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Repression, Derepression and Activation of Nitrogenase in *Azotobacter vinelandii*

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Abstract

Ammonium ions repressed nitrogenase in cells fixing N_2 gas. Immunological tests and electrophoresis in various gels show that component I (Fe–Mo–S protein) was completely repressed by ammonium, whereas component II (Fe–S protein) apoprotein was not markedly affected. Component II from ammonium-grown cells, however, was inactive since it did not cross react with component I to reduce C_2H_2 to C_2H_4. The inactive component II apoprotein is immunologically identical to its active counterpart from cells fixing N_2. Identical protein patterns were also observed in various gel-electrophoresis systems. Oxygen-inactivated component II may be reactivated with $FeSO_4$. This salt is preferable to ferrous ammonium sulphate which inactivated component I. Immunodiffusion under aerobic conditions shows that purified component I is composed of aggregated and non-aggregated forms which are antigenically distinct. The aggregate was dissociated by treatment with sodium dodecyl sulphate (SDS) into a single antigenic species which was further resolved into two subunits on SDS disc polyacrylamide gel electrophoresis.

Introduction

Ammonium ions, when added to culture media, repress the synthesis of nitrogenase in various bacteria (Daesch and Mortenson 1972) even in amounts as low as 2 μg N/ml (Patil *et al.* 1967). The nitrogenase enzyme system is not synthesized until all ammonium is depleted from batch cultures of the bacteria (Mahl and Wilson 1968; Strandberg and Wilson 1968). On the other hand, very small amounts of ammonium appeared to stimulate the production of the nitrogenase enzyme complex when cells were grown in a chemostat (Daesch and Mortenson 1967; Dalton and Postgate 1969). Shah *et al.* (1972) found that the synthesis of both the Fe–Mo–S protein (component I) and the Fe–S protein (component II) in *Azotobacter* after exhaustion of ammonium from the media occurred together, as did their degradation on adding ammonium. Gadkari and Stolp (1974) showed that ammonium sulphate repressed nitrogenase in *Azotobacter* and on the depletion of ammonium in the medium the enzyme was resynthesized following a diauxic lag period of 30 min.

Immunological techniques were used by Bishop *et al.* (1975) to detect the presence of nitrogenase components in *Rhizobium japonicum* grown under various conditions. Similar immunological evidence was the basis of the demonstration by Shah *et al.* (1973*b*) that cross-reactive material to one or other component was present in certain non-N_2-fixing mutants of *Azotobacter*.

We suggest on the basis of immunodiffusion and polyacrylamide gel electrophoresis that ammonium repression of nitrogenase primarily affects component I. Although

component II apoprotein was detected by these techniques in cells grown with ammonium, it was not effective in reducing C_2H_2 when combined with component I from N_2-grown cells. Oxygen-inactivated component II may, however, be reactivated by incubating it with ferrous salts. These results amplify a preliminary report (Deering and Nicholas 1974).

Methods

Culturing the Bacterium

Cultures of *Azotobacter vinelandii* strain 0 were grown in a modified Burk's medium in 10-litre containers and vigorously sparged with sterile air. When the cultures attained an optical density of 0·42 at 650 nm, sterilized NH_4Cl was added to a final concentration of 0·3M. The ammonium content of the cultures was monitored by the Markham distillation method. Aliquots of the cultures (1–2 litres) were withdrawn and harvested at intervals both before and after the addition of NH_4Cl, as indicated in Fig. 4. Cells were collected in a Sorvall RC2B refrigerated centrifuge at 2°C and 7500 *g* for 10 min. Growth of the cultures was monitored throughout by measuring the optical density at 650 nm in a Shimadzu spectrophotometer.

Preparation of Cell Extracts

The cells were washed in 0·025M tris–HCl buffer (pH 7·5), resuspended in the same buffer, and passed through a French Pressure Cell (40-ml capacity) at 2°C with an Aminco motor-driven press at 200 MPa. The homogenate was centrifuged in a Spinco model L ultracentrifuge at 150 000 *g* for 30 min and the supernatant fraction (S105) containing 35–40 mg protein/ml was used as crude extract.

From this stage onwards, all preparative operations were conducted under strictly anaerobic conditions using a continuous flow of high-purity N_2. This gas had been further purified from the commercially available product by passing it through a solution of 10% (w/v) pyrogallol in 20% (w/v) KOH. Buffers and other solutions were vigorously sparged with this O_2-free N_2. Cellulose and Biogel chromatography columns were continuously sparged with this gas and fractions were collected and sealed in special specimen tubes under N_2 (see below for details).

Purification of Nitrogenase Components I and II

The fractionation scheme for components I and II from crude extract to separated purified proteins is shown in Table 1.

Components I and II were co-purified as far as fraction 3, and separation from each other was achieved at the anion-exchange step in fraction 4. Component I at the fraction-4 stage was completely free of component II activity, but component II at the fraction-4 stage always contained some contaminating component I activity. Hence, further fractionation on Biogel P200 was necessary to completely remove component I activity from component II (fraction 5). Passage of separated component I on Biogel P200 resulted in removal of traces of other contaminant proteins.

The first three steps in the purification procedure shown in Table 1 are common to both components I and II. The S105 fraction was treated with cetyltrimethylammonium bromide (CET) according to the method of Moustafa (1970). Thus, S105 was made to 4% (w/v) CET with respect to its protein content and then the nucleic acid precipitate formed was centrifuged at 105 000 *g* for 30 min. The supernatant fraction was made to 10% (w/v) CET to precipitate the nitrogenase complex (components I and II) which was collected by centrifugation at 20 000 *g* for 10 min. To this sedimented fraction was added purified yeast RNA, prepared according to the method of Ralph and Bellamy (1964) (38 mg/g protein), contained in 0·025M tris–HCl buffer (pH 7·5) with 2 mM dithiothreitol. Stirring was continued in an anaerobic environment under N_2 for 60 min. The CET–RNA complex was centrifuged at 105 000 *g* for 15 min, leaving the nitrogenase components I and II in the supernatant (fraction 2). This fraction was heated at 60°C for 10 min and, after cooling, was centrifuged at 105 000 *g* for 15 min to remove the precipitated denatured protein. Components I and II remained in the supernatant (fraction 3).

Fraction 3, containing 15–20 mg protein/ml, was applied to a 2·0 by 15 cm column of DEAE-cellulose 32 equilibrated with 0·025M tris–HCl buffer (pH 7·5) which had been sparged with N_2 to remove air and containing 2 mM dithiothreitol and 2 mM sodium dithionite. The column was first eluted with the buffer and then successively eluted with 150-ml volumes each of 0·125, 0·25 and 0·35M

NaCl contained in the O_2-free buffer. The eluted protein fractions were monitored via a flow-through cell in an LKB Uvicord II absorptiometer unit at 280 nm. The appropriate fractions were collected in specimen tubes under O_2-free N_2 (fraction 4). Component I (fraction 4) was completely free of component II activity, but component II (fraction 4) contained a small contaminant of component I activity.

Components I and II separated and eluted from the DEAE-cellulose 32 column were each concentrated by ultrafiltration under O_2-free N_2 (PM 10 Amicon membrane). Each component was then chromatographed separately on Biogel columns (2·0 by 25 cm Biogel P200, mesh 100–200), kept under strict anaerobic conditions by sparging with N_2. The columns were first equilibrated with O_2-free 0·025M tris–HCl buffer (pH 7·5) containing 2 mM each of dithiothreitol and sodium dithionite, and 0·1M NaCl in the case of component I, which was chromatographed on two separate columns. The components were eluted with the same buffer at a flow rate of 5 ml/h and the elution patterns of the proteins followed in a Uvicord unit at 280 nm. Fractions of the appropriate protein peaks were collected in closed specimen tubes (fractions 5) under N_2.

Assay for C_2H_2 Reduction

Assay mixtures were contained in 10-ml conical flasks fitted with Quickfit necks (C14) and with single sidearms with an entry port each fitted with a Suba-seal (Freeman, Yorks., England). The enzyme was injected through the Suba-seals into the sidearm by means of gas-tight syringes. Quickfit taps connected the flasks to the six-place manifold which was connected to the evacuation and gassing system as described by Elleway *et al.* (1971). The assay mixture, in 0·40 ml final volume, contained (in μmol): creatine phosphate 15; ATP 2; $MgCl_2$ 4; tris–HCl buffer 12·5; NaOH 2·5 (to neutralize the $Na_2S_2O_4$) and creatine kinase 0·125 mg. The flasks were evacuated and flushed with a gas mixture containing 5% C_2H_2 and 95% helium using the apparatus described by Elleway *et al.* (1971). The reaction was started by injecting, from gas-tight syringes, first 5 μmol $Na_2S_2O_4$ [freshly prepared in the tris–HCl buffer (pH 7·5) sparged with O_2-free N_2], which was mixed into the reaction mixture and equilibrated at 30°C, and then the enzyme (usually 0·05 ml under anaerobic conditions), which was also thoroughly mixed into the system. The flasks were then incubated in a reciprocating water bath at 30°C for 10 min. The reaction was stopped by injecting 1 ml 10% (w/v) trichloroacetic acid into the flasks. Ethylene formation was determined in a Philips 4000 series gas chromatograph fitted with Poropak R columns (2 m by 2 mm) at 75°C with a flow rate of 24 ml carrier N_2/min. The specific activity of the enzyme is expressed in nanomoles of C_2H_2 reduced per milligram of enzyme per minute.

Electrophoresis

Polyacrylamide disc gel. The technique used was a modification of the procedure of Williams and Reisfeld (1964). When dissociating conditions were used, stacking and separating gels and electrode buffer solutions contained 1·0% (w/v) sodium dodecyl sulphate (SDS). Samples were either heat-dissociated at 100°C in the presence of 1·0% (w/v) SDS and 1·0% (v/v) β-mercaptoethanol for complete dissociation as described by Schnaitman (1973), or dissociated with the same reagents at 30°C. Gels were stained with Coomassie Brilliant Blue R250 and destained in a water–acetic acid–methanol solution (45 : 10 : 45 v/v) at 37°C.

Polyacrylamide slab gel. The separation of proteins by polyacrylamide slab gel electrophoresis was carried out as described by Reid and Bieleski (1968).

Starch gel. Separation of proteins was made in 12·5% (w/v) starch gel in 0·1M tris–citrate buffer (pH 8·1) at 10 V/cm for about 2 h. The purification of components I and II in the various fractions was followed by this technique.

Protein Determination

Trichloroacetic acid (10% w/v) was used to precipitate proteins in the various fractions since tris–HCl buffer, dithiothreitol and sodium dithionite would interfere with the assay. The protein was dissolved in 10% (w/v) NaOH and the microbiuret procedure of Itzhaka and Gill (1964) was followed.

Production and Purification of Antibody

The components I and II used as immunogens were the fractions 5 of Table 1. The criteria of purity for each of the components were single-band homogeneity on electrophoresis in starch gels and complete freedom from contamination by the other component on the basis of enzyme activity.

For the immunodiffusion studies in the repression–derepression experiments, antibodies to components I and II were raised in mice. This was done by intraperitoneal administration of 50 μg of each antigen emulsified in Complete Freund's Adjuvant once weekly for a period of 3 weeks. A second series of once-weekly intraperitoneal injections of 100 μg antigen, also in Complete Freund's Adjuvant, was administered for 3 weeks. The animals were bled from the retro-orbital plexus via a pipette.

The dissociation studies on component I used antibody raised in rabbits. This was elicited by subcutaneous injections at four sites on a rabbit's back of 2 mg component I emulsified in Complete Freund's Adjuvant once weekly for 2 weeks. This procedure was followed by a series of once-weekly intravenous injections of 1 mg component I for a 3-week period. The rabbits were bled from the marginal ear vein.

The IgG-rich fraction of the antiserum was prepared by a neutral salt fractionation technique, a modification of the method described by Deutsch (1967). Dry Na_2SO_4 was added to whole serum to yield 18% (w/v) Na_2SO_4. The precipitate obtained by centrifugation at 12000 *g* was dissolved in 0·025M tris–HCl buffer (pH 7·5) to the original serum volume. The precipitation and resuspension procedure was repeated twice using successively lower Na_2SO_4 concentrations [16% and then 14% (w/v) Na_2SO_4]. The final [14% (w/v) Na_2SO_4] resuspension was stored in small aliquots at −15°C. This was the IgG-rich antibody used in immunodiffusions in agar gels.

Immunodiffusion

Immunodiffusion experiments were carried out by using a modification of the method of Ouchterlony (1949). The 10-cm diameter Petri dishes contained 0·65% (w/v) Noble purified agar, 0·85% (w/v) NaCl, and 0·02% (w/v) sodium azide in 10 ml 0·025M tris–HCl buffer (pH 7·5). Wells 3 mm in diameter were placed 6 mm (centre to centre) from a central well in a circular arrangement. The central well contained antiserum (10 μl) and peripheral wells received either crude extract or purified component I or II (also 10 μl). Antigen solutions were usually diluted [also with 0·85% (w/v) NaCl] to a concentration sufficient to yield a precipitin line. Gels were stored in humid atmosphere at 30°C for 48 h to allow precipitin development. Diffusion patterns were clarified by saline washing, drying and staining the gels with Coomassie Brilliant Blue R250.

Quantitation of Component I and II Proteins in Crude Extracts by Immunodiffusion (Repression and Derepression Experiments)

Titres of component I and II proteins present in individual crude extracts were determined by an end-point dilution method. A series of twofold dilutions of crude extract (10 μl) were placed in neighbouring wells on an agar gel layer and assayed *versus* IgG mouse antibody (10 μl) placed in a parallel row of wells. The end-point titre for each component was arbitrarily chosen as the highest dilution yielding a clearly recognizable precipitin arc not encroaching into the wells containing antigen.

Reactivation of Component II

Reactivation procedures for component II using ferrous salts are described in the legends of the appropriate figures. Inactivation of component II was achieved by exposure to air for 1 h unless otherwise stated.

Chemicals

Creatine phosphokinase, dithiothreitol, SDS and β-mercaptoethanol were purchased from Sigma Chemical Co., St. Louis, U.S.A.; CET from Fluka, Switzerland; DEAE-cellulose was Whatman 32. Hydrolysed starch for gel electrophoresis was from Connaught Medical Research Laboratories, Canada. Creatine phosphate was prepared from creatine (B.D.H. Chemicals Ltd, Poole, Dorset, U.K.) by the method of Ennor (1957). High-purity nitrogen, acetylene and helium gases were obtained from Commonwealth Industrial Gases Ltd, Australia. Acrylamide was purchased from Eastman Organic Chemicals, Rochester, New York, U.S.A.; *N,N'*-methylenebisacrylamide from B.D.H.; *N,N,N',N'*-tetramethylethylenediamine from Canalco, Rockville, Maryland, U.S.A. Ferrous ammonium sulphate was from May and Baker Ltd, Dagenham, England; ferrous sulphate from Ajax Chemical Co., Sydney, Australia, and sodium dithionite from B.D.H.

Results

Identification of Nitrogenase Components I and II

Both starch and polyacrylamide gel electrophoresis and immunodiffusion techniques were used to detect components I and II in extracts of cells that were fixing N_2 and in those of cells transferred to media containing ammonium, and vice versa.

Table 1. Purification of components I and II

Purification was carried out as described in Methods. The activity of component I was assayed at each step in the presence of a saturating amount of purified component II (fraction 5) as determined by titration, and vice versa for assay of component II activity

Fraction	Total protein (mg)		Total units (nmol C_2H_2 reduced/min)		Specific activity (units/mg protein)		Purification		Recovery (%)	
	I	II	I	II	I	II	I	II	I	II
1	8400	8400	733260	260040	87·2	30·9			100	100
2	1130	1130	659930	195030	582	171	6·6	5·6	90	75
3	792	792	630600	182030	796	229	9·1	7·4	86	70
4	156	78	549940	119620	3530	1530	40	49	75	46
5	82	24	505940	65010	6170	2692	70	87	69	25

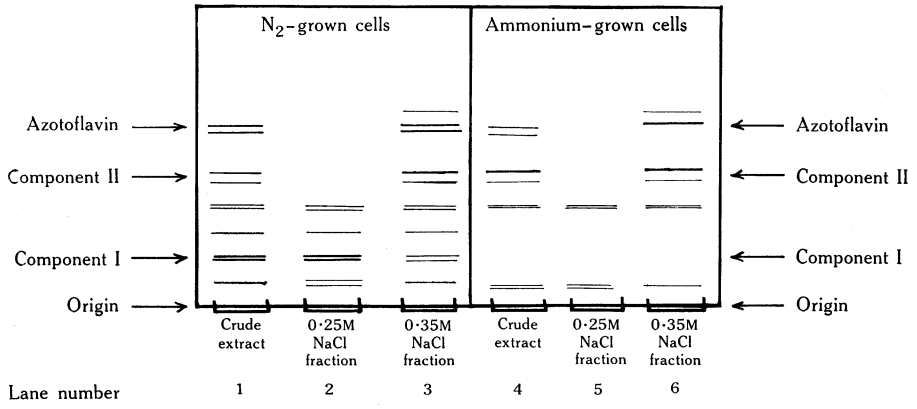


Fig. 1. Starch gel electrophoresis of components I and II in crude extracts and in fraction 4 (Table 1). A comparison is made between cells fixing N_2 and those grown with ammonium. Lane loadings: 1 and 4, 200 μg; 2 and 5, 140 μg; 3 and 6, 150 μg.

Electrophoresis. Components I and II were purified from N_2-fixing cells, as shown in Table 1. The purified fractions were resolved in starch gel as shown in Fig. 1. These served as markers ln subsequent work. Thus, component I was not found in cells grown with ammonium, but component II was detected and, when purified by the same procedure used for cells fixing N_2, it had an identical mobility in starch gel to purified component II from N_2-grown cells (Fig. 1). Similar results were obtained with polyacrylamide gels as shown in Fig. 2.

Ouchterlony immunodiffusion. Both components I and II were identified in crude extracts or in purified preparations by their reactions with specific antisera. The specificity of the antiserum for component II is illustrated in Fig. 3. This antiserum

did not react with either component I or azotoflavin. The non-reaction between normal serum and component II confirms that normal serum does not contain cross-reacting antibody (Fig. 3). The antiserum elicited against component I was similarly used to identify it in cell extracts.

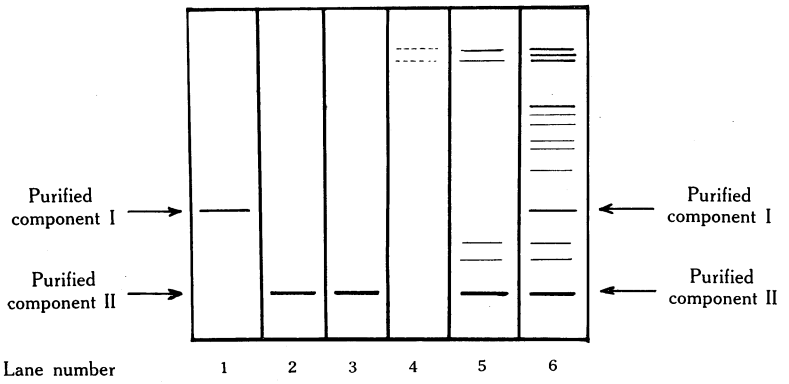


Fig. 2. Polyacrylamide slab gel electrophoresis of crude extracts and purified components I and II (fraction 5, Table 1) prepared from cells utilizing N_2 and from cells grown with ammonium. Lane 1, component I (N_2-grown cells); lane 2, component II (N_2-grown cells); lane 3, component II (ammonium-grown cells); lane 4, component I (ammonium-grown cells); lane 5, crude extract (ammonium-grown cells); lane 6, crude extract (N_2-grown cells). Lane loadings: 1–4, 20 μg; 5 and 6, 200 μg.

Repression of Nitrogenase by Ammonium

In this experiment the changes that occur in N_2-grown cells on the addition of NH_4Cl to the medium in terms of C_2H_2 reduction and the occurrence of components I and II in cell extracts by immunodiffusion are illustrated in Fig. 4. The addition of ammonium resulted in an increased growth rate and a rapid decline in C_2H_2 reduction

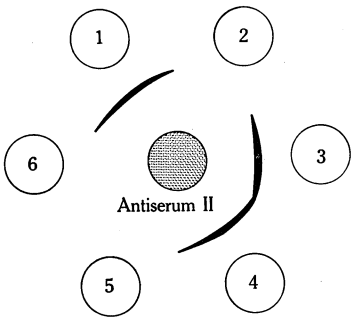


Fig. 3. Diagram of the antigen–antibody precipitin reaction for component II in agar gel. The centre well contained antiserum specific for component II (fraction 5, Table 1). Peripheral wells contained the following antigens (with loadings): 1, crude extract of N_2-fixing cells (200 μg); 2, purified component I (fraction 5, Table 1) (10 μg); 3, crude extract of ammonium-grown cells (200 μg); 4, purified component II (fraction 5, Table 1) (7 μg); 5, normal mouse serum (300 μg); 6, purified azotoflavin (6 μg).

in extracts of cells harvested at various time intervals. The immunological assays indicated loss of component I which paralleled a decline in C_2H_2 reduction. Extracts of cells fixing N_2 formed heavy precipitin lines with the appropriate antiserum for components I and II. On adding NH_4Cl to the cultures, however, the immune reaction for component I in the cell extracts decreased rapidly while component II, after a small initial depression, was maintained at a constant level (Fig. 4).

The effects of supplementing crude extracts of cells harvested during the transition from N_2 to ammonium with either purified component I or II from N_2-fixing cells on C_2H_2 reduction are shown in Fig. 5. It is clear that, after 14 h growth, component I is rate-limiting in extracts of N_2-fixing cells and that supplementing extracts with component I was effective up to 2 h after adding ammonium to the cultures. Subsequently, the addition of component I to extracts of these cells did not enhance C_2H_2 reduction, presumably because component II, although present, was no longer functioning normally. Purified component II stimulated C_2H_2 reduction only slightly when added to extracts of cells up to 2 h after adding ammonium to the cultures.

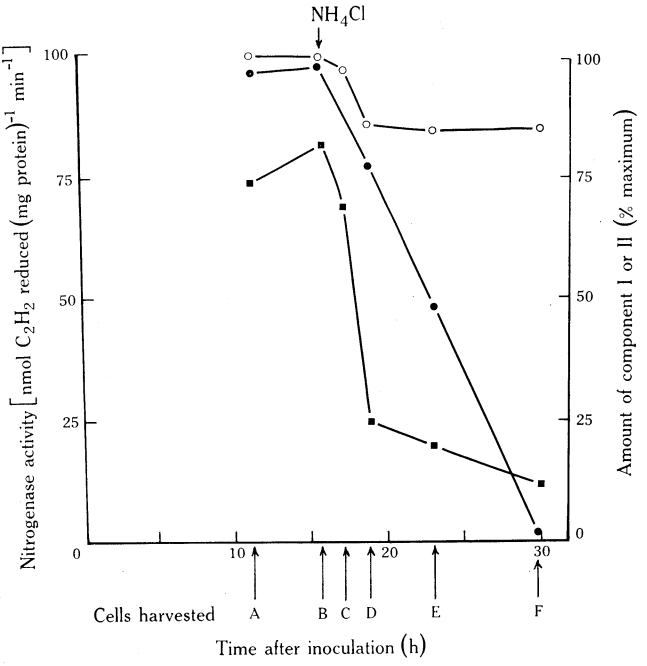


Fig. 4. Effects of adding NH_4Cl to cultures utilizing N_2 on C_2H_2 reduction activity in crude extracts (■) and on levels of component I (●) and component II (○) determined by immunological methods. Cells were harvested at times indicated. Crude extracts were prepared as described in Methods. Quantities of components I and II are expressed relative to the maximum amounts of each present prior to ammonium addition.

Derepression of Nitrogenase on Ammonium Depletion from the Cultures

Results for the transition from the ammonium regime to N_2 are presented in Fig. 6. The parameters monitored were growth, C_2H_2 reduction, immunodiffusion identification of components I and II in cell extracts, and residual ammonium in the culture solutions. When ammonium was included in the cultures, C_2H_2 was not reduced and component I was not detected in cell extracts, either by the immunodiffusion method or by polyacrylamide gel electrophoresis. When ammonium was depleted in the medium, however, there was a dramatic increase in component I and C_2H_2 reduction in cell extracts. Component II was detected in cells grown with ammonium, and this

component increased in amount in extracts of N_2-fixing cells. Polyacrylamide gel electrophoresis also confirmed these specific immunological assays for components I and II.

The immunodiffusion assays for cell extracts during transition from ammonium to the N_2-fixing regime are presented in Fig. 7. Extracts from cells grown with ammonium showed no reaction with a specific antiserum for component I, but when ammonium was depleted, the immune reaction for component I was positive and it appeared as two precipitin lines. Component II, which gave a distinct single precipitin band in cells grown in ammonium, increased in intensity in cells fixing N_2.

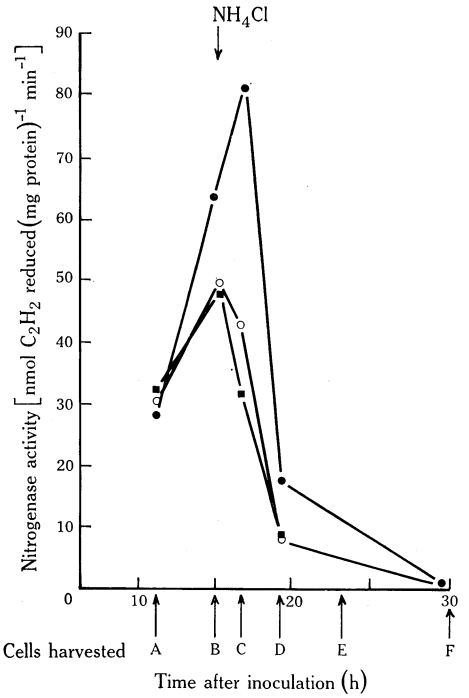


Fig. 5. Effect on C_2H_2 reduction of supplementing crude cell extracts from cultures undergoing a transition from N_2 fixation to the ammonium regime, with purified components I and II (fraction 5, Table 1). The crude extracts were those from cells harvested at various time intervals in the previous experiment (Fig. 4). Crude extracts were assayed for C_2H_2 reduction activity prior to (■) and after supplementing with purified component I (●) or purified component II (○).

Dissociated and Undissociated Component I

The reaction of purified component I (the immunogen) with its specific IgG antibody fraction resulted in the appearance of two distinct precipitin arcs, indicating that there were two antigenic reactions for it (Fig. 8). Purified component I was dissociated on adding 1 % (w/v) SDS and heating at 100°C for 5 min. This preparation produced a single precipitin line which was continuous with the inner arc of the untreated sample, as shown in Fig. 8. Thus the inner arc of the double precipitin lines appears to correspond to the dissociated component, while the outer arc is more likely to be associated with an aggregated form of the component. When component I was treated with a mixture of 1 % (w/v) SDS and 1 % (v/v) β-mercaptoethanol under similar conditions, a single precipitin line was produced in the immunoassay and this fused with the inner precipitin band of the non-dissociated component.

The degree of dissociation of component I appeared to depend upon the nature of the conditions used, as shown in Fig. 9. A gradation in severity of dissociation conditions resulted in formation of a gel pattern intermediate between the untreated and

the fully treated enzyme. Completely untreated component I was composed of large aggregates as evidenced by low mobility in the separating gel. Moderate dissociating conditions promoted disappearance of a large proportion of the material at the top of this gel and the appearance of two closely spaced bands moving midway down. Increasing the severity of the dissociation conditions resulted in the complete disappearance of material from the top of the gel and its appearance solely as the two closely spaced bands.

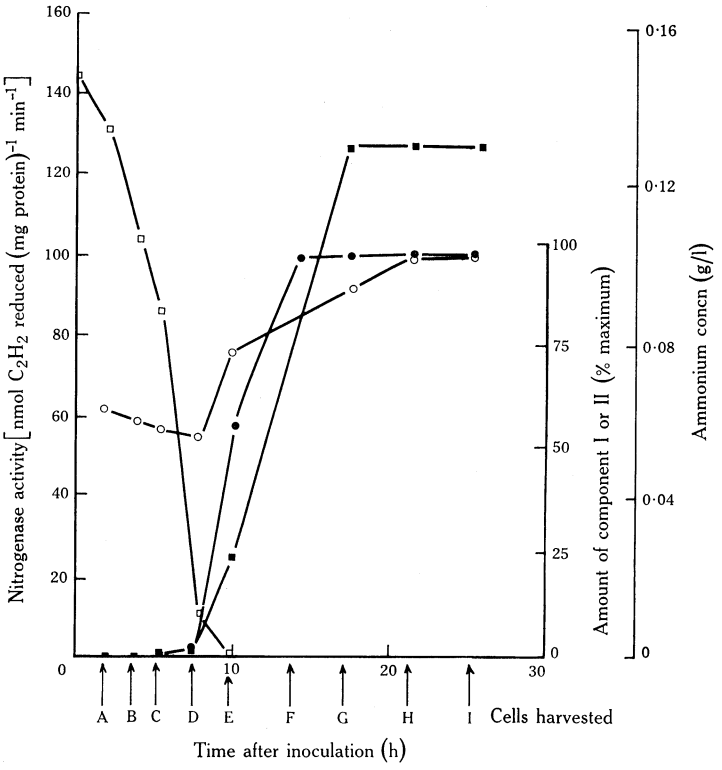


Fig. 6. Effect of depleting ammonium from culture solutions (□) on C_2H_2 reduction activity (■) and on levels of immunologically determined component I (●) and component II (○) in cells harvested at various times after addition of ammonium. Crude extracts were prepared as described in Methods. Quantities of components I and II are expressed relative to the maximum amounts of each present after complete depletion of ammonium.

Reactivation of Component II

Component II was readily inactivated in air. The addition of $FeSO_4$ to component II, which had been exposed to air, reactivated the component since, on recombining with purified component I, the reduction of C_2H_2 was enhanced as shown in Table 2. In this experiment, partially purified component II (fraction 4, Table 1), first inactivated in air, was incubated with 0·028 μg Fe^{2+}/μg protein for various times and then tested for C_2H_2 reduction with and without the addition of purified component I (fraction 4,

Table 1). The incubation of component II for 30 min with $FeSO_4$ increased C_2H_2 reduction by 90% (since component II contained a little component I) and by about 200% when additional component I was added. The effect of varying the time of incubating component II with $FeSO_4$ under anaerobic conditions indicates that 30 min is optimum, either with or without supplementing with component I.

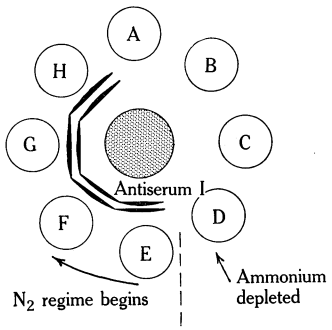


Fig. 7. Diagram of the antigen–antibody precipitin reaction in agar gel for component I present of extracts of cells harvested in the previous experiment (Fig. 6). The centre well contained IgG mouse antiserum specific for component I (fraction 5, Table 1). Peripheral wells contained the crude extracts sampled at times 0–30 h.

The effects of exposing component II (fraction 4, Table 1) to air for 1 h and its subsequent reactivation with $FeSO_4$ are shown in Table 3. After exposure to air, a sample of component II was maintained at 25°C under O_2-free N_2 for a further 48 h, and then aliquots were removed and reactivated with $FeSO_4$ for various time periods

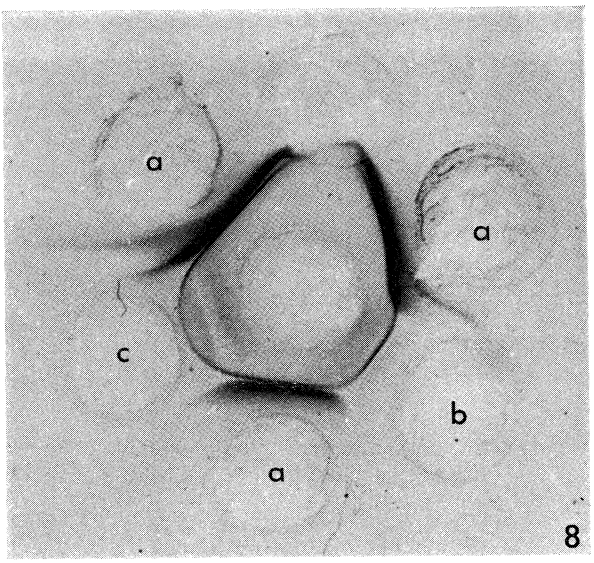
Table 2. Effects of incubating component II with $FeSO_4$ for various times on the reduction of C_2H_2

Component II (fraction 4, Table 1) (4·4 mg) was injected into Warburg flasks containing in 0·9 ml: 46 mM $Na_2S_2O_4$, 39 mM dithiothreitol, 2·3 mM $FeSO_4.7H_2O$ (0·028 μg Fe^{2+}/μg component II), 8 mM tris–HCl buffer (pH 7·5) under 5% C_2H_2 in helium. The flasks were incubated at 30°C and assayed at the times specified. Assays were carried out by injecting aliquots of component II into flasks both with and without addition of component I (fraction 4, Table 1), which had been stored in vials sparged with high-purity dry N_2. Assay procedures were as described in Methods

Time of incubation (min)	Component II (T)[A]		Components II (T)[A] plus I	
	(nmol C_2H_2 reduced/min)	Relative activity (%)	(nmol C_2H_2 reduced/min)	Relative activity (%)
0	48·5	100	89·5	100
5	69·0	142	240·5	268
30	93·2	190	264·8	296
115	97·0	200	274·1	306

[A] T = incubation with $FeSO_4$.

(sample B). The results show that a greater degree of reactivation of component II was achieved when it was incubated with $FeSO_4$ immediately after 1-h exposure to air (sample A). The maximum activation was achieved after a 30-min incubation period with $FeSO_4$. When component II was kept under O_2-free N_2 for a further 48 h, however, the reactivation was only about one-tenth of that attained for a 1-h exposure to air.



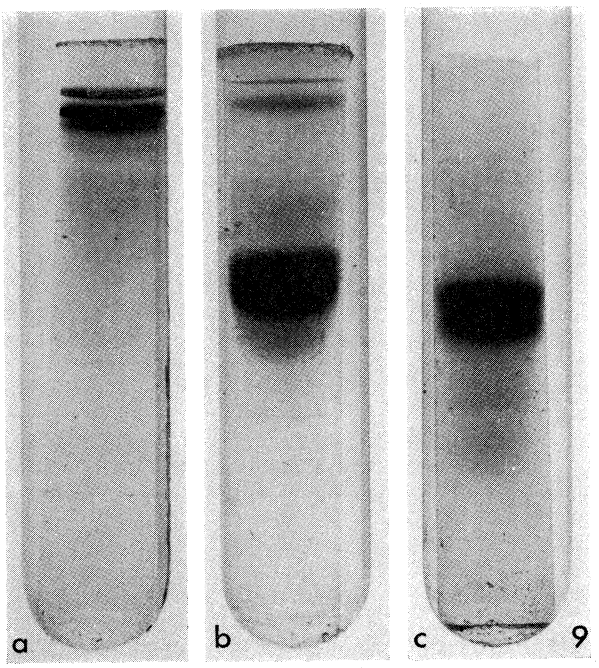


Fig. 8. Antigen–antibody precipitin reaction of component I in agar gel. The centre well contained IgG rabbit antibody specific for component I (fraction 5, Table 1). Component I used as an antigen was fraction 5, Table 1. (*a*) Untreated component I; (*b*) component I incubated with 1·0% (w/v) SDS at 100°C for 5 min; (*c*) component I incubated with 1·0% (w/v) SDS and 1·0% (v/v) β-mercaptoethanol at 100°C for 5 min.

Fig. 9. Polyacrylamide disc gel electrophoresis of component I (fraction 5, Table 1). (*a*) Untreated component I in normal gel; (*b*) component I incubated with 1·0% (w/v) SDS and 1·0% (v/v) β-mercaptoethanol at 30°C for 3 h; (*c*) component I incubated with 1·0% (w/v) SDS and 1·0% (v/v) β-mercaptoethanol at 100°C for 5 min. The electrode buffer, stacking and separation phases of gels 2 and 3 each contained 1·0% (w/v) SDS.

When ferrous ammonium sulphate was used to reactivate component II, instead of $FeSO_4$, there was a 700% stimulation after 100 min with component II, which contained a little bound component I (fraction 4, Table 1) as shown in Table 4. On supplementing with component I, however, the ferrous ammonium sulphate treatment inhibited C_2H_2 reduction, which was probably associated with ammonium inhibition.

Table 3. Reactivation by $FeSO_4$ of component II which had been inactivated in air for 60 min

Incubation with $FeSO_4$ and assay procedures were carried out as described in the legend to Table 2. Sample A component II was reactivated immediately following its inactivation by exposing it to air for 60 min. Sample B component II was inactivated by exposure to air for 60 min but was then stored in O_2-free N_2 for 48 h prior to the reactivation procedure

Time of incubation (min)	Sample A component II (T)[A]		Sample B component II (T)[A]	
	(nmol C_2H_2 reduced/min)	Relative activity (%)	(nmol C_2H_2 reduced/min)	Relative activity (%)
0	28·0	100	48·5	100
5	117·5	406	69·0	142
30	343·1	1178	93·2	190
80	251·7	867	100·7	214
115	149·2	513	97·0	200

[A] (T) = incubation with $FeSO_4$.

Table 4. Effects of incubating component II with ferrous ammonium sulphate for various times on the reduction of C_2H_2

Component II (fraction 4, Table 1) (4·8 mg) was injected into Warburg flasks containing in 0·9 ml the same mixture as in Table 2. Incubation and assay conditions were also the same as for Table 2

Time of incubation (min)	Component II (T)[A]		Components II (T)[A] plus I	
	(nmol C_2H_2 reduced/min)	Relative activity (%)	(nmol C_2H_2 reduced/min)	Relative activity (%)
0	2·3	100	13·4	100
5	9·0	484	5·4	40
15	17·9	768	3·2	25
30	17·3	744	5·4	40
100	18·8	808	5·2	39

[A] (T) = incubation with ferrous ammonium sulphate.

Discussion

The repression of the fixation of N_2 by ammonium *in vivo* is well documented, but its effect on the nitrogenase enzyme complex is less clear. It is well established that the nitrogenase system in *Azotobacter* and other organisms is composed of at least two enzymes, namely component I (Fe–Mo–S protein) and component II (Fe–S protein). These may be detected by immunodiffusion methods (Davis *et al.* 1972; Shah *et al.* 1973*a*) as well as by starch and polyacrylamide gel electrophoresis (Deering and Nicholas 1974; Eady and Postgate 1974).

Component I is markedly repressed in cells when ammonium is added to the culture solutions, whereas component II, although depressed slightly initially, is still present in considerable amounts. These results are not in agreement with those of other workers. Bishop *et al.* (1975), using the technique of immunodiffusion, report that cell extracts of *A. vinelandii* growing under repression conditions with ammonium showed a precipitin reaction with antibody to component I and concluded that this precipitin reaction is due to a component other than component I. The present study indicates that the growth conditions used in their experiments may not have been adequate for complete repression of nitrogenase. If this were so, then their ammonium-grown cultures may well have contained component I.

Although component II was similar to that from cells fixing N_2, based on immunological tests and on patterns in polyacrylamide gel electrophoresis, it did not, on recombining with component I, reduce C_2H_2 to C_2H_4. These results differ from those of Shah *et al.* (1972) who found that the synthesis of components I and II in *Azotobacter* was coordinate as was their degradation on adding ammonium. However, confirmation of the presence of component II apoprotein in repressed cells is suggested by reappraisal of the immunological evidence of Shah *et al.* (1973*b*). The authors' interpretation of the interaction between the reactions of component II from repressed and derepressed wild-type cells is a reaction of non-identity but the photograph appears to reveal a pattern characteristic of a reaction of partial identity.

The loss of activity during the purification of component II and during storage of crude extracts and of the purified protein has been a major problem in work with nitrogenase from most sources; this is attributed to an inactivation by O_2. Bergersen and Turner (1973) reported that the loss of activity of component II prepared from root nodule bacteroids could be restored by incubating these preparations with Fe^{2+} as ferrous ammonium sulphate. This treatment usually increased the activity by about 50–100% within 1 h.

Our experiments with *A. vinelandii* confirm the reactivation of inactive component II after incubation with ferrous salts. Ferrous ammonium sulphate inhibits component I and so it is preferable to use $FeSO_4$ to reactivate component II. The reason for the inactivity of the component II produced in the absence of component I in ammonium-grown cells may be deactivation *in vivo* by O_2.

It is also of interest that, in the immunological studies with purified component I, two antigenic reactions were produced for it since two precipitin lines were detected (Fig. 8). Treatment of the purified component I with SDS and heating at 100°C before immunoassay resulted in formation of one precipitin line, which probably represents reaction with the non-aggregated form of the protein. This SDS-, fully heat-treated component was resolved into two subunits on SDS disc polyacrylamide gels, thus confirming the presence of two subunits as reported by Eady *et al.* (1974). The use of a discontinuous electrophoresis system and rigorous dissociation conditions seemed to be necessary for unequivocal resolution of two component I subunits. Generally, more moderate dissociation conditions (3 h at 30°C) did not result in complete dissociation, nor was clear resolution obtained on a continuous electrophoresis system. Such were the techniques used by workers who have reported *A. vinelandii* component I as being composed of only one type of subunit. Where more rigorous dissociation conditions have been employed for component I from this organism (Fleming and Haselkorn 1973), a similar two-subunit composition was

reported. Thus *A. vinelandii* component I appeared to behave as a membrane-associated protein insofar as its requirement for rigorous conditions to promote complete dissociation was concerned.

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