THE PROBABLE NATURE OF THE INFECTIOUS AGENT OF TRACHOMA*

By L. A. JULIANELLE, Ph.D., R. W. HARRISON, Ph.D., and M. C. MORRIS, Ph.D.

(From the Oscar Johnson Institute, Washington University School of Medicine, St. Louis)

(Received for publication, February 1, 1937)

The confusion and contradiction attending upon the etiology of trachoma when this investigation was first undertaken in 1931 dictated the desirability of establishing certain fundamental conditions regarding the nature of the disease. Consequently, it was necessary in the earlier studies to repeat a number of experiments already described in the literature[1] and to extend them when warranted. In addition, other experiments have been done for the first time in this laboratory, and as a result, the causative agent of trachoma appears to be more tangible and more closely definable.

In the work conducted thus far in this laboratory, it has been demonstrated, as others showed before us (1), that material derived from the conjunctiva of patients with trachoma, while completely innocuous for dogs, rabbits, hogs, guinea pigs, rats, and mice, induces on the conjunctiva of monkeys an infection characterized essentially by folliculosis (2). Preceded by an incubation period of several days to a month, the experimental disease frequently extends from the inoculated to the uninoculated eye and endures for a few weeks to many months, in some instances 2 and 3 years. The histological changes, although simulating those in trachoma, are not emphasized, because follicular reactions of the conjunctiva in general lack any features distinguishing the one from the other. Inoculation of

* Conducted under a grant from the Commonwealth Fund of New York.

[1] It would be impossible in a report of this kind to attempt more than a cursory reference to the extensive literature bearing on the various aspects of the etiology of trachoma. A thorough review of the literature, with a more or less complete bibliography, will be found in a forthcoming monograph on the etiology of trachoma by one of us, to be published by the Commonwealth Fund Division of Publications of New York.

trachomatous material in tissues other than the conjunctiva was found to be without effect. Tissues from different patients vary greatly in infective capacity, and despite the caution exercised in selecting only recent and clinically active disease, about half the tissues only are infectious. Conversely, individual animals (*Macacus rhesus*) exhibit a marked difference in susceptibility, approximately half the monkeys being resistant. It has not been possible to adapt the infection permanently in monkeys, since usually by the third serial passage the infectious agent is either lost or completely inactivated. Upon recovery, no resistance to infection or immunity to the experimental disease is demonstrable. Up to the present time, a hundred or more infected animals have been examined for the epithelial cell inclusions frequently observed in human beings, but neither scrape smears nor histological sections have revealed their presence.

Since the experimental disease lacks the two distinguishing signs of spontaneous trachoma (*i.e.*, cicatrization and pannus), experiments were performed (3) to indicate by virtue of its specificity the genuineness of the disease. Thus, human folliculosis, which in appearance may resemble the experimental disease, is not transmissible to monkeys; the formation of follicles is not stimulated by non-specific infection with a variety of bacteria, by irritation, or by autoinoculation of follicles occurring spontaneously in certain monkeys.

Experiments devised to elucidate the effect of faulty diet on the experimental disease (4) consisted of inoculating monkeys maintained on diets deficient in vitamin A, or inadequate qualitatively and quantitatively in protein, or inadequate in protein and high in fat, or high in fat. The data indicate clearly that a state of malnutrition, as described with its resultant debilitation, does not predispose monkeys to trachoma; on the contrary, the animals appear to be less reactive than those maintained on an adequate diet.

Subsequently, in attempting to define the infectious agent of trachoma, an elaborate study was made of the bacteria cultivable from the disease. A number of workers have ascribed the causation of trachoma to different specific organisms (5), while other authors failed to find any characteristic bacteria (6). It was found in this laboratory (7) that trachoma is not characterized by any particular flora, since the same bacteria may be isolated in similar frequency from other conditions of the eye, and to a less extent even from normal eyes. The variety of bacteria is extensive, and it remains unchanged in the different stages of the uncomplicated disease and is unrelated to the presence of inclusions. Inoculation of the bacteria cultivated, either individually, pooled, or in conjunction with Berkefeld filtrates of infectious material, is without effect on monkeys, even though the tissues from which they are derived are of demonstrable specific infectivity.

The question of whether the infectious agent of trachoma is filterable has occupied the attention of previous workers. Some have succeeded (8) in transmitting trachoma with bacteriologically sterile filtrates, while others found such filtrates to be inactive (9). In any case, however, the number of experiments conducted by the majority of the workers has been too small to include the different variables present in tissue infectivity and animal resistance. Filterability of

the infectious agent was also studied in this laboratory by filtration through Berkefeld V filters. Of 14 experiments performed (10), the original material was infectious in 11. Filtrates of these tissues failed to infect monkeys of demonstrated susceptibility in 10 experiments, and in one the filtrate was as actively infectious as the material from which it was obtained.

The evidence suggests, therefore, that the infectious agent of trachoma is not dependent upon a previously existing state of malnutrition for implantation on the conjunctiva, that it is not bacterial in nature, and that under special conditions it is capable of traversing kieselguhr filters. Because of the obvious indications, a concentrated effort has been made to determine whether the incitant of trachoma may be considered a virus. The experiments undertaken in this connection are presented in the present report.

EXPERIMENTAL

The methods employed both in obtaining material from patients and in inoculating animals[2] have been described in detail in previous communications, so that a brief recapitulation will be sufficient at the present time. The tissue, removed under novocaine anesthesia from the conjunctiva of each patient by grattage, was suspended in 1.5 cc. of veal infusion broth (pH 7.8). Suspensions from different patients were frequently pooled and triturated under sterile conditions and the ground material was inoculated conjunctivally in monkeys. The inoculations consisted of application of trachomatous tissue by swabbing the everted conjunctiva, or subconjunctival injection alone; or first effecting multiple pricking of the conjunctiva with the charged needle, and then injecting subconjunctivally. No particular differences were noticed in infectivity referable to method of inoculation.

Purification of the Infectious Agent by Testicular Passage

The expedient employed by Noguchi (11) in adapting vaccine virus to rabbit testicular tissue offered the possibility of purifying the infectious agent of trachoma by similar means. For this purpose, material from patients was inoculated intratesticularly in rabbits (12), monkeys, and guinea pigs. Since, however, the most consistent results were obtained in rabbits, the routine of inoculating these

[2] The human material employed in this study was obtained from the Trachoma Hospital at Rolla, Missouri, and Richmond, Kentucky, and we express our indebtedness with gratitude to Drs. C. E. Rice and J. E. Smith, and Dr. Robert Sory, respectively.

animals alone was employed. To illustrate the effectiveness of this method, a typical experiment will be described in detail.

Material obtained by grattage from two patients was collected in infusion broth as described above. The suspensions were pooled, and after 4 hours required for transportation to the laboratory, two rabbits were injected into the left testicle with 0.5 cc. each, and several monkeys were inoculated by swabbing the upper conjunctiva of both eyes. Within 2 weeks, typical follicles began to appear at the internal angles of the upper conjunctiva, and within a month there was a scattering of follicles over the conjunctiva of all four lids. The infection subsided within 2 months. 1 week following inoculation, the inoculated testicle of one rabbit was removed, with precautions for sterility, and subsequently ground in a sterile mortar. The emulsified tissue was then inoculated in monkeys by swabbing and by injection. 2 weeks following inoculation, the second rabbit was studied in the same way, and inoculations were repeated in monkeys. The results of the rabbit passage showed that the testicular material 1 week following inoculation was moderately infectious and the disease persisted from 1 to 2 months; the material 2 weeks after inoculation was most virulent of all, the incubation period being shortened to 5 to 7 days and the infection persisting for several months. This increased virulence is only apparent, however, since other materials showed differences in one direction or the other which were undoubtedly referable to variation in individual susceptibility of monkeys.

Four typical experiments are summarized in Table I. An analysis of the data reveals that in 2 experiments the human material was originally infectious and retained its infectivity after testicular passage in one but not the other instance. The material retaining its activity showed no inclusions, while that losing its infectivity contained typical inclusions. In 2 other experiments the human material did not infect monkeys, but in one the infectious agent was demonstrable following testicular passage. The material exhibiting infectious properties following passage contained no inclusion bodies, while the other did. In no case, however, were inclusion-like structures seen in preparations from either rabbits or monkeys.

Twenty experiments in all were performed in similar fashion. The material came from single cases, or that from 2 to 9 patients was pooled. In 10 experiments, the original material proved non-infectious, while in the remaining 10, follicular reactions, characteristic of experimental trachoma, were induced in monkeys following their inoculation. Of the 10 materials originally non-infectious, 2 were capable of infecting monkeys specifically after testicular passage, and

8 remained non-infectious. With the 10 materials of demonstrated infectivity, 4 were unable to infect monkeys after testicular passage, and 6 retained their ability to infect. In attempting to transmit the infectious agent in series, it was found that by the third intratesticular passage it was lost or completely attenuated. The discrepancies measurable in terms of infectivity preceding and following testicular passage serve to demonstrate in another way the difference in resistance of individual monkeys, rather than an acquisition or loss of infectivity by the original tissues. In fact, it is because of this marked variation in susceptibility, as already pointed out, that in order to avoid inaccurate conclusions, experiments on trachoma should be carried out on a statistical basis.

TABLE I

Purification of the Infectious Agent of Trachoma by Rabbit Testicular Passage

Experiment No.	No. of cases pooled	Presence of inclusions	Infectivity		Presence of inclusions in	
			Before passage	After passage	Rabbit	Monkey
T36	2	Both negative	Positive	Positive	Negative	Negative
T58	2	Both positive	"	Negative	"	"
T49	2	Both positive	Negative	"	"	"
T53	3	All negative	"	Positive	"	"

In addition to the experiments with material from human beings, a study was also made on the intratesticular passage of infected tissues from monkeys (*Macacus rhesus*). Material was obtained in the same way, and 4 experiments were done in all. In each instance, the presence of the infectious agent after passage was demonstrated by subsequent infection of monkeys.

For purposes of control, material obtained in the same way from ten patients with folliculosis and from two patients with a chronic infectious conjunctivitis of undetermined etiology was studied in a similar manner. But on no occasion was it possible to demonstrate the presence of an infectious agent in rabbit testicle. Since it has been reported (13) that epithelial cell inclusion bodies indistinguishable from those encountered in trachoma may be found in scrape smears of the

conjunctiva during hog cholera, another experiment was performed with hog cholera virus, which was first proved fatal for hogs. When inoculated directly or following passage in rabbit testicle, it failed to invoke any significant reaction in the conjunctiva of monkeys.

Before preparing the testicular tissue for inoculation, sufficient tissue was removed for histological and bacteriological study. Tissue cultures were also made, as will be described later. Impression smears from cut surfaces were stained by Gram for bacteria and by Giemsa for inclusion bodies. Except for a minor degree of inflammatory reaction in occasional rabbits, probably referable to adventitious bacteria present in the original tissues, the tissue changes were insignificant. This is in agreement with the clinical observation that none of the rabbits injected with trachomatous tissue gave any evidence of a general or local reaction. Inoculations made on several different bacteriological media yielded no growth, except on rare occasion when a colony or two of *Staphylococcus albus* was isolated. Tissue cultures made at the same time were also free of bacteria following incubation. Bacteria were never demonstrated in the impression smears, and neither the elementary nor the initial bodies associated with the inclusion body of trachoma were ever found.

It is interesting in this connection that in attempting to preserve the infectious agent of trachoma under a variety of conditions, Nicolle and Cuenod (14) found that in a single experiment trachomatous material retained its activity 37 days after inoculation into rabbit testicle. Subsequently, Nicolle reported (15) that infectivity was not demonstrable 30 days after testicular inoculation. In each instance, details are lacking as to infectivity of the trachomatous tissues preceding inoculation of the rabbit, presence of bacteria before or after passage, clinical effect in rabbits, and histology of the inoculated testicle.

The purification and survival of the infectious agent in rabbit testicle suggested the possibility of adapting it to brains of rabbits. Material from patients was therefore injected intracerebrally, and after an interval of 2 weeks the animal was sacrificed and the brain employed for serial passage, inoculation of monkeys' eyes, and further study. In 5 experiments of this kind, it was found that the infectious agent survived once, although inducing no tissue alteration. Further

attempts at cerebral adaptation indicated that, although occasionally encephalitic lesions due to *Encephalitozoon cuniculi* (16) were encountered, the infectious agent became lost in serial passage. Similar efforts to adapt the infectious agent to brains of monkeys were also failures.

So, also, passing infectious testicular tissue intraperitoneally through mice rendered the infectious agent recoverable only rarely within the first 24 hours by washing out the peritoneal exudate. The mice suffered no noticeable inconvenience, and impression smears of several organs revealed nothing of note.

Consequently, the evidence indicates, as observed in the one positive filtration experiment cited above, that it is possible to infect monkeys with experimental trachoma with material completely liberated from extraneous bacteria as determined by smear, bacteriological and tissue cultivations. The absence of any reaction in the rabbit, the inability of the infectious agent to survive serial passage, and the maintenance of infectivity in many instances at a given level, all suggest that the infectious agent of trachoma does not propagate in the testicle but is merely preserved. During the period of intratesticular passage, however, purification from the cultivable bacteria present in trachomatous eyes is achieved. Therefore, while this method of demonstrating the presence of the agent of trachoma is irregular in occurrence, it nevertheless justifies its original purpose of excluding bacteria a sufficient number of times (12) to indicate that the organisms cultivable from the trachomatous conjunctiva are not responsible for the infection.

Additional Studies on Filterability

With the tentative elimination of bacteria as involved in the evolution of trachoma, it became desirable to restudy the question of filterability of the incitant of the disease. In retrospect it seemed that several possibilities, all amenable to experimentation, might explain the frequent failure of successful filtration. Adsorption on the filter, reverse potential of the infectious agent, the presence of the infectious agent within or closely adherent to the epithelial cell, etc., theoretically, at least, might interfere with the filtration of an otherwise filterable agent. A publication in the meantime, by Thygeson and his associates, suggested that non-filterability was in fact due to

the adsorptive properties of kieselguhr filters. While in a previous study (17) with Berkefeld and Chamberland filters he had reported that the infectious agent of trachoma was not filterable, he demonstrated at this time (18, 19) that collodion filters as devised by Elford (20) allowed the agent to pass. Both Cattaneo (21) and Stewart (22), on the contrary, failed to show that filtration was any more successful with collodion membranes.

Subjecting the question of filterability to restudy, then, it was decided to consider the various possibilities suggested and to conduct filtrations, when material was sufficient, under different conditions. Accordingly, four different filters were employed: Berkefeld V, Seitz, plaster of Paris as fashioned by Kramer (23), and collodion membranes.[3]

In the majority of instances, the material was collected in infusion broth, but on occasion in Tyrode solution. Scrapings from several patients were pooled and ground in a sterile mortar 3 to 4 hours after collection. After grinding, the suspension was centrifugated for 5 minutes at about 1500 R.P.M. This was sufficient to sediment the various tissue cells and yet not destroy the infective capacity of the supernatant fluid, which was subsequently used for filtration. In each case, filtration was accomplished in a few minutes under a pressure of about 20 cm. of Hg. Cultures were made of the filtrates, and the integrity of the filters was tested later with broth cultures of *Bacillus prodigiosus*; the plaster of Paris filters were controlled by passage of Congo red. Inoculations were then made in monkeys with unfiltered material, with supernatant after centrifugation, and with filtrate.

Twenty-two experiments were done in all, but since the unfiltered material was infectious in only 9, obviously the other experiments have little significance. In all 9, filtration was done with collodion membrane of an average pore size close to 0.6μ in the different tests. In all cases the filtrates failed to infect monkeys. In 5 experiments, parallel filtrations were carried out with Berkefeld V and collodion filters, and in 4, filtrations were done simultaneously with all four varieties of filter. To illustrate experiments conducted on parallel filtration. a summary protocol is given in Table II. It will be seen on examination that in the 4 experiments material was pooled from 4 to 9 patients, with inclusion bodies varying in a percentage incidence of 20 to 75.

[3] The filtering apparatus used was a replica of a model kindly supplied for reproduction by Dr. J. H. Bauer of The Rockefeller Foundation (24).

In each instance, the unfiltered material and the supernatant following centrifugation were infectious in monkeys, thus showing that grinding and centrifugation did not alter measurably the original infectivity. In each case, however, the filtrates from Berkefeld V, Kramer, Seitz, and Elford filters were incapable of inducing experimental trachoma. A number of the monkeys which proved to be non-reactive to filtrates were infected at a later date with unfiltered material, thus eliminating the possibility that their natural resistance was responsible for the original failure of infection. Since the study of filtration is still in

TABLE II

Filterability of Infectious Agent of Trachoma

Experiment No.	No. of cases pooled	Presence of inclusions	Infectivity of					
			Unfiltered material	Supernatant	Berkefeld filtrate	Kramer filtrate	Seitz filtrate	Elford filtrate
T64	9	Present in 6 of 9 patients	Positive	Positive	Negative	Negative	Negative	Negative
T65	4	Present in 3 of 4 patients	"	"	"	"	"	"
T66	5	Present in 1 of 5 patients	"	"	"	"	"	"
T67	5	Present in 2 of 5 patients	"	"	"	"	"	"

progress, it is not desirable to draw final conclusions, although the indications are that filterability appears to be as difficult of achievement as was originally stated.

In addition to the experiments with human tissues, a number of attempts were made to filter infected animal tissues. Scrapings from the conjunctiva of monkeys (*Macacus rhesus*), as well as ground rabbit testicle, were filtered similarly to the human material through both Berkefeld V and Elford filters. The results were uniform in indicating a complete loss of infectivity by the filtrates of tissues from monkeys and rabbits.

There are several reasons for assuming that trachoma is preeminently an infection of the epithelial cell. If this assumption is correct, it should follow logically that the infectious agent is contained within

the cell or attached to its surface. Since the methods employed for filtration retain all tissue cells, it is possible that in consequence the virus, if not entirely withheld, may be diminished to a point beyond its range of infectivity. In order to test this hypothesis, several experiments were undertaken in which lysis of the epithelial cell was caused by supposedly gentle means before attempting filtration. Thus, rupture of the cells was obtained with ox bile, dilute alkali, and alternate freezing (−15°C.) and thawing (+8–10°C.). However, it has been impossible as yet to gain any information on filterability under these conditions, because all the methods thus far attempted have inactivated the infectious agent. Materials tested after the manipulation in question, and before filtration, have always failed to infect monkeys of proved susceptibility.

Cultivability of the Infectious Agent

The results already described in connection with testicular passage reveal the inability of the infectious agent of trachoma to multiply in the usual bacteriological media. It was proposed, therefore, to study cultivability in tissue cultures. For this purpose, the technique devised by Maitland and Maitland of minced rabbit kidney (25), and as modified by Li and Rivers (26) of minced chick embryo, the developing chick egg (Woodruff and Goodpasture (27); also Burnet and Galloway (28)), and various tissue fragments in plasma, were particularly utilized. In order to control both the methods and conditions of growth, similar experiments were carried out with the virus of St. Louis encephalitis, as already reported (29). These methods of tissue cultivation were supplemented later by cultures made in plasma clots with human placental tissue extract and plasma from patients or normal individuals. In attempting to approximate very closely the conditions found in the eye, epithelial cells containing both the infectious agent (as demonstrated by inoculation of monkeys), and in some cases even inclusion bodies, were seeded by this method.

The technical difficulties accompanying the studies in tissue cultivation were numerous, the most consistent being the presence of adventitious bacteria in the original tissues. In the earlier seedings, they were almost always present in the inocula, and they grew rapidly in culture, with the result that the growing cells were quickly destroyed or suppressed, thus defeating the purpose of the experi-

ment. The use of several methods and chemicals, to inactivate selectively the bacteria only, was of no avail, since the infectious agent proved to be less resistant to the different substances, as will be brought out later. This obstacle was circumvented, however, by careful irrigation of the eye with salt solution, and then making multiple seedings of essentially epithelial scrapings collected in Tyrode solution. While it was not possible to eliminate bacterial growth entirely in this way, bacteriologically sterile tissue cultures were frequently obtained. The seeded cultures were incubated both at 30° or 32°C. (to approximate the temperature of the conjunctiva) and at 35° or 37°C., and incubation was carried out for 3 to 7 days before transplants were made or before the cultures were nourished. Other cultures, under similar conditions, were made from infected eyes of monkeys and from testicular tissue carrying the infectious agent. In testing for infectivity, tissue cultures were inoculated in monkeys before and after transplanting, and in every case the inoculum was derived from several cultures which had been pooled and triturated.

The data bearing on tissue cultivation have been summarized in Table III. Examination of the protocol shows that cultivation was tested in 261 cultures of minced chick embryo representing 43 patients whose conjunctival scrapings were pooled to form 12 experiments, in which the original material was infectious 6 times. In the fertile egg, 91 seedings were made from 26 patients, so pooled as to comprise 10 experiments, in 4 of which the human tissues were of demonstrated infectivity. In minced rabbit testicle, 254 inocula were made from 59 patients, for a total of 18 experiments, in which the original tissues were capable of infecting monkeys 6 times. So, also, in rabbit testicle in plasma clot, 217 separate cultures were grown from material supplied by 68 patients, comprising 20 experiments, in 8 of which the original material induced experimental trachoma. In cultures of minced rabbit kidney, 118 attempts were made to cultivate the infectious agent of trachoma, in 8 different trials, with the human material infectious twice. Cells from the human conjunctiva of 48 patients, pooled to make 13 experiments, were cultivated in clotted plasma derived from the same patient and from normal individuals. In 5 of these experiments, conjunctival cells were derived from patients of proved infectivity. In summary, then, 81 experiments, in 31 of which the original human material was infectious for monkeys, were conducted by cultivating and inoculating tissue from different animal species. From 30 to 40 per cent of the trachomatous materials employed in the different experiments on cultivation were found to

contain epithelial cell inclusions. In none of the cultures, however, was it possible to demonstrate the presence of the infectious agent of trachoma by inoculation of monkeys.

Cultivations attempted on a lesser scale in minced mouse kidney, in guinea pig testicle, in the Brown-Pearce tumor of rabbits, in testicle and conjunctiva of monkeys, were all uniformly negative. In several instances, infectious material was inoculated in rabbit testicle simultaneously with a ground suspension of the rabbit tumor described by Brown and Pearce. Again the results indicate a failure of the infectious agent to grow. At other times, tissue cultivation was carried

TABLE III

Attempts to Cultivate the Infectious Agent of Trachoma in Tissue Culture

Method of tissue culture	No. of patients		No. of experiments with pooled material		Total No. of cultures	Infectivity of tissue cultures
	Studied	With inclusions	Infectious	Non-infectious		
Minced chick embryo	43	13	6	6	261	All negative
Fertile egg	26	10	4	6	91	" "
Rabbit testicle						
(*a*) Minced	59	20	6	12	254	" "
(*b*) In plasma clot	68	24	8	12	217	" "
Minced rabbit kidney	17	7	2	6	118	" "
Human conjunctiva	48	19	5	8	234	" "
Totals	261	93	31	50	1175	All negative

out anaerobically, as suggested by Dochez, Mills, and Kneeland (30), without, however, affecting the results already observed with other techniques. A few other experiments were attempted with cultivation of human placenta, but these were discontinued because of the difficulties encountered in growing this tissue.

The evidence is clear, then, that despite numerous attempts to cultivate the infectious agent of trachoma under a variety of conditions, in tissues from six different animal species, including man, and a cultivable rabbit tumor, it has not been possible to create *in vitro* the proper conditions for multiplication. In a recent preliminary communication from India, successful cultivation of the trachomatous agent

in the fertile egg has been reported (31). The evidence presented, however, is not convincing, since the measure of propagation was not infectivity in man or monkey, but the appearance of gross lesions on the chorioallantoic membrane. There was no information in the report to indicate whether even these changes might not have been due to bacterial contamination.

Characteristics of the Infectious Agent of Trachoma

The studies presented up to this point have dealt mainly with the grosser characters of the infectious agent of trachoma, particularly its ability to induce specific infection in monkeys. While at the same time they reflect the nature of the agent, they do not furnish sufficient detailed information to make clear its character. Consequently, experiments were performed on its inactivation or ability to survive under different circumstances. Recognizing the danger of generalization, since the variation both in infectivity of tissues and in susceptibility of animals is great, it nevertheless seems fair to depict the characteristics to be described as typical of the infectious agent of trachoma.

Inactivation by Heat.—As in all the experiments to follow, the reactions were studied with material obtained from patients by grattage and suspended in infusion broth. Consequently, the infectious agent was present in a mixture of lachrymal secretion, blood and tissue cells, consisting chiefly of epithelial cells and to a much less extent of lymphocytes and monocytes. How much of a protective influence the different constituents afforded the infectious agent is obviously indeterminable under the present conditions of experimentation. Such suspensions, then, were placed in a water bath regulated at different temperatures (40°, 45°, 50°, 55°, 60°C.) and for varying intervals of time (15, 30, 45 minutes). In later experiments, the two higher temperatures and the 45 minute interval were discontinued. At the end of the exposure, the material, after cooling, was inoculated in monkeys simultaneously with the unexposed material in control animals. The experiment was repeated a sufficient number of times to allow the conclusion that exposure at 45–50°C. for 15 minutes regularly inactivates the infectious agent. So, also, Hess and Römer (32), and Botteri (33), found that $\frac{1}{2}$ hour at 50°C. destroys the infectious agent.

Preservation.—Preservation of the infectious agent of trachoma in glycerine (34) has been reported by several workers, with a remarkable difference of opinion (35). In most cases, however, the data are difficult of analysis, since little effort was made to determine either the original infectivity of the material or preservation under the same conditions without glycerine. In these experiments, the infectivity of the original suspensions was tested, and when they were found to be non-infectious, the data were discarded. Since variations were encountered in different brands of glycerine, that prepared by Schering-Kahlbaum of Berlin was used, as best adapted for this work. The results of repeated experiments indicate that at ice box temperature glycerine does not maintain the infectious agent active any longer than preservation without glycerine. Ordinary preservation at this temperature varies, with different suspensions, from 1 or 2 days to a week or more. In general, however, tissues lose their activity within 3 or 4 days under these conditions. Similar variations in maintenance of infectivity were observed at room temperature, the infectious agent remaining active for a few hours, varying from 2 or 3 to rarely 24 hours. At incubator temperature (37°C.), inactivation occurs within a few hours.

Effect of Chemical Agents.—Cell suspensions containing the infectious agent were tested for its ability to survive some of the commoner chemical agents. Ox bile (36), added to the amount of 1/4 to 1/3 the volume of the suspension, was found to inactivate the infectious agent after an exposure of 15 minutes at 37°C. At the end of this interval, practically all the cells were lyzed, and the inclusion bodies, when present, were dissolved, thus disappearing, while, as might be expected, many of the bacteria originally present were still cultivable.

Silver nitrate, which is commonly used in the treatment of trachoma, was also tested for its effect on the infectious agent. In a final concentration of 2 per cent, this reagent causes a heavy coagulation of the suspensions, and the infectious agent is inactivated regularly within 3 to 4 hours.[4] So, also, cocaine was found to be deleterious, so that

[4] The regularity of time exposure in these experiments is accounted for by the fact that after collecting the material at the Rolla hospital, it had to be transported to St. Louis before inoculation in monkeys was possible. This was done in most of the experiments recorded in this report, and the material was kept on ice during transportation.

this anesthetic was never employed when obtaining material from patients. In concentration of 4 per cent, cocaine inactivated regularly, while in 2 per cent concentration inactivation was effected after an interval of 3 to 4 hours in about one-half the tissue specimens. Gentian violet in final dilution of 1:100,000 also destroyed the infectivity of active tissues within the same time interval. Tartar emetic inactivated the agent within this period, when present in an ultimate dilution of 1:1000, as did also a 0.25 per cent solution of phenol.

Immunogenic Properties.—The common clinical history of repeated infection in patients with trachoma was experienced in the experimental work when it was shown that monkeys acquire no active immunity to infection after recovery from the artificially induced disease. Nevertheless, an effort was made to determine whether human sera may act to prevent or diminish experimental infection. Conjunctival scrapings from patients, of verified infectivity, were mixed with sera, whole blood, or plasma, derived in some experiments from the patients themselves, and in others from normal individuals. In most of the experiments, the mixture was kept at 20°C. for periods varying from 4 to 12 hours. In a few experiments, incubation was carried out for ½ hour at 37°C. It was not possible to lengthen the incubation period at body temperature because of the injurious effect of the higher temperatures. The mixtures were then inoculated in monkeys to determine whether they were still capable of causing experimental infection. Not desiring to prolong unnecessarily the presentation of these experiments, it is important to state only that blood from patients with trachoma contains no demonstrable substance capable of inactivating or neutralizing the infectious agent, as measured by the development of experimental trachoma in monkeys.

Relation of the Infectious Agent to the Epithelial Inclusion

The epithelial cell inclusion, first described by Prowazek and Halberstädter, was considered by them as the causative agent of trachoma (37). It will not be possible to review in this report the tremendous literature bearing on this structure. For purposes of orientation, however, it is necessary to point out that the inclusion is composed of heterogeneous elements which may appear relatively large, pleomorphic, and basophilic (*i.e.*, initial bodies), or minute, uniform, coccoid,

and acidophilic (*i.e.*, elementary bodies). Both appear extra- or intracellularly, frequently forming over the nucleus a cap composed entirely of either one form or the other, or even in common agglomeration. As a rule, elementary bodies are less common and are more often extracellular, while the initial bodies are more numerous and more frequently occur intracellularly. The inclusions are found in few numbers in trachoma, and in only roughly half the patients, although their incidence is more frequent in the recent infections.

Attempts made in this laboratory to correlate the presence of inclusions with infectious activity of given tissues indicate that materials containing inclusions are not necessarily infectious for monkeys, and that materials lacking inclusions may be actively infectious. Inclusions have never been found in monkeys successfully infected, in infectious rabbit testicle or brain, in tissue culture, or in various organs of mice injected intraperitoneally.

The literature indicates a division of opinion among different workers, the earlier apparently finding inclusions in infected animals (38), while the later have been unsuccessful (39). The inclusions found by the earlier investigators were found, in general, shortly after inoculation with whole material, even in the absence of the experimental disease (Halberstädter and von Prowazek, Leber and von Prowazek, Herford, etc.), and their presence was transient. In successful transmission in monkeys with filtered material, inclusions either were not found (Bertarelli and Cechetto, Julianelle and Harrison) or they were not sought for (Nicolle, Cuenod, and Blaizot; Olitsky, Knutti, and Tyler; and Thygeson and Proctor). In this connection, however, it is important to recall the study of Thygeson, Proctor, and Richards (19), in which they showed that a bacteriologically sterile filtrate of human trachomatous tissue obtained by filtration through a collodion membrane of 0.6μ A.P.D. not only induced trachoma in a human volunteer, but stimulated the formation of epithelial cell inclusions from the 5th day on. The filtrate contained minute structures which the authors considered to be elementary bodies.

The evidence to be gained from the literature indicates that in all the successful transmissions practiced in man, with unfiltered material, to be sure, inclusions were always found when sought. The information available at the present time, however, does not allow a categorical statement regarding the nature of the inclusion body. Whether it represents the infectious agent itself, as the experiment of Thygeson, Proctor, and Richards suggests, or a reaction product between cell and

agent; or whether, as some believe by inference rather than experimentation, it is another agent concurrently present, or consists of nests of phagocyted bacteria, must await future work for elucidation.

DISCUSSION

As a result of continuing the preceding studies on the infectivity of tissues from patients with trachoma, it has been possible to visualize more concretely the infectious agent of this disease. Adapting to this problem the technique of testicular passage in rabbits, it was found that trachomatous tissues, from both man and monkey, may be purified of the extraneous bacteria usually present on the conjunctiva. While irregular in its execution, this method permits the infectious agent to retain its infectivity a sufficient number of times to support the conclusion that trachomatous tissues liberated of bacteria may still be specifically infectious for monkeys. The evidence indicates, however, that the infectious agent does not multiply during this passage.

Further experiments on filtration, employing Seitz, Kramer, Berkefeld, and Elford filters, as described above, justify the impression, gained in earlier studies, that successful filtration is accomplished only rarely and with difficulty.

Except for the studies on filtration reported by Stewart and ourselves, too few experiments have been done by each investigator to warrant the opinion that the infectious agent of trachoma is readily filterable. Thus, Bertarelli and Cechetto, who first reported successful filtration, did a single experiment; Nicolle, Cuenod, and Blaizot did 2 experiments, both successful; Thygeson and his associates reported 6 tests in human beings, with Berkefeld and Chamberland filters, all negative, 4 experiments with collodion membranes, in baboons, all positive, and later a single test, as cited above, in a human volunteer. Of the others reporting filtration, Olitsky, Knutti, and Tyler demonstrated filterability in 1 of 6 trials, and we, in 1 of 20 trials. This makes no allowance, however, for the imposing number of experiments by ten groups of workers who did not establish filterability. It is suggested, therefore, not that the infectious agent is incapable of filtration, but rather that it is not readily filterable, regardless of the method of filtration employed.

Upon reconsideration, it may be that filtration of the infectious

agent depends upon a certain degree of epithelial cell degeneration for its liberation into the surrounding menstruum. Since cellular degeneration is slight, particularly in the uncomplicated stages, it is not unlikely that the unattached infectious agent occurs in quantities insufficient for successful filtration. In the rarer cases of marked cellular degeneration, on the other hand, the infectious agent is filterable, thus contributing the few examples reported in the literature. In inclusion blennorrhea, a related ocular condition, filtration has been reported with greater regularity. In this infection, excoriation and degeneration of epithelial cells is commonly present, and the inclusion, which is indistinguishable from that in trachoma, occurs not only in greater numbers but frequently extracellularly. Since grinding, with its partial cellular disruption, does not suffice in improving filterability, further experiments were undertaken in this connection, which yielded no information, since the attempts to lyze the cells *in vitro* resulted in inactivation of the infectious agent. Consequently, with experimental evidence lacking, the explanation advanced above remains only speculation.

Inability of the infectious agent to multiply in various bacteriological media suggested attempted propagation in tissue cultures. A number of techniques were employed for this purpose, with both infectious and non-infectious human material, which in 35 per cent of the cases contained inclusions, as reported in preceding pages. None of the methods, however, supplied the proper conditions for growth. The conclusion is inevitable, therefore, that the infectious agent of trachoma possesses an exquisite tissue specialization. Unable to infect lower animals at all, the modified disease it induces in apes and monkeys is confined to the conjunctiva. In man, the infectious agent finds the best conditions for growth, and yet the disease is of protracted onset and an extremely circumscribed localization. It is not surprising that it fails to grow in cultures of avian or mammalian tissue. It was for this reason that the technique was conceived of cultivating, in homologous plasma, epithelial cells from the trachomatous conjunctiva, which were frequently infectious and even contained inclusion bodies. While this appears to be a close *in vitro* approximation of the agent's normal habitat, propagation did not ensue.

Upon careful reflection, the evidence suggests that the infectious agent of trachoma is a virus, filterable with difficulty, under conditions

not yet understood. Its characteristics of low infectivity, marked tissue specialization, poor immunogenic properties, rare filterability, weak propagative power, fragility before different agents, all classify the virus as an extremely unusual variety. Indeed, even the inclusions accompanying its presence in human tissues differ from the virus inclusions heretofore recognized. If future investigation succeeds in confirming the viral nature of the infectious agent of trachoma, it will have to be regarded as possessing properties differing considerably from those of viruses now generally known.

SUMMARY AND CONCLUSIONS

1. The infectious agent of trachoma can be freed from extraneous bacteria by passage through rabbit testicle.

2. The infectious agent multiplies little, if at all, during such passage, but in many instances retains its infectivity undiminished.

3. No specific changes occur in the rabbit testicle incidentally to the passage.

4. On rare occasion the trachoma agent may be freed from bacteria by intracerebral passage. The brain tissues show no specific reaction.

5. Filtration experiments with Seitz, Kramer, Berkefeld, and Elford filters confirm the general observation that the infectious agent is filterable with difficulty.

6. Tissue culture experiments, with tissues containing the infectious agent (conjunctiva, rabbit testicle, brain, etc.), conducted under a wide variety of conditions, proved uniformly unsuccessful in the cultivation of the agent.

7. The agent is inactivated by bile, $AgNO_3$, phenol, cocaine, tartar emetic, and gentian violet. Its heat inactivation temperature is between 45° and 50°C., at a time interval of 15 minutes.

8. Attempts to preserve the infectious agent in glycerine were unsuccessful.

9. The accumulated evidence suggests that the infectious agent of trachoma is a virus.

BIBLIOGRAPHY

1. Hess, C., and Römer, P., *Arch. Augenheilk.*, 1906, **55**, 1. Halberstädter, L., and Prowazek, S., *Deutsch. med. Woch.*, 1907, **33**, 1285. Herford, E., *Klin. Monatsbl. Augenheilk.*, 1909, **47**, 225. Morax, V., *Ann. ocul.*, Paris, 1911, **145**, 414. Heyman, E., *Klin. Monatsbl. Augenheilk.*, 1911, **49**, 417.

Nicolle, C., Cuenod, A., and Blaizot, L., *Arch. Inst. Pasteur Tunis*, 1911, **3,** 185. Addario, La Ferla, *Ann. ottal.*, Pavia, 1912, **14,** 278. Olitsky, P. K., and Tyler, J. R., *Science*, 1930, **71,** 564; *J. Exp. Med.*, 1931, **54,** 31.

2. Julianelle, L. A., and Harrison, R. W., *Am. J. Ophth.*, St. Louis, 1933, **16,** 867; 1934, **17,** 1035.
3. Julianelle, L. A., and Harrison, R. W., *Am. J. Ophth.*, St. Louis, 1935, **18,** 11.
4. Hetler, R. A., and James, W. M., *Am. J. Ophth.*, St. Louis, 1934, **17,** 1048.
5. Sattler, H., *Ber. Versamml. ophth. Ges.*, 1881, **13,** 18. Koch, R., *Wien. klin. Woch.*, 1883, **33,** 1550. Michel, J., *Arch. Augenheilk.*, 1885, **16,** 348. Müller, L., *Wien. klin. Woch.*, 1897, **10,** 920; *Arch. Augenheilk.*, 1900, **11,** 13. Raehlman, E., *Beitr. Augenheilk.*, 1908, **7,** 35. Edwards, P. T., *J. Am. Med. Assn.*, 1910, **44,** 965. Williams, A. W., *Arch. Ophth.*, New York, 1913, **42,** 506. Noguchi, H., *J. Exp. Med.*, 1928, **48,** suppl. 2. Lumbroso, U., *Arch. Inst. Pasteur Tunis*, 1931, **20,** 253.
6. Cazalis, C. A., Trachome, Thèse de Montpellier, 1896. Lawson, A., *Roy. London Ophth. Hosp. Rep.*, 1897, **14,** 484. Uthoff, W., *Zentr. prakt. Augenheilk.*, 1898, **22,** 357. Wakizaka, K., *Klin. Monatsbl. Augenheilk.*, 1909, **47,** 793. Pacalin, G., *Arch. Ophth.*, Paris, 1930, **47,** 690. Seidler, M., and Stasinska, J., *Klin. Monatsbl. Augenheilk.*, 1931, **86,** 261. Taborisky, J., *Folia ophth. orient.*, 1932, **1,** 34. Thygeson, P., *Arch. Inst. Pasteur Tunis*, 1933, **22,** 157.
7. Harrison, R. W., and Julianelle, L. A., *Am. J. Ophth.*, St. Louis, 1936, **19,** 118.
8. Bertarelli, E., and Cechetto, E., *Centr. Bakt., 1. Abt., Orig.*, 1908, **47,** 432. Marongiu, L., *Policlinico, sez. prat.*, Rome, 1908, **15,** 805. Nicolle, C., Cuenod, A., and Blaizot, L., *Compt. rend. Acad.*, 1912, **155,** 214. Olitsky, P. K., Knutti, R. E., and Tyler, J. R., *J. Exp. Med.*, 1931, **54,** 557.
9. Pfeiffer, R., and Kuhnt, H., *Z. Augenheilk.*, 1905, **13,** 321. Hess, C., and Römer, P., *Arch. Augenheilk.*, 1906, **55,** 1. Baiardi, P., *Clin. ocul.*, 1907, **8,** 2719. Fermi, C., and Repetto, R., *Berl. klin. Woch.*, 1907, **44,** 1197. Szafnicki, *Zentr. ges. Ophth.*, 1929, **21,** 45. Trapesontzewa, C., *Rev. internat. trachome*, 1930, **7,** 65. Lumbroso, U., and Thygeson, P., *Arch. Inst. Pasteur Tunis*, 1933, **22,** 178. Stewart, F. H., *Ann. Rep. Giza Mem. Ophth. Lab.*, 1933, **8,** 142. Candian, F. L., *Atenca Parmense*, 1933, **5,** 224.
10. Julianelle, L. A., and Harrison, R. W., *Am. J. Ophth.*, St. Louis, 1935, **18,** 133.
11. Noguchi, H., *J. Exp. Med.*, 1915, **21,** 539.
12. Julianelle, L. A., and Harrison, R. W., *Tr. Am. Acad. Ophth. and Oto-Laryngol.*, 1935, 221.
13. Uhlenhuth, P., and Böing, W., *Berl. klin. Woch.*, 1910, **47,** 1514. Himmelberger, L. R., *J. Am. Vet. Med. Assn.*, 1916, N.S. **1,** 450.
14. Nicolle, C., and Cuenod, A., *Arch. Inst. Pasteur Afrique Nord*, 1921, **1,** 149.
15. Nicolle, C., *Bull. Inst. Pasteur*, 1921, **19,** 881.
16. Levaditi, C., Sanchis-Bayarri, V., and Lepine, P., *Ann. Inst. Pasteur*, 1929, **43,** 1064.
17. Lumbroso, U., and Thygeson, P., *Arch. Inst. Pasteur Tunis*, 1933, **22,** 178.

18. Thygeson, P., and Proctor, F. I., *Arch. Ophth.*, New York, 1935, **13,** 1018.
19. Thygeson, P., Proctor, F. I., and Richards, P., *Am. J. Ophth.*, St. Louis, 1935, **18,** 811.
20. Elford, W. J., *J. Path. and Bact.*, 1933, **36,** 49.
21. Cattaneo, D., *Boll. ocul.*, 1932, **11,** 377.
22. Stewart, F. H., *Ann. Rep. Giza Mem. Ophth. Lab.*, 1933, **8,** 142; 1934, **9,** 95.
23. Kramer, S. P., *J. Infect. Dis.*, 1927, **40,** 343.
24. Bauer, J. H., and Hughes, T. P., *J. Gen. Physiol.*, 1934–35, **18,** 143.
25. Maitland, H. B., and Maitland, M. C., *Lancet*, 1928, **2,** 596.
26. Li, C. P., and Rivers, T. M., *J. Exp. Med.*, 1930, **52,** 465.
27. Woodruff, A. M., and Goodpasture, E. W., *Am. J. Path.*, 1931, **7,** 209.
28. Burnet, F. M., and Galloway, I. A., *Brit. J. Exp. Path.*, 1934, **15,** 105.
29. Harrison, R. W., and Moore, E., *Proc. Soc. Exp. Biol. and Med.*, 1936, **35,** 359.
30. Dochez, A. R., Mills, K. C., and Kneeland, Y., Jr., *J. Exp. Med.*, 1936, **63,** 559.
31. Pandit, C. G., Wright, R. E., Rao, R. S., and Satyanathan, *Indian J. Med. Research*, 1935, **23,** 475.
32. Hess, C., and Römer, P., *Arch. Augenheilk.*, 1906, **55, 1.**
33. Botteri, A., *Klin. Monatsbl. Augenheilk.*, 1912, **50,** 653.
34. Julianelle, L. A., *Am. J. Path.*, 1936, **12,** 775.
35. Nicolle, C., Cuenod, A., and Blaizot, L., *Arch. Inst. Pasteur Tunis*, 1913, **3** and **4,** 157. Baroni, V., and Michail, D., *Rev. internat. trachome*, 1932, **9,** 31. Candian, F. L., *Ann. ottal.*, Pavia, 1933, **61,** 890. Stewart, F. H., *Ann. Rep. Giza Mem. Ophth. Lab.*, 1933, **8,** 142; 1934, **9,** 95. Busacca, A., *Folia clin. biol.*, 1935, **7,** 63.
36. Morax, V., Les conjunctivites folliculaires, Paris, Masson et Cie, 1933.
37. Halberstädter, L., and Prowazek, S., *Deutsch. klin. Woch.*, 1907, **33,** 1285.
38. Greef, R., Frosch, H., and Clausen, W., *Arch. Augenheilk.*, 1908, **59,** 203. Herford, E., *Klin. Monatsbl. Augenheilk.*, 1909, **47,** 225. Flemming, *Arch. Augenheilk.*, 1910, **66,** 63. Botteri, A., *Klin. Monatsbl. Augenheilk.*, 1912, **50,** 653. Leber, A., and von Prowazek, S., *Arch. Ophth.*, Leipsic, 1913, **85,** 204. Bertarelli, E., and Cechetto, E., *Centr. Bakt., 1 Abt., Orig.*, 1908, **47,** 432.
39. Böing, W., *Arb. k. Gsndhtsamte*, 1912, **40,** 235. Löhlein, W., *Arch. Augenheilk.*, 1912, **70,** 392. Stewart, F. H., *Ann. Rep. Giza Mem. Ophth. Lab.*, 1933, **8,** 142; 1934, **9,** 95. Wilson, R. W., *Brit. J. Ophth.*, 1931, **15,** 433. Schuurman, C. J., *Centr. Bakt., 1. Abt., Orig.*, 1932, **125,** 158. Julianelle, L. A., and Harrison, R. W., *Am. J. Ophth.*, St. Louis, 1933, **16,** 867; 1934, **17,** 1035.
40. Greef, R., Frosch, H., and Clausen, W., *Arch. Augenheilk.*, 1908, **59,** 203. Mijaschita, S., *Klin. Monatsbl. Augenheilk.*, 1908, **46,** pt. 2, 626. Wakizaka, K., *Jahrb. ophth.*, 1914, **45,** 290. Taboriski, J., *Arch. Ophth.*, Leipsic, 1929, **123,** 140. Thygeson, P., *Am. J. Ophth.*, St. Louis, 1933, **16,** 409.