REDUCTION OF VITAMIN B₁₂

DISSERTATION

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By

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Vitamin $\text{B}_{12}$ is a dark red crystalline compound with the formula $\text{C}_{63}\text{H}_{88}\text{O}_{14}\text{N}_{14}\text{PCo} - 5\text{-}6 \text{H}_2\text{O}$.\(^1\) Its structure was determined by chemical and X-ray crystallographic techniques.\(^2\) (See Figure 1) The molecule is divided into two major portions, the planar group which resembles the porphyrins, and the nucleotide. The cobalt atom is located in the center of the planar group and is linked to four reduced pyrrol rings which form a larger ring. The base of the nucleotide is formed by 5:6 dimethylbenzimidazole which is linked to the cobalt atom through one of its nitrogen atoms. A cyanide group occupies the sixth coordination position on the cobalt atom.

Diehl, Vander Haar, and Sealock\(^3\) determined by magnetic susceptibility measurements that cobalt was present in the trivalent state.


Figure 1

Structure of the Vitamin $\text{B}_12$ Molecule
Vitamin B₁₂

Figure 1
The trivalent cobalt can be reduced in a number of ways. Kaczka, Wolf, and Folkerś\textsuperscript{4} reported that by shaking $\textit{B}_{12}$ with hydrogen gas in the presence of platinum oxide catalyst, the bright red color changed to dark brown, but returned to bright red on contact with air. A subsequent cobalt and phosphorus analysis showed that the compound was not grossly altered in the process. The end product was designated $\textit{B}_{12}$ which presumably contains the original molecule intact with the exception that an OH group has been substituted for the cyanide group. A later study by the same group\textsuperscript{5} substantiated their earlier findings.


Diehl, Sealock, and Morrison\textsuperscript{6} reduced the trivalent cobalt in the $\textit{B}_{12}$ molecule polarographically. They found an irreversible, but well-defined, reduction wave with a half-wave potential, hereafter abbreviated $E_{\frac{1}{2}}$, of $-1.12$ volts versus the saturated calomel electrode. The $E_{\frac{1}{2}}$ was independent of pH in the range 5.2 to 11.7 and had essentially the same value in $\textit{KNO}_{3}$, $\textit{K}_{2}\text{SO}_{4}$, $\text{N}($\textit{CH}_{3})_{4}\text{SO}_{4}$, $\text{N}($\textit{CH}_{3})_{4}\text{OH}$, and $\text{N}($\textit{CH}_{3})_{4}\text{Cl}$ supporting electrolytes. The diffusion current was

proportional to $B_{12}$ concentration. Using a diffusion coefficient obtained with an Anson and Stokes\textsuperscript{7} cell and the Ilkovic equation, an $n$


value of 2.04 was determined.\textsuperscript{8} This indicated a reduction of Co(III)


to Co(I).

A later improvement was made in the method for determining the
diffusion coefficient by Jaselskis, Foster, and Diehl.\textsuperscript{9} The


coefficients reported were $2.9 \times 10^{-6}$ cm$^2$ sec$^{-1}$ for $B_{12}$ and $2.3 \times 10^{-6}$ cm$^2$ sec$^{-1}$ for $B_{12a}$. Using the new coefficients and the Stokes-

Einstein method for calculating molecular weights,\textsuperscript{10} the molecular


weights of $B_{12}$ and $B_{12a}$ were calculated to be 1380 and 2225 respectively. The authors concluded that $B_{12a}$ was a dimer, caused by the

union of the vitamin $B_{12}$-hydroxy compound with molecular oxygen.

X-ray diffraction data supported this conclusion.
Diehl and Murie\textsuperscript{11} reduced the vitamin with hydrogen in the presence of platinum and obtained the same brown color reported by Kaczka and co-workers. The brown material, designated Bi$_{12r}$, required 1 equivalent of ferricyanide for oxidation back to the original red color, indicating that cobalt was present in Bi$_{12r}$ in the divalent state.

Jaselskis and Diehl\textsuperscript{12} also prepared Bi$_{12r}$ by catalytic hydrogenation. In the reduction the cyanide group was released from the molecule, and was reported to be hydrogenated to methylamine. A titration of the Bi$_{12r}$ with ferricyanide and with iodine in an acidic solution showed the cobalt atom to be divalent. Upon oxidation an OH group was added to the cobalt and Bi$_{12a}$ was formed. Polarographic waves were recorded for Bi$_{12}$, Bi$_{12r}$, and Bi$_{12a}$, with 0.1 M potassium sulfate as supporting electrolyte. Bi$_{12}$ gave a single two-electron reduction wave with an $E_1$ of -1.11 volts versus the saturated calomel electrode, hereafter abbreviated SCE. Bi$_{12r}$ gave two one-electron waves with $E_1$ values of -0.04 volts (anodic) and -0.95 volts (cathodic). Bi$_{12a}$ gave two one-electron cathodic waves with $E_2$ values of -0.06 volts and -1.02 volts. All of the waves were independent of pH.


The following reactions were interpreted from the polarographic data by the authors: In $B_{12}$, cobalt(III) is reduced to cobalt(I); in $B_{12r}$, cobalt(II) is oxidized to cobalt(III) in the anodic wave and cobalt(II) is reduced to cobalt(I) in the cathodic wave; in $B_{12a}$, cobalt(III) is reduced to cobalt(II) in the first step, and cobalt(II) is reduced to cobalt(I) in the second.

Boos, Carr, and Conn$^{13}$ performed an amperometric titration of $B_{12}$

$^{13}$R. N. Boos, J. E. Carr, and J. B. Conn, Science, 117, 603 (1953).

with chromium(II) ion at pH 9.5 in 0.1 M ethylenediaminetetraacetic acid, hereafter abbreviated EDTA. The original vitamin $B_{12}$ gave a reduction wave with an $E_1$ of -1.021 volts and the reduction product, which was brown, gave an anodic wave with an $E_1$ of -0.311 volts versus the SCE. Results of the titration gave an equivalent weight of 1340, indicating a 1 electron reduction. Therefore, the reduced product contained divalent cobalt, in agreement with the reports of previous workers. The authors concluded that the anodic wave could be attributed to the oxidation of the divalent cobalt. The wave was actually due to cyanide released from $B_{12}$ on reduction, as will be shown later.

Boos, Carr, and Conn$^{14}$ obtained an absorption spectrum for their

$^{14}$Ibid.
Novel $B_{12}\text{r}$ which differed from that reported by Diehl and Murie.\textsuperscript{15} Later, Beaven and Johnson\textsuperscript{16} discussed the discrepancy. They stated that the difference represented changes in the cobalt oxidation state only and indicated that the organic part of the molecule of vitamin $B_{12}$ was not altered appreciably in either case. Beaven and Johnson suggested that the $B_{12}\text{r}$ of Boos, Carr, and Conn contained increased conjugation of double bonds, accompanied by the release of the benzimidazole from coordination. The statement that the two products differ in cobalt oxidation state is surprising since both titrimetric and polarographic data of both research groups indicate a reduction of cobalt(III) to cobalt(II) in the preparation of $B_{12}\text{r}$ from $B_{12}$.

Schindler\textsuperscript{17} performed chemical reductions with three different reductants. Both NaH$\text{SO}_3$ and Na$_2$S$_2$O$_4$ reduced $B_{12}$ to an orange color. However, the addition of zinc to an ammonium chloride solution of vitamin $B_{12}$ in a nitrogen atmosphere first gave a brown solution which later turned to blue. Grun and Haas\textsuperscript{18} measured the magnetic

\textsuperscript{15}Diehl and Murie, \textit{loc. cit.}


susceptibility of the blue material and suggested that the cobalt was divalent. They reported that the blue reduction product was very unstable, and decomposed even in a sealed tube in a nitrogen atmosphere.

E. Lester Smith and co-workers\textsuperscript{19} achieved a unique reduction of the cobalt atom while attempting alkaline hydrolysis of B\textsubscript{12}. When they heated a solution of B\textsubscript{12} in 0.1 M sodium hydroxide in the absence of air, the solution turned brown and finally to a greenish tint. This suggested reduction of the cobalt valency and simultaneous oxidation of some other part of the molecule. When air was admitted, the color reverted to red. The resulting product was crystallized, then studied. Its physical properties closely resembled those of B\textsubscript{12} but X-ray crystallography showed that it contained a lactam ring formed from an acetamide chain attached to one of the active carbon atoms. Therefore, the carbon atom must have been oxidized when cobalt was reduced.

STATEMENT OF PROBLEM

Up to now relatively little work has been concentrated on the reduction of vitamin B\textsubscript{12}, and the studies have often been contradictory. This research includes a thorough study of vitamin B\textsubscript{12} reduction by chromium(II) ion and constant potential electrolysis, and it clarifies some of the discrepancies reported by others.

Consideration was first given to a study of the anodic polarographic wave which appears on reduction of vitamin B\textsubscript{12}. Previous workers have attributed this wave to the oxidation of cobalt(II) in the reduced product to cobalt(III), and have ignored the fact that the cyanide group is released from the molecule on reduction.

Vitamin B\textsubscript{12} was reduced by amperometric titration with chromium(II) ion and constant potential electrolysis in 0.1 M EDTA, and qualitative and quantitative determinations of the cyanide released were made. It was unequivocally proven that the anodic wave was due to the oxidation of mercury in the presence of cyanide ions, and not to the reduced vitamin.

Further studies led to the discovery of a new anodic wave which was attributed to the oxidation of a new, highly reduced form of vitamin B\textsubscript{12}. The balance of the experimental work was directed to a study of this oxidation process. Even though there has been no previous report of a cobalt(II) oxidation wave, this research indicates
that the anodic wave is due to the oxidation of cobalt(I) in the highly reduced form of vitamin B$_{12}$ to cobalt(II).
EXPERIMENTAL

Reagents

The crystalline vitamin B₁₂ sample was purchased from the Nutritional Biochemical Corporation, Cleveland, Ohio. It was assayed spectrophotometrically according to the method of Cords and Ratycz.¹


The 0.1 M EDTA solution was prepared from reagent grade disodium salt of EDTA, purchased from the G. F. Smith Company, Columbus, Ohio. The pH of the solution was adjusted to the desired value by the addition of 0.1 M sodium hydroxide solution.

The standard solution of 0.1000 M chromium(II) ions in 0.1 M hydrochloric acid was prepared "determinately" from reagent grade potassium dichromate by the procedure of Lingane and Pecsok.² The chromium(II) solution was stored in contact with amalgamated zinc and in a hydrogen atmosphere. (See Figure 2) A potentiometric titration of an aliquot of a standard copper(II) solution was made with each chromium(II) solution as a check on its concentration. In cases where the two values differed, the standardized value was used in the calculations.

Figure 2
Microburet and Chromium(II) Storage Flask
The mercuric nitrate solution used in the cyanide analysis was prepared from reagent grade salt and was standardized by two different methods. First, it was titrated with standard EDTA solution using Eriochrome Black T indicator by the method of Schwarzenbach. Second, it was standardized by titrating amperometrically an aliquot of a standard cyanide solution. The cyanide solution had been previously standardized by the procedure given by Kolthoff and Sandell.

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In the major part of this research nitrogen gas was used for deaeration of the samples. Ordinary tank nitrogen was used, and at first it was purified only by passing it over hot copper. Later a chromium(II) solution in 1 M hydrochloric acid was added to the purification train to remove the last traces of oxygen.

In parts of this research tank hydrogen gas was used. The gas was purified by passing it through a Deoxo catalytic purifier manufactured by Baker and Company, Newark, New Jersey.

Tank argon gas was used for sample deaeration during the hydrogen gas analyses. No attempt was made to purify the argon. Any oxygen present would be reduced during the electrolysis of the sample, and a
correction was made for the small amount of hydrogen impurity present by running a blank determination on the mass spectrometer.

The developing solvent used in the descending paper chromatography experiments was water-saturated secondary butanol containing 1 per cent acetic acid. It was prepared according to instructions reported by Cords and Ratycz.5

5Cords and Ratycz, loc. cit.

The boric acid-sodium hydroxide buffer solutions were prepared by adding 9 M sodium hydroxide solution to 0.05 M boric acid solution to give the desired pH. This buffer system can be used in the pH range of 8-10 according to Kolthoff.6


**Apparatus**

All polarograms were recorded on chart paper with a Leeds-Northrup Electrochemograph, Type E. The polarizing voltage was standardized regularly, and the current response was frequently checked with a built-in standard resistor. The cell resistance was approximately 100 ohms, and since the current measured was only a few microamperes at most, the IR drop was negligible.

All reductions were performed in a polarographic cell of the usual H-type. One side of the cell contained a saturated calomel
electrode, and the other side was used as sample compartment. The two sides were separated by a sintered glass disc and a saturated potassium chloride-agar plug. A gas inlet was located near the bottom of the sample compartment. A tight-fitting rubber stopper was used to seal the sample compartment, since the reduced forms of B₁₂ are quite unstable in air. The stopper was fitted with a dropping mercury electrode, the microburet tip, and a mercury bubbler used as a gas outlet.

The microburet used in the vitamin B₁₂ titration consisted of three major parts. The barrel of the buret was a 0.25 milliliter hypodermic syringe. The plunger of the syringe was driven by a microdrive mechanism, model SB2, manufactured by the Micrometric Instrument Company, Cleveland, Ohio. The microdrive instrument was graduated directly down to 0.001 inch, and had a total displacement of one inch. One inch displacement corresponded to 0.19 milliliters delivered from the syringe. The buret tip was fashioned from a three-way microstopcock with a 1 millimeter bore. One arm of the stopcock was fitted with a female ground glass joint, ground to fit the taper of the syringe. The other horizontal arm was bent downward at a 90° angle, and the end was pulled out to form the fine buret tip, which was ground smooth with carborundum. The third arm of the stopcock was extended upward and connected to the chromium(II) reservoir. (See Figure 2)

The syringe plunger was lightly lubricated with Cello-Seal stopcock grease to help prevent the diffusion of oxygen into the chromium(II) solution. However, stopcock grease was not trusted in the
connection of the syringe and the tip assembly. For this, Kronig cement was used, and the joint was permanently cemented. Since the micro-stopcock-buret was connected rigidly at 3 different points (to the micro-titrationator, to the chromium(II) reservoir, and to the rubber stopper in the polarographic cell), the strain precluded the use of stopcock grease alone.

A different buret was used for the cyanide titration with standard mercuric nitrate solution. In this buret the tip consisted of a piece of flexible stainless steel capillary tubing about a foot long, fitted at one end with a hypodermic needle nub to match the standard syringe joint. The other end of the capillary tubing was passed through a short piece of glass capillary tubing (about 2 inches) and cemented tightly with epoxy cement. This was used to make an airtight seal between the buret tip and the rubber stopper in the polarographic cell. The barrel of the buret was another 0.25 milliliter syringe and the plunger was driven by the microdrive mechanism described above.

All vitamin B₁₂ samples were weighed on an air-damped Sartorius Microbalance. The weights were calibrated.

The spectrophotometric assay of vitamin B₁₂ was performed with a Beckman DU spectrophotometer and 1 centimeter silica cells. However, for scanning a complete spectrum, the Cary model 14 automatic recording spectrophotometer was used. For obtaining the spectra of the unstable B₁₂ reduction products, one of the silica cells was fitted with a rubber stopper containing two small glass tubes extending just to the bottom of the stopper, and to about 1 inch above it. To make it completely airtight the stopper was cemented into place.
The electrolysis potential was furnished by a 6 volt lead storage battery equipped with a 10 ohm, 50 watt potential divider. The mercury-pool cathode potential was monitored constantly with a Keithley model 610 electrometer, using the SCE as reference. The SCE was placed in the anode compartment of the polarographic cell rather than being positioned close to the mercury pool surface. This meant that the measured potential contained an iR component, as it was measured in the current path of the generator electrodes. Due to the small mercury surface and low concentration of vitamin B\textsubscript{12}, the current flow was small. This small current, coupled with the low cell resistance would lead to an iR value of 0.1 volts maximum. The diffusion plateau was sufficiently broad, however, to allow the electrolysis to proceed normally in spite of the uncertainty of the measured potential.

The hydrogen analyses were performed with a model 21-620 mass spectrometer, manufactured by the Consolidated Engineering Corporation, Pasadena, California.

**Amperometric Titration**

Vitamin B\textsubscript{12} was titrated amperometrically with 0.1 M chromium(II) ion in the presence of 0.1 M EDTA at pH 7.0 and pH 9.6. The method used was as follows: approximately a 5 milligram sample of B\textsubscript{12} was accurately weighed and placed in a polarographic cell. Then, 10.00 milliliters of 0.1 M EDTA pH 7.0 or 9.6 were added, and the cell was closed with the tight-fitting rubber stopper containing the dropping mercury electrode, the mercury bubbler, and the microburet. After 15
minutes of deaeration with purified nitrogen, a polarographic wave was recorded. For the titration, the chromium(II) solution was added in 0.0095 or 0.0190 milliliter portions, and the solution was stirred for about 2 minutes with the nitrogen stream. A complete polarographic wave was recorded after each addition of chromium(II).

The method just described gave good results for titrations involving only one equivalent of reduction per mole of vitamin B₄₂. However, when it was later discovered that a two-equivalent reduction could be obtained, the procedure had to be modified slightly to be of value. The changes are as follows: after deaeration of the sample solution, both the nitrogen inlet tube and exit tube were closed by means of a screw clamp over the rubber tubing placed as close as possible to the polarographic cell. Stirring was accomplished by moving a small teflon-coated stirring bar inserted in the polarographic cell before closing it. After each addition of titrant, a small horseshoe magnet was moved back and forth under the sample cell to effect thorough mixing prior to recording the polarographic wave.

Determination of $E_{1/2}^o$ of the Cr⁴⁺⁺ / Cr³⁺ Couple

The $E_{1/2}^o$ of the Cr⁴⁺⁺ / Cr³⁺ couple was determined in the following manner: ten milliliters of 0.1 M EDTA solution was placed in the polarographic cell, the rubber stopper containing the dropping mercury electrode, the mercury bubbler, and the microburet was placed tightly in the top of the cell, and the solution was deaerated for 15 minutes with purified nitrogen. Next, 0.10 milliliters of 0.1 M chromium(II) solution were added with the microburet, and the solution was stirred
with the nitrogen stream. The anodic chromium(II) wave was recorded first by applying the polarizing voltage in the negative direction, and then in the positive direction.

The chromium(II) in the cell was oxidized to chromium(III) by bubbling air through the cell. After this, the solution was again deoxygenated, and the cathodic chromium(III) wave was recorded in the same manner as above. Another 0.10 milliliters of 0.1 M chromium(II) solution were added to the previous solution, and a composite anodic-cathodic wave was recorded as above.

**Determination of Cyanide Released from B<sub>12</sub> on Reduction**

When vitamin B<sub>12</sub> was reduced with chromium(II) ion in 0.1 M EDTA at pH 7.0, cyanide was detected in the effluent nitrogen gas stream. One trap charged with 8.0 milliliters of 0.1 M sodium hydroxide solution and a second one charged with 4.0 milliliters were placed in series with the polarographic cell. Then, an amperometric titration of B<sub>12</sub> with chromium(II) was performed in EDTA at pH 7.0 in the usual manner. After the reduction was complete, a purified nitrogen stream was passed through the polarographic cell and the scrubbers at a rate of about two bubbles per second. The completeness of removal of the cyanide was checked periodically by scanning the anodic cyanide wave. The bubbling was continued for one hour, which was sufficient to lower the cyanide concentration in the B<sub>12</sub><sup>-</sup> solution below the limits of detectability.
The cyanide collected in the scrubbers was determined by an amperometric titration with a standard mercuric nitrate solution in the following manner: after placing the combined solutions in the polarographic cell, the cell was stoppered and the solution was deaerated 15 minutes with nitrogen. A polarographic wave was recorded. Next, the standard mercuric nitrate solution was added with the micro-buret in 0.019 milliliter increments, and a complete polarographic wave was recorded after each addition.

A check on the effectiveness of the removal of cyanide from a solution at pH 7.0 by bubbling nitrogen through was made by placing an aliquot of a standard cyanide solution in the polarographic cell. The sweeping, and subsequent analysis of the contents of the traps, performed in exactly the same manner used for the B12 sample described above, showed that 92 per cent of the initial cyanide was transferred to the traps.

In the cyanide titration, the electrical connection was made between the polarographic cell and the SCE by means of a saturated potassium nitrate-agar salt bridge. This was done to prevent diffusion of chloride ions into the cell during the titration with mercuric nitrate.

*Constant Potential Electrical Reduction*

The polarographic cell used for the electrical reduction contained a gas exit tube near the top of the sample compartment, in addition to the usual gas inlet tube. A mercury bubbler was attached to the gas exit tube with a short piece of gum rubber tubing. A
coiled platinum wire anode was placed in the reference electrode compartment, and a mercury pool about 5 millimeters in depth was placed in the sample compartment. Next, a weighed sample and 5.00 milliliters of 0.1 M EDTA of the desired pH were added. In a few cases where a more dilute solution was desired, 10.00 milliliters of EDTA were used. A small teflon-coated stirring bar was placed in the sample compartment, and the compartment was closed with a rubber stopper containing a dropping mercury electrode.

The sample solution was deaerated 15 minutes with purified nitrogen, and the gas inlet and exit tubes were closed tightly with a screw clamp. The desired potential was applied with the storage battery and voltage divider, and monitored constantly with the electrometer. The potential was kept constant by manual adjustment of the voltage divider.

The sample was stirred by moving the stirring bar with a small horseshoe magnet waved back and forth below the sample cell. In the first part of the research the magnet was moved by hand. Later, it was attached to the arm of a mechanical shaker. The electric shaker motor was equipped with a control for adjusting the rate of oscillation. This permitted a constant stirring rate and proved to be much more satisfactory.

The progress of the electrolysis was followed periodically by checking the polarographic wave.

In a few of the electrolyses, a milliammeter was connected in series in the electrolysis circuit, and the current flowing was recorded at intervals.
Internal Reduction

Vitamin B\textsubscript{12} was reduced by heating it in 0.1 M sodium hydroxide solution in the absence of air in the following manner: a trap consisting of a 50 milliliter erlenmeyer flask fitted with a gas inlet tube extending to the bottom and with a short gas outlet tube was inserted in the nitrogen line immediately in front of the polarographic cell. A 10 milliliter aliquot of a standard vitamin solution was placed in the trap. The solution in the trap and the polarographic cell were deaerated simultaneously with a stream of purified nitrogen. Next, the solution was heated to near boiling for about three minutes, and then cooled to room temperature with a water bath. The trap was then tipped up and some of the solution was forced into the polarographic cell. A polarogram was recorded.

An aliquot of the same standard B\textsubscript{12} solution was deaerated and a polarogram was recorded on the same chart.

Absorption Spectra

The spectrum of vitamin B\textsubscript{12} was obtained on a sample which had been weighed accurately and diluted to volume in a volumetric flask with distilled water. After thorough mixing, a portion of the sample was transferred to a 1 centimeter silica cell and a spectrum was recorded on the Cary spectrophotometer using an identical silica cell filled with distilled water as a reference.

Spectra of the reduction products were obtained in the following manner: the silica spectrophotometric cell fitted with the rubber stopper containing two glass tubes was connected in series in the
nitrogen gas stream just in front of the polarographic cell by means of gum rubber tubing. The $B_{12}$ sample and EDTA solution were placed in the polarographic cell and it was closed. The silica cell and the sample solution were deaerated simultaneously with purified nitrogen.

Next, the reduction, either electrical or by chromium(II) solution, was carried out in the manner previously described. After reduction was complete, the direction of nitrogen flow was reversed and some of the solution containing the reduced product was forced from the polarographic cell over into the spectrophotometric cell. The cell was sealed by closing a screw clamp over the rubber tubing, as close as possible to the glass tubes. The rubber tubing was then cut just above the screw clamp. A spectrum was recorded using an identical silica cell filled with distilled water as a reference. The cell proved to be sufficiently sealed to prevent oxidation during the time required for recording the spectrum.

Spectra of air-oxidized products were obtained as follows: the screw clamp was removed from the cell containing the reduced product, and air was passed through a hypodermic needle into the solution for 15 minutes. A spectrum was then recorded for the resulting solution.

**Paper Chromatography**

The paper chromatographic experiments were performed by the usual descending chromatographic technique. The solvent, water-saturated secondary butanol containing 1 per cent acetic acid, was prepared, and
the actual chromatograms were done according to the method of Cords and Ratycz.

7Cords and Ratycz, loc. cit.

The samples of $\text{B}_12$ in EDTA solution were reduced in the polarographic cell by the addition of solutions of chromium(II) ions by the method previously described. Three different products were obtained. The first product was brown, obtained by the addition of one equivalent of reductant per mole of vitamin. With a second sample, the brown product was first formed and then the cyanide was removed by bubbling nitrogen gas through the sample for one hour. From a third sample a green product was formed by the addition of two equivalents of reductant per mole of vitamin.

After obtaining the desired reduction products, the polarographic cells were opened and air was passed through the solutions until the samples were oxidized back to the red color.

The samples were then spotted on 3 MM Whatman chromatographic paper. Reference spots of a solution of vitamin $\text{B}_{12}$ in water were placed on each strip of paper. The strips of paper were hung in a chromatographic jar previously equilibrated with the developing solvent, and were allowed to run overnight.

**Decomposition Rate Study**

The green reduction product of vitamin $\text{B}_{12}$ formed by the addition of two equivalents of reductant per mole of vitamin, decomposed upon
standing. Its concentration was determined by measuring the anodic polarographic wave produced.

The reduced product was formed by constant potential electrolysis in the manner previously described. After all of the vitamin was reduced, the electrolysis circuit was disconnected, and the magnitude of the anodic current was measured with the polarograph at a potential of -0.6 volts versus the SCE. The solution was stirred intermittently, and the current was measured at 5 to 15 minute intervals for a period of about two hours.

Measurements were made in buffers in the pH range 7.0-9.6. All measurements were made in a constant temperature room. The maximum temperature variation was about one degree centigrade.

Hydrogen Analysis

The vitamin B_{12} sample was reduced by constant potential electrolysis to the green reduction product by the method previously described, with the exception that argon was used instead of nitrogen for sample deaeration. After reduction was complete, the magnitude of the anodic current was measured at -0.6 volts. The sample was allowed to stand a measured length of time, and the current was again checked at -0.6 volts. The cell was then opened to an evacuated, 50 milliliter gas sample bulb, and a gas sample was drawn into the bulb from above the solution.

The hydrogen analysis was performed as follows: the gas sample bulb was attached to the mass spectrometer inlet, and the bulb was immersed in liquid nitrogen. After most of the argon was condensed,
the stopcock was opened, and the gas sample was allowed to enter the instrument. The instrument sample pressure was noted, and the hydrogen band was recorded on chart paper. An instrument blank was run before and after running a sample.

A standard hydrogen sample was prepared by electrolyzing 0.1 M sulfuric acid in the polarographic cell for a known length of time at a known current level. It was sampled and analyzed in the same manner described above.

A blank determination with only the vitamin B₁₂ sample omitted was performed exactly as described above.

**Preparation of Pentacyanocobalt(III) Hydride**

Pentacyanocobalt(III) hydride was prepared by King and Winfield by the liquid phase hydrogenation of pentacyanocobalt(II) ion.⁸ They


reported an absorption spectrum for the compound. Griffith and Wilkinson showed the presence of the hydride by nuclear magnetic resonance.⁹

Pentacyanocobaltate(II) ion had been prepared and was thoroughly studied by Hume and Kolthoff.


In this research pentacyanocobalt(III) hydride was prepared from pentacyanocobaltate(II) ion by controlled potential electrolysis and by the addition of chromium(II) ion in the absence of air, in the following manner: a small trap was made from a test tube. It contained a gas inlet tube extending just to the bottom of the test tube. The gas exit tube was connected to the gas inlet tube of the polarographic cell with a short piece of pure gum rubber tubing.

To the polarographic cell were added 10.000 milliliters of 0.1 M EDTA pH 9.6, and 1.00 milliliter of 0.01 M cobalt(II) chloride solution. The trap contained 1.00 milliliter of 0.1 M potassium cyanide solution, and 1 drop of 0.1 M sodium hydroxide solution to prevent volatilization of the cyanide. Both solutions were deaerated simultaneously by passing purified nitrogen first through the trap and then through the polarographic cell. After 20 minutes the trap was tipped up and the deaerated potassium cyanide solution was forced over into the polarographic cell and mixed with the cobalt(II) chloride solution. Next, either a potential was applied or 0.15 milliliters of 0.1 M chromium(II) solution were added. The absorption spectrum of the resulting reduced material was then obtained in exactly the same
manner used for obtaining spectra for the reduced products of vitamin B₁₂ described previously.
RESULTS

Polarography

Vitamin B$_{12}$ forms a bright red solution and gives a single two-electron reduction wave with a diffusion current proportional to the vitamin concentration as reported previously. When polarograms of B$_{12}$ were recorded in this research the $E^*_2$ varied with the supporting electrolyte. (See Table 1)

Table 1

$E^*_2$ for Vitamin B$_{12}$ in Various Supporting Electrolytes

<table>
<thead>
<tr>
<th>Supporting Electrolyte</th>
<th>pH</th>
<th>$E^*_2$ (volts vs. SCE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 M EDTA</td>
<td>6.0</td>
<td>-1.00</td>
</tr>
<tr>
<td>0.1 M EDTA</td>
<td>7.0</td>
<td>-1.01</td>
</tr>
<tr>
<td>0.1 M EDTA</td>
<td>8.5</td>
<td>-1.03</td>
</tr>
<tr>
<td>0.1 M EDTA</td>
<td>9.6</td>
<td>-1.03</td>
</tr>
<tr>
<td>0.1 M NaOH</td>
<td>-</td>
<td>-1.07</td>
</tr>
<tr>
<td>0.1 M KCl</td>
<td>-</td>
<td>-1.09</td>
</tr>
<tr>
<td>0.05 M H$_3$BO$_3$- NaOH</td>
<td>8.1</td>
<td>-1.14</td>
</tr>
<tr>
<td>0.05 M H$_3$BO$_3$- NaOH</td>
<td>9.1</td>
<td>-1.11</td>
</tr>
<tr>
<td>0.05 M H$_3$BO$_3$- NaOH</td>
<td>9.5</td>
<td>-1.00</td>
</tr>
<tr>
<td>0.1 M tris* - HCl</td>
<td>7.0</td>
<td>-1.04</td>
</tr>
<tr>
<td>0.1 M tris* - HCl</td>
<td>8.1</td>
<td>-1.08</td>
</tr>
</tbody>
</table>

*tris(hydroxymethyl)aminomethane

A typical polarogram obtained for vitamin B$_{12}$ is shown in Figure 3.
Figure 3

Polarograms of Vitamin $B_{12}$, $B_{12r}$, and the Green Reduction Product in 0.1 M EDTA pH 9.6

1. Vitamin $B_{12}$
2. Vitamin $B_{12r}$
3. Green Reduction Product
Figure 3: Current (μA) vs. Volts vs. SCE
When vitamin B\textsubscript{12} was reduced by one equivalent per mole the red color changed to brown. This product was previously designated B\textsubscript{12r}. The reduction wave mentioned above dropped to one-half of its original height and the $E_0$ shifted to about -0.9 volts versus SCE. (See Figure 3) In addition, an anodic wave appeared. At pH 9.6 the anodic wave had an $E_0$ of -0.33 volts versus SCE, but at pH 7.0 it was shifted to a more positive potential and was practically coincident with the mercury oxidation wave. (See Figure 4) This anodic wave was due to the cyanide released from the vitamin on reduction, and not due to the oxidation of cobalt(II) as reported previously. Proof of this will be given later.

When vitamin B\textsubscript{12} was reduced by two equivalents per mole the color changed to green and the cathodic polarographic wave disappeared completely. In addition, to the anodic cyanide wave listed above, a true vitamin anodic wave appeared. (See Figure 3) Its $E_0$ was a constant -0.87 volts versus SCE in all supporting electrolytes used. The magnitude of the anodic wave was equal to the magnitude of the cathodic wave for B\textsubscript{12r} and was one-half of the magnitude of the original B\textsubscript{12} wave. The anodic wave was due to the oxidation of cobalt(I) to cobalt(II), as will be shown later.

A diffusion current constant for vitamin B\textsubscript{12} was measured using a capillary for which $\frac{m^2}{3^+1/6}$ was 2.27 mg$^{2/3}$sec$^{-1/2}$. Duplicate results gave a value of 1.35 $\mu$A/mg cc$^{-1}$sec$^{-1/2}$, or 1.76 $\mu$A/millimoles
Figure 4

Polarograms of Vitamin $B_{12}$ and $B_{12r}$ in 0.1 M EDTA pH 7.0

1. Residual current
2. Vitamin $B_{12}$
3. Vitamin $B_{12r}$
Figure 4

Volts vs. SCE

Current (µA)
liter⁻¹ mg²/₃ sec⁻¹/₂. This differs greatly from the value of 0.219 μA/mg cc⁻¹ sec⁻¹/₂ reported by Boos, Carr and Conn.¹

¹Boos, Carr and Conn, loc. cit.

Amperometric Titration

Vitamin B₁₂ was titrated with 0.1 M chromium(II) solution in the presence of 0.1 M EDTA at pH 7.0 and 9.6 as outlined previously, and the endpoint was located amperometrically. A series of polarographic waves obtained in a titration at pH 9.6 is shown in Figure 5. The current was measured from the polarographic wave for each increment of titrant added; at -1.35 volts when the titration was performed at pH 9.6, and -1.20 volts when the pH was 7.0. It can be seen in Figure 5 that on passing the endpoint the entire polarographic wave decreased in height. This resulted in a further anodic current in the region from -0.4 volts to -0.9 volts. The drop in cathodic current at -1.35 volts, however, was just equal to the increase in anodic current at -0.6 volts. The cathodic current measured at -1.35 volts was corrected for this, and for the residual current measured at -1.35 volts.

Data for an amperometric titration curve taken from the titration shown in Figure 5 by the procedure listed above are shown in Table 2. The titration curve constructed by plotting the corrected current versus volume of titrant is shown in Figure 6.
Figure 5

Polarograms Recorded for a Titration of Vitamin B\textsubscript{12} in 0.1 M EDTA pH 9.6 with Chromium(II) ion

<table>
<thead>
<tr>
<th>Wave</th>
<th>Volume of Cr\textsuperscript{II} (ml.) (residual current)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.0</td>
</tr>
<tr>
<td>2</td>
<td>0.0095</td>
</tr>
<tr>
<td>3</td>
<td>0.0190</td>
</tr>
<tr>
<td>4</td>
<td>0.0285</td>
</tr>
<tr>
<td>5</td>
<td>0.0380</td>
</tr>
<tr>
<td>6</td>
<td>0.0475</td>
</tr>
<tr>
<td>7</td>
<td>0.0570</td>
</tr>
<tr>
<td>8</td>
<td>0.0760</td>
</tr>
</tbody>
</table>
Figure 5 Volts vs. SCE
Figure 6

Amperometric Titration Curve for a Titration of Vitamin B$_{12}$
in 0.1 M EDTA pH 9.6 with Chromium(II)
Table 2

Amperometric Titration of Vitamin B₁₂ in 0.1 M EDTA pH 9.6 with Chromium(II) Ion

<table>
<thead>
<tr>
<th>Volume of Cr (milliliters)</th>
<th>Corrected Current (microamperes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.804</td>
</tr>
<tr>
<td>0.0095</td>
<td>0.679</td>
</tr>
<tr>
<td>0.0190</td>
<td>0.565</td>
</tr>
<tr>
<td>0.0285</td>
<td>0.440</td>
</tr>
<tr>
<td>0.0380</td>
<td>0.372</td>
</tr>
<tr>
<td>0.0475</td>
<td>0.363</td>
</tr>
<tr>
<td>0.0570</td>
<td>0.356</td>
</tr>
<tr>
<td>0.0760</td>
<td>0.354</td>
</tr>
</tbody>
</table>

In this titration, as in all amperometric titrations, the graph shows curvature near the equivalence point. Usually in an amperometric titration a few readings are taken well before the equivalence point, and sufficiently after it so that they lie on the linear portions of the curves. The endpoint is obtained then by extrapolating these linear branches to a point of intersection. In the titrations performed in this research the points farthest from the equivalence point were assumed to lie on the linear branches of the curve, and they were used in extrapolation to obtain the end point. (See Figure 6)

Several titrations were performed in 0.1 M EDTA at pH 9.6, and the end points were located by the procedure just described. A molecular weight was calculated for vitamin B₁₂ for each titration, based on a one-equivalent reduction. The results are shown in Table 3. Based on these results, the mean value for the molecular weight of B₁₂ was found to be 1300 ± 20.
**Table 3**

Molecular Weight of Vitamin B<sub>12</sub> Obtained by Amperometric Titration

<table>
<thead>
<tr>
<th>Sample Number</th>
<th>Corrected Weight (mg)</th>
<th>Volume of Cr&lt;sup&gt;+++&lt;/sup&gt; (ml)</th>
<th>Molecular Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>4.594</td>
<td>0.0378</td>
<td>1245</td>
</tr>
<tr>
<td>16</td>
<td>4.388</td>
<td>0.0346</td>
<td>1299</td>
</tr>
<tr>
<td>17</td>
<td>5.616</td>
<td>0.0462</td>
<td>1245</td>
</tr>
<tr>
<td>19</td>
<td>4.417</td>
<td>0.0334</td>
<td>1355</td>
</tr>
<tr>
<td>21</td>
<td>5.514</td>
<td>0.0416</td>
<td>1358</td>
</tr>
</tbody>
</table>
When B₁₂⁺ formed in the titrations above was exposed to air it immediately turned back to red, and the anodic cyanide wave disappeared. Boos, Carr and Conn used this as proof for their statement that the anodic wave was due to cobalt(II) being oxidized to cobalt (III) in the vitamin. They also made the statement that once a sample had been reduced and oxidized back, no end point could be reached on subsequent titration.

A B₁₂ sample weighing 4.842 milligrams (corrected for moisture) in 10.00 milliliters of 0.1 M EDTA pH 9.6 was titrated with 0.0877 M chromium(II) solution. After the titration was completed, air was bubbled through the solution for 30 minutes to effect oxidation. The sample was deaerated with purified nitrogen and retitrated. Polarograms recorded during the retitration are shown in Figure 7. Both amperometric titration curves are shown in Figure 8. The break in the second curve is just as sharp as the break in the first, and comes at almost the same point, in direct contradiction with the report of Boos, Carr and Conn. The molecular weight of the vitamin calculated from these end points was 1412 and 1384 respectively.

On retitration of the sample above the anodic cyanide wave returned in its original form. (See Figure 7) The anodic wave disappeared on oxidation because the cyanide present in the solution recombined with the cobalt. Since the cyanide is released from the vitamin molecule on reduction, its concentration is 0.37 millimolar.
Figure 7

Polarograms for Retitration of Vitamin $B_{12}$ in 0.1 M EDTA pH 9.6 with Chromium(II) Ion

<table>
<thead>
<tr>
<th>Wave</th>
<th>Volume of Cr$^{II+}$ (ml.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>(residual current)</td>
</tr>
<tr>
<td>2</td>
<td>0.0</td>
</tr>
<tr>
<td>3</td>
<td>0.0190</td>
</tr>
<tr>
<td>4</td>
<td>0.0380</td>
</tr>
<tr>
<td>5</td>
<td>0.0570</td>
</tr>
<tr>
<td>6</td>
<td>0.0760</td>
</tr>
<tr>
<td>7</td>
<td>0.0950</td>
</tr>
</tbody>
</table>
Figure 8
Amperometric Titration Curves for Titration of Vitamin B_{12} in 0.1 M EDTA with Chromium(II) Ion

a. Titration at pH 9.6
b. Retitration at pH 9.6
c. Titration at pH 7.0
d. Retitration at pH 7.0
Figure 8A
Volume of Cr\(^{3+}\) (mL)

Figure 8B
Volume of Cr\(^{3+}\) (mL)

Figure 8C
Volume of Cr\(^{3+}\) (mL)

Figure 8D
Volume of Cr\(^{3+}\) (mL)
in the solution listed above. At pH 9.6 the hydroxyl ion concentra-
tion is approximately 0.04 millimolar. It has been assumed in the
past that on oxidation of cobalt(II) to cobalt(III) in the molecule,
the sixth coordination position becomes occupied by a hydroxyl ion.
In view of the fact that cyanide forms a stronger bond with cobalt
than does hydroxyl ion, and that cyanide is present in 10 times the
concentration of hydroxyl ion, the cyanide should be taken up instead.
This is indeed indicated by the previous experiment.

In another experiment, 5.426 milligrams of vitamin B₁₂ were ti-
trated with 0.0677 M chromium(II) in 0.1 M EDTA pH 7.0. Cyanide was
then removed by bubbling a stream of purified nitrogen through the
solution for an hour. On oxidation, no cyanide was present, so the
sixth coordination position on the cobalt was occupied by a hydroxyl
group and vitamin B₁₂a was formed. After deaeration the B₁₂a was ti-
trated with the chromium(II) solution. (See Figure 9) The ampero-
metric titration curves for the B₁₂ titration, and for the B₁₂a ti-
tration, are shown in Figure 8.

Even though the end point break for the retitrination at pH 7.0 was
quite sharp, it did not agree with the first end point obtained, as
can be seen in Figure 8. The molecular weight of the vitamin, cal-
culated from the first curve, was 1302, while that weight calculated
from the second curve was 1628.

The B₁₂a formed in this experiment gave two one-electron re-
duction waves, with E₁₂ values of -0.10 volts and -0.95 volts versus
the SCE in 0.1 M EDTA at pH 7.0. Diehl and Murie prepared B₁₂a and
recorded polarograms of it in 0.1 M potassium sulfate supporting
**Figure 9**

Polarograms for Titration of Vitamin B\(_{12}\) in 0.1 M EDTA pH 7.0 with Chromium(II) Ion

<table>
<thead>
<tr>
<th>Wave</th>
<th>(Volume of Cr(^{3+}) (ml.))</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>(residual current)</td>
</tr>
<tr>
<td>2</td>
<td>0.0</td>
</tr>
<tr>
<td>3</td>
<td>0.0190</td>
</tr>
<tr>
<td>4</td>
<td>0.0380</td>
</tr>
<tr>
<td>5</td>
<td>0.0570</td>
</tr>
<tr>
<td>6</td>
<td>0.0760</td>
</tr>
<tr>
<td>7</td>
<td>0.0950</td>
</tr>
</tbody>
</table>
They reported \( E_2 \) values of -0.06 volts and -1.02 volts versus the SCE for the two waves. This is satisfactory agreement, considering that different supporting electrolytes were used. The value, -0.10 volts determined above, is only an approximate value, since the first wave of \( \text{B}_{12} \) is almost coincident with the mercury oxidation wave in EDTA at this pH.

When \( \text{B}_{12} \) was titrated, the anodic wave did not appear. This further indicated that the anodic wave was due to cyanide.

In the first part of this research all titrations were performed solely for the determination of the point at which one equivalent of reductant had been added per mole of vitamin \( \text{B}_{12} \). Later, when precautions were taken to exclude all possible traces of oxygen, it was discovered that a second end point could be obtained at the point where two equivalents of reductant had been added per mole of vitamin. Evidence of this second product had been seen from the beginning of the work; however, it was so unstable in the presence of traces of air that the anodic wave shown by this product was erroneously assumed to be due to excess chromium(II).

When two equivalents of chromium(II) ion were added per mole of vitamin \( \text{B}_{12} \) the solution turned dark green, and gave an anodic wave with an \( E_2 \) of -0.87 volts versus SCE as described in the previous section. Polarograms for a typical titration of vitamin \( \text{B}_{12} \) in 0.1 M

3Diehl and Murie, *loc. cit.*
EDTA pH 9.6 with chromium(II) ion to the second end point are shown in Figure 10.

Data were taken from the polarograms for the construction of an amperometric titration curve. The first end point was located by plotting the corrected current versus volume of titrant; the current was corrected in exactly the same manner as described previously. Since the final polarographic wave dropped slightly below the residual current wave at -1.35 volts, the former was used as zero in measuring the current for each polarogram. A plot was made of uncorrected current versus volume of titrant and the end point was taken as the point where the current dropped to zero. The titration curve showing both the first and second end points is shown in Figure 11.

The corrected sample weight was 5.974 milligrams. Using the first end point of 0.0479 milliliters, the molecular weight of the vitamin was calculated to be 1247. Using the second end point of 0.095 milliliters, the molecular weight was 1258. Using the difference between the first and second end points of 0.0471 milliliters the molecular weight obtained was 1268. The latter value should be more nearly correct than the first two, since any oxygen present in the solution will use up chromium(II) before the vitamin starts reacting. It is difficult to remove all traces of oxygen from the solution merely by bubbling purified nitrogen through it. From the data listed above, 0.0479 milliliters of chromium(II) were required for the first equivalent of reduction, while only 0.0471 milliliters were required for the second. The difference between the two, 0.0008 milliliters, is the volume of 0.1 M chromium(II) that would be required to reduce
Figure 10

Polarograms for a Titration of Vitamin B₁₂ in 0.1 M EDTA pH 9.6 with Chromium(II) Ion to the Second End Point

<table>
<thead>
<tr>
<th>Wave</th>
<th>Volume of Cr⁺⁺ (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.0</td>
</tr>
<tr>
<td>2</td>
<td>0.0095</td>
</tr>
<tr>
<td>3</td>
<td>0.0190</td>
</tr>
<tr>
<td>4</td>
<td>0.0380</td>
</tr>
<tr>
<td>5</td>
<td>0.0570</td>
</tr>
<tr>
<td>6</td>
<td>0.0760</td>
</tr>
<tr>
<td>7</td>
<td>0.0950</td>
</tr>
<tr>
<td>8</td>
<td>0.1140</td>
</tr>
<tr>
<td>9</td>
<td>0.1330</td>
</tr>
<tr>
<td>10</td>
<td>0.1520</td>
</tr>
<tr>
<td>11</td>
<td>0.1900</td>
</tr>
<tr>
<td>12</td>
<td>(residual current)</td>
</tr>
</tbody>
</table>
Figure 10

Volt vs. SCE

Current (µA)
Figure 11

Amperometric Titration of Vitamin B₁₂ in 0.1 M EDTA pH 9.6 with Chromium(II) Ion to the Second End Point
Figure 11  Volume of Cr\textsuperscript{3+} (ml.)

Current (\mu A)
the oxygen present in 10 milliliters of solution if the oxygen concentration were 0.002 millimolar. Assuming that the original oxygen concentration was about 0.2 millimolar, which is reasonable, this would still represent a decrease in oxygen concentration of 100 fold during the deaeration process. With such small samples being used, however, greater efficiency is needed for the deaeration process.

Another experiment was obtained to verify the reduced state of the green product. For this, the vitamin was first reduced to the green state by titrating it with 0.1 M chromium(II) in the presence of 0.1 M EDTA at pH 9.6. Then the reduced material was titrated with 0.1 M potassium ferricyanide. Curves for both the chromium(II) titration and the ferricyanide titration are shown in Figure 12. The current for the chromium(II) titration was measured at -1.35 volts in the usual way, whereas the current for the ferricyanide titration was measured at -0.6 volts and was corrected for residual current only. The two breaks shown in the ferricyanide case represent a one- and a two-equivalent oxidation, while the single break for chromium(II) represents a two-equivalent reduction.

An inspection of the curves in Figure 10 reveal that the two two-equivalent breaks do not come at the same place, as they should. The agreement, however, is satisfactory in view of the errors which may have been introduced through the use of unstandardized solutions, or by a small amount of oxygen leaking into the titration cell. An oxygen leak into the cell is a common source of error in this determination since the green product is rapidly oxidized by oxygen. The experiment served its purpose in further indicating that the formation
Figure 12

Amperometric Titration of Vitamin B₁₂ in 0.1 M EDTA pH 9.6 with 0.1 M Chromium(II) to the Green Product and Amperometric Titration of the Green Product with 0.1 M Potassium Ferricyanide
Figure 12: Volume of Titrant (mL) vs. Current (µA).
of the green product required two equivalents of reduction per mole of vitamin B<sub>12</sub>.

E<sub>1/2</sub> for the Cr<sup>+++</sup>/Cr<sup>++</sup> Couple

The halfwave potential for the Cr<sup>+++</sup>/Cr<sup>++</sup> couple was determined in 0.1 M EDTA at pH 9.6 in the manner previously described. A 1.0 millimolar solution of chromium(III) gave a single one-electron reduction wave with an E<sub>1/2</sub> of -1.34 volts versus the SCE. This wave corresponds to the reduction of chromium(III) to chromium(II).

When 1.0 millimolar chromium(II) was polarographed it gave a single one-electron anodic wave with an E<sub>1/2</sub> of -1.33 volts versus the SCE. This wave was due to the oxidation of chromium(II) to chromium (III). When the solution contained both 1.0 millimolar chromium(III) and 1.0 millimolar chromium(II) it gave a composite anodic-cathodic polarographic wave with an E<sub>1/2</sub> of -1.33 volts versus the SCE.

The chromium(III) reduction wave is irreversible and is somewhat drawn out, while the chromium(II) oxidation wave tends to be more sharp and reversible. This accounts for the difference in E<sub>1/2</sub> values obtained.

The polarograms were recorded on chart paper by the Electrochemograph, and the E<sub>1/2</sub> values were measured directly from the charts. The polarograms were recorded in the usual manner by allowing the polarizing voltage to change in the negative direction. As a precaution, all of the polarograms were repeated with the polarizing voltage changing in the positive direction. The E<sub>1/2</sub> values determined in this
manner were identical to the values obtained above, indicating that instrument lag was negligible.

**Determination of Cyanide Released from Vitamin B₁₂ on Reduction**

A 4.809 milligram sample of vitamin B₁₂ in 0.1 M EDTA at pH 7.0 was reduced to B₁₂⁺ in the polarographic cell by the addition of 0.1 M chromium(II) solution, and the cyanide released from the vitamin was swept into 0.1 M sodium hydroxide solution, in the manner previously described.

The sodium hydroxide solution containing the cyanide was transferred to a polarographic cell, and after deaeration, was titrated amperometrically with 0.0531 M mercuric nitrate solution. A complete polarographic wave was recorded after each addition of titrant, as in the vitamin titrations. The polarograms recorded in the titration listed above are shown in Figure 13.

The cyanide trapped in the scrubbers was identified by the polarographic wave it produced. The E_d,e. value cannot be used for identification purposes, since it changes with cyanide concentration. Newman, Cabral, and Hume⁴ defined a term, E_f, characteristic of the cyanide wave, by the equation:

$$E_{d,e} = E_f + 0.030 \log \left( \frac{i_d - 1}{i_e} \right)$$

where E_d,e. is the potential of the dropping mercury electrode, i_d is

Figure 13
Polarograms Recorded in the Titration of Cyanide Released from Vitamin B\textsubscript{12} on Reduction with Mercuric Nitrate Solution

<table>
<thead>
<tr>
<th>Wave</th>
<th>Volume of Hg(NO\textsubscript{3})\textsubscript{2} (ml.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>(residual current)</td>
</tr>
<tr>
<td>2</td>
<td>0.0</td>
</tr>
<tr>
<td>3</td>
<td>0.019</td>
</tr>
<tr>
<td>4</td>
<td>0.038</td>
</tr>
<tr>
<td>5</td>
<td>0.057</td>
</tr>
<tr>
<td>6</td>
<td>0.076</td>
</tr>
<tr>
<td>7</td>
<td>0.095</td>
</tr>
<tr>
<td>8</td>
<td>0.114</td>
</tr>
</tbody>
</table>
Figure 13 Volts vs. SCE
the diffusion current, and $i$ is the average current flowing through the cell for each $E_{d,e}$. The $E_f$ of the cyanide wave obtained here was $-0.286$ volts versus the SCE. This is in excellent agreement with the $E_f$ of $-0.284$ volts versus the SCE reported by Newman, Cabral, and Hume, as an average value for the anodic mercury wave in the presence of cyanide at pH's above 10.

The current was measured at $-0.20$ volts versus the SCE for each polarogram shown in Figure 13, and each value was corrected for the residual current. An amperometric titration curve constructed from these data is shown in Figure 14. Using the end point from the graph, and using 1300 as the equivalent weight of the vitamin, a calculation showed that 96 per cent of the cyanide present in the sample was transferred to the trap.

As stated previously, when an aliquot of a standard cyanide solution in 0.1 M EDTA at pH 7.0 was placed in a polarographic cell and subjected to analysis according to the above procedure 92 per cent of the cyanide was recovered.

The $E_f$ value calculated for the anodic wave obtained during vitamin reduction in EDTA at pH 9.6 in the titration mixture was $-0.33$ volts versus the SCE. Polarograms observed for solutions prepared by adding pure potassium cyanide to 0.1 M EDTA at pH 9.6 gave $E_f$ values of $-0.34$ to $-0.37$ volts versus the SCE, in agreement with the value listed above.

The results presented here, coupled with the previously stated fact that the anodic wave disappeared when cyanide was removed from
Figure 14

Amperometric Titration Curve for the Titration of Cyanide Released from Vitamin B₁₂ on Reduction with Mercuric Nitrate Solution
Figure 14: Volume of Hg(NO₃)₂ (ml.) vs. Current (μA)
the titration mixture, show conclusively that the anodic wave was due to cyanide and not due to the oxidation of cobalt(II) claimed by Boos, Carr, and Conn.\(^5\) The disappearance of the anodic wave on air oxidation of the reduced vitamin, used as evidence by Boos, Carr, and Conn for their claim, has already been shown to be due to cyanide uptake by the vitamin on oxidation.

Constant Potential Electrical Reduction

At the start of this research vitamin $\text{B}_12$ was titrated amperometrically with chromium(II) ion and the end point showed that one equivalent of reduction was obtained per mole of vitamin. This seemed rather incongruous since the polarograms of vitamin $\text{B}_12$ showed only one reduction wave, and it was due to a two-equivalent reduction of cobalt(III) to cobalt(I). At this point it was decided to employ controlled potential electrolysis with a potential value on the diffusion current plateau, and attempt a two-equivalent electrical reduction. The apparatus and techniques used have been described previously.

A vitamin $\text{B}_12$ sample weighing 9.15 milligrams was dissolved in 5.00 milliliters of 0.1 M EDTA at pH 9.6, and was reduced at the mercury pool cathode at a potential of $-1.40$ volts versus the SCE for 45 minutes. The bright red color of the vitamin changed first to brown and finally to green. The course of the electrolysis was
followed polarographically. The two-electron reduction wave, having an $E'_d$ of -1.03 volts versus the SCE, decreased during the electrolysis and finally disappeared completely upon formation of the green color. This indicates that the green product is most probably the product of reduction at the dropping mercury electrode during the polarography of vitamin B$_{12}$.

Two anodic polarographic waves appeared during the electrolysis. The first one was formed during the first phase of the electrolysis while the brown product was forming, and was due to the oxidation of mercury in the presence of cyanide released from vitamin B$_{12}$ on reduction. This wave is discussed in detail in another section. The second anodic wave appeared during the formation of the green product. It had an $E'_d$ of -0.87 volts versus the SCE, and appeared to be caused by a one-electron oxidation of the green product. To check on this the cathode potential was adjusted to -0.60 volts versus the SCE, a value on the anodic diffusion current plateau, and the electrolysis was continued for 40 minutes. In controlled potential electrolysis a potential which lies on an anodic diffusion current plateau will bring about an oxidation of the active substance at the electrode surface, in the same manner as a potential on a cathodic diffusion plateau brings about a reduction. During the course of electrolysis, at -0.60 volts the green color changed back to brown.

Polarograms for the original B$_{12}$, the green reduction product, and the final brown oxidation product are shown in Figure 15. It can be seen that the polarogram for the green reduction product is
Figure 15

Polarograms of Vitamin B$_{12}$, Green Reduction Product Formed by Electroreduction of Vitamin B$_{12}$, and Vitamin B$_{12}$* Formed by Electrooxidation of the Green Product

1. Vitamin B$_{12}$

2. The green reduction product formed by constant potential electroreduction of vitamin B$_{12}$ at -1.40 volts

3. The brown product formed by constant potential electrooxidation of the green product at -0.60 volts
identical to the polarogram for the green product obtained by a two-equivalent reduction of B$_{12}$ with chromium(II) ion shown in Figures 3 and 10. The polarogram for the brown oxidation product is identical to the polarogram for B$_{12}$, formed by the addition of one equivalent of chromium(II) per mole of vitamin shown in Figures 3 and 5.

In the experiment just described coulometry was used to determine the actual amount of electricity required to reduce the vitamin B$_{12}$ sample to the green product, and to oxidize the green product back to the brown product. A microammeter of high sensitivity was placed in series in the electrolysis circuit, and the current flowing through the cell was recorded periodically. A plot of current versus time was made for each electrolysis, and the number of coulombs, used were measured by approximating the area under the curve. For the electrolysis at -1.40 volts approximately 1.52 coulombs of electricity were consumed. This corresponded to $15.7 \times 10^{-6}$ equivalents of reduction. Using 1300 as the molecular weight of the vitamin, the sample contained $7.04 \times 10^{-6}$ moles, or $14.1 \times 10^{-6}$ equivalents based on a two-electron reduction. From these results it was concluded that vitamin B$_{12}$ was reduced by two equivalents per mole, and that the current efficiency was 90 per cent.

From the current-time plot for electrolysis at -0.60 volts it was determined that approximately 0.52 coulombs of electricity were used in the oxidation of the green product. This corresponded to $5.4 \times 10^{-6}$ equivalents of oxidation. From this it was concluded that a one-equivalent oxidation per mole of B$_{12}$ occurred. From these data
only $5.4 \times 10^{-6}$ equivalents of electricity appeared to have oxidized
7.04 $\times 10^{-6}$ equivalents of vitamin $B_{12}$, which indicates a current ef-

ciency of 130 per cent. This apparent high efficiency, and the low
efficiency observed in the first step are to be expected, since the
highly reduced green product is unstable and decomposes chemically,
i.e., without passage of current, to the brown product on standing.
The rate of decomposition was studied and is reported in another
section.

From the coulometric and polarographic data obtained here it was
concluded that cobalt(III) is reduced to cobalt(I) in the vitamin on
electrolysis at $-1.40$ volts versus the SCE, and that cobalt(I) is
oxidized to cobalt(II) at $-0.60$ volts versus the SCE. The anodic
polarographic wave produced by the green reduction product had an $E_0$
of $-0.87$ volts versus the SCE, and was due to the oxidation of
cobalt(I) to cobalt(II) in the vitamin. There has been no previous
report of a cobalt(I) oxidation wave.

**Internal Reduction**

Smith and co-workers reported that vitamin $B_{12}$ could be inter-

mally reduced by heating a solution of $B_{12}$ in $0.1 \, M$ sodium hydroxide
in the absence of air.$^6$ They reported that the color first turned

$^6$Smith et al., loc. cit.

brown and finally to green. When the reduction was performed in this
research according to the procedure outlined earlier, the solution
turned brown, but it did not turn green. Polarograms for vitamin B\textsubscript{12} and for the brown reduction product obtained in 0.1 M sodium hydroxide are shown in Figure 16.

The polarogram for the brown product gave an anodic wave with an \( E^a \) of -0.32 volts versus the SCE and a cathodic wave with an \( E^c \) of about -0.9 volts versus the SCE. The polarographic wave is almost identical to the B\textsubscript{12} wave shown in Figure 3. Furthermore, the anodic wave obtained here is identical to the cyanide wave obtained for the scrubber solution in the cyanide determination shown in Figure 13.

It was concluded that vitamin B\textsubscript{12} was produced without the addition of an external reducing agent simply by heating vitamin B\textsubscript{12} in 0.1 M sodium hydroxide in the absence of air, and that cyanide was released from the B\textsubscript{12} molecule on formation of B\textsubscript{12r} as observed previously when the vitamin was reduced with chromium(II) ions or by constant potential electrolysis. Vitamin B\textsubscript{12} contains five active carbon atoms, any one of which could serve as a reducing agent for such a reaction. Smith and co-workers showed by X-ray crystallography that the final product after internal reduction under these conditions contained a lactam ring.\textsuperscript{7} This indicates that one of the active carbon atoms had indeed been oxidized and had undergone ring closure with one of the acetamide groups.
Figure 16

Polarograms of Vitamin B_{12} and Vitamin B_{12r} formed by internal reduction in 0.1 M Sodium Hydroxide

1. Vitamin B_{12}

2. Brown reduction product

3. Residual current
Figure 16
Absorption Spectra

Absorption spectra obtained for vitamin B12, for the brown reduction product, for the highly reduced green product, and for the air-oxidized product are shown in Figure 17. The vitamin B12 solution contained 5.10 milligrams of the vitamin in 100.0 milliliters of distilled water. The brown product was obtained by adding 0.1 M chromium(II) solution to a deaerated solution of the vitamin containing 5.10 milligrams of vitamin per 100.0 milliliters of distilled water. The green product was formed by adding 0.1 M chromium(II) solution to a deaerated solution of 0.1 M EDTA pH 9.6 containing 5.10 milligrams of vitamin per 100.0 milliliters of solution. The red air-oxidized product was obtained by bubbling air through the brown reduction product.

The absorbance was measured from the recorded chart for each peak, and absorption coefficients were calculated. Since one centimeter cells were used, and the concentration of the vitamin was expressed as per cent by weight, the absorption coefficient was expressed by the term E(1%, 1 cm). Corrections were made for the absorption of the reagent.

The absorption peaks and absorption coefficients obtained for vitamin B12, the brown product, and the green product are shown in Tables 4, 5, and 6 respectively.
Figure 17

Absorption Spectra of Vitamin B\textsubscript{12}, B\textsubscript{12\_red}, the Green Reduction Product, and the Air-Oxidized Product
Figure 17 Wavelength (millimicrons)
<table>
<thead>
<tr>
<th>Table 4</th>
<th>Absorption Spectrum of Vitamin B₁₂ in Water</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absorption Peak (millimicrons)</td>
<td>E(1%, 1 cm)</td>
</tr>
<tr>
<td>278</td>
<td>110</td>
</tr>
<tr>
<td>360</td>
<td>204</td>
</tr>
<tr>
<td>550</td>
<td>63</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Table 5</th>
<th>Absorption Spectrum of B₁₂ in Water</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absorption Peak (millimicrons)</td>
<td>E(1%, 1 cm)</td>
</tr>
<tr>
<td>312</td>
<td>191</td>
</tr>
<tr>
<td>405</td>
<td>41</td>
</tr>
<tr>
<td>473</td>
<td>62</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Table 6</th>
<th>Absorption Spectrum of the Green Reduction Product in Water</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absorption Peak (millimicrons)</td>
<td>E(1%, 1 cm)</td>
</tr>
<tr>
<td>383</td>
<td>234</td>
</tr>
<tr>
<td>455</td>
<td>22</td>
</tr>
<tr>
<td>553</td>
<td>25</td>
</tr>
</tbody>
</table>
The absorption spectrum obtained for the brown product was in agreement with that reported by Diehl and Murie for \( \text{B}_{12} \) which they formed by the reduction of vitamin \( \text{B}_{12} \) with hydrogen gas in the presence of platinum.\(^8\) They were unsuccessful in their attempt to measure the amount of hydrogen used in the reduction; however, by a ferricyanide titration they determined that cobalt was present in \( \text{B}_{12} \) in the divalent state. This is in agreement with our finding that the brown product was formed by the addition of one equivalent of reductant per mole of vitamin \( \text{B}_{12} \).

The absorption spectrum obtained for the green product was identical to a spectrum reported by Boos, Carr, and Conn for a product formed by the reduction of vitamin \( \text{B}_{12} \) with chromium(II) ion in 0.1 M EDTA pH 9.5\(^9\). They also called their product \( \text{B}_{12} \), and stated that it was formed by a one-equivalent reduction per mole of vitamin \( \text{B}_{12} \).\(^{10}\)

Amperometric titration of vitamin \( \text{B}_{12} \) with chromium(II), amperometric titration of the green product with ferricyanide, and coulometry have all shown that two equivalents of reduction per mole of vitamin are required to form the green product.
Beaven and Johnson reported absorption spectra for vitamin B\(_{12}\), B\(_{12r}\), and the green reduction product which are identical to the ones obtained in this research.\(^{11}\) (See Figure 17) They discussed the discrepancy between the spectrum of B\(_{12r}\) reported by Diehl and Murie, and that reported by Boos, Carr, and Conn. According to Beaven and Johnson, the B\(_{12r}\) of Boos, Carr, and Conn contained cobalt in a more highly reduced state than the B\(_{12r}\) of Diehl and Murie, in agreement with our findings. They further stated that the B\(_{12r}\) of Boos, Carr, and Conn contained increased conjugation of double bonds, and that the benzimidazole was released from conjugation, but that the organic part of the molecule was not altered appreciably in either of the reduced products.

Schindler reported the formation of a blue reduction product by the reaction of zinc with vitamin B\(_{12}\) in an ammonium chloride solution under a nitrogen atmosphere.\(^{12}\) Perhaps his blue product was the same as the green product obtained here. He reported that the blue product was extremely unstable and that he was unable to obtain a characteristic absorption spectrum. However, the instability indicates a similarity to the green product obtained in this research.

\(^{11}\) Beaven and Johnson, loc. cit.

\(^{12}\) Schindler, loc. cit.
When the brown and the green reduction products were brought into contact with air both were rapidly changed back to the original bright red color. Absorption spectra for the two air-oxidized products were identical, although the spectra for the reduced products are quite different. (See Figure 17) The absorption spectrum for the product obtained by air-oxidation of the brown reduction product is shown in Figure 17. Data taken from this wave are shown in Table 7.

<table>
<thead>
<tr>
<th>Absorption Peak (millimicrons)</th>
<th>E(1%, 1 cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>273</td>
<td>118</td>
</tr>
<tr>
<td>350</td>
<td>178</td>
</tr>
<tr>
<td>523</td>
<td>51</td>
</tr>
</tbody>
</table>

It can be seen in Figure 17 that the absorption spectrum of the air-oxidized product is similar to the spectrum of vitamin $B_{12}$, but the peaks of the oxidized product are shifted to slightly shorter wavelengths.

When a sample of vitamin $B_{12}$ was reduced internally by heating it in the absence of air in 0.1 M sodium hydroxide and then air-oxidized back to the red color, it gave an absorption spectrum which was identical to that of the original vitamin. According to Smith and co-workers, heating vitamin $B_{12}$ in 0.1 M sodium hydroxide in the absence of air produces a lactam which has chemical and physical
properties identical to those of vitamin B₁₂. The lactam was called dehydrovitamin.

Paper Chromatography

Four vitamin B₁₂ reduction products were subjected to air-oxidation and were chromatographed as outlined previously to determine whether the reduction and oxidation altered the organic portion of the molecule. An $R_c$ value was calculated for each substance, instead of the usual $R_f$ value. $R_c$ is defined as the ratio of the distance moved by the spot in question to the distance moved by a spot of vitamin B₁₂ which was used as a reference on each chromatogram. The $R_c$ values obtained are listed in Table 5.

Table 5

Paper Chromatography of Air-Oxidized Reduction Products

<table>
<thead>
<tr>
<th>Sample</th>
<th>$R_c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin B₁₂</td>
<td>1.00</td>
</tr>
<tr>
<td>Air-oxidized brown product (cyanide not removed)</td>
<td>1.03</td>
</tr>
<tr>
<td>Air-oxidized brown product (after cyanide removal)</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>0.4 - 0.8</td>
</tr>
<tr>
<td></td>
<td>1.03</td>
</tr>
<tr>
<td>Air-oxidized green product (cyanide not removed)</td>
<td>1.02</td>
</tr>
<tr>
<td>Air-oxidized brown product formed by heating B₁₂ in 0.1 M NaOH</td>
<td>1.05</td>
</tr>
</tbody>
</table>
For purposes of comparison, $R_c$ values for some compounds closely related to vitamin $B_{12}$ reported by Cords and Ratycz are listed in Table 9.\textsuperscript{14}

\textsuperscript{14}Cords and Ratycz, \emph{loc. cit.}

### Table 9

\textbf{Literature $R_c$ Values for Some Vitamin $B_{12}$ Related Compounds}

<table>
<thead>
<tr>
<th>Substance</th>
<th>$R_c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydroxocobalamin ($B_{12a}$)</td>
<td>0.4 - 0.6</td>
</tr>
<tr>
<td>Dehydrovitamin</td>
<td>1.05</td>
</tr>
<tr>
<td>Factor B</td>
<td>1.1</td>
</tr>
<tr>
<td>Cyanocobalamin—monocarboxylic acid</td>
<td>1.26</td>
</tr>
<tr>
<td></td>
<td>1.4</td>
</tr>
<tr>
<td></td>
<td>1.8</td>
</tr>
<tr>
<td>Cyanocobalamin—dicarboxylic acid</td>
<td>1.7</td>
</tr>
<tr>
<td></td>
<td>2.1</td>
</tr>
</tbody>
</table>

Dehydrovitamin is the vitamin $B_{12}$ molecule containing a lactam ring, which has been previously discussed. Factor B is the porphyrin-like portion of the molecule which remains intact after removal of the nucleotide by hydrolysis. The carboxylic acids result from hydrolysis of the amide groups and subsequent loss of ammonia.

The $R_c$ value for the product obtained when vitamin $B_{12}$ was heated in 0.1 sodium hydroxide in the absence of air and then air-oxidized is identical to the literature $R_c$ value for dehydrovitamin listed above.
This is in agreement with the report of Smith and co-workers that a lactam results from this treatment.15

15Smith et al., loc. cit.

The $R_c$ values for the air-oxidized brown product and the air-oxidized green product fall between the $R_c$ values for vitamin $B_{12}$ and the dehydrovitamin. The values are quite different from the values for factor $B$ and for the carboxylic acids indicating that hydrolysis did not take place during the reaction. At most, the vitamin molecule was converted to dehydrovitamin by the reaction. Thus, reduction and air-oxidation did not appreciably alter the organic portion of the molecule. Furthermore, when the brown product and the green product were oxidized without removing the cyanide released on reduction, the resulting product was not $B_{12a}$ (hydroxocobalamin) but rather a cyano compound. This is in agreement with the statement made in a previous section that on oxidation of a vitamin reduction product the cyanide reoccupies the sixth coordination position on the cobalt.

When the cyanide was removed from the brown product prior to oxidation a mixture of products resulted on oxidation. The chromatogram contained one rather drawn-out spot which had an $R_c$ range of 0.4 - 0.8 (measured from the front to the back of the spot). This was presumably due to $B_{12a}$ (hydroxocobalamin), which should be the product formed. Polarographic data reported in another section indicated that the product was $B_{12a}$. In addition to this spot, though, the chromatogram showed two other minor spots; one with an $R_c$ value of 1.03 which
indicated that some vitamin B₁₂ or dehydrovitamin had been formed, and
one with an $R_f$ value of 0.3 which was not identified.

In another experiment an $R_f$ value was determined for a solution
of vitamin B₁₂ in water. The value was 0.24, in agreement with the
$R_f$ value of 0.25 reported by Cords and Ratycz.¹⁶ When solutions of

¹⁶ Cords and Ratycz, loc. cit.

vitamin B₁₂ in 0.1 M sodium hydroxide and in 0.1 M EDTA pH 7.0 and 9.6
were chromatographed, the $R_f$ values obtained were identical to the $R_f$
value for vitamin B₁₂ in water, indicating that the electrolyte did
not alter the spot movement rate.

**Decomposition Rate Study**

The highly reduced green product of vitamin B₁₂ was quite un-
stable and decomposed even in the absence of air. It was observed
that hydrogen gas was given off during the decomposition. The de-
composition was studied in several buffers in the pH range 7.0 to 9.6
to determine the effect of hydrogen ion on the decomposition rate.

The green product was prepared by controlled potential electrol-
ysis and the concentration change was followed polarographically by
the technique previously described.

The pH of the solution increased during the course of the de-
composition. The total amount of increase depended upon the extent of
the reaction and upon the effectiveness of the buffer system. It
varied from a change of 0.26 pH units for two hours standing at pH
7.0 in 0.1 M EDTA, down to a change of only 0.03 pH units for two hours standing in 0.1 M EDTA at pH 9.6. The increase is further evidence that hydrogen ion is a reactant in the decomposition reaction.

Data obtained in the decomposition study are shown in Table 10. Graphs with the per cent decomposition plotted against time are shown in Figure 18. The pH value listed for each electrolyte listed in Table 10 is an average of the pH measured before the reaction was started and immediately after it was stopped.

A slope was measured from each curve at the 60 minute point. A graph of \( \log \left( \frac{1}{G} \frac{dG}{dt} \right) \) plotted versus pH was made and is shown in Figure 19. In the term above \( [G] \) is the concentration of the highly reduced green product at 60 minutes, and \( \frac{dG}{dt} \) is the slope of the decomposition curve at 60 minutes. It can be seen in Figure 19 that above pH 8.6 a straight line with a slope of approximately unity was obtained. This indicates that the decomposition was first order with respect to hydrogen ion.

At the lower pH's studied the points fell below the straight line in Figure 19. The exact reason for this is unknown. However, at the 60 minute time picked for the plot the green product was 60-80 per cent decomposed at the lower pH's. A shorter time was not picked due to the inaccuracy in measuring the slope on the sharply rising portion of the curve. The experiments were satisfactory though, since they did show the pH dependence of the decomposition reaction.
Table 10

Decomposition of the Green Reduction Product with Time in Various Buffers

<table>
<thead>
<tr>
<th>Time (minutes)</th>
<th>0.1 M EDTA pH 7.18</th>
<th>0.1 M *tris-HCl pH 7.12</th>
<th>0.1 M *tris-HCl pH 8.11</th>
<th>0.1 M EDTA pH 8.60</th>
<th>0.05 M H$_3$BO$_3$-NaOH pH 9.18</th>
<th>0.1 M EDTA pH 9.46</th>
<th>0.05 M H$_3$BO$_3$-NaOH pH 9.64</th>
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<td>5</td>
<td>7.9</td>
<td>13.1</td>
<td>8.0</td>
<td>6.0</td>
<td>3.7</td>
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<td>10</td>
<td>15.5</td>
<td>23.6</td>
<td>14.6</td>
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<tr>
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<td>21.4</td>
<td>32.7</td>
<td>19.6</td>
<td>--</td>
<td>9.9</td>
<td>6.8</td>
<td>5.6</td>
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<tr>
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<td>27.3</td>
<td>40.5</td>
<td>26.2</td>
<td>18.5</td>
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<tr>
<td>25</td>
<td>32.8</td>
<td>46.9</td>
<td>31.1</td>
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<td>53.4</td>
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<td>26.6</td>
<td>17.5</td>
<td>12.3</td>
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<td>55</td>
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<tr>
<td>60</td>
<td>61.6</td>
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<td>54.1</td>
<td>45.9</td>
<td>29.7</td>
<td>20.2</td>
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<td>--</td>
</tr>
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<td>75</td>
<td>70.8</td>
<td>88.8</td>
<td>60.4</td>
<td>54.5</td>
<td>35.8</td>
<td>24.3</td>
<td>18.8</td>
</tr>
<tr>
<td>90</td>
<td>78.4</td>
<td>95.6</td>
<td>66.1</td>
<td>62.2</td>
<td>41.4</td>
<td>28.1</td>
<td>21.7</td>
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<tr>
<td>105</td>
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<td>71.9</td>
<td>68.8</td>
<td>46.2</td>
<td>31.2</td>
<td>25.1</td>
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<tr>
<td>120</td>
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<td>--</td>
<td>76.3</td>
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<td>50.6</td>
<td>33.8</td>
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<td>--</td>
<td>--</td>
<td>33.0</td>
</tr>
</tbody>
</table>

*tris(hydroxymethyl) aminomethane.
Figure 18
Decomposition of the Green Product with Time in Various Buffers

<table>
<thead>
<tr>
<th>Curve</th>
<th>Buffer</th>
<th>pH</th>
<th>$C_{B12}$ (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>0.05M tris*</td>
<td>7.02 - 7.22</td>
<td>1.38</td>
</tr>
<tr>
<td>2.</td>
<td>0.1 M EDTA</td>
<td>7.06 - 7.32</td>
<td>1.38</td>
</tr>
<tr>
<td>3.</td>
<td>0.05M tris*</td>
<td>8.07 - 8.15</td>
<td>1.40</td>
</tr>
<tr>
<td>4.</td>
<td>0.1 M EDTA</td>
<td>8.53 - 8.67</td>
<td>1.25</td>
</tr>
<tr>
<td>5.</td>
<td>0.05M $\text{H}_3\text{BO}_3-\text{NaOH}$</td>
<td>9.13 - 9.23</td>
<td>1.38</td>
</tr>
<tr>
<td>6.</td>
<td>0.1 M EDTA</td>
<td>9.45 - 9.48</td>
<td>1.32</td>
</tr>
<tr>
<td>7.</td>
<td>0.05M $\text{H}_3\text{BO}_3-\text{NaOH}$</td>
<td>9.60 - 9.68</td>
<td>1.00</td>
</tr>
</tbody>
</table>

*tris(hydroxymethyl)aminomethane
Figure 18
Figure 19

Plot of \( \log \left( \frac{1}{G} \frac{dG}{dt} \right) \) Versus pH for Decomposition of the Green Reduction Product of Vitamin B\(_{12}\) with Time in Various Buffers.
Figure 19
Hydrogen Analysis

The highly reduced green product, prepared in 0.1 M EDTA at pH 7.0 and 9.6 by controlled potential electrolysis, was allowed to stand for a period of time and the amount of hydrogen produced was determined with the mass spectrometer as outlined previously. The instrument gave a broad band for hydrogen in the low mass region with a height proportional to the hydrogen concentration. Four typical instrument charts obtained in these determinations are shown in Figure 20.

The height of the hydrogen band measured from the instrument chart was corrected for instrument background, i.e. the small residual hydrogen band shown by the instrument when no sample was present, and for a blank prepared and analyzed in exactly the same manner used for the samples. Data obtained in this manner are shown in Table 11.

Table 11
Hydrogen Analysis After Decomposition of the Green Reduction Product

<table>
<thead>
<tr>
<th>pH</th>
<th>Time of Standing (hours)</th>
<th>Micromoles Decomposed</th>
<th>Micromoles H2 Found</th>
</tr>
</thead>
<tbody>
<tr>
<td>9.6</td>
<td>1</td>
<td>1.12</td>
<td>0.56</td>
</tr>
<tr>
<td>9.6</td>
<td>1</td>
<td>1.49</td>
<td>0.70</td>
</tr>
<tr>
<td>9.6</td>
<td>2 1/2</td>
<td>2.64</td>
<td>1.13</td>
</tr>
<tr>
<td>7.0</td>
<td>1 3/4</td>
<td>4.04</td>
<td>2.95</td>
</tr>
<tr>
<td>9.6</td>
<td>(oxidized at -0.6 volts for 45 minutes)</td>
<td>7.70</td>
<td>0.60</td>
</tr>
<tr>
<td>9.6</td>
<td>(oxidized at -0.6 volts for 40 minutes)</td>
<td>7.04</td>
<td>0.47</td>
</tr>
</tbody>
</table>
Figure 20

Mass Spectrometer Charts Showing the Hydrogen Band

a. Instrument background

b. Hydrogen produced in the electrolysis of 0.1 N sulfuric acid for 50 seconds at 5 milliamperes

c. Hydrogen produced by the green reduction product on standing 2 1/2 hours at pH 9.6

d. Hydrogen produced by the green reduction product on standing 1 3/4 hours at pH 7.0
Figure 20
When the highly reduced green product decomposed the result was
the brown reduction product. Since the green product was formed by a
two-electron reduction per mole of vitamin B₁₂, the decomposition
represents a one-electron oxidation of the green product. Inspection
of data given in the first part of Table 11 shows that approximately
one-half of a mole of hydrogen gas was formed per mole of the green
product which decomposed. It was shown in an earlier section that the
decomposition reaction was first order with respect to hydrogen ion.

The green product formed from the last two samples listed in
Table 11 was completely oxidized to the brown product by controlled
potential electrolysis at -0.6 volts versus the SCE. Inspection of
the data shows that a relatively small amount of hydrogen gas was
produced during the electrolysis. This small amount of hydrogen gas
was formed in a side reaction by chemical decomposition in the solu-
tion while the electrolysis was in progress. In fact, it was this
chemical decomposition which led to the apparent high current effi-
ciency shown by coulometric data reported in another section.

The chemical reaction occurring during the decomposition was most
probably:

\[ \text{RCo(I)} + \text{H}^+ \rightarrow \text{RCo(II)} + \text{H}_2 \]

where R stands for the rest of the vitamin B₁₂ molecule.

Pentacyanocobalt(III) Hydride

The anodic polarographic wave with an \( E^* \) of -0.87 volts versus
the SCE shown by the green reduction product was attributed to the
oxidation of cobalt(I) to cobalt(II). Hume and Kolthoff reported that
they had prepared pentacyanocobalt(II) ion by reduction of pentacyano-
cobalt(II) ion with sodium amalgam, and that the cobalt(I) complex did
not show a polarographic wave. Later, King and Winfield reported

15 Hume and Kolthoff, loc. cit.

that they had prepared pentacyanocobalt(III) hydride by liquid phase
hydrogenation of pentacyanocobalt(II) ion. From the discussion of

16 King and Winfield, loc. cit.

King and Winfield it appears that the reduction product obtained by
Hume and Kolthoff was actually the cobalt(III) hydride rather than
the expected cobalt(I) complex.

In the present research there appeared to be two possible expla-
nations for the anodic polarographic wave. One possible explanation
was that the green product formed by a one-electron reduction of
cobalt(II) in B12 was a cobalt(III) hydride, and in this case the
anodic wave observed would be due to oxidation of the hydride. A
second possibility was that the cobalt(I) compound could have been
formed, and the anodic wave observed would then be due to its oxi-
dation back to cobalt(II). Results obtained in this research showed
the latter possibility to be true.

The pentacyanocobalt(III) hydride was prepared by reduction of
the pentacyanocobalt(II) ion by controlled potential electrolysis and
by reduction with chromium(II) ion as described previously. This
compound had not been prepared, prior to this research, by either of these methods. Polarograms of the pentacyanocobalt(II) ion and of the pentacyanocobalt(III) hydride formed by controlled potential electrical reduction are shown in Figure 21. The absorption spectrum for pentacyanocobalt(III) hydride formed by reduction with chromium(II) is shown in Figure 22.

The cobalt(III) hydride was identified by its absorption spectrum. The spectrum showed a single absorption peak at 305 millimicrons, in excellent agreement with the spectrum reported by King and Winfield.\textsuperscript{17}

\textsuperscript{17}Ibid.

They reported a molar absorption coefficient of 610 for the 305 millimicron peak. A value of 608 was calculated from the peak shown in Figure 22.

It can be seen in Figure 21 that the cobalt(III) hydride did not give a polarographic wave. Therefore, the reduction product of Hume and Kolthoff, in all probability, was the cobalt(III) hydride and not the cobalt(I) complex as believed.
Figure 21

Polarograms of Pentacyanocobalt(II) Ion and Pentacyanocobalt(III) Hydride, in 0.1 M EDTA pH 9.6

1. Pentacyanocobalt(II) ion

2. Pentacyanocobalt(III) hydride
Figure 21 Volts vs. SCE
Figure 22
Absorption Spectrum of Pentacyanocobalt(III) Hydride
DISCUSSION OF RESULTS

Anodic Cyanide Wave

When vitamin B\textsubscript{12} was reduced by one equivalent per mole the red color changed to brown and an anodic polarographic wave appeared with an $E_{1/2}$ of -0.33 volts versus the SCE in 0.1 M EDTA pH 9.6. This wave had been attributed to the oxidation of cobalt(II) in the reduced product to cobalt(III) by Boos, Carr, and Conn\textsuperscript{1}, an interpretation that is now found to have been in error.

A cyanide analysis showed that the cyanide group had been quantitatively released as the cyanide ion from the vitamin B\textsubscript{12} molecule on reduction. It was observed that the characteristics of the anodic wave were identical to those of a wave produced by a solution of pure potassium cyanide, and that the anodic wave disappeared on removal of the cyanide ion from the titration mixture. This shows that the anodic wave was due to the oxidation of mercury in the presence of cyanide ion and not due to the oxidation of cobalt(II) as claimed.

Green Reduction Product

When vitamin B\textsubscript{12} was reduced by two equivalents per mole the color changed to green, and a true vitamin anodic polarographic wave
appeared. The anodic wave had an E₂ of -0.87 volts versus the SCE, which was independent of both the supporting electrolyte and pH.

Amperometric titration of vitamin B₁₂ with chromium(II) ion, and coulometric measurement during controlled potential electrolysis of vitamin B₁₂ showed that two equivalents of reductant per mole of vitamin B₁₂ were required to form the green product. The first equivalent of reductant per mole of vitamin B₁₂ reduced cobalt(III) to cobalt(II) and formed the brown reduction product, B₁₂⁻. Thus, the green product was formed by a one equivalent reduction of B₁₂⁻.

Conversely, coulometric measurement during controlled potential oxidation of the green product, and amperometric titration of the green product with ferricyanide showed that one mole of the green product yielded vitamin B₁₂ upon reaction with one equivalent of oxidant.

Paper chromatographic experiments and absorption spectra showed that formation of the green product and its oxidation did not alter the organic portion of the molecule. Also, Beaven and Johnson reported that the cobalt was more highly reduced in the green product than in the brown product². The reduction, therefore, affected the cobalt atom, and not the organic part of the molecule of vitamin B₁₂.

When cobalt(II) in vitamin B₁₂ is reduced in a one electron step, the process may yield (1) cobalt(I) in a straightforward reduction of the cobalt atom, or (2) a complex cobalt hydride, analogous to the

²Beaven and Johnson, loc. cit.
pentacyanocobaltate hydride ion discovered by King and Winfield.\(^3\)

\(^3\)King and Winfield, \textit{loc. cit.}

All of the experimental results have been considered in an effort to select the more probable product from these two possibilities.

Since the electrooxidation of the green product gives one electron, the process could be one of the following:

\[ \text{RCO(I)} \rightarrow \text{RCO(II)} + e \quad (a) \]
\[ \text{RCO(III)H} + \text{OH}^- \rightarrow \text{RCO(II)} + \text{H}_2\text{O} + e \quad (b) \]

It was concluded that (a) is the electrode reaction because the \( E^\circ \) for the oxidation was found to be pH independent, and the presence of \( \text{OH}^- \) in reaction (b) indicates that if it were the reaction, the \( E^\circ \) of the anodic wave would change with changing pH. It should also be noted that the pentacyanocobaltate ion is not oxidizable at the dropping mercury electrode. While this fact does not rule out the possibility that (b) takes place, it renders it less likely.

Hume and Kolthoff reported that cobalt(I) was not electrooxidizable at the dropping mercury electrode.\(^4\) In light of the present knowledge, we believe that this reference and all other references to the existence of cobalt(I), with one exception,\(^5\) involve cobalt(III)

\(^4\)Hume and Kolthoff, \textit{loc. cit.}

hydride rather than the cobalt(I) compound as reported. In the ex-
ception, Cotton and Wilkinson reported that the only known cobalt(I)
complexes were formed by the reaction of cobalt carbonyls with
isonitriles and subsequent disproportionation to cobalt(I) and co-
balt(-1) according to the reaction:
\[
\text{Co}_2(\text{CO})_8 + 5 \text{RNC} \rightarrow \left[\text{Co}(\text{CNR})_5\right]^+ \left[\text{Co}(\text{CO})_4\right]^+ + 4 \text{CO}
\]
The polarographic behavior of the complex was not reported.

The chemical decomposition reaction which yields hydrogen could
be formulated
\[
\text{RCO(I)} \xrightarrow{H^+} \text{RCO(II)} + \frac{1}{2} \text{H}_2
\]

or
\[
\text{RCO(III)H} \rightarrow \text{RCO(II)} + \frac{1}{2} \text{H}_2
\]
Alternative (c) is accepted because it shows a dependence on hydrogen
ion, which was found to be first order at pH values above 8.5. If re-
action (d) is an intramolecular oxidation-reduction reaction, as
written, its rate should be independent of hydrogen ion concentration,
contrary to the experimental finding. If reaction (d) consists of
steps, one or more of which do depend on hydrogen in concentration,
for example,
\[
\text{RCO(III)H} \xrightarrow{H^+} \text{RCO(II)} + \text{H}_2
\]
some other steps must be written to account for the observation that
the final product is not RCo(III), (the unreduced vitamin), but
RCo(II), (vitamin $B_{12r}$). A possibility is:
\[
\text{RCO(III)H} \xrightarrow{\text{fast}} \text{RCo(II)} + \text{RCo(III)} + \frac{1}{2} \text{H}_2
\]
This reaction does not, however, reduce the amount of RCo(III) product, and would predict a final mixture of RCo(II) and unreduced vitamin upon standing, which is not found. The reaction of RCo(III) with water to give RCo(II) and oxygen is ruled out, because the vitamin is known to be stable in aqueous solution. Thus, no decomposition reaction involving RCo(III)H fits the experimental results as well as the cobalt(I) reaction given above.

To compare the vitamin with pentacyanocobaltate(III) ion in this respect, it should be noted that the latter was formed by a reversible, un-catalyzed, liquid phase hydrogenation by King and Winfield. Vitamin B₁₂, in aqueous solution, underwent no change upon treatment with hydrogen for 48 hours. Thus, there is no analogy, between the vitamin and the pentacyanocobalt(III) hydride with respect to hydrogenation, the reverse of the hydrogen evolution reaction.

On the basis of the pH independence of the anodic polarographic wave and the pH dependence of the rate of decomposition of the highly reduced form of the vitamin, it must therefore be concluded that the material most likely contains cobalt in the plus-one oxidation state, and that, although there is some analogy between the pentacyanocobaltate ion and the vitamin, the reduced form of the vitamin does not contain a cobalt-hydrogen bond.

The green reduction product could not be prepared in a solution of high enough concentration to apply nuclear magnetic resonance spectroscopy to the study of this substance. Griffith and
Wilkinson have obtained the N. M. R. spectrum for the pentacyanocobaltate ion, and report the cobalt hydride band at 695 cycles per second (positive, versus proton resonance of water at 25° C.). If the problem of preparation of a sufficient quantity of the green reduction product in a solution concentrated enough could be solved, it would be of considerable interest to examine the N. M. R. spectrum of it. This experiment would give important confirmation of the results obtained by purely chemical means in the present research.
BIBLIOGRAPHY


I, Stanford Lee Tackett, was born near Virgie, Kentucky, September 5, 1930. I received my elementary education in the public schools of Pike County, Kentucky, and McDermott, Ohio. I was graduated from McDermott High School, Ohio, in 1949, and enrolled in the College of Arts and Sciences at Ohio State University, Columbus, Ohio, in September, 1949. In August, 1950, I enlisted in the U. S. Air Force and served four years, one of which was spent in Saudi Arabia. Upon my discharge in August, 1954, I returned to Ohio State University where I received the degree of Bachelor of Science in 1957. I immediately enrolled in the Graduate School of Ohio State University. While at Ohio State I held the following positions: Assistant in the Chemistry Department in 1957, 1958, and 1959, Assistant Instructor, Analytical Division, 1959-1960, DuPont Teaching Fellow, 1960-1961, Instructor of Chemistry, 1961-1962.

I have accepted a position as Assistant Professor of Chemistry at Arizona State University.