

CYTOLOGY OF "PINK-EYE"* OF SHEEP, INCLUDING A REFERENCE TO TRACHOMA OF MAN, BY EMPLOYING ACRIDINE ORANGE AND IODINE STAINS, AND ISOLATION OF *MYCOPLASMA* AGENTS FROM INFECTED SHEEP EYES

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[Manuscript received August 21, 1967]

Summary

Numerous forms of a pleomorphic microorganism were observed associated with the cytoplasm of epithelial cells in conjunctival smears from sheep with pink-eye when stained by the Giemsa method. The microorganism was coccobacillary in shape during the acute stage of infection, and stained purplish blue. Various peripheral forms, and dispersed, crescent-, horseshoe-, and ring-shaped figures were present in post-acute smears; all these pleomorphic forms stained blue. Peripheral forms were filaments, blebs, stalked and club-shaped figures, and ring forms. Intracytoplasmic red spherical bodies were the characteristic entity in the epithelial cells of "normal"‡ sheep smears. The developmental forms of the pink-eye and trachoma agents were morphologically different.

Acridine orange staining of representative forms of the pink-eye agent indicated the presence of ribonucleic acid only, in contrast to the demonstration of ribonucleic and deoxyribonucleic acids which appear in sequence during the developmental cycle of the trachoma agent. The iodine method stained the glycogen matrix of trachoma inclusions a brown colour, but a similar stain reaction was not visible in pink-eye or normal sheep smears.

Changes in the cytology of pink-eye smears suggested a gradual replacement of neutrophils successively by large, medium, and small lymphocytes as infection progressed; sometimes plasma cells were present.

Mycoplasma agents were isolated from conjunctival scrapings of sheep with pink-eye. A transmission experiment with one of the isolates produced a mild pink-eye condition. Coccobacillary forms were evident in smears from sheep which developed pink-eye; eye smears from control sheep were normal.

It is postulated that the aetiological agents of pink-eye in sheep are *Mycoplasma* spp. The significance of this finding is discussed in this paper and the syndrome is described.

I. INTRODUCTION

The disease of sheep known in Australia as pink-eye has global distribution, affecting a considerable proportion of flocks in spring and summer. The term pink-eye refers to the bloodshot appearance of the bulbar conjunctiva. Other clinical features are hyperaemia of the palpebral conjunctiva, lachrymation, and photophobia. Inflammation often extends from the bulbar conjunctiva into the cornea (pannus).

* A non-specific term describing the clinical appearance of the eye disease of sheep described in this paper.

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‡ Apparently normal.

Opacity of the cornea is common, and in severe cases purulent keratitis and ulceration develop. Permanent blindness of an eye, surprisingly, only occurs on the rare occasion when ulceration leads to perforation of the anterior chamber, or due to additional accidental injury.

This disease is not considered to be of major economic importance, but afflicted sheep must be culled or treated before they may be exported alive overseas or, in some instances, moved interstate within Australia. Carcass quality is reduced by bruising and there is loss of condition due to bilateral blindness. The disease does not affect wool growth, but the incidence of twinning is reduced when the infection occurs at or just prior to mating (Axelsen 1961). Death can ensue as a result of misadventure.

Coles (1931) was the first worker to describe the microorganism of pink-eye seen in the epithelial cells of conjunctival smears; he considered it to be a rickettsia and proposed the name *Rickettsia conjunctivae*. Rake suggested the name *Colesiota conjunctivae* (see Bergey's "Manual of Determinative Bacteriology", 6th Ed., p. 1119). Further studies were described by Donatien and Lestoquard (1938, 1939), Beveridge (1942), Dickinson and Cooper (1959), Livingston, Moore, and Hardy (1965), and others. This agent has not been isolated (Seddon 1965), but it is believed to be the aetiological agent on the basis of the great numbers present in infected eye smears. Transmission experiments, in which lachrymal secretions from cases of pink-eye are introduced into eyes of normal sheep, have evoked the disease, but the presence of bacteria and other possible unidentified agents make a conclusive interpretation impossible.

Dickinson and Cooper (1959) expressed doubts concerning the classification of this agent which other workers still believe to be a rickettsia. Their observations of infected smears led them to suggest that the agent has a similar growth cycle to the trachoma-inclusion conjunctivitis (TRIC) agent, a member of the psittacosis group of microorganisms, all of which possess a common group antigen.

The purpose of this investigation was to ascertain, by cytological examination and isolation attempts, whether the pink-eye agent was related to the TRIC agent.

II. MATERIALS AND METHODS

(a) *Conjunctival Smears*

Eye smears, on microscope slides, were prepared from eye curettings of pure and crossbred Merino sheep and of humans as follows (the stimulation of tears, which destroys cell morphology, was avoided):

- (1) 41 sheep with obvious clinical symptoms of pink-eye, culled from sheep destined for overseas;
- (2) 32 normal (asymptomatic) sheep;
- (3) 6 sheep purchased at the local market, and penned at the Northfield Field Laboratories of this Institute. Smears were coded according to the numbers given to the sheep in order of their examination, and the suffix L or R added for left or right eye origin.
- (4) Australian aborigines of whom 151 were children and 3 adults examined during trachoma surveys at Coober Pedy (survey CP. 2), Yalata (survey Y. 4), Koonibba Missions (survey K. 1), and Ceduna school (survey C. 2). The Coober Pedy smears had been previously, but unsuccessfully, stained by the iodine method (see later) to show

the presence of brown staining glycogen associated with TRIC inclusions. TRIC inclusions were seen in Giemsa-stained "replicates"* of these smears and, because of a dearth of TRIC-positive smears, were re-examined after storage for 3 years at room temperature.

(b) *Staining of Smears*

The techniques employed are listed below:

- (1) Giemsa's method as described by Hardy, Surman, and Howarth (1967a), using Giemsa stain manufactured by British Drug Houses Ltd. and a staining time of 8 hr.
- (2) Acridine orange (AO) method of Dart and Turner (1959). Since the gradual colour change of AO-stained smears is not so rapid by ultraviolet excitation compared with blue-wavelength light, the former method was used.
- (3) Iodine stain of Rice (1936) as modified by Gilkes, Smith, and Sowa (1958).
- (4) Gram stain; the carbol fuchsin counterstain was diluted 1:5 in 1% neutral red.
- (5) Carbol basic fuchsin method of Giménez (1964).
- (6) Spore stain of Schaeffer and Fulton (1933), as modified by Ashby (1938).
- (7) The lipid stain of Burdon (1946).
- (8) Neisser stain, using chrysoïdin in the modified method described by Gurr (1957).

Some smears were sequentially stained by iodine, AO, and Giemsa's method. After examination, the iodine-stained wet-mounted smears were washed briefly in distilled water, blotted, and decolorized for 24 hr with absolute methanol. AO staining was performed in entirety, and the smears decolorized in 50% ethanol for 24 hr. Finally, the smears were stained by the Giemsa method without further fixation.

(c) *Microscopy*

Microscopes employed were a Wild M 20 with a tungsten light source and a Leitz Laborlux with an additional ultraviolet light source provided by an HBO 200-W lamp. A darkground condenser, primary filters UG 1/1.5 and BG 38/4, and a secondary filter K 430 were inserted for fluorescence microscopy.

Films used for colour photography were Kodachrome 2A (ASA 40) and Ektacolor CPS 404 (ASA 100) for tungsten light illumination, the colour balance of the latter corrected by an 80 B filter, and Ektachrome EH (ASA 160) and Ektacolor CPS 404 (ASA 100) with ultraviolet light. Adox KB 14 (ASA 20) and a green filter were used for black and white photography.

(d) *Measurements*

A Leitz screw micrometer (519061), calibrated with a Shimadzu (Kyoto) 0.01 mm objective micrometer, was used to estimate the size of morphologic entities in smears and of isolated microorganisms.

(e) *Egg Culture Used to Isolate Trachoma Agents*

Left and right conjunctival scrapings were pooled in 1.0 ml of sucrose-potassium glutamate (SPG) of Bovarnick, Miller, and Snyder (1950), containing 20 mg of streptomycin sulphate and held at 4°C for 2-3 hr. Three fertile chick eggs, 6-7 days old, were inoculated each with 0.3 ml of this inoculum by the yolk sac route and incubated at 35°C. Yolk sacs were harvested from embryos immediately after death, and from embryos remaining alive after 10 days' incubation. Homogenized 20% yolk sac suspensions were prepared in SPG containing streptomycin, and inoculated immediately to further eggs as described above. Three consecutive passages were performed in attempts to isolate a trachoma agent. The third passage was terminated after incubation for 12 days.

* Smears prepared from the same eye at the same time.

(f) *Artificial Media used to Isolate Mycoplasma Agents*

The media used to isolate the pleuropneumonia-like organisms (PPLO) were as follows:

- (1) Soft PPLO agar in glass Petri dishes (50 by 15 mm), except for a short intervening period when Falcon cell-culture Petri dishes (60 by 15 mm) were used, or semi-fluid PPLO media in $\frac{1}{4}$ -oz screw-cap glass bottles. The media was prepared as described by Marmion (1967); the base for the soft PPLO agar was prepared with 0.98 g of Difco agar and 1.47 g of Difco PPLO medium (without crystal violet) made up to 70 ml with double glass-distilled water, and for the soft fluid media the Difco agar was reduced to 0.245 g. The KH_2PO_4 was omitted from both media to give a pH of 6.8 (Shepard and Lunceford 1965).
- (2) Soft cystine trypticase agar (CTA) medium (Baltimore Biological Laboratories Inc., 1965) in glass Petri dishes was prepared with CTA as the base, but otherwise was the same as (1) above. This base was 0.735 g of Difco agar added to CTA, containing 0.5% dextrose, to a final volume of 70 ml.

Media were seeded with one conjunctival scraping, or 0.1 ml of SPG inoculum. Further passages were performed as described by Kleineberger-Nobel (1962, pp. 59, 60). Seeded media were incubated in a humid atmosphere, usually at 35°C, aerobically or anaerobically in the presence of hydrogen, except for a few occasions at 37°C. Initially, if growth was absent after 20 days, the media were discarded; later the incubation time was reduced to 10 days. Media from each batch were tested by seeding with a human PPLO strain (*Mycoplasma fermentans*), incubated separately from pink-eye specimens at 37°C, under aerobic conditions.

(g) *Colony Counts*

Colonies were enumerated by microscopic examination of unopened agar plates with the aid of a grid marked out on their bases with a glass marking pencil. Each grid encompassed the seeded areas, each approximately 3 by 3 cm.

III. RESULTS

(a) *Giemsa Staining Method*

Pink-eye smears, stained by the Giemsa method, showed forms associated with the epithelial cells, believed to be development phases of the aetiological agent. The categories in which they have been placed are given below.

(i) *Coccobacillary Forms*

These forms are purple to purplish blue, bipolar stained, with the central portion unstained (Figs. 7b, 8b, 9b, 24, and 25). They were 0.25–0.35 μ by 0.5–0.6 μ , associated with the cytoplasm in massive concentrations or widely scattered; sometimes the location was peripheral (Fig. 8b). Less frequently, these forms were extracellular (Fig. 13); the largest group was rectangular in form, 15 by 60 μ . Closely packed extracellular forms were particulate either due to the overlying of the coccobacillary forms, or the formation of elementary bodies about 0.25 μ in size. Some coccobacillary forms were ovoid in shape with no bipolar staining reaction. Coccobacillary forms, considered synonymous with the acute stage, were present in smears from eyes depicted in Figures 3–6, shown in order of clinical severity. Conjunctival follicles were not observed macroscopically in this disease. Sometimes, grouped coccobacillary forms (Fig. 24), or even overlying pyknotic neutrophils, resembled TRIC inclusions when examined at low magnification.

(ii) *Crescent- and Horseshoe-shaped Forms*

These are deep blue (Figs. 10*b*, 25) or paler blue (Fig. 14) forms $1.0\text{--}2.0\ \mu$ by $0.5\text{--}1.5\ \mu$, observed invariably in the post-acute stage, seldom together with coccobacillary forms. A transition form appears to be a horseshoe figure, closed at the "open end" by a thin membrane to produce an uneven-stained ring.

(iii) *Peripheral Forms*

There are several types of peripheral forms, staining blue, and present in post-acute specimens:

(1) *Ring Forms*.—Single, $0.6\text{--}5.0\ \mu$ in diameter, grouped as a single peripheral layer or in a mass associated with the cytoplasm. The central core of each ring was unstained. Membrane-like strands sometimes connected individual rings, or were present as small projections (Figs. 16, 25). Large ring forms with a membrane thickness of $0.4\text{--}0.5\ \mu$ (Fig. 26) were uncommon. Network-like strands (Fig. 23) might be senescent ring forms, or toxic artefact.

(2) *Stalked Forms*.—The stalked forms, which were of maximum length $8.0\ \mu$, had small ring-like or solid-staining spherical heads $0.6\text{--}1.0\ \mu$ in diameter (Fig. 22).

(3) *Club-shaped Forms*.—These forms, which extended $4\text{--}15\ \mu$ from cell peripheries (Figs. 11*b*, 12*b*, 22), had a maximum head width of $26\ \mu$. Their structure was either amorphous (without or with embedded ring forms occasionally interspersed with blue particles $0.25\text{--}0.30\ \mu$ in size), network-like, or ring-like.

(4) *Blebs*.—Blebs were broadly attached ($3.0\text{--}16.0\ \mu$) to the cell margin, projecting a distance of $2.0\text{--}6.0\ \mu$, and were amorphous in appearance, with or without embedded ring forms.

(5) *Filaments*.—Filaments were of varying length (maximum $46\ \mu$), thickness, and shape, and were either amorphous, granular, network-like, or ring-like in structure (Figs. 22, 26, and 27).

(iv) *Other Forms*

These forms, described below, might not be growth phases of the pink-eye agent, because they were distinctly different from those already considered.

(1) *Intracytoplasmic Eosinophilic Globules*.—These were $0.4\text{--}1.0\ \mu$ in diameter (Fig. 15) and were interspersed with coccobacillary forms in acute specimens, or in vacuolated and granular cells of the post-acute phase; and present to a lesser extent in normal specimens.

(2) *Intracytoplasmic Spherical Bodies*.—In normal smears, large intracytoplasmic red spherical bodies $1\text{--}18\ \mu$ in diameter, similar to those described by Donatien and Lestoquard (1938), were significantly present. One normal smear had as many as 99 out of 331 epithelial cells containing these forms, whose average diameter was $8\text{--}14\ \mu$. The number of these forms fluctuated in smears taken at intervals from the same normal sheep. Usually one was present per cell, sometimes two or three, and four or more were increasingly rare. Many single red forms displaced the nucleus (Fig. 18), and frequently an unstained halo circumscribed them adjacent to the cytoplasm. A few of these red forms were present in acute and post-acute specimens. They bore some resemblance to goblet cells seen in human eye smears.

Forms of similar shape and size range did not stain or were stained pink; occasionally scattered indistinct particles were in the matrix of these forms.

(3) *Deep-staining Eosinophilic Particles*.—A varying number of deep-staining eosinophilic particles, about 0.25μ in size, were present in epithelial cell cytoplasm of normal smears.

(4) *Blue Granular Material*.—There was some blue granular material in the vicinity of cells in smears of the post-acute stage. These granules might be staining artefacts.

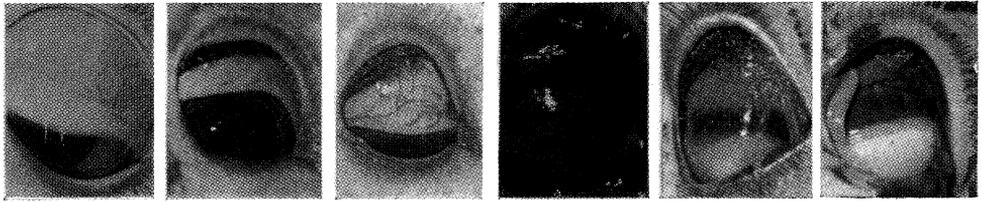
(5) *Blue-staining Spherical Forms*.—These forms, $2-5 \mu$ in size, occasionally with protuberances, and frequently honeycomb-like in structure, were mostly extracellular and rarely peripheral (Fig. 23). These forms have been seen in trachoma and other human smears from cases of chronic conjunctivitis.

“Normal” sheep smears appeared to be characterized by the distinctive red spherical bodies described above in (2), and epithelial cells of normal appearance. An occasional inflammatory cell was present.

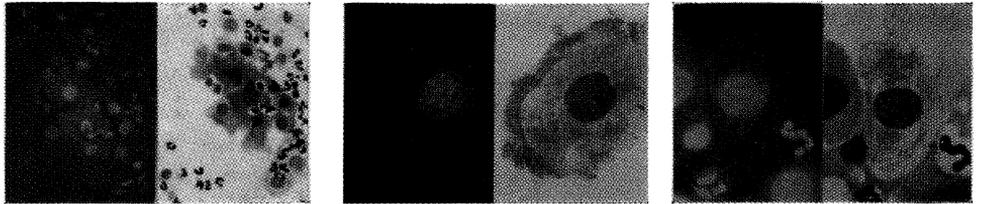
Figs. 1-6.—Sheep eyes. 1, normal sheep eye (60 L); 2, pink-eye: slightly hyperaemic bulbar conjunctivae (61 R recovering 24 days after experimental inoculation); 3, pink-eye: hyperaemic palpebral and bulbar conjunctivae, no pannus, cornea with a slightly hazy appearance (61 R at the most acute clinical stage, 7 days after experimental infection); 4, pink-eye: hyperaemic palpebral and bulbar conjunctivae, pannus 4 mm, cornea opaque (17 R); 5, pink-eye: hyperaemic palpebral and bulbar conjunctivae, pannus 3 mm, cornea opaque with small infiltrate (65 R); 6, pink-eye: hyperaemic palpebral and bulbar conjunctivae, cornea with massive infiltrate (21 L).
Figs. 7-17.—Eye smears from sheep with clinical pink-eye. 7a, AO stain: epithelial cells heavily infected with coccobacillary forms fluorescing red (RNA); the cytoplasm of uninfected cells shows virtually no fluorescence (21 R); $\times 125$; 7b, Giemsa stain: coccobacillary forms in 7a retained purple. Note that the cytoplasm of the uninfected, single cell at top centre did not fluoresce a deep red colour in 7a; $\times 125$; 8a, AO stain: coccobacillary forms with a distinct peripheral location, fluorescing red (RNA) (21 R); $\times 650$; 8b, Giemsa stain: coccobacillary forms in 8a retained purple; $\times 650$; 9a, AO stain: coccobacillary forms in an epithelial cell, with no distinct peripheral location, fluorescing red (RNA) (43 R); $\times 650$; 9b, Giemsa stain: restrain of 9a; $\times 650$; 10a, AO stain: brilliant red (RNA) fluorescence of crescent and horseshoe forms; a few scattered coccobacillary forms are present, but red fluorescence is not intense (46 R); $\times 650$; 10b, Giemsa stain: restrain of 10a; $\times 650$; 11a, AO stain: peripheral small club-shaped forms, fluorescing red (RNA) (23 R); $\times 650$; 11b, Giemsa stain: restrain of 11a; 12a, AO stain: large club-shaped form with brilliant red (RNA) fluorescent ring forms (58 R); $\times 420$; 12b, Giemsa stain: restrain of 12a; 13, extracellular mass of coccobacillary forms (Giemsa stain, 4 R); $\times 450$; 14, pale blue stained crescent forms (Giemsa stain, 28 L); $\times 450$; 15, eosinophilic globules and vacuolated epithelial cell (Giemsa stain, 68 R); $\times 450$; 16, ring forms at the periphery of an epithelial cell—note interconnecting membrane-like strands and projections (Giemsa stain, 56 R); $\times 650$; 17, blue granulation of epithelial cell, and surrounding necrotic mycelial-like nuclei (Giemsa stain, 61 L); $\times 450$.

Fig. 18.—Eye smear from normal sheep. Red spherical bodies displacing epithelial cell nuclei (Giemsa stain, 30 L); $\times 450$.

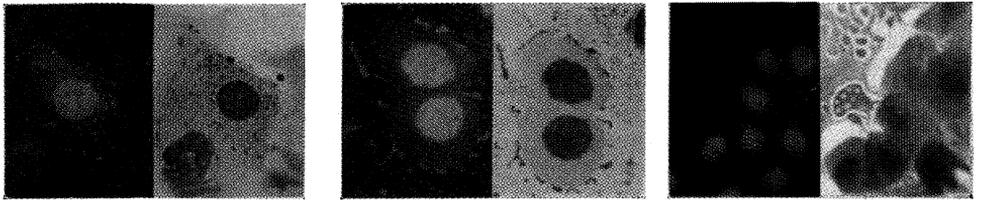
Figs. 19-21.—Eye smears from humans with trachoma. 19a, AO stain: trachoma particles fluorescing red (RNA), prior to the development of the final elementary body growth stage, in the cytoplasm of an epithelial cell (CP.2.643); $\times 1200$; 19b, Giemsa stain: restrain of 19a; $\times 1200$; 20, AO stain: a compact trachoma inclusion fluorescing red (RNA), at a similar growth stage depicted in 19 (CP.2.616); $\times 450$; 21a, iodine stain: the brown stained glycogen matrix of a trachoma inclusion at the elementary body stage (CP.2.616); $\times 450$; 21b, AO stain: restrain of 21a showing dull green fluorescence (DNA) of elementary bodies in the vacuolated cytoplasm; $\times 450$; 21c, Giemsa stain: restrain of 21b; $\times 450$.



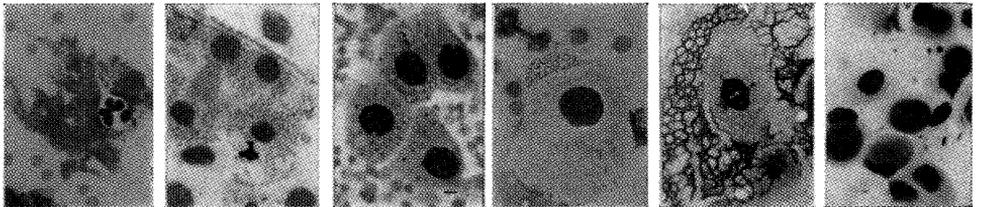
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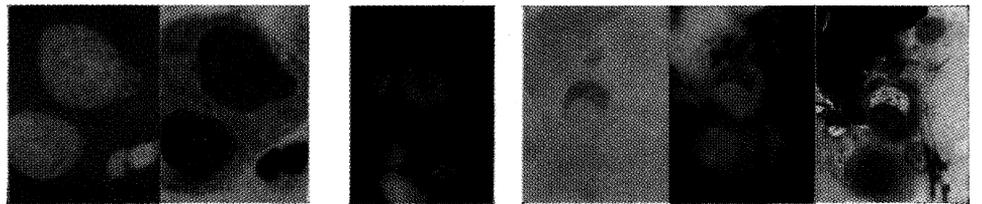
7a 7b 8a 8b 9a 9b



10a 10b 11a 11b 12a 12b

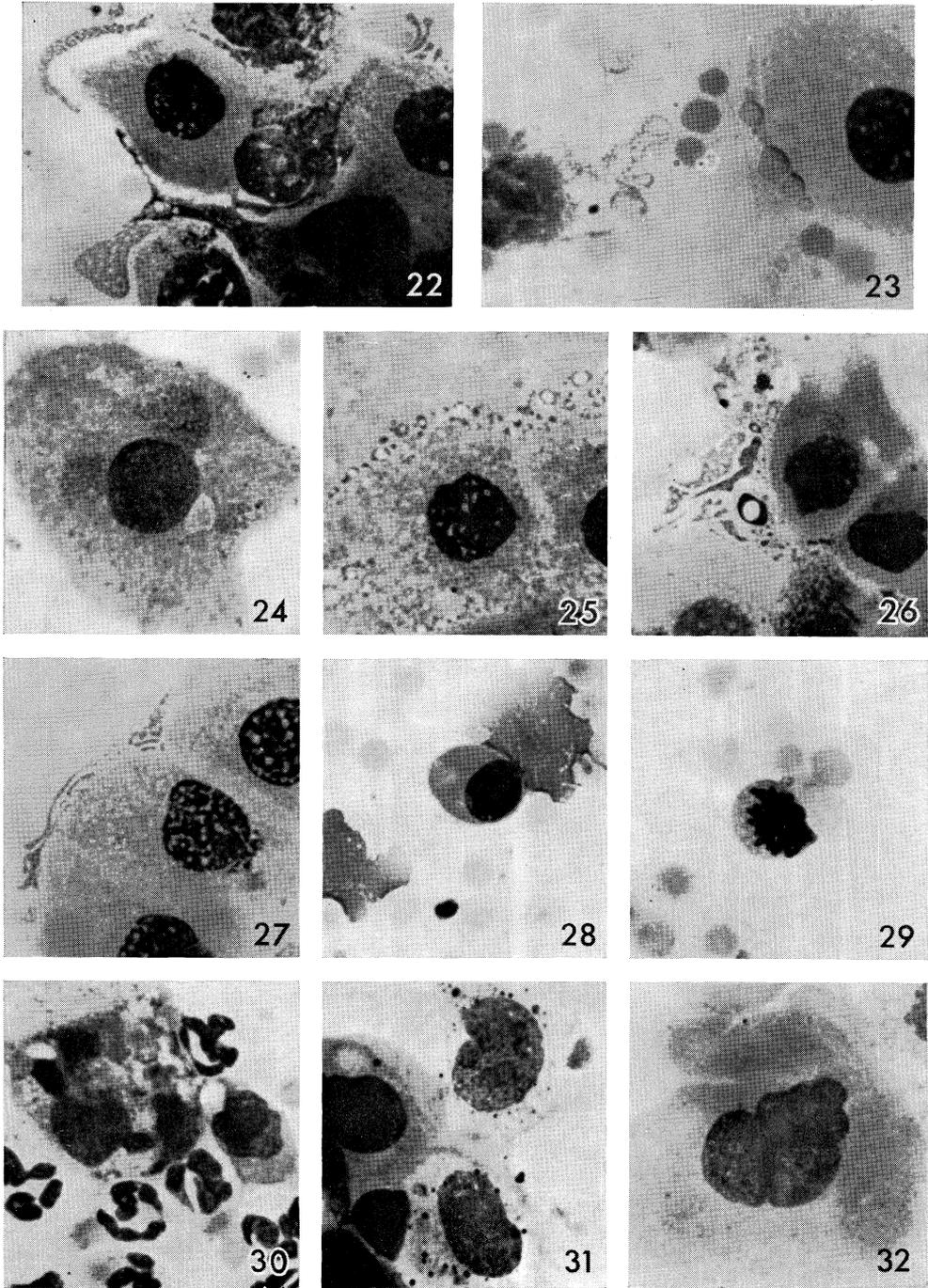


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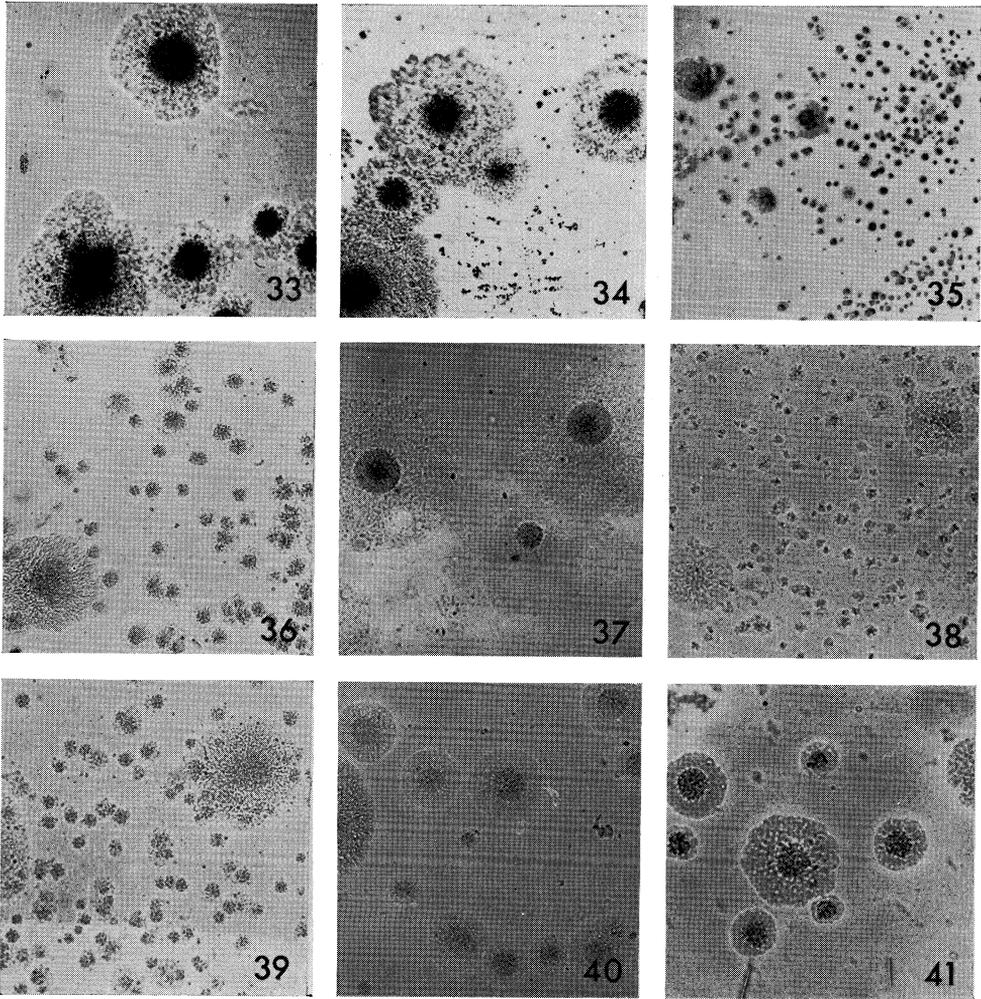
19a 19b 20 21a 21b 21c

[For explanation of figures, see opposite page.]



Figs. 22-32.—Pink-eye smears stained by the Giemsa method, all magnifications $\times 1000$: 22, peripheral forms: stalked forms, either with a ring-like head, filament-like, or club-shaped

[For continuation of legends to figures, see opposite page.]



Figs. 33-41.—*Mycoplasma* colonies from pink-eye specimens grown on PPLO agar: 33, specimen 4 L+R; incubation 14 days; from SPG at -70°C ; $\times 100$; 34, specimen 5 L+R; incubation 14 days; from SPG at -70°C ; $\times 100$; 35, specimen 61 R; incubation 2 days; direct seeding; $\times 40$; 36, specimen 61 R; incubation 2 days; direct seeding; $\times 100$; 37, specimen 67 R showing finer-textured colonies compared to the others presented in this plate; incubation 5 days; second passage; $\times 100$; 38, specimen 69 R; incubation 1 day; direct seeding; $\times 100$; 39, specimen 70 R; incubation 1 day; direct seeding; $\times 100$; 40, specimen 70 R; incubation 1 day; sixth passage without penicillin and thallos acetate present; $\times 100$; 41, specimen 77 R; incubation 2 days; fourth passage without penicillin and thallos acetate present; $\times 100$.

(12 L); 23, blue-staining spherical forms, and network-like strands which might be senescent ring forms (57 R); 24, coccobacillary forms grouped together to form trachoma-like inclusions. A red blood cell overlies the cytoplasm (78 R); 25, coccobacillary, crescent, horseshoe, and ring forms (51 R); 26, one large ring and small ring forms, and peripheral filaments (58 L); 27, peripheral filaments (58 R); 28, plasma cell (78 R); 29, mitotic cell (78 R); 30, Leber-like macrophage cell with large ingested particles, one distorted large lymphocyte and neutrophil (72 L); 31, a macrophage cell with eosinophilic particles (4 L); 32, a multinucleate epithelial cell (58 L).

(b) *Acridine Orange Staining Methods*

TRIC inclusions were seen only in the eye smears of two aborigines from Coober Pedy stained by this method. The early growth stages of the TRIC inclusions, fluoresced red (Figs. 19a, 20) and the final growth stage—consisting of elementary bodies (0.2–0.5 μ diameter) in the inclusion—fluoresced green (Fig. 21b); this indicated the presence of single-stranded RNA and double-stranded DNA, respectively (Mayor 1962; Thompson 1966). This cytochemical sequence in the replication of the trachoma agent was shown previously, using the AO staining method, in psittacosis and trachoma-infected cell culture studies (Pollard, Moore, and Tanami 1961; Pollard and Tanami 1962; Bernkopf, Mashiah, and Becker 1962). The red fluorescing inclusions were brilliant in intensity and distinctive; the green fluorescent inclusions were dull and harder to locate than RNA inclusions. In “replicate” smears 1 and 2 of CP.2.616, 12 and 6 fluorescent inclusions were found, respectively, and TRIC inclusions relocated at the same sites after Giemsa restaining; replicate smear 3 of CP.2.616, not previously stained by the iodine method or fixed before the 3-year storage period, showed fluorescent TRIC inclusions, but the overall colour reaction of the smear was red. One TRIC inclusion was identified in smear CP.2.643. The identification of TRIC inclusions correlated by the AO and Giemsa staining methods.

Pink-eye forms fluoresced red only (Figs. 7a–12a) at all stages. The Giemsa-stained red forms (Fig. 18) did not fluoresce, appearing as black vacuoles.

It was found that fixation prior to storage was necessary for satisfactory AO staining. Compared to the Giemsa stain, the AO-stained smear has the disadvantage of fading on examination during exposure to ultraviolet alone or with blue wavelength light.

(c) *Iodine Staining Method*

Brown glycogen staining of TRIC inclusions was observed only in replicate smears of CP.2.616, and could be relocated by AO and Giemsa restaining (Figs. 21a, 21b, and 21c). In the unfixed replicate smear 3 of CP.2.616, the maximum number of 14 brown-stained TRIC inclusions was located, prior to AO staining. No glycogen staining was found to be associated with the pink-eye staining.

(d) *Other Staining Reactions with Smears from Eyes of Sheep*

Coccobacillary and other post-acute forms of the pink-eye agent were faintly Gram-negative. Carbol basic fuchsin stained the coccobacillary forms a red to purplish blue, contrasting with the blue staining of post-acute forms and cells. The Neisser, lipid, and spore stains gave negative results.

(e) *Associated Cytology*

Cytology associated with pink-eye infection was distinct, in that inflammatory cell types passed through definite phases. In the acute phase, neutrophils were present in overwhelming numbers. Gradually large lymphocytes, sometimes follicular in distribution, followed and these were replaced later, in sequence, by medium and small lymphocytes. Finally, in normal smears only a few pyknotic neutrophils and

TABLE 1

CORRELATION BETWEEN EYE-SMEAR OBSERVATIONS AND RESULTS OF ISOLATIONS ON PPLO AGAR SEEDED WITH PINK-EYE SCRAPINGS STORED IN SPG AT -70°C

Specimen	Pink-eye Forms and Associated Cells in Giemsa-stained Smears	Storage Time (days)	PPLO Colonies*
3 L	Many coccobacilli and neutrophils; a few large, and occasional medium and small lymphocytes; one plasma cell	208	Present
3 R	As above, but no plasma cells		
4 L	Many intra- and extracellular coccobacilli and neutrophils; a few crescent- and horseshoe-shaped and ring forms; an occasional macrophage (Fig. 31); a few large and small lymphocytes	208	Present
4 R	Coccobacilli as above; a few blebs with rings, rings, small club-shapes; large to small lymphocytes; an occasional macrophage; 19 plasma cells		
5 L	None	223	Absent
5 R	A few coccobacilli, crescent, ring, and solid-staining spherical forms. Occasional necrotic neutrophils and small lymphocytes		
6 L	Not done	223	Absent
6 R	A few coccobacilli, amorphous stained blebs, neutrophils, medium and small lymphocytes; an occasional macrophage (Fig. 31); 21 plasma cells		
24 L	A few coccobacilli, and crescent- and horseshoe-shaped, irregular short filaments; many necrotic neutrophils, and an occasional medium lymphocyte	171	Absent
24 R	A few coccobacilli; many irregular short filaments, ring and horseshoe shapes, and solid-stained, ring-sized forms and crescents; neutrophils only		
56 L	A few coccobacilli, blebs, stalked forms, neutrophils, and medium lymphocytes; four plasma cells	94	Present
56 R	A few coccobacilli, rings, neutrophils, and small lymphocytes; one eosinophil		
63 L	Coccobacilli, many with no bipolar staining; neutrophils; medium and small lymphocytes	10	Present
64 R	A few coccobacilli; intra- and extracellular blue granules; neutrophils; medium and small lymphocytes; one plasma cell; two eosinophils	2	Absent

* Incubated aerobically at 35°C .

small lymphocytes were present, or entirely absent. Plasma cells (Fig. 28) were present in many smears of the acute and post-acute stages of infection, as were a few eosinophils.

Necrosis of epithelial cells was widespread in smears of the post-acute stage. Necrotic nuclei frequently had a smeared mycelial-like appearance (Fig. 17). Nuclei with a coiled, globular structure were seen. A few macrophage cells with eosinophilic particles (Fig. 31) were present in acute and post-acute smears, and Leber-like cells (Fig. 30) were rare. Mitotic figures (Fig. 29) and multinucleate epithelial cells (Fig. 32) were present infrequently.

Melanin granules were seen as greenish black rods, $0.25 \times 0.4 \mu$ and dark green spherical particles $0.25-0.65 \mu$ in diameter.

According to the system described by Hardy (1966), and Hardy, Surman, and Howarth (1967*b*), points were allotted for associated cytology considered typical for trachoma infection: 4-6, 3-6, and 2-4 for acute, post-acute, and normal sheep smears, respectively.

(f) *Additional Microorganisms*

The bacterial and yeast content of smears was generally low. Gram-positive cocci and rods, frequently in packets and sometimes club-shaped, and Gram-negative, bean-shaped cocci were present. Pus from a corneal ulcer at autopsy showed a mixed culture of Gram-positive bacteria composed of single and grouped cocci, cocci in chains, and varying forms of rods, some beaded or club-shaped in appearance. The study of the bacterial flora was not pursued further. Forms of the pink-eye agent were not identified.

(g) *Isolation*

No TRIC agents were isolated in eggs from pooled L + R specimens of sheep 3-8.

After cytological examination, the pleomorphism and frequent peripheral location of the agent resembling those of the T strains of Shepard (1957) and *Mycoplasma* agents depicted by Kleineberger-Nobel (1962, Ch. 3), especially in electron micrographs, suggested that the causative agent of pink-eye might be due to similar or different strains of *Mycoplasma*. Specimens from storage at -70°C were seeded on PPLO agar and incubated aerobically at 35°C . Colonies grew which were *Mycoplasma*-like in appearance and which were observed initially 3-4 days after seeding (Table 1; Figs. 33 and 34). In addition, specimen 56 L + R was seeded on CTA, but no colonies appeared. No colonies grew on PPLO agar seeded with specimen 3 L + R, but three colonies grew on the PPLO agar seeded with semi-fluid PPLO agar initially inoculated with the specimen. Direct seeding with conjunctival scrapings to PPLO agar produced much heavier growth (Table 2; Figs. 35, 36, 38, and 39). The colonies in many instances appeared to emanate from epithelial cells. In a further experiment, colony counts were performed on seeded PPLO agar after incubation for 2 days under different conditions (Table 3). Also conjunctival scrapings from three of the sources listed in Table 3 were either seeded directly on PPLO agar or stored at 4 or -70°C in SPG for various periods prior to seeding. The seeded plates were incubated aerobically at 35°C and the colonies counted on day 6 or day

14. To allow comparison between the colonies counted from the direct seeding of one conjunctival scraping and the seeding of 0.1 ml SPG inoculum from storage, the count for the latter was increased tenfold (Table 4).

(h) *Colony Morphology*

Colonies grew to a size of 130–200 μ in diameter. Colonies from most specimens examined at $\times 50$ magnification were steeply dome-shaped with rough granular-like surfaces which were greenish in tint, their central buttons becoming brown with continued incubation. Examination at $\times 125$ magnification revealed a vacuole-like structure in the peripheral part of the colonies, and colour was no longer discernible. Colonies of specimens 63L and 67R (Fig. 37) differed from the other strains in

TABLE 2

CORRELATION BETWEEN EYE-SMEAR OBSERVATIONS AND RESULTS OF ISOLATIONS ON PPLO AGAR DIRECTLY SEEDED WITH PINK-EYE SCRAPINGS

Specimen	Pink-eye Forms and Associated Cells in Giemsa-stained Smears	PPLO Colonies*
65 R	Coccobacilli; a few similar-sized, solid blue forms; many neutrophils; large and medium lymphocytes; one monocyte	Extremely numerous
66 R	Coccobacilli; a few ring forms; neutrophils mainly large lymphocytes with dark blue peripheral areas and blebs	Extremely numerous
67 R	Coccobacilli; a few large lymphocytes	Extremely numerous
68 R	Many coccobacilli; granular cytoplasm; many neutrophils; large, medium, and occasional small lymphocytes	Extremely numerous

* Incubated aerobically at 35°C.

appearance, being flatter and more finely textured, and were without the greenish colouring as they grew slightly larger. The colonies were brittle, breaking into segments when scraped along the agar surface, leaving the central button *in situ*. Strains 70 R (Fig. 40), 77 R (Fig. 41), 78 R, and 79 R after 7, 5, 2, and 3 successive passages, on PPLO agar without inhibitors, retained their original colony morphology. Serial passage of the isolated strains on soft agar was best performed after the second or third day of growth.

(i) *Experimental Infection*

Initially the eyes of sheep 61 and 62 were normal. After the right eye of sheep 61 was bathed with pooled agar and semi-fluid medium shown to be infected with strain 63 L by further passage on PPLO agar, coccobacillary forms became apparent in the smears, and *Mycoplasma* agents were isolated (Table 5). Clinically the infection did not become extremely acute (Fig. 3). After 24 hr post-infection the right eye of this sheep was almost clinically normal, except for slight hyperaemia of the upper palpebral conjunctiva (Fig. 2), compared to a normal eye (Fig. 1). At this time

TABLE 3 (Continued)

Specimen	Pink-eye Forms and Associated Cells in Giemsa-stained Smears	No. of PPLO Colonies under Anaerobic Conditions		No. of PPLO Colonies under Aerobic Conditions			
		35°C	37°C	35°C	37°C	Room Temp.*	Plate from Room Temp. in Previous Column to 35°C
73 L	Cocci bacilli; a few neutrophils and small lymphocytes; an occasional medium lymphocyte; six eosinophils	2†	n.d.	0	0†	0(11)	0
74 R	Many coccobacilli and neutrophils; many large, and a few medium and small lymphocytes; two macrophages (Fig. 30); one monocyte	2,650	n.d.	0	0	0(4)	No count‡
76 R	Cocci bacilli; many pyknotic neutrophils; a few large lymphocytes	13,626	n.d.	5,835	n.d.	n.d.	n.d.
77 R§	A few coccobacilli; many pyknotic neutrophils; a few large lymphocytes	4,259 (1) 10,658 (2) 13,792¶ (3)	n.d.	4,674	n.d.	n.d.	n.d.
78 R§	A few coccobacilli; neutrophils with large, medium, and small lymphocytes; 65 plasma cells; 1 Leber-like cell; 2 mitotic cells	1,711 (1) 2,422 (2)	n.d.	313	n.d.	n.d.	n.d.

* Room temperature (21–25°C), in a non-humid atmosphere. Number of days sealed plate was at room temperature given in parenthesis.

† Plastic plate.

‡ Many colonies present but plate contaminated; no count was attempted.

§ Seeded plates held at 4°C for 4 days before incubation.

|| Counts on replicate plates each seeded with one conjunctival scraping.

¶ Penicillin and thallous acetate absent; no bacterial growth.

pink-eye forms seen in acute and post-acute smears had disappeared. The right eye of sheep 62 remained normal following introduction of the same medium as a control, without the presence of a *Mycoplasma* isolate. The characteristic red spherical forms

TABLE 4
RESULTS OF ISOLATIONS, REPRESENTED BY COLONY COUNTS, ON PPLO AGAR SEEDED WITH PINK-EYE SCRAPINGS STORED IN SPG COMPARED TO DIRECT SEEDING

Specimen	Inoculum	Storage Time	Storage Temp. (°C)	Colony Count*
69 R	Direct†	—	—	2,760
	SPG	4 hr	4	1,240
		8 hr	4	3,110
		14 days	-70	0
70 R	Direct	—	—	32,476
	SPG	4 hr	4	24,680
		8 hr	4	15,920
		14 days	-70	970
71 L	Direct	—	—	0
	SPG	4 hr	4	0
		8 hr	4	0

* Colonies counted 6 or 14 days after seeding according to colony density. Corrected for SPG specimens to 1 ml (equivalent to one scraping). Incubation aerobically at 35°C.

† See Table 3.

TABLE 5
RESULTS OF EXPERIMENTAL TRANSFER OF *MYCOPLASMA* STRAIN 63L TO THE RIGHT EYE OF SHEEP 61

No. of Days after Transfer	Observations
3	A few coccobacilli; ring forms and neutrophils; five medium lymphocytes; hyperaemia of palpebral and bulbar conjunctivae
7	Coccobacilli; a few rings; neutrophils; an occasional large and a few medium and small lymphocytes; hyperaemia at maximal development, cornea slightly hazy and pannus absent (Fig. 3)
8	Pleuropneumonia-like organisms isolated after incubation at 35°C, aerobically
10	A few coccobacilli; neutrophils; medium and small lymphocytes; bulbar conjunctiva normal, palpebral conjunctiva slightly hyperaemic
17	Rings, blebs (some with rings), and short filaments
24	Red forms; no inflammatory cells; no pleuropneumonia-like organisms isolated; clinical appearance as on day 10 (Fig. 2)

of a normal smear were present, and an occasional inflammatory cell; no *Mycoplasma* agent was isolated. The left eyes of these sheep remained normal clinically, cytologically, and by culture, throughout the experimental period. In this preliminary trial the sheep were penned together.

(j) *Culture of Pink-eye Isolates on Bacteriological Media*

Strains 70 R, 74 R, 76 R, 77 R, and 78 R were seeded to blood agar, chocolate agar, Hoyle's tellurite agar, and Loeffler's slopes. Most cultures did not grow any bacteria and it was inferred that the pink-eye agents were not L-forms of bacteria. A species of *Corynebacterium* was isolated during passages of strain 74 R, but this was considered to be a commensal present in the original specimen. These *Corynebacterium* colonies, grown on a Loeffler's slope, were seeded to PPLO agar containing penicillin and thallos acetate; no *Mycoplasma* colonies appeared.

IV. DISCUSSION

Contrary to the original belief that the aetiological agent of pink-eye is a rickettsial agent, the finding presented here indicates that it is a *Mycoplasma* agent. Evidence to support this suggestion includes:

- (1) The morphological appearance and frequent peripheral cellular location of the pink-eye agent in smears.
- (2) The isolation of *Mycoplasma*-like colonies from eye specimens in large numbers. On a plate seeded with specimen 77 R (Table 3) without the presence of inhibitors, the same type of *Mycoplasma*-like colonies grew, and not any bacteria.
- (3) The retention of colony morphology after several passages on agar in the absence of penicillin and thallos acetate. The presence of penicillin in the medium could prevent an L-form of bacterium reverting to its parent state. Dienes (1959) suggests that PPLOs are related to small Gram-negative bacteria of the *Haemophilus-Pasteurella* group. Reversion of a *Mycoplasma* agent to a species of *Corynebacterium* was reported by Wittler, Cary, and Lindberg (1956), but initial attempts to induce reversion of the pink-eye agent to bacterial forms were considered unsuccessful.

Lysozyme is present in lachrymal secretions, and has a similar action to penicillin on the cell walls of sensitive bacteria, producing protoplasts (Stewart 1965) under suitable conditions. It is noted by Marmion (1967) that *Mycoplasma* agents are resistant to both these inhibitors. Lysozyme might have been the influencing factor in the evolution of these apparently stable *Mycoplasma* strains derived from one or more species of bacteria.

A further finding to support *Mycoplasma* aetiology could be the result of the experimental infection described in this study. However, Koch's postulate might not have been fulfilled, since the colony morphology of strain 61 R seemed to differ from that of seeded 63 L. Such a study should include more sheep, and methods for differentiating strains by morphology, growth requirements, chemical structure, and serological characteristics must be available. This is because the possibility exists that sheep can be carriers (Beveridge 1942), or that all sheep harbour the agent in latent form, with activation occurring due to trauma or conditions of stress. The latter concept would explain the ubiquitous presence of this agent in sheep. During the unusually dry winter months of this study the incidence of pink-eye was almost nil, but

clearly flared up about a week after periodic dust storms were experienced in Adelaide and farming areas. The accepted theory is that the disease, being highly contagious, is transmitted by contact with contaminated flies, dust, grass seeds, long pasture, or crops (e.g. Durham 1963; Toop 1964) and outbreaks are aggravated by close grouping and handling of sheep. This is supported by the finding in this study that the pink-eye agent survives at least for a 3-week period at room temperature.

Isolation results from sheep with clinical pink-eye were consistent on agar in glass Petri dishes (Table 3), except for some variation between different scrapings from the same eye. A shortage of glass Petri dishes induced the author to try plastic Petri dishes. However, when plastic and a few glass Petri dishes were used for specimens from sheep 71-73, no *Mycoplasma* colonies grew (Table 3); this was unexpected since many coccobacilli were present in their eye smears. The reason for the lack of colony growth on PPLO agar in the glass Petri dishes in this instance was not discovered. An immediate return to the use of only glass dishes rectified the problem. *M. fermentans* seemed to grow vigorously on the agar in plastic Petri dishes, and Marmion (1967) used Falcon Petri dishes suitable for cell culture. Mr. B. W. Moore of this Institute has shown that *M. pneumoniae* (Boston strain) and *M. hominis* type 1 were affected by probable toxic factors released from the plastic, resulting in the early disintegration of colonies which appeared. He recommends caution in the use of plastic Petri dishes. However, growth of these microorganisms might be improved by alterations to media constituents, pH, and even more rigorous cleansing of glassware.

The pink-eye agent bore no resemblance to the trachoma agent by morphology and differed by the presence of one nucleic acid (RNA) instead of two.

Storz *et al.* (1967) described a follicular conjunctivitis of sheep from which they isolated members of the psittacosis group via the yolk sac route in eggs, and observed characteristic inclusions, similar to trachoma, in eye smears.* A complete cytological and cultural study would enable comparisons to be made between these infections and pink-eye. For pink-eye, the maximum score in a cytology study was 6 for acute or post-acute stage smears according to the point system of Hardy (1966). In this system, which has value in trachoma survey studies for estimating the degree of infection, a score of 8-10 points classifies a smear as positive for trachoma. The system also has value in indicating a lower intensity of infection after the introduction of improved conditions of hygiene and antibiotic treatment (Hardy, Surman, and Howarth 1967*b*, 1967*c*). But its value as a diagnostic method appears to be limited, e.g. in isolated cases of human chronic conjunctivitis of unknown aetiology or possible trachoma influenced by good hygiene and antibiotic treatment, when the cytology score is less than 8.

* Cello (1967) also has reported the presence of inclusions indicative of the psittacosis group of agents in sheep, and failed to recover the agent in eggs because of the presence of *Mycoplasma* in infected washings. Furthermore, in conjunctivitis of cats, he has observed a dual infection, with a psittacosis-like microorganism being followed by the appearance of *Mycoplasma* sp.

Many smears from patients with chronic conjunctivitis have large to small lymphocytes (no follicular distribution), neutrophils, scattered plasma cells, an occasional eosinophil, and sometimes indistinct peripheral ring-like forms; the cytology scores did not exceed 7 points. A concurrent study to grow *Mycoplasma* sp. from eye specimens of 17 such patients in PPLO agar (pH 6·8, a few at pH 7·8) has failed. If *Mycoplasma* agents are the cause of many of these chronic conjunctivides, failure to isolate them might be due to inadequate culture conditions or previous treatment of the patient with antibiotics. However, for pink-eye this score system, or a modification, is unnecessary since the acute and post-acute clinical stages show the presence of the aetiological agent in the eye smears.

The isolation of the aetiological agent will permit fluorescent antibody studies on pink-eye smears to confirm the identity of the aetiological agent, cell culture, or other host-system studies to elucidate the cycle of development of the agent, antibiotic sensitivity studies to find a suitable method of treatment, and investigation into prophylaxis by vaccination.

Finally, reclassification of the pink-eye agent is necessary, and probably for members of the genus *Ricolesia* Rake and *Colettsia* Rake (see Bergey's "Manual of Determinative Bacteriology", 7th Ed. pp. 959-61) which are possible *Mycoplasma* agents. Obtaining clones of pink-eye isolates to ascertain whether the agent is in pure culture or is a mixture of strains, their characterization, and comparative studies between them and other *Mycoplasma* agents will take time. It is a "wiser policy to restrain one's taxonomic efforts" (Sub-committee on the Taxonomy of Mycoplasmatales 1967), but in the interim it might be useful to employ the name *Mycoplasma conjunctivae* var. *ovis* to prevent confusion with the original classification of a rickettsia.

V. ACKNOWLEDGMENTS

The author is deeply indebted to the Management of the Metropolitan and Export Abattoirs Board, to Mr. W. G. Peevie, Mr. W. L. B. Bell, and other veterinary inspectors of the Department of Primary Industry, and to Mr. M. Quinlivan and Mr. P. Sellars of the Metropolitan Wholesale Meat Co., for their invaluable assistance in providing facilities and supply of normal and pink-eye affected sheep.

Grateful acknowledgment is expressed to Dr. D. Hardy, Microbiology Department, University of South Australia, and Dr. W. H. Howarth, formerly Medical Virologist of this Institute, for encouragement and provision of eye smears from Australian aborigines. I am also grateful to Mr. J. W. Macaulay of this Institute for confirming the presence of coccobacillary forms in smear 4 L indicative of the pink-eye agent as experienced in Kenya, to Mr. B. W. Moore also of this Institute for culturing *Mycoplasma pneumoneae* (Boston strain) and *M. hominis* type 1 on PPLO agar in plastic plates, and to the South Australian Department of Agriculture for the loan of the Leitz screw micrometer.

Finally, I wish to thank Mr. W. K. Nolan and Mr. A. J. Smith, for advice and facilities, and the former for providing photographs reproduced in Figures 4 and 6.

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