the studies reported were all capable of growing in butter under favorable conditions. From the standpoint of visible growth, Cospora lactis was the least noticeable. It appeared very seldom on the surface of the inoculated butter but evidently was able to develop in the unsalted butter as shown by the fact that in nearly every instance the odor of the butter became distinctly cheesy, resembling most closely the odor of Cheddar or Brick cheese. Aspergillus flavus produced a slight, white felt upon the butter and also fruited extensively to produce yellow, or yellowish-green sporangia above the surface of the butter. It produced a rather indefinite change in the aroma of the butter which was suggestive of fat hydrolysis and bordered on that produced in Roquefort cheese. Aspergillus niger likewise formed a white, cottony layer of mycelium from which a large number of chocolate-brown or black sporangia arose. The odor produced in the butter was much the same as that observed in the case of Aspergillus flavus. Alternaria humicola grew extensively in the form of a white, surface mycelium which eventually became dark green and penetrated into the butter to cause a very dark, green or black smudge.

The odor produced was peculiar, with a suggestion of acetic and butyric acids as well as a slight cheesiness, resembling a rather poor quality of Cheddar. Hormodendrum cladosporioides grew rapidly and extensively, producing dark green fruiting bodies promptly and sending dark green to black hyphae into the butter and spreading widely over the surface. The principal change in the odor of the butter was the development of a mustiness, with a faint suggestion of the aroma of an old cottage cheese. Mucor sylvaticus sent up an extensive aerial mycelium terminating in numerous greyish or black sporangia. The odor of the butter was rather indefinite but resembled acetic and butyric acids, and also suggested tallowiness. Penicillium biforme and Penicillium expansum produced white, cushion-like spots which in time, became grey-green to blue-green. The aroma with both species was decidedly like that of Roquefort cheese, altho there were some other indefinable odors noticeable. The observation that the Penicillium species were capable of producing the characteristic odor of Roquefort cheese in butterfat and butter when there were no visible signs of growth deserves mention. In a similar way other molds may bring about alterations in the flavor or aroma of butter without affecting the appearance. Rhizopus nigricans grew well on the unsalted butter and appeared as a mass of aerial mycelium topped with many black sporangia. The butter

was not markedly affected in odor altho a slight acetic acid aroma appeared in time. As may be seen, most of the mold species studied grew quite well in butter when conditions of humidity, temperature, atmosphere and salt concentration were favorable, and produced evident changes in the appearance and odor of butter.

It is quite evident that, qualitatively and quantitatively, butter contains a satisfactory supply of food for mold growth. Whether or not molds will develop upon a given lot of butter will then depend upon a number of factors among which must be considered especially, the extent of the contamination, the species of molds, the supply of oxygen, the temperature, and the concentration of salt in the aqueous portion of the butter carrying the major portion of the most useful food constituents. A great deal of work remains to be done to elucidate some of these points.

S U M M A R Y

1. Studies were made of some of the factors influencing the growth of molds in butter.

2. The ten species of molds isolated from butter and selected for the investigations, were as follows: Alternaria humicola, Aspergillus flavus, Aspergillus niger, Hormodendrum cladosporioides, Mucor sylvaticus, Oospora lactis var. A., Oospora lactis var. B., Penicillium biforme, Penicillium expansum, and Rhizopus nigricans.

3. The influence of the food supply, moisture, atmosphere, temperature, and salt concentration upon the growth of the ten species was studied.

4. Purified butterfat did not serve as a readily utilizable food, unless water was present. In that case, growth was only moderate, however.

5. Lecithin proved to be a reasonably satisfactory food for the molds studied.

6. The hydrolytic products of fat which provided fairly satisfactory nutriment were glycerol, palmitic acid and oleic acid. Alternaria humicola made slight growth on media containing stearic acid as the only source of food. No growth occurred on a one per cent solution of butyric acid.

7. Compounds containing nitrogen, such as peptone, curd from butter and serum from cream, were found to be excellent sources of food. Growth was luxuriant on the substrata containing these nitrogenous substances.

8. Lactose and lactic acid in one per cent solutions did not furnish especially good nutriment.

9. Solutions of milk ash were not readily utilized by the molds studied unless they were neutralized.

10. Combinations of fat, lecithin and water; fat and ash; fat, ash and water; and lactose and peptone, provided for better growth than that obtained on the individual substances.

11. The molds grew most extensively on the surfaces of media containing nitrogen-bearing compounds.

12. The growth on substrata containing fats, fatty acids, glycerol, lactose or lactic acid was largely below the surface of the medium.

13. Unsalted butter containing a mixture of fat, protein, carbohydrate and ash, supported active growth.

14. The humidity of the atmosphere had a marked influence upon the growth of the molds on butter, especially when the surface of the butter was contaminated. At low humidities growth was checked. When the molds were actively growing in the cream before

the butter was made, the humidity did not have such a pronounced effect upon the growth.

15. Temperature exerted a marked influence upon the growth of the species in various substrata. Growth was active in all cases at 20 to 25° C. Aspergillus flavus was checked at a temperature of 10° C. or lower; Aspergillus niger, and Rhizopus nigricans at 0° C. Mucor sylvaticus grew in whey media and buttermilk but not in butter kept at 0° C. The other species were able to develop at 0° C. but the growth was not as extensive or as rapid as that at 10° C. and 20° C. Time is a factor influencing the growth at low temperatures.

16. The species of molds studied were able to grow on butter at 20 to 25° C. when under a vacuum of 25 inches.

17. A partial removal of carbon dioxide from the atmosphere did not prevent the growth of the molds on butter.

18. None of the molds were able to develop when the oxygen was exhausted from the atmosphere.

19. Salt exerted a marked effect upon the growth of certain species.

20. Oospora lactis, Mucor sylvaticus and Rhizopus nigricans were most readily effected and their growth was inhibited when the concentration of salt exceeded five per cent.

21. Alternaria humicola, Aspergillus flavus, Aspergillus niger, Hormodendrum cladosporioides, Penicillium biforme and Penicillium expansum were capable of growing in media containing 15 per cent of salt. In some cases, the species of Penicillium showed slight growth when the salt percentage was as high as 20 per cent.

22. The extent to which salt inhibited the growth of the molds studied depended upon the species of molds, and the temperature of incubation.

23. All of the species of molds studied were able to grow on butter when conditions were favorable.

24. The appearance of the butter was marred most extensively by Alternaria humicola and Hormodendrum cladosporioides. The flavor and odor of the butter were effected seriously by all of the other species.

25. Since an ample food and water supply is provided, the growth of molds in butter appears to depend largely upon the species of mold, the humidity of the atmosphere, the supply of oxygen, the temperature of storage, time, and the concentration of salt. These influences may act separately or collectively.

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