BIOLOGICAL RELATIONSHIPS OF DIPLOCOCCUS INTRACELLULARIS AND GONOCOCCUS.[1]

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Recent studies by Brickner and Cristéanu[2] having shown a marked similarity between *Diplococcus intracellularis* and gonococcus, as instanced by their agglutinin and precipitin reactions, as well as their effect on inoculated animals, it seemed interesting to follow the same lines of work and carry them further by testing for possible specificity of the immune bodies developed in the sera of animals immunized to these two organisms. Some of the experiments made by Dr. Flexner[3] on the biology of the diplococcus were repeated with the gonococcus in order to detect possible differences.

BIOLOGY OF THE GONOCOCCUS.

Like the intracellularis, the gonococcus is a coffee-bean shaped diplococcus occurring more frequently within leucocytes than outside them. Comparison of smears from the pus of a recent case of gonorrheal vaginitis and from the cerebro-spinal fluid of an early case of cerebro-spinal meningitis (both in infants) shows a marked similarity, in that both present many polymorphonuclear leucocytes containing from one to ten or more pairs of Gram-negative diplococci, and some pairs of cocci lying extra-cellular. There is, however, a decided difference in the size of the two varieties of organisms, the intracellularis being much the larger of the two. This relatively larger size is also seen in smears made from the peritoneal exudate in guinea-pigs killed by inoculations of diplococcus and gonococcus respectively. In agar cultures, twenty-four hours old, on the other hand, the gonococcus is larger, possibly because its growth is so much less profuse.

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[2] Brickner and Cristéanu, *Compt. rend. de la Soc. de Biologie,* 1906, lx, 846; 942; 988; 1070.

[3] Flexner, *Jour. of Exper. Med.,* 1907, ix, 105.

Gonococcus grows best upon glucose-serum-agar, prepared by adding about one third its volume of human pleuritic or ascitic fluid to the melted agar. The reaction of the medium proved unimportant unless very alkaline to phenolphthalein, a faintly acid, neutral, or faintly alkaline medium giving about equal growths.

The cultures of gonococcus studied were isolated from cases of vaginitis occurring in young infants at the Babies' Hospital. Smears from the discharge in such cases show the gonococcus to be the only organism present in almost every instance, so that glucose-serum-agar plates made from the pus obtained by passing a platinum loop into the vaginal canal gave, in the majority of instances, an abundant and almost pure growth of gonococcus colonies.

Glucose-agar and dog's serum-agar proved useless as a medium for the cultivation of the first generation of gonococci, but subsequent plants from human serum-agar to these media gave a faint growth consisting of separate colonies surviving from two to five days, and bearing transplantation for from two to six generations only. Sheep's serum-agar proved an excellent culture medium, providing that fully one third volume of serum was added. The sheep's serum-glucose-agar ordinarily used for the growth of *Diplococcus intracellularis* was useless, because it contained too little serum. Thalmann's[4] agar gave excellent growths of all the strains of gonococci, but the cultures survived only from seven to twelve days. Picker[5] found that not all strains of gonococcus grew on Thalmann's agar, but some survived twenty-one days, and some even as long as six months, if the tubes did not become dry. The advantage of Thalmann's agar would seem to lie in its reaction, obtained by neutralizing two thirds of the quantity of agar to phenolphthalein and then adding the other, acid, third. Vannod[6] found that plain agar made slightly alakaline to litmus paper is a very suitable medium for the gonococcus, while if the agar is alkaline to phenolphthalein the organism will not grow upon it. I did not repeat Vannod's experiments.

On human serum-glucose-agar slants gonococci remained viable frow twenty-six to thirty-eight days when capped with rubber and

[4] Thalmann, *Cent. f. Bakt.*, 1900, xxvii, 828; 1902, xxxi, 678.
[5] Picker, *Wien. klin. Woch.*, 1906, xix, 1282.
[6] Vannod, *Cent. f. Bakt.*, 1906, xl, 162; 1907, xliv, 10.

kept at 37° C., or as long as the tubes remained moist. The corresponding uncapped cultures in the thermostat usually survived from sixteen to twenty-one days. The addition of a drop of a suspension of calcium carbonate did not prolong the viability of the gonococcus beyond the period of survival on moist serum-glucose-agar, while the danger of making the medium too alkaline with the carbonate is a drawback to its employment.

Gonococcus did not grow in sheep's serum-water containing sugars, but in human serum-water litmus medium to which dextrose or maltose had been added gonococci caused slight reddening without coagulation. Lactose, saccharose, mannite and dextrine were unaffected. In their ability to ferment sugars, gonococcus and intracellularis acted alike. Dunn and Gordon[7] found that the gonococcus did not affect maltose, thus differentiating the two varieties of cocci. The ten strains I isolated from infants all fermented maltose.

Some of the experiments in viability and autolysis made by Dr. Flexner[8] with diplococcus were repeated with gonococcus, suspensions being made in salt solution and also in Ringer's fluid, a duplicate series kept in the ice chest and in the thermostat at 37° C. The suspensions were of four different strengths: the original turbid suspension, and the same diluted twice, five and ten times. The results were almost parallel with those obtained by Dr. Flexner with the *Diplococcus intracellularis,* more cocci remaining viable in the concentrated salt solution suspensions kept at 7° C., and more in the weaker suspensions at 37° C. While in Ringer's fluid the larger number of cocci survived in the concentrated suspension at 37° C., and in the weaker suspension at 7°C. Cover slips were made daily from these tubes, and the cocci found to be less disintegrated in the lower than in the higher salt solution suspensions kept in the thermostat, while in the tubes kept in the ice chest there was remarkably less disintegration, many cocci staining well on the sixth day, although growth had ceased on the third from both salt and Ringer's solution suspensions. Growth took place after six days in sub-cultures made from the salt solution suspensions kept at 37°

[7] Dunn and Gordon, *Brit. Med. Jour.,* 1905, ii, 421.
[8] Flexner, *loc. cit.*

C., and after seven days from the Ringer's solution suspensions. Cover slips showed rather less daily disintegration in the Ringer's solution than in the salt solution. Among the surviving cocci the larger, more resistant pairs, taking on a deep safranin stain, described by Dr. Flexner, were distinctly seen in the cover slips. As in the case of the intracellularis, cold is more injurious than warmth to the gonococcus. But unlike the intracellularis, Ringer's fluid did not prolong the viability of the gonococcus beyond or even up to the period of survival in ascitic-glucose-agar. Even when kept in the ice chest (at 37° C.) growth was obtained in sub-cultures on the seventh day from the serum-agar tubes, while such tubes kept in the thermostat gave excellent sub-cultures on the twenty-seventh to thirty-ninth days. Cover slips showed many deeply staining cocci at that late day, but most of the cocci had been disintegrated.

Two different strains of the diplococcus grew in sub-cultures made on the thirty-fifth day after inoculation on moist sheep's serum-agar, the tubes having been kept constantly at 37° C. A parallel set of tubes kept in the ice chest gave growth for seven days only and no well-staining cocci could be detected in cover slips made on the eighth day. Thus while gonococcus survives longer on solid media kept at 37° C. than does the *Diplococcus intracellularis,* the viability of both organisms on such media at 7° C. is about the same.

As in the case of the diplococcus, the autolytic ferment of the gonococcus is destroyed by exposure to a temperature of 65° C. for thirty minutes. Suspensions in water, salt solution and Ringer's fluid gave the same results in this respect.

PATHOGENESIS OF THE GONOCOCCUS.

Cultures of gonococcus isolated from cases of vaginitis at the Babies' Hospital were inoculated into white mice and young guinea-pigs. The first or second generation was used to inoculate the surface of a pint Blake bottle of serum-glucose-agar. After twenty-four hours at 37° C. the growth was suspended in four cubic centimeters of 0.9 per cent. salt solution and one half injected into the peritoneal cavity of each of two guinea-pigs weighing between 170 and 200 grams. In this way nine different strains were injected in

the second or third generation. All the pigs died within twenty to twenty-four hours. White mice succumbed to smaller doses of recent cultures, one serum-agar slant being sufficient as a rule to cause death over night, half a tube failing to do this. As the mice reacted very irregularly to the inoculations, they were not used extensively.

Cultures lose their virulence readily. It was found that where a second or third generation had caused death in a guinea-pig (170 to 200 grams) in twenty hours, when given doses of half the surface growth of a pint Blake bottle, the sixteenth or eighteenth generation proved not to produce a fatal result when the entire growth in the bottle was injected.

It has been shown by Bail[9] that sub-lethal doses of bacteria may become lethal under the influence of fluids containing aggressins, so-called, which remove the natural protective powers of the organism. While Bail and Weil[10] maintain that aggressins are formed and found chiefly, though not exclusively, in the body fluids, and first at the point of inoculation where the bacteria are proliferating most rapidly, Wassermann and Citron[11] were able to achieve apparently the same results with bacterial extracts made by shaking cultures in normal rabbit's serum or distilled water; and they insist that aggressins are not newly formed in the animal organism, but are merely a dissolved bacterial substance, which is itself toxic. Bail[12] holds the opinion that the natural aggressins described by him, and the artificial ones obtained by Wassermann and Citron are not identical; but into this discussion I shall not enter.

Only artificial gonococcus and diplococcus aggressins, so-called, were used in my work, the extracts being prepared by suspending the twenty-four hours old growth on the surface of a Blake bottle in five cubic centimeters of salt solution, adding a few drops of toluol and leaving the cocci to autolyze over night at 37° C., after which the resulting fluids were preserved in the refrigerator. Just

[9] Bail, *Arch. f. Hyg.*, 1905, lii, 272.

[10] Weil, *Cent. f. Bakt.*, 1906, xli, 121. Bail and Weil, *Cent. f. Bakt.*, 1906, xlii, 51.

[11] Wassermann and Citron, *Deut. med. Woch.*, 1905, xxxi, 1101. Citron, *Cent. f. Bakt.*, 1906, xli, 230.

[12] Bail and Weil, *Cent. f. Bakt.*, 1906, xlii, 51.

before inoculating, the extract was centrifuged until clear or nearly so, and the toluol removed by evaporation in the thermostat (37° C.). Intracellularis extracts were made with ten cubic centimeters of salt solution because the growths were so much more profuse than those of the gonococcus. Whether the addition of this extract to a sub-lethal dose of the coccus would increase its pathogenicity was tested in two ways: First, non-fatal doses of the coccus and its own extract were used; second, the extract of a recent culture (third generation) was given with the cocci of an old culture (forty-ninth generation) and vice versa.

White mice were used for the cross experiments between old and recent strains. It became apparent that the addition of half a cubic centimeter of extract to a non-fatal dose of a twenty-four hour old culture of its own or the other strain of gonococcus caused the death of white mice within twenty-four hours. Specificity of the aggressin is not limited to the homologous strain of gonococcus.

Working with a strain which did not kill a guinea-pig, weighing 170 to 200 grams, in doses of two cubic centimeters, it was found that the addition of a quarter of a cubic centimeter of its extract made the dose a fatal one. Conversely, two cubic centimeters of the extract proving sub-lethal, the addition of a quarter of a cubic centimeter of a surface growth in a Blake bottle suspended in five cubic centimeters of salt solution caused death within ten to twenty hours. But on decreasing the maximum sub-lethal dose of extract or culture in these combinations the animals did not die regularly within twenty-four hours, so that the invariably fatal dose proved to be two cubic centimeters of suspension of the culture plus one quarter of a cubic centimeter of extract, or vice versa.

Working with a diplococcus which was not fatal in doses of half a cubic centimeter (of a ten cubic centimeter salt solution suspension of a twenty-four hour growth on sheep's serum-agar in a pint Blake bottle), the addition of half a cubic centimeter of the extract of the same coccus caused death within eighteen hours, a smaller dose of either extract or culture proving non-fatal. Nothing less than one cubic centimeter of this extract alone killed over night.

The fatal dose of both the gonococcus and the intracellularis combinations having been determined, cross reactions were made. The

suspensions of intracellularis and gonococcus were always made as nearly equal in strength as possible. It was found that a larger dose of gonococcus culture, two cubic centimeters, was required to make half a cubic centimeter of diplococcus extract fatal, while only half a cubic centimeter of diplococcus culture sufficed to make two cubic centimeters of gonococcus extract kill within twenty hours; more was needed than of intracellularis culture to raise the power of the other organism to the fatal point. Thus it becomes evident that the aggressive action of the extracts of gonococcus and diplococcus is more potent for its own than for the other variety of coccus, though the two may act interchangeably in larger doses, and hence are not specific. Dörr[13] has shown the lack of specificity of many bacterial aggressins (coli, dysentery, cholera, pyocyaneus, staphylococcus), and Paul and Lotti[14] found a certain quantitative but not qualitative specificity among them. Bail[15] and Salus,[16] on the other hand, maintain that the natural aggressins are strictly specific.

To prove whether inoculation with living gonococcus cultures or with their extracts protected against intracellularis cultures and extracts, and vice versa, pigs which survived the above experiments were later given a lethal (or larger) dose of the other organism. The diplococcus extract alone, and also non-fatal combinations of extracts and culture did not protect against a fatal dose of a recent diplococcus culture given five to twenty-eight days later. Sublethal doses of intracellularis culture (0.05 to 0.2 cubic centimeter) protected against a fatal dose given seven to nine days later, while less (0.025 cubic centimeter) did not protect. The gonococcus culture alone, and the culture plus the extract, enabled pigs to survive a fatal dose of diplococci injected thirteen to thirty days later. Combinations of gonococcus cultures and intracellularis extract protected against a lethal intracellularis dose administered two to five days later. Not only is specificity lacking here, but the gonococcus alone or in combination with its extract seems to be a more powerful

[13] Dörr, *Wein. klin. Woch.*, 1906, xix, 759; 1038; 1081.
[14] Paul and Lotti, *Cent. f. Bakt.*, 1907, xliii, 718; 809.
[15] Bail, *loc. cit.*
[16] Salus, *Wien klin. Woch.*, 1906, xix, 870.

protection against fatal doses of living diplococci than the diplococcus itself.[17]

The anatomical lesions found in guinea-pigs dying within twenty-four to thirty-six hours after inoculation with living gonococcus cultures are very similar to those described by Dr. Flexner[18] in pigs which succumbed to intracellularis injections. There are marked œdema of the pancreas and surrounding tissues, congestion or hæmorrhage of the adrenals, small hæmorrhages into the mesentery, serous coat of the intestines and the parietal peritoneum, with more or less clear or turbid fluid in the peritoneal cavity, and a layer of pus and fibrin over the liver, spleen and omentum. An increased amount of clear fluid in the pleural cavities is often noted. Cover slips from the peritoneum and omentum show varying numbers of polymorphonuclear leucocytes and of diplococci, within and outside these cells. Multiplication of the cocci is more in evidence after inoculation with both culture and extract than when culture alone is injected, and phagocytosis is much less marked under those conditions. When the extract alone has been administered neither cocci nor leucocytes appear in the cover slips, and cultures remain sterile.

SERUM REACTIONS.

Precipitins.—Four sera were tested for precipitin reactions. Three were from rabbits immunized to the gonococcus, and one from a rabbit inoculated ten times with the intracellularis. The sera were tested from twenty to twenty-seven hours after bleeding and the cocci were prepared in four different ways: the sodium hydrate (0.15 per cent.) macerations recommended by Brickner and Cristéanu;[19] salt solution macerations prepared in the same way; salt solution toluol extract described by Flexner;[18] and the filtrate of Thalmann's broth used by Torrey.[20] Cultures of diplo-

[17] The reactions just described call for a special study in order to establish their significance. The failure of the *Diplococcus intracellularis* to induce resistance or immunity, may be due to a slower final recovery period than in the case of the gonococcus. The reaction noted of the gonococcus versus the diplococcus may, possibly be of the nature of the non-specific reactions of resistance produced by such an indifferent body as bouillon which also endure for a brief period of time.

[18] Flexner, *loc. cit.*

[19] Brickner and Cristéanu, *loc. cit.*

[20] Torrey, *Jour. of Med. Research,* 1907, xi, 329.

coccus, gonococcus and *Micrococcus catarrhalis* were used, and normal fresh rabbit's serum as control. Only one serum (from a rabbit receiving nine inoculations of living gonococci) gave any precipitin reaction, and that only in dilutions of one to ten and one to twenty for both gonococcus and diplococcus. It gave no reactions with *M. catarrhalis.* The normal serum and salt solution controls were always negative. Two other anti-gonococcus sera and one anti-diplococcus serum gave negative precipitin reactions, yet all these sera showed the presence of immune body as demonstrated by the deviation of complement (*vide infra*). Muir and Martin[21] have shown that the formation of precipitate is not a necessary accompaniment of the phenomena of complement deviation in antisera. Brickner and Cristéanu[19] found the precipitin reactions with gonococcus and intracellularis in anti-gonococcus serum to be identical. Torrey[22] finds an appreciable difference between the two.

Agglutinins were not high in amount in any serum obtained by immunizing rabbits with increasing doses of gonococcus and diplococcus over periods of eight to ten weeks. Six sera were examined: (*a*) agglutinated both gonococcus and intracellularis in dilutions of one to ten before inoculation, and in one to fifty after seven injections of living cocci; (*b*) agglutinated one to twenty before inoculation and both gonococcus and diplococcus in dilutions of one to four hundred after ten injections of living cocci; (*c*) agglutinated one to ten before inoculation; after ten doses of gonococcus extract, gonococci were agglutinated in dilutions of one to one hundred, diplococci only in one to fifty dilutions; (*d*) agglutinated in one to ten before treatment, ten doses of living gonococci and extract injected, after which both gonococcus and diplococcus were agglutinated in dilutions of one to fifty; (*e*) did not agglutinate either coccus before treatment; eleven inoculations of living diplococci developed agglutinins for both gonococcus and diplococcus in dilutions of one to four hundred, one to six hundred was negative; (*f*) agglutinated in dilutions of one to twenty before inoculation, and after nine doses of living gonococci the serum gave positive agglutination with gonococci in dilutions of one to one hundred, and with intracellularis, one to twenty.

[21] Muir and Martin, *Jour. of Hyg.,* 1906, vi, 265.
[22] Torrey, *loc. cit.*

Bruck's[23] statement that inoculations with living cultures produce a serum rich in agglutinins but poor in amboceptors seems to be borne out in only two of the four sera so produced in my experiments. But the sera showing the highest agglutination reactions were both obtained with living cultures. One of them, however, showed the highest amboceptor content of any serum studied. On the other hand, the sera produced by means of inoculations with extracts with or without living cocci showed very low agglutinations.

The anti-diplococcus serum kindly supplied by Dr. Jobling was from a horse which is being inoculated with cultures and extracts of *Diplococcus intracellularis*. It agglutinated with the intracellularis in dilutions of one to one hundred, and gonococcus, one to fifty.

Brickner and Cristéanu[24] obtained exactly the same amount of agglutination with diplococcus and gonococcus with the serum of a horse inoculated with gonococci. Vannod,[25] on the other hand, found a marked difference in the degree to which these two organisms agglutinated in their respective specific sera, and believed that while group agglutinins exist, a large number of specific ones also exist. Torrey's[26] results led him to the opinion that the agglutinins for gonococcus and intracellularis are common in very low dilutions only. As no one of my sera agglutinated its homologous coccus (grown on Thalmann's agar) in dilutions higher than one to four hundred, the contrast with Torrey's positive reactions in dilutions of one to two thousand to one to seven hundred thousand after nine or ten inoculations is very marked.

Deviation of Complement.—Müller and Oppenheim[27] were the first to demonstrate specific anti-bodies in the serum of a male (adult) case of gonorrheal arthritis by means of the complement deviation test, normal human serum being used as controls.

Bruck showed the presence of specific immune bodies for gono-

[23] Bruck, *Deut. med. Woch.*, 1906, xxxii, 1368.
[24] Brickner and Cristéanu, *loc. cit.*
[25] Vannod, *Deut. med. Woch.*, 1906, xxxii, 1984.
[26] Torrey, *loc. cit.*
[27] Müller and Oppenheim, *Wien. klin. Woch.*, 1906, xix, 894.

cocci in the serum of three adult cases of gonorrhœal disease (two females and one male). Agglutinins and precipitins were lacking in all of them. He also called attention to the existence of such amboceptors in the serum of inoculated rabbits.

Vannod[28] used gonococcus nucleo-proteid for the immunization of rabbits, and obtained a serum which agglutinated gonococcus in dilutions of one to three hundred and contained sufficient specific immune bodies to inhibit hæmolysis by deflecting complement when added in proportion of 0.01 cubic centimeter to the serum. Another serum, agglutinating at one to four hundred, prevented hæmolysis when present in a dilution of 0.025 and 0.001 cubic centimeter. He thinks that agglutinins and specific anti-body develop side by side, not independently, as Bruck[29] has stated.

Four sera were tested for specific immune bodies by means of Bordet and Gengou's[30] method of complement deviation in the presence of antigen, using a hæmolytic system as the indicator. The free receptors in the extract (antigen) having been bound to the complement by the amboceptor (specific) present in the immune serum to be tested, the addition (after one hour) of an inactivated lytic serum and its corresponding corpuscles caused no hæmolysis. In the present tests, washed hen's corpuscles in five per cent. solution and rabbit's serum made lytic to hen's corpuscles were used. Fresh guinea-pig serum was employed as complement, in amounts varying from one twentieth to one fortieth of a cubic centimeter. Normal horse serum and normal rabbit serum were used as controls, as three of the immune sera were from rabbits and one from a horse. The immune sera and the extracts were tested with corpuscles alone and with hæmolytic system alone, with and without complement, before being combined with either.

1. Serum 494, from a rabbit inoculated with gonococcus extract in salt solution. This serum inhibited hæmolysis by deviating complement in dilutions of one to fifty, with one tenth of a cubic centimeter of extract (Table I). The results were identical when diplococcus extract was used. Having found the minimum amount of amboceptor necessary to bind the complement to the receptors in

[28] Vannod, *loc. cit.*

[29] Bruck, *loc. cit.*

[30] Bordet and Gengou, *Ann. de l'Inst. Pasteur,* 1901, xv, 289.

the extract and thus prevent hæmolysis, this amount was doubled and the antigen titrated against it. Then it developed that 0.05 cubic centimeter of immune serum deviated complement in dilutions of antigen from 0.1 to 0.002 of a cubic centimeter (Table II); and, going further by titrating the serum against the smaller dose of extract, 0.005 cubic centimeter sufficed to inhibit hæmolysis in the presence of 0.05 to 0.0005 cubic centimeter of immune serum (Table III). Below this dilution hæmolysis was complete. As the results were identical with gonococcus and diplococcus extracts with anti-gonococcus serum, it follows that the amboceptor in that serum was as readily bound to the pre-receptors in the intracellularis extract as to those in the gonococcus extract, and strict specificity is, therefore, lacking. I am indebted to Dr. Jobling for the suggestion to titrate immune serum and antigen successively against double the smallest binding dose of either. In this way smaller amounts of immune body were demonstrable. It was hoped, also, to bring out specific differences between the effect of the two extracts upon the serum, but none such were obtained.

Normal rabbit serum with similar dilutions of antigen showed no inhibition of hæmolysis. The following tables embody the above results.

TABLE I.

Immune Serum No. 494.	Complement Guinea-pig	Antigen		Anti-hen Rabbit's Serum.	Hen's Corpuscles.	Results.
		Gonococcus.	D. Intracellularis.			
0.1	0.05	0.1	0	0.01	0.05	—
0.05	"	0.1	0	"	"	—
0.02	"	0.1	0	"	"	—
0.01	"	0.1	0	"	"	±
0.005	"	0.1	0	"	"	+
0.1	"	0	0.1	"	"	—
0.05	"	0	0.1	"	"	—
0.02	"	0	0.1	"	"	—
0.01	"	0	0.1	"	"	±
0.005	"	0	0.1	"	"	+

The sign + means complete hæmolysis; — no hæmolysis; and ± incomplete hæmolysis.

The following controls, nine in number, were made with every series of tests. I give them here instead of repeating them in each

Controls.

No.	Immune Serum.	Guinea-pig Complement.	Antigen.	Anti-hen Serum.	Hen's Corpuscles.	Results.
1	0	0.05	0.1	0.01	0.05	++
2	0.5	0	0.1	0.01	0.05	—
3	0.5	0.05	0	0.01	0.05	++
4	0	0	0	0.01	0.05	—
5	0	0.05	0	0.01	0.05	++
6	0	0	0.1	0	0.05	—
7	0.5	0	0	0	0.05	—
8	0	0.05	0	0	0.05	—
9	0	0	0	0	0.05	—

TABLE II.

Immune Serum No. 494.	Guinea-pig Complement.	Antigen.		Anti-hen Rabbit's Serum.	Hen's Corpuscles.	Results.
		Gonococcus.	D. Intracellularis.			
0.05	0.025	0.1	0	0.01	0.05	—
"	"	0.05	0	"	"	—
"	"	0.02	0	"	"	—
"	"	0.01	0	"	"	—
"	"	0.005	0	"	"	—
"	"	0.002	0	"	"	—
"	"	0.001	0	"	"	+
"	"	0	0.1	"	"	—
"	"	0	0.05	"	"	—
"	"	0	0.02	"	"	—
"	"	0	0.01	"	"	—
"	"	0	0.005	"	"	—
"	"	0	0.002	"	"	—
"	"	0	0.001	"	"	+

TABLE III.

Amboceptor Immune Serum No. 494.	Guinea-Pig Complement.	Antigen.		Anti-hen Serum.	Hen's Corpuscles.	Results.
		Gonococcus Extract.	D. Intracellularis Extract.			
0.05	0.025	0.005	0	0.01	0.05	—
0.02	"	"	0	"	"	—
0.01	"	"	0	"	"	—
0.005	"	"	0	"	"	—
0.002	"	"	0	"	"	—
0.001	"	"	0	"	"	—
0.0005	"	"	0	"	"	—
0.0002	"	"	0	"	"	+
0.05	"	"	0.005	"	"	—
0.02	"	"	"	"	"	—
0.01	"	"	"	"	"	—
0.005	"	"	"	"	"	—
0.002	"	"	"	"	"	—
0.001	"	"	"	"	"	—
0.005	"	"	"	"	"	—
0.002	"	"	"	"	"	+

table. It is hardly necessary to say that only when these controls were correct were the results of the experiments admitted. The quantity of immune serum and antigen varied in each series of controls according to the test to be made. The amount of complement, corpuscles and antigen serum were the same throughout.

2. Serum 495. A second anti-gonococcus rabbit's serum, obtained after ten injections of gonococcus extract and cultures, was found to be anti-hæmolytic without antigen whenever guinea-pig complement was employed. As the serum had been inactivated by heating to 54° C. for thirty minutes, it was thought that anti-hæmolytic substances might have developed under the influence of heat. But on using rabbit complement, hæmolysis was complete, even with 0.2 cubic centimeter of the serum.[31] One tenth cubic centimeter of antigen inhibited hæmolysis in the presence of 0.001 cubic centimeter of immune serum (Table IV). In titrating anti-

TABLE IV.

Amboceptor Immune Serum No. 495.	Rabbit Serum Complement.	Antigen.		Anti-hen Rabbit's Serum.	Hen's Corpuscles.	Results.
		Gonococcus Extract.	D. Intracellularis Extract.			
0.1	0.05	0.1		0.01	0.05	—
0.05	"	0.1		"	"	—
0.02	"	0.1		"	"	—
0.01	"	0.1		"	"	—
0.005	"	0.1		"	"	—
0.002	"	0.1		"	"	—
0.001	"	0.1		"	"	—
0.1	"	0	0.1	"	"	—
0.05	"	0	0.1	"	"	—
0.02	"	0	0.1	"	"	—
0.01	"	0	0.1	"	"	—
0.005	"	0	0.1	"	"	—
0.002	"	0	0.1	"	"	—
0.001	"	0	0.1	"	"	—
1.0005	"	0	0.1	"	"	—

[31] Presumably the guinea-pig serum contained free receptors to which the amboceptor in the immune rabbit serum anchored the complement, thus preventing hæmolysis when the hæmolytic serum was added. On saturating the immune serum with guinea-pig corpuscles over night in the ice chest, and centrifuging the next day, the anti-hæmolytic power of the serum was found to have been lost. The same result was brought about by using rabbit instead of guinea-pig serum as complement.

gen against 0.002 cubic centimeter of immune serum, some differences in the results with the two extracts were noted, the gonococcus being the stronger of the two (Table V).

TABLE V.

Amboceptor Immune Serum No. 495.	Rabbit Serum Complement.	Antigen.		Anti-hen Rabbit's Serum.	Hen's Corpuscles.	Results.
		Gonococcus Extract.	D. Intercellularis Extract.			
0.002	0.05	0.1	0	0.05	0.05	—
"	"	0.05	0	"	"	—
"	"	0.02	0	"	"	—
"	"	0.01	0	"	"	—
"	"	0.005	0	"	"	—
"	"	0.002	0	"	"	—
"	"	0.001	0	"	"	—
"	"	0.0005	0	"	"	—
"	"	0.0002	0	"	"	±
"	"	0	0.1	"	"	—
"	"	0	0.05	"	"	—
"	"	0	0.02	"	"	—
"	"	0	0.01	"	"	—
"	"	0	0.005	"	"	—
"	"	0	0.002	"	"	—
"	"	0	0.001	"	"	±
"	"	0	0.0005	"	"	+

3. Serum 4. A third anti-gonococcus serum was obtained from a rabbit immunized with living cocci. Amboceptor for gonococcus was present in a dilution of one to ten thousand in the presence of one tenth cubic centimeter of extract, and one to two thousand for intracellularis. Table VI gives the results of this and further titrations of the extract.

It is evident that the highest amboceptor content for gonococcus was present in the anti-serum obtained by immunizing a rabbit with living gonococcus cultures, agglutinins being also higher than in the other two sera from rabbits immunized with extracts, and with extracts plus cocci. In these high dilutions a distinct difference between the amount of gonococcus and diplococcus amboceptor was apparent, although the two ran parallel until a dilution of one to two thousand was reached.

The anti-diplococcus horse serum obtained from Dr. Jobling inhibited hæmolysis completely in dilutions of one to five hundred with one tenth cubic centimeter of diplococcus antigen, and incompletely with that amount of gonococcus antigen. Doubling the

TABLE VI.

Amboceptor Immune Serum No. 4.	Guinea-Pig Serum Complement.	Antigen.		Anti-hen Serum.	Hen's Corpuscles.	Results.
		Gonococcus Extract.	D. Intracellularis Extract.			
0.1	0.05	0.1	0	0.01	0.05	—
0.05	"	"	0	"	"	—
0.02	"	"	0	"	"	—
0.01	"	"	0	"	"	—
0.005	"	"	0	"	"	—
0.002	"	"	0	"	"	—
0.001	"	"	0	"	"	—
0.0005	"	"	0	"	"	—
0.0002	"	"	0	"	"	—
0.0001	"	"	0	"	"	—
0.00005	"	"	0	"	"	±
0.1	"	0	0.1	"	"	—
0.05	"	0	0.1	"	"	—
0.02	"	0	0.1	"	"	—
0.01	"	0	0.1	"	"	—
0.005	"	0	0.1	"	"	—
0.002	"	0	0.1	"	"	—
0.001	"	0	0.1	"	"	—
0.0005	"	0	0.1	"	"	±
0.0002	"	0	0.1	"	"	+
0.0001	"	0	0.1	"	"	—
0.001	"	0.1	0	"	"	—
0.001	"	0.05	0	"	"	—
0.001	"	0.02	0	"	"	—
0.001	"	0.01	0	"	"	—
0.001	"	0.005	0	"	"	—
0.001	"	0.002	0	"	"	—
0.001	"	0.001	0	"	"	—
0.001	"	0.0005	0	"	"	—
0.001	"	0.0002	0	"	"	—
0.001	"	0.0001	0	"	"	—
0.001	"	0	0.1	"	"	±
0.001	"	0	0.05	"	"	—
0.001	"	0	0.02	"	"	—
0.001	"	0	0.01	"	"	—
0.001	"	0	0.005	"	"	—
0.001	"	0	0.002	"	"	—
0.001	"	0	0.001	"	"	—
0.001	"	0	0.0005	"	"	—
0.001	"	0	0.0002	"	"	±
0.001	"	0	0.0001	"	"	+

inhibitory amount and titrating the two extracts, the results proved to be identical, deviation being complete with 0.02 cubic centimeter of antigen, incomplete with 0.005 cubic centimeter, and absent with a smaller quantity. On testing the inhibitory amount of antigen with diminishing amounts of amboceptor, it was found that 0.01 cubic centimeter was required to prevent hæmolysis with intracellularis, and 0.02 cubic centimeter for gonococcus. Neither the

amboceptor content nor the agglutinins of this serum were high, and the differences for the two varieties of cocci were very small.

Extracts of *Micrococcus catarrhalis,* of *Streptococcus pyogenes,* of a Gram negative diplococcus from a dog, of the typhoid bacillus and of two varieties of dysentery bacilli (Shiga and Flexner types) did not inhibit hæmolysis in combination with this diplococcus immune serum. Normal horse serum controls with gonococcus, diplococcus, streptococcus, *Micrococcus catarrhalis,* and the typhoid and dysentery bacilli were all completely hæmolyzed.

An anti-typhoid serum obtained from Dr. Park at the Board of Health deviated complement in a combination of one tenth cubic centimeter with one two hundredth cubic centimeter of typhoid antigen, but it had no effect upon hæmolysis when combined with intracellularis, gonococcus, streptococcus or *Micrococcus catarrhalis.*

On several occasions extracts of gonococcus and of diplococcus became useless after about two weeks, because they inhibited hæmolysis in doses of one tenth cubic centimeter or less. This was found to be due to the presence of protectin,[32] which fact was demonstrated by shaking the extract with ether for two hours, decanting, evaporating over a water bath and taking up the residue in salt solution. Two tenths and one tenth of a cubic centimeter of this suspension protected blood corpuscles from solution in the presence of their inactivated lytic serum and fresh complement. I am indebted to Dr. Noguchi for this demonstration.

CONCLUSIONS.

The most marked differences, exclusive of pathogenic effects in man, between gonococcus and *Diplococcus intracellularis* are cultural ones, and consist chiefly in abundance of growth and choice of medium.

Relatively larger doses of gonococci than of diplococci are required to kill young guinea-pigs, but the lesions are very similar in the two cases, and both organisms lose pathogenic power rapidly when cultivated artificially.

Agglutinins, aggressins, protective power, and the amboceptors

[32] Noguchi, *Jour. of Exper. Med.,* 1906, viii, 726.

developed in the serum of immunized animals seem to be largely common to both diplococcus and gonococcus.

Neither other Gram negative cocci nor *Streptococcus pyogenes* have any receptors in common with intracellularis and gonococcus.

My thanks are due to Dr. Flexner for suggesting and supervising the work, and to Dr. Jobling for many courtesies.