

Resistance of *Salmonella typhi* to chloramphenicol¹

Part I A preliminary report

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SYNOPSIS Resistance of *Salmonella typhi* to chloramphenicol has not been reported so far except in strains made resistant in the laboratory. While examining 52 smooth strains of *S. typhi* and three smooth strains of *S. paratyphi* A 10 strains of *S. typhi* were found to be resistant to 50 to 500 µg. chloramphenicol. Of these 10 strains, eight appeared to be tolerant of the antibiotic, but the remaining two strains appeared to produce a substance that antagonizes or destroys chloramphenicol.

Chloramphenicol has been used in treating typhoid fever for a long time, and Smith, Joslyn, Gruhzit, McLean, Penner, and Ehrlich (1948), Woodward, Smadel, Ley, Green, and Mankikar (1948), Murgatroyd (1949), and Bradley (1949) reported that it was effective therapeutically. All strains of *S. typhi* are believed to be uniformly sensitive to chloramphenicol and McLean, Schwab, Hillegas, and Schlingman (1949) showed that a concentration of 0.75 to 2.5 µg. per ml. is sufficient to inhibit the growth and multiplication of the organism. They also showed that by serial subculture in the presence of chloramphenicol, the organism could be rendered resistant. Alexander, Leidy, and Redman (1949) showed that in a large population of *S. typhi* a few cells resistant to 10 µg. per ml. might be present but they were of the opinion that drug resistance would not play an important part in therapy. Meads, Harris, Haslam, and Cline (1950) reported chloramphenicol resistance in *E. coli*, *Ps. aeruginosa* and *A. aerogenes* from urinary infections. According to Meads *et al.* resistance might emerge suddenly or stepwise, and they suggested that the drug selects the resistant cells in a population of largely susceptible cells. Schneierson (1952) studied the emergence of resistance to chloramphenicol and aureomycin over a period of three years and found that salmonellae did not acquire resistance to chloramphenicol to any marked degree, all strains tested being sensitive to 10 µg. per ml.

We could not find any report in the literature about the natural resistance of *S. typhi* to chloramphenicol except one by Colquhoun and Weeth (1950), who reported increased resistance of *S. typhi* to chloramphenicol isolated from blood and faeces in a case of relapsed typhoid fever when compared with the resistance of the strain isolated before treatment with chloramphenicol. However, Good and Mackenzie (1950) and Rankin and Grimble (1950) could not find any increase in resistance in strains isolated from cases of relapsed typhoid fever.

Weiner and Swanson (1960) reported morphological, cultural, and antigenic changes in one strain of *S. typhi* artificially made resistant to chloramphenicol. Voureka (1951a) showed the emergence of morphological and cultural variants of *E. coli* and *Ps. pyocyanea* in patients being treated with chloramphenicol for urinary tract infections and also that such variation could be hastened *in vitro* by specific antisera (Voureka, 1951b). We have not been able to find any reports in the literature about the natural resistance of *S. typhi* to chloramphenicol.

MATERIALS AND METHODS

The strains investigated were 52 smooth strains of *S. typhi* and three smooth strains of *S. paratyphi* A. All were isolated from blood and identified by biochemical and serological tests. Throughout the investigation chloramphenicol prepared by Parke, Davis & Company²

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²By the time the manuscript of this article was ready Messrs. Parke Davis & Co. pointed out that their chloramphenicol capsules contain extraneous substances used as excipients. So all aspects of the work were repeated with pure chloramphenicol supplied by them and the results confirmed.

was used, 250 mg. being dissolved in 250 ml. of broth by boiling. This was stored in the refrigerator and further dilutions made from it in broth.

Resistant strains were subcultured on nutrient agar every few days and tested for resistance to the antibiotic at every third subculture.

The achromycin used was a water-soluble injectable preparation (Lederle Laboratories, India, Ltd.); 2 ml. of the solvent was added to a vial of 100 mg. of achromycin. This sterile solution was used for making further dilutions in nutrient broth.

Terramycin solution was prepared by grinding 250 mg. of the antibiotic in broth until dissolved and then making up the volume to 250 ml. with broth. It was filtered through a Seitz filter for sterilization.

EXPERIMENTAL WITH RESULTS

The resistance of *S. typhi* to chloramphenicol was tested as follows:—

TESTS IN BROTH CULTURES Fifty-two strains were tested by two methods.

1 A volume (0.5 ml.) of an 18-hour broth culture of *S. typhi* diluted 10^{-8} was added in a series of tubes to an equal volume of broth containing antibiotic ranging from 0.02 to 500 μg . Readings were made after 24 and 48 hours' incubation at 37°C. and the concentration of the antibiotic in tubes showing turbidity was noted.

2 To tubes containing 1.0 ml. of similar concentrations of antibiotic one drop of an 18 to 24-hour broth culture was added.

The highest concentration of the antibiotic in the tubes showing turbidity was regarded as the concentration resisted by the strain. The results were identical by each method and so the second was used throughout.

The range of sensitivity of the 52 strains is shown in Table I. There was considerable variation in the

TABLE I

SENSITIVITY OF *S. TYPHI* TO CHLORAMPHENICOL

Antibiotic Inhibiting ($\mu\text{g./ml.}$)	No. of Strains	Percentage of Strains
<2.5	5	9.6
2.5- 5	8	15.4
> 5- > 10	29	55.8
50-100	5	9.6
> 100-250	3	5.8
<250	2	3.8

degree of resistance of those strains which were isolated before antibiotic treatment was begun in the hospital and hence it is unlikely that the patients had been exposed previously to chloramphenicol, although it is possible that some had received small doses before admission. Ten of these strains (19%) were resistant to more than 50 μg . per ml. which is

regarded as being significant therapeutically since the blood level of chloramphenicol under adequate therapy is stated to be about 40 μg . per ml. These strains are therefore designated as 'resistant' and the remaining 42 (81%), which were inhibited by 10 μg . per ml. or less, as 'sensitive'.

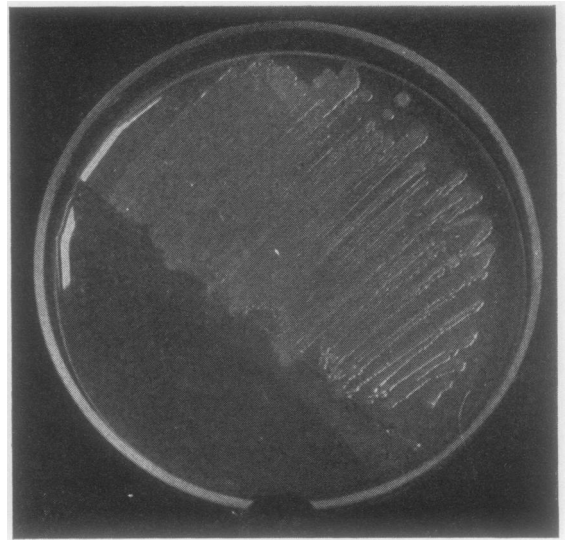
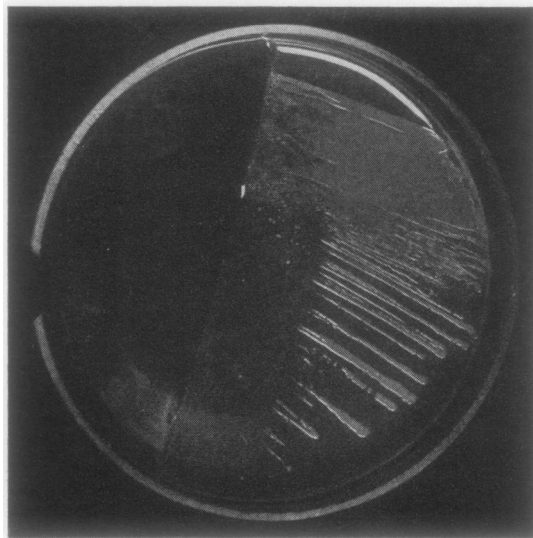
The three strains of *S. paratyphi* did not show a significant degree of resistance.

TESTS IN DITCH PLATES The technique was similar to that described by Barber (1957). About one-third of the area of the plate at one side was filled with agar containing antibiotic. A heavy inoculum from a 24-hour agar culture was spread over the rest of the plate, covering the whole area, in such a way that there was a gradation in the amount of inoculum from one side of the plate to the other along the line of the ditch. The plates were examined after incubation for 48 hours at 37°C.

Resistant strains were re-tested by this method incorporating in the agar the highest concentration of the antibiotic known to be resisted by the strain from tests in the broth. Two sensitive strains were also tested and were inhibited in a zone of uniform width adjacent to the edge of the ditch (Fig. 1). All but two of the resistant strains grew up to the margin of the ditch (Fig. 2). The two exceptional strains (nos. 875 and 1379) grew up to the edge of the ditch where the inoculation was heaviest but as the inoculum thinned out across the plate the growth receded by an increasing distance from the edge of the ditch. The margin of growth nearest the ditch was thin with the appearance of ground glass but farther away it was normally opaque. A few colonies which were prominent because of their opacity developed in the area of the thin growth (Fig. 3).

EFFECT OF SIZE OF INOCULUM ON DEGREE OF RESISTANCE Tubes containing amounts of antibiotic ranging from 5 to 500 μg . in 1.0 ml. of broth were inoculated with one drop of an overnight broth culture undiluted and diluted 10^{-1} to 10^{-5} . After incubation at 37°C. for 48 hours the degree of turbidity was noted and compared with controls in broth only.

The results agree with the findings on the ditch plates. With all the resistant strains, except nos. 875 and 1379, the amount of inoculum had no influence on the concentration of chloramphenicol which they resisted and turbidity was equal in all tubes showing growth. In the exceptional strains the amount of chloramphenicol required to suppress their growth depended on the strength of inocula, progressively less antibiotic being required as the amount of inoculum diminished. In addition growth was partly suppressed, as shown by less turbidity in cultures, when the concentration of antibiotic was somewhat below that required for total suppression

FIG. 1. *S. typhi* sensitive to chloramphenicol.FIG. 2. *S. typhi* tolerant of chloramphenicol.FIG. 3. *S. typhi* destroying or antagonizing chloramphenicol.

and this was the case with all dilutions of inoculum. The results for strain 875 are shown in Table II.

COMPARISON OF RESISTANCE TO CHLORAMPHENICOL, ACHROMYCIN, AND TERRAMYCIN The 10 strains resistant to chloramphenicol and the two sensitive strains (nos. 215 and 334) were tested in broth by a similar technique for resistance to achromycin and

terramycin, as representatives of other broad-spectrum antibiotics. The results are shown in Table III.

There was a considerable range of resistance to both achromycin and terramycin among these strains but no indication that they could be divided into clearly differentiated sensitive and resistant strains as was found with chloramphenicol. The

TABLE II
EFFECT OF AMOUNT OF INOCULUM ON CONCENTRATION OF CHLORAMPHENICOL REQUIRED TO SUPPRESS GROWTH OF A RESISTANT STRAIN OF *S. TYPHI* (NO. 875)

Dilutions of Inoculum	Concentration of Chloramphenicol ($\mu\text{g./ml.}$)						Control
	500	250	100	50	10	5	
0	—	+++ ¹	++	+++	+++	+++	+++
10 ⁻¹	—	+	++	++	+++	+++	+++
10 ⁻²	—	—	+	++	+++	+++	+++
10 ⁻³	—	—	—	++	+++	+++	+++
10 ⁻⁴	—	—	—	+	+++	+++	+++
10 ⁻⁵	—	—	—	—	++	+++	+++

¹The number of plus signs indicates the degree of turbidity.

resistance or sensitivity of any particular strain to chloramphenicol was not an indication of how it would be affected by the other two antibiotics (see particularly strains 215, 234, 875, 1379, 232). Gocke, Finland, and Wilcox (1951) showed cross resistance of organisms to chloramphenicol and the tetracyclines in respect of strains made artificially resistant by serial subcultures in the presence of chloramphenicol.

peared to be a diminution in fermenting power. Glucose continued to be fermented but only two strains retained the ability to ferment xylose and all lost the power to ferment maltose and mannitol. The significance of this finding is not known. Chloramphenicol is believed to interfere with the protein metabolism of bacteria. Whether it has any action on carbohydrate metabolism is not known.

TABLE III

COMPARISON OF RESISTANCE OF STRAINS OF *S. TYPHI* TO CHLORAMPHENICOL, ACHROMYCIN, AND TERRAMYCIN

No. of Strain	Lowest Concentration of Antibiotic Producing Resistance ($\mu\text{g./ml.}$)		
	Chloramphenicol	Achromycin	Terramycin
28	100	50	10
274	50	25	25
279	50	50	25
646	50	10	10
875	250	250	50
1106	50	50	25
1227	100	25	10
1379	250	25	10
135	50	50	25
232	50	500	250
215	1.0	25	250
234	2.5	25	10

CULTURAL AND BIOCHEMICAL CHARACTERISTICS AND AGGLUTINABILITY During repeated subculture on agar and broth no changes in morphology, cultural characteristics, or biochemical reactions were noted and they agglutinated with stock H and O sera by the slide method. The highly resistant strain no. 875 agglutinated slowly with O antiserum compared with other strains. Two strains (nos. 1379 and 1227), originally resistant to 250 and 100 $\mu\text{g.}$ respectively, lost their resistance to some extent.

Resistant strains grown in the presence of chloramphenicol did not show any morphological changes or loss of motility or altered cultural characteristics. They continued to be indole and Voges-Proskauer negative. However, the M.R. reaction which was originally positive became negative and there ap-

DISCUSSION

One strain (no. 875) which is highly resistant was isolated from a girl aged 13 years, admitted for the treatment of myxoedema, who developed fever six weeks after admission. Presumably this was a hospital infection. But it was not possible to trace the source of infection as strains isolated from the same ward at about the same time were not available for study in the laboratory. Since the strains were not in contact with the antibiotic in the laboratory they must be regarded as naturally resistant or might have become resistant *in vivo*.

The appearance of the growth of strains of *S. typhi* on a chloramphenicol ditch plate shows a close resemblance to the growth of staphylococci on penicillin ditch plates (Barber, 1957). Near the ditch the concentration of the antibiotic is high; it gets less and less as it diffuses from the ditch outwards to the end of the plate (strains 875 and 1379 grew near the ditch at the site of primary inoculation) and growth is further and further away from the ditch as the inoculum gets less and less. This appearance closely resembles that of penicillin destroying staphylococci on penicillin ditch plates. It is possible that these two strains produced a substance destroying or antagonizing chloramphenicol. At the site of primary inoculation there are more organisms and more of the substance is produced with the result that more of the antibiotic is destroyed or antagonized. As the inoculum diminishes, less of the substance is produced and more of the antibiotic is left behind near the ditch, with a high concentration of the antibiotic, and so growth is suppressed.

Away from the ditch, where the antibiotic concentration is less, growth takes place. This observation, that the degree of resistance depends upon the number of cells in the inoculum, is supported by the observation that high dilutions of the inoculum containing a smaller number of organisms result in resistance to a smaller amount of the antibiotic.

Smith and Worrel (1950) showed that some organisms (not *S. typhi*) could decompose chloramphenicol.

Other strains grew up to the ditch in all parts of the plate and resembled penicillin-tolerant staphylococci in penicillin ditch plates. It is possible that the mechanism of resistance in this case is similar in that the individual cells are tolerant of the antibiotic. It is interesting to note that these strains, like penicillin-tolerant staphylococci, readily lose resistance. Probably their resistance does not depend upon the production of any substance antagonizing or destroying chloramphenicol.

Part II The mechanism of resistance¹

SYNOPSIS A study of the action of the culture filtrates of 10 strains of *S. typhi* resistant to chloramphenicol showed that two of the strains produced an extracellular substance which enabled a strain of *S. typhi* sensitive to chloramphenicol to grow in a mixture of the filtrate and the antibiotic. A study of the properties of this substance suggested that it was an enzyme.

There are no reports on the resistance of *S. typhi* to chloramphenicol in the literature except concerning strains made resistant in the laboratory by serial subculture in the presence of increasing concentrations of the antibiotic. In Part I we reported resistance in 10 strains of *S. typhi* which have not been made resistant in the laboratory. The present study postulates the mechanism of resistance.

MATERIALS AND METHODS

The 10 chloramphenicol-resistant strains and one strain sensitive to 5 μg . of the antibiotic were inoculated into 250 ml. of broth and incubated for 48 hours at 37°C. The cultures were then filtered through Seitz filters and 0.5 ml. of filtrate was inoculated into 5 ml. of broth and incubated for 48 hours to test for sterility. The filtrates were stored at 0° to 4°C.

A broth culture filtrate of a strain of *E. coli* resistant

¹A paper read before the twelfth annual meeting of the Indian Association of Pathologists in 1961.

to 750 μg . and another sensitive to 10 μg . of chloramphenicol were also prepared in the same way.

The chloramphenicol used in this study was pure chloramphenicol powder supplied by Messrs. Parke Davis (India) Ltd. Five hundred mg. of the antibiotic was dissolved in 250 ml. of glass-distilled water (pH 7.0) and autoclaved at 10 lb. for 10 minutes. The solution was then aseptically added to double-strength nutrient broth of pH 7.6. The mixture thus had 1,000 μg . of antibiotic per ml. of nutrient broth. This stock solution was used to make further dilutions in nutrient broth.

EXPERIMENTAL

PRELIMINARY TEST WITH FILTRATES Different concentrations of chloramphenicol each in 0.5 ml. broth were distributed into rows of sterile tubes and 0.5 ml. of one filtrate was added to one row of tubes. The final concentrations of the antibiotic varied from 500 to 2.5 μg . All mixtures were then seeded with an 18 to 24-hour broth culture of the sensitive strain of *S. typhi*. Readings were made after 48 hours' incubation at 37°C.

TABLE I

ACTIVITY OF FILTRATES OF *S. TYPHI* CULTURES RESISTANT TO CHLORAMPHENICOL

Filtrate Strain No.	Concentration of Chloramphenicol (μg . per ml.)										Filtrate Control	
	500	250	125	100	75	50	25	10	5	2.5		
28	—	—	—	—	—	—	—	—	—	—	+++	+++
274	—	—	—	—	—	—	—	—	—	—	+++	+++
279	—	—	—	—	—	—	—	—	—	—	+++	+++
646	—	—	—	—	—	—	—	—	—	—	+++	+++
875	—	—	++	++	+++	+++	+++	+++	+++	+++	+++	+++
1106	—	—	—	—	—	—	—	—	—	—	+++	+++
1227	—	—	—	—	—	—	—	—	—	—	+++	+++
1379	—	—	—	+	+	++	+++	+++	+++	+++	+++	+++
135	—	—	—	—	—	—	—	—	—	—	+++	+++
232	—	—	—	—	—	—	—	—	—	—	+++	+++
Sensitive strain	—	—	—	—	—	—	—	—	—	—	+++	+++