*REDUCTION OF N_2 BY COMPLEMENTARY FUNCTIONING OF TWO COMPONENTS FROM NITROGEN-FIXING BACTERIA**

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Asymbiotic nitrogen fixation is a common property of a dissimilar group of microorganisms ranging from obligate anaerobes through the facultatives to the obligate aerobes. Representatives are found in the nutritional groups of the chemoorganotrophs, the photoorganotrophs, and the photolithotrophs, including both bacteria and the blue-green algae.

The nitrogenase enzyme complexes from the bacteria have remarkable similarities as demonstrated by the requirement of anaerobiosis, a low-potential electron donor, and adenosine 5′-triphosphate for the reduction of N_2 to ammonia by *Clostridium pasteurianum*,[1-3] *Azotobacter vinelandii*,[4] *Rhodospirillum rubrum*,[4] *Klebsiella pneumoniae*,[5] and *Bacillus polymyxa*.[6] Purification of the nitrogenase enzyme complex from *C. pasteurianum*,[7] *A. vinelandii*,[8] and *B. polymyxa*[9] has demonstrated that at least two components are necessary for the reduction of nitrogen. This communication reports investigations designed to determine whether a fraction prepared from one organism will function with a fraction from another.

Materials and Methods.—Azotobacter vinelandii OP, *Clostridium pasteurianum* W-5, *Bacillus polymyxa* Hino, and *Klebsiella pneumoniae* M5A1 were grown and harvested as described previously.[5, 6, 10] Cell-free extracts from *K. pneumoniae*, *B. polymyxa*, and *A. vinelandii* were prepared by passing cell suspensions (10–20 gm cells) through a French pressure cell and centrifuging at 30,000 × *g* for 30 min. Extracts of *C. pasteurianum* were prepared by treating cell suspensions with lysozyme and deoxyribonuclease,[6] then centrifuging at 30,000 × *g* for 30 min. Because of the oxygen sensitivity of nitrogenase, all operations were performed in an atmosphere of helium or nitrogen. The cell-free extracts contained 30–50 mg protein/ml.

Column chromatography: Chromatography on *O*-(diethylaminoethyl)cellulose (DEAE-cellulose) (Sigma 0.99 mEq/gm) was performed on either 1.4 × 10-cm columns (*C. pasteurianum*) or 2.5 × 10-cm columns for the other organisms. The DEAE-cellulose, suspended in 0.025 *M* phosphate buffer, was packed into the columns by gravity. After the columns had been made anaerobic by washing with N_2-saturated 0.025 *M* phosphate buffer (100 ml), dithionite (dry powder) was added to the buffer (0.2–0.3 mg/ml) and washing was continued with this reduced buffer until the column eluate was reduced.[11, 12] Anaerobic NaCl solutions were prepared in the same manner as described for the phosphate buffer. Up to 1.5 gm protein was syringed onto the large columns and up to 500 mg protein was syringed onto the small columns. The columns were then eluted sequentially with 0.025 *M* phosphate buffer, 0.175 *M* NaCl, 0.3 *M* NaCl, and 0.5 *M* NaCl. The NaCl solutions were prepared in 0.025 *M* phosphate buffer, pH 7.0.

Assays: N_2 reductions were estimated by the method of Bulen *et al.*[4] with minor modifications. The assays were made in 20-ml serum bottles incubated on a reciprocal shaker for 30 min at 30°C. The nitrogen fixed (corrected for that formed in a helium control) was estimated by the technique described by Dilworth *et al.*[3] Other details of the analytical procedures have been previously described.[5, 10, 13]

Results.—Splitting of components I and II: Figure 1 illustrates a typical elution pattern for extracts of *B. polymyxa*. Component I, eluted by 0.3 *M* NaCl,

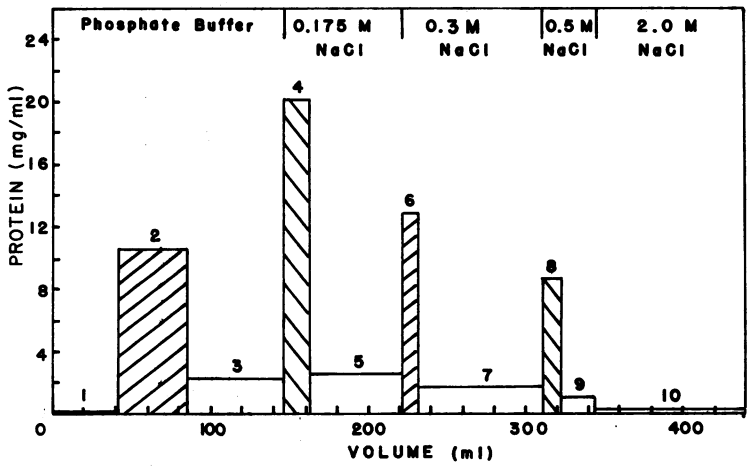

FIG. 1.—Fractionation of crude extract of *B. polymyxa*.

is dark brown (fraction 6). Component II, eluted by 0.5 *M* NaCl, is yellow (fraction 8). None of the other fractions obtained stimulated N_2 reduction when recombined. Components I and II are found always in N_2-grown cells, but neither is present in detectable amounts in NH_4^+-grown cells. The elution patterns for the other organisms are essentially the same as that shown for *B. polymyxa;* with each organism the components could be followed visually on the column. Component I had no N_2-fixing activity, but component II generally had a small amount of activity, presumably because of contamination with component I. The residual activity of component II can be reduced considerably by washing the column with large amounts of 0.3 *M* NaCl after collection of component I. The purification achieved in this one-step fractionation is four- to sixfold when the specific activity is based on the total recombined protein of components I and II. Table 1 summarizes the splitting of the nitrogenase enzyme complex into two components for each of the four bacteria used in this study. The components were obtained in approximately equal volumes from the DEAE columns; because of the limited amount of the components, only 1:1 (v/v) mixtures of two were used in the assays.

Tables 2, 3, and 4 present three typical reactions obtained with certain recombinations. Each test included controls of the components assayed separately and in homologous recombination. The heterologous crosses between the frac-

TABLE 1. *Splitting of crude cell-free extracts into components I and II.*

	Specific Activity				Purification (-fold increase)
	Crude extract	I	II	I and II	I and II
C. pasteurianum	7.0	0	3.0	38.4	5.4
B. polymyxa	5.0	0	5.4	19.4	3.9
K. pneumoniae	4.2	0	5.4	23.8	5.7
A. vinelandii	3.6	0	0	20.7	5.8

Specific activity: nanomoles N_2 fixed/mg protein/min. I, 0.3 *M* NaCl fraction; II, 0.5 *M* NaCl fraction.

TABLE 2. *Recombination between components of cell-free extracts of Clostridium pasteurianum and Azotobacter vinelandii.*

Component			Protein (mg/ml)	N-fixed (μg/ml)	Increase in N-fixed (μg/ml)
C. pasteurianum		*A. vinelandii*			
I			1.2	0.3	—
II			0.5	1.5	—
I and II			1.7	24.0	22.2
		I	3.8	0	—
		II	1.3	0	—
		I and II	5.1	19.1	19.1
I	and	II	2.5	0	0
II	and	I	4.3	0	0

TABLE 3. *Recombination between components of cell-free extracts of Bacillus polymyxa and Klebsiella pneumoniae.*

Component			Protein (mg/ml)	N-fixed (μg/ml)	Increase in N-fixed (μg/ml)
B. polymyxa		*K. pneumoniae*			
I			1.1	0.6	—
II			1.5	0.2	—
I and II			2.6	16.4	15.6
		I	1.1	0	—
		II	1.7	5.6	—
		I and II	1.8	30.3	24.7
I	and	II	1.8	10.5	4.3
II	and	I	2.6	11.7	11.5

TABLE 4. *Recombination between components of cell-free extracts of Bacillus polymyxa and Clostridium pasteurianum.*

Component			Protein (mg/ml)	N-fixed (μg/ml)	Increase in N-fixed (μg/ml)
B. polymyxa		*C. pasteurianum*			
I			2.1	0	—
II			0.6	0.8	—
I and II			2.7	18	17.2
		I	1.3	0	—
		II	0.2	1.8	—
		I and II	1.5	24	22.2
I	and	II	2.3	14.3	12.5
II	and	I	1.9	0.3	−0.5

TABLE 5. *Recombination between components of cell-free extracts of four N_2-fixing bacteria.*

Component I	Component II	Protein (mg/ml)	N-fixed* (μg/ml)
B. polymyxa	*A. vinelandii*	3.5	−0.3
A. vinelandii	*B. polymyxa*	4.4	6.7
K. pneumoniae	*A. vinelandii*	2.5	7.8
A. vinelandii	*K. pneumoniae*	4.3	7.3
K. pneumoniae	*C. pasteurianum*	1.7	−0.8
C. pasteurianum	*K. pneumoniae*	1.7	−1.5

* Corrected for controls as in Tables 2, 3, and 4.

tions from the strict aerobe, *A. vinelandii*, and the strict anaerobe, *C. pasteurianum*, were both negative, whereas the reciprocal crosses between the fractions from the facultative anaerobes, *K. pneumoniae* and *B. polymyxa*, were both positive. *B. polymyxa* and *C. pasteurianum* are related organisms, both belonging to the Bacillaceae, but only one of the heterologous crosses was positive, fraction I from *B. polymyxa* and fraction II of *C. pasteurianum*. Table 5 summarizes the results of tests of the other crosses; in all these the homologous cross control was always positive, and the controls on the individual fractions gave results similar to those shown in Tables 2, 3, and 4.

TABLE 6. *Summary of cross reactions between components from N-fixing bacteria.*

	Component I			
Component II	*A.v.*	*K.p.*	*B.p.*	*C.p.*
A.v.	+	+	+	−
K.p.	+	+	+	−
B.p.	−	+	+	+
C.p.	−	−	−	+

A.v. = *Azotobacter vinelandii*; *B.p.* = *Bacillus polymyxa*; *K.p.* = *Klebsiella pneumoniae*; *C.p.* = *Clostridium pasteurianum*.
\+ = complementary functioning.

Discussion.—Table 6 is a summary of the complementary functioning of components I and II of the N_2-fixing enzyme complex from the four bacteria. Six of the twelve possible crosses were positive with respect to nitrogen fixation as measured by production of NH_3. The facultative anaerobic bacteria, *K. pneumoniae* and *B. polymyxa*, exhibit the widest range of complementarity; the obligate aerobe, *A. vinelandii*, and the obligate anaerobe, *C. pasteurianum*, have more restricted ranges.

Current research is directed toward examination of the possible origin of the observed negative crosses. These may have physiological explanations or arise merely from deficiencies in the techniques. For example, each organism may construct the essential proteins in a sufficiently different form that some cannot bind together in an active heterologous complex. Significantly, the activity of the positive heterologous crosses was always less than that of the homologous control. It is recognized that an implicit assumption in this study is that the two fractions always contained all necessary components. The controls demonstrate that this is true for homologous combinations but may not be so for a heterologous combination if more than two proteins are required for the functioning of the system. Another factor may be that the optimum stoichiometric ratio of the fractions is not 1:1. Finally, the heterologous combinations may be so inefficient that nitrogenase activity was not detected by estimation of ammonia by the microdiffusion technique but could have been by a more sensitive test such as reduction of acetylene.

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