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Dimerization of vitamin B12a

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NOTE TO USERS

DIMERIZATION OF VITAMIN B_{12a}

by

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I. INTRODUCTION

In 1926 it was found that the whole liver was active against pernecious anemia (1). This discovery instigated research in the fractionation of the whole liver and the isolation of the chemical responsible for the medicinal effect. After twenty-two years of tedious and often frustrating labor, the pure material was isolated in crystalline form. The isolation was achieved practically simultaneously by three research groups (2, 3, 4). The pure principle was designated vitamin B_{12}. It was found to have an extraordinary potency, for one microgram per day proved sufficient to cure pernecious anemia (5).

Further investigations revealed that vitamin B_{12} was produced in culture broths by a strain of S. Griseus (6). Crystalline B_{12} is produced commercially from the broths remaining after the extraction of streptomycin. The vitamin is also found in a sewage and is also prepared commercially from sewage. Large quantities of vitamin B_{12} are now manufactured and the price has been dropping steadily. At present (1955), the price is $245 per gram of crystalline material.

A considerable body of work has now been published on the chemistry of vitamin B_{12}. Early investigations revealed that the molecular weight of B_{12} was somewhere between 1300 and 1575 (7), and that vitamin B_{12} contained cobalt and phosphorus (8, 9, 10).

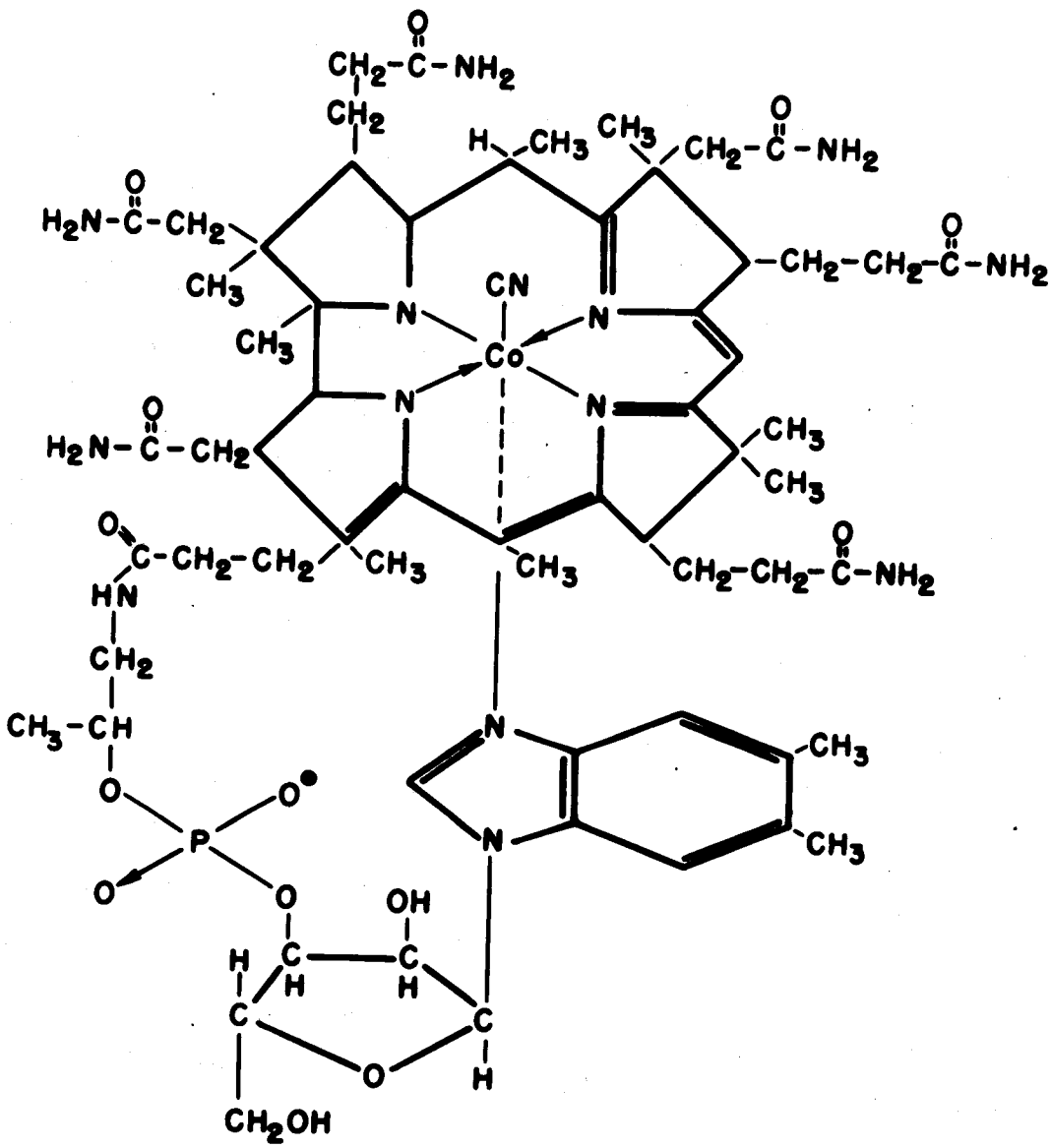
On strenuous acid hydrolysis with hydrochloric acid vitamin B_{12} yielded 5,6-dimethylbenzimidazole, ribose, phosphoric acid, ammonium chloride, 1-amino-2-propanol and a red acidic, cobalt-containing pigment of high molecular weight. The presence of the ammonia and phosphate ions was recognized in the early studies (11, 12). The 5,6-dimethylbenzimidazole was isolated from the hydrochloric acid hydrolyzate (13,14), and the structure was confirmed by the comparison to the pure 5,6-dimetylbenzimidazole (15, 16). The mild acid hydrolysis of vitamin B_{12} yielded 1-α-D-ribofuranosido-5,6-dimethylbenzimidazole (17, 18). The structure was proved by synthetic methods. The presence of 1-amino-2-propanol was observed in acid hydrolyzates subjected to paper chromatography (4, 19). The structure of 1-amino-2-propanol was proved by classical chemical methods (19). Some doubt exists as to the number of 1-amino-2-propanol groups present; there have been postulated one and two moles of 1-amino-2-propanol per mole of vitamin B_{12} (20, 21, 22). It was found that the first group of phosphoric acid was attached to the ribose, and the second phosphate group was attached through 1-amino-2-propanol to the red acid fragment (23). The remaining free acid group of the phosphate is neutralized by a positive charge on the cobalt atom (24).

The presence of a cyanide group in the molecule of vitamin B_{12} was first found in the course of a permanganate oxidation (25). The cyanide group was proved to be attached

directly to the cobalt atom (26), and to be easily replaced by a hydroxide group upon exposing an aqueous solution of vitamin B_{12} to visible or near ultraviolet radiation (27). The cyanide group can also be replaced by other negative radicals (28, 29). All of the derivatives are converted into vitamin B_{12} by the treatment with cyanide.

The nature of the red acid fragment was a mystery until the end of 1954. Early attempts to obtain the pure red acid fragment failed. The red acid fragment produced by hydrochloric acid hydrolysis indicated presence of as many as seven carboxylic acid groups (30). Application of the Plimer modification of the Van Slyke amino nitrogen method showed the presence in the molecule of five primary amide groups (31). However, in the latest structure proposed by the group at Cambridge University (32, 33) there are shown six primary amide groups. The chemical methods of the degradation of the red acid fragment led practically nowhere until the hexacarboxylic acid, the degradation product, was isolated as a pure crystalline material (34). This crystallization enabled the elucidation of its structure by X-ray crystallographic methods (35) and, in turn, to a formulation of the structure of the vitamin itself, Fig. 1 (32). The actual structure was elaborated from the X-ray crystallographic work on the red fragment and the chemical work on the peripheral material (33). The structure of vitamin B_{12} is not entirely solved. The positions of the double bonds in the ring structure, and

Fig. 1 Structure of Vitamin B_{12}.



the stereo chemical configuration are unsettled. There is serious doubt as to the existence of six free amide groups and to the exact nature of the phosphate linkages.

The presence of a cobalt atom in the molecule of vitamin B_{12} presented number of problems in coordination chemistry which has been the primary concern of the group at Iowa State College. The valence state of the cobalt was established as three by magnetic susceptibility measurements (36, 37) and polarographic studies (38, 39). A purple derivative of B_{12} obtained by treatment with excess cyanide was discovered and its properties investigated (40). The valence state of the reduced form of vitamin B_{12} (called vitamin B_{12r}) was established by the means of oxidation reactions and by polarographic studies (41, 42, 43). It was proved also that the cyanide ion in vitamin B_{12} was reduced to methylamine by hydrogen in presence of platinum (44). The ammonia liberated on acid hydrolysis was found to be five molecules per molecule of vitamin B_{12} (45). The absence of primary amino groups, and the presence of five primary amide groups was shown (31). Vitamin B_{12a} was found to act as a catalyst in the air oxidation of iodide to iodine (46). It was shown also that the vitamin was not hydrolyzed by the enzyme phosphatase (47).

In the evaluation of the polarographic studies of vitamins B_{12}, B_{12a}, and B_{12r} it was necessary to determine the diffusion coefficients of these materials. Early values obtained for the diffusion coefficients of B_{12} and B_{12a} gave

erroneous results for the molecular weights as calculated by the Stokes-Einstein equation, less than half of the actual value of 1370 for B_{12} and an acceptable value for B_{12a}. Although no great claim is made for the applicability of the Stokes-Einstein equation to such small molecules, it was puzzling that the method should fail for B_{12} but yield a satisfactory molecular weight for the closely related material, B_{12a}. The primary purpose of the work described in the present thesis is to resolve this difficulty. Briefly, the diffusion coefficients have been redetermined by a more acceptable method. The apparent specific volumes of the vitamins have been measured by a precise pycnometric method. It was found that B_{12a}, but not B_{12}, unites with oxygen in a reversible fashion. A quantitative method has been devised for studying the union of B_{12a} and oxygen. Finally, the effects of this oxygenation on the absorption spectrum, conductivity, and catalytic effects of B_{12a} have been studied.

II. DIFFUSION COEFFICIENTS OF VITAMINS B_{12} AND B_{12a}

A. Introduction

Miscible materials in nonhomogeneous solution tend to migrate from regions of higher concentration to lower concentration. If this migration is caused only by thermal agitation, this phenomenon is called diffusion or concentration diffusion. Fick's first law relates diffusion to the flow of heat by the equation:

$$Q = -D \frac{dc}{dx}$$

where "Q" is the flux - the amount of material crossing a plane of unit area, "$\frac{dc}{dx}$" is the concentration gradient, and "D" is the diffusion coefficient.

Fick's second law is obtained by combing Fick's first law with the hydrodynamic equation of continuity:

$$\frac{dc}{dt} = - \frac{dQ}{dx}$$

After substitution of "Q" and assuming that "D" is constant in the given region the final equation is derived:

$$\frac{dc}{dt} = D \frac{d^2c}{d\ x^2}$$

The last equation is used in calculations of the diffusion coefficients.

Studies of diffusion coefficients in liquid systems find useful application in the determination of molecular weights, particle size, friction coefficients, and solution rates.

Values of the diffusion coefficients of vitamins B_{12} and B_{12a} have been reported as 4.46×10^{-6} and 3.42×10^{-6} cm.^{2}/sec., respectively (38). These values were obtained by the Stokes modification (48) of the Northup and Anson method (49). Molecular weight computations by the Stokes-Einstein (50) equation, using the above values of the diffusion coefficients and the value 1.34 for the density of the crystal, give values of 550 and 1230 for B_{12} and B_{12a}, respectively. This value for B_{12} is less than half the molecular weight, 1370, calculated from the cobalt content of the dry material. There was no apparent reason why the method should have given a reasonable value for B_{12a} and failed entirely for B_{12}. It seemed wise, therefore, to redetermine the diffusion coefficients by another method. Because the apparatus was available (51, 52), the diffusion coefficients were redetermined using the free diffusion method.

B. Experimental Work

1. Materials and reagents

Vitamin B_{12} was recrystallized from carbon dioxide-free water and dried at room temperature over anhydrous magnesium perchlorate. A sample of this material on further drying in vacuum at 80° for 4 hours lost 12.35 per cent of the total weight, presumably owing to the loss of water.

Vitamin B_{12a} was prepared by the hydrogenation of B_{12} (53). In our preparation, acetone was added up to 75 per cent

before the filtration and oxidation. Carbon dioxide was excluded throughout the preparation. Crystals were obtained at room temperature in yields of about 70 per cent. The pH of an aqueous solution of this preparation was 9.1 which was in good agreement with previous observations (26). Inasmuch as, vitamin B_{12a} could not be crystallized from water without the addition of acetone, it was considered possible that acetone of crystallization was included in the crystalline B_{12a}. However, Mr. J. L. Ellingboe of our laboratory, showed by the 2,4-dinitrophenylhydrazine method (54, 55) that no acetone of crystallization was present.

Reagent grade potassium sulfate was recrystallized from carbon dioxide-free water.

2. Measurement of diffusion coefficients

Diffusion coefficient measurements were carried out in a standard 11 ml. Klett electrophoresis cell. The conventional Philpot-Svenson cylindrical-lens optical system with diagonal slit was used for both observation and recording of the concentration gradients. The gradients were recorded on 35 mm. film by means of a Leica camera equipped with a focal plane shutter. The time of exposure was determined by trial and error. A tungsten light source with an orange-red filter gave the best contrast in the pattern of the concentration gradient. Curves for analysis were made by tracing the image under a projection enlarger at a magnification of approximately nine-

fold. In tracing the patterns, great care was taken to follow the center portion of the gradient outline. The overall magnification factor, from the cell to the projected image, was determined directly by photographing a glass plate with etched rulings placed in the position of the cell and measuring the spacings in the projected image under the same conditions as employed for patterns. Measurements were conducted at two temperatures, 2° and 25°. Temperature regulation was within approximately 0.01° at each temperature. Areas under the patterns of concentration gradient were measured with the planimeter and, in some cases, by the weight method as a check for the planimeter measurements.

Known amounts of the crystalline vitamins were dissolved in the solvent, either 0.1 N potassium sulfate or 0.1 N potassium sulfate plus 0.005 M potassium cyanide, so that the final concentration was 0.3 to 0.4 per cent. In all cases, the solutions and solvent were brought to the temperature of the thermostat prior to filling the cell. The cell was filled in the conventional manner. The two boundaries were formed between the vitamin solution (below) and solvent of identical composition (above). After equilibration, the boundaries were made by opening the cell and moving them to the approximate center of the cell. A very slow (2.1 cm./hr.) compensation was used by withdrawing the electrolyte from the appropriate side by a synchronous motor-driven syringe compensator. The cell was then carefully closed for the duration of the

run which extended for several days. The boundaries were not sharpened. In general, both boundaries were recorded and analyzed at intervals of 10 hours for about three days.

The calculations were based on the maximum ordinate area method assuming a mono disperse system (52). The equation used for evaluation of the diffusion coefficient was obtained by the solution of Fick's second law by Wiener's method (56):

$$\frac{dn}{dx} = \frac{n - n_o}{2\sqrt{\pi Dt}} \quad e^{-x^2/4Dt}$$

This equation was simplified by taking the "x" value equal to zero, giving the final form of the equation as follows:

$$D = \frac{k A^2}{4\pi t H_m^2}$$

"D" - diffusion coefficient (cm^2/sec.),

"A" - area under the pattern of concentration gradient (cm^2),

"t" - time elapsed from the formation of the boundary (sec.),

"H_m" - maximum height between base line and the peak (cm.),

"k" - reciprocal of the overall magnification coefficient square (unitless).

The true value of the diffusion coefficient at the steady state was obtained by plotting measured diffusion coefficient values against reciprocal time; the intercept at the infinite time being the true value of diffusion coefficient.

Diffusion coefficients at different temperatures were calculated by the relation:

$$D_{25^\circ} = D_{2^\circ} \frac{(298)}{(275)} \frac{\eta_2}{\eta_{25}}$$

Inasmuch as, the solutions were dilute the viscosity of water was used in the above equation.

The maximum probable error in the measurement of the diffusion coefficients was estimated to be between 6 to 10 per cent.

C. Results and Discussion

The values of the apparent diffusion coefficients for the individual runs are summarized in Tables I through VII. Actual conditions for the given run are specified in the footnote of each table.

The data obtained for B_{12} are shown in Fig. 2 and that for B_{12a} in Fig. 3. The summary of the results and the calculated molecular weights by the Stokes-Einstein-Longsworth equations are shown in Table VIII.

Table I. Apparent diffusion coefficients of vitamin B_{12} (Run A)[a,b,c]

Time sec.	Reciprocal Time 1/sec. x 10^6	H cm.	Area $cm.^2$	Diffusion Coefficient $cm.^2$ /sec. x 10^6
25200	39.5	4.55	11.40	2.24
73200	13.6	2.90	10.90	1.73
92700	10.8	2.75	11.70	1.68
162900	6.1	2.05	11.40	1.59
186000	5.4	1.95	10.50	1.45

[a]Concentration of B_{12} 54.5 mg. per 15 ml. of 0.1 N potassium sulfate.

[b]Overall optical magnification factor: 2.979.

[c]Bath temperature: 2.00°.

Table II. Apparent diffusion coefficients of vitamin B_{12} (Run B)[a,b,c]

Time sec.	Reciprocal Time 1/sec. x 10^6	H cm.	Area $cm.^2$	Diffusion Coefficient $cm.^2/sec. \times 10^6$
82800	12.1	6.08	22.8	1.82
106800	9.2	5.48	22.5	1.66
150900	6.6	4.62	22.3	1.64
195000	5.1	4.11	22.2	1.60
231600	4.3	3.76	22.0	1.58
279000	3.5	3.53	22.4	1.56

[a]Concentration of B_{12}: 47.0 mg. per 15 ml. of 0.1 N potassium sulfate.

[b]Overall optical magnification factor: 2733.

[c]Bath temperature: 3.00°.

Table III. Apparent diffusion coefficients of vitamin B_{12} (Run C)[a,b,c]

Time sec.	Reciprocal Time 1/sec. x 10^6	H cm.	Area $cm.^2$	Diffusion Coefficient $cm.^2/sec.$ x 10^6
60900	11.4	8.20	38.3	3.21
106200	9.4	6.12	37.3	3.13
146700	6.8	5.25	36.7	2.98
176400	5.7	4.80	36.2	2.89
237400	4.2	5.81	48.7	2.65
281800	3.5	5.20	46.2	2.51

[a]Concentration of B_{12}: 49.0 mg. per 15 ml. of 0.1 N potassium sulfate.

[b]Overall optical magnification factor: 2.998.

[c]Bath temperature: 25.0°.

Table IV. Apparent diffusion coefficients of vitamin B_{12} (Run D)[a,b,c]

Time sec.	Reciprocal Time 1/sec. x 10^6	H cm.	Area $cm.^2$	Diffusion Coefficient $cm.^2/sec. \times 10^6$
66900	14.9	4.95	21.8	3.10
95400	10.5	4.17	21.8	3.07
148800	6.7	3.55	22.8	2.97
174300	5.7	3.28	22.7	2.94
197700	5.0	3.17	23.4	2.93

[a]Concentration of B_{12}: 66.0 mg. per 15 ml. of 0.1 N potassium sulfate and 0.005 N potassium cyanide.

[b]Overall optical magnification factor: 2.733.

[c]Bath temperature: 25.00°.

Table V. Apparent diffusion coefficients of vitamin B_{12a} (Run E)[a,b,c]

Time sec.	Reciprocal Time 1/sec. x 10^6	H cm.	Area $cm.^2$	Diffusion Coefficient $cm.^2/sec. \times 10^6$
50400	19.8	1.85	4.90	1.59
73200	13.6	1.68	4.50	1.33
117200	8.6	1.51	5.00	1.17
134300	7.4	1.52	5.30	1.13

[a]Concentration of B_{12a}: 53.4 per 15 ml. of 0.1 N potassium sulfate.

[b]Overall optical magnification factor: 2.536.

[c]Bath temperature: 2.00°.

Table VI. Apparent diffusion coefficients of vitamin B_{12a} (Run F)[a,b,c]

Time sec.	Reciprocal Time 1/sec. x 10^6	H cm.	Area $cm.^2$	Diffusion Coefficient $cm.^2/sec. \times 10^6$
45300	22.1	3.70	13.8	3.29
86100	12.5	2.80	13.2	2.76
103200	9.7	3.55	18.1	2.69
177000	5.7	2.76	17.9	2.54
213600	4.7	2.45	17.2	2.49

[a]Concentration of B_{12a}: 52.5 mg. per 15 ml. of 0.1 N potassium sulfate.

[b]Overall optical magnification factor: 2.733.

[c]Bath temperature: 25.00°.

Table VII. Apparent diffusion coefficients of vitamin B_{12a} (Run G)[a,b,c]

Time sec.	Reciprocal Time 1/sec. x 10^6	H cm.	Area $cm.^2$	Diffusion Coefficient $cm.^2/sec. \times 10^6$
115200	8.7	6.82	36.9	2.74
172500	5.8	5.33	34.5	2.56
212100	4.7	4.50	31.9	2.52
254700	3.9	3.98	30.9	2.48
309000	3.2	3.65	30.6	2.46

[a]Concentration of B_{12a}: 50.0 mg. per 15 ml. of 0.1 N potassium sulfate.

[b]Overall optical magnification factor: 2.733.

[c]Bath temperature: 25.00°.

Fig. 2 Apparent Diffusion Coefficients of Vitamin B_{12} as a Function of Time.

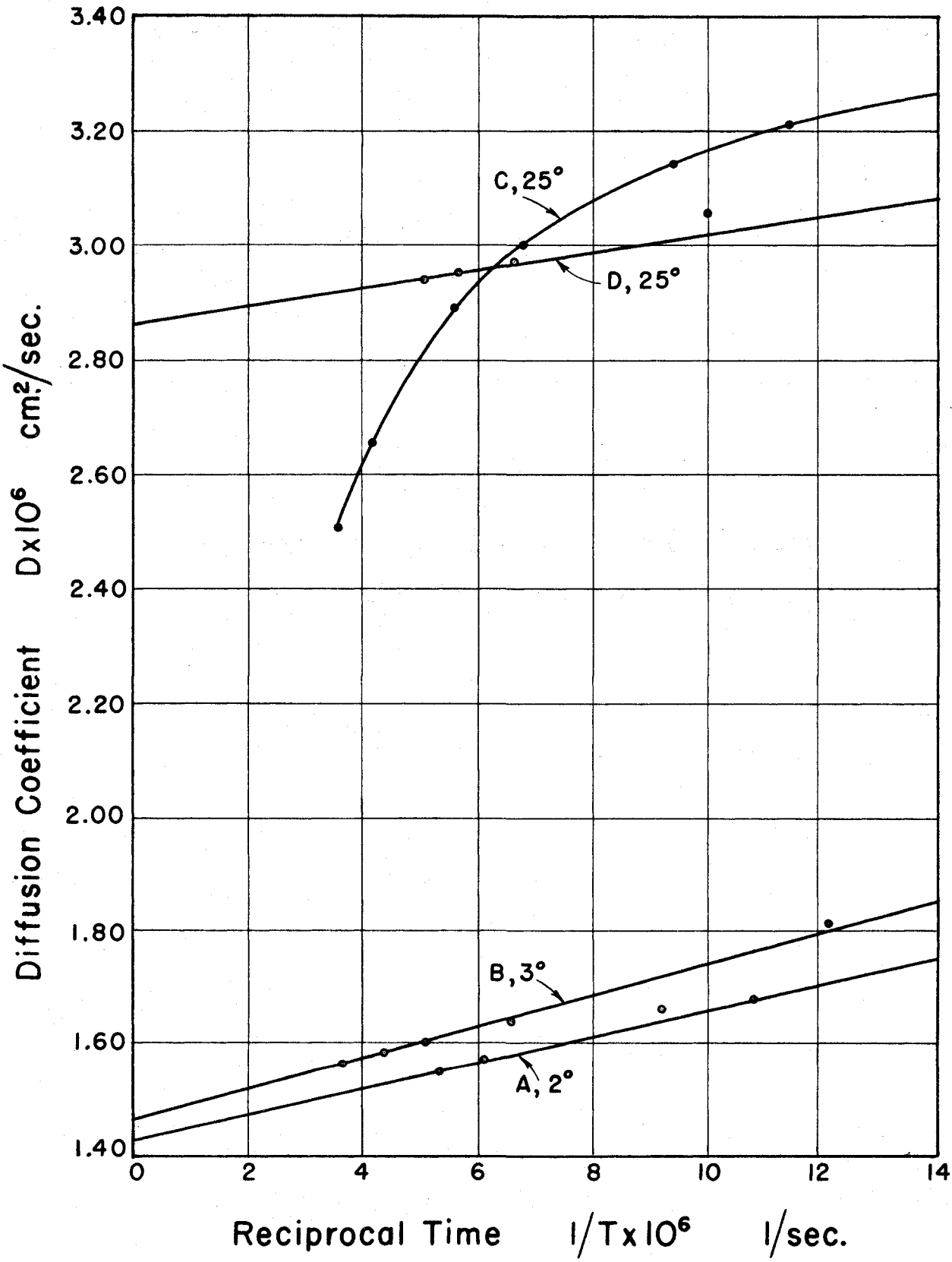

3.40
3.20
3.00
2.80
2.60
2.40
2.20
2.00
1.80
1.60
1.40
0
2
4
6
8
10
12
14
C, 25°
D, 25°
B, 3°
A, 2°
Diffusion Coefficient Dx10⁶ cm.²/sec.
Reciprocal Time 1/Tx10⁶ 1/sec.

Fig. 3 Apparent Diffusion Coefficients of Vitamin B_{12a} as a Function of Time.

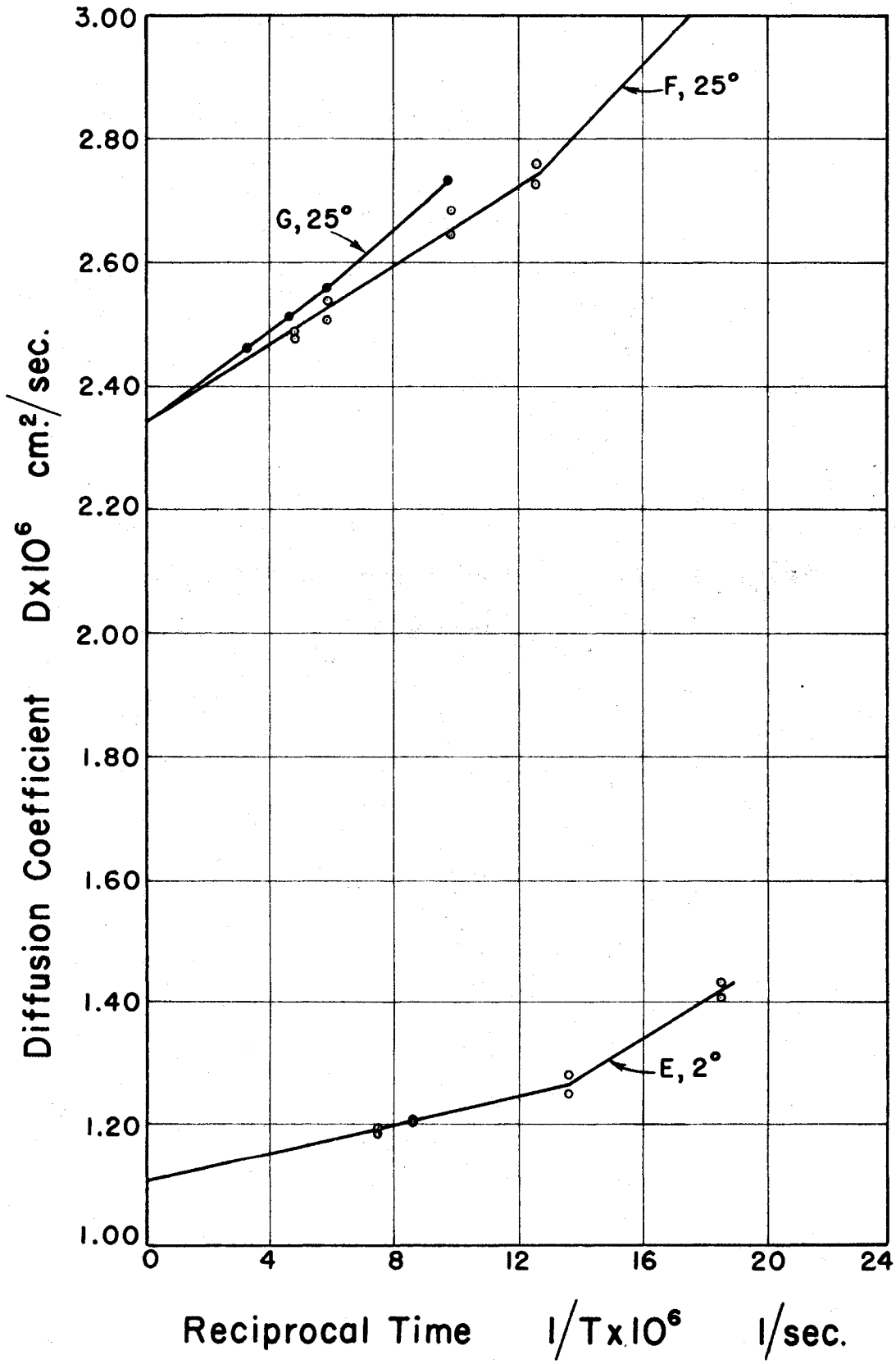

3.00
2.80
2.60
2.40
2.20
2.00
1.80
1.60
1.40
1.20
1.00
Diffusion Coefficient Dx10⁶ cm.²/sec.
F, 25°
G, 25°
E, 2°
0
4
8
12
16
20
24
Reciprocal Time 1/Tx10⁶ 1/sec.

Table VIII. The diffusion coefficients of vitamins B_{12} and B_{12a}

Run No.	Material	Solution	D at 2°C Exper.	D at 25°C Calc.	D at 25°C Exper.	Mol. Wt. (Stokes-Einstein)	Mol. Wt. (Stokes-Einstein-Longsworth)
A	B_{12}	0.1 N K_2SO_4	1.4(3)	2.9(0)		2244	1335
B	B_{12}	0.1 N K_2SO_4	1.4(6)[a]	2.9(7)		2088	1254
C	B_{12}	0.1 N K_2SO_4			2.7(3)[b]	2703	1563
D	B_{12}	0.1 N K_2SO_4 0.005 N KCN			2.8(7)	2316	1373
E	B_{12a}	0.1 N K_2SO_4	1.1(0)	2.2(4)		4496	2426
F	B_{12a}	0.1 N K_2SO_4			2.3(4)	3934	2160
G	B_{12a}	0.1 N K_2SO_4			2.3(7)	3792	2088

[a]Temperature for this run was 3.0°.

[b]This value is an average value for the run C; it should not be averaged with the results A, B, and D because of the evident decomposition of vitamin B_{12} at 25.0° in the absence of excess cyanide.

It is evident from run "C" that B_{12} decomposes somewhat in water, probably with the liberation of cyanide and the formation of B_{12a}, the latter having a lower diffusion coefficient. Such a dissociation has been recognized previously (28). That this dissociation would be repressed by the presence of an excess of cyanide, as found in run "D", was expected. In the presence of excess cyanide, B_{12} is converted to $B_{12}CN$ (containing a total of two cyanides) which is purple. Although a large excess of cyanide is required to convert B_{12} to $B_{12}CN$ quantitively (40), an appreciable amount of $B_{12}CN$ was present in the solution of run "D", for the solution was purplish in color. B_{12} and $B_{12}CN$ differ only slightly in weight (by 26 in 1370) and because the diffusion coefficient is inversely proportional to the molecular weight, the diffusion coefficients of the two would be expected to differ by less than the experimental error (6 to 10 per cent) in the measurement. Runs "A", "B", and "D" may be averaged, therefore.

Using the values of the diffusion coefficients at 25°, the apparent molecular weights were calculated using the Stokes-Einstein equation:

$$D = \frac{RT}{N} \quad \frac{1}{6\pi\eta r}$$

which on substitution of "N" Avogadro's number, "R" - the universal gas constant, "T" - the absolute temperature, "η" the viscosity of water and "r" - the radius of B_{12} molecule

expressed in terms of apparent specific volume "V_m" is simplified to:

$$D = \frac{3.32 \times 10^{-5}}{(V_m)^{1/3}}$$

A value of 1.50 was used for the density of B_{12} in solution, and the value of 1.38 for the density of B_{12a} in solution as obtained from apparent specific volume measurements. The value 8.93×10^{-3} dyne-cm./sec.^{2} was used for the viscosity of water at 25°. The value obtained for the molecular weight of B_{12}, 2085, is considerably higher than the value of 1370 calculated from the cobalt analysis (57) of the material dried in vacuum at 80°.

Application of the empirical modification of the Stokes-Einstein equation recently developed by Longsworth (58)

$$D \times 10^6 = 33.06 / (1.367\ V^{1/3} - 1.750)$$

leads to the average molecular weights of B_{12} and B_{12a} 1380 and 2225, respectively. At request Dr. Howard Schachman of the Virus Laboratory, University of California, determined the sedimentation coefficients of vitamins B_{12} and B_{12a} in 0.1 N potassium sulfate. The measurements were made using the synthetic-boundary cell recently described by Schachman and coworkers (59). Dr. Schachman reports the following sedimentation coefficients (corrected to water at 20°): for B_{12} 0.50 Svedberg units and for B_{12a}, 0.52 units. Each of these values is the average of three independent observations conducted over a concentration range of approximately 5 to 9 mg./ml.

The molecular weights of vitamins B_{12} and B_{12a} were calculated by the Svedberg equation:

$$M = RTs/D(1 - \rho V)$$

where "M" is the molecular weight, "R" - the gas constant, "s" - the sedimentation coefficient, "D" - the diffusion coefficient, "V" - the apparent specific volume, and "ρ" - the density of solution. These calculations yield molecular weights of 1420 for vitamin B_{12} and 2230 for vitamin B_{12a}. It is important to note that these results should be independent of any assumption as to the shape or hydration and should represent the unhydrated molecules. The agreement with the chemical molecular weight of B_{12}, 1370, is very satisfactory and the contention that B_{12a} exists as a dimer seems substantially confirmed.

It should be noted that values of "s" and "D" which have been extrapolated to zero concentration should be used. It seems probable, however, that concentration effects are relatively small for those small molecules.

D. Conclusions

New measurements of the diffusion coefficients of vitamins B_{12} and B_{12a} by the free diffusion method give

B_{12}	2.9 (1)	x 10^{-6}	$cm.^2/sec.$	25°
B_{12a}	2.3 (3)	x 10^{-6}	$cm.^2/sec.$	25°

B_{12} decomposes slightly in water solution at room temperature. This decomposition is repressed by cyanide and by

lowering the temperature.

Values for the molecular weights of the two vitamins calculated from the diffusion coefficients indicate that B_{12a} is a dimer in solution. Such dimerization occurs only after dissolution. The dimeric character is also indicated by measurements of the sedimentation coefficient.

As with the earlier values for the diffusion coefficients, the new values lead to a molecular weight of B_{12a} almost twice that of B_{12}. However, the X-ray crystal pattern of B_{12a} is practically identical (60) with that of B_{12} and the symmetry group into which both fall is such that the molecule cannot be other than a monomer. Any dimerization of B_{12a} must then occur after dissolution in water.

Assuming that the maximum probable error in the measurements of the diffusion coefficients is 10 per cent; the error in molecular weight calculated is not more than 30 per cent. This accuracy is sufficient to justify the conclusion that the molecule of B_{12a} dimerizes but that B_{12} does not.

III. APPARENT SPECIFIC VOLUMES OF VITAMINS B_{12} AND B_{12a}

A. Introduction

In connection with the calculations of molecular weights by the Stokes-Einstein-Longsworth and Svedberg equations it was necessary to determine apparent specific volumes of vitamins B_{12} and B_{12a} in aqueous solution. Rapidity, precision, availability of material and equipment were considered in selecting an appropriate method for the determination of the density of the solution.

The density measurements were made by the pycnometer method, and the calculations were made using the usual relationship:

$$v_s = \frac{1}{\rho_o} - \frac{\rho_o - \rho_s}{\rho_o} \frac{V}{g}$$

in which "v_s" is the apparent specific volume, "ρ_o" and "ρ_s" - the densities of water and solution respectively, "V" - the volume of the pycnometer, and "g" - the weight of the solute.

The molecular weight calculated indicated that vitamin B_{12a} dimerized in aqueous solution but offered no mechanism by which the dimerization had occurred. A clue to this was obtained during the course of the density measurements. Erratic results were obtained in the initial density measurements on B_{12a}, although no difficulty was experienced with B_{12}. The variation was traced to the time of contact of the solutions with the atmosphere and ultimately to dissolved

oxygen.

B. Experimental Work

1. Materials

Vitamin B_{12}, obtained from the Squibb Institute for Medical Research, New Brunswick, N. J., was recrystallized from carbon dioxide-free water.

Oxygen-free nitrogen was prepared by passing tank nitrogen through two scrubbers of vanadous sulfate, one scrubber of sodium hydroxide, and one of water.

Vitamin B_{12a} was prepared from the crystalline vitamin B_{12} by the hydrogenation procedure previously described.

2. Apparatus and procedure

A 5.0 ml. pycnometer was used. Weighings were made on the Ainsworth TCX semimicro balance using tares of identical weight and volume. Solutions were kept in a water bath at $25.00^{\circ} \pm 0.01^{\circ}$. The balance room was thermostated to slightly below 25°.

The pycnometer was charged with liquid already brought to equilibrium with oxygen-free nitrogen, air, or oxygen. Water was placed on a small conical flask bearing a two-holed rubber stopper carrying lengths of glass tubing one of which reached the bottom of the flask, and the second of which served as a gas outlet. The gas was bubbled through the solution for about 15 minutes. The crystalline vitamin was added through the gas outlet tube and thus dissolved in the

water without the stopper having been removed. The gas stream was then continued an additional 30 minutes. The solution was then transferred to the pycnometer with a hypodermic syringe. In oxygen-free experiments the pycnometer and the syringe were well flushed with nitrogen and the operations were carried out in a large beaker filled with continuously flowing nitrogen gas. The pycnometer was then kept in the water bath for 45 minutes. After the pycnometer was taken out of the bath it was rinsed with alcohol and dried with a moist chamois skin and handled without contact with the hands. Weighings were made after 10 minutes with the usual precautions to minimize static electrical effects.

After weighing, and aliquot of 0.200 ml. was taken for cobalt analysis by the colorimetric method with 2-nitroso-1-naphthol-4-sulfonic acid (57).

The maximum probable error in this determination of apparent specific volume is about 3 per cent on the basis that the error in the individual measurements was not greater than 0.01° in temperature, 0.00006 ml. per ml. in the volume of pycnometer, 0.00002 in the weights, and 0.60 per cent of vitamin as obtained from the cobalt determination.

The effect of dissolved air on the density of water is noticeable only in the 6th place.

C. Results and Discussion

The results of the various measurements of the apparent

specific volumes of B_{12} and B_{12a} are presented in Table IX. The average values obtained are:

B_{12} (Runs 1, 2, 3, and 4):	0.665
B_{12a}, deaerated (Runs 10, 12, and 13):	0.650
B_{12a}, in contact with oxygen (Runs 5, 6, 7, 9, 11, 14 and 15):	0.713

The apparent specific volume of B_{12} is independent of the gas with which the solution is equilibrated as shown by Runs 1, 2, 3, and 4 of Table IX. The apparent specific volume of B_{12a}, however, is dependent upon the presence of oxygen and leads to a higher value, 0.713, as contrasted to 0.650. In the absence of oxygen the apparent specific volume of B_{12a} is the same as that of B_{12}. The union of oxygen with B_{12a} is labile for on sweeping out the oxygen with a stream of nitrogen the apparent specific volume dropped to that of B_{12}, Run 10. A fifteen minute period sweeping with nitrogen gas proved to be insufficient time for complete removal of the oxygen, Run 8.

The densities of solutions are linear with concentration as expected, shown in Fig. 4.

Table IX. Apparent specific volume of vitamins B_{12} and B_{12a}

Run No.[a]	Material	Equilibrating Gas	Density of Solution	Weight of Vitamin mg./ml.	Volume ml./g.
1 - 1	B_{12}	Air	1.00031	9.557	0.662
2 - 2	B_{12}	Air	0.99978	8.545	0.670
3 - 3	B_{12}	Nitrogen	0.99903	5.751	0.662
4 - 3	B_{12}	Oxygen	0.99907	5.751	0.669
5 - 5	B_{12a}	Air	0.99876	6.127	0.727
6 - 6	B_{12a}	Air	0.99840	4.648	0.715
7 - 7	B_{12a}	Oxygen	0.99834	4.545	0.720
8 - 7[b]	B_{12a}	Nitrogen	0.99842	4.545	0.706
9 - 9	B_{12a}	Air	0.99832	4.325	0.716

[a]The second number indicates which solution was used for the measurement; thus runs 3 and 4 were made on the same solution.

[b]In Run 8, nitrogen was bubbled through the solution for 15 minutes only; the oxygen was apparently incompletely removed in this time.

Table IX. (Continued)

Run No.[a]	Material	Equilibrating Gas	Density of Solution	Weight of Vitamin mg./ml.	Volume ml./g.
10 - 9(10)[c]	B_{12a}	Nitrogen	0.99844	3.775	0.639
11 - 10	B_{12a}	Oxygen	0.99821	3.775	0.703
12 - 12[d]	B_{12a}	Nitrogen	0.99969	7.379	0.651
13 - 12[e]	B_{12a}	Nitrogen	0.99961	7.379	0.660
14 - 14[f]	B_{12a}	Oxygen	0.99965	8.425	0.707
15 - 14[g]	B_{12a}	Oxygen	0.99956	8.425	0.702

[c]In Run 10, nitrogen was bubbled through solution 9 for 14 hours: the resulting solution was given the new number 10 because of the dilution on transfer.

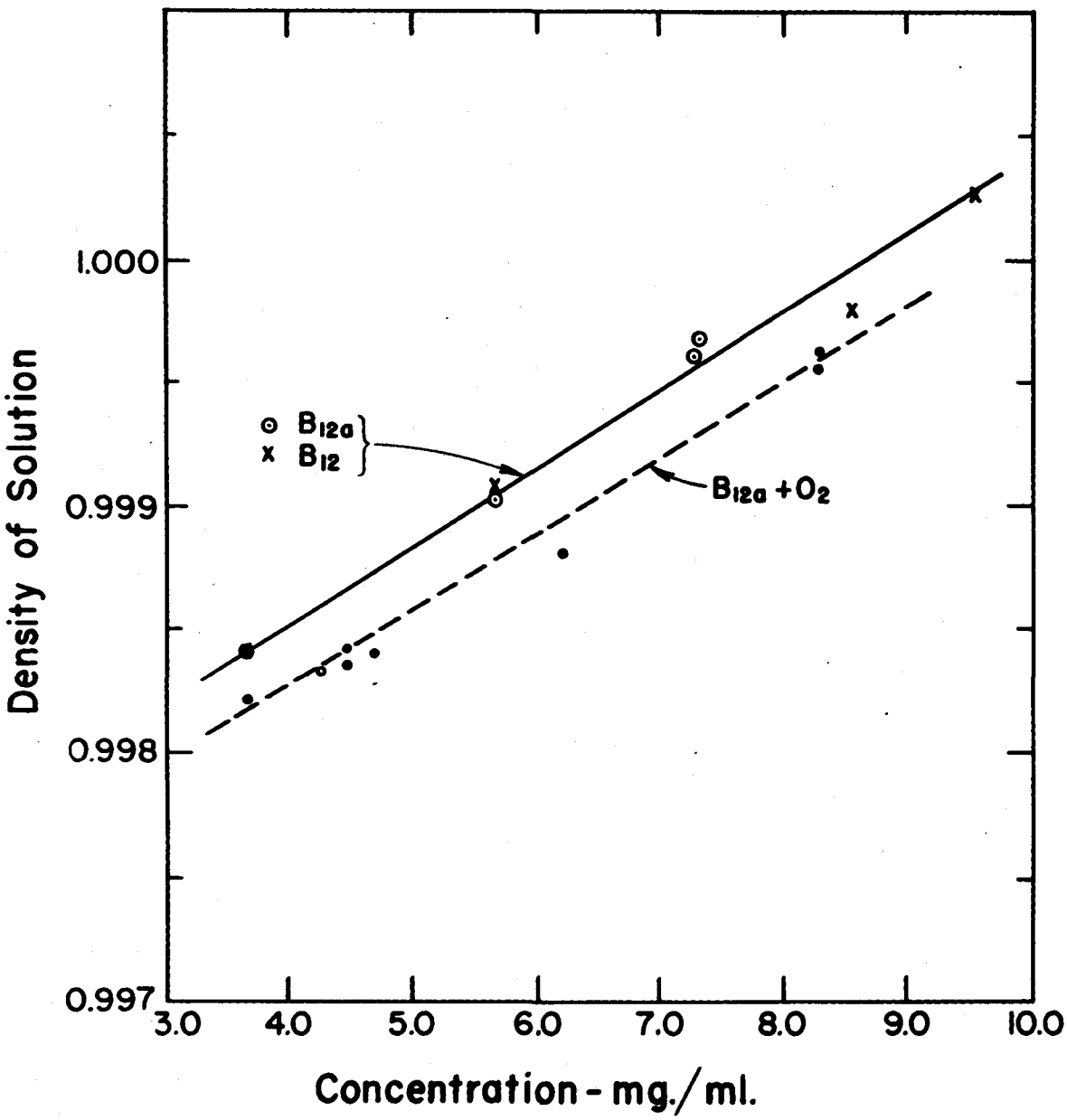
[d]In Run 12, the water was thoroughly deaerated before the B_{12a} was dissolved.

[e]Solution 12 was allowed to stand for 14 hours and the measurements were repeated.

[f]In Run 14, oxygen was bubbled through a fresh solution for 4 hours.

[g]Overnight treatment with oxygen.

Fig. 4 Density as a Function of Concentration.


Density of Solution
1.000
0.999
0.998
0.997
3.0
4.0
5.0
6.0
7.0
8.0
9.0
10.0
Concentration - mg./ml.
B12a
B12
B12a + O2

The dimerization of B_{12a} first observed in diffusion coefficient measurements is caused by the union of B_{12a} with oxygen in a sense of the reaction:

$$2\ R\ Co(III)\ OH + O_2 \xrightleftharpoons{H_2O} [R\ Co(III) - O_2 - Co(III)\ R]^{++} + 2\ OH^-$$

This union is reversible and undoubtedly accounts for the catalytic effect of B_{12a} on air oxidation of iodide (46).

Inasmuch as, B_{12a} cannot be crystallized from water solution without the addition of acetone it is likely that the function of acetone is to expel the oxygen from the dimer in a fashion similar to the expulsion of oxygen from the oxygenated Bis-(disalicylalethylenediimido)-aquo dicobalt (61).

D. Conclusions

In presence of oxygen the apparent specific volume of B_{12a} is larger than that of B_{12}, the values being 0.713 and 0.665, respectively. In absence of oxygen there is no perceivable difference in the apparent specific volume of B_{12} and B_{12a} value being 0.650. The change of the apparent specific volume of B_{12a} in presence of oxygen can be accounted by the interaction of molecular oxygen with B_{12a}. The union of oxygen with B_{12a} is labile and oxygen can be removed from the dimer.

IV. AMPEROMETRIC TITRATION OF B_{12}, B_{12a}, AND B_{12r} WITH OXYGEN

A. Introduction

Measurements of the apparent specific volumes proved the necessity of the presence of oxygen for the dimerization of B_{12a} in aqueous solution. However, this information was not sufficient for the determination of the stoichiometry of the dimerization reaction. Various methods of investigation were considered; finally, the polarographic method was selected as being most applicable with the small amounts of material available.

The polarography of vitamins B_{12} and B_{12a} has already been investigated (38, 39, 43). B_{12} shows a single two-electron reduction wave at a half potential of -1.12 v. toward the S.C.E. Vitamin B_{12a} shows two one-electron reduction waves at half-wave potentials -0.04 and -1.02 v. toward the S.C.E. Oxygen dissolved in water shows two two-electron reduction waves, half-wave potentials -0.08 v. and -0.96 v. toward the S.C.E., corresponding respectively to the reduction of oxygen to hydrogen peroxide and of the latter to water. If B_{12a} and oxygen were present in the same solution and if no interaction were to occur, the wave heights of the first reduction wave would be simply additive, inasmuch as, the half-wave potentials of the first reduction waves of the two substances are the same. On the other hand, if the interaction occurs then a shift should be observed in the half

wave potential and the wave heights should not be additive but characteristic of a new chemical species.

In order to vary the concentration of one component in the mixture, a saturated oxygen solution of the supporting electrolyte was used as a titrant. In effect, this study became an amperometric titration of vitamin B_{12a} with oxygen.

Similar amperometric studies were carried out on the vitamins B_{12} and B_{12r}.

B. Experimental Work

1. Materials and reagents

Vitamin B_{12}, obtained from The Squibb Institute for Medical Research, New Brunswick, N. J., was recrystallized from deionized water.

Vitamin B_{12a} was prepared from the crystalline B_{12} by the hydrogenation procedure previously described.

Oxygen-free nitrogen was prepared by passing tank nitrogen through two scrubbers of vanadous sulfate, one scrubber of sodium hydroxide and one of water. The gas was led to the apparatus through all glass tubing.

Electrolytic hydrogen was obtained from the low temperature laboratory of the Department of Physics, Iowa State College. The impurities in the hydrogen were determined by Dr. Harry J. Svec using the mass spectrograph. Oxygen, carbon dioxide, methane and other gaseous impurities commonly found in commercial cylinder hydrogen were absent.

Potassium sulfate, used for the preparation of supporting electrolyte solutions, was recrystallized from deionized water.

Cylinder oxygen was passed through a tower of ascarite to remove acidic gasses present.

A standard chromous chloride was prepared from chromic chloride dissolved in 0.1 N hydrochloric acid by keeping it over amalgamated zinc in a Machlett buret. A rubber balloon inflated with nitrogen was attached to the upper opening of the buret.

A standard solution of oxygen was prepared by bubbling purified oxygen gas through 0.1 N potassium sulfate solution for 6 hours. The solution was stored in a Machlett buret over which pure oxygen was maintained slightly above atmospheric pressure by a balloon inflated with oxygen and attached to the upper side arm of the buret.

A standard potassium iodate solution was prepared by dissolving reagent grade potassium iodate in boiling deionized water. The hot solution was transferred to the Machlett buret and it was stored over nitrogen atmosphere.

A standard thio-sulfate solution was prepared by dissolving reagent grade sodium thiosulfate in distilled water.

Manganous sulfate solution was prepared from the reagent grade manganous sulfate tetrahydrate. The absence of manganic and ferric salts was checked by the addition of this solution to an acidified potassium iodide solution. Only a slight

trace of iodine was liberated.

Alkaline iodide reagent was prepared by dissolving 700 g. of reagent grade potassium hydroxide and 150 g. of reagent grade potassium iodide in one liter of deionized water. The reagent was checked for free iodine by the addition of starch to an acidified portion of the reagent. This test indicated the absence of nitrites, iodates, and ferric salts.

2. Apparatus

A Sargent Model XXI polarograph was used. The functional operation of this instrument was checked frequently against a standard resistance. The polarograph cell used was the usual type. The tip of the Machlett buret containing the standard oxygen solution was inserted along side of the capillary and the salt bridge of the saturated calomel electrode.

A Beckman DU spectrophotometer was used in making the colorimetric determinations of cobalt.

A Beckman G pH meter employing calomel and platinum electrodes was used for the potentiometric titrations.

3. Determination of the oxygen in the standard solution

In preliminary work the concentration of the oxygen in the standard solution was obtained by interpolating the values for the solubility of oxygen at various temperatures as given in Lange's Handbook, and checked by the calculations involving polarographic data. In later work, the oxygen concentration was determined by direct chemical measurement,

either by a measured volume of standard oxygen solution (62), or by the Winkler method (63). The agreement between various methods was quite satisfactory as shown in Table X.

Chromous chloride method. The chromous chloride method proved to be a rather tedious and time consuming method for the determination of oxygen. Throughout the titration oxygen had to be excluded not only from the titration vessel but also from the standard solutions. The chromous chloride solution was standardized against the standard ferric perchlorate solution which was prepared by dissolving iron wire in perchloric acid. This solution was heated until the dense fumes of perchloric acid appeared. The end point was determined potentiometrically using calomel and platinum electrodes.

The results obtained were not as reproducible as in the titration of the chromous chloride solution with the oxygen-free iodate. An excess of iodate was added to the chromous chloride, and the excess of iodine was back titrated by the same chromous chloride, or by the standard thiosulfate using starch as the indicator. The latter modification did not require exclusion of oxygen after the excess of iodate was added.

A wide mouth titration vessel containing a four-holed rubber stopper giving enough space for the three tips of Machlett burets and a glass tubing for nitrogen was used for the titration of oxygen solution. After deaeration of the titration vessel 5.00 ml. of the standard chromous

Table X. Determination of oxygen concentration by different methods

Standard Oxygen Solution Number	Winkler Method		Polarographic Method		Chromous Oxidation Method	
1	29.7		30.6		29.0	
	29.9	29.8*	30.4		29.1	
			29.8		29.8	29.3*
			29.9	30.2*		
2	27.2		26.1		26.6	
	27.4	27.3*	27.4		27.5	
			27.6		26.2	
			26.8	26.9*	27.9	27.1*
3	30.5		31.2		29.9	
	30.3		30.4		29.4	
	30.4	30.4*	30.3		30.4	29.9*
			31.3	30.8*		

*The average value for the determination.

chloride solution were added from the Machlett buret following in succession with 5.00 ml. of standard oxygen solution. The excess of chromous chloride was oxidized with the standard iodate. The starch solution was added, and the excess of iodine was back titrated with the standard thiosulfate. Oxygen concentration was calculated from the milliequivalents of chromous chloride used for the reduction of oxygen.

The results obtained by the chromous chloride method had greater deviation, and the average values on the whole were lower than the values obtained by the Winkler or polarographic

methods.

Winkler method. The Winkler method was the most reliable method for the determination of oxygen. Aliquots of 200 ml. were taken for oxygen analysis. The size of the sample was the only draw back in this determination. The results obtained by this method were in good agreement with the polarographic determination, and the deviation from the average value was rather small.

The procedure from the "Standard Methods for the Examination of Water and Sewage" was followed.

Polarographic method. The polarographic method proved to be the quickest of the three methods involving the actual determination of oxygen. Exactly 5.00 ml. of the supporting electrolyte, 0.1 N potassium sulfate, were placed in the polarographic cell. The cell was flushed thoroughly with oxygen-free nitrogen. A small portion of the standard oxygen solution was then added, the solution was stirred gently, and the polarogram was then recorded. A faint stream of nitrogen was passed over the surface of the liquid during these operations. A further volume of the standard oxygen solution was then added and the polarogram was recorded. The temperature was maintained at $25^{\circ} \pm 0.2^{\circ}$. The concentration of oxygen in the solution was calculated by the Ilkovic equation using the value 2.60×10^{-5} cm.^{2}/sec. for the diffusion coefficient of oxygen (64) and the diffusion current for the first reduction wave of oxygen.

4. Determination of cobalt

Aliquots of various solutions were analyzed for cobalt by first destroying the organic matter by fuming with perchloric acid and then determining the cobalt with 2-nitroso-1-naphthol-4-sulfonic acid (57).

5. Amperometric titration of B_{12a}

Exactly 5.00 ml. of the supporting electrolyte, 0.1 N potassium sulfate, was placed in the polarograph cell. A platinum boat containing the crystalline B_{12a} was hung above the liquid in the cell. The cell was flushed thoroughly with oxygen-free nitrogen. Boat and B_{12a} were then dropped into the solution. After sufficient time for mixing and dissolution, the polarogram was recorded. A small portion of the standard oxygen solution was then added, the solution was stirred gently, and the polarogram was then recorded. A faint stream of nitrogen was passed over the surface of the liquid during these operations. A further volume of the standard oxygen solution was then added and the polarogram again recorded. This sequence was repeated until sufficient oxygen had been added to have combined with the B_{12a}. At the end of the titration the solution was diluted with measured amount of the standard oxygen solution to exactly 10.0 ml. and an aliquot of 0.200 ml. was taken for cobalt analysis. The temperature throughout the titration was maintained at $25^{\circ} \pm 0.2^{\circ}$.

The diffusion current was measured on the polarograms

recorded by the standard procedure. Using the values for the diffusion currents obtained experimentally, values were calculated for the diffusion current which would have been obtained had there been no dilution:

$$i_{d_{corrected}} = i_{d_{measured}} (V + v)/V$$

in which "V" - is the initial volume of the supporting electrolyte, and "v" - the volume of the titrant added.

Typical polarograms are shown in Fig. 5. The results of a representative titration are summarized in Table XI and shown graphically in Fig. 6. The end-point for this titration was found to be at 2.18 ml.

Fig. 5 Polarograms of Vitamin B_{12a}, Oxygen, B_{12a} plus Oxygen (at End-point), and Supporting Electrolyte.

Curves for B_{12a} and B_{12a} plus oxygen were obtained on the same solutions; that of oxygen was obtained on a solution containing less oxygen than required to reach the end-point.

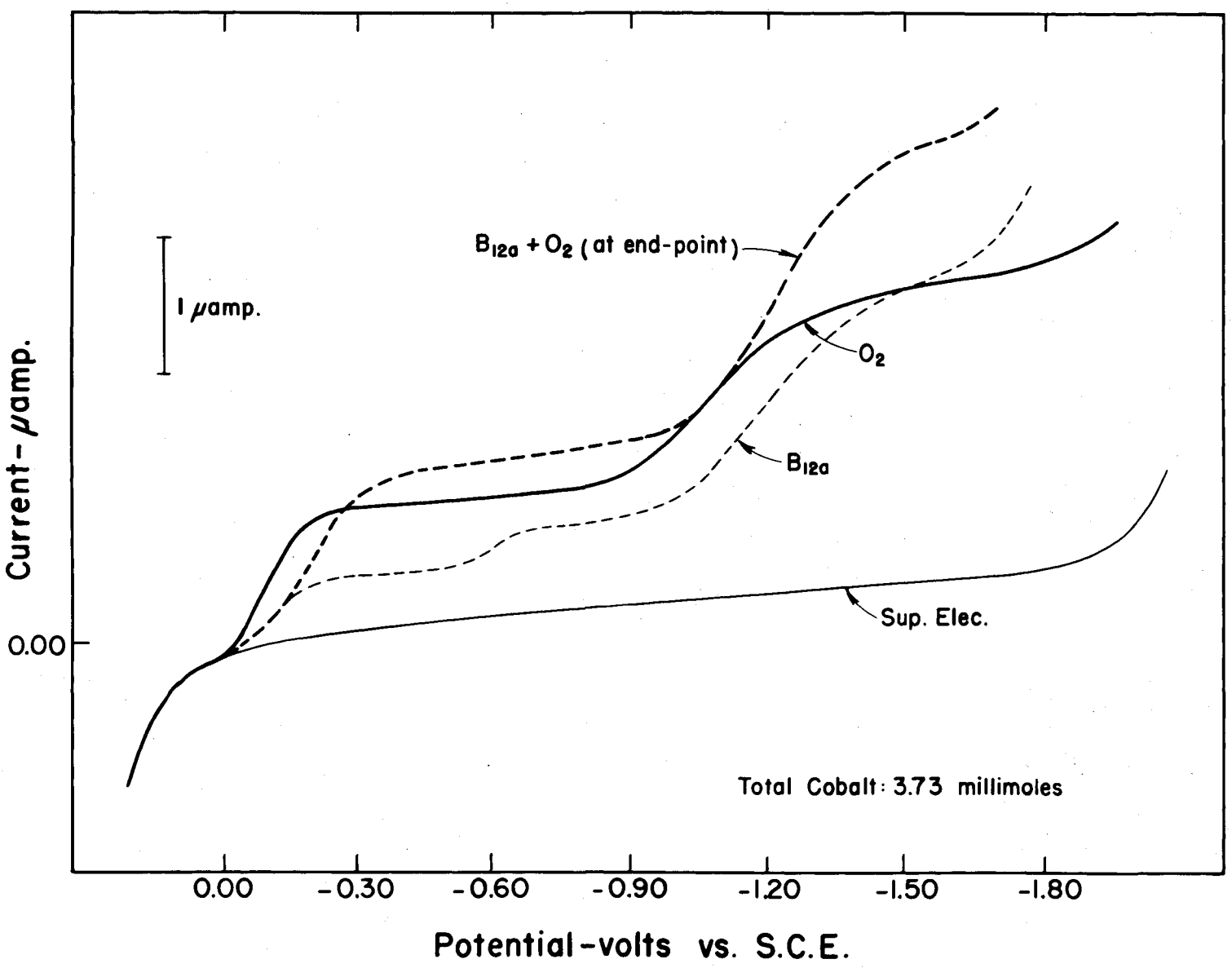

1 μamp.
$B_{12a} + O_2$ (at end-point)
O_2
B_{12a}
Sup. Elec.
Total Cobalt: 3.73 millimoles
Current - μamp.
0.00
0.00
-0.30
-0.60
-0.90
-1.20
-1.50
-1.80
Potential - volts vs. S.C.E.

Table XI. Polarographic behavior of solutions of oxygen and of B_{12a} plus oxygen. (Initial volume of the supporting electrolyte, 5.00 ml.; initial concentration of B_{12a}, 0.746 mmolar)

Oxygen Added		Diffusion Current Observed	
Volume of Standard Solution[a] ml.	Quantity mmoles	Oxygen Alone[b] μamp.	B_{12a} plus Oxygen μamp.
0.00	0.000	0.00	0.51
0.25	0.220	0.43	0.60
0.50	0.436	0.88	0.70
0.75	0.654	1.30	0.96
1.00	0.872	1.75	1.07
1.25	1.090	2.05	1.12
1.50	1.308	2.45	1.24
2.00	1.744	2.92	1.46
2.25	1.960	3.14	1.61
2.50	2.180	3.36	1.77
3.00	2.620	3.80	2.10
3.50	3.050	4.12	2.39
4.00	3.490	4.53	2.74

[a]Concentration of the standard solution: 1) 26.9 p.p.m. (from the polarographic data and calculations using Ilkovic equation); 2) 27.4 p.p.m. (Winkler method).

[b]As determined by blank run.

Table XI. (Continued)

Oxygen Added		Diffusion Current Calculated		
Volume of Standard Solution	Quantity mmoles	Oxygen Alone[c]	B_{12a} Plus Oxygen Assuming No Interaction[d]	B_{12a} Plus Oxygen Observed Values Calculated to Original[e]
0.00	0.000	0.00	0.51	0.51
0.25	0.220	0.46	0.97	0.63
0.50	0.436	0.97	1.48	0.77
0.75	0.654	1.50	2.01	1.10
1.00	0.872	1.99	2.50	1.28
1.25	1.090	2.56	3.07	1.40
1.50	1.3080	3.08	3.59	1.61
2.00	1.744	4.00	4.51	2.04
2.25	1.960	4.50	5.01	2.34
2.50	2.180	4.85	5.46	2.65
3.00	2.620	6.08	6.59	3.36
3.50	3.050	6.80	7.31	4.06
4.00	3.490	8.05	8.56	4.93

[c]Diffusion current calculated from the diffusion current observed to the diffusion current which would have been observed had no dilution occurred during the titration

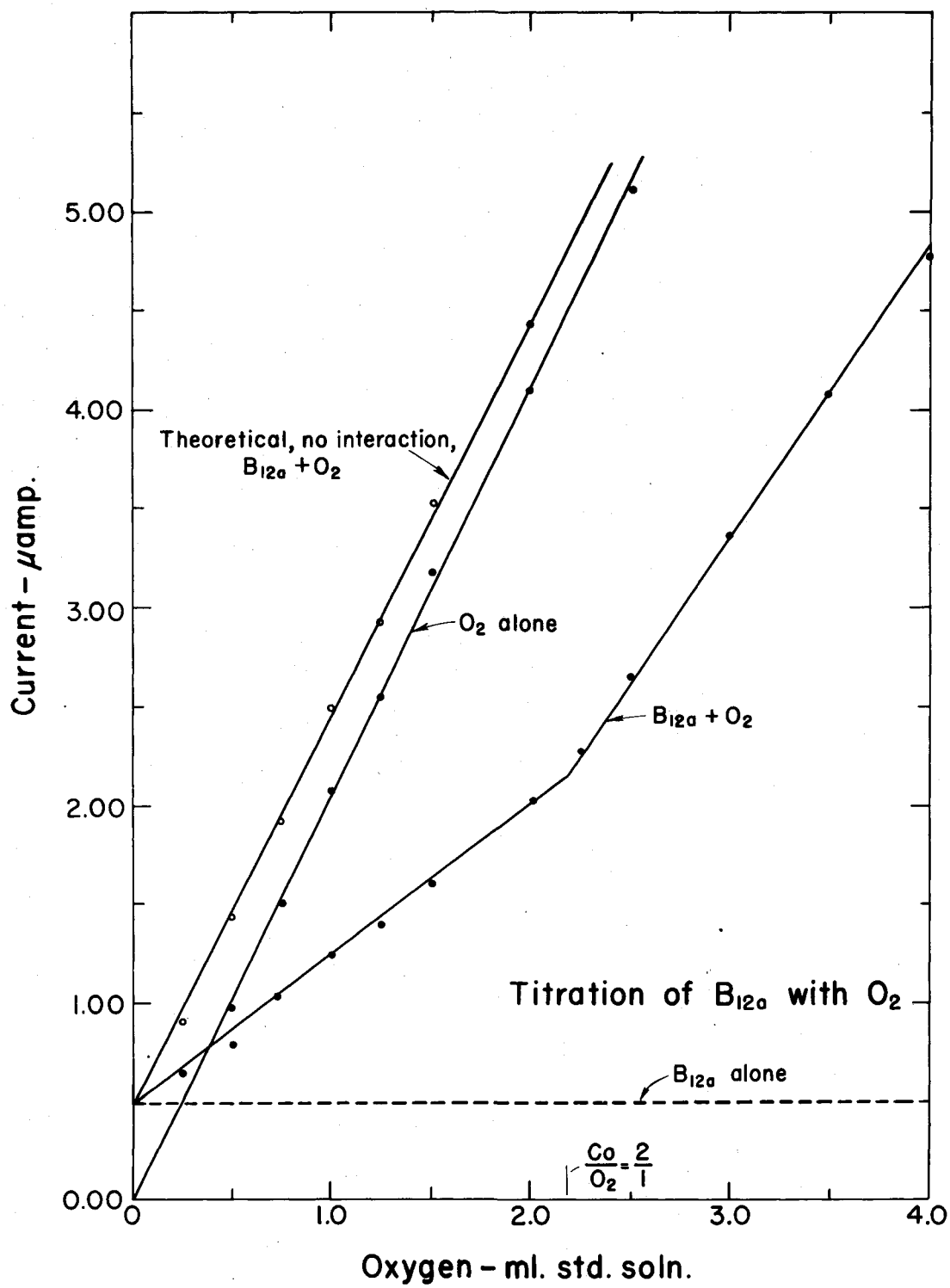
$$i_{d_{corrected}} = i_{d_{measured}} \quad (V + v)/V$$

"V" - the initial volume (5.00 ml.) and "v" - the volume of the titrant added.

[d]Diffusion current of oxygen corrected to original volume (5.00 ml.) plus diffusion current of B_{12a}.

[e]Observed diffusion current of B_{12a} plus oxygen corrected to original volume.

Fig. 6 Amperometric Titration of B_{12a} with Standard Oxygen Solution.


5.00
4.00
3.00
2.00
1.00
0.00
Current – μamp.
Theoretical, no interaction,
B12a + O2
O2 alone
B12a + O2
Titration of B12a with O2
B12a alone
Co/O2 = 2/1
0
1.0
2.0
3.0
4.0
Oxygen – ml. std. soln.

6. Amperometric titration of B_{12r}

Crystalline B_{12} was dissolved in about 5.2 ml. of 0.1 N potassium sulfate, and it was hydrogenated for seven hours using platinum as a catalyst. The hydrogenated solution was transferred from the hydrogenation vessel through a fritted glass filter equipped with all glass connections to the polarograph cell. The cell and all the apparatus were continuously flushed with a stream of nitrogen and hydrogen. Nitrogen was bubbled for additional five minutes through the solution and then the polarogram was recorded. A small portion of the standard oxygen solution was then added, the solution was stirred gently, and the polargram was recorded. This sequence of operations was repeated until sufficient oxygen had been added to have passed theoretical end-point. Throughout the titration a faint stream of nitrogen was swept over the surface of the solution. The temperature was maintained at $25^{\circ} \pm 0.2^{\circ}$.

At the end of the titration the solution was transferred to a volumetric flask. The cell was rinsed with small portions of the standard oxygen solution, measured from the Machlett buret, until the volumetric flask was filled to the mark. The difference in the volume of the volumetric flask and the volume added during and after the titration gave the initial volume of B_{12r} before the titration. An aliquot of 0.200 ml. was taken for cobalt analysis.

Blank polarograms were recorded in a separate series of

runs for the supporting electrolyte alone and were treated with successive portions of the standard oxygen solution. Typical polarograms are shown in Fig. 7.

The diffusion current was measured on the polarograms recorded by the standard procedure. The diffusion currents obtained experimentally were corrected for the dilution as described previously. The results for a representative titration summarized in Table XII and shown in Fig. 8 indicated presence of two end-points, at 1.17 ml. and at 3.48 ml. of standard oxygen solution.

Fig. 7 Polarograms of B_{12r}, Oxygen, B_{12r} plus Oxygen, and Supporting Electrolyte.

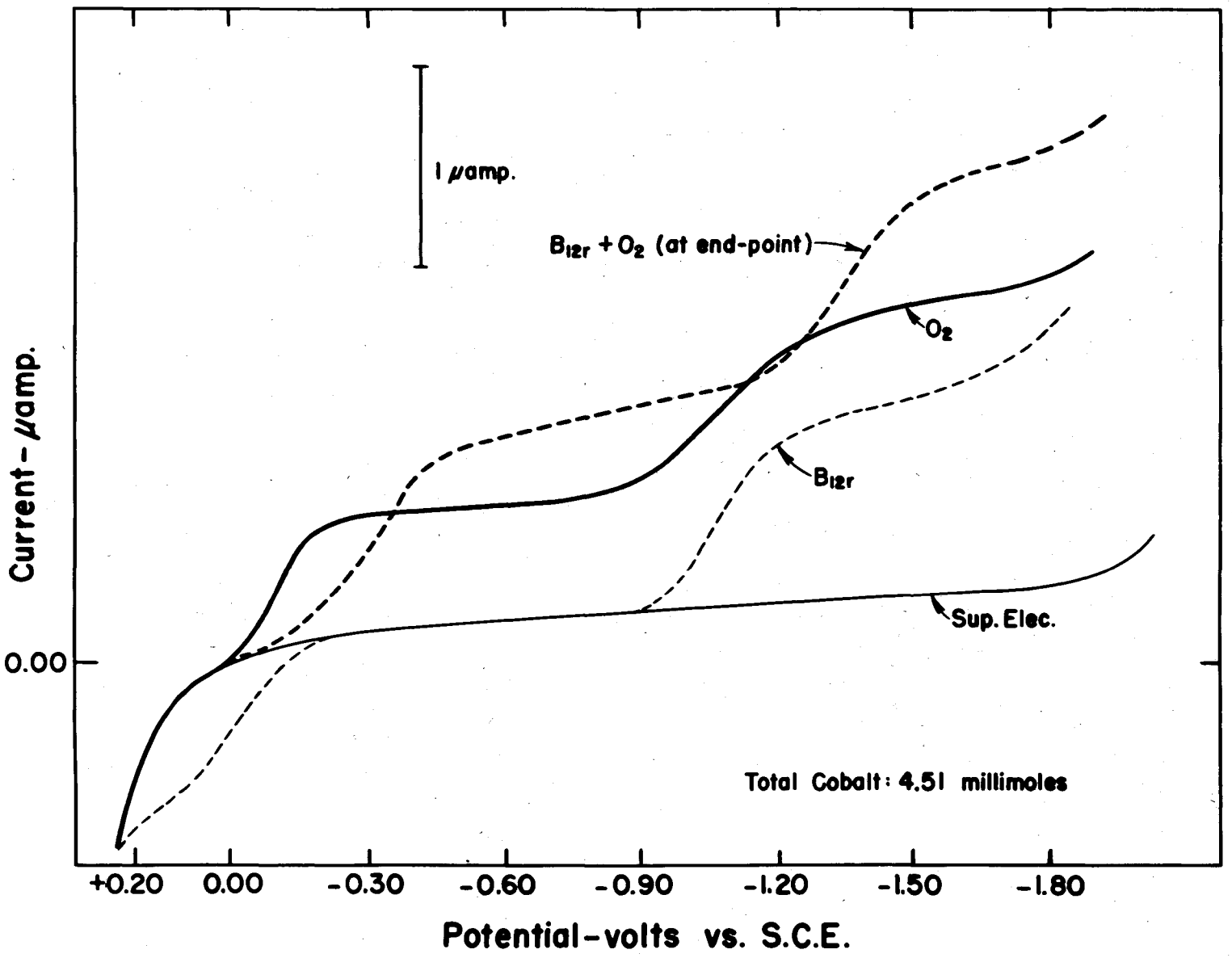

1 μamp.
B12r + O2 (at end-point)
O2
B12r
Sup. Elec.
Total Cobalt: 4.51 millimoles
Current - μamp.
0.00
+0.20
0.00
-0.30
-0.60
-0.90
-1.20
-1.50
-1.80
Potential - volts vs. S.C.E.

Table XII. Polarographic behavior of solutions of oxygen and B_{12r} plus oxygen. (Initial volume of the supporting electrolyte, 4.97 ml.; initial concentration of B_{12r}, 0.906 mmolar)

Oxygen Added		Diffusion Current Observed				
			B_{12r} Plus Oxygen			
Vol. of Stand. Sol.[a] ml.	Quant. mmoles	Oxygen Alone[b] μamp.	Anodic Wave μamp..	1st Cath. Wave μamp.	Impur. Wave μamp.	2nd Cath. Wave μamp.
0.00	0.000	0.00	0.95	0.00	0.00	1.00
0.25	0.230	0.49	0.53	0.02	0.04	0.99
0.50	0.465	0.94	0.32	0.07	0.10	0.96
0.75	0.700	1.34	0.23	0.14	0.16	0.93
1.00	0.934	1.74	0.07	0.22	0.22	0.90
1.25	1.170		0.00	0.32	0.32	0.91
1.50	1.400	2.38	0.00	0.43	0.26	1.02
2.00	1.870	2.94	0.00	0.63	0.10	1.26
2.50	2.340		0.00	0.97	0.00	1.44
3.00	2.820	3.94	0.00	1.14	0.00	1.59
4.00	3.740		0.00	1.92	0.00	2.11
4.50	4.220		0.00	2.40	0.00	2.56

[a]Concentration of the standard solution: 1) 30.2 p.p.m. (from the polarographic data and calculations using Ilkovic equation); 2) 29.8 p.p.m. (Winkler method).

[b]As determined by blank run.

Table XII. (Continued)

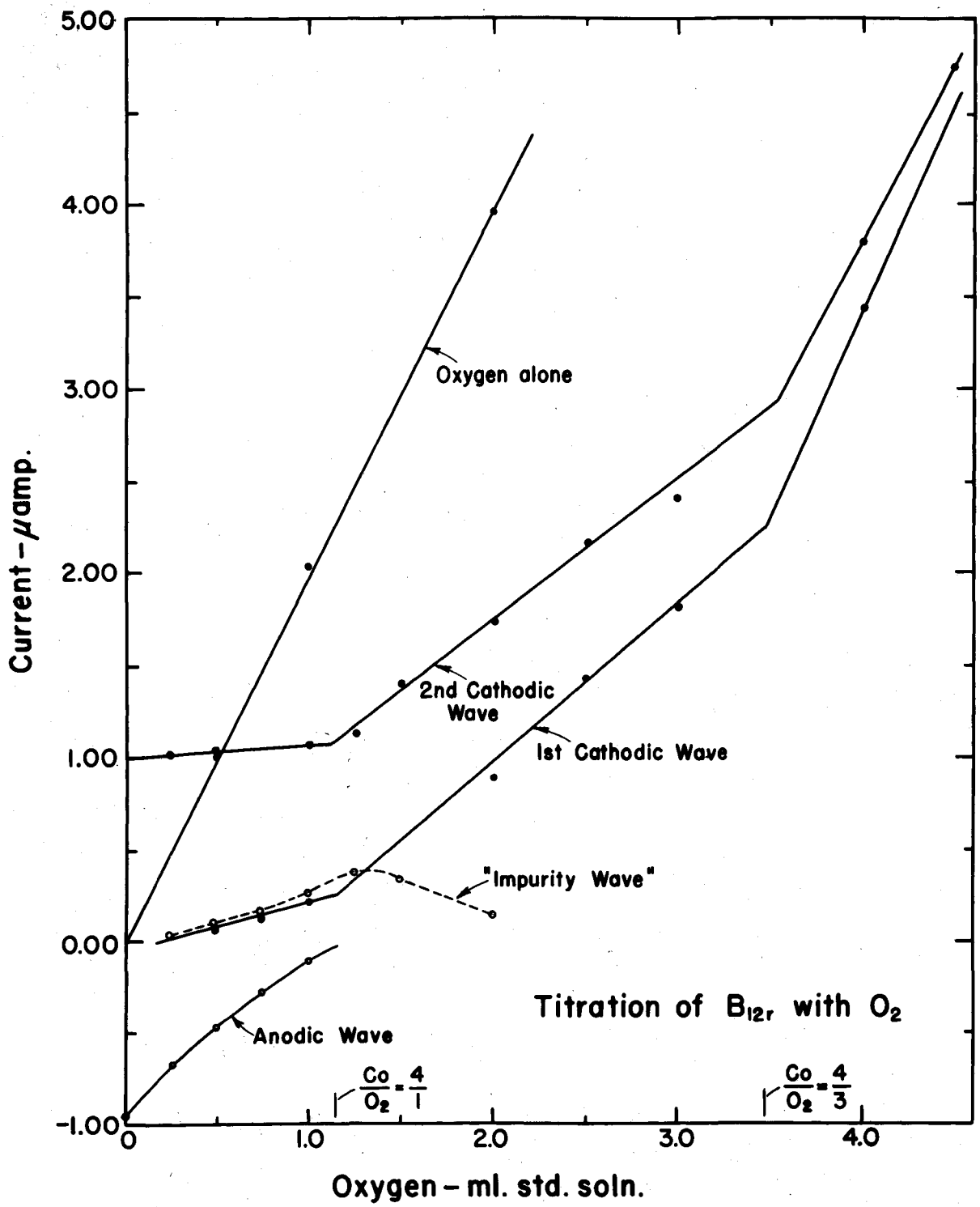
Oxygen Added		Diffusion Current Calculated[c]				
			B_{12r} Plus Oxygen			
Vol. of Stand. Sol.	Quant. mmoles	Oxygen Alone μamp.	Anodic Wave μamp.	1st Cath. Wave μamp.	Impur. Wave μamp.	2nd Cath. Wave μamp.
0.00	0.000	0.00	0.95	0.00	0.00	1.00
0.25	0.230	0.51	0.55	0.02	0.04	1.00
0.50	0.465	1.03	0.35	0.08	0.11	1.05
0.75	0.700	1.56	0.27	0.16	0.18	1.06
1.00	0.934	2.06	0.09	0.26	0.26	1.08
1.25	1.170		0.00	0.40	0.40	1.13
1.50	1.401	3.10	0.00	0.57	0.34	1.35
2.00	1.870	4.12	0.00	0.88	0.14	1.76
2.50	2.340		0.00	1.45	0.00	2.16
3.00	2.820	6.30	0.00	1.82	0.00	2.55
4.00	3.740		0.00	3.45	0.00	3.80
4.50	4.220		0.00	4.55	0.00	4.85

[c]Diffusion current calculated from the diffusion current observed to the diffusion current which would have been observed had no dilution occurred during the titration

$$i_{d_{corrected}} = i_{d_{measured}} \quad (V + v)/V$$

"V" - the initial volume (4.97 ml.) and "v" - the volume of the titrant added.

Fig. 8 Amperometric Titration of B_{12r} with Standard Oxygen Solution.


5.00
4.00
3.00
2.00
1.00
0.00
-1.00
Current – μamp.
0
1.0
2.0
3.0
4.0
Oxygen – ml. std. soln.
Oxygen alone
2nd Cathodic Wave
1st Cathodic Wave
"Impurity Wave"
Anodic Wave
Titration of B_{12r} with O_2
$\frac{Co}{O_2} = \frac{4}{1}$
$\frac{Co}{O_2} = \frac{4}{3}$

7. Amperometric titration of B_{12}

Crystalline B_{12} was dissolved in 5.00 ml. of 0.1 N potassium sulfate which was placed in the polarograph cell. The first polarogram was obtained after the deaeration and mixing of the solution. The titration was carried out in the same manner as that used for the titration of B_{12a} as described earlier.

Blank polarograms were obtained on the supporting electrolyte treated with the successive portions of the standard oxygen solution. The results of a representative titration are given in Table XIII.

C. Results and Discussion

The treatment of B_{12a} with oxygen shifted the half-wave potentials of the first and second waves in the negative direction. In addition, the diffusion currents observed were less than the sum of B_{12a} and oxygen diffusion currents. The diffusion currents continued to be smaller to the point where one molecule of oxygen had been added for every two molecules of B_{12a}. Beyond this point, the diffusion current increased at the rate found for the addition of oxygen to the supporting electrolyte; see the titration curve of Fig. 6. The results of four titrations are shown in Table XIV. The polarogram obtained at the end-point is shown in Fig. 5. As will be seen, the first wave of the oxygen-bearing dimer is well defined with a half-wave potential of -0.22 v.

Table XIII. Polarographic behavior of oxygen and of B_{12} plus oxygen. (Initial volume of the supporting electrolyte, 5.00 ml.; initial concentration of B_{12}, 0.750 mmolar)

Oxygen Added		Dif. Current Observed		Dif. Current Calc.[c]	
Vol. of Standard Solution[a] ml.	Quantity mmoles	Oxygen Alone[b] μamp.	B_{12} Plus Oxygen μamp.	Oxygen Alone μamp.	B_{12} Plus Oxygen μamp.
0.00	0.00	0.00	0.00	0.00	0.00
0.50	0.436	0.88	0.88	0.97	0.96
1.00	0.872	1.74	1.71	2.09	2.05
1.50	1.308	2.42	2.42	3.15	3.14
2.00	1.744	2.88	2.87	4.04	4.01
2.50	2.180	3.34	3.32	5.03	4.98
3.00	2.620	3.77	3.74	6.04	5.97
3.50	3.050	4.12	4.05	7.00	6.89
4.00	3.490	4.51	4.45	8.12	8.00

[a]Concentration of the standard solution 27.9 p.p.m. (Winkler method).

[b]As determined by blank run.

[c]Diffusion current calculated from the diffusion current observed to the diffusion current which would have been observed had no dilution occurred during the titration

$$i_{d_{corrected}} = i_{d_{measured}} \quad (V + v)/V$$

"V" - the initial volume (5.00 ml.) and "v" - the volume of the titrant added.

Table XIV. Titration results of B_{12a} with oxygen

Titration No.	Cobalt Taken mmoles	Oxygen Required to Reach End-point mmoles	Ratio Co:O_2	Diffusion Current[a] μamps	"n" Electrons[b]
1	3.73	1.93	1.95	2.17	3.38
2	2.85	1.44	1.98	1.91	3.84
3	4.67	2.23	2.09	3.05	3.78
4	4.39	2.38	1.85	2.52	3.32

[a]Diffusion current at the end-point; corrected for dilution during titration.

[b]Calculated by the Ilkovic equation, $n = i_d/(605\ C\ D^{1/2}\ m^{2/3}\ t^{1/6})$, in which "$i_d$" - is the diffusion current at the end-point, "C" - the milli molar concentration of B_{12a} dimer, "D" - the diffusion coefficient of B_{12a} dimer (2.33×10^{-6} $cm.^2/sec.$), and "$m^{2/3}\ t^{1/6}$" - the capillary constant at -0.1 v. toward S.C.E. ($1.875\ mg.^{2/3}\ sec.^{1/2}$).

(contrasted with -0.04 v. for the B_{12a} monomer), and the second wave is spread out with a half-wave potential of -1.24 v. (as contrasted to -1.02 v. for the monomer).

The reaction between B_{12r} and oxygen apparently took place stepwise, a first step in which the cobalt is oxidized to the trivalent state (conversion of B_{12r} to B_{12a}), and a second step in which the dimerization is effected. B_{12r} itself is characterized by an anodic wave of half-wave potential -0.94 v. toward the S.C.E. In the early part of the titration with oxygen the anodic wave disappeared and was

replaced by a cathodic wave characteristic of B_{12a}; the second cathodic wave of B_{12r} did not change in height or position (this wave is essentially in the same position as the second cathodic wave of B_{12a} and presumably represents the same reaction, that is, the reduction of bivalent cobalt to univalent cobalt). This first reaction ended abruptly at the ratio $B_{12r}:O_2 = 4:1$. The color of the solution at this point had turned from brown to red-orange. Up to this point, the reaction consisted simply in the oxidation of the cobalt from the bivalent to the trivalent state. Beyond the first end-point the two cathodic waves were shifted to more negative potentials and the behavior was identical with that observed in the titration of B_{12a} with oxygen. The second end-point occurred at the ratio $B_{12a}:O_2 = 4:3$, and thus the second part of the titration consisted in the dimerization produced by the union of one molecule of oxygen to two molecules of B_{12a}. The results of three titrations of B_{12r} with oxygen solution are shown in Table XV.

There is present in the polarogram of B_{12a} a small, unexplained wave of half-wave potential -0.55 v., the diffusion current for which represents about 0.25 electrons per molecule of B_{12a}. This wave is designated here for convenience as the impurity wavelet. On treatment of B_{12a} with oxygen, the impurity wavelet is shifted progressively to more positive potentials and decreased in height, finally, at the end-point of the titration, it disappeared at the potential at which

Table XV. Titration results of B_{12r} with oxygen

Titration No.	Cobalt Taken mmoles	Oxygen Required to Reach End-point: 1st mmoles	2nd mmoles	Ratio Co:O_2 at End-points: 1st	2nd	Diffusion Current[a] at End-points: 1st γamp.	2nd γamp.	"n" Electrons[b] at End-point 1st	2nd
1	4.51	1.11	3.26	4.08	1.38	1.10	2.92	0.63	3.74
2	5.25	1.32		4.03		1.31		0.64	
3	4.23	1.07	3.25	3.95	1.30	1.11	2.80	0.67	3.82

[a]Diffusion current at the end-point; corrected for dilution. Data for run 2 was taken beyond the end-point.

[b]Calculated by the Ilkovic equation; $n = i_d/(605\ C\ D^{\frac{1}{2}}\ m^{2/3}\ t^{1/6})$ in which "i_d" is the diffusion current at the end-point; "C" - is the millimolar concentration of B_{12r}:1) in calculations at the first end-point, concentration of B_{12r} was used, 2) in calculations at the second end-point concentration, that equivalent to the B_{12a} dimer was used; "D" - is the diffusion coefficient:1) in the calculations at the first end-point, the diffusion coefficient that of B_{12} (2.95×10^{-6} cm.^{2}/sec.) was used, in the calculations at the second end-point, the diffusion coefficient that of B_{12a} dimer (2.33×10^{-6} cm.^{2}/sec.) was used; "$m^{2/3}t^{1/6}$" - is the capillary constant at -0.1 v. toward S.C.E. (1.875 mg$^{2/3}$ sec.$^{1/6}$).

it would merge with the wave of the oxygen-bearing dimer ($Co:O_2 = 2:1$). The impurity wavelet is not present in the polarogram of B_{12r}. It appeared on the first addition of oxygen to B_{12r} and increased in height with each successive addition of oxygen, reaching a maximum at the first end point ($Co:O_2 = 4:1$). Beyond the first end-point the impurity wavelet decreased in height, shifted toward the left, and merged with the wave of the dimer at the second end-point. That is, the behavior is identical to that of B_{12a} during this part of the titration.

The titration of B_{12} with standard oxygen solution showed no interaction between the two substances. The diffusion currents throughout the titration were simply those indicated by the addition of the diffusion currents of the two materials.

D. Conclusions

The amperometric titration confirmed the earlier findings that vitamin B_{12a} dimerized through the agency of oxygen. It was further established that the combining ratio was two molecules of B_{12a} with one molecule of oxygen.

Vitamin B_{12} failed to combine with oxygen which is also in agreement with the earlier measurements of diffusion coefficients and apparent specific volumes.

The results obtained from the titration of B_{12r} with oxygen are in accord with the concept of B_{12r}. That is, B_{12r}

is the bivalent cobalt compound which is easily oxidized to give B_{12a}. The amperometric titration showed two end points corresponding first to the oxidation of the cobalt and second to the dimerization of B_{12a}.

V. CONDUCTIVITY OF VITAMIN B_{12a}

A. Introduction

In the early studies on vitamin B_{12} at Iowa State College by Dr. Diehl and co-workers, measurements were made of the electrical conductances of solutions of vitamins B_{12} and B_{12a}. Erratic and unexplainable results were obtained, the conductivities changing with time. The present studies on the dimerization of B_{12a} in presence of oxygen indicate that oxygen perhaps was responsible for the earlier difficulties. For this reason the electrical conductance measurements were repeated with attention to the exposure of the solutions to oxygen.

B. Experimental Work

1. Materials

Vitamin B_{12a} was prepared from crystalline B_{12} by the hydrogenation procedure (previously described).

Reagent grade potassium chloride was recrystallized from deionized water.

Oxygen- and hydrogen-free nitrogen was prepared by passing tank nitrogen through two scrubbers of vanadous sulfate, one scrubber of sodium hydroxide, and one of water. This purification train was followed by a hot tube of cuprous oxide and metallic copper. The hot stream of nitrogen was cooled and finally passed through a water scrubber.

2. Apparatus and procedure

Conductimetric measurements were carried out using a 30 ml. Leeds and Northrup Type LC conductivity cell and Jones bridge equipped with a signal generator, amplifier, oscilliscope as a detector, and oil bath controlled within 0.01.

The conductivity cell constants were determined at various concentrations of potassium chloride in the range of 0.02 M to 0.0001 M, using specific conductance values cited in literature (65).

Exactly 25.0 ml. of conductivity water were placed into the hydrogenation vessel containing sealed in glass and saturated calomel electrodes for the purpose of measuring pH of B_{12a} solution in presence and in absence of oxygen. A platinum boat with the crystalline B_{12a} was hung above the surface of water. The water was deaerated by the passage of nitrogen for 1.5 hours. The boat was dropped into the deaerated water, and the solution was mixed well. The pH was measured at intervals for six hours. Meanwhile, the conductivity cell was well flushed with pure nitrogen. The cell was kept in a large beaker filled with continuously flowing nitrogen. The solution of B_{12a} was transferred from the hydrogenation vessel to the conductivity cell through glass tubing with ground glass connections. After the cell was filled, it was closed with a glass stopper and sealed with paraffin wax. The cell was placed in the oil bath, and

the first readings were obtained after 45 minutes using 2000 cycles per second signal. The resistance of the solution was continuously measured for 18 hours. The contents of the cell were then transferred to the hydrogenation vessel and oxygenated by passing a pure oxygen stream through the solution for 15 minutes. Again, the same solution was placed into the conductivity cell and the measurements were repeated following the resistance values for 18 hours. An aliquot of 1.00 ml. was then taken for cobalt analysis. The contents of the cell were then transferred into the 50.0 ml. volumetric flask and diluted to the mark. An aliquot of 30.0 ml. was taken for titration using the standard hydrochloric acid.

C. Results and Discussion

Conductance measurements of oxygen free B_{12a} and oxygenated B_{12a} solutions revealed spectacular differences. The results are shown in Table XVI.

Table XVI. Conductance measurements of B_{12a} solutions

Material	pH	Resistance ohms	Specific Conductance $cm.^{-1}$ ohm^{-1}	Molar Equivalent Conductance $cm.^2$ ohm^{-1} $eq.^{-1}$
B_{12a} oxygen free	7.98	3335.68	6.38×10^{-5}	75.1
B_{12a} oxygen free		3532.50[a]	6.42×10^{-5}	75.5
B_{12a} oxygenated	8.40	1669.84	12.87×10^{-5}	151.2
B_{12a} oxygenated		1635.27[a]	13.02×10^{-5}	153.5
		1635.27[a]	13.02×10^{-5}	307.0[b]

[a]Resistance measurements were made 18 hours after the run was started.

[b]Molar equivalent conductance was computed using the concentration, that of B_{12a} dimer (4.25×10^{-4} M.).
Concentration of B_{12a}: 8.50×10^{-4} M.
Bath temperature: $25^{\circ} \pm 0.01^{\circ}$.
Cell constant: 0.213.

The actual knowledge of the effective diffusion coefficients enables calculation of the equivalent conductances of the B_{12a} monomer and B_{12a} dimer by the equation (66):

$$D = \frac{RT}{z F^2} \lambda = 2.67 \times 10^{-7} \frac{\lambda}{z_i}$$

The calculated values for the equivalent conductances of B_{12a} monomer $(RCo(III)H_2O)^+$ and B_{12a} dimer $(RCo(III)\text{-}O_2\text{-}Co(III)R)^{++}$ ions are respectively, 11.0 and 17.5 $cm.^2$ ohm^{-1} $equiv.^{-1}$. These calculations are based on the values of diffusion coefficients determined by the free diffusion method: 2.95×10^{-6} $cm.^2/sec.$ for B_{12a} monomer (the same as for B_{12}), and 2.33×10^{-6} $cm.^2/sec.$ for B_{12a} dimer (as obtained experimentally).

Furthermore, the equivalent conductances at infinite dilution are equal to the sum of the ionic conductances giving the values: 209 for B_{12a} monomer and 413.5 for B_{12a} dimer. These values were obtained assuming following equations:

$$RCo(III)OH + H_2O \rightleftharpoons (RCo(III)H_2O)^+ + OH^-$$

and $$2\ RCo(III)OH + O_2 \rightleftharpoons (RCo(III)\text{-}O_2\text{-}Co(III)R)^{++} + 2OH^-$$

In a qualitative way, it is evident that in absence of oxygen, vitamin B_{12a} stays as a monomer in solution with a considerable part of the hydroxyl ions directly bound to the cobalt atom. In case of the dimer the equilibrium is shifted more to the right, which is evident from the higher values of the equivalent conductances.

D. Conclusions

The specific conductance of B_{12a} solutions changes drastically in presence of oxygen. The change in specific conductance is attributed to the formation of B_{12a} dimer. The equivalent conductance values for B_{12a} monomer and B_{12a} dimer at infinite dilution were calculated from the diffusion coefficient constants being 209 and 413.5, respectively. It is apparent that an appreciable amount of the hydroxyl ions are bound directly to the cobalt atom. During the formation of B_{12a} dimer in presence of oxygen, two hydroxyl ions are liberated by one molecule of B_{12a} dimer which causes the increase in pH and in specific conductance.

VI. SPECTROPHOTOMETRIC MEASUREMENTS

A. Introduction

Certain unexplained variations in the absorption spectrum of vitamin B_{12a} solutions have been observed earlier (67). The spectrum of B_{12a} appeared to be pH dependent, and also some changes were observed in the absorption peaks with time. Dr. E. A. Kaczka and co-workers observed leveling off the peak at 280 mμ and some changes at 355 mμ and 320 mμ. It appeared possible that the basis of these changes might be the dimerization of B_{12a} in presence of oxygen found in the foregoing work.

B. Experimental Work

1. Materials

Vitamin B_{12a} was prepared from the crystalline B_{12} by the hydrogenation procedure.

2. Apparatus and procedure

Spectra of B_{12a} solutions were measured by the Beckman DU spectrophotometer.

Crystalline B_{12a} was suspended in a platinum boat over deionized water in a closed vessel. Oxygen was removed from the liquid and the gas space by passage of a stream of oxygen-free nitrogen. The B_{12a} was then dissolved in the water, and a portion of the solution transferred to a 1.0 cm. silica cell previously flushed with nitrogen. The transfer was accom-

plished without contact with air. The cell was then closed with a glass stopper and the joint sealed with paraffin wax. A stream of oxygen was passed through a second portion of the B_{12a} solution. The solution was then placed in an identical silica cell.

The absorption spectra of these solutions were obtained using water as a reference.

C. Results and Discussion

The absorption spectra of the oxygenated and oxygen-free solutions of B_{12a} were practically identical over the visible and the ultraviolet range. The oxygenated solution, however, showed a definite shoulder at 320 mμ, a small shoulder at 280 mμ, and a large peak at 354 mμ. The changes of the peak intensity and its position at 280 mμ have been attributed to the changes of bond strength between N^3 benzimidazole and the cobalt atom (68). The changes in spectra of B_{12a} solutions are likely to be dependent upon the formation of B_{12a} dimer in presence of oxygen.

VII. CATALYTIC EFFECTS OF B_{12a}

A. Introduction

The air oxidation of iodide to iodine has been reported (46), however, the nature of this phenomenon was not understood. Inasmuch as, the B_{12a} solution did not oxidize iodide to iodine in absence of oxygen, it appeared likely that this oxidation was caused by B_{12a} dimer. In addition, it seemed reasonable that the catalytic effect of B_{12a} could be extended to a number of inorganic reactions having reduction potentials close to that of iodine. The presence of free hydrogen peroxide was suspected, and a series of spot tests were made.

B. Experimental Work

1. Materials

Vitamin B_{12}, obtained from the Squibb Institute for Medical Research, New Brunswick, N. J., was recrystallized from deionized water.

Vitamin B_{12a} was prepared from the crystalline B_{12} by the hydrogenation procedure.

2. Ferrous sulfate and oxygen

Two identical aliquots of a standard ferrous sulfate solution, 0.1 N in hydrochloric acid, were placed in conical flasks. To one of the flasks was added 0.8 mg. of B_{12a}. Both solutions were agitated with air for 14 hours. The

solutions were then titrated with standard ceric sulfate using ferrous o-phenanthroline sulfate as indicator. No difference was observed in the amount of oxidizing agent consumed.

3. Sodium arsenite and oxygen

A solution of sodium arsenite was divided into two identical parts. To one portion was added 0.7 mg. of B_{12a}. Both solutions were agitated with air for 14 hours. The arsenite was then titrated with standard ceric sulfate using osmic acid as catalyst and ferrous o-phenanthroline sulfate as indicator.

The experiment was carried out at pH values 5.1, 7.2, and 8.5. There were no differences observed in the volumes of ceric sulfate consumed.

4. Methylene blue and oxygen

The oxidation of methylene blue (reduced form) was checked in a qualitative manner. A neutral solution of methylene blue was reduced with hydrogen sulfide. The excess of hydrogen sulfide was eliminated by boiling the solution for about 20 minutes and bubbling nitrogen through the solution. Two identical aliquots of the colorless methylene blue solutions were placed in conical flasks. To one of the flasks was added 0.6 mg of B_{12a}. Both solutions were agitated with air. The solution containing B_{12a} changed color in a few minutes, while that without B_{12a} changed color gradually.

5. Potassium ferrocyanide and oxygen

Two identical aliquots of a standard potassium ferrocyanide solution were placed in conical flasks. To one of the flasks was added 0.5 mg. of B_{12a}. Both solutions were agitated for 28 hours and were kept in the dark. The solutions were then acidified with 1.0 ml. of concentrated perchloric acid and were titrated with standard ceric sulfate using diphenylaminesulfonic acid as indicator. No difference was observed in the amount of oxidizing agent consumed.

6. Spot tests for free hydrogen peroxide

The presence of free hydrogen peroxide was checked by a number of sensitive spot tests: 1. reduction of ferric-ferricyanide to Turnbull's blue or Prussian blue (69); 2. reduction of higher nickel oxides to nickelous oxide (70); 3. oxidation of cerous carbonate to perceric carbonate (71). These tests were compared to a blank, a solution containing traces of hydrogen peroxide, and an aqueous solution of B_{12a}. A drop of the reagent solution was placed on a spot plate. In an adjacent depression a drop of the reagent and a drop of the solution tested were used for comparison. The solutions of B_{12a} failed to reduce higher nickel oxide, and it failed to oxidize cerous carbonate. However, the ferric-ferricyanide solution in presence of B_{12a} produced some bluish-green precipitate. In absence of B_{12a} the precipitate was pronounced somewhat green.

C. Results and Discussion

The catalytic effect of B_{12a} was checked for the reactions having the reduction potentials close to iodide-iodine (0.53 v.): potassium ferricyanide (0.36 v.), sodium arsenate (0.56 v.), ferric sulfate (0.77 v.), and methylene blue (0.01 v.). The results are shown in Table XVII.

Table XVII. Catalytic effect of B_{12a} solutions

Material Used	Time of Agitation with Oxygen hours	Amount of B_{12a} Used mg.	pH of solution	Results
Ferrous-sulfate	14	0.8	1.0	No effect
Potassium ferricyanide	28	0.5	7.1	No effect
Sodium arsenite	14	0.7	5.1	No effect
Sodium arsenite	14	0.7	7.2	No effect
Sodium arsenite	14	0.7	8.5	No effect
Methylene blue	1/30	0.6	7.1	Intense blue color appeared

There was no effect on oxidation of ferrous sulfate, sodium arsenite, and potassium ferrocyanide. Methylene blue (reduced form) was oxidized almost instantaneously. This rapid change could be due to the reduction of B_{12a} in preference of oxygen.

The presence of free hydrogen peroxide in B_{12a} solutions was not substantiated as shown in Table XVIII.

Table XVIII. Tests for hydrogen peroxide

Reagent Solution	Observations in Presence of		
	B_{12a}	H_2O_2	H_2O
Ferric-ferricyanide	Bluish-green precipitate	Intense blue precipitate	Greenish-blue precipitate
Nickel higher oxide paste	Black no color change	Pale green	Black no color change
Cerous carbonate	Pale white	Brown	Pale white

Tests with ferric ferricyanide gave inconclusive results since the difference in blues consisted only in a shade difference.

D. Conclusions

The catalytic effect of B_{12a} on air oxidation of ferrous sulfate, sodium arsenite, and potassium ferrocyanide is negligible. The reduced methylene blue was oxidized in presence of B_{12a} many times faster than by air alone.

The presence of free hydrogen peroxide was not proven.

VIII. GENERAL DISCUSSION

Measurements of the diffusion coefficients of vitamins B_{12} and B_{12a} by the free diffusion method gave the values 2.9 (1) x 10^{-6} and 2.3 (3) x 10^{-6} $cm.^2/sec.$, respectively. Values for the molecular weights of two vitamins calculated from the values of the diffusion coefficients indicated that B_{12a} was a dimer in solution. Furthermore, it was observed that vitamin B_{12a} decomposed somewhat and formed the dimer at 25°. This decomposition could be repressed by the addition of cyanide or by lowering the temperature.

These observations indicated that B_{12a} dimerized in aqueous solution but did not offer a mechanism by which the dimerization occurred. A clue to this was obtained during the course of the density measurements. It was observed that the apparent specific volumes of B_{12a} in presence of oxygen were consistently larger than that of B_{12}, the values being 0.713 and 0.665, respectively. Consequently, the change of the apparent specific volume of B_{12a} in the presence of oxygen could be accounted by the interaction of the molecular oxygen with B_{12a}.

Knowing that oxygen was involved in the formation of the B_{12a} dimer, it was necessary to develop a quantitative method for the determination of the stoichiometry of this reaction. This problem was solved by the amperometric titration method. Solutions of B_{12}, B_{12a}, and B_{12r} were titrated

with the standard oxygen solution. Throughout the titration entire polarograms were recorded, and the diffusion currents were evaluated by the conventional methods. The titration results clearly indicated that oxygen had interacted with B_{12a} in the molecular ratio of $2B_{12a}$: O_2. Similar titrations of B_{12} with oxygen indicated no apparent interaction. The solution of B_{12r} reacted with oxygen in two steps. The first step was attributed to the oxidation of bivalent to trivalent cobalt; the second step was equivalent to the formation of the B_{12a} dimer.

Number of electrons for the B_{12a} dimer at the end-point were calculated by the Ilkovic equation. The calculated value of electrons involved was somewhat lower than four: giving two electrons for oxygen reduction to hydrogen peroxide and two electrons for the reduction of two atoms of cobalt (III) to cobalt (II). The deviation from four electrons probably could be attributed to the incompleteness of the interaction of oxygen with B_{12a}.

The remarkable change in conductivity and an increase in pH on the addition of oxygen to B_{12a} solutions indicated that in the process of dimerization the hydroxyl ions were liberated. The equivalent conductance of oxygen free B_{12a} solution and the same oxygenated solution (8.5×10^{-4} M) were found to be 75.5 and 307.0, respectively.

The values of the equivalent conductance at infinite dilution were calculated to be 209 for B_{12a} and 413 for B_{12a}

dimer. These calculations were based on the following reactions:

$$R\ Co(III)OH + H_2O \rightleftharpoons (RCo(III)H_2O)^{+} + OH^{-}$$

$$2R\ Co(III)OH + O_2 \rightleftharpoons (RCo(III)\text{-}O_2\text{-}Co(III)R)^{++} + 2OH^{-}$$

The spectra of B_{12a} and B_{12a} dimer were almost identical; B_{12a} dimer had a shoulder at 320 mμ. The likeness of spectra excluded a possible existance of a cobalt (IV) oxygen dimer.

The catalytic effect of B_{12a} was not observed on air oxidation of ferrous sulfate, sodium arsenite, and potassium ferrocyanide solutions. The reduced form of methylene blue was oxidized by B_{12a} solution instantaneously.

The presence of the hydrogen peroxide was not observed as indicated by the spot tests used.

All above observations led to the proof that the dimerization of B_{12a} in solution was caused by oxygen and that it interacted in the ratio of $2B_{12a}:O_2$.

IX. SUMMARY

1. The diffusion coefficients of vitamin B_{12} and of vitamin B_{12a} have been determined using the free diffusion method. The new values are:

   $2.9(1) \times 10^{-6}$ cm.^{2}/sec. for B_{12} at 25°,

   $2.3(3) \times 10^{-6}$ cm.^{2}/sec. for B_{12a} at 25°.

2. A slight decomposition of vitamin B_{12} in solution has been observed. It was found that it could be repressed by the addition of cyanide or by lowering the temperature.

3. The molecular weights of vitamin B_{12} and of vitamin B_{12a} have been calculated by the Stokes-Einstein-Longsworth equation using the new values of the diffusion coefficients. The results obtained are:

   1380 for B_{12} and 2225 for B_{12a}.

4. The molecular weights of vitamin B_{12} and of vitamin B_{12a} have been calculated by the Svedberg equation using the values of the sedimentation coefficients determined by Dr. Schachman, the new values of the diffusion coefficients and newly measured values of the apparent specific volumes. The values are:

   1420 for B_{12} and 2330 for B_{12a}.

5. That the dimerization of B_{12a} occurs after dissolution in water has been shown.

6. The apparent specific volumes of vitamin B_{12} and vitamin B_{12a} have been determined by the pycnometric method. The

values obtained are:

0.665 for B_{12},

0.713 for B_{12a} in presence of oxygen,

0.650 for B_{12a} in absence of oxygen.

7. The interaction of oxygen with the aqueous solutions of vitamins B_{12}, B_{12a}, and B_{12r} has been investigated by an amperometric titration of these materials with standard solution of oxygen.

8. It has been found that B_{12} and oxygen do not interact.

9. Vitamin B_{12a} has been found to react with the molecular oxygen in the ratio $2B_{12a}:O_2$.

10. Vitamin B_{12r} has been found to react with oxygen in two steps. B_{12r} is first oxidized to B_{12a} and then dimerized by union with oxygen.

11. The equivalent conductance of oxygen free and oxygenated B_{12a} solutions have been determined. The values obtained are:

75.5 cm^2/ohm eq. for oxygen free B_{12a} (8.5×10^{-4} M.),

307.0 cm^2/ohm eq. for oxygenated B_{12a} (4.25×10^{-4} M.).

12. The equivalent conductances of oxygen free and oxygenated B_{12a} solutions at infinite dilution have been calculated using the values of the diffusion coefficients. The values obtained are:

209 $cm.^2$/ohm. eq. for oxygen free B_{12a}, and

413.5 $cm.^2$/ohm. eq. for oxygenated B_{12a}.(B_{12a} dimer).

13. It has been found that on addition of oxygen the pH of

B_{12a} solutions increases.

14. The dimerization of vitamin B_{12a} in solution has been shown to follow the reaction

$$2RCo(III)OH + O_2 \rightleftharpoons (RCo(III)\text{-}O_2\text{-}Co(III)R)^{++} + 2OH^-.$$

15. The spectra of oxygen free and oxygenated B_{12a} solutions have been obtained. The oxygenated solution of B_{12a} shows a definite shoulder at 320 mμ.

16. The catalytic effect of B_{12a} on certain air oxidations has been studied. There is no catalytic effect on air oxidation of ferrous sulfate, sodium arsenite, and potassium ferrouscyanide solutions.

17. It has been observed that the reduced form of methylene blue was oxidized instantaneously in presence of B_{12a} and oxygen.

18. The presence of free hydrogen peroxide in a B_{12a} solution has been disproven by sensitive spot tests.

X. LITERATURE CITED

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