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Studies on Vitamin B12a and related compounds

Byron George Kratochvil
Iowa State University

STUDIES ON VITAMIN B_{12a} AND RELATED COMPOUNDS

by

Byron George Kratochvil

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Signature was redacted for privacy.

Head of Major Department

Signature was redacted for privacy.

Dean of Graduate College

Iowa State University
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TABLE OF CONTENTS

INTRODUCTION

In 1926 it was discovered that people suffering from pernicious anemia improved in a remarkable manner when fed large quantities of liver. The active material in liver, referred to as anti-pernicious anemia factor, was not isolated in crystalline form until 1948 and then practically simultaneously by Rickes, Brink, Koniuszy, Wood and Folkers at Merck Laboratories in this country and by Smith and Parker at Glaxo Laboratories in England (1, 2). In the intervening twenty-two years a prodigious amount of time and effort was expended on this problem, the work being especially hampered by the necessity of using human anemia patients for assay of purification schemes. The deep scarlet crystals finally isolated of pure factor were called vitamin B_{12}.

Since 1948 an equal or even greater expenditure of energy has been made in attempts to unravel the structure and physiological function of the vitamin. The presence in it of a metal, cobalt, has intrigued, in addition to biochemists, a number of investigators whose interests in coordination chemistry led them to wonder about the manner in which the metal was held in the molecule. It was early shown that the cobalt atom was tied up with exceeding firmness, so tightly in fact that attempts to remove it without disintegration of the molecule have even to the present time been fruitless.

In the structure as finally determined by X-ray crystallography the cobalt is linked to four nitrogen atoms in a planar arrangement, the nitrogen atoms constituting a ring identical in structure to porphyrin except that fewer double bonds are present and one of the four bridging carbons between the pairs of pyrrole rings is absent (3). The similarity between this ring and the one to which iron is coordinated in hemoglobin is so great that it has been suggested that the pathways of synthesis for hemin and for B_{12} may be quite closely related (4). The fifth coordination position of the cobalt is occupied by a nitrogen atom in a dimethylbenzimidazole structure, while the sixth is occupied by a cyanide group. In addition to the ring surrounding the cobalt, the molecule contains a phosphorylated ribose group linked to the second benzimidazole nitrogen, and a molecule of 1-amino-2-propanol esterified through the phosphate. The amino group of the alcohol is in turn joined to a propionic acid side chain on one of the pyrroles through an amide linkage. The resulting molecule, a complex structure indeed, has a molecular weight of 1357.

This work is concerned with that portion of the molecule in the immediate vicinity of the cobalt, with special emphasis on the compound vitamin B_{12a}, in which the cyanide in B_{12} is replaced by a hydroxyl group. Vitamin B_{12} is sometimes called cyanocobalamin, the term cobalamin referring to all of the

molecule except for the cyanide group. Then B_{12a} can be conveniently named hydroxocobalamin, or sometimes aquocobalamin, as the hydroxyl group is replaced by water if the material is allowed to stand for a few hours in aqueous solution.

Hydrogenation of vitamin B_{12} with hydrogen gas in the presence of platinum dioxide catalyst results in removal of the cyanide group as methylamine, along with reduction of the cobalt from three to two. This hydrogenated material, called vitamin B_{12r}, has powerful reducing properties, and upon exposure to oxygen is rapidly oxidized to B_{12a}, in which form the cobalt is trivalent as in B_{12}.

Magnetic susceptibility measurements on solid B_{12} and B_{12a} indicate that both materials are diamagnetic, corresponding to trivalent cobalt (5, 6). Coordination compounds containing divalent cobalt are paramagnetic. The only exceptions are those compounds of divalent cobalt which have the property of combining reversibly with molecular oxygen. These are diamagnetic in the oxygenated form and paramagnetic in the unoxygenated form (7, 8). One of the simplest compounds of this type is that formed when a salt of divalent cobalt is dissolved in ammonia. The resulting solution will absorb or release oxygen, depending upon the temperature of the solution and the concentration of ammonia.

The polarographic behavior of B_{12}, B_{12a} and B_{12r} has been

carefully studied by Diehl, Morrison, Sealock and Jaselskis. B_{12} gives a two electron reduction wave at -1.12 v. vs. the saturated calomel electrode, corresponding to reduction of the cobalt from three to one (9). In cyanide supporting electrolyte this wave remains the same height but is shifted to -1.35 v. (10). B_{12} in cyanide-containing solutions is converted into a purple $B_{12}CN^-$ compound in which the second cyanide group is attached to the cobalt atom, having displaced the benzimidazole nitrogen (11). This material would be expected to be reduced with more difficulty; that such is the case is indicated by the more negative half-wave potential.

B_{12a} gives two one electron waves at half-wave potentials of -0.04 v. and -1.02 v., corresponding to the stepwise reduction of the cobalt from three to two and from two to one (12). In B_{12r}, on the other hand, the cobalt is already in the divalent state, and displays one anodic wave at -0.04 v. and one cathodic wave at -1.02 v. (12). Both involve one electron, the first corresponding to oxidation of divalent cobalt to the trivalent state and the second to reduction of the divalent state to the univalent state. It is interesting to notice that reduction of cobalt in all three cases involving the cobalamins proceeds only to the univalent state. This unusual situation gives again an indication of the remarkable tenacity with which the cobalt is held. The only other case known in which polarographic reduction of cobalt stops at the plus one

state is that of the very strong cyanide compounds (13).

Another application of polarography to the study of this series of compounds involves use of an amperometric titration in an ingenious way. A solution of B_{12a} was titrated with a standard solution of oxygen dissolved in water to show that an oxygenated compound was formed in the ratio of one oxygen to every two B_{12a} molecules (14).

The work described above has provided valuable information concerning the chemistry of the cobalamins. However, during the course of some other investigations of these compounds, a number of questions were raised which could not be satisfactorily explained. In particular, two observations were especially baffling. The first of these concerned an experiment employing the Warburg manometric method, which was designed to measure quantitatively the oxygen consumed in the oxidation of B_{12r} to B_{12a} (15). Instead of the expected decrease in volume of oxygen over the B_{12r} as it was oxidized to B_{12a}, a slight increase in volume was observed. That B_{12a} was indeed formed was shown by the change in color of the solution from the characteristic red-brown of B_{12r} to the orange-red of B_{12a}.

A second experiment involved the bubbling of a quantity of pure oxygen through a solution of B_{12r} and analysis of the

resulting gas in an Orsat apparatus (16). After absorption of carbon dioxide, carbon monoxide, oxygen and unsaturated hydrocarbons, a quantity of gas amounting to 0.6 mole per mole of B_{12} remained.

One of the possible explanations for the unexpected results of these experiments was the following: There is previous evidence that B_{12a} combines reversibly with molecular oxygen. Now if B_{12r} or B_{12a} combined in a similar fashion with nitrogen, one might visualize a reaction in which the combined nitrogen could in the presence of oxygen be converted into one of the oxides of nitrogen. The formation of nitrous oxide, N_2O, would be especially favorable because the volume of gas produced would be equivalent to the volume of oxygen consumed.

Accordingly, a series of experiments were designed to determine whether such fixation was taking place. Because of the cost of the pure crystalline vitamin and its high molecular weight, the amount of material which could be used per experiment was necessarily limited. For this reason specialized glassware was designed for the reactions, and a mass spectrometer was used for the analysis of the small volumes of gas involved. These experiments are described in Part I.

In later phases of the work some experiments were performed with the Warburg apparatus. These included repetition

of the previous experiments measuring the oxygen uptake of B_{12r}, using a somewhat more refined technique. Part II includes this work, along with some visible and ultraviolet absorption studies on B_{12a} and B_{12r} and magnetic susceptibility measurements on B_{12a}. All of these seemingly unrelated techniques were aimed at elucidation of the oxygenation of B_{12a}.

In Part III is described work done on the so-called 5,6-dimethylbenzimidazolylcobamide coenzyme, the physiologically active form of vitamin B_{12}. First isolated in 1959 by Barker, Weissbach and Smith, it had escaped notice previously because of its sensitivity to light (17). This material became available in research quantities only near the end of this work. Polarographic and magnetic susceptibility measurements were carried out on two samples of the compound. Further discussion of the coenzyme will be deferred to Part III.

PART I. NITROGEN FIXATION STUDIES

Gas Analysis Investigations

Method using reduced pressure

Introduction The approach employed in determining whether any of the B_{12} materials catalyzed the conversion of atmospheric nitrogen to some other nitrogen containing compound is outlined below.

Apparatus For the gas analyses a mass spectrometer, Consolidated-Nier Model CEC 21-201, was used. Voltages were recorded directly from the panel meter, using the number 2 collector. All samples were adjusted to an inlet pressure of 5 cm. of mercury.

To calibrate the machine with respect to nitrous oxide, the compound considered most likely to be formed if fixation was taking place, a sample of the pure gas was prepared. This was done by heating in a vacuum a mixture of ammonium sulfate and sodium nitrate along with a little sulfuric acid. The gas evolved was freed of water by slow passage through a cold trap, held at -50 degrees with an ethanol-water slush, then collected in a sample bulb and fed into the mass spectrometer. The gas was found to be essentially free of oxygen, nitrogen and argon. The ratios of the parent mass/charge peak, 44, to the daughter peaks for nitric oxide (NO, mass 30) and nitrogen (N_2, mass 28)

were 1.00:0.58:0.27 respectively; the mass/charge (M/e) peak 30 is the most indicative of the presence of nitrous oxide because of the interference of carbon dioxide at the 44 M/e peak and nitrogen at the 28 M/e peak. With a sensitivity of 2.76 volts for the pure gas at a M/e ratio of 30, the presence of 0.1 per cent nitrous oxide would give a peak of about 3 mv. above background, a detectable amount.

pH measurements were made with a Beckman Model G pH meter. The instrument was standardized against Beckman standard buffer of appropriate pH before each use.

The apparatus used for the combination of B_{12a} with various gas mixtures is shown in Figures 1 and 2, and is described later in conjunction with the procedures.

Chemicals The nitrogen used in all the B_{12} experiments was taken from a cylinder whose contents were analyzed mass spectroscopically and found to contain less than three parts in ten thousand of argon or carbon dioxide and no detectable oxygen. Pure oxygen was prepared as needed in one to two liter quantities by thermal decomposition of potassium chlorate in a vacuum on a vacuum rack. Traces of manganese dioxide were added in the earlier preparations as a catalyst, but was found to be of no significant effect, for even in its presence the mixture required heating above the melting point before significant gas evolution occurred. For later oxygen preparation,

therefore, potassium chlorate alone was used. Each batch of oxygen was checked mass spectroscopically for impurities before use. Argon was taken from a cylinder whose contents had been analyzed mass spectroscopically. The gas was found to be free from carbon dioxide and to contain less than two parts per ten thousand of oxygen and about two parts per thousand of nitrogen.

Procedure In Figure 1 is shown the apparatus used in the hydrogenation and subsequent filtration of vitamin B_{12}. The hydrogenation compartment was of the design described by Diehl and Murie (15). The B_{12}, dissolved in 0.1 N potassium sulfate, was placed into compartment A and platinum oxide catalyst equivalent in weight to about one half the weight of B_{12} added. The potassium sulfate is necessary to prevent the passage of colloidal catalyst through the filter. After four to six hours hydrogenation the B_{12} was completely converted to B_{12a}, and the solution had changed in color from a bright scarlet to a deep red-brown. The stopcocks were then turned in such a manner that the B_{12r} solution was pushed by hydrogen pressure through filter B into flask C. Stopcock D was then closed, the cell removed from the hydrogenation apparatus and attached to a vacuum rack at E. The vacuum rack assembly is shown in Figure 2. Here the B_{12r} solution was frozen by immersion of the cell into liquid nitrogen and the hydrogen gas over the solution pumped off. Pure nitrogen gas was introduced

Figure 1. Apparatus for reduction of B_{12} and filtration of resulting B_{12r} without exposure to air

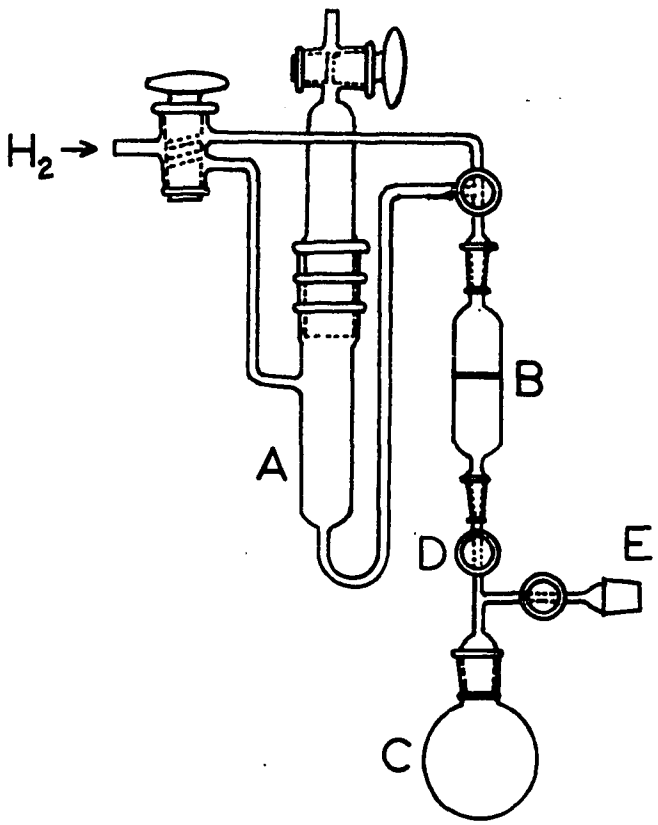
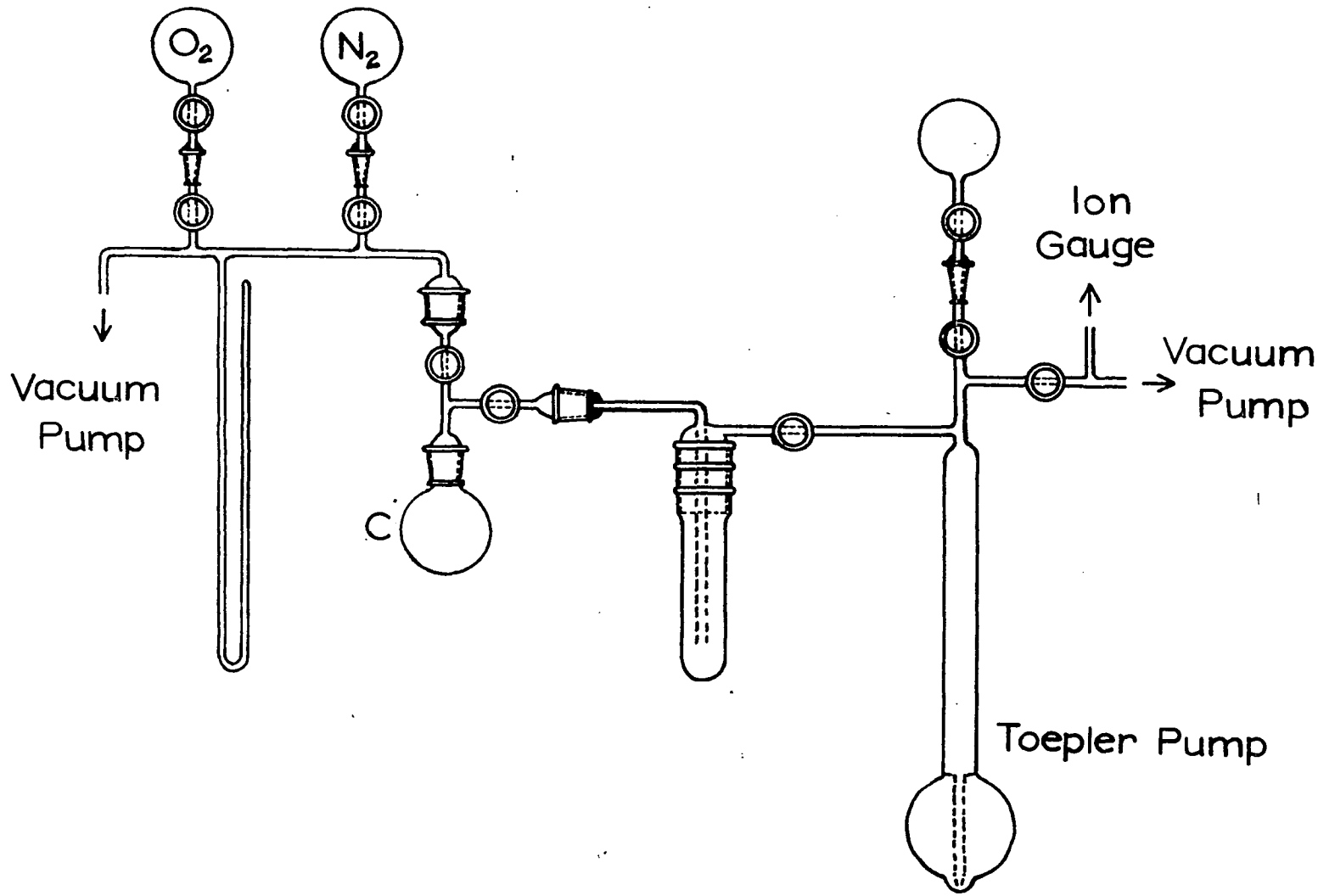

H_2
A
B
C
D
E

Figure 2. Vacuum rack assembly for introduction of nitrogen and oxygen into vitamin B_{12r} solutions and isolation of the resulting gases


O_2
N_2
Vacuum Pump
C
Ion Gauge
Vacuum Pump
Toepler Pump

corresponding to about 12 cm. of mercury on the manometer and the solution stirred in contact with the nitrogen gas for several minutes with a magnetic stirrer. Then pure oxygen, approximately equivalent in amount to the nitrogen, was added and the solution stirred vigorously another several minutes. At this point the red-brown B_{12r} color changed to the orange-red color of B_{12a}. The solution was again frozen with liquid nitrogen and the gases in the cell drawn by a Toepler pump into a sample bulb, which was taken to the mass spectrometer for analysis.

Results Results of the gas analysis for two runs are shown in Table 1. It can be seen that the only significant peaks are those corresponding to the gases originally introduced, nitrogen and oxygen, at M/e ratios of 28 and 32.

Method using atmospheric pressure

Introduction Experiments with the apparatus shown in Figure 1 employed nitrogen pressures over the B_{12} solutions on the order of a sixth of an atmosphere. It seemed possible that nitrogen complex formation could well be dependent upon the partial pressure of the gas over the solution and that higher pressures might encourage nitrogenation, so the equipment was redesigned to permit work at approximately atmospheric pressures.

Table 1. Results of mass spectrometric gas analysis over B_{12a} solutions for two runs at reduced pressures

Mass/charge ratio	Background, volts	Sample, volts	Probable ions
28[a]	0.065	3.0	N_2^+, CO^+(from CO or CO_2)
30	0.002	0.002	NO^+(from N_2O, NO or NO_2)
32	0.016	1.6	O_2^+
40	0.002	0.004	Ar^+
44	0.004	0.007	CO_2^+ , N_2O^+
12[b]	0.001	0.008	C^+
14	0.003	0.218	N^+, N_2^{++}
15	0.002	0.003	N^{15+}
16	0.003	0.116	O^+, O_2^{++}
17	0.010	0.002	OH^+
18	0.036	0.017	H_2O^+
28	0.060	1.88	N_2^+, CO^+
29	0.010	0.021	$N^{14}N^{15+}$
30	0.002	0.002	NO^+
32	0.016	0.660	O_2^+
40	0.002	0.003	Ar^+
44	0.003	0.007	CO_2^+, N_2O^+
45	0.005	0.003	$CH_3OCH_2^+$, $C_2H_5O^+$

[a]Run 1. 0.1231 g. B_{12} in 12 ml. 0.1 N potassium sulfate

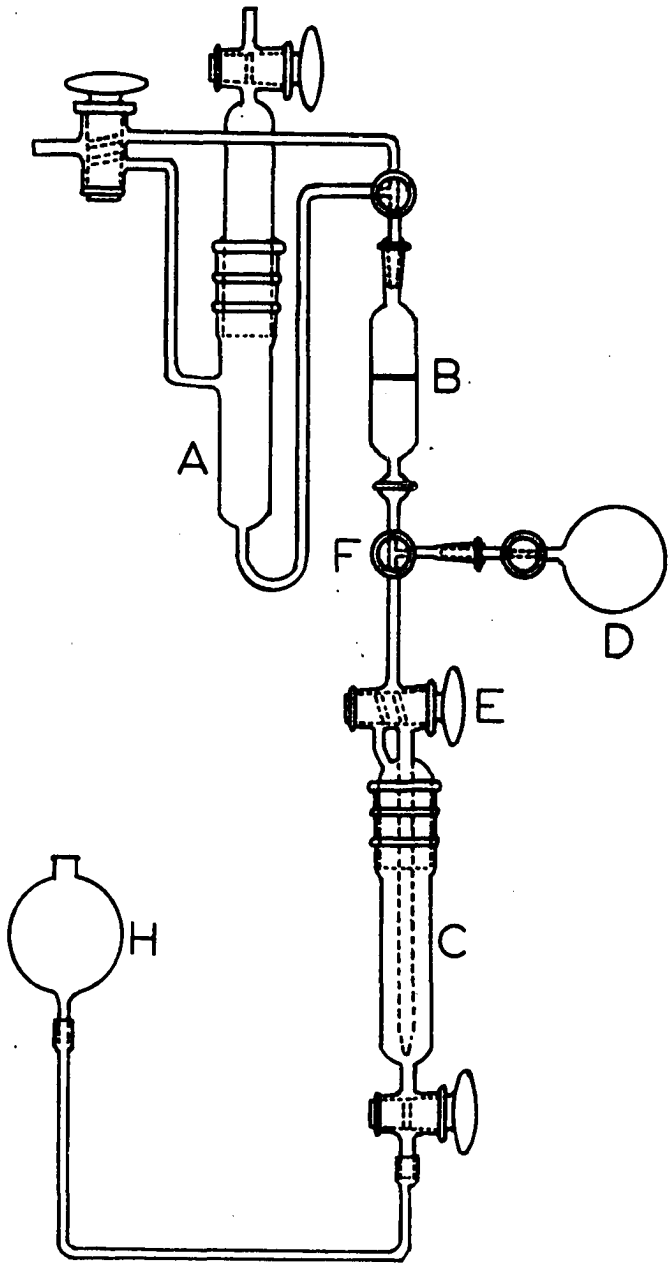
[b]Run 2. 0.1026 g. B_{12} in 10 ml. 0.1 N potassium sulfate

Procedure Portions of crystalline B_{12a}, dissolved in deionized water or in buffer solutions, were reduced to B_{12r} with hydrogen in the presence of platinum oxide catalyst in the apparatus shown in Figure 3. After hydrogenation, the reduced solution was pushed by hydrogen gas as described before from the reduction compartment A through a fine fritted glass filter B into the oxygenation compartment C. The apparatus had previously been thoroughly flushed with hydrogen except for bulb D, which contained approximately 50 ml. of air at atmospheric pressure. Stopcock F was then closed, the filter and hydrogenation cell removed, and the hydrogen in compartment C displaced with mercury by raising leveling bulb H and venting through E and F. Air from D was bubbled slowly through the B_{12r} solution by lowering the leveling bulb, then returned to D by turning stopcock F 180 degrees and raising the leveling bulb. This cycle was repeated from six to ten times for all runs. Bulb D was then detached and a portion of the enclosed gas analyzed with the mass spectrometer.

After each run the B_{12a} solutions were removed from the cell, the pH measured, and tests made for the possible presence of reduced nitrogen in the form of hydroxylamine, hydrazine or ammonia.

Hydroxylamine was tested for by the addition of salicylaldehyde and copper(II) acetate. A yellow precipitate of

Figure 3. Modified apparatus for the preparation of vitamin B_{12r} solutions, their treatment with oxygen and nitrogen at atmospheric pressures, and isolation of the resulting gases


A
B
F
D
E
C
H

copper(II) salicylaldehyde indicates a positive test (18). Hydrazine was checked for by the addition of salicylaldehyde and scrutiny under an ultraviolet light for the intense yellow fluoresence of salicylaldazine (18). Ammonia was tested for by the addition of strong base and absorption of the gases evolved by moist litmus paper. A procedure using Nessler's reagent, K_2HgI_4, was also employed (18).

Results The results of three gas analysis runs, at pH values of 4, 7 and 10, are tabulated in Table 2.

Table 2. Results of mass spectrometric analysis of gases over B_{12a} solutions for three runs at atmospheric pressure

Mass/charge ratio	Background	Voltage, gas sample before run	Voltage, gas sample after run
27[a]	0.013	0.048	0.048
28	0.130	20.9	20.2
29	0.016	0.280	0.277
30	0.003	0.016	0.015
31	0.004	0.006	0.006
32	0.036	5.5	5.5
40	0.006	0.455	0.480
44	0.006	0.048	0.037
46	0.001	0.001	0.001
27[b]	0.008	0.014	0.014
28	0.160	17.4	17.3
29	0.010	0.142	0.143
30	0.004	0.032	0.028
31	0.002	0.005	0.004
32	0.037	3.3	3.3
40	0.006	0.265	0.270
44	0.011	0.039	0.024
45	0.005	0.005	0.005
46	0.001	0.001	0.001

[a]Run 1. 0.0803 g. B_{12a} in 5 ml. deionized water

[b]Run 2. 0.1222 g. B_{12a} in 5 ml. pH 10.0 borate buffer

Table 2. (Continued)

Mass/charge ratio	Background	Voltage, gas sample before run	Voltage, gas sample after run
27[c]	0.006	0.014	0.014
28	0.158	18.6	17.5
29	0.008	0.148	0.145
30	0.002	0.041	0.040
31	0.002	0.004	0.005
32	0.041	3.4	3.2
40	0.006	0.263	0.280
43	0.005	0.006	0.006
44	0.013	0.046	0.036
45	0.004	0.005	0.005
46	0.000	0.001	0.001

[c]Run 3. 0.1235 g. B_{12a} in 5 ml. pH 4.0 potassium acid phthalate buffer

No increase in the mass/charge ratios corresponding to formation of any of the oxides of nitrogen was observed.

Negative results were obtained in all cases with the tests for hydrazine and hydroxylamine. A rather weak test for ammonia was given with all of the solutions, both with litmus and with Nessler's reagent. However, tests on solid B_{12a} gave similar results, so it is probable that ammonia is absent.

Method using cyanide addition

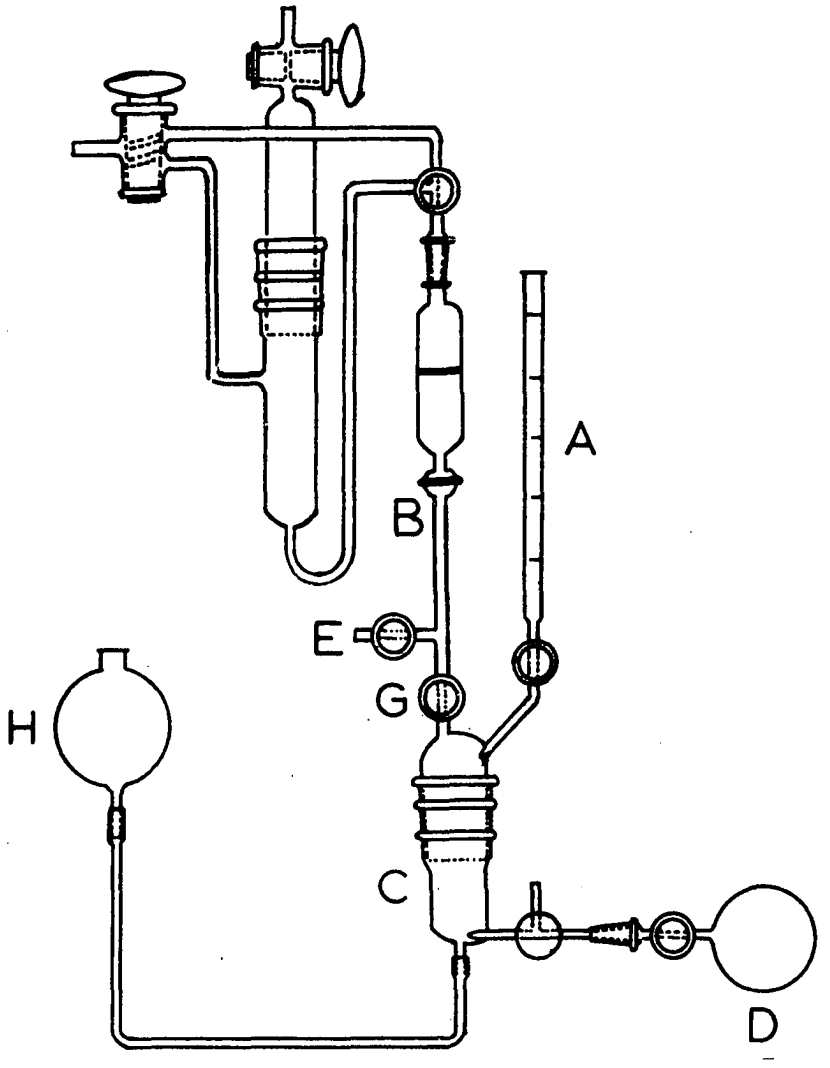
Introduction To determine whether some of the gases which might be evolved upon oxygenation of B_{12r} could be combining with the B_{12a} produced, a modified apparatus was built whereby cyanide could be introduced after the oxygenation to displace any gases which might be coordinating with the cobalt

in the vitamin. The apparatus is shown in Figure 4.

Procedure The apparatus was thoroughly flushed with hydrogen before the start of each run. B_{12r} solution, freshly reduced and filtered, was pushed into oxygenation compartment C and the solution level brought up to stopcock G to vent the hydrogen by raising the mercury filled leveling bulb H. The leveling bulb was then lowered and air drawn into C from bulb D. The solution was mixed thoroughly with the air by shaking the apparatus for about five minutes. Potassium cyanide solution, deaerated with hydrogen, was introduced into the compartment via buret A and the apparatus shaken another five minutes. Next the hydrogenation and filtration assembly was detached at B and replaced by an evacuated bulb. The connecting space between the bulb and compartment was evacuated by attaching a vacuum pump at B. Then the gas in the compartment was drawn into the bulb by opening stopcock G and replacing the gas with B_{12} solution and mercury.

Since the addition of cyanide might cause a rise in pH due to the release of the hydroxyl group coordinated to the cobalt atom in B_{12a}, a buffer was used to maintain a constant pH. A pH 6.86 buffer of ionic strength 0.2 was prepared from a mixture of disodium hydrogen phosphate and potassium dihydrogen phosphate. The ionic strength was kept purposely low

Figure 4. Apparatus for the treatment of vitamin B_{12r} solutions with gas mixtures, followed by addition of potassium cyanide and subsequent isolation of the gaseous products


A
B
E
G
H
C
D

to minimize salt effects upon the vitamin and upon the gases dissolved in the solutions. It was found that the addition of three times as much base in the form of potassium hydroxide to ten ml. of the buffer as would be released by the concentrations of B_{12a} employed changed the pH by less than 0.5 pH unit.

In the first run 0.1416 g. of B_{12}, dissolved in 10 ml. of the phosphate buffer, was reduced with hydrogen in the presence of 0.0693 g. platinum oxide catalyst. The B_{12r} solution was filtered, mixed with air as in the technique described in the previous section, then treated with 1.10 ml. of 0.0929 M potassium cyanide solution. The cyanide solution, freshly prepared and standardized against silver nitrate, was added from buret A in the amount required to just combine with the B_{12a} in a 1:1 ratio. After mixing, the gases over the solution were drawn off and analyzed in the mass spectrometer.

Results An increase in the M/e 30 peak from 2 to 20 mv. was observed. This peak could be attributed to nitric oxide, fixed from the nitrogen and oxygen in the air, or to methylamine formed during reduction of the cyanide group of B_{12}. Another run, using B_{12a} as starting material in place of B_{12} to eliminate the presence of methylamine in the solution, and even a repeat run using B_{12} as starting material gave no significant increase in the M/e 30 peak, so that the first results

could not be reproduced. It was concluded that traces of methylamine must have carried over into the sample bulb, perhaps dissolved in a bit of spray. No nitrous oxide or nitrogen dioxide was detected in any of the gases from any of the runs.

Discussion

During the course of this work a report was published which gave at least indirect support to the hypothesis that B_{12} may be directly connected with fixation of atmospheric nitrogen. Iswaran, Sundara Rao and Mathur observed that the addition of vitamin B_{12} to cultures of Azotobacter chroococcum gave pronounced increases in the amount of nitrogen fixed (19). That these increases were not due solely to the cobalt in the vitamin was shown by the fact that addition of cobalt salts alone at the same concentrations gave much lower fixation, although still significantly higher than the control.

However, we were not able to show that B_{12} alone catalyzes the fixation process. It may be that there is another form of the vitamin in the cells which is active in this respect, or that other materials are necessary to certain steps of the process.

PART II. OXYGEN ABSORPTION STUDIES

Oxygen Uptake by the Warburg Manometric Method

Method using modified cell with side bulb

Introduction As previously mentioned, one of the experiments that led to the hypothesis of nitrogen fixation by B_{12} was an adaptation of the Warburg manometric method. In it there was used a Warburg cell modified by the addition of a side arm bulb attached to the cell through a ground glass joint and a stopcock. The side arm bulb was filled with oxygen, the main cell flushed with nitrogen and a given amount of B_{12r} solution pipeted into the cell. After equilibration in a water bath, the stopcock was opened and the changes in cell pressure recorded. Instead of the expected drop in the pressure in the cell, a slight increase in pressure was observed, even though the B_{12r} was oxidized to B_{12a}.

Because no evidence of nitrogen fixation had been observed by the experiments outlined in Part I, the Warburg experiments were repeated in a manner which would permit analysis of the gases in the cell.

Apparatus The volumes of the Warburg cells and side bulbs were determined by weighing them empty and filled with mercury. Two side bulbs, with volumes of 3.14 and 18.14 ml., were used with a cell of 18.56 ml. capacity. Most of the runs

were made in a commercial Warburg thermostatted water bath with provision for shaking, at either 38.5 degrees or at room temperature (25-26 degrees). A few of the later runs starting with B_{12a} were done at 20±0.2 degrees in a small water bath with a fabricated shaking device.

Procedure The side bulb was first evacuated with a vacuum pump, then filled with oxygen. The main cell, with side bulb and manometer attached, was connected to the hydrogenation apparatus and well flushed with hydrogen. After reduction, the B_{12r} solution was pushed into the cell by hydrogen pressure, the hydrogenation apparatus and filter detached and the closed cell with manometer removed to the water bath for equilibration. Direct addition of the B_{12r} solution to the Warburg cell in this manner avoided any possibility of air oxidation during transfer, a problem that existed with the previous procedure. The stopcock was then opened to allow the oxygen in the bulb to diffuse into the cell. A similar cell without a side bulb was carried along as a thermobarometer to correct for volume changes due to variations in bath temperature or barometric pressure.

The cell pressure did not drop as expected, but remained essentially constant. A repetition of the experiment with replacement of the hydrogen by nitrogen gave the same results. Mass spectrometric analysis of the gases over the solution

after oxygenation showed only the originally added nitrogen and oxygen.

Since hydrogen gave the same results as nitrogen, the system became suspect and was therefore checked for inherent error. This was done by placing a few ml. of 0.1 N potassium sulfate into the cell, flushing with nitrogen, then equilibrating and allowing the oxygen to diffuse into the cell as before. The pressure in the cell increased with time. This experiment was repeated several times, always with the same result. The conclusion was that the constant, or nearly constant, pressures noted in the previous experiments were a combination of two effects; the first a reduction in volume due to the uptake of oxygen by the oxidation of B_{12r} to B_{12a}, and the second an increase in volume due to some change in the system independent of the B_{12r} present.

After some time the pressure change was finally traced to uptake of water by the dry oxygen being used in the side bulb. The increase was evidently caused by absorption of water vapor by the gas, which resulted in a corresponding increase in the vapor pressure in the cell. This effect was eliminated by the introduction of a few drops of water into the side bulb to saturate the oxygen with water vapor before introduction into the main cell. Blank runs with potassium sulfate solution in cells treated in this manner gave no pressure change after

equilibration.

The experiments on B_{12r} were now repeated, and a pressure drop was observed. The volume decrease was greater than that expected solely due to oxygen consumption corresponding to oxidation of B_{12r} to B_{12a}, but the added decrease depended on the size of the side bulb used.

Results Results of runs using each of the side bulbs are given in Table 3 at the end of Part II.

Method using internal oxygen generation

Introduction Because of the trouble experienced in the use of the separate side bulb containing oxygen for the B_{12r} oxidation studies, the possibility of generating oxygen directly in the Warburg cell was considered. It was found that quantities of oxygen could be generated rapidly and reproducibly through catalytic decomposition of hydrogen peroxide, using the iron(III) complex of triethylenetetramine as catalyst. The rapid rate of breakdown of hydrogen peroxide by this complex was first noted by Wang, who estimated the turnover number to be about 100,000 (20).

Apparatus A double Warburg cell with a partial divider across the middle to prevent intermixing of solutions in the two halves of the main compartment was used. Its volume was determined by weighing it empty and filled with mercury. The

water bath was the same as that described in the previous section. The B_{12r} solutions, prepared by hydrogenation in the apparatus described in Part I were pushed directly from the filtration compartment into one side of the Warburg cell, using ground glass connections throughout to avoid any exposure to air.

Chemicals Triethylenetetramine was distilled and the 269-275 degree fraction taken. The recorded boiling point is 272 degrees. A quantity of catalyst solution was prepared by adding 1 ml. of triethylenetetramine to 10 ml. of approximately 5×10^{-4} M iron(III) nitrate. A 0.12 per cent solution of hydrogen peroxide was prepared by diluting 10 ml. of a 3 per cent solution to 250 ml. and was stored in a dark bottle.

Procedure The amount of oxygen released by a given volume of the hydrogen peroxide solution was determined in the following way. A quantity of 1.0 ml. of the 0.12 per cent hydrogen peroxide solution was pipeted into one compartment of the Warburg cell and 2.5 ml. of the catalyst solution measured with a delivery pipet into the side arm next to that compartment. The cell with manometer was equilibrated in a water bath, the manometer reading recorded, then the cell tipped so that the catalyst came in contact with the peroxide. After about 5 minutes the manometer readings again became steady and were recorded. From the volume of the cell and the pressure increase the volume of oxygen liberated was calculated.

For the runs with B_{12r}, the procedure was the same as for the blanks except that after the catalyst and peroxide solutions had been placed in the cell, freshly prepared B_{12r} solution was added before removal to the water bath for equilibration. The pressure changes were recorded before and after the oxygen had been liberated. The amount of oxygen uptake by the B_{12r} was then calculated from the difference in pressure increase for the blank and the B_{12r} runs, taking into account the decrease in gas volume due to the space occupied by the B_{12r} solution. This solution volume was estimated after the run by transferring the B_{12a} solution as completely as possible into a 10 ml. volumetric flask by means of a fine tipped medicine dropper, then filling the flask to mark with deionized water from a 10 ml. buret and obtaining the solution volume by difference. The total amount of B_{12} in the cell was determined colorimetrically by diluting an aliquot of the 10 ml. to an appropriate volume and reading the absorbancy at 551 millimicrons in a Beckman Model DU spectrophotometer, using a value of 175 for $E_{1\ cm}^{1\%}$.

Using this internal generation method, the amount of oxygen liberated from 1.0 ml. of hydrogen peroxide was 0.30 ml., which in the cell used was 0.82 per cent of the total gas volume. This was near the practical upper limit of oxygen concentration, because larger amounts of gas caused pressures

which forced the manometer fluid off scale.

Results The results of this section are tabulated and discussed in conjunction with those of the next section at the end of the next section.

Method using solid vitamin B_{12a}

Introduction Because of the sensitivity of B_{12r} to oxidation it must be freshly prepared for each Warburg run by reduction of B_{12} or B_{12a} with hydrogen. B_{12a} is much more rapidly reduced than is B_{12}, but preparation of quantities of B_{12a} from B_{12} through reduction and reoxidation results in loss of almost a third of the B_{12}, so that the use of B_{12a} as a starting material is not very economical. For this reason most of the runs were made starting with B_{12}.

But after it had been shown that there was a reduction in volume upon oxidation of B_{12r}, and that the partial pressure of oxygen over the solutions had an effect upon the amount of oxygen taken up, as evidenced by the difference in volume decrease with different oxygen volumes, it was of interest to see whether a large increase in oxygen pressure would result in 100 per cent oxygenation of B_{12a}.

Procedure The method employed was to place solid B_{12a} in the side arm of a Warburg cell, add water to the main compartment, purge all nitrogen from the cell with pure oxygen,

then put the B_{12a} into solution by tipping the cell. The cell and contents were equilibrated at 20 degrees in a water bath and the manometer pressure recorded before the B_{12a} was put into solution.

This procedure was tried several times, both in 0.1 N potassium sulfate and in a pH 4 buffer of 0.0500 M potassium acid phthalate. There was no change in the gas volume in the cell after dissolution of the B_{12a} in any of the trials. From this the conclusion was drawn that B_{12a} either does not absorb oxygen in solution or that the solid material in 100 per cent oxygen was already present in the oxygenated form and could not take up further oxygen.

To test the second possibility, a gravimetric experiment was devised to see whether solid B_{12a} would gain in weight in an atmosphere of oxygen. A quantity of 0.4544 g. of B_{12a} was placed in the bottom of a U-shaped Schwartz drying tube (Kimble Glass Co., No. 46050). These lightweight tubes have a small side arm on each side, and hollow ground glass stoppers with a hole bored in one side which, by turning the stopper, can be made to coincide with the side arm opening. In this way the contents of the tube can be conveniently isolated from the atmosphere, yet the whole assembly is light enough to be weighed easily on a conventional analytical balance. To prevent a loss in weight due to removal of water from the B_{12a} by the

dry gas stream, one arm of the tube was filled with anhydrous magnesium perchlorate, separated from the B_{12a} by a tight plug of glass wool. All gases flowing through the tube passed through the dehydrating agent after passing over the B_{12a}; in this way any water removed from the B_{12a} was retained in the tube. An empty tube was carried along as a weighing tare.

After the tube was loaded, pure oxygen, freed of water by passage through a separate tube of magnesium perchlorate, was passed through the tube for about fifteen minutes. The tube was then weighed to the nearest 0.1 mg. After weighing, the oxygen was displaced by pure nitrogen and the tube weighed again. This cycle was repeated a second time. To determine the difference in weight caused by the differing densities of oxygen and nitrogen, the same procedure was carried out on the tube without B_{12a}.

The weight difference between the tube filled with oxygen and with nitrogen for two blank runs was 5.0 and 5.1 mg. With B_{12a} present the difference was 5.3 mg. for each of two runs. This amounts to an observed increase in weight of 0.25 mg.; the theoretical increase calculated for the uptake of one mole of oxygen by each two moles of B_{12a} is 4.8 mg. It was therefore concluded that at room temperatures and at atmospheric pressure B_{12a} in the solid form does not take up oxygen.

Results of Warburg experiments A tabulation of the results of a series of Warburg runs by the techniques outlined above is given in Table 3.

Table 3. Results of oxygen uptake measurements by B_{12r} solutions using the Warburg manometric method

Volume fraction of oxygen over B_{12r} solution[a]	Per cent oxygenation[b]
0.494	41.4, 43.8; ave. 42.6
0.104	23.4, 29.1; ave. 26.3
0.008	11.9, 10.2; ave. 11.1

[a]Remainder of gas primarily nitrogen, with some traces of argon

[b]Calculated on the basis of all oxygen taken up in excess of that required for oxidation of B_{12r} to B_{12a} going into formation of an oxygenated B_{12a} dimer

Spectrophotometric studies of B_{12a}

Introduction A series of experiments were performed to ascertain whether there was any difference in the spectrum of B_{12a} in the presence or absence of oxygen and of nitrogen. In addition, the effects of carbon monoxide and of nitric oxide upon the absorption spectrum in the visible and ultraviolet regions were examined.

Apparatus A Cary Model 12 spectrophotometer was used to record the visible and ultraviolet spectra. Silica 1 cm.

cells were used. For some measurements at single wavelengths a Beckman Model DU was employed, using 1 cm. Pyrex cells in the visible and 1 cm. silica cells in the ultraviolet regions.

Procedure The spectrum of an air saturated solution containing 0.049 mg. B_{12a} per ml. was recorded. Argon was then bubbled through the solution by means of a stainless steel hypodermic needle inserted through a tight fitting rubber cap on the cell. A small glass tube inserted into the cap provided a gas outlet which could be removed during recording of the spectrum. The spectrum of the argon saturated solution was recorded, followed by a spectrum of the solution saturated with nitrogen.

In the same manner the spectrum of a B_{12a} solution of the same concentration was recorded before and after bubbling carbon monoxide through it.

Nitric oxide when bubbled through an air saturated solution of B_{12a} gave four intense peaks in the ultraviolet at wavelengths of 346, 357, 370 and 384 millimicrons. These were at first thought to be due to the formation of a complex between the gas and B_{12a}, but it was later found that the same spectrum could be obtained by bubbling nitric oxide through an air saturated solution of deionized water without the B_{12a}. However, it was not obtained by bubbling nitric oxide through either water or B_{12a} if the solutions had been previously de-

oxygenated with nitrogen or argon. Presumably the spectrum observed was due to the formation of nitrous acid through oxidation of nitric oxide by dissolved oxygen to nitrogen(III) oxide, N_2O_3, and combination of this gas with water.

Results It was concluded that there was no evidence from the absorption spectra for the reaction of oxygen, nitrogen, carbon monoxide or nitric oxide with B_{12a}. In all cases the spectra were comparable to that obtained in air.

Magnetic susceptibility studies of B_{12a}

Introduction Vitamin B_{12a} cannot be obtained in crystalline form by evaporation or cooling of aqueous solutions, precipitating instead as an amorphous scale. That the crystals do not contain any acetone of crystallization has been shown (21). It was thought therefore that the scale might be a solid oxygenated form of B_{12a}, the oxygen forming a dimer between pairs of B_{12a} molecules and so preventing their orientation in a definite crystal pattern.

As was mentioned previously, divalent cobalt coordination compounds are paramagnetic unless they are combined with molecular oxygen, when they become diamagnetic. Trivalent cobalt coordination compounds, including B_{12a}, are diamagnetic. Upon oxygenation, however, these compounds might be expected to become paramagnetic, with a magnetic moment corresponding to the

two unpaired electrons of the oxygen molecule. This would reverse the pattern of divalent cobalt behavior, and would be good evidence for the occurrence of an oxygenated species.

Magnetic susceptibility measurements were therefore made on vitamin B_{12a} crystallized from acetone-water solution, and on the same material after dissolution in water and evaporation to dryness in an air stream.

Apparatus Measurements were made by the Gouy method, using a magnetic susceptibility apparatus provided by the Institute for Atomic Research. The material to be examined was placed in the upper half of a double glass tube with a septum at the center. This tube was suspended from an Ainsworth TCX semimicro balance such that the septum was precisely between the magnet poles. The magnet was a Consolidated Electrodynamics Corporation Model 23-104A capable of developing a field of 11,000 gauss at 10 amp. Magnet current was drawn from a selenium rectifier whose input voltage was stabilized by a voltage regulator. The currents were determined precisely by measuring the voltage drop across a precision resistor with a Leeds and Northrup Model K-2 potentiometer. The tube used had an internal diameter of 0.19 cm., and when suspended was enclosed by a thermostatted water jacket which maintained the sample temperature at 20 ± 0.2 degrees.

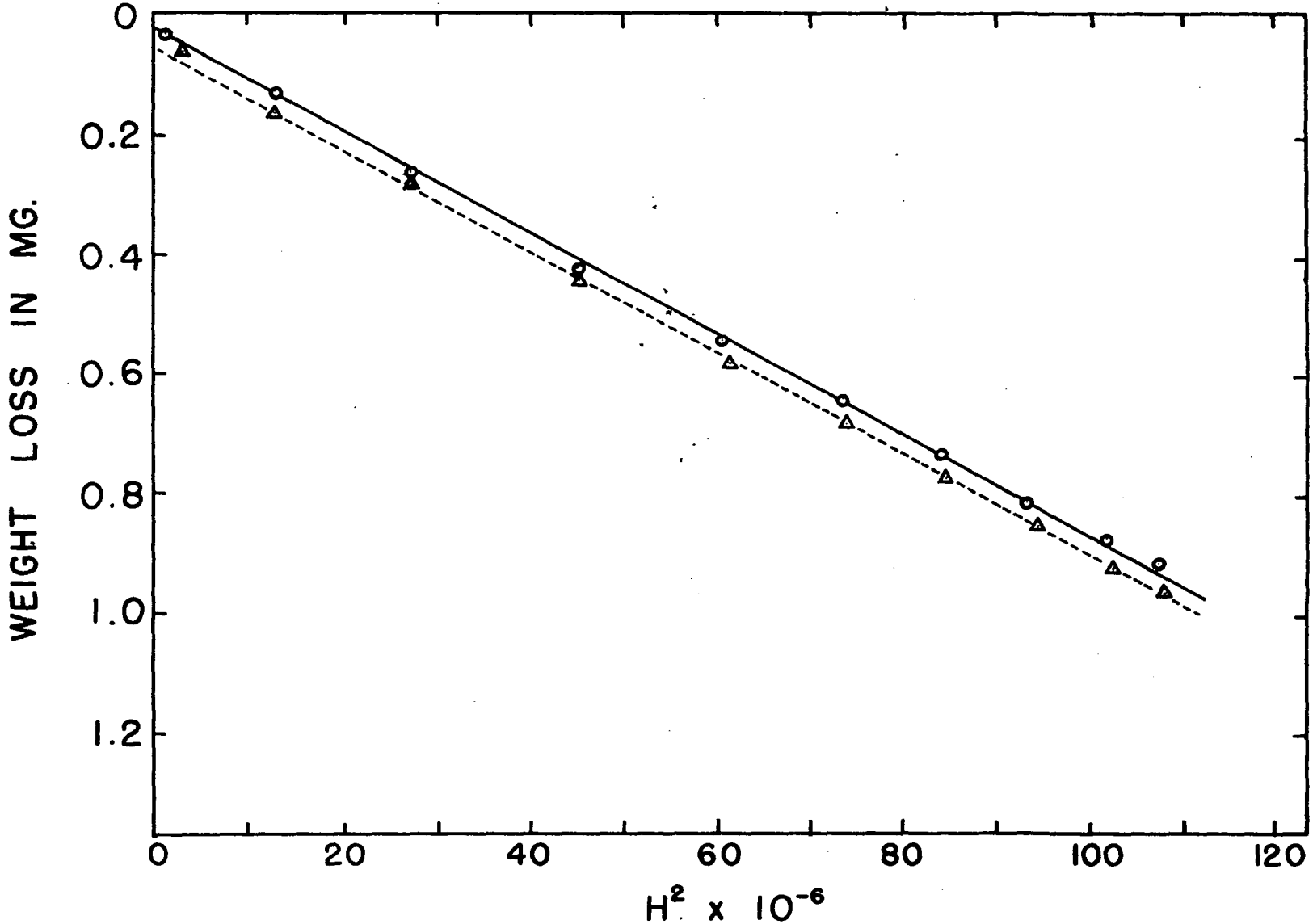
Chemicals B_{12a} was prepared from B_{12} by hydrogenation and subsequent oxidation as previously described, and was obtained as large deep red crystals from acetone-water solution. The scale for the second set of measurements was obtained by dissolving the crystals in deionized water and carefully evaporating the solution to dryness in a stream of air.

Procedure The tube was filled by the addition of small increments of material, and was tapped well after each addition. It was then wiped with an acetone moistened lens tissue and suspended in the apparatus for at least an hour before the first measurements to insure complete equilibration. Weight and magnet current measurements were made at 1 amp. intervals to give a check on the presence of possible ferromagnetic impurities. After the final measurement a reverse current of 0.75 amp. was passed through the magnet windings to remove the residual field and a check made on the original weight of the tube. A run with the tube empty showed it to be magnetically symmetrical and free from ferromagnetic impurities. The tube was calibrated by making a similar series of measurements on a 30 per cent solution of nickel chloride, prepared from 99.9 per cent pure Fisher reagent grade $NiCl_2.H_2O$ and standardized gravimetrically by electrolysis from ammoniacal solution. Plots of the change in weight vs. the square of the field strength for each run are shown in Figure 5.

Figure 5. Magnetic susceptibility plot of weight loss vs. square of the field strength for vitamin B_{12a}

o Vitamin B_{12a} crystals from water-acetone solution

Δ Vitamin B_{12a} scale from evaporation of aqueous solution


WEIGHT LOSS IN MG.
0
0.2
0.4
0.6
0.8
1.0
1.2
0
20
40
60
80
100
120
H² x 10⁻⁶

Results From Figure 5, it may be seen that the difference between the two runs is negligible. It is of interest to note, though, that the B_{12a} in both cases lost weight right from the start. This behavior deviates from that observed previously for B_{12a}, and indicates that the material used in this work is substantially free of any ferromagnetic impurities (6).

Discussion

In the calculation of the per cent oxygenation for the Warburg experiments it was assumed that 0.25 mole of oxygen would be consumed per mole of B_{12r} in the oxidation to B_{12a},

$$4\ RCo(II) + O_2 + 2\ H_2O \rightarrow 4\ RCo(III)OH$$

and that the remainder of the gas used would be oxygen reacting with the B_{12a} produced to yield the oxygenated dimer:

$$2\ RCo(III)OH + O_2 \rightleftharpoons [RCoR\ {-O_2}\ - CoR]^{++} + 2\ OH^-$$

In the above equations the R term designates the remainder of the B_{12} molecule.

It may be seen that with 50 volume per cent oxygen over the solution, the amount of oxygen absorbed is about 43 per cent of theoretical. This behavior is compatible with that observed in other oxygen carrying systems, such as hemoglobin,

and so from this standpoint is not surprising.

What was unexpected, however, was the failure of B_{12a} to pick up oxygen when put into solution. From the work of Jaselskis and Diehl (14), along with the B_{12r} oxygen uptake studies mentioned above, it seems definite that oxygen uptake is taking place in those instances. But why not starting with B_{12a}? One possibility, that B_{12a} solid may take up oxygen, has been eliminated.

A second possibility is that B_{12a} freshly prepared from B_{12r} is chemically different in behavior from B_{12a} crystallized from acetone-water solutions. That the spectrum of B_{12a} in solution changes on standing has long been known, and as it is accompanied by an increase in the pH of the solution it is considered to be due to replacement of the hydroxyl group coordinated to the cobalt by a water molecule. If the aquo group forms a more stable compound, it may not be as easily removed by oxygen as the hydroxyl group. This then could explain why freshly prepared B_{12a} takes up oxygen while solutions prepared from crystalline B_{12a} do not.

PART III. B_{12} COENZYME STUDIES

Magnetic Susceptibility Measurements

Method using Gouy apparatus

Introduction In 1959 Barker, Weissbach and Smith first reported the isolation of a crystalline light sensitive material related to vitamin B_{12} which they called 5,6-dimethylbenzimidazole cobamide coenzyme. This compound, referred to here for convenience as B_{12} coenzyme, is one of three cobamide coenzymes separated and characterized thus far (22). The coenzymes are isolated from bacterial cultures by a combination of extraction and ion exchange procedures, carried out in the dark or in dim light at reduced temperatures (23). They are all active in the catalytic conversion of glutamate to β-methyl aspartate. Barker states that over 90 per cent of the vitamin B_{12} in rabbit liver is present in the coenzyme form, and has been previously overlooked because in the presence of cyanide and upon exposure to light the coenzyme is converted to cyanocobalamin. This proportion very likely extends to other sources of the vitamin also.

That B_{12} coenzyme is closely related to B_{12} is evidenced by the close similarity of the absorption spectra of the two in cyanide solution. It appears that the principal difference in structure is in the replacement of the cyanide in B_{12} by an adenine moiety in B_{12} coenzyme (22). The coenzyme, however,

contains in addition some other material, as the molecular weight has been determined to be about 1660, 170 more than that accounted for by the combined molecular weights of adenine and B_{12a}.

Although the coenzyme structure has already been fairly well characterized, there have been conflicting reports as to the valence of the cobalt (24, 25). A magnetic susceptibility study was therefore undertaken to determine, if possible, the actual oxidation state of the cobalt atom.

Material Two samples of B_{12} coenzyme were used, both provided by the Squibb Institute for Medical Research. The second sample had been specially purified by phenol-water extraction to remove as completely as possible all traces of iron.

Apparatus The apparatus used was the same as that described in Part II for the magnetic susceptibility measurements on B_{12a}.

Procedure The procedure was also identical to that described previously for B_{12a}, except that filling of the tube and all subsequent operations were carried out in the dark or in dim red light to prevent decomposition of the coenzyme.

Results The run on the first batch of coenzyme showed a weight gain, abrupt at first but leveling off at higher field strengths. This behavior suggested that ferromagnetic impuri-

ties might be present, and that the material might be actually diamagnetic in character. The presence of only traces of iron, especially in the form of iron oxides, is sufficient to obscure the diamagnetic properties of many substances. To test for possible iron contamination a 25 mg. sample of the coenzyme was wet ashed with a mixture of equal volumes of nitric, sulfuric and perchloric acids and was analyzed for iron colorimetrically with bathophenanthroline (26). It was found to contain 0.0325 per cent iron, an amount quite likely sufficient to cause anomalous magnetic susceptibility results.

A new portion of the coenzyme, handled and purified in a manner to minimize trace contamination by iron, was obtained from the Squibb Institute. The iron content of this material was found upon wet ashing and analysis to be only 0.008 per cent. A magnetic susceptibility run was carried out on this sample and a plot of weight gain vs. the square of the field strength made. This plot, shown in Figure 6, indicates that the compound is paramagnetic, for the weight gain, though still greater at lower magnet current values than at higher ones, is continuous throughout the range of field strengths.

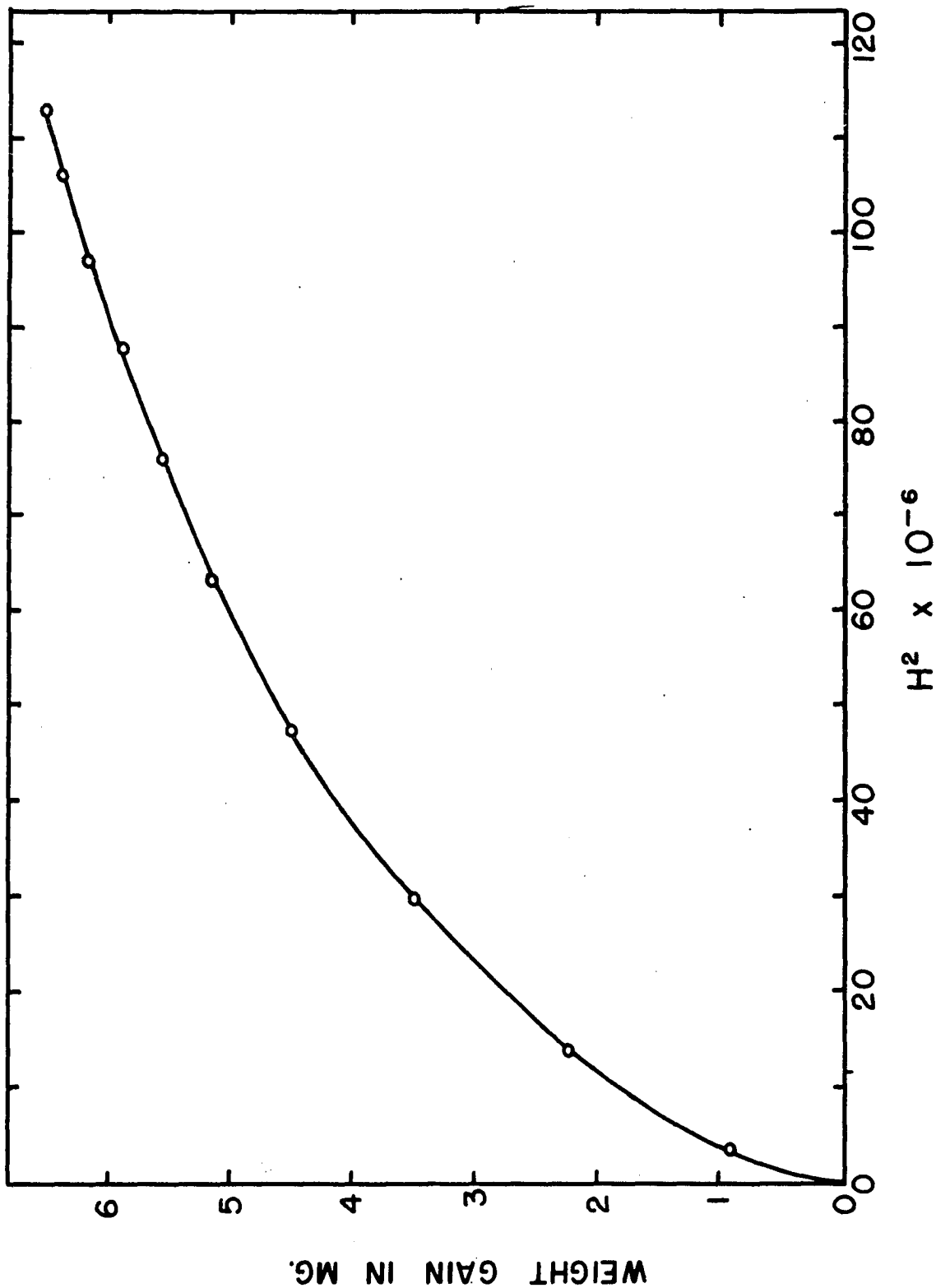
Conclusions The magnetic moment for cobalt(II) corresponds to three unpaired electrons, or 3.8 Bohr magnetons. However, values for the magnetic moment vary widely in the presence of different complexing agents, ranging from 5.3 for

the violet cobaltous bipyridyl compound (27) to 1.7 for biscyclopentadienylcobalt(II) (28). Exceptions here are the cobalt(II) cyanides, which are diamagnetic (29). Cobalt(III) coordination compounds are diamagnetic, though here too there are exceptions reported involving some strongly paramagnetic cobalt chelates with a Schiff's base as ligand (30).

B_{12} and B_{12a} both contain cobalt(III) and are, as expected, diamagnetic (31). The fact that the coenzyme is paramagnetic seems excellent proof that the cobalt in this case is divalent. Using the density of B_{12}, 1.34 g. per cc., as the density of the coenzyme and assuming the material to contain 10 per cent moisture, the magnetic moment of the coenzyme was calculated to be 2.75 Bohr magnetrons. This value is probably somewhat high because of the contribution to the weight gain by ferromagnetic impurities. Extrapolation of the linear upper portion of the curve in Figure 6 to zero field strength in an attempt to correct for the ferromagnetism present gave a weight gain of 3.3 mg. and a magnetic moment of 1.56 Bohr magnetons.

B_{12} coenzyme is the first known case of a strong coordination compound of cobalt(II) existing in stable form in contact with oxygen. In contrast, the reduction potential of B_{12r} in pH 7.4 buffer is in the neighborhood of 0.1 volt, equivalent to that of titanium(III) in solution, and is oxidized rapidly upon exposure to the atmosphere.

Figure 6. Magnetic susceptibility plot of weight gain vs. square of the field strength for purified B_{12} coenzyme


WEIGHT GAIN IN MG.
0
1
2
3
4
5
6
0
20
40
60
80
100
120
$H^2 \times 10^{-6}$

This unusual behavior is but another in the series of unexpected results that seem to be the rule rather than the exception with B_{12} related compounds. The fact that the coenzyme is light sensitive suggests that other cobalt(II) complexes might be stable if kept from exposure to light. It may well be that through B_{12} another fertile field for research in coordination chemistry may be opened.

Polarographic Investigations

Behavior at the dropping mercury electrode

Introduction A further study of B_{12} coenzyme was carried out through the use of polarography. Past applications of polarographic techniques to vitamin B_{12} and related compounds have been surveyed in the introduction preceding Part I and will not be repeated here. Worth emphasizing again, however, is the fact that in all of the B_{12} compounds the cobalt is reduced at the dropping mercury electrode to only the plus one oxidation state. It was therefore of interest to see how readily the cobalt in the coenzyme would be reduced, how far it would be reduced, and incidentally to obtain some confirmation of the magnetic susceptibility finding that it is present in the divalent form.

Chemicals The B_{12} coenzyme used was part of the second batch obtained from the Squibb Institute for Medical Research.

Concentrations were corrected for the moisture content reported by Squibb. Molar concentrations were calculated on the basis of a molecular weight of 1660. The crystalline adenine sulfate used was also provided by Squibb.

As supporting electrolyte a 0.1 M solution of potassium sulfate was prepared from recrystallized reagent grade potassium sulfate and deionized water. A solution of tetraethylammonium perchlorate of the same concentration was prepared from the pure salt (provided through the courtesy of Dr. Lawrence A. Knecht) and deionized water.

Apparatus A Sargent Model XXI polarograph was used to record the polarograms. The anode was a laboratory type saturated calomel electrode with an agar-potassium chloride salt bridge. At 40 cm. head height the drop time of the dropping mercury electrode was 4.74 sec. per drop and the flow rate was 1.18 mg. mercury per sec. The cell was a small weighing bottle of about 20 ml. capacity, fitted with a rubber stopper containing holes for the dropping mercury electrode, the saturated calomel electrode, and the nitrogen inlet. The nitrogen inlet tube was of a smaller diameter than the hole in the stopper to provide a space to serve as a gas outlet. The cell was wrapped in aluminum foil during the coenzyme runs to exclude light.

Oxygen was removed from the solutions in the cell by

bubbling pure tank nitrogen through them. The nitrogen was passed through a wash bottle containing supporting electrolyte before introduction into the cell. Precautions to remove oxygen from the nitrogen were not necessary, as mass spectroscopic analysis of the nitrogen showed the presence of only traces of oxygen; passage of the gas through solutions of supporting electrolyte for 10 minutes gave polarograms with no sign of the oxygen reduction waves.

Procedure The supporting electrolyte was deaerated with nitrogen before addition of the coenzyme or introduction of the electrodes. The electrodes were then inserted through the stopper into the solution and a polarogram recorded. In some cases the pH of the supporting electrolyte was adjusted by the addition of 2 N potassium hydroxide or sulfuric or perchloric acid.

Results In 0.1 M potassium sulfate a reduction wave occurs at a potential of -1.44 v. vs. the saturated calomel electrode, but it is followed so closely by a second wave having a pronounced maxima that the wave heights are not measurable with any degree of accuracy. However, at higher pH values it was found that the second wave is shifted toward more negative potentials so that the two are separated by a well defined diffusion current plateau above pH 10. In addition the maxima on the second wave disappears under these

conditions. A polarogram of the coenzyme at a pH of 11.6 is shown by the solid line in Figure 7.

The first wave is preceded by a small sloping prewave which commences at about -1.0 v., and which is equivalent at its maximum to about 10 per cent of the total height of the first wave. This is thought to be a kinetic prewave caused by absorption of the large coenzyme molecule on the surface of the mercury drop, but its presence is by no means well understood.

The number of electrons involved in each of the reductions was determined by application of the Ilkovic equation, and was found to be equivalent to two in each case. It is necessary to include the height of the prewave in the height of the first wave to arrive at meaningful values in the calculation of n. The diffusion coefficient of the coenzyme was assumed to be the same as that of B_{12} for this calculation, 2.95 cm.^{2}/sec. x 10^6.

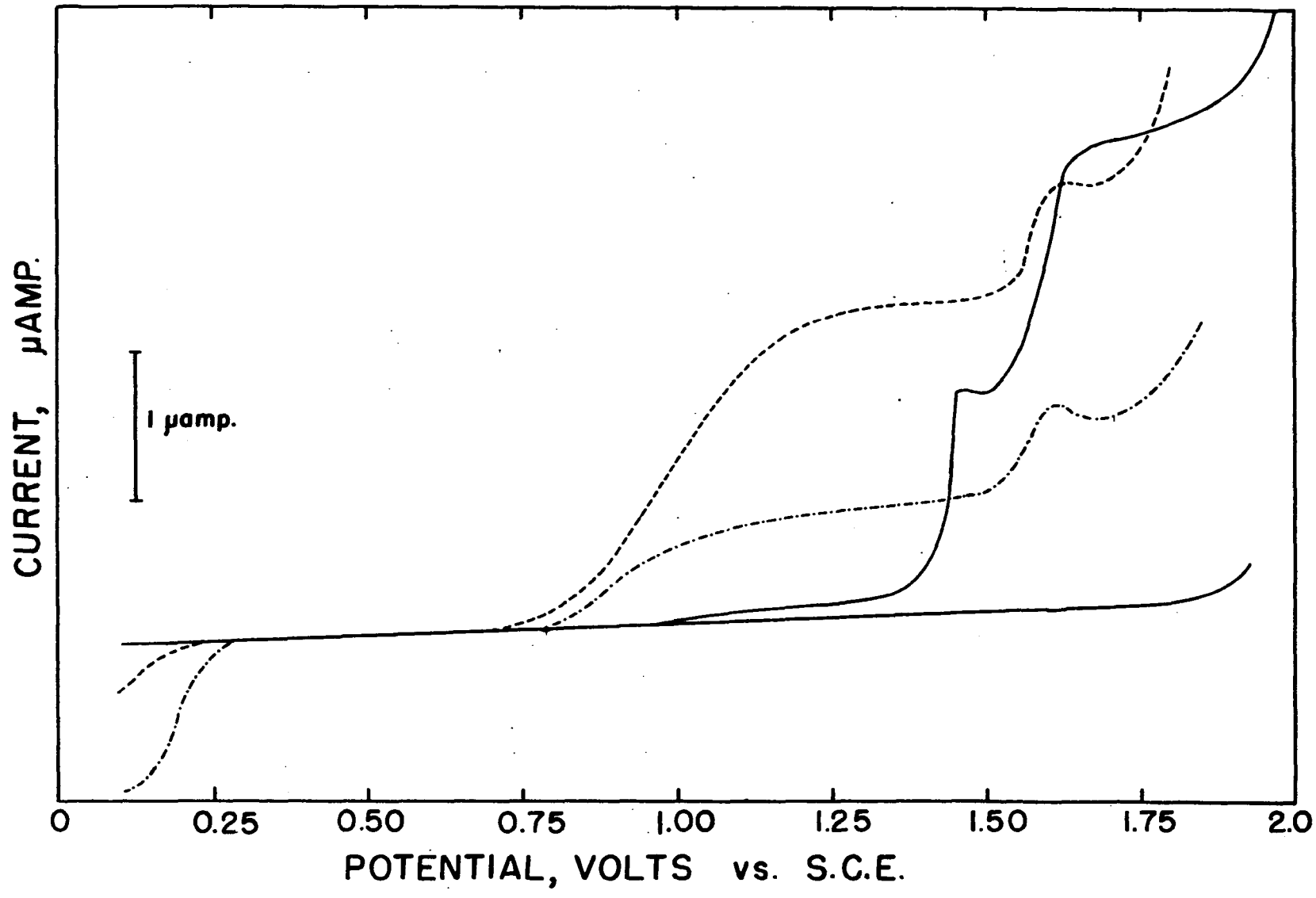
Polarograms of the coenzyme were also recorded using tetraethylammonium perchlorate as supporting electrolyte. The first wave occurred at a half wave potential of -1.31 v. vs. the saturated calomel electrode, but was of peculiar shape and did not appear to be completely diffusion controlled. No other work was done in this medium other than to determine that the wave shape was not altered nor the half wave potential

Figure 7. Polarograms of B_{12} coenzyme in 0.100 M potassium sulfate, adjusted to pH 11.6 with 2.0 N potassium hydroxide

Solid line: 0.402 mM B_{12} coenzyme

Dash-dot line: Same solution, exposed to light for 75 minutes

Dash line: Oxygen bubbled through the light exposed B_{12} coenzyme solution for 5 minutes


CURRENT, μAMP.
1 μamp.
0
0.25
0.50
0.75
1.00
1.25
1.50
1.75
2.0
POTENTIAL, VOLTS vs. S.C.E.

shifted at a pH of 10 to 11.

In an attempt to clarify the relation of B_{12} coenzyme to the other cobalamins, the aluminum foil light shield was removed from the cell after one of the coenzyme runs and the solution exposed to daylight plus fluorescent illumination for 75 minutes. During this time a stream of nitrogen was maintained over the solution to exclude oxygen. A polarogram run on the resulting solution, which had changed from a yellowish orange to a reddish brown, is shown by the dash-dot line in Figure 7. This curve corresponds to that of B_{12r}, except that the anodic wave occurs some 0.16 v. more negative at -0.2 v. Both the anodic wave and the cathodic wave at -0.91 v. are one electron steps as calculated by the Ilkovic equation. The red-brown color was similar to the color of B_{12r} solutions.

After the polarogram of the light exposed coenzyme solution had been obtained, oxygen was bubbled through the cell contents for about five minutes, then the excess gas removed by purging with nitrogen and another polarogram recorded. The color of the solution at this point was red-orange, as would be expected for solutions of B_{12a}. The polarogram, however, was not that of B_{12a}, but consisted of a large multielectron reduction wave at -0.99 v. This wave is shown by the dash line in Figure 7. The cathodic one electron reduction wave at -0.04 v. observed in solutions of B_{12a} is absent here.

A small wave was present in the polarograms of the light exposed solution at -1.56 v., both before and after the oxygen treatment. A similar wave has been observed with B_{12} and B_{12r}; its nature is not understood.

A comparison of the results of the coenzyme runs with previous polarography of the cobalamins is given in Table 4.

Table 4. Comparison of polarographic characteristics of the cobalamins with B_{12} coenzyme solutions

Material	$E_{\frac{1}{2}}$, volts vs. S.C.E.	Concentration, millimoles	i_d, μamp.	n	Reference
B_{12} coenzyme	-1.43	0.402	1.24	2.03	
	-1.62	0.402	1.22	1.99	
B_{12}	-1.11	0.416	1.44	1.98	12
B_{12} coenzyme[a]	-0.2(anodic)	0.402	0.68	1.11	
	-0.91	0.402	0.65	1.06	
B_{12r}	-0.04(anodic)	0.434	0.81	1.06	12
	-0.95	0.434	0.76	1.01	
B_{12} coenzyme[b]	-0.99	0.402	1.98	3.25	
B_{12a}	-0.06	0.303	0.39	0.84	12
	-1.02	0.303	0.45	0.86	

[a]Same solution of coenzyme as above, but exposed to light for 75 minutes in a nitrogen atmosphere

[b]Same solution of coenzyme after exposure to light and passage of oxygen for 5 minutes

SUMMARY

1. A study was made of the possibility of catalysis of nitrogen fixation by some form of vitamin B_{12}. Oxygen and nitrogen were brought into contact with solutions of B_{12r} and the gases over the solutions trapped and analyzed mass spectrometrically. None of the oxides of nitrogen were detected. Addition of potassium cyanide to B_{12r} solutions after the introduction of oxygen and nitrogen to drive off any gases coordinated to the cobalt atom in the vitamin also gave negative results.

2. Previous studies of the oxygen uptake of B_{12r} by the Warburg manometric method were demonstrated to give incorrect results due to non-equilibration of the gas in the side bulb with water vapor. Using two modifications of the Warburg technique, one employing pure oxygen gas in a side bulb and the other internal generation of oxygen by catalytic decomposition of hydrogen peroxide, it was shown that oxygen is taken up in excess of that required for the oxidation of the divalent cobalt in B_{12r} to trivalent cobalt in B_{12a}, the amount in excess depending upon the partial pressure of the oxygen over the B_{12a} solution. This excess oxygen uptake is attributed to the formation of an oxygenated species.

3. Addition of solid B_{12a} to dilute solutions of potassium sulfate in a 100 per cent oxygen atmosphere does not re-

sult in an uptake of oxygen. It is suggested that this may be due to the existence of B_{12a} in either a hydroxo or an aquo form, depending upon which group is coordinated to the cobalt atom, and that B_{12a} crystals from acetone-water solutions may be in the aquo form and therefore not form an oxygenated species.

4. Solid B_{12a} had been shown by a gravimetric method to not take up oxygen at room temperatures and at atmospheric pressure.

5. Magnetic susceptibility measurements on B_{12a} crystals formed from acetone-water solution and on B_{12a} scale obtained by evaporation of an aqueous solution to dryness have shown both compounds to be diamagnetic, so that the scale is not an oxygenated form of the vitamin.

6. The visible and ultraviolet spectra of B_{12a} in the presence of nitrogen, oxygen, carbon monoxide, argon and nitric oxide have been compared and found to be alike.

7. B_{12} coenzyme has been found from magnetic susceptibility measurements to be paramagnetic. From this it is inferred that the cobalt atom in this molecule is divalent.

8. A polarographic study of the coenzyme has been made. In 0.1 M potassium sulfate supporting electrolyte, adjusted to

pH 11.6 with potassium hydroxide, two reduction waves are observed at -1.43 v. and -1.62 v. vs. the saturated calomel electrode. By application of the Ilkovic equation each was found to correspond to a two electron reduction. Exposure of solutions of the coenzyme to light in the absence of oxygen produces a product which gives a polarogram corresponding to that of B_{12r} except that the anodic wave is shifted from -0.04 v. to -0.2 v. Passage of oxygen through light-exposed solutions of the ocenzyme gives a multi-electron reduction wave at -0.99 v., the nature of which is not yet fully understood.

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