may be considered established by previous investigations and are not inconsistent with results obtained with other salts: (*a*) optimal stability at room temperature is obtained at a pH of about 4 (3, 9); (*b*) no improvement in stability can be had by buffering the solution (9); (*c*) heat sterilization causes only slight loss of activity, but should be avoided since the optimal pH for stability at sterilization temperatures is lower than at room temperature (9).

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Effect of the Thiazole Moiety of Thiamine Hydrochloride and Selected Model Compounds on Cyanocobalamin Stability

By ROBERT F. DOERGE*, LOUIS J. RAVIN, and HENRY C. CALDWELL

Data are presented indicating that the thiazole moiety of thiamine hydrochloride, the 3-benzyl derivative of the thiazole moiety, the 3-(4-nitrobenzyl) derivative of the thiazole moiety, and dimethylformamide, a structurally related possible breakdown product of the thiazole moiety, had no adverse effect on the stability of cyanocobalamin in aqueous buffered solutions after storage for periods up to 1 year at 45°. On the other hand, cyanocobalamin was unstable in the presence of cysteine hydrochloride, another structurally related possible breakdown product of the thiazole moiety, under similar conditions.

A PREVIOUS report (1) indicated that crystalline cyanocobalamin (B_{12}) is stable in aqueous solutions with thiamine hydrochloride (B_1) at pH 3.0 to 4.5 during prolonged storage at room temperature. This has also been found to be the case for a flavored and colored liquid form containing B_{12} and B_1 (2).

In contrast to the satisfactory stability of this vitamin combination at room temperature, there are reports that at elevated temperatures (120, 100, 45, and 40°) there is extensive breakdown of B_{12} (3, 4). Thus, data obtained under these conditions are not necessarily indicative of the stability that these combinations will show at normal storage conditions (1, 5).

There are several reports that the decomposition products of B_1 adversely affect B_{12} stability. This is especially marked if nicotinamide is also present. Ponci (6) reported that B_{12} alone in solution was more stable at 120° than when it was in the presence of B_1. He also reported that the extent of loss of B_{12} potency was dependent on both pH and B_1 concentration. Blitz *et al.* (4) also showed that the extent of B_{12} loss was dependent on B_1 concentration when the level of B_1 was over 25 mg./ml. Dony and Conter (7) found that B_{12} was stable at 100° for 4 hr. in the presence of nicotinamide or vitamin B_1 in concentrations up to 10 mg./ml. of B_1. They also reported that B_{12} alone or with nicotinamide at 120° for 20 min. showed only very slight loss, while if all three vitamins were present the solutions could not be autoclaved without considerable loss of B_{12}. Mukherjee and Sen (8) reported that at pH 4 to 4.5 there is a progressive loss of B_{12} in the presence of these two vitamins, but that it can be prevented by certain iron salts. They found that the iron salts protected the B_{12} without preventing the decomposition of B_1. They speculated that the decomposition products of B_1 in the presence of nicotinamide were different from those of B_1 alone.

Gambier and Rahn (5) stated that the presence of nicotinamide accelerated B_1 decomposition and that the thiazole moiety, as one of the decomposition products, promoted the decomposition

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of B_{12}. However, no evidence was given to prove the presence of the thiazole moiety. Feller and Macek (1) considered the effect of both the thiazole and the pyrimidine moieties of B_1 on the stability of B_{12}. They showed that after storage at 40° for 4 months there was a 19 and 3% loss, respectively, the latter being no different from the loss in potency of the B_{12} control. In the same study they also reported that the B_{12} sample, to which was added partially decomposed B_1 (62% intact B_1), showed a 28% loss under the same conditions, compared to an 18% loss when intact B_1 was used. Thus, it appears that under the conditions of the experiment, B_1 decomposition product(s) other than the thiazole moiety also influenced the stability of B_{12}. They reported that samples stored at room temperature for 6 months showed no significant loss in any case. Blitz *et al.* (9) found that the thiazole moiety was not responsible for all of the B_{12} destruction. Thiochrome, an oxidation product of B_1, has been eliminated as being responsible for B_{12} decomposition (10).

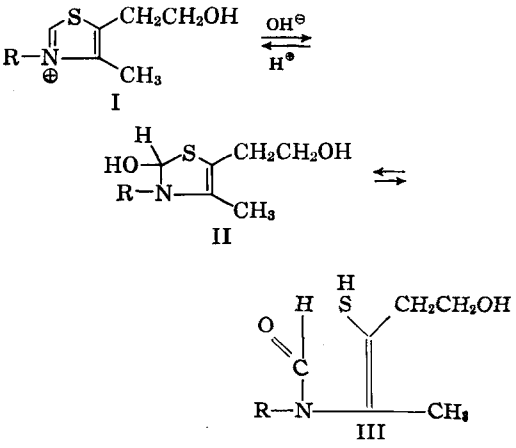
Windheuser and Higuchi (11), in a study of the kinetics of B_1 hydrolysis, found that under non-oxidative conditions in the weakly acid to neutral range, simple hydrolytic cleavage occurs at the methylene bridge between the pyrimidine and thiazole groups. This does not preclude, however, the formation of other decomposition products under the conditions that exist during the usual stability studies of liquid dosage forms.

A recent review (12) mentions several compounds which have been suggested as stabilizers of B_{12}, but some of these are not useful because they have an adverse effect on B_1 or other vitamins that may be present in a liquid dosage form. It appears then, that further studies of the problem of B_1 stability as it affects the stability of B_{12} are desirable.

DISCUSSION

This report deals with a reinvestigation of the effect of the thiazole moiety on B_{12} stability as reported by Feller and Macek (1); the stability of B_{12} and B_1 combinations using B_1 U.S.P. and B_1 ampul grade; and the effect of cysteine hydrochloride and of dimethylformamide. Thiochrome has also been included. To further study the possibility that the thiazole ring may open to give the free thiol (III) which then adversely influences vitamin B_{12}, model compounds, 3-benzyl-5-(2-hydroxyethyl)-4-methylthiazolium chloride (I, R = $C_6H_5CH_2$—) and 3-(4-nitrobenzyl)-5-(2-hydroxyethyl)-4-methylthiazolium chloride (I, R = 4-NO_2-$C_6H_4CH_2$—), have been included. (Scheme I.)

If the thiol (III) is responsible for the breakdown, it should be possible to demonstrate that the rate at which the thiazole ring cleaves is related directly



Scheme I

to the rate of B_{12} breakdown. The benzyl and 4-nitrobenzyl derivatives of the thiazole moiety were selected to test this premise. Since the 4-nitrobenzyl group provides a stronger inductive electron-withdrawing effect than the benzyl group, it was expected that the 4-nitrobenzyl derivative would be more quickly converted to III than would the benzyl derivative. Thus, the 4-nitrobenzyl compound should degrade B_{12} faster than the benzyl compound. In contrast, one would expect the methyl derivative and the free thiazole to be much more slowly hydrolyzed to give III. Breslow and McNelis (13) published similar reasoning to explain why the benzyl derivative was more effective than the methyl derivative for catalyzing the conversion of pyruvic acid to acetoin. However, Yount and Metzler (14) reported that the *o*-, *m*-, and *p*-nitrobenzyl derivatives were practically ineffective in this conversion.

EXPERIMENTAL

Materials

Citric acid U.S.P., sodium citrate U.S.P., methylparaben U.S.P., propylparaben U.S.P., thiamine hydrochloride U.S.P., thiamine hydrochloride, ampul grade, cysteine hydrochloride, reagent, dimethylformamide, reagent, 5-(2-hydroxyethyl)-4-methylthiazole, Merck and Co., cyanocobalamin U.S.P., and thiochrome, Bios Chemical Co., were used in these studies.

Procedure

Experiment A.—Aqueous buffered solutions containing approximately 0.0005% of B_{12} and 2% of B_1 or amounts of possible breakdown products of the thiazole moiety equivalent to 2% of B_1 were prepared, filled into 2-oz. amber bottles, and placed at 45°. Samples were assayed periodically for B_{12} content by the U.S.P. microbiological assay method.

Experiment B.—Aqueous solutions containing 0.0025% of B_{12} and a concentration of 5-(2-hydroxyethyl)-4-methylthiazole equivalent to 1% of B_1 were prepared. Approximately 10 ml. of solution was placed in 10-ml. ampuls, sealed, and placed at

TABLE I.—EFFECT OF THIAMINE HYDROCHLORIDE AND RELATED COMPOUNDS ON THE STABILITY OF B_{12} AT 45°

Test Soln.[a]	Orig.	1 Wk.	4 Wk.	3 Mo.	6 Mo.	1 Yr.
Vitamin B_{12} control	107	120	97	...	105	109
Vitamin B_{12} + thiamine hydrochloride U.S.P.	107	95	65	0	...	...
Vitamin B_{12} + thiamine hydrochloride, ampul grade	107	118	64	50	...	...
Vitamin B_{12} + cysteine hydrochloride	107	34	0[b]	...	...	...
Vitamin B_{12} + thiazole moiety	113	107	100[b]	98	96	...
Vitamin B_{12} + dimethylformamide	113	111[c]	102[d]	96	93	...
Vitamin B_{12} + thiochrome	113	108[c]	109	106	117	...

[a] All assays expressed as per cent of label claim, 5 mcg./ml. [b] Five weeks. [c] Three weeks. [d] Six weeks. All solutions were at pH 4.0.

45° and assayed periodically for B_{12}. In all cases control solutions containing only B_{12} were prepared and assayed. All assays were by the U.S.P. microbiological method for B_{12}.

Preparation of Model Compounds

3-Benzyl-5-(2-hydroxyethyl)-4-methylthiazolium Chloride.—A mixture of 7.2 Gm. (0.05 mole) of 5-(2-hydroxyethyl)-4-methylthiazole and 6.3 Gm. (0.05 mole) of benzyl chloride in 50 ml. of anhydrous toluene was heated at reflux temperature for 24 hr. The solution was cooled and there was obtained 8.6 Gm. (63.5%) of the desired product. An analytical sample, from alcohol–acetone–ether melted at 139–140.5°.[1]

Anal.—Calcd. for $C_{13}H_{16}ClNOS$: C, 57.87; H, 5.98. Found: C, 57.75; H, 6.11.

3-(4-Nitrobenzyl)-5-(2-hydroxyethyl)-4-methylthiazolium Chloride.—Following the above procedure, 7.2 Gm. (0.05 mole) of 5-(2-hydroxyethyl)-4-methylthiazole and 8.6 Gm. (0.05 mole) of 4-nitrobenzyl chloride gave a black tar. The tar was dissolved in alcohol, treated with activated charcoal, and precipitated with ether. After several of these treatments an analytical sample, m.p. 170–171°,[1] was obtained. [Lit. m.p. 172–173° (15).]

Anal.—Calcd. for $C_{13}H_{15}ClN_2O_3S$: C, 49.60; H, 4.80. Found: C, 49.49; H, 4.97.

RESULTS AND DISCUSSION

Table I summarizes the data obtained in the study of the effect of B_1 and various compounds selected because they may be structurally related to possible breakdown products of the thiazole moiety of B_1.

The data show that B_1 of two different grades caused significant degradation of cyanocobalamin at 45° in aqueous buffered solution. These data confirm the observations of Feller and Macek (1). However, the fact that the thiazole moiety, dimethylformamide, and thiochrome did not cause any appreciable loss of potency of cyanocobalamin under similar conditions and the cysteine hydrochloride did adversely affect the stability, suggests that the thiazole ring may be destroyed, liberating a compound with a sulfhydryl group. In view of the data obtained with cysteine hydrochloride, this could conceivably happen and be the cause of the loss of potency.

As previously stated, two model compounds were prepared, and the effect of their presence on stability of cyanocobalamin was determined. The data obtained are summarized in Table II.

After storage at 45° for 1 month, there was no significant difference in potency of cyanocobalamin in the mixtures as compared with their respective controls.

The spectrophotometric behavior of these model compounds was tested in borate buffer at pH 8.0. It was felt that because of the nature of the compounds, the rate at which the thiazole ring may be destroyed liberating a breakdown product with a free sulfhydryl group would be different. There was no significant difference between the behavior of these compounds. It would appear then, that under the conditions of the study, there is no significant difference in the effect of these model compounds on the stability of cyanocobalamin in aqueous buffered solution.

Table III lists the data obtained when the experiments of Feller and Macek (1) were repeated.

The above data indicate that the thiazole moiety has little effect on the stability of cyanocobalamin under the conditions set forth in this experiment. This is consistent with the results obtained by Feller and Macek. Thus, it would appear that the intact thiazole moiety has no adverse effect on the stability of cyanocobalamin after prolonged storage at elevated temperatures. The data obtained do

TABLE II.—EFFECT OF SUBSTITUTED THIAZOLE MOIETIES OF THIAMINE HYDROCHLORIDE ON THE STABILITY OF B_{12} AT 45°

Test Soln.	Original Assay[a]	1 Wk.	2 Wk.	4 Wk.
Vitamin B_{12} + benzyl derivative	109	126	122	116
Vitamin B_{12} control	118	117	111	120
Vitamin B_{12} + 4-nitrobenzyl derivative	133	104	111	108
Vitamin B_{12} control	133	106	101	111

[1] Melting points are uncorrected.

[a] All assays expressed as per cent of label claim, 5 mcg./ml.

TABLE III.—EFFECT OF THE THIAZOLE MOIETY OF THIAMINE HYDROCHLORIDE ON THE STABILITY OF B_{12} AT 45°

Test Soln.	Original Assay[a]	1 Wk.	2 Wk.	3 Mo.	6 Mo.	1 Yr.
Vitamin B_{12} control	97	93	97	92	80	113
Vitamin B_{12} + thiazole moiety	97	78	91	82	82	97

[a] All assays expressed as per cent of label claim, 25 mcg./ml.

suggest that during storage the thiazole ring may rupture, giving rise to a degradation product which does adversely affect cyanocobalamin stability.

SUMMARY

Data are presented to show that the thiazole moiety of thiamine hydrochloride, the 3-benzyl derivative of the thiazole moiety, the 3-(4-nitrobenzyl) derivative of the thiazole moiety, or dimethylformamide, a structurally related possible breakdown product of the thiazole moiety, had no adverse effect on the stability of cyanocobalamin in aqueous solution at pH 4.0. Cysteine hydrochloride, on the other hand, caused significant breakdown of cyanocobalamin, thus suggesting that a thiol-containing degradation product of thiamine hydrochloride may be responsible for losses in B_{12} potency during storage.

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Synthesis and Pharmacological Screening of 3-Aminoalkyl-Sydnones

By TIBERIO BRUZZESE, SILVANO CASADIO, ERNESTA MARAZZI-UBERTI, and CARLA TURBA

Fourteen 3-aminoalkyl-sydnones have been synthesized and submitted to comprehensive pharmacological screening. Some of the compounds show an analgesic, hypoglycemic, and anti-inflammatory activity.

COMPOUNDS containing the sydnone *meso*-ionic ring have for many years been studied for their synthesis and structure (1–4). However, the pharmacological aspect of such compounds has been investigated only recently. In particular, Daeniker and Druey (5) have found that some polymethylene-bis-sydnones show a certain degree of antitumoral activity, while Greco *et al.* (6) have observed a similar action for 3-(*p*-methoxybenzyl)-sydnone. It has been reported that other sydnones stimulate the central nervous system (7, 8) or display a saluretic activity (9).

This paper reports the synthesis of a series of 3-aminoalkyl-sydnones and their comprehensive pharmacological screening. The compounds have been prepared by the classical technique (3), *i.e.*, nitrosation of the appropriate *N*-aminoalkyl-glycine and treatment of the *N*-nitroso derivative with acetic anhydride. The *N*-nitroso derivatives have been isolated as the hydrochlorides and are difficult to crystallize. (See Table I. Other compounds required have not been characterized.) Cyclization necessitates a very short initial heating, otherwise a resinous product which cannnot be purified is obtained.

3-Aminoalkyl-sydnone hydrochlorides are

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