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DEVELOPMENT OF STREPTOMYCIN RESISTANT STRAINS OF TUBERCLE BACILLI IN PULMONARY TUBERCULOSIS

RESULTS OF SIMULTANEOUS SENSITIVITY TESTS IN LIQUID AND ON SOLID MEDIA

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A series of cases of pulmonary tuberculosis has been treated with streptomycin under the auspices of the Medical Research Council. In some of the cases the antibiotic was given intermittently, a period of treatment alternating with a rest period. The results of this investigation will be published elsewhere. Sputa from a group of 18 of these patients treated at the Brompton Hospital, London, were investigated in greater detail in an attempt to obtain further information about the strains of tubercle bacilli excreted during the development of streptomycin resistance. The sputa were cultured directly on solid medium containing streptomycin in various concentrations and sensitivity tests in liquid medium were done at the same time.

Method of Investigation

The patients were aged from 17 to 30 years, and suffered from acute progressive bilateral pulmonary tuberculosis of recent origin. The condition of the disease on admission is comparable to that in an earlier series of patients (Medical Research Council, 1948a). There were 3 men and 15 women.

Dosage of Streptomycin.—Patients were divided into four treatment groups.

Group I.—Every six hours 0.5 g. was given intramuscularly. This was continued for a week and was then followed by a week without treatment. An alternation of a week's treatment and a week's rest period was continued over six months. This group contained four patients, Nos. 1–4.

Group II.—The same dosage was given for 28 days and was followed by 28 days without treatment. These alternating periods were continued for six months. This group contained four patients, Nos. 5–8.

Group III.—Every six hours 0.25 g. was given intramuscularly. This was continued without rest periods for six months. This group contained four patients, Nos. 9–12.

Group IV.—A single injection of 1 g. was given every day for six months. This group contained six patients, Nos. 13–18.

Sensitivity Tests.—Each week a specimen of sputum was collected over 24 hours. Its volume was measured and it was treated in a screw-capped bottle by adding three

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to four times its volume of 4% sodium hydroxide, shaking in a mechanical shaker for 10 minutes at 37° C., centrifuging for 20 minutes at 3,500 r.p.m., discarding the supernatant, and neutralizing the deposit with 6% sulphuric acid. About 10 ml. of M/20 phosphate buffer at *p*H 7.0 was added and the bottle was again centrifuged for about 30 minutes. The supernatant was discarded and 1 or 2 ml. of phosphate buffer was added together with a few glass beads. The bottles were then mechanically shaken for about 30 minutes and an even emulsion was usually formed. From this emulsion serial tenfold dilutions were made.

Herrold's glycerine egg agar medium (Herrold, 1931) was adjusted to a final *p*H of 7.3 and streptomycin was added to batches of it to make concentrations of 0, 1, 4, 16, 64, 256, and 1,024 μg. per ml. These were dispensed in 3 ml. amounts in sterile 3 × 1 in. flat-bottomed tubes plugged with cotton wool so that the agar formed a flat disc at the bottom of the tube. The tubes were placed in an incubator for about four to six hours to remove any water of condensation and were then corked and incubated for a further 18 hours as a sterility test. To each of two tubes at each streptomycin concentration a drop from a 50-dropping pipette (0.02 ml.) was added from the sputum emulsion and the process was repeated from a suitable number of the sputum dilutions, determined on the basis of the number of acid-fast bacilli seen in a smear made from the emulsion. The tubes were then sealed with corks dipped in paraffin wax and were incubated for eight weeks. The number of colonies present in each tube was counted. In this way a very rough viable count was made of the number of tubercle bacilli capable of growth in the various streptomycin concentrations. Accurate counting was often not possible because of the difficulty of distinguishing between individual colonies when the growth was of a spreading character. In a number of cases the concentration of bacilli in the sputum became too small to provide a sufficient number of colonies during the period when streptomycin resistance was appearing. Throughout the period of testing there was a falling, but still rather high, contamination rate, in part due to organisms surviving in the inoculum. Most contaminatory organisms were fungi.

The remainder of the sputum emulsion was inoculated on to slopes of Lowenstein-Jensen medium. When growth occurred on these a sensitivity test in Tween 80-albumen medium was performed according to the technique recommended by the Medical Research Council (1948b). Tests were read after ten days' incubation. The levels of sensitivity to streptomycin among resistant strains appear to occur in three main groups (Mitchison, 1949) and strains falling into these groups are called of low, medium, or high degrees of resistance. A strain of low degree of resistance is four to eight times less sensitive than H37Rv, the standard strain. A strain of medium degree of resistance is 32–128 times less sensitive than H37Rv, and a strain of high degree of resistance is capable of growing in at least 1,000 μg. streptomycin per ml., the highest concentration used.

During part of the period tests on solid medium were performed once a fortnight only, and owing to holidays and other interruptions, there were short periods when no tests were performed.

Effective Concentration of Streptomycin in Herrold's Medium.—The distribution of streptomycin between Herrold's medium and water in contact with it and the degree to which streptomycin is destroyed by long periods of incubation at 37° was investigated as follows:

Streptomycin was incorporated in the medium in concentrations of 0, 4, 16, 64, 256, and 1,024 μg. per ml.; 8 ml. of each concentration was added to a sterile, screw-capped "medicine flat" bottle and was allowed to set with the bottle lying on its side, thus creating a layer 3–4 mm. thick. A further 8 ml. of sterile distilled water, containing

TABLE I

STREPTOMYCIN CONCENTRATION IN WATER AFTER INCUBATION WITH AN EQUAL VOLUME OF MEDIUM CONTAINING STREPTOMYCIN

Original Concentration of Streptomycin in Herrold Medium (μg./ml.)	Streptomycin Concentration in Water (μg./ml.: Period of Incubation in Days)				
	3		7		43
	Experiment 1	Experiment 2	Experiment 1	Experiment 2	Experiment 1
1,024	705	382	594	166	71
256	94	50	82	31	11
64	31	20	18	8.7	3.8
16	6.3	4.1	5.4	2.4	

no streptomycin, was then added to each bottle. Three such bottles were set up from each streptomycin concentration and they were incubated lying flat at 37° C. After three days, when one might expect a stable distribution between the medium and the distilled water, one set of bottles was taken and the streptomycin in the water assayed against standards made up from the bottle containing no streptomycin by the method of Mitchison and Spicer (1949). Further assays were done after seven and 43 days' incubation. The results of two such experiments are given in Table I. They indicate a fall in the streptomycin concentration over a period of six weeks, so that the final concentration is only about 1/10 of its original value. Furthermore there appears to be some tendency for streptomycin to be bound in the medium, although the results of these and other similar experiments indicate that this may occur to a variable extent.

RESULTS

Pre-treatment Strains.—As reported previously (Mitchison, 1949) strains tested in the liquid medium were usually inhibited by 0.5 or 0.25 μg. streptomycin per ml. They were inhibited by a concentration twice (two times less sensitive), half (two times more sensitive) or the same as (equal) the least concentration which inhibited the standard strain H37Rv whose sensitivity was always tested under the same conditions.

On the solid medium growth in tubes containing 1 μg. per ml. was usually similar to that on medium without streptomycin. There were occasional colonies on the medium containing 4 μg. per ml. but none on medium with higher concentrations. The difference between the inhibitory concentrations on solid and liquid media may be explained partly by the presence of Tween 80 in the liquid medium, which tends to increase the sensitivity of strains to streptomycin (Fisher, 1948a; Williston and Youmans, 1949), and partly by the tendency of streptomycin to be bound in the solid medium and gradually to fall in concentration as described above.

Resistant Strains.—During the course of treatment the sensitivity of strains from all the patients showed some change. There was a fairly sharp distinction between two groups. In one of these (10 patients, Nos. 1, 2, 4, 6, 7, 9, 10, 15, 16, 18) the strains developed only a low degree of resistance, becoming on the average four to eight times less sensitive than H37Rv when tested in liquid medium. On the solid

TABLE II

DEVELOPMENT OF LOW DEGREE OF STREPTOMYCIN RESISTANCE DEMONSTRATED ON SOLID AND LIQUID MEDIA IN CASE 10

Days After Starting Treatment	Sensitivity in Liquid Medium × H37Rv	Log_{10} No. of Organisms on Solid Medium without Streptomycin	"Resistant Fraction": Number of Organisms on Medium with Streptomycin/ Number on Medium without Streptomycin (Streptomycin Concentration μg./ml.)					
			1	4	16	64	256	1,024
0	Equal	5.06	1.12	<0.0005				
7	,,	4.04	0.95	<0.005				
17	2× less sensitive	4.30	0.32	0.016	<0.0003			
24	Equal	4.23	0.62	0.006	<0.003			
31	,,	4.30	0.38	<0.001				
49	,,	3.10	0.96	0.02*	<0.01			
67	8× less sensitive	2.55			0.18	<0.04		
89	4× ,, ,,	3.26	1.00	1.07	0.20	<0.007		
115	8× ,, ,,	5.04	1.00	0.66	0.11	<0.0001		
122	4× ,, ,,	4.40	0.85	1.00	0.76	<0.0005		
129	4× ,, ,,	5.72	0.86	0.64	0.18	0.00005*	<0.00005	
136	8× ,, ,,	3.80	1.36	1.12	0.83		<0.003	
143		6.12	0.39	0.34	0.19	0.00002*	0.00001*	0.00002*
151	4× ,, ,,	5.61	0.49		0.50	<0.00006		
157	8× ,, ,,	5.96	0.67	1.10	0.47	<0.00001		
172	4× ,, ,,	2.44	0.45	0.60		<0.09		
178	8× ,, ,,	4.23	0.60	0.67	0.63	<0.001	<0.001	
186	2× more sensitive	4.35	1.56	1.33	0.80	<0.001	<0.001	
192	8× less ,,	4.53	0.41	1.19	<0.0004			
199	8× ,, ,,	5.98			0.24	<0.00003		
206		4.48	0.50		<0.008			

* Only one or two colonies present.

TABLE III

DEVELOPMENT OF A MODERATE DEGREE OF STREPTOMYCIN RESISTANCE DEMONSTRATED ON SOLID AND LIQUID MEDIA IN CASE 3

Days after Starting Treatment	Sensitivity in Liquid Medium ×H37Rv	Log_{10} No. of Organisms on Solid Medium without Streptomycin	"Resistant Fraction" Number of Organisms on Medium with Streptomycin/ Number on Medium without Streptomycin (Streptomycin Concentration μg./ml.)					
			1	4	16	64	256	1,024
0	Equal	6.10	0.92	<0.00008				
9	,,	3.70	0.60	<0.005				
23	4× less sensitive	3.35	0.53	0.022*	<0.01			
30	4× ,, ,,	3.63	0.27	0.29	<0.01			
51	4× ,, ,,	4.90		0.11	0.0013	0.0013	<0.00063	
72	4× ,, ,,	5.20	1.13	1.19	0.0064	0.0013	0.00016*	<0.00016
79	128× ,, ,,	4.70		0.045	0.035	0.030	0.025	<0.0005
86	128× ,, ,,	5.69		0.5	0.055	0.0045	0.0007	<0.00005
114	256× ,, ,,	5.49	1.04	0.96	?1.0	?1.0	?1.0	<0.00072
121	64× ,, ,,	5.65	1.06	0.50	0.56	0.50		<0.000028
156	256× ,, ,,	7.00	0.95	0.97	?1.0	?1.0	?1.0	0.0021
163	256× ,, ,,	5.70	1.02	1.08	1.35	0.90	0.42	<0.00005

* One or two colonies present only. ? This symbol indicates that this estimate of the fraction is only approximate.

TABLE IV

DEVELOPMENT OF HIGH DEGREE OF STREPTOMYCIN RESISTANCE ON SOLID AND LIQUID MEDIA

Days after Starting Treatment	Sensitivity in Liquid Medium ×H37Rv	Log_{10} No. of Organisms on Solid Medium without Streptomycin	"Resistant Fraction": Number of Organisms on Medium with Streptomycin/ Number on Medium without Streptomycin (Streptomycin Concentration μg./ml.)					
			1	4	16	64	256	1,024
0	2× more sensitive	8.10	1.33	<0.000001				
7	2× less ,,	7.78	0.95	0.000008	0.000003	<0.0000004		
14	Equal	5.40	0.70	0.002	<0.0002			
21	2× less sensitive	5.74		<0.000045				
49	512× ,, ,,	*	1.0	0.82	0.76	0.61	0.52	0.10
71	>1,000× ,, ,,	5.39	1.50	0.90	0.75	?1.0	?1.0	<0.0001
77	>1,000× ,, ,,	5.72	1.09	1.05	0.52	0.71	0.48	0.43
162	>1,000× ,, ,,	7.55	0.72	0.92	0.88	0.77	0.79	0.49

* The tubes without streptomycin were contaminated. The value of 1.0 has been assumed for the medium containing 1 μg. streptomycin/ml. ? Indicates that this estimate of the fraction is only approximate.

medium they were completely inhibited usually by 16 or 64 μg. streptomycin per ml. In one patient, although strains in liquid medium were four to eight times less sensitive than H37Rv, on solid medium growth was inhibited only by 256 μg. per ml. or more on a number of occasions. The average duration of treatment after which a strain four or more times less sensitive than H37Rv (liquid medium) was detected in this group was 63.6 days (range 44–75 days). Throughout the remainder of the period of treatment there was no tendency to develop any higher degree of resistance. An example of the results on one of these patients is given in Table II. In column 3, the $\log_{10}$ of the number of organisms growing on solid medium without streptomycin is shown. In each of the remaining columns a figure is given which is the ratio between the number of organisms growing on medium with the indicated streptomycin concentration and the number growing on the medium without streptomycin. This will be called the " resistant fraction." Where no colonies appeared the fraction which would have been detectable if present is indicated after the symbol $<$.

In the second group (seven patients, Nos. 3, 5, 8, 11, 12, 13, 14, 17) the strains developed a moderate or high degree of resistance, becoming at least 32 times less sensitive than H37Rv when tested in liquid medium. The average duration of treatment after which a strain four or more times less sensitive than H37Rv was detected in this group was 62.5 days (range 23–180 days). An example of the results on one of the patients in this group is given in Table III. In this patient a strain exhibiting a low degree of resistance first appeared on the twenty-third day of treatment. From the thirtieth day onwards growth on solid medium containing 4 μg. per ml. was of the same order as on medium without streptomycin. However, during the period from the fifty-first to the one hundred and fourteenth day of treatment a strain exhibiting a moderate degree of resistance appeared and eventually became predominant, this change on the solid medium being accompanied by a similar one in the liquid medium. From the one hundred and fourteenth to the one hundred and sixty-third day there was no apparent tendency to develop a highly resistant strain (one capable of growing in at least 1,024 μg. per ml.).

In some patients the development of a moderate or highly resistant strain occurred more rapidly. An example of results from a patient showing this is given in Table IV.

In this patient a strain obtained on the twenty-first day was still sensitive, both on solid and liquid media, whereas on the fourty-ninth day the strain was composed largely of highly resistant organisms. It should be noted that this patient received streptomycin up to the twenty-eighth day, but that, during the twenty-ninth to the fifty-sixth days, no streptomycin was given (treatment Group II).

One patient, Case 17, developed a low degree of resistance first appearing on the twenty-sixth day (liquid medium). On the seventy-fourth and ninety-sixth days a highly resistant strain appeared, which was demonstrated both in liquid and on solid medium. After this there again appeared strains with a low degree of resistance, except on one occasion on the one hundred and thirty-seventh day when a strain of a moderate degree of resistance appeared. A portion of the results from this patient is given in Table V.

Results in Liquid and on Solid Media Correlated.—In many strains tested a sharp end-point in the solid medium tests was indicated by a large drop in the

TABLE V

REPRESENTATIVE TESTS FROM CASE 17 SHOWING FLUCTUATIONS IN DEGREE OF SENSITIVITY SIMULTANEOUSLY ON SOLID AND IN LIQUID MEDIUM TESTS

Days after Starting Treatment	Sensitivity in Liquid Medium ×H37Rv	Log_{10} No. of Organisms on Solid Medium without Streptomycin	"Resistant Fraction": No. of Organisms on Medium with Streptomycin/ No. on Medium without Streptomycin. (Streptomycin Concentration μg./ml.)					
			1	4	16	64	256	1,000
0	Equal	5.63	0.71	<0.00006				
5	2× more sensitive	5.04	0.014	<0.0005				
12	Equal	3.30	0.50	<0.013				
19	,,	4.70	0.21	0.020	<0.001			
40	4× less sensitive	3.48	2.08	0.013	0.017	<0.004		
68	8× ,, ,,	3.18	1.67	2.33	0.112	<0.02		
74	>4,000× ,, ,,	2.60	0.25	0.56	0.38	0.25	0.033	0.094
96	>4,000× ,, ,,	4.13	0.74	0.81	0.62	0.83	0.95	0.63
116	4× ,, ,,	2.64	0.46		<0.057			
137	64× ,, ,,	2.35	0.89	0.56	0.39	0.50	0.11	0.11
144	4× ,, ,,	4.24	1.43	0.17	<0.0007			
151	4× ,, ,,	4.78	0.81	0.16	0.0046	0.00042*	<0.0042	
158	4× ,, ,,	3.40	0.70	0.28	<0.005			
179	4× ,, ,,	4.38	0.63	0.85	0.021	<0.00053		
186	2× ,, ,,	3.11	0.58	0.97	0.039	<0.01		
193	4× ,, ,,	3.80	0.68	0.64	0.13	0.006	<0.002	

* One colony present only.

number of bacilli capable of growth or by their absence at a particular streptomycin concentration. Among these it could be said that strains of a low degree of resistance in liquid medium were inhibited by 16, 64, or occasionally 256 μg. streptomycin per ml. in solid medium, and that strains of a moderate degree of resistance were either inhibited by 1,024 μg. streptomycin per ml. or grew at this concentration (the highest used). Strains of a high degree of resistance invariably grew on medium with 1,024 μg. streptomycin per ml.

In other strains the "resistant fraction" on solid medium became gradually smaller as the streptomycin concentration in the medium increased. To interpret the results of the liquid medium tests one would like to know the limiting value of the "resistant fraction" which would just cause growth in the corresponding tube of the liquid medium. However, since the bacteriostatic activity of streptomycin in liquid and solid media is not the same, and since there is considerable variability in the results by both methods, we do not know exactly which are the corresponding tubes. To overcome this difficulty the correlation between results on liquid and solid media was calculated for different values of the "resistant fraction" varying from 0.1 to 0.0001. The value of the "resistant fraction," yielding the best correlation, should be equal to the proportion of resistant to sensitive organisms which would just allow growth in liquid medium containing enough streptomycin to inhibit the sensitive population.

A system of scoring was used as follows:

LIQUID MEDIUM TESTS

Sensitivity Test Result	Score
4× more sensitive than H37Rv	0
2× ,, ,, ,, ,,	1
As sensitive as H37Rv	2
2× less sensitive than H37Rv	3
4× ,, ,, ,, ,,	4
2,048× ,, ,, ,, ,,	13
≥2,048× ,, ,, ,, ,,	14

SOLID MEDIUM TESTS

"End-point" of Growth Less than Appropriate Fraction in Medium Containing	Score
1 μg. streptomycin/ml.	0
4 μg. ,,	1
16 μg. ,,	2
1,024 μg. ,,	5
>1,024 μg. ,,	6

As an explanation of the procedure let us consider the results of tests on two hypothetical strains in which we will suppose there is no appreciable error in either test.

	Sensitivity in Liquid Medium ×H37Rv	"Resistant Fraction" (Streptomycin Concentration μg./ml.)					
		1	4	16	64	256	1,024
Strain 1	4× less sensitive	1.0	0.05	0.02	0.002	0.0002	0.00002
Strain 2	4× ,, ,,	1.0	0.9	<0.00001	<0.00001	<0.00001	<0.00001

For different values of the "resistant fraction" from 0.1 to 0.0001 the score on solid medium for Strain 1 would vary from 1 to 5 whereas the score for Strain 2 would be, in each case, 2. Since the scores for both strains on liquid medium tests would be the same, the best correlation would be obtained by the limiting value of 0.03 for the "resistant fraction." This would give Strain 1 a score of 2. From inspection of the results it would also appear that if the "resistant fraction" is less than 0.03, these resistant organisms will not be capable of causing growth in the liquid medium tests in the 10-day incubation period since the results in the liquid medium test are the same for both strains.

Eighty strains were considered in which a fraction as small as 0.0001 of the growth on the medium without streptomycin could have been detected on the media with the various streptomycin concentrations. Correlation coefficients were obtained for limiting values of the "resistant fraction" from 0.1 to 0.0001.

"Resistant Fraction"	Correlation Coefficient
0.0001	0.778
0.0003	0.804
0.001	0.802
0.003	0.821
0.01	0.836
0.03	0.869
0.1	0.840

As can be seen the highest correlation coefficient was obtained by considering the limiting fraction as 0.03, and there is a definite tendency for the coefficients to increase over the range of "resistant fraction" values from 0.0001 to 0.03. There is thus some slight evidence that, if resistant organisms are present in 0.03 of the total (i.e., 3%), they will just cause visible growth.

Further evidence on this point has been given in another publication (Mitchison, 1950) and can also be obtained by considering the results from Case 13 (Table VI). By the thirty-fourth day of treatment the sensitivity tests in liquid medium indicated a strain of a low degree of resistance. However, on solid media there began to appear a small proportion of more highly resistant organisms. The proportion of these organisms gradually increased until at about the eighty-third day most of the organisms were inhibited on solid medium only by 1,024 μg. per ml. There remained a small proportion capable of growth at this concentration. Sensitivity tests in liquid medium read at 7 and 14 days, as well as the usual 10 days, are included in this Table as they show in many cases that this small proportion of highly resistant organisms will just initiate growth after 10 days' incubation. In a number of cases, however, final end-points were not available for readings on the seventh or fourteenth days.

By examination of these results on solid medium, in conjunction with those in liquid medium, it seems that the limiting fraction just capable of causing growth

TABLE VI

DEVELOPMENT OF STREPTOMYCIN RESISTANCE IN CASE 13

Days after Starting Treatment	Sensitivity in Liquid Medium* (Days of Incubation)			Log_{10} No. of Organisms on Solid Medium without Streptomycin	"Resistant Fraction": No. of Organisms on Medium with Streptomycin/ Number on Medium without Streptomycin (Streptomycin Concentration μg./ml.)					
	7	10	14		1	4	16	64	256	1,024
1	=	=	=	4.88	1.0	<0.0007				
8	=	=	=	3.00	1.13	0.025‡	<0.0125			
15	=	=	=	4.70	0.30	0.002	<0.0003			
22	=	=	=	3.35	0.67	0.33	0.011‡	<0.005		
34		L	L	3.98	0.56	0.31	0.30	0.0052	0.0078	0.0039
41		L	L	3.96	1.15	1.43	0.031	0.0055	0.0027	0.0014
48		L	M	4.40	1.40	1.20	0.50	0.065	0.010	<0.0005
55	=	=	MorH†	3.63	0.82	0.85	0.82	0.047	0.012	0.018
62	M	M	M	3.69	1.33	0.90	0.69	0.082	0.092	0.0026
76	M	M	M	4.89	0.89	1.29	0.48	0.097	0.037	0.0011
83	M	M	M	5.82	0.66	0.49	0.76	0.10	0.11	0.0015
91	M	H	H	5.71			0.76	0.49	0.39	0.15
97	M	H	H	6.58	0.60		0.13	0.13	0.10	0.0011
104				7.18	0.75	0.63	0.57	0.31	0.17	0.0012
111		M	M	5.18		0.83	0.50	0.83	0.27	0.010
118	MorL†	H	H	6.05		0.80	1.07	0.74	0.84	0.0025
125	H	H	H	6.40	0.70	0.70	0.60	0.55	0.35	0.0070
132	M	H	H	6.10		0.66	1.00	0.86	0.90	0.0057
139	M	H	H	6.33		0.705	0.35	0.56	0.082	0.0030
146	M	H	H	6.18		0.48	0.80	1.12	0.33	0.020
153		H	H	4.44		0.91	1.09	1.50		0.023
160	M	M	M							

= As sensitive as H37Rv. * L = 4–8 times less sensitive than H37Rv. M = 32–128 times less sensitive than H37Rv. H = 2,000 times less sensitive than H37Rv.
† The end-point in these tests was not finally determined. ‡ One or two colonies present only.

TABLE VII

REPRESENTATIVE TESTS SHOWING DEVELOPMENT OF STREPTOMYCIN RESISTANCE IN CASE 11

Days after Starting Treatment	Sensitivity in Liquid Medium ×H37Rv	Log_{10} No. of Organisms on Solid Medium without Streptomycin	"Resistant Fraction": No. of Organisms on Medium with Streptomycin/No. on Medium without Streptomycin. (Streptomycin Concentration μg./ml.)					
			1	4	16	64	256	1,024
0	2× less sensitive	5.37	1.06	<0.00021				
16	Equal	5.88	<0.0013					
23	,,	6.40	0.011	<0.000072				
37	2× ,, ,,	4.62	0.73	0.0025*	<0.0013			
44	64× ,, ,,	4.45	0.18	0.0036*	<0.0018			
58	2× ,, ,,	4.56	0.70	0.22	<0.0023			
100	128× ,, ,,	3.40	1.00	0.60	0.14	<0.01		
107	4× ,, ,,	3.10	0.38	0.25	0.02*	0.02*	0.03*	<0.01
149	2× ,, ,,	3.02	0.78	0.57	0.56	1.01	0.58	<0.024
156	2× ,, ,,	3.93	0.97	0.88	0.44	0.68	0.50	0.046
163	2× ,, ,,	4.40	0.65	0.51	0.50	0.25	0.46	0.21
			End of streptomycin treatment					
170	128× ,, ,,	4.94	0.84	0.51	0.37	0.26	0.54	0.11
191	128× ,, ,,	6.20	0.82	0.78	0.76	1.01	0.76	0.85
198	128× ,, ,,	5.73	0.79	0.70	0.61	1.02		0.44

* 1–3 colonies present only.

after 10 days' incubation varies from 0.06 to 0.001 but clearly there is considerable variation from strain to strain. A point that should be noticed is that from about the fifty-fifth to the one hundred and fifty-third day the proportion of highly resistant organisms (capable of growing on solid medium containing 1,024 μg. streptomycin per ml.) remained about the same. It would appear that there was little selective influence tending to cause the predominance of highly resistant organisms over those of a moderate degree.

A number of discrepant results between tests on solid and in liquid media were found in Case 11, Table VII. Tests in liquid medium showed wide fluctuations throughout the treatment. Many of the strains were of a moderate degree of resistance, but, on a number of occasions after considerable periods of treatment, sensitive strains reappeared. In particular attention is drawn to the strains obtained on the one hundred and forty-ninth, one hundred and fifty-sixth, and one hundred and sixty-third days. On solid medium these strains appeared uniformly highly resistant. In liquid medium, however, they were still sensitive. The Lowenstein-Jensen slopes from which subcultures were made for the liquid medium tests yielded a moderate to heavy growth, so that the discrepancy could not have been due to a chance sampling of sensitive colonies.

A possible explanation is that towards the end of treatment the strain was composed mainly of a mixture of streptomycin dependent (Spendlove, Cummings, Fackler, and Michael, 1948) and sensitive organisms possibly with a smaller number of organisms of a moderate degree of resistance. *In vitro* some strains, at least, of dependent tubercle bacilli, are unstable and tend to revert to being resistant (Yegian, Budd, and Vanderlinde, 1949). The dependent organisms would account for growth on solid media containing high concentrations of streptomycin, but, being unable to grow on medium without streptomycin, they would not manifest themselves in the liquid medium. Unfortunately the possibility was not considered at the time that these experiments were done so that the individual strains were not tested further. It should be noted that this patient was receiving streptomycin in four doses during the day and there were no rest periods during treatment.

Variations in Total Numbers of Bacilli Excreted during Treatment.—The number of bacilli growing on the solid medium without streptomycin was used to estimate the number of bacilli excreted in 24 hours, taking into account the total volume of sputum and the volume of the concentrate. Clearly such an estimate is subject to very great variation, since it is difficult to be certain that all the sputum over a 24-hour period is collected by the patient and the treatment of the sputum with sodium hydroxide will probably kill a proportion of the tubercle bacilli. However, these sources of error remain the same during the period of treatment.

The $\log_{10}$ of the number of bacilli excreted over 24 hours was plotted against the number of days of treatment for each case. Examples are given in Figs. 1 and 2.

The data from three patients were not used for the following reasons.

Case 14.—Thirteen out of 25 specimens yielded a contaminatory organism which resisted treatment with sodium hydroxide. This organism was not identified but was of similar colonial morphology throughout.

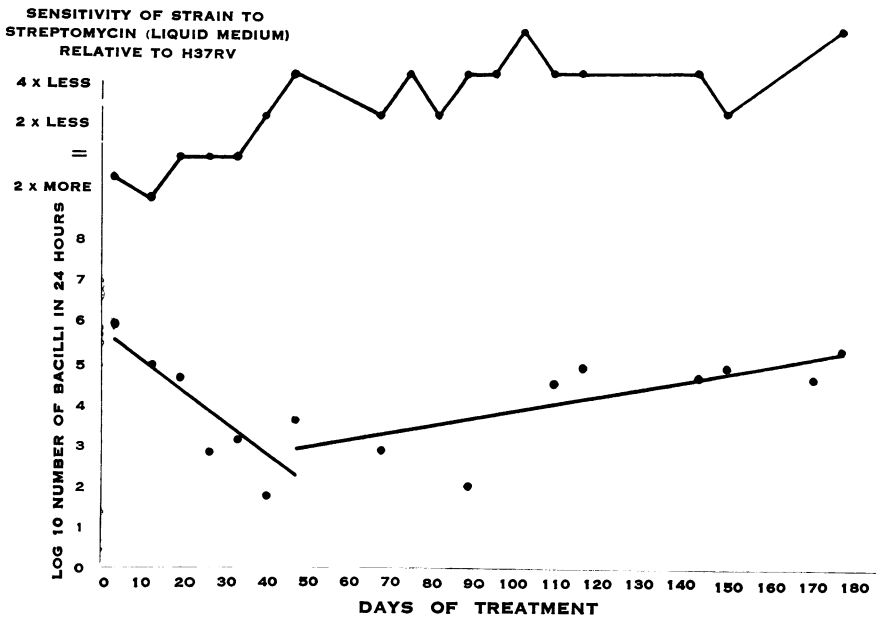

FIG. 1.—Development of low degree of resistance in Case 1.

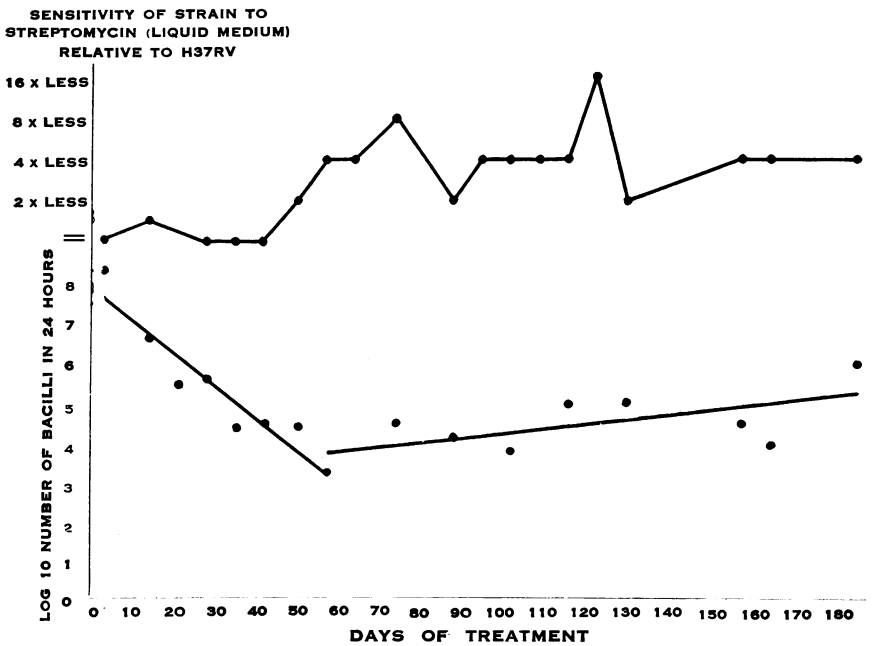

FIG. 2.—Development of low degree of resistance in Case 18. In both graphs the top line shows the results of streptomycin sensitivity tests in liquid medium, and the bottom line $\log_{10}$ of the number of tubercle bacilli in the sputum coughed up over 24 hours. Two lines are fitted to these points, one for the period before the development of resistance, which shows a fall in numbers, and one for the period after the development of resistance, which shows a rise.

TABLE VIII

SUMMARY OF ANALYSIS BEFORE AND AFTER DEVELOPMENT OF RESISTANT STRAINS

Strains Developing a Low Degree of Resistance

	Case No.	1	2	6	7	9	10	15	16	18
Before resistance developed	Regression coefficient*	−0.0734	−0.0881	−0.0314	−0.0762	−0.0141	−0.0324	−0.0284	−0.0213	−0.0797
	Regression mean square	7.871	11.723	2.031	13.047	1.023	2.561	4.183	1.553	14.976
	Error mean square	0.815	2.067	0.516	0.779	1.140	0.193	0.141	0.417	0.283
	Degrees of freedom	5	4	5	6	7	4	8	6	6
	P	0.05–0.02	0.1–0.05	0.2–0.1	0.01–0.001	0.4–0.3	0.05–0.02	0.001	0.2–0.1	<0.001
After resistance developed	Regression coefficient*	0.0167	0.0127	0.0110	0.0049	0.0205	0.0215	0.0008	0.0074	0.0110
	Regression mean square	4.775	3.164	1.779	0.466	3.665	10.645	0.005	0.608	1.864
	Error mean square	0.6277	1.330	1.469	0.925	0.719	3.395	1.288	0.804	0.419
	Degrees of freedom	7	7	8	9	6	14	5	7	7
	P	0.02–0.01	0.2–0.1	0.3	0.5–0.4	0.1–0.05	0.1–0.05	>0.9	0.5–0.4	0.01–0.5

Strains Developing a Moderate or High Degree of Resistance

	Case No.	3	5	8	11	13	17
Before resistance developed	Regression coefficient*	−0.1191	−0.0632	−0.0356	−0.0260	−0.0403	−0.0531
	Regression mean square	3.811	6.049	4.015	4.470	1.217	1.231
	Error mean square	0.824	0.557	0.766	0.570	1.377	0.384
	Degrees of freedom	1	1	3	7	4	3
	P	0.3–0.2	0.2–0.1	0.2–0.1	0.05–0.02	0.5–0.4	0.2–0.1
After resistance developed	Regression coefficient*	0.0241	0.0234	0.0141	0.0254	0.0196	0.0022
	Regression mean square	11.111	3.800	2.644	2.991	8.859	0.182
	Error mean square	0.229	0.532	0.373	2.575	0.774	1.045
	Degrees of freedom	8	5	11	7	15	16
	P	<0.001	0.05–0.02	0.05–0.02	0.4–0.3	0.01–0.001	>0.9

* The regression coefficient is the change in $\log_{10}$ number of bacilli in 24 hours per day of treatment.

ANALYSIS OF VARIANCE

	Degrees of Freedom	Mean Square	F	t	P
Strains Developing a Low Degree of Resistance					
Before resistance developed:					
Joint regression	1	41.575		4.37*	0.01–0.001
Between regressions	8	2.174	3.29		0.01–0.001
Error	51	0.660			
Joint regression coefficient, -0.04015 ± 0.00506					
After resistance developed:					
Joint regression	1	21.817		3.90	<0.001
Between regressions	8	0.644	2.23		0.2–0.1
Error	70	1.438			
Joint regression coefficient, 0.01267 ± 0.00325					
Strains Developing a Moderate or High Degree of Resistance					
Before resistance developed:					
Joint regression	1	17.008		4.64	0.01–0.001
Between regressions	5	0.757	1.00		>0.2
Error	19	0.754			
Joint regression coefficient, -0.03648 ± 0.00768					
After resistance developed:					
Joint regression	1	20.200		4.77	<0.001
Between regressions	5	1.878	2.12		0.1–0.02
Error	62	0.886			
Joint regression coefficient, 0.01379 ± 0.00289					

* Using between regressions mean square as the error term.

Case 12.—Cultures from this case became negative after about 100 days' treatment, and with the exception of three positive ones, remained so up till the two hundred and first day.

Case 4.—The number of bacilli excreted by this patient was never large and cultures were sporadically negative. The data obtained were incomplete.

Each set of data was divided into two portions, a period before the development of resistant strains and a period after the development of resistant strains. The dividing line was taken as the first culture which exhibited a degree of sensitivity of four or more times less sensitive than H37Rv in the liquid medium test. Lines were fitted to the dates from each case for both periods by the method of least squares.

In all cases there was a fall in the numbers of bacilli excreted during the period that the strains remained sensitive. However, following the development of resistant strains, there was rise in the number excreted in every case. Some of the essential data of the calculations are shown in Table VIII. It will be seen that the rise or fall in numbers of bacilli are often not significant in an individual case owing to the high degree of error of the estimations of numbers of bacilli excreted. However, if the joint tendency to rise or fall is calculated as a joint regression coefficient, then the general tendency for numbers to fall before the development of resistance, and to rise after it, is highly significant.

The calculations for those cases which only developed a low degree of resistance have been presented separately from those in which a moderate or high degree of resistance occurred. The rate of fall of numbers of bacilli before resistant strains appeared and the rise afterwards, are the same, within the limits of error, in these two groups.

The average number of bacilli excreted in 24 hours before treatment was started was calculated for the cases developing a low degree of resistance and for those developing a higher degree. These figures were also calculated for the time when the first resistant strain appeared. As can be seen from Table IX, the values for cases developing higher degrees of resistance are, in both cases, slightly greater, but the difference is not significant.

TABLE IX

THE MEAN OF THE LOG_{10} NUMBER OF BACILLI EXCRETED IN 24 HOURS BEFORE TREATMENT AND ON FIRST APPEARANCE OF RESISTANT STRAINS IN CERTAIN CASES

	Low Degree of Resistance	Higher Degree of Resistance	S.E. of Difference	Probability of Occurrence by Chance
Mean of Log_{10} number of bacilli/24 hours before treatment	5.551	6.230	0.628	0.3–0.2
When resistant strain first appeared	3.051	3.790	0.514	0.2–0.1

DISCUSSION

The development of resistant strains in patients treated with streptomycin can be considered as occurring in two stages. In the first stage strains of a low degree of resistance appear and rapidly form the majority of the organisms present. Whether a strain of moderate or high resistance may replace this seems to vary from case to case. What little evidence is available suggests that there is no marked difference between strains of tubercle bacilli as to their capabilities of producing moderately or highly resistant variants. Thus Fisher (1948b), in carrying out sensitivity tests on Herrold's egg agar medium containing varying concentrations of streptomycin, noted the proportion of pre-treatment strains in which variant colonies occurred on medium containing 10 or more μg. streptomycin per ml. and found that there was no relation to the ultimate development of resistant strains. Yegian and Vanderlinde (1948) investigated the proportions of resistant bacilli capable of growth on 1 and 100 μg. streptomycin per ml. in Tween 80-albumen solid medium found in two strains of tubercle bacilli. One of these became resistant after 30 days' treatment and the other remained sensitive after 120 days. They claim that the strain which became resistant had a higher proportion of resistant variants. On the other hand the difference as indicated in their table was less than tenfold and the results, as might be expected, showed considerable variation. Whether the tendency to replace strains of low resistance by those of moderate or high degree is due to differences between strains or to differences in the number of bacilli in lesions and the effective streptomycin concentrations in different patients one cannot say. However, it seems that in some patients a strain of moderate or high resistance

gradually (Case 3, Table III) or suddenly (Case 8, Table IV) replaces the strain of low resistance. In other patients there is an alternation between strains of high and low resistance, noted both on solid and in liquid medium (Case 17, Table V). This suggests that there might be some foci in the lungs in which highly resistant organisms were predominant and others in which there were mainly organisms of low resistance. Differences in the levels of sensitivity in different parts of the lung at necropsy have been shown to occur in one case by Armstrong and Walker (1949). The results on Case 13, Table VI, suggest that there is little tendency to favour highly resistant as against moderately resistant strains.

Case 11, Table VII, is of interest in that there is the possibility that a streptomycin dependent strain was present in the sputum. Lenert and Hobby (1948) injected the tubercle strain H37Rv intracerebrally in mice and gave streptomycin treatment for up to 31 days. At death "the mice were cultured." One hundred and eighty-four out of 196 gave a growth of sensitive bacilli when isolation was performed on media without streptomycin. Thirteen out of the 184 also gave a growth in Tween 80-albumen liquid medium containing 500 μg. streptomycin per ml. and all these strains were found to be dependent. Thus a mixture of sensitive and dependent organisms was present during the course of treatment. Furthermore, passage of these strains *in vivo* yielded only streptomycin dependent and resistant organisms, never sensitive organisms.

The group of cases in which organisms of only a low degree of resistance were isolated is of importance. These organisms would not have been classified as resistant by many American authors (Barnwell, Bunn, and Walker, 1947; Report to Council on Pharmacy and Chemistry, 1947; Wolinsky, Reginster, and Steenken, 1948; Bernstein, D'Esopo, and Steenken, 1948; Blattberg and Ehrhorn, 1949; Howard, Maresh, Mueller, Yannitelli, and Woodruff, 1949), since their resistant strains have been classified as being capable of growth in 10 or more μg. streptomycin per ml. However, in cases where such strains occurred the fall in numbers of bacilli excreted in 24 hours until the strain became resistant, and the subsequent rise, would suggest that these strains are in fact capable of growth in the presence of the antibiotic. Steenken and Wolinsky (1948) injected a series of such strains into guinea-pigs and treated these with streptomycin. The therapeutic results varied from excellent to none. However, since the type of lesion is very different in man and guinea-pig results from animal experiments on strains which are of border line significance in man should be treated with considerable caution.

To conclude, it seems probable that in the form of pulmonary tuberculosis studied treatment with streptomycin results after a period of about 60 days in the production of strains of tubercle bacilli consisting almost entirely of organisms capable of growth in the effective streptomycin concentrations found in the lesions. These strains may not be homogeneous, but if they are not, they are still composed mainly of resistant strains among which the levels of sensitivity to streptomycin differ. Similar cases may respond well to streptomycin and the excretion of bacilli will then become scanty and irregular. In such circumstances sensitive organisms will often continue to appear after long periods of treatment. Apart from these, however, the persistence of a truly sensitive strain in a patient excreting numerous bacilli throughout a six months' period of treatment must be very rare, if indeed it ever occurs.

SUMMARY

Tubercle bacilli were isolated at intervals from the sputum of 18 patients with acute progressive pulmonary tuberculosis treated with streptomycin. The numbers capable of growth at various streptomycin concentrations were estimated by surface viable counting on Herrold's egg agar medium. Simultaneously cultures were tested for streptomycin sensitivity in Tween 80-albumen liquid media. In all cases some degree of streptomycin resistance developed. In 10 patients strains of a low degree of resistance appeared and persisted throughout the six months' course of treatment. In eight patients the strain of a low degree of resistance became replaced, slowly or rapidly, by one of a higher degree of resistance, although in one case it seemed probable that this occurred only in some foci within the lungs. In one case discrepancies between the results on solid and in liquid media might have been explained by the development of a streptomycin dependent strain. In the liquid medium test approximately 3% of resistant organisms will cause growth after a ten-day incubation period.

The number of tubercle bacilli excreted in 24 hours fell during the period when strains were sensitive and rose after the development of resistance. This occurred at the same rate both in cases excreting bacilli of a low degree of resistance and in cases where the bacilli were more highly resistant.

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The streptomycin used was the calcium chloride complex (Merck, Rahway, N.J., U.S.A.). The term μg. is equivalent to 1 μg. of the pure base.

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