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The Isotope Effect in the Fixation of Nitrogen by *Azotobacter*

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The relative rates of fixation of $N^{14}N^{14}$ and $N^{15}N^{14}$ by *Azotobacter* have been studied. The ratio of these rates is 1.000 ± 0.001. The isotope effect in a heterogeneous reaction, the catalytic hydrogenation of the double bond in azobenzene, has been studied also. No isotope effect was found. The rate-determining step in the mechanism of these reactions does not involve a change in bonding to nitrogen. The geochemical implications are discussed.

Introduction

The biological fixation of molecular nitrogen is a very important process from many points of view and information on the mechanism of this reaction is of interest. This paper reports on the relative rates of biological fixation of the isotopic forms of molecular nitrogen. Isotope effects in biological systems have been reported for several elements including hydrogen, carbon, oxygen and sulfur. These isotope effects are important in determining the distribution of the stable isotopes of these elements in nature.

Experiments on the isotope effect in the heterogeneous hydrogenation of azobenzene to hydrazobenzene were undertaken to see if there were any similarities between the mechanism of this reaction and the fixation reaction.

Experimental

Burk's nitrogen-free media was prepared by adding the following to each liter of distilled water: 0.2 g. of KH_2PO_4, 0.8 g. of K_2HPO_4, 0.2 g. of $MgSO_4 \cdot 7H_2O$, 0.1 g. of $CaSO_4 \cdot 2H_2O$, 20 g. of sucrose, 1 mg. of ferric sulfate and 0.1 g. of molybdic acid. Two hundred milliliter volumes of the medium was placed in 500-ml. Kjeldahl flasks and sterilized at 18 pounds pressure for 20 to 30 minutes.

The original stocks of *Azotobacter agile*, *Azotobacter chroococcum*, *Azotobacter indicum* and *Azotobacter vinelandii* were obtained from American Type Culture Collection, Washington, D. C. The flasks were innoculated by sterile techniques and stored for growth and fixation at room temperature out of the direct sunlight. Growth was quenched at varying intervals by adding concentrated sulfuric acid, and nitrogen determinations were made by the Kjeldahl method. Innoculated blank samples showed less than 0.3 mg. nitrogen present. The ammonium sulfate isolated from the Kjeldahl analysis was used for the isotopic analysis of the fixed nitrogen. The procedures for the isotopic analysis of nitrogen have been described previously.[2]

Since the *Azotobacter* used only an infinitesimal fraction of the N_2 available to them, the kinetic isotope effect β, which is defined as

$$\beta = \frac{r_{14}}{r_{15}} \qquad (1)$$

is given by

$$\beta = \frac{R_a}{R_f} \qquad (2)$$

where r_{14} and r_{15} are the rates of fixation of $N^{14}N^{14}$ and $N^{15}N^{14}$, respectively, and R_a and R_f are the ratios of N^{15} to N^{14} in the air and in the fixed nitrogen, respectively.

Pure *trans*-azobenzene was prepared by the elution of a solution of commercially available azobenzene from a chromatography column of alumina with petroleum ether.[3] A platinum–charcoal catalyst was prepared by the method described by Baltzly.[4] It has been shown[4] that azobenzene in sodium methoxide reacts with hydrogen in the presence of platinized charcoal to yield only hydrazobenzene.

In these experiments, samples of azobenzene were partially hydrogenated under the conditions mentioned. The reaction mixture was then acidified to permit the rearrangement of the hydrazobenzene to isomeric benzidines. The unreacted azobenzene was isolated by the chromatography method described previously. The samples of the starting azobenzene and the unreacted fraction from the hydrogenation were treated with sodium hyposulfite and then subjected to a Kjeldahl digestion for the isolation of the nitrogen for isotopic analysis and for a measurement of the fraction reacted in the reduction reaction. The ammonium sulfate from the Kjeldahl analysis was converted to N_2 for isotopic analysis as before. Under these conditions, the kinetic isotope effect β is given by[5]

$$\beta = \frac{\ln(1-f)}{\ln\left[\frac{R_{at}}{R_{ao}}(1-f)\right]} \qquad (3)$$

where f is the fraction of the azobenzene reacted, R_{ao} is the ratio of N^{15} to N^{14} in the starting azobenzene and R_{at} is the isotope ratio in the remaining azobenzene. All steps in this procedure were tested to ensure quantitative conversion to prevent isotope fractionation in the preparation of nitrogen for isotopic analysis.

The results of these experiments are given in Tables I and II. Within the precision of the experiments, both reactions have no isotope effect.

Discussion

Since the net result of both chemical reactions studied is a change in the chemical bonding to nitrogen, an isotope effect may be expected. The kinetics of nitrogen fixation by *Azotobacter* have been studied by Wilson and co-workers[6] and the data has been fitted to the rate law corresponding to the Michaelis–Menten expression

$$E + N_2 \rightleftharpoons EN_2 \qquad K_1 \text{ rapid and reversible} \qquad (4)$$

$$EN_2 \xrightarrow{k_3} E + \text{Products} \qquad \text{slow} \qquad (5)$$

The isotope effects in a system with a weak preassociation have been discussed by Bigeleisen and Wolfsberg.[7] The observed isotope effect is the product of the isotope effects in step 4 multiplied by the effect in (5)

$$\frac{k'_{(obsd)}}{k''_{(obsd)}} = \frac{K_1'}{K_1''} \times \frac{k_3'}{k_3''} \qquad (6)$$

where the single and double primes refer to N^{14} and N^{15} containing molecules, respectively. Lineweaver has shown[8] that the interaction in step 4 is probably a weak one. Little isotope effect is

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expected in such cases. For example, there is no isotope effect in the solubility of N_2 in water.[9]

The nature of the chemical change in step 5 is not known. Evidence indicates that the first recognizable compound formed is ammonia, but the intermediates, X,Y,Z are not known.

$$E + N_2 \rightleftharpoons EN_2 \longrightarrow X \longrightarrow Y \longrightarrow Z \longrightarrow NH_3 \quad (7)$$

Gest[10] has summarized some very speculative possibilities for the reaction in (5). They are

$$N_2 \longrightarrow N_2^+ + e \quad \text{ionization} \quad (8)$$

$$N_2 \longrightarrow 2N \quad \text{dissociation} \quad (9)$$

$$H_2 + N_2 \longrightarrow HN{=}NH \quad \text{hydrogenation} \quad (10)$$

$$^1/_2O_2 + H_2O + N_2 \longrightarrow HON{=}NOH \quad \text{oxidation and hydrolysis} \quad (11)$$

Each of these reactions involves some change in the bonding to a nitrogen atom and thus may be accompanied by an isotope effect. An attempt was made to estimate the magnitude of this isotope effect in reactions 8 to 10 by the method of Bigeleisen.[7] The necessary data for vibrational frequencies of the nitrogen fourteen containing molecules has been tabulated by Herzberg.[11] The calculation of the isotope effect for reaction 10 was made using the nitrogen to nitrogen double bond frequency of diazomethane and the nitrogen to hydrogen bond frequency of ammonia. The frequency shifts on isotopic substitution were calculated using the harmonic oscillator approximation and it was assumed that only the frequencies of the bonds directly connected with the isotopic center were affected by the substitution. No attempt was made to calculate the "effective mass" term of the Bigeleisen equation as this would require a greater knowledge of the geometry of the activated complex than is available.[12] Only a value for the "temperature dependent" term of the equation was calculated. Since the "effective mass" term varies from one to values larger than one, the calculated isotope effect is a minimum value which is all that is required for the purposes of this study. The results are shown in Table III. All predict a measurable isotope effect. The calculations for reaction 11 were not made.

Since there is probably no isotope effect in step 4 and since any change in bonding to nitrogen in step 5 would probably have an isotope effect, it is concluded that either (4) and (5) do not adequately describe the system or that the change in (5) is of an unknown kind that does not involve any change in bonding to a nitrogen.

The rate-determining step of the fixation reaction may be connected with the formation of the enzyme–substrate complex in a heterogeneous, catalytic process. Wang[13] has shown that the isotope effect in the catalytic decomposition of hydrogen peroxide can vary with the size of the catalyst particle, being small for the diffusion of a

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TABLE I

THE ISOTOPE EFFECT IN THE FIXATION OF ATMOSPHERIC N_2 BY *Azotobacter*

	Fixation period, days	Nitrogen fixed, mg.		β
Azotobacter agile	12	7.3		0.9997
	13	8.9		1.0014
	14	10.1		1.0002
	15	9.9		1.0013
	17	9.3		1.0031
	19	9.6		1.0035
			Av.	1.0015
Azotobacter chroococcum	11	1.5		0.9992
	12	1.9		0.9985
	14	3.2		1.0009
	15	3.2		1.0002
	17	4.6		1.0004
	19	14.6		1.0004
	21	12.8		1.0019
	25	14.6		1.0015
			Av.	1.0007
Azotobacter indicum	38	4.8		0.9920
	38	3.2		.9910
	38	3.1		.9967
	48	2.4		.9973
	48	2.1		.9992
	48	2.4		.9989
	48	4.9		.9993
			Av.	0.9963
Azotobacter vinelandii	9	4.9		1.0015
	11	9.4		1.0012
	13	6.0		1.0008
	14	9.1		1.0030
	16	8.1		1.0041
	18	9.0		1.0031
	19	14.3		1.0021
			Av.	1.0022

TABLE II

THE ISOTOPE EFFECT IN THE CATALYTIC REDUCTION OF AZOBENZENE

Fraction reacted	β
0.372	0.9965
.626	.9972
.777	.9971
.432	1.0033
.725	1.0032
.945	1.0000
.637	0.9973
.809	1.0000
.857	1.0000
Av.	0.9994

TABLE III

ISOTOPE EFFECTS CALCULATED FOR REACTIONS 8, 9 AND 10 AT 25°

	Reaction	Ratio of specific rate constants for N^{14} and N^{15} compounds
(8)	Ionization	1.006
(9)	Dissociation	1.084
(10)	Hydrogenation	1.027

reactant molecule up to a large, planar catalytic surface and being large for reactions with catalysts of molecular dimensions (a homogeneous reaction).

An extension of the theory of Michaelis and Menten has been given by Chance[14] for the case where the reaction of the enzyme-substrate complex is second order

$$E + N_2 \rightleftharpoons EN_2 \quad (12)$$

$$EN_2 + AH_2 \longrightarrow E + \text{Products} \quad (13)$$

If it is assumed that the formation of the reactant AH_2 is the slow step in the sequence rather than reaction 13, then the over-all process of nitrogen fixation by *Azotobacter* will not have an observable nitrogen isotope effect. This assumption requires that the activation energy for other reactants in the system is larger than for the breaking of a very stable nitrogen to nitrogen bond in N_2. The anomalies between the large bond energy and the rate of reaction of N_2 has been pointed out by Kamen.[15]

The sulfur isotope effect in the reduction of sulfate ion by *Desulphovibrio desulphuricans* has been studied by Harrison and Thode.[16] They explained the occurrence or non-occurrence of an isotope effect according to where the rate-determining step of their reaction sequence appeared.

The lack of an isotope effect in the catalytic hydrogenation of azobenzene can be explained similarly. The slow step in the process may be connected with the adsorption of hydrogen. The step in which there is a change in bonding in the nitrogens follows and is more rapid than the adsorption or formation of the catalyst–substrate complex. The carbon isotope effect in the catalytic hydrogenation of stilbene to bibenzyl was studied by Bonner and Collins.[17] They found that the ratio of the rates of reduction of the C^{12} compound to the C^{14} compound was 1.02 using Raney nickel in benzene or platinum in ethanol as the catalyst. They also studied the relative rates of hydrogenation of C^{12} and C^{14} acetophenone to 1-phenylethanol. The ratio of the rates was 1.115 using platinum in ethanol as the catalyst and 1.08 using lithium aluminum hydride in ether. In these processes, the rate-determining step is the change of bonding at the carbon undergoing isotopic substitution.

Biological nitrogen fixation is a very important geochemical process and represents the chief means by which atmospheric nitrogen enters the biosphere. If one assumes that symbiotic nitrogen fixation has the same isotope effect as non-symbiotic, then this step partially sets the average N^{15}–N^{14} ratio in the biosphere. Unpublished results from this Laboratory show that the nitrogen in naturally occurring amino acids and in sedimentary rocks has a greater N^{15}–N^{14} ratio than the atmosphere. It is postulated that the kinetic processes of fixation and de-nitrification for the isotopic forms of nitrogen can be schematically represented as in equation 14

$$\text{Atmosphere} \underset{k_1''}{\overset{k_1'}{\rightleftarrows}} \text{biosphere} \underset{k_2''}{\overset{k_2'}{\rightleftarrows}} \text{atmosphere} \quad (14)$$

$$\text{biosphere} \uparrow\downarrow \text{sediments}$$

fixation de-nitrification

where the single and double primes indicate specific rate constants for N^{14} and N^{15} containing molecules, respectively. Hutchinson's inventory[18] of terrestrial nitrogen, as modified by Scalan,[19] is shown in Table IV. In equation 14, k_2 is probably greater than k_1; fixation is the slow step in the general process. A straightforward application of the kinetic laws for successive, pseudo-first-order reactions with the facts that $k_1'/k_1'' = 1$ and that the amount of N in the biosphere and sediments is less than the amount in the atmosphere leads to

TABLE IV

INVENTORY OF TERRESTRIAL NITROGEN

Region	Grams nitrogen per cm.² of the earth's surface
Core	26
Mantle	5660
Crust	102
Atmosphere	755
Sediments	67–108
Nitrate deposits	2×10^{-5}
Biosphere	Very small

$$\frac{B'}{B''} = \frac{A'k_2''}{A''k_2'} \quad (15)$$

where B' and B'' are the N^{15} and the N^{14} content of the biosphere and sediments, A' and A'' are the N^{15} and N^{14} content of the atmosphere. Thus the average N^{15}–N^{14} ratio in the biosphere and sediments is determined by the isotope effect in the denitrification process and since

$$\frac{B'}{B''} < \frac{A'}{A''} \quad (16)$$

then

$$\frac{k_2''}{k_2'} < 1 \quad (17)$$

Isotope effects are to be expected in the circulation of nitrogen in the biosphere and differences in nitrogen isotope ratios in biogenic materials have been observed. The isotope ratio calculated in equation 15 is the average isotope content of the biosphere and sediments.

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