GROWTH OF SMALL NUMBERS OF TUBERCLE BACILLI, H37, IN LONG'S LIQUID SYNTHETIC MEDIUM AND SOME INTERFERING FACTORS

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Very small amounts of substances inhibiting the growth of amounts less than about 10^{-2} mgm. of well-dispersed and suspended tubercle bacilli can be readily and unknowingly introduced into Long's synthetic medium.

This fact is of great importance when investigating probable growth factors for these bacilli. It is of equal importance in *in vitro* studies of the probable inhibiting or lethal effects of substances deliberately added to the culture medium.

If injurious contaminating agents are kept out of Long's liquid synthetic medium, growth of the strain, H37, of human tubercle bacilli will result from amounts at least as small as 10^{-7} to 10^{-8} mgm.

If contamination occurs, growth may be limited to somewhere between 10^{-1} and 10^{-7} mgm. depending upon the amount and kind of contamination.

I

Drea (1940) reported that amounts of the H37 strain "at least as small as 10^{-6} mgm." could be cultured in a slightly modified form of Long's medium. It seemed unnecessary to discuss the self-evident irregularities recorded in a table in that paper for growth from the 10^{-6} to and including 10^{-10} mgm. plantings, since the series was a brief preliminary one, and no conclusions were drawn regarding growth with less than 10^{-6} mgm. However the failure to discuss this point seems to have made the table appear misleading. The present article will clarify the matter.

Continuation of the research following the observations already reported resulted finally in noting that growth of the bacilli was limited to amounts greater than 10^{-6}, 10^{-5}, 10^{-4} or 10^{-3} mgm. with the same technique, in general, as previously used.

The most probable explanation was that contaminating substances inhibiting growth were now present that were previously absent.

The glassware at the beginning of the research was new and after each growth experiment was cleaned and prepared in the usual way of this laboratory for the next plantings of bacilli. It seemed probable, therefore, that injurious invisible films on the glass surfaces had been gradually built up in the tubes used to grind and suspend the bacilli, in the flasks containing the culture medium and in the pipettes.

Elimination of these possible sources of contamination has resulted in demonstrating that growth of the bacilli will result in Long's synthetic medium after estimated 10^{-7} and 10^{-8} mgm. amounts have been planted by taking 0.1 ml.

of 10^{-6} and 10^{-7} mgm. per ml. dilutions respectively. (See Technique, Section IV.)

II

Sources of contamination

Four probable sources of contamination were as follows: (1) persistent adsorption by the glass of soap and other organic films incidental to the cleaning of the glassware in spite of repeated final rinsings with hot tap water; (2) adsorption of the paraffin used to lightly seal the cotton stoppers after planting the bacilli; (3) adsorption of distillates from the bleached non-absorbent cotton used as stoppers for the tubes, flasks and pipettes; and (4) adsorption of metabolic products produced by the tubercle bacilli.

Emphasis is laid upon this fact: that the glassware after the final rinsing with hot water and when dry appeared to be thoroughly clean. There were no visible adsorbed films present.

III

Technique for preparing flasks containing synthetic medium free from contaminating adsorbed films

The glassware must be thoroughly cleaned. When thought advisable, a saturated solution of KNO_3 in concentrated H_2SO_4 was used as a cleaning agent. Bichromate-acid solution was not used because of the possible introduction of chromic compounds (reaction products) into the glassware.

Bleached non-absorbent cotton stoppers when sterilized with the flasks in the hot air oven and autoclave were demonstrated to give off growth-inhibiting distillates. Because non-bleached non-absorbent and bleached absorbent cotton also give off distillates when heated, the use of cotton has been discontinued in this work.

Long's synthetic medium (Long and Seibert, 1926) was used with 2.33 grams of anhydrous Na_2CO_3 per 1000 ml. instead of the amount called for in the original formula. The pH was about 7.1 after autoclaving. Twenty ml. of the medium were placed in each 50 ml. Erlenmeyer flask to be planted.

As substitutes for cotton stoppers, either plain transparent Cellophane, No. 300, well washed with distilled water, was fastened with cleaned rubber bands over the tops of the flasks, or aluminum foil of thickness, 0.00065 inch, previously held over a Bunsen flame, was folded over the tops of the flasks, or flame-cleaned, loosely fitting aluminum cylinders were placed over the tops. As compared with loosely fitting Pyrex glass caps the cellophane and aluminum proved to be equally inert. The same method of capping was used for the parent cultures from which the transplants were secured and for the stock solution from which the culture flasks were filled. The flasks with the culture media were then autoclaved at 120° C. for 20 minutes.

Pyrex glassware, except for the pipettes, was used.

IV

Technique for preparing bacterial suspensions and planting the bacilli

All procedures were planned to avoid contamination by other microorganisms. The air currents in the room were kept to a minimum for several hours before planting the bacilli.

Some of the three-weeks-old surface growth from a vigorously growing culture of the H37 strain on Long's medium was removed and dried on fat-free well-washed absorbent paper. About 6 mgm. were weighed to within 0.1 mgm. in the grinding tube. The grinding tube was a rimless Pyrex tube of 15 x 125 mm. outside dimensions, into the bottom of which was ground, with the aid of medium coarse carborundum powder, one end of a 28 x 1 cm. Pyrex glass rod, thus providing good mortar-pestle, closely fitting, rather rough grinding surfaces. The technique for grinding, suspending and preparing diluted suspensions was in general that of Corper and Cohn (1936) using a 0.5 per cent solution of sodium taurocholate (Eastman, practical) in water for grinding the bacilli. The suspending fluid was 0.8 per cent NaCl in water, Long's medium or Long's medium with the magnesium sulphate, ferric ammonium citrate and glycerol omitted. Each of these solutions was satisfactory.

The grinding was done by hand. The first suspension was of 1.0 mgm./ml. It was thoroughly mixed and allowed to stand for five minutes, when 0.5 ml. was transferred from the middle of the suspension to 4.5 ml. of the fluid in the next decimal dilution tube. Decimal dilutions down to the desired limit were made.

A fresh 1.0 ml. pipette was used for each dilution and before taking up the suspended bacteria its interior was washed once with suspension fluid from a separate flask. Transferrence of possible surface bacterial films was avoided by wiping the end of the charged pipette with oil-and-fat-free linen. The suspension was then slowly discharged into the next dilution tube not allowing the pipette to touch the tube or its contents. Agitation of the new suspension was done by a fresh pipette before withdrawing the desired amount to be transferred to the next tube.

Pasteur pipettes, prepared and calibrated as described by Fildes (1931), were used for planting the bacilli. Three drops (0.1 ml.) of the bacillary suspension were slowly dropped from above the surface into the medium. The pipettes were first washed with suspension fluid to which no bacilli had been added and a fresh one was used for each decimal dilution. The estimated amounts of planted bacilli ranged from 10^{-1} mgm. in decimal steps to the smallest amount to be planted. The bacilli were shielded from direct light rays when suspended and planted.

After planting, aluminum foil of thickness, 0.00065 inches, was flamed over a Bunsen burner and folded over the top and neck of the flask. The aluminum foil cap was not sealed to the glass with paraffin or any other substance. Cleaned filter paper, sealed with paraffin, was used to cap the planted flasks in the early experiments.

The planted flasks were then placed in the incubator at 37.5°C.

V

Results

During the past fifteen months twenty-eight separate plantings were made using sodium taurocholate as the dispersing agent for the bacilli.   The plantings were mostly controls for other investigations under way.   Four of these plantings were made in 2 parallel rows of flasks, two in 3 parallel rows of flasks and one in 5 parallel rows.   The other twenty-one plantings were in single rows.

Plantings were made from decimal dilutions calculated to give 10^{-1}, 10^{-2}, etc. mgm. bacilli.

Of the four 2-parallel-row plantings, one resulted in growth to 10^{-8} mgm. bacilli in each row, two resulted in growth down to 10^{-7} mgm. in one row and 10^{-8} mgm. in the other, and the fourth planting resulted in growth in one row to 10^{-8} and in the other to 10^{-9} mgm.

TABLE 1

NUMBER OF TIMES SMALLEST AMOUNT OF BACILLI GREW	MGM. OF BACILLI PLANTED (ESTIMATED)										
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}	10^{-11}
1	+	+	+	+	+	−	−	−			
1	+	+	+	+	+	+	−	−			
17	+	+	+	+	+	+	+	−			
18	+	+	+	+*	+	+	+	+	−		
2	+	+	+	+	+	+	+	+	+	−	
1	+	+	+	+	+	+	+	+	+	+	−
40 Total											

* There was no growth in one flask.   No other break in continuity noted.
+ = growth.   − = no growth.

Of the two 3-parallel-row plantings, one resulted in growth in all 3 rows to 10^{-7} mgm. and the other had growth down to 10^{-7}, 10^{-7} and 10^{-8} mgm.

From the one 5-parallel-row planting, there was growth to 10^{-8} in 3 rows and 10^{-7} mgm. in 2 rows.

There were no discontinuities of growth in the above parallel rows accounted for, growth taking place in all flasks planted with amounts greater than the minimum amounts of bacilli from which growth developed.

In one of the 21 single row plantings there was no growth from the 10^{-4} mgm. planting when there was growth from all the others to and including the 10^{-8} mgm. planting in that row.

Altogether, there were 40 plantings (table 1).   These are non-selected, consecutive examples.

Figure 1, based on these figures for minimum amounts from which growth resulted, approximates a normal frequency distribution curve.

From the forty plantings, 97.5 per cent resulted in growth from amounts at least as small as 10^{-6} mgm. of the H37 strain, confirming the similar statement

made in my 1940 report.   Ninety-five per cent developed growth from amounts at least as small as 10^{-7} mgm. and it appears safe to state that growth may be expected with regularity from some amount between 10^{-7} and 10^{-8} mgm.   The surprising growth occuring in two plantings with 10^{-9} mgm. and in one planting with 10^{-10} mgm. was also reported in the previous paper.

The average times of first definite appearances of growth were as follows: 10^{-1} mgm. in 6 days; 10^{-2} in 9 days; 10^{-3} in 10 days; 10^{-4} in 14 days; 10^{-5} in 17 days; 10^{-6} in 20 days; 10^{-7} in 27 days and 10^{-8} in 28 days.   One of the 10^{-9} mgm. growths developed in less than 53 days and the other sometime between
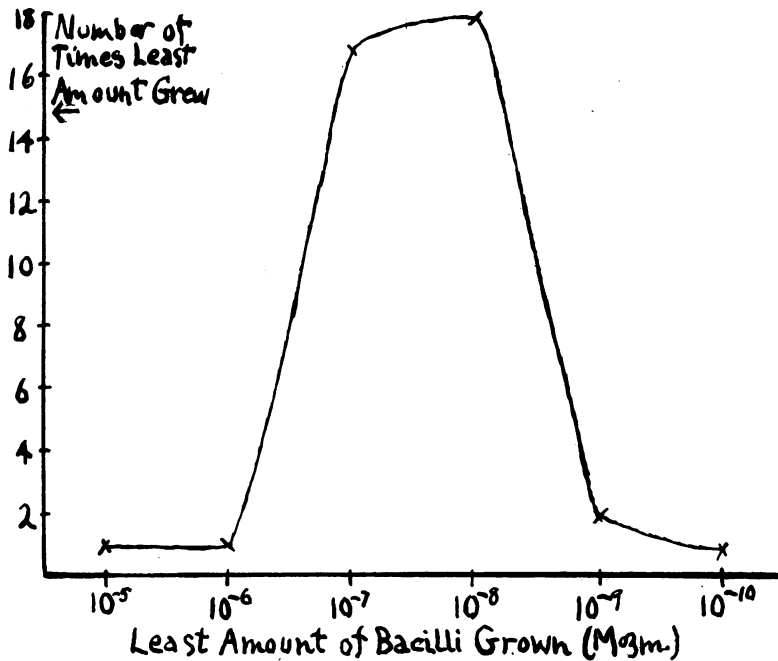

FIG. 1

62 and 76 days.   The 10^{-10} mgm. growth developed at some time less than 54 days.

An incubation time of 75 days should be sufficient to establish final results as to presence or absence of growth.

The growth always began at the bottom of the medium.   Later, growth extended to the surface of the medium from plantings of 10^{-5} mgm. or more of bacilli.

From smaller plantings, there was usually failure of surface growth to develop after the depth growth had been definitely established.   Transplanting a small amount of the depth growth when there was failure of surface growth, to fresh synthetic media resulted in renewed growth and later development of surface growth.

The mass of bacilli at the bottom of the medium when there was no surface growth did not exceed about 21.0 mgm. Generally it was less than this amount, when the growth had extended to the surface. When growth was established on the surface there appeared to be no further growth in the depth. The surface growth when most profuse was about 960 mgm. The bacilli at the bottom were acid-fast to the Ziehl-Neelsen stain.
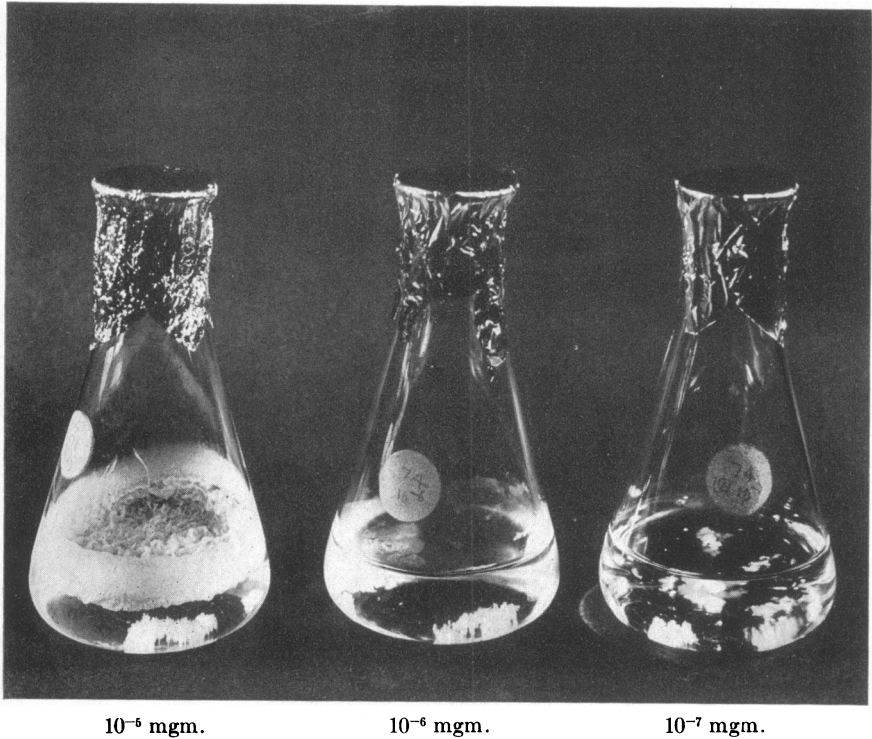

10⁻⁵ mgm.   10⁻⁶ mgm.   10⁻⁷ mgm.

FIG. 2. This is a single photograph of 3 flasks to help make more clear, growth from small numbers of tubercle bacilli in Long's liquid synthetic medium.
H37 was the strain used. The incubation period was 64 days.
The flasks contain growth from 10^{-5}, 10^{-6} and 10^{-7} mgm. bacilli. Growth was also present as a result of plantings with 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-8} mgm. bacilli (not photographed).
The 10^{-5} mgm. flask shows a profuse surface growth and part of the bottom growth.
The 10^{-6} mgm. flask shows a thin surface growth not quite covering the surface of the liquid and depth growth.
The 10^{-7} mgm. flask has no surface growth but does have a considerable amount of depth growth.

Growth from the heavier plantings is of flocculated form at the bottom of the media. With the smaller plantings there exist at first colonies or clumps which go on to produce a more diffused growth. Because of this, and more especially because of the fluidity of the medium, it now appears more accurate to record the positive findings as "growth" and not as "colonies."
Since sodium taurocholate labeled "practical," had been used as a dispersing agent in making the bacillary suspensions it was decided to use an especially purified sample of the dioctyl ester of sodium sulfosuccinic acid with the trade

name *Aerosol OT* (100 per cent),[1] (Caryl and Ericks, 1939) for the same purpose. Thus a dissimilar, more simple compound, was contrasted with the more complicated and less pure taurocholate. Five separate tests were made. In three tests the limit of growth was 10^{-7} mgm. bacilli and in two tests the limit was 10^{-8} mgm. All flasks with amounts greater than 10^{-7} mgm. and 10^{-8} mgm. respectively developed growth. The taurocholate and the dioctyl ester were equally effective as dispersing agents.

The effect upon growth of the bacilli when these two dispersing substances were added to the synthetic medium in decimal dilutions from 10^{-1} to 10^{-7} per cent was observed. In each flask was planted 10^{-3} mgm. (about one million) bacilli. Two separate tests were made on each. The taurocholate in the first test and the dioctyl ester in the other were used as dispersing agents for the bacilli. With each substance in both tests, there was no growth with concentrations of 10^{-1} and 10^{-2} per cent. These two dispersing agents, when added to the synthetic medium, had the same effect on the growth of 10^{-3} mgm. bacilli.

Since, however, growth of the relatively large amount of 10^{-3} mgm. bacilli was inhibited by some amount between 100 and 10 p.p.m. of both substances, it seemed possible that these dispersing agents exert an inhibiting effect upon small numbers of bacilli when they are used only as dispersing agents and that growth could be demonstrated from less than 10^{-7} to 10^{-8} mgm. (estimated) if dispersing agents with a lesser inhibiting growth power were used.

Long's synthetic medium of pH 8.0 as a dispersing agent for the bacilli was tried three times. Growth resulted down to 10^{-6} mgm. in one experiment, down to 10^{-7} in a second, and 10^{-7}, 10^{-8} and 10^{-9} in a third.

Gelatin as the dispersing agent in one test resulted in growth down to 10^{-8} mgm. bacilli.

An M/15 solution of Na_2HPO_4 as the dispersing agent resulted in growth down to 10^{-7} mgm. bacilli in two tests.

A diverse group of agents for dispersing the bacilli thus resulted in similar numbers of the bacilli producing growth and ruled out any specific property of the taurocholate in growth promotion.

The suspensions of 1.0 mgm./ml. for the experiments recorded above were allowed to stand for five minutes to permit clumps of bacilli to settle before transfers of bacilli were made for the next decimal dilution.

This then should actually result in smaller amounts of bacilli being transferred than those indicated by the recorded figures.

The suspending of the bacilli results only in an approximate approach to the ideal suspension of isolated bacilli and many clumps of two or more bacilli must be present.

The effect of not permitting the clumps to settle was now studied. The same technique as before, with sodium taurocholate as the dispersing agent was used, except that when the first 1.0 mgm./ml. suspension had been made and agitated,

[1] The especially purified sample of Aerosol OT, 100 per cent, was provided through the courtesy of Dr. G. B. Ayers of the American Cyanamid Company.

the required amount of this suspension was immediately transferred and decimal dilutions down to 10^{-9} prepared.

Plantings were now made in six parallel rows of flasks.

Four of the rows produced growth down to 10^{-8} mgm. and two down to 10^{-7} mgm. at the end of 75 days incubation. Apparently it did not make any difference whether or not the first suspension was allowed to settle for five minutes before the first dilution was made.

It appears then to be established that growth of the strain, H37, of human tubercle bacilli will result with regularity from an amount somewhere between 10^{-7} and 10^{-8} mgm. when planted in Long's synthetic medium.

Corper and Cohn (1933) estimated that 1 mgm. in moist culture contained about 10^9 (one billion) bacilli.

VI

To check the effect of unheated blood serum upon the inhibiting effect of cotton the following experiment was done.

Four rows of flasks were used. The first row contained synthetic medium only and the flasks were not in contact with cotton at any time. The second contained synthetic medium only and the flasks had non-absorbent cotton stoppers in the oven, autoclave and incubator. The third had 5 per cent serum added to the autoclaved synthetic medium and the flasks were not in contact with cotton at any time. The fourth had 5 per cent serum added to the autoclaved synthetic medium and the flasks had cotton stoppers in the oven, autoclave and incubator. Each row of flasks was planted with decimal dilutions of the tubercle bacilli with amounts from 10^{-1} to 10^{-6} mgm. After planting, the cotton stoppers were lightly impregnated with paraffin and the flasks without cotton were capped with aluminum foil and were not sealed with paraffin. The result was that the cotton-stoppered flasks with no serum had growth develop only from 10^{-1} and 10^{-2} mgm. bacilli and no growth from the 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} mgm. plantings. The other three rows of flasks all developed growth from 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} mgm. bacilli.

Since growth occurred also in the synthetic medium without the serum where there had been no exposure to cotton down to the smallest amount planted, it seems reasonable to assume a neutralization of the inhibitory effects due to the cotton as possible in addition to the growth promotion by the serum as postulated by Boissevain (1940).

VII

The technique now in effect makes use of either aluminum cylinders or foil for capping the flasks when autoclaving the culture media and aluminum foil for capping during incubation. Evaporation of water during the prolonged incubation period is not excessive. Any evaporation can be counter-balanced by preliminary addition of 5 per cent water to the medium. Cotton is not used at any time.

DISCUSSION

Long's synthetic medium, because of its simplicity, lends itself well to such studies with tubercle bacilli, especially since it is now established that amounts of the human strain, H37, as small as 10^{-7} to 10^{-8} mgm. will grow in it.

The H37 strain used in these investigations is well conditioned to laboratory growth and is used by many investigators in the field of tuberculosis research.

An index of its virulence was secured during this past year by making subcutaneous injections. In groups of four guinea pigs, 10^{-2}, 10^{-4} and 10^{-6} mgm. of the bacilli resulted in death from advanced tuberculosis with average life durations of 140, 166 and 177 days respectively.

It has generally been believed that less than about 1.0 or 0.1 mgm. tubercle bacilli would not grow in Long's medium if accessory factors were not added, that growth must necessarily take place on the surface and that if a transplant of bacilli sank beneath the surface, growth failed to occur. However, Kahn (1929) stated incidental to his study of the developmental cycle of the tubercle bacillus, "In this way a number of young vigorous forms develop in the shallow depths of the medium and there is less chance of isolating non-viable forms." He had incubated for 4 days, suspensions of H37 prepared by shaking a small amount of growth membrane with glass beads in 4 ml. of Long's medium. He also found it possible to grow a single tubercle bacillus or small groups of from 2 to 6 microorganisms in individual microdroplets of the same medium. It is now established that with fine suspensions, growth always begins at the bottom of Long's liquid medium.

The importance of "depth" growth in liquid media was stressed by Kirchner (1931) who added serum or plasma to a liquid synthetic medium in order to secure it.

Growth of the tubercle bacilli in the depth of the liquid medium is more comparable to that in animal tissues than is surface growth on the culture media. Constant immersion insures a more constant environment than does surface growth where bacilli are superimposed on their fellows and are exposed directly to the atmosphere above. This applies, of course, only before growth has extended to the surface of the fluid synthetic medium.

Other strains of tubercle bacilli, especially more recently isolated ones, may not grow from such small numbers in Long's medium as does the H37 strain. It is with such strains that accessory growth factors may be sought if such are needed by the bacilli. For example, strains that will grow on egg media or in serum from amounts as small as 10^{-6}, 10^{-7} or 10^{-8} mgm. and grow in Long's medium only when much larger amounts are planted undoubtedly require the addition of some other substance or substances to the medium.

New strains to be established from infected tissues or sputa require complex media such as egg or serum. Investigations as to the necessary additional factor or factors that must be added to Long's medium to establish growth there also, may be very profitable.

Mueller (1937) demonstrated the importance of small amounts of pimelic acid

and nicotinic acid and Mueller and Cohen (1937) of B-alanine as growth-promoting factors for the diphtheria bacillus.   Wadsworth and Wheeler (1934) reported that they obtained growth from thirteen out of twenty recently isolated virulent strains of the diphtheria bacillus in a synthetic medium which did not contain β-alanine and nicotinic acid.

This same condition may prevail for tubercle bacilli, some strains requiring added accessory growth factors and other strains not.

In order, however, to secure growth from small numbers of tubercle bacilli in Long's medium it is necessary to insure the absence of adsorbed, organic growth inhibiting films from the glassware.   Especially inhibiting were the distillates from the bleached non-absorbent cotton stoppers in the flasks.   H. Braun (1939) described technique for insuring clean glassware and stated that the cleaned dry flasks are stoppered "mit fettfreier Watte."   Fat-free cotton is probably satisfactory as a stopper, but instead of cotton, aluminum cappings for the flasks were used in this work when the harmful effects of cotton were to be avoided.   It was demonstrated, however, that blood serum added to the synthetic medium permitted growth of the bacilli when the flasks were stoppered with the cotton.   In estimating possible growth promotion, it is necessary to consider the probable neutralization by the added substance of inhibiting substances accidentally present in the culture media.   Added blood serum can combine with a poison such as copper to neutralize the growth-inhibiting effect of the latter when the inoculum consists of small numbers of various pathogens (O'Meara and Macsween, 1936, 1937).   It was also stated by Gordon and McLeod (1926) that serum protects bacteria against concentrations of amino-acids which would otherwise inhibit their growth.   Similarly, the growth promotion of small numbers of tubercle bacilli by serum added to Long's medium in the flasks with the cotton stoppers may be at least partly due to neutralization of inhibitory substances.

There is one other kind of investigation that should be of value using this technique of cultivating tubercle bacilli.   The bacteriostatic or lethal effects of known substances added to the liquid synthetic medium can be studied.   For example, sodium oleate inhibited the growth of tubercle bacilli when present in the amount of 1 p.p.m. according to a personal communication by Dr. C. H. Boissevain. This also gives an idea of the extreme importance of small amounts of inhibitory substances that may readily be acquired by the glassware incidental to incomplete cleaning as well as to the distillates from the cotton stoppers.

A speculation is now perhaps permissible.   What effect, if any, has relatively unclean glassware upon the virulence of tubercle bacilli, when small numbers of them are injected in animals?

Because practically all bacterial diseases result from infection with relatively small numbers of organisms, it would appear that *in vitro* studies of relatively small numbers of bacilli are of importance, especially when the amount of growth factors carried over from the parent culture, either within the cells or from the parent culture medium is much diminished.

Amounts of tubercle bacilli, strain H37, as small as 10^{-7} and 10^{-8} mgm. grow in Long's medium and it appears reasonable to expect growth in exceptional

cases from amounts as small as 10^{-9} and 10^{-10} mgm. dilutions as recorded both in this paper and the previous one by Drea (1940). This is because of the inherent errors due to sampling when small numbers of organisms are involved. See Berg (1941).

Corper and Cohn (1933) consistently obtained growth on good non-synthetic media from diluted suspensions of recently isolated virulent avian tubercle bacilli, after plantings containing calculated amounts of 10^{-9} mgm. Since this was before they introduced sodium taurocholate as a dispersing agent, human cultures could not be so finely dispersed, though they did state that occasionally a final dilution suspension was obtained containing 10^{-9} mgm. per ml. The taurocholate and other wetting agents make possible finer dispersions of the human cultures than those produced when they are not used. It appears reasonable, therefore, to suppose that 10^{-9} mgm. per ml. suspensions of the human strains can now be prepared more often.

Let it be assumed that there are 10^{9} bacilli in 1 mgm. of the culture and that we have actually prepared a suspension of 10^{-9} mgm. per ml. or what amounts to the same thing, 100 ml. of suspension fluid containing 100 bacilli (1 bacillus per ml.). There is, of course, a random scattering of the bacilli in the suspension fluid resulting in a non-uniform distribution of the organisms. In such discontinuous distributions Poisson's series of probabilities is of the first importance. The probability chance of a 1 ml. sample of this suspension containing bacilli is as follows: 0 bacilli, 37 per cent; 1 bacillus, 37 per cent; 2 bacilli, 18 per cent; 3 bacilli, 6 per cent; and 4 bacilli, 2 per cent. These figures are based on table 1 of Berg (1941).

If now to the above 10^{-9} mgm. per ml. suspension are added 900 ml. of fluid, the 1000 ml. of the resulting 10^{-10} mgm. per ml. suspension will have on an *average* 0.1 bacillus per ml. The same kind of statistical treatment shows that there is a probability chance of 90 per cent that a 1 ml. sample of this suspension will contain no bacilli; a 9 per cent chance that a similar sample will contain 1 bacillus; and a 0.5 per cent chance that it will contain 2 bacilli.

Thus, even if it is further assumed that each bacillus is viable and capable of producing growth, it can be readily understood there must be irregularities of growth when very small numbers of bacilli are planted. There will be failure of growth after some plantings with 1 ml. samples from the 10^{-9} mgm. per ml. suspension and occasionally there will be growth from similar amounts of the 10^{-10} mgm. per ml. suspension.

Since the last paper was published, it appears to be more accurate to record the growth of the bacilli as "growth" and not as numbers of "colonies" even for the smaller amounts of bacilli planted. Because of the fluidity of the medium the organisms may have their positions changed, either to unite in larger clumps or to disperse into smaller ones.

It was also found preferable not to use the slightly modified form of Long's medium previously employed, which was however a synthetic medium. In the investigation now reported, Long's medium was used with only sufficient Na_2CO_3 to give the desired pH value. The reasons for the change from the modified me-

dium previously used are (1) the better buffer action of Long's medium and (2) its much more general use by other investigators.

No investigations were made of substances that may be contributed by the glass itself.

From this work it appears that one well-known strain of tubercle bacilli will grow in Long's synthetic medium from amounts at least as small as 10^{-7} mgm. whereas previously it was thought that amounts greater than about 10^{-2} mgm. were necessary before growth resulted. The irregularities of growth reported in the first paper for amounts of bacilli from 10^{-6} to 10^{-10} mgm. but from which no conclusions were drawn, are now accounted for by the presence of previously unrecognized growth-inhibiting substances adsorbed by the glassware as well as by the errors necessarily associated with random samples where small numbers of bacilli are planted.

SUMMARY

1. Growth of the H37 strain of human tubercle bacilli in Long's liquid synthetic medium can develop when amounts at least as small as 10^{-7} to 10^{-8} mgm. are planted.

2. Very small amounts of contaminating organic substances, hitherto unsuspected, are readily adsorbed by the glassware, and may act as growth inhibitors. The smallest amounts of these bacilli that will grow when such inhibitors are present varies from 10^{-2} to 10^{-6} mgm.

3. A soap, sodium oleate, will inhibit the growth of tubercle bacilli when present in something greater than 0.1 p.p.m. and less than 1.0 p.p.m. Soaps may be a part of the contaminating adsorbed films.

4. Especially inhibiting to growth are the distillates from bleached, non-absorbent cotton stoppers in the glassware.

5. The elimination of inhibitory substances from a liquid synthetic medium will provide a valuable means of investigating: (1) accessory growth factors and (2) bacteriostatic and bactericidal properties of deliberately added agents in *in vitro* studies of tubercle bacilli.

6. The elimination of inhibitory substances from culture media is of importance in the cultural studies of other microorganisms and it is probable that organic films including the distillates from cotton stoppers adsorbed on the glassware are inhibitory to the growth of small numbers of these organisms especially in synthetic media which otherwise permit growth. This may also be of importance in virulence studies where small numbers of microorganisms are used.

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