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Photocatalytic Interaction of Resazurin *N*-Oxide with Cysteine Optimizes Preparation of Anaerobic Culture Media

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For the growth of several strictly anaerobic microorganisms, cysteine is the preferred reducing agent because of its low toxicity, but cysteine reduces media only slowly, making it inconvenient if the medium is needed immediately. A culture medium employed to cultivate ruminal anaerobic bacteria supplied with cysteine achieved the desired degree of anaerobiosis (measured spectrophotometrically by the bleaching of the indicator, resazurin) in considerably less time when exposed to high light intensity from a halogen lamp. The effect of light intensity on time to reduce media was quadratic; tubes kept under no illumination (dark) took more than 12 h to become colorless while tubes kept under normal laboratory light, took more than 2 h. On the other hand, media exposed to high light intensity (equivalent to a regular 100 W bulb lamp) became completely bleached in less than 20 min. Separate experiments demonstrated a direct correlation between the photocatalytic effect on medium reduction and a decrease in dissolved O_2 content. However, media gassing with CO_2 is still required, as the photocatalytic reaction requires semi-reduced conditions. A comparison of growth parameters for five species of ruminal bacteria cultivated in media prepared under high light *vs* normal light conditions showed no differences, indicating no adverse affect of cysteine catalysed medium reduction on growth. Aside from this benefit, other procedures such as extensive heating, cooling, evacuation and degassing with O_2-free gases can be eliminated, thereby further reducing the time required for media preparation. Thus, use of high light intensity on cysteine/resazurin containing media greatly accelerated the establishment of anaerobic conditions, so that the media can be used almost immediately.

Introduction

While many anaerobes can tolerate transient exposure to oxygen, others are killed by brief exposure, and growth of these strains usually requires not only the

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absence of oxygen but also a relatively low redox potential (E_h). Laboratory studies with strict anaerobes are thus complicated by precautions required to obtain sufficiently reduced culture media. Preparation of culture media for strict anaerobes typically involves extensive purging with N_2, CO_2, or a noble gas to displace O_2 followed by sealing vessels with butyl rubber closures [1,2]. In addition, for many strains, the redox potential must be further lowered by adding chemical reducing agents prior to inoculation. Some reducing agents (e.g., sodium dithionite) act rapidly but generate toxic reaction products. Others, such as sodium sulfide, precipitate essential metal cations, and are fairly toxic to some anaerobes (e.g., many ruminal bacteria) and to higher life forms. Ti (III) salts, while relatively non-toxic, are expensive and somewhat difficult to handle. Cysteine (Cys) is often used as a reducing agent because of its low toxicity and its lack of ancillary chemical reactions, but the rate of medium reduction by this compound is particularly slow (requiring several hours at room temperature), and this further lengthens the time required for medium preparation. The vagaries of anaerobic medium preparation have prompted the use of redox dyes to allow visual assessment of the redox state of culture media. Resazurin (RNO; 7-hydroxy-3*H*-phenoxazin-3-one-10-oxide) is the most widely used indicator of reducing conditions in these culture media.

An observation of a laboratory technician (P.A. Tirabasso, pers. comm.) that anaerobic medium preparation was faster on sunny days than on cloudy days prompted us to examine the effect of light on the Cys-dependent preparation of anaerobic culture media containing RNO as an indicator. Although some reports have described an influence of light on the reduction of RNO by reducing agents [3–5] none have focused on using the photocatalytic reaction to accelerate anaerobic media preparation.

Materials and methods

Culture vessels, media, and chemicals

Experiments were conducted in 18×150 mm Balch-type anaerobic culture tubes sealed with flanged butyl stoppers (Bellco, Vineland, NJ) and aluminum crimp seals (Wheaton, Millville, NJ). Tubes contained 9.6 mL of Modified Dehority Medium (MDM) (equivalent to the medium of Scott and Dehority [6], modified by removal of casein hydrolysate and the use of the indicated carbohydrate). The basal medium containing trace minerals and volatile fatty acids was gassed under CO_2 (rendered O_2-free by passage through hot copper filings) for 15–90 min, depending on the experiment, and was dispensed into tubes under continuous CO_2 gassing. The sealed tubes were autoclaved (1.2 kg/cm^2), and after cooling KH_2PO_4, Na_2CO_3, and carbohydrate were added via N_2-flushed hypodermic syringes from stock solutions prepared under N_2 and autoclaved. For growth experiments, yeast extract was added via syringe to a final concentration of 2.0 g/L. Cysteine was added to the tubes of medium via N_2-flushed hypodermic syringes from stock solutions of cysteine hydrochloride (2.5% w/v, prepared under N_2); unless otherwise indicated, 0.4 mL of stock solution was added to 9.6 mL of MDM. Except for experiments to assess the effects of dye concentration, resazurin (Na salt, ~85% dye content; Sigma, St. Louis, MO) was used in the medium at a concentration of 2.0 mg/L (~6.8 μM).

Light exposure

Culture medium was illuminated in a shaded room to eliminate outdoor and hallway light. Light generally was provided via two 500 W quartz-halogen T3 lamps. The lamp housings were angled 45° normal to the bench surface, and the vertical distance of the light source to the benchtop was 38 cm. Anaerobic tubes were laid horizontally onto white xerography paper placed on the benchtop between the lamp housings. A rheostat switch was used to vary light intensity between 10 and 360 μE/m^2/s, and 115 V power was supplied to the rheostat through a Patriot line conditioner (Best Power Systems, Necedah, WI) to stabilize input voltage. Some illuminations were conducted in parallel in a laboratory fitted with ceiling-mounted overhead fluorescent bulbs (three 160-W F48T12/CW/VHO Cool White bulbs; GTE-Sylvania Lighting Services, Kent, WA), and the measured light intensity on the benchtop varied slightly around a mean value of ~10 μE/m^2/s. For all experiments, light intensity was measured with a model LI-189 quantum photometer (LI-COR, Lincoln, NE). Temperature measurements were made with a mercury thermometer calibrated to a primary standard thermometer (certified by the National Institute of Standards and Technology, Gaithersburg, MD).

Effect of preparation procedures and reagent concentrations on medium reduction

(i) *Light intensity.* Culture tubes, prepared as described above, were gassed for 90 min with CO_2 after which 0.4 mL of a 10% glucose solution and 0.4 mL of a 2.5% cysteine hydrochloride solution were injected with a hypodermic syringe just prior to the start of the trial. Tubes were illuminated in triplicate at: 0 (dark), 10, 45, 90, 180 or 360 μE/m^2/s. Control tubes were subjected

to the same light regime, but had no reducing agent. RNO reduction was determined by measuring A_{540} at 2 min intervals, with a Milton Roy spectrophotometer model Spectronic 21.

(ii) *Gassing time*. Tubes were gassed with O_2-free CO_2 for either 15, 30, 45, 60, 75 or 90 min prior to dispensing. Tubes were kept in the dark or exposed to light at 360 μE/m^2/s ('high light'). A_{540} values were recorded every 2 min, except for those tubes kept in the dark where readings were recorded every 30 min.

(iii) *Resazurin and cysteine concentrations*. Media that contained 1, 2 or 3 mg of RNO/L (final concentration) were gassed with CO_2 for 90 min prior to dispensing. After autoclaving and addition of reagents, either 0.5, 1.0 or 1.5 g of Cys/L was added; control tubes had no Cys. Tubes were exposed to 'high light' and A_{540} recorded every 2 min.

Measurement of oxygen uptake

O_2 uptake was measured with a Clark-type oxygen electrode (Rank Bros., U.K.) at 30°C containing 0.5 mL of air-saturated medium and RNO (100 μg/mL). The stimulation in O_2-consumption upon adding Cys (1.25 mg/mL) was recorded in the absence (by covering with aluminum foil) and presence of incandescent light (Tensor^R lamp, model 1500, [Boston, MA] supplied 15 candlepower incandescent bulb) placed 2 cm from the reaction cuvette.

Bacterial growth experiments

Lachnospira multipara 40, *Prevotella ruminicola* $B_1$4, *Ruminococcus flavefaciens* FD-1, *Selenomonas ruminantium* D and *Streptococcus bovis* JB1 were revived from 50% (v/v) glycerol stock cultures maintained under CO_2 at −80°C. Microbial growth was evaluated by measuring optical density at 540 nm. Growth experiments were conducted for each bacterial strain using media that had been reduced with Cys at normal laboratory light intensity (10 μE/m^2/s, 'low light') or high intensity (360 μE/m^2/s; 'high light'). Four replicate tubes were used for each condition. Cultures were grown for 4–12 h (to an OD_{540} of 0.6–0.8), prior to inoculation of 0.3 mL of culture into each tube (previously warmed to 39°C). Cultures were incubated in the dark at 39°C without shaking and were mixed by inversion just prior to each OD reading (10–60 min intervals); for *L. multipara*, vigorous vortexing was required prior to OD reading to break up the masses of bacterial cells and exopolysaccharide.

Growth rates were calculated as the slope of the linear portion of a graph of ln OD_{540} *vs* time. Because of the difficulty of directly measuring the length of the lag time prior to onset of growth, lag time was estimated as

$$\text{Discrete lag time} = [(y\text{intercept of ln OD}_{540}) - (\ln \text{OD}_{540} \text{ at zero time})]/\mu_{\max}.$$

Results and discussion

Effect of light intensity

Light (≥10 μE/m^2/s) accelerated the reduction of RNO by Cys (Figure 1). At the two highest intensities (360 and 180 μE/m^2/s), virtually total reduction took place in about 20 min (for comparison, an ordinary 100-W clear-bulb incandescent lamp produced a light intensity of ~370 μE/m^2/s measured 11 cm away from the light source). When tubes were kept in the dark or at 10 μE/m^2/s (equivalent to regular illumination from ceiling-mounted fluorescent lamps) complete RNO reduction never took place during the experimental period (Figure 1), perhaps due to the limited precautions taken to remove O_2 during medium preparation. Our observation of the effect of light intensity is in accord with the reported insignificant increase in RNO reduction in any of several mammalian cell culture media tested during a 2-day storage period in the dark [5].

The time required to fully reduce RNO was directly related to light intensity (Figure 2). There was essentially no difference between fluorescent and incandescent light in the ability to reduce RNO at 10 μE/m^2/s (Figure 3). On the other hand, because reduction of RNO by cell culture media was significantly enhanced by exposure to diffuse fluorescent light for 8 h per day, Rasmussen and Nicolaisen [5] recommended that exposure of RNO-containing

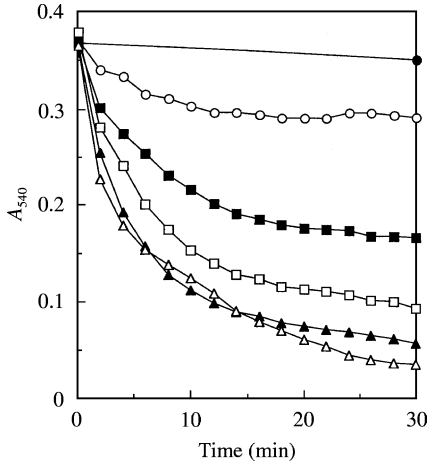

Figure 1. Effect of light intensity on reduction of resazurin by cysteine: (—●—) dark; (—○—) 10 μE; (—■—) 45 μE; (—□—) 90 μE; (—▲—) 180 μE; (—△—) 360 μE.

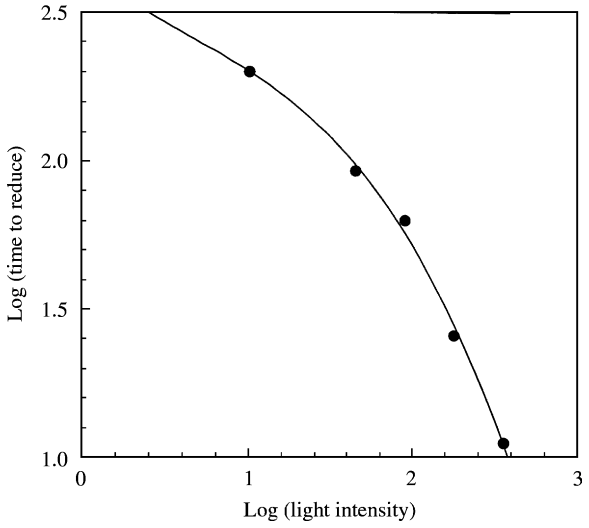

Figure 2. Effect of light intensity (μE/m^2/s) on time (min) required to fully reduce resazurin in the presence of cysteine.

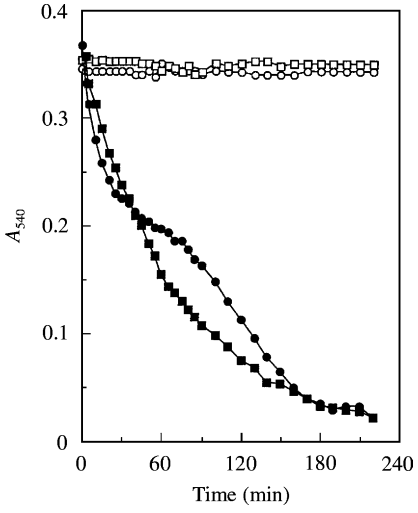

Figure 3. Effect of cysteine (Cys) on reduction of RNO under incandescent (I) or fluorescent (F) light at 10 μE/m^2/s. (—□—) I-Cys; (—○—) F-Cys; (—■—) I + Cys; (—●—) F + Cys.

media to light and reducing agents be minimized if RNO was intended to be used as a viability indicator in mammalian cell cultures. As expected, reducing agent was necessary for the reaction to occur (Figure 3); the Cys probably acted as an electron donor, yielding cystine and resorufin (the intermediate reduced form of RNO). However, the role played by light and the exact mechanism by which it accelerates RNO reduction in the presence of Cys is unclear at this moment. RNO has been shown to catalyse very efficiently the photooxidation of other reducing agents such as NADH, GSH, and dopa [4]. High molecular weight soluble polymers produced by heating amino acids in a modified sea medium catalysed the coupled reaction between dehydrogenation of NADH and reduction of RNO, and this reaction was also accelerated by light [3].

Exposure of media to light at successively higher intensity resulted in a modest increase in culture medium temperature. At the highest light intensity, measured on the benchtop surface adjacent to the tubes, temperature reached 31°C (~10°C above ambient). The temperature of the media was likely to be somewhat lower, due to the heat capacity of the water. Although this slight temperature increase contributed to the reduction, we observed that the reduction at high light intensity still proceeded considerably faster than reduction observed under room light in a hot water bath (60°C).

Effect of CO_2 gassing time

CO_2 gassing during medium preparation is intended to displace dissolved O_2, and the concentration of dissolved CO_2 is time dependent until a saturation plateau is reached. When tubes were left in the dark, CO_2 gassing times had no substantial effect on RNO reduction by Cys (Figure 4 top panel—only 15, 45 and 75 min shown); however, at high light intensity, RNO reduction was faster in tubes gassed at 75 min than in those gassed at 45 or 15 min. At 75 min tubes were virtually reduced ($A_{540} < 0.05$) at 10 min of light exposure whereas at 45 and 15 min gassing times, tubes required about 20 min and almost 30 min, respectively, of light exposure (Figure 4 bottom panel). These results may be a reflection of dissolved O_2 concentration in the media—the lower the O_2 content the faster the RNO reduction. Prütz *et al.* [4] observed that, upon illumination in N_2-saturated solution, RNO was bleached very efficiently with an equivalent increase of resorufin absorption and loss of NADH absorption at 340 nm. These authors also reported that addition of oxygen to the buffers caused a drastic decrease in photocatalytic rates, particularly those of reduction of RNO and NADH oxidation, and they concluded that this was most likely due to quenching of an excited RNO* species by O_2 competing with the oxidation/reduction reaction.

Effect of cysteine and resazurin concentration

At any RNO or Cys concentration, tubes were all reduced in less than 25 min of light exposure, except at RNO and Cys concentrations of 2 and 1 g/L, respectively (Figure 5). In this particular case, it remains unclear as to why RNO reduction happened in this unexpected manner and considering that all three tubes (replicates) behaved similarly suggests that some kind of interaction among reagents may be

taking place. Independently of this set of tubes, apparently neither the concentration of RNO and/or that of Cys seemed to have a major influence on the time required to reduce virtually all RNO. Although the rate of RNO reduction was faster at higher RNO concentration, which seemed to be independent of Cys concentration (Figure 5), this observation may not be relevant; first, because the extent of time to almost completely reduce RNO was the same in all but one set and second, this faster rate may merely reflect a higher RNO concentration. However, the presence of RNO is necessary, because it apparently serves two complementary functions: it acts as a redox potential indicator and it can accelerate the rate of reduction of the medium by Cys under strong light. Interestingly, the first absorbance readings (at 2 min after Cys addition) showed a steep decline in all tubes and this effect could not solely be accounted for by the dilution resulting from addition of Cys solution.

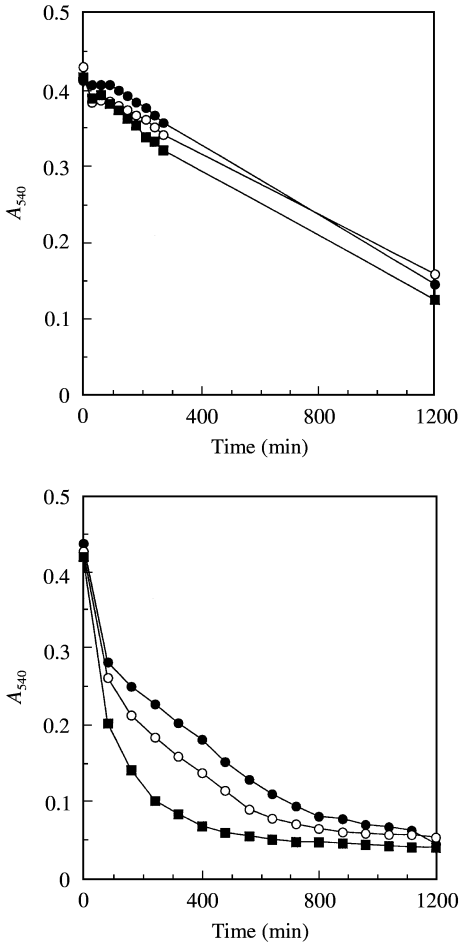

Figure 4. Effect of CO_2 gassing time on reduction of RNO. Tubes were incubated in the dark (top panel) or under light (bottom panel) at 360 μE/m^2/s. Gassing time (—●—) 15 min; (—○—) 45 min; (—■—) 75 min.

Effect of light on RNO-catalysed O_2 uptake

In order to establish that the light-accelerated reduction of culture media in the presence of RNO and Cys was accompanied by a simultaneous reduction in dissolved O_2 content, the effect of light on O_2 consumption was measured in an O_2 electrode under simulated reaction conditions. Photocatalytic reaction took place only after the medium was partially reduced. Figure 6 shows that in non-reduced medium (saturated prior to RNO and Cys addition by gassing with compressed air) light had a minimal, if any, effect on O_2 uptake; however, after partial reduction with dithionite O_2 uptake was strongly influenced by light. It is noteworthy that 15 min of purging with CO_2 appears sufficient to achieve this 'partial reduction' of medium (see Figure 4 bottom panel), and thus is in accord with data relating light intensity to the chemical reduction of RNO determined spectrophotometrically (Figure 1).

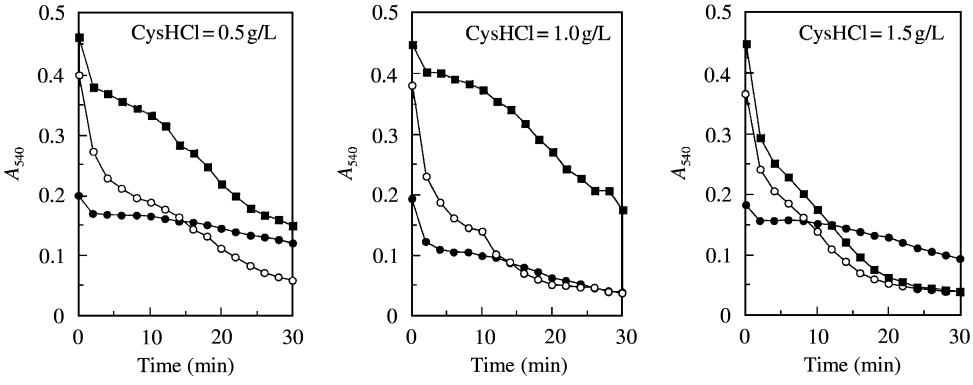

Figure 5. Effect of resazurin and cysteine concentrations on reduction of RNO under light at 360 μE/m^2/s. Resazurin: (—●—) 1 mg/L; (—○—) 2 mg/L; (—■—) 3 mg/L.

Table 1. Growth parameters for five strains of ruminal bacteria in media reduced at low or high light intensity[1]

Culture	Substrate[2]	Lag time (h)		Growth rate (h^{-1})		Maximum OD_{540}	
		Low	High	Low	High	Low	High
Lachnospira multipara 40	Glucose	3.25±0.47	3.04±1.20	0.49±0.06	0.46±0.06	1.18±0.05	1.17±0.03
Prevotella ruminicola B_14	Glucose	0.08±0.03	0.25±0.30	0.56±0.02	0.56±0.03	1.41±0.01	1.42±0.03
Ruminococcus flavefaciens FD-1	Cellobiose	1.76±0.93	0.93±0.72	0.27±0.01	0.27±0.01	0.70±0.04	0.79±0.06
Selenomonas ruminantium D	Glucose	1.22±0.24	1.09±0.06	0.61±0.01	0.61±0.03	1.19±0.03	1.21±0.04
Streptococcus bovis JB1	Glucose	1.57±0.18	1.33±0.16	1.23±0.03	1.23±0.03	1.41±0.04	1.37±0.03

[1]Media were reduced in the presence of low (10 μE/m^2/s) or high (360 μE/m^2/s) light. Results are mean values of four replicate cultures±S.E.M.

[2]Cultures were grown in MDM supplied with 1 g of yeast extract/L, plus glucose (10 g/L) or cellobiose (4 g/L), under a CO_2 gas phase.

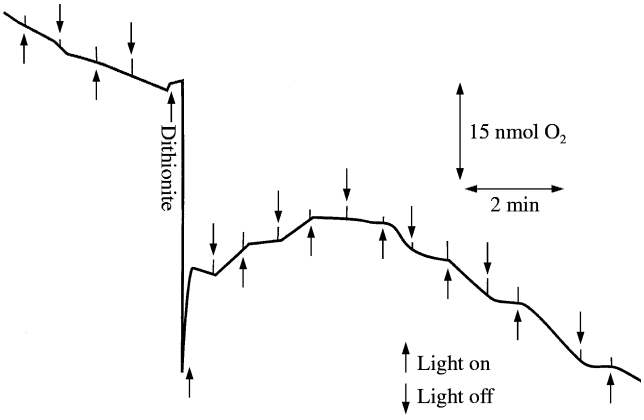

Figure 6. Effect of light on O_2 consumption in medium supplied RNO and Cys. At the times indicated light was introduced (or removed) and Na dithionite (~50 mg) added.

Culture assays

While the above experiments establish the effectiveness of light in RNO reduction and in concomitant disappearance of dissolved O_2 in the media, it remained to be demonstrated that anaerobic microorganisms could grow as well on media exposed to high light intensity as on the same media exposed to room light. Therefore, five strains of anaerobic ruminal bacteria were grown in MDM reduced with Cys at either high (360 μE/m^2/s) or low (room fluorescent light, 10 μE/m^2/s) light intensity. Results are shown in Table 1. For all five strains, no significant differences were observed between the two light intensities in terms of growth rate, extent of growth or lag time prior to the initiation of growth. The data indicate that the benefits of using light to rapidly reduce the culture medium do not compromise the utility of the medium.

Conclusions

Achieving anaerobiosis in culture media supplied with RNO as a redox indicator and Cys as a reducing agent can be optimized by illumination under intense light. Aside from the time saved in media preparation, other procedures intended to diminish the O_2 content can be eliminated, such as: (i) boiling and cooling prior to dispensing, (ii) extensive sparging, and (iii) evacuation and filling of culture tubes with O_2-free gases. This acceleration of medium reduction does not result in the formation of inhibitory compounds, as growth (measured by lag time, growth rate, or extent of growth) of a number of strictly anaerobic bacteria was the same as in the same media reduced more slowly at lower light intensity. Thereby, we suggest the use of this simple technique to accelerate anaerobic media preparation.

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