Spirochete from the Rabbit Tick, *Haemaphysalis leporispalustris* (Packard)

I. Isolation and Preliminary Characterization

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An unidentified spirochete, referred to as the 277F agent, was isolated from *Haemaphysalis leporispalustris* ticks from two cottontail rabbits by inoculation of the tick suspension into embryonated chicken eggs. Because of its minute width (0.1 μ), the organism was difficult to see when stained by the Giemsa method, but was readily demonstrated by silver impregnation or fluorescent-antibody procedures. In dark-field microscopy, the spirochetes appeared uniformly and rather tightly coiled, and exhibited typical corkscrewlike motility. After yolk-sac inoculation, the agent was highly lethal for chick embryos and was recovered in large quantity from several embryonic tissues and fluids. It could also be maintained in nonfertile eggs and in an enriched liquid medium. This previously undescribed spirochete could pass through Berkefeld N but not Seitz EK filters. It was relatively resistant to heat and to penicillin or sulfadiazine, but was markedly inhibited by streptomycin, broad-spectrum antibiotics, and homologous neutralizing antiserum. Of several species of animals tested for susceptibility to this spirochete, only the snowshoe hare gave evidence of infection.

During a study planned for isolating the Rocky Mountain spotted fever agent, *Rickettsia rickettsii*, from the rabbit tick, *Haemaphysalis leporispalustris*, pools of ticks collected from cottontail rabbits (*Sylvilagus nuttallii*) were routinely triturated and inoculated into embryonated chicken eggs (10). In one instance, tick suspension yielded an agent highly lethal for chick embryos but without apparent effect on any of several laboratory animals tested. Structures resembling bacteria or rickettsiae were not seen in yolk-sac smears, and the agent was not cultivable in any of several commercial bacteriological media. These characteristics, plus infectiousness of some filtrates, suggested that the agent was viral in nature, and it was considered such until spirochete-like bodies were observed by phase microscopy of infected chorioallantoic fluid. Dark-field microscopy, fluorescent-antibody staining, and electron microscopy confirmed that they were spirochetes of extremely small width. All studies, both before and after the first observation of the spirochete, indicated that this was the only agent involved and that it was present in the original isolation.

The present communication reports isolation

and preliminary characterization of this heretofore undescribed microorganism, which we refer to as the 277F agent.

MATERIALS AND METHODS

The pool of *Haemaphysalis leporispalustris* from which the 277F agent was isolated consisted of 85 adult and 40 nymphal ticks collected from two cottontail rabbits taken in the Bitterroot Valley of western Montana. The ticks were soaked for 30 min in 1:1,000 aqueous Merthiolate, rinsed three times in saline, and triturated with mortar and pestle in 10 ml of saline containing 1,200 units of penicillin per ml. The suspension was injected into yolk sacs of eight 5-day-old chick embryos, six of which died 3 to 7 days later. The agent responsible for death could be maintained thereafter by routine yolk-sac passage.

For serial passage in embryonated eggs and for inoculation into animals, yolk sac and other embryonic tissues were homogenized with a Waring Blender to an initial concentration of 10% (w/v) in 0.42% NaCl buffered at pH 7.4 with 0.067 M phosphate buffer. Further dilutions, where specified, were made in the same diluent. Seed material to be frozen was prepared by similarly homogenizing tissues to 50% concentration in sterile skim milk, pH 7.4. For routine yolk-sac inoculation, 5- or 6-day-old embryos were given 0.5 ml of suspension. In titrations, 10 eggs were inoculated with each logarithmic dilution.

Inoculated eggs were incubated at 34.5 C and were

¹ Deceased 27 November 1966.

candled daily. Embryos dying on the first 2 days were omitted. Chick embryo LD₅₀ of suspensions containing the agent were calculated by the method of Reed and Muench (12). Number of LD₅₀ per gram of infected tissue or unit volume of culture in liquid medium could then be computed. In some instances, the 50% death time (DT₅₀), rather than LD₅₀, was determined. The DT₅₀ signified the time postinoculation when half the embryos in a group were dead. Interpolation was made when necessary.

Culture of the 277F agent was attempted in various artificial media at 30, 34, or 36 C. The most satisfactory medium, designated no. 10, consisted of pooled embryonic fluids (chorioallantoic and amniotic) harvested from 12-day-old embryos and fortified with 0.2% Casamino Acids (Difco), 0.3% crystallized bovine plasma albumin (Armour and Co.), and 0.5% (v/v) of blood cells from 13-day-old chick embryos. Before addition of a sterile suspension of blood cells, the medium base was adjusted to pH 6.5 (found preferable to 7.0 or 7.5) with 1 N HCl, and was sterilized by passage through a sintered-glass filter. Blood cells added to the sterile base settled to the bottom and gradually lysed.

The 277F agent in artificial medium or in chick embryonic fluids was sometimes concentrated and partially purified by differential centrifugation to obtain suspensions more suitable for microscopy, especially electron microscopy. Preliminary centrifugation at 600 × *g* for 10 min in conical tubes in a horizontal rotor removed large particles of debris but relatively few spirochetes. Supernatant fluid was then centrifuged at 12,000 × *g* for 30 min in an angle rotor. The pellet was washed by resuspension in 0.01 M tris-(hydroxymethyl)aminomethane (Tris)-chloride, pH 7.4, with 0.2 M sucrose, and was centrifuged again at 12,000 × *g* for 30 min.

Morphology and motility of the 277F agent were studied by dark-field microscopy of infected embryonic fluids or artificial medium; by microscopy of fixed smears of concentrates or of yolk-sac tissues impregnated with silver stain according to Krajan's method (7); and by fluorescent-antibody staining by a method (2) using fluorescein isothiocyanate conjugates prepared from anti-277F hamster serum. Electron microscopy, to a limited extent, was also used to ascertain morphological appearance of the agent. For this purpose, drained pellets from concentrated, partially purified preparations of spirochetes were resuspended in a mixture of equal parts of 0.2 M sucrose in 0.01 M Tris-chloride buffer, pH 7.4, and negative stain. The latter consisted of 3% phosphotungstic acid adjusted to pH 6.8 with 1 N NaOH. Immediately after mixing, portions of suspension were dispensed to Formvar-coated specimen grids. After 30 sec, the aqueous phase was removed and the specimens were examined after drying.

Animals examined for susceptibility to the 277F agent included suckling and 21-day-old Swiss mice, hamsters, guinea pigs, domestic rabbits, varying hare (*Lepus americanus*), chicken, pheasants, and pigeons. Depending on species tested, the intraperitoneal (ip), subcutaneous (sc), intracranial, intravenous (iv), or intranasal routes were used for injection of infected yolk-sac material.

To determine effect of heat on the 277F agent, portions of 1% suspension of infected yolk sac containing 10^{6.4} chick-embryo LD₅₀ per ml were submerged for 30 min in water at 23, 40, 45, 50, 55, and 60 C. After exposure, the suspensions were immediately cooled to 23 C and each was inoculated into the yolk sacs of 40 embryonated eggs to determine DT₅₀. In addition, a portion of the original 1% yolk-sac suspension was stored at room temperature (21 to 27

FIG. 1. Accumulation of 277F granules in Macchiavello-stained smear of infected yolk-sac tissues. × 1,700.

C) for 24 hr and, at intervals, a sample was removed for passage to embryonated eggs.

Effects of penicillin, sodium sulfadiazine, streptomycin, and chloramphenicol were investigated by treating inoculated embryonated eggs with each of these agents administered via the yolk sac or chorioallantoic cavity. In one experiment, the drugs were added to the diluent used for titrating infected yolk-sac material.

Guinea pig and hamster antisera were tested for complement-fixing (CF) antibody against 277F and control antigens prepared by a method of ether extraction (14). For neutralization tests, antiserum was prepared in New Hampshire red roosters by the method of Hilleman, Haig, and Helmold (6). Each bird received 32 ip inoculations of yolk-sac suspension containing $4 \times 10^{7.5}$ chick embryo LD₅₀ per dose, at 3- to 4-day intervals. Serum was collected 2 weeks after the final inoculation.

RESULTS

Morphology. The first morphological entity that appeared to be associated with the 277F agent consisted of groups of basophilic granules seen in Macchiavello-stained smears of infected yolk sacs (Fig. 1). These granules were spherical, 300 to 500 μ m in diameter, and were observed frequently but not consistently in tissues of the third and succeeding egg passages. Although more frequently seen in yolk sacs, they were also detectable in chorioallantoic fluid. They occurred both intra- and extracellularly, singly and in clusters. Hence, they resembled the elementary bodies of *Chlamydia* species (9) but were less uniform in size, frequently larger, and not always accompanied by developmental structures comparable to chla-

mydial inclusions. Evidence of serological relationship between this agent and chlamydiae also was lacking.

When chorioallantoic fluid or washings of yolk-sac fragments from infected embryos were examined by dark-field microscopy, numerous spirochete-like organisms were seen. They were usually 6 to 12 μ long, but occasionally up to 30 μ , and were extremely thin. They appeared uniformly and rather tightly coiled from end to end, lacked a distinct hook at either end, and exhibited typical corkscrew-type motility. In silver-impregnated and fluorescent antibody-treated smears, the spirochete-like morphology of the 277F agent was again clearly seen (Fig. 2-5) and division forms, as indicated by binary fission of spirochetes, were regularly observed.

An outstanding morphological feature was a granule-like protuberance at one end or anywhere along the length of many spirochetes. Sometimes a single granule would appear to have multiple spirochetes attached to or emanating from it. Isolated granules were detected wherever spirochetes were present, but were especially abundant in yolk-sac tissues from dead embryos.

By fluorescent microscopy, the antigenic similarity between granules and spirochetes could readily be demonstrated, since the two morphological forms reacted identically with labeled immune serum (Fig. 2-4). Brilliantly stained granules were seen both intra- and extracellularly, occasionally as completely spherical bodies (Fig. 3) but more often as spherules elongating into short, curved rods (Fig. 4). These rods usually

FIG. 2. Fluorescent antibody-stained 277F spirochetes in smear of infected yolk-sac tissues. $\times 1,300$.

FIG. 3. *Fluorescent antibody-stained 277F antigen in yolk-sac tissues of dead chick embryos. $\times 1,300$.*

FIG. 4. *Fluorescent antibody-stained 277F antigen in form of spherical bodies elongating into curved rods. $\times 2,700$.*

projected outward from the spherules but sometimes were seen curving around them and lying in close proximity with their periphery. Fluorescent staining reactions were highly specific and did not occur with heterologous conjugates or after treatment of smears with unlabeled 277F antiserum.

Electron microscopy of negatively stained preparations added final confirmation to the typical

spirochetal morphology of the 277F agent. Individual organisms, uniformly about 0.1μ in width but varying tremendously in length, consisted of numerous spirals (Fig. 6).

Related to its minute width is the filterability of the 277F spirochete. Two portions of infected yolk-sac suspension, partially clarified by centrifugation, were passed through separate Berkefeld

FIG. 5. 277F spirochetes and numerous granules in silver-impregnated smear of infected yolk-sac tissues. $\times 2,700$.

FIG. 6. Electron micrograph of a single 277F spirochete in semipurified concentrate of a culture in medium no. 10. $\times 15,000$.

N filters, and part of each filtrate was inoculated into 20 embryonated eggs. By the 12th day, all embryos were dead. Yolk sacs passed from each group on the 8th day killed all embryos within 6 days, and spirochetes were regularly found in embryonic fluids. Repetition of this experiment with Seitz EK filters yielded completely different

results; embryos of eggs inoculated with these filtrates remained alive, as did those in two serial blind passages of yolk sac.

Behavior of the 277F agent in chicken eggs. Since its original isolation, the 277F agent has been maintained through 52 yolk-sac passages in which dilution 10^{-1} or 10^{-2} served as inoculum.

At all passage levels, the death pattern was nearly the same and DT_{50} was reached in approximately the same interval after inoculation. Composite results indicate that the agent manifested near maximal virulence for chick embryos immediately after isolation and did not require a long period of adaptation. Embryos seldom died before the 4th day and were essentially all dead by the 5th or 6th day. However, in other studies, life of embryos could be extended to 12 days by decreasing the dose of inoculum, for example, to 10^{-9} dilution of infected yolk sac.

Inoculation of the 277F spirochete into yolk sac was followed by its migration into all parts of the embryonated egg. Infectious titer of chorioallantoic membrane was often equal to that of yolk sac, whereas that of embryo was usually about one-tenth as high. Highly infectious embryonic fluids, both chorioallantoic and amniotic, could also be prepared by inoculating 8-day-old embryonated eggs via the chorioallantoic sac; in this case embryos survived longer.

Since the relatively clear embryonic fluids were ideal for visual detection of spirochetes by dark-field illumination, embryonated eggs were always preferred for 277F culture. At first they were considered essential, but later studies demonstrated that multiplication of the agent is not dependent on presence of the embryo and its developing auxiliary tissues. In one experiment, the organisms were transferred four times through nonfertile eggs by passage of yolk fluid; that used in the final passage was lethal to all embryos inoculated with 10^{-7} dilution, the highest tested. This dilution represented 10^{-43} dilution of the original inoculum obtained from embryonated eggs.

The number of spirochetes seen in embryonic fluids increased markedly when these fluids were incubated at 30 C for 3 to 5 days after harvest from eggs.

Behavior of the 277F agent in artificial media. The 277F agent was successfully maintained by continuous passage in medium no. 10. Chick blood cells, although not essential, enhanced growth of the spirochetes and appeared desirable for continuous maintenance through serial passages. The effect of certain other ingredients added to embryonic fluid was investigated to a limited extent. These included rabbit serum and blood cells, Tween 80, yolk fluid from fresh eggs, and crystallized egg albumin. None yielded growth more luxuriant than that obtained with medium no. 10. Likewise, Eagle's Minimum Essential Medium (4) was not satisfactory as a substitute for normal embryonic fluid in medium no. 10.

Cultures were incubated at 30 C in screw-cap tubes; growth also occurred at 34 and 36 C but was less abundant. When cultures were routinely

diluted 10^{-3} between passages, near maximal populations were achieved in 3 days and passages could be made at such intervals. After the organisms were cultured thus for five passages, a portion of the final suspension (representing 10^{-19} dilution of the original inoculum from eggs) was titrated in embryonated eggs; the LD_{50} was $10^{-9.8}$, and spirochetes were still detectable in fluids of eggs inoculated with 10^{-9} dilution.

Behavior of the 277F agent in laboratory animals. Attempts to establish the 277F agent in a host other than the chick embryo were generally unsuccessful. The animals and birds previously mentioned, in groups of 4 to 10 individuals, were given massive doses (e.g., 10^7 to 10^9 chick embryo LD_{50}) by various routes; at appropriate intervals thereafter, blind passages were made of tissues selected according to the route of inoculation. Only the hare showed significant evidence of susceptibility. A single animal was given 10 chick embryo LD_{50} by the iv route. On days 7 and 10, blood was drawn for passage to embryonated eggs. Five serial yolk sac passages were made and, in all beyond the first, the DT_{50} occurred between 4 and 11 days. However, death patterns were always somewhat atypical of 277F, and spirochetes were found in embryonic fluids only with difficulty.

Stability in various aqueous solutions. Morphology of the spirochetes was relatively stable in pooled embryonic fluids at 30 C, since typical, motile organisms were observed for as long as 3 weeks in this environment. In 4 weeks, however, disintegration was evident. Removal of spirochetes from this fluid by centrifugation and resuspension in a variety of solutions revealed that they are very fragile. They quickly lysed in distilled water. In solutions of NaCl varying from 0.15 to 0.5 M, or in 0.05 M $MgCl_2$, they were more stable but lysed within 1 hr. Addition of phosphate buffer did not benefit; i.e., in the exact buffered saline routinely used as diluent in 277F titrations, spirochetes rapidly disappeared or became atypical. Their morphology was maintained longer, for at least 6 hr, in 0.01 M Tris-chloride buffer, pH 7.4, with 0.2 M sucrose. They were somewhat less stable when the sucrose concentration was reduced to 0.1 M and markedly less stable with 0.03 M.

Effect of temperature. After exposure of infected yolk sac suspensions to 23, 40, 45, and 50 C, as previously described, the DT_{50} of inoculated embryos was 4.2, 4.3, 4.9, and 6.5 days, respectively, and spirochetes were found in eggs from all four groups. The DT_{50} was never attained with suspensions exposed to 55 and 60 C, and attempts on the 8th and 13th days to recover the agent by yolk-sac passage were unsuccessful. Resistance of

the 277F agent to a temperature of 50 C, but not of 55 C, was thus indicated.

At room temperature, the agent appeared relatively stable, although a gradual reduction in infectivity was evident over a 24-hr period. The DT_{50} obtained with a zero-hour sample (control) was 4.3 days. After 2, 4, 9, 14, and 24 hr of storage at 21 to 27 C, it increased 2, 6, 13, 14, and 16%, respectively.

We have repeatedly observed that the infectivity of yolk-sac suspension in skim milk can be maintained for many months at -70 C. When a partially purified suspension of spirochetes in medium no. 10 was stored at this temperature for 24 hr and then thawed, the number and morphology of spirochetes remained essentially unchanged. Thus, in spite of their fragility under many circumstances, they appeared resistant to mechanical damage by freezing and thawing.

Effect of chemotherapeutic agents. Such agents

TABLE 1. *Chemotherapy of 277F infection in embryonated eggs treated via the chorioallantoic cavity*

Drug	Dose/egg	Infection-to-treatment interval hr	Deviation in DT_{50} ^a
Penicillin . . .	1,250 units	0.5	6% Decrease ^b
Sodium sulfadiazine	5 mg	0	5% Decrease
Streptomycin	5 mg	48	29% Increase
Chloramphenicol	0.5 mg	24	98% Increase

^a DT_{50} = 50% death time. Each group contained 40 eggs.

^b Average of two separate determinations.

were first administered via the chorioallantoic cavity to eggs that had received 10^6 LD₅₀ of 277F via the yolk sac. Only streptomycin and chloramphenicol (Table 1), particularly the latter, lengthened survival of the embryos. Similar results were obtained in another experiment in which drugs were administered via the yolk sac immediately after inoculation of eggs with the 277F agent (Table 2). Resistance to penicillin and sulfadiazine, but not to streptomycin and the broad-spectrum antibiotic, chlortetracycline, was indicated. Doses of streptomycin less than 0.5 mg per egg were relatively ineffective. Larger amounts protected almost all embryos; in those that died, spirochetal infection was readily demonstrated.

In an additional experiment, three titrations of 1% infected yolk-sac suspension were performed simultaneously in embryonated eggs. Buffered saline was used as diluent throughout, but for the second titration, 500 units of penicillin and 400 µg of streptomycin per ml were added; for the third titration, 1,500 units of penicillin and 1,200 µg of streptomycin per ml were added. The LD₅₀ values obtained were: $10^{-6.8}$, $10^{-4.9}$, and $10^{-3.7}$, respectively. Gradual reduction of titers with increasing concentration of these antibiotics was probably owing to the effect of streptomycin.

Serological studies. Complement-fixation and neutralization tests were conducted before the spirochetal nature of the 277F agent was recognized. The results indicated that CF antibodies were produced in guinea pigs and hamsters and that the agent was neutralized by hyperimmune rooster serum.

Of several guinea pigs inoculated ip, or sc and ip, with infected yolk-sac suspensions, two produced sera without normal yolk-sac antibody and were thus useful for CF tests. Titers obtained with these sera are shown in Table 3. Antigens and antisera representing two rickettsiae and a chlamydia were included for specificity control. Ho-

TABLE 2. *Chemotherapy of 277F infection in embryonated eggs treated via the yolk sac*

Drugs	Dose/egg	DT_{50} ^a days	Deviation in DT_{50}
Penicillin G	1,000 units	5.5	6% Decrease
Sodium sulfadiazine	2 mg	5.1	2% Increase
Streptomycin	0.5 mg	10.5	94% Increase
Chlortetracycline	1 mg	>12	>122% Increase
Control	—	5.4	—
Streptomycin	0.25 mg	7.3	20% Increase
Streptomycin	0.5 mg	11.7	92% Increase
Streptomycin	1 mg	>12	>97% Increase
Streptomycin	2 mg	>12	>97% Increase
Control	—	6.1	—

^a DT_{50} = 50% death time. Each group contained 35 eggs.

TABLE 3. Complement fixation between antigens and antisera prepared with 277F agent and other microorganisms

Antigen	Reciprocal of serum dilution giving complete fixation					
	277F		Psittacosis	Rocky Mt. spotted fever	Q fever	Normal guinea pig
	Serum A	Serum B				
277F (1)	24	16	0	0	0	0
277F (2)	64	16	0	0	0	0
277F (3)	64	12	0	0	0	0
Psittacosis (1)	0	0	192	16	0	0
Psittacosis (2)	0	0	384	16	0	0
Psittacosis (3)	0	0	256	0	0	0
Rocky Mt. spotted fever	0	0	0	256	0	0
Q fever	0	0	0	0	1,024	0

TABLE 4. Neutralization of 277F agent by homologous and heterologous antisera

Antiserum	Chick embryo LD ₅₀ (negative log) of 277F	
	8th day	10th day
277F.....	1.1	3.7
Ornithosis.....	6.3	7.7
Bovine encephalomyelitis....	6.1	6.9
Normal yolk sac.....	6.8	7.4

mologous titers were lower in the 277F system than in the others, but the reactions appeared to be specific. Evidence of antigenic similarity between 277F and the other agents was completely absent.

In neutralization tests, suspensions of 277F-infected yolk sac were titrated in the presence of homologous antiserum prepared in New Hampshire roosters; titrations in the presence of similarly prepared antisera against the agents of psittacosis and bovine encephalomyelitis and against normal yolk sac were included. Whole serum and logarithmic dilutions of yolk sac were mixed and incubated at 23 C for 30 min and at 4 C for 18 hr prior to inoculation into embryonated eggs. On either the 8th or 10th day after inoculation, neutralization by the 277F antiserum, but not by the others, was clearly evident (Table 4).

DISCUSSION

At this laboratory, thousands of ixodid ticks from a variety of host animals, including rabbits and hares, have been injected into embryonated eggs for isolation of rickettsiae or other microorganisms; yet, to our knowledge, records do not exist which would indicate previous isolation of an organism similar to the 277F spirochete. Steinhilber and Hughes (13) did recover an unidentified spirochete from chicken eggs but, in their study, eggs were inoculated with liver tissue from hens rather than with ticks. Although their spirochete was much wider (width 0.4 to 0.6 μ) than the 277F agent, they also described granules in the cytoplasm of yolk-sac cells in conjunction with the spirochete.

Whether the 277F agent originated from *Haemaphysalis leporispalustris* injected into embryonated eggs or from the eggs themselves cannot be stated with certainty. If its origin from ticks is assumed, the question remains whether it existed as a contaminant on the exterior, was a permanent inhabitant of the interior, or was occurring temporarily in one or several ticks after being ingested with blood of a rabbit on which the ticks were feeding. Origin from the interior of the ticks is supported by the following considerations.

(i) The eggs were obtained from flocks of healthy hens owned by a single commercial concern. Eggs from these flocks have been used in many different studies at this laboratory, without evidence of naturally occurring microbial contaminants in them.

(ii) The rapidity with which the 277F agent adapted itself to embryonated eggs and its high lethality for embryos suggest that it would not readily have been missed in other usages of eggs from the source cited, if it were occurring frequently in the eggs.

(iii) In the study in which the 277F agent was isolated, 24 pools of ticks, consisting of 1,667 individuals, were tested but only one yielded the isolate. If its occurrence as an external tick contaminant had been widespread, repeated isolation would have been expected, since the same procedure for exterior sterilization of ticks was used throughout the study.

(iv) Reports by other investigators (1) emphasize the fact that latent infections are rarely found in eggs.

The apparent resistance to 277F of all animals tested, except the hare, seems noteworthy in view of the fact that the agent was originally isolated from ticks taken from cottontail rabbits. The combined findings suggest that the agent might be a parasite of lagomorphs and that

the ticks, in this instance, were merely mechanical vectors.

Ever since spirochetes were first recognized and described as the etiological agents of syphilis and relapsing fevers, their life cycle has been the subject of intensive investigations. In general, two hypotheses exist concerning multiplication of these organisms. One considers transverse division or binary fission as the only means of reproduction, whereas the other postulates an additional, rather complex developmental cycle consisting of the production of "granules" from which, by elongation, new spirochetes may develop. The voluminous literature regarding this controversial issue has been reviewed by several authors for the louse- and tick-borne relapsing fever agents (5, 8), as well as for other spirochetal organisms (3, 11). The present consensus is that spirochetes in general do not have a complex life cycle but multiply by binary fission only.

Pillot et al. (11) investigated the development of species of *Treponema*, *Borrelia*, and *Leptospira* by various techniques, including electron microscopy. According to them, spherical and granular formations similar to those described for the 277F agent represent localized detachments of the spirochetal envelope or the products of spirochetal autolysis, and they do not constitute resistant forms or elements of an obligatory cycle. Although this hypothesis is not questioned by the present authors, certain observations suggest that the above-described granular forms of the 277F agent may still be infectious and capable of producing typical spirochetes. Such observations include disappearance of spirochetes from cultures before loss of infectivity during heat treatment; high infectivity of Berkefeld filtrates, even though relatively few spirochetes pass the filters; high infectivity of embryonic fluids, even though spirochetes cannot be found until after several days of incubation at 30 C; and lysis of spirochetes in buffered saline, the diluent that has been satisfactorily used in many titrations of infectivity.

Electron microscopic studies are being continued to determine in greater detail the morphology of the 277F agent and the relationship of the "atypical" granules to spirochetes; findings will be reported later.

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