STUDIES OF THE CHEMICAL DETERMINATIONS OF VITAMIN B_1

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by

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Table of Contents

Introduction

Thiamin hydrochloride (Vitamin B_1) is a white crystalline powder having an empirical formula of $C_{12}H_{18}ON_4SCl_2$ and a melting point of 245°C. Its synthesis in 1937 by R. R. Williams (1) was the culmination of a half century of effort to identify and isolate the factor responsible for beriberi, a disease evidenced in its acute stages by a generalized edema, or peripheral neuritis or enlargement of the heart. As early as 1884 Takaki, a Japanese doctor, recognized that beriberi was amenable to dietary treatment but it was not until 1905 to 1910 that the dietary origin of this disease became accepted to any great extent. In 1911 Funk (2) succeeded in extracting from rice polishings a crystalline material which cured polyneuritis in pigeons and three years later published his book "Die Vitamine" which first gave that name to the accessory food factors. This publication provided a distinct impetus to investigations in the dietary field and many methods for isolation of the polyneuritic factor (Vitamin B_1) were proposed and tried. Most notable among these was the use of Fullers earth as an adsorbent by Seidell (3) and quinine sulfate elution by Williams (4). Meanwhile Jansen and Donath in 1926 (5) and Ohdake in 1932 (6) succeeded in isolating enough crystalline material to propose a tentative formula for this factor. In 1936 Williams proposed a revised formula which accurately denoted the molecular structure of the crystalline substance and one year later announced the synthesis of a compound, thiamin hydrochloride, having the same biological activity and chemical properties as the natural occurring polyneuritic factor.

History

Until 1934 all assays of vitamin B_1 potency were made with pigeons or rats. However, in that year Kinnersly and Peters (7) published the first chemical test for this vitamin. They had found that diazotized sulfanilic acid in the presence of a buffer and formaldehyde formed a pink colored compound with vitamin B_1. This test was applicable only qualitatively in fractionation experiments.

In the same year W. H. Schopfer (8) proposed a quantitative test using mold growth. As early as 1932 he believed that some "vitamin" influenced bacterial growth on media containing maltose. He later identified one of the factors as vitamin B_1 and then developed the method. It consisted essentially of growing the mold Phycomyces Blakesleeanus on a synthetic media to which had been added varying amounts of the vitamin. The weight of the mycelium developed varied directly with the amount of vitamin B_1 in the substrate. This method was applicable only within narrow limits, being dependent upon the possibility of growth by the mold. It was Schopfer's theory that the organism used vitamin B_1 to synthesize its own growth factor.

The following year Peters (9) found that the purest vitamin B_1 preparations available could be converted by oxidation in aqueous solution into substances showing an intense sky-blue fluorescence. Barger, Bergel and Todd (10) then found that this fluorescence was due to a pale yellow sulfur containing compound (m.p. 221°) having all the recorded properties of "Thiochrome" ($C_{12}H_{14}ON_4S$) which was first found by Kuhn (11) and his colleagues. The fluorescence of this compound was also used as a qualitative test for vitamin B_1.

In 1936 the first quantitative chemical methods for the estimation of vitamin B_1 were published. One of these was a gravimetric procedure proposed by Naiman (12). He found that bismuth iodide in potassium iodide formed an orange-red precipitate with thiamin chloride which could be filtered, dried and weighed. The weight of the precipitate was found to be proportional to the amount of vitamin B_1 present.

Far more important than this, however, were several modifications of previously developed qualitative methods which made possible their application to quantitative determinations. The less important of these were colorimetric procedures consisting of two modifications of the Kinnersly Peters color producing reaction (13, 14) and the use of a new color producing reagent described in a preliminary report by Prebluda and McCollum (15). The other line of attack was the quantitative measurement of fluorescence produced after the oxidation of thiamin chloride to thiochrome. Jansen (16) developed the latter method which has up to the present time found the widest application of any method. He found that the thiochrome produced upon the oxidation of thiamin chloride with potassium ferricyanide could be extracted by isobutanol, in which the fluorescence of thiochrome is much brighter than in water solution. By visual comparison of this fluorescence with that of a series of standards it was possible to determine the amount of vitamin B_1 originally present.

It should be remembered that up until this time vitamin B_1 was obtained solely from natural sources. Its concentration and purification were accomplished almost entirely through adsorption on Fullers earth, Lloyds reagent or acid clays followed by elution with alkalis, aqueous alcoholic hydrochloric acid, quinine sulfate, pyridine or the acid salts of the nitrogen bases. All of these methods were slow and recovery was not quantitative. For that reason, the discovery by Cerecedo and Hennessy (17) that thiamin could be quantitatively adsorbed on and eluted from synthetic zeolites provided a definite step toward more accurate analysis. It made possible the separation of thiamin chloride from some impurities which might interfere with any of the quantitative determinations previously proposed. Whitehorn (18) in 1923 had shown that organic bases of moderate strength are removed from solution by passing through a bed of permutit, one of the synthetic zeolites. Furthermore he showed that the organic base could subsequently be removed from the bed by a salt solution such as potassium chloride. The application of this knowledge by Cerecedo and Hennessy to the problem of purificatinn of vitamin B_1 was of importance because permutit adsorption has several distinct advantages over that of Fullers earth or acid clays. One of the most important is the use of neutral salt solutions for recovery of the vitamin rather than an alkali in which thiamin chloride is relatively unstable. Further advantages are the known composition of the adsorbing agent, the controlled size of the particles, the possibility of modifying the composition of the zeolite and the possibility of regeneration of the adsorbent. Through the use of this agent Cerecedo and co-workers were able to prepare crystalline vitamin B_1 from yeast (17) rice (19) and wheat bran (20).

Another quantitative method which is gradually gaining wider acceptance was also proposed in 1937. Schultz, Atkin, and Frey (21) observed that crystalline vitamin B_1 exerted a pronounced influence on the rate of alcoholic fermentation. By using carefully controlled conditions it is possible to detect as little as one microgram of vitamin B_1 through the measurement of the volume of gas produced by yeast in a certain period of time.

Since 1937, three colorimetric methods have been proposed. A Japanese worker (22) converted thiamin chloride to thiochrome with potassium ferricyanide. The resultant ferrocyanide was then converted to prussian blue. This method involves a notable weakness in its lack of specificity for any reducing substance might change ferricyanide to ferrocyanide. Villela and Leal (23) found that treating thiamin chloride with ammonium molybdate in 3 N sulfuric acid and aminonaphthosulfonic acid resulted in an intense blue color. Since this is the same procedure used in the Fiske and Subbarow determination of inorganic phosphorus, it is necessary to run two determinations, one on the original sample and one after the destruction of organic matter (including thiamin) with a sulfuric acid - nitric acid mixture. The difference between the two determinations would then represent the color due to thiamin. It appears that any organic phosphorous present would make this method inaccurate. A third colorimetric method was one advanced by Melnick and Field (24) using the reagent introduced by Prebluda and McCollum (25). It is this method and the thiochrome method of Cerecedo and Hennessy (26) which have been investigated for this thesis.

Object

The object of these experiments was to study the effectiveness and applicability of the chemical methods for determining thiamin chloride. Two of these, the thiochrome method and a colorimetric method were studied because they have merited the widest application and attention.

Principle

Thiamin pyrophosphate (cocarboxylase) a naturally occurring form of thiamin is converted to free thiamin by enzymatic hydrolysis. The thiamin solution is then purified by adsorption on and elution from a zeolite. This is followed by the determination of the total thiamin (free + phosphoralated) by one of two methods, thiochrome or colorimetric.

Experimental Procedure

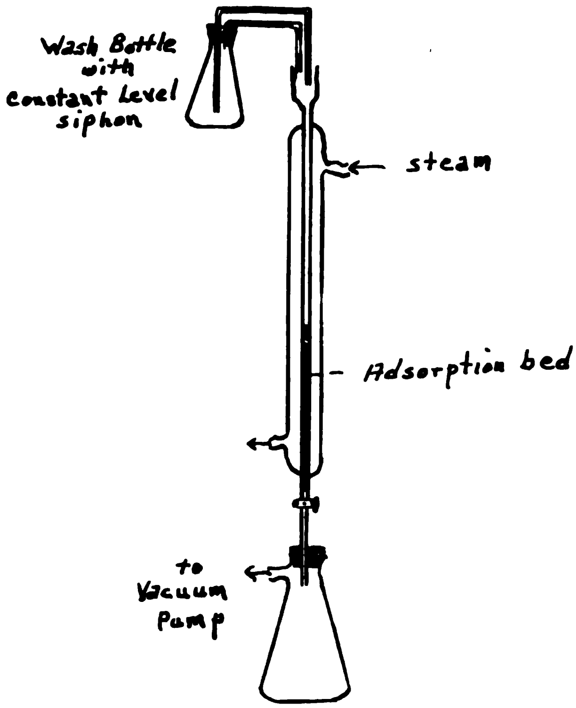
Preparation of the Zeolite

Permutit was originally used in this investigation but was discarded when it became apparent that it would not give satisfactory results. Decalso (30-40 mesh), a sodium aluminum silicate produced by the Permutit Company of New York was finally adopted. A preliminary treatment of this compound is necessary to remove any excess alkali and to replace the sodium with potassium. Two methods have been proposed to accomplish these ends.

Method A - The zeolite is placed in the adsorption tube and fifteen ml of hot 2 percent acetic acid are passed through it. This is followed by fifteen ml of hot 25 percent potassium chloride solution. This alternate washing is repeated four times after which one hundred mls of boiling water are passed through the zeolite bed. It is then ready for use.

Method B - Decalso is suspended in water and while being thoroughly stirred is adjusted to a pH of 4.5 with concentrated sulfuric acid. This pH is maintained by the further addition of sulfuric acid for fifteen minutes. The suspension is allowed to settle and the liquid is decanted. After repeating the acid wash three times, the zeolite is washed five times with water, three times with 95 percent ethyl alcohol, once in acetone and three times in ethyl ether. It is dried in a flat pan at $37^{\circ}c$ for three days. The dried material is placed in the adsorption tube and washed with 30 mls of 25 percent potassium chloride solution followed by 500 mls of hot distilled water. The Decalso bed is then ready for use.

Adsorption Tube



Hydrolysis of Sample

A sample which will contain approximately 150 gamma of total thiamin is refluxed for fifteen minutes with twenty volumes of 2 percent acetic acid. After cooling, one tenth volume of 1 N NaOH and one tenth volume of freshly prepared 6 percent Takadiastase (Parke-Davis) are added. The mixture is incubated at 38°C for one and one half hours, brought to a boiling point, cooled and filtered. The total filtrate or an aliquot thereof is then ready for passage through the adsorption tube.

Adsorption and Elution

A volume of thiamin chloride solution containing not more than 200 gamma of the vitamin is introduced into the adsorption tube at room temperature. If the concentration is low, the solution is permitted to run through the Decalso bed at a fairly rapid rate (10 ml/minute). If the concentration is higher, the rate of flow should be such that the solution is passing through the bed for a minimum of 7 minutes. When this is completed, the adsorption tube is heated by steam and the Decalso bed washed with 30 ml of distilled water. This wash serves two purposes. It removes any impurities which might have been left in the tube and also helps heat the Decalso bed preparatory to elution. Immediately after the last of the wash water has been sucked out, the stopcock is closed and while steam is still passing through the outer jacket, 25 percent potassium chloride solution adjusted to pH 2.0 with sulfuric acid is added to the tube. This is then permitted to pass through the Decalso bed at such a rate that 20 mls of eluate are collected in 6 to 9 minutes. The potassium chloride solution is added to the tube in 10, 5, 3, and 2 ml portions to facilitate complete elution. The eluate is cooled and is then ready for a quantitative analysis of the thiamin chloride content by either the fluorometric (thiochrome) or colorimetric procedure. The Decalso bed is washed with 500 ml of hot distilled water using the constant level siphon arrangement (see diagram) to prepare it for the next sample.

Determination of thiamin

A. Fluorimetric. A volume of potassium chloride eluate containing not more than 20 gamma of thiamin chloride is diluted to 5 mls. 3 ml of 15 percent sodium hydroxide are added followed immediately by 0.05 to 0.2 ml of 1 percent potassium ferrocyanide. The thiochrome is extracted at once with 20 mls of isobutanol by shaking one minute, centrifuging and separating into the two layers. The isobutanol is treated with anhydrous sodium sulfate until it is perfectly clear. The fluorescence of the isobutanol solution is compared either visually or in a fluorometer with standards of known thiamin chloride solution which have been similarly treated. For visual comparison a General Electric Vapor Lamp producing black light was used.

B. Colorimetric. The color-producing reagent, diazotized p-aminoacetophenone, was prepared according to the directions of Prebluda and McCollum (25) as follows:

Solution A - 3.18 gm of p-aminoacetophenone (Eastman Kodak Company, No. 631) are dissolved in 45 ml of concentrated hydrochloric acid (37 percent) and made up to a final volume of 500 ml in a volumetric flask with distilled water. Precautions are taken to keep the solution in a glass-stoppered flask and to avoid the presence of strong light when not in use. The activity of this solution is unaltered after standing for six months.

Solution B - 22.5 gm of C.P. sodium nitrite are dissolved in distilled water and made up to a final volume of 500 ml in a glass stoppered volumetric flask. This reagent begins to deteriorate after standing for a month. It is best preserved at refrigeration temperatures.

Solution C - 20 gm of C.P. sodium hydroxide are dissolved in 600 ml of distilled water; 28.8 gm of C.P. sodium bicarbonate are then added and the solution made up to a final volume of one liter with distilled water.

Diazotization and Final Reagent - The diazotization reaction is carried out in an ice bath with use of one part by volume each of Solution A and of Solution B. This reaction requires a period of about 10 minutes. The mixture is agitated by means of an electric stirrer. At the end of this period 4 parts of Solution B are added to the resultant mixture. The solution is stirred and maintained at a temperature of 0 - 5°C for at least 20 minutes. When the reaction is completed, the diazotized solution remains satisfactory for use at least 12 hours at refrigeration temperatures. In making the final reagent for reaction with the vitamin, add 20 ml of freshly diazotized p- aminoacetophenone to a flask containing 275 ml of Solution C. This mixture is stirred for a period of 5 to 10 minutes after which the reagent is ready to use.

<u>Preparation of Standards</u>. Since a photelometer is used in this work it is necessary to prepare a reference standard curve from pure thiamin hydrochloride (Merck). A stock solution containing 100 gamma of thiamin chloride per ml is prepared. This solution, when acidified to pH 2.0 with sulfuric acid, is stable for at least 2 months at refrigeration temperatures. In a series of 50 ml centrifuge tubes are placed 0.2, 0.4, 0.6, 0.8 and 1.0 ml of the stock thiamin solution. Each is diluted to 5 ml with potassium chloride solution (25 percent) which has passed through the adsorption tube when no thiamin was present. To

each is added 5 ml of an alcohol-phenol solution containing 5 mgs of phenol per ml and two drops of thymol blue. While this mixture is being stirred with a fine stream of nitrogen, N sodium hydroxide is added drop by drop until the first faint blue color appears and this is followed immediately by the addition of 2 volumes (10 ml) of the diazo reagent. After thorough mixing, the solution is allowed to stand from 15 to 24 hours at room temperature. The red compound is extracted with 4 ml of redistilled xylene (B.P. 134° - 138°) by adding the xylene directly to the tube containing the reaction mixture and agitating by means of a stream of nitrogen for $1\frac{1}{2}$ minutes. The layers are allowed to separate and the red color in the xylene layer is determined with the photelometer.

Standardization of the Photelometer. The photelometer was standardized using a green filter having maximum transmission between 520 - 580 mu. The standard xylene solutions prepared above contain the color produced by 5, 10, 15, 20 and 25 gamma of thiamin chloride per ml of xylene. These solutions were read in the photelometer and the gamma per ml xylene (ordinate) plotted against percent transmission (abscissa). A straight line results which was used as a standard curve for the thiamin diazotate.

Preparation of Sample. An aliquote of potassium chloride eluate containing 30 to 70 gamma of thiamin was used for the actual determination. It was treated the same way as previously described for the standard solutions. In some cases a heavy precipitate was formed in the reaction mixture upon standing overnight. When this occurred, the reaction mixture plus the xylene was treated with 5 ml of 20 percent sulfuric acid and thoroughly shaken. Ten ml of 15 percent sodium hydroxide were then added, the mixture again shaken vigorously and allowed to separate. The color in the xylene layer was then read in the photelometer and the amount of thiamin present calculated from the standard curve.

TABLE A

Zeolite - Permutit

Preliminary Preparation - Method B

Exp. No.	γ to tube	Vol. of soln.	Adsorption	Elution	γ Found	% Recovery	Remarks
1	100	10	Hot	Hot	30	30	
2	100	10	Hot	Hot	37	37	
3	200	20	Hot	Hot	56	28	
4	250	25	Hot	Hot	155	62	
5	600	10	Cold	Hot	504	84	Color in 2nd 10 ml eluate.
6	600	10	Cold	Hot	522	87	
7	600	10	Cold	Hot	558	93	
8	75	10	Cold		35	47	
9	100	10	Cold		57	57	
10	125	10	Cold	Hot	73	58	
11	150	6	Cold		60	40	
12	175	7	Cold		40	23	
13	200	8	Cold	Hot	60	30	
14	75	25	Warm	Hot	55	73	
15	100	25	Warm	Hot	67	67	
16	125	25	Warm	Hot	66	53	

TABLE B

Zeolite - Decalso

Preliminary Preparation - Method A

Exp. No.	pH of Adsorption	γ to tube	Vol. of soln.	Adsorption	Elution	γ Found	% Recovery	
17	7.0	150	100	Hot	Hot	92	61	
18	7.0	Filtrate from #1		Hot	Hot	None		Adsorption complete
19	7.0	100	100	Hot	Hot	59	59	
20	4.5	200	100	Hot	Hot	192	96	Color in 2nd 10 ml of eluate
21	4.6	200	100	Hot	Hot	176	88	
22	5.2	200	100	Hot	Hot	158	79	
23	7.5	100	50	Hot	Hot	91	91	
24	7.0	100	50	Hot	Hot	89	89	
25	4.2	100	50	Hot	Hot	78	78	
26	3.8	200	25	Hot	Hot	180	90	
27	4.0	100	25	Hot	Hot	87	87	
28	2.6	100	25	Hot	Hot	90	90	
29	7.4	100	25	Hot	Hot	78	78	
30	7.3	200	25	Hot	Hot	164	82	
31	5.0	100	25	Hot	Hot	78	78	
32	6.3	100	25	Hot	Hot	90	90	
33	6.9	150	25	Hot	Hot	132	88	
34	6.2	200	25	Hot	Hot	168	84	
35	6.4	100	25	Cold	Hot	88	88	
36	7.0	150	25	Cold	Hot	132	88	
37	6.2	200	25	Cold	Hot	180	90	

TABLE C

Zeolite - Decalso

Preliminary Treatment - Method B

Exp. No.	pH of Adsorption	γ to tube	Vol. of soln.	Adsorption	Elution	γ Found	% Recovery	
38	7.3	100	25	Hot	Hot	64	64	
39	7.2	150	25	Hot	Hot	102	68	
40	7.2	200	25	Hot	Hot	144	72	
41	4.2	200	10	Hot	Hot	144	72	
42	4.1	Filtrate from 41		Hot	Hot	None		Adsorption complete
43	4.0	200	25	Hot	Hot	156	78	
44	4.1	200	25	Hot	Hot	144	72	
45	4.0	200	10	Hot	Hot	136	68	
46	4.0	Filtrate from 45		Hot	Hot	None		
47	7.0	200	2	Cold	Hot	160	80	
48	7.1	Filtrate from 47		Cold	Hot	None		
49	7.0	200	2	Cold	Hot	172	86	
50	7.0	200	2	Cold	Hot	120	60	Elution rate too fast
51	7.0	200	2	Cold	Hot	144	72	KCl not adjusted to pH2.0
52	7.1	200	2	Cold	Hot	154	77	
53	4.0	200	2	Cold	Hot	184	92	
54	4.1	200	2	Cold	Hot	174	87	
55	4.1	200	2	Cold	Hot	178	89	
56	3.9	200	2	Cold	Hot	188	94	
57	3.7	200	2	Cold	Hot	188	94	
58	3.7	200	2	Cold	Hot	176	88	
59	3.9	200	2	Cold	Hot	192	96	
60	3.7	200	2	Cold	Hot	184	92	
61	3.8	200	2	Cold	Hot	184	92	
62	3.8	200	2	Cold	Hot	184	92	
63	7.2	200	2	Cold	Hot	192	96	
64	3.7	200	2	Cold	Hot	184	92	
65	7.0	200	2	Cold	Hot	184	92	
66	7.0	150	1.5	Cold	Hot	129	86	
67	7.1	100	1.0	Cold	Hot	84	84	
68	7.0	200	2	Cold	Hot	184	92	
69	7.1	150	1.5	Cold	Hot	132	88	
70	7.2	100	1.0	Cold	Hot	84	84	
71	7.0	200	2	Cold	Hot	192	96	
72	3.7	150	1.5			144	96	
73	3.5	100	1.0			90	90	

TABLE D

Photelometer Readings of Standards

Gamma of thiamin/ml xylene	Low	High	Average
5.0	65	66	65.7
10.0	45	48.5	46.5
12.5	37	40.0	38.3
15.0	29.5	33.2	31.3
17.5	26.0	28.0	27.0
20.0	22.0	24.1	23.4
25.0	16.0	18.9	17.4

TABLE E

Progressive Percent Recovery of Thiamin

Experiment Number	Tube Number 1	2	3
38	64	68	72
41	72	-	78
44	72	68	-
47	80	-	86
50	60*	72	77
53	92	87	89
56	94	94	88
59	96	92	92
62	92	96	92
65	92	86	84
68	92	88	84
71	96	96	90

* Elution too fast

TABLE F

Effect of Temperature of Adsorption

Experiment number	pH of Adsorption	γ thiamin to tube	Volume of solution	Adsorption	Elution	γ thiamin found	% Recovery
32	6.3	100	25	Hot[x]	Hot	90	90
35	6.4	100	25	Cold[*]	Hot	88	88
33	6.9	150	25	Hot	Hot	132	88
36	7.0	150	25	Cold	Hot	132	88
34	6.2	200	25	Hot	Hot	168	84
37	6.2	200	25	Cold	Hot	180	90

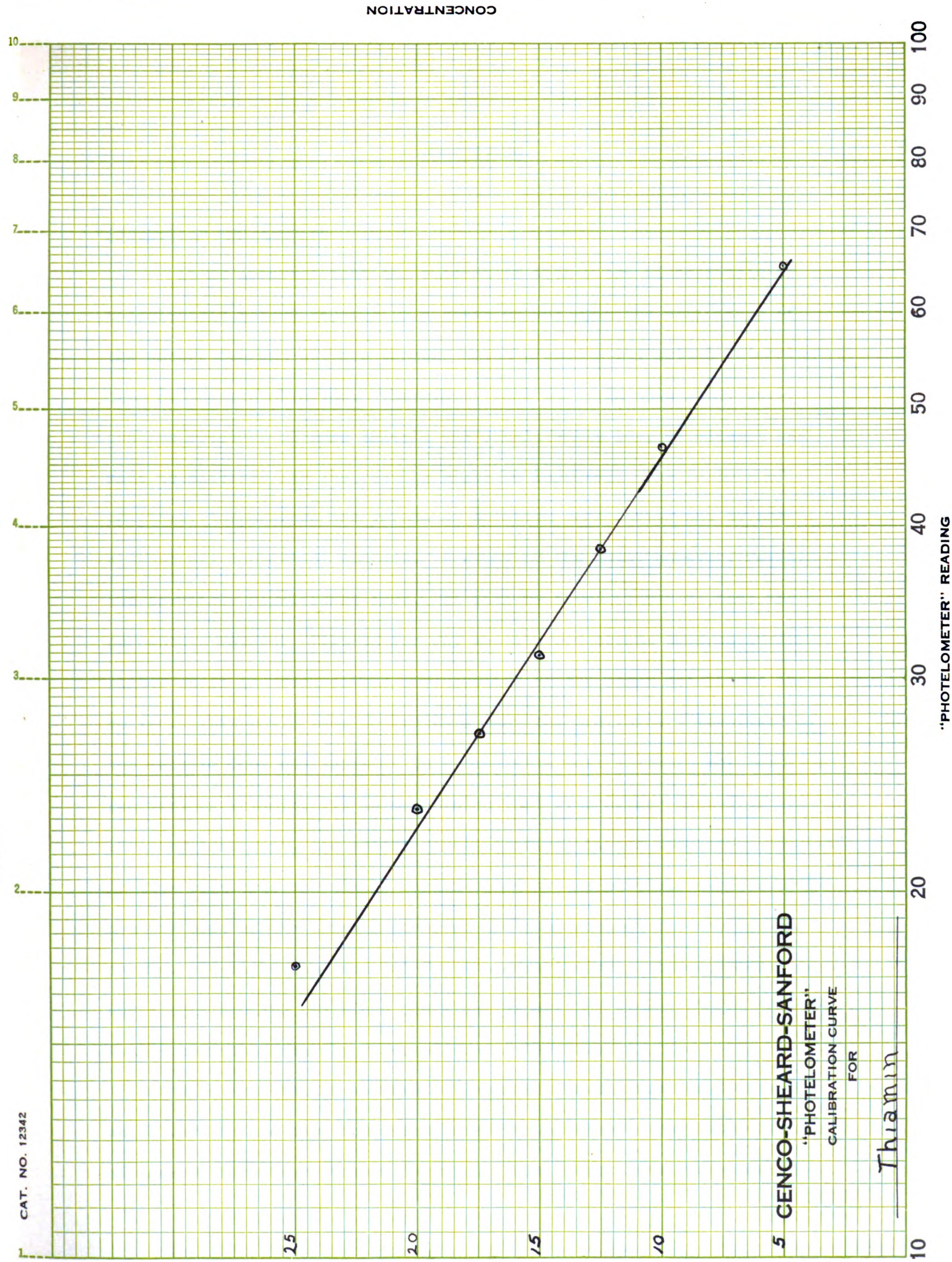
x Hot - 90^{o} - 95^{o} C

* Cold- 22^{o} - 25^{o} C

TABLE G

Effect of pH of Adsorption

pH of Adsorption	4.0	3.9	3.9	3.8	7.0	7.0	7.0
% Recovery	92	94	96	92	92	92	96



Gamma of Thiamin per ml

Discussion

Adsorption and Elution

It is apparent that the success of either the fluorometric or colorimetric method depends to a great extent upon the efficiency of the adsorption and elution process which precedes the actual determination of thiamin chloride. Therefore, when early in this study percentage recovery of pure thiamin was consistently low, an attempt was made to discover how the results might be improved. The factors of pH, time, temperature and volume of solution were varied one by one without any consistent success. A study of the data, however, reveals one seemingly significant fact. It will be noted (Table E) that the percentage recovery figures for any zeolite bed become progressively higher as it is used, almost irrespective of the other conditions involved. This would seem to indicate that the preliminary treatment of the zeolite does not leave it in a condition most suitable for complete recovery of thiamin. It is my opinion that with either method of preliminary treatment, the excess alkali (sodium) is not completely removed. If this were true the alkali concentrated at the particle surface might destroy some of the thiamin adsorbed and thus account for the low recovery. It might also account for the progressively higher results from any single zeolite bed since one might expect the excess alkali to eventually be washed away by repeated use of the tube.

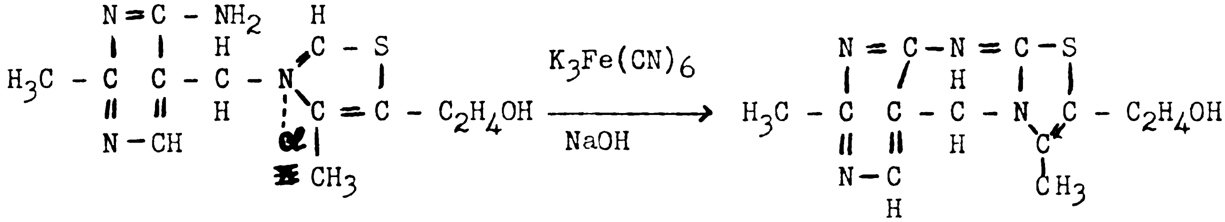
With that belief in mind, selection of data from zeolite beds which were in the same condition makes it possible to show the influence of some of the other factors involved. The temperature of adsorption used by other workers varied between room temperature (22^{o} - 25^{o} C) and steam temperature (90^{o} - 95^{o} C). Both of these extremes were tried. No evidence of any difference could be found (Table F). Cold adsorption was finally adopted because of the theoretical considerations involved.

Many attempts were made to find what difference if any the pH of the zeolite bed and of the thiamin solution might make. Alkaline conditions were excluded because of the instability of thiamin in alkalies. Table G shows that other conditions being kept the same, adsorption is equally efficient at pH 4.0 or pH 7.0.

Elution was judged complete when the addition of more potassium chloride to the adsorption tube showed no evidence of thiamin. Twenty ml of potassium chloride was found to completely elute up to 250 gamma of thiamin in a period of 5 minutes.

Fluorometric Method.

Thiamin or its hydrochloride upon treatment with potassium ferrocyanide in the presence of an alkali yields thiochrome, a compound which gives a sky - blue fluorescence in ultra violet light.

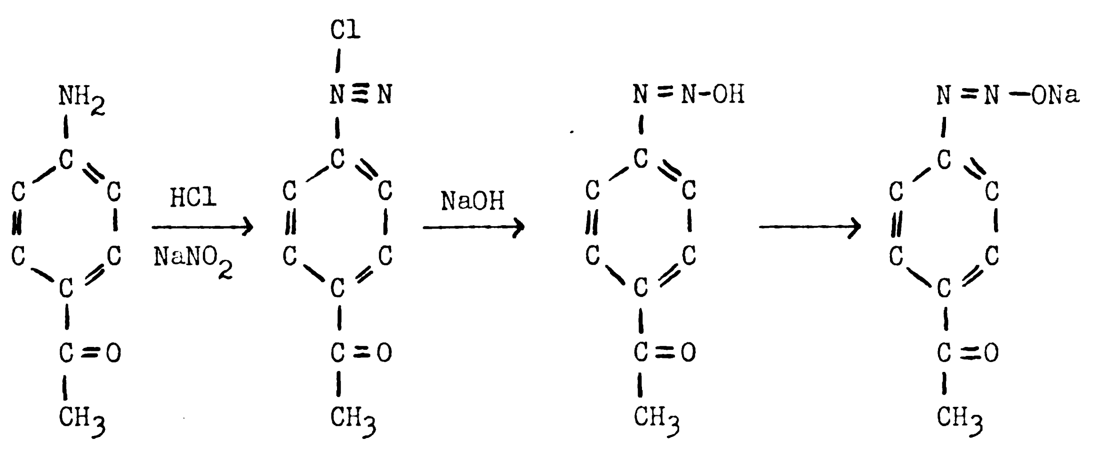


Thiamin Thiochrome

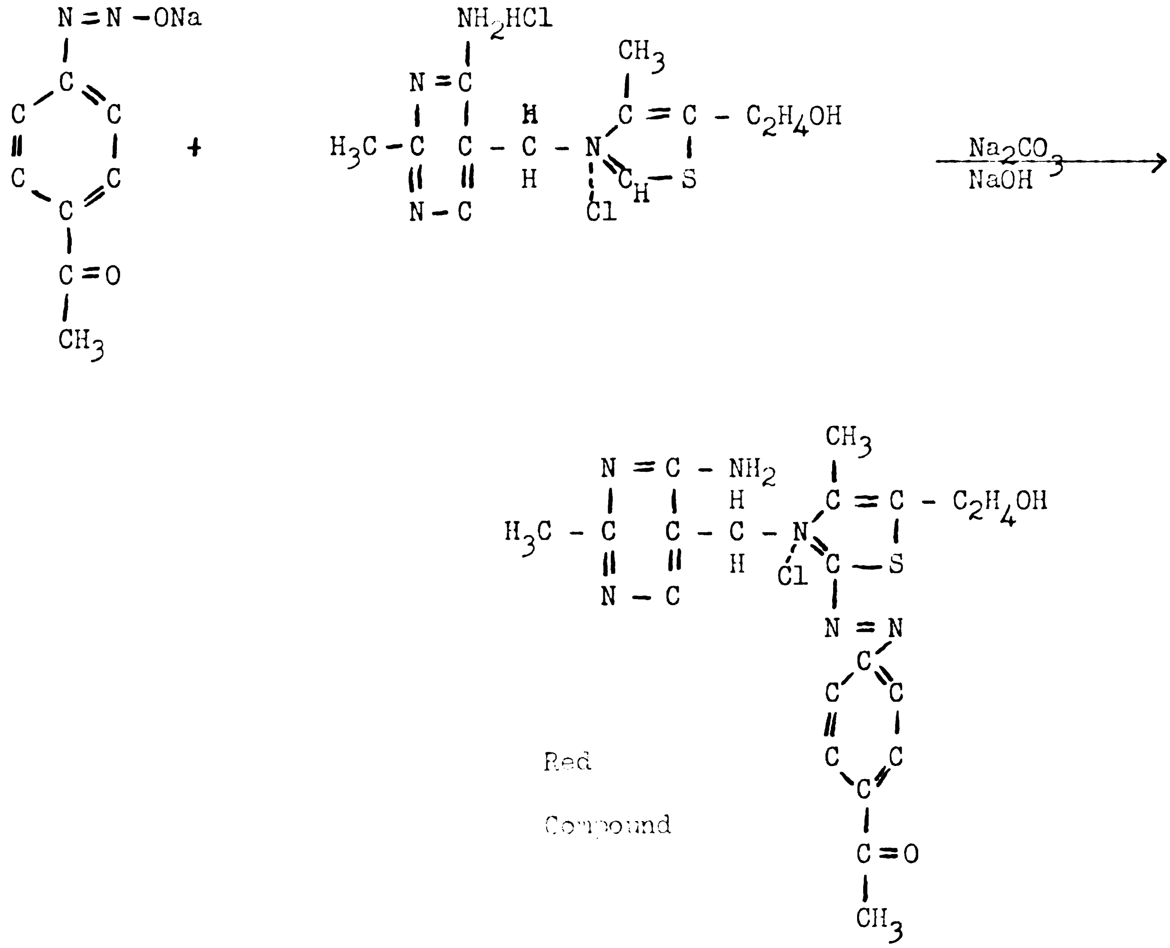
The fluorescence of the thiochrome is best read in a fluorometer, an instrument using ultra violet light at 3685 Å which is changed by thiochrome to the visible range between 4300 Å and 5000 Å. The fluorescent light is measured at right angles to the incident light by means of a photocell, galvanometer arrangement. Such an instrument was not available so an attempt was made to compare the sample with standards visually. It was found that isobutanol will not quantitatively extract one gamma of thiochrome per ml. The fluorescence from that amount of thiochrome is too slight to read accurately by eye so the method was abandoned in favor of the colorimetric method.

Colorimetric method.

Thiamin hydrochloride reacts with the diazo compound of p-aminoacetophenone to form a red, stable, water-insoluble, xylene soluble compound having a maximum absorption at 520 millimicrons.



p-aminoacetophenone　　diazonium　　diazo



Red Compound

The reagent does react with some other compounds to yield colored substances but none of them are extractable by xylene.

The use of the photelometer makes it unnecessary to prepare a fresh standard with each determination, but necessitates working with small samples because of the intensity of the color developed. For that reason special care must be taken to avoid excess alkali (pH 7.5 to 8.5 required for reaction) when adding sodium hydroxide to the reaction mixture and also to insure complete extraction of the thiamin compound by thorough mixing with xylene. When these precautions are observed the method is very satisfactory.

Conclusions

1. Adsorption and elution take place best on Decalso which has been given extended acid treatment.

2. The Fluorometric method of analysis is rapid and accurate but requires a fluorometer for accurate reading of results.

3. The colorimetric method of analysis is complex and requires more time than the above but is accurate and applicable to research problems.

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