Purification and Properties of the Constituents of the Nitrogenase Complex from 
*Clostridium pasteurianum*

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A new procedure for a rapid and extensive purification of the FeMo protein and the Fe protein of the nitrogenase complex from *Clostridium pasteurianum* is described. Specific activities of 345 and 460 nmoles of N₂ reduced per mg of protein per min for the FeMo protein and for the Fe protein, respectively, have been obtained. Preparations of the FeMo protein contained 0.96 atom of molybdenum and 15 atoms of iron per molecule, whereas those of the Fe protein contained 2.86 atoms of iron per molecule. Experiments suggest that a definite association of two Fe proteins and one FeMo protein is functional in the active enzyme complex. No individual role could be ascribed to either of the two proteins, but the fact that hydrogenase inhibits N₂ fixation but not the reductant-dependent adenosine triphosphate hydrolysis supports the idea that there are two distinct sites on nitrogenase, one concerned with N₂ activation and the other with activated electron transport.

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Purification of the N₂-fixing systems from *Azotobacter vinelandii* by Bulen and LeComte (2) and from *Clostridium pasteurianum* by Mortenson et al. (17), Moustafa and Mortenson (18), and Kelly et al. (15) has yielded two proteins which are required for N₂ fixation and adenosine triphosphate (ATP)-dependent H₂ evolution. The first protein, containing molybdenum and iron, has been called enzyme I or molybdoferredoxin, and the second, containing iron, has been called enzyme II or azoferredoxin. In this text, these two proteins are referred to as the FeMo protein and the Fe protein, respectively. These designations are explicit and seem advisable until the functions of the individual components are established clearly (1).

This paper describes a rapid procedure for preparing the FeMo protein and the Fe protein from *C. pasteurianum* and discusses the possible involvement of a third component in the clostridial nitrogen-fixing system (14, 16, 22). The relationship between nitrogenase and hydrogenase (26) is examined, and the respective roles of the FeMo and Fe proteins in nitrogen fixation and the question of their association are considered.

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MATERIALS AND METHODS

 Cultures. Cultures of *C. pasteurianum* strain W-5 were grown with N₂ in the nitrogen-deficient medium of Westlake and Wilson (25). In the final stage, a 150-liter glass-lined fermentor with medium at 35 °C and pH 6.4 was used, and phosphates were omitted from the medium. Cells were harvested and dried at 35 °C under vacuum as described by Carnahan et al. (3). They were stored at −15 °C in evacuated, sealed ampoules.

 Extracts. Extracts were prepared by autolysis of dried cells (0.8 to 1.2 g of cells per 10 ml of 20 mM tris(hydroxymethyl)aminomethane (Tris) buffer, pH 7.4). The suspension was shaken under H₂ at 30 °C for 1 hr. The crude extract obtained after centrifugation (25,000 × g for 20 min) contained 25 to 30 mg of protein per ml. All manipulations at all stages of preparation and purification of the extracts were conducted under strictly anaerobic conditions. All flasks and solutions to be used were evacuated and gassed with H₂ or A three or more times. Transfers between flasks under A, H₂, or N₂ were made with hypodermic syringes through serum stoppers. Whenever a system containing a nitrogenase preparation was open (for introduction of samples on chromatographic columns, during collection of fractions from these columns, during introduction of samples in manometric flasks), a stream of N₂ was kept flushing through the system.

 Assays of nitrogenase activity. A typical assay mixture for the determination of nitrogenase activity
contained, for 1 ml of reaction mixture, creatine phosphate, 40 μmoles; creatine phosphokinase, 0.4 mg; ATP, 5 μmoles; MgCl₂, 10 μmoles; Na₂SO₄, 20 μmoles; potassium 2-(N-morpholino) ethanesulfonate (MES) buffer (pH 6.6), 40 μmoles; and 1 atm of N₂ or A, or 0.4 atm of CH₄ plus 0.6 atm of A, or 5 to 10 μmoles of azide and 1 atm of A, according to the assay to be run. Vessels were incubated with shaking (150, 4-cm strokes/min) at 30 C in a water bath for 10 to 15 min. Nitrogen fixation was measured by NH₃ formation as described by Dilworth et al. (6). Acetylene reduction was measured by gas chromatographic determination of the ethylene produced; a Varian Aerograph 600D gas chromatograph with Porapak R column was used as described by Stewart et al. (21). Azide reduction was measured by ammonia formation and manometric measurements. H₂ evolution was measured manometrically. All manometric determinations were performed with Gilson all-glass volumometers (23) which allow convenient evacuation and gassing. The specific activities of the FeMo and Fe proteins and defined as the amount of nitrogen (nanomoles of N₂) fixed per minute per milligram of the specified protein in the presence of an excess of a purified preparation of the complementary protein. The specific activity of nitrogenase is expressed as nanomoles of N₂ fixed per minute per milligram of total protein for a crude extract or for the optimal combination of purified FeMo and Fe proteins in a reconstituted enzyme system.

Analytical methods. ATP hydrolysis was measured by determination of the creatine liberated from creatine phosphate (7). These determinations were performed in the reaction mixtures after the reaction had been stopped by addition of a saturated potassium carbonate solution. Protein was measured by the microbiuret method of Gornal (9) with serum albumin as a standard. Molybdenum was determined with the dithiol reagent (4) by the method described by Bulen and LeComte (2). Iron was determined with batho-phenanthroline (24). In experiments involving exchange with D₂, determinations of the three molecular species, H₂, HD, and D₂, were made with a Consolidated Nier isotope-ratio mass spectrometer; D₂ served as an internal standard or measurements were made at a standard manifold pressure. Disc electrophoresis of the purified fractions was run in polyacrylamide gels according to Davis (5).

Reagents. All reagents were purchased from commercial dealers, except that creatine phosphate was prepared according to Ennor and Stocken (8). The diethylaminoethyl (DEAE) cellulose used was Whatman DE 52.

RESULTS

Purification of proteins. Crude extracts had a specific activity of about 14 nmols of N₂ fixed per min per mg of protein. They were first fractionated on an anaerobic DEAE-cellulose column (19). A volume of 60 to 100 ml of extract (1.5 to 2.5 g of protein) was put on top of a DEAE-cellulose column (2.7 cm in diameter by 8 cm in height) which had been equilibrated anaerobically with 0.02 M Tris buffer, pH 7.4. The flow rate was 0.8 to 1.0 ml/min. After the extract had entered the column, it was washed with 25 ml of Tris buffer to complete elution of the large quantity (about 50% of total protein) of weakly adsorbed yellowish proteins. Elution then was started with 0.065 M MgCl₂ in 0.02 M Tris buffer, pH 7.4. This eluted a dark brown band; it was collected as fraction 1 and contained the FeMo protein. The front of this band contained hydrogenase, and the first 6 to 8 ml was collected separately. Elution then was continued with 0.09 M MgCl₂ in 0.02 M Tris buffer (pH 7.4), and the brown-orange band eluted was called fraction 2. It contained the Fe protein. A dark brown band of ferredoxin was left on the column; it could be eluted with 0.3 M MgCl₂. Table I gives the results of a representative fractionation experiment.

Fraction 1 contained the FeMo protein and hydrogenase. Hydrogenase was separated by gradient elution on DEAE cellulose. The column (2.9 by 11 cm) was equilibrated with anaerobic 0.02 M Tris buffer, pH 7.4. Fraction 1 (usually about 200 mg of protein) was diluted with buffer, so that the final concentration of MgCl₂ was 0.04 M, and then was applied to the top of the column. Elution was effected with a linear gradient of MgCl₂ (0.04 to 0.08 M) in 0.02 M Tris buffer, pH 7.4. Two distinct brown bands formed, and fractions were collected in a series of anaerobic bottles. The results of such an experiment are

<table>
<thead>
<tr>
<th>Table 1. Fractionation of a crude extract on a DEAE-cellulose column</th>
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<tbody>
<tr>
<td>Determination</td>
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<tr>
<td>-------------------</td>
</tr>
<tr>
<td>Crude extract</td>
</tr>
<tr>
<td>Fraction 1</td>
</tr>
<tr>
<td>Residual activity</td>
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<tr>
<td>Supplemented with F₁</td>
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<tr>
<td>Fraction 2</td>
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<tr>
<td>Residual activity</td>
</tr>
<tr>
<td>Supplemented with F₁</td>
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<tr>
<td>Reconstituted enzyme</td>
</tr>
</tbody>
</table>

a Expressed as nanomoles of N₂ fixed/(minutes × milligrams of protein).

b A 13.65-g amount of dried cells in 166 ml of 0.02 M Tris, pH 7.4, gave 90 ml of extract.

c Corrected for the residual activity of the fraction 2 added.

d Fraction 1 (F₁) was used to supplement fraction 2 (F₂) in the assay. If, a purified FeMo protein preparation was used instead, the specific activity of the same F₂ was 310.
given in Fig. 1 and Table 2. The first band had the hydrogenase activity, and the second band contained the FeMo protein. The volume of the pooled fractions containing the FeMo protein was reduced by ultrafiltration under N₂ through an Amicon filter.

Immediately after collection from the initial fractionation on DEAE cellulose, fraction 2 was placed on an anaerobic column (2.7 by 33 cm) of Sephadex G-100 equilibrated with 0.02 M Tris buffer (pH 8.0), containing 2 mM dithionite and 2 mM diethiothreitol. Two bands were formed when it was eluted with the same buffer. The first band was yellow and contained the Fe protein; the second band contained a red protein. Table 3 gives the results of such a separation. Specific activities of 130 to 140 were obtained for nitrogenase reconstituted from purified FeMo and Fe proteins.

Properties of the constituents of nitrogenase: stability. The FeMo protein concentrated after purification as described was kept in the refrigerator under N₂ for as long as 1 week with a 20% loss of activity. It was kept in liquid nitrogen for longer storage. The Fe protein was much more labile, and a precipitate formed within 1 hr in fraction 2 from the initial DEAIE column. All activity was lost in 10 hr unless the preparation was frozen in liquid nitrogen. After purification on Sephadex, the Fe protein was less cold-labile and in general was more stable. No precipitate formed during weeks of storage in the refrigerator, and about 80% of the activity was retained for 2 days in the refrigerator (5 C). If not used immediately, the Fe protein was preserved in liquid nitrogen.

Activity. No preparations of FeMo protein showed any residual activity when assayed alone for reduction of N₂ or other substrates or when assayed for ATP hydrolysis. Fraction 1 evolved H₂ from dithionite independent of ATP because it contained hydrogenase, but purified FeMo protein did not evolve H₂ from dithionite with or without ATP. Fraction 2, however, had residual activity for all nitrogenase reactions. Purification with Sephadex yielded an Fe protein preparation which had no residual activity for N₂ fixation, C₃H₂ reduction, or ATP hydrolysis.

![Graph](http://jb.asm.org/Downloaded from)

**Fig. 1.** Chromatography of fraction 1 by gradient elution from a column of DEAE-cellulose. Symbols: large dashes, MgCl₂ concentration; solid line, protein concentration; small dashes, hydrogenase activity; alternating dots and dashes, FeMo protein activity. The hydrogenase activity was measured by H₂ evolution from dithionite at pH 6.6 at 30 C without the addition of an electron carrier. The classical hydrogenase assay of Peck and Gest (20) (in the presence of methyl viologen as an electron carrier) indicated a Q₁₀(N) of 6 × 10⁴ for the most active hydrogenase fraction.
TABLE 2. Purification of the FeMo protein on a DEAE-cellulose column

<table>
<thead>
<tr>
<th>Determination</th>
<th>Vol (ml)</th>
<th>Protein concn (mg/ml)</th>
<th>Specific activity of FeMo protein</th>
<th>Purification (no. of times)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fraction 1 before purification</td>
<td>20</td>
<td>15.7</td>
<td>76</td>
<td>1</td>
</tr>
<tr>
<td>Purified FeMo protein</td>
<td>42.75</td>
<td>1.45</td>
<td>318</td>
<td>4.2</td>
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</table>

TABLE 3. Purification of the Fe protein on a column of Sephadex G-100

<table>
<thead>
<tr>
<th>Prepn</th>
<th>Vol (ml)</th>
<th>Protein (mg/ml)</th>
<th>Specific activity*</th>
<th>Supplemented with FeMo protein*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Residual</td>
<td></td>
</tr>
<tr>
<td>Fraction 2 before purification</td>
<td>10</td>
<td>6.9</td>
<td>20.7</td>
<td>250</td>
</tr>
<tr>
<td>Purified Fe protein</td>
<td>11</td>
<td>4.0</td>
<td>0</td>
<td>460</td>
</tr>
</tbody>
</table>

* Refers to N₂ fixation.
† The supplementary FeMo protein preparation had a specific activity of 235 for N₂ fixation.
‡ Refers to C₂H₄ reduction.

Metal analysis of the FeMo and Fe proteins. A purified FeMo protein preparation with a specific activity of 235 had 0.585 μg of Mo per mg of protein or 6.1 nmoles of Mo per mg of protein. Although the reported molecular weight of the FeMo protein has varied between 90,000 and 100,000 (16, 17), the currently accepted value appears to be about 160,000 (1 n mole equals 0.16 mg, or 1 mg of protein is equivalent to 6.25 nmoles). Thus, 6.1 nmoles of Mo per mg of protein corresponds to 0.98 atom of Mo per molecule of the FeMo protein. Analysis for Fe indicated 15 atoms of Fe per molecule. A purified preparation of the Fe protein with a specific activity of 460 contained 2.86 atoms Fe per molecule based on a molecular weight of 40,000 (16).

The hydrogenase fraction from the second chromatographic separation on DEAE cellulose had a Q₉₂ (N) of 6 x 10⁶ and showed no FeMo protein activity, although it carried Fe and a fraction of an atom of Mo per molecule.

Purity. Electrophoretic separations on polyacrylamide gels were run with purified preparations of the FeMo and Fe proteins. The FeMo protein showed two main bands which may be caused by the splitting of the FeMo protein into subunits (17). The Fe protein showed several minor impurities.

Molar ratios of FeMo and Fe proteins in combinations for optimal nitrogenase activity. When nitrogenase was reconstituted by adding increasing amounts of FeMo protein to a given amount of Fe protein, activity per unit of Fe protein increased (Fig. 2), but with excess FeMo protein the specific activity decreased on the basis of total protein of the two fractions. From these data, the respective specific activities of the FeMo and Fe proteins and of the nitrogenase complex can be derived for any combination of the FeMo and Fe proteins. Figure 2 also gives the optimal combination of the FeMo and Fe proteins for nitrogen-fixing activity. A molar ratio approximating 1 FeMo protein to 2 Fe proteins to re-
constitute the N₂-fixing complex was indicated by applying molecular weights of 160,000 and 40,000, respectively, and by correcting for the estimated purity of the proteins. (Experiment 1, 0.77 mg of 80% pure FeMo protein and 0.35 mg of 95% pure Fe protein were combined; 0.62/0.16 mg per n mole = 3.87 n moles; 0.33/0.04 = 8.25 n moles; 8.25/3.87 = 2.13 molar ratio of Fe protein to FeMo protein. Experiment 2, 0.92 mg of 80% pure FeMo protein and 0.40 mg of 95% pure Fe protein were combined; 0.74/0.16 = 4.63 n moles of FeMo protein; 0.38/0.04 = 9.5 n moles of Fe protein; 9.5/4.63 = 2.05 molar ratio of Fe protein to FeMo protein.)

**Inhibition of N₂ fixation by hydrogenase.** The fractions derived from separation of the crude extract on DEAE-cellulose were assayed in the way illustrated in Fig. 2. If fraction 1 instead of purified FeMo protein was used to complement fraction 2, lower values were obtained for fraction 2 activities. In addition, the curve for fraction 2 specific activity instead of reaching a plateau when larger amounts of fraction 1 were added showed a maximum followed by a decrease. Figure 3 illustrates this fact and also shows that ATP hydrolysis, in contrast to N₂ fixation, is not inhibited by excess fraction 1. The same phenomenon was observed when C₂H₂ reduction instead of N₂ fixation was measured. If the complementary experiment was run (measurement of N₂ fixation and ATP hydrolysis when increasing amounts of fraction 2 were added to a constant amount of fraction 1), a normal saturation curve was obtained. Because the inhibition of N₂ fixation was no longer observed when a purified FeMo protein preparation was used to complement fraction 2, it appeared likely that hydrogenase caused the inhibition. This was verified by observing the effect of the addition of increasing amounts of a purified hydrogenase preparation to a given mixture of the FeMo protein and fraction 2 protein. N₂ fixation was strongly inhibited but ATP hydrolysis was not (Fig. 4). A possible explanation is that hydrogenase is able to accept electrons from nitrogenase by an ATP-dependent process. Experiments reported below on H₂ evolution are in agreement with this view.

**Experiments on H₂ evolution.** The hydrogenase fraction obtained by the gradient elution of fraction 1 from a DEAE-cellulose column was free from demonstrable ferredoxin and the FeMo protein, but still evolved H₂ from dithionite at a reduced rate. Addition of the FeMo or Fe protein to a hydrogenase preparation, in the presence or the absence of ATP, did not enhance this H₂ evolution. This shows that neither the FeMo nor Fe protein is able to act alone as an electron carrier (dependent or independent of ATP) from dithionite to hydrogenase. However, when the ATP-dependent H₂-evolving system, consisting of the FeMo and Fe proteins plus an ATP-gen-

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**Fig. 3.** Inhibition of N₂ fixation by adding increasing amounts of fraction 1 to a constant amount of fraction 2. The fraction 1 used had a specific activity of 44. Specific activity of fraction 2 (●) is shown on the left y axis; the amount (µg) of creatine formed per minute per milligram of protein of fraction 2 (□) is shown on the right y axis.

**Fig. 4.** Inhibition of N₂ fixation by hydrogenase. The reaction mixture contained, in addition to the standard components in 1 ml: hydrogenase (Q₉₅(N) = 5 × 10⁵), 0.75 mg of FeMo protein (specific activity, 210), and 0.5 mg of protein of fraction 2 (specific activity, 245). Dithionite was 40 instead of 20 mM. The time of incubation was 8 min. Specific activity, expressed as n moles of N₂ fixed per minute per milligram of fraction 2 protein (●), is shown on the left y axis; amount (µg) of creatine formed per minute per milligram of fraction 2 protein (□) is shown on the right y axis.
erating system (Fig. 5), was added to a hydrogenase preparation evolving H₂ from dithionite independent of ATP, the H₂ evolution was only slightly increased, and total H₂ evolution was much less than the sum of the two H₂-evolving systems measured separately. This suggests that, under these conditions, hydrogenase may function as an electron acceptor from nitrogenase.

**DISCUSSION**

Initial fractionation of nitrogenase into its two proteins markedly shortens the purification procedure. One quickly obtains both proteins from the same crude extract and can apply suitable purification steps to each independently. The method yields a purified Fe protein preparation in 10 to 12 hr after starting with dried cells. The purified FeMo protein requires 16 hr of preparation, but the procedure can be interrupted after the initial fractionation step. In several preparations of the FeMo protein, hydrogenase contamination could be detected. Hence, further purification on a DEAE-cellulose or on a Sephadex G-200 column may be desirable. No evidence was found for the involvement of a third component in the nitrogen-fixing system from *C. pasteurianum*, and the requirement for a third component appears unlikely at the stage of purification attained (14, 16, 22).

Hydrogenase at high concentrations inhibits N₂ fixation but not ATP hydrolysis; this suggests that hydrogenase (CO sensitive) competes with the N₂-fixing system for the ATP-dependent electron flow. As reported by Winter and Burris (26), addition of a complete ATP-dependent H₂-evolving system to a preparation of hydrogenase produces only a small enhancement in H₂ evolution instead of the large increase expected if the systems are running independently. It is clear, however, that both the FeMo and Fe proteins are required for electron donation to produce H₂ by the ATP-dependent route. This can be interpreted as a donation of electrons from the reduced-activated nitrogenase complex.

The scheme presented in Fig. 6 indicates the relationship between hydrogenase and nitrogenase. In this scheme, X and Y, respectively, represent the reductant site and the N₂ binding site of nitrogenase proposed by Hardy et al. (11) and discussed in a recent review (10). The evidence for the existence of two distinct sites is based mainly upon the effects of CO on nitrogenase. Because N₂ fixation is inhibited by CO but ATP-dependent H₂ evolution is not, it is logical to assume that CO binds to site Y. Thus, in the presence of CO, site X will evolve H₂ by

![Fig. 5.](http://jb.asm.org/) Hydrogenase and ATP-dependent H₂ evolution. Each flask contained, in a volume of 1 ml: dithionite, 40 μM; MES (pH 6.6), 40 μM; and, when indicated, the standard ATP-generating system, 1.7 mg of hydrogenase, 0.56 mg of FeMo protein (specific activity, 210), and 0.5 mg of protein of fraction 2 (specific activity, 245). Addition of either FeMo protein or fraction 2 to hydrogenase (not represented here) did not increase the H₂ evolution. Symbols: ○, hydrogenase; □, hydrogenase, FeMo protein, fraction 2; ATP; Δ, hydrogenase, ATP ○, FeMo protein, fraction 2, ATP; Λ, FeMo protein, ATP.

![Fig. 6.](http://jb.asm.org/) Electron flow between hydrogenase and N₂ and scheme for N₂ fixation in Clostridium pasteurianum.
an ATP-dependent process instead of transferring electrons to site Y. The residual ATP-dependent H₂ evolution in the presence of N₂ may also be interpreted as evidence for the existence of two sites, but alternative explanations are conceivable. For example, this H₂ evolution could result from a reaction at the site of N₂ binding analogous to the addition of N₂ on metal hydride complexes of the type CoH₃(PPh₃)₃ + N₂ ⇌ CoH(N₂)₃(PPh₃) + H₂. The inhibition by hydrogenase of N₂ fixation but not of ATP hydrolysis provides new evidence for the existence of two different enzymatic sites; the inhibition can be attributed to the ability of site X to transfer its electrons to hydrogenase instead of donating them to site Y.

Finally, the fact that any manifestation of nitrogenase activity requires both the FeMo and Fe proteins can be mechanistically expressed in several fashions. In the scheme shown (Fig. 6), sites X and Y have been located on the same protein A which may be either the FeMo or Fe protein. The other protein, B in the scheme, is depicted as mediating the activation of reduced X through ATP hydrolysis.

The role of ATP in the nitrogenase reactions is not understood. The ATP/2e ratios, as measured for N₂ fixation and H₂ evolution, were found between 4.5 and 5; this is in reasonable agreement with recent reports (2, 26; K. L. Hadfield and W. A. Bulen, 1968, Fed. Proc. 27: 593). N₂ inhibited the ATP-dependent H₂ evolution by 65%, but the overall activity of the nitrogenase complex was unchanged. The determination of the ATP/2e ratio for azide reduction was more difficult because of the complexity of the reaction. With an azide concentration of 5 to 10 mM and a gas-to-liquid ratio of 15 to 1, much of the azide reduced was not released as N₂ but was further reduced, suggesting that it remained bound for direct reduction. Experiments with acetylene suggested that the ATP/2e ratios may be below 4 for CH₄ production. However, there is insufficient evidence yet to conclude whether the ATP/2e ratios differ for various substrates, and definitive information on this point may aid in interpreting the mechanism of action of ATP.

The individual role of each protein and the location of the N₂ binding site are of particular interest. H₂ is a competitive inhibitor of N₂ fixation, and there are indications that an enzyme-bound nitrogen intermediate is responsible for the formation of HD from D₂ in reactions mixtures which fix N₂ (12, 13); hence it was of interest to determine whether HD production required the FeMo or Fe protein or both. However, little HD production from D₂ was found under N₂ or A in the presence of dithionite, the ATP-generating system, and the FeMo or Fe protein or their combinations.

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LITERATURE CITED