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Semi-solid Agar Media for Rapid Drug Sensitivity Tests on Cultures of *Mycobacterium tuberculosis*

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SUMMARY: Semi-solid agar media prepared as previously described (Knox, 1955; Knox, Swait & Woodroffe, 1956) were found especially useful for studying the sensitivity of cultures of *Mycobacterium tuberculosis* to isoniazid, streptomycin and *p*-aminosalicylic acid (PAS). Kirchner, Fisher and Dubos media were compared both as semi-solid and as liquid media. In the semi-solid media when large inocula of *M. tuberculosis* H37Rv strain were used, results could be read in 2 days with a hand-lens and were usually easily read with the naked eye in 3-5 days. Results were easier to read and interpret in semi-solid than in the corresponding liquid media both because of the sharpness of the initial end-point defining the minimum inhibitory concentration of the drug (M.I.C.), and because the later growth of numerous or isolated colonies gave a measure, not obtainable with liquid media, of the variability within a culture. Such presumptively resistant colonies appeared most frequently with isoniazid in Kirchner semi-solid medium (in which the drug decayed rapidly), and less frequently with streptomycin and PAS. Colonies which appeared in isoniazid-containing tubes were not always found to consist of drug-resistant organisms on subculture. But when 2-3 days were allowed for microcolonies to appear before the drug was added, then the few large colonies which subsequently developed were found to contain truly resistant organisms. Strains resistant to isoniazid or streptomycin were distinguished from sensitive strains more easily than in liquid media. PAS sensitivity tests gave sharp and consistent end-points in Fisher and Kirchner semi-solid agar even when large inocula were used, though the actual level of the M.I.C. varied greatly with inoculum size, and with different strains. This may be partly accounted for by the reversal of PAS by *p*-aminobenzoic acid (PAB). In Dubos medium readable end-points were often obtained only with small inocula. For all three drugs it seemed that Fisher semi-solid agar gave the most satisfactory results.

The use of semi-solid agar media for the rapid culture of *Mycobacterium tuberculosis* has been previously described (Knox, 1955; Knox, Swait & Woodroffe, 1956). It was mentioned there that such media were found to be particularly useful for the performance of drug sensitivity tests. The present paper gives the results of further experience supporting this, and emphasizes the advantages of this type of medium for assessing the drug susceptibility of *M. tuberculosis*.

METHODS

The methods used were essentially the same as in the previous papers, but will be briefly summarized here. The media mainly used were those of Kirchner, prepared as described by Mackie & McCartney (1953) but without penicillin, of Fisher (1952) and of Dubos (Dubos & Davis, 1946). These media were used with the addition of agar (0.125 %) as semi-solid media, or without it as liquid media.

The organisms used were *Mycobacterium tuberculosis* H37Rv strain, variants of this strain made resistant in the laboratory to isoniazid or streptomycin, and *p*-aminosalicylic acid (PAS) resistant strains of *M. tuberculosis* obtained from Dr W. Steenken, Jun., Trudeau, New York, and from Mr E. M. Bavin, Smith and Nephew Research Ltd., Hunsdon, Hertfordshire. The inoculum was 0.02 ml. of a 10- to 14-day culture in Dubos liquid medium, unless otherwise stated. In some experiments more dilute inocula than this were used, and viable counts were frequently done as well by inoculating serial dilutions in semi-solid Kirchner agar.

Fig. 1. A diagram of the apparatus used for observing the growth of *Mycobacterium tuberculosis* in semi-solid agar.

Results were recorded by inspection of tubes at frequent intervals. Both observation and recording of results were greatly helped by the improved viewing method described previously (Knox, *et al.* 1956) and shown in Fig. 1, while in many experiments the photographic apparatus described by Engel & Knox (1957) made it easy to obtain permanent serial records.

RESULTS

General appearance of drug sensitivity tests in semi-solid media

The general features of the growth of *Mycobacterium tuberculosis* in semi-solid media have been fully described in the previous papers, and the differences between media with and without Tween 80 have been emphasized. Here, certain general features of drug-sensitivity tests in Kirchner semi-solid agar

medium (without Tween) will first be described. When a large inoculum was used, growth could first be seen in the drug-free control tube in the form of uncountable minute colonies visible with a hand-lens ($\times 6$) under proper illumination as early as 48 hr. after inoculation. This early growth could be easily distinguished from complete inhibition of growth which occurred in tubes containing high drug concentrations and, although the sharpness of the transition between the two extremes varied with the size of inoculum, with the drug used and with the spacing of the drug dilutions, a fairly accurate preliminary reading could be obtained in 2 days. After 3–4 days of incubation growth appeared as a diffuse band easily seen by naked eye and extending for some distance below the surface of the medium, and an end-point giving the 'minimum inhibitory concentration' (M.I.C.), at least for the majority of the organisms inoculated, was sharply defined. On longer incubation growth began to appear in tubes beyond the initial end-point though this itself could still be defined. Depending on the exact conditions used, this delayed growth ranged from innumerable minute colonies to a few large isolated colonies, but it was seldom as profuse, even after long incubation, as growth in the drug-free control tube or in other tubes containing drug concentrations below the initial M.I.C. In general, it may be said that the initial end-point indicated the average drug sensitivity of a bacterial culture (M.I.C.), while the delayed growth of numerous or isolated colonies was partly at least a measure of the variability among different individuals within a bacterial population.

These general features of drug sensitivity tests in semi-solid media are illustrated by several photographs showing the sensitivity to isoniazid of *Mycobacterium tuberculosis* H37 Rv as read after different periods of incubation in Kirchner semi-solid agar (Pl. 1). Although, as mentioned above, a sharp end-point was definable in 2 days at a magnification of $\times 6$ and easily readable at 3 days with the naked eye, it was not easy to reproduce these early readings photographically. Photographs taken on the fourth day, however, show the initial end-point clearly. Photographs taken after longer periods of incubation show the nature of the 'shift to the left' which occurred in this medium. The appearance of isolated colonies in increasing drug concentrations on continued incubation is well shown.

Comparison between drug sensitivity tests in semi-solid and liquid media

Table 1 illustrates the contrast between drug sensitivity tests in liquid and in semi-solid media of the same basal composition. In the experiment recorded in Table 1 isoniazid-sensitivity tests were performed with the H37 Rv strain and an isoniazid-resistant variant of it in Kirchner liquid and semi-solid agar media. In the liquid medium the M.I.C. of isoniazid for the sensitive strain was hard to determine. Growth in the control tube consisted of coarse granules, which were replaced in tubes containing increasing drug concentrations by finer or less numerous granules, but there was no sharp line of demarcation. In the semi-solid medium, on the other hand, a sharp end-point was readable at 2–3 days and, as shown in Pl. 1, could be photographed at 4 days, while the subsequent 'shift to the left' could be expressed

quantitatively, consisting as it did of decreasing numbers of colonies as the drug concentration was increased. Differences between the sensitive and resistant strain were also shown more clearly in the semi-solid medium since they too could be expressed quantitatively. In the liquid medium both sensitive and resistant strains eventually grew in 10 μ g. isoniazid/ml. and were distinguished mainly by the time taken for growth to occur in that concentration

Table 1. *Isoniazid sensitivity tests in liquid and semi-solid media*

In this experiment doubling dilutions were used, but for brevity only fourfold dilutions are reported.

Strain of <i>M. tuberculosis</i>	Medium	Period incubated at 37° (days)	Concentration of isoniazid (μ g./ml.)						
			10.0	2.5	0.625	0.156	0.039	0.009	0
H37Rv	Liquid	4	Tr	Tr	Tr	Tr	Tr	±	+
		11	±	±	±	±	+	+ ±	+ ±
		30	+	++	++	++	+++	+++	+++
	Semi-solid	4	0	0	0	0	0	SC	SC
		11	0	0	0	0	Unc	SC	SC
		30	1 colony	Unc	SC	C	C	C	C
I.N.H.-resis- tant variant (R1)	Liquid	4	±	±	±	±	±	±	±
		11	+	+	+	+	+	+	+
		30	++	++	++	++	++	++	++
	Semi-solid	4	Unc	Unc	Unc	Unc	Unc	Unc	Unc
		11	SC	SC	SC	SC	SC	SC	SC
		30	C	C	C	C	C	C	C

In liquid medium, + signs indicate approximate amount of granular growth, Tr=trace of growth.

In semi-solid medium, C=confluent, SC=semi-confluent growth, Unc=uncountable colonies.

of the drug. In the semi-solid medium, however, the two strains were easily distinguished since the growth of the resistant strain in that concentration was rapid and equal to its growth in the control tube, whereas growth of the sensitive strain occurred only after a long delay and when it did occur, consisted of only one colony.

Sensitivity patterns with different drugs

Isoniazid. One of the characteristic features of isoniazid-sensitivity tests in most media is the 'shift to the left' which makes the reading of an end-point in liquid media difficult (Knox, King & Woodroffe, 1952). As shown above, semi-solid media combine the advantages of a sharp early end-point with a clear picture of the development of presumptively resistant colonies on continued incubation (see Pl. 1). It was found, however, that there were considerable differences between different media. In all the three media here used (Kirchner, Fisher, Dubos) the initial end-point for the H37Rv strain was 0.05–0.01 μ g. isoniazid/ml., when read on the first possible occasion (2–4 days), but a shift to the left occurred rapidly in Kirchner medium, rather more slowly in Dubos medium and more slowly still in Fisher medium. A typical experiment is illustrated in Table 2.

In several experiments isoniazid-sensitivity tests were carried out with inocula of different sizes. The end-point was somewhat higher with very heavy

Table 2. *Isoniazid sensitivity of Mycobacterium tuberculosis H37Rv strain in three semi-solid media*

In this experiment doubling dilutions of drug were used but for brevity not all of these have been included.

Medium	Period incubated at 37° (days)	Concentration of isoniazid (μg./ml.)						
		2.5	0.625	0.156	0.039	0.019	0.009	0
Kirchner	3	0	0	0	0	0	SC	SC
	7	0	0	0	0	0	SC	SC
	17	0	0	0	Unc	Unc	C	C
	31	Unc	Unc	SC	C	C	C	C
Dubos	3	0	0	0	0	0	SC	SC
	7	0	0	0	0	SC	SC	SC
	17	0	0	Unc	SC	C	C	C
	31	0	Unc	SC	C	C	C	C
Fisher	3	0	0	0	0	0	SC	SC
	7	0	0	0	0	0	SC	SC
	17	0	0	0	SC	C	C	C
	31	0	0	50 colonies	C	C	C	C

C = confluent, SC = semi-confluent growth, Unc = uncountable colonies.

than with dilute inocula, but the extent of this effect varied a good deal with the period of incubation. When readings were made at the earliest possible moment, end-points were around 0.01–0.05 μg./ml. for a wide range of inocula. But after incubation, even for only 10 days, differences became obvious, and the shift to the left was very rapid with large but negligible with small inocula.

It has already been shown (Table 1) that in spite of this shift to the left, drug-sensitive and drug-resistant strains were more easily distinguished in semi-solid than in the corresponding liquid media. The isoniazid-resistant variant of the H37Rv strain grew well and rapidly in 10 μg. isoniazid/ml., but not, usually, much above 20 μg./ml. When more closely spaced drug dilutions were used, however, it was seen that a slight 'shift' occurred, although relatively much less than with the sensitive strain. This is illustrated in Table 3.

Table 3. *Isoniazid sensitivity of drug-resistant variant of Mycobacterium tuberculosis H37Rv strain*

Period incubated at 37° (days)	Concentration of isoniazid (μg./ml.)						
	28	26	24	22	20	10	0
3	0	0	0	Unc	SC	C	C
21	37 colonies	Unc	C	C	C	C	C

C = confluent growth, SC = semi-confluent growth, Unc = uncountable colonies.

Streptomycin. Table 4 shows the streptomycin sensitivity of the H37Rv strain in Kirchner, Fisher and Dubos semi-solid media; the initial end-point at 3 days was about 0.15 μg./ml. in all 3 media. On continued incubation the

end-point shifted slightly in Fisher and in Dubos media, but more extensively in Kirchner medium. But in none of the media was the shift as great as in the corresponding media containing isoniazid. The initial end-point varied with the size of inoculum more conspicuously with streptomycin than with isoniazid. On the other hand, the subsequent shift to the left was much less conspicuous with streptomycin, even when heavy inocula were used, than it was with isoniazid with much smaller inocula. Streptomycin-resistant strains grew well in semi-solid agar and could easily be distinguished from sensitive strains.

Table 4. *Streptomycin sensitivity of Mycobacterium tuberculosis H37 Rv strain in three different semi-solid media*

Medium	Period incubated at 37° (days)	Concentration of isoniazid ($\mu\text{g./ml.}$)					
		2.5	0.625	0.156	0.039	0.009	0
Kirchner	3	0	0	0	SC	SC	SC
	7	0	Unc	SC	SC	SC	SC
	17	0	C	C	C	C	C
	31	50	C	C	C	C	C
Dubos	3	0	0	0	SC	SC	SC
	7	0	0	0	SC	SC	SC
	17	0	0		SC	SC	SC
	31	0	0	Unc	C	C	C
Fisher	3	0	0	0	SC	SC	SC
	7	0	0	SC	SC	SC	SC
	17	0	0	SC	C	C	C
	31	0	4	C	C	C	C

C=confluent growth, SC=semi-confluent growth, Unc=uncountable colonies. Figures give number of colonies when these were countable.

p-Aminosalicylic acid (PAS). The sensitivity of the H37 Rv strain to PAS was about the same in all the three semi-solid media (though the M.I.C. was usually slightly less in Kirchner and Fisher than in Dubos semi-solid agar). When cultures were examined daily after inoculation large numbers of minute colonies (visible only with a lens $\times 6$) could be seen in the first 2 or 3 days in tubes containing high PAS concentrations. In these tubes, however, this 'growth' did not progress, and by the fifth or sixth day it was usually possible to establish a M.I.C. which shifted very little on further incubation. The exact position of this end-point varied considerably with the size of inoculum and the medium, ranging from 0.5 $\mu\text{g./ml.}$ with an undiluted inoculum to 0.05 $\mu\text{g./ml.}$ with a 10^{-4} dilution. The end-point was usually much sharper in Kirchner and Fisher semi-solid media than it was either in the corresponding liquid media or in Dubos medium, either liquid or semi-solid, in which when large inocula were used it was often impossible to define an end-point at all. Table 5 shows the effect of inoculum size on the PAS sensitivity of the H37 Rv strain in liquid and semi-solid Kirchner agar read at 8 and 29 days. Table 6 shows the PAS sensitivity of 4 strains of *Mycobacterium tuberculosis* in Kirchner semi-solid agar. When readings were taken at 15 days, the H37 Rv strain was

found to be sensitive to 0.45 μg . PAS/ml. or less, whether the inoculum was undiluted or diluted 1 in 100. With one resistant strain (R. 10) growth occurred in 125 μg . PAS/ml. from both inocula, but the M.I.C. for two other strains was found to be highly dependent on inoculum size, decreasing with strain R. 14 from about 125 to 8 μg ./ml., and with strain R. 15 from 31 to 2 μg ./ml. when undiluted inocula were compared with 1 in 100 dilutions.

Table 5. *Sensitivity of Mycobacterium tuberculosis H37 Rv strain of p-aminosalicylic acid (PAS) in liquid and semi-solid media*

Medium	Inoculum	Period incubated at 37° (days)	Concentration of PAS (μg ./ml.)							
			10.0	1.0	0.5	0.1	0.05	0.01	0.001	0
Liquid Kirchner	Undiluted	8	0	0	±	±	+	+	+	+
		29	±	±	±	+	++	++	++	++
	10 ⁻²	8	0	0	0	0	0	±	±	±
		29	0	0	0	±	+	+	++	++
	10 ⁻⁴	8	0	0	0	0	0	0	0	0
		29	0	0	0	Tr	Tr	±	±	±
	Semi-solid Kirchner agar	8	0	0	0	SC	C	C	C	C
		29	0	0	0	C	C	C	C	C
	10 ⁻²	8	0	0	0	0	0	Unc	SC	SC
		29	0	0	0	SC	C	C	C	C
	10 ⁻⁴	8	0	0	0	0	0	0	0	0
		29	0	0	0	22	29	50	50	50

C=confluent growth, SC=semi-confluent growth, Unc=uncountable colonies. Figures give number of colonies when these were countable.

Table 6. *Sensitivity of 4 strains of Mycobacterium tuberculosis to p-aminosalicylic acid (PAS) in Kirchner semi-solid agar*

Inoculum		Concentration of PAS (μg ./ml.)					
		1.25	31.2	7.8	1.95	0.45	0
H37 Rv	Undiluted	0	0	0	0	0	SC
	1/100	0	0	0	0	0	SC
R. 15	Undiluted	0	0	Unc	SC	SC	SC
	1/100	0	0	0	0	Unc	Unc
R. 14	Undiluted	0	SC	SC	SC	SC	SC
	1/100	0	0	0	Unc	Unc	Unc
R. 10	Undiluted	SC	SC	SC	SC	SC	SC
	1/100	SC	SC	SC	SC	SC	SC

Readings after 15 days of incubation. Doubling dilutions of PAS were used but for brevity only fourfold dilutions are shown. C=confluent growth, SC=semi-confluent growth, Unc=uncountable colonies. 0=no growth.

It was shown by Youmans, Raleigh & Youmans (1947) that the action of PAS on *Mycobacterium tuberculosis* could be reversed by *p*-aminobenzoic acid (PAB), and the variability in PAS sensitivity tests may well be related to differences in the concentration of PAB in different media or in its production by different strains. Table 7 shows that when PAB was incorporated in

semi-solid Kirchner agar it reversed the action of PAS in what appears to be a competitive way. But it was not necessary to incorporate the PAB into the medium. It could be added (in a strength calculated to give a required concentration by diffusion through the medium) to tubes which had already been inoculated and incubated up to several days. Table 8 shows that even when added to PAS-containing tubes after incubation for 5 days PAB showed some annulling action, though this was much slower than after shorter periods of incubation.

Table 7. *Effect of p-aminobenzoic acid (PAB) in annulling effect of p-aminosalicylic acid (PAS)*

Medium: Kirchner semi-solid. Readings after 6 days of incubation at 37°.

Concentration of PAB ($\mu\text{g./ml.}$)	Concentration of PAS ($\mu\text{g./ml.}$)									
	10.0	5.0	1.0	0.5	0.1	0.05	0.01	0.005	0.001	0
0	0	0	0	0	SC small	SC small	SC small	SC	SC	SC
1.0	0	0	SC	SC	SC	SC	SC	SC	SC	SC
5.0	0	SC	SC	SC	SC	SC	SC	SC	SC	SC
10.0	SC	SC	SC	SC	SC	SC	SC	SC	SC	SC

SC = semi-confluent growth.

Table 8. *Annulment of effect of p-aminosalicylic acid (PAS) by p-aminobenzoic acid (PAB) added at intervals after inoculation*

Organism: *Mycobacterium tuberculosis*, strain H37 Rv. Medium: Kirchner semi-solid.

Period of incubation (days) before adding PAB (10 $\mu\text{g./ml.}$)	Period incubated at 37° (days)	PAS concentration ($\mu\text{g./ml.}$)										
		8.0	4.0	2.0	1.0	0.5	0.25	0.125	0.062	0.031	0.016	0
0	7	C	C	C	C	C	C	C	C	C	C	C
	12	C	C	C	C	C	C	C	C	C	C	C
	19	C	C	C	C	C	C	C	C	C	C	C
1	7	0	C	C	C	C	C	C	C	C	C	C
	12	C	C	C	C	C	C	C	C	C	C	C
	19	C	C	C	C	C	C	C	C	C	C	C
2	7	0	0	0	0	C	C	C	C	C	C	C
	12	C	C	C	C	C	C	C	C	C	C	C
	19	C	C	C	C	C	C	C	C	C	C	C
4	7	0	0	0	0	0	0	C	C	C	C	C
	12	0	0	0	C	C	C	C	C	C	C	C
	19	SC	C	C	C	C	C	C	C	C	C	C
5	7	0	0	0	0	0	0	C	C	C	C	C
	12	0	0	0	0	C	C	C	C	C	C	C
	19	0	C	C	C	C	C	C	C	C	C	C
No PAB added	7	0	0	0	0	0	0	C	C	C	C	C
	12	0	0	0	0	0	0	C	C	C	C	C
	19	0	0	0	0	0	SC	C	C	C	C	C

0 = no growth, SC = semi-confluent, C = confluent growth.

Summary of the differences between different drugs

The results here described show that the drug-sensitivity patterns for isoniazid, streptomycin and PAS in semi-solid agar showed characteristic differences. With isoniazid, the initial end-point (M.I.C.) was about 0.01–0.05 $\mu\text{g./ml.}$ and was often little affected either by inoculum size or the formula of the medium. But on continued incubation 'resistant' colonies developed far more rapidly in Kirchner than in Dubos or Fisher semi-solid agar, and their number was roughly proportional to the size of the inoculum. With streptomycin, on the other hand, it was the initial end-point which was affected both by the size of the inoculum and by the nature of the medium. The M.I.C. for streptomycin was four- to eightfold higher in Kirchner than in Dubos or Fisher semi-solid agar in which it was 0.15–0.3 $\mu\text{g./ml.}$ With streptomycin the development of 'resistant' colonies did not spread over so wide a range of concentrations as with isoniazid. However, as will be seen later, it was easier to select variants permanently resistant to streptomycin than to isoniazid. With PAS the initial end-point was not always as clearly defined in the first 3–4 days of incubation as it was with the other two drugs, but usually sharp end-points were obtained at 7 days, especially in Kirchner and Fisher semi-solid agar, even when large inocula were used. Although the M.I.C. varied greatly with inoculum size it showed little change on further incubation and the development of 'resistant' colonies was much slower than with either of the other two drugs. In several experiments it was found that, with isoniazid or streptomycin, when growth occurred in increasing concentrations of drug it consisted of progressively smaller numbers of colonies, but the colonies which did develop eventually reached normal size. With PAS, on the other hand, the size of the colonies decreased steadily with increasing drug concentration, but the numbers were much less affected. This interesting effect was not consistently observed and requires further investigation.

Inactivation of isoniazid, streptomycin and p-aminosalicylic acid in uninoculated media

It was natural to suspect that differences in initial end-point and in its stability might be related to the chemical stability of the different drugs in different media. Solutions containing known initial concentrations (4–5 $\mu\text{g./ml.}$) of isoniazid, streptomycin or PAS were incubated at 37°; samples were removed at intervals and the drug concentration determined by the serial dilution method using for each assay the end-point given by the H37Rv strain in Dubos liquid medium after 7 days of incubation. The results are shown in Fig. 2. None of the drugs decayed significantly in distilled water at 37° over a period of 4 weeks. Streptomycin decayed slowly in all 3 media. Isoniazid decayed very rapidly in Kirchner and in Dubos medium but more slowly in Fisher medium. PAS showed no significant decay in any of the media (Fig. 2). The presence of PAS did not affect the decay of isoniazid, nor did isoniazid affect the stability of PAS. In one experiment the decay of isoniazid in semi-solid Kirchner medium was followed directly by inoculating

the H37Rv strain on to the surface of drug-containing tubes incubated for different times at 37°. The decay was of the same order as was found in liquid Kirchner medium.

The drug sensitivity of organisms growing in drug-containing semi-solid agar

On many occasions attempts were made to measure the drug sensitivity of organisms growing in semi-solid media containing various concentrations of isoniazid or streptomycin. Several methods were used. Agar at the concentration of 0.125 % used, while solid enough to give discrete colonies when

Fig. 2. Decay of drugs in uninoculated media. K = Kirchner; F = Fisher; D = Dubos.

suitably inoculated, was also liquid enough to be sucked up in a Pasteur or dropping pipette. A single colony could thus be picked up and transferred to another tube and then shaken up with Dubos or other suitable liquid medium. When separate colonies were not available, then a confluent or semi-confluent growth could be similarly transferred in a pipette. Inoculated tubes were then incubated and after 7–14 days the drug sensitivity of the culture was determined in semi-solid Dubos agar. It was found that colonies growing in high drug concentrations were not necessarily as resistant as would be suggested by the drug concentration in which they originally grew, though many of them did show increased drug resistance when tested in this way. It was interesting to find that although presumptively 'resistant' colonies occurred with greater frequency with isoniazid than with streptomycin, drug resistance in the subcultures was more often found with streptomycin than with isoniazid. It is possible of course that any truly resistant variants, which might have grown in drug-containing media, might have been largely eliminated by the process

of subcultivation in drug-free medium involved in the method of sensitivity testing here used. Preliminary experiments have shown that single colonies can be transferred from semi-solid agar medium and, after shaking in Dubos or other suitable medium, can be used without subculture in drug-free medium for sensitivity tests. Similarly a 4-day culture of the H37Rv strain in Dubos semi-solid agar could be used for direct inoculation by a Pasteur pipette with a set of tubes containing serial drug dilutions in semi-solid agar and the drug sensitivity estimated in 2 days with a hand-lens and in 3–4 days by naked eye. This technique is being developed to investigate afresh this problem of the true drug sensitivity of presumptively resistant colonies growing in drug-containing media.

Selection of drug-resistant variants in semi-solid agar media

The early development of microcolonies of *Mycobacterium tuberculosis* in semi-solid agar offered an opportunity to study the effect of drugs added to known numbers of organisms at different stages in their development. Only a few preliminary experiments have been done, but some interesting results have been obtained by adding isoniazid, streptomycin or PAS to 3-day cultures of the H37Rv strain in semi-solid agar. It was mentioned earlier that with a heavy inoculum growth could be seen at this stage to consist of innumerable minute colonies. Drugs added to such cultures diffused rapidly through the medium and arrested or delayed further growth. With PAS the colonies continued for 2–3 days to increase slowly in size as compared with colonies in drug-free medium, but usually no large colonies developed. With isoniazid or streptomycin, on the other hand, the growth of nearly all colonies was completely arrested, but a few colonies continued to grow at about the normal rate. These colonies, when their drug sensitivity was determined by the method described above, were found to be much more resistant than similar colonies growing in media which had contained drugs from the time of inoculation. This would suggest that a more accurate picture of the mutation rate to drug resistance might be obtained from adding drugs to young cultures in a medium in which they are actively multiplying rather than to older cultures which are exposed to new medium and drug at the same time.

DISCUSSION

The differences obtained with the three media here used show clearly some of the difficulties in the interpretation of drug sensitivity tests on cultures of *Mycobacterium tuberculosis*. Both the initial end-point and the number of presumptively resistant organisms are greatly influenced by the medium used, and it is not possible at present to say which medium gives the more accurate picture of the average sensitivity of a culture and of the distribution of resistant individuals in it. These difficulties, however, are inherent in any of the tests at present used and can be to some extent overcome by using a strain such as the H37Rv strain of *M. tuberculosis* as a standard for comparison.

The method of serial drug dilutions in liquid media frequently used for

drug-sensitivity tests gives at best a fairly accurate picture of the average drug sensitivity of the majority of the organisms in a bacterial culture. If a culture is fairly homogeneous in this respect, then the method gives a sharp end-point between 'no growth' and 'growth equal to the drug-free control', even when closely spaced (e.g. doubling) dilutions are used. But when there is a good deal of variation within the bacterial population the method loses much of its value. A sharp end-point can then be obtained only by increasing the spacing of the dilutions (say to tenfold) with a great loss of accuracy. If doubling or fourfold dilutions are still used, then a sharp end-point is no longer obtained. These difficulties are especially troublesome with sensitivity tests for tubercle bacilli in liquid media. Provided a small inoculum is used and conditions are carefully standardized it may be possible to obtain a fairly sharp end-point if sensitivity tests are read at the earliest possible moment. But all strains do not grow at the same speed and when on continued incubation a shift in the end-point occurs, it is impossible to tell with liquid media whether this is due to delayed growth of most of the organisms in the culture or only of a few individuals different from the average. Further, no information is given about the frequency of drug-resistant mutants in a bacterial population.

Fully solid media, on the other hand, which do give this information have many disadvantages which have already been discussed (Knox *et al.* 1956). Growth is usually rather slow, the risks of contamination or dehydration of the medium are difficult to avoid and cultures or sensitivity tests cannot conveniently be repeatedly inspected and assessed at a glance. Semi-solid media, however, combine the advantages of liquid and solid media without the disadvantages of either. The results shown in this paper suggest that though one medium might be the best with one drug but not so good with another, the medium most suitable for all three drugs was Fisher semi-solid agar.

Since both viable counts and drug-sensitivity tests can be so easily and conveniently performed, semi-solid media open up new possibilities for the intensive study of drugs and combinations of drugs, both for bacteriostatic and bactericidal activity. Since many substances added to low agar concentrations diffuse rapidly through the media, they can also be used to investigate the action of reversing agents added at different periods after inoculation, for example, the effect of *p*-aminobenzoic acid in reversing the action of *p*-aminosalicylic acid or of haemin in reversing the action of isoniazid. In the examination of clinical material too, semi-solid media have shown great promise. Rapid results have been obtained both with 'indirect' tests on cultures isolated from patients' sputa and also with direct tests on sputum concentrates. These results are reported in a separate communication (Knox & Skinner, 1957).

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R. KNOX & R. WOODROFFE—DRUG SENSITIVITY OF *M. TUBERCULOSIS*. PLATE 1
(Facing p. 659)

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EXPLANATION OF PLATE

Isoniazid sensitivity of H37Rv strain of *M. tuberculosis* recorded after different times of incubation. The tubes contained the following isoniazid concentrations ($\mu\text{g./ml.}$): 7, 0.6; 6, 0.3; 5, 0.16; 4, 0.08; 3, 0.04; 2, 0.02; 1, 0.01; C (control) 0. Note that in the last two photographs tubes 2 and 1 have been omitted, so that tube 3 is now next to the control tube.

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