Laboratory diagnosis of psittacosis.

REPORTS
ON
PUBLIC HEALTH AND MEDICAL SUBJECTS
No. 80

Laboratory Diagnosis of Psittacosis

MINISTRY OF HEALTH

LONDON
HIS MAJESTY'S STATIONERY OFFICE
1937

PREFATORY NOTE BY THE CHIEF MEDICAL OFFICER.

To the Right Hon. Sir Kingsley Wood, M.P.,
Minister of Health.

SIR,

I beg to submit a report on the laboratory diagnosis of a disease of birds communicable to man (Psittacosis), which has been prepared in the Department with the help of Professor W. Levinthal and Professor S. P. Bedson. Professor W. Levinthal provided a detailed statement of his own laboratory observations many of which are as yet unpublished. The Department are much indebted to him for this information and for the assistance he has given in the detection of actual outbreaks of psittacosis in this country. Professor Bedson has also rendered much help in similar ways and in the critical revision of the report.

The report describes as concisely as is practicable the technical procedures necessary in the examination of pathological material from birds, from experimentally inoculated mice and from human beings suspected of infection with the virus of psittacosis. The microscopical appearances of the virus in infected tissues are discussed and illustrated.

A previous account of the disease, which also included its epidemiology and clinical manifestations, was given by Dr. E. L. Sturdee and Dr. W. M. Scott in 1930 (Reports on Public Health and Medical Subjects No. 61). The present document may be regarded as supplementary to that report.

It is hoped that the report will encourage public health bacteriologists and clinical pathologists to study the laboratory diagnosis of the disease, and that it may aid them to give a confident report of the presence or absence of the virus in both human and animal cases of doubtful illness.

It is most desirable that close watch should be kept for the occurrence of anomalous lung inflammation or continued fever in persons in association with cage birds and that material from such persons and from the suspected birds should be examined in the manner described.

I have the honour to be,

Sir,

Your obedient Servant,

ARTHUR S. MACNALTY.

Whitehall,

March, 1937.

Laboratory Diagnosis of Psittacosis.

In 1929, a Memorandum (151 Med.) was issued by the Minister of Health in which, after calling attention to the existence of psittacosis in this country and to the precautions to be adopted in preventing its spread, he asked Medical Officers of Health (1) to inquire, in all notified cases of enteric fever or pneumonia, into the possible association of the patient with sick or recently dead birds, (2) to instruct Sanitary Inspectors and Health Visitors to look out for illness among pet parrots, budgerigars, etc., and (3) to forward to the Laboratory of the Ministry (*a*) bodies of birds which seemed to have been associated with cases of human illness and (*b*) portions of liver, spleen, lungs, bone marrow and lymph glands, in 50 per cent. glycerine diluted with normal saline, from fatal human cases of psittacosis.

These recommendations still hold good, but, as the result of recent investigations both in Europe and America, it has become possible to give somewhat more precise indications as to the detection of the disease both in man and in animals.

In the following paragraphs the technique of laboratory diagnosis is described in sufficient detail to permit of its being followed by clinical and public health pathologists.

I. *Precautions.*

Any material containing psittacosis virus must be regarded as highly pathogenic and dangerous to handle unless proper precautions are taken. Psittacosis-infected birds (parrots, parrakeets, budgerigars, etc.), in particular, scatter infected material freely around them in the form of dried faeces and beak discharges attached to down and feather particles. Birds suspected of psittacosis, therefore, should be killed at once by asphyxiation with coal gas or chloroform and plunged complete with cage into 5 per cent. lysol solution. It may be remarked that experimental inoculation of birds with psittacosis material is to be deprecated except in specially equipped laboratories. Transport of living birds in which infection is suspected should never be attempted. Dead birds, wrapped in lysol-soaked muslin or wool, may be sent by post, provided the Post Office Regulations for transmission of pathological material are strictly followed.

In the laboratory the usual precautions in dealing with infective material should, of course, be observed, special care being taken that no chance is given for it to become dried and to enter the atmosphere in the form of dust. A separate fly-proof room is necessary for experimentally infected mice; access to

this should be restricted to the pathologist and one well-instructed attendant. The mice must be kept in glass jars containing raised floors of perforated metal and with perforated metal lids covered with several layers of gauze to prevent escape of dust. The use of a special overall with rubber gloves is advisable during handling, feeding and cleaning.

II. *Examination of suspected birds.*

The ventral feathers should be plucked with the bird immersed in lysol solution; it should then be pinned out on a board and the abdomen and thorax opened. Findings suggestive of psittacosis are (1) pericardial effusion with fibrinous flakes or complete fibrinous covering of the heart, (2) greatly enlarged spleen, sometimes with visible yellow necrotic nodules, and (3) pale or yellow liver with spots of necrosis sometimes surrounded by haemorrhagic zones. The lungs are most commonly free from obvious pathological changes. The pericardial fluid is first collected by means of a capillary pipette; a portion of the *pericardium* is cut out and its serous surface smeared on a slide. The *heart blood* is then collected by means of a capillary pipette, a thin blood film being made at the same time. The *lungs* are then removed as completely as possible and any portions showing inflammation cut out and placed in a footed tube (" Griffith tube "); smears and impression preparations on slides are made from any pneumonic portion. The serous surface of the *liver* should be scraped so as to collect any fibrinous exudate, from which smears are then made. Any piece of the liver showing focal necrosis is cut out; smears are made from it and it is placed similarly in a Griffith tube. Finally the *spleen* (a round purple body lying behind the gizzard in a fold of the duodenum) is similarly collected and treated.

The various smears, which can all be made on two or three slides, are then examined (*vide infra*). If direct microscopical examination gives a clear positive result, no further work is necessary and the various pieces of organs can be destroyed, though histological sections of the organs offer valuable confirmatory evidence. If microscopical examination is negative or doubtful, the combined pieces of tissue are ground up with a roughened glass rod inside a Griffith tube, at first without added fluid, then, when reduced to a paste, with the addition of sterile broth or tap water so as to make about 5 ml. of a rather thick suspension. Cultures from this suspension and from the heart blood should be made so as to detect bacterial infection (chiefly Salmonellosis or Pasteurellosis) as a possible alternative cause of death. Then about 0·5 ml. of the suspension is injected intraperitoneally into each of at least two mice. Should the organ emulsion be grossly contaminated with bacteria, it will

be necessary to remove these, partially or completely, before further inoculations. Light centrifugation (about five minutes at 4,000 revolutions) may suffice, 0·5 ml. of the supernatant fluid being then injected intraperitoneally, again into each of two mice. Alternatively, coarse filtration through sand and paper pulp may be substituted.

[Into a glass tube with funnel outlet, either mounted in a collecting flask with a side-tube for attachment to a pump or provided with a rubber bung and tube for applying positive pressure, paper pulp, prepared by shredding filter-paper in water to make a mash (which can be kept in stock with chloroform as a preservative), is packed lightly with a glass rod to a depth of 1 to 2 cm.; such a layer, when the tube is filled with water, should allow about six drops to pass per minute. Finally a layer of 1 cm. of silver sand is put on top and the whole apparatus sterilised by steaming.]

One or two passages of the suspension (first diluted to 10 ml. with broth and cleared of gross particles by standing for 10 minutes) through such a filter should give a fairly clear filtrate of which 0·5 ml. can be injected as with the centrifugate above-mentioned. Finally such a filtrate or centrifugate may be passed through a Berkefeld V filter or, preferably, through an Elford gradocol membrane of 1·0μ average pore size; this final filtrate should be injected intraperitoneally, again into at least two mice, in 1·0 ml. quantity. There is always some loss of virus in filtration; hence inoculation of the original emulsion should never be omitted.

The inoculated mice should be kept under observation for one month, but one of each pair should be killed at the end of 10 days and an emulsion of its spleen inoculated into a fresh pair of mice. Should they all survive (apart from deaths from intercurrent infection), the test has given a negative result. This may be confirmed or disproved by further passage of their spleens into fresh mice or by their inoculation with a known virulent emulsion of the spleen of mice dead of psittacosis infection. Should any survive this, the indication is that the original inoculum contained psittacosis virus in amount insufficient to cause fatal infection but sufficient to produce active immunity. Should they all die, the negative result is confirmed.

III. *Examination of suspected human cases.*

The material available may be either tissues, etc., obtained at autopsy, or blood, pleural fluid and sputum obtained during the illness.

Post-mortem material should consist of any pneumonic portions of the lung, any pleural and pericardial effusions, the liver and, especially, the spleen. These should be examined in

the same manner as described for the organs of birds. Negative results, however, are almost the rule with human material obtained *post mortem*, probably because death usually occurs late in the course of the infection in man and the virus has largely disappeared.

Blood obtained during the first four days of illness may contain the virus; it should be inoculated (defibrinated) in 0·5 to 1·0 ml. quantity intraperitoneally into mice. Later in the disease than the fourth day, blood practically always gives negative results on such inoculation. Microscopical examination of blood films is extremely unlikely to give any useful information at any stage of the illness, though virus particles have been seen in them (Coles). Blood obtained from convalescent patients may be used to give a retrospective diagnosis by complement-fixation tests with the serum and an emulsion rich in virus (mouse-spleen). Such tests, however, can hardly be said to lie within the competence of routine laboratories and, for the present, blood to be tested in this way should be sent to Professor Bedson at the London Hospital, Whitechapel, E.1.

Pleural fluid may be inoculated in similar amounts into mice with more hope of positive results. The deposit from larger quantities (when available), obtained by prolonged high speed centrifugation, can also be recommended.

Sputum, particularly in the early stages of the disease, is the most likely material to give positive results. Unfortunately it is apt to be scanty or unobtainable but every effort should be made to get a specimen (not saliva), however small in amount. After making cultures on blood agar plates the sputum should be emulsified in about ten times its volume of broth by thorough shaking with glass beads in a stoppered bottle. The emulsion is then injected intraperitoneally into at least three mice in quantities of 0·25 to 0·5 ml. in the hope that at least one of the mice may escape the almost inevitable concomitant infection with pneumococci and survive long enough for the development of virus. Filtrates of the emulsion should be made, in addition, in the manner described for tissue emulsions; both the sand-and-pulp coarse filtrate and the filtrate obtained by passage through a Berkefeld V filter or gradocol membrane should be inoculated intraperitoneally in 1 to 2 ml. quantity into several mice. Such inoculation may be repeated on three successive days into the same mice and this procedure gives the best chance of detecting the often minute amounts of psittacosis virus. The mice are kept under observation, as described above; at least one further passage is advisable, using the spleen of one or more of the mice at the end of ten days and again at the end of a month for inoculation of fresh mice.

IV. *Microscopic appearances of virus.*

(a) *Staining methods.*—The smears and impression preparations, made on perfectly clean, grease-free slides, should be fixed for five minutes with methyl alcohol and stained either with Giemsa's stain or with Bedson and Bland's or Lépine's modification of the Castañeda stain used for rickettsia, as recommended by Levinthal. In using Giemsa's stain, which gives perhaps the best preparations, a reliable brand of stain (Grübler or Gurr) must be used; it is diluted in the proportion of one drop of the stain to 1 ml. of distilled water which must be neutral in reaction (orange with neutral red); a trace of sodium carbonate solution (1 per cent.) will usually suffice to give the desired reaction to stock distilled water. The diluted stain is placed in a Petri dish and the slides immersed in it, face down, supported by pieces of capillary glass (or Coles' curved glass basins may be used), for three to 20 hours after which they are washed in distilled water to differentiate, dried, and examined directly with an oil-immersion lens. Permanent preparations may be made by applying a coverslip, using as the mounting medium either Zeiss's cedar oil or Gurr's neutral mountant.

For Bedson and Bland's modification of Castañeda take

Phosphate buffer solution (pH 7·0)	95 ml.
Formalin (neutral)	5 ml.
Borrel blue	10 ml.

Stain for two minutes, rinse with tap water and counter-stain for a few seconds with 1·0 per cent. aqueous safranin; dry by blotting.

For the Lépine modification, Azur II (Grübler or Gurr) is dissolved to make a 1 per cent. solution in 0·5 per cent. phenol in water; this stock solution keeps for several months. To use, add to 10 ml. of distilled water

5	drops of 1 per cent. potassium carbonate
10	,, of Azur II solution
10	,, of neutral formalin

and heat gently till steaming. Stain with hot solution for five to 10 minutes; wash with tap water; stain with safranin (1 in 2,000 in distilled water) for five to 10 seconds; wash with tap water; dry by blotting.

For staining sections, Heidenhain's classical iron-haematoxylin method is best and requires no detailed description; with it the virus bodies stain densely black like nuclear matter.

(b) *The virus particles.*—The commonest form in which the virus appears in smears from infected exudates or organs is that of a minute coccus of about 0·25μ in diameter, diffusely scattered by the disruption of infected cells or in pairs and short chains staining purple with Giemsa and deep blue with

Castañeda. It is in this form that the virus passes through bacteria-tight filters. Much larger virus bodies also appear, perhaps most definitely within undisrupted cells; these are oval or circular in shape and may reach 1μ in size; they often stain blue rather than purple with Giemsa and may show polar-staining or signs of simple division. Especially in smears from infected exudates or organs of parrots and parrakeets, bodies of all gradations in size from the minutest to the largest can be seen. The interpretation of this pleomorphism is still a matter of opinion. Bedson and Bland see in it a cycle of multiplication, the large forms being the early stage, while Levinthal regards the large form as an abnormal development of the small virus particles produced by the defensive reaction of the parasitised cell. In any case the important point is that the virus particle grows within the cytoplasm of susceptible cells (chiefly endothelial phagocytes) and there forms colonies, some of which are apparently homogeneous, perhaps owing to a veiling basophilic matrix, others clearly revealing their structure. The disruption of the cell, which occurs in making smears, breaks up and scatters some of the colonies in the form of free virus particles.

In " natural " infections in birds the virus colonies may occur in all the different stages of development. Smears made from the pericardial exudate and from the spleen pulp are the most likely to give positive findings, but blood smears sometimes show numerous detached endothelial cells packed with virus colonies as well as free virus particles which have escaped by disintegration of such cells.

The staining reactions and morphological appearance of psittacosis virus bodies are so characteristic that identification in smears from infected tissue (bird or mouse) is usually easy, though some experience is no doubt necessary before a confident report can be given. The attached plate will be of service for comparison with preparations made for diagnosis. The blue or purple staining of the pleomorphic virus bodies contrasts with the red of the cell cytoplasm and the reddish purple of the cell nuclei; erythrocytes are stained intensely blue with the Castañeda method.

In all psittacosis infections, whether of birds, rodents or man, the essential lesion is the invasion and destruction of the reticulo-endothelial system; attention should, therefore, be directed especially to the cells of this system in searching for virus bodies.

V. *Examination of experimental mice.*

The experimental disease in the mouse is not very characteristic: its symptoms consist of ruffled fur, closed eyes, often with some discharge, apathy, occasionally diarrhoea. Its

EXPLANATION OF PLATE.

(The Plate is reproduced by kind permission of the publishers of the British Journal of Experimental Pathology.)

FIG. 1.—Group of virus lying free in an impression preparation made from the spleen of a mouse removed 24 hours after infection. Giemsa differentiated in orange G-tannin. × 1500. Both elementary bodies and larger forms present. Large forms appear to be dividing and intermediate forms occur. Note gradation in staining.

FIG. 2.—Group of virus lying free in an impression preparation made from the spleen of a mouse removed 15 hours after infection. Giemsa differentiated in orange G-tannin. × 1500. Note the irregularity in the shape of the large particles, unlike what one would expect were they merely large forms of a coccus.

FIG. 3.—Virus at same stage of development as in Fig. 2, but inside a cell. (Morula) Impression preparation of mouse spleen removed 15 hours after infection. Castaneda. × 1500. Note that the large virus forms, like elementary bodies, retain the blue stain Cell cytoplasm and chromatin take the safranin used as counterstain.

FIG. 4.—Virus in same stage of development as in Fig. 1, but intracellular. Impression preparation of mouse spleen removed 24 hours after infection. Giemsa differentiated in orange G-tannin. × 1200.

FIG. 5.—Intracellular mass of virus intermediate in stage between morula and elementary bodies. Impression preparation of mouse spleen removed 24 hours after infection. Giemsa differentiated in ornage G-tannin. × 1200.

FIG. 6.—Small morula inside a cell. Impression preparation of a mouse spleen removed 24 hours after infection. Giemsa. × 1200.

FIG. 7.—Plaque (plasmodium) in which there are indications of commencing division. Smear of mouse spleen removed 15 hours after inoculation. Castaneda. × 1200.

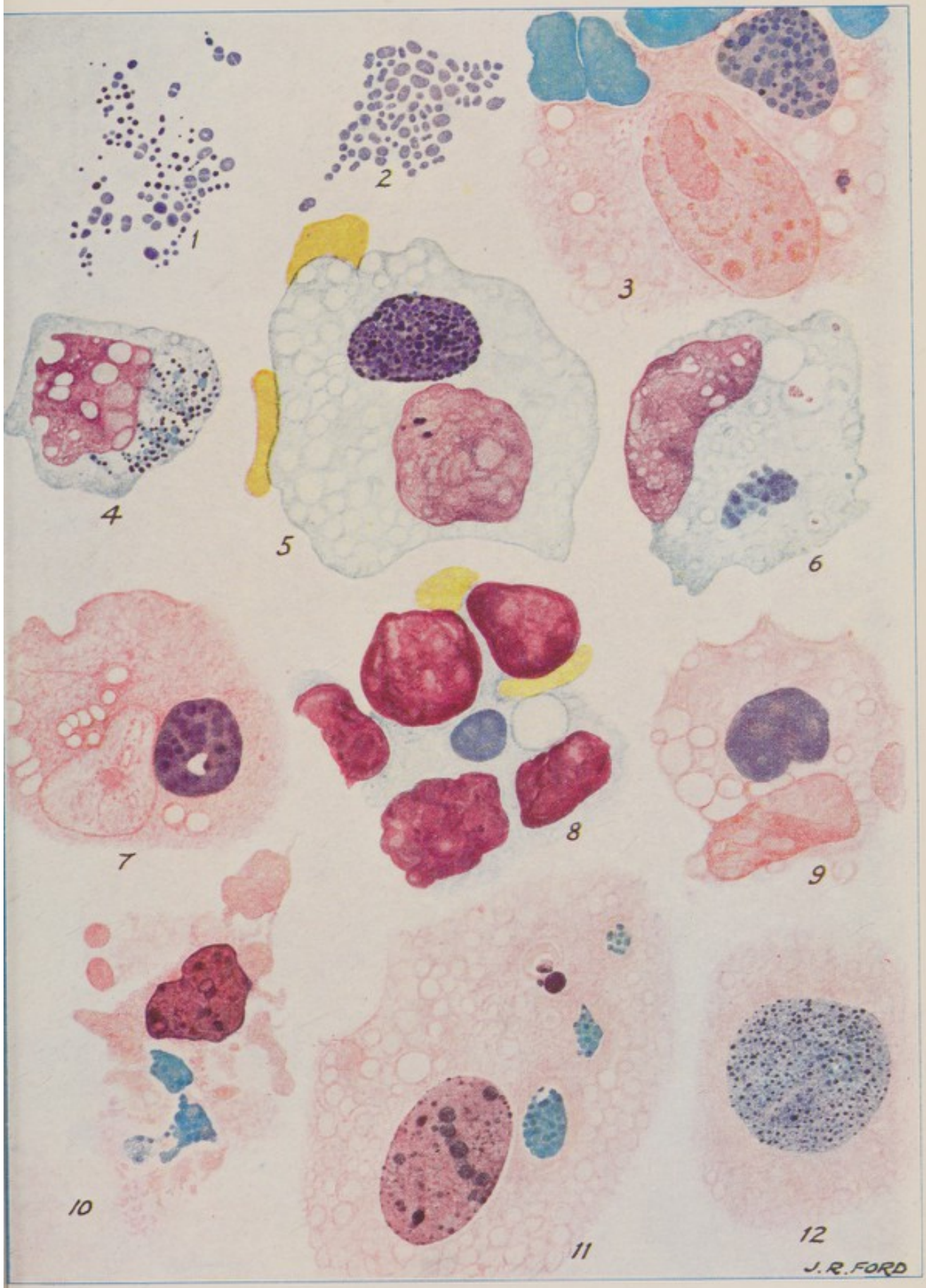
FIG. 8.—Plaque (plasmodium) which appears homogeneous. Impression preparation of mouse spleen removed 15 hours after inoculation. Giemsa differentiated in orange G-tannin. × 1200. The plaque is stained a dirty greenish blue.

FIG. 9.—Plaque (plasmodium) in same stage as in Fig. 8. Impression preparation of mouse spleen removed 15 hours after inoculation. Castaneda. × 1200. The plaque is stained blue, differentiating it from cytoplasm or nuclear material, which take the safranin by this staining method.

FIG. 10.—Plaque (plasmodium) just beginning to divide. Tissue culture made from normal mouse spleen soaked in virus filtrate and grown in mouse serum and chick embryo extract. 24 hours' incubation at 32°C. Fixation : Schaudinn. Giemsa. × 1200.

FIG. 11.—Cell showing three masses of virus in morula stage. Tissue culture. From same experiment as Fig. 10, but after 48 hours' incubation at 32°C. Fixation : Schaudinn. Giemsa. × 1200.

FIG. 12.—Cell showing mass of elementary bodies. Tissue culture. From same experiment as Figs. 10 and 11, but after 4 days' incubation at 32°C. Fixation : Schaudinn. Giemsa. × 1200.



Bedson and Bland.

duration depends on the amount and virulence of the virus injected. Virulent material from parrots or parrakeets injected intraperitoneally causes death of the mouse in from five to 30 days; occasionally animals recover. Passage through a series of mice increases the virulence for this species of animal, producing death in two to three days when injected in adequate dose.

The conditions found on post-mortem examination are of three main types:—

(1) If *death occurs in two to three days* after inoculation there may be little abnormal to see with the naked eye; the spleen is usually normal in size and appearance, as is the liver though the latter may show some mottling. Microscopically both these organs show small haemorrhages and necrotic foci. The virus particles are most abundant in endothelial cells of peritoneal exudate and especially of the spleen. These cells are packed with them and may show obvious signs of degeneration.

(2) If *death occurs in three to* 15 *days* the abdominal cavity will be found full of thick fibrinous exudate; the intestines are matted together and the liver and spleen coated with a thick layer of fibrin easily stripped off. The fluid in the peritoneum and pericardium is in excess of the normal and is turbid with suspended fibrin flakes and endothelial cells. The virus particles are abundant in these cells, especially those from the perihepatic, perisplenitic and pericardiac exudates; they are less numerous in the spleen.

(3) If *death occurs in* 15 *to* 30 *days* turbid serous effusions in peritoneum, pleura and pericardium are conspicuous. The liver and spleen may be enlarged and friable. Virus bodies, however, are usually scanty both in exudate and spleen pulp; the most favourable smears are those from the pericardium.

In all three types lung lesions are exceptional. When they occur, they are usually in the form of an acute haemorrhagic oedema of the alveolar walls which, when extensive, may simulate lobar pneumonia; a true broncho-pneumonia, however, is occasionally met with. The blood of the mouse at any stage is poor in virus, though endothelial cells in it may be found infected; this is in contrast to the blood of parrots, dead after natural or experimental infection, in which virus bodies are often abundant.

When the illness of inoculated mice is doubtful and death delayed, as not infrequently happens when the inoculum is poor in virus, as, for example, in much of the human material, it may be difficult to find typical virus bodies. Further passage

in mice will then be desirable and this should be done by injecting two or more fresh mice intraperitoneally with 0·5 ml. of a fairly thick emulsion of the spleen of each of the mice originally inoculated. Should such emulsions contain a bacterial infection (salmonella or pasteurella), filtration through Berkefeld V or gradocol membrane filters must first be done and the bacterial sterility of the filtrate determined. Such passage mice, if the psittacosis virus is present, ought to die with typical appearances within 15 days and further passages may reduce this period to three days. It must be borne in mind, however, that, in such passage experiments, there is always the risk of activating some other infection latent in the mouse. In addition to the bacterial infections, the virus disease, ectromelia, is common in mouse stocks and may simulate psittacosis in that it produces death with liver and spleen necrosis in which no bacterial infection can be found. Moreover, it is associated, like psittacosis, with cell-inclusions superficially like the psittacosis virus bodies, though those of ectromelia are found more in epithelial cells than in reticulo-endothelium, and do not reach full development in the liver and spleen cells. In ectromelia the staining reaction, both of inclusions and free virus particles, differs from that of the psittacosis virus bodies, being definitely acidophil (red in Mann's method and with Giemsa) as contrasted with the basophil staining of the latter. The pathologist should be familiar with the appearances in both in order to make a confident report.

VI. *Examination of budgerigar flocks.*

It is established that the budgerigar (love-bird) is subject to psittacosis, not only in domesticated stocks in all countries where these have been examined, but also in its native wild state in Australia. Furthermore the budgerigar is more resistant to the disease under ordinary conditions than the tropical parrots and parrakeets and, instead of dying more or less rapidly when infected, as these birds do, may survive and harbour the virus while appearing in perfect health. Such birds carrying virus not only maintain the infection in aviaries, but are a constant danger to human beings who come in contact with them. Data as to the extent of psittacosis infection in budgerigar stocks in this country are somewhat scanty, though its existence has been amply proved in a few instances. Pathologists who have reason to suspect the budgerigar origin of a case of human psittacosis should, therefore, make as many observations as possible on the suspected stock. Should proof of infection be obtained by the laboratory examination of sick or dead birds, it would be useful to extend the investigation to the rest of the stock or as many as practicable, to determine in

how many of them virus can be detected. Inoculation of two mice with the spleen of each bird would suffice, as a rule, and may be expected to detect a percentage of " carriers "—varying with the ages of the birds and the amount of cross-infection —of from 5 to 20 per cent. or more in an infected aviary.

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