Sulfhydryl oxidation using procedures and experimental conditions commonly used for *Treponema pallidum*

T J FITZGERALD, R C JOHNSON, AND E T WOLFF

From the Department of Medical Microbiology, University of Minnesota Medical School, Minneapolis, Minnesota, USA

SUMMARY Certain reducing agents containing sulfhydryl groups are important to the in-vitro survival of *Treponema pallidum*. Discrepancies occur, however, concerning the agents and the concentrations that are optimal. To clarify some of this confusion, sulfhydryl oxidation was determined using procedures and experimental conditions commonly used for *T pallidum*. Sulfhydryl oxidation varied according to the type of culture medium, the size of the culture vessels, the volume of the culture medium, and the gaseous environment within the culture vessels, as well as the method of extracting treponemes from infected testicular tissue. Dithiothreitol maintained highly reduced conditions by reducing disulfide groups to sulfhydryl groups. Lastly, the organisms influenced the sulfhydryl concentration by either direct oxidation or specific uptake. The sulfhydryl content was sharply decreased in the presence of viable preparations of *T pallidum* compared with heated preparations or membrane filtrates of viable preparations.

Introduction

The presence of sulfhydryl (SH) agents prolongs the in-vitro survival of *Treponema pallidum*. Brewer¹ first reported the beneficial effects of sodium thioglycollate on treponemes. Cysteine, glutathione, and thioglycollate were important components of Nelson's basal medium.² Weber³ characterised the influences of SH agents on *T pallidum*. Glutathione, cysteine, thioglycollate, mercaptosuccinate, and homocysteine enhanced survival; mercaptoethanol, ethanethiol, and mercaptoethylamine did not affect survival. Metzger and Smogor⁴ reported the effects of various oxidation-reduction potentials on the survival of treponemes. Different combinations and concentrations of glutathione, cysteine, and thioglycollate were used. Optimum survival occurred at Eh -230 to -240 mV (pH 7-25). Another SH agent, dithiothreitol, has recently been added to the list of beneficial agents.⁵

In the last five years, there has been a resurgence of interest in attempts to cultivate *T pallidum* in the presence of tissue culture cells. The diverse findings in published reports on the use of SH agents are

shown in table I. In nine separate reports a variety of SH agents at different concentrations was used, whereas in two other reports SH agents were not used.

Reducing agents oxidise in solution. Since the in-vivo generation time of *T pallidum* has been estimated to be 30 hours¹⁶ successful multiplication is likely to require prolonged incubation. Thus, it is important to consider the oxidation of SH groups during this incubation period. In the modified Nelson's medium used by Weber,³ the glutathione and cysteine were 50% oxidised within four days of incubation. If these agents were partially oxidised before addition of the treponemes, the beneficial effects were sharply decreased. Norris and co-workers¹⁷ also reported that oxidised SH agents were far less beneficial, or even detrimental, to treponemal survival.

The purpose of this report is to clarify some of the present confusion regarding SH agents in tissue culture systems. To this end, SH oxidation was determined using procedures and experimental conditions commonly used for *T pallidum*.

Materials and methods

SULFHYDRYL ASSAY

A modified nitroprusside assay¹⁸ was used to determine low molecular weight SH compounds. A test

Address for reprints: Dr T J Fitzgerald, Department of Medical Microbiology, 2205 East Fifth Street, Duluth, Minnesota 55812, USA

Received for publication 25 June 1979

TABLE 1 Reducing agents and optimum concentrations for incubation of *T pallidum* with tissue culture cells

Report	Concentration (mmol/l)			
	Glutathione	Cysteine	Sodium thioglycollate	Dithiothreitol
Fieldsteel <i>et al</i> ⁶	0.50	0.64		0.25
Fitzgerald <i>et al</i> ⁷				
Fitzgerald <i>et al</i> ⁸	4.00	1.00		1.00
Fitzgerald <i>et al</i> ⁵	4.00	1.00		1.00
Hayes <i>et al</i> ⁹		6.61	6.14	
Jones <i>et al</i> ¹⁰				
Kiraly and Horvath ¹¹	0.63	0.82	9.61	
Rathlev ¹²	1.11			
Sandok <i>et al</i> ¹³	3.91	6.61	6.14	
Sandok <i>et al</i> ¹⁴	0.40	0.68	0.64	
Sandok <i>et al</i> ¹⁵	1.95			

sample of 0.5 ml added to 2 ml of water was mixed with 1 ml of 13% (u/v) sulfosalicylic acid. After incubation for 10 minutes in a water bath at 37°C the samples were centrifuged for 10 minutes at 1000×g. A volume of 1.5 ml of the supernatant was removed and 1 ml of K₂CO₃·NaCN and 1 ml of sodium nitroprusside were added. After six minutes at 22°C, the percentage transmittance was read at 525 nmol/l using a Zeiss PM Q11 spectrophotometer. This procedure was highly reproducible; similar readings were recorded within triplicate samples and within control preparations in different experiments.

SH AGENTS

Crystalline-reduced glutathione, cysteine, and dithiothreitol were obtained from Sigma. These reagents were stored in a desiccator at 4°C in an atmosphere of 95% nitrogen and 5% carbon dioxide. Fresh solutions were prepared on the day of the experiment.

GASEOUS ENVIRONMENTS

To test various gaseous environments, experiments were performed in 10-litre desiccator jars that were evacuated and flushed five times with the appropriate gas mixture. One mixture contained 95% nitrogen and 5% carbon dioxide; it is referred to as 0% oxygen. Another mixture contained 2.5% oxygen, 92.5% nitrogen, and 5% carbon dioxide; it is referred to as 2.5% oxygen. These two commercially prepared gas mixtures were obtained from and analysed by Ohio Medical Supply (Cleveland, Ohio).

RABBITS

Adult Dutch Belt rabbits weighing approximately 1.81–2.72 kg were used. Animals were screened for *Treponema parvulus cuniculi* infection by physical examination and by serological testing with the rapid plasma reagin circle card test (Hynson, Westcott, and Dunning, Baltimore, Maryland). Rabbits were maintained at 19–20°C and given antibiotic-free food and water in unrestricted quantities.

T. PALLIDUM

The Nichols strain of *T pallidum* was passaged intratesticularly. Each testis received 1×10⁷ to 3×10⁷ treponemes. Daily intramuscular injections of cortisone acetate (Merck Sharp and Dohme, West Point, Pennsylvania) at 6 mg/kg were given three days after inoculation of testes. After a satisfactory orchitis had developed, usually within 9–11 days, the animals were killed by intracardiac injection of sodium pentobarbital. Testes were removed, rinsed in physiological saline, and placed in tissue culture medium. The basal tissue culture medium contained Eagle's minimal essential medium (MEM) with Hank's balanced salts, 2× amino acids, 2× vitamins, and 4 mmol/l NaHCO₃. It was buffered with 30 mmol/l N-2-hydroxyethyl-piperazine-N-2-ethane-sulfonic acid. A supplement of 10% (v/v) heat-inactivated (56°C, 30 minutes) fetal bovine serum (FBS) (Reheis Chemical Co, Kankakee, Illinois) was added. This medium is referred to as Eagle's MEM with 10% FBS. The tissue was sliced and extracted for 10–20 minutes at room temperature under aerobic conditions without shaking. The suspension was centrifuged at 1000×g for seven minutes at 22°C to sediment particulate matter, and the supernatant containing the organisms was decanted. For experiments requiring concentrated suspensions of organisms, this supernatant was then centrifuged at 12 000×g for 30 minutes at 4°C. The pelleted treponemes were resuspended in smaller volumes of the supernatant.

SOURCE AND MAINTENANCE OF CULTURED CELLS

Primary cultures of normal rabbit testis were established as previously described.⁷ The basal medium for growth of the cultured cells was identical to that used for the extraction of *T pallidum*.

Results

A standard curve was established using different concentrations of glutathione (GSH) and cysteine

FIGURE Standard curve for relating percentage transmittance in the nitroprusside reaction to sulfhydryl (SH) concentration.

(cySH) in saline. Previous studies with tissue culture cells had indicated optimum GSH and cySH concentrations of 4 mmol/l and 1 mmol/l respectively for maintaining survival of *T pallidum*.⁵ To establish a standard curve, GSH was tested at 0.33, 0.98, 1.63, 2.61, 3.26, 3.91, and 4.89 mmol/l; cySH was tested at 0.08, 0.83, 1.65, and 2.48 mmol/l. A third preparation contained GSH and cySH at a 4 : 1 ratio at final concentrations of 0.10, 0.37, 1.13, 1.88, 2.62, 3.00, 3.76, 4.88, and 5.00 mmol/l SH. The SH moiety of GSH is cySH. Thus, the nitroprusside assay of the three preparations measures cySH. When the data were plotted on a graph (figure), each of the three curves closely overlapped. To determine SH concentration within test samples, the percentage transmittance was compared to the standard curve.

Standard curves were also established with the SH agents dissolved in basal tissue culture medium instead of saline. Identical data were recorded. Only one difference was apparent. When standing, the SH agents oxidised more quickly in basal medium; oxidation even occurred at -20°C . Thus, in all experiments crystalline GSH and cySH were dissolved in the appropriate medium just before the experiment was begun.

DIFFERENT TREPONEMAL MEDIA

SH oxidation in different treponemal suspending media was examined (table II). GSH (4 mmol/l) and

cySH (1 mmol/l) were added to Eagle's MEM, McCoy's medium, 50% normal rabbit serum plus 50% saline, and 50% FBS plus 50% saline. Incubation was performed in air at 36°C in 50-ml screw-cap test tubes containing 20 ml of medium.

The most rapid oxidation occurred in rabbit serum (table II). Within four hours, its SH content had decreased to 2 mmol/l compared with 3.4 mmol/l for the other three media. Slowest oxidation occurred in McCoy's medium. After 24 hours, pronounced differences were observed. The SH concentration had decreased to 0.6 mmol/l in rabbit serum, to 1.3 mmol/l in Eagle's MEM, to 1.6 mmol/l in FBS, and to 2.2 mmol/l in McCoy's medium. With continued incubation, SH oxidation slowed considerably. After 170 hours, the SH concentration was 0.7 mmol/l in rabbit serum, 0.9 mmol/l in FBS, and 1.3 mmol/l in both Eagle's MEM and McCoy's medium.

FLUID SURFACE AREA

SH oxidation is related to the amount of fluid surface area in direct contact with the atmosphere. To this end, 15 ml of medium was added to a 600-ml bottle, a T-30 culture flask (75-ml volume), and a 15-ml screw-cap test tube. The medium contained GSH (4 mmol/l) and cySH (1 mmol/l) in Eagle's MEM with 10% FBS. These vessels were incubated in air at 36°C . Owing to the smaller area interface between air and medium, slowest SH oxidation occurred in the test tube (table III). After seven hours, the SH

TABLE III Oxidation of SH agents in different containers*

Time (h)	SH concentration (mmol/l)		
	600-ml bottle	T-30 flask	15-ml test tube
0	5.0	4.9	5.0
7	1.6	2.4	3.6
19	1.3	1.3	3.2
25	1.3	1.3	2.6
44	1.4	1.4	2.1
66	1.4	1.4	1.6

*Air incubation of vessels containing 15 ml of Eagle's MEM with 10% FBS

TABLE II Oxidation of SH agents in different treponemal culture media*

Time (h)	SH concentration (mmol/l) in different media			
	NRS/saline	FBS/saline	Eagle's MEM	McCoy's
0	5.0	5.0	5.0	4.9
4	2.0	3.4	3.4	3.4
12	1.7	2.5	2.2	3.0
24	0.6	1.6	1.3	2.2
48	0.5	1.0	1.0	1.1
170	0.7	0.9	1.3	1.3

*Air incubation in 50-ml screw-cap test tubes containing 20 ml of medium

NRS = normal rabbit serum

FBS = fetal bovine serum

TABLE IV Oxidation of SH agents in different volumes*

Time (h)	SH concentration (mmol/l)	
	5-ml volume	15-ml volume
0	4.9	4.9
5	3.7	3.8
10	3.2	3.7
20	2.2	3.3
48	0.8	2.5

*Air incubation of 15-ml screw-cap test tubes containing Eagle's MEM with 10% FBS

content within the 600-ml bottle was 1.6 mmol/l compared with 2.4 mmol/l for the T-30 vessel and 3.6 mmol/l for the test tube. After 19 hours, SH concentration in the large bottle and the T-30 flask was 1.3 mmol/l compared with 3.2 mmol/l in the test tube. A plateau of SH oxidation was detected with continued incubation. This plateau was reached within 19 hours in the bottle and T-30 vessel and within 66 hours in the test tube.

DIFFERENT VOLUMES OF MEDIA

SH oxidation should also be related to the volume of medium within the culture vessels. In the next set of experiments, 5 ml and 15 ml of medium were added to 15-ml screw-cap test tubes. The medium contained GSH (4 mmol/l) and cySH (1 mmol/l) in Eagle's MEM with 10% FBS. The tubes were incubated in air at 36°C. Oxidation of SH was much faster in the smaller volume (table IV). After 20 hours, the 5-ml sample contained 2.2 mmol/l SH compared with 3.3 mmol/l in the 15-ml sample. After 48 hours, the 15-ml sample contained three times as much SH as the 5-ml sample (2.5 mmol/l and 0.8 mmol/l respectively).

GASEOUS ENVIRONMENT

In previous tissue culture experiments, treponemes were incubated in different gaseous environments.⁵ Experiments were performed to assess SH oxidation within these environments. GSH (4 mmol/l) and

cySH (1 mmol/l) in Eagle's MEM with 10% FBS were exposed to air (approximately 21% oxygen), 2.5% oxygen, or 0% oxygen. T-30 culture vessels containing 7 ml of medium were incubated with loose-fitting metal caps at 36°C.

Oxidation of SH was very rapid in air, minimal in 0% oxygen, and intermediate in 2.5% oxygen (table V). After five hours, 2.2 mmol/l SH remained in the medium exposed to air compared with 3.6 mmol/l in 2.5% oxygen and 4.7 mmol/l in 0% oxygen. Pronounced differences were apparent after 20 hours. The sample in air contained 0.9 mmol/l SH compared with 2.6 mmol/l SH in 2.5% oxygen and 4.7 mmol/l SH in 0% oxygen. SH oxidation decreased to its lowest level after 20 hours in air compared with 170 hours in the other two environments.

ADDITION OF DITHIOTHREITOL

Previous studies had indicated that dithiothreitol (DTT) at 1.2 mmol/l prolonged treponemal survival.⁵ The beneficial effect of this reducing agent might be related to its ability to reduce disulfide (SS) groups to SH groups.¹⁹ To test this possibility, various concentrations of DTT (0.1-2 mmol/l) were added to Eagle's MEM with 10% FBS containing GSH (4 mmol/l) and cySH (1 mmol/l). Incubation was performed in air in 50-ml screw-cap test tubes containing 15 ml of medium. Far less SH oxidation was observed in the presence of 1 and 2 mmol/l DTT. SH oxidation was marginally decreased at 0.5 mmol/l DTT. No effects on SH oxidation were detected with DTT at 0.1 mmol/l. DTT alone was not reactive in this assay. Apparently, the sulfosalicylic acid precipitated this compound.

SEQUENTIAL ADDITIONS OF DTT

Treponemal survival in the presence of tissue culture cells is enhanced by the addition of 1 mmol/l DTT every 24 hours.⁵ Experiments were undertaken to assess SH oxidation in the absence of treponemes. A volume of 15 ml of Eagle's MEM with 10% FBS with

TABLE V Oxidation of SH agents in different gaseous environments*

Time (h)	SH concentration (mmol/l)		
	Air	2.5% Oxygen	0% Oxygen
0	4.9	5.0	4.9
5	2.2	3.6	4.7
20	0.9	2.6	4.7
48	0.9	1.7	2.7
72	0.9	1.3	2.3
96	0.9	1.0	2.3
170	0.9	0.9	2.0

*Incubation in T-30 culture vessels containing 7 ml of Eagle's MEM with 10% FBS

TABLE VI Delay of SH oxidation by sequential addition of dithiothreitol (DTT)*

Time (h)	SH concentration (mmol/l)				
	Flask 1	Flask 2	Flask 3	Flask 4	Flask 5
0	4.9	5.0†	5.0†	5.0†	5.0†
20	3.2	4.7	4.6†	4.7†	4.7†
44	2.0	2.8	4.6	4.6†	4.6†
66	1.6	2.2	2.7	4.5	4.6†
93	1.6	2.2	2.7	4.5	4.6
126	1.2	1.8	1.9	2.5	3.2

*Air incubation in T-30 culture vessels containing 15 ml of Eagle's MEM with 10% FBS and cultured rabbit testis cells

†Added 1 mmol/l DTT

GSH (4 mmol/l) and cySH (1 mmol/l) was incubated in 2.5% oxygen in T-30 culture vessels containing confluent monolayers of normal rabbit testis cells. DTT at 1 mmol/l was added to the various flasks at the times indicated in table VI. Assays for SH groups were performed immediately after the addition of DTT (table VI).

The periodic addition of DTT maintained reduced conditions for extended periods. Fastest SH oxidation occurred in flask 1; slowest SH oxidation occurred in flask 5. The length of incubation required for each flask to decrease to approximately 3.2 mmol/l SH was noteworthy: flask 1, 20 hours; flask 2, 20-44 hours; flask 3, 44-66 hours; flask 4, 93-126 hours; and flask 5, 126 hours.

Two comments about DTT are pertinent. Firstly, the addition of this agent had an immediate effect on SH concentration. For example, samples that contained 1.2 mmol/l SH increased to 2.1 mmol/l SH 30 minutes after addition of DTT. This reduction of SS may have been quicker but the nitroprusside assay required at least 26 minutes to complete. Secondly, the storage of DTT at -20°C did not adversely influence the effectiveness of DTT in reducing SS groups. After storage for two months this agent remained as potent as freshly prepared DTT.

TISSUE EXTRACTION PROCEDURES

Experiments were then performed to investigate SH oxidation using different extraction procedures for harvesting treponemes from testicular tissue. Two infected testes were sliced centrally and equivalent amounts of tissue were placed in different Petri dishes. In one dish, the tissue was carefully sliced with a sharp scalpel. In a second dish, the tissue was cut with scissors and minced with forceps. The tissue in the third dish was cut with scissors and finely ground for 5 minutes with a pestle and mortar. The culture medium contained Eagle's MEM with 10% FBS with GSH (4 mmol/l) and cySH (1 mmol/l). Treponemes were extracted for 30 minutes in air. Each preparation was centrifuged at $1000\times g$ for seven minutes to remove particulate matter. A volume of 5 ml was then placed into T-15 culture vessels without cultured cells and incubated in air. At various intervals, samples were removed and assessed for treponemal motility and for SH content. As a control, culture medium was used that was not exposed to tissue extraction.

The data from the four experiments are presented in table VII. The fastest oxidation was detected in the sample that was finely ground with pestle and mortar. The slowest oxidation, other than the control, was detected in the sliced tissue. After 11 hours, the sliced tissue sample contained 2.2 mmol/l

TABLE VII Oxidation of SH groups as a function of tissue extraction procedures*

Time (h)	SH concentration (mmol/l)			
	Control	Sliced	Cut and minced	Finely ground
2	4.0	4.0	4.0	4.0
5	3.9	3.3	2.4	2.4
11	3.3	2.2	1.1	0.8
21	1.5	1.3	0.7	0.5
47	0.8	0.8	0.5	0.2

*Air incubation in T-15 culture vessels containing 5 ml of Eagle's MEM with 10% FBS

SH compared with 1.1 mmol/l SH in the cut and minced sample and 0.8 mmol/l SH in the finely ground sample. In addition, treponemal motility was apparently affected by the extraction procedure. Best motility occurred in the tissue samples that were sliced; poorest motility occurred in the finely ground samples.

OXIDATION/UPTAKE BY *T. PALLIDUM*

To assess the role of *T. pallidum* in decreasing the SH content within the culture medium treponemes were extracted from infected tissue in a volume of 55 ml of Eagle's MEM with 10% FBS containing GSH (4 mmol/l) and cySH (1 mmol/l). After extraction and slow-speed centrifugation to remove particulate matter the organisms were pelleted by high-speed centrifugation. The concentrated treponemes were resuspended in 10 ml of the supernatant at approximately 1×10^8 treponemes/ml. This treponemal suspension was equally divided; one-half was heated at 50°C for five minutes to immobilise the organisms. Four T-15 flasks with confluent monolayers of normal rabbit testis cells were used. Viable treponemes were added to one, heated treponemes were added to another, and the supernatant from the high-speed centrifugation that was membrane-filtered (0.2μ pore size) was added to another. The control flask contained culture medium not exposed to testicular extraction. Incubation was performed in 2.5% oxygen. To maintain treponemal survival, DTT was added periodically to each of the four flasks as indicated in table VIII. The data represent the mean of four separate experiments.

The SH content remaining in the flasks containing heated treponemes, high-speed supernatant, and control medium was similar at each time period. The viable treponemal preparation either utilised or was responsible for oxidation of SH groups. This was apparent after incubation for 90 hours. At this time, the viable preparation contained 1.8 mmol/l SH compared with 2.7-3.0 for the other three preparations. After 138 hours, the viable preparation contained only one-third to one-half as much SH as

TABLE VIII Oxidation or uptake of SH agents by *T pallidum* or both*

Time (h)	SH concentration (mmol/l)				% Motility
	Control suspending medium	Heated treponemes	Supernate without treponemes	Viable treponemes	Viable treponemes
0	5.0	4.9	4.9	4.9	98
18†	3.7	3.5	3.5	3.5	98
50†	3.2	3.0	3.0	3.0	90
66†	3.7	3.7	3.7	3.5	72
90†	3.0	2.7	2.8	1.8	16
114†	2.9	2.5	2.5	1.3	8
138†	2.0	1.5	1.5	0.7	0

*Incubation in 2.5% oxygen in T-15 culture vessels containing 5 ml of Eagle's MEM with 10% FBS and cultured rabbit testis cells.

†Added 1 mmol/l DTT

the other three preparations (0.7 mmol/l compared with 1.5-2.0 mmol/l). Motile treponemes in the viable preparation were observed for 114 hours.

Discussion

It is well established that certain SH agents are vital to prolong the in-vitro survival of *T pallidum*. As shown in table I, however, differences exist between laboratories concerning the agents and the optimum concentrations. The purpose of this paper was to demonstrate the wide variation in SH content within different experimental systems. Oxidation to SS groups was related to the size of the culture vessels, the volume of fluid in the vessels, the gaseous environment within the vessels, and the treponemal suspending medium. SH oxidation was also dependent on the method of extracting treponemes from infected tissue. These observations emphasise the importance of clearly defining experimental conditions. For example, a specific concentration of glutathione may be beneficial to treponemal survival when incubation is performed in 5-ml test tubes containing 3 ml of medium. This same concentration of glutathione, however, will probably not be optimal if the 3 ml of treponemes are in a larger test tube or a T-30 flask. Furthermore, concentrations of SH agents that are optimal for treponemes in Nelson's medium may not be optimal for treponemes in Eagle's MEM.

Two examples within our laboratory illustrate the problems associated with SH content using different experimental conditions. When treponemes are incubated in a T-30 culture vessel and in a Sykes-Moore chamber, similar periods of survival occur in each vessel (5-7 days). With the T-30 vessel, but not the chamber, it is necessary to add DTT daily. This may reflect faster SH oxidation in the larger vessel. A second example concerns different incubation systems. In a small test tube containing 1 ml of treponemes, better survival occurs in 3% oxygen in

the presence of DTT; poorer survival occurs with the combination of DTT, GSH, and cySH.¹⁷ In a T-30 vessel containing cultured cells in 2.5% oxygen this was not observed; best survival occurred with the combination of DTT, GSH, and cySH.

The influences of DTT on SH oxidation were noteworthy. This agent is an important addition to the tissue culture-*T pallidum* system. The initial report of the beneficial effects of DTT indicated that the minimum concentration for prolonging treponemal survival was approximately 1 mmol/l.⁵ The data in this report agree with this observation. DTT at 1 mmol/l effectively slowed the oxidation of SH groups. Previous observations had also demonstrated the beneficial influences of daily addition of DTT to the treponeme-cell culture system.⁵ This procedure resulted in a significant delay in SH oxidation (table VI).

A major advantage of DTT is its stability. Solutions of glutathione and cysteine rapidly oxidise in air; DTT does not.¹⁹ Furthermore, DTT is oxidised in the process of reducing other SS groups, and oxidised DTT does not further influence the system.¹⁹ The opposite applies to glutathione and cysteine. When these two agents oxidise, they enhance the chemical reaction favouring SS formation.²⁰⁻²³ This may be one of the reasons why Weber³ failed to show that sequential additions of cysteine had beneficial effects on treponemal survival.

The precise role of SH agents in prolonging treponemal survival is unknown. Two possibilities have been suggested:²⁻⁴ (a) SH agents poise the redox potential of the suspending medium, partially neutralising oxygen toxicity; and (b) *T pallidum* requires certain SH agents for active metabolic functioning.

In determining optimum concentrations of SH agents, it is important to consider the oxygen-*T pallidum* interaction. Previously, this organism was considered a strict anaerobe. In an important

finding, Cox and Barber²⁴ demonstrated that *T pallidum* (Nichols strain) takes up oxygen, suggesting an aerobic nature. Baseman and co-workers²⁵ reported maximum retention of treponemal survival in 10-20% oxygen. Fitzgerald *et al*,⁵ Fieldsteel *et al*,⁶ Norris *et al*,¹⁷ and Sandok *et al*¹⁵ reported maximum treponemal survival in relatively low concentrations of oxygen (less than 5%). SH content, affected by differing experimental conditions, may be the reason for these discrepancies. Two comments are pertinent; firstly, the concentration of dissolved oxygen within the culture medium is related to the concentration of reducing agents. Molecular oxygen reacts with SH groups to yield SS groups and water, thereby decreasing the amount of dissolved oxygen present.^{22 23 26} Secondly, the toxicity of oxygen to micro-organisms appears to be mediated through free radicals.²⁷ SH groups are able to scavenge free radicals.^{22 23 28} In the process of interacting with the SH moiety, the free radical is neutralised, resulting in SS formation. Thus, SH agents conceivably minimise oxygen toxicity by inactivating free radicals. Incubation in the presence of increasing concentrations of oxygen would probably require increasing concentrations of SH agents to counteract oxygen toxicity.

In support of this, Norris and co-workers¹⁷ recently examined the survival of *T pallidum* (Nichols strain) in 21%, 3%, and 0% oxygen in the presence and absence of SH agents. Poor survival was observed under aerobic conditions (21% oxygen) with and without SH agents. Interestingly, directly opposing effects were apparent in the presence of two lower oxygen concentrations. Maximum treponemal survival occurred in 0% oxygen without SH agents. In contrast, with dithiothreitol the treponemes survived for much longer periods in 3% oxygen than in 0% oxygen.

Besides control of oxygen toxicity, SH groups may be critical to metabolic functioning of *T pallidum*. Weber³ examined the effects of different SH agents on treponemal survival. Some were beneficial and some were not. Since the ineffective agents also lower redox potential, he stated that control of redox potential could not be the sole function of these agents. In the present report the data in table VIII indicate the direct interaction of SH agents with *T pallidum*. SH content in viable treponemal preparations was sharply decreased compared with non-viable preparations or with membrane filtrates of viable preparations. Viable *T pallidum* either oxidises or takes up the SH agents, thereby decreasing their concentration in the suspending medium.

Thus, it appears as if successful in-vitro multiplication will require a delicate balance provided by specific concentrations of various SH agents. These concentrations will have to be carefully balanced and

maintained during prolonged periods of in-vitro incubation.

Funds for this study were provided by Public Health Service grants AI-08124 and AI-12978 from the National Institutes of Health, Allergy and Infectious Diseases Branch.

References

1. Brewer JH. Clear liquid mediums for "aerobic" cultivation of anaerobes. *J Amer Med Assoc* 1940; **115**:598-600.
2. Nelson RA. Factors affecting the survival of *Treponema pallidum* in vitro. *Amer J Epidemiol* 1948; **48**:120-32.
3. Weber MM. Factors influencing the in-vitro survival of *Treponema pallidum*. *Amer J Epidemiol* 1960; **71**:401-17.
4. Metzger M, Smogor W. Study of the effect of pH and Eh values of the Nelson-Diesendruck medium on the survival of virulent *Treponema pallidum*. *Arch Immunol Ther Exp* 1966; **14**:445-53.
5. Fitzgerald TJ, Johnson RC, Sykes JA, Miller JN. Interaction of *Treponema pallidum* (Nichols strain) with cultured mammalian cells: effects of oxygen, reducing agents, serum supplements, and different cell types. *Infect Immun* 1977; **15**:444-52.
6. Fieldsteel HA, Becker FA, Stout JG. Prolonged survival of virulent *Treponema pallidum* (Nichols strain) in cell-free and tissue culture systems. *Infect Immun* 1977; **18**:173-82.
7. Fitzgerald TJ, Miller JN, Sykes JA. *Treponema pallidum* (Nichols strain) in tissue cultures: cellular attachment, entry, and survival. *Infect Immun* 1975; **11**:1133-40.
8. Fitzgerald TJ, Johnson RC, Miller JN, Sykes JA. Characterization of the attachment of *Treponema pallidum* (Nichols strain) to cultured mammalian cells and the potential relationship of attachment to pathogenicity. *Infect Immun* 1977; **18**:467-78.
9. Hayes NS, Muse KE, Collier AM, Baseman JB. Parasitism by virulent *Treponema pallidum* of host-cell surfaces. *Infect Immun* 1977; **17**:174-86.
10. Jones RH, Finn MA, Thomas JJ, Folger C. Growth and subculture of pathogenic *T pallidum* (Nichols strain) in BHK-21 cultured tissue cells. *Br J Vener Dis* 1976; **52**:18-23.
11. Kiraly K, Horvath I. Survival of *T pallidum* under microaerophilic conditions in cell and tissue cultures. *Zentralbl Bakt (Orig A)* 1976; **235**:500-5.
12. Rathlev T. Investigations of in-vitro survival and virulence of *Treponema pallidum* under aerobiosis. *Br J Vener Dis* 1975; **51**:296-300.
13. Sandok PL, Jenkin HM, Graves SR, Knight ST. Retention of motility of *Treponema pallidum* (Nichols virulent strain) in an anaerobic cell culture system and in a cell-free system. *J Clin Microbiol* 1976; **3**:72-4.
14. Sandok PL, Knight ST, Jenkin HM. Examination of various cell culture techniques for coinoculation of virulent *Treponema pallidum* (Nichols strain) under anaerobic conditions. *J Clin Microbiol* 1976; **4**:360-71.
15. Sandok PL, Jenkin HM, Matthews HM, Roberts MS. Unsustained multiplication of *Treponema pallidum* (Nichols virulent strain) in vitro in the presence of oxygen. *Infect Immun* 1978; **19**:421-9.
16. Magnuson HJ, Eagle H, Fleischmann R. The minimal infectious dose of *Spirochaeta pallida* (Nichols strain) and a consideration of its rate of multiplication in vivo. *Amer J Syph* 1948; **32**:1-18.
17. Norris SJ, Miller JN, Sykes JA, Fitzgerald TJ. Influence of oxygen tension, sulfhydryl compounds, and serum on the motility and virulence of *Treponema pallidum* (Nichols strain) in a cell-free system. *Infect Immun* 1978; **22**:689-97.
18. Mortensen E. The nitroprusside method for the determination of reduced glutathione. *Scand J Clin Lab Invest* 1964; **1**:87-97.
19. Cleland WW. Dithiothreitol, a new protective reagent for SH groups. *Biochem* 1964; **3**:480-2.

20. Eldjarn L. Some biochemical effects of S-containing protective agents and the development of suitable SH/SS systems for the in vitro studies of such effects. *Prog Biochem Pharmacol* 1965;1:173-85.
21. Jellum E. The prevention of thio autoxidation in biological systems by means of thiolated sephadex. *Acta Chem Scand* 1964;18:1887-95.
22. Jocelyn PC. *Biochemistry of the SH Group*. New York: Academic Press, 1972.
23. Trowell OA. Cells and tissues in culture. In: Willmer EM, ed. *Methods, Biology, and Physiology*, vol 3. New York: Academic Press, 1963:64-149.
24. Cox CD, Barber MK. Oxygen uptake by *Treponema pallidum*. *Infect Immun* 1974;10:123-7.
25. Baseman JB, Nichols JC, Hayes NS. Virulent *Treponema pallidum*: aerobe or anaerobe? *Infect Immun* 1976;13:704-11.
26. Horn RS, Haugaard N. Inhibition of carbohydrate metabolism by oxygen and N-ethylmaleimide in rat heart homogenates. *J Biol Chem* 1966;241:3078-82.
27. Morris JG. The physiology of obligate anaerobiosis. *Adv Microb Physiol* 1975;12:169-246.
28. Guzman-Barron ES. Oxidation of some oxidation-reduction systems by oxygen at high pressures. *Arch Biochem Biophys* 1955;59:502-10.