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.

REFERENCES

(1) Gakenheimer, W. C., and Feller, B. A., THIS JOURNAL 38, 660(1949).
(2) Trenner, N. R., Buhs, R. P., Bacher, F. A., and Gakenheimer, W. C., *ibid.*, 39, 361(1950).
(3) Rosenblum, C., and Woodbury, D. T., *ibid.*, 41, 368(1952).
(4) Loy, H. W., Jr., Haggerty, J. F., and Kline, O. L., *J. Assoc. Offic. Agr. Chemists*, 35, 169(1952).
(5) Skeggs, H. R., U. S. pat. 2,584,627, Sharp & Dohme (1952).
(6) Leffler, M. T., U. S. pat. 2,579,679, Abbott Laboratories (1951).
(7) Stewart, E. M., Ferrer, E. B., and Stubberfield, L., THIS JOURNAL, 41, 587(1952).
(8) Organon, N. V., British pat. 692,968 (June 17, 1953).
(9) Stapert, G. M., Ferrer, E. B., and Stubberfield, L., THIS JOURNAL, 43, 87(1954).
(10) Campbell, J. A., and McLeod, H. A., *ibid.*, 44, 263 (1955).
(11) Hutchins, H. H., Cravioto, P. J., and Macek, T. J., *ibid.*, 45, 806(1956).
(12) Rosenburg, J. A., *J. Biol. Chem.*, 219, 951(1956).
(13) DeMerre, J. L., and Wilson, C., THIS JOURNAL, 45, 129(1956).
(14) Screenivasamurthy, V., Swaminathan, M., and Subrahmanyan, V., *J. Sci. Ind. Research India*, 16C, 83(1957).
(15) Michel, G. H., and Knight, W. K., U. S. pat. 2,778,-771, American Cyanamid, (1957).
(16) Newmark, H. L., U. S. pat. 2,823,167, Vitarine (Feb. 11, 1958).
(17) Conine, J. W., and Zuck, D. A., U. S. pat. 2,835,627, Eli Lilly and Co., (1958).
(18) Ponci, R., *Farmaco Pavia Ed. Part.*, 13, 71(1958); through *Chem. Abstr.*, 52, 17618d (1958).
(19) Mukerjee, S. L., and Sen, S. P., *J. Pharm. Pharmacol.*, 9, 759(1957); *ibid.*, 11, 26(1959).
(20) Zuck, D. A., U. S. pat. 2,874,089, Eli Lilly and Co., (1959).
(21) "Inorganic Chemistry", 6th ed., Interscience Publishers, Inc., 1954, pp. 287–327.
(22) D'Amore, G., and Bellomo, A., *Atti Soc. Peloritana Sci. Fis. Mat. Nat.*, 5, 449(1958–1959); through *Chem. Abstr.*, 54, 17004e (1960).
(23) Asperger, S., *Trans. Faraday Soc.*, 48, 617(1952).
(24) MacDiarmed, A. G., and Hall, N. F., *J. Am. Chem Soc.*, 75, 5204(1953).
(25) Conine, J. W., and Zuck, D. A., THIS JOURNAL, 52, 63(1962).

Stabilization of Vitamin B_{12} II

α-Hydroxynitriles

By JAMES W. CONINE and DONALD A. ZUCK

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

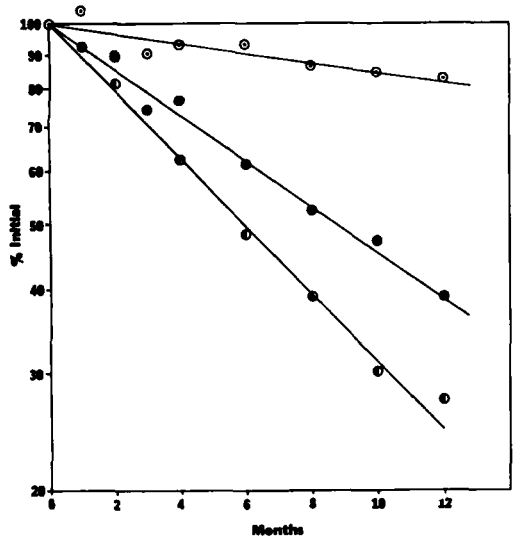

Fig. 1.—First-order decomposition of vitamin B_{12} at 26° in a liquid multiple vitamin preparation containing esters of mandelonitrile. ⊙, 0.1% acetylmandelonitrile; ●, 0.1% benzoylmandelonitrile; ◐, control.

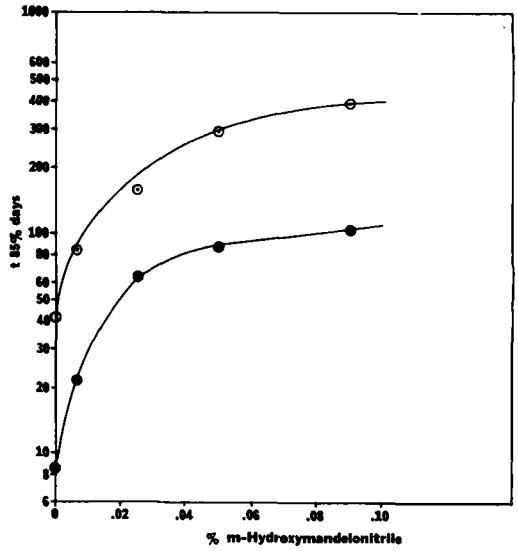

Fig. 2.—The relationship of $t_{85\%}$ of vitamin B_{12} in a liquid multiple vitamin preparation (logarithmic scale) and concentration of *m*-hydroxymandelonitrile. ⊙, at 26°; ● , at 37°.

1-pt. bottles and stored at 26 and 37° in controlled temperature rooms. Samples were removed periodically and assayed for vitamin B_{12} activity by the microbiological method of the U.S.P.[1]

Solution Cyanocobalamin.—Solutions of cyanocobalamin, 15 mcg. per ml., were prepared. Before the solution was made up to final volume the α-hydroxynitriles were added from a stock solution. Water was used to dissolve *m*-hydroxymandelonitrile. Ethanol, 95%, was used as a solvent for carbethoxymandelonitrile, and the solutions in this series were prepared at a final concentration of 5% ethanol. The solutions were filled into 5-ml. No. 1 flint glass ampuls and irradiated in the Fade-Ometer[2] at a temperature of 52° for periods of up to 96 hours. The ampuls were assayed for vitamin B_{12} activity by microbiological assay method of the U.S.P.[1]

The substituted mandelonitriles were prepared by the method of Buck (8). The esters of mandelonitrile were prepared by the method of Francis and Davis (9). Lactonitrile,[3] amygdalin, and aniline-free benzonitrile were also used in this study.

DISCUSSION AND RESULTS

The effect of five α-hydroxynitriles and four esters of mandelonitrile upon the stability of vitamin B_{12} in a liquid multiple vitamin preparation was studied and the results are listed in Table I.

The decomposition of vitamin B_{12} follows first-order kinetics as indicated by the straight line relationship obtained when the concentration of vitamin B_{12} was plotted on a logarithmic scale against the age of the sample (Fig. 1). In order to determine the effect of concentration of α-hydroxynitrile upon the rate of vitamin B_{12} decomposition, several concentrations of *m*-hydroxymandelonitrile were studied and these results are listed in Table II.

TABLE I.—PER CENT OF VITAMIN B_{12} REMAINING AFTER AGING SAMPLE

Preparation, 0.1%	1 yr. at 26°, %	6 mo. at 37°, %
Control	21.7	3.2
Benzoylmandelonitrile	39	10.8
Carbethoxymandelonitrile	76	67.5
Benzene sulfonyl mandelonitrile	76.6	68
Acetylmandelonitrile	82	85
Lactonitrile	89	76
p-Methoxymandelonitrile	79	77
o-Chloromandelonitrile	89	84.5
m-Hydroxymandelonitrile	87	80
3-Methoxy-4-hydroxymandelonitrile	90.3	81

TABLE II.—EFFECT OF CONCENTRATION OF *m*-HYDROXYMANDELONITRILE ON STABILITY OF VITAMIN B_{12}

	Vitamin B_{12} % Initial Assay, 12 mo. at 26°	k, day^{-1}	Vitamin B_{12} % Initial Assay, 6 mo. at 37°	k, day^{-1}
Control	27.2	0.00392	3.2	0.0188
m-Hydroxymandelonitrile, %				
0.1	89	0.00043	77.3	0.00159
0.05	88.6	0.000575	71.5	0.00189
0.025	90.2	0.00108[a]	67	0.00255
0.00625	67	0.00201	34.1	0.00724

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

[2] Atlas Electric Devices Co.

[3] American Cyanamid Co.

[a] The plot of the log concentration *vs.* storage time changes from one rate to another at 10–12 months; the more rapid reaction rate is given here.

Figure 2 is a graph of $t_{85\%}$ (the time required for vitamin B_{12} concentration to reach 85% of the initial assay value) plotted on a logarithmic scale against the concentration of *m*-hydroxymandelonitrile. The $t_{85\%}$ was calculated from *k* values obtained by drawing the best straight line through the points obtained by plotting log concentration of vitamin B_{12} against the sample age in days. The most rapid change in reaction rate occurred at the lowest concentrations of nitrile. As the amount of of *m*-hydroxymandelonitrile was increased up to 0.1%, successively less change was produced for each unit of α-hydroxynitrile added. This indicates that the reaction rate may be related to the equilibrium reaction between two forms of vitamin B_{12}, cyanocobalamin and a noncyano form.

$$\text{cyanocobalamin} \rightleftharpoons \text{noncyanocobalamin} + [CN^-]$$

$$k = \frac{[\text{noncyanocobalamin}]\,[CN^-]}{\text{cyanocobalamin}}$$

It has been reported that at pH 2.5–3.0 at room temperature pure cyanocobalamin decomposes at the rate of about 1.5% a day in the presence of ascorbic acid, compared with almost complete destruction of hydroxocobalamin (vitamin B_{12a}) in 1 day under similar conditions (2). In the presence of cyanide the equilibrium is shifted toward the more stable cyanocobalamin and away from the less stable compound.

Equilibrium constants have been reported for the dissociation of α-hydroxynitriles (10). These compounds dissociate to a greater extent in aqueous solutions than when water is absent (11). If the dissociation is sufficiently in favor of the formation of cyanide, this in turn will favor the formation of cyanocobalamin

$$\begin{array}{l} \text{RCH} - \text{CN} \rightleftharpoons \text{RCHO} + \text{HCN} \\ \quad\;\; | \qquad\qquad\qquad\quad \Uparrow \\ \quad\;\; \text{OH} \qquad\qquad\qquad\quad \Downarrow \end{array}$$

$$\text{cyanocobalamin} \rightleftharpoons \text{noncyanocobalamin} + \text{HCN}$$

All the compounds employed in this study demonstrated this stabilizing effect on vitamin B_{12} except amygdalin and benzonitrile. Amygdalin would be expected to undergo dissociation more slowly than either free α-hydroxynitriles or the esters. Benzonitrile should not dissociate to form cyanide. Benzoyl mandelonitrile was not as satisfactory a stabilizer as the other esters or free nitriles, possibly because of a slower rate of hydrolysis.

Aqueous cyanocobalamin solutions are stable for long periods of time under ordinary storage conditions, but decompose readily upon exposure to ultraviolet light (12). Solutions which contained 15 mcg. per ml. of cyanocobalamin and from 0.1 to 0.001% of *m*-hydroxymandelonitrile were exposed to ultraviolet irradiation on the Fade-Ometer for periods of up to 96 hours. The results were plotted in Fig. 3 as log per cent of the initial assay against time of exposure.

The rate of decomposition of cyanocobalamin in the presence of α-hydroxynitrile did not follow first-order kinetics as did the control sample. Plots of the decomposition of cyanocobalamin in the

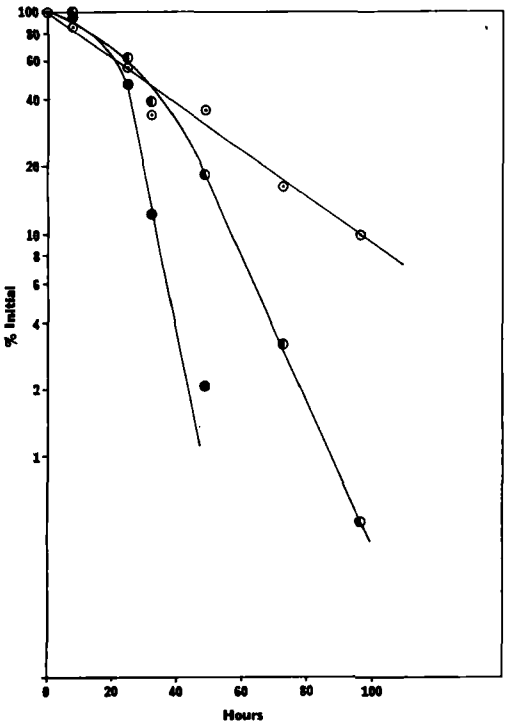

Fig. 3.—Decomposition of vitamin B_{12} in the presence of *m*-hydroxymandelonitrile following exposure to ultraviolet irradiation in the Fade-Ometer. ●, 0.1% *m*-hydroxymandelonitrile; ◐, 0.001% *m*-hydroxymandelonitrile; ⊙, control.

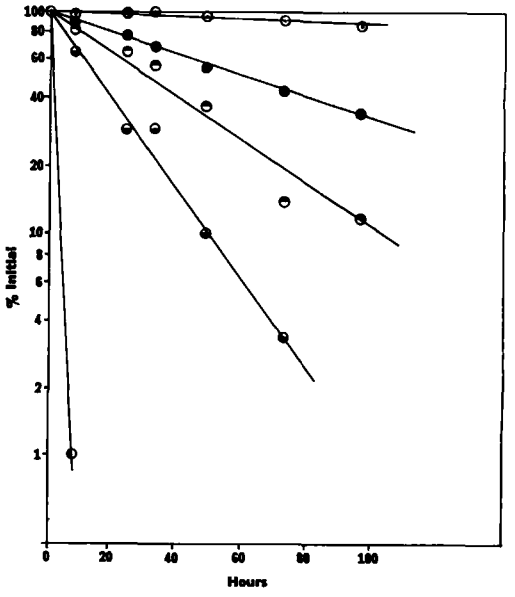

Fig. 4.—Decomposition of vitamin B_{12} in the presence of potassium cyanide or *m*-hydroxymandelonitrile following exposure to ultraviolet irradiation in the Fade-Ometer. ⊙, 0.001% potassium cyanide; ●, 0.0002% potassium cyanide; ◐, 0.1% *m*-hydroxybenzaldehyde; ◒, 0.001% *m*-hydroxybenzaldehyde; ◓, control.

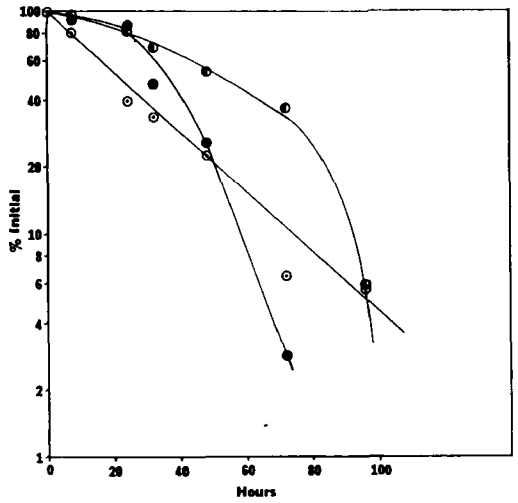

Fig. 5.—Decomposition of vitamin B_{12} in the presence of carbethoxymandelonitrile following exposure to ultraviolet irradiation in the Fade-Ometer. ●, 0.1% carbethoxymandelonitrile; ◐, 0.005% carbethoxymandelonitrile; ⊙, control.

presence of potassium cyanide and *m*-hydroxybenzaldehyde are illustrated in Fig. 4. The reactions in both of these cases follow first-order kinetics. The fact that solutions containing *m*-hydroxymandelonitrile do not follow first-order kinetics suggests that the rate at which equilibrium is reached in the dissociation of the nitrile to the aldehyde and hydrogen cyanide is the factor controlling the rate of decomposition of cyanocobalamin. In the cases where carbethoxymandelonitrile was used, the change in reaction rate was much slower (Fig. 5). This would be expected since hydrolysis of the ester precedes the dissociation of the mandelonitrile. The hydrolysis rate is the rate-controlling step in this reaction. The eventual reaction rate at which vitamin B_{12} decomposes should be that for the equilibrium mixture of aldehyde, hydrogen cyanide and α-hydroxynitrile.

Cyanocobalamin solutions containing α-hydroxynitriles after short periods of irradiation are more stable than the control solution of cyanocobalamin alone. After longer periods of irradiation they are less stable than the control. One explanation for this is that a competition exists between the cobalamin and the aldehyde for the cyanide in solution and that the ratio of cobalamin to free aldehyde is an important factor in the loss of vitamin B_{12} activity. If the effect of cyanide and aldehyde upon the rate of decomposition of cyanocobalamin were independent, the change from a rate slower than the control to one which is faster would not be expected to occur. Cyanide salts are much more effective in stabilizing cyanocobalamin in the presence of ultraviolet light than is cyanide in the form of an α-hydroxynitrile.

Acute oral toxicity in the mouse was obtained for two compounds (Table III). Although these

TABLE III.—ACUTE ORAL TOXICITY OF α-HYDROXYNITRILES IN MICE

	LD_{50}, mg. per Kg.
m-Hydroxymandelonitrile	25.40 ± 1.47
Carbethoxymandelonitrile	34.86 ± 2.34

compounds have a low LD_{50}, they may still be useful because of the low concentration required for vitamin B_{12} stabilization. The optimum concentration for *m*-hydroxynitrile based on both efficacy and toxicity is close to 0.05%. No toxicity studies have been made on pharmaceutical preparations.

SUMMARY

1. α-Hydroxynitriles and esters of mandelonitrile were shown to be effective stabilizers of vitamin B_{12} in a liquid multiple vitamin preparation.

2. *m*-Hydroxymandelonitrile and carbethoxymandelonitrile demonstrated some stabilizing action on vitamin B_{12} solutions which were exposed to ultraviolet irradiation. The initial stabilizing effect is reversed upon longer exposure and, at or before 96 hours, all the samples containing α-hydroxynitriles were decomposing at a greater rate than the control samples.

REFERENCES

(1) Gakenheimer, W. C., and Feller, B. A., THIS JOURNAL, **38**, 660(1949).
(2) Trenner, N. R., *et al.*, *ibid.*, **39**, 361(1950).
(3) Macek, T. J., *Am. J. Pharm.*, **132**, 433(1960).
(4) Organon, N. V., British pat. 692,968 (June 17, 1953).
(5) Stepert, E. M., *et al.*, THIS JOURNAL, **43**, 87(1954).
(6) Daniel, L. E., and Woodruf, H. B., U. S. pat. 2,650,-896 (September 1, 1953).
(7) Conine, J. W., and Zuck, D. A., U. S. pat. 2,874,089 (May 20, 1958).
(8) Buck, J. S., *J. Am. Chem. Soc.*, **55**, 3388(1933).
(9) Francis, F., and Davis, C. M., *J. Chem. Soc.*, **95**, 1404(1909).
(10) Migrdichian, V., "The Chemistry of Organic Cyanogen Compounds," Reinhold Publishing Corp., New York, N. Y., 1947, pp. 173–175.
(11) Jones, W. J., *J. Chem. Soc.*, **105**, 1560(1914).
(12) DeMerre, L. J., and Wilson, C., THIS JOURNAL, **45** 129(1956).