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NITROGENASE

II. CHANGES IN THE EPR SIGNAL OF COMPONENT I (IRON–MOLYBDENUM PROTEIN) OF *AZOTOBACTER VINELANDII* NITROGENASE DURING REPRESSION AND DEREPRESSION

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SUMMARY

The purified, native iron–molybdenum protein (Component I) of *Azotobacter vinelandii* nitrogenase shows EPR signals at g values of 4.32, 3.65, and 2.01, when observed below 40°K. The resonances near $g = 4.32$ and 3.65 are unobscured when whole nitrogen-fixing cells are observed, and we have compared the appearance and disappearance of these resonances with nitrogenase activity as well as Component I protein determined immunochemically, during derepression and repression of nitrogenase. The characteristic resonances are absent in cells grown on ammonium salts. During derepression the activity, EPR signal, and cross-reacting material appear and pass through a maximum in parallel. During repression the immunochemically-detectable protein decays inversely with cell growth, while the activity and signal are depressed considerably more rapidly. This suggests that a short-term control mechanism, including the substantial alteration of the paramagnetic center of Component I without elimination of the antigenic moiety, is responsible for rapid repression of nitrogenase by ammonia.

EPR signals similar to those of Component I in Azotobacter were also observed at low temperatures in nitrogen-fixing cells of *Clostridium pasteurianum*, *Klebsiella pneumoniae*, and *Bacillus polymyxa*, suggesting that *in vivo* studies of Component I of nitrogenase may be feasible generally.

INTRODUCTION

NICHOLAS *et al.*[1] examined fractions from *Clostridium pasteurianum* and *Azotobacter vinelandii* and noted several EPR signals near $g = 2$, from cells grown under N_2-fixing conditions. Changes in these signals were seen in spectra of ammonia-grown cells. In the intervening period SHETHNA and co-workers[2–4] and DERVARTANIAN *et al.*[5] have described iron sulfur proteins and a flavoprotein from Azotobacter which on reduction yield signals in this spectral region. PALMER *et al.*[6] and HARDY *et al.*[7] have also described EPR signals near $g = 2$ from purified iron-sulfur proteins from *C. pasteurianum*. Some or all of these proteins may be electron carriers during nitrogen

fixation, but only recently have EPR spectra of purified nitrogenase components been reported. HARDY *et al.*[8] have detected EPR signals at g values of 4.33, 3.67, 2.01, and 1.94 in crystalline Component I of *A. vinelandii* nitrogenase, at 4°K. DALTON *et al.*[9] subsequently reported an EPR spectrum of purified Component I of *C. pasteurianum* nitrogenase.

The repression and derepression of the nitrogenase system in Azotobacter was studied by STRANDBERG AND WILSON[10], who reported growth rates of whole cells and activity of cell-free extracts. More recently SHAH *et al.*[11] have determined quantitatively the time-course of derepression and repression of the Azotobacter nitrogenase. The technique of growing cells on limiting ammonia[10,12] can be conveniently applied in large- or small-scale cultures with comparable results. It has also been used under conditions where active fixation of N_2 is impossible, under $He-O_2$ with wild-type organisms[10] and under air with mutants[13].

We have examined the EPR signals of whole cells of Azotobacter grown on N_2 and ammonium acetate over the range of temperatures from 4.2–100°K. In this communication we describe the changes in the signals near $g = 4.3$ and 3.6, observed at relatively low temperatures, during the course of repression and derepression, and we compare these observations on whole cells with results we obtained using crystalline iron–molybdenum protein obtained from N_2-grown cells. We also report the presence of similar signals, which we believe are due to Component I of nitrogenase, in nitrogen-fixing cells of *C. pasteurianum*, *Klebsiella pneumoniae*, and *Bacillus polymyxa*.

METHODS

A. vinelandii OP, *C. pasteurianum* W5, *B. polymyxa* (Hino), *K. pneumoniae* M5a1, were all obtained from the collection of P. W. Wilson. They were grown as previously described[10,14,15]. Details of derepression and repression are given by SHAH *et al.*[11].

Preparations of extracts[16] and chromatographic separation of the iron–molybdenum (Component I) and iron–protein (Component II) by the method of BULEN AND LECOMTE[17] was carried out with the modifications described by SHAH *et al.*[11]. Crystallization of the Component I was done by a modification (V. K. SHAH AND W. J. BRILL, unpublished result) of the method of BURNS *et al.*[18].

Assays for N_2 and acetylene-reduction were carried out as described by SHAH *et al.*[11]. Activities are expressed as 2-electron μequivalents (μequiv) transferred per min, per ml of packed cells or per mg purified Component I, measured at 30° with saturating levels of substrates and Component II. Small amounts of Component I were titrated against an excess of Component II to determine the specific activity of Component I. The most active preparations had an activity of more than 1.15 and 0.9 μequiv/min per mg protein of Component I and II, respectively. Activities in whole cells were estimated from the activities in extracts, assuming better than 95% breakage and equipartition of activity between supernatant fraction and pellet during centrifugation of the osmotic lysate (ref. 16; L. C. DAVIS, unpublished experiments). The highest observed activity was about 5 μequiv/min per ml packed cells (sedimented 6 min at 800 $\times$ g). One ml packed cells contains $1.4 \cdot 10^{11}$ cells and this is equivalent to 65 mg of extract protein when lysed by osmotic shock.

Antiserum to Component I was prepared in female New Zealand white rabbits 3 months old at the first injection. Four rabbits received subcutaneous injections each with 1 ml of emulsion made up of 0.5 ml (1.0 mg) of purified Component I in 0.025 M Tris–HCl buffer (pH 7.4), containing 0.25 M NaCl *plus* 0.5 ml of complete Freund's adjuvant. After 1 week, another 1 mg of purified Component I suspended in Freund's complete adjuvant was injected subcutaneously at 4 sites in each rabbit. Four more intravenous injections of 0.5 mg Component I in 0.025 M Tris–HCl buffer (pH 7.4), containing 0.25 M NaCl, were administered at weekly intervals. 5-ml blood samples were taken from the marginal ear vein 10 days after the final injection. The animals were bled by heart puncture 8 weeks after the first injection and the whole serum separated by centrifugation at 20000 × *g* for 20 min after clotting. The serum from each rabbit was collected separately and 0.1 mg/l merthiolate was added. Each rabbit's serum was divided into several 2.5-ml fractions so that freezing and thawing of the serum sample would not cause deterioration of an entire lot.

Quantitation of the levels of Component I present in extracts was done using Preer tubes[19]. Whole serum was diluted with an equal quantity of 4 M glycerol in 0.025 M Tris–HCl buffer (pH 7.4) and 20 μl layered into the bottom of a 4-mm (outer diameter) Preer tube. Over this was layered a 6-mm column (about 30 μl) of agar medium prepared with 0.025 M Tris–HCl buffer (pH 7.5), containing 0.9% NaCl, 0.02% NaN_3 and 1.5% Bacto purified agar. Appropriate dilutions of extract or purified Component I were layered on the agar and the tubes were sealed with plasticine. The positions of precipitin lines were determined at 48 and 72 h. Concentrations were estimated by comparison to dilutions of extract and purified Component I of known activity and protein concentrations.

Acrylamide-gel electrophoresis in the Ortec electrophoresis system was carried out on 8% cross-linked gel with 4% cross-linked streaking gel with 0.065 M Tris–borate buffer (pH 9.0) in the reservoirs and 0.025 M Tris–HCl buffer (pH 7.4) in the gels. The upper reservoir contained 0.3 mg/ml of dithionite. Iron containing bands were detected using bipyridyl (J. WESTPHAL, L. C. DAVIS, V. K. SHAH AND W. J. BRILL, unpublished results) and protein was stained with aniline black. Protein concentrations were determined by the method of LOWRY *et al.*[20].

EPR spectroscopy with 100 kHz field modulation was carried out at X-band essentially as previously described[21–23]. Portions of cell paste were suspended in an equal weight of 0.025 M Tris buffer, transferred to 4.8-mm (outer diameter) quartz tubes with a Pasteur pipette, and centrifuged 6 min at 800 × *g* in a clinical centrifuge to give a firmly packed pellet. The supernatant solution was removed with a pipette and the pellet was frozen in liquid N_2. Samples were stored under liquid N_2 until use.

Cell extracts and purified enzyme fractions were transferred to quartz tubes under Ar. The tubes were placed in a pyrex tube fitted with a rubber septum in the top and a ground-glass sidearm connecting to a vacuum-gas manifold. After evacuating and flushing 5 or 6 times, the sample was injected into the quartz tube through the rubber septum from a syringe fitted with a 22-gauge needle. While a brisk flow of Ar was maintained, the septum was removed and the quartz tube was withdrawn with a fine tapered rubber stopper. The sample was then frozen in liquid N_2 and stored under liquid N_2 until use. Experiments in which material was recovered from the tubes, after EPR spectra were taken, indicated that this arrangement excluded O_2 sufficiently to preserve the activity of extracts and enzyme fractions. More elaborate

apparatus (*cf.* ref. 24) is required for experiments in which the oxidation state of purified components is adjusted, and this will be described in a subsequent publication.

RESULTS AND DISCUSSION

Purified Component I

Purified Component I of Azotobacter nitrogenase was examined at several temperatures from 4.2 to 40°K. Fig. 1 shows EPR spectra from a sample observed at 4.2 and 13°K. Three prominent spectral features can be seen at $g = 4.32$, 3.65, and 2.01. Addition of sodium dithionite to Component I did not produce additional resonances nor did it alter the signal already present (*cf.* ref. 8). At 13° one could discern indications of other smaller signals on either side of the $g = 2.01$ resonance

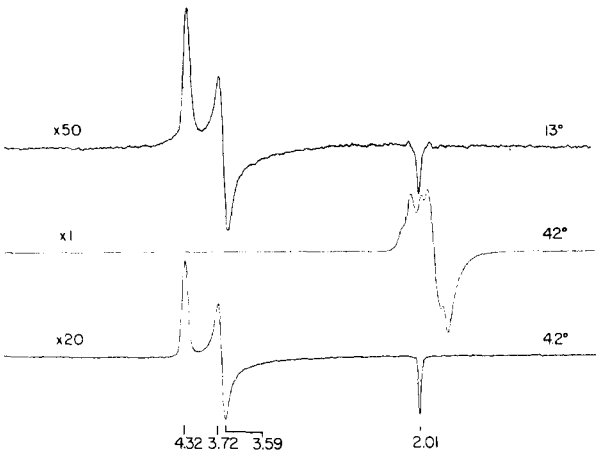

Fig. 1. EPR spectra of purified *A. vinelandii* nitrogenase Component I as well as reduced *C. acidi urici* ferredoxin. The ordinate is a linear function of dX''/dH (ref. 24); the abscissa is linear in applied magnetic field. The corresponding g values at several field positions are marked along the abscissa. Top: Component I, 7 mg/ml in 0.25 M NaCl in 0.025 M Tris–HCl buffer (pH 7.4); temperature, 13°K; microwave power, 2.7 mW; relative instrument gain, 50. Middle: *C. acidi-urici* ferredoxin[27], 0.82 mM in 0.1 M Tris–HCl buffer (pH 8), reduced with excess sodium dithionite; temperature, 4.2°K; microwave power, 0.27 mW; gain, 1. Bottom: Component I as in top spectrum, but at 4.2°K; microwave power, 0.27 mW; gain, 20. Other conditions of EPR spectroscopy were: modulation amplitude, 6 gauss; time constant, 0.25 sec; field sweep rate, 1000 gauss/min; klystron frequency, 9100 MHz.

but we could not elicit these in more definite form by alterations of power or temperature. They may represent small amounts of contaminating iron–sulfur proteins, and a similar phenomenon may explain the observation of signals near $g = 1.94$ in addition to the $g = 2.01$ resonance, reported by others[8,9]. To illustrate the possibilities in this direction we have included in Fig. 1 a spectrum of reduced ferredoxin from *C. acidi-urici*[25,26] which because of its width and complexity also shows how the region near $g = 2$ can be obscured in spectra from whole cells when ferredoxins and other iron–sulfur proteins are present. An additional complication is provided by the fact, also reported by others[8,9,27], that O_2 denaturation of Component I leads to disappearance of the signals shown, and the appearance of a large narrow signal at

$g = 2$, as well as a signal near $g = 4.3$. This latter signal is of a type found in compounds containing high-spin iron in a rhombic site (*cf.* ref. 28). Partial O_2 inactivation of Component I during preparation can, of course, contribute spectral features of this type, further complicating the interpretation of the properties of less pure preparations. We will report later our studies now in progress, on the quantitation of unpaired spins, oxidative titrations, and the interaction of the protein with other components of the nitrogenase reaction.

We have also examined frozen suspensions of crystals of Component I, and find only a weak signal of the type shown in Fig. 1, compared to the expected size of the signal based on the protein present. Solution of the crystals yielded a normal sized spectrum expected from the corresponding protein concentration. Whether these observations are an artifact due to an unfavorable position of the crystals in the microwave field caused by fractionation of the suspension during freezing, or whether they indicate a difference between the crystal and the dissolved states of the protein, we are at present unable to say.

The nature of the center responsible for the EPR signal is not yet clear[8,9,27] although the large anisotropy of the g values and the departure of the average g value from the free-electron value certainly suggest a transition metal complex containing the iron and/or the molybdenum atoms found in this protein. The reported magnetic susceptibility results on Component I of Azotobacter (3 Bohr magneton per iron atom[8]) are consistent with a number of schemes involving isolated atoms, some in the high-spin and some in the low-spin state, and/or clusters of the type now thought prevalent in ferredoxins[29].

Observations on whole cells—general features

Whole cells of *A. vinelandii* exhibit readily observable EPR signals near $g = 4.3$

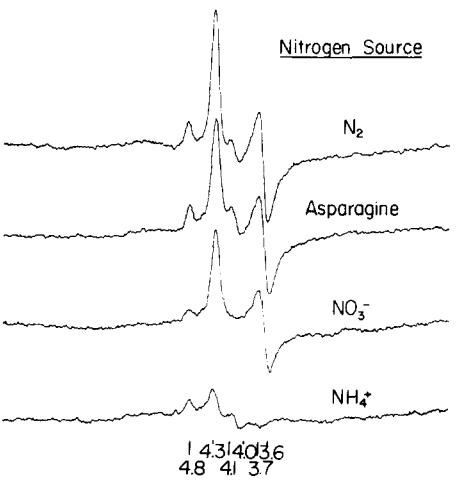

Fig. 2. EPR spectra of *A. vinelandii* cells grown under air with the following additions (quantities are 400 μg/ml ammonia-N equivalents): Top spectrum, no addition; second spectrum, L-asparagine; third spectrum, potassium nitrate; bottom spectrum, ammonium acetate. The samples were prepared as described in the text. Conditions of EPR spectroscopy were, for all spectra: temperature, 13° K; microwave power, 2.7 mW; instrument gain, the same for all samples; modulation amplitude, 6 gauss; time constant, 0.5 sec; field sweep rate, 1000 gauss/min; klystron frequency, 9100 MHz; presentation of spectra, as in Fig. 1.

when grown on N_2 as the sole source of nitrogen and subsequently examined at temperatures below 40° K. Fig. 2 depicts the signals seen at $g = 4.3$ and 3.65 in packed Azotobacter cells thus prepared. In confirmation of previous studies[30,31] we have found that there is no detectable nitrogenase synthesis so long as there is an adequate supply of ammonia in the medium. It is to be expected that when cells are grown on poor nitrogen sources such as nitrate or asparagine, the nitrogenase is derepressed to some extent. Fig. 2 also shows the signals obtained from cells of Azotobacter grown in the presence of air as well as the indicated amounts of asparagine, potassium nitrate, or ammonium acetate. As evidenced by the major EPR signals at g is 4.3 and 3.65, the presence of asparagine and nitrate at these levels does not fully repress Component I synthesis, whereas the absence of signal intensity near $g = 3.65$ in the ammonia-grown cells suggests that Component I synthesis is fully repressed under these conditions in agreement with previous work[30,31]. The remaining signal at $g = 4.3$, as well as the smaller signals near 4.8 and 4.0, may be associated with iron compounds[28] other than nitrogenase. The absence of the small signal near $g = 4$ in spectra from nitrate grown cells is at present unexplained.

We examined cells of *C. pasteurianum*, *K. pneumoniae*, and *B. polymyxa*, comparing the EPR spectra at 13° K from cells grown in the presence of ammonia as well as cells grown under conditions where nitrogen fixation was taking place. The apparent g values for what we believe is the nitrogenase Component I in these organisms, present when the cells fix nitrogen, are given in Table I. The values for *C. pasteurianum* agree with those of purified Component I (M.-Y. TSO AND W. H. ORME-JOHNSON, unpublished results). For these organisms, as for Azotobacter, a number of other resonances obscure the expected third resonance at $g = 2$, when whole cells are examined.

TABLE I

PROMINENT LOW-FIELD EPR RESONANCES IN N_2-GROWN ORGANISMS

The organisms were grown as described under METHODS on minimal medium unsupplemented with combined nitrogen. Appropriate controls were grown with added ammonium salts and examined for EPR. g values were measured with a proton NMR gaussmeter and klystron frequency counter[24].

Organism	*g value of EPR extrema*
A. vinelandii OP	4.34; 3.71, 3.60*
C. pasteurianum W5	4.29; 3.85, 3.70*
K. pneumoniae M5a1	4.32; 3.69, 3.57*
B. polymyxa (Hino)	4.37; 3.62, 3.43*, **

* Minima; *cf.* Fig. 1.

** This spectrum contains additional prominent peaks at $g = 4.94$ and 3.75.

We concluded from these studies that EPR signals in this spectral region would serve as useful indicators of the levels of Component I of nitrogenase, particularly since the resonance near $g = 3.65$ is relatively free of interference from the residual signals found with fully repressed cells, and we proceeded to examine the time-course of appearance and disappearance of this center during repression and derepression of nitrogenase as previously described[11].

Derepression and repression of nitrogenase

SHAH *et al.*[11] have devised precise methods for quantitation of activity in extracts during derepression and repression of nitrogenase activity. The determination of acetylene reduction is sensitive to activities of 1 nmole/30 min which is about 1/10000 the fully derepressed activity. Reliability of this measurement on different extracts is only about ± 5 % because of variations in breakage rate, extract volume, as well as possible O_2 inactivation. N_2 reduction assays are 1/20 as sensitive and may be considerably less accurate because of relatively high levels of free ammonia in extracts, which give large blank values. The immunodiffusion technique with Preer tubes is sensitive to about 1 % of the fully derepressed level of Component I but accuracy is lowered by the necessity of measuring small changes in precipitin band position. Errors of ± 5 % in a dilution series are not uncommon. The advantage of the Preer-tube estimation of iron–molybdenum protein is that it does not require active enzyme since the reaction is carried out aerobically and oxygenation of an extract does not change the precipitin pattern. The accuracy of EPR measurements, assuming the use of calibrated tubes, good temperature control, and careful positioning within the EPR cavity, may be better than ± 5 % (ref. 24). The precision of measurements on a given sample from day to day were found to be ± 3 % or better. EPR

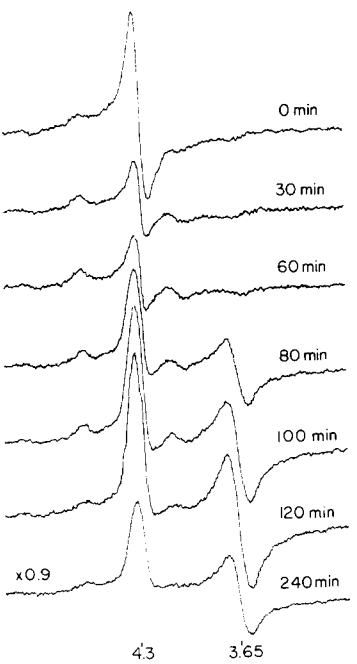

Fig. 3. EPR spectra of *A. vinelandii* cells grown under air in the presence of ammonium acetate (90 μg/ml ammonia-N) and subsequently allowed to deplete the combined nitrogen to a level where derepression of nitrogenase took place. Time zero is defined as when ammonia is exhausted from the medium. The times at which samples were taken are marked along the right side of the figure. Preparation of samples, presentation of spectra, and conditions of EPR spectroscopy were the same as in Fig. 2, except that the sweep rate was 400 gauss/min, and the bottom spectrum was recorded at 0.9 times the gain of the other spectra.

spectroscopy is thus comparable in accuracy to the other methods of evaluation of nitrogenase Component I, and the sensitivity (1 % of fully derepressed levels of nitrogenase in whole cells is detectable) is adequate for the present purposes.

Fig. 3 shows the low-field signals of whole cells before and after derepression. The earliest timepoint corresponds to a level of ammonia-N in the growth medium of 24 μg/ml, which is insufficient to repress nitrogenase in continuous cultures[30] but which is sufficient to maintain repression in batch cultures[10].

The signal observed at $g = 4.3$ is clearly different from that at $g = 4.3$ in N_2-grown cells (Fig. 2) in its temperature dependence and saturation with power. The signal seen at the outset of derepression also has a much greater intensity than the $g = 4.3$ signal seen in cells grown with excess ammonia at the same cell density. It may in part represent an iron uptake or binding material in cells depleting their source of combined nitrogen. The presence of this signal in cells during derepression makes ambiguous the interpretation of spectra obtained at only one temperature. We therefore have examined the behavior of the signal as a function of temperature and power. There does not appear to be significant interference by other signals with the portion of the Component I signal observed at $g = 3.65$, when the temperature was varied from 4.2 to 27° K and the power was varied from 3 to 3000 μW at 4.2°. We have therefore used the relative size of the $g = 3.65$ signal as a measure of the nitrogenase activity in the cells and we have compared these determinations with the activity obtained in extracts and with Preer-tube quantitation of the concentration of the Component I in extracts. The results of this comparison are summarized in Table II. There appears to be reasonable agreement between the several measures of activity.

The rise and fall in the levels of nitrogenase activity found in extracts at long times after derepression appear to be quite real. We are unable to distinguish between several possible causes: (1) Derepression may lead to a rapid increase in nitrogenase activity which sufficiently raises the level of ammonia internally to subsequently

TABLE II

DEREPRESSION OF NITROGENASE IN AZOTOBACTER

All values are expressed relative to the activity of the 120-min sample. This corresponds to approx. 4.8 μequiv (2-electron change) substrate reduced per min per g cells, based on the average of acetylene and nitrogen reduction activities. The extract at 120 min showed an activity of 68 nequiv/min per mg protein, assayed as described by SHAH *et al.*[11]. Cross-reacting material was determined by serial dilution in Preer tubes.

Time (min)	*Acetylene reduction*	*Nitrogen reduction*	*Cross-reacting material*	*EPR signal**
0	0	0	0	0
30	0	0	0	0
60	5.6	5.6	5	5.5
80	34	42	43	43
100	79	82	75	76
120	100	100	100	100
180	90	97	85	108
240	79	75	73	69

* Peak to peak amplitudes of $g = 3.65$ signal.

partially delay synthesis of further material. (2) The high cell density at later times may lead to changes in physiological state of the cells, principally due to changes in O_2 tension in the culture (J. L. PATE AND W. J. BRILL, unpublished observation). (3) The specific activities could vary by initial depletion of the soluble protein pool and subsequent re-enrichment, giving an artifactual increase in specific activities. Other experiments, such as the repression experiment shown in Table III, suggest that the first explanation is the most likely, since steady growth on N_2 to the same cell density as found in the early times of derepression yields extracts with specific activities no higher than those found at the latest point in the derepression experiment. Cultures grown in small baffled flasks on N_2 have activities like those at time zero of repression, as do cells obtained from 60-l fermenters.

TABLE III

REPRESSION OF NITROGENASE IN AZOTOBACTER

Activities are all expressed relative to the activity of the 0-h samples, as determined at optimum activity as described by SHAH *et al.*[11]. The 0-h sample had an activity of 3.2 μmoles electrons transferred per min per g cells based on the average of acetylene and nitrogen reduction. The extract of this sample had a specific activity of 45 nmoles/min per mg protein. Cross-reacting material was determined by serial dilution in Preer tubes.

Time (min)	*Dithionite oxidation*	*Acetylene reduction*	*Nitrogen reduction*	*Cross-reacting material*	*EPR signal* *
0	100	100	100	100	100
60	60	64	76	73	67
120	40	41	37	60	42
180	26	24	32	50	25
240	10	8.5	15	40	10
300	3	4.5	4	33	4.5

* Peak to peak amplitude of $g = 3.65$ signal.

In contrast to the results obtained during derepression of the nitrogenase system where all the measures of activity were in agreement, the results obtained during repression showed a marked difference (Table III). Activity as measured by dithionite oxidation, acetylene reduction or nitrogen fixation, fell rapidly after the addition of ammonia to the growth medium[11]. The parallel fall of all the activities indicated that the repression did not lead to specific inhibition of the ability to use reducible substrates but affected all the measurable functions of the nitrogenase. The EPR signal (Fig. 4) fell in direct proportion to the activity measured in these three ways.

The loss of the EPR signal in parallel to activity after repression suggests that the paramagnetic center being observed is present only in the active species of Component I of nitrogenase. After 5 h there was a 95 % loss of activity, although less than two generations had elapsed. The cross-reacting material, when detected by Preer tube dilutions, fell at a much slower rate which was completely consistent with simple dilution. It appears then, that during repression of nitrogenase there is a rapid mechanism of Component I inactivation, including alteration of the paramagnetic site near $g = 4$, followed by a slower process in which the protein portion is finally broken down and reutilized. The most obvious mechanism by which the rapid in-

activation would occur is O_2 denaturation (*cf.* refs. 21 and 30), but these studies do not permit us to say if this happened. SHAH *et al.*[11] presented evidence that the loss of activity was not caused by destruction of Component II alone, although it is known to be more O_2 sensitive than Component I. J. WESTPHAL, L. C. DAVIS, V. K. SHAH AND W. J. BRILL (unpublished observations) have observed changes in the electrophoretic mobility of the iron-containing band corresponding to Component I when the extract is subject to oxidizing conditions. A similar change occurs after long times of repression (more than 0.5 generation) suggesting that the nitrogenase system is no longer protected against O_2 after repression of synthesis.

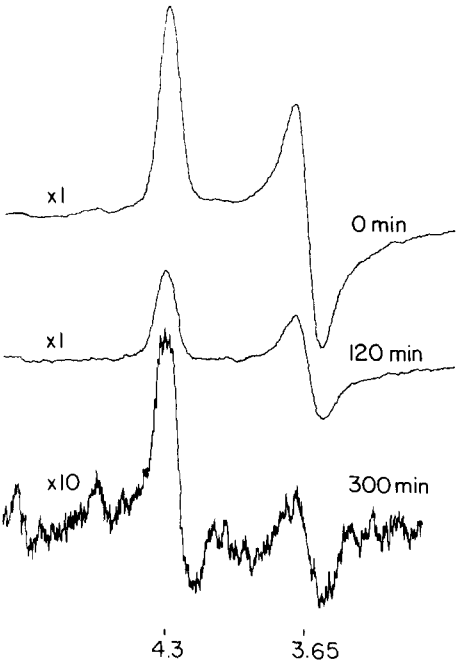

Fig. 4. EPR spectra of *A. vinelandii* cells grown in the presence of air under N_2-fixing conditions to a turbidity of $3.7 \cdot 10^8$ cells/ml, after which ammonium acetate (130 μg/ml N) was introduced to repress nitrogenase. The times after addition of ammonia are noted along the right-hand edge of the figure, and the instrument gains utilized are marked along the left-hand edge. Preparation of samples, presentation of spectra, and conditions of EPR spectroscopy were as noted in the legend to Fig. 3.

The EPR signals described here do not appear to require the presence of Component II. The isolated, purified Component II does not appear to have any signals in the low-field region[32] that can be conveniently detected in whole cells and we can thus say nothing on the basis of EPR about the appearance and disappearance of Component II during derepression and repression. Determination of activities in extracts previously described[11] indicate that the two components appear and disappear in parallel, within the limits determined by our assay systems. The only exception to this might be a mutant such as UW10 which lacks Component I activity[13], but which possesses Component II.

Studies of changes in the signal of Component I as a function of oxidation state

and during *in vitro* nitrogen fixation, as well as further studies on the component under various physiological conditions *in vivo* are all obvious potential sequels to these findings. Although the signal, or a similar one, is present in other N_2-fixing organisms examined, interpretation of spectra awaits purification of Component I from these sources since the high-field resonances are not easily resolvable in whole cells. Examination of the EPR properties of Azotobacter mutants unable to fix nitrogen[13] in particular should lead to identification of classes of mutants having Component I but deficient in some other part of the system, or with a partially effective, modified Component I.

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