ANTIBODY FORMATION AGAINST TREPONEMA PALLIDUM—AGGLUTINATION.*

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Plates 52 to 54.

Although active immunization in syphilis has been extensively attempted by many workers, the results seem to show generally unsuccessful experiments. It is the opinion of Neisser and others that true immunity in syphilis has not so far been demonstrated and possibly does not exist. It seems that a human being is immune to reinoculation only while still diseased, but that susceptibility is again established after clinical recovery. As regards animals experimentally inoculated these matters are still uncertain, because of the unavoidable technical difficulties; for in none of the animals easily and cheaply accessible to most laboratory workers can syphilis be produced in anything like the generalized or prolonged course observed in human beings.

It has been the aim of many workers to attempt the demonstration of antibodies in the circulation of animals experimentally inoculated or treated with extracts of either syphilitic tissues or treponema culture material. Before the cultivation of *Treponema pallidum* by Noguchi's methods had made possible extensive experimentation with pure cultures, many investigators attempted active immunization of animals with tissues rich in treponemata, but with entirely unsatisfactory results.

Neisser[1] and Bruck summarize their opinion as follows: "It is plain, therefore, that parasiticidal antibodies do not occur in the course of syphilis, and that we may at most count only upon the occurrence of specific complement-fixing and agglutinating substances, although the occurrence of such antibodies can be determined with accuracy only when we shall be able to work with pure cultures of treponemata."

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[1] Neisser, A., *Arb. a. d. k. Gsndhtsamte.*, 1911, xxxvii, 187, 206.

In a later summary Bruck[2] states that complement fixation in syphilis can be looked upon as only in part depending upon a specific antibody reaction. He states also that Fornet and Schereschewski's demonstration of precipitins in syphilitic sera cannot be confirmed, and that the formation of true agglutinins could not be determined by Uhlenhuth and Mulzer, who treated rabbits, goats, and monkeys with materials containing considerable amounts of treponemata. He adds, however, that this question will probably remain unsettled until the work can be repeated with materials richer in microörganisms.

Noguchi,[3] in April, 1912, published a paper on the fixation of complement when aqueous extracts of syphilis cultures were used as antigen, and the sera of patients with syphilis and of experimentally inoculated rabbits were used. He found positive fixation in the majority of the cases of secondary, tertiary, and hereditary syphilis, also in one of five syphilitic rabbits, using as antigen the emulsion from syphilitic testicles or from pure cultures. The validity of the reactions obtained with syphilitic testicles he regarded as doubtful, since emulsions from normal testicles gave, with some of the sera, equally strong reactions.

Craig and Nichols,[4] using alcoholic extracts of pure cultures of the syphilis organism and some other spirochete, found occasional fixations in syphilitic cases, but these were weaker than similar fixations obtained with the ordinary lipoid tissue extracts used in the common Wassermann technique.

Kolmer, Williams, and Laubaugh,[5] working both with aqueous and alcoholic extracts of concentrated pure cultures, found that such antigens gave positive fixation reactions with the serum of a considerable number of human syphilitic cases, and strong reactions with the serum of rabbits treated with treponema cultures.

Kolmer[6] also observed that treponemata from cultures in horse serum broth were agglutinated by the sera of rabbits which had received injections of such cultures either intravenously or into the testicle.

These later researches seem to indicate definitely that circulating antibodies may be formed in the course both of spontaneous syphilis of human beings and in animals treated with cultures of the treponema. Noguchi in summing up the problem in his paper concludes that his experiments seem to show that the ordinary Wassermann reaction is not determined by specific antibodies; that, however, the fixation produced with the culture pallida antigen seems to signify the presence of the true antibodies. He remarks upon the peculiar fact that the concentration of antibodies in syphilitic patients as determined by the pallida antigen seems to be surprisingly slight when compared with antibody formation in many other infectious diseases.

[2] Bruck, C., in Kolle, W., and von Wassermann, A., Handbuch der pathogenen Mikroorganismen, 2d edition, Jena, 1913, vii, 1060.

[3] Noguchi, H., *Jour. Am. Med. Assn.*, 1912, lviii, 1163.

[4] Craig, C. F., and Nichols, H. J., *Jour. Exper. Med.*, 1912, xvi, 336.

[5] Kolmer, J. A., Williams, W. W., and Laubaugh, E. E., *Jour. Med. Research*, 1913, xxviii, 345.

[6] Kolmer, J. A., *Jour. Exper. Med.*, 1913, xviii, 18.

That specific circulating antibodies for *Treponema pallidum* may be formed in syphilitic human beings and in experimentally inoculated animals has thus been rendered likely and has to some extent found support in experimental observation.

The observations which we desire to report in the present communication deal entirely with the appearance of specific agglutinating antibodies in the sera of rabbits intravenously treated with emulsions in salt solution of *Treponema pallidum,* killed by heating to 56° C. for thirty minutes. This temperature was chosen after it had been ascertained by Dr. Hopkins (in experiments as yet unpublished) that the thermal death point of these microörganisms lay between 50° and 55° C.

We have been studying antibody formation in syphilitic infection for some time, but our work could not be carried out on a sound experimental basis until we had developed a technique by which we could obtain larger quantities of treponema material which could be centrifugalized and washed free of culture fluid in the same way as this is done in similar experiments with bacteria. These methods have been in essence described in a previous paper.[7]

In all our antibody work in which the serum of treated rabbits was used, we took care to treat animals with treponema material that had been grown in sheep serum mixtures with salt solution or broth, whereas the treponemata used in the final reactions were grown on human ascitic fluid media. Such a technique was used in order to avoid the possibility of false reactions which might have been caused by the production of protein antibodies by antigen injected into the animals with remnants of the culture fluids. This is a precaution which we think should be taken in all experiments with this microorganism.

The material for agglutination was obtained by decanting the culture fluid from 250 cubic centimeter flasks in which the treponemata had been grown for from four to six weeks. Relatively slow centrifugation was first carried out for a short time to throw down coarse particles. After this, rapid and prolonged centrifugation yielded a sediment which when suspended in salt solution gave an evenly turbid emulsion very rich in treponemata. It should be noted

[7] Zinsser, H., Hopkins, J. G., and Gilbert, R., *Jour. Exper. Med.,* 1915, xxi, 213.

for the benefit of others undertaking these experiments that it is often difficult to free the treponema emulsion entirely from minute particles of precipitate likely to occur in old ascitic fluid mixtures. Such a precipitate often adds considerable turbidity to the final emulsion and may result in disturbing irregularities of reaction. In many of our experiments it will be noticed that ± is used, and this often signifies very slight flake formation in the tubes when such an unclean suspension was used. The reactions are as sharp and distinct as similar reactions on the agglutination of bacteria when the emulsions are unmixed with such precipitate. We have found it of advantage to discard material in which such a disturbing precipitate was present to any extent. When, as in a very few experiments, spontaneous agglutination occurred in salt solution this was of extremely slight degree only and of such a character that it was easily and sharply differentiable from that occurring in the positive tubes.

EXPERIMENT I.

Suspension Strain A (Macroscopic).

Serum of Rabbit 609 (Intravenously Treated with Five Injections of Suspension A Ranging from 1 to 5 Cubic Centimeters).

Each Tube Contained 0.5 of a Cubic Centimeter of Serum Dilution and 0.25 of a Cubic Centimeter of Suspension.

Concentration.	Serum 609. First bleeding.		Serum 609. Second bleeding.		Normal rabbit serum.	
	2 hrs.	15 hrs.	2 hrs.	15 hrs.	2 hrs.	15 hrs.
Undiluted	+++	+++	+++	+++	+++	+++
1 : 2	+++	+++	+++	+++	+++	+++
1 : 5	+++	+++	+++	+++	+++	+++
1 : 10	+++	+++	+++	+++	+++	+++
1 : 20	+++	+++	+++	+++	±	±
1 : 50	+++	+++	+++	+++	0	0
1 : 100	+++	+++	+++	+++	0	0
1 : 200	+++	+++	+++	+++	0	0
1 : 500	++	+++	+++	+++	0	0
1 : 1,000	±	0	Not set up		0	0
1 : 2,000	0	0			0	0
1 : 4,000	0	0			0	0
Salt solution (control)	0	0	0	0	0	0

EXPERIMENT II.

Suspension Strain A (Macroscopic).

Serum of Rabbit 611 (Intravenously Treated with Six Injections of Suspension A Ranging from 1 to 4 Cubic Centimeters).

Each Tube Contained 0.5 of a Cubic Centimeter of Serum Dilution plus 0.25 of a Cubic Centimeter of Treponema Suspension.

Concentration.	Serum 611.		Normal rabbit serum.	
	2 hrs.	15 hrs.	2 hrs.	15 hrs.
Undiluted	+++	+++	+++	+++
1 : 2	+++	+++	+++	+++
1 : 5	+++	+++	+++	+++
1 : 10	+++	+++	+++	+++
1 : 20	+++	+++	±	±
1 : 50	+++	+++	0	0
1 : 100	+++	+++	0	0
1 : 200	+++	+++	0	0
1 : 500	+++	+++	0	0
1 : 1,000	+++	+++	0	0
1 : 2,000	±	+++	0	0
1 : 4,000	0	±	0	0
Salt solution (control)	0	0	0	0

Since it seemed important to establish, if possible, the ordinary limits of the agglutinating properties of normal rabbit serum, we carried out the following experiment with a larger number of normal rabbit sera and a suspension of strain A. For comparison, rabbit sera 609 (five injections), 610 (five injections), and 619 (five injections) were set up with the same suspension.

EXPERIMENT III.

Concentration.	Serum 609.	Serum 610.	Serum 619.	Normal 1.	Normal 2.	Normal 3.	Normal 4.	Normal 5.	Normal 6.	Normal 7.
Undiluted	+++	+++	+++	++	++	+++	++	++	+++	++
1 : 10	+++	+++	+++	±	±	+	±	±	±	±
1 : 20	+++	+++	+++	±	±	+	±	±	±	±
1 : 50	+++	+++	+++	±	±	+	±	±	+	±
1 : 100	+++	+++	+++	±	±	+	±	±	+	±
1 : 200	+++	+++	+++	±	±	+	±	±	+	±
1 : 500	+++	+	+++	±	±	+	±	±	+++	±
1 : 1,000	+	+	+++	±	±	+	±	±	+	±
1 : 2,000	+	+	+++	±	±	+	±	±	+	±
1 : 4,000	+	+	±	±	±	+	±	±	+	±
Salt solution (control)	±	±	±	±	±	+	±	±	±	±

In interpreting this experiment it is necessary to note that the ±, or very slight flocculation, which occurred throughout in the normal

sera, occurred also in the salt solution controls. It must, therefore, be looked upon as spontaneous clumping, in our opinion due, in part at least, to the fact that the emulsion of treponemata on this day contained not inconsiderable amounts of the precipitate granules mentioned above. Moreover, this flocculation in the normal sera and in the salt solution was slow and in character recognizably different from that appearing in the tubes with immune serum, in that in the last named the flakes formed rapidly and settled out in a short time.

The irregularity in normal serum 6 we cannot explain, but feel that in spite of it our results are sufficiently convincing.

Since at this time we happened to possess good material from a mass culture of one of the strains which Dr. Noguchi had kindly sent us, his No. 9, our Noguchi I, we carried out an experiment to determine whether the serum of a rabbit treated with our strain A would agglutinate Noguchi I.

EXPERIMENT IV.

Suspension Strain Noguchi I. Serum of Rabbit 609.

Each Tube Contained 0.5 of a Cubic Centimeter of Serum Dilution and 0.25 of a Cubic Centimeter of Spirochete Suspension.

Concentration.	Serum 609. First bleeding.		Serum 609. Second bleeding.		Normal rabbit serum.	
	2 hrs.	15 hrs.	2 hrs.	15 hrs.	2 hrs.	15 hrs.
Undiluted	+++	+++	±	+++	0	++
1 : 2	+++	+++	++	+++	0	+
1 : 5	+	+++	++	+++	Not set up	
1 : 10	±	+++	0	+++	0	±
1 : 20	0	+++	0	+++		
1 : 50	+++	+++	0	+++		
1 : 100	+++	+++	±	+++	Not set up	
1 : 200	+++	+++	++	+++		
1 : 500	+++	+++	+	+++		
Salt solution (control)	0	0	0	0	0	0

DISCUSSION.

The protocols which are reported above are based entirely upon experiments done with macroscopic agglutination carried out in small test-tubes. The sharpness with which these can be read is apparent from figure 1. We have also carried out a number of microscopic agglutinations in which small drops of the mixtures of serum

dilution and treponema emulsion were placed on slides, covered with cover-slips, and rimmed with vaselin. In preparations so made it was very easy to follow the agglutination in a way exactly similar to that habitual in the usual Widal reaction in typhoid fever. The clumping was sharp and distinct, and false clumping, that is, that which would depend upon the presence of disturbing precipitate in the preparations, could easily be ruled out by this method, although our microscopic reactions thus far done were set up with very clean treponema preparations and were interpreted with absolutely no difficulties, as, we think, will be apparent from the photographs attached to this paper (figures 2 to 5).

The results we have obtained, of course, obviously suggest the possibility of working out an agglutination reaction with emulsions of *Treponema pallidum* prepared by our technique, and human sera, with possibly some diagnostic value.

We are especially encouraged in the hope, since Zabolotny and Maslakowetz[8] have observed clumping of the microörganisms in drops of exudate taken directly from primary lesions. In order to obtain some light upon this we immediately carried out a number of microscopic and macroscopic agglutination reactions with sera taken from a series of cases, some of them showing positive and some of them negative Wassermann reactions. It was revealed that many human sera used in concentration and in dilutions as high as 1 to 10 will agglutinate strain A. It will be necessary to examine a large series of normal and syphilitic sera, carefully controlled by clinical observation.

The last experiment we have reported also suggests the possibility that by agglutination it may be possible to determine that a given treponema belongs to the species pallidum. However, it will be necessary first to ascertain whether sera prepared by injections of pallidum may not also agglutinate spirochetes of other species.

SUMMARY.

It has been shown by our experiments that the serum of rabbits treated with emulsions of *Treponema pallidum* contains agglutinating substances.

[8] Zabolotny, D., and Maslakowetz, *Centralbl. f. Bakteriol., 1te Abt., Orig.*, 1907, xliv, 532.

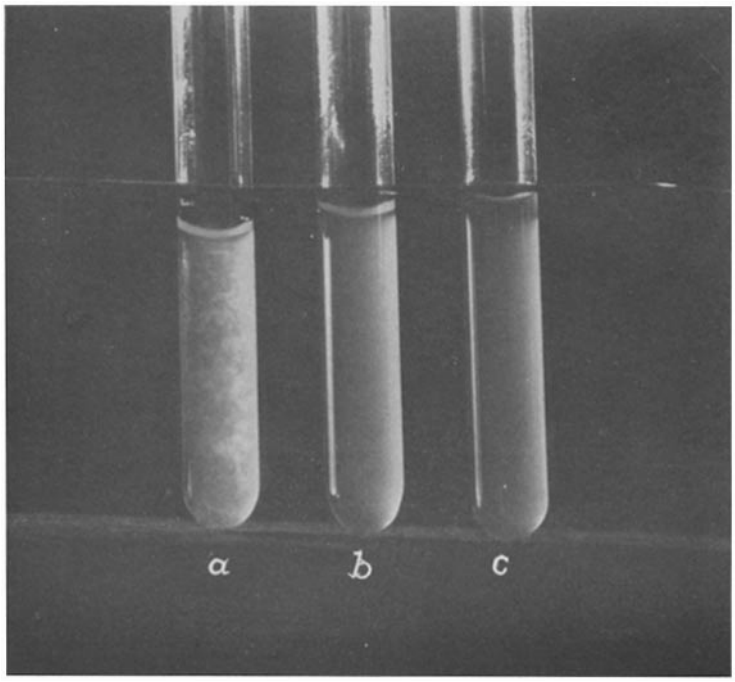

FIG. 1.

(Zinsser and Hopkins: Antibody Formation.)

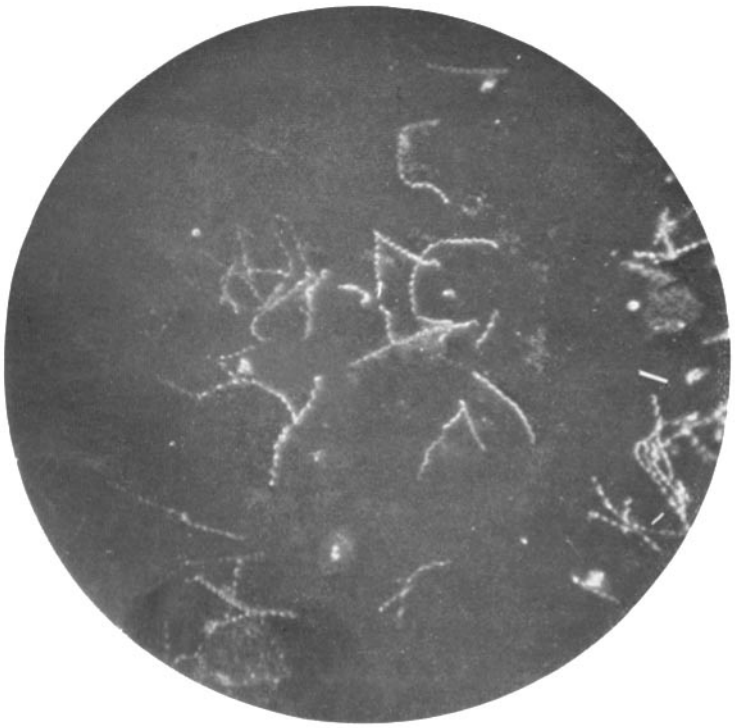

FIG. 2.

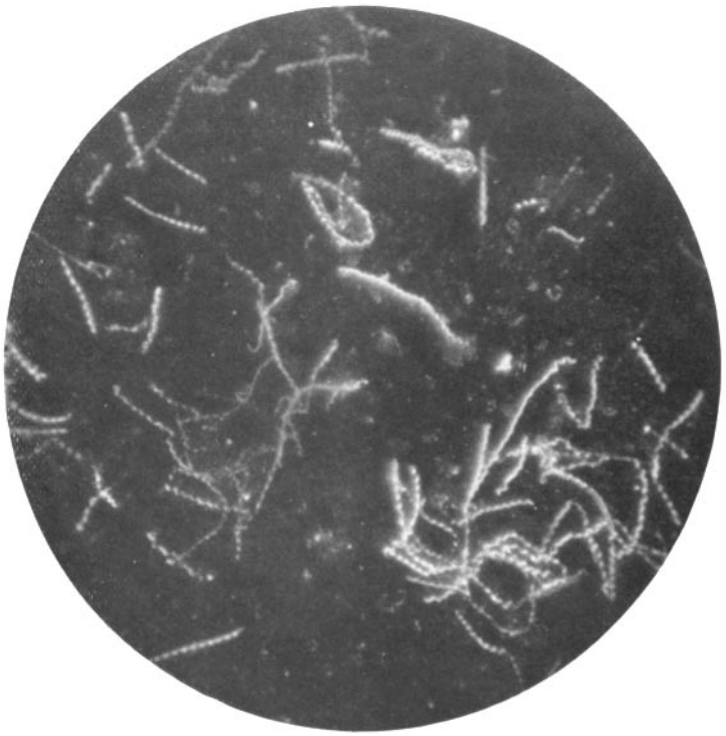

FIG. 3.

(Zinsser and Hopkins: Antibody Formation.)

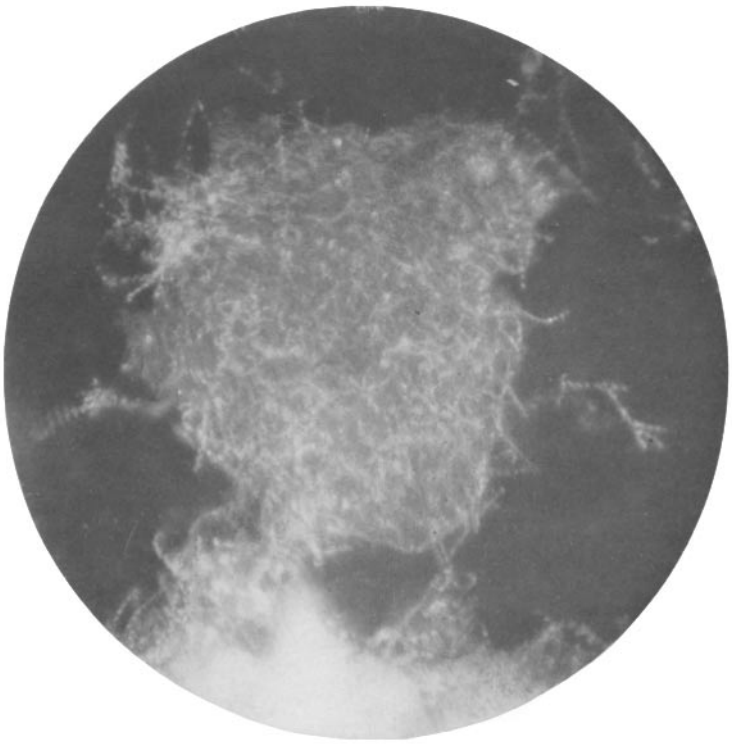

FIG. 4.

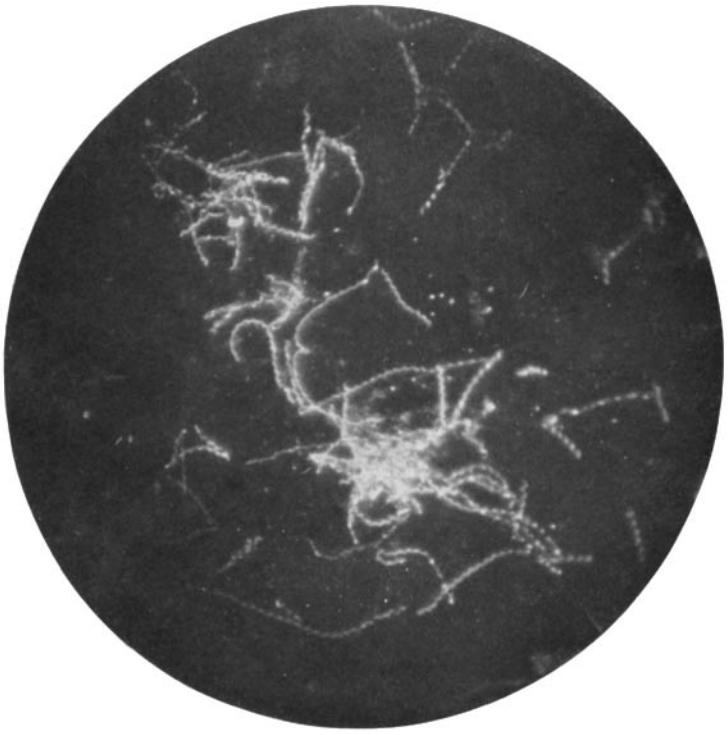

FIG. 5.

(Zinsser and Hopkins: Antibody Formation.)

Normal rabbit serum also possesses agglutinating power for this organism, but, as in the case of normal bacterial agglutinins, to an extent very much inferior to that possessed by the sera of immunized animals. Normal human sera will agglutinate similar pallidum emulsions, as will the sera of certain syphilitic patients with positive Wassermann reactions. Whether or not there is a quantitative difference of diagnostic value between the sera of normal human beings and those of syphilitics remains to be seen.

The sera of rabbits immunized with strain A agglutinate Noguchi's strain 9 in dilutions as high as 1 to 500.

We regard as the most important result of these experiments the demonstration of definite antibodies in the circulation of animals treated with dead emulsions of *Treponema pallidum*. Since it is our belief[9] that the agglutinating effect is due to an antibody essentially the same as that which produces bactericidal, precipitating, and opsonic effects, *i. e.*, that there is probably one type of antibody only, we believe that the demonstration of agglutinins establishes the fact that in syphilis as in bacterial diseases the host responds by the formation of antibodies or sensitizers specific for the treponema.

Spirocheticidal experiments with these sera, both *in vitro* and *in vivo*, are in progress.

EXPLANATION OF PLATES.[10]

PLATE 52.

FIG. 1. Microscopic agglutination. A, immune serum 1 : 50. B, normal serum 1 : 50. C, salt solution control.

PLATE 53.

FIG. 2. Microscopic appearance of the salt solution control.

FIG. 3. Microscopic appearance of the preparation with normal rabbit serum 1 : 50.

PLATE 54.

FIG. 4. Microscopic appearance of the preparation containing immune serum 1 : 50.

FIG. 5. Microscopic appearance of the preparation containing immune serum 1 : 2,000.

[9] Zinsser, H., *Jour. Exper. Med.*, 1913, xviii, 219.

[10] We are indebted to Dr. John E. McWhorter for the photomicrographs reproduced in figures 2 to 5.