form a sulfonyl chloride and cyanamide hydrochloride.

2. N-Alkyl substituted isothioureas interact with chlorine under similar conditions to form sulfonyl chloride and the corresponding alkyl cyanamide derivatives.

3. N-Aryl substituted isothioureas interact with chlorine to give cyanamides containing chlorine substituted in the aryl grouping of the cyanamide.

4. N,N'-Diarylisothioureas interact with chlorine to form halogenated diaryl ureas and sulfonyl chlorides.

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The Determination of Free and Phosphorylated Thiamin by a Modified Thiochrome Assay[1]

BY DOUGLAS J. HENNESSY AND LEOPOLD R. CERECEDO

The assay of thiamin, through its quantitative conversion to thiochrome which can be determined fluorometrically, was proposed by Jansen in 1936.[2] Since that time, several investigators[3-7] have applied the method with little modification. The method is based on the oxidation of thiamin by potassium ferricyanide in an alkaline medium, extraction of the thiochrome formed by isobutanol, and estimation of the intensity of the violet-blue fluorescence in ultra-violet light. Adsorption on Franconite or similar adsorbents has been resorted to in an attempt to separate the thiamin from materials interfering in the assay. There is, however, need for a more efficient purification of the thiamin. Base-exchange, replacing the adsorption technique, allowed of a simpler isolation of thiamin from three natural sources.[8-10] It is also more effective in preparing samples for the thiochrome assay. By means of this step, materials which interfere chemically or physically, are eliminated.

Lohmann and Schuster[11] proved that cocarboxylase is a pyrophosphoric ester of thiamin and that it cures polyneuritis in pigeons. In many yeasts and animal tissues this is the pre-dominant form of vitamin B_1. Although not affecting the conversion to a fluorescent thiochrome, the pyrophosphoric acid group prevents extraction by isobutanol.[12] The presence of interfering materials in the aqueous layer precludes the accurate fluorometric measurement of the non-extractable thiochrome pyrophosphate. Tauber,[13,14] Weijlard and Tauber[15] and Lohmann and Schuster[11] have found that cocarboxylase can be hydrolyzed enzymatically. Tauber, using kidney tissue, measured the decrease in cocarboxylase activity, but did not show the hydrolysis to be a complete removal of the pyrophosphate group. Lohmann and Schuster state that a kidney phosphatase hydrolyzed the pyrophosphate to orthophosphate, and only with an enzyme from prostate were they able, after several days, to obtain thiamin from the hydrolysate. Extraction of beef kidney, clarification of the extract, and fractional precipitation with acetone, has given us a stable preparation, practically free of vitamin B_1, which will in two or three hours quantitatively convert cocarboxylase and thiamin orthophosphate to what is probably thiamin. The thiochrome formed from this hydrolysate is extractable by isobutanol in the usual way.

Experimental

Preparation of the Extracts.—The finely divided sample is refluxed for three minutes with five to twenty parts of 2% acetic acid solution. After cooling and centrifuging, the residue is reëxtracted and the filtrates are combined. In case of gelation, where good separation is not accomplished by centrifuging, the solution is made 30% with

(1) Presented at the Dallas Meeting, April, 1938, and at the Milwaukee Meeting, September, 1938, of the American Chemical Society.

(2) B. C. P. Jansen, Rec. trav. chim., 55, 1046 (1936).

(3) (a) H. G. K. Westenbrink and J. Goudsmit, Rec. trav. chim., 56, 803 (1937); (b) W. Karrer and V. Kubli, Helv. Chim. Acta, 20, 369 (1937).

(4) F. Widenbauer, O. Huhn and G. Becker, Z. ges. exptl. Med., 101, 178 (1937).

(5) K. Ritsert, Deut. med. Wochschr., 64, 481 (1938).

(6) M. A. Pyke, Biochem. J., 31, 1958 (1937).

(7) G. Hongo, J. Pharm. Soc. Japan, 58, 361 (1938).

(8) L. R. Cerecedo and D. J. Hennessy, THIS JOURNAL, 59, 1617 (1937).

(9) L. R. Cerecedo and F. J. Kaszuba, ibid., 59, 1619 (1937).

(10) L. R. Cerecedo and J. J. Thornton, ibid., 59, 1621 (1937).

(11) K. Lohmann and P. Schuster, Biochem. Z., 294, 188 (1937).

(12) H. W. Kinnersley and R. A. Peters, Biochem. J., 32, 697 (1938).

(13) H. Tauber, J. Biol. Chem., 123, 499 (1938).

(14) H. Tauber, ibid., 125, 191 (1938).

(15) J. Weijlard and H. Tauber, THIS JOURNAL, 60, 2263 (1938).

alcohol. If the extract is to be used under Method II or Method III (cf. below), the alcohol is later removed by vacuum distillation and the volume made up with water. In most cases the extract does not represent the total volume of acetic acid solution added. Correction is made for this in the calculation. Liquids such as milk, blood, and animal tissue extracts are prepared for analysis by precipitation of the proteins with just enough 2% trichloroacetic acid solution, refluxing of the resulting suspension, and centrifuging after cooling.

In the case of adsorbates such as the International Standard or the U. S. P. Reference Standard, approximately 100 mg. is extracted with four 5-cc. portions of boiling two normal hydrochloric acid. The residue is separated each time by centrifuging. Finally, the residue is extracted twice by mixing with 10 cc. of cold two normal sodium hydroxide. The acid and alkaline eluates are combined and, after adjustment to the required pH, the volume is made up to 50 cc. with water.

Concentrates which are water soluble are merely diluted to a convenient volume.

In the case of urine the sample is adjusted to the proper acidity and assayed by Method II.

The Assay.—As we perform the thiochrome assay, there are three alternate procedures: direct assay (Method I), assay following base-exchange (Method II), and assay following enzymatic hydrolysis with or without base-exchange (Method III). Method I is used whenever the blank is comparatively small and the recovery of vitamin, added in an equal quantity to that present, is practically quantitative. Further, there should be left in the aqueous layer following the isobutanol extraction not more fluorescence, as observed visually, than is in the same layer of the blank or of a sample containing an equal quantity of pure thiamin. If direct assay is not practical, though no unusual unextractable fluorescence is noted, Method II is used. When obvious residual fluorescence is noticed in the alkaline aqueous layer, then Method III is employed.

Method I.—Such a volume of solution as will contain 0.2–20.0 micrograms of the vitamin is made up to 5 cc. in a 25-cc. glass-stoppered graduated cylinder. To the sample 0.05–0.20 cc. of 1% potassium ferricyanide solution is added. Three cc. of 15% sodium hydroxide solution is added with mixing and immediately followed by 13 cc. of isobutanol. The mixture is shaken vigorously for one minute and centrifuged for one-half minute to separate the two layers. It is then carefully poured into a separatory funnel, and the lower layer removed and set aside for comparison with the same solution in the blank determination to ascertain whether Method III be necessary. The isobutanol solution is transferred to a test-tube and 2–4 g. of anhydrous sodium sulfate is added. After mixing well, the sodium sulfate is allowed to settle and 10 cc. of the perfectly clear isobutanol solution is decanted into the cuvette of the fluorometer. The same procedure is repeated for the blank except that potassium ferricyanide is omitted, and the order of addition of the sodium hydroxide and the isobutanol is reversed.

When thiamin chloride is added to a sample in an amount equal to that already present, 93–102% is recovered in this method.

Method II. The Exchange Tube.[16]—A tube of 7 mm. inside diameter surrounded by an outer jacket over 40 cm. of its length is used. A constriction at the lower end of the jacketed portion holds a plug of glass wool. On this is filled a 20 cm. column of 30-mesh decalso.[17] At the upper end of the tube is a funnel for introduction of liquids, at the lower end a two-way stopcock for gravity flow and suction.

When newly introduced, the decalso is freed of excess alkali and converted to the potassium form by alternately passing through four 15-cc. portions each of boiling 2% acetic acid and 25% potassium chloride solution. During this treatment steam is passed through the outer jacket so as to keep the temperature near 100°. The column is then washed by passage of three 15-cc. portions of boiling water, which leaves the tube ready for use.

The Exchange.—From 5 to 20 cc. of the solution containing up to 10.0 micrograms of thiamin as indicated in a rough direct assay is adjusted to pH 4.0–4.5 with acetic acid. It is brought to a boil and introduced into the exchange tube which is kept near 100°. The solution, which is allowed to pass through the decalso bed in three to five minutes, is followed by three 5-cc. portions of boiling water. The stopcock is shifted to suction and the excess water drawn from the tube.

For the removal of the vitamin from the decalso bed, boiling 25% potassium chloride solution is introduced into the funnel at the top of the exchange tube. The flow of the solution through the decalso bed should be near 1 cc. per minute, 10–25 cc. being collected. The larger volume is used when the sample contains the larger quantities of thiamin. For 0–1 μg. collect 10 cc., for 1–3 μg., 15 cc., for 3–7 μg., 20 cc. and for 7–10 μg., 25 cc. The approximate content of the sample can be judged, after some experience, by visual observation of the oxidized sample in ultraviolet light. Aliquots of the potassium chloride effluent (usually 5 cc.) are assayed by Method I. The amount of the 1% potassium ferricyanide solution required is 0.05 cc.

Since the decalso tube may be used repeatedly by merely washing with hot water for removal of the potassium chloride left in the tube, it is essential, when a sample of low thiamin content follows a sample of high content, that all the thiamin of the former sample be removed. Refilling the exchange tube is resorted to when a determination on a pure sample of thiamin shows either a large blank or a low recovery. Recovery of thiamin chloride added to samples is 91–97% when Method II is used.

Method III. Preparation of the Enzyme.—Ground defatted beef kidney is extracted by shaking or stirring with an equal weight of 1% sodium chloride solution for two hours. The mixture is centrifuged and the centrifugate put aside in the refrigerator. The residue is reëxtracted in the same way. The combined extracts are stirred with sufficient 300-mesh Volclay[18] to give a thin paste which is centrifuged. On filtration of the centrifugate a clear liquid is obtained, the volume of which is about 60% of that of the original extract. One-half volume of acetone is stirred into the extract and the precipitate re-

(16) Obtained from E. Machlett and Son, New York City.

(17) Obtained from The Permutit Company, New York City.

(18) Obtained from the American Colloid Co., Chicago, Ill.

moved by centrifuging. A second portion of acetone, equal in volume to that already added, is stirred into the centrifugate. The precipitate which forms is collected by centrifuging and is dried in a vacuum desiccator over sulfuric acid. The dry residue is powdered and preserved in a brown stoppered bottle.

Enzymatic Hydrolysis.—The solution to be tested is adjusted to pH 6.5–7.0, 5–20 cc. mixed with 20–80 mg. of the powdered enzyme and kept at 37° for three hours. The hydrolysate is acidified by the addition of one drop of acetic acid, boiled, cooled and centrifuged. An aliquot of the centrifugate is assayed under Method I or, if necessary, Method II.

The recovery of thiamin chloride and of cocarboxylase is 90–97% when added to samples assayed by Method III.

Fluorometry.[19]—In operating the fluorometer, the incident beam passes through the thiochrome solution, causing it to fluoresce. The fluorescent light strikes a photocell at the side of the cuvette and is transformed into electrical energy, the current of which is measured by a galvanometer. With a constant incident intensity, there is a linear relationship between the deflection of the galvanometer and the thiamin chloride up to twenty micrograms.

The instrument is calibrated by the following procedure. With a fully opened iris diaphragm in the path of the incident ultraviolet light, Q, T and B, respectively, are the galvanometer deflections measuring the fluorescence of a standard quinine sulfate solution in tenth normal sulfuric acid, of a thiochrome solution prepared according to Method I from N micrograms of thiamin chloride and of isobutanol obtained in the same way without any thiamin chloride being used. With Q_0 as the quinine deflection for a lower incident intensity, the deflection per microgram of thiamin chloride is

$$Q_0 \frac{T - B}{NQ}$$

A photocell at the rear of the empty cuvette allows measurement of the incident intensity. These deflections plotted against the various readings for Q_0 may be used conveniently to set the deflection per microgram of thiamin chloride. In the measurement of the unknown, the difference between the deflections induced by the oxidized and unoxidized aliquots is divided by the deflection per microgram of thiamin chloride as set directly by reference to Q_0 or indirectly to the rear photocell deflection just before pouring the thiochrome solution into the cuvette.

Discussion

An examination of the results summarized in the table shows that although the vitamin contents of the International Unit and of the U. S. P. Reference Unit differ widely when the free thiamin alone is determined, the vitamin activity of the two adsorbates is nearly identical if the phosphoric esters of thiamin are also included in the assay. The table also reveals that thiamin in its phosphorylated form has no more biological

(19) The fluorometer and accessories may be obtained from Pfaltz and Bauer, New York City.

potency than free thiamin as measured in rat curative, rat growth and mouse tests. These results are in contrast with the report of Lohmann and Schuster,[11] according to which the pigeon curative test shows the cocarboxylase to be of significantly higher potency. A previous report by Lohmann and Schuster[20] indicated that thiamin was not more potent in the phyrophosphate form.

Nothing is known regarding the amount of thiamin orthophosphate present in natural sources. This substance might also be formed during the acid extraction which is used in preparing the samples for the assay. We were interested, therefore, in ascertaining whether this compound could also be estimated by our method. Lohmann and Schuster[11] have shown that cocarboxylase on hydrolysis with boiling one normal hydrochloric acid for fifteen minutes is split into phosphoric acid and thiamin orthophosphate. We have used a hydrolysate prepared in this manner for the assay. An examination of the table shows that the thiochrome method includes thiamin in this form. No accurate estimate of the biological potency of thiamin orthophosphate can be made from the data in the table. It is realized, of course, that both phosphoric esters behave similarly in the thiochrome method and that, to distinguish them, cocarboxylase activity must be determined. This latter measurement, however, is as yet uncertain because of the many factors other than cocarboxylase concentration which influence the result.

It is of interest to note that the concentration of thiamin in blood and in urine is of the same magnitude.

With the exception of yeast, plant materials seem to be relatively low in thiamin phosphoric ester content. This is in general agreement with the findings of Tauber,[21] which show that such materials have low cocarboxylase activity. The age and previous treatment of the samples may have some significance in this respect, as shown in the case of the different wheat germ samples, only one of which gives an increase in free thiamin after hydrolysis.

The cocarboxylase activity of animal tissues as reported by various investigators is borne out by the high proportion of the phosphoric esters found in liver.

(20) K. Lohmann and P. Schuster, *Naturwissenschaften*, **25**, 26 (1937).

(21) H. Tauber, *Proc. Soc. Exptl. Biol. Med.*, **37**, 541 (1937).

Comparison of Results of Bioassay and Fluorometric Assay of Various Materials

Material assayed	Vitamin B₁/g. found in bioassay	Vitamin B₁/g. calcd. as thiamin chloride as found in Method		
		I	II	III
Cocarboxylase,[a] (72% thiamin chloride)	66%[b]	1%	0	69%
Thiamin orthophosphate (86% thiamin chloride)	.....	3%	...	80%
International standard	170–500 μg.[c]	240 μg.	...	290 μg.
Proposed U. S. P.[d] reference standard	100 I. U.[e]	60 μg.	...	280 μg.
Yeast I	8 I. U.[f]	14 μg.	18 μg.	24 μg.
Yeast II	15 I. U.[f]	28 μg.	33 μg.	46 μg.
Yeast III	21 I. U.[g]	4 μg.	5 μg.	59 μg.
Yeast IV	53 I. U.[g]	28 μg.	30 μg.	160 μg.
Yeast V	160 I. U.[f]	230 μg.	240 μg.	490 μg.
Rice polishings	6 I. U.[h]	...	19 μg.	...
Whole wheat	1.3–1.9 I. U.[h]	...	4.2 μg.	...
Wheat germ I	7 I. U.[h]	...	22 μg.	21 μg.
Wheat germ II	7 I. U.[j]	...	21 μg.	20 μg.
Wheat germ III	10 I. U.[i]	...	25 μg.	30 μg.
Fortified cereal I	1.5 I. U.[i]	...	4.2 μg.	...
Fortified cereal II	0.6 I. U.[i]	...	1.6 μg.	...
Vitavose	7 I. U.[g]	...	20 μg.	20 μg.
Sirup concentrate	45 I. U.[g]	...	80 μg.	130 μg.
Dry concentrate	135 I. U.[g]	...	390 μg.	380 μg.
Cow's milk	0.17 I. U.[j]	...	0.38–.51 μg.	...
Rat liver	2.6 I. U.[k]	...	1.0 μg.	7.2 μg.
Human urine	30–90 μg.[l]	...	70–160 μg.	...
Human blood	0.07–0.14 μg.[m]	...	0.09–.12 μg.	...

[a] Generously furnished by Dr. R. T. Major, Merck and Co., Rahway, N. J.   [b] Mouse growth assay. The method was reported by us at the Chapel Hill Meeting, American Chemical Society, April, 1937.   [c] This represents the range reported in the literature. The most widely accepted values are all within 10% of 300 μg.   [d] Generously supplied by Mr. R. F. Light, Fleischmann Laboratories, New York City.   [e] From Bulletin 25, U. S. Pharmacopeial Convention, Vitamin Committee, with the kind permission of Dr. E. Fullerton Cook, Chairman.   [f] Rat growth assay conducted by Fleischmann Laboratories. Data kindly supplied by Dr. C. N. Frey.   [g] Rat curative assay conducted by E. R. Squibb and Sons, Brooklyn, N. Y. Data kindly supplied by Dr. W. S. Jones.   [h] A. F. Morgan and M. J. Hunt, Cereal Chem., 12, 411 (1935), quoted by E. P. Daniel and H. E. Munsell, U. S. Dept. of Agric. Misc. Bull. No. 275, June, 1937.   [i] Rat growth assay. Data kindly supplied by Dr. J. S. Andrews, General Mills Laboratories, Minneapolis, Minn.   [j] Borden's Grade B Milk. Data kindly supplied by Dr. G. C. Supplee, The Borden Co., Research Division, Bainbridge, N. Y.   [k] P. C. Leong, Biochem. J., 31, 367 (1938).   [l] This represents the daily output. L. J. Harris and P. C. Leong, Lancet, 1, 886 (1936).   [m] A. P. Meiklejohn, Biochem. J., 31, 1441 (1938).

In general the table shows that with a wide variety of materials assayed, the results are in good agreement with biological tests. Although further work is needed to settle the point with finality, it would seem that the International Unit is equivalent to 2.9 micrograms of thiamin chloride and 4.0 micrograms of cocarboxylase. All the laboratories whose biological assays are reported in the table in terms of International Units use a conversion factor within 10% of our factor of 2.9 μg. of thiamin chloride per International Unit, except the Fleischmann Laboratories whose factor is 4.0 μg.

Although conversion factors significantly larger or smaller than 2.9 μg. have been reported, the explanation may be found in the statement of Sampson and Keresztesy[22] that "any estimation of vitamin activity in terms of the present International Unit must be weighted in terms of the method of assay employed; before such a comparison is made it should be determined that the vitamin present in both standard and test substance is available to the test animal to the same extent."

Summary

Materials interfering in the thiochrome method for determining vitamin B₁ activity are efficiently eliminated by the use of a base-exchanging zeolite.

The use of a more sensitive fluorometer allows the accurate determination of 0.1 μg. of vitamin B₁.

An enzyme preparation, which is obtained from kidney as a stable powder, converts thiamin

(22) W. L. Sampson and J. C. Keresztesy, Proc. Soc. Exptl. Biol. Med., 36, 30 (1937).

phosphoric esters to thiamin, thus allowing materials containing cocarboxylase to be assayed by the thiochrome method. Such an assay gives results agreeing with biological tests.

According to our findings one International Unit is equivalent to 2.9 μg. of thiamin chloride and 4.0 μg. of cocarboxylase.

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Synthesis of Isoquinoline Acids

BY FLOYD T. TYSON

Only two of the seven possible isoquinoline acids have thus far been reported in the literature, i. e., the 1-isoquinoline carboxy acid[1] and the 5- or 8-isoquinoline carboxy acid.[2] Since the derivatives of the isoquinoline acids may have useful physiological properties, it seemed desirable to proceed to synthesize members of the series not yet reported.

The 4-isoquinoline acid was prepared from the readily available 4-bromoisoquinoline[3] by reaction with cuprous cyanide to form the nitrile, followed by hydrolysis of the nitrile with concentrated hydrochloric acid.

For the preparation of the 5-, 6-, 7- and 8-isoquinoline carboxy acids by a procedure similar to that used for the 4-acid, the necessary intermediate bromoisoquinolines were prepared by the condensation of the three bromobenzalaminoacetals, using a modification of the method of Pomeranz and Fritsch.[4] The necessary acetals were prepared by the reaction of the bromobenzaldehydes with amino acetal.

The position of the carboxyl in the 5, 6, 7 and 8-acids herein described is based upon the assumption that there is no change of position of substituent groups due to intramolecular change. The substituted isoquinoline derived from o-bromobenzaldehyde then is 8, that derived from p-bromobenzaldehyde is 6, and that from m-bromobenzaldehyde is either 5 or 7, or a mixture of the two. A mixture of bromoisoquinolines actually is obtained from m-bromobenzaldehyde, as proved by the separation of derivatives of the 5- and 7-bromoisoquinoline, by fractional crystallization from aqueous dioxane of the sodium salts of the mixture of isoquinoline carboxy acids obtainable from the bromoisoquinolines.

The limiting yields obtained in the necessary reactions in each series are found in the condensation of the bromobenzalaminoacetals to the bromoisoquinolines. The yields, based upon the theoretical, for this step range from 6% in the case of the 8-bromoisoquinoline to 65% in the case of the mixture of bromoisoquinolines obtainable from m-bromobenzalaminoacetal.

The 5- or 8-isoquinoline acid was prepared by Jeiteles[2] from the 5- or 8-nitrile derived from the 5- or 8-isoquinoline sulfonic acid. The author has, in addition, prepared the 5- or 8-isoquinoline nitrile by the action of cuprous cyanide upon 5- or 8-bromoisoquinoline. The 5- or 8-bromoisoquinoline was obtained from 5- or 8-nitroisoquinoline as described by Claus and Hoffman.[5]

Since both the 5- and 8-acids would yield the same tricarboxybenzene,[2] by oxidation, the question of the position of the carboxyl has remained undecided. In this paper the preparation of an acid identical with Jeiteles' 5- or 8-acid from m-bromobenzaldehyde indicates that the acid is the 5-isoquinolinecarboxylic acid.

The methyl esters of the series of isoquinoline acids were obtained readily by the preparation of the chlorides using thionyl chloride, followed by reaction with methyl alcohol. The melting points of the methyl esters confirmed the conclusion obtained from the acids. The acid named the 5- or 8-isoquinolinecarboxylic acid,, and obtained by Jeiteles from the isoquinolinesulfonic acid,[2] is the 5-isoquinolinecarboxylic acid.

Further work is in progress in this Laboratory on the investigation of derivatives of the series of isoquinoline acids.

Experimental

Preparation of Bromobenzalaminoacetals.—Aminoacetal prepared by the procedure of Marckwald and Wohl[6] was

(1) Reissert, Ber., 38, 3429 (1905); Kaufmann, ibid., 46, 2929 (1913).

(2) Jeiteles, Monatsh., 18, 810 (1894).

(3) Edinger and Bossung, J. prakt. Chem., 43, 192 (1891).

(4) Pomeranz, Monatsh., 14, 116 (1893); 15, 299 (1894); Fritsch, Ber., 26, 421 (1893).

(5) Claus and Hoffman, J. prakt. Chem., 47, 252 (1893).

(6) Marckwald, Ber., 25, 2355 (1892); Wohl, ibid., 39, 1953 (1906).