

A CRITIQUE ON THE VIOLABILITY OF FACTORIA.

A CRITIQUE ON THE FILTRABILITY OF TYPHUS
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Thesis

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A Critique on the Filtrability of Bacteria

Introduction

In the commercial world a proposal must have an immediate inherent material value, whereas in pure science any scientific fact irrespective of its practical value is important. Frequently observations that apparently have no material worth, later become essential stepping stones to objectives of positive economic importance. The proof of the existence of filtrable stages in bacterial life cycles may be the basis of important economic discoveries in all phases of bacteriology.

In 1923 Löhnis and Smith presented a rather intricate life cycle for the *Azotobacter* which included a filtrable stage. They postulated the formation within the bacterial cells of gonidia of such size that they would pass through a Berkefeld filter candle. More recently Handuroy, Hadley, and Kendall have presented data on other organisms that appear to validate the findings of Löhnis and Smith. Further observations are necessary both as to proof of the existence of filtrable forms and of their relation to the non-filtrable bacteria, as the data at the present are extremely fragmentary and highly unorganized as to continuity.

Historical

Bacteriologists are divided into two schools of thought concerning the existence of life cycles in bacteria. The first consists of the followers of pleomorphism, the ability of the bacterial cell to present many varied features of structure and size; the second composed of those who believe in monomorphism, the fixity of the bacterial cell in only one form.

Häggell (55) in 1877 presented the view that bacteria are extremely pleomorphic. He believed that all bacteria belonged to a single type of fission fungi, highly variable, greatly adaptable morphologically, fermentatively, and biochemically. On the other hand, Cohn (11) in 1875 presented the theory of monomorphism with a classification of fission fungi based on the above characteristics. Koch agreed with Cohn and was able to present enough proof to swing the pendulum of thought definitely to this view. Koch's teachings have dominated the trend of bacteriology until recently. During this period manifestations of dissociation were attributed to contamination, involution forms, and even to poor technique.

About 1911 the tide changed and from this time the consensus of opinion has gradually turned towards the opposite viewpoint. Jones (33) 1929 summarized his former paper of 1913. He noticed that some *Azotobacter* possessed a complex life cycle. One observation was that the cells of these organisms formed two types of

granules, a stainable and a non-stainable. The former were apparently reproductive bodies or gonidia. When the mother cell disintegrated these granules were liberated, whereupon they grew into normal cells, reproduced indefinitely by binary fission, then each cell again formed gonidia and disintegrated thus completing the cycle. However, he stated that they were not filtrable and made no mention of the size of the colonies produced by the gonidial forms.

Löhnis and Smith in a preliminary note (40) 1916 showed that the life history of the *Azotobacter* and other bacteria was very complex. In a later communication (41) 1923 they pointed out that the *Azotobacter* possessed seven distinct cell types. They reported that all types of bacterial reproductive organs were found in these organisms, gonidia and gonidangia, zygo-spores, arthro-spores, microcysts, endospores and exospores. They stated that when the nuclear material was accompanied by scant amounts of other cell elements, gonidia were formed. They also stated that these gonidia were in part filtrable or had ultramicroscopic particles associated with them which were capable of reproducing *Azotobacter* colonies after filtration. This paper is a classic on the dissociation of bacteria and is the basis of practically all work on dissociative phenomena.

Ludd and Barron (51) in 1923 reported the isolation of a readily cultivable vibrio. They attributed the filtrability of this organism in part at least to its

motility.

Handuroy (26) in 1927 presented a technic for the cultivation and production of visible organisms from the filtrates of filtrable forms of bacteria. This process consisted essentially in placing one or two cc. of the filtrate on a lactose gelatin plate, incubating the seeded plate for 24 hours, washing the surface of the medium with sterile broth, and transferring the washing to a sterile lactose gelatin plate. This was repeated five or six times until fine, pin-point colonies appeared. He stated that at this stage it was possible to grow the filtrable forms in broth. This was the only method by which he was able to grow these forms.

Hadley et al. (22) in 1931 were perhaps the first to generate artificially and subsequently to cultivate in pure culture the filtrable virus-like state of any bacterial species. This filtrable or G form was produced during dissociative changes by rapid transfer and aging. The G forms were grown as pure cultures; their filtrability was demonstrated and by means of special technique, the rapid serial plate transfer of Handuroy, they were recovered from the filtrate. The different reactions of the G forms were found to be different from the normal R or S type, but after suitable manipulation they reverted to the normal form. G forms for other organisms were also demonstrated.

Kendall (26) in 1931 reported a culture medium which was lacking in protein digestion products. This medium

was capable of changing ordinary bacteria to primitive forms which were able to pass filters. These filtrable organisms were uncultivable in ordinary media but grew well in K medium.

Varney and Bronfenbrenner (66) in 1952 extended the observations of Mendell and demonstrated that the cause of the filtrability might be attributed to the suspended material present in K medium and adsorbed by the filter. They noted that the organisms in the filtrate were equally cultivable in K or other media.

Experimental

(A)

Attempts to Produce G Forms of Bacteria by Physical Methods

1. The effect of rapid transferring and aging bacteria in different media

There exists in the literature much confusion as to the exact import carried by the term G form. Hadley et al. (23) in 1931 introduced this designation applying it to colonies, microscopic in size, produced by coccoid forms which were filtrable through various types of filters. Aruechner and Sherman (7) in 1932 isolated organisms which they called primitive bacteria uncultivable by ordinary methods. Other names further cloud the situation. In this paper the following terminology will be used.

1. G forms which consist of any organism in a form different from the normal and which will pass filters and produce colonies microscopic in size upon the application of rapid serial plate transfer or aging.

2. Filtrable forms which consist of any organism in the normal or changed form which will pass filters, and will produce colonies and have reactions normal for that organism upon the application of suitable technic.

3. L forms which consist of any organism which will not pass filters, but will produce colonies microscopic in size upon the application of suitable technic.

The nature of this study required the use of several controls in order to evaluate correctly the data obtained.

1. Control and method of filtration. The Berkefeld filters were cleaned before each filtration by passing through them under negative pressure 250 cc. of a solution of potassium permanganate (1 gram $KMnO_4$ + 6.5 grams concentrated HCl + 1 liter of distilled water), 250 cc. of 1 per cent oxalic acid solution, 500 cc. of hot distilled water, and 500 cc. of cold distilled water. The filter was then connected to the accompanying flask, the outlets plugged with cotton, and the apparatus sterilized in the autoclave at 15 pounds steam pressure for 20 minutes. The Seitz filter pads were renewed after each filtration and the apparatus sterilized as above. Each filter was tested for its ability to retain Serratia marcescens after every 10 filtrations.

The pressure and time of filtration were adjusted to permit the filtrate to pass at the rate of one or two drops per second. For 20 cc., the amount generally filtered, the pressure was 150 to 200 mm. of Hg , and the time was three to five minutes. These methods were applied to all filtration studies.

2. Control of sterility of media. The glassware was sterilized twice at $180^{\circ}C$. for 10 minutes or longer. The media were prepared in the usual manner and sterilized according to the standard procedure. Test tubes containing media were placed at $37^{\circ}C$. for 48 hours, then at

room temperature for one week. All tubes showing growth were discarded. The agar plates were poured aseptically, incubated at 27°C. for 48 hours and at room temperature 24 hours before using; those showing contamination were discarded.

3. Control of test material. The K medium tubes seeded with the filtrates were controlled by duplicate filtrates placed in sterile cotton stoppered test tubes, in plain nutrient beef extract broth, or in differential media for the particular organism studied.

Rapid transferring and aging of bacteria under various conditions have been studied by several authors. Hadley (23) 1961 observed that rapid transfer of Shigella dysenteriae in media which had slight inhibitory effects on this organism caused a change from the normal colony size to one which could only be seen with the aid of the microscope. He reported that S forms of Shig. dysenteriae arose 16 times during spontaneous or enforced dissociation of the S or the R forms of this organism. These cultures originated from both R and S types undergoing rapid transfer and aging in broth, with and without lithium chloride, in pancreatin broth, from bacteriophage, and by animal injection.

The former methods will be considered in detail. Plain beef infusion broth, pH 6.8 to pH 7.8, was used for spontaneous dissociation. Twenty-five hundredths to 0.5 per cent lithium chloride was added to broth, pH 7.5 to pH 7.8, and 5 per cent pancreatin (Squibbs)

was likewise placed in broth, pH 6.8 to pH 7.8, for studying enforced dissociation. Both R and S type cultures underwent rapid transfer and aging in these media.

The technic of rapid transfer consisted essentially in transferring from one loop to several drops from tube to tube at 24 hour intervals. After 24 to 48 hours' incubation at 37°C. the culture was placed on a sterile agar plate and smeared over the surface with a sterile glass L-shaped rod. These seeded plates were incubated from 24 to 48 hours and differential colony plate counts made. The studies in aging consisted in part in placing the organisms to be studied in the various media and making subculture plates at intervals for differential colony counts. Hadley found that on about the seventh to tenth transfer microcolonies appeared in large numbers, sometimes preceded by a sterile plate. These appearances were very regular in recurrence. With repeated transfer the colonies disappeared, returning again when further plantings were made. The cultural, morphological, and chemical reactions of the G colonies were obtained. These differed from the normal Shig. dysenteriae reactions. The cultures were found to be filtrable and the filtrate under proper manipulation produced microcolonies or were true G forms.

Murox (54) 1931 obtained results with Salmonella aertrycke which confirmed Hadley's work. The technic used closely followed that of the latter. At the termination of the experiment six cultures of the G forms

of Sal. aertrycke were seeded on veal agar plates, sealed with adhesive tape, and placed in the ice box for five months. At the beginning of this period the cultures were microscopic in size, but after the period of aging only yellow, opaque colonies 2 to 3 mm. in diameter could be discovered. An explanation of this phenomenon will be offered later in this paper.

A. The effect of rapid transferring and aging Shig. dysenteriae in different media. In an attempt to obtain G forms the influence of rapid transferring and aging a strain of Shig. dysenteriae was tested in different media. The culture was obtained from Hadley of the University of Michigan. This strain was the one used by Hadley to obtain the G forms that he has reported (25) 1931. In order to simulate the conditions under which his work was conducted the following media were selected and prepared according to his recommendations: (1) Plain beef infusion broth, pH 7.8, (2) 0.25 per cent lithium chloride broth, pH 7.6, (3) 5 per cent pancreatin broth, pH 6.8, (4) 5 per cent pancreatin broth, pH 7.6, (5) plain beef infusion agar, and (6) litmus lactose agar (Difco). A sufficient amount of each medium was made at one time for the entire experiment. Before using each tube or plate of medium was incubated for sterility as outlined previously.

Daily transfers were made in each of the four broth media. All tubes were incubated at 37°C. After 48 hours' incubation each culture was streaked on litmus lactose

and plain beef infusion agar plates. The plates were incubated 48 hours at 37°C. for 5 to 10 days at room temperature and examined for colony formation at a magnification of 100 diameters.

The initial tube of each series was placed at room temperature, after the incubation period at 37°C., to determine the effect of aging. Every 24 hours each culture was streaked on litmus lactose and plain beef infusion agar. The plates were incubated and examined as outlined above. Although Shig. dysenteriae was aged for 50 days, and in spite of 50 rapid transfers in four different broth media, no effect of dissociation was observed. The colonies present on each plate in the series were rough and opaque throughout the course of the experiment.

B. Spontaneous and enforced dissociation of bacteria by rapid transferring and aging. Although no G forms could be obtained from Shig. dysenteriae, the method of rapid transferring and aging was extended to other bacteria and to several other media. Seven organisms of different genera, both pathogenic and nonpathogenic, motile and nonmotile, pigment and nonpigment formers were selected: (1) Shigella paradysenteriae, (2) Serratia indica, (3) Chronobacterium violaceum, (4) Escherichia coli, (5) Salmonella pullorum, (6) Aerobacter aerogenes, and (7) Shigella dysenteriae.

The media were prepared as outlined below: All media were sterilized in the autoclave at 15 pounds

steam pressure for 15 to 20 minutes and checked for sterility by incubating as described previously. The seven organisms were planted into each of the broth media: (1) Plain beef extract broth, pH 7.8, (2) plain veal infusion broth, pH 7.2, (3) 0.5 per cent lithium chloride broth, (4) 1.0 per cent lithium chloride broth, (5) 2.0 per cent lithium chloride broth (6) K medium (Difco), and (7) 0.007 per cent brilliant green broth. In addition a tube of each was used as a control on the absence of G forms from the media. The culture tubes together with the controls were incubated at 27°C. for 24 hours. Daily transfers were made from each tube in each series. After 48 hours' incubation the cultures and controls were streaked on veal infusion agar plates that had been previously checked for sterility. The seeded plates were incubated at 27°C. for 48 hours, followed by 5 to 10 days at room temperature, and examined for colony formation at a magnification of 100 diameters. Colonies resembling G forms were subcultured to veal infusion broth, incubated 48 hours at 27°C., and filtered through Berkefeld N filters according to the technic previously outlined. The filtrates were studied for the presence of G forms.

To determine the effect of aging the initial tubes of each series were placed at room temperature after the incubation period at 27°C. At the intervals given in Tables 2, 4, 6, 8, 10, and 12 the material in each tube was streaked on veal infusion agar plates previously

checked for sterility, and after incubation at 37°C., followed by room temperature, were examined for colony formation. The control tubes remained sterile for the entire experiment. The plates producing microcolonies were incubated one week to observe any change in colony size. Microcolonies which remained constant in size were carefully transferred under a magnification of 100 diameters, to veal infusion broth, pH 7.0, and incubated at 37°C. for 48 hours. Broth cultures which showed faint, opalescent growth were filtered according to the method previously described. The filtrate was clear and colorless. One cc. amounts of the filtrate were placed in: (1) Veal infusion broth, pH 7.2, (2) K medium (Difco), and (3) a sterile cotton stoppered test tube. These tubes were incubated at 37°C. for 72 hours, followed by one week at room temperature, when they were streaked on veal infusion agar plates. These were incubated at 37°C. for 48 hours, followed by one week at room temperature, and then examined at a magnification of 100 diameters for colony formation. No colonies were discovered on any of the subculture plates; the media containing filtrates from the cultures forming microcolonies remained sterile macroscopically and microscopically. This demonstrated that these organisms were not filtrable, and for this reason the term S form could not be applied to any culture isolated by rapid transferring or aging. According to the terminology outlined previously, the microcolonies formed were labeled H colonies.

1. Dissociation in plain beef extract broth, pH 7.8. Plain beef extract broth was made in the usual manner, adjusted to pH 7.8 with N/1 NaOH, tubed in 10 cc. amounts, sterilized and seeded with the test organisms.

a. Rapid Transfer. Data in Table 1 show that only three organisms produced M colonies, Shig. para-dysenteriae, Esch. coli, and Sal. pullorum. They appeared when S colonies were the predominating type.

b. Aging. Data in Table 2 show that only three organisms produced M colonies, Chron. violaceum, Esch. coli, and Sal. pullorum. With the exception of Chron. violaceum in which the appearance of the M types was preceded by three sterile plates, they arose when S colonies were the predominating type.

2. Spontaneous dissociation in veal infusion broth, pH 7.2. Veal infusion broth was prepared in the usual manner, checked for sterility by incubation, and seeded with the test organisms.

a. Rapid transfer. Data in Table 3 show that four organisms, Serr. indica, Shig. dysenteriae, Chron. violaceum, and Sal. pullorum, produced M type colonies, the first two when R colonies were the predominating type, the others when S colonies were in excess.

b. Aging. Data in Table 4 show that the production of M colonies in Chron. violaceum and Sal. pullorum occurred when R colonies were predominant in the former, and when S colonies were in excess in the latter.

Table 1. Dissociation by Rapid Transfer in Plain Beef Extract Broth, pH 7.8.

Day	Shig.			Serr.			Chrom.			Esch.		
	E	R	M	E	R	M	E	R	M	E	R	M
1	100	100	100	100	100	100	100	100	100	100	100	100
2	10	90	100	100	100	100	100	100	100	100	100	100
3	20	80	100	100	100	100	100	100	100	100	100	100
4	30	70	100	100	100	100	100	100	100	100	100	100
5	30	70	100	100	100	100	100	100	100	100	100	100
6	10	90	100	100	100	100	100	100	100	100	100	100
7	10	90	100	100	100	100	100	100	100	100	100	100
8	10	90	100	100	100	100	100	100	100	100	100	100
9	10	100	100	100	100	100	100	100	100	100	100	100
10	10	100	100	100	100	100	100	100	100	100	100	100
11	10	100	100	100	100	100	100	100	100	100	100	100
12	10	100	100	100	100	100	100	100	100	100	100	100
13	10	100	100	100	100	100	100	100	100	100	100	100
14	10	100	100	100	100	100	100	100	100	100	100	100

B = Broth culture appearance
R = Rough colonies in per cent
S = Smooth colonies in per cent
M = Microcolonies
H = Homogeneous clouding
G₂ = Granular precipitate
P = Pellicle
C = Clear
F = Flocculent precipitate

Table 1. Dissociation by Rapid Transfer in Plain Beef Extract Broth, pl 7.9
(continued)

	Sal.			Micro.			Shig.			Control		
	pullorum			aerovences			dysenteriae			B R S		
1st	B	R	S	B	R	S	B	R	S	B	R	S
1	H	-	100	H	100	-	G	100	-	C	-	-
2	H	-	100	H	100	-	G	100	-	C	-	-
3	H	10	90	H	100	-	G	100	-	C	-	-
4	H	20	70	H	100	-	G	100	-	C	-	-
5	H	40	60	H	80	20	G	100	-	C	-	-
6	H	-	100	H	60	40	G	100	-	C	-	-
7	H	-	100	H	50	50	G	100	-	C	-	-
8	H	-	-	H	50	50	G	100	-	C	-	-
9	H	-	-	H	50	50	G	100	-	C	-	-
10	H	-	-	P	-	100	G	100	-	C	-	-
11	H	-	100	P	-	100	G	100	-	C	-	-
12	H	-	100	P	-	100	G	100	-	C	-	-
13	H	-	100	P	-	100	G	100	-	C	-	-
14	H	-	100	P	-	100	G	100	-	C	-	-

B - Broth culture appearance
R - Rough colonies in per cent
S - Smooth colonies in per cent
M - Microcolonies
H - Homogeneous clouding
G - Granular precipitate
P - Pellicle
C - Clear

Table 2. Dissociation by aging in Plain Beef Extract Broth, pH 7.8.

Day	Shig.			Serr.			Chrom.			Hsch.		
	paratyph. coli			indica			violaceum			coli		
	R	S	B	R	S	B	R	S	B	R	S	B
1	100	-	-	100	-	-	-	-	-	-	-	-
2	50	50	-	100	-	-	-	-	-	-	-	-
4	100	-	-	50	50	-	100	-	-	-	-	-
7	100	-	-	50	50	-	-	-	-	-	-	-
10	100	-	-	-	-	-	-	-	-	-	-	-
14	100	-	-	-	-	-	-	-	-	-	-	-
18	100	-	-	-	-	-	-	-	-	-	-	-
23	100	-	-	-	-	-	-	-	-	-	-	-
27	100	-	-	-	-	-	-	-	-	-	-	-
32	100	-	-	-	-	-	-	-	-	-	-	-
40	100	-	-	-	-	-	-	-	-	-	-	-
50	100	-	-	-	-	-	-	-	-	-	-	-

B - Broth culture appearance
 R - Rough colonies in per cent
 S - Smooth colonies in per cent
 M - Microcolonies
 I - Homogeneous clouding
 P - Pellicle
 C - Clear
 F - Flocculent precipitate

Table 2. Dissociation by Aging in Plain Leaf Extract Broth, pl 7.8.
(Continued)

Day	Sal. phlorum			aero.			Control		
	B	R	S	B	R	S	B	R	S
1	100	100	100	100	100	100	100	100	100
2	100	100	100	100	100	100	100	100	100
4	100	100	100	100	100	100	100	100	100
7	100	100	100	100	100	100	100	100	100
10	100	100	100	100	100	100	100	100	100
14	100	100	100	100	100	100	100	100	100
18	100	100	100	100	100	100	100	100	100
22	100	100	100	100	100	100	100	100	100
27	100	100	100	100	100	100	100	100	100
32	100	100	100	100	100	100	100	100	100
40	100	100	100	100	100	100	100	100	100
50	100	100	100	100	100	100	100	100	100

B - Broth culture appearance
R - Rough colonies in per cent
S - Smooth colonies in per cent
M - Microcolonies
H - Homogeneous clouding
C - Clear

Table 3. Spontaneous Dissociation by Rapid Transfer in Veal Infusion Broth, pH 7.2.

Day	Sis. paratyphenteriae	Serr. indica	Chrom. violaceum	Resch. coli
1	100	100	100	100
2	100	100	100	100
3	100	100	100	100
4	100	100	100	100
5	100	100	100	100
6	100	100	100	100
7	100	100	100	100
8	100	100	100	100
9	100	100	100	100
10	100	100	100	100
11	100	100	100	100
12	100	100	100	100
13	100	100	100	100
14	100	100	100	100

B - Broth culture appearance
R - Rough colonies in per cent
S - Smooth colonies in per cent
M - Microcolonies
H - Homogeneous clouding
G - Granular precipitate
P - Pellicle
F - Flocculent precipitate

Table 3. Spontaneous Dissociation by Rapid Transfer in Veal Infusion Broth, pH 7.2
(Continued).

Day	Sal. pallidum			Aero. aerogenes			Shig. dysenteriae			Control		
	B	R	S	M	B	R	S	M	B	R	S	M
1	H	90	10	-	P	100	-	-	H	100	-	-
2	H	90	10	-	P	100	-	-	H	100	-	-
3	H	90	10	-	G	100	-	-	H	70	30	-
4	H	80	20	-	G	100	-	-	H	90	10	Few
5	H	70	30	-	G	100	-	-	H	100	-	Few
6	H	50	50	-	G	100	-	-	H	100	-	Few
7	H	50	50	Many	G	100	-	-	H	100	-	Few
8	H	50	50	Many	G	100	-	-	H	100	-	Few
9	H	-	100	Many	G	100	-	-	H	100	-	Few
10	H	-	100	Many	G	100	-	-	H	100	-	Few
11	H	-	100	Few	G	100	-	-	H	100	-	Few
12	H	-	100	-	G	100	-	-	H	100	-	-
13	H	-	100	-	G	100	-	-	H	100	-	-
14	H	-	100	-	G	100	-	-	H	100	-	-

B = Broth culture appearance
R = Rough colonies in per cent
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M = Microcolonies
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P = Pellicle
F = Flocculent
C = Clear

Table 4. Spontaneous Dissociation by Aging in Veal Infusion Broth, pH 7.2.

Day	Shig. paradyssenteriae						Serr. indica						Chrom. violaceum						Esch. coli					
	B	R	S	M	B	R	S	M	B	R	S	M	B	R	S	M	B	R	S	M	B	R	S	M
1	H	-	100	-	F	100	-	-	F	100	-	-	F	100	-	-	F	-	100	-	-	-	100	-
2	H	100	-	-	F	100	-	-	F	100	-	-	F	100	-	-	F	10	90	-	-	-	10	90
4	H	100	-	-	F	50	50	-	F	100	-	-	F	100	-	-	F	50	50	-	-	-	50	50
7	H	100	-	-	F	-	100	-	F	100	-	-	F	100	-	Few	F	50	50	-	-	-	50	50
10	H	100	-	-	F	-	100	-	F	100	-	-	F	100	-	Few	F	50	50	-	-	-	50	50
14	H	100	-	-	F	-	100	-	F	100	-	-	F	100	-	Few	F	50	50	-	-	-	50	50
18	H	100	-	-	C	-	100	-	C	100	-	-	F	100	-	Few	F	50	50	-	-	-	50	50
23	H	100	-	-	C	-	100	-	C	100	-	-	F	100	-	Few	F	-	100	-	-	-	-	100
27	H	100	-	-	C	-	100	-	C	100	-	-	F	100	-	Few	F	-	100	-	-	-	-	100
32	H	100	-	-	C	-	100	-	C	100	-	-	F	100	-	-	F	-	100	-	-	-	-	100
40	H	100	-	-	C	-	100	-	C	100	-	-	F	100	-	-	F	-	100	-	-	-	-	100
50	H	100	-	-	C	-	100	-	C	100	-	-	F	100	-	-	F	100	-	-	-	-	-	100

B = Broth culture appearance
 R = Rough colonies in per cent
 S = Smooth colonies in per cent
 M = Microcolonies
 H = Homogeneous clouding
 P = Pellicle
 F = Flocculent
 C = Clear

**Table 4. Spontaneous Dissociation by Aging in Veal Infusion Broth, pi 7.2
(Continued).**

[illegible]

B	-	Broth culture appearance
R	-	Rough colonies in per cent
S	-	Smooth colonies in per cent
M	-	Microcolonies
H	-	Homogeneous clouding
P	-	Pellicle
F	-	Flocculent
C	-	Clear

3. Enforced dissociation in 0.5 per cent lithium chloride broth. To veal infusion broth, prepared in the usual manner, was added LiCl q.s. to make a concentration of 0.5 per cent. The medium was sterilized in 10 cc. amounts and seeded with the test organisms.

a. Rapid transfer. Data in Table 5 show that M type colonies were produced when S types were in excess in Serr. indica, and when R types predominated in Chron. violaceum and Shig. dysenteriae.

b. Aging. Data in Table 6 show that S types and R types were equal in amount when M colonies arose in Serr. indica, and that S types predominated when M types appeared in Esch. coli and Sal. pullorum.

4. Enforced dissociation in 1.0 per cent lithium chloride broth. The medium was prepared as in part 3, except that the concentration of LiCl was made 1.0 per cent.

a. Rapid transfer. Data in Table 7 show that M types were obtained when S colonies predominated in Serr. indica and Sal. pullorum.

b. Aging. Data in Table 8 show that R and S types were equal in Serr. indica, and that S types predominated in Sal. pullorum when M colonies appeared.

5. Enforced dissociation in 2.0 per cent lithium chloride broth. The concentration of LiCl was made 2.0 per cent in veal infusion broth.

a. Rapid transfer. Data in Table 9 show that M colonies were produced in Serr. indica and Esch. coli.

Table 5. Dissociation by Rapid Transfer in 0.5 per cent LiCl Broth.

Day	Shig.			Serr.			Esch.			Chrom.		
	paratyphenteriae			indica			coli			violaceum		
	B	R	M	B	R	M	B	R	M	B	R	M
1	H	100	-	H	100	-	P	50	50	P	50	50
2	H	100	-	H	100	-	P	50	50	C	-	100
3	H	100	-	H	100	-	P	60	40	C	-	-
4	H	100	-	H	80	20	P	60	40	C	-	-
5	H	100	-	H	90	10	P	60	40	C	100	-
6	H	100	-	H	80	20	P	60	40	C	100	-
7	H	50	50	H	50	50	P	30	70	C	100	-
8	H	50	50	H	50	50	P	-	100	C	Few	Many
9	H	-	100	H	50	50	P	-	100	C	Few	Many
10	H	-	100	H	-	100	P	-	100	C	-	Few
11	H	-	100	H	10	90	P	-	100	C	100	-
12	H	-	100	H	-	100	P	-	100	C	100	-
13	H	-	100	H	-	100	P	-	100	C	-	-
14	H	-	100	H	-	100	P	-	100	C	-	-

B = Broth culture appearance

R = Rough colonies in per cent

S = Smooth colonies in per cent

M = Microcolonies

H = Homogeneous clouding

F = Flocculent

C = Clear

Table 5. Dissociation by Rapid Transfer in 0.5 per cent LiCl Broth (Continued).

Day	Sal. pullorum			Aero. aerogenes			Shig. dysenteriae			Control		
	B	R	S	B	R	S	B	R	S	B	R	S
1	H	50	50	P	100	-	F	100	-	C	-	-
2	H	50	50	P	100	-	F	100	-	C	-	-
3	H	40	60	P	100	-	F	100	-	C	-	-
4	H	40	60	P	100	-	F	90	Few	C	-	-
5	H	30	70	P	100	-	F	100	-	C	-	-
6	H	30	70	P	100	-	F	100	-	C	-	-
7	H	-	100	P	100	-	F	100	-	C	-	-
8	H	-	100	P	20	80	F	100	-	C	-	-
9	H	-	100	P	-	100	F	100	-	C	-	-
10	H	-	100	P	-	100	F	100	-	C	-	-
11	H	-	100	P	-	100	F	100	-	C	-	-
12	H	-	100	P	-	100	F	100	-	C	-	-
13	H	-	100	P	-	100	F	100	-	C	-	-
14	H	-	100	P	-	100	F	100	-	C	-	-

B = Broth culture appearance
R = Rough colonies in per cent
S = Smooth colonies in per cent
M = Microcolonies
H = Homogeneous clouding
P = Pellicle
F = Flocculent precipitate
C = Clear

Table 6. Dissociation by Aging in 0.5 per cent LiCl Broth.

Day	Shik. paratyphenteriae	Serr. indica	Chrom. violaceum	Esch. coli
1	B 100'	B 100'	B 100'	B 100'
2	H 100'	H 100'	H 100'	H 100'
4	H 100'	H 100'	H 100'	H 100'
7	H 50'	H 50'	H 50'	H 50'
10	H 100'	H 50'	H 100'	H 100'
14	H 100'	H 50'	H 100'	H 100'
18	H 100'	H 50'	H 100'	H 100'
23	H 100'	H 50'	H 90'	H 100'
27	H 100'	H 50'	H 100'	H 100'
32	H 100'	H 50'	H 100'	H 100'
40	H 100'	H 50'	H 100'	H 100'
50	H 100'	H 50'	H 100'	H 100'

B - Broth culture appearance
 R - Rough colonies in per cent
 S - Smooth colonies in per cent
 M - Microcolonies
 H - Homogeneous clouding
 F - Flocculent precipitate
 C - Clear

Table 6. Dissociation by Aging in 0.5 per cent L101 broth.
(Continued).

Day	Sal.			Aero.			Control		
	1	2	3	1	2	3	1	2	3
127	1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1	1
2	1	1	1	1	1	1	1	1	1
4	1	1	1	1	1	1	1	1	1
7	1	1	1	1	1	1	1	1	1
10	1	1	1	1	1	1	1	1	1
14	1	1	1	1	1	1	1	1	1
18	1	1	1	1	1	1	1	1	1
22	1	1	1	1	1	1	1	1	1
27	1	1	1	1	1	1	1	1	1
32	1	1	1	1	1	1	1	1	1
40	1	1	1	1	1	1	1	1	1
50	1	1	1	1	1	1	1	1	1

B = Broth culture appearance
R = Rough colonies in per cent
S = Smooth colonies in per cent
1 = Microcolonies
1 = Homogeneous clouding
2 = Pellicle
3 = Flocculent precipitate
0 = Clear

Table 7. Dissociation by Rapid Transfer in 1.0 per cent LiCl Broth.

Day	Shig.			Serr.			Chrom.			Esch.		
	paradysenteriae			indica			violaceum			coli		
	B	R	S	B	R	S	B	R	S	B	R	S
1	C	100	-	H	100	-	C	-	-	F	100	-
2	H	100	-	H	90	10	C	-	-	F	100	-
3	H	100	-	H	90	10	C	-	-	F	100	-
4	H	80	20	H	80	10	C	-	-	F	100	-
5	H	60	40	H	50	50	C	-	-	F	100	-
6	H	50	50	H	50	50	C	-	-	F	100	-
7	H	-	100	H	50	50	C	-	-	F	100	-
8	H	-	100	H	50	50	C	-	-	F	50	50
9	H	-	100	H	10	90	C	-	-	F	-	100
10	H	-	100	H	10	90	C	-	-	Many	F	-
11	H	-	100	H	-	100	C	-	-	Few	F	-
12	H	-	100	H	-	100	C	-	-	F	-	100
13	H	-	100	H	-	100	C	-	-	F	-	100
14	H	-	100	H	-	100	C	-	-	F	-	100

B = Broth culture appearance
 R = Rough colonies in per cent
 S = Smooth colonies in per cent
 H = Microcolonies
 I = Homogeneous clouding
 P = Pellicle
 F = Flocculent precipitate
 C = Clear

Table 7. Dissociation by Rapid Transfer in 1.0 per cent L1J1 Broth (Continued).

Day	Sal.			Aero.			Shig.			Control		
	B	R	S	B	R	S	B	R	S	B	R	S
1	H	100	-	F	100	-	H	100	-	C	-	-
2	H	-	100	F	100	-	H	-	100	C	-	-
3	H	-	100	F	100	-	H	-	100	C	-	-
4	H	50	50	F	100	-	H	-	100	C	-	-
5	H	-	100	F	100	-	H	-	100	C	-	-
6	H	-	100	F	100	-	H	-	100	C	-	-
7	H	-	100	F	100	-	H	-	100	C	-	-
8	H	-	100	F	100	-	H	-	100	C	-	-
9	H	-	100	F	100	-	H	-	100	C	-	-
10	H	-	100	F	100	-	H	-	100	C	-	-
11	H	-	100	F	100	-	H	-	100	C	-	-
12	H	-	100	F	100	-	H	-	100	C	-	-
13	H	-	100	F	100	-	H	-	100	C	-	-
14	H	-	100	F	100	-	H	-	100	C	-	-

B = Broth culture appearance
 R = Rough colonies in per cent
 S = Smooth colonies in per cent
 H = Microcolonies
 F = Homogeneous clouding
 P = Flocculent precipitate
 C = Pellicle
 - = Clear

Table 8. Dissociation by Aging in 1.0 per cent LiCl Broth.

Day	Shig.			Serr.			Chrom.			Esch.		
	paratyphenteriae			indica			violaceum			coli		
	B	R	S	B	R	S	B	R	S	B	R	S
1	C	100	-	H	100	-	C	-	-	F	50	50
2	H	100	-	H	90	10	C	-	-	F	100	-
4	H	100	-	H	50	50	C	-	-	F	50	50
7	H	150	50	H	50	50	C	-	-	F	-	100
10	H	50	50	H	50	50	C	-	-	F	-	100
14	H	50	50	H	50	50	C	-	-	F	-	100
18	H	50	50	H	50	50	C	-	-	F	-	100
23	H	50	50	H	50	50	C	-	-	F	-	100
27	H	50	50	H	50	50	C	-	-	F	-	100
32	H	50	50	H	50	50	C	-	-	F	-	100
40	H	50	50	H	50	50	C	-	-	F	-	100
50	H	100	-	H	100	-	C	-	-	F	-	100

B = Broth culture appearance
 R = Rough colonies in per cent
 S = Smooth colonies in per cent
 M = Microcolonies
 H = Homogeneous clumping
 C = Clear
 F = Flocculent precipitate

Table 8. Dissociation by Aging in 1.0 per cent LiCl Broth (Continued).

Day	Sal.			Aero.			Control		
	B	R	S	B	R	S	B	R	S
1	H	100	-	F	100	-	C	-	-
2	H	-	100	F	100	-	C	-	-
4	H	-	100	F	100	-	C	-	-
7	H	-	100	F	100	-	C	-	-
10	H	-	100	Few	50	50	C	-	-
14	H	-	100	Many	F	100	C	-	-
18	H	-	100	Many	F	100	C	-	-
23	H	-	100	Many	F	100	C	-	-
27	H	-	100	-	F	-	C	-	-
32	H	-	100	-	F	-	C	-	-
40	H	-	100	-	F	-	C	-	-
50	H	-	100	-	F	-	C	-	-

B = Broth culture appearance
R = Rough colonies in per cent
S = Smooth colonies in per cent
M = Microcolonies
H = Homogeneous clouding
F = Flocculent precipitate
C = Clear

Table 9. Dissociation by Rapid Transfer in 2.0 per cent LiCl Broth.

Day	Shig.			Serr.			Chrom.			Esch.		
	B	R	S	B	R	S	B	R	S	B	R	S
1	C	-	-	C	-	-	-	-	-	P	50	50
2	C	-	-	H	90	10	-	-	-	P	50	50
3	C	-	-	H	60	40	-	-	-	P	50	50
4	C	-	-	H	60	40	-	-	-	P	50	50
5	C	-	-	H	60	40	-	-	-	P	50	50
6	C	-	-	H	50	50	-	-	-	P	50	50
7	C	-	-	H	50	50	-	-	-	P	50	50
8	C	-	-	H	50	50	-	-	-	P	100	-
9	C	-	-	H	Few	Few	-	-	-	P	50	50
10	C	-	-	H	Few	Few	-	-	-	P	10	90
11	C	-	-	H	Few	Few	-	-	-	P	-	100
12	C	-	-	H	Few	Few	-	-	-	P	-	100
13	C	-	-	H	100	-	-	-	-	P	-	100
14	C	-	-	H	100	-	-	-	-	P	-	100

B - Broth culture appearance
 R - Rough colonies in per cent
 S - Smooth colonies in per cent
 M - Microcolonies
 H - Homogeneous clouding
 P - Pellicle
 C - Clear
 F - Flocculent precipitate

Table 9. Dissociation by Rapid Transfer in 2.0 per cent LiCl Broth (Continued).

Day	Sal. pullorum			Aero. aerogenes			Shig. dysenteriae			Control		
	B	R	S	B	R	S	B	R	S	B	R	S
1	C	100	-	P	100	-	P	100	-	C	-	-
2	C	-	-	P	100	-	P	-	-	C	-	-
3	C	-	-	P	100	-	C	Few	-	C	-	-
4	C	-	-	P	100	-	C	100	-	C	-	-
5	C	-	-	P	100	-	C	100	-	C	-	-
6	C	-	-	P	100	-	C	100	-	C	-	-
7	C	-	-	P	100	-	C	100	-	C	-	-
8	C	-	-	P	50	50	C	100	-	C	-	-
9	C	-	-	P	10	90	C	100	-	C	-	-
10	C	-	-	P	-	100	C	100	-	C	-	-
11	C	-	-	P	-	100	C	100	-	C	-	-
12	C	-	-	P	-	100	C	100	-	C	-	-
13	C	-	-	P	-	100	C	100	-	C	-	-
14	C	-	-	P	-	100	C	100	-	C	-	-

B - Broth culture appearance
R - Rough colonies in per cent
S - Smooth colonies in per cent
M - Microcolonies
H - Homogeneous clouding
P - Pellicle
C - Clear
F - Flocculent precipitate

b. Aging. Data in Table 10 show L types appeared when H types predominated in Serr. indica.

6. Dissociation in K medium (Difco). A 2.0 per cent suspension of powdered K medium (Difco) was made in distilled water, sterilized, checked for sterility, and seeded with the test organisms.

a. Rapid transfer. Data in Table 11 show that H types appeared when R types predominated in Shron. violaceum, and when S types predominated in Sal. pullorum.

b. Aging. 10 H types were produced.

7. Dissociation in brilliant green broth. Brilliant green broth was prepared by adding peptone, 10 grams and meat extract, 20 grams to 1 liter of distilled water. This suspension was heated until dissolved, adjusted to pH 7.0, autoclaved for one-half hour, filtered, and to it was added 7 cc. of a 1.0 per cent solution of brilliant green. The medium was tubed and sterilized.

a. Rapid transfer. Data in Table 12 show that H types were obtained when S types were predominant in Sal. pullorum.

b. Aging. Data in Table 13 show that H types were obtained when S colonies were predominant in Esch. coli and Sal. pullorum.

Discussion:

Seven different species of bacteria were transferred daily into seven different media and the appearance, persistence, and disappearance of H colonies noted.

Table 10. Dissociation by Aging in 2.0 per cent LiCl Broth

Day	Shig.			Serr.			Chrom.			Esch.		
	paratyphenteriae			indica			violaceum			coli		
	B	R	S	B	R	S	B	R	S	B	R	S
1	C	-	-	C	100	-	C	-	-	P	50	50
2	C	-	-	H	90	10	C	-	-	P	50	50
4	C	100	-	H	50	50	C	-	-	P	100	-
7	C	100	-	H	100	-	C	-	-	P	100	-
10	C	100	-	H	100	-	Many	C	-	P	100	-
14	C	100	-	H	100	-	C	-	-	P	100	-
18	C	100	-	H	100	-	C	-	-	P	100	-
23	C	100	-	H	100	-	C	-	-	P	100	-
27	C	100	-	H	50	50	C	-	-	P	100	-
32	C	100	-	H	50	50	C	-	-	P	100	-
40	C	100	-	H	50	50	C	-	-	P	100	-
50	C	100	-	H	50	50	C	-	-	Many	P	100

B = Broth culture appearance
 R = Rough colonies in per cent
 S = Smooth colonies in per cent
 M = Microcolonies
 H = Homogeneous clouding
 P = Pellicle
 C = Clear

Table 10. Dissociation by Aging in 2.0 per cent LiCl broth (Continued).

Day	Sal.			Aero.			Control		
	b	R	S	b	R	S	b	R	S
1	100	-	-	100	-	-	100	-	-
2	-	-	-	100	-	-	-	-	-
4	-	-	-	100	-	-	-	-	-
7	-	-	-	100	-	-	-	-	-
10	-	-	-	100	-	-	-	-	-
14	-	-	-	100	-	-	-	-	-
18	-	-	-	100	-	-	-	-	-
23	-	-	-	100	-	-	-	-	-
27	-	-	-	100	-	-	-	-	-
32	-	-	-	100	-	-	-	-	-
40	-	-	-	100	-	-	-	-	-
50	-	-	-	100	-	-	-	-	-

b - Broth culture appearance
R - Rough colonies in per cent
S - Smooth colonies in per cent
M - Microcolonies
H - Hazy mucous clouding
C - Clear
F - Flocculent precipitate

Table 11. Dissociation by Rapid Transfer in 5.0 per cent K Medium (Iifco).

Day	Shig.			Serr.			Chrom.			Each.		
	A	B	C	A	B	C	A	B	C	A	B	C
1	100	100	100	100	100	100	100	100	100	100	100	100
2	100	100	100	100	100	100	100	100	100	100	100	100
3	100	100	100	100	100	100	100	100	100	100	100	100
4	100	100	100	100	100	100	100	100	100	100	100	100
5	100	100	100	100	100	100	100	100	100	100	100	100
6	100	100	100	100	100	100	100	100	100	100	100	100
7	100	100	100	100	100	100	100	100	100	100	100	100
8	100	100	100	100	100	100	100	100	100	100	100	100
9	100	100	100	100	100	100	100	100	100	100	100	100
10	100	100	100	100	100	100	100	100	100	100	100	100
11	100	100	100	100	100	100	100	100	100	100	100	100
12	100	100	100	100	100	100	100	100	100	100	100	100
13	100	100	100	100	100	100	100	100	100	100	100	100
14	100	100	100	100	100	100	100	100	100	100	100	100

E = broth culture appearance
R = Rough colonies in per cent
S = Smooth colonies in per cent
A = Microcolonies
H = Homogeneous clouding

Table 11. Dissociation by Rapid Transfer in 3.0 per cent K Medium (Diffco)
(Continued).

Day	Sal.			aero.			dysenteriae			Shig.			Control		
	L	H	S	L	H	S	L	H	S	L	H	S	L	H	S
1	-	-	100	-	-	100	-	-	100	-	-	100	-	-	-
2	-	-	100	-	-	100	-	-	100	-	-	100	-	-	-
3	-	-	100	-	-	100	-	-	100	-	-	100	-	-	-
4	-	-	100	-	-	100	-	-	100	-	-	100	-	-	-
5	-	-	100	-	-	100	-	-	100	-	-	100	-	-	-
6	-	-	100	-	-	100	-	-	100	-	-	100	-	-	-
7	-	-	100	-	-	100	-	-	100	-	-	100	-	-	-
8	-	-	100	-	-	100	-	-	100	-	-	100	-	-	-
9	-	-	100	-	-	100	-	-	100	-	-	100	-	-	-
10	-	-	100	-	-	100	-	-	100	-	-	100	-	-	-
11	-	-	100	-	-	100	-	-	100	-	-	100	-	-	-
12	-	-	100	-	-	100	-	-	100	-	-	100	-	-	-
13	-	-	100	-	-	100	-	-	100	-	-	100	-	-	-
14	-	-	100	-	-	100	-	-	100	-	-	100	-	-	-

B - Broth culture appearance
R - Rough colonies in per cent
S - Smooth colonies in per cent
M - Microcolonies
H - Homogeneous clouding
P - Pellicle
C - Clear

Table 12. Dissociation by Rapid Transfer in 0.007 per cent Brilliant Green Broth

Day	Shis. 'paradycenteriae'	Serr. infecta	Chrom. violetogen	Lech. coli
1	100	100	100	100
2	100	100	100	100
3	100	100	100	100
4	100	100	100	100
5	100	100	100	100
6	100	100	100	100
7	100	100	100	100
8	100	100	100	100
9	100	100	100	100
10	100	100	100	100
11	100	100	100	100
12	100	100	100	100
13	100	100	100	100
14	100	100	100	100

B - Broth culture appearance
R - Rough colonies in per cent
S - Smooth colonies in per cent
M - Microcolonies
H - Homogeneous clouding
C - Clear
P - Flocculent precipitate

Table 12. Dissociation by Rapid Transfer in 0.007 per cent Brilliant Green Broth

Day	Shig. paratyphenteriae	Serr. indica	Chron. violacea	Mech. coli
1	100	100	100	100
2	100	100	100	100
3	100	100	100	100
4	100	100	100	100
5	100	100	100	100
6	100	100	100	100
7	100	100	100	100
8	100	100	100	100
9	100	100	100	100
10	100	100	100	100
11	100	100	100	100
12	100	100	100	100
13	100	100	100	100
14	100	100	100	100

B - Broth culture appearance
R - Rough colonies in per cent
S - Smooth colonies in per cent
M - Microcolonies
H - Homogeneous clouding
C - Clear
P - Flocculent precipitate

Table 12. Dissociation by Rapid Transfer in 0.007 per cent Brilliant Green broth (Continued).

Day	Sal. pullorum			Aero. aerogenes			Shig. dysenteriae			Control		
	B	R	S	B	R	S	B	R	S	B	R	S
1	H	-	100	H	50	50	F	100	-	C	-	-
2	H	-	100	H	100	-	F	-	-	C	-	-
3	H	-	100	H	100	-	F	-	-	C	-	-
4	H	-	90 Few	H	100	-	C	-	-	C	-	-
5	H	-	100	H	100	-	C	-	-	C	-	-
6	H	-	100	H	10	90	C	-	-	C	-	-
7	H	-	100	H	20	80	C	-	-	C	-	-
8	H	20	80 Few	H	-	100	C	-	-	C	-	-
9	H	20	80 Few	H	-	100	C	-	-	C	-	-
10	H	-	100 Few	H	-	100	C	-	-	C	-	-
11	H	-	100	H	-	100	C	-	-	C	-	-
12	H	-	100	H	-	100	C	-	-	C	-	-
13	H	-	100	H	-	100	C	-	-	C	-	-
14	H	-	100	H	-	100	C	-	-	C	-	-

B = Broth culture appearance
 R = Rough colonies in per cent
 S = Smooth colonies in per cent
 F = Microcolonies
 H = Homogeneous clouding
 C = Clear
 P = Flocculent precipitate

Table 13. Dissociation by Aging in 0.007 per cent Brilliant Green Broth

Day	Shig.			Serr.			Chrom.			Esch.		
	paratyphenteriae			indica			violaceum			coli		
	B	R	S	B	R	S	B	R	S	B	R	S
1	100	-	-	100	-	-	100	-	-	100	-	-
2	-	-	-	100	-	-	-	-	-	-	-	-
4	-	-	-	50	50	-	-	-	-	-	-	-
7	-	-	-	100	0	50	50	-	-	-	-	-
10	-	-	-	100	0	50	50	-	-	-	-	-
14	-	-	-	100	0	50	50	-	-	-	-	-
18	-	-	-	100	0	50	50	-	-	-	-	-
23	-	-	-	100	0	50	50	-	-	-	-	-
27	-	-	-	100	0	50	50	-	-	-	-	-
32	-	-	-	100	0	50	50	-	-	-	-	-
40	-	-	-	100	0	50	50	-	-	-	-	-
50	-	-	-	100	0	50	50	-	-	-	-	-

B = Broth culture appearance
 R = Rough colonies in per cent
 S = Smooth colonies in per cent
 M = Microcolonies
 H = Homogeneous clouding
 P = Pellicle
 C = Clear
 F = Flocculent precipitate

Table 13. Dissociation by Aging in 0.007 per cent Brilliant Green Broth (Continued).

Day	Sal.			Aero.			Control		
	B	R	S	B	R	S	B	R	S
1	H	50	50	H	100	-	-	-	-
2	H	100	-	H	100	-	-	-	-
4	H	-	100	H	100	-	-	-	-
7	H	-	100	H	100	-	-	-	-
10	H	-	100	Few	H	50	50	-	-
14	H	-	100	Many	H	-	100	-	-
18	H	-	100	Many	H	100	-	-	-
23	H	-	100	Many	H	100	-	-	-
27	H	-	100	-	H	100	-	-	-
32	H	-	100	-	H	100	-	-	-
40	H	-	100	-	H	100	-	-	-
50	H	-	100	-	H	100	-	-	-

B = Broth culture appearance
 R = Rough colonies in per cent
 S = Smooth colonies in per cent
 M = Microcolonies
 H = Homogeneous clouding
 C = Clear

It was observed that in most cases R colonies appeared on the plates made from the fourth to sixth transfer tube, persisted through five to seven transfers and then disappeared. They also appeared after 10 days aging and disappeared from the 25th to 50th day of aging.

The regularity with which the G forms arose in experiments by Hadley on spontaneous and enforced dissociation of Shig. dysenteriae by rapid transfer and aging, and confirmed by Lurox with Sal. aertrycke, was not observed either with Shig. dysenteriae or other organisms. None of the media produced M types in all of the cultures; veal infusion broth itself spontaneously gave rise to more cases of M colonies than any of the special enforcing media. These colonies occurred haphazardly, and no relationship between the presence of R or S colonies, the type of media, the variations in the characteristics of the organisms, or the technic and the appearance of M colonies could be discovered.

Representative colonies selected at random and filtered as outlined previously did not pass Lorkelold N filters. For this reason the writer did not feel justified in applying the term G form to any of the microcolonies obtained by rapid transfer or aging.

2. Attempts to obtain pure cultures of S types by serial dilution.

Although rapid transfer and aging of pure cultures, as indicated previously, in various media produced microcolonies which resembled G types very closely they were

not filtrable. Several authors, Hadley et al. (23) 1931, Varney and Bronfenbrenner (66) 1932, and Linsser (70) 1932 stated that a true G culture must be filtrable. The data obtained in the experiments on pure cultures indicated that these forms were not present or associated with the organisms studied and could not be demonstrated by rapid transfer or aging. Consequently a different method, serial dilution, was employed on material known to contain several different types of organisms in naturally occurring substances, milk, sewage, etc.

The method of procedure was similar to that employed by Erneckner and Sherman (7) 1932 who reported a high incidence of primitive forms of bacteria in many naturally occurring substances, milk, soil, sewage, etc. They were able to obtain these forms in pure culture by serial dilutions, 1×10^{-13} cc. and higher, obtaining growth by special methods as rapid serial plate transfer. They did not conclude that these forms were filtrable, but they did state that they would not grow under ordinary cultural methods. In a study of milk from normal cows they demonstrated that primitive types of bacteria occurred in larger numbers than the ordinary type of organisms. This was shown by obtaining growth of primitive forms from high dilutions of the material studied, 1×10^{-8} cc. and higher, although the usual forms of bacteria could not be isolated.

Six samples of sewage and four samples of skim milk were used for the test material. Dextrose beef infusion broth was tbed in 9.0 cc. amounts, sterilized in the

autoclave at 15 pounds steam pressure for 20 minutes, and checked for sterility by incubating before using. One cc. of the sample was placed in tube 1, this tube was carefully shaken, and 1.0 cc. of the mixture transferred to tube 2 with a sterile pipette and continued until the 20th transfer had been made giving a series of dilutions from 0.1 cc. to 1×10^{-20} cc. of the original sample. Duplicate dilution series were run on each sample and on a control tube for each batch of media. One set of dilutions was placed at 25°C., the other at 37°C. for 72 hours. All tubes showing growth at the expiration of this period were discarded; the remainder were incubated at 25°C. for three weeks, after which they were streaked on dextrose beef infusion agar. In each series the lowest dilution tube that had not shown growth at the end of the preliminary incubation period was examined by the rapid serial plate transfer method of Hadley (23) 1931. At the time the samples were diluted they were also filtered fractionally through a Berkefeld N candle. No difference could be shown between the samples incubated at 25°C. for the entire period and those receiving preliminary treatment at 37°C. The material treated with rapid serial plate transfer did not show growth macroscopically or microscopically. Data in Table 14 show that no growth was obtained on subculture plates at the end of three weeks at 25°C. from tubes which did not show growth after 72 hours at 37°C. This did not disprove the presence of C types in the materials studied, but merely showed that they were not present in the samples in greater numbers

Table 14. Growth obtained on Subculture Plates from
Serial Dilution Tubes of Sewage and Skim Milk.

Material	Dilution = 1×10^{-x}					
	-1	-2	-3	-4	-5	-6 to -20 inc.
Sewage	+	+	+	+	-	-
Sewage	+	+	+	+	+	-
Sewage	+	+	+	+	-	-
Sewage	+	+	+	+	-	-
Sewage	+	+	+	+	+	-
Sewage	+	+	+	+	-	-
Skim Milk	+	+	+	+	+	-
Skim Milk	+	+	+	-	-	-
Skim Milk	+	+	+	+	-	-
Skim Milk	+	+	+	+	-	-
Control	-	-	-	-	-	-

+ = Growth obtained in dilution tubes after preliminary incubation at 25°C. or 27°C. for 72 hours

- = No growth obtained by rapid serial plate transfer or on streak subculture plates of the material after final incubation at 25°C. for 3 weeks.

than the usual bacteria. The attempt to demonstrate the presence of G types in any concentration in the sample by the methods of fractional filtration gave negative results as shown in Table 15. The organisms present in the sample did not pass the filter until the fourth to eighth fraction, and were the usual types found in milk or sewage. Either filtrable forms or G forms would have passed in the first or second fraction and according to Bruckner and Sherman (7) 1930 and Hadley et al. (23) 1931 would have had different characteristics from the organisms actually obtained.

Discussion of Section A.

1. Attempts were made to enforce the production of G forms from spontaneous and enforced dissociations by rapid transfer and aging of seven different microorganisms in various media. A rough strain of Shig. dysenteriae was aged for 30 days and carried through 30 daily transfers. No evidences of dissociation were noted in any of the four media used. The regularity noted by Hadley with which G forms occurred in Shig. dysenteriae was not observed in any of the seven organisms studied in any of the media. No relationships could be established between the hazardness of the appearance of microcolonies and the type of media, the characteristics of the organism, or the state of dissociation. Apparently these results confirmed those obtained by Hadley et al. (23) 1931 and Purox (34) 1931; but when representative microcolonies were transferred to veal infusion broth and filtered, they did not

Table 15. Growth in Fractional Filtrates of Sewage and Skim Milk.

Material	Filtrate Fraction (10 cc. in amount)														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Sewage	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sewage	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sewage	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sewage	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sewage	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sewage	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Skim Milk	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Skim Milk	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Skim Milk	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Skim Milk	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

♦ = Growth obtained in filtrate fraction and in subcultures

- = No growth obtained in filtrate fraction and in subcultures

pass Porkefold H filters. Because these microcolonies were not filtrable, the term B colonies rather than G form was applied.

2. The technic of serial dilution reported by Bruckner and Sherman (7) 1932 was applied to six samples of sewage and four samples of skim milk. No G forms or B types could be demonstrated in these samples which were also filtered fractionally. The organisms recovered were the usual types present in sewage or in skim milk.

B forms of bacteria rather than G types were demonstrated in organisms undergoing dissociation and in two natural substances, sewage and skim milk.

(B)

Attempts to Demonstrate the Presence of G Forms in the
Bacteriophage Filtrates of Salmonella pullorum and
Staphylococcus aureus

Whether or not G forms may be obtained from the bacteriophage filtrates of various organisms is still a matter of controversy. Varney and Bronfenbrenner (36) in 1932 were unable to obtain G forms from 25 strains of bacteriophage filtrates studied. Hadley et al. (23) in 1931 reported the presence and isolation of a G form from Shig. dysenteriae bacteriophage filtrate. In the literature may be found other references either affirming or denying the presence of these forms in the bacteriophage filtrates of various organisms. However, it seems unnecessary to cite these references at this time. In view of these conflicting reports it was thought expedient to make further studies on the bacteriophage filtrates lytic for Sal. pullorum and Staph. aureus.

1. Attempts to demonstrate G forms by rapid serial plate transfer of the bacteriophage filtrate of Sal. pullorum aged in K medium

The materials used in these studies consisted of serial filtrates obtained from 30 transfers of a bacteriophage on Sal. pullorum. The filtrate in each case was added in amounts of 0.1 cc. and 1.0 cc. in duplicate, to plain nutrient meat extract broth, pH 7.7, to which Sal. pullorum had previously been added. After 24 hours' incubation at 37°C., the tubes showing the greatest lytic

action were filtered through Seitz filters. This transfer was repeated daily using the freshly obtained bacteriophage filtrate. At the time each of the above transfers were made, 1.0 cc. portions of the filtrate were placed in K medium and plated on liver infusion agar plates. The K medium was prepared from fresh hog intestines according to the method recommended by Kendall (38) 1951. A 3.0 per cent suspension was made in Tyrode solution and tubed in 10 cc. amounts. The tubes were autoclaved at 15 pounds steam pressure for 20 minutes and incubated to check sterility. The K medium tube and the liver infusion agar plate were used as controls to check the sterility of the bacteriophage filtrate at the time of its preparation. The broth culture containing Sal. gallinarum and the bacteriophage filtrate was filtered by diluting it with 20 cc. of sterile physiological salt solution and filtering under a negative pressure of 100 to 150 mm. of Hg. The time of filtration was less than three minutes.

At the time the bacteriophage filtrate was planted in K medium, 8 to 10 cc. of the filtrate was also placed in a sterile cotton stoppered test tube. These 60 tubes constituted the material for studying the effect of rapid serial plate transfer on bacteriophage filtrates aged in K medium.

Immediately after preparing, these tubes were incubated at 37°C. for 72 hours, then at room temperature until the completion of the experiment. In the studies made on these filtrates, the ones aged in K medium will be designated as series a and the others as series b. All tubes

were sterile macroscopically at the time the rapid serial plate transfer method was applied to each tube of both series, which represented periods of aging from 1 to 30 days. The procedure consisted in placing 1.0 cc. of the material in each tube of series a and b on a sterile veal infusion agar plate which had been checked for sterility. The filtrate was spread over the surface of the agar with a sterile L-shaped glass rod. After incubating the seeded plates at 37°C. for 24 hours they were examined at a magnification of 100 diameters for the presence of colonies; the surface of the agar was then washed with 2.0 cc. of sterile veal infusion broth. One cc. of this washing was transferred to a sterile veal infusion agar plate. This procedure was repeated until the colonies appeared. Those were fished and planted in sterile veal infusion broth for further study and identification. Where no growth could be discovered the series was continued for at least 16 transfers.

Data in Table 16 show that growth could be obtained from only 16 of 60 macroscopically sterile filtrates to which rapid serial plate transfers had been applied. If K medium enhances the growth of G forms, colonies should be obtained from filtrates aged in K medium a shorter period of time than from the corresponding filtrates aged alone. This, however, was not the case, indeed no regularity of appearance was noted in either series a or b, thus the occurrence of colonies on the rapid serial plates from filtrates aged alone and in K medium must be

Table 16. Growth Obtained by Rapid Serial Plate Transfer of Sal. pullorum Aged in K Medium.

Filtrate Number	Number of days aged	Growth obtained by serial plate transfer	
		K medium + filtrate Series a	Filtrate alone Series b
1	30	-	-
2	29	-	-
3	28	-	-
4	27	-	-
5	26	-	-
6	25	-	-
7	24	-	-
8	23	-	-
9	22	-	-
10	21	+	-
11	20	-	-
12	19	-	-
13	18	-	-
14	17	+	-
15	16	-	-
16	15	-	+
17	14	-	+
18	13	+	+
19	12	-	+
20	11	+	-
21	10	-	+
22	9	-	-
23	8	+	+
24	7	+	-
25	6	-	-
26	5	+	+
27	4	+	-
28	3	+	-
29	2	-	-
30	1	-	-

+ = Growth obtained by rapid serial plate transfer

- = No growth obtained by rapid serial plate transfer

attributed to other factors than aging. It is moreover not dependant on the medium used. Data in Table 17 show that there were three main types of colonies recovered:

Type 1 was a translucent, colorless colony measuring about one mm. in diameter, consisting of short rods which were Gram negative. These were probably filtrable forms of M. mullorum and passed the filter in either the normal form or in one which quickly reverted to the normal, because they were recovered on the first transfer from the tubes of series a, aged 5 and 11 days, and from the tubes of series b, aged 13 and 14 days.

Type 2 - The majority of cultures recovered formed translucent, colorless colonies, measuring from 0.01 mm. to 0.1 mm. in diameter at the end of the incubation period at 37°C. The organisms were minute Gram positive cocci. These were very similar to the G types described by Hadley et al. (23) 1931 and Luror (24) 1931 but after standing one or two weeks at room temperature the colonies became yellow and opaque, measuring 2 to 4 mm. in diameter, consisting of cocci, in the arrangement of tetrads which were Gram positive. These organisms resembled those of the genus Larcia and were probably introduced from the air during the rapid serial plating technic.

Type 3 formed spreading, white, and opaque colonies. The organisms contained central endospores. These organisms were unidentified and were probably either air contaminants or were present on the agar plate before it was seeded with the filtrate, but due to their slow growth did not appear until after the plate had been incubated several

Table 17. Culture Types Obtained by Rapid Serial Plate Transfer of Lacteriophage Filtrates of Sal. pullorum used in K Medium.

Culture Number	Growth on serial plate trans-fer number	Diameter of colony in mm. after incubat-ing 72 hours	Color of colony after 1 week at room temperature	Gram stain and shape of organism	Type and remarks
10a	2	0.01-0.05	2.0 yellow	+ cocc1	2 sarcina
14a	2	0.01-0.1	3.0 yellow	+ cocc1	2 sarcina
10b	2	0.1	2.0 yellow	+ cocc1	2 sarcina
17b	1	0.1	0.5 colorless	- rods	1 sal. pullorum
13a	4	0.01	2.0 white	+ rods	3 spore former
10b	1	0.1	0.5 colorless	- rods	1 sal. pullorum
10b	2	0.01	2.0 yellow	+ cocc1	2 sarcina
20a	1	0.01	0.5 colorless	- rods	1 sal. pullorum
21b	4	0.01	2.0 yellow	+ cocc1	2 sarcina
23a	3	0.1	2.0 yellow	+ cocc1	2 sarcina
22b	6	0.1	2.5 yellow	+ cocc1	2 sarcina
24a	4	0.1	2.0 yellow	+ cocc1	2 sarcina
20b	3	0.1	4.0 white	+ rods	3 spore former
20a	1	0.01	0.5 colorless	- rods	1 sal. pullorum
27a	3	0.01	2.0 white	+ rods	3 spore former
20a	2	0.01-0.1	2.0 white	+ rods	3 spore former

Culture number - numeral refers to filtrate number, letter to series of Table 16.

days longer than the period used for checking sterility.

With the exception of the four cultures in type 1, the organisms recovered did not revert to Sal. mullorum. The aged filtrates themselves were lytic for Sal. mullorum, while filtrates of the organisms recovered by rapid serial plate transfer were not. All of the colonies of type 2 and 3 obtained closely resembled G colonies upon their first appearance and during subsequent rapid serial plate transfer, but upon standing at room temperature for one to two weeks their appearance was greatly changed and ordinary types of colonies arose from the microcolonies. When these G colony forms of ordinary bacteria arose it was difficult to differentiate them from the G colonies described by Hedley (25) in 1951. The rapid serial plate transfer technique was suspected of having been the source of the colonies obtained from the filtrates. For this reason a check experiment was planned which eliminated this factor.

2. Attempts to Demonstrate G Forms by subculture plate technique in K medium bacteriophage filtrates of Sal. mullorum

In part 1 the presence of colonies resembling G forms, as demonstrated by rapid serial plate transfer in the bacteriophage filtrates of Sal. mullorum aged in K medium, was studied. Part 2 demonstrates the absence of G forms in the K medium bacteriophage filtrate of Sal. mullorum when the influence of the rapid serial plate

transfer method was eliminated. The preparation of the filtrates was similar to that outlined previously. To duplicate series of K medium and plain nutrient beef extract broth, pH 7.7, tubes, previously seeded with Sal. pullorum, was added bacteriophage filtrate in amounts of 0.1 and 1.0 cc. Also 1.0 cc. of the filtrate of each of the 4 series was added to sterile plain nutrient beef extract broth, pH 7.7, sterile K medium (Difco), a sterile cotton stoppered test tube, a glass sealed ampule, and a veal infusion agar plate checked for sterility by incubation. These cultures served as a control of the sterility of the bacteriophage filtrate at the time of filtration. The first three mentioned also furnished the material for the experiment. After 24 hours' incubation at 37°C. the K medium and plain broth tubes containing the bacteriophage filtrate and Sal. pullorum were filtered through Berkofeld N candles and Seitz filters previously checked for their ability to retain Sorr. marcescens. A total of 10 transfers were made. The K medium and control tubes containing both Berkofeld and Seitz filtrates remained free from macroscopic changes for six months; thus we may assume that neither ordinary contamination nor Sal. pullorum was present in the filtrate at the time of filtration or subsequently.

As mentioned above, the results of part 1 indicated that the rapid serial plate transfer method might be susceptible to contamination. For this reason a variation in treatment of the filtrates was made. In place of using

the rapid serial plate transfer method, subculture plates were made at intervals of four weeks from each of the tubes of the four series of the bacteriophage filtrates aged in K medium and of the controls. These plates were examined for growth after being incubated for 48 hours at 37°C. and again after standing two weeks at 25°C. No growth was obtained on any of the subculture plates made either from the K medium or the controls. Although in part 1 cultures aged for a similar period showed growth when the method of rapid serial plate transfer was applied. These data indicate that colonies resembling G types may appear from the extraneous sources of contamination inherent in the rapid serial plate transfer technic.

3. Attempts to demonstrate G forms in the bacteriophage filtrates of Staph. aureus

The material for this experiment was obtained from 10 daily transfers of the bacteriophage of Staph. aureus. Bacteriophage filtrate in amounts of 0.1 and 1.0 cc. was added to plain nutrient beef extract broth, pH 7.7, previously seeded with Staph. aureus. One cc. amounts of the filtrate were placed in sterile K medium (Difco), sterile cotton stoppered test tubes, and on sterile veal infusion agar plates. After 24 hours' incubation at 37°C. the tubes containing bacteriophage and Staph. aureus showing the greatest lytic action were filtered and the filtrate treated as above. This was repeated 10 times. The filtrate in sterile K medium and in the cotton stoppered test tubes (control) was incubated at 37°C.

for 72 hours then at room temperature until the completion of the experiment. No visible growth had appeared in either series during an incubation period of six months. At intervals of four weeks subculture plates were made, which were incubated for 48 hours at 37°C. followed by one week at 25°C. No growth was obtained from the subculture plates of either series.

Summary of Section B.

1. Bacteriophage filtrates of Staph. aureus and Sal. pullorum aged in K medium together with controls in plain nutrient beef extract broth, pH 7.7, and in cotton stoppered test tubes were studied for the presence of G forms.

2. Organisms forming microcolonies (X types) resembling G forms were obtained by using rapid serial plate transfer technic on the bacteriophage of Sal. pullorum aged in K medium. These upon aging were shown to be slow growing air contaminants.

3. The presence of G forms in the bacteriophage filtrates of Sal. pullorum or Staph. aureus could not be demonstrated by subculturing portions of the filtrate, aged in K medium or in controls, on sterile veal infusion agar plates at intervals of four weeks.

4. It was shown that rapid serial plate transfer must be applied with caution and be carefully controlled for accurate results.

The Production of Microcolonies of Bacteria by Physical Influences

1. The effect of temperature on colony size.

Hadley (23) in 1931 advised that plates containing inoculum of the G types of organisms should be incubated for 1 to 10 days at 37°C. before examination for the formation of G colonies. The cultures with which he worked possess an optimum temperature of 37°C. as do the cultures in Sections A and B of this paper. Brueckner and Sherman (7) 1932 reported the isolation of G or primitive forms of bacteria by a method of dilution. They advised that the tubes be incubated for 72 hours at 37°C. and then placed at 25°C. for two weeks before they were plated.

To determine the effect of temperature on the size and structure of bacteria grown at other than their optimum temperature, 16 cultures were selected for study that were obtained from experiments on the bacteriophage of Sal. pullorum. Five cultures of the G forms of Sal. aertrycke obtained from Lurox (54) 1931 and 1 culture of Aero. aertrycke dissociated on 0.25 per cent lithium chloride veal infusion broth. These cultures were planted into veal infusion broth, incubated 24 hours at 37°C., and streaked on veal infusion and litmus lactose agar plates. These plates were incubated for 72 hours at 37°C., followed by two weeks at 25°C. The average diameter of the

colonies of each culture was measured at the end of 72 hours and at the end of the two weeks' incubation period. The data in Table 18 show that there is a marked difference in the colony size before and after the incubation period at room temperature. Several of the organisms very closely resemble Sarcina lutea. All of the colonies at the end of the incubation at 37°C. resembled G forms in size and structure but were greatly changed at the end of room temperature incubation. Duplicate plates incubated only at room temperature showed normal size colonies at the end of 72 hours. This suggested the use of a pure culture of Sar. lutea in an effort to determine the effect of temperature on colony formation, because it is a slow growing organism with an optimum temperature of approximately 25°C. Two plates were streaked with a 24 hour culture of this organism. One was incubated at 37°C. for 72 hours and at room temperature for 14 days; the other was incubated at room temperature for 17 days. The results are given in Table 19. It can be seen that similar results were obtained with a pure culture of Sar. lutea as with several of the suspected G forms from various sources. Bacteria under adverse temperature conditions may produce micro- or H colonies. This experiment offers an explanation of the phenomena observed with G cultures of Eurox page 10.

2. The influence of rapid serial plating on colony size. Two cultures were selected, Serr. indica having an optimum temperature of 25°C. When these organisms are grown under their optimum temperature requirements they

Table 18. The Effect of Temperature on the Structure and Size of Colonies.

Culture Number	Broth '24 hours	Colony diameter in millimeters		Gram stain	Organism
		72 hours 37°C.	14 days 25°C.		
10a	clear	0.01-0.05	2 mm.	+ cocci	
14a	clear	0.01-0.1	2 mm.	+ cocci	
16b	clear	0.01	2 mm.	+ cocci	
17b	cloudy	0.1	2 mm.	- rods	Sal. pullorum
18a	clear	0.01	2 mm.	+ rods	
18b	cloudy	0.1	2 mm.	- rods	Sal. pullorum
19b	clear	0.01	2 mm.	+ cocci	
20a	cloudy	0.1	3 mm.	- rods	Sal. pullorum
21b	clear	0.01	3 mm.	+ cocci	
23b	clear	0.01	3 mm.	+ cocci	
24a	clear	0.01	3 mm.	+ cocci	
26b	clear	0.1	3 mm.	+ rods	
26a	cloudy	0.1	2 mm.	- rods	Sal. pullorum
27a		0.01	2 mm.	+ rods	
28a		0.01	2 mm.	+ rods	
17p	clear	0.01-0.1	2 mm.	+ cocci	
18p	clear	0.01-0.1	2 mm.	+ cocci	
19p	clear	0.01-0.1	2 mm.	+ cocci	
20p	clear	0.01-0.1	2 mm.	+ cocci	
21p	clear	0.01-0.1	2 mm.	+ cocci	
22p	clear	0.01-0.1	2 mm.	+ cocci	
23p	clear	0.01-0.1	2 mm.	+ cocci	

Table 19. The Effect of Temperature on the Colony Size
of Sar. lutea.

Plate 1			Plate 2		
24 hour broth culture	Colony diameter after 72 hours 27°C.	14 days 22°C.	24 hour broth culture	Colony diameter after 72 hours 22°C.	14 days 22°C.
clear	less than 0.01 mm.	3 mm.	clear	2.5 mm.	3 mm.

form approximately the same size colonies, about 1 to 3 mm. in diameter. The protocol consisted in running serial plate transfers on each culture at 37°C. and at 25°C. The cultures were planted into plain beef extract broth. One cc. of the latter was placed on a sterile veal infusion agar plate after 24 hours' incubation and smeared with a sterile L-shaped glass rod. The plates were incubated for 24 hours and examined for colony formation. They were then washed with 5.0 cc. of sterile veal infusion broth and the washings were diluted 1 to 10,000. One cc. of the dilution was placed on a sterile veal infusion agar plate, and smeared with an L-shaped glass rod. This was repeated 12 times. Sar. lutea formed microcolonies at 37°C., and normal types at room temperature. Sarr. indica formed normal size colonies at room temperature and at 37°C., but after the second transfer the colonies at 25°C. lost their pigment forming properties. Data in Table 20 show that rapid serial transfer apparently has little effect on colony size except when temperature is also a factor. The important application lies in the fact that in the serial plate transfer of Landurey (26) 1927 and Hadley (23) 1931 plates are incubated at 37°C. Sar. lutea in particular and other air contaminants in general having an optimum temperature of 25°C. under the conditions of this technique are liable to form microcolonies which may be easily confused with the true G type.

Table 20. The Influence of Rapid Serial Plate Transfer on Colony Size.

Day of transfer	<u>Serr. indica</u>		<u>Sar. lutea</u>	
	37°C.	25°C.	37°C.	25°C.
1	'2 mm.(red)'	'2 mm.(red)'	'M (0.01 mm)'	'3 mm.'
2	'2 mm.(red)'	'2 mm.(colorless)'	'M (0.01 mm)'	'3 mm.'
3	'2 mm.(red)'	'2 mm.(colorless)'	'M (0.01 mm)'	'3 mm.'
4	'2 mm.(red)'	'2 mm.(colorless)'	'M (0.01 mm)'	'3 mm.'
5	'2 mm.(red)'	'2 mm.(colorless)'	'M (0.01 mm)'	'2 mm.'
6	'2 mm.(red)'	'2 mm.(colorless)'	'M (0.01 mm)'	'1 mm.'
7	'2 mm.(red)'	'2 mm.(colorless)'	'M (0.01 mm)'	'1 mm.'
8	'2 mm.(red)'	'2 mm.(colorless)'	'M (0.01 mm)'	'1 mm.'
9	'2 mm.(red)'	'2 mm.(colorless)'	'M (0.01 mm)'	'1 mm.'
10	'2 mm.(red)'	'2 mm.(colorless)'	'M (0.01 mm)'	'1 mm.'
11	'2 mm.(red)'	'2 mm.(colorless)'	'M (0.01 mm)'	'1 mm.'
12	'2 mm.(red)'	'2 mm.(colorless)'	'M (0.01 mm)'	'1 mm.'

3. The effect of selective plating on colony size. A culture of Serr. indica was planted in plain beef extract broth and in 0.25 per cent lithium chloride veal infusion broth. These tubes were incubated 24 hours at 37°C. and streaked on sterile litmus lactose and veal infusion agar plates with a straight needle. The plates were incubated 24 hours at 37°C. and examined at a magnification of 100 diameters; the largest and the smallest colonies were transferred to plain beef extract broth and to 0.25 per cent lithium chloride veal infusion broth. These tubes were incubated 24 hours and restreaked. This process was repeated 12 times. Observations were made on the colony size and structure throughout the experiment. Data in Table 21 show that the only characteristic tendency is toward a median size of colony. Cultures which produced a greater number of colonies of 0.1 mm. when picked to broth and streaked on agar plates may show a majority of 3.0 mm. colonies. The same effect holds true if a colony of 3.0 mm. is picked from a plate containing a maximum of this size; there the tendency is towards the smaller colony size. There was no apparent permanent effect on colony size produced by selective plating.

4. Comparison of serial plating transfer and serial test tube transfer. The filtrates of the bacteriophage of the 21st and 23rd transfers of Sal. mullorum grown in bacteriophage were planted in 1.0 cc. amounts in K medium and in veal infusion broth. Together with the filtrates, they were incubated for 72 hours at 37°C. At the end of

Table 21. The Influence of Selective Plating on Colony Size.

Date of plate readings	Plated on									
	Mittus lactoso agar					Veal infusion agar				
	Plain broth					Plain broth				
	Original size					Large small				
	Colony size on plates in ml.					Colony size on plates in ml.				
	large	small	large	small	large	large	small	large	small	large
6/16/1932	3.0	0.1	3.0	0.1	3.0	0.1	0.1	3	0.1	
6/16/1932	1.0	0.3	2.0	0.2	1.0	0.2		2	0.3	
6/20/1932	0.1	3.0	0.1	3.0	1.0	1.0		1	1.0	
6/22/1932	0.1	2.0	1.0	0.5	3.0	0.1		3	0.1	
6/24/1932	3.0	3.0	1.0	2.0	3.0	1.0		1	1.0	
6/26/1932	1.0	1.0	1.0	1.0	1.0	1.0		1	1.0	
6/28/1932	3.0	3.0	0.1	3.0	0.1	0.0		3	0.1	
6/29/1932	0.1	3.0	3.0	0.1	3.0	0.1		3	1.0	
7/7/1932	3.0	0.1	0.1	2.0	0.5	3.0		2	1.0	
7/9/1932	2.0	0.5	0.1	3.0	1.0	3.0		1	3.0	
7/11/1932	1.0	1.0	3.0	3.0	3.0	1.0		2	1.0	
7/13/1932	3.0	0.1	3.0	0.1	1.0	3.0		2	3.0	

this time they showed no growth visible to the naked eye.

One cc. of the material in each tube, K medium, veal infusion broth, and the filtrate, were placed on a sterile veal infusion agar plate and smeared with a sterile L-shaped glass rod. After 24 hours' incubation the surface of the agar plate was covered with 2.0 cc. of the sterile veal infusion broth. This was rotated carefully to mix thoroughly, one cc. of the washing being transferred to another sterile veal infusion agar plate and the process repeated until growth appeared or until 16 transfers had been made.

At the same time 1.0 cc. of K medium, of veal infusion broth, and of the filtrate was placed on sterile veal infusion agar slants. These were rotated carefully and incubated 24 hours at 37°C. Two cc. of sterile veal infusion broth was added to each tube. One cc. was transferred to a sterile veal infusion agar plate and 1.0 cc. to a sterile veal infusion agar slant. The tube and plate were incubated 24 hours at 37°C. At the end of this time the broth was added to the tube and the plate inspected for colonies. Each day a new series was also started. Any colonies discovered on the plates were planted on K medium and into veal infusion broth. Part of the tubes were placed at room temperature and the remainder at 37°C. At the end of three days' incubation they were streaked on sterile veal infusion agar plates and incubated at the respective temperatures. At the end of 48 hours microcolonies were found on the plates incubated at 37°C., while duplicate plates at room temperature showed yellow,

pigmented colonies 2 to 4 mm. in diameter. These colonies were composed of Gram positive cocci occurring in tetrads, and were obtained only from the rapid serial plate transfers and not from the test tube series which remained sterile throughout the experiment. The results are given in Table 21. This experiment does not prove that true G forms can not be obtained by test tube transfers, but that this method is not so susceptible to extraneous contamination as the rapid serial plate transfer method.

Summary of Section C.

1. Adverse temperature conditions were shown to favor the production of microcolonies.

2. Rapid serial plating had little effect on colony size when considered alone, but when temperature was also a factor, the production of microcolonies was favored.

3. Selective plating was shown to have little effect on colony size.

4. Serial test tube transfer was shown to be free from many of the difficulties inherent in the rapid serial plate transfer method.

Table 22. Comparison of the Rapid Serial Plate Transfer and Test Tube Transfer Methods.

Transfer number	Rapid Serial Plate transfer	Rapid Test Tube transfer series 1 to 10
1	-	-
2	-	-
3	-	-
4	Film	-
5	Film	-
6	Film	-
7	M	-
8	M	-
9	transfers discontinued	-
10		-
11		-
12		-
13		-
14		-

- = no growth

M = microcolonies

(D)

Studies on the Filtration of Bacteria

The removal of bacteria by passing the suspending liquid through a membrane or structure impermeable to the ordinary forms of microorganisms is of value in preparing water and other liquids for human consumption, in sterilizing thermolabile substances, purifying filtrable viruses, and in preparing vaccines, toxins, etc. However, the literature is replete with references which indicate that in many instances there may be failure of numerous types of filters to prevent the passage of microorganisms, and ^{to} the difficulties which are encountered in their use. Hesse (31) 1856 confirmed by Pflaeg (60) 1886 stated that clay and asbestos filters retained microorganisms, preventing direct transmission, but that they allowed the organisms to grow through the pores permitting indirect transmission. v. Samarch (15) 1902 presented photomicrographs of stained sections of filters which showed the course of the bacteria between the pore walls.

An excellent discussion of the history and technic of filtration is given by Hauduroy (27) 1929. He mentions some of the following filters.

The Pasteur-Chamberland filter composed of dense biscuit porcelain was the first one prepared which was more or less impermeable to bacteria. Fulloch and Caw (3) 1906 stated that these filters retained Sapp. purcoscons during five days continuous filtration, but that they allow

indirect transmission of bacteria.

Forkofold filters made of diatomaceous earth were produced in three graded pore sizes, N (normal), V (coarse), and W (fine). Killech et al. (9) 1908 stated that they permit bacteria, Shorthella typhi and Shig. dysenteriae, to pass in 50 per cent of the instances studied.

Handker filters, similar in structure to Forkofolds, were produced in the United States during the World War because of the difficulty of obtaining the latter. Filters have also been made from inorganic substances other than siliceous material, e.g., plaster of Paris, etc. Collodion sacs, parchment membranes, and colloidal jelly filters have likewise been constructed. The chief disadvantage of the Seitz filter, fitted with asbestos discs, is the difficulty of preventing contamination due to air leaks.

There have been many theories advanced as to the reason why bacteria are retained by filters or permitted to pass. These may be classified in four groups:

1. Properties of the filter, 2. Properties of the organism, 3. Properties of the medium of suspension, and 4. The technic of filtration.

1. Properties of the filter.

- a. The electrical charge of the filter. There exists between the filter and the surrounding liquid an electrical field, the Helmholtz-Lamb layer. Fudd and Fudd (52) 1924, Eliava and Smaroz (14) 1927 stated that

colloidion membranes possessed a positive electric charge when an acid substance was filtered and a negative potential with an alkaline mixture. Kramer (37) 1927 found that filters of siliceous earth were negatively charged and did pass certain organisms. By making filters with plaster of Paris, a calcium sulfate and calcium carbonate mixture, the charge became positive and the filter retained the organisms filtrable through the siliceous earth filters. A neutral filter did not retain any of the substances filtered. Kramer (32) 1929 reported the construction of an amphoteric filter, that is one capable of removing both positively and negatively charged colloids and particles. This was made by adding a water insoluble, thermostable basic material, FeO , carrying a positive charge to the siliceous material in the filter. He stated that this would retain the virus of tobacco mosaic, bacteriophage, and filtrable bacteria.

b. The method of testing the filter. Prohisher (21) 1926 found that testing filters by air pressure was inadequate. Filters tested by this method allowed organisms to pass.

c. Adsorption. Eliava and Suarez (14) 1927 reported that adsorption played an important part in the ultra-filtration of bacteriophage through colloidion membranes. The first 50 cc. contained no bacteriophage; later fractions contained the lytic agent with the same titer as the residue. They reported more adsorption in a positive suspension than

in a negative. Fudd (53) 1927 reported that adsorption played an important part in filtration. Larkum and Normes (39) 1930 reported that bacteriophage was adsorbed to the protein fraction.

d. Mechanical retention. Fudd (51) 1923 reported that mechanical retention due to organisms being caught among the pores of the filter assisted in their removal from the suspension.

e. Pore size. Fudd (51) 1923 reported that Vibrio parcolana did not filter through Berkefeld N filter candles under a pressure of 0.0 to 76.0 cm. of Hg but did through Berkefeld V filter candles.

2. Properties of the organism. Fudd (53) 1927 found size, motility, and flexibility to be very important in the filtrability or retention of organisms.

3. Properties of the medium of suspension.

a. Hydrogen ion concentration. Larkum and Normes (39) 1930 reported that bacteriophage was removed by Fandler and Seitz filters from suspensions at pH 4.5 to 5.0 and pH 9.0 to 10.0, by plaster of Paris filters at pH 7.0, but passed at pH 4.5 to 5.0.

b. K medium. Kendall (36) 1931, Varney and Ironfong-brenner (66) 1932 reported that cultures in K medium passed more quickly and in larger amounts than in ordinary culture media. A similar effect was obtained by merely suspending the organisms in K medium. They claimed that this was not due to fat, but to adsorbed proteins.

4. The technic of filtration. The time, pressure,

volume filtered, and manipulation of the process of filtration is very important. Probisher (21) 1923 attributes ultrabacteria or filtrable forms to poor filters, technical error, or imperfect autoclaving. Eliava and Suarez (14) 1928, Bronfenbrenner and Muckenfuss (6) 1927, Rudd (53) 1927, and other authors reported retention of bacteria followed by passage as volume filtered was increased. Others have reported passage of bacteria with increased pressure.

Many authors did not give sufficient data concerning the method and conditions of filtration used in filtering a particular organism to permit a complete interpretation of their results. Thus we discover in the literature conflicting reports on the filtrability of various organisms visible through the microscope in their classical morphological state. In many instances we may assume that due to one or more of the reasons previously outlined that passage of viable bacteria was accomplished. Among the organisms reported as being filtrable or having filtrable forms associated with them are:

<u>B. cereus</u>	Andervont and Simon (2) 1924
<u>B. diphtheriae</u>	Hellon (46) 1921
<u>B. fusiformis</u>	Hellon (47) 1926
<u>B. of Johne's disease</u>	Morin and Valtis (49) 1926
<u>B. of Schweinsauche</u>	Lourens (42)(43) 1907
<u>B. pestis</u>	Burnet (10) 1926
<u>B. tuberculosis</u>	Fontes (19) 1910, Valtis (65) 1924, Durand (13) 1924, Durand and Vaudremer (12) 1924, Arloing and Dufort (3) 1925, Veber (68) 1926
<u>B. typhosus</u>	Almquist (1) 1911, Friedberger and Meissner (20) 1923, Hauduroy (24)(25) 1924, & Herelle and

	Hauduroy (29) 1925, Wojgin (17) 1925
<u>Diplococcus reninitidis</u>	Hort (32) 1917
<u>Lontospira icteroides</u>	Hoguchi (56) 1919
<u>Spirocheta recurrentis</u>	Kovv and Knapp (59) 1906, Ireinl and Kinghorn (5) 1906, Nicolle and Blanc (57) 1914, Wohlbach (69) 1915
<u>Spirocheta elusa</u> and <u>biflora</u>	Wohlbach (69) 1915
<u>Streptococcus</u> from <u>encephalitis</u>	Rosonow (61) 1924, Evans (16) 1923
<u>Streptothrix</u>	Mellon (45) 1920
<u>E. coli</u>	Izar (34) 1921, d'Herelle (28) 1922, Hauduroy (24)(25) 1924, Tomassoli (64) 1923
<u>E. dysenteriae</u> <u>Shiga</u> , <u>Flexner</u> , and <u>Liss</u>	d'Herelle (28) 1922, Hauduroy (24)(25) 1924, d'Herelle and Hauduroy (29) 1925, Hadley (23) 1931
<u>E. nestis</u>	d'Herelle (30) 1926
<u>Staphylococcus albus</u> and <u>aureus</u>	d'Herelle and Hauduroy (29) 1925
<u>Mycobacterium tuberculosis</u>	Fonis (4) 1923, Mellon and Jost (42) 1924, Hollis (56) 1926, Fossi (62) 1926, Sweeney (63) 1923, Vasilin and Irminciu (67) 1926
<u>E. proteus</u> <u>X₁₉</u>	Wojgin (17) 1924

The filtrations reported in this paper may be divided into two classifications, those in which small amounts of material were used and those involving larger amounts.

1. Filtrations of amounts involving less than 40 cc. of material. The conditions under which the materials were filtered is very important. Two types of filters were used: Berkefeld V (coarse), N (normal), and W (fine) and Seitz. The Berkefeld filters (2 1/2 inches by 3/4 inch) were tested for cracks and leaks under air pressure. Filters satisfactory by this test were cleaned as previously

outlined. The fittings were applied to the filter flask and the entire apparatus tested again. It was then sterilized and tested as outlined on page 7.

All cultures filtered were diluted three to five times with sterile physiological salt solution, placed in the filter aseptically, and filtered under conditions of temperature and pressure, such that one to two drops of filtrate passed per second. The filtrate was collected in sterile test tubes, inserted in the filter flask (Plate 1), which were removed and incubated at 37°C. for 48 to 72 hours, at 25°C. for one week or longer, and the rapid serial plate transfer or the streak subculture plate method was applied. The series of filtrations reported extended over a period of approximately 12 months, consisting in tests on a number of different organisms from a variety of sources, and under different methods of preparation.

A. Filtration of Sal. mullorum and Staph. aureus grown in the presence of bacteriophage

Daily filtrations of Sal. mullorum grown in the presence of bacteriophage were made through Seitz filters. The filtrates were placed in sterile R medium and sterile cotton stoppered test tubes. After suitable periods of aging the rapid serial plate transfer methods were used; on another series of filtrations of Sal. mullorum and Staph. aureus bacteriophage streak plate subcultures were used.

Data in Table 23 show that in 40 filtrations where the method of rapid serial plate transfer was applied a

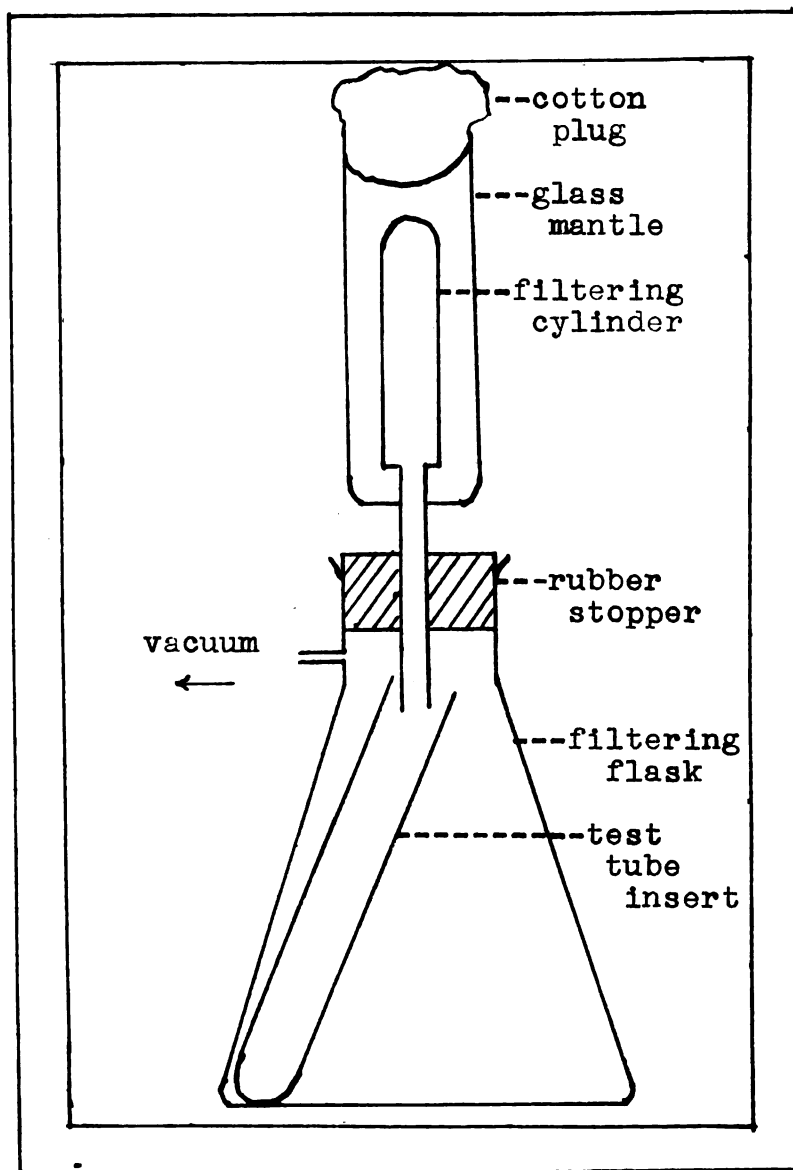


Plate 1. Apparatus for collecting small amounts of filtrate. (1/2)

Table 22. Growth Recovered from the Bacteriophage Filtrates of Sal. pullorum.

Number of filtrations	Cultures recovered by rapid serial plate transfer		Cultures recovered on subculture plates	
	Sal. pullorum filtrate'K medium'	contamination filtrate'K medium'	Sal. pullorum filtrate'K medium'	contamination filtrate'K medium'
40	2	2	5	7
	1	1	1	1
			2	2
			1	1
			0	0

filtrable form of Sal. pullorum was recovered in 4 instances and contamination in 12 instances, also that where streak plate subcultures were used no contaminating organisms were recovered. In a similar series of Staph. aureus filtrations like results were obtained. A discussion of the forms obtained was presented in Section B - Attempts to demonstrate the presence of G forms in the bacteriophage filtrates of Sal. pullorum and Staph. aureus.

B. Filtration or organisms recovered by rapid serial plate transfer from filtrates of Sal. pullorum grown in bacteriophage

The 16 organisms obtained in part 1 (including organisms shown to be contaminations), together with 5 cultures of the G forms of Sal. aertrycke obtained from Hurox, and 4 H cultures of Aero. aerogenes dissociated on 0.25 per cent lithium chloride veal infusion broth, were planted into veal infusion broth, and incubated 24 hours at 37°C. The cultures were diluted with 24 cc. of sterile physiological salt solution and filtered through Seitz filters. The filtrates were placed in sterile K medium, veal infusion broth, and sterile cotton stoppered test tubes. After suitable periods of incubation rapid serial plate transfers were applied. Data obtained showed that of 25 cultures filtered over 80 per cent of the filtrates remained sterile. After the methods of rapid serial plate transfer were applied, cultures were recovered from each filtrate after the first to fifth transfers. The Gram

stain and the sugar reactions of the cultures recovered were different from those filtered in each case. After a period of 16 months aging at room temperature, these organisms have not yet recovered the Gram stain or sugar reactions of the original organisms filtered. It is probable that these organisms represented contaminations and were the result of the rapid serial plate technique because they were not obtained on the streak plate sub-culture plates.

C. The effect of K medium on the filtrability of Esch. coli

Esch. coli was planted into 20 cc. of K medium and plain beef extract broth; these tubes were incubated 24 hours at 37°C. The same filters, Forksfold H, V, and W, and Boitz, were used for each filtration series. They were cleaned, tested for air leaks, and sterilized, as given before, after each filtration. The following series of filtrations were made:

- a. A tube of sterile K medium followed by a plain beef extract broth culture
- b. A K medium culture
- c. A tube of sterile beef extract broth followed by a K medium culture
- d. A plain beef extract broth culture
- e. A suspension in physiological salt solution
- f. A suspension in K medium

One cc. of the filtrates was placed in K medium, plain beef extract broth, lactose beef extract broth, a

sealed ampule, and a sterile cotton stoppered test tube. No cultures of Esch. coli were recovered from any of the six filtration series. Apparently K medium does not enhance the filtrability of Esch. coli in amounts less than 40 cc.

D. Filtration of combined cultures of Esch. coli and Serr. indica.

Cultures of Esch. coli and Serr. indica were planted to separate tubes of media. After 24 hours' incubation at 37°C. these were combined, diluted to 40 cc. with sterile physiological salt solution, and filtered through a Berkefeld N candle. The filtrate was placed in duplicate in lactose beef extract broth, plain beef extract broth, sterile cotton stoppered test tubes, and on dextrose beef extract agar.

Data in Table 24 show that Serr. indica was retained when both organisms were grown in plain beef extract broth, was filtrable when Esch. coli was grown in K medium, and when both were grown in K medium. Esch. coli was retained in all three cases.

E. Filtration of miscellaneous cultures

Cultures were selected at random from the collection of stock cultures used for class work in elementary bacteriology. These were planted into K medium (Difco) and plain beef extract broth, incubated at 37°C. for 24 hours, diluted with 20 cc. of sterile physiological salt solution, and filtered through Berkefeld N and Seitz filters according to the procedure previously outlined.

Table 24. Filtration of Combined Cultures of Esch.
coli and Serr. indica.

<u>Esch. coli</u> grown in	<u>'Serr. indica'</u> in	Time in 'minutes'	'Pressure' in 'mm. of Hg'	Recovered	
				<u>'Esch. coli'</u>	<u>'Serr. indica'</u>
Plain broth	'Plain broth'	4	-160	-	-
K medium	'Plain broth'	15	-260	-	+
K medium	'K medium'	10	-300	-	+

Volume filtered 40 cc.

The filtrates were placed in plain beef extract broth, K medium (Difco), lactose beef extract broth, sealed ampules, sterile cotton stoppered test tubes and dextrose beef extract agar plates. These were incubated for 48 to 72 hours at 37°C., for two weeks at 25°C. and streak plate subcultures were made. Growth due to contamination was present in 3.0 per cent of the 300 filtrations made on stock cultures. The per cent growth resulting from filtrations in this experiment was considerably less than that reported by Probsthor (21) 1928. The latter was able to obtain as high as 40 per cent growth from filtrates of bacteriophage of sterile broth and approximately 20 per cent from filtrates passed through air tested filters.

Summary of filtrations using small amounts of suspending medium.

1. A total of 496 filtrations were made using less than 40 cc. of suspending medium. Growth was obtained by the technic of rapid serial plate transfer or streak plate subculture from approximately 3.0 per cent of the filtrates. The cultures recovered from these filtrates consisted of either filtrable forms or K colonies.

2. True G forms were not recovered from the filtrates of cultures suspended in less than 40 cc. of material.

3. K medium was shown to enhance the filtrability of Sorr. indica in the presence of Esch. coli.

4. Refiltration of the forms recovered gave only sterile filtrates.

2. Fractional filtration of amounts involving more than 40 cc. of material

Bullock et al. (9) 1908, Budd (53) 1927, Ironfenbrenner and Muckenfuss (6) 1927, and Hadley et al. (23) 1931 used fractional filtration in investigations of the filtrability of microorganisms. As this method of filtering offers slightly more difficulties in manipulation it should have advantages which the ordinary method does not possess. Fractional filtration makes it possible to compare the filtrability of identical or different microorganisms under the same or varied conditions, especially with reference to a determination of the moment of passage of the organism.

The apparatus used for making the filtrations was constructed from glass water stills, Liebig's condensers, (Plate 2). A ring of glass was removed from the middle of the outer jacket, the central core was cut transversely, and made one inch shorter than the jacket. The filter was placed in a one-hole rubber stopper and fitted to the outer jacket so that the outlet projected into the central core. The small end of the jacket was inserted into one end of a six inch section of heavy vacuum rubber tubing, to the other end was fastened a sterile media tubing shield, Hellmann (44) 1931. A clamp was placed in the middle of the tubing. The inner jacket was calibrated to deliver 10 cc. and the apparatus cleaned and sterilized. To show that the apparatus excluded all sources of extraneous contamination, 400 cc. of sterile plain beef extract broth

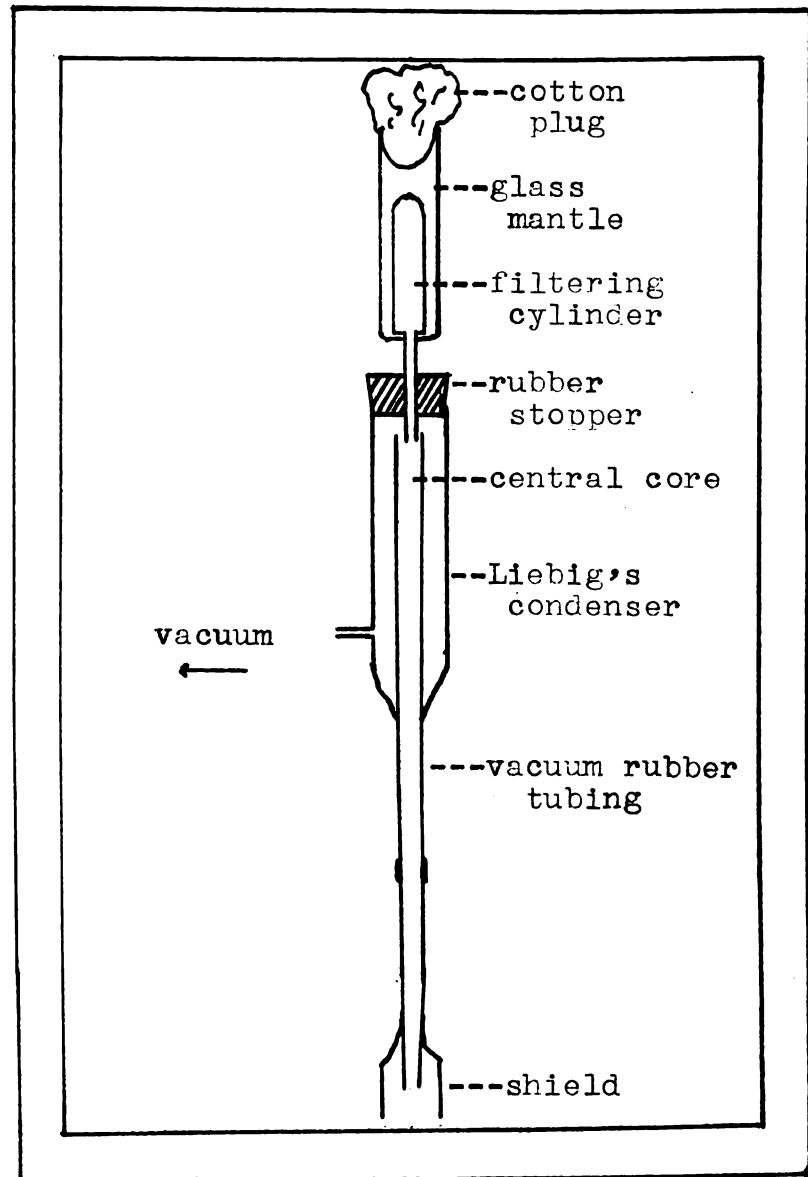


Plate 2. Apparatus used for fractional filtration. (1/4)

was filtered in 10 cc. amounts (barometric pressure 100 mm. Hg, time of filtration of each fraction 1 to 2 minutes). These fractions were incubated at 37°C. for three days and at room temperature for two months. No growth appeared in any of the fractions.

A. Effect of subculturing filtrate fractions

Fromfenbrenner and Duckenfass (6) 1927 reported that so few bacteria passed the filter pores that the entire fraction had to be incubated in order to demonstrate viable organisms. A filtration was made to determine whether this phenomenon could be observed using 10 cc. filtrate fractions of a plain beef extract broth culture of Esch. coli. The culture was planted into 200 cc. of plain beef extract broth, pH 7.0. This was incubated 24 hours at 37°C. and filtered fractionally through a Forkedfold H filter. The 10 cc. fractions were incubated in toto, and at the end of one week the material in each tube was streaked on eosin-methylene blue agar plates, from which typical colonies were transferred to lactose beef extract broth. The lactose tubes showing gas formation were planted into Kosser's sodium citrate medium and checked for Esch. coli. Table 25 shows that Esch. coli was recovered from the eighth to twelfth fraction inclusive.

The effect of subculturing the filtrate to other media at the time of filtration was demonstrated as follows. Esch. coli was transferred to 200 cc. of plain beef extract broth, incubated 24 hours at 37°C., and filtered through the same filter as was used in the previous experiment.

Table 25. Growth Obtained from Fractions
Incubated in Toto.

Fraction of 10 cc.	Each, coli recovered from plain broth filtrates
1	-
2	-
3	-
4	-
5	-
6	-
7	-
8	+
9	+
10	+
11	+
12	+

Time of filtration of each fraction = 3 minutes

Filtration pressure = -200 mm. Hg

One cc. of each 10 cc. fraction was transferred immediately after filtering to plain beef extract broth, K medium, lactose beef extract broth, a sealed ampule, to plain beef extract agar plates, and the remaining portion of the filtrate to a sterile cotton stoppered test tube. These were incubated 48 hours at 37°C. and for two weeks at 25°C. Data in Table 26 show that Bach. coli was recovered from the eighth to twelfth fraction inclusive. Thus little or no difference in the growth appearing from the filtrates could be shown whether the fraction was incubated in its entirety or by subcultures in various media.

There was no apparent macroscopic difference in the appearance of the fraction at the moment of collection nor in the manner in which it came through the filter. It was impossible to tell which fraction would remain sterile and which would later show growth.

B. Effect of K medium on the filtrability of Bach. coli.

Kendall (38) 1931 reported that K medium enhanced the filtrability of microorganisms. Ironfontronner and Puckenfuss (6) 1927 reported that organisms grown in K medium passed the filter more quickly and in larger amounts than those grown in plain beef extract broth, also that subcultures grew more quickly in K medium than in plain beef extract broth. We have already shown that this is not necessarily true when small amounts of material are filtered. The following series of experiments were planned to demonstrate the presence or absence of this effect when

Table 26. Growth Obtained from Subcultured Filtrate Fractions.

Fraction	Each, coli recovered after 1 week incubation in	of 10 cc.	plain	K	lactose	sealed	filtrate	colonies on	broth	medium	broth	ampule	alone	plain agar
1	-	-	-	-	-	-	-	0						
2	-	-	-	-	-	-	-	0						
3	-	-	-	-	-	-	-	0						
4	-	-	-	-	-	-	-	0						
5	-	-	-	-	-	-	-	0						
6	-	-	-	-	-	-	-	0						
7	-	-	-	-	-	-	-	0						
8	-	-	-	-	+	+	+	0						
9	+	+	+	+	-	+	+	3						
10	+	+	+	+	+	+	+	8						
11	+	+	+	+	+	+	+	20						
12	+	+	+	+	+	+	+	45						

larger amounts of material were filtered fractionally.

To show whether or not K medium has an effect on the filtrability of a microorganism, Bach. coli was planted in 200 cc. each of K medium and plain beef extract broth. These were incubated 48 hours at 37°C. and filtered through a Berkefeld B filter. The fractions were collected aseptically in 5.0 cc. amounts and incubated at 37°C. for 72 hours. Extreme difficulty was experienced in filtering the K medium. The time for each fraction increased rapidly as did also the air pressure necessary. In both cases the rate of filtration did not exceed one drop per second nor fall less than one drop in 10 seconds. No air bubbles were apparent. The appearance of the filtration was normal. The point at which the organisms passed could not be foretold at the time the filtration was completed. The data presented in Table 27 show that Bach. coli grown in K medium passed the filter after 25 cc. of material had been filtered, however, growth was delayed for one week. Organisms which passed after 55 cc. had been filtered grew out in 48 hours. Organisms grown in plain beef extract broth did not pass until 80 cc. had been filtered. It is thus apparent that K medium does have a marked effect on the filtrability of this organism. In order to demonstrate whether this effect is due to a change produced in the organism itself or to the mere physical presence of the K medium, growth was transferred from plain beef extract agar slants to K medium until a suspension of the same turbidity as in the previous experiment

Table 27. Comparative Filtrability of Esch. coli Grown in Plain Beef Extract Broth and in K Medium.

Fraction of 5 cc.	Time of Filtration		Pressure in cc.	Esch. coli recovered from			
	P.B.	K.M.	K.M.	P.B.	Plain broth filtrate 48 hours	K medium filtrate 48 hours	1 week
1	3	2	5	20	-	-	-
2	3	5	11	20	-	-	-
3	3	8	16	20	-	-	-
4	3	12	24	20	-	-	-
5	3	14	40	20	-	-	+
6	3	12	30	20	-	-	+
7	3	27	40	20	-	-	+
8	3	50	40	20	-	-	+
9	3	25	40	20	-	-	+
10	3	45	74	20	-	-	+
11	3		74	20	-	+	+
12	3		74	20	-	+	+
13	3		74	20	-	+	+
14	3			20	-	-	-
15	3			20	-	-	-
16	3			20	+	+	+

P.B. = plain broth
 K. M. = K medium
 + = recovered
 - = not recovered

was obtained. This suspension was filtered fractionally and the filtrate placed in the various media in 1.0 cc. amounts. Data presented in Table 28 show that Esch. coli passed the filter when 55 cc. of material had been filtered, this was about half way between the amount for plain beef extract broth culture and K medium culture. The effect was not so marked when K medium was merely present as when Esch. coli was actually grown in the material.

C. Attempts to determine the factors responsible for the effect of K medium on the filtrability of Esch. coli.

The next experiments were designed to determine if possible what factor present in K medium would have the effect of increasing the filtrability of organisms especially with reference to Esch. coli. In order to eliminate the filter clogging due to gross particulate material a 48 hour culture was filtered through sterile absorbent cotton, then fractionally through a Berkefeld N filter. The filtrate was incubated in 5.0 cc. fractions. Data presented in Table 29 show that the clumps present in K medium neither increase nor decrease markedly the filtrability of Esch. coli. To eliminate the possibility of the physical properties of the K medium and to establish the fact that the soluble or filtrable portions of this substance had an effect on the filtrability of the organism two companion experiments were attempted.

Table 28. Fractional Filtration of Esch. coli
Suspended in K Medium.

Fraction of 5 cc.	1		2		Esch. coli recovered from the filtrates of	
					1	2
	T	B.P.	T	B.P.	Plain broth culture	K medium suspension
1	3	20	2	5	-	-
2	3	20	2	5	-	-
3	3	20	2	10	-	-
4	3	20	2	10	-	-
5	3	20	2	15	-	-
6	3	20	3	15	-	-
7	3	20	3	15	-	-
8	3	20	3	15	-	-
9	3	20	3	15	-	-
10	3	20	4	15	-	-
11	3	20	5	20	-	+
12	3	20	5	20	-	+
13	3	20			-	
14	3	20			-	
15	3	20			-	
16	3	20			+	

T = Time in minutes

B.P. = Barometric pressure in centimeters

Table 29. Effect of Clumps of K Medium on Filtrability of Esch. coli.

Fraction of 5 cc.	1		2		Esch. coli recovered			
	T	B.P.	T	B.P.	Cleared with cotton		Not cleared with cotton	
					48 hours	1 week	48 hours	1 week
1	1	5	2	5	-	-	-	-
2	1	7	5	11	-	-	-	-
3	1	10	8	16	-	-	-	-
4	2	20	12	24	-	-	-	-
5	3	20	14	40	-	-	-	-
6	3	30	12	30	-	-	-	-
7	5	40	27	40	-	-	-	-
8	20	40	50	40	-	+	-	+
9	30	40	25	40	+	+	-	+

T = Time in minutes

B.P. = Barometric pressure in centimeters

Two hundred cc. of sterile K medium was filtered through a sterile Berkefeld N filter. This was followed by a 24 hour culture of Esch. coli in plain beef extract broth. The data in Table 30 show that although the organisms passed more quickly than when plain beef extract broth cultures are filtered alone, it did not compare with the filtration of a culture grown in K medium. The K medium filtrate from the above experiment was collected in a sterile container and seeded with Esch. coli, incubated for 24 hours and filtered fractionally. If any difference is noted in the filtrability of the organisms under these conditions, it must be due to a single factor, that is, a change produced in the organism itself by soluble, or at least filtrable, portions of K medium. Data in Table 31 show that the clumps of insoluble material have no effect on increasing the filtrability of Esch. coli. Indeed, the organisms grown in the sterile filtrate of K medium passed the filter more quickly than those grown in unclarified K medium.

D. Fractional filtration of combined cultures of Esch. coli and Sorr. indica.

Esch. coli and Sorr. indica were selected to show the effect of the presence of another organism on the filtrability of bacteria. One hundred cc. of plain beef extract broth was seeded with Esch. coli and a like amount with Sorr. indica. After being incubated at 37°C. for 24 hours the cultures were combined and filtered fractionally.

Table 30. Effect of Clogging Filter with Sterile K Medium on Filtration of Esch. coli.

Fraction of 5 cc.	1		2		Esch. coli recovered			
	T	B.P.	T	B.P.	Plain broth		K medium	
					alone		first	
					24	48	24	48
					hours	hours	hours	hours
1	3	20	2	5	-	-	-	-
2	3	20	2	5	-	-	-	-
3	3	20	2	10	-	-	-	-
4	3	20	2	10	-	-	-	-
5	3	20	2	15	-	-	-	-
6	3	20	3	15	-	-	-	-
7	3	20	3	15	-	-	-	-
8	3	20	3	15	-	-	-	-
9	3	20	3	15	-	-	-	-
10	3	20	4	15	-	-	-	-
11	3	20	5	20	-	-	-	+
12	3	20	5	20	-	-	+	+
13	3	20	5	40	-	-	-	-
14	3	20			-	-	-	-
15	3	20			-	-	-	-
16	3	20			+	+	-	-

T = Time in minutes

B.P. = Barometric pressure in centimeters

Table 31. Filtration of Esch. coli grown in filtered sterile K Medium.

Fraction of 3 cc.	T	B.P.	Esch. coli recovered from				
			broth	K medium	lactose	ampule	agar
1	2	5	-	-	-	-	0
2	2	10	+	+	+	+	15
3	4	10	+	+	+	+	469
4	5	10	+	+	+	+	529
5	5	10	+	+	+	+	584
6	8	20	+	+	+	+	645
7	10	30	+	+	+	+	786
8	15	30	+	+	+	+	892
9	20	30	+	+	+	+	904
10	20	74	+	+	+	+	1310

T = Time in minutes

B.P. = Barometric pressure in centimeters

The following combinations were filtered. (1) Both grown in plain beef extract broth, (2) Esch. coli in K medium, Serr. indica in plain beef extract broth, (3) Both in K medium.

Data in Table 32 show that in combination (1) Esch. coli passed in the ninth fraction, Serr. indica was retained; (2) Esch. coli was retained, Serr. indica passed in the fifth fraction; (3) Both organisms passed in the third fraction.

Serr. indica was probably more filtrable than Esch. coli. K medium was shown to enhance the filtrability of both organisms, however, its mere presence was sufficient to cause the passage of Serr. indica, while Esch. coli had to be grown therein, in order to effect the passage. The passage of Esch. coli in the ninth fraction (1) was probably not significant for the fact that it was beyond the range of the other combinations.

E. The effect of aging Esch. coli in K medium.

A culture of Esch. coli grown in K medium at room temperature for 43 days showed no apparent change in filtrability from a 24 hour culture. The data in Table 33 show that the organisms passed the filters in direct ratio to the graded pore size of the particular filter used. Seitz filters were shown to retain the organisms for the longest period of time.

In Table 34 is given a summation of the various experiments on the effect of K medium on the filtrability of Esch. coli. These data show that a soluble or filtrable

Table 32. Fractional Filtration of Combined Cultures of Esch. coli
and Serr. indica.

<u>Esch. coli</u> <u>Grown in</u>	<u>Serr. indica</u> <u>Grown in</u>	<u>Time in</u> <u>minutes</u> <u>elapsed</u> <u>until</u> <u>pressure</u>	<u>Pressure</u> <u>in</u> <u>mm. of Hg.</u>	<u>Volume</u> <u>of each</u> <u>fraction</u>	<u>Recovered in fraction No.</u>
Plain broth	Plain broth	69	-260	10 cc.	9
K medium	Plain broth	67	-400	10 cc.	5
K medium	K medium	29	-160	10 cc.	3

Table 34. Fractional Filtration of Esch. coli.

Table number	Description of the conditions of filtration	Moment of passage in co.
25	Plain broth filtrate fraction incubated in toto	80
26	Plain broth filtrate fraction subcultured	80
27	K medium (grown in)	25
28	K medium (suspended in)	55
29	K medium (cleared through cotton)	40
30	Filter clogged with sterile K medium followed by a plain broth culture	55
31	Grown in K medium previously filtered through a Berkefeld B filter	10

substance present in the medium affects the filtrability of the organism. From an examination of this table it is apparent that there is present in K medium a soluble, or filtrable, substance which has a marked effect on the filtrability of Esch. coli, Table 31. The mere presence of K medium will cause this effect to a lesser extent as shown by Tables 28 and 30. The gross particles present in the suspension inhibit the filtrability, Tables 28, 29 and 30. The effect on the organism may be sufficient to overcome this inhibition, Table 27 or 28.

Summary of Section D.

1. In a series of approximately 500 filtrations involving amounts less than 40 cc. growth was obtained in three per cent of all cases.
2. Refiltration gave sterile filtrates.
3. K medium enhanced the filtrability of Serr. indica in the presence of Esch. coli.
4. The effect of K medium on the filtrability of Esch. coli was shown by a series of fractional filtrations.
5. A soluble or filtrable portion of K medium was shown to enhance the filtrability of Esch. coli.
6. The solid, gross, particulate material inhibited this effect.
7. Aging of Esch. coli in K medium before filtration had no effect on the filtrability of the organism.

Summary and Discussion

(A)

1. Attempts were made to produce G forms by spontaneous and enforced dissociation of various bacteria undergoing rapid transfer and aging in a series of media. Both organisms and media were selected so that there was presented a wide variation of factors; pH of medium, concentration of lithium chloride, dye, characteristics of organisms, etc. In many cases colonies which bore a striking resemblance to the G colonies reported by Hadley were discovered on plates seeded with cultures in the various stages of rapid transfer. These colonies generally appeared after the fourth or sixth transfer. Because the organisms forming the microcolonies could not be demonstrated as being filtrable, they were labelled M colonies according to the terminology introduced in this paper. The M colonies formed during the stages of dissociation of the various organisms did not appear with the regularity noted by Hadley or Purox. The data did not show any relationship between the production of M colonies and any of the various factors considered. There seems to be indications that the appearance of M colonies in dissociative phenomena may be due to an inherent characteristic of the organism, rather than to external influences which may not be strong enough to break down the sturdy mechanism which controls the dissociation of the bacterial cell.

2. The technic of serial dilution and fractional filtration was applied to a series of six samples of sewage

and four samples of skim milk. The report of Iraceclnor and Shorman that primitive forms were present in milk in vastly greater numbers than were ordinary bacteria was not confirmed. No H colonies or filtrable forms were discovered. The number of samples studied was not large enough to justify conclusions as to the occurrence of primitive forms of bacteria in milk, however, there is a suggestion that many of the organisms recovered may be contamination resulting from the very delicate technique employed, that is, rapid serial plate transfer.

(B)

Serial bacteriophage filtrates lytic for Sal. pullorum and Staph. aureus were studied by rapid serial plate transfers and by streak plate subcultures for the presence of G forms. The filtrates were aged in sterile cotton stoppered test tubes and in K medium. H colonies resembling G colonies were obtained by the rapid serial plate transfer method from 16 of the 60 filtrates. There was no regularity as to their appearance. Three main types were found, two of which were air contaminants. When streak plate subcultures were used no colonies were recovered. The data suggest that K medium in this particular instance had no effect on the production of G colonies from the filtrates of bacteriophage. There is also an indication that rapid serial plate transfer is responsible in many cases for the production of G and similar forms of bacteria.

(C)

The production of H colonies, which simulate the appearance of G colonies, by various physical influences, temperature, rapid serial plating, and selective plating was studied. The data show that bacteria having an optimum temperature of approximately 25°C. may form H colonies if grown at higher temperatures. This is of special importance because the technic for demonstrating G forms requires the incubating of plates at 37°C. Neither rapid serial plate transfer nor selective plating were shown to have marked effects on the production of G forms or H forms of bacteria. Data obtained when rapid serial plate and test tube transfers were run coincidentally showed that the former was very susceptible to contamination. It is suggested that the serial test tube transfer be substituted because of its comparative freedom from contamination.

(D)

A review of some of the filters with their disadvantages and faults which have been used for removing bacteria from suspensions and a discussion of the theories of filtration are presented. Approximately 500 filtrations were made using less than 40 cc. of suspension. The report of Prohisher was not confirmed. Only three per cent growth was obtained from these filtrations consisting of Sal. pullorum and Staph. aureus grown in the presence of bacteriophage, organisms grown in K medium, combined cultures

of Esch. coli and Sour. indica and miscellaneous cultures. Prohisher has reported as high as 40 per cent growth in filtrates of bacteriophage or of sterile broth. The data indicate that under proper conditions and controls, and where the capacity of the filter is not exceeded, sterile filtrates may be expected in practically all cases. Refiltrations of the organisms recovered (three per cent of the total filtered) gave sterile filtrates.

A study of fractional filtration was made with special reference to determining the effect that K medium exerts on the filtrability of Esch. coli. It was demonstrated that there is present in K medium a soluble or filtrable fraction which increases the filtrability of Esch. coli to a marked extent, while the gross particulate matter has a slight inhibitory effect. Similar results were shown whether the filtrates were incubated in toto or in subcultures. No particular medium was demonstrated as possessing the ability to decrease the period of time necessary for the appearance of growth. In many cases one to three weeks elapsed before macroscopic growth appeared in filtrates or before it could be demonstrated by streak plate subculture methods. It is well known that in many cases single cells obtained by the various single cell techniques require periods of one or more weeks for growth. We suggest that, as was demonstrated by the data presented in Section D, only one or two organisms may pass the filter. There would then be present in the

filtrate conditions, except for the excess of suspending medium, essentially the same as found in single cell technic and the extended time period would be expected. This is very likely the condition in the earlier fractions where growth is delayed, while in later fractions where greater numbers pass through the filter pores growth is obtained in 24 to 48 hours. This is demonstrated by the data of many of the fractional filtrations. A 43 day old culture of Esch. coli and a 24 hour culture possessed identical filtrability properties.

Constantly but haphazardly throughout the experiments presented there have been observed microcolonies. These were similar in size, appearance, and behavior to the G colonies reported by Hadley and others. There was also a striking similarity in the morphology of the organisms. Lönnis and Smith mentioned that gonidia were microscopic granules formed by bacterial cells in one stage of their reproductive cycle. The gonidiangia upon disintegration released the gonidia which then grew into normal size cells. Other authors postulate that from the gonidia were produced colonies microscopic in size, G colonies, which after suitable technic would form normal colonies coincident with the formation of the normal cells. In the literature may be found scattered references stating that the gonidia are filtrable but no mention is made as to the size of colonies formed from them. Thus microscopic demonstration of the granules, proof of their filtrability, and ability

to form normal cells rather than size of colony is the criterion for G types. Kendall reported filtrable forms but did not give evidence of the presence of gonidia.

Thus in literature much confusion as to the terminology exists, and the difficulty of determining precisely with what particular phase or phenomenon the author is concerned is apparent. More exact descriptions and definitions of the terms applied would clarify the situation and with this intention we again offer a terminology which it is hoped will aid rather than add to the confusion.

1. G colonies formed from organisms in the gonidiangia phase, possessing filtrable gonidia, which upon the application of suitable technic will produce colonies microscopic in size. The various reactions of the culture are different from the normal form.

2. Filtrable forms - organisms capable of passing filters under carefully controlled conditions but which revert so quickly to the normal form that colonies microscopic in size are not produced.

3. H colonies - organisms normal in form which produce colonies microscopic in size due to the influences of adverse physical conditions.

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A Critique on the Filtrability of Bacteria.

(Abstract)

The term M colony was introduced to distinguish between colonies closely resembling the G types, reported by Hadley, except for filtrability, which were produced haphazardly by rapid transfer and aging 7 species of microorganisms in 7 different media, and the true G type. No relationship could be established between the appearance of the M colonies and the pH of the media, concentration of chemical (LiCl), dye, characteristics of the organism, or the state of dissociation.

The serial dilution method of Brueckner and Sherman did not demonstrate the presence of primitive forms or G types in sewage or skim milk.

The appearance of M colonies from the bacteriophage filtrates of Salmonella pullorum and Lactylosoccus aureus upon the application of the rapid serial plate transfer method of Mandurey was not dependent upon the factor of aging but apparently upon the method of technique itself. Similar series of bacteriophage filtrates to which streak subculture plates were applied remained sterile.

Adverse temperature conditions were shown to favor the production of M types. Rapid serial plating, selective plating, had no effect upon the production of M types.

An historical review of filtration and the factors influencing filtration were presented.

It was shown that when amounts less than 40 cc. were filtered through Berkefeld filters (2 1/2 x 3/4 inch) sterile filtrates were obtained in 97 per cent of 500 filtrations.

The method of fractional filtration was applied to the study of the effect of K medium on the filtrability of Esch. coli. A filtrable or soluble portion of K medium was shown to enhance the filtrability of this organism.

The following terminology was used to differentiate the three forms of organisms commonly encountered in filtrations. 1. G colonies - formed from organisms in the conidiangia phase, possessing filtrable conidia, which upon the application of suitable technique will produce colonies microscopic in size. The various reactions of the culture are different from the normal

form. 2. Filtrable forms - organisms capable of passing filters under carefully controlled conditions but which revert so quickly to the normal form that colonies microscopic in size are not produced. 3. B colonies - organisms normal in form which produce colonies microscopic in size due to the influences of adverse physical conditions.

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