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Results of Comparative Experiments Using Different Methods for Determining the Sensitivity of *Neisseria gonorrhoeae* to Penicillin G

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Between 1957 and 1960 a decrease in the sensitivity of Neisseria gonorrhoeæ *to penicillin was observed in many parts of the world, but the ranges of sensitivity and the distribution of strains within these ranges varied in different countries. It was not known, however, whether these differences were real or due to the different methods of sensitivity determination used. The collaborative study described in this paper was sponsored by WHO with the object of developing an international reference method for use in epidemiological and interlaboratory studies. Fourteen gonococcal strains, representing a wide range of sensitivity to penicillin, were tested in the three participating laboratories, using two plate dilution methods and a tube dilution method. When the strains were arranged in order of sensitivities, the order was the same whichever method was used, but the levels of sensitivity showed considerable differences, particularly for the less sensitive strains. Good agreement was obtained when the technique of one laboratory was repeated in another. On the basis of the results, it is recommended that an international reference method should use a plate dilution technique and a provisional medium is suggested for this method. It is probable that better agreement between different methods could be achieved by the use of reference strains which would enable a correction factor to be applied. Three gonococcal strains that might prove suitable for this purpose are suggested.*

INTRODUCTION

Several different methods have been used for the determination of the sensitivity of *Neisseria gonorrhoeae* to penicillin. These are summarized in Appendix Table 1, from which it is seen that most workers have used dilution methods (tube or plate); however, during recent years diffusion methods have also gained favour. The media employed show great variation and many of them contain biological substances such as serum, ascitic fluid or hydrocele fluid. Until about 1955, it was generally agreed that the sensitivity of *N. gonorrhoeae* to penicillin had remained unchanged, since treatment with penicillin was still successful. Subsequently reports of treatment failure became more frequent, and *in vitro* experiments showed a tendency to decreasing sensitivity of the gonococcal strains to penicillin. However, the different and changing methods used and the lack of control experiments performed with strains isolated before the introduction of penicillin left the *in vitro* results open to discussion for some time. Later it was shown by Reyn et al. (1958) that the sensitivity of strains isolated in 1944 differed significantly from that of strains isolated in 1957, and that the range of sensitivity had increased, the extremes now differing by a factor of 200 instead of a factor of 10. The least sensitive strains now required for inhibition about twenty times the concentration of penicillin needed in 1944. Recently, similar studies have been made by others (Cole et al., 1961; Kaiser & Rinderknecht, 1961;

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TABLE 1
PERCENTAGE DISTRIBUTION OF STRAINS ACCORDING TO MIC VALUES[a]

Reference	Strains isolated in	Number of strains	Percentage of strains sensitive to the following minimum inhibitory concentrations (IU/ml) of penicillin							
			>1.0	1.0	0.5	0.25	0.125 0.100	0.06 0.05	0.03 0.025	≤0.015
Reyn, Korner & Bentzon (1958)	1944	90							49.3	50.7
Lankford (1945)	1945	100							11	89
Romansky & Robin (1947)	1947	53						9.4	54.7	35.9
Del Love & Finland (1955)	1947	104							1	99
Del Love & Finland (1955)	1949	52				2	2	10	37	49
Marcuse & Hussels (1954)	1950-52	232					0.4	15.9	74.5	9.1
Schümmer & Hubbes (1951)	1951	100					2	5	27	66
Schreus & Schümmer (1951)	1951	100					1	7	53	39
Del Love & Finland (1955)	1954	106							3	97
Reyn, Korner & Bentzon (1958)	1957	103		12	3	3	8	46	28	
Cradock-Watson et al. (1958)	1957-58	200			2.5	8	8.5	6	23	52
Curtis & Wilkinson (1958)	1957	302			1.6	12.6	5.3	5.6	6.6	68.3
Hirsch & Finland (1960)	1958-59	157				0.6	8.3	15.9	10.8	64.2
Roiron et al. (1961)	1958-60	327	1	0.3	4.6	5.5	16.2	22	50.4	

[a] Slightly modified from the table published by Roiron et al. (1961).

Bakker et al., 1962) and clinical failures have also become more apparent.[1]

The decreased susceptibility makes it desirable to determine the sensitivity of the gonococcal strains quantitatively, not only at intervals in epidemiological surveys (to test the over-all sensitivity level) but also in individual patients as a guide to therapy and to distinguish better between relapse and re-infection or between " false and true contact pairs ".[2]

If the same gonococcal strains were tested in different laboratories (using different methods) one would expect to obtain somewhat differing values, even if all the laboratories used a dilution method and the results were expressed as the minimum inhibitory concentration per ml (MIC). If different diffusion techniques (agar cup or disc) were used, the results would not be directly comparable either with one another or with those obtained by a dilution method.[3]

Table 1 (Roiron et al., 1961) illustrates the decreasing trend of sensitivity which has been observed. The ranges of sensitivity and the distribution of the strains within these ranges vary from one country to another. It was not known, however, whether the differences (both in range and distribution) seen in the years from 1957 to 1960 were due to the use of different methods or whether such differences really did occur. Differences up to 16-fold were found among the less sensitive strains, e.g., when the strains isolated in 1957 by Reyn et al. (1958) were compared with those isolated in 1958-59 by Hirsch & Finland (1960). Recently, however, Reyn (1963) and Reyn et al. (1963) found great geographical differences in the ranges of sensitivity and in the distributions of sensitivity in 300 strains collected from Ceylon, Greenland, the

[1] See: Schümmer & Hubbes (1951); Cradock-Watson et al. (1958); Curtis & Wilkinson (1958); King (1958); Stepniak (1961); *Brit. J. vener. Dis.* (1961); Gjessing & Ødegaard (1962b); Rantasalo (1962); Rantasalo et al. (1962); Röckl (1962); Willcox (1962).

[2] See: Catterall (1962); Gjessing & Ødegaard (1962a); Reyn (1962); Schmidt & Olesen Larsen (1962a,b); Reyn & Bentzon (1963).

[3] Ringertz (1961) and Rantasalo et al. (1962) are so far the only workers who, when determining the sensitivity of gonococci, have " translated " zone diameters in mm into concentrations. This procedure has recently been discussed by Reyn et al. (1963).

Philippines, Poland and West Africa; these strains were all tested by the same method.

As a logical consequence of the increasing therapeutic failure rates, the incidence of reported cases of gonorrhoea showed an increase in many countries (Guthe, 1961; Guthe & Idsøe, 1962). This development revived interest in sensitivity determinations of gonococcal strains. Thus, in 1959 the Medical Research Council of Great Britain formed a working party to examine the situation in Great Britain. An interim report of the results has been published (Great Britain, 1961). The sensitivity to penicillin of 1984 strains of gonococci was determined in nine centres during 1959 and the first three months of 1960. Sensitivities were determined either by a tube dilution technique, using a hydrocele-broth or a modified Levinthal-broth medium, or, in two centres, by plating on a series of chocolate-agar or horse-serum-agar plates. Some strains required from 0.125 to 0.5 international units (IU) per ml for complete inhibition, and such strains were found in all areas. Among other things it was found that the incidence of relatively insensitive strains varied widely between different areas and at different times in the same neighbourhood. Strains were exchanged between the various centres to act as an interlaboratory check on the sensitivity of the methods used and good agreement was found. However, a common set of strains was not systematically studied in all the laboratories.

The picture thus emerging in many parts of the world over the last few years reveals, on the one hand, a changing susceptibility of gonococcal strains to penicillin as observed by laboratory methods in local use, and on the other hand, an increasing number of failures of penicillin dosages previously effective clinically in the treatment of gonococcal infections. A correlation between these two aspects was not always evident. This gradually led to further studies of the methods used in various countries, their differences and comparability. In June 1960, on the initiative of the Royal Swedish National Health Service, a committee on the culture and sensitivity determination of gonococci was established. In six laboratories in four countries (Denmark, Finland, Norway and Sweden) comparative experiments were performed using a diffusion method (Ericsson et al., 1954) and a plate dilution method (Reyn et al., 1958).[1] Ten gonococcal strains, *Sarcina lutea* ATCC 9341 and *Staphylococcus aureus* FDA 209 P were repeatedly tested, and two different media were employed, one of which was common to all the laboratories. The two methods established the relative sensitivities of the strains with a substantial degree of uniformity. Both methods were found to be of practical value. The plate method gave more reproducible results than did the diffusion method; the difference in reproducibility can be explained by the difficulty in specifying the size of the inoculum, which is most important for a diffusion technique. The reading of the results of the diffusion method is also difficult to standardize (Reyn et al., 1963).

As long ago as 1959, a WHO Expert Committee on Venereal Infections and Treponematoses drew attention to the need for developing as soon as possible an international reference method for determining penicillin sensitivity of gonococcal strains so as to allow comparison of findings in different parts of the world. As a step in this direction WHO subsequently sponsored a collaborative study by a group of microbiologists—Reyn in Denmark, Wilkinson in the United Kingdom and Thayer in the USA. The statistical analysis of the results was undertaken by Bentzon, who also helped in the planning of some of the experiments. The object of this study was to develop an international reference method to be used primarily in epidemiological and interlaboratory studies where it is essential to compare results.

The use of a dilution method as a reference procedure was considered as the most direct way of determining the inhibitory effect *in vitro* of a given antibiotic. Hence, it was decided not to include a diffusion procedure in the comparative experiments.

MATERIAL

Gonococcal strains

Fourteen gonococcal stock strains were tested in all three laboratories and 9 of these strains had also been included in the Scandinavian study (Reyn et al., 1963). The strains were either lyophilized and dispatched in ampoules, or they were dispatched in screw-cap tubes on chocolate agar slants. Table 2 contains a preliminary description of the fourteen gonococcal strains tested in all three laboratories; a wide range in sensitivity to penicillin was covered by these strains which also comprised strains of varying sensitivity to streptomycin. Altogether 26 stock strains and 21 fresh strains were used in

[1] For convenience, these experiments are subsequently referred to as the Scandinavian study.

TABLE 2
SUMMARY OF STRAINS EXAMINED IN ALL LABORATORIES

Source of strains	Strain No. used in study [a]	Original strain No.	Isolated in	IC_{50} of penicillin [b] (IU/ml)
Thayer	I	P-1	1958	0.35
	II	P-5	1958	0.088
	III	P-26	1955	0.044
	IV	P-78	1960	0.71
	V	P-83	1960	0.71
Reyn	VI	11413	1940	0.0078
	VII	11421	1940	0.0093
	VIII	12990	1960	≥0.71
	IX	17732	1940	0.0111
Wilkinson	W I	W 7420	1961	0.50
	W II	M 8736	1961	0.022
	W III	A 4516	1961	0.50
	W IV	W 5252	1961	0.0111
	W V	A 5172	1961	≥2.0

[a] The numbers of Thayer's and Reyn's strains are the same as those used in the Scandinavian study.
[b] Results of a single test performed in Copenhagen.

the experiments; however the fresh strains were only tested by Reyn. *Sarcina lutea* ATCC 9341, the Oxford *Staphylococcus* and *Staphylococcus aureus* FDA 209 P were included in some of the comparisons.

Media

The composition of the media used in the collaborative study is shown in Table 3.

METHODS

Method 1 (Reyn)

This is a plate dilution method which was first described by Reyn, Korner & Bentzon in 1958 and in more detail by Reyn, Bentzon and Ericsson in 1963. The results are expressed in terms of the 50% inhibitory concentration (IC_{50}). In the present

TABLE 3
COMPOSITION OF MEDIA USED IN THE COLLABORATIVE STUDY

Method	Agar		Broth	NaCl (%)	Na_2HPO_4, H_2O (%)	Haemoglobin or blood	Enrichment	Glucose (%)
	Brand	%						
Plate dilution (Reyn)	Japanese (threaded)	1.1	Beef-heart without peptone [a]	0.3	0.2	6.7% heated horse blood	10% ascitic fluid	No extra addition
Plate dilution (Thayer)	Proteose Agar Special Exp. control 442097 (Powdered) (Difco) [b]	1.5	None	0.5	0.5	1% haemoglobin (Difco) [b]	1% Bacto-Supplement C (Difco) [b]	0.3
Tube dilution (Wilkinson)	None	—	Tryptone Soya Broth (Oxoid) CM 129	None added	None added	None	12.5% hydrocele fluid	1

[a] Prepared at the Statens Seruminstitut, Copenhagen.
[b] Difco Laboratories, Detroit, Mich., USA.

study, both the IC_{50} and the minimum inhibitory concentration (MIC) were recorded, the concentrations being expressed in international units (IU) per ml. Twofold dilution steps were employed.

Method 2 (Thayer)

This method, which is also a plate dilution method, is described in detail in *Gonococcus—Procedures for Isolation and Identification* (US Public Health Service, 1962). The results are given as MIC values and twofold or smaller dilution steps are used.

Method 3 (Wilkinson)

This method was described in detail by Curtis & Wilkinson in 1958 and by Wilkinson in 1962. It is a tube dilution procedure using twofold dilution steps, and the results are read as MIC values. Purity of growth is checked by Gram staining and phenol red is added to facilitate the readings.

Apart from the differences in the media, the techniques employed by Reyn and by Thayer were much the same. The design of the Thayer-Reyn comparison was different from that of the Reyn-Wilkinson and Wilkinson-Thayer comparisons for the reasons given below.

THAYER-REYN COMPARISON

The exchange of strains between Thayer and Reyn dates back to 1959, when Thayer became interested in obtaining some of Reyn's lyophilized strains, especially those that had been isolated in and before 1944. Later, both Reyn and Thayer agreed to participate in the WHO collaborative study. In 1960 12 strains were sent from Thayer to Reyn and 14 strains from Reyn to Thayer; both sets of strains were accompanied by information on the results obtained in the home laboratory. The Thayer strains were tested by Reyn, who found that her results showed a positive correlation with those obtained by Thayer, but that in her laboratory about twice as much penicillin was needed for complete inhibition as in Thayer's laboratory. This finding made it desirable to exchange methods and media, but circumstances prevented adequate planning and this is the explanation of the difference in design of the three comparisons. Thus, Reyn did not use Thayer's method but she used Thayer's medium, whereas Thayer used both Reyn's medium and Reyn's method. Owing to circumstances it was not possible for Thayer either to pre-test Reyn's method before commencing his experiments or to estimate the IC_{50} concentrations, criteria for reading the amounts of growth not having been published at that time.

In order to exclude the possibility of a deficiency in the Danish penicillin (penicillin G " Leo "), a sample was sent to the Food and Drug Administration (FDA) for potency determination. It was found to contain 1638 IU per mg, in good agreement with FDA standard crystalline penicillin G sodium which contains 1654 IU per mg.

Altogether 40 gonococcal strains plus two control strains were tested; 21 of the gonococcal strains were recently isolated. *Sarcina lutea* ATCC 9341 was also included, because it is used by Thayer to control the technical procedures. Appendix Tables 2 and 3 present the results of the experiments in IU per ml; both IC_{50} and MIC values are given. The strains were divided into two groups having different levels of sensitivity, and the grouping was made so that the two groups contained about the same number of strains. Group 1 comprised the less sensitive strains with an $IC_{50} \geq 0.074$ IU per ml ($\log IC_{50} = -1.13$), and group 2 comprised the more sensitive strains with an $IC_{50} \leq 0.063$ IU per ml ($\log IC_{50} = -1.20$) (column 1 in Appendix Table 2).

Table 4 shows the results (log MIC values) of a series of comparisons obtained by testing the same strains repeatedly in the same way and in different ways. In order to be able to demonstrate the identity of two methods it is necessary to estimate the standard error of the differences found by repeated testing of the same strains. This report contains only results of repeated testing performed in Copenhagen by the Reyn method (see columns 2 and 5 in Appendix Table 3). The standard error of differences between the log MIC values was calculated to be 0.14 (Table 4), whereas in the Wilkinson-Reyn study it was 0.16 (Table 6). The corresponding standard error for the Thayer method performed by Thayer (T-T) was about 0.11 in replicated testing of strain P-1, and 0.16 in replicated testing of the *Sarcina lutea* strain.

In Fig. 1 the results of the Thayer method performed by Thayer (T-T, Appendix Table 3, column 7) are plotted against the results of the Reyn method performed by Reyn (R-R, Appendix Table 3, column 5). It is evident that the level of susceptibility is different for the two methods; the mean differences between the log MIC values are shown in Table 4. The mean difference for all strains (−0.48) corresponds to an average ratio of the MIC values of

TABLE 4

PAIRED COMPARISONS BETWEEN LOG MIC VALUES OBTAINED USING DIFFERENT PLATE DILUTION METHODS

Methods compared [a]	Group 1: less sensitive strains		Group 2: more sensitive strains		All strains		Standard deviations of individual differences			
	No. of strains	Mean difference in log MIC values	No. of strains	Mean difference in log MIC values	No. of strains	Mean difference in log MIC values	Group 1	Group 2	Mean [b]	Duplicate testing (experimental error)
T-T and R-R (5) [c]	9	–0.54	10	–0.42	19	–0.48	0.14	0.20	0.18	
T-T and R-T [d]	9	–0.77	10	–0.33	19	–0.54	0.07	0.21	0.16	0.11
R-T and R-R	9	0.23	10	–0.09	19	0.06	0.13	0.28	0.23	
RT-R and R-R (2)	16	–0.38	21	–0.29	37	–0.32	0.20	0.20	0.20	0.14 [e]

[a] R-R (2) = Reyn method performed by Reyn, Appendix Table 3, column 2.
R-R (5) = Reyn method performed by Reyn, Appendix Table 3, column 5.
R-T = Reyn method performed by Thayer, Appendix Table 3, column 6.
RT-R = Reyn method using Thayer medium performed by Reyn, Appendix Table 3, column 4.
T-T = Thayer method performed by Thayer. Appendix Table 3, column 7.

[b] Calculated as the square root of the mean of the variances for groups 1 and 2.

[c] See also Fig. 1.

[d] See also Fig. 2.

[e] Comparison between R-R (5) and R-R (2).

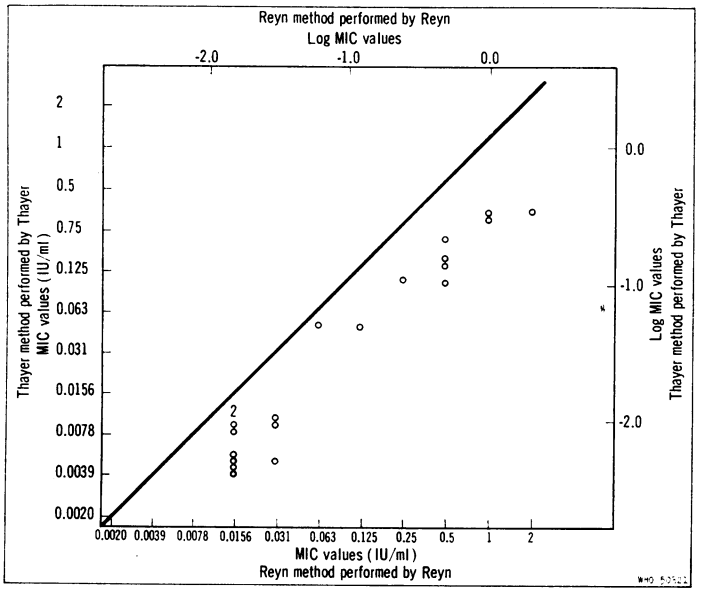
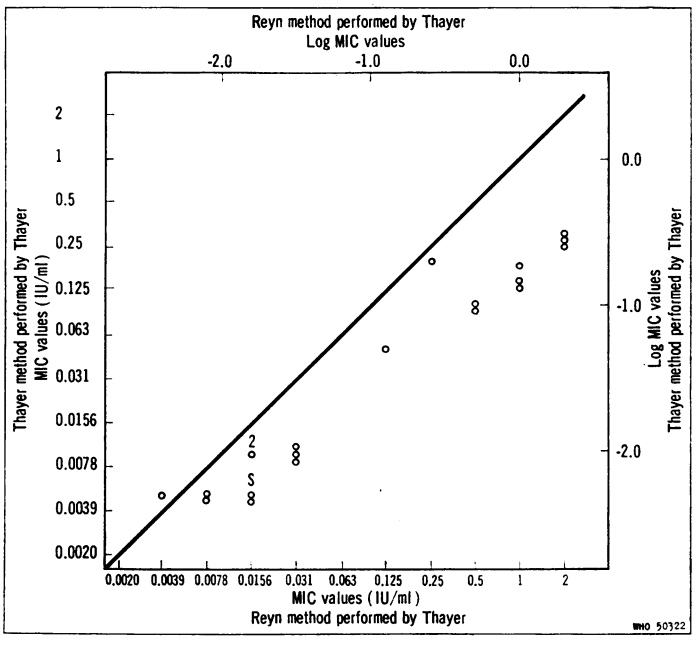


FIG. 1

COMPARISON OF MIC VALUES OBTAINED BY THE PLATE DILUTION METHODS OF REYN PERFORMED BY REYN AND OF THAYER PERFORMED BY THAYER

2 = control strain No. 2

FIG. 2
COMPARISON OF MIC VALUES OBTAINED BY THE PLATE DILUTION METHODS OF REYN PERFORMED BY THAYER AND OF THAYER PERFORMED BY THAYER



2 = control strain No. 2
S = *Sarcina lutea*

about 1:3. The mean difference in the log MIC values is nearly the same for the two sensitivity groups, being slightly greater for group 1 than for group 2.

The result of Reyn's comparison between the Thayer medium (RT-R, Appendix Table 3, column 4) and Reyn's medium (R-R, Appendix Table 3, column 2), using Reyn's method, indicates that part of the observed difference in susceptibility may be ascribed to the difference between the media. This comparison also shows that the difference is slightly greater for group 1 than for group 2. The assumption that the difference is due to differences in the media is supported by the results of the comparison between the Thayer method by Thayer (T-T, column 7) and the Reyn method by Thayer (R-T, column 6); this comparison is illustrated in Fig. 2.

In this case, however, a distinct and significant difference[1] between the two sensitivity groups is observed, the average MIC ratios being about 1:5 for group 1 and about 1:2 for group 2; yet, as demonstrated by the comparison between R-T and R-R (columns 5 and 6), this difference may possibly be ascribed to the fact that Thayer had not had enough time to become thoroughly familiar with the Reyn method.

[1] The difference is 0.44 with a standard error of $0.16\sqrt{1/9+1/10} = 0.07$.

The results showed some variations between the strains, the standard errors due to differences between strains (within sensitivity groups) being in all cases larger than the corresponding experimental errors. It is also remarkable that the standard errors are higher in sensitivity group 2 than in group 1.

REYN-WILKINSON AND WILKINSON-THAYER COMPARISONS

The exchange of strains between Wilkinson and Reyn took place late in 1961. Wilkinson agreed to participate in the WHO collaborative study and based on the experience gained in the Thayer-Reyn study and in the Scandinavian study it was planned to exchange both strains and methods, including media, at once. All materials for the preparation of the media, with the exception of penicillin, and detailed descriptions of the methods were exchanged. During the experiments care was taken to copy the technique of the other laboratory as closely as possible, but small deviations did occur. Thus, Reyn incubated the tubes in a CO_2-atmosphere and made the readings after 72 hours and sometimes also after 48 hours, whereas Wilkinson did not incubate in a CO_2-atmosphere and read both the plate and the tube dilution results after 48 hours.

The CO_2-atmosphere is considered to be of minor importance, but the different reading times would probably tend to make the MIC values higher in Copenhagen than in London; the 72-hour values were used in the evaluation of the results. When the 48- and 72-hour values were compared in Copenhagen, the MIC values were twice as high as in London in 5 of the 34 cases in which both 48- and 72-hour readings were made; in the other 29 cases no difference was observed. In addition, in Copenhagen it was found most practical to add the penicillin to a whole batch of fluid medium before it was dispensed into tubes, whereas in London the penicillin was added to a smaller portion of the medium and dispensed into tubes immediately before these were inoculated.

Fifteen gonococcal strains (the 10 strains used in the Scandinavian study and 5 strains isolated by Wilkinson) were repeatedly tested in the two laboratories. The Oxford *Staphylococcus* was included by Wilkinson as a control, and *Staphylococcus aureus* FDA 209 P was included by Reyn; both strains are sensitive to penicillin and both were isolated before the introduction of penicillin. It was decided to have all the strains tested at least twice in each of the two laboratories. The number of test days, six in all, was paired, each pair consisting of two days on which the same set of strains was tested. This procedure made it possible to estimate the standard deviations due to experimental errors of the two methods in question. The design of the experiments is shown in Table 5.

The results in IU per ml are presented in Appendix Tables 4 and 5. Both the IC_{50} and MIC values are given for the plate dilution method, and the readings after 24, 48 and 72 hours are included as well. The six-day plan was not followed exactly; both laboratories included some extra testing. It had also been suggested that three batches of each medium should be prepared and two experiments a week performed, but again neither laboratory followed these suggestions completely.

In this study the experimental error of replicated testing was estimated for both methods (and for the IC_{50} and MIC separately) in both laboratories (Table 6). It is seen that the results obtained in Copenhagen showed a distinct day-to-day variation, many of the mean differences exceeding two to three times their standard errors. In London mean differences of the same order of magnitude were found, but none of these were more than twice their standard errors. It is also seen that the experimental

TABLE 5
DESIGN OF THE EXPERIMENTS

	Test days	Strain numbers [a]					
1st week	1	I	W I	W III	II	III	IV
	2	I	W I	W IV	V	VI	VII
2nd week	3	I	W I	W V	VIII	IX	X
	4	I	W II	W III	II	III	IV
3rd week	5	I	W II	W IV	V	VI	VII
	6	I	W II	W V	VIII	IX	X

[a] The numbers prefixed by "W" refer to Wilkinson's strains; the remaining numbers are those used in the Scandinavian study.

error (s_d) was somewhat lower for the IC_{50} than for the MIC values.

The comparison between the methods and between the investigations performed in London and Copenhagen was based on the geometrical means of two determinations for each strain (an exception being the results from Chamblee, which are based mainly on single determinations) (Table 7). The results of the tests that were additional to those originally planned were omitted; had they been included, the precision would have been only slightly greater and it is easier to compare the results when all strains are tested in the same way. The results obtained with the Oxford *Staphylococcus* and with *Staphylococcus aureus* FDA 209 P were also excluded. The latter strain, which had been included in the Scandinavian study, was used in Copenhagen instead of the Oxford *Staphylococcus* in the mistaken belief that the two strains were identical.

Both for the gonococcal and for the staphylococcal strains the MIC values tended to be higher when the incubation time was prolonged (Appendix Tables 4 and 5). Furthermore, it was observed in Copenhagen that the tube dilution method for *Staphylococcus aureus* FDA 209 P gave consistently higher values than did the plate method, the opposite being the case for the gonococcal strains (Fig. 6.) Thus, the statistical analysis was based solely on the results obtained with gonococcal strains.

Fig. 3-5 illustrate pairwise comparisons of results obtained with each of the two methods performed in London and in Copenhagen; nearly the same sensitivity differences were found between the strains,

TABLE 6

COMPARISON OF INHIBITORY CONCENTRATIONS (LOG VALUES) DETERMINED ON TWO DIFFERENT DAYS BY THE METHODS OF REYN AND WILKINSON

Test days [a]	Age of media [b] (days)	No. of strains (n)	$\bar{d}$ [c]	SE ($\bar{d}$) [d]	Age of media [b] (days)	No. of strains (n)	$\bar{d}$ [c]	SE ($\bar{d}$) [d]
Log IC_{50} determined by Reyn method (plate dilution)								
Reyn method by Reyn					Reyn method by Wilkinson			
4 and 1	5 and 4	5	−0.17	0.05	2 and 2	5	0.08	0.07
5 and 2	4 and 7	5	0.15	0.05	6 and 1	5	0.14	0.07
6 and 3	8 and 1	5	0.15	0.05	3 and 3	5	0.06	0.07
Standard deviation of individual differences (s_d):			0.10				0.16	
Log MIC determined by Reyn method (plate dilution)								
Reyn method by Reyn					Reyn method by Wilkinson			
4 and 1	5 and 4	5	−0.18	0.07	2 and 2	5	−0.06	0.09
5 and 2	4 and 7	5	0.18	0.07	6 and 1	5	0.12	0.09
6 and 3	8 and 1	5	0.06	0.07	3 and 3	5	0.18	0.09
Standard deviation of individual differences (s_d):			0.16				0.20	
Log MIC determined by Wilkinson method (tube dilution)								
Wilkinson method by Reyn					Wilkinson method by Wilkinson			
4 and 1	7 and 4	5	0.12	0.06	2 and 2	5	0.18	0.13
5 and 2	4 and 7	4	0.08	0.06	1 and 2	5	0.18	0.13
6 and 3	8 and 3	5	0.00	0.06	3 and 3	5	0.12	0.13
Standard deviation of individual differences (s_d):			0.13				0.30	

[a] Days compared: thus, results obtained on day 1 were subtracted from those obtained on day 4.

[b] The two figures give the ages of the media on the two test days: thus, the medium used by Reyn on day 4 was 5 days old, that used on day 1 was 4 days old; similarly, the medium used by Wilkinson both on day 4 and on day 1 was 2 days old.

[c] Mean difference in log IC_{50} or log MIC values.

[d] Standard error of mean differences (SE ($\bar{d}$)) $= \frac{S_d}{\sqrt{n}}$

but the level of sensitivity was somewhat different.

In contrast to this Fig. 6 and 7 show that the relation between the methods was different for strains of different sensitivity levels. The strains were divided into two groups according to their sensitivities and using the IC_{50} values obtained in Copenhagen, the principle being the same as that used in the Thayer-Reyn comparison. Group 1 comprised 8 strains (Scandinavian study I, II, IV, V, VIII, W I, W III, W V), and group 2 comprised 7 strains (Scandinavian study III, VI, VII, IX, X, W II, W IV). The mean difference was calculated for each of the comparisons illustrated in Fig. 3-7 (Table 8).

Table 8 shows that on the average the Copenhagen results are 0.14 and 0.20 log values lower than the London results for the plate method and 0.16 log values higher than the London results for the tube

TABLE 7

SUMMARY OF MEAN LOG IC_{50} AND MEAN LOG MIC VALUES FOR THE STRAINS EXAMINED IN ALL THREE LABORATORIES[a]

Strain No.[b]	Sensitivity group[c]	Mean log IC_{50} values[d]		Mean log MIC values[d]		Mean log MIC values[e]		Log MIC values[f]
		R-R	R-W	R-R	R-W	W-R	W-W	T-T
I	1	−0.53	−0.27	−0.30	0.00	−0.90	−0.90	−0.82
II	1	−1.02	−0.94	−0.75	−0.60	−0.90	−1.35	−1.30
III	2	−1.24	−1.05	−1.05	−0.90	−1.35	−1.20	−1.30
IV	1	0.00	0.15	0.30	0.45	−0.60	−0.45	−0.52
V	1	−0.04	0.08	0.30	0.45	−0.75	−0.45	−0.52
VI	2	−2.33	−2.22	−1.95	−1.95	−2.10	−2.40	−2.30
VII	2	−2.14	−1.99	−1.95	−1.80	−2.10	−2.40	−2.30
VIII	1	0.04	−0.19	0.30	0.30	−0.30	−0.90	−0.52
IX	2	−1.88	−1.62	−1.65	−1.35	−1.80	−1.80	−2.30
X	2	−1.95	−1.58	−1.80	−1.35	−2.10	−2.25	
W I	1	−0.30	−0.30	0.00	0.00	−0.60	−0.75	−0.45
W II	2	−1.88	−1.73	−1.65	−1.35	−1.80	−1.95	−1.80
W III	1	−0.49	−0.27	−0.15	0.00	−0.75	−1.05	−0.82
W IV	2	−2.10	−1.80	−1.80	−1.35	−1.80	−2.25	−2.00 and −1.30 (mean: −1.65)
W V	1	0.08	0.08	0.30	0.60	−0.30	−0.45	−0.35 and −0.30 (mean: −0.32)

[a] The mean log IC_{50} and mean log MIC values for the methods of Reyn and Wilkinson were computed by taking the geometric mean of two values from Appendix Table 4 or Appendix Table 5 and expressing this as the logarithm. Thus, from Appendix Table 5, the IC_{50} for strain III is 0.074 in experiment I and 0.044 in experiment IV. The geometric mean of these two values is 0.058, the logarithm of which is −1.24. As this is less than −1.20, strain III is placed in sensitivity group 2. The results of experiments IIIb and IVb in Table 4 and of experiment VIb in Table 5 have not been taken into consideration.

[b] The numbers prefixed by " W " refer to Wilkinson's strains; the remaining numbers are those used in the Scandinavian study.

[c] cf. Table 4 and explanation on page 481.

[d] Plate dilution method of Reyn: R-R = performed by Reyn; R-W = performed by Wilkinson.

[e] Tube dilution method of Wilkinson: W-R = performed by Reyn; W-W = performed by Wilkinson.

[f] Plate dilution method of Thayer performed by Thayer.

method. In terms of inhibitory concentrations the ratios between the Copenhagen and the London results were 1: 1.4, 1: 1.6 and 1.5: 1.

Greater mean differences are found when the different methods are compared. The differences −0.42 and −0.78 (Copenhagen and London) correspond to ratios of 1: 2.6 and 1: 6 between inhibitory concentrations obtained by the Reyn and the Wilkinson methods. The largest deviations were observed for the less sensitive strains; the difference between groups is significant in both cases. The comparison between the Thayer method and the Wilkinson method showed a small uniform difference of −0.10 corresponding to a ratio of 1: 1.3.

Similarly, as observed under A, the results showed some variation between the strains, the standard errors due to differences between strains (within sensitivity groups) being in all cases larger than the corresponding experimental errors.

The IC_{50} results (R-R — R-W) were slightly closer to each other than the corresponding MIC results. A similar observation was made in the Scandinavian study (Reyn et al., 1963).

DISCUSSION AND CONCLUSIONS

The comparative experiments in which repeated sensitivity determinations were made on the same

FIG. 3

COMPARISON OF IC_{50} VALUES OBTAINED BY THE PLATE DILUTION METHOD OF REYN PERFORMED BY REYN AND BY WILKINSON

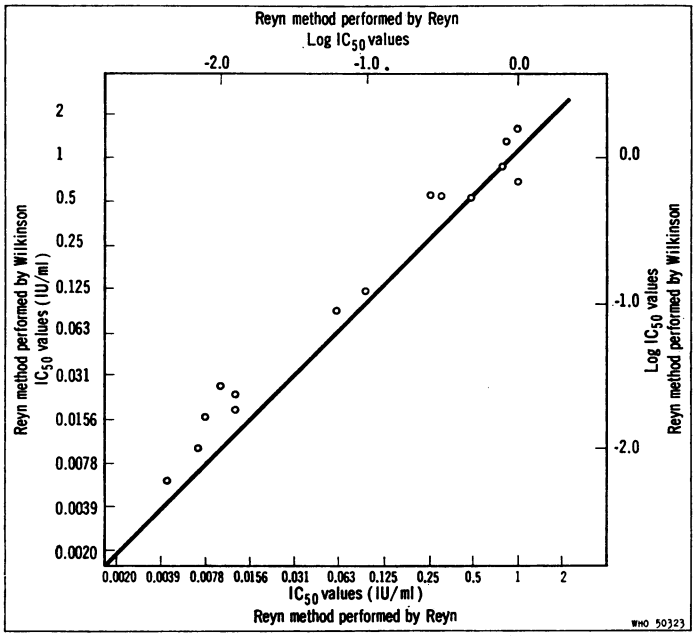
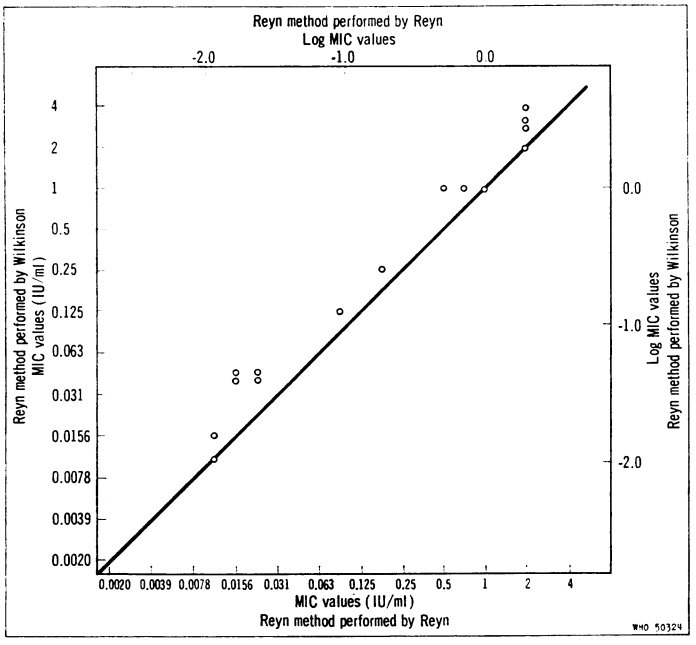


FIG. 4

COMPARISON OF MIC VALUES OBTAINED BY THE PLATE DILUTION METHOD OF REYN PERFORMED BY REYN AND BY WILKINSON



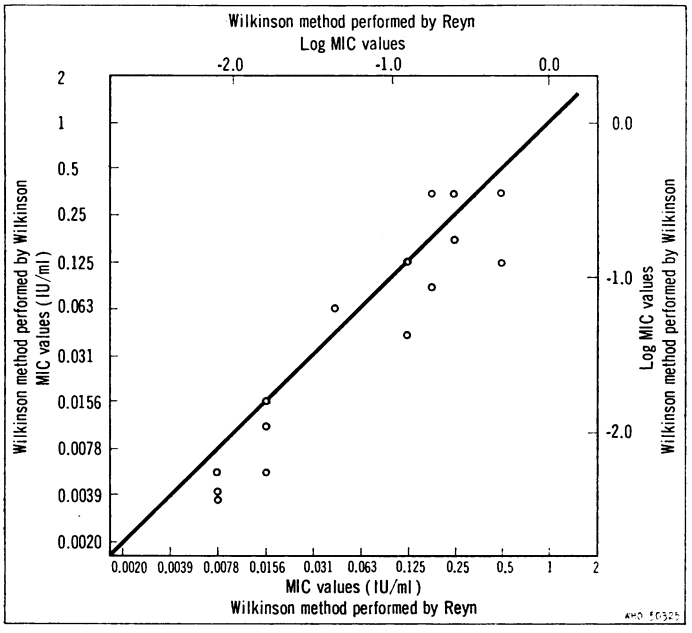


FIG. 5

COMPARISON OF MIC VALUES OBTAINED BY THE TUBE DILUTION METHOD OF WILKINSON PERFORMED BY REYN AND BY WILKINSON

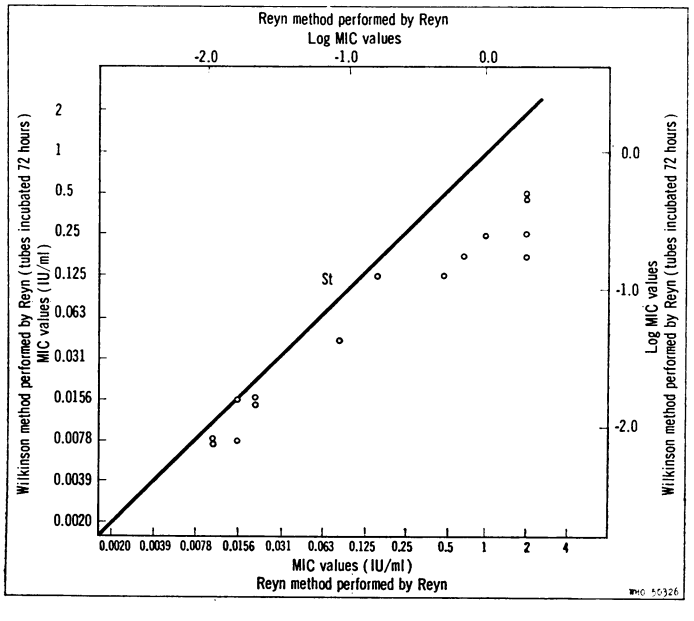


FIG. 6

COMPARISON OF MIC VALUES OBTAINED BY THE PLATE DILUTION METHOD OF REYN AND THE TUBE DILUTION METHOD OF WILKINSON, BOTH PERFORMED BY REYN

St = *Staphylococcus aureus* FDA 209 P

FIG. 7

COMPARISON OF MIC VALUES OBTAINED BY THE PLATE DILUTION METHOD OF REYN PERFORMED BY REYN AND BY THE TUBE DILUTION METHOD OF WILKINSON PERFORMED BY WILKINSON

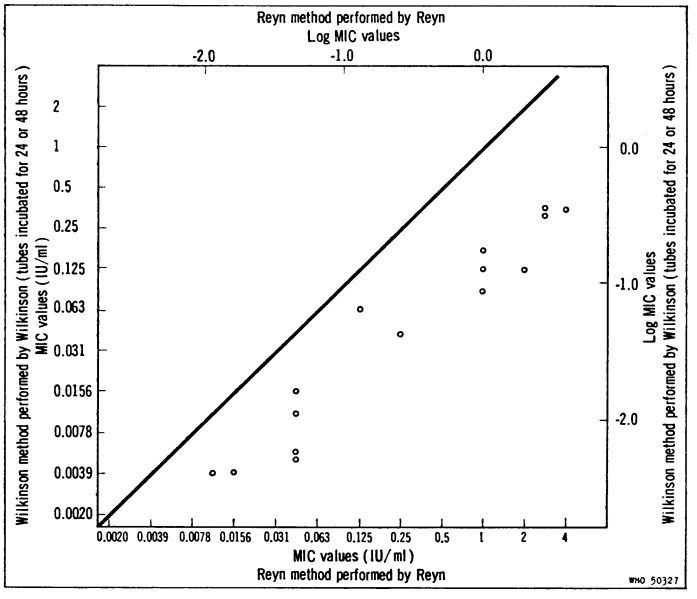


TABLE 8

MEAN DIFFERENCES IN THE RESULTS OF THE SENSITIVITY DETERMINATIONS PERFORMED IN THE THREE LABORATORIES [a]

Comparisons [b]	Mean differences in log values for:			Standard deviations of individual differences			
	Group 1 [c] (8 strains)	Group 2 [c] (7 strains)	All strains	Group 1 [c]	Group 2 [c]	Mean [d]	Duplicate testing (experimental error) [e]
Plate method: R-R/R-W (log IC_{50})	–0.08	–0.22	–0.14	0.15	0.09	0.13	0.09
R-R/R-W (log MIC)	–0.15	–0.26	–0.20	0.11	0.17	0.14	0.13
Tube method: W-R/W-W (log MIC)	0.15	0.17	0.16	0.30	0.20	0.26	0.16
Copenhagen: W-R/R-R (log MIC)	–0.64	–0.17	–0.42	0.26	0.10	0.21	0.10
London: W-W/R-W (log MIC)	–0.94	–0.60	–0.78	0.16	0.23	0.19	0.18
London/Chamblee: W-W/T-T (log MIC)	–0.13	–0.06	–0.10	0.17	0.36	0.26	—

[a] Calculated from the mean log IC_{50} and mean log MIC values given in Table 7 after grouping the strains according to sensitivity.

[b] Plate method: R-R/R-W (log IC_{50})=plate method of Reyn performed by Reyn compared with same method performed by Wilkinson; results expressed as log IC_{50} values.

Copenhagen: W-R/R-R (by MIC)=comparison in Copenhagen of Wilkinson method and Reyn method, both performed by Reyn; results expressed as log MIC values.

See also Table 7.

[c] cf. Table 4 and explanation on page 481.

[d] Calculated as the square root of the mean of the variances for groups 1 and 2.

[e] Derived from Table 6. Standard error (SE) of difference between mean of two determinations.

Examples: Plate method: R-R/R-W (log IC_{50}): $SE = \sqrt{1/4\ (0.10^2+0.16^2)} = 0.09$

London: W-W/R-W (log MIC): $SE = \sqrt{1/4\ (0.20^2+0.30^2)} = 0.18$

strains using three different dilution methods showed that all three methods placed the gonococcal strains in nearly the same order of sensitivity (Fig. 1, 6 and 7). However, the levels of sensitivity were different. The greatest differences in level were between Reyn's plate dilution method and Wilkinson's tube dilution method (a ratio of 1:6) and between Reyn's and Thayer's plate dilution methods (a ratio of 1:2 or 1:3). Wilkinson's and Thayer's methods gave more uniform results.

In experiments in which the method was kept constant but the medium was changed, it was shown that the difference between Thayer's and Reyn's methods was mainly due to the differences in the media employed (Table 4).

Another remarkable result was that the difference in level was greater for the less sensitive strains than for the sensitive strains, and this applied both to the comparison between Reyn's and Thayer's methods and to that between Reyn's and Wilkinson's methods.

The three media employed in the study were all different (Table 2); that of Reyn contained the highest amount and that of Thayer the lowest amount of protein, the only protein source in the latter being 1.0% of haemoglobin. It has been shown[1] that penicillin is readily adsorbed on serum proteins, especially on albumin, possibly resulting in both *in vitro* and *in vivo* inactivation of the drug. It was natural to ascribe the difference in level of sensitivity to the fact that in Reyn's medium part of the penicillin is bound to albumin originating from the horse blood and from the ascitic fluid. When the Reyn medium was compared with the other two media, the less sensitive strains required relatively more penicillin for inhibition than did the sensitive strains.

It was later shown (Reyn et al., 1965) that a medium, prepared with 0.45-1.0% of haemoglobin as the only protein source (Møller & Reyn, 1965) and which supported the growth just as well as did the Reyn medium, resulted in nearly as high MIC values as did the usual Reyn medium.[2] In this case also, a difference was observed between the sensitive and less sensitive strains, but it was much smaller. Furthermore, it has been shown by Wilkinson (unpublished data) that his tube method and Reyn's plate method (omitting the ascitic fluid from the medium) showed a difference in the level, but that in this case the difference was about the same for the less sensitive strains as for the sensitive strains. (Wilkinson used the same fifteen strains as those used in the present experiments). The results of the Thayer and Wilkinson methods were in close agreement, in spite of the fact that a solid medium is used in the former and a fluid one in the latter, and that the two media used contain different kinds of protein, haemoglobin and hydrocele fluid. However, other factors than proteins may also play a role and further studies are needed. At present, we can only state that differences were observed and keep this observation in mind. This problem is also discussed by Reyn et al. (1965).[3]

The number of strains studied by all three methods was limited, and the strains employed were mainly older laboratory strains which had been repeatedly subcultured, involving the risk of the development of changed growth requirements, etc. In an additional experiment, in which the Reyn method was used for comparing Reyn's and Thayer's media, 21 more recently isolated strains were employed and in this case also a difference between the sensitive and the less sensitive strains was observed (Table 2), but it should be added that the difference was not statistically significant. However, as already mentioned a similar (but smaller) and statistically significant difference has been demonstrated by Reyn et al. (1965),[3] who used more than 200 fresh strains for the comparison. They also found that the variance of the sensitive strains was greater than that of the less sensitive strains, both variances being greater than that of the old control strains.

In order to achieve greater uniformity in the results, two steps could be envisaged: (1) the development of a *reference method* in which the technique and medium are standardized and/or (2) the inclusion of *reference strains* in the testing procedures. No synthetic medium is satisfactory and at the present stage it is only possible to specify a provisional medium. Therefore, the second step should be considered.

Reference strains are thought to be useful because when the strains used in the present study were arranged in order of sensitivity the order was the same whichever of the three methods was used. In the Scandinavian study, in which a diffusion

[1] See: Chow & McKee (1945); Tompsett et al. (1947); Oeff et al. (1955); Anderson et al. (1955-56); Lightbown & Sulitzeanu (1957); Marner & Lund (1957); Gourevitch et al. (1960); Bunn & Knight (1961); Yakobson & Grigoreva (1961); Bond et al. (1963).

[2] See the articles on page 471 and page 503 of this issue.

[3] See the article on page 503 of this issue.

method was compared with one of the dilution methods included in the present study, the same observation was made and the use of three reference strains was recommended.

When the present results are considered, it is evident that *the application of an average correction determined by means of reference strains cannot bring about complete identity of the results with all three media throughout the whole range of sensitivities.*

In principle, this may be achieved by the use of several reference strains which cover the range of sensitivities encountered. For example, the results presented in Fig. 6 might be used to convert the results obtained by Wilkinson's method into those obtained by Reyn's method. In this way, systematic differences would be eliminated, but at the same time a certain " conversion error " would be introduced, owing partly to random errors (day-to-day variation) and partly to variation between strains.

If the experimental errors of the different methods were known, it would be possible to decide upon a certain number of strains and a certain number of test days on which both methods should be used side by side in order to ascertain the accuracy wanted in the conversion. In practice, however, it is necessary to limit the number of strains. Thus, three strains of differing sensitivity were recently recommended as a result of the Scandinavian study (Reyn et al., 1963). Of course, these strains will necessarily be stock strains and greater variation in the conversion is to be expected when freshly isolated strains are tested (Reyn et al., 1965).[1]

All the same, the differences due to this variation are hardly of any importance in relation to the differences in level of sensitivity observed in different countries. Thus, a conversion by means of reference strains would apparently permit a reasonable comparison of the sensitivity levels in such countries.

Good agreement was obtained when the laboratories tried to copy the techniques used by the other laboratories. The mean difference between the results obtained with the same method performed in different laboratories ranged from about 0.06 (Reyn method performed by Thayer) to −0.20 (Reyn method performed by Wilkinson, MIC results). Thus, a standardized technique should have a good chance of success, provided that the difficulty of standardizing the medium can be overcome. Hence, efforts should be made to specify the medium and the technique as closely as possible, to study the relevant factors responsible for the observed differences, and to extend future experiments to include more resistant strains—both those that occur naturally (if possible) and strains made artificially resistant.

[1] See the article on page 503 of this issue.

RECOMMENDATIONS

On the basis of the results of the present study and those recently obtained in the Scandinavian study, an *international preliminary reference method* employing a *provisional medium* is proposed for use in epidemiological studies and for the evaluation of locally employed quantitative methods. The reference method should be a dilution method and should include the testing of common *international reference strains* covering the range of sensitivities encountered in practice. This would make possible the cross-checking of results obtained with the same method in different laboratories.

Choice of method

As already mentioned, this should be either a tube or a plate dilution procedure; both methods have advantages and disadvantages, depending on the circumstances.

(*a*) *Tube dilution.* This procedure has certain advantages. Comparison of the present techniques used by Reyn and Thayer for the preparation of the antibiotic-containing plates with the procedure used by Wilkinson for the preparation of his fluid medium indicates that it is perhaps easier and more economical to prepare this medium in small batches than it is to prepare a small series of plates. It also is possible to add the penicillin immediately before use, so that no inactivation due to storage can take place. However, in this method the reading of the results is more laborious than in the plate dilution method. The reading has to be made after a constant period of incubation (48 hours) and IC_{50} values cannot be obtained. The addition of phenol-red facilitates the reading, and the colour change due to the fermentation of glucose is also of some value in distinguishing between pure growth and contamination. However, it is necessary to control the results by Gram-staining and preferably also by platings. Recently, in the hands of experienced workers, this method (in Wilkinson's modification) has given useful information in the experimental study performed in Great Britain by the Medical Research Council working party.

(*b*) *Plate dilution.* For this method, it is most practical to prepare relatively large batches of medium at a time. According to experience, plates containing penicillin will give constant results when kept for less than a week at about 4°C. It might be necessary, when used in a reference method, to stipulate three or four days as the maximum storage time. The plates have the advantage that six results can be read at a time and that the distribution of the colonies in accordance with their individual sensitivities can be directly observed. Contamination is easier to control and if a " rich " medium is used the reading can be performed after only 20-24 hours' incubation; the results do not change if the plates are incubated for 48 hours or longer. In the Scandinavian study, the plate dilution method (in Reyn's modification) has given reproducible results when used in different laboratories, the IC_{50} values being somewhat more uniform than the MIC values. A similar observation was made in the present study.

Used on the gonococcal strains at present in circulation it is a common feature of the two methods that inoculum variation does not influence the results to any important degree; this would not be the case if penicillinase-producing strains should appear. Another common feature of the methods is the difficulty of giving a specification for the medium.

Thus, it is concluded that a *plate dilution* procedure should be used in the proposed reference method. At present this reference method cannot be described in detail, mainly because it is not as yet possible completely to specify a provisional medium which contains a minimum of proteins and which is enriched with haemoglobin and liver and yeast autolysates (Reyn, 1965; Møller & Reyn, 1965).[1]

It is very difficult, if not impossible, to decide upon the source of agar to be used in the provisional medium; control procedures should be described, especially for the gel strength and the toxicity of the agar. A simple apparatus for the measurement of gel strength has recently been developed by Møller, but the toxicity of the agar can be estimated only in bacteriological experiments with different batches of agar. The specification of the broth (which should not necessarily contain peptone) and the salts (to control pH and osmotic pressure) is not particularly difficult; glucose should most probably not be added.

In this sketchy attempt at proposing a medium, care has been taken to reduce the use of commercial products to a minimum. The salts, the agar and the haemoglobin are the exceptions, but it should be possible to develop a simple recipe for the preparation of haemoglobin from horse blood or from some other source of animal blood.

Choice of preliminary reference strains

It was shown in the Scandinavian study (Reyn et al., 1963) that *Sarcina lutea* ATCC 9341 and *Staphylococcus aureus* FDA 209 P varied more than did the gonococcal strains, and it was observed in the present study that *Staphylococcus aureus* FDA 209 P gave consistently higher values in the tube dilution method than in the plate dilution method, the opposite being the case for the gonococcal strains. If is, therefore, preferable to use gonococcal strains as reference strains and the following three strains (also recommended in the Scandinavian study), are proposed: Scandinavian study Nos. III, V and VII. A stock of ampoules containing lyophilized cultures of these strains is stored at the Statens Seruminstitut, Copenhagen.

It should be added that the first report of the WHO Expert Committee on Gonococcal Infections (1963) recommends that " international reference methods be established through WHO for the determination of sensitivities of circulating strains of the gonococcus to various drugs, initially penicillin and streptomycin; and that inter-laboratory assays be continued between the competent reference laboratories in Chamblee, London, Paris and Moscow, with the Neisseria Centre in Copenhagen acting as the WHO reference laboratory; the latter should also function as a bank for established international reference strains needed for the definition of provisional and definitive reference methods."

[1] See the articles on page 449 and page 471 of the present issue.

ACKNOWLEDGEMENT

Dr Reyn and Dr Wilkinson wish to record their thanks to Mrs L. Olesen and to Miss M. Joan Whittington for their valuable help in the performance of the experiments carried out in Copenhagen and in London, respectively.

RÉSUMÉ

L'apparition, depuis 1957, de souches de gonocoques moins sensibles à la pénicilline pose de sérieux problèmes épidémiologiques et thérapeutiques et a rendu nécessaire une détermination quantitative de la sensibilité de *Neisseria gonorrhoeae* à cet antibiotique. Plusieurs laboratoires se sont attachés à cette étude, mais les résultats suivant les pays ont montré des divergences, portant à la fois sur la gamme des sensibilités et sur la position respective au sein de cette gamme de différentes souches de gonocoques, qu'il était difficile d'attribuer à des différences réelles entre souches plutôt qu'à la diversité des méthodes.

Dès 1959, un Comité OMS d'experts des maladies vénériennes et des tréponématoses soulignait l'intérêt d'une méthode internationale de référence pour l'évaluation comparative de la sensibilité des souches de *N. gonorrhoeae* à la pénicilline. Le présent travail donne les résultats d'une étude inter-laboratoires due à la collaboration de microbiologistes du Danemark, des Etats-Unis d'Amérique et du Royaume-Uni, et menée sous le patronage de l'OMS, pour tenter de définir une telle méthode.

Dans trois laboratoires de ces pays, on a recherché la sensibilité à la pénicilline G d'un lot commun de 14 souches de *N. gonorrhoeae*, de sensibilités très différentes, et dans certains essais comparatifs, la sensibilité de *Sarcina lutea* ATCC 9341, *Oxford Staphylococcus* et *Staphylococcus aureus* FDA 209 P. Deux méthodes de dilution sur plaque, celles de Reyn (Copenhague) et de Thayer (Chamblee), et une méthode de dilution en tube (Wilkinson, Londres) furent utilisées. Les milieux d'essais étaient différents, celui de Reyn ayant la plus forte teneur en protéines, celui de Thayer la plus faible. Chaque laboratoire employa, outre sa technique habituelle, une ou deux autres méthodes. Les résultats furent donnés par la mesure de la concentration inhibitrice minimum (CIM) et de la concentration inhibitrice 50% (CI_{50}), exprimées en unités internationales de pénicilline G par ml.

Le classement des souches obtenu par ces différentes méthodes fut quasiment identique, mais on nota cependant des écarts dans les niveaux de sensibilité: les plus importants donnaient un rapport de 1-6 entre les méthodes de Reyn et de Wilkinson et de 1-2 et 1-3 entre les méthodes de Reyn et de Thayer. La comparaison des méthodes de Wilkinson et de Thayer montra des résultats plus uniformes. Les divergences entre les niveaux de sensibilité étaient surtout accentuées pour les souches les moins sensibles. D'autre part, en employant une technique identique mais des milieux différents, on démontra que les variations entre les méthodes de Thayer et de Reyn devaient être attribuées en majeure partie aux particularités des milieux employés. La concordance fut satisfaisante lorsqu'un laboratoire utilisa la technique habituelle d'un autre: le rapport entre les CIM ainsi obtenues varia de 1,2 (méthode de Reyn appliquée par Thayer) à 1,6 (méthode de Reyn mise en œuvre par Wilkinson).

A l'opposé de *N. gonorrhoeae*, *Staphylococcus aureus* FDA 209 P montra constamment des niveaux de sensibilité plus élevés par la méthode de dilution en tube que par la méthode de dilution sur plaque.

Les résultats sont discutés et la nécessité de poursuivre les recherches est soulignée. Il faudrait utiliser, lors des essais ultérieurs, des souches récemment isolées, et des souches plus résistantes (naturellement ou artificiellement). Certaines, de sensibilité connue, seraient sélectionnées comme souches de référence, ce qui permettrait d'introduire dans les déterminations effectuées par des laboratoires différents un indispensable facteur de correction.

Les auteurs proposent pour la méthode de référence provisoire la technique de la dilution sur plaque, plus avantageuse, et énumèrent un certain nombre de qualités que l'on doit exiger d'un milieu standard. Enfin, ils suggèrent de choisir, comme souches de références, trois souches de gonocoques conservées, sous forme lyophilisée, au Statens Seruminstitut de Copenhague.

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Appendix Table 1

SURVEY OF METHODS

Reference	Country	Method [a]	Medium
Cohn & Seijo (1943, 1944)	USA	Tube and plate dilution	Inactivated citrated human blood
Frisch et al. (1944)	USA	Plate dilution	Chocolate agar
Bahn et al. (1945)	USA	Tube dilution	Douglas broth + 5 % of rabbit broth
Carpenter et al. (1945)	USA	Tube dilution	Douglas broth + 5 % of rabbit broth
Lankford (1945)	USA	Plate dilution	Proteose No. 3 agar (Difco) + 5 % of starch
Meads et al. (1945)	USA	Tube dilution	Broth with 1 % horse blood
Miller & Bohnhoff (1945)	USA	Plate dilution	Trypsine digest of casein + 10 % of rabbit blood
Nell & Hill (1947)	USA	Tube dilution	Beef heart infusion broth
Romansky & Robin (1947)	USA	(1) Tube dilution	(1) Inactivated human blood in Alsever's solution
		(2) Plate dilution	(2) Proteose peptone starch agar
Hawks & Grey (1948)	Canada	Plate dilution	Solid media, not specified
Hughes & Carpenter (1948)	USA	Tube dilution	Douglas broth + 5 % of rabbit blood
Cohn et al. (1949)	USA	Plate dilution	Peizer's medium
Mills (1949)	USA	Plate dilution	Proteose No. 3 haemoglobin agar (Difco)
Storck et al. (1949)	Switzerland	Tube dilution	Semi-solid agar + 1 % of glucose, 0.06 % of phenol red, and ascitic fluid
Zierz & Eckert (1949)	Germany	Plate dilution	Neumann's 20 % " blood-water " agar
Gocke et al. (1950)	USA	(1) Tube dilution	(1) 10 % horse-blood agar
		(2) Plate dilution	(2) 20 % sheep-blood agar
Golotina et al. (1950)	USSR		
Hagerman (1950)	Sweden	Diffusion (" drops on plates ")	Chocolate agar or other " appropriate " medium
Schümmer & Hubbes (1951)	Germany	Plate dilution	Neumann's 20 % " blood-water " agar
Gartmann & Knapp (1954)	Germany	Plate dilution	Neumann's 20 % " blood-water " agar
Marcuse & Hussels (1954)	Germany	Plate dilution	25 % ascitic-fluid-glucose plates
Del Love & Finland (1955)	USA	Plate dilution	Heart-infusion agar
Marchionini & Röckl (1957)	Germany	Tube dilution	Semi-solid ascitic-fluid-agar
Thayer et al. (1957)	USA	Tube dilution	Semi-solid trypticase phytone (BBL) + phenol red
Anderson & Troyanosky (1958)	USA	Tube dilution Plate dilution	GC base with haemoglobin and supplement B (Difco)
Cradock-Watson et al. (1958)	Great Britain	Plate dilution	Hydrocele chocolate agar (10 % horse blood)
Curtis & Wilkinson (1958)	Great Britain	Tube dilution	Hydrocele broth
Reyn et al. (1958)	Denmark	Plate dilution; diffusion; tablets (RMC [b]), 25 IU	Chocolate agar (6.7 % of horse blood); 10 % of ascitic fluid (not 30 % as originally used by a mistake)

[a] All authors using dilution methods (except Reyn et al.) express their results in terms of minimum inhibitory concentrations (MIC).

[b] Roskilde Medical Company (now know as " Rosco ").

Appendix Table 1 (*continued*)

SURVEY OF METHODS

Reference	Country	Method	Medium
Barile et al. (1959)	USA	Tube and plate dilution	Bacto brain-heart + haemoglobin and yeast extract (Difco)
Röckl (1959)	Germany	Tube dilution	Semi-solid broth-agar + 30 % of ascitic fluid and 1 % of glucose
Ujvary et al. (1959)	Romania	Diffusion (Tablets ? units)	Peizer-Steffen's medium
Hirsch & Finland (1960)	USA	Plate dilution	Heart-infusion agar
Mead et al. (1960)	USA	Diffusion (2 units); Sensi-discs (BBL)	Chocolate agar
Ødegaard (1960)	Norway	Plate dilution	Chocolate agar + 8 % of horse blood
Anderson (personal communication, 1961)	USA	Plate dilution	GC base with haemoglobin and Supplement B (Difco)
Cole et al. (1961)	USA	Tube and plate dilution	Semi-solid trypticase + chocolate agar; both media enriched with Supplement C (Difco)
McCarthy et al. (1961)	USA	Plate dilution	Chocolate agar
Great Britain, Medical Research Council (1961)	Great Britain	Tube dilution; [a] plate dilution	Various media
Reyn (1961)	Denmark	Diffusion; tablets (RMC), 25 IU; plate dilution	Chocolate agar + 6.7 % of horse blood + 10 % of ascitic fluid
Ringertz (1961)	Sweden	Diffusion; paper discs, 20 IU (H. Ericsson)	Chocolate agar + 8.5 % of horse blood + 7.1 % of horse serum
Roiron et al. (1961)	France	(1) Plate dilution (2) Diffusion	Beef-broth-gelatin + 20 % of horse plasma
Letchner & Nicol (1961)	Great Britain	Plate dilution	Chocolate agar + hydrocele fluid
Thayer et al. (1961)	USA	Plate dilution	Proteose agar Special + haemoglobin and Supplement C (Difco)
Ziprkowski et al. (1961)	Israel	Not known [b]	Not known [b]
Kaiser & Rinderknecht (1961)	Switzerland	Plate dilution	Ascitic agar
Bakker et al. (1962)	Netherlands	Plate dilution	Levinthal agar + 10 % of ascitic fluid
Gjessing & Ødegaard (1962a)	Norway	Diffusion; paper discs, 20 IU	Chocolate agar + 8 % of horse blood
Rantasalo et al. (1962)	Finland	Diffusion; paper discs, 20 IU	Chocolate ascitic-fluid-agar
Röckl (1962)	Germany	Tube dilution	Semi-solid broth-agar + 30 % of ascitic fluid and 1 % of glucose
Reyn et al. (1963)	Denmark, Finland, Norway, Sweden	Plate dilution; paper discs, 2 IU, 20 IU	Various chocolate agar types
Snell et al. (1963)	Canada	Plate dilution	Chocolate agar

[a] Various techniques.

[b] Paper published in Hebrew.

Appendix Table 2

RESULTS OF EXPERIMENTS PERFORMED ON 24 AUGUST 1960 BY REYN, COPENHAGEN

	Strain Nos,	Inhibitory concentrations of penicillin (IU/ml)			
		R-R [a]		RT-R [b]	
		IC_{50} (1)	MIC (2)	IC_{50} (3)	MIC (4)
Control 1	50700	0.25	0.50	0.088	0.125
Sensitivity group 1 (less sensitive strains)	61001/02	0.21	0.50	0.044	0.125
	60849/50	0.149	0.25	0.088	0.125
	60897	0.149	0.25	0.044	0.125
	61080	0.088	0.125	0.053	0.125
	61525	0.125	0.25	0.022	0.063
	61526/27	0.35	0.50	0.053	0.125
	57129	0.35	0.50	0.088	0.125
	57977	0.71	1.00	0.21	0.50
	59199	0.35	0.50	0.088	0.125
	60071/72	0.21	0.50	0.088	0.125
Control 2	43562	0.0111	0.0156	0.0111	0.0156
Sensitivity group 2 (more sensitive strains)	40818	0.0093	0.0156	0.0046	0.0078
	61000	0.0111	0.0156	0.0039	0.0078
	61132	0.0111	0.0156	0.0055	0.0078
	61263	0.0186	0.031	0.0039	0.0078
	61374	0.0078	0.0156	0.0046	0.0078
	61523	0.0093	0.0156	0.0020	0.0039
	61524	0.0111	0.0156	0.0055	0.0078
	61528	0.0156	0.031	0.0093	0.0156
	61678	0.0055	0.0078	<0.0020	<0.0020
	56467	0.044	0.063	0.022	0.063
	60105	0.0156	0.031	0.0078	0.0156
	Sarcina lutea ATCC 9341	0.0046	0.0078	0.0039	0.0078

[a] Reyn method by Reyn.

[b] Reyn method, Thayer medium by Reyn.

Appendix Table 3

RESULTS OF EXPERIMENTS PERFORMED BY REYN, COPENHAGEN AND BY THAYER, CHAMBLEE

	Strain Nos.[a]		Inhibitory concentrations of penicillin (IU/ml)						
			R-R [b]		RT-R [c]		R-R [b]	RR-T [d]	T-T [e]
			IC_{50} (1)	MIC (2)	IC_{50} (3)	MIC (4)	MIC (5)	MIC (6)	MIC (7)
Control 1	50700		0.21	0.50	0.085	0.13	0.50	0.50	
Sensitivity group 1 (less sensitive strains)	P-1	I	0.35	0.50	0.125	0.25	0.50	1.0	0.15
	P-5	II	0.088	0.125	0.063	0.125	0.125	0.25	0.05
	P-28		0.21	0.50	0.125	0.25	0.50	0.50	0.10
	P-35		0.35	0.50	0.125	0.25	0.50	1.0	0.15
	P-58		0.35	0.50	0.177	0.25	0.50	1.0	0.20
	P-78	IV	⩾0.71	>0.50	0.25	0.50	1.0	>2.0	0.30
	P-83	V	0.59	>0.50	0.21	0.50	1.0	2.0	0.30
	P-92		0.177	0.25	0.088	0.125	0.25	0.50	0.10
	12990	VIII	⩾0.71	>0.50	0.30	0.50	2.0	2.0	0.30
Control 2	43562		0.0111	0.0156	0.0093	0.0156	0.0156	0.0156	0.01
Sensitivity group 2 (more sensitive strains)	P-26	III	0.063	0.125	0.025	0.063	0.063	0.125	0.05
	P-82		0.0093	0.0156	0.0066	0.0156	0.0156	0.0156	0.005
	C-123		0.0078	0.0156	0.0046	0.0078	0.0156	0.0078	0.005
	11413	VI	0.0078	0.0156	0.0039	0.0078	0.0156	0.0039	0.005
	11421	VII	0.0093	0.0156	0.0055	0.0078	0.0156	0.0078	0.005
	12954		0.0111	0.0156	0.0111	0.0156	0.0156	0.0156	0.01
	17732	IX	0.0111	0.0156	0.0111	0.0156	0.031	0.0156	0.005
	17769		0.0093	0.0156	0.0078	0.0156	0.031	0.031	0.01
	17941		0.0125	0.031	0.0111	0.0156	0.031	0.031	0.01
	43354		0.0078	0.0156	0.0020	0.0039	0.0156	0.031	0.01
	Sarcina lutea ATTC 9341		0.0066	0.0156	0.0055	0.0156		0.0156	0.005

[a] The Roman figures are the strain numbers used in the Scandinavian study.
[b] Reyn method by Reyn (Column 5: 15/6 1960).
[c] Reyn method, Thayer medium by Reyn.
[d] Reyn method, Reyn medium by Thayer (Column 6: 1961).
[e] Thayer method by Thayer (Column 7: 1961).

Appendix Table 4

RESULTS OBTAINED BY WILKINSON, LONDON

Experiment	Age of medium [a]	Strain Nos.[b]	Inhibitory concentrations of penicillin (IU/ml)		
			Plate dilution		Tube dilution
			MIC [c]	IC_{50}	MIC [c]
I	CA 2 days	I	1.0	0.50	0.125
	TSB 2 days	II	0.25	0.105	0.016
		III	0.125	0.088	0.063
		IV	>2.0 [d]	1.19	0.5
		W I	1.0	0.42	0.125
		W III	1.0	0.50	0.063
		Oxford *Staphylococcus*			24 h: 0.063 48 h: 0.125
II	CA 1 day	I	0.5	0.35	0.063
	TSB 2 days	V	2.0	0.84	0.25
		VI	0.016	0.0066	0.004
		VII	0.016	0.0093	0.004
		W I	1.0	0.71	0.25
		W IV	0.032	0.0131	0.004
		Oxford *Staphylococcus*			24 h: 0.063 48 h: 0.125
IIIa	CA 3 days	I	1.0	0.5	0.125
	TSB 3 days	VIII	2.0	0.59	0.125
		IX	0.032	0.022	0.016
		X	0.032	0.0186	0.004
		W I	1.0	0.35	0.125
		W V	>2.0	1.68	0.25
		Oxford *Staphylococcus*			24 h: 0.063 48 h: 0.125
IIIb	CA 1 day	I	1.0	0.59	0.25
	TSB 4 days	VIII	1.0	0.35	0.125
		IX	0.032	0.022	0.008
		X	0.032	0.0186	0.008
		W I	0.5	0.30	0.125
		W V	0.5	0.21	0.5
IVa	CA 2 days	I	1.0	0.59	0.125
	TSB 2 days	II	0.25	0.125	0.125
		III	0.125	0.088	0.063

For footnotes, see next page.

Appendix Table 4 (*continued*)

RESULTS OBTAINED BY WILKINSON, LONDON

Experiment	Age of medium [a]	Strain Nos.[b]	Inhibitory concentrations of penicillin (IU/ml)		
			Plate dilution		Tube dilution
			MIC [c]	IC_{50}	MIC [c]
IVa (continued)	CA 2 days	IV	>2.0	1.68	0.25
	TSB 2 days	W II	0.063	0.0186	0.008
		W III	1.0	0.59	0.125
		Oxford *Staphylococcus*			24 h: 0.063 48 h: 0.125
IVb	CA 1 day	I	1.0	0.71	0.125
	TSB 5 days	II	0.125	0.088	0.063
		III	0.063	0.037	0.032
		IV	0.25	0.125	0.25
		W II	0.032	0.0156	0.016
		W III	1.0	0.42	0.125
		Oxford *Staphylococcus*			24 h: 0.063 48 h: 0.125
V	CA 6 days	I	1.0	0.59	0.125
	TSB 1 day	V	>2.0	1.68	0.5
		VI	0.008	0.0055	0.004
		VII	0.016	0.0111	0.004
		W II	0.032	0.0156	0.008
		W IV	0.063	0.0186	0.008
		Oxford *Staphylococcus*			24 h: 0.063 48 h: 0.125
VI	CA 3 days	I	2.0	0.71	0.125
	TSB 3 days	VIII	2.0	0.71	0.125
		IX	0.063	0.026	0.016
		X	0.063	0.037	0.008
		W II	0.063	0.022	0.016
		W V	>2.0	0.841	0.5
		Oxford *Staphylococcus*			24 h: 0.063 48 h: 0.125

[a] CA = Time from preparation of chocolate ascitic-fluid plates with penicillin.
TSB = Time from preparation of batch of tryptose-soya-glucose-hydrocele broth. Penicillin incorporated in this at the time of the test.

[b] Oxford *Staphylococcus*: This is put up with each batch of tube tests as a check on the accuracy of the dilution.

[c] 48 hours.

[d] >2.0 is included in the calculations as 4.0, because this value is the lowest concentration which, in a prolonged twofold dilution row, would give complete inhibition.

Appendix Table 5

RESULTS OBTAINED BY REYN, COPENHAGEN

Experiment	Age of medium[a]	Strain Nos.	Inhibitory concentrations of penicillin (IU/ml)			
			Plate dilution [b]		Tube dilution	
			MIC	IC_{50}	MIC (72 h)	MIC (48 h)
I [c]	CA 4 days	I	0.50	0.35	0.125	0.125
	TSB 4 days	II	0.25	0.105	0.125	0.125
9/1/62		III	0.125	0.074	0.031	0.031
		IV	2.0	1.19	0.25	0.25 (0.125?)
		W I	1.0	0.50	0.25	0.25
		W III	1.0	0.42	0.125	0.125
II	CA 7 days	I	0.50	0.35	0.125	
	TSB 7 days	V	2.0	1.0	0.125	
12/1/62		VI	0.0078	0.0033	0.0078	
		VII	0.0078	0.0055	contaminated	
		W I	1.0	0.50	0.25	
		W IV	0.0156	0.0066	0.0156	
III	CA 1 day	I	1.0	0.42	0.125	
	TSB 3 days	VIII	2.0	0.84	0.50	
19/1/62		IX	0.0156	0.0111	0.0156	
		X	0.0156	0.0111	0.0078	
		W I	1.0	0.50	0.25	
		WV	2.0	1.0	0.50	
IV	CA 5 days	I	0.50	0.25	0.125	0.063
	TSB 7 days	II	0.125	0.088	0.125	0.125
23/1/62		III	0.063	0.044	0.063	0.063
		IV	2.0	0.84	0.25	0.25
		W II	0.0156	0.0111	0.0156	0.0078
		W III	0.50	0.25	0.25	0.063
V	CA 4 days	I	1.0	0.50	0.125	0.125
	TSB 4 days	V	2.0	0.84	0.25	0.125
27/1/62		VI	0.0156	0.0066	0.0078	0.0078
		VII	0.0156	0.0093	0.0078	0.0078
		W II	0.0156	0.0111	contaminated	
		W IV	0.0156	0.0093	0.0156	0.0078
VIa	CA 8 days	I	1.0	0.50	0.125	0.125
	TSB 8 days	VIII	2.0	1.41	0.50	0.50
31/1/62		IX	0.031	0.0156	0.0156	0.0078
		X	0.0156	0.0111	0.0078	0.0078
		W II	0.031	0.0156	0.0156	0.0156
		W V	2.0	1.41	0.50	0.25
VIb	—	II	0.25	0.177	0.125	0.125
		III	0.125	0.088	0.031	0.031
31/1/62		W III	1.0	0.59	0.25	0.25
		VII	0.0156	0.0093	0.0078	0.0078
I	—	Staph. [d]	0.063	0.031	0.25	0.125
II	—	Staph.	0.063	0.037	0.125	
III	—	Staph.	0.063	0.026	0.125 (0.25)	
IV	—	Staph.	0.063	0.026	0.125	0.063
V	—	Staph.	0.063	0.026	0.125	0.031
VI	—	Staph.	0.063	0.026	0.125	0.125

[a] CA = Time from preparation of chocolate ascitic-fluid plates.

TSB = Time from preparation of batch of tryptose-soya-glucose-hydrocele broth. Penicillin incorporated at the time of preparation. Tubes and plates were stored in the cold.

[b] 24-hour readings. The 72-hour readings were used in the results shown in Tables 6, 7 and 8 in the main text.

[c] Readings difficult in tube test. Tubes out of incubator for several hours after 48 hours' incubation.

[d] *Staphylococcus aureus* FDA 209 P.