STUDIES OF PPLO INFECTION

I. The Production of Cerebral Polyarteritis by Mycoplasma galli- septicum in Turkeys; the Neurotoxic Property of the Mycoplasma*

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Jungherr (1), in 1949, noted the occurrence of encephalitis during several outbreaks of the chronic respiratory disease (CRD) of turkeys, then considered to be a virus disease, and demonstrated necrotizing and inflammatory lesions of the cerebral arteries in affected birds. Adler et al. (2), in 1954, cultivated a mycoplasma, now designated *Mycoplasma gallisepticum*, from the tissues of turkeys with CRD, and established the capacity of this organism to reproduce the sinusitis and aerocystitis characteristic of the natural disease. A neurotropic strain of *M. gallisepticum* was recovered by Zander in 1954 from the brain of a turkey with torticollis. This strain was shown by Cordy and Adler (3) to produce a fatal encephalopathy in young turkey poults, with the predominant lesions confined to the arteries of the central nervous system.

This paper is concerned with studies of the pathology of cerebral polyarteritis at various stages of its development, with certain factors involved in the pathogenicity of *M. gallisepticum*, and with a neurotoxic property associated with concentrated suspensions of this organism. The therapeutic efficacy of gold salts, and tetracycline, previously established in other types of mycoplasma infection, will be demonstrated. Evidence will be presented indicating that the polyarteritis in this experimental model does not depend upon an immunologic mechanism, and more probably arises as the result of direct, toxic injury to the walls of cerebral arteries by the mycoplasma.

Materials and Methods

The S6 strain of *M. gallisepticum* was obtained from Dr. Henry E. Adler, University of California, Davis. A broth culture demonstrated to be pathogenic for turkeys was divided into 1 ml aliquots; these were stored at −70°C and used for the preparation of all cultures employed

in these experiments. The organisms were grown in broth or agar media (4) consisting of 7 parts Difco PPLO broth (Difco Laboratories, Inc., Detroit) or agar base, 2 parts unheated horse serum, and 1 part 25% yeast extract supplemented with 0.5% glucose and 10^3 penicillin units per cc. To prepare cultures for injection an overnight subculture was made with 1 ml of the stored culture in 20 cc of fresh broth, and from this a 5% subculture was made in 200 to 1000 ml broth; the cultures were harvested after 16 to 20 hr in logarithmic growth phase, concentrated by centrifugation at 13,800 g for 10 min, and resuspended in the desired volume. All cultures were grown aerobically at 37°C. The titer of *M. gallisepticum* in broth cultures was determined by plating serial 10-fold dilutions on PPLO agar and counting colonies after 5 days.

The turkeys were young line bred Beltsville white poults, usually 2 to 3 wk old, weighing 750 to 1000 g, obtained from Truslow Farms, Inc., Chestertown, Maryland. All birds were used within a few days after arrival, and most experiments were completed within 10 days. They were killed by chloroform inhalation or by exsanguination. Tissues were prepared for histological examination by fixation in 10% formalin or in Vandergriff's fixative. For determining titers of *M. gallisepticum*, tissues were homogenized as 10% suspension in broth, centrifuged 500 g to remove gross tissue fragments, and diluted serially for plate inoculation. Colony counts were made after 5 days' incubation.

Antiserum against *M. gallisepticum* was prepared in rabbits by repeated intradermal inoculations of 1 ml of a suspension containing approximately 5×10^{10} organisms combined with complete Freund's adjuvant (Difco); the organisms were harvested from a 24 hr broth culture and washed three times in saline. The serum was conjugated with fluorescein isothiocyanate on Celite 10% (California Corporation for Biological Research, Los Angeles), according to the method of Rinderknecht (5). After conjugation, the serum was centrifuged for 30 min at 30,000 g, passed through a column of Sephadex G-25 to remove unconjugated fluorescein, and then through a column of DEAE-cellulose to eliminate nonspecific staining reactants (6). Several samples of turkey antiserum obtained from adult birds recovering from *M. gallisepticum* infection were supplied by Dr. Henry Adler.

EXPERIMENTAL

Cerebral Arteritis

Course of Disease.—Groups of 6 turkey poults were injected in the wing vein with 1 ml of various concentrations of *M. gallisepticum* in broth. In most instances, a 24 hr broth culture of the organism yielded 10^9 colony-forming units per ml. In order to obtain suspensions with 10^{10} and 10^{11} organisms per ml, broth cultures were centrifuged at 13,800 g for 10 min, and the pellet resuspended in amounts of broth to make 10- or 100-fold concentrations

The typical disease was elicited with doses of 10^6 through 10^9 organisms, with variations in the incubation period depending on the number of mycoplasmas injected, as indicated in Table I. Two of the birds receiving 10^6 organisms became ill on the 8th day; all of those given 10^9 organisms became ill between the 3rd and 5th day. The illness began suddenly, with neurological manifestations consisting of ataxic gait, paralysis of one or both legs, and torticollis. Some of the birds were unable to stand, and exhibited convulsive floundering and rolling movements. In most cases, death occurred within less than 24 hr after onset.

Neurotoxic Effects of High Doses.—The injection of 10^{10} or 10^{11} mycoplasmas

was followed in all cases by the appearance of signs indicating the presence of a neurotoxin in the suspensions. With the 10^{11} dose, these developed within 1 to 3 hr (Table I). At this time all birds, which until then appeared entirely well, suddenly became ataxic, fell to the floor of the cage, exhibited torticollis and paralysis of the extremities, and usually died within less than an hour. Some of them underwent constant rolling movements, rather reminiscent of the "rolling disease" caused by *Mycoplasma neurolyticum* in mice (7). The time between the injection of mycoplasmas and the onset of neurological signs varied depending on the dose employed. With 10^{11} organisms, all birds were dead within 4 hr; with 5×10^{10}, death occurred in 5 to 10 hr; with 10^{10} the latent period became still longer, ranging from 9 to 28 hr.

TABLE I

Relation of Time of Appearance of Neurological Signs to Dose of Mycoplasmas Injected Intravenously

	Dose of mycoplasmas*					
	10^4	10^6	10^8	10^9	10^{10}	10^{11}
Time of onset of disease	—	8 days	7 days	3 days	9 hr	1 hr
	—	8 days	7 days	4 days	9 hr	1 hr
	—	—	7 days	4 days	20 hr	1 hr
	—	—	14 days	4 days	20 hr	2 hr
	—	—	14 days	5 days	20 hr	3 hr
	—	—	—	5 days	28 hr	3 hr
No. dead/No. in group	0/6	2/6	5/6	6/6	6/6	6/6

* Each of 6 turkeys received 1 ml of a suspension of *M. gallisepticum* in the dose indicated. Times refer to onset of paralysis or gross ataxia. Birds indicated by (—) remained well during a 3 wk period of observation.

When titrations were performed with increments of mycoplasma dosage more closely arranged than in the conventional 10-fold dilution technique, it became evident that the latent interval between injection and illness could be varied by relatively small variations in the number of mycoplasmas injected. The point is illustrated in Table II, showing the outcome when 6 different doses were prepared from a single suspension of mycoplasmas, spanning the range between 1.25×10^9 and 10^{11} organisms, and given to groups of 3 turkeys each. After the highest dose, the average survival time was 3 hr, after the lowest, 96 hr, and in between the survival times conformed in a fairly orderly fashion to the numbers of organisms used. All of the birds appeared to be healthy throughout the latent period indicated, and then presented the same acute onset of neurological signs and died in much the same manner. Birds

dying after 3 or 4 days showed severe polyarteritis in brain sections; those dying after 1 or 2 days often had fibrinoid necrosis of the cerebral arteries with little or no inflammation; those dying in less than 1 day had only equivocal vascular lesions. It is probable that these differences in histopathology reflect differences in the time required for the development of histological evidence of reaction to injury.

The neurotoxicity of concentrated suspensions was dependent on the presence of intact, viable mycoplasmas. Heating the suspension at 50°C for 1 hr eliminated toxicity, as did repeated freezing (−70°C) and thawing of suspensions of mycoplasmas in distilled water; both procedures caused major reductions in the numbers of viable organisms, as is shown in Table III.

Exposure of the mycoplasmas to specific rabbit antibody also eliminated neurotoxicity. A neurotoxic suspension was mixed with an equal volume of

TABLE II

Relation of Dose to Time of Death, Within Narrow Range of Doses

	Dose of mycoplasma*					
	1.25×10^9	2.5×10^9	5×10^9	10^{10}	2×10^{10}	10^{11}
Time of death, hr	96	78	48	20	9	2
	96	120	96	20	9	3
	96	120	120	20	27	4
Average time of death, hr.........	96	106	88	20	15	3

* Each of 3 turkeys received 1 ml of the dose indicated.

undiluted rabbit anti-gallisepticum serum, incubated for 1 hr at 37°C and 2 ml of the mixture was then injected intravenously in 4 turkeys. As controls, similar mixtures were prepared with normal rabbit serum, and with rabbit antiserum against an unrelated mycoplasma species, Mycoplasma orale. The results are shown in Table III. Complete protection was provided by the antiserum for M. gallisepticum, while none was observed with normal or anti-orale serum.

No soluble neurotoxin was detectable in broth cultures of M. gallisepticum. Intravenous injections of 20 ml of culture broth from which the organisms had been removed by centrifugation caused no symptoms. In one experiment, a liter of broth in which the organisms had been cultivated for 20 hr, then removed by centrifugation, was lyophilized and reconstituted in 10 ml of distilled water. Injection of 5 ml of this concentrated broth failed to produce any neurological manifestations, and the birds survived without ill effects.

Effect of Intracerebral Injection.—The neurotoxic action of M. gallisepticum

seemed best accounted for by an effect on the blood vessels of the brain, in view of the general correlation between neurologic manifestations and cerebral polyarteritis in birds surviving longer than 24 hr. If this were the case, and if the toxic action were exerted by mycoplasmas arriving by way of the circulation, one might expect an intracerebral injection of equivalent doses of organisms to be no more effective, and perhaps less effective, than when given by vein. The following experiment was done in order to obtain information on this point.

Two turkeys were given a dose of 1.6 × 10¹¹ mycoplasmas contained in 1 ml, by vein; at the same time, 2 turkeys received the same dose, contained in 0.1 ml, intracerebrally. The times of death, shown in Table IV, were significantly different. Both of the intravenously

TABLE III

Properties of Mycoplasma gallisepticum Neurotoxin

Treatment of mycoplasmas	No. dead/No. in group	Viable mycoplasmas in inoculum
Anti-*gallisepticum* rabbit serum*..................	0/4‡	4 × 10⁸
Anti-*orale* rabbit serum*........................	4/4	10¹¹
Normal rabbit serum*..........................	4/4	10¹¹
Frozen-thawed§................................	0/4	10⁶
Heated 50°C‖..................................	0/4	10³
Control suspension (10¹¹ organisms)..............	4/4	10¹¹

* Inoculum: 1 ml containing 10¹¹ mycoplasmas mixed with equal volume of serum, incubated 37°C 1 hr before injection.

‡ Numbers refer to turkeys dying within 24 hr after injection.

§ Inoculum: 1 ml containing 10¹¹ mycoplasmas in distilled H₂O, frozen and thawed 10 times in dry ice-alcohol.

‖ Inoculum: 1 ml containing 10¹¹ mycoplasmas, heated at 50°C for 1 hr.

injected birds died within 10 hr, while the other 2 died at 22 and 24 hr, respectively. A similar experiment was performed with a somewhat smaller dose, 5 × 10¹⁰ organisms, administered to 2 birds by vein and to 2 intracerebrally. The intravenously injected pair were dead within 19 hr; 1 of the intracerebrally injected died after 40 hr, while the other survived without evident illness for 8 days.

The results indicated that the lethal effect of *M. gallisepticum* can be brought about more rapidly and uniformly when the organisms are injected into the wing vein than when the same dose is administered intracerebrally.

Pathology of the Disease.— The brains of 90 turkeys infected with various doses of *M. gallisepticum* and dying at various intervals were examined. In 20 of the birds with severe brain disease, examination was also made of other organs, including heart, liver, lung, spleen, kidney, adrenal, gastrointestinal tract, and muscle.

The essential and primary pathological alteration was arteritis, involving prin-

cipally and often exclusively the arteries of the brain. A remarkable feature of these lesions in some animals was the extent of involvement; in many of the birds virtually all arteries in both the parenchyma and meningeal space were involved. Most of the involved vessels were readily identifiable as arteries. In some cases with extensive involvement of small vessels, the possibility that veins were also affected could not be excluded. The histologic features of the vascular lesions were quite variable and appeared to depend on the duration of the disease in part, and in part on the length of the latent period between the injection of mycoplasmas and the onset of neurological signs. Thus, with the more concentrated neurotoxic suspensions, causing death within 24 hr or less, minimal or equivocal evidence of structural damage to vessels was present, in the form of swelling of endothelial cells, and there were no inflammatory cells. With smaller doses, leading to death on the 3rd or 4th day, necrosis and inflammation were more conspicuous. At this time, some of the vessels showed fibrinoid necrosis of the media with little or no cellular infiltrate; in others the necrosis was accompanied by leukocytic infiltration, principally adventitial. Accumulation of large amounts of fibrinoid material in the adventitia was often seen. Although neutrophiles and eosinophiles were sometimes conspicuous in the infiltrate, mononuclear cells generally predominated. Identification of the different types of mononuclear cells was not always possible; however, in the early lesions (2 to 3 days) most of them appeared to be macrophages; within the next few days increasing numbers of immature and then mature plasma cells appeared. Finally, with doses small enough to permit survival for 7 days or longer, the arteries showed dense adventitial infiltration by plasma cells, often with many small lymphocytes and a few macrophages. In these later lesions, fibrinoid necrosis was still a characteristic feature, but it appeared less severe than earlier.

Illustrative lesions of the cerebral arteries from turkeys receiving various doses of mycoplasmas are shown in Figs. 1 to 6. In addition to the necrotizing and inflammatory lesions of the walls of arteries, some of the vessels were partly or completely occluded by thrombi; the latter seemed to be composed of the same sort of fibrinoid material as that deposited within the walls together with enmeshed leukocytes. Some of the turkeys also showed irregular areas of fresh infarction of the brain, associated with mild degrees of leukocytic infiltration.

In 3 of 20 turkeys exhibiting severe arteritis of cerebral vessels, similar vascular lesions were also encountered in vessels outside the central nervous system. The arteries of the pectoral muscle were involved in all 3, and one also had lesions in the renal arteries. In each case the lesions were less extensive, and were generally less severe, than those in the cerebral vessels of the same birds.

Apart from polyarteritis and the evidently secondary lesions of parenchymal infarction in the brain, the only striking abnormality found was a glomerular lesion (Fig. 7), characterized by swelling and proliferation of intracapillary cells; an occasional glomerulus contained a few neutrophiles. This glomerular alteration was found in all of the 20 turkeys whose kidneys were examined. In 2 instances fibrin thrombi were seen in glomerular capillaries (Fig. 8).

Attempts to demonstrate the presence of mycoplasmas in the affected arteries of the brain were unsuccessful. Giemsa stains revealed no bodies resembling mycoplasmas, and fluorescein-conjugated rabbit and turkey antiserum produced no stain-

ing in frozen sections of brains taken at various intervals after infection. In 2 birds injected intravenously with 10^{11} organisms and killed within 1 hr, bright fluorescence was demonstrated with rabbit antiserum in the Kupffer's cells of the liver and in phagocytic cells of the spleen; no areas of fluorescence were demonstrable in sections of the brains of these turkeys.

Effects of Treating Turkeys with Gold Thiomalate, Tetracycline, and Other Agents

Gold salts have been reported to be effective in preventing infection of animals by a variety of mycoplasma species, including several strains causing arthritis in mice and rats (8), and the "Eaton agent" (*Mycoplasma pneumoniae*) in hamsters, cotton rats, and chick embryos (9). Gold thiomalate was found by Sabin and Warren (8) to be a useful preparation for this purpose because of its relatively low toxicity.

TABLE IV

Comparison of Intravenous and Intracerebral Injection of Mycoplasmas

Route of injection*	Time of death	
	5×10^{10}‡	1.6×10^{11}‡
Intravenous	18 hr, 19 hr	9 hr, 10 hr
Intracerebral	40 hr, >8 days	22 hr, 24 hr

* Mycoplasma suspensions, in indicated doses, were contained in 1 ml for intravenous injection, 0.1 ml for intracerebral injection.

‡ Dose of mycoplasma injected by indicated route.

Turkeys were given intramuscular injections of gold thiomalate, in a dose of 50 mg per kilo per day. After 3 days of pretreatment, they were injected with suspensions of *M. gallisepticum* in doses sufficient to produce acute manifestations of neurotoxicity and death within 3 hr. Other birds received the same treatment with gold thiomalate, but were injected with smaller numbers of mycoplasmas in order to determine the effect of treatment on the development of polyarteritis.

The results are shown in Table V. It will be seen that gold provided some protection, although not complete, against acute neurotoxicity, while it protected completely against the neurological disease caused by injections of 10^9 mycoplasmas. Histological sections of the brains of gold-treated birds, examined 2 wk after injection, showed a few lesions of relatively mild polyarteritis; none of these turkeys appeared ill at any time during a 10 day period of treatment, and none developed cerebral infarction in association with the arteritis.

Tetracycline (Sumycin, E. R. Squibb & Sons, New York) was administered to 14 turkeys in a dose of 10 mg per kilo, 1 day before the injection of mycoplasmas and each day thereafter for 3 days. The doses of mycoplasmas were 10^9 (6 birds) and 10^{11} (8 birds); similar groups of untreated turkeys were given the same doses.

The results, included in Table V, showed complete protection of all treated birds; none became ill during the first 4 hr, during which all of the controls given 10^{11} organisms died, none subsequently developed neurological manifestations during an 8 day period of observation, and none showed cerebral arteritis on postmortem examination at the end of this period.

It was also found that tetracycline was therapeutically effective when administered after the onset of neurological disease. Twelve turkeys were infected by an intravenous injection of 10^9 organisms, and on the 4th day, when weakness, ataxia, and prostration had developed in most of the birds, 6 were given 50 mg tetracycline. The 6 selected for treatment were those with the most obvious neurological manifestations. On the following day, 4 of these had improved, and tetracycline was repeated. By the 7th day the results were clear;

TABLE V

Effects of Treatment of Turkeys with Gold, Tetracycline, and Cortisone

Treatment*	Dose of mycoplasmas	
	10^9	10^{11}
	No. dead/No. in group	
Gold thiomalate...................	0/6	3/8
Tetracycline......................	0/6	0/8
Cortisone........................	6/6	8/8
Methotrexate.....................	6/6	6/6
6-Mercaptopurine.................	6/6	6/6
None............................	6/6	8/8

* See text for details of treatment of turkeys.

all 6 untreated birds were dead, 2 of the tetracycline treated were dead, and the other 4 now appeared to be completely healthy. Examination of the brains of the treated birds showed mild arteritis in 3 of the 4 animals which were clinically cured; in the 4th very severe arteritis was found with fibrinoid necrosis and severe inflammation involving numerous plasma cells. In the 2 treated turkeys which died on the 5th day mild arteritis was found which did not differ in character or severity from that in the untreated group. It thus appeared that the therapeutic action of tetracycline was not directly associated with reversal or amelioration of the arterial lesions.

Cortisone, in a daily dose of 20 mg per kilo, was found to be entirely lacking in protective action against both the neurotoxicity of large doses and subsequent infection with polyarteritis following smaller doses of mycoplasmas. The outcome of a typical experiment is shown in Table V. Histological examination of the brains of the cortisone-treated birds revealed lesions of polyarteritis of the same extent and severity as in the untreated controls.

Methotrexate (aminopterin) and 6-mercaptopurine were tested for therapeutic effect employing the same doses of mycoplasmas used in the experiments with tetracycline. The drugs were administered 2 hr before the injection of mycoplasmas, and each day thereafter until death of the birds. The dosage of methotrexate was 10 mg per kilo per day; that of 6-mercaptopurine 100 mg per kilo per day.

As indicated in Table V, neither drug had any effect on the mortality caused by 10^9 or 10^{11} mycoplasmas. The time of onset and nature of neurological manifestations were no different in the treated turkeys. The vascular lesions in

TABLE VI

Recovery of Mycoplasmas from Brain Homogenates in Treated and Untreated Turkeys

Dose of mycoplasmas	Treatment	Time of culture	Condition of turkey	Brain titer*
3×10^{10}	Gold	12 days	Well	2×10^4
3×10^{10}	"	12 "	"	1×10^4
3×10^{10}	None	1 day	Dead	5×10^5
3×10^{10}	"	1 "	"	5×10^6
2.5×10^9	Tetracycline	9 days	Well	10^3
2.5×10^9	"	10 days	"	10^3
2.5×10^9	"	10 days	"	10^2
2×10^9	None	1 hr	Well	10^5
2×10^9	"	1 "	"	10^5
2×10^9	"	1 day	"	2×10^5
2×10^9	"	1 "	"	2×10^5
2×10^9	"	2 days	"	2×10^4
2×10^9	"	2 "	"	8×10^3
2×10^9	"	4 "	Moribund	1.6×10^5
2×10^9	"	4 "	Moribund	1.4×10^5
2×10^9	"	4 "	Moribund	5×10^4

* Numbers of colony-forming units per gram (wet weight) of brain tissue.

these birds appeared to show as much necrosis as in untreated controls, but inflammatory cell infiltration was much scantier. Plasma cells were seen only in 1 animal, and even in this case only a few were present.

Culture of Mycoplasmas from Brains of Infected Turkeys

Although both gold thiomalate and tetracycline afforded striking degrees of protection against the development of cerebral polyarteritis, neither drug was effective in eliminating viable mycoplasmas from the brains of infected birds. Indeed, the correlation between the numbers of mycoplasmas demonstrable in homogenates of brain and the incidence or severity of neurological disease, although present, was not impressive. This is illustrated by the following experiment.

A group of 16 turkeys was studied for the content of viable organisms in brain tissues after intravenous injection of mycoplasmas. Two were treated with gold thiomalate, and 3 with tetracycline, by the schedule outlined above. At various periods after injection, sample groups of birds were killed by exsanguination and the brains were removed and homogenized in sufficient broth to make 10% suspensions. These were clarified by low speed centrifugation, and the supernate titered in serial 10-fold dilutions, and immediately plated on PPLO agar. Colony counts were made after 5 days' incubation. The results are shown in Table VI.

It will be seen that the higher concentrations of viable mycoplasmas (10^5 or more) tended to occur in the brains of birds moribund or dead from the infection. However, none of the brains were free of mycoplasmas, even in birds which had been completely protected against neurological manifestations by gold or tetracycline. In 9 untreated birds, injected with 2×10^9 organisms, an attempt was made to learn whether intracerebral multiplication of mycoplasmas was temporally associated with the development of cerebral arteritis. The results, grouped in the lower portion of Table VI, indicate that this is not a feasible approach to the problem. The same numbers of mycoplasmas were present in brain homogenates obtained 1 hr or 1 day after injection as were demonstrable on the 4th day when the birds had become moribund. These results are difficult to interpret; it is quite possible that significant changes may be occurring in the population of viable mycoplasmas in or around the affected arteries, without being reflected in the total population in homogenates of whole brain.

Attempts to Produce Disease with M. Gallisepticum in other Animals

Suspensions of *M. gallisepticum* were injected by vein in mice, rats, syrian hamsters, and cotton rats, in doses ranging from 10^9 to 10^{11} organisms. None of these animals developed evidence of neurological disease, and all remained healthy during an observation period of 10 days.

Eight baby chicks were injected by vein 3 days after hatching, with a dose of 10^{10} mycoplasmas. One chick became ill on the 4th day and was killed. Brain sections showed a mild inflammatory infiltration in the walls of several cerebral arteries, composed mainly of large mononuclear cells; fibrinoid necrosis of the arteries was not observed. The remaining chicks remained well and showed no arterial lesions when killed 3 wk later.

DISCUSSION

In confirmation of the report of Cordy and Adler (3), it has been shown that intravenous injections of suspensions of *Mycoplasma gallisepticum* into young turkey poults result in extensive arteritis affecting almost exclusively the arteries of the brain. The lesions involve vessels of all sizes, and are characterized by fibrinoid necrosis of all portions of the vessel walls, with infiltration by inflammatory cells. In the earliest forms of arteritis, occurring after large doses of mycoplasmas, some of the vessels exhibit fibrinoid necrosis with little inflammation; lesions developing later show milder degrees of necrosis and a

more conspicuous cellular infiltrate principally composed of plasma cells and lymphocytes. Some of the arteries undergo thrombosis, and infarction of the brain is occasionally observed.

The arterial lesions are strikingly similar to those seen in experimental serum sickness, although their distribution is obviously different. In serum sickness of rabbits, polyarteritis occurs most frequently in vessels of the heart, kidney, gastrointestinal tract, and pancreas; cerebral polyarteritis has not been described. The glomerular lesions in the turkey disease are also morphologically similar to the glomerulonephritis associated with serum sickness. However, the lesions of serum sickness appear to differ with respect to the role of polymorphonuclear leukocytes as the mediators of damage to blood vessels. It has been shown by other investigators that leukocytic infiltration occurs in the walls of arteries before the appearance of necrotizing lesions, and measures which eliminate polymorphonuclear cells from the circulating blood provide protection against arterial damage (16). In contrast, the earliest arterial lesions in the turkey disease consist of fibrinoid necrosis involving all elements of the vessel walls, with little or no leukocytic infiltration. It is not until several days later that dense accumulations of cells, chiefly mononuclear leukocytes and plasma cells, appear in the arteries. The development of severe arterial necrosis without leukocytic infiltration was particularly striking in the birds treated with methotrexate.

These differences aside, there are other reasons to doubt that an immunologic mechanism analogous to that of serum sickness is implicated in the arteritis caused by *M. gallisepticum*. The early onset of the arteritis argues against hypersensitivity as a basis. The situation cannot be explained by assuming the presence of a large amount of antigen to which the birds are already hypersensitive, with arterial depositions of antigen-antibody complexes. The turkey poults are from uninfected stock, without evidence of prior contact with this mycoplasma and lacking demonstrable antibody in their serum. It was demonstrated that the mycoplasmas must be alive if arteritis is to occur; no lesions are produced by organisms killed by heating, freeze-thaw disruption, or by exposure to specific rabbit antibody. Moreover, the protection afforded by tetracycline and gold thiomalate indicates that the lesions are more likely the result of infection than a reaction of hypersensitivity. The failure to alter the onset, course, or mortality with large doses of methotrexate, 6-mercaptopurine, or cortisone is compatible with this view (17). The conspicuous infiltration of arterial walls by plasma cells in the later stages of polyarteritis suggests the involvement of an immunologic mechanism at this time, but this probably represents a secondary reaction, either to mycoplasma antigens or to products of tissue injury.

There are a number of other infectious agents known to be capable of producing arteritis. A filterable agent, believed to be a virus, has been demonstrated to cause infectious polyarteritis in horses, affecting chiefly the visceral

arteries (18). Necrotizing arteritis has been described in rickettsial infections (19). Angiitis has been reported in association with granulomatous lesions of various tissues in bovine pleuropneumonia (20), and also in other PPLO infections of animals and chick embryos (21).

However, a special difficulty arises if one attempts to account for the effects of *M. gallisepticum* in terms of an infectious process. How are the neurotoxic reactions to this organism to be dissociated from its infectious properties, or can the two be dissociated? When concentrated suspensions are injected, in doses large enough to produce acute neurological reactions and death within an hour or 2, it is clear that some sort of a toxin is involved. The time seems too short for invasion and multiplication to occur. Yet, the organisms must be alive to be toxic, and tetracycline therapy affords complete protection. Moreover, by injecting progressively smaller doses, the latent interval before the appearance of neurological signs becomes correspondingly longer, and one can arrange for any incubation period ranging from a few hours to as long as 14 days by appropriately adjusting the dose of mycoplasmas. It is, in fact, impossible to determine any point in time at which intoxication ceases and infection takes over as the cause of death. The brains of birds dying within the first few hours do not exhibit any histologic abnormality, while those from birds surviving for longer than 1 day show progressively increasing degrees of necrotizing arteritis, but these differences do not necessarily signify different mechanisms. Since the only histologic evidence of brain damage, even after many days, is in association with blood vessels, it is a reasonable assumption that the initial neurotoxic effect of large doses is mediated through vascular damage; possibly either spasm or increased permeability. The latter mechanism is suggested by the observation that accumulation of fibrinoid, presumably representing plasma proteins, in the adventitia is often one of the conspicuous early manifestations of arteritis.

Indirect evidence that the toxin acts directly on blood vessels, by way of the blood stream, was obtained in experiments in which mycoplasmas were injected directly into the brain. The number of organisms required to cause acute neurological reactions and death was less when injected into the wing vein than when given intracerebrally, and the interval between injection and the onset of disease was longer with the latter route of injection.

If the early deaths caused by large doses, and the lesions of cerebral arteritis observed several days later with smaller doses, are both due to a direct toxic action of *M. gallisepticum*, the absence of a fixed incubation period (or the changeability of the latent interval depending on the dose of mycoplasmas) can be accounted for by three possible explanations: (a) a certain threshold population of mycoplasmas must be reached before damage to the arteries can occur, in which case the longer latent period after smaller doses is explainable by the time needed for replication; (b) the toxic action on arteries is inflicted entirely at the time of intravenous injection, and the issue of early or

late death is determined by the total toxin dose in the same sense as with other microbial toxins; or (c) the injected mycoplasmas continue to produce a toxin after becoming established in the host, perhaps in the walls of the arteries, and the cumulative effects of the toxin are determined by the number of organisms remaining viable. The first possibility is not supported by evidence of progressive multiplication of mycoplasmas to a recognizable threshold population in the brains of turkeys sampled at various intervals after infection. Although in general the brains contained larger numbers of organisms at the time of death than in the intervening periods of apparent good health, the differences were not great. Admittedly, the methods for quantitating mycoplasmas in homogenates of whole brain may not yield meaningful information about the numbers affecting the arteries. The second possibility is unlikely in view of the protective action of tetracycline and gold thiomalate, and especially in view of the demonstrated reversal of the disease by tetracycline when the latter was given 4 days after infection. It is more likely that the acute lethal neurotoxicity produced by large doses, as well as the more protracted illness associated with polyarteritis following smaller doses, are the result of a toxic property continually manifested by living mycoplasmas. Thus, 10^{11} organisms may be postulated to have available enough toxin to cause death within an hour or so, while 10^9 organisms produce enough during a 4 day period to cause arterial damage and death at the end of this time, and 10^8 require 7 days or longer for the same outcome. Whether toxin is produced by organisms lodged within the vessel walls, or reaches the arteries from other sites, cannot be stated. The failure to demonstrate organisms by immunofluorescence suggests that if mycoplasmas are present they are not aggregated in collections large enough to be visualized.

Suspensions of nonviable mycoplasmas, killed by heating at 50°C for 1 hr or disrupted by repeated cycles of freezing and thawing, were devoid of neurotoxicity as well as the capacity to cause arteritis. It is probable that the inactivation observed with specific rabbit antibody is on the same basis, in view of the considerable reduction in viable organisms observed after incubation in the presence of antibody. No soluble toxin could be demonstrated in broth cultures after removal of the mycoplasmas by centrifugation, even when very large amounts of the supernatant fluid were injected.

The nature and mode of action of the toxin remain to be determined. Turkey erythrocytes have been shown to be agglutinated by *M. gallisepticum* (10), and the affinity between these organisms and the surface of turkey cells appears to be determined by sialic acid receptors on the host cells (11). Erythrocytes to which mycoplasmas have become attached can be shown to be damaged by hydrogen peroxide, which this organism produces without an associated catalase (12). It is possible that attachment of mycoplasmas to cell surfaces in cerebral blood vessels may be a factor in neurotoxicity. Studies of the problem by electron microscopy are in progress.

The lack of clear differentiation between the toxic and infectious properties of *M. gallisepticum* makes this a microbe of unusual interest. Other pathogens with recognizable toxins do not present this problem. With influenza virus, for example, or certain of the rickettsiae, lethal toxicity can be demonstrated by the injection of very large doses (it is of interest that these also require the presence of intact, viable organisms), but the toxic reactions seem to be clearly dissociated, both in the time of their occurrence and in their clinical manifestations, from the disease caused by infection. Rickettsial toxin, if given in a large enough dose, will kill mice within 18 hr or less, but if the dose is below the threshold for toxicity, so that the animals survive for the 1st day, a fixed asymptomatic incubation period of several days always intervenes before the signs of rickettsial infection appear (13).

In certain respects the toxic properties of *M. gallisepticum* for the turkey resemble those of *M. neurolyticum*, the agent causing "rolling disease" in mice (7). In both, neurotoxicity is associated with the intact, viable organism, although there is also a filterable "exotoxin" demonstrable in broth cultures of *M. neurolyticum*. In both, toxic properties are eliminated by heating at 50°C. With *M. neurolyticum*, we have found that intravenous injections in mice are much more lethal than injections into the brain. Extensive bilateral lesions of cerebral infarction have been observed in the hemispheres of mice which survive rolling disease for 24 hr or longer, but arteritis does not occur (14). It is possible that this agent may also exert selective effects on the arteries of the brain, perhaps by inducing intense arterial constriction.

The mechanism by which gold thiomalate protects turkeys against both the acute neurotoxicity and the development of polyarteritis nodosa is not clear, although the therapeutic effect of gold is uniform and striking. The results are comparable with those observed by Sabin and Warren (8), and Findlay et al. (15), with mycoplasmas causing arthritis in mice and rats, and by Marmion and Goodburn (9) with *M. pneumoniae* in hamsters and chick embryos. There is little evidence for a mycoplasmacidal action of gold in vivo, although a slight reduction in the number of viable mycoplasmas in brain homogenates was observed.

Various neurologic syndromes associated with polyarteritis of cerebral vessels have been described in human beings (22), with lesions comparable to those observed in turkeys. No evidence exists for the implication of mycoplasmas in such cases, or in other forms of polyarteritis in man. Nevertheless, the possibility must be given consideration as a speculative alternative to the widely held view that such lesions are necessarily based on reactions of hypersensitivity.

SUMMARY

Turkey poults injected intravenously with suspensions of *Mycoplasma gallisepticum* develop a fatal neurologic disease associated with polyarteritis affect-

ing almost exclusively the cerebral arteries. The incubation period depends on the dose of organisms. With high doses (10^{10} to 10^{11} mycoplasmas) the birds become ill and die within a few hours; with lower doses (10^6 to 10^8) neurologic manifestations appear after 7 days. The rapid onset of neurologic signs after high doses indicates the presence of a toxin in the mycoplasma, but efforts to extract toxin from disrupted organisms or to demonstrate its presence in culture fluid free of mycoplasmas have been unsuccessful. The toxin appears to be associated only with living mycoplasmas.

The toxic component of *M. gallisepticum* is inactivated by heating the organisms at 50°C, disruption by repeated cycles of freezing and thawing, and exposure to specific antibody.

Treatment of turkeys with gold thiomalate furnishes partial protection against the toxic effects of large doses of mycoplasmas, and protection against the development of cerebral arteritis. Treatment with tetracycline protects completely against both toxicity and arteritis, and, when delayed, restores diseased birds to a healthy state. Cortisone, methotrexate and 6-mercaptopurine have no effect on the course or outcome of the disease.

Intracerebral injections of *M. gallisepticum* are less toxic and lethal than when the same dose was given by vein, indicating that the organism exerts its damaging action on blood vessels by way of the blood stream.

The arterial lesions resemble those of serum sickness, except for their distribution, and are associated with glomerular inflammatory lesions. However, for various reasons discussed, it is considered more likely that they result from a direct toxic action of living mycoplasmas on the vessels concerned than from an immunologic mechanism.

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EXPLANATION OF PLATES

All sections were stained with hematoxylin and eosin.

PLATE 81

FIG. 1. Meningeal artery of a turkey which died 96 hr after intravenous injection of 5×10^9 mycoplasmas. Fibrinoid necrosis of the vessel wall is present, with only minimal leukocyte infiltration. $\times$ 400.

FIG. 2. Intracerebral artery from a bird which died 8 days after injection of 5×10^8 mycoplasmas. In addition to fibrinoid necrosis of the media, moderate perivascular infiltration by mononuclear cells and plasma cells is present. $\times$ 750.
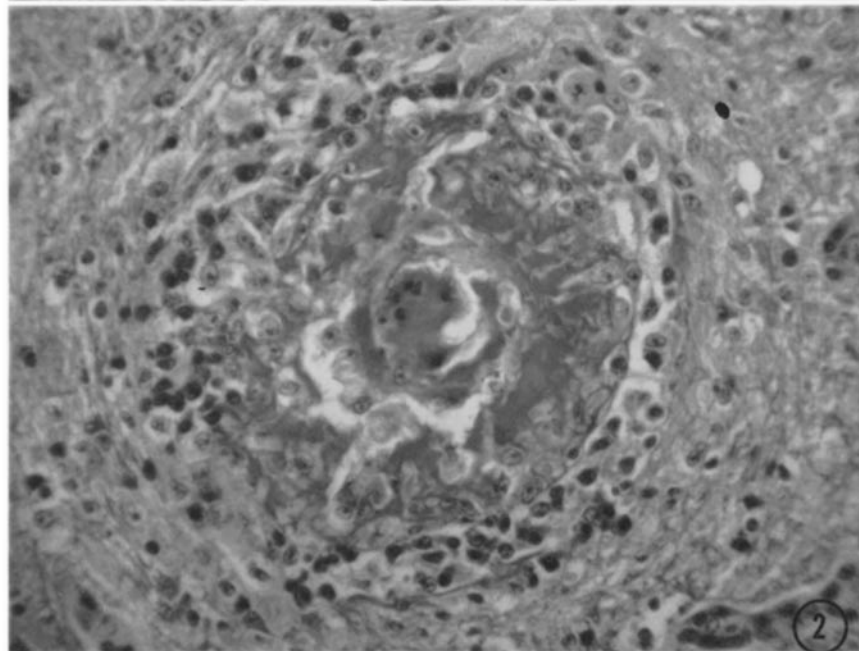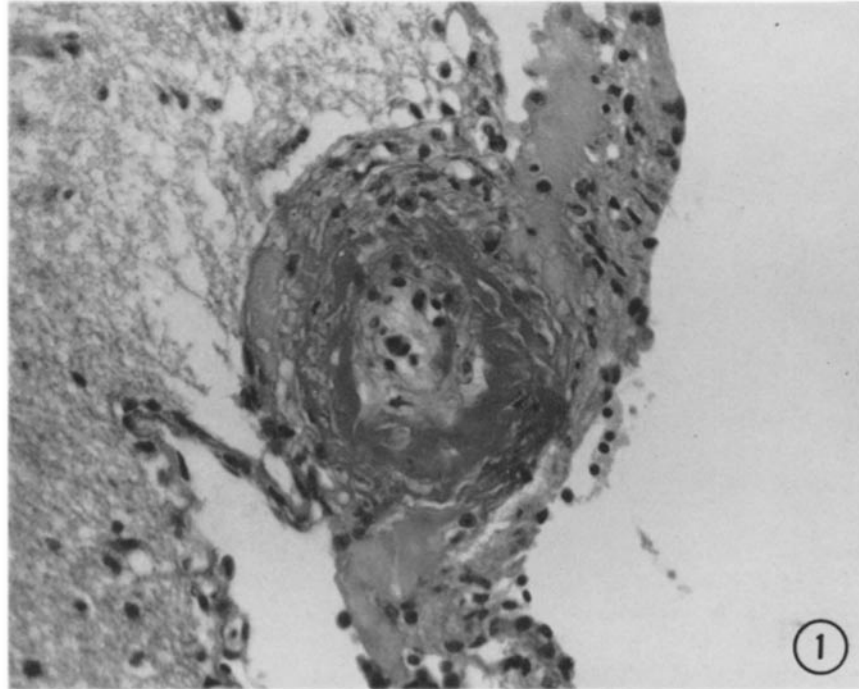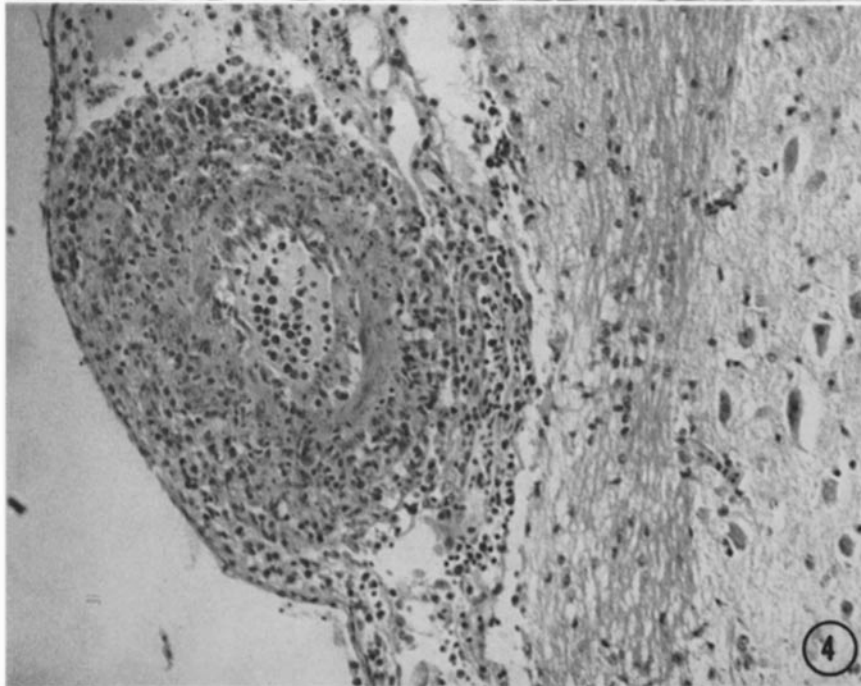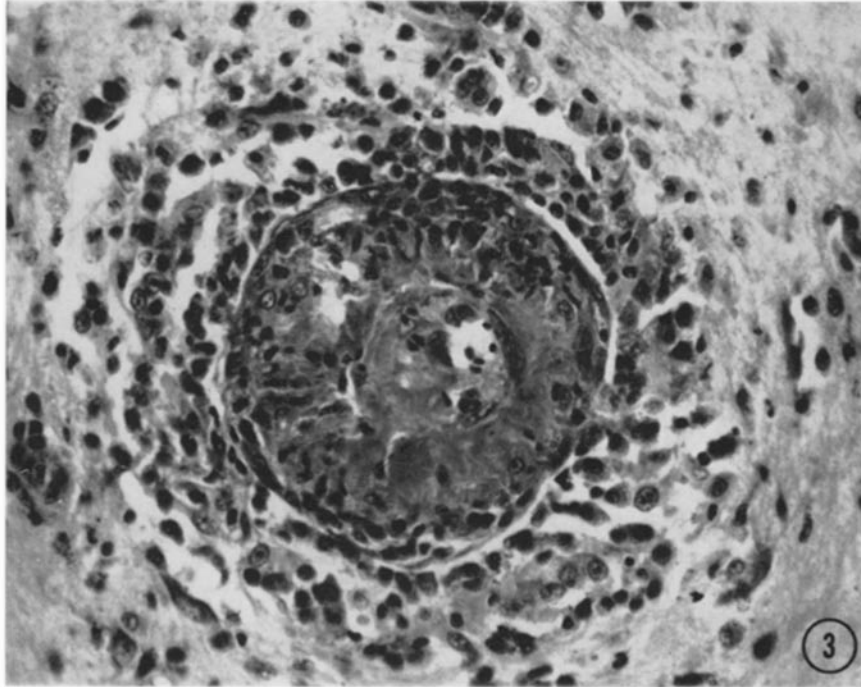
(Thomas et al.: Studies of PPLO infection. I)

PLATE 82

FIG. 3. Intracerebral artery from a turkey treated with tetracycline for 4 days after the onset of neurological signs, with apparent clinical recovery; killed on the 12th day. Complete necrosis of the vessel wall with marked leukocytic accumulation. × 650.

FIG. 4. Meningeal artery from a turkey killed 14 days after an injection of 10^7 mycoplasmas. Intense leukocyte infiltration throughout the entire wall is present with necrosis of the media and fragmentation of leukocytes. × 300.
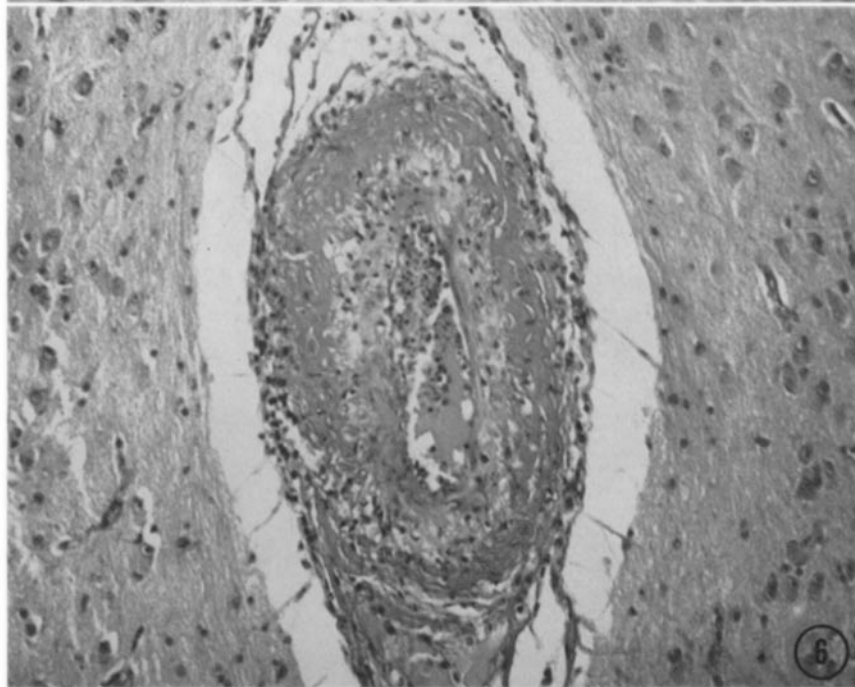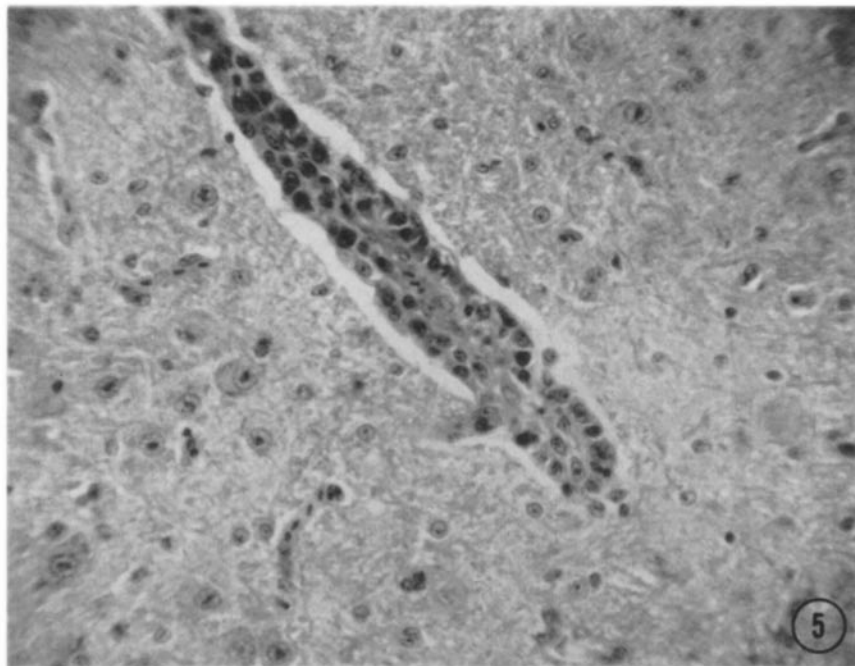
(Thomas et al.: Studies of PPLO infection. I)

PLATE 83

FIG. 5. Small intracerebral vessel from a bird which died 8 days after an injection of 4.5×10^{10} mycoplasmas. The vessel is surrounded by mononuclear cells and plasma cells. $\times$ 700.

FIG. 6. Medium sized intracerebral artery from a bird killed 8 days after injection of 10^{10} mycoplasmas. The most striking change is accumulation of fibrinoid material in the adventitia. $\times$ 350.
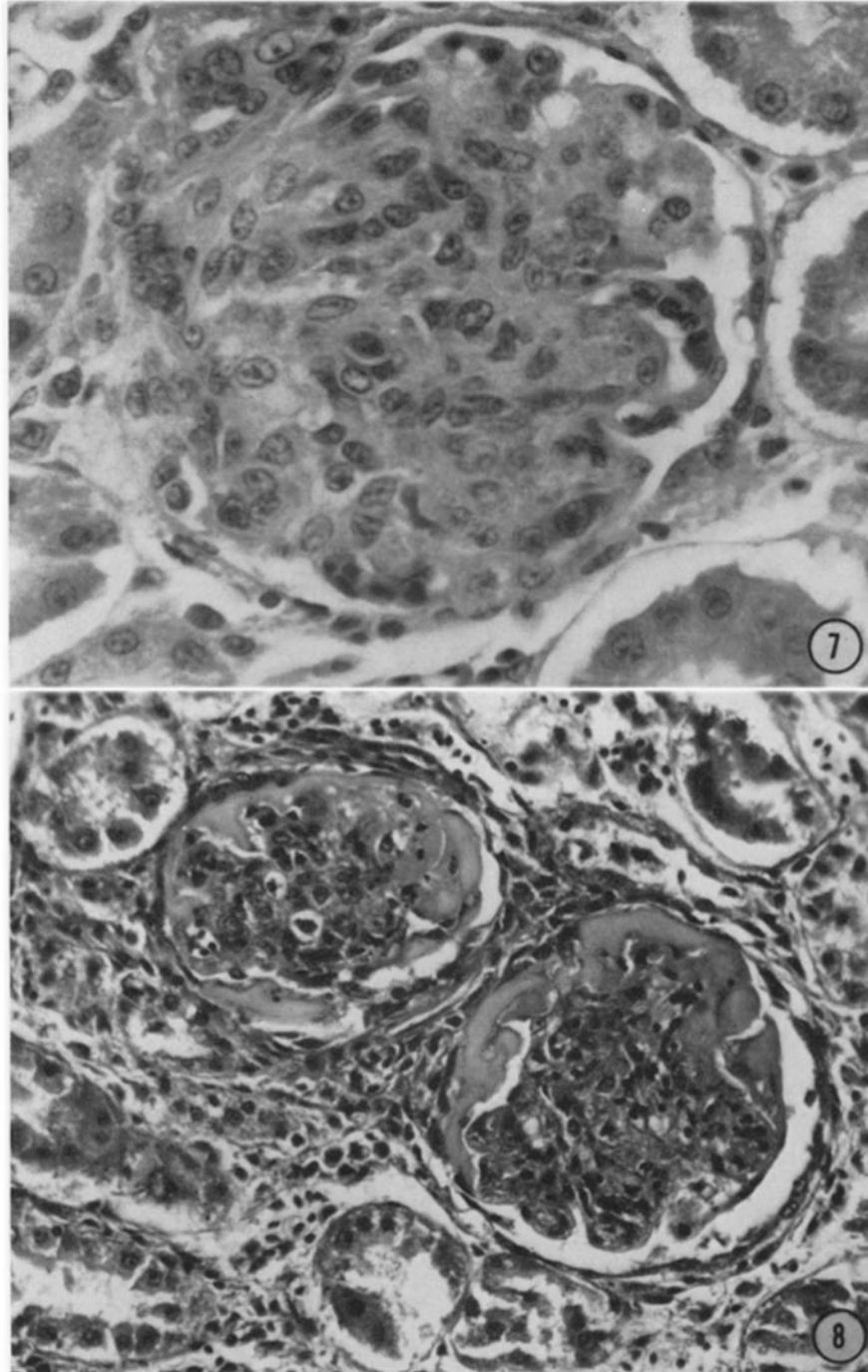
(Thomas et al.: Studies of PPLO infection. I)

PLATE 84

FIG. 7. Glomerulus from a turkey killed 8 days after injection of 10^9 mycoplasmas. The glomerulus is enlarged and shows swelling and proliferation of intracapillary cells. $\times$ 950.

FIG. 8. Glomeruli from a turkey which died 7 days after an injection of 10^9 mycoplasmas. In addition to some hypercellularity deposits of fibrin are seen in Bowman's space and in peripheral capillary loops. $\times$ 400.

(Thomas et al.: Studies of PPLO infection. I)