NITROGEN FIXATION IN CELL-FREE EXTRACTS OF *CLOSTRIDIUM PASTEURIANUM*

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SUMMARY

Reproducible nitrogen fixation has been achieved in cell-free enzyme preparations. Extracts of *Clostridium pasteurianum* underwent enrichments averaging 0.7 atom % excess ^{15}N when exposed to 60 atom % excess $^{15}N_2$ for 1 h. Activity was not sedimented at 144,000 $\times g$ in 4 h, demonstrating that the nitrogenase is non-particulate.

Several experimental conditions were critical in securing active extracts. These included: (a) use of particular cell-breaking methods, (b) addition of an energy source, preferably sodium pyruvate, (c) close control of pH, and (d) strict exclusion of O_2. Consistently active preparations were obtained by disrupting frozen cells in a Hughes press or by autolysis of specially dried cells resuspended in phosphate buffer.

Nitrogen fixation in *C. pasteurianum* involves reduction to ammonia, since the $^{15}N_2$ taken up by the extracts could be recovered quantitatively as $^{15}NH_3$. Coupling of fixation to the clostridial-type phosphoroclastic reaction is inferred. Differential spectral responses at 300–365 mμ in the presence and absence of N_2 are observed and may represent interaction of nitrogen with the enzyme system.

INTRODUCTION

Progress in establishing the mechanism of biological nitrogen fixation has been slow because the enzyme system has appeared too labile for study outside the cell[1–6]. Existing information has been obtained largely from studies with living organisms. The end-product has been generally conceded to be ammonia[7–10], though there are differences of opinion about its pathway of formation[10–12]. The immediate source of hydrogen for the reduction is obscure[13], particularly in view of the inhibitory effects of molecular hydrogen[14–16]. Substances recognized to play some part in nitrogen fixation in growing cells include molybdenum[17], iron[18–19], and biotin[19].

In this report we describe methods for extracting and activating the nitrogen-fixing enzyme system from *Clostridium pasteurianum*. Availability of active extracts

Abbreviations: CoA, coenzyme A; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; DPN, diphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide; ATP, adenosine triphosphate.

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now opens the way for investigating the mechanism of nitrogen fixation at the enzyme level, and first results are presented. A preliminary note on this work has been published[20]. Details were communicated to Professors R. H. BURRIS and P. W. WILSON, who with their coworkers have confirmed the major findings and have extended the methods successfully to *Rhodospirillum rubrum*[21].

EXPERIMENTAL

Culture methods

Cultures of *C. pasteurianum* strain W-5 were obtained from the American Type Culture Collection (Catalog 6013), Washington, D.C., and grown under nitrogen-fixing conditions as described previously[19] except that the iron was added as alcoholic $FeCl_3$ solution. 2 ml of stock solution (5.0 g $FeCl_3 \cdot 6H_2O$ in 100 ml absolute ethanol) was used per liter of culture medium. Cultures with a generation time of 3 h or less were produced. Various size cultures were used: 0.5 l, 8 l, and 40 l in glass vessels (12-gallon solution bottles, A. H. Thomas Co., for the 40-l cultures). The inoculum was maintained as 500-ml cultures transferred daily. After about one hundred serial transfers, a fresh inoculum was started as previously described[19]. A 2% inoculum was used in each instance. After 16 h growth, the O.D. usually was 0.7–0.8 (650 mμ in a standard Klett tube in a "Lumetron" colorimeter), and the cultures were generally found to be at pH 5.4–5.8. The yield of cells after being washed and dried in a "Rinco" evaporator as described below was 0.63–0.75 g/l. During growth N_2 was bubbled through the cultures at 10–30 l/h to provide agitation and maintain anaerobiosis.

Buffer

Unless otherwise stated, the buffer employed throughout this work was 0.05 *M* potassium phosphate, pH 6.8, prepared by dissolving 6.80 g (0.05 mole) KH_2PO_4 in 400 ml distilled water, adjusting the pH to 6.8 with KOH, and diluting to 1 l.

Resting cells

For resting-cell experiments, 400 ml of a culture was centrifuged at 3,000 × *g* for 15 min. The sedimented cells were washed in 50 ml of buffer, recentrifuged, and suspended in 25 ml of buffer. Exclusion of air and maintenance of low temperatures were not essential during these steps. Fixation experiments were made in the absence of O_2.

Preparation of nitrogen-fixing extracts

Hughes press: The cells from 400 ml of a culture at O.D. 0.7–0.8 (650 mμ in a standard Klett tube in a "Lumetron" colorimeter) were sedimented at 27,000 × *g* for 15 min at 5°. The paste was washed with 25 ml of cold buffer, centrifuged, and resuspended in 9 ml of cold buffer. The suspension was poured into a Hughes press[22] previously cooled to — 35°. The piston was inserted, and after 5 min the frozen material was driven through the press by application of a Carver press or by 20–25 blows with a 4.5-kg weight dropped 2.4 m through a pipe guide. The press was opened in air, and the frozen preparation was rapidly removed without thawing. It was either transferred directly to a flask for assay of its nitrogen-fixing activity

or was thawed under N_2 and centrifuged to remove debris and intact cells. Centrifugation was at 27,000 × *g* at 0° in a "Servall" refrigerated centrifuge for 15 min. The activity was in the cell-free supernatant solution and required protection from O_2.

Autolysis of dried cells: 10 l of a culture at O.D. 0.7–0.8 (650 mμ in a standard Klett tube in a "Lumetron" colorimeter) was centrifuged at 5–10° in a Sharpless supercentrifuge with N_2 pressure being used to force the liquid from the culture bottle into the centrifuge bowl. The cell paste was washed by suspension in 500 ml of cold buffer and recentrifuged. Enough distilled water, usually about 5–20 ml, at 5° was added to the cell paste so that the paste could be poured into a 5-l round-bottomed flask. The flask was attached to a "Rinco" rotating evaporator and the cells were dried *in vacuo* without freezing in a water bath at 30–40°. After 1 to 2 h the pressure had dropped to 0.5 to 0.25 mm. The flask was left *in vacuo* overnight without rotation. The dry cells, which weighed 6.3–7.5 g, were stored in sealed bottles at — 15° (see below).

10 g of dried cells were pulverized in a mortar and added to 100 ml of buffer in a 250-ml suction flask. Air was deleterious beyond this point. The flask was thoroughly purged with H_2, sealed with 0.8 atm of H_2 and placed on a Brunswick rotary shaker at 30° for 45 min. It was then cooled to 0° in an ice bath, and the contents were immediately poured into 50-ml centrifuge cups for centrifugation at 27,000 × *g* for 15 min at 0°. The supernatant solution was the cell-free nitrogen-fixing preparation.

Ultracentrifugation of extracts: When more rigorous centrifugation of the nitrogen-fixing preparations was desired, it was performed at 144,000 × *g* in a "Spinco" model L preparative ultracentrifuge for 3 to 4 h at 0°. All activity remained in solution.

Storage: The extracts were reasonably stable to storage provided they were kept under H_2 or N_2 and below 5°. 24-h storage under these conditions resulted in 20–50 % loss in activity. Storage beyond a few days resulted in total loss of activity. Freezing the extracts gave some improvement in storage life.

Dried cells prior to autolysis retained their full capacity to yield nitrogen-fixing extracts for over 2 months under air at — 15°, provided moisture was excluded. In the presence of moisture, air was rapidly destructive.

Assay for nitrogen-fixing activity

Extracts were assayed for nitrogen-fixing activity by adding sodium pyruvate and exposing to $^{15}N_2$, which was prepared and handled by the methods of BURRIS AND WILSON[23]. The proportions of extract, buffer, and sodium pyruvate were chosen so that the solution contained 10–20 mg of protein-nitrogen (biuret method) and 100–200 mg of sodium pyruvate in a total volume of 5–8 ml. Typical examples are presented in Tables IV, VI, and VII.

The sodium pyruvate (100 mg) and buffer (4 ml) were placed in a 25-ml round-bottomed flask, frozen, and then the extract (4 ml) was added. Purging with A was begun immediately. The flask was alternately evacuated and filled with A until thawing was completed and all entrapped air was released. At least five purgings were made. Finally, the flask was filled to 0.5 atm with 60 % enriched $^{15}N_2$, sealed, and agitated for 40–60 min at 30° on a "Gyrotory Shaker" (New Brunswick Scientific Co., New Brunswick, N.J.). Considerable CO_2 and H_2 accumulated in the flask during reaction. The solution was digested by Kjeldahl procedure[23], and the resulting NH_3 was converted to N_2 by the procedures of BURRIS AND WILSON[23]. The Fredericks

Modification[24] of the Kjeldahl procedure gave identical results. Dumas combustion of the lyophilized extracts produced erratic results probably due to the difficulty in combustion.

The $^{15}N/^{14}N$ ratio was determined with a Type 21-103C mass spectrometer (Consolidated Engineering Co., Pasadena, Calif., U.S.A.).

Spectral differentiation procedure

Difference spectra of the cell-free nitrogen-fixing extracts were obtained with the aid of the apparatus shown in Fig. 1. The "capillary bleed" in Fig. 1 was a capillary tube which was attached by a rubber sleeve to the tube at the mouth of the 25-ml flask, and it projected through the stopcock leading to the spectrophotometer cell. The capillary was snapped when this stopcock was closed to seal the cell

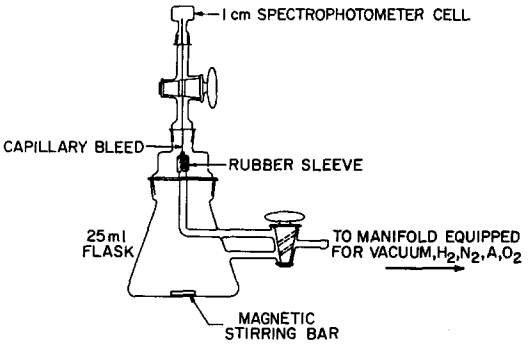

Fig. 1. Spectral differentiation apparatus.

just before transferring it to the spectrophotometer. Four of these assemblies were connected to a vacuum manifold which was supplied with H_2, N_2, and A, each scrubbed with aqueous chromous chloride. These gases analyzed less than 2 ppm O_2 (continuous flow O_2 amperometric analyzer, Lockwood and Mc Lorie, Inc., Hatboro, Pa., U.S.A.). In a typical experiment 3.5 ml of buffer containing 160 mg sodium pyruvate was placed in each 25-ml flask and degassed by stirring *in vacuo* at room temperature for 5 min. The flasks were then filled with H_2 and cooled to 5°. The flasks were opened momentarily and 0.5 ml of a nitrogen-fixing extract, centrifuged at 100,000 × *g* for 15 min and typically containing 4-6 mg protein-N/ml, was added. The flasks were evacuated, then filled and flushed with H_2 six times. Evacuations were conducted with the stopcock turned so that access to the flask was through the larger tubing (Fig. 1). The contents of the flasks were gently stirred and warmed to 25° for 15 min and again cooled to 5°. With the stirrer stopped, each flask was flushed twice and filled with the appropriate gas. The contents of each flask were again gently stirred at 5° for 15 min. The flasks were then inverted in an ice bath and the contents transferred into the spectrophotometer cell by application of reduced pressure (3-5 mm) through the capillary bleed. The spectrophotometer cell was sealed by closing its stopcock snapping the "capillary bleed" tube in the process. The cell together with its stopcock was detached as a unit from the rest of the assembly and transferred to the spectrophotometer without exposing the sample to air. The difference spectrum was obtained by using the sample under H_2 as the blank in a Beckman Model DU Spectrophotometer.

Tests for fixation intermediates

Experiments seeking evidence of fixation intermediates were concerned chiefly with hydrazine, hydroxylamine, nitrite, and dihydropyridazinone-5-carboxylic acid[11].

A cell-free extract (37 ml) was prepared from cells from 3 l of culture, and 300 mg of sodium pyruvate and 113 mg of $(NH_4)_2SO_4$ were added. The $(NH_4)_2SO_4$ was intended to repress ammonia synthesis and force accumulation of precursors. The solution was placed under N_2 and agitated at 30° with a magnetic stirrer. A control was assayed with $^{15}N_2$ to assure that fixation was occurring. Samples (5 ml) were withdrawn at intervals and freed of protein by treatment with 70% ethanol. Tests for hydrazine were made with Ehrlich's reagent[25]. Tests for hydroxylamine and nitrite were by the method of ENDRES AND KAUFMANN[26].

Evidence for involvement of dihydropyridazinone-5-carboxylic acid was sought by determining whether it is altered by active extracts. The compound was added to an extract at a concentration of 10 μmoles/ml, a level found to be noninhibitory to fixation in these extracts. The concentration of the compound[11] was measured at intervals by means of the Ehrlich reagent applied to aliquots before and after acid hydrolysis for 30 min at 100°.

Fractionation of ^{15}N-labeled compounds

An extract (60 ml) was prepared from 6 l of culture, and 0.7 g of sodium pyruvate was added. After exposure to $^{15}N_2$ for 1 h, the extract was freed of proteins with 70% ethanol. Ethanol was removed in a "Rinco" rotating evaporator at 2 mm pressure. The solution which contained 4.93 atom % excess ^{15}N and 16 mg total nitrogen was introduced to a 55 × 2.5 cm column of "Dowex" 50 resin. The compounds on the column were fractionated by elution with increasing concentrations of HCl (see ref. 27). Elution was followed by u.v. absorption measurements and by ninhydrin reaction. Initial identification was made by paper chromatography with butanol–acetic acid–water (1:3:4), acetone–urea–water (60:0.5:40), butanol–urea–water (60:0.5:40) and lutidine–water[28].

RESULTS AND DISCUSSION

Search for consistently active nitrogen-fixing extracts

The choice of *C. pasteurianum* for this work was based largely on the facts that it is an anaerobe and its nitrogen-fixing activity is relatively insensitive to hydrogen[15].

The design of experiments on enzyme extraction was aided by first establishing conditions for nitrogen fixation in resting cells. With the $^{15}N_2$ technique[23], cell suspensions of *C. pasteurianum* were found not to fix nitrogen unless supplied with a substrate, *e.g.*, sucrose or, better, pyruvate. Supplementary iron, molybdenum, and biotin, substances needed for fixation in growing cells, were not beneficial. The amount of fixation obtained relative to the initial nitrogen content was approximately 1 to 2% measured by the $^{15}N_2$ method and 2 to 5% in other experiments in which the less precise Kjeldahl method was used. Typical experiments are presented in Table I.

On the basis of the foregoing results, pyruvate was used in conjunction with the $^{15}N_2$ assay to investigate enzyme extraction methods.

Of a variety of cell-breaking techniques examined, only two have consistently given active nitrogen-fixing extracts; these are the Hughes press and the autolysis of dried cells. With 60 atom % excess $^{15}N_2$, extracts from either procedure gave enrichments averaging 0.7 atom % ^{15}N which corresponds to 12 μg N fixed/mg protein-N initially present. In the absence of pyruvate, no fixation occurred. The Hughes press method is simpler to control, but the autolysis method is less laborious and affords a larger capacity. Both are highly reproducible with due care to operating variables. Details are discussed in other sections.

TABLE I

NITROGEN FIXATION IN RESTING CELLS OF *C. pasteurianum*

*Additions**	*Percent nitrogen increase*	*Analytical method***
None	None	$^{15}N_2$ or Kjehdahl
Pyruvate	1.9	$^{15}N_2$
Pyruvate	4.5 ± 1.1	Kjeldahl
Pyruvate, biotin, Fe, Mo	4.4 ± 1.0	Kjeldahl
Sucrose	2.0 ± 1.0	Kjeldahl

* Cells were suspended in 25 ml buffer, pyruvate was added as 0.25 g sodium salt, and other concentrations were as in growth medium. Time of incubation was 1 h.

** Kjeldahl results are presented as average and range of 6 determinations.

Disruption with sonic oscillations proved highly erratic, though enrichments of 0.35 % in ^{15}N content were occasionally obtained. A homogenizer[29] operating with powdered glass was more consistent than the sonic oscillator though less effective than the Hughes press in both the fraction of cells disrupted and activity levels obtained. Little or no success was had in a limited number of experiments with acetone-drying, enzymic lysis, detergent lysis, freezing, or pressure changes.

Evidence for cell-free state of active extracts

That the nitrogen-fixing activity of these extracts was not due to intact cells or large particles was demonstrated by centrifugation. Supernatant fractions retained full activity and contained no visible particles by microscopic examination after 2 to 4 h at 144,000 × *g* (Table II). Activity was not lost in a series of centrifugations first at 27,000 × *g* and then at 144,000 × *g*. In each step only a small upper portion of the preceding supernatant liquid was taken by pipette to avoid resuspension of sedimented cells. Centrifugation seemed to enhance both fixation activity (Table II) and efficiency of pyruvate utilization (Table VI) in the extracts, and it was adopted as routine procedure (27,000 × *g* for 15 min).

Broken-cell preparations before centrifugation contained intact cells ranging from 0.7 % to 10 % but usually 2 to 5 % of the original population. Although some of the cells were viable as shown by culture experiments, they contributed little nitrogen-fixing activity. Thus, extracts containing 2 to 5 % of their original cells were up to seven times as active as their intact-cell controls (Table II).

General properties of the enzyme extracts

The optimum hydrogen ion concentration for fixation activity was pH 6.3–6.5 (Fig. 2). On either side of this range, activity decreased sharply. Inactivation in the

range pH 5.5–6.3 could be reversed by readjusting to pH 6.3–6.8, but, after inactivation below pH 5.5, the system was not reactivated by pH adjustments.

TABLE II

NONDEPENDENCE OF ACTIVITY ON INTACT CELLS IN EXTRACTS

Hughes press method. 1 and 2 were from one preparation, 3 and 4 were from another. Intact-cell content is given relative to initial count. % ^{15}N enrichment = % ^{15}N final minus 0.36. Time of incubation was 1 h.

System and treatment	*Content intact-cells*	*% ^{15}N enrichment*
1. Resting cells	100%	0.10
2. Broken cells not centrifuged	8%	0.64
3. Extract 27,000 × *g*/15 min	None	0.54
4. Extract 144,000 × *g*/90 min	None	0.78

Air sensitivity is marked, although serious losses in activity were not encountered during brief exposures to air provided the enzyme solution was kept cold and not agitated during the exposure. Particular care must be taken to exclude O_2 during the fixation assay procedure. For example, a significant drop in fixation occurred from air released from small ice particles not melted prior to sealing the preparation with $^{15}N_2$.

Ionic strength also affects activity. Good fixation can be achieved in the 0.05 *M* phosphate buffer (pH 6.8), but this concentration apparently represents a compromise

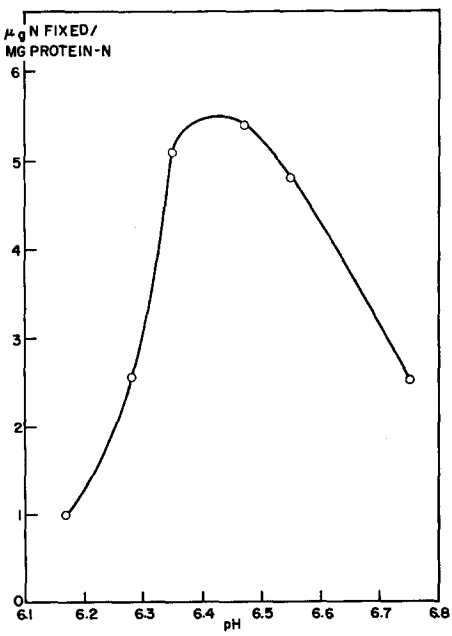

Fig. 2. Effect of pH on nitrogen-fixing activity. The reaction mixture contained 12 mg protein-N as cell-free extract, 100 mg sodium pyruvate, and 0.05 *M* phosphate buffer at the desired pH in total volume of 8 ml. The solutions were incubated under 0.5 atm of 60 atom% $^{15}N_2$ 60 min at 30°. Extracts were prepared in the buffer at pH 6.8 as usual and adjusted to desired pH just before use.

between the requirements for pH control and sensitivity to ionic strength. Higher concentrations of phosphate were deleterious. Addition of sodium chloride to the buffer to bring it to 0.05 *M* NaCl and 0.05 *M* phosphate similarly caused substantial losses of activity.

A point of significance relative to the mechanism of fixation is that the enzyme system apparently does not effect the isotope exchange reaction of N_2.

TABLE III

ISOTOPE EXCHANGE EXPERIMENTS $^{14}N_2 + {}^{15}N_2 \leftrightharpoons 2\,{}^{14}N - {}^{15}N$

Conditions	% $^{14}N_2$	% ^{14}N–^{15}N	% $^{15}N_2$
1. Control sample before exposure to cell-free preparation	94.10	3.57	2.39
2. Control sample exposed 1 h to cell-free preparation with pyruvate added*	94.00	3.63	2.41
3. As above without pyruvate	94.00	3.69	2.39
4. Calculated equilibrium composition	91.86	7.97	0.17

* This preparation fixed 0.14 atom % excess ^{15}N when exposed to 60 atom % excess $^{15}N_2$ under the condition of 2.

Experiments (Table III), which should be repeated with refinements, have thus far failed to provide evidence that this reaction occurs under influence of the extracts either in the presence or absence of added sodium pyruvate. Accordingly, dissociation of N_2 may not be an early step in adsorption.

Dependence of fixation activity on pyruvate metabolism

Nitrogen fixation in the extracts is closely coupled with pyruvate metabolism though the mode of coupling has yet to be learned. A search for other compounds capable of supporting nitrogen fixation turned up only one, α-ketobutyrate, but this compound is only about half as effective on a molar basis as pyruvate. The majority of compounds tested (Table IV) were selected on the possibility that they might

TABLE IV

COMPOUNDS TESTED FOR PYRUVATE REPLACEMENT

Compound added	*Atom % excess* ^{15}N
None	0.00
Pyruvate (100 mg)	0.64
α-ketobutyrate (100 mg)	0.25
Hydrogen (0.7 atm)	0.00
Hydrogen (0.7 atm) + pyruvate (100 mg)	0.60

* Flasks contained in addition to the compounds mentioned, the cell-free extract (15 mg protein-N), 0.3 atm of 60 atom % $^{15}N_2$ and buffer to make 8 ml. Time of incubation was 1 h.

Other compounds tried which would not support nitrogen fixation were sodium or potassium salts of formic, acetic, propionic, butyric, glycolic, glyoxylic, lactic, crotonic, malic, oxalacetic, succinic, fumaric, mesaconic, α-ketoglutaric, phosphopyruvic, and phenylpyruvic acids, ethanol, propanol, glycerol, 2,3-butanediol, acetoin, biacetyl, acetaldehyde, ornithine, sucrose, ethyl acetoacetate, and the substrate-enzyme combinations, malic acid/malic acid dehydrogenase and glucose-6-phosphate/glucose-6-phosphate dehydrogenase.

serve as hydrogen donors, ammonia acceptors or pyruvate precursors. Also tested were various products of pyruvate metabolism, a few of which proved inhibitory to fixation (Table V). Presumably the function of pyruvate is to supply reducing capacity to the fixation system. A detailed understanding of pyruvate chemistry in the extracts should assist in unraveling the mechanism of coupling to fixation.

TABLE V

EFFECTS OF METABOLITES ON NITROGEN FIXATION IN EXTRACTS

*Compound added**		*Nitrogen fixation as % of control*
Sodium butyrate	(100 mg)	71
Sodium acetate	(100 mg)	75
Lithium acetyl phosphate	(12 mg)	50
Lithium acetyl phosphate	(25 mg)	0
ATP	(4 mg)	80
ATP	(20 mg)	0
Phosphate	(300 μmoles)	100
Phosphate	(400 μmoles)	100
Phosphate	(900 μmoles)	45
Phosphate	(1400 μmoles)	15

* Each flask also contained cell-free extract (15–20 mg protein-N), 100 mg sodium pyruvate 0.3 atm of 60 atom % $^{15}N_2$, and total volume was adjusted to 8 ml with buffer. Time of incubation was 1 h. Control fixed 0.51 atom % excess ^{15}N.

There is an optimum concentration of pyruvate for maximum fixation activity, and the amount varies with the concentration of protein in the extracts. Normally, the fixation assay system contained 2 mg protein-N/ml, and maximum fixation was attained with sodium pyruvate at 12 mg/ml. For more dilute extracts, the optimum pyruvate concentration was lower. These relationships are illustrated in Tables VI and VII and in Fig. 3.

Time-course experiments revealed that the start of nitrogen fixation lagged behind the start of pyruvate metabolism, which began with a burst several times

TABLE VI

EFFECTS OF PYRUVATE CONCENTRATION AND CENTRIFUGATION ON NITROGEN FIXATION IN EXTRACTS

*Flask**	*Pyruvate (mg)*	*Preparation***	*^{15}N fixed (μg)*
1	200	Uncentrifuged	50
2	100	Uncentrifuged	48
3	50	Uncentrifuged	32
4	200	Centrifuged	33
5	100	Centrifuged	84
6	50	Centrifuged	46

* Each flask contained also 0.6 atm of 60 atom % $^{15}N_2$ and enough buffer to give 8 ml total volume. Time of incubation was 1 h. Equivalent volumes of uncentrifuged to centrifuged preparation were used; 20.2 mg protein-N/flask for the uncentrifuged preparation and 14.5 mg protein-N/flask for the centrifuged preparation.

** The uncentrifuged preparation metabolized pyruvate at a rate 150 % that of the centrifuged preparation as measured by H_2 evolution.

faster than the rate it maintained after fixation got underway. Data are presented in Fig. 4. The rates of nitrogen fixation and pyruvate metabolism were linear after the induction period. The significance of the initial lag in fixation and of the initial high rate of pyruvate consumption has not been established. The induction period may represent the time required to generate a key substrate or to condition in some way an enzyme essential to the nitrogen fixation system. Spectral responses discussed below point to the latter possibility.

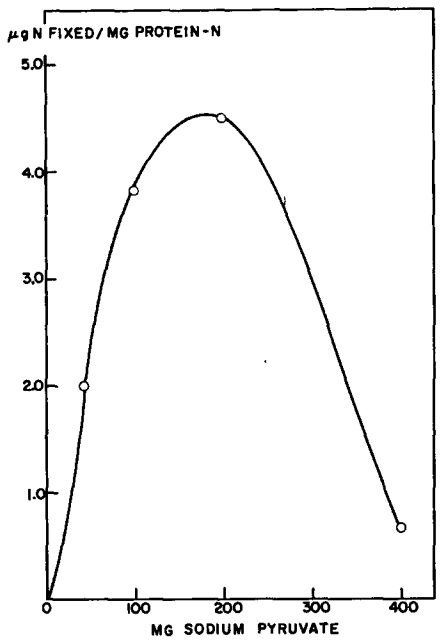

Fig. 3. Effect of concentration of pyruvate on nitrogen-fixing activity. The system was identical to that in Fig. 2 except 20 mg protein-N as cell-free extract was used in each determination and the solution was buffered at pH 6.5.

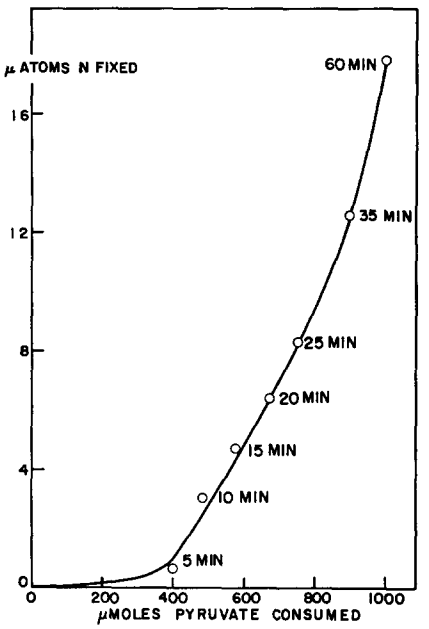

Fig. 4. Time-course of nitrogen fixation and pyruvate consumption. The reaction mixture of 8 ml total volume contained 14 mg protein-N as cell-free extract and 100 mg sodium pyruvate in 0.05 *M* phosphate buffer pH 6.5. The mixture was incubated with 0.5 atm of 60 atom % $^{15}N_2$ for the varying periods indicated and then analyzed for ^{15}N and pyruvate[36].

That it was the metabolism of pyruvate rather than the presence of pyruvate *per se* that gave rise to nitrogen fixation in the extracts seems fairly well indicated by the time-course data and by the fact that α-ketobutyrate also was active.

Chemistry of pyruvate in the extracts

In view of the requirement for pyruvate and the nature of some of the products formed, it would seem that nitrogen fixation may be coupled to the phosphoroclastic reaction. The phosphoroclastic reaction, which occurs in various clostridia[30,31], appears to operate in the nitrogen-fixing extracts. H_2 and CO_2 are copiously evolved in 1:1 ratio when the extracts are supplied with pyruvate. Formation of acetyl phosphate in substantial quantities has also been observed by the hydroxamic acid method[32] (Fig. 5). Acetyl phosphate is an inhibitor for nitrogen fixation (Table V),

but its effect is limited by reactions converting it to other products and preventing its accumulation. Coenzyme A, required by the phosphoroclastic system, was frequently stimulatory to nitrogen fixation by these extracts (Table VIII). Other supplements implicated in pyruvate metabolism such as iron, FAD, FMN, cocarboxylase, DPN, TPN, lipoic acid, and folic acid were not effective in the crude extracts.

Chemistry of $^{15}N_2$ in the nitrogen-fixing extracts

In the *C. pasteurianum* extracts the product of nitrogen fixation is NH_3. During early stages of these fixation experiments, all of the $^{15}N_2$ fixed could be recovered as $^{15}NH_3$ by distillation from dilute alkali (Table IX). That the freshly fixed nitrogen was present essentially as NH_3 rather than as easily hydrolyzable amide was indicated

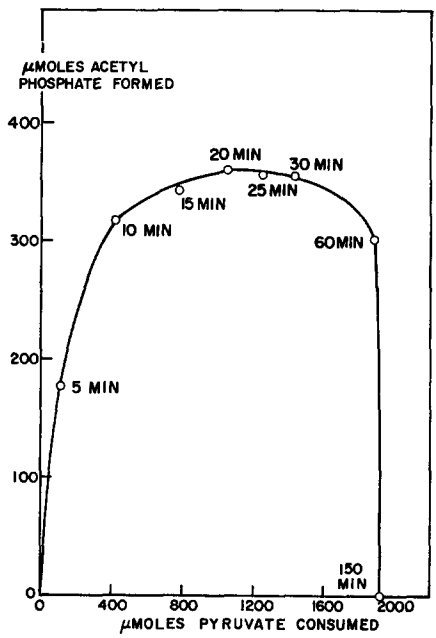

Fig. 5. Time-course of pyruvate consumption and acetyl phosphate content. Procedure was similar to that in Fig. 4. Fixation was interrupted after the varying periods indicated, and the solutions were analyzed for pyruvate[36] and acetyl phosphate[32].

TABLE VII

EFFECT OF PROTEIN CONCENTRATION ON NITROGEN FIXATION IN *C. pasteurianum* EXTRACTS

Pyruvate (mg)	*Protein-N* (mg)*	*Atom % excess ^{15}N*
100	21.7	0.48
100	15.5	1.05
100	9.3	1.39
100	3.1	0.02

* Extract was centrifuged at 144,000 × *g* and aliquots were taken to give protein concentrations indicated. Each flask was made to 8 ml with buffer, and 0.6 atm of 60 atom % $^{15}N_2$ was used. Time of incubation was 1 h.

TABLE VIII

EFFECT OF SUPPLEMENTS IN *C. pasteurianum* EXTRACTS

*Additions to standard assay system**	*Atom % excess ^{15}N*
None	0.53
10 μmoles CoA	0.65
5 μmoles Fe^{++}	0.51
Cocarboxylase (1 μmole)	0.53
Riboflavin-5-PO_4 (10 μmoles)	0.53
Cocarboxylase, CoA, FMN, Fe^{++}	0.61
CoA, (5 μmoles), 2 μmoles Fe^{++}, 2 μmoles FMN	0.63
CoA (5 μmoles), 2 μmoles FMN	0.63
2 μmoles Mo as $Na_2MoO_4 \cdot 2H_2O$	0.56
2 μmoles Mo + 2 μmoles Fe^{++}	0.53
2 μmoles DPN	0.52

* Each flask contained cell-free extract (15 mg protein-N), 100 mg sodium pyruvate, buffer to make the total volume 8 ml, and 0.6 atm of 60 atom % $^{15}N_2$. Time of incubation was 1 h.

TABLE IX

CONVERSION OF $^{15}NH_3$ TO DERIVATIVES WITH TIME DURING FIXATION

Each flask contained cell-free extract (15 mg protein-N), 100 mg sodium pyruvate, enough buffer to make the total 8 ml, and 0.6 atm of 60 atom % $^{15}N_2$. After incubation at 30° for the periods indicated, the solutions were divided into two parts for determination of total ^{15}N content and of ^{15}N content recoverable by alkaline distillation. The difference represented nonhydrolyzable ^{15}N derivatives.

Time (min)	*Total ^{15}N fixed (μg)*	*^{15}N recovered by alkaline distillation (μg)*	*% ^{15}N as $^{15}NH_3$*	*% ^{15}N not hydrolyzable to $^{15}NH_3$*
12	109	109	100	0
20	190	85	45	55
30	244	10	4	96

TABLE X

DISTRIBUTION OF ^{15}N AMONG NH_3, AMIDE AND OTHER FORMS IN NITROGEN-FIXING EXTRACTS

Fixation mixture comprised cell-free extract (16.8 mg protein-N), 100 mg sodium pyruvate, and buffer to make 8 ml.

Forms of nitrogen	*Amounts of nitrogen*		
	Before fixation (μg)	*After fixation (μg)*	*Gain (μg)*
As NH_3*	22.4	258	236
As amide*	437	442	5
Unavailable by alkaline distillation	—	—	6
Total fixed**	—	247	247

* Ammonia and ammonia-plus-amide contents before and after fixation were determined on separate aliquots by distillation from K_2CO_3 and from NaOH, respectively, in micro-Conway dishes[33]. The difference represented amide content.

** Total nitrogen gain of 247 μg was determined from ^{15}N enrichment of 0.94 % resulting from exposure for 1 h to 60 atom % excess $^{15}N_2$. Difference between 247 and sum of ammonia and amide nitrogen contents (241) gave amount of fixed nitrogen converted to forms not recoverable as NH_3 by alkaline distillation.

by tests summarized in Table X. Of the total nitrogen fixed, 95 % was present as ammonia and about 2 % as amide, with the remainder in non-hydrolyzable though Kjeldahl-digestible forms.

Whether there are intermediates between N_2 and NH_3 remains undecided. Certainly, unless a 6-electron transfer occurs, partially reduced forms of N_2 must intervene, although they may be retained on the enzyme until converted to NH_3. Attempts to detect possible fixation intermediates such as hydrazine, hydroxylamine, and nitrite have been unsuccessful. The data on $^{15}NH_3$ recovery argue against a carbon derivative such as dihydropyridazinone-5-carboxylic acid[11] as an intermediate of nitrogen fixation. If it were involved, only 50 % of the ^{15}N fixed should be recoverable as free NH_3 by simple hydrolysis. Furthermore, tests indicated that this compound was not metabolized by the extracts.

TABLE XI

RETENTION OF ^{15}N AMMONIA BY TRAPPING AGENTS IN NITROGEN-FIXING EXTRACTS

Expt.	*Additions to standard assay system*	*^{15}N fixed*	
		Total (μg)	*Recovered by alkaline distillation (μg)*
1a	None	244	10
1b	1.7 mg N as NH_3	214	182
1c	4.0 mg N as amide in glutamine	240	250
2a	None	157	2
2b	4 mg N as NH_3	78	51
2c	4 mg N as amide in glutamine	138	118
3a	None	173	163
3b	4 mg N as NH_3	55	54
3c	100 mg α-ketoglutarate	66	2
3d	100 mg α-ketoglutarate + 4 mg N as NH_3	14	12

Procedures were as for Table IX. Time of incubation 1 h.

Cell-free fixation experiments allowed to proceed more than 10–15 min often contained most of the ^{15}N in a form no longer released by alkaline distillation (Table IX). This effect did not always occur, as apparently the extracts varied in their ability to incorporate NH_3 into organic compounds. Conversion of $^{15}NH_3$ into organic derivatives could be largely suppressed by supplying an initial pool of NH_3 or glutamine to trap by dilution the freshly fixed nitrogen (Table XI). Glutamine had little or no effect on nitrogen fixation, but NH_3 was inhibitory at high concentrations (Table XII). From this, the NH_3 acceptor mechanism could presumably have an important role in determining fixation yield. However, the minimum inhibitory concentration of NH_3 exceeded the levels normally produced in the extracts, and it would therefore appear that NH_3 acceptors were not a rate-limiting factor in nitrogen fixation under present conditions. Specifically, inhibition by NH_3 became detectable when the concentration reached about 200 μg ammonia-N/ml whereas the normal yield was about 30 μg fixed-N/ml.

Extracts varied considerably in ability to convert freshly fixed nitrogen to nonhydrolyzable derivatives; an example is seen in Table XI, 3a, wherein over 90 %

of the fixed nitrogen remained as NH_3 or amide after 60 min. Conversion of freshly fixed nitrogen to nonhydrolyzable derivatives was accelerated by addition of α-ketoglutarate, a known NH_3 acceptor (Table XI, Expt. 3); at the same time, nitrogen fixation was strongly inhibited. An interference with the reduction system is assumed to have occurred, perhaps by antagomism of pyruvate or interception of hydrogen, but the mechanism is not known.

TABLE XII

AMMONIA-INHIBITION OF NITROGEN-FIXATION IN EXTRACTS

*Ammonia-N added/flask**	*% Inhibition*
1.7 mg	10–20 %
4.0 mg	50–60 %
20.0 mg	94–98 %

* Mixture consisted of cell-free extract (15–20 mg protein-N), 100 mg sodium pyruvate, buffer to make 8 ml, and 0.5 atm of 60 atom $^{15}N_2$. Inhibition was determined by comparison of ^{15}N fixed with controls.

To provide an indication of the types of organic nitrogen compounds formed in the crude extracts, the distribution of ^{15}N among various free amino acids was determined[27] by fractionation of the deproteinized extract. The highest concentration of label was in glutamic acid, 21.7 atom % excess ^{15}N, indicating that about one-third of the glutamic acid present was formed from freshly fixed nitrogen. The next most heavily labeled amino acid was not identified but contained only 5.56 atom % excess ^{15}N.

Spectral effects of nitrogen in the extracts

SHUG, HAMILTON AND WILSON[4] have shown that sonic extracts of *C. pasteurianum* undergo spectral changes at 390–450 mμ on exposure to N_2. These authors[34] and later BERGERSEN AND WILSON[35] observed similar effects in sonic extracts of *Azotobacter*

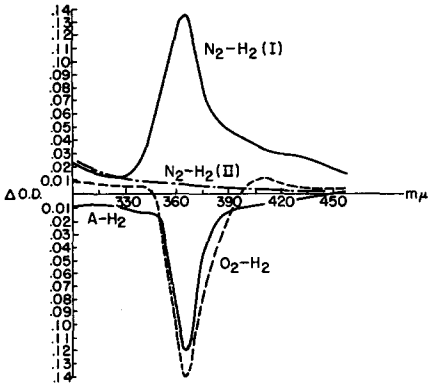

Fig. 6. Difference spectra of pyruvate-conditioned extracts exposed to N_2, A, and O_2 relative to H_2. The extracts contained 1.1 mg protein-N in total volume of 3.0 ml, and their spectra were determined within 2 h after preparation. Extracts were incubated for 15 min at 25° with 40 mg sodium pyruvate except for Curve II where 20 mg was used.

vinelandii and soybean root nodules, in which N_2-induced spectral changes were found at 400 and 550 mμ.

N_2 has now been found to cause characteristic spectral changes in the nitrogen-fixing enzyme preparations from *C. pasteurianum*. Freshly prepared extracts conditioned with sodium pyruvate (40 mg/ml) or sodium α-ketobutyrate exhibit an increase in absorption at 365 mμ when exposed to N_2. The effect is illustrated in Fig. 6, which gives the difference spectra of such preparations measured under N_2, A, and O_2 relative to H_2. Responses to A and O_2 (decreased absorption at 365 mμ) were also obtained and may have been caused by desorption of H_2 since they were reversed by replacing A or O_2 with H_2 in the overlying atmosphere (Fig. 7). The N_2 response was not similarly reversible. In the absence of pyruvate or α-ketobutyrate, the N_2-induced spectral change was located at about 300 mμ rather than at 365 mμ and has been rather erratic in occurrence.

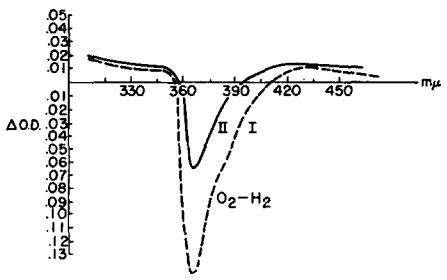

Fig. 7. Difference spectra of pyruvate-conditioned extracts exposed to O_2 reversed by H_2. Curve I represents the difference spectrum after 15 min exposure to O_2 at 0°. Curve II represents the difference spectrum as in I followed by exposure to H_2 for 10 min.

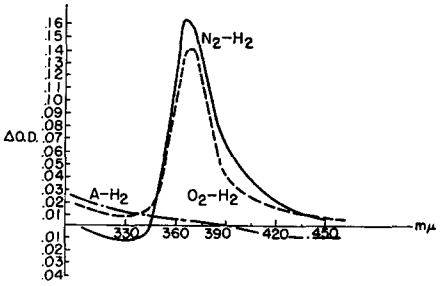

Fig. 8. Difference spectra of pyruvate-conditioned extracts under N_2, A, and O_2 relative to controls under H_2. Extracts were stored 16 h under H_2 before use.

In extracts stored more than 7 h at 5° under H_2, the N_2-induced spectral change decreased with time until after 36 to 48 h it was no longer detectable. Also, the apparent reversible adsorption of H_2 disappeared and the O_2 response became indistinguishable from that of N_2 (Fig. 8). Nitrogen-fixing activity of the extracts decreased to zero at approximately the same time.

These spectral changes are believed to be evidence for the interaction of N_2 with the nitrogen-fixing enzyme system. Their significance to the mechanism of nitrogen fixation is being investigated.

Trial extraction of other organisms

A few attempts were made to obtain nitrogen fixation in extracts of *Azotobacter vinelandii* and *Nostoc muscorum* G.

While activity was obtained, it was weak and variable in contrast to the results with the *C. pasteurianum* extracts. Disruptions were with the Hughes press and also with sonic oscillations. Pyruvate was the only energy source tried. Enrichments were less than 0.06 atom % excess ^{15}N after exposure to 60 atom % excess $^{15}N_2$ for 1 h. One might expect that success in obtaining active extracts from other nitrogen-fixing organisms will perhaps depend as much on finding the proper activator as on using the proper extraction techniques.

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