*FERREDOXIN AND ATP, REQUIREMENTS FOR NITROGEN FIXATION IN CELL-FREE EXTRACTS OF CLOSTRIDIUM PASTEURIANUM**

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Because N_2 fixation in cell-free extracts of *Clostridium pasteurianum* is coupled to the metabolism of pyruvate,[1] and because the conversion of N_2 to ammonia obviously requires reductive steps, it has been assumed that electrons released during pyruvate metabolism in some way served as the source of electrons for N_2 reduction. Pyruvate oxidation in extracts of this organism is coupled to the reduction of ferredoxin (Fd), the iron sulfide containing electron carrier isolated from *Clostridium pasteurianum*.[1] Recently, it was demonstrated that Fd is an indispensable requirement for N_2 fixation when pyruvate serves as the electron donor.[2] If all that is required for N_2 fixation is a source of high-energy electrons, present results suggest that any oxidation resulting in the production of reduced Fd should support N_2 fixation in these extracts. However, in previous experiments H_2, which readily serves as the electron donor for reducing Fd via the enzyme hydrogenase, did not substitute for pyruvate when mixed with N_2 in the gas phase above an active extract. This suggested that in these experiments either H_2 was inhibitory to N_2 fixation as had been shown with whole cells of many N_2-fixing organisms,[3] or that a component in addition to reduced Fd is required. Several pieces of indirect evidence suggested that there was an additional component needed and that it might be ATP.[1, 4] For example,[4] arsenate inhibited N_2 fixation, and it is known that arsenate indirectly inhibits the synthesis of ATP in these extracts by competing with phosphate in the phosphotransacetylase reaction. Although many attempts were made to obtain N_2 fixation with H_2 and ATP as supporting substrates, no measurable N_2 was fixed when either a high or low level of ATP was added to active cell-free extracts of *C. pasteurianum* under an atmosphere of H_2 and N_2. However, Arnon *et al.*[5] have reported that fixation of N_2 by an extract from *Chromatium* sp. is enhanced by H_2.

This report describes the procedures for obtaining N_2 fixation with ATP as energy source and reduced Fd as electron source as well as a new, rapid, and quantitative method for measuring N_2 fixation based on these findings.

Materials and Methods.—*C. pasteurianum* was grown on a modified Winogradsky medium containing 1% sucrose and 1% $CaCO_3$,[6] or at a controlled pH of 5.5 without $CaCO_3$. Cells were harvested, washed, dried, and extracted by methods previously described.[7] The crude cell extract was diluted to 30 mg protein per ml, and treated with 60 mg protamine sulfate per gm protein to remove nucleic acids. The clear protein solution obtained after centrifugation was stored under H_2. It is referred to as the protamine sulfate preparation (PS-1). To show an ATP requirement, the crude or PS-1 preparations were dialyzed against 0.01 *M* tris-HCl buffer at pH 7.5 anaerobically under H_2 at 25° for 4–6 hr; N_2-fixing activity lost by further dialysis was not restored by ATP and Mg^{++}. The uptake of hydrogen and nitrogen was measured quantitatively in standard Warburg flasks[8] gassed through h-tubes fitted to the manometers. Gases were freed of oxygen[9] by passing them through reduced copper columns at 170°. Ferredoxin was removed from the crude or PS-1 preparations (without the loss of N_2-fixing activity) by titration with DEAE cellulose.[2] Protein was determined by the biuret method,[10] and acetyl phosphate by the method

of Lipmann and Tuttle as modified by Stadtman.[11] Ferredoxin was prepared by the method of Mortenson[12] modified by adding DEAE batchwise to the acetone supernatant solution. The components contaminating the Fd were removed from the DEAE cellulose on a Buchner funnel by washing with 0.2 *M* tris-HCl at pH 7.5, and the Fd was eluted with the same buffer at 0.5 *M* concentration. Acetyl phosphate was prepared by the method of Avison as described by Stadtman.[11]

Measurement of N_2 fixed: The amount of N_2 fixed was determined by the ammonia synthesized.[13] In addition, a second method was developed from the information reported in this paper; the combined μl of H_2 and N_2 consumed was measured and was corrected for the μl of H_2 consumed in a similar flask containing H_2 and argon. The μmoles ammonia synthesized was obtained by dividing the corrected total μmoles of H_2 plus N_2 consumed by 2, i.e., $N_2 + 3H_2 \rightarrow 2NH_3$. The methods agreed well.

Results and Discussion.—N_2 fixation with H_2 gas as electron source and acetyl phosphate as the source of high-energy phosphate: Figure 1 shows that N_2 fixation occurs readily with H_2 as the electron source and acetyl phosphate as the source of high-energy phosphate for generation of ATP. No measurable N_2 was fixed when either H_2 or acetyl phosphate was omitted. In other experiments, rates of N_2 fixation from 0.32 to 1.70 μmoles NH_3 synthesized/mg protein/hr have been obtained. These rates are similar to those obtained with pyruvate as the electron donor.

The total ammonia synthesized with 150 μmoles acetyl phosphate as the high-energy phosphate source was from 6.5 to 7.4 μmoles. After this amount of ammonia was synthesized, no acetyl phosphate remained, and N_2 fixation stopped abruptly. From 15 to 20 μmoles of acetyl phosphate were consumed for each μmole of ammonia synthesized. In the control flasks, with 0.5 atm argon and 0.5 atm H_2, the rate of acetyl phosphate consumption was the same as in 0.5 atm N_2 and 0.5 atm H_2, but when the atmosphere contained only argon or N_2 (or argon-N_2 mixtures), the rate of acetyl phosphate consumption was much less. Since, under the conditions of these experiments, acetyl phosphate utilization occurs only through ATP:acetate phosphotransferase, the nitrogen-fixing system in the absence (and to some extent in the presence) of N_2 acts like a H_2-requiring "ATPase."

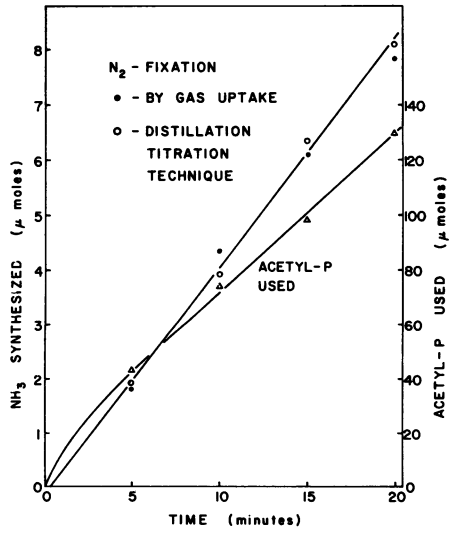


FIG. 1.—Time course of N_2 fixation versus acetyl phosphate utilization. The complete system in a total volume of 3 ml in a Warburg flask at 30°C consisted of: 40 mg extract protein (PS-1) at pH 6.5, 1 μmole Mg^{++} and water to 2.6 ml in the main compartment, and 150 μmoles lithium acetyl phosphate in 0.4 ml water in the side arm. The gas phase contained 0.5 atm N_2 and 0.5 atm H_2 in the experimental flask, and 0.5 atm argon and 0.5 atm H_2 in the control flask. When H_2 was omitted, a full atm of N_2 was used, and the control flask contained an atm of argon. The reaction was initiated by tipping the acetyl phosphate. The μmoles NH_3 synthesized was determined as described in *Methods*. No N_2 was fixed when either H_2 or acetyl phosphate was omitted.

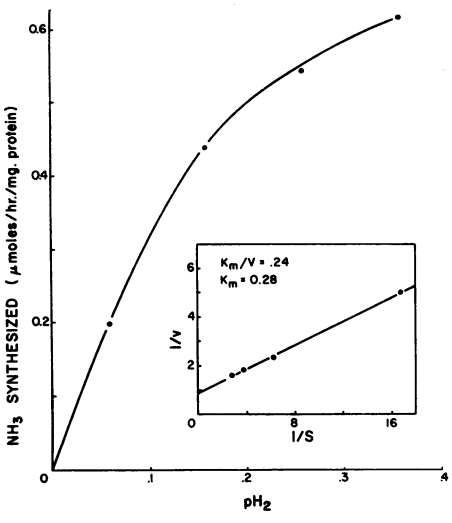


FIG. 2.—Pressure of H_2 required for maximum N_2 fixation. Conditions were the same as those in Fig. 1 with the exception that 75 mg protein as crude extract was used. N_2 was maintained at 0.6 atm, and H_2 was varied as indicated.

Partial pressures of H_2 and N_2 required for maximum nitrogen fixation: In Figure 2, N_2 was maintained at 0.6 atm, and H_2 was varied from 0.0 to 0.4 atm; the pressure then was made to 1 atm with argon. The rate of N_2 fixation increased from 0.0 to 0.6 μmoles/mg protein/hr when the H_2 was increased from 0.0 to 0.4 atm. The rate was the same at 0.5 atm H_2 and 0.5 atm N_2 as it was at 0.4 atm H_2 and 0.6 atm N_2, but it decreased when the atmosphere contained 0.6 atm H_2 and 0.4 atm N_2. When the data were plotted according to Lineweaver and Burk, the K_m as shown in Figure 2 was 0.28 atm. This result was based on the best straight-line approximation obtained in the plot of $1/v$ versus $1/s$; the K_m was calculated from the slope and intercept of this line.

The effect of increasing pressure of N_2 on N_2 fixation with H_2 as electron donor and acetyl phosphate as high-energy phosphate donor is presented in Figure 3. In these experiments, H_2 was maintained at 0.4 atm, and the N_2 varied from 0.0 to 0.3 atm. When plotted on the basis of initial velocities according to Lineweaver and Burk, the K_m was found to be 0.17 atm. This K_m is the same as the K_m (0.17–0.20 atm) found for N_2 fixation with the same extract but with pyruvate as the indirect source of reduced Fd and acetyl phosphate.

When we assay N_2 fixation manometrically, the gas phase above the reaction mixture is adjusted routinely to 0.5 atm N_2 and 0.5 atm H_2, and a control flask containing 0.5 atm argon and 0.5 atm H_2 is always included.

Acetyl phosphate requirement: H_2 will serve as the source of electrons for N_2 reduction. However, even with H_2 present, no N_2 is fixed unless a continuous source of ATP is available. At present purity, our cell-free extracts require 15–20 μmoles of high-energy phosphate for each μmole of ammonia synthesized. Because the assay is not sensitive enough to detect the synthesis of less than 0.3 μmoles of ammonia, and because ATP inhibits N_2 fixation at concentrations greater than 5 μmoles/ml when the protein concentration is 20 mg/ml, a continuous supply of ATP was maintained by adding to the reaction mixture substrate amounts of acetyl phosphate in the presence of ATP: acetate phosphotransferase and 1 μmole of either ATP or ADP. These extracts already contain this ATP-generating system (up to 100 μmoles of ATP are synthesized from acetyl phosphate/mg extract protein/hr).

In Figure 4, the μmoles of ammonia synthesized at increasing concentrations of acetyl phosphate were plotted against time. The rate at which N_2 was fixed was the same at acetyl phosphate concentrations of 37, 75, and 112 μmoles/3 ml, and the total nitrogen fixed increased linearly within this range. At concentrations of acetyl phosphate of 150, 187, and 225 μmoles/3 ml, the rate of N_2 fixed decreased,

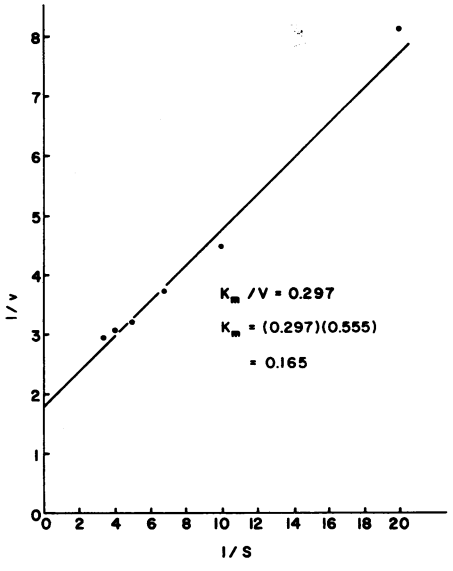


FIG. 3.—Pressure of N_2 required for maximum N_2 fixation, Lineweaver-Burk plot. Conditions the same as Fig. 1 with the exception that 60 mg protein as crude extract was used in each reaction flask. H_2 was maintained at 0.5 atm, and the atm of N_2 (S) was varied as indicated. The final atm was made to 1 with argon. Velocity is expressed as μmoles NH_3 synthesized/mg protein/hr.

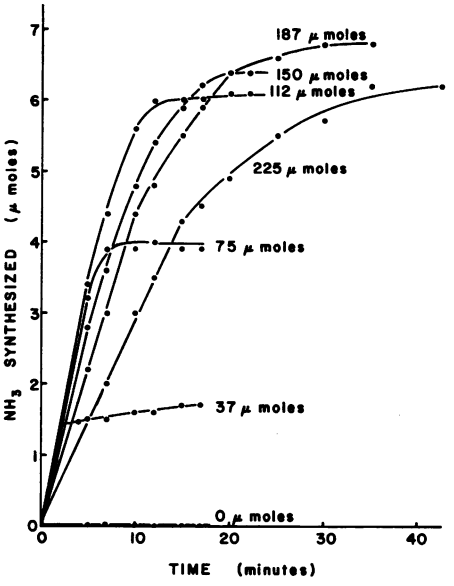


FIG. 4.—Effect of acetyl phosphate on nitrogen fixation with H_2 as electron donor. Conditions as in Fig. 1. Concentration of acetyl phosphate as indicated. Protein content per flask was 82 mg.

and at these concentrations the total ammonia synthesized, 6.2–6.7 μmoles, during the limited period of measurement, was only slightly higher than the 6.1 μmoles synthesized with 112 μmoles acetyl phosphate present.

Acetyl phosphate at a concentration of 21 μmoles/ml was reported to be 100 per cent inhibitory to N_2 fixation when pyruvate was the electron donor.[7] In contrast, in the present experiments with H_2 as the electron donor not only is acetyl phosphate not inhibitory but it is required. Perhaps in our earlier experiments[7] with pyruvate as electron donor, an inhibitory component was present in the sample of acetyl phosphate used. In recent experiments with pyruvate as electron and energy donor, even though we still found an inhibition of N_2 fixation by acetyl phosphate, the inhibition was considerably less than that previously reported, particularly when high concentrations of the enzyme were used. It also was found that a high concentration of Li^+ inhibited N_2 fixation when pyruvate served as electron and energy source; possibly Li^+ may inhibit some step in the pyruvic acid dehydrogenase system, but not acetokinase, hydrogenase, or the enzymes involved in N_2 fixation.

Concentration of cell-free extract: When pyruvate served as the source of electrons and high-energy phosphate for N_2 fixation, fixation was obtained with cell extracts with as little as 1–2 mg protein/ml. In contrast, with H_2 as the electron donor and acetyl phosphate as the energy source, little or no ammonia is synthesized from N_2 by cell-free extracts even with as much as 5 mg protein/ml. In one experiment,

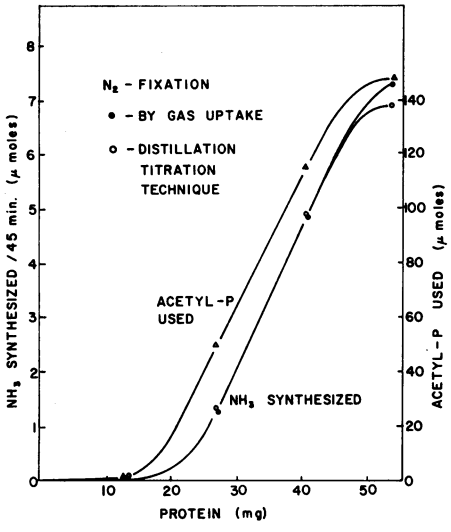


FIG. 5.—Effect of enzyme concentration on N_2 fixation and acetyl phosphate consumption. Conditions as in Fig. 1. PS-1-type preparation was used.

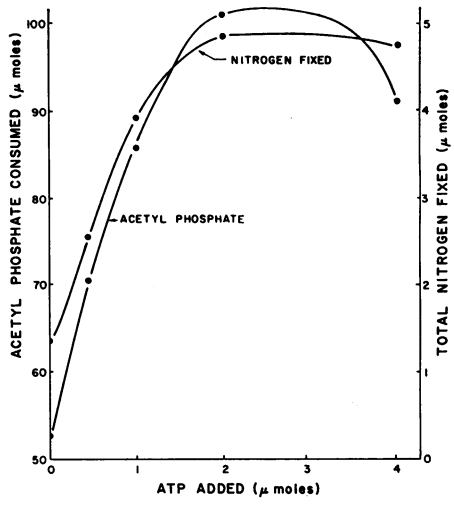


FIG. 6.—Comparison of effect of ATP on nitrogen fixation and on acetyl phosphate consumption. Conditions as in Fig. 1. Fifty-six mg protein as dialyzed enzyme was added per flask.

the rate of NH_3 synthesized increased with increasing protein concentration from 5 to 22 mg protein/ml. At 5 mg protein/ml not only was there no N_2 fixed but little if any acetyl phosphate was consumed (Table 1 and Fig. 5). For example, the efficiency of N_2 fixation, expressed as μmoles acetyl phosphate used per μmole NH_3 synthesized, increased with increasing protein concentration of the cell extract (Table 1 and Fig. 5). These results show that certain components must be present in high concentration to function in H_2 and acetyl phosphate-supported N_2 fixation. One explanation is that the equilibrium of the reaction, Fd + H_2 + hydrogenase $\rightleftharpoons$ reduced Fd + $2H^+$, is greatly in favor of H_2 evolution and therefore a large excess of hydrogenase and H_2 is required to maintain an optimal reduced Fd level. This is not a problem when pyruvate is the electron donor for N_2 fixation because Fd is reduced during pyruvate oxidation by the pyruvic dehydrogenase system and the equilibrium of this reaction is far to the right: CoA·SH + Fd + pyruvate $\rightleftharpoons$ reduced·Fd + acetyl·S·CoA. One might expect that when pyruvate serves as the electron donor, hydrogenase would inhibit by competing with "nitrogenase" for reduced Fd. When pyruvate, in contrast to H_2, is the elec-

TABLE 1

EFFECT OF CELL EXTRACT ON N_2 FIXATION AND ACETYL PHOSPHATE CONSUMPTION*

Protein (mg/ml)	μMoles NH_3 Synthesized/mg Protein/hr	μMoles NH_3 Synthesized/mg Total	Acetyl phosphate used (μmoles)	μMoles acetyl phosphate used/μmole NH_3
5.4	0.00	0.00	15	..
8.1	0.30	2.55	79	31
10.7	0.47	5.42	117	22
13.4	0.55	6.35	150	23
16.1	0.62	6.82	150	22
21.5	0.68	7.35	150	20

* Conditions as in Fig. 1. Time of experiment was 30 min. The rate is based on the first 10 min during which time the acetyl phosphate was nonlimiting.

tron donor, optimal fixation occurs at protein concentrations as low as 1–2 mg/ml, whereas at 2.5 mg protein/ml and higher, considerable reduction in fixation occurs.

ATP partially alleviates the effect on N_2 fixation of the dilution of cell extracts with H_2 as electron donor. For example, the rate of N_2 fixation by a cell extract with 26 mg protein/ml was 0.36 μmoles NH_3 synthesized/mg protein/hr with no ATP added and 0.43 with ATP added; with 13 mg protein/ml as cell extract the rate was 0.22 without ATP and 0.45 with ATP. At a concentration of 6.5 mg protein/ml, however, no N_2 fixation occurred with or without added ATP. Other factors, such as Fd, DPN, TPN, CoA, Mg^{++}, and $KHCO_3$, did not restore the fixation activity lost when the cell extracts were diluted, and TPN and CoA proved inhibitory to N_2 fixation even at optimal protein concentrations. However, addition of 2 μmoles of ATP and 10 μmoles of Mg^{++}/3ml restored activity when the protein concentration was diluted to 6.5 mg/ml.

ATP requirement for N_2 fixation: Logical roles that acetyl phosphate could play in N_2 fixation include formation of acetyl·S·CoA which could function in formation of an ammonia acceptor, and service as a high-energy phosphate pool for the synthesis of ATP from ADP catalyzed by ATP:acetate phosphotransferase. As evidence for the latter hypothesis it was found that ATP restored the N_2-fixing activity lost by a cell extract on dialysis (Fig. 6). Complete removal of ATP or ADP was not attempted because prolonged dialysis removed factors required for N_2 fixation in addition to ATP and Mg^{++}. About 90 per cent of the N_2-fixing activity was lost after 6–8-hr dialysis, and half of it was restored by the addition of ATP. The restoration was specific for ATP (or ADP), since the triphosphates of guanosine, uridine, and cytidine were inactive when added at the same (or higher) concentration optimal for fixation with ATP. GTP and CTP markedly inhibited the residual 10 per cent N_2-fixing activity and acetyl phosphate utilization.

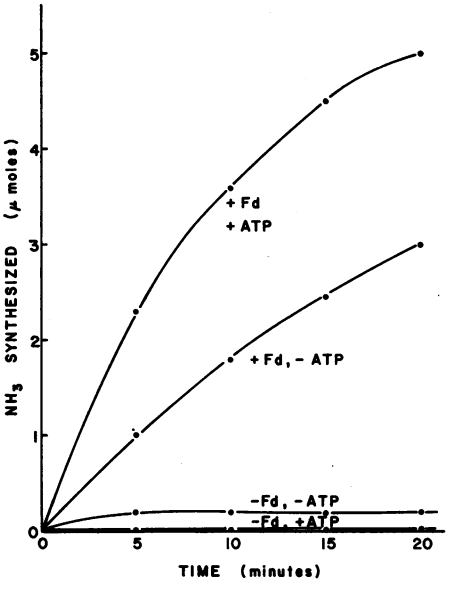


FIG. 7.—Fd and ATP requirements for N_2 fixation with H_2 as electron donor and acetyl phosphate as high-energy phosphate donor. Conditions as in Fig. 1. Fd-free extract was added at 50 mg protein per flask. Where designated, 2 μmoles ATP was added.

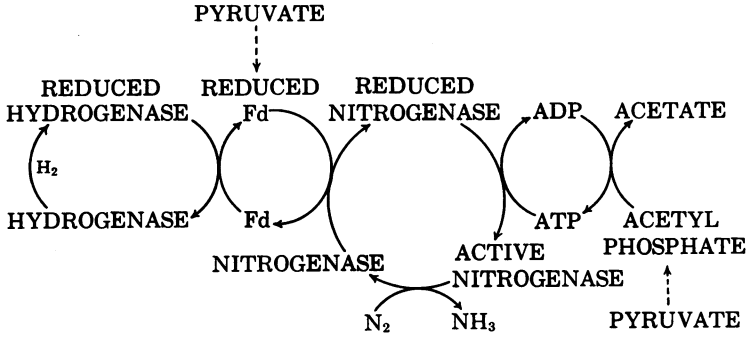
Figure 6 provides evidence that ATP rather than acetyl phosphate is required directly for N_2 fixation since both N_2 fixation and acetyl phosphate consumption are dependent upon the presence of ATP (ADP).

Ferredoxin requirement: Just as Fd is required for N_2 fixation when pyruvate serves as the electron donor, so also Fd is an absolute requirement for N_2 fixation with H_2 as the electron donor. This is not unexpected because previous reductions with H_2 as electron donor also required Fd[1] (e.g., the reduction of NO_2^- to NH_3). Figure 7 shows that a Fd-free extract does not fix N_2 when H_2 is present as electron

donor and acetyl phosphate as a source of high-energy phosphate for ATP synthesis. Fd restored N_2-fixing activity. Although this was not a dialyzed preparation, under the conditions of this experiment, the addition of ATP greatly increased N_2 fixation. The rate of consumption of acetyl phosphate in the absence of Fd, but with ATP added, was less than half the rate observed with Fd restored; at least part of the consumption of acetyl phosphate is Fd-dependent.

Discussion.—In extracts of *C. pasteurianum*, H_2 serves as the source of electrons for the synthesis of NH_3 from N_2, but because ferredoxin is required, it is obvious that the more immediate source of electrons for N_2 reduction is reduced Fd. In addition to H_2, a continuous low level of ATP is required. In these extracts this is accomplished by adding substrate levels of acetyl phosphate to the enzyme preparations which already contain acetate:ATP phosphotransferase and a low level of ADP. Enzyme preparations of present purity require 15–20 μmoles of ATP (based on acetyl phosphate utilization) for each μmole of NH_3 synthesized. Because many enzyme systems in these extracts may compete for acetyl phosphate, the amount actually needed for N_2 fixation is undoubtedly less than that needed to maintain fixation in the crude extracts. We are presently purifying the extracts to determine the stoichiometry of the reaction.

The observation that much less acetyl phosphate is consumed when cell extracts are incubated under an atmosphere of argon or nitrogen than under an atmosphere of H_2 or H_2 mixed with argon or nitrogen suggests that ATP utilization depends upon H_2. Since at least part of the acetyl phosphate consumed is reduced Fd-dependent and because N_2 fixation is reduced Fd-dependent, it is possible that ATP is required to activate "reduced nitrogenase." Present results suggest the following mechanism:



ATP also could activate nitrogenase prior to the reductive steps. Phosphorylated nitrogenase might then "activate" N_2 which could be reduced *in situ*. The fact that N_2 has no apparent effect on ATP utilization in the absence of H_2, whereas H_2 in the absence of N_2 appears to be required for ATP utilization, suggests that ATP is activating "reduced" nitrogenase rather than "oxidized" nitrogenase.

Summary.—ATP and reduced Fd have been shown to be components required for converting N_2 to ammonia in cell extracts of *Clostridium pasteurianum*. In our system acetyl phosphate is used as the source of high-energy phosphate for continuous ATP synthesis, and H_2 is used as the source of electrons for maintaining a

constant supply of reduced Fd. The Michaelis constants (K_m) for H_2 and N_2 are 0.28 and 0.17 atm, respectively.

Evidence is presented for the hypothesis that ATP activates "reduced" nitrogenase which in turn reacts with N_2.

A method is described for measuring N_2 fixation in this system by following the uptake of N_2 and H_2 manometrically.

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*PRODUCTION OF ANTIBODIES TO DENATURED DEOXYRIBONUCLEIC ACID (DNA)**

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In 1956, when we initiated our study of DNA as an antigen, relatively few decisive data were available,[1, 2] and there was no evidence that chemically pure DNA by itself could induce the formation of antibody. However, the possibility remained that some natural complexes containing DNA, or DNA coupled to a suitable carrier, might elicit the formation of antibodies as a hapten. Initially, therefore, we employed DNA-rich preparations from Brucella that had been isolated by the phenol procedure of Braun *et al.*[3] These contained DNA-complexes that, in rabbits, elicited the formation of precipitins against several antigens in the DNA-rich preparation. At least one of these was sensitive to the action of DNA-ase[4–6] and was found to be a species-specific complex of DNA and polysaccharide.[6]

In the meantime, evidence accumulated that antibodies capable of reacting with pure DNA are formed under certain conditions. Thus, antibodies that react