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A Comparison of the Sensitivity to *p*-Aminosalicylic Acid of Tubercle Bacilli from South Indian and British Patients

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In a comparison of home and sanatorium treatment for tuberculous patients in India, pretreatment cultures of tubercle bacilli showed a higher average level of resistance to p-*aminosalicyclic acid (PAS) than pretreatment cultures from a representative sample of patients in Great Britain. The investigation described in the present paper was therefore undertaken to find out the nature of the difference in the PAS sensitivity of cultures from Indian and British patients. In this investigation, carried out jointly at the Tuberculosis Chemotherapy Centre, Madras, and the Postgraduate Medical School of London, sputum specimens from 147 Indian and 93 British patients were cultured and subjected to sensitivity tests. The tests were set up on slopes containing various concentrations of PAS and inoculated with* 10^5 *viable units of the cultures, and the minimal concentrations of PAS inhibiting the growth of 20, 50 and 100 colonies were determined. According to the 20-colony end-point—the one commonly used in routine sensitivity tests—the Indian strains were significantly more resistant than the British strains. This difference in sensitivity was not apparent, however, in either the 50-colony or the 100-colony results. The presence of a small proportion of resistant organisms was found to be a general characteristic of the Indian strains, but did not appear to be related to any special tendency for the patient to fail to respond to treatment with PAS. Since a chance increase in the inoculum size might well affect the 20-colony results, the authors recommend a tenfold decrease in the size of the inoculum used in routine PAS-sensitivity tests on Indian patients.*

The Tuberculosis Chemotherapy Centre, Madras, was established, under the joint auspices of the Indian Council of Medical Research, the Madras Government, the World Health Organization and the Medical Research Council of Great Britain, to investigate the chemotherapy of tuberculosis in India. Chemotherapeutic drugs have been studied mainly in patients in Europe and in North America, and laboratory research has largely been confined to strains of tubercle bacilli isolated from patients in these regions. One of the essential investigations in Madras was therefore a comparison of the susceptibility of South Indian and British strains of tubercle bacilli to the drugs commonly employed for treatment. Differences, in particular, in susceptibility to *p*-aminosalicylic acid (PAS) were suspected for the following reasons. First, a sample of pretreatment cultures from Indian patients in a controlled comparison of home and sanatorium treatment (Tuberculosis Chemotherapy Centre, 1959) had been found to show a higher average level of resistance to PAS than a representative sample of cultures from previously untreated patients in Great Britain using the same method of testing (Mitchison & Selkon, 1957). Secondly, the demonstration of PAS-resistance in pretreatment cultures, according to definitions based on strains from British patients, was less closely related to the subsequent emergence of isoniazid-resistance, or to continuing bacterial positivity during treatment with PAS and isoniazid, in Indian patients (Tuberculosis Chemotherapy Centre, 1959) than in British patients (Great Britain,

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Medical Research Council, 1955; Selkon & Mitchison, 1958). The aim of the investigations reported here was to study the nature of any differences in the PAS sensitivity of cultures from Indian and British patients.

METHODS

Cultures of tubercle bacilli

Sputum specimens from South Indian patients attending the Tuberculosis Chemotherapy Centre, none of whom, as far as could be discovered, had received more than two weeks of previous chemotherapy for tuberculosis (the great majority had received none) were obtained before the start of treatment in the Centre and were cultured in the Centre's laboratory (TCC). Sputum specimens from previously untreated British patients attending selected chest clinics in Great Britain (see acknowledgements) were cultured at the Postgraduate Medical School of London (PGMS). The specimens were cultured by the same method at the TCC and the PGMS—namely, homogenization with 4% sodium hydroxide followed by washing of the deposit with water (Tuberculosis Chemotherapy Centre, 1959). The deposit was cultured on Löwenstein-Jensen medium [1] slopes, which were examined weekly for the occurrence of growth.

Sensitivity tests

A suspension was prepared by adding approximately 2 mg (moist weight) of a representative sample of the bacillary mass from the primary culture to 0.5 ml of sterile water in a ¼-oz. (7-g) screw-capped bottle containing about six glass beads of 3-mm diameter, and then shaking the bottle mechanically for one minute. A 3-mm loopful of this suspension was inoculated on a series of slopes of Löwenstein-Jensen medium [1] containing various concentrations of PAS and on one slope without PAS. In investigation 1, these slopes contained 0.25, 0.5, 1, 2, 4, 8, 16 and 64 μg/ml sodium PAS dihydrate, the same concentrations as were used in routine sensitivity tests at the TCC; and, in investigations 2 and 3, the concentrations were increased, by twofold steps, from 0.0625 to 32 μg/ml sodium PAS dihydrate. The inoculated slopes were examined for growth after four weeks' incubation at 37°C. " Growth " on a slope was defined in three different ways: (1) the presence of 20 or more colonies; (2) the presence of 50 or more colonies; and (3) the presence of 100 or more colonies. The lowest concentration of sodium PAS inhibiting each of these degrees of growth was recorded (the minimal inhibitory concentration, or MIC). Thus three endpoints were obtained for each test, which will be referred to as the 20-colony MIC, the 50-colony MIC, and the 100-colony MIC. Since it was known that the original inoculum contained about 10^5 viable units, the 20-colony MIC represented the concentration of sodium PAS which inhibited 99.98% of the viable units in the inoculum; the corresponding proportions for the 50-colony MIC and the 100-colony MIC were 99.95% and 99.90%, respectively. The standard sensitive strain, H37Rv, was also titrated similarly with each batch of test strains. The ratio of the MIC of the test strain, defined in any one of the three ways mentioned above, to the MIC of the strain H37Rv, defined in the same way, is referred to as the resistance ratio (RR). There are thus three resistance ratios for each test—namely, the 20-colony RR, the 50-colony RR, and the 100-colony RR. The 20-colony RR has been used in earlier studies (Mitchison & Selkon, 1957; Tuberculosis Chemotherapy Centre, 1959; East African/British Medical Research Council Sulphone Investigation, 1959; East African / British Medical Research Council Isoniazid Investigation, 1960), and reasons for its use have been given by Mitchison & Monk (1955).

[1] Medium without potato starch (Jensen, 1955).

RESULTS

The results of three separate investigations are reported here. Investigations 1 and 2 consisted of a general comparison of the sensitivities to PAS of strains from Indian and British patients, one being undertaken at the TCC and the other at the PGMS. In investigation 3, a more complex experimental design was used, in an attempt to discover to what extent the differences found in investigations 1 and 2 lay in the strains themselves, in the patients studied, or in the techniques used in the two laboratories.

Investigation 1

The aim of the first investigation was to discover whether strains from Indian and British patients differed in their sensitivity to PAS, when both were tested at the TCC. A total of 133 strains from 81 Indian and 52 British patients were examined in the course of five months. Each week, cultures from about five Indian and three British patients (the latter cultures having been flown out from Britain)

TABLE 1

COMPARISON OF THE SENSITIVITIES TO PAS OF INDIAN AND BRITISH STRAINS USING 20-COLONY AND 100-COLONY END-POINTS
(Investigation 1)

Minimal inhibitory concentration of sodium PAS (μg/ml)	Number of strains							
	20-colony MIC				100-colony MIC			
	Indian patients		British patients		Indian patients		British patients	
	No.	%	No.	%	No.	%	No.	%
>64	1	1	0	0	0	0	0	0
64	2	2	0	0	0	0	0	0
16	4	5	0	0	0	0	0	0
8	16	20	0	0	0	0	0	0
4	15	19	1	2	5	6	1	2
2	15	19	10	19	6	7	0	0
1	13	16	15	29	14	17	7	13
0.5	10	12	13	25	11	14	14	27
0.25 or less	5	6	13	25	45	56	30	58
Total	81	100	52	100	81	100	52	100

were tested in parallel with the routine weekly batch of sensitivity tests at the TCC. In reading the tests, particular note was made of the 20-colony MIC, since this was the usual end-point used in routine sensitivity tests. However, although the 100-colony MIC was recorded, it was determined less precisely because its value in the investigation had not been realized at the time.

The distributions of the minimal concentrations of sodium PAS inhibiting these strains are set out in Table 1. Considering the 20-colony MIC values, the Indian strains were on the average more resistant than the British strains. Values of 4 μg/ml or more were given by 38 (47%) of the 81 Indian strains and by only one (2%) of the 52 British strains. The range of the MIC values also appears to have been wider. The distributions for the 20-colony resistance ratios (not tabulated here) showed similar differences between the Indian and British strains; 12 (15%) of the 81 Indian strains had resistance ratios of 8 or more, whereas none of the 52 British strains had resistance ratios of this order. With the 100-colony MIC, in contrast to the above findings, the distributions showed much less difference between the Indian and British strains. Values of 2 μg/ml or more were given by 11 (14%) of the 81 Indian strains and by one (2%) of the 52 British strains. Growth of between 20 and 99 colonies was found on an average of 2.3 PAS-containing slopes per Indian strain, but only on an average of 0.8 slopes per British strain.

Two possible sources of bias existed in this investigation:

1. The Indian cultures were tested within one week of first showing growth, whereas the British cultures were often stored for longer or were tested after subculture.

2. The identity of the strains was known to the person setting up the tests, since the culture medium was contained in different-sized bottles at the TCC and the PGMS, but it was unknown to the person reading them.

These two sources of bias were eliminated in planning investigation 2.

Investigation 2

In this investigation, a total of 87 strains from 56 Indian and 31 British patients were tested for their sensitivity to PAS, but this time at the PGMS, in three large batches of tests. The Indian and the British strains were both subcultured three weeks before the tests were inoculated, the inoculum being prepared from the subcultures. The cultures were arranged in a random order, and their identity was concealed, both in the setting up and in the reading of the tests. In this investigation, as in the first, the 20-colony MIC was again determined more precisely than the 100-colony MIC.

The distributions of PAS sensitivity, set out in Table 2, were similar to those found in the first investigation. The geometric means of the 20-colony MIC were 1.45 μg/ml sodium PAS for the Indian strains and 0.80 μg/ml for the British strains, a difference attaining statistical significance at the 5% level. With the 100-colony MIC values, little difference was found between the Indian and the British strains. Growth of between 20 and 99 colonies was found on an average of 2.8 PAS-containing slopes per Indian strain, and on an average of 1.6 slopes per British strain.

The differences between the results with the 20-colony MIC and the 100-colony MIC obtained in investigations 1 and 2 suggest that some of the Indian strains contained a small proportion of bacilli capable of growth on slopes containing the higher PAS concentrations, these resistant organisms

TABLE 2
COMPARISON OF THE SENSITIVITIES TO PAS OF INDIAN AND BRITISH STRAINS USING 20-COLONY AND 100-COLONY END-POINTS
(Investigation 2)

Minimal inhibitory concentration of sodium PAS (μg/ml)	Number of strains							
	20-colony MIC				100-colony MIC			
	Indian patients		British patients		Indian patients		British patients	
	No.	%	No.	%	No.	%	No.	%
32	1	*2*	0	*0*	0	*0*	0	*0*
16	0	*0*	0	*0*	0	*0*	0	*0*
8	3	*5*	0	*0*	0	*0*	0	*0*
4	10	*18*	2	*6*	0	*0*	0	*0*
2	16	*29*	2	*6*	2	*4*	0	*0*
1	12	*21*	15	*48*	2	*4*	1	*3*
0.5	10	*18*	9	*29*	13	*23*	11	*35*
0.25	2	*4*	3	*10*	8	*14*	12	*39*
0.12 or less	2	*4*	0	*0*	31	*55*	7	*23*
Total	56	*101*	31	*99*	56	*100*	31	*100*
Mean MIC (geometric)	1.45		0.80		—		—	

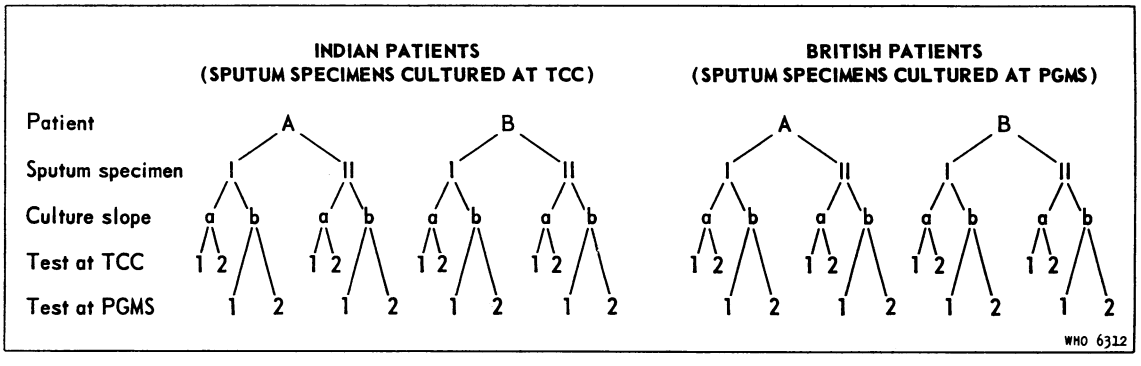
being present in such small numbers that they rarely gave rise to 100 colonies, but fairly frequently produced as many as 20 colonies. As indicated above, it can be estimated that they usually comprised less than 0.1% of the total bacterial population of the strain.

Investigation 3

The third investigation was planned to answer two main questions. The first of these was whether the difference in the PAS sensitivities of Indian and British strains would still be apparent if the tests were carried out on recently isolated strains which had not been subcultured. The second question was whether the presence of PAS-resistant organisms in Indian strains was a characteristic of all Indian strains, or only of strains from particular patients, or only of tests carried out on particular days or on particular batches of medium. If it was characteristic of all Indian strains, then it might influence the response of Indian strains in general to chemotherapy with PAS, as compared with British strains. If it was characteristic only of strains from particular patients, then the responses of patients with such strains might differ from those of patients with fully sensitive strains. Finally, if it was characteristic of particular batches of medium, attention would have to be directed to the standardization of the technique and medium employed in the tests.

The investigation consisted of a series of five similar experiments. The arrangement of each experiment is set out diagrammatically in Fig. 1. Two sputum specimens, obtained within a few days of each other from each of two previously untreated Indian patients, were cultured at the TCC, and two, obtained similarly from each of two previously untreated British patients, were cultured at the PGMS. The culture medium was prepared in similar bottles in the two laboratories. One slope from each sputum culture was retained at the laboratory which cultured the sputum and another was sent by air to the other laboratory. The parcels of

FIG. 1
ARRANGEMENT OF EACH EXPERIMENT IN INVESTIGATION 3*



* There were five experiments in all, with different patients in each.

cultures were dispatched in opposite directions within a few days of each other. It was thus possible to perform PAS sensitivity tests almost simultaneously in both laboratories on Indian and British strains within a week of their first becoming positive. Two sensitivity tests were set up from the growth on each slope. The same batch of medium was used in each laboratory in each experiment, so that a total of 10 batches was employed in the whole investigation. The strain H37Rv was also tested in each batch, so that resistance ratios could be determined. When counting the colonies, care was taken to define accurately all three end-points—namely, the 20-colony MIC, the 50-colony MIC (read only at the TCC), and the 100-colony MIC. The order of setting up and reading the tests was randomized, and the identity of the strains tested was concealed.

By virtue of the experimental design, a number of comparisons may be made:

1. A comparison of the average sensitivities of Indian and British strains according to the various end-points.

2. A study of the relative magnitudes of the three main sources of variation in the results of sensitivity tests—namely, (*a*) differences in the PAS sensitivity of strains from different patients (" between patients "), (*b*) differences in the sensitivity of cultures from different sputum specimens obtained from the same patient (" between specimens ") and (*c*) technical variation between duplicate tests set up on the same culture (" between tests ").

3. A study of the variations in average PAS sensitivity in different batches of tests.

4. The effect of any differences in technique in the two laboratories on these comparisons.

All results were evaluated after transformation to a logarithmic scale in which a twofold increase in PAS concentration (one dilution step in the test) has been given a value of one working unit. The transformed values were examined by analysis of variance. The full analyses of variance for the 20-, 50-, and 100-colony MIC values are summarized in Appendix Table 1, and those for the batch differences are shown in Appendix Table 2. The main conclusions to be drawn from these analyses are discussed below. Except where indicated in the text, the conclusions were similar for the results expressed as resistance ratios and for those expressed as MICs.

1. *Difference between cultures from Indian and British patients.* In Table 3 are set out the averages

TABLE 3
MEAN MINIMAL INHIBITORY CONCENTRATIONS OF PAS FOR INDIAN AND BRITISH STRAINS, ACCORDING TO THE END-POINT READ
(Investigation 3)

End-point	Mean MIC of sodium PAS (μg/ml)	
	Indian patients	British patients
20-colony	2.13	0.83
50-colony*	1.00	0.62
100-colony	0.39	0.38

* Read only at the TCC.

of the sensitivities of the strains from Indian and British patients according to the three MIC end-points employed. With the 20-colony MIC end-point the mean sensitivity to sodium PAS of the Indian strains was 2.13 μg/ml and that of the British strains was 0.83 μg/ml, a difference significant at the 1% level (see Appendix Table 1, term *a*). In contrast, no significant differences between the mean sensitivities of Indian and British strains appeared with the 50-colony MIC or with the 100-colony MIC (see Appendix Table 1, term *a*). Thus, in confirmation of the results of investigations 1 and 2, Indian strains were found, on the average, to be less sensitive to PAS than British strains, owing solely to the presence of a small proportion of Indian organisms capable of yielding less than 50 colonies on slopes containing the higher concentrations of PAS.

2. *Variation between patients.* There was greater variation in the sensitivity of the strains of tubercle bacilli from patient to patient than in the sensitivity of the strains from the same patient. This tendency for strains from different patients to differ in their sensitivity was found with both the 20-colony and the 100-colony MIC values and was significant for each at the 0.1% level (see Appendix Table 1, term *d*).

Estimates of the variation from patient to patient are presented, as standard deviations, in Table 4 (these are the square roots of the components of variation due to this source in the analyses of variance). The estimated values ranged from 1.31 dilution steps for 50-colony MIC values on British strains to 0.73 dilution steps for 20-colony MIC values on Indian strains. It is apparent that the extent of the variation between patients is small, and

TABLE 4
ESTIMATES OF VARIATION IN PAS SENSITIVITY FROM DIFFERENT SOURCES IN STRAINS FROM INDIAN AND BRITISH PATIENTS
(Investigation 3)

Source of variation	Square root of component of variance (standard deviation *)		
	20-colony MIC	50-colony MIC **	100-colony MIC
Indian patients			
Between patients	0.73	0.77	0.80
Between specimens from same patient	0.37	0.28	0.41
Between tests on same specimen	0.94	0.92	0.72
Total †	1.25	1.23	1.15
British patients			
Between patients	0.91	1.31	0.87
Between specimens from same patient	0.16	0.32	0.11
Between tests on same specimen	0.51	0.59	0.60
Total †	1.05	1.47	1.06

* The working unit is one twofold dilution step.
** The 50-colony MIC was read only at the TCC.
† Total variation estimated for single test on single sputum specimen from each patient.
Note. Identical results were obtained for resistance ratios, except for minor differences due to the fitting of a missing value for one Indian strain.

is no larger in Indian than in British patients. Thus, the difference in sensitivity between Indian and British strains cannot be attributed to the presence of resistant strains in a larger *proportion* of Indian patients.

3. *Variation between specimens from the same patient.* There is little evidence of important differences in the sensitivity of the two strains obtained from each patient. The variation between specimens was little or no greater than the variation between tests on the same specimen, that is, than the technical error of the test. Only with the 100-colony MIC values for Indian strains did the " between specimens " term attain significance (see Appendix Table 1, term *h*, P=0.01-0.05). Estimates of the variation between specimens are set out in Table 4 and are small in value, ranging from a standard deviation of 0.41 dilution steps for Indian strains to one of 0.11 dilution steps for British strains, both with the 100-colony MIC values.

4. *Variation between tests on the same specimen.* The standard deviations of the differences between duplicate tests on the same culture are set out in Table 4. With the 20-colony MIC, the standard deviation was higher for Indian strains (0.94 dilution steps) than for British strains (0.51 dilution steps). This difference was significant at the 0.1% level (see Appendix Table 1, term *u*). With the 50-colony MIC, the difference between the estimates for Indian and British strains was slightly smaller. However, with the 100-colony MIC, the standard deviations were 0.72 dilution steps for Indian strains and 0.60 dilution steps for British strains, a difference that did not attain statistical significance.

5. *Variation between batches of tests.* No significant differences were found between the average sensitivity of batches of strains set up on different lots of medium and on different days (see Appendix Table 2), with the exception, commented on below, of minor differences in the 100-colony MIC values. Separate analyses for Indian and British strains, which are not tabulated here, have also failed to show batch differences.

6. *Difference between the two laboratories.* No significant differences were found between the mean levels of PAS sensitivity measured at the TCC and at the PGMS laboratories, in terms of either the 20-colony or 100-colony end-points, whether expressed as MIC values or as resistance ratios (see Appendix Table 1, term *j*). There was no evidence that smaller differences occurred with resistance ratios than with MIC values.

With the 20-colony MIC values, the variation between duplicate tests on Indian cultures appeared to be greater at the PGMS than at the TCC (see Appendix Table 1, term *w*). With the 100-colony MIC values, there was some variation in average sensitivity between different batches of tests in each laboratory (see Appendix Table 1, term *l*) and the amount of variation between specimens was also different at the two laboratories (see Appendix Table 1, term *q*). Each of these differences attained significance at the 5% level, but, in view of the large number of differences tested in the analysis, it is to be expected that some of them would attain significance at this level purely by chance.

7. *Size of colonies at the 20-colony end-point.* In each sensitivity test, the number of large colonies—that is, colonies with a diameter of at least 0.5 mm—

TABLE 5

COMPARISON OF 20-COLONY AND 100-COLONY END-POINT RESULTS OBTAINED IN PAS SENSITIVITY TESTS DONE IN THE 1957 AND 1958 PERIODS

(Indian patients)

Minimal inhibitory concentration of sodium PAS (μg/ml)	Number of strains								Resistance ratio *	Number of strains			
	20-colony MIC				100-colony MIC					20-colony RR			
	1957		1958		1957		1958			1957		1958	
	No.	%	No.	%	No.	%	No.	%		No.	%	No.	%
>64	0	*0.0*	8	*2.4*	0	*0.0*	1	*0.3*	64 or more	0	*0.0*	9	*2.7*
64	0	*0.0*	9	*2.7*	0	*0.0*	0	*0.0*	32	0	*0.0*	9	*2.7*
16	10	*5.4*	21	*6.2*	3	*1.6*	0	*0.0*	16	4	*2.2*	1	*0.3*
8	3	*1.6*	62	*18.3*	0	*0.0*	6	*1.8*	8	9	*4.9*	32	*9.4*
4	41	*22.2*	61	*18.0*	10	*5.4*	18	*5.3*	4	23	*12.4*	65	*19.2*
2 or less	131	*70.8*	178	*52.5*	172	*93.0*	314	*92.6*	2 or less	149	*80.5*	223	*65.8*
Total	185	*100.0*	339	*100.1*	185	*100.0*	339	*100.0*	Total	185	*100.0*	339	*100.1*

* 100-colony RR results are not presented, since the 100-colony MIC for H37Rv was not always available.

was counted on the slope containing the highest concentration of PAS which allowed the growth of at least 20 colonies. The proportion of large colonies in the growths from Indian strains was no lower than in the growths from British strains. Thus, those organisms in the Indian strains responsible for their increased resistance to PAS showed no special tendency to yield small colonies.

8. *Nature of the PAS-resistance of Indian strains.* Single colonies of Indian strains growing on the sensitivity-test slopes containing 0.5 μg/ml and 2 μg/ml sodium PAS were subcultivated on further slopes containing 2 μg/ml sodium PAS and on drug-free (control) slopes. Of 19 colonies transferred from the sensitivity-test slopes containing 2 μg/ml sodium PAS, 14 yielded between 20 and 100 colonies on further slopes containing the same PAS concentration and the remaining five yielded fewer than 20 colonies. In contrast, only one of 14 single colonies transferred from the sensitivity-test slopes containing 0.5 μg/ml sodium PAS yielded more than 20 colonies when subcultivated on 2 μg/ml sodium PAS slopes. This difference is significant at the 0.1% level. All of the 33 colonies gave confluent growth on the drug-free slopes. Thus, the colonies of Indian strains growing on 2 μg/ml sodium PAS, which were responsible for the difference in PAS sensitivity of Indian and British strains, contained a proportion of organisms more resistant to PAS than those growing on lower concentrations.

STABILITY OF THE METHOD FOR TESTING PAS SENSITIVITY

Routine PAS sensitivity tests were undertaken at the TCC on pretreatment strains from South Indian patients in two chemotherapeutic studies; the intake for the first study was from September 1956 to September 1957 (the 1957 period) and for the second, from October 1957 to December 1958 (the 1958 period) (Tuberculosis Chemotherapy Centre, 1959, 1960 [1]). The distribution of the sensitivities of single pretreatment strains from the patients in each study are set out in Table 5, in terms of 20-colony MIC and resistance ratio values, and of 100-colony MIC values only. (In these routine tests the 100-colony MIC value for H37Rv was not always available, since the test was designed for reading with the 20-colony end-point. In consequence, the corresponding 100-colony resistance ratios could not be determined.) There was a considerable increase in the proportion of strains giving high MIC values and resistance ratios using the 20-colony end-point between the two periods of testing, but there was little change in the 100-colony MIC values. Thus, the 20-colony MIC was 8 or more μg/ml sodium PAS in 13 (7%) of 185 strains in the 1957 period and in 100 (29%) of 339 strains in the 1958 period. On the other hand, 100-colony MIC values of 4 μg/ml sodium PAS or more were found in

[1] See article on page 535 of this issue.

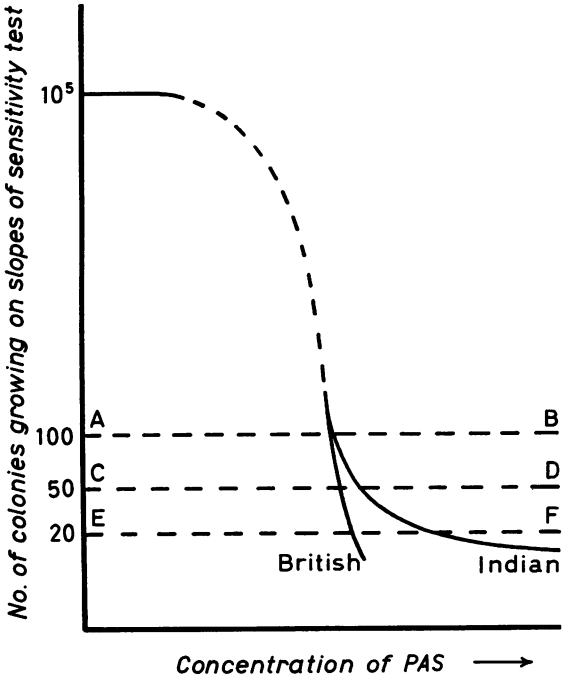
13 (7%) of the 185 strains in the 1957 period and in 25 (also 7%) of the 339 strains in the 1958 period. Since there was no reason to expect a marked increase in infections with PAS-resistant strains during these two years, the results indicate that the 20-colony end-point could not be relied upon to give consistent results, whereas the 100-colony end-point appeared to be reasonably stable.

DISCUSSION

A model of the PAS-sensitivity structure of Indian and British strains

The differences found between the behaviour of Indian and that of British strains of tubercle bacilli in PAS sensitivity tests are illustrated in Fig. 2. This figure has been drawn from the data in Table 3, though not to scale. It shows diagrammatically the relationship between the number of colonies which grow on the sensitivity-test slopes and the concentration of PAS, using an inoculum containing approximately 10^5 organisms. A detectable difference in the survivor curves for the Indian and the British strains apparently occurs only if the concentration of PAS is sufficiently high to allow less than 50 colonies to grow (the portion of the curves under the line CD). A small proportion of the bacilli in Indian strains is apparently capable of growth in a medium containing high PAS concentrations, whereas such bacilli are very much scarcer or non-existent in British strains. The presence of this resistant "tail" accounts for the difference in the average sensitivities of Indian and British strains, using the 20-colony end-point, that was found in all three investigations reported here. According to this representation of the findings, the slope of the survivor curve for Indian strains must be much flatter for colony counts below 50 than the corresponding curve for British strains. Thus, if a 20-colony end-point is used (Fig. 2, line EF), for example, small changes in the size of the inoculum in the test would cause considerable variation in the 20-colony MIC values. Even in the carefully performed experiments of investigation 3, the variation between duplicate tests on Indian strains was higher than that on British strains. Under conditions of routine testing, greater variations in inoculum size would be expected, and these could account for the particularly high 20-colony MIC and resistance ratio values found in a small proportion of Indian strains both in investigation 1 and in the routine tests for the chemotherapeutic trial of 1958. Similarly, the apparent large increase in the incidence of PAS-resistance in pretreatment strains in the 1958 trial, compared with that in 1957, might have been due to a very slightly larger inoculum having been employed on the average in the 1958 tests.

FIG. 2
DIAGRAMMATIC SURVIVOR CURVE FOR INDIAN AND BRITISH STRAINS IN SENSITIVITY TESTS



Apart from this resistant tail, the organisms in the Indian and British strains appeared to have a closely similar pattern of sensitivity to PAS. No differences were found in the average sensitivities of Indian and British strains, as measured by the 50-colony and 100-colony end-points in investigations 2 and 3, and only a slight excess of apparently resistant Indian strains was found with the 100-colony end-point in investigation 1. This indicates that the slope of the survivor curve (Fig. 2) is probably fairly steep where it is crossed by line AB, which represents growth of 100 colonies, so that small variations in inoculum size would make less difference to the sensitivities of Indian strains with this end-point than with the 20-colony end-point. This explains the tendency for the variation between duplicate tests on Indian strains to decrease as the end-point increases from 20 to 100 colonies and the similar values for this variation in Indian and

British strains with the 100-colony end-point (Table 4). It also explains the absence of any marked differences in the distribution of 100-colony MIC values for Indian strains during the testing of strains in the two trials of 1957 and 1958.

Interpretation of previously reported PAS sensitivity tests

The existence of differences in the PAS sensitivity of strains from different *patients*, demonstrated in investigation 3, is surprising in view of earlier conclusions that no such differences occurred (Mitchison & Selkon, 1957). The analysis of variance approach used in investigation 3 is, however, a more sensitive method of detecting whether such differences exist than that used previously; moreover, the variation found between patients was small, the standard deviation for Indian and British patients combined being 0.82 dilution steps [1] with 20-colony MIC values. Despite the difference in *average* sensitivity of Indian and British strains (employing the 20-colony end-point) this variation from patient to patient was similar for Indian and for British strains. It may therefore be concluded that the presence of the resistant tail, which was responsible for the apparent increased resistance of the Indian strains, is a general property of these strains and is not merely a characteristic of the organisms from a few individual Indian patients. In other words, a finding of resistance, using the 20-colony end-point, in a strain from an Indian patient is not likely to indicate that this particular patient is harbouring a large proportion of resistant organisms, but only that in the particular test some factor, such as a chance large inoculum, was responsible for demonstrating the small proportion naturally present in all Indian strains. For similar reasons, little relationship would be expected between pretreatment PAS-resistance (again using the 20-colony end-point) and the subsequent appearance of resistance to isoniazid among Indian patients treated with isoniazid plus PAS. PAS-resistant strains, on the same criterion, would be expected to occur sporadically during the early months of treatment. Both these findings were observed in the 1957 trial of isoniazid plus PAS at home and in sanatorium in Indian patients (Tuberculosis Chemotherapy Centre, 1959), using 20-colony resistance ratios in the PAS sensitivity tests. Furthermore, the rate of 2.6% for the prevalence of patients with pretreatment resistance to PAS reported in this trial is likely to be an overestimate, since a proportion of the strains identified as resistant could have appeared so merely as a result of an unusually large inoculum in the test.

There is no reason to believe that these inconsistencies in the results of the PAS sensitivity tests in the 1957 trial were due to abnormally high technical variation in the laboratory at the TCC. All of the main conclusions of investigation 3 as to the differences in the sensitivity patterns of Indian and British strains were reached in both laboratories. In investigation 3, the estimates of technical variation derived from differences between pairs of tests on the same culture were closely similar in the two laboratories with British strains, and were lower in the TCC laboratory than in the PGMS laboratory with Indian strains. In an earlier study at the PGMS laboratory (Mitchison & Selkon, 1957), the standard deviation of single tests on single sputum specimens from British patients was found to be 1.11 dilution steps. The corresponding estimate (derived from the 20-colony MIC values in Table 4) obtained in investigation 3 was 1.05 dilution steps—a value in close agreement.

Proposal for modification of PAS sensitivity tests on Indian strains

Definitions of resistance based on the 20-colony end-point, with an inoculum of about 10^5 viable units, have proved unreliable for the demonstration of PAS-resistance during controlled clinical trials on Indian patients. Definitions using the 100-colony end-point appear to have been more stable, that is, less affected by such factors as inoculum size. It would thus seem desirable to use 100-colony resistance ratios in future, but it is more difficult to read the 100-colony end-point than the 20-colony end-point. However, decreasing the inoculum size, but continuing to read the 20-colony end-point, should have an equivalent effect. It is therefore proposed to make the standard inoculum contain approximately 10^4 organisms (a dilution of one part in 10) in future work with Indian patients and to continue to use 20-colony resistance ratios. There is no reason known at present for employing this modification with patients of other nationalities.

Treatment of Indian patients with PAS

The presence of a small proportion of PAS-resistant organisms in Indian strains of tubercle bacilli suggests that PAS might be less effective in preventing the development of resistance to

[1] The square root of the component of variance.

isoniazid, during treatment with isoniazid plus PAS, among Indian patients than among British patients. Studies of Indian patients treated with isoniazid plus PAS (Tuberculosis Chemotherapy Centre, 1959, 1960 [1]) have shown that this combination is remarkably efficient, though in the first study 12% and in the second 8% of the patients were classified, on very stringent criteria, as having bacteriologically active or relapsed disease at the end of one year. It is impossible to say whether these results are any less satisfactory than would be obtained among patients in other countries, because of differences in the type of disease, in the nutritional state of the patients, in their inherent racial immunity, and in the virulence of their tubercle bacilli (Mitchison et al., 1960). It must be appreciated that the resistant organisms found in the Indian strains, but not in the British strains, comprised only about 0.02%, or somewhat less, of their bacterial populations. There is little evidence as to whether such a low proportion of PAS-resistant organisms would be sufficient to permit the development of isoniazid-resistant mutants in the presence of both drugs, and further experiments to investigate this possibility are being carried out.

The nature of the resistant organisms present in the Indian strains is still obscure. Although they were capable of producing large colonies on a medium containing 2 μg/ml sodium PAS, only a limited amount of growth occurred when these colonies were subcultured on a medium containing the same PAS concentration. A similar phenomenon has been described by Tsukamura (1957) for a variant made PAS-resistant by multiple transfers in PAS-containing medium.

SUMMARY

1. The sensitivities to PAS of strains of tubercle bacilli obtained before the start of antituberculosis chemotherapy from totals of 147 Indian and 93 British patients have been compared in three investigations, one in Madras, one in London and one simultaneously in both places.

2. Sensitivity tests were set up on slopes inoculated with about 10^5 viable units, and the minimal concentrations of PAS inhibiting the growth of 20, 50 or 100 colonies were read. In each investigation, more of the Indian than of the British strains were resistant to PAS with the 20-colony end-point, but no differences were apparent with the 50-colony and 100-colony end-points. Thus, Indian strains contained a small proportion (0.02% or slightly less) of resistant organisms not present in British strains.

3. Variation from patient to patient in the sensitivity of these strains was of similar magnitude for Indian and British patients. Thus the presence of the resistant organisms was a general characteristic of Indian strains and there was no evidence that a higher proportion of Indian patients had been infected with resistant strains.

4. A chance factor, such as an increase in inoculum size in the sensitivity test, would allow the resistant organisms characteristic of Indian strains to yield 20 or more colonies on slopes containing the higher PAS concentrations, thus causing the strain to be called resistant in routine tests. Under these circumstances resistant strains would appear sporadically and would not be related to any special tendency for the patient to fail to respond to treatment with PAS. Similarly, a considerable increase in the apparent prevalence of PAS-resistant strains, which occurred between two controlled chemotherapy trials on Indian patients, could be accounted for by a slight increase in the average size of the inoculum used in the tests during these trials.

5. A tenfold decrease in the inoculum size in PAS sensitivity tests on strains from Indian patients is recommended for future work.

[1] See article on page 535 of this issue.

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RÉSUMÉ

Au cours de l'étude comparative de la chimiothérapie de la tuberculose en sanatorium et à domicile, organisée à Madras, Inde, au cours des dernières années, il est apparu que les bacilles tuberculeux isolés de malades avant le début du traitement étaient plus résistants au PAS (acide *p*-aminosalicylique) que les bacilles isolés dans les mêmes conditions de malades en Grande-Bretagne. Une étude a donc été entreprise, conjointement par le Centre de chimiothérapie antituberculeuse de Madras et le Postgraduate Medical School of London, pour élucider les causes de cette différence.

Les bacilles cultivés à partir des crachats de 147 malades de l'Inde et de 93 malades de Grande-Bretagne, ont été soumis aux épreuves de résistance au PAS. On a déterminé dans des tubes contenant chacun 10^5 unités microbiennes viables, la concentration minimum de PAS inhibant la croissance de 20, 50 et 100 colonies, soit de 99,98%, 99,95% et 99,90% des bacilles viables. D'après le résultat de la première de ces épreuves (20 colonies) qui est utilisée comme épreuve de routine dans les tests de sensibilité, les souches indiennes étaient significativement plus résistantes que les souches britanniques. Cette différence ne se manifestait plus dans les tests de 50 et 100 colonies. Ainsi, au contraire des souches britanniques, les souches indiennes contiennent une faible proportion (0,02%) de souches résistantes, mais il n'a été nullement prouvé qu'une proportion plus élevée de malades indiens soit infectée par des souches résistantes, ou que ces malades réagissent moins bien au traitement par le PAS.

En raison du fait que dans les tests de résistance, une augmentation de l'inoculum pourrait fausser l'interprétation des résultats en accroissant le nombre relatif des bacilles résistants, les auteurs conseillent de ramener au dixième de sa valeur actuelle la quantité d'inoculum requise pour les tests de sensibilité au PAS.

REFERENCES

East African/British Medical Research Council Sulphone Investigation (1959) *Tubercle (Lond.)*, **40**, 1

East African/British Medical Research Council Isoniazid Investigation (1960) *Tubercle (Lond.)*, **41**, 83

Great Britain, Medical Research Council, Tuberculosis Chemotherapy Trials Committee (1955) *Brit. med. J.*, **1**, 435

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

Mitchison, D. A. & Monk, M. (1955) *J. clin. Path.*, **8**, 229

Mitchison, D. A. & Selkon, J. B. (1957) *Tubercle (Lond.)*, **38**, 85

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

Selkon, J. B. & Mitchison, D. A. (1958) In: *Transactions of the 17th Conference on the Chemotherapy of Tuberculosis held February 3rd to 6th, 1958, at Memphis, Tennessee*, Washington, D.C., US Government Printing Office, p. 258

Tsukamura, M. (1957) *Amer. Rev. Tuberc.*, **75**, 608

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

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

APPENDIX TABLE 1

ANALYSES OF VARIANCE OF 20-COLONY MIC, 50-COLONY MIC AND 100-COLONY MIC VALUES
(Investigation 3)

Term	Source of variation	Degrees of freedom	20-colony MIC			50-colony MIC *		100-colony MIC	
			mean square	term tested against	P (F test)	mean square	P (F test**)	mean square	P (F test **)
a	Indian *versus* British-Race (R)	1	72.900	*c* + *d*	*0.001-0.01*	9.800	>0.2	0.056	NS
b	Between experiments (E)	4	4.741	*d*	NS †††	6.081	>0.2	3.634	NS
c	Interaction R × E	4	8.228	*d*	>0.2	4.644	NS	8.478	>0.2
d	Between patients of same race in same experiment (P)	10	6.300	*g*	*0.001*	5.400	*0.001*	6.356	*0.001*
e	Indian	5	5.663	*h*	*0.01-0.05*	3.350	*0.05*	6.250	*0.01-0.05*
f	British	5	6.938	*i*	*0.001*	7.450	*0.001*	6.463	*0.001*
g	Between specimens from same patient (S)	20 ††	0.900	*t*	0.1	0.763	>0.2	0.794	*0.05*
h	Indian	10 ††	1.438	*u*	0.1-0.2	1.000	>0.2	1.175	*0.01-0.05*
i	British	10	0.363	*x*	>0.2	0.550	0.2	0.413	>0.2
j	Between laboratories (L)	1	0.900	*l*	NS			28.056	0.05-0.1
k	Interaction L × R	1	0.625	*m*	NS			0.506	NS
l	Interaction L × E	4	3.728	*n*	0.05-0.1			4.728	*0.01-0.05*
m	Interaction L × R × E	4	3.703	*n*	0.05-0.1			1.522	>0.2
n	Interaction L × P	10	1.325	*q*	0.1-0.2			1.181	>0.2
o	Indian	5	1.013	*r*	NS			1.200	NS
p	British	5	1.638	*s*	0.05-0.1			1.163	>0.2
q	Interaction L × S	19 †	0.816	*t*	0.1-0.2			0.967	*0.01*
r	Indian	9 †	1.097	*u*	>0.2			1.306	*0.01-0.05*
s	British	10	0.563	*x*	*0.05*			0.663	0.05-0.1
t	Between duplicate tests	79 † 39 †	0.570			0.590		0.437	
u	Indian	39 †	0.885	*x* ***	*0.001*			0.513	>0.2
v	TCC tests	19 †	0.474	*y* ***	>0.2	0.842	0.05-0.1	0.368	NS
w	PGMS tests	20	1.275	*z* ***	*0.001*			0.650	0.05-0.1
				v ***	*0.01-0.05*				>0.2
x	British	40	0.263					0.363	
y	TCC tests	20	0.275	*z* ***	>0.2	0.350		0.450	>0.2
z	PGMS tests	20	0.250					0.275	
	Total	157	1.979			1.873		1.568	

* The 50-colony MIC was read only at the TCC.

** Against term indicated in the 20-colony MIC column.

*** Tested with two-tail distribution of F.

†, †† The tests for one Indian strain were contaminated at the TCC. Estimates for these values were made by application of standard missing plot techniques; † indicates those terms whose degrees of freedom were reduced in consequence for all three end-points; †† indicates that one was subtracted from the given degrees of freedom for 50-colony MIC analysis only.

††† NS indicates that the variance ratio is less than 1.0.

APPENDIX TABLE 2

BATCH DIFFERENCES WITH 20-COLONY MIC AND 100-COLONY MIC VALUES AT THE TCC AND PGMS LABORATORIES

Source of variation	Degrees of freedom	TCC laboratory				PGMS laboratory			
		20-colony MIC		100-colony MIC		20-colony MIC		100-colony MIC	
		mean square	P (F test)	mean square	P (F test)	mean square	P (F test)	mean square	P (F test)
Between batches of tests	4	5.394	NS *	6.394	>0.2	3.075	>0.2	1.969	NS
Between patients of same race tested in same batch	10	5.563		4.000		2.063		3.538	

* NS indicates that the variance ratio is less than 1.0.