Observations on the Pigment of *Streptomyces coelicolor*

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Several studies have been made on the conditions which determine the formation of pigments by microorganisms (Reid, 1936), their possible practical application (Beijerinck, 1913), their role in bacterial metabolism (Birkinshaw, 1937), their correlation with some morphological or physiological variations, and their chemical structures (Brazhnikova, 1946; Friedheim and Michaelis, 1931; Tobie, 1936; Waksman, 1950). The characteristic of pigment production seems to be more widely present in the actinomycetes than in any other of the bacterial groups and, as a consequence, there are many works tending to define the identity between the pigments formed and the bacteria producing them, for taxonomic purposes (Conn and Conn, 1941).

The present paper deals with some of the factors which determine the production of a blue pigment by a *Streptomyces coelicolor* strain, indicating its purification and some of its more important characteristics.

Materials and Methods

The *Streptomyces* strain was isolated from soil by means of the dilution method. Microscopic observations were made on colored slides obtained by a Drechsler's technic modification (Drechsler, 1919) in Czapek's medium. This strain was found to be *Streptomyces coelicolor*, following Waksman and Curtis's key in *Bergey's Manual* (Breed, Murray and Hitchens, 1948). It was plated in Czapek's medium, incubated at 28 C and, after periodic observations, colonies showing more rapid pigment production were selected and transferred. After repeated selection and transfer, pigment production by the selected final strain was studied in the following media: Oxford (1946), agar-Oxford, cotton-Oxford, Gause (1946), cotton-Gause, Czapek, cotton-Czapek, semisolid-Czapek, and Bottcher-Conn (1942). In each case the inoculum was a loopful of sporulated culture in Czapek's medium; the cultures were incubated at 28, 30, and 33 C. Observations were made after 2, 5, 10, 15, 20, and 30 days.

Pigment intensity. After the proper medium for pigment production had been selected, a method for measuring pigment intensity in an adequately approximate manner was developed. The following procedure was found to be satisfactory. The solution containing the pigment was filtered through glass wool and then through filter paper; pH was adjusted to neutrality and 0.5 ml of the solution was filtered and complemented to 10 ml with a pH 6.4 to 6.8 phosphate buffer solution. The light transmission of the solution was read with a Klett-Summerson photoelectric colorimeter with a green filter. The highest reading was considered to have a value of 100 per cent intensity and other readings were referred to this figure by calculation.

Influence of pH on pigment production. A series of twenty 300-ml Erlenmeyer flasks, each containing 40 ml of Bottcher-Conn's medium, was used. The medium was sterilized for 30 minutes under 15 pounds of steam pressure, and the pH was adjusted to give a range from pH 4 to 11. All media were inoculated with a loopful of a sporulated culture in Czapek's medium and incubated at 28 C for 20 days. The final pH and relative pigment intensities were then determined.

Influence of temperature. Six 300-ml Erlenmeyer flasks containing 40 ml of Bottcher-Conn's medium were inoculated, as above, and incubated at 25, 28, 30, 33, and 37 C. After 20 days the final pH, growth, and relative pigment intensities were determined.

Influence of carbon and nitrogen sources. For the determination of the influence of carbon source on pigment production, a series of Erlenmeyer flasks was prepared with 40 ml of Bottcher-Conn's medium without glycerol. The carbon sources under test were added in a proportion of 1 per cent; the pH was adjusted to 7.5 and the flasks were sterilized at 5 pounds for 1 hour. The media were inoculated as above, and incubated at 28 C for 20 days. The pigment intensities were then measured. The influence of nitrogen sources was studied in a similar manner using Bottcher-Conn's medium modified as follows: Yeast extract 0.5 g, K_2HPO_4 1 g, glycerol 5 g, and sufficient water to 1000 ml.

Aeration. The effects of aeration were studied using 500-ml Erlenmeyer flasks. Each flask contained 150 ml of Bottcher-Conn's medium which had been inoculated with 4 loopfuls of a sporulated culture grown in Czapek's medium. The flasks were aerated by means of an aquarium pump. The air used was passed through a washing flask, a filter, and then to the cultures. Aloxite stone spargers were used. Aeration was at a rate of 150 ml per minute and was continued for 120 hours at 28 C. A control was maintained under the same conditions.

Influence of the amount of inoculum. As it is difficult, if not practically impossible, to control the inoculum in

[1] A summary of this work was presented at the Sixth International Congress of Microbiology, Rome, 1953.

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actinomycetes, it was thought advisable to determine the influence of inoculum size on the results. Two series of Erlenmeyer flasks were prepared as in the pH experiments with the exception that a range of pH 6.9 to 10.5 was used. These flasks were inoculated with 1 and 2 loopfuls of inoculum for comparison. After 20 days incubation at 28 C, the final pH and pigment intensities were determined.

Pigment purification. After several assays were tried using modifications of Brazhnikova's (1946) method for litmocidin purification, and chromatographic procedures, the following purification technique, which is similar in some respects to Oxford's (1946) method, was adopted. A series of 300-ml Erlenmeyer flasks with 50 ml of Bottcher-Conn's medium at a pH of 7.5 was inoculated as indicated previously, and incubated at 28 C for 3 to 4 weeks; the pigment-containing solution from these flasks was collected, liquids from the cotton being extracted under pressure and by washing with a diluted ammoniacal solution, and placed in a larger flask. The whole liquid was filtered through glass wool and then through filter paper. The pigment was then precipitated by the addition of concentrated HCl and refrigerated overnight. This was followed by decantation, repeated washing of the precipitate with dilute HCl, and centrifugation to eliminate the liquid of lavation. The precipitate was then redissolved with a diluted ammoniacal solution and again precipitated with dilute HCl, this process being repeated for at least five times. A dilute ammonia solution was again added to dissolve the precipitate, together with two volumes of amyl acetate, acetone or carbon disulphide, and the whole liquid was stirred for 30 minutes. The solvent, which contained a yellow impurity, was eliminated and fresh solvent was added under constant stirring. The process was repeated until the solvent remained completely colorless and then it was removed by centrifugation or decantation. Now, five to six volumes of distilled water were added, and the pigment again precipitated by the addition of concentrated HCl. The precipitate was then washed several times with dilute HCl, followed by centrifugation each time to remove the supernatant fluid. Five to six volumes of glacial acetic acid were used to dissolve this precipitate, and centrifuged to remove the insolubles. The acetic solution of the pigment was diluted by the addition of distilled water and then precipitated with concentrated HCl. Repeated centrifugation and redissolution in acetic acid followed, until a negative Nessler reaction was obtained. Finally, the precipitate was dissolved in acetone, any insoluble portions which may have been formed having been removed by centrifugation and the solution was evaporated at room temperature.

Antimicrobial properties of the pigment. Antimicrobial properties were studied following Waksman and Reilly's (1945) method. A solution containing 20 mg of the purified pigment in 6 ml of methanol was diluted with distilled water to obtain a final concentration of 1 mg per ml. From this solution, aliquots were taken for the activity tests. Readings were made at the end of 48 hours for *Rhizobium*, 72 hours for *Mycobacterium*, 5 days for *Fusarium*, and 24 hours for the other microorganisms. Fred and Waksman's medium "79" (Fred and Waksman, 1928) was used for *Rhizobium*, Sabouraud's for *Fusarium*, and broth-agar for the others.

Results and Discussion

The strain of *Streptomyces* used in this study showed all the morphological and biochemical characteristics of *Streptomyces coelicolor*. It is noteworthy that it produced acid from glucose, lactose, sucrose, and mannitol; results which differ from Müller's (1908) but agree with Conn's (1943) findings.

Some variants attacked agar as observed by Stanier (1942); others lost the aerial mycelium and did not produce pigment, but later they recovered both properties as pointed out by Erikson (1948). Occasionally some lysis appeared in a medium containing Czapek's salts and 30 g of mannose per 1000 ml. Stanier (1942) and Dimitrieff (1937) observed a similar phenomenon in other media. Erikson, on the contrary, made no such observation.

There appeared to be some possibility of increasing pigment production by use of selective cultures. However, after two or four subcultures the time necessary for the pigment to appear in the medium was increased and the intensity lowered.

The morphology of the *Streptomyces* colonies was extremely variable and showed no relationship to pigment production. Colonies of three different morphological types which developed in the same Petri dish produced very similar pigment intensities, whereas identical colonies showed various intensities (figures 1 to 4).

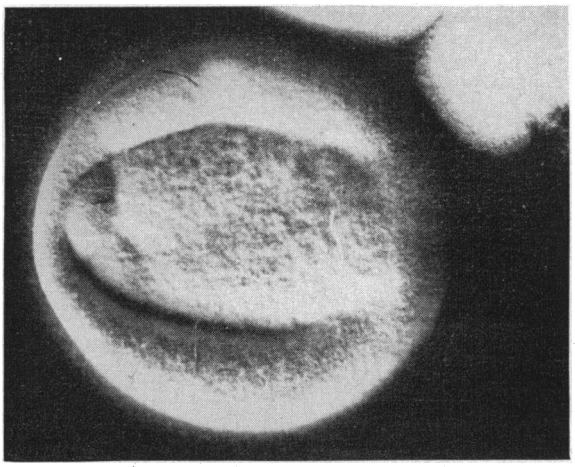

Fig. 1. A colony, two of which appeared on a single plate during observations. This one formed abundant pigment which diffused into media with a deep-blue color; the other produced no pigment.

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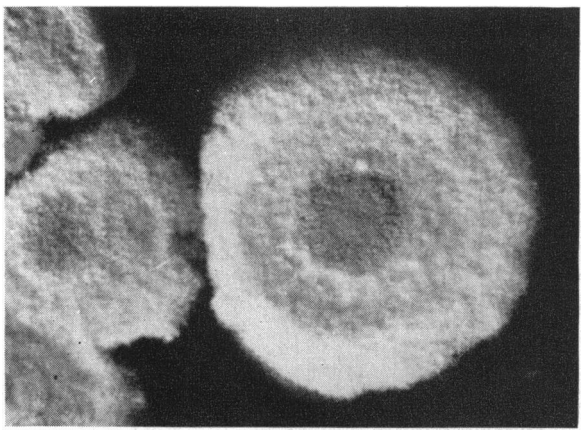

FIG. 2. Another type of colony, four of which appeared on one plate, similar in pigmentation to that in figure 1. One colony lacked pigment.

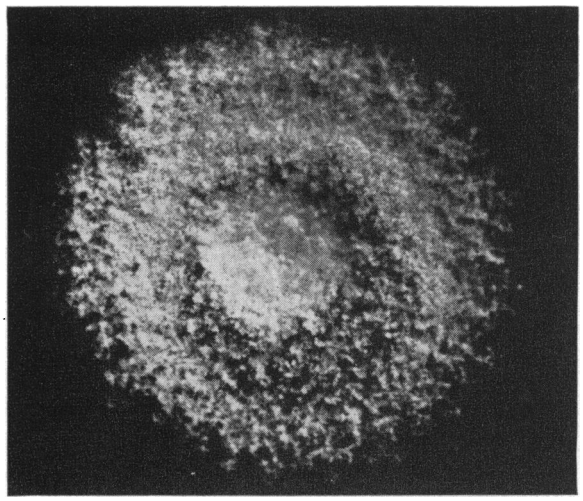

FIG. 3. The third type of colony, more than 30 colonies on one plate, with various pigment intensities.

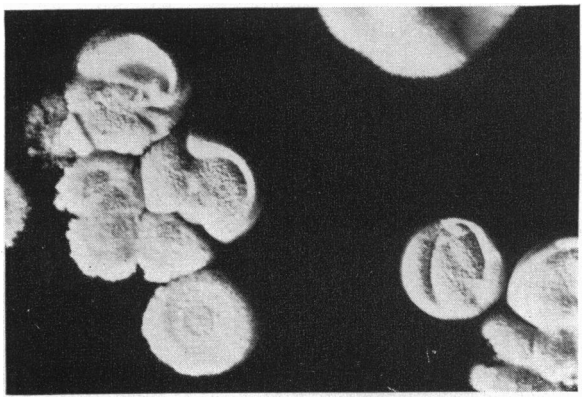

FIG. 4. Coexistence of three types of colonies on the same plate.

Pigment production. Results are expressed in table 1. The most suitable media for pigment production were Bottcher-Conn's and Czapek's. We preferred the former to the latter for two reasons: 1) the pigment was produced very rapidly and in large quantities, and 2) after slight modification, it made possible the performance of all of the experiments. It seemed inadvisable to study the influence of carbon sources by using Czapek's medium because the toxic effects of nitrites, in the case of acid production, would have given false results. The optimum temperature appeared to be 28 to 30 C.

TABLE 1. *Pigment production by* Streptomyces coelicolor *in various culture media*

CULTURE MEDIA	INCUBATION TEMPERATURE	DAYS OF INCUBATION					
		2	5	10	15	20	30
	C						
Oxford	28	−	−	−	−	+	++
	30	−	−	−	−	+	++
	33	−	−	−	−	+	+
Oxford (agar)	28	−	−	−	+	++	++
	30	−	−	−	+	++	++
	33	−	−	−	−	−	+
Oxford (cotton)	28	−	−	−	−	+	++
	30	−	−	−	−	+	++
	33	−	−	−	−	+	+
Czapek's (agar)	20	−	+	++	+++	++++	++++
	30	−	+	++	+++	++++	++++
	33	−	−	−	+	+	+
Czapek's (semi-solid)	28	−	+	++	+++	+++	++++
	30	−	−	++	+++	+++	++++
	33	−	−	−	+	+	+
Czapek's (cotton)	28	−	−	++	++	+++	++++
	30	−	−	++	++	+++	++++
	33	−	−	−	−	+	+
Gause (agar)	28	−	−	−	−	+	+
	30	−	−	−	−	+	+
	33	−	−	−	−	−	−
Bottcher-Conn	28	+	++	+++	++++	+++++	+++++
	30	+	++	+++	++++	+++++	+++++
	33	−	−	+	+	++	++

−: no pigment; + to +++++: various intensities of pigment.

Relative intensity of the pigment. It was possible to establish a correlation between the dilutions of the culture medium containing the pigment and the photocolorimetric readings. It would have been better to prepare standard solutions of known concentration, but acceptable pigment purification had not been obtained at that time.

Influence of pH. Table 2 shows that the optimum pH lies between 7.3 and 7.7. This factor appears to be of paramount importance because deviations of less than a unit with reference to the optimum point showed great differences in the relative pigment intensity. A pH lower than 5.4 or higher than 9.9 allowed neither growth nor pigment production.

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TABLE 2. *Influence of pH on the production of pigment by* Streptomyces coelicolor

pH		PHOTOCOLORIMETRIC READINGS	RELATIVE PIGMENT INTENSITY	GROWTH
Initial	Final			
4.20	4.30	0	0	−
4.70	4.75	0	0	−
5.00	5.00	0	0	−
5.60	5.45	13	11.11	±
6.20	6.30	45	38.46	+
6.45	6.75	78	66.66	++
7.15	6.60	85	72.64	++
7.35	6.90	105	89.74	++
7.40	6.90	105	89.74	++
7.45	6.60	109	91.88	++
7.75	7.05	117	100.00	++
8.30	7.45	80	68.37	++
8.65	7.45	60	51.13	++
9.15	7.60	56	47.86	++
9.35	7.75	45	38.46	+
9.95	8.00	9	7.69	−
10.95	8.65	0	0	−

−: no growth; ±: scarce growth; + and ++: various intensities of growth.

TABLE 3. *Influence of temperature on the production of pigment by* Streptomyces coelicolor

ROOM TEMPERATURE	pH		PHOTOCOLORIMETRIC READING	RELATIVE PIGMENT INTENSITY	GROWTH
	Initial	Final			
C					
	7.5				−
25	7.5	6.9	40	72.72	+
28	7.5	7.0	55	100.00	++
30	7.5	7.2	55	100.00	++
33	7.5	6.5	38	69.09	+
37	7.5	6.55	7	12.72	±

−: no growth; ±: scarce growth; + and ++: various intensities of growth.

Temperature. As presented in table 3, the optimal temperature for both growth and pigment formation lies between 28 and 30 C. A similar conclusion was drawn from table 1.

Carbon and nitrogen sources. The best carbon sources for pigment production were: D-mannose, glycerol, raffinose, and D-xylose, as shown in table 4. Mannitol gave scant pigmentation, L-arabinose almost negligible, and inulin none. These results agree with those of Cochrane and Conn (1947).

The best nitrogen sources were sodium caseinate and peptone; gelatin, nitrate, and ammonia salts gave very poor results.

Inoculum. Table 5 shows that results obtained in the experiments could not be greatly altered even if the inocula were not quantitatively uniform, since the size of the inoculum appeared to have no influence on pigment production.

Aeration. Under the conditions of our experiments, aeration seemed to inhibit pigment production. This fact is difficult to explain because it is known that pigmentation appears under aerobic conditions.

TABLE 4. *Influence of carbon and nitrogen sources on the production of pigment by* Streptomyces coelicolor

SOURCES	PHOTOCOLORIMETRIC READING	RELATIVE PIGMENT INTENSITY
Carbon (1%):		
None	10	9.52
Glucose	57	54.28
Galactose	60	57.14
D-Mannose	105	100.00
Fructose	52	49.52
D-Xylose	80	76.19
L-Arabinose	17	16.19
Glycerol	97	92.38
Mannitol	31	29.52
Inositol	60	57.14
Raffinose	78	74.28
Inulin	0	0
Starch	65	61.90
Nitrogen:		
$NaNO_3$ 0.64 (g/L)	0	0
Urea* 0.24 (g/L)	32	62.74
$(NH_4)_2HPO_4$* 0.50 (g/L)	8	15.68
CH_3COONH_4* 0.58 (g/L)	0	0
Asparagine* 0.50 (g/L)	38	74.50
Peptone 1.00 (g/L)	48	94.11
Tryptone 1.00 (g/L)	40	78.43
Gelatin 1.00 (g/L)	17	33.33
Sodium caseinate 1.00 (g/L)	51	100.00

* Total nitrogen 0.6 g per liter.

TABLE 5. *Influence of size of inoculum on the production of pigment by* Streptomyces coelicolor

CULTURES	INITIAL pH	INOCULUM (LOOPFULS)	FINAL pH	PHOTOCOLORIMETRIC READING	RELATIVE PIGMENT INTENSITY
1	6.90	1	6.55	74	61.66
1	6.90	2	6.60	78	65.00
2	7.40	1	6.95	111	93.00
2	7.40	2	6.95	109	90.83
3	7.70	1	6.95	120	100.00
3	7.70	2	7.05	116	96.66
4	8.05	1	7.35	71	59.16
4	8.05	2	7.35	73	60.83
5	8.40	1	7.45	26	21.66
5	8.40	2	7.40	58	48.33
6	8.70	1	7.50	56	46.50
6	8.70	2	7.55	60	50.00
7	9.20	1	7.60	50	41.66
7	9.20	2	7.55	47	39.16
8	9.50	1	7.80	40	33.33
8	9.50	2	7.85	35	29.16
9	10.50	1	8.50	10	8.33

Purification. A number of workers have studied this pigment. Müller (1908) developed an extraction procedure using a potato medium; Kriss (1936) reported another method and indicated anthocyanidin properties

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TABLE 6. *Antimicrobial spectrum of the purified pigment of* Streptomyces coelicolor

TEST MICROORGANISMS	ACTIVITY (UNITS PER MG)
Escherichia coli W*	0.0
Micrococcus pyogenes var. aureus W	0.0
Bacillus subtilis 27 W	0.0
Bacillus subtilis 33 W	0.0
Bacillus mycoides W	0.0
Micrococcus lysodeikticus W	0.0
Mycobacterium phlei W	0.0
Rhizobium meliloti RM 38 DA**	10.10
Rhizobium japonicum RJ DA	30.30
Fusarium sp. 4 DA	0.0

* W: Waksman Collection.
** DA: Dirección de Defensa Agricola (México, D. F.).

for his preparations; Erikson *et al.* (1938) suggested that it could be a polyhydroxiphenazine. Frampton and Taylor (1938) concluded that not all of the pigments are alike; some were similar to Müller's preparations and at the same time different from Waksman's *Actinomyces violaceus-ruber* pigment. Dr. Waksman mentions a similarity in chemical structure between azolitmin and his strain pigment on one hand and that of Müller on the other. Oxford (1946) obtained some preparations with a total protein N content of 1.9 per cent which he considered a residual protein contamination. The pigment produced by the strain of *Streptomyces coelicolor* used in this study had the same N content (Kjeldahl) and the same solubility characteristics, which suggests that such nitrogen would form a part of the pigment molecule itself. By repetition of the purification process on a portion of the purified pigment, the same amount was obtained. That this suggestion could be valid, however, depends on further verification.

In our procedure for purification, using 2 liters of Bottcher-Conn's medium, it was possible to obtain 100 mg pigment per liter; Oxford obtained only 5 mg per liter. Whether this higher yield was due to the strain or to the medium used, was not determined. The nitrogen data indicate, in agreement with Oxford, that the pigment is not an anthocyanidin, nor phenazine and that it does not have azolitmin characteristics.

Antimicrobial spectrum. Table 6 shows that the pigment had only antirhizobic activity. This simple biological test differentiates the pigment from litmocidin (Gause, 1946) and possibly it could be used to solve Conn's problem in the sense that not all of the pigments produced by different *Streptomyces coelicolor* strains are the same.

SUMMARY

A strain of *Streptomyces coelicolor*, isolated from Mexican soils, was studied with special reference to the production of its pigment and the antibiotic properties of this substance.

It was found that the quantity of inoculum had no influence on pigment production. Bottcher-Conn's medium gave the best results; the optimum temperature for growth was 28 to 30 C; and the most favorable pH was between 7.3 and 7.7. This strain used mannose and glycerol as sources of carbon, and sodium caseinate and peptone as sources for nitrogen. Aeration of the cultures inhibited the production of pigment.

For the purification of the pigment, a procedure based fundamentally on the property of the precipitation of the substance from its aqueous solution with HCl followed by redissolution in ammoniacal, acetic or acetonic media was used. During the first steps of purification the use of organic solvents allowed for the extraction of a great deal of impurities.

In the preparations obtained by this procedure, a nitrogen content of 1.9 per cent was found, which is similar to the value obtained by Oxford.

The substance was found active only against some species of *Rhizobium* and it is assumed that this property might be useful as a test in the differentiation of similar pigments.

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Coliform Bacteria in Soluble Oil Emulsions[1]

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Cutting oils are commonly used in machine shop operations as lubricants in the cutting and grinding of metals. Two types of cutting fluids are used, the straight oils and the soluble oils. The straight oils are usually mineral oils with or without chemical additives and admixtures of fatty oils. They are used as they are prepared by the manufacturer or they may be diluted with light mineral oils. The soluble oils are mineral oils solubilized with such materials as soaps of rosin, tall oil, petroleum sulfonates or other emulsifying agents. They are usually mixed with varying amounts of water to form stable, milky emulsions. The straight oils do not support microbial life but the soluble oils, when dispersed in water for use in machine shop operations, are excellent media for bacterial growth.

Many species of bacteria have been found growing in soluble oil emulsions (Duffett *et al.*, 1943; Lee and Chandler, 1941) and previous investigators (Duffett *et al.*, 1943; Weirich, 1943) have reported the isolation of coliform bacteria from them. Whether the coliforms are of importance from a public health consideration is not yet known but one investigator (Dolge) has reported that feces and other body discharges have been found in industrially used emulsions.

This investigation was undertaken to study: a) the number and types of coliform bacteria in soluble oil emulsions used in industry, b) the growth of organisms from feces inoculated into soluble oil emulsions in the laboratory, and c) the antagonism of nonlactose fermenting bacteria (predominantly *Pseudomonas* species) towards coliform bacteria in soluble oil emulsions.

EXPERIMENTAL METHODS

The numbers of coliform bacteria in soluble oil emulsions obtained from several industrial sources were determined by the Most Probable Number technique (A.P.H.A., 1946) and total bacterial populations were determined by the plate-count method using M/20 phosphate buffer at pH 7.0 as diluent and nutrient agar as the plating medium. Colonies were counted after 48-hr incubation at room temperature. Types of coliform bacteria were determined by examination on eosin-methylene blue agar and by the IMViC reactions.

The growth of fecal organisms in soluble oil emulsions was determined by distributing two g of feces in an apparatus (Pivnick and Fabian, 1953) containing an emulsion composed of 2 per cent soluble oil in tap water and circulating the mixture through iron chips. Plate counts of the bacterial population were made at appropriate intervals. After 40 days, counts of lactose-fermenting organisms were made on Endo medium and of the 6,800,000 organisms per ml present, 75 per cent fermented lactose.

Industrial samples of soluble oil emulsions, which contained predominantly members of the genus *Pseudomonas*, were mixed with inocula from the above-mentioned emulsion in which coliform bacteria had grown

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