THE GROWTH OF HUMAN TUBERCLE BACILLI, H37, IN SYNTHETIC MEDIUM WITH AND WITHOUT AGAR

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It is generally believed that tubercle bacilli will not grow on synthetic media when less than 10^{-1} mgm. of bacilli are planted. It is also a general belief that they must be planted on the surface of the liquid medium for growth to occur: that if they sink beneath the surface they do not multiply. Agar when combined with synthetic media has been assumed to be inert toward the growth of tubercle bacilli.

The purpose of this paper is to record that a liquid synthetic medium without agar will support growth of a human tubercle bacillus strain, H37, and a subcolony strain from the latter when amounts at least as small as 10^{-6} mgm. are planted, that growth of these bacilli occurs consistently beneath the surface of this liquid medium and that agar, combined with the synthetic media when the latter was desired in a nearly solid form, is inhibitory to the growth of small amounts of the bacilli.

Corper and Uyei (1927) stated that 3 or 4 drops (about 0.3 ml.) of suspensions containing from 10 to 10^{-1} mgm. per milliliter of tubercle bacilli placed on synthetic media combined with agar resulted in questionable growth and that similar plantings from 10^{-2} mgm. per milliliter or less produced no growth. Uyei (1930) remarked that tubercle bacilli would grow under these conditions when similar plantings from 1 mgm. per milliliter suspensions were made. Boissevain and Schultz (1938) reported similar findings.

TECHNIQUE

A slightly modified form of Long's synthetic medium was used. The formula is as follows:

	grams
Asparagin	5.00
Ammonium citrate	5.00
Dibasic sodium phosphate ($\bar{c}12H_2O$)	4.00
Potassium chloride	2.00
Magnesium sulphate (anhydrous)	1.00
Ferric ammonium citrate	0.05
Glycerine	50.00
Water q.s. ad	1000.00

The pH of the above was made 7.0 by adding a saturated solution of sodium hydroxide (about 1.3 ml.).

This medium supports the growth of human tubercle bacilli well.

When solid tube slants were used sufficient agar to make a 2 per cent gel was added.

The agar used was of U.S.P. quality in a granual powder form.

The bacilli used for growth tests were always secured from surface cultures growing on the synthetic medium. The strains were H37 and a strain referred to as Q control no. 7 which was derived from a colony of H37 and shown to be more virulent for guinea pigs than the latter. The cultures used had grown from 16 to 27 days.

The weighed bacilli with excess moisture removed were ground in a test tube with the rounded end of a glass rod and gradually suspended using various watery diluents. Suspensions containing from 1 mgm. per milliliter down to the desired smallest amounts of bacilli were made.

Most of the growth tests were made with 25 ml. of medium in 50 ml. Erlenmeyer flasks. The non-absorbent cotten stoppers in the latter experiments were impregnated lightly with paraffin to prevent excessive evaporation during incubation.

Incubation was at 37.5°C.

EXPERIMENTAL

I

Synthetic media plus 2 per cent agar slants were planted with suspended amounts of bacilli varying from 1.0 to 10^{-6} mgm. and

the same done for Corper and Cohn's egg medium. In 3 months there was no growth with less than 10^{-1} mgm. bacilli on the agar slants while there was growth with all the gradings down to 10^{-6} mgm. on the egg medium.

This experiment confirms the experience of others but does not prove that synthetic media without agar will not support the growth of smaller amounts than 10^{-1} mgm. bacilli.

II

Synthetic medium without agar was placed in flasks. Clumps of bacilli were washed 3 successive times with distilled water and 3 successive times with fresh synthetic medium. They were suspended in synthetic medium to give the desired graded amounts. From these various dilutions 0.5 ml. were transferred for growth tests. Three flasks were used for each of 1.0, 10^{-2}, 10^{-4}, 10^{-6}, 10^{-8} and 10^{-10} mgm. and 1 flask for each of the 10^{-1}, 10^{-3}, 10^{-5}, 10^{-7} and 10^{-9} mgm. transplants. Also, the same number of flasks with the same medium but with filter papers (C. S. & S., no. 589, blue label) at the bottom of the medium were similarly planted.

Some weeks before the end of the experiment the 1.0, 10^{-1}, 10^{-2} and 10^{-3} mgm. flasks were overgrown and discontinued. At the end of 67 days, growths from the other amounts of bacilli were as recorded in table 1.

Growth occurred with all plantings from 1.0 to and including 10^{-5} mgm. Uncertainty of growth began with 10^{-6} mgm. though with this, 5 out of 6 flasks showed growth.

It is notable that growth begins at the bottom of the medium and only with amounts of bacilli greater than 10^{-4} is there rather promptly-appearing surface growth which appears after the bottom growth is well established.

The above findings have been confirmed by other experiments at least up to 10^{-6} mgm. plantings. Filter papers floated on the surfaces of the synthetic media also supported growth from similar small amounts of bacilli.

In the absence of paper the growths with 1.0 and 10^{-1} mgm. uniformly cover the glass bottoms and are rather thin. With smaller plantings, freely-moving small flocculent colonies develop on the bottoms.

On the paper bottom the colonies adhere, though not tenaciously, to the paper. These colonies are not as flocculent and with the 2 heaviest plantings the papers are soon covered. It could not be decided that the paper promoted growth and visual examination did not show it had been attacked in any way.

TABLE 1

BACILLI PLANTED	NUMBER OF PAPERS			PAPERS AT BOTTOM OF MEDIUM		
	Surface growth		Growth at bottom (number of colonies)	Surface growth		Growth on paper (number of colonies)
mgm.						
10^{-4}	1	–	Numerous	4	–	Numerous
	2	–	Numerous	5	–	Numerous
	3	–	Numerous	6	–	Numerous
10^{-5}	1	–	5	2	–	Numerous
10^{-6}	1	–		5	++++	
	2	–	8	6	–	7
	3	–	12	7	–	Numerous
10^{-7}	1	–		2	–	2
10^{-8}	1	–	2	4	–	
	2	–		5	–	6
	3	–		6	–	5
10^{-9}	1	–	6	2	–	
10^{-10}	1	–		4	–	
	2	–		5	–	
	3	–	10	6	–	

– = negative; ++++ = profuse.

Some of the clustered colonies at the bottom of the medium were transferred to fresh synthetic medium and to egg slants where they grew. Bacilli from these submerged growing colonies were acid-fast to the Ziehl-Nielsen stain.

In addition to the experiments with suspended bacilli, clumps of bacilli were transferred directly to flasks containing synthetic medium. Some of the clumps were forced with considerable

difficulty to the bottom of the medium where they grew. It appears that if clumps of bacilli sink spontaneously or easily to the bottom they are water-logged and that the bacilli in them are incapable of multiplying since apparently such clumps of bacilli do not grow.

III

A possible explanation for failure of 2 per cent agar in synthetic media to favor growth of small amounts of tubercle bacilli is that the agar contained injurious inorganic elements.

A spectrum analysis of the agar ash was made with a quartz spectrograph and a comparison made with a similar study of egg yolk ash previously reported by Drea (1935).

Traces of Al, Ba(?), B, Cu, Fe, Pb, Li(?), Mn(?), Si, Na, Sr, Sn, Zn(?) and Ti(?) were found in the agar ash. Ca and Mg were present in larger amounts. Of these, only Sn was absent from the yolk. The yolk contained traces of Ba, Mn, Zn and Ti which were questionably present in the agar. The yolk also contained Cr(?), Mo(?), P, K, Rb and V which were not found in the agar. P and K are in the synthetic medium and need not be further considered.

Fe, Mg and Pb were present in greater amounts in the agar than in the yolk. Fe and Mg were eliminated from the synthetic medium and 2 per cent agar was added.

No improvement in growth promotion was noted. This leaves Pb for consideration but it is doubtful if sufficient of it is present to be injurious.

IV

The effects of various amounts of agar in the synthetic media were now studied. The following percentages of agar were prepared: 2.0, 1.0, 0.5, 0.25, 0.12, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}. Twenty-five ml. amounts were placed in 50 ml. flasks. Horizontal surfaces were thus provided for the bacteria to settle upon.

The suspension was planned to give 10^{-2} mgm. per drop from a glass pipette. One drop was placed in each flask.

Incubation for 2 months showed that greater percentages than 0.1 or 0.25 per cent of agar were definitely inhibiting to growth. Growth was more profuse and appeared earlier with the least amounts of agar and was most profuse and earliest with no agar.

V

A single colony appeared beneath the surface of a 0.1 per cent agar synthetic medium sol in a flask and was observed to develop over a period of 6 months. Starting as a compact, irregularly-shaped and somewhat angular little mass it became flocculent and in appearance like those recorded for the colonies on the glass bottoms of the flasks containing synthetic medium only. After about 2.5 months, extremely numerous smaller colonies began to develop about this original colony until finally they occupied a roughly spherical region of about 0.7 cm. radius. Before the 6th month of incubation there were small islands of growth on half of the surface.

A series of flasks was prepared containing 0.1 per cent agar in synthetic medium. They have been planted with amounts of bacilli varying from 10^{-1} to 10^{-10} mgm. bacilli.

After 24 days incubation there are quite numerous colonies beneath the surface associated with quite profuse surface growths in the 10^{-1} mgm. flasks and smaller numbers of colonies in the 10^{-2} mgm. flasks.

Thus, it has been shown that colonies of tubercle bacilli can develop at various levels in liquid agar as well as at the bottom of synthetic media.

SUMMARY

The strain H37 and a subcolony strain of the latter will grow in synthetic media when planted with amounts less than 10^{-1} mgm. and down to 10^{-6} mgm. at least. This may take place because the H37 strain by long continued laboratory transplanting is especially conditioned to do this. Other strains of bacilli, especially the more recently isolated ones, may not be capable of growth from such small amounts of bacilli. But it is also possible that a search for such results has not been made,

since it is generally believed that tubercle bacilli sinking beneath the surface of a liquid synthetic medium do not grow.

It is also possible that the slight modification of Long's medium had something to do with these results.

Excessive evaporation of water from the medium when incubation occurs over a long period of time must be avoided.

Synthetic medium plus 1.5 or 2 per cent agar questionably supports the growth of tubercle bacilli from amounts of 10^{-1} to 10^{-2} mgm. and does not support the growth of smaller amounts. Agar is generally considered to be an inert material when combined with synthetic medium. This is not true. Agar is mainly a mixture of polysaccharide sulphuric esters combined with calcium and magnesium. It has the general formula $(RO \cdot SO_3)_2$ Ca, R representing the polysaccharide residue (Neuberg and Ohle, 1921; Percival and Somerville, 1937). Solubility differences produce partial separation of the mixture and it has been reported by Rippel and Lehmann (1936) that different activities in stimulating bacterial growth are associated with the separated fractions.

The surfaces of the solid agar media may be unfavorable for the growth of small amounts of tubercle bacilli because of (1) the inhibiting power of the agar itself; (2) impaired permeability of the surface to the passage of nutrient substances; (3) excessive and unrecognized deposit of salts on the surface; (4) poisoning of the surface in some manner, and (5) flaming of the cotton stoppers which results in contamination of the agar media with volatile and harmful substances. So certain is the latter that the technique is now designed to make the flaming of stoppers unnecessary. It is possible that some form of agar different from that generally used in the laboratory will be more favorable to growth.

That colonies may develop in a 0.1 per cent agar synthetic medium anywhere between the surface and bottom was expected since its density is such that only a very few if any of the planted bacilli will sink to the bottom.

The colonies oscillate about a mean position when the flask is disturbed and are free to develop and project their field of

influence in all three dimensions of space. The transparency and viscosity of this medium permit continued observations of growing colonies and the probable development of daughter colonies.

That tubercle bacilli will grow throughout Long's medium when suspensions of kephalin or lecithin have been added to it has been reported by Boissevain (1931).

Growth of bacilli in the depths of other media observed by other investigators is accounted for by Calmette (1928).

Growths of small numbers of acid-fast bacilli in liquid substrates composed of blood or serum diluted with water or with Long's medium and of the washed cellular fraction of blood suspended in saline or dilute Long's medium have been reported by Evans and Hanks (1939).

CONCLUSIONS

1. Human tubercle bacilli of the strain H37 and of a subcolony strain of the latter will grow in a synthetic medium when amounts at least as small as 10^{-6} mgm. are planted in it.

2. These bacilli grow on the bottom of the synthetic medium and later there is growth on the surface.

3. Agar is definitely inhibiting to the growth of these bacilli when amounts greater than 0.1 or 0.25 per cent are added to the synthetic medium and less inhibiting when smaller amounts are used.

4. Colonies of these bacilli can be grown at different levels of a 0.1 per cent agar in the synthetic medium.

5. The above facts will be of value in the study of probable accessory growth factors for tubercle bacilli.

REFERENCES

BOISSEVAIN, C. H. 1931 Some relations between immunity and biochemistry in tuberculosis. Am. Rev. Tuberc., **23**, 66–70.

BOISSEVAIN, C. H., AND SCHULTZ, H. W. 1938 A lipoid growth factor for the tubercle bacillus. Am. Rev. Tuberc., **38**, 624–628.

CALMETTE, A. 1928 L'infection bacillaire et la tuberculose. Troisième édition, Masson et Cie, Editeurs, Paris.

CORPER, H. J., AND UYEI, N. 1927 The cultivation of tubercle bacilli. J. Lab. Clin. Med., **13**, 469–480.

DREA, W. F. 1935 Spectrum analysis of hen eggs and chick tissues. J. Nutrition, **10**, 351–355.

EVANS, B., AND HANKS, J. H. 1939 Growth of small numbers of acid-fast bacilli in blood and serum. Proc. Soc. Exptl. Biol. Med., **40**, 112–114.

NEUBERG, C., AND OHLE, H. 1921 Uber einen schwefelgehalt des agars. Biochem. Z., **125**, 311–313.

PERCIVAL, E. G. V., AND SOMERVILLE, J. C. 1937 Part II. Acetylation and methylation of agar-agar. J. Chem. Soc., 1615–1619.

RIPPEL, A., AND LEHMANN, B. 1936 Uber die wirkung von geringen mengen agar auf wachstum und stickstoffbingung von axotobacten und auf andere microbiologische vorgänge. Arch. Microbiol., **7**, 210–234.

UYEI, N. 1930 The nature of the growth promoting active principle in potato. Am. Rev. Tuberc., **22**, 203–217.