Stabilization of Vitamin B_{12} I

Complex Cyanides

By DONALD A. ZUCK and JAMES W. CONINE

The destruction of cyanocobalamin in liquid multiple vitamin preparations can be greatly reduced by the addition of small amounts of complex cyanides or iron salts. Cyanocobalamin stabilization with iron salts is not as effective as with complex cyanides under conditions designed to exclude air. The superiority of complex cyanides over iron salts was also demonstrated in partly filled containers where a large volume of air overlaid the product. The complex cyanides were also effective in reducing the destruction of cyanocobalamin induced by ultraviolet irradiation.

THE PROBLEM of deterioration of vitamin B_{12} in aqueous and multiple vitamin preparations was recognized shortly after its isolation as a pure crystalline compound (1, 2). Mention of stabilizers was first made in 1952 by Rosenblum (3) who reported that ferrous and ferric ions showed definite stabilizing properties on vitamin B_{12} in a multiple vitamin capsule. Additives such as sodium bisulfite (4), saccharated iron oxide (5), and thiodipropionic acid (6) found use as stabilizers with some degree of success.

Analogs of cyanocobalamin have been recognized (2, 11) as less stable forms. Other factors contributing to the instability of vitamin B_{12} in the complex preparations were air volume above the liquid and/or the area of the liquid-air interface (7), presence of copper ions with ascorbic acid (12), exposure to sunlight, direct ultraviolet light, or incandescent light (13).

The use of cyanide as a stabilizer was first mentioned in 1953 (8, 9). The process involved addition of hydrogen cyanide to the vitamin solution. Excess hydrogen cyanide was then removed by vacuum.

The question of vitamin B_{12} stability in multiple vitamin preparations has continued to be of major concern. Campbell (10) pointed out from a survey on marketed products that vitamin B_{12} was below label in all liquid and tablet formulations which were tested by his group.

Sodium pyrosulfite and sodium nitrite reduced vitamin B_{12} decomposition by 50% in liver preparations which were exposed to diffused light. This rapid decomposition of vitamin B_{12} in liver preparations, when exposed to air and light, was also reversed by the addition of sodium cyanide. Screenivasamurthy (14) found that light favored the conversion of the cyanocobalamin to the hydroxo form. The decomposition was then due to the instability of the hydroxo form in the presence of oxygen.

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More recently reported stabilizers were ammonium sulfite (15), iron compounds (16, 18, 19), organonitriles of the R—CH(OH)CN type (17), and metallocyanide complexes (20). This report deals with a comparative study of the iron salts and complex cyanide salts as stabilizers for vitamin B_{12} in a liquid multiple vitamin preparation in presence and absence of air. The effect of complex cyanides on the stability of an aqueous cyanocobalamin solution on exposure to ultraviolet light was also studied.

EXPERIMENTAL

A homogenized liquid multiple vitamin formulation was made up to 95% of the final volume of the preparation. Each milliliter of the finished preparation was to contain the following: vitamin A synthetic, 600 units; thiamine hydrochloride, 0.20 mg.; riboflavin, 0.24 mg.; pyridoxine hydrochloride, 0.20 mg.; nicotinamide, 2.0 mg.; ascorbic acid, 12.0 mg.; and vitamin D synthetic, 200 units.

The bulk preparation was stored under refrigeration. Cyanocobalamin, to give a final concentration of 1.0 mcg. per ml., and different stabilizers at predetermined amounts were dissolved in a minimum amount of purified water. This solution was then added to the bulk vitamin preparation which was then made up to 100% of volume with purified water. Such a multiple vitamin preparation was then filled under nitrogen cover into 4-oz. amber screw-capped bottles, and partly filled (40 oz.) under air cover into 1-gal. amber screw-capped bottles. These containers were stored at 26, 37, 46,[1] and 65° in constant temperature storage rooms or ovens. Assays were carried out by control laboratory using the U.S.P. microbiological method of assay for vitamin B_{12}.[2] The sampling schedule was based on results received in order to obtain the best distribution of data log-concentration-time plots.

For the ultraviolet irradiation study, solutions of 15 mcg. of cyanocobalamin containing potassium ferrocyanide 0.1 to 0.00005% and potassium ferricyanide 0.1 to 0.00001% were irradiated in a Fade-

[1] Upon completion of experiment it was found that the temperature in the 50° storage room was not uniform throughout. The area in which the bottles were stored was found to be 46°.

[2] The microbiological assays were performed by Mr. J. T. Stephenson and his associates of the Microbiological Testing Department, Eli Lilly and Co.

TABLE I.—EFFECT OF IRON COMPOUNDS ON STABILITY OF VITAMIN B_{12}

Stabilizer	Concn. Iron, mcg./ml.	Concn. Compd., mcg./ml.	% Initial at 1 yr. at 26° Nitrogen Cover Full Bottle	% Initial at 1 yr. at 26° Air Cover 1/4 Full Bottle	% Initial at 6 mo. at 37° Nitrogen Cover Full Bottle	% Initial at 6 mo. at 37° Air Cover 1/4 Full Bottle
None	0	0	10.5	21.4	31	<5
Peptonized iron	170	1000	88	56.5	82	41
Ferrous sulfate	170	850	77	51	67	29
Ferric ammonium citrate	170	1130	86	51	74	32
Ferrous gluconate	170	1460	76	50	75	35
Ferrous dihydrogen EDTA	170	1325	80	52.5	65	44
Ferric betaine citrate	170	1550	84	64	...	31
Potassium ferrocyanide	170	1320	98	100	90	81

TABLE II.—EFFECT OF CONCENTRATION OF IRON COMPOUNDS ON STABILITY OF VITAMIN B_{12}

Stabilizer	Concn. Iron, mcg./ml.	Concn. Compd., mcg./ml.	% Initial at 1 yr. at 26° Nitrogen Cover Full Bottle	% Initial at 1 yr. at 26° Air Cover 1/4 Full Bottle	% Initial at 6 mo. at 37° Nitrogen Cover Full Bottle	% Initial at 6 mo. at 37° Air Cover 1/4 Full Bottle
None	0	0	30	26	38	5
Ferrous gluconate	160	1370	79	50	86	38
Ferrous gluconate	120	1030	81	56	79	41
Ferrous gluconate	80	685	76	57	68	38
Ferrous gluconate	40	344	78	36	68	33
Potassium ferrocyanide	160	1250	93	89	95	77
Potassium ferrocyanide	120	937	91	94	95	74
Potassium ferrocyanide	80	625	96	96	97	84
Potassium ferrocyanide	40	312	87	91	95	79

Ometer[3] for periods of up to 96 hours at 52°. Samples were assayed over this period for vitamin B_{12} activity. Physical observations were also made and the pH was measured.

Reagents.—Cyanocobalamin concentrate B_{12} activity oral (3000 mcg. per Gm.) (Merck and Co.); vitamin B_{12} crystalline (Merck and Co.); iron peptonized N.F. IX powder; ferrous sulfate U.S.P.; ferric ammonium citrate N.F. XI; ferrous gluconate U.S.P.; ferrous dihydrogen ethylenediamine tetraacetic acid (Alrose Chemical Co.); ferric betaine citrate (Fleming Laboratories Inc.); potassium ferrocyanide reagent; potassium ferricyanide reagent; potassium cobalticyanide $K_3Co(CN)_6$ (Amend Drug & Chemical Co.); potassium manganocyanide $K_3Mn(CN)_6$ (Donald Axelson, U. of Ill.); potassium molybdocyanide $K_4Mo(CN)_8$ (Robert Novak, U. of Ill.); potassium nickelocyanide $K_2Ni(CN)_4 \cdot H_2O$ (City Chemical Corp.); potassium cuprocyanide $K_3Cu(CN)_4$ (City Chemical Corp.).

RESULTS AND DISCUSSION

Liquid Multiple Vitamin Product, Stabilization of Vitamin B_{12} in Presence of Vitamin C.—The results tabulated in Table I and Table II were from data obtained at the time and temperature indicated from full and partly filled containers for each of the stabilizers listed. Table I is a comparison of a number of stabilizers containing iron at 170 mcg. of iron per ml. Table II represents a study of ferrous gluconate and potassium ferrocyanide at four different concentrations. This was an attempt to determine the minimum effective concentration of stabilizer that could be used advantageously. Although the difference between the two stabilizers is quite striking, no appreciable change is observed in the effect of potassium ferrocyanide concentrations between 160 mcg. and 40 mcg. of iron per ml. Very little difference in stabilizing effect was noted with ferrous gluconate between the concentrations of 170 and 40 mcg. of iron per ml. It has been observed that potassium ferrocyanide and other metalocomplexes were effective at a metal-concentration as low as 10 mcg. per ml.

Because of amber glass containers which were used, light (13, 14) cannot be considered as a contributing factor in the conversion of the cyano-form to the relatively unstable hydroxo form in these experiments. It appears that a compound like potassium ferrocyanide, by furnishing cyanide ions, interferes with the transformation of the cyanocobalamin to the hydroxo or other less stable analogs of vitamin B_{12}. This action has been demonstrated in the study with ultraviolet irradiation. The analogs, once formed, were readily oxidized in presence of oxygen (14). A similar situation exists in the presence of ascorbic acid (1) where the cyano-form was found to be more stable than the other vitamin B_{12} analogs. Ferrous sulfate appears to inhibit the oxidation of vitamin B_{12}; however, this protection is reduced by an increase in air volume and/or surface area.

In Table III a comparison of six different metalocyanide complexes was made. The amounts added were such as to give 20 mcg. of metal per ml. of the vitamin preparation. There were no marked differences with respect to their stabilizing properties with the exception of $K_3Cu(CN)_4$. This complex exhibited a somewhat lesser stabilizing effect which became more evident in the partially filled containers at the higher temperatures. This behavior supports a previous observation (12) made with respect to copper ions, ascorbic acid, and vitamin B_{12}.

With the exception of $K_3Cu(CN)_4$ it would be difficult to select one of these complexes as a stabilizer for vitamin B_{12} on the basis of these results. But toxicity results show that potassium ferrocy-

[3] Atlas Electric Devices Co.

TABLE III.—EFFECT OF COMPLEX CYANIDES ON STABILITY OF VITAMIN B_{12}

Stabilizer	Concn. Iron, mcg./ml.	Concn. Compd., mcg./ml.	% Initial at 1 yr. at 26° Nitrogen Cover Full Bottle	% Initial at 1 yr. at 26° Air Cover 1/4 Full Bottle	% Initial at 6 mo. at 37° Nitrogen Cover Full Bottle	% Initial at 6 mo. at 37° Air Cover 1/4 Full Bottle
None	0	0	16	26	19	7
$K_3Co(CN)_6$	20	112	79	78	72	70
$K_2Ni(CN)_4$	20	88	84	68	77	48
$K_3Cu(CN)_4$	20	90	80	51	74	10
$K_3Mn(CN)_6$	20	119	94	72	79	66
$K_4Mo(CN)_8$	20	104	93	74	79	63
$K_4Fe(CN)_6$	20	150	93	81	81	72

TABLE IV.—DECOMPOSITION OF VITAMIN B_{12} FOLLOWING EXPOSURE TO ULTRAVIOLET IRRADIATION

$K_4Fe(CN)_6$, % Concn.	pH Initial	pH 8 hr.	% Loss 8 hr.	% Loss 8 to 96 hr.	% Loss Total	k, hr.$^{-1}$
0.1	6.4	8.1	97.3	1.8	99.1	...
0.02	6.45	8.6	84.0	2.0	86.0	0.00153
0.005	6.55	8.45	46.7	3.8	50.5	0.00107
0.001	6.15	6.70	7.8	14.4	22.2	0.00193
0.002	...	...	16.8	35.1	51.9	0.00416
0.00005	...	...	12.4	70.0	82.4	0.0182
0.00001	...	...	13.8	68.0	81.8	0.0172
0	5.82	5.90	14.5	75.5	90.0	0.0142

anide and potassium ferricyanide are the least toxic of the compounds studied with an LD_{50} oral in mice greater than 2 Gm. per Kg. Potassium cobalticyanide had an LD_{50} of 1529 ± 196 mg. per Kg. The LD_{50} of potassium molybdocyanide was found to be approximately 1500 mg. per Kg. Potassium nickelocyanide and potassium manganocyanide and potassium zinc cyanide had an LD_{50} of less than 275 mg. per Kg. This large variation in degree of toxicity is directly related to the ease with which the complex salts dissociate in solution (21). For example, the double nickelocyanide ($K_2[Ni(CN)_4]$) gives a precipitate with ammonium sulfide whereas potassium ferrocyanide gives no precipitate with either ammonium sulfide or with sodium hydroxide.

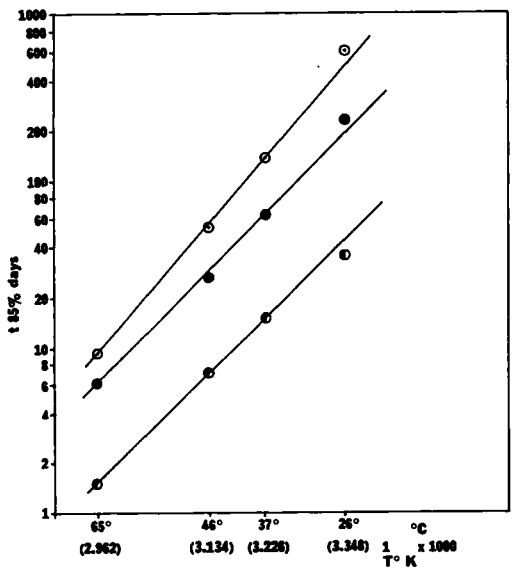

Fig. 1.—The relationship of $t_{85\%}$ (logarithmic scale) of vitamin B_{12} in a liquid multiple vitamin preparation and the reciprocal of the absolute temperature under nitrogen cover. ⊙, Potassium ferrocyanide; ●, ferrous gluconate; ◐, control.

The addition of iron at a concentration of 170 mcg. per ml. will stabilize vitamin B_{12} quite satisfactorily in both filled and partly filled containers. The superiority of potassium ferrocyanide as a stabilizer is not as pronounced in the full containers as it is in partly filled containers. This effect can be more readily seen in Fig. 1 and Fig. 2 in which the t_{85} (time for material to reach 85% of the initial concentration) was plotted against the reciprocal of the absolute temperature. The t_{85} values were obtained directly from the concentration-time plots at four different temperatures. Straight line plots indicated first-order reactions.[4]

There was no indication that changes in concentration of the stabilizer affected the decomposition rate for vitamin B_{12} between concentrations of potassium ferrocyanide at 20 to 170 mcg. of iron per ml. The t_{85} values for potassium ferrocyanide are averages of five separate experiments: 170, 40, 40, 20, and 20 mcg. of iron per ml. The t_{85} values representing ferrous gluconate are averages of three experiments: 170, 160, and 40 mcg. of iron per ml.

Figures 1 and 2 clearly demonstrate the superiority of potassium ferrocyanide over that of ferrous gluconate, particularly where contact with air is a factor. For example, under air cover, the time in days to reach 85% of initial at 26° was 320 days using potassium ferrocyanide *vs.* 90 days using ferrous gluconate (a ratio of 3.5:1.0); and under nitrogen cover the time was 560 days using potassium ferrocyanide *vs.* 230 days with ferrous gluconate (a ratio of 2.4:1.0).

Cyanocobalamin Solution, Effect of Cyanides on Decomposition Due to Ultraviolet Irradiation.—Results obtained from the ultraviolet degradation studies are tabulated in Tables IV and V.

A rapid destruction of vitamin B_{12} was observed at the higher concentrations of the complex. Over the limits of this particular study this decomposition

[4] The assay at 1 month for the control experiment at 26° in partly filled containers showed a greater drop than was expected. Thereafter, a first-order decomposition followed.

TABLE V.—DECOMPOSITION OF VITAMIN B_{12} FOLLOWING EXPOSURE TO ULTRAVIOLET IRRADIATION

$K_4Fe(CN)_6$, % Concn.	pH		% Loss			k, hr.$^{-1}$
	Initial	8 hr.	8 hr.	8 to 96 hr.	Total	
0.1	6.75	9.6	92.0	6.3[a]	98.3	...
0.02	6.7	9.3	48.3	6.7	55.0	0.00157
0.005	6.85	8.95	18.7	7.8	26.5	0.00115
0.001	6.40	6.9	16.3	9.8	26.1	0.00143
0.0002	...	...	21.4	47.7	69.1	0.0106
0.00005	...	...	19.5	67.6	87.1	0.0208
0	...	...	13.9	78.5	92.4	0.0405

[a] Extrapolated value.

was proportional to the amount of cyanide added. The initial reaction appeared to be destruction of the vitamin B_{12} activity. After this, a stabilizing effect on the remaining vitamin B_{12} activity was observed. The optimum concentration for potassium ferricyanide was 0.001% and for potassium ferrocyanide it was from 0.001 to 0.005%. The stabilizing effect decreased rapidly below these concentrations. The optimum stabilizing effect of potassium cyanide under the same conditions was 0.001% (25). With concentrations greater than this, there was no improvement in the stabilizing effect. Tables IV and V give the per cent of initial concentration lost in the first 8 hours and the per cent of initial concentration lost in the remaining 88 hours. The total per cent loss is also given. Figure 3 illustrates the type of curve obtained when the logarithm of per cent of the initial vitamin B_{12} concentration was plotted against time. After 8 hours' exposure, a precipitate giving a positive test for iron was formed, the pH changed as listed in Tables IV and V, and a pronounced odor of cyanide was present in solutions.

At this time the reasons that the two complexes accelerate the degradation of vitamin B_{12} during their photochemical decomposition are not clear.

The photochemical degradation of potassium ferrocyanide has been studied previously (22–24). Although our work was done on less concentrated solutions, it confirms the previously reported results.

The reaction for potassium ferrocyanide in presence of air is believed to be

$$2Fe^{++} + 4OH^- + H_2O + {}^1/_2O_2 \rightarrow 2Fe(OH)_3$$

In the absence of air the reaction is

$$Fe(CN)_6^{----} + HOH \rightleftharpoons Fe(CN)_5 \cdot H_2O^{---} + CN^-$$

and then followed by

$$CN^- + HOH \rightleftharpoons HCN + OH^-$$

Since the glass-sealed ampuls had a limited supply of air, both reactions could have occurred.

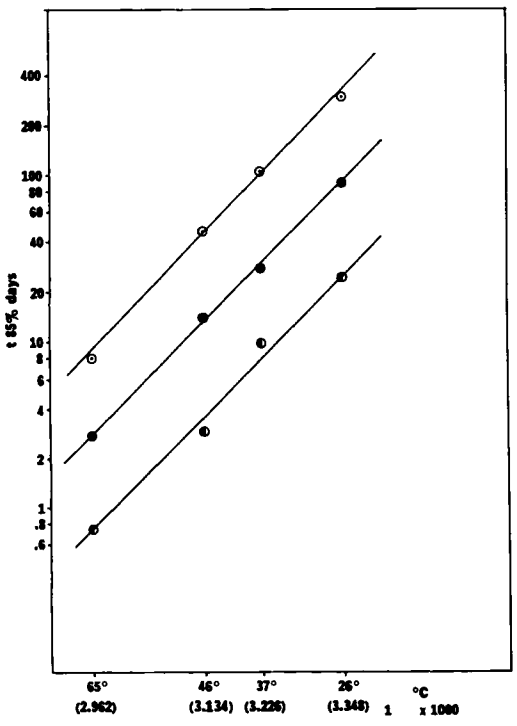

Fig. 2.—The relationship of $t_{85\%}$ (logarithmic scale) of vitamin B_{12} in a liquid multiple vitamin preparation and the reciprocal of the absolute temperature in presence of air. ⊙, Potassium ferrocyanide; ●, ferrous gluconate; ◐, control.

SUMMARY

1. A comparative study of the stabilizing effect of ferrous salts and complex cyanides on

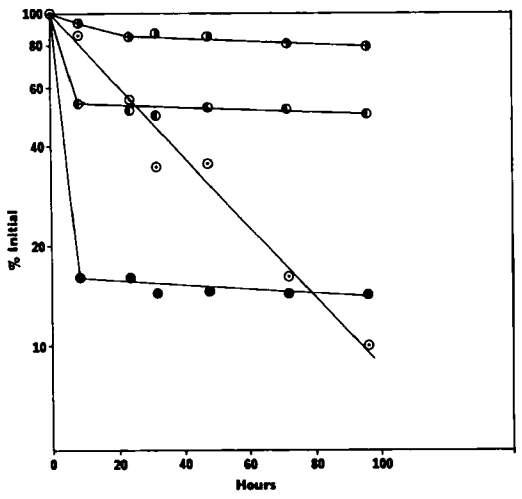

Fig. 3.—Decomposition of vitamin B_{12} in the presence of potassium ferricyanide following exposure to ultraviolet irradiation in the Fade-Ometer. ●, 0.02% potassium ferricyanide; ◐, 0.005% potassium ferricyanide; ◑, 0.001% potassium ferricyanide; ⊙, control.

vitamin B_{12} in multiple vitamin preparations in the presence and absence of air was made.

2. The superiority of potassium ferrocyanide over iron salts as a vitamin B_{12} stabilizer in multiple vitamin preparations was demonstrated.

3. The destruction of vitamin B_{12} by ultraviolet light was investigated. Under the conditions of the experiment it was found that the optimum stabilizing concentration of potassium ferrocyanide and potassium ferricyanide was 0.001 to 0.005% and 0.001%, respectively.

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Stabilization of Vitamin B_{12} II

α-Hydroxynitriles

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The destruction of vitamin B_{12} in liquid multiple vitamin products can be greatly reduced by the addition of α-hydroxynitriles or their esters. The stabilizing effect appears to be due to decomposition of the α-hydroxynitrile into hydrogen cyanide and the corresponding aldehyde. An initial stabilization of cyanocobalamin was seen in solutions which contained α-hydroxynitriles and were exposed to ultraviolet irradiation. However, upon long exposure to ultraviolet light, cyanocobalamin solutions containing α-hydroxynitriles eventually decomposed more than the control.

VITAMIN B_{12} has been shown to be extremely susceptible to decomposition in the presence of ascorbic acid (1, 2). A recent review (3) has covered a number of compounds which have been suggested as stabilizers to reduce the rate of decomposition of vitamin B_{12}. Some of these, such as nitrites or bisulfites, are not completely satisfactory because they have an adverse affect upon the stability of thiamine or some of the other vitamins. Hydrogen cyanide has been used successfully to stabilize vitamin B_{12} injections (4, 5), and cyanides have been utilized in the production of cyanocobalamin (6). α-Hydroxynitriles and their esters are potential sources of cyanide and as such have been shown to be effective stabilizers of vitamin B_{12} in pharmaceutical preparations (7). The effect of α-hydroxynitriles and their esters upon the rate of decomposition of vitamin B_{12} is the subject of the present study.

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EXPERIMENTAL

Liquid Multiple Vitamin Preparation.—A homogenized liquid multiple vitamin formulation was made up to contain 95% of the final volume of the preparation. Each milliliter of the finished preparation was to contain: vitamin A, 600 units; vitamin B_1, 0.2 mg.; vitamin B_2, 0.24 mg.; vitamin B_6, 0.2 mg.; nicotinamide, 2.0 mg.; ascorbic acid, 12 mg.; and vitamin D synthetic, 200 units. To this preparation was added a triturate of crystalline cyanocobalamin in mannitol to give the preparation a concentration of 1 mcg. per ml. of vitamin B_{12}. The α-hydroxynitrile was dissolved in distilled water or aqueous alcohol and added in the appropriate concentration. The product was brought up to final volume with distilled water and stirred until uniform. The samples were filled into amber