had been desensitized(26). While their results are similar to the data reported herein utilizing cells from tolerant animals, the mechanisms by which cells are rendered inert in the two situations make further speculation unwarranted.

Summary. Picryl chloride was found to exert a lethal effect *in vitro* on leukocytes, principally lymphocytes, from guinea pigs exhibiting delayed-type skin reactivity to this hapten in the absence of antibody and complement. This reaction was found to be immunologically specific. PCl caused no discernible effect on leukocytes from guinea pigs rendered specifically tolerant to this substance.

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Trachoma Viruses Isolated in the United States. VIII. Separation of TRIC Viruses from Related Agents by Immunofluorescence.* (29385)

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For many years the similarity of trachoma-inclusion conjunctivitis (TRIC) viruses to agents of the psittacosis-LGV group has been evident on the basis of size, morphology, developmental and chemical characteristics and the possession of a common "group" antigen. The behavior of all these organisms in the commonly employed serologic tests (complement-fixation, neutralization) has been so uniform that the antigenic expression of species-specificity has been questioned. Such species-specific antigens were first demonstrated in the cell walls of meningopneumonitis (MP) organisms by Jenkin(1), but no similar specific antigens have yet been obtained from TRIC agents. The serologic results of Woolridge *et al*(2), which appeared

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to demonstrate species-specific reactions, have not been reproduced in other laboratories.

Fluorescent antibody (FA) technics were applied to meningopneumonitis-psittacosis agents by Ross and Borman(3) and to trachoma agents by Nichols and McComb(4). These studies again indicated considerable cross-reactivity among all members of the group, attributable to the shared "group" antigen, but a few results(4) suggested additional species-specific components encountered by the sensitive FA technic.

In view of the probable location of species-specific antigens in the cell wall of these microorganisms, it appeared desirable to "unmask" the possible specific antigens by removal of the "group" antigen, and then to examine the intact particles by immunofluorescence. Bernkopf *et al*(5,6) found that fluorocarbon purification for removal of nonviral protein and lipids provided a method for preparing antigens from TRIC agents to be tested by microagglutination or immunofluorescence. Initially, they questioned the specificity of the immunofluorescent technic, since antisera prepared against ornithosis and psittacosis reacted with TRIC antigens. The present study was undertaken in an attempt to evaluate such a method for separation of TRIC viruses from other members of the psittacosis-lymphogranuloma venereum-trachoma (PLT) group, and was designed to include representative isolates from several types of disease.

This preliminary report summarizes a large number of examinations performed with unabsorbed, high-titer rabbit antisera.†

Materials and methods. Viruses: Five trachoma isolates and 2 inclusion conjunctivitis isolates were employed, together with one strain each of psittacosis, lymphogranuloma venereum and meningopneumonitis virus in the following yolk sac (YS) passages:

Trachoma strains:‡ BOUR YS 10-16; ASGH YS 9-14; APACHE #2 YS 6-7; APACHE #4 YS 9 (all isolates from the United States in this laboratory(7)); TE 55 YS 6 (derived from the original Peking isolate of T'ang *et al*(8)) after an unknown number of YS passages.

Inclusion conjunctivitis strains: IC-Cal 3 YS 9-10 (isolated in California)(7), LB-1 YS 33 (isolated in London)(9).

Psittacosis strain 6BC YS 48, *lymphogranuloma venereum* strain Jackson YS 4-8, and meningopneumonitis YS 4-6 (all obtained through the courtesy of Dr. B. Eddie, Hooper Foundation).

Antigens: Yolk sacs harvested from embryonated eggs dying between the 6th and 8th day after infection served as source of the antigen. To a 10% saline suspension of infected yolk sac ⅓ volume of fluorocarbon-heptane mixture (Freon TF, Dupont + Heptane NM F.W. 100.20) adjusted to specific gravity of 1.3 was added. The material was blended in a cooled Waring blendor for 3 two-minute periods, with pauses to prevent undue warming(6). The blended material was centrifuged for 5 minutes at 1800 rpm, after which the supernatant fluid was removed and kept at 4°C. Two additional saline extractions of the residual blended yolk sac material were carried out. To the supernatant fluids ⅓ by volume of fluorocarbon was added, blended for 3 two-minute periods, and centrifuged for 5 minutes at 1800 rpm. The supernate was removed, and to it was added ½ the volume of fluorocarbon used in the previous step. Again the mixture was blended and centrifuged as described. The fluorocarbon treatment was repeated a third time, the supernatant fluid spun for 45 minutes at 6000 rpm in a Spinco Model L centrifuge. The supernate was decanted, the tubes drained, and the sediment suspended in 0.5 ml saline containing merthiolate 1:10,000; an additional 1.5 ml saline-merthiolate was

† The observation of possible species-specific reactions encountered with fluorocarbon-treated TRIC antigens was first mentioned at a meeting held in 1961 (Bernkopf, H., *Ann. N. Y. Acad. Sci.*, 1962, v98, 346).

‡ According to recommendations of Trachoma Group, International Congress of Microbiology, Montreal, 1962, nomenclature would be as follows: BOUR = TRIC/ /USA-Cal/Cal-1/OT; ASGH = TRIC/ /USA-Cal/Cal-2/OT; APACHE #2 = TRIC/ /USA-Ariz/Cal-4/OT; APACHE #4 = TRIC/ /USA-Ariz/Cal-6/OT; IC-Cal-3 = TRIC/ /USA-Cal/Cal-9/ON;LB 1 = TRIC/ /GB/MRC-1/G; TE 55 = TRIC/ /China/Peking-2/OT.

added, and the antigen was stored temporarily at 4°C. The preparation was checked by Giemsa stain and adjusted to contain approximately 2.5×10^9 particles per ml as determined by direct count(10).

Antisera: Rabbits were immunized in two ways: (1) Four subcutaneous injections of 50% suspensions of crude yolk sac from live embryos (harvested when 50% of the group were dead) were administered over a period of 74 days; or (2) four injections of 50% yolk sac suspension + complete Freund's adjuvant (Difco) were given intramuscularly + 4 injections of 20% yolk sac suspension intravenously over a period of 22 days, followed by 3 subcutaneous injections of 50% yolk sac over a period of more than 50 days. Animals were bled one week after the last injection, the sera were separated aseptically and stored at —20°C without preservative.

Immunofluorescence: One loopful of antigen was placed on a slide, air-dried, and fixed in methyl alcohol at —50°C for one hour. The slide was again air-dried and stored at —20°C. Slides were thawed immediately before staining.

Two-fold dilutions of rabbit antisera were made in phosphate buffered saline (PBS) pH 7.4; one drop of diluted serum was laid over the antigen; slides were incubated for 30 minutes at 37°C in a moist chamber, rinsed 3 times for 5 minutes in PBS pH 9.4 and air-dried or blotted with filter paper. One drop of fluoroescein-conjugated anti-rabbit gamma globulin§ previously diluted 1:4 or 1:6 with PBS pH 9.4 was added to each spot, incubated for 30 minutes at 37°C in a moist chamber, and rinsed 3 times with PBS pH 9.4. The slides were dried, covered with buffered glycerol pH 7.6 and coverslips were added. The slides were refrigerated until examined in a Zeiss fluorescence microscope illuminated with an Osram HBO 200 mercury vapor burner. A darkfield condenser, 40 × oil immersion objective, 8 × oculars, an OG 1 barrier filter and a BG 12 exciter filter were used. Slides were read independently by at least 2 observers, who were unaware of the identity of the sera. In most instances, a single antigen was tested against multiple sera in any one experiment.

Degree of fluorescence was recorded as follows: Elementary bodies inapparent or dim = negative; elementary bodies seen, but with blurred outline, and with faint fluorescence pale green in color = doubtful; elementary bodies fluorescing brightly with brilliant yellow color, sharply demarcated in outline = positive. The highest dilution of antiserum which yielded a "positive" result was considered the titer of the serum. Usually the highest titer was obtained with the homologous antiserum. It was graded "++." A serum titer which was more than 1:16 but at least 4-fold less than the maximal titer was graded "+." A serum yielding no FA reaction in a dilution of 1/16 was considered non-reactive "—." Fig. 1 shows preparations of BOUR antigen with (a) normal yolk sac (NYS) antiserum and (b) the homologous antiserum in an indirect test by FA.

Results. All rabbit antisera tested showed significant amounts of antibody against the homologous antigens as demonstrated by immunofluorescence. In contrast, neither normal rabbit sera nor sera from rabbits immunized with normal yolk sac gave demonstrable reactions with any of the purified antigens.

There was little evidence of strain-specificity as a practical method of identification. However, a distinct pattern of reactivity was apparent (Table I). This pattern was reproducible with different lots of antigen, varying egg passage levels and with sera from many rabbits. Some strains were virtually indistinguishable. For example, LGV, IC-Cal 3 (an inclusion conjunctivitis isolate), and BOUR (a trachoma isolate) cross-reacted with each other. However, the BOUR strain was easily differentiated from ASGH, a trachoma isolate from a Pakistani living in California. No anti-BOUR serum reacted with ASGH antigen, and anti-ASGH sera showed little or no reactivity with BOUR antigen. Psittacosis 6BC and one strain of meningopneumonitis could be separated unequivocally from the LGV and TRIC antigens and showed slight cross-reactivity with each other.

§ Obtained from Baltimore Biological Labs or Microbiological Associates.

Rabbits receiving the least intensive immunizations showed lower antibody titer but an apparent higher specificity, whereas those receiving many injections together with adjuvant had high titers but lower specificity. Examples of antibody levels in individual rabbit sera are shown in Table II.

Some variation was noted in the appearance of the antigens. BOUR- and LGV-infected yolk sacs consistently gave the most satisfactory antigens, with clean preparations free from background material. With other strains results were more variable, and an occasional lot had to be discarded because of the presence of non-staining material which interfered with evaluation of a slide.

Discussion. The separation of these re-

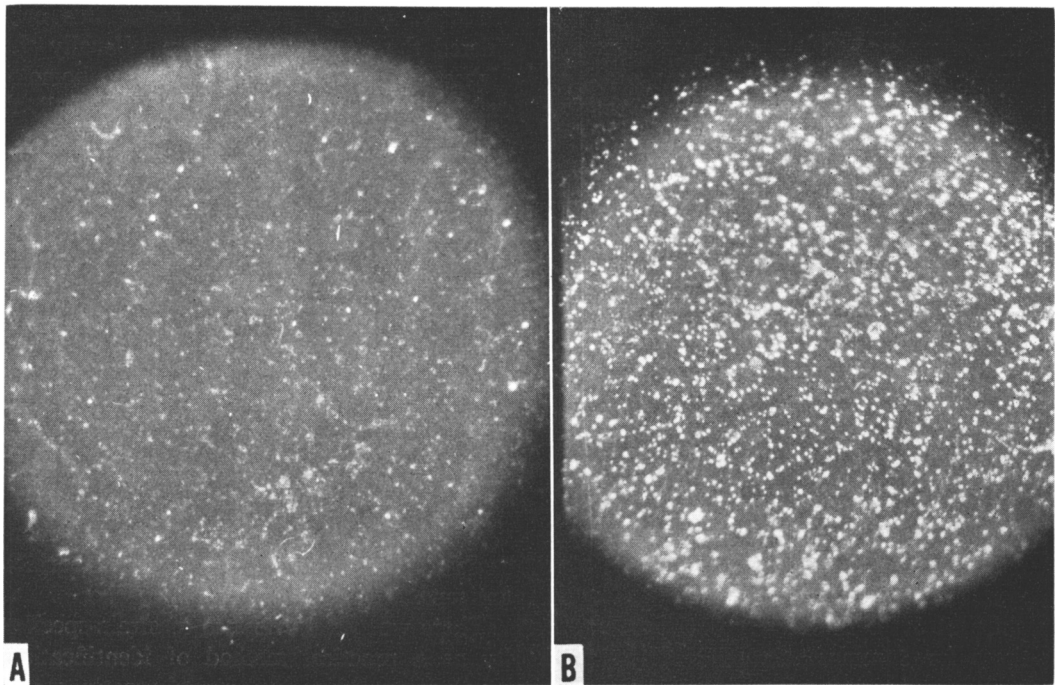

FIG. 1. Photomicrographs of fluorocarbon-purified TRIC (BOUR) anti stained by indirect immunofluorescence. (a) With normal yolk sac antiserum; (b) with homologous antiserum.

TABLE I. Pattern of Reactivity as Indicated by Immunofluorescence Between Fluorocarbon-Treated Antigens and Their Corresponding Antisera.

Antiserum (rabbit)	Homologous titer	Antigen					
		IC-Cal 3	LGV	BOUR	ASGH	6BC	MP
IC-Cal 3	256*	++†	+	+	+	—	—
LGV	512	+	++	++	+	—	+
BOUR	128	+	++	++	—	—	+
ASGH	256	+	+	+	++	—	—
6BC	64	+	—	—	—	++	+
MP	256	—	+	—	—	+	++
TE 55	ND	++	++	++	+	—	ND
LB 1	ND	++	ND	++	++	ND	ND
APACHE #2	ND	+	ND	+	—	—	ND
APACHE #4	ND	ND	+	+	ND	—	ND
NYS	---	—	—	—	—	—	—

* Reciprocal of highest serum dilution giving positive reaction by FA.

† ++ = homologous or maximal; + = positive, but 4-fold less than maximal; — = no reaction.

TABLE II. Examples of Individual Titrations of Reactivity by Immunofluorescence.

Antisera	Antigen LGV	BOUR	ASGH	6BC
Rabbit #295—LGV	512*	256	128	16
" #167—BOUR	128	128	<16	<16
" #233—ASGH	128	<16	256	16
" #284—6BC	<16	<16	<16	128
" #190—NYS	<16	<16	<16	<16

* Reciprocal of highest dilution of serum giving positive reaction.

lated agents by immunofluorescence as described above provides one method of studying their antigenic relationship. By the technics employed it was quite clear that yolk sac preparations of meningopneumonitis and of psittacosis 6BC were antigenically separable from several inclusion conjunctivitis and trachoma strains. It is more difficult to interpret the apparent strain specificity which could be demonstrated between BOUR and ASGH.

Correlation of results by fluorescent antibody technics with the antigenic groupings reported by Bell and Theobald(11) and Chang *et al*(12) is not feasible at present, since few of the isolates have been included in all studies. However, it is interesting to note that BOUR and ASGH, clearly separated by immunofluorescence, are also clearly separated by mouse toxicity tests. On the other hand, Chang *et al*(12) found no cross-protection between BOUR and either IC-Cal 3 or the APACHE isolates. Psittacosis, LGV and MP have not been included in cross-neutralization studies of TRIC mouse toxins reported by others.

Nichols *et al*(4) considered that they were dealing with group antigens in their FA studies. However, a high-titer human LGV antiserum might be the most satisfactory reagent for screening conjunctival scrapings, in view of its broad spectrum of reactivity as observed in our studies.

The total number of animals immunized against any one isolate was small, usually 5. It appeared that subcutaneous injection of crude yolk sac material resulted in moderate antibody levels, with good specificity, while intensive immunization with Freund's adjuvant and *via* several routes resulted regularly in higher antibody levels, but in a broader spectrum of reactivity. This is in keeping with general experience with other antigens.

Summary. Yolk sacs infected with trachoma, inclusion conjunctivitis, lymphogranuloma venereum, psittacosis 6BC or meningopneumonitis were extracted repeatedly with fluorocarbon. The resulting suspension of elementary bodies contained little egg material. The particles were distinct, and reacted with strain-specific rabbit antisera as measured by indirect fluorescence. The homologous antiserum usually yielded the highest titer; heterologous antisera showed equal, intermediate or no reaction; antisera to normal yolk sac were negative. Psittacosis 6BC and meningopneumonitis could be separated easily from TRIC agents. LGV, an IC isolate, and BOUR, a trachoma isolate, showed much cross-reaction, but BOUR could be differentiated regularly from another trachoma isolate (ASGH).

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