DISCUSSION

It was believed in the past that an epitheliotropic virus like that of trachoma would require epithelial tissues for growth and only scant attention was given the yolksac medium as compared with the great deal of attention given the chorioallantois and tissue culture. Inoculations on the chorioallantoic membrane and into the amniotic sac yielded only negative results in the present investigations. Smears from the harvested chorioallantoic and amniotic materials showed no virus elementary bodies. It was not possible even to find evidence of infection or presence of inclusion bodies in the conjunctiva of the embryo from eggs inoculated through the amniotic sac route. By inoculation through the yolksac route three strains of trachoma virus could be isolated from 130 conjunctival specimens and and serially passaged in 6-8 day old embryonated eggs.

The presence of bacteria in the conjunctival specimen constituted the main difficulty in isolation experiments. Bacteria most commonly encountered were diphtheroids and micrococci. An antibiotic was chosen which would inhibit bacteria but would not affect the growth of virus or the viability of embryo. 5-10 mg. of streptomycin allowed to act upon 0.5 ml. of the inoculum kept inside the refrigerator at 4°C for 3-4 hours provided satisfactory results. T'ang et al.¹ found that streptomycin could be used without affecting virual multiplication but penicillin was
antiviral; they used streptomycin in the concentration of 2.5 mg. per ml. Sowa and Collier\textsuperscript{113}, however, showed that concentration of streptomycin could be raised to 20 mg. per ml. without affecting the virus or the embryo.

The problem of fungal contamination, occurring at times, could be effectively dealt with by adding Mycostatin in the concentration of 50 mg. per ml. The use of penicillin for the purpose of decontaminating the inocula was probably responsible for many failures reported in literature; it interferes with multiplication of trachoma virus.

The temperature of $35^\circ$C proved to be optimum for incubation of eggs following inoculation. This was so, probably because human conjunctival sac which is the natural habitat of trachoma virus has the same temperature.

Repeated blind passages were necessary for the primary isolation of trachoma virus. In the first or second passages, the number of elementary bodies was too few to be detected unequivocally. Earlier attempts at laboratory cultivation of trachoma virus which did not include repeated blind passages for each specimen had generally met with failure.

Growth of the virus in yolksac was indicated by sluggish movement or death of the embryo. Death mostly occurred after 4 - 8 days of inoculation but such lethality effect of virus proliferation was not always regular. Even at 12 days after inoculation live embryos could be seen in some of the infected eggs. On harvest, embryos from infected eggs looked stunted as compared to the live ones of the same
Infected yolksac membranes were found to be congested, sometimes even haemorrhagic. Lethality effect on the chick embryo and haemorrhagic changes were, however, consistently observed when well adapted strains were passaged for preparing the antigen.

Elementary bodies could be demonstrated in smears from infected yolksac membranes stained by Macchiavello's method. They looked red in the blue background, occurring singly or in small irregular clumps, mostly extracellularly. Intracellular elementary bodies were rare and no inclusion body could be seen in yolksac cells. Collier and Sowa, however, reported that with a particular Gambian strain, there were yolksac cells packed with virus particles which stained characteristically with Giemsa and with Castaneda. The number of elementary bodies in infected yolksac tissue increased with repeated passages of the isolated strains but such increase was not always consistent.

With view to facilitate primary isolation of trachoma virus adjuvants such as lysozyme, sodium fluoride, cortisone, desoxyribonucleic acid and glutathione were added to the inoculum for the yolksac but there was no favourable effect with any of them. All the positive isolations were through the use of inocula without adjuvant.

All the positive isolations were from clinically established cases of trachoma. Conjunctival specimens from 9 clinically normal persons inoculated through the yolksac route yielded negative results.
Identification of the strains isolated was based on microscopy, animal inoculation and serology.

Morphology in regard to size, shape, distribution and staining character was studied by microscopic examination of smears from infected yolksac membranes stained by Macchiarvello's method. Similarly stained smear of TW 10 strain of trachoma virus was obtained from Dr. Grayston and examined for the purpose of comparison. Under the microscope, a close identity between the three strains and TW 10 was evident. TW 10 strain, available at a later date from Dr. Grayston was also grown in the laboratory and it closely resembled strains P23, P24 and P36 in regard to morphology and growth character including the effect on the chick embryo.

Monkeys inoculated with one P36 of the isolated strains developed follicular conjunctivitis without any obvious corneal involvement. The disease in monkeys was not associated with pannus and regressed without scar formation. No inclusion bodies were observed in conjunctival scrapings of the infected monkeys. However, sera from them, tested with purified elementary body antigen of trachoma (TW 10 purified with trypsin and polymyxin; obtained from Dr. Grayston) showed the presence of specific complement fixing antibodies.

Antigen made from P36 by the method of phenolization and boiling gave positive complement fixation reaction with sera from clinically established cases of trachoma and the experimentally infected monkeys.
It was thus clear that the strains isolated were the viruses of trachoma, obtainable only from trachoma patients and not from clinically normal persons.

In recent years, fluorocarbon has been used by many investigators\textsuperscript{200-205} for purification of viral antigens. In view of the fact that trachoma virus grown in yolk sac invariably contains adhering yolk material predominantly fatty in nature, the fat solvent fluorocarbon (Genetron 113) was chosen as the purifying agent. Gesseler et al.\textsuperscript{200} used a mixture of Freon 112 and n-heptane for purification of vaccinia virus grown on the chorioallantoic membrane and noted that if Genetron 226 was chosen, n-heptane was not required; there was no difference between the results obtained with Freon 112 mixture and Genetron 226. It was because of its suitability for use without the possible complicating factor of n-heptane that Genetron 113 was selected for purifying the trachoma antigen.

Epstein\textsuperscript{206} showed that treatment with fluorocarbon removed formed host cell constituents but nevertheless the product contained other important host substances; the gelatinous material of host origin could not be removed even after repeated treatments with fluorocarbon. Fluorocarbon purification, though inadequate for chemical analysis of virus, is well suited to the study of viral antigens. Diagnostic complement fixation antigens have been prepared from animal viruses grown in different host systems by this method.
Viral antigens are known to vary in their susceptibilities to the fluorocarbon treatment. Hamparian et al. showed that the coxsackie and poliomyelitis viruses tolerated repeated treatment of fluorocarbon without loss in titre whereas mumps and influenza viruses were rendered antigenically inactive by two treatments. The difference is probably due to the chemical structures of viruses; mumps and influenza viruses have a relatively high lipid content compared to coxsackie and poliomyelitis viruses.

In the absence of precise knowledge about the chemical composition of trachoma virus, in the beginning, it was not possible to predict the suitability of Genetron in the purification of trachoma antigen. Single as well as repeated treatments using varying amounts of Genetron were adopted with a view to determine the optimum amount which would remove anticomplementary factors and nonviral yolk-sac antigens but would not affect the specific viral antigen. Products of repeated Genetron treatments were free of anticomplementary activity, rather procomplementary but antigenically inactive. The total amount of Genetron employed in repeated treatments was obviously excessive. In view of the drastic effect on the viral antigen caused by the excessive amount of Genetron used in repeated treatments, single treatment using less amount of Genetron was adopted. Purification employing single treatment yielded the antigen which was devoid of anticomplementary
effect and yet exhibited the capacity to fix complement in presence of specific antiserum. 1 part of Genetron proved to be optimum for 4 parts of 10% crude antigen in preparing antigen of unimpaired complement fixing activity. The antigen prepared by Bernkopf et al using 1 part of fluorocarbon (Arcton) for 3 parts of 10% crude antigen showed some impairment of complement fixing activity as evident in their results of complement fixation tests.

The essential role of lecithin in the complement fixing activity of trachoma antigen was discovered during purification experiments employing repeated Genetron treatments. Antigen purified with more than optimal amount of Genetron showed little or no complement fixing activity, although the number of virus particles in such antigen remained unaffected. Obviously some essential component of the viral antigen was removed by the excess of Genetron. Addition of lecithin to preparations with little or no complement fixing activity enhanced or restored the antigenicity. Similar observations were made by Hilleman and Nigg in connection with their work on complement fixation antigen of lymphogranuloma venereum virus grown in yolksac and extracted with ether. The low potency of Bernkopf et al.'s antigen might be due to low content of lecithin because they used fluorocarbon in amounts more than the optimum as determined in the current study. As in case
of Wassermann antigen, lecithin seems to be an essential component of the complement fixation antigen of trachoma. Lecithin by itself had neither anticomplementary effect nor complement fixing capacity. Appropriate amounts of lecithin could be added to Genetron purified antigens without rendering them anticomplementary. Such amount of lecithin added to the antigen purified with optimal amount of Genetron caused enhancement of complement fixing reactivity but the enhanced sensitivity was associated with appearance of nonspecific reactivity for normal human serum. Those normal sera which uniformly gave negative reactions with the optimally purified antigen yielded positive reactions with lecithin fortified antigen. In view of the finding that lecithin enhanced sensitivity at the expense of specificity, Genetron purified antigen fortified with lecithin could not be recommended for diagnostic serology.

Investigations on the aqueous supernatant revealed that apart from the virus particles a soluble component was associated with the infected yolksac; it showed ability to fix complement in presence of specific antisera. The soluble antigen could not be deposited by high speed centrifugation but was completely withheld when passed through Seitz EK filters. Such antigen which contained abundant nonviral yolksac elements was obviously not suited for diagnostic work. It was
group specific, showing the capacity to fix complement in presence of sera from both trachoma and lymphogranuloma venereum patients.

The method of phenolization and boiling is commonly employed to enhance the potency of antigens from psittacosis-lymphogranuloma group of viruses. Such method employed for trachoma viruses yielded an antigen, which was capable of fixing complement in presence of specific antisera, but had little or no enhanced reactivity. This antigen reacted in high titres with the lymphogranuloma sera but in low titres or not at all with the trachoma sera. It was clear that the method of phenolization and boiling rendered the trachoma antigen group specific rather than species specific without any appreciable enhancement of reactivity.

Study of the effect of treatment with potassium periodate indicated that purified elementary bodies contained a component, probably, carbohydrate in nature which was oxidised by the periodate. Periodate treatment caused reduction of antigenicity. This antigen of reduced sensitivity appeared to be species specific; it did not react with lymphogranuloma serum. However, in view of poor sensitivity such antigen was not deemed suitable for diagnostic work in detecting low level trachoma antibody in human infections.

Vaccinia and Rous sarcoma viruses purified with Genetron exhibited higher infectivity (Gessler et al.200) whereas trachoma virus, purified similarly, proved to be
noninfectious. Similar observation in case of trachoma virus has been reported by Bernkopf et al. who, however, noted that the preparations remained infectious if all manipulations were carried out in the cold. In the present investigation, homogenisation with Genetron was carried out at the room temperature and the heat generated was probably responsible for the loss of infectivity; because Genetron by itself showed no deleterious effect on virus infectivity.

Woolridge and Grayston made extensive studies by complement fixation tests using the antigen purified with trypsin and polymyxin and obtained significant results. Bernkopf et al. adopted agglutination test, complement fixation test and fluorescent antibody technique using the antigen purified with fluorocarbon (Arcton) and found complement fixation test less sensitive that the other two methods. As pointed out earlier, the defect was with the antigen used. In preparing the antigen for diagnostic complement fixation test in the current study, paramount considerations were given to potency and specificity. Potency of the antigen was all the more important in the investigation of trachoma which is associated with a low antibody particularly in the early stage.

Even though complement fixation test is by far the most common serologic test conducted in virological diagnosis, techniques vary from laboratory to laboratory. In an evaluation of several complement fixation techniques in the laboratory diagnosis of rickettsioses, Schubert et al. showed that sensitivity and specificity were
inversely related; a gain in one property led to a concomitant loss in the other. The modified Kolmer technique was adopted in the optimal proportion method of Friedwald was adopted in the current work; a happy middle position of balance between sensitivity and specificity could be achieved by this method. For primary incubation overnight refrigeration at 4°C was used; it was found to be more sensitive than the incubation at 37°C for 1 hour.

Evaluation of the Genetron purified antigen was made with sera from immunized rabbits and vaccinated monkeys. Contrary to the erroneous view of Julianelle, elementary bodies of trachoma proved to be antigenically potent; showed the capacity of inducing production of antibodies and of specifically reacting with them. Compared to the antigen purified with trypsin and polymyxin the antigen purified with optimal amount of Genetron contained less of nonviral antigen. The Genetron purified antigen was also better in regard to nonspecific reactivity with normal human sera.

Compared with the group antigen of trachoma prepared by the method of phenolization and boiling Genetron purified elementary body antigen proved more potent in terms of frequency as well as height of antibody titres detected. Genetron purified antigen was species specific, giving positive reaction with trachoma sera, but not with normal human sera or lymphogranuloma
sera. One hundred "normal" sera were obtained from healthy U.S. marines stationed at Okinawa who presumably had no trachoma in view of the country of origin and the close medical supervision. Some of them were, however, likely to have had past experience with lymphogranuloma venereum. Uniform negative findings with these sera indicated that the antigen was highly specific and there would be no non-specific reaction with normal human sera. Negative reactions even at the lowest dilution of 1 in 4 with the lymphogranuloma sera provided the additional proof for specificity. Ross and Gogolak's method of preparing the antigen by treatment of sonically disrupted virus with potassium periodate has been recommended by K.F. Meyer but such antigen of unpredictable reactivity could not be considered suitable for detecting low level trachoma antibodies.

Ordinarily it is impermissible to draw a diagnostic conclusion on the basis of a test of one serum specimen, but in a superficial and chronic disease like trachoma the mere presence of antibody in the serum may safely be regarded as diagnostically significant provided the antigen used is highly specific. It is not yet possible to say with certainty what level of serum dilution should be taken as the one beyond which a positive reaction indicates prior and continuing infection with trachoma virus. However, in the current study fixation at dilutions of 1 in 8 or above has been considered as reliable evidence of infection. Rake believes fixation at 1 in 4 dilution to be diagnostic in
lymphogranuloma venereum.

When the Genetron purified elementary body antigen was used in complement fixation tests on 306 sera from Aligarh area, an overall 63.6 per cent showed antibody titres ranging from 1 in 4 to 1 in 256. Broken down according to clinical diagnosis of the eye conditions positive results were as follows: early trachoma 30.0 per cent, late trachoma 68.1 per cent, doubtful 19.2 per cent and "normal" 37.0 per cent. Corresponding antibody titres are significant; 6 out of 36 positive sera in the early trachoma group had antibody titres of 1 in 32 or more, while 64 out of 92 in the late trachoma group had similar high titres. Evidently, late trachoma group not only had larger number of positives but also higher antibody titres. Frequency and height of antibody titre seem to be related to the duration of illness. Similar correlation of the antibody with severity and activity of trachoma has also been reported by Woolridge and Grayston.

In experiments with human volunteer, they observed that complement fixing antibodies tend to fall or even disappear prior to the cessation of activity of the disease. Seronegativity in the late trachoma group conform to the experimental observation, whereas, seropositivity with high antibody titres signifies that in natural infections antibodies persist at a high level even to the late stage, possibly due to a booster effect consequent upon repeated
antigenic stimulation occurring in the highly endemic area of Aligarh.

The finding of large number of seronegative cases in the early trachoma group poses a problem which remains to be solved by further investigations. Complement fixation test is undoubtedly by far the most sensitive of all serological methods and it is difficult to attain further improvement in the potency of the antigen without affecting the specificity. The superficial lesion localised to the eye and the long protracted course of the disease with occasional flare-ups after several years suggest that general immunity reaction of trachoma would be rather weak and every case may not have circulating antibodies detectable by any kind of serological test. Based on clinical impression of the ophthalmologist many asymptomatic cases were included in the early trachoma group and these cases associated with a weak antigenic stimulus are likely to have no circulating antibodies at all. Furthermore, in the early stage of trachoma if positive reactions at 1 in 4 dilution is taken into consideration then the number of seronegatives would be fewer. It is also possible that some cases clinically labelled as trachoma were in reality infections by other viruses like adenoviruses.

In view of the possible mistake in the clinical diagnosis it was not unlikely that there would be some seropositive cases in the doubtful group; they might be real cases of trachoma which could not be correctly diagnosed without appropriate laboratory aid. This finding, in fact,
illustrates the need for the laboratory aid in the diagnosis of trachoma. Cases with clinical diagnosis of chronic follicular conjunctivitis included in doubtful group might as well prove to be cases of inactive trachoma.

Considering the specificity of Genetron purified antigen which reacts only with trachoma antibodies, the large number of positive cases in the "normal" group of Aligarh area was, indeed, a finding of paramount significance. It is all the more so because earlier tests on 100 "normal" sera with the same antigen did not yield a single positive reaction even at the lowest titre of 1 in 4. In view of high incidence of trachoma in Aligarh area it was quite likely that the "normal" inevitably exposed to the virus in the family or in the community had infections of mild nature without clinical manifestations. This fact was fully borne out by the results of 20 "normal" sera from Coonoor area; only 2 were positive and the antibody titre in both cases as low as 1 in 4. It is well known that in countries like Japan, Thailand and Taiwan there are very mild cases of trachoma which heal spontaneously without leaving visible permanent changes and these pose a problem of diagnosis to the ophthalmologist. Results of "normal" sera from 3 different regions (America, Aligarh and Coonoor) suggest that very mild form of trachoma does occur in India particularly at Aligarh, an area of high trachoma index.

Seropositivity in the "normal" group is not limited to Aligarh area of this investigation; 5 - 6 per cent of
"normal" cases in Taiwan showed antibody titres of 1 in 10 or greater in the investigation conducted by Woolridge and Grayston. Positive agglutination and complement fixation reactions were also observed in 11 - 13 per cent of "normal" cases in Jerusalem by Bernkopf et al. In a personal communication Terrizzo corroborated the finding of similar seropositivity amongst the clinically "normal" cases of Tunisia by complement fixation test.

The complement fixation test using the Genatron purified antigen provides measurement of low level trachoma antibodies in persons infected with trachoma virus and represents a reliable aid in the laboratory diagnosis of trachoma. Diagnostic significance of complement fixing antibodies could be better appreciated by a study of serial samples of sera and in that case it would be possible to determine the diagnostic titre as well as the time appropriate for collecting the serum.

The skin test has proven most useful in the diagnosis of lymphogranuloma venereum, although it is less sensitive than the complement fixation test. In their study on trachoma virus, Grayston et al. observed that intradermal injection of purified trachoma antigen elicited delayed tuberculin type reaction in persons with various stages of trachoma, but the Frei antigen used as control failed to elicit positive reactions even in human volunteers with active trachoma. The preliminary study of Collier and Sowa also indicated the usefulness of skin test in trachoma;
positive skin reactions with trachoma antigen were found only in trachoma patients, not in clinically normal subjects. Skin test with Genetron purified trachoma antigen provided results only of doubtful significance. Considering the large number (70 per cent) of early trachoma cases with no detectable complement fixing antibody, the need for a sensitive test was very real but the skin test could not serve as the proper substitute for complement fixation test. Skin test results bore no relation either to the clinical diagnosis or to the severity of infection; the number of positive skin reactors is equal in early (56 per cent) and late (57 per cent) trachoma groups while more (69 per cent) in doubtful group. Results in the "normal" group were of some interest; all those individuals who had complement fixing antibodies were also positive skin reactors. It appears that persons with subclinical infection of trachoma develop antibodies as well as delayed hypersensitivity.

Because of the extreme divergence in the results of microscopic examination for finding trachoma inclusion bodies, reported in the literature, it has been rather difficult to assess the value of such examination in the laboratory diagnosis of trachoma. In fact, Snyder et al. stated that wide differences exist in the occurrence of inclusions in conjunctival scrapings from different geographic areas, or indeed, from two villages only one kilometer apart. When the results of microscopic
examination were compared with those of complement fixation tests and skin tests performed on the same 308 individuals of Aligarh area, it became clear that diagnosis by demonstration of Halberstaedter-Prowazek type of inclusion bodies is the least sensitive of the three methods; fewer number of inclusion positive cases in both early and late trachoma groups and none in either doubtful or in "normal" groups. Inclusion bodies were demonstrable neither in Stage IV, nor in low grade infections even in Stage I. Even if inclusion bodies of trachoma are taken as sufficiently pathognomonic such lowly sensitive method cannot be regarded as of much aid even in confirming the clinical diagnosis of trachoma.