III MATERIALS AND METHODS

CULTURE OF VIRUS

Egg: White leghorn and Rhode Island eggs supplied by the Government Poultry Farm, New Delhi were used for isolation experiments. After receiving in the laboratory, eggs were washed in weak soap solution and then in running tap water. After drying they were kept inside egg incubator at 37°C. Eggs were candled to select out those possessing developing embryos; sterile and dead eggs were discarded. Eggs of the same batch received on a particular day were used for an experiment.

Collection of specimen: The specimen consisted of swab rubbed over the scraped upper tarsal conjunctiva. The clinical diagnosis was based on the criteria laid down by the W.H.O. Expert Committee on Trachoma (1956). Active cases in stages 1 & 2, particularly those with copious exudation from eyes were preferred. The patients belonged to Delhi Area including Kotla Mubarakpur and Gautam Nagar; most of them were children. Specimen could not always be collected under ideal conditions of hospital operation theatre; a number of them were collected in the local dispensaries where the risk of outside contamination was high.

The patient was made to lie on a table and 1-2 drops of anethane (1%) were instilled in infected eye. After 3-5 minutes the eye was washed with sterile normal
saline. The upper lid was everted and the upper tarsal conjunctiva was scraped with a single sharp but light sweep so as to collect only superficial cells. The scraped material was smeared on clean glass slides. The sterile swabs were then rubbed on the scraped surface and put back into screw capped vials. One of the swab was placed in a vial containing 1 ml. of Snyder's solution for isolation of virus. The other swab was used for bacteriological culture. Terramycin eye ointment was then applied in the eyes.

The specimens were transported to the laboratory in a wide-mouthed thermos flask containing salted ice. Swabs for virus isolation were squeezed dry by sterile forceps and discarded; the fluid material was kept inside the refrigerator for 2-4 hours before inoculation in eggs.

Inoculation and harvest: Inoculations were made on the following lines:—

1. On the chorio-allantoic membrane.
   Age of the embryo ............... 11 days.
   Dose of inoculum .................. 0.1 ml.
   Harvest ..................... After 6 days.

2. Into the amniotic sac.
   Age of the embryo ............... 7 days.
   Dose of inoculum .................. 0.1 ml.
   Harvest ..................... After 6 days.
3. Into the yolksac.

Age of the embryo .................. 7 days.
Dose of inoculum .................. 0.25 - 0.50 ml.
Harvest ............................ Immediately after death of the embryo or after 12 days.

In some experiments, adjuvants were added and inoculated through the yolksac route. Adjuvants used per 0.5 ml. of the inoculum were:

i) Sodium fluoride .......... 0.5 mgm.
ii) Lysozyme ........... 0.25 ml.
iii) Cortisone .......... 1 microgram.
iv) Desoxyribonucleic acid ......... 5 "
v) Glutathione ........ 5 "

In all experiments, eggs inoculated with equal volume of Snyder's solution were included as controls. When adjuvants were used, the control eggs were inoculated with equal volume of Snyder's solution containing equivalent amounts of adjuvants.

For inoculation by the yolksac route the airsac was marked out and the area was sterilized with rectified spirit and Tr. iodine; at the centre a needle hole was made with a pointed punch. The streptomycin treated inoculum was injected, pushing the needle down straight along the longitudinal axis to a depth of 1.5 cm. The piston was drawn up a little to verify whether the needle was inside yolksac.

The needle holes were sealed with melted paraffin and the
inoculated eggs were incubated at 35°C. The eggs were candled twice daily; those dying within 48 hours of inoculation were discarded. The inoculated eggs were harvested immediately after death of the embryo or killed after 12 days of inoculation.

During harvest, the yolksac was squeezed with forceps to get rid of excess yolk fluid and the membrane was preserved in screw capped vial. A piece from the yolksac membrane was cut, lightly touched on sterile blotting paper and then smeared on clean glass slides. The smear stained by Macchialvallo's method was examined for elementary bodies. The membranes were also tested for bacteriological sterility by routine inoculation on sheep blood agar.

**Egg passage:** Harvested membranes which did not show evidence of gross bacterial contamination were pooled, weighed and grinded in sterile mortar and pestle. Appropriate amount of Snyder's solution was added to make approximately 10-15% suspension. The suspension was lightly centrifuged at 300 - 1000 rpm. for 5 minutes and the supernatant was removed with sterile pasteur pipette carefully avoiding the floating fatty material at the top.

The supernatant after treatment with streptomycin as above was reinoculated on the same day into another batch of eggs. Irrespective of any evidence of multiplication of the virus, three blind passages were made for each specimen. The specimens were regarded negative only when after three passages no elementary body could be seen in the smear of
Microscopic examination: Impression smears of harvested yolksac membranes were stained by Macchiavello's method and examined under oil immersion objective of ordinary light microscope.

Macchiavello's method: Solutions:

1) 0.25 per cent basic fuchsin in distilled water.
2) freshly prepared 0.25 to 0.50 per cent citric acid.
3) 1 per cent Methylene blue in distilled water.

After drying in air, the smear was fixed by heat. The basic fuchsin solution first passed through filter paper in a small funnel, was dropped onto the film and allowed to remain for 5 minutes. The fuchsin was then drained off and the slide quickly dipped for 15-20 seconds into the citric acid solution, held in a Coplin jar. The slide was thoroughly washed with tap water and then stained with Methylene blue for 1-1 minute. The slide was washed again in tap water and dried by blotting.

Animal inoculation: Two Rhesus (Genus - Macaca) monkeys were first examined for evidence of spontaneous conjunctival folliculosis and spontaneous follicular conjunctivitis. They were free from follicles as well as hyperaemia and discharge.

The eyes of monkeys were inoculated with the strain P36. A cotton swab soaked in the inoculum (grinded yolksac membrane suspension) was rubbed over the upper tarsal conjunctiva which was lightly scraped beforehand. Each eye received approximately 0.2 ml. of the suspension. The animals were examined twice a week. Conjunctival changes
such as follicular hypertrophy, inflammatory infiltration, conjunctival hyperaemia and purulent discharge were recorded. Conjunctival scrapings from them, stained by Giemsa's stain were examined for inclusion bodies. Serum collected after 6 weeks of experimental disease was tested for complement fixing antibodies.

**Seroology:** Complement fixation test.

**Preparation of antigen:** The method of Nigg, Hilleman and Bowser was used. The infected yolksac membranes were grinded and to this was added saline containing 0.5 per cent phenol to make a 10 per cent suspension. The material contained in a screw capped vial was incubated at 37°C for 4 weeks with daily shaking to resuspend ground particles deposited at the bottom. After 4 weeks the suspension was centrifuged at 1000 rpm for 10 minutes. The deposit was discarded and the supernatant was placed in boiling waterbath for 30 minutes; and then centrifuged again at 1500 rpm for 10 minutes. The straw coloured supernatant was used as the group specific antigen.

**Procedure of the test:** a) Reagents.

1) 0.85% saline containing 1 ml. of 10% MgSO₄ and 3 ml. of 1% CaCl₂ per litre.

2) Sera were inactivated for 30 minutes in a water bath at 56°C. The initial dilution used in the test was 1 in 4 and serial 2-fold dilutions made with saline beginning with this starting dilution.
3) Antigen - 2 full units contained in 0.25 ml.
4) Complement - Fresh or lyophilised guinea-pig serum restored to the original volume. 2 full units contained in 0.50 ml. were used.
5) Haemolytic system - 0.50 ml.
Cells - Defibrinated sheep red blood cells washed in saline 10 minutes at 2000 rpm. for 3 successive times; packed for 15 minutes at 2000 rpm. and made to 2% suspension.
Haemolysin - 2 units contained in 0.25 ml. were used for sensitization of 0.25 ml. of cell suspension.
Sensitized cell suspension, prepared by adding equal amounts of haemolysin and cell suspension was kept at room temperature for 10 minutes.

b) Test: Protocol of complement fixation test.

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The modified Kolmer technique as described by Rosenbaum and Woolridge was employed. In all tests 2 units of antigen and 2 full units of.
complement were mixed with serial dilutions of test sera and incubated overnight at 4°C. The following day, the tubes were incubated at 37°C for 10 minutes and 2 units of haemolysin and 2% sheep cells were added. The tubes were again incubated at 37°C for 30 minutes and readings taken. The total volume in each tube was 1.5 ml.

Titration of complement in the presence of antigen was performed along with each batch of tests.

The highest serum dilution in which 3+ fixation (75 per cent) occurred was taken as the end point and reported as the reciprocal of the original serum dilution.

PURIFICATION OF ANTIGEN

**Virus:** The antigens subjected to purification were made only from well adapted virus strains (TW 29 and ND 115) isolated earlier by the U.S. Naval Medical Research Unit No.2 at Taipei. The virus seeds were in their 18th and 6th passages in eggs. These strains inoculated in eggs through yolksac route killed majority of embryos within 6 - 8 days. When harvested, yolksac membranes were congested, even haemorrhagic in some cases and impression smears from them invariably showed large number of elementary bodies.

**Crude antigen:** Selected yolksac membranes rich in elementary bodies found bacteriologically sterile were pooled and weighed. Measured amount of Mc-IIlvaine's citrate buffer solution (pH 7.4) was added and the mixture homogenised in waring
blender operating at high speed for 2 minutes. The pH of the homogenate was adjusted to 7.2. The homogenate was then clarified by low speed (1000 rpm, for 10 minutes) centrifugation. The fat material which floated at the top was removed by suction. The supernatant collected by means of pipette represented the crude antigen. The concentration of virus suspension or antigen which varied according to the plan of experiment were calculated in terms of initial weight of yolksac membranes.

Procedure of purification: Purification method consisted of fluorocarbon treatment and differential centrifugation. Fluorocarbon is a potent fat solvent as well as a deproteiniser. Genetron 113 (trifluoro-trichloro ethane, \(\text{CCl}_2F - \text{CClF}_2\)) obtained from Allied Chemicals, U.S.A. was used.

 Different volumes of Genetron were used depending on the experimental set up; the requisite volume was added to the crude antigen contained in a waring blender already cooled at the refrigeration temperature. Homogenisation in presence of Genetron was carried out for 2 minutes and the whole assembly was transferred inside refrigerator for 10 - 15 minutes. The process was repeated three times. The homogenate was centrifuged at 1800 rpm. for 5 minutes and it separated into three well defined layers.

Most of the nonviral yolksac protein wetted with
Genetron settled at the bottom in the form of thick reddish brown deposit. The supernatant aqueous layer was straw or light yellow coloured showing very little or no fatty material ordinarily observed in the preparation of crude antigen. The interphase layer between the supernatant and deposit was thin and grayish in colour.

The supernatant which was rich in virus elementary bodies was carefully pipetted and stored in the refrigerator. The interphase layer which also contained virus particles was carefully pipetted off, resuspended in buffer solution, homogenised and centrifuged as before; the resulting supernate was removed and stored as before. The bottom layer which contained very few virus particles was also similarly treated.

The three supernatant aliquots were pooled together and virus particles were thrown by high speed centrifugation at 10,000 rpm. for 30 minutes using a spinco refrigerated centrifuge. The deposited virus was suspended in measured volume of buffer solution to make a 40 per cent suspension. The suspension after thorough agitation was centrifuged again at low speed (2000 rpm. for 10 minutes). The high and low differential centrifugation was at times repeated. The final supernatant representing the purified antigen was stored at 4°C without any preservative.

The purified antigen was subjected to the following tests:
a) **Viral content** - A drop of the purified virus suspension was smeared on clean glass slide, stained by Macchiavello's method and examined under light microscope using oil immersion objective for the approximate number of elementary bodies.

b) **Anti-complementary activity** - Complement was titrated in the presence of purified antigen. A (1) one plus (25) twentyfive dilution of complement was prepared by adding 6.25 c.c. of complement fixation saline (0.85 per cent saline containing 1 c.c. of 10% MgSO₄ and 3 c.c. of 1% CaCl₂ per litre) and 0.25 c.c. of complement. The dilution (1 in 26) of complement was used in complement titration.

<table>
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<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
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<td>0.25cc</td>
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Titration was set up in 2 rows; with and without antigen. After preliminary overnight incubation in the refrigerator 1 c.c. of haemolytic system (prepared by adding equal amounts of haemolysin and sheep red blood cell suspension (2%) and keeping at room temperature for 10 minutes) was
added in all tubes; incubated at 37°C for 30 minutes and reading taken.

Complement titre was calculated as 2 full units represented in 0.50 ml. volume. Because the volume of haemolytic system in complement titration was twice that used in complement fixation test, the tube showing complete haemolysis was taken as 2 exact units and the next tube as 2 full units. Calculation was made as follows:

If there was complete haemolysis in the tube containing 0.20 ml. of 1 in 26 dilution of complement. Then 0.25 ml. of 1 in 26 dilution = 2 full units.
.
. 0.50 ml. of 1 in 52 dilution = 2 full units.

The antigen was regarded as free of anticomplementary activity only when complement titre in the presence of antigen equalled that in the absence of antigen or more than 1:30.

c) Complement fixing reactivity - Sera from rabbits and monkeys immunized with purified trachoma antigen were tested.

d) Nonviral yolksac antigen - Serum from rabbit immunized with nonviral yolksac antigen was tested.

e) Specificity - Sera from clinically normal persons,
syphilis and lymphogranuloma patients were tested to show that the purified antigen reacted only with trachoma antiserum.

f) Sensitivity - Sera from clinically established cases of trachoma were tested simultaneously with purified elementary body antigen and the group specific antigen to show that the former gave positive reaction in higher percentage of cases with higher antibody titres.

Preparation of antisera:

Adjuvant: Aluminium hydroxide - 20 mg/ml.
Gamma globulin - 200 gamma/ml.
Formalin - 0.02 per cent.

An amount of 10 ml. of antigen-adjuvant mixture (7 parts of antigen added to 5 parts of adjuvant) was injected intramuscularly on the back of the rabbit at 5-6 different sites. After 4 weeks rabbits were bled and sera tested for complement fixing antibody. When the titre rose to 1 in 128 or higher, the rabbit was bled to death. The serum was separated and stored in the frozen state in the refrigerator at -20°C.

Five rabbits were immunized with the purified elementary body (Tw 29) antigen and one rabbit with nonviral yolksac antigen. Uninfected yolksac membranes from embryonated eggs, 16-18 days old, were used for preparation of the nonviral antigen. The procedure of immunization was exactly the same as above.
LABORATORY DIAGNOSTIC PROCEDURES

1. Complement fixation test: The following sera were used:

1) Sera from immunized rabbits.
   a) Rabbit No. 95 - immunized with trachoma antigen purified by trypsin and polymyxin (obtained from NAMRU-2)
   b) Rabbit Nos. (104, 105, 106, 107 and 111) immunized with trachoma antigen purified by Genetron treatment and differential centrifugation.
   c) Rabbit No. 112 - immunized with nonviral yolksac antigen.

2) Sera from monkeys which were immunized and later on challenged with trachoma virus (obtained from NAMRU-2).

3) Sera from one hundred American Navy personnel posted at Okinawa, who were clinically normal, with no evidence of past or present eye infection (obtained from NAMRU-2).

4) Sera from highly trachomatized population of Aligarh area which included all 4 stages of trachoma, trachoma dubium, chronic follicular conjunctivitis and clinically normal subjects (non-trachomatous).

5) Sera from clinically normal (non-trachomatous) persons of South India.

Titration of antigen: Before use in diagnostic tests
the antigen (purified) was titrated by the method of optimal proportion to discover the appropriate dilution to be used in complement fixation test. Serial dilution of antigens were set up in a chess board titration against a series of two-fold dilution of a positive serum ranging from 1 in 4 to 1 in 256. To each serum-antigen mixture the appropriate amount of complement, and after overnight fixation the haemolytic system was added. The end point of titration was that antigen dilution which reacted with the greatest dilution of the serum.

2. Intradermal test:

Procedure of test: A volume of 0.1 ml. of the purified antigen was injected intradermally in the flexor aspect of the forearm. Control test was similarly done on the other forearm using 0.1 ml. of normal yolksac antigen. Reading was taken after 48 hours. An induration of 5 m.m. or more was regarded as positive reaction.

Preparation of antigen: The purified elementary body suspension (40%) which was used as complement fixation antigen was diluted, treated with 0.5% phenol and kept in the incubator at 37°C for 4 weeks.

Titration of skin test antigen: The skin test titration was carried out in 3 serologically proved trachoma cases, all of whom had the complement fixing antibody titre of 1 in 128. 0.1 ml. volumes of antigen containing 0.2, 0.4, 0.8, 1.6 or 3.2 C.F. antigen units were injected intradermally on the ventral aspect of the arm.
Normal yolksac antigen was also given in the other arm. Results of the test was observed at 2 and 5 days after the injection. An induration of 5 mm. size was taken as positive. The skin test antigen titre was expressed as the least amount of C.F. antigen, in units, which elicited the positive reaction.

*Microscopic examination for inclusion bodies:* The conjunctival scrapings smeared on clean slides were dried in air and fixed in absolute methyl alcohol for 5 minutes. Giemsa powder (BDH) was used in preparation of the stock Giemsa's solution. The dilution was made with buffer solution (pH 6.8). The slide was placed in a coplin jar in a dilute Giemsa's solution (1 drop to 2 ml. of neutral distilled water). The jar was kept inside incubator at 37°C for one hour. The slide was then rapidly rinsed in two changes of 75% alcohol to remove stain debris. After drying in air the slide was examined for inclusion bodies.

**Solution:** Composition:-

1. **Snyder's solution:**

   Sucrose ... 37.5 g.
   \(\text{KH}_2\text{PO}_4\) ... 0.26 g.
   \(\text{Na}_2\text{HPO}_4\) ... 0.61 g.
   Glutamic acid ... 0.36 g.
   Distilled water ... 500 ml.
   1.5 ml. of 2N NaOH added to pH 7.6.
2. McIlvaine's buffer solution:

Stock solution A: 0.1 M Citric acid
\( \text{C}_6\text{H}_8\text{O}_7 \) solution.

Stock solution B: 0.2 M Disodium phosphate
\( \text{Na}_2\text{HPO}_4 \) solution.

Stock solution C: Stock A 1.83 ml. + Stock B
18.17 ml. pH adjusted to 7.4.

Final solution: (1:50 dilution).

1 part of stock C solution + 49 parts of distilled water.