EFFECTS OF PENICILLIN, STREPTOMYCIN, AND TETRACYCLINE ON *N. GONORRHOEAE* ISOLATED IN 1944 AND IN 1957*†

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N. gonorrhoeae is a species which was originally highly sensitive to penicillin (Cohn and Seijo, 1944; Frisch, 1944; Carpenter, Bahn, Ackerman, and Stokinger, 1945; Lankford, 1945; Meads, Ory, Wilcox, and Finland, 1945; Romansky, 1946; Kenedy and Alföldy, 1947; Romansky and Robin, 1947; Hughes and Carpenter, 1948; Hawks and Greey, 1948; Cohn, Grunstein, Goldberg, and Crane, 1949; Mills, 1949; Storck, Rinderknecht, and Fries, 1949; Zierz and Eckert, 1949); however, by serial subcultivation on media containing increasing concentrations of penicillin (Bahn, Ackerman, and Carpenter, 1945; Carpenter and others, 1945; Miller and Bohnhoff, 1945; Mills, 1949), the sensitivity can be reduced. In accordance with earlier findings with many other micro-organisms, it is rather difficult to increase the *in vitro* resistance to penicillin compared with, for example, streptomycin (Miller and Bohnhoff, 1945, 1946).

When the present investigations were started, only a few reports indicated the *in vivo* and *in vitro* existence of strains with a decreased sensitivity to penicillin (Franks, 1946; Duemling and Horton 1947). It was generally agreed, however, that very resistant strains did not occur (Dowling, 1954; Gartmann and Knapp, 1954; Marcuse and Hussels, 1954; Mark, 1954; Fischer, 1955; Del Love and Finland, 1955; Finland, 1955, 1958; Lodin, 1956a,b; World Health Organization, 1957a,b; Marchionini and Röckl, 1957).

Some authors had reported a change in the distribution of the *in vitro* concentrations necessary for inhibition or killing of *N. gonorrhoeae* (Gocke, Wilcox, and Finland, 1950; Schreus and Schümmer,

1951; Schümmer and Hubbes, 1951; Storck, Rinderknecht, and Flury, 1951; Schreus, Schümmer, Gahlen, and Jaeckel, 1953; Siebert, 1953); none of these papers reported simultaneous tests of strains isolated before and during the penicillin era.

Until late in 1955 only two-fold or four-fold increases of the "usual" inhibitory *in vitro* concentrations had been observed in the Gonococcal Department of this Institute. About that time, four gonococcal strains were isolated from a case* of uncomplicated gonorrhoea, which was refractory to repeated "ordinary" penicillin treatment. All four strains showed a penicillin sensitivity twenty times less than usual. The strains were tested by means of a modified agar-cup technique (Bang, to be published), a plate dilution method, and a tablet method, all the results being in agreement. The lowered sensitivity was retained on subculture. After "ordinary" penicillin treatment, the patient's serum showed penicillin levels similar to those commonly observed. Penicillinase formation could not be shown by the *B. subtilis* method (Gots, 1945).†

The above case and the increasing use of oral penicillin together with the rising number of allergic cases made it desirable to review the sensitivity pattern of gonococci to penicillin. The remarkable constancy of the incidence of gonorrhoea in spite of the wide use of penicillin is a further indication of the need for such a study to be made.

In a preliminary experiment, some 300 strains isolated in 1956 were tested with penicillin by the tablet method (see below). About 10 per cent. of the strains appeared to possess a somewhat reduced sensitivity in comparison to most of the strains

(Reyn, 1957). Hence, it was decided to compare the sensitivity of strains isolated in 1944 with that of strains isolated in 1957. Sensitivity to streptomycin and tetracycline was also determined.

The connexion between the *in vitro* and the *in vivo* results was investigated by discriminating between strains sent in for diagnostic purposes only and strains with a request for drug-sensitivity determina• tion. An evaluation of the two methods chosen for the experiments was also undertaken.

Material and Methods

Diagnostic Criteria.—The following criteria were chosen. The organisms must be Gram-negative diplococci forming typical colonies on McLeod's medium enriched with 33 per cent. ascitic fluid. They must be oxidase-positive and form acid on 1 per cent. glucose ascitic agar plates and no acid on either 1 per cent. maltose or 1 per cent. laevulose ascitic agar plates. A few strains from 1957 did not ferment glucose; in these cases the diagnosis was supported by either complement-fixation tests or by the absence of growth at 22°C. on plain nutrient agar plates.

Strains

(1) *Ninety Strains isolated in 1944.*—These came from ninety patients and had been stored as lyophilized cultures.

(2) *103 Strains isolated in 1957.*—These came from 101 patients and were picked out at random from among the routine diagnostic cultures. Two strains from each were included in the case of two patients. Both had sensitive strains at first, but the second isolations showed decreased sensitivity.

(3) *103 Strains also isolated in 1957.*—These were isolated from 96 patients, and a request for drug-sensitivity determination had been sent in with the specimen. Included in this series are thirteen strains from six patients. These thirteen strains showed the same distribution of sensitivity as the whole group. Six were strains from the original specimens, six were from second isolations, and one was a third specimen from one of these cases. In three patients the second specimens were significantly different in sensitivity; the first specimen showed reduced sensitivity, but the second was sensitive. This suggested re-infection.

(4) *27 "Special" Strains isolated in 1956.*—These showed different degrees of sensitivity as measured by the tablet method.   ..

(5) *Three Control Strains*

    (*a*) No. 750: isolated before 1944; very sensitive to penicillin.

    (*b*) No. 50700: isolated in 1955 from the treatment-refractory case cited above.

    (*c*) No. 30603: isolated in 1956; showed "normal" sensitivity in preliminary tablet tests.

All these strains had been kept in a lyophilized state and were stored in this way between the different experimental series. On subculturing, which was carried out on

control plates without any added antibiotic, care was taken to avoid selection.

Media.—Routine cultures were obtained on McLeod's heated horseblood ("chocolate") agar plates enriched with 33 per cent. ascitic fluid. Lyophilized cultures were made from 25 per cent. ascitic broth. The sensitivity tests were carried out on McLeod's chocolate agar plates enriched with 10 per cent. ascitic fluid, but containing no peptone, the latter being omitted because an occasional sulphonamide determination was carried out on this medium.

Antibiotics

Tablet Method.—The tablets were prepared by R.M.C. (Roskilde Medical Company, Denmark) (Lund, Funder-Schmidt, Christensen, and Dupont, 1951; Lund, 1953). the following doses were employed: 15 μg. crystalline penicillin G (25 units), 3 μg. streptomycin and tetracycline 1 μg. The plates were inoculated by flooding the surface with a saline suspension of about 2×10^8 organisms per ml. (applied by means of a Pasteur pipette). The plates were tilted to distribute the suspension, the superfluous fluid was sucked off, and the tablets were put on the plates at once. Petri dishes 9 to 10 cm. diameter were used. They were incubated at 36–37°C. for 24 hrs in an atmosphere of 8 per cent. CO_2. As a rule two or three tablets were placed on each plate; duplicate tests were often performed.

The diameters of the inhibition zones were measured in millimetres. When the growth was heavy, the limits of the zones were clearcut; when the growth was scanty, the limits were blurred and delicate growth was observed within the "zone" itself. The zones were read where the sharpest change from normal to inhibited growth could be seen. Rings of thickening were often noticed about 5 mm. outside the edge of the zone and concentric with it. These were presumably due to heavier growth; this was rather frequent with strains giving a scanty growth. In the present material most of the strains grew abundantly although there were some poorly growing strains, especially among the most recently isolated cultures. The reproducibility of the results is discussed below.

Plate Dilution Method.—Two-fold dilutions of the following three antibiotics were added to the medium:

    (1) Penicillin from 0·0024 μg. (0·004 units) to 0·6 μg. (1·00 units) per ml.

    (2) Streptomycin from 0·25 μg. to 128 μg. per ml.

    (3) Tetracycline from 0·08 μg. to 42·7 μg. per ml.

Thus nine plates + one control without antibiotic were used for each determination. To avoid heat-destruction, care was taken to cool down the agar as much as possible before the drugs were added to the medium. Six strains were tested on each set of plates. The inoculum was about 5×10^6 organisms spread on a square centimetre. Confluent growth resulted after incubation for 48 hrs at 36–37°C. (the first 24 hrs with 8 per cent. CO_2, the last 24 hrs without added CO_2). The growth on the control plates was read as 4 plus and the

decreasing degrees of growth on the other plates were read as 3 plus, 2 plus, and 1 plus. Four plus was counted as 100 per cent., 3 plus as 75 per cent., and so on.

The 50 per cent inhibitory concentrations were calculated by means of the *Kärber* method (Finney, 1947). The $\log_{10}$ values corresponding to the reciprocal dilutions (the plates with the highest concentrations giving a $\log_{10}$ value of zero) were used for the determination of the variances, see below.

The dose response curves were rather steep and 100 per cent. inhibition was generally obtained at a concentration 50 per cent. higher than that giving 50 per cent. inhibition; the latter value could be more accurately estimated than the 100 per cent. value.

Experimental Series.—The main experiments were carried out in five periods:

(1) *December, 1956*, to *February, 1957.*—Experiments with penicillin were performed in which the medium was varied in a way which would be unavoidable in practice.

The three control strains + three sets of nine "special" strains, which were selected after the preliminary tablet experiments, were repeatedly tested on 12 experimental days in all, according to the plan shown in Table I.

TABLE I

PLAN OF EXPERIMENTS WITH DELIBERATE MEDIA VARIATION TABLET METHOD AND PLATE DILUTION METHOD

Day	Broth	Horse Blood	Controls	Strains "Special" Strains		
				Set 1	Set 2	Set 3
1	A	a	3	9		
2	A	a	3		9	
3	A	a	3			9
4	A	b	3	9		
5	A	b	3		9	
6	A	b	3			9
7	B	c	3	9		
8	B	c	3		9	
9	B	c	3			9
10	B	d	3	9		
11	B	d	3		9	
12	B	d	3			9

The medium was varied by using two different batches of broth (A and B) and four different portions of blood from the same horse (a, b, c, d).

The same ascitic fluid and agar batch were used in these experiments. In our routine work ascitic fluid from some particular patient is used for long periods.

(2) *March to May, 1957.*—In this period ninety strains isolated in 1944 were tested against penicillin.

(3) *June to July, 1957.*—103 strains picked out at random from the strains isolated early in 1957 and for which no drug-sensitivity determination was requested.

(4) *September to October, 1957.*—In this period experiments were made with 103 strains from 1957 for which drug-sensitivity determination had been requested.

(5) *May, 1958.*—88 of the ninety strains from 1944 were tested against streptomycin and tetracycline, by the plate dilution method only.

Results

Evaluation of Variability of Methods

Variation within the Single Experimental Series

Plate Dilution Method.—Duplicate tests on single experimental days were not performed, but the three control strains were tested every day in all this work. The 27 "special" strains were repeatedly tested in the preliminary experiments. From these results were estimated the variances (s^2) for the three antibiotics used (Table II).

TABLE II

VARIANCE OF PLATE DILUTION METHOD

Experiment		1	2	3	4
Date		Dec. 56–Feb. 57	March May 57	June July 57	Sept. Oct. 57
Strains		(1) Control (2) "Special"	Control	Control	Control
Peni-cillin	Variance ..	(1) 0·0052 (2) 0·0032	0·0055	0·0003	0·0142
	Standard deviation	(1) 0·072 (2) 0·057	0·074	0·017	0·119
	Degrees of freedom	(1) 30 (2) 78	17	15	14
Strepto-mycin	Variance ..	—	0·0115	—	0·0136
	Standard deviation	—	0·107	—	0·117
	Degrees of freedom	—	17	—	15
Tetra-cycline	Variance ..	—	0·0028	—	0·0016
	Standard deviation	—	0·053	—	0·040
	Degrees of freedom	—	17	—	15

In the case of penicillin no variations in common could be demonstrated for the three strains during each of the experimental periods, except in the first series, where the media were varied on purpose. Estimates of the standard deviations were made from only two experimental periods with streptomycin and tetracycline. The standard deviations (s) varied to a rather high degree from one experimental series to the other. This variability was probably due to the fact that the intervals between the concentrations from one plate to the next one were great compared with the magnitudes of the standard deviations: with penicillin they varied about 0·075 $\log_{10}$ values or about one-fourth of a two-fold dilution step; those obtained with streptomycin and with tetracycline varied about 0·11 and 0·05 $\log_{10}$ values respectively or about one-third and one-sixth of a two-fold dilution step; with tetracycline they appeared to be very low and distinct day-to-day variations were observed.

Tablet Method.—In most of the experiments two tablets of each drug were employed; the variances based on these duplicates varied from 0·5 to 1·0 mm. It was possible to

demonstrate a day-to-day variation in the September to October, 1957 experiments, but only with streptomycin and tetracycline.

The control and the "special" strains were repeatedly tested on different days and the mean results from two tablets were used to estimate the variances, which were found to vary from 1 to 4 mm. with penicillin and from about 0·5 to 3 mm. with streptomycin and tetracycline. The standard deviation was about 1·5 mm. with penicillin and about 1·2 mm. with the other two drugs.

In plotting the values for the plate dilution tests against values for the tablet tests, it was seen that a difference of 1 mm. corresponded to a concentration difference of about 0·1 $\log_{10}$ values. Thus, the tablet method (using two tablets per day) had a somewhat greater standard deviation than the dilution method. The standard deviation with penicillin and tetracycline was about twice as high as that found with the dilution method, whereas the increase was only slight with streptomycin. However, the estimates for streptomycin and tetracycline were based on rather few degrees of freedom.

Variation of the Control Strains from One Period to Another

As already mentioned the control strains were the same in all the experiments; they were chosen so as to cover the range from the highest to the lowest degree of sensitivity which had been encountered when the experiments were started.

Penicillin.—The average results from the various experimental periods showed that the control strains did not vary in any constant way from one period to the other. By the dilution method only Strain 750 showed an appreciable degree of variation, *i.e.* 0·17 $\log_{10}$ values or about one-half of a dilution step; by the tablet method Strain 30603 showed the greatest variation.

Streptomycin.—The greatest difference was found for Strain 30603 in the dilution method (0·255 $\log_{10}$ values).

Tetracycline.—The three strains varied in parallel, but the differences went in opposite directions with the two methods, being about 2·5 mm. with the tablet method and about 0·15 $\log_{10}$ values with the dilution method.

It is concluded that no major variation occurred from one experimental period to the other, and this means that the comparison of results from two different periods is valid.

The control strains were included in the experiments for the purpose of adjusting any possible variation from one day to the next and from one series to the next. Adjusting factors can be achieved from the difference between the actual results and average ("standard") results, calculated from repeatedly testing the control strains. By the use of

one control strain only, the variance of the adjusted values would be increased by 40 per cent., but by using three control strains, the variance would only be increased by 15 per cent.

With tetracycline the observed variations from one day to another were of such an order of magnitude that the three control strain values were used to correct the results within one of the experimental periods. With penicillin and streptomycin no appreciable day-to-day variations were observed; hence the results were not corrected.

Comparison between the Dilution Method and the Tablet Method

Penicillin.—As shown in the next section, the distribution of the strains, arranged according to their quantitative sensitivities in the dilution method, was found to have two distinct peaks. The material was divided into four groups, dividing each of the two "peaks" into two parts of about equal size. The results obtained with the tablet method were divided into two groups based on some preliminary results not reported here. All the results obtained with the strains from 1957 are compiled in Table III.*

TABLE III

PENICILLIN
201 strains from 1957

50 per cent. Inhibitory Concentrations (μg./ml.)	Zone Diameters (mm.)				Total
	$\geqslant$35		<35		
	No.	Per cent.	No.	Per cent.	
<0·010	59	43	1	2	60
0·010–0·035	73	53	4	6	77
0·036–0·150	5	4	13	20	18
>0·150	0	0	46	72	46
Total ..	137	100	64	100	201

A division of the results of the tablet test into zones $\geqslant$35 mm. and <35 mm. corresponds fairly well with a division of the results of the dilution test into 50 per cent. inhibitory concentrations < > 0·036 μg. per ml.

When these strains with zones <35 mm. and 50 per cent. inhibitory concentrations >0·036 μg. per ml. were classified as "less sensitive" and the rest as "sensitive", disagreement between the two methods was observed in only ten out of 201 cases.

Streptomycin and Tetracycline.—These results were not distributed in such a way that a classification into four groups according to their sensitivities naturally presented itself. Hence, the results obtained with both methods were divided into two fairly equal groups as shown in Tables IV and V (opposite).

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* Because of missing data the total numbers vary from 200 to 203 in Tables III, IV, V, VI, and VIII, and in Figs 1, 2, 3, and 4.

TABLE IV

STREPTOMYCIN
201 strains from 1957

50 per cent. Inhibitory Concentrations (μg./ml.)	Zone Diameters (mm.)				Total
	⩾36		<36		
	No.	Per cent.	No.	Per cent.	
<3·7	68	78	39	34	107
>3·7	19	22	75	66	94
Total ..	87	100	114	100	201

TABLE V

TETRACYCLINE
202 strains from 1957

50 per cent. Inhibitory Concentrations (μg./ml.)	Zone Diameters (mm.)				Total
	⩾36		<36		
	No.	Per cent.	No.	Per cent.	
<0·6	63	65	47	45	110
>0·6	34	35	58	55	92
Total ..	97	100	105	100	202

With streptomycin the 50 per cent. inhibitory concentrations were >3·7 μg. per ml. in 75 (66 per cent.) strains out of 114 strains with zone diameters <36 mm.; for the remaining 87 strains with zones ⩾36 mm. only nineteen (22 per cent.) showed values >3·7 μg. per ml. Thus a positive correlation between the methods was observed.

Some correlation, though weaker, was found with tetracycline; here 58 (55 per cent.) strains out of 105 strains with zone diameters <36 mm. showed 50 per

cent. inhibitory concentrations >0·6 μg. per ml., whereas this was the case for only 34 (35 per cent.) of the rest of the strains with zones ⩾36 mm.

Comparison between the Sensitivities of the 1944 and 1957 Strains

As mentioned above, the results obtained with the two methods were in good agreement. Only the dilution method which gave the most reproducible results is discussed in this section.

Fig. 1 shows the distribution of ninety strains isolated in 1944 in relation to the 50 per cent. inhibitory *penicillin* concentrations. The distribution is "normal", with the peak corresponding to 0·01 μg. per ml. with a range from 0·0038 to 0·024 μg. per ml. (6-fold).

Fig. 2 (overleaf) shows the distribution of 103 strains from 1957 which were sent in for diagnostic purposes only. Here two peaks are observed, one at 0·01 μg. per ml. as in Fig. 1, and one at about 0·2 μg. per ml. The range is from 0·0055 to 0·43 μg. per ml. (80-fold). Fig. 2 also shows the distribution of 100 strains which were sent in with a request for sensitivity determination (the requests mainly concerned sensitivity to penicillin). Here, also, two peaks are seen, but the peak at about 0·20 μg. is larger than that seen above. The range is from 0·0028 to 0·50 μg. per ml. (180-fold).

Among strains sent in for diagnosis only, the incidence of 50 per cent. inhibitory values >0·036 μg. per ml. is 17 per cent., as against 46 per cent. among strains sent in for sensitivity determination. This difference is significant and demonstrates the correlation between clinical treatment failure and *in vitro* "resistance".

Of the ninety strains isolated in 1944, 88 were tested with *streptomycin*. The distribution of 50 per cent. inhibitory concentrations was "normal" (range from 3 to 11 μg. per ml.). The distribution of streptomycin

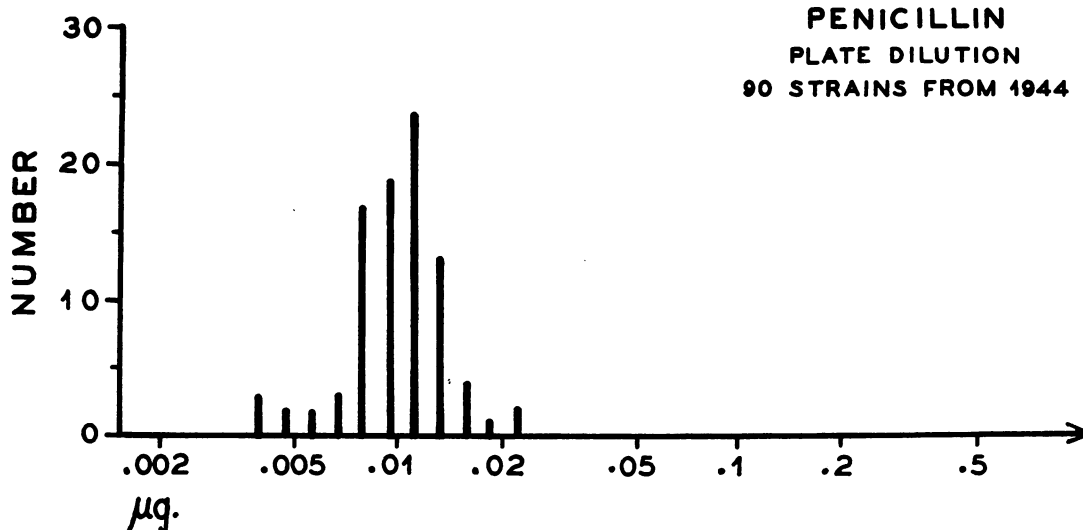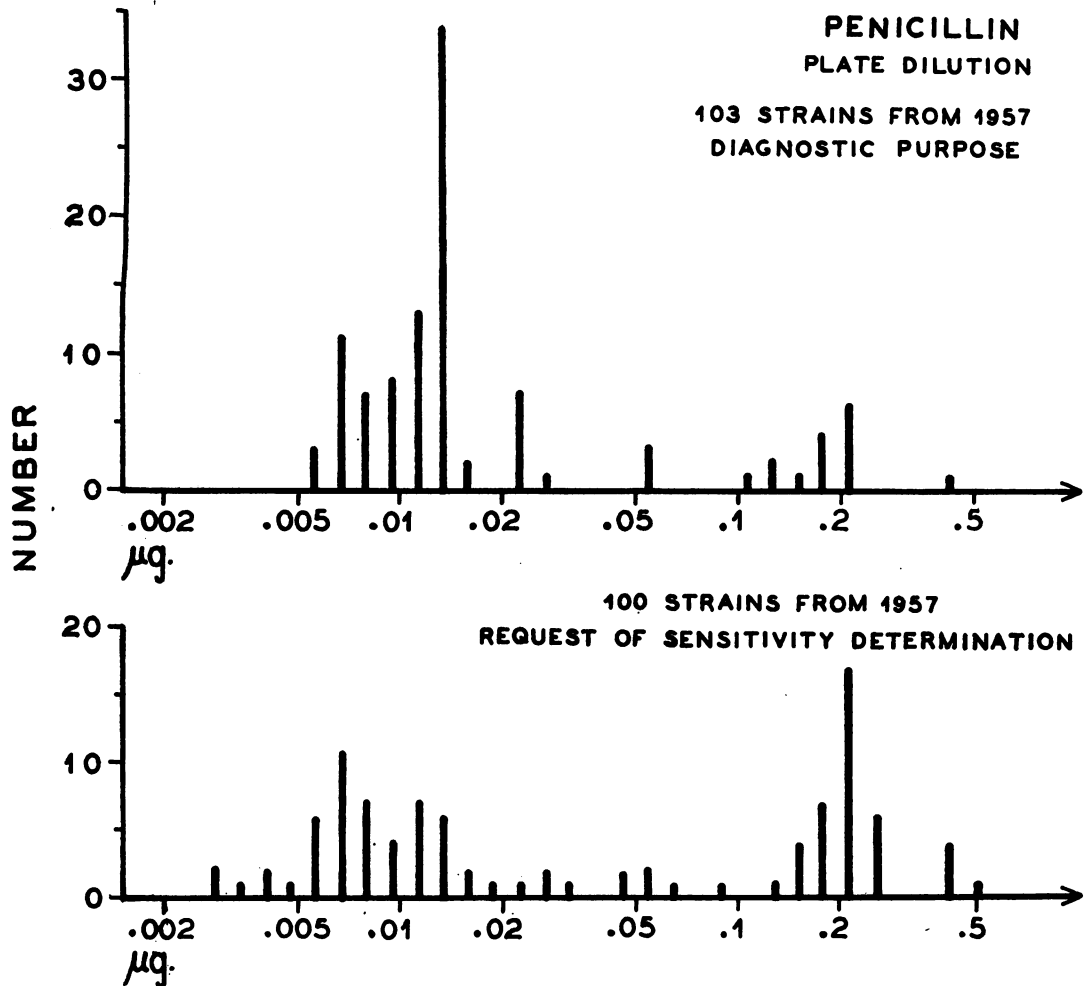## PENICILLIN
PLATE DILUTION
90 STRAINS FROM 1944

FIG. 1.

FIG. 2.

sensitivity for 103 strains sent in for diagnosis only in 1957 is shown in Fig. 3 (opposite); within a narrow range (from 1·3 to 7·0 μg. per ml.) two peaks are seen at 3 and 6 μg. per ml. respectively. One strain was very resistant, growing on a medium containing 2,048 μg. per ml.

The distribution of 100 strains sent for sensitivity determination in 1957 is shown in Fig. 4 (opposite); here also, there are two peaks, but the peak at 6 μg. per ml. is higher than that seen in Fig. 3. The range is from 2 to 10 μg. per ml., and three very resistant strains were found above that range, which grew abundantly on media containing 2,048 μg. per ml. The incidence of 50 per cent. inhibitory concentrations >3·7 μg. per ml. was here significantly greater than that observed for strains sent in for diagnosis only (65 per cent. ~ 38 per cent.).

With *tetracycline* the range for the 1944 strains was very narrow (from 0·4 to 0·9 μg. per ml.); the distribution was "normal".

All the 1957 strains showed one peak distributions at about 0·6 μg. per ml. The incidence of 5 per cent. inhibitory concentrations > 0·6 μg. per ml. was greater for strains sent in for sensitivity determination (56 per cent. ~ 45 per cent.), but the difference was not significant. The values ranged from 0·1 to 1 μg. per ml. (10-fold).

Comparison between the Different Antibiotics

In order to show any correlation between the inhibitory effects of these drugs the results were grouped, and the sensitivity of all the strains were compared for two of the drugs at a time.

(1) *Tablet Method.*—No comparisons were made with the 1944 strains, as these were not tested for sensitivity to streptomycin and tetracycline. The results of the three
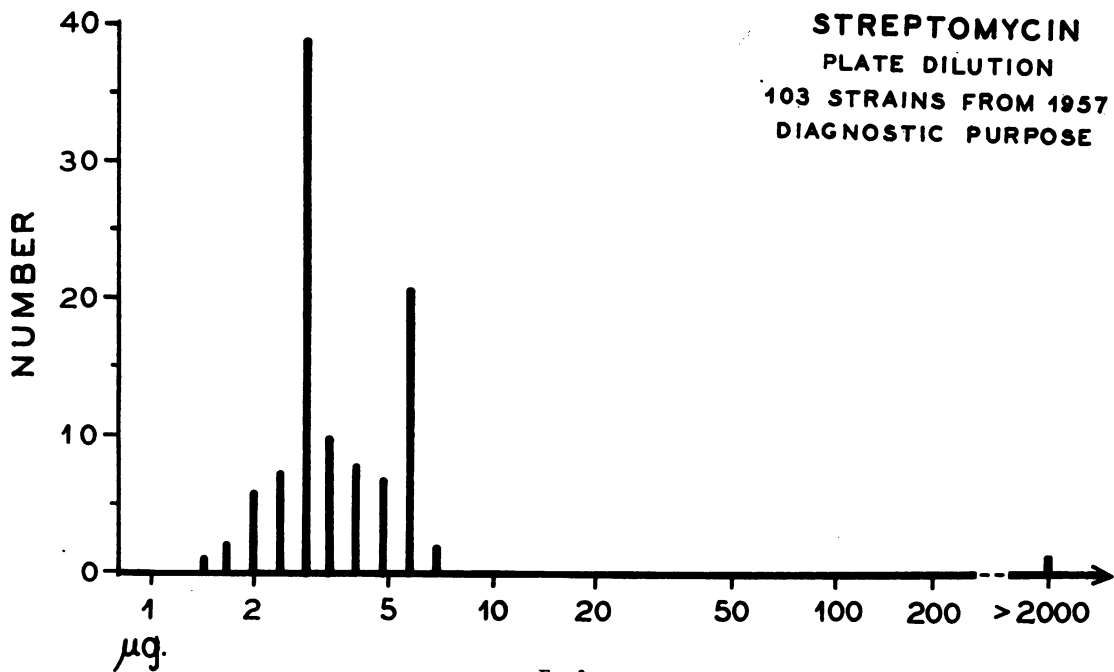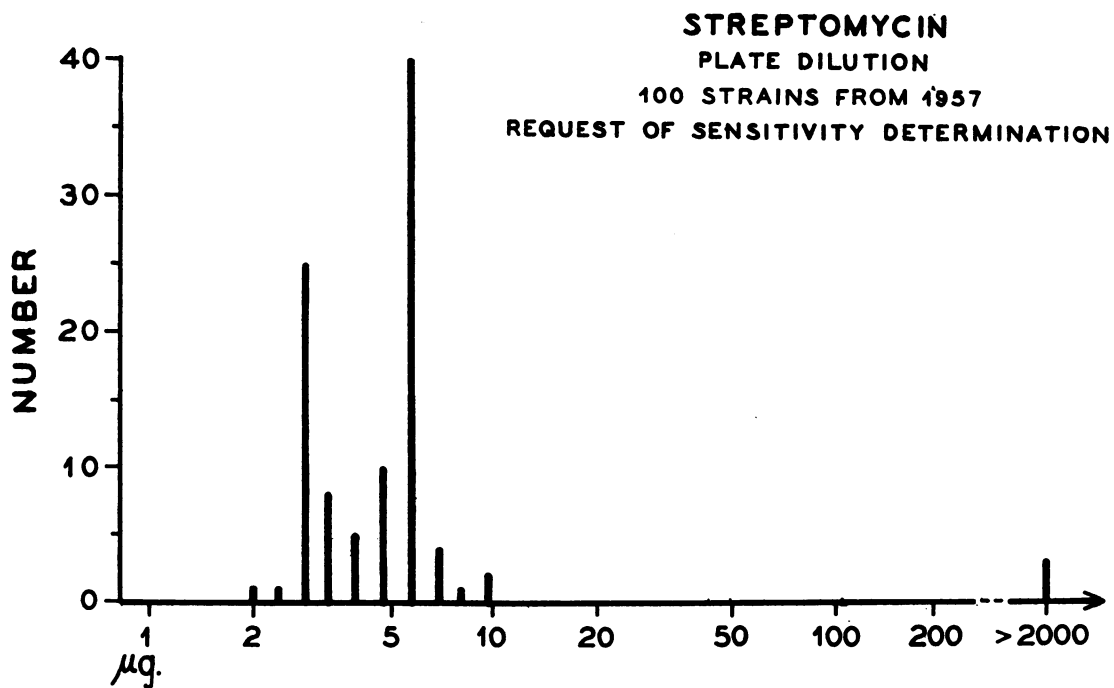
FIG. 3.

FIG. 4.

TABLE VI

200 STRAINS FROM 1957
TABLET METHOD.
Zone Diameters (mm.)

Penicillin	Streptomycin					Tetracycline				
	≥36		<36		To-tal	≥36		<36		To-tal
	No.	Per cent.	No.	Per cent.		No.	Per cent.	No.	Per cent.	
≥45	30	97	1	3	31	30	97	1	3	31
35–44	55	52	50	48	105	51	49	54	51	105
<35	1	2	63	98	64	15	23	49	77	64
Total ..	86		114		200	96		104		200

Streptomycin	Tetracycline				
	≥36		<36		Total
	No.	Per cent.	No.	Per cent.	
≥36	69	80	17	20	86
<36	27	24	87	76	114
Total ..	96		104		200

TABLE VII

88 STRAINS FROM 1944
PLATE DILUTION
50 per cent. Inhibitory Concentrations (µg./ml.)

Penicillin	Streptomycin					Tetracycline				
	<3·7		>3·7		To-tal	<0·6		>0·6		To-tal
	No.	Per cent.	No.	Per cent.		No.	Per cent.	No.	Per cent.	
<0·010	2	4	44	96	46	26	57	20	43	46
>0·010	10	24	32	76	42	13	31	29	69	42
Total ..	12		76		88	39		49		88

Streptomycin	Tetracycline				
	<0·6		>0·6		Total
	No.	Per cent.	No.	Per cent.	
<3·7	3	25	9	75	12
>3·7	36	47	40	53	76
Total ..	39		49		88

comparisons for the 1957 strains are given in Table VI. The penicillin and streptomycin results are obviously positively correlated; 98 per cent. of the strains with penicillin zone diameters of <35 mm. belong to the group with streptomycin zones <36 mm., whereas only 2 per cent. of the penicillin zone diameters ≥45 mm. fall into this group. The tetracycline results are also positively correlated with the penicillin results; 77 per cent. of the strains with penicillin zones <35 mm. give tetracycline zones <36 mm., whereas only 4 per cent. of the penicillin zones ≥45 mm. fall into this group.

The streptomycin and tetracycline values were also positively correlated; 76 per cent. of the strains with streptomycin zones <36 mm. give tetracycline zones <36 mm., against 20 per cent. of the strains with streptomycin zones ≥36 mm.

All these correlations are statistically significant.

(2) Dilution Method

(a) 1944 Strains.—Altogether 88 strains were tested with all three drugs. Each of the three sets of results was divided into two groups using the same intervals as those used for the results obtained from the 1957 strains. The classifications turned out to be convenient, dividing the material into groups of nearly equal size, except in the case of streptomycin where the part with low inhibitory concentrations turned out to be rather small (Table VII). This means that the distribution in 1944 was slightly different from that in 1957.

The penicillin and streptomycin values were negatively correlated, i.e. low streptomycin values were significantly more frequent when the penicillin values were high than when they were low (24 per cent. ~ 4 per cent.).

The penicillin and tetracycline values, however, were positively correlated, i.e. low tetracycline values were more frequently associated with low penicillin values

than with high penicillin values (57 per cent. ~ 31 per cent.).

The streptomycin and tetracycline values were found to be negatively correlated, but this finding was not statistically significant.

(b) 1957 Strains.—With penicillin, four groups were formed: <0·010, 0·010–0·035, 0·036–0·150, and >0·150 µg. per ml., and the streptomycin and tetracycline series were divided into two parts each: streptomycin <>3·7 µg. per ml. and tetracycline <>0·6 µg. per ml. (Table VIII, opposite).

As with the tablet method, the penicillin and streptomycin values were positively correlated, but in contrast to the results obtained with the 1944 strains, the incidence of penicillin values <>0·010 µg. per ml. was equal for high and low streptomycin values. It is worth mentioning that one of the strains which was very resistant to streptomycin was inhibited by only 0·04 µg. per ml. penicillin.

Comparison of penicillin with tetracycline showed a similar but not significant tendency; 52 per cent. of the high tetracycline values were associated with penicillin values >0·036 µg. per ml. as opposed to 42 per cent. associated with penicillin concentrations <0·036 µg. per ml.

The streptomycin and tetracycline values were also positively correlated; i.e. 57 per cent. of the high tetracycline values were found among the high streptomycin values and only 33 per cent. of the high values among the low streptomycin values. The correlation was statistically significant.

Discussion

Although the tablet and dilution methods used in this work corresponded fairly well, the dilution

TABLE VIII

203 STRAINS FROM 1957
PLATE DILUTION
50 per cent. Inhibitory Concentrations (μg./ml.)

Penicillin	Streptomycin					Tetracycline				
	<3·7		>3·7		To-tal	<0·6		>0·6		To-tal
	No.	Per cent.	No.	Per cent.		No.	Per cent.	No.	Per cent.	
<0·010	43	69	19	31	62	80	58	59	42	139
0·010–0·035	53	69	24	31	77					
0·036–0·150	2	11	16	89	18	31	48	33	52	64
>0·150	1	2	45	98	46					
Total ..	99		104		203	111		92		203

Streptomycin	Tetracycline				Total
	<0·6		>0·6		
	No.	Per cent.	No.	Per cent.	
<3·7	66	67	33	33	99
>3·7	45	43	59	57	104
Total ..	111		92		203

method is preferred both for its higher degree of accuracy and because it is supposed to be less influenced by growth conditions. Hence, only the results obtained with the dilution method are discussed.

Penicillin.—The first reports describing a tendency to a change in the distribution of the sensitivity pattern appeared about 1950 (Gocke and others, 1950; Storck and others, 1951; Schreus and Schümmer, 1951).

This altered distribution pattern was considered a sign of decreasing sensitivity of some gonococcal strains and eventually it was found that relapses were correlated with the isolation of strains of reduced sensitivity (Schreus and others, 1953). A few early papers contained reports on very resistant strains, but later similar findings were not reported. Most recent reports (Dressler and Gumpesberger, 1955; Finland, 1955; Terrial and Chabbert, 1955; Wiesmann, 1955; Flodén, 1956; Lodin, 1956a,b; Marchionini and Röckl, 1957; Finland, 1958) conclude that *N. gonorrhoeae* is still very sensitive to penicillin. Thayer, Field, Magnuson, and Garson (1957), Thayer, Perry, Magnuson, and Garson (1957), and Thayer, Perry, Field, and Garson (1957) found the mean inhibitory dose of penicillin to be from one and a half to ten times greater than that reported by Lankford (1945) and by Del Love and Finland (1955).

In the present investigation, gonococcal strains isolated in 1944 and in 1957 were tested for sensitivity to penicillin by two different methods.

The range of sensitivity was about 6-fold for the 1944 strains, and by use of the dilution method these strains were found to be normally distributed with the peak at about 0·01 μg. per ml.; this is in agreement with previous findings (Cohn and Seijo, 1944; Frisch, 1944; Carpenter and others, 1945; Lankford, 1945; Meads and others, 1945; Hughes and Carpenter, 1948; Mills, 1949; Storck and others, 1949; Zierz and Eckert, 1949). The 1957 strains were divided into two groups: those sent in for routine testing, and those sent in with a request for drug-sensitivity determination. In the strains sent in for diagnostic purposes only, two peaks were observed, one at about 0·01 μg. per ml. and one at about 0·2 μg. per ml., the range here being 80-fold; in the strains sent in for sensitivity testing there were two similar peaks, but the one at 0·2 μg. per ml. was greater and the range was 180-fold. Thus, a correlation was found between the clinical results and the results *in vitro*; among the strains for sensitivity determination the incidence of 50 per cent. inhibitory concentrations >0·036 μg. per ml. was significantly greater than that found in strains for diagnosis (46 per cent. ~17 per cent.) The highest concentration needed for complete inhibition was 0·75 μg per ml.

Quite recently, Cradock-Watson, Shooter, and Nicol (1958) found that 19 per cent. of 200 gonococcal strains isolated in 1957–58 needed a penicillin concentration more than ten times as high as had been commonly used before, and that some needed 0·307 μg. per ml. for *in vitro* inhibition. The *in vitro* findings corresponded to the *in vivo* findings and, ignoring the possibility of re-infection, it was found than thirteen of 38 patients (34 per cent.) infected with "resistant strains" relapsed, as opposed to only twelve of the 162 patients (7 per cent.) infected with sensitive strains. Schamberg, Kalodner, and Lentz (1958) recently reported a case of female gonorrhoea refractory to repeated treatment with long-acting penicillin. The corresponding strain was resistant to 0·12 μg. per ml. and inhibited by 0·2 μg. per ml. Curtis and Wilkinson (1958) examined 302 strains of gonococci obtained before penicillin treatment; 19·5 per cent. of these strains showed *in vitro* sensitivity to between 0·075 and 0·3 μg. per ml. King (1958) found that 19 per cent. of 115 gonococcal strains showed minimal inhibitory concentrations ⩾0·075 μg. per ml. The clinical outcome showed a correlation between increased resistance and failure of treatment. At a WHO seminar held in Tokyo in March, 1958, failure-rates of up to 30 per

cent. were noted with higher doses than those which had given 95 per cent. success only a few years ago (W.H.O., 1958).

These results agree with our *in vitro* findings and with the clinical results reported by Jensen (1957), who found that, among 181 penicillin-treated cases of gonorrhoea in 1956, 155 of the 181 corresponding strains were sensitive when tested by the tablet method, whereas 26 showed decreased sensitivity (see below). Thirteen of the "relapses" were found among the 155 sensitive strains (8 per cent.), whereas five "relapses" were found among the 26 strains with decreased sensitivity (19 per cent.).

A tendency to two-peak distributions similar to those described in this paper for the 1957 strains is mentioned in recent papers dealing with the changed resistance of gonococci to penicillin (Cradock-Watson, Shooter and Nicol, 1958; King, 1958; Curtis and Wilkinson, 1958). In these reports, also, the ranges between the two "peaks" are said to be about 20-fold. This indicates a stepwise change in sensitivity to penicillin and supports the assumption that resistance to penicillin may develop as an "obligatory multiple-step resistance" (Welsch, 1955).

Streptomycin.—The first reports of the effect of streptomycin on gonococci were those of Miller and Bohnhoff (1945) and Mortara and Saito (1946); Miller and Bohnhoff found that gonococci were inhibited within a 5-fold range (8 to 40 units). The *in vitro* resistance could be increased to 7,500 units per ml. after only four to six subcultures in media with increasing concentrations of streptomycin, but the resistance to penicillin was not increased at the same time.

Streptomycin has not been much used for the treatment of gonorrhoea, although several authors agree that streptomycin can cure more than 90 per cent. of cases of acute gonorrhoea (Chinn, Putnam, Taggart, and Herwick, 1947; Pulaski, 1947; Taggart, Hirsh, Hendricks, Gable, Puzake, and Greaves, 1949; Taggart, Putnam, Greaves, and Watson, 1950; Jacoby, Goldberg, Sobel, and Rosenthal, 1950; Putkonen and Rouhunkoski, 1951; Hagerman, 1952; Fischer, 1955). Ryan (1952) reported one case of streptomycin resistant gonorrhoea in which the corresponding strain resisted more than 1000 µg. per ml. *in vitro*. Zierz and Jacob (1954) found streptomycin resistant gonococci after treatment with streptomycin and Davey (1957) observed one case of streptomycin resistant gonorrhoea; recently Alergant (1958) reported an increased failure rate when cases from 1954 were compared with cases from 1956 (2·3 per cent. ~7 per cent.). Willcox (1957), however, did not find any increased failure rate when cases from 1951 were compared with cases from 1956, and Lodin (1956a) observed only one relapse out of 76 cases after treatment with 0·5 g. streptomycin in a single dose.

In the present study, most of the 50 per cent. inhibitory concentrations of the gonococcal strains were found to be distributed normally around 4·5 µg. per ml. within a 5-fold range. However, four strains, resistant to more than 2,048 µg. per ml. were found among the 1957 strains; all four strains were isolated after streptomycin treatment. Some degree of correlation between the clinical results and the *in vitro* findings was also found, the incidence of 50 per cent. inhibitory values >3·7 µg. per ml. being significantly greater than that found among strains with a drug sensitivity determination request (65 per cent. ~38 per cent.) than among strains sent in for diagnosis only.

Tetracycline.—The literature contains only a few reports on the *in vitro* sensitivity of gonococci to tetracycline or to other related drugs. The most important is that of Gocke and others (1950). There are also rather few clinical reports (Perkins, Koch, Gara, Stephens, and David, 1955; Collins, Trousdale, Kaiser, Regan, and Finland, 1949; Braff, David, Perkins, Koch, Gara, and Stephens, 1956; Marmell and Prigot, 1955; and, most extensive, Mendell, Wornas, and Foxworthy, 1954). Most authors agree that tetracycline can be used in the treatment of gonorrhoea, but that the cure rate is less satisfactory than that found with penicillin, and that the broad antibiotic spectrum of tetracycline makes it unsuitable for routine treatment. No tetracycline resistant strains have been reported.

In the present study we found narrow ranges of sensitivity both in 1944 and in 1957, *i.e.* about 2-fold in 1944 and 10-fold in 1957, with the most frequent 50 per cent. inhibitory concentrations around 0·6 µg. per ml.

Inter-Relationship between Drugs.—Only a few papers contain comparative studies of the *in vitro* sensitivity of gonococcal strains to penicillin, streptomycin, and tetracycline; Miller and Bohnhoff (1945, 1946) found no correlation between increased resistance to streptomycin in strains artificially made resistant to penicillin in the laboratory and *vice versa*; their results were not published in detail and the number of strains tested is unknown.

Schümmer and Hubbes (1951) found a statistically significant correlation between decreased sensitivity to penicillin and streptomycin; on this basis they advised against the use of streptomycin for the cure of gonorrhoea. Recently, Curtis and Wilkinson (1958) compared the sensitivity to penicillin

and streptomycin in 81 gonococcal strains; they found no evidence of cross-resistance.

In the present study, the 50 per cent. inhibitory concentrations of penicillin and streptomycin were found to be negatively correlated for the 1944 strains, but positively correlated for the 1957 strains, both correlations being statistically significant.

The tetracycline and streptomycin values showed a similar shift from negative to positive correlation, but the correlation was not significant for the 1944 and on the borderline for the 1957 strains.

A correlation was also found between penicillin and tetracycline, and for both the 1944 and 1957 strains the correlations were positive.

It seems most natural to ascribe the change in the type of correlation between the penicillin and streptomycin values to the extensive use of penicillin which had a selective (and perhaps also inducing) action and to the fact that a certain number of the "penicillin resistant" cases were treated with streptomycin. It is emphasized that the negative correlation found in the 1944 material was not very strong and that one of the highly streptomycin resistant strains from 1957 was inhibited by only 0.04 μg. per ml. penicillin.

The reason for the increased resistance to more than one drug found in some of the strains is uncertain. It may be due to clones within the single strains with differing degrees of sensitivity, some being resistant to one, some to the other, and some perhaps to both drugs. It seems most likely that all combinations do exist. As mentioned above, Miller and Bohnhoff did not find that decreased sensitivity to streptomycin was correlated with decreased sensitivity to penicillin or *vice versa*; this indicates that the positive correlation observed in the 1957 series can most probably be explained by the increased resistance of *different* clones to each of the two drugs. Presumably, the observed correlation has different causes from one strain to the other, though the use of both drugs will tend to select clones resistant to both. In Denmark penicillin has been widely used and streptomycin, as far as is known, only in relatively few cases.

The question may be answered by means of experiments using the replica technique of Lederberg and Lederberg (1952) to select resistant clones from strains which have never been exposed to either of the two drugs, followed by crosswise resistance-determination by means of, for example, the gradient plate (Szybalski, 1952a,b). A study along these lines is in preparation.

The change, which is less marked, from negative to positive correlation for streptomycin and tetracycline may be due to a selection by streptomycin and/or possibly penicillin, the correlation being significant for the 1957 strains only. No change was observed when tetracycline was compared with penicillin, and this agees with the fact that the two substances had already shown positively correlated values in the 1944 series.

These tentative explanations should not be given too much credit; many uncontrollable selective and inducing factors play a part in sensitivity determination. Changes in sensitivity may be accompanied by changes in growth requirements (Welsch, 1955) and such changes may influence the quantitative results obtained in the tests.

Both the 1944 and the 1957 strains were kept as lyophilized cultures, and selection may have taken place by this means, though it seems very unlikely that this would influence the inter-relationship between the drugs.

Eventual Effects of Antibiotic Therapy.—Antibiotic treatment may lead to selection not only directly by preferentially favouring the more resistant clones but also indirectly by killing the most easily available organisms. Organisms protected either by phagocytosis or by inflammatory tissue may be spared.

Suggestive facts in this connexion are the prolonged incubation period for gonorrhoea in males reported by several authors and the less pronounced symptoms when compared with classical gonorrhoeal urethritis (Bittiner and Horne, 1955; Lodin, 1955; Nørgaard, 1955; *Brit. med. J.*, 1955). The protection against penicillin of gonococci phagocytized in tissue culture also supports the above assumption (Thayer and others, 1957a,b,c).

In this laboratory, a striking change has recently been observed in the type of growth. About 30 per cent. of all positive cultures received for diagnosis have shown poor growth on the routine medium and weak or absent fermentation of glucose. The viability and growth rate of these atypical strains seem to be less than those of the typical strains. Thirty of these strains which were tested with penicillin, streptomycin, and tetracycline were found to be sensitive with a few exceptions. In spite of this, these weakly growing strains may have been evoked by penicillin therapy because the action of penicillin is connected with the division process (Lederberg, 1950, 1957). Further details will be published in a subsequent paper.

Revision of Treatment Schedule.—These changes in penicillin sensitivity suggest that the treatment schedule should perhaps be reviewed. After the usual intramuscular dose of 300,000 units of aqueous procaine penicillin the average blood concentration

will be about 0·6 μg. or 1 unit per ml. for at least 4 hrs, giving a 25-fold inhibitory margin for gonococci requiring not more than 0·0225 μg. per ml. for complete* *in vitro* inhibition (Miescher, 1949; Cohen 1950; Florey, 1952; Hassing, Juncher, and Raaschou 1955). However, about 25 per cent. of the 1957 strains needed more than 0·225 μg. per ml., which gives a rather narrow safety margin. The wide range of penicillin levels found in the blood after treatment should be taken into consideration (Cohen, 1950) as well as differences between various aqueous procaine penicillin preparations (Rein, Buckwalter, Mann, Landy, and Flax, 1953; Tommila and Savolainen, 1955). There is already a tendency to use increased doses especially in female gonorrhoea Thayer and others, 1957a,b,c; Kaalund-Jørgensen, 1958; Curtis and Wilkinson, 1958; Schamberg and others, 1958). It appears advisable also to reinvestigate the most convenient scheme of dosage and whether short or long-acting penicillin or both should be used (Curtis and Wilkinson, 1958); the latter method has lately been recommended by Schamberg and others (1958).

As yet, no strains highly resistant to penicillin have been isolated from patients and in reporting our results to physicians we have used the term "decreased sensitivity" for strains requiring more than 0·05 μg. per ml. for complete inhibition.

It is doubtful whether the prophylactic treatment of contacts with long-acting penicillin (Schamberg and others, 1958) should be recommended in view of possible future effects on the penicillin sensitivity of the gonococcus; sub-inhibitory penicillin concentrations in the tissues may lead to the selection of strains with decreased sensitivity. There is also some reason to suggest that an extensive use of streptomycin should not be recommended because of the easily acquired high grade of resistance to this drug.

Summary

296 gonococcal strains isolated in 1944 and 1957 have been tested for their sensitivity to penicillin, streptomycin, and tetracycline. A tablet method and a plate dilution method were used and they agreed well, although the latter proved more accurate; the results of the plate dilution method were determined as the 50 per cent. inhibitory concentration expressed in μg. per ml. The variance of the methods was estimated by repeatedly testing three control strains and 27 strains isolated in 1956.

The strains fall into three groups:
  (i) Ninety strains from 1944,
  (ii) 103 strains from 1957 isolated from specimens sent in for diagnosis only ("diagnostic" strains),
  (iii) 103 strains from 1957 from specimens accompanied by a request for sensitivity determination ("sensitivity" strains).

Penicillin.—The sensitivity was found to have changed between 1944 and 1957. The 50 per cent. inhibitory concentrations of the strains from 1944 showed a normal distribution around 0·01 μg. per ml. with a 6-fold range. The 1957 strains showed a two-peak distribution with one peak at about 0·01 μg. per ml. and the other at about 0·2 μg. per ml. The range of the "diagnostic" strains was 80-fold, but the "sensitivity" strains had a 180-fold range and a higher percentage of the strains were in the peak at 0·2 μg. per ml.

Streptomycin.—The sensitivity of the 1944 and 1957 strains was about the same. All the strains were distributed normally around 4·5 μg. per ml., the range being approximately 5-fold in both cases. However, there were in addition four strains from 1957 which were very resistant, and all came from patients previously treated with streptomycin.

Tetracycline.—The sensitivity was about the same for the 1944 and the 1957 strains. The most frequent 50 per cent. inhibitory concentrations were about 0·6 μg. per ml.; the range was 2-fold in 1944 and 10-fold in 1957.

Correlations.—The penicillin and streptomycin values were negatively correlated for the 1944 strains, but positively correlated for the 1957 strains; both correlations were statistically significant.

The penicillin and tetracycline values were positively correlated for both the 1944 and the 1957 strains, and both correlations were significant.

The streptomycin and tetracycline values showed a non-significant negative correlation for the 1944 strains. There was a positive correlation for the 1957 strains, which was significant. The explanation of the observed correlations is discussed.

Atypical strains with low growth rates have been observed, and there has been an increase in the incidence of these strains since June, 1957. It is suggested that the appearance of these strains is a result of penicillin therapy. This suggestion is put forward despite the fact that thirty of those atypical strains which were tested to penicillin were, with a few exceptions, sensitive.

In view of these and other findings in recent reports, it is recommended that the treatment schedule of gonorrhoea should be altered.

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* cf. p. 228, 14 lines from bottom.

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CORRIGENDUM

It is regretted that in the previous article from the Statens Seruminstitut, Copenhagen (by A. Reyn and M. W. Bentzon (*Brit. J. vener. Dis.*, 1958, **34**, 169), the two figures on pages 172 and 173 do not correspond to the captions. The figure on p. 172 refers to *chronic* type syphilitic sera and should be numbered Fig. 2, and that on p. 173 refers to *acute* type syphilic sera and should be numbered Fig. 1.