INHIBITION OF NITROGEN FIXATION BY FLUOROACETATE

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The reactions involved in the activation and reduction of molecular nitrogen to ammonia by nitrogen fixing bacteria are probably coupled to known reactions of the cell for energy, and for a source of coenzymes and intermediate compounds. The dependence of nitrogen fixation by cell free extracts of Clostridium on metabolism of pyruvate is an example of a necessary coupling in the anaerobic system (Mortenson, 1961). In the aerobic Azotobacter the tricarboxylic acid (TCA) cycle of reactions is considered to be a major pathway of cell metabolism. In addition it provides intermediate compounds for cellular synthesis and as such, may be involved in the nitrogen fixing process. Therefore, the effect on nitrogen fixation of inhibiting the operation of the TCA cycle was evaluated.

The data presented below demonstrate that nitrogen fixation is inhibited completely by fluoroacetate. Whether or not the inhibition is direct or through suppression of the TCA cycle has to be ascertained.

Methods: Cultures of Azotobacter vinelandii were grown on Burk's sucrose medium (Newton et al., 1953) in 500 ml shake flasks from a 2% inoculum in 16 hours. Aliquots of the cultures in the logarithmic phase of growth were exposed to a 95% N^{15}-enriched gas mixture (N_2:O_2:He::2:2:6) or to $(NH_4)_2$ SO_4 enriched 97% with N^{15} and the effect of fluoroacetate on N^{15} incorporation was determined. Samples were processed and N^{15} was analyzed by the method of Burris and Wilson (1957).

Results and Discussion: Fluoroacetate is a competitive inhibitor of aconitase through the formation of fluorocitrate and as such, can regulate the oxidation of substrates through the tricarboxylic acid cycle (Peters, 1957).

Azotobacter cultures respond to fluoroacetate by lowered oxygen uptake which declines 60% at 5 x 10^{-3} M and by accumulation of citrate which is apparent at 5 x 10^{-4} M fluoroacetate and higher concentrations.

Nitrogen fixation was completely inhibited by 1 x 10^{-3} M fluoroacetate. NH_4^+, a postulated intermediate compound in the fixation of N_2 (Newton et al., 1953), was incorporated into cellular protein at this concentration of fluoroacetate but its rate was reduced about 75%. Figure 1 shows that 5 times this concentration of inhibitor did not suppress $N^{15}H_4^+$-incorporation completely. At 5 x 10^{-5} M fluoroacetate, $N^{15}H_4^+$-incorporation was inhibited 54% whereas N_2^{15}-fixation was only mildly affected (14%).

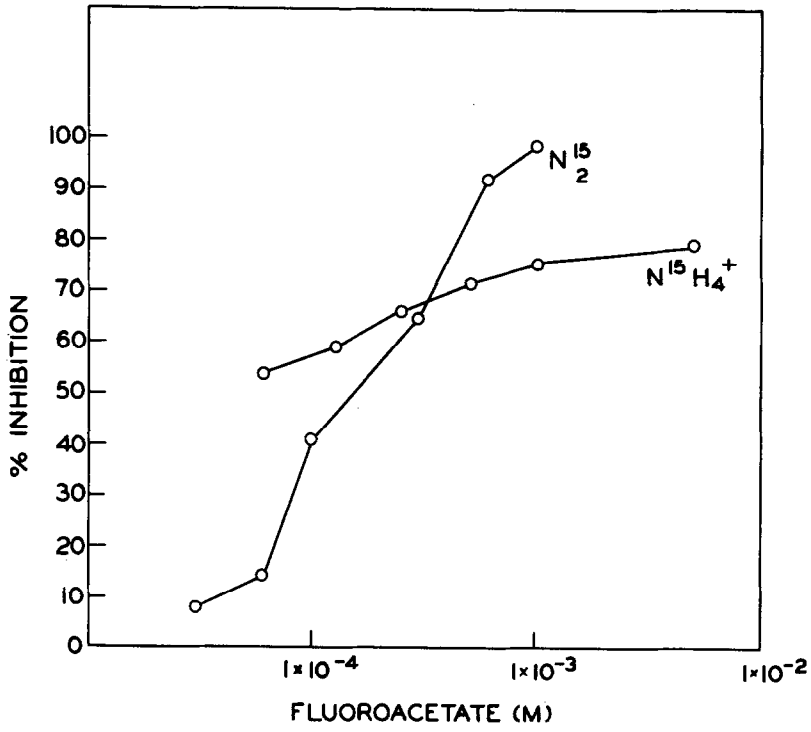

Figure 1. Fluoroacetate Inhibition of Nitrogen Fixation. N_2-fixation: Eight-ml aliquots of Azotobacter culture were exposed to N^{15} gas mixture for 15 minutes. Fluoroacetate was added 3 minutes before the gas. Total culture was processed for N^{15} analysis. NH_4-incorporation: Ten-ml aliquots of Azotobacter culture were incubated with 17 μmoles of N^{15} enriched $(NH_4)_2SO_4$ for 15 minutes. Fluoroacetate was added at zero time. Only twice washed cell residue, precipitated with N/10 H_2SO_4, was processed for N^{15} analysis.

The complete suppression of N_2-fixation by the same concentration of fluoroacetate that inhibited NH_4^+-incorporation 75% indicates that N_2-fixation is inhibited prior to the formation of the intermediate compound that is in equilibrium with exogenous NH_4^+. Furthermore, the selective inhibition of NH_4^+-incorporation by 5×10^{-5} M fluoroacetate suggests that the equilibrium reaction between exogenous NH_4^+ and the intermediate compound in the fixation of nitrogen is also sensitive to fluoroacetate.

The time course of inhibition of $N^{15}H_4^+$-incorporation is given in Table I. Inhibition was almost constant between 10 and 30 minutes at both 5×10^{-5} M and 1×10^{-3} M fluoroacetate. Fluoroacetate inhibition of $N_2{}^{15}$-fixation also remained nearly constant after 10 minutes (Table II). Thus, the data plotted in figure 1 were obtained under steady state conditions of inhibition.

Table I

INCORPORATION OF $N^{15}H_4^+$ WITH TIME INTO ACID-INSOLUBLE COMPOUNDS*

Fluoroacetate	N^{15} Enrichment in Acid Insoluble Cell Residue Atom % Excess N^{15}			% Inhibition		
	10 min.	20 min.	30 min.	10 min.	20 min.	30 min.
none	3.07	5.31	8.96	0	0	0
5×10^{-5} M	1.72	3.03	5.25	44.0	42.9	41.4
1×10^{-3} M	.88	1.34	2.08	71.3	74.8	76.8

*Ten-ml aliquots of a 16-hour shaken culture of Azotobacter were incubated with 17 μmoles of N^{15} enriched $(NH_4)_2SO_4$ for 10, 20 and 30 minutes. Fluoroacetate was added at zero time. After incubation the culture was acidified to N/10 H_2SO_4, the residue recovered and washed twice with N/10 H_2SO_4.

Table II also shows that the fixation of $N_2{}^{15}$ into the acid-soluble, amino acid fraction and into the acid-insoluble, protein fraction was inhibited to about the same extent. This agreement between the fractions in % inhibition supports the contention that fluoroacetate affects directly the reactions associated with the fixation of N_2 and incorporation of exogenous NH_4^+ rather than indirectly by inhibiting N-assimilation into proteins. When

Table II

DISTRIBUTION WITH TIME OF N_2^{15} INTO ACID-SOLUBLE AND ACID-INSOLUBLE COMPOUNDS*

Fluoroacetate	N^{15} Enrichment Atom % Excess N^{15}		% Inhibition	
	10 min.	18 min.	10 min.	18 min.
	Acid-Soluble Extract			
none	1.56	2.29	0	0
5×10^{-5} M	1.30	1.78	16.7	22.3
1×10^{-3} M	.08	.01	94.9	99.6
	Acid-Insoluble Residue			
none	.53	.97	0	0
5×10^{-5} M	.42	.78	20.8	19.6
1×10^{-3} M	00	00	100	100

*Eight-ml aliquots of a 16-hour shaken culture of Azotobacter were exposed to N_2^{15} gas mixture for 10 and 18 minutes. Fluoroacetate was added 3 minutes before gas. After exposure cultures were acidified to N/10 H_2SO_4 and the residue separated and washed by centrifugation.

protein synthesis of Azotobacter cells fixing N_2^{15} was inhibited with chloramphenicol, isotopic enrichment in the acid-insoluble fraction decreased and the N^{15} content of the acid-soluble compounds increased. The net effect of the disproportionate inhibition was a 10-fold increase in the ratio of enrichment of the acid-soluble fraction (Bruemmer and Rinfret, 1960).

Acetate at 10^{-2} M completely counteracted the inhibition of N_2-fixation by 10^{-3} M fluoroacetate but α-ketoglutarate did not. However α-ketoglutarate is not metabolized by sucrose-grown cells without prior adaptation. Presently fluoroacetate inhibition is being studied with cells adapted to oxidize all the TCA cycle substrates to ascertain whether any of them can relieve the inhibition or whether fluoroacetate is inhibiting a reaction specific to the nitrogen fixation process.

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