STABILITY OF VITAMIN B_{12}.

PART II. PROTECTION BY AN IRON SALT 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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The decomposition products of aneurine in solution at pH 8·0 and aneurine and nicotinamide at pH 4–4·5 destroy vitamin B_{12} activity. Ferric chloride has a protective effect. The decomposition products have reducing properties, and it is thought that these are responsible for the loss of B_{12} activity. Solutions containing the reducing substances heated to dryness and redissolved lost the ability to destroy the vitamin. Cysteine and H_2S are shown to destroy the activity of vitamin B_{12}, and again ferric chloride offers protection.

PREVIOUSLY[1] we observed that vitamin B_{12} deteriorates progressively in association with aneurine and nicotinamide in solution at a pH range between 4 to 4·5 when stored at room temperature or subjected to accelerated test conditions; the deterioration can be satisfactorily prevented by the use of iron salts. The total loss of aneurine was shown to be the same when aneurine alone in solution and aneurine together with nicotinamide in solution are subjected to accelerated test conditions at a pH range between 4 and 4·5. The decomposition product or products of aneurine alone have no destructive action on vitamin B_{12}, while those of aneurine and nicotinamide together have.

This led us to suppose that under the test conditions the decomposition products of aneurine and aneurine plus nicotinamide were not the same. The effect of the decomposition products was further investigated and the protective effect of an iron salt, $FeCl_3$, assessed on vitamin B_{12} in their presence. Therefore the pH of the solutions was varied, and as vitamin B_{12} activity can be destroyed by reducing substances the reducing effect of the decomposition products was investigated. Feller and Macek[2] suggested the thiazole moiety of aneurine to be responsible for the destruction of B_{12}, and polarographic studies[3,4] have shown that aneurine in alkali releases an –SH group and in higher concentrations of alkali finally decomposes releasing H_2S[5]. We therefore investigated the reducing effect of both cysteine and H_2S on vitamin B_{12}, and also the protective effect of $FeCl_3$ in these solutions.

EXPERIMENTAL

Solutions. Aneurine and nicotinamide were dissolved in 5 ml. of distilled water and the pH adjusted with HCl or NaOH; no buffer was added. When aneurine (150 mg.) and nicotinamide (1 g.) were dissolved in 5 ml. of water the pH of about 4·3 was not adjusted. The solutions tested in Table II were heated and then cooled and after acidification titrated with 0·01N iodine solution. Some, after adjusting the pH to

4·5, had 50 μg. of vitamin B_{12} added and the solution was reheated for 4 hours at 100°, after which the B_{12} was assayed.

Cysteine and H_2S solutions in amounts shown in the Tables were freshly prepared.

The "test" solution used in Table IV was made as follows.

Aneurine (150 mg.) was dissolved in 5 ml. of water and the pH adjusted to 8·0. The solution was heated in an open test tube on a water bath at 100° for 15 minutes and the pH, now about 7, was adjusted to 4·5. The solution was either diluted to 10 ml. and vitamin B_{12} 50 μg. (or the B_{12} plus ferric chloride, 5 mg.) added and heated for 4 hours at 100° and the B_{12} assayed, or the solution was evaporated to dryness and the residue dissolved in 10 ml. of water. The solution so formed was then titrated with iodine or 5 μg./ml. of B_{12} added and the solution heated for 4 hours at 100° and the B_{12} assayed.

Iodimetric titration. The usual procedure of iodimetric titration was followed by titrating aneurine or aneurine and nicotinamide solution, after acidification with HCl, with N/100 iodine solution using mucilage of starch as internal indicator. To the 10 ml. of solution described in Tables II and III, was added 1 ml. of concentrated HCl in the cold and 1 ml. of 1 per cent starch solution. To this, N/100 iodine solution was added dropwise with stirring. The end point occurred when the entire solution became blue for about 2 seconds. Due to the transient character of the blue colour, difficulties were experienced with the end point, particularly with the cysteine solution and with the aneurine and nicotinamide solution. Hence the end point chosen was the first appearance of the blue colour throughout the solution and which lasted for about 2 seconds.

Tests for Mercaptans. Colour tests for mercaptans were made with $FeCl_3$, $CuSO_4$ and nitroprusside solutions, and although cysteine solution gave characteristic colour reactions with all three, the decomposition products of aneurine and aneurine and nicotinamide did not. They reacted only with alkaline nitroprusside solution to give a pink colour.

Assays. The microbiological potency of vitamin B_{12} was determined by the "Cup Plate Assay Method" using *Escherichia coli* mutant M200 as test organism, developed by Bessel and others[6] and Cuthbertson and others[7]. 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

Table I shows the effect of aneurine in solution and aneurine with nicotinamide in solution at different ranges of pH on the stability of vitamin B_{12} when subjected to heating for 4 hours at 100° in 10 ml. rubber capped vials, and also shows the protection offered by $FeCl_3$.

It seems that aneurine solution subjected to an elevated temperature test with vitamin B_{12} at pH ranges between 4 to 6·5 causes no destruction of the B_{12} although there is substantial destruction of aneurine. But aneurine solution at pH 7·5 and 8 under similar conditions of heating

destroys B_{12}, and $FeCl_3$ offers significant protection. Also aneurine with nicotinamide in the concentrations indicated in the Table destroy vitamin B_{12} at pH ranges between 4 to 8, $FeCl_3$ offers nearly full protection at pH 4 and significant protection at higher pH ranges. The total loss of aneurine in aneurine and nicotinamide solution at various pH ranges is more or less equivalent to the total loss of aneurine when aneurine solution alone was heated at similar pH ranges.

TABLE I

EFFECT OF ANEURINE (15 MG./ML.) IN SOLUTION AND ANEURINE (15 MG./ML.) WITH NICOTINAMIDE IN SOLUTION, AT DIFFERENT RANGES OF pH, ON THE STABILITY OF VITAMIN B_{12} (5 μG./ML.) WHEN SUBJECTED TO HEATING FOR 4 HOURS AT 100° IN 10 ML. RUBBER CAPPED VIALS. ALSO THE PROTECTION OFFERED BY $FeCl_3$

Initial nicotinamide mg./ml.	$FeCl_3$ mg./ml.	Initial pH	Potency after test		pH after test
			Aneurine mg./ml.	Vitamin B_{12} μg./ml.	
—	—	4·0	11·0	4·5	3·9
—	—	5·5	10·5	4·5	5·1
—	—	6·5	10·2	4·5	5·1
—	—	7·5	8·4	0·05	5·6
—	0·5	7·5	7·8	1·25	5·8
—	—	8·0	7·5	0·5	5·6
—	0·5	8·0	6·2	2·5	6·0
100	—	4·0	10·6	Nil	3·9
100	0·5	4·0	10·6	4·5	3·9
100	—	6·0	11·0	Nil	5·4
100	0·5	6·2	11·0	3·0	5·5
100	—	7·9	7·0	0·5	5·4
100	0·5	8·1	5·1	2·5	6·0

TABLE II

THE IODINE TITRE VALUES OF SOLUTIONS OF ANEURINE, NICOTINAMIDE, ANEURINE AND NICOTINAMIDE, CYSTEINE AND H_2S AND THEIR EFFECT ON STABILITY OF VITAMIN B_{12} (5 μG./ML.) AFTER HEATING AT 100° FOR 4 HOURS

Substance in solution in open test tube	pH adjusted to	Time of heating min.	Iodine solutions ml. of N/100	Vitamin B_{12} potency after elevated temperature test μg./ml. approx.
Aneurine 150 mg./5 ml.	4·5	30	0·1	4·5
,,	4·5	60	0·1	4·5
,,	4·5	120	0·1	4·5
,,	4·5	240	0·1	4·5
,,	6·5	30	0·5	4·5
,,	6·5	60	0·3	4·5
,,	6·5	120	0·2	4·5
,,	6·5	240	0·1	4·5
,,	8·0	15	5·0	Nil
,,	8·0	30	4·0	Nil
,,	8·0	60	2·4	0·5
Nicotinamide 1 g./5 ml.	4·5	60	0·1	4·5
,,	4·5	240	0·1	4·5
,,	8·0	60	0·1	4·5
,,	8·0	240	0·1	4·5
Aneurine 150 mg. and nicotinamide 1 g./5 ml.	4·3	60	1·6	2·0
,,	4·3	120	3·3	Nil
,,	4·3	240	4·2	Nil
Cysteine 2 mg./10 ml.	—	—	3·8	Nil
H_2S solution 0·5 mg./10 ml.	—	—	3·0	Nil

The iodine titre values of solutions of aneurine, nicotinamide and aneurine and nicotinamide and their effect on stability of vitamin B_{12} after heating at 100° for 4 hours, and also the effect of cysteine and H_2S on vitamin B_{12} stability, are shown in Table II.

Results in Table II indicate that aneurine solution heated for 4 hours at pH 4·5 to 6·5 does not produce any appreciable iodine titration value and also does not destroy vitamin B_{12}. Similarly, nicotinamide solution heated for 4 hours at a pH between 4·5 and 8·0 does not produce any

TABLE III

THE IODINE TITRE VALUES AND VITAMIN B_{12} POTENCY OF VITAMIN B-COMPLEX INJECTION* WHEN STORED AT ROOM TEMPERATURE (27–37°) IN 10 ML. VIALS

pH	Time stored months	Iodine in ml. of N/100	Vitamin B_{12} potency
4·5	16	3·0	Nil
4·5	12	2·7	Nil
4·5	8	2·6	Nil
4·5	6	2·4	Nil
4·5	3	2·0	0·5
4·5	Fresh	0·8	4·5

* Composition of B-Complex Injection in mg./10 ml. in rubber capped vials—aneurine 150; riboflavine 15; pyridoxine 50; nicotinamide 1000; panthenol 50; choline HCl 50; benzyl alcohol 150 and also vitamin B_{12} 50 μg.

TABLE IV

CHANGES IN IODINE TITRATION VALUE OF THE "TEST" SOLUTION BEFORE AND AFTER EVAPORATION AND THE EFFECT OF THIS ON THE STABILITY OF VITAMIN B_{12} (5 μG./ML.) HEATED AT 100° FOR 4 HOURS IN COMPARISON WITH THE EFFECTS OF CYSTEINE AND H_2S SOLUTION. ALSO THE PROTECTION OFFERED BY $FeCl_3$

Substance in solution	Potency of aneurine before heating at 100° mg./ml.	Iodine titre ml. N/100	$FeCl_3$ mg./ml.	Potency of aneurine after test mg./ml.	Potency of vitamin B_{12} after test μg./ml.
"Test" solution diluted to 10 ml. with added vitamin B_{12} 50 μg.	10·6	5·0	—	9·2	Nil
"	10·6	5·0	0·5	8·0	4·5
"Test" solution evaporated to dryness. Residue in 10 ml. water with vitamin B_{12} 50 μg.	10·0	0·9	—	7·0	4·5
Cysteine 2 mg. in 10 ml. with vitamin B_{12} 50 μg.	—	3·8	—	—	Nil
"	—	3·8	0·5	—	4·5
H_2S solution, 0·5 mg. in 10 ml. with vitamin B_{12} 50 μg.	—	3·0	—	—	Nil
"	—	3·0	0·5	—	4·5

appreciable iodine titration value nor has any effect on vitamin B_{12}. Cysteine and H_2S solution cause destruction of vitamin B_{12} and give appreciable iodine titre; also aneurine at pH 8 and aneurine and nicotinamide solution at pH 4 to 4·5 when heated, produce significant iodine titration values and destroy vitamin B_{12}. It is suggested that the iodine titre of the solution gives an indication of the presence of reducing substances, perhaps containing an –SH group or H_2S, which are likely to bring about B_{12} destruction.

Table III gives the iodine titre values and vitamin B_{12} potency of a vitamin B-complex injection when stored at room temperature (27–37°) in

10 ml. vials. The results indicate that the iodine titre of the vitamin B-complex injection increases with time of storage, and is paralleled by the progressive destruction of vitamin B_{12}.

Table IV shows the changes in iodine titration value of the "test" solution before and after evaporation and the effect of this on the stability of vitamin B_{12} heated at 100° for 4 hours in comparison with cysteine and H_2S solution, and also the protection offered by $FeCl_3$. The results indicate that on heating aneurine solution at pH 8 for 15 minutes, decomposition products are produced which possess properties which destroy vitamin B_{12}. $FeCl_3$, however, offers protection to the B_{12}. The decomposition products were volatile in nature, for when the solution containing them is evaporated to dryness the residue in solution gives much lower iodine titre and also loses its vitamin B_{12}-destroying property. 0·2 mg./ml. of freshly prepared cysteine solution and 0·05 mg./ml. of H_2S solution also possess vitamin B_{12}-destroying properties, when subjected to elevated temperature, which can be satisfactorily protected by 0·5 mg./ml. of $FeCl_3$.

Discussion

Although the thermal decomposition product or products of aneurine alone under the conditions of the experiment between pH ranges of 4 to 6·5 were not detrimental to vitamin B_{12} stability, the decomposition product or products of aneurine alone at pH 7·5 and 8 did destroy vitamin B_{12} activity. This was prevented significantly by ferric chloride. Heating aneurine solution alone for short intervals at pH 8·0 resulted in significant loss of aneurine, and the solution adjusted to pH 4 to 4·5 for better stability and subjected to heating at 100° for 4 hours after adding vitamin B_{12} can destroy the B_{12} activity (see Table IV). Other workers[8] have shown that vitamin B_{12} can be reduced by a variety of reducing substances, and our studies on the thermal decomposition product or products of aneurine in solution at pH 8·0 revealed the presence of certain titratable reducing substances which from the changes in pH appear to be of an acidic nature. When aneurine and nicotinamide are heated in solution at pH 4 to 4·5, similar titratable reducing substances are formed and both solutions can destroy B_{12} activity. (See Table II.) The protective effect of ferric chloride in the presence of aneurine and nicotinamide is interesting, and the mode of action is perhaps by sparing the vitamin B_{12} from the reductive influence of the decomposition product or products. When a solution containing decomposition product or products derived from heating aneurine solution alone at pH 8·0 is evaporated on water bath to dryness, and the residue taken into solution, its titratable reducing properties are reduced and so also is its ability to destroy B_{12} activity. During the evaporation the solution was found to liberate H_2S as evidenced by blackening of the moist lead acetate paper. Feller and Macek[3] suggested that the thiazole moiety of aneurine is responsible for destruction of vitamin B_{12}, and it has also been shown[4,5] that aneurine in alkali releases an –SH group and with higher concentration of alkali finally decomposes into H_2S[5]. The initial decomposition product, therefore, is perhaps of a thiol nature, which on further heating decomposes into H_2S.

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