

Microbial Detection Method Based on Sensing Molecular Hydrogen

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A simple method for detecting bacteria, based on the time of hydrogen evolution, was developed and tested against various members of the *Enterobacteriaceae* group. The test system consisted of (i) two electrodes, platinum and a reference electrode, (ii) a buffer amplifier, and (iii) a strip-chart recorder. Hydrogen evolution was measured by an increase in voltage in the negative (cathodic) direction and recorded on a strip-chart recorder. Hydrogen response curves consisted of (i) a lag period, (ii) a period of rapid buildup in potential due to hydrogen, and (iii) a period of decline in potential. A linear relationship was established between inoculum size and the time hydrogen was detected (lag period). Lag times ranged from 1 h for 10^6 cells/ml to 7 h for 10^0 cells/ml. For each 10-fold decrease in inoculum, length of the lag period increased 60 to 70 min. Mean cell concentrations at the time of hydrogen evolution were 10^6 /ml. Based on the linear relationship between inoculum size and lag period, these results indicate the potential application of the hydrogen-sensing method for rapidly detecting coliforms and other gas-producing microorganisms in a variety of clinical, food, and other samples.

Recently, Wilkins (2) described a method for measuring gas production by microorganisms by using pressure transducers and based on the linear relationship between inoculum size and the time to buildup in gas pressure; it was suggested that this method could be used to detect gas-producing microorganisms. In an extension of this observation, it was noted that by sensing molecular hydrogen the detection sensitivity was markedly increased over the pressure transducer method. This report describes a method for rapidly detecting certain groups of bacteria that is based on sensing a buildup in molecular hydrogen. The apparatus required is easy to assemble and use and has the added advantage that hydrogen evolution can be measured automatically and accurately. This system was used to detect and enumerate various members of the *Enterobacteriaceae* group.

MATERIALS AND METHODS

Cultures. The following cultures were obtained from the American Type Culture Collection (Rockville, Md.): *Escherichia coli* 12014, *Enterobacter aerogenes* 13046, *Serratia marcescens* 13880, *Citrobacter intermedium* 6750, *Citrobacter freundii* 8090, and *Proteus mirabilis* 12453. Cultures were main-

tained at 5 C on Trypticase soy agar slants (TSA, BBL) and transferred monthly.

Inoculum preparation, viable counts, and media. Inocula for the hydrogen measurements were prepared by making 10-fold dilutions of a 24-h Trypticase soy broth culture (BBL) in sterile 0.05% peptone broth and adding 3 ml of appropriate dilutions to 27 ml of phenol red broth base with 1.0% glucose (Difco) prewarmed to 35 C. In a limited number of tests, members of the coliform group were tested in lauryl tryptose broth (Difco). Viable counts were made by spreading appropriate dilutions from the 10-fold series on TSA and counting colonies after 24 h of incubation at 35 C. Viable counts were also made on each organism at the time of hydrogen evolution and at the end of 24 h of incubation.

Hydrogen measurements. The experimental setup for measuring hydrogen evolution by the test organisms is shown in Fig. 1. It consisted of a test tube (25 by 90 mm) containing two electrodes plus broth and organisms and positioned in a 35 C water bath. Leads from the electrodes were connected to a dc buffer amplifier (type 122, Neff, Inc., Duarte, Calif.) which in turn was connected to a strip-chart recorder (model 194, Honeywell Industrial Div., Fort Washington, Pa.). The dc buffer amplifier served to match the high impedance of the electrode test system with the strip-chart recorder. Hydrogen evolution was measured by an increase in voltage in the negative (cathodic) direction and was recorded on the strip-chart recorder. Details of the electrodes are shown in

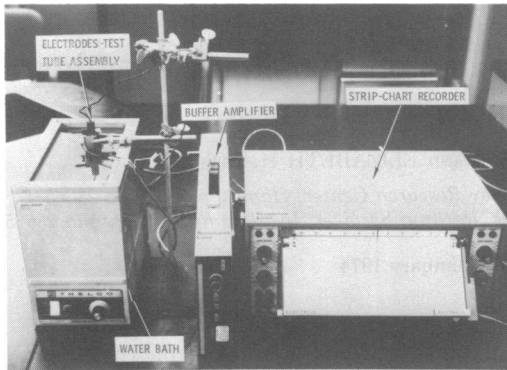


FIG. 1. Experimental setup for performing hydrogen response measurements.

Fig. 2 and consisted of a standard calomel electrode (SCE) (Beckman Instruments, Inc., Fullerton, Calif.) cemented to the plastic cap. The platinum electrode was formed by shaping a strip of platinum to fit the circumference of the test tube; a section of the platinum was positioned outside of the test tube for attachment to the amplifier lead. During operation, the platinum electrode and test tube were steam-sterilized by conventional autoclave procedures. The reference electrode (SCE), attached to the plastic cap, was sterilized by exposure for 30 min to two ultraviolet lamps (15T8, General Electric Heights, Ohio) housed in a clear plastic box. A number of tests demonstrated this technique to be effective in sterilizing the reference electrodes.

RESULTS

A strip-chart tracing of a hydrogen response curve for 1.9×10^6 cells of *E. coli* per ml is shown in Fig. 3. Characteristically, a hydrogen response curve, as determined by an increase in voltage, consisted of a lag period, followed by a period of relatively rapid buildup in potential due to hydrogen, and then a decline in the measured potential. For the organisms tested, the maximal change in potential was between 0.4 and 0.5 V. In addition, the decline period usually took place between 3 and 4 h after termination of the lag period.

The relationship between inoculum size and length of the lag period for various inocula of *E. coli* is shown in Fig. 4. When inoculum size versus lag times for the organisms tested were plotted on semilogarithmic paper, the relationship was linear, as is shown in Fig. 5. Lag times ranged from 1 h for 10^8 cells/ml to 7 h for 10^6 cells/ml. Studies with a range of inoculum levels from 10^0 to 10^8 cells/ml indicated (i) that each 10-fold increment of cells reduced the lag time by 60 to 70 min and, (ii) that the mean cell concentration at time of rapid buildup in hydro-

gen was 1×10^6 cells/ml. A summary of the least squares method of analyzing lag time data for the organisms tested is shown in Table 1. Although some differences were noted in the slopes and intercepts, the high levels of the correlation coefficients indicated an excellent fit of the data.

Because initial studies showed no differences in the response curves for washed or unwashed cells, these studies were conducted with unwashed cells. In addition, no differences in response curves or lag times were noted for the coliforms, *E. coli*, *E. aerogenes*, and *C. intermedium*, when tested in lauryl tryptose broth or phenol red broth supplemented with glucose. By using the methods described by Wilkins (2), gas chromatography analysis of headspace gas showed that for the cultures used in this study, the level of H_2 for 24-h cultures was between 4 and 10% by volume. The only exception was *S. marcescens* in which H_2 was

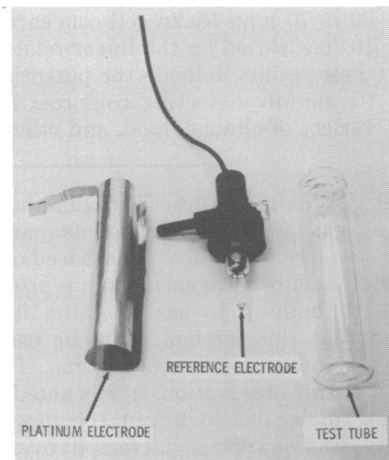


FIG. 2. Details of electrodes used for sensing hydrogen.

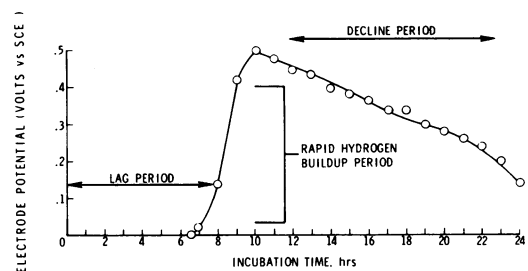


FIG. 3. Strip-chart tracing of hydrogen response curve for 1.9×10^6 cells/ml of *Escherichia coli* showing various periods used for interpreting curves. *, Standard calomel electrode.

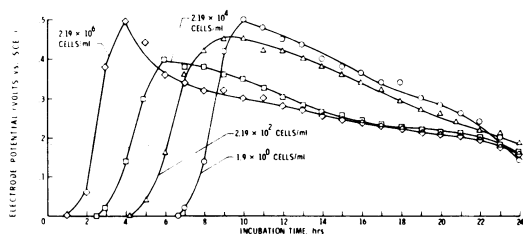
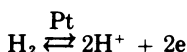


FIG. 4. Variations with time of hydrogen response curves for various inocula of *Escherichia coli*. *, Standard calomel electrode.

estimated as a trace ($\leq 1\%$). Limited studies indicated that pH did not change markedly before or during the time H_2 was detected.

DISCUSSION

The principle of hydrogen (H_2) detection with a platinum electrode is based on the observation that hydrogen ions (H^+) and molecular hydrogen (H_2) establish an equilibrium with noble metals, such as platinum, and is characterized as:



For noble metals (gold, rhodium, palladium, etc.) or other metals in which $H_2 \rightleftharpoons H^+$ is reversible, the equilibrium between $H_2 \rightleftharpoons H^+$ is very fast. When this occurs, and equilibrium is established, one has what is termed a "nonpolarizable" or reference electrode. The normal hydrogen (reference) electrode (NHE) is platinum in an acid solution (pH = 0) with a saturated solution of molecular hydrogen (being bubbled continuously). This reference electrode is defined to have a potential of 0.00 V and is the basis of the electromotive series. Now, due to the fact that H^+ ions are involved in this equilibrium, the hydrogen electrode is pH dependent via the Nerst equation (1).

Calomel ($Hg-Hg_2Cl_2$) is another reference electrode which does not depend on H^+ concentration (i.e., pH) and its potential is 0.23 V (with respect to NHE). Therefore, thermodynamically, at pH 7.0, a hydrogen electrode should be at $(-0.42) - (+0.23) = -0.65$ V or 0.65 V negative to calomel. However, one only has a reversible hydrogen electrode when both H^+ and H_2 are present, in which case the electrode will try to attain its reversible potential. Thus, ideally, when hydrogen is produced, the "sensing" platinum will "plunge" towards -0.65 V versus calomel. In reality, one never reaches $P_{H_2} = 1$ atmosphere due to CO_2 , nitrogen, etc.; thus, one reaches a leveling-off potential of approximately -0.4 to -0.5 V versus calomel.

A comparison of the hydrogen-sensing method with the pressure transducer technique (2) indicated a number of interesting similarities between the two procedures. In both cases, response curves consisted of a lag period, a period of rapid buildup in pressure or hydrogen, and a leveling-off period or, in case of the hydrogen method, a period of decline. A linear

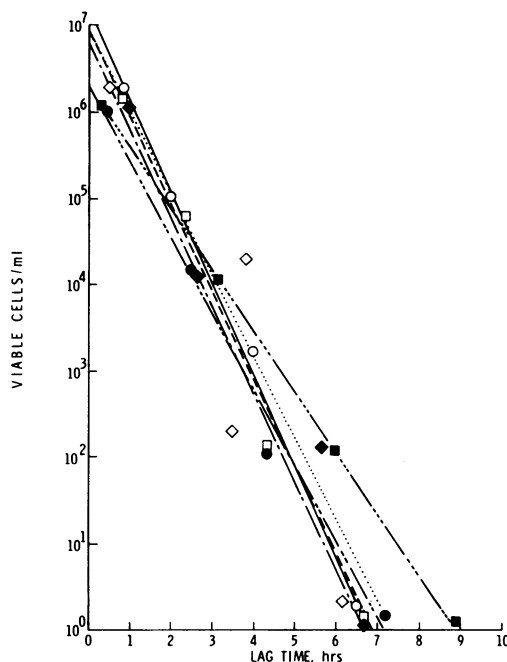


FIG. 5. Relationship between inoculum size and length of the lag period. Symbols and correlation coefficients for the lines fitted by the method of least squares: \circ , *Escherichia coli*, 0.9940; \square , *Enterobacter aerogenes*, 0.9950; \diamond , *Serratia marcescens*, 0.9140; \bullet , *Citrobacter intermedium*, 0.9929; \blacksquare , *Citrobacter freundii*, 0.9998; and \blacklozenge , *Proteus mirabilis*, 0.9765.

TABLE 1. Summary of analysis of lag time data by method of least squares^a

Organism	Parameters		
	Slope	Intercept	Correlation coefficient
<i>Escherichia coli</i>	-0.04089	1.62×10^7	0.9940
<i>Enterobacter aerogenes</i>	-0.03935	9.83×10^6	0.9950
<i>Serratia marcescens</i>	-0.03897	6.37×10^6	0.9140
<i>Proteus mirabilis</i>	-0.03628	8.97×10^6	0.9765
<i>Citrobacter intermedium</i>	-0.03424	2.20×10^6	0.9929
<i>Citrobacter freundii</i>	-0.02662	2.07×10^6	0.9998

^a Based on four to six data points for an inoculum range of 10^5 to 10^8 cells/ml.

relationship between inoculum size and length of the lag period was noted for both methods. Each 10-fold increment of cells reduced lag time by 60 to 70 min for the two techniques. The hydrogen-sensing method, however, was considerably more sensitive in detecting numbers of cells than was the pressure transducer method, i.e., for 10^9 cells/ml, 7 and 12 h, respectively. Cell concentrations at the end of the lag period were 10^9 /ml for the hydrogen method and 10^8 /ml for the pressure transducer technique. In neither method were differences noted between washed and unwashed cells or tests conducted in a limited number of difference media. Studies are currently under way to correlate the hydrogen-sensing results with the pressure transducer measurements in an attempt to understand the interesting relationship between

cell numbers and the time of rapid buildup in pressure and hydrogen.

The linear relationship between cell concentrations and the time hydrogen is sensed with the described apparatus suggests several areas of potential application such as rapidly detecting coliforms and other gas-producing microorganisms in clinical, food, and other samples. The hydrogen-sensing method, coupled with the pressure transducer technique (2), could be used in various experiments where gas production by microorganisms is being measured.

LITERATURE CITED

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