Aids for *In Vitro Mycobacterium lepraemurium* Investigations: Estimation of Oxidation-Reduction Potentials and pO_2 with 2, 6 Dichlorophenol Indophenol[1]

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Mycobacterium lepraemurium has been considered to be an obligate, intracellular parasite and is used as a model for studying "host-dependent" microbes. Recently, bacterial counts showed a limited multiplication of the organism in the NC-5 medium of Nakamura ([7, 12, 14, 16]). Modifications of the original medium have resulted in ND-5 medium ([13]) in which the doubling time was found to be 1.4-2.6 days. However, since subcultivation and growth resulting in turbidity was not successful, it is apparent that limitations still are present in the formulation of the medium. One limitation may be the proper oxidation-reduction potential (ORP) and another may be the oxygen tension (pO_2) of the growth medium ([5, 12]).

This investigation is concerned with methods to measure the ORP ([2, 9]) of the NC-5 medium. Cysteine and autoclaved glucose which contribute to the "poising" ([2]) of the NC-5 medium were studied and both compounds provide means for adjusting the ORP of the growth medium. Also, a general method for estimating the pO_2 with the redox dye, 2, 6 dichlorophenol indophenol was developed.

MATERIALS AND METHODS

Kirchner's formulation with the following additives: serum, α-keto-glutaric acid, cytochrome C, hemin and L-cysteine constitutes NC-5 medium ([14]). The "EK" components are phosphate buffered and autoclaved glucose, glutamate, citrate, glycerol, pyruvate and Mg SO_4 at pH 7.0. The medium was used as described by Nakamura ([14]) except that sterile, solid L-cysteine HCl was added last ([5]).

Others ([1, 2, 9]) have determined that, based on the hydrogen standard, the redox dye, 2, 6 dichlorophenol indophenol, is 50% reduced (E'_0) at +217 millivolts (mV). Henceforth, this compound will be referred to as the dye. The dye was supplied by Sigma (lot 8B-2180). Aqueous 0.125% or 0.8% (w/v) solutions of the dye were stored at 8°C. Dilutions of the stock were sterilized by millipore filtration (0.22 μ pore size) before use. Autoclaving destroyed the dye.

The state of oxidation of the dye in buffer was examined in two ways. Addition of H_2O_2 at a final concentration of 0.06% (v/v) to the dye in a 0.05M phosphate buffer pH 7.1, decreased the optical density (O.D.) at 600 mμ to 83% and 20% of the initial values in 2 and 21 hours, respectively. Also, reduced temperatures (8°C) which increase the solubility of O_2 and elevate the mV potentials (Fig. 2) did not increase the O.D. of the dye solution. It was concluded that at normal atmospheric pressure (20% pO_2) the dye was completely oxidized. Therefore, with various treatments, any reduction of the dye may be expressed directly without adjustments.

A combination Ag, AgCl redox electrode with a platinum disc (Beckman #38186D7) containing 4M KCl saturated with AgCl electrolyte (Beckman #4787) was used. A pH-mV meter (Fisher, Model 320) with an expanded scale was used to measure mV and pH. The stability of the redox electrode was measured before use with Zobell's solution ([17]). The range of values over a seven month period was +232 to +255 mV. For comparison, a second electrode (Ingold P.T. 4865-35) in Zobell's solution showed +255 mV, when the Beckman read +244 mV. These variations were greater than reported for other electrodes and test solutions ([8]). In commercial pH buffer solutions (pH 4, 7 and 9), each pH unit resulted in an average change of 54.2 mV, which was 3.5 mV lower than reported

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values ([1, 9]).

Measurements of mV potential of NC-5 and glucose solutions were conducted in 15 ml beakers. The solutions were aerated by slowly stirring with a magnetic bar. Without constant stirring the mV values here and elsewhere ([3]) were inconsistent and end points could not be determined. The time required to reach a steady state was 5-10 minutes. As in other studies ([3, 4, 8, 10]) conversion of mV, pH and temperature data to Eh values ([2, 9, 11]) was not done as all experiments were conducted at pH 7.0-7.1 and at room temperatures (27°-29°C) unless stated otherwise.

Standard methods were used for determining pO_2 and pCO_2 with a pH/gas analyzer (Instrumentation Laboratories, Model 113). Samples were withdrawn with 10 ml glass syringes from 95 mm × 25 mm stoppered tubes. The tubes were inoculated with 1×10^6 *M. lepraemurium* per ml ([5, 7]) and contained 22 ml of NC-5. Air above the medium occupied 20% of the total volume. The syringes were flushed with N_2 before use and capped in a stream of N_2. The syringes containing inoculated and uninoculated NC-5 were held no longer than one hour prior to pO_2 and pCO_2 determinations.

Optical density measurements were recorded from cuvettes (1 cm light path) with a Cary Model 14 spectrophotometer or in cuvettes or 13 mm screw cap tubes using a Bausch and Lomb Spectronic 70.

RESULTS

Effects of cysteine on millivolt potentials of phosphate buffer and NC-5. In certain experiments a rapid increase in mV was observed when NC-5 was aerated. The compound responsible for the increase in mV was sought because of the possible effect of "poising" NC-5. The increase occurred when the temperature of NC-5 was decreased to 8°C. The reduced temperature would increase the concentration of O_2. Cysteine, a relatively unstable amino acid present in NC-5 at 0.004M concentrations ([12, 14]) was known to increase the number of organisms ([12]) and the amount of adenosine triphosphate ([6]) present in cultures of *M. lepraemurium* ([5, 7]). Therefore, cysteine was added (0.004M final concentration in all experiments) to 6 ml of phosphate buffer (0.05M, pH 7.1) and the mV were recorded while the solution was aerated (Fig. 1). A rapid decrease of 288 mV occurred when cysteine was added. A return to the initial mV level was seen approximately after one hour of aeration. In contrast, when the reductant $Na_2S_2O_4$ was added to phosphate buffer the mV did not return to initial levels. The increase in mV occurred when cysteine was oxidized, and the "poising" of NC-5 would in part depend upon the extent of this oxidation.

To test if 2, 6 dichlorophenol indophenol would measure the ORP of NC-5 the dye was added to 6 ml of the medium at a concentration of 2 mg%. With constant stirring a three mV decline occurred in seven minutes. Following the addition of cysteine the dye changed from the blue oxidized form to the colorless reduced form (Fig. 2). Visually, the color change occurred between +85 and +40 mV. With exposure to air the color began to reappear at +55 mV. A constant +64 mV level was reached after 3 hours 46 minutes. The solution was held at 8°C for 42 hours and the mV increased to +248 which was followed by a return to +74 when the temperature of the solution increased to 27°C. An equivalent amount of cysteine was again added. The mV rapidly decreased and again the color disappeared (Fig. 2). Thus, the dye in NC-5 can be reduced by cysteine, and when cysteine became partially oxidized, the dye was also reoxidized. The dye readily de-

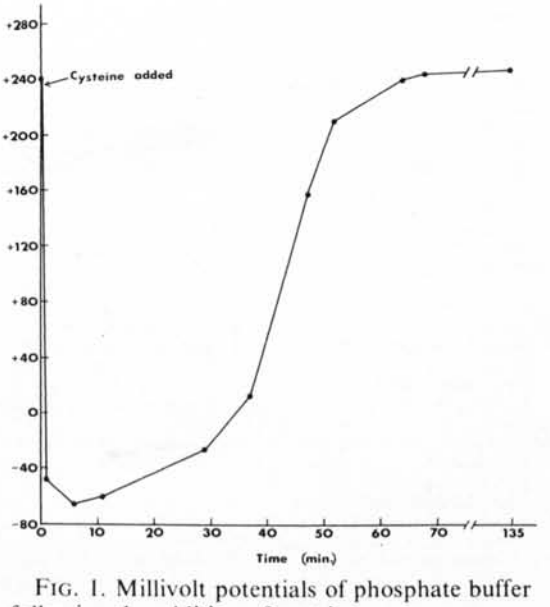

FIG. 1. Millivolt potentials of phosphate buffer following the addition of cysteine.

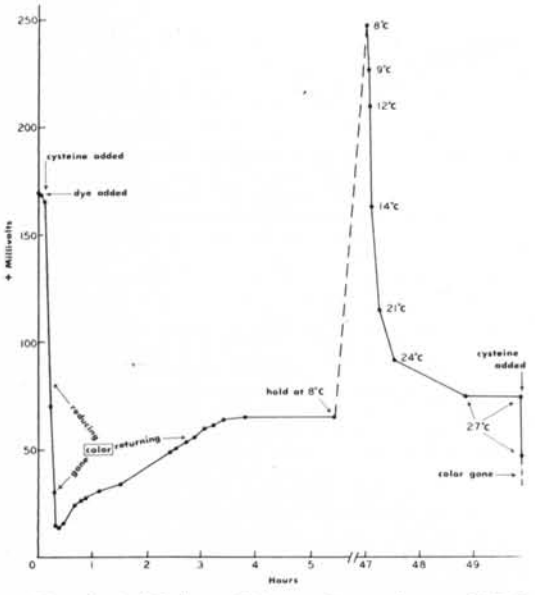

FIG. 2. Addition of dye and cysteine to NC-5; millivolt potentials during reduction and partial reoxidation of the dye and the effects of temperature changes.

tects changes in the ORP of the medium.

Optical density measurements were also used to detect the reducing effects of cysteine on the dye in the medium. NC-5 has absorption peaks at 410 mμ and 560 mμ, whereas the dye has a rather broad peak at 600-610 mμ. Thus, noting changes at 600-610 mμ while employing NC-5 as a blank provided a sensitive method for measuring the O.D. of the dye. Cysteine was added to open cuvettes containing NC-5 and the dye. The O.D. rapidly decreased to 20% of the initial value. The O.D. increased from 20% to 47% of its initial value when the solution was held at 8°C for 51 hours. Similarly, a rapid decline to 22% and an increase to 54% of original O.D. values in 60 hours was observed in closed 100 × 13 mm tubes containing 7 ml of NC-5 and cysteine. The air above the liquid phase was 24% of the total volume. The restriction of 0_2 had no effect on the degree to which the dye returned to its original O.D. In contrast to buffer (Fig. 1), the mV or O.D. of NC-5 containing dye and added cysteine never returned to initial values. There was no change in the absorption peak of the reoxidized dye.

Effect of reduced pO_2 on the dye. The effects of reduced pO_2 on the dye were further examined by placing graded concentrations of dye in the "EK" components ([14]) of NC-5 (see Materials and Methods). Tubes containing the dye plus the "EK" components were placed in air-tight containers, evacuated to 740 mm Hg, and filled to 20 mm Hg with N_2. The dye was reduced approximately three-fold in three hours as compared with solutions of dye held in 20% pO_2. The dye was reoxidized in four to five hours when exposed to 20% pO_2. Some component(s) was affected by reduced pO_2 which in turn influenced the state of oxidation of the dye. These results indicated that the dye could be used in a general method to monitor reduced pO_2. If the component(s) was known which was responsive to the reduced pO_2 the solution for monitoring reduced pO_2 could be simplified. Others have used glucose ([15]). Therefore, glucose at 4% concentrations in H_2O or phosphate buffer (0.05M) was boiled and then autoclaved. Aliquots of each solution were removed at timed intervals and held for 24 hours. The reducing substances were titrated by adding the dye at 1.6 mg% concentrations to 0.8% solutions of glucose and waiting for two hours before the O.D. measurements were taken (Fig. 3). Autoclaved glucose has a sharp absorption peak at 520 mμ, and dilute solutions contribute little to the 600-610 mμ O.D. measurements.

The decline in pH during the heating of the buffered and aqueous 4% glucose solutions was from 7.1 to 5.7 and from 7.3 to 3.9, respectively. Buffer molarities of 0.003 and above maintained the pH of a 0.1% solution

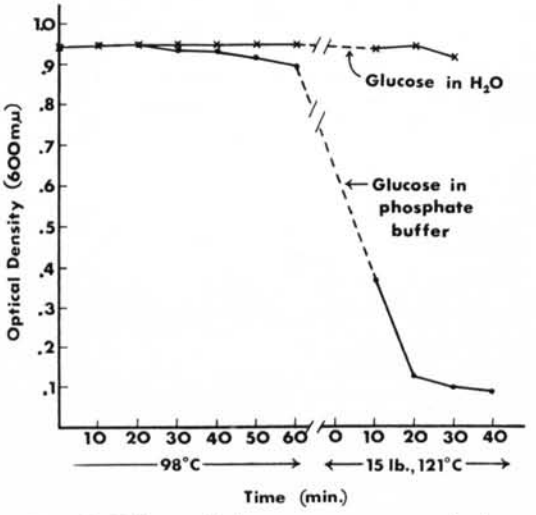

FIG. 3. Effects of time, temperature and phosphate buffer on the production of reducing substance(s) from glucose.

of glucose which was autoclaved for 40 minutes at 7.1. Similarly, a 7.0-7.1 pH was maintained by 0.05M buffer containing autoclaved glucose at the various concentrations used to monitor pO_2 (see Fig. 6).

The reducing capacity of solutions which were autoclaved for 0, 10, 20, 30 and 40 minutes (excluding the time required for decompression) were measured at graded concentrations of (0.8% to 0.025%) glucose (Fig. 4). Although the reducing capacity increased following every 10 minute period of autoclaving (Fig. 3), the increase was nearly linear with each dilution when glucose was autoclaved for 40 minutes. Presumably many reducing substances were produced when phosphate-buffered glucose was autoclaved.

The requirements for producing the reducing substance(s) from glucose were found to be autoclaving (121°C, 15 lb) in phosphate buffer. In contrast to the observations of others ([15]), autoclaving in H_2O or boiling the phosphate-buffered glucose produced only minimal amounts of the substance(s) which would reduce the dye.

The responsiveness to 1% pO_2 and the reversibility of the reduction of the dye was examined by adding the dye (0.8 mg%) to glucose (autoclaved in 0.05M phosphate buffer for 20 minutes at 4% and then diluted to 0.1% and 0.05% concentrations). The solutions were held in cotton stoppered tubes. Prior to exposure to 1% pO_2 the O.D. for 0.1% and 0.05% glucose-dye solutions was 0.20 and 0.27, respectively. The solutions were held for four days in 1% pO_2 and the O.D. declined to 0.03 and 0.1. This reduction and the rates of reoxidation with exposure to 20% pO_2 were measured by rapidly pipetting one half (0.5 ml) of the solution into a cuvette and recording the O.D. at 600 mμ (Fig. 5). The O.D. slowly increased to the initial values in five hours for the 0.1% solution, and in 22 hours or less for the 0.05% solution. Thus, the reoxidation of the dye was complete. The rate of return to the initial O.D. values for the 0.1% glucose solution was 0.034 O.D. units per hour, or 20% of the total change in O.D. per hour. Only a few seconds were required to pipette from tubes containing the indicator solution to cuvettes and any error involved would be minor. Given this slow rate of reoxidation matched cuvettes can be used to measure O.D. thus providing greater sensitivity and accuracy.

A range of pO_2 (20%, 10%, 2%, 1%) and glucose concentrations (0%-0.48%), each con-

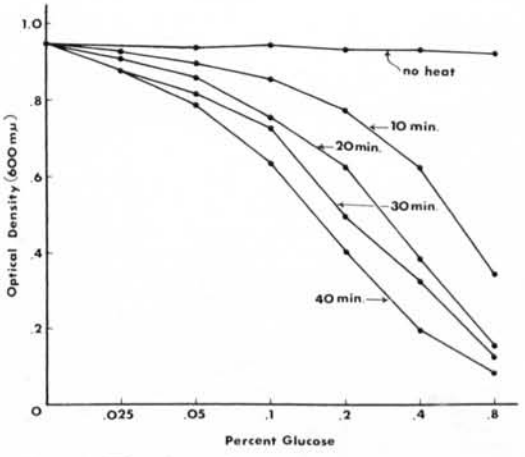

FIG. 4. Titration of reducing substance(s) produced by autoclaving phosphate-buffered glucose for varying times.

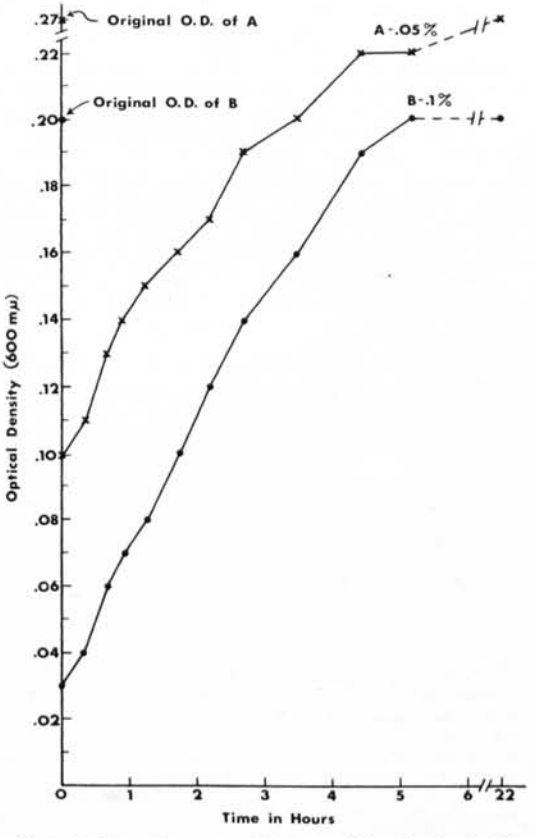

FIG. 5. Complete reoxidation of the dye in 0.1% (B) and 0.05% (A) glucose solutions held in 1% pO_2 and then exposed to 20% pO_2. A and B designate the O.D. in 20% pO_2 before and after exposure to 1% pO_2.

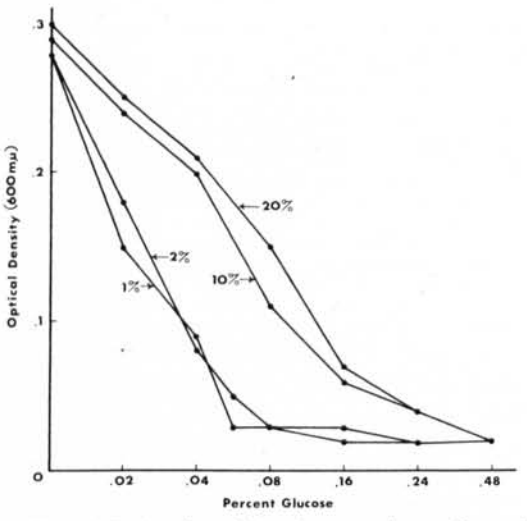

FIG. 6. Reduction of the dye at various pO_2 and glucose concentrations. The pO_2 and glucose concentrations are both expressed in percent. The concentration of the dye was 1 mg%.

taining 1 mg% dye, were held at 28°C for ten days (Fig. 6). The airtight containers were hydrated with an aqueous solution of 1% zephiran as evaporation was found to concentrate the dye during long-term experiments. The O.D. measurements were conducted in matched cuvettes as described above. Glucose at 0.06% and 0.04% were found to be most responsive to decreased pO_2. The solutions would distinguish between 20%, 10%, and 1-2% pO_2. The O.D. measurements could not accurately distinguish between 1% and 2% pO_2 because of the small differences in mV as described below.

To examine the relationships between the concentrations of glucose, O.D. and mV, the mV of solutions held in 20% pO_2 (illustrated in Fig. 6) were recorded during constant stirring (Fig. 7). The mV range was from +60 (0.48% glucose and 0.02 O.D.) to +170 (.00% glucose and 0.3 O.D.). Over this mV range each 0.1 O.D. unit was equivalent to 39.3 mV (110 mV/2.7 O.D.). However, as with the reductants produced by autoclaving glucose (Fig. 4), the change in mV was not linear with O.D. or glucose concentrations.

A further measurement recorded the change in mV and O.D. when the 0.04% glucose solution which had been held in 1% pO_2 (Fig. 6) was exposed to 20% pO_2 (Fig. 7). Each 0.1 O.D. unit was equivalent to 39.4 mV (67 mV/1.7 O.D.) which agrees with the value above, and each % O_2 in the atmosphere produced a change of 3.5 mV (67 mV/19% O_2) in the solution. The 0.4% glucose solution required 1.5 hours to attain maximum O.D. The results of these experiments have been expressed as mV versus percent reduction of dye. The occurrence of large changes in mV with respect to changes in the O.D. is best illustrated in this matter.

At pH higher or lower than 7, the dye is reduced at higher or lower potentials, respectively ([1]). In borate buffer at pH 8.5 and at glucose concentrations of 0.025%, 0.05% and 0.1%, atmospheres containing 1% pO_2 decreased the O.D. to an average of only 87% of 20% O_2 values. In contrast, with solutions at pH 7.1 the O.D. was decreased to an average of 37% of 20% O_2 values. In citrate buffer at pH 5.5 the O.D. of the dye at 600 mμ was 49% of the O.D. at pH 7.1. With the lower pH the color was red to pink. The effects of reduced pO_2 were not determined on this dye which had undergone a tautomeric change ([1]). Thus, with this dye the useful pH is in the range of neutrality. If differentiating between low pO_2 levels such as 1% and 2%

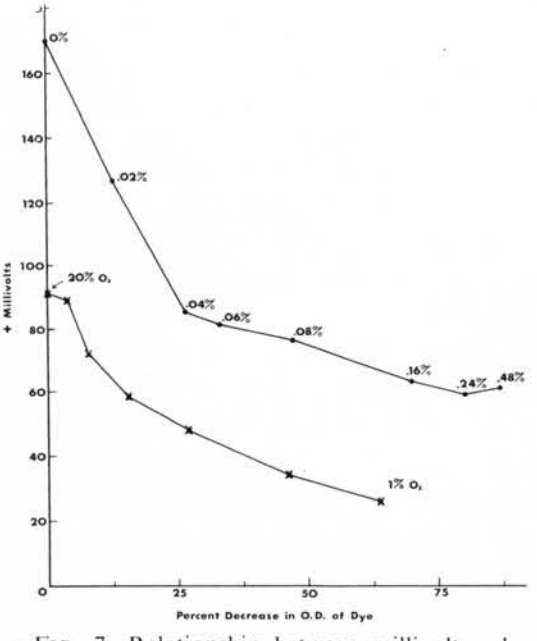

FIG. 7. Relationship between millivolts, glucose concentrations and the reduction of the dye in solutions held in 20% pO_2 (•) and in a 0.04% glucose solution held in 1% pO_2 and then exposed to 20% (X). The glucose concentrations of solutions held in 20% pO_2 are expressed in percent along the curve at the top, and the pO_2 concentrations are expressed in percent on the curve at the bottom.

O_2 is desirable a redox dye with a lower E'_0 will provide greater sensitivity.

An interesting though puzzling effect observed here and elsewhere in more complex ORP systems involving tissue cultures ([3]), was the increase in mV of solutions with stirring. In tissue cultures the increase was followed by a partial decrease ([3]). The O.D. for the various glucose and pO_2 levels shown on Figure 6 were taken before aeration. The O.D. and percent increase after aeration appear in Figure 8. With increasing concentrations of autoclaved glucose the O.D. of the dye in the stirred solutions also increased. However, no partial return to values approaching those of solutions which had not been aerated was seen.

Others have suggested that the increase in mV with stirring may be due to a faster utilization of dissolved O_2 by the cells in the culture ([3]). Here, no cells were present nor did evaporation during aeration account for the increased O.D. (Fig. 8). Since the increase in O.D. was related to the concentration of redox substance(s) in autoclaved glucose an explanation for the increase may reside in an undescribed property of the substance(s) which, with aeration, shift the equilibrium to a more oxidized state. The effect was not observed in 0.05% and 0.1% glucose solutions illustrated in Figure 5. Perhaps these glucose concentrations were too low to produce a significant increase in O.D. with aeration.

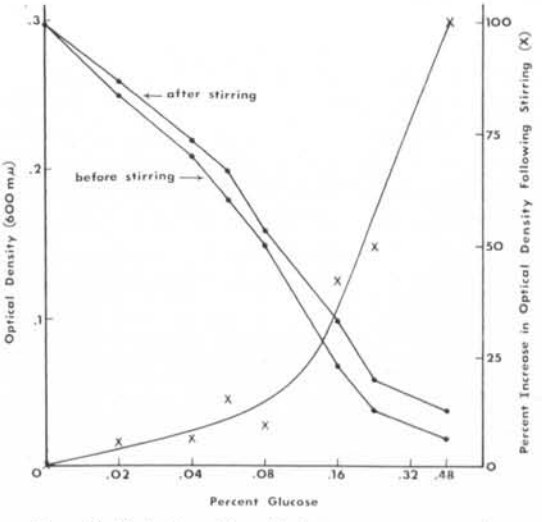

FIG. 8. Relationship of glucose concentrations and increase in optical density of the dye following stirring; X plots the percent difference in O.D. before and after aeration.

Other approaches to millivolt and O_2 measurements. The continuous measurement of mV in NC-5 requires placing a combination electrode in a sealed tube. Pressure differences between NC-5 and the electrolyte in the electrode will cause movement of the electrolyte into the NC-5, or the NC-5 will enter the electrolyte. Large variations in mV values of NC-5 occurred when an electrode was sealed into a tube with a rubber stopper. Another approach was opening tubes and rapidly inserting the combination electrode into the medium without stirring. Here, the problem of determining end points arises since time is required to equilibrate the electrode and any changes in pO_2 will influence the mV values (Fig. 7). This approach was abandoned because interpretation of the results was most tenuous.

The possibility of generating a reference potential was investigated by placing a silver wire coated with chloride as well as a platinum wire in various media. In Dubos medium inoculated with *Mycobacterium phlei* the mV dropped from +6 to −120 in 40 hours (35°) and *Escherichia coli* in thioglycolate medium resulted in a drop in mV from +20 to −340 in two hours at 35°C. This rapid decline with *E. coli* may be due to hydrogen being generated ([10]). With 1.4 mg chloride electrolytically deposited over a distance of 15 mm on a #20 silver wire, the concentration of chloride ions in NC-5 was too low to generate a potential. Following the addition of NaCl (final concentration of 0.9%) to NC-5 the mV attained +48. When increased to 1.8% NaCl a potential of +68 mV resulted. The Beckman combination electrode read +160 mV. Thus, this approach would require modifying the NC-5 by adding large amounts of chloride. In addition, with an uncoated and hence unprotected AgCl surface, any adverse effects of sulfydryls present in NC-5 may produce spurious results ([1]).

Measurements with the pH/gas analyzer of pO_2 in NC-5 and NC-5 inoculated for 28 days with *M. lepraemurium* showed a pO_2 of 133 mm and 148.5 mm, respectively. This was 90% (133/148.5) and 92% (136/148.5) of the normal 20% pO_2 values for that day. The solutions were aerated for one hour and the pO_2 increased to 140 mm in NC-5 and to 138 mm in inoculated NC-5. The mV potentials for both were +152. The solutions were held at 8°C for 18 hours and the pO_2 in-

creased to 230 mm and 225 mm, respectively. The pCO_2 values were all less than 10 mm and could not be accurately determined. Nor was there evidence for or against growth of *M. lepraemurium*. However, the findings confirm Nakamura's inference that NC-5 is an aerobic medium ([12]), and indicate that dissolved O_2 levels are well within the range of the instruments' ability to accurately measure pO_2 but not pCO_2.

DISCUSSION

Soon after the initial reports of the *in vitro* cultivation of *M. lepraemurium* ([14]), Nakamura inferred that the medium was aerobic and also found enhancement of growth when L-cysteine HCl was added to the medium ([12]). Others confirmed this requirement for cysteine by measuring the increase in adenosine triphosphate ([6]) in cultures with and without cysteine ([5,7]). Also important was the amount of air above the cultures containing varying amounts of NC-5 inoculated with a constant number of bacteria ([5,7,12]). Here, cysteine rapidly decreased the mV of buffer (Fig. 1) as well as NC-5 (Fig. 2). Return to initial mV values with aeration occurred only in buffer. The ORP level imposed by cysteine may be governed by other unknown factors in NC-5 and not entirely by the availability of O_2 in uninoculated cultures.

Another component which contributes to the ORP of NC-5 is glucose. It is present in NC-5 ([14]) at 0.154% concentrations (autoclaved for 20 min at 115°C in phosphate buffer). In phosphate buffer this concentration reduced the O.D. of the dye to 23% of control values (Fig. 6) which corresponded to a drop of 106 mV (Fig. 7). In addition, a 0.154% glucose concentration was responsive to reduced pO_2 levels. At 1% pO_2 the O.D. of the dye at 0.16% glucose concentrations was ±0.02 as compared with 0.07 O.D. for solutions held in 20% pO_2 (Fig. 6). Thus, if bacterial growth and/or oxidation of components of NC-5 decreases the availability of O_2, the reducing substance(s) produced by autoclaving glucose will enter the reactions with a resultant decline in ORP. In glucose buffered solutions the decrease in O.D. of the dye due to low pO_2 (Fig. 5) was reversible. The reversibility was also reflected by the return of mV to initial values (Fig. 7). Perhaps with long incubation the amount of air above cultures of *M. lepraemurium* ([5,7,12]) mediated, in part, the ORP of NC-5 through the reductants produced by autoclaving glucose.

The dye is a helpful reagent to monitor and to ultimately determine the optimal ORP for the growth of *M. lepraemurium* and perhaps other "host-independent" organisms as well. The useful concentration of dye when added to NC-5 will be determined by the concentration and duration of autoclaving glucose (Figs. 3, 4), the amount of cysteine in its nonoxidized form (Figs. 1, 2), and the amount of O_2 in the cultures (Fig. 6). Considering these many known variables and the complexity of NC-5, an index of consistency is needed. Addition of a minimal but constant amount of dye to an aliquot of each formulation of NC-5 and recording the O.D. at 600 mμ before inoculation provides this measurement of consistency.

Changing the concentrations of autoclaved glucose in NC-5 will have a large effect on the ORP level (see 20% pO_2, Figs. 4, 6). If alterations in glucose levels seem inadvisable, the length of time the glucose is autoclaved should also result in raising or lowering the ORP of NC-5. For instance, when glucose at a concentration of 4% was autoclaved and diluted to 0.15%, autoclaving times of 10, 20, 30 and 40 minutes resulted in the reduction of the dye to 86%, 74%, 64% and 55% of control (nonautoclaved glucose) values, respectively (Fig. 4). These reductions with autoclaving times were linear. However, it is noteworthy that the mV and O.D. measurements at graded concentrations of autoclaved glucose were not linear throughout the entire range of glucose concentrations (Figs. 6, 7). Large changes in O.D. with less change in mV occurred in the range of 0.48%-0.04% glucose than at lower concentrations (Fig. 7). It may be expected that alterations of ORP in NC-5 with changing concentrations or the duration of autoclaving of glucose will not necessarily be linear responses.

Another means of adjusting the ORP of NC-5 would be altering the concentration of cysteine. Cysteine as well as other reducing substances have been used to adjust the ORP in other systems ([3,4,5,7]). With increased concentrations the ORP is reduced (Fig. 2). However, the effects of redox substance(s) from autoclaved glucose are more stable (Figs. 3-7) as compared to those effects obtained from cysteine (Figs. 1, 2).

Visually, the dye detected changes in the ORP of NC-5 cultures when the mV passed through the +85 to +40 mV range (Fig. 2) and greater sensitivity was attained with spectrophotometric measurements. With the aerobic *M. phlei* grown in closed tubes containing NC-5, the O.D. of the dye (610 mμ) decreased to 51% of initial values in three days incubation at 35°C. The O.D. (610 mμ) of the organisms increased to 0.35. Thus, the dye was reduced by bacterial growth. When the culture was exposed to 20% pO_2 the dye became partially reoxidized. The O.D. of the dye in NC-5, with or without *M. lepraemurium,* was still detectable after incubation for six months at 30°C. The ability of the dye to monitor the ORP of NC-5 containing *M. lepraemurium* has been presented elsewhere ([5]).

Ulrich and Larsen ([15]) described a single solution indicator for anaerobiosis containing glucose, $Na_2HAsO_4{:}7H_2O$, and methylene blue. Methylene blue has a E_0' of +11 mV ([1, 2, 9]) and remained colorless in the absence of O_2. Only gentle heating was required to reduce the dye before placing it in an anaerobic atmosphere. Here, autoclaving glucose in phosphate buffer was required to produce a significant amount of reducing substance(s) (Figs. 3, 4). Without this substance(s) the reduced pO_2 had little effect on the dye (Fig. 6). Therefore, at least one function of the reducing substance(s) was to reduce the dye to the point where alterations of the pO_2 resulted in large changes in state of oxidation of the dye (Figs. 6, 7). When compared with 20% pO_2, reductions of the dye clearly visible to the eye occurred at glucose concentrations of 0.02%-0.08% and pO_2 of 1% and 2%. If certain bacterial cultures grow best at reduced pO_2 but not under anaerobic conditions, the dye in 0.04% glucose (autoclaved for 20 minutes) will indicate when the cultures approach O_2 depletion (Figs. 6, 7).

When small (15 mm × 5 mm) tubes were sealed against the upper inside walls of larger tubes the intensity of color in the glucose-dye indicator solutions declined rapidly with growth of *E. coli.* With growth of *Mycobacterium phlei* longer incubations were required to visibly reduce the dye. A convenient O.D. of the indicator solution was 0.3. However, in small indicator tubes greater O.D. (which sacrifices sensitivity of the system) is needed to visually assess the changes in color intensity. As a visual comparison for dye reduction, tubes of equal size containing a range (0.02%-0.08%) of glucose concentration may be placed in 1% pO_2. Another application is incubating bacterial cultures in reduced pO_2 with other tubes containing dye and a range of autoclaved glucose concentrations. For comparison, duplicate indicator tubes are placed in the same pO_2. When the O_2 approaches depletion in vessels containing the cultures, the dye will be reduced further than in tubes held for comparison at a constant pO_2. The reduction is completely reversible (Figs. 5, 7) and addition of O_2 will reoxidize the dye. Thus, monitoring and replacing O_2 when needed may be conducted over long periods of time. No aberrant color changes have been detected over periods of two months.

The advantages of the present indicator system are that the pO_2 levels in cultures may be visually estimated without opening the tubes and many tubes may be monitored with little apparatus. Additionally, if fine comparisons between pO_2 of cultures are required a spectrophotometer may be used. Rapid transfer of the indicator solutions to matched cuvettes caused little or no increase in O.D. (Fig. 5). Also, indicator solutions in matched tubes may be used for O.D. measurement. Thus, the dye in autoclaved glucose solutions provides a general method for estimating pO_2 at levels above anaerobiosis.

SUMMARY

The partial success in cultivating a "host-dependent" microbe provided the incentive to develop methods which may aid the growth of the organism. The oxidation-reduction potential (ORP) of NC-5, an aerobic, cysteine containing medium which supports the limited *in vitro* growth of *Mycobacterium lepraemurium,* is measurable with the redox dye, 2, 6 dichlorophenol indophenol. Both cysteine and autoclaved glucose can be used to adjust to ORP. Glucose autoclaved in phosphate buffer but not in aqueous solutions reduced the dye. The dye was also reduced in glucose solutions by atmospheres containing 10% and 1%-2% pO_2. With exposure to 20% pO_2 the reoxidation of the dye was slow but complete. Thus, the dye in glucose solutions provides a general method for estimating pO_2 above the level of anaerobiosis. Proper adjustment and mon-

itoring of the ORP and pO_2 may enhance growth.

RESUMEN

El éxito parcial alcanzado en el cultivo de un microbio "huésped-dependiente" ha servido de incentivo para el desarrollo de nuevos métodos que pueden ayudar en el cultivo del microorganismo. Con el colorante de óxido-reducción, 2,6-diclorofenol indofenol, es posible medir el potencial de óxido-reducción (POR) del medio NC-5, un medio para aeróbios que contiene cisteína, el cual permite un crecimiento limitado *in vitro* del *Mycobacterium lepraemurium*. La glucosa sometida al autoclave en regulador de fosfátos redujo al colorante cosa que no sucedió cuando la glucosa estuvo en solución acuosa. El colorante también se redujo en soluciones de glucosa bajo atmósferas conteniendo 10% y 1-2% de pO_2. En atmósferas con 20% de pO_2, la reoxidación del colorante fue lenta pero completa. Así, la reducción del colorante en soluciones de glucosa proporciona un método general para medir los valores de pO_2 por arriba del nivel de anaerobiosis. El control y ajuste apropiados del POR y del pO_2, pueden ayudar a mejorar el crecimiento.

RÉSUMÉ

Le succès partiel rencontré en cultivant un germe dépendant de l'hôte (host-dependent) a fourni le prétexte pour développer des méthodes qui pourraient stimuler la croissance de cet organisme. Le potentiel d'oxydation-réduction de NC-5, un milieu de croissance aérobie contenant de la cystéine, et qui permet la croissance limitée *in vitro* de *Mycobacterium lepraemurium*, peut être mesurée avec le colorant redox, 2, 6 dichlorophenol indophenol. La cystéine, tout comme du glucose autoclavé, peuvent être utilisés pour ajuster le potentiel d'oxydation-réduction. Le glucose autoclavé en tampon phosphate réduit le colorant alors que le glucose autoclavé en solution aqueuse ne le permet pas. Le colorant est également réduit en solution glucosée dans des atmosphères contenant 10%, et 1 à 2% d'oxygène (pO_2). Lorsque le colorant est exposé à de l'oxygène (pO_2) à 20%, la réoxydation est lente mais complète. Dès lors, le colorant en solution glucosée fournit une méthode générale qui permet l'estimation du pO_2 au-dessus du niveau d'aérobiose. Des ajustements adéquats et le contrôle permanent du potentiel d'oxydation-réduction et de la pression d'oxygene (pO_2) peuvent stimuler la croissance.

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