Dihydrostreptomycin and Anaerobiosis—Comparison with Other Antibiotics and its Selectivity with regard to Obligate Anaerobes

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SUMMARY: The sensitivity to various antibiotics of certain facultatively anaerobic bacteria when growing under aerobic or anaerobic conditions was compared. The antibiotics tested were: dihydrostreptomycin, penicillin, chloramphenicol, erythromycin, carbomycin, chlortetracycline, oxytetracycline, tetracycline. Dihydrostreptomycin was unique amongst the antibiotics tested in being less active against facultative anaerobes when they were growing anaerobically than when growing aerobically. Dihydrostreptomycin was relatively inactive against *Clostridium welchii* and *C. novyi*, and its action was governed by the inoculum size. Dihydrostreptomycin is not a useful selective agent for the isolation of *C. welchii*.

It was observed during routine sensitivity examinations by the ditch-plate technique that streptomycin appeared to have less effect on certain sensitive facultative anaerobes when they were growing anaerobically than upon the same organisms when growing aerobically. There have been several similar observations (Bondi, Dietz & Spaulding, 1946; Geiger, Green & Waksman, 1946; May, Vourekla & Fleming, 1947; Grumbach, 1950; Lightbown, 1954). These observations have led to the assumption that streptomycin or dihydrostreptomycin interfere with the oxidative mechanisms of bacterial metabolism. As far as is known, penicillin does not possess this property, but there is little information about other antibiotics (Stokes, 1955). We thought it of interest to compare the effect of penicillin and other antibiotics on facultative anaerobes when growing aerobically or anaerobically.

METHODS

Organisms. With the exception of Friedländer's bacillus (*Klebsiella pneumoniae* strain 41; May *et al.* 1947) and *Staphylococcus aureus* Oxford strain, the organisms used (*Neisseria meningitidis*, *Haemophilus influenzae* type b, *Escherichia coli*, *Aerobacter aerogenes*, *Streptococcus pyogenes*, *Diplococcus pneumoniae*, *Clostridium welchii* and *C. novyi*) were all stock laboratory strains; all were typical by routine laboratory tests. In experiments to test the efficiency of dihydrostreptomycin as a selective agent for the isolation of obligate anaerobes, in particular *C. welchii*, samples of faeces sent to the laboratory for routine examination were used.

Media. Heated blood (10 %, v/v) agar (pH 7·8; chocolate agar) was used as it satisfied the needs of the most fastidious members of the collection. When dihydrostreptomycin was tested as a selective agent for obligate anaerobes,

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the medium was Robertson's cooked meat medium, either alone or in conjunction with fresh blood (10 %, v/v) agar and sterilized milk.

Antibiotics. Dihydrostreptomycin, penicillin, chloramphenicol, erythromycin, carbomycin, chlortetracycline, oxytetracycline and tetracycline were examined. The dihydrostreptomycin sulphate (Glaxo) was dispensed in sterile distilled water in amounts necessary to give the required concentration when 1.0 ml. of the antibiotic solution was added to the suspending medium. The other antibiotics were made up in sterile distilled water to give penicillin 100 i.u./ml., and the remainder 100 µg./ml.; these solutions were further diluted with nutrient agar, when required, to give a final concentration of 20 i.u./ml., and 20 µg./ml. respectively.

Comparison of the activity of dihydrostreptomycin with other antibiotics

Heated blood agar (25 ml.) was poured into Petri dishes of 9 cm. diameter, cooled and dried; a ditch was then cut on one side. Eight groups of six ditch plates were used and the ditches in each group filled with nutrient agar, at 45°, containing either penicillin 20 i.u./ml. or 20 µg./ml. of one of the other antibiotics under examination. One standard loopful of an 18 hr. culture of the organism under test grown in suitable fluid medium (diluted 1/10⁴ in nutrient broth in the case of the more vigorously growing organisms, e.g. *Escherichia coli* and 1/10² in the case of the less vigorous, e.g. *Streptococcus pyogenes*) was streaked across the dry surface of the plate, beginning from the edge of the ditch. This procedure ensured that the heaviest inoculum was the first to be exposed to the diffusing antibiotic. Each group of six plates was divided into two sets of three. One set was incubated aerobically and the other anaerobically in a McIntosh and Fildes anaerobic jar for 48 hr.

Examination of the action of dihydrostreptomycin on Clostridium welchii

As previously, triplicate heated blood agar ditch plates were prepared containing 1000, 500, 200, 100 or 50 µg. dihydrostreptomycin/ml. nutrient agar in the ditch. 1/10¹ to 1/10⁸ dilutions of an 18 hr. broth culture of *Clostridium welchii* were prepared and one standard loopful of the undiluted culture and of each dilution streaked across the plate. Parallel experiments were carried out in Robertson's cooked meat medium. Series of tubes containing 1000, 500, 100, 50 and 25 µg. dihydrostreptomycin/ml. medium were inoculated with 0.034 ml. of parent culture and of the 1/10², 1/10⁴, 1/10⁶ and 1/10⁸ dilutions used in the experiments on solid media. The plates were examined after 48 hr. of anaerobic incubation and cultures in fluid media after 24 hr. and 5 days of incubation.

*A possible selective medium containing dihydrostreptomycin
for the isolation of Clostridium welchii*

Since obligate anaerobes are not inhibited by dihydrostreptomycin, its use as a selective agent for the isolation of *Clostridium welchii* was investigated. Tubes of cooked meat medium containing decreasing concentrations of dihydrostreptomycin were inoculated with a sample of faeces. A conventional

test for the isolation of anaerobes was set up in parallel: a tube of cooked meat medium was inoculated and then heated at 65° for 30 min. (Mackie & McCartney, 1953). After overnight incubation in air each cooked meat broth culture was subcultured to a fresh-blood agar plate. After further anaerobic incubation for 18–24 hr. these plates were examined for the presence of *C. welchii*. Typical colonies were examined microscopically and for 'stormy clot' fermentation in sterilized milk.

RESULTS

Comparison of the activity of dihydrostreptomycin with other antibiotics

The activity of the antibiotic under investigation toward a number of representative facultative anaerobes growing under aerobic and anaerobic conditions is recorded in Table 1. As controls, a strict aerobe (*Neisseria meningitidis*) and two obligate anaerobes (*Clostridium welchii* and *C. novyi*) were included. From this survey it is seen that dihydrostreptomycin was unique among the antibiotics tested in being more effective against facultative anaerobes when

Table 1. *Inhibition of growth of a number of anaerobes (facultative and obligate) separately exposed to eight different antibiotics, under aerobic or anaerobic conditions*

Organism	Conditions of growth	Linear extent (mm.) of complete inhibition of growth measured from ditch containing antibiotic							
		Dihydrostreptomycin, 20 µg./ml.	Penicillin, 20 i.u./ml.	Chloramphenicol, 20 µg./ml.	Erythromycin, 20 µg./ml.	Carbomycin, 20 µg./ml.	Chlortetracycline, 20 µg./ml.	Oxytetracycline, 20 µg./ml.	Tetracycline, 20 µg./ml.
<i>Neisseria meningitidis</i>	Aerobic	8.0	26.0	15.0	16.0	5.0	10.0	15.5	5.0
<i>Haemophilus influenzae</i> type b	Aerobic	10.0	15.5	17.0	14.0	5.0	8.0	11.0	2.0
	Anaerobic	6.0	18.0	19.0	11.0	5.0	8.0	12.0	3.0
<i>Staphylococcus aureus</i>	Aerobic	8.0	22.0	11.0	15.5	9.5	10.0	12.0	6.0
	Anaerobic	3.0	24.0	12.0	17.0	11.0	11.5	13.0	6.0
<i>Klebsiella pneumoniae</i> strain 41	Aerobic	7.0	0.0	8.0	2.0	0.0	3.5	8.0	0.0
	Anaerobic	4.0	0.0	9.0	2.0	0.0	6.0	9.5	0.0
<i>Escherichia coli</i>	Aerobic	7.0	5.0	6.0	1.0	0.0	3.5	10.0	0.0
	Anaerobic	3.0	5.5	10.0	1.0	0.0	5.5	11.0	0.0
<i>Aerobacter aerogenes</i>	Aerobic	5.0	0.0	6.5	1.0	0.0	3.0	7.5	0.0
	Anaerobic	3.0	0.0	10.0	1.5	0.0	5.5	9.0	0.0
<i>Streptococcus pyogenes</i>	Aerobic	3.0	22.5	9.0	15.5	11.5	10.5	10.0	0.0
	Anaerobic	1.0	25.5	12.0	17.5	12.0	10.0	10.0	0.0
<i>Diplococcus pneumoniae</i>	Aerobic	3.0	24.0	14.0	22.0	13.0	10.5	15.0	5.0
	Anaerobic	1.0	27.0	15.0	22.0	14.0	12.5	16.0	6.0
<i>Clostridium welchii</i>	Anaerobic	0.0	19.0	11.0	15.0	10.0	13.5	14.0	12.0
<i>C. novyi</i>	Anaerobic	0.0	23.0	15.0	17.0	11.0	16.0	16.0	12.0

Each figure is the average of the results of at least three experiments.

they were growing aerobically than against the same organisms when growing anaerobically. This is the more significant when it is realized that facultative anaerobes grow at a slower rate under anaerobic as compared with aerobic conditions (Monod, 1942). No doubt the slower rate of growth of facultative anaerobes when growing anaerobically contributes to their greater sensitivity to the other antibiotics, tested under anaerobic as compared to aerobic conditions. The resistance to dihydrostreptomycin of the two species of obligate anaerobes used as controls, though not absolute, was found experimentally to be well beyond the limit at which any organism is regarded as sensitive to the antibiotics tested. Indeed, in the case of *C. welchii* an inoculum of one organism resisted the activity of dihydrostreptomycin 50 $\mu\text{g./ml.}$ (Table 3). Taken together these observations confirm previous reports on the mode of action of streptomycin and dihydrostreptomycin under anaerobic conditions (Robinson, Smith & Graessle, 1944; Bondi *et al.* 1946; Geiger *et al.* 1946; May *et al.* 1947; Grumbach, 1950; Lightbown, 1954). They also emphasize a difference, as yet undefined, between the mode of action of dihydrostreptomycin and that of the other antibiotics tested. This difference may explain the synergistic action of streptomycin when mixed with other antibiotics (see Demerec, 1948; Zinnemann, 1950).

The action of dihydrostreptomycin on Clostridium welchii

The resistance to dihydrostreptomycin of the two species of obligate anaerobes examined led to a fuller investigation of the factors underlying this resistance. The results are recorded in Tables 2 and 3. As observed with

Table 2. *Inhibition of growth of Clostridium welchii exposed to decreasing concentrations of dihydrostreptomycin*

Dihydro- streptomycin in ditch ($\mu\text{g./ml.}$)	Linear extent (mm.) of complete inhibition of growth, from culture diluted						
	1/1	1/10 ¹	1/10 ²	1/10 ³	1/10 ⁴	1/10 ⁵	1/10 ⁶
1000	0	1.5	2.0	2.5	3.0	3.0	6.0
500	0	2.0	2.0	2.5	3.0	3.0	4.0
200	0	0	1.0	1.0	1.0	1.0	2.5
100	0	0	0	0	0	0	1.0
50	0	0	0	0	0	0	1.0

Figures are average of three experiments.

numerous other organisms, the bactericidal concentration of dihydrostreptomycin for *Clostridium welchii* depends upon the inoculum size. The broth culture of *C. welchii* from which the various inocula were prepared contained approximately 30×10^8 viable organisms/ml. In the experiments with fluid media a Pasteur pipette delivering 29 drops/ml. was used, one drop constituting the inoculum; different dilutions were used to give inocula containing 1 to 1×10^8 organisms/drop. An inoculum of 1×10^6 organisms grew out in 1000 $\mu\text{g.}$ dihydrostreptomycin/ml. and an inoculum containing one organism grew out in 50 $\mu\text{g.}$ dihydrostreptomycin/ml. The results on solid media were similar.

Table 3. *Relationship of inoculum size of Clostridium welchii to concentration of dihydrostreptomycin*

Dihydro- streptomycin in medium ($\mu\text{g./ml.}$)	Growth of <i>C. welchii</i> when number of organisms in inoculum was				
	10^8	10^6	10^4	10^2	1
1000	+	+	—	—	—
500	+	+	—	—	—
200	+	+	—	—	—
100	+	+	+	+	—
50	+	+	+	+	+
25	+	+	+	+	+

+ = growth; — = no growth.

The possible use of dihydrostreptomycin as a selective agent for the isolation of Clostridium welchii

An inoculum as small as one *Clostridium welchii* organism resists the action of dihydrostreptomycin at a concentration which is normally lethal to most facultative anaerobes growing anaerobically, e.g. an inoculum of 10,000 organisms of *Escherichia coli* is killed by dihydrostreptomycin 4–16 $\mu\text{g./ml.}$, depending on the strains used, when growing anaerobically. The results recorded in Table 4 reveal, however, that dihydrostreptomycin was less efficient as a selective agent than heating at 65° for $\frac{1}{2}$ hr. Furthermore, unless high concentrations of dihydrostreptomycin are used the growth of *Proteus*

Table 4. *A comparison of the effectiveness of dihydrostreptomycin with that of heat treatment (65° for 30 min) for the isolation of Clostridium welchii from faeces*

Faeces sample no.	Degree of growth of <i>C. welchii</i> on subculture from heated cooked meat medium	Growth of <i>C. welchii</i> on subculture from unheated cooked meat medium containing dihydrostreptomycin $\mu\text{g./ml.}$				
		1000	500	200	100	50
1 to 6	3	0	nt	nt	nt	nt
7	3	nt	1	2	2	nt
8	0	nt	0	0	0	nt
9	3	nt	Few cols.	1	1	nt
10	0	nt	0	0	0	nt
11	3	nt	0	0	0	nt
12	3	nt	1	2	2	nt
13	3	nt	nt	0	1	1
14	3	nt	nt	0	2	3
15	3	nt	nt	2	3	3
16	3	nt	nt	1	1	3
17	Few cols.	nt	nt	0	0	0
18	3	nt	nt	0	1	1

3 = heavy; 2 = moderate; 1 = light; 0 = no growth of *C. welchii*; nt = not tested. After a few hours on the bench *Proteus vulgaris* appeared on the fresh blood agar plate inoculated from the dihydrostreptomycin-cooked meat broths; the dihydrostreptomycin had exerted only a bacteriostatic effect on it.

vulgaris and *Streptococcus faecalis* is not inhibited. As these organisms occur frequently, together with *Clostridium welchii*, in infections, any advantage that might be gained from the use of dihydrostreptomycin is lost in the presence of *Proteus vulgaris* and/or *Streptococcus faecalis*.

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REFERENCES

- BONDI, A., DIETZ, C. C. & SPAULDING, E. H. (1946). Interference with the anti-bacterial action of streptomycin by reducing agents. *Science*, **103**, 399.
- DEMEREK, M. (1948). Origin of bacterial resistance to antibiotics. *J. Bact.* **56**, 63.
- GEIGER, W. B., GREEN, S. R. & WAKSMAN, S. A. (1946). The inactivation of streptomycin and its practical applications. *Proc. Soc. exp. Biol., N.Y.* **61**, 187.
- GRUMBACH, A. (1950). Zum Wirkungsmechanismus der Antibiotica. *Schweiz. Z. Path.* **13**, 586.
- LIGHTBOWN, J. W. (1954). An antagonist of streptomycin and dihydrostreptomycin produced by *Pseudomonas aeruginosa*. *J. gen. Microbiol.* **11**, 477.
- MACKIE, T. J. & MCCARTNEY, J. E. (1953). *Handbook of Practical Bacteriology*, 9th ed., p. 514. Edinburgh: E. and S. Livingstone, Ltd.
- MAY, J. R., VOUREKA, A. E. & FLEMING, A. (1947). Some problems in the titration of streptomycin. *Brit. Med. J.* **i**, 627.
- MONOD, J. (1942). *La Croissance des cultures bactériennes*, pp. 87–96. Paris: Hermann et Cie.
- ROBINSON, H. J., SMITH, D. G. & GRAESSLE, O. E. (1944). Chemotherapeutic properties of streptomycin. *Proc. Soc. exp. Biol., N.Y.* **57**, 226.
- STOKES, E. J. (1955). *Clinical Bacteriology*, p. 160. London: Edward Arnold, Ltd.
- ZINNEMANN, K. (1950). Sulphonamide sensitivity of *H. influenzae* strains with special reference to the combined use of antibacterial drugs. *Brit. Med. J.* **ii**, 705.

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