Redox potential and survival of virulent Treponema pallidum under microaerophilic conditions

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SUMMARY A strongly reduced culture medium, capable of maintaining the virulence of *Treponema pallidum* (Nichols) for several days, was exposed to an atmosphere of 3% oxygen in nitrogen for 2-3 days before inoculation with *T pallidum*. By using various volumes of medium in uniform tubes a range of redox potentials (E_{cal}) from -94 mV to -325 mV was produced depending on the surface area-to-volume ratios of the medium. The anaerobic medium had an E_{cal} value of -387 mV.

The medium was inoculated with *T pallidum* and incubated in an atmosphere of 3% oxygen. The survival of treponemes at different redox potentials was monitored by observing the retention of motility and by measuring the latent period of infection after inoculation of the cultures into the shaved backs of rabbits.

Under these conditions *T pallidum* survived longest at low (electronegative) redox potential. An inverse linear relationship was observed between the redox potential of the culture medium and the survival of *T pallidum,* as measured by the time required for a 90% reduction of virulent organisms. No optimum redox potential was detected, the most electronegative medium (-325 mV, E_{cal}) giving the best survival.

Introduction

Despite an effort extending over 70 years successful cultivation of *T pallidum* in vitro has not been achieved (see Addendum), although several laboratories have recently reported slight but notable growth of this pathogen.[1] [2] The difficulties of in-vitro cultivation have limited attempts to develop a vaccine against syphilis. Present research has included studies of the short-term metabolism of *T pallidum* in the hope of establishing conditions under which sustained growth of this bacterium in vitro could be obtained.

T pallidum was previously considered to be an obligate anaerobe. The demonstration in recent years of oxygen consumption similar to an aerobic *Leptospira,*[3] the presence of a functional cytochrome system,[3 4] and oxidative phosphorylation[5] have indicated the aerophilic character of the organism. Degradation of glucose and pyruvate,[6] [7]

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incorporation of amino acids into protein,[8] uridine into nucleic acids,[9] [10] and glucose into TCA precipitable material,[11] and decarboxylation of pyruvate[12] all occurred under aerobic conditions, although some studies have also shown anaerobic metabolism.[13] [14]

The optimum concentration of oxygen for *T pallidum* metabolism has been determined by monitoring its survival under varying concentrations of atmospheric oxygen. Using a tissue culture system Fieldsteel *et al*[15] established an oxygen gradient in which maximum survival (21 days) was observed in a region neither fully aerobic nor anaerobic. Under microaerophilic conditions (2·5% oxygen) Fitzgerald *et al*[16] were able to demonstrate prolonged retention of virulence and the synthesis of treponemal surface acid mucopolysaccharide in a tissue culture system. Recently, Graves and Billington[17] examined the survival of *T pallidum* in a cell-free system under a range of dissolved oxygen concentrations from 0·7% to 12·6% (atmospheric equivalents). Maximum survival was seen at 3% oxygen. Norris *et al*[18] found 3% oxygen to be superior to either strictly anaerobic or aerobic conditions. That oxygen was a mixed blessing for *T pallidum* was demonstrated by the

requirement for reducing agents for survival under microaerophilic conditions.[18] Research using strictly anaerobic conditions indicated that the retention of motility by *T pallidum* required a highly reduced environment.[19] *T pallidum* is now known to be much less active metabolically under anaerobic conditions.[20] The present study was undertaken to determine the optimum conditions of oxidation-reduction potential for the retention of virulence in *T pallidum* under what is now considered to be the optimum level of oxygen in the atmosphere above the culture medium (3%).

Materials and methods

MAINTENANCE OF *T PALLIDUM*

Adult male rabbits were used for the propagation of *T pallidum* (Nichols). They were inoculated with 5×10^7 treponemes per testis. An orchitis usually developed in 11 days. The rabbit was killed by intravenous injection of 3 ml of sodium pentobarbitone (200 mg/ml), and the testes were minced and eluted with six successive 10-ml aliquots of reduced medium under aerobic conditions. The extraction period was approximately 30 minutes. The first 10 ml were discarded since they contained large amounts of tissue debris and erythrocytes. The yield was approximately 50 ml of inoculum with a concentration of approximately 10^7 treponemes/ml. These bacteria were used immediately as inoculum for the tubes of medium at various redox potentials (see below), 10% of the medium volume being added as inoculum giving a final concentration of approximately 10^6 treponemes/ml.

PREPARATION OF REDUCED MEDIUM

The medium was a modification of that used by Graves and Billington.[17] It was prepared in two parts (A and B) as shown in table I. Part A was prereduced (before the addition of reducing agents and HEPES buffer) by autoclaving at 121°C for eight minutes followed by slow exhaust. It was cooled under sterile oxygen-free nitrogen and the reducing agents and HEPES buffer added as powders and dissolved by stirring. The medium was adjusted to pH 7·6-7·7 with 10mol/l NaOH, the flask tightly stoppered, placed in a clamp, and sterilised by autoclaving at 121°C for 15 minutes followed by fast exhaust.

Part B was prepared from solutions sterilised separately by filtration (amino acids, vitamins, solution D) or autoclaving (fructose, glucose, and phosphate solutions). When part A had cooled to ambient temperature part B (excluding glutamine and solution D) was added under sterile nitrogen. The final pH was 7·2-7·4. Ten-millilitre aliquots were dispensed into nitrogen flushed tubes (Bellco)

TABLE I *Composition of reduced medium*

Components	Amount/l (final total medium)
Part A	
Balanced salt solution concentrate*	60 ml
$(NH_4)_2SO_4$ (50 g/l)	2 ml
H_2O	614 ml
HEPES buffer†	7·15 g
Glutathione (reduced)	2·38 g
Cysteine	1·58 g
Sodium thioglycolate	1·00 g
Part B	
Glucose‡	1·60 g
Fructose‡	1·60 g
Glutamine (29·2 g/l)	10 ml
Amino acid concentrate A§	10 ml
Amino acid concentrate B‖	10 ml
Amino acid concentrate C¶	10 ml
Vitamin concentrate**	10 ml
Solution D††	10 ml
Fetal calf serum (heat inactivated)	250 ml

*NaCl (40 g/l), KCl (4 g/l), $CaCl_2$ (1·4 g/l), Na_2HPO_4 (0·6 g/l). KH_2PO_4 (0·6 g/l), $MgSO_4$ $7H_2O$ (2 g/l).
†N-2-Hydroxyethylpiperazine -N^1 -2-ethanesulfonic acid (HEPES).
‡Added as sterile 50% solutions.
§Composition per 10 ml: arginine HCl, 105 mg; histidine HCl H_2O, 31 mg: isoleucine, 52 mg; leucine, 52 mg; lysine HCl, 58 mg; methionine, 15 mg; phenylalanine, 32 mg; threonine, 48 mg; tryptophan, 10 mg; and valine, 46 mg.
‖Composition per 10 ml; cystine, 24 mg and tyrosine, 36 mg
¶Composition per 10 ml: alanine, 8·9 mg; asparagine H_2O, 15 mg; aspartic acid, 13·3 mg; glutamic acid, 14·7 mg; proline, 11·5 mg; serine, 10·5 mg; and glycine, 7·5 mg.
**Composition per 10 ml: choline chloride, 1 mg; folic acid, 1 mg; i-inositol, 2 mg; nicotinamide, 1 mg; calcium pantothenate, 1 mg; pyridoxal HCl, 1 mg; riboflavin, 0·1 mg; and thiamine HCl, 1 mg.
††Composition per 10 ml: sodium pyruvate, 250 mg; adenine, 5 mg; cocarboxylase, 3 mg; coenzyme A, 50 µg; isobutyric acid, 10 mg; α-lipoic acid, 5 mg; p-amino benzoic acid, 5 mg; biotin, 50 µg; nicotinic acid, 500 µg; pyridoxine HCl, 2·5 mg; pyridoxamine HCl, 5 mg; putrescine 2HCl, 10 mg; and cyanocobalamin, 50 µg; uracil 5 mg; folinic acid 0·8 mg; and pyridoxal phosphate 1 mg.

and tightly stoppered. These became the anaerobic control tubes. The remainder of the medium was placed in an anaerobic hood (Germ Free Laboratories) under an atmosphere of 3% oxygen in nitrogen and dispensed into sterile glass tubes (Corex, 150 × 23 mm, 50-ml volume). A redox gradient was established by placing varying volumes of medium into the tubes and allowing them partially to equilibrate with the 3% oxygen atmosphere. The range of surface-to-volume ratios resulted in different degrees of oxidation of the medium by the 3% oxygen atmosphere. The volumes used were 3, 5, 10, 20, and, in some experiments, 7 ml, giving surface-to-volume ratios of 1·38, 0·83, 0·42, 0·21, and 0·59 respectively. The tubes, with cottonwool plugs, were allowed to remain at 34°C for 2-3 days before inoculation with freshly harvested *T pallidum*. Immediately before inoculation glutamine and solution D were added to each individual tube because of the chemical instability of certain components of each of these additives.

MEASUREMENT OF DISSOLVED OXYGEN CONCENTRATION

An oxygen meter (Model LR-30, Titron Australia, Melbourne), fitted with a Clark-type oxygen electrode modified to allow measurements in static solutions, was used. The meter gave readings in "parts per million" (μg/l) dissolved oxygen in water. The electrode was calibrated at 34°C with 100% nitrogen (oxygen-free), 3% oxygen in nitrogen and air, giving a linear relationship between oxygen meter reading (μg/l) and oxygen concentration in the gases as previously shown.[17] Since the media in which dissolved oxygen concentration was being measured had been exposed to an atmosphere of 3% oxygen for two or three days, the calibration of the electrode with these three oxygen/nitrogen gas mixtures was considered satisfactory, although we appreciated that the electrode-based measurements of the culture media were not as accurate as a chemical determination of dissolved oxygen concentration. (The dissolved oxygen concentrations given in table II correspond to the equivalent percentage of oxygen in the atmosphere above the medium.)

MEASUREMENT OF REDOX POTENTIAL

The redox potential of the medium was determined with a Platinum-Calomel electrode.[17] Tubes were stoppered before removal from the 3% oxygen hood and kept closed until the redox electrode was inserted for redox measurement. Duplicate samples were measured at each determination.

DETERMINATION OF VIABILITY OF *T PALLIDUM*

Survival of *T pallidum* was monitored by determining motility and virulence. Motility was determined by darkfield microscopical examination of 50 randomly selected treponemes from each of a pair of tubes at a given sample time and the percentage of actively motile treponemes recorded. Virulence was determined by inoculating the *T pallidum* samples into rabbits. Before removal from the 3% oxygen atmosphere, 0·8 ml of the sample to be tested was placed in a 1-ml syringe and 0·4 ml immediately inoculated into the shaved backs of each of two male rabbits. The backs of the rabbits had been previously divided into a 4 × 5 grid of 2·5 cm squares, and for each sample 0·1 ml was inoculated into each of four

TABLE II *The relationship between volume of medium per tube, redox potential of the medium, percentage of motile T pallidum, and the virulence of T pallidum present in the medium over the three-day experimental period**

Time (hours)	Medium volume (ml)	Redox potential†† (mV)	Dissolved oxygen (%) (atmospheric equivalent)	T pallidum survival Motility (%)	Virulence‡
0	3	− 171	3·2	100	1·11
	5	− 207	2·4	100	1·06
	10	− 282	2·0	100	0·99
	20	− 305	1·8	100	1·00
	AnO$_2$§	− 397	0·4	100	1·05
24	3	− 121	3·2	20	1·40 (12/20)‖
	5	− 179	3·1	28	1·23 (16/20)‖
	10	− 282	2·6	38	0·97
	20	− 330	2·5	49	1·00
	AnO$_2$	− 380	1·1	80	0·73
48	3	− 102	3·6	0	NL
	5	− 158	3·2	11	1·40
	10	− 270	2·4	19	1·05
	20	− 323	2·4	21	1·00
	AnO$_2$	− 367	0·7	72	0·65
72	3	− 75	2·9	0	NL
	5	− 114	2·4	0	NL
	10	− 255	1·6	0	NL
	20	− 345	2·0	5	1·00
	AnO$_2$	− 399	0·6	55	0·79

*Averaged data from six independent experiments.
†Daily readings given in table. The mean values over the three-day experimental period were: 3 ml: − 94mV; 5 ml: − 166mV; 10 ml: − 268mV; 20 ml: − 325mV; and AnO$_2$: − 387mV.
‡Expressed as the ability of the sampled *T pallidum* to cause syphilitic lesions on rabbits compared with treponemes in the 20-ml medium sample. Calculated as the mean latent period of infection of the sample divided by the mean latent period of infection produced by the 20-ml tube sample. Numbers >1 indicate longer latent periods and consequently fewer virulent *T pallidum* present than in the 20-ml sample. Latent period is the time (in days) between rabbit inoculation and the appearance of syphilitic induration at the inoculation site.
§Anaerobic control; volume irrelevant.
‖Denominator: total number of inoculated sites on rabbits; numerator: number of inoculated sites that developed into syphilitic lesions. Unless otherwise stated all sites developed into lesions.
NL = No lesions developed from any inoculated sites.

sites on the grid. The samples from media with different redox potentials were inoculated into different rows on the back of the same rabbit. Duplicate rabbits were inoculated at each time sample to minimise any effects of individual variations in their susceptibility to *T pallidum*. A total of eight rabbits was used for each experiment. The rabbits were kept cool (18°C), shaved, and examined daily for the appearance of induration at the site of inoculation. The number of virulent *T pallidum* in the sample was determined from the latent period of the infection using a standard curve.[21]

Results

GENERATION OF A RANGE OF REDOX POTENTIALS

With the method used the survival of *T pallidum* was tested in a range of redox potentials. The results of six separate experiments (averaged) are given in table II. Under the 3% oxygen atmosphere, concentrations of dissolved oxygen equivalent to between 1·8% and 3·2% atmospheric oxygen were present in the tubes of medium at the start of the experiment, depending on the particular surface-to-volume ratio, while the redox potentials ranged from − 171 mV to − 305 mV (table II).

EFFECT OF REDOX POTENTIAL ON *T PALLIDUM* SURVIVAL

Survival of *T pallidum* as monitored by both motility and virulence under various redox conditions is shown in table II and fig 1.

Survival times were longest in tubes which were the most electronegative. Survival was considerably better in the anaerobic control than would have been expected by virtue of its lower redox potential (see Discussion). Survival was markedly diminished in the 3-ml and 5-ml tubes where the redox potential was more electropositive than − 200 mV (E_{cal}) (table II). This was also shown by the marked delay in lesion formation following inoculation of treponemes into rabbits after only short incubation periods in vitro at these redox potentials. A typical example of one such experiment is given in table III. In two other experiments no viable treponemes were observed in media more electropositive than − 150 mV (E_{cal}) after only 24 hours' incubation in vitro.

Overall, an inverse linear relationship between the redox potential of the culture medium (under 3% oxygen) and the survival of virulent *T pallidum* was observed when treponemal survival was measured as the time (in hours) required for a 90% fall in the concentration of viable *T pallidum* in the culture (fig 2).
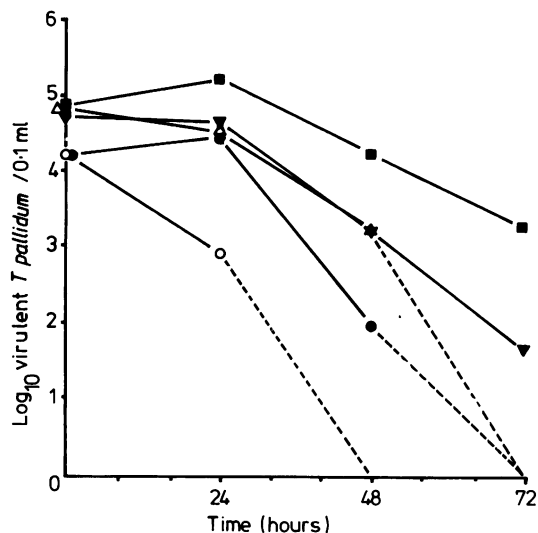

FIG 1 *The fall in concentration of virulent T pallidum (per 0·1 ml) in media with different redox potentials:* ■ *anaerobic tubes: average redox potential, − 387 mV (E_{cal});* △ *20-ml tubes: average redox potential, − 325 mV (E_{cal});* ▼ *10-ml tubes: average redox potential, − 268 mV (E_{cal});* ● *5-ml tubes: average redox potential, − 166 mV (E_{cal}); and* ○ *3-ml tubes: average redox potential, − 94 mV (E_{cal}).*

Discussion

The oxygen concentration and the redox potential of the medium can be of great importance to anaerobic and microaerophilic organisms which have little capacity for controlling the toxic effects of oxygen on the living cell.[22] In this study with *T pallidum* incubated in vitro we have found a clear relationship between treponemal survival and the redox potential of the medium at low oxygen concentrations (≤3%). The more electropositive the medium, the poorer was the survival of *T pallidum* in the cell-free medium

TABLE III *The virulence of T pallidum removed after one hour in culture medium with different redox potentials under microaerophilic conditions.*

Volume of medium (ml)	Redox potential (mV)	Latent period of lesion formation (days)*
3	− 152	17·0
5	− 168	15·3
7	− 230	9·5
10	− 252	8·4
20	− 271	8·6

*Latent period is the time (in days) between rabbit inoculation and the appearance of syphilitic induration at the inoculation site. Longer latent periods indicate fewer virulent *T pallidum* present in the inoculum.
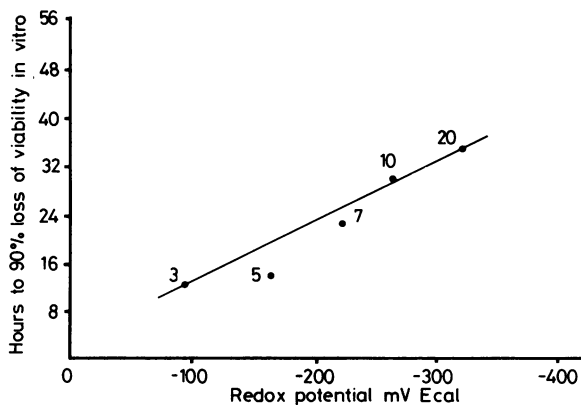
FIG 2 *Inverse linear relationship between redox potential of culture medium (under 3% oxygen) and survival of virulent T pallidum, as measured by the time required for the concentration of virulent T pallidum to fall by 90%. (Key: 3, 5, 7, 10, and 20 = No of ml of medium per tube.)*

used. The electropositive medium appeared to be toxic since a large proportion of the bacteria died within a few hours of exposure. Under strictly anaerobic conditions Metzger and Smogor,[19] using retention of motility of *T pallidum* as the criterion of survival, reported an optimum redox potential of −240 mV (E$_{cal}$). In our study using a different medium and microaerophilic conditions an inverse relationship between redox potential and survival was evident, but no optimum was observed (fig 2).

The effects of redox potential on treponemal survival could be explained either by the medium becoming toxic or by the lack of some component necessary for long-term survival. Toxicity of the medium would best explain the very rapid death of treponemes seen at redox potentials above approximately −150 mV (E$_{cal}$). With an in-vivo generation time estimated at 33 hours,[23] it is very unlikely that nutrient deficiency would cause death in such a short period. The most probable explanation is the previous production of toxic substances[24] (for example, superoxide anions, hydrogen peroxide, hydroxyl radicals, and singlet oxygen) from the action of oxygen on certain medium components.[25 26] At more negative redox potentials molecular oxygen is not as readily available in the medium to cause production of these toxic compounds. Survival in the anaerobic control medium was noticeably better than predicted from fig 2 on the basis of its redox potential alone. This suggests that by-products of oxidation reactions may have been detrimental to the survival of *T pallidum* under the microaerophilic conditions used.

Media with redox potentials more electronegative than −150 mV showed stationary periods of 24 hours, when the number of virulent *T pallidum* remained almost constant, before the onset of a rapid decline in numbers (fig 1). This suggests that the bacterium was unable to synthesise some key compound during in-vitro incubation in this medium. The stationary period might be expected to be shorter in a more aerobic medium, since metabolism is more rapid in higher concentrations of oxygen[20] and thus leads to an earlier death due to the deficiency in some key compound. This may be more complicated than a simple absence of a nutrient or factor necessary for macromolecular synthesis, since a dependency on the host could mean that *T pallidum* may have faulty transport systems that break down outside of the host. This phenomenon is seen with intracellular parasites such as the *Rickettsia*.[27]

The apparent preference of *T pallidum* for a microaerophilic but strongly electronegative environment in vitro may be related to several observations concerning *T pallidum* in vitro and in vivo. In vivo *T pallidum* grows well in oxygenated actively metabolising tissues which would, by virtue of rapid oxygen consumption, be quite electronegative. Such tissues would also be microaerophilic, since although oxygen flows into them from the blood circulation it is being rapidly utilised by the metabolising cells. The actual extracellular pO$_2$ surrounding most mammalian tissues is low. In the uninfected rabbit testis it is 11·6 mmHg[28] and in the human epidermis 5 mmHg at 32°C epidermal temperature.[29] Both microaerophilic conditions and electronegative redox potentials may well occur concurrently in those locations in vivo in which *T pallidum* grows well, such as the germinal epithelium of the rabbit testis,[30] the human fetus in utero, and granulating wound tissue of rabbits and humans.[31] *T pallidum*, when inoculated intravenously into rabbits, showed a marked preference for localising in granulating wounds on the shaved back where active cell growth was apparent.[31] Furthermore, the injury to the skin may have resulted in the redox potential becoming more electronegative and, as such, more suitable for the metabolism of *T pallidum*. Injuries to other tissues have resulted in the development of more electronegative redox potentials.[32 33]

The curative effect of hyperbaric oxygen on rabbit syphilis[34] may be explained in terms of rises in the redox potential of the animal tissues. Cater *et al*[35] have shown that the breathing of oxygen causes a rise in tissue redox potential, especially subcutaneous tissue.

Attachment of *T pallidum* to tissue culture cells and its postulated intracellular location appears to be

associated with prolonged survival *in vitro*.[36][37] This may reflect conditions of optimum redox potential for *T pallidum* at these sites. Coburn *et al*[38] found the intracellular oxygen tension to be 4-6 mmHg and to remain constant over an extracellular range of 35-400 mmHg oxygen tension. A survey of published intracellular redox potentials by Shapiro[39] showed that the E_h* varied from -240 mV (based on lactate/pyruvate ratio) to -285 mV (based on oxidized/reduced pyridine nucleotide ratio) to -307 mV within the mitochondrion. These values are considerably more reduced than those obtained in our most reduced microaerophilic culture medium, where the 20-ml tube averaged -325 mV (E_{cal}) (table II) or -84 mV(E_h). Extracellular redox potentials have been determined by the insertion of platinum or gold electrodes into intact tissue. Cater *et al*[35] found E_h values of approximately $+250$ mV in muscle, $+130$ mV in testis, and $+90$ mV in brain (E_{cal} equivalents; $+9$ mV; -111 mV, and -151 mV respectively). These extracellular values seem to be too electropositive for the survival of *T pallidum*, based on our in-vitro studies reported in this paper. *T pallidum* is known to survive primarily as an extracellular parasite, at least in the early stages of syphilis. Perhaps a redox gradient exists in host tissue, from more electropositive extracellularly to more electronegative intracellularly. A redox potential most suitable to *T pallidum* may occur at the membrane of the metabolising mammalian cell, thus explaining the enhanced survival of that fraction of exogenously added *T pallidum* that attaches to cells in tissue culture.[36][37] Of course, other explanations are also possible for this phenomenon (for example, nutritional or enzymic factors), but the role of redox potential is likely to be as important as that of oxygen concentration and nutritional components in future attempts to grow and subculture virulent *T pallidum* in vitro.

Addendum

Fieldsteel and co-workers[41] have just reported the successful cultivation of *T pallidum* in tissue culture under 1·5% oxygen.

*To convert E_h to E_{cal} the potential of the saturated calomel electrode, which at 25°C and with saturated KCl is $+241$ mV,[40] must be taken into account. Thus $E_h = (E_{cal} + 241)$ mV.

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