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In vitro cultivation of *Treponema pallidum*: a review

THOMAS FITZGERALD[1]

In vitro *cultivation of* Treponema pallidum *would facilitate many different aspects of syphilis research. This review summarizes developments in this field that have been published since 1975. Findings are discussed in terms of treponemes and the oxygen question, treponemal metabolism involving proteins, nucleic acids, and fatty acids, and treponemal interaction with tissue culture cells. Suggested future approaches and potential problem areas pertinent to successful cultivation are discussed.*

The *in vitro* cultivation of *Treponema pallidum* would be a major breakthrough in studies aimed at the control of syphilis. In 1966, Willcox & Guthe (*99*) stated, "the conclusion is reached that there is hardly any problem of the vast subject of treponematosis research for which an advance would not be made towards solution by the successful *in vitro* culture of virulent *T. pallidum*". They specified at least ten areas of research that would immediately benefit: morphological observations to resolve the question of different life-cycle forms; comparative studies of syphilis, yaws, and pinta; identification of treponemes within tissues after treatment failures; development of better serological tests based on "purer" organisms; characterization of treponemal antigenic structure and chemical make-up; assessment of enzyme activities; organ distribution of treponemes in latent stages; purification of organisms for radioactive labelling; antibiotic sensitivity; and lastly the development of effective vaccines. "Purer" treponemes growing *in vitro* would result in better antigenic preparations and thus facilitate characterization of host immunity in terms of both the humoral and the cellular responses. One other important benefit would follow successful cultivation. If the organisms could be grown in test-tubes, the need for rabbit passage of *T. pallidum* would be avoided. Currently, rather large animal facilities and access to numerous rabbits are required for syphilis research. Thus, test-tube grown organisms would simplify experimental procedures and enable more investigators to enter the field.

In 1905, Schaudinn & Hoffman (*82*) discovered that *T. pallidum* caused syphilis. An intense interest then evolved in growing this organism *in vitro* (*99*). An analysis of numbers of publications demonstrates the rise and fall of interest: between 1905 and 1920, 78 reports; between 1920 and 1940, 29 reports; and between 1940 and 1966, 20 reports. There are two probable reasons for the decline in interest. First, in the late 1940s penicillin was shown to be very effective in the treatment of syphilis and it was thought that the disease would soon disappear. In the past 30 years, however, the continued epidemic of syphilis demonstrates the fallacy of this thinking. Secondly, the frustration of attempting to grow *T. pallidum* is indicated by the accumulated failures over the past 75 years. Many intensive, well planned investigations have been performed and many different extracts, culture media, nutrients, and additives have been tested under a variety of experimental conditions, but still *T. pallidum* has not been successfully cultured *in vitro*.

Many reports have claimed cultivation of *T.*

[1] Associate Professor, Department of Medical Microbiology and Immunology, School of Medicine, University of Minnesota at Duluth, MN 55812, USA.

pallidum. In 1906, Volpino & Fontana (*96*), and in 1909 Schereschewsky (*83*), initially reported *in vitro* growth. Since then, other claims have been made. In each case, two problems occurred. Other laboratories were unable to reproduce the original findings, or the cultured organisms lost their ability to induce lesions. Subsequent research has shown that some past "successes" were the result of contamination with non-pathogenic treponemes.

Recently, interest in the *in vitro* cultivation of *T. pallidum* has been rekindled. In 1975, Fitzgerald et al. (*24*) published findings involving the use of tissue culture techniques. The survival of *T. pallidum* was significantly improved in the presence of the cultured cells. Subsequent studies by others have confirmed and expanded on the interaction between cultured cells and treponemes. Three of these have suggested minimal but significant *in vitro* multiplication (*21*, *45*, *81*). More research will be required to confirm these promising results.

In addition to tissue culture, an interest in the metabolism of *T. pallidum* has recently emerged. In 1974, Baseman & Hayes (*4*) developed procedures for assessing treponemal protein synthesis. A number of studies by others have followed. Such knowledge should be immensely useful for establishing optimal conditions for *in vitro* multiplication.

The purpose of this report is to evaluate the current status of research involving *in vitro* cultivation of *T. pallidum*. It will focus primarily on findings published in the last 8–10 years. The older literature has been reviewed in detail by Willcox & Guthe (*99*). Studies on the physiology, growth, metabolism, and composition of non-pathogenic treponemes will not be cited. These organisms seem to be so different from *T. pallidum* that direct comparisons are not always valid.

This review is arranged in 3 major sections: the first assesses the problems associated with the treponemal oxygen question and the sulfhydryl requirement and oxygen toxicity are also discussed; the second reviews the metabolic processes of *T. pallidum*, in particular as regards protein synthesis, nucleic acid synthesis, and fatty acid involvement; the third section evaluates the interaction of treponemes with tissue culture cells and the gradual development of this promising culture technique. Finally, suggested future approaches and potential problem areas are discussed. It is hoped that this report will stimulate further research directed towards the successful multiplication of *T. pallidum*.

THE OXYGEN QUESTION

Resolution of the oxygen question is crucial to the successful cultivation of *T. pallidum*. Do the organisms require oxygen? What levels of oxygen are toxic? What is the mechanism of oxygen toxicity? Inasmuch as sulfhydryl (SH) agents directly influence oxygen toxicity (*42*, *44*, *92*) and prolong treponemal survival, they are intimately associated with the oxygen question. This section will focus on the interaction of SH agents with treponemes and also on recent developments pertinent to specific mechanisms of oxygen toxicity.

T. PALLIDUM AND THE SULFHYDRYL REQUIREMENT

In 1911, Noguchi (*69*, *70*) reported that anaerobiosis and small fragments of tissue were beneficial to the *in vitro* survival of *T. pallidum*. The tissue probably added to the reducing potential of the medium. Brewer (*12*) and Kast & Kolmer (*48*) initially observed that SH agents improved treponemal survival. Brewer used sodium thioglycollate and petrolatum seals to bypass the need for anaerobic conditions; Kast & Kolmer used cysteine and glutathione. Sodium thioglycollate, cysteine, and glutathione were also important components of the basal medium formulated by Nelson (*64*). Subsequent reports (*18*, *20*, *26*, *37*, *38*, *61*, *63*, *65*, *75*, *77*, *79*, *80*, *97*, *98*,) have confirmed the beneficial influence of SH agents.

Nelson (*64*), Weber (*97*), and Metzger & Smogor (*61*) proposed two beneficial roles for SH agents: (*a*) they poise the redox potential (Eh) thereby minimizing oxygen toxicity, and (*b*) they are required by the organisms for active metabolic functioning. In attemping to cultivate *T. pallidum* both roles should be considered.

The estimated generation time of *T. pallidum in vivo* is 30–33 h (*57*). In order to detect significant multiplication *in vitro*, prolonged incubation will probably be required. During extended incubation, oxidation of SH agents will occur. Metzger & Smogor (*61*) recognized the need for control of SH oxidation during incubation of treponemes. In the modified Nelson's medium used by Weber (*97*), half the glutathione and cysteine was oxidized within 4 days. If these agents were partially oxidized prior to the addition of treponemes, beneficial affects were sharply decreased. Norris et al. (*71*) observed that partially oxidized SH agents were far less beneficial, or were even detrimental to treponemal survival.

Recent papers involving tissue culture and *T. pallidum* indicate the difficulties associated with the

use of SH agents. In 10 reports (*20, 21, 26, 27, 40, 49, 76, 79, 80, 81*) different SH agents and different optimal concentrations were used; in 2 other reports (*24, 45*) SH agents were not used.

The specific influences of SH agents on *T. pallidum* have been documented. Weber (*97*) tested a variety of different agents; glutathione, cysteine, thioglycollate, mercaptosuccinate, and homocysteine were beneficial and mercaptoethanol, ethanethiol, and mercaptoethylamine were not beneficial. Since the ineffective agents also lowered Eh, the beneficial influences could not be solely attributed to maintenance of Eh. In further experiments, if the glutathione was oxidized prior to addition to the treponemes, it lost its beneficial effects. The method of preparation of SH agents was also important. Agents that were sterilized by membrane filtration were partially oxidized whereas agents that were autoclaved were fully reduced. During incubation with treponemes the SH agents became oxidized. Efforts were made to compensate for this oxidation by adding cysteine at various times during incubation, but this failed to extend the duration of treponemal survival. Ascorbic acid and sodium sulfhydrate did not enhance survival. In combination with glutathione and cysteine, however, these reducing agents were beneficial. This suggested that the ascorbic acid and sodium sulfhydrate acted by re-reducing the oxidized glutathione and cysteine. This concept was adapted later by Fitzgerald et al. (*26, 31, 71*) using dithiothreitol to maintain SH agents in the reduced form.

Metzger & Smogor (*61*) examined the survival of *T. pallidum* as reflected by changes in Eh and pH. These two parameters were altered by using different combinations and concentrations of cysteine, glutathione, and thioglycollate. Maximum survival occurred with initial values of pH 7.2–7.3 and Eh −230 to −240 mV. This work demonstrated an important concept: a specific range of Eh was compatible with treponemal survival. Previously it was known that a high Eh was detrimental; Metzger & Smogor showed that Eh could also be too low. Beyond 40 h, rapid SH oxidation occurred elevating Eh and accelerating treponemal death. When the level of SH oxidaton was partially controlled by addition of SH agents during incubation, the organisms survived longer. Furthermore, in confirmation of Weber's findings (*97*), oxidized SH agents failed to improve treponemal survival.

Sandok et al. (*79*) reported the influence of Eh on treponemal survival in the presence of rat glial tissue culture cells. Their results were similar to those of Metzger & Smogor (*61*). Best survival occurred at Eh −232 mV and pH 7.6. Further modifications of their basal tissue culture medium resulted in better survival at Eh −275 mV and pH 7.3.

Fitzgerald et al. (*24*) initially demonstrated that tissue culture cells extended the *in vitro* survival of *T. pallidum*. These authors suggested that one of the beneficial effects might be attributed to partial neutralization of oxygen toxicity by the cultured cells. Attempts were made to further negate oxygen toxicity by adding SH agents and by altering the oxygen concentration within the tissue culture system (*26*). The affects of glutathione, cysteine, and thioglycollate on treponemal survival were assessed in the absence of cultured cells. The combination of glutathione at 4mmol/litre and cysteine at 1mmol/litre was optimal under the defined conditions. These findings were then applied to tissue culture and significant extensions of treponemal survival occurred.

Based on the findings of Weber (*97*) and Metzger & Smogor (*61*) that SH agents became oxidized during incubation, experiments were performed to evaluate the role of dithiothreitol in maintaining highly reduced conditions. The addition of this agent markedly improved treponemal survival, probably through maintenance of cysteine and glutathione in the reduced form (*31*). Four subsequent studies (*14, 20, 71*, and Norris et al., unpublished data, 1980) have confirmed the beneficial influences of dithiothreitol.

Additional studies were performed to assess the effects of different concentrations of oxygen (0–21%) on the growth and survival of cultured mammalian cells. It was important to determine the minimum oxygen concentration compatible with cultured cell survival. The exclusion of oxygen was rapidly detrimental to the cultured cells; growth did not occur and within 48 h, the monolayers began to detach from the glass surface and die. In the presence of 3% oxygen in the atmosphere above the culture, however, the cultured cells readily adapted and multiplied; subsequent incubation for 2–3 weeks was not detrimental. This concentration of oxygen was then utilized for further experiments involving incubation of *T. pallidum* with tissue culture cells.

Oxygen at 3% was a fortunate choice. In subsequent work, Norris et al. (*71*) compared the influences of 21% (air), 3%, and 0% oxygen on *T. pallidum* with and without SH agents. In all experiments the organisms survived poorly in 21% oxygen. Without SH agents and also with glutathione, the treponemes survived better in 0% oxygen than in 3% oxygen. In direct contrast, however, in the presence of dithiothreitol far better survival occurred in 3% oxygen. This important observation indicates that the concept of anaerobiosis based on oxygen sensitivity depends on the experimental conditions. Without dithiothreitol, *T. pallidum* survived best in 0% oxygen suggesting an anaerobic nature; with dithiothreitol *T. pallidum* survived best in 3% oxygen suggesting a microaerophilic nature.

Graves & Billington (*36*) arrived at similar conclusions by examining survival of *T. pallidum* after gently bubbling either air or a mixture of oxygen and nitrogen through reduced medium. Variation in time of bubbling resulted in differences in dissolved oxygen concentrations ranging from 0.7% to 12.6% oxygen. Treponemal survival, as shown by retention of motility and virulence, was at a maximum in 3% oxygen. In 0.7% and 12.6% oxygen treponemal survival was similar. The authors suggested that *T. pallidum* possessed mechanisms for neutralizing oxygen toxicity that are efficient at 12.6% oxygen but overwhelmed at 21% oxygen (air). They confirmed the findings of Norris et al. (*71*) and concluded that *T. pallidum* was probably a microaerophilic organism.

Chalmers & Taylor-Robinson (*14*) compared the effectiveness of cysteine, glutathione, sodium thioglycollate, and dithiothreitol in extending treponemal survival with an atmosphere of 3% oxygen. Highly reduced conditions were maintained by daily addition of 1 mmol of dithiothreitol per litre. In the presence of all four reducing agents best treponemal motility was observed in 20% foetal calf serum and 0.5% bovine serum albumin. Each SH agent was then selectively omitted from the medium to determine potential toxicity. Cysteine and dithiothreitol were beneficial and glutathione and thioglycollate were detrimental. Further experiments indicated that foetal calf serum partially neutralized the toxicity of glutathione and thioglycollate. In a final series of experiments the effects of storage were assessed on medium containing all four reducing agents. Far better motility was observed in medium that had been stored at 4°C for 10 days. The authors suggested that toxicity resulted from the oxidation of the sulfhydryl groups in thioglycollate.

Later experiments (Norris et al., unpublished data, 1980) added sodium metabisulfite to the list of beneficial reducing agents. In an atmosphere of 3% oxygen, metabisulfite extended the duration of treponemal motility. Concentrations between 0.6 and 1 mmol/litre were beneficial. Treponemal adenine incorporation into trichloroacetic acid (TCA) insoluble material was then assessed in 0%, 1%, 3%, 5%, 10%, and 21% oxygen. Maximum incorporation was detected in 3–5% oxygen without metabisulfite and in 3–10% oxygen with metabisulfite. Finally, the influence of transferring *T. pallidum* into fresh medium containing metabisulfite was examined in an atmosphere containing 3% oxygen. Fresh medium added at 3-day intervals significantly extended treponemal survival, motile organisms being detected for 9 days without additions and for 18 days with additions of medium. Inoculation of rabbits revealed no significant decrease in the numbers of virulent treponemes after incubation for 13 days.

The effects of metabisulfite were compared with the effects of dithiothreitol. Both agents exhibited similar activities in enhancing treponemal survival. The authors suggested that the beneficial influences of metabisulfite and dithiothreitol resulted from quenching of the toxic forms of oxygen.

In other work by Norris et al. (*71*), oxidized SH agents were not beneficial to *T. pallidum* and in some cases were actually detrimental. Dithioerythritol was also added to the list of SH agents that extended treponemal survival.

Fieldsteel et al. (*20*) used two approaches to demonstrate the influences of oxygen on *T. pallidum*. In one, treponemes were added to cultured cells in Leighton tubes containing different volumes of tissue culture medium. Colour changes of resazurin showed that this produced variations of Eh within each tube. This work also showed that extremes of redox potential were detrimental to *T. pallidum*, thus confirming the work of Metzger & Smogor (*61*). In a second approach, gradient tissue cultures in vertical Leighton tubes were used. Different amounts of SH agents were added and incubation was performed in the presence or absence of air. At the bottom of the tubes in the most reduced area, both treponemal survival and cultured cell survival was poor. At the top of the tubes in the most oxidized area, the cultured cells survived quite well but the treponemes did not. The area of best treponemal motility shifted downward during incubation in air or with decreasing concentration of SH agents. These observations indicate the complexity of the interaction between *T. pallidum*, oxygen, and SH agents.

Fitzgerald et al. (*31*) reported that SH oxidation varied depending on experimental conditions. Two examples emphasize the problems associated with the oxidation of SH agents during incubation. Treponemes added to cultured cells in either T-30 culture vessels or in Sykes-Moore chambers retained motility for 5–7 days. It was necessary to add dithiothreitol each day to the T-30 vessels. Daily addition of this SH agent to the chambers, however, resulted in rapid inactivation of the organisms.

A second example involves incubation of treponemes in either small test-tubes without cultured cells or T-30 vessels with cultured cells. When 1 ml of treponemes was placed into 3-ml test-tubes and incubated in an atmosphere of 3% oxygen, best survival occurred with the addition of dithiothreitol alone. The addition of glutathione, cysteine, and dithiothreitol was detrimental. In contrast, when 15 ml of treponemes were placed into T-30 vessels and incubated in 2.5% oxygen, best survival occurred with the combination of glutathione, cysteine, and dithiothreitol.

In further experiments (*31*), large fluctuations

occurred in SH oxidation depending on the experimental conditions. SH oxidation varied in relation to the treponemal suspending medium, the size of the culture vessel, the volume of fluid in the vessel, the gaseous environment within the vessel, and the method of extraction of organisms from testicular tissue. In addition, dithiothreitol maintained highly reduced conditions by re-reducing oxidized glutathione and cysteine.

These findings emphasize the importance of carefully defining experimental conditions. Metzger & Smogor (*61*) showed that *T. pallidum* survived within a specific range of Eh. SH oxidation is related to the experimental conditions. Thus, the different findings concerning optimal SH concentrations for *T. pallidum* are probably a result of differences between experimental systems. Those systems that favour SH oxidation would require correspondingly higher concentrations of SH agents. As an example, certain concentrations of glutathione may be beneficial to treponemal survival when incubation is performed in 5-ml test-tubes containing 4 ml of medium. This same concentration of glutathione, however, may not be optimal if the 4 ml of treponemes are in a larger test-tube or a T-30 flask. Also, concentrations of SH agents that are optimal for treponemes suspended in Nelson's medium may not be optimal for treponemes suspended in Eagle's MEM medium.

A further series of experiments was performed to investigate the role of *T. pallidum* in decreasing SH concentration (*31*). Briefly, SH content was monitored in (*a*) viable, (*b*) heat-inactivated, and (*c*) membrane-filtered preparations of treponemes. After extended incubation, far less SH was present in the viable preparation, suggesting a specific treponeme-mediated SH oxidation and/or SH uptake. This observation supports the postulate of Nelson (*64*), Weber (*97*), and Metzger & Smogor (*61*) that SH agents, besides poising the Eh, also contribute to the metabolic functioning of *T. pallidum*.

OXYGEN TOXICITY

Treponemes are sensitive to oxygen. An understanding of the basis of oxygen toxicity will provide direction for future studies involving *in vitro* cultivation; it will also help to improve procedures for laboratory manipulation of the treponemes. *T. pallidum* was previously assumed to be a strict anaerobe. However, Cox & Barber (*16*) showed that this organism does use oxygen. In further work, Lysko & Cox (*54*) demonstrated a functioning electron transport system. This research, which is fully discussed in the section on metabolism (on page 796) indicated the importance of oxygen to *T. pallidum*.

There are three excellent reviews concerning specific mechanisms of oxygen toxicity. Haugaard (*39*) evaluated oxygen toxicity from a biochemical standpoint. He emphasized the paradox that oxygen is basically detrimental to all forms of life; but that protective mechanisms have evolved that counteract excessive cellular oxidation. Morris (*62*) applied the problems of oxygen toxicity to bacteria. He attempted to develop an understanding of the physiology of obligate anaerobiosis. A third review in the *Federation proceedings* (*19*) emanated from a symposium on free-radical pathology. This series of papers summarized the chemical and biochemical mechanisms of oxygen toxicity and generated important concepts relevant to biological systems. These findings will be briefly outlined with a view to potential application to the culture of *T. pallidum*.

Free radical pathology and lipid peroxidation

Oxygen toxicity is complex. The harmful influences on biological systems appear to be mediated through oxidation of tissue constituents, primarily lipids, and through oxidation of cellular agents vital to metabolic functioning, such as glutathione, coenzyme A, lipoic acid, and various enzymes. Molecular oxygen is not the active factor; rather, the deleterious effects result from intermediates formed by the biological one-electron reduction of molecular oxygen and complexes, or reaction products of these species.

Gerschman et al. (*35*) noted parallels between oxygen toxicity and the effects of ionizing radiation. They initially postulated that toxicity could be attributed to free radicals. Most chemical species have electron orbitals filled with paired electrons spinning in opposite directions. Free radicals, however, have a lone unpaired outer orbital electron; they are quite unstable and highly reactive. Within biological systems there is a predilection for metabolic reduction of oxygen through univalent pathways, thus generating the superoxide radical and the hydroxyl radical. As an example, oxygen reacts with reduced flavins, flavoproteins, quinones, thiols, and various proteins including oxidases to form superoxide radicals. In turn, singlet oxygen may be generated. This is not a free radical but is highly reactive and very unstable. Theories of oxygen toxicity centre on the complex interactions of these three oxygen species with cellular constituents. Owing to their unstable nature, free radicals generate other free radicals resulting in chain reactions that lead to highly destructive free radical pathology.

Lipids are major targets for superoxide radicals, hydroxyl radicals, and singlet oxygen. These oxygen species react with polyunsaturated fatty acids to form

semi-stable peroxides and free radical intermediates. Semistable peroxides in turn, initiate free radical chain reactions that extend and enhance the original reaction. Lipid peroxidation is a highly destructive reaction that rapidly damages cellular constituents. Because membranes contain relatively large amounts of polyunstaturated fatty acids, they are especially susceptible to lipid peroxidation. This has been demonstrated using cellular, mitochondrial, and microsomal membranes. When membrane integrity is disrupted, various membrane systems are inactivated including those responsible for ATP synthesis, electron transport, nucleic acid synthesis, and the Krebs cycle. The accumulation of these effects results in loss of viability and lysis of the cell.

Free radicals that are generated during cellular metabolism, must be rigorously controlled because of their highly reactive nature. Cellular mechanisms neutralize free radicals as they evolve. These free radical traps include mannitol and formate, anti-oxidants such as glutathione, vitamin E, phenols, and specific enzymes such as peroxidases, catalases, reductases, and superoxide dismutases. Three of these are of particular interest. Glutathione,as the most abundant thiol in biological systems (*50*), participates in a variety of cellular reactions. One of its functions is to scavenge free radicals. Vitamin E is the primary biological antioxidant concerned with membrane integrity. It is incorporated into cellular membranes where it acts as a chain-breaking antioxidant to minimize lipid peroxidation. Superoxide dismutase has recently been implicated as a primary control mechanism that neutralizes superoxide anions (*56*). These free radicals induce lipid peroxidation, oxidize sulfhydryl groups, denature proteins, and damage nucleic acids.

In mammalian tissues, there is a correlation between superoxide dismutase, lipid peroxidation, and free radical pathology. If this enzyme is important in preventing lipid peroxidation, larger quantities should be found in those tissues that contain the highest levels of polyunsaturated fatty acids. Relatively large concentrations of superoxide dismutase are present within the adrenals, liver, and testes. These three tissues also contain the highest levels of polyunsaturated fatty acids. This suggests an *in vivo* role for superoxide dismutase in preventing lipid peroxidation.

Biochemistry of sulfhydryl agents

It is important to emphasize the complexity of SH–disulfide (SS) reactions. Much of the published data involves simplified *in vitro* systems such as an enzyme incubated with its substrate in the presence of an SH agent. The resulting effects may have some *in vivo* application. However, the complexity of *in vivo* systems frequently negates the *in vitro* findings. Jocelyn (*44*) has published an excellent review on the biochemistry of SH–SS reactions.

Many important cellular components contain SH groups that function only in the reduced form. These include glutathione, lipoic acid, ferridoxin, coenzyme A, and enzymes such as hexokinase, superoxide dismutase, arginase, papain, urease, cathepsins, dehydrogenases, and oxidases. In the presence of oxygen these compounds rapidly oxidize, and their biological activity decreases. The addition of SH agents restores the activity.

Glutathione is an especially important thiol and an adequate supply is necessary for many cellular metabolic processes. One major role of glutathione is to act as a redox buffer that maintains highly reduced conditions within cells (*86*). Oxidants that are formed during cellular metabolism are absorbed via glutathione; in the process this SH agent is oxidized. In turn, specific enzymes such as glutahione reductase re-reduce the oxidized glutathione. In addition, glutathione is an important factor in maintaining the integrity of membranes against extracellular chemical challenge and oxidizing agents.

Another interesting biochemical aspect of SH groups is their specific interaction with oxygen, as follows:

$$4\,RSH + O_2 \rightleftharpoons 2RSSR + 2H_2O.$$

This interaction in which the SH groups are oxidized is directly proportional to the partial pressure of oxygen. At pH 7–9 the amount of oxygen consumed can be quite substantial.

SH agents can absorb or scavenge oxygen and free radicals such as superoxide anions, hydroxyl anions, and fatty acid peroxides. In the process SH groups are oxidized to SS groups. Lipid peroxidation involves polyunsaturated fatty acids which are important constituents of membranes. Free radicals induce peroxidative chain reactions that are highly destructive to cellular membranes. In order to minimize lipid peroxidation, SH compounds in the cytoplasm and vitamin E within membranes terminate these chain reactions by absorbing the fatty acid peroxides. Furthermore, certain SH agents chelate heavy metals such as iron and copper which are potent catalysts of lipid peroxidation. Thus, SH agents indirectly minimize lipid peroxidation by removing heavy metals. Extensive reviews of lipid peroxidation have been published by Barber & Bernheim (*3*) and in *Federation proceedings* (*19*).

Relation to culture of T. pallidum

Pathogenic and saprophytic treponemes have been classified as strict anaerobes with the exception of

Treponema hyodysentariae. Recent findings, however, question whether this classification applies to *T. pallidum*. There are a number of definitions of anaerobiosis that are based on growth or killing in the presence of oxygen, the requirement for low redox potential, or the inability to use oxygen as the terminal electron acceptor in energy reactions. Further definitions distinguish between obligate anaerobes, facultative anaerobes, aerotolerant organisms, and microaerophilic organisms. A functional definition was proposed by Morris (*62*): "an obligate anaerobe exhibits adverse oxygen sensitivity that renders it unable to grow in air; also, it has energy and biosynthetic capabilities that by-pass molecular oxygen".

Inasmuch as successful *in vitro* cultivation of *T. pallidum* has not been achieved, the inability to grow in air cannot be used as the basis for anaerobic classification. Previous observations that suggested an anaerobic nature were predicated on maintenance or survival *in vitro*—the organisms survived far longer in the absence of air. It should be noted, however, that anaerobiosis is also eventually detrimental to *T. pallidum*.

The concept of anaerobiosis has subjective connotations. *Clostridium tetani*, a strict anaerobe, grows in the presence of air when cobalt is added to the culture medium (*17*). Two microaerophiles, *Campylobacter fetus* and *Spirillum volutans*, can also grow in air when norepinephrine, $FeCl_3$, superoxide dismutase, or catalase are added to the medium (*11*, *34*, *41*). *In vivo*, anaerobes are not restricted to poorly oxygenated areas such as the intestinal tract, but in fact, are frequently prevalent in aerobic locations. Anaerobes may outnumber aerobic forms on the skin surface by 10 to 1, and mucous membranes may contain 30 times as many anaerobes (*62*). One explanation involves the presence of other microorganisms. Their growth might significantly alter the *in vivo* environment and result in a low Eh that would be conducive to anaerobic growth. Talley et al. (*91*) examined the oxygen sensitivity of clinical specimens of freshly isolated anaerobic bacteria. Interestingly, all of the organisms tolerated exposure to air without adverse affects for at least 8 hours. Strict anaerobes failed to grow at oxygen concentrations above 0.5%. Moderate anaerobes grew in the presence of 2–8% oxygen, and also exhibited limited survival when exposed to air for 60–90 min. Loesche (*52*) reported similar findings.

Norris et al. (*71*) demonstrated that the subjective connotations of anaerobiosis apply to *T. pallidum*. In the absence of reducing agents, the treponemes survived far longer in 0% oxygen than in 3% or 21% oxygen. In direct contrast, in the presence of dithiothreitol, maximum survival occurred in 3% oxygen.

The work of Cox & Barber (*16*) is especially important in that it stimulated a re-examination of the classification of *T. pallidum* as a strict anaerobe. Recent studies both with (*20*, *21*, *26*, *49*, *81*) and without tissue culture (*36*, *71*) have demonstrated maximum treponemal survival in the presence of low levels of oxygen. This confirms the findings of Cox & Barber (*16*) that the organism requires oxygen. Furthermore, *T. pallidum* exhibits a predilection for tissues such as the testis and the skin, which are well vascularized. Subcutaneous tissues contain 2–3% oxygen (*13*, *85*), and it is likely that *T. pallidum* would evolve oxygen requirements similar to those found within these tissues.

Lipid peroxidation is conceivably one of the primary factors responsible for the *in vitro* demise of *T. pallidum*. During incubation the proportion of motile organisms remains high for a number of days. Once motility begins to decrease, the decline is rapid and cannot be reversed or even stabilized. Since lipid peroxidation involves reactions with polyunsaturated fatty acids, evidence for their involvement would require demonstrations of these compounds within *T. pallidum* but this is hampered by the inability to grow the organisms *in vitro*, and by the inability to remove contaminating testicular tissue from organisms grown *in vivo* (*43*). However, testis contains relatively large amounts of polyunsaturated fatty acids and it is well-established that the lipid content of bacteria directly reflects their growth environment. A recent paper by Matthews et al. (*59*) shows that the fatty acid composition of *T. pallidum* was quite similar to that of the rabbit testis and included a number of polyunsaturated fatty acids.

Because of the highly reactive nature of lipid peroxides, chain reactions within the suspending medium could be transmitted to the organisms. At least three sources of lipid peroxidation may occur during extraction of *T. pallidum* and subsequent *in vitro* incubation. First, the extraction process involves cutting and mincing of the testis. Disruption of the integrity of tissues will generate lipid peroxidation and this will be especially prominent in tissues with large amounts of polyunsaturated fatty acids. Secondly, if the extraction and/or subsequent incubation are not performed anaerobically, peroxides of lipid material will result from the interaction of oxygen with the unsaturated fatty acids of testicular origin that contaminate the treponemal preparation. As mentioned previously, maximum survival of *T. pallidum* in tissue culture occurs in the presence of low levels of oxygen. Thus during incubation, in addition to interacting with lipids of testis origin, oxygen may also interact with the polyunsaturated fatty acids within the membranes of *T. pallidum*. Thirdly, a

standard component of treponemal media is serum which also contains polyunsaturated fatty acids, and lipid peroxidation may also result from oxygen interaction with these. This last possibility may be the basis for better treponemal survival using freshly harvested as opposed to frozen serum that has been stored for weeks, since serum exposed to air will deteriorate due to lipid peroxidation even while stored at −20°C.

One important role of SH agents may be to minimize oxygen toxicity by scavenging free radicals that subsequently induce lipid peroxidation. Such peroxidation occurs whenever tissues are disrupted, for example when testicular tissue is minced for extraction of *T. pallidum*. While a certain amount of disruption is probably beneficial owing to release of tissue constituents required by the organisms (*64*), excessive mincing of tissue is detrimental (*31*). Experiments were performed in which infected tissue was extracted using two different methods. In one, the tissue was carefully cut with a sharp scalpel, in the other, the tissue was cut with a scalpel, and finely minced for 5 min with a mortar and pestle. After extraction in medium containing 5 mmol of SH per litre, both preparations were centrifuged to remove particulate matter. Treponemal motility and the quantity of SH remaining within the medium were monitored during subsequent incubation in air.

The time before motility decreased to 50% was 15 h after cutting only and 6 h after mincing. Furthermore, the SH content was rapidly depleted in the latter preparation. After 11 h, the preparation from cut tissue contained 2.2 mmol of SH per litre compared with 0.8 mmol of SH per litre in the minced preparation. These findings may reflect increased lipid peroxidation induced by excessive disruption of the testicular tissue, and oxidation of the SH groups after reaction with lipid peroxides.

The beneficial influences of dithiothreitol on treponemal survival could be attributed to the re-reduction of SH groups that have been oxidized (*20*, *26*, *71*). Fitzgerald et al. (*26*) first suggested that dithiothreitol was gradually oxidized during incubation. Subsequent experiments (*31*) revealed that dithiothreitol was oxidized and also effectively maintained glutathione and cysteine in the reduced form during prolonged incubation of treponemes.

In unpublished observatons, iron ($FeCl_3$) was added to *T. pallidum* at 1, 2, and 3 times the physiological concentration in rabbit blood. The three concentrations were proportionately detrimental to retention of treponemal motility and virulence. Norris et al. (unpublished data, 1980) recently confirmed this, showing that, in the absence of iron, motility was reduced to 50% after incubation for 68 h; in the presence of iron, 50% motility was reached after 12 h. The iron may have been harmful to *T. pallidum* because of increased peroxidation of the polyunsaturated fatty acids in its membranes.

In order to determine the potential influence of serum polyunsaturated fatty acids on treponemal survival, freshly prepared rabbit serum was incubated for 3 h in air or in 95% nitrogen: 5% carbon dioxide. Treponemes were added to each preparation and retention of motility was observed during incubation in air. Poorer survival occurred in the serum pre-incubated in air. The addition of free radical traps, such as superoxide dismutase (superoxide anion scavenger), mannitol or formate (hydroxyl anion scavengers), vitamin E (lipid peroxide scavenger), and various SH agents (free radical scavengers) extended the duration of treponemal motility during incubation in air.

In addition to lipid peroxidation, a second factor responsible for the demise of *T. pallidum* during incubation might be oxygen-mediated depolymerization of treponemal mucopolysaccharides. *T. pallidum* (Nichols strain) has an outer capsule (*28*, *101*) that specifically reacts with ruthenium red indicating that it is comprised of acidic mucopolysaccharides (*53*). This material also reacts with the plant lectins, wheat germ agglutinin, and soybean agglutinin, demonstrating the presence of *N*-acetyl-D-glucosamine and *N*-acetyl-D-galactosamine (*22*). Lectin agglutination is decreased after treatment of the treponemes with hyaluronidase (*22*). These observations suggest that the acidic mucopolysaccharides are hyaluronic acid and chondroitin sulfate, or closely related compounds.

Zeigler et al. (*101*) showed that the treponemal capsule rapidly dissipated during *in vitro* incubation. Acidic mucopolysaccharides are long straight-chain polymers of high relative molecular mass. When exposed to oxygen in the presence of reducing agents, these polymers undergo oxidative-reductive depolymerization (ORD) (*74*, *87*). It appears to be mediated through free radical reactions. Fitzgerald & Johnson (*22*) postulated that maintenance of capsular integrity is crucial to treponemal survival, and that the ORD reaction is the basis for capsular dissipation during *in vitro* incubation.

A third factor responsible for the *in vitro* demise of *T. pallidum* could be the oxidation of SH groups within important metabolic components. The oxygen intolerance of the organism and the partial neutralization of this intolerance by the addition of SH agents might be related to maintenance of functional SH groups. Certain metabolic compounds have strategically located SH groups that must remain reduced in order to function. These include cofactors such as lipoic acid, ferridoxin, and coenzyme A, and thiols such as glutathione and cysteine. In addition,

there are many SH-containing enzymes that are inactivated by oxidation of these SH groups.

Little research has been performed to characterize the enzymes associated with *T. pallidum*. Recently, Fitzgerald & Johnson (*23*) showed that *T. pallidum* exhibits mucopolysaccharidase activity. They postulated that this enzyme, besides providing substrates for capsular biosynthesis, also mediated treponemal attachment to host tissues. Unpublished preliminary findings suggested that this enzyme contains SH groups that must remain reduced to mediate attachment. If further evidence substantiates this, the inactivation of this important enzyme as a result of oxidation may partially explain the oxygen sensitivity of *T. pallidum*.

THE METABOLISM OF *TREPONEMA PALLIDUM*

The inability to cultivate *T. pallidum in vitro* has hindered studies of its metabolism, but, nevertheless, in the past 6 years, at least 17 papers have been published. Comparisons between studies are difficult, however, because procedures and techniques have varied considerably over this period.

The 17 reports on *T. pallidum* metabolism will be summarized in separate sections involving general observations, protein synthesis, nucleic acid synthesis, and fatty acid involvement. These summaries will be presented in chronological order to indicate the gradual development of the appropriate techniques and procedures. Specific details of the experimental protocols will be listed, since differences could be responsible for some of the observed discrepancies.

GENERAL OBSERVATIONS

As a forerunner to metabolic studies, Metzger & Michalska (*60*), in 1970, compared the incorporation of ^{14}C-sodium acetate into *T. phagedenis* biotype reiter, a non-pathogenic treponeme, and *T. pallidum* (Nichols strain). The organisms were incubated for 4 days at 35°C in Nelson-Diesendruck medium in an atmosphere of hydrogen and carbon dioxide. The non-pathogen incorporated the label much faster in the logarithmic phase of growth than in the stationary phase. Glucose, which stimulated treponemal growth, correspondingly increased the incorporation of acetate. Most of the label was found in carbohydrates, some was found in proteins, and very little was found in lipids. Two control preparations were used. Organisms were suspended in Ringer's solution, a non-growth medium, or were heat inactivated. Label was not detected in either control.

In direct contrast to the non-pathogen, *T. pallidum* failed to incorporate acetate after 4 days of incubation. The authors emphasized a key point that applies to all further metabolic studies—*T. phagedenis* was actively growing whereas *T. pallidum* was not.

In 1975, Nichols & Baseman (*67*) observed the evolution of CO_2 after substrate degradation by *T. pallidum*. The Nichols strain was extracted from infected tissue in phosphate-buffered saline containing $CaCl_2$, $MgCl_2$, NH_4Cl_2, and glutathione at pH 7.5. After slow-speed centrifugation the organisms were incubated at 34°C in an atmosphere of nitrogen, carbon dioxide, hydrogen, and argon. Treponemes lost motility within 7 h probably because of the lack of serum components. Thus, the majority of observations were made during a relatively short (2 h) period of incubation. Two control preparations were used because of problems associated with rabbit tissue cell contamination. The treponemal preparation was centrifuged at high speed to pellet the organisms, and the high-speed supernatant was used as one control. The red blood cells, white blood cells, testicular cells, and sperm cells were sedimented by slow centrifugation, and the second control consisted of the slow-speed pellet resuspended in the high-speed supernatant. Background activity attributed to the contaminating rabbit cells was then subtracted from activity attributed to treponemes.

Only pyruvate and glucose were degraded to CO_2 during 2 h of incubation. CO_2 did not evolve during treponemal incubation with the following compounds: galactose, ribose, fructose, formate, acetate, lactate, succinate, citrate, α-ketoglutarate, acetyl coenzyme A, oleic acid, palmitic acid, tripalmitin, glycerol, ethanol, aspartate, asparagine, glutamate, glutamine, and methionine. Further research indicated that the treponemes degraded pyruvate linearly only in the initial 12 h of incubation. Pyruvate degradation was also related to the concentration of organisms. Decreases in treponemal numbers from 10^7 to 1.3×10^6 organisms/ml resulted in corresponding decreases in rates of degration. The carboxyl group of pyruvate was preferentially degraded to CO_2, rather than the C-2 or C-3 carbons. In related studies, the C-1, C-3, and C-4 carbons of glucose were preferentially degraded to CO_2. End product analysis revealed that pyruvate was degraded primarily to CO_2 and acetate.

The authors emphasized the difficulties associated with these techniques. In many instances compounds were significantly degraded by the control

preparations of rabbit tissue cells. This necessitated rigorous control of each experiment. Furthermore, the relatively poor survival of *T. pallidum* under these conditions (less than 7 h) emphasizes the need for caution in the interpretation of the data. For example, if the organisms survived for longer periods, some of those compounds that were not degraded during the 2 h incubation might have eventually been degraded.

In 1977, Schiller & Cox (*84*) studied the catabolism of glucose and fatty acids by *T. pallidum*. The Nichols strain was extracted in sodium citrate with 100 ml of rabbit serum per litre or in phosphate-buffered saline with glutathione. A new technique was used to further purify the treponemal preparations. After slow-speed centrifugation to sediment gross particulates, the supernatant was passed through a Nucleopore filter (0.8 μm pore size) that retained host tissue cells but passed the treponemes. This procedure resulted in a reduction in contaminating host cells to less than 200 per ml. These preparations were then centrifuged at high speed. Controls consisted of either the pellet of host cells from the slow-speed centrifugation resuspended in the high-speed supernatant, or the high-speed supernatant alone. Enzyme assays were performed on sonicated high-speed supernatants (soluble enzymes) and on the sonicated high-speed pellet (insoluble enzymes).

Uptake experiments were initially performed at 25, 30, and 37 °C. No differences were apparent and further studies were performed at 25 °C. Since motility was rapidly lost, uptake was assessed after 2 h of incubation. Palmitic acid and oleic acid were incorporated by the treponemes; glucose was not. Palmitic acid and oleic acid, however, were not degraded to CO_2. Determination of CO_2 evolution from glucose labelled in different carbon positions suggested that *T. pallidum* possessed functional Embden-Meyerhoff-Parnas (EMP) and hexose-monophosphate (HMP) pathways but lacked an Entner-Doudoroff (ED) pathway. Degradation of pyruvate, α-ketoglutarate, and citrate in addition to assays for Krebs cycle enzymes also indicated the lack of a complete Krebs cycle in treponemes.

Four papers were published relating to electron transport in *T. pallidum*. In 1974, Cox & Barber (*16*) first suggested a functioning treponemal electron transport system. After making the critical observation that *T. pallidum* consumes oxygen, they reported inhibition of oxygen uptake by cyanide, azide, chlorpromazine, and amobarbital. They suggested that oxygen was a final electron acceptor through a cyanide-sensitive cytochrome oxidase. Additional data suggested a flavoprotein–cytochrome mediated oxidation of nicotinamide adenine dinucleotide to oxygen that might be coupled to oxidative phosphorylation.

In 1976, Baseman et al. (*7*) extended these findings. Cyanide and carbon monoxide, which inhibit cytochrome oxidase, were assessed for influences on treponemal metabolism. Only relatively high concentrations of cyanide interfered with treponemal degradation of glucose and pyruvate to CO_2. Protein synthesis was inhibited by cyanide, but again only at high cyanide concentrations. Carbon monoxide failed to affect treponemal metabolism. The authors suggested that electron flow might terminate at an early step in oxidative phosphorylation; alternatively, *T. pallidum* might possess a cyanide-insensitive cytochrome oxidase.

Lysko & Cox published a report in 1977 (*54*) involving terminal electron transport as a further evaluation of energy systems in *T. pallidum*. The Nichols strain was extracted in phosphate-buffered saline containing glutathione. Treponemes were prepared by means of slow-speed centrifugation, Nucleopore filtration (0.8 μm pore size), and high-speed centrifugation. Control preparations were similar to those previously published (*84*) with one addition—Nucleopore filtration through a 0.2 μm pore size to remove treponemes. These controls were important to minimize the possiblity that detectable cytochromes were from rabbit tissue.

Sonically treated preparations of treponemes were examined at different wavelengths to demonstrate typical absorbance-spectrum characteristics of cytochromes. Large amounts of flavoproteins were detected; these flavoproteins have been found in other spirochaetes such as *T. hyodysenteriae*, *Spirochaeta aurantia*, and *Leptospira* species. Cytochromes of the b- and c- types were also found. Cytochrome O, a b-type cytochrome, is the terminal oxidase; it was the only cytochrome oxidase detected in *T. pallidum*. Further studies suggested that this organism was capable of aerobic respiration. This supports the original contention by Cox & Barber (*16*) that *T. pallidum* is not an anaerobe. There appears to be a functioning flavoprotein-cytochrome electron transport system that is driven by the oxidation of NADH and reduced NADP.

In 1978, Lysko & Cox (*55*) extended their previous findings involving the role of oxygen and the energy capabilities of *T. pallidum*. Experimental procedures were similar to those previously published. An additional control consisted of uninfected rabbit testicular extract. Many findings involved sonicated preparations of treponemes. Various dehydrogenases important to electron transport appeared to be coupled to oxidative phosphorylation. Aerobic respiration in *T. pallidum* apparently occurred through NADH dehydrogenase coupled to terminal electron transport. NADPH oxidation also occurred via a dehydrogenase coupled to electron transport.

The organisms seemed to possess catalase activity, but more research is required to demonstrate clearly that this enzyme is not of rabbit origin. Oxidative phosphorylation in treponemes exhibited P:O ratios that varied between 0.5 and 1.1. The organisms probably contain a membrane bound ATPase that requires inorganic phosphorus.

Two other findings apply to whole cells rather than sonicates of *T. pallidum*. Bubbling air through the cultures resulted in increased production of ATP compared with bubbling a mixture of nitrogen and carbon dioxide. In addition, the treponemes appeared to have an adenylate kinase that was involved in recycling AMP back to the ATP system via conversion of AMP to ADP. The authors concluded that *T. pallidum* derives large amounts of energy from terminal electron transport coupled to oxidative phosphorylation. The inability to cultivate these organisms *in vitro* is probably not due to intrinsic problems with overall energy metabolism.

PROTEIN SYNTHESIS

In 1974, Baseman & Hayes (*4*) published the first study of *T. pallidum* metabolism. They presented techniques for monitoring protein synthesis. The Nichols strain was extracted in phosphate-buffered saline containing bovine serum albumin, bovine serum ultrafiltrate, pyruvate, and glutathione. The treponemes were centrifuged at slow speed and then incubated in an atmosphere of nitrogen, carbon dioxide, and hydrogen in the presence of a labelled amino acid mixture or specifically labelled amino acids. After incubation, the treponemes were membrane-filtered, then washed with 5% trichloroacetic acid to isolate label incorporated into protein. To differentiate between radioactivity in protein of treponemes as distinct from protein of rabbit tissue, control experiments were performed with two antibiotics. Cycloheximide inhibits incorporation into eukaryotic cells; erythromycin inhibits incorporation into prokaryotic cells. In the presence of this latter antibiotic, treponemal synthesis was almost completely (90%) inhibited; this occurred without reducing motility of the treponemes.

Temperature was the initial parameter investigated. Optimum protein synthesis occurred at 32, 34, and 36 °C. At 28, 38, and 40 °C very little label was incorporated. In fact, treponemes lost motility within a few hours at 38 and 40 °C. Protein synthesis was then evaluated in the presence of different pH levels. Optimum synthesis occurred at pH 7.6–7.9. Treponemes retained good motility for the duration of incubation at pH 7.2, 7.4, and 7.6. At other pH levels, however, including pH 7.9, poor retention of motility was recorded. This was puzzling since optimum protein synthesis occurred at pH 7.9 and yet motility was readily lost.

Protein synthesis was linear over the initial 24 h of incubation; further incorporation was not detected after 24 h. When individual amino acids were evaluated for incorporation into treponemal proteins, the following results were recorded: serine and valine were incorporated in large amounts; arginine, phenylalanine, tyrosine, glutamic acid, lysine, and histidine were incorporated in small amounts; glycine, aspartic acid, threonine, leucine, isoleucine, and alanine were incorporated in intermediate amounts.

Optimum conditions for protein synthesis by *T. pallidum* in this system were 34°C, pH 7.6, and incubation for 24 h. One of the inherent difficulties pointed out by the authors concerns the endogenous amino acid pools within the organisms. These pools would affect subsequent uptake and incorporation of labelled exogenous amino acids. Nevertheless, these observations provided the foundation for future studies of the metabolism of *T. pallidum*.

In 1976, Baseman et al. (*7*) demonstrated that treponemal metabolism was directly affected by oxygen. Substrate degradation and protein synthesis were examined in the presence of various concentrations of oxygen. *T. pallidum* (Nichols strain) was extracted anaerobically in phosphate-buffered saline containing salts, glutathione, glucose, pyruvate, bovine serum albumin, bovine serum ultrafiltrate, and calf serum. Control preparations consisted of the high-speed supernatant of the treponemal suspension and the slow-speed pellet of rabbit tissue that was then resuspended in a membrane filtrate of the treponemal suspension. Incorporation of labels was assessed during incubation for 4–24 h.

Protein synthesis was quantified in the presence of different oxygen concentrations. After 24 hours maximum incorporation of amino acids was detected in 10, 15, and 20% oxygen. Far less protein synthesis was detected in 0% oxygen. Protein synthesis was then measured in the presence of 0% and 20% oxygen. Without oxygen, synthesis was linear for 3 h, then stopped. In contrast, with 20% oxygen synthesis was linear for 24 h. Finally, the motility of *T. pallidum* was retained far longer in the presence of oxygen. After 24 h of incubation, 75–90% of the treponemes exposed to 1–20% oxygen were active. In the absence of oxygen, 10–30% of the treponemes were sluggish. Thus, substrate degradation, protein synthesis, and retention of motility were optimum in 10–20% oxygen.

These observations concerning the beneficial role of oxygen reinforce the important findings of Cox & Barber (*16*) concerning the aerobic nature of *T.*

pallidum. Baseman et al. (*7*) suggested that motility was not a good criterion for characterizing treponemal activity. Marked reduction in metabolism occurred at lower oxygen concentrations without adversely affecting motility. This corroborates their previous findings (*67*) in which erythromycin inhibited protein synthesis but did not affect motility.

Baseman & Hayes in 1977 (*5*) published a note concerning the anabolic potential of *T. pallidum*. The Nichols strain was extracted in medium as previously described (*7*) and *T. phagedenis* biotype reiter, an anaerobe, was used for comparative purposes. Both organisms were incubated for 21 h at 34 °C with a labelled amino acid mixture. *T. pallidum* was incubated aerobically and *T. phagedenis* was incubated anaerobically. Cycloheximide and erythromycin were used as appropriate controls to inhibit synthesis by rabbit tissue cells or by treponemes.

After incubation, acrylamide gel autoradiography of labelled proteins was performed. *T. pallidum* synthesized a spectrum of proteins of high relative molecular mass. Similar protein profiles were detected with *T. phagedenis*. These observations indicated that *T. pallidum* possesses significant anabolic competence. Further research by Baseman & Hayes (*6*) confirmed these findings of treponemal protein biosynthesis.

In 1978, Sandok & Jenkin (*78*) published important observations on protein and RNA synthesis by *T. pallidum*. For the first time metabolic experiments were carried out over extended periods of incubation (96 h). Since rapidly degenerating treponemes may exhibit abnormal metabolism, data from extended incubations should more closely approximate normal metabolic functioning of the organisms. Long-term incorporation experiments are also advantageous in that they can be performed with lower concentrations of treponemes. In these experiments 5 x 10^6 treponemes/ml were used.

T. pallidum (Nichols strain) was extracted from infected tissue in a complex serum-free medium. Gross particulate matter was sedimented by slow-speed centrifugation. Treponemal uptake experiments were performed anaerobically. As a control, uptake was monitored in a preparation of uninfected rabbit testicular tissue in the presence and absence of cycloheximide. After incubation with substrates, the preparations were membrane-filtered then assayed for residual radioactivity. Thus, the data indicate counts from whole treponemes rather than from specific fractions of organisms. This technical limitation does not differentiate between uptake and incorporation of substrates.

Serine was taken up linearly during the 96 h of incubation; erythromycin inhibited uptake whereas cycloheximide did not. Linear uptake over 96 h also occurred with a labelled amino acid mixture. Different results were reported with uracil. Uptake was linear for 96 h in the absence of erythromycin. In the presence of this antibiotic, uptake was linear only for the initial 48 h of incubation; further uptake was not detected. In related studies, thymine, pyruvate, and uridine were not taken up by *T. pallidum*.

These results differ from those of Baseman & Hayes (*4*, *5*) in that metabolic activity was detectable under anaerobic conditions. Linear rates of uptake indicated efficient metabolism for at least 96 h. Sandok & Jenkin emphasized, however, that these observations applied to *T. pallidum* only in the "resting" state and may not reflect metabolism of growing organisms. The presence of erythromycin did not affect motility of the organisms. This confirms the original observations of Baseman & Hayes (*4*) that motility does not directly parallel treponemal biosynthetic activity.

NUCLEIC ACID SYNTHESIS

In 1978, Nichols & Baseman (*68*) published the initial findings about nucleic acid synthesis of *T. pallidum*. They monitored ribosomal RNA synthesis as reflected by uridine incorporation. The Nichols strain was extracted from infected testicular tissue in Dulbecco's MEM containing glucose, tryptose phosphate broth, foetal bovine serum, cysteine, and sodium thioglycollate. After slow-speed centrifugation, uridine was added to either the treponeme-containing supernatant or to the rabbit cells contained in the pellet that were resuspended in the high-speed supernatant of the testicular extract. Subsequently RNA extraction and analysis were performed.

Uridine incorporation was linear for the initial 12 h of incubation; little additional label was incorporated between 12 and 48 h. The majority of organisms remained actively motile over the 48 h of incubation. Uridine incorporation was inhibited 90–95% by actinomycin D. Incorporation was then determined after 24 h of incubation at different temperatures. Maximum activity was detected at 39 °C; very little activity was detected at 4 or 42 °C; and intermediate activity was detected at 24, 33, and 37 °C. Interestingly, at 39 °C high rates of uridine incorporation were observed; most of this incorporation, however, occurred in the initial 8 h, and thereafter incorporation declined. In contrast at 24, 33 and 37 °C continued increases in radioactivity were recorded during the 24 h of incubation.

The effects of oxygen on RNA synthesis were then examined. Organisms were incubated for 45 h in different atmospheres. Optimal uridine incorporation

occurred in the presence of 20% oxygen, and reduced incorporation occurred in 15% oxygen. Levels of incorporation failed to increase in 10, 5, and 0% oxygen, in spite of good retention of treponemal viability for the 45 h of incubation. In further experiments involving pulse-labelling, most of the RNA synthesis was confined to the initial 3 h of incubation. This suggested a rapid shutdown of RNA synthesis well before loss of treponemal motility, again emphasizing the basic disparity between motility and efficient metabolic functioning.

In 1979, Baseman et al. (*8*) described a method for assessing DNA synthesis in *T. pallidum*. The Nichols strain was extracted in Dulbecco MEM containing 10% foetal bovine serum. After slow-speed centrifugation, the treponemal preparation was centrifuged over a Methocel-Hypaque gradient to remove additional contaminating rabbit cells. Treponemes in this culture medium remained motile for at least 48 h.

Assays were performed to detect thymidine kinase activity in lysates of *T. pallidum*. No activity was found, indicating that the enzyme was lacking, inactivated by the experimental methods employed, or present at such low levels that is was undetectable. Thymidine uptake was not demonstrated under a variety of experimental conditions. The lack of thymidine kinase may be related to the inability to take up thymidine. As an alternative assay system for DNA synthesis, DNA polymerase activity was investigated. Significant but very low levels of this enzyme were detected in *T. pallidum*.

Labelled uridine was taken up by treponemes only during the initial 24 h of incubation. Of the total amount incorporated, 46% was degraded by RNAase and the remainder was degraded by DNAase. Radiolabelled DNA from treponemes exposed to uridine was further demonstrated by cesium chloride gradient centrifugation. In temperature experiments, appreciable activity was detected after incubation of organisms at 34 °C in contrast to the negligible activity found after incubation at 4 °C.

These results demonstrate that *T. pallidum* is able to synthesize DNA during *in vitro* incubation. Compared with other microorganisms, *T. pallidum* has relatively low levels of DNA polymerase. The authors suggested that this may be related to its prolonged *in vivo* generation time. Under these experimental conditions, DNA biosynthesis by *T. pallidum* occurred for the initial 24 h of incubation. This synthesis may reflect the completion of synthesis initiated *in vivo*; or alternatively, it may reflect *de novo* synthesis under suboptimal *in vitro* conditions.

In 1980, Norris et al. (*72*) published a detailed report of both DNA and RNA synthesis by *T. pallidum*. The Nichols strain was extracted from infected tissues in Eagle's MEM containing 500 ml of rabbit serum per litre and dithiothreitol. After slow-speed centrifugation, organisms were adjusted to 0.2–3 x 10^7 treponemes/ml and incubated at 34 °C, usually in an atmosphere of 3% oxygen. As controls to assess rabbit cell contamination, the pellet remaining after slow-speed centrifugation was suspended in the high-speed supernatant.

The uptake of a number of nucleic acid precursors by treponemes was tested and incorporation into TCA insoluble material after incubation for 24 h was measured. Maximum incorporation occurred using adenine and somewhat less incorporation was found with 2'-deoxyadenosine and uridine. Little activity was detected with thymine or thymidine. Further experiments were performed with adenine.

In these studies a number of different antibiotics were tested for their influence on adenine incorporation into nucleic acids. Treponemes were exposed to antibiotic levels of 10 mg/litre for 4 h then incubated for 24 h with adenine. Penicillin G was most effective in decreasing label incorporation (99% inhibition) followed by mitomycin C (95% inhibition), actinomycin D (87% inhibition), and erythromycin (46% inhibition). Rifampicin, streptomycin, and cycloheximide did not affect adenine incorporation. Inhibition of adenine incorporation correlated with decreased treponemal motility. Penicillin G, mitomycin C, and actinomycin D markedly reduced motility; the other agents including erythromycin did not affect motility.

After incorporation of adenine, the labelled products were purified on a cesium chloride gradient and exposed to RNase and DNase; 20% of the label was degraded by DNase and 80% by RNase.

To simplify experimental procedures, a batch technique was developed for rapid assessment of adenine incorporation. Treponemal RNA and DNA synthesis were determined in the presence of air, 3% oxygen, and 0% oxygen. Maximum synthesis of both nucleic acids occurred in 3% oxygen; far less synthesis occurred in air or 0% oxygen. Retention of motility correlated with metabolic activity. Pulse-labelling experiments demonstrated that DNA and RNA synthesis were continuous over 6 days of incubation. Subsequently, synthesis stopped and motility was greatly decreased.

FATTY ACID INVOLVEMENT

It is well established that non-pathogenic treponemes require long-chain fatty acids for *in vitro* growth since they cannot beta-oxidize, desaturate, or synthesize fatty acids. Nevin et al. (*66*) showed that

oleic and butyric acids improved the *in vitro* survival of *T. pallidum*. Vaczi et al. (*95*) noted that treponemes contained large amounts of lipid that was primarily composed of palmitic, oleic, and oleinic acids. *T. pallidum* contained cardiolipin and significant amounts of lysolecithin. Nichols & Baseman (*67*) did not detect the release of CO_2 during the degradation of oleic acid, palmitic acid, and tripalmitin by *T. pallidum*. Schiller & Cox (*84*) confirmed that CO_2 was not released from palmitic and oleic acids; they also demonstrated the incorporation of oleic and palmitic acids into *T. pallidum*. They failed to find enzymes associated with beta-oxidation of fatty acids. These observations point to incomplete fatty acid degradation by *T. pallidum* similar to that found in the non-pathogenic treponemes.

Three recent studies have been concerned with either the fatty acid composition of *T. pallidum* or the influences of specific fatty acids on treponemal survival. In 1976, Smibert (*88*) analysed the lipid composition of *T. pallidum*: 41% of the total lipids were phospholipids, 27% were glycolipids, and 22% were neutral lipids. Phosphatidyl choline, phosphatidyl inositol, and lysophosphatidyl choline were demonstrated. The organisms contained the following fatty acids: C16 = 0, C16 = 1, C16 = 2, C17 = 2, C18 = 0, C18 = 1, C18 = 2, and C19 = 2. The C16 = 2, C17 = 2, and C19 = 2 were treponemal fatty acids not found in rabbit testicular tissue. These analyses were performed on the Beckman commercial preparation of *T. pallidum* used for FTA-ABS studies. Smibert indicated that certain fatty acids may have been lost during preparation.

In 1978, Matthews et al. (*58*) reported the deleterious effects of various fatty acids on the survival of *T. pallidum*. Palmitic, stearic, oleic, or linoleic acids caused rapid loss of motility at concentrations as low as 5 mg/litre. Surprisingly, the combination of palmitic and oleic acids was not toxic. Assessments of the types and quantities of fatty acids found within uninfected rabbit testis were performed. Based on these studies, palmitic, stearic, oleic, and linoleic acids were combined and tested for treponemal toxicity. This combination, however, was not toxic. In further studies, Tween 40, Tween 60, and Tween 80 were inhibitory to *T. pallidum* survival. Sodium oleate that was freshly prepared was toxic to the organisms. This toxicity was lost when oleate was stored for 3–5 months. Analysis of the stored oleate revealed degradation to 7 other fatty acids and only 8% of the original oleate was still present.

In 1979, Matthews et al. (*59*) determined the lipid composition of *T. pallidum* (Nichols strain). In order to remove as much contaminating host material as possible, the treponemes were centrifuged at slow speed, then at high speed; resuspended organisms were filtered with Nucleopore filters then centrifuged over a Hypaque gradient.

The lipid composition of *T. pallidum* was as follows: phospholipids, 68% of total lipids (mostly cardiolipin, sphingomyelin, and phosphatidyl choline); neutral lipids, 32% of total lipids (mostly cholesterol). As expected, the individual fatty acids of *T. pallidum* reflected the fatty acids found within testicular tissue: palmitic, stearic, oleic, and linoleic comprised 76% of the total. Compared with rabbit testis, *T. pallidum* contained twice as much palmitic acid and two to three times as much 20-carbon chain fatty acids. In addition, monoglycosyldiglyceride, a component of all non-pathogenic treponemes, was not found in *T. pallidum*. The authors suggested that this fatty acid could be used as a marker to identify *T. pallidum* after successful *in vitro* multiplication. The best method available for definitive identification of *T. pallidum* is dermal injection of the organisms into rabbits and observation of any lesions produced. This technique will not detect the emergence of an "avirulent" population of *T. pallidum* that is unable to induce lesions. If this occurs, the lack of monoglycosyldiglyceride would indicate the presence of *T. pallidum* rather than of a "contaminating" non-pathogenic treponeme (*81*).

IN VITRO STUDIES IN THE PRESENCE OF TISSUE CULTURE CELLS

This section summarizes research involving the interaction of *T. pallidum* with cultured mammalian cells. Findings will be presented in chronological order to indicate the progress achieved. Eight reports were published between 1913 and 1963 and 18 have appeared since 1975. In reviewing the older manuscripts, the frustrations of this type of research are apparent. The two major problems were reproducibility in other laboratories and loss of virulence during incubation. Despite numerous claims of success, when identical procedures were attempted by different investigators, multiplication was not detected. Two comments apply to loss of virulence during incubation. First, many of the false claims to have achieved successful multiplication resulted from contamination with non-pathogenic treponemes that are morphologically indistinguishable from *T. pallidum*. Second, loss of virulence may be a natural

consequence of *in vitro* growth. Many bacteria exhibit a loss of virulence during *in vitro* growth. Usually, however, virulence can be fully restored by re-inoculation into animals. This does not apply to *T. pallidum*, since its loss of virulence is apparently irreversible and cannot be restored by animal inoculation. As stated by Fieldsteel et al. (*20*), loss of virulence during incubation of *T. pallidum* probably results from loss of the ability of the organisms to divide. This apparently occurs well before loss of motility (*15*).

EARLY STUDIES

The 8 early reports will be briefly reviewed since they have been somewhat overlooked. The first attempt to culture *T. pallidum* in the presence of cultured cells was made by Steinhardt in 1913 (*89*). Hanging-drop preparations of rabbit testicular tissue previously inoculated with treponemes were used. After 25 days, extensive outgrowth of the testis cells occurred. At the time of initiation of these cultures, no treponemes were detected. After the first few days of incubation, some organisms were observed; within 25 days numerous motile organisms were present. Virulence determinations were not performed. Unfortunately, Steinhardt obtained the strain of *T. pallidum* from Noguchi who had indicated that it had already lost its virulence. Subsequently, successful cultivation of this strain has been achieved and it has been identified as a non-pathogenic treponeme. Nevertheless, these techniques of Steinhardt were the forerunners of future *T. pallidum*–tissue culture interaction. Others had attempted to culture the organisms prior to Steinhardt, most notably Schereschewsky in 1906, but these attempts utilized fragments of host tissue in nutrient broth rather than tissue culture methods based on outgrowth of the cultured cells.

In 1920, Levaditi (*51*) attempted to adapt *T. pallidum* to tissue culture techniques used for growth of poliovirus and rabies virus. Fragments of infected rabbit testis were grown in rabbit plasma. Outgrowth of these tissue cells occurred and numerous motile treponemes were detected. Outgrowth of the cultured cells was also observed in three subsequent passages. Motile treponemes, however, were reported only in the primary passage. Samples of material from the fourth passage failed to produce testicular lesions, indicating loss of virulence.

In 1929, Bessemans & Vlaeyen (*10*) cultured infected testicular tissue and observed outgrowth of fibroblastic cells. They used anaerobic conditions and human isolates of *T. pallidum* that were recently injected into rabbits. Although the treponemes survived for a short time, they did not multiply.

In 1932, Foldvari (*32*) summarized his studies of the influences of tissue explants on the survival of *T. pallidum*. The human tissue explants were derived from uninfected skin or from syphilitic tissues (condylomata latum and papules) isolated from eight patients. The explants were solidified by adding human plasma and hen embryo extract. Samples from one patient yielded increased numbers of treponemes. Interestingly, large numbers of organisms clumped around the tissue fragments and also attached to the fragments. This provided the initial evidence that *T. pallidum* attaches to host tissues. In material obtained from another patient, outgrowth of epidermal cells was observed, but increases in treponemal numbers were not detected. The author did not claim successful *in vitro* multiplication, but indicated that these studies were quite promising.

In 1933, Kast & Kolmer (*47*) described their attempts to culture *T. pallidum* using techniques previously employed for cultivation of different viruses. This was the first detailed description of tissue culture techniques applied to *T. pallidum*. Explants of infected rabbit testis and lymph nodes, and of uninfected rabbit testis and embryo were used. Extracts of chick embryo and rabbit spleen, testis, and embryo provided additional nutrients to the basal medium of Maitland. In a departure from other studies, incubation was performed aerobically. Within 48 hours, cellular outgrowth of tissue occurred in most of the primary cultures. These were subsequently passaged 2–3 times. Multiplication of *T. pallidum*, however, was not detected. The morphology and motility of some of the organisms were well preserved, but infectivity was lost.

In 1934, Bessemans & de Geest (*9*) tested suspension-drop cultures of testis and popliteal lymph nodes from uninfected rabbits and from rabbits infected with the Gand strain of *T. pallidum*. These cultures were incubated aerobically in Locke's medium supplemented with rabbit plasma. Tissue cell proliferation with fibroblastic morphology occurred. Better tissue outgrowth was observed with the uninfected tissues. The cultured cells were passaged four times every fourth day. The treponemes within the infected tissue lost motility within 48 h.

In 1948, Perry (*73*) described a tissue culture system, which he described as partially successful for growth of *T. pallidum*. Explants of uninfected rabbit testicular tissue were obtained from newly born or young adult animals. Within 24 h outgrowth of fibroblastic and epithelial cells was detected. Tissue from infected testis was also explanted, and outgrowth of this was more difficult to establish. In two of these cultures, large numbers of motile treponemes were detected 5 and 7 days later. After 10

days of incubation, medium from these was inoculated intratesticularly into rabbits. Syphilitic chancres that were dark-field positive resulted. Unfortunately, the data specifying increases in treponemal numbers were not provided. These increases may have represented transient *in vitro* multiplication. Without specific data, however, it was not possible to distinguish multiplication from the gradual passage of treponemes from the infected tissue into the culture medium during incubation. Perry did not publish any subsequent reports to confirm this finding.

In 1962, Wright (*100*) published results of her detailed studies of the interaction of treponemes with cultured mammalian cells. These cells were derived from rabbit testis, liver, and kidney, and human amnion and embryonic liver. An established cell line, Hela, was also tested. The survival of *T. pallidum* was not affected by the cultured cells. In like manner, the survival of *T. pertenue* and *T. phagedenis* biotype reiter was also not influenced by the cultured cells. Each treponemal preparation lost motility within 6–12 h.

Most of this work was performed under aerobic conditions. Attempts were made to decrease oxygen concentrations by overlaying with agar after adding treponemes to the cultured cells. Although the tissue cells survived well, the organisms did not. The effects of reducing agents and different "reducing media" were also tested. Glutathione, Brewer's (*12*), Nelson's (*64*), and Weber's (*97*) media failed to extend treponemal survival. In addition, these media were highly toxic to the cultured cells.

One interesting series of experiments was tried. Cultured cells were incubated with *T. pallidum* for 30 days. Wright thought that treponemal multiplication might occur intracellularly and require prolonged incubation. Although multiplication was not detected, the idea of intracellularity was ahead of its time. Several years later, Sykes & Miller (*90*) and Azar (*2*) demonstrated intracellular *T. pallidum* within samples of infected human and rabbit tissue. Twelve years after Wright's study, Fitzgerald et al. (*24*) showed that *T. pallidum* attained intracellularity after incubation with tissue culture cells.

Despite the negative overtones of Wright's research, one important, and often overlooked observation was made. Up to one-third of the inoculum of *T. pallidum*, and apparently of *T. pertenue* also, readily attached to the surface of the culture cells. The attachment was firm for both motile and non-motile treponemes; these organisms could not be dislodged by the currents caused by pressure on the coverslip. Wright minimized this observation and relegated it to "fortuitious collision with a sticky surface".

MORE RECENT STUDIES

In 1975, Fitzgerald et al. (*24*) reported further observations on the interaction of *T. pallidum* with cultured mammalian cells. They used cells derived from rabbit testis and from a human cervical carcinoma. Incubation was performed aerobically. Contrary to the findings of Wright (*100*), the presence of cultured cells significantly extended the time of survival of *T. pallidum*. Motile organisms were detected for 10 h with cultured cells and 4 h without cultured cells. Duration of virulence was also prolonged by cultured cells. After 24 h of incubation with cultured cells, the initial inoculum of 10^7 treponemes/ml had decreased to 10^4 treponemes/ml; in contrast, without cultured cells, virulent organisms were not detected after 9 h of incubation.

Microscopic observation confirmed Wright's (*100*) preliminary finding of attachment. Within minutes organisms began to attach to the surface of the cultured cells. Attachment was very firm and the attached organisms remained motile. Detachment of some organisms occurred after addition of fresh culture medium. *T. pallidum* rendered non-motile by heating failed to attach. This indicated that attachment was due to active participation of the organisms as opposed to phagocytosis by the fibroblast-like rabbit testis cells. In addition, two non-pathogenic treponemes, *T. denticola* and *T. phagedenis* biotype reiter failed to attach. This was the first implication that attachment might be associated with the pathogenicity of *T. pallidum*. Vital staining showed that attachment of the organisms did not harm the cultured cells. Finally, *T. pallidum* rapidly attained intracellularity during incubation with the cultured cells as demonstrated by transmission electron microscopy. Intracellular treponemes were not observed after inoculation of the tissue culture cells with *T. denticola* or *T. phagedenis*.

In 1976, Sandok et al. (*79*) published a preliminary note involving tissue culture and *T. pallidum*. A pre-reduced culture medium and rat glial cells were used. They confirmed the earlier findings concerning the attachment of *T. pallidum* to cultured cells. Organisms that were attached retained motility longer than those that were not attached. In evaluating various redox potentials, optimal treponemal motility occurred at -232 mV, pH 7.6. In further experiments, treponemal survival was assessed in the absence of tissue culture cells with fresh medium or with spent medium previously incubated with rat glial cells. Better survival occurred in the spent medium indicating that the tissue culture cells elaborated a factor beneficial to *T. pallidum*.

In 1976, Fitzgerald et al. (*25*) discussed previous

findings relative to the tissue culture-treponeme interaction. Improvements in the system resulted in extending the retention of virulence to 48 h without decreases in treponemal numbers. Comparisons were made between *in vivo* activities of *T. pallidum* and *in vitro* observations in the presence of cultured cells. It was initially suggested that attachment was the first step in the syphilitic disease process. The contributions of cultured cells responsible for extending treponemal survival both *in vitro* and *in vivo* were discussed. The beneficial effects appeared to be due to direct interaction of the organisms with the cultured cells. It was hypothesized that cultured cells neutralized oxygen toxicity thereby prolonging treponemal survival.

In 1975, Rathlev (*76*) published observations on the survival and virulence of *T. pallidum* under aerobiosis. A portion of this work involved incubation with tissue culture cells derived from rabbit testis. Attachment of the treponemes to the cultured cells was reported. The antibiotic gentamicin significantly increased treponemal survival. Virulence of the organisms was maintained for at least 6 days.

Successful *in vitro* cultivation of *T. pallidum* was reported in 1976 by Jones et al (*45*) using baby hamster kidney (BHK-21) cells. Their procedure was unique in that reducing agents were not used and incubation was performed in air with 7% carbon dioxide. The organisms were subcultured 9 separate times over a period of 10 days. Approximately 30 generations of *T. pallidum* resulted. The authors attributed their success to 3 important factors: initial numbers of cultured tissue cells (2+ confluence), subculturing frequency (every 24 h), and the duration of the infection from which the virulent organisms were initially obtained. This last factor is certainly unusual. Multiplication did not occur if the inoculum was obtained from a 12–18-day testicular infection but did occur if the inoculum was from a 43-day-old testicular infection. The authors suggested that a cytotoxic factor was present at higher levels in inocula from early infections. This observation of multiplication may reflect non-pathogenic treponemal contamination. It is difficult, however, to reconcile contamination with the induction of dark-field positive syphilitic lesions produced after 2, 5, 6, 8, and 9 days of incubation.

In 1976, Sandok et al. (*80*) described modifications in their system that improved the survival of *T. pallidum*. Whereas in their first experiments (*79*) motility of the organisms in the presence of rat glial cells had been retained for at least 24 h, in the modified system motility and virulence were extended to 120 h. Cultured cells derived from human prepuce tissue were more beneficial than rat glial cells. An anaerobic environment comprised of nitrogen, hydrogen, and carbon dioxide was superior to nitrogen and hydrogen in the absence of cultured cells; no differences, however, were detected in the presence of cultured cells. Furthermore, the effects of different tissue culture media, both freshly prepared and spent (previously incubated aerobically only with cultured cells) were assessed. Freshly prepared Richter medium was generally better than freshly prepared or spent Eagle's MEM.

The authors observed increases in treponemal numbers during incubation with cultured cells. In one experiment the initial inoculum of 18 treponemes per field increased to 30 treponemes per field, but the authors were careful to avoid specific claims of *in vitro* multiplication. Instead, they suggested that increases may have resulted from the release of treponemes from particulate debris or from clumps of organisms; increases may also have resulted from the completion of treponemal multiplication that was initiated within the rabbit testis prior to harvesting.

Observations on the interaction of *T. pallidum* with cultured cells were published by Kiraly and Horvath in 1976 (*49*). Their research differed from previous reports in that it involved the Budapest strain of *T. pallidum*, basal medium comprised of modified Nelson's medium supplement with heated human serum, and low levels of oxygen (1.1%). These studies were the forerunner of research by others (*20*, *26*, *71*, *81*) in which maximum survival of *T. pallidum* occurred in low levels of oxygen.

Treponemal survival was similar in the presence of different cell types including HeLa, HEP-2, and KB cells lines and primary cultures derived from human amnion, human embryo, and pig kidney. Motility was retained for at least 96 h. Better survival, however, occurred in the absence of cultured cells. With explants of infected testicular tissue, motile organisms were detected for only 48 h. The Budapest strain readily attached to the cultured cells. This is a noteworthy observation, since all other reports of treponemal attachment involved the Nichols strain of *T. pallidum*.

In some of these experiments, floccules of treponemes were detected. These consisted of a core of tissue debris surrounded by an extremely large accumulation of swarming treponemes. Human serum, modified Nelson's medium, and rabbit testicular constituents were essential for floccule formation, In contrast, cultured cells were not essential. The authors suggested that these floccules may have represented transient multiplication as opposed to heavy agglutination of organisms.

In 1977, Fitzgerald et al. (*26*) reported improvements in their tissue culture system that significantly extended treponemal survival. Anaerobic conditions in confirmation of Sandok et al. (*80*) were better than

aerobic conditions. The cultured cells, however, failed to survive beyond 48 h. The minimal oxygen concentration compatible with cultured cell survival was 3%. Additions of cysteine and glutathione were beneficial to treponemal survival. Serum supplements of 100ml of normal rabbit or fetal bovine serum per litre were superior to bovine serum albumin, bovine serum albumin low in fatty acids, or bovine serum albumin low in lipids. Besides cultured cells derived from normal rabbit testis, the treponemes also benefited from other cell types such as HSE, TRK-1, rat glial, NRK, and SFIEpNBL-11 cells. The addition of dithiothreitol every 24 h significantly extended treponemal survival. In earlier work, these authors had shown that treponemes were maintained for 2 days without decreases in the number virulent. Subsequent improvement resulted in survival for 6 days without decreases in the number virulent; after 7 days the number of virulent organisms decreased from 10^7 to 3×10^4 treponemes per ml.

In 1977, Fitzgerald et al. (*28*) used scanning electron microscopy to demonstrate the topographical features of *T. pallidum* attached to cultured cells. The treponemes were randomly distributed on the cell surface and did not exhibit a predilection for any particular area. The morphological integrity of the cultured cells remained intact despite heavy infestation by the organisms. With larger treponemal inocula, as many as 50–70 organisms were attached per cultured cell without damaging the cell. Usually, one end of the organism was attached, but occasionally attachment was seen at both ends. No difference between the two ends in treponemal morphology was apparent at magnifications of 100 000x, and hooks or attachment organelles were not observed. At the site of attachment, the cell surface did not show any indentations or swellings around the treponemes. The attached end of the organism was in close physical proximity to the cell surface but did not alter the cell surface. A coat of mucopolysaccharide was demonstrated on the surface of the treponemes by staining with ruthenium red. This treponemal outer layer confirmed the initial observation of a capsule (*101*).

In 1977, Hayes et al. (*40*) reported on the parasitism of host cell surfaces by *T. pallidum*. Although these observations do not apply to the *in vitro* cultivation of the organisms, they do contribute further knowledge of the interaction of treponemes with cultured cells. HEP-2 cells, a human cell line, and NRT cells derived from rabbit testis were used and incubation was performed aerobically. In confirmation of research by Fitzgerald et al. (*24*), *T. phagedenis* biotype reiter failed to attach to cultured cells and the attachment of *T. pallidum* was dependent on actively metabolizing organisms. Heat inactivated or formaldehyde-treated *T. pallidum* did not attach. Another indicator of the association between treponemal metabolism and attachment was temperature dependence. Far more organisms attached at 25, 33, and 37°C than at 4°C. Based on transmission electron microscopy, the authors suggested that the tapered ends of *T. pallidum* were specialized organelles that mediated attachment to host cells.

Further research assessed treponemal effects on the surface of the cultured cells. NRT cells were incubated for 2 h with 14×10^6 organisms/ml. The medium was then replaced with different concentrations of treponemes and after 2 h, the number of attached organisms was counted. Fewer organisms became attached than was anticipated. The authors postulated that the membranes of the host cells were modified by prior attachment making them somewhat refractory to further attachment.

The influences of various detergents, enzymes, and metabolic inhibitors on the adherence of *T. pallidum* to cultured cells were also investigated. The two detergents sodium deoxycholate and digitonin restricted treponemal attachment without interfering with treponemal motility. Various enzymes as well as the metabolic inhibitors did not affect attachment, except for potassium cyanide which was highly toxic to the organisms.

An important observation was the blockage of attachment of *T. pallidum* to cultured cells by immune rabbit serum. Treponemes were preincubated for 1 h with heated sera obtained from previously infected rabbits. After incubation with NRT cells for 4 h, the number of attached organisms was counted. No blockage was observed with two of the infected sera, while blockage was observed with two other sera obtained from rabbits infected 30 or 135 days previously. Half as many organisms were attached compared with the control. This blockage of attachment occurred without interfering with treponemal motility.

In 1977, Fieldsteel et al. (*20*) studied the effects of twelve different cultured cell types derived from rabbit, man, rat, mouse, and dog. The initial experiments were performed under anaerobiosis and this resulted in a rapid demise of the tissue culture cells. *T. pallidum* attached to each cell type and attachment extended survival. SfIEp[a] cells were chosen for further study because of their slow growth and consequent reduced need for medium changes. As indicated on page 790 redox potential, a function of oxygen concentration, was altered by using different volumes of culture medium in vertical test tubes and horizontal Leighton tubes and incubating aerobically. Extremes of redox potential resulted in

[a] A rabbit cell line from epidermal tissue.

shorter survival of *T. pallidum* in these cell-free systems. Different results occurred in the presence of cultured cells and the authors suggested that one effect of the cultured cells was on the redox potential. This corroborates the original suggestion of Fitzgerald et al. (*25*) that tissue culture cells partially neutralized oxygen toxicity.

In another series of experiments, gradient tissue cultures were used. Cells were grown on cover-slips in horizontal Leighton tubes, the medium was removed, the tubes were placed in a vertical position, and enough medium was added to cover the cultured cells completely. Incubation was performed in air or in nitrogen and carbon dioxide with different concentrations of reducing agents. Resazurin indicated areas with different redox potentials. Optimal treponemal motility was recorded only within certain areas. These optimal zones shifted downwards during air incubation or in the presence of reducing agents. The authors pointed to an inherent problem with this procedure. In the lower portions of the tubes (lower oxygen concentration), the cultured cells survived poorly, whereas in the upper portions the cells survived well. This produced a gradient of tissue culture growth, the depth of the oxidized region corresponding to the depth of confluent cell growth and always including the area of maximum *T. pallidum* survival. Thus, that area, besides reflecting an optimum redox potential for the organisms, may also reflect the presence of cultured cells. Attempts to clarify this problem by excluding the tissue culture cells are complicated by the settling of treponemes to the bottom of the tubes during extended incubation, As an alternative procedure, cultured cells were grown horizontally on cover-slips in flat-bottom shell vials. Treponemes were added, then covered with different volumes of medium. Best survival of organisms was detected with intermediate volumes, again demonstrating that extremes of redox potential are harmful. In subsequent experiments, vigorously motile organisms were detected after 21 days of incubation and importantly, these organisms retained virulence.

In 1977, studies of Fitzgerald et al. (*27*) reinforced the previous hypothesis that attachment of *T. pallidum* to tissue culture cells is an integral part of the infective process (*25*). Inasmuch as syphilis is a generalized infection in which the organisms infect most tissues, attachment studies were expanded to 19 different cell types derived from testis, kidney, spleen, lung, epidermis, cervix, urethra, and nerve tissue. The treponemes interacted with each of the different cell types. Although slight variations occurred in numbers of treponemes attached per individual cell, one cell type did not emerge as clearly superior to the others. With each cell type, organisms attached to virtually all of the cells within the culture vessel; large numbers attached without overt damage to the cells; and the presence of the cells significantly extended the time of treponemal survival. In a previous paper (*24*), heated *T. pallidum* failed to attach; in this study treponemes that were rendered non-motile by exposure to air also failed to attach. In addition, cultured cells that were rendered non-viable by freezing and thawing were far less beneficial to treponemal survival and far fewer treponemes attached per cell. When inocula containing 10^6, 10^7, or 10^8 organisms/ml were added to the cells, approximately 50–60% of the organisms within each preparation attached. This suggested that only some of the treponemes were capable of attaching to the cultured cells. Importantly, *T. pallidum* appeared to attach *in vivo* in an identical manner to that observed *in vitro* with cultured cells. Samples removed from testicular or dermal infections revealed numerous organisms attached to small pieces of tissue. These organisms were actively motile and attached at their distal end.

Previous results with two non-pathogenic treponemes suggested that attachment reflected a virulence determinant. These studies were extended to nine other non-pathogenic treponemes, and in each case no attachment was detected. Finally, a factor was demonstrated within immune rabbit serum that blocked treponemal attachment. In order to demonstrate this factor, it was necessary to pre-incubate *T. pallidum* for approximately 20 h. These "sensitized" preparations were then added to cultured cells and the number of attached organisms was assessed after 1.5–3 h of incubation. Immune rabbit serum reduced the proportion of treponemes capable of attaching to cultured cells from 27% to zero without interfering with active motility. The authors postulated that the immune serum component, presumably antibody, was an important factor in the healing of treponemal lesions.

In 1978, Sandok et al. (*81*) reported unsustained multiplication of *T. pallidum* using a modification of their previous cell culture systems (*79*, *80*). The organisms were incubated in the presence of low levels of oxygen with and without human prepuce cells. With oxygen (5–6%) 2–4-fold increases in treponemal numbers were observed in the absence of the cultured cells; without oxygen no significant increases in treponemal numbers were observed.

Slightly different results were detected in the presence of cultured cells. With oxygen (3–4%) 2–5-fold increases in treponemal numbers were observed; without oxygen 2.5–3.5-fold increases were observed, but the latter were not significant. Thus multiplication appeared to be dependent on low levels of oxygen.

Four other papers by Fitzgerald et al. (*22*, *23*, *29*, *30*) concern the attachment of *T. pallidum* to tissue

culture cells. Briefly, the treponemes elaborated a mucopolysaccharide material during attachment to the cultured cells; the mucopolysaccharide outer layer of *T. pallidum* contains *N*-acetyl-D-glucosamine and *N*-acetyl-D-galactosamine and appears to be highly labile; *T. pallidum* exhibited a predilection for host ground substance *in vivo*. Finally, a mechanism of attachment of *T. pallidum* to cultured cells was hypothesized. The organisms possess a mucopolysaccharidase enzyme at their outer surface which specifically interacts with its substrate mucopolysaccharide found at the surface of the cultured cells. Thus, attachment appears to involve an enzyme–substrate reaction.

In 1979, Fieldsteel et al. (*21*) compared the attachment of the Nichols strain of *T. pallidum*, which had been passaged in rabbits for 67 years, and of the KKJ strain, which had been passaged in rabbits 4–6 times. Similar rates of attachment were observed with each of five different cultured cell types. This observation extends the attachment phenomenon to three strains of *T. pallidum*.

In further work, the Gauthier and FB strains of *T. pertenue* also attached to the five cultured cell types, although relative to *T. pallidum* fewer organisms were attached per cultured cell. This important finding confirms the suggestion of Fitzgerald et al. (*11*) that attachment may be a virulence determinant related to pathogenicity.

During these experiments, Fieldsteel et al. (*21*) noted what appeared to be 4-fold increases in the numbers of treponemes attached to the cultured cells. To exclude the possiblity that this had resulted from the gradual settling of the organisms, they added *T. pallidum* to Sf1Ep cells for 2 h; unattached treponemes were then removed by washing and fresh medium was added. In 25 consecutive experiments, 3–5-fold increases in treponemal numbers were detected after 7 days. This may reflect transient *in vitro* multiplication, or it may reflect completion of multiplication cycles initiated *in vivo* just prior to harvesting from the infected testes. Certainly, more research is required to extend this promising observation.

The authors also detected swarms of *T. pallidum*, consisting of thousands of vigorously motile treponemes, associated with cells. This phenomenon was previously described by Kiraly & Horvath (*49*) and termed floccule formation.

CONCLUSIONS

Resolution of the oxygen question will facilitate research involving the *in vitro* multiplication of *T. pallidum*. Studies of treponemal oxygen uptake and treponemal survival in different concentrations of oxygen combined with the demonstration of a functional electron transport system indicate that *T. pallidum* is not a strict anaerobe. Recent research points to a microaerophilic nature with an optimal range of 1–3% oxygen. This is reasonable since treponemes multiply extensively in the dermis and testis and these tissues contain about this concentration of oxygen.

Oxygen toxicity is, however, a complex matter. Free radical species of molecular oxygen, such as superoxide anions, hydroxyl anions, and lipid peroxides, mediate toxic influences at the cellular level. Lipid peroxidation plays a prominent role in oxygen toxicity, especially in membranes. In treponemal research, lipid peroxidation may occur as a result of fatty acid composition of treponemes, the procedures used for harvesting organisms from infected testis, or the presence of polyunsaturated fatty acid in the extraction medium.

Besides peroxidation of lipids, disruption of treponemal capsular integrity may be related to oxygen toxicity. This capsule is comprised of acidic mucopolysaccharides that degrade in the presence of oxygen. The degradation of this protective capsule may in turn make the membrane lipids more accessible to oxygen species, leading to damaging peroxidation.

The requirement of *T. pallidum* for sulfhydryl (SH) agents appears to be related to oxygen toxicity. SH agents may limit capsular dissipation and subsequent lipid peroxidation by absorbing the highly reactive free radicals of molecular oxygen. In the process, the SH groups are oxidized to disulfide (SS) groups. The rates of SH oxidation are extremely variable with different experimental protocols. In developing media for suspension of *T. pallidum*, numerous different combinations and concentrations of SH agents have been used. The variations between laboratories probably result from differences in experimental protocol. Conditions that favour oxygenation of the medium would generate more free radicals of oxygen, and higher concentrations of SH agents would be required to absorb these additional free radicals that are detrimental to *T. pallidum*.

In the future, successful multiplication of *T. pallidum* will depend on minimizing both areas of potential oxygen toxicity. Lipid peroxidation can be somewhat neutralized by excluding heavy metals, using free-radical traps including SH agents,

minimizing testicular tissue damage, and employing reduced oxygen concentrations both during extraction and during incubation. Depolymerization of the mucopolysaccharide treponemal capsule may be retarded by poly-cations that stabilize the poly-anionic mucopolysaccharides, by SH agents, and by reduced oxygen concentrations.

Investigations of the metabolism of *T. pallidum* should be most helpful in establishing optimal conditions for *in vitro* incubation. Previously, experiments aimed at multiplication were evaluated in terms of retention of motility, assuming that motility directly reflected viability. Conditions that increased the duration of motility were then used for further cultivation experiments. Although motility does reflect viability, it does not always indicate optimal metabolic functioning. Long-term retention of motility may also indicate minimum maintenance of the treponemes. Fieldsteel et al. (*20*) maintained *T. pallidum in vitro* for 24 days without detecting increases in treponemal numbers. If conditions were conducive to multiplication, 24 days should have been sufficient time for both acclimatization to the *in vitro* environment and for significant multiplication. Many bacteria can be maintained at reduced temperatures apparently because of marked decreases in metabolic activity. In similar fashion, prolonged survival of *T. pallidum* may be a result of reduced metabolic activity. Therefore, optimum conditions for retention of motility may not be best for *in vitro* cultivation. The effects of erythromycin demonstrate this problem. Treponemal motility is not influenced by this antibiotic although protein synthesis is rapidly inhibited. Metabolic observations then provide a better assessment of the status of the treponemes. In contrast, motility observations do not differentiate between treponemes that are being maintained and treponemes that are actively metabolizing.

It will be important to develop procedures for evaluating treponemal metabolism during extended periods of incubation. The early studies of metabolism involving short-term incubation (2–7 hours) were valuable for the development of methods and for demonstrating that metabolic experiments were possible. One drawback to these studies, however, was that abnormal rather than normal metabolism may have resulted. Sandok & Jenkin (*78*) suggested that death of *T. pallidum* within hours could produce a burst of metabolic activity that is not typical. Thus, metabolic studies performed over days should be more representative of normal metabolism.

Further investigations of DNA synthesis are extremely important. DNA biosynthesis should directly reflect treponemal multiplication. After laboratory procedures for demonstrating DNA synthesis have been developed, it will be possible to decide upon the optimum levels of oxygen concentration and temperature. Other parameters can also be determined. For example, most studies are performed with concentrations of treponemes adjusted to 5×10^6 or 10^7 organisms/ml. Is this an upper limit for multiplication? Once these conditions are established, further research will be required to assess whether optimal RNA and protein synthesis also occur at these concentrations.

Metabolic studies of *T. pallidum* are difficult to perform as evidenced by the divergent findings obtained in different laboratories. The effects of temperature provide a good example. Best retention of motility of treponemes occurs during incubation at less than 37°C (*94*, *99*). In metabolic studies, glucose was taken up at similar rates at 25, 30, and 37°C (*84*); protein synthesis was optimum at 32, 34, and 36°C but greatly reduced at 28°C (*4*); nucleic acid synthesis was optimum at 39°C (*68*). The effects of oxygen also produced conflicting reports. Baseman et al. (*7*) reported best protein synthesis in 10–20% oxygen and little synthesis at 0% oxygen. In contrast, Sandok & Jenkin (*78*) demonstrated a linear rate of protein synthesis during 96 hours of incubation in 0% oxygen. Norris et al. (*72*) observed little nucleic acid synthesis at 20% oxygen and optimum synthesis at 3% oxygen. These divergent findings probably reflect different experimental conditions. Some laboratories, for example, incubated treponemes in a simple medium composed of salts and buffer; under these conditions the organisms survived poorly. Other laboratories utilized a complex medium that supported better treponemal survival. Thus, metabolic assessments will vary depending on the survival of the organisms.

The observations on electron transport and oxidative phosphorylation were noteworthy in that they confirmed the original hypothesis of Cox & Barber (*16*) that *T. pallidum* is not a strict anaerobe. The fatty acid make-up and requirements of the treponemes seem to be important in relation to lipid peroxidation as major factors in the death of *T. pallidum* during incubation. Control of peroxidation together with incorporation of specific fatty acids should improve the chances of *in vitro* multiplication.

Tissue culture seems to hold much promise for *in vitro* multiplication of *T.pallidum*. The three recent reports of successful multiplication will be briefly discussed in terms of the problem areas of reproducibility, retention of virulence, and counting difficulties.

So far, no attempts have been made to reproduce the work of Sandok et al. (*81*) or Fieldsteel et al. (*21*). In 1977, Foster et al. (*33*) was not able to corroborate claims of Jones et al. (*45*) regarding successful cultivation and subculturing of virulent *T. pallidum* following the procedures described (*45*) or the

variations in these procedures communicated to them later by the authors verbally.

Another problem concerns retention of virulence. With freshly harvested preparations of *T. pallidum*, there is a good correlation between virulence and motility. Intradermal inoculation of these treponemes will produce lesions within the expected time periods. When treponemes are incubated *in vitro*, however, virulence is lost well before motility. For example, with a preparation adjusted to 2×10^7 treponemes/ml, 50% of the organisms may remain motile after four days of incubation. The injection of 0.1 ml of this material representing 1×10^6 motile treponemes should result in an incubation period of 4–5 days. However, the incubation period may extend to 10–12 days indicating 1×10^3 virulent treponemes.

Increases in microscopic counts are important for demonstrating multiplication. These increases in counts should be related to virulence. The credibility of successful multiplication is still dependent on retention of virulence. Where increases in microscopic counts are associated with decreases in virulence contamination with non-pathogenic treponemes must be suspected.

Jones et al. (*45*) attempted to correlate increases in numbers with virulence. Rabbits were injected intradermally with samples of culture medium after each subculture. Treponemal lesions occurred in less than half of the sites injected (18 of 46). These results are difficult to explain. For example, the samples injected after 24 h did not produce any syphilitic lesions indicating that less than 5×10^2 virulent treponemes were present. (Treponemal preparations at or below this concentration may not produce lesions in 100% of the animals injected (*94*)). After an additional 24 h of incubation, 10^7 virulent treponemes were detected corresponding to an increase of at least 5×10^4 organisms. The virulence of the organisms may have been temporarily lost, then regained. However, such a return of virulence for *T. pallidum* has not been previously reported.

The procedures of Sandok et al. (*81*) also failed to retain virulence of the organisms. In one set of experiments, during the initial 48 h of incubation treponemal numbers increased 4-fold. When samples of these were injected intradermally, the 0-h sample exhibited an incubation period of 7 days whereas the 48-h sample exhibited an incubation period of 14 days. According to previously published data by these authors (*37*), this represents an almost 100-fold decrease in numbers of treponemes virulent.

Turner & Hollander (*94*) determined the sensitivity of rabbit dermal injection as an indicator of virulent numbers of *T. pallidum*. In general, for each 10-fold difference in numbers, a 4-day difference in the incubation period results. In these studies by Sandok et al. (*81*) microscopic counts revealed a 2–5-fold increase. Thus, unless an unusually large number of rabbits was injected, the dermal test would not be expected to demonstrate these slight but significant differences. However, the incubation periods resulting from the injection of the 0-h and 48-h samples should at least be closely similar. They were not. In order to explain this lack of correlation between microscopic counts and virulence, the authors suggested a preferential stimulation of growth of an avirulent subpopulation of treponemes normally associated with the virulent microorganism *in vivo*.

Fieldsteel et al. (*21*) did not attempt to assess the retention of virulence in those experiments in which multiplication has occurred.

A third problem associated with *in vitro* multiplication involves counting of organisms. In order to count treponemes on a microscopic slide, large numbers of organisms are required. In our laboratory, a count of 10 treponemes per microscopic field at 400 x magnification is equivalent to approximately 1×10^7 treponemes/ml. Jones et al. (*45*) reported increases from an initial inoculum of 2×10^5 to 22×10^5 organisms/ml. The actual count per field must have been extremely low. Artley & Clark (*1*) have reviewed the difficulties of counting treponemes. They statistically evaluated inherent errors in counting procedures. With low numbers of organisms exact counting is difficult because of tendencies to clump. In addition, if 2-fold increases in numbers are detected it may reflect completion of multiplication cycles begun *in vivo* as pointed out by Sandok et al. (*81*). The microscopic counts in the work of Sandok et al. (*81*) were higher. They reported increases from an initial inoculum of 5×10^6 to 19×10^6 organisms/ml. Fieldsteel et al. (*21*) utilized the highest concentration of treponemes. In four separate experiments, the initial inoculation of 150 treponemes per microscopic field increased to 572 treponemes per microscopic field.

Despite these limitations, each of the three most recent reports of successful multiplication are certainly promising. More research will have to be performed to confirm these encouraging results. It will be important to determine the precise contributions of tissue cells to extending treponemal survival. When this is accomplished, it should be possible to utilize the parameters developed via metabolic studies to obtain reproducible *in vitro* multiplication of *T. pallidum* in nutrient broth.

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RÉSUMÉ

ÉTUDE SUR LA CULTURE *IN VITRO* DE *TREPONEMA PALLIDUM*

La culture *in vitro* de *Treponema pallidum* faciliterait bien des aspects différents de la recherche sur la syphilis. Cette étude résume les progrès accomplis dans ce domaine et publiés depuis 1975. Les résultats sont examinés du point de vue des tréponèmes et de la question de l'oxygène, du métabolisme des tréponèmes relatif aux protéines, aux acides nucléiques et aux acides gras, et de l'interaction des tréponèmes avec les cellules de cultures de tissu. Les approches futures proposées et les domaines où la culture des tréponèmes risque de rencontrer des problèmes sont examinés.

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