THE STABILITY OF VITAMIN B_{12}

PROTECTION BY IRON SALTS AGAINST DESTRUCTION BY ANEURINE AND NICOTINAMIDE

BY S. L. MUKHERJEE AND S. P. SEN

From the Microbiological Section, Albert David (Laboratories) Ltd., Calcutta

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VITAMIN B_{12} has been found to deteriorate progressively in association with aneurine and nicotinamide in solution over a pH range of 4 to 4·5. The deterioration can be satisfactorily prevented by the use of iron salts, in ferrous, ferric and complex form. Salts of cobalt, manganese, copper and lead do not protect the vitamin from the combined destructive influence of aneurine and nicotinamide in solution.

Whereas solutions of pure crystalline vitamin B_{12} have been found to be stable over a period of eighteen months at an optimum pH range between 4 to 4·5 and under normal storage conditions, we have observed, with others, that vitamin B_{12} progressively deteriorates when mixed with other vitamins. In some instances the loss is complete in three months in sterile vitamin B-Complex and liver extract solutions prepared according to prescribed specifications[1] and stored under similar conditions. Light, oxygen or air in the containers, temperature[2], reducing agents[3], and ascorbic acid[4,5] have been shown to be possible factors responsible for the deterioration of vitamin B_{12} potency in liver extract and other pharmaceutical preparations. Blitz, Eigen and Gunsberg[6] in an elaborate study of various commercial B-Complex preparations containing vitamin B_{12} have found it unstable, steadily losing its potency at pH 4·25 in B-Complex solution containing aneurine and nicotinamide, and that perhaps oxidation is not a possible factor for such loss. They have also found that loss of Vitamin B_{12} is a function of the concentration of both aneurine and nicotinamide present. Dony and Conter[7] and Feller and Macek[8] also confirmed the observation of Blitz and his colleagues[6], and have shown that stability of vitamin B_{12} in solution with aneurine and nicotinamide is affected, particularly at elevated temperature when the destruction of B_{12} is at a maximum. Feller and Macek[8] have presented evidence that this destruction of vitamin B_{12} may be due to aneurine decomposition products or to the thiazole moiety.

The role of iron as a stabiliser of vitamin B_{12}, particularly in liver extract solutions, has been elaborated by Shenoy and Ramsarma[9], who have stabilised B_{12}-activity in fractionated liver extract solutions, containing insufficient naturally present iron salts, by the addition of ferric chloride over wide ranges of pH. Smith[10] has also suggested that the presence of iron salts in liver extract exerts a protective action on vitamin B_{12}.

The authors of the present paper, while confirming the work of Blitz and others[6] on the destructive influence of aneurine and nicotinamide on vitamin B_{12}, have also observed that iron salts in general exert a satisfactory protective action in stabilising vitamin B_{12} in B-Complex solution,

containing aneurine and nicotinamide[12]. The concentration of iron salts required is so low that their adoption to stabilise vitamin B_{12} in pharmaceutical preparations like vitamin B-Complex or liver extract solution seems practical.

The procedures, methods, technique and experimental findings are described below.

Experimental

The simple elevated temperature test proposed by Gakenheimer[11] was used to assess the compatibility of the various substances with vitamin B_{12}. This consisted of heating the samples in a suitable buffer of pH 4–4·5 for 4 hours at 100°. Blank compatibility experiments were made with the vials, caps and the individual components before use. Three to 4 ml. of a 2 per cent sodium acetate (analar) solution adjusted to pH 4–4·5 with glacial acetic acid was used as buffer in 10 ml., and care was taken that the pH remained within this range.

TABLE I

Stability of vitamin B_{12} in vitamin B-complex injectable solutions* stored in room temperature, and also in the refrigerator

Time stored at room temperature (27°—33°) months	Vitamin B_{12} µg./ml.	Time stored in the refrigerator (0°—4°) months	Vitamin B_{12} µg./ml.
1	5	1	5
2	2·6	2	5
3	0·5	3	5
4	none	4	5

* 10 ml. in rubber-capped vials: aneurine, 15; riboflavine, 1·5; pyridoxine, 5; nicotinamide, 100; panthenol, 5; choline HCl, 10 mg./ml., vitamin B_{12}, 5 µg./ml., benzyl alcohol, 1·5 per cent.

Room temperature (27–33°) and refrigerator (0–4°) storage stability experiments for a period of 4 months were made. Storage at room temperature was found to affect the stability of vitamin B_{12} similarly to heating at 100° for 4 hours.

The microbiological potency of vitamin B_{12} was determined by the "Cup Plate Assay Method" using *E. coli* Mutant M200 as test organism, developed by Bessel and others[12] and Cuthbertson and others[13]. The accuracy of this microbiological method is $\pm$ 10 per cent. Aneurine was assayed fluorimetrically by the thiochrome method of the U.S.P. XV.

Results

From Table I it can be seen that potency of vitamin B_{12} in B-Complex solution on storage at room temperature progressively deteriorates and is destroyed completely within 3 to 4 months, but its potency is stable when kept at 0–4° for 4 months. After heating a similar preparation at 100° for 4 hours the initial B_{12}-activity of 5 µg./ml. was reduced to no activity, and the initial aneurine content from 15 mg./ml. to 11 mg./ml.

In an attempt to ascertain the effect of individual constituents of a vitamin B-Complex solution, 5 µg. of vitamin B_{12} was heated at 100° for 4 hours with, respectively, aneurine, 15 mg./ml., riboflavine 1·5

mg./ml., pyridoxine, 5 mg./ml., nicotinamide, 100 mg./ml., panthenol, 5 mg./ml., choline HCl, 5 mg./ml., and benzyl alcohol, 15 mg./ml. The B_{12}-activity after this treatment was found to be approximately unchanged. Some aneurine loss occurred, the original 15 mg. assaying at 10·6 mg.

TABLE II

EFFECT OF DIFFERENT CONCENTRATIONS OF ANEURINE AND NICOTINAMIDE ON THE STABILITY OF VITAMIN B_{12} IN AN ELEVATED TEMPERATURE TEST

Aneurine mg./ml.	Nicotinamide mg./ml.	Vitamin B_{12} before test μg./ml.	Vitamin B_{12} after test μg./ml.	Loss of Vitamin B_{12} activity per cent	Aneurine after test mg./ml
15	5	5	4	20	11·0
15	10	5	3·75	25	10·8
15	20	5	0·25	95	10·7
15	100	5	none	100	10·8
7·5	100	5	none	100	5·6
2	100	5	0·2	96	1·44
1	100	5	2·0	60	0·8

The results in Table II indicate the effect on vitamin B_{12} potency of varying the concentration of aneurine and nicotinamide in the original solution. It appears that over 95 per cent loss of B_{12} occurs if the aneurine content is between 2–15 mg./ml. and the nicotinamide content 20–100 mg./ml.

To investigate the protective action of iron salts in the elevated temperature test, eight iron salts were heated separately with 15 mg./ml. of aneurine, 100 mg./ml. of nicotinamide and 5 μg./ml. of vitamin B_{12}. The salts used were (0·5 mg./ml.) iron and ammonium citrate, ferrous gluconate ferric alum, ferrous alum, ferric chloride, potassium ferro- and ferricyanides, and ferrous sulphate. There was no loss in B_{12} activity after the test. The aneurine content, however, fell to between 10·6 and 11 mg./ml.

TABLE III

EFFECT OF FERRIC CHLORIDE SOLUTION ON THE STABILITY OF VITAMIN B_{12} IN VITAMIN B-COMPLEX INJECTABLE SOLUTION* AT THE ELEVATED TEMPERATURE TEST AND WHEN STORED AT ROOM TEMPERATURE

Ferric chloride mg./ml.	Initial vitamin B_{12} activity μg./ml.	Vitamin B_{12} activity (μg./ml.) after storage at room temperature for				Vitamin B_{12} activity after test μg./ml.
		1 month	2 months	3 months	4 months	
0·5	5	5	5	5	5	5
0·25	5	5	5	5	5	5
0·1	5	5	5	4	3·5	4
0·05	5					3·5
0·02	5					0·5

* Composition same as in Table I.

Table III shows that the ferric chloride solution of about 0·25 mg./ml. gave protection to vitamin B_{12} over a period of 4 months or under elevated temperature conditions.

The protective effect of 0·5 mg./ml. of the sulphates of cobalt, manganese and copper and also lead acetate was investigated, using the same quantities and conditions as those for iron salts. The B_{12}-activity was completely destroyed.

Discussion

According to Feller and Macek[8] decomposition products of aneurine are likely to cause deterioration of vitamin B_{12}. Our observations indicate that the total loss of aneurine is more or less the same when it is subjected to elevated temperature test conditions in association with vitamin B_{12} alone or in combination with nicotinamide with or without the presence of iron salts, whereas B_{12} potency deteriorates only in presence of aneurine and nicotinamide (Table II). Hence it is felt that the decomposition products of aneurine and nicotinamide are different from those of aneurine alone. Iron salts in association with aneurine and nicotinamide without preventing the decomposition of aneurine protect vitamin B_{12} in a specific way.

Further studies on the mechanism of action of iron salts in preventing or arresting decomposition of vitamin B_{12} in combination with aneurine and nicotinamide are in progress.

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