TWO-DIMENSIONAL GEL ELECTROPHORESIS OF *IN VIVO* AND *IN VITRO* SYNTHESIZED PROTEINS, ANTIGENIC PROTEINS, AND CROSS-REACTIVE ANTIGENS IN *TREPONEMA PALLIDUM* SUBSP. *PALLIDUM* NICHOLS STRAIN AND *TREPONEMA PHAGEDENIS* BIOTYPE REITER

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DISSERTATION

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Two-dimensional electrophoretic protein profiles of *in vivo* and *in vitro* propagated *T. pallidum* subsps. *pallidum* Nichols strain were analyzed and compared. This comparative analysis revealed two *in vitro* synthesized, cytoplasmic cylinder-associated polypeptides with molecular masses 29.5 and 34.7 kDa, pI 5.62, and one *in vitro* "lost" polypeptide with molecular mass 34.7 kDa, pI 5.34. Integral membrane proteins of *in vitro* and *in vivo* propagated *T. pallidum* was identified by phase partitioning with the nonionic Triton X-114, and twelve outer membrane-associated, antigenic proteins were identified in western blots probed with pooled human secondary syphilitic sera. The solubilization of the outer membrane of *T. pallidum* with Triton X-114 were monitored by electron microscopy. Treatment of freshly harvested ^{35}S labeled *T. pallidum* with 1% Triton X-114 resulted in solubilization of the outer membrane and reduction of the diameter of the treponemes from 0.14 ± 0.02 μm to 0.095 ± 0.003 μm. Examination of thin sections of untreated organisms showed integrity of outer and cytoplasmic membranes. In contrast, thin sections of Triton X-114-treated treponemes showed integrity of the cytoplasmic membrane but the loss of the outer membrane. The cytoplasmic cylinders generated by detergent treatment retained their periplasmic flagella, as judged by electron microscopy and immunoblotting. Integral membrane proteins of *Treponema phagedenis* were also identified by phase partitioning with Triton X-114, and sixteen cross-reactive, outer membrane-associated, antigenic polypeptides were identified

in western blots probed with pooled human secondary syphilitic sera. The results of this study indicate that tow-dimensional protein profiles of *in vivo* and *in vitro* propagated *T. pallidum* are almost identical except for the differences mentioned. This results also indicate that 1% Triton X-114 selectively solubilizes the outer membrane, and the antigenic hydrophobic proteins present in the detergent phase are located exclusively in the outer membrane.

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CHAPTER I

INTRODUCTION

The Origin of Human Treponematoses. A close relationship between the four human treponematoses is suggested by their clinical and epidemiological characteristics. No treponemes of this group (except for that of the rabbit) is known other than in man, but the human treponemes, probably arose from an animal infection long ago (49). The long period presence of pinta suggests that it may have been the earliest human treponematoses (49). It may have been spread out by about 1,5000 B.C., being subsequently isolated in the Americas when the Bering Strait was flooded (49). About 10,000 B.C. in the Afro-Asian land mass, environmental conditions might have favored treponeme mutants leading to yaws. From yaws, about 7,000 B.C., in Southwest Asia endemic syphilis perhaps developed. Finaly, endemic syphilis gave rise to venereal syphilis about 3,000 B.C. in Southwest Asia as big cities developed there (49). Toward the end of the fifteenth century A.D. a further mutation may have resulted in a more severe venereal syphilis in Europe which, with European exploration and geographical expansion, was subsequently carried throughout the then treponemally uncommitted world (49).

Etiological Agents of Treponematoses. The four treponematoses in man are caused by organisms which are microscopically indistinguishable by present techniques. The appearance of the *Treponema pallidum* is typical of them. These four human treponematoses may be grouped according to the usual way they are transmitted as follows (49): one, nonvenereal (often called endemic) treponematoses; transmitted mostly among children. In this group are two subgroups. One consists of pinta, which has different clinical manifestations and a long period of infectiousness; its causal organism is

Treponema pallidum subsp. *carateum.* The other subgroup contains yaws and endemic syphilis. These, like pinta, are diseases of less developed rural populations and are usually contracted in childhood. The causal organisms of yaws and endemic syphilis are *Treponema pallidum* subsp. *pertenue* and *Treponema pallidum* subsp. *endemicum*, respectively. Two, venereal (sometimes called sporadic) treponematoses; transmitted mostly among adolescents and adults. The only subgroup in venereal treponematoses is venereal or sporadic syphilis, the venereal disease of urban populations in any climate. The bacterial origin of syphilis has been known since 1905 when the syphilis spirochete, *Treponema pallidum* was described as the etiological agent of this disease (141).

Morphology of *T. pallidum* subsp. *pallidum* Nichols Strain. The cells of *T. pallidum* are unicellular helical rods (147), 5 - 15 μm long and at the thickest point about 0.15 μm wide (72). Several studies have been done to determine if *T. pallidum* possesses a cell envelope. Zeigler and co-workers (171) used ruthenium red in their thin section studies of *T. pallidum* infected testies. Ruthanium red is known to enhance the electron density of acid mucopolysaccharides. Fitzgerald and co-workers (44) also published an electron microscope study of *T. pallidum* using ruthenium red. However, in both studies, useful controls such as stainig of infected tissue with no treponema, or noninfected tissue were not included, which made the interpretation of the electron micrographs somewhat difficult. Presently electron microscopy has not been able to provide convincing evidence for the presence of a capsule or slime layer on cells of *T. pallidum.*

Cross-sections of *T. pallidum* show that organisms are surrounded by a triple-layered outer membrane consisting of two dark layers with a narrow light gap in between. The two dark layers are of equal electron density and each is approximately 3 nm wide (72).

Cross-sections of *T. pallidum* also demonstrate a peptidoglycan layer as a dark linein close apposition to the outer leaflet of the cytoplasmic membrane. The indirect evidence for presence of such a layer comes from studies done by Hovind-Hougen (67, 69) where he demonstrated that *T. pallidum* cells treated with *Myxobacter* AL-1 protease I loose their helical shape, while the cells treated with sodium deoxycholate are only slightly influenced. It is known that protease I isolated from *Myxobacter* AL-1 splits the muramyl-alanine bonds of the peptidoglycan whereas sodium deoxycholate has no effect on these bonds (76). Further evidence for the presence and position of peptidoglycan layer is obtained from studies on other spirochetes. Joseph and co-workers isolated the peptidoglycan layer from *Spirochaeta stenosterepta* (76). They found that, when isolated, the layer maintained a helical shape, thus proving its shape-conferring role in the spirochete.

The electron micrographs of cytoplasmic matrix of *T. pallidum* demonstrate the presence of ribosomes, mesosomes, nuclear regions, and cytoplasmic tubules (69). A few vacuoles are sometimes observed in the cytoplasm of the organism and in general, with the exception of the presence of cytoplasmic tubules; the cytoplasm of *T. pallidum* appears to be similar to that of most Gram-negative bacteria (68).

The Disease Course of the Human Venereal Syphilis. The course of venereal syphilis in man might be taken as typical of that of treponematoses, because in it may occur all the manifestations of endemic syphilis and yaws (49). Syphilis in humans develops in stages. Stages with clinically characteristic symptoms alternate with latent stages in which virulent treponemes can destroy tissues in the infected body without clinical signs. Many years after an untreated infection, late manifestations can appear in blood vessels and the central nervous system which are then irreparable (108).

The treponeme enters the body through the mucosa or through a break in the epidermis. This penetration may take only a few minutes. Magnuson and co-workers

(88) calculated that the 50 percent infectious dose for humans was less than 57 treponemes. In his studies, as few as 10 treponemes produced a generalized infection which developed slowly. In contrast, Schöbl (144) reported that inoculation of two treponemes into animals caused infection. However, Magnuson (87) also suggests that in man one to two organisms may suffice to cause infection once they have passed through the epithelial layers of the skin or mucous membranes.

Where the treponemes enter the skin, they divide by transverse binary fission about every 30 hours (157) and the dermal and epidermal tissues react by cellular proliferation and infiltration to produce the initial lesion of primary syphilis. From infection to initial lesion is about three weeks. While the initial lesion is developing, treponemes reach the neighboring lymph glands, which become enlarged, sometime later treponemes enter the blood-stream and are carried throughout the body, enter the skin and multiply there to produce the papillomata of the secondary syphilis. In these lesions, the treponemes continue to multiply until interrupted by the immune processes (157). As the immune response increases, the lesions heal but some treponemes persist through the latent stage that follows. Later, when treponemes again multiply, a relapse occurs.

Pathological Characteristics of the Human Venereal Syphilis

Primary Syphilis. Fourteen to twenty-one days after the initial infection, a red painless papule appears at the site of inoculation. Histologic examination (110) of the papule reveals a relatively avascular center which contains large amounts of mucopoly-saccharide and a few inflammatory cells, and a more peripheral infiltrated area containing polymorphonuclear leukocytes, lymphocytes, and macrophages. Within a few days, the papule ulcerates, producing the typical chancre of primary syphilis. Enlargement of inguinal lymph nodes, frequently bilaterally, is observed in 80 percent of patients with a syphilitic chancre (103). As the papule evolves into a chancre, the central area necrosis and plasma cells and macrophages come to predominate at the periphery. Endothelial

proliferation and perivascular infiltration by lymphocytes and plasma cells are characteristically present (110).

Antibody to cardiolipin detected by the Venereal Disease Research Laboratories (VDRL) or Rapid Plasma Reagin (RPR) reaction is present in 70 to 80 percent of patients with primary syphilis (103). Antibody to treponemal antigen detected by the fluorescent treponemal antibody postabsorption with *Treponema phagedenis* (FTA-ABS) test is said to be present in up to 90 percent of patients with primary syphilis. The microtreponemal heamagglutination (MHA-TP) test is also positive. Once the FTA-ABS test becomes positive, it persists for many years, often throughout life; MHA-TP test might become negative over a prolonged period after treatment. Since the VDRL is a nonspecific test and the FTA-ABS and MHA-TP might indicate past infection, dark-field microscopy of lesion exudate remains as the definitive test.

Secondary Syphilis. If untreated, syphilitic chancres heal spontaneously within three to eight weeks, due to some kind of local immunity (110). The so-called "secondary" lesions of syphilis appear as a result of the dissemination of treponemes from syphilitic chancres, and the term "disseminated syphilis" is probably more appropriate. At least three weeks, and often longer, elapses between the initial infection and the emergence of these macular and reddish brown lesions. Condylomata (condyloma lata) are large, whitish confluent lesions which are found in warm, moist areas such as the axilla or groin of patients with secondary syphilis (110). Inflammatory cells are regularly seen in the secondary lesions, but the nature of the inflammation varies considerably from case to case. In some lesions, plasma cells are the prominent cells, however, frequently a mixed infiltrate is present including lymphocytes, macrophages and polymorphonuclear leukocytes, and any of these cells may predominate (1, 28).

Disseminated or secondary syphilis is a systemic disease and involvement of almost

the entire body is possible. Recent studies suggest that pain in the joints may be due to synovitis and structures resembling treponemes have been detected in synovial tissues (47, 136). Bones are also commonly affected with the skull, tibia, sternum and ribs being the most frequent (153). Perhaps as many as 10 percent of nonspecific hepatitis detected by laboratory studies and supported by biopsy are caused by syphilitic infection (38, 77, 112, 148). Nephritic syndrome has been recognized and is presumably due to antigen-antibody deposition in the glomerulus (17, 22, 37, 78). The VDRL and RPR reactions are uniformly positive, usually in high titer (>1:16); the FTA-ABS and MHA-TP tests are also always positive.

Latent Syphilis. Untreated secondary syphilis is resolved spontaneously after a period of three to twelve weeks, leaving the patient free of lesions and without symptoms. This naturally attained asymptomatic stage is called latency. If untreated, 20 percent of patients will develop relapses with recurrence of a full blown picture of secondary syphilis within one year of the onset of latency (27); accordingly, this period is called early latency. Almost every organ of the body can be affected, but lesions are most often seen in skin, mucous membranes, bones and joints. The skin lesions are nodular, asymmetrical, sometimes grouped and found anywhere on the skin with a slow cycle of healing, scaring and development of new lesions. If recurrence does not occur within one year, the patient is said to have entered the late latent period. It has long been recognized that patients with late latent syphilis are immune to reinfection with *T. pallidum* (88).

Tertiary Syphilis. In approximately half of the untreated patients, clinical manifestations of tertiary syphilis develops when more than one year of latency has elapsed (27). Tertiary syphilis may not cause trouble if present as gummas of the skin or bones. Gummata lesions are characterized by prominent endothelial proliferation and infiltration of the involved tissues by plasma cells, lymphocytes, histocytes, and fibroblasts. Serious manifestations, however, usually result from lesions in the central

nervous system, causing meningomyelitis, tabes dorsalis, and general paralysis of the insane. In the cardiovascular system, where they affect the aortic valves or cause aortic aneurysms; and in the eyes, where they may produce permanent blindness. Tertiary lesions contain very few organisms but frequently result in necrosis, scar formation and extensive tissue damage, probably involving a delayed hypersensitivity response to products of the small number of organisms that persist (68). In 25 percent of untreated cases, the tertiary stage is asymptomatic and is recognized only by the persistence of antibodies in the serum; in the remaining 25 percent, the tertiary syphilis never develops (54).

Congenital Syphilis. Congenital syphilis is the major exception to the rule that syphilis usually develops as a result of venereal contact with an infected person. This infection is acquired by transplacental passage of *T. pallidum* from an infected pregnant woman to her fetus. Stillbirth may occur or the newborn may be born with a variety of signs of disseminated disease. Generally, the disease appears in the second to sixth week of life. The first symptom is usually "sniffles" resulting from involvement of the mucous membranes; skin lesions and mucous patches then appear. The infant may also be febrile. Ultimately, in the absence of treatment, waisting develops, culminating in marasmus and death. This set of symptoms is rapidly reversed by treatment (56). Late manifestations of congenital syphilis are those which appear after two years of age and resemble the lesions of tertiary syphilis in the adult.

Treatment.

Arsenicals, bismuth and mercury have been superceded in the treatment of syphilis by penicillin (54). The sensitivity of *T. pallidum* to penicillin has been known since 1943 (89). Patients in the early stages of the disease may be treated adequately within one to two weeks (89). Short-acting penicillin preparations, even when given as infrequently as once every 24 hours, will usually cure early syphilis, doubtless due to the slow multiplica-

tion of *T. pallidum* in the tissues. Penicillin is less effective in the late stages of the disease and it sometimes fails to eradicate infection when administered to patients with tertiary lesions (54). Such failures are probably due to the presence of nongrowing treponemes, as there is no evidence that *T. pallidum* ever becomes penicillin-resistant (54). Within three to four hours after treatment, there often occurs transient fever (2 to 4 hours up to 105°F), shaking chills, malaise, and a brief exacerbation of visible lesions. This reaction was first described by Jarisch in 1895 (71) and later by Herxheimer and Krause in 1901 (59). Today this reaction is known as Jarisch-Herxheimer reaction. It is apparently caused by an allergic response to antigens released by the organisms being killed in the tissues. An alternative explanation is that some of the products released have the properties of endotoxins (68).

Immunity in Syphilis.

Natural Immunity. It is generally considered that man does not possess any significant resistance or immunity to syphilitic infection. It has been agreed that man's principal defense against syphilitic infection has been the maintenance of an intact skin and mucous membrane barrier, since effective natural humoral or cellular resistance have not been demonstrated (24).

Immune Response. The phagocytosis of *T. pallidum* in the early stage of syphilis is questionable. Several lines of evidence seem to indicate the failure of the afferent limb of the immune response due to the biological properties of the virulent treponemes (164, 142). It was oce postulated that organism's ability to evade host defense mechanisms may be attributed to the fact that *T. pallidum* coats itself with mucoid material and host proteins (10, 127, 145, 165). During the incubation period, an accumulation of mucoid material parallels the multiplication of *T. pallidum* at the site of the entry (157). It has been assumed for many years that this material provides a physical barrier against the host defense mechanism; only recently has the material been shown to

have immunosupp-ressive activity (42, 167) which delays the appearance of the inflammatory process at the site of entry.

Although phagocytosis in the early stage of syphilis is questionable, treponemal antigens are ingested and processed by the phagocytic cells, as demonstrated by the production of antibodies. In the naturally infected host, anti-*T. pallidum* IgM appears first and then simultaneously appears IgG, IgA, and IgE antibodies (21, 91). The fact that the humoral response does not seem to be effective in abolishing syphilitic infection has led investigators to examine the cellular response (111, 120). During early syphilis when *T. pallidum* multiplies and spreads freely throughout the body, depletion of lymphocytes in thymus-dependent areas of lymphoid tissues has been observed in man (81, 158). T-dependent cell-mediated responses are held in restraint as indicated by negative skin reaction to treponemal antigens and by *in vitro* tests (111, 120). Some aspects of the humoral response (B dependent) progress undisturbed, whereas suppression can be demonstrated in yet others (68). The mechanism by which the suppression of cell-mediated responses occurs remains unknown. Despite the production of high titer antibodies and sensitized lymphocytes, *T. pallidum* persists in the host for long periods of time and disease progresses and enters a noninfective latent stage. The duration of the latent stage cannot be predicted, and there can be no firm prognosis as to whether the disease may cease, whether secondary infection recurs or whether the tertiary stage will begin. The outcome is most likely determined by the balance between the host's immune stage, expressed by sensitization and the virulence of the invading strain.

Investigators have presumed that, as with other bacterial pathogens, surface-exposed molecules mediate the interactions between treponemes and their human host. Studies in several loboratories suggest that various outer membrane proteins of *T. pallidum* may have a major role in the pathogenesis of syphilis by mediating motility, the binding of

certain host serum proteins, and the attachment of treponemes to host tissues.(11, 12, 23, 39, 43, 57). For this reason, characterization of the outer membrane of *T. pallidum* and analysis of its proteins have become the subject of several treponemal research efforts (3, 11, 31, 57, 134, 135, 150). Many of these outer membrane proteins have been shown to be strongly immunogenic in both humans and rabbits. Determination of those antigens of *T. pallidum* that are capable of eliciting systemic resistant is important in understanding the induction of resistance and in practical applications, including diagnosis and immunization.

Despite the efforts of Fieldsteel and co-workers in cultivation of *T. pallidum* (40); critical studies that might help to define the role of various *T. pallidum* proteins in the pathogenesis of syphilis have been complicated by the fact that *T. pallidum* remains one of the few important pathogens of humans that cannot be maintained by continuous *in vitro* cultivation. To circumvent this problem, molecular approaches such as recombinant DNA and monoclonal antibody methodologies have been used to analyze a number of treponemal immunogens (75, 86, 93, 115, 132, 151, 156). Also some investigators (124, 126, 130, 131) have used nonpathogenic, cultivatable *Treponema phagedenis* to identify cross-reactive antigens for the possible use of serodiagnostics or antisyphilis vaccine.

The elucidation of surface macromolecules with specific biological functions such as mediating host cell surface parasitism is essential to the generation of rational vaccine candidates to prevent or limit disease progression. Likewise, characterization of the outer membrane of *T. pallidum* in a manner analogous to other parasite systems is prerequisite for future studies dealing with ligand-receptor interactions as well as understanding the parasite's ability to evade immune surveillance.

Up to now, investigators in this field have mainly used one-dimensional sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as the method for

separating *T. pallidum* polypeptides. This method separates proteins on the basis of their molecular weight, and usually a single protein band contains several co-migrating polypeptides. Two-dimensional SDS-PAGE is a technique that combines isoelectric focusing and molecular weight sieving to separate proteins in two dimensions. Therefore, in the present study, two-dimensional SDS-PAGE was utilized to resolve and characterize further the proteins, antigenic proteins, and the cross-reactive antigens of *T. pallidum* Nichols strain and *T. phagedenis* biotype Reiter. Studies were conducted to solubilize and isolate the outer membrane proteins of *T. pallidum* and *T. phagedenis* using the nonionic detergent, Triton X-114. The integral membrane proteins present in the detergent phase of Triton X-114 extraction, the aqueous phase proteins, and the proteins present in Triton X-114 nonsoluble material (cytoplasmic cylinders) were analyzed for their protein composition. The antigenic proteins in each group of polypeptides were identified in Western blots using pooled human sera from patients with secondary syphilis. The cross-reactive antigens between *T. pallidum* and *T. phagedenis* were identified in Western blots by probing *T.phagedenis* polypeptides with antibodies present in pooled sera from patients with secondary syphilis. Similar studies also were conducted using the virulent *in vitro* propagated *T. pallidum.* The outer membrane proteins present in the detergent phase of Triton X-114 extraction, the aqueous phase polypeptides, and the polypeptides of cytoplasmic cylinders were compared to the polypeptides of the *in vivo* propagated polypeptides.

CHAPTER II

METHODOLOGY

Cultures of *Treponema phagedenis*

T. phagedenis biotype Reiter was originally obtained from Dr. James Miller (UCLA Medical Center, Los Angeles, CA) and maintained in Hanson's medium (53) supplemented with 10% heat inactivated rabbit serum (Irvine Scientific, Lot #503284). Refer to Appendix A for the components in the medium.

The seven day old cultures were harvested by centrifugation at 12,000 x *g* for 15 minutes at 4°C. The supernatant was discarded and the pellet resuspended in 10 ml of phosphate buffered saline (PBS, pH 7.6) (101). Refer to Appendix A for the components of PBS. The bacteria were collected again by centrifugation and processed according to need.

Cultures of *Treponema pallidum* subsp. *pallidum*

The virulent Nichols strain of *T. pallidum* subsp. *pallidum* was originally obtained from Dr. James Miller (UCLA Medical Center, Los Angeles, CA) and propagated by intratesticular passage in 7-9 pound, male New Zealand white rabbits.

Motile *T. pallidum* subsp. *pallidum* were frozen at a concentration of 4 x 10^8 per ml in *T. pallidum* cultivation medium (TpCM), containing 10% fetal bovine serum (FBS) (Hyclone Laboratories, Inc., Logan, UT, Lot #633) and 15% glycerol. The treponemes were frozen at a rate of -2°C per minute and stored in liquid nitrogen. Refer to Appendix A for the ingredients of TpCM; unless otherwise stated TpCM always contained 10% FBS. The inoculum was thawed quickly by warming to 34°C and 0.25 ml was injected into each rabbit testes.

Ten days following inoculation, at peak orchitis, rabbits were sacrificed by intracardial injection of 0.5 ml T-61 euthanasia solution (American Hoescht Corporation, Somerville, NJ), and the testes were aseptically removed, minced, and placed in stoppered flasks with 5-10 ml TpCM. The testes were shaken vigorously on a reciprocal shaker for 15-20 minutes. The liquid was removed and centrifuged at 500 x *g* for 7 minutes to remove gross tissue debris. The treponemes in the supernatant were counted by dark-field microscopy and the treponemes needed to inoculate the tissue culture flasks were set aside. The remaining liquid was centrifuged at 12,000 x *g* for 10 minutes to make the testes extract (TEx). The TEx was heated to 56° C for 30 minutes to inactivate any complement present. Some precipitate was formed which was removed by centrifugation at 20,000 x *g* for 15 minutes at 4° C.

Tissue culture flasks (T-150, Corning Glass Works, Corning, NY) were seeded with 2.4 x 10^6 Sf1Ep (cotton tail rabbit epithelial, American Tissue Culture Collection, Rockville, MD) cells in 20 ml of Eagle's MEM (33) (American Biorganics Inc., North Tonawanda, NY 14120) with 10% FBS two days prior to inoculation with *T. pallidum*. To inoculate the flasks with treponemes, the medium was removed from the flask and replaced with 50 ml TpCM containing 1.7 ml TEx and 2 x 10^7 freshly extracted, motile *T. pallidum*. The flasks were then placed in a humidified incubator at 34°C with an atmosphere of 3.5% O_2, 4% CO_2 and 92.5% N_2. After seven days, an additional 10 ml of freshly prepared TpCM was added to each flask.

Thirteen day old cultures of *T. pallidum* were harvested in the following manner: The T-150 flasks were shaken on a rotatory shaker for 20 minutes at low setting. The suspensions from all flasks were pooled and to each flask, 10 ml PBS containing 0.04% (w/v) EDTA was added. The flasks were shaken in the same manner for another 10 minutes to dislodge the Sf1Ep cell monolayer from the flasks. The second suspension

from all the flasks were pooled and both groups of suspensions were centrifuged at 500 x *g* for 7 minutes to remove the Sf1Ep cells. To the supernatant recovered from the monolayer, EBSS (10x) (Appendix A) was added at ratio of 1 ml EBSS (10x) per 100 ml supernatant to neutralize the EDTA. Both supernatants were pooled and centrifuged in 4°C at 12,000 x *g* for 15 minutes. The supernatant was discarded and the tubes were drained upside down on an adsorbent towel. The *T. pallidum* pellets were then immediately processed according to the need.

Sample Preparation

T. pallidum and *T. phagedenis* pellets were suspended in cell suspending solution (CSS, Appendix B) which consisted of 1 mM $MgCl_2$ in 1 mM Tris-HCl buffer, pH 7.8, to a concentration of 100 mg wet cells per ml. The cells were then disrupted by sonic oscillation (45 seconds pulse and 45 seconds pause for 6 cycles) using SONIFIER cell disruptor model W-350 at setting 5. Fifty mg per 100 mg wet cells of RNase and DNase were added and incubated at room temperature for 15 minutes. These samples were designated whole cell sonicate (WCS) and stored at -20°C until used.

Extraction and phase partitioning of treponemal polypeptides with Triton X-114

Extraction and phase separation of treponemal polypeptides were performed using the method of Bordier (20) with modifications by Radolf (134).

T. pallidum or *T. phagedenis* pellets in portions of 5 x 10^9 organisms were added to 1 ml of ice cold 1% (v/v) Triton X-114 in PBS. After a 20 minute incubation at 4°C with frequent gentle agitation, the insoluble material was separated by centrifugation at 4°C for 30 minutes at 20,000 x *g*. The pellet was sonicated as described above and stored at -20°C. The supernatant containing the detergent soluble material was either stored at -70°C or immediately phase separated. Phase separation was performed by warming the supernatant for 10 minutes in a 37°C water bath followed by centrifugation at room temperature for 10 minutes at 13,000 x *g*. The detergent phase developed below the

aqueous phase. The separated detergent and aqueous phases were then washed at least four times in the following manner: The detergent phase (approximately 50 µl) was diluted to 1 ml in the ice cold PBS and incubated at O°C with occasional mixing for five minutes, rewarmed to 37°C for 10 minutes and centrifuged at room temperature at 13,000 x *g* for 10 minutes. The aqueous phase was cleansed by the repeated addition of fresh 10% Triton X-114 to a final concentration of 4% and phase separated as above. The aqueous phase was dialyzed over night in distilled water using a dialysis membrane tubing (American Scientific Products, m.w. cutoff 6000-8000), then lyophilized and stored at -20°C until used. The detergent phase proteins were precipitated using equal volume of cold acetone at -70°C for 30 minutes. The precipitated proteins then were collected by centrifugation at 13,000 x *g* for 10 minutes at room temperature and stored at -20°C for later use.

Protein Assay

Protein content of samples was determined before storage using a Protein Assay Kit (Bio-Rad, Richmond, CA).

Preparation of Samples for Electrophoresis

Preparation of samples with the presence of SDS in solubilization buffer. Samples were thawed and solubilized using the method of O'Farrell (118) with the modification by Ames and Nikaido (4). Samples were diluted 1:2 in working solubilization solution (Appendix B), which consisted of 2% sodium dodecyl sulfate (SDS) (Bio-Rad, Richmond, CA) and 0.5 mM $MgCl_2$ (Fisher Scientific, Pittsburgh, PA) in 50 mM Tris-HC1 buffer, pH 6.8. Diluted samples were heated for 5 minutes in 95°C water bath and two volumes of sample preparation solution (SPS, Appendix B) were immediately added (sample's dilution is now 1:4). SPS consisted of 9.5 M urea (Bio-Rad), 2.5% ampholines (equal amounts of pH 5-7 and pH 3.5-10, LKB Instruments, Rockville, MD), 5% 2-mercaptoethanol (Bio-Rad) and 8% Nonidet P-40 (NP-40) (Sigma

Chemical Co., St. Louis, MO).

Preparation of samples without the presence of SDS in solubilization buffer. Samples were thawed and solubilized as described by O'Farrell (118) with minor modifications. Lyophilized samples were dissolved in solubilization buffer containing 9.5 M urea (Bio-Rad), 4% (w/v) Nonidet P-40 (NP-40, Sigma Chemical Co., St. Louis, MO), 5% (v/v) Ampholines (comprised of 2.5% pH range 5 to 7 and 2.5% pH range 3.5 to 10, LKB Instruments, Rockvill, MD), and 5% (v/v) 2-mercaptoethanol (Bio-Rad).

Two-Dimensional Gel Electrophoresis

SDS-PAGE was done by the method described by O'Farrell (118). Solubilized samples containing 60-100 μg of protein were first subjected to isoelectric focusing on 4% polyacrylamide tube gels (0.15 cm x 10 cm) containing 2% NP-40, 2% ampholines (equal amounts of pH 5-7 and pH 3.5-10), and 9.2 M urea. The second dimension was run in 10% polyacrylamide (30% T, 2.67% C), 0.1% SDS, 16 cm x 15 cm x 0.15 cm slab gels.

First Dimension, Isoelectric Focusing (IF)

The solubilized samples were subjected to isoelectric focusing in 10 cm x 0.15 cm 4% polyacrylamide tube gels containing 2% NP-40, 2% ampholines, and 9.2 M urea, using GT2 Tube Gel Unit by Hoeffer, as follows:

Preparation of IF gel tubes. A square of parafilm was cut and drawn several times over one end of an IF gel tube to make a water-tight seal. A mark was made with a pen 10 cm from the sealed end and the prepared tubes were placed in the stand (Hoeffer).

Preparation of IF gel. The following solutions (Appendix C) were warmed to room temperature before use: IF bis acrylamide (IF Bis Acr); 10% NP-40 (NP-40); lysis buffer (LB); Sample Overlay Solution (SOS). The IF gel was prepared in a 50 ml side-arm flask as described in Table 1. (Gloves must be worn at all times when working with

polyacrylamide since this chemical is a potent neurotoxin and carcinogen).

After the urea was dissolved, the amount of ammonium persulfate (see appendix C for preparation) indicated in Table 1 was added and the flask was deaerated for 45 seconds using a water aspirator. The vacuum was released slowly before the water was turned off. When the IF gels were ready to pour, TEMED (Bio-Rad) was added. Using a 10 cc syringe fitted with an eighteen gauge needle and a 10 cm piece of flexible polyethylene tubing (Intramedic #7420), the IF gel solution was introduced into the tube. The polyethylene tubing was inserted nearly to the bottom of the IF tube and slowly

TABLE 1. Components and the quantities of each that are necessary for the preparation of IF gels (4% acrylamide, 2% NP-40, 2% ampholines)

Component	Number of Gels			
	3	6	8	12
Urea (9.2 M)	1.435 g	2.85 g	3.8	5.7 g
3 x D.W.	0.487 ml	0.975 ml	1.3 ml	1.95 ml
10% NP-40	0.5 ml	1.0 ml	1.33 ml	2.0 ml
IF BIs Acryl	0.332 ml	0.665 ml	0.89 ml	1.33 ml
Ampholine, pH 5-7	0.0625 ml	0.125 ml	0.17 ml	0.25 ml
Ampholine, pH 3.5-10	0.0625 ml	0.125 ml	0.17 ml	0.25 ml
	Stir flask contents to dissolve urea. Slight amount of heat might be used to speed the dissolving process.			
10% Ammonium persulfate	2.5 µl	5 µl	6.7 µl	10 µl
	Deaireat flask for about 45 seconds.			
TEMED	3.5 µl	7 µl	9.3 µl	14 µl

withdrawn as the IF solution was injected into the tube. The bottom of the IF tube was

tapped to release any air bubbles. The gel solution was then overlaid with 20 µl of 3x D.W. using an Eppendorf pipetter. The water was allowed to run down the side of the tube to prevent air bubbles. The IF gels were incubated at room temperature for two hours to polymerize. (These could be stored at room temperature for one day if parafilm seals were placed on top of the tubes. If the room temperature was too cold, on occasion the urea precipitated and urea crystals formed in the gels. The crystals were redissolved by placing the gels in front of a heat lamp.)

Once the gels polymerized, the parafilm seals were removed and the marks were removed using ethanol. The water overlay was poured off the tubes and the bottom of each tube was rinsed in 0.1 N H_3PO_4 solution (Appendix C) and inserted into the stopper of the Hoeffer cell. After all the tube gels were in place and the empty holes had been plugged, 20 µl of lysis buffer (Appendix C) was added to the top of each IF tube gel and the rest of the tube was filled with 0.02 M NaOH solution (Appendix C). Some NaOH solution was added to the upper chamber of the Hoeffer cell to check for leaks. If no leaks were detected, then the lower chamber was filled with the 0.1 N H_3PO_4 and after the upper chamber was in place, the rest of the upper chamber was filled with 0.02 M NaOH. The NaOH solution was at least one cm above the top of the tube gels. The cell was placed on a stirrer for constant mixing of the lower chamber buffer.

IF gel prefocusing. The IF cell containing the tube gels was connected to the power supply (Buchler, Model 3-1500). The power supply was set on constant voltage. The gels were prefocused as follows: at 200 volts for 15 minutes, followed by 30 minutes at 300 volts, and finally at 400 volts for 30 minutes. During the last 30 minutes samples were prepared according to the procedures outlined in the sample preparation section.

Sample addition. After the IF gels were prefocused, the power supply was turned off and the lead was removed from the IF chamber. The NaOH was siphoned from the upper chamber into a side-arm flask. The flask was deaerated and solution was held under

vacuum. The IF tube overlays were removed using a 1 cc syringe fitted with a 26 gauge needle and a small piece of polyethylene tubing. The samples were then introduced into the tubes by allowing them to flow down the side of the tubes. The samples were overlaid with 10 µl of sample overlay solution (Appendix C) and the rest of the tubes were filled with 0.02 M NaOH. The upper chamber was refilled with 0.02 M NaOH and placed into the lower chamber of the Hoeffer cell. The lead was placed on the cell and then the cell was returned to the stirrer.

Isoelectric focusing. The power supply was set on constant voltage so that a total of 9000 volt hours (VH) were attained over a period of at least 12 hrs (i.e. power supply was set on 474 volts and the gels were focused over a period of 19 hrs). When the IF gels were run for the desired amount of time, the power supply voltage was turned up for an additional 1000 volts for one hour. After one hour, the cell was turned off, the upper and lower chamber buffers was discarded, the IF tubes were removed from the stoppers, and the gels were extruded from the tubes as described below.

Extruding the IF tube gels. The overlay solution was poured off the IF tubes. Using a 1 ml syringe fitted with a 25 gauge needle, the surface of the IF gels were gently flushed with 3x D.W. to remove lipids and DNA. To separate the gel from each tube, a Hamilton syringe filled with 3x D.W. was used. The needle was inserted into the tube between the gel and the glass, and water was injected while the tube was being rotated. This was done at both ends of each tube. Then the top of the IF tube was attached to a short length of tygon tubing connected to a 50 ml syringe (tygon tubing should fit the IF tube tightly). Using air pressure, the gels were forced out of the tubes into a pan filled with 3x D.W. After being rinsed, the worms were equilibrated in sample equilibration buffer (Appendix C).

Equilibration of the IF gels. The IF gels were equilibrated for 30 minutes in

screw cap tubes with 5 mls of sample equilibration buffer containing dithiothreitol (DTT) (65 mM final concentration) with constant gentle agitation. At the end of the 30 minutes, the tubes were either processed further or frozen at -20°C for later use. At that time, the IF gels were thawed and equilibration was continued in fresh equilibration buffer containing DTT (65 mM) and iodoacetamide (260 mM final concentration) (48) for another 30-45 minutes.

Determining pH gradient of the IF gel. A blank gel (without sample) was run with each isoelectric focusing which was sliced into 0.5 cm pieces and each piece was placed in a screw cap tube containing 2 mls degassed 3x D.W. The tubes were agitated for 10 minutes, and the pH of the solution was determined using a Corning pH meter model 120 (118). Each time the gradient ranged from 4.45-7.42 pH units

Second Dimension, Sodium dodecyl sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Using the protean II multigel system (Bio-Rad), the second dimension was run in 10% polyacrylamide (30% T, 2.67% C)[1], 0.1% SDS resolving slab gels. The slab gels were 0.15 cm x 25 cm x 16 cm in size. Two reference lines for one dimensional electrophoresis profile were run on each side of each gel. One line contained the prestained molecular weight markers (Sigma) and the other contained the sample.

Preparation of the casting chamber. Using the multigel casting chamber (Bio-Rad), the needed number of slab gels were casted as follows. The glass plates and the spacers were cleaned with a lent free tissue (Kimwipes) and absolute ethanol and sandwiched in the chamber in the following manner: One sheet of sandwich separator, one long glass plate, two spacers, one short notched plate and another sandwich

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[1] $\%C = \frac{\text{grams Bis}}{\text{grams bis + grams acrylamide}} \times 100$

$\%T = \frac{\text{grams Bis + gram acrylamide}}{\text{Total volume (ml)}} \times 100$

separatorsheet were layered in the order mentioned. The layers were repeated until the desired number of sandwiches were achieved. The rest of the chamber was filled with the plastic blocks provided with the casting chamber.

Preparation of the resolving slab gel mixture. The following solutions (Appendix C) were warmed to room temperature before use: Acrylamide/Bis (30% stock); 10% SDS; 1.5 M Tris-HCl, pH 8.8; 0.5 M Tris-HCl, pH 6.8. The resolving gel mixture was prepared in a 500 ml side-arm flask as described in Table 2.

All solutions except ammonium persulfate (APS) and TEMED were combined and deaerated for ≥ 15 minutes. The APS and TEMED were added, the flask was swirled to

TABLE 2.Components and the quantities of each that are necessary for the preparation of two resolving slab gels (10% acrylamide, 0.1% SDS, 0.375 M Tris-HCl, pH 8.8, 30% T, 2.67% C)

Component	% Acrylamide				
	5%	7.5%	10%	12%	15%
3x D.W. (ml)	20.1	11.8	40.2	33.5	23.5
1.5 M Tris-HCl,pH 8.8 (ml)	25.0	25.0	25.0	25.0	25.0
10% (w/v) SDS stock (ml)	1.0	1.0	1.0	1.0	1.0
Acrylamide/Bis (30% stock) (ml)	16.70	25.0	33.30	40.0	50.0
Deaerate for ≥15 minutes					
10% Ammonium persulfate (µl)	500	500	500	500	500
TEMED (µl)	50	50	50	50	50
Total monomer^{+} (ml)	100	100	100	100	100

mix well and the monomer mixture was poured to the mark (16 cm from the bottom of the chamber) in the previously prepared casting chamber. The gels were overlaid with 2 mls of 1-Butanol (J.T. Baker). Care was taken to remove any air bubbles that were introduced

during the casting of the gels. The gels were allowed to polymerize for 20-30 minutes at room temperature. At the end of the polymerization, the gels were removed from the chamber, and the butanol was removed. One by one the surfaces of the gels were washed under distilled water and placed in the clamps provided . All the excess water was blotted using adsorbent paper. If the gels were to be used the same day, they were stacked. If they were to be used the next day, they were overlaid with the 1:4 lower gel buffer (Appendix C) (Table 3) and stored overnight at room temperature.

Preparation of stacking gel. The stacking gel (4% acrylamide, 0.125 M Tris-HCl, pH 6.8, 0.1% SDS, 30% T, 2.67% C) was prepared in a 125 ml side-arm flask as described in Table 4. The monomer solution was prepared by mixing all reagents except APS and TEMED. The flask was deaerated for 45 seconds. To initiate polymerization, APS and TEMED were added. The flask was swirled gently to mix, and using a serological pipit, the stacking gel was poured up to the top of each slab gel. Immediately the stacking gels were overlaid with few drops of 1-butanol using a pasture pipet. Care was taken to remove any air bubbles that were introduced during pouring of the gels. The gels were allowed to set at room temperature for 20-30 minutes to polymerize.

TABLE 3. Components and quantities of each that are necessary for the preparation of the 1:4 lower gel buffer overlay.

Component	Number of Gels					
	2	4	5	6	7	8
Lower gel buffer, ml	2	4	5	6	7	8
3x D.W., ml	6	12	15	18	21	24
10% Ammonium persulfate, µl	24	48	60	72	84	96
TEMED, µl	8	16	20	24	28	32

Assembling the IF gels onto the slab gels. After polymerization of the stacking gel, the slab gels were washed to remove the butanol and were connected to the cooling coils following the manufacturer's guide. Two teflon combs (Bio-Rad) were placed at both ends of each slab gel to form the reference wells. The equilibrated IF gel was removed from the equilibration buffer and placed into a scoopula so that the acid side (the positive end) was toward the lip of the scoopula. The IF gel was then positioned along the notched short plate (positive end to the left) and warm, molten 1% agarose (Appendix C) was quickly pipetted over the IF gel using a serological pipet. Care was taken to remove any air bubbles that were introduced. When all the IF gels were transferred to the slab gels and the agarose had hardened, the teflon combs were gently removed from the agarose. The prestained molecular weight standards (Sigma) were placed into the left reference well (at the positive end of the IF gel) and the reference

TABLE 4. Components and quantities of each that are necessary for the preparation of stacking gel.

Component	Number of Gels		
	2	4	6
3x D.W. (ml)	18.3	30.5	48.8
0.5 M Tris-HCl,pH 6.8 (ml)	7.5	12.5	20.0
10% (w/v) SDS stock (ml)	0.3	0.5	0.8
Acrylamide/Bis (30% stock) (ml)	3.9	6.5	10.4
Deaerate for 45 seconds			
10% Ammonium persulfate (µl)	150	250	400
TEMED (µl)	30	50	80

sample was placed in the right well (the negative end of the IF gel). The upper and lower chambers were filled with running buffer (Appendix C) and the cell was assembled according to manufacturer's guide. A few drops of tracking dye (Appendix C) were mixed with the buffer in the upper chamber and the cooling coils were connected to the cooling pump (HAAKE, Model F2).

Electrophoresis. The gels were electrophoresed at constant current using a Buchler power supply overnight so that a K[2] value of 256 per gel was achieved. (i.e. the tracking dye was 1-2 cm from the bottom of the slab gel). At the end of the electrophoresis, the cooling pump was turned off, the electrophoresis cell was disconnected from the pump, the buffer in the upper chamber was discarded and the slab gels were removed from the cooling coils. The buffer in the lower chamber was stored at 4°C and reused with fresh running buffer at 1:1 ratio in the subsequent runs.

Removing the gels from the glass plates. The slab gels were separated from the cooling coils following the manufacturer's guide. The spacers were removed by pushing the end of each spacer that laid on the longer glass plate away from the gel. Then the glass plates were separated by inserting a spatula between them and gently prying them apart. The stacking gel was cut off. A small portion of the left corner of the slab gel was also cut off to mark the positive end of the first dimension. The gel was then placed in a Pyrex baking dish and processed according to the need.

Silver Stain for Slab Gels.

After electrophoresis, the gels were silver stained using the method of Morrissey (104). Throughout the procedure, the gels were incubated at room temperature with gentle agitation (60 revolutions/minute on a reciprocal shaker). The gels were handled with gloved hands at all times. Refer to Appendix D for solutions involved. Use enough of each solution so that the gel is freely floating during staining (usually five to six times

[2] K per gel = Time (h) x current (mA) per gel

the volume of the gel is sufficient). The gels were prefixed in 50% methanol-10% acetic acid solution for 30 minutes. This solution was removed by aspiration and a solution containing 5% methanol - 7% acetic acid was added. After 30 minutes, this solution was aspirated and 10% glutaraldehyde was added. After another 30 minutes this solution was aspirated. Depending on the time of day, the gels were either washed for 2 hours in 3x D.W. with change of water every 15 minutes or were soaked in large amounts of 3x D.W. overnight. After the gels were rinsed in water, they were placed in a solution containing 5 mg/ml DTT for 30 minutes followed by a 30 minute soaking in a 0.1% silver nitrate solution. The gels were then rinsed for 1 minute in 3x D.W. and once in developer which consisted of 50 ml of 37% formaldehyde in 100 ml 3% sodium carbonate. After the gels were rinsed with the developer, they were soaked in 250 mls of developer with occasional swirling until the desired amount of staining had been obtained (5-7 minutes were usually adequate for a good stain). The reaction was stopped by addition of 12.5 mls of 2.3 M citric acid followed by 10 minutes of agitation on a shaker. Finally, the gels were rinsed in 3x D.W., photographed, and dried.

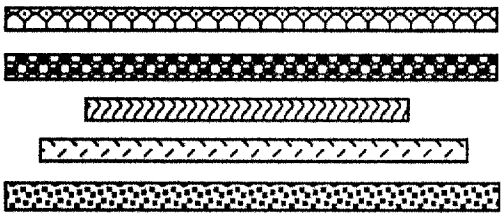
Drying Gels.

Each gel was soaked in 500 ml of 3% glycerol (w/v)-40% methanol-10% acetic acid solution for about 3 hrs with constant gentle agitation. Each gel was placed on a piece of filter paper (Whatman #3 presoaked in 3x D.W.) and was covered with Saran Wrap. This sandwich was dried for two hrs at 60°C using Bio-Rad model 443 gel drier. Figure 1 shows the arrangement for drying gels.

Silver Stain for IF Gels

To assure quality, isoelectric focusing at each run on of the IF tube gels were silver stained using a method introduced by Merril et al (94). Refer to Appendix E for solutions After the IF gel was removed from the glass tube, it was placed in 50% methanol-12% acetic acid solution for 30 minutes on a shaker. It was then rinsed in 10% ethanol-5%

Figure 1. Schematic representation of the arrangement for drying SDS-polyacrylamide gels.


→ Transparent Sealing Gasket
→ Saran Wrap
→ Polyacrylamide Gel
→ Filter Paper
→ Porous Gel Support

acetic acid solution for 10 minutes. This rinse was repeated 3 times. Next, the gel was soaked for 5 minutes in a solution containing 0.0034 M sodium dichromate and 0.0034 N nitric acid followed by four washes, one minute each, in 3x D.W. The gel was then stained for 30 minutes in 0.012 M silver nitrate followed by 1 minute rinse in 3x D.W. Next, the gel was rinsed quickly in the developer (containing 0.5 ml 37% formaldehyde per one liter 0.28 M sodium carbonate), and placed on the shaker in developer until the image reached the desired intensity. The developer was then aspirated and the gel was soaked in 1% acetic acid for 10 minutes followed by two washes in 3x D.W.

Immunoblotting (Western Blotting)

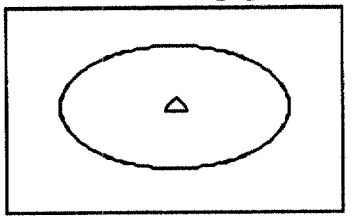
Immunoblotting was done by the method of Towbin et al (154) using an electroblot apparatus (Bio-Rad) and 0.2 mm pore size nitrocellulose membranes (Bio-Rad). The antigenic proteins were detected by reacting the blots with pooled sera from patients with secondary syphilis. The visualization of the immune complexes was done with horse radish peroxidase conjugated goat anti-human IgG, or goat anti-human IgM (Hyclone) using the Monroe method (102). The entire method can be observed schematically in Figure 2. Refer to Appendix F for solutions involved. All steps were carried at room temperature unless indicated otherwise.

Equilibration of slab gels. The slab gels that were to be blotted were equilibrated for 30 minutes on a Shaker (60 laps/minute) in Tris-glycine buffer (25 mM Tris, 192 mM glycine, 20% methanol, pH 7.8).

Blotting sandwich preparation. Using the blotting grids provided, the sandwich was assembled as shown in Figure 3. All layers were pre-wetted in the Tris-glycine buffer prior to the assembly of the sandwich. Nitrocellulose membranes were handled with gloved hands at all times. To wet the nitrocellulose membranes, the sheets were slowly placed into the buffer at a 45° angle to prevent entrapment of air bubbles

Figure 2. Schematic representation of Immunoblotting (Western Blotting) technique. All steps are performed at room temperature. Steps 3 and 5 may be done overnight if necessary.

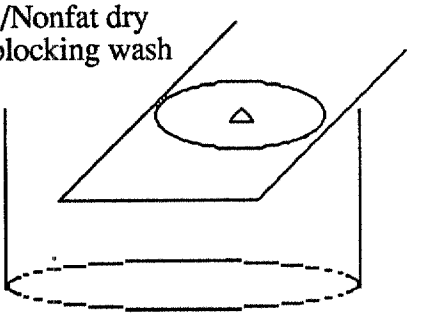
1. Antigens transferred from SDS-polyacrylamide gels onto nitrocellulose paper



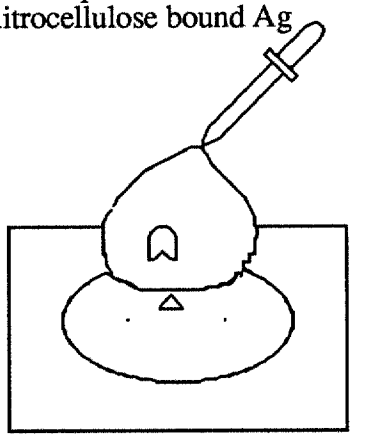
- Antigen
- Patient Antibody
- HPR-Congugated Anti-Ig Antibody

Reduced / Oxidized — 4 Cl N Chromogenic substrate

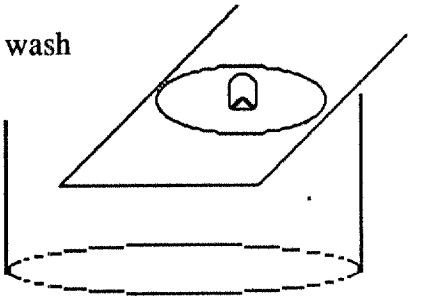
2. TTBS/Nonfat dry milk blocking wash



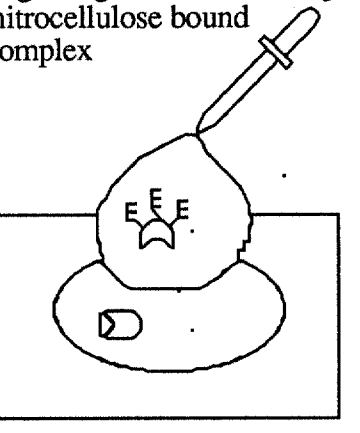
3. TTBS/syphilitic patient serum added to nitrocellulose bound Ag



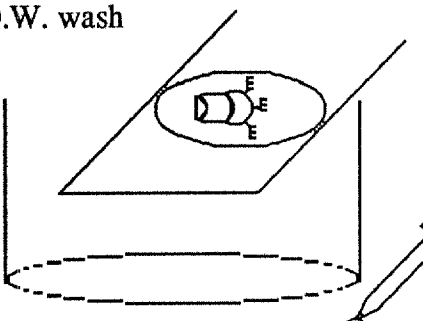
4. TTBS wash



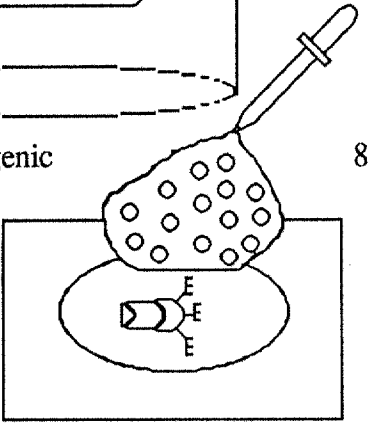
5. HRP-conjugated goat anti-human Ig added to nitrocellulose bound immune complex



6. TTBS wash followed by 3x D.W. wash



7. Reduced chromogenic substrate added



8. Positive test or color change when chromogen substrate oxidized

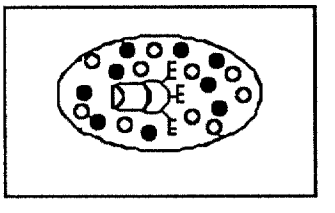
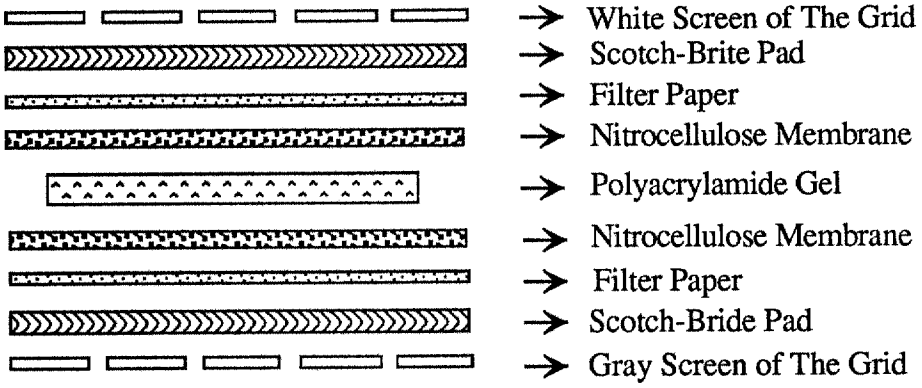


Figure 3. Schematic representation of an electroblot sandwich. All layers were pre-wetted in the Tris-glycine buffer prior to assembly of the sandwich.


White Screen of The Grid
Scotch-Brite Pad
Filter Paper
Nitrocellulose Membrane
Polyacrylamide Gel
Nitrocellulose Membrane
Filter Paper
Scotch-Bride Pad
Gray Screen of The Grid

under the membranes (the trapped air bubbles would prevent the proteins from being transferred to the nitrocellulose). After layering the first Scotch-Brite pad, the first filter paper (Whatman #3), the first nitrocellulose membrane, the polyacrylamide gel, the second nitrocellulose membrane, and the second filter paper; the trapped air bubbles were removed by firmly rolling a stirring glass rod in one direction over the top layer. The second Scotch-Brite pad was placed and the grid was closed. The sandwich was placed in the electroblot chamber (gray screen facing the black cathode) and the chamber was filled with Tris-glycine buffer. The chamber was placed on a stirring plate for constant mixing of the buffer during the transfer period.

Transferring the proteins. To transfer the proteins from the gel onto the nitrocellulose membrane, an electrophoretic ramping procedure introduced by Bio-Rad was used. The proteins were transferred at 4°C at constant voltage (15 V) for 15 minutes. The voltage was increased to 35 V for the next 30 minutes followed by 30 minutes at 80 volts. The transfer continued at constant current of 400 mA for another 2 hours. The efficacy of transfer was assured by using low and high prestained molecular weight standards (Sigma and Amersham). In addition, the gels were also silver stained after the transfer was completed.

Blocking the nitrocellulose membranes. After the transfer was completed, the nitrocellulose sheet at the anode side (immediately under the white screen of the transfer grid) was removed and washed twice for ten minutes each in Twin -20 tris buffered saline (TTBS, Appendix F) on a shaker. The membranes were blocked for one hour in "Blotto" (Appendix F) with constant agitation.

Probing the proteins. After the blocking step, membranes were rinsed in two changes of 3x D.W., 10 minutes each, and placed in the primary antibody solution (i.e. pooled human secondary syphilitic sera diluted in TTBS 1:50 to 1:100 depending on the

reactivity of the pool determined by VDRL test for syphilis) overnight with gentle agitation.

Detection of the immune complexes. Following overnight incubation, the membranes were removed from the primary antibody solution and washed twice in TTBS for 10 minutes each. Secondary antibody, the horseradish peroxidase conjugated goat anti human IgG,or goat anti-human IgM (Hyclone, 1:5000 dilution in TTBS), was added and the membranes were agitated for 2-3 hours on a shaker. The incubation period was followed by two washes, 10 minutes each in TTBS to remove excess antibody, and two washes, 10 minutes each in 3x D.W. to remove all the present Tween-20.

Developing the immunoblots. The working chromogenic solution was prepared fresh just prior to use (Appendix F). The last water wash was removed and the chromogenic solution was added to the membranes and the trays containing the membranes were rocked gently until the desired color was achieved. The chromogenic solution was then removed and the membranes were washed in 3x D.W. twice for 10 minutes each. Following the last wash, the membranes were hung to air dry for a few hours. The color of the immunoblots was stable for several months if stored in the dark.

Secondary staining (i.e. reprobing) of the same electroblots. Secondary staining of the same electroblots with monospecific antibodies directed against the endoflagella of *T. phagedenis* biotype Reiter (generous gift of Dr. Michael Norgard at the University of Texas Southwestern Medical Center, diluted 1:200) were carried out after the elution of the primary antibodies in glycine hydrochloric (pH 2.0 for one hour). The above procedure was followed in the same manner, except that the secondary antibody was horseradish peroxidase labeled goat anti-rabbit antibody and diaminobenzidine (Sigma) was used as the substrate.

In vitro Radiolabeling of *Treponema pallidum* polypeptides

20 hours radiolabeling. *T. pallidum* ssp. *pallidum* were extracted from the

rabbit testes in the manner previously described using a modified extraction media introduced by Stamm and Bassford (149). See Appendix G for the components of the extraction media. Extracted treponemes were centrifuged at 500 x g for 7 minutes to remove most of the rabbit tissue. Supernatant containing the treponemes was centrifuged at 12,000 x g for 20 minutes. The pelleted treponemes were suspended in the extraction medium to a density of 3 x 10^8 to 6 x 10^8 cells per ml. The treponemes were placed into a sterile, glass screw cap tube. Cycloheximide (sterile 5 mg/ml stock; Sigma Chemical Co., St. Louis, MO) was added to a final concentration of 100 µg/ml to inhibit protein synthesis by any contaminating cells of rabbit origin. Treponemal proteins were radiolabeled with 65 µCi of [^{35}S] methionine and [^{35}S] cysteine (80:20 mixture, Trans ^{35}S-label; ICN Radiochemicals, Irvine, CA) per ml at 34°C overnight (approximately 20 hrs). The treponemes were pelleted at 12,000 x g for 25 minutes and immediately extracted and phase partitioned as previously described.

Thirteen days radiolabeling. *T. pallidum* ssp. *pallidum* were cultured in T-150 tissue culture flasks as previously described. The proteins were radiolabeled by addition of 40 µCi Trans [^{35}S]-label (ICN Radiochemicals) per flask. Thirteen day old cultures were harvested, extracted and phase partitioned as previously described.

Autoradiography

Following electrophoresis, the slab gels were fixed in a solution containing 50% methanol - 10% acetic acid for one hour. They were then washed twice for 5 minutes each in 3x D.W. followed by 45 minutes in 1 M sodium salicilate solution. The slab gels were then dried as previously described. Next, a piece of X-OMAT AR film (Kodak) was laid on top of each dried gel and placed in an exposure cassette (Sigma) and incubated at -70°C for 1-4 days. Following the incubation, the films were developed as follows:
1 minute in Dektol developer (Sigma); 30-45 seconds in indicator stop bath (Sigma); 4 minutes in rapid fixer (Sigma); and 5-10 minutes wash under running water. All steps

were performed with constant agitation in a dark room. The developed film was then air dried.

Electron microscopy

Specimens were prepared for whole-mount electron microscopy using the single droplet method of Valentine (159). Approximately 10^{10} freshly harvested *T. pallidum* were suspended in 2 ml of ice cold 1% (v/v) Triton-X-114 in PBS (pH 7.4) and incubated at 0°C with frequent gentle agitation. Samples of *T. pallidum* (15 µl) were taken out at 0, 10, 15, and 20 minutes and were applied to carbon coated nickel grids (200 mesh per grid) (Polysciences Inc., Warrington, PA) for 5 minutes at 23°C in a moist chamber. The grids were then dried by being blotted on filter paper. For the Control samples, organisems were processed in PBS without Triton X-114. The grids were washed with ice cold PBS prior to negative staining at room temperature with 1% (v/v) uranyl acetate (polyscinces). Micrographs were taken at 75 KV accelerating voltage on a Hitachi H-600 electron microscope. A portion of the samples were also taken at the same time intervals and processed for thin sectioning as described below. Refer to Appendix H for solutions involved. Samples were placed in centrifuge filter units (Spin-X cellulose acetate, Costar, Cambridge, MA) and centrifuged at room temperature for 5 minutes at 13,000 x g. The filters then were fixed over night in 2% (v/v) glutaraldehyde in 0.02 M piperazine buffer (PIPES, pH 7.4, Polysciences). The next day, fixation was continued for one hour in 2% (v/v) glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4, Polysciences); rinsed three times 5 minutes each in 0.1 M cacodylate buffer. The filters were postfixed with 2% (w/v) osmium tetroxide (Polysciences) in D.W. for one hour in the dark; rinsed three times 5 minutes each in D.W. The filters were dehydrated in series of 10%, 30%, 50% (v/v) ethanol 10 minutes each; 70%, 95% (v/v) ethanol 15 minutes each; 100% ethanol three times 15 minutes each. The filters were embedded in plastic following Luft's method

(82). Filters were placed in a 50% absolute ethanol, 50% Epon 812 (Polysciences) solution for 30 minutes, followed by 30 minutes in a 25% absolute ethanol, 75% Epon 812 solution. At the end of the 30 minutes, the filters were incubated overnight in 100% Epon 812. The plastic was polymerized for 14-16 hours at 70°C and sectioned using an HT2-B ultramicrotome (Ivan Sorvall Inc., Norwalk, CN) with diamond knife. Silver sections, about 75 nm thick, were placed on nickel grids (150 mesh, Polysciences) and stained using the method of Sato (139). Grids were placed in a 70% ethanol saturated with uranyl acetate for 5 minutes at room temperature; rinsed with D.W. for one minute, and stained with Sato's lead citrate for 5 minutes. The grids were rinsed with warm D.W. for one minute and micrographs were taken as before.

CHAPTER III

RESULTS

Preliminary Studies

Establishment of pH gradient for the IF tube gels. Several experiments were conducted in which the total ampholine concentration was kept constant at 2%. However, the ratio of the ampholine mixture (ampholine pH 5-7 to ampholine pH 3.5-10) was varied. The goal was to establish a smooth pH gradient between pH 4.5 and 7.0. This goal was achieved when ampholines pH 5-7 and pH 3.5-10 were present at the ratio of 1:1 in the IF gels.

Establishment of total volt hour needed for IF. These experiments were conducted to determine the total volt hour needed for efficient isoelectric focusing of treponemal polypeptides. In these experiments total volt hours of 4800, 5800, 6800, 7800, 9000, and 10000 were tested. The efficiency of focusing increased as the total volt hour increased. However, the IF gels were brittle and hard to handle, if total volt hour exceeded 9000. Therefore, 9000 total volt hours over 15-19 hours was determined to be optimal.

Establishment of polyacrylamide concentration for slab gels. Eight, 10, 12.5, and 15 percent isotropic polyacrylamide gels were tested for their ability to efficiently resolve treponemal polypeptides. The 8% polyacrylamide gels separated the high molecular weight polypeptide fairly well. However, most of the lower molecular weight polypeptides were lost. The 15% polyacrylamide gel separated the low molecular weight polypeptides fairly well ,but, the intermediate and high molecular weight polypeptides were poorly resolved. The resolving ability of 12.5% and 10%

polyacrylamide gels were similar. However, since the majority of treponemal polypeptides were within 60-25 kDa, and the 10% polyacrylamide gel showed a better resolving ability in this range, it was used in the further analysis of treponemal proteins.

Establishment of a solubilizing system for treponemal polypeptides. Two kind of solubilization buffers were tested for their ability to solubilize treponemal polypeptides. One contained the nonionic detergent, NP-40, and the other contained the ionic detergent, SDS. These studies demonstrated that SDS solubilized treponemal proteins more efficiently than NP-40, resulting in a higher number of polypeptides detected by autoradiogrophy. A series of experiments were conducted where the concentration of the SDS present in the solubilization buffer varied. SDS concentrations of 0.5, 1, 2, 3, 4, and 5 percent were tested. It was found that 3% SDS (final concentration) solubilized treponemal samples the best, resulting in the highest number of polypeptides. The same kind of experiments were conducted to determine the effect of SDS on the ability of the poly peptides to react in western blots. These experiments demonstrated that the highest number of antigenic proteins were detected when NP-40 was used to solubilize treponemal polypeptides. The number of antigenic polypeptides decreased as the concentration of SDS increased. Since the identification of antigenic polypeptides was one of the major goals of this study, the solubilization buffer containing NP-40 was used.

Efficacy of polypeptide transfer from polyacrylamide gel to nitrocellulose membrane. Several experiments were conducted to determine the length of the time needed for complete transfer of proteins to the nitrocellulose membrane. A mixture of prestained molecular weight standards was used which allowed for immediate visualization of an efficient transfer. In these experiments, the voltage was maintained at a constant voltage of 80 volts and the time varied from 1 to 3 hours. The longer the transfer

time, the more efficiently the proteins transferred to the nitrocellulose membrane. Three to three and a half hours were found to be sufficient for the transfer of polypeptides to nitrocellulose membrane.

Experimental Results

Release of the *T. pallidum* outer membrane by Triton X-114. The effect of Triton X-114 (TX-114) on the structure of thirteen day old freshly harvested *T. pallidum* cultures was examined by whole-mount electron microscopy. Untreated control organisms at 0, 10, 15 and 20 minutes time intervals retained both outer membrane and the tightly adherent periplasmic flagella on their cytoplasmic cylinders. Control organisms had a diameter of 0.14 ± 0.02 μm at 20 minutes incubation (Figure 4 A-D). In contrast, at the same time intervals, treatment with TX-114 caused the removal of the outer membrane, unwound but did not release the periplasmic flagella from the morphologically intact cytoplasmic cylinders. Treatment reduced the diameters of the organisms to an average of 0.095 ± 0.003 μm after 20 minutes incubation (Figure 4 A-D). The difference in diameter between TX-114 treated and untreated *T. pallidum* was further demonstrated when detergent treated and untreated organisms were placed on the same grid. Figure 5 shows that the differences in the diameters of the cells are not artifact of processing on separate grids for electron microscopy.

Examination of thin sections of 13 day old freshly harvested *T. pallidum* cultures revealed identical results to that of the whole-mount samples, at the same time intervals. Figure 6 shows representative thin sections indicating that a readily discernible outer membrane found on untreated control organisms (Figure 6 A-D) was lost upon treatment with TX-114 (Figure 6 A-D), while the appearance of the cytoplasmic membrane was unchanged. Therefore, it is reasonable to say that one of the reasons for the decrease in diameter of TX-114 treated *T. pallidum*, shown in Figure 4, is the selective release of the outer membrane

Figure 4. Effects of TX-114 treatment on *in vitro T. pallidum* ultrastructure. Whole-mount electron micrographs of untreated *T. pallidum* at time 0 (A), 10 (B), 15 (C), 20 (D) minutes and TX-114 treated *T. pallidum* at 0 (a), 10 (b), 15 (c), 20 (d) minutes, demonstrating the differences in diameters of untreated and TX-114 treated *T. pallidum.* The arrow dead in panel (a) indicates the outer membrane blebbing of the TX-114 treated organisms at time less than one minute. Unwound periplasmic flagella (arrow) attached to the cytoplasmic cylinder are shown in panels (c) and (d).

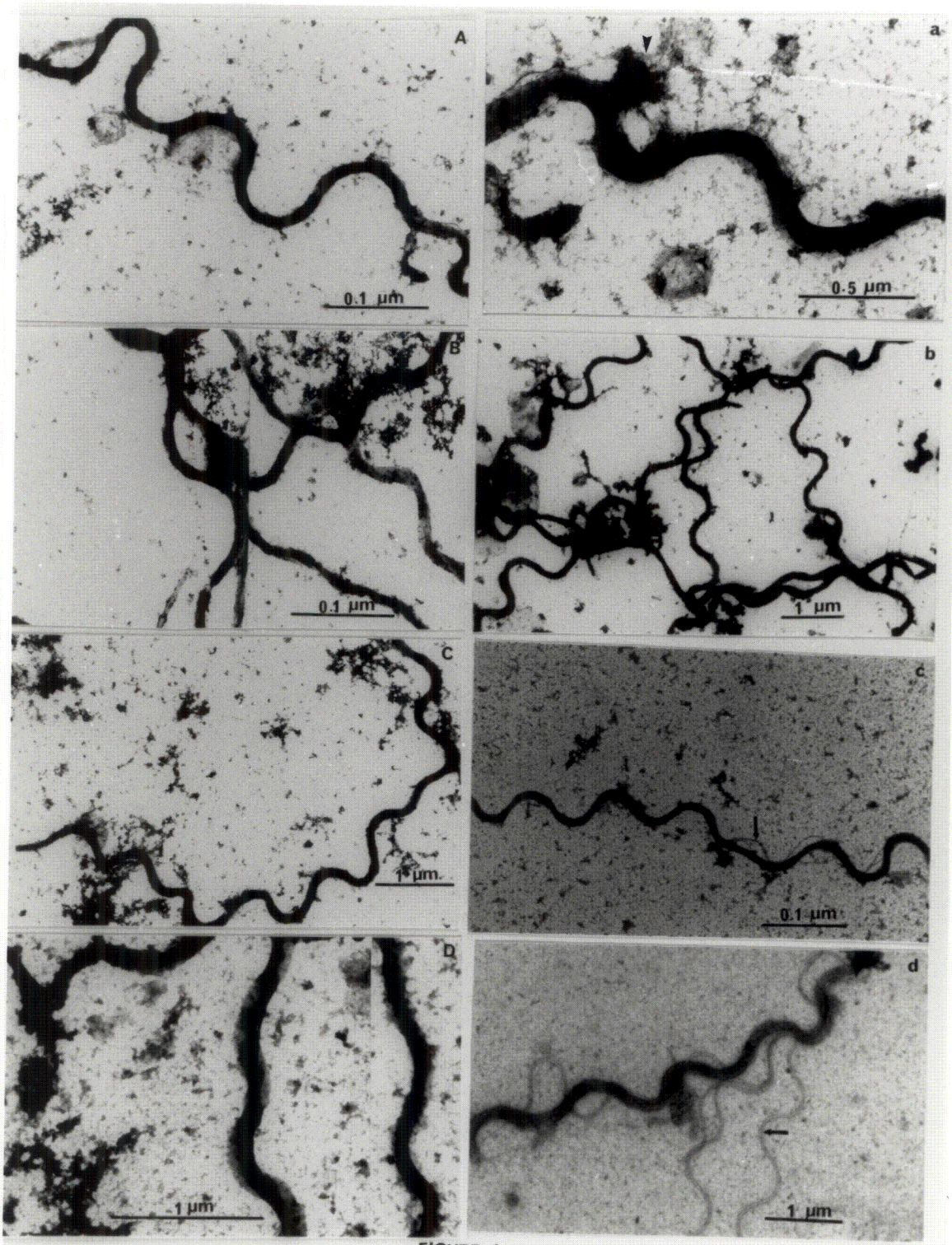


FIGURE: 4

Figure 4 continued. Effects of TX-114 treatment on *in vitro T. pallidum* ultrastructure. Higher magnification of whole-mount electron micrographs of untreated *T. pallidum* at time 0 (A'), 10 (B') minutes and TX-114 treated *T. pallidum* at 0 (a'), 10 (b'), minutes, demonstrating the differences in diameters of untreated and TX-114 treated *T. pallidum.* The arrow head in panels (A') and (B') indicates the outer membrane present in the control groups.

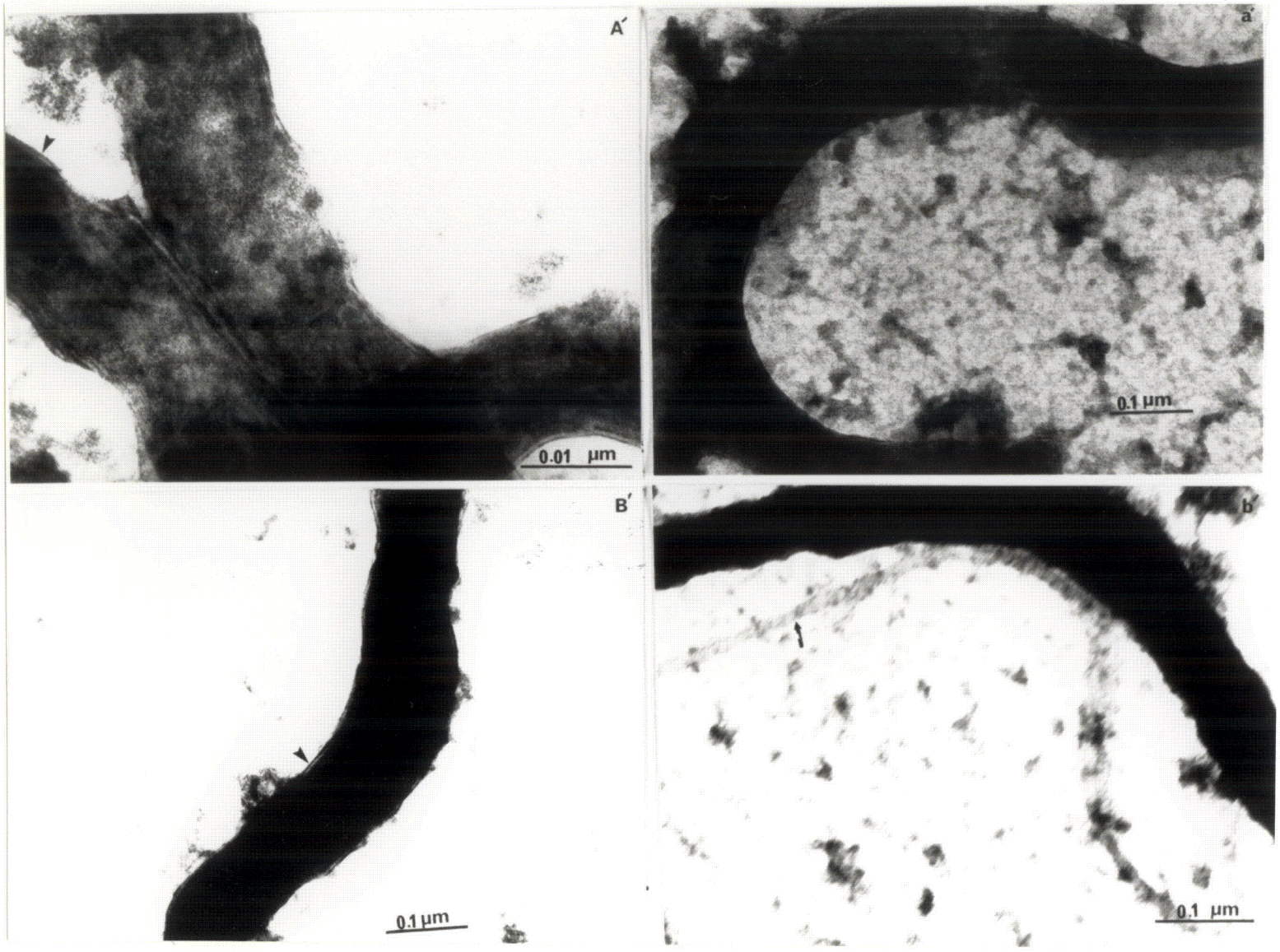

A´
0.01 µm
a´
0.1 µm
B´
0.1 µm
b´
0.1 µm

Figure 4 continued. Effects of TX-114 treatment on *in vitro T. pallidum* ultrastructure. Higher magnification of whole-mount electron micrographs of untreated *T. pallidum* at times 15 (C'), 20 (D') minutes and TX-114 treated *T. pallidum* at 15 (c'), 20 (d'), minutes, demonstrating the differences in diameters of untreated and TX-114 treated *T. pallidum.* The arrow head in panel (C) indicates the intact outer membrane. Unwound periplasmic flagella (arrow) attached to the cytoplasmic cylinder are shown in panels (c') and (d'). Parallel striations in panel C' are adherent periplasmic flagella (arrow).

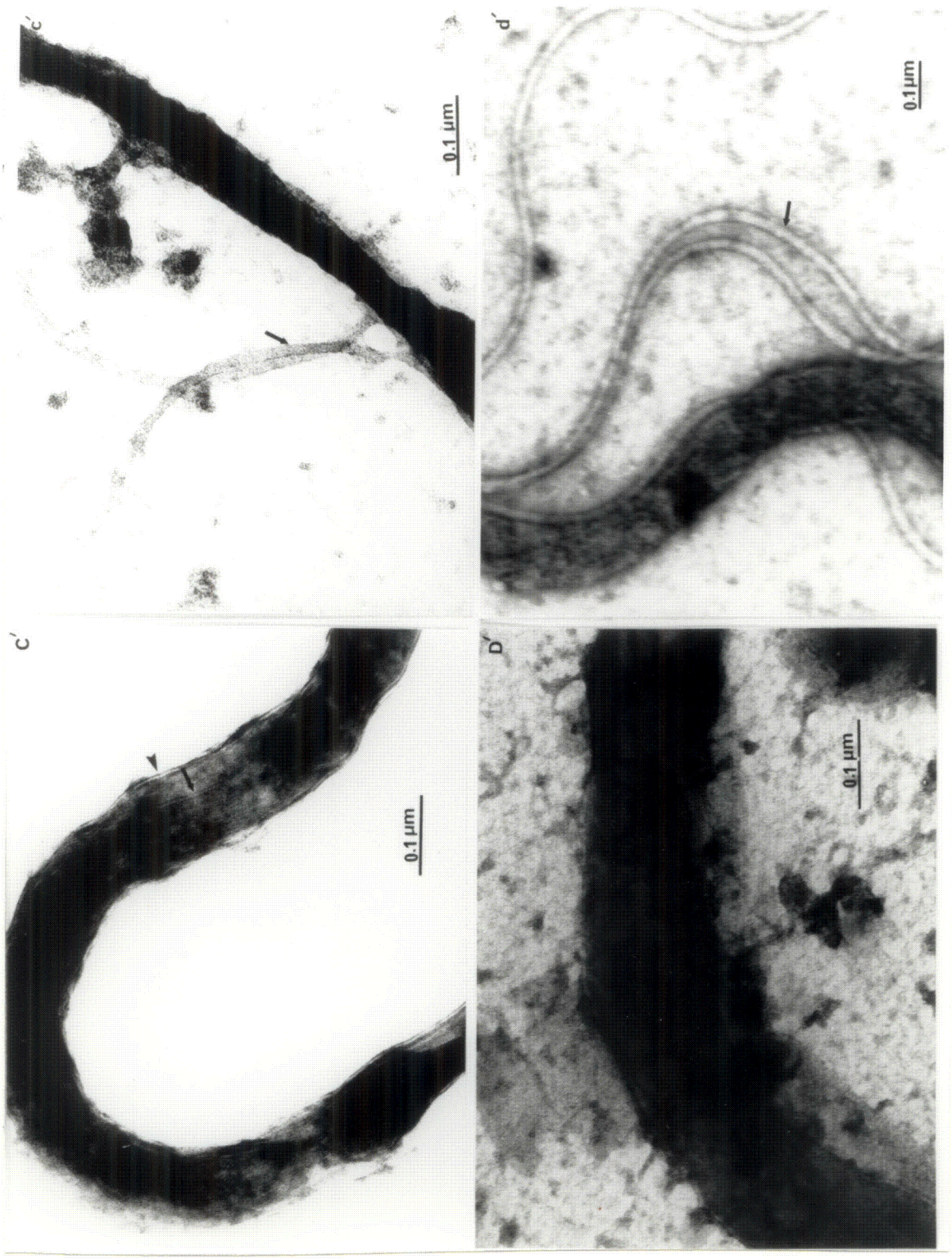

c'
0.1 µm
d'
0.1µm
C'
0.1 µm
D'
0.1 µm

Figure 5. Effects of TX-114 treatment on *T. pallidum* ultrastructure. Electron micrographs of *T. pallidum* before and after treatment with TX-114. Untreated (arrow-head) and 20 minutes TX-114 treated (arrow) organisms were mixed on the same grid demonstrating that differences in diameters were not artifacts of processing on separate grids for electron microscopy.

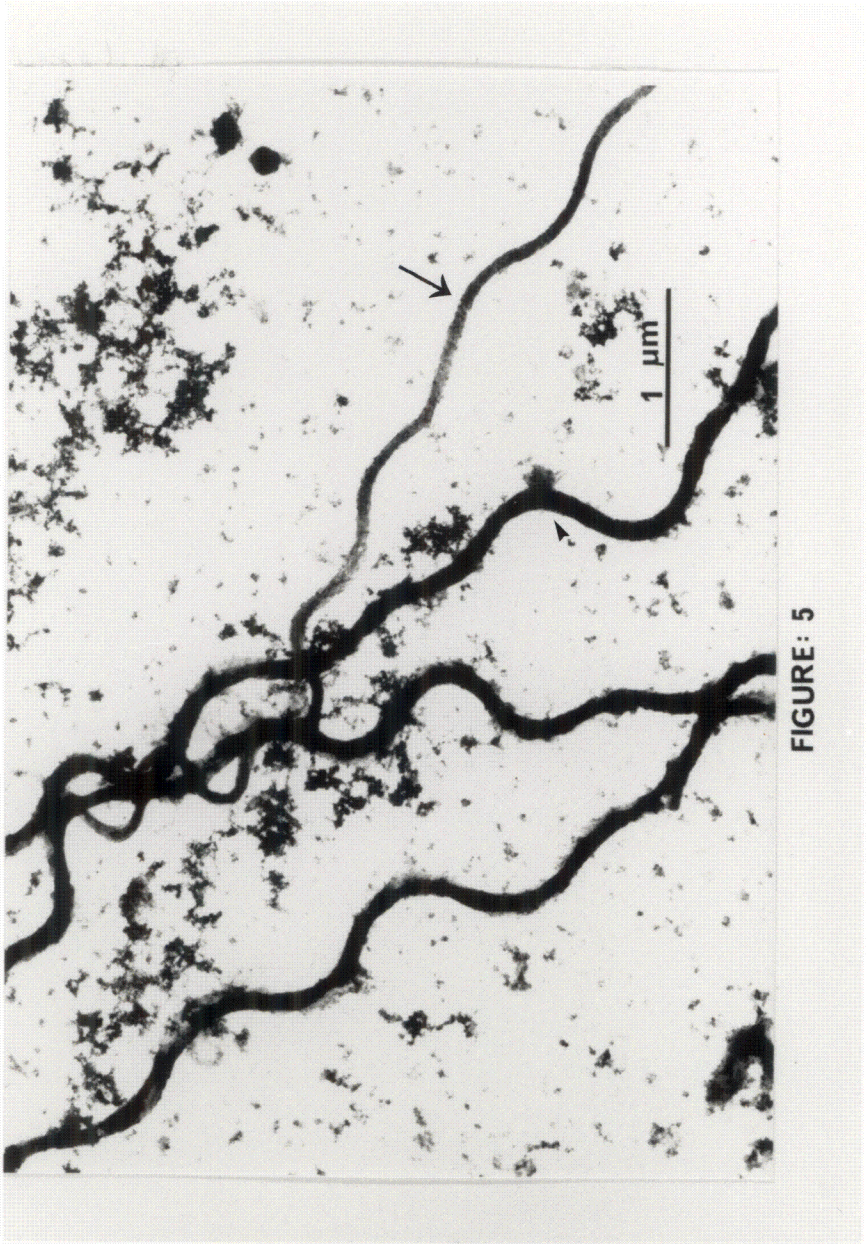


FIGURE: 5

Figure 6. Release of the *T. pallidum* outer membrane by TX-114. Electron micrographs show thin sections of freshly harvested *in vitro T. pallidum.* Untreated control organisms retained both cytoplasmic (arrow) and outer membranes (arrowhead) at 0 (A), 10 (B), 15 (C) and 20 (D) minutes. The TX-114 treated organisms retained only the cytoplasmic membrane. The partial loss of outer membrane is seen in panel (a) at time 0. The total loss of outer membrane is shown at 10 (b), 15 (c), and 20 (d) minutes.

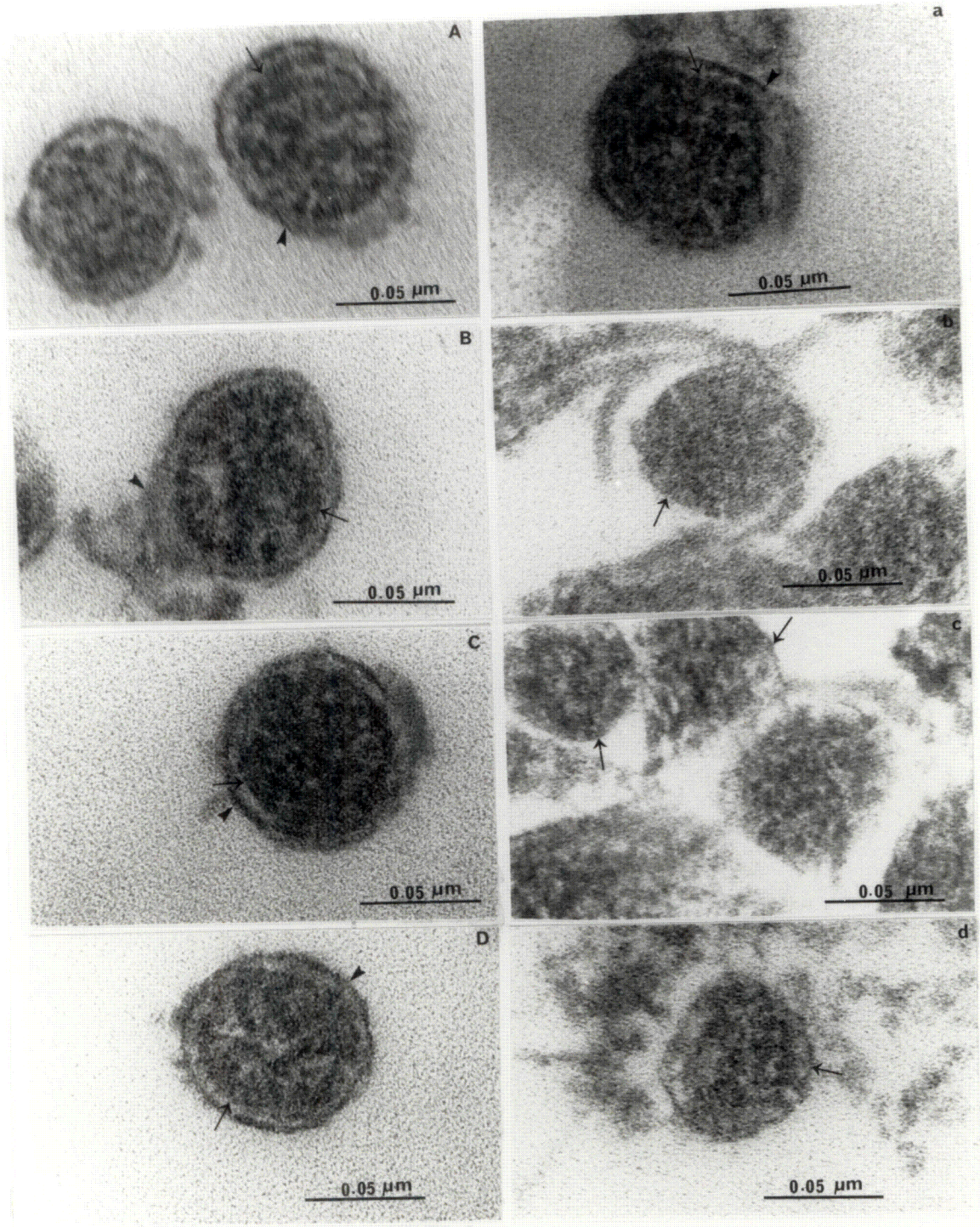


FIGURE : 6

Composition of fractions derived from TX-114 extraction and phase partitioning of *in vivo T. pallidum*. Figure 7 outlines the major steps in extraction and phase partitioning of freshly harvested *T. pallidum* with TX-114 as described in Materials and Methods. The panels in Figure 8 are silver stained two-dimensional SDS-PAGE, which show the results of TX-114 extraction and phase partitioning of 20 hour ^{35}S labeled *T. pallidum*. A total of 150 protein species with molecular weights sizing from 141.3 to 14.0 kDa with pI ranging from 4.86 to 7.48 (Figure 8A) were detected by silver stain. Most of the cellular proteins of *T. pallidum* cytoplasmic cylinders remained in the insoluble pellet (Figure 8B) after TX-114 treatment. However, polypeptides of 40, 38.5, 27.5, 25.7, 24.8, and 16 kDa (all with pI 4.86); 38, 34.7 kDa (pI 5.15); 120.2, 56.2, 38.5, and 35.5 kDa (pI 5.34); two 141.3, two 104.3, 74.1, 51.3, two 45.7, two 41.7, 36.3 kDa (all with pI 5.63); 22 kDa (pI 6.32); 43.7 kDa (pI 5.72); 38.5 (pI 5.90); 34.7, 28.8 (pI 6.05); and 38 kDa (pI 6.58) were consistently extracted in all the six experiments performed with TX-114. The aqueous phase (Figure 8C) contained all the 120.2 kDa (pI 5.34); 74.1 and 51.3 kDa (pI 5.62); 22 kDa (pI 6.32); 16 kDa (pI 4.60) polypeptides and part of the 38 kDa (pI 6.58) polypeptide. The rest of the polypeptides including part of 38 kDa (pI 6.58) were present in the detergent phase (Figure 8D). The detergent and the aqueous phase polypeptides do not represent the actual molar concentration present in the organism, since both samples were enriched for the purpose of detection. Figure 8E is a silver stained two-dimensional SDS-PAGE of the FBS used in cultivation of *T. pallidum*. It is quite obvious that all the contaminating FBS polypeptides [marked as (▲) in all the panels in figure 8] end up in the aqueous phase after extraction and phase partitioning. The detergent phase contains exclusively *T. pallidum* polypeptides.

Figure 7. Schematic presentation of TX-114 extraction and phase partitioning procedure. Freshly harvested *T. pallidum* was extracted in 1% TX-114 at 4°C for 20 minutes as described in Materials and Methods. Particulate material (i.e. cytoplasmic cylinders) were removed by centrifugation (20,000x *g* for 30 minutes at 4°C). A second centrifugation (13,000x *g* for 10 min. at 25°C), followed incubation (10 minin in a 37°C water bath) of TX-114 extracted material, resulted in the formation of an aqueous phase and a detergent phase.

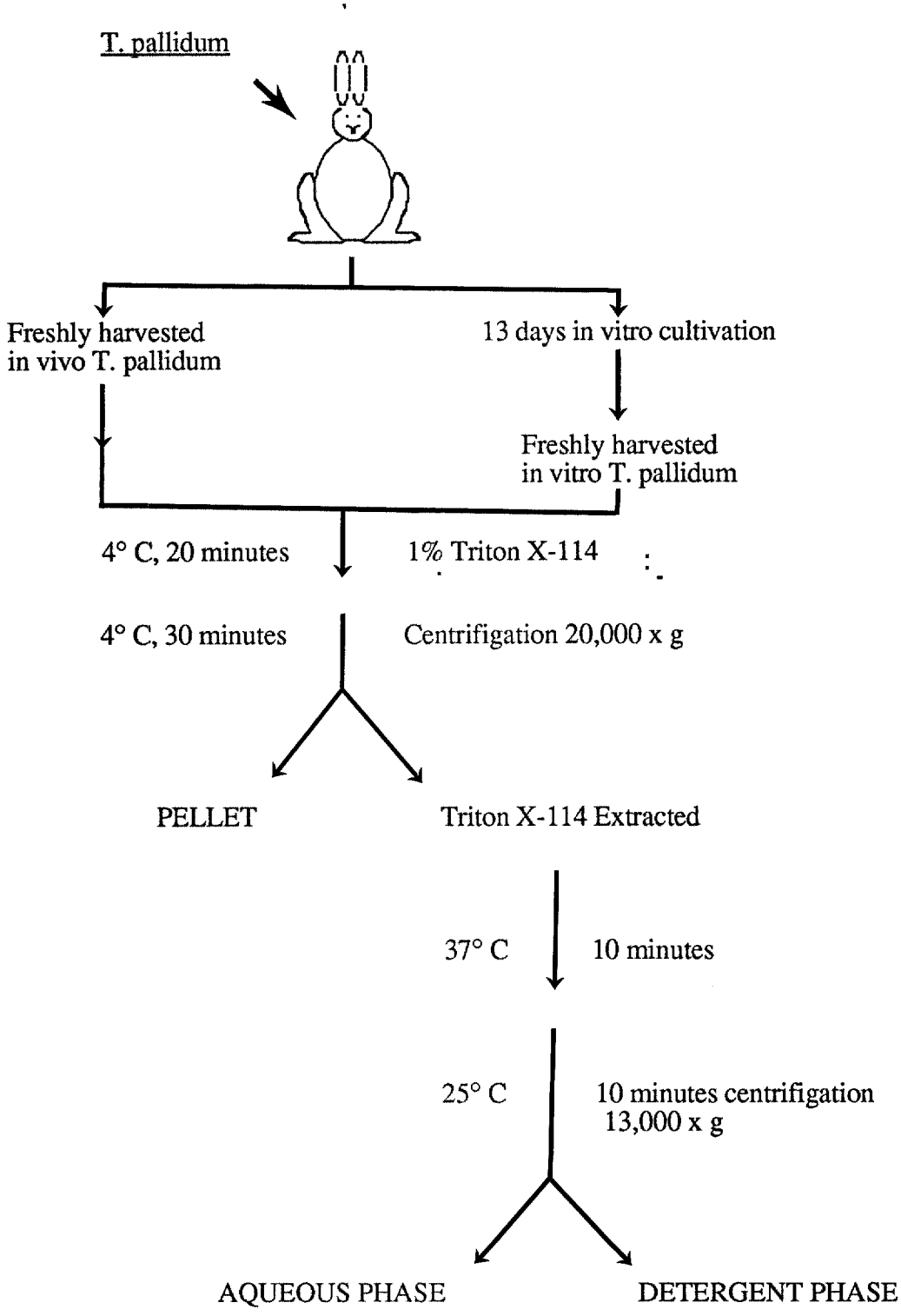
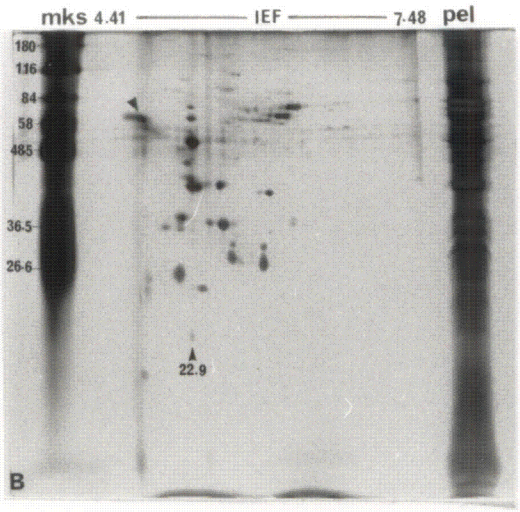

T. pallidum
Freshly harvested
in vivo T. pallidum
13 days in vitro cultivation
Freshly harvested
in vitro T. pallidum
4° C, 20 minutes
1% Triton X-114
4° C, 30 minutes
Centrifigation 20,000 x g
PELLET
Triton X-114 Extracted
37° C
10 minutes
25° C
10 minutes centrifigation
13,000 x g
AQUEOUS PHASE
DETERGENT PHASE

Figure 8. Composition of fractions derived from TX-114 extraction and phase partitioning of 20 hour ^{35}S labeled *in vivo* proteins of *T. pallidum* subsp. *pallidum*. Figure shows two dimensional protein profiles of whole *T. pallidum* (A), pellet (cytoplasmic cylinders) (B), aqueous phase (C) and detergent phase (D) as detected by silver staining. Isoelectric focusing (IEF, pH 4.41 - 7.48) was performed in 4% polyacrylamide tube gels which were then applied to 10% SDS-PAGE gels for the second dimension. As a reference, one-dimension SDS-PAGE separations of *T. pallidum* whole cell sonicate (wsc), pellet (pel), aqueous phase (aqu), detergent phase (det), and prestained sigma M_r standards (mks) were also run in the same gel. Panel E is a silver stained two dimensional 10% SDS-PAGE of the FBS used in cultivation of *T. pallidum*. The contaminating serum proteins in all panels are indicated by (▲). Each gel in this figure is representative of six separate experiments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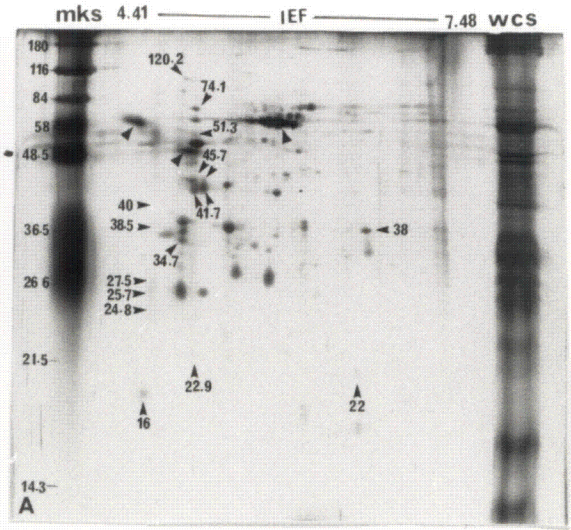
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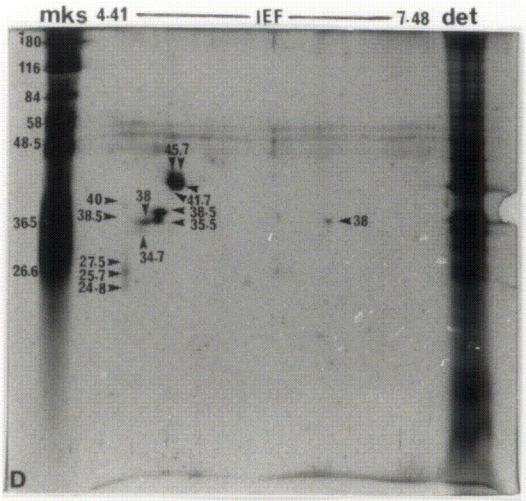
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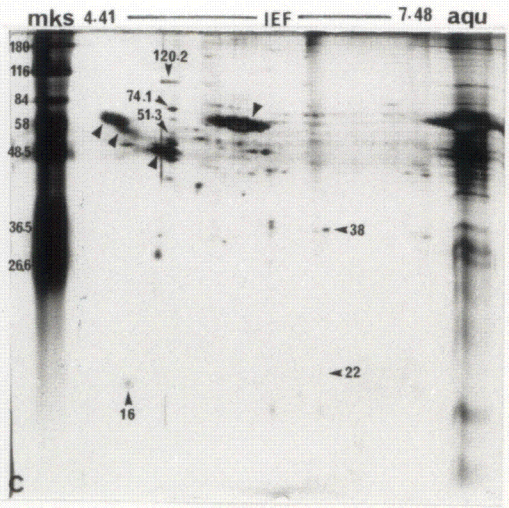
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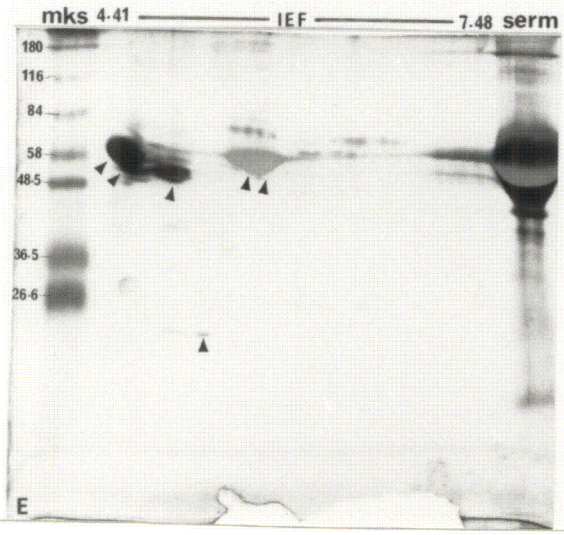
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E

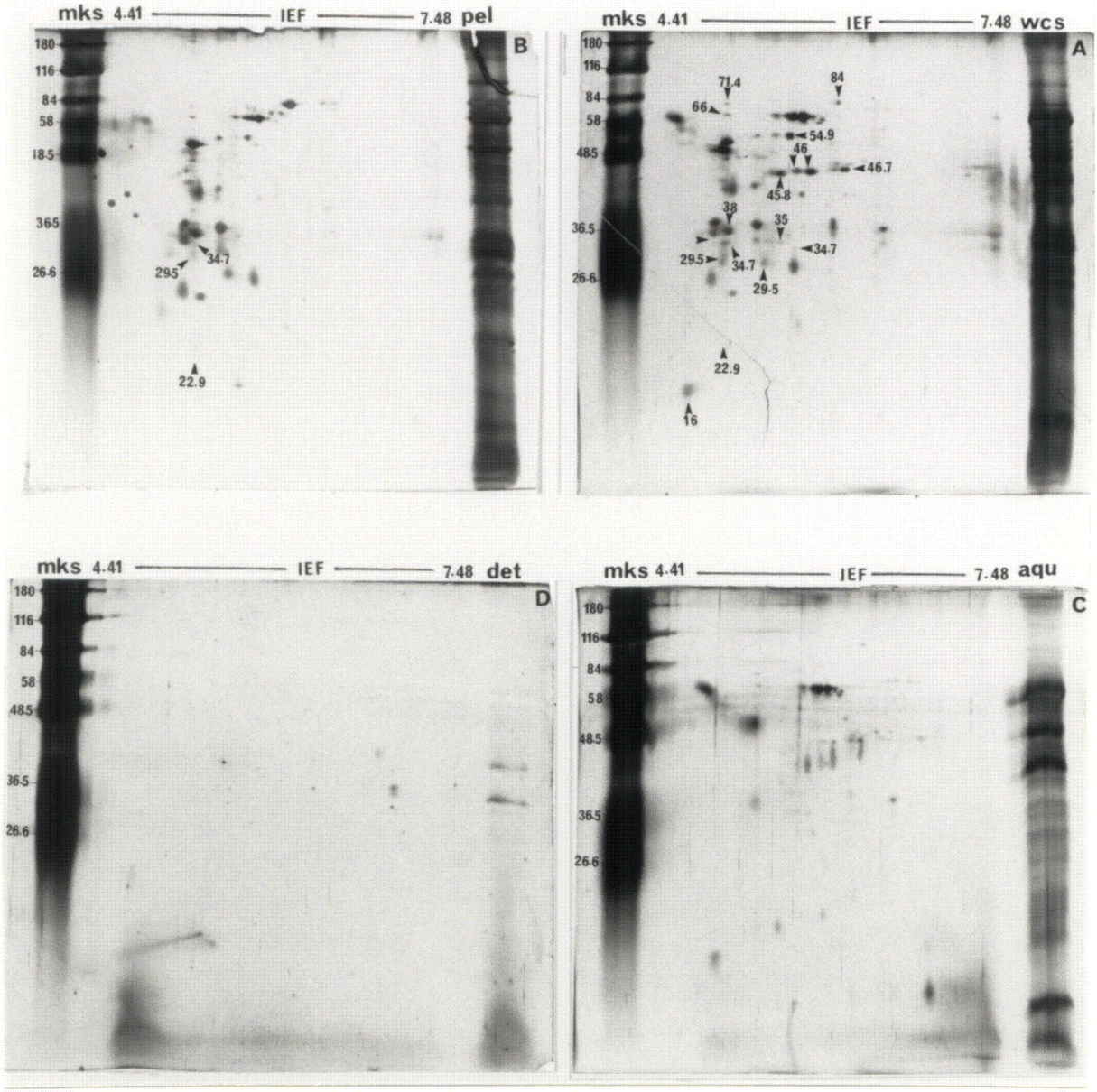
Composition of fractions derived from TX-114 extraction and phase partitioning of *in vitro T. pallidum*. Freshly harvested thirteen day old cultures of *in vitro* ^{35}S radiolabeled *T. pallidum* were extracted with TX-114 and phase partitioned as previously described. Silver stained two dimensional SDS-PAGE panels presented in Figure 9 demonstrate the differences between polypeptide profiles of the *in vitro* and *in vivo T. pallidum*. A total of 151 protein species (M_r 141.3-14.0, pI 4.86-7.48) were detected by silver stain (Figure 9A). An increase in the concentration of several polypeptides, mainly the 54.9 kDa (pI 6.05), 46.7 kDa (pI 6.32), two 46 kDa (pI 6.05), and 45.8 kDa (pI 5.96) was observed in the *in vitro* cultured *T. pallidum* (Figure 9A). Some polypeptide concentrations, including the 35 kDa (pI 5.96), 66 and 74.1 kDa (pI 5.62), 29.5 kDa (pI 5.90) and 34.7 kDa (pI 6.05) decreased in the *in vitro* cultured organisms (Figure 9A). Most importantly, two newly *in vitro* synthesized polypeptides with molecular weight of 29.5 and 34.7 kDa (both with pI of 5.62) were detected (Figure 9A). Also, of equal importance was the total loss of an antigenic (as judged by immunoblot, Figure 11A) polypeptide with molecular weight of 34.7 kDa (pI 5.34) (Figures 9A and 8A). Both *in vitro* synthesized polypeptides remained associated with the pellet as shown in Figure 9B. The autoradiograms of ^{35}S labeled proteins of *in vitro* (Figure 10 a-d) and *in vivo* (Figure 10 A-D) organisms represent the same differences. Although it seems that the 20 hour labeled *in vivo* organisms took up less label, the presence of the two *in vitro* synthesized polypeptides (29.5 and 34.7 kDa, pI 5.62) and the absence of 34.7 kDa (pI 5.34) are clearly evident (Figure 10 A and a). Moreover, in addition to all the contaminating serum proteins, five polypeptides: 38 kDa (pI 5.34), 45.8 kDa (pI 5.96), two 46 kDa (pI 6.05) and 46.7 kDa (pI 6.32) were not labeled with ^{35}S in both *in vitro* and *in vivo* organisms (Figure 10A and a) giving a total of approximately 120 ^{35}S labeled polypeptides (M_r 84 - 14 kDa, pI 4.86 - 7.48) for the *in vivo* organisms

and 121 ^{35}S labeled polypeptides (with the same M_r and pI ranges as the *in vivo* polypeptides) for the *in vitro* organisms (Figure 10 A and a).

Fate of *in vitro* and *in vivo T. pallidum* Antigens in TX-114 Extraction and Phase Partitioning.

Antigenic polypeptides detected by human IgG antibodies. Immunoblots of whole *T. pallidum* and the fractions derived from TX-114 extraction and phase partitioning was prepared. Figure 11 shows the two dimensional SDS-PAGE profile (with flanking single dimension *T. pallidum* and M_r standard lanes) of *T. pallidum* as determined by immunoperoxidase staining. The electroblots were probed with pooled human sera from patients with secondary syphilis at a 1:100 dilution and detected with horseradish peroxidase conjugated goat anti-human IgG at 1:5000 dilution. The human sera bound to a total of 59 polypeptide species of the *in vivo* whole *T. pallidum* (Figure 11A) and a total of 56 polypeptide species of *in vitro* whole *T. pallidum* (Figure 11A) (all molecular weights ranging from 141.3 - 22.9 and pI 4.86 -6.58). The immunoblot of the *in vitro* whole *T. pallidum* (Figure 11a) clearly demonstrates the absence of the binding of human syphilitic sera to the two *in vitro* synthesized polypeptides (29.5 and 34.7 kDa, pI 5.62) and the *in vitro* lost 34.7 kDa (pI 5.34) polypeptide. The immunoblot of the *in vivo* whole *T. pallidum* (Figure 11A) clearly demonstrates the binding of human syphilitic sera to the *in vitro,* "lost", 34.7 kDa (pI 5.34) polypeptide as expected. The human syphilitic sera did not show any binding to the four polypeptides: 45.8 kDa (pI 5.96), two 46 kDa (pI 6.05) and 46.7 kDa (pI 6.32) (Figure 11 A and a). These polypeptides, however, were present in both *in vitro* (Figure 9A) and *in vivo* (Figure 8A) *T. pallidum* as stated before. Interestingly, human syphilitic sera binds to a 14 kDa polypeptide in the one-dimension flanking reference lane while it does not bind to any polypeptide of the same molecular weight in the two dimension profiles of both *in vitro* and *in vivo T.pallidum.* This 14 kDa polypeptide seems to be one of the detergent phase

Figure 9. Composition of fractions derived from TX-114 extraction and phase partitioning of *in vitro* grown, ^{35}S labeled proteins of *T. pallidum* subsp. *pallidum*. Figure shows two-dimensional protein profiles of whole *T. pallidum* (A), pellet (B), aqueous phase (C), and detergent phase (D) (with flanking single dimension reference and M_r standard lanes) as detected by silver staining. The gels were prepared as described in Figure 8. The positions of the two *in vitro* synthesized polypeptides (29.5 and 34.7 kDa, pI 5.62) and the *in vitro* lost polypeptide (34.7 kDa, pI 5.34) are indicated in panels(A) and (B). Each gel in this figure is representative of six separate experiments.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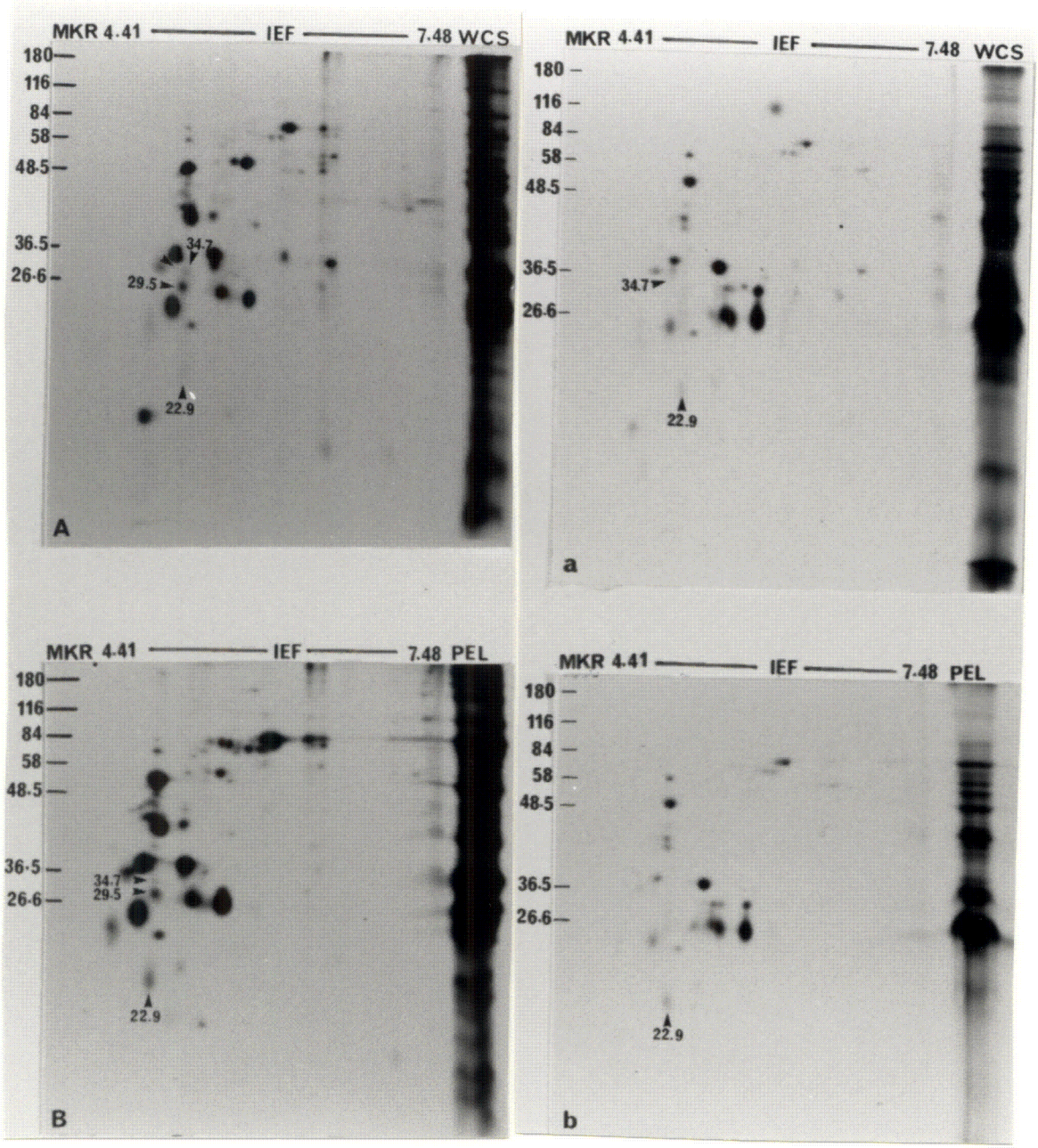
35
34.7
29.5
34.7
29.5
22.9
16
mks 4.41 IEF 7.48 det
D
mks 4.41 IEF 7.48 aqu
C

Figure 10. Composition of fractions derived from TX-114 extraction and phase partitioning of *in vitro* and *in vivo T. pallidum* subsp. *pallidum*. Figure shows the autoradiograms of whole (A) and pellet (B) of *in vitro* proteins of *T. pallidum*; whole (a) and pellet (b) of *in vivo* proteins of *T. pallidum*. The two dimensional SDS-PAGE was performed as described in Figure 8. The presence of the two *in vitro* synthesized polypeptides (29.5 and 34.7 kDa, pI 5.62) and the absence of *in vitro* lost 34.7 kDa (pI 5.34) polypeptide, are shown in panels (a) and (A). Panel (B) demonstrates the association of these polypeptides with the cytoplasmic cylinders. Each gel in this figure is representative of six separate experiments.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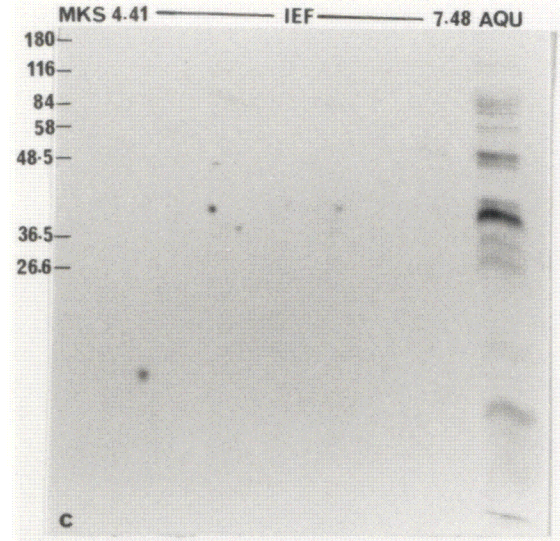
116
84
58
48.5
36.5
26.6
34.7
29.5
22.9
A
MKR 4.41
IEF
7.48 WCS
180
116
84
58
48.5
36.5
26.6
34.7
22.9
a
MKR 4.41
IEF
7.48 PEL
180
116
84
58
48.5
36.5
26.6
34.7
29.5
22.9
B
MKR 4.41
IEF
7.48 PEL
180
116
84
58
48.5
36.5
26.6
22.9
b

Figure 10 continued. Composition of fractions derived from TX-114 extraction and phase partitioning of *in vitro* and *in vivo T. pallidum* subsp. *pallidum*. Figure shows the autoradiograms of aqueous phase (C) and detergent phase (D) of *in vitro* proteins of *T. pallidum*; aqueous phase (c) and detergent phase (d) of *in vivo* proteins of *T. pallidum*. The two dimensional SDS-PAGE was performed as described in Figure 8. Each gel in this figure is representative of six separate experiments.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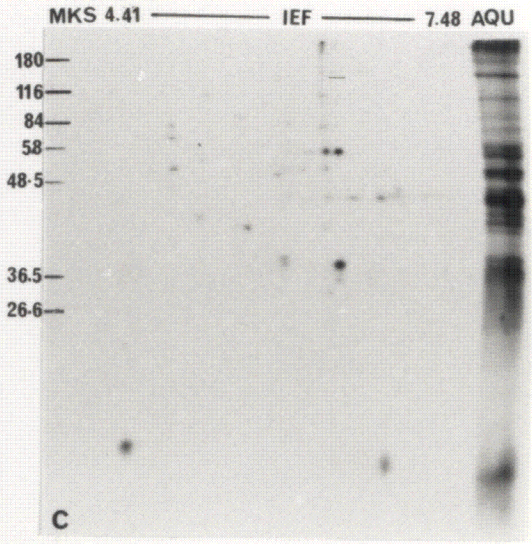
IEF
7.48 AQU
180
116
84
58
48·5
36·5
26·6
c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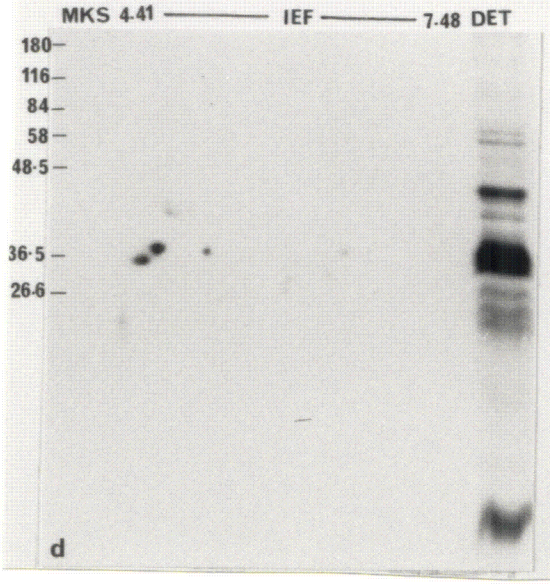
IEF
7.48 AQU
180
116
84
58
48·5
36.5
26·6
C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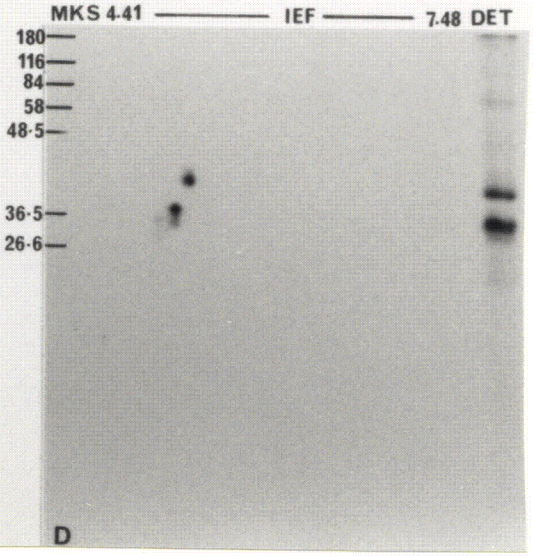
IEF
7.48 DET
180
116
84
58
48·5
36·5
26·6
d

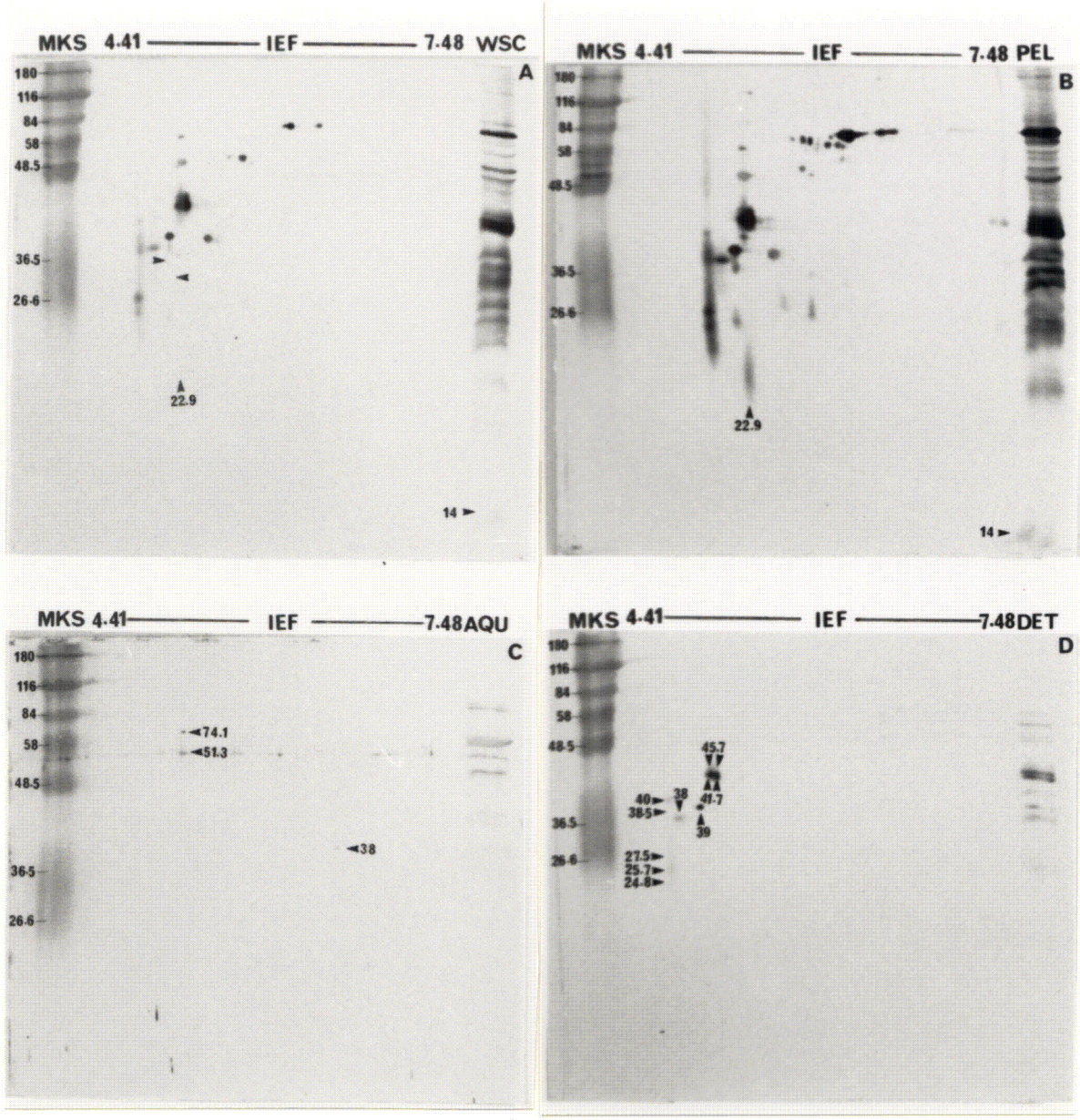

MKS 4.41
IEF
7.48 DET
180
116
84
58
48·5
36·5
26·6
D

polypeptides extracted with TX-114. The immunoblots of the *in vivo* (Figure 11a) and *in vitro* (Figure 11A) whole *T. pallidum* demonstrate seven highly antigenic [compared to their molar concentration as judged by the silver staining (Figures 8A and 9A) and autoradiograms (Figure 10 A and a)] polypeptides of molecular weights: 40, 38.5, 27.5, 25.7 and 24.8 kDa (pI 4.86), 38 kDa (pI 5.15) and 22.9 kDa (pI 5.62). The immunoblot of the *in vivo* (Figure 11B) and *in vitro* (Figure 11B) pellets indicate that the 22.9 kDa (pI 5.62) polypeptide remained associated with the cytoplasmic cylinders of *T. pallidum* after TX-114 extraction (Figure 11B and b). The immunoblot of the *in vivo* (Figure 11d) and *in vitro* (Figure 11D) detergent phase indicate that the 40, 38.5, 27.5, 25.7, 24.8, kDa (pI 4.86) and 38 kDa (pI 5.15) were associated with the detergent phase after TX-114 extraction and phase partitioning. In addition to these polypeptides, the immunoblot of the detergent phase of the *in vivo* and *in vitro T. pallidum* demonstrated at least five more antigenic polypeptides of molecular weights 39, 41.7, 45.7 kDa (pI 5.34) and 41.7, 45.7 kDa (pI 5.62). The immunoblot of the detergent phase of the *in vivo T. pallidum* demonstrated nine polypeptides in addition to the ones mentioned above. Apparently the TX-114 extraction had lasted slightly longer than 20 minutes, which is sufficient to solubilize some of the proteins in the cytoplasmic membrane. To determine whether any of the detergent phase proteins represented endoflagellar contaminants, immunoblots were reprobed with monospesific antiserum directed against the antigenically conserved endoflagella of the nonpathogenic *T. phagedenis* biotype Reiter (generous gift from Dr. V. Norgard at the University of Texas Southewestern Medical Center). This antiserum did not react with any of the proteins found in the detergent phase (Figures 11 d and D). The immunoblots of the aqueous phase of the *in vitro* (Figure 11C) and *in vivo* (Figure 11c) *T. pallidum* after TX-114 extraction and phase partitioning indicates three antigenic polypeptides with molecular weights 74.1, 51.3 kDa (pI 5.62) and 38 kDa (pI 6.58).

Antigenic polypeptides detected by human IgM antibodies. Immunoblots of whole *T. pallidum* and fractions derived from TX-114 extraction and phase partitioning were prepared as previously mentioned, with the exception that the secondary antibody was horseradish peroxidase conjugated goat anti-human IgM antibody. The immunoblot of whole *in vitro T. pallidum* demonstrated five antigenic polypeptides of approximate molecular weights 45.7 kDa (pI 5.34),41.7 kDa (pI 5.34), 38 kDa (pI 5.15), 27.5 kDa (pI 4.86), and 22.9 kDa (pI 5.34). All of these polypeptides except the 22.9 kDa, were extracted by TX-114 and were present in the detergent phase after phase partitioning. The 22.9 kDa polypeptide remained associated with the cytoplasmic cylinders. The aqueous phase polypeptides did not show any binding to human anti-treponemal IgM antibodies. Since, the IgM antibody response seemed to be weak and not extensive the IgM studies were not done with the *in vivo T. pallidum* or with *T. phagedenis*.

Composition of fractions derived from TX-114 extraction and phase partitioning of nonpathogenic *Treponema phagedenis* biotype Reiter. The panels in Figure 12 A-D are silver stained two-dimensional SDS-PAGE of *T. phagedenis* one of the nonpathogenic counter parts of *T. pallidum*. The silver stain of ^{35}S labeled proteins of *T. phagedenis* detected 173 polypeptide species with molecular weights ranging from 200 - 20.9 kDa with pI sizing from 4.60 to 7.48 (Figure 12A). Figure 13A represents the autoradiogram of ^{35}S labeled proteins of *T. phagedenis* detecting 237 polypeptide species with molecular weights sizing from 200 to 12 kDa and pI ranging from 4.60 to 7.48. Figure 12B represents two-dimensional silver stained profile of *T. phagedenis* cytoplasmic cylinders which remained insoluble in TX-114 after 20 minutes extraction. A total of 107 polypeptide species with molecular weights sizing from 200 to 24 kDa and pI ranging from 4.60 to 7.48 were detected by silver stain.

Figure 11. Fate of antigens in TX-114 extraction and phase partitioning of *in vitro T. pallidum.* Figure shows immunoperoxidase staining of two dimensional electroblots of whole (A), pellet (B), aqueous phase (C), detergent phase (D) of *in vitro T. pallidum.* Electroblots of two dimensional protein profiles obtained with *T. pallidum* fractions were prepared as described in Figure 8. The electroblots were reacted sequentially with a 1:100 dilution of pooled sera from patients with secondary syphilis, a 1:5000 dilution of peroxidase conjugated goat anti-human IgG, and 4-chloro-naphthol as the peroxidase substrate. The absence of reactivity of the two *in vitro* synthesized (29.5 and 34.7 kDa, pI 5.62) and the *in vitro* lost (34.7 kDa, pI 5.34) polypeptides are demonstrated in panel A. The electroblot of the detergent phase (D) was reprobed with monospecific antibody directed against the endoflagella of *T. phagedenis* biotype Reiter using diaminobenzidine as the substrate. This antibody did not detect any flagellar antibody in the detergent phase proteins. Each gel in this figure is representative of six separate experiments.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A
180
116
84
58
48.5
36.5
26.6
22.9
14
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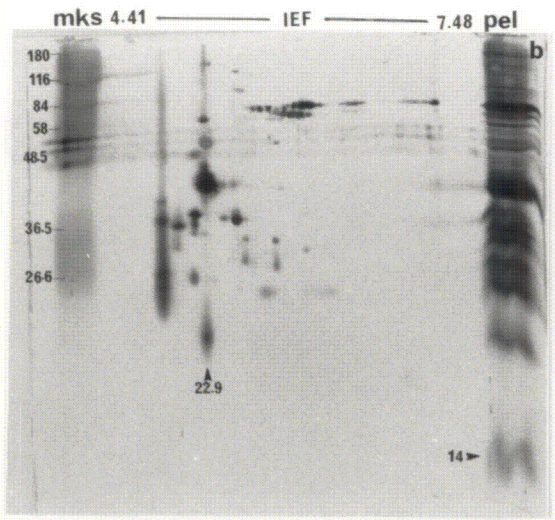
B
180
116
84
58
48.5
36.5
26.6
22.9
14
MKS 4-41 IEF 7-48AQU
C
180
116
84
58
48.5
36.5
26.6
74.1
51.3
38
MKS 4-41 IEF 7-48DET
D
180
116
84
58
48.5
36.5
26.6
45.7
41.7
40
38.5
38
39
27.5
25.7
24.8

Figure 11 continued. Fate of antigens in TX-114 extraction and phase partitioning of *in vivo T. pallidum.* Figure shows immunoperoxidase staining of two dimensional electroblots of whole (a), pellet (b), aqueous phase (c), detergent phase (d) of *in vivo T. pallidum.* Electroblots of two dimensional protein profiles obtained with *T. pallidum* fractions were prepared as described in Figure 8. The electroblots were reacted sequentially with a 1:100 dilution of pooled sera from patients with secondary syphilis, a 1:5000 dilution of peroxidase conjugated goat anti-human IgG, and 4-chloronaphthol as the peroxidase substrate. The presence of reactivity of the *in vitro* lost (34.7 kDa, pI 5.34) polypeptide is demonstrated in panels (a) and (b). The electroblot of the detergent phase (d) was reprobed with monospecific antibody directed against the endoflagella of *T. phagedenis* biotype Reiter using diaminobenzidine as the substrate. This antibody did not detect any flagellar antibody in the detergent phase proteins. Each gel in this figure is representative of six separate experiments.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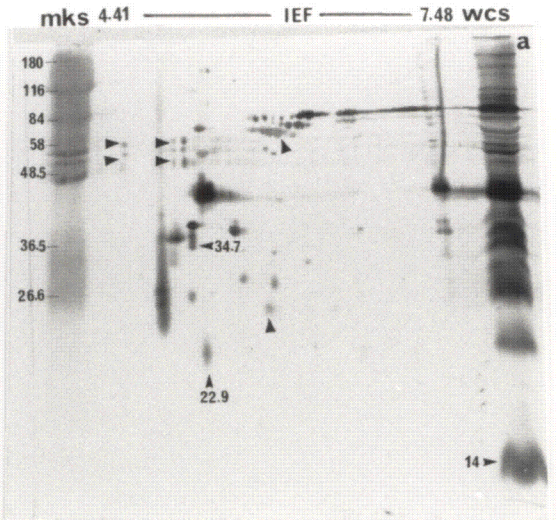
IEF
7.48 pel
b
180
116
84
58
48.5
36.5
26.6
22.9
14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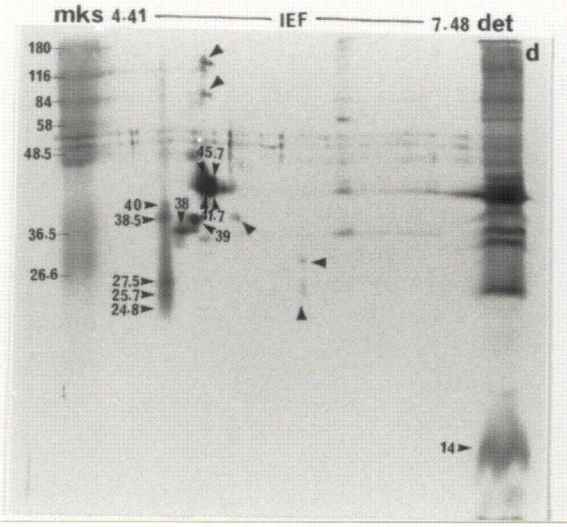
48.5
36.5
26.6
34.7
22.9
14


mks 4.41
IEF
7.48 det
d
180
116
84
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48.5
36.5
26.6
45.7
40
38.5
38
39
27.5
25.7
24.8
14

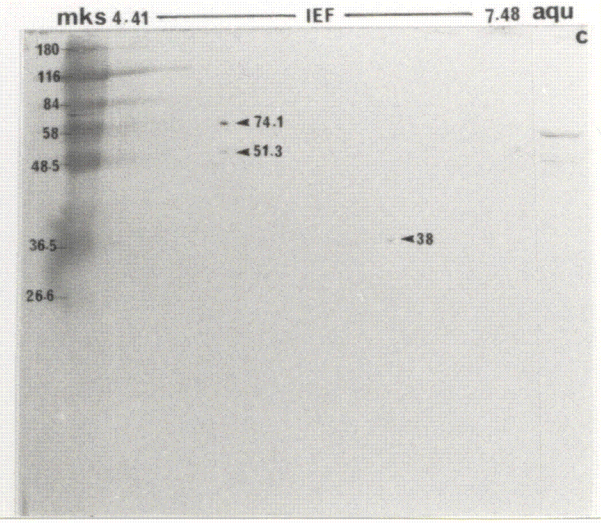
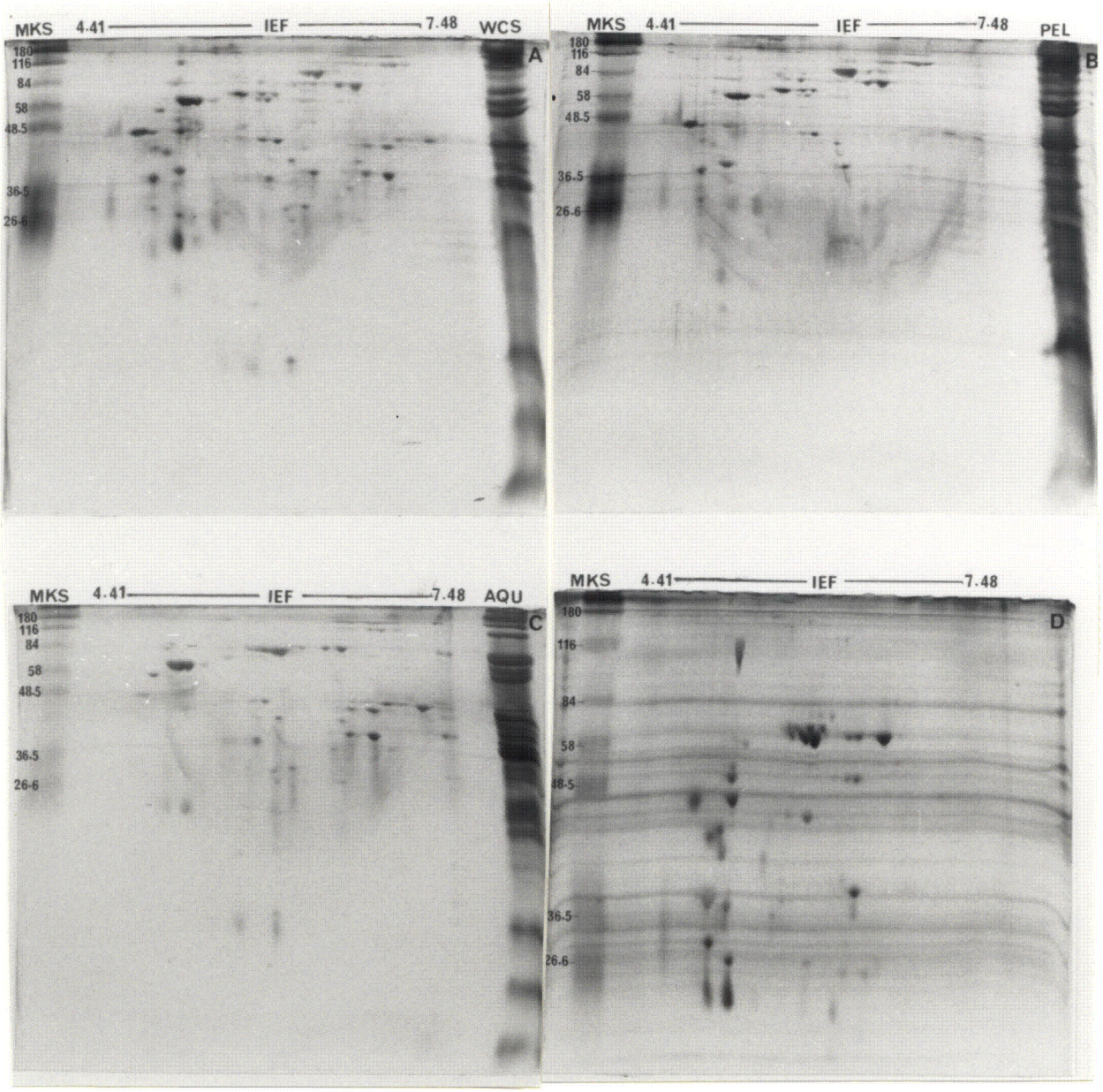

mks 4.41
IEF
7.48 aqu
c
180
116
84
58
48.5
36.5
26.6
74.1
51.3
38

Figure 13B represents the autoradiogram of the cytoplasmic cylinders detecting 128 polypeptide species with molecular weights sizing from 200 to 12 and pI ranging from 4.60 to 7.48. Figure 12D represents the detergent phase polypeptide species after TX-114 extraction and phase partitioning of *T. phagedenis*. A total of 79 polypeptide species were detected by silver stain with molecular weights sizing from 112.2 to 12.0 and pI ranging from 4.60 to 7.48. The autoradiogram (Figure 13D) of the detergent phase demonstrated the same polypeptides. The silver stained aqueous phase (Figure 12C) of TX-114 extraction and phase partitioning detected 100 polypeptides with molecular weights sizing from 200 to 20.9 kDa and pI ranging from 4.60 to 7.48. Figure 13C is the autoradiogram of the aqueous phase detecting 155 polypeptides with molecular weights ranging from 200 to 12 kDa and pI ranging from 4.60 to 7.48.

Fate of *T. phagedenis* biotype Reiter cross-reactive antigens with *T. pallidum* in TX-114 extraction and phase partitioning. Immunoblots of whole *T. phagedenis* and the fractions derived from TX-114 extraction and phase partitioning were prepared. Figure 14 A-D shows the two-dimensional SDS-PAGE of cross-reactive antigenic protein profiles of *T. phagedenis* as determined by immunoperoxidase staining. The electroblots were probed with pooled sera from patients with secondary syphilis at a 1:100 dilution and detected with peroxidase conjugated goat anti-human IgG at 1:5000 dilution. The human sera bound to a total of 46 cross-reactive polypeptide species of *T. phagedenis* (Figure 14A). Most of the cross-reactive polypeptides remained in the insoluble pellet (Figure 14B) after TX-114 treatment. However, polypeptides of 35.5 kDa (pI 4.60); 41.7, and 14.1 kDa (pI 4.86); 69.2, 45.7, 38, 35.5, 33.1, and 12.0 kDa (pI 5.34); 112.2,and 41 kDa (pI 5.62); 4l, and 50.1 kDa (pI 5.72); two 54 kDa (pI 6.18); two 52.5 kDa (pI 6.25); two 45.7, and 44.7 kDa (pI 6.32); 45, and 56.2 kDa (pI 6.49); 87.1 kDa (pI 6.92) and 41 kDa (pI 7.48) were consistantly extracted with TX-114. The

aqueous phase (Figure 14C) contained the cross-reactive proteins 69.2, 45.7 kDa (pI 5.34) 41 kDa (pI 5.62); 41 kDa (pI 5.72); two 45.7 kDa (pI 6.32); 87.1 kDa (pI 6.92) and 41 kDa (pI 7.48). The rest of the cross-reactive polypeptides were present in the detergent phase (Figure 14D). The detergent and the aqueous phase polypeptides do not represent the actual molar concentration present in *T. phagedenis*, since both samples were enriched for the purpose of the detection.

Figure 12. Composition of fractions derived from TX-114 extraction and phase partitioning of nonpathogenic *Treponema phagedenis* biotype Reiter. Figure shows silver stained two-dimensional SDS-PAGE profiles of ^{35}S labeled proteins of whole*T. phagedenis* (A), pellet (B), aqueous phase (C), and detergent phase (D). The gels were prepared as described in Figure 8 (with flanking one-dimension reference and M_r standard lanes). Each gel in this figure is representative of six separate experiments.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IEF
7.48
WCS
A
180
116
84
58
48.5
36.5
26.6
MKS
4.41
IEF
7.48
PEL
B
180
116
84
58
48.5
36.5
26.6
MKS
4.41
IEF
7.48
AQU
C
180
116
84
58
48.5
36.5
26.6
MKS
4.41
IEF
7.48
D
180
116
84
58
48.5
36.5
26.6

Figure 13. Composition of fractions derived from TX-114 extraction and phase partitioning of nonpathogenic *T. phagedenis*. Figure shows two dimensional autoradiograms of ^{35}S labeled proteins of whole *T. phagedenis* (A), pellet (B), aqueous phase (C), detergent phase (D). The gels were prepared as described in Figure 8 (with flanking one-dimension reference and M_r standard lanes). Each gel in this figure is representative of six separate experiments.

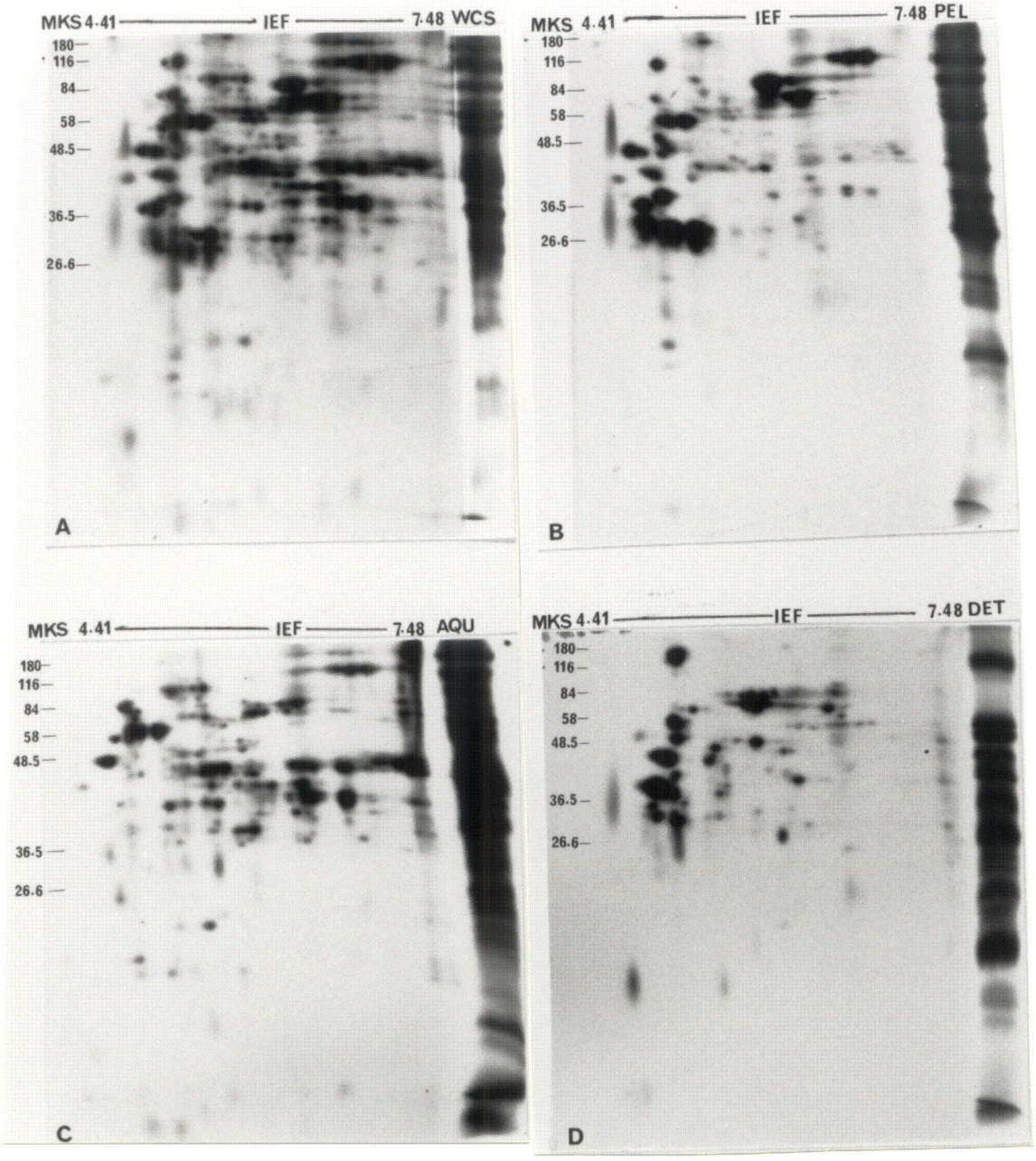
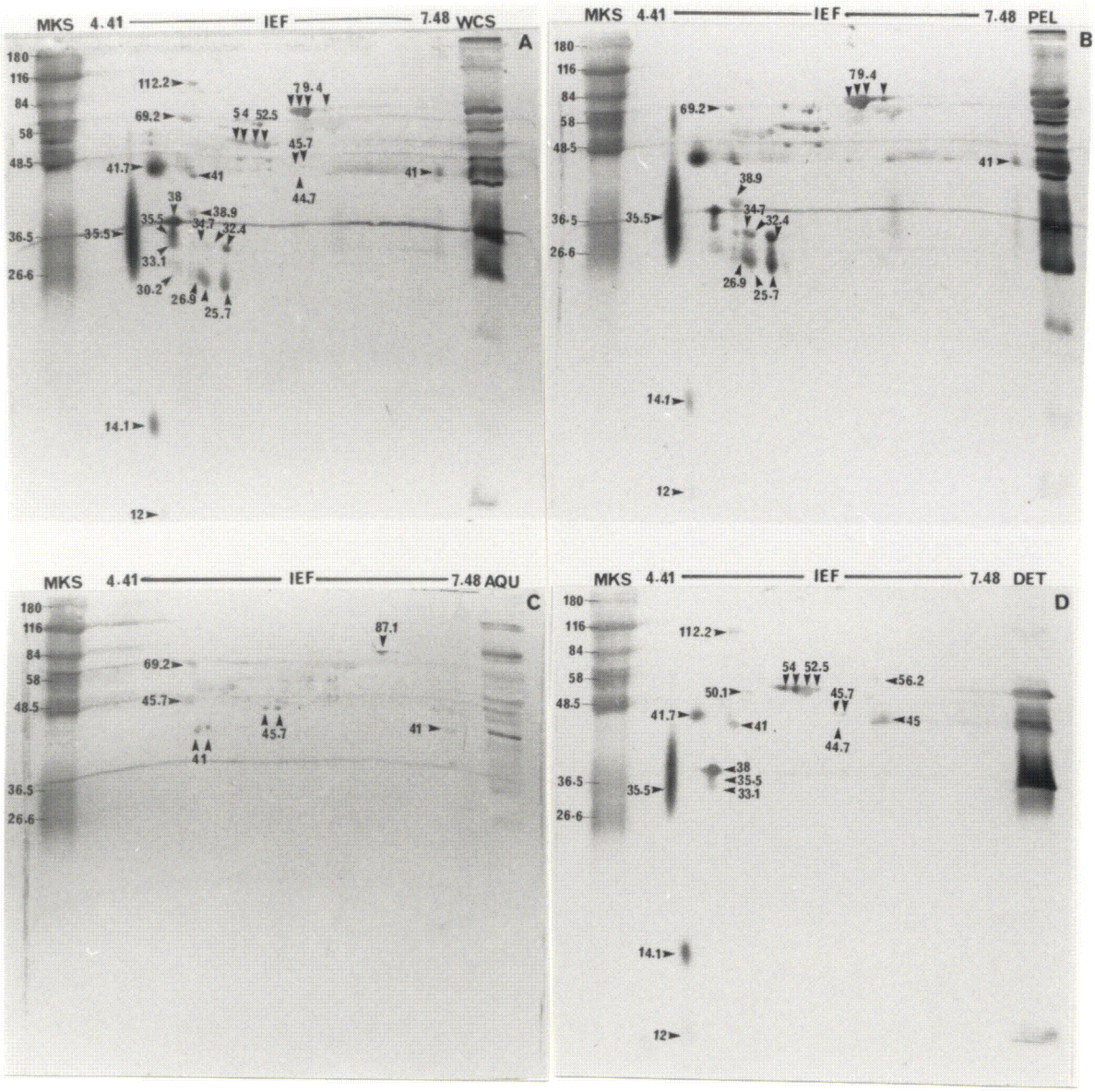

MKS 4·41
IEF
7·48 WCS
180
116
84
58
48.5
36.5
26.6
A
MKS 4·41
IEF
7·48 PEL
180
116
84
58
48.5
36.5
26.6
B
MKS 4·41
IEF
7·48 AQU
180
116
84
58
48.5
36.5
26.6
C
MKS 4·41
IEF
7·48 DET
180
116
84
58
48.5
36.5
26.6
D

Figure 14. Fate of *T. phagedenis* biotype Reiter cross-reactive antigens with *T. pallidum* Nichols strain, in TX-114 extraction and phase partitioning. Figure shows the two dimensional protein profiles of cross-reactive antigens in *T. phagedenis* and *T. pallidum*, as determined by immunoperoxidase staining with pooled sera from patients with secondary syphilis. Immunoblots of whole *T. phagedenis* (A), pellet (B), aqueous phase (C), detergent phase (D) were prepared as described in Figure 11. Each gel in this figure is representative of six separate experiments.


MKS 4.41 IEF 7.48 WCS
A
180
116
84
58
48.5
36.5
26.6
112.2
69.2
54 52.5
79.4
45.7
41.7
41
44.7
38
35.5
38.9
34.7
32.4
33.1
30.2
26.9
25.7
14.1
12
MKS 4.41 IEF 7.48 PEL
B
180
116
84
58
48.5
36.5
26.6
79.4
69.2
41
38.9
35.5
34.7
32.4
26.9
25.7
14.1
12
MKS 4.41 IEF 7.48 AQU
C
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116
84
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48.5
36.5
26.6
87.1
69.2
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41
45.7
41
MKS 4.41 IEF 7.48 DET
D
180
116
84
58
48.5
36.5
26.6
112.2
54 52.5
50.1
45.7
56.2
41.7
41
45
44.7
38
35.5
33.1
14.1
12

CHAPTER IV

DISCUSSION

Treponema. pallidum is highly evolved for coexistence with its hosts. Reflecting this adaptation are many differences in outer membrane properties of *T. pallidum* from those of other Gram-negative pathogens, mainly the absence of at least a smooth type of LPS (128). This may contribute to the low reactivity towards the host of this pathogen and its adaptation to long term survival in chronic infection despite host immune responses to internal antigens. It has been long appreciated that non-aged, freshly extracted treponemes do not react in serological tests (55, 98). It has also been shown by immuno-electron microscopy that the surface of the virulent organism is resistant to the binding of anti- treponemal antibodies in the absence of complement (65, 66, 135). Another indication for this resistance to antibody binding is the time, 4 hours or longer, required for *in vitro* complement-dependent serum bactericidal reactions in the *T. pallidum* immobilization and *in vitro-in vivo* neutralization tests (18).

Stamm and co-workers provided further evidence demonstrating the unusual nature of the surface of *T. pallidum* (150). They reported that the outer membrane could be removed by 0.04 percent SDS resulting in exposure of only periplasmic flagella, identified as the most susceptible to antibody binding. In their view, this implied that the outer membrane is relatively devoid of protein. Another possibility to explain the antigenic inertness of the surface is a model of outer membrane architecture with transient discontinuities owing to the motility of the organism and membrane fluidity. In support of the hypothesis that a fluid outer membrane allows at least transient surface exposure of subsurface structures, Radolf and co-workers (133) found that antibodies to *T. pallidum*

endoflagella, which are located in the periplasmic space (61), have treponemacidal activity. Freeze fracture and deep-etching studies recently done by Radolf (personal communication; in press) demonstrate the paucity of intramembranous particals (IMPs) in the outer membrane. He reported that the outer membranes of nonpathogenic treponemes and *E. coli* contained 10-15 and 300-500-fold more IMPs than *T. pallidum.* This implys that the paucity of integral membrane proteins serves as a novel parasitic strategy for evasion of host humoral defenses.

In recent years the application of molecular biological techniques has greatly facilitated the identification and functional characterization of important *T. pallidum* antigenic proteins. Polyacrylamide gel electrophoresis (PAGE) and Western blot techniques have been used by several investigators (7-9, 15, 50-52, 84, 161) to identify as many as 22 antigenic molecules of *T. pallidum.* The use of two-dimensional SDS-PAGE and Western blot techniques have revealed 67 antigenic polypeptides (116). Despite this, uncertainty remains with regard to the identities of those polypeptides which are integral membrane proteins and their precise locations in either the outer or cytoplasmic membranes. Several investigators have reported the successful removal of the outer membranes of spirochetes using detergents or chaotropic reagents (5, 74). Penn and co-workers in two separate reports (128, 129) indicated 0.2 percent and 1.0 percent (w/v) concentration of the nonionic detergent Triton X-100 could solubilize *T. pallidum* outer membranes. Cunningham and co-workers (131) and Radolf and co-workers (134) used closely related detergent Triton X-114 at 1 percent concentration to solubilize *T. pallidum* outer membrane. One advantage to using Triton X-114 is its low cloud point (20°C) allowing for separation of detergent solubilized proteins into distinct aqueous and detergent phases at room temperature.

In this study, the outer membranes were successfully extracted. The extraction was

monitored at time intervals by electron microscopy in two ways: whole-mount and thin sectioning. It was found that the nonionic detergent TX-114 removes the outer membrane without visual damage to the cytoplasmic membrane, reducing the diameter of *T. pallidum* from 0.14 μm to 0.095 μm. These measurements are in agreement with Hovind-Hougen's measurement (68) of 0.15μm, and are in total disagreement with Cunningham (31), who measures the diameter of the intact *T. pallidum* to be 0.25 - 0.35 μm and the TX-114 treated to be 0.1-0.15 μm. Whole-mount electron microscopy of treponemes treated with one percent TX-114 demonstrated extensive blebbing of the outer membrane preceding its removal. This blebbing is demonstrated at time 0 minutes (Figure 4a) of treated organisms. Considering the processing time after addition of the TX-114, the blebbing must have taken place within less than one minute. These electron microscopy findings are in accord with the findings of Radolf and co-workers (134) and Cunningham and co-workers (31). However, these findings are in contrast to the well-described susceptibility of the cytoplasmic membrane of isolated *Escherichia coli* cell envelopes to nonionic detergent solubilization (143). In *E. coli*, covalent and strong noncovalent forces link outer membrane proteins to peptidoglycan, and thus, stabilize the association of the outer membrane with the cells. In contrast, *lpp* and *ompA* mutants of *E. coli* (*lpp* lacks the genes for bound and free lipoproteins; *ompA* lacks the gene for OmpA protein; all three proteins seem to be involved in stabilization of the outer membrane structure and in anchoring the outer membrane to the peptidoglycan) exhibit blebbing (83). Hovind-Hougen (68) demonstrated a layer in thin sections of *T. pallidum* whose appearance is thought to be consistent with that of peptidoglycan juxtaposed to the outer leaflet of the cytoplasmic membrane. The stability of the cytoplasmic membrane of *T. pallidum* following TX-114 treatment may be based on strong protein-protein interactions, or on tight links to peptidoglycan, in a manner analogous to the association of the *E. coli* outer membrane with peptidoglycan. If the latter is the case, movement

mediated by the periplasmic flagella might be impossible.

In this study, silver stained two-dimensional SDS-PAGE protein profiles of *in vitro* and *in vivo T. pallidum* revealed a total of 151 and 150 polypeptides respectively (M_r 141.3 - 14 kDa; pI 4.687 - 7.86). In addition, to increase or decrease in concentration of certain polypeptide species as described in Results section, special attention should be given to the two *in vitro* synthesized polypeptides with molecular weights 29.5 and 34.7 kDa (pI 5.62) (Figure 9A). These two proteins remained with the cytoplasmic cylinders (pel, Figure 9B) after the removal of the outer membrane of *T. pallidum*. Their presence was confirmed by ^{35}S radiolabeling of proteins of *in vitro* and *in vivo T. pallidum*. As expected, the autoradiograms in Figure 10 (A) and (B) demonstrate their presence in the *in vitro T. pallidum* profile, while the autoradiograms (a) and (b) in Figure 10 demonstrate their absence in the *in vivo T. pallidum* protein profile. Further support was given by the immunoblots presented in Figure 11 (A) and (B), where pooled sera from patients with secondary syphilis did not show any binding to these polypeptides.

Equally important was the absence of synthesis of an antigenic polypeptide with molecular weight 34.7 kDa (pI 5.34) in the silver stained two-dimensional SDS-PAGE of *in vitro T. pallidum* protein profile. This absence of synthesis was further supported by the autoradiograms (Figure 10 A and B) and immunoblots (Figure 11 A and B).

Comparison between silver stained two-dimensional protein patterns of whole *in vitro* (Figure 9A) and *in vivo* (Figure 8A) *T. pallidum* revealed that treponemes cultivated *in vitro* possessed increased concentrations of five polypeptides: 38 kDa (pI 5.34), 46.7 kDa (pI 6.32), two 46 kDa (pI 6.05), and 45.8 kDa (pI 5.69). However, the presence of these polypeptides were not detected in the autoradiograms or in the immunoblots implying the possibility that these polypeptides do not have treponemal origin. The possibility of them being part of serum contamination is ruled out by the silver stained two-dimensional SDS-PAGE of FBS (Figure 8E). Two possibilities exist as of

the origin of these polypeptides. One, they could represent degradive products of serum or host components. Two, since no protease inhibitors were used during sample preparation, these proteins could represent protease degradive products of treponemal origin. However, considering the label, that contained 80% [^{35}S]methionine and 20% [^{35}S]cysteine, a treponemal protein must be devoid of both cysteine and methionine in order not to get labeled. It is unlikely that proteins of M_r 38 - 47 kDa lack both cysteine and methionine residues. At any rate, these hydrophilic polypeptides appear in the aqueous phase (Figures 8C and 9C) after TX-114 extraction and phase partitioning. In general, from gel to gel at the lower and higher molecular weight ranges, it was difficult to correlate the one-dimensional SDS-PAGE patterns with the two-dimensional patterns.

A relatively small number of *T. pallidum* polypeptides were extracted by TX-114, most of which were present in the hydrophobic detergent phase. Two-dimensional SDS-PAGE and immunoblot analysis revealed eleven antigenic polypeptides that have been partially identified and characterized previously by other investigators. Among these was a molecule that appeared as a tetramer in the two-dimensional profile with molecular weights 41.7 and 45.7 kDa (pI 5.34) and 41.7 and 45.7 kDa (pI 5.62). This molecule was described by Peterson (132), Jones (75), Norgard (114), Stamm (149), Norris (116), Penn (128), Radolf (134) and Cunningham (31). Also, a 39 kDa (pI 5.34) described by Norris (116), Penn (128) and Moskophidis (105, 106); a 38 kDa (pI 5.15) described by Norris (117), Baseman (11), Stamm (149), Penn (128), and Embden and co-workers (60,160). Finally, a family of five very lightly silver stained polypeptides with molecular weights 40, 38.5, 27.5, 25.7 and 24.8 kDa (pI 4.86) were strongly stained on the immunoblots indicating their high antigenicity. These five molecules were previously detected by Radolf (134), Embden and co-workers (60, 160), Norris (116) as one diffused band. In addition, an antigenic polypeptide with apparent M_r of 14 kDa (Figure

11d) was detected in the one-dimensional protein profile of the detergent phase. This polypeptide also has been described by Radolf (134), Lukehart (84), Hensel (58) and Hanff (50). The absence of this polypeptide from the two-dimensional immunoblot is probably due to the urea present in the IF gels. Urea dissociates and denatures the polypeptides. The antibody directed against epitopes in their native conformation may not bind to a denatured polypeptide. The antigenic polypeptides of the detergent phase are probably of the outer membrane origin since the endoflagellar proteins were not detected by monospecific anti-flagellar antibodies in the reprobed electroblots.

The silver stained two-dimensional protein profiles of the aqueous phase demonstrated the presence of the contaminating serum proteins in addition to several treponemal polypeptides (FIgures 8C and 9C). The autoradiograms of the aqueous phase facilitated the separation between the contaminating proteins and the treponemal proteins (Figure 10 C and c). The radiolabeled aqueous phase polypeptides with molecular masses at and below 16 kDa appear to correspond to proteins that Radolf (134) and Stamm (149) described previously. Stamm (149) proposed that these polypeptides represent extra cellular secretion products of *T. pallidum*; however, Radolf (134) disagrees in view of the fact that many of these organisms have suffered variable amounts of outer membrane damage, probably as a consequence of physical manipulations (e.g., centrifugation and resuspension) during the radiolabeling procedure, and proposes that these polypeptides possibly represent soluble periplasmic components released from disrupted organisms. In this study, we tend to agree with the latter proposal. Of all the aqueous phase radiolabeled polypeptides, only three demonstrated antigenicity in the two-dimensional protein profiles. These included antigenic polypeptides with molecular masses 74.1 and 51.3 kDa, pI 5.62, and a 38 kDa, pI 6.58. The one-dimensional profile demonstrated at least six antigenic polypeptides, none of which, below the molecular mass 38 kDa. This observation disagrees with findings of Radolf (134) and Stamm (149) in that they both had found

antigenic aqueous phase polypeptides with M_r at or below 16 kDa.

Of the 237 polypeptide species of *T. phagedenis* identified by autoradiogram only 46 were cross-reactive with *T. pallidum* as indicated in Western blots probed with pooled sera from patients with secondary syphilis (Figure 14A). Utilizing sera from syphilitic patients and one-dimensional SDS-PAGE and Western blotting technique, Hanff (51) characterized eight cross-reactive protein antigens with M_r of 30, 33, 35.5, 40, 45, 47, 73 and 82 kDa. Using crossed immunoelectrophoresis technique, Pedersen (123) has identified six cross-reactive protein antigens. Lukehart and co-workers (84) have identified five common antigens in one-dimensional SDS-PAGE using pooled rabbit anti-*T. pallidum* serum and the Western blotting technique. Finally, Moskophidis (105) has identified 15 cross-reactive antigens between *T. phagedenis* and *T. pallidum* in one-dimensional SDS-PAGE using human syphilitic sera and Western blotting technique.

Among the hydrophobic outer membrane associated (detergent phase) proteins of *T. phagedenis* were two cross-reactive antigens of molecular masses 12 and 14.1 kDa pI 5.34 (Figure 14D) previously identified by Hensel (58).

In the present study, all the treponemal antigens and *T. phagedenis* cross-reactive polypeptides detected by the Western blotting technique represent polypeptides recognized by human IgG antibodies. Some studies were done to demonstrate the antigens recognized by human IgM antibodies. Basically, the IgM response seemed to be weaker and less extensive toward *T. pallidum* polypeptides than the IgG response. However, the IgM reactivity may be underestimated by the Western blot technique using unfractionated pooled serum because of potential competitive inhibition of IgM binding to treponemal antigens by IgG, thereby, masking IgM reactivity (29). IgG antibodies generally have higher avidity and maintain a more stable combination with Gram-negative bacterial antigens than do IgM antibodies (138). The continued presence of IgM in latent and even late syphilis has been demonstrated previously by serological testing (109,119,146).

Baker-Zander and co-workers (9) were able to show IgM activity in patients with primary, secondary, early latent and late latent syphilis. They demonstrated gradual loss of IgM activity from primary to late latent syphilis. Eijk and Embden (161) did not detect any IgM response in patients with late latent syphilis and Moskophidis (105) and Hanff (51) did not investigate IgM reactivity beyond the secondary stage.

There appeares to be much variation exists between the number and the M_r of *T. pallidum* polypeptides and antigenic proteins reported by different laboratories. Several important points must be considered in the interpretation of the results described in this study and the literature cited. First, the starting material (purified *T. pallidum*) can be a good source of variation in the protein profiles. Manipulation of the organism (e.g., centrifugation, resuspension) can cause damage to the outer membrane and loss of some soluble periplasmic proteins and even outer membrane polypeptides. Second, the method of solubilization and preparation of the sample of SDS-PAGE (e.g., solubilization time and temperature, presence or absence of SDS or NP-40) can alter the protein profile as was demonstrated in the preliminary studies presented in this project . Third, the sample size, 15 - 100 μg, can alter the protein profiles (e.g., proteins with low concentration in cell may be detected if 100 μg of protein is loaded onto the gel while they may be absent if 15 μg is loaded). Fourth, the choice of the IF, stacking, and resolving gels (e.g., isotropic vs. gradient; percent polyacrylamide; IF gradient) may alter the protein profile by retaining some high molecular weight molecules at the application point or loss of some low molecular weight from the gel. Fifth, the methods of detection (e.g., silver stain vs Coomassie blue) is important in visualizing all the resolved molecules. Sixth, the methods of molecular weight determination (e.g., best fit straight line of all M_r standards vs. 'point to point' interpolation of molecular weight from the nearest M_r standard) is another source of variation which is even more complicated by the manufacturers giving different M_r values to the same standard molecule. Seventh, technical variables in Western

blotting, such as the conditions of electrophoretic transfer of proteins form the gel to the nitrocellulose, method of detection of the antigenic proteins (e.g., HRP-labeled vs. biotin-labeled secondary antibodies vs. staphylococcal protein A) will influence the number of protein bands that become visible. Eighth, the time and condition of the film exposure during autoradiogrophy may alter the protein profiles (i.e. over or under exposure). Therefore, conducting preliminary studies in an effort to find the best conditions for the efficient analysis of a given protein population is a must in this line of research.
In this study the treponemal proteins would have been more efficiently resolved if a gradient gel with polyacrylamide concentration ranging from 8% to 15% would have been used.

In conclusion, it appears that most of the major proteins of *T. pallidum* are recognized by human as foreign and induce an antibody response during the course of the infection. These antigenic moieties elicit an immune response to a multitude of epitopes, and immune response is stimulated further by continued antigenic exposure during long term latent infection. It is likely, however, that only those immune elements (either immunoglobulin or cellular) that react with surface components are important in the destruction of the bacteria. Identification of the outer membrane polypeptides (detergent phase) is promising and may lead to immunization studies, utilizing immunogens produced through *in vitro* cultivation of *T. pallidum.* Further research on the antigenicity of *T. pallidum* will show which of the antigens identified so far represents surface exposed, true species specificity, and whether antibodies to the specific antigens are responsible for elimination of the organisms and for immunity. At this time it seams that the family of five hydrophobic detergent phase polypeptides associated with the outer membrane; the 40, 38.5, 27.5, 25.7, and 24.8 kDa (pI 4.86) are probably good candidates for farther studies for the following reasons: 1) They are outer membrane

associated. 2) Considering their low concentration they elicit a very strong humoral immune responce which leads to the thought that they might be surface exposed. 3) Their concentration fits the concentration of surface exposed IMPs in freez fracture studies of Radolf. Meanwhile, since the *in vitro* cultivation of *T. pallidum* is limited, the identified *T. phagedenis* cross-reactive outer membrane polypeptides especialy the polypeptide with the M_r 35.5 kDa, pI 4.60 may prove useful for diagnostic purposes and immunization studies.

APPENDIX A

CULTURE MEDIA

The following media were used to culture and harvest *Treponema pallidum* and *Treponema phagedenis*.

Hanson's medium (500 ml)

Component	Amount/500 ml	
Spirolate Broth (BBL)	6.5	g
Brain Heart Infusion (DIFCO)	8.2	g
Sodium Thioglycollate, grade V, anhydrous (Sigma Chemical Co.)	125.0	mg
Bacto-Trypton (DIFCO)	125.0	mg
3x D.W.	450.0	ml

Stir to dissolve; aliquot 90 mls in 100 ml screw cap bottles; autoclave for 30 minutes and let cool at room temperature. Add 10 mls of heat inactivated rabbit serum and inoculate.

Phosphate Buffered Saline (PBS) (10 X)

Component	Amount/2 liter	
$NaH_2PO_4.H_2O$	5.12	g
Na_2HPO_4	23.88	g
3x D.W.	11.00	ml

Stir to dissolve and adjust pH to 7.2 - 7.4 then add:

NaCl	175.32	g

Q.S. to 2 liter with 3x D.W.

Dilute stock 1:10 prior to use. Final Concentrations: 0.01 M phosphate; 0.15 M NaCl

TPCM (*Treponema pallidum* Cultivation Medium)

Component	Amount/100 ml	
Earls balanced salt solution (10x)	10.0	ml
Essential amino acids (50x) (Flow Laboratories, Inc.)	0.5	ml
Nonessential amino acids (100x) (Flow Laboratories, Inc.)	0.5	ml
Vitamins (100x) (Flow Laboratories, Inc.)	0.5	ml
Glucose	250.0	mg
L-Glutamine (200 mM) (Sigma Chemical Co.)	1.0	ml
$CaCl_2$ (0.5 mg/100 ml) (Sigma Chemical Co.)	1.0	ml
Cocarboxylase (O.2 mg/100 ml) (Sigma Chemical Co.)	0.1	ml
Mannitol (10 g/100 ml) (Sigma Chemical Co.)	0.1	ml
Histidine (5g/100 ml) (Sigma Chemical Co.)	0.1	ml
Sodium Bicarbonate (Sigma Chemical Co.)	253.5	mg
MOPS Buffer (1 M, pH 7.5) (Sigma Chemical Co.)	2.5	ml
Sodium Pyruvate (Sigma Chemical Co.)	10.0	ml
Dithiothreitol [DTT] (Sigma Chemical Co.)	15.0	mg
Superoxide dismutase [SODase] (Sigma Chemical Co.)	2500.0	units
Catalase (Sigma Chemical Co.)	1000.0	units

Mix all the components except DTT, SODase, and catalase. Adjust pH to 7.5 using 10 N NaOH and Q.S. to 100 ml. Sterilize by filtration and store under atmosphere of 95% N_2/5% CO_2. Hydrate the DTT, SODase, and catalase; filter sterilize separately and add to TPCM just prior to use.

Earle's Balanced Salt Solution (EBSS) (10 X)

Component	Amount/liter	
$CaCl_2.2H_2O$	2.7	g
KC1	4.0	g
$MgSO_4.7H_2O$	2.0	g
NaCl	68.0	g
$NaH_2PO_4.H_2O$	1.4	g
Glucose	25.0	g
Phenol Red, Na (optional)	0.2	g

Dissolve all the components except $CaCl_2.2H_2O$ and $MgSO_4.7H_2O$ in pyrogen-free water, Q.S. to 500 ml. In a separate container, dissolve $CaCl_2.2H_2O$ in pyrogen-free water, Q. S. to 250 ml. In a third container, dissolve $MgSO_4.7H_2O$ in pyrogen free water and Q. S. to 250 ml. With rapid mixing, slowly add the Ca Cl_2 and $MgSO_4$ solutions to the first solution. Filter sterilize the final mixture.

APPENDIX B

SAMPLE PREPARATION SOLUTIONS

The following solutions are necessary for preparing the samples for Two-dimensional electrophoresis (Ames and Nikaido 1976). Unless otherwise indicated, all solutions should be stored at 4°C. The CSS and solubilization solution #1 should not be kept longer than three months.

Cell Suspending Solution (CSS) (1 mM $MgCl_2$, 1 mM Tris, pH 7.8)

Component	Amount/100 ml
Tris Base (Bio-Rad)	0.02 g
$MgCl_2.6H_2O$ (Fisher)	0.02 g

Dissolve in about 90 ml of 3x D.W. then adjust pH to 7.8 with 1 N HCl . Q. S. to 100 ml with 3x D.W.

Solubilization Solution #l (0.5 M Tris, pH 6.8)

Component	Amount/50 ml
Tris Base (Bio-Rad)	3.03 g

Dissolve in 40 ml of 3x D.W. and then adjust pH to 6.8 with concentrated HCl. Q. S. to 50 ml with 3x D.W.

Solubilization Solution #2 (10% SDS)

Component	Amount/10 ml
SDS (Bio-Rad)	1.0 g

Dissolve in 10 ml 3x D.W. and store at room temperature.

Solubilization Solution #3 (0.1 M $MgCl_2$)

Component	Amount/10 ml
$MgCl_2.6H_2O$ (Fisher)	0.21 g

Dissolve in 10 mls of 3x D.W.

Sample Preparation Solution (SPS) (9.5 M Urea, 2.5% Ampholines, 5% 2-ME, 8% NP-40)

Component	Amount/10 ml	
Urea (Bio-Rad)	5.71	g
Ampholine, pH 5-7 (LKB)	0.13	ml
Ampholine, pH 3.5-10 (LKB)	0.13	ml
2-mercaptoethanol (2-ME) (Sigma)	0.50	ml
Nonidet P-40 (NP-40) (Sigma)	0.80	ml

Dissolve in 3x D.W. and Q. S. to 10 mls. Warm the solution to completely dissolve the urea. Dispense in 0.5 ml aliquots and store at -20°C. Thaw and use each aliquot only once, stable indefinitely.

Working Solubilization Solution (2% SDS, 0.5 mM $MgCl_2$, 0.05 M Tris-HCl, pH 6.8)

Component	Amount
Solubilization Solution #1	20 µl
Solubilization Solution #2	40 µl
Solubilization Solution #3	1 µl
3x D.W.	139 µl

Mix well and use.

Solubilization Buffer (9.5 M Urea, 5% Ampholines, 5% 2-ME, 4% NP-40)

Component	Amount/10 ml	
Urea (Bio-Rad)	5.71	g
Ampholine, pH 5-7 (LKB)	0.25	ml
Ampholine, pH 3.5-10 (LKB)	0.25	ml
2-mercaptoethanol (2-ME) (Sigma)	0.50	ml
Nonidet P-40 (NP-40) (Sigma)	0.40	ml

Dissolve in 3x D.W. and Q. S. to 10 mls. Warm the solution to completely dissolve the

urea. Dispense in 0.5 ml aliquots and store at -20°C. Thaw and use each aliquot only once, stable indefinitely.

APPENDIX C

ELECTROPHORESIS SOLUTIONS

All buffers should be kept for no longer than three months. Acrylamide solution should be checked for precipitate formation and should be kept for no longer than one month. All solution should be stored at 4°C unless indicated otherwise.

Note: Unpolymerized acrylamide is carcinogenic and neurotoxic. Use a mask when weighing acrylamide and gloves when handling gel solutions or wet gels.

IF Bis Acrylamide, 30% (IF Bis Acryl.)

Component	Amount/50 ml
Acrylamide (Bio-Rad)	14.19 g
Bis Acrylamide (Bio-Rad)	0.81 g

Dissolve in 3x D.W. and Q. S. to 50 ml. Then filter the solution through Whatman No. 1 filter paper and store in brown bottle at 4°C.

Acrylamide for SDS Slab Gels, 30% (SDS-Bis Acryl.)

Component	Amount/250 ml
Acrylamide (Bio-Rad)	73.0 g
Bis Acrylamide (Bio-Rad)	2.0 g

Dissolve in 3x D.W. and Q. S. to 250 ml. Then filter the solution through Whatman No. 1 filter paper and store in brown bottle at 4°C.

Lysis Buffer

Component	Amount/10 ml
Urea (Bio-Rad)	5.7 g
3x D.W.	2.7 ml
10% NP-40 (Sigma)	2.0 ml
Ampholine, pH 5-7	0.3 ml
Ampholine, pH 3.5-10	0.3 ml

Heat the mixture at 37°C to dissolve urea. May take up to 4 hrs to dissolve. After the urea

has dissolved, add:

Component	Amount/10 ml
2-mercaptoethanol	0.5 ml

Dispense in 0.5 ml aliquots and store at -20°C. The buffer may be kept indefinitely; however, each aliquot should be thawed and used only once.

Sample Overlay Solution (SOS)

Component	Amount/10 ml
Urea (Bio-Rad)	5.400 g
3x D.W.	5.750 ml
Ampholine, pH 5-7 (LKB)	0.125 ml
Ampholine, pH 3.5-10 (LKB)	0.125 ml

Heat the mixture at 37°C to dissolve urea. Dispense in 0.25 ml aliquots and store at -20°C. The solution may be kept indefinitely, but each aliquot should be thawed and used only once.

Equilibration Sample Buffer (pH 6.8)

Component	Amount/250 ml
Glycerol (J. T. Baker)	19.75 ml
Tris Base (Bio-Rad)	1.89 g
Sodium dodecyl sulfate (Bio-Rad)	5.75 g

Dissolve in 230 ml 3x D.W., adjust pH to 6.8 using concentrated HCl. After the pH has been adjusted, add 3x D.W. and Q. S. to 250 ml.

Lower Gel Buffer (LGB) (1.5 M Tris, 0.4 % SDS, pH 8.8)

Component	Amount/10 ml
Tris Base (Bio-Rad)	18.17 g
Sodium Dodecyl Sulfate	0.40 g

Dissolve in 90 ml 3x D.W. and adjust pH to 8.8 with concentrated HCl. High concentrations of Tris make pH reading difficult. Be certain that the meter has the proper type of electrode. When reading, do so quickly. After each reading, rinse the electrode with distilled water, then immerse it in the standard buffer until it becomes depolarized. The next reading in Tris buffer can then be done. If the pH is over adjusted, it can be brought to pH 8.8 with NaOH. Q. S. to 100 ml with 3x D.W.

Running Buffer (RB) (0.025 M Tris, 0.192 M Glycine, 0.1% SDS, pH 8.3)

Component	Amount/8 liter
Tris Base (Bio-Rad)	24.22 g
Glycine (Sigma)	115.30 g
SDS	8.00 g

Dissolve in 3x D.W. The pH should be approximately 8.3. If it varies significantly from this value, remake the solution. The pH has been adjusted with NaOH without seeming to alter the final results; however, the use of HCl is not permitted. Chloride ions (from any source) may lead to poor resolutions. RB can be stored at room temperature for several weeks.

Agarose Gel (for one gel) (Prepare when needed)

Component	Amount/8 ml
Agarose (Bio-Rad)	0.1 g
Equilibration Sample Buffer	9.5 ml

Microwave for a total of 3 minutes. Interrupt microwaving to prevent boiling over. This

should be maintained at 100°C using a hot plate. In a fume hood, add 0.5 ml of 2-ME and mix.

Tris-HCl (1.5 M, pH 8.8)

Component	Amount/300 ml
Tris Base (Bio-Rad)	54.5 g

Dissolve in 280 ml 3x D.W. Adjust pH to 8.8 with concentrated HCl. Q. S. to 300 ml with 3x D.W.

Tris-HCl (0.5 M, pH 6.8)

Component	Amount/100 ml
Tris Base (Bio-Rad)	6.0 g

Dissolve in 90 ml 3x D.W. Adjust pH to 6.8 with concentrated HC1. Q. S. to 100 ml with 3x D.W.

10% SDS Solution

Component	Amount/100 ml
SDS (Bio-Rad)	10.0 g

Dissolve SDS in 3x D.W. with gentle stirring. Q. S. to 100 ml with 3x D.W.

10% Ammonium Persulfate Solution (prepare fresh daily)

Component	Amount/1 ml
Ammonium Persulfate (Bio-Rad)	0.1 g

Dissolve ammonium persulfate in 1 ml 3x D.W.

Bromphenol Blue Tracking Dye

Component	Amount/10 ml
Bromphenol Blue (Bio-Rad)	0.01 g

Dissolve in 10 ml of 3x D.W. and store at room temperature. This solution is stable indefinitely.

Sodium Hydroxide (0.02 M) (prepare when needed)

Component	Amount
NaOH	0.6 g
3x D.W. (previously boiled deaerated, and cooled)	750.0 ml

Dissolve and boil solution, deaireatate, and swirl occasionally until bubbling stops. Allow to cool before use.

Phosphoric Acid Solution (0.1 N) (prepare when needed)

Component	Amount/3 liter
H_3PO_4 (85%)	3.45 ml
3x D.W	2996.55 ml

Mix and use.

Nonidet P-40 (10% Solution)

Component	Amount/100 ml
NP-40 (Sigma)	10.0 ml
3x D.W.	90.0 ml

Mix and use.

APPENDIX D

SILVER STAIN SOLUTIONS FOR SLAB GELS

The following solution are necessary for the silver stain developed by Morrissey (32). The developer, silver nitrate, and dithiothreitol solutions should be prepared when needed. The other solutions may be kept until used. One slab gel requires 250 ml of each solution. **Fixative (50% Methanol - 10% Acetic Acid)**

Component	Amount/liter	
Methanol (reagent grade) (J. T. Baker)	500.0	ml
Acetic Acid (glacial) (J. T. Baker)	100.0	ml
1x D.W.	400.0	ml

Mix and use.

Rinse Solution (5% Methanol - 7% Acetic Acid)

Component	Amount/liter	
Methanol (reagent grade) (J. T. Baker)	50.0	ml
Acetic Acid (glacial) (J. T. Baker)	70.0	ml
1x D.W.	880.0	ml

Mix and use.

10% Glutaraldehyde

Component	Amount/liter	
Glutaraldehyde (50%) (Kodak)	200.0	ml
1x D.W.	800.0	ml

Mix and use.

Dithiothreitol (DTT) (5 μg/ml)

Component	Amount/liter	
DTT (Sigma)	5.0	mg
1x D.W.	1.0	l

Mix and Use.

Silver Nitrate (0.1% w/v)

Component	Amount/liter
Silver Nitrate (Sigma)	1.00 g
1x D.W.	0.99 l

Mix to dissolve, Q. S. to 1 liter.

Developer (3% Sodium Carbonate, 0.05% Formaldehyde)

Component	Amount/liter
Sodium Carbonate (Anhydrous) (J. T. Baker)	30.0 g
Formaldehyde (37%) (J. T. Baker)	0.5 ml

Dissolve sodium carbonate in 1x D.W., Q. S. to 1 liter, then add 0.5 ml of 37% formaldehyde.

Citric Acid Solution (2.3 M)

Component	Amount/100 ml
Citric Acid (Monohydrate) (Sigma)	44.25 g

Dissolve in 1x D.W. and Q. S. to 100 ml.

APPENDIX E

SILVER STAIN SOLUTIONS FOR IF TUBE GELS

The following solution are necessary for the silver stain developed by Merril (5). Only the soaking solution should be prepared ahead of time. The rest should be made when needed. One tube gel requires 40 ml of each solution

Fixative (50% Methanol - 12% Acetic Acid)

Component	Amount/200 ml
Methanol (reagent grade) (J. T. Baker)	100.0 ml
Acetic Acid (glacial) (J. T. Baker)	24.0 ml
1x D.W.	76.0 ml

Mix and use.

Rinse Solution (10% Ethanol - 5% Acetic Acid)

Component	Amount/600 ml
Ethanol (100%) (J. T. Baker)	60.0 ml
Acetic Acid (glacial) (J. T. Baker)	30.0 ml
1x D.W.	510.0 ml

Mix and use.

Soak Solution (0.0034 M Sodium Dichromate, 0.0034 N Nitric-Acid)

Component	Amount/liter
Nitric Acid (1 N) (J. T. Baker)	35.00 ml
Sodium Dichromate (Sigma)	0.98 g

Dissolve sodium dichromate and Q. S. to 1 liter with 1 x D.W.

Stain (0.012 M Silver Nitrate)

Component	Amount/200 ml
Silver Nitrate	0.4 g

Dissolve the silver nitrate in 1x D. W. and Q. S. to 200 ml. Keep in brown bottle until used.

Developer (0.28 M Sodium Carbonate, 0.05% Formaldehyde)

Component	Amount/liter	
Sodium Carbonate (Anhydrous) (J. T. Baker)	30.0	g
37% Formaldehyde (J. T. Baker)	0.5	ml

Dissolve the sodium carbonate in 1 liter 1x D.W. then add 0.5 ml 37% formaldehyde.

APPENDIX F

WESTERN BLOT SOLUTIONS

These solutions are used in Western blotting. The blotting buffer should be prepared one day prior to each run and stored at 4°C. The saline solution should be stored at room temperature. Do not keep this solution for more than one month.

Tris-Glycin Buffer, pH 7.8 (25 mM Tris, 192 mM Glycine 20% Methanol)

Component	Amount/liter
Tris Base (Bio-Rad)	3.03 g
Glycine (J. T. Baker)	14.40 g

Add about 500 ml of 3x D.W. and dissolve the tris and glycine. Add 200 ml of absolute methanol and then bring the final volume to 1 liter with 3x D. W. The pH; should be about 7.8. No adjustment is necessary.

Tris Buffered Saline Solution (TBS) (500 mM NaCl, 20 mM Tris, pH 7.5)

Component	Amount/liter
NaCl (Sigma)	29.24 g
Tris Base (Bio-Rad)	2.42 g

Add approximately 800 ml of 1x D. W. and dissolve the NaCl and tris. Adjust the pH to 7.5 with 5 N HCl and then add 1x D. W. to a final volume of 1 liter.

Tween 20 - Tris Buffered Saline (TTBS) (500 mM NaCl, 20 mM Tris, 0.05% Tween 20, pH 7.5)

Component	Amount/liter
NaCl (Sigma)	29.24 g
Tris Base (Bio-Rad)	2.42 g
Tween -20 (Sigma)	0.50 ml

Add approximately 800 ml 1x D.W. and dissolve the NaCl and Tris. Adjust the pH to 7.5 with 5 N HCl and then add 0.5 ml Tween -20 and Q. S. to 1 liter.

Blocking Solution (Blotto) (500 mM NaCl, 20 mM Tris, 5% Nonfat Dry Milk, 0.01% Antifoam A, pH 7.5)

Component	Amount/liter
NaCl (Sigma)	29.24 g
Tris Base (Bio-Rad)	2.42 g
Tween -20 (Sigma)	0.50 ml
Nonfat Dry Milk (Carnation)	50.00 g
Antifoam A (Sigma)	0 .10 ml

Add approximately 800 ml 1x D.W. and dissolve the NaCl and Tris. Adjust the pH to 7.5 with 5 N HCl and then add 0.5 ml Tween -20, 0.1 ml antifoam A and 50 grams of nonfat dry milk. Dissolve the milk and Q. S. to 1 liter.

Chromogenic Substrate Stock Solution (3 mg/ml 4-chloro-1-napthol in methanol)

Component	Amount/liter
4-chloro-1-napthol (Sigma)	60.0 mg
Methanol (reagent grade) (J. T. Baker)	20.0 ml

Mix to dissolve. Prepare in brown bottle just prior to use.

Chromogenic Working Solution

Component	Amount
Chromogenic Stock Solution	20.0 ml
TBS	100.0 ml
30% Hydrogen peroxide (Sigma)	60.0 µl

Mix thoroughly in a brown bottle and use immediately

Chromogenic Substrate Stock Solution for Secondary Staining (3 mg/ml diaminobenzidine in TBS)

Component	Amount/100 ml	
Diaminobenzidine (Sigma)	60.0	mg
TBS	100.0	ml

Mix to dissolve in a brown bottle. Add 60 µl of 30% hydrogen peroxide just prior to use.

APPENDIX G

IN VITRO RADIOLABELING MEDIUM

The following medium was used to radiolabel *T. pallidum* and *T. phagedenis* polypeptides overnight. Replace the FBS for rabbit serum when *T. phagedenis* is to be labeled. The amino acids in the "stock amino acid solution" may not readily go into solution. Slight amount of heat will help to dissolve the amino acids. Every component of this medium, except for the amino acid stock solution, should be prepared fresh prior to use.

Component	Amount/100 ml	Total
3x D.W.	66 ml	
EBSS (10x)	5 ml	
Glucose (20%)	4 ml	0.77%
Thiamine (0.1%)	2 ml	1.92 mg
Glycerol (12%)	1 ml	0.12%
Sodium Pyruvate (5%)	1 ml	0.05%
FBS	7.5 ml	7.20%
Cysteine (1%) and Thioglycollate (0.5%)	10 ml	48.03 mg
Stock Amino Acid (1.26 mg/ml) @	4 ml	
Aspartate		0.05 mg
Glycine		0.05 mg
Glutamine		0.05 mg
Histidine		0.05 mg
Isoleucine		0.05 mg
Lysine		0.05 mg
Phenylalanine		0.05 mg
Proline		0.05 mg
Tryptophan		0.05 mg

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APPENDIX H

ELECTRON MICROSCOPY SOLUTIONS

The following solutions were used to prepare samples for electron microscopy.

Piperazine buffer (0.3 M)

Component	Amount/100 ml
Piperazine N-N-bis 2 ethanol sulfonic acid (PIPES, Polysciences)	9 g
3x D.W.	50ml

Stir to dissolve, adjust pH to 7.4 using 0.1 N NaOH. Q.S. to 100 ml with 3x D.W. To make 0.02 M PIPES, mix 2 ml of 0.3 M PIPES with 28 ml 3x D.W.

Cacodylate buffer (0.2 M)

Solution A: 0.2 M sodium cacodylate is made by dissolving 4.28 g of $Na\ (CH_3)_2\ AsO_2.3\ H_2O$ in enough 3x D.W. to make 100 ml total volume.

Solution B: 0.2 N HCl is made by mixing 1.66 ml concentrated (36-38%) HCl with 98.34 ml 3x D.W.

pH	6.4	6.6	6.8	7.0	7.2	7.4
Solution A (ml)	25	25	25	25	25	25
Solution B(ml)	9.2	6.7	4.7	3.2	2.1	1.4

Mix the indicated amounts of solution A and B to make 0.2 M cacodylate buffer with desired pH. To make 0.1 M cacodylate buffer, mix 10 ml 0.2 M buffer with 10 ml 3x D.W.

Sato's lead citrate

Component	Amount
3x D.W.	82 ml
Sodium citrate (Polysciences)	2.0 g
Lead nitrate (Polysciences)	1.0 g
Lead acetate (Polysciences)	1.0 g
Lead citrate (Polysciences)	1.0 g

Add the components in the order listed above, stir to dissolve. At this point, solution is milky in color. To clear solution, add 18 ml (all at once) 4% NaOH in 3x D.W.

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