COMPARISON OF SOLID AND LIQUID MEDIUM SENSITIVITY TESTS OF TUBERCLE BACILLI TO PARA-AMINOSALICYLIC ACID

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In the treatment of tuberculosis *para*-aminosalicylic acid (P.A.S.) is now chiefly used in combination with streptomycin or isoniazid with the aim of preventing or delaying the emergence of drug-resistant strains of tubercle bacilli (Medical Research Council, 1950, 1953c). Such combined therapy is only effective in preventing drug resistance when the strains obtained before the start of treatment are sensitive to P.A.S. Thus resistance to streptomycin has been found to emerge more frequently during treatment with the combination of streptomycin and P.A.S. when the pre-treatment strains were resistant to P.A.S. than when they were sensitive to it (Medical Research Council, 1953b).

The selection of the most suitable chemotherapy for any patient should involve a test of the P.A.S. sensitivity of pre-treatment strains, since resistance would prejudice the results of treatment with a combination of drugs that included P.A.S. Initial P.A.S. resistance occurs most frequently as a result of previous treatment with P.A.S. alone. Less frequently it is found as a result of therapy with a combination of two drugs, one of which was P.A.S. Djang, Bogen, and Will (1952) have found some bovine strains to be naturally resistant to P.A.S. Finally, the patient may have been infected by another person who harboured a P.A.S.-resistant strain (Medical Research Council, 1953a; Thomas, Borthwick, Horne, and Crofton, 1954). Routine P.A.S. sensitivity tests during chemotherapy are less valuable, but, if the patient has remained culture positive, a test at the end of a course of treatment may be very helpful in planning further treatment. Thus cultures from the majority of patients receiving chemotherapy should be tested on at least one occasion for P.A.S. sensitivity. The number of these tests may be a considerable burden for the pathologist, who will, therefore,

look for a simple yet reliable technique of performing them.

During the Medical Research Council (1950, 1952b, 1953b) trials of streptomycin and isoniazid, P.A.S.-sensitivity tests were carried out in Tween-albumin medium. This method was convenient since many of the strains were also tested for streptomycin sensitivity in the same medium. On the other hand, American and French workers (American Trudeau Society, 1952; Coletsos, 1952) have advised the use of solid medium tests, claiming that these were more reliable and easier to use. A comparison of the two methods was clearly required both to establish their inherent accuracies and to provide the data necessary for recommending a simple technique that could be relied upon in testing an increasing number of cultures. Such a comparison has therefore been made, using, in the main, strains from patients treated in the Medical Research Council (1952b) trials of isoniazid.

Methods

Sensitivity Tests.—Liquid medium sensitivity tests to P.A.S. were carried out in Tween-albumin liquid medium by the Medical Research Council (1953a) method. In the solid medium tests, sodium *para*-aminosalicylate dihydrate (Na P.A.S.) was added to Löwenstein-Jensen medium before inspissation to make a series of final concentrations from 0.12 µg./ml., increasing by serial twofold steps, to 128 µg./ml. This medium was distributed in 4 ml. amounts in screw-capped bottles and inspissated at 80° C. for one hour. The inoculum was prepared by grinding up a representative loopful of the growth from a two to three week Löwenstein-Jensen slope culture in sterile distilled water to make a suspension approximately equal in opacity to a 10-day growth in Tween-albumin medium. A 3 mm. loopful (0.005 ml.) of this suspension was spread on each of the series of slopes containing P.A.S. and on a drug-free slope. Each batch

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of tests included one on the standard sensitive strain, H37Rv. Results were read after 14, 21, 28, and 42 days' incubation.

Cultures.—A total of 130 strains of tubercle bacilli was obtained from the sputa of patients with pulmonary tuberculosis. These were divided into three groups :—

Group I.—Forty-five pre-treatment strains from 45 patients who had received no previous chemotherapy.

Group II.—Thirty-six strains from 29 patients which were resistant to P.A.S. (resistance ratio of 8 or more) when tested in liquid medium.

Group III.—Forty-nine strains from 37 patients which were sensitive to P.A.S. in liquid medium, but, for reasons indicated in Table I, might have been resistant if tested by a more delicate technique.

TABLE I
REASONS FOR SUSPECTING P.A.S. RESISTANCE IN
GROUP III STRAINS

	No. of Strains
P.A.S. resistance ratio of 2 or 4 in liquid medium test	8
P.A.S. resistance found by other laboratories	5
Previous culture from same patient found resistant to P.A.S.	6
Later culture from same patient found resistant to P.A.S.	3
Resistant to streptomycin	6
Previous chemotherapy with P.A.S. and isoniazid for 4-5 months	10
Previous chemotherapy with P.A.S. and isoniazid for 6 or more months	11
Total	49

The sensitivity of the majority of the strains was tested once in liquid medium. Each strain was then subcultivated on a Löwenstein-Jensen slope, and from the growth a further liquid medium test and a solid medium test were set up in parallel. The results of these parallel tests were compared.

Tests on 23 out of the 130 strains were carried out only once in liquid medium, and the strains were then subcultivated and tested on solid medium. The results of these tests were also included in the comparison. It might be thought that the inclusion of these results would bias a comparison between the methods, since it will be shown that some resistant strains tend to revert to sensitivity during subcultivation. However, no evidence of reversion was found among these strains; five of them were highly resistant in both tests and a sixth was more resistant in the second test than in the first. The remainder consisted of 11 pre-treatment strains which were unlikely to alter in sensitivity during subcultivation and six strains which were unequivocally sensitive in both tests.

End-points.—The minimal concentration of P.A.S. inhibiting the growth of resistant strains was frequently difficult to define, both in liquid and on solid media, since increasing P.A.S. concentrations only gradually decreased the rate of growth. In liquid

medium tests it was important to read the presence or absence of growth under constant lighting conditions. The minimal concentration completely inhibiting growth was taken as the end-point. In solid medium tests two end-points were used. One of these was the minimal concentration necessary to inhibit growth completely. The other was the minimal concentration that prevented the growth of 20 or more colonies (subsequently called the "20 colonies end-point"). Scanty, minute colonies often appeared first on slopes containing P.A.S., and were then followed by a profuse growth over the whole inoculated surface. Since it was difficult to be certain that the minute colonies were composed of tubercle bacilli, the "20 colonies end-point" was found easier to use.

Results

Pre-treatment Strains.—Fig. 1 shows the results of sensitivity tests on pre-treatment strains (Group I) in terms of the minimal concentration of P.A.S. inhibiting growth. The shape of these distributions approximates to that of the normal curve of errors. The scatters of the distributions for tests in liquid or on solid medium were very similar. However, inhibitory concentrations were slightly lower in liquid medium. Thus, after 14 days' incubation in liquid medium, all the strains were inhibited by 4 µg./ml. Na P.A.S., whereas, after 28 days' incubation on solid medium, they were all inhibited by 16 µg./ml. Na P.A.S.

During incubation on solid medium growth appeared in progressively higher concentrations of P.A.S. Thus, between readings at 14 and 42 days, the minimal inhibitory concentrations increased on the average about four times. This was not due to inactivation during incubation, since the H37Rv strain was inhibited by the same P.A.S. concentration when the slopes were incubated for two, four, six, or eight weeks before inoculation. Singh (1955) has also shown that P.A.S. was not inactivated during incubation in liquid medium. Examination of the 28-day readings on solid medium showed that the "20 colonies end-point" was, on the average, one concentration lower than the "no-growth end-point."

Fig. 2 illustrates the results of sensitivity tests on the same strains, in terms of the resistance ratio which is the ratio between the minimal inhibitory concentrations of the test strain and of the standard sensitive strain, H37Rv. Again, the shape and scatter of the distributions are very similar. Both in liquid and on solid medium all the strains had a resistance ratio of less than 8. Resistance ratios of $\frac{1}{2}$ were more common than those of 2, due to slightly lower minimal inhibitory concentrations for test strains than for H37Rv. It was presumed that test strains were less well adapted to growth

FIG. 1.—The sensitivities of 45 pre-treatment strains, under various conditions of testing, expressed as the minimum inhibitory concentrations of Na P.A.S. The result on one strain was not read after 42 days' incubation.

on artificial media than was H37Rv. The distributions of resistance ratios determined on solid medium after 21 or 42 days' incubation were similar to those at 28 days, but in about a third of the tests read after 14 days a resistance ratio could not be defined because of inadequate growth either of the test strain or of H37Rv.

Differences in Sensitivity of Pre-treatment Strains.—Sensitivity tests in liquid medium were carried out twice on 24 of the 46 pre-treatment strains. This made it possible to determine whether the apparent differences of sensitivity of these strains were due to the technical error of the tests or to real differences in the sensitivities of the

strains. Resistance ratios of $\frac{1}{8}$, $\frac{1}{4}$, $\frac{1}{2}$. . . were scored as 1, 2, 3 . . . , and an analysis of variance was then carried out on these scores. The results are shown in Table II. The mean square for variation between strains was not significantly different from the error mean square, which is a measure of the technical error. One can therefore conclude that, as far as can be determined by the method

FIG. 2.—The sensitivities of 45 pre-treatment strains on solid and in liquid medium tests expressed as resistance ratios.

used, pre-treatment strains had the same degree of sensitivity to P.A.S., any apparent differences being due to technical error.

The standard deviation of the test is seen to be ± 1.083 , so that approximately 19 out of 20 tests

TABLE II
ANALYSIS OF VARIANCE OF DUPLICATE TESTS ON PRE-TREATMENT STRAINS IN LIQUID MEDIUM

	Sum of Squares	Degrees of Freedom	Mean Square
Between repeat tests ..	2.083	1	2.083
Between strains ..	35.667	23	1.551
Error ..	25.917	23	1.083

lay within the range of four times more to four times less than the true value of their resistance ratio. An estimate, by similar means, of the standard deviation in streptomycin sensitivity tests carried out in Tween-albumin medium yielded a value of ± 0.636 (Mitchison, 1949) so that the P.A.S. sensitivity test had about half the accuracy of the corresponding streptomycin sensitivity test.

Medium Batch Differences.—During the period of the experiments pre-treatment strains were tested on nine different batches of solid medium and in 12 different batches of liquid medium, all prepared in the same laboratory. Since pre-treatment strains had the same degree of sensitivity, an analysis could be made to determine whether the minimal concentration of P.A.S. that inhibited growth varied from batch to batch of medium. The results of solid medium tests read after 28 days, using the “20 colonies end-point,” and of liquid medium tests read after 14 days were given scores as before. Minimal inhibitory concentrations of 0.12, 0.25, 0.5 . . . $\mu\text{g./ml. Na P.A.S.}$ were scored as 0, 1, 2, . . . Analyses of variance were carried out, with the results shown in Table III. The mean square for variation between batches was significantly greater than the error mean square in the solid medium tests, but not in the liquid medium tests. One can conclude that for the purpose of these tests Löwenstein-Jensen medium was more difficult to standardize than was Tween-albumin medium.

Resistant Strains.—A resistant strain can be defined as one requiring a higher drug concentration to inhibit growth than did pre-treatment

TABLE III
ANALYSIS OF VARIANCE OF BATCH DIFFERENCES IN SOLID AND LIQUID MEDIUM TESTS OF PRE-TREATMENT STRAINS

	Sum of Square	Degrees of Freedom	Mean Square	Variance Ratio	P
Solid medium tests: Between batches	11.88	8	1.485	2.9	0.05-0.1
Error ..	19.10	37	0.516		
Liquid medium tests: Between batches	8.34	11	0.758	0.7	0.2
Error ..	34.99	34	1.031		

strains from the same patients. In theory, therefore, one should carry out sensitivity tests on a number of pre-treatment strains from the patients to obtain an estimate of the variation of the tests, and one should then see whether the sensitivity of the resistant strain lies outside the distribution of the sensitivities of these pre-treatment strains. However, it is sufficient to use the distribution of single tests on pre-treatment strains from different patients, since it has been shown that all these strains had the same degree of sensitivity.

The most important criterion of a method of performing sensitivity tests is its ability to detect resistant strains. This ability can be tested by first obtaining a definition of a resistant strain from the distribution of the sensitivities of pre-treatment strains and then, while using this definition, seeing how many out of a batch of strains with varying degrees of sensitivity are found to be resistant. Such a procedure was used to compare the efficiencies of liquid and solid medium tests, with readings taken after various periods of incubation and using either the "no-growth" or the "20 colonies" end-point in solid medium. The definition of resistance was obtained by fitting a normal curve to the results, by each method, of the tests on the pre-treatment strains (Group I). This curve was used to find the concentration of P.A.S. or the resistance ratio below which the sensitivities of 99 out of 100 would fail (standard deviation $\times 2.326$). Any strain whose minimal inhibitory concentration or resistance ratio was greater than this value was then defined as resistant. Using these definitions, the number of strains found to be resistant by each method was determined among the 85 strains that had previously been found resistant (Group II) or were suspected of possibly being resistant (Group III). The results are shown in Table IV.

The number of resistant strains that could be detected became larger as the period of incubation of the tests was increased. Thus, in liquid medium tests, 25 strains defined as resistant by their resistance ratios were detected after 10 days' incubation and 29 after 14 days. Similarly, on solid medium, using the "20 colonies" end-point, 19, 27, 30, and 31 resistant strains were found after, respectively, 14, 21, 28, and 42 days' incubation. A reading at 28 days on solid medium is probably the optimal period of incubation, because there was little gain in efficiency in increasing the period of incubation from 28 to 42 days and because of the desirability of an early report.

An examination of the results of 14-day readings in liquid medium and 28-day readings on solid medium shows that about the same number of

TABLE IV
DEFINITIONS OF RESISTANCE AND NUMBERS OF GROUPS II AND III STRAINS FOUND RESISTANT ACCORDING TO METHOD OF TESTING

	End-point	Period of Incubation (Days)	Lowest Result Indicating Resistance		No. of Strains in Groups II and III		
			Minimal Inhibitory Concentration (μ g. ml. NaP.A.S.)	Resistance Ratio	Unreadable*	Sensitive	Resistant
Liquid	No growth	10	2		6	56	23
"	"	14	4		1	55	29
"	"	10		8	6	54	25
"	"	14		8	1	55	29
Solid	"	14	8		3	59	23
"	"	21	16		2	58	25
"	"	28	16		1	54	30
"	"	42	16		0	52	33
"	"	14		16	3	62	20
"	"	21		16	2	60	23
"	"	28		8	1	54	30
"	"	42		8	0	54	31
"	20 colonies	14	8		4	62	19
"	"	21	8		2	56	27
"	"	28	8		1	54	30
"	"	42	16		0	54	31
"	"	14		8	22	42	21
"	"	21		8	2	55	28
"	"	28		4	1	52	32
"	"	42		8	0	54	31

* No growth in control tube.

resistant strains were found, irrespective of the end-point used or whether the results were expressed in terms of the minimal inhibitory concentrations or of resistance ratios. There was an apparent slight advantage to solid medium tests, since they detected 30 to 32 resistant strains, whereas only 29 were detected by liquid medium tests. However, five of the seven strains that were resistant on solid medium but sensitive in liquid medium yielded resistance ratios of 4 in liquid medium, a result sufficiently high to suggest the necessity of retesting. On the other hand, only two of the six strains that were resistant in liquid medium, but not on solid medium, yielded resistance ratios on solid medium over 1; the values of their ratios were 2 and 4. Thus, if we take into account not only those strains definitely found resistant but also those with results on the borderline between sensitivity and resistance, it can be concluded that solid and liquid medium tests were about equally efficient in detecting resistant strains.

Only four of the Group III strains were found to be resistant in the parallel tests. Of these, three had resistance ratios of 2 or 4 in the original liquid medium tests and were resistant in at least two readings of the parallel tests. The fourth strain was just resistant in one reading on solid medium, but was sensitive in all other readings, and should probably be classified as sensitive.

The degrees of resistance found among the 36 strains that were resistant in liquid and on solid medium tests, or in either alone, are shown in Table V. The distribution of these strains according to their degrees of resistance was similar whichever method of testing was used. The resistance ratios in liquid and solid medium tests were in agreement with 21 strains, where agreement means that the ratio in one medium was not more than four times the ratio in the other. However, there was disagreement with 15 strains, seven yielding a higher result in liquid medium and eight on solid medium. Some of these discrepancies were very large: for instance, a strain with resistance ratios of 64 in liquid medium and 1 on solid medium and another with resistance ratios of 4 in liquid medium and more than 64 on solid medium. It can be concluded that individual determinations of the resistance ratios of resistant strains are frequently very inaccurate.

TABLE V
DEGREES OF RESISTANCE IN 36 STRAINS TESTED IN LIQUID AND ON SOLID MEDIUM

Resistance Ratio	No. of Strains	
	Liquid Medium*	Solid Medium*
>64	12	15
64	2	4
32	4	0
16	3	3
8	8	6
4	6	4
2	0	1
1-†	1	3

* Liquid medium tests were read after 14 days' incubation and solid medium tests after 28 days' incubation using the "20 colonies" end-point.

Stability of Resistant Strains.—Liquid medium tests were done twice on 32 strains that were found resistant in one of the two tests. The period between these tests varied from one to six months, during which the strains were subcultivated at least three times. A comparison of the resistance ratios, read after 14 days, shows agreement between the results of the two determinations in 19 strains and disagreement in 13 strains. In all cases of disagreement, the ratio at the repeat test was lower than the original value, and in No. 6 strain, originally found resistant, the repeat test indicated a sensitive result.

This loss of resistance was investigated in a further experiment in which nine strains, which had been found resistant in at least one previous test, were subcultivated every 10 days in Tween-albumin medium. Sensitivity tests on solid medium were set up at the start and from the third, sixth, and ninth subcultures. The results, shown in

TABLE VI
STABILITY OF RESISTANT STRAINS DURING SUBCULTIVATION IN TWEEN-ALBUMIN MEDIUM

Strain	Resistance Ratio after Successive Subcultivations			
	No. of Subcultures			
	0	3rd	6th	9th
1	1	1	1	2
2	1	1	1	8
3	1	4	1	16
4	8	4	64	16
5	32	8	64	16
6	32	16	16	32
7	128	>64	128	>128
8	128	4	32	32
9	>128	32	256	64

Table VI, fail to demonstrate any general tendency towards a decrease in the degree of resistance during the period of subcultivation.

The apparent discrepancy between these results might be accounted for in two ways. It is possible that only a proportion of resistant strains are unstable. These unstable strains would tend to become less resistant during the first few subcultures in artificial media, thus accounting for the results of the original and repeat tests. On the other hand, those strains which were resistant at the beginning of the subcultivation experiment would be stable since they had retained their resistance until then. An alternative explanation is that some strains contain only a small proportion of resistant bacilli. During storage between the original and repeat tests these bacilli might have died out, with a resulting loss of resistance. Such an occurrence would be less likely during successive subcultivations at 10-day intervals.

Inoculum Size.—The effect of altering the size of the inoculum in the liquid and solid medium

TABLE VII
EFFECT OF ALTERATIONS OF INOCULUM SIZE ON MINIMAL INHIBITORY CONCENTRATION OF P.A.S. USING H37Rv AND A RESISTANT STRAIN

Strain	Volume of Inoculum (ml.)	Minimal Inhibitory Concentration of Na P.A.S. (μ g./ml.)					
		Solid Medium Test*			Liquid Medium Test		
		Period of Incubation (Days)			Period of Incubation (Days)		
H37Rv		14	21	28	14	21	28
	0.01	4	8	32	8	16	32
	0.002	0.12	0.5	1	0.5	2	2
	0.0004	0.12	0.5	1	0.25	0.5	1
	0.00008	<0.12	0.12	0.5	<0.12	0.5	1
129		16	64	>128	16	16	16
	0.002	2	8	16	4	4	8
	0.0004	1	8	8	2	4	4
	0.0008	0.5	4	8	NG†	0.5	2

* 20 colonies end-point used. † NG=no growth in control tube of test.

tests was investigated using H37Rv and six resistant strains. Cultures grown in Tween-albumin medium were concentrated or diluted so that the bacilli which would be contained in volumes ranging from 0.01 to 0.00008 ml. of a 10-day growth were added in the drop or loopful used as the inoculum. Typical results are shown in Table VII. Alterations in the inoculum size had a marked effect on the minimal inhibitory concentrations of P.A.S. This effect was usually least between the inoculum sizes of 0.002 ml., the normal volume used, and 0.0004 ml. Thus it is most important to standardize the inoculum in P.A.S. sensitivity tests, although small decreases from the normal size are unlikely to cause serious error.

Discussion

For routine purposes two types of indirect sensitivity test have been suggested. In one of these (Medical Research Council, 1953b) a rather large number of tubes containing closely graded drug concentrations are inoculated, and the result is expressed as a resistance ratio. In the other (American Trudeau Society, 1952; U.S. Veterans Administration, 1954), a few tubes containing widely spaced drug concentrations are inoculated and, on the basis of experience with sensitive strains, growth on the medium containing a particular drug concentration is said to indicate resistance. Although the former method requires a larger number of tubes, the expression of the result as a resistance ratio has certain advantages. We have found that the end-point in P.A.S. sensitivity tests varied markedly according to the size of the inoculum, and to some extent according to the batch of solid medium prepared in the same laboratory. In spite of every effort to standardize the test, there are likely to be small variations in the inoculum used and in the preparation of the medium from one laboratory to another. These variations would be unimportant if a resistance ratio were obtained, since they would affect the titration of the standard sensitive strain to the same extent as the test strain.

We have shown that there was little difference between solid and liquid medium tests in the efficiency with which they detect resistant strains. It is probably slightly easier to prepare a wide range of drug concentrations in liquid than in solid medium. On the other hand, the solid medium test dispenses with the need for the preliminary subcultivation employed in the liquid medium test, during which contamination of the strains may occur. The time between obtaining a positive culture and reading the test is about the

same for both methods, namely a month. Testing the P.A.S. sensitivity of isoniazid-resistant strains in Tween-albumin medium presents a problem, since these strains may grow poorly in this medium (Mitchison, 1953). However, modification of the medium by the addition of iron, keto-acids, haemin, or catalase (Cohn, Kovitz, Oda, and Middlebrook, 1954) may be sufficient to allow satisfactory growth of these strains. If solid medium tests are to be used, it is suggested that a suitable range of concentrations of Na P.A.S. would be 0.25, 0.5, 1, 2, 4, and 8 $\mu\text{g./ml.}$ with one higher concentration, say 100 $\mu\text{g./ml.}$ A resistance ratio for sensitive and slightly resistant strains could then be obtained. The results of the test should be read, using the "20 colonies" end-point, after four weeks' incubation, though readings at two and three weeks might be of value in the early detection of resistant strains. A resistance ratio of 8 would definitely indicate resistance and a ratio of 4 would be sufficiently suspicious to necessitate retesting the strain or others from the same patient.

Smaller laboratories might think it necessary to sacrifice the safeguards provided by the use of resistance ratio in favour of a test with a smaller number of tubes. In this case solid medium slopes containing 4 and 10 $\mu\text{g./ml.}$ Na P.A.S. (approximately 3 and 7.5 $\mu\text{g./ml.}$ P.A.S.) would probably be the most satisfactory. Our observation that approximately 10% of sensitive strains yielded some growth on slopes containing 4 $\mu\text{g./ml.}$ Na P.A.S. after six weeks' incubation could be used as a check against variations in techniques. Thus readings at six weeks could be taken on the 4 $\mu\text{g./ml.}$ slopes inoculated with the standard sensitive strains and, over a period, approximately 10% of them should show some growth. If growth were to occur on a smaller percentage or none of these slopes then some resistant strains would have been classified as sensitive. On the other hand if a larger percentage were to show growth then some sensitive strains would have been classified as resistant.

A laboratory subcommittee of the U.S. Veterans Administration (1954) has recently recommended that indirect P.A.S. sensitivity tests should be carried out by subculturing on to egg medium slopes containing 0, 10, and 100 $\mu\text{g./ml.}$ P.A.S. Strains were to be considered resistant when the growth on the slope containing 10 $\mu\text{g./ml.}$ P.A.S. was as good as on the control slope. If such a definition of resistance had been

employed in our test at least nine out of 30 resistant strains would have been misclassified as sensitive, since none of these nine strains yielded 20 or more colonies on slopes containing 16 $\mu\text{g.}/\text{ml}$. Na P.A.S. (approximately equal to 10 $\mu\text{g.}/\text{ml}$. P.A.S.).

Since six out of 32 strains reverted from resistance to sensitivity during storage, it would seem important to carry out sensitivity tests on primary cultures as soon as they have grown. Furthermore, nearly 20% of resistant strains tested both on solid and in liquid medium tests yielded sensitive results in one of these tests. Thus a sensitive result on a single strain from a patient cannot be relied upon. Since the presence of a P.A.S.-resistant strain is a contraindication to the use of chemotherapy with streptomycin and P.A.S. or isoniazid and P.A.S., it would be advisable to carry out two or preferably three tests on strains from every patient before the start of treatment.

Summary

Sensitivity tests to P.A.S. were carried out in Tween-albumin liquid medium and on slopes of Löwenstein-Jensen medium. Distributions of the sensitivities of 45 pre-treatment strains of tubercle bacilli, employing different methods, end-points, and periods of incubation, were obtained and used for defining resistant strains. All pre-treatment strains had the same degree of sensitivity; apparent

variations were due to the technical error of the tests, which was about twice that of similar tests for streptomycin sensitivity. The number of strains found to be resistant, out of a total of 85 strains previously found to be resistant or suspected of being resistant, was very similar whether the tests were carried out in liquid or on solid medium. Individual determinations of the degree of resistance were frequently very inaccurate. The minimal inhibitory concentration of P.A.S. was markedly affected by inoculum size and slightly by the method of preparing the solid medium. Some resistant strains reverted to sensitivity during storage.

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