The thiopyrimidine is slightly soluble in boiling water (1 g. in 300 cc.) in boiling ethanol (1 g. in 90 cc.) and soluble in alkali and acids. It sublimes readily below its melting point.

2-Mercaptoacetic Acid 5-Carbethoxypyrimidine, XIII.—A solution of 1 g. of thiopyrimidine XII and 1 g. of chloroacetic acid in 125 cc. of hot water was boiled for half an hour. On cooling, 0.95 g. of the mercaptopyrimidine XIII separated as long needles which melted at 175–176.5° after one recrystallization from water. One gram of the substance was soluble in about 100 cc. of boiling and in 500 cc. of cold water.

Anal. Calcd. for $C_9H_{10}O_4N_2S$: N, 11.52; S, 13.25. Found: N, 11.42; S, 12.85.

The pyrimidine dissolved in acid with the evolution of thioglycolic acid. The residue left after acid hydrolysis has not yet been identified.

Summary

1. Salt derivatives of carbethoxymalonic aldehyde were obtained by application of a Claisen condensation with ethyl β,β'-diethoxypropionate and ethyl formate. Crystalline copper and potassium salts were isolated in addition to an amorphous sodium salt.

2. The interaction of the sodium salt of carbethoxymalonic aldehyde with (a) urea, (b) thiourea, and (c) pseudoethylthiourea hydrobromide resulted in the formation of (a) a monoureide, (b) a monothioureide and (c) a molecular addition product of pseudoethylthiourea and carbethoxymalonic aldehyde. Each of these substances, when dehydrated, formed the corresponding (a) 2-keto, (b) 2-thio and (c) 2-mercaptopyrimidines.

NEW HAVEN, CONN. RECEIVED SEPTEMBER 26, 1933

[CONTRIBUTION FROM THE FERTILIZER INVESTIGATIONS UNIT, BUREAU OF CHEMISTRY AND SOILS, UNITED STATES DEPARTMENT OF AGRICULTURE]

The Dissociation Constant of Nitrogen-Nitrogenase in Azotobacter

BY HANS LINEWEAVER, DEAN BURK AND W. EDWARDS DEMING

This paper presents the results of an investigation concerning the nature of the dissociation of the complex formed between nitrogen gas and the enzyme nitrogenase in nitrogen fixation by Azotobacter at ordinary temperatures and pressures.

Methods for the evaluation of dissociation constants of enzymic and other reactions have been extended recently by Lineweaver and Burk.[1] The results to be presented here show that the mechanism of nitrogen fixation by Azotobacter may be represented by

$$N_2 + E \underset{v_2}{\overset{v_1}{\rightleftharpoons}} N_2E \xrightarrow{v} E + P \qquad (1)$$

$$v = k'(N_2)(E) = k(N_2E) \qquad (2)$$

$$K_{N_2} = (E)(N_2)/(N_2E) \qquad (3)$$

where one molecule of N_2 combines reversibly with one molecule, or independently reacting group, E (nitrogenase) in the Azotobacter cells. v and k are the velocity and velocity constant of the irreversible decomposition of N_2E (nitrogen-nitrogenase), v_1 and v_2 are the velocities of reversible formation and dissociation of N_2E, K_{N_2} is the respective dissociation constant at equilibrium, and P is the reaction product. (N_2E) is a rectangularly hyperbolic function of (N_2).

This type of mechanism leads to the equation

$$1/v = K_{N_2}/V_{max.}(N_2) + 1/V_{max.} \qquad (4)$$

where $V_{max.}$ is a numerical constant representing the maximum velocity at high nitrogen pressures where the enzyme exists completely in the form N_2E ($V_{max.} = k(E_{total})$). A plot of the reciprocal of the velocity against the reciprocal of the nitrogen pressure ($1/v$ against $1/(N_2)$) yields a straight line whose slope $K_{N_2}/V_{max.}$ and ordinate intercept $1/V_{max.}$ evaluate K_{N_2}, as illustrated in Fig. 1. K_{N_2} is established as a thermodynamic dissociation constant if it does not vary as a function of k, or $V_{max.}$, that is, if v is negligible compared to v_2 (*cf.* also Haldane,[2] p. 40). In this study, several factors influencing k have been varied, *viz.*, P_H, humic acid concentration, oxygen pressure and temperature.[3] Several other factors have also been varied, *viz.*, concentrations of calcium, strontium and oxalate, and P_H, which were previously shown[3,4] to be specific in fixation as distinguished from Azotobacter growth. These might alter $V_{max.}$ by altering the concentration

(1) H. Lineweaver and D. Burk, "The Determination of Enzyme Dissociation Constants," in press.

(2) J. B. S. Haldane, "Enzymes," Longmans, Green and Co., London, 1930.

(3) D. Burk and R. T. Milner, *Ind. Eng. Chem., Anal. Ed.*, **4**, 3 (1932).

(4) D. Burk, H. Lineweaver and C. K. Horner, *J. Bact.*, in press; *cf.* also D. Burk, "Azotase and Nitrogenase in Azotobacter," a review chapter in "Ergebnisse der Enzymforschung," by F. F. Nord and R. Weidenhagen, Vol. III, in press, Leipzig, 1934.

of total nitrogenase (E + N_2E) per unit concentration of Azotobacter but would not thereby alter K_{N_2}.

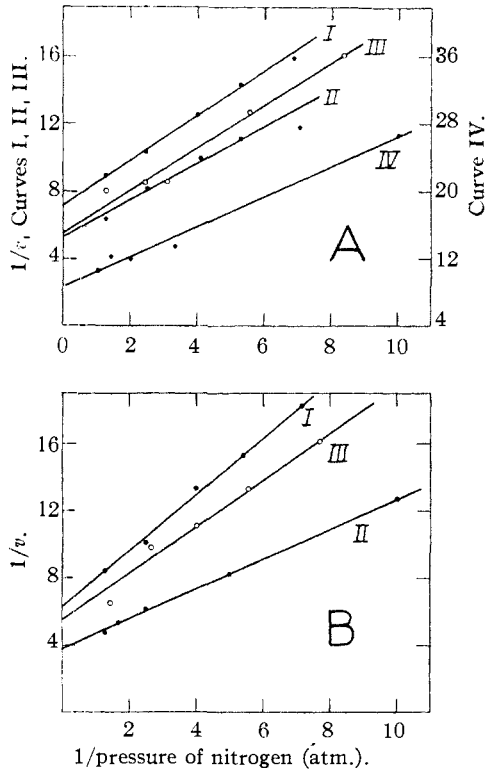

Fig. 1.—The influence of pressure of nitrogen on the velocity of fixation (illustrative data for individual experiments): A, curves I and II, 14-hour *A. vinelandii* at 22.5 and 31.0°, K_{N_2} = 19 ± 2 and 22 ± 2; Curve III, 1-day *A. vinelandii* at P_H 6.15, K_{N_2} = 22 ± 4; Curve IV, 2-day *A. chroococcum*, 5% O_2, K_{N_2} = 21 ± 3. B, curve I, 1-day *A. vinelandii* (previously grown for three transfers in Ca-free medium containing Sr), K_{N_2} = 25 ± 3; Curve II, 1-day *A. vinelandii*, low Ca concentration (5.8×10^{-5} *M*), K_{N_2} = 23 ± 3; Curve III, 2-day *A. vinelandii*, very low Ca concentration (5.8×10^{-6} *M*), K_{N_2} = 23 ± 2. K_{N_2} values in vol.-%.

Apparatus and Methods

The Warburg manometric apparatus, technique, and methods of culture have been described in detail previously.[5,6] The total gas pressures employed were atmospheric so that 100 vol.-% = 1 atm. A multiple all-glass flowmeter was used to prepare gas mixtures of varying N_2, O_2 and H_2 content. Unless otherwise stated, *Azotobacter vinelandii* has been employed, and a temperature of 31°, a P_H of 6.9 ± 0.2, a partial pressure of oxygen of 21%, and a culture medium consisting of 0.055 *M* glucose, 0.00035 *M* $CaSO_4$, 0.00000025 *M* $Fe_2(SO_4)_3$, 0.00081 *M* $MgSO_4$, 0.0034 *M* NaCl, 0.0037 *M* K_2HPO_4 and 0.0012 *M* KH_2PO_4.

The fixation of nitrogen is an autocatalytic process under the experimental conditions employed. The nitrogen-fixing system Azotobacter, as measured by either amount of nitrogenase, cell nitrogen (nitrogen fixed), cell number, dry matter, turbidity or respiration rate (*cf.* Ref. 3, Fig. 3), increases logarithmically with time. v in equation 4, for any constant N_2 pressure and unit concentration of Azotobacter, is therefore the monomolecular velocity constant of fixation (P formation in equation 1), or the fractional increase per unit of time, and is evaluated in the present experiments from the slope of a plot of the logarithm of the rate of respiration in cu. mm. O_2/cc./hr. against the time in hours (*cf.* Ref. 4, Fig. 2; Ref. 5, Fig. 7).

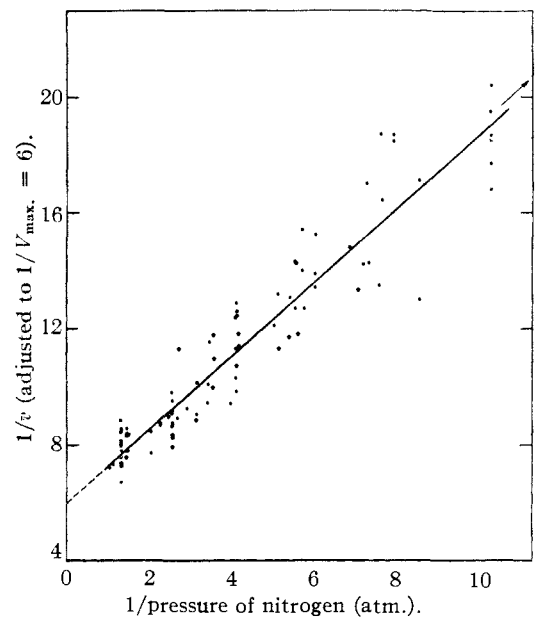

Fig. 2.—The influence of pressure of nitrogen on the velocity of fixation (collected experiments).

Experimental Results

The average of twenty-one values of K_{N_2}, obtained by the graphical method of Fig. 1, was 21.86 ± 0.25 vol.-% (*cf.* Table I) in a large series of experiments of varying $V_{max.}$, concentration of calcium, concentration of strontium, temperature, P_H, oxygen pressure and certain incidental physiological factors such as species (2), age of culture (one-half to three days),

5) D. Burk, *J. Phys. Chem.*, **34**, 1174 (1930).

6) O. Meyerhof and D. Burk, *Z. physik. Chem.*, **A139** (Haber-Band), 117 (1928).

TABLE I

THE INFLUENCE OF TEMPERATURE, LOW CONCENTRATION OF CALCIUM OR STRONTIUM, P_H AND OXYGEN PRESSURE ON K_{N_2}

Variable	Date	Age of culture, days	Initial resp. rate, cmm./cc./hr.	$V_{max.}$	K_{N_2} (vol.-%)	$v_{78\%\ N_2}$	$v_{78\%\ N_2}/V_{max.}$
A. Temp., °C.							
31	1/8/32	1	14	0.24 ± 0.02[b]	22 ± 3[b]		0.79
31	1/22/32	1	22	.26 ± .01	23 ± 3[d]		.81
31	11/14/32	2	30	.19 ± .01	21 ± 2		.76
31	1/27/33	1	10	.28 ± .04	23 ± 5		.74
31	2/27/33	0.5	43	.19 ± .02	22 ± 2		.80
28[a]	9/26/29[c]	2	22	.27 ± .02	20 ± 2		.81
28	9/26/29[c]	2	20	.17 ± .02	22 ± 2		.78
22.5	1/11/33	1	4	.12 ± .01	21 ± 4		.80
22.5	1/11/33	3	24	.11 ± .01	22 ± 2		.80
22.5	1/27/33	1	6	.16 ± .01	21 ± 4		.81
22.5	1/27/33	1	20	.14 ± .01	26 ± 5		.74
22.5	2/27/33	0.5	20	.14 ± .02	19 ± 2		.80
Average					21.9		.787
B. Ca or Sr concn. moles per liter × 10^5							
5.8	1/22/32	1	22	0.26 ± 0.01	23 ± 3	0.21	
0.58	1/6/33	2	26	.18 ± .01	23 ± 2	.16 (0.21)[f]	
1.16	1/6/33	2	70	.13 ± .02	23 ± 4	.10 (.17)[f]	
5.4[e]	4/12/33	1	20	.19 ± .02	20 ± 4		
5.4[e]	4/12/33	1	22	.16 ± .01	25 ± 3		
Average					22.8		
C. P_H							
6.05	12/8/32	1	16	0.13 ± 0.01	23 ± 4	0.10 (0.17)[g]	
6.1	12/8/32	1	16	.18 ± .02	20 ± 4	.13 (.17)[g]	
6.15	12/2/32	1	20	.18 ± .02	22 ± 4	.13 (.20)[g]	
Average					21.7		
D. O_2 pressure (vol.-%)							
5	2/24/30[c]	2	25	0.14 ± 0.01	21 ± 3		
10	8/9/29[c]	3	10	.13 ± .01	20 ± 2		
Average					21.5		

[a] 0.0125% (125 p. p. m.) natural soil humate employed.
[b] The limits of error in K_{N_2} and $V_{max.}$ values refer to estimated experimental limits of error, not to probable error.
[c] *Azotobacter chroococcum* (all others *A. vinelandii*) employed, at 28°.
[d] 5.8×10^{-5} *M* calcium (this experiment same as first one in section B).
[e] Cultures maintained in Ca-free medium containing Sr for three previous culture transfers.
[f] Values at normal Ca concentration (5.8×10^{-4} *M*).
[g] Values at normal P_H (7.0).

Azotobacter concentration (respiration rate = 4 to 70 cu. mm./cc./hr.) and date (extending over four years). All these factors were varied over a range as wide as possible within the limits of experimental convenience and without involving irreversible inactivation of the system.

The estimated limits of experimental error set by the observers, in individually obtained K_{N_2} values, are indicated in the tables, and, if used to obtain an average value of K_{N_2} based on a weighting according to the reciprocals of the squares of the assigned deviations, yield 21.42 ± 0.23 vol.-%. The calculated probable errors of a single unweighted determination and of a determination of unit weight are ±0.93 and ±1.58. An average of K_{N_2} values obtained in each experiment by the method of least squares, instead of graphically as in Fig. 1, yielded, upon assigning equal weights to $1/v$ values, 21.67 ± 0.49 vol.-% (P. E. of single determination = ±2.25). The average of the three average K_{N_2} values is 21.64 ± 0.16 vol.-%. A slightly different calculated K_{N_2} value, 21.01 vol.-%, obtained later makes a finally accepted average value of 21.5 ± 0.2 vol.-%.

K_{N_2} does not vary with $V_{max.}$ and its value therefore represents the ratio of the velocity constants of reversible decomposition and formation of the intermediate N_2E in equation 1, inappreciably complicated by the velocity con-

stant of formation of product P from N_2E. The free energy of formation of N_2E from N_2 is thus zero, in view of the close approximation to equilibrium. If it is assumed that within experimental error K_{N_2} actually decreases as much as 1 in 21.6 (to 20.6) when $V_{max.}$ decreases 50%, it can be shown (*cf.* Ref. 1, Case VI) that precise equilibrium does not obtain to the extent that $v_1/v_2 = 1.12$ instead of unity, and that ΔF is only RT ln 1.12 or 100 cal. absorbed.

The weighted averages of K_{N_2} at 31 and 22.5° are 21.89 ± 0.24 and 20.91 ± 0.61 vol.-% (*cf.* Table I). This corresponds, by the van't Hoff equation, to a heat of dissociation ($N_2E \longrightarrow N_2 + E$) of 960 ± 600 calories absorbed, or zero within the limits of significance for the probable error involved (normally accepted significance = 3.2 × 600 = 1920 cal.). This value has been confirmed by data of a different nature. As indicated in Table II the temperature coefficient of k, the constant of Azotobacter increase, is the same between 20 and 30° whether the increase occurs when E is maintained only partially saturated at 78% N_2, or whether some form of fixed nitrogen (instead of free nitrogen) is employed at a concentration (7×10^{-3} M) at least as great as needed to obtain maximum velocity of increase at any of the temperatures studied. This would not be the case if the dissociation were affected by temperature over the range studied. Row B, Table II, shows that for any given temperature $V_{max.}$ in free nitrogen is substantially the same as the maximum velocities in the forms of fixed nitrogen tested; assuming an experimental error of ±1%, $\Delta H = 0 \pm 1100$.

$v_{78\%\ N_2}$ values reported in Column 7 of Table I, show that cultures whose $V_{max.}$ is low in the presence of low concentrations of calcium or hydroxyl ion yield normal velocities at normal concentrations. These specific fixation factors are presumably directly involved in, or constituents of, the enzyme E, affecting the total concentration thereof per unit concentration of Azotobacter and thereby $V_{max.}$.

Azotobacter increase in free nitrogen has been found[4] to cease abruptly at P_H 5.97 but in fixed nitrogen (KNO_3, NH_4Cl, urea) it occurs even below P_H 5.0. Similarly, increase in free nitrogen requires a 10–100-fold greater concentration of calcium or strontium than in fixed nitrogen. The absence of any increase in K_{N_2} with respect to calcium, strontium or hydrogen-ion concentrations has been confirmed using Erlenmeyer flask technique (Ref. 5, p. 1182). The ratio of v values (based on turbidimetric measurements) obtained at 8 and 0.8 atmospheres of nitrogen at different concentrations of these variables (each varied independently) remained substantially constant. 2.5×10^{-3} M oxalate causes approximately 50% inhibition of $v_{78\%\ N_2}$ (by virtue of calcium inactivation), but no increase in K_{N_2} was observed in similar experiments. Likewise no increase in K_{N_2} occurred when the concentration of iron was varied over a wide range, from 4.8×10^{-8} to 1.8×10^{-5} M. This element was tested in view of its general biochemical importance.

Statistical Analysis of Aggregate Data.—The probability of the correctness of the mechanism of fixation proposed in equations 1, 2 and 3, involving strict linearity of the plot of $1/v$ against $1/(N_2)$, was tested further by considering the 21 experiments (each involving five to seven nitrogen pressures) collectively rather than individually. The $1/V_{max.}$ values were multiplied by factors to make them coincide at a value of 6, and the corresponding $1/v$ values were then multiplied by the same respective factors. Figure 2 shows the collected data, the line being drawn with $1/V_{max.} = 6$, and $K_{N_2} = 21.01$, these values having been obtained by the method of

TABLE II

THE INFLUENCE OF TEMPERATURE UPON AZOTOBACTER INCREASE IN FREE AND VARIOUS FORMS OF FIXED NITROGEN[a]

	Temperature, °C.	20	21	22	23	24	25	26	27	28	29	30
A	$v_{78\%\ N_2}$	0.075	0.085	0.095	0.109	0.123	0.137	0.153	0.167	0.184	0.200	0.215
B	$V_{max.}$[b]	.096	.108	.121	.139	.157	.175	.195	.213	.235	.256	.275
C	v_{KNO_2}	.088	.100	.115	.130	.145	.160	.177	.195	.214	.232	.257
D	v_{KNO_3}	.092	.104	.117	.131	.149	.166	.185	.205	.225	.247	.267
E	v_{NH_3}	.095	.108	.122	.138	.155	.172	.190	.210	.229	.252	.271
F	C/A	1.17	1.17	1.21	1.19	1.18	1.17	1.16	1.17	1.16	1.16	1.19
G	D/A	1.23	1.22	1.23	1.20	1.22	1.21	1.21	1.23	1.22	1.23	1.24
H	E/A	1.27	1.27	1.28	1.27	1.26	1.25	1.25	1.26	1.24	1.26	1.26
I	(C + D + E)/3A	1.22	1.22	1.24	1.22	1.22	1.21	1.21	1.22	1.21	1.22	1.23

[a] *Cf.* Ref. 3, Fig. 6. [b] $V_{max.} = v(K_{N_2} + (N_2))/(N_2) = v_{78\%\ N_2}(21.5 + 78)/78 = 1.28\ v_{78\%\ N_2}$.

least squares in which each v (not $1/v$) value was equally weighted (see later). It is assumed in Fig. 2 that none of the factors varied in Table I affects the form of the nitrogen pressure function. There appears to be no definite trend from linearity (*cf.* also Fig. 1).

where r is the number of observations in any one array (*i. e.*, at any one $1/(N_2)$ value) on which m, the mean thereof, depends, whereas $\overline{m}$ is the theoretical (graphical) value of the assigned function. σ is the standard deviation (root mean square deviation) of an indefinitely great number

TABLE III

THE "CHI" TEST OF THE LINE IN FIG. 2 ("GOODNESS OF FIT")

No.	$1/(N_2)$		σ^2[a]	$\overline{m} = 1/v$ graph.	$m = 1/v$ obs.	$\frac{r(m-\overline{m})^2}{\sigma^2}$	Segment values: Nos.	χ^2	n[b]	P[b]
1	33.3	1	35.44	48.56	47.90	0.0012 (−)	1–3	0.1761	3	0.99
2	16.7	1	35.38	27.38	25.20	.1343 (−)				
3	10.0	6	7.82	18.83	18.60	.0406 (−)	3–7	.9612	5	.97
4	8.03	4	4.38	16.32	16.82	.2283 (+)	4–11	3.8179	8	.87
5	7.39	3	3.56	15.50	16.18	.3897 (+)				
6	7.11	3	3.24	15.15	15.14	.0001 (−)				
7	6.77	2	2.88	14.71	14.05	.3025 (−)				
8	5.88	3	2.08	13.58	14.84	2.2898 (+)	8–13	3.4340	6	.75
9	5.52	10	1.81	13.12	13.35	0.2923 (+)				
10	5.27	2	1.64	12.80	12.38	.2151 (−)				
11	4.97	3	1.45	12.42	12.20	.1001 (−)				
12	4.00	12	0.94	11.18	11.28	.1277 (+)	12–17	7.3217	6	.29
13	3.40	6	.71	10.41	10.63	.4090 (+)				
14	2.84	7	.53	9.70	9.44	.8928 (−)	14–20	9.8274	7	.20
15	2.50	10	.44	9.26	8.85	3.8205 (−)				
16	2.22	2	.37	8.91	8.70	0.2384 (−)				
17	2.00	2	.33	8.63	8.08	1.8333 (−)				
18	1.44	8	.23	7.91	8.03	0.5009 (+)				
19	1.28	15	.21	7.71	7.86	1.6071 (+)				
20	1.08	2	.18	7.45	7.16	0.9344 (−)				
							1–20	14.3851	20	.81[b]

[a] $\sigma^2 = 6 \times 10^{-5}(1/v)^4$.

[b] P is given in Fisher's table[8] as a function of χ^2 and n, and in Elderton's table[7c] as a function of χ^2 and n', where $n' = n + 1$. When, as in the present analysis, n is the number of arrays in the segment or line being tested, the value of P has the following significance: *if* the assigned function, including the adjustable constants (parameters), is assumed to represent the fixation mechanism, then P is the fraction of a large number of sets of data (each similar to the set in Fig. 2) in which as bad or worse agreement is to be expected. When, on the other hand, n is the number of arrays diminished by the number of adjustable constants (in this case 2), P is the fraction of a large number of sets of data in which one would expect as bad or worse agreement with curves fitted in each set by least squares, which is essentially a method for minimizing χ^2. Any other method of fitting that is efficient in Fisher's sense might be employed. The second interpretation of P involves the assumption concerning the form of the function but not the values of the constants. In the present case we wish to test not only the linearity but also the particular constants of the curve in Fig. 2; the former interpretation of P has therefore been employed. Furthermore, the second interpretation cannot be employed with respect to P values for segments of a particular assigned line, since such segments are not fitted individually by some efficient method. If P for the whole line is derived on the basis of $n = 20 - 2 = 18$, instead of 20, it is actually only a little smaller in the present instance—0.64 instead of 0.81. The distinction between the two interpretations of P has often been neglected or misunderstood, because emphasis has been placed on the choice of n rather than on the meanings of P in relation to the values of n chosen and the completeness of the hypothesis under test (*cf.* Ref. 8, p. 231).

It is possible to apply an additional, analytical criterion, the "Chi" test, or "Goodness of Fit."[7,8] The data may be tested by the equation

$$\chi^2 = \Sigma\,(r(m - \overline{m})^2/\sigma^2)$$

of measurements in a particular array. χ^2 is related to P, the probability that χ^2 shall exceed any specified value, by a complicated algebraic equation (*cf.* Ref. 7c, p. xxxi; Ref. 8, Table III). The χ^2 test indicates when a given curve does not represent a set of data. If P is small (perhaps 0.02 or less) it indicates that the assigned function fails to account for the whole of the facts (Ref. 8); if P is equal to or greater than 0.1 there is no reason to reject the function. Ex-

(7) (a) K. Pearson, *Phil. Mag.*, (5) **50**, 157 (1900). A small table appeared in this article, but a more complete one was published by (b) W. P. Elderton, *Biometrika*, **1**, 155 (1901). Elderton's table is reproduced in the (c) "Tables for Statisticians and Biometricians," Part I, edited by Karl Pearson and published by University College, London. Examples were worked out by (d) Pearson in *Biometrika*, **11**, 239 (1915).

(8) R. A. Fisher, "Statistical Methods for Research Workers," 4th edition, Oliver and Boyd, London, 1932.

amination of the unsummed χ^2 values of any given array, or of P values calculated from three or more consecutive arrays, indicates the portion of the curve of maximum improbability.

Table III gives the evaluation of χ^2 from the data previously described. An estimate of σ^2 in any array is $\Sigma u^2/(r-1)$, and this is, of course, subject to statistical fluctuations. u is the deviation of a single observation from m. Values of σ^2 estimated from arrays containing 6 to 15 points were scattered statistically about the line $\sigma^2 = 6 \times 10^{-5} (1/v)^4$, which is equivalent to an equal weighting of v values. The $1/(N_2)$ values were assumed to be free from error. None of the P values reported, for either the whole curve or any of its segments, casts any appreciable doubt on the assigned function. The relatively low value, 0.20, at the lowest $1/(N_2)$ segment, although indicating acceptable probability of the line drawn over this range, suggests the possibility of a very slight upward concavity which might be more definitely indicated with a large number of additional experiments. The absence of any portion of the curve with significant consistent maximum improbability ($P = < 0.02$) is also evident from the random variations in Column 7, Table III. The signs of the deviations $(m - \bar{m})$ from the theoretical mean are indicated in parentheses. P is an increasing function of σ^2 and thus variations therein might be important. In this case σ^2 values would need to be decreased by over half (62%) before P for the whole curve would approach improbability (0.02 or less).

According to equation 4, the ratio $v/V_{max.}$ should be constant at any given nitrogen pressure, and at 78% N_2 (air) is 0.783. It is of interest to note in Table I, Column 8, how closely this value is attained experimentally in the various experiments and in their average.

Summary

1. The fixation of nitrogen at ordinary temperatures and pressures by Azotobacter, as a function of nitrogen pressure, corresponds to one N_2 molecule combining reversibly with one enzyme molecule E (nitrogenase) to form a compound N_2E whose thermodynamic dissociation constant, $K_{N_2} = (E)(N_2)/N_2E)$, is 21.5 ± 0.2 volume per cent. (0.215 ± 0.002 atm.).

2. This constant is highly characteristic, being independent of wide variations in the following important factors, the first four of which are specific in the fixation process; concentrations of calcium, strontium and oxalate, P_H: oxygen pressure, concentration of iron, $V_{max.}$ (maximum velocity of irreversible decomposition of N_2E to form protein, at saturating nitrogen pressures) and certain physiological factors (species, Azotobacter concentration, culture age, and date of experiment).

3. Statistical treatment of the collected data, involving Pearson's "Chi" test, was carried out to substantiate the fixation mechanism and related constants were obtained. The importance of employing this seldom-used test in analyzing chemical and physical data is indicated.

4. The molal heat and free energy of the dissociation of N_2E are zero within experimental errors of about 1000 and 100 cal., respectively.

WASHINGTON, D. C. RECEIVED SEPTEMBER 28, 1933