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The Virulence in the Guinea-pig of Tubercle Bacilli Isolated before Treatment from South Indian Patients with Pulmonary Tuberculosis

1. Homogeneity of the Investigation and a Critique of the Virulence Test

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A series of studies on the virulence in the guinea-pig of tubercle bacilli isolated before treatment from Indian tuberculous patients admitted to a controlled comparison of different regimens of domiciliary chemotherapy has recently been undertaken by the Tuberculosis Chemotherapy Centre, Madras. The main object of these studies was to determine whether the differences in virulence of the tubercle bacilli obtained from Indian patients before the start of chemotherapy were related to the severtiy or type of the patients' disease at that time and to the subsequent response to treatment. Before these relationships could be' investigated, however, it was necessary to find out whether the results of the virulence tests, which were carried out over a period of two-and-a-half years at the Centre and at the Microbiological Research Establishment, Porton, England, could be considered as a unified whole-that, is, as if they had all been done on the same day in the same laboratory.

A proportion of the cultures was stored at – 20°C for 44-78 weeks, but this did not affect their virulence. Inter-experimental variation was found to be small in the Porton series of tests and undetectable in the Madras series, and the results in the latter series could be successfully adjusted to those in the former by allowing for differences in the means and standard deviations of the distributions for the two series. The measure of virulence used was found to be reasonably acceptable for the analysis of variance technique. Suggestions are made as to ways of improving the efficiency of the experimental design in future studies.

INTRODUCTION

Cultures of tubercle bacilli obtained from untreated South Indian and British patients with pulmonary tuberculosis were compared by Mitchison et al. (1960) for their virulence in the guinea-pig. The cultures were sensitive to isoniazid and streptomycin and had been identified as *Mycobacterium tuberculosis* by a number of *in vitro* tests. In agreement with the earlier work of Frimodt-Mølller, Mathew & Barton (1956) and Frimodt-Møller (1957), the average virulence of the cultures from the Indian patients was found to be lower than that of the cultures from the British patients and also the range of virulence was wider, only about 30 % of the Indian cultures being as virulent as those from British patients. These findings have been confirmed and extended in the second of the present series of three papers (Bhatia et al., 1961a[3]). Further, Bhatia et al. (1961b) have shown that the variation in virulence among cultures obtained over a 6-week period from the same untreated Indian patient was no greater than the natural

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[3] See article on page 313.

variation in the response of the guinea-pigs to the virulence test and was considerably less than the variation in virulence from patient to patient. Thus, individual Indian patients yielded strains of a consistent degree of virulence. As a result of these findings, it became meaningful to investigate whether the differences in virulence of the tubercle bacilli obtained from Indian patients before the start of chemotherapy were related to the severity or type of the patients' disease at that time, and to the subsequent response to treatment with antituberculosis drugs. Such an investigation has now been made, and is reported in the third of the present series of papers (Ramakrishnan et al., 1961 [1]).

From October 1957 to December 1958 a total of 341 South Indian patients were admitted to a controlled comparison of four regimens of chemotherapy in the domiciliary treatment of tuberculosis (Tuberculosis Chemotherapy Centre, 1960). After the exclusion of 22 patients because they yielded isoniazid-resistant cultures on admission, and four for other reasons, there remained 315 patients in the main analysis of the results of this comparison. Virulence tests in the guinea-pig were carried out on single cultures obtained before the start of the prescribed regimen (except in one patient, whose culture was obtained three months after the start of treatment) from 281 (89.2 %) of the 315 patients.

The results of the virulence tests on these 281 patients are related to assessments of the extent and type of their disease on admission to the chemotherapy trial, and to their progress during one year's treatment with the prescribed regimen, in the third paper in this series (Ramakrishnan et al., 1961 [1]). However, for any associations found to be meaningful, it was essential to be able to consider the results of the virulence tests as a unified whole, that is, as if they had all been done on the same day in the same laboratory. There were certain features of the investigation which might be expected to introduce serious heterogeneity into the results of these tests. We therefore report here, as a preliminary to the third paper, the findings on experiments which were incorporated in the investigation to measure the degree of homogeneity, and also the measures taken to standardize the results where a source of heterogeneity was found.

The more important possible sources of heterogeneity and the measures taken to estimate them, or to adjust for them, were as follows:

1. Facilities for large-scale experimental work on animals were not available until the latter part of the period of intake of patients to the chemotherapy trial, so that the majority of the cultures from patients admitted at the beginning of the period were stored at -20°C in a deep-freeze cabinet. The effect of storage in the deep-freeze was examined by comparing the virulence of pairs of cultures from the same Indian patient, one culture in each pair having been tested shortly after isolation and the other after storage in the deep-freeze.

2. The virulence tests were carried out in two series of experiments, the larger series at the Microbiological Research Establishment, Porton, Wiltshire, England (Porton) and the other at the Tuberculosis Chemotherapy Centre, Madras, India (Madras). These experiments were done over a period of two-and-a-half years. The extent of the variation in the results from experiment to experiment was examined in several ways:

(a) In both series of experiments, the results of the tests on the cultures from the Indian patients themselves provided evidence on inter-experimental variation.

(b) A sample of cultures of tubercle bacilli was obtained from untreated British patients, and these cultures were included in both series of experiments. Since the virulence of British cultures was known to vary less than the virulence of Indian cultures (Mitchison et al., 1960), the results of the tests on the British cultures would be a particularly sensitive indicator of inter-experimental variation.

(c) In the majority of the experiments at Porton, a standard strain, H37Rv, of moderate virulence, was included among the cultures tested.

3. The breed of guinea-pig used at Porton was different from that used at Madras. Although the same method of virulence testing was used, the responses in the two breeds differed substantially. The results of the smaller Madras series were therefore adjusted to make them comparable with those obtained at Porton.

These findings are followed in the present paper by a statistical critique of the virulence test, concerned principally with *(a)* the measure of virulence and the validity of its use in analysis of variance, *(b)* the advantages of various possible arrangements of guinea-pigs in the tests, and *(c)* the relative efficiencies of the two breeds of guinea-pig in measuring virulence.

[1] See article on page 323.

METHODS

NOMENCLATURE

The Tuberculosis Chemotherapy Centre will be referred to as Madras and the experiments carried out there as Mad. 1, 2..., the Microbiological Research Establishment as Porton, with experiments Por. 1, 2..., and the MRC's Unit for Research on Drug Sensitivity in Tuberculosis as London. Some of the results of the virulence tests described here-namely, those on cultures from 73 of the Indian and 22 of the British patients-have already been reported by Mitchison et al. (1960), using the same nomenclature.

PATIENTS

Indian patients

Virulence tests were carried out on single cultures of tubercle bacilli from 281 South Indian patients. All of these patients had been admitted to a chemotherapeutic study at Madras (Tuberculosis Chemotherapy Centre, 1960) and had contributed to the main analysis on 315 patients. Virulence tests on cultures from the remaining 34 patients were not done, since 23 cultures were contaminated during storage or transport from Madras to England, eight were mislaid, and three failed to grow on subculture after storage in the deep-freeze. The 281 patients with virulence-test results conformed to the following important criteria :

(a) The patients were aged 12 years or more and were living in Madras City.

(b) All the patients had pretreatment cultures that were sensitive to isoniazid and all except three had organisms sensitive to streptomycin. The emergence of streptomycin-resistance is not associated with loss of virulence (Feldman, Karlson & Hinshaw, 1948; Steenken & Wolinsky, 1948; Karlson & Gainer, 1951). No attempt was made to exclude cultures resistant to p-aminosalicylic acid (PAS) for reasons reported elsewhere (Selkon et al., 1960; Tuberculosis Chemotherapy Centre, 1960). Sensitivity tests were carried out on Löwenstein-Jensen medium (Tuberculosis Chemotherapy Centre, 1959). (The Löwenstein-Jensen medium referred to here, and throughout the text, did not contain potato starch (Jensen, 1955).)

(c) The great majority of the patients had not had any previous antituberculosis chemotherapy so far as was known. Up to two weeks of such chemotherapy had been received by 11 patients. In one patient an isoniazid-sensitive culture, obtained three months after the start of the prescribed chemotherapy, was tested for virulence, because a pretreatment result was not available. The results of virulence tests on cultures from these 12 patients have been included, since Subbaiah et al. (1961) have shown that the virulence of cultures from Indian patients is unaffected by three months of chemotherapy provided that the organisms remain sensitive to isoniazid.

(d) The patients had subsequently followed the initially prescribed regimen of treatment in the trial for 12 months with, at most, minor variations, unless chemotherapy was stopped or changed owing to death, deterioration or major toxicity.

British patients

Virulence tests were done on single cultures from 93 patients of British extraction, aged 12 years or more, and with newly diagnosed, untreated, and moderately or far advanced pulmonary tuberculosis. A test was done, by mistake, on one culture from a patient who had received previous antituberculosis chemotherapy, but the organisms were sensitive to isoniazid, streptomycin and PAS. The results of this virulence test have been included for the reason given above.

CULTURES OF TUBERCLE BACILLI

The sputum specimens obtained from Indian patients were cultured at Madras, and those from British patients at London, the same method (Tuberculosis Chemotherapy Centre, 1959) being used-namely, homogenization with 4% sodium hydroxide, followed by inoculation on to slopes of Löwenstein-Jensen medium.

Cultures from Indian patients

As soon as the cultures from the Indian patients had become positive they were stored in Madras, either for a short period at 37°C (138 "fresh cultures ") or for 44-78 weeks (average, 62 weeks) at –20°C (143 " deep-freeze-stored cultures "). After storage, the cultures for testing at Porton were sent by air to London for subcultivation in the virulence test, in parallel with the cultures from British patients. Of the 138 fresh Indian cultures, 132 were stored for up to eight weeks (usually less than six weeks), three for nine weeks, and three for 10 weeks. One of the 143 deep-freeze-stored

cultures had to be decontaminated by treatment with sulfuric acid at London.

Cultures from British patients

All of the cultures from the British patients were stored at 37°C for periods of up to eight weeks. Those to be tested for virulence at Madras were sent by air, to be set up as fresh cultures in parallel with the cultures from Indian patients.

Strain H37Rv

A standard strain of *Myco. tuberculosis* var. *hominis,* H37Rv, was obtained from Dr R. J. W. Rees, National Institute for Medical Research, London. This strain, which had recently been passaged through mice, was inoculated on to a number of slopes of Löwenstein-Jensen medium, which were then stored at –20°C in a deep-freeze. In each experiment one of these slopes was removed from the deep-freeze and subcultivated in the virulence test.

IDENTIFICATION TESTS

The wide range of identification tests employed for 73 of the cultures from Indian patients included in this study, and already described by Mitchison et al. (1960), was slightly restricted for the remaining cultures to: *(a)* a smear from the growth on Löwenstein-Jensen medium stained by the Ziehl-Neelsen method; *(b)* colonial morphology on 7H-10 oleic-acid-albumin agar medium and on Löwenstein-Jensen medium; *(c)* examination for ability to grow at 23°C; *(d)* abnormal pigmentation on Löwenstein-Jensen medium in the dark and after exposure to light; *(e)* the niacin test; *(f)* a qualitative catalase test; and *(g)* sensitivity to p-acetamidobenzaldehyde thiosemicarbazone (thiacetazone). The methods employed are described fully elsewhere (Thomas et al., 1961). In addition, Mantoux tests with 0.1 ml of 1: 100 Old Tuberculin were done four weeks after infection on all animals tested at Madras. Identification tests were not done systematically on the cultures from British patients, since they were fairly uniformly of high virulence in the guinea-pig.

VIRULENCE TESTS

The origin and diet of the guinea-pigs and the procedures for the virulence test have been described in detail by Mitchison et al. (1960) so that the methods will only be presented briefly here.

Guinea-pigs

At Porton, a total of 1138 Duncan Hartley (DH) breed albino animals (99% males) was used, of average weight 426 g (range, 300-580 g). At Madras a total of 166 M-breed, mixed-colour guinea-pigs (69% males) was used, of average weight 370 g (range, 258-608 g).

Infecting dose

The initial diagnostic culture, after storage as described earlier, was subcultivated on Löwenstein-Jensen medium. After three weeks' incubation at 37°C, growth from the subculture was weighed out into screw-capped bottles containing glass beads. Sterile distilled water (at Madras), shown not to be bactericidal to tubercle bacilli, or 0.1% bovine albumin in water (at London) was added and the bottles were shaken to prepare a suspension of the bacilli. Either two or four guinea-pigs (depending on the particular virulence test) were each injected in the right thigh muscle with 0.5 ml of the suspension, which contained 1 mg (moist weight) of bacilli. From some of the suspensions, a viable count was set up in 7H-10 medium (Cohn, Middlebrook & Russell (1959), as modified by Subbaiah, Mitchison & Selkon (1960)), solidified with silica gel according to the method of Selkon & Mitchison (1957).

Root-index of virulence

When two guinea-pigs were infected, one was killed at 6 weeks and the other at 12 weeks; when four animals were infected, two were killed at each time. If, when two guinea-pigs were infected, one of them died before six weeks, then it was counted as a 6-week guinea-pig and the surviving guinea-pig was killed at 12 weeks. If both guinea-pigs died, then the one that died first was counted as the 6-week guinea-pig and the other was counted as the 12-week guinea-pig. Similar rules applied when four guinea-pigs were infected. At post-mortem examination the amount of visible disease was assessed as a score ranging from 0 to 100. The maximum score for the spleen was 40, for the liver 30, for the lungs 20, and for the site of inoculation, and its draining lymph-nodes, 10. Animals that died, either of tuberculosis or from natural causes, were scored in the same way. The total score for each animal (whether dying or killed) was divided by its survival time in days to give an index. This index is a measure of the rate at which lesions develop in the organs and, by inference, it also measures the approximate rate at which tubercle bacilli multiply in the animal body.

Further, it combines the results of score and mortality. For reasons given later in the present report (see page 299 *et seq.),* the square roots of the 6-week and the 12-week indices were calculated and termed the " 6-week root-index " and the " 12-week root-index ", respectively. The mean of the root-indices for all the animals infected with a culture was termed the " root-index of virulence " and has been employed as the measure of virulence in the present report.

Non-tuberculous deaths

Non-tuberculous deaths were defined as those occurring in animals with a total score of less than 40, and a lung score of less than 20 (the maximum for this organ). Defined in this way 16 non-tuberculous deaths (1.4 % of 1138 animals) occurred in the experiments at Porton and two (1.2 % of 166 animals) in the experiments at Madras. If such a death occurred 30 or more days after infection, and if there was no evidence of an infection or other cause of death which could be confused with tuberculosis, then a root-index was calculated as described earlier, and the result was included. If the guinea-pig died less than 30 days after infection, or if the nature of the lesions was obscure, then the root-index was estimated as follows. When the guinea-pig concerned was one of four injected with the culture, the value of the root-index of the paired 6-week or 12-week guinea-pig was taken as the estimate. When the missing value was from, say, the single 6-week guinea-pig injected with a culture, then its root-index was estimated by adding to the root-index of the corresponding 12-week guinea-pig the mean difference between the 6-week and 12-week root-indices of all the remaining guinea-pigs in the particular experiments, whether their results are reported here or not (see below). The results on 10 animals at Porton and on two animals at Madras had to be estimated in this manner.

ARRANGEMENT OF EXPERIMENTS

Both at Porton and at Madras a series of experiments was set up, the interval between successive

TABLE 1
ARRANGEMENT OF EXPERIMENTS IN THE PORTON SERIES (DH GUINEA-PIGS)

Experiment No.	Results in the present report								Total number of guinea-pigs in experiment (including results not in the present report)
	Indian cultures					British cultures (tested in 4 guinea-pigs)	Strain H37Rv (8 guinea-pigs in each experiment)	Number of guinea-pigs	
	Tested in 2 guinea-pigs		Tested in 4 guinea-pigs		Total				
	Fresh	Deep-freeze-stored	Fresh	Deep-freeze-stored					
Por. 1	0	0	21	0	21	5	0	104	104
„ 2	0	0	27	0	27	5	0	128	136
„ 3	0	0	20	0	20	5	0	100	108
„ 4	31	3	8	1	43	5	1	132	164
„ 5	3	15	1	9	28	5	1	104	164
„ 6	0	15	0	8	23	5	1	90	164
„ 7	0	21	0	8	29	5	1	102	168
„ 8	0	3	0	4	7	5	1	50	158
„ 9	0	2	0	0	2	5	1	32	84
„ 10	0	15	0	4	19	5	1	74	174
„ 11	0	11	0	7	18	5	1	78	152
„ 12	0	8	0	7	15	5	1	72	166
„ 13	0	2 2[a]	0	0	22	5	1	72	190
Total	34	115	77	48	274	65		1138	1932

[a] Including the 29 cultures in the comparison of the virulence of fresh and deep-freeze-stored cultures.

experiments being six weeks. Consequently, in all except the first and the last experiment of each series, the 6-week guinea-pigs from one experiment and the 12-week guinea-pigs from the previous experiment were killed and scored together. In both series, virulence tests on cultures not reported here were also carried out (by the same method) in nearly all of the experiments. The order of preparing the infecting suspensions, of injecting the doses, and **of** killing the guinea-pigs was randomized, and the identity of the infecting organisms was not known to the observer who assessed the score.

Porton series

The Porton series (Table 1) consisted of 13 experiments, in which 274 Indian cultures were tested. These 274 cultures were from 254 of the 281 patients, two cultures from each of 20 patients being tested twice, for the comparison of fresh and deep-freeze-stored cultures (see below). In experiments Por. 1 to Por. 3 all the Indian cultures were fresh, and each culture was injected into four guinea-pigs. Some of the cultures in experiments Por. 4 and Por. 5, and all of them in Por. 6 and later experiments, had been stored in the deep-freeze. In Por. 4 and subsequent experiments, only a sample averaging 28% of the cultures was set up in four guinea-pigs per culture. The purposes of these samples were to obtain estimates of the duplicate error of the test for 6-week and 12-week guinea-pigs and to facilitate certain comparisons, reported in the accompanying paper by Bhatia et al. (1961a [1]), with the British cultures which were also injected into four guinea-pigs each (see below). For each of the remaining cultures, two guinea-pigs were infected per culture. In all, 125 Indian cultures were each injected into four guinea-pigs and 129 were each injected into two guinea-pigs.

The effect of storage in the deep-freeze was investigated in pairs of cultures from 20 Indian patients. One of the two cultures from each patient was tested fresh, in experiment Por. 4, and is included among the other Indian cultures. The second culture of the pair was stored in the deep-freeze and was then tested in experiment Por. 13, each culture being injected into two guinea-pigs. The results of the tests on the second cultures are excluded from all analyses other than that of this comparison.

In each experiment at Porton, five British cultures were tested, totalling 65 cultures in the series. Each British culture was injected into four animals. As a further control on inter-experimental variation, strain H37Rv was tested in eight animals in each experiment from Por. 4 onwards. In addition to serving as a test for inter-experimental variation, these animals were also used for a further study, described later (page 296), of the variations in virulence due to preparation of the infecting suspension, and of the effect of a 10-fold decrease in the size of the dose.

Madras series

The Madras series (Table 2) consisted of 12 experiments, in which 55 Indian cultures were tested. Of these 55 cultures, 27 were tested only at Madras and, together with the 254 cultures in the Porton series, make up the total of 281 cultures from the same number of Indian patients in the investigation. Of the remaining 28 cultures, 23 were also tested in the Porton series; the other five were from patients from whom alternative pretreatment sputum cultures were tested in the Porton series.

TABLE 2

ARRANGEMENT OF EXPERIMENTS IN THE MADRAS SERIES (M GUINEA-PIGS)

Experiment No.	Results in the present report					Total number of guinea-pigs in experiment (including results not in the present report)
	Indian cultures			British cultures	Number of guinea-pigs	
	Tested only at Madras	Tested at Madras and Porton	Total			
Mad. 1	4	0	4	2	12	12
" 2	3	0	3	1	8	24
" 3	2	0	2	1	6	18
" 4	4	0	4	2	12	20
" 5	4	0	4	2	12	14
" 6	7	0	7	3	20	24
" 7	0	3	3	2	10	17
" 8	0	7	7	3	20	36
" 9	2	9	11	5	32	50
" 10	0	5	5	3	16	44
" 11	0	0	0	0	0	20
" 12	1	4	5	4	18	41
Total	27	28	55	28	166	320

[1] See article on page 313.

These 28 cultures do not, therefore, contribute to the total of 281 patients. They provide a check on the adjustment of the results obtained with, cultures tested only at Madras. All of the 55 Indian cultures were fresh. A total of 28 British cultures was tested in the series. Each culture in the series, whether Indian or British, was injected into two guinea-pigs.

RESULTS

The results of the virulence tests are described in two sections. Section A deals with the homogeneity of the investigation, principally with the effect on the root-index of virulence of such factors as conditions of storage of the culture, inter-experimental variation, variations in the preparation of the infecting suspension, and differences in the responses of the two breeds of guinea-pig. Section B consists of a critical analysis of the virulence test itself, together with suggestions for its modifications in future work. The results of the tests were examined by analysis of variance. This statistical method is based on certain assumptions–namely, additivity of effects and homogeneity of variance in a normally distributed population. The extent to which the results of virulence tests reported here satisfied these conditions is described in section B.

A. HOMOGENEITY OF THE INVESTIGATION

Identification tests

Of the total of 306 cultures from the 281 Indian patients (duplicate cultures being obtained from 20 patients in the comparison of fresh and deep-freeze-stored cultures and from five patients in the comparison between virulence tests in the Porton and the Madras series), 287 (93.8%) were examined for their identity. The results on 262 cultures were among those reported fully by Thomas et al. (1961), and the findings on the remaining 25 cultures were similar. In brief, all 287 were found to be *Myco. tuberculosis* and, of 279 examined, all were of the human type, as indicated by a positive niacin test.

Comparison of fresh and deep-freeze-stored cultures

The effect of storage in the deep-freeze on the virulence of cultures was investigated in pairs of cultures from 20 Indian patients. The pairs of sputum specimens which yielded the cultures were obtained at intervals, on the average, of two days from each other. One of the cultures from each patient was tested as a fresh culture, in experiment Por. 4, and the other was stored in the deep-freeze for, on the average, 57 weeks (range, 51-59 weeks) before being tested in experiment Por. 13. This period of storage is similar to the mean period for all deep-freeze-stored cultures in the investigation (62 weeks; range, 44-78 weeks). The results are set out in full, as a representative sample of virulence-test data, in Table 3. A few of the cultures had been tested in four animals in experiment Por. 4, and for these one 6-week and one 12-week root-index were selected at random for simplicity in analysis. The means of the root-indices of virulence were 0.64 for the fresh cultures in Por. 4 and 0.71 for the deep-freeze-stored cultures in Por. 13. This difference does not attain statistical significance (Table 3, term b, $P = 0.1$). However, the difference measures not only any tendency for deep-freeze-stored cultures to differ in mean virulence from fresh cultures, but also any difference in the average virulence of cultures tested in Por. 4 and Por. 13. Separate analyses were therefore done of the tests on British cultures and on strain H37Rv included as controls in Por. 4 and Por. 13, and these showed no evidence of a significant difference between the experiments. Nevertheless, examination of the means of the tests on the British cultures and on strain H37Rv in Por. 4 and Por. 13 (Table 3) suggests that, if these means indicate any systematic difference between the two experiments, it is in a direction that would imply a greater mean virulence in the deep-freeze-stored than in the fresh cultures.

Further evidence that storage in the deep-freeze did not alter the virulence of Indian cultures is provided by the close similarity of the mean of the root-indices of virulence with fresh cultures (0.73) to the mean with deep-freeze-stored cultures (0.74) in the entire Porton series (Table 4). In summary, evidence was obtained that deep-freeze-stored cultures did not differ from fresh cultures in virulence.

TABLE 3. COMPARISON OF FRESH AND DEEP-FREEZE-STORED INDIAN CULTURES IN EXPERIMENTS POR. 4 AND POR. 13

Indian patient No.	Fresh cultures (Por. 4)			Deep-freeze-stored cultures (Por. 13		
	6-week root-index	12-week root-index	Root-index of virulence	6-week root-index	12-week root-index	Root-index of virulence
1	0.84	1.08	0.96	1.10	0.93	1.02
2	0.45	0.32	0.38	0.70	0.49	0.60
3	0.45	0.32	0.38	0.62	0.55	0.58
4	0.81	0.62	0.72	0.45	0.46	0.46
5	0.45	0.40	0.42	0.79	0.67	0.73
6	0.84	1.02	0.93	0.79	1.02	0.90
7	0.81	0.94	0.88	0.96	0.92	0.94
8	0.67	0.32	0.50	0.89	0.73	0.81
9	0.81	0.89	0.85	0.94	0.88	0.91
10	1.15	0.57	0.86	0.66	0.66	0.66
11	0.84	0.45	0.64	0.89	0.62	0.76
12	0.63	0.35	0.49	0.45	0.32	0.38
13	0.45	0.26	0.36	0.78	0.60	0.69
14	1.01	0.62	0.82	0.89	0.62	0.76
15	0.45	0.72	0.58	0.45	0.55	0.50
16	0.71	0.63	0.67	0.82	0.61	0.72
17	0.87	0.56	0.72	0.66	0.62	0.64
18	0.63	0.32	0.48	0.75	0.55	0.65
19	0.45	0.77	0.61	0.87	0.75	0.81
20	0.84	0.32	0.58	0.62	0.77	0.70
Mean	0.71	0.57	0.64	0.75	0.67	0.71
Mean for British cultures	1.11	1.11	1.11	1.11	0.96	1.03
Mean for H37Rv	**1.02**	**0.82**	**0.92**	**0.94**	**0.71**	**0.82**

ANALYSIS OF VARIANCE

Term	Source of variation	Sum of squares	DF	Mean square	Term tested against	F	P
a	Patients (P)	**1.8950**	**19**	**0.0997**	**c**	**3.52**	**0.005**
b	Storage-fresh and deep-freeze - (S)	0.0952	1	0.0952	**c**	3.36	0.1
c	Interaction S x P	0.5379	19	0.0283	**g**	1.51	0.2
d	6 and 12 weeks (W)	0.2464	1	0.2464	**e**	9.51	0.005
e	Interaction W x P	0.4915	19	0.0259	**g**	1.38	>0.2
f	Interaction W x S	0.0106	1	0.0106	**g**	–	N S[a]
g	Interaction W x P x S	0.3572	19	0.0136			
	Total	3.6338	79	0.0460			

[a]NS indicates that the variance ratio is less than 1.0.

TABLE 4

VIRULENCE OF FRESH AND DEEP-FREEZE-STORED CULTURES FROM INDIAN PATIENTS IN THE PORTON SERIES

Experiment	Fresh cultures		Deep-freeze-stored cultures	
	Number	Mean root-index of virulence	Number	Mean root-index of virulence
Por.1 - Por. 3	68	0.74	0	—
Por. 4	39	0.71	4	0.60
Por. 5	4	0.71	24	0.74
Por.6-Por.13	0	—	115	0.74
Total	111	0.73	143	0.74

Inter-experimental variation

Indian cultures. The results of virulence tests on single cultures from Indian patients were divided into three groups, which were analysed separately: *(a)* Porton series, four guinea-pigs per culture; *(b)* Porton series, two guinea-pigs per culture; *(c)* Madras series, all with two guinea-pigs per culture. The analyses of variance for these three groups are set out in Tables 5 and 6. In the Porton series where each culture was injected into four animals (Table 5), 125 cultures were tested in 11 of the 13 experiments. There is no evidence that the variation in mean virulence from experiment to experiment (inter-experimental variation) was greater than the variation from culture to culture in the same experiment (Table 5, terms b and c). In the Porton series where each culture was injected into two animals (Table 6), a further 129 cultures

TABLE 5

INDIAN AND BRITISH CULTURES TESTED IN FOUR GUINEA-PIGS IN THE PORTON SERIES: ANALYSIS OF VARIANCE

Design of the investigation
(1) Indian cultures: 125 cultures in 11 experiments; each culture Injected into four guinea-pigs; total of 500 guinea-pigs.
(2) British cultures: 65 cultures; five cultures in each of 13 experiments; each culture injected into four guinea-pigs; total of 260 guinea-pigs.

Term	Source of variation	Indian cultures					British cultures			
		DF	Mean square	Term tested against	F	P	DF	Mean square	F [a]	P
a	Cultures (C)	124	0.1978				64	0.0388		
b	Experiments (E)	10	0.1625	c	—	*N S* [b]	12	0.0723	2.33	0.01-0.02
c	Cultures in same experiment C(E)	114	0.2009	h	8.85	<0.001	52	0.0310	1.95	0.001
d	6 and 12 weeks (W)	1	3.6006	f	183.70	<0.001	1	1.1246	34.50	<0.001
e	Interaction W x C	124	0.0310	h	1.37	0.02	64	0.0201	1.26	0.1-0.2
f	Interaction W x E	10	0.0196	g		NS	12	0.0326	1.90	0.05
g	Interaction W x C(E)	114	0.0320	h	1.41	0.01	52	0.0172	1.08	>0.2
h	Duplicate guinea-pigs	245 [c]	0.0227				126 [d]	0.0159		
	6 weeks	120	0.0215	j	1.11	0.5 [e]	62	0.0110		
	12 weeks	125	0.0238	i			64	0.0207	1.88	0.02 [e]

[a] Against the term indicated in the " Indian cultures " column.
[b] NS indicates that the variance ratio is less than 1.0.
[c] For five non-tuberculous deaths (all in 6-week guinea-pigs) missing observations were estimated as described in the text (page 289).
[d] For four non-tuberculous deaths (three in 6-week guinea-pigs) missing observations were estimated as described in the text (page 289).
[e] Two -tail probability.

TABLE 6

INDIAN AND BRITISH CULTURES TESTED IN TWO GUINEA-PIGS: ANALYSIS OF VARIANCE

Design of the investigation

(1) Indian cultures: *(a)* Porton series: 129 cultures in 10 experiments; total of 258 guinea-pigs. *(b)* Madras series: 55 cultures in 11 experiments; total of 110 guinea-pigs.

(2) British cultures: Madras series: 28 cultures in 11 experiments; total of 56 guinea-pigs.

Term	Source of variation	Indian cultures									British cultures			
		Porton series					Madras series				Madras series			
		DF	Mean square	Term tested against	F	P	DF	Mean square	F [a]	P	DF	Mean square	F [a]	P
a	Cultures (C)	128	0.0988				54	0.2158			27	0.0576		
b	Experiments (E)	9	0.1943	c	2.12	0.04	10	0.2449	1.17	>0.2	10	0.0426	–	NS [b]
c	Cultures in same experiment C(E)	119	0.0915	e	3.43	< 0.001	44	0.2092	8.47	<0.001	17	0.0664	1.69	0.1
d	6 and 12 weeks (W)	1	1.9745	e	73.95	<0.001	1	1.8331	74.21	<0.001	1	0.4866	12.41	<0.005
e	Interaction W x C	128	0.0267				54	0.0247			27	0.0392		
f	Interaction W x E	9	0.0317	g	1.20	>0.2	10	0.0145	–	NS	10	0.0275	-	NS
g	Interaction W x C(E)	119	0.0264				44	0.0270			17	0.0460		

[a] Against the term indicated in the " Porton series " column.
[b] NS indicates that the variance ratio is less than 1.0.

were tested in 10 of the experiments. Statistically significant evidence of inter-experimental variation is apparent here (Table 6, term b, P = 0.04). Finally, in the Madras series, 55 cultures were tested in 11 experiments;' no inter-experimental variation is apparent (Table 6, term b).

British cultures. In each of the 13 experiments in the Porton series, five cultures from different British patients were tested, each in four guinea-pigs. The analysis of variance is set out in Table 5. The variation in mean virulence from experiment to experiment is significantly greater than the variation from culture to culture in the same experiment (Table 5, terms b and c, P=0.01–0.02). Since the variation from culture to culture in the same experiment is smaller for the British cultures (mean square, 0.0310) than for the Indian cultures (mean square, 0.2009), the results on the British cultures are likely to provide a more sensitive test for inter-experimental variation, even though the total number of British cultures tested was fewer.

The analysis of variance of the virulence-test results on the 28 British cultures tested in 11 experiments at Madras is set out in Table 6. No significant inter-experimental variation was found (Table 6, term b).

Strain H37Rv. In the Porton series, strain H37Rv was tested in Por. 4 and in all subsequent experiments. The culture to be tested in each experiment was taken from the deep-freeze and inoculated on to two Löwenstein-Jensen medium slopes. After the usual 3-week incubation period, two separate infecting suspensions were prepared from each slope. From each suspension, two guinea-pigs were infected with 1.0 mg of bacilli and a further two with 0.1 mg of bacilli. Thus eight animals were used in each experiment. The full analysis of variance of the results of the virulence tests is set out in Table 7. Statistically significant evidence of inter-experimental variation exists (Table 7, term b, P=0.01).

Estimates of inter-experimental variation. Estimates of the extent of the variation from experiment to experiment, expressed as a standard deviation (the square root of the component of variance due to this source in the analyses of variance in Tables 5, 6 and 7), are set out in Table 8. In addition, the probability that these estimates differ from 0.00 is shown. The estimates range from 0.00 to 0.07.

In summary, the presence of inter-experimental variation has been sought for in six analyses and demonstrated in only three. The estimated magni-

TABLE 7

STRAIN H37Rv IN THE PORTON SERIES : ANALYSIS OF VARIANCE

Design of the investigation { 10 experiments ; in each experiment two infecting suspensions, each in two doses, each injected into two guinea-pigs ; total of 80 guinea-pigs.

Term	Source of variation	Sum of squares	DF	Mean square	Term tested against	F	P
a	Infecting suspensions (I)	0.6908	19	0.0364			
b	Experiments (E)	0.5375	9	0.0597	**k**	2.83	0.01
c	Infecting suspensions in same experiment I (E)	0.1533	10	0.0153	**i**		N S[a]
d	Doses of 1.0 and 0.1 mg (D)	0.5136	1	0.5136	**f**	12.62	<0.005
e	6 and 12 weeks (W)	0.2453	1	0.2453	**j**	10.85	<0.005
f	Interaction D x I	0.7726	19	0.0407	**i**	1.81	0.1
g	Interaction W x I	0.4305	19	0.0227	**i**	1.01	>0.2
h	Interaction W x D	0.0256	1	0.0256	**i**	1.14	>0.2
i	Interaction W x D x I	0.4267	19	0.0225			
j	(g + h + i)	0.8828	39	0.0226			
k	(c + g + h + i)	1.0361	49	0.0211			
	Total	3.1051	79	0.0393			

[a] NS indicates that the variance ratio is less than 1.0.

TABLE 8

ESTIMATES OF INTER-EXPERIMENTAL VARIATION

Series	Type of culture	Number of guinea-pigs per culture	Degrees of freedom		Inter-experimental variation	
			Experiments	Cultures in same experiment	P	Square root of component of variance (standard deviation)
Porton	**Indian**	**4**	**10**	**114**	**NS** [a]	**0.00**
	Indian	**2**	**9**	**119**	**0.04**	**0.07**
	British	**4**	**12**	**52**	**0.01-0.02**	**0.05**
	H37Rv	**8**	**9**	**49** [b]	**00.1**	**0.07**
Madras	**Indian**	**2**	**10**	**44**	**>0.2**	**0.06**
	British	**2**	**10**	**17**	**NS**	**0.00**

0.01?

[a] NS indicates that the variance ratio is less than 1.0.
[b] Term k in Table 7.

tude of the variation is very small, and probably contributed less than 0.07 to the standard deviation of the root-index of an individual guinea-pig.

Variation between infecting suspensions

As mentioned above, two separate infecting suspensions of strain H37Rv were used in each experiment. No evidence was obtained of differences between the root-indices of the animals infected with these suspensions in the same experiment (Table 7, term c). In each experiment the infecting suspension was injected in two doses, 1.0 and 0.1 mg. The mean of the root-indices was 0.93, for animals receiving 1.0 mg and 0.77 for those receiving 0.1 mg. The difference attains significance at the 0.5% level (Table 7, term d). Thus, the variation in dose resulting from miscellaneous errors in the preparation of the infecting suspension was inconsiderable, whereas a known lo-fold decrease in dose decreased to a definite (though small) extent the values of the root-indices of virulence.

Further evidence on the effect of variation in the infecting suspensions is provided by relating the viable count on the infecting suspension to the root-index of virulence obtained with it, separately for tests on 24 Indian cultures (Fig.1) and on 58 British cultures (Fig. 2). No association is evident (Fig. 1: r =0.20, P> 0.3; Fig. 2: r =0.10,P> 0.4). Thus,variation in the numbers of bacilli in the infecting suspensions, as measured by the viable counts, did not influence the values of the root-indices of virulence. Clumps of bacilli were sometimes visible in the infecting suspension, and it will be appreciated that the viable counts cannot, therefore, be considered as accurate measures of either the number or the weight of bacilli present. In summary, no evidence of the existence of variation between infecting suspensions was obtained, and, if such variation existed, it was apparently unrelated to the root-index of virulence.

FIG. 1
VIABLE COUNTS ON INFECTING SUSPENSIONS FROM 24 INDIAN CULTURES RELATED TO ROOT-INDICES OF VIRULENCE

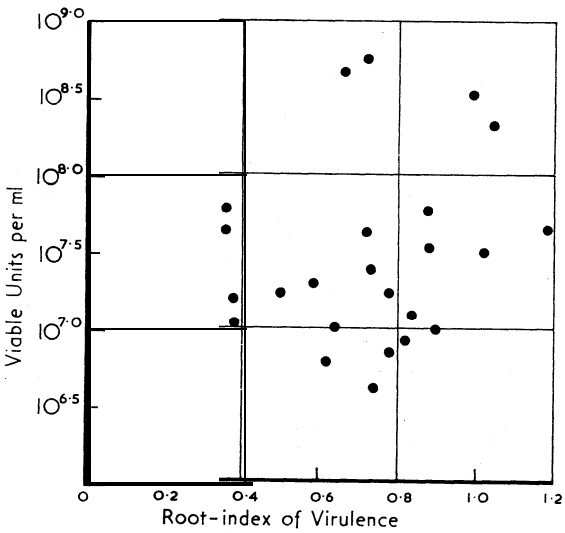
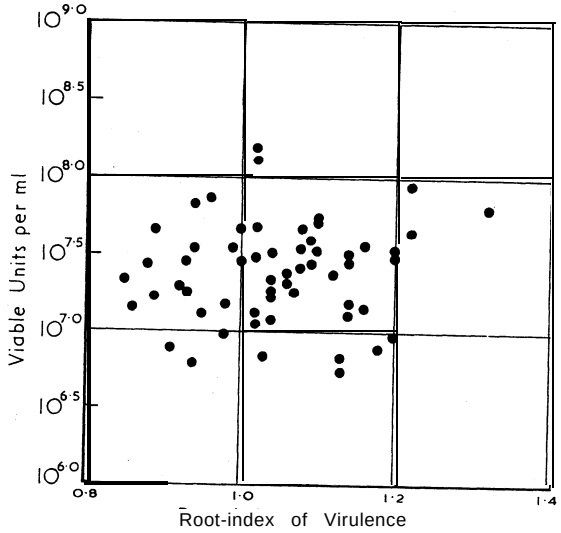


FIG. 2
VIABLE COUNTS ON INFECTING SUSPENSIONS FROM 58 BRITISH CULTURES RELATED TO ROOT-INDICES OF VIRULENCE



Further terms in the analyses of variance

The further terms in the analyses of variance set out in Tables 5, 6 and 7 will be considered in section B.

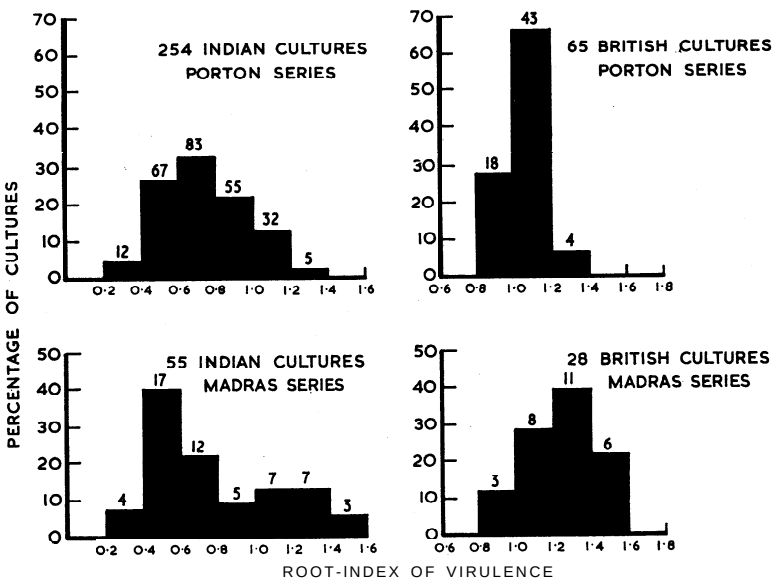
Adjustment of the Madras to the Porton Series

Histograms of the root-indices of virulence on single cultures from the 254 Indian patients in the Porton series and from the 55 Indian patients in the Madras series are set out on the left in Fig. 3 (the data are also given in Table 2 of Bhatia et al. (1961a [1])). It is apparent, particularly in the Porton series, that the distributions approximate to normal-

[1] See article on page 313.

FIG. 3

DISTRIBUTIONS OF ROOT-INDICES OF VIRULENCE OBTAINED WITH INDIAN AND BRITISH CULTURES IN THE PORTON AND THE MADRAS SERIES



ity, but that they differ both in their means and in their standard deviations, which are:

	Mean	*Standard deviation*
Indian cultures, Porton series	0.7285	0.223
Indian cultures, Madras series	0.7886	0.336

Since cultures from 27 of the Indian patients were tested only in the Madras series, their root-indices were adjusted to correspond to the Porton series by allowing for the differences both in the means and in the standard deviations of the two distributions, using the following equation:

$$\frac{Y - 0.7285}{0.223} = \frac{X - 0.7886}{0.336} \quad \ldots\ldots\ldots\ldots\ldots \quad (1)$$

where X is the root-index of virulence before adjustment and Y is the adjusted value.

From (1) $Y = 0.66X + 0.21$ . . . . . (2)

Cultures from 28 of the 55 patients in the Madras series were also tested in the Porton series, and in 23 of these patients the same culture was tested in both series. For these 28 patients, the values of the root-indices of virulence obtained in the Madras series both before (X) and after adjustment (Y) are compared in Table 9 with the root-indices obtained in the Porton series. The success of the adjustment was evaluated by comparing the variation between the adjusted root-index in the Madras series and the root-index obtained on the (usually) same culture in the Porton series, with the natural variation in response from guinea-pig to guinea-pig estimated from the 6-week and 12-week root-indices of the same 28 cultures in the Porton series. The adjustment appears to have been successful, since the former variation was practically the same as the latter.

In a previous publication (Tuberculosis Chemotherapy Centre, 1960), which presented the main findings of a study of three different regimens of isoniazid alone compared with isoniazid plus PAS, the results of the virulence tests reported here were used to assess more precisely the response to the four different regimens. In this connexion, the results of the tests on the cultures from the 27 patients tested only in the Madras series were adjusted to correspond to the Porton series, but the procedure used for adjustment differed slightly from the method described above. The adjustment equation, corresponding to equation 2, was derived in the same manner, but from the distributions of the *square roots of the means* of the 6-week and 12-week indices (score divided by survival period)

TABLE 9
EFFECT OF ADJUSTMENT OF ROOT-INDICES OF VIRULENCE ON CULTURES FROM 28 INDIAN PATIENTS TESTED IN BOTH THE MADRAS AND THE PORTON SERIES

Patient No.	Madras series		Porton series
	Observed root-index of virulence (X)	Adjusted root-index of virulence (Y)	Observed root-index of virulence
1	0.44	0.50	0.58
2	0.46	0.51	0.83
3	0.50	0.54	0.48
4	0.54	0.57	0.52
5	0.58	0.59	0.36
6	0.58	0.59	0.97
7	0.59	0.60	0.47
8	0.60	0.61	0.64
9	0.63	0.63	0.66
10	0.64	0.63	0.43
11 [a]	0.68	0.66	0.52
12	0.68	0.66	0.47
13	0.70	0.67	0.41
14	0.76	0.71	0.54
15	0.80	0.74	0.58
16	0.90	0.80	0.65
17 [a]	0.93	0.82	0.48
18	0.98	0.86	0.82
19	1.06	0.91	1.00
20	1.08	0.92	0.89
21	1.10	0.94	0.83
22	1.14	0.96	0.93
23 [a]	1.21	1.01	0.99
24	1.22	1.02	1.08
25	1.28	1.05	1.08
26 [a]	1.38	1.12	1.18
27	1.41	1.14	0.90
28 [a]	1.46	1.17	1.10

Source of variation	DF	Mean square	F	P
Between adjusted Madras root-index and observed Porton root-index for same patient	28	0.0140	1.01	> 0.9
Residual [b]	27	0.0138		

[a] Different cultures from the same patient tested in the two series.
[b] Interaction weeks x patients obtained from the results at Porton for the 28 patients.

and not, as above, from the distributions of the *means of the square roots* of these indices. The differences found between the adjusted values obtained by these two methods were small.

B. CRITIQUE OF THE VIRULENCE TEST

In the following section the root-indices of Indian cultures tested in the Madras series are the observations unadjusted by the procedure described above.

Variation between cultures

Among the cultures obtained from Indian patients the variation in the root-indices of virulence from culture to culture was greater than the natural variation in the response of the guinea-pigs in the tests. This difference attains significance at the 0.1% level in both the Porton and the Madras series (Table 5, terms c and h, and Table 6, terms c and e). Among the cultures from British patients, significant variation from culture to culture was found in the Porton series Table 5, term c, P = 0.001), but not in the smaller Madras series (Table 6, term c, P = 0.1). The implications of these findings are considered further in the accompanying paper by Bhatia et al. (1961a).[1]

Precision of the estimates of virulence of' Indian cultures

The 95% confidence limits for root-indices of virulence in tests done on Indian cultures in four guinea-pigs in the Porton series and on two guinea-pigs in the Porton and the Madras series have been calculated from the residual mean squares in the analyses of variance (Table 5, term h, and Table 6, term e). They are as follows:

Series	*Number of guinea-pigs per culture*	*Residual*	*95% confidence limits of root-index of virulence*
Porton	4	Duplicate guinea-pigs (Table 5)	±0.151
Porton	2	Interaction: weeks x cultures (Table 6)	±0.231
Madras	2	Interaction: weeks x cultures (Table 6)	±0.222

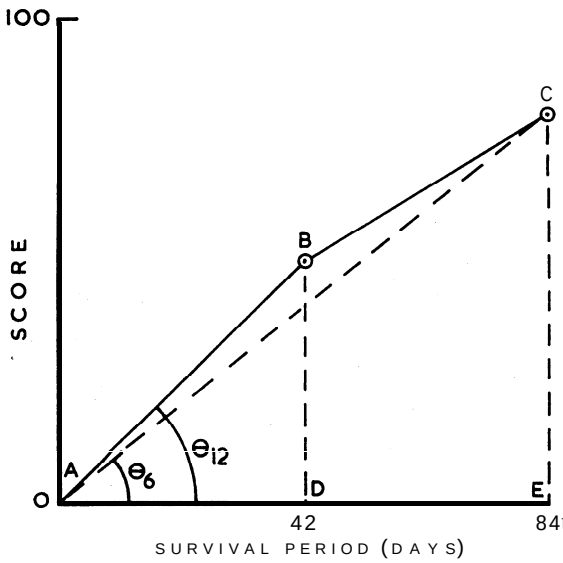
Relationship between post-mortem score and survival period

The relationship between the post-mortem score and the survival period of the guinea-pigs in a

[1] See article on page 313.

FIG. 4

RELATIONSHIP BETWEEN POST-MORTEM SCORE AND PERIOD OF SURVIVAL OF GUINEA-PIGS



virulence test is represented diagrammatically in Fig. 4, where B and C represent the findings on 6-week and 12.week animals, respectively. The index of the 6-week guinea-pigs is the score, BD, divided by the survival period, AD, or tan θ_6. Similarly, the index of the 12-week guinea-pigs is tan θ_{12}. If a linear relationship between score and survival period exists, then $\theta_6 = \theta_{12}$, and the 6-week and 12-week indices would be equal. However, the 6-week indices were found to be greater, on the average, than the 12-week indices (and similarly for the root-indices), indicating a non-linear relationship between score and survival period (A-B-C in Fig. 4). The difference between weeks attains a high degree of statistical significance in all of the analyses both of the indices (not tabulated here) and of the root-indices (term d of Tables 5 and 6 and term e of Table 7).

The square root transformation

In an earlier publication (Mitchison et al., 1960) the post-mortem score divided by the survival period in days was defined as the index and the

TABLE 10

RESIDUAL MEAN SQUARES RELATED TO INDICES OF VIRULENCE

Race of patient	Index of virulence	Porton series						Madras series	
		4 guinea-pigs per culture				2 guinea-pigs per culture		2 guinea-pigs per culture	
		Between duplicate 6-week indices		Between duplicate 12-week indices		Weeks x cultures interaction		Weeks x cultures interaction	
		DF	Mean square	DF	Mean square	DF	Mean square	DF	Mean square
Indian	0.0 –	36	0.0368	37	0.0178	47	0.0315	19	0.0110
	0.4 –	48	0.0615	49	0.0573	53	0.0504	14	0.0474
	0.8 or above	36	0.0817	39	0.0652	26	0.1138	19	0.1221
	Total	120	0.0601	125	0.0481	126	0.0564	52	0.0614
British	0.7 –	28	0.0495	29	0.0455		— [a]		— [b]
	1.1 or above	34	0.0581	35	0.1153				
	Total	62	0.0542	64	0.0837			27	0.2370
Bartlett's test	Corrected χ^2	35.573				14.911		39.729	
	DF	9				2		3	
	P	<0.001				<0.001		<0.001	

[a] Estimates of mean squares have not been tabulated since there were no cultures.

[b] Estimates of mean squares have not been tabulated since 25 of the 28 cultures had indices of virulence of 1.1 or above.

TABLE 11
RESIDUAL MEAN SQUARES RELATED TO ROOT-INDICES OF VIRULENCE

Race of patient	Root-index of virulence	Porton series						Madras series	
		4 guinea-pigs per culture				2 guinea-pigs per culture		2 guinea-pigs per culture	
		Between duplicate 6-week root-indices		Between duplicate 12-week root-indices		Weeks x cultures interaction		Weeks x cultures interaction	
		DF	Mean square	DF	Mean square	DF	Mean square	DF	Mean square
Indian	0.0 -	34	0.0261	35	0.0199	43	0.0265	20	0.0160
	0.6 -	54	0.0224	55	0.0311	59	0.0268	13	0.0201
	0.9 or above	32	0.0152	35	0.0162	24	0.0251	19	0.0263
	Total	**120**	**0.0215**	**125**	**0.0238**	**126**	**0.0263**	**52**	**0.0208**
British	**0.8 –**	**32**	**0.0105**	**33**	**0.0166**		— [a]		— [b]
	1.05 or above	**30**	**0.0115**	**31**	**0.0250**				
	Total	62	0.0110	64	0.0207			27	0.0392
Bartlett's test	Corrected χ^2	20.469				0.030		4.915	
	DF	9				2		3	
	P	0.01-0.02				0.98		0.1-0.2	

[a] Estimates of mean squares have not been tabulated since there were no cultures.
[b] Estimates of mean squares have not been tabulated since 25 of the 28 cultures had root-indices of virulence of 1.05 or above.

mean of the 6-week and 12-week indices (termed here the *index* of virulence) was employed as a measure of virulence. In the present report the mean of the square roots of the 6-week and 12-week indices has been used instead, and is called the *root-index* of virulence. The extent to which the square root transformation resulted in data showing additivity of effects together with normal and independent distribution of the errors with constant variance, conditions necessary for the valid application of the analysis of variance technique, is considered below.

Homogeneity of error variance. The residual mean squares in the analyses of variance are set out in Tables 10 and 11, related to values of the indices of virulence in the former and to values of the root-indices of virulence in the latter. High mean squares were associated with high values of the indices of virulence, especially in the Madras series, in the Porton series with two guinea-pigs per culture and in the 12-week duplicate guinea-pigs on British cultures in the Porton series. Bartlett's tests applied to the data tabulated yielded significant evidence of heterogeneity ($P < 0.001$) in all the three series. After application of the square root transformation there appeared to be little association between the values of the residual mean squares and the root-indices of virulence. Bartlett's tests yielded evidence of heterogeneity only in the Porton series with four guinea-pigs per culture, and even here the chance probability ($P = 0.01$-0.02) was larger than the corresponding probability ($P < 0.001$) based on the indices of virulence.

The variation among residual mean squares obtained in the 13 experiments of the Porton series was also studied, and the findings are presented in Table 12. The mean squares tabulated were calculated from the results with Indian and British cultures on duplicate 6-week and duplicate 12-week guinea-pigs. There was greater variation among the residual mean squares based on indices of virulence than among the corresponding mean squares based on root-indices of virulence. Bartlett's

TABLE 12
RESIDUAL MEAN SQUARES FOR EXPERIMENTS IN THE PORTON SERIES

Experiment No.	Degrees of freedom	Between duplicate guinea-pigs mean square	
		Index	Root-index
Por. 1	52	0.0410	0.0163
" 2	64	0.0928	0.0307
" 3	50	0.0421	0.0163
" 4	27	0.0604	0.0230
" 5	27	0.0488	0.0223
" 6	23	0.0504	0.0161
" 7	25	0.0591	0.0199
" 8	17	0.1366	0.0291
" 9	18	0.0525	0.0223
" 10	24	0.0240	0.0091
" 11	24	0.0421	0.0157
" 12	10	0.0667	0.0181
" 13	10	0.0666	0.0166
Por. 1-13	371	0.0592	0.0204
Bartlett's test	Corrected χ^2	30.477	17.810
	DF	12	12
	P	0.001-0.01	0.1-0.2

test yielded evidence of heterogeneity among the former (P = 0.001-0.01), but no evidence of heterogeneity among the latter (P = 0.1-0.2).

Additivity of effects. Additivity of the two main effects of the analysis of variance-namely, between cultures and between weeks-would correspond to a similar relationship between the score and the survival period at different levels of virulence. The mean differences between the 6-week and the 12-week results, related both to the index and to the root-index of virulence, are set out in Table 13. In general, there is a trend, not easy to see, indicating that the mean difference was low for low values of the *index* and for high values of the *root-index.* This trend implies that the condition of additivity was not satisfied completely either for the index or for the root-index.

In the Porton series with four guinea-pigs per culture, the constancy of the difference between weeks was tested by comparing the interaction between cultures and weeks with the residual mean square between duplicate guinea-pigs; in the Porton series with two guinea-pigs per culture and in the Madras series, it was studied by a test developed by Tukey (1949). The results of these tests of additivity, considered separately for the Indian and the British cultures, are set out in Table 14. Among the five sets of tests on indices, significant non-additivity was found in two-namely, in the results in the Porton series on Indian cultures set up on four guinea-pigs each (P = 0.001) and in the Madras series on British cultures (P = 0.03). In the corresponding tests on root-indices, there was also evidence of non-additivity in two of the sets of tests, again in the Porton series on Indian cultures (P = 0.02), and in the Madras series on Indian cultures (P = 0.01-0.02).

The reasons for non-additivity with the transformed data were examined further in the Madras series on Indian cultures, by plotting the products obtained in Tukey's test for individual cultures against the values of the root-index, and it seems probable that it was mainly due to tests in which the 12-week guinea-pig had died from tuberculosis. The proportion of 12-week guinea-pigs which died from tuberculosis was high in the Madras series, there being 12 (21.8 %) deaths among 55 guinea-pigs infected with Indian cultures (Table 15). In the tests where the 12-week guinea-pig died before 12 weeks, the 12-week root-index was not very different from the 6-week root-index, and consequently the difference between weeks was smaller than usual. A further consequence of tuberculous deaths is that the indices or root-indices of virulence obtained in those tests in which they occurred are slightly biased in an upward direction, owing to the non-linear relationship between score and survival period. Thus, if the 12-week guinea-pig died early, its root-index would be similar to the 6-week root-index, which is, on the average, higher than the root-index of virulence.

Although additivity between main effects is considered to be the most essential characteristic of the population for the valid application of the analysis of variance technique (Snedecor, 1956), the root-index of virulence, employed as a measure of virulence, is an average of the responses in 6-week and 12-week guinea-pigs and is meaningful despite minor variations in the size of the difference between weeks at different levels of virulence.

Normality of distributions. The distributions of the indices of virulence obtained with Indian and British cultures in the Porton and the Madras

TABLE 13
DIFFERENCES BETWEEN 6-WEEK AND 12-WEEK INDICES AND ROOT-INDICES, RELATED TO THE VIRULENCE OF CULTURES

Series	Race of patient	Index of virulence	Difference between 6-week and 12-week indices		Root-index of virulence	Difference between 6-week and 12-week root-indices	
			Number of cultures	Mean		Number of cultures	Mean
Porton	Indian	0.0–	85	0.167	0.0-	79	0.185
		0.4-	103	0.279	0.6–	115	0.188
		0.8 or above	66	0.283	0.9 or above	60	0.126
		Total	254	0.243	Total	254	0.172
	British	**0.7–**	**29**	**0.306**	**0.8–**	**33**	**0.171**
		1.1 or above	**36**	**0.229**	**1.05 or above**	**32**	**0.091**
		Total	65	**0.263**	Total	65	0.132
Madras	Indian	0.0–	20	0.268	**0.0–**	21	0.312
		0.4-	15	0.551	**0.6–**	14	0.356
		0.8 or above	20	0.324	0.9 or above	20	0.133
		Total	55	0.366	Total	55	0.258
	British	0.8 or above	28 [a]	0.489	0.9 or above	28 [b]	0.186

[a] Including 25 cultures with an index of 1.1 or above.
[b] Including 25 cultures with a root-index of 1.05 or above.

series are shown in Fig. 5. The corresponding distributions of the root-indices of virulence are illustrated in Fig. 3. It is apparent that the square root transformation has resulted in distributions that are more nearly symmetrical. This is particularly true of the large series of Indian cultures tested at Porton; the distribution of the indices of virulence showed positive skewness ($g_1 = 0.81$, $P < 0.001$) and negative kurtosis ($g_2 = -0.09$, non-significant), whereas the distribution of the root-indices of virulence was less skewed ($g_1 = 0.31$, $P = 0.05$) but had more kurtosis ($g_2 = -0.65$, $P = 0.04$).

In summary, the results of applying the square root transformation were:

(a) Heterogeneity of error variance was substantially decreased, especially in the Madras series and in the Porton series with two guinea-pigs per culture.

(b) Non-additivity was decreased slightly in the Porton series and increased slightly in the Madras series. The Porton series contributed the major portion of the virulence tests, so that, on balance, a slight gain may have been obtained by the transformation.

(c) A more symmetrical distribution of the results of the virulence tests was obtained.

Thus, more reliance can be placed on the results of the analysis of variance using the root-indices in place of the indices. However, when all the analyses of variance considered in section A were done on the indices, none of the conclusions that could be drawn from them differed from those reported here, nor were the probability levels altered appreciably.

The arrangement of guinea-pigs in the test

The main purpose in killing guinea-pigs at two set dates (separated by six weeks) after infection was to obtain evidence on the progress of the disease. Thus, it has been reported that Indian cultures of low virulence produce disease which tends to regress

FIG. 5

DISTRIBUTIONS OF INDICES OF VIRULENCE OBTAINED WITH INDIAN AND BRITISH CULTURES IN THE PORTON AND THE MADRAS SERIES

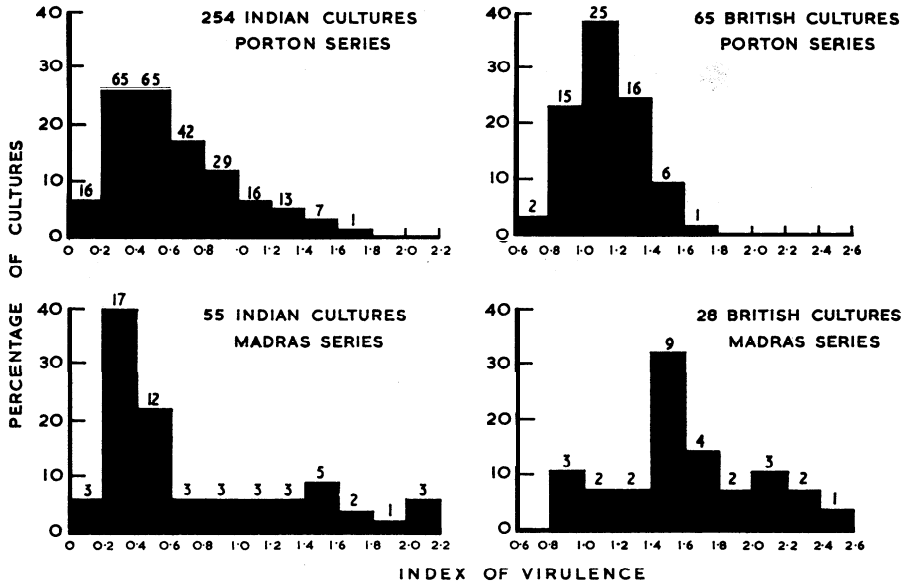


TABLE 14

RESULTS OF TESTS OF ADDITIVITY IN THE PORTON AND THE MADRAS SERIES

Series	Race of patient	Number of guinea-pigs per culture	Source of variation	Index DF	Index Mean square	Index F	Index P	Root-index Mean square	Root-index F	Root-index P
Porton	Indian	4	Interaction: week x cultures	**124**	**0.0841**	1.56	0.001	0.0310	1.37	0.02
			Between duplicate guinea-pigs	**245**	**0.0540**			0.0227		
		2	Interaction: week x cultures	128	0.0586			0.0267		
			Non-additivity	1	0.0291	-	NS[a]	0.0482	1.81	0.2
			Residual	127	0.0588			0.0266		
	British	**4**	**Interaction: weeks × cultures**	**64**	**0.0844**	1.22	0.2	0.0201	1.26	0.1-0.2
			Between duplicate guinea-pigs	**126**	**0.0692**			0.0159		
Madras	Indian	2	Interaction: week x cultures	54	0.0660			0.0247		
			Non-additivity	1	0.0203	—	NS	0.1407	6.25	**0.01-0.02**
			Residual	53	0.0668			0.0225		
	British	2	Interaction: weeks x cultures	27	0.2370			0.0392		
			Non-additivity	1	1.0630	5.30	0.03	0.0820	2.19	0.1-0.2
			Residual	26	0.2044			0.0375		

[a] NS indicates that the variance ratio is less than 1.0.

TABLE 15

DEATHS FROM TUBERCULOSIS IN GUINEA-PIGS IN THE PORTON AND THE MADRAS SERIES

Series	Guinea-pig group	Indian cultures			British cultures		
		Number of guinea-pigs	Deaths from tuberculosis		Number of guinea-pigs	Deaths from tuberculosis	
			No.	%		No.	%
Porton	6-week	379	8	2.1	130	8	6.2
	12-week	379	49	12.9	130	69	53.1
	Total	758	57	7.5	260	77	29.6
Madras	6-week	55	6	10.9	28	9	32.1
	12-week	55	12	21.8	28	19	67.9
	Total	110	18	16.4	56	28	50.0

between 6 and 12 weeks (Mitchison et al., 1960). Having obtained this evidence, it is pertinent to inquire whether any further advantage was gained by this procedure and, if not, whether killing all the animals at either 6 weeks or 12 weeks would be an improvement in future virulence tests.

Inter-experimental variation. A subsidiary reason for killing the guinea-pigs at 6 weeks and at 12 weeks was to reduce inter-experimental variation due to systematic changes in scoring from one day of post-mortem examination to another, by taking the average of the two test results for the same culture obtained on successive days of examination. Estimates of inter-experimental variation, expressed as standard deviations, were derived separately for 6-week root-indices, 12-week root-indices and root-indices of virulence, and are set out in Table 16. These estimates range from 0.00 to 0.07 for the 6-week root-indices, from 0.00 to 0.08 for the 12-week root-indices and from 0.00 to 0.07 for the root-indices of virulence. No apparent reduction in inter-experimental variation has resulted from the use of 6-week and 12-week guinea-pigs in the tests, nor is there any clear evidence that it is smaller with either 6-week or 12-week animals.

Efficiencies of various arrangements. The efficiencies of various possible arrangements of guinea-pigs were studied, using the root-indices obtained in the Porton series. By efficiency is meant the ability of an arrangement to distinguish between the virulence of different cultures. It has been expressed (Table 17) as the relative numbers of guinea-pigs required to equalize the variance ratio (the ratio of the mean square for cultures in the same experiment to the residual mean square), that is, to equalize the relative efficiencies of the various arrangements. The arrangements considered were:

(a) All guinea-pigs killed at 6 weeks.

(b) All guinea-pigs killed at 12 weeks.

(c) Half the guinea-pigs killed at 6 weeks and the other half at 12 weeks.

In a design with two guinea-pigs per culture, the relative numbers of guinea-pigs required for equal efficiency with Indian cultures are 100, 97 and 136,

TABLE 16

ESTIMATES OF INTER-EXPERIMENTAL VARIATION WITH 6-WEEK ROOT-INDICES, 12-WEEK ROOT-INDICES AND ROOT-INDICES OF VIRULENCE

Series	Type of culture	Number of guinea-pigs per culture	Inter-experimental variation (square root of component of variance)		
			6-week root-indices	12-week root-indices	Root-indices of virulence
Porton	Indian	4	0.00	0.00	0.00
	Indian	2	0.04	0.08	0.07
	British	4	0.03	0.07	0.05
	H37Rv	6	0.07	0.08	0.07
Madras	Indian	2	0.07	0.00	0.06
	British	2	0.00	0.00	0.00

TABLE 17

EFFICIENCIES OF VARIOUS ARRANGEMENTS OF GUINEA-PIGS IN THE VIRULENCE TESTS

(Derived from root-indices of virulence in the Porton series)

Race of patient	Relative number of guinea-pigs required to equalize the variance ratios					
	2 guinea-pigs per culture			4 guinea-pigs per culture		
	Both killed at 6 weeks	Both killed at 12 weeks	1 killed at 6 weeks and 1 at 12 weeks	All killed at 6 weeks	All killed at 12 weeks	2 killed at 6 weeks and 2 at 12 weeks
Indian	100[a]	97[a]	136[a]	100[b]	97[b]	104[b]
British	100[a]	128[a]	309[a]	100[b]	128[b]	129[b]

[a] Derived from mean squares in Table 5 and analysis of variance done on one 6-week guinea-pig and one 12-week guinea-pig, selected at random from two at 6 weeks and two at 12 weeks.

[b] Derived from mean squares in Table 5.

respectively, for experimental arrangements *a*, *b* and *c*. The corresponding figures with British cultures are 100, 128 and 309, respectively. In a design with four guinea-pigs per culture, the relative numbers are 100, 97 and 104 for Indian cultures, and 100, 128 and 129 for British cultures. Thus, it appears that arrangement c, employed in the Porton series (two animals per culture) and in the Madras series, is the least efficient, and that the most efficient design is probably to kill all the guinea-pigs at six weeks, whether two or four guinea-pigs are used in the test.

Homogeneity of error variance, and normality of distributions in the separate 6-week and 12-week results. The residual mean squares (between duplicate guinea-pigs) in the Porton series were related (separately for 6-week and 12-week results and for Indian and British cultures) to values of indices of virulence and root-indices of virulence in Tables 10 and 11. The results of Bartlett's tests on these residuals for Indian cultures are set out in the upper half of Table 18. The mean squares based on 6-week root-indices show least evidence of heterogeneity (P = 0.3). For British cultures, the mean squares based on 6-week indices and 6-week root-indices were least heterogeneous (lower half of Table 18, P > 0.2).

The distributions of the indices and root-indices obtained with 'Indian and British cultures at 6 and 12 weeks are set out in Table 19. For Indian cultures, the distribution of the 6-week root-indices was more nearly normal than the other distributions, with neither the skewness nor kurtosis attaining statistical significance. For the British cultures, none of the distributions showed significant evidence of skewness or kurtosis. However, the estimates of g_1 and g_2 were closer to zero for the distribution based on the 6-week root-indices than for that based on the 6-week indices.

In summary, considering both homogeneity of variance and normality of the distributions, the 6-week root-indices appear most amenable to analysis of variance, both for Indian and for British cultures. This suggests that it would be an advantage in the design of future virulence tests to kill all guinea-pigs at six weeks.

The breeds of guinea-pig

A comparison of the results obtained from Indian cultures with the DH-breed guinea-pigs (two animals per culture) at Porton and the M-breed guinea-pigs at Madras showed that the variation from culture

TABLE 18

TESTS ON RESIDUAL MEAN SQUARES

Race of patient		Index		Root-index	
		6-week results	12-week results	6-week results	12-week results
Indian	Corrected χ^{2a}	5.437	16.167	2.263	4.958
	DF	2	2	2	2
	P	0.07	<0.001	0.3	0.09
British	F	1.17	2.53	1.10	1.51
	DF	34, 28	35, 29	30, 32	31, 33
	P	>0.2	0.005-0.01	>0.2	0.1-0.2

[a] Bartlett's test.

TABLE 19

DISTRIBUTIONS OF INDICES AND ROOT-INDICES FOR 6-WEEK AND 12-WEEK GUINEA-PIGS IN THE PORTON SERIES

Race of patient	Index	6-week guinea-pigs		12-week guinea-pigs		Root-index	6-week guinea-pigs		12-week guinea-pigs	
			%	No.	%		No.	%	No.	%
Indian	0.0-	12	*4.7*	59	*23.2*	0.0-	0	*0.0*	2	*0.8*
	0.2-	39	*15.4*	71	*28.0*	0.2-	6	*2.4*	49	*19.3*
	0.4-	53	*20.9*	42	*16.5*	0.4-	38	*15.0*	66	*26.0*
	0.6-	56	*22.0*	31	*12.2*	0.6-	74	*29.1*	64	*25.2*
	0.8-	32	*12.6*	23	*9.1*	0.8-	75	*29.5*	46	*18.1*
	1.0-	25	*9.8*	16	*6.3*	1.0-	46	*18.1*	22	*8.7*
	1.2-	20	*7.9*	5	*2.0*	1.2-	13	*5.1*	5	*2.0*
	1.4-	8	*3.1*	5	*2.0*	1.4 or above	2	*0.8*	0	*0.0*
	1.6-	5	*2.0*	2	*0.8*					
	1.8 or above	4	*1.6*	0	*0.0*					
	Total	254	*100.0*	254	*100.1*	Total	254	*100.0*	254	*100.1*
	g_1	0.80		0.99		g_1	0.17		0.36	
	P	<0.001		<0.001		P	>0.2		0.01-0.02	
	g_2	0.46		0.45		g_2	*-0.44*		-0.68	
	P	0.1-0.2		0.1-0.2		P	0.1-0.2		0.03	
British	0.2-	0	*0*	1	*2*	0.6-	0	*0*	4	*6*
	0.4-	0	*0*	2	*3*	0.8-	8	*12*	31	*48*
	0.6-	1	*2*	12	*18*	1.0-	45	*69*	25	*38*
	0.8-	*7*	*11*	20	*31*	1.2-	12	*18*	5	*8*
	1.0-	18	*28*	15	*23*	1.4 or above	0	*0*	0	*0*
	1.2-	24	*37*	8	*12*					
	1.4-	11	*17*	6	*9*					
	1.6-	2	*3*	1	*2*					
	1.8 or above	2	*3*	0	*0*					
	Total	65	*101*	65	*1 0 0*	Total	65	*99*	65	*100*
	g_1	0.38		0.23		g_1	0.10		0.06	
	P	0.2-0.3		0.4-0.5		P	0.7-0.8		0.8-0.9	
	g_2	0.38		-0.30		g_2	-0.08		-0.19	
	P	0.5-0.6		~ 0.6		P	0.8-0.9		0.7-0.8	

TABLE 20

EFFICIENCIES OF DH-BREED AND M-BREED GUINEA-PIGS IN THE VIRULENCE TESTS

(Derived from root-indices of virulence)

Race of patient	Relative number of guinea-pigs required to equalize the variance ratios	
	DH breed	M breed
Indian	308[a]	100[a]
British	347[b]	100[b]

[a] Derived from mean squares in Table 6.

[b] Derived from mean squares in Table 6 and analysis of variance done on one 6-week guinea-pig and one 12-week guinea-pig, selected at random from two at 6 weeks and two at 12 weeks.

to culture among the root-indices obtained with the DH-breed animals was less than that which occurred with the M-breed animals (Fig. 3; Table 6, term c). Furthermore, the natural variation in the response of individual guinea-pigs of the two breeds was similar (Table 6, term e) and the average of the root-indices of virulence with the DH breed (0.728) was not very different from the average with the M breed (0.794). These findings indicate that, with cultures of high virulence, the root-indices of the DH-breed guinea-pigs were smaller than those of the M-breed guinea-pigs (this tendency was also apparent in the root-indices of virulence, shown in Fig. 3, and in the deaths from tuberculosis, shown in Table 15, for the highly virulent cultures from British patients carried out in both series), whereas, with cultures of low virulence, the root-indices of the DH-breed guinea-pigs were larger than those of the M-breed guinea-pigs. Thus, the tests on DH-breed guinea-pigs were less effective in distinguishing differences of virulence than were those on M-breed guinea-pigs.

The efficiency with which the two breeds distinguished differences in virulence was again expressed as the relative numbers of guinea-pigs of the two breeds required for equal efficiency (Table 20). For every 100 M-breed animals the number of DH-breed guinea-pigs which would result in equal efficiency was estimated as 308 for Indian cultures and 347 for British cultures. In making this comparison of the relative efficiencies of the DH-breed and the M-breed guinea-pigs, it has been assumed that the scoring procedure was uniform at Porton and Madras, even though it was necessarily done at the two centres by different observers. This assumption is supported by evidence presented previously (Mitchison et al., 1960).

DISCUSSION

The conditions under which the present investigation of virulence was carried out might have introduced factors, in addition to the virulence of the cultures, affecting the results of the tests. The initial plan was to do all tests in M-breed guinea-pigs. Difficulties in breeding these animals under tropical conditions made this impossible, and large-scale facilities at Porton only became available towards the end of the study. In consequence, the tests were done in two breeds of guinea-pig, the M breed at Madras and the DH breed at Porton, and about half of the Indian tiultures were tested after storage at -20°C. Furthermore, they were tested in 25 experiments, extending over a period of two-and-a-half years. However, the results of the Madras series were successfully adjusted, to permit amalgamation with those of the much larger Porton series, storage at -20°C was found not to have affected the virulence of the cultures, and inter-experimental variation was shown to have been very small. Thus, the results of the tests appear to give a true measure of virulence, little influenced by these potential external sources of variation.

In an earlier publication (Mitchison et al., 1960) the index-that is, the post-mortem score divided by the survival time in days-was adopted as the measure of virulence for four reasons. First, it indicated the rate of development of the lesions and was thus related to a more fundamental measure, the rate of multiplication of the bacilli in the organs of the guinea-pig. Secondly, it had an advantage over the use of the score alone, since it allowed comparison of the results of different tests, irrespective of whether deaths had occurred before the appointed day for the sacrifice of the animals. Thirdly, the results obtained with 6-week and 12-week guinea-pigs could be combined to give a single measure of virulence, thus gaining information on the progress of the disease with little loss in precision of the estimates of virulence. Finally, the indices obtained in the virulence tests, which were less numerous than those reported here,

appeared acceptable for the standard statistical technique of analysis of variance. In the present report, on a much larger number of tests, the conditions underlying the use of analysis of variance with the indices did not seem to be entirely satisfied. In consequence, the square root of the index-that is, the root-index-was adopted as the measure of virulence. The results of the analyses of variance of the root-indices can be accepted with greater confidence since the square root transformation largely eliminated heterogeneity of error variance, and the root-indices were more nearly symmetrical in distribution than the indices. Nevertheless, additivity of the difference between weeks and the differences between cultures was not entirely satisfied by the root-indices of virulence in the tests on Indian cultures. However, the degree of non-additivity appeared to be small, so that the conclusions in the present report and in the papers by Bhatia et al. (1961a),[1] Ramakrishnan et al. (1961)[2] and Subbaiah et al. (1961), based on analyses of variance applied to root-indices, can be considered as reliable. It will be appreciated that the other three reasons listed above for using the index also apply to the root-index.

The effect of the square root transformation is to expand the lower range of values of the index and contract the higher range. Equally spaced indices of 0.0, 0.5 and 1.0 correspond approximately to root-indices of 0, 0.7 and 1.0, in which the interval 0.0-0.7 is more than twice the interval 0.7-1.5). In a given series of tests, low values of the root-index will affect the mean *more* than low values of the index, and high values of the root-index will have *less* influence on it than high values of the index. Virulence in a system of the type used here is difficult to define in quantitative terms, and there is no definite evidence that either of these alternative measures is preferable in a biological sense. With markedly attenuated cultures, the disease produced is confined mainly to the site of inoculation and its draining lymph-nodes. The lesions in these sites, with the scoring system used in the present paper, are allocated only a possible total of 10 out of a maximum score of 100 for the whole guinea-pig. Thus, variation in virulence among such cultures results in only small changes in the score and therefore in the index, but causes relatively larger changes in the root-index. The square root transformation can therefore be considered as a simple alternative to the more cumbersome (but equally empirical) procedure of reallocating the arbitrary score values in different proportions among the possible sites of the lesions to give more weight to variation among attenuated cultures.

TABLE 21

SCORING SYSTEMS IN EXPERIMENTAL TUBERCULOSIS OF GUINEA-PIGS

Reference	Maximum score (expressed as a percentage of the total score) for extent of tuberculosis in:			
	Spleen	Liver	Lungs	Site of inoculation and draining lymph-nodes
Feldman (1943)	35	25	30	10
Steenken & Wolinsky (1947)	25	25	25	25
Bloch et al. (1949)	33	33	33	0
Dessau, Yeager & Kulish (1949)	12	12	12	62
Marshak & Kuschner (1950)	33	33	33	0
Mitchison et al. (1961) a	40	30	20	10

[a] Present report.

Scores have frequently been used in experimental tuberculosis of guinea-pigs to assess the extent of the lesions visible to the naked eye in the organs (Table 21). The allocation of the scores among the organs has always been empirical and has varied from one investigation to another. The proportion of the total score which could be allocated to the site of inoculation and the lymph glands varied from 0 % to 62 %. Equal weights were usually given to the spleen, liver and lungs. The scoring system used in the present report resembles the system used by Feldman (1943), except for minor differences in the relative weights of the lesions in the spleen, liver and lungs. So far as can be discovered, no investigation of whether scores are acceptable for the analysis of variance technique has previously been described.

The main purpose of the investigation described here and in the companion papers was to relate the virulence in the guinea-pig of the Indian cultures to assessments of the patients' condition. Under these circumstances, and with limited numbers of guinea-pigs, it is more efficient to test a large number of cultures, each in a small group of guinea-pigs,

[1] See article on page 313.

[2] See article on page 323.

than to make accurate estimates of virulence with large groups on a few cultures. In the basic test, two guinea-pigs were therefore infected. Of these two guinea-pigs one was killed at 6 weeks and one at 12 weeks, the latter in order to gain additional information on the progress of the disease. A reason for seeking this information was to make possible a comparison between the biological characteristics of attenuation in these isoniazid-sensitive cultures and attenuation in their isoniazid-resistant variants (the latter were tested in the same set of experiments and the results will be reported elsewhere). The arrangement with two guinea-pigs, one killed at 6 weeks and the other at 12 weeks, was found to be less efficient in detecting differences in virulence between cultures than the other possible arrangements, in which the two animals are both killed either at 6 weeks or at 12 weeks; the loss in efficiency was small in the tests on Indian cultures, but larger in those on British cultures. A further advantage in killing both animals at the same time in future tests would be that non-additivity of the two main effects would cease to be a cause for lack of confidence in the analyses of the results. As to the choice between 6 and 12 weeks as the time for killing both animals, the 6-week period is preferable since the 6-week root-indices were found to be more homogeneous and their distribution did not depart significantly from normality. In addition, the proportion of deaths from tuberculosis, which is here shown to be a source of minor bias, would be lowered and, finally, the results of the tests would be available earlier. In the future design of virulence tests on cultures comparable to those reported here, it might well not be necessary to gain additional information on the progress of the disease during more than one interval after infection. Under such circumstances the procedure which would be most efficient and also most amenable to analysis of variance would be to kill two or more animals, all at six weeks.

The differences in efficiency found between the DH and the M breeds of guinea-pig is of interest. Cultures of high virulence produced lower root-indices and fewer deaths from tuberculosis in the DH-breed than in the M-breed animals, whereas cultures of low virulence produced higher root-indices in the DH-breed animals. Consequently, with similar arrangements and numbers of guinea-pigs in the tests, about three of the DH-breed animals were required for every one of the M-breed animals to achieve equal ability in discriminating between the virulence of cultures. In the interests of economy in guinea-pigs, it would clearly be an advantage to use only the M breed. In this breed, to kill all animals at six weeks would be of particular importance, since the heterogeneity and bias introduced by the high proportion of deaths from tuberculosis in 12-week animals was higher in the Madras than in the Porton series.

SUMMARY

1. Virulence tests in the guinea-pig were done on 281 isoniazid-sensitive cultures obtained from the same number of Indian patients on admission to a study of various regimens of domiciliary chemotherapy in the treatment of pulmonary tuberculosis, and on 93 cultures from newly diagnosed, untreated British patients. The tests on 254 of the Indian cultures and on 65 of the British cultures were in DH-breed guinea-pigs at Porton, and the remaining 27 Indian cultures and 28 British cultures were tested in M-breed guinea-pigs at Madras.

2. In the test, 1 mg of each culture was injected by the intramuscular route into two guinea-pigs or, for 125 Indian cultures and all 65 British cultures in the Porton series, into four guinea-pigs. Half the animals were sacrificed at 6 weeks and the other half at 12 weeks; the extent of tuberculosis in the organs was scored at the post-mortem examination, the maximum score per guinea-pig being 100. Animals dying before the appointed day were similarly scored. The score on each animal was divided by its survival period to give an index. The mean of the square roots of the 6-week index and the 12-week index (the root-index of virulence) was taken as the measure of virulence since it was found to be more acceptable for the analysis of variance technique than the index of virulence used by Mitchison et al. (1960).

3. Of the 254 Indian cultures in the Porton series, 143 were stored at –20°C for 44-78 weeks (average, 62 weeks) before being tested. A comparison carried out on pairs of cultures, one stored at –20°C and the other tested fresh, from 20 Indian

patients showed no clear evidence of alteration in virulence.

4. The tests were done in 13 experiments at Porton and in 12 experiments at Madras over a period of two-and-a-half years. The results on the Indian and British cultures in both series, and on strain H37Rv, set up as a control in the majority of the experiments at Porton, indicated that inter-experimental variation was small in the Porton series and could not be detected in the Madras series.

5. In the tests on strain H37Rv, variation in the preparation of the infecting suspension did not appear to influence the root-indices of virulence, nor were the viable counts on the suspensions of Indian and British cultures associated with the values of the root-index. However, a known 10-fold decrease in the dose of bacilli lowered the root-index to a small extent.

6. To obtain comparable results throughout the study, the root-indices of virulence in the Madras series were adjusted to those in the Porton series by allowing for differences in the means and standard deviations of the distributions for the two series. The adjustment appeared to be successful, since the adjusted root-indices in the Madras series were the same, within the limits of error of the test, as the root-indices in the Porton series obtained in tests done in both laboratories on cultures from the same 28 Indian patients.

7. The results of the tests in the Porton series indicate that to kill all guinea-pigs six weeks after infection would have the advantages of greater efficiency in detecting differences in the virulence of the cultures, of yielding results more acceptable for the analysis of variance technique, and of rapidity.

8. Cultures of high virulence produced fewer deaths from tuberculosis and lower root-indices in the DH-breed than in the M-breed guinea-pigs, whereas cultures of low virulence produced higher root-indices in the DH-breed guinea-pigs. In consequence, differences in virulence were shown less efficiently with the DH-breed guinea-pigs, about three DH animals being of equal efficiency to one M-breed animal

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RESUME

Poursuivant la comparaison de la virulence de souches de bacilles tuberculeux isolés de malades de l'Inde méridionale d'une part, de Grande-Bretagne d'autre part, les auteurs ont étudié la virulence de ces diverses souches pour le cobaye.

Pour ce faire, ils ont utilisé 281 cultures sensibles à l'isoniazide provenant de 281 malades participant aux recherches comparées sur le traitement à domicile et en sanatorium. L'autre lot de souches comprenait 93 cultures provenant de cas récents, non traités, de Grande-Bretagne. Les tests ont été effectués à Porton d'une part, avec 254 cultures de l'Inde et 65 cultures britanniques, sur des cobayes de lignée DH, et à Madras d'autre part, avec 27 cultures de l'Inde et 28 cultures britanniques, sur des cobayes de lignée M. Un mg de chacune des cultures a été inoculé par voie intramusculaire à deux cobayes et, pour 125 cultures de l'Inde et 65 cultures britanniques des séries de Porton, à quatre cobayes. La moitié des animaux fut sacrifiée après 6 semaines, l'autre moitié après 12 semaines. L'importance des lésions tuberculeuses fut estimée post mortem, le nombre maximum de points étant de 100. L'infection des animaux morts avant l'autopsie fut aussi évaluée. La somme des points pour chaque animal fut divisée par sa période de survie de façon à obtenir un indice. La moyenne des racines carrées de l'indice de 6 semaines et de l'indice de 12 semaines (racine de l'indice de virulence) fut prise comme mesure de la virulence. Cet indice parut en effet convenir mieux que l'indice de virulence utilisé par Mitchison et al. à l'analyse de la variance.

Sur les 254 cultures indiennes de la série de Porton, 143 avaient été conservées à −10°C pendant 44-78 semaines (moyenne 62 semaines) avant d'être soumises au test. La comparaison de couples de cultures, l'une conservée à −20°C, l'autre fraîche, provenant de 20 malades indiens, ne permit pas de déceler une différence de virulence entre ces deux groupes de cultures. Des essais témoins utilisant la souche de bacilles tuberculeux H37Rv montrèrent que les variations au sein de la série de Porton étaient faibles, et, dans la série de Madras, indécelables.

Dans les tests avec la souche H37Rv, des variations dans la préparation de la suspension n'ont pas paru affecter la valeur « racine de la virulence ». Cette dernière a cependant été légèrement abaissée par l'emploi d'une dose infectante qui n'était que le dixième de la dose normale.

Afin de rendre les résultats comparables, les indices « racine de la virulence » de la série de Madras ont été ajustés à ceux de la série de Porton, en tenant compte de l'écart moyen et de l'écart type de la distribution dans les deux séries. Cet ajustement a paru convenable, puisque les indices de la série de Madras étaient les mêmes que ceux de la série de Porton, dans les limites d'erreur du test, dans les essais faits dans les deux laboratoires avec les mêmes cultures (28 malades de l'Inde).

Ces divers tests ont permis de vérifier que les variations entre les deux séries d'expériences et la variabilité d'origine externe étaient minimums ou nulles, ce qui est d'une importance considérable pour les recherches futures.

Les résultats des tests de la série de Porton indiquent que le sacrifice des animaux après 6 semaines a l'avantage de mettre mieux en évidence les différences de virulence et de donner des résultats se prêtant mieux à l'analyse de la variance, sans parler d'une plus grande rapidité.

Les cultures hautement virulentes ont produit moins de morts par tuberculose et de plus faibles indices « racine de la virulence » dans la lignée de cobayes DH que dans la lignée M. Les cultures faiblement virulentes, au contraire, ont donné des indices plus élevés dans la lignée DH. Il s'ensuit que la lignée DH se prête moins bien à la mise en évidence de la virulence que la lignée M, trois animaux de la première correspondant à un animal de la seconde.

REFERENCES

Bhatia, A. L., Csillag, A., Mitchison, D. A., Selkon, J. B., Somasundaram, P. R. & Subbaiah, T. V. (1961a) *Bull. Wld Hlth Org.*, **25,** 313

Bhatia, A. L., Devadatta, S., Mitchison, D. A., Radhakrishna, S., Selkon, J. B. & Subbaiah, T. V. (196lb) *Tubercle (Lond.)*, **42,** 317

Bloch, R. G., Vennesland, K., Ebert, R. H. & Gomori, G. (1949) *Amer. Rev. Tuberc.*, **59,** 554

Cohn, M. L., Middlebrook, G. & Russell, W. F. (1959) *J. clin. Invest.*, **38,** 1349

Dessau, F. I., Yeager, R. L. & Kulish, M. (1949) *Amer. Rev. Tuberc.*, **60,** 223

Feldman, W. H. (1943) *Amer. Rev. Tuberc.*, **48,** 248

Feldman, W. H., Karlson, A. G. & Hinshaw, H. C. (1948) *Amer. Rev. Tuberc.*, **57,** 162

Frimodt-Møller, J. (1957) In: Indian Council of Medical Research, *Technical Report of the Scientific Advisory Board*, New Delhi, p 153

Frimodt-Møller, J., Mathew, K. T. & Barton, R. M. (1956) In: Tuberculosis Association of India, *Proceedings of the Thirteenth Tuberculosis Workers' Conference, held in Trivandrum . . .*, New Delhi, p. 157

Jensen.K. A., (1955) *Bull. int. Un. Tuberc.*, **25,** 89

Karlson, A. G. & Gamer, J. H. (1951) *Proc. Mayo Clin.*, **26,** 5 3

Marshak, A. & Kuschner, M. (1950) *Publ. Hlth Rep.* (Wash.), **65,** 131

Mitchison, D. A., Wallace, J. G., Bhatia, A. L., Selkon, J. B., Subbaiah, T. V. & Lancaster, M. C. (1960) *Tubercle (Lond.)*, **41,** 1

Ramakrishnan, C. V., Bhatia, A. L., Fox, W., Mitchison, D. A., Radhakrishna, S., Selkon, J. B., Subbaiah, T. V., Velu, S. & Wallace, J. G. (1961) *Bull. Wld Hlth Org.*, **25,** 323

Selkon, J. B. & Mitchison, D. A. (1957) *J. gen. Microbiol.*, **16,** 229

Selkon, J. B., Subbaiah, T. V., Bhatia, A. L., Radhakrishna, S. & Mitchison, D. A. (1960) *Bull. Wld Hlth. Org.*, **33,** 599

Snedecor, G. W. (1956) *Statistical methods*, 5th ed., Ames, Ia., Iowa College Press, p. 315

Steenken, W. & Wolinsky, E. (1947) *Amer. Rev. Tuberc.*, **56,** 227

Steenken, W. & Wolinsky, E. (1948) *Amer. Rev. Tuberc.*, **58,** 353

Subbaiah, T. V., Bhatia, A. L., Geraghty, E., Mitchison, D. A., Radhakrishna, S. & Selkon, J. B. (1961) *Bull. Wld Hlth Org.*, **25** (In press)

Subbaiah, T. V., Mitchison, D. A., & Selkon, J. B. (1960) *Tubercle (Lond)*, **41,** 323

Thomas, K. L., Joseph, S., Subbaiah T. V. & Selkon, J. B. (1961) *Bull. Wld Hlth Org.*, **25** (In press)

Tuberculosis Chemotherapy Centre (1959) *Bull. Wld Hlth Org.*, **21,** 51

Tuberculosis Chemotherapy Centre (1960) *Bull. Wld Hlth Org.*, **23,** 535

Tukey, J. W. (1949) *Biometrics*, **5,** 232

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