REPORT

of

COMMITTEE ON EXAMINATION

This is to certify that we the undersigned, as a Committee of the Graduate School, have given Edmond Newell Nelson final oral examination for the degree of Master of Science. We recommend that the degree of Master of Science be conferred upon the candidate.

Minneapolis, Minnesota

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GRADUATE SCHOOL

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Date

10-20 5M

IMMUNOLOGIC STUDIES OF ACTINOMYCETES, WITH SPECIAL REFERENCE TO THE ACID-FAST SPECIES.

A Thesis submitted to

The Faculty of The Graduate School of

The University of Minnesota

By

Edmond Nelson

In partial fulfillment of the requirements for the

Degree of Master of Arts.
[Science?]

June

1922

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Introduction

The use of serological reactions for the differentiation of the non-acid-fast forms of Actinomycetes from the acid-fast forms and also for the differentiation of the acid-fast of Actinomycetes, and the acid-fast bacteria, especially bacillus tuberculosis, have not been very extensive. There has, however, been a considerable amount of investigation toward the classification of the different species and strains of the Actinomyces group, by their culturaland morphological characteristics. There is an extensive literature on this subject and the work has led to a more or less complete classification. San Felice (1) and (2) included in his classification the acid-fast bacteria, such as the leprosy and tubercle bacillus. He divided the genus into three groups, one non-acid-fast, a second partly acid-fast, and a third acid-fast.

Serum reactions have been used by Choukevitch (3) who found that by the use of agglutination and precipitin tests with rabbits immune serum, against the various types of Actinomyces, group reactions could be demonstrated. Similar investigations were carried on by Harbitz and Grondahl (4), who obtained negative results.

The grouping of the various strains of the Actinomycetes and their relation to some of the acid-fast bacteria was carried on by Claypole (5) by means of the complemental fixation test. In her studies thirteen different strains of organisms were used representing three characteristic groups:- a non-acid-fast group, a representative of this group being Actinomyces bovis; a partly acid-fast group, a member of this group being Actinomyces asteroides; and an acid-fast group, represented by bacillus tuberculosis.

Complement fixation tests were carried on with members

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of the same group, and also cross fixation tests with members of the other groups. Six strains were non-acid-fast and four were acid-fast types of the Actinomyces. The other organisms were bacillus leprae (Duval) and bacillus tuberculosis.

It was found by Miss Claypole that a sharp division could not be drawn between the non-acid fast actinomyces, and those that are partly acid-fast. Reactions between rabbit immune serum against a non-acid-fast form and bacillus tuberculosis were negative. Cross fixation tests between the partly acid-fast strains of Actinomyces and bacillus tuberculosis and bacillus leprae were positive, and no distinction could be made between these forms by means of the qualitative fixation test.

Henrici and Gardner (6) found that the serum of a patient infected with Actinomyces gypsoides gave a positive fixation reaction with antigen prepared from this organism, while the serums of twelve other persons with the same antigen gave negative results. Agglutination tests were also successful in a 1:20 dilution and a partial agglutination in a dilution of 1:100.

Agglutination and fixation tests were carried on by Widal, who found that the serum of patients infected with the Actinomyces gave positive results, when the spores of Sporothrix Beurmanni were used as antigen.

Owing to the close relationship that exists between the acid-fast actinomycetes and bacillus tuberculosis, a few of the investigations by means of serum reactions in tuberculosis should be mentioned.

The methods used by different people in performing the fixation test in tuberculosis and in the preparation of antigen

have varied considerably and therefore the results obtained have been quite variable. Wills (7) has shown by means of the fixation reaction that a close relationship exists between the different strains of bacillus tuberculosis and other acid-fast bacteria. Much and Hoessli (8) observed that the complement fixing substances of the tubercle bacillus are the same as those of the other acid-fast bacteria and that cross fixation occurred with quantitative differences. The investigations of Cooke (9) have shown that a close serological relation exists between the bacilli of the acid-fast group, especially between the bacillus tuberculosis and bacillus leprae. The results of different investigators in regard to the use of the fixation reaction in the diagnosis of tuberculosis have been quite variable. This is possibly due to some extent at least to the variations in the technique and also to the different methods used in the preparation of the antigens.

The work here reported has had for its purpose the following:-

(1) To observe the relationship between the non-acid-fast actinomycetes and the acid-fast types and also the relation of the acid-fast actinomycetes to bacillus tuberculosis, by means of the complemental fixation test. (2) To determine if the serum of cattle infected with actinomycosis will give a fixation reaction with Actinomyces bovis antigen. (3) To produce a protective immunity against virulent strains of Actinomyces (A.gypsoides).

Material and Methods

Material for the isolation of the bovine cultures was obtained from infected animals at the packing plants at South

St. Paul. The pus was taken from the lesions under as sterile conditions as possible, and a small amount of the pus was emulsified in sterile broth and 1 cc. of the emulsion was plated on plain agar. The saprophytic Actinomyces colonies which often appear after incubation can be differentiated from the true bovine colonies by the fact that the colonies of A.bovis do not generally appear until about the seventh or eighth day of incubation. The colonies are small and grow partially under the surface of the agar. The mycelium appear as small regular growths at the surface and the color is, as a rule, white. The colonies of the saprophytic forms develop in from two to four days and are much larger. The growth of the aerial mycelium is quite profuse and the colors are variable. When colonies have grown on Sabouraud's agar for some time, the saprophytic types give off a distinct musty odor, while the bovine forms have very little odor. When colonies from plates are transferred to Sabouraud's agar slants the development of the saprophytic types is much more rapid than the bovine forms.

Antigen: For the preparation of antigen the organisms were grown in flasks containing 100 c.c. of 3 % dextrose broth. The flasks were incubated for about fifteen days, or until growth was exhausted. The cultures were then heated for one hour at 70°C. and then placed in Larson's (10) apparatus and treated with CO_2 under high pressure for about twelve hours. Upon releasing the pressure, the mycelium were found to be broken into small fragments and a uniform suspension was obtained. For the preservation of this antigen a small crystal of thymol was added. The antigen as prepared by this method proved to be satisfactory. It was not anti-complementary except in amounts of three to four times the dose used. It did,

however, upon standing for some time develop anti-complementary bodies. It was non-hemolytic. A constant amount of two drops of the antigen was used in all of the tests.

Immune Serum: Normal adult rabbits were immunized by injecting into their marginal ear vein 1 c.c. of the antigen in three doses, at four day intervals. About seven days after the last injection the animals were bled and the serum inactivated by heating to 56°C. for thirty minutes. No thymol was added to the antigens which were used for immunization. A constant amount of two drops of serum was used in all of the tests, except in the quantitative fixation reactions between the acid-fast Actinomyces and bacillus tuberculosis. In the quantitative reactions the serum was used in different dilutions. Rabbits were immunized to a representative of each type of organism, one to bacillus tuberculosis, to Actinomyces gypsoides and to Actinomyces bovis.

Complement: The complement was prepared by diluting normal guinea pig serum 1:10 with normal salt solution.

Hemolytic Amboceptor: The dilution used was one part of rabbits serum immunized against sheep cells to fifty parts of normal salt solution and the titration so adjusted that 0.1 c.c. was used in all of the tests. The sheep blood cells were diluted 1:100 and 0.5 c.c. used in all tests.

Controls: Two control tubes were used, containing all of the reagents in proper amounts, except in one tube no antigen was added and in the other tube one drop of normal inactivated rabbit serum replaced the immune serum.

Results: The following table contains the results of fixation experiments, with serum of animals infected with Actinomyces

bovis, and antigens prepared from cultures of A. bovis. The controls consist of serum from normal animals.

Table I.

Details of a Fixation Experiment with Bovine Serums

	Serum from Actinomycotic Cattle								Serum from Normal Cattle		
Antigen	1	2	3	4	5	6	7	8	1	2	3
Bovine (1)	+	+	-	+	+	-	+	+	-	-	-
Bovine (2)	+	+	-	+	+	-	+	+	-	-	-
Bovine (3)	+	+	-	+	+	-	+	+	-	-	-
Bovine (4)	+	+	-	+	+	-	+	+	-	-	-

Results of fixation experiments with the non-acid-fast and acid-fast Actinomycetes and bacillus tuberculosis are given in the following table.

Table II.

Results of Fixation Tests with the Actinomycetes and Bacillus Tuberculosis

Antigen	Type	Rabbit Immune Serum Against A.Bovis	Rabbit Immune Serum Against A.Gypsoides	Rabbit Immune Serum Against Human T.B.
A.Bovis	(1)	+ + + +	+	-
A.Bovis	(2)	+ + + +	+	-
A.Bovis	(3)	+ + + +	+	-
A.Bovis	(4)	+ + + +	+	-
A.Aesteroides	Acid Fast	+	+ + + +	+ + + +
A.Gypsoides	Acid Fast	+	+ + + +	+ + + +
B.Tuberculosis	Human	-	+ + + +	+ + + +

+ 25 to 50% fixation. + + + + complete fixation.

It is apparent from this that there is a fairly sharp differentiation between the acid-fast group and the non-acid-fast group, and a close relationship between the various acid-fast species of Actinomyces and bacteria. To study this relationship more closely, quantitative tests were made. Serial dilutions of the serums immune to A.gypsoides and B.tuberculosis were used, the dilutions ranging from 1:20 to 1:1280 so that the tubes contained from 0.1 cc. down to 0.0016 cc. of serum. The results of these experiments are shown in the following table.

Table III.

Details of Quantitative Fixation Ractions

Immune Serum	Dilution of Serum	Actinomyces Gypsoides Antigen	Actinomyces Aesteroides Antigen	Bacillus Tuberculosis Antigen (Human)
To Tuberculosis	1:20	+	+	+
" "	1:40	+	+	+
" "	1:80	+	+	+
" "	1:160	+	+	+
" "	1:320	-	-	+
" "	1:640	-	-	+
" "	1:1280	-	-	-
To A.gypsoides	1:20	+	+	+
" " "	1:40	+	+	+
" " "	1:80	+	+	+
" " "	1:160	+	+	+
" " "	1:320	+	+	-
" " "	1:640	+	-	-
" " "	1:1280	-	-	-

The acid-fast Actinomycetes are remarkable for their high and constant virulence for laboratory animals, particularly guinea pigs, and this is especially true of A.gypsoides. Even small quantites injected intraperitoneally produce death in from three to six days with the production of a pseudotuberculous peritonitis. Apparently no studies of the mechanism of infection or the possibility of protective immunity have been made.

Because of the high virulence it was thought that possibly a soluble toxin was secreted. The broth filtrate of a three weeks old culture, however, proved harmless to guinea pigs in doses as high as 2 cc. The broth filtrate produced no immunity, as these same guinea pigs succumbed two weeks later to an inoculation of the living organisms, at the same time as controls.

The dead organisms, however, are quite toxic and invariably cause death even in small doses. A three weeks old culture heated to 70°C. for one hour and emulsified by the CO_2 pressure method proved lethal in doses of 0.05 cc. in which the emulsion was very heavy. The animals died in from ten to twelve days. During this incubation period there occurs marked emaciation. No lesions can be demonstrated at autopsy, save congestion of the mesenteric lymph nodes.

For the purpose of determining the possibility of protective immunity, a rabbit was immunized by injecting 1 cc. of a dead culture of the A.gypsoides into the marginal ear vein. All attempts to actively immunize guinea pigs were unsuccessful because of the toxicity of the dead organisms. Four injections were made at four day intervals. Seven days after the last injection 0.5 cc. of a culture attenuated by heating for half an hour at 50°C. was given

subcutaneously, and as a result a small abscess was formed, but soon disappeared. One cc. of the attenuated culture was now given intravenously, and after five days another 0.5 cc. dose was given subcutaneously, and there was no abscess formation. A small amount of living culture was given intravenously. Four days later 0.5 cc. of the living organisms were given, and still later 1 cc. of the living culture was injected. Seven days following the last injection the rabbit was bled, and about 5 cc. of serum obtained. In an attempt to obtain a passive immunity a guinea pig was inoculated intraperitoneally with a very small amount of a virulent culture of A.gypsoides, and a few hours later the serum was given. Two control pigs were also injected with a small amount of the virulent culture but no serum was given. Four days following injection the control pigs died, and on autopsy showed the tubercle-like lesions. The pig given the culture and the serum did not show any of the effects of the inoculation, at this time, but died fourteen days later, and on autopsy no lesions were found. The internal organs appeared to be normal except for a congestion of some of the lymph nodes. This experiment was repeated several times, with identical results, except that the time of death varied somewhat. An active protective immunity can be produced in the rabbit, which can be passively transferred to the guinea pig. The serum of the rabbit seems to prevent the development of infection, but does not prevent ultimate death from toxemia.

Discussion.

There is considerable confusion in literature as to whether the Actinomyces bovis grow best under aerobic or anaerobic conditions. Some investigators maintain that growth takes place

best when cultivated by aerobic methods, while others have the opposite view. The general impression is that they are very hard to grow. It was found in this study that they could be cultivated aerobically quite readily, by simple plating of the pus on plain agar. Since A.bovis is not pathogenic for laboratory animals and cultural characteristics ill defined, there is no very good criterion for the identification of the species by this method. Positive complement fixation with the serums of actinomycotic cattle indicates that the true Actinomyces bovis was really isolated. However, this cannot be settled until the degree of specificity of the complemental fixation reaction is more accurately determined. The possibility that several species of Actinomycetes may cause lumpy jaw should be considered. It was not the purpose of this study to develop a procedure for the diagnosis of actinomycosis, but the ease with which a satisfactory antigen can be prepared, and the sharpness of the reaction obtained indicate that this test may be of possible value not only in bovine but also in human cases.

The cross fixation experiments are of interest because of the phylogenetic relationships of the Actinomycetes and the acid-fast bacteria. The systematic position of the Actinomycetes has been quite uncertain until recently, and they have been included with the bacteria by some authors and with the higher fungi by others. On a purely morphologic basis they must be included with the fungi as has been shown by Dreschsler (11), who states that "owing to the absence of any well defined bacterial characteristics, the view that the Actinomycetes represent a transition stage between the Hyphomycetes and the Schizomycetes may be safely abandoned." He also points out that "it is doubtful whether far reaching toxonomic

generalizations can be based on the 'acid-fast' staining reaction, especially as this reaction has not played a very important role in mycological research." However, with organisms so low in organization and simple in structure as these it is doubtful whether morphologic criteria can be so strictly adhered to as with higher organisms. Foulerton (12 and 13) is a warm advocate of the generic identity of organisms of tuberculosis and leprosy with the streptothrices, and his extensive and valuable contributions to the subject entitle his views to a careful consideration. Complement fixation studies indicate a closer relationship between acid-fast bacteria, and acid-fast Actinomyces than between the latter and non-acid-fast Actinomycetes. This confirms the work of Claypole. Quantitative tests show almost complete identity between Actinomyces gypsoides and Actinomyces aesteroides, but a well defined differentiation between the acid-fast Actinomyces and acid-fast bacteria. Protective immunity against Actinomyces gypsoides was established in some cases, but with difficulty because of the virulence of the organism. Rabbits were apparently completely protected, as intravenous injections of live organisms are invariably fatal in non-immunized animals. Passive immunization of guinea pigs with rabbits serum is only partially successful as the pigs die from eight to fourteen days after intraperitoneal injection, but the serum seems to inhibit growth of the organisms and prevents the development of lesions, but does not neutralize the effect of the endotoxin.

Summary and Conclusions.

(1) Actinomyces bovis was isolated from pus by simple plating on plain agar.

(2) Satisfactory antigens for complement fixation can

be prepared by the "explosion" method of Larson, Hartzell and Diehl.

(3) Serums of actinomycotic cattle fix complement in some cases, with antigens prepared from A.bovis.

(4) Non-acid-fast Actinomycetes (A.bovis and A.madurae) give cross complement fixation. Acid-fast Actinomycetes (A.gypsoides and A.asteroides) and acid-fast bacteria (B.tuberculosis) give cross complement fixation, with some quantitative differentiation. Only a partial cross fixation is obtained between the non-acid-fast Actinomycetes, and acid-fast species. It is concluded, therefore, that there is a closer relationship between the acid-fast Actinomycetes and acid-fast bacteria, than between the acid-fast Actinomycetes and the non-acid-fast Actinomycetes.

(5) Actinomycetes gypsoides does not produce a soluble toxin but does form a potent endotoxin.

(6) Protective immunity against A.gypsoides can be established in some instances in rabbits. This can be partially transferred to guinea pigs.

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