The kinetic mechanism of alanine dehydrogenase from soybean nodule bacteroids was studied by initial velocity experiments with or without product inhibitors, dead-end inhibitors, or alternate substrates. Without inhibitors, double-reciprocal plots of initial velocity experiments showed intersecting lines, indicating a sequential mechanism. These initial velocity experiments also revealed rapid-equilibrium ordered binding of NH₂⁺ prior to pyruvate. When NAD was varied at changing-fixed concentrations of L-alanine, a nonlinear, concave down double-reciprocal plot was obtained. Substrate inhibition by pyruvate or L-alanine with cosubstrates varied was uncompetitive giving further support to an ordered mechanism. Product inhibition studies showed that both NAD and NADH and pyruvate and L-alanine were competitive. This suggested a Theorell-Chance mechanism. When product inhibition by L-alanine was studied with NH₃ varied in a series of experiments at increasing concentrations of pyruvate, the inhibition was eliminated, as expected for a Theorell-Chance mechanism. Furthermore, when NADH, NH₂⁺, and pyruvate were varied simultaneously, maintaining their concentrations at a constant ratio to each other, an infinite Vₘₐₓ was obtained. pH studies of the kinetic parameters indicated that NH₂⁺, rather than NH₃, was the true substrate that binds to a residue on the enzyme with a pK of 8.1. In conclusion, the kinetic mechanism at pH 8.5 was determined to be a Ter-Bi Theorell-Chance. In the amination direction, the substrates add in the order: NADH, NH₂⁺, pyruvate, with NH₂⁺ binding in rapid-equilibrium. In the reverse direction, NAD adds first, followed by L-alanine.

Alanine dehydrogenase (EC 1.4.1.1) catalyzes a reversible reaction converting pyruvate to alanine using NADH as an oxidation/reduction cofactor.

\[ \text{NADH} + \text{NH}_2^+ + \text{pyruvate} \leftrightarrow \text{L-alanine} + \text{NAD} + \text{H}_2\text{O} \quad (\text{Eq. 1}) \]

The reaction has a pH optimum of 8.5 in the amination direction and 10.0 in the direction of deamination. The enzyme exhibits substrate inhibition with pyruvate or alanine and apparent substrate inhibition at high concentrations of NH₂⁺. A study of partially purified alanine dehydrogenase from soybean bacteroids shared some of these characteristics (1, 2). The enzyme exists in several microorganisms and usually has a high Kₘ for NH₂⁺ in the range of 20-300 mM. In \textit{Bradyrhizobi um japonicum} bacteroids, however, the apparent Kₘ for NH₂⁺ is 10-100 times less (4-7 mM). Since the enzyme potentially plays an important role in nodule nitrogen metabolism, an attempt was made to determine the kinetic mechanism in order to better understand how the enzyme functions.

The kinetic mechanism of alanine dehydrogenase has been previously studied in \textit{Bacillus subtilis} (3, 4), \textit{Bacillus sphaericus} (5), and \textit{Propionibacterium freudenreichii} subspecies \textit{shermanii} (6). This is the first attempt to elucidate the mechanism of the enzyme from a nitrogen-fixing organism; however, preliminary kinetic studies have been carried out with the enzyme from \textit{Anabaena cylindrica} (7), \textit{Rhodobacter capsulatus} (8, 9), \textit{Methylococcus capsulatus} (10), \textit{Bacillus licheniformis} (11), and soybean bacteroids (1, 2).

### EXPERIMENTAL PROCEDURES

**Enzyme Assays**—Assays were done as described previously.¹ Alanine dehydrogenase purified from soybean nodule bacteroids was assayed by following the oxidation of NADH or reduction of NAD at 340 nm in a 1-cm quartz cuvette. Assays were done in 100 mM TAPS² buffer, pH 8.5, or CAPS buffer, pH 10.0. Buffers and NH₄Cl stock solutions were kept at 25 °C, as were the assays. The rest of the substrates and enzyme were kept on ice until use. The ionic strength was kept constant with NaCl. Assays were done on a Gilford 250 spectrophotometer, using a deuterium lamp. The spectrophotometer was equipped with a Gilford 6051 chart recorder and a Gilford 2451-A automatic cuvette positioner. Full scale sensitivity of A₃₄₀ and a chart speed of 1 cm/min were used.

**pH Studies**—A mixture of BisTris, HEPES, and Tricine buffers or TAPS, CHES, and CAPS buffers was used at 50 mM concentration each. Assays at overlapping pH values between the two mixtures were used. The pH of the reaction mixture without added enzyme was measured, and the final pH at the end of the assay was checked. The enzyme was stable throughout the pH range as determined previously.¹

**Data Processing**—The nomenclature used is that of Cleland (12). Reciprocal initial velocities were plotted versus reciprocal substrate concentrations, and the experimental data were fitted to Equations 2-10.

¹ Smith, M. T., and Emerich, D. W. (1993) \textit{Arch. Biochem. Biophys.}, in press.
² The abbreviations used are: TAPS, 3-[(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)amino]-1-propanesulfonic acid; CAPS, 3-(cyclohexylamino)ethanesulfonic acid; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-propane-1,3-diol; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; CHES, 2-(cyclohexylamino)ethanesulfonic acid.
by the least squares method (13), using an IBM XT computer and the compiled Fortran programs of Cleland (14), except Equations 3 and 10, which were fitted using the least squares Minas program by MicroMath (Salt Lake City, UT), kindly supplied by Dr. Peter Tipton. Except where noted, the points in the reciprocal plots are the experimentally determined values, while the lines are calculated from the fits of these data to the appropriate rate equation. Data were usually the average of four replicates, and the error analysis was expressed as the standard deviation. Linear double-reciprocal plots were fitted to Equation 2, while sequential initial velocity data were fitted to Equation 4. Nonlinear concave up intercept replots, indicating uncompetitive substrate inhibition, were fitted to Equation 3. Data conforming to linear competitive, linear noncompetitive, and linear uncompetitive inhibitions were fitted to Equations 5-7, respectively. The pH dependence of log V/K for pyruvate was fitted to Equation 8, describing a half-ball-shaped curve with a drop in activity at high pH, with a slope of 1. The pH dependence of log V/K for NH₄⁺ was fitted to Equation 9, which describes a bell-shaped curve with slopes of 1. Equation 10 describes a rapid-equilibrium Theorell-Chance mechanism and was derived (15) from an equation used to describe the mechanism for malic enzyme (16).

RESULTS

Initial Velocity Patterns—In the forward direction, intersecting lines were obtained with all combinations of substrates, confirming a sequential mechanism (Table I). In the reverse direction, when L-alanine was varied at different levels of NAD, intersecting lines were also obtained, indicating a sequential mechanism in this direction as well (Fig. 1). However, statistical analysis of the data gave kinetic constants with a relatively large error, and when the same data was plotted with NAD as the varied substrate, a curved line concave down at the 1/y axis was obtained (Fig. 2). A similar pattern has been obtained, when NAD was varied, with the enzyme from A. cylindrica (7). This apparent negative cooperativity (17) has been demonstrated in several dehydrogenases. This property was not investigated further in this study and should be considered tentative until confirmed by binding studies. In the amination direction with pyruvate varied and NH₄⁺ as the changing-fixed substrate, lines that intersect on the 1/y axis were obtained (Fig. 3). This type of pattern in an initial velocity plot occurs when the changing fixed substrate (NH₂⁺) adds in rapid-equilibrium fashion to the enzyme. The same data plotted with ammonia as the varied substrate gave lines intersecting to the left of the 1/y axis (Table I). The slope in this plot became nearly horizontal at high concentrations of pyruvate, making the velocity appear independent of NH₂⁺ concentration. To confirm the rapid-equilibrium binding of NH₂⁺, the slope replot was examined (Fig. 4), which extrapolated through the origin, as predicted. This type of pattern can only be obtained in a rapid-equilibrium ordered mechanism with pyruvate adding after NH₂⁺ (18).

Product Inhibition—Product inhibition by NADH with NAD varied (in the region of linear response) gave competitive inhibition, demonstrated by lines intersecting on the 1/y axis (Table II). This is consistent with NADH and NAD adding first and coming off last, respectively (Scheme 1), as occurs in many other dehydrogenases with ordered mechanisms. The reverse experiment, with NADH varied and NAD the changing-fixed product inhibitor, gave a similar pattern (Table II). When pyruvate was varied at changing-fixed levels of L-alanine, a competitive inhibition pattern was also obtained (Table II). This is consistent with either a Theorell-Chance or random mechanism with dead-end complexes (18). However, a random mechanism would not be consistent with the rapid-equilibrium ordered pattern observed with NH₂⁺ and pyruvate. It is also inconsistent with an ordered mechanism, which would show only one competitive product inhibition pattern (18).

If product inhibition by L-alanine with NH₂⁺ as the variable substrate were examined at saturating and nonsaturating concentrations of pyruvate, a distinction between mechanisms could be made. For an ordered mechanism with pyruvate adding last, saturation with pyruvate would convert noncompetitive inhibition to uncompetitive inhibition. In an ordered mechanism with pyruvate adding second, saturation with pyruvate would have no effect, and the inhibition by alanine would remain noncompetitive. In a random mechanism, the inhibition would also remain noncompetitive, as NH₂⁺ can add last and remain reversibly connected to alanine. A Theorell-Chance mechanism would give a unique pattern upon saturation with pyruvate. That is, the noncompetitive inhibition by alanine would be eliminated by saturation with pyruvate (18), causing the intersecting lines to converge to a single line.

However, substrate inhibition by pyruvate prevents saturation with this substrate. Nevertheless, a series of experiments with NH₂⁺ varied versus changing-fixed concentrations of L-alanine at increasing concentrations of pyruvate (below substrate inhibition levels) should indicate a trend toward elimination of inhibition (18). The patterns obtained with 0.5 and 2.3 mM pyruvate are shown in Figs. 5 and 6, respectively. The plots of these experiments, which were otherwise carried out under the same conditions, show a compression of the lines at the higher pyruvate concentration. This implies that at saturating pyruvate, the lines would compress into a single line, indicating no inhibition. When a tertiary plot of the slopes of these plots, including data from an experiment at 1.25 mM pyruvate was made, the trend at infinite pyruvate concentration approached zero (no change in slope). This is consistent with an elimination of inhibition at saturating pyruvate. These experiments provide further evidence for the existence of a Theorell-Chance mechanism.

Determination of the True Maximum Velocity—A prediction of the Theorell-Chance mechanism is that at infinite substrate concentration, the Vₘₐₓ should also become infinite (16). The true Vₘₐₓ in a ter-reactant mechanism can be determined by varying all the substrates simultaneously, maintaining their concentrations at a constant ratio to each other (18). Extrapolation to infinite substrate concentration in a double-reciprocal plot should give the true Vₘₐₓ. This was done for alanine dehydrogenase in the amination direction,
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TABLE I

Kinetic parameters from initial velocity experiments with alanine dehydrogenase

| Variable substrate | Cosubstrates | $K_m$ $mM$ | $K_i$ $mM$ | Data fit to equation |
|--------------------|--------------|------------|------------|----------------------|
| Pyruvate           | NADH (7.5-80 mM) | 0.43 ± 0.06 | 0.082 ± 0.016 | 4 |
|                    | NH$_3$ (58.4 mM) | 0.098 ± 0.012 | 0.021 ± 0.004 | 4 |
| NADH               | Pyruvate (0.120-0.599 mM) | 5.5 ± 0.5 | 3.7 ± 0.7 | 4 |
|                    | NH$_3$ (58.4 mM) | 0.022 ± 0.002 | 0.015 ± 0.003 | 4 |
| NH$_3$             | Pyruvate (0.120-0.599 mM) | 0$^a$ | 7.4 ± 1.8 | 11 |
|                    | NADH (160 μM) | ND | 0$^a$ | 11 |
| Pyruvate           | NH$_3$ (2.1-10.0 mM) | 0.37 ± 0.06 | 1.0 ± 0.5 | 4 |
|                    | NADH (0.050-1.5 mM) | 0.098 ± 0.012 | 0.021 ± 0.004 | 4 |

$^a$ Theoretical values interpreted from the double-reciprocal plots.

FIG. 1. Initial velocity double-reciprocal plot with L-alanine varied at different fixed concentrations of NAD. Individual lines were fit to Equation 2 (see "Experimental Procedures"). The $\sigma$ value for the fit to Equation 2 ranged between 0.015 and 0.065.

FIG. 2. Initial velocity double-reciprocal plot with NAD varied at different fixed concentrations of L-alanine. Data set is the same as in Fig. 1.

FIG. 3. Initial velocity double-reciprocal plot with pyruvate varied at different fixed concentrations of ammonium. Data were fit to Equation 10 (see "Experimental Procedures"). The $\sigma$ value for the fit of different lines to Equation 10 was between 0.0025 and 0.050.

FIG. 4. Slope replot of ammonium versus pyruvate double-reciprocal plot. Slopes calculated by nonlinear least squares analysis of individual lines in double-reciprocal plot, using Equation 2 (see "Experimental Procedures"). $\sigma$ value for the fit to Equation 2 was between 0.035 and 0.050.

maintaining a ratio between the substrates similar to that of their individual $K_m$ values (1:0.064:14 for pyruvate: NADH:NH$_3$) and keeping pyruvate below the substrate inhibition level (2.0 mM). The results of two experiments shown
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TABLE II
Kinetic parameters resulting from product inhibition of alanine dehydrogenase

Standard deviation indicated by (±) was the result of four replicates. Ks values are apparent values.

| Varied and Non-varied substrate | Product inhibitor | Ks (mM) | Km (mM) | Ki (mM) | Type of inhibition* |
|--------------------------------|-------------------|---------|---------|---------|---------------------|
| NADH (22.5 μM) & NADP (1.5 mM) | NADH (22.5 μM) | 0.093 ± 0.010 | 0.0123 ± 0.001 | | C |
| NADH (38-90 μM) & Pyruvate (2.3 mM) | NADH (38-90 μM) | 0.073 ± 0.010 | 0.81 ± 0.09 | | C |
| Pyruvate (0.120-0.599 mM) & NH4+ (45 mM) | L-Alanine (0-6.0 mM) | 0.604 ± 0.030 | 1.02 ± 0.03 | | C |
| NADH (160 μM) & NH4+ (7.0 mM) | Pyruvate (0.120-0.599 mM) | 3.4 ± 0.2 | 1.26 ± 0.070 | 4.2 ± 0.4 | NC |
| NH4+ (7.0 mM) & NADH (32 μM) | NADH (160 μM) | 4.4 ± 0.3 | 3.8 ± 0.5 | 4.8 ± 0.6 | NC |
| NADH (32 μM) & Pyruvate (2.30 mM) | NADH (160 μM) | 4.0 ± 0.1 | 3.2 ± 0.2 | 5.0 ± 0.2 | NC |

*C, competitive; NC, noncompetitive.

SCHEME 1. Proposed mechanism for alanine dehydrogenase from soybean nodule bacteroids.

FIG. 5. Product inhibition by L-alanine with ammonium varied. Pyruvate concentration was 0.5 mM. Data were fit to Equation 6 (see "Experimental Procedures"). The σ value for the fit to Equation 6 was 0.013.

in Fig. 7 describe a curve that is concave up at low pyruvate concentrations, as expected for an ordered mechanism (18). The dashed line that is tangent to this curve passes through the origin, indicating that the velocity extrapolates to infinity at infinite substrate concentration.

Substrate Inhibition—L-Alanine and pyruvate both give substrate inhibition at higher levels of substrate. Ammonium also shows apparent substrate inhibition at concentrations greater than 50 mM. When NH4+ was varied at different fixed levels of pyruvate sufficient to give substrate inhibition, the pattern in Fig. 8 was obtained. At noninhibitory levels of pyruvate (dashed line), the pattern was intersecting to the left of the 1/v axis as expected. As pyruvate was increased to substrate inhibition levels (solid lines), the lines eventually became parallel, indicating uncompetitive substrate inhibition. The uncompetitive substrate inhibition was confirmed by the concave up intercept replot. This is consistent with an ordered mechanism (18) with pyruvate adding to the E-NAD complex, the enzyme form to which L-alanine normally binds (Scheme 1). The combination of pyruvate as a dead-end inhibitor with any other enzyme form would give noncompetitive or competitive inhibition. It is also inconsistent with a random mechanism, which would give a noncompetitive pattern.

When substrate inhibition by L-alanine was analyzed at variable concentrations of NAD (64-170 μM), which were within the range that results in linear response on double-reciprocal plots, a similar pattern was obtained (Fig. 9). At higher concentrations of alanine, the lines again became parallel, indicating uncompetitive substrate inhibition. The replot of the intercepts confirmed the uncompetitive substrate inhibition since it was concave up at the 1/v axis. This is
pyruvate and NH:\.

This pattern as being due to an alternate reaction rather than true substrate inhibition.

They were maintained at a concentration ratio of 1:0.064:14 for pyruvate:NADH:NH\.

Substrate inhibition patterns imply that the substrate inhibition patterns were consistent with an ordered mechanism, with alanine binding to the form of the enzyme that normally binds pyruvate. Thus, the substrate inhibition patterns imply that the substrates bind in a compulsory ordered manner in both directions.

When the apparent substrate inhibition by NH\(\text{I}\) was examined, the pattern in Fig. 10 was obtained. This is similar to the pattern obtained by Grimshaw and Cleland (3) with alanine dehydrogenase from B. subtilis. The intercept replots were concave up as expected for uncompetitive inhibition (18). This pattern has been shown (3) to result from an alternate reaction sequence due to the nonenzymatic combination of pyruvate and NH\(\text{I}\).

Pyruvate + NH\(\text{I}\) \(\rightarrow\) iminopyruvate \hspace{1cm} \text{(Eq. 11)}

The iminopyruvate may then undergo catalysis by the enzyme to yield the normal products. Their analysis thus identifies this pattern as being due to an alternate reaction rather than true substrate inhibition.

Dead-end Inhibition—To provide further support for the addition of pyruvate following the addition of NH\(\text{I}\), dead-end inhibition experiments were carried out with the pyruvate analogs: oxamate, propionate, and methylglyoxal. When pyruvate was varied in the presence of each of these analogs, however, lines intersecting to the left of the \(1/\text{Pyruvate}\) axis were obtained, rather than the expected competitive inhibition pattern (Table III). There are two ways such a pattern could be generated. First, if the pyruvate analog were adding to the enzyme before the addition of pyruvate and reversibly connected to it, a noncompetitive inhibition pattern would result.Second, these patterns could be generated, if the pyruvate analogs are adding to two different enzyme forms. The pyruvate analogs could add to the same enzyme form as pyruvate, giving a slope effect, as well as adding to the E-NAD complex, the form which normally binds L-alanine, giving an intercept effect (Scheme 1). The combined effect of binding to both enzyme forms would give a slope and intercept effect, produc-
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**TABLE III**

Kinetic parameters for alternate substrate and dead-end inhibition of alanine dehydrogenase

| Varied and non-varied substrate | Dead-end inhibitor or alternate substrate | $K_s$ (mM) | $K_i$ (mM) | $K_u$ (mM) | Type of inhibition |
|--------------------------------|------------------------------------------|----------|----------|----------|-------------------|
| Pyruvate (0.3-1.5 mM)          | Oxamate (0-10 mM)                        | 0.27 ± 0.02 | 10.5 ± 2.2 | 38.6 ± 9.6 | NC               |
| NADH (300 μM)                  |                                          |          |          |          |                  |
| NH$_2$ (58.4 mM)               |                                          |          |          |          |                  |
| Pyruvate (0.3-1.5 mM)          | Propionate (0-40 mM)                     | 0.62 ± 0.03 | 54.1 ± 6.6 | 33.4 ± 2.6 | NC               |
| NADH (190 μM)                  | Methylglyoxalate (0-40 mM)               | 0.52 ± 0.05 | 30.9 ± 6.5 | 58.2 ± 16.9 | NC               |
| NH$_2$ (3.5-17.5 mM)           | β-Hydroxypropionate (0-40 mM)            | 0.34 ± 0.02 | 20.0 ± 0.3 | 10.5 ± 0.9 | NC               |
| Pyruvate (0.5 mM)              |                                          |          |          |          |                  |
| NADH (18-90 μM)                |                                          |          |          |          |                  |
| NH$_2$ (58.4 mM)               |                                          |          |          |          |                  |

*NC, noncompetitive; UC, uncompetitive.

A similar experiment with NADH varied and oxamate as the dead-end inhibitor also gave uncompetitive inhibition (Table III). These results are consistent with the proposed order of addition: NADH, NH$_2$, and pyruvate.

Alternate Substrate—To give further support to the dead-end inhibition experiments, an alternate substrate, β-hydroxypropionate, was used (Table III). β-Hydroxypropionate has about 15% of the activity of pyruvate. Thus it would be expected to bind to the same form of the enzyme as pyruvate. When pyruvate was varied at changing-fixed concentrations of β-hydroxypropionate (Table III), a noncompetitive pattern was obtained, consistent with the results of the dead-end inhibition experiments.

These results, together with the dead-end and substrate inhibition experiments, and the initial velocity data, confirm the ordered addition of NH$_2$ and pyruvate.

**pH Profiles of Kinetic Parameters**—In order to gain further insight into the mechanism of alanine dehydrogenase, the variation of kinetic parameters with pH was studied. The log $V/K$ and log $V$ plots for NH$_2$ are shown in Fig. 11. The log $V/K$ profile fit Equation 9, which describes a bell-shaped curve with slopes equal to 1. The profile gave a pK of 9.0 on the basic side of the curve, which is very close to the pK of 9.2 for NH$_2$. The acidic side of the curve gave a pK of 8.1, due, presumably, to a group on the enzyme which is responsible for binding NH$_2$. Since the log $V/K$ rises with an increase in pH, this group must be unprotonated in order to bind NH$_2$. The decline in log $V/K$ after the pK of NH$_2$ implies that NH$_2$ rather than NH$_2^-$ is the species that binds to the enzyme. The log $V$ plot (Fig. 11), however, was relatively independent of pH, compared with the other pH profiles. The pH dependence of $V/K$ for pyruvate is shown in Fig. 12. The

**FIG. 11. Variation in log $V/K$ and log $V$ with pH for ammonium.** Data were fit to Equation 9 for log $V/K$ (see "Experimental Procedures"). The $\epsilon$ value for the fit to Equation 9 was 0.22. The curved line in the log $V$ plot was not fit but represents the best curve through the data points.

**FIG. 12. Variation in log $V/K$ with pH for pyruvate.** Data was fit to Equation 8 for log $V/K$ (see "Experimental Procedures"). The $\epsilon$ value for the fit to Equation 8 was 0.40.
log V/K profile fit Equation 8, which describes a single group that must be protonated in order to bind the substrate. The apparent pK of this group was 8.8. The results of the pH studies are listed in Table IV.

**DISCUSSION**

**Kinetic Mechanism**—The intersecting patterns obtained with the initial velocity experiments clearly indicated a sequential as opposed to a ping-pong mechanism (Table I). Thus, all of the substrates must be bound to the enzyme before any products are released.

The mechanism appears to be ordered as opposed to random on the basis of several lines of evidence. Substrate inhibition by both L-alanine (Fig. 9) and pyruvate (Fig. 8) was uncompetitive, which would be predicted for an ordered rather than a random mechanism (18). The initial velocity experiment with pyruvate and NH₃ gave a unique pattern expected for a rapid-equilibrium ordered mechanism (Fig. 3). This pattern would not be obtained with a random mechanism. Product inhibition by alanine with NH₃ varied at increasing concentrations of pyruvate (Figs. 5 and 6) also supported an ordered addition, as discussed previously, rather than random addition. This ordered addition of NH₃ followed by pyruvate was also supported by dead-end inhibition studies, which showed that pyruvate analogs added after NH₃ and NADH (Table III). This was further supported by experiments with the alternate substrate, β-hydroxypyruvate (Table III).

Although the substrates add in a compulsory order to the enzyme, the mechanism is not a simple ordered one. The product inhibition studies gave two competitive inhibition patterns: one with the substrate-inhibitor pair NAD-NADH, and the other with pyruvate and alanine (Table II). A simple ordered mechanism, however, would only give one competitive product inhibition pattern, due to the two reactants binding to the free enzyme. That is, the first substrate to add and the last product to come off the enzyme would give the competitive pattern. The two competitive inhibition patterns are consistent with either a Theorell-Chance or a random mechanism with dead-end complexes forming (18). In the Theorell-Chance mechanism, the additional competitive inhibition pattern is obtained with the last substrate to add and the first product to be released. This is due to the rapid breakdown of the central complexes to undetectable levels, so that the enzyme forms to which these reactants bind appear to interconvert directly. A rapid-equilibrium random mechanism would give all competitive product inhibition patterns unless substrates are able to bind to enzyme forms as dead-end inhibitors. In that case, they would produce apparently non-competitive inhibition patterns due to the combination of competitive product inhibition and uncompetitive dead-end inhibition. However, as demonstrated under "Results," a random mechanism does not hold for this enzyme. Therefore, the Theorell-Chance fits best with the data. The product inhibition pattern with NH₃ varied at changing-fixed concentrations of pyruvate gives strong support to this conclusion. As pyruvate concentration was extrapolated to infinity, product inhibition by alanine was eliminated. As demonstrated under "Results," this would only hold in a Theorell-Chance mechanism (18). This is because alanine cannot convert E-NAD back to E-NADH-NH₃ when pyruvate is saturating, which forces the flux of enzyme forms back to E-NAD. The Theorell-Chance mechanism was further confirmed by the fact that the velocity extrapolates to infinity at infinite substrate concentrations (Fig. 7). A Theorell-Chance mechanism was suggested for alanine dehydrogenase from *Mycobacterium tuberculosis* (19). Departure from a simple ordered mechanism was also indicated by the rapid-equilibrium rather than steady-state addition of NH₃. This was demonstrated by the initial velocity patterns of NH₃ and pyruvate (Figs. 3 and 4, Table I). The Theorell-Chance mechanism has been demonstrated for only a few enzymes (Ref. 22 and references therein). Recently, a Theorell-Chance mechanism with one substrate adding in rapid-equilibrium has been demonstrated for a bireactant enzyme (20-22).

Since NADH and NAD are the first to add and the last to be released (Table II), and pyruvate adds after NH₃ (Figs. 3 and 4, Table II), the order of addition for the forward reaction is: NADH, NH₃, and pyruvate (Scheme 1). For the reverse reaction, since NAD was the first to add and alanine is reversibly connected to pyruvate (Table II), the order is: NAD, followed by alanine (Scheme 1).

Thus, the mechanism determined for alanine dehydrogenase from soybean bacteroids at pH 8.5 is an ordered Ter-Bi Theorell-Chance with rapid-equilibrium binding of NH₃ in the second position (Scheme 1). This mechanism was further supported by fitting the initial velocity data of pyruvate and NH₃ to Equation 10 (Fig. 3). This mechanism differs somewhat from the mechanism determined for the enzyme from *B. subtilis* (3), which has a Ter-Bi ordered mechanism with NH₃ adding after pyruvate. In this organism, as in most other organisms, the enzyme appears to favor the deamination reaction due to its high K₆ for NH₃. In fact, NH₃ does not actually bind to the enzyme but simply chemically reacts with the E-NADH-pyruvate complex (3). This would explain the binding order (NADH, pyruvate, NH₃), because the NH₃ cannot react until the other substrates are in place.

The kinetic mechanisms of alanine dehydrogenase from *B. sphaericus* (5) and *P. freudenreichii* (6) are also reported to be different from that of *B. subtilis* (3). The proposed mechanism of the enzyme from *B. sphaericus* is similar to that determined for soybean bacteroids. Ohashima and Soda (5) found a completely ordered mechanism with NADH, NH₃, and pyruvate adding in that order. The deamination reaction was also the same, with NAD adding first, followed by alanine. Their data, however, showed linear NAD versus alanine initial velocity plots and no Theorell-Chance mechanism at pH 9.0.

The mechanism for alanine dehydrogenase from *P. freudenreichii* was reported to be partial random with alanine and NAD adding in ordered fashion and NAD adding second (6). In the amination reaction, NH₃ was reported to add first, followed by random addition of pyruvate and NADH.

**pK values from pH profiles of the kinetic parameters for alanine dehydrogenase**

| Parameter | Equation fit | pKᵢ | pKᵢ' | pKᵢ'' |
|-----------|--------------|-----|------|-------|
| Log (V/Kpyruvate) | 8 | 9.08 ± 0.20 | |
| Log (V/Kalanine) | 9 | 8.13 ± 0.12 | 5.96 ± 0.13 |
B. japonicum alanine dehydrogenase may be due to the binding of NH$_3^-$, since it must bind before pyruvate. The log V/K for pyruvate for the B. subtilis enzyme, on the other hand, had a pK of 7.9 (4).

In conclusion, there are several characteristics of the alanine dehydrogenase mechanism from B. japonicum bacteroids that would influence its role in the nodule. Rather than simply diffusing in and chemically reacting with the enzyme-substrate complex, NH$_3^-$ appears to bind directly to the enzyme. This may partially explain why the apparent $K_m$ for NH$_3^-$ was substantially lower in this organism than it is in other organisms. The fact that the enzyme binds NH$_3^-$ rather than NH$_3$ would enable the enzyme to use the form of nitrogen that would be most abundant at physiological pH. Finally, the Theorell-Chance mechanism would help make most efficient use of the rapid-equilibrium binding of NH$_3^-$.

Acknowledgments—We thank Dr. Peter A. Tipton and Dr. Warren Zahler for many helpful discussions with regard to this work.

REFERENCES
1. Dunn, S. D., and Klucas, R. V. (1973) Can. J. Microbiol. 19, 1493-1499
2. Muller, P., and Werner, D. (1982) Z. Naturforsch. 37c, 927-930
3. Grimshaw, C. E., and Cleland, W. W. (1981) Biochemistry 20, 5650-5655
4. Grimshaw, C. E., Cook, P. F., and Cleland, W. W. (1981) Biochemistry 20, 5655-5661
5. Ohashima, T., and Suda, K. (1979) Eur. J. Biochem. 100, 29-39
6. Crow, V. L. (1987) Appl. Environ. Microbiol. 53, 1685-1692
7. Rowell, P., and Stewart, W. D. P. (1976) Arch. Microbiol. 107, 115-124
8. Caballero, F. J., Cejudo, F. J., Florencio, F. J., Cadenas, J., and Castillo, F. (1985) J. Bacteriol. 162, 804-809
9. Tolkstorf-Neutzling, R., and Klemme, J.-H. (1982) FEMS Microbiol. Lett. 13, 153-159
10. Murrell, J. C., and Dalton, H. J. Gen. Microbiol. 129, 1197-1206
11. McCowen, S. M., and Phibbs, P. V., Jr. (1974) J. Bacteriol. 118, 590-597
12. Cleland, W. W. (1963) Biochim. Biophys. Acta 67, 153-157
13. Wilkinson, G. N. (1961) Biochem. J. 80, 334-332
14. Cleland, W. W. (1979) Methods Enzymol. 63, 153-158
15. Smith, M. T. (1991) Alanine Dehydrogenase from Soybean Nodule Bacteroid. Ph.D. dissertation, University of Missouri
16. Schimertik, M. I., Grimshaw, C. E., and Cleland, W. W. (1977) Biochemistry 16, 571-578
17. Neet, K. E. (1980) Methods Enzymol. 64, 139-192
18. Cleland, W. W. (1970) in The Enzymes (Boyer, P. D., ed) Vol. 2, pp. 1-66, Academic Press, New York
19. Goldman, D. (1959) Biochim. Biophys. Acta 34, 527-530
20. Gates, C. A., and Northrop, D. B. (1988) Biochemistry 27, 3826-3833
21. Gates, C. A., and Northrop, D. B. (1988) Biochemistry 27, 3834-3842
22. Gates, C. A., and Northrop, D. B. (1988) Biochemistry 27, 3820-3825