

Isolation and Antigenic Characteristics of Axial Filaments from the Reiter Treponeme

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Received for publication 18 November 1974

Axial filaments were isolated and purified from Reiter treponemes after detergent solubilization of the cells' outer envelope. The axial filaments were separated from the spirochetal cells by shearing, purified by density gradient centrifugation, and fragmented by ultrasonication. Acrylamide gel electrophoresis of dissociated filaments revealed two major protein bands. Gel diffusion precipitin tests and immunoelectrophoresis between a purified axial filament suspension and anti-Reiter treponeme serum gave a single precipitin line. Checkerboard complement fixation tests also gave results consistent with a single antigen-antibody system. Tests with immune sera to other cultivable spirochetes were positive with some and negative with others. In addition, strongly positive reactions were obtained in complement fixation and precipitin tests with sera from rabbits and humans with syphilis and other treponematoses. However, both serological tests gave reactions of partial identity between the antigen(s) of Reiter treponeme axial filaments and those of the pathogenic treponemes. It was concluded from these studies that the axial filaments were probably the cellular locus of the so-called "Reiter protein" antigen of syphilis serology.

Immunological studies of syphilis have always been handicapped by the lack of well-defined treponemal antigens. The inability to culture pathogenic treponemes *in vitro* has prevented the acquisition of *Treponema pallidum* in sufficient quantity to permit exploratory immunochemical studies. In efforts to circumvent this difficulty, nonpathogenic cultivable treponemes have been investigated, either for the purpose of identifying and extracting antigenic components shared with pathogens or as models for the development of isolation procedures adaptable to the small quantities of pathogenic treponemes that can be recovered from infected tissues. Thus, D'Alessandro et al. (7), working with the Reiter treponeme, extracted and partially characterized a protein component that reacted with syphilitic serum. However, despite attempts by a number of investigators to extract and identify this antigen, it has never been obtained in an unquestionably pure form.

In earlier studies from this laboratory, a method was developed for the isolation and purification of a soluble polysaccharide from the ghosts of lysed Reiter treponemes (22). Although this substance was highly antigenic, it did not react with syphilitic serum and the isolation procedure was not adaptable to extraction of a comparable antigen from small quan-

ties of pathogenic treponemes. Subsequent studies proved unsuccessful in recovering other soluble antigens from cultivable treponemes, and because of this we recently began to investigate particulate treponemal antigens, especially those associated with specific cellular organelles. This paper describes the isolation, purification, and serological activity of Reiter treponeme axial filaments—the spirochetal analogues of bacterial flagella.

MATERIALS AND METHODS

Reiter treponeme. This organism has been maintained as a stock culture in our laboratory for many years. It is one of a small group of spirochetes reputed to be cultivable, avirulent strains of *T. pallidum*. The Reiter treponeme, like the other members of this group, has a very obscure pedigree and it seems likely that the claims for its origin are incorrect. It is probably one of the indigenous spirochetes of the human alimentary tract. Moureau (20) concluded from morphological and biochemical considerations that the Reiter treponeme was a strain of *T. refringens*. In several recent publications (1, 25), this spirochete has been designated *T. phagedenis*, but no evidence to justify the use of this terminology has been presented. Also, the Reiter treponeme morphology is markedly different from that of the organism originally named *Spirochaeta phagedenis* by Noguchi (23). In view of this rather confused status, we feel it is inappropriate to use a species designation at this time. Instead, in this paper we shall continue to use

the strain name that workers in this field have used for more than 30 years.

The Reiter treponeme was grown in Spirolate broth (Bioquest) supplemented with normal rabbit serum (10%, vol/vol). For bulk cultures, screw-cap bottles were filled to within 1 cm of the lip after inoculation and then sealed; the inoculum for each bottle was a log-phase culture approximately 0.1% of the total volume. Bottle cultures were stirred continuously by a magnetic mixer during incubation at 35 C for 3 days. The final yield was 2×10^9 to 5×10^9 cells/ml.

Electron microscopy. All steps in the preparation of axial filament suspensions were monitored by electron microscopic examination of negatively stained samples. Specimens to be examined were mounted on grids with carbon-coated Formvar films and stained with either 1% potassium phosphotungstate or 1% neutral ammonium molybdate. The preparations were examined with an AEI EM6B microscope at 60-kV accelerating voltage.

Immune sera. Antisera to the Reiter treponeme and other cultivable spirochetes were prepared as previously described (22). Antisera to pathogenic treponemes were pooled bleedings from rabbits infected with one of the pathogens or individual bleedings from humans with early infectious syphilis.

Protein determinations. These were performed by the Folin-Ciocalteu procedure on trichloroacetic acid precipitates as described by Chase and Williams (6). Crystalline bovine serum albumin, standardized by Kjeldahl nitrogen determination, was used as the reference.

CF tests. The procedure described by Osler et al. (24) for complement fixation (CF) tests with 5 CH₅₀ units of guinea pig complement was modified for use in a microtiter system. The volume of each reagent was: 25 μ liters of antibody dilution; 50 μ liters of complement; 25 μ liters of antigen dilution; and 25 μ liters of standardized suspension of sensitized sheep erythrocytes.

Gel diffusion precipitin reactions. Wells punched in films of 1% agarose on microscope slides were filled with reactants and incubated in a moist chamber at room temperature until precipitin lines had fully developed. The gels then were soaked for 3 days in phosphate-buffered saline, stained with Buffalo black, decolorized in 5% acetic acid, and air dried.

Immuno-electrophoresis. One percent agarose in barbital buffer, pH 8.6, 0.1 μ ionic strength, was used for electrophoresis of the antigen. Current was applied for 1.5 h at 2 mA per slide. After serum was added to the troughs, the slides were stored in a moist chamber at room temperature until lines developed.

Acrylamide gel electrophoresis. Gel electrophoresis with 10% acrylamide in 0.129 M phosphate buffer (pH 7.2) and 0.1% sodium dodecyl sulfate was carried out according to the method of Weber and Osborn (26). Protein bands were revealed by staining with Coomassie blue.

RESULTS

Treponeme cells were collected by centrifugation at $2,000 \times g$ and washed once in 0.15 M NaCl solution containing 0.01 M CaCl₂ and

MgCl₂. The washed cells were resuspended in deionized water to a volume of approximately 1% of the original culture and lysed by alkaline shock, pH 11.6, as previously described (22). Cell ghosts were removed from the lysate (fraction A) by sedimentation ($2,500 \times g$ for 60 min) and washed twice in saline. In experiments where separation of fraction A and the treponemal outer envelope was not desired, this initial lytic step was omitted.

The outer envelope of the cells was removed by detergent "solubilization" (15). Cells were suspended in 0.03% aqueous sodium dodecyl sulfate, incubated at room temperature for 30 min, and then sedimented by centrifugation in a Beckman Spinco L3-50 ultracentrifuge at $17,400 \times g$ for 45 min (rotor 42.1). After aspiration of the supernatant containing the outer envelope fraction, the cell bodies were resuspended in saline and sedimented again to remove any remnants of the detached envelope.

Electron microscopic examination of cells at this point revealed most of the axial filaments still attached at their basal body end but no longer wrapped around the cell. The filaments were broken from the cells by shearing in a blender (Sorvall Omnimixer) for 2 min. The cells were removed by centrifugation at $30,900 \times g$ for 30 min, and the blending step was repeated several times, as long as electron microscopic examination of the supernatant fluid revealed a high concentration of detached filaments. The crude filament suspensions were pooled, and after addition of sodium dodecyl sarcosinate (Sarkosyl NL-97; Geigy) to 0.2% the fibrils were sedimented by centrifugation at $94,600 \times g$ for 60 min.

The filament pellets were resuspended in a small volume of saline and evenly dispersed by placing the suspension for several minutes in an ultrasonic cleaning bath (Branson H10). The suspension was then mixed with CsCl solution to yield a 26% (wt/wt) solution of the salt. After addition of Sarkosyl to 0.2% the mixture was centrifuged in a swinging bucket rotor (SW 41) at $150,000 \times g$ for 45 to 48 h. Under these conditions the filaments formed a sharply defined, opaque band at a density of approximately 1.289 g/cm³; this was clearly separated from several lighter bands of contaminating material. After aspiration of the various fractions, the filament band was collected and diluted in water, and the filaments were resedimented at $94,600 \times g$ for 60 min and finally resuspended in saline to the desired concentration. Electron microscopic examination of such preparations revealed a dense aggregation of uniformly sized filaments free of any recognizable contaminating material (Fig. 1a). Moreover,

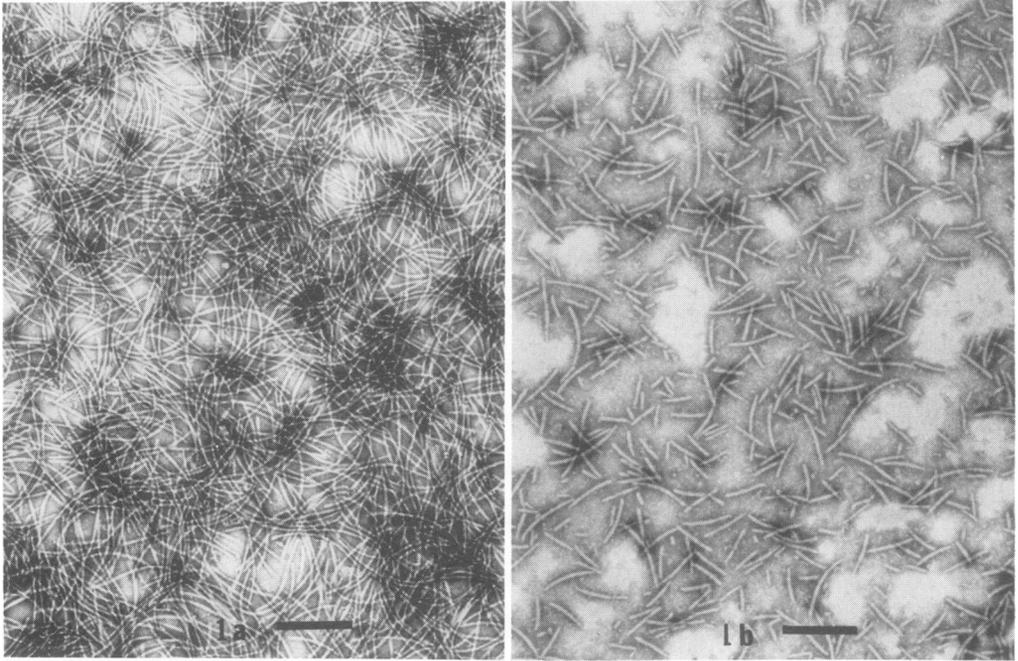


FIG. 1. Purified Reiter treponeme axial filaments negatively stained with 1% potassium phosphotungstate before (a) and after (b) ultrasonic fragmentation. Calibration bar represents 400 nm.

neither basal bodies nor the adjacent hooked portion of the filaments (13) were observed. The filaments had an average diameter of 18.5 nm and were therefore of a size compatible with the sheathed forms described by Hovind Hougen and Birch-Anderson (13). They were completely soluble at low pH.

When purified filaments were subjected to acrylamide gel electrophoresis, two major, closely migrating protein bands were observed (Fig. 2). In addition, there was a third, barely detectable band that migrated somewhat faster than the other two. Under the conditions of this study, it was not possible to ascertain whether this was a contaminant or a minor filament component.

For immunological studies, filaments were chopped into small pieces in order to increase the number of antigenic particles and to promote diffusion through gels. Chopping was achieved by prolonged (up to 15 min) intermittent ultrasonication with a microprobe cell disruptor (model W410; Heat Systems-Ultrasonics, Inc.). The resulting fragments varied in length (Fig. 1b); they ranged from 20 to 400 nm with a mean length of 190 nm. When these were tested against whole Reiter treponeme antiserum by gel diffusion precipitins and immunoelectrophoresis, a single precipitin line devel-

oped (Fig. 3a, 4). However, the immunoelectrophoretic precipitin line was long and trailing, indicative of heterogeneity in either charge or size (Fig. 4). Further evidence for the antigenic purity of the filament preparation was obtained from precipitin reactions with sera of rabbits immunized with purified filaments in Freund adjuvant. Here again only a single line developed.

Chopped filaments were also examined by gel diffusion precipitins with antisera to a variety of other spirochetes. Reactions were obtained not only with antisera to the closely related Kazan and PK strains, but also with that of the FM strain of *T. microdentium* and the N-9 strain of *Borrelia vincentii* as well (Fig. 3a-d). Quite surprisingly, strong lines were also obtained with sera from rabbits infected with various pathogenic treponemes—*T. pallidum*, *T. pertenue*, and *T. cuniculi* (Fig. 3c, e). When antisera to cultivable and pathogenic treponemes were in adjacent wells, the resulting precipitin lines occasionally developed spurs at the point of junction (Fig. 3c). Sera from a small group of humans with early syphilitic infection were also examined and all but one gave a strong precipitin line upon overnight incubation. The exception was from an individual treated for a primary infection 10 days before bleeding, and

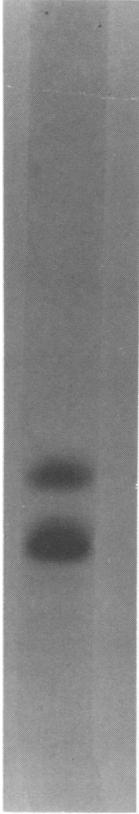


FIG. 2. Acrylamide gel electrophoresis, pH 7.2, of axial filaments after dissociation with sodium dodecyl sulfate and mercaptoethanol.

even this serum was found to give a very faint reaction when the slide was subsequently washed and stained with Buffalo black (Fig. 3f).

To obtain quantitative information on antigenicity, purified filaments were assayed by checkerboard block CF titrations. These were performed with both homologous, i.e., Reiter treponeme immune serum, and with serum from syphilitic rabbits and humans. The results of two such blocks are recorded in Table 1. It will be noted that an antigen concentration giving optimal CF was obtained with both types of serum and that antigen concentrations below this level gave little or no CF, a feature typical of a single antigen-antibody system (18). However, it will also be observed that in the homologous system optimal fixation occurred with only 6.0 ng of filament protein per reaction mixture, whereas in the cross-reactive system four times as much antigen was required. This clearly indicated that the two systems were not identical.

DISCUSSION

Studies of some of the antigenic characteristics of axial filaments from the Reiter treponeme have been described in this paper. Because these are not surface structures like flagella, their bacterial counterparts, axial filament antigens could not be investigated by serological reactions with intact cells, as in the case of H agglutination of eubacteria. For this reason we undertook to separate the axial filaments from spirochetal cells and to obtain them in suspension free of other spirochetal antigens. The success of our efforts was evident in the single precipitin line that developed in Ouchterlony reactions between axial filament preparations and antiserum to whole Reiter treponemes. Similarly, a single precipitin line was also formed in immunoelectrophoresis with the same reagents. However, in this situation, the precipitin line was quite extended, indicating variation in migration distance of different antigen particles. Since the antigen was particulate, and the particles were heterogeneous in size, variation in electrophoretic migration through a gelled medium was not surprising.

Further evidence for antigen purity was obtained by checkerboard CF reactions. In these, maximum CF (i.e., highest serum titer) in Reiter treponeme antiserum occurred with 6.0 ng of antigen protein per reaction mixture (240 ng/ml), but with half this amount of antigen little or no CF occurred. Such a sharp cut-off in antigen titer is characteristic of a single antigen-antibody system (17).

In contrast to the immunological evidence for a single antigen, acrylamide gel electrophoresis revealed the presence of two major proteins and a trace amount of a third that could possibly have been a non-filament contaminant. For this examination, however, the filaments were solubilized by strong detergent treatment and reductive cleavage. Therefore, the finding of two proteins by electrophoresis is not incompatible with antigenic purity as indicated immunologically. Even if the protein subunits possessed different antigenic ligands, they would have behaved as a single antigen serologically in the polymerized filamentous form used in the immunological studies.

The acrylamide electrophoretic procedure was that described by Weber and Osborn (26) for protein molecular weight determinations. Although it was not our original goal to determine molecular weights, calculations based on the formula of Weber and Osborn were made, and these indicated that the protein subunits were in the range of 15,000 to 25,000 daltons.

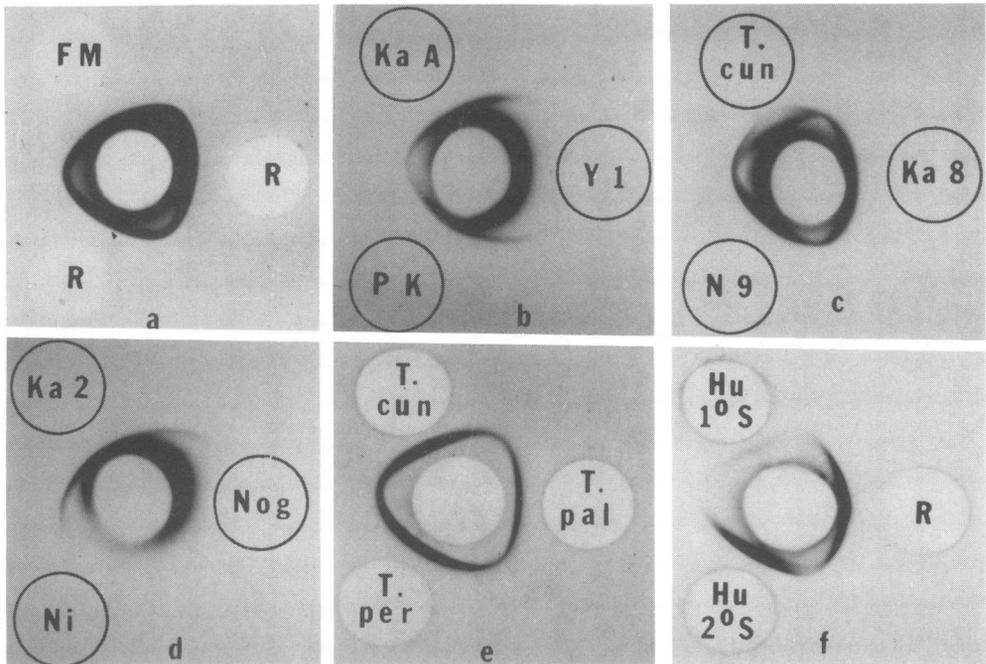


FIG. 3. Gel diffusion precipitin reactions with axial filament fragments and various immune sera. Filament fragments were in the center well and sera in the outer wells of each group. The spirochetes against which the immune sera were prepared are as follows. (a) FM, *T. microdentium*, strain FM; R, Reiter treponeme. (b) Ka A, Kazan A; Y1, small oral treponeme, strain Y1; PK, oral treponeme strain PK 1. (c) T. cun, *T. cuniculi*, strain A; Ka 8, Kazan 8; N9, *B. vincentii*, strain N9. (d) Ka 2, Kazan 2; Nog, cultivable *T. pallidum*, strain Noguchi; Ni, cultivable *T. pallidum*, strain Nichols. (e) T. pal, *T. pallidum*, strain Nichols; T. per, *T. pertenue*, strain Haiti B. (f) Hu 1°S, serum from human with primary syphilis; Hu 2°S, serum from human with secondary syphilis.

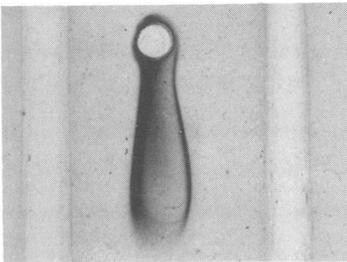


FIG. 4. Immunoelectrophoresis of axial filament fragments in agarose-pH 8.6 barbital buffer. Anti-Reiter treponeme serum in lower trough, syphilitic rabbit serum in upper trough.

The finding of two major protein bands for Reiter treponeme axial filaments was in agreement with observations on two other anaerobic spirochetes, *S. stenostrepta* (16) and *T. zuelzeræ* (4), but differed from numerous studies on bacterial flagella of various species that have all yielded a single protein band (2, 17, 19, 21). In contrast to both, the single study of leptospiral filaments produced six protein bands (21). In all of the anaerobic spirochetes

studied thus far, the axial filaments have been found to consist of a filamentous core surrounded by a readily distinguishable sheath (3, 10-14, 16). Bacterial flagella, on the other hand, have been sheathed in some species and unsheathed in others, and even when sheathed this outer covering has sometimes been lost in the course of flagellar purification (17). Such substructural differences could explain the electrophoretic findings in these organelles. In the present study the diameter of the purified axial filaments was compatible with retention of the sheath (13), although this covering was not actually identified.

The antigenic specificity of the filaments was examined with immune sera to both cultivable and pathogenic spirochetes. Strongly positive reactions were obtained not only with sera against the closely related Kazan strains and the oral isolate, PK 1, but also with two distinctly different spirochetes, the FM strain of *T. microdentium* and N-9, a *B. vincentii* strain. On the other hand, there were negative results with sera against the small oral treponeme, Y-1, and the large treponemes, the Nichols and

Noguchi strains of so-called cultivable, avirulent *T. pallidum*.

Most surprising were the strong reactions given by sera from rabbits and humans with treponemal infections. No differences were observed between sera from *T. pallidum*, *T. pertenuae*, and *T. cuniculi* infections. However, when such sera were tested in checkerboard CF reactions, the antigen titer was significantly less than that obtained with homologous immune serum (or that of closely related organisms). Instead of the 6.0 ng of filament protein per reaction found with the latter, 24 ng per reaction was required to give maximum CF with sera from treponeme-infected hosts. The explanation for this was revealed by Ouchterlony precipitins in which the two types of sera were in adjacent wells. Spur formation at the point where the precipitin lines from the two sera joined (Fig. 3c) indicated a reaction of partial identity by the pathogenic treponeme antibodies. This finding bolstered the argument for different antigens on the two filament protein subunits and further suggested that only one was shared by the cultivable and pathogenic organisms. This possibility is currently under investigation.

As noted in the Introduction, the Reiter

treponeme has long been known to share a protein antigen with the pathogenic treponemes. However, heretofore this antigen has not been recovered in a pure form and no evidence has been presented concerning its location within the cell. It has usually been separated from spirochetal cells by cryolysis, and it has always been recovered in a macromolecular form that is readily sedimentable in the ultracentrifuge (5). These are the same conditions under which axial filaments could be freed, though inefficiently, and sedimented. Although the protein nature of the filament antigen(s) was not established in the present study, it seems reasonable to assume that this is the case. It is therefore probable that the "Reiter protein" and the axial filament antigen are the same.

In the past the Reiter protein has been used extensively in CF tests for the serological diagnosis of treponemal infections. Its lack of use today is due in part to the cumbersome nature of CF reactions, in part to lack of sensitivity in antibody detection, and in part to nonspecific results arising from the impure status of available antigen preparations. With increasing recognition that the FTA-ABS test does not possess the specificity previously claimed (8, 9). There

TABLE 1. Two-dimensional complement fixation tests with Reiter treponeme axial filaments versus anti-Reiter and human syphilitic serum

Antigen protein (ng)	Serum dilution ^a									
	10	20	40	80	160	320	640	1,280	2,560	Buffer control
Anti-Reiter serum										
384	0	0	0	0	0	0	0	3	4	4
192	0	0	0	0	0	0	0	4	4	4
96	0	0	0	0	0	0	0	4	4	4
48	0	0	0	0	0	0	0	4	4	4
24	0	0	0	0	0	0	0	3	4	4
12	0	0	0	0	0	0	0	2	4	4
6	0	0	0	0	0	0	0	2	4	4
3	0	1	2	3	3	4	4	4	4	4
Buffer control	4	4	4	4	4	4	4	4	4	
Human serum-secondary syphilis										
384	0	0	4	4	4	4	4	4	4	4
192	0	0	0	4	4	4	4	4	4	4
96	0	0	0	4	4	4	4	4	4	4
48	0	0	0	0	4	4	4	4	4	4
24	0	0	0	0	1	4	4	4	4	4
12	4	1	1	2	3	4	4	4	4	4
6	4	4	4	4	4	4	4	4	4	4
Buffer control	4	4	4	4	4	4	4	4	4	

^a Expressed as reciprocal. Conditions were: serum, 25 μliters; complement, 5 CH₅₀/50 μliters; antigen, 25 μliters; fixation, 18 h, 4 C; sensitized cells, 25 μliters; lysis, 60 min at 37 C. 0 indicates no lysis; 4 indicates complete lysis.

may still be a place in syphilis serology for a Reiter protein test, providing the antigen is pure and the test is more simple than CF. The filament preparation described in this paper fulfills the first of these criteria. Its application, either in native form or as protein subunits, to hemagglutination and other simple but sensitive serological tests may fulfill the second.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service research grant E-2336 from The National Institute of Allergy and Infectious Diseases and by The World Health Organization.

LITERATURE CITED

- Abramson, I. J., and R. M. Smibert. 1971. Inhibition of growth of treponemes by antimicrobial agents. *Br. J. Vener. Dis.* **47**:407-412.
- Ada, G. L., G. J. V. Nossal, J. Pye, and A. Abbot. 1964. Antigens in immunity. I. Preparation and properties of flagellar antigens from *Salmonella adelaide*. *Aust. J. Exp. Biol. Med. Sci.* **42**:267-282.
- Bharier, M. A., F. A. Eiserling, and S. C. Rittenberg. 1971. Electron microscopic observations on the structure of *Treponema zuelzeriae* and its axial filaments. *J. Bacteriol.* **105**:413-421.
- Bharier, M. A., and S. C. Rittenberg. 1971. Chemistry of axial filaments of *Treponema zuelzeriae*. *J. Bacteriol.* **105**:422-429.
- Cannefax, G. R. 1963. Reiter protein antigen. I. Relationship of some physical and chemical characteristics to serological activity. *Br. J. Vener. Dis.* **39**:121-127.
- Chase, M. W., and C. A. Williams. 1968. Chemical analyses. A. Protein analysis, p. 273-275. In C. A. Williams and M. W. Chase (ed.), *Methods in immunology and immunochemistry*, vol. 2. Academic Press Inc., New York.
- D'Alessandro, G., F. G. Oddo, R. Comes, and L. Dardanoni. 1949. Sulla struttura antigene del *T. pallidum*. Ricerche sullo stiptite coltivabile di Reiter. *Riv. Ist. Sieroterap. Ital.* **24**:134-166.
- Department of Health, Education and Welfare. 1973. Technical problems with FTA-ABS test for syphilis. CDC Morbidity and Mortality Weekly Report **22**:102, 107.
- Hardy, P. H., Jr., and E. E. Nell. 1972. A study of the properties of Sorbent, the reagent employed in the fluorescent treponemal antibody-absorption test. *Am. J. Epidemiol.* **96**:141-152.
- Hovind Hougen, K. 1972. Further observations on the ultrastructure of *Treponema pallidum* Nichols. *Acta Pathol. Microbiol. Scand. Sect. B* **80**:297-304.
- Hovind Hougen, K. 1974. The ultrastructure of cultivable treponemes. 1. *Treponema phagedenis*, *Treponema vincentii* and *Treponema refringens*. *Acta Pathol. Microbiol. Scand. Sect. B* **82**:329-344.
- Hovind Hougen, K. 1974. The ultrastructure of cultivable treponemes. 2. *Treponema calligyrum*, *Treponema minutum* and *Treponema microdentium*. *Acta Pathol. Microbiol. Scand. Sect. B* **82**:495-507.
- Hovind Hougen, K., and A. Birch-Anderson. 1971. Electron microscopy of endoflagella and microtubules in *Treponema* Reiter. *Acta Pathol. Microbiol. Scand. Sect. B* **79**:37-50.
- Jackson, S., and S. H. Black. 1971. Ultrastructure of *Treponema pallidum* Nichols following lysis by physical and chemical methods. II. Axial filaments. *Arch. Microbiol.* **76**:325-340.
- Johnson, R. C., M. S. Wachter, and D. M. Ritz. 1973. Treponeme outer envelope: solubilization and reaggregation. *Infect. Immun.* **7**:249-258.
- Joseph, R., and E. Canale-Parola. 1972. Axial fibrils of anaerobic spirochetes: ultrastructure and chemical characteristics. *Arch. Microbiol.* **81**:146-168.
- Joys, T. M. 1968. The structure of flagella and the genetic control of flagellation in Eubacteriales. A review. *Antonie van Leeuwenhoek J. Microbiol. Serol.* **34**:205-225.
- Kabat, E. A., and M. M. Mayer. 1961. *Experimental immunochemistry*, p. 212-214. Charles C Thomas, Publishers, Springfield, Ill.
- Martinez, R. J., D. M. Brown, and A. N. Glayer. 1967. The formation of bacterial flagella. III. Characterization of the subunits of the flagella of *Bacillus subtilis* and *Spirillum serpens*. *J. Mol. Biol.* **28**:45-51.
- Moureau, M. 1955. Recherches biochimiques sur les tréponèmes anaérobies. I. *T. comandoni*, *T. phagedenis*, *T. refringens*, and *T. ambigua*. *Ann. Inst. Pasteur Paris* **88**:231-233.
- Nauman, R. K., S. C. Holt, and C. D. Cox. 1969. Purification, ultrastructure, and composition of axial filaments from *Leptospira*. *J. Bacteriol.* **98**:264-280.
- Nell, E. E., and P. H. Hardy, Jr. 1966. Studies on the chemical composition and immunologic properties of a polysaccharide from the Reiter treponeme. *Immunochemistry* **3**:233-245.
- Noguchi, H. 1912. Pure cultivation of *Spirochaeta phagedenis* (new species), a spiral organism found in phagedenic lesions on human external genitalia. *J. Exp. Med.* **16**:261-268.
- Osler, A. G., J. H. Strauss, and M. M. Mayer. 1952. Diagnostic complement fixation. I. A method. *Am. J. Syph. Gonorr. Vener. Dis.* **36**:2-15.
- Smibert, R. M. 1973. Spirochaetales, a review. *Crit. Rev. Microbiol.* **2**:491-552.
- Weber, K., and M. Osborn. 1969. The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. *J. Biol. Chem.* **244**:4406-4412.