steel wire loop is required. This process of inspection has the distinct advantage that with very little experience it is possible to obtain reproducible results which agree with known concentrations of NaCl within 0.02% without plotting cooling curves or removing samples for analysis. Furthermore, frequent inspection gives a quick and sure test of the integrity of the results with respect to possible precipitation of solute as well as to the possible increase in concentration due to excessive ice formation.

DISCUSSION

There is good evidence that the thermistor thermometer is the instrument of choice for this work. Several modifications of the instrument have been used by classes of students who seem to prefer it to ordinary thermometers because of the ease of reading the scale. The method of quick freezing and subsequent inspection is greatly facilitated by a thermistor probe which allows freedom to remove the freezing tube from the air bath while maintaining a permanently located reading scale.

REFERENCES

(1) Goyan, F. M., and Reck, D., THIS JOURNAL, **44**, 43 (1955).
(2) Zeffert, B. M., and Witherspoon, R. R., *Anal. Chem.*, **28**, 1701(1956).
(3) Szekely, I. J., and Goyan, F. M., THIS JOURNAL, **41**, 30(1952).
(4) Lyman, R. A., and Sprowls, J. B., "Textbook of Pharmaceutical Compounding and Dispensing," 2nd ed., J. B. Lippincott Co., Philadelphia, 1955, p. 138.
(5) Scatchard, G., and Prentiss, S. S., *J. Am. Chem. Soc.*, **55**, 4355(1933).
(6) Harned, H. S., and Owen, B. B., "Physical Chemistry of Electrolytic Solutions," 2nd ed. Reinhold Publishing Corp., New York, 1950, p. 556.

Cyanocobalamin (Vitamin B_{12}) II.

Further Studies of the Effect of Ascorbic Acid Degradation Products on Cyanocobalamin*

By A. J. BARTILUCCI, R. DiGIROLAMO, and H. EISEN

The data presented seem to indicate that the stability of cyanocobalamin is affected by dehydroascorbic acid, a decomposition product of ascorbic acid. This study further corroborates the initial findings of the effect of decomposed ascorbic acid on cyanocobalamin.

IN A PREVIOUS PUBLICATION (1) it was suggested that the decomposition products of ascorbic acid are significantly involved in the decomposition of cyanocobalamin in aqueous solution.

Further investigations are reported indicating the effect of varying concentrations of ascorbic acid decomposition products on cyanocobalamin; and the effect of dehydroascorbic acid, the reversible oxidative product encountered in the stepwise degradation of ascorbic acid, on cyanocobalamin.

EXPERIMENTAL AND RESULTS

Ascorbic acid was determined by titration in a 1% oxalic acid solution with 0.1 *N* iodine, using starch T. S. as the indicator. The cyanocobalamin was assayed by a procedure described in the Difco Manual (2) using *L. leichmannii* 4797 ATCC as the test organism. The possible effect of the decomposition products of ascorbic acid on the microbial assay was not determined. Generally, determinations were discontinued when 50 to 75% decomposition had taken place.

The cyanocobalamin used was in the form of a 0.1% trituration with mannitol as the diluent. The mannitol in the concentration present did not appear to affect the results. The ascorbic acid used in the study was of U. S. P. quality in a fine crystalline form. The water used as a solvent was distilled water redistilled from an all-glass still. The dehydroascorbic acid was obtained from the

* Received May 3, 1957, from St. John's University, College of Pharmacy, New York, N. Y.
Presented to the Scientific Section, A. PH. A., New York meeting, April-May 1957.
Project financially supported by Charles Pfizer & Co., Inc., Brooklyn, N. Y.

TABLE I.—PERCENTAGE LOSS OF CYANOCOBALAMIN IN ASCORBIC ACID SOLUTION AND DECOMPOSED ASCORBIC ACID SOLUTIONS AT 40°

	A.A.	B_{12}	A.A.	B_{12}	A.A.	B_{12}	A.A.	B_{12}
Initial	0.0	0.0	9.2	0.0	23.5	0.0	68.1	ca 60
1 week	19.4	10.6	15.5	36.7	31.4	52.8	..	..
2 weeks	25.3	50.6	19.2	78.7	34.8	100.0	..	..
1 day	..	..	..	..	..	..	68.8	67.3
2 days	..	..	..	..	..	..	68.7	79.5

TABLE II.—PERCENTAGE LOSS OF CYANOCOBALAMIN IN SOLUTIONS OF DEHYDROASCORBIC ACID AT 40°

	—Dehydroascorbic Acid, mg./ml.—		
	5	12.5	25
Initial	5	24	44
1 day	15	40	60
2 days	26	54	72
3 days	36	70	..
4 days	46	79	..
5 days	54	..	..
6 days	64	..	..
7 days	76	..	..

National Biochemicals Co., Cleveland, Ohio, and decomposed at 225°. Containers, pipets, and all glassware used were thoroughly washed, rinsed several times with glass-distilled water, and air dried before use.

Five-hundredths per cent of propylparaben was included in all samples as an antimicrobial preservative. The solutions were subdivided into one-ounce flint glass, screw-capped bottles and stored in cardboard boxes at 40°.

The stability data of a solution initially containing 50.0 mg./ml. of ascorbic acid and 11.0 mcg./ml. of cyanocobalamin stored at 40° appear in the first two columns of Table I.

The effect of varying concentrations of ascorbic acid decomposition products on the stability of cyanocobalamin was then investigated.

A stock solution of ascorbic acid containing 51 mg./ml. as determined by assay, was stored at 40°. Progressive decomposition was followed by periodically removing aliquot portions and determining the ascorbic acid content remaining therein. Three test solutions indicating 9.2, 23.5, and 68.1% decomposition of ascorbic acid were removed.

An amount of cyanocobalamin was added to these solutions which would be expected to yield a solution containing 10 mcg./ml. Initial assays indicated a cyanocobalamin content in the first two test solutions of 10.7 and 11.0 mcg./ml., respectively. In the test solution containing the 68.1% decomposed ascorbic acid, the initial microbial assay indicated a cyanocobalamin content of 4.0 mcg./ml.

All solutions were stored at 40° and the ascorbic acid and cyanocobalamin content determined periodically. The first two solutions were assayed at weekly intervals. The third solution was assayed daily. The results appearing in Table I indicate a progressive loss of cyanocobalamin in direct ratio to the degree of ascorbic acid decomposition.

On the basis of the change in color observations previously reported (1) and the experiments reported above, an investigation of the effect of one of the known decomposition products of ascorbic acid on cyanocobalamin seemed warranted. Dehydroascorbic acid is the initial oxidation product of ascorbic acid. While this reaction is reversible, further decomposition of dehydroascorbic acid eventually leads to extensive degradation with the formation of oxalic acid and 2,3-diketogulonic acid.

Accordingly, then, each of three solutions was prepared to contain 10 mcg./ml. of cyanocobalamin and 5 mg., 12.5 mg., and 25 mg./ml. of dehydroascorbic acid, respectively. The quantity of dehydroascorbic acid used was calculated to be equivalent to the amount present in solutions containing respectively 10, 25, and 50% decomposed ascorbic acid based upon the assumption that dehydroascorbic acid was the only decomposition product present. Initial microbial assays indicated from possibly slight to marked destruction of the cyanocobalamin in the solutions.

The solutions were stored at 40° and the cyanocobalamin content determined daily. The assay data summarized in Table II indicate that the rate of decomposition of cyanocobalamin increases as the initial concentration of dehydroascorbic acid increases.

SUMMARY

The stability of cyanocobalamin was shown to be decreased in the presence of decomposed ascorbic acid. Dehydroascorbic acid, the first decomposition product of ascorbic acid, caused greater instability of cyanocobalamin in solution than ascorbic acid. Investigations are continuing on the effect of other known decomposition products of ascorbic acid on cyanocobalamin, and it is expected that the results will be published.

REFERENCES

(1) Bartilucci, A., and Foss, N. E., THIS JOURNAL, **43**, 159 (1954).

(2) "Difco Manual," 9th ed., Difco Laboratories, Inc., Detroit, Mich., 1953, p. 223.