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THE FATE OF THE BACILLI IN INCUBATED LEPROMATOUS TISSUES AND THE QUESTION OF MICROSCOPIC GROWTH

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In the hope of finding some situation in which it might be possible to demonstrate *in vitro* multiplication of the leprosy bacillus, a general survey has been made of the potentialities of a wide range of media, using material from various kinds of clinical cases. This material included: excised lepromatous nodules, tissue scrapings from tuberculoid leproids, tissue scrapings from lesions of both tuberculoid and lepromatous cases in a state of lepra reaction, pus from lepra reaction blisters, bloods from reaction and nonreaction lepromatous cases, and a few miscellaneous tissues obtained at autopsy. Although several types of micro-organisms reported by earlier workers have been obtained, none was found to be characteristically associated with any given type of leprous lesion or condition or to be consistently cultivable from material containing an abundance of leprosy bacilli. Since the findings with lepromatous nodules were representative of those from other materials, only that part of the work has been published (1).

The article referred to recorded a study of the bacilli from emulsified lepromata inoculated into media composed of fresh blood, serum, egg yolk, chick embryo juice, and extracts of leprous organs, all of which had been supplemented with various substances known to promote the growth of acid-fast bacteria, other fastidious micro-organisms, or tissue cells. The inoculum used ensured that all of the components of the nodules were included in each medium. Quantitative microscopic counts failed to demonstrate multiplication of the bacilli under any of the conditions studied.

In connection with this work, interest was aroused by the many claims that the bacilli grow microscopically on media inoculated with small fragments of leprous tissues. Temporary or limited growth within incubated pieces of tissue has been affirmed so frequently that careful students of the literature have been led to

emphasize its actual or probable occurrence. For example, among recent reviewers Klingmüller (2) explained the growth in certain circumstances as due to accompanying leprous tissue. Jeanselme (3) stated, in italics:

> The multiplication of the bacillus at the expense of a fragment of human leprous tissue has been demonstrated many times. But, to this day, it has not been possible to obtain a culture capable of being transferred from tube to tube.

Topley and Wilson (4) subscribed tentatively to the findings of Duval (5), Duval and Holt (6), and Schlossman (7, 8), who have published the most convincing reports concerning the growth of leprosy bacilli in tissue fragments.

Judging from the diverse circumstances under which this type of growth has been described, it may be concluded that the alleged multiplication does not depend on the presence of living cells, the gas environment, the temperature of incubation, or the type of medium on which the tissues are incubated. The sole factor that has been universally present is the lepromatous tissue. Such tissues are frequently so full of bacilli that almost one-third of the area in stained sections may appear to be occupied by bacillary masses. If we assume that the bacilli constitute 20 per cent of a richly-loaded leproma—Unna (9) went so far as to say that they may be one-half to three-fourths of the mass—it is apparent that a ten-fold increase in their original numbers would approximately double the size of the tissue used, and thus would certainly result in definitely visible growth. Incubated tissues do not, however, increase in size. Hence the earlier reports of multiplication in incubated tissues would indicate, on the basis of our assumption, a total increase of the general order of only two to five times the original number of bacilli. This is not a very significant degree of multiplication, and it is not capable of experimental proof by methods hitherto employed.

The present paper reports quantitative estimation of the bacilli in incubated lepromatous tissues. In view of the variables and difficulties which will be pointed out, it is impossible to speak of estimating the numbers of bacilli within the limits of a specified error. It was possible, however, to develop methods whereby a two-fold increase should be detected with certainty if it were to occur consistently in any given medium or throughout the course of any single experiment.

METHODS

Nodules were obtained and tested for sterility as described in the preceding paper, and cut into cubes approxmiately 2×2×2 mm. in size. For control counts, 24 or more tissue cubes were ground

in a Pyrex glass mill (10) until completely suspended, and then diluted to a concentration of two tissue cubes per cubic centimeter. At first the control suspensions were spread on slides immediately, and not counted until the incubated cultures were sampled. It was found better, however, to store the control suspensions in the presence of 0.25 per cent formaldehyde and count them when the cultures were sampled. If subjected to slight grinding just before being used, these controls were satisfactory.

The tissue cubes for incubation were placed in groups of four on the surfaces of a series of agar slants, and in groups of two to twelve in tubes or flasks of the various liquid media previously employed.[1] The cultures on solid media were incubated both in air and in an atmosphere containing 10 per cent carbon dioxide and 40 per cent oxygen; those in liquid media were incubated under added carbon dioxide and (with the exception of one experiment) 40 per cent oxygen.

After the chosen periods of incubation, four or more cubes from each medium were ground until suspended homogeneously in fifteenth molar Na_2HPO_4 containing 0.5 per cent pancreatin and diluted in the same way as the controls; i.e., so that each cubic centimeter of the suspension represented two tissue cubes. These suspensions were digested at 37°C. until they became homogeneous, which required from one to four hours.

Drops of the suspensions were then transferred to clean glass slides with a standard 2 mm. loop and allowed to spread over an area 6 mm. in diameter. Ordinarily, six films were prepared from each sample. After drying, each film was covered carefully with one loopful of 0.5 per cent gelatin in distilled water containing 0.5 per cent phenol. (The gelatin film must not extend beyond the border of the original smear, lest some of the bacilli be carried outside of the area to be counted.) After the gelatin had dried, the slides were fixed for five minutes in rubber stoppered Coplin jars containing a few drops of formalin; before staining, the formalin vapor was allowed to diffuse out of the films for a few minutes. The slides were stained in carbol-fuchsin at room temperature for five minutes, and counterstained in 1:5 Gabbett's stain for the same length of time.

In counting, five fields were selected on both the vertical and the horizontal diameters of each of four films, thus totalling ten fields per film or forty fields for each sample. Since the bacilli com-

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[1] The preceding paper contains details concerning diluting fluids, control of pH, and the preparation of media and gas mixtures.

monly occur in clumps which prevent exact counting, the masses of differing sizes were classified and recorded as follows:

A: 1- 5 bacilli, regarded as averaging 2 bacilli per group;

B: 6-25 bacilli, regarded as averaging 15 bacilli each;

C: 26-75 bacilli, regarded as averaging 40 bacilli each.

Exceptionally large clumps were scored as two or more *C* groups. The total numbers of the *A*, *B*, and *C* groups in each film were multiplied by the corresponding factors, and the counts in four or more films were then averaged in order to express the results as the *estimated average number of bacilli per 10 fields* (see Table 4).

TECHNICAL ASPECTS OF SAMPLING AND COUNTING

Among the sources of error peculiar to estimation of the bacillary content of leprous tissues, the following are important and will be discussed seriatim.

1. Marked and unpredictable irregularities in the distribution of the bacilli in certain nodules.

2. Rapid spontaneous flocculation of suspensions prepared from incubated tissues.

3. Loss of bacilli from the slides during staining.

4. Occurrence of the bacilli in clumps so large that the individual bacilli cannot be counted.

5. Fading or loss of staining capacity of bacilli from control slides during storage.

(1) Irregularity of distribution in the nodule is the only source of error which was not satisfactorily minimized by the procedures used. Homogenization of the entire nodule—the ideal method—is of course impossible in work with tissue slices. Small groups of cubes represent the general situation only when the bacillary distribution is fairly uniform; otherwise, the counts from small portions of the nodule are likely to be untrue of other parts. This difficulty was frequently not overcome by using from four to six cubes per sample (see experiments 5 and 6, Table 7). Errors of interpretation, however, were avoided by a study of sections of each nodule, which in several instances indicated that the results obtained with an ordinary number of cubes were open to question.

In more recent work, care was taken to ensure that pieces of tissue cut from adjacent parts of the nodule should not be placed in the same medium but would be spread equitably among all of the cultures. For simply testing the question of growth in incubated leprous tissues, one could use one-half of the total number of cubes for a control suspension and incubate the remainder under chosen conditions, the cubes being distributed at random between the two samples. This was not practicable in these experiments, however, because of the desire to test a number of media.

(2) The fact that suspensions made from incubated tissues produce floccules which collect and conceal the bacilli, is an important source of trouble. Digestion is necessary to produce satisfactory suspensions; pancreatin is superior to pepsin for this purpose.

(3) Loss of bacilli during staining is greater with smears of digested

suspension than with ordinary smears. Special methods of fixing the bacilli to the slide, always important for quantitative counting of acid-fast bacteria (11), are indispensable. Table 1 shows the counts obtained when digested suspensions were placed in parallel rows on slides and formalinized with a gelatin coating on one row, and without this protection on the other. The average counts in the unprotected films were only 59 per cent as high as in the coated ones.

TABLE 1.—*Control of loss of bacilli from slides by means of a gelatin coating hardened by formaldehyde.*

With coating		Without coating	
Film No.	Bacilli estimated	Film No.	Bacilli estimated
1	806	1a	97 [a]
2	486	2a	252
3	471	3a	470
4	514	4a	492
5	249	5a	30 [a]
6	491	6a	444
TOTAL	3,027		1,785
Retained in the absence of fixative coating			59 per cent

[a] More completely digested than other preparations; only 12 per cent of the bacilli were retained.

The method of protecting the films is important, especially, since the losses are apparently from the uppermost layers of the preparations. Placing a protective substance—gelatin, serum, or albumin—on the slide beneath the smears is unsatisfactory. Mixing such fixatives with the suspension improves retention of the bacilli, but is inferior to the coating method. With the bacilli beneath the protective coating, the losses are minimal (Table 2).

TABLE 2.—*Comparing retention af bacilli on slides by methods of staining and of protecting smears with serum*

Method of decolorizing and counter-staining	Method of applying protective substance		Totals by staining methods
	Serum 1:10 mixed with equal volume of suspensions [a]	Fixation coat serum 1:25 on dried smears [b]	
Usual: acid decolorization, rinse, counterstain, rinse	542	616	1,158
Gabbett's solution 1:5, 5 minutes	712	964	1,676
Totals for each filming method	1,254	1,580	

[a] The figures in this column are the actual counts multiplied by two, to correct for the dilution.

[b] An additional series of 13 slides with the serum coating and 13 with gelatin coating showed no choice between the two filming agents.

Even with fixatives so used, the slides lose some bacilli because of the soaking and swelling of the films during the complicated process of staining. Rinsing should be done gently, in a series of dishes. Losses can be reduced further by allowing the preparations to dry before counterstaining in the usual technique or, preferably, by using Gabbett's simultaneous decolorizer and counterstain[2] to shorten and simplify the procedure.

The results shown in Table 2 indicate that, irrespective of the staining procedure, the fixative retained more bacilli when applied as a protective coating than when mixed with the suspension. It also shows that, with either fixation method, the Gabbett's counterstain produced higher counts than the usual procedure. The latter, when applied to films in which the fixative was mixed with the suspension, permitted finding only 56 per cent as many bacilli as by the method recommended.

(4) The occurrence of clumps of bacilli in which it is impossible to count the individual organisms made it necessary to classify the masses into only the three categories that have been mentioned. The *A* (1-5) and *B* (6-25) groups are easily distinguished. In order to permit differentiation between the *B* and *C* (26-75) clumps, the individual bacilli in medium sized aggregates were repeatedly counted as accurately as possible. With continual checking at the 25-bacillus level, it was possible to rely on the judgment of the scorer for rapid classification of the masses. The 75-bacillus limit of the *C* groups corresponded to the size of three 25-bacillus groups. The average values for each class were assigned only after considerable data had been accumulated. It was found that the same suspensions, after standing for months, could be recounted with essential agreement in the estimates.

(5) Fading or loss of staining capacity of bacilli in control slides was responsible for defects in the data of several long-term experiments, before it was realized that the numbers of recognizable bacilli decreased continuously in films stored at room temperature whether unstained, stained, or covered with cedar oil (Table 3). Hygroscopic materials present in the films cause them, if unstained, to appear translucent within a short time at room temperature. This change doubtless produces a solution of the concentrated salts and, in stained slides, also permits ionization of residual traces of acid. If stained films are to be preserved for future study, rinsing in one-hundred and fiftieth molar Na_2HPO_4 after decolorization with acid is an essential precaution. Either stained or unstained films should be stored at 37°C, or in a desiccator (Table 3).

To obtain quantitive results, it is also important to place tissues of equal size in each group from which suspensions are made, to fill the measuring loop uniformly, and to make extra films on the slides so that only those having the standard diameter need be counted. Accuracy is also dependent on observing enough fields to ensure adequate sampling of the films and to permit counting enough bacilli to make the estimate reliable; within certain limits these factors are reciprocals of each other. Both present and past experience (6) indicate that to count 40 fields containing a total of 200 to 400 bacteria or clumps permits one to make fair estimates.

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[2] This solution works equally well in the original strength or in a 1:5 dilution. Although ideal for counting acid-fast bacteria, neither strength counterstains sufficiently to be reliable for the detection of nonacid-fast bacteria.

TABLE 3.—*Examples of fading or loss of staining capacity of bacilli stored on glass slides.*

First count	Subsequent counts	Storage		Previous treatment
		Temperature	Time	
115	66	Room	1 week	Stained (cedar oil)[a]
	33	"	2 weeks	Stained (cedar oil)
287	115	"	3 months	Stained (cedar oil)
	86	"	5 months	Stained (cedar oil)
303	22	"	5 months	Stained (cedar oil)
	85	"	5 months	Stained (no oil)
410	72	"	2 weeks	Not stained
137	83	"	4 months	Not stained
146	136	37°C.	4 months	Not stained
287	206	37°C.	5 months	Not stained

[a] The slides which were stained and covered with cedar oil were simply recounted after storage. In all other cases the counts made after storage were compared with earlier counts of other slides of the same series.

The counting may be limited to fewer fields (20 or 30) if 500 bacteria have been found in them, with similar results from each film (Table 4). On the other hand, if only 50 or 60 bacteria are found in 40 fields, counting should be continued until at least 100 to 200 bacilli or clumps have been seen.

EXPERIMENTAL RESULTS

Estimates of the bacillary content of incubated leprous tissues were made in *(a)* four experiments in which bacteriological media were employed, and in *(b)* two experiments which involved both bacteriological and tissue-culture solutions. Each experiment was made as comprehensive as possible in order to simplify the problem of control counts and to permit ready comparisons between the different media and conditions tested.

EXPERIMENTS WITH BACTERIOLOGICAL MEDIA

Tissues were placed on a hormone glycerol agar in all six experiments, in five of which the influence of 10 per cent carbon dioxide and 40 per cent oxygen (12) was tested. Results from one such experiment have been given in Table 4. The 1,815 bacilli found after incubation in air and the 1,522 counted from tissues maintained in the gas mixture are values essentially identical with the control count of 1,583 bacilli. Other results obtained with this combination of medium and gas mixture are shown in Table 5 and 7. These, likewise, fail to indicate multiplication of the bacilli.

The results of another bacteriological experiment are shown in detail in Table 5 to illustrate the fact that neither the gas mixture nor the media influenced the number of bacilli found after incuba-

TABLE 4.—*Illustrating: (a) the method of scoring and calculation used in all experiments; (b) the accuracy of estimates based on two and on four tissue cubes; (c) the similarity of counts after incubation in air and in 10 per cent carbon dioxide and 40 per cent oxygen.*

Sample [a]	Clumps of each class seen in each 10 fields, and totals				Total bacilli	Average bacilli per 10 fields based on		Remarks
	A	B	C	Total		2 cubes	4 cubes [b]	
Tube I, tissues 1+2	551	35	42	628	3,307			Culture on liver-infusion hormone glycerine agar, pH 7.2, incubated for two months in presence of air.
	589	44	21	654	2,678			
	438	39	20	497	2,261			
	521	43	20	584	2,487			
	2099	161	103	2363				
	×2	×15	×40					
	4198	2415	4120		10,733	2,683		
Tube I, tissues 3+4	161	25	5	191	897		1,815	
	123	24	4	151	766			
	240	28	14	282	1,460			
	95	13	7	115	665			
	619	90	30	739				
	×2	×15	×40					
	1238	1350	1200		3,788	947		
Tube II, tissues 1+4	120	29	29	178	1,835			Culture on the same medium, incubated for two months in presence of 10% CO_2 and 40% O_2, maintained at pH 7.2 by means of $NaHCO_3$
	239	10	52	301	2,708			
	114	29	21	164	1,503			
	139	7	19	165	1,143			
	612	75	121	808				
	×2	×15	×40					
	1224	1125	4840		7,189	1,797		
Tube II, tissues 2+3	256	27	9	292	1,277		1,522	
	330	24	27	381	2,100			
	117	9	4	130	529			
	205	18	10	233	1,080			
	908	78	50	1036				
	×2	×15	×40					
	1816	1170	2000		4,986	1,247		
Control count (based on 84 cubes):							1,583	

[a] The four tissues on each agar slant were arranged and numbered thus: $\frac{1|2}{3|4}$; the numbers in this column show how tissues from each tube were paired for sampling.

[b] Average for each tube if all four tissue cubes are taken as a single sample.

tion for three or six months. The average counts of 349, 354, 316, 315, and 330 in the several parts of the experiment did not differ significantly from the control count of 287. In view of the data presented in Table 3, and of the demonstration that the bacilli were disappearing from the control slides, this control count must have been too low.

TABLE 5.—*Number of bacilli recovered from tissue cubes incubated under several conditions for three and six months.*

Medium and atmosphere	Bacilli estimated		Average count for each medium
	3 months	6 months	
Liver-infusion hormone glycerol agar; air	300	398	349
Same, plus gas mixture[a]	459	249	354
Serum, serum digest, Long's medium and 929pH; [b] plus gas mixture	316	—	(316)
Serum digest, Long's medium and 929pH; gas mixture	216	414	315
Peptic globulin digest, Long's medium, 929pH and yeast extract; gas mixture.	289	370	330
Average counts at 3 and 6 months:	316	358	
General average count:	337		
Counts in control slides stained and stored:	287	115	

[a] Gas mixture: 10 per cent CO_2 and 40 per cent O.

[b] See preceding paper for details concerning these media.

The two remaining bacteriological experiments confirmed the foregoing results with the solid medium and also provided tests on nine liquid media other than those shown in Table 5. One or another medium sometimes gave higher counts than the others, but any apparent superiority was always contradicted by low counts in other samples or in the other experiment.

Since the conditions tested did not influence the counts, nothing would be gained by presenting in detail the results from each part of each of these bacteriological experiments. The question is simply whether or not the bacilli grow on nutrient materials furnished by the nodules. Consequently, the results from all media at each sampling period have been pooled and averaged, and the results of the four bacteriological experiments (arranged chronologically) are summarized in Table 6.

The data of the first experiment (Table 5) require no further comment. In the second experiment the average counts of 336 at three months, 402 at six months, and 356 at eight months show that there was no multiplication between the third and eighth months. These counts are but slightly higher than the control count of 303, which is believed to be too low.

TABLE 6.—*Summarizing the average number of bacilli in four experiments with tissue cubes in bacteriological media for periods up to eight months.*

Experiment No.	Bacilli estimated [a] after incubation for						Average for each experiment	Control counts
	1 mo.	2 mos.	3 mos.	4 mos.	8 mos.	6 mos.		
1			316(18)			358(12)	337(30)	287 [b]
2			336(24)		356(8)	402(20)	365(52)	303 [b]
3		1,668(8)					(1,668)(8)	1,583 [b]
4	42(16)		103(16)				73(32)	146

[a] Figures in parentheses indicate the total number of tissue fragments on which each estimate was based. The counts shown are averages obtained by pooling the results from several media.

[b] These control counts from stored slides are probably too low (see Table 3).

Since in these two experiments the only suggestion of increase of the bacilli was due to differences between the faulty control counts and the experimental counts at three months, shorter incubation periods were used in the next two experiments. The data of the third experiment have been presented in Table 4 and discussed. In the fourth experiment the formalinized control counted 146, while the average of the experimental cubes was 42 at one month and 103 at three months. However, as shown by examination of histological sections, and also by counts as high as 389 in groups of tissue stored in formaldehyde and in phenol for other purposes, the low counts in the incubated tissues were probably not due to actual disappearance of bacilli, but to their irregular distribution in the nodule.

EXPERIMENTS WITH TISSUE CULTURE SOLUTIONS

Since it was not possible to obtain evidence of growth in the preceding experiments, in the next two the liquid media were renewed periodically so that any essential but quantitatively deficient components might be made available in larger amounts. Tissue-culture solutions were known to be suitable for the growth of acid-fast bacteria and—if changed more frequently—for the cells of the leproma; hence they appeared to be of interest. In these experiments the cubes on hormone glycerol agar were not transplanted from tube to tube but were employed as controls.

In experiment 5 (Table 7) the solution containing Long's medium was renewed each month for five months, while the monocyte and fibroblast media were changed every two weeks for a period of four months (six renewals). The high count of 2,305 bacilli in the monocyte medium at two months was contradicted by counts of 154 at four months and 802 at six months. Similar sharp fluctuations occurred also in the bacteriological medium, in which the numbers of bacilli in previous experiments had been stable. Since the average counts obtained in each medium did not

TABLE 7.—*Influence of tissue culture solutions and periodic renewals of media.*

Experiment No.	Medium	Bacilli estimated a after			Average for each medium	Remarks
		2 mos.	4 mos.	6 mos.		
5	Liver-infusion hormone-glycerol agar ..	953(6)	—	968(9)	960(15)	Medium not changed
	Fresh serum 10%, peptic fibrin digest, Long's medium	995(6)	62(4)	497(6)	518(16)	Renewed each month for 5 mos.
	Fresh serum 50%, proteose N 0.01% (monocyte medium)	2,305(6)	154(4)	802(8)	1,087(18)	Renewed every 2 weeks for 4 mos.
	Fresh serum 10%, proteose N 0.10%, embryo extract 15% (fibroblast medium)..	1,005(6)	1,337(4)	—	1,171(10)	Renewed every 2 weeks for 4 mos.
6	Average for each sampling period	1,315(24)	518(2)	756(23)		
	Liver-infusion hormone-glycerol agar ..	47(4)	—	302(4)	175(8)	Medium not changed
	Monocyte medium as above	301(4)	—	99(6)	200(10)	Renewed every 3 weeks for 4 mos.
	Fibroblast medium as above	73(6)	—	246(6)	160(12)	Renewed every 3 weeks for 4 mos.
	Average for each sampling period	140(14)		216(16)		

a The figures in parentheses indicate the total number of tissue fragments on which each estimate was based. The counts shown are averages and, therefore, are not proportional to the number of tissues used.

differ significantly from the general average, it appears probable that the high and low counts were due to the markedly irregular distribution of the bacilli which was observed in histological sections of this nodule.

In experiment 6 (Table 7) the tissue-culture solutions were renewed every three weeks during the first four months (five renewals). Due to irregular distribution of the bacilli in this tissue also, the two counts for each medium at the different sampling periods were inconsistent, but the average counts for the three media were similar: 175, 200, and 160 for the agar, monocyte, and fibroblast media, respectively.

Comparison of these two experiments indicates that in the fifth one the counts decreased from 1,315 at two months to 756 at six months while in the sixth they rose from 140 to 216. In view of the irregular distribution of the bacilli in the tissues, no special significance can be assigned to the differing trends of the two experiments. In spite of renewal of the culture solutions, there was no

evidence that the media were capable of inducing multiplication of the bacilli.[3]

DISCUSSION

Since the enumeration of leprosy bacilli both in liquid media inoculated with completely homogenized nodules (1) and in incubated tissue cubes have not revealed multiplication, the results of these two studies appear to invalidate the widespread impression that this micro-organism grows in the first inoculations of nodule suspensions or in pieces of leprous nodules, because of special substances derived from the tissue. On the other hand, it has been seen how the hypothesis of "microscopic growth" is readily suggested by use of the direct smear method. It is desirable to indicate why the impressions so readily gained by this method are incorrect.

In fresh nodules the bacilli are for the most part encased in tissue cells, and they are liberated less readily than from the disintegrated cells and softened tissues of incubated cultures. Consequently, ordinary *smears* from the latter must be expected actually to contain more bacilli. Furthermore, the cells and proteins of fresh tissue preparations (scrapings or suspension) are readily fixed to slides and furnish a conspicuous background. Autolysis, on the other hand, by decreasing the amount of tissue debris and making the proteins more soluble, often results in smears in which as much as 90 per cent of the material appears to be bacilli, thus increasing the illusion that they have grown.

Certain of the procedures which have been said to promote multiplication of the bacilli in tissue fragments definitely increase this deception. For example, Duval (5) and Duval and Holt (6) have stressed the favorable effect of bacterial or tryptic digestion of the nodule. In the present work, tissue cubes contaminated with extraneous bacteria sometimes showed in direct smears what appeared to be phenomenal growth, but actually the number of acid-fast bacteria had not increased. The advantage ascribed by Duval to the digestion of nodule proteins must be attributed to the liberation of the bacilli rather than to their multiplication.

In Schlossman's work (7, 8), tissues were incubated in various media for as long as six months before growth seemed to occur. The control tissues were suspended in phenol solutions, and the

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[3] In the monocyte medium the two pieces of tissue in each tube fused into a single, small round mass during the first few weeks of incubation. From recent observations it appears that this change may be ascribed to growth of the tissue cells. It is probable that viable cells persisted for more than one month, in spite of the infrequent changes of the medium.

results were judged by smearing the fluids surrounding the tissues, not by actual sampling of the tissues themselves. Several factors play a role in producing illusory results in such experiments. The liberation of large numbers of bacilli into culture fluids requires more complete autolysis than is necessary for the recovery of corresponding numbers by rubbing or crushing. Phenolized control tissues do not undergo autolysis and cannot release their bacilli. Under certain circumstances the phenol may even destroy some of the bacilli in the controls.[4]

It has been stated (see Schlossman, 8) that small colonies of leprosy bacilli appear at the margins of leprous tissues which are incubated on solid media. It is true that, as drying occurs at the margins of the tissues, small particles which suggest colonies often appear. However, if they are carefully fished out and stained without crushing or smearing, they can be seen to be minute fragments of the original tissue cuttings. Schlossman observed the association of tissue fragments with these "growths," but he felt that the bacilli were multiplying there because these small tissue particles furnished nutrients for the bacilli. A better explanation is that the tissue fragments themselves are the "colonies" and thus account for the presence of the bacilli.

When small bits of infected tissue from tuberculous animals are placed in liquid media, growth of the bacilli causes the tissue to become studded with minute colonies. On several occasions during the present study, incubated leprous tissues assumed a similar appearance. Examination of stained fragments, however, always revealed that these apparent colonies were actually small collections of lipoid material which became visible only after autolysis and clearing of the tissues.

In connection with this question of pseudocolonies, it should be remembered that the usual conditions for the incubation of long-term cultures for acid-fast bacteria in certain types of media may promote the formation of pseudocolonies composed of spherocrystals of lipoid substances. Brown, Swift, and Watson (13), who

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[4] On one occasion the writer homogenized a large volume of sputum and divided it into two portions, one preserved with 0.5 per cent phenol and the other with 0.25 per cent formaldehyde. After refrigeration for six months the bacilli in the formalinized suspension were present in approximately their original numbers, but only a small percentage of those treated with phenol could be detected. In the present work, however, phenolized leprous suspensions and tissues were sampled over periods of six months in two experiments without demonstrating destruction of the bacilli. It is not known whether the discrepancy between the results with these two micro-organisms was due to differences inherent in the bacteria or to the presence in certain phenols of extraneous substances which may influence the lytic action.

recently confirmed and extended Laidlaw's observation on this subject (14), state:

> After inoculating a serum agar plate with minced tissue or exudate, the pseudocolonies usually developed more numerously along the inoculated steaks, a phenomenon which might easily mislead the observer into thinking that they had been derived from the inoculum. Moreover, simply disturbing the surface of the medium by rubbing with a spud, or by "inoculating" it with sterile salt solution or broth, sometimes similarly caused the pseudocolonies to appear and "grow" most vigorously along the "inoculated" areas. . . .

In bacteriological studies on leprosy, lipoids suitable for pseudocolony formation may be contributed by substances from the media, such as egg yolk, blood serum, tissue extracts, unfiltered meat juices as in hormone broths, and also by the suspensions or fragments of lepromata themselves. Because of the well-known affinity of acid-fast bacteria for lipoids and lipoid solvents (15, 16), the bacilli of the inoculum should be found to be concentrated in these lipoid aggregates in greater numbers than elsewhere. Hence, apparent colonies rich in bacilli may be formed in the complete absence of actual multiplication.

If one considers the potential delusion afforded by increased numbers of bacilli in smears from autolyzed tissue, the decreased background of blue-staining material, and the existence of visible pseudocolonies due to tissue fragments or arising from the inoculum or the medium, it is not surprising that difficulty has been experienced in judging the presence or absence of growth of the bacilli by the ordinary qualitative methods.

The judgment of many workers has doubtless been influenced in part by the very attractive hypothesis that slight growth was to be expected because of special nutrient substances assumed to be present in the nodules. This idea has been most frequently expressed in words which imply great virtue in human leproma-nodule proteins. Such implications have no basis in terms of a chemical specificity of enzyme action or of bacterial nutrition.

The hypothesis of slight growth of leprosy bacilli in first inoculations on a medium, or in tissue cubes, also seems contrary to what is known concerning the physiology of bacterial growth. When bacteria are freshly transplanted to a new environment, the culture experiences a variable period of stationary or declining viability. This "lag" phase is crucial in the life of the culture, and it persists until the micro-organisms establish suitable control over the hydrogen ion concentration, oxidation-reduction potential, carbon dioxide pressure, and other factors in the vicinity of viable bacterial cells. During this period the original micro-organisms of the inoculum

must be able to synthesize, or to find in the medium, growth accessory substances required for respiration and for the formation of new protoplasm. This process, in its entirety, can be accomplished only by successful utilization of nutrient materials provided by the medium. In short, the critical problem for bacteria is to begin to grow. By the time the number of bacteria in a culture increases even slightly, they have mastered their environment and thenceforth proceed to multiply with logarithmic rapidity. Bacterial growth may be compared with the physical phenomena of inertia and momentum; it may start slowly under certain circumstances, but once it has started it is not readily stopped under the conditions which provided the acceleration.

These considerations suggest that whenever suitable conditions for the multiplication of leprosy bacilli outside of the host tissues may be provided, their growth is not likely to require quantitative microscopic methods such as those discussed here for its demonstration. In the meantime, however, such methods offer the only means of ascertaining with certainty whether growth has or has not occurred. The futility of observing ordinary smears for this purpose is amply illustrated by the results of this study.

SUMMARY AND CONCLUSIONS

The impression of earlier workers that leprosy bacilli seem to grow *in vitro* in small bits of lepromatous tissue have been confirmed by use of the direct smear method. However, when a method was developed for complete disintegration and suspension of the tissues and for actually estimating the numbers of bacilli recoverable from the tissues, it became evident that growth did not occur.

The conditions which were studied included incubation of tissues on a standard solid medium, in liquid media particularly well adapted to the growth of small numbers of other acid-fast bacteria, and in tissue-culture solutions suitable for the cultivation of fibroblasts or of blood monocytes. They also included incubation in the presence of added carbon dioxide and oxygen.

Several procedures which have been thought to promote growth have been found simply to increase the erroneous impressions which result from nonquantitative microscopic methods. Other sources of error are pointed out. It is concluded that the supposed microscopic growth of leprosy bacilli must be attributed to reliance on methods which are incapable of dealing with an unusual combination of misleading circumstances.

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