Stability of Cyanocobalamin in Liver Preparations for Use in the Treatment of Pernicious Anemia

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A deficiency of vitamin B_{12} has been found in a significant number of commercially available injectable products containing liver extract with added cyanocobalamin, with or without other added ingredients. In a study of the stability of cyanocobalamin when added to liver injectable preparations, with and without added folic acid, mixtures were stored in the dark at room temperature and examined at intervals throughout 1 year. No appreciable loss in cyanocobalamin occurred during the first month. Solutions stored at pH 5.0 were relatively stable for 12 months. At pH 6.5, destruction occurred within 3 months in some samples. Greater destruction occurred when the pH was raised to 8.0. Progressive destruction was observed in unstable samples after 6- and 12-month periods. With some preparations, destruction increased when folic acid was present, and in some instances when solutions were in contact with rubber stoppers.

CYANOCOBALAMIN in aqueous preparations has been found to be more stable than the hydroxo analog (1, 2) and, also, more stable in the absence of its analogs (3). Its stability in solutions decreased under certain conditions in the presence of other water-soluble vitamins (4–12) and in the presence of degradation products of these other vitamins (9, 13–15). The effect of impure phenol (16), other components of aqueous solutions (2, 17–21), light (22, 23), and oxygen (4, 24) on the stability of vitamin B_{12} also has been reported.

Procedures for predicting the stability of vitamins, including vitamin B_{12}, have been devised (25, 26) which involve studies at elevated temperatures. A report of studies on oral multivitamin preparations revealed that potencies of several vitamins, including vitamin B_{12}, were markedly affected by shelf age and storage conditions for certain types of products (27). The authors called attention to the responsibility of the manufacturer for maintenance of potency of these products throughout normal shelf life, and suggested that an expiration date on the label would be helpful in ensuring that a product meets its label claim.

Despite the availability of much information relating to the stability of vitamin B_{12} in various pharmaceutical preparations, that dealing with the stability of cyanocobalamin when added to liver solutions is limited.

There are on the market injectable preparations containing liver extract with added cyanocobalamin that are labeled for use in the treatment of pernicious anemia. Some of these products also contain added minerals, folic acid, and other vitamins. It has been shown conclusively in this laboratory that cyanocobalamin is unstable in many of the products with labeled potencies varying from about 10 to 120 mcg. of vitamin B_{12} per ml. It is not unusual to find deficiencies of vitamin B_{12} ranging from 20 to 50% and, in some instances, as great as 90% or more. A marked difference may occur in the degree of deterioration among vials bearing the same code. Deterioration frequently progresses during storage, even at 10°. We also have found that when a 1- or 2-ml. portion has been withdrawn from a 10-ml. multiple-dose vial, the vitamin B_{12} potency in the remainder may decrease as much as 25% after a 1-week storage period at 10°. Gakenheimer (16) has called attention to the influence of liver solutions upon the stability of added cyanocobalamin and pointed out that in a given liver extract which may retain its vitamin B_{12} activity for 1 year, added cyanocobalamin may not be stable. He emphasized that liver solutions must be pretested for compatibility when vitamin B_{12}, with or without folic acid, is to be added.

Our findings led us to carry out a study on the stability of cyanocobalamin when added to liver solutions, with and without the addition of folic acid.

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EXPERIMENTAL

Assay Methods

Vitamin B_{12}.—The A.O.A.C. (microbiological) method (28) was used. There was good agreement between the microbiological results and those obtained by the U.S.P. (spectrophotometric) method (29), slightly modified, on the cyanocobalamin stock solution and the control samples of cyanocobalamin and cyanocobalamin-folic acid.

Folic Acid.—The U.S.P. (chemical) method (30), slightly modified, was used. There was good agreement between the chemical results and those obtained spectrophotometrically on the folic acid stock solution and the control samples of cyanocobalamin-folic acid.

Stock Solutions

Cyanocobalamin and folic acid were of U.S.P. XV grade and all reagents were of analytical grade. Distilled water used as a solvent had a specific conductance of 0.5 micromho. Sterile solutions were used and all operations (including the subsequent preparation of sample solutions and filling and stoppering of vials) were conducted under aseptic conditions.

Liver, cyanocobalamin, and folic acid solutions were buffered with 0.05 *M* sodium acetate. Redistilled phenol was used at 0.5% concentration as the bacteriostatic agent in the preparation of each solution since it was already present in the liver solutions.

Acetate Buffer.—A 0.05 *M* solution of sodium acetate was adjusted to pH 6.5 with acetic acid.

Liver.—Solutions were taken from intact vials of U.S.P. XV grade liver injection and liver injection crude. These solutions were from various lots of samples that had accumulated in the laboratory and represented preparations from a number of manufacturers. Selection of the lots was arbitrary and stock solutions prepared from these lots did not represent any one manufacturer's product, and also were not necessarily identical with solutions used commercially. Each of six stock solutions was prepared by compositing three different lots of liver preparations and adjusting the slightly acidic solutions to pH 6.5 with sodium hydroxide solution.

Cyanocobalamin.—This solution, adjusted to pH 6.5 with acetic acid, contained 150 mcg. of cyanocobalamin per ml.

Folic Acid.—This solution contained the equivalent of 15 mg. of folic acid per ml. Folic acid was suspended in water, sodium hydroxide solution added until solution was complete (about pH 8.0), and adjustment to pH 6.5 was made with acetic acid.

Sample Solutions

At pH 6.5.—This pH was selected because it was near the value we had obtained for many liver-cyanocobalamin injectable solutions examined, some of which also contained folic acid. Most of the samples examined were within a pH range of 6.2–6.9, with an extreme range of 5.0 to 9.0.

(*a*) *Liver.*—Each of the six samples consisted of a portion of the respective liver stock solution.

(*b*) *Cyanocobalamin Control.*—Stock solutions of acetate buffer and cyanocobalamin were mixed in the ratio of 2:1.

(*c*) *Cyanocobalamin-Folic Acid Control.*—Stock solutions of acetate buffer, cyanocobalamin, and folic acid were mixed in the relation of 1:1:1.

(*d*) *Liver-Cyanocobalamin.*—Stock solutions of acetate buffer, liver, and cyanocobalamin were mixed in the relation of 1:1:1.

(*e*) *Liver-Cyanocobalamin-Folic Acid.*—Stock solutions of liver, cyanocobalamin, and folic acid were mixed in the relation of 1:1:1.

At pH 5.0.—A portion from the control under (*b*) and from each of samples 1, 3, and 6 under (*d*) was adjusted to pH 5.0 by the addition of acetic acid (about 0.3 ml./100 ml. solution).

At pH 8.0.—Portions similar to those taken for pH 5.0 adjustment were adjusted to pH 8.0 by the addition of 0.5 *M* tribasic sodium phosphate (about 0.2 ml./100 ml. solution).

Procedure

An 11-ml. portion of the respective sample or control solution was added to each vial used in the different phases of the study and the vial sealed immediately with a rubber stopper. Vials were Kimble[1] serum bottles, Neutraglas, narrow mouth, short form, No. 14196 for the 10-ml. size, and No. 14200 for the 20- and 50-ml. sizes. The respective volume of air in the 10-, 20-, and 50-ml. vials was 3, 16, and 50 ml. The 20- and 50-ml. vials were used to determine if any increase in the amount of enclosed air affected the stability of the cyanocobalamin control solution and the liver-cyanocobalamin sample solutions 1, 3, and 6 when stored at pH 6.5. Rubber stoppers were West[2] No. 124 (pink) stock. They were washed in a warm solution of sodium lauryl sulfate and rinsed repeatedly with distilled water. They were then immersed in distilled water, autoclaved, and rinsed. This latter process was repeated. A sufficient number of vials was prepared so that after portions were removed for assay, the remaining contents of an entered vial were discarded.

Determinations were made of the initial vitamin B_{12} and folic acid potencies of the vial contents from each series of solutions under study. Thereafter, determinations were made at intervals of 1, 3, 6, and 12 months with the vials stored upright so that the rubber stoppers were never in contact with the solutions. Storage was in the dark at 23.5 ± 1.5°. One set of vials for each phase of the study was stored in an inverted position to determine if potency was affected by contact of the solution with the vial stopper. These were examined only at the end of the 12-month storage period.

RESULTS

Liver Solutions.—Considerable destruction of vitamin B_{12} occurred in four of the six samples during twelve months storage in vials in upright position as shown in Table I. In fact, some loss was apparent at the end of the first month. Two of these unstable solutions were appreciably affected by contact with the stopper in the inverted vials.

Cyanocobalamin in Control Solutions.—There was no loss of cyanocobalamin activity in the control solutions throughout the 12-month storage period

[1] Kimble Glass Co., Subsidiary of Owens-Illinois, Toledo 1, Ohio.
[2] West Co., Phoenixville, Pa.

TABLE I.—STABILITY OF VITAMIN B_{12} IN LIVER SOLUTIONS AT pH 6.5[a]

Sample No.	Mcg. of Vitamin B_{12} per ml. after Storage, mo. Initial	Vials Upright 1	3	6	12	Vials Inverted 12
1	1.2	1.2	1.2	1.2	1.2	1.2
2	2.1	2.1	2.1	1.8	1.8	1.8
3	2.7	2.3	2.3	2.2	2.1	1.7
4	3.6	3.1	2.8	2.7	2.1	2.2
5	8.6	7.4	6.8	5.9	4.4	4.2
6	11.1	9.6	9.6	7.8	6.6	5.0

[a] Stored in 10-ml. vials in the dark at room temperature.

(Tables II–V). Cyanocobalamin alone in aqueous solution was stable at pH 5.0, 6.5, and 8.0; at pH 6.5 in the presence of folic acid; and in all solutions in contact with vial stoppers.

Liver Solutions with Cyanocobalamin.—Stability of vitamin B_{12} in the six samples stored at pH 6.5 in 10-ml. upright vials is shown in Table II. No appreciable destruction of vitamin B_{12} occurred during the first 3 months. At the end of 6 months, there was a loss in potency of 10%, or more, in four samples. Three of these samples continued to lose potency during the next 6 months. Vitamin B_{12} in the four unstable solutions was markedly affected by contact with the stopper.

TABLE II.—STABILITY OF VITAMIN B_{12} IN LIVER SOLUTIONS CONTAINING CYANOCOBALAMIN AT pH 6.5[a]

Sample No.	Mcg. of Vitamin B_{12} per ml. after Storage, mo. Initial	Vials Upright 1	3	6	12	Vials Inverted 12
Control	50	50	49	49	50	50
1	50	50	47	45	38	23
2	51	51	49	45	44	32
3	51	51	48	42	37	19
4	51	51	49	45	40	26
5	53	52	53	50	49	45
6	54	53	52	50	50	47

[a] Stored in 10-ml. vials in the dark at room temperature.

It is shown for the three samples listed in Table III that the increase in amount of air enclosed in vials has no deleterious effect upon vitamin B_{12}. The results obtained on these samples correspond closely with those from the same three samples contained in 10-ml. vials included in Table II.

There was a marked change in the stability of two of these same three solutions stored in 10-ml. vials when the pH was altered, as shown in Table IV. At pH 5.0, the loss of vitamin B_{12} was 10% or less in samples 1 and 3, even when stored 12 months in contact with the vial stopper. The loss was drastic, however, in these two samples when the pH was raised to 8.0. The greatest stability occurred in sample 6. Even when inverted for 12 months, at pH 8.0, the loss in vitamin B_{12} was only 15%.

Liver Solutions with Cyanocobalamin and Folic Acid.—During the first month, as shown in Table V, there was little effect upon stability of vitamin B_{12} from the addition of folic acid to the six samples stored at pH 6.5 in upright 10-ml. vials. A loss in

TABLE III.—STABILITY OF VITAMIN B_{12} IN LIVER SOLUTIONS CONTAINING CYANOCOBALAMIN AT pH 6.5[a]

Sample No.	Vial Size, ml.	Mcg. of Vitamin B_{12} per ml. after Storage, mo. Initial	Vials Upright 1	3	6	12	Vials Inverted 12
Control	20	50	50	50	50	50	49
	50	50	50	50	50	50	50
1	20	50	49	47	44	38	23
	50	50	49	47	45	38	23
3	20	51	51	48	43	38	18
	50	51	50	47	43	37	19
6	20	54	53	52	49	49	48
	50	54	53	52	50	49	47

[a] Stored in 20- and 50-ml. vials in the dark at room temperature.

TABLE IV.—STABILITY OF VITAMIN B_{12} IN LIVER SOLUTIONS CONTAINING CYANOCOBALAMIN AT pH 5.0 and 8.0[a]

Sample No.	pH	Mcg. of Vitamin B_{12} per ml. after Storage, mo. Initial	Vials Upright 1	3	6	12	Vials Inverted 12
Control	5.0	50	50	50	51	50	51
	8.0	50	49	50	50	50	48
1	5.0	50	50	49	50	49	49
	8.0	50	49	38	20	13	3.4
3	5.0	51	50	49	50	48	46
	8.0	51	50	5.7	2.3	0.97	0.50
6	5.0	54	52	52	50	50	50
	8.0	54	53	51	50	47	46

[a] Stored in 10-ml. vials in the dark at room temperature.

TABLE V.—STABILITY OF VITAMIN B_{12} IN LIVER SOLUTIONS CONTAINING CYANOCOBALAMIN AND FOLIC ACID AT pH 6.5[a]

Sample No.	Mcg. of Vitamin B_{12} per ml. after Storage, mo. Initial	Vials Upright 1	3	6	12	Vials Inverted 12
Control	50	50	50	49	50	50
1	50	50	41	40	10	10
2	51	51	44	44	26	27
3	51	50	45	38	8.7	4.9
4	51	50	44	38	21	12
5	53	52	46	45	44	2.7
6	54	53	48	48	41	3.8

[a] Stored in 10-ml. vials in the dark at room temperature.

TABLE VI.—STABILITY OF FOLIC ACID IN LIVER SOLUTIONS CONTAINING CYANOCOBALAMIN AND FOLIC ACID AT pH 6.5[a]

Sample No.	Mg. of Folic Acid per ml. after Storage, mo. Initial	Vials Upright 1	3	6	12	Vials Inverted 12
Control	5.0	5.0	4.7	3.9	3.5	3.4
1	5.0	5.0	4.7	4.1	3.5	3.4
2	5.0	5.0	4.3	3.9	3.5	3.5
3	5.0	5.0	4.6	3.9	3.6	3.5
4	5.0	5.0	4.4	3.6	2.8	2.8
5	5.0	5.0	3.7	2.9	2.8	2.8
6	5.0	5.0	4.3	3.3	3.2	3.2

[a] Stored in 10-ml. vials in the dark at room temperature.

potency was apparent, however, in all samples at the end of 3 months. There was no further loss between 3 and 6 months except in samples 3 and 4, but there was marked destruction of vitamin B_{12} in the first four samples at the end of 12 months. Greater destruction occurred in some solutions when the vials were inverted. This was most noticeable in samples 5 and 6.

Folic acid was stable in all solutions in upright vials during the first month (Table VI). A loss in potency was apparent at the end of 3 months and continued throughout further storage. Except for samples 4 and 5, the loss of folic acid activity observed in the liver-vitamin solutions was the same as in the control solution. Inversion of the vials had no noticeable effect on the folic acid activity of any of the solutions.

DISCUSSION

In the examination of the effect of liver solutions upon stability of cyanocobalamin, no single commercial product has been used. It was convenient to utilize combinations of commercial products available as samples in this laboratory, combined arbitrarily to provide a sufficient amount of test material for the six stock solutions that were studied. Therefore, the data presented here do not relate specifically to any particular manufacturer.

The factors, aside from composition, considered of possible importance in this study were the amount of air space, the pH of the solution, time of storage, and the possible effect of the rubber closures. It is apparent that a variation in the amount of oxygen contained in the vial was not directly related to the degree of deterioration of vitamin B_{12}. On the other hand, stability was markedly affected by pH of the solution. It was clear also that in some instances, particularly in the presence of folic acid, the rubber closure did affect the rate and degree of deterioration.

The most important factor, however, is some characteristic of the liver products used. There is no basis in results of this study for suggesting the nature of the variant in the liver products that is related to cyanocobalamin deterioration. Without knowledge of the method of manufacture or the source material, it is not possible to do other than point to the liver preparation as a principal source of the factor responsible for the decreased stability of vitamin B_{12} in these preparations.

The study reported here has been carried out under well-controlled conditions and demonstrates, beyond question, the magnitude of the losses of vitamin B_{12}, and in some cases folic acid, that can be expected when liver preparations that are incompatible with these compounds are used. It is emphasized again, as Gakenheimer (16) stated in 1952, that liver solutions to be used in injectable preparations with added cyanocobalamin require pretesting for compatibility.

SUMMARY

1. A study was conducted for a period of 12 months to obtain further information on the stability of cyanocobalamin when added to liver injectable preparations, with and without the addition of folic acid.

2. Loss of vitamin B_{12} potency in some of the liver solutions occurred during 3 months storage and continued in these preparations during further storage.

3. In the solutions tested, potency decreased when the pH was increased from 5.0 to 6.5 and from 6.5 to 8.0.

4. The loss of vitamin B_{12} was greater in some preparations when folic acid was added.

5. There was further loss of vitamin B_{12} in some samples when the solutions in the vials were in contact with the rubber stopper.

REFERENCES

(1) Loy, H. W., Haggerty, J. F., and Kline, O. L., *J. Assoc. Offic. Agr. Chemists*, **35,** 169(1952).
(2) Merck Service Bulletin, "Vitamin B_{12}," Part 1, Pharmaceutical Information, Merck and Co., Inc., Rahway, N. J., 1953, pp. 3–9.
(3) Hutchins, H. H., Cravioto, P. J., and Macek, T. J., THIS JOURNAL, **45,** 806(1956).
(4) Blitz, M., Eigen, E., and Gunsberg, E., *ibid.*, **43,** 651 (1954).
(5) Ponci, R., *Farmaco Pavia Ed. Sci.*, **10,** 997(1955).
(6) Ponci, R., *ibid.*, **10,** 1003(1955).
(7) Brumfield, P. E., and Gross, H. M., *Drug & Cosmetic Ind.*, **77,** 46(1955).
(8) Macek, T. J., and Feller, B. A., THIS JOURNAL, **44,** 254(1955).
(9) Blitz, M., Eigen, E., and Gunsberg, E., *ibid.*, **45,** 803 (1956).
(10) Mukherjee, S. L., and Sen, S. P., *J. Pharm. and Pharmacol.*, **9,** 759(1957).
(11) Gambier, A. S., and Rahn, E. P. G., THIS JOURNAL, **46,** 134(1957).
(12) Gambier, A. S., and Rahn, E. P. G., *ibid.*, **47,** 356 (1958).
(13) Bartilucci, A., and Foss, N. E., *ibid.*, **43,** 159(1954).
(14) Feller, B. A., and Macek, T. J., *ibid.*, **44,** 662(1955).
(15) Bartilucci, A. J., DiGirolamo, R., and Eisen, H., *ibid.*, **47,** 42(1958).
(16) Gakenheimer, W. C., *Bull. Parenteral Drug Assoc.*, **6,** No. 5, 2(1952).
(17) Ravin, L. J., and Doerge, R. F., THIS JOURNAL, **48,** 425(1959).
(18) Shenoy, K. G., and Ramasarma, G. B., *Arch. Biochem. Biophys.*, **55,** 293(1955).
(19) Gerber, C. F., Hetzel C. P., Klioze, O., and Leyden, A. F., THIS JOURNAL, **46,** 635(1957).
(20) Barr, M., Kohn, S. R., and Tice, L. F., *ibid.*, **46,** 650 (1957).
(21) Smith, E. L., "Vitamin B_{12}," John Wiley & Sons, Inc., New York, N. Y., 1960.
(22) Baxter, N., Horsford, J., Wokes, F., Norris, F. W., and Fernandez, S. J. G., *J. Pharm. and Pharmacol.*, **5,** 723 (1953).
(23) Demerre, L. J., and Wilson, C., THIS JOURNAL, **45,** 129(1956).
(24) Ponci, R., *Farmaco Pavia Ed. Sci.*, **9,** 467(1954).
(25) Garrett, E. R., THIS JOURNAL, **45,** 171(1956).
(26) Garrett, E. R., *ibid.*, **45,** 470(1956).
(27) Campbell, J. A., and McLeod, H. A., *ibid.*, **44,** 263 (1955).
(28) "Official Methods of Analysis of the Association of Official Agricultural Chemists," 8th ed., Association of Official Agricultural Chemists, Washington, D. C., 1955, p. 835.
(29) "United States Pharmacopeia," 15th rev., Mack Publishing Co., Easton, Pa., 1955, p. 187.
(30) *Ibid.*, p. 300.