Analysis of Acetylene Reduction Rates of Soybean Nodules at Low Acetylene Concentrations

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ABSTRACT

It has been previously proposed that acetylene reduction data at subsaturating acetylene concentrations could be interpreted by use of the Michaelis-Menten equation, based on the acetylene concentration external to the nodules. One difficulty of this view is that the assumption that the system is not diffusion limited is violated when studying intact nodules. The presence of a gas diffusion barrier in the nodule cortex leads to an alternate expression for the gas exchange rates at subsaturating gas concentrations. A theoretical comparison of the ‘apparent’ Michaelis-Menten model and diffusion model illustrated the difficulties observed in the former model of overestimating the Michaelis-Menten coefficient and yielding a correlation between the Michaelis-Menten coefficient and the maximum rate. On the other hand, use of a diffusion model resulted in (a) estimates of the Michaelis-Menten coefficient consistent with enzyme studies, (b) stability of the estimates of the Michaelis-Menten coefficient independent of treatment, and (c) a sensitivity of the diffusion barrier conductance to plant drought stress. It was concluded that all studies of nodule gas exchange need to consider possible effects caused by the presence of a diffusion barrier.

The acetylene reduction assay for examining nitrogen fixation potential has gained wide acceptance since its introduction and initial evaluation for quantifying nitrogenase activity. Commonly, the acetylene reduction assay has been performed at saturating acetylene concentrations. However, for extended observations there may be deleterious effects either from toxicity or other metabolic effects. Mederski and Streeter (10) proposed using low concentrations of acetylene as an approach to eliminating these deleterious effects and allowing long term, in situ observations of acetylene reduction.

At subsaturating acetylene concentrations, interpretation of acetylene reduction rates in terms of nitrogen fixation potential is more difficult. To convert acetylene reduction rates obtained under subsaturating acetylene concentrations to an estimate for saturating acetylene concentrations, use of a rectangular hyperbola model in the form of an ‘apparent’ Michaelis-Menten equation has been suggested (2, 5).

\[ V = \frac{A V_m}{A + K_m} \]

The values of the Michaelis-Menten constant \( K_m \) and the maximum rate \( V_m \) can be evaluated by measuring acetylene reduction rates \( V \) over a range of acetylene concentrations \( A \). A Lineweaver-Burk plot or similar regression analysis of the data can be used to estimate \( V_m \) and an apparent \( K_m \) for the intact nodule system. In subsequent measurements, the value of the apparent \( K_m \) could be used with acetylene reduction rates at subsaturating acetylene concentration to estimate \( V_m \) by rearranging equation 1.

\[ V_m = V (1 + K_m/A) \]

However, a fundamental assumption in the use of the apparent Michaelis-Menten equation is that there are no diffusion processes limiting the supply of substrate. For the nodule system, it seems quite likely that this assumption is violated since several studies have indicated that there may be a substantial barrier to gas diffusion in the cortex of intact nodules (11, 12, 14, 15). This barrier may simply be a layer of cells in the inner cortex of the nodules devoid of air spaces. Such a layer that forces gas to diffuse through a liquid phase over a distance of a few cells would introduce a significant resistance to gas availability inside a nodule. Consequently, the acetylene concentration to which the nitrogenase is exposed would be less than the external concentration. The presence of such a diffusion barrier raises considerable doubt about the adequacy of the apparent Michaelis-Menten analysis.

In this paper, an alternate model for interpreting acetylene reduction rates at subsaturating acetylene concentrations is presented. This model includes the possibility of a barrier to gas diffusion. The analytical approach presented for interpreting the data provides a numerical evaluation of the conductance of the diffusion barrier, \( V_m \), and \( K_m \). A theoretical and experimental comparison is made between the use of the apparent Michaelis-Menten model and the diffusion barrier model at subsaturating acetylene concentrations.

MATERIALS AND METHODS

Experimental Techniques. In situ acetylene reduction rates were observed on field-grown soybean plants using the system described by Denison et al. (4). Briefly, this system used opened long chambers in which plants were grown from seeding. The chambers were 16 cm long so that much of the root system grew out the bottom of the chamber and explored the soil in a normal fashion. Yet a substantial fraction of the nodules were contained in the chamber. When acetylene reduction rates were to be measured, a lid was put on the top of the chamber just above the soil surface. Gas was introduced at the bottom of the chamber and continuously flowed through the chamber at a rate of 1.7 ml/s. The concentration of acetylene was usually kept well below saturation at about 80 μmol/l (0.002 atm). The return
gas mixture was injected into a gas chromatograph with a flame
ionization detector to measure acetylene and ethylene concentra-
tion. Usually 40 chambers were studied at one time and gas from
each chamber was analyzed every 30 min. The system was
controlled by a minicomputer and operated continuously, 24
h/d.
In normal operation, the acetylene concentration of the gas
flowing to the chambers was varied each night to three additional
concentrations of approximately 40, 200, and 400 μmol/l (0.001,
0.005, and 0.010 atm, respectively). Each concentration was
maintained for 1 h allowing two observations for each plant at
each altered concentration. In addition, in special tests the system
was reprogrammed to monitor only a few plants over a greater
number of acetylene concentrations.

Data were collected during three years at two locations. In
1979 and 1980, the soybean (Glycine max [L.] Merril) cv
‘Chippewa 64’ was grown at Ithaca, NY, on Darien gravelly silt
loam soil. The field was broadcast with 200 kg/ha of 6:24:24
(N-P205:K20) fertilizer prior to seeding each year. The seeds were
inoculated with a commercial inoculant that contained a
rhizobial serotype identical to USDA 110. The seeds were seeded
at 33 seeds/m of row in rows spaced 76 cm apart. The root
chambers were installed and seeded immediately after the rows
were machine seeded.

In 1982, the soybean cv ‘Biloxi’ was studied at Gainesville,
FL. The soil was Arredondo fine sand and fertilizer was broadcast
at 700 kg/ha of 0:10:20 (N-P205:K20) prior to seeding. No
inoculant was added to the seeds since soybeans previously grown
on the field had been well nodulated. The field was seeded on
March 23 in rows 91 cm apart with 33 seeds/m. The root
chambers were installed and seeded immediately following the
machine seeding.

Conductance Model. The simplest model for defining gas flow
into a nodule is a steady state, first-order diffusion equation
coupled with the Michaelis-Menten equation defining the reac-
tion rate inside the nodule. In this model, it is assumed that the
diffusion barrier exists completely exterior to the bacteroid-
containing volume where nitrogenase is active. Further, it is
assumed the dimensions of the diffusion barrier are small relative
to the dimensions of the bacteroid-containing sphere so that a
one-dimensional form of the diffusion equation is used. Both of
these assumptions are supported by anatomical study (6). Let

\[ J = k (A_e - A_i) \]  

(3)

where: \( J \) = flux of gas into the nodule (mol/s),
\( k \) = conductance of the diffusion barrier (l/s),
\( A_e \) = acetylene concentration outside of the nodule
(mol/l), and
\( A_i \) = acetylene concentration in the intercellular air
spaces interior to the diffusion barrier of the
nodule (mol/l).

Internal to the diffusion barrier, it is assumed that diffusion is
sufficiently rapid so that no significant concentration gradients
exist within this internal volume. The existence of air spaces
within the bacteroid-containing volume supports this assumption
(1, 12, 13). Consequently, the Michaelis-Menten equation is used
to describe enzyme kinetics inside the bacteroid-containing cells
of the nodule.

\[ J = \frac{sA_e V_m}{sA_i + K_m} \]  

(4)

where \( s \) = solubility coefficient of acetylene in water (\( s = 1.0 \)
at 21.5°C).

In the measurements of acetylene reduction rates, \( A_e \) and \( J \)
are measured leaving four unknown variables—\( k, V_m, K_m, \) and
\( A_i \). Equations 3 and 4 are equivalent expressions of acetylene
flux under steady state conditions and can be combined to
eliminate the unknown acetylene concentration inside the nodule
(\( A_i \)). Both equations are solved for \( A_i \) and then set equal to
each other. The resultant expression can be rearranged to give
the following equation.

\[ \frac{A_i}{J} = \frac{K_m}{V_m - J} + \frac{1}{k} \]  

(5)

Equation 5 defines a line with a slope \( K_m/s \) and intercept \( 1/k \),
when \( A_i/J \) is plotted against \( 1/(V_m - J) \). If \( V_m \) were known,
linear regressions would allow \( K_m \) and \( k \) to be readily determined.

If only subsaturating acetylene concentrations are used, then
\( V_m \) is not known and an iterative solution to equation 5 is
required. A binary search algorithm was used to find the value
of \( V_m \) which gave the smallest error sum of squares with equation
5. At each stage in the iteration, two regressions were performed
and the range of possible \( V_m \) values was cut in half based on
whether the error sum of squares increased or decreased for a
very small increase in \( V_m \). Ten iterations found the best value
of \( V_m \) in a given range with a precision of 0.1%. A reasonable range
for the search was from the highest observed value of acetylene
reduction to four times that value.

RESULTS AND DISCUSSION

Theoretical Comparison of Models. An initial theoretical anal-
ysis was done to examine the apparent success (i.e. high regres-
sion coefficients) of the Michaelis-Menten approach (equation
1) in dealing with subsaturating acetylene concentration ex-
change rates if a diffusion barrier actually existed. To do these
analyses, simulated data were generated from the diffusion-
barrier model (equations 3–5) for acetylene reduction rate versus
external acetylene concentration. Four different combinations of
the parameters \( V_m \) (2.05 or 4.10 nmol/s), \( K_m \) (82 or 164 μmol/

![Fig. 1. Simulated ethylene production rates versus external acetylene concentrations calculated for various assumed values of \( K_m \), \( V_m \), and \( k \). The assumed values were: (●), \( K_m = 164 \) μmol/l, \( V_m = 2.05 \) nmol/s, \( k = 10 \) μl/s; (○), same as (●) except \( K_m \) is halved; (△), same as (●) except \( V_m \) is doubled; (+), same as (●) except \( k \) is halved.](image-url)
(5), and $k$ (5 or 10 $\mu$l/s) were studied. Using these defined parameters and a series of seven assumed internal acetylene concentrations (20–2000 $\mu$mol/l), the flux rates for acetylene reduction were calculated from equation 4 and the external acetylene concentrations were calculated from equation 3. These simulated data are plotted in Figure 1.

The data in Figure 1 were first analyzed using the conductance model and the binary search algorithm. In each of the four cases, the iterative procedure found the initial value of $V_m$ with an error of less than 1 per cent. The estimates of $K_m$ and $k$ were similarly found to match the input values. The small errors resulted from round-off errors and a finite number of iterations in the search algorithm.

The data in Figure 1 are replotted in Figure 2 as double-reciprocal plots using the Lineweaver-Burk analysis. While these plots are slightly concave upwards, the departure from linearity does not appear to be great. H. J. Mederski (personal communication, Ohio Agricultural Experiment Station, Wooster, OH) has observed this concave tendency in attempting to analyze acetylene reduction data obtained from intact plants with double-reciprocal plots.

The linear regression coefficient ($r^2$) for each simulated curve in Figure 2 was greater than 0.99. However, the resultant estimates of $K_m$ and $V_m$ agreed very poorly with the initial input values. For example, the case where the input values were $K_m = 164$ $\mu$mol/l, $V_m = 4.10$ $\mu$mol/s, and $k = 10$ $\mu$l/s, the Lineweaver-Burk estimates were $K_m = 1040$ $\mu$mol/l and $V_m = 7.5$ $\mu$mol/s.

The results of this analysis from the apparent Michaelis-Menten approach are also consistent with experimental observations. Generally, apparent $K_m$ values from intact nodules are greater than that observed for the isolated enzyme. Davis and Wang (3) reported the $K_m$ of acetylene for nitrogenase isolated from K. pneumoniae as 40 to 200 $\mu$mol/l and from C. pasteurianum as 60 to 240 $\mu$mol/l. Previously, Hardy et al. (7), 2000 $\mu$mol/l by Fishbeck et al. (5), and 400 to 900 $\mu$mol/l in the Lineweaver-Burk analysis of our experimental data. Because of the diffusion barrier, the apparent $K_m$ of the intact nodules does not reflect the actual $K_m$ of the enzyme system.

An important problem demonstrated in this theoretical analysis of the apparent Michaelis-Menten approach is that the apparent $K_m$ estimates were found to be correlated with $V_m$. Contrasting the results from the above example, a decrease in $V_m$ from 4.10 to 2.05 $\mu$mol/l caused the apparent $K_m$ derived from a linear regression of the reciprocal plot to decrease from 1040 to 533 $\mu$mol/l. The use of the apparent Michaelis-Menten approach results in a close linkage between $V_m$ and $K_m$ even though in principle the apparent $K_m$ should be independent of $V_m$. Mederski (9) raised this concern about the linkage he found between $V_m$ and apparent $K_m$ when illumination on plant shoots was changed. Rather than a true linkage between $V_m$ and actual $K_m$, this apparent linkage is a consequence of the apparent Michaelis-Menten analysis which ignores the existence of a diffusion barrier. Similar tests using the analysis procedure based on the conductance model showed that it did not lead to such spurious correlations, either between $V_m$ and $K_m$ or between $V_m$ and $k$. A correlation between $J$ and $k$ was found for low acetylene concentrations, as would be expected.

The conductance model was also tested to assure that for systems which were not diffusion limited, a finite value of $k$ would not be erroneously predicted. In a diffusion-free system, $k$ approaches infinity and the intercept in equation 5 should not differ statistically from zero. Six data sets were simulated as described above with a true $K_m$ of 140 $\mu$mol/l, $V_m$ ranging from 1.64 to 6.56 $\mu$mol/s, and with $A_i$ equal to $A_i$ in equation 3 as would occur if no diffusion barrier existed. The conductance model and iterative search procedure was used to solve for each parameter. The values of $K_m$ and $V_m$ were predicted correctly in each case with a mean error of 0.3 per cent. The intercept for the regression through each data set did not significantly differ from zero ($\alpha = 0.05$) indicating that a diffusion barrier did not exist. It was concluded that the conductance model correctly predicted the values of $K_m$, $V_m$, and $k$ values for diffusion-free systems, as well as for those in which a barrier exists.

**Experimental Comparison of Models.** Two plants were subjected to nine concentrations of acetylene ranging from 45 to 470 $\mu$mol/l during the night of July 1, 1982. Twenty min were allowed to establish equilibrium at each concentration before measuring acetylene reduction rates. Four measurements of acetylene reduction rate were made at each concentration producing

![Diagram](https://example.com/diagram.png)

**Fig. 3.** Lineweaver-Burk plot obtained from ethylene production rates measured through a range of acetylene concentrations. The $1/A_i$ plot is based on external acetylene concentrations. The $1/A_i$ plot is based on internal acetylene concentrations calculated by assuming the conductance of the diffusion barrier was 14 $\mu$l/s.
a total of 36 observations. The Lineweaver-Burk plot for only the mean responses at each concentration for one plant is presented in Figure 3. As noted in the test using simulated data, there was a slight tendency of upward concavity at the high acetylene concentrations (low values of 1/A.). Yet a linear regression for these data was excellent with a r² of 0.999. The regression yields an estimate of \( K_m = 817 \text{ \mu mol/l} \) and \( V_m = 8.2 \text{ nmol/s}. \)

Solution of the model incorporating a diffusion barrier as represented by equation 5 gave quite different results. Using the binary search algorithm, the following estimates were obtained: \( K_m = 129 \text{ \mu mol/l}, V_m = 4.2 \text{ nmol/s}, \) and \( k = 14 \text{ \mu l/s}. \) (Use of the Statistical Analysis System's 'NLIN' procedure resulted in similar estimates for these three variables.) A Lineweaver-Burk plot of the results from the diffusion model is also included in Figure 3 by calculating \( A_i \) from the conductance of 14 \( \mu l/s \) and plotting the inverse.

The apparent Michaelis-Menten analysis on the intact plant yielded a high \( K_m \) estimate consistent with previous attempts to use this model. It also produced a \( K_m \) estimate much greater than expected from enzyme studies. On the other hand, the diffusion model \( K_m \) of 129 \( \text{ \mu mol/l} \) is consistent with enzyme studies (3, 7).

In normal use, the \textit{in situ} system used to measure acetylene reduction was programmed to subject all plants to three or four concentrations of acetylene during a 4-h period each night. The data for these varied acetylene concentrations were used to compute \( K_m, V_m, \) and \( k. \) Even though only three or four concentrations were not adequate to yield small standard errors in the analysis of these variables, in all three years most estimates of \( K_m \) were in the range of 50 to 250 \( \text{ \mu mol/l}. \) For instance, in 1982, for 262 estimates of \( K_m \) the mean was 158 \( \text{ \mu mol/l} \) with a 95 per cent confidence interval for the mean of 140 to 175 \( \text{ \mu mol/l}. \)

Not surprisingly, considerable variability in response to treatment in the value of \( k \) and \( V_m \) was observed during each of the 3 years. As an example, acetylene reduction data and conductance estimates for a plant being subjected to drought stress from August 21 to 28, 1980, are plotted in Figure 4. There was a steady decline in acetylene reduction rate and computed gas conductance by the nodule as the drought became more severe. The decrease of nodule conductance with drought stress is consistent with the conclusion of Pankhurst and Sprent (1975) that a diffusion barrier becomes greater with stress. This may result because decreased turgor of the cortical cells might further reduce the air spaces for gas diffusion and thereby decrease conductance.

In conclusion, we have rejected the apparent Michaelis-Menten approach for analyzing nodule gas exchange. The assumption that there is no restriction to gas diffusion is inconsistent with nodule morphology. The apparent Michaelis-Menten analysis produces \( K_m \) estimates that are higher than the \( K_m \) of nitrogenase, and are positively correlated with the \( V_m \) estimate. Additionally, a model incorporating a diffusion barrier (equation 5) produces \( K_m \) values that agree with enzyme \( K_m \) values and are relatively stable. We conclude that any analysis of nodule gas exchange rates must consider the effects of a gas diffusion barrier. Certainly, analysis of acetylene reduction rates under subsaturating acetylene concentration must incorporate such a gas diffusion model.

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