TITLE THE NUTRITION AND FACTORS AFFECTING THE GROWTH OF BACTERIA IN SOLUBLE OILS

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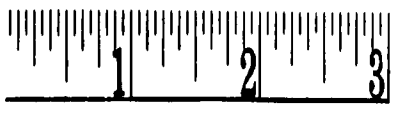

THE NUTRITION AND FACTORS AFFECTING THE GROWTH OF BACTERIA IN SOLUBLE OILS

by

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A THESIS

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ACKNOWLEDGEMENTS

Scientic matters are readily expressed in easily understood terms which have very exact meanings but there are no words which can possibly express the depths of a man's thoughts. It is with this in mind that I humbly offer the following acknowledgements.

To Dr. F. W. Fabian for his wise and experienced guidance in the pursuit of these studies and for personal counselling in the daily struggle, I am most grateful. I am likewise greatly indebted to the many men in industry without whose help this project could not have been carried out: such men as Dr. Edwin Place, Industrial Physician, and Mr. L. Garlieb, Lubrication Engineer, of the Oldsmobile Division, General Motors Corporation, Lansing, and Dr. E. W. Adams and Mr. A. W. Lindert of the Standard Oil Company (of Indiana) for materials, emulsion samples and advice; to the great number of chemical and pharmaceutical companies for generously supplying materials for investigation; to my friends in the Ontario Department of Health, C. Christie, A. Miller and M. Magis, for emulsion samples and cultures; to the "media room" in the bacteriology department for expertly filling the many orders for media.

It is an especial pleasure to acknowledge the cheerful encouragement of my wife--a silent but stimulating partner in these many research projects.

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Introduction

The ability of bacteria to utilize hydrocarbons has been studied by microbiologists for over a half of a century, but it is only recently that the subject has received the attention which it deserves. The work of Zobell and others has emphasized the role that microorganisms play in the breakdown and, to a smaller degree, in the synthesis of petroleum — an urgent study in a mechanized world.

As a basis for orientation towards the work which follows, some of the fundamental aspects of the petroleum industry will be discussed after which a description of cutting oils, especially soluble oils, will be given.

Refining of Petroleum

Crude petroleum exists in subterranean deposits usually in the free or liquid state but also often in close association with sands or shale. When a well is drilled, the petroleum is forced to the surface by gas stored under high pressure in association with the subterranean oil pools. If the gas pressure is not adequate or becomes depleted with time, then other methods must be used to bring the oil to the surface.

The crude oil is often in the form of an emulsion with brines which are chemically similar to sea water; and before they are refined, the emulsion must be broken to separate the petroleum from the water.

The refining procedure may be divided into two phases, (a) the distillation and fractionation of the petroleum to break the complex mixture into its desired components and (b) the treatment of these components to

remove undesirable materials. The distillation depends on the heating of the crude oil with fractionation to produce the various "cuts". Vacuum may be used. The number of fractions depends on the manufacturer's equipment and market demands. However, the fractions may be roughly grouped as gasoline, kerosene, diesel oils, fuel oils, lubricating oils, asphalt and road oils. Treatment of the oils following distillation is usually necessary to remove undesirable compounds which are unstable or malodorous. The most common method of treatment is to mix the fraction with concentrated sulfuric acid to form a sludge of petroleum sulfates with the unsaturated hydrocarbons and also to precipitate organic bases, phenols, organic sulfur compounds and other undesirable substances. After the sludge has been removed, the residual acid is neutralized with sodium hydroxide and washed with water. A few other methods of treatment include extraction of undesirable compounds with various solvents such as furfural, acrolein and nitrobenzene and treatment with materials such as sodium plumbite or cupric chloride to convert foul-smelling mercaptans to non-smelling disulfides.

Chemistry of Petroleum

The chemistry of petroleum is so complex that even today, after many decades of intensive research, there are huge gaps in our knowledge concerning the nature of many of its components. The composition of crude oil varies in different oil fields and even in wells in the same fields. Indeed, a single well may produce crude oil of varying composition at different times.

Generally crude oils are classified according to their components into paraffin base, asphaltic (naphthene) base and mixed base. The paraffin base crudes consist predominately of aliphatic hydrocarbons with some cyclic compounds while the asphaltic base consists mainly of naphthenic or saturated ring structures with some aliphatics. The mixed base crude oils contain a more equal mixture of the two. If wax is present, the crude oil is called "wax-bearing".

The aliphatic hydrocarbons are mixtures of saturated and unsaturated, straight and branched chains. The carbocyclics or ringed compounds are of two general classes; the aromatics (unsaturated compounds) and the cycloparaffins or naphthenes (saturated compounds). Both classes of carbocyclics may or may not have aliphatic side chains of varying number, size and complexity.

Cutting Fluids

Cutting fluids are lubricating and cooling compounds used to facilitate metal-cutting operations. The best lubricating action is furnished by 100 per cent fatty oil such as lard oil or rape seed oil, while the best cooling action results from 100 per cent water. Petroleum oils lack the lubricating properties of fatty oils and the cooling properties of water. However, they are the most common component of cutting fluids because of their cheapness, rust-preventing characteristics, relative inertness and ability to be synthesized into compounds with good lubricating properties.

Cutting fluids have been classified by many workers, but they may be conveniently divided into three groups:

(a) The alkaline solutions which may consist of a soap solution, with or without added base; or they may be solutions of such substances as borax, sodium carbonate or trisodium phosphate. The use of such cutting fluids is limited principally to cooling and laying dust in grinding operations.

(b) The soluble or emulsifiable oils. These oils consist of mineral oils, with or without fatty oils, that have been compounded with emulsifying agents to make them miscible with water. A few emulsifying agents are saponified fatty acids, abietic acid, petroleum sulfonates, phenols and naphthenates. Mixed with water these soluble oils are used in cutting and grinding operations where cooling requirements predominate, but where lubricating and anti-corrosive activity are also required.

(c) The straight oils. These oils consist of mineral oils, with or without fatty oils. They are used where greater lubrication than that afforded by soluble oil emulsions is required.

All oils used, whether mineral or fatty, may or may not be treated chemically or contain additives to improve their lubricity. Such treatment is employed to increase the film strength of lubricants which are used in high pressure work and also to give anti-weld properties to the oils. The commonest chemical treatment is with sulfur or chlorine.

Additives which are used may contain arsenic or phosphorous (5). These compounds, under extremes of temperature and pressure, form iron oxides which serve as a lubricating film when the organic lubricant breaks down and thus prevent welding of chips to the tools.

Bacteria are not known to grow in straight oils as water is necessary for their existence. However, they do grow in the soluble or emulsifiable oils (62, 36, 34, 25) when these have been mixed with water. Growth in this medium is thought to be responsible for several undesirable occurrences such as obnoxious odors, discoloration of the emulsion and an increase in acidity with resultant breaking of the emulsion. There is also considerable opinion, but no proof, that these bacteria are responsible for dermatitis of workers in contact with the oils. For these reasons studies of the flora of soluble oil emulsions and factors which affect their growth have been undertaken.

PART I. METHOD USED IN DETERMINING NUMBERS OF BACTERIA IN SOLUBLE OILS

Review of Literature

It is true, but nonetheless unfortunate, that much quantitative bacteriology is carried on with little regard for suitability of the diluent and media employed. Workers in water and milk bacteriology have well established methods (3) (4) based on considerable research, but in many other fields there are no authoritative works to guide the researcher. It has been observed that some laboratories use distilled water, tap water, 50-50 mixtures of the two or physiological saline

with apparently little consideration for the pH or osmotic pressure of the material being investigated. Supposedly, the ideal diluent would be some of the material being investigated. But since such diluents are rarely feasible, it is desirable to use a diluent which will have the least effect on the bacteria being investigated.

Zobell (64), searching for a diluent which would be suitable for marine bacteria, found that the bacteria in marine mud perished rapidly in tap water, distilled water, 0.85 per cent saline and a solution formulated upon the average composition of river water. Using the counts obtained when autoclaved sea water was used as a diluent as 100 per cent, he found that when sea water and distilled water were mixed in varying ratios, there was the least survival in those diluents having the greatest proportion of distilled water. The mixture giving the greatest survival at 60 min contained 75 per cent of sea water and 25 per cent of distilled water. In work with petroleum products, there has been a variety of methods employed for growing and enumerating the bacteria concerned. Stone, Fenske and White (54) used nutrient agar as a plating medium because this medium gave slightly higher counts than a mineral-salt oil agar and because all organisms which they transferred from the latter medium grew when transferred to nutrient agar. They did not mention the diluent which they used. Bushnell and Haas (15) used a mineral-salt agar containing hydrocarbons but did not mention the diluent. In investigations of soluble oil emulsions Lee and Chandler (34) used distilled water as a diluent and nutrient agar as a plating medium while

Liberthson (36) mentions neither the medium nor the diluent. Duffett, Gold and Weirich (25) used the standard agar plate method for obtaining counts.

Experimental

The experiments reported here embraced a study of materials and methods for obtaining plate counts of bacteria in soluble oils. However, the following procedure was common to all this work. Media were held at 45°C before pouring; unless otherwise stated, duplicate plates were poured; decimal dilutions were so made that one ml of suspension was added to each petri dish; dilution bottles were shaken 25 times by hand; plates were incubated at 30°C for 48 hours and the colonies counted on a Quebec colony counter.

Media tested were nutrient agar (Difco) and nutrient agar to which 1 per cent of a soluble oil had been added. The diluent was distilled water. Nine samples of soluble oil emulsions from industrial sources were plated and duplicate plates poured with each of the media. Three samples were plated with a medium composed of 1.5 per cent agar and 1 per cent of a soluble oil.

Diluents tested were those frequently encountered in bacteriological work; distilled water, 0.85 per cent saline, M/20 phosphate buffers and M/20 phosphate buffers containing 0.85 per cent saline. Buffers ranged from pH 7.0 to pH 8.0 in 0.2 pH unit increments while buffered salines were used at pH 7.0, 7.6 and 8.0.

The inoculum used for investigation of diluents was usually a mixture of 20 different soluble oil emulsions obtained from industrial sources. Occasionally, as noted, individual samples were used. All samples were refrigerated at 50°F upon arrival and held at this temperature.

The following procedure was used to minimize the amount of work and equipment necessary for the experiments with diluents. Plate counts using nutrient agar, with distilled water as a diluent, were made on refrigerated samples of emulsions. When the bacterial count was obtained, a portion of the sample was removed from the refrigerator and so diluted that a one ml portion would give plates ideal for counting. Plate counts in quadruplicate, of the final suspension were made at one minute and at intervals thereafter. The average of the four plates was used to calculate the percentage change which occurred after one minute.

Results and Discussion

Table 1 shows the effect of adding one per cent of soluble oil to nutrient agar. Six of the nine samples had higher counts on the nutrient agar without oil, and five of the six counts were significantly higher. Since the soluble oil produced a very opaque medium when added to nutrient agar, probably the decreased count in the presence of the oil was due to masking of colonies rather than inhibition of growth by the oil. Several colonies growing on the nutrient agar with oil grew well when fished to nutrient agar without oil.

Plating media composed only of distilled water, 1.5 per cent agar and one per cent soluble oil showed no growth after two days at 30°C.

Table 1

A comparative count of bacteria in soluble oil emulsions using nutrient agar and nutrient agar containing one per cent soluble oil as plating media.

	Bacteria per ml	
Sample	Nutrient agar	Nutrient agar + oil
1	760,000	310,000
2	2,200,000	1,040,000
3	2,710,000	1,620,000
4	2,065,000	805,000
5	244,350,000	295,600,000
6	14,350	13,300
7	78,400	82,200
8	16,500	8,250
9	750	1,450

Table 2 shows the effect of distilled water, as a diluent, on organisms in soluble oil emulsions. There was a definite decrease in bacterial population of Sample 6 and Mixture A with increased time. However, the changes in Sample 1 and Mixtures B and C are within experimental error (43). Table 3 is representative of two experiments identical in all respects except that in the experiment not shown counts were also made after 15 mins. Both experiments showed identical trends. Physiological saline was decidely toxic. Buffer at pH 7.0 gave the most consistent results

Table 2

Per cent change in number of bacteria from soluble oil emulsions suspended in distilled water

Emulsion	Per cent change						
	5 min	15 min	30 min	60 min	90 min	120 min	240 min
Sample 1	- 8.1	- 2.8	2.5	-25.5	-	1.7	- 4.6
Sample 6	-12.5	- 7.0	-18.6	-22.9	-	-33.1	-52.2
Mixture A	-	-	-11.5	-23.0	-	-	-
Mixture B	-	- 7.8	- 5.1	- 5.3	- 9.7	-	-
Mixture C	-	-	13.7	5.0	1.6	-	-

- = no experiments made

Table 3

Per cent change in number of bacteria from mixture of soluble oil emulsions suspended in various diluents

Diluent		Bact. per ml at 1 min	Per cent change 30 min	60 min	90 min
1 Distilled water		160	13.7	5.0	1.6
2 0.85 per cent saline		171	-33.9	-85.4	-96.4
3 Buffer	pH 7.0	165	3.0	1.2	2.4
4 Buffer	pH 7.2	165	- 1.2	-12.1	2.4
5 Buffer	pH 7.4	211	- 0.9	6.2	3.8
6 Buffer	pH 7.6	172	-14.5	- 8.1	4.6
7 Buffer	pH 7.8	157	5.7	19.1	9.5
8 Buffer	pH 8.0	158	3.2	0.6	-17.7
9 Buffered saline	pH 7.0	77	118.2	106.6	62.4
10 Buffered saline	pH 7.6	51	233.4	263.0	202.0
11 Buffered saline	pH 8.0	89	28.1	74.2	76.4

with a mean deviation for the two experiments of 2.6 per cent from the count at one minute. Buffers at the other pH's gave deviations which were generally within experimental error. However, buffered saline gave results totally unlike those given by saline alone or buffer alone. Although all diluents received identical inocula, the mean count at one minute in diluents 1 to 8 inclusive was 170 per ml; while in diluents 9 to 11 inclusive, buffered salines, the mean count was 72 per ml. This indicated that buffered salines caused a rapid decrease in bacterial population as determined by the plate count. The decrease was not due to a killing effect because at 30 minutes and thereafter the counts were considerably greater than at one minute. This would definitely indicate that the buffered saline caused an immediate clumping, but that on standing, the clumps of bacteria broke apart. The buffer had a sparing activity towards the toxic effect of sodium chloride because in one experiment there were six bacteria per ml in diluent 2 (0.85 per cent saline) at 90 minutes while diluents 9, 10 and 11 (buffered salines) had a mean count of 145 bacteria per ml at the same time. The other experiment showed similar results.

A rather extraordinary phenomenon involving diluents is included in these studies as an example of some of the difficulties encountered. An emulsion containing 0.05 per cent of parachlorometacresol, inoculated with a mixed inoculum, when plated after 68 days, yielded about 6,000 colonies if 0.1 ml of emulsion were plated directly but if dilutions of 1: 100 were plated no colonies appeared. It was thought that dilution

of a nutritional factor was involved and the following were added to the nutrient agar without benefit: parachlorometacresol in eight concentrations, soluble oil, heat killed bacterial suspensions, vitamin mixtures, yeast extract and incubation in a carbon dioxide-air mixture. It was suggested that the diluent might be at fault and experimentation showed that if soluble oil emulsion, or phosphate buffer at pH 9.0 was used as diluents the count remained stable for one hour: but buffer at pH 7.0 caused a decrease of 84 per cent in one minute and a decrease of 99.6 per cent in 30 minutes. In this particular case the toxic effect of the diluent was traced directly to hydrogen ion concentration.

It can be concluded that nutrient agar is suitable for enumeration of aerobes in soluble oil emulsions. However, one must be constantly on guard concerning the diluent used for plating work. Saline and buffered saline are definitely useless: distilled water and M/20 phosphate buffers should be used with caution. Soluble oil emulsions are not usable because they make the medium too cloudy for counting if one ml is added to a petri dish. A wise precaution would be the direct plating of 0.1 ml of an emulsion as well as plating dilutions of the emulsion.

PART II. THE GROWTH OF BACTERIA IN SOLUBLE OIL EMULSIONS

Review of Literature

It has been known for many years that bacteria were present in cutting fluids. However, early workers were mainly concerned with health hazards, especially the role that cutting fluids played in causing and spreading dermatitis.

Contemporary workers are chiefly interested in cutting fluids as a cause of dermatitis, but there are a few who are interested in the detrimental effects that organisms have on the soluble oil emulsions. Lee and Chandler (34) investigated samples from various departments of one company for five months and found that bacterial counts were usually around 25 million per ml, seldom lower than 15 million and sometimes greater than 50 million. They found practically a pure culture of the same organism in all the departments of this plant and in another plant some distance away. Chandler (17) in a personal communication stated that this organism was still present in practically pure culture in this same plant several years later. They (34) found that replacing the emulsion or sterilizing it by heat was useless because circulation of the emulsion through the machines recontaminated it. Fresh emulsion in use for two hours contained 14,480,000 bacteria per ml and in four days they had increased to 37,800,000. The organism, a Pseudomonas, grew equally well in emulsions made with mineral oils alone or mineral and lard oil combinations. Duffett, Gold and Weirich (25) conducted a very extensive survey of the bacteriology of soluble oil emulsions but unfortunately only a short note was published and further information was unobtainable (60). They found that the majority of 634 samples taken from 325 different factories in northeastern United States contained over one million bacteria per ml. The highest count was 350 million.

Most of the work reported does not state the source of contamination of the emulsions. Westveer (62) suspected the source of contamination to be river water used to make the emulsion, aerial contamination, con-

taminated metal parts and the workers' hands. He also suspected that refuse thrown into the emulsion by workers might serve as a source of contamination. Stone, Fenske and White (54) and Gray and Thornton (29) showed that some soil organisms were capable of fermenting hydrocarbons similar to those found in petroleum, but they did not work with soluble oil emulsions.

Experimental

Experiments were designed to grow mixed cultures in emulsions of several soluble oils and also pure cultures in a single soluble oil. The ability of organisms from several suspected sources of contamination to grow in soluble oil emulsions was investigated. Also the increase in bacterial population was followed in fresh emulsions in a local machine shop. Counts on several emulsions obtained from industrial sources were made.

The growth curves of a mixture of four emulsions obtained from machine shops were obtained in fresh emulsions of 13 soluble oils. All 13 oils were mineral oils emulsified with petroleum sulfonates. One also contained saponified abietic acid as an emulsifier. Fifty ml of a four per cent emulsion of each oil were sterilized in 150 ml Erlenmeyer flasks at 15 pounds steam pressure for 15 minutes and two gm of heat-sterilized iron chips was added to each flask. Each flask was then inoculated with 0.1 ml of the mixture and incubated at room temperature (23 to 27°C). Plate counts were made immediately and at intervals up to 21 days.

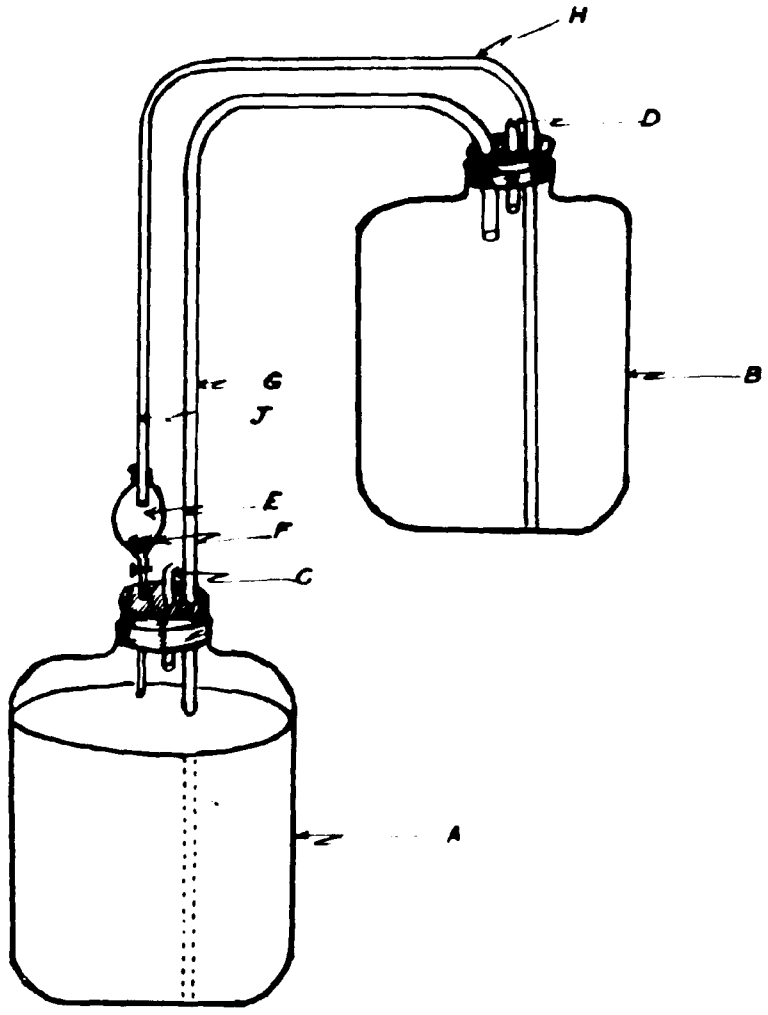

Fig. 1 Diagramatic sketch of single unit for growing bacteria in soluble oils.

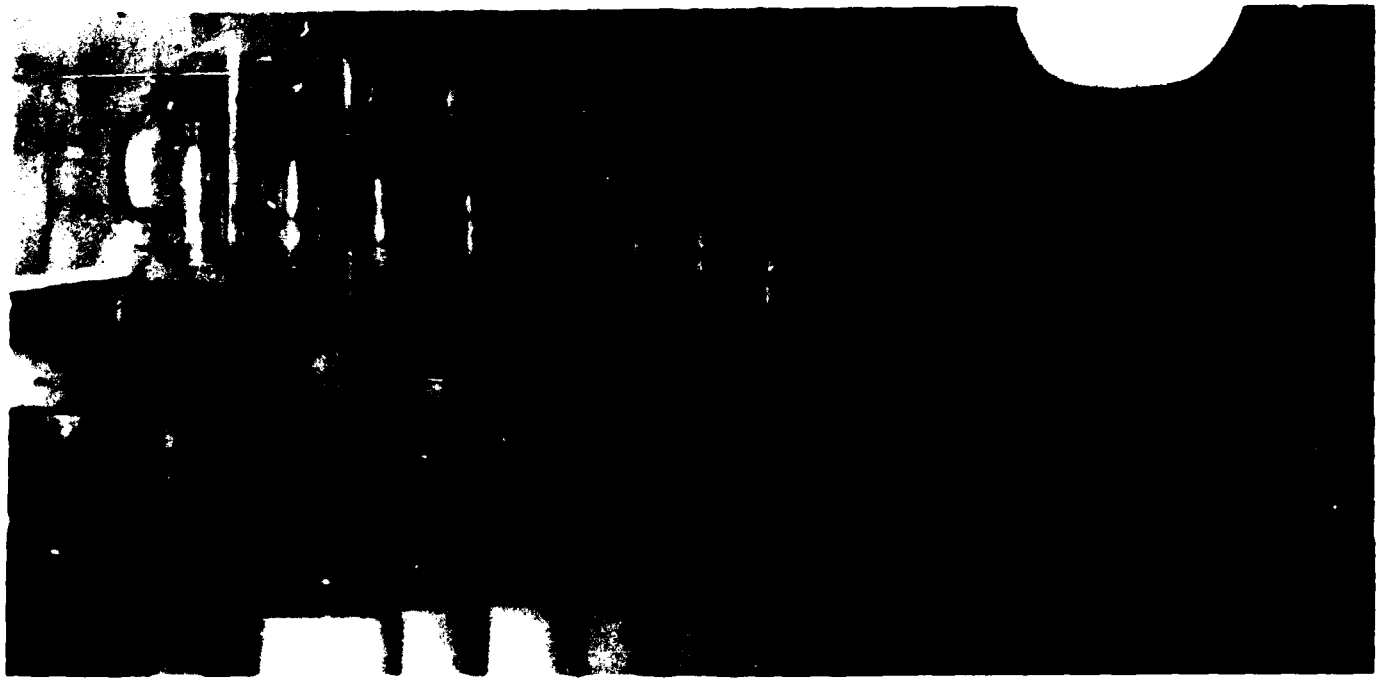

Fig. 2 Photograph of multiple units of the single unit shown in Fig. 1.

The above work was repeated using only 11 oils and a different technique. When the work was repeated, the apparatus was designed to eliminate some of the variables such as aeration and shaking which were encountered with the use of the Erlenmeyer flask. The apparatus shown in Fig. 1 was constructed as follows:

A and B were one-gallon jugs each fitted with a three-holed rubber stopper which was wired securely. B stood on a shelf above A. C and D were inlets for compressed air to jugs A and B respectively. E was a 250 ml separatory funnel containing 30 gm of iron chips resting on glass wool located at point F. G and H were 7 mm glass tubes with H being constricted to approximately 2 mm at J. The operation of the apparatus was simple. Compressed air was used to force the emulsion from A to B and also to start a siphoning action which returned the emulsion from B to A. With the stop-cock of funnel E closed, compressed air was introduced at C and this forced the emulsion through tube G into flask B until flask A was emptied. To empty flask B, compressed air was introduced at D and this started a siphoning action. As soon as the siphon was started, the stop-cock of funnel E was opened allowing the emulsion to return through the iron chips F into flask A. The apparatus shown in Fig. 1 was set up in groups of three, each member of the group being interconnected by air lines. The whole apparatus which is shown in Fig. 2 consisted of 12 units set up in four groups.

Aseptic precautions were observed in sterilizing, assembling and maintaining the system. The apparatus was sterilized by filling for 24 hours with a 1: 1250 solution of Roccal (high molecular alkyl dimethyl benzyl-

ammonium chlorides) and was flushed out several times with tap water immediately before filling with soluble oil emulsion. The soluble oil emulsion was a four per cent emulsion in tap water prepared in gallon jugs which had been sterilized with a 200 p.p.m. chlorine solution. The air supply was filtered through sterile cotton, a method which was found, by occasional checks, to remove all microorganisms.

After filling the apparatus with the emulsion, the emulsion was immediately inoculated with two ml of an emulsion which consisted of a mixture of 20 samples obtained from industry. Plate counts were made at the beginning and at intervals thereafter. The emulsions were circulated twice daily but occasionally a day was missed. However, the emulsions were always circulated twice immediately prior to removing samples from E for plate counts, and all were treated exactly the same to minimize variables.

Thirteen pure cultures were isolated from three samples of emulsion. They were inoculated into flasks containing 50 ml of sterile emulsion made with a single oil and incubated at room temperature. Plate counts were made immediately after inoculating and at intervals thereafter.

The possible sources of contamination of soluble oil emulsions were investigated by introducing probable contaminants into soluble oil emulsions. The contaminants were river water, sputum, human feces, unfiltered air, floor sweepings and sludge from an oil pit in a local factory. Washing the hands in the emulsion was not tried as a source of inoculum because most of the above materials would probably be on the hands at some time.

The oil emulsions were circulated in a system shown in Fig. 3 which was designed to simulate industrial conditions. This entailed aeration of the emulsion by means of compressed air and circulation of the emulsion through iron chips. A single unit of the apparatus is shown in Fig. 3 and a photograph of the whole apparatus is shown in Fig. 4 . Details of the apparatus are as follows:

A - Air inlet.
B - Screw clamp for adjustment of air flow.
C - Metal screw cap.
D - Oil emulsion flow tube.
E - Gooch funnel set in screw cap C, plugged with cotton.
F - Rubber band encircling the funnel E to keep it from slipping through the hole.
G - Three grams of iron chips (50 chips per gm).
H - Stainless steel wire screen.
I - Gallon bottle containing 3600 ml of two per cent oil emulsion.
J - Water manometer.

Aseptic precautions were observed in setting up the many units of the system. The gallon bottles were treated with 200 p.p.m. of chlorine for 48 hours and washed thoroughly with hot tap water immediately before use. The iron chips and the screens were sterilized in a hot air oven and all of the other apparatus was sterilized in the autoclave. The compressed air supply was filtered through a sterilized adsorption column packed with cotton. It was then bubbled through sterile distilled water to decrease moisture loss in the emulsion caused by the aeration. All holes in the cap C were sealed with melted paraffin after the necessary glass tubing and funnel had been installed. The Gooch funnels, C, were plugged with cotton except for flask No. 4 which was exposed to aerial contamination. Flask 5 served as a control on the sterility of equipment and emulsion.

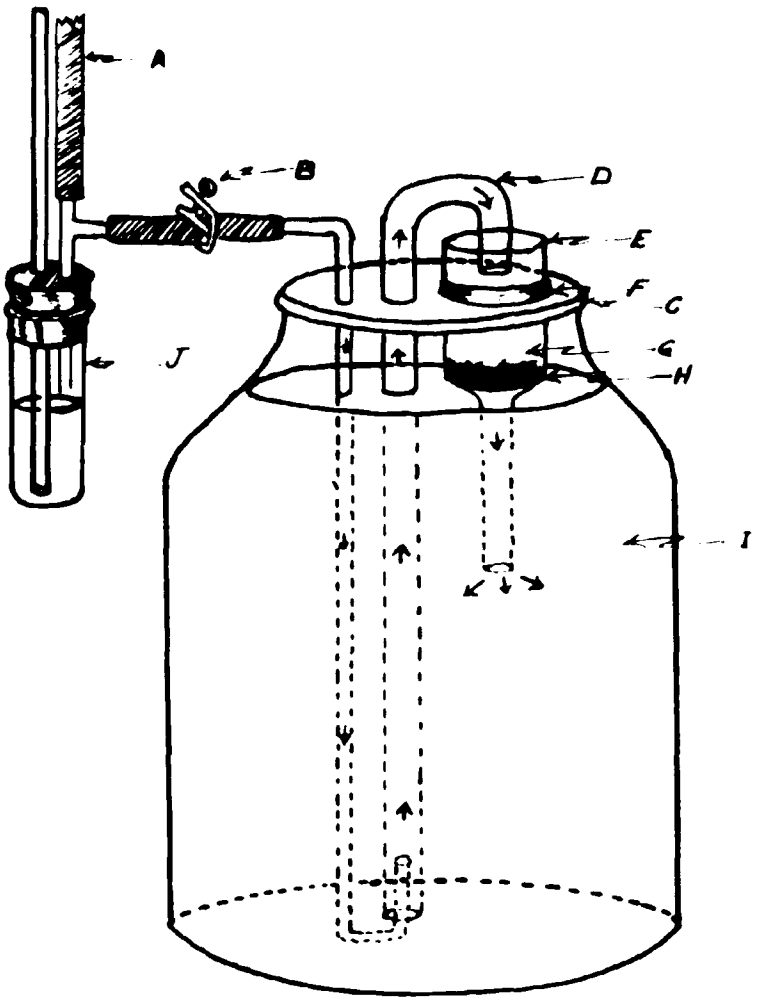

Fig. 3 Diagramatic sketch of single unit for growing bacteria in soluble oils.

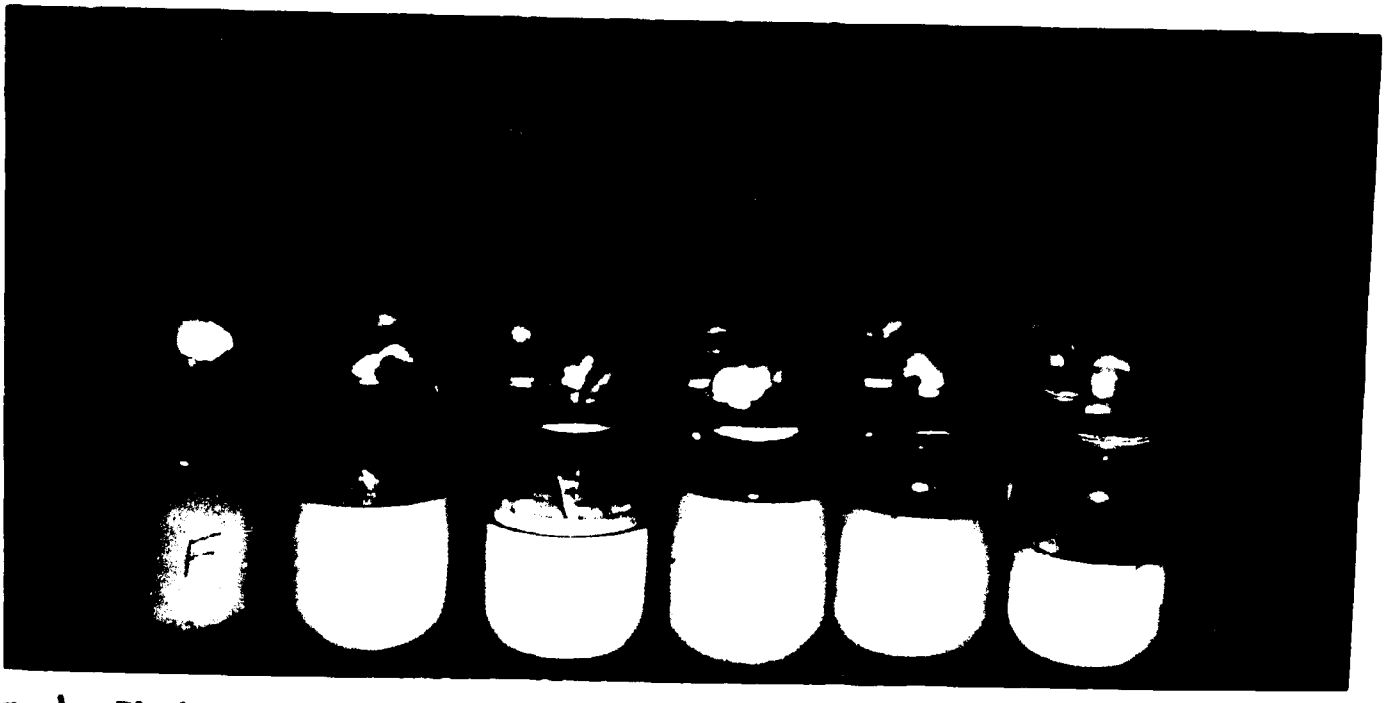

Fig. 4 Photograph of multiple units of the single unit shown in Fig. 3.

The emulsions, containing two per cent of a soluble oil, were made with tap water except in flask No. 1 where river water was used. The inoculum was placed in funnel E and the air was adjusted to circulate the emulsions in the various systems at approximately the same rate. Circulation was carried on for 45 minutes to distribute the organisms before the initial samples were taken for a plate count.

Plate counts were made at 0, 9, 19, 43, 86, 161, 353 and 780 hours using a beef extract-peptone agar containing two per cent agar instead of the customary 1.5 per cent agar. This modification was employed in order to inhibit the spreading which frequently occurs when soil organisms are grown on agar plates. Diluent employed was the previously described phosphate buffer. Plates were incubated for 72 hours at 30°C.

The ability of bacteria to grow in emulsions in machine shops was investigated in two different types of distributing systems in a local automobile factory. These systems will be designated as system "A" and "B". The fresh emulsions in these systems were not inoculated but were seeded by bacteria left when the pits were cleaned out. System A was filled with a two per cent emulsion of a petroleum oil emulsified with petroleum sulfonate, and system B contained a translucent solution, possibly a soap-type coolant. A sample of emulsion from System A, taken when it was being cleaned out, contained 16,500,000 bacteria per ml, and a sample of sludge in the bottom of the pit contained 710,000 bacteria per gm. Samples were plated using methods described in Part I.

Samples from eight machine shops in different localities were obtained and plated to make a survey of the bacterial populations in emulsions used in industry. The oils used to make the emulsions had been manufactured by several different companies and the emulsions had been in use for various lengths of time before plating. Thirty-six samples were plated by the methods previously described in Part I.

Results and Discussion

The growth curves of mixed cultures in the 13 soluble oils run in the Erlenmeyer flasks were very similar to the growth curves obtained in the more complicated apparatus. Moreover, there was very little difference in the growth curves obtained in the emulsions of the 13 soluble oils irrespective of the oil used. For this reason only three curves, representative of the group, are shown in Fig. 5 . Although all three emulsions contained approximately the same number of bacteria after two days, emulsions A and B showed greater numbers at three days and emulsion A was able to sustain a far greater bacterial population than the other two.

The pure cultures grew well in the soluble oil emulsion. Only one of them did not increase to at least one million per ml. The other 12 organisms reached their maximum populations at various times. Growth curves of three cultures, representative of the group, are shown in Fig. 6 . Both Figs. 5 and 6 show the extreme rapidity with which these organisms multiply — almost a hundred fold increase within 24 hours.

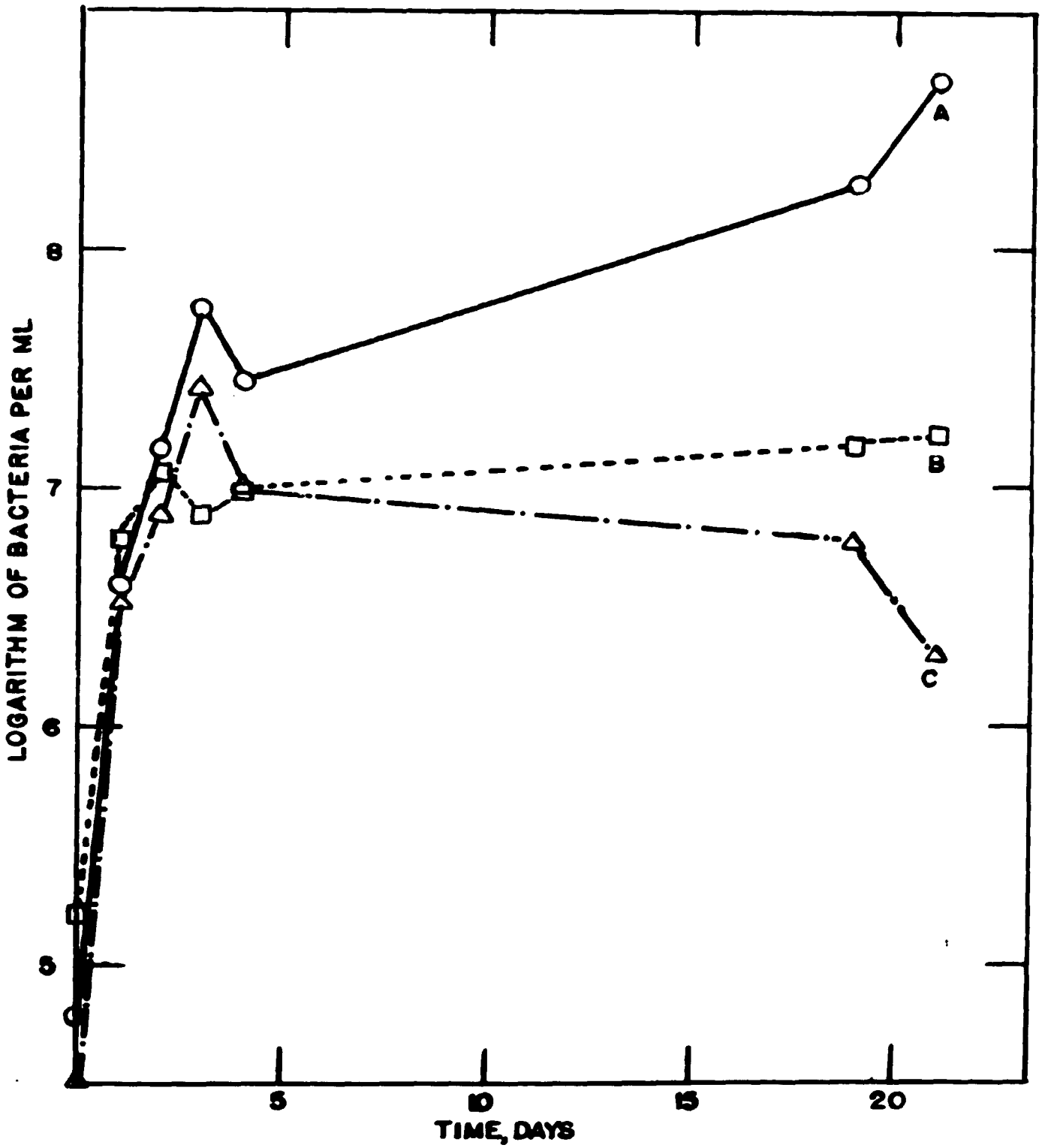

Fig. 5 Typical growth curves of mixed cultures in three soluble oils out of thirteen soluble oils studied.

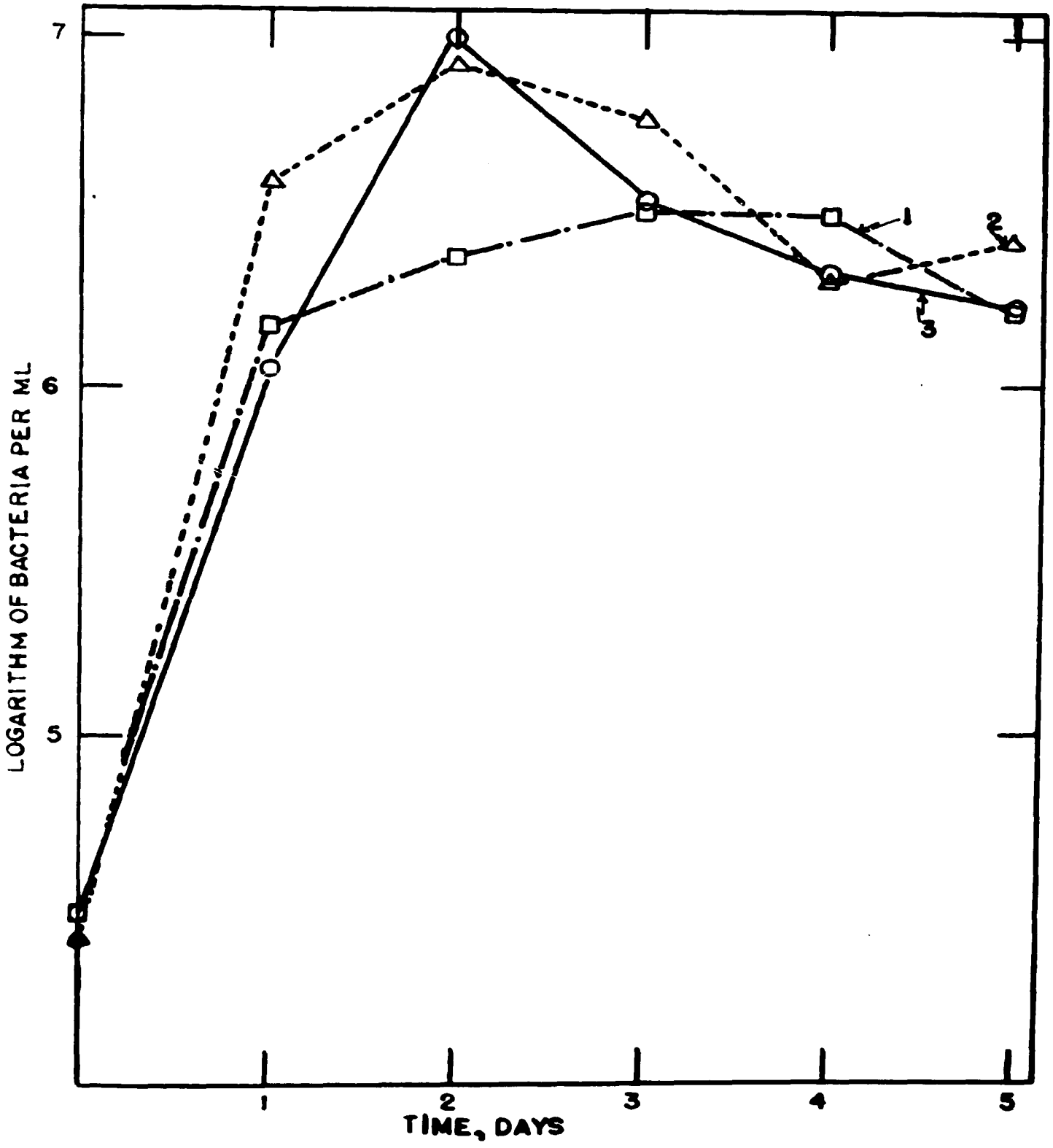

Fig. 6 Typical growth curves in soluble oil of three pure cultures out of thirteen pure cultures studied.

The sources of contamination of soluble oils are shown very well in Fig. 7 . The results for sputum, aerial contamination and control are not graphed because the flask containing the sputum was sterile at nine hours, the control remained sterile throughout the experiment and the flask exposed to aerial contamination contained no bacteria up to 161 hours but had 2,600,000 per ml at 353 hours and 395,000 at 780 hours. Fig. 7 requires little discussion. It is obvious that the sources of contamination are numerous. The greater numbers in the flask inoculated with feces may be due to the nature of the organisms or the relatively large amount of organic matter introduced with the inoculum.

The decline in population with time is characteristic of these organisms in a closed system. Under industrial conditions either the emulsions are replaced every few weeks or additional emulsion is added each day to compensate for evaporation of the water and loss of oil which forms a film on the finished work. One large system investigated contained 35,000 gallons of emulsion and this required a daily addition of about 1,000 gallons. It serviced several dozen machines. The lubricating engineer in charge said that the main reservoir had not been emptied in more than a year. Under such conditions, where the emulsion is renewed continuously the bacterial population tends to remain static.

The growth of bacteria in soluble oil emulsions under industrial conditions is shown in Table 4 . The translucent coolant in system B caused a slight lag phase and it was not able to support as much growth as the emulsion in system A. It was impossible to obtain a sample from system A at the time it was being filled.

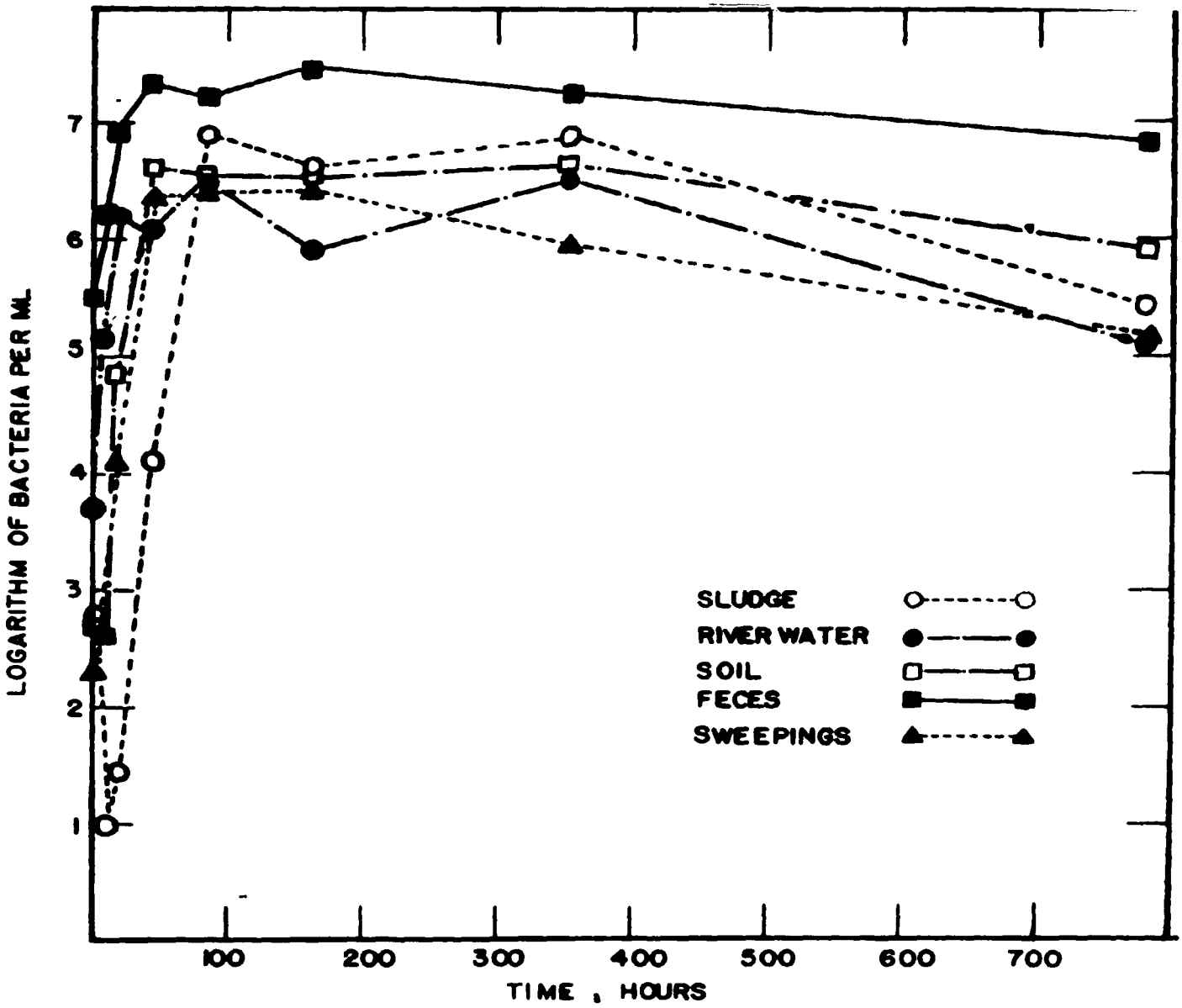

Fig. 7 Growth curves of possible contaminants in soluble oils.

Table 4

Increase in bacterial populations of 2 systems in a machine shop

Days in Use	Bacteria per ml	
	System A	System B
0		200,000
1		
2	16,000,000	
3		270,000
4		
5	46,000,000	
6		12,300,000
7		
8		
9	91,000,000	
10		9,000,000

The levels of bacterial populations in 36 industrial samples of soluble oil emulsions are well demonstrated in the histogram shown in Fig. 8 . Thirteen of the samples contained between one and 10 million bacteria per ml and 11 contained from 10 to 100 million.

It may be concluded that bacteria, whether normal to soluble oil emulsions or from such sources as soil, water and feces, grow with considerable rapidity when introduced into fresh emulsions. Some soluble oils maintain a higher level of bacterial population than others, and there are indications that pure cultures of bacteria, or even mixed inocula from different sources, may attain different levels of population in the same emulsion.

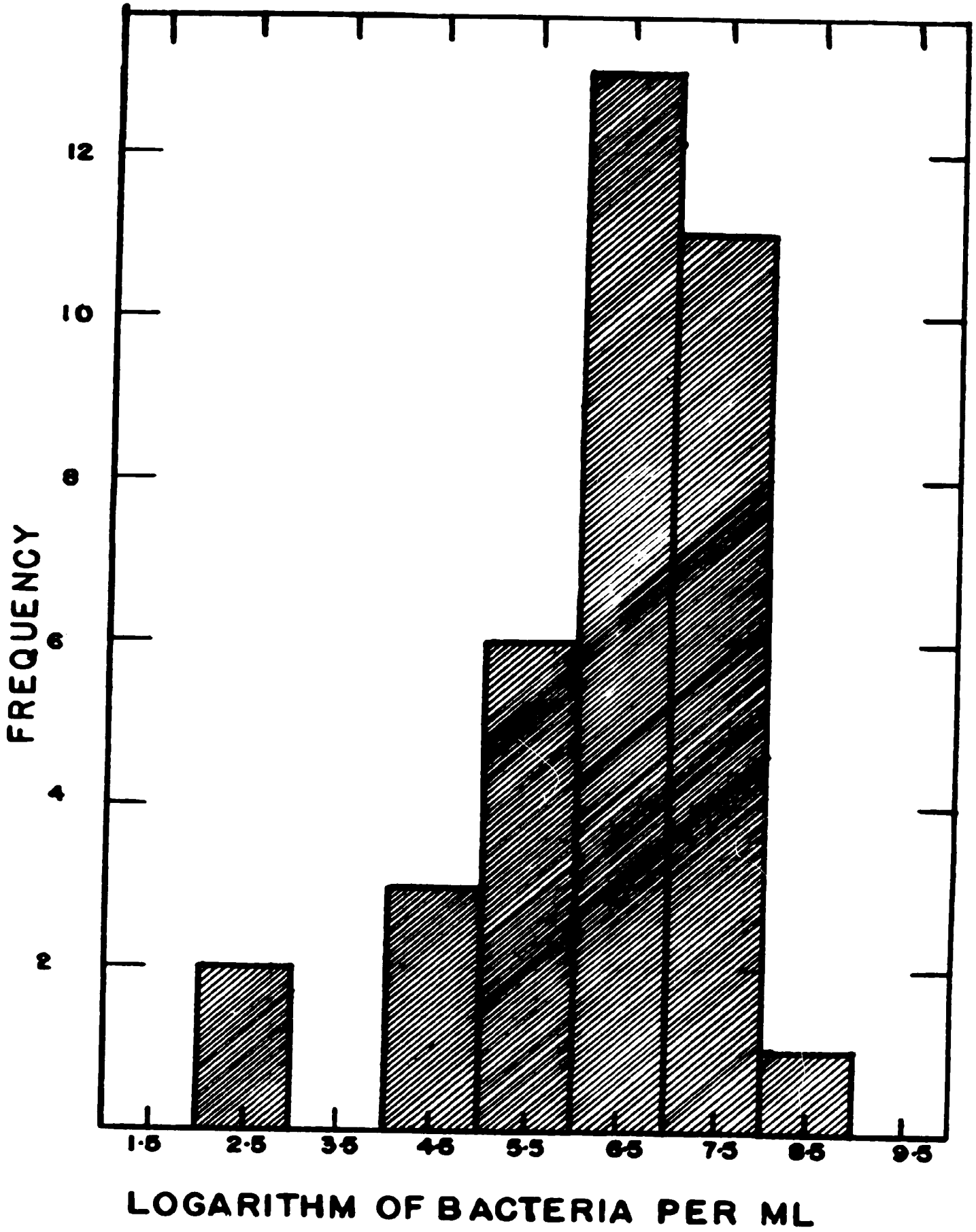

Fig. 8 Frequency distribution of bacterial populations in 36 samples of soluble oil emulsions obtained from industrial sources.

PART III. COLIFORM BACTERIA IN SOLUBLE OIL EMULSIONS

Review of Literature

Coliform bacteria are ubiquitous in nature, and it is, therefore, not surprising that they have been found in soluble oil emulsions. Page and Bushnell (41) found Bacillus aerogenes and Bacillus coli communis in cutting compound. Duffet, Gold and Weirich (25) found Aerobacter aerogenes and Escherichia coli in soluble oil emulsions and Weirich (59) has stated that B. coli is the second or third most frequently found organism in cutting oils.

Antagonists of coliform bacteria which are particularly active are two members of the genus Pseudomonas, Ps. fluorescens and Ps. aeruginosa. Of these two organisms only the letter has been reported in soluble oil emulsions (25).

Ps. aeruginosa produces two substances which are antagonistic to E. coli. They are an enzyme-like substance called pyocyanase and a pigment, pyocyanin.

The antagonism of Ps. aeruginosa toward E. coli is very pronounced as the two following examples will demonstrate. Havens and Dehlers (30) found that Ps. aeruginosa was always present in the gut of a minnow, Gambusia affinis, and that when this minnow was introduced into polluted water, the coliform rapidly disappeared. One loopful of a pure culture of the Ps. aeruginosa isolated from the minnow, when introduced into a 24 hr culture of E. coli, completely destroyed that organism within 72 hrs.

Rochaix and Vieux (44) found that if B. pyocyaneus and E. coli were mixed in equal quantities in nutrient broth, and the mixture serially subcultured daily they could still isolate both organisms after 13 days, but if the two organisms were allowed to remain in the same tube for 13 days, then only Ps. aeruginosa could be recovered.

There are many other members of the genus Pseudomonas reported to be present in soluble oil emulsions but their ability to act antagonistically has not been reported. However, their ability to so act must be suspected from a consideration of the previously discussed work of Lee and Chandler (34) and Chandler (17).

Experimental

These experiments were designed to find out whether the "normal" flora of soluble oil emulsions from industrial sources were capable of acting antagonistically toward coliform organisms. The inocula were soluble oil emulsions in which either coliform or "normal" organisms had grown for long periods of time. As a source of coliforms, an emulsion (designated No. 3) was used which had been inoculated 40 days previously with about two gm of feces. (See Part II). It had a total count of 6,850,000 bacteria per ml of which 75 per cent were capable of fermenting lactose in Endo medium. Flora "normal" to soluble oil suspensions was obtained from two sources. One was from an emulsion (designated No. 7) which had been inoculated 40 days previously with sludge taken from a machine shop pit. It contained 270,000 bacteria per ml. The other was a mixture (des-

ignated M) of soluble oil emulsions obtained from several machine shops. It contained 81,000,000 bacteria per ml.

The ratio of coliform to "normal" organisms was varied. In three experiments the inocula were introduced into fresh, two-per cent soluble oil emulsions to give approximately the following ratios of coliform to non-coliform: Flask A, 1:1; Flask B, 10:1; Flask C, 1:10. In the fourth experiment the coliforms and non-coliforms were not introduced into fresh emulsion but 1800 ml of emulsion No. 3 in which coliforms had grown for 40 days was mixed with 1800 ml of emulsion No. 7 in which "normal" bacteria had grown for the same length of time. The initial inoculum of bacteria per ml of emulsion and the per cent of coliforms are shown in Table 5 for all four experiments.

Table 5

Initial inoculum in experiments designed to show the antagonism of bacteria "normal" to soluble oil emulsions toward coliform bacteria.

Flask	Coliform per ml	Non-coliform per ml	Source of Coliform	Source of Non-Coliform	Per cent Coliform
A	2,250	1,950	No. 3	Mixture M	53.6
B	27,500	5,000	No. 3	Mixture M	84.6
C	3,400	27,100	No. 3	Mixture M	10.8
D	2,600,000	750,000	No. 3	No. 7	77.7

Total counts of bacteria were made at 0, 7, 17, 26 and 70 days by the previously described methods, and coliform organisms were determined by plating appropriate dilutions of the emulsions with Endo medium. At 26 days, the most probable number (MPN) of coliforms was determined in lauryl sulfate tryptose broth (37) to compare the method of coliform determination with that of the Endo plate method (See Table 6); and at 70 days, only the MPN was used because as little as 0.1 ml of the two per cent emulsion rendered the Endo plates useless for detection of coliform organisms.

As a check on the Endo plate method 17 colonies which were thought to be lactose fermenters and 17 which were thought to be non-fermenters were fished from the 26th day plating to brilliant green bile lactose broth fermentation tubes. Sixteen of the supposed lactose fermenters produced gas and one did not. Conversely, of the 17 supposed non-fermenters, 16 did not produce gas in brilliant green bile lactose broth, but one did. As a check on the MPN, all positive tubes of lauryl sulfate tryptose broth from the 70 day determination were inoculated into brilliant green bile lactose broth and streaked on eosin methylene blue agar. All were confirmed on these media.

The presence of coliforms in soluble oil emulsions in machine shops was also investigated. The MPN of coliform bacteria was determined in 22 samples obtained from one plant and the presence of coliforms, but not the number, was determined in eight samples from another machine shop.

Results and Discussion

The ability of the flora "normal" to soluble oil emulsions to act antagonistically toward coliform bacteria is shown beyond doubt in Fig. 9 . In all instances the relative numbers of coliforms was very small after 26 days. At 70 days, Flasks A and D contained .003 and .0004 per cent of the total number of bacteria as coliforms while Flasks B and C contained no coliforms.

When the coliform and "normal" organisms were inoculated into fresh emulsion (Flasks A, B and C) both types of bacteria grew well. However, even though the absolute number of coliforms per ml increased in all three flasks, the per cent coliform increased only in Flask C and in this flask only up to 17 days. In Flasks A and B there were very rapid decreases in the relative numbers of coliforms.

The antagonism appears to be a one-sided antagonism in the sense of Waksman (58). Regardless of the ratio of coliform to "normal" organisms at the beginning, the organisms which were considered "normal" to soluble oil emulsions were able to suppress the coliforms.

A mixture of equal volumes of emulsion No. 3 and No. 7 was contained in Flask D. Emulsion No. 3 had been inoculated 40 days previously with feces, and emulsion No. 7 had been seeded with bacteria from a machine shop source for the same time. As can be seen from Fig. 9 , Flask D, there was very little increase in total count and none of this was due to coliforms. On the contrary the coliforms decreased rapidly.

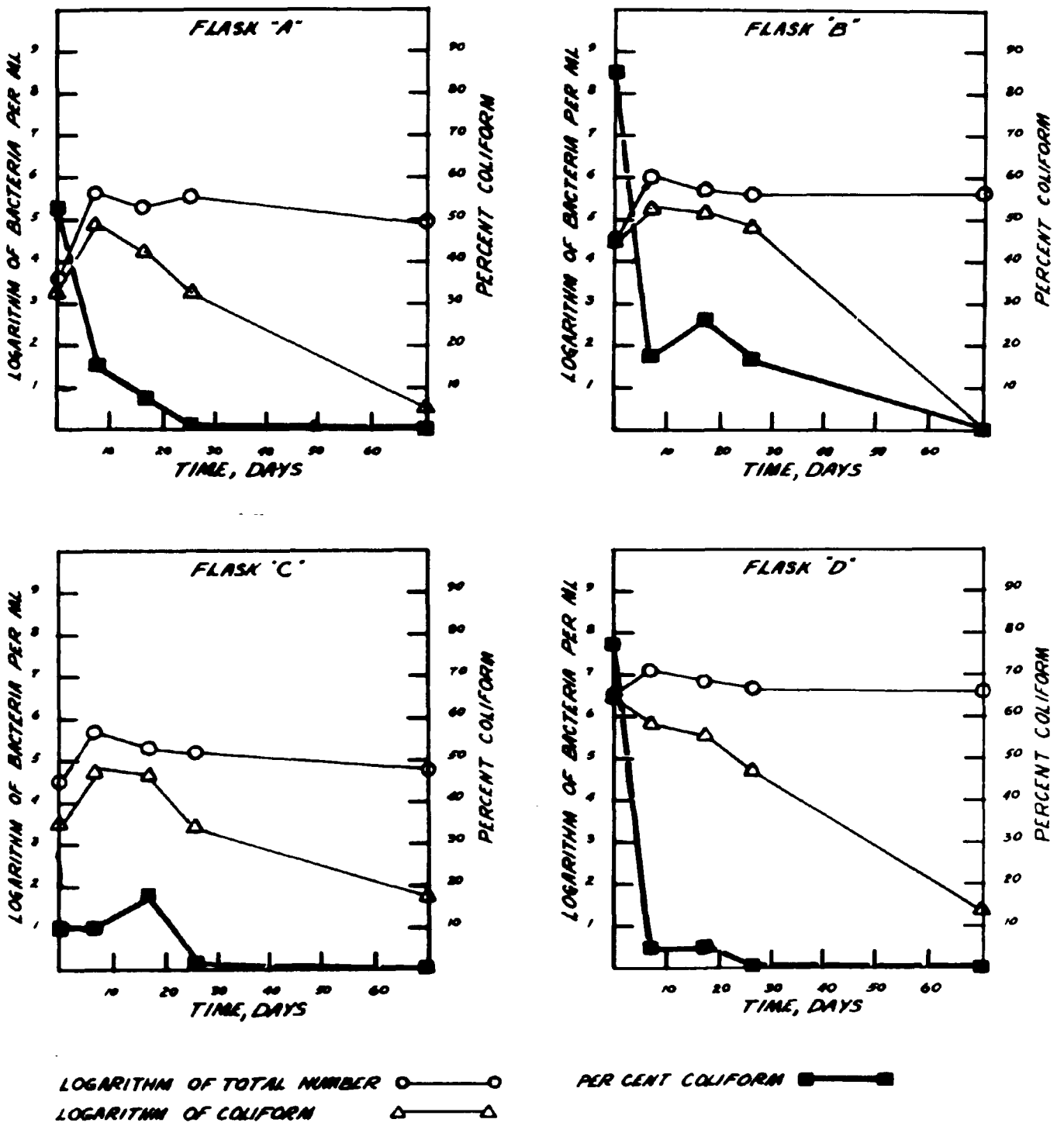

Fig. 9 Antagonism of bacteria "normal" to soluble oils towards coliform bacteria.

A comparison of the MPN method and the Endo agar plate method for determining coliform organisms in soluble oil emulsions is shown in Table 6 .

Table 6

Flask	Bacteria per ml	
	Endo	M.P.N.
A	1,900	430
B	70,000	4,300
C	2,600	430
D	46,500	25,000

In all instances the Endo medium gave a higher count than the M.P.N. This would indicate a supression of the coliform by the "normal" flora even in lauryl sulfate tryptose broth.

The presence of coliform organisms in soluble oil emulsions obtained from industrial sources tends to substantiate the results of the experiments concerned with antagonisms. Of the 22 samples examined by the M.P.N. method, only 11 contained any coliforms and these were present only in very small numbers as is shown in Table 7 .

Table 7

Coliform bacteria in industrial samples of soluble oil emulsions

Sample Number	Total Bacteria per ml	Coliforms per ml	Per cent Coliforms
1	2,350,000	2,500	.1
3	650,000	250	.04
5	300,000	1.5	.0005
6	4,300,000	92	.002
14	105,000	14.7	.01
15	7,650,000	250	.003
17	5,575,000	10,000	.2
18	17,500,000	920	.005
19	6,870,000	92	.001
20	43,400	2,500	5.8
22	20,310,000	92	.0005

The presence but not the number of coliforms was determined in eight samples of emulsions obtained from one factory. The eight samples, inoculated into lauryl sulfate tryptose and brilliant green bile lactose broths, all gave presumptive evidence, and this was confirmed for the lauryl sulfate tryptose medium by streaking on eosin methylene blue agar.

It may be concluded that coliform bacteria are capable of growing in the presence of bacteria normal to soluble oil emulsions when both are introduced into fresh emulsion at the same time. But the normal flora acts antagonistically towards the coliform and, regardless of the ratio of coliform to non-coliform bacteria at the beginning, they cause the coliform to die out within a few weeks to a few months.

PART IV. DERMATITIS AND CUTTING OILS

Review of Literature

The history of cutting fluids is paralleled by a history of industrial dermatitis. Schwartz (49) has stated that a study by the U.S. Public Health Service showed that 18.8 per cent of 41,628 cases of occupational dermatosis were due to petroleum oils and Weslager (61) has said that 65 per cent of all occupational diseases reported are caused by or related to dermatosis. This would indicate that approximately 12 per cent of all occupational diseases are due to petroleum oils.

Annually, one per cent of the workers in the basic industries suffer from dermatitis at an estimated total cost of $200 per case (19). The contribution of cutting fluids to the overall cost of industrial dermatosis is considerable.

The Committee on Healthful Working Conditions, National Association of Manufacturers (19) defines industrial dermatosis as any disease of the skin due to industrial work, and they call dermatitis any skin disease which results in an inflammation of the skin. Dermatitis is the most frequently encountered of all skin diseases.

The dermatitis found bn workers exposed to cutting fluids may be of two general types, the non-infectious and the infectious. The former, may be in the form of a vesicle, and this may, at a later stage, become infected. Or dermatitis due to infection may result from the entrance of pyogenic organisms into minute abrasions, skin cracks or malfunctioning hair follicles.

The research staff of the E.F. Houghton Company (32) recognized four types of infection which involved especially the extensor surface of the arms and perhaps the backs of the hands and the skin between the fingers. They were: (a) A leathery condition of the skin showing comedones (black heads) thickly dotted over the skin's surface; (b) Red blotches on the skin varying in size from 1/64 inch to a quarter-of-a-dollar, which became elevated; (c) Progression of the red blotches to pustules; and (d) The true furunculosis or boil stage, usually exhibiting several lesions. Schwartz (47) stated that the acute stages of industrial dermatitis were characterized by erythema, edema, papules and vesiculation, but that pus formation and parasitic infection might entirely change the picture of the original disease.

Dermatitis of persons exposed to cutting fluids may be caused by one or more of the following: (a) primary irritants, (b) sensitization, (c) folliculitis, (d) defatting of the skin and (e) cutting and puncturing the skin.

Primary irritants in cutting fluids may be of several types. Adams and co-workers (1) have found that several chlorinated hydrocarbons and even non-chlorinated petroleum oils can cause acneform dermatitis in man, and they believed that these compounds might be the cause of related papular and warty eruptions. Schwartz and Barlow (50) stated that workers exposed to the mists of chlorinated cutting oils developed lesions, which resembled chloracne both clinically and microscopically on their face and other parts of their body. Schwartz (48) stated

that the chlorine or sulfur content of cutting oils may be sufficiently high to irritate the skin; or that hydrogen sulfide or sulfur dioxide formed by the heat of cutting operations might cause dermatitis. He did not believe (49) that alkaline coolants such as solutions of soaps and/or sodium carbonate were often the cause of dermatitis.

Disinfectants have been occasionally incriminated as primary irritants. Schwartz (49) stated that excessive amounts of germicides which contain phenol or formaldehyde are more conducive to dermatitis than the bacteria in the oil, and he reported (48) finding as much as five per cent of a phenolic disinfectant in cutting fluids. Duckham (24) also blamed excessive concentrations of germicides in cutting fluids as causes of dermatitis. Duckham did not believe that petroleum, per se, caused skin trouble because in 40 years he never found a single case of skin irritation among workers in crude oils in the oil fields or in persons handling fuel oils.

Sensitization causes trouble only among susceptible individuals, whereas primary irritants are likely to affect all workers equally (19). Sensitization, leading to allergic dermatitis, only occurs when an individual's skin is supersensitive to a particular substrate even in weak concentration. During the sensitization period there is usually no dermatitis, but skin disorders appear on contact subsequent to the sensitization. Schwartz (49) stated that such compounds as phenols, cresols and nitrobenzene may act as sensitizers and cause allergic eczema. In higher

concentrations than are present in cutting fluids, they would function as primary irritants. Lee and Chandler (34) did not believe that dermatitis was generally of the allergic type.

Acne and folliculitis due to the plugging of the sebaceous glands and the ducts supplying the hair follicles is thought to be one of the most common causes of dermatitis among workers exposed to cutting fluids (21). The resulting irritation furnishes conditions suitable for invasion by infectious bacteria. Schwartz (49) has stated that these types of dermatitis occur especially where oil-soaked sleeves and trousers contact the skin. Folliculitis occurs more frequently on hairy workers than on workers with little hair.

Defatting of the skin by the solvent action of the cutting fluid has been mentioned as a factor contributing to dermatitis (61, 40, 6). The defatted skin cracks more readily thus allowing bacteria to enter. As a result, people with dry skins are less resistant to cutting fluid dermatitis than people with oily skins.

The cutting and puncturing of the skin by metal particles in the cutting oils or in dirty towels and cotton wastes lays it open to infection (49, 32, 21, 48, 40, 26).

Besides the dermatitis arising from exposure to cutting fluids, there is also dermatitis which results from the harsh cleaning methods used by some workers to remove the cutting fluids from their bodies. Such treatment as washing hands in gasoline or kerosene, use of sand soaps or high-alkali soaps, bleaching powders, and other harmful agents often results in dermatitis which is attributed to the cutting fluids.

Bacteria responsible for dermatitis among workers exposed to cutting fluids have been the subject of some irresponsible comment. Some workers have blamed the indigenous flora growing in soluble oil emulsions for inciting dermatitis, and others have postulated that contaminated water used for making emulsions has carried the responsible organism. However, Schwartz (48), former Medical Director of the United States Health Service and outstanding worker in the field of industrial dermatitis, believes that the most common infective agent in cases of oil dermatitis is Micrococcus aureus found on the healthy skin or in ordinary boils or in cellulitis. Streptococci have been isolated (41) from lesions in machine shop workers, but such occurrences are not common.

The role of cutting oils in the distribution of Micrococcus aureus has received considerable attention though there is no evidence that Micrococcus aureus can grow in cutting fluids. Schwartz (48) stated that the United States Public Health Service found no significant numbers of staphylococci or streptococci in straight cutting oils used by workers with cutting oil dermatitis, and Davis (20) reported that regular checks in a very large plant over a period of years found pyogenic bacteria on only two occasions and then only in negligible numbers. In contrast to the above opinions, Weirich (59) stated that streptococci have frequently been found in grinding compounds, especially in the winter, and mist from grinding may cause sore throat infection. Albaugh (1) believed that cutting compounds were carriers of pus-forming bacteria and were responsible for spreading the wound infections from worker

to worker. He found 110,000 organisms per gm of oil — almost a pure culture of Staphylococcus aureus. This opinion was shared by others (28, 9,40,39). Shie (52) also found pyogenic organisms in "vast numbers" in the cutting compounds though he found none in the straight oils. Guinea pigs inoculated with isolates from the compound and with cultures obtained from boils of infected workers displayed identical syndromes. Disinfection of the cutting compounds with cresol reduced incidence of boil affliction from five per cent to 0.5 per cent in this particular group. Another extensive outbreak of furunculosis (39) in a machine shop was curbed by centrifuging the fluid to remove metal chips and pasteurizing to destroy the bacteria. Two years after this practice was begun, an examination of 400 men failed to show any furunculosis.

Besides dermatitis, a less frequent type of infection may be incited by bacteria in cutting fluids. Weirich (59) stated that minor conjunctivitis outbreaks occur in machine shops. He mentioned the possibility that Bacillus pyocyaneus, growing in oil emulsions, might be the cause of such outbreaks. Recently, an outbreak of conjunctivitis in a Canadian machine shop was attributed to M. aureus, the same strain having been isolated from all cases (38). However, no additional information concerning this subject has been encountered.

The methods of control of cutting fluid dermatitis are numerous. Possibly all are necessary for a well regulated safety program.

Scrupulous cleanliness with adequate washing facilities for workers appears to be the most important. Schwartz (48) recommended that the workers be supervised during their ablutions to ensure adequate personal cleanliness. He believed that a soap of sulfonated castor oil with two per cent fatty alcohol sulfate was desirable as a cleansing agent. Harsh cleaning methods such as washing with gasoline, kerosene, sand soaps, high-alkali soaps or bleaching powder should be prohibited. Clean clothing at frequent intervals and protective clothing such as impervious aprons to prevent the body from becoming soaked with oil were recommended. Clean cotton waste and towels must be available in order that workers need not be subjected to the hazards of wiping their hands with rags impregnated with metal slivers.

Bland protective ointments should be applied to the skin before exposure to cutting fluids. Susceptible workers should be transferred to departments where they would not be in contact with cutting fluids. Persons with dermatitis should be removed from contact with the oils, and this is especially important where infections such as boils may contaminate the oils. The metal chips and slivers in the cutting fluids should be removed constantly by centrifugation or settling.

Fluids should be pasteurized frequently or should contain disinfectants in sufficient amount to control the bacteria but not in such concentrations that they would act as primary irritants.

Prohibition of expectoration into the cutting fluids by the machinists has been advocated by most workers.

Experimental and Results

The following experiments were designed to investigate the effect of soluble oil emulsions on a few common pathogens, some of which are known to cause cutaneous and wound infections. More specifically it was desired to find out whether the organisms could grow or could be adapted to grow in soluble oil emulsions.

Four cultures were obtained from the stock culture collection of the Department of Bacteriology at Michigan State College. They were M. aureus, alpha and Beta hemolytic streptococci and Pseudomonas aeruginosa. Another culture of M. aureus was obtained from the Ontario Department of Health. It had been isolated from several cases of conjunctivitis which had occurred in a Canadian machine shop (38), and it was thought that the organism may have been disseminated in the soluble oil mist which arises during grinding operations. To check this possibility, eight samples of soluble oil emulsions were obtained from the factory in question.

Media used were made with Difco products and C.P. chemicals. The soluble oil was a petroleum oil with a petroleum sulfonate emulsifier. Soluble oil emulsions and nutrient solutions were made with tap water. Unless otherwise indicated, M/20 phosphate buffer at pH 7.0 was used as a diluent and brain heart infusion agar was used as a plating medium. Colonies were counted after two days at 37°C.

All growth experiments were carried out in 500 ml Erlenmeyer flasks containing 150 ml of solution or emulsion. Flasks were incubated at room

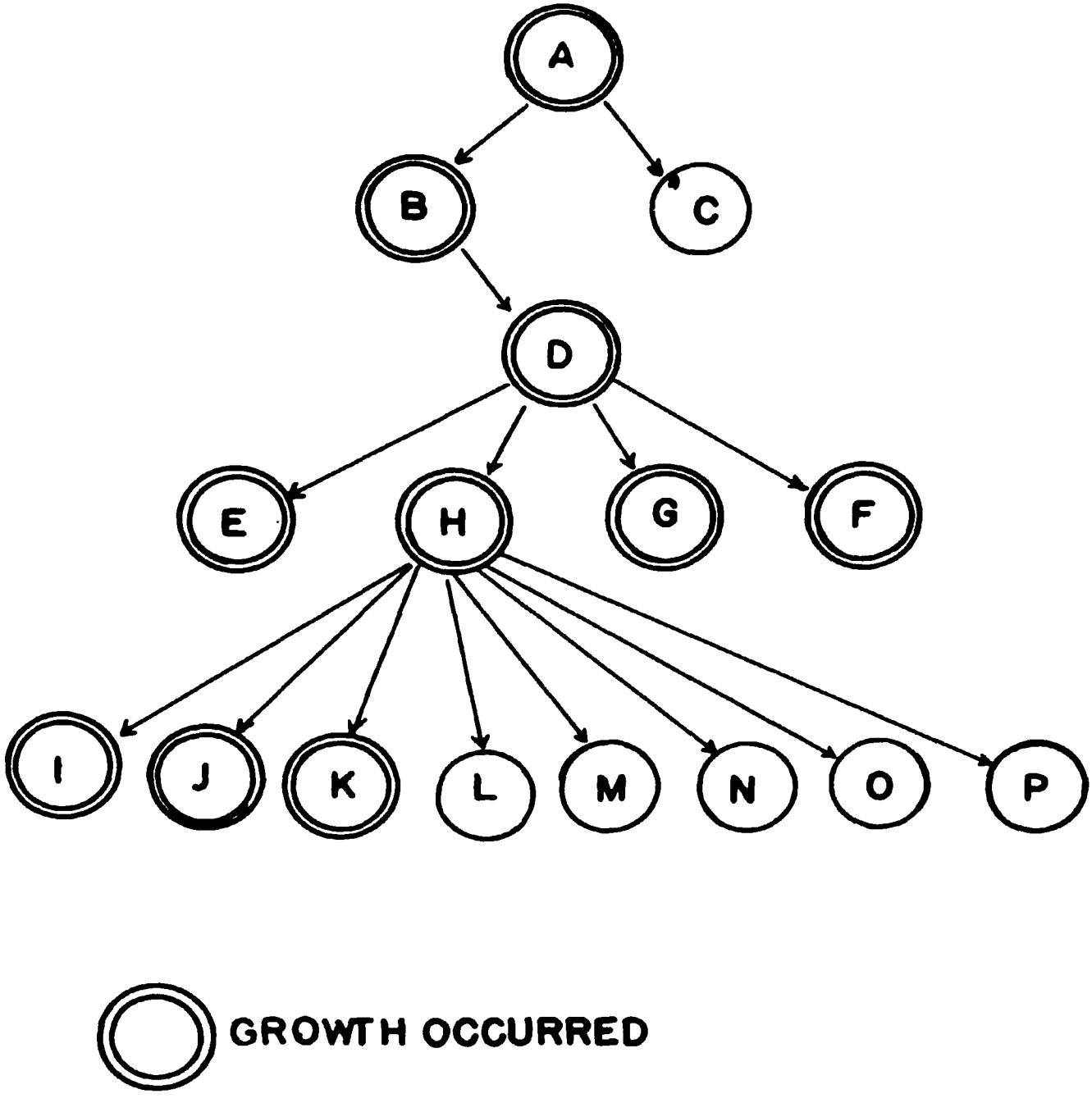

Fig. 10 Flow sheet of the adaptation of M. pyogenes var aureus to presence of soluble oil.

temperature (23-27°C) to simulate conditions which would be obtained in industry. Immediately before plating, the flasks were shaken for five minutes on a reciprocating shaker travelling at 108 strokes per minute.

Inoculation of the four organisms from the Michigan State College collection into a one per cent soluble oil emulsion and into the same emulsion containing 0.1 per cent each of peptone and dextrose showed that the streptococci were quickly destroyed by the medium. None were found in the first plating although they were in contact with the oil less than 30 minutes. The M. aureus was present at the first plating but not at two days. The Ps. aeruginosa, however, not only tolerated the oil but grew well, after a period of adaptation, in the emulsion without added nutrients. The results are shown in Table 8 .

Table 8

Growth of Ps. aeruginosa in soluble oil emulsion

Medium	Bacteria per ml x 10^3			
	0	2 days	7 days	14 days
One per cent oil emulsion	460	1.4	93	6,187
One per cent oil emulsion with 0.1 per cent peptone and dextrose	490	440	6,000	45,000

Since Ps. aeruginosa readily adapted itself to grow in soluble oil emulsion it was not included in any further experimentation.

The next step was to increase the concentration of nutrients and decrease the concentration of oil. M. aureus and the streptococci were inoculated in 3.7 per cent brain heart infusion with and without 0.1 per

cent of soluble oil. Table 9 shows the very immediate lethal effect of as little as 0.1 per cent oil on the streptococci and the lack of inhibition of M. aureus in the oil-containing medium.

Table 9

Effect of soluble oil on growth of pathogens in brain heart infusion medium

Culture	Bacteria per ml x 10^3					
	Brain heart infusion			Brain heart infusion + oil		
	0 days	2 days	7 days	0 days	2 days	7 days
M. aureus	970	26,300	90,000	1,040	10,000	95,000
Alpha streptococcus	960	24,400	84,000	80	0	0
Beta streptococcus	560	8,100	73,000	30	0	0

Further work was concerned only with the two strains of M. aureus. However, since the two strains responded in an almost identical manner to the presence of soluble oil, only the results obtained from the culture isolated from the conjunctivitis outbreak are reported.

To facilitate understanding of the experimental procedure carried out in adapting M. aureus to grow in the presence of soluble oil a flow sheet is given (Fig. 10) and the results are tabulated in Table 10. M. aureus, inoculated from a nutrient agar slant (A) grew well in the presence of 0.5 (B) but not in 1.0 (C) per cent oil. However, the growing bacteria after in 0.5 per cent oil grew readily when transferred to 1.0 per cent oil (D). Increasing the oil concentration (E) or decreasing the brain heart infusion (F, G, H) did not prevent inoculum from D from growing. Inoculum from H (0.37 per cent brain heart infusion and 1.0 per cent oil) grew

readily in the presence of 2.0 per cent oil only if appreciable brain heart infusion were present. Growth on nutrient agar slants inoculated from contaminated soluble oil emulsions could not serve as a substitute for brain heart infusion (L, M) nor would small concentrations of brain heart infusion support growth. Sixty-day-old emulsion with organisms "normal" to soluble oils did not supply the necessary nutrients when sterilized with the growth medium. However, inoculum from H into media L to P survived for two days but not seven days.

In a search for M. aureus in eight emulsion samples taken from the site of the conjunctivitis outbreak Chapman and Stone and M. aureus media (22) were used. These media are selective for micrococcus while inhibiting most other bacteria. However, Gram stains of the organisms which did grow showed only sporulating rods. Gram stains of suspensions washed from growth of these emulsions on nutrient agar plates also gave no evidence of micrococci. Plating the emulsions with brain heart infusion agar containing 0.02 per cent sodium azide resulted in the growth of many yeasts, and there was evidence that one sample might contain micrococci. Enrichment of this sample in dextrose azide broth with subsequent inoculation on to blood agar plates containing 0.02 per cent sodium azide (46) furnished many colonies of gram positive cocci or coccobacilli which were non-hemolytic. Since the strain isolated from the conjunctivitis outbreak was hemolytic, it was concluded that the non-hemolytic strain could not have been the cause of this outbreak.

Table 10

Adaptation of M. aureus to presence of soluble oil

Flask	Per cent brain-heart infusion	Per cent oil	Other constituents	Inoculum	Bacteria per ml x 10^3 0 days	2 days	7 days
A	3.7	0.1	-	Agar culture	49	295	1,035,000
B	3.7	0.5	-	Flask A - 2 days	1.6	440,000	920,000
C	3.7	1.0	-	Flask A - "	0.8	0	0
D	3.7	1.0	-	Flask B - 9 days	430	1,550	35,000
E	3.7	2.0	-	Flask D - "	40	275	8,000
F	1.85	1.0	-	" "	69	205,000	220,000
G	0.92	1.0	-	" "	174	75,000	315,000
H	0.37	1.0	-	" "	24	148	885
I	1.85	2.0	-	Flask H - "	0.59	6	1,250
J	0.92	2.0	-	" "	0.59	7	500
K	0.37	2.0	-	" "	0.59	30	121
L	0.037	1.0	bacteria*	" "	0.59	0.70	0
M	0	1.0	bacteria*	" "	0.59	0.80	0
N	0	1.0	old emulsion**	" "	0.59	0.50	0
O	0	1.0	-	" "	0.59	0.25	0
P	0	2.0	-	" "	0.59	0.60	0

* suspension of mixed organisms from oil emulsion grown for two days on nutrient agar.

** 25 ml of an emulsion which had been inoculated 60 days previously with emulsion from a machine shop.

Discussion

There hasn't been sufficient work done with Micrococcus aureus to state definitely whether or not it will grow in soluble oil emulsions without additional nutrients. Perhaps fatty oils such as lard oil would support growth even though the petroleum oils used in this experiment did not.

It has been conclusively demonstrated that M. aureus is capable of growing in 0.37 per cent brain heart infusion broth in the presence of a soluble oil emulsion containing two per cent of soluble oil and that this growth takes place readily at room temperature. It has also been shown that a strain adapted to the presence of soluble oil can exist in a two per cent soluble oil emulsion without brain heart-infusion for at least two days.

The two strains of streptococci were so susceptible to the toxic action of 0.1 per cent soluble oil that they do not appear to be capable of withstanding the usual concentrations of soluble oils which may vary from 2 to 15 per cent.

The growth of Ps. aerugenosa in soluble oil emulsions confirms the work of Weirich (59) who isolated this organism from industrial samples.

PART V. DISINFECTION OF SOLUBLE OIL EMULSIONS

There are two reasons why disinfection of soluble oil emulsions are of considerable interest. These reasons are health and economic.

From the health viewpoint, proof is lacking that pyogenic organisms can not be disseminated by cutting fluids. And the work of Shie (52) and Albaugh (2) furnishes evidence that cutting compounds can act as carriers for Micrococcus aureus. Also, there is no proof that M. aureus will not grow in at least some of the several hundred different soluble oils which are available.

Economic reasons for disinfecting the oil are several. Growth of bacteria in the emulsions results in acid and hydrogen sulfide production. The acid must be neutralized frequently at some expense. If it is not neutralized adequately, the pH drops to the point where the emulsion breaks and the whole batch must be thrown out. The hydrogen sulfide and other disagreeable odors are objectionable to the workers and unless steps to eliminate such odors are successful, labor-management difficulties occur. Such steps which have had success are aeration of the emulsions and frequent pasteurization. Both are expensive as regards initial cost of installation, and pasteurization is expensive to carry out. Moreover, the efficacy of pasteurization is sometimes in doubt unless very thoroughly performed.

Disinfection of soluble oil emulsions has been the object of considerable comment. Some workers advocate it with reserve and some think its

value is questionable. Few think that it is a panacea. Schwartz (49) stated that if disinfectants were added they should be added strictly according to the manufacturer's specifications. He said that the germicides were usually either phenolic or contained formaldehyde and that excessive concentrations of these were more conducive to dermatitis than the bacteria in the oil. He cited an instance (48) of finding five per cent of a phenolic disinfectant in a cutting fluid. It had been added from time to time over a long period. Ballard (9) was of the opinion that disinfectants should be added only on the advice of the plant physician. Beard (11) stated that automobile plants used phenol type disinfectants in an initial concentration of 1 to 400 and maintained the disinfectant by adding one gallon per day for each 1000 gallon of cutting fluid. The Department of Scientific and Industrial Research in Great Britain (21) advocated 0.5 per cent of water soluble disinfectants for emulsions and this has been advocated by others (6). Varley (56) stated that cutting oil disinfectants were usually high boiling cresylic acids in combination with suitable coal-tar hydrocarbons. He recommended a 1 to 200 to a 1 to 400 use dilution. Weirich (59) stated that heating the entire body of oil at 140-160°F for 30 min. would destroy the bacteria, but he cautioned about the dangers of recontamination and recommended the use of disinfectants which would act under operating conditions. Westveer (62) recommended sodium o-phenylphenate in concentrations of 0.1 per cent as an adequate disinfectant in soluble oil emulsions and the Dow Chemical Company (23) recommended 0.1 per cent of

either 2, 3, 4, 6 tetrachlorophenol, sodium o-phenylphenate or sodium 2, 3, 4, 6, tetrachlorophenate. They stated that the sodium tetrachlorophenate could be used alone or in combination with the other two but advised against using it in emulsions which would be in contact with the workmen. Lee and Chandler (34) recommended resorcinol as a suitable disinfectant and stated that as little as 15 lbs per month in a 500 gal. tank would prevent spoilage of the emulsion under conditions of use. Larger quantities were necessary for sterilization. They found that coal tar disinfectants would kill bacteria in soluble oil emulsions in the laboratory although the same concentrations were ineffective in the factory. Chlorine as a gas or as chlorine water destroyed the bacteria but also broke the emulsion. Iodine sterilized the oil but on re-inoculation, the bacteria grew. Acriflavine in 0.15 per cent concentration was not effective, and boric acid in high concentrations was also ineffective. Copper sulfate broke the emulsion. Liberthson (36) stated that formaldehyde was satisfactory for rendering the emulsions stable to bacteria, but it was subject to volatilization and loss in hot summer weather.

On the other hand, some think that disinfection was of little value. Yates (63) thought that personal hygiene and keeping the oil free from contamination were more important in the prevention of dermatitis than heat sterilization or disinfection. Albaugh (2) concluded that chemical disinfection was not practical because of the following reasons:

lack of effectiveness of the disinfectant in the emulsion; the disinfectant had to be so strong that it would act as an irritant; objectionable odors of the disinfectants. He recommended heat sterilization. Paine (40) stated that germicides were not too practical because some were corrosive and phenols were irritating. He also recommended heat sterilization. Contrary to these beliefs, Shie (52) found that disinfection of cutting oils with cresol reduced infection of workers by more than 90 per cent.

Methods of laboratory testing the effectiveness of disinfectants in soluble oil emulsions have not occupied the attention of many workers. Lee and Chandler (34) used plate counts to determine the effectiveness of added disinfectants. Westveer (62) inoculated 0.1 ml of a 24 hr culture of an organism isolated from soluble oil emulsion into 50 ml of emulsion which contained the disinfectant. After incubation for 24 hrs at 37°C, plates were streaked. No growth on the plates after 24 hrs indicated an adequate concentration of the disinfectant.

Experimental

The experimental work reported here was designed first to find a suitable screening method for eliminating useless compounds: and then to subject the compounds which had appeared suitable in the screening test to conditions similar to those obtained under actual use.

The screening method was devised after considerable work with changes in oxidation-reduction potentials of mixed cultures growing in soluble oil

emulsions. The oxidation-reduction potential curves were followed with a Model G Beckman potentiometer using platinum electrodes and a calomel half cell, (55). The experiment was set up in the following manner. Five-hundred ml florence flasks were filled to within two inches of the top of the neck with a four per cent soluble oil emulsion and three gm of iron chips were added. The flasks were then inoculated with one ml of a mixture of several soluble oil samples obtained from industrial sources. Then a platinum electrode and a potassium chloride agar bridge, held in a rubber stopper, were put into the flask, and Eh readings were taken.

The potential dropped 540 mv in two days changing from +335 to -205 mv with respect to the hydrogen half cell. When methylene blue thiocyanate was used in the concentration recommended for testing milk (3) there was a change in redox potential from +310 mv to -90 mv and the methylene blue thiocyanate was reduced to the colorless state. There was little change in the above values after six days indicating a poising action of the methylene blue. However, this poising action was insufficient to interfere with reduction of the indicator.

When methylene blue thiocyanate is used in dairy bacteriology, one ml of a 1:200 concentration is added to 10 ml of milk, and the approximate number of bacteria per ml of milk can be determined by the length of time required to reduce the methylene blue to its colorless or leuco form. However, when methylene blue thiocyanate was added in the above concentration to twelve emulsions, which had been inoculated 11 days pre-

viously with a mixed inoculum from machine shop sources, there was no appreciable reduction after 2.5 days even though these emulsions contained from several hundred thousand to a few million bacteria per ml. In these experiments tubes containing 0.3 gm of iron chips to 5 ml of emulsion were used as well as tubes which contained emulsion only. Experimentation eventually showed that if a sterile emulsion containing methylene blue and iron chips were inoculated with a mixture of organisms normally found in soluble oil emulsions, the methylene blue would be reduced, but, if no iron were present, reduction would occur slowly or not at all.

In this work the methylene blue test was not used as an indicator of the number of bacteria per ml of emulsion. Rather, it was used only to indicate whether or not growth of an inoculum was taking place in an emulsion which contained a disinfectant. If the methylene blue were reduced, the disinfectant was considered ineffective. If no reduction occurred, the disinfectant appeared to be useful in the concentration used and further experiments were carried out. In short, it was used only as a screening test.

Details of the test were as follows:

The culture tubes, 16 mm x 150 mm, containing about 0.3 gm of cast iron chips (approximately 50 chips per gm), were plugged with cotton and sterilized in a hot air oven.

Methylene blue thiocyanate solution was prepared by adding one tablet to 200 ml of sterile distilled water which had been heated to boiling immediately before addition of the indicator.

The soluble oil used was an oil emulsified with petroleum sulfonate. A four per cent emulsion of this oil was made with tap water and sterilized in the autoclave at 15 lb pressure for 20 min.

The inoculum was 0.5 ml of a mixture of soluble oil emulsions obtained from industrial sources.

The disinfectant was in a one per cent solution made by dissolving 0.1 gm of the disinfectant in 10 ml of four per cent soluble oil emulsion contained in a dilution bottle.

Ten ml of the autoclaved emulsion was pipetted aseptically into each tube and this was followed by one ml of the methylene blue thiocyanate solution. Then the disinfectant solution was pipetted into duplicate tubes to give concentrations of 0.1, 0.05 or 0.01 per cent.

The tubes containing the disinfectants were held for one week at room temperature with occasional shaking before they were inoculated, and then one tube was inoculated with 0.5 ml of the inoculum while the duplicate tube was held as a sterile control. This control was necessary because occasionally a disinfectant reduced the methylene blue. A second control was always used to check the organism and the method. This was done by inoculating a tube which contained no disinfectant.

The tubes were held at room temperature (23 - 27°C). The methylene blue in the inoculated control, which contained no disinfectant, was reduced in one to one and a half days. Disinfectants which inhibited dye reduction for one week were considered for further tests. If the dye was reduced by the disinfectant (sterile control) then plate count methods were used to study the disinfectant.

In this work, although 0.5 ml of a mixed growth in soluble oil emulsion was used, it was found that as little as 0.1 or as much as 1.0 ml would work. The age of the inoculum and the exact number of bacteria per ml was of little consequence: emulsions stored several months in the refrigerator served very well as inoculum. Pure culture studies with 90 different organisms showed that 92 per cent of them reduced the dye to some extent under the conditions of the test but only 33 per cent of the organisms brought about complete reduction. However, when a mixed inoculum from industrial sources was used, reduction was always complete and rapid.

The promising disinfectants were subjected to more severe tests. These tests, designed to simulate conditions of use in industry, included aeration of the emulsion containing the disinfectant and circulation of the liquid through iron chips. Also, the emulsion was subjected to repeated inoculation with contaminated soluble oil emulsions obtained from industrial sources.

The test was run in the following manner: The apparatus described in Fig. 3 was filled with 3600 ml of two per cent soluble oil emulsion and sufficient disinfectant was added to furnish the concentration required. The emulsion was circulated for one week and then inoculated with 10 ml of a mixture of contaminated soluble oils obtained from industry.

Plate counts were made on the inoculum to determine the resultant concentration of organisms in the flask. Plate counts were also made one day after the initial inoculation using 0.1 ml of the emulsion directly and decimal dilutions to 1:100,000. Further plate counts were made at 10 and 18 days. Those emulsions found to be sterile at 18 days were reinoculated at 22 days with 10 ml of the inoculum and a final plate count was made at 29 days. In a few instances plate counts were also made at 44 days.

Results and Discussion

Many compounds were screened by the methylene blue reduction technique, but only a few were considered sufficiently effective to be tried in the circulation apparatus.

Compounds which failed to inhibit reduction of methylene blue under conditions of the test when used in 0.1 per cent concentration were orthophenyl phenol and its sodium salt; 2, 4, 6 trichlorophenol; chloro-2-phenylphenol; 2 chloro-4-phenylphenol; 2 bromo-4-phenylphenol; 2, 3, 4,

6- tetrachlorophenol, technical grade, and its sodium salt; pentachlorophenol and its sodium salt; mixture of 4-chloro- and 6-chloro-2-phenylphenol; parachlorometaxylenol; dehydroacetic acid and its sodium salt; trichloroacetic acid; sodium benzoate; methyl parahydroxybenzoate; paracresyl benzoate; benzoyl peroxide; alpha alanine; beta alanine; sorbic acid; malonic acid; thiourea; sodium tetraborate; hexylene glycol; Hycol (mixture of phenols, coal tar neutral oils and soap sold by the Creolin Co., N.J.); octyl phenol; nonyl phenol; dinonyl phenol; dodecyl phenol; orthobenzyl parachlorophenol; succinchlorimide; 1, 3-dichloro 5, 5-dimethyl hydantoin; dodecylamine; tetradecylamine; 4, 4-dichlorobenzophenone.

The following compounds appeared promising: 2, 4, 5, trichlorophenol and its sodium salt in 0.1 per cent concentration; parachlorometacresol in 0.1 per cent concentration; nitromersol (4-nitro anhydro-hydroxy-mercuri ortho cresol) in 0.01 per cent concentration; mixture of sodium salt of 2-mercaptobenzothiazole (58 per cent) and lauryl pyridinium salt of 2 mercapto thiazole (42 per cent) in 0.05 but not 0.01 per cent concentration; bis 3, 5, 6-trichloro-2 hydroxy phenyl methane in 0.1 per cent concentration; and chloramine T and dichloramine B in 0.1 per cent concentrations.

Several compounds were tried but were abandoned for various reasons. Quaternary ammonium compounds were incompatible with anionic constituents of soluble oils as was expected. Compounds of iodine such as potassium iodide and biniodate broke the emulsion as did copper acetate. Some compounds were not soluble.

The compounds which appeared promising in the methylene blue test were subjected to the circulation test. The results of the circulation test are shown in Table 11 .

It is obvious that ability of the disinfectant to inhibit reduction of methylene blue did not necessarily mean that the disinfectant would be suitable under more severe conditions. As shown in Table 11 Chloramine T in 0.05, and 3, 4, 5, trichlorophenol in 0.1 and 0.05 per cent concentrations had little or not inhibitory effect on bacterial growth. Dichloramine B and nitromersol caused immediate decreases in the bacterial populations, but their action was not sustained. Parachlorometacresol in 0.05 per cent concentration killed the initial inoculum but not the inoculum which was added at 22 days. Bis 3, 5, 6, trichloro, 2 hydroxy phenyl methane gave sustained killing action in 0.1 per cent concentration and inhibited growth even in 0.05 and 0.01 per cent concentrations.

The use of a disinfectant in soluble oil emulsions is governed by many factors. Besides being effective under the conditions of use, it must be non-toxic to the workers and inexpensive. It must be long lasting or else an easy analytical method must be available to the plant chemist in order that he can maintain the desired concentration of disinfectant. Of the compounds tested only bis 3, 5, 6 trichloro, 2 hydroxy phenyl methane was effective for a long period of time. Since this compound is a component of toilet soap, it must be suitable from a toxicological viewpoint. If its cost were not prohibitive, it might be valuable

in industry. Parachlorometacresol might have limited activity under conditions of use. The others did not appear of much value when tested by the above methods.

Table 11

Effect of disinfectants on the bacterial populations of soluble oil emulsions aerated and circulated through iron chips

Disinfectant	Per cent conc.	Bacteria per ml x 10^3						
		∓0 day*	1 day	10 days	18 days	22 days*	29 days	44 days
Nitromersol	.05	15	0	-	-	-	-	260
Chloramine T	.05	15	38	950	-	-	-	-
Parachlorometacresol	.05	15	0	0	0	13	0.1	60
Dichloramine B	.05	118	6	25	260	-	-	-
2,4,5 trichlorophenol, sodium salt	.1	118	930	6,400	3,400	-	-	-
" " " "	.05	118	6,700	7,000	3,000	-	-	-
bis 3,5,6 trichloro, 2 hydroxy phenyl methane	.1	118	0	0	0	80	0	-
"	.05	2.8	0	8.7	123	-	30	-
"	.01	2.8	2,700	680	450	-	135	-
Control - no disinfectant	-	2.8	5,700	4,900	4,400	-	1,500	-

* Inoculum added at these days.

\- No data obtained

∓ The various compounds were investigated in three different groups as can be seen by the bacteria per ml at 0 days.

PART VI. NUTRITIONAL STUDIES OF THE BACTERIA IN SOLUBLE OIL EMULSIONS

Review of Literature

The chemical composition of soluble oils may be extremely variable because the molecular structure of the petroleum component is unknown: and the petroleum may be made water soluble with a large number of emulsants whose chemical composition may or may not be known. Also many of the soluble oils contain a coupling agent and a water softener.

The oil portion of the soluble oil may be either a petroleum oil, a fatty oil, or a mixture of the two. The petroleum oil may be a paraffin, a napthene, or a mixture of the two, while the fatty oil may be lard oil or one of several vegetable oils. Both types of oil may be untreated or they may be treated with such materials as sulfur or chlorine. Even the method of treatment may vary, e.g. lard oil sulfurized with flowers of sulfur by a high temperature, short-time process replaces hydrogen with sulfur while a low temperature, long time process results in sulfur adding at the double bond (45).

The compounds used for emulsifiers and stabilizers are numerous. Schwartz and Perry (51) stated, "The usual emulsifying agents for soluble cutting oils include the alkylolamine soaps of fatty acids, rosin acids, and naphthenic acids, the mahogany sulfonates, and the sulfated oils.

These are most often used in mixtures rather than singly, and the mahogany sulfonates are probably used in larger quantities than the other products mentioned. Other emulsifying agents that have been used are the Igepon A class of sulfonates, the soaps of carboxylic acids from oxidized paraffin wax, and the polyethylene oxide type of non-ionic agent." Besides the above-mentioned emulsifying agents the sludge base extracts from the acid refining process of petroleum (28) and tall oil (13), a by-product of the sulfate process for making wood pulp, are often used.

In addition to the emulsifying agents most soluble oils contain a coupling agent or mutual solvent to aid in solubilizing additives. Such coupling agents may be glycol ethers, terpene solvents, butyl and amyl alcohols, etc. (51).

Obviously the composition of any soluble oil emulsion is so complex that it eludes exact chemical definition. Perhaps a combination of the definitions of Schwartz and Perry (51) and Philipp (42) are as comprehensive as we can expect. Schwartz and Perry stated, "Soluble oils are usually mixtures of oil and a surface active agent together with a blending agent or mutual solvent which serves to give a homogeneous preparation", and, concerning the emulsion, Phillips said, "Der Grad der Emulgierbarkeit erstreckt sich vom volligen Klarbleiben beim Verdunnen mit beliebigen Mengen Wasser uber das Opalisieren der Losung hinweg bis zur Bildung einer milchigen Suspension von grosserer oder geringerer Bestandigkeit, die also mehr oder weniger zum Aufrahmen neigt".

The growth of bacteria in soluble oil emulsions has been demonstrated but there has been little attempt to study the nutritional factors affecting such growth. Lee and Chandler (34) found that the bacteria isolated from soluble oil emulsions (presumably Ps. oleovorans) could grow in Locke's Basal Solution containing one per cent of crude naphthenic acids only if a nitrogen source was added. They used 0.1 per cent potassium nitrate. The bacteria did not grow in a medium containing only carbohydrate as a carbon source thus proving that the crude naphthenic acids acids were utilized by the bacteria as a carbon source. Lee and Chandler were unable to concentrate the oil used in their experiments sufficiently to demonstrate any nitrogen in it but they believed that the nitrogen source was nitrogenous compounds in the oil itself. Such compounds have been discussed by Ellis (27).

The debris which frequently finds its way into the soluble oil emulsions used in factories may serve as a source of nutrients. Liberthson (36) reported finding metal filings, powder, abrasives and such organic contaminants as tobacco juice, remnants of abandoned lunches and other putrefieable materials. And the C.B. Dolge Company (16) stated that feces and other body discharges have been found in emulsions taken from some shops.

Work with petroleum bacteriology, outside the field of soluble oil emulsions, has been more abundant and fruitful. Recently it has been the occasion for two very excellent reviews (65 , 66) which may be summarized briefly.

Contrary to popular belief the ability of microorganisms to attack hydrocarbons is quite general, over 100 species representing about 40 genera of yeasts, molds and bacteria having been shown to possess hydrocarbonoclastic properties.

Almost all types of hydrocarbons are susceptible, aliphatic, olefinic, naphthenic and aromatic compounds having been investigated. Aliphatic compounds are generally more easily attacked than aromatics and some organisms attack aliphatics but not aromatics of similar molecular weight. If dispersed adequately long-chain hydrocarbons are more susceptible to attack, within certain limits, than short-chain compounds. Unsaturation and branching enhance the vulnerability of the molecule to attack. Nearly 100 pure hydrocarbons ranging in complexity from methane to dibenzanthracene and several hundred mixtures of hydrocarbons have been investigated. "The latter include natural gases, ligroins, gasolines, kerosenes, benzines, lubricating oils, fuel oils, paraffin waxes, tars, crude oils, asphalts, asphaltenes, synthetic and natural rubbers, etc." (66). Even compounds commonly used as bactericides, eg. phenol, cresols and toluene, are metabolised in low concentrations by some bacteria.

Experimental

The experiments described in this section were designed to study some of the nutritional factors involved in the growth of bacteria in soluble oil emulsions. Four projects were carried out (a) the effect of soluble oil concentration on growth curves (b) the effect of added pep-

tone on growth curves (c) determination of the organic nitrogen content of soluble oils and (d) the growth of pure cultures in various components of soluble oil emulsions.

The effect of the concentration of oil was studied by inoculating emulsions containing 10, 1, 0.1, 0.01 and 0.0 per cent of soluble oil and following the growth curves. The medium consisted of the above concentrations, soluble oil, tap water and iron chips. The soluble oil was a light mineral oil emulsified with petroleum sulfonates. Three hundred ml of the different emulsion concentrations was dispensed into one-litre flasks and autoclaved at 15 lbs for 20 min. To each flask was added aseptically three gm of oil-free, heat-sterilized cast iron chips.

The inoculum was prepared by growing a mixed culture (mixture of 20 emulsions obtained from industry) on nutrient agar containing one per cent of a soluble oil, for two days at 30°C. The growth was harvested in M/20 phosphate buffer at pH 7.0, shaken 30 min at 240 strokes per min, centrifuged, and resuspended in fresh buffer. The washing process was carried out three times. The final suspension was plated, after which it was refrigerated at 0°C. The plates made from the suspension were incubated and counted. Sufficient suspension was then added to each flask to give a concentration of about 10^5 cells per ml of emulsion.

The flasks were incubated at room temperature (23 - 27°C) and plate counts were made at intervals. Immediately before withdrawing the samples for plate counts the flasks were shaken at 108 strokes per min for five min.

The effect that added peptone had on the growth curves of bacteria in soluble oil emulsions was determined by growing an inoculum of the above-mentioned bacterial mixture in a four per cent emulsion of the above-mentioned soluble oil containing Difco peptone.

Three runs were made. In two of them concentrations of 0.5, 0.05, 0.005 and 0.0 per cent peptone were used. And in the third run concentrations of 0.5, 0.25, 0.1, 0.05, 0.01, 0.005, 0.001 and 0.0 per cent were used.

The apparatus and methods employed were the same as those used for determining the effects of soluble oil concentrations on growth curves.

The kjeldahl nitrogen was determined on duplicate samples of four of the oils by the Kjeldahl-Gunning method (8). A slight modification was necessary which used only about 0.5 gm of oil due to the difficulty of digesting larger amounts of the oil by the described method. Also, the ammonia content of one sample of soluble oil was determined.

The ability of bacteria to utilize the various materials which have been reported to be found in soluble oils was investigated. The bacteria were four pure cultures isolated from soluble oils. They are described in Part 7. The soluble oil components were six compounds commonly used as emulsifiers and a light paraffin oil which was representative of those used in industry as the main component of soluble oils. The emulsifiers were sodium naphthenate designated as A (about a 40 per cent mixture of naphthenate in mineral oil); polyoxyethylene sorbitan trioleate (Tween 85), B; mahogany soap (petroleum sulfonates), C; triethanolamine, D; tall oil

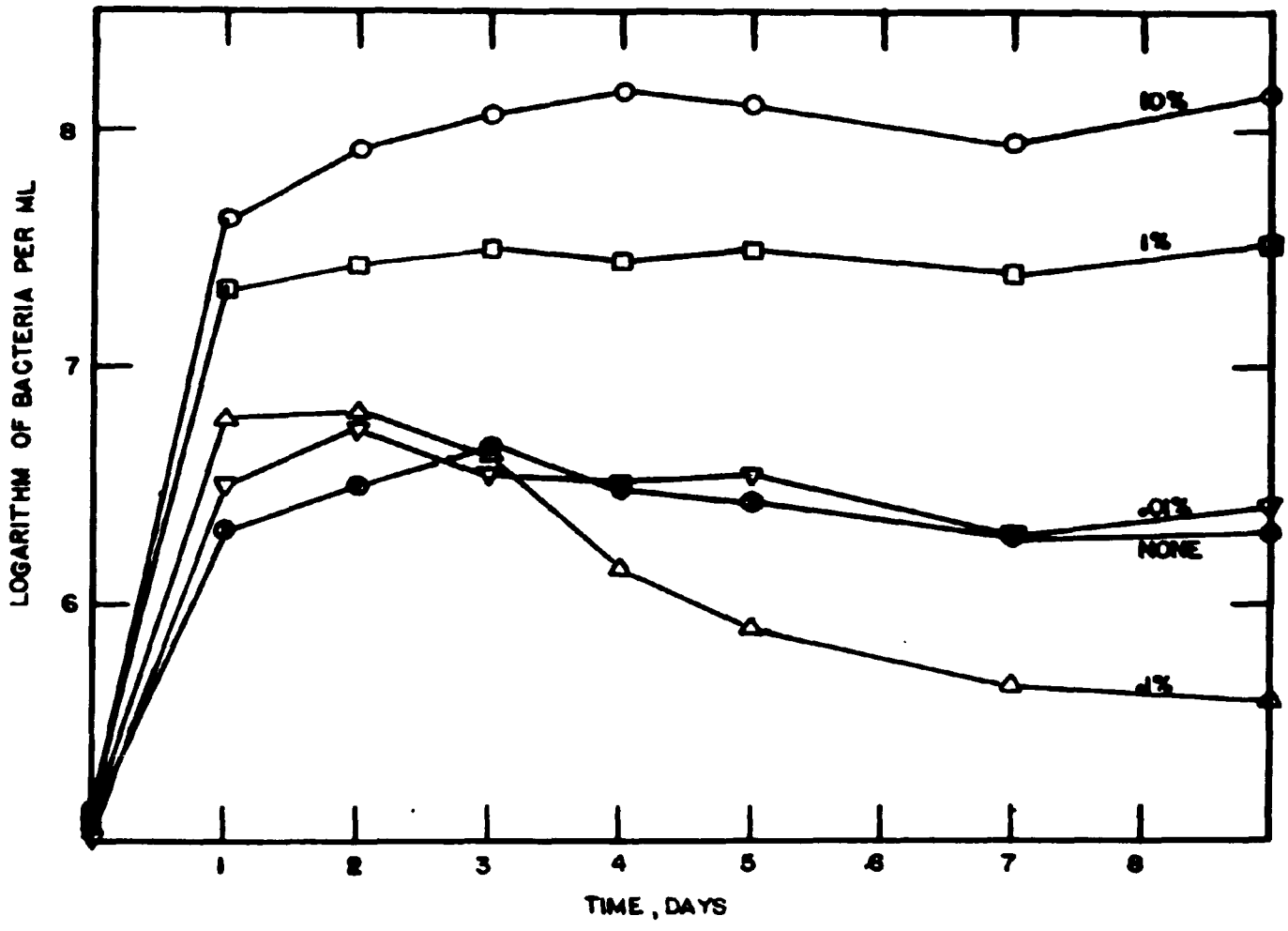

Fig. 11 Growth curves of mixed cultures in emulsions containing different percentages of soluble oil.

soap, E; sodium salt of abietic acid, F. Besides the components of soluble oils another compound, G, used in place of soluble oils for machine shop work, was investigated. G was the sodium salt of an alkylated glycine having the formula $RNHCH_2COONa$ where R is a C_{12} to C_{16} hydrocarbon chain.

Three media were used -- tap water; tap water plus 0.1 per cent of compound A to G; and tap water plus 0.1 per cent of compound A to G plus one per cent of light paraffin oil. The media were agitated in a Waring Blendor, dispensed in 10 ml quantities in dilution bottles and autoclaved.

The inocula were the four previously mentioned pure cultures. They were prepared by suspending a small amount of growth from a soluble oil-nutrient agar slant in sterile distilled water. Each bottle containing medium was inoculated with 0.1 ml of bacterial suspension to give an initial bacterial population of 10^2 to 10^3 cells per ml. A plate count was made on the inoculum in order to determine the bacteria per ml of medium immediately after inoculation.

The inoculated bottles were incubated at room temperature and shaken four hours daily on a reciprocating shaker running at 120 strokes per minute. After five days the bacterial population of each medium was determined by the plate count method.

Results and Discussion

Fig. 11 shows growth curves prepared from the averaged results of two identical experiments, both of which showed identical trends.

It can be seen that an increase in soluble oil concentration resulted in an increased growth rate. As shown below the mean generation times during the first 24 hrs decreased as the oil concentration increased.

Per cent oil	Mean generation time in minutes
10.0	173
1.0	193
0.1	247
0.01	289
0.0	367

The mean generation time of 173 minutes is considerably greater than that obtained in work with enteric organisms and many other groups of bacteria in laboratory media. It would appear that something in the soluble oil itself was responsible for the stimulation of growth because of the inverse relationship of oil concentration and generation time but it is very unlikely that this stimulant was a hydrocarbon. The oil was very well dispersed, globules being generally only a few microns in diameter, and therefore the hydrocarbon would not be the limiting factor. The nitrogen present in the soluble oil might be the limiting factor because nitrogen was present to only a very small degree.

The ability of increased concentrations of soluble oil to maintain increased bacterial populations is apparent from an examination of the curves for 10, 1.0 and 0.01 per cent concentrations. The decrease in

bacterial population with time found in 0.1 per cent concentration of the oil can only be explained by the rapid production of antagonistic conditions, perhaps pH. A higher concentration of oil might have exerted a buffering action while a lower concentration would not have sufficient oil to cause much change when metabolized.

The effect of added peptone is shown in Fig's. 12 and 13. Fig. 12 shows growth curves prepared from the averaged results of two identical experiments and Fig. 13 was obtained by using the bacterial populations per ml at four days for the above two experiments, and also a third experiment which used eight concentrations of peptones. In all instances an increased peptone concentration resulted in maintenance of a greater bacterial population but this population increase was relatively greater with small concentrations of peptone. The data represented in Fig. 13, when plotted as logarithm of bacterial population against logarithm of peptone concentration, gave a linear relationship between 0.005 and 0.25 per cent peptone concentrations. The curve had the formula $y = 1.47x$ where y was the logarithm of the bacterial population at four days and x was the logarithm of the peptone concentration in the oil emulsion.

The addition of peptone to the soluble oil emulsion resulted in the production of foul odors. At four days oils containing 0.5 per cent peptone possessed very objectionable odors while those containing 0.05 per cent peptone possessed the same odor but it was less pronounced. Emulsions containing 0.005 per cent peptone smelled like the control which contained no peptone.

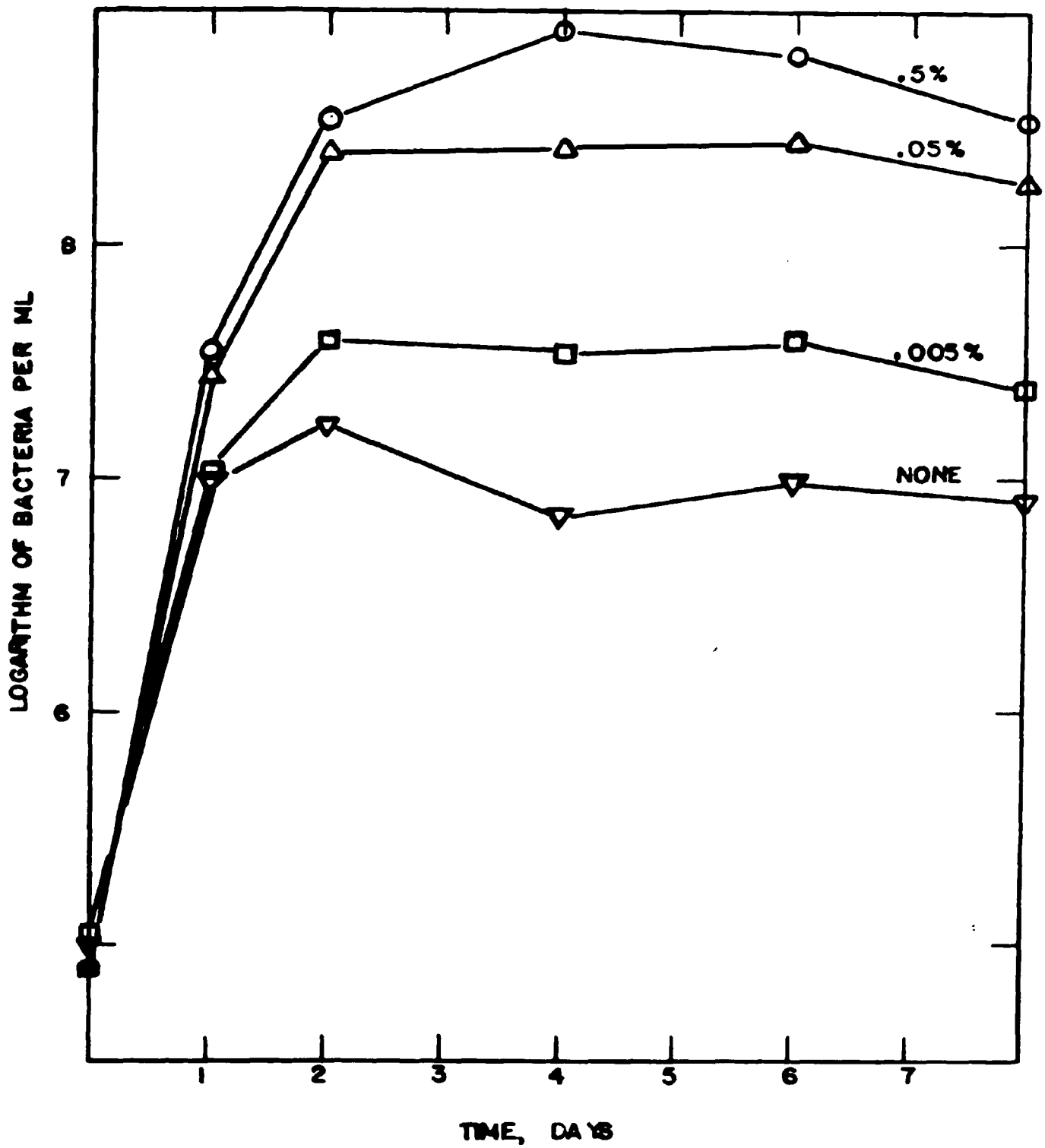

Fig. 12 Growth curves of mixed cultures in soluble oil containing various amounts of peptone.

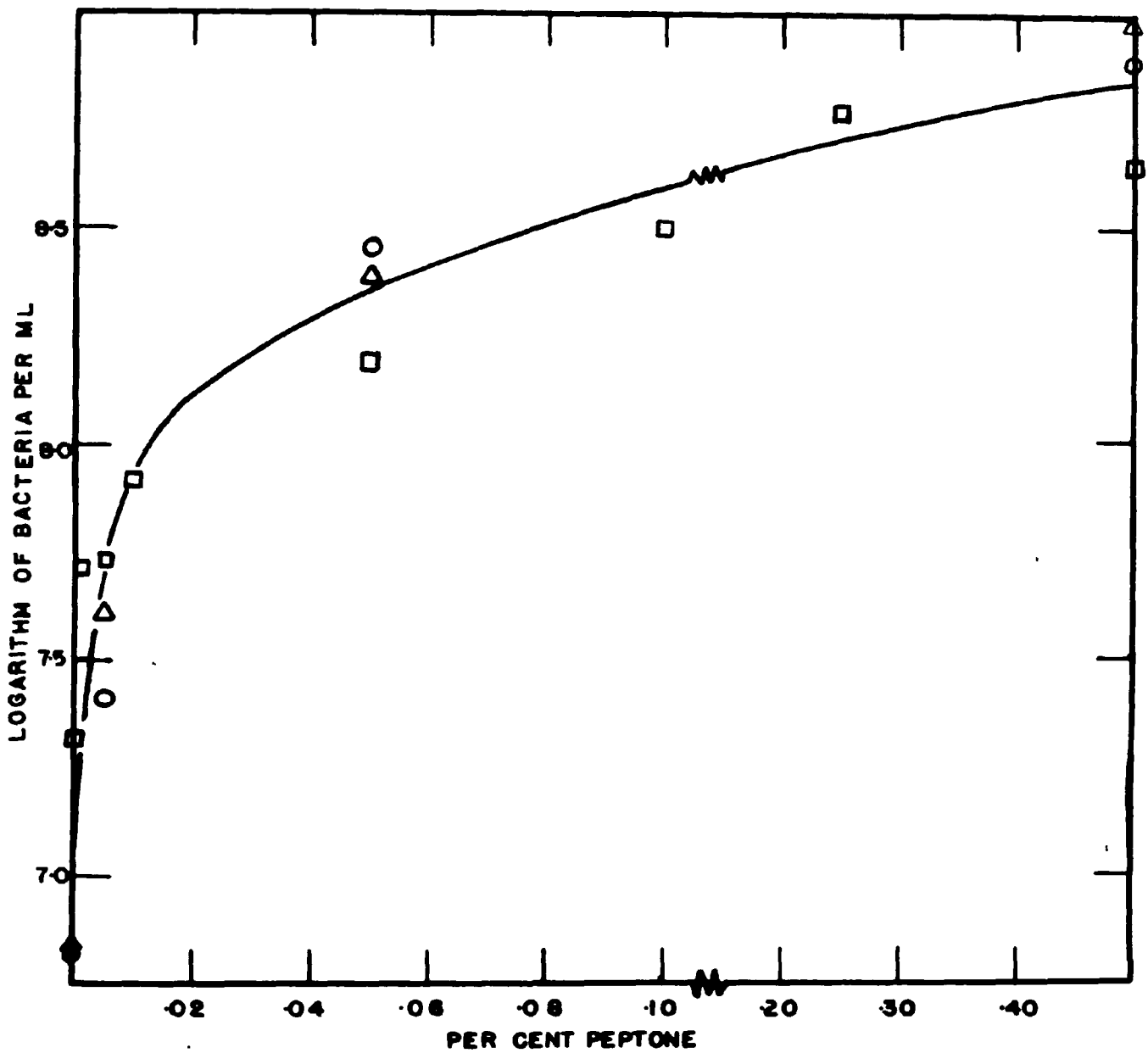

Fig. 13 Effect of different percentages of peptone on the bacterial population of a mixed culture grown for four days in soluble oil emulsion.

The kjeldahl determinations of four soluble oils showed that all contained some amino nitrogen. Oils designated 1, 3, 5 and 9 contained 0.0276, 0.0672, 0.1458 and 0.1306 per cent kjeldahl nitrogen respectively. It is interesting to note that growth curves in oils 1, 3 and 5 were very similar while oil 9 supported about 2.5 times the bacterial population that the other three oils did. Oil 5 contained the greatest amount of nitrogen and it was known to contain triethanolamine. Data of experiments not given here in which eight different concentrations of triethanolamine were added to soluble oil emulsions showed that triethanolamine did not cause an increase in growth. This may be the reason why oil No. 5, with the greatest nitrogen content, did not support as much growth as oil No. 9. Oil No. 9 did not contain triethanolamine but the kjeldahl nitrogen which it did contain evidently was available to the bacteria.

The effect of the several components of soluble oils on the growth of the four pure cultures is shown in Table 12, which is based on the averages of two identical experiments. The bacteria per ml of tap water for cultures 6, 12, 72 and 94 at five days were 15.5, 9.3, 5.1 and 8.3 million respectively. The bacterial populations in the mixtures of water and emulsifier or water, emulsifier and oil at five days are reported in Table 12 as percentages of the populations in water.

It can be seen that the four cultures did not grow equally well in all of the soluble oil components. Sodium naphthenate enhanced growth of cultures 6 and 12 but not 72 and 94. Tween 85 appeared to cause increased growth of culture 72 but not the others. Mahogany soap did not affect

cultures 6 and 12 to any appreciable extent but it was toxic to 72 and 94 when used alone or with oil. Triethanolamine with water, or with water and oil had little effect on cultures 6 and 94 but was toxic to 12. Tall oil soap alone had little effect on cultures 6 and 12 but when oil was added to the mixture growth of these two organisms was stimulated. In contrast when oil was added to the sodium naphthenate - water mixture growth of 6 and 12 was inhibited. Tall oil soap had little effect on culture 72 but it was toxic to 94. Sodium abietate stimulated cultures 6, 12 and 72 but the addition of oil caused little additional stimulation to cultures 6 and suppressed 12 and 72. Sodium abietate was toxic to culture 94. The compound $RNHCH_2COONa$ had no effect except on culture 12. Apparently cultures 6 and 12 were stimulated far more than cultures 72 and 94 by the compounds investigated and culture 94 was frequently destroyed by compounds which were stimulatory to 6 and 12.

Table 12

Growth of pure cultures isolated from soluble oil emulsions in components of soluble oils after five days

Soluble oil component	Growth expressed in terms of per cent of growth in tap water*							
	Culture 6		Culture 12		Culture 72		Culture 94	
	Emulsifier	Emulsifier + oil	Emulsifier	Emulsifier + oil	Emulsifier	Emulsifier + oil	Emulsifier	Emulsifier + oil
Sodium naphthenate	387	86	208	159	49	135	96	99
Tween 85	72	194	141	0	216	0	131	78
Mahogany soap	54	108	147	204	0	0	0	0
Triethanolamine	97	119	0	0	-	-	86	48
Tall oil soap	148	404	58	269	43	74	0	0
Sodium abietate	175	197	163	86	255	63	0	0
$RNHCH_2COONa$	113	61	226	-	69	74	147	157
Tap water - control	100	100	100	100	100	100	100	100
* Bacteria per ml in tap water - control	15,500,000		9,300,000		5,100,000		8,300,000	

PART VII. BACTERIA IN SOLUBLE OIL EMULSIONS

Review of Literature

Page and Bushnell (41) found no obligate anaerobes by any of the several methods which they used. They isolated Bacillus aerogenes, Bacillus coli communis, and an organism similar to, but not identical with, Proteus vulgaris. They found no staphylococci or spore formers.

Lee and Chandler (34) found a short motile rod with a single polar flagellum, which did not ferment sugars, liquefy gelatin, or produce indole. Nitrates were reduced and starch was hydrolyzed. Growth in stab culture was only to about two-thirds of the depth of the stab. One of the outstanding characteristics of the organism was that it produced no water-soluble fluorescent pigment though the colonies themselves were markedly fluorescent. This organism which was present in almost pure culture in all the machine shop emulsions which they investigated was named Pseudomonas oleovorans.

Duffett, Gold and Weirich (25) examined the flora of thirty samples of soluble oil emulsions. The predominating colonies on most pour plates produced greenish or reddish iridescence by transmitted light without the production of true pigment. Unlike Lee and Chandler they found several species and genera contained in single samples of oil. The

predominating flora was members of the genus Pseudomonas, Ps. oleovorans and six new species of Pseudomonas being most common. They also found Ps. aeruginosa, Aerobacter aerogenes, Escherichia coli, Bacillus alvei, and two species of unidentified pink-producing gram-negative rod bacteria, as well as yeasts and molds. Unfortunately there was no published description of the six new species and the original experimental data were not available (60).

In order to gain additional knowledge of the flora of soluble oils the following work was undertaken.

Experimental

Methods employed for pure culture study were generally those advocated by the Society of American Bacteriologists (53). However, deviations from these methods were occasionally necessary.

Ninety cultures were isolated from the previously mentioned samples obtained from industries in Michigan, Illinois, Wisconsin and Ontario, a total of 14 samples being investigated. After plate counts had been made on the refrigerated samples the emulsion was diluted and quantities plated in duplicate so as to obtain 30, 40 or 50 colonies per plate. Colonies were fished then to nutrient agar slopes and after growth had occurred loop inoculations were made into about 50 ml of sterile water. This suspension was shaken 225 times

per minute for 30 minutes and then streaked on nutrient agar plates. Well separated colonies were fished and the process repeated until at least two successive platings showed only one type of colony. Final isolates were stabbed into nutrient agar and held in the refrigerator for identification.

All work, except microscopical studies, was done at least twice. Gram stains were made at one, two, four and six days; capsule and acid-fast stains at six days; flagella stains at 18 to 24 hours and at two days. Flagella stains were made by the Leifson method (35).

Colonies on agar plates incubated at 30° and gelatin plates at 20° were examined at one, two, three and seven days with the aid of a dissecting microscope (X43) and at seven days only with a "black daylight" ultra violet (U/V) lamp. Only where marked fluorescence under ultra violet was observed is it discussed in the results. Fluorescence by transmitted light (T/L) was observed on the Quebec colony counter; by holding the plates or tubes between the eye and a 150 watt frosted electric light bulb; and by the method of Huddleson (31). It was necessary, in observing fluorescence, to move the investigated material until the light struck it at an oblique angle because if the plate was on a straight line between

the frosted electric light bulb and the eye colonies rarely appeared fluorescent. Observations by reflected light (R/L) were made under the above-mentioned electric light bulb or by daylight.

Agar strokes, nutrient broth and gelatin stabs (10 per cent Difco gelatin in distilled water) were examined at one, two, three and seven days. The gelatin stabs were also examined at intervals up to eight weeks.

Motility medium (22) was incubated at room temperature, 30° and 37° and observed at one, two and eight days. Pigmentation as well as motility was observed. True pigment production was observed in many organisms on this medium but not on other media.

Potato slants, incubated at room temperature were observed at one, two, four and seven days.

Peptone iron agar, incubated at room temperature, had a lead acetate paper strip inserted with the cotton plug.

Litmus milk, nitrate broth, indole test medium, Voges-Proskauer test medium and citrate broth were Difco products. All were incubated at room temperature. Litmus milk was examined at frequent intervals up to 28 days; nitrate reduction was tested at one, two, four, seven and 21 days; indole production was tested at one, two and four days; methyl red

and Voges-Proskauer tests, when made, were made at one, two and four days. Citrate broth was examined at one, two, four and ten days.

All cultures were grown in Uschinsky's solution which was found by Clara (18) to be very reliable for elaboration of water-soluble green pigments and also in Georgia and Foe's medium. However, after three weeks at room temperature no green or blue water soluble pigments had been observed.

Results and Discussion

All cultures except two were gram-negative rods. None were acid fast or capsulated. Five of them (No's. 81, 97, 99, 101, 103) fermented lactose with the production of acid and gas and are members of the Enterobacteriaceae. One was a gram-positive Micrococcus (No. 4), one a Sarcina (No. 105), one a Flavobacterium (No.106), and one a Vibrio (No. 55). The remaining 81 organisms were almost all members of the genus Pseudomonas because of the presence of polar flagella although it was not possible to state definitely whether some of the cultures belonged to this genus. Twenty of the 81 cultures regularly produced opaque, white daughter colonies within the translucent, fluorescent parent colonies and because it was impossible to secure cultures free from these variants, regardless of the number of successive platings employed, they are not described. No organisms studied could

be classified as Pseudomonas oleovorans because this organism hydrolyzes starch and the few organisms of our collection which did hydrolyze starch differed considerably from Ps. oleovorans in other respects. Cultures No's. 38, 46, 87 and 93 may belong to the genus Phaeomonas proposed by Kluyver and Van Niel (33) because of the production of a water soluble brown pigment. However, Bergey (14) describes no bacteria which resembles this group. Cultures (No's. 83, 88, 94) were bright pink in all media tested and this characteristic was maintained in culture for over a year. They are polar-flagellated and produce a yellowish, florescent, water soluble pigment in motility medium, Uschinsky's solution and gelatin stab but this pigment is not noticeable in any other media. Undoubtedly this group and the group of organisms producing a brown water soluble pigment will be classified in the genus Pseudomonas if additional work shows that they are stable. Cultures No's. 12, 72, 75, 76, 77, 89, 92 reduced nitrate to nitrogen gas and in many ways resembled Ps. eudomonas denitrificans. However, they differ from this organism in several respects and actual comparison with Ps. denitrificans will be made in order to determine whether these organisms may be classified as Ps. denitrificans. Cultures No's. 1, 2, 3, 4, 5, 6, 7, 9, 10 resemble Ps. oleovorans but whereas Ps.

oleovorans is described as reducing nitrates and hydrolyzing starch these organisms produce only slight amounts of nitrite and that only after prolonged incubation and they do not hydrolyze starch. Here again actual comparison with Ps. oleovorans must be employed to determine whether the difference is apparent or real.

Many of the cultures do not resemble exactly any described species and have so few outstanding characteristics that they can not be grouped together. Cultures No's. 13, 18, 21, 32, 35, 37, 40, 41, 43, 47, 49, 50, 51, 54, 56, 59, 65, 66, 68, 71, 73, 86, 90 are tentatively identified as Ps. eudomonas desmolyticum Gray and Thornton (29), a species which may or may not reduce nitrates and ferment dextrose. Culture No. 27 resembles Ps. eudomonas dacunhae Gray and Thornton. Culture No. 55 resembles Vibrio percolans Mudd and Warren (14) but it was non-motile and no flagella were observed. Culture No. 82 produced no water soluble pigment in motility medium and gave no indication of motility. It is not similar to any organism described in Bergey and is probably a hitherto unidentified Achromobacter.

Cultures No's. 11, 15, 16, 17, 49, and 50 which do not resemble any organisms described in Bergey could be paired according to their characteristics into No's. 11 and 15; 16 and 17; and 49 and 50. No's. 11, 15, 49 and 50 possessed

a single polar flagellum and are members of the genus Pseudomonas. Numbers 16 and 17 were non-fluorescent, did not produce water soluble pigment and were not flagellated. However, insufficient work was done to determine whether they belonged in the genus Bacterium. They resembled none of the Achromobacteria described by Bergey.

Culture No. 8 resembles Ps. oleovorans in many respects but it turns litmus milk alkaline and does not hydrolyze atarch.

No. 98 slowly produced a small gas bubble in lactose broth indicating that it may be a coliform. However, its predominating flagellation is polar indicating a Pseudomonas. Further work must be done to determine to which genus this organism belongs.

No. 100, a member of the genus Pseudomonas, has several very distinctive characteristics. It is not described in Bergey and if a few more cultures can be isolated it may be named as a new species. Cultures No's. 102 and 104 are also Pseudomonas the species of which differ from each other and also from No. 100. They, too, may be new species if further work and additional isolates substantiate the work which has been done already.

In general it may be concluded that soluble oil emulsions contain a large number of species hitherto not described and that at least five genera are represented. This

work confirms the findings of Duffett, Gold and Weirich (25) and goes further in that it gives accurate and detailed descriptions of at least a few of the species. It also presents several genera not found by these workers. It is expected that future work will result in naming these organisms.

Cultures No's. 81, 97, 99, 101, 103

These five cultures could be classified as Escherichia intermedium (Werkman and Gillen) Vaughn and Levine. They gave a positive Voges-Proskauer test. On eosin methylene blue agar all five cultures fitted descriptions of Vaughn and Levine's colony types 1 or 2 (57).

Culture No. 44

Vegetative cells: Gram-positive cocci, 0.6 u diameter, mainly in clusters, occasionally in pairs or chains of three or four cells.

Agar colonies: Punctiform, smooth, entire, convex, translucent at one day enlarging to 1.0 - 1.5 mm at two days and 3.0 mm at seven days. Colonies are greyish becoming whitish by seven days.

Agar stroke: Moderate, filiform, glistening, greyish.

Nutrient broth: Slight clouding. Scanty granular sediment at two days becoming viscid by seven days.

Gelatin stab: Uniform, filiform growth with slow saccate liquefaction becoming stratiform. Three-quarters liquefied at eight weeks with no sediment.

Motility medium: No pigmentation.

Litmus milk: Acid by one week but no curd at four weeks.

Potato: Creamy growth on one potato, colorless on another.

Nitrate: Nitrates not reduced.

Indole and hydrogen sulfide negative.

Starch not hydrolyzed. Acid but no gas from dextrose, fructose, galactose, lactose, sucrose, maltose. Very slight acid from dextrin at 21 days. No action on arabinose, xylose, raffinose, inulin, mannitol, dulcitol, salicin, aesculin.

No growth in citrate broth and Uschinsky's medium.

Discussion: Similar to M. candidus but differs with regard to gelatin liquefaction, color and size of colonies and growth on potato.

Culture No. 105

Very similar to [illegible] Sarcina flava, DeBarry, except that litmus milk does not become alkaline.

Culture No. 106

Very similar to [illegible] Flavobacterium breve, (Frankland and Frankland).

Cultures No's. 38 and 46

Vegetative cells: 0.6 by 1.0 to 1.5 u shortening to 0.8 to 1.0 u at two days with no change from two to six days. No. 46 is motile at two days at 30° and 37° while No. 38 is doubtful at 37° after eight days and non-motile at 30°. Single undulate polar flagellum in No. 46. None observed on No. 38. Gram-negative.

Agar colonies: At one day, punctiform, smooth, entire, convex, translucent, greyish enlarging to 1.5 mm at two days and 2.0 to 3.0 mm at seven days. Fluorescent by T/L. No. 38, colonies become rugose and centres drop precipitously at two days. Medium becomes browned, especially in the case of No. 38.

Gelatin colonies: Punctiform, convex, entire, smooth, blue by T/L. Medium browned, especially by No. 38. Colonies increase to about one mm at seven days.

Agar stroke: No. 38, abundant filiform, glistening, brownish growth, with medium becoming browned. No. 46, abundant, filiform changing to echinulate, glistening, greyish, medium not browned.

Nutrient broth: No. 38, strong clouding with scanty, viscid sediment by seven days. No. 46, like 38, but abundant sediment. Medium brown.

Motility medium: Profuse brown pigmentation throughout medium at two days at 30° and 37°.

Litmus milk: No. 38, slowly alkaline; No. 46, no change.

Potato: In one potato medium both cultures made the medium reddish brown and there was glistening, brownish growth. On another medium the growth was very sparse and the potato became tan.

Nitrate: Reduced to nitrite.

Indole and hydrogen sulfide negative. Peptone iron agar becomes dark brown in upper half.

Starch hydrolyzed to achroodextrin by 10 days by No. 46 but not No. 38. Dextrose not fermented.

No growth in citrate broth.

Culture No's. 87 and 93

Vegetative cells: 0.5 by 0.6 u, with rounded ends. No change in size or shape from one to six days. Motile in two days at 30 and 37° in motility test medium. Single undulate polar flagellum.

Agar colonies: Punctiform, smooth, entire, convex, translucent, greyish to brownish at one day enlarging to 1.0 to 2.0 mm by two days. At seven days colonies of No's. 87 and 93 were 4.0 and 2.0 mm respectively and were brown. Colonies were translucent, fluorescent by T/L; and by U/V

both colonies and media fluoresced yellowish green. Medium became brown.

Gelatin colonies: Punctiform, convex, entire, smooth greyish colonies at two days enlarging to 1.0 mm at seven days and No. 93 becoming brown. Colonies were translucent and fluorescent by T/L and by U/V both colonies and media were yellow. Media became brown.

Agar stroke: From one to seven days abundant, filiform, glistening greyish growth, strongly fluorescent by T/L. Medium is brown.

Nutrient broth: Strong clouding with abundant viscid sediment by seven days. Medium is brown at seven days. No. 93 has a pellicle at three days.

Gelatin stab: Filiform growth, medium becoming brown by 14 days.

Motility medium: Brown at 30° and 37°.

Litmus milk: Becomes very slightly alkaline by three weeks with reduction by four weeks.

Potato: Variable pigment production on different potatoes. No. 94 did not grow on one potato medium.

Nitrate is not reduced.

Indole and hydrogen sulfide negative.

Citrate: No. 87 did not grow, No. 93 did.

Culture No's. 83, 88, 94

Vegetative cells: 0.5 by 0.6 u, with rounded ends. No change in size and shape by six days. Motile in motility test medium at one day at 30° but not at eight days at 37° although there is good growth at 37°. Single polar flagellum may be quite straight or slightly undulate.

Agar colonies: Circular, pink, smooth, entire, convex, translucent, strongly fluorescent by T/L, 1.0 to 1.5 mm at one day increasing to 7.0 to 12.0 mm by seven days. At seven days, by U/V, colonies fluoresce olive green.

Gelatin colonies: Considerable cup-shaped liquefaction at seven days. By U/V colonies fluoresce yellowish, media silverish.

Agar stroke: Abundant, filiform, glistening, pink growth. Strongly fluorescent by T/L.

Nutrient broth: Moderate clouding in 83 and 88 at three and seven days. No. 94 shows strong clouding and a pellicle at three days. No. 83 has scanty sediment, No. 88, abundant and viscid, and No. 94, abundant but not viscid at seven days.

Gelatin stab: Filiform growth, best at top with slight napiform liquefaction in No's. 83 and 88, crateriform to saccate in No. 94 at two days. At eight weeks No's. 83 and 88 have stratiform liquefaction, two thirds completed

with the unliquefied medium yellowish and the line of stab black. At two weeks No. 94 was two-thirds liquefied with stratiform liquefaction and liquefaction was completed before the observation at eight weeks.

Motility medium: Pronounced yellow in the medium at 30° for cultures 83 and 88 and at 37° for culture 94. Cultures 83 and 88 caused a brownish pigmentation at 37° and culture 94 produced only a slight yellowing at 30°C.

Litmus milk: All cultures reduced litmus milk within 16 hours and No's. 88 and 94 caused noticeable proteolysis within 10 days. No. 83 did not cause noticeable proteolysis by 21 days.

Potato: These cultures gave variable characteristics on the two tests, perhaps due to the difference in potatoes. All grew but No. 94 grew more profusely than the other two.

Nitrate: All reduced nitrate to nitrite, No. 94 more slowly than the others. At 21 days but not at seven days tests for nitrogen, nitrite and nitrate were negative.

Indole negative.

Hydrogen sulfide was produced in the medium in two to four days by No's. 83 and 88 and in one day by No. 94. Lead acetate test strips were blackened by No. 94 in one day but the other cultures caused little blackening even after 28 days.

Starch not hydrolyzed and glucose not fermented.

No growth in citrate broth.

Cultures 12, 72, 75, 76, 77, 89, 92

Vegetative cells: 0.5 x 0.6 u, with rounded ends. Do not change from one to six days. No. 72 motile in motility test medium at 30° at eight days. Not motile at 37°. No. 72 motile in two days at 30° and 37°. All except No. 75 had one to three very long, slender, straight to slightly curved polar flagella. No's. 12, 89 and 92 had occasional cells with a flagellum at each end. Gram negative.

Agar colonies: 24 hr. colony is punctiform, greyish, smooth convex and translucent with edges which may be entire or erose. At two days colonies are 1.0 to 2.0 mm in diameter and are rugose or (X43) coarsely granular. At seven days all colonies have yellowish-grey central areas. No's. 12, 72, 76, 89, and 92 have wide, effuse transparent marginal zones of about five mm radius while the central portion of the colony has a radius of 1.0 to 1.5 mm. No's. 75 and 77 are 3.0 to 4.0 mm diameter with small or no transparent marginal zones. All colonies except No. 12 fluoresced by T/L, especially one to three day old colonies.

Gelatin colonies: At two days punctiform, convex, entire smooth, translucent colonies, circular to slightly irregular, fluorescent by T/L. At seven days colonies were 1.0 - 1.5 mm in diameter, coarsely granular (X43), smooth, entire to erose, convex, translucent, blue to bluish green by T/L.

Agar stroke: Abundant, glistening, filiform growth with parts of the streak exhibiting spreading growth. By seven days some cultures have yellowish grey growth.

Nutrient broth: Moderate clouding, no sediment at one day becoming strongly clouded with abundant viscid sediment by seven days.

Gelatin stab: Uniform stab, filiform at one day becoming villous to arborescent by seven days. At eight weeks growth is best at top and has spread throughout the medium to give a cloudy appearance for about two-thirds of the diameter of the tube. Edge of the diffuse growth is echinutate. No's. 12, 89 and 92 did not produce spreading growth and No. 12 liquefied the gelatin slowly with an infundibuliform to saccate liquefaction.

Motility medium: After eight days at 37° upper half of the medium is moderately yellow while the surface growth of No's. 75 and 77 is faintly pink.

Litmus milk: No change for about two weeks then becomes moderately to strongly alkaline.

Potato: Glistening pinkish orange growth by seven days with the potato becoming definitely grey. None of these cultures grew on one potato medium.

Nitrate is reduced to nitrogen gas with no detectable nitrite or nitrate left at seven days.

Indole and hydrogen sulfide negative.

Starch hydrolyzed to reducing sugars by 10 days by all except No's. 12, 76, 89 and 92 which gave a positive test for erythrodextrin at seven days and achroodextrin at 10 days. Acid hydrolysis of these four at 10 days gave reducing sugars. Dextrose not fermented.

Good growth in citrate broth at 10 days.

Culture No. 82

Vegetative cells: 0.5 by 0.8 u, with rounded ends. No change in size or shape up to six days. Non-motile and no flagella observed. Gram-negative.

Agar colonies: Punctiform, smooth, entire, convex, greyish, translucent at three days becoming glistening white, pulvinate, 2.0 to 2.5 mm in diameter by seven days. At seven days they were pale violet by U/V.

Gelatin colonies: No growth at seven days.

Agar stroke: At seven days, moderate, beaded, glistening creamy white.

Nutrient broth: Slow growth. At seven days there was no surface growth or clouding and only a scanty sediment.

Gelatin stab: After eight weeks very sparse, greyish filiform growth in upper half of stab.

Litmus milk: No change.

Potato: No growth.

Nitrate: Slight reduction to nitrite at 21 days.

Indole, hydrogen sulfide, starch hydrolysis, glucose fermentation negative.

No growth in citrate broth.

Culture No. 1

Vegetative cells: 0.6 by 1.0 to 1.3 u, with rounded ends. No change in size or shape up to six days. Motile in motility test medium at one day. Single undulate polar flagellum. Gram-negative.

Agar colonies: 24 hr. colony is circular, about one mm in diameter increasing to 1.5 to 2.0 mm at two days and 6.0 to 7.5 mm at seven days. Colony is smooth, convex, translucent with entire edges up to three days but seven day colony has a convex slightly opaque central portion of 2.0 to 2.5 mm diameter and a transparent effuse marginal zone of 2.0 to 2.5 mm which is somewhat undulate with finely erose edge. At seven days colonies macroscopically show some radiate ridging while microscopically (X43) they are coarsely granular, especially in central part. By oblique transmitted light (T/L) colonies are fluorescent, especially those one to three days old while by reflected light (R/L) colonies are greyish.

Gelatin colonies: Invisible at one day but at two days are punctiform, circular in shape, convex, smooth, translucent,

greyish by R/L, fluorescent by T/L. At seven days colonies are much like those at two days but are circular to slightly irregular in shape and blue by T/L.

Agar stroke: Abundant filiform growth becoming echinulate at two days. Growth is greyish by R/L, fluorescent by T/L. Fluorescence is pronounced up to three days but not at seven days.

Nutrient broth: Strong clouding which is persistent. Abundant sediment which becomes viscid by seven days.

Gelatin stab: Growth is best at top, filiform, yellowish-brown along line of stab. No liquefaction.

Motility medium: After eight days at 37° upper half of the medium is slightly yellow and surface growth is light pink. At 30° medium is unchanged and surface growth is only slightly pink.

Litmus milk: Becomes slightly alkaline in about two weeks and markedly alkaline by three weeks.

Potato: No visible growth at one day but glistening, tan growth at seven days. Potato becomes greyish at two days and is tan at four and seven days.

Nitrate is reduced to nitrite to a slight extent by seven days and to a considerable extent by 21 days.

Indole and hydrogen sulfide negative.

Starch not hydrolyzed and glucose not fermented.

Good growth in citrate broth at 10 days but not at four days.

Culture No. 2

Vegetative cells: 0.6 by 0.8 to 1.2 u, with rounded ends. Shortening to 0.8 to 1.0 u at two days with no further change up to seven days. Gram-negative. Non-motile in motility test medium at two days but shows doubtful motility test at 8 days at 30°C and positive test at 37°. Single polar flagellum is undulate and is present in only a few cells of an 18 hr. culture.

Agar colonies: 24 hr. colony is circular, about one mm in diameter, increasing gradually to 3.0 to 3.5 mm by seven days. From one to seven days colonies are circular, smooth, convex and translucent with entire edges. By R/L colonies are greyish. Colonies are fluorescent by T/L at 1, 2 and 3 days. At seven days they have a yellowish centre and translucent bluish marginal zone by T/L and by R/L or T/L show one to several wedge-shaped areas extending from the border towards the centre. These wedge-shaped areas possess less optical density than the rest of the colony and microscopically (X43) they appear as if a gouge had cut a concave, smooth, amorphous, shining channel in an otherwise finely granular colony.

Gelatin colonies: No visible growth at 24 hr. but two day old colonies are punctiform, convex, smooth, and with entire edges. They are greyish by R/L and translucent.

By T/L they are fluorescent. Seven-day colonies are much like those at two days but by T/L are blue. They are circular to slightly irregular in shape.

Agar stroke: Abundant, glistening, greyish, butyrous, filiform, strongly fluorescent by T/L, from one to seven days.

Nutrient broth: clouding is slight at one day, strong at seven days. Sediment is scanty at one and two days, abundant at seven, and is viscid from one to seven days.

Gelatin stab: Identical with culture No. 1.

Motility medium: After eight days at 37° the upper half of the medium is fairly yellow and surface is light pink. No change in medium at 30°.

Litmus milk: Identical with culture No. 1.

Potato: Glistening creamy yellow growth at two days becoming brownish by seven days. Potato becomes tan by seven days.

Nitrate: No nitrite at seven days but slight nitrite by 21 days.

Indole and hydrogen sulfide negative.

Starch and glucose negative.

Scanty growth in citrate at four days and fair at 10 days.

Culture No. 3

Vegetative cells: Identical with culture No. 2.

Agar colonies: Identical with description of culture No. 2 except that they were considerably smaller at one day

and slightly larger at seven days.

Gelatin colonies: No visible growth at two days but at seven days they were identical with culture No. 2.

Agar stroke: Identical with culture No. 2.

Nutrient broth: Identical with culture No. 2 except that at seven days there was a slightly flocculent as well as a viscid sediment.

Gelatin stab: Identical with culture No. 1.

Motility medium: Identical with No. 2 except surface growth is only slightly pink.

Litmus milk: Identical with No. 1.

Potato: Sparse tan growth by seven days. Potato becomes tan.

Nitrate: No nitrite at seven days and doubtful results at 21 days.

Indole, hydrogen sulfide, starch and glucose identical with No. 1.

Citrate broth: Negative at 10 days.

Culture No. 4

Vegetative cells: 0.6 to 0.8 u thick by 0.8 to 1.4 u long. Longer cells are most numerous at one day while shorter cells are most numerous at seven days. Cells have rounded ends. Gram-negative. Motile in motility test medium within two days at 30° and 37°. One or two undulate flagella on

one or both ends.

Agar colonies: Are identical with colonies in culture No. 2 except that seven-day old colonies are only 2 to 3 mm in diameter.

Gelatin colonies: Identical with culture No. 2.

Agar stroke: Identical with culture No. 2 except that growth is filiform to echinulate.

Nutrient broth: Identical with culture No. 2 except that clouding is moderate at one day and strong at two days.

Gelatin stab: Identical with culture No. 1.

Motility medium: Identical with No. 3 except only slightly yellow in top half.

Litmus milk: Identical with No. 1.

Potato: At seven days profuse, glistening tan growth. Potato becomes tan.

Nitrate: Identical with No. 2.

Hydrogen sulfide: Negative in medium but strongly positive on lead acetate test strip at seven days.

Indole, starch and glucose: Identical with No. 1.

Culture No. 5

Vegetative cells: Ovoid rods 0.6 to 0.8 by 0.8 to 1.0 u changing little from one to six days. Gram-negative. Non-motile in motility test medium at two days but motile at eight days at 30° and 37°. Single polar flagellum is straight.

Agar colonies: Identical with culture No. 2 except for the following: smaller at one day. By seven days a thin effuse growth with undulate margin had spread out from some of the wedge-shaped areas in the original colony. This growth was greyish by T/L.

Gelatin colonies: No visible growth at 48 hr. but at seven days colonies are one mm in diameter, circular to slightly irregular, convex, smooth, translucent and with entire edge. By T/L they are bluish.

Agar stroke: Identical with colony No. 2.

Nutrient broth: Clouding is slight at one day, strong at two days. Sediment is identical with culture No. 2.

Gelatin stab: Identical with culture No. 1.

Motility medium: Identical with No. 3.

Litmus milk: Slightly alkaline at 28 days.

Potato: Moderate tan growth by seven days. Potato tan.

Nitrate: Identical with No. 3.

Indole, hydrogen sulfide, starch and glucose identical with No. 1.

Citrate: Negative at 10 days.

Culture No. 6

Vegetative cells: 0.6 by 0.8 to 1.3 u at one day shortening to 0.8 to 1.0 u by six days. Rounded ends. Gram-negative. Non-motile at eight days at 30° and 37°. Single

straight polar flagellum is present on only a few cells of an 18 hr. culture.

Agar colonies: Colonies from one to seven days are smooth, circular, convex, translucent, with entire edge. Increase from one mm diam. at one day to eight mm at seven days. Seven-day colonies have wedges similar to those in culture No. 2. Microscopically the seven-day colony is coarsely granular. By T/L one to three day old colonies fluoresce strongly while seven-day colonies have yellowish centre and bluish marginal zone. By U/V seven-day colonies are a very pale rusty orange.

Gelatin colonies: Identical with culture No. 2.

Agar stroke: Identical with culture No. 6.

Nutrient broth: Clouding is slight at one day, strong at three days. Sediment is scanty up to three days but at seven days it is abundant and viscid.

Gelatin stab: Identical with culture No. 1.

Motility medium: Identical with No. 2.

Litmus milk: Identical with No. 1.

Potato: Moderate yellowish tan growth at seven days. Potato tan.

Nitrate: Slight nitrite at 21 days but not at seven.

Indole, hydrogen, sulfide, starch and glucose identical with No. 1.

Citrate: Scanty growth at four days, fair at 10 days.

Culture No. 7

Vegetative cells: 0.6 to 0.8 by 0.8 to 1.0 u; with rounded ends. Do not change from one to six days. Gram-negative. Non-motile at eight days at 30° and 37°. Flagella as in culture No. 6.

Agar colonies: At one day colonies are one mm in diameter, circular, smooth, greyish, translucent, convex, and with entire edge. At two days colonies (X43) are finely granular and have darker centres. Some centres have one to several deep fissures. The fissures may or may not radiate from the centre. At three days colonies (X43) range from convex to umbilicate and all have pointed, transparent bristles extending from an otherwise entire edge. At seven days colonies are 3 to 4 mm in diameter, have shallow, crater-like centres and (X43) have small colonies surrounding and usually adhering to the parent colonies. All colonies are more or less coarsely granular, greyish by T/L or R/L and very pale orange by U/V.

Gelatin colonies: At two days punctiform, convex, smooth, translucent colonies with entire edges. Greyish by R/L, fluorescent by T/L. At seven days colonies were one mm in diameter, circular to irregular, raised to convex, smooth, translucent and with entire edge. By T/L, blue and by R/L, grey.

Agar stroke: Identical with No. 2.

Nutrient broth: Identical with No. 4.

Gelatin stab: Identical with No. 1.

Motility medium: Identical with No. 5.

Nitrate: Identical with No. 1.

Indole, hydrogen sulfide, starch, glucose, citrate: Identical with No. 1.

Culture No. 9

Vegetative cells: 0.6 by 0.8 to 1.0 u, with rounded ends. No change in size or shape from one to six days. Non-motile in motility medium at eight days but several have single, undulate polar flagellum. Gram-negative.

Agar colonies: Circular, smooth, entire, convex, translucent, one mm diameter at one day increasing to three mm at seven day. Greyish by R/L, fluorescent by T/L.

Gelatin colonies: Punctiform, convex, entire, smooth, greyish colonies. By T/L they were fluorescent at two days, bluish at seven days.

Agar stroke: Abundant, filiform, glistening strongly fluorescent greyish growth from one to seven days.

Nutrient broth: Slight clouding and no sediment at one day becoming strongly clouded with abundant viscid sediment by seven days.

Gelatin stab: Identical with No. 1.

Motility medium: Faint yellowing of the medium at 37° but not at 30°. Surface growth slightly pink at 30° and 37°.

Litmus milk: Identical with No. 1.

Potato: Moderate tan growth. Potato tan.

Nitrate: Identical with No. 2.

Indole, hydrogen sulfide, starch, glucose, citrate: Identical with No. 1.

Culture No. 10

Vegetative cells: 0.6 by 0.8 to 1.0 u at one day shortening to 0.6 by 0.8 u at six days. Non-motile in motility test medium at eight days. Single undulate polar flagellum. Gram-negative.

Agar colonies: At one day circular, one mm diameter, smooth, entire, convex, translucent, greyish by R/L, fluorescent by T/L. By two days many of the colonies' centres have dropped to give a rugose crater-like depression and colonial size is 1.5 to 2.0 mm. Fluorescent by T/L. Seven day old colonies are much the same as two day old colonies.

Gelatin colonies: From two to seven days are punctiform, convex, entire, smooth, translucent, greyish by R/L. By T/L they are fluorescent at two days, bluish at seven.

Agar stroke: Abundant, filiform, glistening, greyish growth fluorescent by T/L from one to seven days.

Nutrient broth: Slight clouding at one day, strong at three days. At seven days there is a stringy surface growth. Sediment is scanty up to three days but is abundant and viscid by seven.

Gelatin stab: Identical with culture No. 1.

Motility medium: Identical with No. 6.

Litmus milk: Identical with No. 1.

Potato: Glistening tan growth. Potato tan.

Nitrate: Slight amount of nitrite at four days with no increase by 21 days.

Indole, hydrogen sulfide, starch, glucose, citrate: Identical with No.1.

Culture No's. 11, 15

Vegetative cells: 0.6 by 2.0 to 2.4 u at one day shortening to 1.0 to 1.5 u by six days. No. 11 shows positive motility at four days but No. 15 shows only doubtful motility at eight days. Single polar flagellum is undulate. Gram-negative.

Agar colonies: Punctiform, smooth, entire, convex, translucent colonies increasing to one mm diameter at three days at which time colonies are raised with a peak-like central area and (X43) are coarsely granular with many bristles extending from the edge. Colonies, macroscopically, are rough at two days. They are not fluorescent by T/L.

Gelatin colonies: Punctiform, convex, entire, smooth, translucent, bright blue by T/L, greyish by R/L.

Agar stroke: Moderate, filiform, glistening, greyish, non-fluorescent by T/L.

Nutrient broth: Slight clouding and no sediment at one day. By seven days there is a ring, moderate clouding, abundant sediment which rises as floccules when agitated (No. 11) or has some viscid and some flocculent sediment, No. 15.

Gelatin stab: Uniform, beaded, brownish growth. Liquefaction is stratiform to slightly crateriform and is very slow, about one-third of the medium being liquefied in eight weeks.

Motility medium: Moderate brown discoloration of medium at 30° and 37°.

Litmus milk: Becomes alkaline after about two weeks.

Potato: Orange to reddish tan growth varying with the medium.

Nitrate: Slowly reduce nitrate to nitrogen gas without detectable nitrite.

Indole and hydrogen sulfide negative.

Starch not hydrolyzed and glucose not fermented.

No growth in citrate broth.

Culture No's. 16 and 17

Vegetative cells: 0.5 by 1.0 to 1.2 u. No change in size or shape up to six days. Non-motile and no flagella observed.

Agar colonies: Circular to slightly irregular, smooth to rough, entire to undulate, convex, greyish, translucent. One mm at one day increasing to 4.0 to 7.0 mm by seven days. At seven days colonies are lobate and (X43) are coarsely granular with amorphous marginal zones. Greyish by T/L.

Gelatin colonies: Little growth at two days. At seven days they are circular to slightly irregular, convex, entire to slightly undulate, smooth, translucent and bluish by T/L.

Agar stroke: Abundant, echinulate, glistening, greyish, non-fluorescent by T/L.

Nutrient broth: Slight clouding with little sediment at one day increasing to moderate clouding and abundant, viscid sediment at seven days.

Gelatin stab: Uniform, filiform growth. Both cultures produce feathery outgrowths from the line of stab, especially in the top half of the medium but at eight weeks No. 16 has spread completely throughout the top 5 mm of the medium.

Motility medium: No pigmentation.

Litmus milk: No. 16 caused noticeable proteolysis by 10 days. No. 17 was moderately alkaline by 16 days and com-

pletely reduced by 21 days.

Potato: Variable pigment production. At seven days growth and potato were both tan.

Nitrate is not reduced, starch not hydrolyzed, dextrose not fermented, indole not formed, and no growth in citrate broth.

Discussion: Although these cultures were not found to be motile under the conditions of testing and flagella were absent it is felt that the growth in gelatin stabs precludes any possibility of calling these organisms non-motile.

Cultures No's. 49 and 50

Vegetative colonies: 0.6 by 0.8 to 1.0 u at one day shortening to 0.8 u at six days. Rounded ends. Single undulate polar flagellum. Gram-negative.

Agar colonies: Punctiform at one day increasing to four to six mm by seven days. Circular, smooth, entire, convex, greyish, translucent, fluorescent by T/L losing the fluorescent properties by seven days. By U/V, yellowish to rusty orange in large colonies.

Gelatin colonies: At two days, punctiform, greyish, convex, undulate, smooth, translucent, fluorescent by T/L. At seven days circular to slightly irregular, 1.0 to 1.5 mm, smooth, convex, greyish, blue by T/L.

Agar stroke: Abundant, filiform, glistening, greyish, butyrous becoming decidedly viscid by seven days.

Nutrient broth: Slight clouding and scanty sediment at one day becoming strongly clouded with abundant viscid sediment by seven days.

Gelatin stab: Uniform, filiform growth developing feathery puffs from the stab line by two weeks.

Motility medium: Slight yellowing of the medium at 37° at eight days. Surface growth of No. 50 is slightly pink at 30° and 37°.

Litmus milk: Becomes alkaline slowly.

Potato: Creamy tan growth on one medium, orange tan on another. Medium tan.

Nitrate reduced to nitrate in one day.

Indole negative.

Hydrogen sulfide on lead acetate strip but not in stab.

Starch not hydrolyzed and glucose not fermented.

Moderate growth in citrate by 10 days.

Culture No. 8

Vegetative cells: 0.6 by 1.0 to 1.6 u with shorter forms predominating by six days. Rounded ends. Motile in motility medium at one day. Single, undulate polar flagellum. Gram-negative.

Agar colonies: At one day colonies are circular, smooth,

entire, convex and translucent, greyish by R/L. Microscopically they are coarsely granular. Colonies increase in size to 2.5 - 3.0 mm at seven days and from two to seven days many of them have precipitously sunken centres which are rugose.

Gelatin colonies: Punctiform, convex, entire, smooth colonies at two days becoming irregular, flat and lobate by seven days.

Agar stroke: Abundant, ecninulate, glistening, greyish growth, fluorescent by T/L from one to three days..

Nutrient broth: Identical with No. 4.

Gelatin stab: Identical with No. 1.

Motility medium: Faint yellowing of the medium at 37° but not at 30°. No pink colour in surface growth.

Litmus milk: Slightly alkaline at two weeks, strongly alkaline at four weeks.

Potato: Glistening tan growth. Potato tan.

Nitrate: Reduced to nitrite in one day.

Indole, hydrogen sulfide, starch, glucose, citrate: Identical with No. 1.

Culture No. 98

Vegetative cells: 0.6 by 0.8, with rounded ends. No change up to six days. Motile in motility test medium. Flagellation extremely varied, most common types being one

flagellum at one or both ends or from the side, or two flagella from one end: more rarely five flagella from one end, two from the side, or a variable number from one end with one flagellum on one or both sides. Gram-negative.

Agar colonies: Circular, rough, entire, convex, 1.0 - 1.5 mm at one day increasing to 2.0 to 2.5 mm at seven days, greyish by R/L or T/L.

Gelatin colonies: Circular, smooth, entire, convex, punctiform at three days and one mm at seven days.

Agar stroke: Abundant, filiform, glistening, greyish, becoming viscid by three days.

Nutrient broth: Strong clouding, putrefactive odor.

Gelatin stab: Filiform, growth best at top, with numerous small colonies scattered throughout top cm of medium at 14 days.

Motility medium: No pigmentation.

Litmus milk: Acid at one day, coagulated at three weeks.

Potato: Abundant, glistening creamy growth. Potato is reddish-brown at two days.

Nitrate: Reduced to nitrite at one day.

Indole: Negative.

Hydrogen sulfide: Slight blackening of lead acetate test strip at 14 days.

Starch not hydrolyzed.

Acid and gas in arabinose, xylose, dextrose, fructose, galactose, mannose, sucrose, maltose, mannitol. Slow acid production with slight gas bubble in lactose, raffinose, inulin, dextrin, dulcitol, salicin and aesculin.

Culture No. 100

Vegetative cells: 0.9 by 0.7 u. Motile in motility at one day at 30° and 37°. Single undulate polar flagellum is common. Occasionally two or five polar flagella. Gram-negative.

Agar colonies: Circular, smooth, entire, convex, greyish, fluorescent by T/L. At seven days colonies are 1.5 to 2.0 mm, smooth to slightly rough, raised to convex.

Gelatin colonies: At one day punctiform, convex, entire, smooth, greyish, fluorescent by T/L. At two days colonies are 2.5 mm with cup-shaped liquefaction advanced. At three days liquefaction is complete and by U/L the liquefied and non-liquefied portions of the medium fluoresce silvery grey.

Agar stroke: Abundant, filiform, greyish, glistening.

Nutrient broth: Pellicle, strong clouding, scanty sediment from one to seven days with slight odor of putrefaction at seven days. Abundant granular sediment at 14 days.

Gelatin stab: Rapid infundibuliform liquefaction complete at seven days with flocculent, greyish sediment.

Motility medium: After eight days upper half of medium is moderately or slightly yellowed at 30° and 37° respectively. No pigmentation of surface growth.

Litmus milk: Acid at one day, neutral at two days with proteolysis occuring at two days and completed at 10 days.

Nitrate: Reduced to nitrite at one day. At 21 days but not at seven days, nitrogen gas present but nitrate and nitrite absent.

Indole present at one day. Hydrogen sulfide negative.

Starch hydrolyzed in two days.

Considerable acid in dextrose, fructose, galactose, sucrose, maltose, inulin, mannitol, salicin and aesculin. Slight acid in arabinose, xylose, mannose, lactose, raffinose, inulin, and dulcitol. No gas produced.

Growth in citrate broth.

Culture No. 102

Vegetative cells: 0.2 by 0.4 to 1.0 u, motile in one day at 30° and 37°. Two undulate polar flagella. Gram-negative.

Agar colonies: Circular, smooth, entire, convex, greyish, strongly fluorescent by T/L increasing in size to 5.0 to 6.0 mm and containing wedge-shaped areas cf. culture No. 2 at three days.

Gelatin colonies: Circular, convex, entire, smooth, with cup-shaped liquefaction advanced considerably at two days.

Agar stroke: Abundant, echinulate, glistening, greyish, from one to seven days.

Nutrient broth: Strong clouding, scanty sediment, developing a slightly putrefactive odor at two days, a slight ring and an abundant sediment by seven days.

Gelatin stab: Filiform growth with infundibuliform liquefaction complete at seven days. Greyish sediment.

Motility medium: No water soluble pigment produced. Surface growth at 37° is slightly pink.

Litmus milk: Acid at one day but no curd at 28 days.

Potato: Scanty, glistening, creamy growth.

Nitrate: Reduced to nitrite by one day.

Indole: Present at one day.

Hydrogen sulfide: Negative.

Starch not hydrolyzed.

No growth in citrate broth.

Acid from dextrose, fructose, galactose, lactose, sucrose, maltose, raffinose, dextrin, mannitol, salicin, aesculin. Slight acid production from dulcitol: none from arabinose, xylose, mannose, inulin. No gas.

Culture No. 104

Vegetative cells: 0.2 to 0.3 by 1.0 to 1.5 u, from one to six days. Non-motile in motility medium at 30° and 37°. Flagellated, most commonly with a single polar flagellum on one or both ends, three flagella on each end, rarely with two flagella on one end or two flagella on one end and two originating on one side.

Agar colonies: Circular, smooth to rough, greyish, translucent, convex to raised, with thin, effuse marginal zone which is erose, lobate, or very ragged. At seven days colonies are 8.0 to 15.0 mm and (X43) are coarsely granular.

Gelatin colonies: At seven days are circular, 1.0 mm, convex, entire, smooth and translucent.

Agar stroke: Abundant, filiform, glistening, greyish, becoming somewhat echinulate and spreading by three days. Fluorescent by T/L.

Nutrient broth: Moderate clouding and scanty sediment at one day with pellicle, strong clouding and slight putrefactive odor at seven days.

Gelatin stab: Filiform growth with feathery puffs from line of stab by 14 days.

Motility medium: Slight yellowing of medium at seven days. No pigmentation of surface growth.

Litmus milk: Slightly alkaline at two weeks, markedly alkaline at three weeks.

Nitrate: Reduced to nitrite in one day.

Indole and hydrogen sulfide negative.

Starch not hydrolyzed and glucose not fermented.

Slow growth in citrate broth.

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