1953

Chemical studies on vitamin B12

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NOTE TO USERS

CHEMICAL STUDIES ON VITAMIN B_{12}

by

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A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of
The Requirements for the Degree of
DOCTOR OF PHILOSOPHY

Major Subject: Physiological and Nutritional Chemistry

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Iowa State College
1953

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I. INTRODUCTION

The chemical nature of vitamin B_{12}, a material of extraordinary pharmacological potency, has not as yet been elucidated. The molecule of this vitamin is so large that some uncertainty even exists in its empirical formula as based on the most careful ultimate analyses. Fragments amounting to about one-third of the whole have been stripped from the molecule by digestion with hydrochloric acid and have been identified. Some information about their linkage to each other and to the whole has been secured. The metal cobalt, and the acid radical cyanide, have been shown to be present, occurrences which are unique among biochemical materials. The major portion of the molecule, however, that part bearing the metal, is a material of remarkable stability, resistant to the usual methods of attack, and after four years of effort by numerous investigators still largely unknown. The significance of vitamin B_{12} in medicine and nutrition alone is ample to justify intensive research to unravel its chemistry; the unexpected composition and unique chemical behavior present a challenging problem worthy of the best intellectual and scientific effort.

The work presented in this thesis is a further investigation of the fragments of vitamin B_{12} produced by hydrochloric acid hydrolysis. The quantities of these

fragments and their chemical and physical characterization constitute the bulk of the work. Certain reactions of the intact molecule of the vitamin were investigated in detail, as they bore on the problems of structure and composition. In particular, the catalytic activity of the vitamin in oxidation reactions was studied.

II. HISTORICAL

The presence of an anti-pernicious anemia factor in whole liver extracts has been known since 1926 (1). The isolation of the pure factor proved to be an exceptionally difficult task and twenty-two years elapsed before the factor, now known as vitamin B_{12}, was isolated (2, 3). It was necessary to follow each step in the isolation by the slow, erratic and expensive clinical response of pernicious anemia patients. The factor proved to be rather inert chemically and to be present in liver in only minute quantities. Following the discovery of a microbiological method of assay (4), the isolation and crystallization of the factor was rapidly achieved by chemists of Merck and Company in New Jersey.

At present the vitamin is obtained from fermentation broths of S. griseus and S. aureofaciens, rather than by the lengthy and cumbersome isolation from liver.

In addition to its use in the treatment of Addisonian pernicious anemia (5), vitamin B_{12} has also been found beneficial in the treatment of sprue and nutritional macrocytic anemia. It is known also to be identical with the so-called "animal protein factor", that is, a growth-promoting substance involved in animal nutrition. Evidence has also been presented that it may be effective in treatment of chronic allergic bronchitis (6).

Vitamin B_{12} appears to be involved in a variety of physiological functions. It is believed to be involved in transmethylation. There appears to be an interrelationship between the folic acid and the B_{12} requirements of organisms. It is possibly implicated in purine and pyrimidine syntheses. There is evidence that ascorbic acid may aid in B_{12} utilization.

Vitamin B_{12} is one of the most potent physiological materials known; as little as a microgram a day may be sufficient in the treatment of anemia. It is also one of the least toxic.

The formula of vitamin B_{12} is approximately $C_{61-64}H_{86-92}N_{14}O_{13}PCo$, the minimum molecular weight being about 1300 (7). It is soluble in water. It crystallizes from a water-acetone mixture in birefringent crystals which fail to melt up to 300° but darken around 210° to 220°. The material is l-rotatory with a specific rotation of $[\alpha]_{6563}^{23} = -59 \pm 9°$ (7). In aqueous solution it shows absorption maxima at 278 mμ ($E_{1\ cm.}^{1\%} = 115$), 361 mμ ($E_{1\ cm.}^{1\%} = 204$) and 550 mμ ($E_{1\ cm.}^{1\%} = 63$).

A related material, vitamin B_{12a}, may be obtained from vitamin B_{12} by catalytic hydrogenation (8). Vitamin B_{12a} may also be prepared by illumination of an acidic, aqueous solution of B_{12} (9). It is believed that B_{12a} differs from B_{12} only by the replacement of the cyanide group of B_{12} (10) by a hydroxyl group (11).

Other B_{12} analogues have been prepared by replacement of cyanide by various anions such as nitrite, sulfate, chloride and cyanate (11, 12). Because of the various analogues possible, it was suggested that the name cobalamin be assigned to all of the B_{12} molecule excepting the cyanide (11, 12). Then the compounds could be referred to by Werner nomenclature as cyano-cobalamin, nitrito-cobalamin, hydroxo-cobalamin, etc. Such compounds have high biological activity, although not usually as high as that of B_{12} itself.

The presence of cyanide in B_{12} was discovered during an oxidation of the vitamin in dilute sulfuric acid with potassium permanganate (10). By alkaline permanganate oxidation, eight organic acids have been isolated, four of which were identified (13). With these exceptions, the most informative studies have been by acid hydrolysis.

The acid hydrolysis of B_{12} yields a number of small, or relatively small fragments, as well as the red acid fragment which amounts to approximately two-thirds of the molecule and still contains cobalt. Ammonia, 1-amino-2-propanol and three benzimidazole compounds have been isolated and identified in varying amounts depending on the conditions for the hydrolysis.

A "ninhydrin-reacting " product was reported from the acid hydrolysis of B_{12} (14). It was first char-

acterized as 2-aminopropanol (15, 16), but it was later shown to be 1-amino-2-propanol (17). The actual amount of this product has been reported as both one and two moles (18, 19).

Three components arising from the acid hydrolysis of B_{12} have been reported and referred to as the α, β and γ components (19). The γ component was shown to be 5,6-dimethylbenzimidazole (21, 22). The β component was shown to be 1-α-D-ribofuranosido-5,6-dimethylbenzimidazole (23) and the α component to be a phosphate ester of the ribose portion of the β component (24). The actual position of the phosphate on the ribose portion is not definitely known yet, but it has been established that it is on the second or third carbon atom. The α component predominates under relatively mild hydrolytic conditions, while the γ component is obtained only with strong acid at elevated temperatures.

Ammonia is produced on hydrolysis or hydrogenation as reported by several workers (13, 16, 18).

The large skeletal portion of the molecule which remains on mild acid hydrolysis is very much a mystery. It is known to be acidic and to contain cobalt (14). It forms a purple reaction product with cyanide in alkaline solution (25). Other than this, little information as to its properties or constitution has been reported.

III. EXPERIMENTAL WORK

A. The Distribution of Nitrogen in Vitamin B_{12}

1. Introduction.

A definitive allocation of the nitrogen atoms present in the molecule of vitamin B_{12} has not yet been reported. The total number of nitrogen atoms, as determined by ultimate analyses, has been definitely established as fourteen (7). Vitamin B_{12a}, in which cyanide has been replaced by hydroxyl, has one less nitrogen atom in the molecule, that is, a total of thirteen, and we confirm this figure by ultimate analysis.

Of the fragments isolated from the products of hydrochloric acid hydrolysis of B_{12}, five contain nitrogen: that is, 5,6-dimethylbenzimidazole (20, 21), ammonia (16), 1-amino-2-propanol (17, 18, 19, 26), a red acidic, cobalt-bearing fragment (14), and cyanide (10). The benzimidazole accounts for two of the nitrogen atoms. A precise determination of the amount of ammonia has not been made, although it has been reported that the ammonia corresponds to five to six atoms of nitrogen (13). Five acid amide groups have been reported (27); these would yield ammonia on hydrochloric acid hydrolysis so that the number of ammonia atoms produced is probably five. The number of 1-amino-2-propanol groups in the B_{12} molecule has been contradictorily reported as two (18) and one (19).

The red acid fragment has not been purified and crystallized and no ultimate analyses of it are available; accordingly its nitrogen content is not known. It is quite definite that only one cyanide group is present. In summary then, the current knowledge of the nitrogen chemistry of vitamin B_{12} shows:

5,6-Dimethylbenzimidazole	2 nitrogen atoms
Ammonia from acid amide	5 or 6
1-Amino-2-propanol	2 or 1
Red acid fragment	?
Cyanide	1
Total (known)	14

By the present work, the uncertainties in this distribution have been removed. The final experiment was actually performed on vitamin B_{12a}, inasmuch as preliminary work showed that competing reactions of the cyanide group during the hydrolysis introduced a degree of uncertainty into the interpretation of the results. That is to say, the cyanide is expelled in part as hydrogen cyanide, hydrolyzed in part to ammonium formate, and oxidized in part to carbon dioxide. By using vitamin B_{12a}, such ambiguity was avoided.

The experiment, in brief, consisted of hydrolyzing a carefully purified and analyzed specimen of B_{12a} with hydrochloric acid in a stream of oxygen-and carbon

dioxide-free nitrogen and collecting any carbon dioxide liberated in the hydrolysis. The hydrolysis mixture was then subjected to a Craig countercurrent distribution between butanol and 1.0 N hydrochloric acid; this separated the various components of the mixture. The material from each tube of the Craig apparatus was then analyzed for benzimidazole, red fragment, cobalt, total nitrogen, and ammonia nitrogen.

2. Experimental.

a. Apparatus and materials. Crystalline vitamin B_{12} obtained from the Squibb Institute for Medical Research, New Brunswick, New Jersey, was recrystallized from water. This material was then converted to vitamin B_{12a} by hydrogenation over a platinum catalyst and oxidation of the resulting B_{12a} (8). The B_{12a} was then crystallized from an acetone-water mixture. The final material was crystalline and of a deep red color. Found: H_2O (80°, vacuum, 4 hrs.), 8.95% (by J. Ellingboe), 8.53% (by E. W. D. Huffman), 9.14% (by J. Alicino); Cobalt: 4.24, 4.30% (colorimetric, by J. Ellingboe), 4.26% (colorimetric, by J. Brierly) 4.42% (residue of $CoSO_4$ by J. Alicino); Nitrogen: 13.03, 13.20, 13.48% (Dumas by E. W. D. Huffman), 13.37% (Dumas by J. Alicino), ave.: 13.27%. Ratio of nitrogen to cobalt (using colorimetric cobalt value) 13.0 to 1.

The standard solution of 1-amino-2-propanol used

for the determination of distribution coefficient and steam-volatility was obtained from Eastman Kodak Company, practical grade. The material was freshly distilled and collected at 159°-160°.

The hydrolysis apparatus consisted of a 200 ml., round bottom flask provided with a gas-inlet tube sealed into the side of the vessel and a reflux condenser. Tank nitrogen was introduced into the system through a train consisting of vanadous sulfate (28), a U-tube containing magnesium perchlorate and a tube containing Ascarite. From the top of the condenser the exiting gases passed through a trap containing silver nitrate, a U-tube containing magnesium perchlorate, and a Turner bulb packed with Ascarite.

The countercurrent distribution was performed in a Craig all-glass apparatus employing equilibrated solutions of 1 N hydrochloric acid and iso-butanol.

All spectrophotometric data were obtained with a Beckman DU spectrophotometer.

b. Hydrolysis procedure and carbon dioxide determination. A carefully weighed amount of B_{12a}, 501.3 mg., containing 8.95% moisture, was dissolved in 50.00 ml. of water in the hydrolysis flask. Two 0.10 ml. aliquots were taken for cobalt and spectrophotometric analyses respectively. Nitrogen, free of carbon dioxide and oxygen, was swept through the system for 3 hours,

at which time the Turner bulb was removed and weighed. The Turner bulb was replaced and 4.8 ml. of 11.6 M hydrochloric acid was added to the solution to give a final hydrochloric acid concentration of approximately 1 N. The solution was then heated at 100° for 22 hours by means of an oil bath. Nitrogen was swept through the system continuously during this period.

The Turner bulb was removed and weighed and the hydrolysis solution quantitatively transferred to a 100 ml. volumetric flask; the solution was then diluted to volume with water. Three 1.00 ml. aliquots of this solution were taken for cobalt analyses and three 1.00 ml. aliquots taken for determination of ammonia nitrogen. The solution, now equivalent to 427.3 mg. B_{12a}, was then transferred to an evaporating dish and evaporated to dryness in a vacuum desiccator over magnesium perchlorate and sodium hydroxide pellets.

c. Countercurrent distribution. The dry residue was dissolved as completely as possible in 1 N hydrochloric acid which had previously been equilibrated with iso-butanol. All of the material would not dissolve in the amount of lower layer required to fill tube zero of the Craig apparatus to the pour-off point (31 ml.), so the remaining red material was dissolved in equilibrated iso-butanol, which constitutes the upper layer of the system, and this was then introduced to tube zero. The

vessel which had contained the residue was finally rinsed with 5 ml. of lower layer and this was introduced to tube 1 of the apparatus. Forty transfers were then performed.

The tubes were drained into correspondingly numbered 100 ml. volumetric flasks, each tube rinsed with 25 ml. of ethanol and the solutions diluted to volume with water. The addition of the25 ml. of ethanol to each flask resulted in a completely homogeneous solution.

d. Analysis of tubes.

(i) Spectrophotometric. The contents of each flask were read at 278 mμ and 346 mμ. The absorption peak for the benzimidazole moiety is at 278 mμ, and the red acid fragment absorbs characteristically at 346 mμ. A plot of the distribution at two wavelengths is shown in Fig. 1 and the actual data are given in Table 1.

(ii) Analyses for total nitrogen. Aliquots of the 100 ml. representing each tube of the Craig distribution were digested in the usual kjeldahl manner. The Kjeldahl ammonia was determined by titration of a 7 minute steam-distillate from alkaline solution. The distillate was collected in 5 ml. of 4 percent boric acid containing 2 drops of methylene blue-methyl red indicator and titrated with standard hydrochloric acid. The hydrochloric acid was standardized by distillation of standard

Fig. 1.

Distribution of the Red Acid Fragment and Benzimidazole as Determined by Spectrophotometric Analysis.

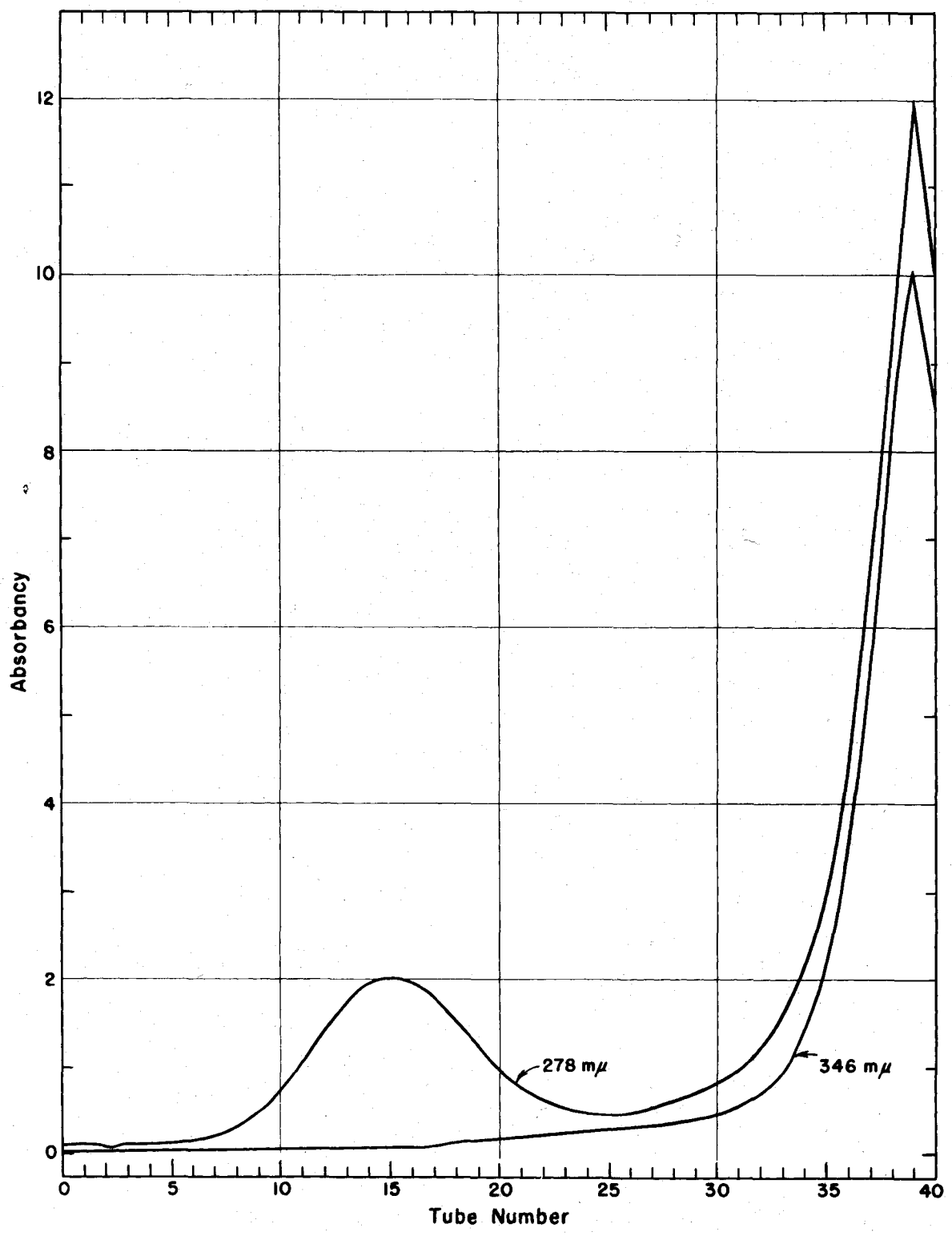

12
10
8
6
4
2
0
Absorbancy
0
5
10
15
20
25
30
35
40
Tube Number
278 mμ
346 mμ

Table 1

Distribution of Benzimidazole and Red Acid Fragment in B_{12a} Hydrolysate from Absorbancy Determinations[a]

Tube No.	$A_{278\ m\mu}$[b]	% of Total	$A_{346\ m\mu}$[c]	% of Total
0	0.073	0.095	0.033	0.071
1	0.065	0.085	0.034	0.073
2	0.046	0.061	0.036	0.078
3	0.099	0.130	0.037	0.080
4	0.110	0.145	0.045	0.097
5	0.108	0.142	0.050	0.108
6	0.132	0.174	0.047	0.102
7	0.202	0.266	0.054	0.117
8	0.326	0.428	0.057	0.123
9	0.467	0.613	0.058	0.125
10	0.736	0.968	0.063	0.136
11	1.10	1.450	0.066	0.143
12	1.41	1.850	0.073	0.158
13	1.75	2.300	0.077	0.166
14	1.91	2.510	0.082	0.177
15	2.07	2.720	0.100	0.216

[a]Benzimidazole absorbs characteristically at 278 mμ with negligible absorption at 346 mμ. Red fragment absorbs at both 278 mμ and 346 mμ.

[b]Slit 1.75

[c]Slit 0.425

Table 1 (Continued)

Tube No.	$A_{278\ m\mu}$	% of Total	$A_{346\ m\mu}$	% of Total
16	1.97	2.590	0.107	0.231
17	1.82	2.390	0.112	0.242
18	1.50	1.970	0.134	0.289
19	1.27	1.670	0.146	0.316
20	0.985	1.295	0.165	0.357
21	0.750	0.985	0.186	0.402
22	0.632	0.830	0.212	0.458
23	0.532	0.700	0.233	0.504
24	0.495	0.650	0.260	0.562
25	0.458	0.602	0.277	0.599
26	0.503	0.661	0.309	0.668
27	0.530	0.696	0.334	0.722
28	0.613	0.806	0.373	0.806
29	0.695	0.913	0.414	0.895
30	0.792	1.040	0.468	1.010
31	0.970	1.270	0.572	1.240
32	1.25	1.640	0.725	1.570
33	1.65	2.170	0.950	2.050
34	2.28	3.000	1.45	3.140
35	3.08	4.050	2.20	4.760
36	4.55	5.980	3.58	7.740
37	6.55	8.610	5.40	11.680

Table 1 (Continued)

Tube No.	$A_{278\ m\mu}$	% of Total	$A_{346\ m\mu}$	% of Total
38	9.60	12.610	8.23	17.790
39	12.00	15.750	10.05	21.750
40	10.01	13.150	8.45	18.250
Total	76.089		46.249	

ammonium sulfate solutions. The distribution of total nitrogen is represented in Fig. 2. The data for the nitrogen curve are given in Table 2.

(iii) Analyses for volatile hydrolytic nitrogen. Aliquots of tubes 0-5 were evaporated to dryness under reduced pressure. The residue was transferred quantitatively to the distillation apparatus and steam-distilled for 5.0 minutes from alkaline solution. The distillate was collected and titrated as in (ii) above. The distribution of ammonia nitrogen is shown in Fig. 2 and the data are given in Table 3.

If the aliquots were distilled for 7.0 minutes, some 1-amino-2-propanol distilled and this was subsequently titrated along with the ammonia. Control distillation of standard 1-amino-2-propanol solutions containing 3.8 mg. and 9.5 mg. of 1-amino-2-propanol showed titratable base in 7 minutes distillation, but no base in 5 minutes for either solution. This amount of 1-amino-2-propanol, 3.8 mg., represents approximately 8.0 percent of the theoretical 1-amino-2-propanol in B_{12a} on the basis of 2 moles per mole of B_{12a}, and is somewhat more than the amount of 1-amino-2-propanol to be encountered in the aliquots taken for analysis from the distribution experiment.

It was attempted to determine ammonia by Nesslerization, but difficulties were encountered. First of all, a good standard curve for nitrogen in the amounts of 10

Fig. 2

Distribution of Nitrogen in B_{12a} Hydrolysate.

(Total nitrogen obtained by Kjeldahl digestion, distillation and titration. Ammonia nitrogen obtained by distillation and titration.)

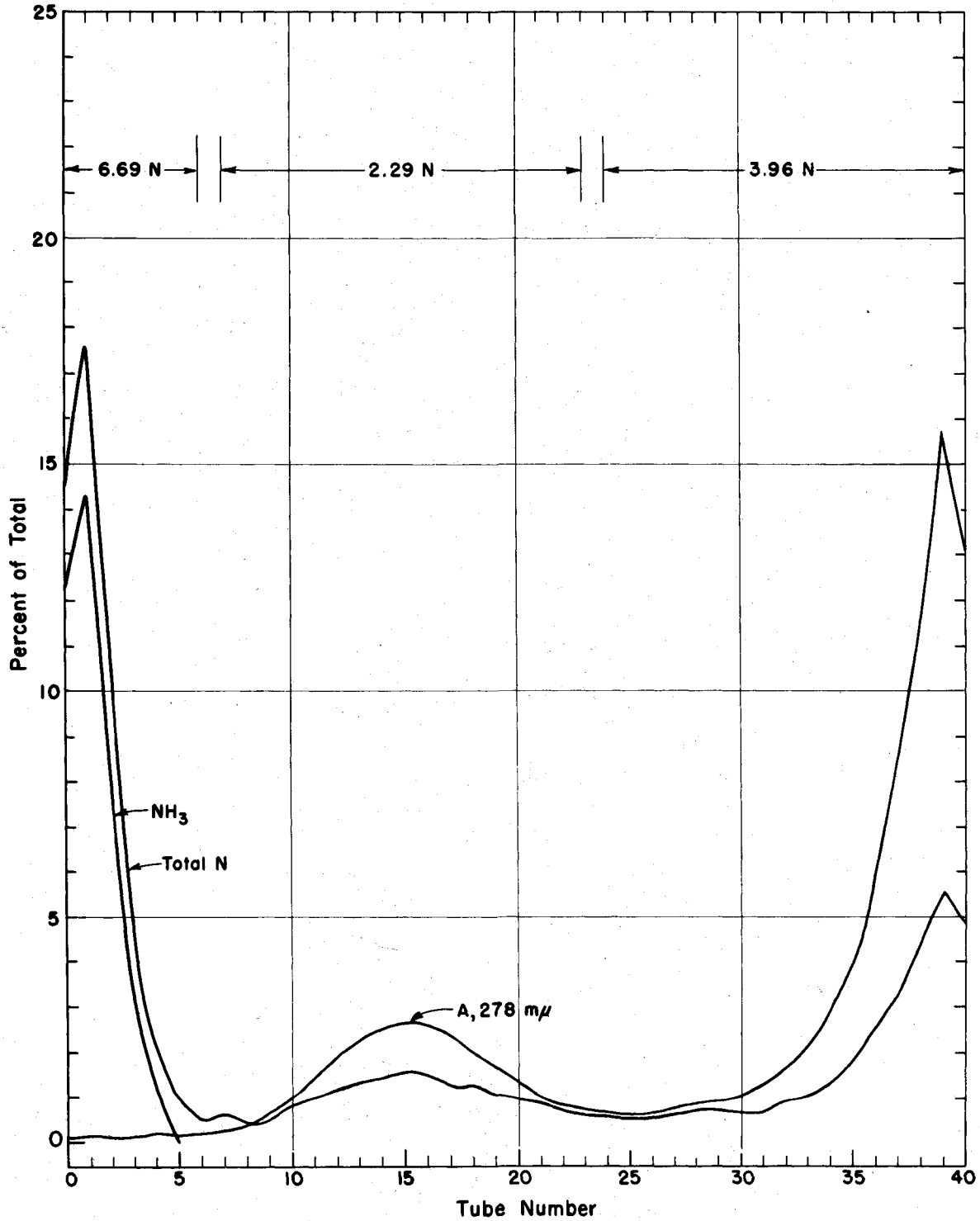

6.69 N
2.29 N
3.96 N
Percent of Total
NH3
Total N
A, 278 mμ
Tube Number
0
5
10
15
20
25
30
35
40

Table 2

Distribution of Total Nitrogen in B_{12a} Hydrolysate[a, b]

Tube No.	Mg. N	% of Total	Moles of N. Corr'd. to 13[c]
0	7.976	14.49	1.884
1	9.750	17.71	2.302
2	6.196	11.26	1.464
3	2.766	5.02	0.653
4	1.156	2.10	0.273
5	0.514	0.93	0.121
6	0.251	0.46	0.060
7	0.328	0.60	0.078
8	0.211	0.38	0.049
9	0.264	0.48	0.062
10	0.457	0.83	0.108
11	0.539	0.98	0.127
12	0.633	1.15	0.150
13	0.704	1.28	0.166
14	0.786	1.43	0.186

[a]Total nitrogen was determined by Kjeldahl digestion, distillation and titration.

[b]Total nitrogen recovered equals 97.3 percent of the nitrogen taken as calculated from a value of 13.24 percent nitrogen in B_{12a}.

[c]Moles of nitrogen per tube corrected for each tube by multiplying the percent of the recovered nitrogen in each tube by 13.

Table 2 (Continued)

Tube No.	Mg. N	% of Total	Moles of N. Corr'd. to 13
15	0.879	1.60	0.208
16	0.836	1.52	0.198
17	0.685	1.24	0.161
18	0.694	1.26	0.164
19	0.600	1.09	0.142
20	0.539	0.98	0.127
21	0.466	0.85	0.111
22	0.379	0.69	0.090
23	0.337	0.61	0.079
24	0.337	0.61	0.079
25	0.282	0.51	0.066
26	0.286	0.52	0.068
27	0.345	0.63	0.082
28	0.392	0.71	0.092
29	0.393	0.71	0.092
30	0.373	0.68	0.088
31	0.353	0.64	0.083
32	0.510	0.93	0.121
33	0.575	1.04	0.135
34	0.721	1.31	0.170
35	1.006	1.83	0.238
36	1.430	2.60	0.338
37	1.836	3.34	0.434

Table 2 (Continued)

Tube No.	Mg. N	% of Total	Moles of N. Corr'd. to 13
38	2.448	4.45	0.578
39	3.078	5.59	0.728
40	2.736	4.97	0.646
Total	55.047	100.01	12.931

Table 3

Distribution of Ammonia and Non-Volatile Nitrogen in the Tubes Containing Ammonia [a, b]

Tube No.	Mg. of N as NH_3	Moles of NH_3 [c]	Mg. of Non-Vol. N	Moles of Non-Vol. N [d]
0	6.962	1.60	1.229	0.283
1	8.078	1.86	1.935	0.446
2	4.798	1.10	1.565	0.361
3	1.890	0.43	0.951	0.218
4	0.754	0.17	0.433	0.113
Total	22.482	5.16	6.113	1.421

[a] Ammonia determined by distillation and titration.

[b] Non-volatile nitrogen obtained by subtraction of ammonia nitrogen from total Kjeldahl nitrogen.

[c] Calculated by multiplying the fraction of the total nitrogen in B_{12a} by 13.

[d] Calculated by multiplying the fraction of the total nitrogen in B_{12a} by 13.

μg. to 150 μg. was not obtained. A Koch and McMeekin reagent was used. Actually absorbance determinations at 480 mμ and 520 mμ amounted to less than the blank at these low concentrations. A reasonable standard curve was obtained for nitrogen concentrations ranging from 0.100 mg. to 1.000 mg. nitrogen per 100 ml. and determined at 500 mμ. However, this required that aliquots of 30.0 ml. from tubes 3, 4 and 5 be taken. When the evaporated aliquots of these tubes were distilled and Nesslerized, immediate turbidity occurred. The reason for this turbidity has not been determined, but it was proven that the 1-amino-2-propanol was not responsible by the Nesslerization of standard 1-amino-2-propanol solutions. In the amounts of 1-amino-2-propanol to be expected, no color change from the blank was found and no turbidity was produced.

The reagent of Vanselow (29) was next tried and again in attempting to prepare a standard curve, absorbance values less than the blank were obtained. However, when no sulfuric acid was employed, a reasonable standard curve for nitrogen in the amount of 10 μg. to 260 μg. was obtained at 500 mμ. Therefore, aliquots from the distribution experiment were evaporated to dryness and distilled 5.0 minutes (to avoid distillation of 1-amino-2-propanol). The distillate was collected in water and Nesslerized. The results were extremely erratic and com-

pletely incapable of consideration.

(iv) Analyses for cobalt. Aliquots representing each tube of the Craig distribution were evaporated to near-dryness and digested with a perchloric acid-nitric acid mixture until a colorless solution was obtained. The solution was then evaporated to near-dryness. In a few cases in which the solution was evaporated to complete dryness, a small amount of hydrochloric acid was added to dissolve any basic cobalt or cobalt oxide before transferring the digest. The acidic digests were then neutralized with sodium bicarbonate after the addition of 2.0 ml. of 0.2 M citric acid. The solutions were transferred to 25.0 ml. volumetric flasks and diluted to volume. Appropriate aliquots of these solutions were taken for determination of cobalt by the nitroso-R method (30). The cobalt results are illustrated in Fig. 3. The data for the cobalt durve are given in Table 4.

e. Determination of 1-amino-2-propanol distribution coefficient. A weighed amount, 0.97 mg., of freshly distilled 1-amino-2-propanol was diluted to 25.00 ml. with 1 N hydrochloric acid previously equilibrated with iso-butanol. A 1.00 ml. aliquot of this solution and 14.00 ml. of equilibrated 1 N hydrochloric acid was shaken thoroughly with 15.00 ml. of equilibrated iso-butanol. The two layers were separated and to each layer an equal volume of the opposite layer was added. To both was

Fig. 3

Distribution of Cobalt in B_{12a} Hydrolysate

(Cobalt determined colorimetrically with nitroso-R salt)

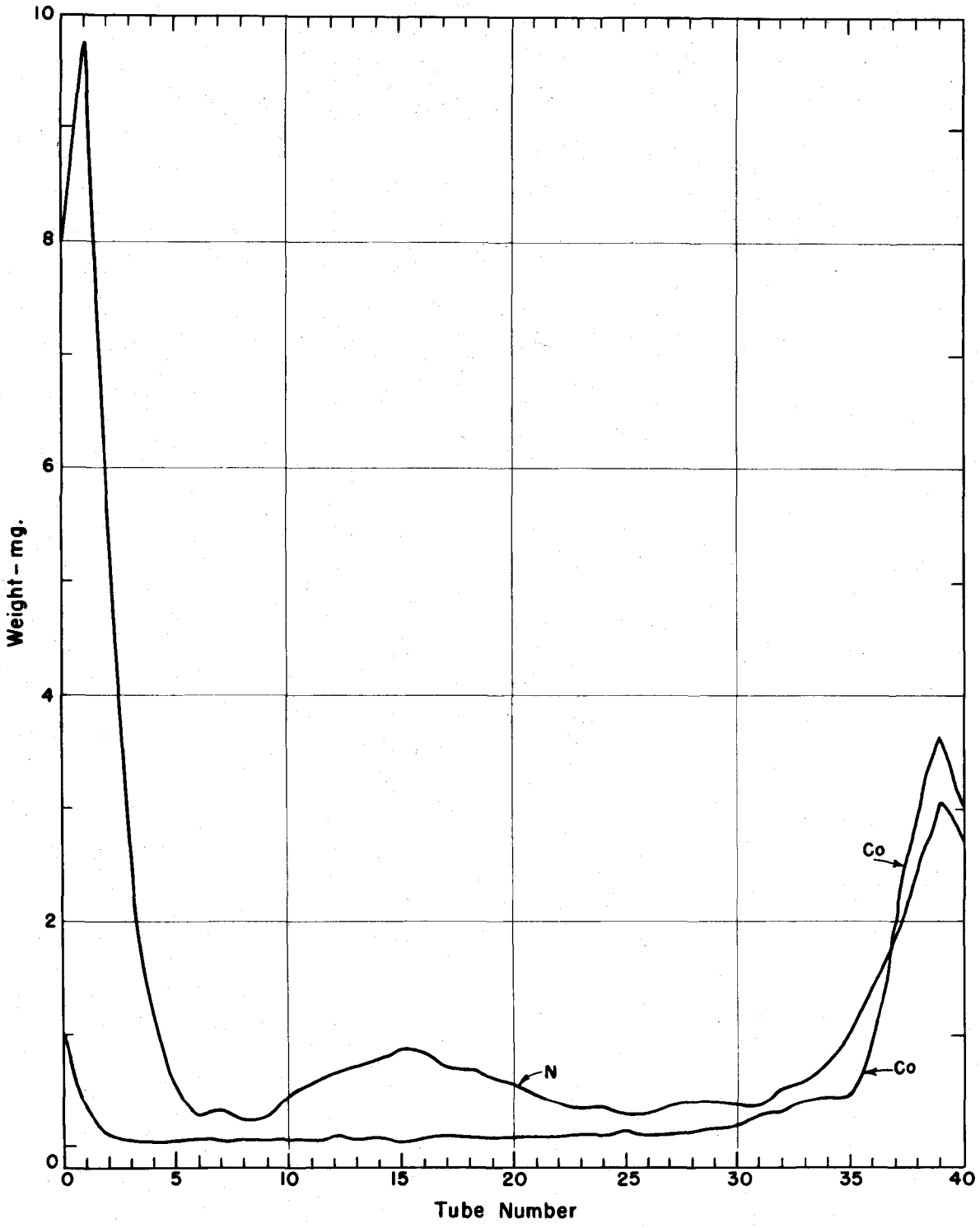

10
8
6
4
2
0
Weight - mg.
0
5
10
15
20
25
30
35
40
Tube Number
Co
N
Co

Table 4

Distribution of Cobalt in B_{12a} Hydrolysate[a]

Tube No.	Mg. Co	Mg. Co Corr'd. to 100%[b]
0	0.969	0.994
1	0.350	0.359
2	0.085	0.087
3	0.039	0.040
4	0.025	0.026
5	0.030	0.031
6	0.052	0.053
7	0.045	0.046
8	0.045	0.046
9	0.033	0.034
10	0.044	0.045
11	0.033	0.034
12	0.074	0.076
13	0.060	0.062
14	0.094	0.096
15	0.033	0.034
16	0.081	0.083
17	0.093	0.095

[a]Cobalt recovery equals 97.4 percent calculated from value of 4.26 percent cobalt in original B_{12a} found by analysis.

[b]Cobalt in each tube corrected to 100 percent by dividing cobalt found by fraction of total actual cobalt (0.974).

Table 4 (Continued)

Tube No.	Mg. Co	Mg. Co Corr'd. to 100%
18	0.070	0.072
19	0.089	0.091
20	0.075	0.077
21	0.088	0.090
22	0.083	0.085
23	0.100	0.103
24	0.089	0.091
25	0.124	0.127
26	0.090	0.092
27	0.118	0.121
28	0.119	0.120
29	0.136	0.140
30	0.173	0.177
31	0.275	0.281
32	0.303	0.309
33	0.420	0.428
34	0.410	0.418
35	0.430	0.439
36	0.938	0.957
37	1.925	1.964
38	2.825	2.882

Table 4 (Continued)

Tube No.	Mg. Co	Mg. Co Corr'd. to 100%
39	3.675	3.748
40	3.000	3.060
Total	17.735	

added 10 ml. of ethanol and each finally diluted to 50.00 ml. with water. Aliquots of each solution were evaporated and digested in the micro-Kjeldahl manner, distilled from alkaline solution and titrated with standard hydrochloric acid. The ratio of the volume of hydrochloric acid required for the upper and lower layers represents the distribution coefficient for 1-amino-2-propanol for the iso-butanol-1 N hydrochloric acid system, and was found to have a value of 0.065. The theoretical distribution curve calculated from this distribution coefficient is shown in Fig. 4, along with the non-volatile nitrogen found in tubes 0 through 7.

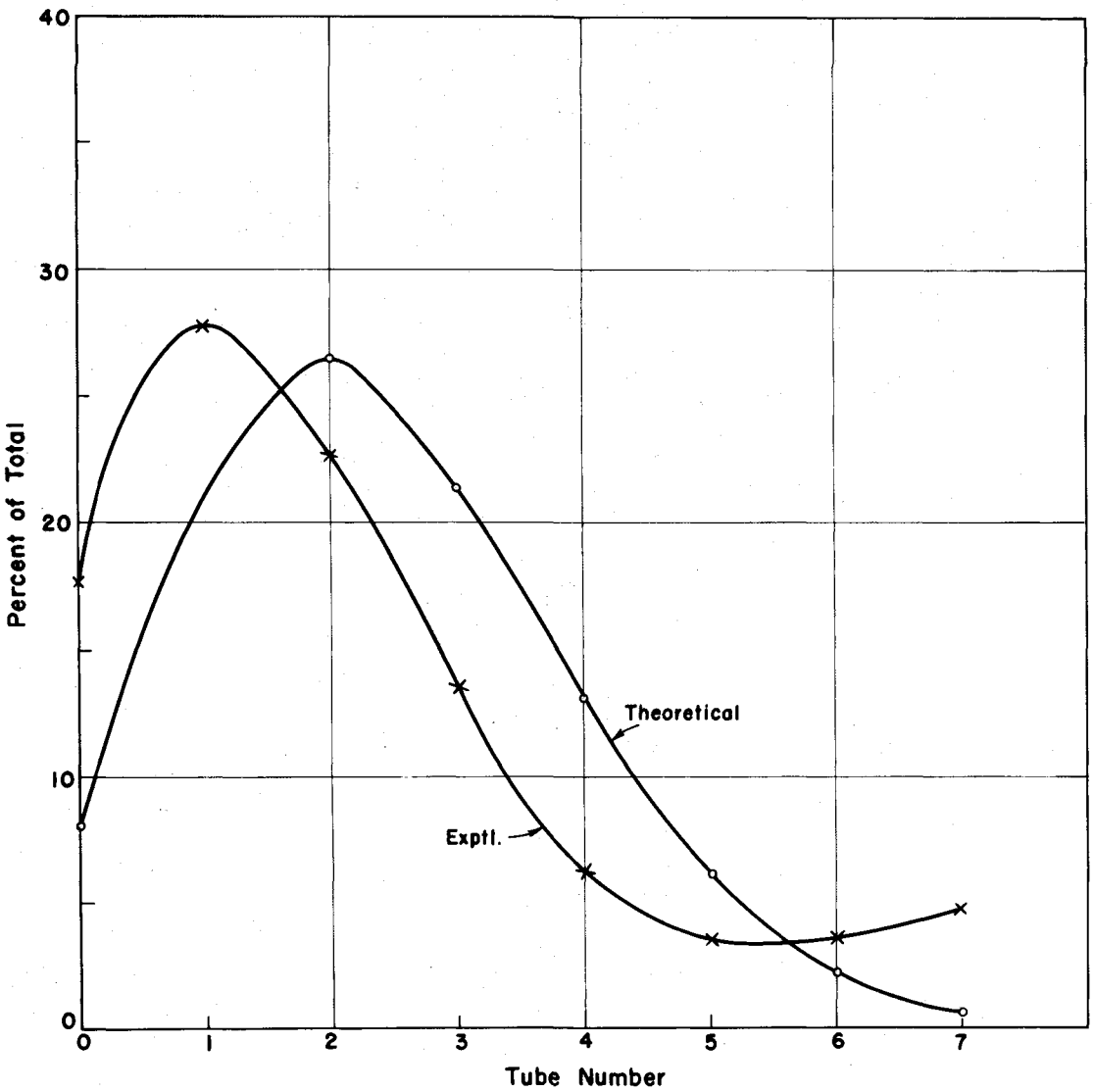
f. Calculations. The total nitrogen value was taken as 13.24 percent of the dry weight of B_{12a} employed. The total cobalt value was obtained from the analysis of aliquots of the hydrolysis solution. The percent recovery of nitrogen and cobalt was calculated on these bases.

The nitrogen in each tube was corrected to 100 percent recovery, thus distributing the error encountered in the total analyses. The nitrogen content of each tube was then calculated as the fraction of the total nitrogen. Multiplication of this fraction by 13 then gave the equivalents of nitrogen per tube. By summation of the equivalents of nitrogen for the tubes concerned, the atoms of nitrogen in the various products of the hydrolysis were

Fig. 4

Distribution of 1-Amino-2-Propanol.

(Theoretical curve calculated from distribution coefficient.
Experimental curve obtained by subtraction of ammonia nitrogen from total Kjeldahl nitrogen.)


40
30
20
10
0
Percent of Total
0
1
2
3
4
5
6
7
Tube Number
Theoretical
Exptl.

ascertained.

3. Results.

The mechanical separation of the various hydrolytic components fulfilled all expectations. The red acid fragment, a visibly evident component, moved rapidly in the system and was concentrated in the latter tubes of the apparatus with a peak concentration in tube 39. The spectrophotometric analyses, along with the total nitrogen determinations, showed the benzimidazole fragment to be confined near the center of the distribution with a maximum concentration in tube 15. A considerable amount of nitrogenous material, namely ammonia and 1-amino-2-propanol was retained in the early tubes. Thus three distinct separations were attained.

The total nitrogen recovery amounted to 97.3 percent of the amount taken. The total cobalt recovery amounted to 97.4 percent of the amount taken; nearly 8 percent of this was found in tubes 0, 1 and 2.

Ammonia nitrogen resulting from hydrolysis was confined to tubes 0 through 5 and amounted to 5.19 nitrogen.

From the distribution coefficient of 1-amino-2-propanol, it was calculated that the 1-amino-2-propanol would be distributed as far as tube 7. An arbitrary allocation of 1-amino-2-propanol nitrogen in tubes 0 through 6, obtained by subtracting the ammonia nitrogen from the total nitrogen in the tubes involved, resulted

in a value of 1.69 nitrogen.

The benzimidazole moiety, which showed a maximum in tube 15, both by nitrogen analyses and absorbance determinations, was assumed to fall in tubes 7 through 23, and the nitrogen in these tubes amounted to 2.28 nitrogen.

The remaining tubes, 24 through 40, contained 3.96 nitrogen. A summation gives a total 12.93 nitrogen atoms.

Actual nitrogen to cobalt ratios as obtained by analysis in the individual tubes assigned to the red acid fragment showed marked variations. Tubes 37 through 40 gave values between 3.6 and 4.0 nitrogen per cobalt, but from there all values were far higher. A summation of mg. nitrogen obtained and mg. cobalt obtained results in an average value of 4.78 nitrogen per cobalt.

4. Discussion.

The spectrophotometric analyses of tubes at 278 mμ and 346 mμ allowed an allocation of the benzimidazole fragment and the red acid fragment within certain tubes. The benzimidazole absorption at 346 mμ is very, very little, so the 346 mμ curve shows the red acid fragment only. At 278 mμ both the benzimidazole and the red acid fragment absorb quite strongly. Thus a comparison of the two curves shows two absorption maxima on the 278 mμ curve, one for benzimidazole and one for the red acid fragment, while the 346 mμ curve shows only red acid fragment

absorption which coincides with the second 278 mμ maximum. These two absorption curves, coupled with the nitrogen distribution curve, leave no doubt as to the location of these two components.

The evidence presented above clearly shows that vitamin B_{12a} contains five nitrogen groups which yield ammonia on hydrolysis with 1 N hydrochloric acid at 100° for 22 hours. As expected, two nitrogen atoms are accounted for by the one mole of the benzimidazole product.

Two nitrogen atoms fall in the place expected for 1-amino-2-propanol. The recent report of one mole of 1-amino-2-propanol, in contrast to the earlier figure of two, leaves some uncertainty as to the true figure. The fact that two nitrogens were found in the region expected for 1-amino-2-propanol cannot settle the controversy, for there is not an independent, unambiguous method for the absolute determination of 1-amino-2-propanol in this system. The best that can be said at this time is, that if there are not two moles of 1-amino-2-propanol, then there is another small nitrogen-containing compound present with distribution characteristics similar to 1-amino-2-propanol which has so far escaped detection.

The red acid fragment bears four nitrogen atoms.

The ratio of nitrogen to cobalt in tubes 24 through 40 following the distribution was not constant; this ratio ran far higher in tubes 24 through 36, but became

3.6 to 4.0 in tubes 37 through 40. The red fragment obtained is thus not a single compound but a mixture of a cobalt-free and cobalt-bearing red material. The presence of significant amounts of cobalt in the tubes 0, 1 and 2, present as free, ionic cobalt, is in accord with this.

5. Summary.

a. Vitamin B_{12a} was hydrolyzed in 1 N hydrochloric acid for 22 hours at 100°, and the hydrolysis mixture distributed through 40 transfers by countercurrent distribution between 1 N hydrochloric acid-iso-butanol.

b. Analysis of the individual tubes of the distribution apparatus showed that five nitrogen atoms were present as ammonia, two nitrogen atoms as 1-amino-2-propanol, or one nitrogen as 1-amino-2-propanol and one as an unknown compound with similar distribution characteristics, two nitrogen atoms as benzimidazole, and four nitrogen atoms as an unidentified, red, acid fragment.

c. About 8 percent of the cobalt was stripped from the molecule during the hydrolysis.

d. The red acid fragment was shown to be not a single specie, but shown to be a mixture of cobalt-free and cobalt-bearing material.

B. The Rate of Ammonia Production in the Acid Hydrolysis of Vitamin B_{12}

1. Introduction.

It was shown in the preceding section that vitamins B_{12} and B_{12a} contain five nitrogen atoms which yield ammonia on acid hydrolysis. It seemed desirable to determine if a difference exists in the nature of these nitrogen atoms. One possible means of detecting such a difference should be the rate of production of the ammonia. The following experiment was devised with this in view.

An auxiliary experiment was performed on the rate of hydrolysis of sodium cyanide as some question arises as to the fate of the cyanide of B_{12} during hydrolysis.

2. Experimental.

a. Hydrolysis procedure. A solution containing 118.8 mg. of B_{12} in 50 ml. of water was employed. Analysis showed the presence of 0.439 mg. of cobalt per each 4.00 ml. of this solution. Aliquots of 4.0 ml. were refluxed in test tubes with 4.00 ml. of 2 N hydrochloric acid under micro Liebig condensers at 95° for given time intervals. The solutions were placed in the water bath with the bath already heated to 95°.

When the allotted time had elapsed, the tubes were removed from the bath and immediately plunged into ice water to quench the hydrolytic action at the elevated temperature. These tubes were then kept at ice bath

temperature until analyzed.

A control experiment to determine the rate of production of ammonia from cyanide was carried out. A freshly prepared aqueous solution of sodium cyanide containing 0.068 mg. per ml. was employed. Five ml. aliquots of this solution were hydrolyzed for various lengths of time in a manner identical with that described above.

b. Analysis for ammonia. The cold hydrolysis solutions were transferred rapidly for steam distillation and quickly neutralized by the addition of 4 ml. of 1 N sodium hydroxide. A volume of 15 ml. of 0.05 M phosphate buffer, pH 8.5, was added and the solution was distilled for 7 minutes into a 4 percent boric acid solution containing 2 drops of methyl red-methylene blue indicator.

3. Results.

The values obtained from the analysis are tabulated in Table 5. Approximately three moles of ammonia were obtained within the first 30 minutes of hydrolysis. Eventually the total approaches six molecules of ammonia, 5.62 moles actually found, but the release was slow, requiring up to 20 hours. The rate of ammonia production is represented in Fig. 5.

Hydrolysis of sodium cyanide solutions under identical conditions for the B_{12} hydrolysis gave only a 36.5 percent conversion to ammonia. A 30 minute hydrolysis of sodium cyanide solutions failed to yield any titratable

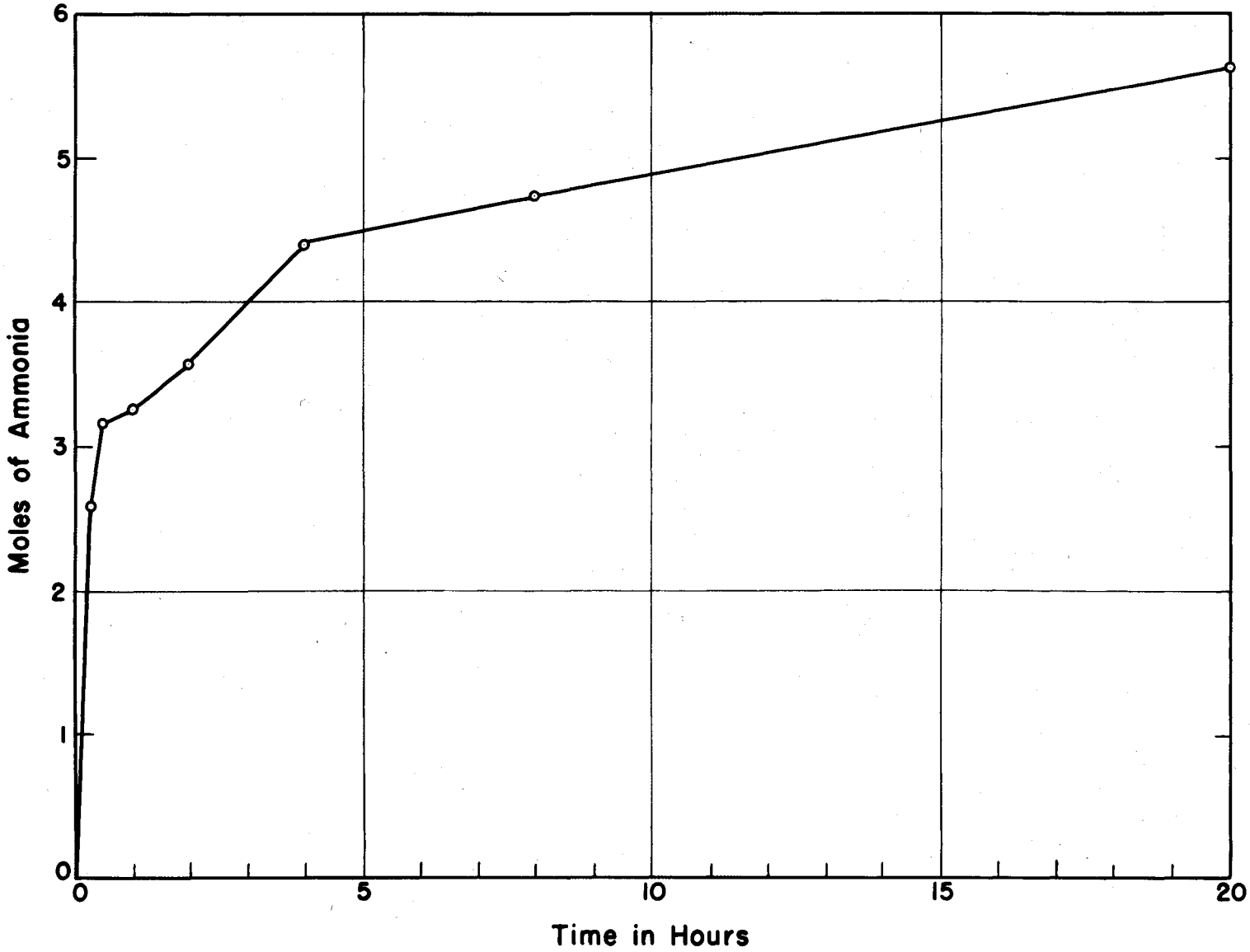
Table 5

Rate of Ammonia Production on B_{12} Hydrolysis

Time Hours	ml. of 0.0102 N HCl	Mg. N	N:Co
0.25	1.80	0.2558	2.59
0.50	2.20	0.3126	3.17
1.00	2.25	0.3197	3.24
2.00	2.47	0.3510	3.56
4.00	3.05	0.4334	4.40
8.00	3.28	0.4661	4.72
20.00	3.70	0.5417	5.62

Fig. 5

Rate of Production of Ammonia During Acid Hydrolysis of Vitamin B_{12}


Moles of Ammonia
0
1
2
3
4
5
6
0
5
10
15
20
Time in Hours

ammonia.

4. Discussion.

That the number of molecules of ammonia released approached but did not actually reach six is probably a result of incomplete conversion of cyanide to ammonia. The results from the hydrolysis of sodium cyanide under the same condition support this. The hydrolysis of sodium cyanide is, of course, not strictly comparable to the hydrolysis of the B_{12} cyanide where the cyanide is coordinated with the cobalt, but at least ought to proceed more rapidly, inasmuch as sodium cyanide is much more extensively ionized.

Taking the cyanide into consideration, it appears that of the five nitrogen atoms which yield ammonia, on hydrolysis, three are released more rapidly than the other two. If these five nitrogen atoms are present as acid amide groups as reported by Ellingboe and Diehl (27), some significant difference in their spatial arrangement must exist.

5. Summary.

a. The amount of ammonia produced by acid hydrolysis of B_{12} approaches six moles.

b. Three moles of ammonia are produced during acid hydrolysis of B_{12} in a relatively short period of time.

c. Control hydrolysis of sodium cyanide shows only 36.5 percent production of ammonia in the time employed for the hydrolysis of B_{12}. This fact is presented in

support of the belief that the failure to attain six moles of ammonia on B_{12} hydrolysis is due to incomplete hydrolysis of the cyanide of B_{12}.

C. Carbon Dioxide and Hydrogen Cyanide Liberation in the Hydrochloric Acid Hydrolysis of Vitamin B_{12}

1. Introduction.

Carbon dioxide might be expected to be produced during the acid hydrolysis of B_{12}, either arising from the cyanide, or as a result of oxidative degradation. Liberation of hydrogen cyanide during this hydrolysis has been reported by others (10). In the present work, the quantities of these two substances liberated on hydrolysis of B_{12} with 1 N hydrochloric acid at 95° have been measured.

2. Experimental.

Into a 200 ml., round bottom flask was sealed an inlet tube designed to allow the introduction into the flask of gas or of liquid as desired. The flask was provided with a water cooled condenser, the outlet of which was connected in series to a trap containing silver nitrate, a U-tube containing anhydrous magnesium perchlorate, and a Turner bulb packed with Ascarite and anhydrous magnesium perchlorate. The Turner bulb was in turn connected to a water aspirator so that air could be drawn through the entire system. The incoming air was passed first through a tube containing Ascarite and then through a water bubbler.

The reaction flask was immersed in an oilbath maintained at a temperature of 94 to 97° by electrical heating.

The vitamin B_{12} was first dissolved in a known volume of water and an aliquot of the solution taken for a spectrophotometric determination of the B_{12} present ($E^{1\%}_{1\ cm.}$ = 207 at 361 mμ). The solution was then transferred to the reaction vessel. A measured volume of 0.1 N silver nitrate was placed in the trap. Air was drawn through the system for 30 minutes and the Turner bulb was then disconnected and weighed. The Turner bulb was then replaced and sufficient 6 N hydrochloric acid added to the reaction vessel to make the resultant solution 1 N in acid. The mixture was then heated and air drawn through the solution at a rate of 2 bubbles per second.

At the end of the hydrolysis (20 to 25 hours) the Turner bulb was again weighed, giving the amount of carbon dioxide liberated, and the cyanide collected along with the chloride in the silver nitrate trap was determined. The latter determination was effected by transferring the contents of the trap to a flask, rinsing the flask thoroughly with ammonium hydroxide which dissolves both silver chloride and silver cyanide. To the ammoniacal solution was added a measured amount of a standard sodium cyanide solution. Potassium iodide was added and the solution was then titrated with standard silver nitrate to the appearance of a silver iodide turbidity. From the total

silver nitrate (the amount initially in the trap and that added in the back titration) and the added cyanide, the cyanide liberated from the B_{12} could be calculated. Chloride does not interfere with the cyanide determination when made in this manner.

3. Results.

Two runs were made using the procedure described above. The results are shown in Table 6.

The carbon dioxide liberated varied considerably in the two experiments (0.40 and 0.70 moles per mole of B_{12}, respectively). It was below one in each case.

Hydrogen cyanide liberated varied considerably also (0.37 and 0.26 moles per mole, respectively).

Carbon dioxide plus hydrogen cyanide together approach one (0.40 and 0.37 or 0.77 in experiment A; 0.70 and 0.26 or 0.96 in experiment B), particularly at the longer reaction time.

4. Discussion.

It is likely that the carbon dioxide found was derived from the cyanide group by hydrolysis and oxidation:

$$B_{12}CN + H^+ = B_{12}^+ + HCN$$
$$HCN + H_2O = HCONH_2 \text{ (formamide)}$$
$$HCONH_2 + H_2O = HCOONH_4 \text{ (ammonium formate)}$$
$$HCOOH + 1/2\ O_2 = CO_2 + H_2O.$$

Slow hydrolysis of formamide to formic acid or slow oxidation of formic acid to carbon dioxide would account for

Table 6

Release of Hydrogen Cyanide and Production of Carbon Dioxide on Acid Hydrolysis of Vitamin B_{12}

	Experiment A		Experiment B	
B_{12} taken[a]	1.0949 g.	(0.811 milli-moles)[b]	1.1478 g.	(0.850 milli-moles)[b]
CO_2 found (moles per mole of B_{12})		0.40 (20 hours)	0.52 (20 hours)	0.70 (25 hours)
CN^- found (moles per mole of B_{12})	0.266 (16 hours)	0.367 (20 hours)	0.16 (20 hours)	0.26 (25 hours)

[a] determined spectrophotometrically

[b] molecular weight taken as 1350

the failure of the 20 hour experiment A to give a value of one for $CO_2 + CN^-$. This is in line with the fact that hydrolysis of sodium cyanide under similar conditions resulted in only 36.5 percent conversion to ammonia.

The fact that the carbon dioxide production is only a part of one mole, coupled with the fact that no carbon dioxide was produced on the hydrolysis of B_{12a}, strongly suggests the carbon dioxide produced arises from partial hydrolysis of the B_{12} cyanide and subsequent oxidation of the formic acid produced.

5. Summary.

a. A fraction of the total cyanide of vitamin B_{12} is evolved as hydrogen cyanide in the acid hydrolysis of the vitamin.

b. Less than one mole of carbon dioxide is produced during the acid hydrolysis of vitamin B_{12}.

c. A summation of the hydrogen cyanide evolved and the carbon dioxide produced during the acid hydrolysis of vitamin B_{12} approaches one.

d. It was concluded that the carbon dioxide produced during B_{12} hydrolysis arises from partial hydrolysis of the cyanide of the vitamin.

D. The Red Acid Fragment

1. Introduction.

The so-called "red acid fragment" of vitamin B_{12}

is produced by hydrochloric acid hydrolysis. The red fragment is approximately two-thirds of the original molecule. It is the portion of the molecule which contributes the color and which carries the cobalt atom. It is a material of unusual stability for only comparatively drastic degradation methods cause any significant breakdown. It has not yet been obtained in crystalline form and is undoubtedly a mixture of a number of closely related substances. Information about it has thus been obtained from impure material.

Vitamin B_{12} has no acidic properties (31) and the acid groups of the red, acid fragment are due to carboxyl groups produced by the hydrolysis of the acid amide groups reported present in B_{12} by Ellingboe and Diehl (27). That the acidic groups can be esterified was first reported by Ellis, et al (14). Oxidation of the red fragment with alkaline permanganate yielded a mixture of at least eight acids, four of which were identified (13). Beyond this, little has been reported about the nature and chemistry of the red fragment.

The material in this section deals with the preparation, chemical and physical properties of the red acid fragment and attempts to separate its components.

2. Hydrolysis procedure.

In all instances, except one, vitamin B_{12} was hydrolyzed with 1 N hydrochloric acid in a round bottom flask

equipped with a reflux condenser. In the one exception, 6 N hydrochloric acid was employed. The temperature was maintained between 95°-105°, and the concentration of B_{12} was varied from 5 mg. to 10 mg. per ml. The time of hydrolysis was 20 to 25 hours.

3. Isolation methods.

a. Precipitation. A typical procedure consisted of concentration of the hydrolysis solution by distillation under reduced pressure. During the concentration water was added at intervals to prevent the acid concentration from becoming too high. The solution was evaporated to near-dryness and the residue extracted with acetone. The red material was readily soluble and some white residue remained. The acetone extract was then evaporated to dryness either under reduced pressure or by air jet, and the residue again extracted with acetone. It was sometimes necessary to add a trace of water to the completely dry residue in order to effect solution of the red material. This extraction further separated the red material from the colorless material. Occasionally some brown, acetone-insoluble material remained.

The extraction-evaporation process was repeated again and the dry residue then dissolved in either 0.1 N hydrochloric acid or 0.1 N sodium hydroxide. Dissolution of the residue in the base was usually accompanied by the formation of a white gelatinous material. When

the pH of the acidic or basic solution of the red material was adjusted to 3 to 4, the red material was precipitated. The yields of red material varied from 30 to 60 percent of the theoretical value obtained by subtracting the other known hydrolytic fragments (ammonia, benzimidazole ribose phosphate, and 1-amino-2-propanol). The precipitate was always amorphous, red to red-brown in color, and sometimes gel-like in appearance.

The red acid fragment was also obtained as an amorphous precipitate from acetone on the addition of ether or petroleum ether.

Another method employed for isolating the red acid fragment involved the addition of barium hydroxide directly to the hydrolysis solution in sufficient amount to bring the pH to about 9. The benzimidazole precipitated on standing and was removed by centrifugation. The red acid fragment was precipitated as the barium salt by the addition of sufficient alcohol to produce a nearly colorless solution; four volumes of alcohol were required. The precipitate was then suspended in water and acidified to Congo Red with dilute sulfuric acid. After removal of the barium sulfate, the material was then isolated by concentration and acetone extraction as described above. The barium method was not as satisfactory as the first method outlined because of the difficulty in removing the barium sulfate and because the

product obtained was frequently rust to brown in color, in contrast to the products obtained by the first procedure.

b. Countercurrent distribution. Several attempts to purify the red acid fragment were made by utilization of the Craig all-glass countercurrent distribution apparatus. Although the smaller known hydrolytic fragments could be separated, no success was achieved in the isolation of a pure, red, cobalt-containing compound. Butanone and iso-butyl alcohol were found to be most effective in the separation of red material from the smaller hydrolytic products (ammonium ion, the benzimidazole moiety and 1-amino-2-propanol), but no pure, red material was obtained even with 175 transfers. It was apparent from visual inspection that the red material consisted of a number of red-colored compounds (usually at least three) but the distribution procedure failed to isolate any one component.

In these distribution experiments the aqueous layer always consisted of dilute hydrochloric acid. It was found that the colored material moved more rapidly when the hydrochloric acid concentration was increased. The distribution coefficient was less than one if the concentration of hydrochloric acid was less than 0.01 N. The details of an actual experiment for distribution of hydrolytic fragments are presented in Experimental A.

c. Ion-exchange. An attempt to purify a hydrolysis mixture of B_{12} by ion-exchange was also made. Both anion and cation exchange resins, Amberlite IRA-45 and IRC-50 respectively, were employed. Although small scale experiments indicated possibility of effecting a separation of the components of the red acid fragment on IRA-45, when the procedure was employed with larger columns, the behavior was not the same. The major part of the red material could not be eluted from the columns. Various solvents, iso-butyl alcohol, ethyl alcohol, acetone, benzene, and phenol, failed to elute the material. Aqueous solutions of sodium chloride, di-sodium phosphate, sodium hydroxide, ammonium hydroxide, and hydrochloric acid were equally ineffective. The red material which was so tenaciously retained was finally freed from the resin only by heating and vigorous stirring of a sodium chloride slurry of the resin with iso-butanol.

d. Chromatography. Various attempts to purify the red acid fragment by adsorption and partition chromatography were made. Magnesium oxide, calcium carbonate, Hyflo Super-Cel and alumina were used as adsorbents. Water, alcohol, chloroform, ethylene chloride and dilute acid of varying concentrations were tried as solvents. No isolation of a red compound which could be crystallized was obtained.

In almost all cases, it was found that all of the

red fragment material was either tenaciously adsorbed or that no appreciable adsorption occurred. The red fragment as the free acid was adsorbed with particular tenacity.

When the red fragment was converted to the methyl or butyl ester for partition, some separation was effected by passing an ethylene chloride solution through an alumina column. Fractions were obtained by elution with mixtures of ethylene chloride and methanol up to 50 percent (v/v). Finally absolute methanol followed by 0.1 N methanolic hydrochloric acid and 0.1 N aqueous hydrochloric acid resulted in removal of nearly all colored material from the column. The fractions varied from pale yellow in the first cut to purple, pink, dark red and orange-red. None of the fractions could be induced to crystallize, however.

4. Crystallization attempts.

Various attempts were made to crystallize the red acid fragment as the free acid. Slow spontaneous concentration of aqueous and acetone solutions was found ineffective. Aqueous solutions of varying concentrations of hydrochloric acid were also used. The addition of non-polar solvents such as ether or benzene to the acetone solutions always yielded amorphous precipitates.

Many attempts to obtain the red acid fragment as a crystalline metal salt were also unsuccessful. The sodium, potassium, ammonium, barium, zinc, mercury and

silver salts were prepared, but no crystalline derivative was obtained. Monovalent metal salts were very soluble in water and precipitation was effected by the addition of acetone or alcohol to the aqueous solution.

In one experiment, an attempt was made to obtain crystalline zinc and mercury salts by utilizing the Leisegang phenomenon in silica gel. Although precipitation and banding occurred, no crystals were obtained.

An attempt was made to obtain a crystalline ammonium salt by slow diffusion of ammonia into an acetone solution of the free acid. Only amorphous precipitates formed. Likewise, only amorphous precipitates formed on slow diffusion of acetone into an aqueous solution of the red acid fragment as the di-cyanide sodium and potassium salts.

5. Properties of the red acid fragment.

a. Solubility. The red fragment as the free acid is soluble in water, acetone and most alcohols. It is insoluble in all non-polar solvents. The material obtained by isoelectric precipitation is soluble only in dilute acid or base. The methyl or butyl ester is quite insoluble in water but soluble in many organic solvents such as chloroform, ethylene chloride, benzene and alcohol.

b. Analytical composition. Analysis of 4 products is given in Table 7.

Table 7

Elemental Analyses of Red Acid Fragment[a]

	C	H	N	Co	O	Empirical Formula	Molecular Weight from Formula	Molecular Weight Based on Co.
1	55.74	6.39	7.00	7.15	23.90	$C_{38-39}H_{52-53}N_4O_{12}Co$	815-828	825
2	56.40	6.88	5.50	5.88	25.20	$C_{46}H_{69}N_4O_{16}Co$	992	1004
3	60.17	6.43	5.88	6.25	21.27	$C_{47-48}H_{60-61}N_4O_{12-13}Co$	931-960	944
4	54.09	6.28	4.56	5.97	29.10	$C_{45}H_{62}N_{3.3}O_{18}Co$		
5[b]	57.46	5.07	5.14	19.29	13.04			
Ave. 1-4	56.57	6.49	5.73	6.31	24.87	$C_{44}H_{61}N_4O_{15}Co$	944	933

[a]Analyses performed by J. F. Alicino.

[b]Hydrolysis in 6 N hydrochloric acid, 20 hours.

In view of the fact that it has been shown that some cobalt may be stripped from the molecule during hydrolysis in 1 N hydrochloric acid, the obvious conclusion for the cobalt figure obtained for 5 in Table 7 is that this is badly contaminated with inorganic cobalt freed from the molecule during hydrolysis in 6 N hydrochloric acid.

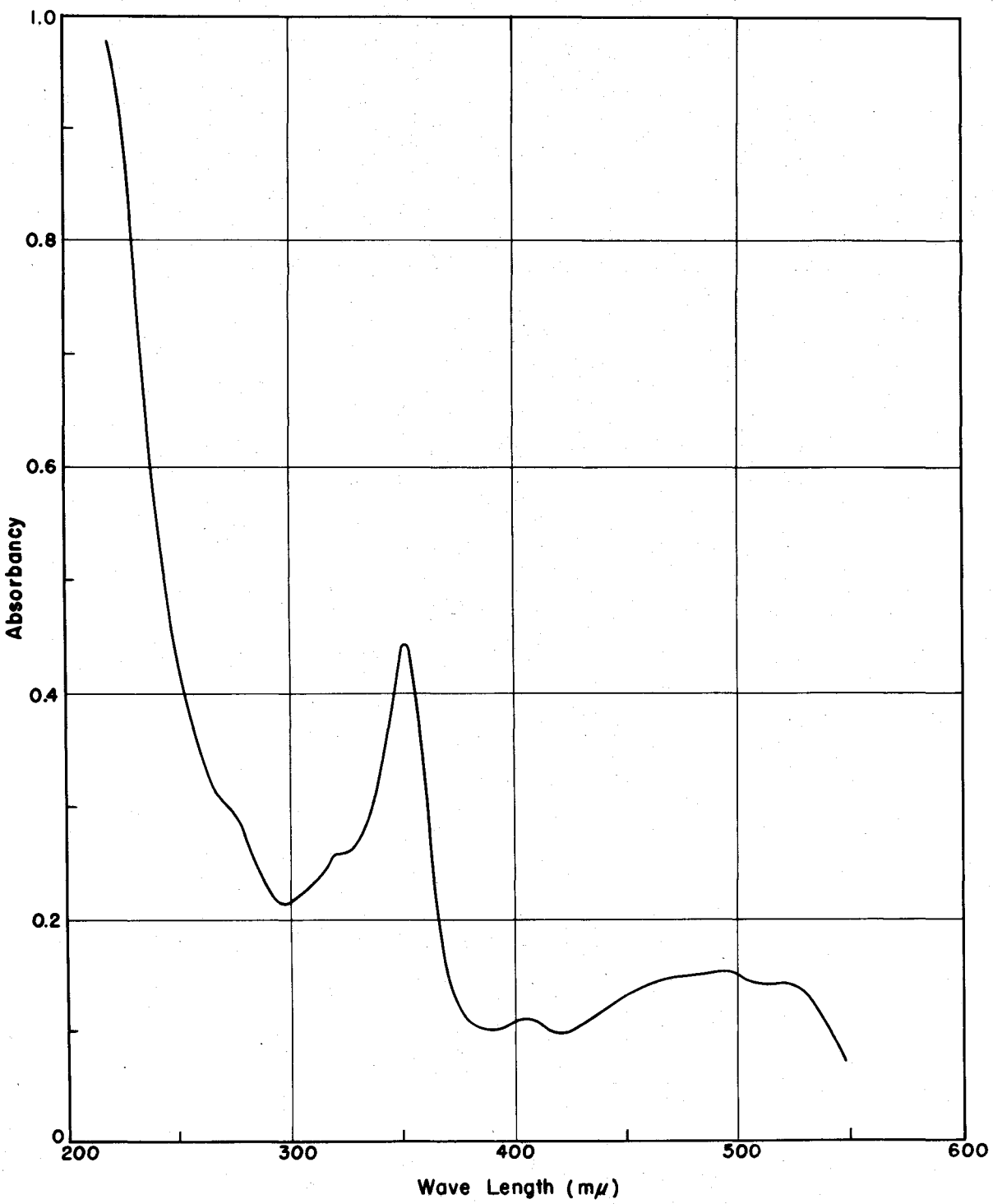
c. Spectra.

(i) Ultraviolet and visible. A typical spectrum of the red acid fragment obtained by 20 hour hydrolysis at 100° in 1 N hydrochloric acid is shown in Fig. 6. It shows maxima at 351, 405 and 495-500 mμ, and a shoulder at about 520-525 mμ. For a given red acid fragment, there is no shift in position of the major peak in the 350 mμ region or the 405 mμ region with major changes of pH, but the position of the major peak varies somewhat in the different products prepared, and may vary from 346 to 353 mμ. For a given product, however, a change in absorbance does occur with changes in pH. $E^{1\%}_{1\ cm.}$ determinations on a product which showed a maximum at 346 mμ gave a value of 183 in phosphate buffer, pH 7.4. On another product with a maximum at 351 mμ, $E^{1\%}_{1\ cm.}$ was 185 in phosphate buffer, pH 7.4, and in approximately 0.01 N sodium hydroxide. The same product in 0.01 N hydrochloric acid gave an $E^{1\%}_{1\ cm.}$ value of 194. The small peak and shoulder at 500 mμ and 525 mμ shift slightly

Fig. 6

Ultraviolet and Visible Spectrum of the
Red Acid Fragment

(Phosphate buffer, pH 7.4)


1.0
0.8
0.6
0.4
0.2
0
Absorbancy
200
300
400
500
600
Wave Length (mμ)

toward shorter wavelengths in acid solutions.

No absorption peak is obtained in the region of 278 mμ, the region in which benzimidazole absorbs. Benzimidazole must be absent in these preparations. If B_{12} is hydrolyzed for shorter periods of time, the benzimidazole is not hydrolyzed away completely; for example, a product from 6 hour hydrolysis shows the typical 278 mμ peak.

(ii) Infrared.[1] The infrared spectra of B_{12}, free red acid fragment and the sodium salt of the cyanide derivative of the red fragment are shown in Figs. 7, 8 and 9. Comparison with B_{12} shows the following: (a) The strong band at 6.0 in B_{12} which is attributed to amides is removed; (b) The free acid shows double band absorption at 5.6μ and 5.8μ; (c) The sodium salt has no bands at 5.6μ and 5.8μ, but has a strong band at 6.3μ.

d. Reactions.

(i) Bromination. The red fragment reacts readily with excess bromine water in a slightly acid environment. An amorphous, red-brown precipitate is produced. The material formed is insoluble in water and it is also insoluble in chloroform and ether. It shows considerable solubility in alcohol and acetone.

In a typical experiment 5 mg. of a red acid frag-

---

[1]All infrared spectra shown in this thesis were obtained with the Baird Associates Infrared Spectrophotometer. The spectra are tracings from the originals.

Fig. 7

Infrared Spectrum of Vitamin B_{12}

(Nujol mull; bands at 3.5μ, 6.9μ and 7.3μ are due to Nujol.)

Fig. 8

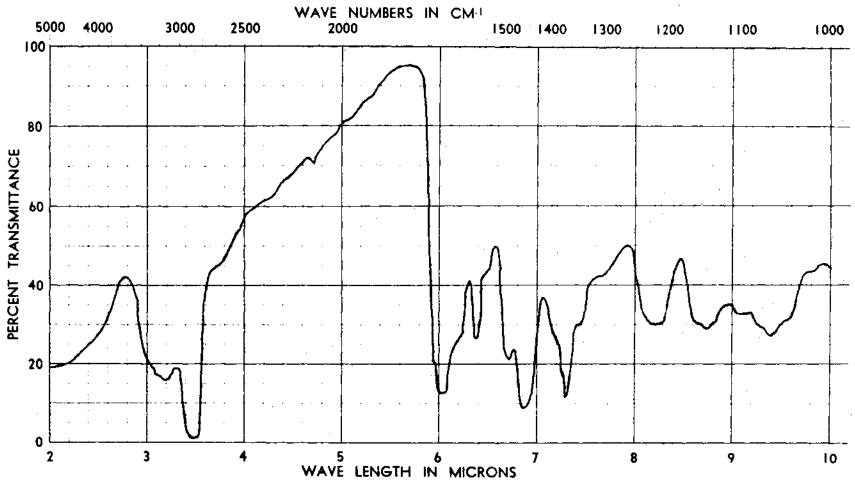
Infrared Spectrum of the Red Acid Fragment

(Nujol mull; bands at 3.4μ, 6.8μ and 7.3μ are due to Nujol.)

Fig. 9

Infrared Spectrum of the Cyanide Derivative of the Red Acid Fragment

(Nujol mull; bands at 3.4μ, 6.9μ and 7.3μ are due to Nujol.)


WAVE NUMBERS IN CM-1
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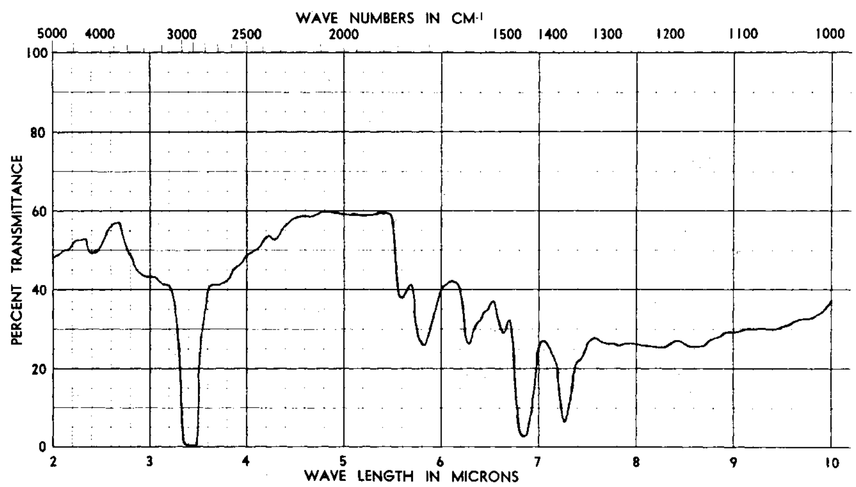
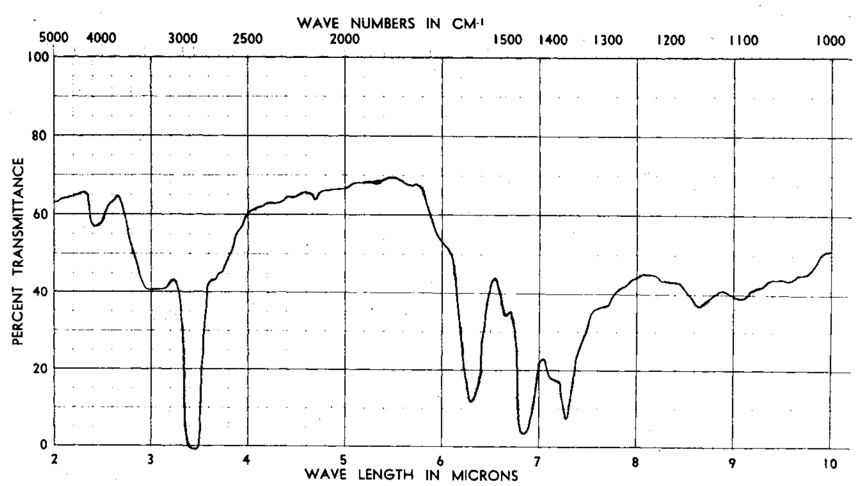
4000
3000
2500
2000
1500
1400
1300
1200
1100
1000
PERCENT TRANSMITTANCE
WAVE LENGTH IN MICRONS


WAVE NUMBERS IN CM-1
5000
4000
3000
2500
2000
1500
1400
1300
1200
1100
1000
PERCENT TRANSMITTANCE
WAVE LENGTH IN MICRONS


WAVE NUMBERS IN CM-1
5000
4000
3000
2500
2000
1500
1400
1300
1200
1100
1000
PERCENT TRANSMITTANCE
WAVE LENGTH IN MICRONS

ment was suspended in water, dissolved by addition of 2 ml. of 0.1 N sodium hydroxide, and then made slightly acid with 0.1 N hydrochloric acid. Three ml. of a one-tenth saturated bromine water solution was added. The red color disappeared instantaneously and the solution became cloudy. A red-brown precipitate was obtained on centrifugation; the supernatant solution was pale yellow in color.

The spectrum of the supernatant was obtained immediately against a bromine water blank. This showed a small hump in the region of 400 mμ, and a broad hump at 470-490 mμ. No absorption peak was present in any other region.

The precipitate obtained was divided into an acetone-soluble fraction and an acetone-insoluble-alkali-soluble fraction. The spectra of these two fractions is shown in Fig. 10. It is interesting to note that the major absorption peak in the 350 mμ region, which is usually attributed to cobalt coordination, is completely missing, as well as the absorption peak in the 500-530 mμ region.

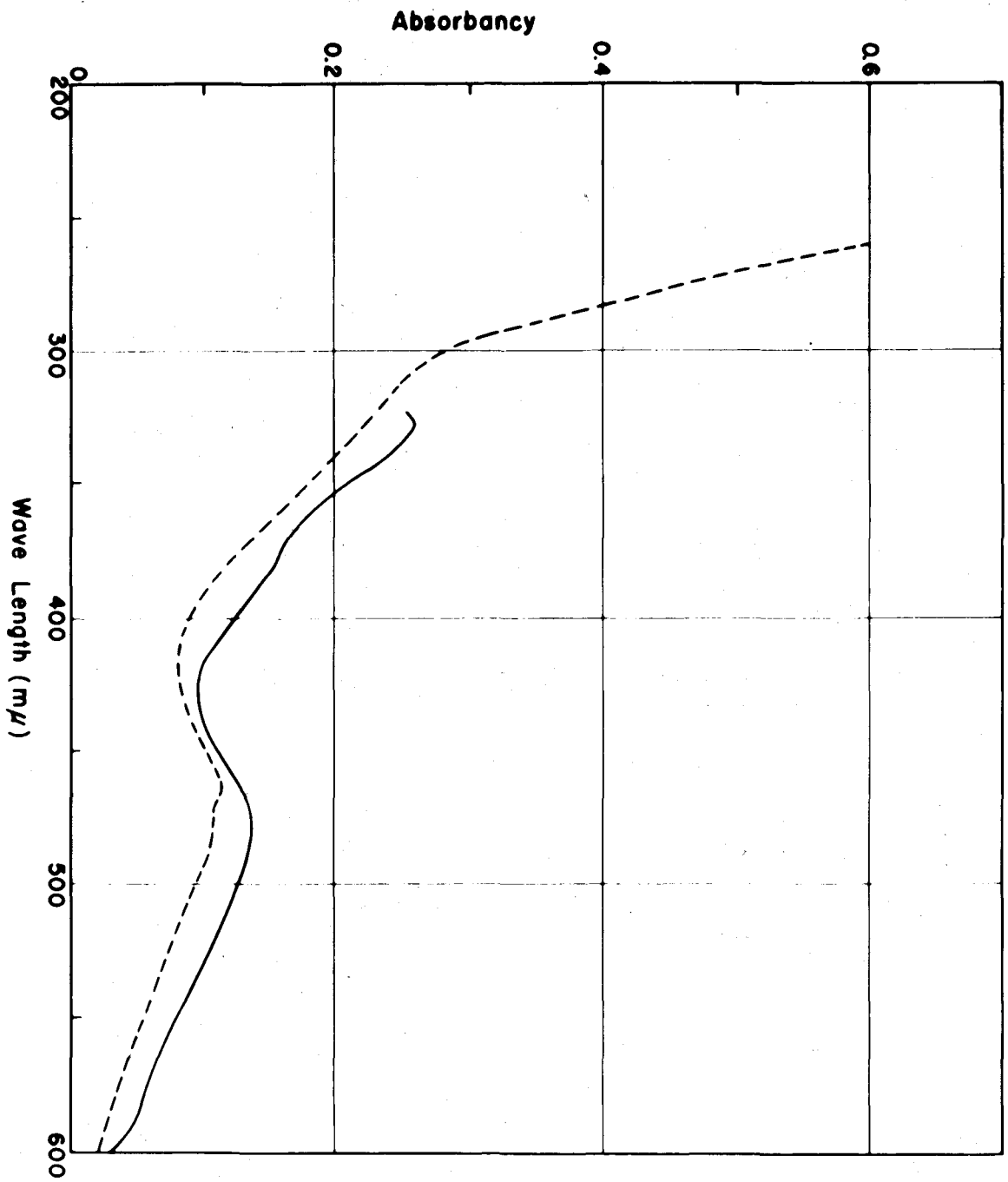
In attempts to quantitatively brominate the red fragment, difficulties similar to those reported earlier with B_{12} and B_{12a} were encountered; the end point was in excess of the blank indicating oxidation of iodide by sources other than excess bromine.

(ii) Reaction with cyanide. Just as B_{12} reacts with excess cyanide in alkaline solution to form a purple

Fig. 10

Ultraviolet and Visible Spectra of Red Acid Fragment-Bromine Products

(----- Acetone insoluble-alkali soluble product, read against water. ——— Acetone-soluble product read against acetone.)


Absorbancy
0
0.2
0.4
0.6
200
300
400
500
600
Wave Length (mμ)

di-cyanide (32), the red acid fragment also forms an intensely purple derivative. The spectrum of this material differs very little from that of B_{12} di-cyanide.

The absorption peak at 278 mμ of the B_{12} cyanide, purple compound is more pronounced than that of B_{12}. This has been interpreted as support for a theory that the benzimidazole is coordinated with cobalt in B_{12} itself, but in the presence of excess cyanide, the benzimidazole is displaced from the cobalt atom and more intense absorption results (25, 33).

The absorption spectrum of a red acid fragment is shown in Fig. 6; that of its cyanide derivative is shown in Fig. 11. The spectrum of the red acid fragment is devoid of any suggestion of benzimidazole, but the cyanide complex shows a very pronounced peak at 278 mμ.

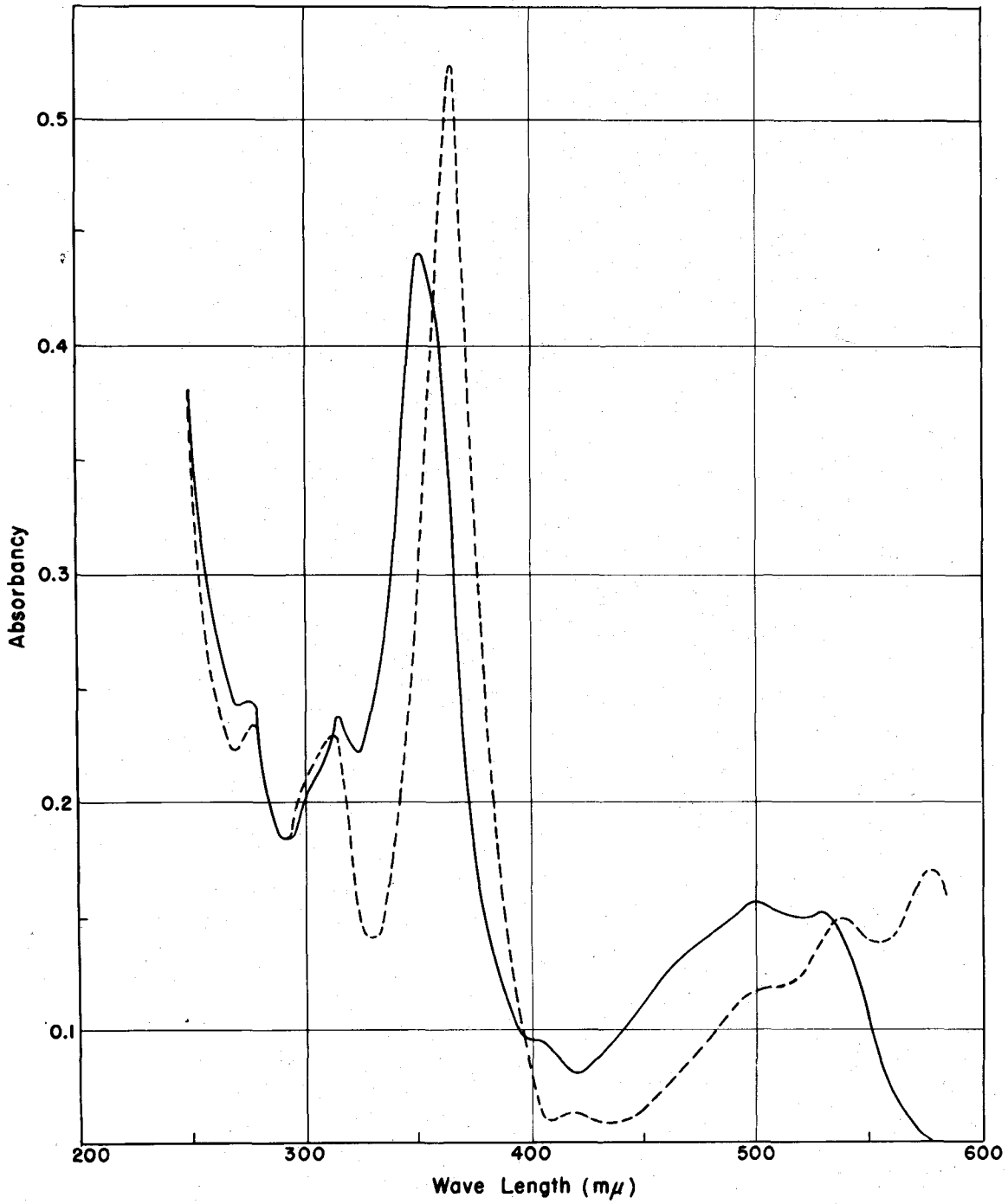
The cyanide compound is stable in alkaline solutions, decomposing slowly at pH values below 7.

It is interesting to note that the spectrum changes as acidified solutions stand, resulting in restoration of the original red acid fragment absorption peaks at 350 mμ, 500 mμ and 530 mμ. However, the peak in the region of 315 mμ found in the cyanide compound, but not present in the red acid fragment remains after this reversion (even after 140 hours). The most interesting point, however, is that the peak at 278 mμ remains, although not quite as intense as the original.

Fig. 11

Ultraviolet and Visible Spectrum of the Cyanide Derivative of the Red Acid Fragment

(-----phosphate buffer, about pH 6, read immediately after formation.
——— same solution read 60 hours later.)


0.5
0.4
0.3
0.2
0.1
Absorbancy
200
300
400
500
600
Wave Length (mμ)

It appears from these studies that cyanide, as well as benzimidazole, may contribute to the absorption in the 278 mμ and 315 mμ regions and that the 1/2 hour spectrum of Fig. 11 represents a di-cyanide derivative, whereas the 60 hour spectrum represents a mono-cyanide derivative.

(iii) Reaction with acetic anhydride. When B_{12} is hydrolyzed with acid, the skeletal portion of the molecule that remains is known to be acidic as a result of the hydrolysis of the amide groups present. The infrared spectrum of this red acid fragment shows double band absorption at 5.6μ and 5.8μ, which is suggestive of anhydrides and lactones. It was felt that it would be interesting to see what would happen to the infrared spectrum if a deliberate attempt was made to prepare an anhydride of the molecule.

Approximately 120 mg. of red acid fragment was dissolved in 2.0 ml. of acetic anhydride and heated to 150° under reflux for 45 minutes. The color of the solution became very dark at the elevated temperature, but on cooling to room temperature, the solution was a dark red-purple color. Some amorphous precipitate settled out on cooling. This precipitate was extracted with two 1 ml. portions of acetic anhydride. Some insoluble, colored material remained.

To the combined acetic anhydride solutions, 40 ml. of dry ether was added. This resulted in the precipitation

of some red-purple, amorphous material which was removed by centrifugation and washed well with ether. To the remaining ether solution an additional 40 ml. of ether was added; further precipitation resulted. This precipitate was removed and washed well with ether. The infrared spectrum of this product, Fig. 12, differed from the original red fragment spectrum (Fig. 8), only in the intensity of the band at 5.8μ.

This change, however, suggested that some alteration had occurred, so the ether-precipitated material was returned to the reaction flask and heated at 130° under reflux with 6 ml. of acetic anhydride for 22 hours. The acetic anhydride was then removed by distillation under reduced pressure to near-dryness. The product was then dried 24 hours over potassium hydroxide pellets in a vacuum desiccator. The residue was then washed with ether and again dried 24 hours. The infrared spectrum of a Nujol mull of this product showed a flat broad band between 5.6μ and 5.8μ (Fig. 13). The material exhibited some solubility in ethylene chloride, so the spectrum in this solvent was obtained (Fig. 14). The broad band of the mull was resolved into two good bands at 5.6μ and 5.7μ. Thus this treatment resulted in a shift of the 5.8 band of the red acid fragment to 5.7μ, the 5.6 band remaining identical with the original. It is apparent that anhydride formation did occur.

Fig. 12

Infrared Spectrum of Red Acid Fragment-Acetic Anhydride Reaction Product

(Reaction time, 45 minutes. Nujol mull; bands at 3.4μ, 6.9μ and 7.3μ are due to Nujol.)

Fig. 13

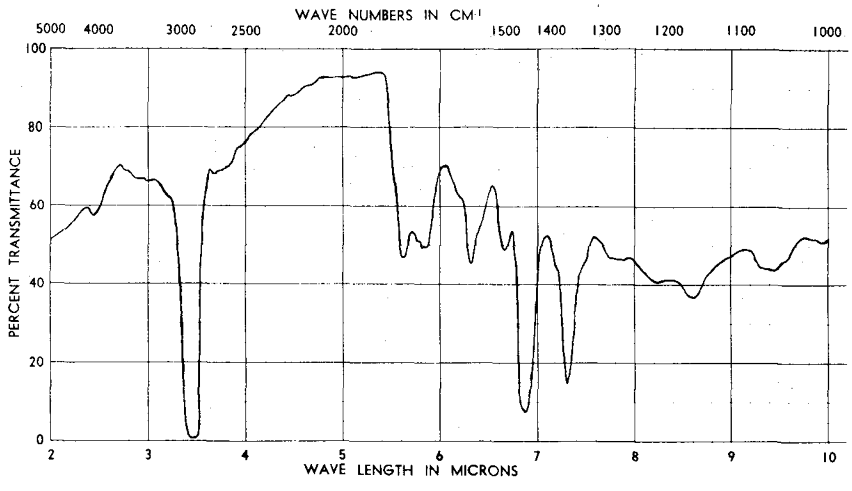
Infrared Spectrum of Red Acid Fragment-Acetic Anhydride Reaction Product

(Reaction time, 22 hours. Nujol mull; bands at 3.4μ, 6.9μ and 7.3μ are due to Nujol.)

Fig. 14

Infrared Spectrum of Red Acid Fragment-Acetic Anhydride Reaction Product

(Reaction time, 22 hours. Ethylene chloride solvent; bands at 3.5μ, 6.8μ and 7.3μ are due to the solvent.)


WAVE NUMBERS IN CM⁻¹
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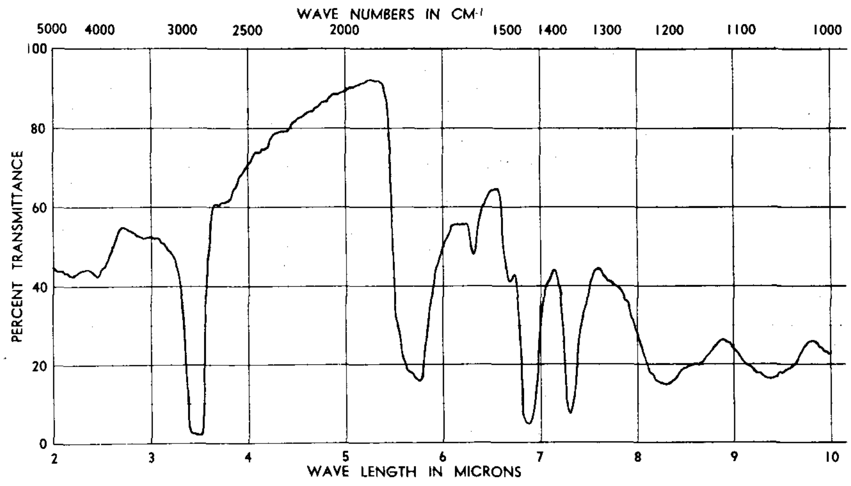
PERCENT TRANSMITTANCE
100 80 60 40 20 0
2 3 4 5 6 7 8 9 10
WAVE LENGTH IN MICRONS


WAVE NUMBERS IN CM⁻¹
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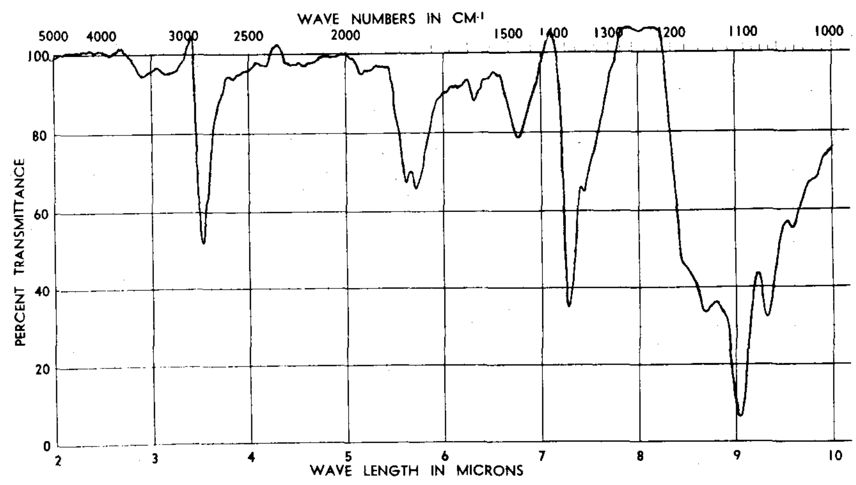
PERCENT TRANSMITTANCE
100 80 60 40 20 0
2 3 4 5 6 7 8 9 10
WAVE LENGTH IN MICRONS


WAVE NUMBERS IN CM⁻¹
5000 4000 3000 2500 2000 1500 1400 1300 1200 1100 1000
PERCENT TRANSMITTANCE
100 80 60 40 20 0
2 3 4 5 6 7 8 9 10
WAVE LENGTH IN MICRONS

(iv) Reaction of B_{12} with acetic anhydride.

It was reasoned that anhydride or cyclic imide formation would result from treatment of B_{12} with acetic anhydride by reaction with the amide groups of B_{12}. An earlier experiment with this objective in view was carried out using phosphorus pentoxide, but the method proved unsatisfactory.

Vitamin B_{12} amounting to 38.0 mg. was heated under reflux with 10.0 ml. of acetic anhydride at 120° to 145° for 4 1/2 hours. The reaction mixture became dark purple and then dark brown at the elevated temperature, but on cooling returned to a dark purple color.

The reaction mixture was evaporated to near-dryness under reduced pressure and the residue washed well with ether. The dry residue was partially dissolved in ethylene chloride and the infrared spectrum obtained (Fig. 16).

The band that B_{12} has at 6.0μ was completely removed but the band at 6.3μ remains. A double band was produced at 5.75μ and 5.9μ, similar to the double band present in both the free red acid fragment and in the reaction product of the red acid fragment and acetic anhydride. However, the relative intensities of these two bands is reversed, for the 5.75μ band is considerably stronger in this instance. It is not possible to decide whether the bands are due to a cyclic imide or an anhydride.

The ultraviolet and visible spectra of the reaction

Fig. 15

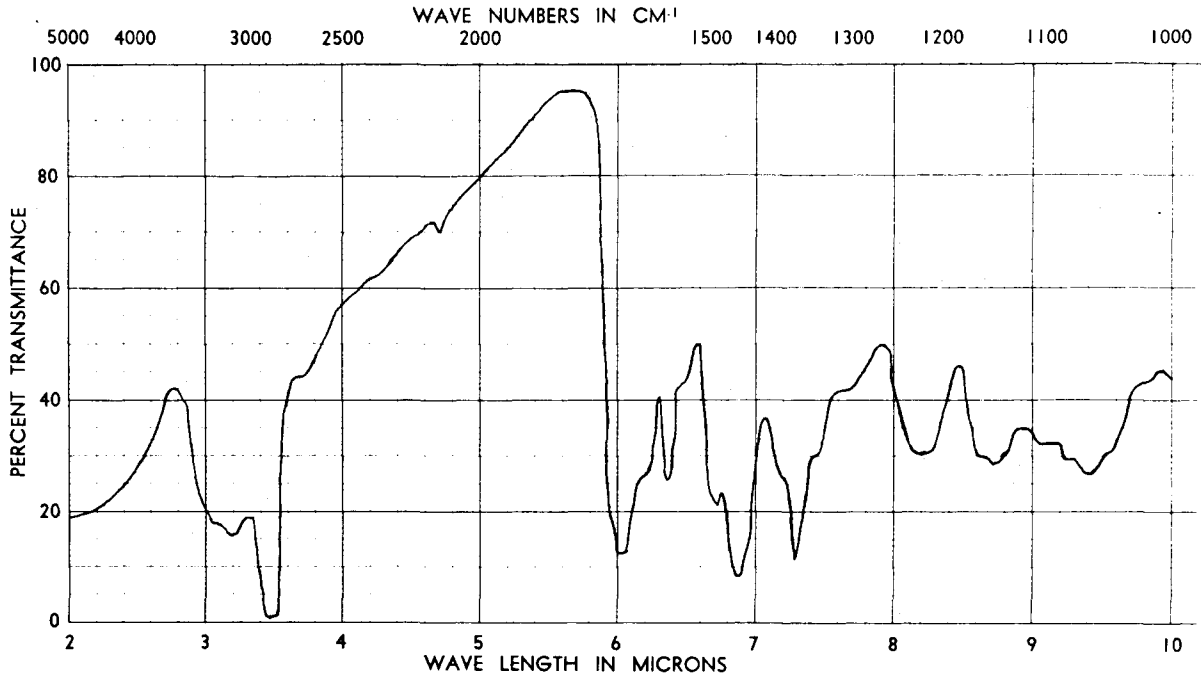
Infrared Spectrum of Vitamin B_{12}

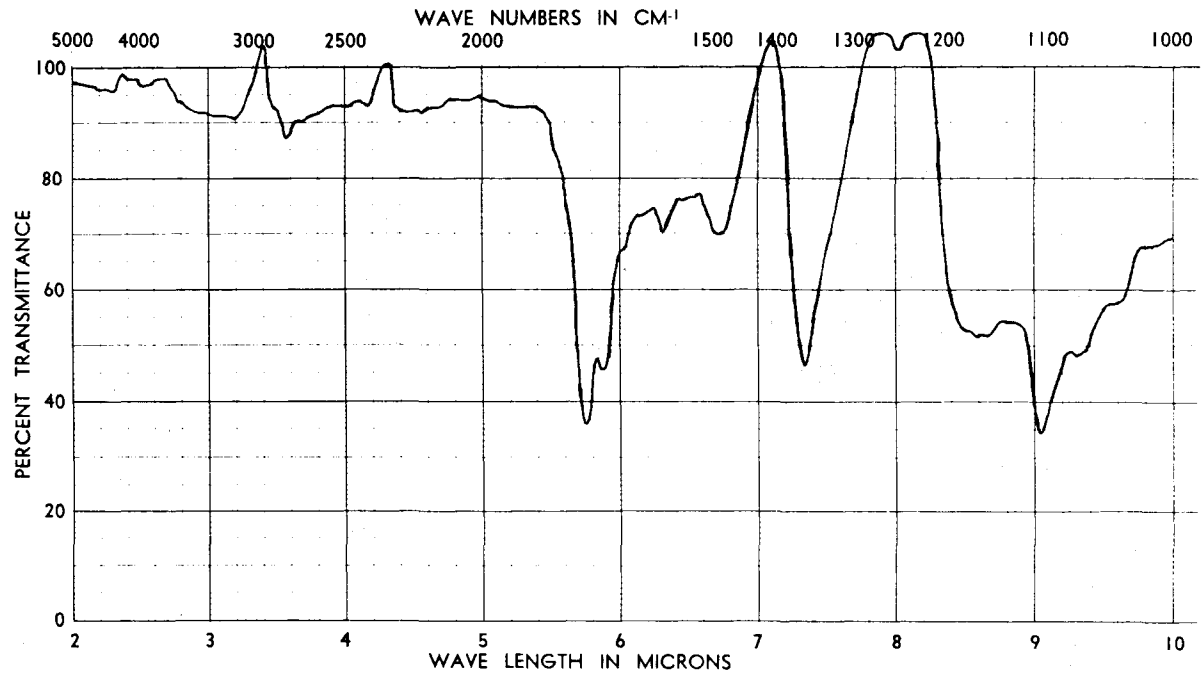
(Nujol mull; bands at 3.5μ, 6.9μ and 7.3μ are due to Nujol.)

Fig. 16

Infrared Spectrum of B_{12}-Acetic Anhydride Reaction Product

(Reaction time, 4.5 hours. Ethylene chloride solvent; bands at 3.5μ, 6.8μ and 7.3μ are due to the solvent.)


WAVE NUMBERS IN CM-1
5000 4000 3000 2500 2000 1500 1400 1300 1200 1100 1000
100
80
60
40
20
0
PERCENT TRANSMITTANCE
2 3 4 5 6 7 8 9 10
WAVE LENGTH IN MICRONS


WAVE NUMBERS IN CM-1
5000 4000 3000 2500 2000 1500 1400 1300 1200 1100 1000
100
80
60
40
20
0
PERCENT TRANSMITTANCE
2 3 4 5 6 7 8 9 10
WAVE LENGTH IN MICRONS

product was obtained in aqueous acetic acid, 5 hours after dissolution (Fig. 17), and in ethylene chloride (Fig. 18). The spectrum in aqueous acetic acid showed a maximum at 350 to 352 mμ, but not as pronounced as in the red acid fragment or in B_{12}. Two small maxima occurred at 475 mμ and 500 mμ.

The spectrum of the reaction product in ethylene chloride showed only an inflection in the 350 mμ region and a small peak at 335 mμ. There was also a peak at 475 mμ and a shoulder around 500 mμ. This spectrum is markedly different from both B_{12} and the red acid fragment.

6. Summary.

a. The method for the hydrolysis of B_{12} to produce the red acid fragment was described.

b. Attempts to purify the red acid fragment by precipitation, countercurrent distribution, ion-exchange and chromatography were presented. No isolation of a pure compound capable of crystallization was attained.

c. Attempts to prepare crystalline metal salts of the red acid fragment failed.

d. The physical properties of the red acid fragment were enumerated.

e. The red acid fragment reacts with bromine to product a product with a markedly different spectrum from that of the red acid fragment.

f. Spectrophotometric studies of the red acid frag-

Fig. 17

Ultraviolet and Visible Spectrum of B_{12}-Acetic Anhydride Reaction Product

(Aqueous acetic acid solution, read 5 hours after dissolution against water blank. ----- solution diluted to obtain ultraviolet spectrum.)

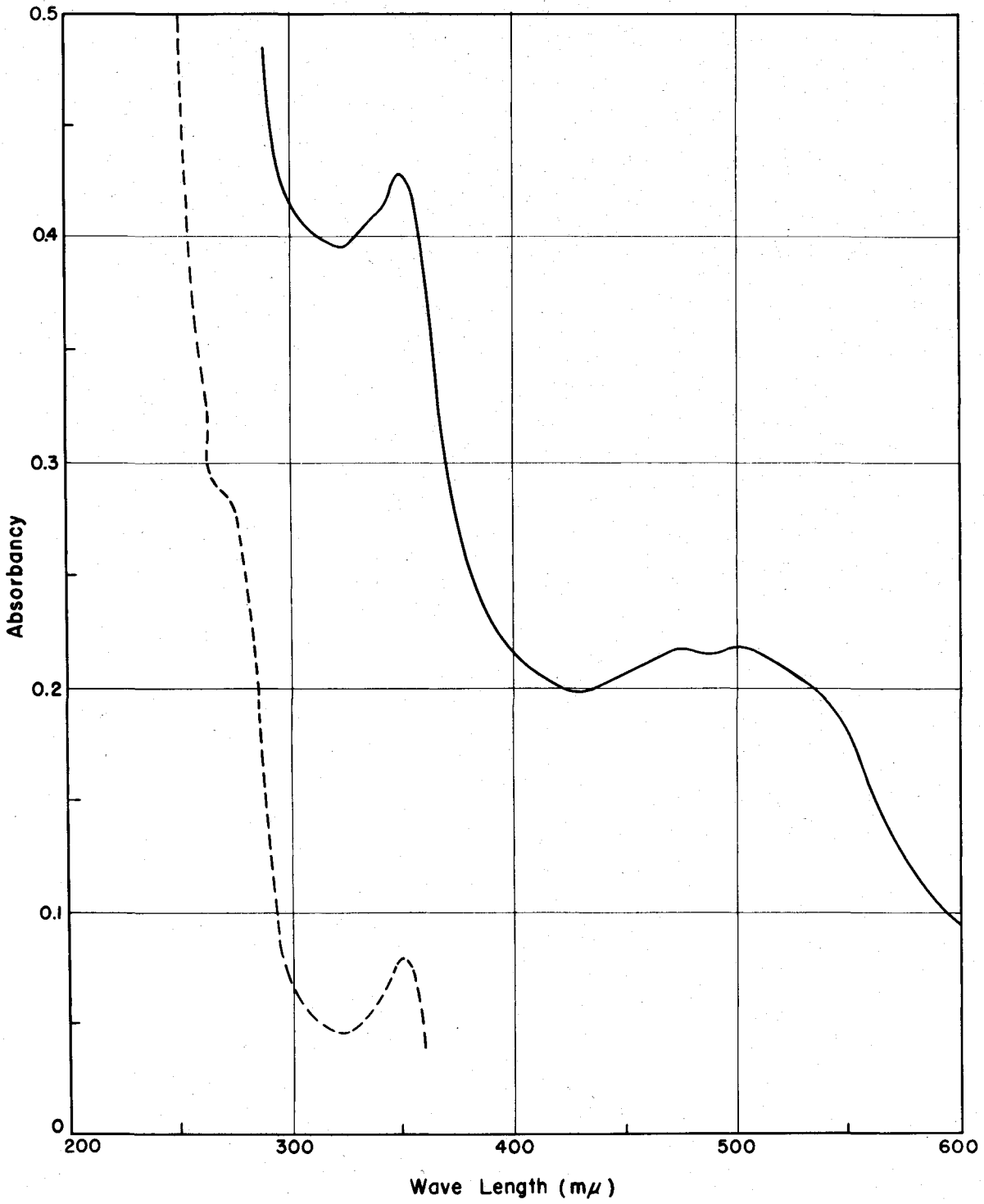
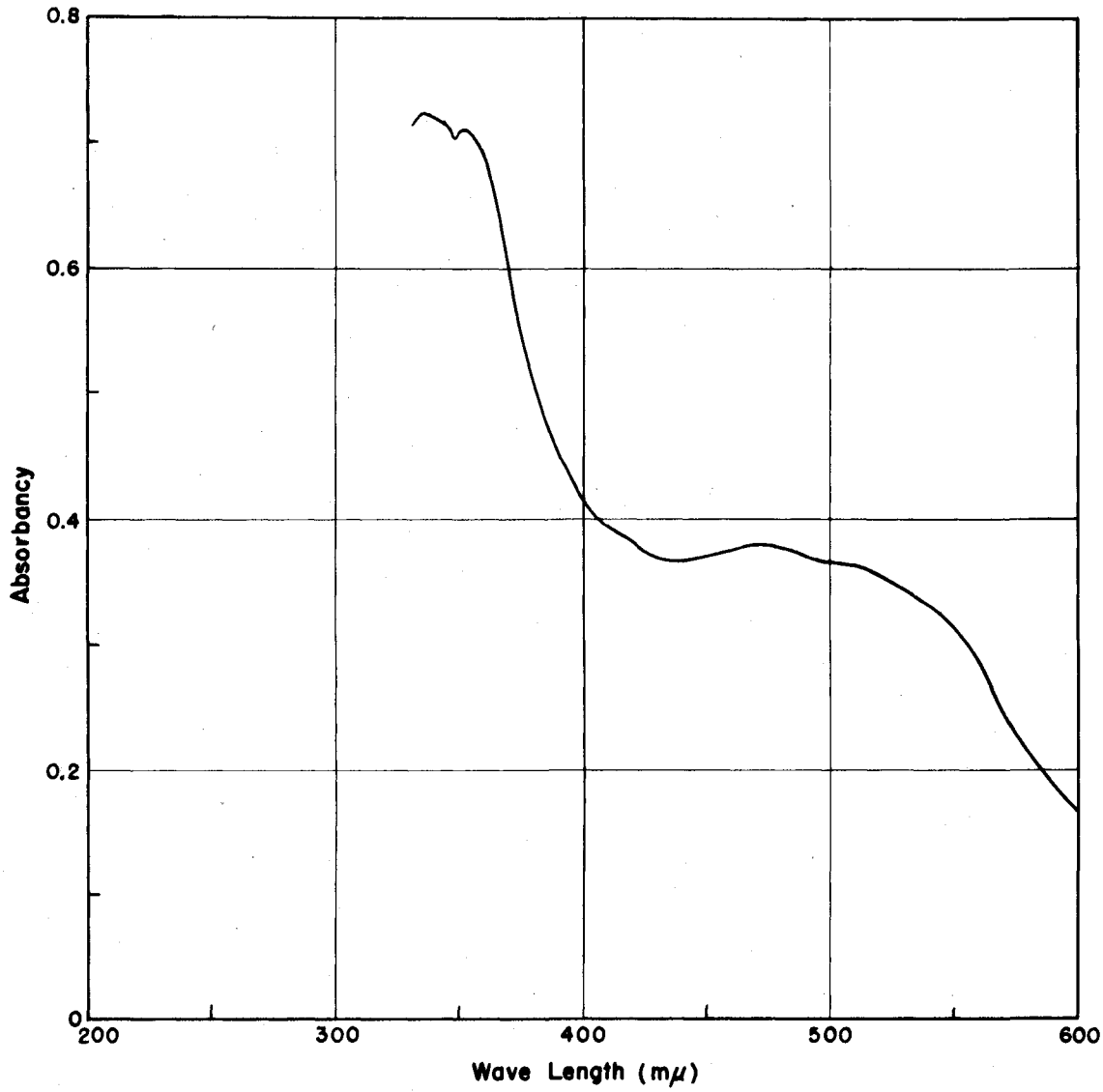

0.5
0.4
0.3
0.2
0.1
0
Absorbancy
200
300
400
500
600
Wave Length (mμ)

Fig. 18

Ultraviolet and Visible Spectrum of B_{12}-Acetic Anhydride Reaction Product in Ethylene Chloride


0.8
0.6
0.4
0.2
0
Absorbancy
200
300
400
500
600
Wave Length (mμ)

ment cyanide derivative were described. Absorption at 278 mμ of this compound suggests that cyanide contributes in this region.

g. The infrared spectrum of the reaction product of the red acid fragment and acetic anhydride indicates that an anhydride was produced.

h. The infrared spectrum of the reaction product of B_{12} and acetic anhydride shows absorption bands which might be interpreted as anhydride or cyclic imide.

E. Reaction of Vitamin B_{12} with Sodium Hypochlorite

1. Introduction.

Vitamin B_{12} has been shown to contain primary amide groups (27). The molecule would therefore be expected to undergo the Hofmann haloamide reaction. With this in mind, an experiment was devised to attempt the preparation of the expected primary amine. Incidental to the primary purpose, the production of ammonia which could be expected if a substituted urea structure is present, was determined. An attempt to determine carbon dioxide evolution went awry.

2. Experimental.

a. Apparatus. A 100 ml. round bottom flask, with a ground glass joint, was fitted with a reflux condenser and a gas inlet tube sealed in the side leading to near the bottom of the flask. Carbon dioxide-free nitrogen was pre-

pared by passing cylinder nitrogen successively through a solution of sodium hydroxide, and a U-tube containing magnesium perchlorate. A delivery tube from the top of the condenser led either to an absorption bulb charged with sulfuric acid, or to an absorption train consisting of a U-tube packed with magnesium perchlorate followed by a Turner bulb containing Ascarite.

b. Method. The system was swept with nitrogen for 30 minutes and a slow stream of nitrogen was maintained throughout the course of the experiment.

An aqueous B_{12} solution containing 57.0 mg. of B_{12} was made up in carbon dioxide-free water and introduced into the reaction flask by means of pipets previously flushed with nitrogen. The flask was cooled in an ice bath and 1.0 ml. of 0.260 N sodium hypochlorite, 6.1 equivalents, was added. This was followed by 2.0 ml. of 40 percent sodium hydroxide. The final hydroxide concentration was about 1.8 N. The absorption flask was charged with 10 ml. of 1 N sulfuric acid.

The reaction mixture was allowed to stand at ice bath temperature for 30 minutes. It was then warmed to room temperature for 1 hour. At the end of this time, the original acid trap was replaced by a second, identical trap and the solution warmed at 78° for 1.5 hours. The Ascarite train was then put in place of the acid trap and the system swept with nitrogen for 30 minutes. The Turner bulb was

removed, weighed and replaced, and the solution acidified with 3.0 ml. of concentrated hydrochloric acid. The final acid concentration was about 1.1 N.

The acid traps were subsequently analyzed for ammonia by Nesslerization, and the Turner bulb was weighed to determine the amount of carbon dioxide produced.

c. Characterization of the reaction mixture. The alkaline reaction mixture failed to yield any colored material extractible by ether, butanol or ethylene chloride.

Evaporation of a 1 ml. portion of the alkaline reaction mixture to near-dryness resulted in solution of considerable colored material on addition of alcohol, but only on the first addition. Solubility probably requires about 70-80 percent ethanol. This material gave appreciable brown-red amorphous precipitate and minute amounts of apparently crystalline material of undetermined color on treatment with alcoholic picric acid. The precipitate was soluble in aqueous alcohol and apparently a picrate. Attempts to isolate a crystalline compound failed.

The addition of potassium cyanide to the alkaline reaction mixture resulted in the intense purple color typical of all B_{12} and cobalt-containing red acid fragments.

Acidification of the alkaline solution caused no noticeable color change. Some color could be extracted by butanol immediately, following acidification, and on

long standing in contact with butanol, the majority of the color moved into the organic layer.

An acid solution of the reaction mixture was insoluble in ethylene chloride, but the addition of ethanol resulted in a homogeneous red solution and salt precipitation. The dry material obtained on evaporation was readily soluble in absolute alcohol.

3. Results and observations.

The carbon dioxide determination failed because hydrochloric acid fumes were swept over when the solution was acidified.

No ammonia was found by Nesslerization of the acid traps.

The reaction mixture slowly became deeper red in color at ice bath temperature, and on allowing to warm to room temperature, it became dark red-violet in color. The color did not apparently intensify on heating at 78°.

A white, gelatinous, water and alcohol-insoluble material settled from the original reaction mixture, probably a benzimidazole moiety cleaved under the alkaline conditions.

4. Discussion.

Some amine formation probably did occur, as evidenced by the formation of an insoluble picrate. However, the lack of solubility in ether and other organic solvents from alkaline solution, plus the solubility of red mater-

ial in butanol and ethanol on acidification strongly suggests that hydrolysis to produce an acidic compound occurred concomitantly. The production of the white gel is further evidence of hydrolysis.

Hydrolysis could not have occurred if all amide groups had reacted with hypochlorite; there was only one equivalent of hypochlorite in excess of five amide groups. If, however, there was competition for hypochlorite by olefinic unsaturation to yield halohydrins, then some amide groups could not undergo reaction for lack of reagent. This is undoubtedly the case, for it is believed that unsaturation does exist as a result of bromination experiments.

5. Summary.

a. No single product was isolated from the Hofmann reaction mixture, although some amine formation probably occurred.

b. No ammonia was produced as a result of reaction of B_{12} with sodium hypochlorite.

c. Some properties of the reaction mixture are recorded.

F. Mercurated Vitamin B_{12}

1. Introduction.

Amides are known to form mercury derivatives in which the mercury is attached to the amide nitrogen. The product formed often depends on whether mercuric

oxide or mercuric acetate is used as the reagent. For example, mercuric oxide reacts with acetamide to give mercury acetamide (34), CH_3-CO-NH-Hg-NH-CO-CH_3. However, the reaction of mercuric acetate with acetamide yields acetoxymercuri acetamide, CH_3-CO-NH-Hg-CO_2-CH_3, even though two moles of acetamide are employed (35). Succinamide reacts with mercuric oxide to give mercury succinamide (36-37), $(CH_2\text{-CO-NH})_2Hg$. Urea reacts with mercuric acetate to form mercury carbamide, $CO(NH)_2Hg$, or di-acetoxymercuri carbamide, $(CH_3\text{-}CO_2\text{-Hg-NH})_2CO$, depending on whether one or two molecular proportions of urea are employed (35).

Being an amide, B_{12} might be expected to yield a mercury derivative, although admittedly the derivative might well be rather complex inasmuch as five amide groups are present.

2. Experimental.

a. Reaction with mercuric oxide. Thirteen mg. of B_{12} and one mg. of mercuric oxide in about 4 ml. of water was warmed over a free flame. A red amorphous precipitate formed in the hot solution within ten minutes and further heating did not increase the amount of precipitate. The supernatant solution was still somewhat red in color and a small amount of unreacted mercuric oxide was visible. The precipitate dissolved readily on warming with a small amount of potassium hydroxide.

In a second experiment, 8 mg. of B_{12} and a large

excess of mercuric oxide was heated over a free flame in about 10 ml. of water. The solution was concentrated by heating to a final volume of about 3 ml. On cooling an amorphous red precipitate settled out along with unreacted mercuric oxide. The precipitate was extracted with about 8 ml. of hot water and filtered while hot to remove unreacted mercuric oxide. When the solution cooled, an amorphous red precipitate settled out along with a small amount of mercuric oxide, which has appreciable solubility in hot water. The supernatant solution was somewhat colored, and on repeating the hot water extraction several times, a point was reached where no red precipitate formed on cooling, but a white solid appeared, presumably mercuric hydroxide.

b. Reaction with mercuric acetate. Fifty mg. of B_{12} was dissolved in 20 ml. of 95 percent alcohol. Fifteen ml. of an alcoholic solution containing 36.0 mg. of mercuric acetate and 3 drops of glacial acetic acid was added to the B_{12} solution with stirring. The mixture was allowed to stand 5 hours and no precipitation occurred. On the addition of 1 drop of 1 N sodium hydroxide, however, immediate, flocculent, amorphous precipitation occurred and very little color remained in the supernatant liquid. The precipitate was removed by centrifugation and washed well with portions of alcohol.

On repeated extraction of a portion of the precipitate with hot water, the material behaved in a manner

similar to the product from the reaction of mercuric oxide with B_{12}; that is, color to the solution and the appearance of a grey-white precipitate.

The red precipitate dissolved quite readily in dilute sulfuric acid, presumably with decomposition, for mercury was readily extracted from the solution by dithizone. The spectrum of a sulfuric acid solution is shown in Fig. 19. The material dissolved only very reluctantly over a long period of time in dilute sodium hydroxide. The infrared spectrum of the dry material as a Nujol mull is shown in Fig. 21.

c. Analysis for cobalt and mercury. Mercury determinations were performed colorimetrically by extraction of a 1 N sulfuric acid solution of the compound with dithizone in chloroform (30). Cobalt determinations were made on a nitric acid-perchloric acid digest of a sample of the compound by the nitroso-R salt method (30).

3. Results and Discussion.

B_{12} reacts with both mercuric oxide and mercuric acetate. The mere fact that reaction does occur may be construed as a confirmation of the presence of primary amide groups, although it is recognized that many other types of compounds are capable of reacting with mercuric ion. No analyses were performed on the product obtained from reaction with mercuric oxide because of the difficulty in obtaining a product free of extraneous mercury.

Mercury to cobalt ratios obtained by analyses of the product obtained by reaction with mercuric acetate gave values of 2.65, 2.70, 2.76, 2.82, 2.82 and 3.17 or an average of 2.82.

With 5 amide groups present, it might be expected that a value of 2.50 would be obtained by formation of 2 intramolecular derivatives with the fifth amide group of two molecules of B_{12} involved in an intermolecular linkage.

Another possibility is that the acetoxymercuri derivative of three amide groups is formed. It should be recalled that the rate of production of ammonia on hydrolysis showed that three of the amide groups are much more susceptible to hydrolysis than the remaining two. Thus, it might be expected that these three groups would be more reactive toward other chemical attack than the remaining two. Unfortunately, it is not possible at this time to positively say which, if either, of the above two possibilities is the actual case, because of the size and complexity of the molecule.

All attempts to obtain a crystalline mercury derivative failed. The best approach to crystallization involved slow diffusion of trimethylamine into an aqueous or alcoholic solution of the product in the presence of a small amount of acetic acid. As the pH increased, precipitation occurred, but the precipitate was always amorphous.

Fig. 19

Ultraviolet and Visible Spectrum of Mercurated Vitamin B_{12}

(----- Solution in 1 N sulfuric acid read immediately.
——— Solution in 1 N sulfuric acid read 24 hours after dissolution.)

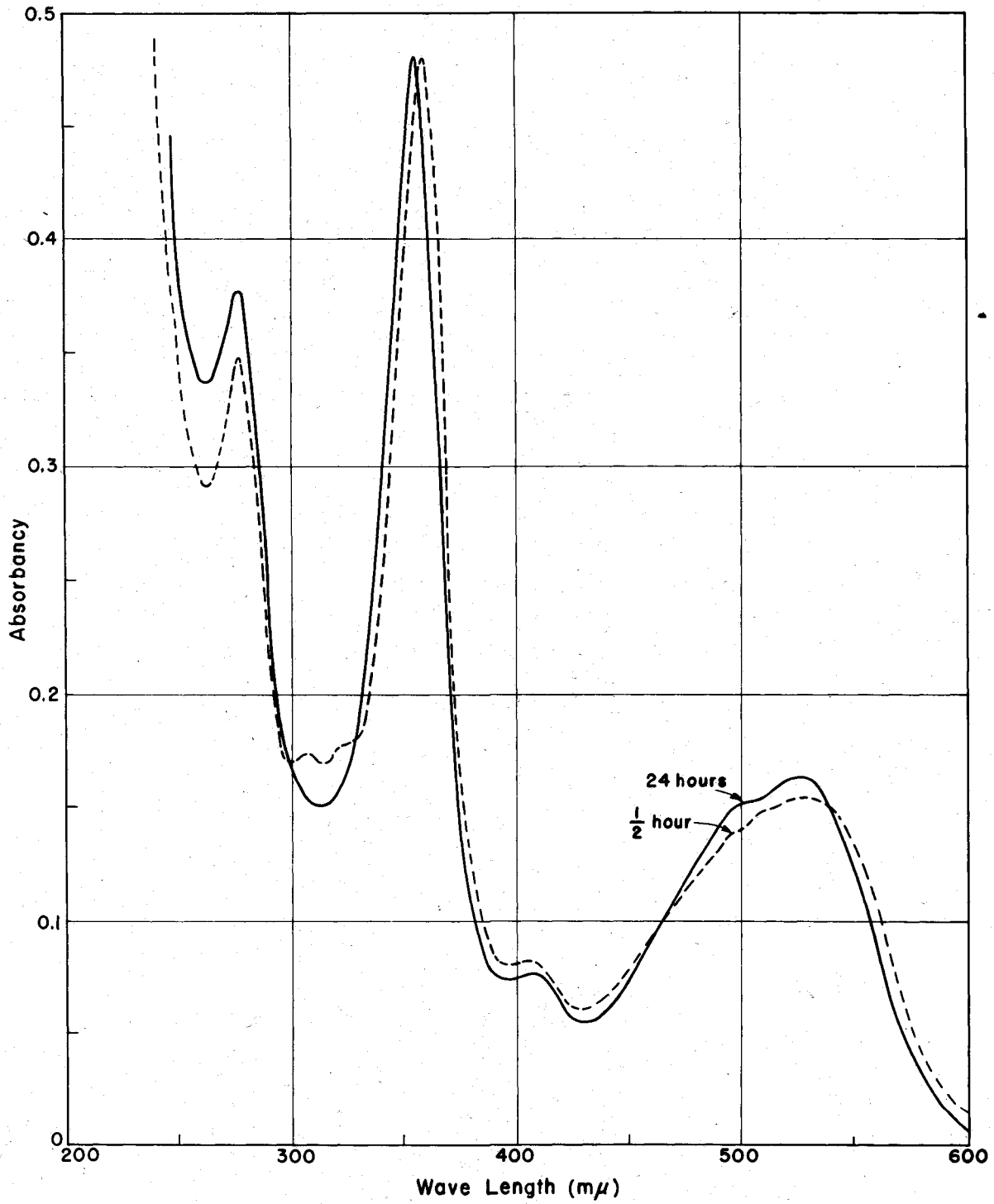

0.5
0.4
0.3
0.2
0.1
0
Absorbancy
200
300
400
500
600
Wave Length (mμ)
24 hours
½ hour

Fig. 20

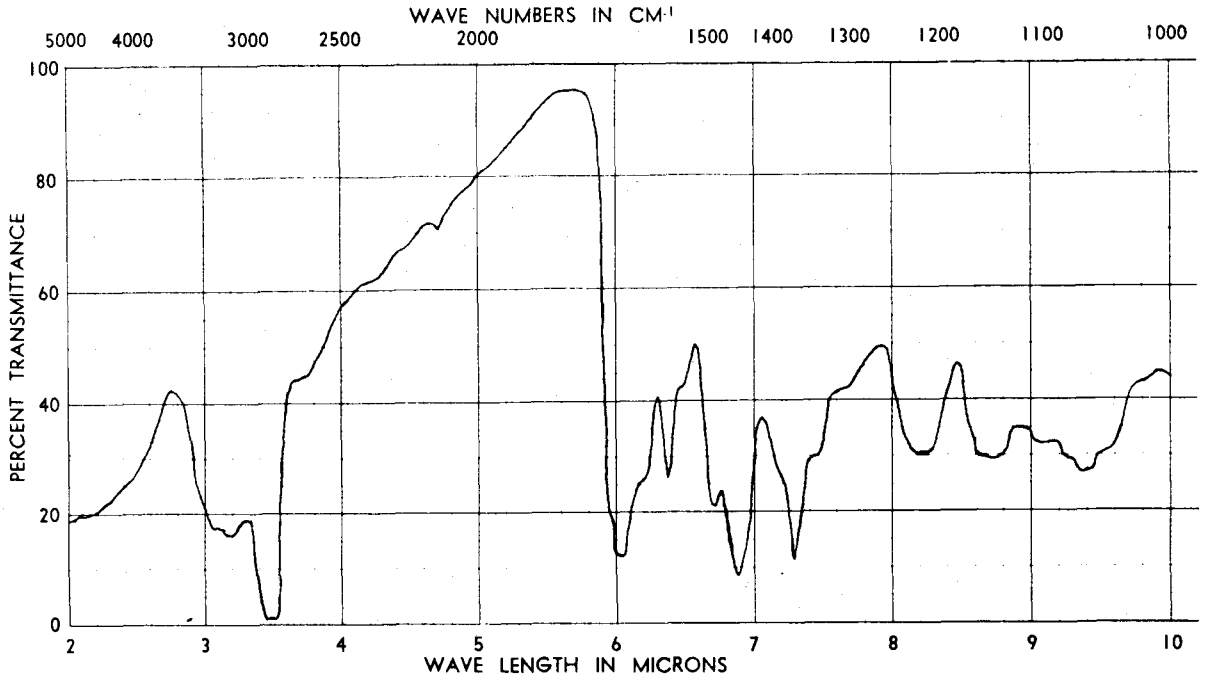
Infrared Spectrum of Vitamin B_{12}

(Nujol mull; bands at 3.5μ, 6.9μ and 7.3μ are due to Nujol.)

Fig. 21

Infrared Spectrum of Mercurated Vitamin B_{12}

(Nujol mull; bands at 3.5μ, 6.9μ and 7.3μ are due to Nujol.)


WAVE NUMBERS IN CM-1
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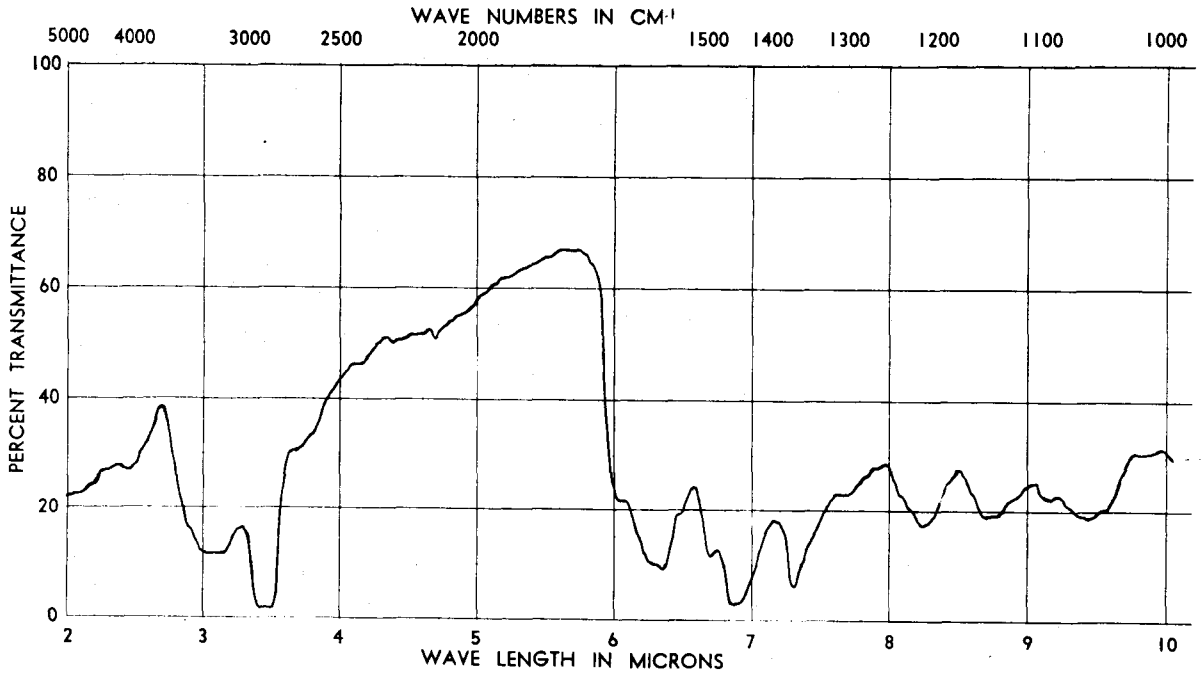
100
80
60
40
20
0
PERCENT TRANSMITTANCE
2 3 4 5 6 7 8 9 10
WAVE LENGTH IN MICRONS


WAVE NUMBERS IN CM-1
5000 4000 3000 2500 2000 1500 1400 1300 1200 1100 1000
100
80
60
40
20
0
PERCENT TRANSMITTANCE
2 3 4 5 6 7 8 9 10
WAVE LENGTH IN MICRONS

The absorption spectrum of a solution obtained by hot water extraction of the reaction product of mercuric oxide and B_{12} showed absorption peaks and absorbancies similar to those of B_{12a}. It is probably that the treatment with hot water caused hydrolysis of the mercury compound to produce mercuric hydroxide and B_{12a}. The excess mercuric oxide apparently removed the cyanide groups during the formation of the mercury derivative.

The spectrum of an aqueous solution of the product from mercuric acetate and B_{12}, prepared with hot water, could not be distinguished from that of B_{12}. In this case, the cyanide is probably still present for the conditions of reaction were much milder than in formation of the mercuric oxide product. Furthermore, the spectrum of the product in 1 N sulfuric acid immediately and 24 hours after dissolution (Fig. 19) shows a progressive shift toward the B_{12a} spectrum. This is expected as a result of displacement of cyanide in acid solution. In any event, it is evident that a solution of the material in either water or acid shows little change in spectrum from that of B_{12} or B_{12a}, and it is concluded that the compound in solution dissociates to yield the original starting material. There is good evidence for this assumption, for bio-assay of the material shows activity approximately 80 percent that of B_{12}.

Finally, examination of Fig. 21 reveals that the

mercuric acetate product still contains cyanide (band at 4.7μ), but the strong band at 6.0μ in B_{12}, which we have interpreted as an amide band, has been nearly completely obliterated. Thus, it seems certain that reaction has occurred with the amide groups.

4. Summary.

a. Both mercuric oxide and mercuric acetate react with vitamin B_{12}, presumably with the amide groups of B_{12}.

b. The reaction product of B_{12} with mercuric oxide and mercuric acetate is probably not the same.

c. Infrared, ultraviolet and visible spectra were obtained for the mercuric acetate reaction product.

d. No crystals of either the mercuric oxide or mercuric acetate product were obtained.

G. Catalytic Behavior of Vitamin B_{12a} in the Oxidation of Iodide by Air

1. Introduction.

In the course of attempts to measure quantitatively the uptake of bromine by vitamins B_{12} and B_{12a}, it was discovered that vitamin B_{12a} is a very active catalyst for the air oxidation of iodide to free iodine, and that to a much lesser extent, B_{12} is also active. The method employed in the bromination experiments involved the addition of an excess standard bromide-bromate reagent to an acid solution of the vitamin. The excess of bromine was determined subsequently by addition of iodide and titration of

the free iodine produced with thiosulfate. However, because of the catalytic effect of the vitamins in the oxidation of iodide, the presence of even trace amounts of dissolved oxygen caused drifting at the end point and the amount of iodine produced by only the excess bromine was thus seriously in error. In the bromination of B_{12a}, the results were extremely erratic and unreasonably high.

Bromination experiments using B_{12}, which was much less active in catalysis, indicated a value in the neighborhood of 4 to 5 bromine atoms consumed per mole of B_{12}. The uncertainty of the absolute value does not even permit speculation as to how the bromine was consumed, however. With the technique developed in this section for the study of the catalytic effect on the air oxidation of iodide, it should be possible at some future time to get reliable results in the bromination of B_{12} and B_{12a}.

The velocity of the B_{12a} catalysis of iodide oxidation and the turnover number of B_{12a} in this reaction have been measured at various concentrations of B_{12a}, iodide and sulfuric acid. Because it seemed possible that this catalytic effect is exerted through the union of B_{12r} (in which the cobalt atom is bivalent) with molecular oxygen, several experiments were conducted to determine if B_{12a} is a binuclear compound in which the cobalt atoms of two molecules of the vitamin are linked through a peroxo group.

2. Experimental.

a. Reagents. Vitamin B_{12a} was prepared by the hydrogenation of crystalline vitamin B_{12}, and treatment of the B_{12r} so obtained with air (8). The concentration of solutions of this product was determined spectrophotometrically using a Beckman DU instrument and the value $E^{1\%}_{1\ cm.} = 150$ at 352.5 mμ (phosphate buffer, pH 7.4).

Oxygen-free nitrogen was obtained by passing tank nitrogen through a train of vanadous sulfate (28).

b. Reaction of B_{12a} with iodide. B_{12a}, potassium iodide and sulfuric acid were brought together in the complete absence of oxygen. Any iodine liberated was titrated with thiosulfate.

The reaction vessel used consisted of a wide-mouth, conical flask with two smaller necks. The large opening was closed by a four-hole rubber stopper through which were passed (a) a buret tip, (b) dropping funnel, (c) a gas inlet tube bearing a fritted glass dispersion cylinder at its lower end, and (d) a gas outlet tube leading to a water trap outside the flask. Platinum and saturated calomel electrodes were introduced through the smaller side arms.

The solution of potassium iodide was placed in the flask and, with magnetic stirring, was deaerated by the passage of a slow stream of oxygen-free nitrogen through the solution for two hours. Concurrently, dilute sulfuric

acid, placed in the dropping funnel, was deaerated by bubbling through it a slow stream of oxygen-free nitrogen. The sodium thiosulfate solution, stored in a Machlett buret, had previously been freed of dissolved oxygen in a similar manner. Immediately preceding the reaction, crystalline B_{12a} was dissolved in carefully deaerated water. Aliquots of this were introduced into the reaction vessel using a pipet previously flushed with nitrogen. Other aliquots were taken for a spectrophotometric measurement of the B_{12a} content. Sulfuric acid was then added to the reaction mixture. After an interval of several hours any iodine liberated was titrated potentiometrically with thiosulfate. The thiosulfate solution (approximately 0.001 N) was standardized potentiometrically by the titration of aliquots of a standard potassium iodate solution treated with potassium iodide and hydrochloric acid. The potassium iodate solution was prepared by weight from primary standard material.

A typical reaction mixture contained 0.0135 g. of B_{12a}, 10.00 g. of potassium iodide and 10.0 ml. of 4.0 N sulfuric acid, all in a total volume of 235 ml.

It was necessary to take rigid precautions to exclude oxygen; in particular, the nitrogen was bubbled through the vanadous sulfate train very slowly and all rubber tubing was eliminated from the gas train.

No iodine was liberated under these conditions. It

was found, however, that the addition of thiosulfate to the completely deaerated reaction mixture resulted in a slow disappearance of the typical orange-red color of B_{12a} and the formation of a yellow-brown color similar to that of B_{12r} obtained by hydrogenation. The B_{12a} color was readily restored on the introduction of air and the formation of free iodine rapidly followed.

c. <u>The Catalytic action of B_{12a} on the oxidation of iodide by air</u>. <u>Effect of varying conditions</u>. A solution of potassium iodide, containing also the starch indicator, was placed in a 500 ml. wide-mouth conical flask equipped with a three-hole rubber stopper through which passed a gas inlet tube, buret tip and outlet tube. The solution was stirred vigorously magnetically. The gas inlet tube ended in a fritted glass dispersion cylinder. The solution was deaerated for 20 minutes with oxygen-free nitrogen. Oxygen-free sulfuric acid was then pipetted into the flask through the outlet tube. The B_{12a} solution was then added. The flow of nitrogen was stopped, a stream of air was started through the solution, and zero time was taken. The air was delivered under constant pressure, atmospheric pressure plus 5 cm. of mercury, obtained by the usual T-tube pressure regulator. After 5.0 minutes, the air stream was replaced abruptly by a stream of nitrogen. This was continued 5.0 minutes. The free iodine was then titrated with

standard thiosulfate, the latter being stored under oxygen-free nitrogen in a Machlett buret and delivered to the reaction vessel without exposure to air. A small stream of nitrogen was continued during the titration.

The total volume in each reaction was 320 ml. In the course of the study, the quantities of B_{12a}, potassium iodide and sulfuric acid were varied as described below. Ten ml. of a 1 percent solution of starch was added in each case. The sodium thiosulfate solution (0.0095 N) was standardized by titrating aliquots of a standard solution of potassium iodate treated with potassium iodide and hydrochloric acid; this thiosulfate solution remained constant in concentration for over a month. A 1 percent solution of starch served as indicator.

The experiments were all run at room temperature, $27^{\circ} \pm 2^{\circ}$. No closer attention was paid to temperature inasmuch as a larger source of error is inherent in the method; see below under results. In several experiments the gases leaving the reaction vessel were bubbled through a solution of potassium iodide; no iodine was collected in this trap in any of the runs.

(i) Variation of B_{12a} concentration. In each reaction mixture the solution contained 10.00 ml. of 4.357 N sulfuric acid (final concentration: 0.136 N), 10.00 g. of potassium iodide and 10.0 ml. of 1 percent starch. The amount of B_{12a} (aliquots of solutions standardized spectro-

photometrically) varied from 11 μg. to 448 μg. The total volume of the solution was 320 ml. The results of this series of experiments are shown in Fig. 22.

(ii) Variation of potassium iodide concentration. In each reaction mixture was placed 79.0 μg. of B_{12a}, 10.00 ml. of 4.357 N sulfuric acid and 10.0 ml. of 1 percent starch. The amount of potassium iodide was varied from 2.0 to 35.0 g. The results are shown in Fig. 23.

(iii) Variation of sulfuric acid concentration. In each reaction mixture was placed 122 μg. of B_{12a}, 10.00 g. of potassium iodide and 10.0 ml. of 1 percent starch. The concentration of sulfuric acid in the final solution was varied from 0.0136 N to 0.272 N. The results are shown in Fig. 24.

(iv) Effect of aging the B_{12a} solution. Crystalline B_{12a} was dissolved in water and aliquots of the solution taken at intervals for spectrophotometric measurement and for a determination of its catalytic effect. The spectrophotometric measurements were made on a solution buffered at pH 7.4 with 0.2 M phosphate. The conditions for catalysis determinations were held constant: 320 ml. total volume, 10.0 ml. of 4.357 N sulfuric acid, 10.0 g. of potassium iodide, 10 ml. of 1 percent starch, 5.0 minutes aeration and 5 minutes sweeping with nitrogen.

Fig. 22

Catalytic Oxidation of Iodide by Air as a Function of the Amount of B_{12a} Present

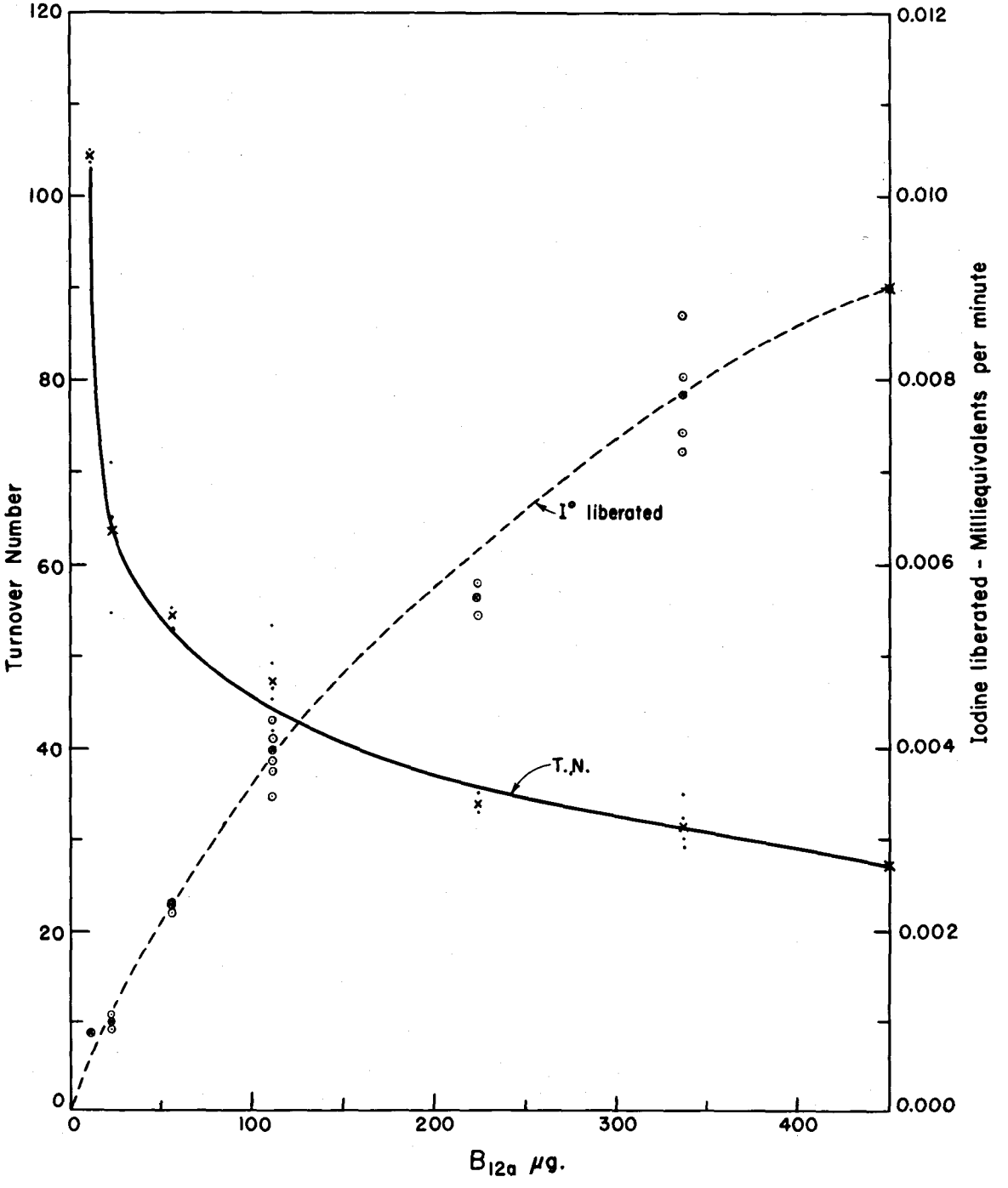

120
100
80
60
40
20
0
0.012
0.010
0.008
0.006
0.004
0.002
0.000
0
100
200
300
400
Turnover Number
Iodine liberated - Milliequivalents per minute
B12a µg.
I° liberated
T.N.

Fig. 23

Catalytic Oxidation of Iodide by Air as a Function of Potassium Iodide Concentration

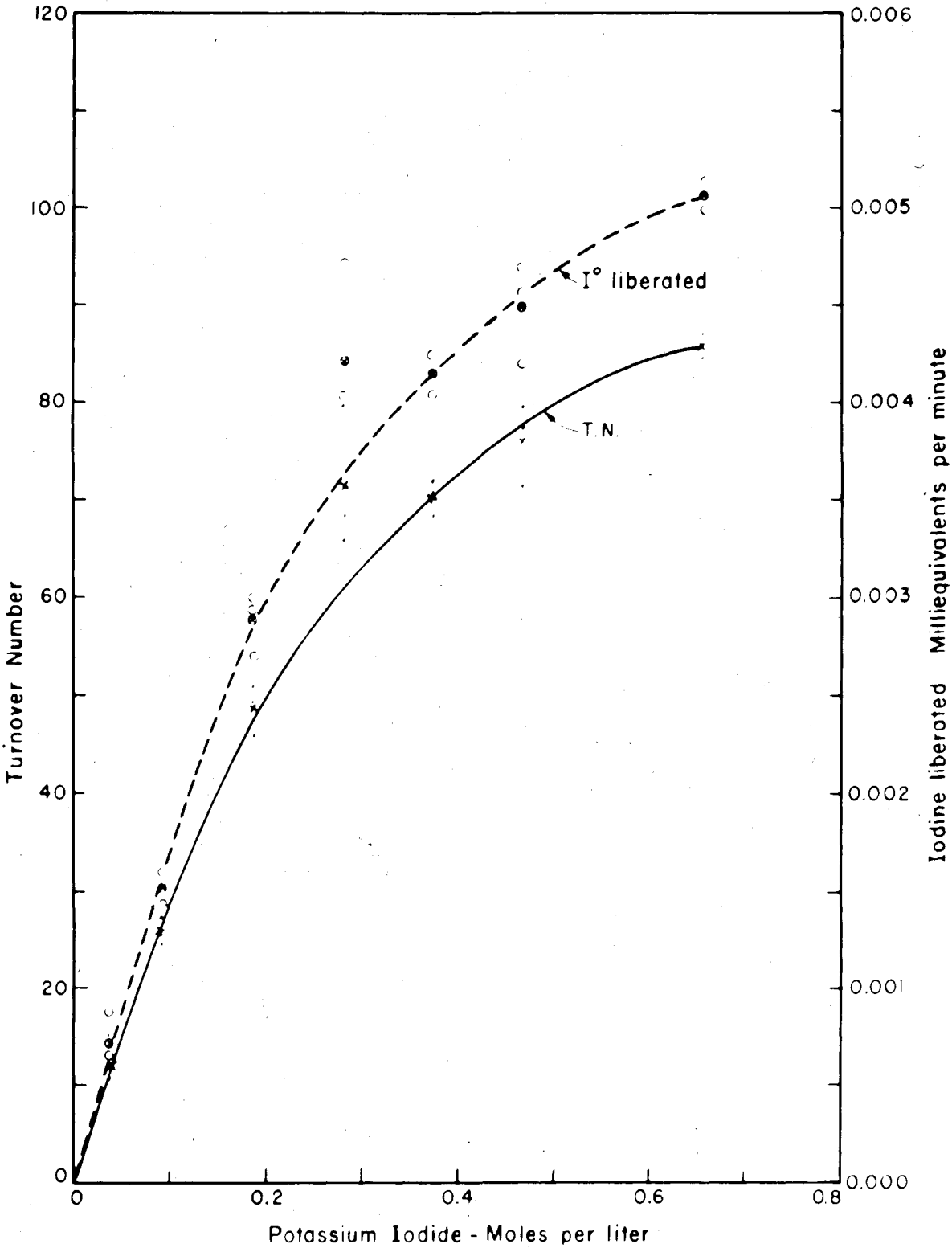
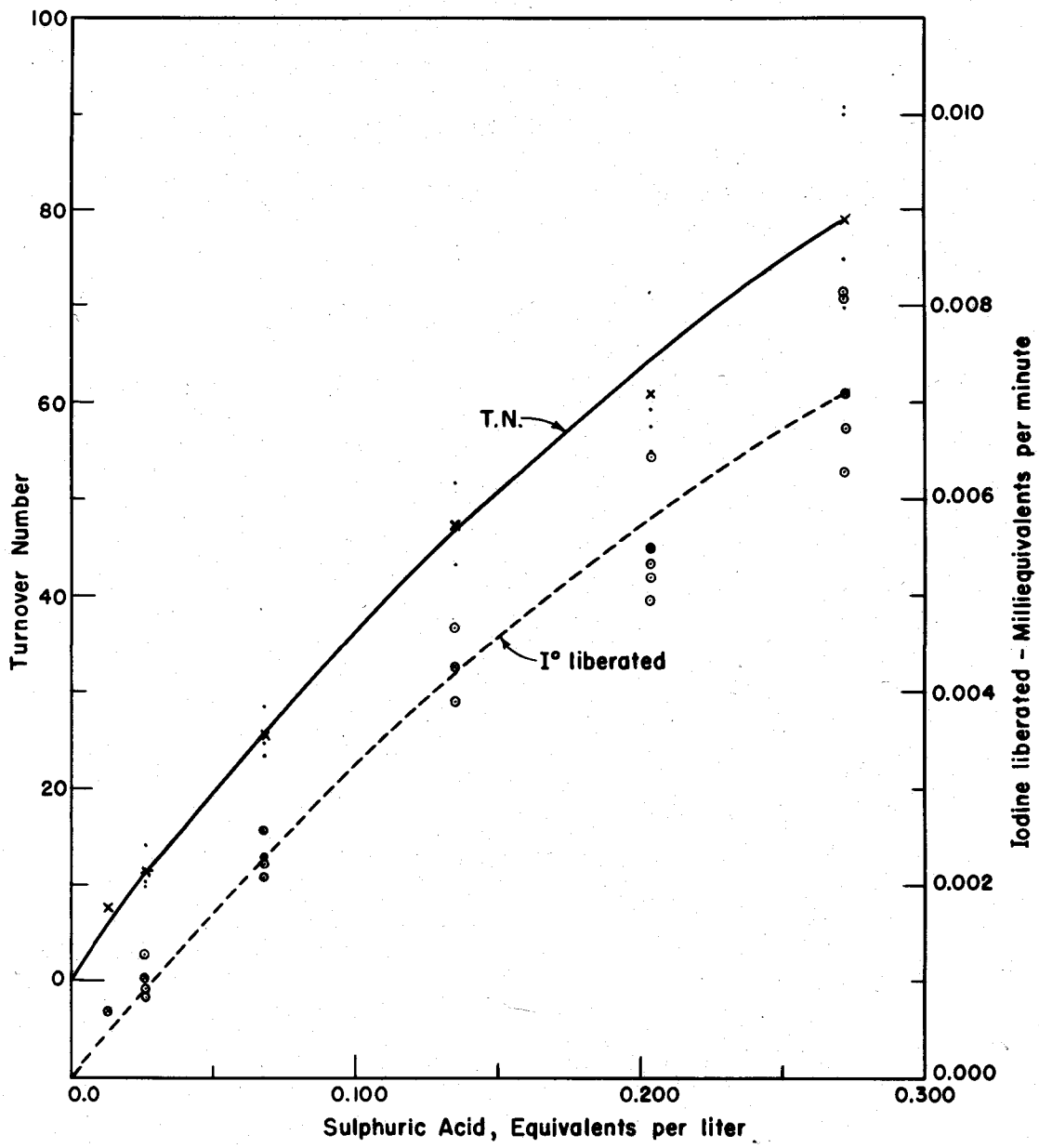

120
100
80
60
40
20
0
0.006
0.005
0.004
0.003
0.002
0.001
0.000
I° liberated
T.N.
Turnover Number
Iodine liberated Milliequivalents per minute
0
0.2
0.4
0.6
0.8
Potassium Iodide - Moles per liter

Fig. 24

Catalytic Oxidation of Iodide by Air as a Function of Sulfuric Acid Concentration


100
80
60
40
20
0
0.010
0.008
0.006
0.004
0.002
0.000
T.N.
I° liberated
Turnover Number
Iodine liberated - Milliequivalents per minute
0.0
0.100
0.200
0.300
Sulphuric Acid, Equivalents per liter

d. B_{12} as catalyst. Using the same conditions given above in (i), B_{12} was substituted for B_{12a}. Only very small amounts of iodine were liberated. An appreciable amount of iodine was liberated when a beam of light from a carbon arc was directed into the flask.

e. Reduction of B_{12a} by thiosulfate. Titration of B_{12r}. An excess of standard sodium thiosulfate was added to an oxygen-free solution of 2.62 mg. of B_{12a} and 10 ml. of 4.0 N sulfuric acid. On standing the solution changed in color from orange-red to yellow-brown. After 3 hours the solution was titrated potentiometrically with iodine. The iodine required was equal to that required for the titration of the thiosulfate alone. The reduction of B_{12a} by thiosulfate is reversible in agreement with the observation reported in section b (last paragraph) above. Thiosulfate is a stronger reducing agent than B_{12r} and both are oxidized by the iodine.

A solution of B_{12r} was prepared by the catalytic hydrogenation of B_{12} using the apparatus of Diehl and Murie (38). An aliquot of this solution containing 4.34 mg. of B_{12}, was transferred to an oxygen-free solution containing 10 ml. of 4.0 N sulfuric acid in 200 ml. of water. The B_{12r} was then titrated potentiometrically with standard iodine. A smooth titration curve was obtained, one equivalent of oxidizing agent per mole of B_{12r} being required. The potential at the mid-point of the titration was +0.25 volts on the hydrogen scale. This

is higher than the +0.09 volts found by Diehl and Murie (38) in the titration of B_{12r} with ferricyanide in neutral solution.

3. Results.

From the volume and concentration of thiosulfate used and the time of the reaction, the velocity of the reaction was calculated as milliequivalents of iodine liberated per minute. The turnover numbers reported were obtained by dividing the velocity by the number of millimoles of B_{12a} present, the molecular weight of B_{12a} being taken as 1350.

The effect of B_{12a} concentration on the velocity of the reaction and on the turnover number is shown in Fig. 22. The velocity increases as a linear function of B_{12a} concentration at lower concentrations of B_{12a}. The turnover number, however, decreases with increasing amounts of B_{12a}. The failure of the velocity to maintain a linear increase, and the decrease in the turnover number at higher concentrations of B_{12a} probably results from the lack of sufficient oxygen to maintain maximum activity at higher concentrations of B_{12a}.

The values reported for velocity and turnover number show considerable variation, as much as twenty percent between the high and low observations of duplicate experiments in one instance. This resulted largely because the method of quenching the reaction was not entirely satis-

factory; that is, the displacement of oxygen from the solution by a stream of nitrogen does not stop the reaction sharply. Auxiliary experiments showed that the reaction continues approximately three minutes after the air stream was replaced by nitrogen. The time intervals and rates of gas flow and stirring were held constant in all of the runs, however.

As seen from Figs. 23 and 24, velocity and turnover number increase with increasing concentration of potassium iodide and with increasing concentration of acid. In a neutral solution, pH 7, phosphate buffer, no oxidation of iodide occurs at all.

Data showing the change in the position of the absorption peak and in absorbancy, together with turnover numbers as a function of time, are shown in Table 8. The differences in turnover number are of the order of accuracy of the experimental work and indicate that no appreciable variation in catalytic power of B_{12a} occurs on aging.

4. Discussion.

a. Vitamin B_{12a} as an oxygen carrier. Oxygen carrying cobalt compounds have been described, notably the derivatives of disalicylalethylenediimine (39), of a cobaltous chloride-ammonia-ammonium chloride mixture (40), and the cobaltous derivatives of histidine (41). That B_{12a}, which results from the action of oxygen on

Table 8

Variation in Absorption Maximum, Absorbancy and Turnover Number of B_{12a} Solution on Standing[a]

Time	Absorption Maximum		Turnover Number
hours	mμ	Absorbancy	Equiv. I° per min. per mole of B_{12a}
0.5	357-58	0.374	73.5
2.5	356-58	.362	64.8
5.2	356-57	.356	64.2
7.2	355-56	.353	65.4
11.2	354-56	.354	48.1
13.5	354-55	.356	62.8
19.3	354-55	.354	67.2
25.6	353-55	.357	60.4
31.0	353-55	.357	70.8
36.0	353-55	.360	63.4
48.0	353-54	.366	64.2
73.0	353	.366	

[a]Phosphate buffer, pH 7.4.

the bivalent cobalt compound B_{12r}, might be a reversible, oxygen-carrier is not inconceivable. As such, it should contain molecular oxygen in the form of a peroxo group linking together two cobalt atoms. Such peroxo groups react with iodide to form free iodine. The failure of B_{12a} to liberate iodine, as shown experimentally above, leads us to believe that B_{12a} does not possess a peroxo linkage. Several other observations which bear on this subject are worth recording.

The diamagnetic character of B_{12a} can be interpreted either as trivalent cobalt or as oxygenated bivalent cobalt (42). The passage of oxygen-free nitrogen through a solution of B_{12a} at room temperature does not effect a change in the absorption spectrum of B_{12a}, either in the visible or the ultra violet, as might be expected if oxygen were being removed and brown B_{12r} reforming. Moreover, the characteristics of the polarograph wave of B_{12a} are not changed on the addition or removal of oxygen from the solution.

A peroxo group present in B_{12a} would be expected to pass to molecular oxygen on the conversion of B_{12a} to B_{12} by treatment with cyanide. No evolution of gas occurs during this conversion, however, providing further support for the absence of a peroxo group.

The molecular weight of vitamin B_{12a} has not been determined. Direct methods for its measurement involving

aqueous solutions are unlikely to succeed owing to the changes B_{12a} undergoes when dissolved in aqueous media. Indirect evidence from measurements of the diffusion constants (43) leads to a value twice as great as for B_{12}, although the value obtained for B_{12} is seriously in error (550 instead of 1350), probably because of departures from the conditions assumed in applying the Stokes-Einstein equation.

Crystalline B_{12a} dissolved in water undergoes a change which is approximately 50 percent complete at the end of the first day and nearly complete after ten days. The electrical conductance increases, the pH rises, and the absorption peak in the neighborhood of 355 mμ is shifted toward the lower wavelengths. Inasmuch as only one titratable hydroxyl is present initially and after standing, and because the values for K_b are somewhat different, the change is quite probably the replacement of a hydroxyl group in the coordination sphere about the cobalt atom by a molecule of water. This has already been suggested by the British Drug House group (44). It might well be expected that the catalytic effect on the air oxidation of iodide would depend on the extent of this change after dissolution of the B_{12a} crystals. As indicated above, however, experiments showed no significant variation with time.

b. Enzyme-like character of B_{12a}. The variation

in the velocity of the oxidation of iodide to free iodine by air with changing iodide concentration, Fig. 23, resembles similar plots for enzymatic reactions. A plot of reciprocal velocity against reciprocal iodide concentration yielded a straight line, Fig. 25, in agreement with simple enzyme theory (45). The value obtained for the dissociation constant of the B_{12a}-I^- complex (enzyme-substrate complex), K_s = 0.396 M, is large in comparison with K_s values of most enzyme substrate complexes.

The overall reaction, of course, involves the hydrogen ion, $O_2 + 4H^+ + 4I^- = 4I^0 + 2H_2O$, so that it is not surprising to find the reaction dependent on the hydrogen ion concentration. A plot of reciprocal velocity against reciprocal acid concentration gave a straight line indicating that only one hydrogen ion enters into the rate controlling step, Fig. 26. Considering sulfuric acid as the substrate, the dissociation constant has the value, K_s = 0.430M.

Cyanide acts as an inhibitor for this system, for B_{12} which is formed by the addition of cyanide to B_{12a} is inactive as a catalyst in concentrations comparable to those of B_{12a}. It has been demonstrated that cyanide is detached from B_{12} by irradiation with ultra violet light (9) and this is in accord with the catalysis observed when the iodide-sulfuric acid-air-B_{12} solution was irra-

Fig. 25

Catalytic Oxidation of Iodide by Air; Plot of Reciprocal Velocity, v, (Moles per Liter per minute) against Reciprocal Iodide Concentration, S, (moles per liter). Calculated from data of Fig. 23. K_s = 0.40 N; V = 8.3×10^{-6} moles I^o per minute (maximum velocity).

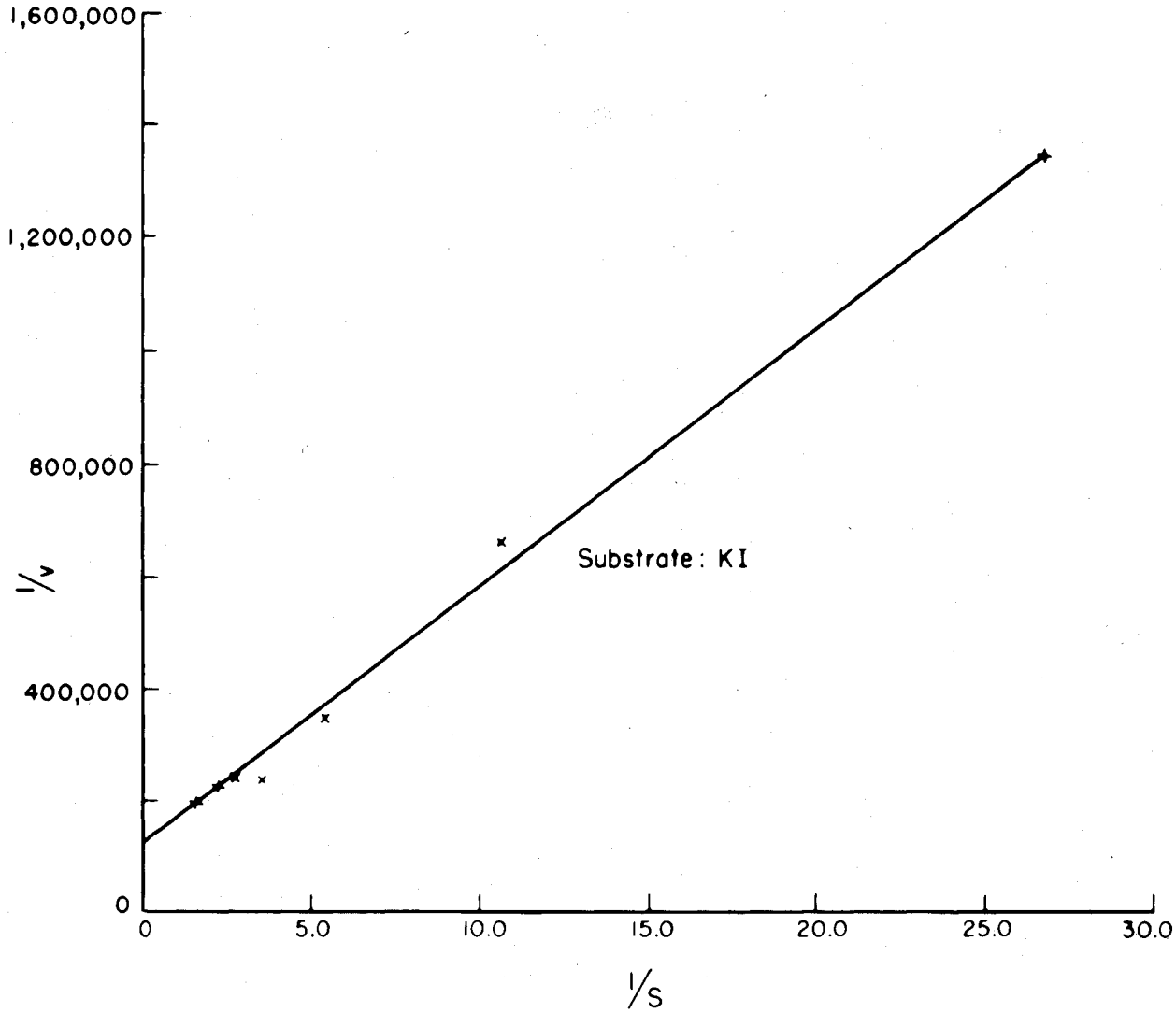
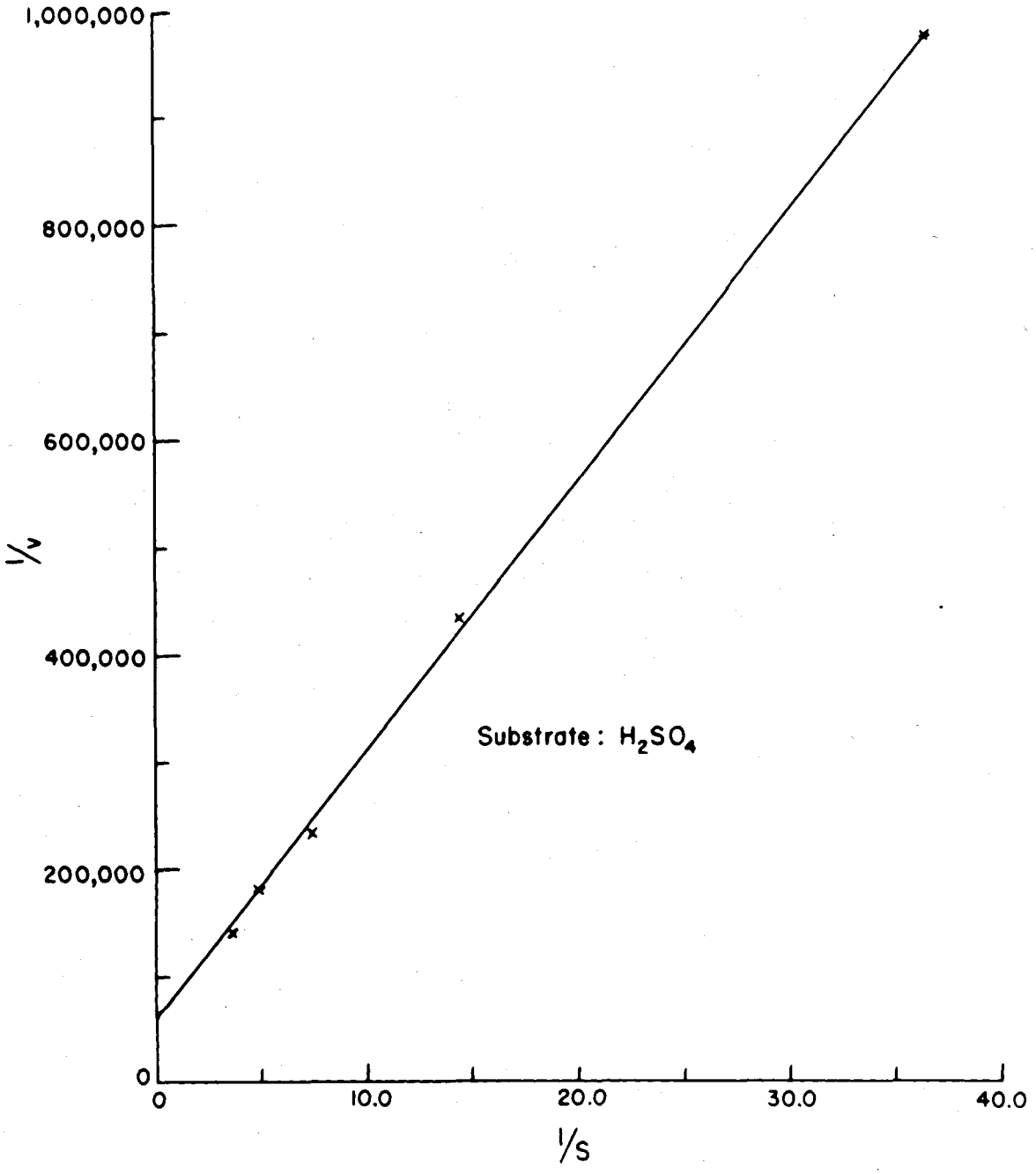

1,600,000
1,200,000
800,000
400,000
0
1/v
Substrate: KI
0
5.0
10.0
15.0
20.0
25.0
30.0
1/S

Fig. 26

Catalytic Oxidation of Iodide by Air; Plot of Reciprocal Velocity, v, (Moles per Liter per minute) against Reciprocal Sulfuric Acid Concentration, S, (equivalents per liter). Calculated from data of Fig. 24. $K_s = 0.430^M$; $V = 1.7 \times 10^{-5}$ moles of I^o per minute (maximum velocity).


1,000,000
800,000
600,000
400,000
200,000
0
1/v
0
10.0
20.0
30.0
40.0
1/S
Substrate : H_2SO_4

diated. Using the technique employed in this work in which a stream of air was passed through an acid solution and from which hydrogen cyanide would be rapidly swept, it is impossible to study this inhibition quantitatively.

Preliminary attempts to obtain oxidation under more nearly physiological conditions were performed by including several pure proteins in the reaction mixture with the objective of providing a carrier which might change the conditions of acidity under which the catalysis occurs. No significant oxidation occurred, even in periods of time up to 25 minutes and pH values down to 2.5. Nor did histidine activate the catalyst in neutral solution. It seems quite possible, however, that B_{12a} might have physiological significance in oxidative processes involving iodine, as well as in other oxidative catalyses.

5. Summary.

a. Vitamin B_{12a} catalyzes the oxidation of iodide to free iodine by air in acid solution.

b. The rate at which the air oxidation of iodide occurs was measured at various concentrations of B_{12a}, potassium iodide and sulfuric acid.

c. The system resembles an enzymatic system inasmuch as plots of the reciprocal velocity versus reciprocal substrate concentration yield a straight line, considering potassium iodide and sulfuric acid as substrates. Values for the dissociation constant of the "enzyme-substrate"

complex were obtained.

d. Vitamin B_{12} does not catalyze the air oxidation of iodide in the concentration employed and therefore cyanide may be considered as an inhibitor in this system.

e. It was postulated that the catalytic behavior of vitamin B_{12a} involves the addition of molecular oxygen to the bivalent cobalt atoms of B_{12r}, forming a binuclear compound in which two cobalt atoms are linked through a peroxo group. Vitamin B_{12a}, however, under completely oxygen-free conditions failed to oxidize iodide in acid solution and therefore itself cannot contain a peroxo group.

IV. SUMMARY

1. Chemical studies have been carried out on vitamin B_{12} and on the related material, vitamin B_{12a}, which increase our knowledge of the composition, structure and behavior of these biologically important materials.

2. The acid hydrolysis of vitamin B_{12} has been studied in some detail and with particular respect to the nature, amounts and rate of formation of the breakdown products.

3. The hydrochloric acid hydrolysis of vitamin B_{12a}, followed by countercurrent distribution of the products, and appropriate analyses of the various fractions has made possible a definitive allocation of the nitrogen atoms of vitamin B_{12a}, and, by implication, those of vitamin B_{12}. Two nitrogen atoms were found as benzimidazole, five nitrogen atoms as ammonia, four nitrogen atoms as an acidic red fragment, and two nitrogen atoms probably as 1-amino-2-propanol.

4. Cobalt is stripped from the B_{12} molecule on acid hydrolysis to the extent of about 8 percent.

5. No carbon dioxide is produced during the hydrochloric acid hydrolysis of vitamin B_{12a} in the absence of oxygen. Carbon dioxide is produced in the hydrolysis of vitamin B_{12}.

6. The red acid fragment obtained on acid hydrolysis

of B_{12a} is a mixture of cobalt-free and cobalt-containing materials.

7. Less than one mole of carbon dioxide and less than one mole of hydrogen cyanide is produced on acid hydrolysis of vitamin B_{12}. The sum of the two approaches one. No carbon dioxide is produced during the acid hydrolysis of vitamin B_{12a}. It is probable that the carbon dioxide produced in B_{12} hydrolysis arises from the cyanide of B_{12}.

8. A method for the preparation of the red acid fragment was described.

9. Attempts were made to purify the red acid fragment by precipitation, countercurrent distribution, ion-exchange and chromatography. No pure, crystallizable, red fragment was obtained by any method.

10. Attempts to prepare crystalline metal salts of the red acid fragment also failed.

11. Certain chemical and physical properties of the red acid fragment were established in so far as could be done on a mixture of related materials.

12. The infrared, ultraviolet and visible spectra of the red acid fragment were obtained.

13. Bromine reacts with the red acid fragment.

14. The bromination product produced has a markedly different spectrum from that of the red acid fragment and is quite insoluble in water.

15. Attempts at quantitative bromination of the red acid fragment failed because of drifting at the starch-iodine endpoint.

16. Spectrophotometric studies of the red acid fragment cyanide derivative were described. Absorption at 278 mμ of this compound suggests that cyanide contributes in this region.

17. The reaction of the red acid fragment and acetic anhydride yielded an anhydride as shown by the infrared spectrum of the product.

18. The reaction product of B_{12} and acetic anhydride yielded a product which could be either an anhydride or cyclic imide.

19. The Hofmann haloamide reaction with vitamin B_{12} was studied.

20. A pure reaction product could not be isolated from the reaction mixture of B_{12} with alkaline hypochlorite. Some amine formation probably occurred, however.

21. No ammonia was produced in the Hofmann reaction with vitamin B_{12}.

22. Vitamin B_{12} reacts with both mercuric oxide and mercuric acetate, probably through the amide groups of the B_{12} molecule. The two products obtained are probably not the same.

23. Analyses of the reaction product of B_{12} and

mercuric acetate gave mercury to cobalt ratios approaching three. It is not possible to say how the mercury is attached.

24. Infrared, ultraviolet and visible spectra of the mercuric acetate reaction product were obtained.

25. Vitamin B_{12a} catalyzes the air oxidation of iodide to free iodine in acid solution. To a much lesser degree, vitamin B_{12} and the red acid fragment are also active as catalysts. It is probable that the catalytic action involves the cobalt atom.

26. The rate of the air oxidation of iodide by B_{12a} catalysis was measured with various concentrations of B_{12a}, iodide and acid.

27. Plots of reciprocal velocity versus reciprocal concentration of iodide or sulfuric acid yield straight lines.

28. Values for the dissociation constant of the catalysis complex were obtained.

29. In the complete absence of oxygen, no oxidation of iodide occurs by B_{12a}. This fact eliminates the possibility of a peroxo group in B_{12a}.

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VI. ACKNOWLEDGMENTS

The author is pleased to make an acknowledgment of gratitude and respect to the late Dr. Robert R. Sealock under whom this research was originally begun.

To Dr. Harvey Diehl, who assumed the guidance of the continuation of this research following the regrettable demise of Dr. Sealock, the author will be forever grateful for the inspiration and suggestions supplied. It was a privilege and a pleasure to be associated with the keen mind of scientist Harvey Diehl.

The generous financial support of, and the vitamin B_{12} supplied by, the Squibb Institute for Medical Research, New Brunswick, New Jersey, is appreciatively acknowledged.

For the typing of this thesis under most difficult and trying conditions, many thanks are expressed to Mrs. Brierly. More important, however, is an expression of appreciation to her for her attitude and moral support during the author's college training.