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BEHAVIOR OF LEPROSY BACILLI IN COMPLEX LIQUID MEDIA WITH HIGHLY AVAILABLE SOURCES OF NUTRIENT AND ACCESSORY SUBSTANCES

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The central problem in the bacteriology of leprosy lies in the simple fact that, though the lesions of lepromatous cases are full of acid-fast microorganisms, the majority of attempts by careful workers to cultivate the leprosy bacillus from such tissues have led to completely negative results. Unlike the situation with respect to almost any other problem in medicine, all claims of positive results in this field have failed of adequate confirmation. Various kinds of bacteria have been cultivated, but none has been proved to be the causative organism of the disease. The result is that, in spite of a tremendous amount of work over a long period of years, promising islands of progress are today recorded on many charts as shoals.

It is well to recognize that there are two different methods of approach to the problem of cultivating this organism. If one uses only a few media in experiments involving an extensive series of nodules, he exhibits confidence that those media are adequate for the purpose, and his procedure implies that the main problem is to find lesions in which the bacilli are alive. On the other hand, if one believes there is necessity for studying a variety of physiological conditions which might make it possible to cultivate the bacilli from each lesion, he subscribes automatically to the premise that a certain portion of the bacilli

in each nodule are alive. Having tested a given medium with material rich in bacilli from several cases of leprosy, he must accept a negative result as indicating once and for all that that medium is inadequate.

The experiments to be reported here were directed along this second line of approach. Because of the uncertainty that surrounds the entire problem, much of the effort has consisted of exploratory work designed to find some one situation in which the bacilli could be shown with certainty to multiply. The preparation of the media used was based on the general principle that highly pathogenic or parasitic bacteria exhibit various deficiencies in their individual abilities to synthesize enzymes and other strategic materials required for the production of new cells. In this connection the reader is referred especially to Knight's Bacterial Nutrition (17) and a review by Koser and Saunders (18).

It was proposed, first, to increase the assortment of potential nutrients in the media until all apparent likelihood of success along purely bacteriological lines had been exhausted. It was proposed, next, to study the fate of leprosy bacilli in tissue-culture solutions in which human blood monocytes and the fibroblasts from leprous nodules are capable of growing. It was hoped that, if the bacilli failed to multiply in any medium devised, it might be possible at least to answer the question: "Do leprosy bacilli grow in solutions which nurture their host cells, or must we anticipate the necessity of trying to propagate appropriate tissue cells as intermediaries between the bacilli and the medium?"

Blood and serum were used as physiological solutions which approximate, more closely than any other readily obtainable substances, the nutritional background to which a strictly parasitic microorganism might be habituated. Since the leprosy bacilli might not be able to utilize the blood and serum in their native state, these substances were supplemented with a variety of combinations of digestion or extraction products of blood, egg yolk, tissues, acid-fast bacteria and yeast; also with peptones, Long's synthetic medium, and other simple sources of nitrogen and carbon. Tissue extracts, sterile egg yolk suspensions, and chick embryo juice were also used as basic solutions, and these were supplemented in similar ways.

Consistent production of positive growths from the smallest

numbers of bacteria is the crucial test of the quality of a medium. The tradition that acid-fast bacteria must be cultivated on a solid medium or by floating pellicles on liquids is a timehonored but erroneous notion. Any convenient combination of blood and Long's medium-used as prepared or, better, diluted -is greatly superior to a first-rank egg-yolk-glycerol medium for the detection of viable acid-fast bacteria in the blood (8, 14). Furthermore, liquid media composed, for example, of 10 percent serum and 10 percent Long's medium equal or excel this standard solid medium for obtaining positive growths from minimal numbers of human, bovine, and avian tubercle bacilli, or of the saprophytic timothy bacillus (9). Drea (5) found that standardized suspensions of the H-37 strain of human tubercle bacillus required only one ten-thousandth as much inoculum to produce growth in liquid Long's medium as on the same medium containing agar. The fact that liquid media are adapted to the initiation of growth from small numbers of acid-fast bacteria has been regarded as of the greatest importance in the present experiments. It permitted not only the rapid and easy combining of sterile components of the media, but also the removal of aliquot portions of the culture from time to time in order to follow the fate of the bacilli by quantitative methods, which have been so badly needed in this field of bacteriology.

The present communication outlines new types of media which have been tested for the cultivation of leprosy bacilli. Although the question of growth of the bacilli in tissue slices placed in such media has also been studied, this paper will refer mainly to the results obtained by the inoculation of finely ground suspensions.

TECHNIQUE

Subcutaneous nodules that were supposed to have existed for at most three to six months were obtained from lepromatous cases in which the disease was believed to be advancing. None of the cases was known to be tuberculous. Those with ulcers were avoided so far as possible, especially if there were ulcers on the same limb or distal to the site of otherwise suitable nodules.

Preparation and seeding of suspensions.—The skin was disinfected by scrubbing and iodine treatment. Frequently, after removal of the iodine the site of operation was covered for two minutes with a patch of cotton fabric about 4 cm. square which had been soaked in half-strength tincture of iodine; this permitted prolonged action of the disinfectant without the possibility of its drying out and losing its effectiveness. One set of instruments was used to incise the skin and another to effect the removal of the nodule itself.

The nodule was dropped into a large, short test tube containing 10 cc. of gelatinized salt solution. This solution was drawn off and renewed once, to wash the tissue thoroughly. The sterility of its surface was tested by transferring 1 cc. of these salt solutions to a tube of freshly boiled "sterility test" medium.¹ The nodule was then trimmed of excess tissue and cut up in sterile Petri dishes to produce small cubes of approximately 2 mm. dimensions. The juices and tissue fragments adhering to the scissors and forceps were rinsed off into sterility test medium to test for secondary infection within the nodule or contamination during the manipulations.

The first few suspensions for inoculating the media were made by grinding the tissue cubes with a pestle in a covered sedimentation jar containing a small amount of salt solution and sand. The fluid obtained was diluted with balanced salt solution (⁶), filtered through sterile Whatman filter paper No. 5, and adjusted to a standard turbidity. Later, the tissue pieces were disintegrated in a Pyrex glass grinding device (¹³) and diluted so that 12 pieces of tissue were suspended completely in 1 cc. of the salt solution.

At least 0.2 cc. of each final suspension was placed in the sterility test medium, and the same amount was spread on tubes of glycerol agar and of egg medium, 0.05 cc. being used per tube. For cultivation, the latter amount (0.05 cc.) was transferred to each of a series of 10×75 mm. tubes, and immediately afterward the various experimental media were added, 0.25 cc. per tube.

Control on original bacterial content.—Adequate controls on the numbers of acid-fast bacilli originally present in the suspensions were difficult to maintain. Stained smears from freshly inoculated tubes were found to fade and deteriorate during the long periods of incubation of the cultures. Heat-fixed, unstained smears, when stored in ordinary slide boxes, acquired moisture from the air and the numbers of stainable bacilli decreased. Fairly satisfactory results were obtained by storing standardized smears in the incubator at 37°C. and staining new slides from time to time as needed. Dilution of the original suspension 1:6—the same concentration as the cultures—in the presence of 0.25 percent formaldehyde, and storage in tightly stoppered tubes, provided the best controls. The numbers of bacilli did not decline, and an abundance of material was available to be sampled in the same way as the experimental materials.

Observation of the cultures.—The inoculated tubes were observed at approximately weekly intervals for one month, at 'The first of the "routine" media to be described shortly.

monthly intervals for the next five months, and then every two months for periods which often exceeded one year of incubation. Appropriate particles from all tubes that suggested the development of positive growths were stained by the Ziehl-Neelsen method, and transfers were made to slants of glycerol agar and egg medium. At several times during the incubation period of each experiment, tubes representing each medium were shaken thoroughly and smears were prepared on standard areas for comparison with controls which had been stored at the outset of the experiment. In five experiments, actual counts of the bacilli were made after grinding the contents of tubes to obtain thorough homogenization. Details of the smearing and counting methods will be given in a subsequent paper.

MEDIA EMPLOYED

The media used in this work may be divided into two classes: (1) those which were used routinely in each experiment, as controls for the detection of readily cultivated microorganisms, and (2) those which were designed as experimental media for the cultivation of the leprosy bacillus.

MEDIA USED ROUTINELY

The media used routinely, for control purposes, were four: (a) the sterility test medium, a meat infusion, neo-peptone² broth containing 0.1 percent of agar, as recommended especially for the cultivation of delicate microorganisms and for the detection of contamination in biological products (11); (b) a hormone liver-infusion agar containing 1 percent of glycerol, different peptones being employed from time to time; (c) an egg-yolk-water-glycerol medium (4) at pH 6.2 with, however, only 1 percent of glycerol; and (d) a similar egg-yolk medium in which an equal volume of egg white was added to the yolk in order to adjust the pH to 7.2.

COMPONENTS OF EXPERIMENTAL MEDIA

The individual components of the experimental media may be classified as (a) undenatured natural products, and (b) simpler supplementary materials. These components are outlined below, and the methods of their preparation are indicated briefly.

Undenatured natural products.—Blood and serum from lepers and nonlepers, both children and adults; chick-embryo juice; sterile egg yolk; and fresh extracts of leprous organs.

²A product of the Difco Laboratories, Inc., Detroit, Michigan.

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Heparinized blood was usually employed after centrifugation; the serum and the cellular portion were tested separately in concentrations of 10 percent by volume. Serum was most commonly used as a base for the incorporation of more available sources of nutrient.

Tissue extract of 11-day chick embryos was used in concentrations corresponding to 10 to 20 percent of minced chick tissue.

Sterile egg yolk was obtained by aspiration with a syringe, and diluted to concentrations of 10 to 20 percent by volume. These suspensions were used both at the original pH, approximately 6.2, and after being adjusted to pH 7.2. Media containing egg-yolk were usually clarified by centrifugation.

Fresh extracts of leprous organs—liver, spleen and testes—were made by grinding the tissues in a food chopper and diluting in salt solution to a 25 percent concentration. They were sterilized by filtration.

Supplementary nutrients.—These may be classified roughly as follows: (1) autoclaved and alkaline digests of blood serum; pancreatic or peptic digests of blood, serum globulin, fibrin and egg yolk; proteoses and peptones; and autolysates and pancreatic digest of leprous organs. (2) Supplementary materials from suspensions and hydrolysates of acid-fast microorganisms. (3) The synthetic medium of Long and Seibert; glucose, glycerol, and the ammonium salts of several organic acids.

1. Two digests were prepared from serum: (a) Autoclaved serum was secured by diluting serum 1:5 in distilled water and autoclaving; it was used in final concentrations corresponding to 10 percent of serum by volume. (b) An alkaline digest of serum (serum digest) was prepared by heating serum at 100° C. for 20 minutes in the presence of 0.5N NaOH, followed by neutralization, filtration through paper, replacement of the traces of cystine which are destroyed by heating with alkali, and autoclaving.

Pancreatic digests of blood and of egg yolk were made by continuing the digestion until drops of the material, on being placed in boiling acidified water, showed that maximal digestion had occurred. They were used in various concentrations.

A series of peptic digests useful in the cultivation of fibroblasts or monocytes was prepared by the methods of Baker and Carrel (see Parker, ²⁰) from human serum, globulin fraction of leper's serum, and fibrin of cow's blood.

Two types of products were made from the suspensions of leprous liver, spleen and testes mentioned: organ autolysates and pancreatic digests of organ autolysates. These tissues were first allowed to autolyze at 37° C. in the presence of toluol for one month. (a) A part of the supernatant fluid was then removed from each, boiled to remove the toluol and the remaining coagulable proteins, adjusted to neutrality and autoclaved. These autolysates were used in final concentrations of 10 to 20 percent, based on the amount of tissue used. (b) At this time pancreatin was added to the remaining portions of the autolysates to promote further di-

gestion. After boiling and filtering, the total nitrogen content of each digest was determined by a micro-Kjeldahl procedure. The solutions were autoclaved and incorporated in various media to contribute 0.01 percent or 0.1 percent nitrogen.

2. Supplementary materials from acid-fast bacteria were prepared by direct heating, by treatment with acid and alkali, and by refluxing suspensions in both aqueous and alcoholic solutions in the presence of acid and of alkali. The products obtained were combined in several ways and used in final concentrations corresponding to 0.2 percent dry weight. Further details will be given below. A concentrated alcoholic extract of yeast³ was used in varying concentrations.

3. The synthetic medium of Long and Seibert (19) was ordinarily used in final concentrations of 10 percent. Glucose, glycerol, and the ammonium salts of acetic, citric, oxalic and tartaric acids were used in concentrations of from 0.2 to 1.0 percent.

General features of the media.-Since it will not be feasible to give the formulae for all of the media tested, certain features should be mentioned at this point as applying in general to their preparation or use. The use of a balanced salt solution as diluent has been mentioned. Isotonic sodium carbonate (1.4 percent) was used almost exclusively in adjusting the pH of the experimental media. These media were stored or incubated with paraffined-cork stoppers, maintenance of the correct pH depending on the presence of CO₂, universally required for the respiration of bacteria. All media except those of egg base contained 0.001 percent phenol red, so that except in the presence of blood the pH could be judged at all times by inspection. In general, a slow acid drift was observed. This was corrected periodically by allowing CO₂ to diffuse from the tubes or, when necessary, by the addition of a drop or two of M/15Na₂HPO₄. Except for purposes of specific inquiry into the effect of hydrogen ion concentration, the media were adjusted to pH 7.2 to 7.4, and an attempt was made to keep them within the range of pH 6.8 to 7.6.

COMBINATIONS USED IN THE EXPERIMENTAL MEDIA

It is believed useful to outline the reasons why certain types of media were regarded as of interest, and to indicate in the tables that follow some of the nutrient combinations which were tested. If it is borne in mind that each of the 109 combinations indicated was inoculated with suspensions of nodules from at least two lepromatous cases and, in some instances, also with

³Kindly supplied by Dr. E. P. Casman, of the Abington Memorial Hospital, Abington, Penna.

material from tuberculoid or lepra-reaction cases, detailed descriptions of individual experiments may be foregone.

Two series of combinations in which serum and blood cells (series A) and chick embryo juice and fresh extracts of leprous tissue (series B) were employed as basic constituents are shown in Table 1. The excellence of blood or serum in combination with Long's medium for the cultivation of minimal inoculations of acid-fast bacteria has been mentioned. Chick-embryo juice contains proteins and other materials readily available to fibroblasts (3), which are unable to grow satisfactorily on the proteins of serum itself. It was thought that the use of fresh extracts from leprous organs might make it possible to include similar materials from man, the natural host of the leprosy bacillus.

TABLE 1.—Blood serum, blood cells, embryo juice and fresh organ extracts employed as media, supplemented with serum derivatives and simple compounds.

Series A	Series B
Fresh blood serum and cells	Embryo and organ extracts
with Long's medium and	with Long's medium and
supplementary nutrients	supplementary substances
Serum & Long's medium	Embryo juice, Long's & autoclaved serum
Serum, Long's & serum digest ^a	Embryo juice, Long's & serum digest
Long's & serum digest (without serum)	Embryo juice, Long's & serum digest
Serum, Long's & embryo juice	with glucose, glycerol, NH4 salts
Serum & Long's with liver, testicle and	of acetic, citric, lactic, oxalic, and
lymphnode autolysates (each separately)	tartaric acids (each separately)
Cells & Long's medium Cells, Long's & serum digest Cells, Long's & embryo juice Cells, Long's & autoclaved serum Long's & autoclaved serum	Fresh organ extracts: Liver, Long's & serum digest Testis, Long's & serum digest Lymph node, Long's & serum digest

^a This medium was used more commonly than any other as a general base-line in many experiments.

These complex natural substances were supplemented with digestion products and simple compounds. It was known that autoclaving in distilled water improves greatly the availability of serum nitrogen for M. phlei (15). Alkaline digests of serum (cystine replaced) satisfy the exacting growth requirements of diphtheria and certain diphtheroid bacilli (28), and offer a means of making the serum nitrogen more available than by autoclaving alone. Since autolysis produces end-products differing from those in fresh extracts or those obtainable through the action of commercial enzymes, tissue autolysates were also tested in the presence of serum and Long's medium.

The supplementary solution most frequently used was Long's

medium, because it furnishes the ammonium ion, apparently universally utilized by cultivable acid-fast bacteria, and also a useful mixture of carbon compounds and inorganic salts. Glucose and the ammonium salts of certain organic acids were employed in view of the fact that some investigators believe that glycerol interferes with the growth of dysgonic strains of bovine tubercle bacilli. If this view is correct, the favorable effect of glycerol on the leprosy bacillus can hardly be taken for granted. It should be noted that the added carbon sources could not be expected to make growth possible in an otherwise inadequate medium, but that if growth occurred to a limited degree in any basic medium it might be modified or enhanced by the presence of such substances.

Egg yolk has always been a useful source of nutrient material for the cultivation of acid-fast bacteria. Because of observations which suggested the occurrence of microscopic growth of leprosy bacilli in tissue slices on solid media containing egg or blood, pancreatic digests of egg yolk and whole blood were prepared in the hope of increasing the suitability of such materials. Table 2 shows media representative of those tested in this connection. In all of the media in series C, sterile egg yolk was tested with Long's medium and also when supplemented with digests of egg yolk, blood, or serum. The media of series D included both serum components and tissue extracts which were enriched with the egg yolk and blood digests.

TABLE	2.—Media	composed	oj	egg-yolk	suspensi	ons, s	erum	derivatives,	and	tissue
	extracts co	mbined w	ith	digests of	egg yolk,	whole	blood	and serum	. a	

Series C	Series D					
Sterile egg-yolk suspensions with Long's	Other basic constituents with					
medium, with and without digests	Long's medium and digests					
Yolk (pH 6.2) & Long's medium	Fresh serum, Long's medium & PED					
Yolk (pH 7.2) & Long's medium	Autoclaved serum, & Long's medium with					
Yolk (pH 7.2) & Long's medium with PED;	PED; PBD					
PBD; SD	Liver extract, Long's medium & PED					
Heated yolk supernatant. (pH 7.2) & Long's	Testis extract, Long's medium & PBD					
medium with PED; PBD	Lymph-node extract, Long's medium & PED					

a/ Symbols: PED = pancreatic egg-yolk digest; PBD = pancreatic whole blood digest; SD = serum digested with alkali. Each was tested individually with the basic media indicated.

Heat-killed suspensions of acid-fast bacteria supply growth factors required by Johne's bacillus and were first used in attempts to cultivate the leprosy bacillus by Twort (27). In

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this study, special interest was attached to testing the effects of acid-fast bacteria which had not been subjected to heat, and also of products of such bacteria which had been partially hydrolyzed.

A dense suspension of a culture (No. 929) which had been freshly isolated from a nodule was divided into two portions. One portion was treated with N/1 HCl and the other with N/1 NaOH for 48 hours. No growths could be obtained from either suspension after three hours, and the cells in both became nonacid-fast. Since acid treatment destroys certain constituents which are not affected by alkali, and vice versa, the two preparations were combined in a single suspension (designated 929pH) in order to overcome the deficiencies inevitable in either preparation alone. A part of this suspension (929pHY) was enriched with alcoholic yeast extract, an excellent source of growth accessory substances for bacteria.

Similar suspensions of tubercle bacilli were boiled under reflux condensers in: 0.5N HCl in water, 0.5N HCl in 95 percent alcohol, 0.5N NaOH in water and 0.5N NaOH in alcohol, thus producing four preparations. In order to include fractions in different stages of hydrolysis, onehalf of each preparation was removed after 30 minutes of heating and the remainder was heated for a total of two hours; these two portions were recombined. Since the fractions of the bacilli which are acted upon, and the type of hydrolysis that occurs, differ in acid and alkaline digestion, the aqueous HCl and NaOH hydrolysates were tested in combination, as well as separately. The alcoholic derivatives were handled in the same way.

From Anderson's extensive studies of the highly purified fractions of acid-fast bacteria, it is known that partial hydrolysis of the proteins, fats, waxes, phosphatides and polysaccharides can be accomplished by these methods.

The combinations of these preparations from acid-fast bacteria with basic media are indicated in Table 3. This table includes only two basic media which had been employed before. The new media, tested separately as well as with the products of acid-fast bacilli, were characterized by the presence of peptic digests of serum, serum globulin, and fibrin. Baker and Carrel have shown that peptic digests rich in proteoses are superior to peptones, tryptic digests, or amino acids for the enrichment of media in which fibroblasts (1, 3) or monocytes (2) are to be cultivated. Witte's peptone, which has a high content of proteoses, is widely used in tissue cultivation and was also of interest because it had been employed by Soule and McKinley (26) in their studies on the cultivation of the leprosy bacillus.

Analysis of the nutrient complexity of some of the media included in these tables shows that many of the media contained all of the nutrient materials or accessory substances known

to be required by fastidious microorganisms, including newly isolated strains of tubercle and Johne's bacilli. Moreover, these strategic compounds were often contributed in differing degrees of availability from multiple sources. It therefore became impossible to justify the preparation of further combinations along the present line of approach.

TABLE 3.-Media composed of leukemic serum, peptic digests, and yeast extract in various combinations with products obtained from acid-fast bacteria.

Basic media	Products from acid-fast bacteria										
Containing Long's medium		Culture			Tubercle bacilli refluxed with ^b						
(tested individually and in	No. 929 ^a			Aqueous			Alcoholic				
combination with each item indicated at the right)	н	pH	pHY	HC1	HCl/ NaOH	NaOH	HCI	HCl/ NaOH	NaOH		
Fresh serum & Witte's peptone	+	+	+	+	+	+	+	+	+		
Serum digest	+	+	+	+	+	+	+	+	+		
Peptic globulin digest	+	+	+	+	+	+	+	+	+		
Peptic serum digest	-	+		+	+	+	+	+	+		
Peptic fibrin digest	_	+	-	+	+	+	+	+	+		
Serum (leukemic)	+	+	+			-	_	-			
Serum (leukemic) & SD ^e	_	+	+	-	-			-			
Serum (leukemic) & PSD	_	+	+	_	-	-	-	-	-		
Serum (leukemic) & PFD	-	+	+	_	-	-		-			
Serum (leukemic) & PDLA	_	+	+	-	-	-	-	-			

^a Product H = autoclaved suspension of this microorganism; pH = acid- and alkali-treated fractions combined; pHY = the same with yeast extract.^b The product indicated as HCl/NaOH is a combination of portions refluxed with acid and with alkali. Each fraction (i.e., HCl and NaOH) had been heated for two different time-intervals and then combined to give a spectrum of the hydrolytic products. ducts ^c SD = serum digest; PSD = peptic serum digest; PFD = peptic fibrin digest; PDLA = pancreatic digest of liver autolysate.

Media of much simpler composition (see Table 4) than the majority of those mentioned above, but shown to be capable of supporting the growth of fibroblasts from lepromata or of blood monocytes, were also tested with nodule suspensions. In addition, the supernatant fluids which were removed periodically from tissue cultures of lepromata and which contained leprosy bacilli were retubed and incubated further in order to take advantage of any end products of cellular metabolism which might assist in the proliferation of the bacilli. Such tubes, though not inoculated with the standard suspensions, furnished tests of the growth of leprosy bacilli in tissue culture solutions containing leproma extract and cell metabolites.

ORGANISMS CULTIVATED

Seventeen nodules were obtained for these experiments. Three showed evidence of surface contamination. The results from two

TABLE 4.—Media found to support the growth of human blood monocytes or of fibroblasts from human lepromata.

Medium for	Fresh	Embryo	Protein digests		
growth of	serum ^a	juice	Total N	Sourcesb	
Fibroblasts	25%	20%	0.10%	W; PFD; PDLA	
Monocytes	50%		0.01%	W; PFD; PDLA	

^a Serum sources: young and adult lepers, young and adult normals, fresh placentas.

b Symbols: W = Witte's peptone; PFD = peptic fibrin digest; PDLA = pancreatic digest of liver autolysate. Each of these was used individually, in conjunction with other items in the same line.

of these were disregarded. The set of tubes first inoculated from the third nodule was discarded, but extra portions of this nodule which still remained were treated with sulphuric acid on the second day and a new suspension was prepared. Thus, 15 nodules were actually useful for the present purpose. The numbers of cultures of various kinds that were recovered are shown in Table 5. Since detailed descriptions of these cultures would not contribute to a solution of the fundamental problem, it will suffice merely to comment on the circumstances under which they were found.

Nodule	Number							
	of - tubes	Acid	Acid-fast		heroid	1		Ulceration
No.	inoe-	Pigment		Pigment		Strepto-	Miscel-	
	ulated -	+	1 -	+	-		Iditeout	
1	75		_	_	_	-	2	Presenta
2	61		-	-		-	_	Above biopsy b
3	37		-		_	-	3	Hands
4	99	1	1	6		1	2	Hands, etc.
5	81		_	-	7		-	Legs, nose
6	52		-		5	-	1	Hands, feet
7	143			-		-	3	None
8	131		-	-	_	-	1	None
9	142		-	-	-	-	5	None
10	124	_	-	-	-	-	3	None
11	56			_	-	-	-	None
12	139	+c		-	_		2	None
13	31	_	_	_	_	-	-	None
14	40	_	_	-				None
15	38	-	-	-	-	-	-	None

TABLE 5.—Cultures obtained from lepromata suspensions.

^a Distribution of ulcers in this case was not recorded.

^b Distribution of ulcers in this case was not recorded. ^b This nodule was contaminated with fungi and treated with acids and alkali before use; the three miscellaneous organisms were fungi.

^c A pigmented acid-fast culture was isolated from tissue cubes of this nodule.

It is apparent that miscellaneous cocci, gram-negative bacilli, spore-bearing bacilli, yeasts, fungi, etc., were encountered

more frequently than the micro-organisms to which special importance has been attached by workers in this field. The incidence of these miscellaneous organisms paralleled roughly the number of the tubes involved in each experiment, and it is impossible to say what proportion of them actually came from the nodules or arose through contamination during the work.

A streptothrix was isolated only once, from a nodule (No. 4) on the arm of a patient with ulcers on the hands. This nodule was a veritable botanical garden and contained all of the microorganisms which have been featured prominently as the causative agents of leprosy.

Two of the three cultures of acid-fast microorganisms cultivated were also obtained from tubes inoculated with material from this nodule. They were readily distinguished from each other by their morphology, pigmentation and other growth characteristics.

One of these cultures (No. 536), nonpigmented, appeared in sterile egg yolk with 10 percent Long's medium (pH 6.2) after five days of incubation. The uninoculated portion of this medium (5 cc., sufficient to make 20 experimental tubes) was distributed into medium-sized tubes, without leproma suspensions, and incubated in a horizontal position for months; no growth of any kind appeared. Although this culture produces colonies of a rough or "worm-cast" type, and will not grow homogeneously in liquids or form stable suspensions without the use of special methods, it is not a tubercle bacillus. From the outset it produced isolated colonies on egg or agar slants in 48 hours, grew rapidly on ordinary media at different temperatures below 45° C., and was acid-fast on plain agar.

The other acid-fast culture (No. 929) obtained from this case, an orange-red chromogen, was first observed after incubation for eleven days in serum digest and 10 percent Long's medium. When streaked directly on liver-infusion-glycerol agar and on egg slants for the isolation of colonies, growth on the agar was more abundant within five days than on the egg within twelve days. The colonies were smooth and moist. The culture made homogeneous suspensions in liquids and developed a low proportion of rough variants only after considerable subcultivation. It grows on a variety of media at different temperatures below 45° C. On plain agar it is less acid-fast than No. 536. Both of these cultures are killed by incubation at 45° C. for three weeks in the presence of adequate moisture.

Although the third acid-fast culture isolated (No. 1077, from nodule No. 12), grew from tissue cubes rather than the suspension, it should be mentioned here since it was the only other culture of this type obtained from this series of nodules.

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After ten days of incubation in peptic serum digest and 10 percent Long's medium, a homogeneous growth of nonacid-fast bacteria was observed. Subcultures were made and the pieces of tissue were treated with sulphuric acid, neutralized, and set up again in a new tube with new medium. The first subcultures showed only one type of colony—pink, smooth and moist. Such colonies were restreaked several times in series and checked by Ziehl-Neelsen staining. In the second subcultures the growth on glycerol agar at 96 hours was nonacid-fast, and that on egg slants was recorded as containing about 30 percent of acid-fast cells. During the course of the first two weeks of subculturing the acid-fast property of the culture became fully developed, and has been retained. The original tissue cubes again gave growth after treatment with acid, the new growth slowly becoming acid-fast in the liquid medium. This culture resembles No. 929 more closely than No. 536, but it is identical with neither.

Diphtheroid bacilli were isolated from three nodules, in a total of 18 tubes. In all instances they occurred at random, without regard to the media employed. The six cultures from nodule No. 4 showed variations in the shade of pigmentation from yellow to red, but were similar in morphology and growth characteristics. The seven nonpigmented cultures from nodule No. 5 showed a series of colony types and morphological forms ranging from small cocco-diphtheroid cells to large "mandolin" Repeated plating cells with swollen ends, or branching filaments. of these different colony types on several media showed that they were all capable of producing transitional as well as the extreme cell forms, and it was concluded that the cultures were variants of each other. The five cultures from nodule No. 6 resembled each other closely and possessed the interesting property of dividing longitudinally, each original cell producing two daughter cells which were often incompletely separated and appeared like mirror images of each other. In these diphtheroid cultures the larger, pleomorphic cells had a tendency to retain tinges of the carbol-fuchsin stain, but could not properly be called acid-fast.

Special interest attaches to the presence of ulcers in the cases from which these diphtheroid organisms were cultivated. A total of six cases had such lesions. In one instance (No. 2) there were only a few small ones on the arm, above the site of the nodule used. In another instance (No. 3) the tissue was treated with acids and alkali before use. Of the four other cases, with moderate degrees of ulceration or with strategically located ulcers, three yielded cultures of diphtheroids.

MICROSCOPIC FINDINGS

Since so much emphasis has been placed on the occurrence of microscopic growth of leprosy bacilli, special attention was given to this matter. The method employed in making the original suspensions caused the emulsification of all the nutrient materials of the nodule tissue, and dilution of these suspensions did not decrease the amount of potential nutrient available to each bacillus present. Thus, except for possible differences between mechanically disrupted and autolyzed tissues, all of the cell products or leproma extractives which may occur in incubated tissue cubes must be considered as present in all of the media tested. The use of liquid media made it possible to sample the cultures in such a way as to obtain reliable information on changes in bacterial numbers.

The general subject of microscopic growth will be discussed in a paper to follow, and it is only necessary to mention here the results obtained. Both promising and doubtful results were observed before satisfactory data were finally obtained. Early in the first experiment, in the absence of microscopic controls or standardized methods of preparing smears, there was considerable optimism. After ten days of incubation it was recorded that the bacilli had apparently multiplied, and that certain media appeared to be more favorable than others. The ten-day set of slides was saved and used as a basis for later comparisons. When new slides were made after two months, they offered no convincing evidence of growth during the interim.

In several succeeding experiments, control samples from the freshly inoculated tubes were placed on glass slides and stored in ordinary slides boxes, to permit making comparisons during the incubation period. In these experiments, later examinations of the cultures gave rise to both optimism and uncertainty, due especially to failure to standardize the smearing methods and also to the fact—appreciated only later—that bacilli disappeared from the control slides during the long periods of storage in a humid atmosphere at room temperature. Since these procedures were found incapable of furnishing clear-cut evidence of growth, or of proving its absence, actual counting⁴ of the bacilli was undertaken.

The average counts obtained in five experiments at different times during the incubation periods are shown in Table 6. ⁴The method of counting and scoring, and the basis for criticizing the controls stored on glass slides, will be given in a later paper. Samples for this purpose were taken from cultures in both bacteriological media and tissue culture solutions. About 50 percent of the tubes had been incubated under increased tensions of carbon dioxide and oxygen. Since these factors did not influence the results, the counts in all media were pooled and averaged in preparing the table.

TABLE 6.—Numbers of leprosy bacilli found in representative liquid media after varying periods of incubation.

Exp. Control	Number of bacilli ^a after incubation for								
NO.	count -	1 mo.	2 mos.	3 mos.	4 mos.	6 mos.	8 mos		
1	303b						463		
2	12 ^b		58			81			
3	137 ^b		372		316	303			
4	410				364				
5	146	152		139					

^a Expressed as the number seen per 10 standard microscopic fields. In each experiment, after each time interval, the counts from all media were averaged to produce the single values shown.

b The control counts in these first three experiments were too low because of fading of the bacilli stored on the control slides. In the last two experiments the control suspensions were stored in 0.25 percent formaldehyde.

In the first three experiments the numbers of bacilli found after incubation were appreciably higher than the numbers in the control slides. Experiments 1 and 2 were not sampled frequently enough to furnish an adequate measure of possible changes in numbers during incubation. In experiment 3, samples were made at regular intervals. The results showed an average of 372 bacilli at two months, 316 at four months, and 303 at six months, which suggests a slow decline in numbers throughout the incubation period. From further observations on the preservation of controls it is known that the control counts in these three experiments were too low.

In the fourth and fifth experiments the control suspensions were stored in formaldehyde. In experiment 4 the control count was 410 bacilli and the average count after incubation for four months was 364. In experiment 5 the control count of 146 bacilli corresponded closely with the average counts of 152 after one month and 139 after three months.

Thus, multiplication of leprosy bacilli in the first transplants from fresh nodules could not be demonstrated when the bacilli were actually enumerated. In general, the bacilli showed a tendency to lose their acid-fastness and to disappear rather quickly in salt solution or in acid fluids (see also Eddy, 7), but per-

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sisted in great numbers for periods of more than one year in neutral media containing serum or tissue extracts.

THE INFLUENCE OF GASEOUS ENVIRONMENT

As already noted, a great many of the tubes were incubated under partial pressures of carbon dioxide as a part of the CO_2 -NaHCO₃ buffer system most compatible with the growth of delicate cells. The concentrations of carbon dioxide varied from 4 to 10 percent by volume. In a total of 341 tubes, 40 percent of oxygen was used along with 8 to 10 percent of carbon dioxide. One of the acid-fast cultures described (No. 1077) was obtained under these conditions, but the others were obtained in ordinary atmosphere. The negative results in the study of the possible occurrence of "microscopic" multiplication of the bacilli indicate that neither oxygen nor carbon dioxide promoted their growth.

DISCUSSION

Taken together, the miscellaneous organisms cultivated in this work outnumbered those to which special importance has always been attached. Since these miscellaneous cultures must have occurred as secondary invaders in the nodules or as contaminants during the manipulations, only the special groups need be discussed.

It is reasonable to assume that if certain microorganisms occur as secondary invaders in leprous tissues, they might be absent from a given nodule or from the portion of the nodule cultivated, while on the other hand they might appear in scattered tubes throughout an experiment or be encountered in goodly numbers. A distribution of this kind has been observed as regards the diphtheroid bacilli. Special importance has been assigned to such organisms by several workers (see Salle, 21). From the experience here reported, however, they seem more likely to be recovered from ulcerated cases than from others, even though there may be no reason to criticize the nodule or its immediate surroundings. In studying lepra reaction cases I have found that the blister pus yields diphtheroid bacilli more frequently than any other kind of microorganism. On one occasion, by direct inoculation of solid media with diluted pus, pigmented colonies grew in such numbers that there must have been at least 60,000 viable cells per cubic centimeter of pus.

In keeping with these observations Salle (21) says, of the leprotic materials which he studied in Honolulu, that "positive results occurred only when broken down nodules were cultured." In one instance the inoculation of nine tissue-culture flasks produced two positive cultures; in three other instances all tissue cultures inoculated (nine, nine, and eight flasks, respectively) were positive. This suggests that there, also, the number of diphtheroid bacilli must have been high.

Three reports have appeared fairly recently of bacteriological investigations of leprotic materials at the national leprosarium at Carville, Louisiana. These materials were obtained with the cooperation of the surgical and pathological staffs of that institution, and it may be assumed that asepsis in the procurement of the nodules was comparable. From eleven nonulcerating nodules, Salle and Moser (25) reported 100 percent successful cultivation of diphtheroid bacilli which were described as acidfast when associated with tissues. In ten instances the cultures were obtained directly on glycerol-veal-hormone-agar, 84 (49 percent) of the 172 tubes becoming positive. At the same time, Eddy (7) worked with twenty nodules and recovered diphtheroid bacilli from "several." Evans (10), without mentioning the total number of nodules studied (apparently many), described the circumstances under which her diphtheroid cultures were obtained. They were recovered: (a) once, together with staphylococci, from a cluster of nodules on the neck, where she believed disinfection and cauterization did not sterilize the folds of the skin; (b) in six of nine tubes inoculated with material from a large nodule with a broken down center; (c) in two of five tubes from two adjacent nodules separated by a depressed area of skin "which may not have been thoroughly cauterized"; (d) in four of five tubes inoculated with pus from an acute abscess on the neck; (e) in two of eight tubes from two small nodules removed after cautery; and (f) in two of eight tubes from another large subcutaneous nodule. In short, she mentioned reasons for misgivings in connection with at least four of the six positive isolations.

This report by Evans, like the earlier work by Fraser and Fletcher (12), illustrates the fact that the probable significance of cultures isolated from leprosy may often be judged in the light of circumstances surrounding the work. To this should be added an imperative suggestion that even bacteriologists should keep brief notes concerning the presence or absence of scabies,

ulcerations, lepra-reaction blisters, and possibly other skin or medical conditions. The present experiments indicate an association of diphtheroid bacilli with ulceration in the cases from which the nodules were obtained. Since microorganisms are carried from open ulcers along lymphatics, one should be especially wary when cultivating from nodules situated along possible lines of lymphatic drainage from such lesions.

Although Salle (21) and Salle and Moser (22-25) maintained that, in the presence of tissues, their diphtheroid cultures possessed acid-fast properties which related them to leprosy bacilli, it would be unfortunate to let these cultures retain a reputation for being essentially different from those isolated by Eddy and by Evans in the same institution, or from those isolated in the present study. We all agree that these cultures are nonacid-fast on bacteriological media. None of us has a sound reason for saying that they are different from the diphtheroids which have long since been isolated from chronic hyperplasias, Hodgkins' disease, lymphosarcomas, urine and feces, laboratory air, or apparently normal areas on the surface of the skin of lepers (see, for example, Harris and Wade 16).

That Salle and Moser ascribed acid-fast properties to diphtheroid bacilli in chick embryo cultures, chick embryo suspensions, and minced mammalian tissues, apparently resulted from a misinterpretation of the results of their experiments. Under the conditions described some bacilli were in and on the tissues, while others were growing free in the liquids. It is unlikely that, when the cultures were brought out of the tubes or Carrel flasks and spread on slides for staining, the bacilli from the fluid (nonacid-fast) always remained at a distance from the tissue fragments and that those adhering to the tissues (acid-fast) were never washed off during the manipulations. It is more likely that the original relationship of the bacilli to the tissues was disturbed. When stained smears showed acid-fast bacilli only "on or near" the tissues, and blue bacilli elsewhere, it can only mean that decolorization was less effective in the immediate vicinity of the tissue fragments, not that the bacilli actually possessed both acid-fast and nonacid-fast properties, controlled by the circumstance of their growth in or outside of the tissue. Without fixation of the plasma cultures in situ and the preparation of sections, no other conclusion is permissible. The disappearance of acid-fastness in cultures of one week's duration and its prompt reappearance in freshly prepared tissue cultures

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may be explained by the alternate disintegration and renewal of organized tissue fragments in conjunction with corresponding changes in the physiological age of the bacterial cells. Some of the diphtheroids isolated during the present experiments, like those of Salle and Moser and of earlier workers, were more resistant to decolorization than most bacterial species; and they were inclined, especially when young, to retain the carbol-fuchsin stain in the vicinity of tissue debris or other particles.

Because the question of nonacid-fast or semiacid-fast microorganisms arising from leprosy bacilli has been discussed at great length by certain earlier workers—and it may appear again—it is well to remember that acid-fast bacteria rarely grow in liquid media as single cells, but that they occur typically in clumps, strands, and skeins from which the new outgrowths should originate. When new growth first appears in tubes of liquid medium inoculated with lepromatous materials, drops of liquid can be transferred to clean slides and allowed to spread without smearing or rubbing, and the original relationship of the new forms to the acid-fast bacteria can be ascertained. From all such examinations made in this study it can be stated that the streptothrix and diphtheroid cultures, though filamentous or "rough" in their growth, were never found to be physically connected with the acid-fast bacteria of the inoculum.

The cultures of acid-fast bacteria obtained in each of three experiments occurred only in single tubes out of many inoculated. With our present information, any argument in favor of their etiological connection with leprosy would be embarrassed by the fact that in one instance two dissimilar cultures were obtained from the same nodule. This nodule was open to suspicion because of its having been unfavorably located with respect to ulcers, and also because it gave rise to cultures of diphtheroids and a streptothrix. Whatever the source or the nature of these acid-fast cultures, their recovery was not due to any special properties of the different media on which they appeared. They are not believed to differ fundamentally from similar cultures which have been isolated by earlier workers.

The observations on the question of "microscopic growth" of leprosy bacilli here recorded are perhaps the first to be made under conditions which permit a positive statement concerning the fate of the bacilli. So-called growth in first transplants has

usually been attributed to the presence of protein or other derivatives from the tissue of the leprous nodule itself. Since, however, the inoculum used in these experiments contained all of the elements of the leproma, hypothetical deficiencies in the media used cannot be invoked to explain the negative results.

The question of possible further lines of bacteriological approach to this problem deserves consideration. Among the most complicated media employed were those which included fresh leukemic serum, pancreatic digest of liver autolysate, products from acid-fast bacteria, yeast extract and Long's medium. The leukemic serum furnished leucocytic extract in addition to the usual serum proteins, enzymes, and nonprotein nitrogen constituents. Liver digests have been shown by Baker and Carrel to supply nitrogen and other factors required for the growth of monocytes and fibroblasts in tissue cultures, and are of classical value as sources of bacterial growth factors. The products from acid-fast bacteria contained not only the growth factor for Johne's bacillus but, as shown by Anderson's work on purified fractions of acid-fast bacteria, the hydrolysates must have contained a most complicated assortment of products from the proteins, waxes, fat phosphatides, and polysaccharides of bacteria which are related to the leprosy bacillus. The yeast extract contained growth factors essential for a comprehensive list of bacteria.

Such media are so complex that they might be criticized by any student of conventional bacterial nutrition. Such a person could point out, quite justly, that growth factors had been supplied in an unnecessary number of ways and also that, if any component of the medium contained traces of some special growth factor for leprosy bacilli, these traces should probably manifest their favorable action in much simpler media. We should not delude ourselves concerning the help to be expected from recent trends in the field of bacterial nutrition. The brilliant and fundamental progress of the past few years in this field has not been directed toward the cultivation of new species of bacteria, but toward learning what items in the complex diet ordinarily offered to bacteria are essential for the growth of particular strains or species already cultivated. A precise list of sources of nutrient and of accessory factors has been compiled for many fastidious microorganisms. Information of this kind would make it possible to compile a long list of identified substances which, when combined in the forms used in the present experiments, were not adequate for the multiplication of the leprosy bacillus. The present situation suggests that, for new lines of approach to the solution of this problem, it may be well to give more attention to the developments appearing continuously in the fields of virus and tissue cultivation.

SUMMARY AND CONCLUSIONS

Completely homogenized suspensions of fifteen leprous nodules were inoculated into complex natural media such as blood, serum, leprous tissue extracts, chick embryo juice, and sterile egg yolk suspensions. These substances were supplemented with a variety of proteolytic digests, autolysates, peptones, products derived from acid-fast bacilli, yeast extract and simpler sources of nitrogen and carbon. Each inoculated medium also contained all of the nutrient substances present in the lepromata.

Diphtheroid bacilli were recovered from three of six cases showing ulcers of the skin. One culture of a streptothrix and two of acid-fast bacilli were also obtained from one of the ulcerated cases. A third acid-fast microorganism was cultivated from a patient who showed no ulcers. These acid-fast cultures were not tubercle bacilli.

By quantitative microscopic studies in liquid media from which aliquot portions were removed for comparison with control smears or formalinized suspensions, it was learned that the leprosy bacilli did not multiply in any of the 109 nutritional combinations tested, or in the presence of added carbon dioxide and oxygen.

Growth did not occur in media shown to be suitable for the cultivation of human blood monocytes or of fibroblasts from lepromata.

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