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R. Samuel-Maharajah
University of Nebraska-Lincoln

Hilliard Pivnik
University of Nebraska-Lincoln

W.E. Engelhard
University of Nebraska-Lincoln

Sheila Templeton
University of Nebraska-Lincoln

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R. Samuel-Maharajah, Hilliard Pivnick, W. E. Engelhard, and Sheila Templeton

Department of Bacteriology, University of Nebraska, Lincoln, Nebraska

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This investigation is concerned primarily with the growth and virulence of pathogenic bacteria introduced into soluble oil emulsions. The soluble oils are petroleum or fatty oils emulsified with soaps of petroleum sulfonic acids, naphthenic acids, fatty acids, rosin, or tall oil. When mixed with water, the soluble oils form stable oil-in-water emulsions and, as such, are used in machine shops as coolants and lubricants in the cutting and grinding of metals. Unfortunately, they serve as excellent substrates for the growth of bacteria.

The predominating microflora indigenous to soluble oil emulsions used industrially belongs to the genus *Pseudomonas* (Lee and Chandler, 1941; Duffett *et al.*, 1943; Pivnick, 1952, 1955; Pivnick *et al.*, 1956; Sabina, 1956). However, members of the genera *Achromobacter*, *Bacillus*, and *Vibrio*, as well as yeasts and molds have also been found (Duffett *et al.*, 1943; Pivnick, 1952; Davis and Updegraff, 1954). In addition, coliform bacteria persist in these emulsions. For example, Page and Bushnell (1921), Duffett *et al.*, (1943), and Bennett (1956) isolated *Escherichia coli* and *Aerobacter aerogenes*, while Pivnick and Fabian (1954) found similar bacteria in about one-half of the emulsion samples obtained from industrial sources. Pivnick and Fabian (1954) also found that the bacteria in fecal matter undergo rapid cell division in soluble oil emulsions.

With regard to the pathogenic bacteria, those of the genera *Salmonella* and *Klebsiella* grow readily in these emulsions and members of the genus *Shigella* survive for several days (Pivnick *et al.*, 1954). Bennett and Wheeler (1954) investigated survival but not growth of pathogens in soluble oils which differed from those used by Pivnick *et al.* They found viable *Shigella paradysen-*

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teriae after 76 days in one emulsion and after 42 days in another. Recently, Bennett (1956) has reported finding a *Paracolobactrum* and a *Salmonella* species in emulsions used industrially. The *Salmonella* species were isolated from the same emulsion on three different occasions.

Although coliform bacteria have been frequently found in emulsions, they comprised less than 0.1 per cent of the total microbial population (Pivnick and Fabian, 1954; Pivnick, *unpublished data*). A notable exception was the discovery by Bennett (1956) that *A. aerogenes* predominated in one sample of emulsion obtained from a machine shop. The usual small numbers of coliform bacteria in soluble oil emulsions used industrially did not correlate with the rapid growth of fecal bacteria inoculated into sterile soluble-oil emulsions. Therefore investigations of microbial antagonism existing in soluble oil emulsions were initiated (Pivnick and Fabian, 1954). It was found that coliform bacteria and pseudomonads indigenous to oil emulsions grew when mixed together in fresh emulsion. After 10 days, however, the coliform bacteria began to decrease in numbers while the pseudomonads remained stationary.

The work reported herein concerns the association of the microflora indigenous to soluble oil emulsions, predominantly pseudomonads, and pathogenic bacteria. The effect of growth in emulsions on the virulence of enteric pathogens was also investigated.

MATERIALS AND METHODS

In order to study the fate of pathogenic bacteria in the presence of the microflora indigenous to soluble oil emulsions, pathogenic bacteria and the bacteria in emulsions used industrially were introduced simultaneously into a fresh, sterile emulsion. Samples were removed at various time intervals and the number of each type of bacteria was determined. Control experiments contained emulsions inoculated with pathogens or with bacteria indigenous to soluble oil emulsions.

Cultures and inoculum. The pathogenic bacteria were obtained from the stock culture collection of the University of Nebraska. After reisolation from nutrient-agar plates, the identity of *Salmonella* and *Shigella* species was checked by agglutination with homologous antisera (Markam Laboratories, Chicago, Illinois), while cultures of *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* were found to have cultural characteristics typical of the species.

The microflora indigenous to soluble oil emulsions was obtained by pooling samples of used emulsions from four machine shops. Numerous studies indicate that these organisms are predominantly pseudomonads, and in this report they are so designated.

The pathogens and pseudomonads were enumerated in order to add known numbers of each to a sterile soluble-oil emulsion. Initially, the pathogens were inoculated into nutrient broth and incubated at 37 C for 18 to 24 hr, after which they were refrigerated. Plate counts of the broth suspensions were made, using nutrient agar as a plating medium and 37 C as the incubation temperature. The bacteria in the pooled, refriger-

TABLE 1

Pathogens and pseudomonads in soluble oil emulsions at 0 and 32 days

Flask No.	Organisms	Bacteria per Ml	
		0 day	32 days
1	*Salmonella schottmuelleri*	30×10^6	11×10^5
	Pseudomonads	30×10^6	64×10^6
2	*S. schottmuelleri*	30×10^6	6×10^5
	Pseudomonads	30×10^4	27×10^6
3	*S. schottmuelleri*	30×10^6	19×10^6
4	*Salmonella paratyphi*	5×10^6	23×10^6
	Pseudomonads	5×10^6	104×10^6
5	*S. paratyphi*	5×10^6	31×10^6
6	*Salmonella typhosa*	5×10^6	14×10^6
	Pseudomonads	5×10^6	78×10^6
7	*S. typhosa*	5×10^6	18×10^6
8	Pseudomonads	5×10^6	110×10^6
9	*Shigella sonnei*	16×10^6	53×10^4
	Pseudomonads	16×10^6	72×10^6
10	*S. sonnei*	16×10^6	52×10^4
	Pseudomonads	16×10^4	28×10^5
11	*S. sonnei*	16×10^6	15×10^5
12	*Shigella dysenteriae*	15×10^6	22×10^5
	Pseudomonads	15×10^6	72×10^6
13	*S. dysenteriae*	15×10^6	5×10^6
	Pseudomonads	15×10^4	36×10^6
14	*S. dysenteriae*	15×10^6	6×10^6
15	*Shigella paradysenteriae*	30×10^6	21×10^6
	Pseudomonads	30×10^6	98×10^6
16	*S. paradysenteriae*	30×10^6	25×10^6
	Pseudomonads	30×10^4	50×10^6
17	*S. paradysenteriae*	30×10^6	32×10^6
18	*Klebsiella pneumoniae*	6×10^6	3×10^6
	Pseudomonads	6×10^6	134×10^6
19	*K. pneumoniae*	6×10^6	20×10^6
20	Pseudomonads	6×10^6	7×10^5
21	*Pseudomonas aeruginosa*	6×10^6	27×10^5
	Pseudomonads	6×10^6	163×10^5
22	*P. aeruginosa*	6×10^6	20×10^6

ated samples of used emulsions were enumerated, using nutrient-agar plates incubated at 30 C.

The pathogens and pseudomonads were added to 100 ml of fresh, sterile emulsion and shaken for about 8 hr per day at room temperature on a reciprocating shaker. In some experiments, equal numbers of each type were inoculated into the sterile emulsion; in others, the pathogens : pseudomonad ratio in the inoculum was 100:1. The organisms and size of inocula are shown in table 1.

Enumeration of pathogens and pseudomonads. The total bacterial population and the population of pathogens in the emulsion were determined at various time intervals. The population of pseudomonads in mixtures was calculated by subtracting the population of pathogens from the total bacterial population.

The pathogens were enumerated by using selective media and higher temperatures of incubation. It was impossible to use the usual selective media, for example, Difco bismuth sulfite and SS agar for *Salmonella* and *Shigella* species, because only a small proportion of known numbers of these organisms grew on such media. In this work, pathogens were grown on medium "B"

TABLE 2. *Characteristics of surface colonies of pathogens on medium "B"*

Cultures	Temperature of Incubation	
	37 C	42 C
Salmonella schottmuelleri		Round dark colony with metallic sheen
Salmonella paratyphi		Round colony with dark green metallic sheen center and lighter green periphery
Salmonella typhosa	Brown to green colony with metallic sheen	
Shigella sonnei	Colony with light-green metallic sheen in center and dark periphery	
Shigella dysenteriae	Brown colony with green metallic sheen in center	
Shigella paradysenteriae	Colony with brown center and light periphery	
Klebsiella pneumoniae		Mucoid type colony with green to brown metallic sheen in center and light periphery
Pseudomonas aeruginosa	Concentric ring-type colony with dark to brown metallic sheen	

TABLE 3. *Relationship of population of pathogens and pseudomonads indigenous to emulsions*

Culture	Millions of Bacteria per Ml		
	Medium "A"	Medium "B"	
	30 C	37 C	42 C
Salmonella schottmuelleri	298		300
Salmonella paratyphi	311		323
Salmonella typhosa	327	301	
Shigella sonnei	165	148	
Shigella paradysenteriae	1100	920	
Klebsiella pneumoniae	720		450
Pseudomonas aeruginosa	181		230
Pseudomonads in used emulsions obtained from machine shops	122	0	0
Pseudomonads in used emulsions obtained from machine shops	140	0	0

(beef extract, 3.0 g; peptone, 5.0 g; sodium chloride, 5.0 g; glucose, 10.0 g; dibasic potassium phosphate, 2.0 g, eosin Y, 0.4 g; methylene blue, 0.065 g; agar, 20.0 g; distilled water, 1000 ml; pH 7.0) incubated at 37 or 42 C depending on the species. When 0.1 ml of a suitable dilution of the emulsion was spread with a bent glass rod over the surface of solidified medium "B," colonies of pathogens developed with characteristic color, shape, and topography (table 2). The pseudomonads, on the other hand, did not grow on medium "B" at 42 C, and grew sparsely as colorless to light-pink colonies at 37 C.

Total bacterial populations in the mixtures were determined by spreading 0.1-ml aliquots of suitable dilutions of the emulsion on medium "A" (beef extract, 3.0 g; peptone, 5.0 g; agar, 20.0 g; distilled water, 1000 ml; pH 7.0) and incubating at 30 C. Pure cultures of pathogens, or pseudomonads not mixed with pathogens, were enumerated using respectively medium "B" at 37 or 42 C and medium "A" at 30 C.

In addition, experiments were carried out to determine if pathogens grew equally on medium "B" at 37 or 42 C and on medium "A" at 30 C. Representative results are shown in table 3.

Serologic reactions. Serologic typing was carried out on *Salmonella* and *Shigella* species grown in soluble oil emulsions for a period of 16 days. Typical colonies were reisolated from medium "B," grown for 24 hr on nutrient agar (Difco), and subjected to slide agglutination with homologous antisera. Cultures which failed to agglutinate were transferred three times in trypticase broth (Baltimore Biological Laboratory) and the agglutination tests were repeated.

Pathogenicity. The effect of growth in soluble oil emulsions on the virulence of the pathogens was determined. Bacteria grown in pure culture in the emulsion for 16 to 24 days were separated from the emulsion by centrifugation. The cells were washed and resuspended in nutrient broth to obtain 10^6 cells per 0.2 ml. Patho-

gens grown in mixture with pseudomonads were reisolated on medium "B" after 16 to 24 days in the emulsion. Typical colonies were fished to nutrient broth, grown at 37 C for 24 hr, and refrigerated. The population was determined and the suspension diluted to obtain 10^6 cells per 0.2 ml. The original cultures of pathogens not exposed to oil emulsions were also grown in nutrient broth and diluted to obtain 10^6 cells per 0.2 ml.

Four mice were injected intraperitoneally with 0.2 ml of each of the suspensions described above. Mice which succumbed within 3 to 5 hr were not indicated in the final tabulation.

Mycobacterium tuberculosis. It is common knowledge that some machinists expectorate into soluble oil emulsions. It would be interesting, therefore, from a public health standpoint, to determine the survival time of *M. tuberculosis* in these emulsions. Strains BCG and H37Rv were grown in Dubos' broth at 37 C for 5 days (Dubos and Middlebrook, 1947) and refrigerated. Plate counts of the suspensions were made by the method of Fenner (1951). Sufficient inoculum was introduced into the emulsion to obtain 20×10^4 cells of BCG per ml, or 25×10^3 cells of H37Rv per ml, and the flasks were incubated at 37 C.

RESULTS

Growth curves. All pathogens, except *M. tuberculosis*, grew in soluble oil emulsions inoculated with pseudomonads. After 32 days, they were still present in appreciable numbers. Table 1 shows the bacteria used, and the bacterial populations at 0 and 32 days. Repeated experiments with *Salmonella typhosa* (*S. typhi*), *Salmonella paratyphi* and *Salmonella schottmuelleri* gave essentially the same results. Figure 1 shows the growth of *S. schottmuelleri* when mixed with equal numbers of pseudomonads, whereas figure 2 shows the effect of inoculating 100 times more pathogens than pseudomonads into the emulsions. Figures 3 and 4 illustrate similar effects for *S. paradysenteriae*. Figures 5 and 6 indicate that *K. pneumoniae* and *P. aeruginosa* also grew in the presence of the pseudomonads indigenous to soluble oil emulsions. After several days, however, the pathogens decreased in number and at all times were present in smaller numbers than when grown in pure cultures.

Similar results were observed with *S. typhosa*, *S. paratyphi*, *Shigella sonnei* and *S. paradysenteriae* (Samuel-Maharajah, 1956).

M. tuberculosis was inoculated into soluble oil emulsion containing no pseudomonads. It failed to grow and death occurred within a few days. Strain BCG decreased from 20×10^4 cells per ml at 0 days to 4×10^3 at 6 days, and it could not be recovered from the emulsion at 12 days. Strain H37Rv decreased from

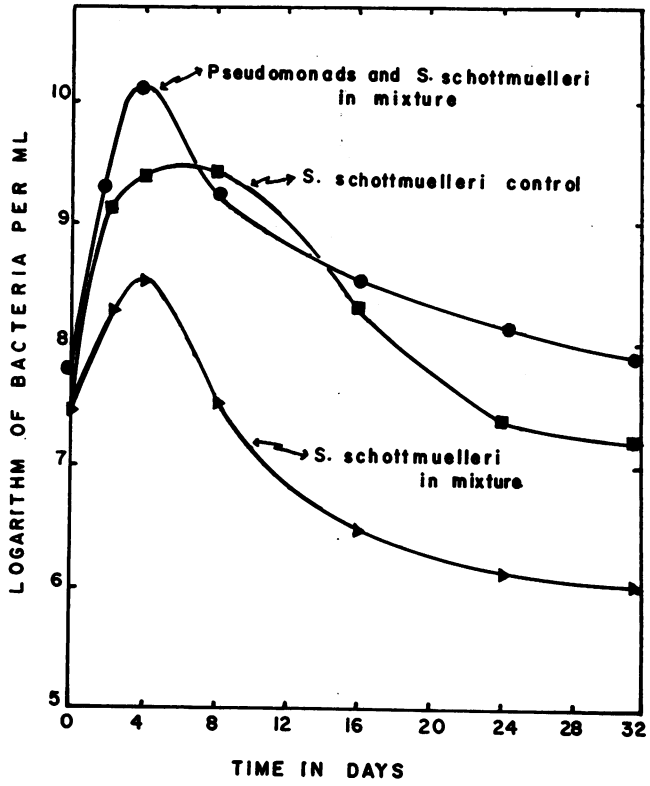

FIG. 1. Growth of pathogens in pure culture and pathogens and pseudomonads in mixed cultures. Inoculum consisted of 30×10^6 bacteria of each type per ml of emulsion.

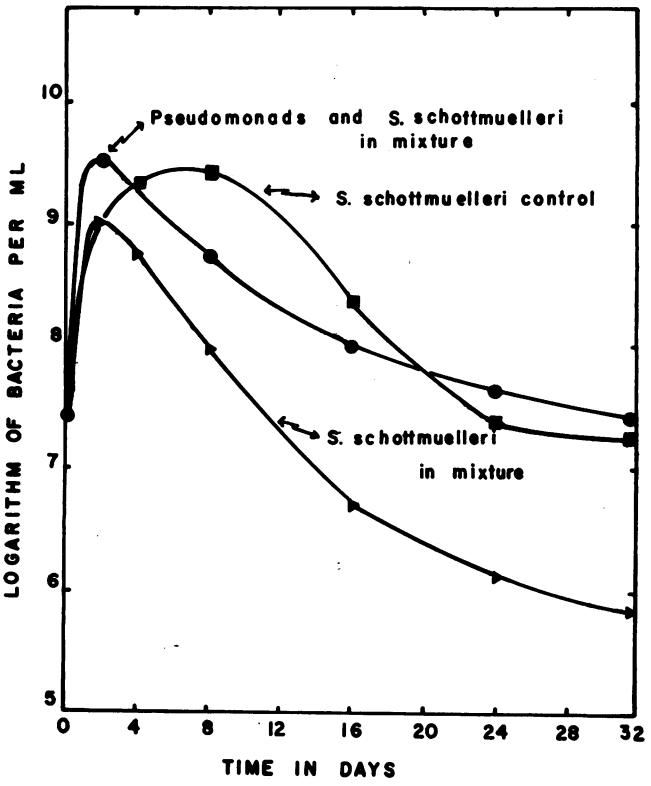

FIG. 2. Growth of pathogens in pure culture and pathogens and pseudomonads in mixed culture. Inoculum consisted of 30×10^6 pathogens and 30×10^4 pseudomonads per ml of emulsion.

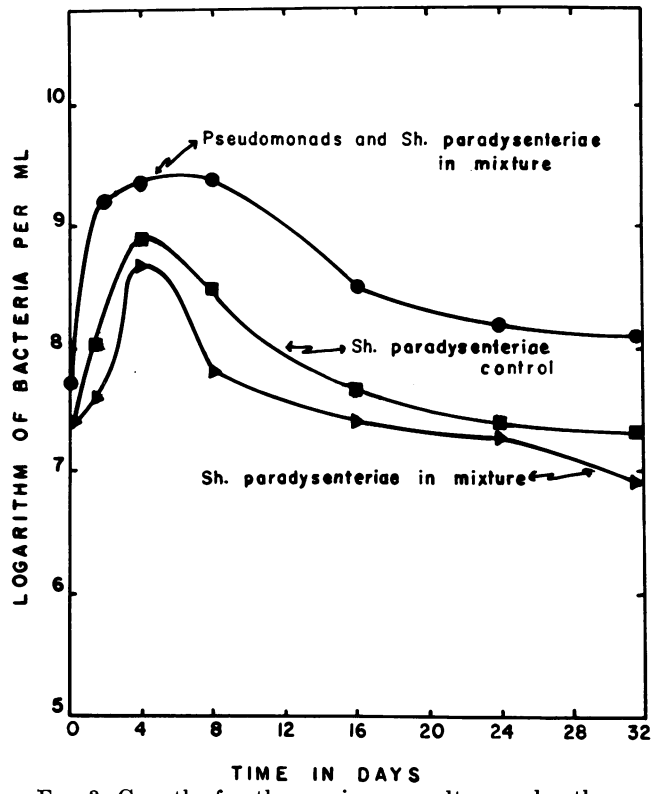

FIG. 3. Growth of pathogens in pure culture and pathogens and pseudomonads in mixed cultures. Inoculum consisted of 30×10^6 bacteria of each type per ml of emulsion.

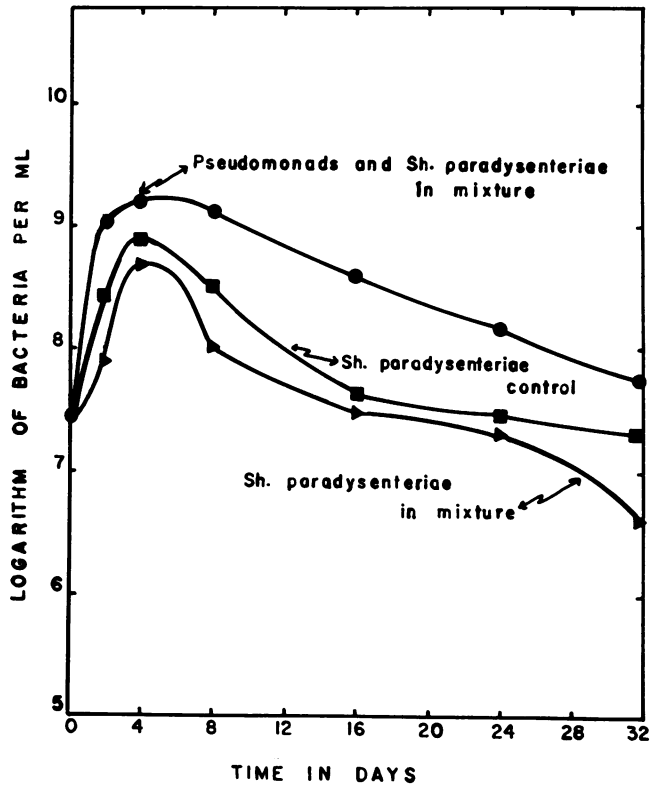

FIG. 4. Growth of pathogens in pure culture and pathogens and pseudomonads in mixed culture. Inoculum consisted of 30×10^6 pathogens and 30×10^4 pseudomonads per ml of emulsion.

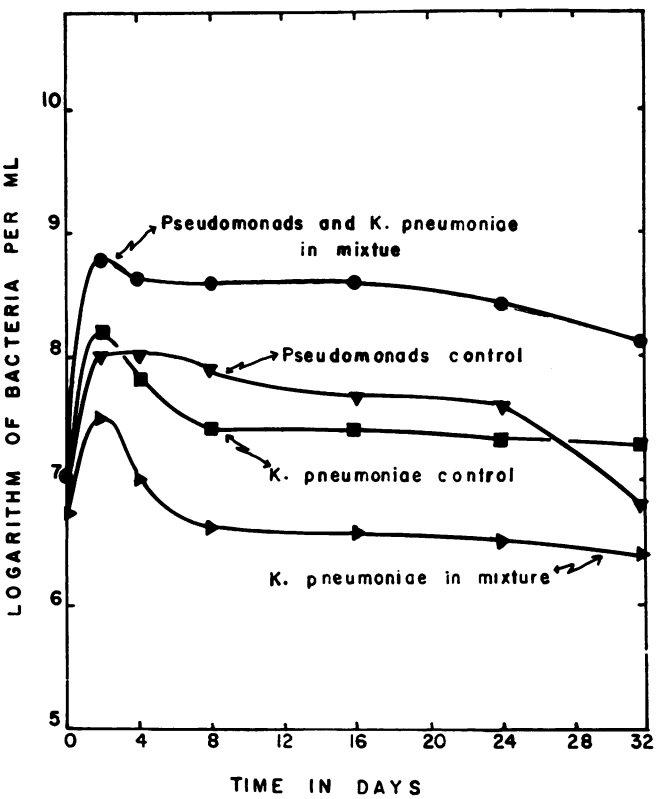

FIG. 5. Growth of pathogens in pure culture and pathogens and pseudomonads in mixed culture. Inoculum consisted of 6×10^6 bacteria of each type per ml of emulsion.

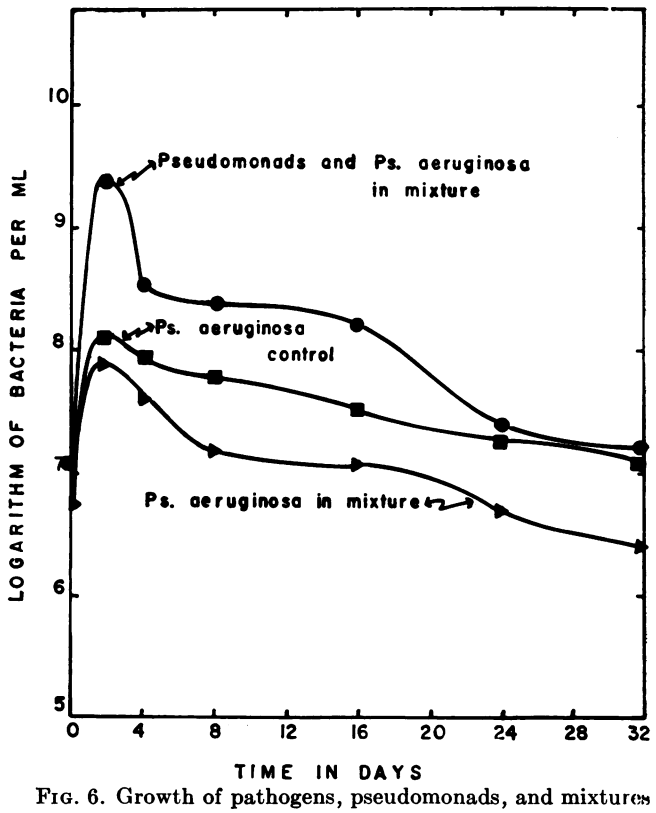

FIG. 6. Growth of pathogens, pseudomonads, and mixtures of both. Inoculum consisted of 6×10^6 bacteria of each type per ml of emulsion.

TABLE 4. *Virulence of pathogenic bacteria grown in pure culture or mixed with pseudomonads in soluble oil emulsions*

Cultures	Ratio of Mice Dead to Mice Inoculated*		
	Original stock cultures (no exposure to emulsion)	Pathogens grown in pure culture in emulsion	Pathogens grown in mixture with pseudomonads in emulsions
Salmonella schottmuelleri	2/4	2/4	2/4
Salmonella paratyphi	2/4	2/4	—
Salmonella typhosa	4/4	4/4	—
Shigella sonnei	2/4	2/4	3/4
Shigella dysenteriae	1/4	2/4	—
Shigella paradysenteriae	4/4	4/4	—
Klebsiella pneumoniae	4/4	4/4	4/4
Pseudomonas aeruginosa	4/4	4/4	—
Control (nutrient broth)	0/4	—	—

* Observation period 8 days.

25×10^3 per ml at 0 days to 19×10^3 at 1 day and was not recovered at 6 days.

Serologic reactions. *Shigella* species which had grown in pure culture or mixed with pseudomonads in the emulsions for 16 days failed to agglutinate when tested with their homologous antisera. *Salmonella* species grown in pure culture exhibited agglutination, but those recovered from the *Salmonella*-pesudomonad mixtures failed to do so.

Pathogenicity. Pathogens which had grown for 16 to 24 days in soluble oil emulsions retained their virulence whether grown in pure culture or mixed with pseudomonads. Bacteria from the emulsions killed about the same number of mice as the stock cultures which had never been exposed to soluble oil emulsions (table 4). All controls survived.

DISCUSSION

The work described in this report agrees with the results of Okawaki (1953) and Pivnick *et al* (1954) who showed that soluble oil emulsions supported the growth of *Salmonella* species and *K. pneumoniae*. It differs from the work of Pivnick *et al.* in that *Shigella* species did not grow in the soluble oil emulsions which they used. A plausible explanation for the difference is variability in the composition of the soluble oils. For example, Bennett and Wheeler (1954) showed that a given organism might survive only a few days in one soluble oil emulsion, but the same organism might survive more than 150 days in a different type of emulsion. *S. paradysenteriae* survived 76 days after inoculation into one soluble oil emulsion, but only 42 days in another emulsion. In the same two emulsions *Salmonella typhimurium* survived 62 days and 7 days, respectively.

The pathogens grew well in the presence of pseudomonads indigenous to soluble oil emulsions used industrially. However, in mixed culture they did not attain the population levels obtained by similar inocula in pure culture. Antagonism by the pseudomonads was not pronounced, and may indicate a simple competition between pathogens and pseudomonads for nutrients.

Increasing the pathogen:pseudomonad ratio in the inoculum to 100:1 did not give the pathogens an advantage in the emulsion. Figures 2 and 4 (100 pathogens to 1 pseudomonad) show trends similar to figures 1 and 3. In the latter experiments, the pathogen:pseudomonad ratio in the inoculum was 1:1.

The inability of *P. aeruginosa* to antagonize the indigenous pseudomonads is interesting and may explain why this organism is encountered rarely in soluble oil emulsions. During several years' work with soluble oil emulsions, this organism has been isolated in our laboratory only once, and then only as a result of selective enrichment procedures. The cultures thus isolated were nonhemolytic (Pivnick, *unpublished data*).

The continued virulence of pathogens in soluble oil emulsions may be of considerable importance, although it is too early to fully evaluate the significance of this discovery. Bennett and Wheeler (1954) reported a case of typhoid fever resulting from accidental contamination of a laboratory worker with *S. typhosa* growing in soluble oil emulsion for 4 weeks, and Bennett (1956) has isolated a *Salmonella* species on three different occasions from an emulsion used industrially. He has also isolated a paracolon organism.

The loss of antigenicity in *Shigella* species grown in pure or mixed culture, and *Salmonella* species in mixed culture, may be due to the selective growth of mutants. Other explanations may become available if work in this field is continued.

The survival of *M. tuberculosis* for only a few days may be of some importance when one considers the ease with which this pathogen could enter the emulsions. Furthermore, the aerosols of emulsion produced by cutting and grinding operations could disseminate this organism readily. In these experiments, however, young cells of H37Rv and the avirulent BCG were used. Such cells are very permeable and this may partially account for their rapid destruction. Experiments are under way to test the survival of *M. tuberculosis* in sputum added to soluble oil emulsions.

SUMMARY

The pathogenic bacteria, *Salmonella schottmuelleri*, *Salmonella paratyphi*, *Salmonella typhosa*, *Shigella sonnei*, *Shigella paradysenteriae*, *Shigella dysenteriae*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* grow well in soluble oil emulsions containing pseudomonads indigenous to emulsions used industrially. They are not readily antagonized by the pseudomonads and are present to the extent of at least 10^5 cells per ml after 32 days.

Pathogens in the emulsion for 16 days retain their

virulence, but in some instances lose their ability to agglutinate with specific antiserum.

Pure cultures of *Mycobacterium tuberculosis* strains H37Rv and BCG survive several days in the soluble oil emulsions.

ADDENDUM

Recently we have observed that the source of inoculum and type of soluble oil used influences the survival of *M. tuberculosis* and *S. pyogenes* var. *aureus*. For example, when 10 g of known positive tuberculous sputum was added to sterile 100 ml amounts of different soluble oil emulsions, viable mycobacteria and staphylococci were isolated six weeks later from some emulsions but not others.

Work is under way to determine whether these soluble oils serve as growth or maintenance media. Virulence studies and the fate of these organisms in the presence of pseudomonads indigenous to oil emulsions is being investigated.

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