QUANTITATIVE DETERMINATION OF MOULDS &C. IN CREAMERY BUTTER

A RAPID METHOD FOR THE QUANTITATIVE DETERMINATION OF MOULDS AND YEASTS IN CREAMERY BUTTER.

by

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GENERAL INTRODUCTION.

Until the last few years, Canada has never devoted much attention to the development of markets for her surplus creamery butter. Although this product has been exported in varying quantities since the introduction of mechanical refrigeration on steamships in 1897, the period of rapid growth prior to the war steadily reduced the volume available for export, until in 1913 this country imported six times as much as she exported. More recently, there has been a decided increase in butter production, not only in Ontario and Quebec, but also in the three Prairie Provinces. In the latter, the phenomenal growth of the dairy industry can be attributed largely to the development of a system of mixed farming, as a result of the poor returns from grain-growing after 1920. Thus these provinces, which not long ago imported butter, now manufacture nearly as much as is made in Ontario. Unlike the latter province, these provinces have few industrial centres to provide a home market, and are forced to seek a market for any surplus farther afield.

For many years, Alberta has found a ready market for most of her surplus butter in Vancouver. Like the British market, the demand here is for a mild flavored, lightly salted butter with a smooth, waxy "body" to it. The Albertans were forced to adopt this type in order to meet the competition of New Zealand butter, and for many years all butter has been made from pasteurized cream, and graded by the Provincial

Government before shipment. Recently, Saskatchewan and Manitoba have been shipping butter to this market, and they have adopted the same type as the Albertans. Once the Vancouver market has been supplied, however, any surplus has to find another outlet. With an eight cent per pound duty (recently increased to twelve cents) as a barrier, little of this butter could be profitably marketed in the United States, and attention naturally turned toward Canada's best customer, Great Britain.

In previous years, all the Canadian butter exported to Britain had been surplus Eastern butter, made primarily for home consumption. This butter, with its higher degree of flavor and salting, together with a lack of uniformity, had not met with much favor with the British butter dealer. The Western butter, however, being of the preferred type, and backed by the grade certificates of the respective provinces, met with a very favorable reception, and increasing quantities have gone forward each year. Noting this favorable reception, and being anxious to develop the British market for Canada's surplus butter as production increased, the Federal Dairy and Cold Storage Branch adopted the Western type as the standard type of butter for all Canada. They also instituted a grading system, whereby all butter or cheese exported would bear a Federal grade certificate. As a result, the reputation of Canadian butter on the British market has been considerably enhanced, and this has been reflected in the increased price offered for it.

In spite of this improvement, the reputation of Canadian butter has suffered from the development of mould on occasional lots. From the standpoint of the British dealer, this is a serious defect, causing him considerable loss and inconvenience, and making Canadian butter less desirable in his eyes. It is therefore highly desirable that every possible step be taken to prevent the export of any butter from this country which is highly contaminated with mould spores, and is likely to develop mould spots when favorable conditions are encountered.

Although complaints regarding mould have been directed largely against Western butter, this is not because this butter is invariably more highly contaminated with mould spores, but because, during the long rail haul to Montreal, conditions are encountered which are favorable to the development of mould on the butter. Thom and Shaw(1), in their studies on mouldiness in butter, concluded that the strength of the brine in the butter, the relative humidity, and the temperature were the most important factors governing mould growth. They found that over two percent of salt was necessary to inhibit the growth of Alternaria, Cladosporium and Oidium, while some Penicillium species required a higher concentration. This has been confirmed by Boekhout and de Vries (2), North and Reddish (3), Davis (4), and many others. As our export type of butter usually carries only one and one-half percent of salt, it is evident that this will not prevent mould growth.

Considering the second factor, humidity, we find that much of this Western butter, when shipped via Montreal, spends a fortnight or longer in a refrigerator car between the date of loading and the date of arrival in the steamer's hold. Not only are high relative humidity conditions frequently encountered all this time, but the temperature, the third factor, often rises above 50° F. (Ruddick, (5)). This, as Thom and Shaw (1) pointed out, is distinctly favorable to mould development. Therefore, if butter contains numerous mould spores, we cannot be certain that mould will not develop in transit. That mould actually does develop under these conditions is borne out by the statement made to the author by Joseph Burgess, Chief, Dairy Produce Division, Dominion Department of Agriculture, who has charge of all grading done at the Port of Montreal. Mr. Burgess states that in a number of cases, butter which had been graded for export at Winnipeg and other Western points, has shown considerable mould growth when reinspected at Montreal.

Although better refrigeration would no doubt lessen the possibilities for mould development, yet when we consider the wide range of temperatures to which butter is subjected before being consumed, it is obvious that improvements in this connection will never completely solve the question. Rather, steps must be taken to reduce the contamination of the butter with mould spores, so that mould will not develop even under the most favorable conditions, and to prevent the export of any butter which is potentially danger-

ous. Considerable attention has been paid to this question during 1925, since the results of the investigations conducted by the Dairy Research Division have become known, but much remains to be done before the mould problem is entirely solved.

All control and educational work in connection with mould in butter is based upon the determination of the number of moulds (and yeasts) present. Investigations by Thom and Ayers (6), Bouska and Brown (7), Lund (8), Nicholls (9), Grimes (10), Hood and White (11), and others have established beyond question that proper pasteurization of cream for buttermaking destroys all moulds and yeasts present, most of them succumbing at temperatures from twenty to forty degrees Fahrenheit lower than those commonly used in cream pasteurization in this country (170-185° F.). If therefore moulds or yeasts are found in a sample plated out from the interior of the butter made from such cream, it is obvious that contamination has taken place subsequent to pasteurization. The mould and yeast count can therefore be used as an index of sanitary conditions in the creamery, for where everything with which the cream or resulting butter comes in contact has been thoroughly cleaned and sterilized, the mould and yeast count should be very close to zero. If the butter from any creamery continues to show high counts, a detailed analysis of the cream and butter after every operation, together with the wash water, salt and air, will locate the source of the contamination, and thus assist the buttermaker in keeping his mould and yeast counts as low as possible.

Routine mould and yeast counts are being carried on not only by the Dairy Research Division of the Dominion Dairy and Cold Storage Branch, but also by the Dairy Branches in the Prairie Provinces and Ontario. The information obtained from this work provides a record of the mould and yeast content of a number of lots of butter, which is of value if complaints are received later in connection with the development of mould. In addition, this information is being used as a medium for educating the buttermakers in the production of butter with a low mould and yeast content. Unfortunately, the scope of this work is limited by the expense and time involved in using the petri dish method of analysis. The time factor is particularly important, for five days must elapse before counts can be obtained; consequently, information regarding high mould contamination in any lot of butter for export may not be available in time to stop this lot from going forward. This situation can best be met by the development of a more rapid method of analysis, whereby results could be obtained in from twelve to twenty-four hours. Such a method would facilitate the detection of high count butter at the grading stations, and such butter could then be refused a certificate for export. In this way, the deleterious effect of mould development upon the reputation of Canadian butter in Great Britain could be reduced to a minimum, and buttermakers, knowing that their butter would not be passed for export unless low in mould content, would be forced to guard against contamination of the cream and butter subsequent to pasteurization.

In addition, such a rapid method, in the hands of a trained field man. could be made to render valuable service in educating the less progressive type of buttermaker. This type of man is not likely to bother visiting a central laboratory to find out more about the mould question: hence the necessity for conducting the analysis right at the creamery, where he may see with his own eyes what has developed from the butter churned the previous day.

Finally, by using such a rapid method to supplement the petri dish method used in routine work at present, a considerable saving could be effected. When we consider that by the latter method, it is necessary to pour duplicate plates in dilutions from 1/1 to 1/1000 for every sample, it is evident that a great saving could be effected by using the rapid method as a guide to the proper dilution to employ.

It is unlikely that such a rapid method would entirely replace the present petri dish method in routine analysis, because of the difficulty involved in obtaining accurate counts on low count butter. Rather, it should be regarded as a supplementary method having certain advantages over the present method in the rapid detection of highly contaminated butter, and for work in the field. It has been with a view to developing such a supplementary method that the present work has been undertaken.

REVIEW OF PREVIOUS INVESTIGATIONS.

Probably the first extensive quantative determinations of the mould and yeast content of creamery butter were commenced by Bouska and Brown (7) in 1913. While seeking the reason for poor keeping quality in butter, these authors conceived the idea of predicting the keeping quality of butter by determining the number of yeasts and Oidium lactis present. Samples were carefully taken to avoid contamination, and one c.c. portions were plated out on whey agar acidified with sterile tartaric acid. The plates were incubated for two or three days at room temperature, and then counted. Based upon investigations covering 135,000,000 lbs. of creamery butter, they state that the mould and yeast count of butter made from properly pasteurized cream and protected from subsequent contamination, should not exceed thirty. In many cases these were less than ten. In view of the higher counts usually obtained by workers since that time both in this country and in the U.S., the possibility suggests itself that Bouska and Brown might have obtained higher counts if they had used beer wort agar; North and Reddish (3) found in their experience that Oidium lactis developed on the latter medium, but not on the whey agar. Stiritz(12) also mentions obtaining more satisfactory results from a medium made from near beer, resembling the beer wort agar of Lund (8)

Lund (8) working at Ontario Agricultural College, was

probably the first in Canada to attempt mould and yeast determinations on a large scale. After considerable preliminary experimentation, he developed the following technique, which is the one used in all routine count work in Canada today. This technique will be referred to in this thesis as the Standard Plate Method.

Sample plugs of butter are obtained with a sterile tryer, and transferred to a sterile screw cap jar with the handle of a scalded teaspoon, discarding the top $\frac{1}{2}$" of each plug drawn. This sample is then heated to 40 - 45° C. to melt the butter, shaken vigorously, and a 10 cc. portion is transferred to a 90 cc. water blank also warmed to 45° C. After thoroughly shaking further dilutions may be made as desired. One cc. portions of the dilute butter are then transferred to sterile petri dishes, and 10 cc. of beer wort agar, acidified with sterile lactic acid, added. These are then incubated at 25° C. The Oidia are counted after 3 days and Penicillium and yeasts after 5 days.

Commencing in 1918, several hundred samples were analysed annually according to the above technique. The beer wort agar was adopted after a number of comparative tests as being the most satisfactory for the purpose. It was hoped at first that the mould and yeast count could be used as a means of distinguishing between butter made from raw and pasteurized cream, but many samples giving a negative reaction to the Storch test showed the presence of numerous moulds and yeasts. Lund concluded, however, that the mould and yeast count afforded a

valuable indication of sanitary conditions in the creamery.

Brown, Smith and Ruehle(13), 1920, in a study of experimental butters. used a similar technique. The sample was melted at 35° C. and stirred with a sterile glass rod: 11.5 c.c. of this was then introduced into 90 c.c. of sterile physiological salt solution at 35-40° C. From this, higher dilutions were prepared. No mention is made of the media employed.

Grimes(10), 1922, used a technique similar to that of Bouska and Brown(7), in studying the action of certain bacteria, moulds and yeasts on the keeping quality of butter.

The American Association of Creamery Butter Manufacturers(26) conduct a large number of bacteriological analyses and mould and yeast count determinations every year. For the latter, they use a whey agar medium. and incubate plates for 3 days at 37° C. They have set the following standards for salted butter:

Less than 10 yeasts and moulds	Excellent
Between 10 and 50 yeasts and moulds	Good
" 50 " 100 " "	Fair
Over 100 yeasts and moulds	Poor

In the author's opinion, the use of whey agar, together with an incubation period of three days at 37° C. would result in a lower count than would be obtained if Lund's method were used.

Within the last two years, Hood and White(11), of

the Dairy Research Division at Ottawa, have done a great deal of work on moulds and yeasts in butter, using the Standard Plate Method evolved by Lund(8), but with a five day incubation period for all moulds and yeasts. They have also made use of the standards established by the American Association of Creamery Butter Manufacturers, and all samples analysed by them are graded on this basis. It will be noted that in this classification, yeasts and moulds are considered of equal importance; not because yeasts are as detrimental to the quality of the butter as the moulds, but because yeasts and moulds alike indicate contamination subsequent to pasteurization. High counts of both usually go hand in hand. Parfitt(24), in a report on work at Purdue University, mentions that the yeast content of butter appears to be a good measure of the methods and care used in the manufacturing process.

All the methods mentioned thus far are macroscopic, and suffer from the disadvantages common to such methods. In the first place, plates must be incubated for from three to five days before being counted. The handicap of this procedure in attempting to detect high count butter and prevent its being exported, has already been referred to. In addition, the high cost of a petri dish method definitely limits the extent to which it can be employed. Finally, for field work, such methods are unsatisfactory, on account of the difficulties involved in carrying about large quantities of water dilution blanks, petri dishes, pipettes, and media.

The problem of facilities for incubation and plate counting also presents real difficulties.

In an endeavour to overcome some of the objections to the petri dish method, Redfield(14), in 1919, adapted the direct microscopic method of Breed(21) to the determination of yeasts and oidia in cream and butter. He melted the butter at 40-45° C., until the curd-whey-brine serum was clearly separated from the fat. 1 c.c. of this serum was then transferred to a clean watch glass, and 0.01 c.c. was measured with a capillary pipette and spread over an area of 1, 2, or 4 cm^2 on a microscopic slide. After air drying, the fat was extracted with xylol, the film fixed with 95% alcohol, and stained with a saturated aqueous solution of methylene blue. Counts were made of 100 fields under the oil immersion lens, the microscope being adjusted to a factor of 500,000. Each cell, separate or as an element in a chain or budding colony, was counted.

Counts so obtained were compared with those obtained by plating out by the use of the following technique, 4 plugs of butter were liquefied in a sterile bottle, 10.1 grams weighed into a sterile porcelain mortar, and enough sterile sand added to give a dry, granular mixture when ground up with a sterile pestle. The material was then scraped into a sterile 500 c.c. wide mouthed glass stoppered bottle; 90 c.c. of sterile distilled water was added, and the bottle shaken thoroughly to insure a uniform mixture. 1 c.c. portions were then plated out on whey or wort agar, and in-

cubated for five days at 30° C.

While in general low grade butter showed high mould and yeast counts, a number of samples revealed great variations in the counts obtained by the two methods. The following extracts from Table 1 in Redfield's paper, bring out this fact very clearly;

EXTRACTS FROM TABLE 1. (Redfield)

Sample No.	Score.	Microscopic Count. Yeasts	Oidia.	Cultural Count. Yeasts.	Oidia.
90	92	135,000	-15,000	5	1
19	91.5	815,000	100,000	-10	-10
31	90.5	300,000	-15,000	3	0
50	86	385.000	135,000	3	4
27	84.5	1,235.000	335,000	-10	-10
54	84	1,715,000	285,000	12	0

From a consideration of the above, it will be obvious that the direct microscopic method cannot be relied upon to give a satisfactory indication of the moulds and yeast content of a sample of butter. In many cases, samples giving extremely high counts by this method showed counts sufficiently low by the plate method to place them in the highest grade of the proposed standards. The reason for this disparity is to be found in the fact that, by the former method, it is not possible to distinguish between viable and dead cells. This, therefore, distinctly limits the value of this

method for determining the mould and yeast content of creamery butter.

In 1920, North and Reddish(3) checked Redfield's technique, using high grade experimental butter. They concluded that, in general, this method can be used to show the relative numbers of yeasts and oidia in samples of butter. Tests were also made to compare the count obtained by plating out 1 cc. of the butter with that obtained from plating out 1 c.c. of the curd-whey-brine serum, in the hope of being able to establish a ratio between the two counts. They found, however, that the counts obtained by plating out the serum ran all the way up to ten times as high as those obtained from the butter, and no ratio that might be universally employed could be established.

The author finds it difficult to agree with the conclusions of these workers in regard to the Redfield method. Any method showing such wide variations from the standard plate count is far too unreliable to be satisfactory. In addition, Redfield's method possesses the disadvantage that, even when 100 fields are counted, -5,000, the lowest count that can be stated, is fifty times the magnitude of the lower limit of the poorest class in the classification in use today. From the results of Redfield(14) and North and Reddish(3), we may safely conclude that the direct microscopic method is of limited value in determining the mould and yeast content of butter, and that we must look to some other method for a rapid test to supplement the present method.

INVESTIGATIONAL

PART 1. DEVELOPMENT OF METHOD.

A. Review of Situation and Statement of Problem.

In the foregoing review, an endeavour has been made to point out the importance of the question of mould growth on Canadian butter, and the necessity for taking all possible steps to overcome this defect. Mention has also been made of the work being carried on at the present time by the Dairy Research Division at Ottawa, and by the Provincial Dairy Branches in the Prairie Provinces and Ontario; of the need for a rapid method for the detection of potentially dangerous butter at the grading station, and for the use of the field man, who is carrying the fight against mould right to the creamery. Lastly, the methods used by various investigators of the mould and yeast content of butter have been critically reviewed. The conclusion arrived at may be stated as follows; the only rapid method for obtaining mould and yeast counts developed thus far is of limited value in relation to the present problem, due to its inability to count below 5,000 per c.c., and to distinguish between dead and living cells.

In brief, then, the problem which the present investigation seeks to solve is the development of a method for determining the mould and yeast content of butter in a much

shorter space of time than that required by the present standard plate method incidentally, one which will be more economical of glassware, media, and labor; which will give counts showing a distinct correlation to those obtained by the standard plate method; and which will enable the worker to count as far below 5,000 per c.c. as possible.

B. Preliminary Consideration of Factors Involved.

Mention has been made of the fact that with the standard plate method, it is necessary to wait from three to five days before the yeast and mould colonies have developed sufficiently to be counted with the naked eye. If counts are to be obtained in a shorter space of time, the macroscopic petri dish method must evidently be replaced by a microscopic method. Of the two best known microscopic methods, the first. Breed's method(21) has already been discussed, and shown to be unsuitable for butter from pasteurized cream, as it also is for pasteurized milk. The other method, known as the Frost little plate method(15), combines certain features of both the Breed direct microscopic method and the petri dish method. The Frost method has been shown by Simmonds(16), Hatfield and Park(17), and others, to give results in routine milk analysis which compare favorably with those obtained by the official plate method. This is due to the fact that only the colonies of bacteria which

develop on the little plates during incubation are counted, while in the Breed method, all cells, dead or alive, are included in the count.

Briefly, tne technique of the Frost little plate meth od as applied to milk analysis is as follows

An area of 2 x 2 cm. is marked off on an ordinary microscopic slide, sterilized in a flame. and 0.05 c.c. of milk deposited thereon. An equal quantity of melted nutrient agar is added. and mixed with the milk. The slide is harden-ed under cover. then incubated in a steriie moist chamber for six hours or longer. The films are then dried, fixed, stained, and examined under a microscope which has been ad-justed to give a convenient microscopic factor. The count is then calculated by the formula

$$\frac{\text{No. of colonies counted}}{\text{No. of fields counted}} \times \text{Reciprocal of quantity} \times \frac{\text{Area of plate}}{\text{Area of field}} = \text{Number of colonies per c.c.}$$

In view of the unsatisfactory results reported with the Breed method, and of the more promising indications of the Frost method, it was decided to try and adapt the latter method to the quantitative determination of moulds and yeasts in creamery butter; then to compare the counts ob-tained by the method mentioned above with those obtained by the standard plate method, on a number of samples. As the development of this method has been the chief aim in the

present work. it has been found most convenient in the arrangement of this thesis to discuss each phase of the work in detail as dealt with, and to collect all tables of data, charts, plates, etc. together at the end, where they may be more readily referred to and compared.

In the modification of the Frost little plate method of milk analysis to the determination of moulds and yeasts in creamery butter, a number of factors must be considered. Some of these, such as the period and temperature of incubation, and the plating of butter vs. the plating of serum, have required considerable experimental work in order to provide sufficient data on which to base decisions and draw conclusions. These factors will now be discussed in detail, followed by a detailed description of the technique finally adopted, which will be equivalent to the results of Part 1. Investigational.

Specific Considerations of Media.

As mentioned in the review of previous work, whey agar acidified with tartaric acid has been used by a number of investigators. Experiments conducted by Lund(8), however, to test out the value of a number of different kinds of media showed the superiority of a beer wort agar made up as follows,

Unhopped wort	400 c.c.
Tap water	600 c.c.
Agar	15 grams.

This medium is filtered and sterilized in the autoclave at 15 lbs pressure for twenty minutes. Before pouring plates, 4 c.c. of sterile 5% lactic acid are added to each 100 c.c. of wort agar. Although Lund makes no mention of the pH value of his medium, determinations by the colorimetric method during the present work, using brom phenol blue as indicator, have shown that the pH of the acidified wort agar ranges between 3.5 and 4.0, depending upon the original acidity of the wort. For the majority of the common moulds, this represents the optimum hydrogen ion concentration for spore germination and growth, as shown by the work of Webb(18) and of Johnson(19).

This wort agar medium of Lund's has been adopted by the Dominion Dairy Research Division, and by the various Provincial Dairy Departments, in their routine mould and yeast determinations. Inasmuch as the microplate method must be compared with the method now in use, it was considered advisable to adopt this medium for the present work also, and not to make any attempt to evolve a new medium. It was found necessary, however, to slightly modify the method of preparing the medium, in order to obtain the clearest possible backgrounds for the microplates. A clear medium is not of paramount importance in the standard plate method, but makes considerable difference in the ease with which yeast colonies in particular can be counted under the microscope with the microplate method. To obtain this clear medium, the wort is first autoclaved and filtered through cotton. This is

repeated a day or two later. By this means as much of the heat coagulable constituents as possible is thrown down before the final sterilization of the wort agar. The agar is washed in distilled water for several days prior to being added to the wort. Finally, the medium, after making up and filtering for the last time, is sterilized by heating in flowing steam for thirty minutes on two successive days. Care must also be taken when melting the agar before using, not to heat it too high, or for too long a period. Wort agar prepared in this manner gives a clear background, which is very satisfactory for counting mould and yeast colonies under the microscope.

Area of Microplate.

A few trials were made with large glass slides having an area of 4 x 4 cm. marked out. It was found that this larger size did not give any advantages to compensate for the disadvantages incidental to the handling, incubation and staining compared with the ordinary 2.5 x 7.5 cm. slide having an area of 2 x 4 cm. marked out. This size takes only one-half the incubator space required by the larger one, and permits the use of Coplin jars for staining the films.

The 2 x 4 cm. area is marked out on a clean microscopic slide with a wax pencil using a metal marker of the correct size. The wax pencil lines prevent the liquid from running off the area marked out, which is a great advantage when being mixed with the agar to form the microplate.

Amount of Material Plated.

With the original technique, a dilution of 1/10 was used, and 0.1 c.c. of this plated on an area of 2 x 4 cm. This enabled counts to be obtained as low as 2,000 per c.c. Later, however, in order to reduce this lower limit, a dilution of 1/5 was used, and 0.2 c.c. of this plated upon the same area. This enabled counts to be obtained as low as 500 per c.c., when 20 fields were counted.

Microscopic Factor.

By the microscopic factor is meant the relation between the area of the microscopic field and the area of the microplate. In order to arrive at this, the diameter of the microscopic field, under the low power objective (2/3"), with the minimum draw-tube length, was determined with a stage micrometer to be 1.6 mm., and the area of the field calculated by the formula πr^2

$$\pi r^2 = 3.1416 \left(\frac{1.6}{2}\right)^2 \text{mm.}$$

$$= 3.1416 \times .64 \text{ mm.}$$

$$= 2.01 \text{ mm}^2, \text{ or approximately } 2 \text{ mm}^2.$$

The area of the microplate being 800 mm^2 (20 x 40 mm.), the microscopic factor is then

$$\frac{800}{2} = 400$$

Counting.

Keeping in mind the purpose for which this test will be used, it is evident that rapidity and convenience are of greater importance than extreme accuracy, where the latter can be obtained only by great refinement of technique. Therefore, it was decided that, as in milk analysis, the counting of 20 fields should be sufficient. Tests in which various numbers of fields have been counted have shown that the counting of more than 20 fields rarely causes any significant change in the count obtained, except in the case of low count butter. Here it has been found advisable to count 40 fields. Where the yeast count runs over 50 colonies per field, the counting of 10 fields is ample; if over 100 colonies per field, 5 fields will be sufficient.

The method employed in counting is as follows;

First, make a rapid preliminary survey of the microplate, to ascertain how evenly distributed the colonies are. Then twenty representative fields are selected and counted, starting from the top left hand corner, proceeding diagonally across to the opposite side, then diagonally back again. This method of zigzagging across the microplate usually givesmore satisfactory results than when counts are made from fields taken diagonally from one corner to another across the full length of the microplate. The use of a mechanical stage also tends to reduce the possibility of error due to the entrance of the personal equation in the select-

ion of fields for counting.

The counts on the duplicate microplates are then averaged and the mould and yeast count of the butter calculated from the formula

$$\frac{\text{Number of colonies counted}}{\text{Number of fields counted}} \text{ X Reciprocal of dilution X}$$

Reciprocal of quantity plated X Microscopic factor

= Number of moulds (or yeasts) per c.c. of butter.

For example, if the average of the duplicate microplates showed 10 moulds and 40 yeasts on 20 fields, we would have

Moulds. $\frac{10}{20} \times \frac{5}{1} \times \frac{5}{1} \times 400 = 5{,}000$ moulds per c.c.

Yeasts. $\frac{40}{20} \times \frac{5}{1} \times \frac{5}{1} \times 400 = 20{,}000$ yeasts per c.c.

With the technique first used. it was not possible to obtain counts any lower than 2,000 per c.c. That is, if no colonies were encountered on twenty fields, where 0.1 c.c. of a 1/10 dilution was plated on a 2 x 4 cm. area, the mould (and yeast) count was stated as less than 2,000 per c.c. (-2,000). This technique was fairly satisfactory with the extremely high count butter used at first (which had been returned from England on account of mould), but proved of limited value in dealing with the ordinary run of samples. The technique was therefore modified by changing the dilution from 1/10 to 1/5, and increasing the quantity of the material

plated from 0.1 c.c. to 0.2 c.c. With this technique, if 20 fields are counted and no colonies encountered, we may regard the mould content as less than 500 per c.c. (-500), and the yeast content the same. If 40 fields are counted with the same result, we may express the count as less than 250 (-250). Attempts were made to obtain lower counts by increasing the quantity of material plated, and also by using dilutions lower than 1/5. In neither case were they successful. If a dilution greater than 1/5 be used, it is difficult to obtain a satisfactory suspension, even temporarily; the greater amount of fat present cannot be mixed with the agar, and large blobs of fat form on the microplate. These obscure the field, hiding many colonies, and making it difficult to obtain accurate counts under the microscope. Similarly, satisfactory microplates cannot be obtained if more than 0.2 c.c. is plated out.

There still remains the possibility of reducing the lower limit of counting by the use of an ocular of lower power in the microscope. If an ocular could be obtained which would give a diameter of field of 2.25 mm. in place of the 1.6 mm. in the present work, the microscopic factor would be reduced to 200, and a minimum count of 250 per c.c. obtained. As such an eyepiece was not available, the author was not able to test out its suitability compared with the one used in this work. Unless it is possible to reduce the minimum count appreciably, as above, the author feels that a change would not compensate for the loss of the present con-

ven.ent microscopic factor, which when multiplied by the reciprocals of the dilution and the quantity plated, gives a factor of 10,000 by which to multiply the average count per field. With this factor, the calculation of the numbers of moulds and yeasts present is as simple as by the standard plate method.

Plating Butter vs. Plating Serum.

Bearing in mind the use of this method in the field, the possibility of further simplifying the microplate technique by dispensing with dilutions and plating out the curd-whey-brine serum from the bottom of the melted butter samples was investigated. This procedure would also enable the worker to secure a lower minimum count. In order to test the reliability of this method in comparison with the butter dilution method, both counts being compared against the standard plate count, some 50 samples of butter were plated out. The data obtained from this experiment appears in Table 1.

From a study of the results obtained by the two modifications of the microplate method, the following conclusions were drawn;

The mould count by the serum microplate method shows much greater variation in comparison with the standard plate count than is shown by the butter dilution microplate count. Consequently, it would be very difficult to establish a satisfactory ratio between the serum count and the standard

plate count, for comparing the count obtained by the one method with that obtained by the other.

2. In a number of cases, the yeast count by the serum method was more closely comparable to that obtained by the standard plate method than was the count on the butter dilution microplates.

3. With the serum microplates, it was not possible to obtain as clear a background as with the butter dilution method. These murky backgrounds make it difficult to obtain satisfactory counts where the mould or yeast colonies are small and lightly stained.

4. With an incubation period of 18 hours, the Oidium lactis colonies on the serum microplates had disintegrated into oidia in many cases; this makes it very difficult to obtain accurate counts, particularly where there are several colonies present per field.

5. Oidia in chains, and yeasts in clumps, in the butter sample are not broken up into individual cells before plating in the serum method, as in the butter dilution method.

6. For field work, the serum method can be used to advantage, as it gives a satisfactory indication of the extent of contamination with moulds and yeasts, and is simpler than the butter dilution method, in that no dilutions are required.

In addition to the above-mentioned comparisons by the microplate method, portions of the serum, the clear melted butterfat, and the butter itself were plated out from seven

samples, the counts from which appear in Table 8. In these tests, the ratio of the mould count of the serum to that of the butter varied from 4.3 : 1 to 17.7 : 1, while the ratio of the count on the pure butterfat compared with that of the butter varied between 1 : 1.4 and 1 : 114. These results are in agreement with those obtained by North and Reddish (3), already referred to.

Inasmuch as the microplate method will be used mainly for catching shipments of export butter having a high mould count, the author decided that it was more important to have a method giving accurate mould counts, even if there were greater discrepancies in the yeast count. Therefore, the butter dilution method has been adopted as meeting these requirements more satisfactorily than the serum method. The latter method, however, may be used to advantage in field work.

Period of Incubation.

In his description of the little plate method for milk analysis, Frost(15) recommends an incubation period of six hours or longer. Hatfield and Park(17), working in the laboratories of the New York Board of Health, have used a fifteen hour period in routine milk analysis with satisfactory results. In order to determine the most suitable period for mould and yeast counts, two series of experiments were conducted. In the first, ten microplates were made from the

same dilution of each sample of butter. These were then incubated at 25° C. At the end of six hours, two plates were removed, dried down, stained and counted. This was repeated after nine. twelve. fifteen and twenty-four hours. Table 2 contains data from this series of experiments. These show that with some samples, it is possible to obtain a count after six hours which will give a fair indication of the mould content: with many. however. germination of the mould spores is too slow, and there is a progressive increase up to the twelfth or fifteenth hour. After the twelfth hour, the mycelium of the mould has usually spread until its diameter is as great as that of the microscopic field, and care must be taken in counting, where the whole of the colony is not within the field. Satisfactory counts of the yeast colonies cannot be made until after the twelfth hour, when they have reached a size large enough to show up clearly when stained with thionin. It should be noted that in a number of cases, the yeast counts at twenty-four hours are considerably higher than at fifteen hours.

In the second series, the microplates were prepared as in the first, duplicate plates being removed and counted after fifteen, eighteen, twenty-one and twenty-four hours. Table 3 contains data for this series. A study of the data reveals the fact that the differences between the mould counts made after these different periods of incubation are not large enough to be considered significant, being no greater than the differences between duplicate plates both

by the standard plate method and by the microplate method. It was noticed however, that from eighteen hours on, the Oidium lactis colonies tended to disintegrate, rendering accurate counting difficult. On the other hand, the yeast counts in some cases showed a considerably higher count after twenty-four than after fifteen hours.

Bearing in mind the question of convenience in routine laboratory work, in addition to the results above noted, it may be stated that a period of between twelve and eighteen hours, preferably fifteen hours gives most satisfactory results for mould counts. Plates made late in the afternoon can be counted fearly on the following morning. Where conditions render it imperative , however, it is possible to obtain valuable information as to the mould content in six to nine hours.

Temperature of Incubation.

Among previous workers, opinion seems divided as to the optimum temperature of incubation for moulds and yeasts. Nicholls(9), states that Oidium lactis, the commonest mould in butter, grows best at 35° C. Hunziker (25) also recommends this temperature. The American Association of Creamery Butter Manufacturers(26) incubate plates at 37° C. Stiritz(12) and Redfield(14) both consider 30° C. the optimum, while Lund(8) used 25° C. and Bouska and Brown(7) room temperature. To settle the question for the microplate method,

several series of experiments were conducted. In the first series, four standard plates and four microplates were prepared from each dilution. Half of these were kept at room temperature (16-19° C.) and the remainder in the incubator at 27° C. Counts obtained appear in Table 4; these show that although room temperature is apparently equally satisfactory for the standard plates, which are incubated for five days, germination of mould spores on the microplates is greatly delayed. It is quite feasible to use the room temperature if the period of incubation is extended long enough, viz. twenty-four hours or longer.

In the second series, the same procedure was followed, except that the temperatures compared were 24° and 29° C. This series was plated with the original technique, which did not permit counting below 2,000 per c.c. Therefore, as only two samples showed any mould count by the microplate method, the results are inconclusive. Counts obtained appear in Table 5.

In the third series, the samples were plated out using the revised technique, and incubated at 25° and 30° C. The counts from this series appear in Table 6. Considerable variation is evident. yet by both methods. the count at 25° exceeds that at 30° in twice as many cases as the reverse holds true. More important with the microplates is the fact that at 25° after sixteen to eighteen hours, both mould and yeast colonies stand out much more clearly, and are more easily counted, than after incubation for a like period at 30°.

Degree of Shaking, Style of Pipette, etc.

In order to determine the effect of these factors, seven samples of butter were plated out as follows; the first two plates(A) were prepared after shaking the dilution blank for fifteen seconds. then with an ordinary 1 c.c. pipette, graduated in 1/100ths, delivering 0.2 c.c.

The second two microplates(B) were prepared similarly, except that the dilution flask was shaken for thirty seconds. The third two(C) were prepared after fifteen seconds shaking, but using a one-mark pipette which delivered 0.2 c.c. The fourth two(D) were the same as (C), except that the dilution was shaken for thirty seconds. Data appear in Table 7.

A study of the data shows that in nearly every case the B and D plates gave the highest counts. This is to be expected, as, with a longer shaking, the chains of oidia and groups of yeasts are more completely broken up. This indicates the desirability of thoroughly shaking the dilutions immediately before pipetting onto the slide. Again, in practically every instance, the counts obtained from the microplates where the one-mark pipette was used were lower than those where the ordinary graduated pipette was employed. The most likely explanation of this is that more fat is deposited upon the slide with the one-mark pipette. With the ordinary graduated 1 c.c. pipette, the mixture of fat and water is drawn up to the highest graduation, the fat has time to

rise to the top, and a clear liquid, practically devoid of fat, is deposited upon the slide in plating. This naturally results in a much clearer type of microplate, which is much more easily counted under the microscope. Where large blobs of fat are deposited, many mould and yeast colonies are obscured, which accounts for the lower counts obtained.

Mention has already been made of the analyses conducted to determine the relative distribution of the mould spores and yeast cells between the butterfat and the serum, data from which appear in Table 8. These showed that the majority of these cells were to be found in the serum. In addition, by the time the butter has been thoroughly shaken in diluting, the organisms remaining in the butterfat itself will be but a small percentage of the original flora of the butter. It was decided that these few cells remaining in the butterfat were unimportant compared with the necessity for obtaining microplates with as clear a film as possible, to facilitate accurate counting.

In view of the fact that the microplates prepared by the use of the ordinary graduated 1 c.c. pipettes were more satisfactory for counting, and gave a higher count, it is recommended that this type of pipette be used in the microplate method, in preference to pippettes of the one-mark type.

C. Detailed Description of Technique.

Having fully discussed the various factors involved in modifying the Frost little plate method of milk analysis to the quantitative determination of moulds and yeasts in butter, it now remains to sum up the foregoing by giving a detailed description of the modified technique as finally adopted.

Samples of butter for analysis are obtained by drawing three or four plugs with a sterile trier, and transferring these to a sterile screw-capped jar with the handle of a scalded teaspoon. The top ½" of each plug is discarded, so that the results will not be affected by contamination upon the surface of the butter; in this way, the results will furnish a valuable indication of sanitary conditions in the creamery manufacturing the butter. The sample jars are next placed in water at 40-45° C. This temperature must not be exceeded, or some mould or yeast cells will be weakened or killed, and not develop on the microplates. When the butter is thoroughly melted, it is shaken vigorously for thirty seconds, and a 10 c.c. portion withdrawn with a sterile pipette. The exterior of the pipette is carefully wiped with a sterile cloth to remove any butter, and the contents introduced into a 250 c.c. Erlenmeyer flask containing 40 c.c. of sterile water warmed to 45° C. The use of a 10 c.c. portion reduces the possibility of error through random

sampling, while the use of a 40 c.c. water blank reduces the possibility of error due to inaccuracies, when compared with smaller samples and water blanks. However, where, through lack of sufficient laboratory equipment or space, the larger sample cannot be used, satisfactory results may be obtained where 2 c.c. of butter are added to 8 c.c. of water in ordinary test-tubes. When these smaller samples are used, it is essential that the butter sample, and the dilution made from it, be exceptionally well shaken, in order to distribute the moulds and yeasts as evenly as possible throughout. For the major portion of the present work. the author has been obliged to use the smaller samples. Before doing so, tests were made comparing the results from both large and small samples, which showed that where sufficient care is taken, entirely satisfactory results be obtained with the smaller samples.

While the butter is melting, clean microscopic slides are prepared by marking out upon them with a wax pencil, an area of 2 x 4 cm. (See Fig.1.) A small insulated metal box or tank with a flat cover is then filled with water at 45° C., (See Fig. 2.) This is used as a "warm table", to prevent the agar from hardening on the microplates before it can be mixed with the diluted butter being plated. $\overset{10}{A}$ c.c. tube of previously melted wort agar, which has been acidified with sterile lactic acid to give a pH of 3.5 to 4.0 (4 c.c. of 5% lactic acid to 100 c.c. of agar), is next placed in in the water through the hole in the top of the warm table,

to keep it at the proper temperature.

Duplicate slides are now sterilized by passing several times through the flame and placed upon the top of the warm table. The dilution flask or test-tube is vigorously shaken again, and the mixture drawn up to the top graduation in a 1 cc. pipette graduated in 1/100ths of a cc.(Fig.3) After wiping off the exterior with a sterile cloth,or paper, 0.2 cc. are spread over the 2 x 4 cm. area on each slide. Four drops of agar are now added to each microplate, thoroughly mixed with the diluted butter and spread evenly over the area with a sterile needle. The microplates are now removed to a cold level surface, covered to protect them from dust contamination while hardening. When the agar has set, they are transferred to a sterile moist chamber, which contains enough moisture to prevent the agar drying out during incubation. The most convenient form of moist chamber is that manufactured by the Central Scientific Co. of Chicago, for use with the Frost little plate method.(Fig.4). If a large number of samples are being examined, as in routine laboratory work, such a chamber is practically a necessity, being very convenient and most economical of space. Where relatively few samples are being plated at one time, a satisfactory form of moist chamber can be improvised in the following manner. The top part of a large sterile glass culture dish, having a diameter of about 20 cm. is placed inside of a larger dish of the same type,(Fig. 5), and 50cc. of sterile water added

to maintain the required humidity. The glass lid is easily removed when putting in or taking out the microplates. The chamber should be washed with a 1: 1000 solution of mercuric chloride once a week, to prevent any growth upon the interior surface. A chamber of this type will hold ten to twelve slides. Four of these chambers were used by the author in the present work and proved very satisfactory.

When the microplates have been prepared, the moist chamber is removed to the incubator. The period of incubation which gives best results has been found to be from twelve to eighteen hours, preferably fifteen hours, at 25° C. This period fits in well with the ordinary laboratory routine. Where necessary, an indication of the mould content may be obtained in less than nine hours, but such a count will be lower than where a longer incubation period is employed. For a short incubation period, it is advisable to raise the temperature of incubation to 30° C; conversely, if the microplates are to be left for more than eighteen hours, a lower temperature should be used.

At the conclusion of the incubation period, the microplates are removed and dried down on a metal sheet placed above boiling water. This takes from five to ten minutes. If dried down too fast, or left drying too long, the films tend to crack. Once dried, the microplates are placed in Coplin jars containing a thionin stain made up as follows;

Thionin	1 gram
Carbolic acid	2½ "

Distilled water 400 c.c.

Filter, and add 5% of glacial acetic acid.

(Conn(22), in a recent paper, states that care should be taken to obtain Thionin (synonym Lauth's Violet) and not Thionin Blue, as originally erroneously specified by Frost.)

With this stain, no preparatory fixation is necessary, as the acetic acid in the stain prevents the agar from staining too deeply. Slides are stained for three minutes, washed carefully in clean water, dried, and examined under the microscope.

The microscope should be so adjusted that the area of the field under the low power (2/3") objective will be 2 mm^2. After a preliminary survey to observe the evenness of distribution of the colonies, twenty representative fields are counted, preferably with the aid of a mechanical stage. Where only a portion of a colony appears within the field, the worker making the count must use his judgement, balancing one field with another. If no colonies are encountered in twenty fields, another twenty may be counted. If the yeast count runs over fifty colonies per field, the counting of ten fields will be sufficient; if over one hundred per field, five fields will be enough to count.

The mould and yeast counts on the duplicate microplates are now averaged, and the count per c.c. calculated by the formula

$\frac{\text{Number of colonies counted}}{\text{Number of fields counted}}$ x Reciprocal of quantity x Reciprocal of dilution x Microscopic factor = Number of moulds (or yeasts) per c.c. of butter.

Thus, if the average count of twenty fields on each duplicate microplate gives 4 moulds and 30 yeasts; if 0.2 c.c. of a 1/5 dilution are plated, and the microscopic factor $\frac{\text{Area of microplate}}{\text{Area of field}}$ $\frac{800}{2}$ 400, we have

$\frac{4}{20}$ x 5 x 5 x 400 2,000 moulds per c.c.

$\frac{30}{20}$ x 5 x 5 x 400 15,000 yeasts per c.c.

If no moulds (or yeasts) are encountered in searching twenty fields. then

$\frac{<1}{20}$ x 5 x 5 x 400 <500 moulds (or yeasts) per c.c.

while if none are found in forty fields. the count may be stated as <250 per c.c.

In actual practice, all that need be done is to find the average count per field, then multiply by 10,000 (5 x 5 x 400). In this way, the calculation is as simple as in the standard plate method.

The technique described above will enable counts to be obtained up to as high as 50,000 moulds and 1,000,000 yeasts per c.c., which will include the majority of the samples encountered. For the higher count butter, a higher dilution may be prepared, or a smaller quantity plated out.

INVESTIGATIONAL.

PART 2. COMPARISON OF THE MICROPLATE WITH THE STANDARD PLATE METHOD.

In a comparison of the microplate with the standard plate method for the determination of moulds and yeasts in butter the reliability of the former method is, of course, the feature of paramount importance. This means, of course, that the microplate counts must afford a reasonably accurate indication of the mould and yeast content of the butter being analysed. We have no way of knowing just what the actual mould and yeast content of a sample of butter is; even the standard plate method gives only an approximation of the number, as is the case with the official plate method in milk analysis. However, when a new method of milk analysis is to be tested out, it is compared with the official plate method. Similarly, in the case of a new method for mould and yeast counts, the comparison must be made with the standard plate method. Part 2, Investigational therefore deals with the comparison of the two methods, not only in regard to reliability and accuracy, but also on several minor points. Each of these will now be discussed in full.

A. Reliability and Accuracy.

In comparing the new microplate method, described in Part 1, with the standard plate method now in use, 186 samples of butter have been plated out by the both methods simultaneously. With the idea of testing out the new method on as wide a range of butters as possible, samples have been secured from Alberta, Saskatchewan, Manitoba, Ontario. Quebec. New Zealand. and Australia: the mould counts on these samples ranging from 3 to 540,000 per c.c. by the standard plate method.

As the microplate method is designed primarily for the detection of butter with a high mould count, attempts were made to secure as many samples of high count butter as possible. Unfortunately, on account of the tremendously increased exportations of butter during the early fall, great difficulty was experienced in obtaining sufficient samples of this nature. Numerous trips were made to Montreal to obtain such samples, usually without success. Faced with the choice of presenting results based upon some fifty samples, or else "manufacturing" high count butter by mixing butter of this type with low count butter, the author, after long consideration, and with some misgivings, chose the latter. Samples of low and high count butters were melted, and varying quantities of the latter added to the former, after which the "inoculated" samples were thoroughly shaken for several minutes. While the necessity for such a step is to

be regretted, there seems to be no reason why such samples should not be satisfactory for comparative analysis by the two methods.

In analysing all the samples, care has been taken to eliminate sources of error as far as possible. The standard plates and microplates were prepared from the same dilution, the same media from the same flask was used for each, and all plates were incubated in a large incubator at the same temperature. The details of technique in both methods have already been given.

In Table 9 have been gathered together the results of all analyses conducted in this investigation, some of which have appeared in connection with tests of the various factors involved in the development of the microplate method. These have been arranged in numerical sequence of mould counts by the standard plate method. In addition, the ratio of the mould count by the microplate method to that obtained by the standard plate method is also shown. The data from Table 9 have also been plotted out in graphical form. In Chart 1, the logarithmic values of the counts obtained by both methods have been plotted on the y-axis, the samples being numbered in numerical sequence of standard plate mould count, and running from left to right on the x-axis. The advantages of using the logarithmic, rather than the arithmetical, values in making such a comparison have been fully set forth by Field(23) and others. In addition, a glance at the range covered by the counts (3 to 1,510,000)

shows the extreme difficulty involved in an attempt to compare all the arithmetical values of the data on a single graph.

A study of this chart shows that, for the butter with a high mould content, there exists a distinct parallelism between the counts obtained by the two methods. As the counts decrease, however, the microplate counts tend to show wider variations from the standard plate counts. This of course is only to be expected. With the microplate method, one mould encountered in counting twenty fields will give a mould count of 500 per c.c. This one mould might chance to be the only one present on the whole microplate, containing an area of 400 fields, or equivalent to a count of 25 moulds per c.c. Consequently, occasional higher counts by the microplate method on the lower count butter, particularly that below 100 per c.c., are not surprising. The really surprising thing is that the counts approach the normal ratio in so many cases. However, with the present technique, it is probably best to state the equivalent of a standard plate count of 100 per c.c. as the lowest reliable limit for the microplate method. This will not greatly handicap the method, in the detection of butter with a dangerously high mould content.

In Chart 2, the close parallelism between the counts by the two methods with high count butter is graphically depicted in a somewhat different manner. Here the dots represent the co-ordinates of the logarithmic values of the

counts obtained by both methods on 128 samples. The logarithmic value of the standard plate count is plotted upon the x-axis, and that of the microplate count on the y-axis. This depicts very clearly the close correlation existing in the high mould counts, and the greater degree of departure from the average, as indicated by the pencil line, in the lower counts.

In Chart 3, the frequency distribution of the ratios of the microplate counts to standard plate counts on 128 samples has been plotted. In this, the grouping of the ratios about the modal value of 2 ; 1 is significant. Of the 128 samples, 99, or 77.3%, give ratios falling between 1 : 1 and 3 : 1, with 53, or 41.4%, falling in the modal value of 2 : 1. In a frequency distribution of this sort, the mode, rather than the median or the arithmetical mean, is the most suitable form of average to use where counts by one method are to be transferred into the terms of the other method, where only the one count is known. From a study of Chart 3, it would appear that the modal value of 2 : 1 is the most suitable ratio for this purpose. Comparisons must be made over a larger number of samples before this value can be finally accepted as the most satisfactory average to use.

Mention was made on a previous page that the microplate method would be concerned mainly with samples showing over 100 moulds per c.c. by the standard plate method. If then, only those samples are considered which gave counts above this figure, and their ratios plotted, (Chart 4), we

obtain a frequency distribution very much closer to the normal. Here the widest ratio obtained is 9.5 : 1, with 84 out of 107 ratios falling between 1 : 1 and 2.5 : 1 inclusive. It should be noted that the class intervals in Chart 4 are but one-half the magnitude of those in Chart 3, which changes the shape of the histogram to some extent.

In connection with the results plotted, it must be borne in mind that the earlier counts were made with the original technique, which gave a lower limit of counting of 2,000 per c.c. No doubt if the revised technique, giving a lower limit of 250 per c.c. had been used on these samples, many of the wide ratios on the lower count samples would not have appeared. Of the ten samples showing ratios wider than 50 : 1, eight of these were plated by the original technique. All ten were on samples giving a count of less than 25 per c.c. by the standard plate method, which is obviously below the satisfactory working range of the microplate method.

Perhaps a word should be said as to the reason for the higher counts by the microplate method. In counting under the microscope, the mycelium of each mould, resulting from the germination of a single spore, is counted; on the standard plates, a mould colony, which after five days generally has a diameter of more than 1 cm., may have resulted from the germination of two or more spores. Again, where the standard plate is crowded, either with moulds or yeasts or both, the growth of the weaker mould colonies may be inhibited, and the count will be lower than it should

be. Proof of this is given in the higher counts obtained from plates poured from the higher dilutions, as compared with the more crowded plates of the lower dilutions. The claim may therefore be made that the microplate method affords a more accurate indication of the actual mould content of the butter than does the standard plate method.

Another point in support of the above contention is that with the high count butter, a larger quantity of the butter is plated on the microplate (0.04 c.c.) than on the 1/100 dilution standard plate (0.01 c.c.). With the 1/1000 dilution, which is necessary for many high count samples, the microplate carries forty times as much butter as the standard plate.(0.04 to 0.001 c.c.) In this way, the possibility of error through the use of high dilutions is avoided in the microplate method. This no doubt explains why the counts from duplicate microplates vary much less than those from standard plates of the higher dilutions. The relation between counts obtained with different dilutions by the standard plate method is also puzzling at times, for occasionally more mould colonies appear on the higher dilution plates than on the lower. The possibility of results being disturbed by mould contamination from the air is also greater with the standard plate method, due to the greater dilutions employed, even though the microplate is exposed to the air for perhaps thirty seconds during plating.

When the yeast counts obtained by the microplate

method are compared with the standard plate counts, a less satisfactory correlation is observed. In several cases, the microplate count is very low in comparison with the standard plate count. In explanation of these discrepancies, a number of reasons suggested themselves. In the first place, it was thought that insufficient shaking of the dilutions when preparing the microplates might be the cause, but the experiments reported in Table 7 failed to substantiate this. The next idea which suggested itself was that in using the graduated 1 c.c. pipette to deposit 0.2 c.c. upon the slide, the yeasts might rise to the top with the butterfat within the bore of the pipette, and thus escape being plated out. The data in Table 7 also dispose of this possibility.

Two other possibilities suggested themselves. One is that in these particular samples, the majority of the yeasts had a very low thermal death-point, and could not stand being held at 45° C. for thirty to forty minutesbetween the plating out on the standard plates and the preparation of the microplates. The second possibility is that the yeasts in these particular samples were very slow growing, and were unable to form recognisable colonies upon the microplates during the fifteen to eighteen hours incubation at 25 - 30°.C. This was supported by an examination of the standard plates of several of these samples showing discrepancies, which revealed the presence of large numbers of extremely small subsurface colonies at the end of the incubation period. Unfortunately, neither of these conjectures could be confirmed

by experiment, as the samples of butter involved had been discarded to make room for fresh ones before the discrepancy in yeast counts became known.

In connection with the explanation based upon slowness of growth, it is interesting to note that in one of the experiments where microplates were counted after six, nine, twelve, fifteen and twenty-four hours of incubation, (Table 2), the yeast count jumped from 65,000 at 15 hours, to 508,750 at 24 hours, while the standard plate gave a count of 300,000 yeasts per c.c. In addititon, it should be noted that, with some samples of butter, it is difficult to obtain microplates with as clear a background as is desirable. This renders it difficult to count very small or lightly stained yeast colonies under the microscope, thus giving a lower yeast count by the microplate method.

Even though the yeast count is of secondary importance in the detection of butter with a high mould content, nevertheless, it is desirable that more work be done to determine definitely the cause of the discrepancies between yeast counts by the two methods. This the author hopes to have an opportunity of doing during the coming year.

Despite the greater irregularity of the yeast counts (which are still a great deal more reliable than those obtained by workers using the Breed direct microscopic method), we may safely conclude that the microplate method will give accurate and reliable results in the detection of butter with a high mould content.

B. Cost.

When the costs of obtaining mould and yeast counts by the microplate and the standard plate methods are compared, the advantage is seen to lie entirely with the former. In plating out a sample of butter by the standard plate method, it is necessary to pour plates at least three, and usually four, dilutions, ranging from 1/1 to 1/1,000. This requires two or three dilution flasks, three or four pipettes, and six or eight petri dishes, together with 10 c.c. of wort agar medium for each plate. With the microplate method, all that is required is a dilution flask or test-tube, two pipettes, two ordinary 2.5 x 7.5 cm. microscopic slides, and eight drops (0.4 c.c.) of wort agar. The upper limit of counting with a 1/5 dilution microplate is as high as that of a 1/1,000 standard plate, so that it will rarely be necessary to prepare microplates of higher dilutions.

In addition to the actual cost of the glassware and media required, as referred to above, there might be mentioned the considerably greater expense by the standard plate method in connection with the washing of glassware, the wrapping and sterilization of petri dishes, and the preparation of media. Likewise, the loss due to breakage of glassware is much greater by the standard plate method, as petri dishes are much more expensive, and more easily broken, than microscopic slides.

Lastly, the great saving in incubator space with the

microplate method is no small consideration, particularly during the busy season. If one of the regulation moist chamber cabinets (Fig. 4) put out by the Central Scientific Co. is used, it may be placed inside an ordinary incubator set at 25° C. The dimensions of the cabinet are 6½" x 4" x 6", and its capacity is 48 microplates, from 24 samples. To plate out this number of samples by the standard plate method, between 144 and 192 plates would be prepared. In addition, the microplates are never incubated for more than twenty four hours, while the standard plates must remain in the incubator for five days. Thus, a laboratory analysing 24 samples per day would have between 720 and 960 plates incubating at one time, in place of 48 microplates by the microplate method. As a result, more samples can be analysed, at a considerably lower cost per sample, by the microplate method.

C. Time Required to Obtain Results.

The importance of being able to obtain mould counts on butter in as short a space of time as possible has already been referred to several times. Here, of course, the microplate method enjoys a distinct advantage over the standard plate method, enabling counts to be obtained in from one seventh to one tenth of the time required by the latter method. The most satisfactory counts are obtained from microplates which have been incubated around fifteen hours at 25°

C., while the standard plates must be incubated for five days before counting. Furthermore, if the necessity arises, a count can be obtained by the microplate method in eight to nine hours, which, although lower than that obtained with a longer incubation period, will still enable the worker to detect butter containing a dangerously high number of mould spores, and prevent its being exported.

D. Further Minor Comparisons.

By obviating the necessity for making several dilutions, and preparing plates from each of them, the technique of the microplate method is simpler than that of the standard plate method. The amount of water-bath accomodation required to keep so many dilution flasks or test-tubes at the proper temperature is therefore much greater with the latter method, and tends to limit the number of samples which can be handled at one time. Thus, through the saving of time and of water-bath accomodation, a worker is enabled to handle a larger number of samples in a shorter space of time by the microplate method.

In making the count at the end of the incubation period, the author is of the opinion that the microplates are counted more easily, and with less eye strain, than are the standard plates. Hatfield and Park(17), of the New York Board of Health Laboratories, reporting on a series of tests

in which the Frost little plate method was compared with the official plate count method in routine milk analysis, state that the workers there expressed a similar opinion.

Another advantage possessed by the microplate method is that the samples of butter may be retained for twenty-four hours, until the counts have been obtained; then if the necessity arises, the samples may be replated. With the standard plate method, samples would have to be stored for five days. In addition, the microplates form a permanent record which may be filed for further reference in case of dispute arising later, while the standard plates cannot be kept for more than a few days.

The value of a method which will enable a trained worker to conduct mould and yeast analyses right at the creamery has been mentioned before. For this purpose, the microplate method possesses many advantages over the standard plate method. Equipped with one of the portable outfits put out by the Central Scientific Co. for field work with the Frost method of milk analysis(which can easily be adapted to mould and yeast count work with butter), a worker could visit at least one town per day. In this manner, a large area could be covered during the season, and a great deal accomplished in educating the buttermakers in the production of butter with a low mould count.

SUMMARY AND CONCLUSIONS.

In order that counts of moulds and yeasts in creamery butter may be obtained in less time than with the present standard plate method, a rapid method, known as the Microplate Method, has been evolved. This method, which is a modification of the Frost little plate method of milk analysis, enables counts to be obtained in from one-seventh to one-tenth of the time required by the standard plate method.

Samples from 186 lots of butter from various sources have been analysed by both the microplate and the standard plate methods, and the results compared. A study of the data obtained leads to the conclusion that the microplate method can be relied upon to furnish a reasonably accurate indication of the mould content of the butter, where this is above 100 per c.c. as determined by the standard plate method. This figure may be regarded as the minimum working limit of the new method.

On the average, mould counts obtained by the microplate method are twice as great as those obtained by the standard plate method. The reason for this higher count is that with the microplate method, each individual colony resulting from the germination of a spore can be counted, while with the standard plate method, two or three spores may germinate and grow together to form a single colony.

A frequency distribution of the ratios of the counts obtained by the microplate method to those by the standard plate method shows a close grouping about the modal value of 2 : 1. More work is necessary in order to verify this as the most common average between counts by the two methods.

Yeast counts obtained by the microplate method showed greater variability, due to factors not fully determined. However, a satisfactory relationship between the counts by the two methods was obtained on 74.6% of the samples analysed.

In addition to the saving of time already referred to, the microplate method is more economical of glassware, media, equipment and labor, and can be readily adapted to field work.

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LITERATURE CITED.

1. Thom, C. and Shaw, R. H. Mouldiness in butter. Jour. Agric. Research, 3; 4, 301-310. 1915.

2. Boekhout, F. W. J. and de Vries, J. J. Ott. Uber angeschimmelte Butter. Centralbl. f. Bakt. Abte II. 52; 39-45. 1920.

3. North, W. R. Jr. and Reddish, G. F. Yeasts and Oidia in high grade experimental butter. Jour. Dairy Sci. 4; 6. 510-520. 1921.

4. Davis, L. M. Calif. Sta. Report, 1916, p. 48.

5. Ruddick, J. A. The butter trade. Paper given at the Alberta Dairymens Ass'n Convention, Calgary, Feb. 3-8, 1925.

6. Thom, C. and Ayers, S. H. Effect of pasteurization on mould spores. Jour. Agric. Res. 6; 153. 1916.

7. Bouska, F. W. and Brown, J. C. The significance of yeasts and Oidia in pasteurized butter. Univ. of Wisconsin Studies in Science, No.2. 131-138. 1921.

8. Lund, T. H. Yeasts and moulds in butter. 44th Annual Report, Ont. Agr. College, 39-41. 1918.

Lund, T. H. Notes on yeast and mould counts on creamery butter. Dairymens Ass'n Ont. Annual Report, 1919, 90-94.

Lund, T. H. Micro-organisms in creamery butter. Sci. Agr., 2; 10, 332-340. 1922.

9. Nicholls, H. M. The deterioration of butter through the agency of Oidium lactis. Tasmania Agr. and Stock Dept. Bul. No. 86. 1919.

10. Grimes, M. A study of the action of certain bacteria, yeasts and molds on the keeping quality of butter in cold storage. Jour. Dairy Sci. 6;5,427-445. 1923.

11. Hood, E. G. and White, A. H. The cause and prevention of mould in Canadian pasteurized butter. Dom. Dept. of Agr. Bul. No. 48. N. S. 1925.

12. Stiritz, B. A. Yeast and mold counts and their relation to pasteurization of cream for buttermaking purposes. Jour. Dairy Sci. 5; 4, 362-371, 1922.

13. Brown, C. W., Smith, L. M. and Ruehle, G. L. A. A bacteriological and biochemical study of experimental butters. Jour. Dairy Sci. 3; 5, 375-405. 1920.

14. Redfield, H. W. The determination of yeasts and Oidia in cream and butter. Jour. Dairy Sci. 5; 1, 14-21, 1922.

15. Frost, W. D. A rapid method for counting bacteria. Jour. Inf. Dis. 19; 273-287, 1916.

Frost, W. D. Improved technique for the little plate method of counting bacteria in milk. Jour. Inf. Dis. 28; 176-184, 1921.

16. Simmons, J. E. A comparison with the standard plate method of some rapid methods for the bacteriological analysis of milk. Jour. Inf. Dis. 24; 322-336, 1919.

17. Hatfield, H. M. and Park, W. H. A study of the practical value of the Frost little plate method in the routine colony count of milk samples. Amer. Jour. Pub. Health, 12; 478-487, 1922.

18. Webb, R. W. Studies in the physiology of the fungi. XV. Germination of the spores of certain fungi in relation to hydrogen ion concentration. Ann. Missouri Bot. Gard. 8; 3, 283-341, 1921. Abstracted in E. S. R. 49; 125, 1923.

19. Johnson, H. M. Some relationships between hydrogen ion, hydroxyl ion, and salt concentration, and the growth of soil molds. Iowa Sta. Res. Bul. No. 76. 1923.

20. Rosenau, M. J., Frost, W. D. and Bryant, R. A study of the market butter of Boston. Jour. Med. Res. 30; 1, 69-85, 1914.

21. Breed, R. S. and Brew, J. D. Counting bacteria by means of the microscope. New York Agr. Exp. Sta. Tech. Bul. No. 49. 1916.

Breed, R. S. and Brew, J. D. The control of bacteria in market milk by direct microscopic examination. New York Agr. Exp. Sta. Bul No. 443, 1917.

22. Conn, H. J. Thionin. Jour. Dairy Sci. 6; 2, 135-136, 1923.

23. Field, J. A. Some advantages of the logarithmic scale in statistical diagrams. Jour. Pol. Econ. 25; 805-841, 1917.

24. Parfitt, E. H. Relationship between yeasts and molds in butter and the butter score and care in manufacture. Creamery & Milk Plant Monthly, 40;1, 1926.

25. Hunziker, O. F. The butter industry. XXII, 665 pp. 113 fig. O. F. Hunziker, LaGrange, Ill. U.S.A. 1920.

26. American Association of Creamery Butter Manufacturers. Personal communication to the author from Dr. G. L. McKay, Sec. April 15th. 1926.

TABLE 1.

RELATION OF STANDARD PLATE COUNTS TO COUNTS OBTAINED BY PLATING BUTTER DILUTIONS AND SERUM BY MICRO-PLATE METHOD.

	Standard Plate.		Micro-plate(Serum)		Micro-plate(Butter)	
	Moulds	Yeasts	Moulds	Yeasts	Moulds.	Yeasts
3/1	13,700	x	88,000	10,000	44,000	6,000
BD	8,250	16,000	x	91,400	20,000	500
BB	6,000	117,750	x	328,800	11,000	19,250
8Xa	5,750	403,000	39,000	3,130,000	8,750	251,000
A2	5,650	590,000	34,000	5,460,000	12,250	226,000
BC	5,000	124,500	x	538,400	17,250	21,750
A4	4,450	138,500	67,500	707,500	9,500	228,000
A5	3,325	166,250	24,300	202,000	6,250	110,000
6/12	3,200		12,530		8,000	
A6	2,500	357,000	19,500	4,426,000	5,500	666,000
7XA	925	1,600,000	8,000	8,800,000	1,500	1,663,000
AN	778	445,000	5,000	x	2,250	520,000
6J	725	2,000,000	20,000	x	2,500	1,718,000
BK	700	x	191,500	x	5,000	2,118,000
67	475	730,000	20,000	x	2,750	622,000
5/2	350	18,000	1,050	295,200	750	250
BG	300	20,500	1,550	x	750	50,250
BM	240	42,500	1,900	x	750	52,750
5/7	75	34,000	-50	225,600	250	43,500
5/6	50	38,500	-50	320,400	500	46,250
NZ13	35	400	100	15,200	-250	-250
BP	28	180	-100	1,300	250	-250
6I	25	x	-250	x	250	1,916,000
6B	15	139;500	-250	x	333	-250

Cont'd.

TABLE 1 (Cont'd)

	Standard Plate. Moulds	Yeasts	Micro-plate (Serum) Moulds	Yeasts	Micro-plate (Butter) Moulds	Yeasts
A3	13	600	-100	16,100	500	250
NZ-28	10	330	300	7,400	-250	375
BE	10	4,525	-100	22,700	-250	4,000
6A	5	180,000	250	x	-250	330
6C	5	9,750	-250	23,200	-250	333
6F	5	25,200	-250	154,400	-250	21,750
6G	5	26,000	-250	320,800	125	-250
6H	5	210,000	-250	x	-250	625
NZ-8	5	145	200	1,300	-250	375
BF	5	155	-500	-500	-250	-250
BQ	5	83	-100	1,100	-250	125
BR	5	20	50	150	250	-250
BU	5	233	-125	-125	125	750
NZ-1	3	563	-100	9;600	-250	250
NZ-1x	3	695	200	11,700	-250	375
5/12	-50	345,500	-50	622,800	-250	157,750
5/11	-50	251,000	-50	524,800	-250	145,000
5/10	-50	120,500	-50	209,200	-250	50,000
5/3	-50	100,000	50	540,800	500	500
5/1	-50	71,750	50	510,400	500	500
5/5	-50	26,250	-50		500	55,000
5/8	-50	21,500	-50	249,200	250	38,250
5/9	-50	750	-50	2,400	500	250
6E	-10	147,000	750	72,000	-250	33,000
6D	-5	140	-250	900	-250	-250
BS	-5	13	-125	-125	125	-250
BT	-5	43	-125	-125	125	750

TABLE 2.

MICRO-PLATE COUNTS FROM DIFFERENT INCUBATION PERIODS COMPARED WITH STANDARD PLATE COUNTS.

	6 hours.	9 hours.	12 hours.	15 hours.	24 hours.	Aver.	Stand.P.C.
M	6000	8,000	10,500	17,060	20,000	12,310	
N	11,000	12,500	14,500	21,000	23,500	16,500	
O.	1,500	10,000	15,000	13,000	18,000	14,000	2,600
P.	12,500	17,000	20,500	19,000	22,000	18,200	
Q.	-2,000	-2,0000	-2,000	-2,000	-2,000		-100
R.	-2,000	-2,000	-2,000	-2,000	-2,000		-100

The above counts are for moulds only.

EA(M)	500	7,500	7,750	7,750	7,000	6,100	5,825
"(Y)	-250	16,250	35,750	47,000	56,750	31,200	715,000
EB(M)	750	12,250	15,000	12,000	14,000	10,800	11,500
"(Y)	9,750	57,500	71,000	123,750	348,500	122,100	x
EC(M)	7,750	14,500	16,250	18,000	19,500	15,200	15,250
"(Y)	x	x	x	x	x		x
ED(M)	2,500	5,250	5,500	7,500	6,750	5,500	3,900
"(Y)		3,750	27,750	65,000	508,750	121,050	300,000
EE(M)	250	500	2,500	3,000	3,250	1,900	700
"(Y)	500	23,000	169,000	311,750	412,750	183,400	63,000
EF(M)	750	7,750	8,000	7,750	7,500	6,350	2,750
"(Y)	x	x	x	x	x	x	x

Note:

x Too numerous to count.
(M) Moulds
(Y) Yeasts

TABLE 3.

MICRO-PLATES FROM DIFFERENT PERIODS OF INCUBATION COMPARED WITH STANDARD PLATE COUNTS.

	15 hours.	18 hours.	21 hours.	24 hours.	Aver.	Stand. P. C.
AK(M)	6,750	6,500	8,250	6,500	7,000	5,650
"(Y)	844,000	785,000	794,000	763,000	796,500	450,000
AL(M)	12,000	13,000	12,500	10,000	11,875	6,625
"(Y)	1,135,000	1,272,000	1,258,000	1,232,000	1224,250	680,000
AM(M)	2,500	2,750	2,250	2,500	2,500	475
"(Y)	190,000	622,000	1,612,000	839,500	665,875	730,000
AN(M)	3,000	2,250	2,750	2,750	2,688	778
"(Y)	?	520,000	453,000	603,000	525,333	445,000
AO(M)	3,500	3,750	3,500	5,150	3,950	2,625
"(Y)	16,000	26,750	16,750	70,750	32,563	500,000
AP(M)	3,500	5,250	5,500	5,250	4,875	3,125
"(Y)	36,000	29,000	31,750	52,750	37,375	575,000
AU(M)	6,500	9,750	9,000	8,250	8,375	2,275
"(Y)	87,500	93,750	113,750	118,500	103,375	37,850
AV(M)	13,250	12,000	11,000	10,750	11,750	4,575
"(Y)	6,500	6,250	10,500	8,750	8,000	35,500
AW(M)	13,750	11,750	9,500	10,250	11,312	7,350
"(Y)	80,750	61,750	73,500	70,500	71,625	131,000
AX(M)	12,500	10,000	11,000	8,750	10,562	2,900
"(Y)	92,500	102,500	97,750	92,250	96,250	27,500
BA(M)	14,500	10,250	?	11,500	12,083	8,500
"(Y)	294,250	245,500	?	89,000	209,583	400,000
BB(M)	10,500	11,000	8,000	7,750	9,312	6,000
"(Y)	13,250	19,250	17,750	21,000	17,800	117,750

TABLE 4.

MOULD COUNTS FROM PLATES INCUBATED AT 16 to 18° C. COMPARED WITH COUNTS FROM PLATES INCUBATED AT 26 to 28° C.

	Standard Plate Counts		Micro-plate Counts.	
	16-18°	26-28°	16-18°	26-28°
F	87,000	78,500	108,000	170,000
G	86,000	90,500	140,000	360,000
H	260,000	225,000	169,000	374,000
J	22,500	26,500	1,000	54,000
K	22,000	20,500	8,000	36,800
L	28,500	30,500	-2,000	46,800
M	393,000	541,000	47,000	1,380,000
N	16,000	12,650	33,000	45,000
O	63,000	19,175	21,000	43,000

TABLE 5.

MOULD COUNTS FROM PLATES INCUBATED AT 24° C. COMPARED WITH COUNTS FROM PLATES INCUBATED AT 29° C.

	Standard Plate Counts.		Micro-plate Counts.	
	24°	29°	24°	29°
Series 2				
A	1,200	1,100	-2,000	2,000
B	170	145	-2,000	-2,000
C	470	460	-2,000	-2,000
D	1,750	1,900	-2,000	3,000
E	50	35	-2,000	-2,000
F	40	15	-2,000	-2,000
G	5	15	-2,000	-2,000
H	30	30	-2,000	-2,000

TABLE 6.

MOULD AND YEAST COUNTS FROM PLATES INCUBATED AT 25°C. COMPARED WITH COUNTS FROM PLATES INCUBATED AT 30° C.

	Standard Plate Counts. 25°	30°	Micro-plate Counts. 25°	30°
CAB (M)	-50	50	-250	-250
" (Y)	10,000	5,000	375	-250
CCD (M)	50	50	-250	375
" (Y)	33,500	18,000	2,250	875
CEF (M)	100	50	-250	125
" (Y)	36,000	21,000	625	1,375
CGH (M)	100	-50	250	125
" (Y)	20,000	650	-250	-250
CIJ (M)	41,500	68,500	70,000	115,000
" (Y)	5,000	-500	-2,000	5,000
CKL (M)	1,000	300	3,500	2,000
" (Y)	530,000	292,500	1,083,000	753,000
CLN (M)	15,000	17,000	39,750	36,750
" (Y)	750	-500	-250	250
COP (M)	21,000	12,500	19,750	32,750
" (Y)	145,000	145,000	32,750	29,500
CQR (M)	2,550	1,550	3,250	3,000
" (Y)	1,550	300	-250	-250
CST (M)	300	150	250	1,250
" (Y)	170,000	114.500	61,000	24,000
CUV (M)	91,500	72,500	150,000	135,000
" (Y)	3,500	1,000	500	1,000

Cont'd.

TABLE 7.

EFFECT OF DEGREE OF SHAKING, STYLE OF PIPETTE, ETC., UPON COUNTS BY THE MICROPLATE METHOD.

	Standard Plate.		Microplate.	
	Moulds.	Yeasts.	Moulds.	Yeasts.
A.	925	1,600,000	1,500	1,663,000
B.	675	1,840,000	1,750	1,668,000
C.	800	1,470,000	1,000	486,000
D.	975	1,720,000	1,500	887,000
A1.	5,750	403,000	8,750	251,000
B1.	6,250	345,000	12,500	367,000
C1.	4,500	385,000	8,500	310,500
D1.	5,500	350,000	10,000	250,000
A2.	5,650	590,000	12,250	226,000
B2.	5,725	470,000	10,500	271,000
C2.	10,100	715,000	9,000	241,500
D2.	5,675	486,000	9,000	287,000
A3.	5	500	750	375
B3.	13	605	500	250
C3.	--	--	250	-250
D3.			-250	-250
A4.	3,150	127,750	10,250	232,500
B4.	4,450	138,500	9,500	228,000
C4.			10,000	206,500
D4.			8,750	204,000

Cont'd.

Table 7, Cont'd.

	Standard Plate.		Microplate.	
	Moulds.	Yeasts.	Moulds.	Yeasts.
A5.	2,750	130,250	5,500	166,250
B5.	3,325	166,250	7,000	53,500
C5.			6,000	175,000
D5.			3,750	32,500
A6.	2,500	357,000	5,500	666,000
B6.	2,100	555,000	4,250	924,000
C6.			3,750	722,500
D6.			4,000	630,000

LEGEND:

Samples marked A - Dilution tubes shaken 15 seconds, 0.2 c.c. pipetted with ordinary 1 c.c. pipette.

" " B - As A, but tubes shaken 30 seconds.

" " C - Tubes shaken as A, but 0.2 c.c. pipetted with graduated special one-mark pipette.

" " D - As C, but tubes shaken 30 seconds.

TABLE 8.

DISTRIBUTION OF MOULDS AND YEASTS IN BUTTER - STANDARD PLATE COUNTS OF SERUM, BUTTERFAT, AND BUTTER.

	Serum.		Butterfat.		Butter.	
	Moulds	Yeasts	Moulds	Yeasts	Moulds	Yeasts
A	3,650	3,772,000	7	x	800	1,720,000
A1	30,500	2,100,000	1,050	268,000	6,000	378,000
A2	69,500	3,900,000	2,400	545,000	5,688	530,000
A3	100	4,400	3	129	9	553
A4	24.000	557,500	2,800	26,100	3,800	133,000
A5	26,000	1,028,000	900	23,000	3,013	148,000
A6	31,000	x	900	403,000	2,300	456,000

TABLE 9.

COMPARISON OF MICROPLATE COUNTS WITH STANDARD PLATE COUNTS.

	Standard Plate Counts.		Microplate Counts.		Mould Ratio
	Moulds	Yeasts	Moulds	Yeasts	1:
1/I	541,000		1,380,000		2.5
1/P	278,500		1,510,000		5.4
1/Q	237,000		900,000		3.8
1/E	225,000		540,000		2.4
7/AR	175,000	280,500	x	x	
7/AT	153,000	90,000	x	x	
7/AQ	133,000	75,000	x	x	
C/UV	91,500	3,500	150,000	50500	1.6
1/D	90,500		290,000		3.2
1/C	78,500		200,000		2.5
CV	72,500	1,000	135,000	1,000	1.9
CI	68,500	-500	115,000	5,000	1.7
7/AS	60,500	30,000	x	x	
CJ	41,500	5,000	70,000	-2,500	1.7
DU	41,000	x	46,500	1,690,000	1.1
DV	33,500	x	39,500	1,590,000	1.1
1/A	30,500		46,800		1.5
4/Q	28,000		47,200		1.7
1/F	26,500		54,000		2.0
DI	25,500	x	45,000	5,040,000	1.8
CO	21,000	145,000	19,750	32,750	0.9
1/G	20,500		36,800		1.8
4/R	19,500		27,000		1.4
DJ	19,500	x	35,000	4,660,000	1.8

Contd

TABLE 9 (Cont'd).

	Standard Plate Counts.		Microplate Counts.		Mould Ratio 1:
	Moulds.	Yeasts.	Moulds.	Yeasts	
1/K	19,175		43,000		2.2
DL	19,000	175,000	25,000	240,000	1.3
CN	17,000	-500	36,750	250	2.1
DK	16,500	x	30,000	295,000	1.8
DW	15,500	x	13,500	1,222,000	0.9
EC	15,250	x	18,000	x	1.2
CM	15,000	750	39,750	-250	2.6
DM	15,000	x	8,750	203,000	0.6
DX	14,500	x	11,500	636,000	0.8
DA	14,000	x	18,250	2,385,000	1.3
1/A	13,700		44,000		3.2
	13,000	x	13,500	357,500	1.0
1/J	12,650		45,000		3.6
CP	12,500	145,000	18,000	29,500	1.4
DF	12,000	x	12,000	856,000	1.0
EB	11,500	x	12,000	71,000	1.0
DE	11,000	x	16,250	1,291,000	1.5
DB	10,500	x	14,500	1,775,000	1.4
DP	10,500	x	20,500	700,000	2.0
DR	10,500	x	11,500	1,030,000	1.1
8/X3	10,100	715,000	9,000	241,500	0.9
DN	10,000	x	15,750	188,000	1.6
DO	9,000	x	16,750	1,027,000	1.8
DC	8,500	x	18,000	780,000	2.1
BA	8,500	400,000	10,250	245,500	1.2

Cont'd.

TABLE 9 (Cont'd).

	Standard Plate Counts.		Microplate Counts.		Mould Ratio 1:
	Moulds.	Yeasts.	Moulds.	Yeasts.	
BD	8,250	16,000	20,000	500	2.4
3/P	8,200		18,200		2.2
7/AW	7,350	131,000	11,750	61,750	1.6
DQ	7,000	x	12,000	965,000	1.7
6/AL	6,625	680,000	11,500	1,187,000	1.7
8/Y2	6,250	345,000	12,500	367,000	2.0
7/BB	6,000	117,750	11,000	19,250	1.8
EA	5,825	715,000	7,750	47,000	1.3
8/Y1	5,750	403,000	8,750	251,000	1.5
8/X2	5,725	470,000	10,300	271,000	1.8
8/X4	5,675	486,000	9,000	245,100	1.6
8/X1	5,650	590,000	12,250	226,000	2.1
8/Y4	5,500	350,000	10,000	250,000	1.8
7/BC	5,000	124,500	17,250	21,750	3.5
AV	4,575	35,500	12,000	6,250	2.6
CX	4,500	x	2,750	1,975,000	0.6
CW	4,500	x	2,500	2,220,000	0.6
8/Y3	4,500	385,000	8,500	310,500	1.9
8/V2	4,450	138,500	9,500	228,000	2.1
ED	3,900	300,000	7,500	65,000	1.9
6/AK	3,650	450,000	7,250	1,207,000	2.0
8/U2	3,325	166,250	7,000	53,500	2.1
1/B	3,200		8,000		2.5
8/V1	3,150	127,750	10,250	232,500	3.2
7/AP	3,125	575,000	5,250	29,000	1.7

Cont'd.

TABLE 9 (Cont'd).

	Standard Plate Counts.		Microplate Counts.		Mould Ratio 1:
	Moulds.	Yeasts.	Moulds.	Yeasts.	
7/AX	2,900	27,500	10,000	102,500	3.4
8/U1	2,750	130,250	5,500	166,250	2.0
EF	2,750	x	7,750	x	2.8
7/AO	2,625	500,000	3,750	26,750	1.4
3/O	2,600		14,000		5.4
8/T1	2,550	1,550	3,250	-250	1.3
	2,500	357,000	5,500	666,000	2.2
7/AO	2,275	37,850	9,750	93,750	4.2
8/T2	2,100	555,000	4,250	924,000	2.0
2/D	1,960		3,000		1.6
DT	1,850	x	2,000	2,455,000	1.1
CR	1,550	300	3,000	-250	1.9
DS	1,200	x	2,500	4,000,000	2.1
2/A	1,100		2,000		1.8
CK	1,000	530,000	3,500	1,083,000	3,5
8/Z4	975	1,720,000	1,500	877,000	1.5
8/Z1	925	1,600,000	1,500	1,663,000	1.6
EN	825	x	2,000	2,080,000	2.4
8/Z3	800	1,470,000	1,000	486,000	1.3
7/AN	778	445,000	2,250	520,000	2.9
6/AJ	725	2,000,000	2,500	1,718,000	3.3
8/BK	700	x	5,000	2,118,000	7.1
EE	700	63,000	3,000	311,750	4.3
8/Z2	675	1,840,000	1,750	1,668,000	2.6
7/AM	475	730,000	2,750	622,000	5.8

Con't'd.

TABLE 9 (Cont'd).

	Standard Plate Counts. Moulds.	Yeasts.	Microplate Counts. Moulds.	Yeasts.	Mould Ratio 1:
2/C	460		-2,000		
6/Z	438	25	750	-250	1.7
5/B	350	18,000	750	250	2.1
8/BG	300	20,500	750	50,250	2.5
CL	300	292,500	2,000	753,000	6.6
CS	300	170,000	250	61,000	0.8
DG	250	x	250	1,818,000	1.0
8/BM	240	42,500	750	52,750	3.1
2/L	180		-2,000		
2/B	150		-2,000		
CT	150	114,500	1,250	24,000	8.0
4/V	115		250		2.2
4/C	105		1,000		9.5
DH	100	x	250	828,000	2.5
CE	100	36,000	-250	-625	
CG	100	20,000	250	-250	2.5
4/D	75		-2,000		
5/G	75	34,000	250	43,500	3.3
5/F	50	38,500	500	46,250	10.0
2/I	50		-2,000		
CB	50	5,000	-250	-250	
CC	50	33,500	-250	2,250	
CD	50	18,000	375	875	7.5
CF	50	21,000	125	1,375	2.5
2/K	45		-2,000		

Cont'd.

TABLE 9 (Cont'd).

	Standard Plate Counts.		Microplate Counts.		Mould Ratio 1:
	Moulds.	Yeasts.	Moulds.	Yeasts.	
2/J	40		-2,000		
7/NZ1	35	400	-250	-250	
2/E	35		-2,000		
2/H	30		-2,000		
8/BP	28	180	250	-250	8.9
5/D	25	23,750	1,250	57,000	50.0
6/A1	25	x	250	1,916,000	10.0
4/E	25		-2,000		
4/P	20		1,000		50.0
2/F	15		-2,000		
2/G	15		-2,000		
4/A	15		-1,000		66.7
4/J	15		2,000		133.3
4/K	15		2,000		133.3
6/AB	15	139,500	333	-250	22.2
8/N2	13	605	500	250	38.0
6/X	13	-5	-250	-250	
4/T	13		-250		
4/U	13		-250		
4/B	10		-2,000		
4/H	10		-2,000		
4/L	10		4,000		400.0
4/M	10		4,000		400.0
4/S	10		-2,000		
7/NZ3	10	330	-250	375	

TABLE 9 (Cont'd).

	Standard Plate Count. Moulds.	Yeasts.	Microplate Count. Moulds.	Yeasts.	Mould Ratio 1:
7/BE	10	4,525	-250	4,000	
6/X	8	540	1,000	-250	125.0
4/F	5		-2,000		
4/N	5		1,000		200.0
4/O	5		2,000		400.0
6/AA	5	180,000	-250	333	
6/AC	5	9,750	-250	333	
6/AF	5	25,200	-250	21,750	
6/AG	5	26,000	125	-250	25.0
7/NZ2	5	145	-250	250	
7/BF	5	155	-250	-250	
8/W1	5	500	750	375	150.0
8/BF	5	155	-250	-250	
8/BQ	5	83	-250	125	
8/BR	5	20	250	-250	50.0
8/BU	5	233	125	750	25.0
7/NZ5	3	695	-250	375	
7/NZ6	3	563	-250	250	
3/Q	-100		-2,000		
3/R	-100		-2,000		
5/A	-50	71,500	500	250	
5/C	-50	100,000	500	500	
5/E	-50	26,250	500	55,000	
5/H	-50	21,500	250	38,250	
5/I	-50	750	500	250	

Cont'd.

TABLE 9 (Cont'd).

	Standard Plate Count.		Microplate Count.		Mould Ratio 1:
	Moulds.	Yeasts.	Moulds.	Yeasts.	
5/J	-50	120,500	-250	50.000	
5/K	-50	251,000	-250	145,000	
5/L	-50	345,500	-250	157,750	
CH	.50	650	125	-250	
4/G	.10		-2,000		
4/I	.10		2,000		
6/AE	.10	147,000	-250	33,000	
6/AD	-5	140	-250	-250	
6/AH	-5	210,000	-250	625	
8/BS	-5	13	125	-250	
8/BT	-5	43	-250	-250	

Note;

x Too numerous to count.

CHART 1

COMPARISON OF MOULD COUNTS OF BUTTER BY THE MICROPLATE AND STANDARD PLATE METHODS.

Moulds per c.c.

1,000
100
10
0

Logarithmic Mould Count.

6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5

0 10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160 170

LEGEND.

Count by Standard Plate Method.

Count by Microplate Method.

CHART 2.

DISTRIBUTION OF LOGARITHMIC CORRESPONDING VALUES OF OBSERVED MOULD COUNTS BY STANDARD PLATE AND MICROPLATE METHODS ON 128 SAMPLES.

Moulds per c.c.

Logarithmic Mould Count

MICROPLATE METHOD

1,000,000 100,000 10,000 1,000 100 10 1 0

65 60 55 50 45 40 35 30 25 20 15 10 05 0

Modal Ratio - Microplate Count / Standard Plate Count = 2/1

STANDARD PLATE COUNT

0 0·5 1·0 1·5 2·0 2·5 3·0 3·5 4·0 4·5 5·0 5·5 6·0 6·5 7·0

Logarithmic Mould Count

CHART 3.

FREQUENCY DISTRIBUTION OF THE RATIOS OF MOULD COUNTS BY THE MICROPLATE METHOD TO COUNTS BY THE STANDARD PLATE METHOD ON 128 SAMPLES OF BUTTER.

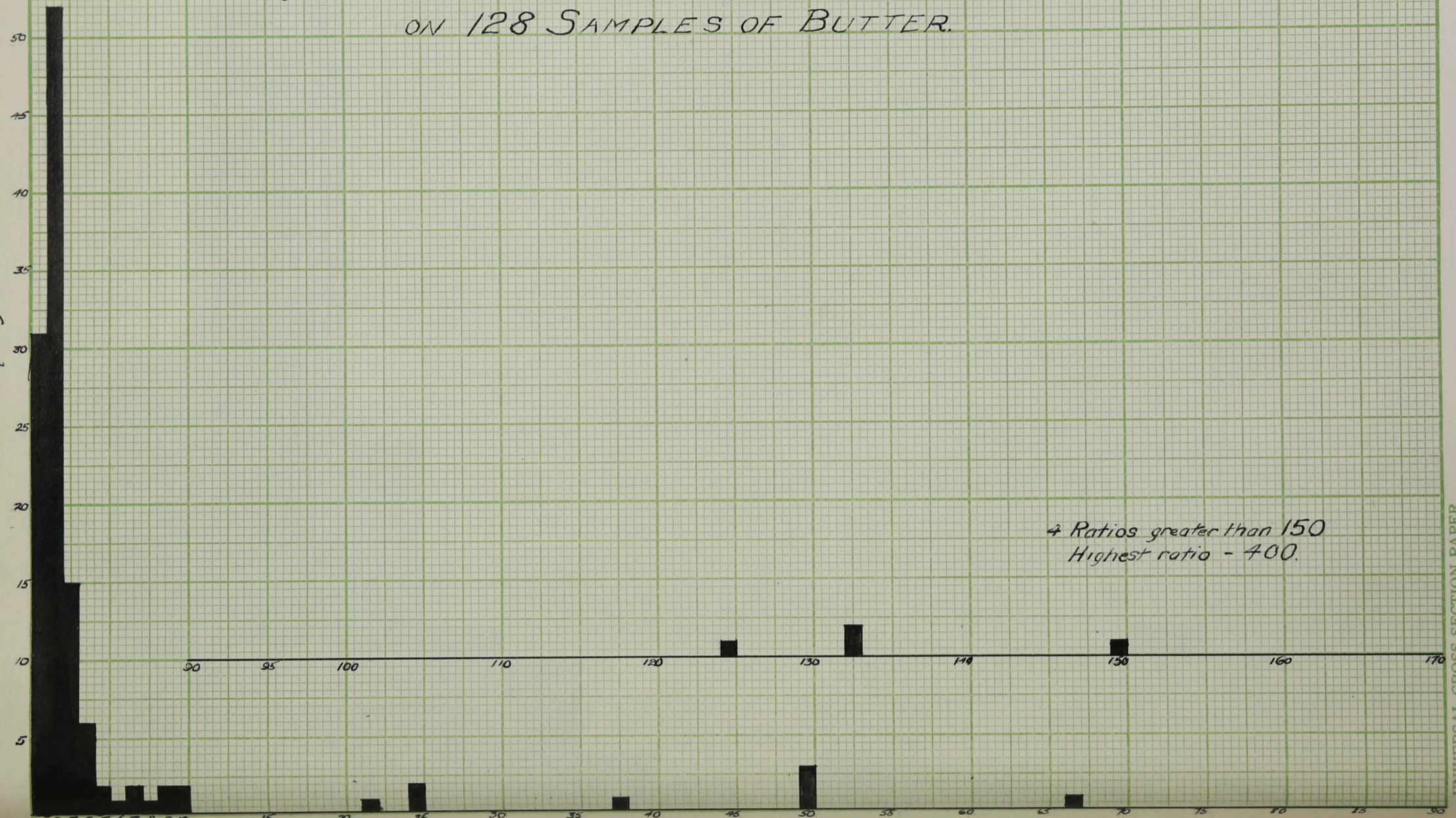

CHART 4

FREQUENCY DISTRIBUTION OF THE RATIOS OF MICROPLATE MOULD COUNTS TO STANDARD PLATE MOULD COUNTS ON 107 SAMPLES (STANDARD COUNTS ABOVE 100 PER C.C.)

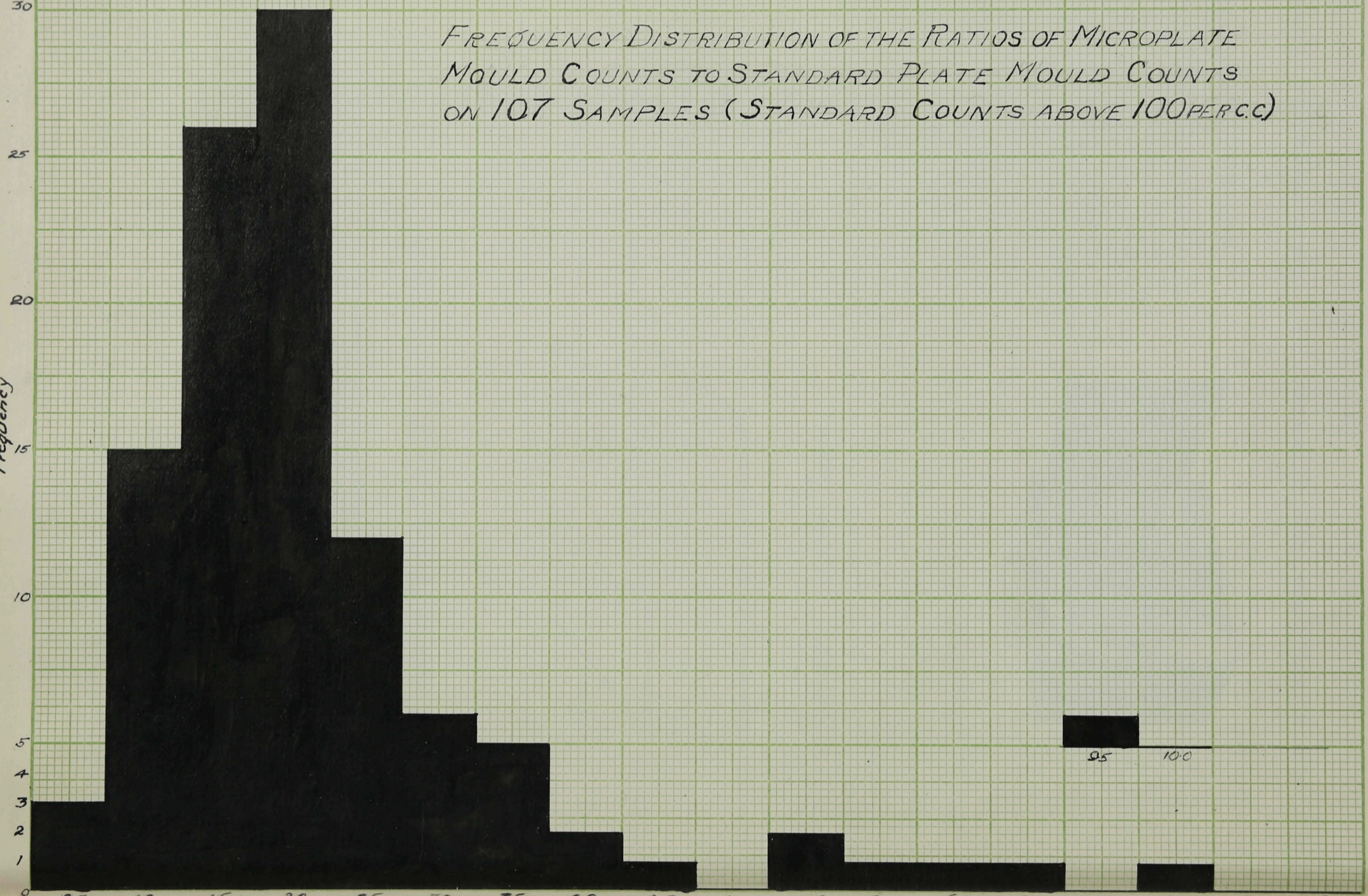

PLATE 1.

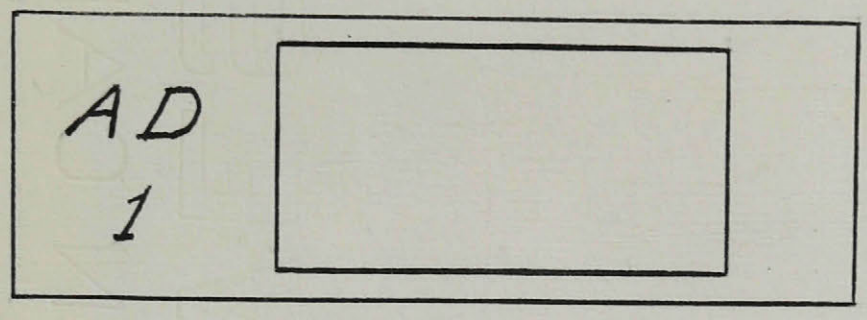

FIG 1. Slide Prepared for Microplate Method.

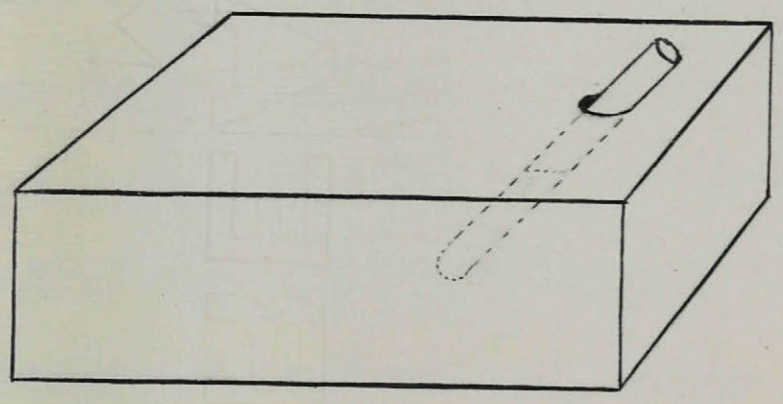

FIG. 2. Warm Table.

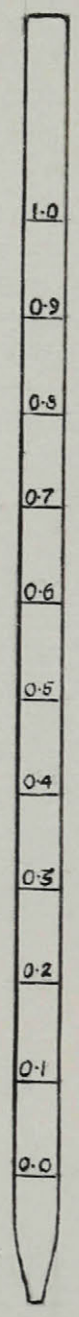

FIG. 3. 1 c.c. Graduated Pipette. (½ Natural Size).

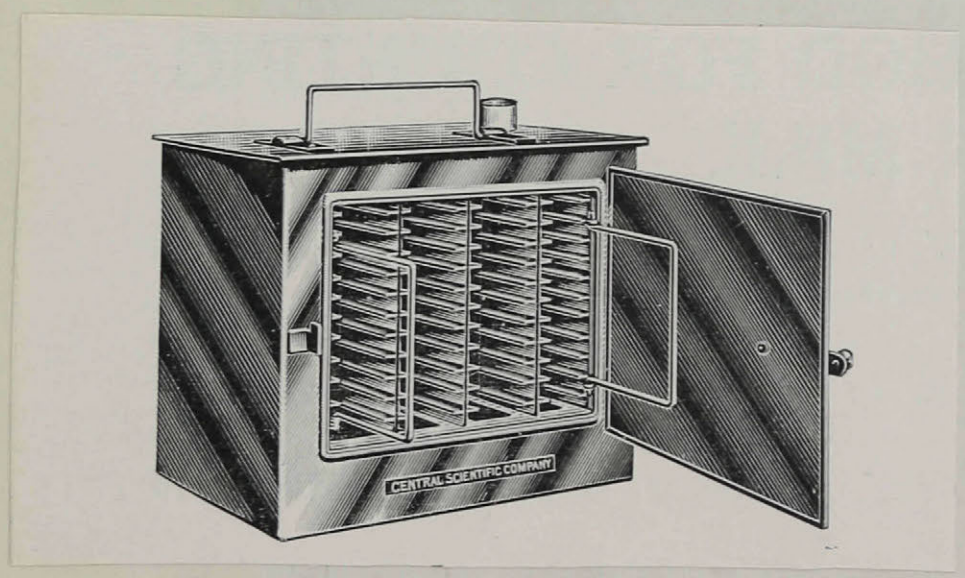

FIG. 4. Regulation Moist Chamber.

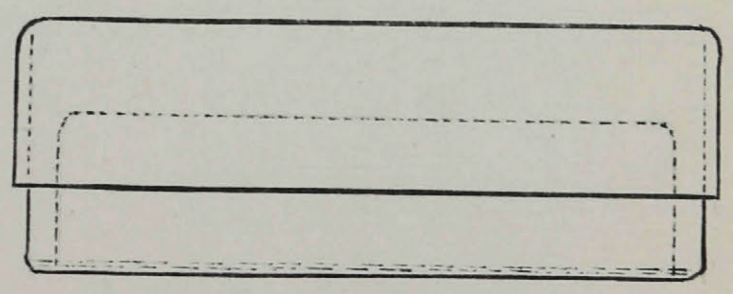

FIG. 5 Improvised Moist Chamber.

PLATE 2.

FIG. 1. Field taken from Sample EC, after incubation at 25° C. for 6 hours. Mould spores have germinated, but yeasts too small to count.

FIG. 2. Field taken from Sample EC, after incubation at 25° C. for 9 hours. Moulds showing considerably greater growth, and yeast colonies visible.

PLATE 2.

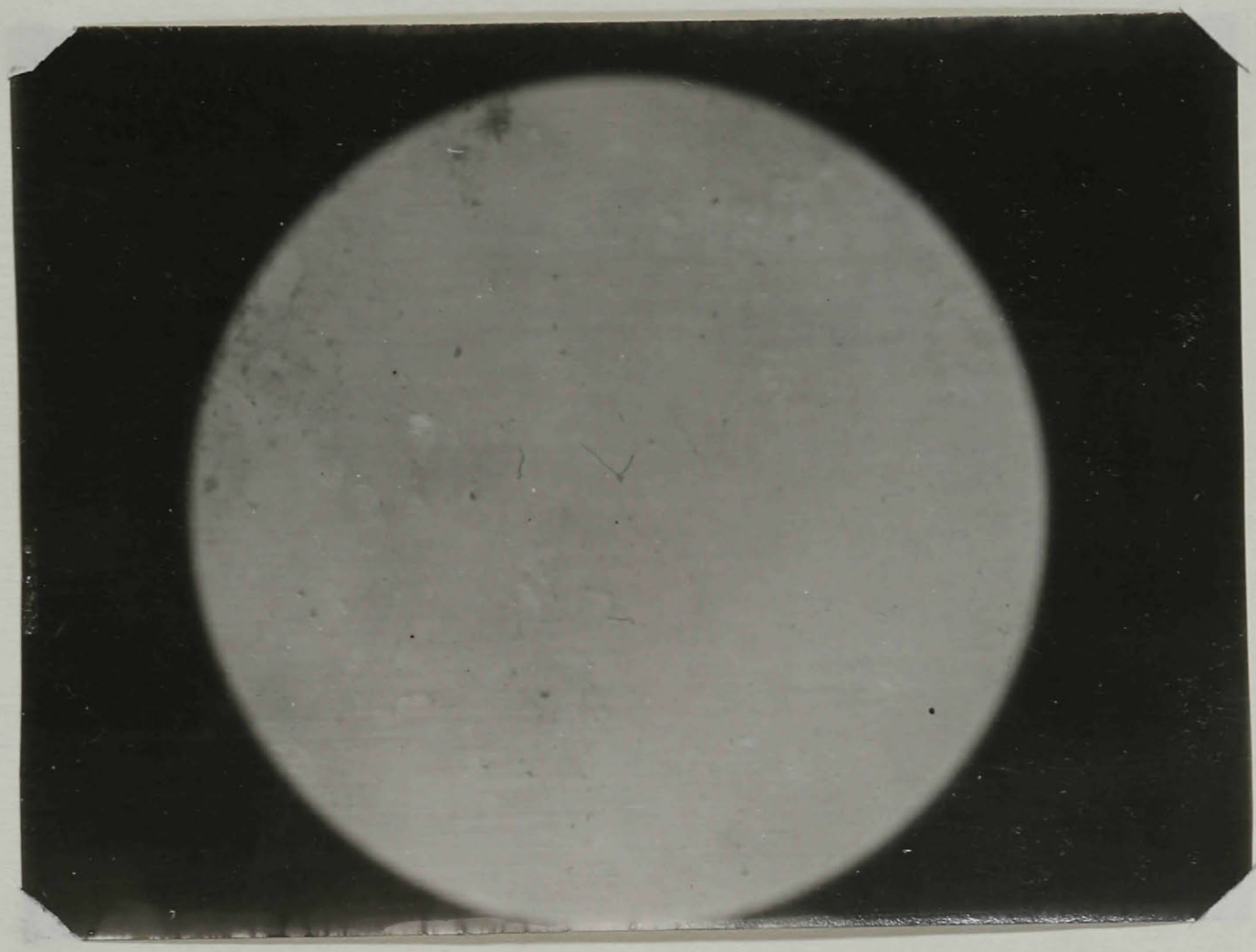

Fig. 1.

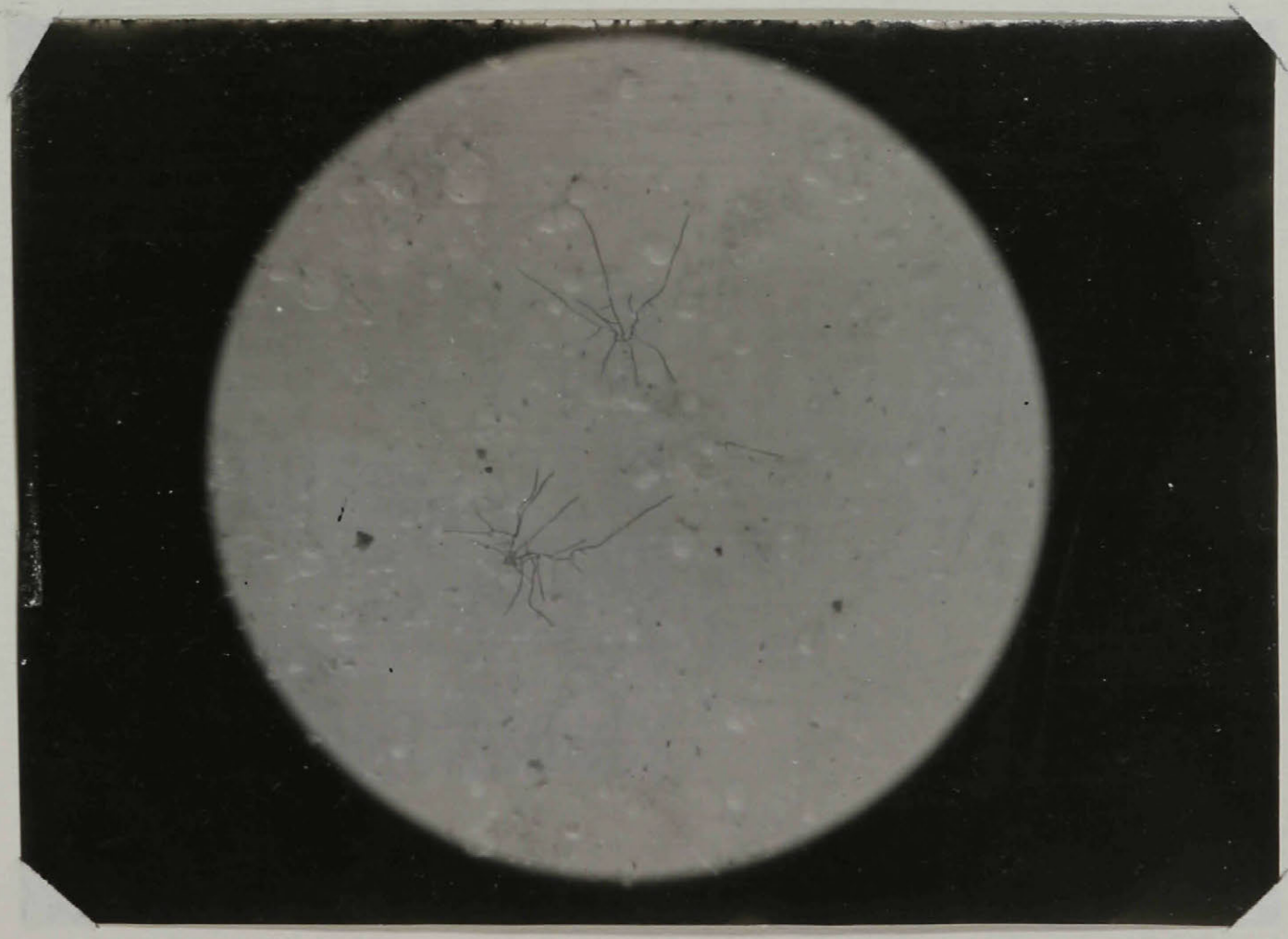

Fig. 2.

PLATE 3.

FIG. 1. Field taken from microplate of sample EC, after incubation for 12 hours at 25° C. Yeast colonies are much more readily counted.

FIG. 2. Field taken from microplate of sample EC, after incubation for 15 hours at 25° C. This is the optimum stage for counting moulds.

PLATE 3.

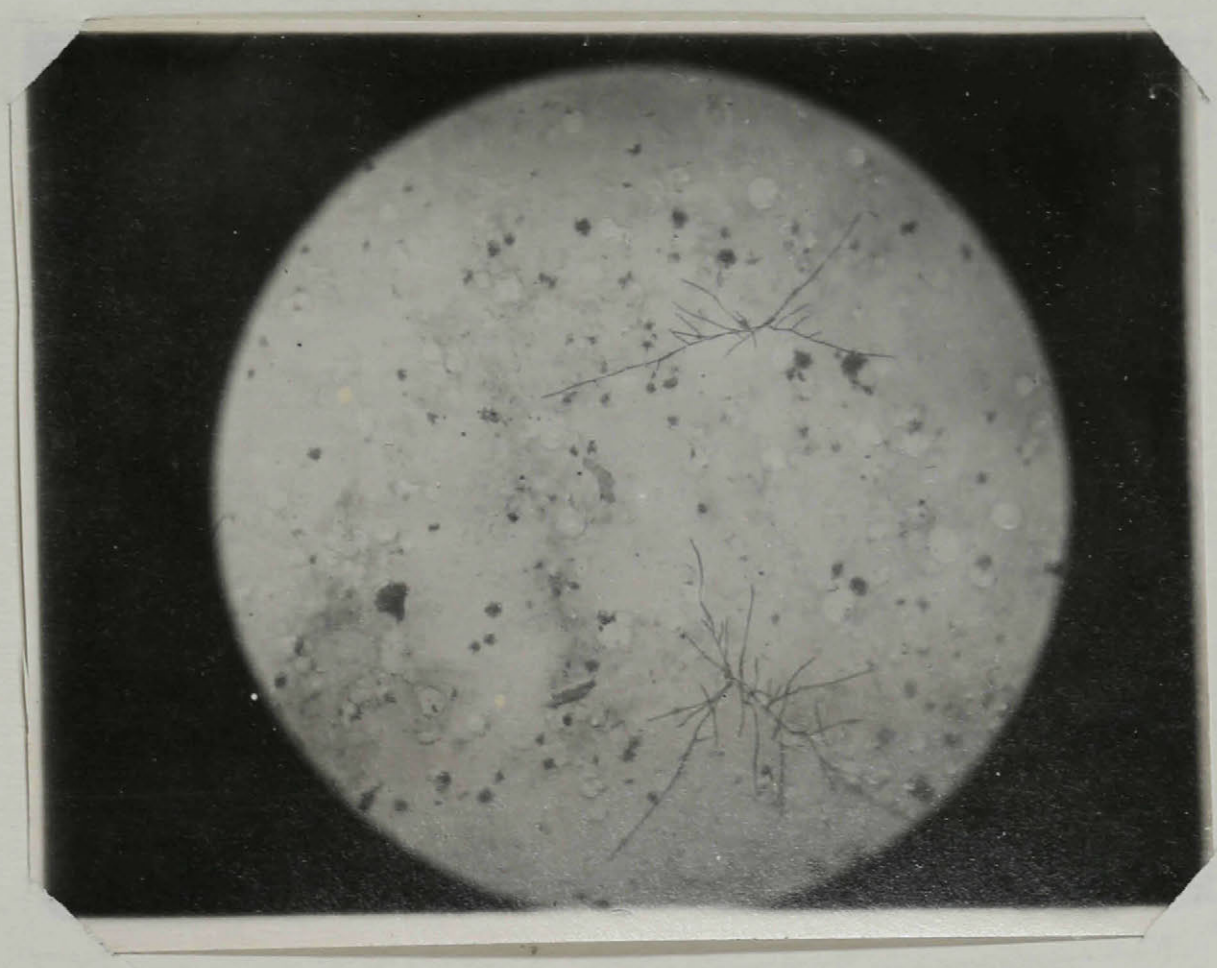

Fig. 1.

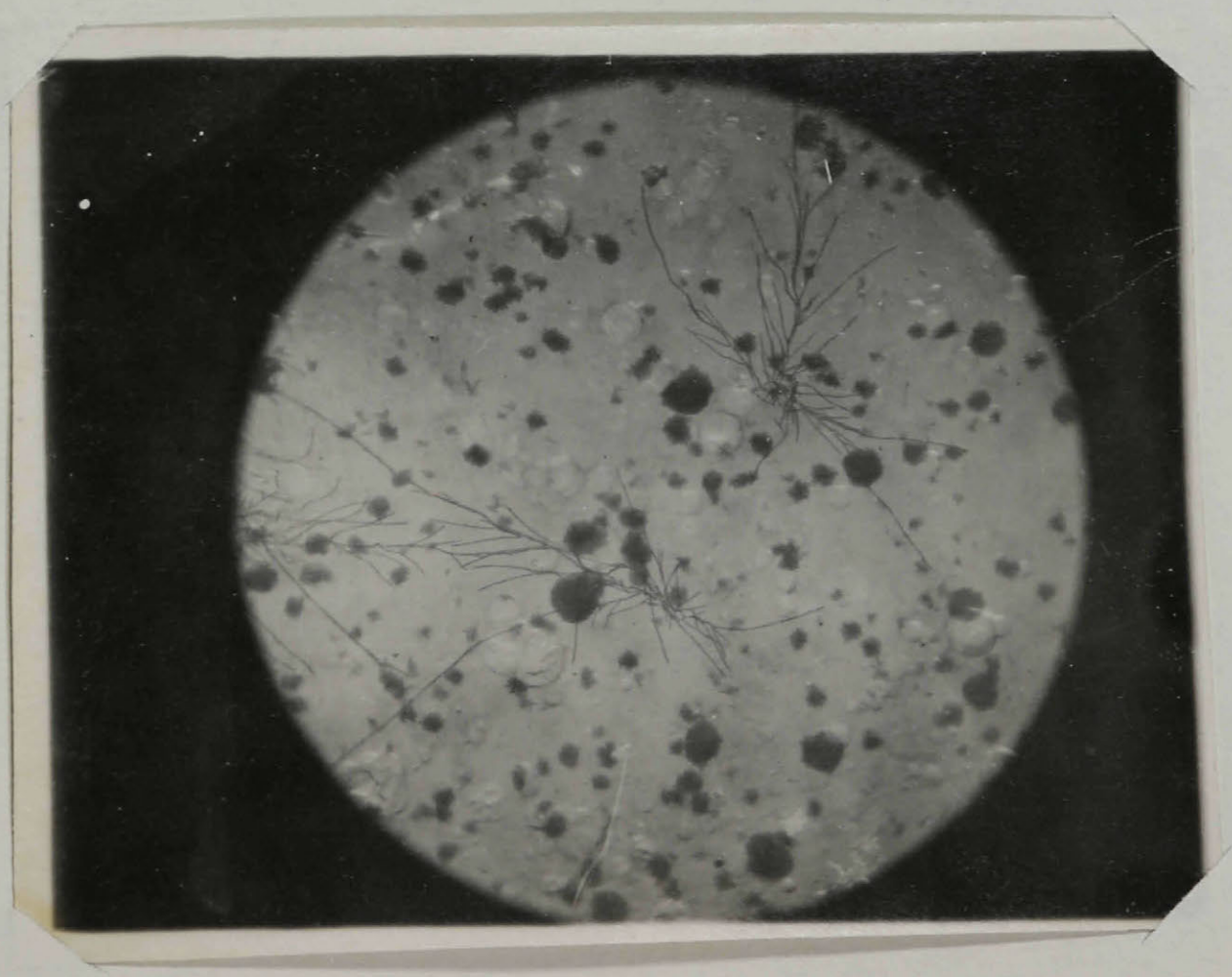

Fig. 2.

PLATE 4.

FIG. 1. Field taken from microplate of Sample DC, after incubation for 18 hours at 25° C. The average spread of the mould colony is about 1.5 mm.

FIG. 2. Field taken from microplate of same sample, after incubation for 18 hours at 30° C. Note the breaking up of the Oidium lactis colony into oidia, making counting more difficult than with fields like that shown in Fig. 1.

PLATE 4.

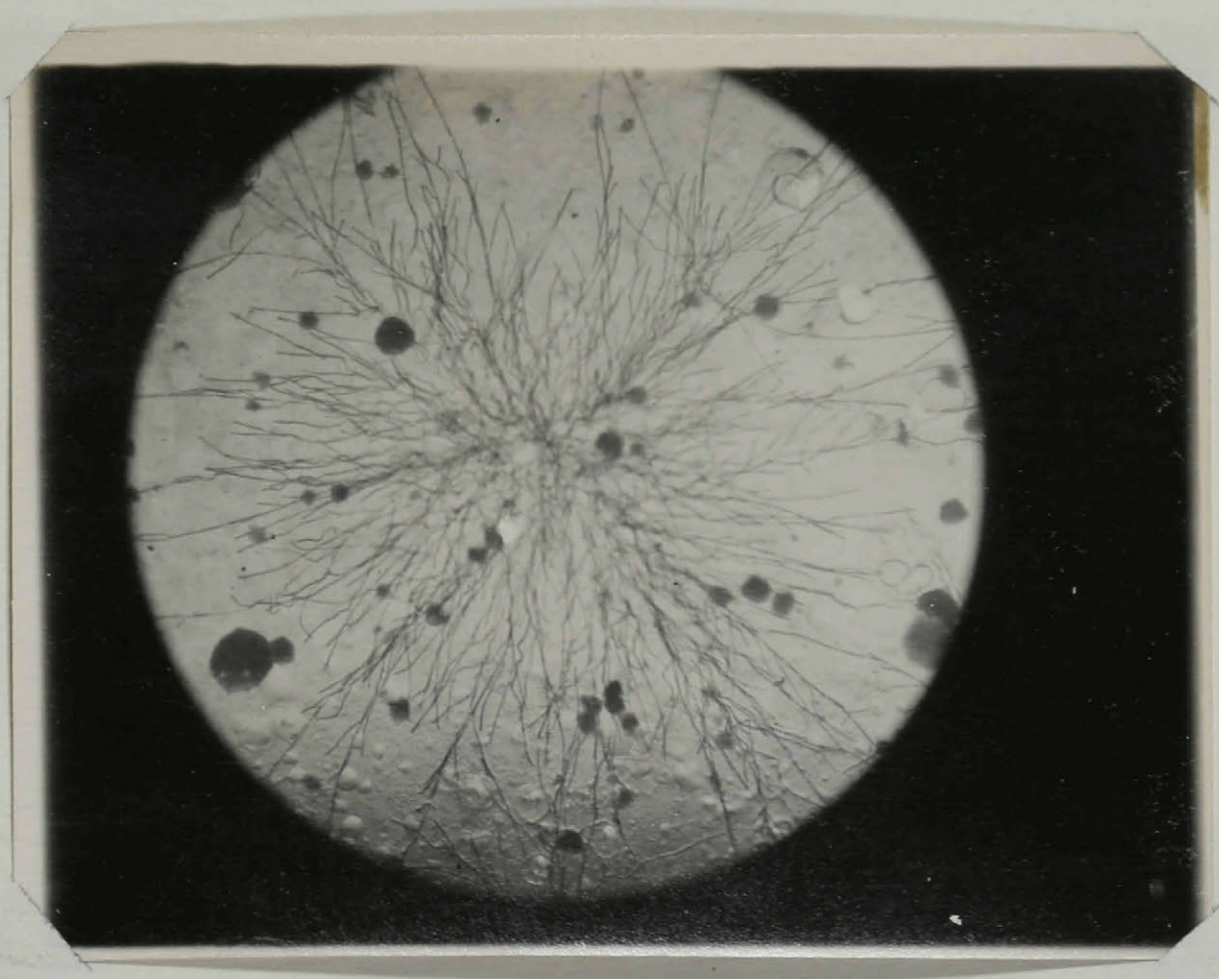

Fig. 1.

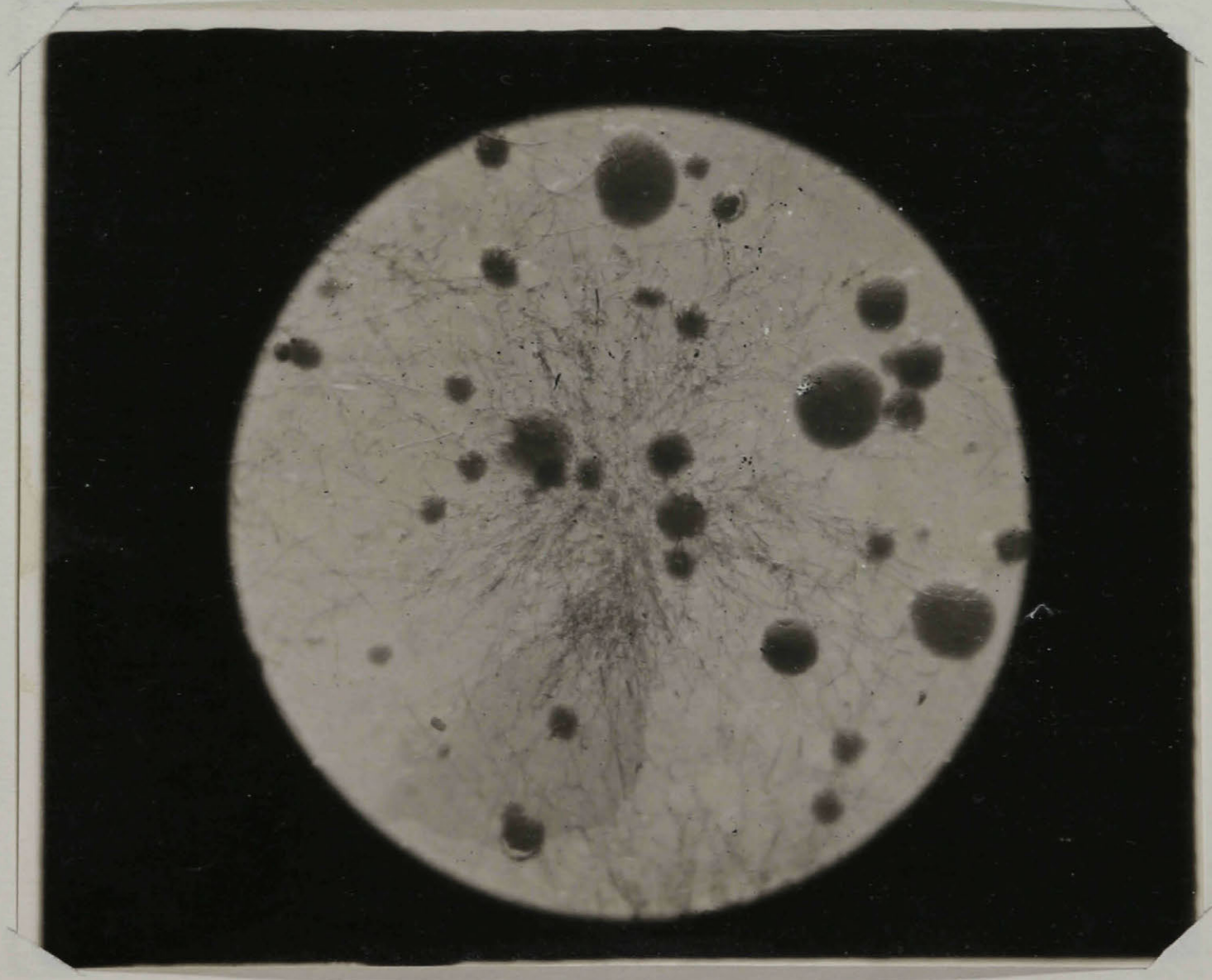

Fig. 2.

PLATE 5.

FIG. 1. Field taken from microplate of Sample EC, after incubation for 24 hours at 25° C.

PLATE 5.

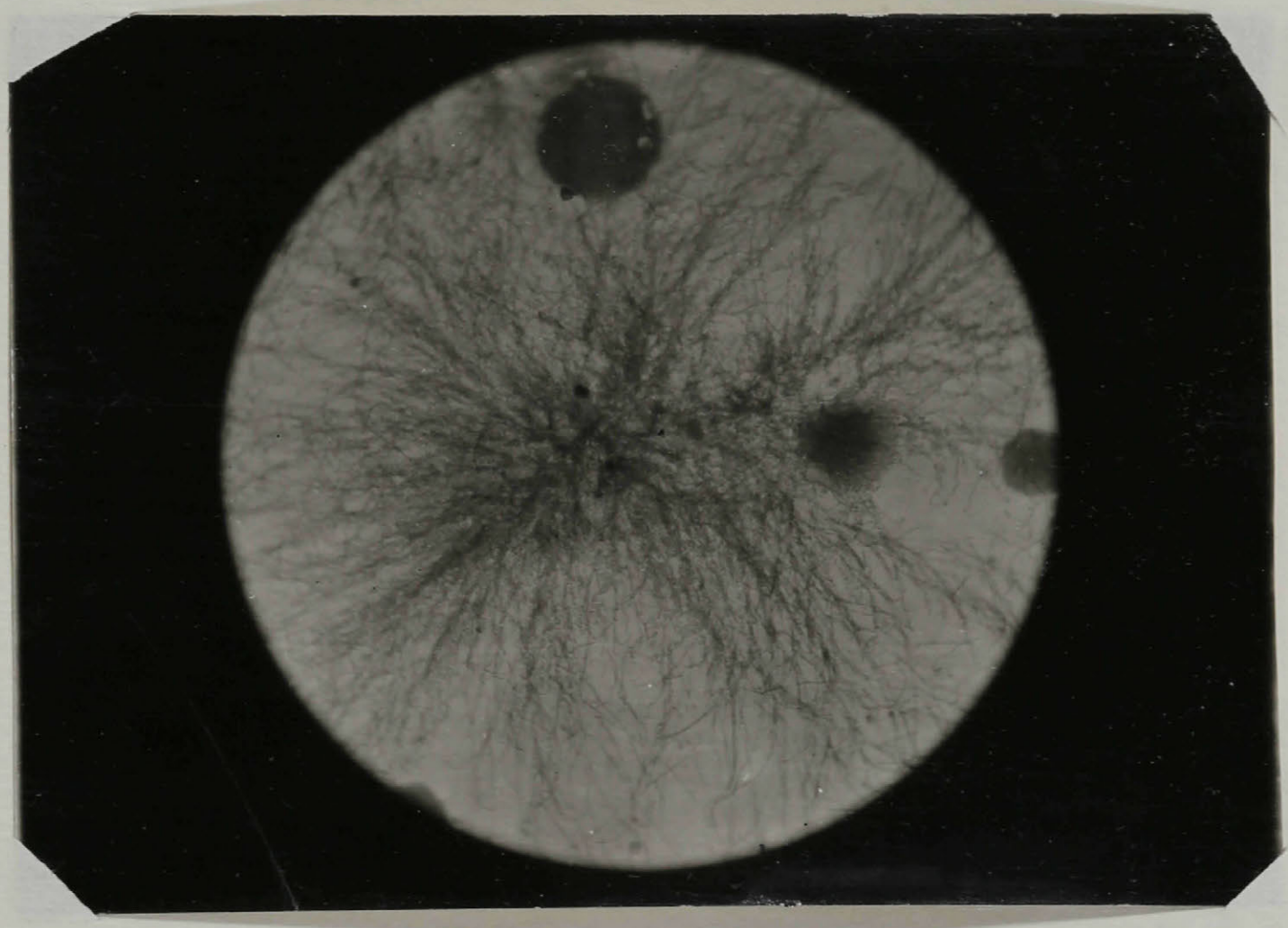

Fig. 1.