BACTERIA ASSOCIATED WITH CERTAIN TYPES OF ABNORMAL LYMPH GLANDS.*

JOHN C. TORREY.

(From the Department of Experimental Pathology, Loomis Laboratory, Cornell University Medical College, New York City.)

The publications of Negri and Mieremet,¹ and in particular those of Bunting and Yates,² appearing within the past two years, awakened the hope that the causative agent in Hodgkin's disease had been discovered. Since the appearance of these papers, however, little confirmatory evidence has been presented that the pleomorphic diphtheroid, which they describe, is concerned in the etiology of the disease; whereas, on the contrary, much of the work stimulated by these papers and published subsequently has shaken confidence in this theory. Shortly after the publication of the Negri and Mieremet article, the writer began a cultural study of such Hodgkin's material as could be obtained and also of abnormal glands from other diseased conditions, the purpose being to determine the frequency with which diphtheroids are present in such conditions and whether they could be referred to a distinct type within this large bacterial group. Very recently a bacteriological study of lymphatic glands has been published by Bloomfield.³ This investigation was animated by much the same purpose as the study presented here. Although our results are quite similar as regards the different types of bacteria encountered, certain new details have been ascertained during the course of this work which it seems desirable to report.

Material. — For most of the material and for the histological diagnoses I am indebted to Dr. James Ewing and the General Memorial Hospital. A number of specimens were also received from the New York Hospital through the courtesy of Drs. Elser and Lee, from Dr. J. H. Hartwell,

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from the Skin and Cancer Hospital by courtesy of Dr. Jessup, and also from a few other sources. In part of the work I have been assisted by Dr. Elizabeth Finch of the Skin and Cancer Hospital.

The following types of abnormal lymph nodes have been submitted to culture:

А.	Cases in which the primary seat of the disease was in the lymphatic glane	ds:
	Hodgkin's disease	10
	Hyperplastic lymphangitis of obscure origin	7
	Tuberculous adenitis	4
	Mikulicz' disease	I
	Lymphosarcoma	5
	Primary endothelioma	I
	Adenocarcinoma	I
	Chronic lymphatic leukemia	I
		30
B.	Cases in which the lymphatic glands were involved secondarily:	
	Sarcoma	4
	Endothelioma	2
	Acanthomatous adamantinoma	I
	Tumor of the thymus	I
	Melanoma	I
	Dermatitis exfoliativa	I

The regional distribution of these lymph nodes was as follows: twenty-one cervical, ten axillary, three supraclavicular, and five inguinal.

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With the exception of two cases in which the glands were removed post-mortem the material was obtained through surgical operations. The material was planted in culture media in the great majority of instances within a few hours of removal. If a somewhat longer interval occurred the glands were kept on ice. In a number of instances, where the size of the gland permitted, the external surface of the tissue was sterilized by dipping momentarily in boiling water and then transferring quickly to cool sterile saline solution. The gland was then placed in a sterile dish and macerated under a glass cover with fine scissors. Not over five minutes elapsed, as a rule, during this procedure. As far as bacteria of the diphtheroid type are concerned, control tests show that misleading contamination of the material from the laboratory air is unlikely. Large culture dishes containing glucose blood agar, exposed for thirty to forty-five minutes to laboratory air and incubated at 37° C., did not reveal more than three or four diphtheroid colonies per plate. Harris and Wade⁴ recovered under similar conditions from the air of their laboratory, in New Orleans, about the same number of diphtheroids. The types recovered from the air were largely chromogenic, forming either yellow, red-orange or brownish colonies. Out of twenty-one cultures, which I isolated, only one or two resembled the types encountered in glands.

Pieces of glandular tissue, ranging in size from wide slices to fine particles, were planted in a variety of media during the course of this investigation; the media including glucose ascitic agar, glucose blood agar, Bordet's glycerine potato blood agar, Loeffler's solidified serum, Dorset's egg medium, egg agar and also in one instance media mixed with an emulsion of the gland heated to 60° C. In nearly every instance anaërobic cultures were made, generally by planting pieces of tissue at various levels in columns of glucose ascitic agar.

Results. — Diphtheroids: As the possible connection of a diphtheroid bacillus with Hodgkin's disease has aroused widespread interest, the findings of bacteria of this type will be discussed first. A diphtheroid bacillus of one type or another was recovered from twenty-two of the forty cases. Lymph glands yielding positive cultures included such diverse pathological conditions as Hodgkin's disease, chronic hyperplastic lymphangitis of obscure nature, lymphosarcoma, sarcoma, melanoma, endothelioma, tuberculous adenitis, and chronic lymphatic leukemia. These findings included diphtheroids of the most divergent types morphologically and culturally. As a systematic classification of the diphtheroid group has not been formulated, they are here distributed among several general, but more or less distinct, groups.

Group I: Here are collected strains which exhibit distinct pleomorphism. Such cultures were recovered from twelve of the cases. The bacilli ranged in morphology from elongated, irregular, more or less curved rods, 2.5 to eight microns in length, showing bars and granules when stained with Loeffler's alkaline methylene blue, to coccoid forms. These granules were frequently positive to Neisser's stain. In five instances the rods were clubbed and in two exhibited short branches, either from the side or as a bifid extremity. None were definitely acid-fast, although after twenty seconds decoloration with Gabbet's stain a few retained the red tint in the granules or, in two instances, in the whole bacillus. All these, however, were decolorized after forty seconds and also after thirty seconds treatment with one per cent HCL in seventy per cent alcohol. On Loeffler's solidified blood serum these cultures, at 37° C., gave rise to a dull white or gravish (in a few instances turning later to a pale canary yellow) growth, which was generally rather thin, but always moist. Most of them grew as well at room temperature as at incubator. In neutral glucose broth no pellicle developed and the medium remained clear, the growth taking the form of a viscid sediment. A few of the strains formed acid in this medium, in one instance as high as +3.3 to phenolphthalein in fourteen days. All but one of the cultures tested fermented levulose and dextrose (Hiss serum water medium), six also saccharose, and one also dextrin, lactose, and maltose, In no instance did the carbohydrate fermentations correspond to that of B. diphtheriæ. In stab cultures in one-half per cent nutrient agar some growth occurred along the line of inoculation, but generally not further below the surface than three centimeters. The strains recovered from six of the cases were especially pleomorphic and resembled the type recently described by Simon and Judd⁵ as occurring in glands from a case of lymphatic leukemia, and also that isolated by Rhea and Falconer⁶ from Hodgkin's disease, especially as regards the number and striking size of some of the granules associated with the bacilli. In some instances the bacillus resembled a short chain of streptococci, in others one large

granule occupied the end of the bacillus, which tapered to a point at the other extremity, while in other instances granules with a diameter of 1.5 to three microns were found in the microscopic field. Cultures exhibiting one or more of these bizarre types were isolated from glands removed from one case each of perivascular endothelioma, chronic hyperplastic lymphangitis, tuberculous adenitis, chronic lymphatic leukemia and two cases of Hodgkin's disease.

Group 2: Here are assembled strains which resembled in morphology B. Hoffmanii rather than B. diphtheriæ. Bacilli of this type were recovered from the following twelve cases: two lymphosarcoma, two Hodgkin's disease, two hyperplastic lymphangitis, two tuberculous adenitis, and one each of Mikulicz's disease, endothelioma, tumor of the thymus and chronic lymphatic leukemia. In morphology these bacilli showed a marked regularity as compared with those of the previous group, being straight rods measuring .4-.5 x 1.5-2 microns. Generally granules were absent or at least much less deeply stained than those of group one. In all, however, the ends stained darker than the rest of the bacillus and more or less distinct barring was evident. In the almost complete absence of acid-fast properties this group resembled the foregoing. About one-half the cultures failed to act on any carbohydrate, whereas four split dextrose, levulose, and saccharose, one dextrose, levulose, and maltose and one dextrose, levulose, maltose, saccharose, lactose, and dextrin. It will be observed that none of the cultures tested resembled B. diphtheriæ in fermentations. The growth on Loeffler's solidified serum was white or grayish white and varied from a thin layer to one of moderate luxuriance. These cultures grew well at room temperature and to a depth of fifteen to thirty millimeters in stab cultures.

Group 3: In this group are collected miscellaneous strains. For example, in three cases there were isolated rather thick irregularly staining rods one to four microns in length which showed a marked tendency to stick together. These bacilli were barred and in one instance exhibited strongly staining granules. From four cases chromogenic

diphtheroids were isolated. One of these was bright yellow and the other three a brilliant orange red. As has been mentioned, types similar to these are not uncommon in the air. Also from two cases a xerosis type was recovered, giving a thin dry growth on Loeffler's serum. It is of interest to note that none of the cultures isolated were of the acidfast type, which have been reported as occurring in leprosy lesions by many observers and one of which has been described recently in detail by Wolbach and Honeij.⁷

Animal inoculations. — The inoculation of monkeys (rhesus) with several representative strains of these diphtheroids has resulted negatively. In these experiments four animals were used. Generally a heavy dose, injected subcutaneously in the axillary and inguinal region, was followed within a few days by a moderate induration at the site of the inoculation, accompanied with a slight enlargement of the neighboring lymph nodes. This did not progressively increase, but on the contrary had entirely subsided after three or four weeks. Repeated heavy inoculations were of no avail, nor were successful results obtained when accompanied by repeated injections of morphine or by changes in diet.

An attempt was made to obtain a toxin by growing selected cultures for nine days in slightly alkaline sugar-free broth. These cultures were inoculated subcutaneously into guinea-pigs, but without definite results.

Discussion. — In the course of this study, as has already been intimated, aërobic diphtheroids of one type or another have been isolated from four of ten typical cases of Hodgkin's disease, but in only two instances were they of the pleomorphic 'granular type which has been claimed by Negri and Mieremet and also Bunting and Yates to stand in etiological relationship to that disease. As a result of the work presented here and also from the evidence of other investigators, the writer has come to the conclusion that the theory of a causal relationship between a diphtheroid and Hodgkin's disease rests at present upon an insecure basis. This seems to be so for the following reasons:

The various investigators who have reported the isolation of diphtheroids from glands presenting the histological picture of Hodgkin's disease and who have been more or less impressed with the etiological significance of their findings have not established the identity of their several strains. The pleomorphism of the bacillus has generally been emphasized, but this is a character common to entirely unrelated members of the diphtheroid group and by no means may be considered a specific characteristic. Again, the description of the colony formation has not been uniform. In one instance Negri and Mieremet¹ describe the colonies as gray becoming later yellow or ochre, round and glistening, whereas Bunting and Yates² speak of their diphtheroids as forming colonies which become finally an opaque white, with some early cultures showing a greenish yellow tint. Rhea and Falconer⁴ describe only the primary growth of their strain as indistinct and stippled, and Rosenow^{8,9} gives no description of the type of colony formed by the strains which he isolated from such glands. None of these investigators have reported on the action of their several cultures on carbohydrates. In the collection of bacteria in the American Museum of Natural History there are three cultures of diphtheroids with a history of having been isolated from Hodgkin's glands; one having been received from Dr. Bunting and the other two from Dr. Rosenow. The writer has had the opportunity of examining these cultures and has found that they are quite distinct from one another in morphology, appearance of growth on Loeffler's serum medium, in their action on carbohydrates, and in their failure to interagglutinate. When one bears in mind the great numbers of similar and yet fundamentally distinct types of microörganisms embraced within the diphtheroid group, the importance of a detailed and comprehensive description of any strain, for which pathogenic properties is claimed, becomes obvious.

In all except two of the cases of Hodgkin's disease in this

series very few or no diphtheroid colonies appeared on the culture media. Generally in the positive cases from one to four or five diphtheroid colonies were all that developed on a series of ten to twenty culture tubes. In view of the fact that these diphtheroid strains are not particularly fastidious in their cultural requirements, it seems reasonable to expect that if they are the inciting agent in Hodgkin's disease, large numbers of the colonies would develop when gland cultures are made on favorable media and under suitable conditions. On the other hand, occasionally, many different types of diphtheroids may be recovered from a single Hodgkin's case. In one case in this series at least five distinct types were recovered from an axillary gland. These types included those described under groups one and two and also a xerosis and a chromogenic type. In other words, although many varieties of diphtheroids might be recovered from a single case, rarely were many of the same type recovered from a single case.

No one has yet advanced any serological evidence that a diphtheroid is concerned in the etiology of this disease. Such reports as have been published have been negative as regards the presence of agglutinins and other antibodies for this bacillus in the blood of the patients. The few tests which the writer has made for agglutinins have also resulted negatively.

The diphtheroids isolated from Hodgkin's glands have been reported, with one notable exception, to be devoid of pathogenic properties for laboratory animals. As is well known, however, Bunting and Yates by enhancing the virulence of their culture through inoculations in a series of monkeys were able finally to establish a progressive systemic glandular enlargement; a condition in the animal in fact which bore a striking resemblance to Hodgkin's disease in man. This interesting finding as yet lacks confirmation from other sources.

No single type of diphtheroid is apparently found alone in the enlarged glands of Hodgkin's disease. Soon after Negri and Mieremet's original publication, Steele¹¹ called attention to the fact that a bacillus evidently identical with their "corynebacterium" might be isolated from enlarged glands in lymphatic leukemia. This observation has since been confirmed by Simon and Judd.⁵ The author has also isolated from glands removed from a case of chronic lymphatic leukemia a markedly pleomorphic "corynebacterium," which is apparently very similar morphologically to that of Simon and Judd, but which differs from it in sugar fermentations. A similar finding has recently been reported by Bunting and Yates.¹⁸ I have also encountered strikingly pleomorphic and granular diphtheroids in such diverse conditions as perivascular endothelioma, hyperplastic lymphangitis of obscure origin, and tuberculous adenitis. Similar findings have been reported by Harris and Wade,⁴ Langford,¹² and Bloomfield.8

Harris and Wade,⁴ in a recent discussion of the significance of the diphtheroids so frequently found associated with abnormal tissues, have emphasized the fact that bacteria of this type are decidedly ubiquitous, not only as semi-parasites upon and within the human body, but also as saprophytes in nature. This wide range of environment has thus given rise to quite as diverse a group of these bacteria, as regards chromogenesis and other cultural characteristics, as is that embraced within the family of the Coccaceæ. The large number of saprophytic types within this group does not, of course, preclude the possibility that pathogenic properties may be demonstrated for certain strains. Although this is obvious, yet very seldom has pathogenicity for a diphtheroid been established by convincing evidence. Fox¹⁴ in a recent review of the literature and of his own experience concludes that in only a few cases of angina has the diphtheroidal nature of the infection been proved. To this may be added the recent work of Rosenow¹⁵ on the relation of a diphtheroidal microorganism with erythema nodosum.

Other aërobic bacteria. - A number of other aërobic bacteria were encountered in these lymph glands but, with the exception of staphylococcus, only sporadically. Staphylococci, as has been noted by others, are very commonly met with in these abnormal glands. In this series the albus type was recovered in twenty-six of the cases. They occurred most abundantly in the glands from the neck and axillary regions. In one instance Staphylococcus aureus and in another Staphylococcus citreus was isolated. Streptococci were isolated from only one Hodgkin's gland, an acute inguinal case. This strain formed chains of moderate length composed of regularly rounded cocci. It fermented lactose, but not inulin, salicin or raffinose and was present in such small numbers as to exclude it as a possible factor in the disease. A cervical gland, removed from a case of Mikulicz's disease, yielded a few colonies of a long chained non-hemolytic streptococcus which clotted milk and split dextrose, but not raffinose. Cervical glands from a case of adenocarcinoma also yielded streptococci of a type similar to the last.

Hyphomycetes. — A granulomatous gland of indefinite nature yielded a sporothrix culture which has been described in detail by Dr. Finch.¹⁶ From a case of Hodgkin's disease an oidium was isolated which developed a thin septate mycelium with single spores budding from the sides and terminally. Pathogenic properties could not be demonstrated.

Anaërobic bacteria. — Early in the course of this work it was noted that on certain of the Loeffler serum tubes, incubated from ten to twenty days, round, acutely convex, opaque, white or flesh colored colonies had appeared, growing out from the tissue. On aërobic sub-cultures no growth developed as a rule, but in stab cultures a slow increase occurred within three or four days. The microörganism forming this growth was found to be a slender, pleomorphic, strongly Grampositive rod with rounded ends, some strains measuring .5 x

1.2-3.2 and others .4 x 1-2. It is not acid-fast and stains rather lightly with alkaline methylene blue and dilute carbolfuchsin. It generally occurs singly, at times in pairs, but never forms chains in any medium. It is not motile. Α smear from a primary culture deep in the medium frequently shows short, rather plump rods which vary little in length, measuring .75-I x I.5-2 microns and generally slightly curved. Stab cultures in one-half per cent nutrient agar, which is a very favorable medium, are composed generally of rods of a more irregular morphology, the bacilli varying in length and staining rather unevenly with ends frequently slightly swollen. They are collected in clumps. Deep staining granules were never observed, nor barred nor clubbed types. Growths on or in dry media may show a few rods with bifid ends.

The best mode of culturing the material in order to bring these anaërobic types to development was found to consist in burying the bits of tissue at various depths in a column of ascitic glucose agar with a reaction of about +2 to phenolphthalein. This acid reaction is decidedly favorable and accelerates the growth of this bacillus. In a medium with a reaction of +.5 to +1 the rate of growth is very slow and is not observable in primary cultures until the eighth or tenth day, whereas with the more acid medium the same result was obtained in two to four days. The addition of ascitic fluid to the medium is not essential. From the tissue planted deep in the medium this bacillus grew out as a white papilla which increased in size until after a week or more a shelf-like structure had been formed, very similar in appearance to a fungus. This growth was tinged a faint pink and clouded the medium. It emitted a sour musty odor. Colonies appeared at various depths of the medium up to within one centimeter of the surface. The isolated colonies were angular, pyramidal in shape, and not more than two millimeters in the diameter.

The following table indicates the relative frequency with which this anaërobic bacillus was isolated from the various types of abnormal glands:

	Positive.	Negative.
Hodgkin's disease	. 10	о
Indefinite hyperplastic lymphangitis	• 4	4
Lymphosarcoma	• 3	2
Tuberculous adenitis	. 2	2
Primary endothelioma	. I	ο
Endothelioma	. г	I
Mikulicz's disease	. г	ο
Adeno-carcinoma	. 0	I
Acanthoma	. о	I
Chronic lymphatic leukemia	. 0	I
Sarcoma	. o	4
Tumor of thymus	· 0	I
Melanoma	. о	I

As this tabulation shows, although this bacillus was not specific for any single pathological condition it was recovered in the highest percentage of instances (one hundred per cent) from Hodgkin's glands. In five cases, three Hodgkin's and two lymphosarcoma, it was practically the only microörganism developing in the culture tubes.

Until very recently I have not encountered any reference to this bacillus in the literature. During the past summer, however, the paper by Bloomfield, already mentioned, appeared in which an anaërobic bacillus, without doubt identical with this one, is described. He recovered this bacillus from ten out of twenty-five abnormal glands, including Hodgkin's disease, lymphosarcoma and arthritis. Colony formation indicated the presence of from one to one thousand of these bacilli in the amount of tissue submitted to culture. This bacillus also bears a resemblance morphologically and in the relation of its growth to oxygen tension to the typhus bacillus of Plotz.¹⁷ It is much more readily cultivated, however, and in other features exhibits important differences. Bloomfield also describes a Grampositive anaërobic coccus which he isolated from three cases, two of them being Hodgkin's disease. This coccus has not been recognized in this series of cases.

Further cultural characteristics of the anaërobic bacillus are the following: The optimum incubation temperature was found to be about 37° C., although a slower growth was

obtained at 30° C., and at 41° C. No growth occurs at room temperature, 20° to 25° C. The most favorable mode of sub-culturing this bacillus consists in deep stab inoculations in one-half per cent nutrient agar with a reaction of about +2 to phenolphthalein. The zone of optimum growth in such cultures varied somewhat with different strains. With some the first growth was observed near the surface, in others half way down and with still others at the bottom of the tube. Contrary to Bloomfield's finding most of the strains would finally grow up to the surface, although rarely in any degree on the surface of the medium. Occasionally certain strains could be induced to develop on the surface of Loeffler's solidified serum medium in the form of a raised white growth, quite similar to that of Staphylococcus albus. A marked peculiarity of this bacillus lies in the fact that it produces very high amounts of acid in fluid medium containing certain carbohydrates. In dextrose broth with an initial acidity of +2.3 the titer was raised by certain strains in ten days up to + 10 or even + 12, producing in fact almost as much acid as B. bulgaricus. This microörganism is thus markedly aciduric and to a considerable extent acidophilic. It was found, in fact, capable of multiplying in glucose broth containing five per cent normal acetic acid. Why a bacillus of such acidophilic propensities should find abnormal lymph nodes a favorable soil is not clear.

All the strains of this bacillus tested were found to split dextrose and glycerine actively and practically only these carbohydrates. The production of acid in glycerine broth generally equalled or even exceeded that in dextrose broth. Lactose, mannit, inulin, and dextrin were not split by any strain and saccharose by only one strain. Growth in broth generally took the form of a clouding of the lower third of the tube with a heavy sediment. With three strains, however, there was an even clouding of the whole medium. Gas was never produced. No more than a very slight growth occurred in a stab culture in sugar-free agar medium. Litmus milk was not affected and gelatin and serum were not fluidified. This bacillus was found to offer no resistance to antiformin.

Culturally all strains of this bacillus exhibited a marked uniformity and the conclusion that they consist of a single species was substantiated by agglutination experiments. It was found that serums with an agglutinating power of two thousand to five thousand could easily be produced by inoculating rabbits. Large amounts of this bacillus in a viable condition could be injected intraperitoneally or intravenously into rabbits without producing toxic symptoms. The greater number of strains agglutinated to the same degree with each one of the several serums produced. Two strains, which gave a lower titer, were shown to be identical with the others through absorption experiments.

Animal inoculations with this anaërobic bacillus were as unsuccessful as with the aërobic diphtheroids recovered from the glands. A number of guinea-pigs and monkeys were inoculated with large doses of recently isolated strains, frequently with the primary growth, but without any evidence of pathogenic effect aside from a moderate transitory enlargement of the lymph nodes. One monkey, which died about ten days after the inoculation but probably from other causes, yielded cultures of this bacillus from the axillary and mesenteric lymph nodes and from the spleen. An equal period of persistence occurred within the body of a guinea-pig.

As has been mentioned, cultural findings in various types of abnormal glands have indicated that this bacillus does not stand in specific relationship to any definite pathological This conclusion is further substantiated by condition. agglutination and complement fixation experiments with the blood of patients. Although serum from Hodgkin's cases and related conditions might cause an agglutination of this bacillus in as high dilutions as 1-80, yet a like or even higher titer was observed with serum from certain patients suffering from conditions entirely distinct from this disease, such as various types of sarcoma. In fact the highest titer obtained, 1-640, was produced by serum from a case of melanoma. The same irregularity of results occurred in complement fixation. These results give added weight to the caution which should be exercised in accepting positive

serological results as evidence of the specific relationship of an organism to a malignant growth.

Although cultivation at various temperatures indicates that this anaërobic bacillus has become restricted to the temperature of the body, I agree with Bloomfield that the evidence suggests it is present there as a parasite rather than as an active pathogen. It is likely that abnormal glandular conditions offer a favorable soil for the growth of this microorganism. As it is a bacillus of such uniform type and occurs so frequently in abnormal states of the lymph glands, it seems desirable that it should be designated by a definite name. I would suggest the designation *Bacillus lymphophilus*.

In seven cases of diverse nature one or more of the columns of ascitic glucose agar planted with the glandular tissue were blown up with gas. In five instances this was due to the development of a Gram-positive bacillus with the morphology of B. aerogenes capsulatus. In one case a small Gram-negative bacillus (not B. coli) was the cause of the gas production and in another instance the bacillus was an anaërobic Gram-positive one, but more slender than the Welch type, producing less gas and giving negative results in the rabbit test.

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DESCRIPTION OF PLATE III.

FIG. 1.— B. lymphophilus, from case of Hodgkin's disease, x 950. Smear from growth on tissue planted deep in glucose ascitic agar.

FIG. 2. — B. lymphophilus, from case of lymphosarcoma, x_{950} . Smear from primary growth out of the tissue, planted deep in glucose ascitic agar, and appearing after about two weeks' incubation.

FIG. 3. — Pleomorphic diphtheroid bacillus with large granules, x 950, recovered from a case of Hodgkin's disease (fibrosing) in a five-year old child. Smear from growth appearing on the original glucose agar culture, incubated four days.

FIG. 4. — Primary deep plant of tissue showing fungus-like outgrowth of B. lymphophilus after three weeks' incubation.

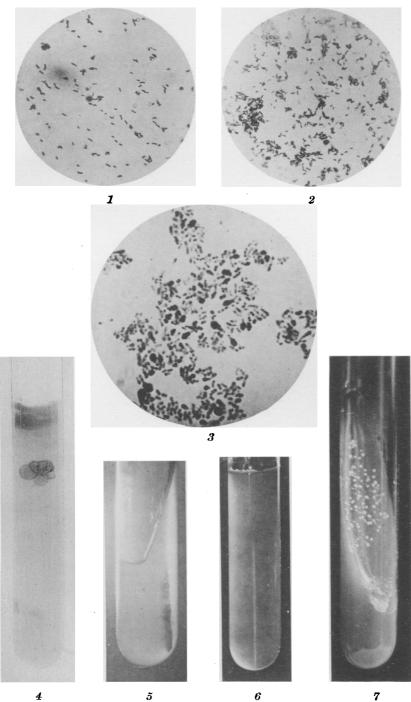
FIG. 5. — Stab culture of B. lymphophilus in glucose ascitic glycerine agar after ten days' incubation. No growth on the surface of the medium.

FIG. 6. — Stab culture of B. lymphophilus in glucose ascitic glycerine agar after five days' incubation, showing the relation of the growth to oxygen tension.

FIG. 7. — Surface growth of B. lymphophilus on glycerine agar after about twenty-five days' incubation.

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