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Optimum concentration of dissolved oxygen for the survival of virulent *Treponema pallidum* under conditions of low oxidation-reduction potential

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SUMMARY A maintenance medium with a low oxidation-reduction (redox) potential, when gently bubbled with 5% oxygen in nitrogen or with air for various periods of time, gave a range of dissolved oxygen concentrations between 1·6 and 5·8 µg/l. Virulent *Treponema pallidum* (Nichols strain) inoculated into these media were assayed 24 and 48 hours later for motility and virulence and were compared with samples taken at zero time. Virulent *T. pallidum* survived best in the presence of 2·4 µg/l dissolved oxygen over a 48-hour period, which corresponded to a gaseous mixture of 3% oxygen in nitrogen. Higher concentrations of oxygen did not give significantly different results from anaerobic conditions over this period. Thus, until it can be grown *in vitro, T. pallidum* would appear to be a microaerophilic bacterium.

Introduction

During this century attempts to grow virulent *Treponema pallidum* (the causative agent of syphilis) *in vitro* have been unsuccessful despite claims to the contrary (Willcox and Guthe, 1966). Growth of avirulent *T. pallidum* from a virulent inoculum has recently been reported (Sandok *et al.*, 1976, 1978). Since the availability of large numbers of *T. pallidum* which are relatively free from host-cell contaminants is probably a prerequisite for the development of a vaccine against syphilis, attempts to grow this bacterium *in vitro* are important steps in reducing the incidence of this disease.

For many years *T. pallidum* has been considered a strict anaerobe with a low oxidation-reduction (redox) optimum (Metzger and Smogor, 1966). Only in reduced media could the organism maintain its viability, as measured by rabbit virulence. Recently this opinion has been challenged. Freshly isolated *T. pallidum* utilises oxygen at a rate equivalent to that of aerobic organisms (Cox and Barber, 1974), and the presence of a cytochrome system (Lysko and Cox, 1977), suggests that this organism may respire aerobically. Glucose oxidation, pyruvate degradation, and protein synthesis were stimulated under aerobic conditions (Baseman *et al.*, 1976). Norris *et al.* (1978), using a reduced culture medium, found that virulent *T. pallidum* survived better under 3% oxygen than under either aerobic or anaerobic conditions, indicating that the bacterium is micro-aerophilic.

The importance of oxygen for the survival and metabolism of *T. pallidum* has also been shown in tissue culture systems where the addition of superoxide dismutase to infected cell monolayers prolonged the virulence of *T. pallidum* (Fitzgerald *et al.*, 1975). With certain cell lines and reduced culture medium an atmosphere of 3% oxygen maintained *T. pallidum* without loss of virulence for six days (Fitzgerald *et al.*, 1977) and 2·5% oxygen enabled a surface acid mucopolysaccharide to be synthesised as a result of the cell-treponeme interaction (Fitzgerald *et al.*, 1978). Fieldsteel *et al.* (1977) established an oxygen gradient in tissue culture and found that *T. pallidum* survived best in a region neither fully aerobic nor anaerobic, indicating the importance of microaerobiosis for the survival of *T. pallidum*. Kiraly and Horvath (1976) used low oxygen conditions (7 mm Hg partial pressure) and found that *T. pallidum* survived well, but in their work

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survival was poorer with, rather than without, a tissue culture system.

In our study, an optimum dissolved oxygen concentration of 2·4 μg/l (corresponding to a gaseous mixture of 3% O_2 in N_2) was required for *T. pallidum* to retain its virulence.

Materials and methods

PROPAGATION OF *T. PALLIDUM*

The bacterium was grown in the testes of serologically negative rabbits by inoculating approximately 5×10^7 viable *T. pallidum* per testis and harvesting it 11 days later, at which time orchitis has developed. Rabbits (of various breeds) were housed at 18°C to permit satisfactory growth of the *T. pallidum*. After the rabbit had been killed with intravenous sodium pentobarbitone, the testes were removed aseptically and minced in successive 5-ml samples of medium (see below) under aerobic conditions. This eluted the *T. pallidum* from the tissue, yielding about 10^7 organisms/ml. The suspension was used without purification and 0·5 ml was inoculated into 10-ml tubes of medium to give a final concentration of *T. pallidum* of about 5×10^5/ml. This concentration varied from experiment to experiment depending on the concentration of treponemes in the rabbit testes. The 1/20 dilution of the inoculum into experimental medium meant that the treponemes were only exposed to air, albeit in a reduced medium, for about 30 minutes.

T. PALLIDUM IN VITRO

After inoculation of the medium with *T. pallidum* under a stream of sterile, oxygen-free nitrogen the tubes were tightly sealed with butyl rubber stoppers (Bellco Glass Inc., Vineland, New Jersey), placed in anaerobic jars to prevent extraneous oxygen entering the medium, and incubated at 35°C.

PREPARATION OF MEDIUM

The medium of Graves *et al.* (1975) was modified and made in two parts (A and B), which were combined immediately before inoculation with *T. pallidum*. The compositions of medium A and B are shown in Table 1. Medium A (without buffered bicarbonate solution or reducing agents) was prereduced by autoclaving at 121°C for eight minutes with slow exhaust. Immediately after removal from the autoclave the medium was continuously flushed with oxygen-free nitrogen until cool; the latter was obtained by passing nitrogen through a hot copper column before use. When the medium was cool, buffered bicarbonate solution and reducing agents (as powders) were added by gently stirring. The pH of the solution was adjusted to 8·6 with 10 mol/l NaOH. The medium was maintained under a stream of nitrogen until it was added to nitrogen-flushed, anaerobic tubes (Bellco Glass Inc.) using a pipette preflushed with nitrogen (4·7 ml/tube). The tubes were tightly sealed with butyl rubber stoppers and autoclaved in a press at 121°C for 15 minutes using fast exhaust. Medium A was never prepared earlier than one day before use. After medium A had been cooled, medium B was added to the tubes of medium A under sterile, oxygen-free nitrogen. To make 10 ml of complete medium, 5·3 ml of medium B was added to 4·7 ml of medium A. All components of medium B were previously sterilised by filtration. The final pH was 7·2-7·4.

Table 1 *Composition of T. pallidum maintenance medium*

Component	*Amount/l (Final total medium)*
Medium A	
Balanced salt solution concentrate (10 ×)*	60 ml
$(NH_4)_2SO_4$ (50 g/l)	2 ml
Glucose	1·60 g
Fructose	1·60 g
H_2O	400 ml
Buffered bicarbonate solution†	10 ml
Glutathione (reduced)	2·38 g
Cysteine	1·58 g
Sodium thioglycolate	1.00 g
Resazurin solution (0·25 g/l)	4 ml
Medium B	
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
Foetal calf serum (heat inactivated)	250 ml
H_2O	220 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)
†$NaHCO_3$ (50 g/l), K_2HPO_4 (40 g/l) and KH_2PO_4 (8 g/l)
‡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; l-inositol, 2 mg; nicotinamide, 1 mg; calcium pantothanate, 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

OXYGEN BUBBLING INTO MEDIUM

Cylinders of 5% oxygen in nitrogen (Commonwealth Industrial Gases, Melbourne, Australia) or of air were used to add oxygen to the medium. When medium A had been prepared, and immediately

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before it was added to the nitrogen-flushed anaerobic tubes, it was bubbled with the appropriate gas mixture for the predetermined time. For example, 5% O_2 × 15 minutes means 15 minutes' bubbling with 5% O_2 for every 10 ml of medium being prepared, that is, 150 minutes for 100 ml final medium. The bubbling rate was approximately 2 bubbles per second from a 21-mm Pasteur pipette (orifice diameter approximately 1 mm). After bubbling, medium A was dispensed in 4·7-ml volumes into nitrogen-flushed anaerobic tubes and sterilised by autoclaving in a press; medium B was then added as before.

MEASUREMENT OF OXYGEN IN MEDIUM

An oxygen meter (Model LR-30, Titron Australia, Melbourne), fitted with a Clark-type oxygen electrode modified to allow measurement in static solutions, was used. The meter gave readings in 'part per million' (μg/l) dissolved oxygen in water. The electrode was calibrated at 22°C with air (being the same as air-saturated water at 22°C), 5% O_2, 3% O_2, and 1% O_2 in nitrogen and 100% nitrogen (oxygen-free). The exact oxygen concentration in the gas mixtures was determined by the suppliers (Commonwealth Industrial Gases, Melbourne) and was accurate to 0·1%. The relationship between oxygen-meter readings (μg/l) and O_2 concentration in the gas was linear (Figure); this was the standard curve from which all subsequent O_2 concentrations in media were determined. We appreciated that the calibration curve, based on dissolved oxygen concentration in water, was not strictly applicable to the culture medium. Chemical determinations of dissolved oxygen concentrations in the culture media were not done. However the method used enabled the most appropriate gaseous O_2-N_2 mixture for optimum retention of *T. pallidum* virulence in the culture medium to be determined.

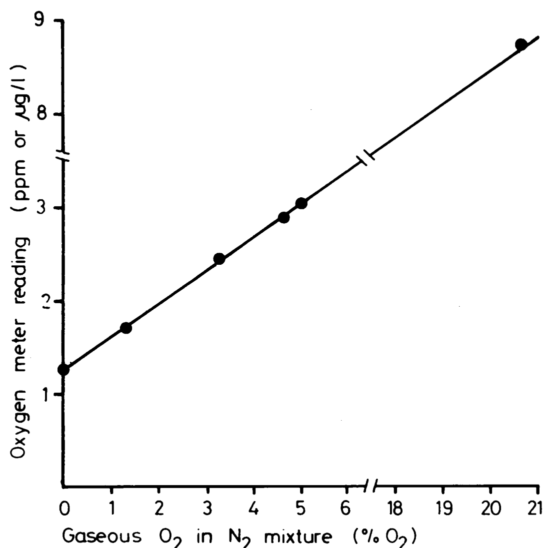

Figure *Relationships between oxygen meter reading and concentration of oxygen in the gaseous O_2-N_2 mixture. This standard curve was used to determine the oxygen concentrations in culture media as equivalent gaseous oxygen concentrations.*

Dissolved oxygen measurements on triplicate tubes were performed at daily intervals. Fresh tubes were used for readings at each time interval because insertion of the oxygen electrode biologically contaminated the medium. The electrode was inserted into the bottom of the 10-ml column of medium and stable readings were obtained within one to two minutes. Exposing the medium to air for this short time did not affect the oxygen reading, as confirmed by flushing control tubes with nitrogen while taking oxygen readings.

MEASUREMENT OF REDOX POTENTIAL IN THE MEDIUM

A platinum calomel combination redox electrode (Model 96-78, Orion, Cambridge, Massachusetts) was connected to an Orion model 701A 'digital ionalyser' and inserted directly into the medium for five minutes. Measurements were made at approximately 22°C and pH 7·3. The redox potential was read directly from the millivolt scale of the meter. The efficacy of the electrode was confirmed with a standard redox system of potassium ferricyanide and potassium ferrocyanide as recommended by the manufacturer. The readings obtained, being based on a calomel reference electrode, are designated Ecal; to convert them to Eh values, approximately 241 mV must be added to the reading (Jacob, 1970)—that is, −276 mV (Ecal) is equivalent to −35 mV (Eh).

DETERMINATION OF *T. PALLIDUM* VIRULENCE

Samples (0·6 ml) of culture medium were taken immediately after inoculation with *T. pallidum* and at 24, and 48 hours thereafter. Each sample was mixed with 0·6 ml of sterile deoxygenated 20% glycerol in saline and stored at −70°C until all samples were available. Samples were thawed immediately before inoculation into the rabbit. The backs of male rabbits were shaved and marked into a 10-cm × 10-cm grid, which was subdivided into 16 equal areas. Into each site 0·1 ml of culture was injected intradermally. For each time sample, quadruplicate samples were injected into each rabbit (4 × 0 h; 4 × 24 h; 4 × 48 h). Rabbits were kept shaved and cool (18°C) and examined daily for

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development of lesions as determined by the detection of induration at the site of inoculation, which subsequently developed into a typical syphilitic lesion. Some of these lesions were abraded and shown to contain treponemes. The time the lesions took to develop for the 24-hour and 48-hour samples was compared with that for the zero time samples. Testing all samples from one experiment on the same rabbit overcame the problem of variability between rabbits in their susceptibility to *T. pallidum*.

DETERMINATION OF *T. PALLIDUM* MOTILITY

Darkfield microscopy was used to examine 100 *T. pallidum* chosen at random in a drop of culture medium on a microscope slide. Each bacterium was scored as motile or non-motile and the percentage motility of the culture determined. Only actively motile *T. pallidum* were considered motile—that is, those with snapping, spinning, or flexing motions. For each experiment motility determinations were carried out in triplicate from three identical tubes of media.

Statistical comparisons were made by Student's *t* test.

Results

The anaerobic medium (Table 1) used for the maintenance of *T. pallidum* could be made partially aerobic by bubbling 5% oxygen in nitrogen or air into it for various periods of time. Maximum oxygenation obtained in this series of experiments was 5·8 μg/l, equivalent to an atmosphere of 12·6% oxygen (Table 2) with a series of lower values down to 1·6 μg/l (equivalent to an atmosphere of 0·7% oxygen), the anaerobic medium.

Redox potentials of the anaerobic medium were not affected by bubbling the medium with 5% oxygen in nitrogen (data not shown), but bubbling with air did cause the redox potential to become more positive (Table 3). Bubbling the medium with air for 60 minutes produced the highest redox potentials, but even so the medium was still 'reduced' relative to a calomel reference electrode.

The oxygenation of the medium did not have a marked effect on the reduction in percentage motility of the *T. pallidum* culture (Table 4); by day 2 the more oxygenated cultures were slightly less motile than the anaerobic controls.

Using the intradermal inoculation test on the shaved back of the rabbit, a trend was observed when the cultures were tested for the presence of virulent *T. pallidum*. In anaerobic medium the latent period of infection was shortened by only 0·2 day over the first 24 hours. The only oxygenated media to

Table 2 *Measurement of dissolved oxygen concentration in medium* following bubbling with 5% oxygen in nitrogen or air for specified periods*

Method of oxygen addition	*Dissolved oxygen concentration in μg/l (%O_2)*		
	Day 0	*Day 1*	*Day 2†*
No oxygenation	1·6 (0·7)	1·6 (0·7)	1·6 (0·7)
5% O_2 in N_2 × 5 min	1·8 (1·3)	1·8 (1·3)	1·7 (1·0)
5% O_2 in N_2 × 15 min	2·4 (3·0)	2·2 (2·4)	2·1 (2·1)
Air × 30 min	2·95 (4·7)	2·6 (3·6)	2·4 (3·0)
Air × 40 min	3·8 (7·3)	3·6 (6·5)	3·4 (5·9)
Air × 50 min	5·0 (10·4)	3·6 (6·5)	2·0 (1·9)
Air × 60 min	5·8 (12·6)	4·2 (8·2)	2·1 (2·1)

*Mean of five independent samples. Measurements made immediately after inoculation of medium with *T. pallidum* (day 0) and on days 1 and 2

†The fall in oxygen concentration over the two-day period is presumably due primarily to removal of the added free oxygen by reducing agents in the medium, as very little would be due to treponemal metabolism

Table 3 *Oxidation reduction (redox) potential (mV) of medium after bubbling with air for specified periods*

Method of oxygen addition	*Redox potential (mV)**		
	Day 0	*Day 1*	*Day 2*
No oxygenation	−276	−272	−254
Air × 30 min	−271	−271	−265
Air × 40 min	−263	−263	−261
Air × 50 min	−259	−258	−227
Air × 60 min	−243	−240	−229

*Mean of four independent samples. Measurements made immediately after inoculation of medium with *T. pallidum* (day 0) and on days 1 and 2

Table 4 *Determination of motility of Treponema pallidum cultures (%) maintained in medium bubbled with 5% oxygen in nitrogen or air for specified periods*

Method of oxygen addition	*Time of motility estimation**			*% decrease (day 0 to day 1)*	*% decrease (day 0 to day 2)*
	Day 0	*Day 1*	*Day 2*		
No oxygenation	100	94	80	6	20
5% O_2 × 5 min	100	96	91	4	9
5% O_2 × 15 min	100	91	77	9	23
Air × 30 min	100	88	67	12	33
Air × 40 min	100	91	73	9	27
Air × 60 min	100	85	62	15	38

*Mean of three samples from each of three identical tubes of media

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alter significantly the difference in the latent period was after being bubbled with 5% O_2 for 15 minutes (Table 5). After bubbling with 5% O_2 for 15 minutes (that is, 2·4 μg/l dissolved O_2 concentration equivalent to a 3% oxygen atmosphere; Table 2), the difference in latent period was 1·4 days (Table 5).

Table 5 *Latent period of syphilitic lesions after inoculation of T. pallidum into the shaved backs of rabbits**

Method of oxygen addition	Latent period of lesions (days)†		Difference between day 0 and day 1 latent periods‡ (days)
	Day 0	Day 1	
No oxygenation	10·7	10·5	−0·2
5% O_2 in N_2 × 5 min	9·6	9·1	−0·5
5% O_2 in N_2 × 15 min	10·4	9·0	−1·4§
Air × 30 min	9·1	7·9	−1·2
Air × 40 min	9·1	8·75	−0·35
Air × 50 min	12·8	11·9	−0·9
Air × 60 min	12·8	12·4	−0·4

*Samples for rabbit inoculation were taken from medium either immediately after additon of *T. pallidum* (day 0) or 24 hours later (day 1) after incubation at 34°C
†Latent period defined as the time between rabbit inoculation and appearance of syphilitic lesion as indicated by induration in the skin. The differences in latent periods of samples taken on day 0 is due to differences in starting concentration of *T. pallidum* from different harvests and to slight differences in susceptibility of rabbits used in different experiments. In each case the data presented are the mean of 16 lesions from four rabbits. Degree of oxygenation of medium would have no effect on latent period of day 0 samples
‡ − means that day 1 samples had shorter latent periods than day 0 samples
§When compared by Student's *t* test, the difference was significant $P<0·05$

When the latent periods at day 0 and 1 were compared by Student's *t* test, the value of 1·4 days was significant at $P<0·05$. In five repeated experiments using 5% O_2 in N_2 for 15 minutes, the difference in latent period from day 0 to day 1 was always greater than for anaerobic medium, indicating better survival in the slightly oxygenated medium compared with the anaerobic medium.

Over a 48-hour incubation period *in vitro,* the latent period of infection always lengthened (Table 6), indicating a loss of virulent *T. pallidum* during this time. However the lengthening of the latent period with 5% O_2 in N_2 for 15 minutes was only 4·8 days compared with 6·6 days in anaerobic medium. Differences in latent period for media with other levels of oxygenation were not significantly different to the anaerobic control.

We conclude that bubbling the anaerobic medium with 5% oxygen in nitrogen for 15 minutes (per 10-ml medium volume), resulting in a dissolved oxygen concentration of 2·4 μg/l (equivalent to a 3% O_2 in N_2 atmosphere), significantly enhanced the survival of virulent *T. pallidum* over a 48-hour period compared with anaerobic controls. Media with higher concentrations of dissolved oxygen (up to 5·8 μg/l, equivalent to a 12·6% O_2 in N_2 atmosphere) behaved no differently from anaerobic controls regarding retention of *T. pallidum* virulence during this time.

Table 6 *Latent period of syphilitic lesions after inoculation of T. pallidum into the shaved backs of rabbits*

Method of oxygen addition	Time at which samples taken for rabbit inoculation*†		Difference between day 0 and day 2 latent periods‡ (days)
	Day 0	Day 2	
No oxygenation	10·1	16·7	+6·6
5% O_2 in N_2 × 5 min	9·6	15·9	+6·3
5% O_2 in N_2 × 15 min	10·4	15·2	+4·8
Air × 30 min	9·0	16·2	+7·2
Air × 40 min	9·0	16·3	+7·3
Air × 60 min	14·5	21·3	+6·8

*Samples for rabbit inoculation were taken from medium either immediately after additon of *T. pallidum* (day 0) or 48 hours later (day 2) after incubation at 34°C
†See Table 5
‡ + means that day 2 samples had longer latent periods than day 0 samples. In each experiment the day 0 and day 2 samples were inoculated into the same rabbit so as to overcome any variability between rabbits

Discussion

From many earlier observations (Willcox and Guthe, 1966) we expected that a dissolved oxygen concentration would be detected above which oxygen was toxic to *T. pallidum*. This is because *T. pallidum* has always retained its virulence best *in vitro* under anaerobic conditions, implying that an atmospheric concentration of oxygen (21%) was toxic to the bacterium. In this study, although we found an atmospheric equivalent of 3% O_2 to be the optimum concentration for the survival of virulent *T. pallidum* (Table 5), higher concentrations (up to 12·6%; Table 2) were no more detrimental to survival than anaerobic conditions. This suggests that *T. pallidum* does have inherent mechanisms for dealing with the toxic end-products of oxygen reduction (Fridovich, 1975, 1978; Haugaard, 1968), a necessary requirement for aerobic metabolism.

Oxygen at atmospheric concentrations (21%) may, however, overwhelm these mechanisms. Since *T. pallidum* is an obligate human parasite it may have partially lost these mechanisms as a result of degenerate evolution associated with specialisation for a parasitic existence. The guanine plus cytosine (G + C) content of *T. pallidum* indicates that it may be closely related to *Spirochaeta zuelzerae* and *Spirochaeta litoralis* (Miao and Fieldsteel, 1978), free-living anaerobic spirochetes. *Spirochaeta aurantia,* which is a facultative anaerobe, may be a close (but non-parasitic) relative of *T. pallidum.*

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Under aerobic conditions it incompletely oxidises carbohydrates mainly to acetate and carbon dioxide. It does not possess a tricarboxylic acid (TCA) (Krebs) cycle but has developed mechanisms of oxidative phosphorylation and a rudimentary electron transport chain involving one or two cytochromes (Canale-Parola, 1977).

By comparison, *T. pallidum* degrades glucose and pyruvate to CO_2 and acetate (Nichols and Baseman, 1975) under both aerobic and anaerobic conditions, with better conversion under aerobic conditions (Baseman *et al.*, 1976). It has a functional electron transport system probably driven by the oxidation of reduced nicotinamide-adenine dinucleotide (NADH) and reduced nicotinamide-adenine dinucleotide-phosphate (NADPH) (Lysko and Cox, 1977) and coupled to oxidative phosphorylation with the production of adenosine triphosphate (ATP) (Lysko and Cox, 1978). The production of ATP in cell-free extracts was stimulated by air. The presence of catalase was also inferred by the release of O_2, presumably from H_2O_2 (Lysko and Cox, 1978). Like *S. aurantia, T. pallidum* lacks a TCA cycle, thereby probably explaining its slow growth rate (Schiller and Cox, 1977). The most likely origin of *T. pallidum* therefore is from an ancestoral spirochete, something like *Spirochaeta aurantia,* capable of growing in and utilising oxygen to a limited extent. Consequently, it is not surprising that *T. pallidum* should have a requirement for an optimum oxygen concentration.

Norris *et al.* (1978), comparing the effect of anaerobic, aerobic, and 3% oxygen conditions in the presence of reducing agents on the survival of virulent *T. pallidum*, found 3% oxygen to be superior.

We have come to the same conclusion after comparing anaerobic conditions and a range of dissolved oxygen concentrations in reduced culture medium. It is probably no coincidence that our determined oxygen optimum of 3% is within the range of normal rabbit tissue (2·6-4% oxygen; Campbell, 1931)—1·5% in the testis (Cross and Silver, 1962)—and human subcutaneous tissue (2·0-3·6% oxygen; Seevers, 1936).

Using cell-free extracts, glucose and pyruvate were degraded under aerobic conditions (Nichols and Baseman, 1975). Baseman *et al.* (1976) found optimum substrate degradation and amino acid utilisation with 10-20% O_2. The synthesis of high molecular weight proteins and incorporation of uridine into RNA was detected under normal atmospheric conditions (Baseman and Hayes, 1977; Nichols and Baseman, 1978), incorporation being poorer with lower oxygen concentrations. However there is no evidence that atmospheric oxygen concentration is compatible with the maintenance of *T. pallidum* viability as measured by the retention of virulence.

Under anaerobic, reduced conditions amino acids have been incorporated into protein (Baseman and Hayes, 1974) and serine and uracil taken up by *T. pallidum* (Sandok and Jenkin, 1978).

It appears likely that when *T. pallidum* is successfully grown *in vitro,* it will grow in quite a wide range of oxygen concentrations, but from our present state of knowledge studies on the *in-vitro* growth of this bacterium should be conducted under microaerophilic conditions.

It is interesting that a human malaria protozoon (*Plasmodium falciparum*), another human parasite which defied cultivation *in vitro* for many years, has recently been grown in a culture system with 1% or 5% oxygen (Trager and Jensen, 1976).

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