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Studies on the structure and catalytic properties of vitamin B12

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NOTE TO USERS

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STUDIES ON THE STRUCTURE AND CATALYTIC PROPERTIES OF VITAMIN B_{12}

by

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Dean of Graduate College

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INTRODUCTION AND REVIEW OF LITERATURE

It has been known for a number of years that the inclusion of liver in the diet is of great aid to persons suffering pernicious anemia (1). Isolation of the principle active in curing the pernicious anemia proved to be exceptionally difficult. The active principle is present in liver in only minute amounts and the assay method by which the steps in its concentration could be followed was poor, the assay method being the assessment of clinical improvement of pernicious anemia patients in relapse. After some 20 years of only moderately successful work, a more rapid bio-assay method was found (2) and the isolation of the pure principle followed rapidly (3, 4). In 1948 a red crystalline compound was isolated by the chemists of Merck and Company. They named this material vitamin B_{12} (5). Microgram quantities of this crystalline material produced a positive hematalogic response in addisonian pernicious anemia, and compared with an arbitrarily selected standard liver concentrate assigned a potency of 1,000 LLD units per milligram the red crystalline compound had a potency of about 11,000,000 LLD units per milligram.

Shortly after the announcement of the isolation by Merck and Company a similar announcement was made by Glaxo Laboratories, Ltd. of England (6).

Vitamin B_{12} was found to contain cobalt (7, 8), phosphorus and nitrogen. The presence of cobalt was quite unusual and is the first

case of cobalt appearing in a biologically active material. The minimum molecular weight of vitamin B_{12} is about 1300 (9) while the formula is approximately $C_{61-64}H_{86-92}N_{14}O_{13}PCo$. It is soluble in water and 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 $[\alpha]_{2563}^{23} = -59 \pm 9°$ (10). 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).

It was disclosed in nearly simultaneous publications from the United States and the Netherlands (11, 12) that vitamin B_{12} contains a cyano group, undoubtedly attached coordinatively to the cobalt atom. Since vitamin B_{12} is not toxic, the cyano group must be tightly bound within the coordination complex.

When a solution containing vitamin B_{12} is hydrogenated over a platinum catalyst, a brown solution results (13). Oxidation of this brown solution produces a product termed vitamin B_{12a}. Vitamin B_{12a} may also be prepared by illumination of an acidic aqueous solution of B_{12} (12). It is believed that B_{12a} differs from B_{12} only by the replacement of the cyanide group of B_{12} (11) by a hydroxyl group (14).

Other B_{12} analogues have been prepared by replacement of cyanide by various anions such as chloride, bromide, sulfate, cyanate and nitrite (15). The B_{12} derivatives are converted to vitamin B_{12} by reaction with cyanide ions. 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 (14, 15). Then the compounds could be referred to by Werner nomenclature as cyano-cobalamin, nitrite-cobalamin, hydroxo-cobalamin, sulfato-cobalamin, etc.

Degradation of vitamin B_{12} by acid hydrolysis yields a number of small fragments and a large red acidic, cobalt-containing fragment designated RAF, red acid fragment. The red acid fragment amounts to approximately two thirds of the molecule.

A "ninhydrin-reacting" hydrolytic fragment first reported to be 2-aminopropanol (16, 17) but later identified as 1-amino-2-propanol has been characterized by structure examination and by synthesis (18). The number of molecules of 1-amino-2-propanol per molecule of vitamin B_{12} has been reported as both one and two (19, 20, 21).

Three benzimidazole compounds have been isolated in varying amounts depending upon the conditions of the hydrolysis (20, 22). 1-2-D-ribofuranoside-5-6-dimethyl-benzimidazole has been obtained by degradation of vitamin B_{12} and by synthesis (23). One nitrogen benzimidazole is thought to fill one coordination position of the cobalt atom (24).

The acid hydrolyzates from vitamin B_{12} were found to contain phosphate (25), and further it was suggested that the phosphate is attached to the C_2 or C_3 in the ribose molecule (26, 27). Recent X-ray studies appear to show the phosphorus attached to the C_3 of the ribose molecule (28).

Much work has gone into attempts to obtain crystalline the red acid fragment. Various methods for the separation and purification of the RAF include the Craig Countercurrent distribution apparatus (21), electrophoresis and chromatography (29). Recently a hexa-basic acid fragment has been obtained as red prisms after rigorous alkaline hydrolysis with 30 per cent sodium hydroxide at 150° for 1 hour (30).

The work presented in this thesis is a further investigation into the nature of the groups attached to the red acidic, cobalt-containing fragment and into methods of their cleavage from the fragment. The isolation of the fragment itself by countercurrent distribution methods was also studied.

INVESTIGATIONS

The Effects of Heating Vitamin B_{12} in a Stream of Dry Nitrogen at Various Temperatures

Introduction

The presence of five amide groups in the molecule of vitamin B_{12} was shown by Ellingboe and Diehl (31). Various experiments with vitamin B_{12} and its red acidic hydrolysis product lead us to believe that at least some of these amide groups are located sufficiently close to permit formation of cyclic anhydrides or imides. The experiments here were designed to determine if vitamin B_{12} could be converted directly to an imide by expulsion of ammonia by direct heating.

Vitamin B_{12} has been dried at temperatures up to 100° C., apparently without detectable decomposition (10). It is reported to darken without melting at 190° to 250° C. (25).

Experimental work

Apparatus and materials. Crystalline vitamin B_{12} obtained from the Squibb Institute for Medical Research, New Brunswick, New Jersey, was recrystallized from water-acetone solution and dried in a vacuum desiccator.

A standard solution of hydrochloric acid was prepared from Baker and Adamson's reagent hydrochloric acid and was standardized against sodium hydroxide which, in turn, was standardized against potassium

acid phthalate. The hydrochloric acid was 0.002271 normal and the sodium hydroxide was 0.001992 normal.

The heating was carried out in a platinum boat which was inserted into a specially constructed glass tube equipped with ground glass joints. This tube was inserted into a heating element made from a larger size tubing wound with nichrome wire. A variac was used to control the temperature between the desired limits.

Commercial cylinder nitrogen was passed through a vanadous sulfate train (32) to remove oxygen and then successively through tubes containing calcium chloride, ascarite and anhydrous magnesium perchlorate to remove water, ammonia and acidic gases.

Heating procedure. A weighed quantity of vitamin B_{12} was placed in the platinum boat which was inserted into the glass tube. The glass tube was then connected to the nitrogen train and on the outlet side to an absorption vessel containing a measured volume of hydrochloric acid. The train was swept with nitrogen and the temperature was adjusted. After the heating periods the system was allowed to cool to room temperature, the nitrogen stream was discontinued, and the tube and boat were weighed. The acid was titrated with the standard base. Thus, the loss in weight was obtained as well as the ammonia liberated.

The titrations were carried out potentiometrically, precautions being observed to avoid the introduction of carbon dioxide from the atmosphere.

Characterization of the heated products. In all, three heating experiments were carried out. In experiment number one the sample of vitamin B_{12} was held for 8 hours successively at each of five temperatures from 109° to 210° C., as shown in Table 1.

The 210° product, a black material, was soluble in water yielding a brown-orange solution. This solution turned purple when treated with an excess of sodium cyanide.

The infra-red spectrum of the 210° product showed some modifications in the bands at 6.0 and 6.2 μ and a new band at 5.7 μ .

The data for the ultraviolet and visible spectra for vitamin B_{12} are shown in Table 2, while the data for the spectra after heating to 210° are recorded in Table 3. A plot of these data is shown superimposed over the spectra of vitamin B_{12} in Figure 1.

The dicyanide complex spectra were obtained (Table 4) and are shown with the spectra of the vitamin B_{12} dicyanide in Figure 2. The data for the ultraviolet and visible spectra of the vitamin B_{12} dicyanide are given in Table 5.

In experiment number two the sample was held for 20 to 24 hours at each successive temperature, the final temperature being 243° C. (See Table 1.)

The 243° product, black in color, was not soluble in water, benzene, methanol, dioxane, acetone, carbon disulfide or chloroform. It yielded

Table 1. Loss in weight and ammonia liberated on heating vitamin B_{12}

Temp. °C.	Initial weight mg.	Final weight mg.	Change in weight mg.	Change in weight %	Ammonia liberated mg.	Ammonia liberated meq.	Mole ratio* NH_3/B_{12}
			Experiment 1				
109	22.280	19.775	-2.505	-11.23	0.00	-	-
123	19.775	20.058	+0.283	+ 1.27	0.00	-	-
155	20.058	19.904	-0.154	- 0.69	0.032	0.00191	0.135
186	19.904	19.437	-0.467	- 2.09	0.033	0.00191	0.135
210	19.437	18.838	-0.609	- 2.73	0.088	0.00517	0.368
Total			-3.452	-15.47	0.153	0.00899	0.638
			Experiment 2				
100	22.607	19.098	-3.509	-15.470	0.0169	0.00100	0.0805
123	19.098	18.998	-0.100	0.442	0.0101	0.00060	0.0482
143	18.998	18.808	-0.190	0.839	0.0169	0.00100	0.0805
183	18.808	17.953	-0.855	3.780	0.0777	0.00457	0.3630
210	17.953	17.215	-0.738	3.260	0.1500	0.00886	0.7090
Total			-5.392	23.791	0.2716	0.0160	1.22
243	17.215	16.213	-1.002	4.320	0.0948	0.00558	0.4440
Total			-6.394	28.1	0.366	0.02161	1.7252
			Experiment 3				
175-80	59.855	48.047	-11.808	19.75	0.5955	0.0350	0.965

*Milliequiv. B_{12} = (Final wt. + wt. NH_3 liberated)/1340.

Table 2. Ultraviolet and visible spectra data of vitamin B_{12}

Wavelength in mμ	Optical density	Wavelength in mμ	Optical density
270	1.130	395	0.350
275	1.225	400	0.340
278	1.245	410	0.335
280	1.220	420	0.305
285	1.065	430	0.300
287	1.035	440	0.300
290	0.985	450	0.315
295	0.790	460	0.350
300	0.745	470	0.390
305	0.745	480	0.440
307	0.730	490	0.480
310	0.705	500	0.540
312	0.660	510	0.625
315	0.635	515	0.655
320	0.645	520	0.660
325	0.630	525	0.660
330	0.600	530	0.665
335	0.670	535	0.675
340	0.890	540	0.705
345	1.110	545	0.715
350	1.360	550	0.710
355	1.860	555	0.690
357	2.090	560	0.620
358	2.155	580	0.240
360	2.150	570	0.415
365	1.800	590	0.150
375	0.800	600	0.100
380	0.520		
385	0.395		
390	0.350		

Table 3. Ultraviolet and visible spectra data of vitamin B_{12} after heating to 210°

Wavelength in mμ	Optical density	Wavelength in mμ	Optical density
273	0.703	395	0.223
274	0.704	400	0.214
275	0.702	406	0.210
276	0.702	410	0.208
277	0.703	420	0.204
278	0.700	430	0.199
279	0.697	440	0.203
280	0.691	445	0.207
281	0.689	450	0.213
285	0.650	455	0.218
290	0.607	460	0.226
295	0.494	465	0.233
300	0.451	470	0.239
305	0.454	480	0.242
310	0.465	490	0.245
317	0.480	495	0.244
320	0.478	500	0.244
325	0.472	508	0.248
330	0.481	510	0.247
335	0.505	515	0.244
340	0.548	520	0.239
345	0.579	525	0.241
350	0.603	530	0.234
353	0.609	540	0.217
360	0.545	550	0.185
365	0.470	560	0.150
370	0.396	570	0.127
375	0.333	580	0.107
380	0.286	590	0.094
390	0.236	600	0.085

Figure 1. Ultraviolet and visible spectra: A, vitamin B_{12}; B, vitamin B_{12} after heating to 210°

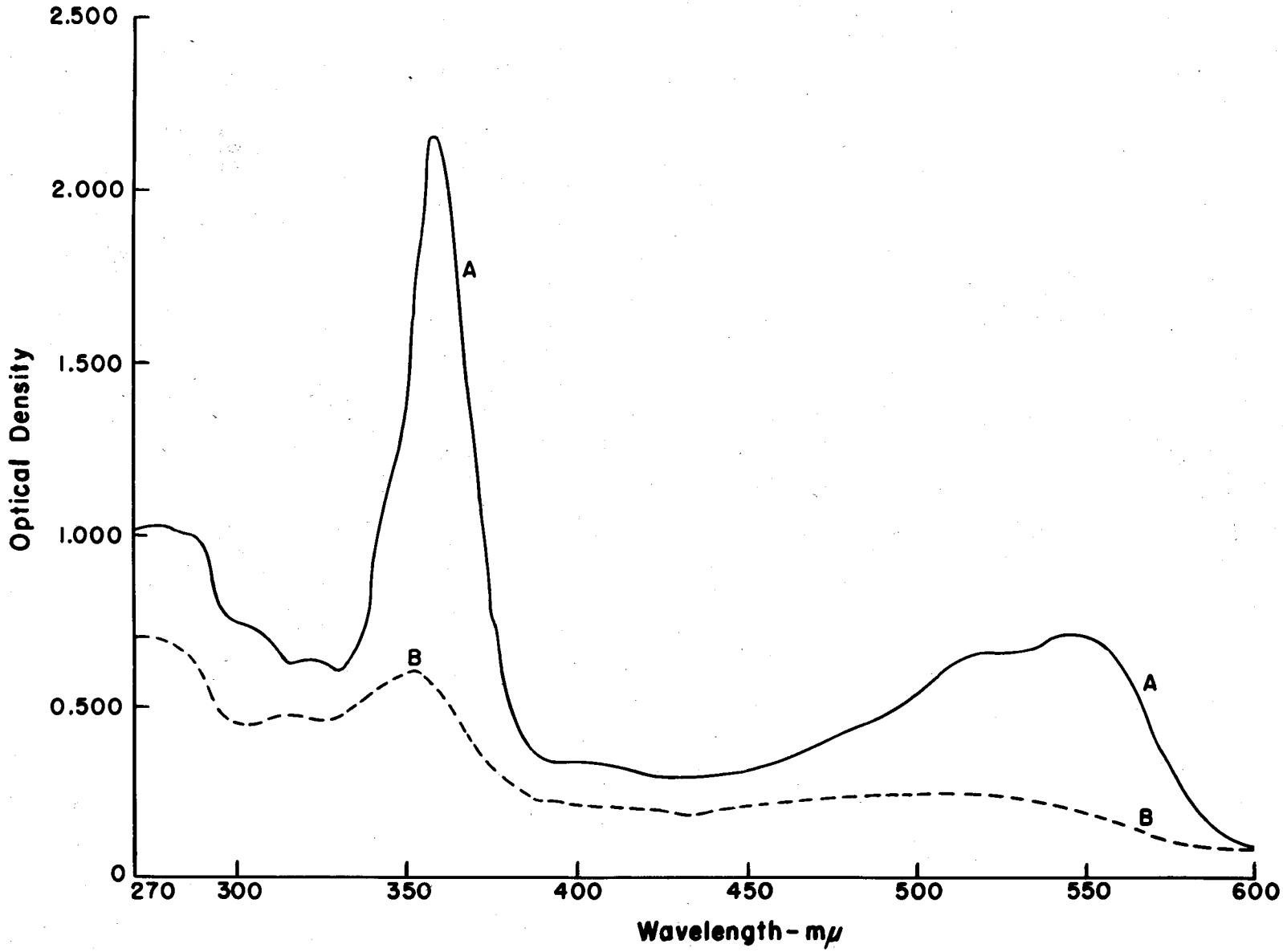

2.500
2.000
1.500
1.000
0.500
0
Optical Density
A
B
A
B
270
300
350
400
450
500
550
600
Wavelength - mμ

Table 4. Ultraviolet and visible spectra data of the dicyanide adduct formed with vitamin B_{12} heated to 210°

Wavelength in mμ	Optical density	Wavelength in mμ	Optical density	Wavelength in mμ	Optical density
272	0.568	375	0.593	530	0.203
274	0.580	380	0.496	535	0.201
276	0.593	385	0.433	540	0.211
278	0.601	390	0.368	542	0.214
280	0.599	395	0.305	544	0.215
284	0.558	400	0.241	546	0.215
286	0.542	405	0.192	547	0.215
290	0.526	410	0.164	550	0.212
294	0.417	415	0.153	552	0.202
298	0.375	420	0.145	554	0.204
300	0.375	425	0.137	556	0.200
305	0.383	430	0.130	558	0.197
310	0.380	435	0.125	560	0.195
315	0.380	440	0.125	564	0.194
320	0.337	445	0.127	568	0.197
325	0.304	450	0.134	570	0.200
330	0.291	455	0.137	575	0.211
335	0.295	460	0.142	578	0.220
340	0.318	465	0.147	580	0.227
344	0.362	470	0.154	582	0.230
348	0.407	475	0.158	584	0.232
350	0.427	480	0.161	586	0.233
355	0.452	485	0.174	588	0.232
360	0.513	490	0.179	590	0.229
364	0.626	500	0.190	600	0.184
366	0.678	505	0.194		
367	0.694	510	0.196		
368	0.700	515	0.195		
369	0.699	520	0.194		
370	0.693	525	0.197		

Figure 2. Ultraviolet and visible spectra: A, vitamin B_{12} dicyanide complex; B, dicyanide complex with the 210° heating product

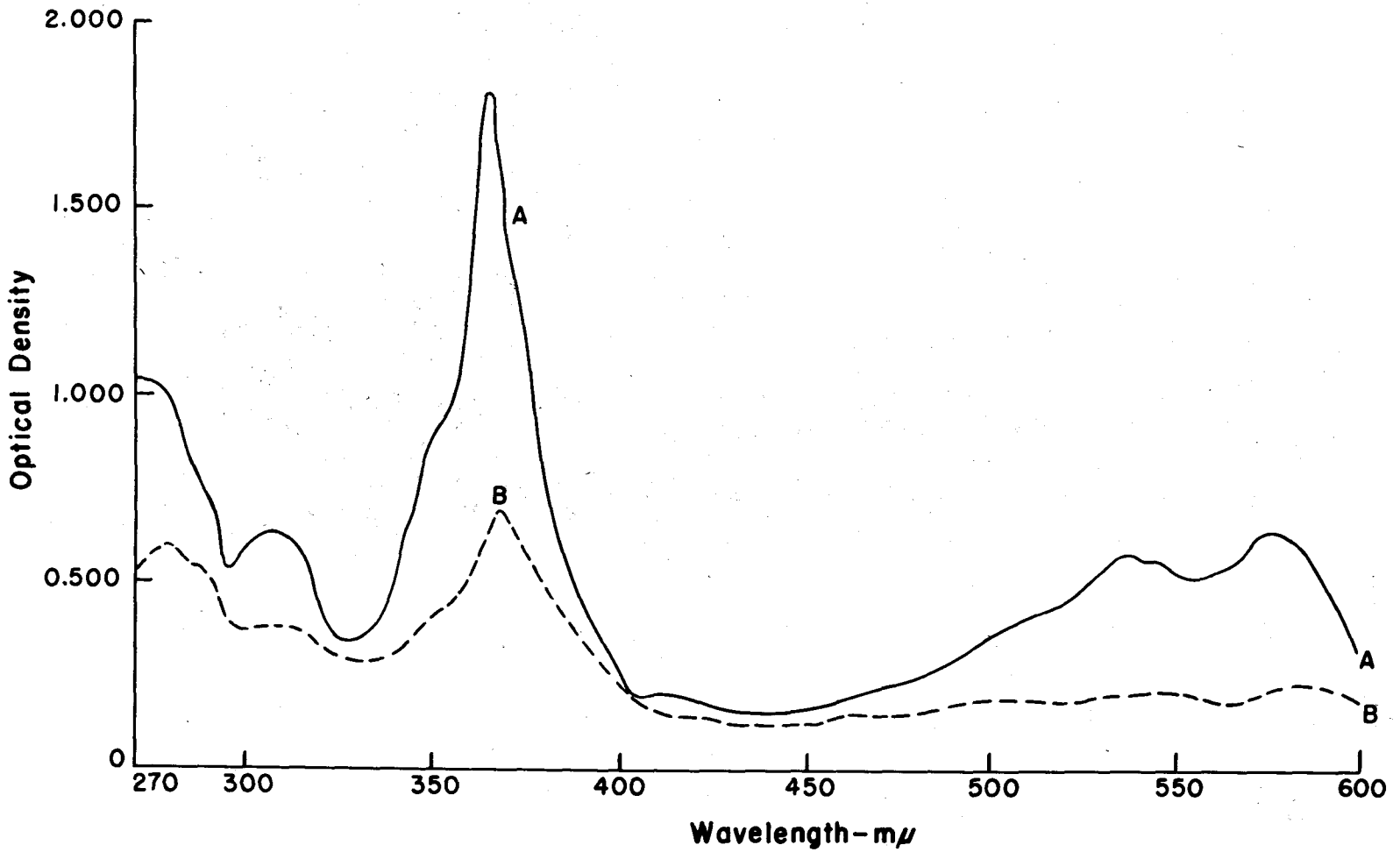

2.000
1.500
1.000
0.500
0
Optical Density
A
B
A
B
270
300
350
400
450
500
550
600
Wavelength-mμ

Table 5. Ultraviolet and visible spectra data of the vitamin B_{12} dicyanide complex

Wavelength in mμ	Optical density	Wavelength in mμ	Optical density
270	1.050	395	0.360
275	1.035	400	0.250
277	1.025	405	0.200
280	0.960	410	0.200
285	0.815	415	0.200
287	0.810	420	0.195
290	0.735	430	0.160
295	0.540	440	0.160
300	0.580	450	0.170
305	0.640	460	0.190
310	0.640	470	0.225
315	0.600	480	0.255
320	0.430	490	0.300
325	0.345	500	0.365
330	0.345	510	0.410
335	0.390	520	0.450
340	0.500	530	0.540
345	0.720	535	0.580
347	0.835	540	0.575
350	0.895	545	0.560
352	0.910	550	0.530
355	0.950	555	0.515
360	1.290	560	0.525
362	1.570	565	0.550
365	1.800	570	0.605
366	1.810	575	0.640
370	1.550	580	0.639
380	0.810	585	0.600
385	0.600	590	0.500
390	0.450	600	0.300

a brown solution when dissolved in a sodium cyanide solution, but a little fine carbonaceous material still failed to dissolve. The infra-red spectrum of the 243° material showed a new band at 5.7μ and some modification in the bands at 6.0 and 6.2μ .

The ultraviolet and visible spectra were obtained on the brown solution which resulted when the 243° product was dissolved in sodium cyanide. The data for these spectra are shown in Table 6, and the spectra are shown in Figure 3 with the spectra of the vitamin B_{12} dicyanide complex. Also shown in Figure 3 are the spectra which which resulted when hydrochloric acid was added to the cyanide solution to effect the removal of the cyanide. The optical density data of the acid solution are listed in Table 7.

The addition of hydrochloric acid to the brown solution formed by dissolving the 243° product in sodium cyanide solution caused a brown precipitate to form. The liquid above the precipitate was quite clear. Some of the precipitate was washed several times with distilled water and placed in a titration flask with some distilled water. Purified nitrogen was passed through the solution for 45 minutes and then the solution was titrated with sodium hydroxide. The titration curve data are shown in Table 8 and are plotted in Figure 4.

In experiment number three the sample was heated for 14 hours at 100° and then for 5 days at 175° to 180° (see Table 1). The material was soluble in 10 per cent sodium hydroxide. A brown solution having a somewhat reddish tint resulted. The material is somewhat soluble,

Table 6. Ultraviolet and visible spectra data of the dicyanide adduct formed with vitamin B_{12} heated to 240°

Wavelength in mμ	Optical density	Wavelength in mμ	Optical density
275	1.17	420	0.345
280	1.19	425	0.327
285	1.14	430	0.312
290	1.10	435	0.298
295	0.918	440	0.286
300	0.820	445	0.274
305	0.816	450	0.261
310	0.791	460	0.249
315	0.771	470	0.236
320	0.741	480	0.225
325	0.701	490	0.212
330	0.670	500	0.203
335	0.644	505	0.185
340	0.624	510	0.183
345	0.621	515	0.177
350	0.620	520	0.172
355	0.610	525	0.168
357	0.610	530	0.165
360	0.609	532	0.164
362	0.612	534	0.165
364	0.630	536	0.164
366	0.639	538	0.163
367	0.641	540	0.162
368	0.644	545	0.160
369	0.644	550	0.156
370	0.642	555	0.150
375	0.607	560	0.145
380	0.563	565	0.141
385	0.529	568	0.140
390	0.497	570	0.137
395	0.463	573	0.138
400	0.423	575	0.137
405	0.384	580	0.135
410	0.353	590	0.130
415	0.363	600	0.114

Figure 3. Ultraviolet and visible spectra: A, vitamin B_{12} dicyanide complex; B, dicyanide complex with the 240° heating product; C, dicyanide complex with the 240° heating product after treatment with hydrochloric acid

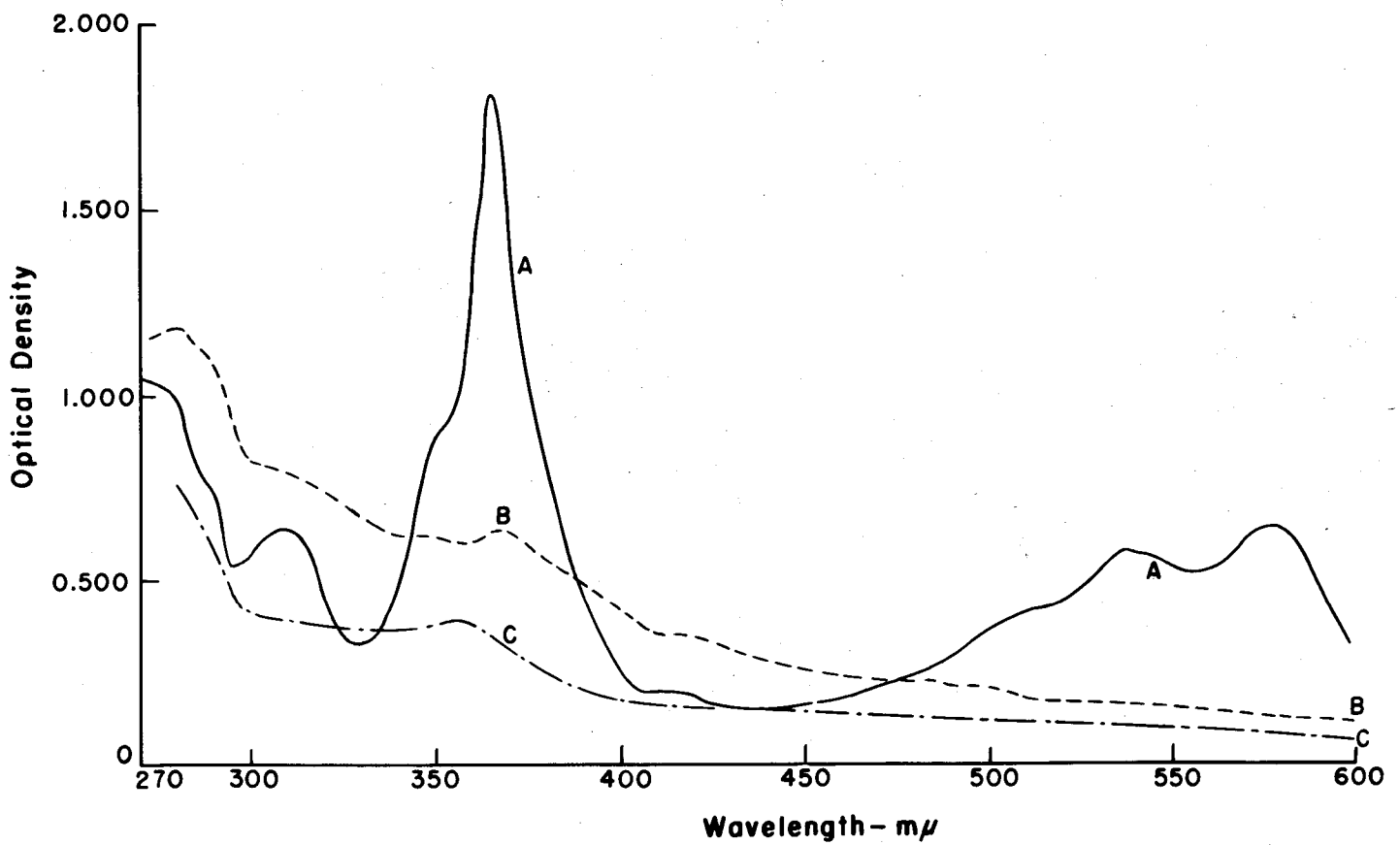

2.000
1.500
1.000
0.500
0
Optical Density
A
B
C
A
B
C
270
300
350
400
450
500
550
600
Wavelength – mμ

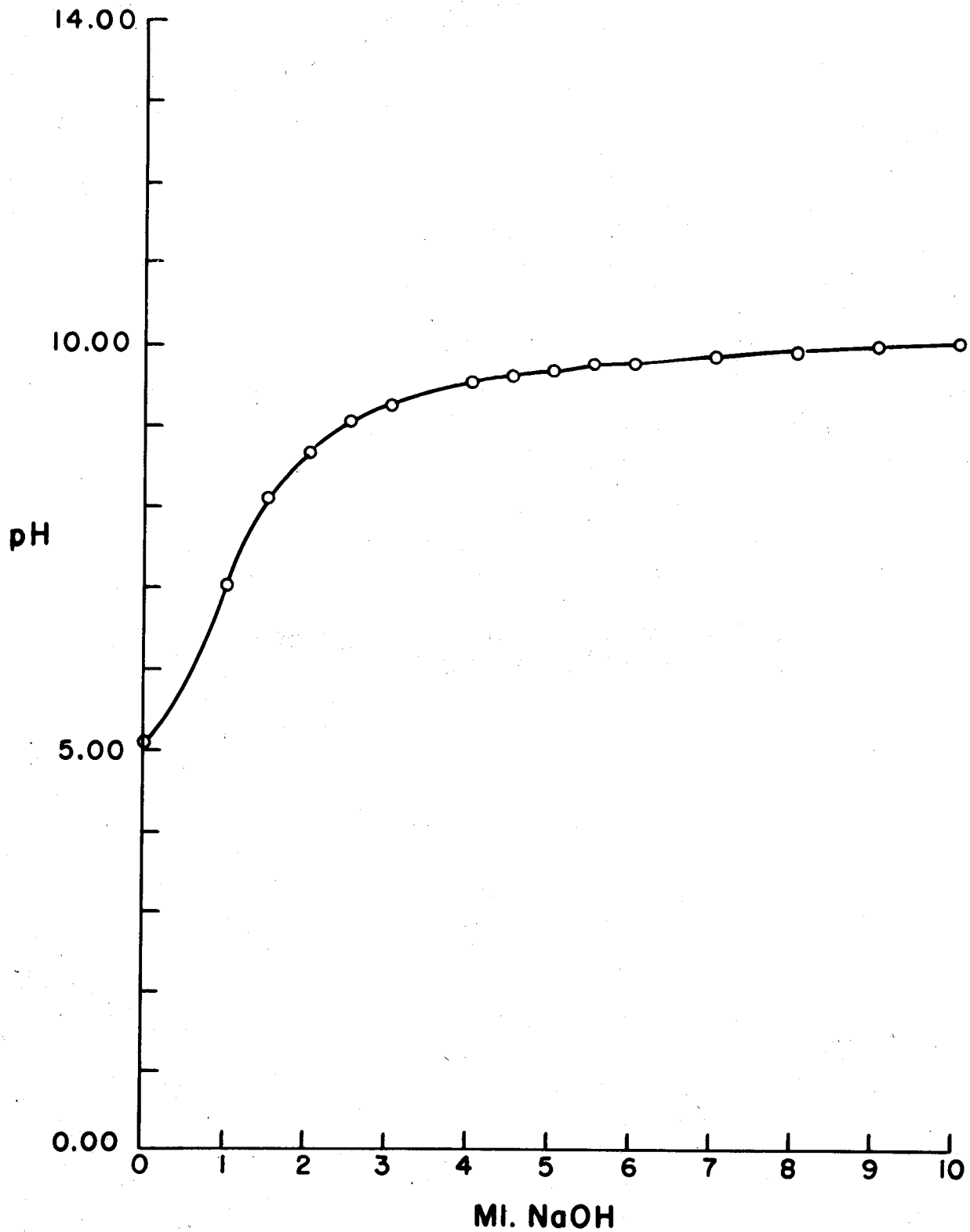
Table 7. Ultraviolet and visible spectra data after treating the dicyanide adduct of the 240* material with hydrochloric acid to effect removal of the cyanide

Wavelength in mμ	Optical density	Wavelength in mμ	Optical density
280	0.767	395	0.198
285	0.670	400	0.183
290	0.580	405	0.177
295	0.450	410	0.172
300	0.413	420	0.168
305	0.403	430	0.157
310	0.392	440	0.154
315	0.393	445	0.151
320	0.389	450	0.149
325	0.382	455	0.143
330	0.377	460	0.142
335	0.375	470	0.136
340	0.374	480	0.127
345	0.379	490	0.121
350	0.387	500	0.117
355	0.395	510	0.113
357	0.395	520	0.110
358	0.392	530	0.106
360	0.385	540	0.104
365	0.358	550	0.098
370	0.325	560	0.090
375	0.294	570	0.083
380	0.266	580	0.075
385	0.239	590	0.070
390	0.217	600	0.066

Table 8. Titration data for the brown precipitate formed upon addition of hydrochloric acid to the brown cyanide complex solution of the 240° product

NaOH ml.	pH
0.00	5.10
1.00	7.00
1.10	7.26
1.20	7.45
1.30	7.75
1.40	7.84
1.50	8.06
1.60	8.15
1.70	8.31
1.80	8.44
1.90	8.60
2.00	8.63
2.10	8.73
2.20	8.83
2.30	8.90
2.40	8.93
2.50	9.00
2.60	9.10
2.70	9.11
2.80	9.18
3.00	9.23
3.20	9.30
3.40	9.32
3.60	9.39
3.80	9.46
4.00	9.50
4.50	9.60
5,00	9.68
5.50	9.73
6.00	9.78
7.00	9.88
8.00	9.91
9.00	9.98
10.00	10.00

Figure 4. Titration curve of brown precipitate after heating to 243°


14.00
10.00
5.00
0.00
pH
0
1
2
3
4
5
6
7
8
9
10
Ml. NaOH

but not completely so in distilled water. A sodium cyanide solution dissolved the product to produce a purple colored solution but, as with the sodium hydroxide, a small amount of material remained undissolved.

A sample of the material was weighed out, placed in a titration flask and nitrogen was passed through the solution for 45 minutes. The solution was titrated with sodium hydroxide. The titration data are listed in Table 9 and are plotted in Figure 5. No potentiometric break was observed in the titration curve.

Results

The infra-red spectrum of the 210° product showed some modifications in the bands at 6.0 and 6.2 μ and a new band at 5.7 μ .

The ultraviolet spectrum of the 210° product was changed. The peak at 278 mμ was missing and the peak at 351 mμ was decreased considerably. The peaks at 516 mμ and at 538 mμ in the visible were not discernible. A low, broad peak covering the region from 450 mμ to 550 mμ was all that remained.

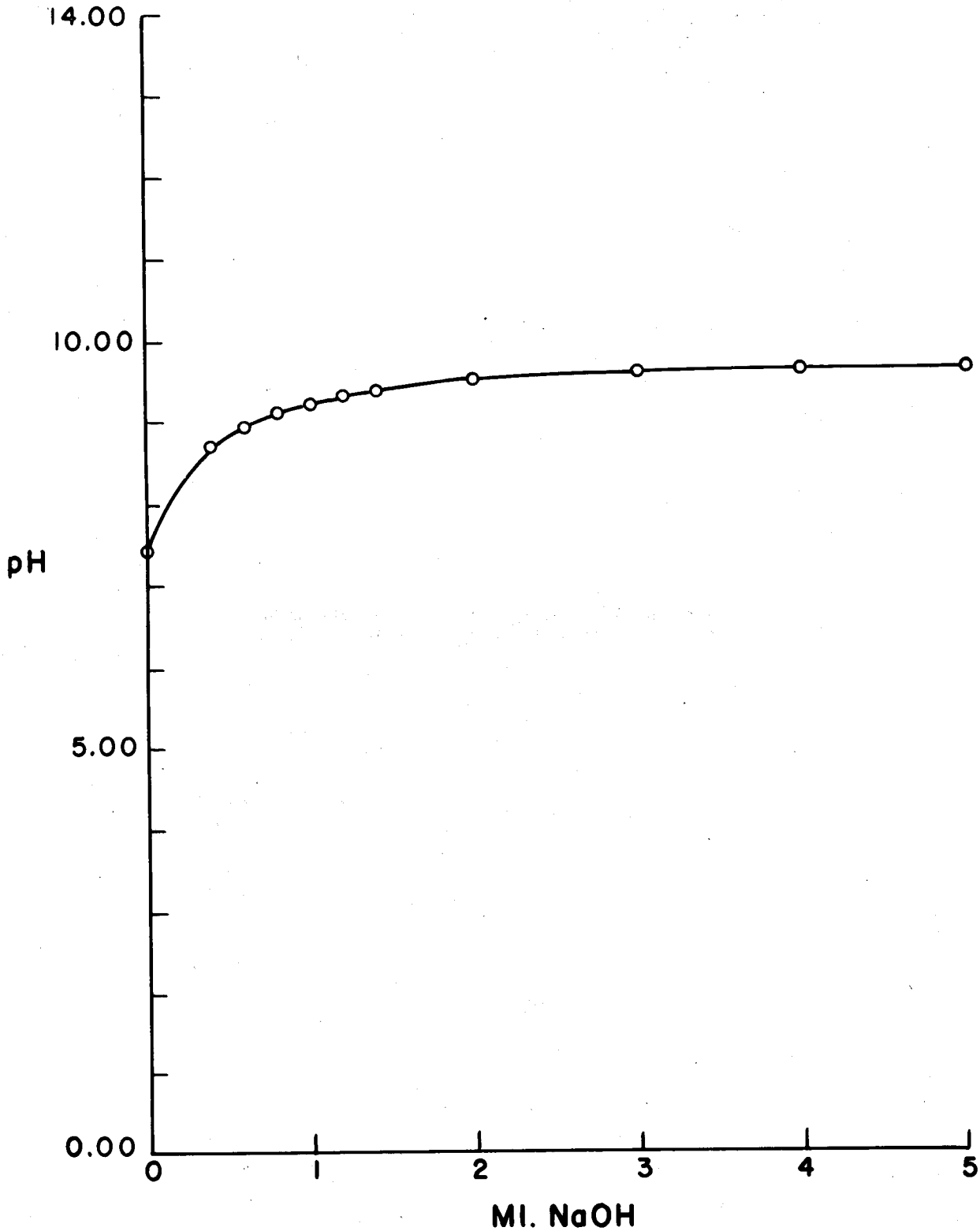
The dicyanide complex of the 210° product had the same general outline as the vitamin B_{12} dicyanide complex except that the maxima were not so pronounced.

The infra-red spectrum of the 240° product showed a new band at 5.7 μ and some modification in the bands at 6.2 and 6.0 μ .

Table 9. Titration data for vitamin B_{12} after prolonged heating at 180°

NaOH ml.	pH
0.00	7.43
0.12	8.00
0.20	8.27
0.32	8.51
0.40	8.70
0.52	8.83
0.60	8.96
0.72	9.04
0.80	9.12
0.92	9.19
1.00	9.21
1.12	9.27
1.20	9.30
1.32	9.32
1.40	9.37
1.52	9.40
1.60	9.42
1.72	9.42
1.80	9.46
1.92	9.50
2.00	9.51
2.20	9.51
2.60	9.58
3.00	9.60
3.52	9.60
4.00	9.60
4.52	9.60
5.00	9.60

Figure 5. Titration curve of vitamin B_{12} after heating to 180°


14.00
10.00
5.00
0.00
pH
0
1
2
3
4
5
Ml. NaOH

The dicyanide complex of the 240° product differed greatly from the vitamin B_{12} dicyanide. No maxima were observed in the visible and only a faint maxima in the ultraviolet at 368 mμ . After treatment with dilute HCl to destroy the dicyanide complex the ultraviolet and visible spectra were obtained. There were no maxima except a low peak at 335 mμ .

Discussion

Vitamin B_{12} loses weight rapidly at temperatures above 180° C. This is partly due to loss of ammonia during the formation of an imide group as is shown by the 5.7μ band which showed up in the infra-red. The remainder of the loss of weight was due to decomposition. The decomposition at temperatures above 180° is evidenced by the change in the absorption spectra especially the spectra of the dicyanide complex which is greatly changed in the visible region between 520 mμ and 590 mμ .

The absence of the maxima at 278 mμ in the 210° and 243° product is indicative of the destruction of the benzimidazole which contributes strongly to this band in vitamin B_{12} (22).

Summary

1. At 180° in an atmosphere of nitrogen, one molecule of ammonia is expelled from vitamin B_{12}. The process is accompanied by a much greater loss in weight than would be expected from the expulsion of ammonia alone--19 per cent instead of 1.0 per cent.

2. The peak at 358 mμ was shifted to 352 mμ and a new band appeared in the infra-red at 5.7μ after heating to 210°. This latter would be expected to appear as the result of cyclic imide or anhydride formation.

3. At 240° two molecules of ammonia are expelled but the other changes in the molecule are much more extensive.

Enzymatic Hydrolysis of Vitamin B_{12}

Introduction

Vitamin B_{12} contains phosphorus which is thought to be a tri-substituted derivative of phosphoric acid (26). Acid hydrolysis causes cleavage of a phosphate linkage which is part of the molecule, the phosphate showing up as free phosphoric acid and in combination with ribose and with ribose linked to benzimidazole (22). Inasmuch as 1-amino-2-propanol is also a product of the acid hydrolysis, it is possible that the phosphate may also be linked to the 1-amino-2-propanol.

It was thought that a cleaner and more complete cleavage of the phosphate linkages might be obtained using the enzyme phosphatase in place of the previous acid hydrolysis. One important result of such an improved hydrolysis, if realised, might well be the crystallization of the acidic cobalt-containing fragment, the failure of all previously obtained cobalt fragments to crystallise undoubtedly being due to an

incomplete rupture of the bonds attaching the various units to it.

In the present work three phosphatase preparations were examined as to their ability to cause release of phosphate from vitamin B_{12}. They were (1) prostate phosphatase, (2) a commercial preparation called polydase and (3) purified diesterase from rattlesnake venom.

Experimental work

Apparatus and materials. A Beckman Model DU quartz spectrophotometer equipped with 1-cm. quartz cells was used to measure the optical density of all solutions.

A Beckman Model G pH meter with micro electrodes was used for all pH measurements.

Vitamin B_{12} obtained from The Squibb Institute for Medical Research was recrystallized from distilled water and dried in a vacuum over anhydrous magnesium perchlorate.

Baker and Adamson reagent-grade ammonium molybdate was used to prepare a 6.6 per cent stock solution. Acid molybdate solutions were prepared from this solution as described by Rockstein and Herron (33).

Baker and Adamson reagent-grade sulfuric acid was used for preparing a 7.5 N solution.

Baker and Adamson reagent-grade potassium cyanide was used to prepare a 10 per cent solution to be used in forming the vitamin B_{12} dicyanide complex.

Baker and Adamson reagent-grade anhydrous sodium sulfate was used.

Baker analyzed reagent ferrous sulfate heptahydrate was the agent used to develop the molybdenum blue color. A solution for color development was prepared by weighing out 5 g. of the solid, adding 1 ml. 7.5 N sulfuric acid and diluting to 100 ml. A fresh solution was prepared each time.

Eastman Kodak benzyl alcohol was used for the extraction of the vitamin B_{12} dicyanide complex.

The compound 1-amino-2-propanolorthophosphate was prepared as described by Cooley *et al.* (20).

Baker and Adamson reagent-grade perchloric acid was used in the wet ashing of samples.

Baker and Adamson reagent-grade nitric acid was used in the wet ashing of samples.

Baker and Adamson reagent-grade potassium dihydrogen phosphate was used to prepare standard phosphate solutions.

Prostate phosphatase was supplied by Dr. G. Schmidt of the Boston Dispensary, Boston, Massachusetts.

The polydase was a solid commercial preparation from the Schwarz Company of Mt. Vernon, New York. The polydase contains a number of

unspecified enzymes. A 0.1 per cent (w/v) solution was prepared fresh before each use.

Purified diesterase from rattlesnake venom was supplied by Dr. Robert Sinsheimer of the Iowa State College Physics Department.

Procedure for preparing standard curves. From the stock solution of phosphate a series of standards were prepared containing 1, 10, 20, 30, 40, 50 and 60 μg. of phosphorus per milliliter. The standard curve was prepared by taking a series of small beakers in which were placed the following amounts of phosphorus: 0, 1, 10, 30, 40 and 60 μg. The pH was adjusted to 0.7 with sulfuric acid. The solutions were transferred quantitatively to a series of 10-ml. volumetric flasks and the flasks were diluted to volume with distilled water. The contents of the flasks were, in turn, transferred to 25-ml. volumetric flasks. The 10-ml. flasks were rinsed with two 2-ml. aliquots of acid molybdate solution. Ten more ml. of acid molybdate were then pipetted into the 25-ml. flasks followed by 1 ml. of freshly prepared ferrous sulfate solution. The flasks were then stoppered and inverted 50 times. The optical density was read at 720 mμ 15 minutes after starting the inverting of the flasks. Distilled water was used as a blank.

The plot of the optical density against the concentration of phosphorus per 25 ml. followed Beer's law in the concentration range investigated. The data are recorded in Table 10 and are shown by curve "A" of Figure 6.

Figure 6. Calibration curves of phosphorus as the molybdophosphate blue: A, direct aqueous solution; B, after extraction with benzyl alcohol

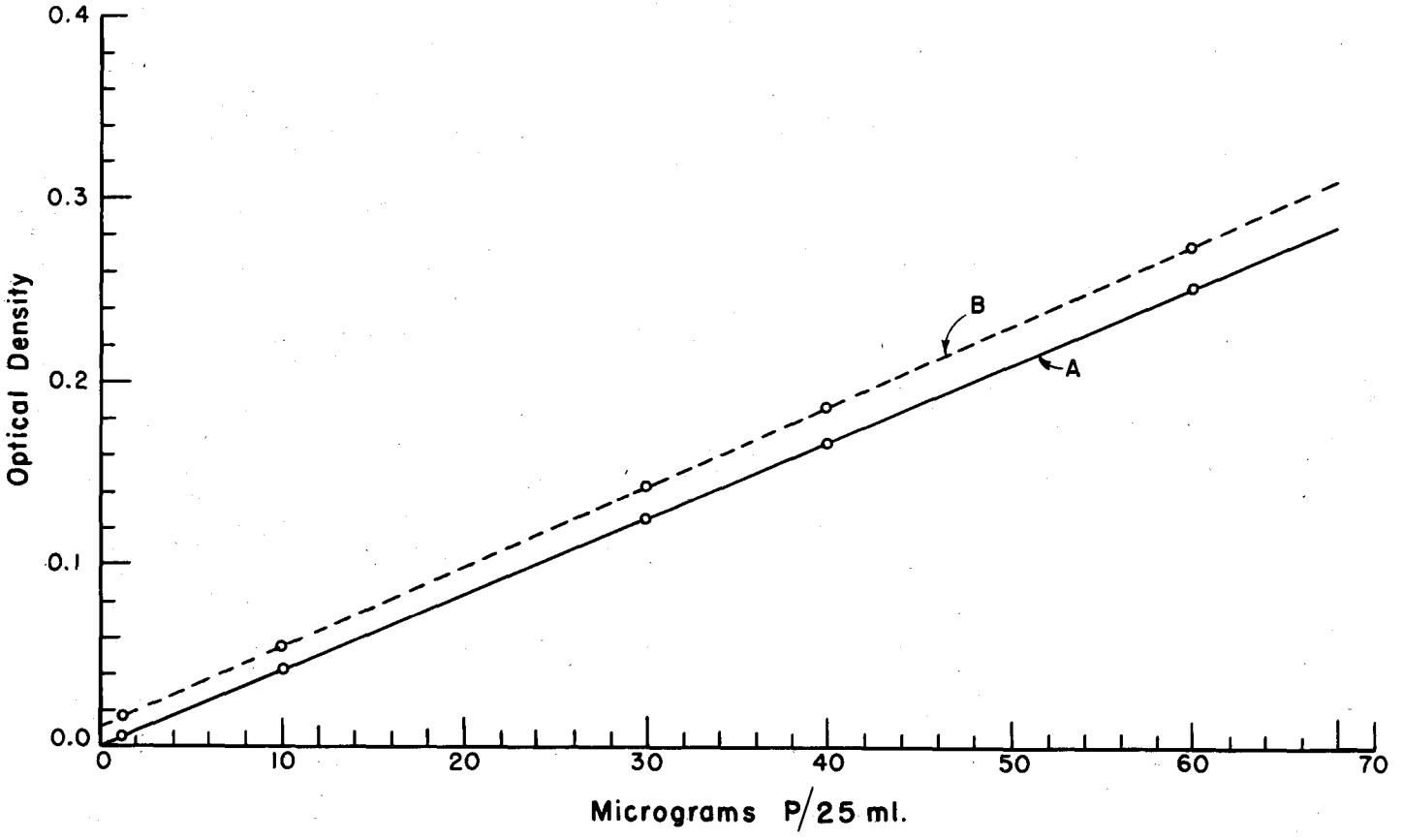

0.4
0.3
0.2
0.1
0.0
Optical Density
B
A
0
10
20
30
40
50
60
70
Micrograms P/25 ml.

Table 10. Determination of phosphorus as molybdophosphate blue calibration data, in μg. per 25 ml.*

μg P in sample	Optical density
1.0	0.006
10.0	0.043
30.0	0.125
40.0	0.168
60.0	0.250

*See Curve "A" of Figure 6.

When solutions containing vitamin B_{12} were prepared for analysis with known amounts of phosphorus added, a pink flocculent precipitate formed upon addition of the acid molybdate reagent. This caused incorrect optical density readings. It was necessary to eliminate the vitamin B_{12} prior to the formation of the complex with acid molybdate. This was effected by extraction of the vitamin B_{12} dicyanide complex with benzyl alcohol (34).

A standard curve was prepared using the extraction procedure. This involved adjusting the pH to 11.5 followed by the addition of 1 ml. of 10 per cent potassium cyanide. The solution was then permitted to stand for 5 hours after the addition of the potassium cyanide. This amount of time was necessary to insure the complete conversion of

vitamin B_{12} to its dicyanide complex (34). The total volume of the solution was 10 ml.

After standing 5 hours, 2 g. of anhydrous sodium sulfate was added to each solution, and the mixture was shaken with 1 ml. of benzyl alcohol. The resulting solution was centrifuged to separate the two layers. The alcohol layer was the top layer and was removed with a hypodermic syringe and needle.

After three alcohol extractions the pH of the aqueous layer was adjusted to 0.6 with sulfuric acid and the liquid carefully evaporated over a steam bath. The residue was taken up in a small amount of water and quantitatively transferred to a 10-ml. volumetric flask by several rinsings with distilled water. The 10-ml. flasks were then diluted to volume with distilled water. The remainder of the determination was carried out as described above with the transfer of the contents of the 10-ml. flasks to 25-ml. volumetric flasks.

The data for the extraction procedure are tabulated in Table 11 and shown graphically by curve "B" of Figure 6. Curve "B" is a straight line lying somewhat above curve "A."

Hydrolysis of vitamin B_{12} and 1-amino-2-propanolorthophosphate. Vitamin B_{12} was recrystallized from water and dried in a vacuum desiccator over anhydrous magnesium perchlorate. A stock solution was prepared by dissolving approximately 60 mg. in distilled water and diluting to exactly 100 ml. Aliquots of this were destroyed by wet

Table 11. Determination of phosphorus as molybdophosphate blue calibration data, in μg. per 25 ml.*

μg P in sample	Optical density
1.0	0.018
10.0	0.055
30.0	0.145
40.0	0.188
60.0	0.273

*See Curve "B" of Figure 6.

ashing with concentrated nitric and perchloric acids. The residue was analyzed for phosphate by the first method described under methods. The phosphorus content was 21.5 μg. of phosphorus per milliliter.

A stock solution of 1-amino-2-propanolorthophosphate was prepared and the phosphorus content determined by wet oxidation followed by analysis of the phosphate produced. The phosphorus content was 58.4 μg. of phosphorus per milliliter.

Two 1-ml. aliquots of the 1-amino-2-propanolorthophosphate were treated with a freshly prepared polydase solution. These solutions and a blank were incubated at 37° for a week. The resulting solutions were analyzed for free phosphate. Practically quantitative release of phosphate was found. These data are tabulated in Table 12.

Table 12. The action of the various enzymes on vitamin B_{12} and 1-amino-2-propanolorthophosphate

	Vitamin B_{12}		1-amino-2-propanolorthophosphate	
Enzyme used	µg. P taken as B_{12}	µg. P found	µg. P taken	µg. P found
Prostate	64.5	0.00	58.4	59.0
	64.5	0.00	58.4	59.0
Polydase	64.5	1.80	58.4	57.2
	64.5	0.00	58.4	57.1
	64.5	0.00		
Diesterase	64.5	0.00		
	64.5	0.00		
	64.5	0.00		

Three 3.0-ml. aliquots of vitamin B_{12} solution were taken to dryness on the steam bath. The dry residue was taken up in 5 ml. of 0.1 per cent polydase solution and then incubated for 7 days. The analyses for the free phosphate were carried out after extraction of the solution with benzyl alcohol as described above. No phosphate was found to have been liberated.

Three aliquots of 1-amino-2-propanolorthophosphate were taken and the pH adjusted to 4, 5 and 6, respectively. To each aliquot was added 0.1 ml. of prostate phosphatase and each sample was incubated for 24 hours at 37°. The analyses for free phosphate showed that at pH 4 and 5 quantitative release of the phosphate occurred. At a pH of 6 the release was not quantitative.

Two aliquots of vitamin B_{12} containing 3 ml. each were adjusted to a pH of 4.0, and 0.1 ml. of prostate phosphatase was added. The solutions were incubated for 24 hours at 37°. After incubation the vitamin B_{12} was extracted and the samples were analyzed for free phosphate. No phosphate was liberated.

The action of purified diesterase prepared from rattlesnake venom was studied on vitamin B_{12}. Aliquots of 3 ml. were taken and evaporated to dryness carefully on a steam bath. The pH was kept at 9.2 by an ammonia-ammonium acetate buffer. The solutions were made 0.02 M in magnesium by the addition of magnesium acetate. After incubation at 37° for 24 hours the solutions were removed and analyzed for free phosphate. No free phosphate was found.

The results of the action of the various phosphatase tried are tabulated in Table 12.

The treatment of vitamin B_{12} with cold concentrated hydrochloric acid followed by treatment with polydase was studied.

Two 2-ml. aliquots of vitamin B_{12} were taken to dryness, dissolved in 2 ml. of concentrated hydrochloric acid and let stand at room temperature for 2 hours. The solutions were then neutralized to a pH of 6.5 to 7.5 and 1 ml. of 0.1 per cent polydase solution was added. The resultant solutions were placed in a 37° box for 33 hours to incubate.

After the incubation period the solutions were made alkaline and 1 ml. of a 10 per cent potassium cyanide solution was added and the solution put aside for 5 hours to permit formation of the dicyanide complex. The dicyanide complex was extracted with benzyl alcohol as described and the phosphate was determined in the aqueous and organic phases.

Table 13 shows the results of the combined concentrated hydrochloric acid and polydase treatment.

Table 13. The action of the concentrated hydrochloric acid on vitamin B_{12} followed by treatment with polydase

Solution	μg. P in aqueous layer	μg. P in organic layer	Total μg. P	Theor. μg. P	% P in aqueous
B_{12} + HCl * + polydase	25.8	0.00	25.8	32.0	80.5
B_{12} + HCl * + polydase	27.5	0.00	27.5	32.0	85.8
Polydase blank	0.00	0.00	0.0	0.0	0.0
Polydase blank	0.00	0.00	0.0	0.0	0.0

* Vitamin B_{12} was treated with concentrated hydrochloric acid at room temperature for 2 hours and then with polydase in a neutral solution at 37° for 33 hours.

Results and discussion

The experiments described show that the three phosphatase enzyme preparations tried had no hydrolytic activity on the vitamin B_{12} molecule as regards the liberation of free phosphate.

When vitamin B_{12} was treated with concentrated hydrochloric acid at room temperature for 2 hours, neutralized and then treated with polydase, 80 per cent or more of the phosphate was liberated.

Two of the preparations, prostate phosphatase and polydase, were found to liberate quantitatively the phosphate group from 1-amino-2-propanolorthophosphate.

The phosphate in vitamin B_{12} is bound very tightly and is perhaps trisubstituted since none of the phosphatase examined were able to release free phosphatase. No report was found of an enzyme preparation which will cleave a trisubstituted phosphate.

Vitamin B_{12} apparently forms an insoluble complex with acid molybdate requiring the extraction of vitamin B_{12} before analyzing for phosphorus. The nature of this complex is not known but is perhaps similar to those formed when phospho-tungstic acid is used to precipitate proteins. Therefore, the vitamin B_{12} must be removed from the solution to be analyzed for phosphate by conversion to the dicyanide complex and extraction into benzyl alcohol.

Summary

1. Three enzyme preparations were examined as a possible means of hydrolyzing the phosphate group in vitamin B_{12}. The preparations examined were polydase, prostate phosphatase and purified diesterase from rattlesnake venom. None of these enzymes exhibited any ability to hydrolyze the phosphate group.

2. Prostate phosphatase and polydase were found to liberate quantitatively the phosphate from 1-amino-2-propanolorthophosphate.

3. Greater than 80 per cent of the phosphate in vitamin B_{12} was liberated when the molecule was treated with concentrated hydrochloric acid for 2 hours, neutralized and then treated with polydase at 37° for 33 hours.

4. The addition of acid-molybdate reagent to vitamin B_{12} hydrolysate solutions caused a pink flocculent precipitate to form. Therefore, the removal of vitamin B_{12} by extraction into benzyl alcohol as the dicyanide complex was necessary before analyzing the hydrolysate solutions for phosphorus.

Combined Enzymatic and Hydrochloric Acid Hydrolysis

Introduction

Vitamin B_{12a} has been subjected to hydrochloric acid hydrolysis and the components of the hydrolysate have been separated using the Craig countercurrent distribution apparatus (21). In this experiment each tube of the countercurrent distribution was analyzed for nitrogen and for

cobalt. This permitted the location and identification of the various hydrolytic products.

In the preceding section it was shown that greater than 80 per cent of the phosphorus can be liberated from vitamin B_{12} by treating the molecule with concentrated hydrochloric acid at room temperature and then following with an enzymatic hydrolysis with polydase at 37° for 30 hours. It was thought that by the use of this combined hydrolysis procedure a cleaner and more complete cleavage of the various fragments would result and that a greater degree of separation could then be achieved in the Craig countercurrent apparatus than was achieved when the simple hydrochloric acid hydrolysis cited above was utilized.

The following experiment was designed with the hopes of obtaining a cleaner cleavage of the hydrolyzable fragments which could then be separated in the Craig apparatus. In the experiment the combined enzymatic and hydrochloric acid hydrolysis was carried out and the hydrolysate was put through 40 transfers in the Craig apparatus employing 1 N hydrochloric acid equilibrated with n-butanol as the stationary phase and n-butanol equilibrated with 1 N hydrochloric acid as the moving phase. A sharp separation in the Craig apparatus would allow the allocation of nitrogen to benzimidazole, 1-amino-2-propanol, free ammonia and the red acidic fragment to be accurately determined.

Experimental work

Apparatus and materials. A Beckman Model DU spectrophotometer equipped with 1-cm. silica cells was used for all absorption measurements.

The hydrochloric acid and nitric acid were reagent-grade materials supplied by the Baker and Adamson Company, New York.

The reagent-grade perchloric acid was obtained from the G. F. Smith Chemical Company, Columbus, Ohio.

The 1-amino-2-propanol used to determine the distribution coefficient was technical-grade material from Eastman Kodak Company, Rochester, New York. The material was freshly distilled and the fraction which distilled at 159° to 160° was used.

Baker and Adamson's reagent-grade ammonium sulfate was employed as a primary standard in determining the distribution coefficient of the ammonium ion in the solvent system employed in the study.

Crystalline 2-nitroso-1-naphthol-4-sulfonic acid was obtained from the G. F. Smith Chemical Company, Columbus, Ohio. One g. of this material was dissolved in 500 ml. of distilled water and used as a colorimetric reagent for cobalt analysis.

The acid-molybdate, sulfuric acid and ferrous sulfate used in the analysis for phosphate were those described in the previous section.

Baker and Adamson's reagent-grade sodium citrate was used to prepare a 1 M solution for use in the cobalt analysis.

The polydase enzyme preparation previously described was employed for the enzymatic hydrolysis of vitamin B_{12a}.

An all-glass semi-micro distillation apparatus was used for nitrogen determinations. Hengar granules supplied by the Hengar Company, Philadelphia, and copper selenite from the Hach Chemical Company, Ames, Iowa, were used in the digestion of the samples for total nitrogen analysis.

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

Crystalline vitamin B_{12} was supplied by the Squibb Institute for Medical Research, New Brunswick, New Jersey.

Hydrolysis procedure. Crystalline vitamin B_{12} (about 280 mg.) was dissolved in distilled water. The solution was made slightly acid (ca. 0.001 N) with hydrochloric acid and nitrogen was bubbled through the solution for 2 days. The solution was placed in the sunlight and also irradiated by strong white light. The nitrogen used was purified by passing it through a vanadous sulfate train (32), through a tube containing anhydrous magnesium perchlorate and through a tube containing ascarite. This treatment of the vitamin B_{12} solution was carried out to effect the removal of the cyanide group attached to the molecule, thus converting the vitamin B_{12} to vitamin B_{12a}.

The resulting solution of vitamin B_{12a} was diluted to 100 ml. and aliquots were taken for the analysis of cobalt, nitrogen and phosphorus. The amounts of each found were 10.84 mg., 33.415 mg. and 5.70 mg.,

respectively. The ratio of nitrogen to cobalt was calculated to be 12.97. The remainder of the solution was taken to dryness in a vacuum desiccator containing sodium hydroxide and anhydrous magnesium perchlorate.

The dry residue was dissolved in a minimum of concentrated hydrochloric acid and was left standing for 2 hours at room temperature. After standing, the solution was diluted to 50 ml. with distilled water and neutralized with 10 per cent sodium hydroxide. The pH was finally adjusted to 7 using weak solutions of sodium hydroxide and hydrochloric acid.

To the neutral solution was added 1 ml. of a 0.1 per cent polydase preparation. The polydase preparation contained 0.371 mg. of nitrogen per milliliter by analysis. The solution was then covered and placed in a constant temperature box at 37° for 4 days to incubate.

After incubation the solution was removed, placed in a 200-ml. round-bottomed flask and enough concentrated hydrochloric acid was added to make the solution 6 N in hydrochloric acid. A long water-cooled condenser was placed on the flask and the flask was placed in a steam bath. The flask was left in the bath for 30 hours, during which time the temperature was 98°.

After removal from the steam bath the solution was quantitatively transferred to a beaker where it was neutralized to a pH of approximately 5.0. The solution was transferred to a 200-ml. volumetric flask and diluted to volume with distilled water. Aliquots were taken

for cobalt, phosphorus and nitrogen analyses. The amounts of each found were 10.40 mg., 5.46 mg. and 32.44 mg., respectively. The ratio of nitrogen to cobalt was calculated after subtracting 0.371 mg. from the value obtained for nitrogen, since 0.371 mg. represents the nitrogen added as enzyme. The ratio was 12.98, which corresponds closely to the theoretical value of 13 nitrogens per cobalt in vitamin B_{12a}. A total of 4 ml. was removed for the analyses. The solution was then transferred to an evaporating dish and evaporated to dryness in a vacuum desiccator over anhydrous magnesium perchlorate and sodium hydroxide pellets.

Countercurrent distribution. The dried residue was dissolved in 1 N hydrochloric acid which had previously been equilibrated with n-butanol. This was transferred to tube zero of the Craig apparatus and the tube was filled with more equilibrated 1 N hydrochloric to the pour-off point (31 ml.). The upper layer of the system (31 ml. of equilibrated n-butanol) was then added and 40 transfers were performed.

Each tube was drained into a correspondingly numbered 100-ml. flask, rinsed with 25 ml. of ethanol and then with 5 ml. of distilled water. The flasks were diluted to volume with distilled water. The 25 ml. of ethanol added to each flask produced a homogeneous solution. The color of the solutions in the flasks containing the red acid fragment was purple, similar to that of a permanganate solution.

Analysis of the various solutions resulting from the countercurrent distribution. The optical density of the solutions from each tube was

measured at 278 mμ and 350 mμ, the wave lengths of maximum absorption of the benzimidazole fragment and the red acid fragment, respectively. The data obtained for the optical density values at the two wavelengths are given in Table 14 and the data are plotted in Figure 7.

Aliquots from each flask were digested in the usual Kjeldahl manner. The Kjeldahl ammonia was determined by titration of a 5-minute steam-distillate from alkaline solution. The distillate was collected in 5 ml. of 4 per cent boric acid and titrated with standard hydrochloric acid. The data for the distribution of nitrogen are given in Table 15 and are plotted in Figure 8.

Aliquots of tubes 0 to 7 were evaporated to dryness under reduced pressure in semi-micro Kjeldahl flasks. The residue was steam-distilled for 5 minutes from a buffered solution at pH 9.5. The data for the distribution of ammonia nitrogen are given in Table 16 and are shown in Figure 9.

Aliquots from tubes 0 to 7 were treated with periodic acid to effect the cleavage of the ammonia from 1-amino-2-propanol. After treatment the solutions were buffered and distilled in the Kjeldahl manner. The ammonia produced over the amount found by analysis for the free ammonia was allocated to 1-amino-2-propanol. The data are given in Table 16 and are shown in Figure 10.

It was found by the distillation of solutions containing a known amount of standard ammonium sulfate and standard 1-amino-2-propanol

Table 14. The optical density of the solutions obtained in the Craig countercurrent distribution of vitamin B_{12} hydrolysates

Tube no.	278 mμ	350 mμ
0	0.048	0.015
1	0.091	0.018
2	0.107	0.023
3	0.144	0.033
4	0.120	0.027
5	0.093	0.015
6	0.085	0.000
7	0.070	0.006
8	0.055	0.006
9	0.062	0.006
10	0.120	0.007
11	0.086	0.006
12	0.040	0.006
13	0.046	0.005
14	0.048	0.006
15	0.052	0.005
16	0.072	0.004
17	0.148	0.004
18	0.478	0.007
19	0.560	0.007
20	1.060	0.009
21	1.240	0.009
22	1.170	0.008
23	0.670	0.009
24	0.490	0.010
25	0.350	0.011
26	0.204	0.013
27	0.157	0.019
28	0.078	0.023
29	0.100	0.025
30	0.186	0.027
31	0.200	0.027
32	0.292	0.035
33	0.375	0.047
34	0.418	0.075
35	0.435	0.108
36	0.583	0.230
37	1.180	0.672
38	3.150	2.74
39	9.200	7.68
40	15.40	13.50

Figure 7. Distribution of the red acid fragment and benzimidazole as determined by spectrophotometric analysis

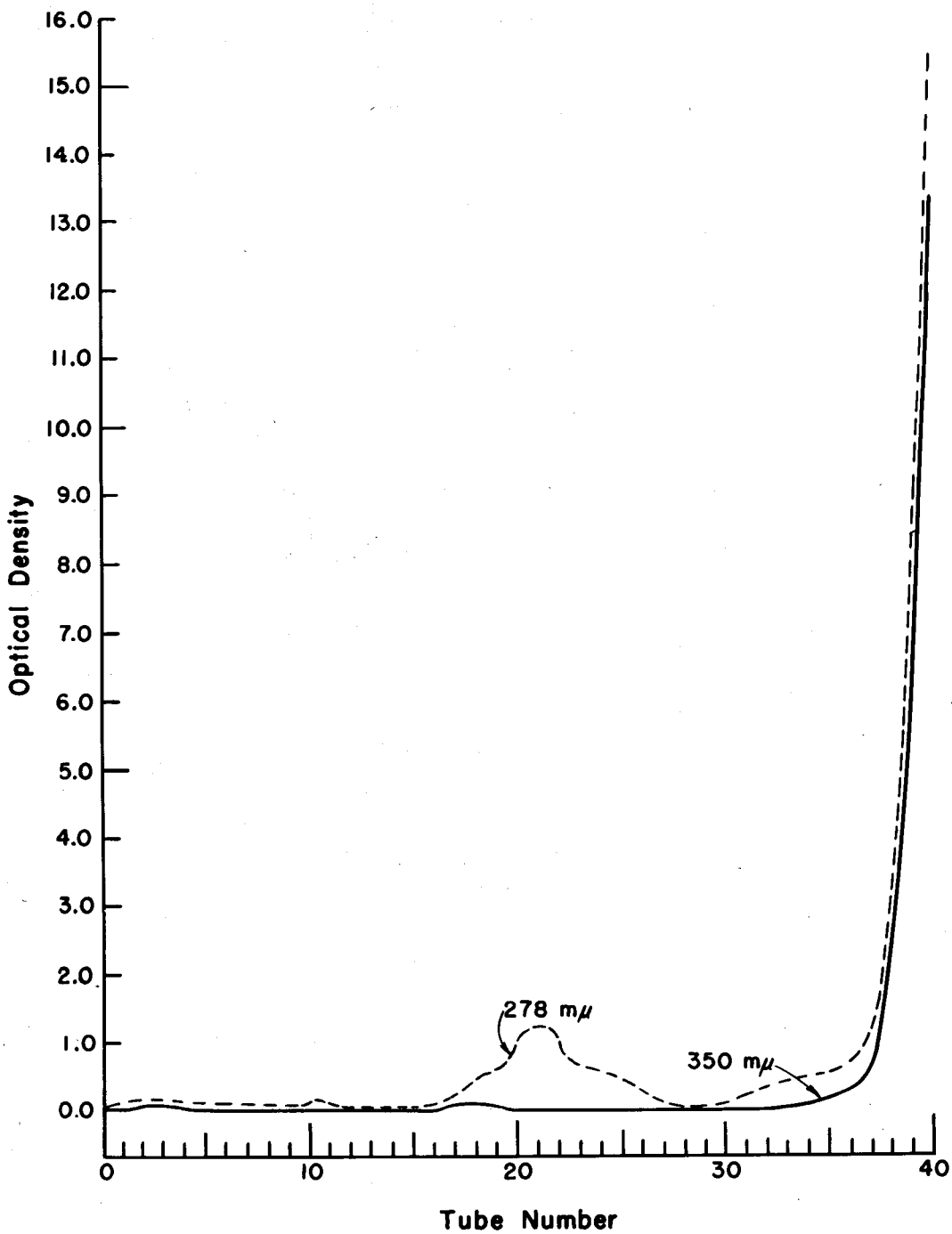

16.0
15.0
14.0
13.0
12.0
11.0
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
Optical Density
278 mμ
350 mμ
0
10
20
30
40
Tube Number

Table 15. Distribution of total nitrogen

Tube no.	Mg. N per tube*	% of total	Atoms N per tube**
0	0.495	1.55	0.201
1	2.517	7.92	1.029
2	5.174	16.26	2.11
3	3.975	12.49	1.62
4	3.824	12.01	1.56
5	1.470	4.63	0.601
6	0.702	2.20	0.286
7	0.184	0.57	0.074
8	0.000	0.00	0.000
9	0.050	0.15	0.019
10	0.118	0.37	0.048
11	0.116	0.36	0.046
12	0.046	0.14	0.018
13	0.000	0.00	0.000
14	0.000	0.00	0.000
15	0.000	0.00	0.000
16	0.000	0.00	0.000
17	0.100	0.31	0.040
18	0.370	1.16	0.150
19	0.396	1.24	0.161
20	0.544	1.70	0.221
21	0.620	1.94	0.252
22	0.552	1.73	0.224
23	0.461	1.44	0.187
24	0.363	1.14	0.148
25	0.307	0.96	0.124
26	0.173	0.54	0.070
27	0.113	0.35	0.045
28	0.082	0.25	0.032
29	0.000	0.00	0.000
30	0.136	0.42	0.054
31	0.196	0.61	0.079
32	0.260	0.81	0.105
33	0.340	1.06	0.137
34	0.390	1.22	0.158
35	0.460	1.44	0.187
36	0.590	1.85	0.240
37	0.660	2.07	0.269
38	1.440	4.53	0.588
39	1.806	5.67	0.737
40	2.790	8.76	1.138
Total	31.820	99.85	12.958

*Obtained by Kjeldahl digestion, distillation and titration.

**Obtained by multiplying the per cent in each tube by 13.

Figure 8. Distribution of total nitrogen in hydrolysate

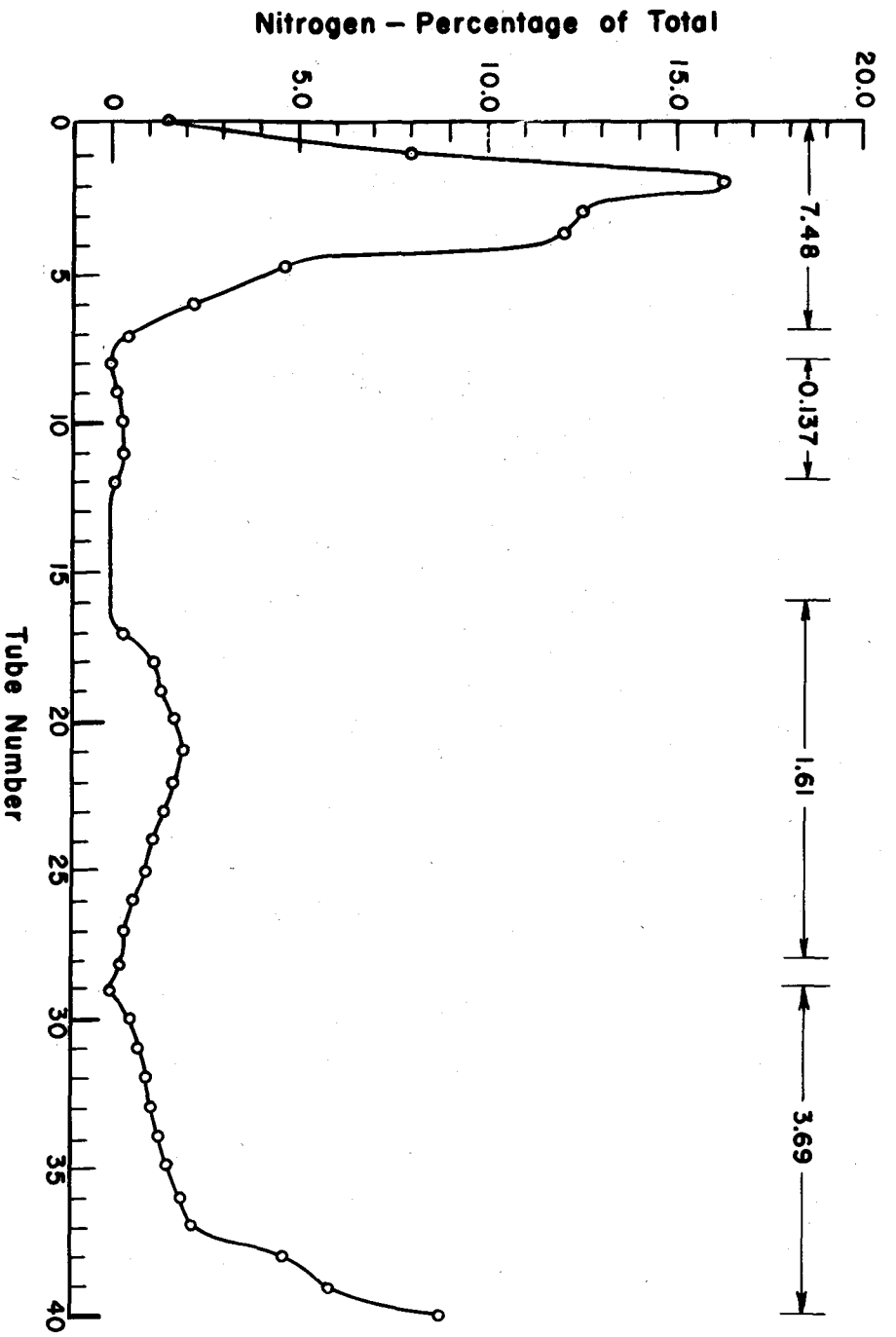

Nitrogen – Percentage of Total
0
5.0
10.0
15.0
20.0
Tube Number
0
5
10
15
20
25
30
35
40
7.48
0.137
1.61
3.69

Table 16. Distribution of 1-amino-2-propanol and free ammonia

Tube no.	Mg. free NH_3	% of total	Mg. free NH_3 + NH_3 released by H_5IO_6	Difference in mg.	% of total
0	0.3882	2.73	0.486	0.1478	7.67
1	1.8902	13.34	2.222	0.3310	17.18
2	4.1192	29.07	4.808	0.6880	35.72
3	3.2232	22.74	3.675	0.4510	23.41
4	3.0602	21.59	3.256	0.1960	11.17
5	1.1752	8.29	1.223	0.0470	2.44
6	0.2680	1.89	0.301	0.0330	1.71
7	0.0452	0.54	0.078	0.0320	1.66
Total	14.1694	100.19	16.049	1.9258	100.96

Figure 9. Distribution of free ammonia in hydrolysate

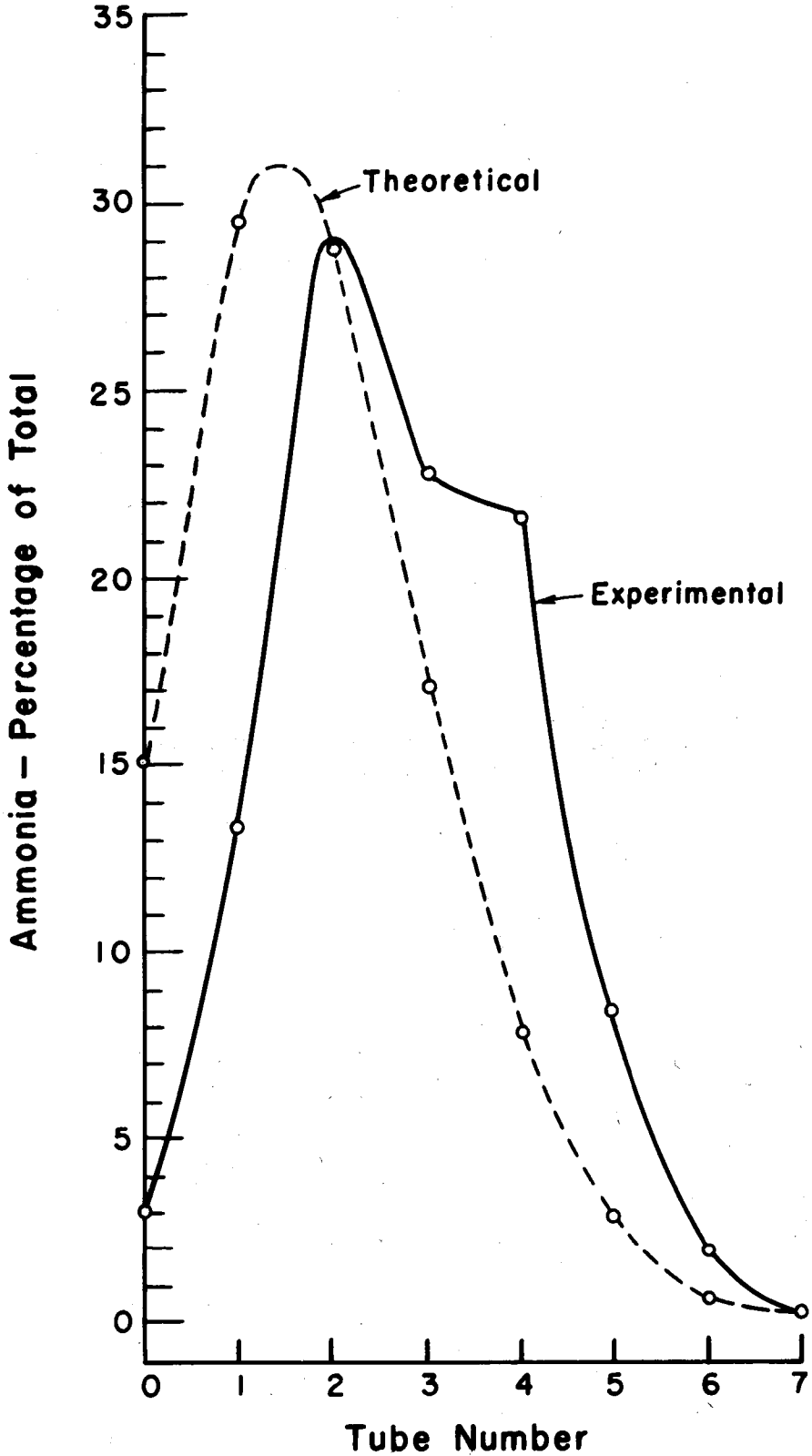
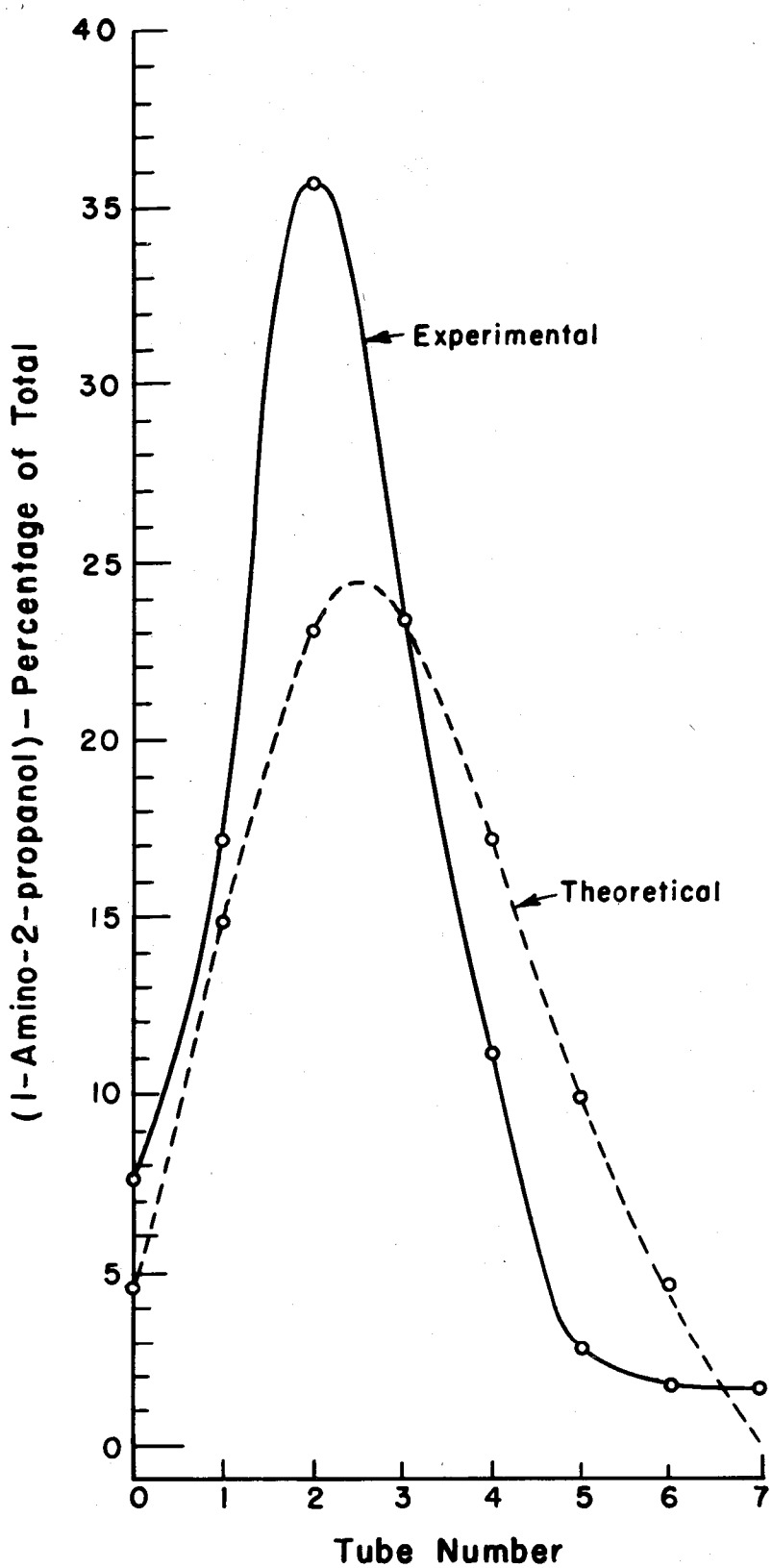

35
30
25
20
15
10
5
0
Theoretical
Experimental
Ammonia – Percentage of Total
0
1
2
3
4
5
6
7
Tube Number

Figure 10. Distribution of 1-amino-2-propanol in hydrolysate


40
35
30
25
20
15
10
5
0
(1-Amino-2-propanol) - Percentage of Total
Experimental
Theoretical
0
1
2
3
4
5
6
7
Tube Number

that 0.0328 mg. of nitrogen distilled with the free ammonia. Therefore, this amount has been subtracted from the amount of nitrogen obtained as free ammonia and the values for free ammonia in each tube of Table 16 have been corrected in this manner.

Aliquots for the cobalt analysis were wet-ashed in nitric-perchloric acid mixtures and determined spectrophotometrically using 2-nitroso-1-naphthol-4-sulfonic acid as described by Brandt and Wise (36). The data are given in Table 17 and shown in Figure 11.

Aliquots for the analysis of phosphorus were wet-ashed in nitric-perchloric acid mixtures and determined as described in the preceding section. The phosphorus distribution is given in Table 18 and shown in Figure 12.

Determination of the distribution coefficient of 1-amino-2-propanol. 1-Amino-2-propanol was freshly distilled and a weighed amount was diluted to 25 ml. with 1 N hydrochloric acid previously equilibrated with n-butanol. A 1.00-ml. aliquot of this solution and 14.00 ml. of equilibrated 1 N hydrochloric acid were shaken with 15.00 ml. of equilibrated n-butanol. The two layers were separated and to each was added an equal volume of the opposite layer and 10 ml. of ethanol, and each was finally diluted to 50.00 ml. with distilled water. Aliquots were digested in the Kjeldahl manner, distilled from an alkaline solution and titrated with standard hydrochloric acid. The ratio of the volume of hydrochloric acid required for the upper layer

Table 17. Distribution of cobalt in hydrolysate

Tube no.	Mg. Co	% of total
0	0.025	0.25
1	0.055	0.55
2	0.050	0.50
3	0.054	0.54
4	0.029	0.29
5	0.010	0.10
6	0.000	0.00
7	0.025	0.25
8	0.000	0.00
9	0.000	0.00
10	0.000	0.00
11	0.000	0.00
12	0.000	0.00
13	0.000	0.00
14	0.000	0.00
15	0.000	0.00
16	0.000	0.00
17	0.000	0.00
18	0.000	0.00
19	0.062	0.62
20	0.030	0.30
21	0.025	0.25
22	0.000	0.00
23	0.000	0.00
24	0.000	0.00
25	0.000	0.00
26	0.000	0.00
27	0.000	0.00
28	0.000	0.00
29	0.000	0.00
30	0.000	0.00
31	0.025	0.25
32	0.025	0.25
33	0.025	0.25
34	0.050	0.50
35	0.062	0.62
36	0.125	1.25
37	0.300	3.01
38	1.062	10.66
39	2.875	28.85
40	5.050	50.60
Total	9.962	99.89

Figure 11. Distribution of cobalt in hydrolysate

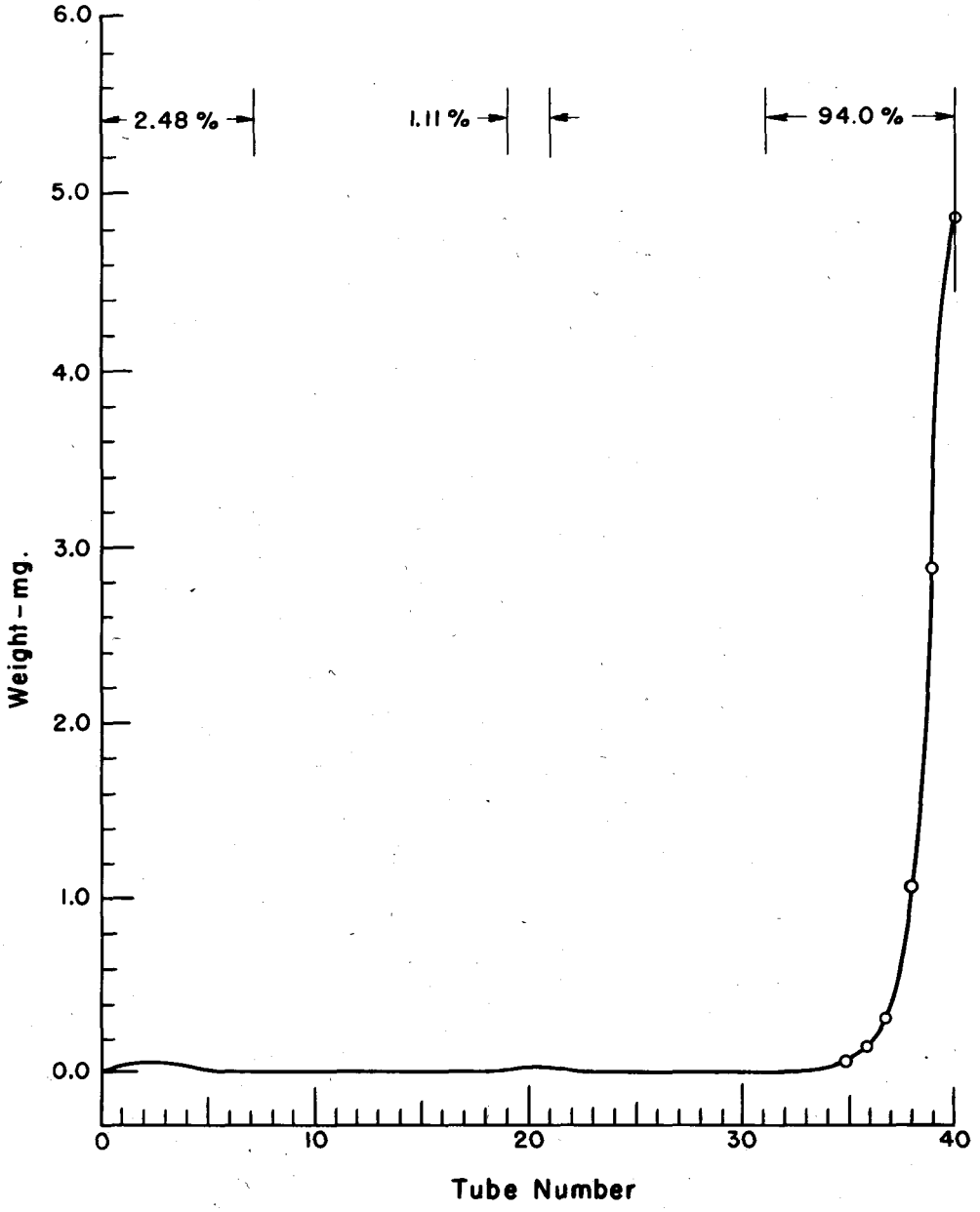
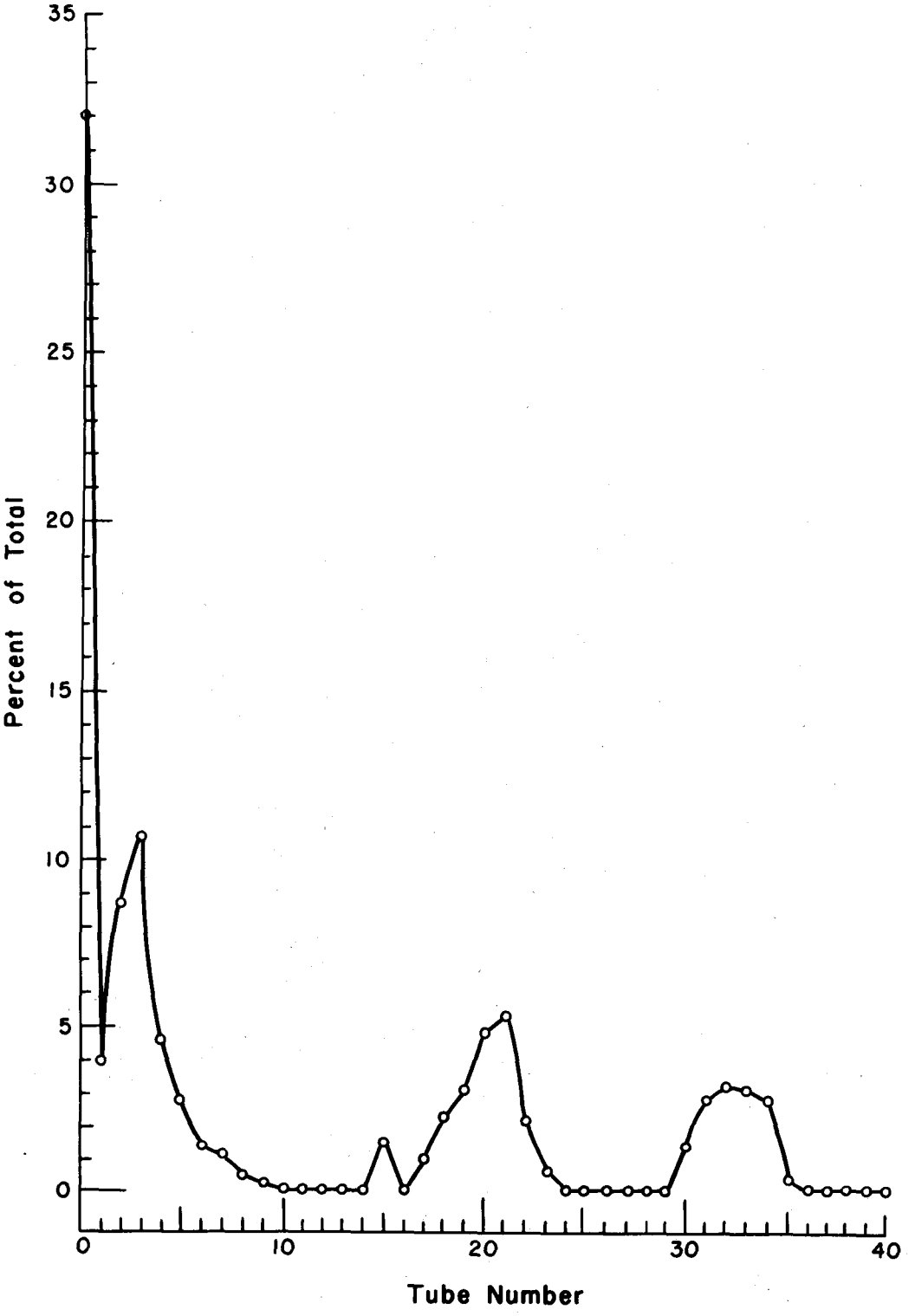

6.0
5.0
4.0
3.0
2.0
1.0
0.0
Weight - mg.
2.48 %
1.11 %
94.0 %
0
10
20
30
40
Tube Number

Table 18. Distribution of phosphorus in hydrolysate

Tube no.	Mg. P	% of total
0	1.690	32.31
1	0.207	3.95
2	0.455	8.70
3	0.560	10.70
4	0.240	4.58
5	0.145	2.77
6	0.072	1.37
7	0.055	1.05
8	0.024	0.45
9	0.010	0.19
10	0.000	0.00
11	0.000	0.00
12	0.000	0.00
13	0.000	0.00
14	0.000	0.00
15	0.077	1.47
16	0.000	0.00
17	0.052	0.99
18	0.117	2.23
19	0.162	3.09
20	0.250	4.78
21	0.275	5.25
22	0.110	2.10
23	0.030	0.57
24	0.000	0.00
25	0.000	0.00
26	0.000	0.00
27	0.000	0.00
28	0.000	0.00
29	0.000	0.00
30	0.070	1.33
31	0.142	2.71
32	0.167	3.19
33	0.160	3.05
34	0.142	2.71
35	0.015	0.28
36	0.000	0.00
37	0.000	0.00
38	0.002	0.03
39	0.000	0.00
40	0.000	0.00
Total	5.229	99.85

Figure 12. Distribution of phosphorus in hydrolysate


35
30
25
20
15
10
5
0
Percent of Total
0
10
20
30
40
Tube Number

and lower layers represents the distribution coefficient for 1-amino-2-propanol for the n-butanol-1 N hydrochloric acid system, and was found to have a value of 0.0796. The theoretical distribution curve was calculated from this distribution coefficient and is shown in Figure 10 along with the nitrogen allocated to 1-amino-2-propanol.

Determination of the distribution coefficient of the ammonium ion. A weighed amount of carefully dried reagent-grade ammonium sulfate was dissolved in equilibrated 1 N hydrochloric acid and diluted to 50 ml. A 5.00-ml. aliquot of this solution and 10.00 ml. of equilibrated 1 N hydrochloric acid were shaken with 15.00 ml. of equilibrated n-butanol. The layers were separated. To each was added an equal volume of the opposite layer and 10.00 ml. of ethanol and finally each layer was diluted to 50 ml. with distilled water. Aliquots of these solutions were evaporated and the residues taken up in 10 ml. of distilled water and distilled from an alkaline solution in the semi-micro Kjeldahl apparatus. The ratio of the volume of standard hydrochloric acid required for the upper and lower layers represents the distribution coefficient for the ammonium ion for the n-butanol-1 N hydrochloric acid system and was found to have a value of 0.0486. The theoretical distribution curve was calculated from this distribution coefficient and is shown in Figure 9 along with the nitrogen allocated to free ammonia.

Calculations

The per cent recovery of cobalt, nitrogen and phosphorus was calculated by dividing the amounts of each found by the summation of the weight of each element per tube by the amount obtained upon analysis of the hydrolysate before performing the countercurrent distribution. The per cent recovery of each is as follows: cobalt, 97.75; nitrogen, 100.08; and phosphorus, 97.73.

The nitrogen content in each tube was calculated as the fraction of the total nitrogen recovered. Multiplication of this fraction by 13 then gave the atoms of nitrogen per tube. The atoms of nitrogen in the various hydrolytic fragments were determined by summation of these atoms. The fractions were multiplied by 13 since this was found to be the ratio of nitrogen to cobalt atoms which entered the countercurrent distribution. The cobalt and phosphorus content of each tube was calculated as the per cent of the total found in all tubes.

Results

The movement of the red acid fragment through the Craig apparatus was easily followed since the fragment is intensely colored. Spectrophotometric measurements at 350 mμ showed the red acid fragment to be concentrated in tubes 30 to 40, with the maximum in tube 40. Similar measurements at 278 mμ showed the benzimidazole to be concentrated in tubes 17 to 30 with its maxima at tube 21. This is also shown by the total nitrogen analysis.

From Figure 8 (distribution of total nitrogen) it is readily seen that three distinct separations were achieved by the Craig apparatus. The separation of the free ammonia from the 1-amino-2-propanol was not clean. The amount of nitrogen obtained as free ammonia is greater than the amount expected and the amount obtained as 1-amino-2-propanol is low. It was expected that a total of 7 nitrogens would be found. Actually the total nitrogen analysis on tubes 0 to 12 gave 7.61 nitrogens.

The total cobalt recovery amounted to 97.75 per cent of the amount put into the Craig apparatus. Nearly 2.5 per cent of this was found in tubes 0 to 7; 94 per cent was found in tubes 31 to 40; and 90.2 per cent was found in tubes 38 to 40.

The total phosphorus recovery amounted to 97.73 per cent of the amount put into the Craig apparatus. Nearly 64.6 per cent was found in tubes 0 to 10; 20 per cent in tubes 11 to 23; and 12.8 per cent in tubes 30 to 38.

The benzimidazole fragment, which showed a maximum in tube 21, both by nitrogen analyses and absorbance determinations, was assumed to fall in tubes 17 through 28, and the nitrogen in these tubes amounted to 1.61 nitrogens.

The remaining tubes, 29 through 40, contained 3.69 nitrogens. A summation gives a total value of nitrogens as 12.91.

The nitrogen to cobalt ratio in tubes 29 through 40 was 3.91 and closely approaches the theoretical value of 4.0.

Discussion

The nitrogen analyses combined with the spectrophotometric analyses at 278 mμ and 350 mμ allocates the benzimidazole to tubes 17 through 28 and the red acid fragment to tubes 30 through 40. The red acid fragment absorbs strongly at 350 mμ where there is no absorption by benzimidazole. The red acid fragment and benzimidazole both absorb at 278 mμ and a comparison of the two curves readily shows where the benzimidazole and red acid fragment are concentrated.

The ammonia and 1-amino-2-propanol are concentrated in tubes 0 through 9. Summation of the nitrogens in these tubes yields a value of 7.48 nitrogens. Assuming that there are five hydrolyzable acid amide groups in vitamin B_{12a} and two 1-amino-2-propanol molecules, this value is 0.48 nitrogens too high. However, the summation of the nitrogens in the tubes allocated to the benzimidazole moiety is only 1.61 nitrogens. Therefore, it appears that part of the benzimidazole may have been retained in the early tubes, possibly linked with the ribosephosphate grouping. If this assumption is accepted, a value of 2.0 nitrogens could be allocated to the benzimidazole moiety.

Previous work from this laboratory (21, 37) showed the presence of five acid amide groups which yield ammonia upon hydrolysis with hydrochloric acid. Two ammonias were accounted for by two 1-amino-2-propanol molecules and five from acid amide groups.

The determination of the free hydrolytic ammonias in this experiment yielded high results--5.89 nitrogens instead of the expected five. It was thought that perhaps a considerable amount of 1-amino-2-propanol was distilling with the ammonia under the conditions employed. Therefore, known amounts of ammonia were distilled from known amounts of 1-amino-2-propanol under the same conditions used in the analysis of the free ammonia. Some 1-amino-2-propanol was found to distill with the ammonia and this amount, as nitrogen, was subtracted from the values of free hydrolytic ammonia in tubes 0 through 7. This value for free ammonia was entered in Table 16. However, this does not serve to explain the high results since subtraction of the amount of 1-amino-2-propanol which distilled over in the control experiments from each of the tubes 0 through 7 still leaves a value of 5.78 nitrogens as free ammonia.

The determination of free ammonia after treatment with periodic acid to liberate 1-amino-2-propanol ammonia yielded 6.55 nitrogens, a difference of 0.77 nitrogens. The difference between the five nitrogens expected for free hydrolytic ammonia and the 6.55 nitrogens obtained after treatment with periodic acid leaves 1.55 nitrogens which could be assigned to 1-amino-2-propanol. This is half-way between the conflicting reports of two and one moles of 1-amino-2-propanol in vitamin B_{12a} and does not serve to settle the controversy.

Analysis of tubes 9 through 12 yields 0.330 mg. of nitrogen. This appears to be the nitrogen due to the enzyme preparation, since a total of 0.371 mg. of nitrogen was added as enzyme.

The phosphorus distribution shows four maxima and indicates that it is not present as a single specie but occurs attached to various fragments. The first maximum is in tube zero and is quite possibly due to free phosphate as phosphoric acid. The second maximum occurs in tube three and may be present in combination with 1-amino-2-propanol as the ester. The third maximum appears in tube 21, where the benzimidazole also peaks. This undoubtedly is attached to the benzimidazole moiety through the ribose group. The fourth maximum is in tube 32 and is attached to some other fragment not identified.

Summary

1. Vitamin B_{12} was converted to vitamin B_{12a} by release of the cyanide group which is attached to vitamin B_{12}. The cyanide was released by bubbling nitrogen through the slightly acid solution while the solution was illuminated with white light.

2. The vitamin B_{12a} solution was treated with concentrated hydrochloric acid for 2 hours at room temperature, polydase enzyme preparation in a neutral solution for 33 hours at 37°, and then refluxed in 6 N hydrochloric acid at 98° for 30 hours.

3. The hydrolysate was put through a 40-tube Craig countercurrent separation employing 1 N hydrochloric acid equilibrated with n-butanol as the stationary phase and n-butanol equilibrated with 1 N hydrochloric acid as the moving phase. The separation fulfilled all expectations and the red acid fragment was concentrated in the last three tubes of the apparatus.

4. Spectrophotometric analysis on the contents of each tube at 278 mμ and at 350 mμ showed the benzimidazole to be concentrated in tubes 17 through 28 and the red acid fragment in tubes 31 through 40, inclusive.

5. The contents of each tube was analyzed for cobalt, nitrogen and phosphorus. About 3.5 per cent of the cobalt occurred as free cobalt. On the basis of the nitrogen analyses, three distinct separations were shown to have been achieved: the free ammonia and 1-amino-2-propanol were in tubes 0 through 7, the benzimidazole was in tubes 17 through 28 and the red acid fragment was in tubes 29 through 40. The phosphorus appeared in four separate places and it appears that a complete hydrolysis of the groups linked to the phosphate group did not occur. The greatest amount of phosphorus appeared in the zero tube, possibly as free phosphoric acid.

6. The ratio of nitrogen to cobalt in the tubes containing the red acid fragment (29 through 40) gave a value of 3.91 which is close to the theoretical value of 4.0.

7. On the basis of the nitrogen analyses four nitrogens were allocated to the red acid fragment, two to the benzimidazole moiety and seven nitrogens to free ammonia and 1-amino-2-propanol. A definite allocation of two nitrogens to 1-amino-2-propanol was not able to be made. The free hydrolytic ammonia was assumed to account for five of the nitrogens. A total of 12.95 nitrogens were found.

8. The distribution coefficient of 1-amino-2-propanol was determined in the solvent system employed. This was calculated to be 0.0796. A theoretical distribution curve was plotted and showed that the 1-amino-2-propanol should peak in tube 3, the experimental curve showing a peak in tube 2.

9. The distribution coefficient of ammonia using reagent-grade ammonium sulfate as a standard was determined in the solvent system employed. Using the value for the distribution coefficient obtained, 0.0486, a theoretical distribution curve was drawn which showed a peak at tube 1, with the experimental curve showing a peak in tube 2.

Vitamin B_{12} as a Catalyst

Introduction

It has been shown (21, 37) that vitamin B_{12a} serves as a catalyst in the air oxidation of iodide to iodine. With this in mind, it was thought that possibly there might be other systems found in which the catalytic behavior of vitamin B_{12a} could be employed. Two experiments were designed to determine the catalytic effect, if any, of vitamin B_{12a} on (a) the oxidative deamination of glycine and (b) the reduction of cyanide to methylamine in the presence of platinum oxide.

Oxidative Deamination of Glycine

Introduction

The oxidative deamination of amino acids by pyridoxal and the appropriate metal salts to yield the corresponding α-keto-acids and ammonia has been studied (38). In the following experiment vitamin B_{12a} replaced pyridoxal in the deamination of glycine and the deamination was studied at pH 4 and pH 9.6.

Experimental work

Apparatus and materials. Reagent-grade chemicals were used to prepare solutions of copper sulfate, sodium acetate and sodium bicarbonate.

Pfanstiehl glycine was used to prepare a standard glycine solution.

A Beckman Model G pH meter was used in all pH determinations.

An all-glass micro Kjeldahl apparatus was used to determine ammonia nitrogen.

Deamination procedure. A solution of glycine was prepared by dissolving 0.080 g. in 50 ml. of distilled water. Solutions were prepared of copper sulfate, sodium carbonate, sodium bicarbonate and sodium acetate in concentrations of 8 μmoles per milliliter, 0.5 M, 0.5 M and 0.5 M, respectively. The vitamin B_{12a} solution employed contained 0.05 mg. per milliliter.

The experiments were carried out at pH 4 and at pH 9.6. The buffer for pH 4 was sodium acetate-acetic acid. The buffer for pH 9.6 was a sodium carbonate-sodium bicarbonate solution. Blanks were prepared with all components present except vitamin B_{12a}. This was replaced with an equal volume of distilled water.

Twelve solutions were prepared and each solution was heated in contact with air for 30 minutes in a micro Kjeldahl flask. In the case of the samples at pH 9.6 a trap consisting of 5 ml. of 4 per cent boric acid was used to catch any ammonia that might have been liberated. After heating, each sample was steam-distilled from an alkaline solution. The distillates were caught in 5 ml. of 4 per cent boric acid and titrated with standard hydrochloric acid. In the case of the samples at pH 9.6 the distillates were caught in the traps used to catch any liberated ammonia. The data for the titrations are given in Table 19.

Table 19. Titration of ammonia liberated in deamination of glycine

pH 4		pH 9.6	
Sample no.	Ml. HCl required	Sample no.	Ml. HCl required
Blank	0.400	Blank	0.400
Blank	0.400	Blank	0.404
Blank	0.406	Blank	0.408
1	0.405	1	0.400
2	0.400	2	0.408
3	0.400	3	0.402

Results and discussion

The deamination of glycine employing vitamin B_{12a} as a catalyst was tried at pH 4 and pH 9.6. No ammonia was liberated. It is possible that some metal ion other than copper might be used in conjunction with vitamin B_{12a} which would bring about deamination.

It is not strange, however, that vitamin B_{12a} did not work as a deaminating oxidase since these materials are very often quite specific substances. For example, l-glutamic hydrogenase catalyses the conversion of l-glutamic acid to the corresponding imino-acid, a reaction that is followed by spontaneous hydrolysis of the imino-acid to yield the corresponding α-keto-acid together with ammonia. This dehydrogenase appears to be absolutely specific for l-glutamic acid.

Catalytic Reduction of Cyanide

Introduction

The hydrogenation of vitamin B_{12} to vitamin B_{12r} with hydrogen in the presence of platinum oxide has been shown to produce some methylamine (40) by the reduction of the cyanide group of vitamin B_{12}. With this in mind, it was of interest to determine whether or not small amounts of vitamin B_{12a} would catalyze the reduction of cyanide to methylamine when hydrogen was bubbled through a solution containing potassium cyanide, vitamin B_{12a} and platinum oxide. The only difference between this experiment and the reduction of vitamin B_{12} to vitamin B_{12r} is the amount of cyanide present.

The possibility of cyanide's being reduced by hydrogen in the presence of platinum oxide was not overlooked. Therefore, two experiments were also carried out to determine the uptake of hydrogen by cyanide in the presence of platinum oxide.

Experimental work

Apparatus and materials. Pure electrolytic hydrogen was used in the hydrogenation experiment. This was obtained from the Physics Department of Iowa State College.

The all-glass apparatus previously described (39) in the catalytic reduction of vitamin B_{12} to vitamin B_{12r} was used to contain a solution of potassium cyanide, vitamin B_{12a} and catalyst while passing hydrogen gas through the solution.

Reagent-grade chemicals were used to prepare solutions of potassium cyanide and silver nitrate.

An all-glass apparatus for measuring the quantitative uptake of hydrogen was used to determine whether or not the cyanide ion would take up hydrogen.

Determination of hydrogen uptake by potassium cyanide. A solution of potassium cyanide was prepared to contain 0.6600 g. per liter and standardized against standard silver nitrate.

Ten ml. of distilled water was placed in a quantitative hydrogenation flask and 8.9 mg. of platinum oxide catalyst was added. The flask was closed and hydrogen was brought into the system to saturate the catalyst. Stirring was achieved by a magnetic stirrer.

After the catalyst was saturated, a 50-ml. aliquot of the standard cyanide solution was added and the apparatus was adjusted to atmospheric pressure. The level in the gas buret was recorded and stirring was started. Stirring was continued for 18 hours. The data recorded are listed in Table 20 under Experiment 1.

Another experiment was performed in the same manner. In this experiment 5.6 mg. of catalyst was placed in the hydrogenation flask, 10 ml. of distilled water was added and the catalyst was saturated with hydrogen. After the catalyst was saturated, a 50-ml. aliquot of the cyanide solution was introduced into the flask. The pressure was equalized, the buret reading was obtained and stirring was started. The data are shown in Table 20 under Experiment 2.

Table 20. Uptake of hydrogen by cyanide ion

Time in hours	Buret reading	Temperature ° C.
	Experiment 1	
0	64.4	23.0
18	64.5	23.1
	Experiment 2	
0	91.4	25.0
1	92.7	25.0
11	93.0	24.0
12	90.8	24.5

In both of the above experiments the data show that no uptake of hydrogen occurred.

Hydrogenation of cyanide in the presence of vitamin B_{12}. The reduction of the cyanide ion was next attempted in the apparatus used in the reduction of vitamin B_{12} to vitamin B_{12r} (39). To the flask was added 10 mg. of vitamin B_{12} which had been recrystallised from a water-acetone solution. A 100-mg. sample of potassium cyanide was added and 25 ml. of distilled water was pipetted in the flask. A platinized-platinum electrode inserted into the flask served as a catalyst. Hydrogen was passed through the solution at a slow rate.

The color of the solution throughout the experiment was the purple color which is produced when the dicyanide complex of vitamin B_{12} is formed.

After hydrogen had been passed through the system for 56 hours, 10 mg. of platinum oxide catalyst was added and the hydrogenation was continued for 16 hours longer.

After the hydrogenation was stopped, aliquots of the solution were taken and an attempt was made to prepare the picrate and phenylisothiocyanate derivatives of methylamine, which should be present if the cyanide ion was reduced. The only crystals which were obtained were those of picric acid and potassium picrate.

Another experiment was carried out in which 1 mg. of recrystallized vitamin B_{12} was first reduced to vitamin B_{12r} before the addition of potassium cyanide. The potassium cyanide was added in 1-ml. increments until a total of 7 ml. had been added. The length of time over which the addition took place was 4 days. The exit hydrogen gas was passed through a solution of sodium hydroxide which acted as a trap for any hydrogen cyanide formed.

After the hydrogenation, the solution was filtered through a sintered glass filter to remove the catalyst. Aliquots of the solution were titrated with standard silver nitrate. The standard sodium hydroxide trap was also titrated with silver nitrate. The total amount of silver nitrate required was 0.91 ml.; the theoretical amount was

5.38 ml. Aliquots were also taken for the preparation of the picrate and phenylisothiocyanate derivatives of methylamine. No derivatives were obtained; however, derivatives were able to be obtained on a standard solution of methylamine having the same concentration range as that expected if reduction had occurred.

It was thought that perhaps a large amount of catalyst would cause the reduction of the cyanide ion to methylamine. Therefore, a 25-mg. sample of catalyst was placed in 20 ml. of distilled water and a potassium cyanide solution containing 1 mg. per milliliter was added in 1-ml. increments for 2 days until a total of 6 ml. had been added.

Again, no derivatives were obtained nor was the amount of standard silver nitrate required equal to the theoretical required amount.

Results and discussion

The hydrogenation of potassium cyanide solutions in the presence of platinum oxide in a quantitative hydrogenation apparatus did not show any uptake of hydrogen. Vitamin B_{12} and vitamin B_{12r} did not act as catalysts and promote the formation of methylamine. The vitamin B_{12} was converted to the dicyanide complex and it remained in that form even after prolonged hydrogenation.

The fact that methylamine is not found in the hydrogenation of cyanide is puzzling and is at variance with the observations obtained from the hydrogenation of vitamin B_{12}.

The quantitative recovery of cyanide ion was not obtained by titration with a standard silver nitrate solution. This was not unexpected since the cyanide ion hydrolyzes to formate and ammonia according to the equation

$$CN^- + 2H_2O = HCOO^- + NH_3 \ .$$

If oxidizing conditions were present, it might be expected that cyanogen would form which would decompose to form oxalate ions and ammonia; however, as the existing conditions were reducing, the formation of oxalate was excluded.

Summary

1. The replacement of pyridoxal by vitamin B_{12a} in the procedure for the oxidative deamination of glycine at pH 4 and at pH 9.6 did not promote deamination. Analysis showed that no ammonia was liberated at either pH.

2. Two like experiments measuring the uptake of hydrogen were conducted to determine if potassium cyanide would be reduced to methylamine by hydrogen in the presence of platinum oxide. The results were negative.

3. Vitamin B_{12} was added to a solution of potassium cyanide and hydrogen was passed through the solution in the presence of platinum oxide. No reduction occurred as was evidenced by the failure to obtain derivatives of methylamine; however, derivatives were obtained on a

standard solution of methylamine containing the amount of methylamine expected if reduction had occurred.

4. Vitamin B_{12} was reduced to vitamin B_{12r} by reduction with hydrogen in the presence of platinum oxide, cyanide was then added and the hydrogenation was continued for 4 days. No derivatives of methylamine were obtained from the resulting solution. Therefore, vitamin B_{12a} and vitamin B_{12r} do not promote the reduction of cyanide to methylamine.

SUMMARY

1. Vitamin B_{12} was heated in a stream of dry nitrogen and the ammonia liberated was determined by passing the exit gases through a standard hydrochloric acid solution and back-titrating the acid.

2. When vitamin B_{12} was heated to 180°, one molecule of ammonia was shown to have been expelled. There is no extensive damage to the molecule when heated to this temperature.

3. When the temperature was raised to 210°, the vitamin B_{12} product exhibited a new band in the infra-red at 5.7μ. This would be expected to appear as the result of a cyclic imide or anhydride formation. The expulsion of one ammonia of two adjacent amide groups with subsequent formation of an imide group would explain the appearance of the new band at 5.7μ.

4. Further heating at a higher temperature, 240°, expelled two molecules of ammonia and resulted in a greater loss in weight than that due to the ammonia alone. Extensive damage was done to the molecule and some charring was observed. The product was not soluble in water, benzene, methanol, dioxane, acetone, carbon disulfide or chloroform. A solution of sodium cyanide dissolved all except some fine carbanaceous material.

5. The spectra of the heated products were altered considerably in the visible region between 500 mμ and 600 mμ.

6. Three enzyme preparations were examined as a possible means for hydrolyzing the phosphate group in vitamin B_{12}. The preparations examined were polydase, prostate phosphotase and purified diesterase from rattlesnake venom. None of the enzymes exhibited any ability to cause hydrolysis of the phosphate group.

7. It was shown that the treatment of vitamin B_{12} with concentrated hydrochloric acid for 2 hours at room temperature followed by treatment with polydase enzyme preparation in neutral solution liberated greater than 80 per cent of the phosphate present in vitamin B_{12}.

8. The compound 1-amino-2-propanol-orthophosphate was prepared. Two of the enzymes, prostate phosphotase and diesterase, quantitatively liberated the phosphate from this compound.

9. A large amount of vitamin B_{12} was hydrolysed by a combined procedure utilizing hydrochloric acid hydrolysis and enzymatic hydrolysis. The enzyme used was polydase. The vitamin B_{12} was treated for 2 hours with concentrated hydrochloric acid at room temperature for 33 hours at 37° in a neutral solution containing polydase and finally with 6 N hydrochloric acid at 98° for 30 hours.

10. The hydrolysate was evaporated to dryness and the residue was put through 40 transfers in a Craig countercurrent distribution apparatus. The solvent system employed 1 N hydrochloric acid equilibrated with n-butanol as the stationary phase and n-butanol equilibrated with 1 N hydrochloric acid as the moving phase.

11. Three distinct separations were attained. The red acid fragment moved rapidly and was concentrated in the tube 40. The ammonia and 1-amino-2-propanol remained in the first seven tubes while the benzimidazole moiety appeared in tubes 17 through 28.

12. Analyses were made on each tube for cobalt, nitrogen and phosphorus. About 3.5 per cent of the cobalt was stripped from the molecule.

13. On the basis of the analysis, two nitrogens were allocated to the benzimidazole, four to the red acid fragment and seven appeared as ammonia and 1-amino-2-propanol. The number of nitrogen allocated to free hydrolytic ammonia was five. This permits two nitrogens to be assigned to 1-amino-2-propanol.

14. The analysis for phosphorus showed that complete hydrolysis of the groups linked to the phosphate group did not occur. The phosphorus appeared in four separate regions. The greatest amount appeared in tube zero, possibly as free phosphoric acid.

15. The distribution coefficient for 1-amino-2-propanol was determined for the solvent system 1 N hydrochloric acid equilibrated with n-butanol as the lower phase and n-butanol equilibrated with 1 N hydrochloric acid as the upper phase. The distribution coefficient was calculated to be 0.0796.

16. The distribution coefficient for the ammonium ion was determined for the same solvent system and was found to be 0.0486.

17. The theoretical distribution curves of 1-amino-2-propanol and ammonia were calculated for the first seven tubes of a 40-transfer separation and were compared with the experimental distribution curves found by analysis.

18. Vitamin B_{12a} was substituted for pyridoxal as a catalyst in the oxidative deamination of glycine at pH 4 and 9.6. It was found not to be effective.

19. Potassium cyanide was not able to be reduced by hydrogen over platinum oxide as a catalyst. Vitamin B_{12} and vitamin B_{12r} were found not to act as promoters for the reduction of potassium cyanide.

20. Quantitative recovery of the cyanide from the attempted reduction of the cyanide ion was not realized. This is attributed to the hydrolysis of cyanide to form ammonium formate.

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