A STUDY OF MICRO-ORGANISMS FROM BUTTER ISOLATED AT 0°C. AND PRESUMABLY CAPABLE OF CAUSING CHANGES IN THE BUTTER AT COLD STORAGE TEMPERATURES.

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A STUDY OF MICRO-ORGANISMS FROM BUTTER ISOLATED AT 0°C. AND PRESUMABLY CAPABLE OF CAUSING CHANGES IN THE BUTTER AT COLD STORAGE TEMPERATURES.

INTRODUCTION AND HISTORICAL

It is generally conceded that the deterioration of butter during storage may be associated with the activity of bacteria, yeasts and moulds. In spite of the fact that butter ordinarily is stored at temperatures below the freezing point, most investigators have used higher incubation temperatures to obtain information as to the types of organisms probably affecting its deterioration. Hammer (6) states that butter stored at 0°C. (32°F.) may contain many organisms that are so definitely psychrophilic that they fail to grow at 37°C. The same reasoning would render the use of 21°C. as of questionable soundness.

The use of ordinary incubation temperatures suggests a reasoning somewhat as follows. Incubation of plates at 21° or 37°C. proves the presence of bacteria in butter. Butter deteriorates at temperatures below 0°C. The same bacteria must be capable of growth and of causing change in the butter at 0°C. It appears obvious that more direct knowledge of what specific types of bacteria might do to butter at 0°C. could be obtained by incubating plates at or near the freezing point. If it can be demonstrated that there are types of bacteria in butter that will grow on laboratory media at this temperature, there is sound reason for believing that these types are more likely to be responsible for the deterioration. Hence a study of the numbers and types of facultative psychrophilic

bacteria in cold storage butter might throw a new light on the relationship of bacteria to the problem of deterioration.

Wallace and Tanner (19) recently published a comprehensive review of the literature dealing with the microbiology of Frozen Foods. The materials covered in their report represent soil, dairy products, fruit and fruit juices, meat products, water and sewage. Their studies deal with the natural flora of the product in many cases and, in others, with pure cultures of well known mesophiles. They state that it has been more or less definitely established that some bacteria grow and function at temperatures as low as from -10°C. (14°F.) to -20°C. (-4°F.). These temperatures are comparable to those of the butter storage room. These investigators quote from a reference by Pennington (1908) dealing with milk held at -0.55°C. (31°F.) for a period of from five to six weeks. There was a steady increase in the number of organisms throughout the experiment. The predominating species were liquefiers that peptonized the casein during the storage period. Ravenel, Hammer and Hastings (1910) confirmed this finding as at 0°C.; but found no increase in the numbers of bacteria in milk held at -9°C. Rogers and Gray (1909) noted that there was a decrease in numbers of bacteria in butter held at -12°C. (10.4°F.) and at -20°C. (-4°F.). Stiles and Pennington (1909) attributed the increase in numbers of bacteria in ice cream held at a temperature varying between -10°C. (14°F.) and -20°C. (-4°F.) to the development of psychrophilic types.

On the other hand Hammer (1912) and Eaton and Mason (1915) did not observe an increase in numbers of bacteria in ice cream frozen hard.

Davis (3) states that the types of bacteria predominating in milk held below 10°C. attack the protein and cause the milk to become "stinking" and that Pseudomonas types can grow at 0°C.

Hiscox (9), studying areas of blackish discoloration in refrigerated butter, isolated an unidentified species of Pseudomonas which grew over a wide temperature range but produced a blackish pigment below 15°C. only.

Jensen (11) reports that Weinzert and Gerdeman found that constant bacterial development takes place in ice cream at a temperature of -10°C. (14°F.). He summarises the investigations of Conn and Eaton (1904) Arthur Wolff, Luxwolda (1911) and Jensen on the flora of milk at different temperatures by the statement that the fluorescent bacteria predominate at temperatures below 5°C.

Discussing the aerobic putrefactive bacteria Jensen considers first the organisms producing a fluorescent green colouring matter (insoluble in chloroform) on neutral or alkaline media. These can grow at temperatures only slightly above 0°C. (32°F.) In this group he makes special mention of Bacterium fluorescens liquefaciens (Ps. fluorescens) which hydrolyses fat and therefore may play an important part in turning butter rancid.

SCOPE OF THE PROBLEM

On the assumption that the isolation of bacteria at a low temperature might throw light on the problem of butter deterioration during storage, the following determinations were made:

I. The numbers of bacteria appearing on plates "incubated" at 0°C. and the relation between them and the counts of bacteria and fungi obtained as a result of incubation at the temperatures usually employed, (20°--37°C.).

II. The characteristics of the bacteria isolated at 0°C.

III. Methods by which the predominant types encountered could be recognized quickly and easily.

For convenience the first two considerations are dealt with in Section A; the third in Section B.

SECTION A

I. The Numbers of Bacteria Appearing on Plates Incubated at 0°C. and Their Relation to the Counts of Fungi and Bacteria Incubated at 25°C.

Material and Methods

Two types of butter were considered in this study. That designated special was made from high quality cream, selected, pasteurized and churned under painstaking methods of sanitation and used for exhibition purposes. Commercial was first-grade butter manufactured under routine conditions of commerce.

Only products from creameries making both types of butter from May to October were included. The operators were not aware that this study was being conducted.

The mould and yeast count was obtained by plating 1 ml. direct and 1 ml. of a 0.1 dilution in Difco malt agar, acidified to a pH of 3.5 with lactic acid--Hood and White (10)--and incubating at 25°C. for five days. For counts of bacteria the butter was plated at 0.1 and 0.01 dilutions in 1% lactose nutrient agar and incubated at 0°C. for 30 days; and at 0.01 and 0.001 dilutions for incubation at 25°C. for five days. In some cases approximate estimation only was possible. Naturally, the samples involved were rated as poor from the standpoint of counts and this failure to obtain an accurate determination would not affect the general results.

Results.

The counts on the 531 samples are presented in the appendix (page 73--100). Yeast and mould counts range from nil to 10,200; counts of bacteria at 0°C. from nil to 34,000; and those of bacteria at 25°C. from nil to 2,000,000 per ml. Since the experiment was not designed on a statistical basis, the counts are arranged in three classes, arbitrarily chosen as a modification of Hood and White's (10) system for mould and yeast in pasteurized butter. Excellent and good are considered as one class, termed good. The other classes are fair and poor. The boundaries of these classes follow:

	Good	Fair	Poor
moulds and yeasts	0 - 50	51 - 100 (51 - 1M)	101 - over (over 1M)
bacteria at 0°C.	0 - 500 (0 - 5M)	501 - 1,000 (5M - 10M)	1,001 - over (over 10M)
bacteria at 25°C.	0 - 5,000 (0 - 50M)	5,001 - 10,000 (50M - 10T	10,001 - over (over 10T)

It may be noted that the boundaries of classes for bacterial counts at 0°C. are ten times as high as the mould and yeast standards and that those for bacteria at 25°C. are 100 times as great.

The frequency distribution of the number of samples of butter in each class for each type of butter for each isolation procedure and for each creamery is presented in Table 1.

82.1% of the special samples fall in the good class on the basis of counts of moulds and yeasts; 78.3% on the basis

Table 1. Frequency distribution of samples in classes for each creamery.

Creamery No.	Mould & Yeast								Bacteria at 0°C.								Bacteria at 25°C.							
	Special				Commercial				Special				Commercial				Special				Commercial			
	#	Frequency			#	Frequency			#	Frequency			#	Frequency			#	Frequency			#	Frequency		
	S.	G.	F.	P.	S.	G.	F.	P.	S	G.	F.	P.	S.	G.	F.	P.	S.	G.	F.	P.	S.	G.	F.	P.
1.	6	6			11	11			6	6			11	11			6	6			11	11		
2.	7	7			16	16			7	7			16	16			7	7			16	16		
3.	7	7			12	12			7	7			8	8			7	7			12	12		
4.	7	6	1		6	3	1	2	7	7			8	8			5	4	1		5	1	2	2
5.	7	6	1		12	11	1		7	6		1	9	9			7	4		3	12	6	1	5
6.	7	7			16	14	2		7	5	1	1	16	15		1	7	5		2	16	15	1	
7.	7	7			12	11		1	7	6	1		16	15		1	7	5	2		16	12	1	3
8.	7	6	1		16	12	1	3	6	6			16	15		1	7	6	1		16	15	1	
9.	4	1	2	1	16	5		11	4	2		2	16	14		2	4	2	1	1	16	1	4	11
10.	7	6	1		16	13	2	1	7	7			16	13		3	7	5	2		16	8	3	5
11.	7	7			12	10	1	1	7	6		1	11	8		3	7	6		1	11	4	2	5
12.	7	7			12	7	4	1	7	7			12	8	1	3	7	7			12	5	3	4
13.	7	7			12	10	1	1	7	4		3	12	10		2	7	4	2	1	12	9	1	2
14.	3	3			12	9	1	2	3	3			12	8	1	3	2	2			12	6		6
15.	7	7			16	6	2	8	6	6			16	13		3	7	4	1	2	16	7	2	7
16.	7	6	1		13	6	2	5	7	6		1	12	8	1	3	7	7			13	5	3	5
17.	7	4	1	2	12	9	2	1	7	3		4	11	7	1	3	7	2	1	4	11	5		6
18.	3	3			12	3	2	7	3			3	12	5	2	5	3			3	12	3		9
19.	6	6			16	12	1	3	6	6			16	9	3	4	6	5		1	16	9	4	3
20.	3	3			8	5		3	3	1		2	8	4	1	3	3	1		2	7	1	2	4
21.	7	5		2	12	10		2	7	6		1	9	5		4	7	4	1	2	11	3	2	6
22.	2	2			12	9		3	2		2		9	4		5	2		1	1	10	3		7
23.	7	7			16	12	1	3	7	6		1	16	10	1	5	7	6		1	16	6	2	8
24.	7	3	1	3	9	4		5	7	5	1	1	9	7	1	1	7	6	1		9	4		5
25.	3	1	1	1	7	2	1	4	3	1	2		7	4	1	2	3		3		7		1	6
26.	7		1	6	12			12	6	1		5	12		5	7	7	3		4	12			12
27.	7	4		3	16		1	15	7	5	1	1	13	1		12	7	3	1	3	14	1		13
28.	3	2	1		16	10	1	5	3	2	1		14			14	3		2	1	16		1	15
Total	166	136	12	18	360	234	27	99	163	127	9	27	343	235	18	90	163	111	20	32	353	168	36	149
Per-cent.		81.9	7.2	10.9		65.0	7.5	27.5		77.9	5.5	16.6		68.5	5.2	26.2		68.1	12.3	19.6		47.6	10.2	42.2

S: Number of samples; G:Good; F: Fair; P: Poor.

of bacteria incubated at 0°C.; and 68.1% on the basis of bacteria incubated at 25°C. Similarly most of the commercial samples place in the same class although the proportions are not as high as for special samples. The percentages are 65.1, 68.5 and 47.4 respectively, for the three types of counts.

The remaining special samples divide about equally into the fair and poor classes in the three systems of classifying, the poor class showing a slightly larger proportion than the fair. Commercial butters show a considerably larger portion in the poor class, especially in the case of counts obtained by incubation at 25°C., where 42.3% of the samples rate poor.

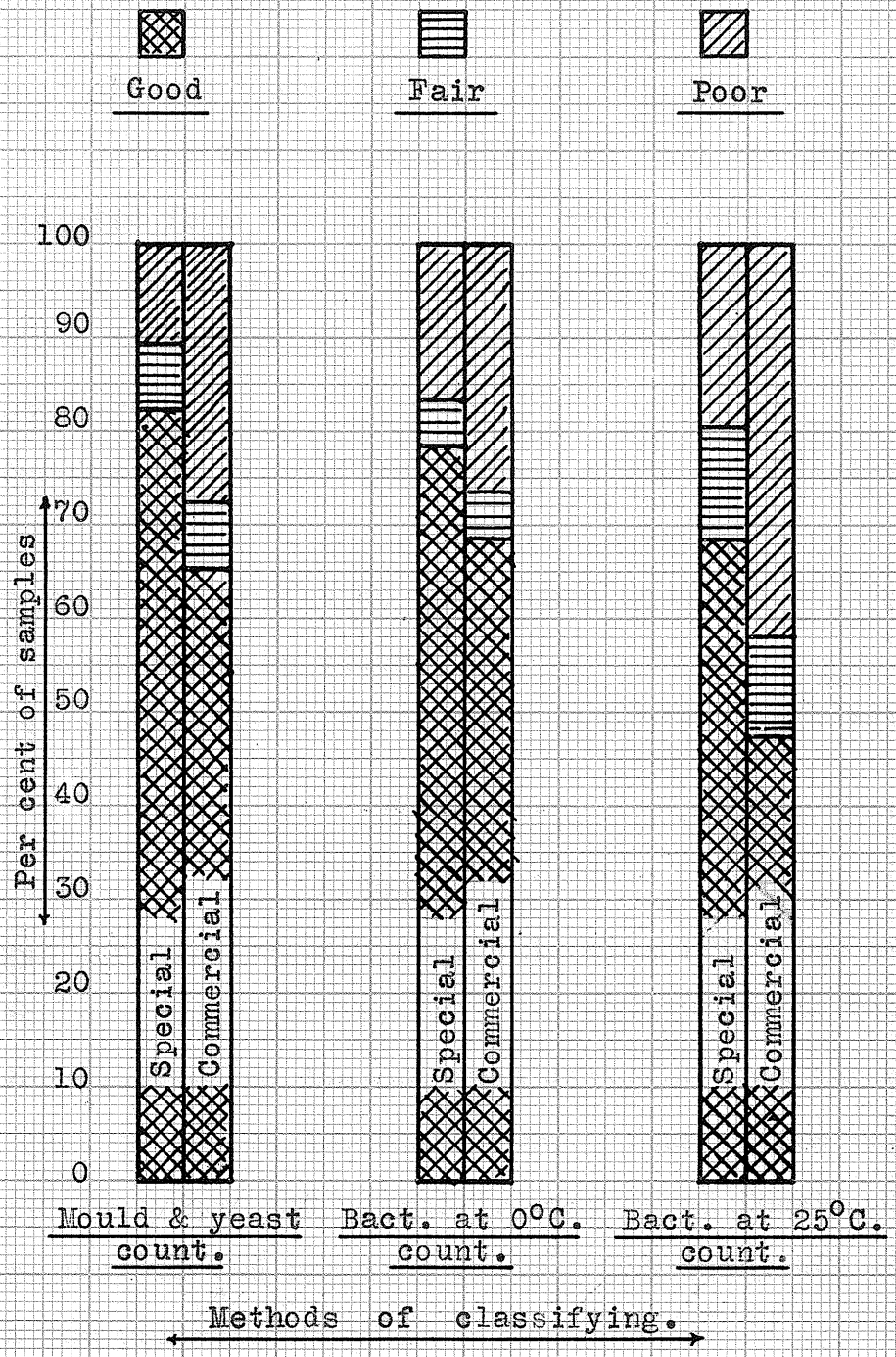
The data from Table 1 may be summarized as follows:

System of Classifying.	Classes.	Type of butter.	
		Special	Commercial.
Moulds & yeasts	Good	82.1%	65.1%
	Fair	7.2%	7.6%
	Poor	10.7%	27.3%
Bacteria at 0°C.	Good	78.3%	68.5%
	Fair	5.4%	5.2%
	Poor	16.3%	26.3%
Bacteria at 25°C.	Good	68.1%	47.4%
	Fair	12.3%	10.3%
	Poor	19.6%	42.3%

Chart 1 presents this summary graphically.

CHART 1.

THE PERCENTAGE OF SPECIAL AND COMMERCIAL SAMPLES OF BUTTER FROM 28 CREAMERIES IN CLASSES AS DETERMINED BY THREE METHODS.



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II. The Characteristics of the Bacteria Isolated at 0°C.

(a) Milk Tube Classification.

The milk tube method of classifying cultures isolated from milk products has been used by Ayers (1), Thurston and Olson (18) and Grimes and Hennerty (7). In this study 1,229 cultures, isolated from butter at 0°C., were incubated in litmus milk at 0°C. for 30 to 35 days and classified as acid formers, alkali formers, inert and peptonizers. This classification served three purposes:

(1) To determine the proportion in each group at 0°C. incubation.

(2) To compare this proportion with those obtained at higher incubation temperatures by others.

(3) To provide specific cultures that peptonized milk for further study.

Results.

The detailed results from this classification as presented in the appendix (pp. 73--101) are summarised in Table 2. The largest proportion of the cultures isolated from these samples at 0°C. proved to be peptonizers. Inert types were slightly less in number and alkali formers still fewer. The acid formers made up the smallest group. The study was continued with the peptonizers only.

Table 2.

Litmus milk-tube classification of Cultures from butter isolated at 0°C.

Classes	Number of cultures in class	Percentage of total cultures
Acid formers	118	9.6
Alkali formers	305	24.8
Inert.	385	31.3
Peptonisers	421	34.3
Totals	1,229	100.0

Before proceeding with their characterisation, the 421 cultures were re-cultured and re-classified twice in duplicate and at two different temperatures of incubation. The purpose of this was:

(1) To obtain only cultures which gave indication of purity by repeated agreement on their physiological reaction in litmus milk.

(2) To determine whether the more applicable and rapid incubation at 25°C. for 15 days could be substituted for 0°C. for 30--35 days.

(3) To carry the cultures over a period of suspended study. By this means the 421 cultures were reduced in number to 180. All of these gave litmus milk reactions at 25°C. similar to those of the original cultures at 0°C.

(b) Classification of peptonisers by the colours produced in litmus milk.

In peptonized litmus milk these cultures produced

definite colours, yellow, green, blue and purple, the majority being in the green. The characterisation was sufficiently marked and consistent to provide a fundamental basis for classification. The colours were graded and named by matching each culture with the Color Standards and Nomenclature of Ridgway (13). By this method the 160 cultures, having the common characteristic of milk peptonisation, were divided into nine groups. For convenience these named colour classes were designated by capital letters A to I.

Results.

The complete results on the classification of peptonisers by the colours produced in litmus milk are presented in the appendix (pp. 73--101). A summary of this classification is given in Table 3.

Table 3.

Classification of peptonisers by colours produced in litmus milk.

Class Designation	Colour classes (Ridgway's Standards)		Number of cultures	
A.	Beru Olive	*Plate XXX	14	
B.	Yellow Olive	" XXX	67	
C.	Mignonette Green	" XXXI	14	149(82.8%)
D.	Jade Green	" XXX	31	
E.	Cress Green	" XXXI	9	
F.	Slate Olive	" XLVII	14	
G.	Dusky Slate Blue	" XLIII	0	
H.	Brownish Drab	" XIV	11	
I.	Snuff Brown	" XXIV	11	
			160	

* Plate number in Ridgway's Standards.

Classes A to F were shades of green and included 82.8% of the total cultures. The colours were outstanding. The odour from these cultures was decidedly that of rotting vegetables and was strongest in all cultures in Class B. The colours in classes G, H, and I were definite but the odour was not as objectionable or easily described.

(c) Sub-classification of the colour classes.

The cultural characteristics on nutrient agar slants, in nutrient broth and gelatin provided the basis for subdivision of the colour classes. The characters are presented in the following diagram:

Cultures used	Media	Characters observed
Each of the 180 peptonized litmus milk cultures.	(1) Nutrient agar slant.	(a) Form of growth (b) surface--smooth or rough.
	(2) Nutrient Broth.	(c) cloudy or clear (d) surface--pellicle or not (e) sediment--viscous or not.
	(3) Nutrient gelatin stab.	(f) liquefaction--form (g) " --rate (h) " --cloudy or clear.

This information, arranged in key form, as presented in the appendix (pp. 102--109), afforded data for differentiating the sub-classes within each colour class. These are summarized in Table 4.

Table 4.

Sub-classification of the colour classes.

Color Classes			Sub-classes	
Desig-nation	Name (Ridgway's Standards)	Total Cultures	Desig-nation	Total Cultures
A.	Ecru Olive	14	1 2 3	8 1 5
B.	Yellow Olive	67	1 2 3 4	13 27 6 21
C.	Mignonette Green	14	1 2 3 4 5	3 3 3 1 4
D.	Jade Green	31	1 2 3 5 6 7	7 5 4 1 5 6
E.	Cress Green	9	1 2 3 4	3 4 1 1
F.	Slate Olive	14	1 2 3	8 4 2
G.	Dusky Slate Blue	9	1 2	4 5
H.	Brownish Drab	11	1	11
I.	Snuff Brown	11	1	11
	Totals	180	30	180

All cultures in classes H and I respectively, appeared to be similar. The number of sub-classes in the other classes varied between 2 and 7. In all there were 30 groupings within the 9 colour classes of 180 individual cultures of peptonizers. One culture picked at random from each of the sub-classes provided 30 individual representatives of the dominant type of bacteria isolated from these samples at 0°C. These cultures were designated by a capital letter followed by a number, representing their class and sub-class. Each was tested for purity by the qualitative plating technique before proceeding further.

(d) Detailed study of 30 type cultures.

The 30 cultures probably represent separate species of bacteria or variants of one or more species. In order that such be proven more conclusively, the following systematic detailed study was conducted.

Procedure.

The general procedure adopted was that outlined by the Society of American Bacteriologists in the "Manual of Methods for the Pure Culture Study of Bacteria," (16). Special methods, used by other investigators or in some cases not found in the literature, were introduced. The tests conducted are listed, or in some instances, described in brief as follows:

A. For optimum growth conditions.

1. Temperature for growth--in broth at 0°, 6°, 21--25°, 37°C.
2. Thermal death point--in broth 10 mins. at 65°, 75°and 85°C.
3. Suitability of standard types of media.
4. Relation to free oxygen.
5. Relation to salt concentration--in Dunham's peptone solution containing the following amounts of salt 3.3%, 6.6%, 9.9% and 13.2%, equalling in butter of 15% moisture 0.5%, 1.0%, 1.5% and 2% respectively.

B.- For morphological characters.

1. Motility--hanging drop preperations from young broth cultures.
2. Flagellation--by Leifson's method (12).
3. Presence of endospores--by malachite green--safranin stain.
4. Gram staining reactions--Hucker's stain.
5. Form of vegetative cells--from simple carbol fuchsin stain.
6. Arrangement of vegetative cells--from simple carbol fuchsin stain.
7. Size of vegetative cells--from simple carbol fuchsin stain.

C.- For cultural characteristics.

1. Agar colonies--from purification platings.
2. Agar stroke.
3. Nutrient broth.
4. Gelatin colonies-- spot colonies on poured plates.
5. Gelatin stab.
6. Potato stroke.

D.- For physiological or biochemical characteristics.

1. Indole formation--in tryptophane broth.
2. Ammonia formation--in 0.1% potassium nitrate broth.
3. Nitrate reduction--in 0.1% potassium nitrate broth.
4. Starch hydrolysis--in 0.1% starch agar slants and starch broth.
5. Hydrogen sulphide formation--on lead acetate agar slants.
6. Carbohydrate fermentation--in 0.5% dextrose, lactose and sucrose broths in Durham fermentation tubes.
7. Protein decomposition--i. e., peptonization or proteolysis.
   (a) Gelatin--by spot colonies on gelatin--agar poured plate as per Frazier (5).
   (b) Casein--spot colonies on casein agar of Frazier and Rupp (6).
   (c) Milk--spot colonies on 1% milk agar.
   (d) Plain milk--peptonization rate.
   (e) Litmus milk--colour of peptonization by Ridgway's Standards and Nomenclature.
   (f) Casein solution--amino nitrogen increase by method of Frazier and Rupp (6).
   (g) Plain milk--amino nitrogen increase by method used in (f).
8. Butterfat lipolysis--streak cultures on plates poured with Nile-blue sulphate-dyed butterfat dispersed in agar by method of Collins and Hammer (2).
9. Pigment production--in nutrient broth and on nutrient agar slants, both media made in tap water. See Sec. 3.

10. Colour of fluorescent pigment--in Sullivan's (17) synthetic medium K, modified. See Sec. 3.

Results.

The results from numerous tests were common to many or even all cultures. Others were of no significance in differentation, whilst some others lacked sufficient uniformity or consistency to justify their inclusion. The common and closely agreeing characteristics are presented in condensed form as follows:

Common or closely agreeing characteristics.

A.- Optimum growth conditions.

1. Temperature--between 0°C. and 25°C., more slowly at the lower temperature.
2. Thermal death point--between 65°C. and 75°C. (149 to 158°F).
3. Relation to standard media--good growth in all.
4. Relation to free oxygen--aerobic, facultative.
5. Relation to salt concentration--good growth in 3.3% (0.5% in butter); fair in 6.6% (1% in butter); very slight in 9.9% (1.5% in butter); no growth in 13.2% (2.0% in butter).

B.- Morphological.

	For cultures A_1 to H_1	For culture I_1
1. Motility--	motile	non-motile
2. Flagellation--	polar	none
3. Endospores--	none	none

4. Gram stain--	negative	positive	
5. Form of cells--	rods	spheres	
	All cultures except B_3 and I_1	For B_3	For I_1
6. Cell arrangement--	singles and pairs.	long chains.	singles.
7. Cell size--	0.5 to 0.6 X 1.4 to 2.5 microns.	0.6 X 2.7 to 3.4 microns.	0.7 to 0.8 microns.

C. Cultural.

	Cultures A_1 to G_2	Culture H_1	Culture I_1
Agar colonies--	circular, entire, flat to convex, variable in smooth and rough, glistening, greyish white to greenish grey, translucent.	Circular, convex, wrinkled, dull, creamy white, opaque.	Circular, convex, smooth, glistening, yellow opaque.
Agar stroke--	filiform to echinulate moderate, variable in rough and smooth, glistening slight metallic sheen, greenish grey, translucent.	echinulate, rough, wrinkled, dull, creamy white, opaque.	filiform, glistening, canary yellow, opaque.
Nutrient broth--	variable in pellicle formation, clouding and sediment.	heavy wrinkled pellicle, viscous sediment.	slight yellow pellicle, clear, flaky sediment.

	A_1 to G_2	H_1	I_1
Gelatin colonies--rapid liquefaction.		slow liquefaction.	very slow liquefaction.
Gelatin stab--infundibuliform to strateform, variable in rate. (See differing characters Table 5.)		napiform slow.	crateriform slow.
Litmus milk--alkaline, peptonized in colours of green to blue as used in classification. Table 5.		alkaline, peptonised colour brownish drab.	alkaline peptonized colour snuff brown.
Potato stroke--abundant, spreading, moist sepia brown to pinkish at bottom.		abundant, spreading, dark brown.	no growth.

D. Physiological. (Common to all cultures.)

1. Indole--not formed.
2. Ammonia--formed in varying amounts.
3. Starch hydrolysis--none.
4. Hydrogen sulphide--not formed.
5. Carbohydrate fermentation--only dextrose acid but no gas.
6. Nitrate reduction--see differing characters Table 5.
7. Protein decomposition in--(a) gelatin, (b) casein, (c) milk, no reliable characters secured, (d) plain milk--peptonization at variable rates.

(e) Litmus milk--alkaline peptonization with development

of colours used in classification, Table 3.

(f) Casein solution--characters not reliable for amino nitrogen increase.

(g) Plain milk--for amino nitrogen increase: see differential characters Table 5.

8. Bufferfat lipolysis--see differential characters Table 5.

9. Pigment production--see differential characters Table 5.

10. Colour of fluorescent pigment--see differential characters Table 5.

Characteristics showing differences in the cultures.

The voluminous data on this phase of the study are presented in concise form in Table 5.

By the characterizations for cell shape, endospore formation, pigment production and optimum growth temperature the 30 type cultures, representing a total of 180 original cultures, were grouped into their respective families, tribes and genera as shown in the summary of Table 6. This indicates that of the 180 pure peptonising cultures from butter isolated at 0°C., 82.6% are of the genus Pseudomonas.

Subdivision of the genera.

On the basis of nitrate reduction, type of gelatin liquefaction and in the case of culture B_8 the arrangement of cells, the 26 Pseudomonas cultures may be further classified into five numbered species. The members of each of these

Table 5.

Differential characteristics of the 30 selected type cultures.

Genus Pseudomonas	Cultures.	Cell Arrangement. Singles	Cell Arrangement. Chains	Nitrate Red Reaction	Nitrate Red Nitrite	Gelatin Liquefaction. Infundi.	Gelatin Liquefaction. Infund. Le cerati.	Gelatin Liquefaction. Stratif.	Fluorescent Colour. Yellow Green	Fluorescent Colour. Green	Fluorescent Colour. Blue Green
Sp. 1. Strain 1	A_1 A_3	✓		✓		✓			✓		
" 2	B_1 B_2	✓		✓		✓				✓	
" 3	B_4	✓		✓		✓				✓	
" 4	A_2	✓		✓		✓				✓	
Sp. 2.	B_3		✓	✓		✓				✓	
Sp. 3. Strain 1	C_1	✓			✓	✓					✓
" 2	C_2	✓			✓	✓					✓
" 3	C_5	✓			✓	✓					✓
" 4	C_4	✓			✓	✓					✓
" 5	C_3	✓			✓	✓					
Sp. 4. Strain 1	D_6	✓			✓		✓				✓
" 2	D_1	✓			✓		✓				
" 3	D_4 D_5	✓			✓		✓				
" 4	D_3	✓			✓		✓				
" 5	D_7	✓			✓		✓				
" 6	D_2	✓			✓		✓				
Sp. 5. Strain 1	E_1	✓			✓			✓			
" 2	E_3 E_4	✓			✓			✓			
" 3	F_3	✓			✓			✓			
" 4	F_1 F_2	✓			✓			✓			
" 5	E_2	✓			✓			✓			
Genus Achromobacter						Crateriform Napiform					
Sp. 1.	G_1 G_2	✓		✓		✓					
Sp. 2.	H_1	✓		✓				✓			
Genus Micrococcus						Crateriform to saccate					
Sp. 1.	I_1	✓		✓		✓					

Table 5 (contd).

Fluorescent Colour			Amino Nitrogen Increase				Lipolysis				Out of 180 original cultures			Already scored as inoculated butter.
Green Blue	Blue	Dark Blue	0-10	10-30	31-45	45-60	Slight	Moderate	Strong	Very Strong	No Apparent	Per Species	Per Genus	
			✓							✓	13			37.0
					✓				✓		40	78		36.0
					✓					✓	21			36.5
						✓				✓	1			
						✓			✓		6	6		37.8
				✓				✓			3			37.5
				✓					✓		3			
						✓			✓		4	14		
						✓				✓	1			
✓				✓				✓			3			
				✓					✓		5			
✓				✓				✓			7			
✓					✓			✓			4	31	149	38.0
✓					✓				✓		4			
✓					✓			✓			6			
✓						✓			✓		5			
	✓		✓					✓			3			37.75
	✓			✓				✓			2			38.8
		✓	✓	✓				✓			2	23		
		✓	✓		✓			✓			12			38.0
		✓				✓		✓			4			38.0
			✓				✓				9	9	20	39.0
					✓		✓				11	11		39.0
				✓			✓				11	11	11	39.0
											180	180	180	

Table 6.

The generic and numerical grouping of the type cultures.

Genera	Out of 30 types			Out of 180 peptonizers.	
	Designation	Number	%	Number	%
Pseudomonas	F_1 to F_5	26	86.7	149	82.8
Achromobacter	C_1 to H_1	3	10.0	20	11.1
Micrococcus	I_1	1	3.3	11	6.1
		30	100.	180	100

species show sufficient differences in fluorescent colour of pigment, amino nitrogen increase and butterfat lipolysis possibly to warrant division into the strains shown in Table 5.

The Achromobacter cultures were divided into two species by their growth on agar slants and by their form of gelatin liquefaction.

Identification of species.

None of the numbered species of these two genera checks with species listed in Bergey's "Manual of Determinative Bacteriology." However, species 4 and 5 of the genus Pseudomonas agree with Ps. chlororaphis (Bergey et al.) except for type of gelatin liquefaction. Identification of the cultures or establishment of probable new species was not considered important in this investigation.

The one species of the genus Micrococcus agrees in detail with Micrococcus subflavescens, Bergey et al., except for the type of gelatin liquefaction.

Frequency occurrence of the species from the contributing creameries.

A knowledge of the occurrence of these species in the butter made in different creameries seemed important. Each of these species represents a definite group of peptonising cultures. A frequency occurrence, Chart 2. was established by tracing backward from the culture to the group it represents, then to the butter samples from which isolated and finally, to the respective creameries. From this the following summary was made:

Out of 28 creameries,

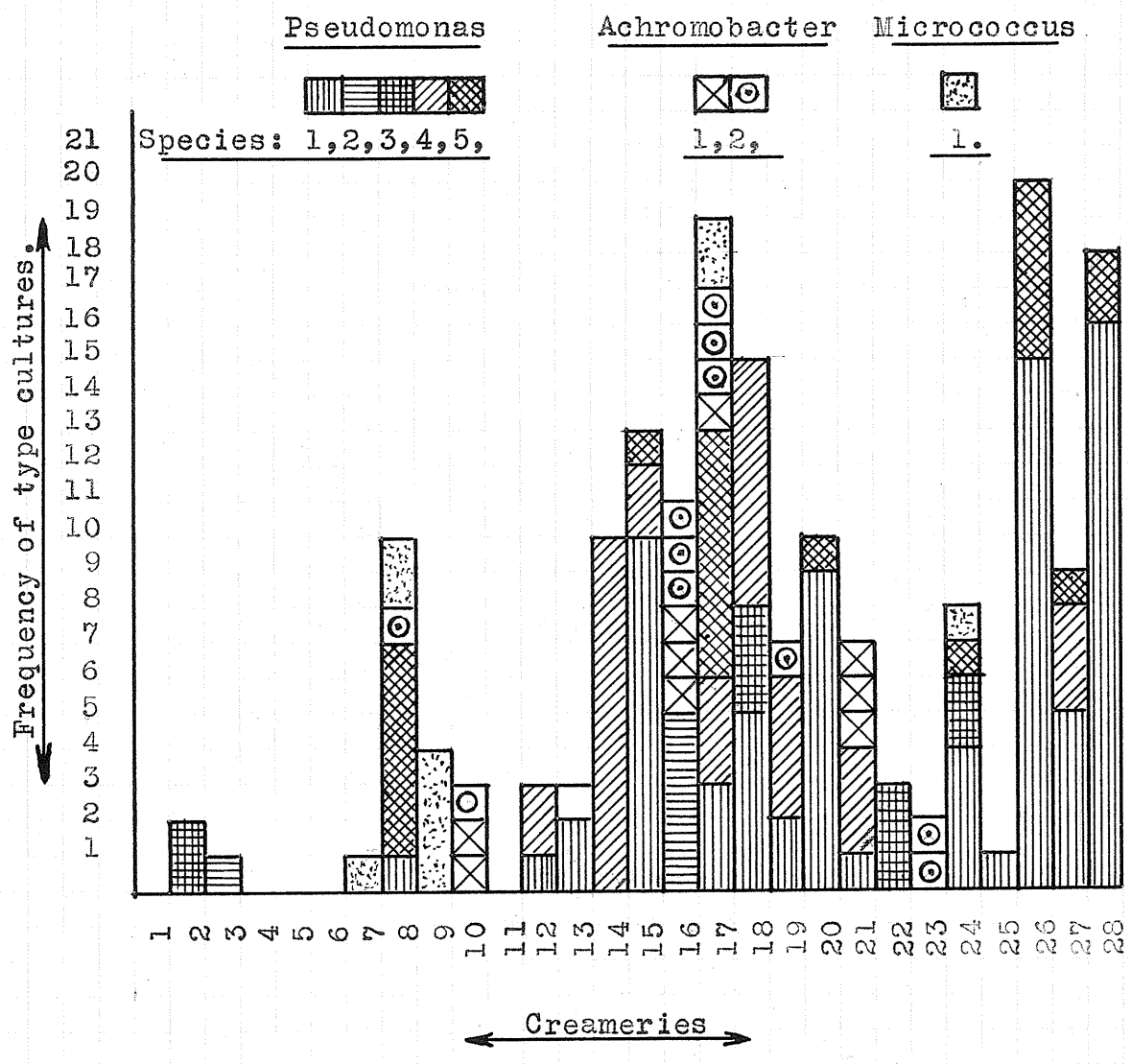
23 contributed all of the 160 cultures.
19 contributed organisms of the Pseudomonas genus.
14 contributed organisms of Pseudomonas, species 1.
2 contributed organisms of Pseudomonas species 2.
4 contributed organisms of Pseudomonas species 3.
7 contributed organisms of Pseudomonas species 4.
8 contributed organisms of Pseudomonas species 5.
6 contributed organisms of the Achromobacter genus.
4 contributed organisms of Achromobacter species 1.
6 contributed organisms of Achromobacter species 2.
6 contributed organisms of the Micrococcus genus.

It may be noted that organisms of the genus Pseudomonas may be isolated from the butter of most creameries and that species 1. appears to be common. Further there is no specialised occurrence of any type of culture.

CHART 2.

FREQUENCY DISTRIBUTION OF THE 180

PEPTONIZING CULTURES IN CREAMERIES.



(c) The relationship of the classified cultures to the flavour of cold-stored butter.

The practical application of this study depended on whether these classified cultures were deleterious to the flavour of cold-stored butter. This was determined by churning cream, sterilized and later inoculated with a specific culture, under aseptic conditions and judging the butter after holding in cold storage for different periods.

Since this phase of the study preceded the final classification of cultures, 15 of the most typical were selected. Later these were found to represent all the species.

In order to remove the effects of bacteria of contamination on the quality of the butter the equipment, cream and wash water were sterilized. This was accomplished by using steam under 15 pounds pressure for 20 minutes, except in the case of the four-quart glass churns. These were sterilized with commercial sterilising solution, "H. T. H." having 150 to 175 p. p. m. of available chlorine, and later rinsed several times with sterile water.

Special grade cream having an acidity of .21% and a fat content of 32% was sterilized, cooled to 5°C. (41°F.) and held overnight at that temperature. It was churned in three-pint lots on a mechanical shaker, strained, washed, worked and packed in triplicate in four-ounce glass jars tightly covered with double ply parchment caps. One churning was used as a control. In each of the others, the cream was inoculated

by adding a 15 ml. plain milk culture of the test organism and the butter was inoculated further by adding the growth from an agar slant culture to the final wash water.

The sanitary condition of the equipment, cream and wash water was checked by plating according to standard procedures. Numbers of bacteria were determined (1) in the fresh butter; (2) in butter held 30 days at 0°C. and (3) in samples held 120 days at 0°C. Finally, the butter was scored after storage for 30 days at 0°C., by Professor R. W. Brown who had no means of identifying the treatments under consideration.

Results.

The counts of bacteria and moulds follow:

(1) Cream ready for churn--bacteria 50 per ml. (B. subtilis type).

(2) Churn ready for cream--bacteria none, mould 1 per ml.

(3) Bulk wash water--bacteria none, moulds 3 per ml. These counts indicate that the equipment was satisfactory. The cream, although not sterile, had a low count of a spore-forming type easily distinguished from the test organism. Associative action with the cultures used was possible. However, since this might be common to all it was disregarded.

(4) The bacterial counts and flavour scorings of the test butters are presented in Table 7.

Bacterial counts on the inoculated butters lacked uniformity and were also inconsistent. In samples C_1, E_1,

Table 7.

Bacterial counts and flavour scores of inoculated butters.

Sample	Counts per ml.			Flavour scores & descriptions	
	Fresh	30 days 0°C.	120 days 0°C.	Score out of 45.	Description
Check	5	50	180	40	clean
A_1	3HT	1HT	395	37.6	cheesy
B_1	1HT	34T	100	36.0	butyric,rancid,bitter
B_3	74T	80T	16H	37.6	slightly butyric
B_4	80T	42T	670	36.5	butyric, rancid
C_1	3HT	4HT	720	37.5	sour, rancid
D_4	58T	46T	267	38.0	stale
E_1	43T	1HT	28T	37.75	cheesy
E_2	58T	6HT	13H	38.0	stale
E_3	2HT	6HT	86T	38.8	slightly stale
F_1	27T	2HT	78H	38.0	stale
G_1	67T	1HT	64H	39.0	quite clean
H_1	23HT	17HT	12HT	39.0	quite clean
I_1	12HT	9HT	6HT	39.0	quite clean

H: Hundred
T: Thousand

E_2, E_3, F_1, and G_1 the counts increased in 30 days at 0°C. while all others decreased. However, with the exception of the control, all showed lowered counts after refrigeration at 0°C. for 120 days. Flavour scores did not appear to be correlated with bacterial population. Sample B_1 which was poorest in flavour, gradually decreased in bacterial content during holding and latterly was the lowest of all the samples. Of the 13 samples the 10 inoculated with cultures, classified as Pseudomonas scored the lowest. Samples B_1 and B_4 scored 36 and 36.5 respectively. The cultures for inoculation of these two butters, appear to belong to closely related strains of a common species.

Discussion.

The development of colonies on plates incubated at 0°C. demonstrates the presence in butter of types of bacteria capable of growth at a temperature closely simulating that of cold storage. This suggests that these organisms may be responsible for deterioration of butter in storage. This assumption is borne out by the experimental butters inoculated with specific cultures. While the increase in the numbers of bacteria in these butters was not consistent, large numbers persisted for 30 days and even for 120 days and the butters deteriorated in quality. Particularly was this shown in the experiment with Pseudomonas types, when all butters scored less than 30. While the counts at 120 days in all samples were

lower than those on the fresh it is conceivable that growth took place. In other words, the bacteria may not have remained dormant and died off gradually. It is reasonable to assume merely that death was occurring more rapidly than reproduction with the resultant lower count.

The significance of numbers of bacteria is primarily dependent on types concerned. Since peptonizers proved to predominate in the isolations at 0°C., one might assume that they are the most detrimental in butter stored at low temperatures. Peptonizers represent the smallest group of organisms in raw or properly pasteurized milk as reported by Ayers (1) and Thurston and Olson (18). Likewise, Grimes and Hennerty (7) found peptonizers to account for only 2.1% of the cultures isolated at 21°C. from cold stored butter, whereas acid-formers represented 57.1%. An isolation temperature of 0°C. appears to reveal the development of a different flora from that observed at the usual incubation temperatures. It seems reasonable to assume that this flora may be responsible for deterioration in butter and that an incubation temperature of 0°C. may provide information of more practical value than those used commonly.

Since 62.6% of the peptonizers isolated represent the Pseudomonas genus, there appears to be sound reason for considering these types the most serious. While the number of experimental butters inoculated with specific types of isolates

at 0°C. was not sufficiently large to warrant definite conclusions on this point, it appears significant that the score on the three butters inoculated with representatives of other genera in each case was higher than that on any of the ten butters contaminated with Pseudomonas types. The practical finding that cultures of the Pseudomonas genus isolated at 0°C. cause deterioration in butter held at 0°C. is a confirmation of somewhat similar observations made by other investigators and reviewed in the historical section.

Shutt (14) and Noble (4) report that species of this genus may be responsible for "surface taint" in butter. Members of the Pseudomonas genus are found in water and consequently are com on contaminants in creamery products. They gain access to butter from the wash water or indirectly from the equipment. Their isolation from both types of butter from most of the creameries included in this study indicates that water may have been their common source. It is reasonable to assume that numbers in water might be used as a reliable index of suitability of water for creamery use. This has been proposed by Shutt (15).

When the butters were placed in arbitrary classes on the basis of counts there is reasonably close agreement between yeast and mould count classes and those of bacterial counts at 0°C. This agreement strengthens the value of the yeast and mould count as an index of sanitation in the manufacture of pasteurized butter.

SECTION B

III. Methods by Which the Predominant Types Encountered Could be Recognised Quickly and Easily.

Introduction and Historical

According to the "Manual of Determinative Bacteriology," by Bergey and al. (20), differentiation between the genera, Pseudomonas and Achromobacter, is based primarily on pigment formation by the former. This pigment may vary in colours from yellow, green, blue, brown to black and is diffused into the medium in or on which the cultures grow.

The critical dependence of pigmentation upon certain constituents of the medium became apparent with the change of distilled water to tap water. In a medium made with the latter, which was of lake or river origin, pigment consistently developed but the same cultures grown on identical media made with distilled water would have been classed as Achromobacter organisms due to the lack of pigmentation. Thus qualification of media appears to be a necessary requisite for the recognition of Pseudomonas cultures.

Investigations on the requirements for pigment production have been made with synthetic media. According to Georgia and Foe (23) Hueppe was the first to use a synthetic medium in 1880. This contained ammonium tartrate, neutral potassium phosphate, magnesium sulphate and calcium chloride. Sullivan (32) reports that Gessard in 1892 studied the production of fluorescence by B. pyocyaneus in a medium similar

to that of Hueppe. From this he found that phosphates were so essential to pigment production that 1/8,000 part of phosphate could be detected in a medium by the production of fluorescence.

Again, Georgia and Poe (23) give credit to Thumm for having studied fluorescence from B. pyocyaneus in a medium similar to that used by Gessard. For the organic constituents he tried a number of compounds and concluded that in addition to a source of organic nitrogen, the best of which was asparagine, a phosphate and magnesium must be present.

Jordan (28), in studying the production of fluorescence with six species of fluorescing bacteria, used ammonium salts of a number of organic acids with magnesium sulphate and sodium phosphate. He concluded that a sulphate and a phosphate were the only inorganic constituents needed and that, regardless of the base, equally good fluorescence resulted.

Sullivan (32) agreed with Jordan. He concluded, "that the fluorescent pigment could be formed whenever, in addition to asparagine, there was present in the solution both phosphates and sulphates, irrespective of the base."

Tanner (33) studied one hundred strains of fluorescent bacteria isolated from water in Uschinsky's medium, Sullivan's medium and Frankel's medium. In addition to other ingredients the first two media contained magnesium sulphate and phosphate but in Frankel's medium neither sulphate nor magnesium were present. Tanner claimed that all three media gave good pigment and so it

appeared that sulphate and magnesium were non-essential in pigment formation.

Georgia and Poe (23) made a comprehensive study of the inorganic constituents necessary for the production of fluorescence using selected chemicals and pyrex glassware carefully checked for impurities. They concluded that along with the generally accepted organic compound, asparagine, the inorganic constituents, sulphates, phosphates and magnesium must be present. They were so certain of the necessity of these as to state that the production of fluorescence could be used in place of chemical tests as a delicate method for the detection of sulphates, phosphates and magnesium. Their conclusions differed from those of Jordan and Sullivan both of whom reported magnesium unnecessary for fluorescence. They believed that slight impurities in chemicals or even the possible magnesium dissolved from soft glass might have been neglected by these earlier investigators.

Georgia and Poe (24) found peptones used in making nutrient broth media to vary greatly in their composition and suitability for pigment production. Some lacked phosphates and others magnesium. They concluded that peptones should be tested to determine the constituents lacking in order that these might be supplied to the medium.

Throughout this review of literature, fluorescence and pigment have been used synonymously. The former is a physical

characteristic apparently associated with these bacterial pigments yet no reference on methods for determining such could be found. Davidson and Gregory (21) investigating cases of ring-worm found that violet light produced a diagnostic fluorescence about areas infected. This means for displaying fluorescence seemed applicable to pigmented bacterial cultures.

From this resume of the literature it seemed apparent that methods designed to provide for the accurate recognition of the genus Pseudomonas might be based upon the use of the following:

1. Media suitable for consistent pigment production.
   (a) Standard nutrient media for routine tests.
   (b) Synthetic media for research purposes.

2. Fluorescence as a method of demonstrating bacterial pigmentation.
   (a) Colours of fluorescence in pigments as a possible means for differentiating species or strains of Pseudomonas.
   (b) Methods for determining the fluorescent colours of Pseudomonas pigments.
      i Spectroscopic analysis.
      ii The pH of the cultures by electrometric method.
      iii The use of standardised buffer solutions.
   (c) The nature and function of fluorescent bacterial pigments.

(d) Fluorescence of bacterial colonies as a method of recognising Pseudomonas organisms.

Procedure.

1. Media Suitable for Consistent Pigment Production.

(a) Standard nutrient media for routine tests.

In the literature, there was general agreement that sulphate, phosphate and magnesium are necessary for pigmentation. These must be supplied in the medium constituents or be added directly. From the demonstration of the production of pigment by cultures grown in nutrient broth or agar made in tap water but not in the same media in distilled water it was assumed that this natural water contained these required constituents. This assumption is borne out by the statement of Georgia and Poe (23), that the production of fluorescence could be used in place of a chemical test for sulphate, phosphate and magnesium; unless, as also was suggested by these investigators (24) the Bacto peptone was unsuitable for pigmentation. The latter possibility was improbable due to the use of the same peptone with both waters.

In order to prove that the tap water supplied the required inorganic constituents for pigmentation, nutrient broth and agar media were prepared from distilled water to which SO_4, PO_4 and Mg were added in concentrations equal to those in Sullivan's (32) synthetic medium K. The comparative pigment producing effect of two peptones was tested also. The specifications of each medium and results on pigmentation are presented in Table 8. The test cultures included G_2 which was similar to the others except that it was non-pigment forming;

and also two stock cultures (Ps. aeruginosa and Ps. fluorescens) both known to be pigment producers. Inoculations were made from cultures grown in nutrient broth made with distilled water.

Results.

Pigment was not produced in any media made with pure distilled water even with the proteose peptone, reported by Georgia and Poe (24) to be the most favourable for pigmentation. Types of peptone did not affect differences in results. The addition of SO_4, PO_4 and Mg to distilled water media stimulated pigment production but did not equal that in media made in tap water. Equality might have been secured by more perfect concentrations or balance of the added constituents. This was not attempted. Pigment was less pronounced on the nutrient agar slants than in the liquid media. The synthetic medium gave the most definite pigmentation. This is believed by Sullivan and others to be due, primarily, to the asparagine which provides a nitrogen base more adequate for pigmentation than that of peptone.

(b) Synthetic media for research purposes.

The results in Table 8 indicate that the synthetic medium favours the production of pigment. Such a medium provides an accurate and unmistakable means of differentiating between organisms of the genera Pseudomonas and Achromobacter.

For determining colours of fluorescence in bacterial

Table 8.

Comparative stimulation of pigment by waters, inorganic constituents and peptones in broth and agar media.

Specifications of Media (Beef extract and reaction pH 7.2 common to all)	Pigmentation Results Test cultures							
	A_2	B_3	E_1	E_3	D_6	G_2	Ps. aerug	Ps. fluor.
Beeto peptone in tap water	++++	++++	++++	++++	++	-	+++++	++++
Proteose peptone in tap water	+++	+++	+++	+++	+	-	++++	+++
Beeto peptone in dis. water + 0.2% $MgSO_4$ + 0.1% K_2HPO_4	++	++	++	++	+	-	++	+
Proteose peptone in distilled water + 0.2% $MgSO_4$ + 0.1% K_2HPO_4	++	++	++	++	+	-	++	+
Beeto peptone in distilled water	-	-	-	-	-	-	-	-
Proteose peptone in distilled water	-	-	-	-	-	-	-	-
Std. Nutr. agar slants in tap water.	++	++	+	+	+	-	+++	+
Std. nutr. agar slants in dist. water	-	-	-	-	-	-	-	-
Std. nutr. agar slants in dist. water + 0.2% $MgSO_4$ + 0.1% K_2HPO_4	+	+	+ -	+ -	+ -	-	++	+ -
Synthetic medium K (Sullivan's) in distilled water	++++	++++	+++	+++	+++	-	++++	+++

Legend: - no pigmentation
± very slight pigmentation
+ slight pigmentation
++ moderate pigmentation
+++ strong pigmentation
++++ very strong pigmentation

pigments, as presented in the following section, a synthetic medium was necessary in order to eliminate the fluorescence displayed by all nutrient media containing peptone. Media suggested by Tanner (33), Sullivan (32), Georgia and Poe (23) were non-fluorescent and suitable for pigment production. Sullivan's "K" medium was the most favourable for the characterisation of the various cultures by means of their colours of fluorescence. After experimentation this medium was modified as follows:

Sullivan "K" medium		Modified Sullivan "K" medium	
asparagin	1.0%	asparagin	1.0%
$MgSO_4$	0.2%	$MgSO_4$	0.2%
K_2HPO_4	0.1%	KH_2PO_4	0.05%
distilled water		K_2HPO_4	0.05%
Reaction pH 7.2		glycerine	1.0%
		distilled water	
		Reaction pH 7.2	

The addition of glycerine produced more marked differences in the colours of fluorescence, permanency of which required buffering by equal amounts of KH_2PO_4 and K_2HPO_4. This synthetic medium supported excellent fluorescence that remained permanent.

2. Fluorescence as a Method of Demonstrating Bacterial Pigmentation.

Fluorescence is defined by Wood (34) as: "The property, according to Stoke's Law, possessed by some substances

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of giving off radiations of longer wave lengths than those of the light which excites them. The radiations given off are in the yellow, green and blue colours while the exciting light is the violet or ultra violet. If the radiations continue after the exciting light is removed the property is then known as phosphorescence."

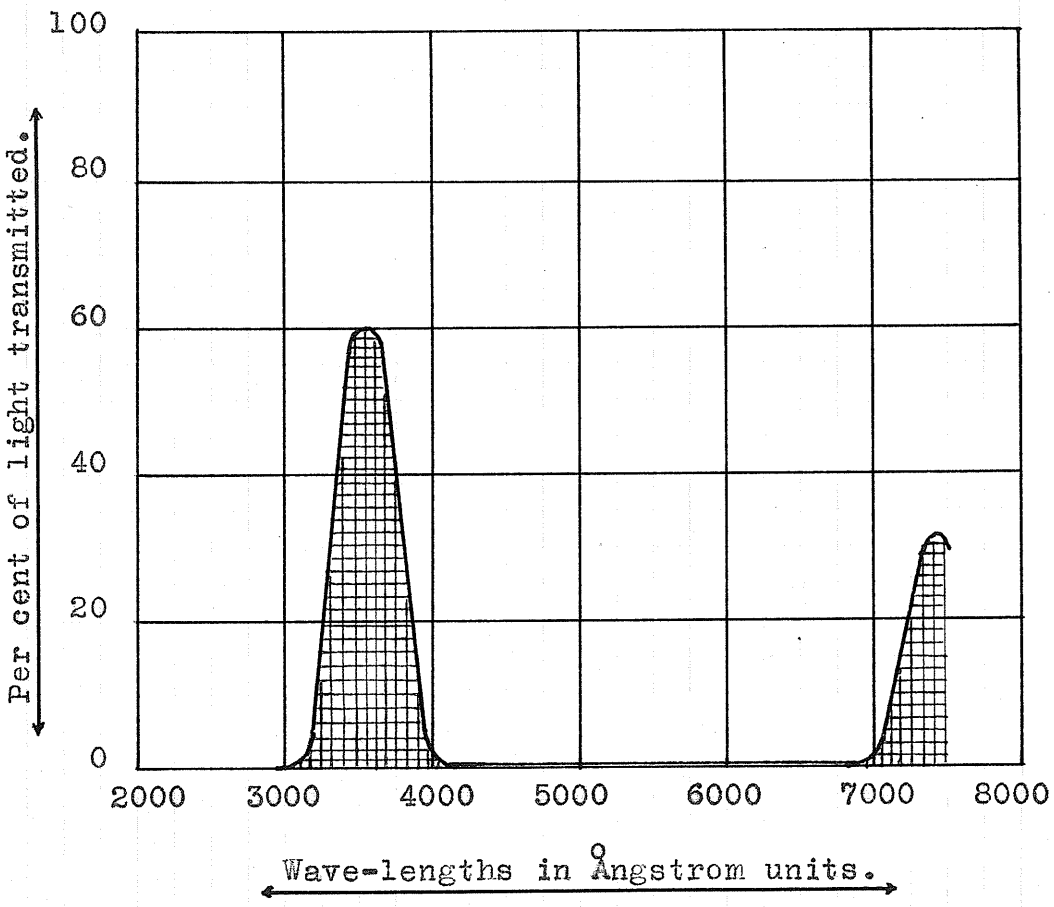
The method of showing fluorescence was suggested by the work of Davidson and Gregory (21). These investigators found the fungi, associated with ring-worm in humans and domestic animals, to cause the hairs about the infected areas to display fluorescence when examined in ultra violet light in a darkened room. Their technique seemed applicable to bacterial pigments.

The exciting light was from a lamp made with a stove pipe. This housed a 400 watt Mazda bulb, the light from which passed through two 4 mm. thicknesses of Corning red purple ultra filters, No. 587. These were obliquely caged on the vertical pipe housing so that the filtered violet light was directed to the cultures in test tubes before the lamp. The intense heat generated by the light bulb necessitated air cooling by a fan. The No. 587 filters were recommended by the Corning Glass Co. for the demonstration of fluorescent effects. As shown by Chart 3. they transmitted ultra violet of a dominant wave length of 3650 Å. and extreme red about 7200 Å.

The cultures grown in some of the media as reported in Table 8 were the first examined for fluorescence under the improvised violet light.

CHART 3.

THE NATURE OF THE LIGHT TRANSMITTED BY TWO "CORNING" RED PURPLE ULTRA FILTERS, NO. 587.



Results.

In daylight, the results were similar to those of Table 8 in that pigment formed only from the cultures in media made from tap water or distilled water plus the required inorganic constituents. The pigments varied in intensity from ones barely visible to those which were pronounced. Their colour was a dull, pale green.

Under the violet light pigmented cultures were bright and sparkling colours of yellow, green, greenish blue, blue and dark blue as shown in Table 9. These colours differed from those in ordinary light and from the exciting violet light. These characters give the bacterial pigments the property of fluorescence. This physical characteristic was unmistakable even in cultures M_3 and D_6 in which pigmentation was scarcely discernible on nutrient agar. All cultures in nutrient media with distilled water and also culture G_2 in all nutrient media were of a dull blue fluorescence (designated "y" in Table 9). In this, they were similar to the nutrient media controls. This is not a pigment fluorescence but due, as reported by Sullivan (32) and Tanner (33), to peptone exhibiting a weak fluorescence. The synthetic media was non-fluorescent, but favourable to the production of pigments which displayed pronounced differences in colours of their fluorescence.

Table 9.

Fluorescence of bacterial pigments.

Media Used. (common reaction pH 7.2)	Fluorescence (colours) Test Cultures								
	Control	A_2	B_3	E_1	E_3	D_6	G_2	Aeruginosa.	Fluor.
Nutr. broth in tap H_2O	?	G	G=	B	B	D	?	YG	DB
Nutr. broth in dist. H_2O	?	?	?	?	?	?	?	?	?
Nutr. broth in dist. H_2O + 0.2% $MgSO_4$ + 0.1% K_2HPO_4	?	G	G	B	B	B	?	G=	B
Nutr. agar slants in tap H_2O	?	G	G	B	B	DB	?	G	DB
Nutr. agar slants in dist. H_2O	?	?	?	?	?	?	?	?	?
Synthetic medium K (Sullivan)	O	G=	G=	BG	GB	B	O	YG=	DB

Legend: G green = strong
B blue ? dull blue
D dark O colourless

(c) Colours of fluorescence in pigments as a possible means for differentiating species or strains of Pseudomonas.

As shown in Table 9, the pigments, produced in Sullivan's medium "k" by different cultures, displayed a variety of colours of fluorescence. By modifying this medium as formulated on page 41, these differences were so accentuated as to provide a possible basic method for demonstrating different species or strains in the 16 cultures of Pseudomonas.

Accordingly all cultures were grown in the modified "K" medium at 25°C. for 15 days, then examined under the U. V. L. (ultra violet light).

Results.

Outstanding differences in the colours of fluorescence were observed, especially, when the cultures were lined closely before the U. V. L. The classes are as follows: yellow green, green, bluish green, greenish blue, blue and dark blue. The results, presented in Table 10 are abbreviated as: YG, G, BG, GB, B and DB. Inconsistencies occurred in the seven tests with the majority of cultures. These persisted even after re-purification of cultures. The average results from the seven tests were used for the classification of cultures presented in Table 11. These results were uned in Section A., Table 5 for the proposed differentiation of species or strains of Pseudomonas studied.

(b) Methods of determining the fluorescent colours of Pseudomonas pigments.

One of the most serious limitations to the use of fluorescent colours of pigments, as a characterisation of bacteria, is their accurate description. The different colours are simple to demonstrate but difficult to describe in a manner that may be understood by all investigators. To formulate some fundamental determination of these colours the following methods were investigated:

Table 10.

Fluorescent colours of Pseudomonas cultures in modified "K" medium.

No.		Tests 1	2	3	4	5	6	7	Average of 7 tests.
A	1	YG	YG	G	YG	YG	YG	G	YG
	2	G	BG	G	YG	G	G	G	G
	3	YG	YG	YG	G	G	YG	YG	YG
B	1	YG	G	G	GB	G	G	G	G
	2	G	BG	G	G	G	G	G	G
	3	YG	YG	G	G	BG	G	G	G
	4	G	BG	G	G	G	G	G	G
C	1	BG	G	G	BG	GB	BG	BG	BG
	2	G	BG	BG	BG	B	GB	BG	BG
	3	G	GB	GB	B	GB	GB	BG	GB
	4	BG	BG	BG	B	BG	BG	BG	BG
	5	BG	BG	GB	GB	BG	BG	BG	BG
D	1	GB	GB	B	B	GB	GB	GB	GB
	2	GB	GB	BG	B	GB	B	GB	GB
	3	GB	GB	B	GB	GB	GB	BG	GB
	4	BG	GB	GB	GB	BG	GB	GB	GB
	5	GB	B	GB	GB	GB	GB	GB	GB
	6	BG	BG	B	BG	BG	BG	BG	BG
	7	BG	GB	GB	B	GB	GB	GB	GB
E	1	B	B	GB	DB	B	B	B	B
	2	DB	DB	B	DB	DB	DB	DB	DB
	3	B	B	B	DB	DB	B	B	B
	4	B	B	GB	B	B	B	B	B
F	1	DB	DB	B	DB	B	DB	DB	DB
	2	DB	DB	B	B	B	DB	DB	DB
	3	DB	DB	B	DB	DB	DB	DB	DB
aerug		YG	YG	YG	G	G	YG	YG	YG
fluor		-	-	-	DB	DB	DB	DB	DB

i Spectroscopic analysis.

ii The pH of the cultures by electrometric method.

iii The use of standardized buffer solutions.

Comparison of these methods is based upon the percentage of cultures similarly classified by each method as correlated to the original classification by visualised colours.

Table 11.

Classification of Pseudomonas cultures by their visualized colours of fluorescence.

Classes of Fluorescent Colours.					
YG	G	BG	GB	B	DB
A_1	A_2	C_1	C_3	E_1	E_2
A_3	B_1	C_2	D_1	E_3	F_1
Ps. aerug.	B_2	C_4	D_2	E_4	F_2
	B_3	C_5	D_3		F_3
	B_4		D_4		Ps. fluor.
			D_5		
			D_6		
			D_7		

i. Spectroscopic analysis.

The U. V. L. was directed through a 10 cm. column of centrifuged culture and into a single prism spectroscope (Baertner). The mean spectroscopic scale reading of the transmitted light band was used to denote the colour of fluorescence produced by each culture. The readings from the arbitrary scale

were converted to approximately Angstrom units by the use of a curve established as a result of the spectroscopic examination of potassium, lithium, sodium, caesium and rubidium as shown in Chart 4.

Results.

By the established wave-lengths of transmitted light, the cultures were classified as presented in Table 12. Comparison between this and the classification by visualized colours of fluorescence in Table 11. shows that 19 out of 28 or 67.9% of the cultures were similarly classified by either method. It was difficult to decide as to which was the more accurate. That by spectroscopic analysis was fundamentally sound except for the following possibilities of error:

(a) Filtration changed the colour of fluorescence. Consequently, cultures were centrifuged and the determinations made on the supernatant liquid. The effect of bacterial cells on the spectroscopic analysis was not investigated.

(b) The Gaertner spectroscope had no accurate provision for comparing and measuring the transmitted light-band.

(c) The method of converting the arbitrary scale readings to Ångstrom units lacked precision.

(d) The U. V. L. was not strong enough to transmit a sharply demarcated light-band.

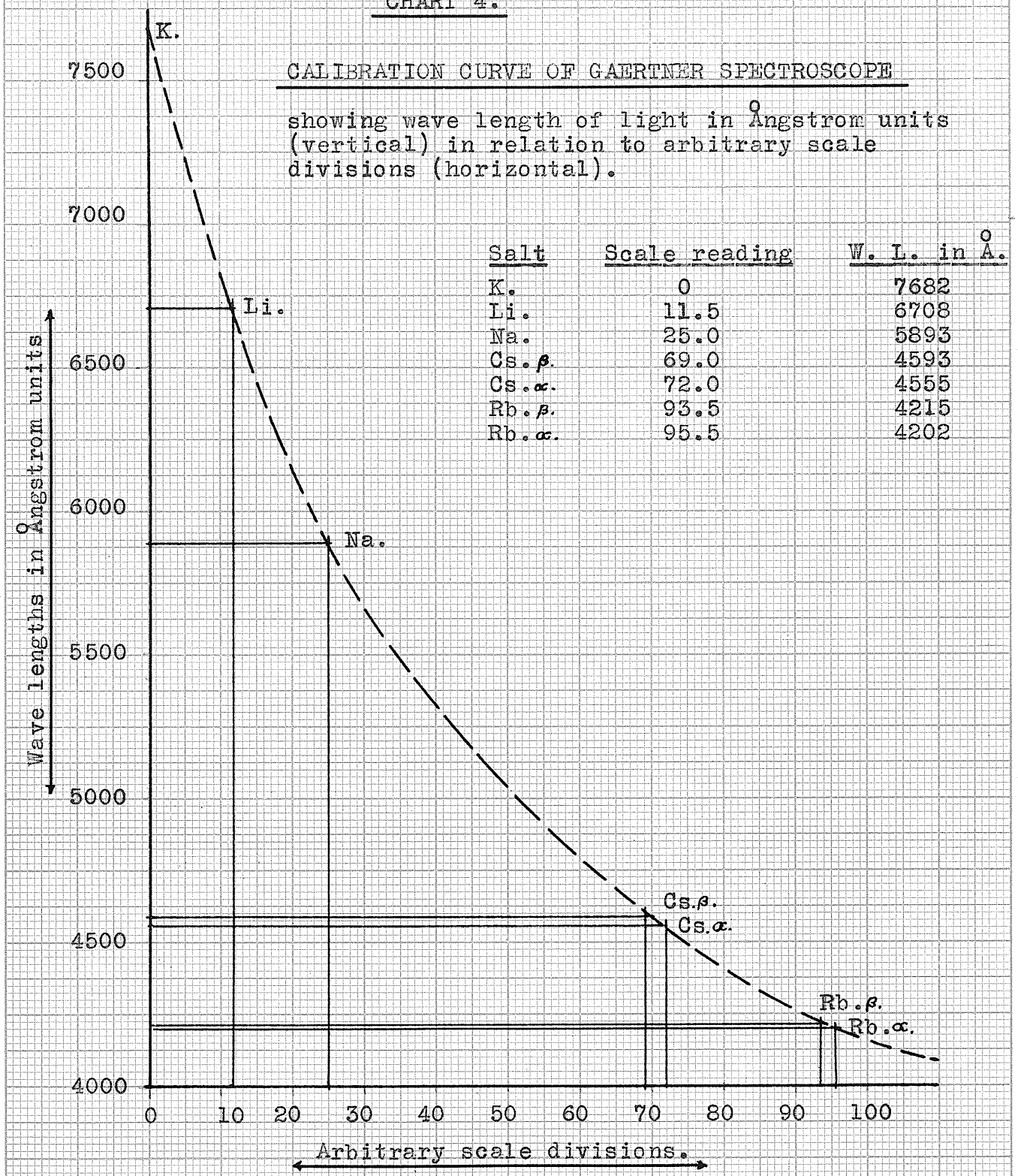
(e) A quartz system of lenses and prisms in the spectroscope and light condenser is essential.

CHART 4.

CALIBRATION CURVE OF GAERTNER SPECTROSCOPE

showing wave length of light in Ångstrom units (vertical) in relation to arbitrary scale divisions (horizontal).

Salt	Scale reading	W. L. in Å.
K.	0	7682
Li.	11.5	6708
Na.	25.0	5893
Cs. β.	69.0	4593
Cs. α.	72.0	4555
Rb. β.	93.5	4215
Rb. α.	95.5	4202



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Table 12.

Classification of Pseudomonas cultures by spectroscope analysis.

Wave-length classes by arbitrary scale.							
48-49	50-51	52-53	54-55	56-57	58-59	60-61	115-116
Wave-length classes in Angstrom units.							
5080	5040	4975	4940	4900	4840	4800	4100
xA_1	xB_1	B_3	C_1	D_3	E_4	xF_1	medium control
A_2	xB_2	xC_4	C_2	xE_1		Ps. $x_{fluor.}$	
xA_3	xB_4	xC_5	xC_3	E_2			
Ps. $x_{aerug.}$			xD_1	xE_3			
			xD_2	F_2			
			xD_4	F_3			
			xD_5				
			xD_6				
			xD_7				

x--similarly classed by visualised fluorescent colour.

Table 13.

Classification of the Pseudomonas cultures by their pH with the quinhydrone electrode.

Classes of pH							
8.80 to 8.80	8.79 to 8.70	8.69 to 8.60	8.59 to 8.50	8.49 to 8.40	8.39 to 8.30	8.20 to 8.20	7.20 to 7.10
xA_1	xA_2	B_3	C_1	xE_1		xF_2	medium control
xA_3	xB_1	C_3	C_2	xE_3		E_4	
Ps. $aerug.$	xB_2	xC_4	C_5	F_2		xF_1	
	xB_4	D_4	xD_1	F_3		Ps. $x_{fluor.}$	
		D_6	xD_2				
		D_7	xD_3				
			xD_5				

x--similarly classed by visualised fluorescent colour.

Despite these inaccuracies, the results demonstrate that the pigmented cultures fluoresce to produce different colours.

ii. The pH of the cultures by electrometric method.

Sullivan (32) states that once a pigment is formed, it is rendered colourless by acid or more vivid by alkali, and that this colouration is regained or lost by the opposite reaction. From this it was apparent that the various colours of fluorescence in the pigmented cultures might be due to the reaction produced by the cultures, the determination of which might provide a method of identifying these colours.

Accordingly, nine electrometric pH tests were made on each centrifuged solution using a quinhydrone electrode.

Results.

The average results from these nine tests are presented in Table 13. By comparison with Table 11, it may be noted that only a fair correlation is indicated between reaction of the culture and colour of fluorescence. A more positive relation no doubt could have been secured by the use of a glass or hydrogen electrode as the quinhydrone electrode is inaccurate above pH 8.0 to 8.2.

iii. The use of standardised buffer solutions.

Assuming that the bacterial pigments acted as "indicators," it seemed possible to ascertain the existing reaction

in any pigmented culture by making each pigment display its optimum reaction for similar fluorescent colour production, by the use of a series of buffered solutions of known pH.

The pigment was water-soluble and permitted a dilution of five times with distilled water without any noticeable change in its fluorescent colour. This dilution, when made with a buffered solution differing from the culture reaction, effected marked changes in the fluorescent colour of the pigment. Therefore, a dilution of one to five (1 ml. of culture to 5 ml. of distilled water or buffer solution of known pH) was made in small vials and used in all comparisons. Clark and Lubs' stock buffered solutions, Group V as per Hawk and Bergeim (27) were prepared by the Chemistry Department of the University of Manitoba. These ranged from pH 7.8 to pH 10.0 in intervals of 0.4.

Colour comparisons were made by matching the fluorescent colour of each centrifuged culture, diluted 1 to 5 with water, through a series of 6 tubes each likewise diluted with buffered solutions having pH's of 8.0, 8.4, 8.8, 9.2, 9.6 and 10.0. The pH of the buffered tube with which the control culture most closely agreed in colour, under the U. V. l., was assumed to be the reaction of the pigment of the respective culture.

Results.

The detailed results are presented in Table 14 and

summarized in the form of a culture classification in Table 15.

The great variance between these pH results and those by electrometric determination no doubt was due to the unsuitability of the quinhydrone electrode for reactions above pH 8.2. At pH 8.2 fair agreement resulted between the two methods. Fundamentally, the electrometric results were questionable and those from the buffer solution method reasonably accurate. It is hoped this can be verified at some future date by means of reliable electrometric methods.

The results in Table 15 indicate a reaction of pH 9.2 common to both of the fluorescent colours visually classed as greenish blue and blue. These differences were comparative only. When these two classes were united and the resulting classification compared with that of Table 15, it is found that 25 out of 28 cultures or 89.3% agree in class placings. This undoubtedly indicates a positive correlation between reaction and colour of fluorescence. This suggests that the variety of colours noted in the fluorescence of pigments from different cultures was caused by the variation in alkalinity produced by the cultures.

(c) The nature and function of fluorescent bacterial pigments.

The information on fluorescence of bacterial pigments may be summarized briefly under two headings.

Table 14.

Reaction of the Pseudomonas cultures determined by the pH of the buffered solution producing the original colour.

Culture No.	Colour at various pH's						
	Control	8.0	8.4	8.8	9.2	9.6	10.0
A_1	YG	DB	B	B	GB	BG	G ->
A_2	G	DB	B	B	GB	BG	G
A_3	YG	DB	B	B	GB	BG	G ->
B_1	G	DB	B	B	GB	BG	G
B_2	G	DB	B	B	GB	BG	G
B_3	G	DB	B	B	GB	BG	G
B_4	G	DB	B	B	GB	BG	G
C_1	BG	DB	B	B	GB	BG	G
C_2	BG	DB	B	B	GB	BG	G
C_3	GB	DB	DB	B	B	GB	BG
C_4	BG	DB	DB	B	GB	BG	G
C_5	BG	DB	B	B	GB	BG	G
D_1	GB	DB	B	B	GB	BG	G
D_2	GB	DB	B	B	GB	BG	G
D_3	GB	DB	DB	B	B	GB	BG
D_4	GB	DB	B	B	GB	BG	G
D_5	GB	DB	B	B	GB	BG	G
D_6	BG	DB	DB	B	GB	BG	G
D_7	GB	DB	B	B	GB	BG	G
E_1	B	DB	DB	B	B	GB	BG
E_2	DB	DB	B	B	B	GB	GB
E_3	B	DB	DB	B	B	GB	BG
E_4	B	DB	DB	B	B	GB	BG
F_1	DB	DB	DB	B	B	GB	BG
F_2	DB	DB	DB	B	B	GB	BG
F_3	DB	DB	DB	B	B	GB	BG
aerug.	YG	DB	B	B	GB	BG	G ->
fluor.	DB	DB	B	B	B	GB	GB

Underline (_) denotes agreement with control.
Arrow (->) denotes colour still not matched.

Table 18.

Classification of the Pseudomonas cultures by their pH with buffered solutions.

Classes of pH by buffer solution.				
Over 10	10.0	9.6	9.2	8.4 to 8.2
x A_1	x A_2	x C_1	x D_1	x E_2
x A_3	x B_1	x C_2	x D_2	x F_1
x Ps. aerug.	x B_2	C_3	x D_4	x F_2
	x B_3	x C_4	x D_6	x F_3
	x B_4	x C_5	x D_7	x Ps. fluor.
		D_3	x E_1	
		D_6	x E_3	
			x E_4	
Corresponding classes of fluorescent colours.				
YG	G	BG	GB & B	DB

x Similarly classed by fluorescent colours.

A. Nature

1. Requirements for optimum production.

a. Medium constituents--a nitrogen source together with SO_4, PO_4 and Mg. River and tap water provide these salts in sufficient quantities. Distilled water does not.

b. Medium reaction--slightly alkaline; pH 7.2.

c. Incubation temperature--25°C. to 0°C; none at 37°C.

d. Oxygen--requires free oxygen, i. e.; aerobic.

2. Solubility.

a. In water--readily soluble.

b. In chloroform--not soluble. This was common to all of the fluorescent pigments involved in the study and also in Ps. fluorescens but not to that from Ps. aerugi-

nosa. The latter produces a pigment, pyocyanin, which is soluble in chloroform but is not fluorescent.

c. In alcohol and ether--not soluble. This is not in accord with the findings of Sullivan (32) who reported the fluorescent pigments to be soluble in these reagents but pyocyanin not soluble.

3. Diffusibility--rapid in liquid media; slower in liquefiable-solid media.

4. Precipitation by salts of heavy metals and ammonium sulphate--the fluorescent pigments, from the cultures studied, were not precipitated. Pyocyanin from Ps. aeruginosa was precipitated.

5. The influence of light on pigment production--the less light the better at the time of pigment formation after which even sun-light seemed to effect no noticeable changes.

6. The colours diffused into the medium in or on which the culture is grown.

a. In daylight--colourless to faint blue or green.

b. In U. V. L.--sparkling dark blue to yellow green and fluorescent.

7. The effect of reaction on the colours of fluorescence:

a. Acid--changes greens to blues and if added in excess renders them colourless and non-fluorescent.

b. Alkali--changes blues to greens and yellow greens; if added in excess renders them colourless, non-fluorescent and followed by general precipitation.

3._ The cause of the various colours noted in the fluorescence of pigments--the formation of different amounts of alkali by the respective cultures. This seemed to be ammonia which was found in higher concentrations in the yellow green and green cultures than in those of blue pigment.

2._ The number of pigments found in each culture.

a. In the cultures of the study and also Ps. fluorescens only one pigment was found. This pigment was common to all cultures including Ps. aeruginosa. It was non-chloroform soluble and was fluorescent in colours varying from blue to yellow green as influenced by the reaction of the culture.

b. In Ps. aeruginosa two pigments were formed. One was chloroform soluble, non-fluorescent, blue in colour but changing to a brownish black on oxidation. This was pyocyanin. The second was non-chloroform soluble, blue green in fluorescence and similar to that from the series of cultures.

These findings are in accord with those of Boland as reported by Tanner (33), Gessard, as reviewed by Sullivan (32), and of Sullivan. These authorities agreed that the maximum number of water-soluble pigments produced by bac-

teria was two, one being fluorescent and the other non-fluorescent.

10. The chemical nature of the pigments.

A consideration of the literature failed to reveal definite information pertaining to the chemistry of the fluorescent pigment. Sullivan (32), in reporting on "The Biochemistry of Bacterial Pigments," states that they react like albuminous bodies in that they are precipitated by salts of heavy metals; give positive Xanthoproteic and Adamkiewicz reactions for albumins and strong reactions with Millon's reagent, thus showing the presence of either tyrosine or an (OH) derivative of benzol.

Positive results were obtained for all of these tests, except that of Millon's reaction, with pigment from cultures of Ps. aeruginosa and negative with all the fluorescent pigments from the series of cultures. From this it appears that Sullivan referred only to the non-fluorescent pyocyanin pigment.

B. Function of fluorescent bacterial pigments.

Various opinions on the function of pigments have been expressed. Jordon (28) states that the fluorescent property of diffusible bacterial pigments may be of no benefit to the cell.

Tanner (33) reports Wassersug's conclusion that the coloured bacterial pigment is merely a waste product resulting

from cellular metabolism. Sullivan (32) concludes that, "The production of pigment is not an essential vital act, as it is of no discoverable advantage to the organism possessing the power of producing it. Its production is purely accidental."

Morgan (29) states that the pigment appearing in ordinary media from cultures of Ps. fluorescens is of little importance in itself except that it draws attention to the presence of a highly undesirable type of organism.

Corbach (26) in studying the proteolytic phenomenon of various strains of B. pyocyaneus (Ps. aeruginosa) concludes that, "Pigment formation goes parallel with that of the proteolytic enzyme. Optimum conditions for enzyme production are also those for pigmentation." Whether this is true also for Ps. fluorescens or the pigmented cultures of this study was not proven but the supposition is supported by the fact that these cultures were active proteolysers.

Shutt (31) states that a distinct greenish-yellow discolouration of the medium surrounding the colonies on a plate identifies the members of the "fluorescens" group of bacteria.

From the foregoing it may be noted that the function of pigments is considered under the following two categories:

(a) to the producing cells. Here the pigment is of no advantage.

(b) to the bacteriologist. The fluorescent pigment provides a means of recognising an undesirable type or group of organisms especially in association with dairy products; butter in particular.

(d) Fluorescence of bacterial colonies as a method of recognizing Pseudomonas organisms.

When the cultures, classified as Pseudomonas, were streaked on agar slants the cultural growth displayed fluorescence in the U. V. L. one to two days in advance of any pigment formation. Similarly, colonies from pure culture platings were easily recognized under the U. V. L. as members of the Pseudomonas genus by their fluorescence. Some of these colonies did not show pigmentation in daylight even after 5 to 15 days of incubation and accordingly would not have been recognized as Pseudomonas.

The use of the U. V. L. for recognizing bacteria of this genus, on routine quantitative platings from butter or water for creamery use, seems to offer valuable possibilities since these organisms generally are considered detrimental in butter.

In this regard Gilruth (25), Meklee (22) and Shutt (30) have reported Ps. fluorescens to be one of the most likely causative agents of Surface Taint in butter. This organism commonly gains access to butter from contaminated water supplies.

Morgan (29) studied four pigment producing organisms, isolated from the water supplies of dairy factories, and concluded that the Ps. fluorescens type produces a marked green pigment in the medium, and is particularly dangerous in butter.

Both Shutt and Morgan claimed that organisms of the Ps. fluorescens group are easily identified by means of the

green to greenish yellow pigment diffused through the medium about the colonies, after four days of incubation at 25°C. The U. V. L. method for detecting organisms of the Pseudomonas genus appears more accurate and rapid.

The method, as first investigated presented limitations for either qualitative or quantitative determinations from plates because only surface colonies display fluorescence. This is due to pigment, the causative agency in fluorescence, requiring free oxygen for its metabolism; consequently only surface colonies are fluorescent. No doubt numerous sub-surface colonies require only the same aerobic condition to show similar characteristics.

In endeavouring to prove this deduction the following simple yet useful technique was developed. This, in a large measure, overcomes the inaccuracies caused by non-fluorescent, sub-surface colonies and provides greater applicability of the U. V. L. method for quantitative determinations of Pseudomonas organisms from routine platings.

Modified U. V. L. Technique.

After one to two day's incubation of the plates at 25°C., all possible sub-surface colonies are given access to free oxygen by aseptically stabbing each with a fine, sterile inoculating needle. In general, only plates with a maximum of 150 colonies permit of this without interfering with those already developed on the surface. This enforced aeration, fol-

lowed by another day of incubation, results in accurate determinations of the number of fluorescent colonies, when observed under the U. V. L.

Results.

The results secured with this technique, from ten creamery water samples, are presented in Table 16. This data indicates the following:

(1) Bacterial colonies must have air or free oxygen to elaborate the fluorescent pigment.

(2) Unless some means of admitting air to sub-surface colonies is provided, accurate determination of Pseudomonas organisms can not be made directly from platings.

(3) The U. V. L. method for spotting Pseudomonas types of colonies seems more accurate and rapid than the usual method which relies upon the observation of pigment diffused into the surrounding medium.

Assuming Pseudomonas types to be detrimental to butter, this technique makes possible more efficient interpretation of routine plate counts from creamery products.

Table 16.

Increases in fluorescent colonies from water due to admitting air to sub-surface colonies.

Sample No.	Dilution Counted.	Colony counts per plate.				Increase due to stabbing.
		Total	Pigmented in Daylight.	Fluorescent under U. V. L.		
				Before stab.	After stab.	
19	.1 ml.	11	0	2	5	2.5
20	.1 ml.	65	3	14	27	2.1
21	.1 ml.	14	0	3	7	2.3
22	.001 ml.	128	5	32	75	2.3
25	.001 ml.	130	2	9	17	1.9
27	.001 ml.	47	1	2	6	3.0
31	.001 ml.	52	4	7	19	2.7
35	.1 ml.	129	3	8	25	3.1
36.	.1 ml.	160	0	14	37	2.6
12A	.1 ml.	72	4	7	22	3.1
						Average 2.56 x

DISCUSSION - SECTION B.

The delicate requirements for the production of characteristic Pseudomonas pigments are believed generally unappreciated. In consequence of this, errors no doubt result in determinative bacteriology. This possibility is deemed of sufficient importance to justify the inclusion of specific information on media for pigment production in the, "Manual of Methods for Pure Culture Study," by the Society of American Bacteriologists.

Colours of fluorescence in bacterial pigments present a possible added key to the differentiation of Pseudomonas species or their variants. With equipment, more efficient than that used, possible valuable results might be obtained from this method.

The results indicate that the variety of fluorescent colours is due to the indicator action of the pigment produced by the culture. If the final reaction or pH of a pigmented culture can be considered a sound characterization, the resulting colour of fluorescence may be used to indicate the reaction.

The fluorescence produced by colonies of Pseudomonas organisms, grown on a medium suitable for pigment production, offers a valuable and practical method of recognising these bacteria quickly and easily. This can be done directly from platings of any suspected material in a much shorter time than that usually required for diffusion of the characteristic pigment. Recognition of sub-surface fluorescent colonies is rendered

possible by puncturing the agar above such colonies and thereby allowing access of air for part of the incubation period.

SUMMARY

1. Five hundred and thirty-one samples, representing commercial and exhibition butter, were plated according to recognized procedures for fungi and bacteria. Incubation was at 25°C. except for a duplicate set for bacterial counts which was held at 0°C. for 30 days.

2. The samples were placed in classes on the basis of counts obtained by each procedure.

3. The classes for bacteria at 0°C. agreed more closely with those for fungi at 25°C. than for bacteria at 25°C.

4. One thousand two hundred and twenty-nine colonies were picked from plates incubated at 0°C. for a study of the types growing at this temperature. Peptonizers were found to predominate. 180 peptonizing cultures were investigated in greater detail and these finally were reduced to 30 representatives. Of these, 26 proved to belong to the genus Pseudomonas, 3 to Achromobacter and 1 to the genus Micrococcus. The 26 type cultures of Pseudomonas were divided into 5 probable species and the Achromobacter into two.

5. Butter was made from sterile cream, inoculated with representatives from each species of the three genera and stored for 30 days at 0°C. The scores on the butters inoculated with Pseudomonas in all cases were lower than those with Achromobacter or Micrococcus cultures.

6. Nutrient media, made in distilled water, was found inadequate for the production of pigment by cultures of Pseudomonas. The same media, prepared in tap water, supported pigmentation

7. The requirements for pigment production were investigated. These proved to be SO_4, PO_4 and Mg. Tap water supplied these constituents.

8. The pigment of Pseudomonas was most easily recognized by its character of fluorescence as displayed in an ultra violet light (U. V. L.).

9. The variety of colours of fluorescence, exhibited by cultures of Pseudomonas, offered a possible method for the differentation of closely related species or variants.

10. The degree of alkalinity produced was shown to be the agency causing the differences noted in the fluorescent colours of the pigment.

11. The nature and function of bacterial pigments was reviewed. The pigment appears to be of no use to the producing cells. To the bacteriologist it furnishes a valuable aid in identifying objectionable organisms in butter.

12. The ultra violet light examination of platings from creamery products provides a rapid, efficient method for recognising the predominant bacterial type isolated at 0°C. from butter.

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APPENDIX

	Page
Data for Table 1.	73 - 100
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CREAMERY NO. 1				CLASSIFICATIONS OF CULTURES ISOLATED AT 0°C.														
	Counts per ml.			By Litmus Milk Tube					By Colours of Peptonized Litmus Milk									
No.	M. & Y.	Bact. 25°C.	Bact. 0°C.	T.	Pept	Acid	Alk.	In-ert	T.	A	B	C	D	E	F	G	H	I
25	0	1M	10	1				1										
74	0	1M	30	3	1	1		1										
118	0	30	0															
204	0	12M	0															
971	0	10	0															
X 1893	0	30	0															
254	1	1M	0															
255	0	3M	0															
257	0	1M	0															
424	0	60	0															
425	0	60	0															
426	0	8M	0															
1000	0	0	0															
1001	0	10	0															
1525	0	0	0															
1526	0	0	0															
1527	0	0	0															
	Note: All data above space "X" refers to special butters; below space refers to commercial butter.																	
17	--- TOTALS ---			4	1	1		2										

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CREAMERY NO. 2				CLASSIFICATIONS OF CULTURES ISOLATED AT 0°C.														
	Counts per ml.			By Litmus Milk Tube					By Colours of Peptonized Litmus Milk									
No.	M. & Y.	Bact. 25°C.	Bact. 0°C.	T.	Pept	Acid	Alk.	In-ert	T.	A	B	C	D	E	F	G	H	I
5	1	30	0															
66	0	20	10	3	3				2			2-1						
127	0	0	0															
289	0	2M	0															
976	0	10	0															
1860	2	0	0															
1900	0	0	0															
216	0	2M	0															
219	0	4M	0															
235	0	4M	10	1				1										
236	0	2M	0															
391	0	30	0															
419	0	30	0															
420	0	20	10															
421	0	20	10															
992	0	0	0															
993	0	20	10	1			1											
994	0	0	0															
995	5	0	0															
1562	0	-	0															
1563	0	1M	10	1			1											
1564	0	0	0						Note: Number following dash (-) designates the sub-class.									
1565	0	0	0															
23	--- TOTALS ---			6	3	0	2	1	2			2-1						

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CREAMERY NO. 3				CLASSIFICATIONS OF CULTURES ISOLATED AT 0°C.														
	Counts per ml.			By Litmus Milk Tube					By Colours of Peptonized Litmus Milk									
No.	M. & Y.	Bact. 25°C.	Bact. 0°C.	T.	Pept	Acid	Alk.	In-ert	T.	A	B	C	D	E	F	G	H	I
6	9	60	0															
44	38	1H	30	3	2		1		1		1-3							
126	21	0	0															
269	20	4H	0															
976	41	0	0															
1670	0	0	0															
1905	16	0	0															
245	0	1H	-															
246	0	4H	-															
247	1	23H	-															
253	0	28H	-															
1360	0	3H	0															
1361	2	0	0															
1366	7	30	0															
1367	1	30	0															
1562	0	0	10	1			1											
1563	9	1H	0															
1564	4	0	10	1			1											
1565	2	0	0															
19	--- TOTALS ---			5	2	0	3	0	1		1-3							

CREAMERY NO. 4				CLASSIFICATIONS OF CULTURES ISOLATED AT 0°C.														
	Counts per ml.			By Litmus Milk Tube					By Colours of Peptonized Litmus Milk									
No.	M. & Y.	Bact. 25°C.	Bact. 0°C.	T.	Pept	Acid	Alk.	In-ert	T.	A	B	C	D	E	F	G	H	I
18	2	13H	0															
92	0	16H	0															
119	0	"	0															
320	4	"	0															
979	14	58H	0															
1861	90	1H	0															
1902	23	1H	0															
222	26	88H	0															
223	56	97H	20	2		2												
440	5H	124T	60	3		3												
441	4H	114T	0															
1112	0	2H	0															
1113	0	"	0															
1574	0	"	0															
1575	0	"	0															
15	--- TOTALS ---			5	0	5	0	0										

CREAMERY NO. 5				CLASSIFICATIONS OF CULTURES ISOLATED AT 0°C.														
	Counts per ml.			By Litmus Milk Tube					By Colours of Peptonized Litmus Milk									
No.	M. & Y.	Bact. 25°C.	Bact. 0°C.	T.	Pept	Acid	Alk.	In-ert	T.	A	B	C	D	E	F	G	H	I
8	30	12T	1H	5	2	2	1											
40	11	15T	6T	10	3		7											
124	70	34H	0															
295	33	75T	80	2				2										
974	1	15H	0															
1675	7	8H	0															
1904	10	10H	0															
242	36	136T	-															
243	10	237T	-	3			3											
244	15	56H	-															
332	21	13T	40	2			1	1										
399	90	32T	0															
432	11	51T	0															
1009	4	36H	10															
1110	24	19H	10	1				1										
1111	16	11H	10	1			1											
1571	0	11H	0															
1572	0	4H	0															
1573	25	5H	20	3			3											
19	--- TOTALS ---			27	5	2	16	4										

CREAMERY NO. 6				CLASSIFICATIONS OF CULTURES ISOLATED AT 0°C.														
	Counts per ml.			By Litmus Milk Tube					By Colours of Peptonized Litmus Milk									
No.	M. & Y.	Bact. 25°C.	Bact. 0°C.	T.	Pept	Acid	Alk.	In-ert	T.	A	B	C	D	E	F	G	H	I
27	4	19H	11H	5			5											
76	6	13H	6H	5			1	4										
121	17	11H	0															
265	19	37H	4H	7				7										
972	1	13T	0															
1669	0	24H	0															
1697	38	16T	20															
342	70	52H	0															
343	8	19H	10	1				1										
344	6	15H	20	1				1										
345	76	12H	40	3	2			1										
692	40	10H	50															
693	9	9H	0															
694	7	10T	18H															
695	4	0	0															
1348	0	0	0															
1349	9	0	40															
1361	16	2H	10															
1362	2	0	0	4	3			1										
1834	21	3H	4H	6				6										
1835	9	1H	0															
1836	2	17H	0															
1837	40	2H	0															
23	--- TOTALS ---			32	5	0	6	21										

CREAMERY NO. 7				CLASSIFICATIONS OF CULTURES ISOLATED AT 0°C.														
	Counts per ml.			By Litmus Milk Tube					By Colours of Peptonized Litmus Milk									
No.	M. & Y.	Bact. 25°C.	Bact. 0°C.	T.	Pept	Acid	Alk.	In-ert	T.	A	B	C	D	E	F	G	H	I
9	8	13H	10	1			1											
56	1	65H	7H	5			5											
128	2	0	0															
291	10	57H	30	3	1	1	1		1									1-1
985	0	0	0															
1863	3	0	0															
1885	7	0	0															
147	-	13H	0															
148	-	33H	0															
149	-	45H	0															
150	-	19H	10	1				1										
404	14	62H	10															
405	0	11H	0															
406	43	41H	1H	4				4										
407	2H	37T	25H	5				5										
1415	0	20H	0															
1416	1	0	0															
1417	1	30H	0															
1419	0	9H	0															
1471	3	2H	0															
1472	0	27T	0															
1473	7	0	0															
1474	0	2H	0															
23	--- TOTALS ---			19	1	1	7	10	1									1-1

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CREAMERY NO. 8				CLASSIFICATIONS OF CULTURES ISOLATED AT 0°C.															
	Counts per ml.			By Litmus Milk Tube					By Colours of Peptonized Litmus Milk										
No.	M. & Y.	Bact. 25°C.	Bact. 0°C.	T.	Pept	Acid	Alk.	In-ert	T.	A	B	C	D	E	F	G	H	I
21	1	34H	70	4	1	1	2		1					1-3				
84	3	7H	20	7	7				4					2-2 1-4				
109	80	0	0															
287	7	87H	2H	4	1	1	1	1	1		1-2							
980	11	13H	1H	6		6												
1878	4	5H	30															
1896	18	24H	-															
260	0	6H	20	2				2										
261	0	8H	2H	5	3	1		1	1								1-1	
262	1	35H	26H	9	1			8	1					1-2				
263	1	11H	4H	5	1			4	1									1-1
687	2H	1H	0															
689	50	74H	0															
690	2H	1H	10															
691	2	1H	0															
1374	2H	10H	1H	1	1				1									1-1
1375	19	3H	10	1				1										
1376	6	3H	20	2			1	1										
1377	29	2H	10	1				1										
1597	48	21H	0															
1598	21	7H	10	2			1	1										
1599	4	1H	0															
1600	4	0	0															
23	--- TOTALS ---			49	15	9	5	20	10		1-2			4-2 1-3 1-4			1-1	2-1

CREAMERY NO. 9				CLASSIFICATIONS OF CULTURES ISOLATED AT 0°C.														
	Counts per ml.			By Litmus Milk Tube					By Colours of Peptonized Litmus Milk									
No.	M. & Y.	Bact. 25°C.	Bact. 0°C.	T.	Pept	Acid	Alk.	In-ert	T.	A	B	C	D	E	F	G	H	I
26	12	5H	0	3	1		2		1									1-1
77	75	12H	0															
293	7H	24T	30H	11	11				2									2-1
989	69	54H	30H	4				4										
226	3H	63H	5H	4				4										
227	1H	19H	20	2				2										
228	40	12T	0															
229	36	122T	30H															
437	6H	43T	60															
438	8H	15T	0															
439	2H	29T	0															
557	20H	53T	80	4				4										
1362	6H	17T	10															
1363	16H	40T	2H	3			3											
1364	5H	51T	14H	7				7										
1365	2H	77H	10	1				1										
1578	40	7H	0															
1579	47	65H	4H	4	3			1										
1580	4H	29T	3H	7	4			3	1									1-1
1581	36	63H	2H	6			6											
20	--- TOTALS ---			56	19	0	11	26	4									4-1

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CREAMERY NO. 10				CLASSIFICATIONS OF CULTURES ISOLATED AT 0°C.														
	Counts per ml.			By Litmus Milk Tube					By Colours of Peptonized Litmus Milk									
No.	M. & Y.	Bact. 25°C.	Bact. 0°C.	T.	Pept	Acid	Alk.	In-ert	T.	A	B	C	D	E	F	G	H	I
23	55	68H	20	5			5											
102	28	85H	0															
139	1	0	0															
321	25	22H	0															
984	0	0	0															
1872	10	8H	0															
1894	18	7H	0															
220	0	64H	0															
221	86	16T	0															
240	81	59H	0															
241	2H	250T	30H	3	3				3							2-1	1-1	
394	1	14H	10	1			1											
396	4	24T	20	2				2										
397	9	20T	10	1			1											
398	0	19H	20	1				1										
1005	7	42H	0															
1006	10	14H	0															
1007	8	30H	0															
1008	9	35H	0															
1601	10	6H	0															
1602	11	350T	26H	5			4	1										
1603	25	17H	0															
1604	12	56H	15H	7			7											
23	--- TOTALS ---			25	3	0	18	4	3							2-1	1-1	

CREAMERY NO. 11				CLASSIFICATIONS OF CULTURES ISOLATED AT 0°C.														
No.	Counts per ml.			By Litmus Milk Tube					By Colours of Peptonized Litmus Milk									
	M. & Y.	Bact. 25°C.	Bact. 0°C.	T.	Pept	Acid	Alk.	In-ert	T.	A	B	C	D	E	F	G	H	I
29	7	11T	44H	5				5										
62	26	10H	70	7	1			6										
100	3	22H	0															
262	0	26H	0															
868	0	1H	3H															
1864	8	2H	0															
1892	0	2H	10															
133	2	51H	80	4				4										
134	9	16T	19H	4				4										
139	0	33T	15H															
411	35	90H	1															
414	8	31T	0															
416	7	16H	-															
1403	3	6H	0															
1404	37	25T	2H	5				5										
1405	97	29T	1															
1483	7H	26T	1H	4			2	2										
1484	1	23H	19H	7			7											
1486	0	0	0															
19	--- TOTALS ---			36	1	0	9	26										

CREAMERY NO. 12				CLASSIFICATIONS OF CULTURES ISOLATED AT 0°C.														
	Counts per ml.			By Litmus Milk Tube					By Colours of Peptonized Litmus Milk									
No.	M. & Y.	Bact. 25°C.	Bact. 0°C.	T.	Pept	Acid	Alk.	In-ert	T.	A	B	C	D	E	F	G	H	I
16	7	60	0															
65	0	1H	0															
117	0	2H	0															
283	0	10H	1H	5				5										
973	0	1H	0															
1862	2	0	0															
1890	2	5H	0															
213	34	34H	0															
214	13	14H	0															
215	52	21H	0															
680	16	74T	19H	4	1	1	1	1	1	1-1								
681	4H	98H	6H	2		2												
682	2	78H	0															
1229	62	330T	36H	5	4		1		2				2-7					
1330	75	33H	20	2				2										
1331	38	63H	10	1				1										
1847	72	11T	0															
1848	16	11T	14H	4				4										
1849	19	9H	0															
19	--- TOTALS ---			23	5	3	2	13	3	1-1			2-7					

CREAMERY NO. 13				CLASSIFICATIONS OF CULTURES ISOLATED AT 0°C.														
	Counts per ml.			By Litmus Milk Tube					By Colours of Peptonized Litmus Milk									
No.	M. & Y.	Bact. 25°C.	Bact. 0°C.	T.	Pept	Acid	Alk.	In-ert	T.	A	B	C	D	E	F	G	H	I
4	1	13T	11T	11		10		1										
87	0	42M	2M	5	1			4	1		1-4							
116	4	33T	13M	6				6										
290	8	49M	30	3			2	1										
983	1	11M	0															
1665	5	3M	1M	3			2	1										
1899	8	70M	30M	6	1		3	2	1		1-4							
157	84	40M	2M	7	1	5	1		1									1-1
158	2M	30T	30M	1	1													
159	36	28M	2M	7	2		4	1										
365	4	13T	48M	6	6													
366	2	33M	0															
367	2	10M	10															
1498	8	2M	0															
1499	8	0	0															
1500	27	0	0															
1550	4	0	0															
1551	19	0	0															
1552	45	14M	0															
19	--- TOTALS ---			55	12	15	12	16	3		2-4							1-1

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CREAMERY NO. 14				CLASSIFICATIONS OF CULTURES ISOLATED AT 0°C.														
	Counts per ml.			By Litmus Milk Tube					By Colours of Peptonized Litmus Milk									
No.	M. & Y.	Bact. 25°C.	Bact. 0°C.	T.	Pept	Acid	Alk.	In-ert	T.	A	B	C	D	E	F	G	H	I
17	0	3H	0	2		2												
31	0	-	0	3	2		1											
364	0	7H	70	3	3				1				1-2					
302	9	13T	0															
303	27	12T	6H	6	2			4										
304	9	31T	12H	8	8				2				2-2					
577	2H	61T	51H	4	4				4				3-4 1-5					
578	2H	30T	20															
579	48	40H	0															
1491	18	0	19H	7	7				2				2-1					
1492	22	0	1H	6	4		2		1				1-1					
1493	12	0	60	3			3											
1556	3	1H	0															
1557	62	0	0															
1559	8	2H	0															
15	--- TOTALS ---			42	30	2	6	4	10				3-1 3-2 3-4 1-5					

CREAMERY NO. 15				CLASSIFICATIONS OF CULTURES ISOLATED AT 0°C.															
	Counts per ml.			By Litmus Milk Tube					By Colours of Peptonized Litmus Milk										
No.	M. & Y.	Bact. 25°C.	Bact. 0°C.	T.	Pept	Acid	Alk.	In-ert	T.	A	B	C	D	E	F	G	H	I
12	16	9H	0															
99	12	9H	0															
112	18	96H	0															
301	0	13H	0															
978	23	26T	2H	6	3		1	2	3		3-2							
1873	11	114T	-															
1898	21	13H	0															
182	13H	17H	37H	4	3		1		3		1-2		2-2					
183	4	46T	0															
184	0	19T	0															
185	9	84H	10															
751	3H	31T	22H	8	4		2	2										
753	19	30H	0															
754	14H	11T	3H	4				4										
755	18	78H	0															
1389	2H	28T	2H	3	3				1						1-1			
1391	90	17H	10	1			1											
1392	39	12T	3H	3	2		1		1		1-2							
1393	4H	16T	60	4				4										
1589	2H	9H	3H	2			1	1										
1590	72	2H	30	1			1											
1591	2H	33H	56H	10	5		4	1	5		5-2							
1593		1H	0															
23	--- TOTALS ---			46	20	0	12	14	13		10-2		2-2		1-1			

CREAMERY NO. 16				CLASSIFICATIONS OF CULTURES ISOLATED AT 0°C.														
	Counts per ml.			By Litmus Milk Tube					By Colours of Peptonized Litmus Milk									
No.	M. & Y.	Bact. 25°C.	Bact. 0°C.	T.	Pept	Acid	Alk.	In-ert	T.	A	B	C	D	E	F	G	H	I
22	30	6H	7Q	3			3											
58	59	9H	4H	5	2		1	2	2		2-3							
125	0	0	0															
286	0	23H	0															
987	0	0	0															
1079	4	46H	0															
1891	8	24H	0															
237	2	19H	0															
238	0	14T	0															
239	2	22H	0															
589	3H	98T	10															
592	6H	35T	50H	5	3		2		3								3-1	
595	3H	56T	40	1		1												
996	22	140T	-															
997	6H	54T	33H	4	4				4		3-3					1-2		
998	63	40H	4H	4				4										
999	6	0	0															
1566	69H	81T	30H	2		1	1											
1567	80	15H	1H	4	4													
1568	39	13H	6H	8	6		2		2							2-1		
20	--- TOTALS ---			36	19	2	9	6	11		5-3					2-1 1-2	3-1	

CREAMERY NO. 17				CLASSIFICATIONS OF CULTURES ISOLATED AT 0°C.														
	Counts per ml.			By Litmus Milk Tube					By Colours of Peptonized Litmus Milk									
No.	M. & Y.	Bact. 25°C.	Bact. 0°C.	T.	Pept	Acid	Alk.	In-ert	T.	A	B	C	D	E	F	G	H	I
15	0	72H	2H	4		1	3											
63	0	5H	1H	5	2	1	2		2									2-1
111	3H	62T	2H	2	1		1		1						1-3			
297	20	43H	16H	9	4	1	4		1				1-7					
869	5	52T	54H	10	5	5			1							1-2		
1874	6H	57T	16H	4	2	1	1		2	2-1								
1901	70	180T	72H	6	6				3					3-1				
191	52	25T	45H	6	3		3		1				1-3					
192	22	22T	40H	4	2		2		1				1-7					
193	10	90T	30H	1	1													
704	4	-	-															
705	1H	40T	9H	6	6				2	1-1							1-1	
706	3	36T	3H	5	3	1		1	1						1-1			
1357	88	11T	1H															
1358	25	7H	2H	4	4				2								2-1	
1359	3	0	0															
1510	5	9H	50	3	3				2						2-1			
1511	1	15H	10															
1512	2	0	0															
19	--- TOTALS ---			69	42	10	16	1	19	3-1			1-3 2-7	3-1	3-1 1-3	1-2	3-1	2-1

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CREAMERY NO. 18				CLASSIFICATIONS OF CULTURES ISOLATED AT 0°C.														
	Counts per ml.			By Litmus Milk Tube					By Colours of Peptonized Litmus Milk									
No.	M. & Y.	Bact. 25°C.	Bact. 0°C.	T.	Pept	Acid	Alk.	In-ert	T.	A	B	C	D	E	F	G	H	I
24	2	197T	18T	10			10											
75	13	29T	13H	2	2				1	1-3								
279	3	124T	70H	8	5		3		1	1-3								
200	1	68T	2H	7	2		5		2				2-3					
201	3	17T	4H	9	9				4				4-1					
202	31	36T	40H	7	4	2	1		3			3-3						
714	82	32H	10															
715	83	18H	0															
716	7H	23T	12H	-														
1336	8H	2H	6H	5				5										
1337	3H	1H	6H	7		3	3	1										
1338	4H	430T	4H	5	3			2	1				1-6					
1451	4H	240T	30H	8				8										
1452	2H	19H	20H	7				7										
1453	10T	200T	30H	6	4		2		3	3-3								
15	--- TOTALS ---			81	29	5	24	23	15	5-3		3-3	4-1 2-3 1-6					

CREAMERY NO. 19				CLASSIFICATIONS OF CULTURES ISOLATED AT 0°C.															
No.	Counts per ml.			By Litmus Milk Tube					By Colours of Peptonized Litmus Milk										
	M. & Y.	Bact. 25°C.	Bact. 0°C.	T.	Pept	Acid	Alk.	In-ert	T.	A	B	C	D	E	F	G	H	I
10	19	14H	10	4	3		1		2		2-4								
93	27	6H	10	4		4													
300	2	17H	0																
1116	0	0	0																
1883	2	1H	0																
1887	1	12T	2H	5	3			2	3			3-2							
264	27	12T	71H	11	11														
265	14	31H	2H	6		1	1	4											
266	10	52H	14H																
267	12	49H	6H	6		1	1	4											
696	6	34H	0																
697	3H	67H	-	4				4											
698	2H	35T	12H	5				5											
699	17	22H	0																
1428	4	7H	90	3				3											
1429	11	31H	65H	8	5		3		1									1-1	
1430	0	67H	17H	7			2	5											
1431	0	2H	6H	6	3			3	1			1-1							
1540	64	84H	20	3			2	1											
1541	22	9H	0																
1542	2H	53T	80	3				3											
1543	0	27H	60	3			1	2											
22	--- TOTALS ---			78	25	6	11	36	7		2-4	1-1 3-2						1-1	

CREAMERY NO. 20				CLASSIFICATIONS OF CULTURES ISOLATED AT 0°C.														
No.	Counts per ml.			By Litmus Milk Tube					By Colours of Peptonized Litmus Milk									
	M. & Y.	Bact. 25°C.	Bact. 0°C.	T.	Pept	Acid	Alk.	In-ert	T.	A	B	C	D	E	F	G	H	I
19	19	156T	30H	8	5	1	2		4		4-1							
48	21	35T	30H	6	3		1	2	2		2-1							
292	6	9H	1H	4	2	1		1	1				1-6					
348	2H	324T	30H	6		4		2										
349	2	31T	0															
352	0	60H	20															
756	6	6H	7H	5			3	2										
1425	3H	-	16H	6	3	1	2		1		1-1							
1426	10	16T	30	1				1										
1537	2H	79T	41H	8	4	1	2	1	2		2-1							
1539	3H	90H	4H	5			5											
11	--- TOTALS ---			49	17	8	15	9	10		9-1		1-6					

3268 83.

CREAMERY NO. 21				CLASSIFICATIONS OF CULTURES ISOLATED AT 0°C.														
	Counts per ml.			By Litmus Milk Tube					By Colours of Peptonized Litmus Milk									
No.	M. & Y.	Bact. 25°C.	Bact. 0°C.	T.	Pept	Acid	Alk.	In-ert	T.	A	B	C	D	E	F	G	H	I
13	3	1H	10	5	1		4											
88	1	4H	1H	3		2		1										
123	6	62H	0															
296	7	7H	10	1				1										
982	13	44H	13H	7	5	2			1				1-3					
1867	2H	12T	0															
1889	5H	184T	3H	5	5				1	1-2								
224	0	52H	-															
225	5	13H	-															
418	22	-	-															
462	0	15T	30H	6	6				4				1-7			3-2		
463	22	258T	30H	4	1		3		1				1-7					
464	5	66T	30H	3	3													
1114	16	12T	50	3				3										
1115	0	2H	10	1				1										
1384	7	37T	0															
1566	2H	26H	2H	4			1	3										
1567	14H	41T	35H															
1568	0	95H	30	5			5											
19	--- TOTALS ---			47	21	4	13	9	7	1-2			1-3 2-7			3-2		

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CREAMERY NO. 22				CLASSIFICATIONS OF CULTURES ISOLATED AT 0°C.														
	Counts per ml.			By Litmus Milk Tube					By Colours of Peptonized Litmus Milk									
No.	M. & Y.	Bact. 25°C.	Bact. 0°C.	T.	Pept	Acid	Alk.	In-ert	T.	A	B	C	D	E	F	G	H	I
14	7	82H	6H	7			7											
57	1	69T	6H	6			6											
211	10	43H	3H	6			6											
212	4	249T	30H	6	6				2			2-S						
210	2	82H	17H	10	6		4		1			1-S						
379	49	21T	14H	4			2	2										
380	0	40T	30	3		3												
381	0	30T	3H	6		6												
1394	0	-	-															
1395	0	-	-															
1427	5	116T	-															
1850	7H	53T	30H	4			4											
1851	2H	91T	30H	3			2	1										
1852	2H	0	30	2			1	1										
14	--- TOTALS ---			57	12	9	32	4	3			3-S						

CREAMERY NO. 23				CLASSIFICATIONS OF CULTURES ISOLATED AT 0°C.															
No.	Counts per ml.			By Litmus Milk Tube					By Colours of Peptonized Litmus Milk										
	M. & Y.	Bact. 25°C.	Bact. 0°C.	T.	Pept	Acid	Alk.	In-ert	T.	A	B	C	D	E	F	G	H	I
2	19	7H	3H	4			4												
41	24	24T	30H	6		4	2												
115	0	0	0																
268	1	14H	20	2				2											
981	0	0	0																
1677	0	1H	0																
1868	0	3H	2H	2			1	1											
135	30	143T	30H	4	4				1									1-1	
136	1	36H	1H	3				3											
137	26H	84H	71H	5				5											
138	29H	12T	94H	5				5											
410	3	22H	0																
412	53	46H	2H	5				5											
415	43	170T	35H	3		3													
417	25	150T	35H	3		3													
1406	11	16T	20	2				2											
1407	2	98H	0																
1408	15	4H	0																
1409	9H	10T	90	2			2												
1481	1	0	0																
1486	1	11H	0																
1487	20	24T	10	1				1											
1488	8	9H	6H	5	5				1									1-1	
23	--- TOTALS ---			62	9	10	9	24	2									2-1	

CREAMERY NO. 24				CLASSIFICATIONS OF CULTURES ISOLATED AT 0°C.															
	Counts per ml.			By Litmus Milk Tube					By Colours of Peptonized Litmus Milk										
No.	M. & Y.	Bact. 25°C.	Bact. 0°C.	T.	Pept	Acid	Alk.	In-ert	T.	A	B	C	D	E	F	G	H	I
3	9	39H	0																
43	55	16H	10H	8	5		3		2			1-5						1-1	
113	5	1H	0																
322	1H	2H	20	2	1			1	1						1-1				
970	2H	74H	40H	10	6	4			2		1-4	1-4							
1866	10H	18H	0																
1886	7	3H	0																
385	43	6H	0																
387	10	16H	0																
388	6	14H	0																
465	5H	12T	6H	4	2	1		1											
466	2H	26T	15T	4	4				3	3-1									
1003	7H	296T	3H	5				5											
1004	4H	55T	10	4				4											
1569	2H	20H	60	2	2														
1570	0	10T	0																
1886	7	3H	0																
1866	10H	18H	0																
18	--- TOTALS ---			39	20	5	3	11	8	3-1	1-4	1-4 1-5			1-1			1-1	

CREAMERY NO. 26				CLASSIFICATIONS OF CULTURES ISOLATED AT 0°C.															
	Counts per ml.			By Litmus Milk Tube					By Colours of Peptonized Litmus Milk										
No.	M. & Y.	Bact. 25°C.	Bact. 0°C.	T.	Pept	Acid	Alk.	In-ert	T.	A	B	C	D	E	F	G	H	I
30	69	67H	6H	7			7											
61	48	53H	8H	3				3										
323	2H	93H	4H	4				4										
249	34	17T	7H	7		3	2	2										
250	49	28T	0															
269	2H	19T	0															
333	35H	64T	60	3			3											
334	7H	67T	23H	5	2		3		1		1-2							
1002		72H	20	2		2												
9	--- TOTALS ---			31	2	5	15	9	1		1-2							

CREAMERY NO. 26				CLASSIFICATIONS OF CULTURES ISOLATED AT 0°C.															
	Counts per ml.			By Litmus Milk Tube					By Colours of Peptonized Litmus Milk										
No.	M. & Y.	Bact. 25°C.	Bact. 0°C.	T.	Pept	Acid	Alk.	In-ert	T.	A	B	C	D	E	F	G	H	I
7	15T	30T	35H	15	14		1		12		12-4							
82	15T	35T	35H	6	5		3		3		3-4							
114	6H	16H	2H	2			1	1										
280	12H	46H	20H	6				6										
988	5H	30H	68H	4				4										
1671	60H	155T	-															
1884	11H	167T	15H	2	2				1						1-2			
160	18H	83T	35H	3	3				2						2-1			
161	10H	12T	57H	9	2	3	2	2	1						1-1			
162	4H	31T	35H	8	3		1	4										
362	27H	34T	57H	6				6										
363	42H	13T	8H	5				5										
364	36H	13T	25H	4				4										
1501	9H	33T	6H	5			5											
1502	12H	33T	7H	5				5										
1503	17H	76T	7H	5				5										
1553	48H	50T	20H	4	4													
1554	10H	300T	20H															
1555	40H	435T	3H	2	2				1						1-3			
19	--- TOTALS ---			95	35	3	13	44	20		15-4				3-1 1-2 1-3			

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CREAMERY NO. 27				CLASSIFICATIONS OF CULTURES ISOLATED AT 0°C.														
	Counts per ml.			By Litmus Milk Tube					By Colours of Peptonized Litmus Milk									
No.	M. & Y.	Bact. 25°C.	Bact. 0°C.	T.	Pept	Acid	Alk.	In-ert	T.	A	B	C	D	E	F	G	H	I
20	2M	35M	30	2	2				1						1-2			
46	14	25M	20	2		1	1											
120	2M	69M	10															
281	2M	31T	7M	7	7				3				3-6					
966	18	32T	11M	9				9										
1903	17	32T	30	4	2		2		2		2-1							
1859	3	3M	2M															
316	9M	45T	63M	9	1		4	4										
316	37M	110T	54M	7	1		1	5	1		1-1							
317	2M	356T	28M	10	9	1			1	1-1								
316	19T	35T	30M	1		1												
467	2M	-	-															
468	19M	346T	30M	4	4													
469	15M	35T	30M	1	1				1		1-1							
570	3M	35T	30M															
718	12T	22T	50M	8			8											
719	13T	210T	86M	6			6											
740	15T	-	35M	4			4											
741	96	10M	0															
1465	91M	350T	21M	6	6													
1466	6M	22T	-															
1467	4M	185T	-															
1468	2M	24T	30M	5	5										-			
23	--- TOTALS ---			85	38	3	26	18	9	1-1	4-1		3-6		1-2			

CREAMERY NO. 28				CLASSIFICATIONS OF CULTURES ISOLATED AT 0°C.														
	Counts per ml.			By Litmus Milk Tube					By Colours of Peptonized Litmus Milk									
No.	M. & Y.	Bact. 25°C.	Bact. 0°C.	T.	Pept	Acid	Alk.	In-ert	T.	A	B	C	D	E	F	G	H	I
38	67	59H	2H	6				6										
78	10	78H	70	4	1		3		1		1-2							
296	26	30T	6H	7	4		2	1	2		2-2							
272	2	397T	12T	9	3			6	3		3-2							
273	1	400T	25T	6				6										
274	6	140T	25T	5		2		3										
275	2	127T	25T	4		2	1	1										
296	26	20T	6H															
445	28	100T	25T	4	3	1			1		1-2							
446	1H	202T	25T	6	3	1	2		3		3-2							
447	49	137T	25T	6		4	2											
449	2H	416T	25T	6	6				3		3-2							
1332	6	365T	25T															
1333	16	90H	24T	5				5										
1334	44	75H	25T	6	4			2	2		2-2							
1335	26	13T	0															
1517	11H	70T	25T															
1518	19H	93T	34T	6	6				2						2-2			
1519	32H	490T	25T															
1520	49H	780T	-															
19	--- TOTALS ---			80	30	10	10	30	17		15-2				2-2			

SUMMARY OF CLASSIFICATIONS OF CULTURES ISOLATED AT 0°C.

Cry. No.	By Litmus Milk Tube					By Colours of Peptonized Litmus Milk.									
	T.	Kept.	Acid	Alk.	Int.	T.	A	B	C	D	E	F	G	H	I
1	4	1	1		2	0									
2	6	3		2	1	2			2-1						
3	5	2		3		1		1-3							
4.	5		5			0									
5	27	5	2	16	4	0									
6	32	5		6	21	0									
7	19	1	1	7	10	1									1-1
8	49	15	9	5	20	10		1-2			4-2 1-3 1-4			1-1	2-1
9	56	19		11	26	4									4-1
10	25	3		18	4	3							2-1	1-1	
11	36	1		9	26	0									
12	23	5	3	2	13	3	1-1			2-7					
13	66	12	15	12	16	3		2-4							1-1
14	42	30	2	6	4	10				3-1 3-2 3-4 1-5					
15	46	20		12	14	13		10-2		2-2		1-1			
16	36	19	2	9	6	11		6-3					2-1 1-2	3-1	
17	69	42	10	16	1	19	3-1			1-3 2-7	3-1	3-1 1-3	1-1	3-1	2-1
18	81	29	5	24	23	15	5-3		3-5	4-1 2-3 1-6					
19	73	25	6	11	36	7		2-4	1-1 3-2					1-1	
20	49	17	8	15	9	10		9-1		1-6					
21	47	21	4	13	9	7	1-2			1-3 2-7			3-2		
22	57	12	9	32	4	3			3-5						
23	52	9	10	9	24	2								2-1	
24	39	20	5	3	11	6	3-1	1-4	1-4 1-5			1-1			1-1
25	31	2	5	15	9	1		1-2							
26	95	35	3	13	44	20		15-4				1-2 1-3 3-1			
27	85	38	3	26	18	9	1-1	4-1		3-6		1-2			
28	80	30	10	10	30	18		16-2				2-2			
TOTALS	929	421	112	303	369	180	6-1 1-2 5-3	15-1 26-2 6-3 20-4	5-1 5-2 3-3 1-4 4-5	7-1 6-2 4-3 3-4 1-5 5-6 6-7	3-1 4-2 1-3 1-4	6-1 4-2 2-3	5-1 4-2	11-1	11-1
							14	67	14	31	9	14	9	11	11

Key to the Sub-Division of Pentoni-ed Milk Colour Classes.

		No. of cultures
Class A: Horu Olive:		**14.**
a. Agar slant:	filiform.	14.
b. " " :	smooth.	
c. Broth:	cloudy.	9.
d. Broth surface:	no pellicle.	
e. " sediment:	viscous.	8.
f. Gel. liquefaction:	infundi.	
ff. " " :	moderate.	
h. " " :	cloudy	8. sub-class: 1.
ee. Broth sediment :	not viscous.	1.
f. Gel. liquefaction:	infundi.	
ff. " " :	moderate.	
h. " " :	cloudy.	1. sub-class: 2.
ee. Broth:	clear.	5.
dd. " surface:	pellicle.	
ee. " sediment:	not viscous.	
f. Gel. liquefaction:	infundi.	
ff. " " :	moderate.	
h. " " :	cloudy.	5. sub-class: 3.
Class B: Yellow Olive:		**67.**
a. Agar slant:	filiform.	46.
b. " " :	smooth.	
c. Broth:	cloudy.	40.

		No. of cultures
d. Broth surface:	no pellicle.	
e. " sediment:	viscous.	
f. Gel. liquefaction:	infundi.	40.
g. " " :	slow.	13.
h. " " :	cloudy.	13. sub-class: 1.
ggg. Gel. liquefaction:	rapid.	27.
h. " " :	cloudy.	27. sub-class: 2.
cc. Broth:	clear.	6.
dd. " surface:	pellicle.	
ee. " sediment:	not viscous.	
f. Gel. liquefaction:	infundi.	
gg. " " :	moderate.	
hh. " " :	clear.	6. sub-class: 3.
aa. Agar slant:	echinulate.	21.
bb. " " :	rough.	
c. Broth:	cloudy.	
d. " surface:	no pellicle.	
e. " sediment:	viscous.	
f. Gel. liquefaction:	infundi.	
ggg. " " :	rapid.	
h. " " :	cloudy.	21. sub-class: 4.

Class C: Mermonette Green: 14.

a. Agar slant:	filiform.	14.

		No. of cultures
b. Agar slant:	smooth.	10
c. Broth:	cloudy.	
d. " surface:	pellicle.	
e. " sediment:	viscous.	9
f. Gel. liquefaction:	infundi.	
g. " " :	slow.	3.
h. " " :	cloudy.	3. sub-class: 1.
gg. Gel. liquefaction:	moderate.	3.
h. " " :	cloudy.	3. sub-class: 2.
ggg. Gel. liquefaction:	rapid.	3.
h. " " :	cloudy.	3. sub-class: 3.
ee. Broth sediment:	not viscous.	1.
f. Gel. liquefaction:	infundi.	
gg. " " :	moderate.	
h. " " :	cloudy.	1. sub-class: 4.
bb. Agar slant:	rough.	4.
c. Broth:	cloudy.	
d. " surface:	no pellicle.	
f. Gel. liquefaction:	infundi.	
ggg. " " :	rapid.	
h. " " :	cloudy.	4. sub-class: 5.

Class D: Jade Green: 31.

a. Agar slant:	filiform.	31.

		No. of cultures
b. Agar slant:	smooth.	16.
c. Broth:	cloudy.	
d. " surface:	no pellicle.	16.
e. " sediment:	viscous.	12.
ff. Gel. liquefaction:	infundi to stratiform.	12.
g. Gel. liquefaction:	slow.	12.
h. " " :	cloudy.	7. sub-class: 1.
hh. Gel. liquefaction:	clear.	5. sub-class: 2.
ee. Broth sediment:	not viscous.	4.
ff. Gel. liquefaction:	strati.	
g. " " :	slow.	
h. " " :	clear.	4. sub-class: 3.
bb. Agar slant:	rough.	15.
c. Broth:	cloudy.	
d. " surface:	no pellicle.	
e. " sediment:	viscous.	
ff. Gel. liquefaction:	infundi to stratiform.	15.
g. Gel. liquefaction:	slow.	4.
h. " "	cloudy.	3. sub-class: 4.
hh. Gel. liquefaction:	clear.	1. sub-class: 5.
gg. Gel. liquefaction:	moderate.	11.

		No. of cultures
h. Gel. liquefaction:	cloudy.	5. sub-class: 6.
Class E: Cress Green:		9.
a. Agar slant:	filiform.	7.
b. " " :	smooth.	3.
cc. Broth:	clear.	
dd. " surface:	pellicle.	
e. " sediment:	viscous.	
ff. Gel. liquefaction:	stratiform.	
gg. " " :	moderate.	
hh. " " :	clear.	3. sub-class: 1.
bb. Agar slant:	rough.	4.
c. Broth:	cloudy.	
d. " surface:	no pellicle.	
e. " sediment:	viscous.	
ff. Gel. liquefaction:	stratiform.	
ggg. " " :	rapid.	
h. " " :	cloudy.	4. sub-class: 2.
aaa. Agar slant:	spreading.	2.
b. " " :	smooth.	
cc. Broth:	clear.	
dd. " surface:	pellicle.	
ee. " sediment:	not viscous.	
ff. Gel. liquefaction:	stratiform.	
g. " "	slow.	

		No. of cultures.
h. Gel. liquefaction:	cloudy.	1. sub-class: 3.
hh. Gel. liquefaction:	clear.	1. sub-class: 4.
Class F: Slate Olive:		**14.**
a. Agar slant:	filiform.	6.
b. " " :	smooth.	
c. Broth:	cloudy.	
d. " surface:	no pellicle.	
ee. " sediment:	not viscous.	
ff. Gel. liquefaction:	stratiform.	
g. " " :	slow.	
h. " " :	cloudy.	8. sub-class: 1.
aa. Agar slant:	echinulate.	6.
b. " " :	smooth.	
c. Broth:	cloudy.	
d. " surface:	no pellicle.	
e. " sediment:	viscous.	4.
ff. Gel. liquefaction:	stratiform.	
g. " " :	slow.	
hh. " " :	clear.	4. sub-class: 2.
ee. Broth sediment:	not viscous.	2.
ff. Gel. liquefaction:	stratiform.	
g. " " :	slow.	
hh. " " :	clear.	2. sub-class: 3.

		No. of cultures
Class G: Dusky Slate Blue:		**9.**
aaa. Agar slant:	spreading.	9.
bb. " " :	rough.	
c. Broth:	cloudy.	4.
dd. " surface:	pellicle.	
e. " sediment:	viscous.	
fff. Gel. liquefaction:	crateriform.	
g. " " :	slow.	
h. " " :	cloudy.	**4. sub-class: 1.**
cc. Broth:	clear.	5.
dd. " surface:	pellicle.	
e. " sediment:	viscous.	
fff. Gel. liquefaction:	crateriform.	
g. " " :	slow.	
h. " " :	cloudy.	**5. sub-class: 2.**
Class H: Brownish Drab:		**11.**
aa. Agar slant:	echinulate.	11.
bb. " " :	rough.	
c. Broth:	cloudy.	
dd. Broth surface:	pellicle.	
ee. " sediment:	not viscous.	
ffff. Gel. liquefaction:	napiform.	
g. " " :	slow.	
h. " " :	cloudy.	**11. Class: 1.**

		No. of cultures
Class I. Snuff Brown:		11.
a. Agar slant:	filiform.	
bb. " " :	smooth.	
cc. Broth:	clear.	
dd. " surface:	pellicle.	
ee. " sediment:	not viscous.	
fff. Gel. liquefaction:	crateriform.	
g. " "	slow.	
h. " "	cloudy.	11. Class: 1.