SOME PHARMACEUTICAL ASPECTS OF VITAMIN B_{12}

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INTRODUCTION

VITAMIN B_{12} is an erythropoietic substance isolated almost simultaneously from liver by Rickes *et al.*[1] in U.S.A. and by Smith[2,3,4] in this country and subsequently by Ellis, Petrow and Snook[5]. It has since been obtained from the products of metabolism of *Streptomyces griseus*[6], *Streptomyces aureofaciens*[7] and other micro-organisms[6] and from the dung of animals[8].

Vitamin B_{12} is obtainable as an intensely red crystalline substance which can be characterised by its absorption spectrum[5,9], by its ability to act as an essential growth factor in the culture of micro-organisms such as *Lactobacillus lactis* Dorner[10], *L. leichmannii*[11] and *Euglena gracilis*[12], and by its ability to serve as the "animal protein factor" in promoting the growth in chicks[13,8]. It is capable of inducing erythropoiesis in humans on injection in microgramme doses[14,15].

Vitamin B_{12} appears to be one member of a group of naturally occurring chemical substances whose relationship has not yet been completely elucidated. Woodruff and Foster[16] have isolated from fermentation broths and from liver extracts a substance termed vitamin B_{12a} which differs from vitamin B_{12} in its absorption spectrum and its lower biological activity. Vitamin B_{12a} was first described by Kaczka *et al.*[17], who obtained it on catalytic hydrogenation of crystalline vitamin B_{12}. Brockman *et al.*[18], however, found that under similar conditions vitamin B_{12} gave a different red crystalline substance which had properties identical with another member of the group named vitamin B_{12b}, which had previously been isolated by Pierce *et al.*[7], from cultures of *Streptomyces aureofaciens* and has since been extracted from liver[20]. A fourth member of the group[21] previously referred to as vitamin B_{12x} has recently been renamed vitamin B_{12c}.

Vitamin B_{12} alone, of the four members of the group, appears so far to have been studied intensively with a view to elucidation of its chemical structure, though recently a report has been published[21] on vitamin B_{12c} which appears to be more highly oxygenated than vitamin B_{12}. Vitamin B_{12} has a molecular weight of about 1400 and contains per molecule one atom of cobalt[9] and one atom of phosphorus[22], its analysis[9] indicating a formula in the range $C_{61-64}H_{86-92}N_{14}O_{13}P$ Co. It is somewhat hygroscopic and is soluble in water to the extent of 1 per cent. or more. It is optically active having $[\alpha]_{6563}^{23^{\circ}C.}$ approximating -60°, though accurate determinations were difficult due to the necessity of working with dilute solutions because of the intensely red colour of its aqueous solutions[9]. On fusion with alkali it forms products which react with *p*-dimethyl-

aminobenzaldehyde, indicative of the presence of certain cyclic 5-membered nitrogen-containing compounds such as pyrroles[9]. Hydrolysis with hydrochloric acid releases a ninhydrin-reacting fragment[5], phosphate[22], and a 5:6-dimethyl-benziminazole residue[23,24]. A *l*-D-ribofuranosido-5:6-dimethylbenziminazole has also been obtained[25] on degradation of vitamin B_{12}. Ammonia has been shown by Ellis and Petrow[26] to be liberated during acid or alkaline hydrolysis of vitamin B_{12}.

The widespread interest that has arisen in vitamin B_{12} both intrinsically and in the search for reliable methods for the quantitative determination of the amounts present in concentrates and other materials appears to be adequate justification for considering the determination of its stability a matter of importance. It is the purpose of this communication to report our experimental work on the stability of vitamin B_{12} in such a variety of conditions as would enable adequate appraisal of its pharmaceutical aspects to become possible. Any studies of the stability of a chemical substance, however, obviously first involve consideration of methods by means of which changes in the physical, chemical or biological properties of the substance may be detected.

Methods of Determination of Vitamin B_{12}

Physical. The physical property most widely used is the absorption spectrum of the aqueous solution. Ellis, Petrow and Snook[5] found that vitamin B_{12} exhibited a characteristic absorption spectrum with maxima at 278, 361 and 548 mμ, their data indicating values for $E_{1\,cm.}^{1\,per\,cent.}$ of 108 at 278 mμ, 183 at 361 mμ and 57 at 548 mμ. Brink *et al.*[9] subsequently reported $E_{1\,cm.}^{1\,per\,cent.}$ 115 at 278 mμ, 204 at 361 mμ and 63 at 548 mμ, and recently the U.S.P. XIII Third Sheet Supplement has adopted the value of $E_{1\,cm.}^{1\,per\,cent.}$ 207 at 361 mμ for the pure anhydrous vitamin B_{12}. We ourselves find values for $E_{1\,cm.}^{1\,per\,cent.}$ at 361 mμ slightly over 200 calculated with reference to the substance dried to constant weight at 90°C. *in vacuo*.

In our experience the determination of the absorption spectrum is of the greatest value in assessing the purity of vitamin B_{12}. Figure 1 shows the type of spectra obtained with impure samples, the curves being adjusted so that they relate to the same total amount of vitamin B_{12} in each case. Curve A represents the spectrum of the solution prepared from a concentrate containing about 0·6 per cent. of vitamin B_{12}; it shows no peak at 278, 361 or 548 mμ, since the presence of relatively highly absorbing impurities masks the characteristic vitamin B_{12} spectrum, leaving a slight shoulder only at *ca.* 520 to 550 mμ. A solid concentrate containing approximately 2 per cent. of vitamin B_{12} (Curve B) begins to show the peaks at 361 and 548 mμ, while for a solid concentrate containing 5 per cent. of vitamin B_{12} (Curve C) these peaks are more apparent and the divided peak can be seen in the visible region. It is necessary to have a much purer sample before the peak at 278 mμ can be realised, and even with samples having a purity of approximately 10 per cent. the value of $E_{max.}$ at 278 mμ is relatively high when compared with the

curve for pure vitamin B_{12}; the peaks at 361 and 548 mμ, however, approximate to those shown by pure vitamin B_{12}. Thus a suitable criterion exists for establishment of the purity of a sample of vitamin B_{12} in that the three peaks at 278, 361 and 548 mμ should be in a definite ratio and the Third U.S.P. XIII Sheet Supplement has taken advantage of this property in fixing a limiting range for the ratios

$E^{1\text{ per cent.}}_{1\text{ cm.}}$ 361 mμ./$E^{1\text{ per cent.}}_{1\text{ cm.}}$ 278 mμ. of 1·62 to 1·88, and for

$E^{1\text{ per cent.}}_{1\text{ cm.}}$ 361 mμ./$E^{1\text{ per cent.}}_{1\text{ cm.}}$ 548 mμ. of 2·83 to 3·45

This U.S.P. Supplement also fixes limits for wavelength values, allowing maxima within ± 1 mμ. at 278 and 361 mμ., and within ± 4 mμ. at 548 mμ., allowance being made in the latter case for the broad maximum showing in the visible region.

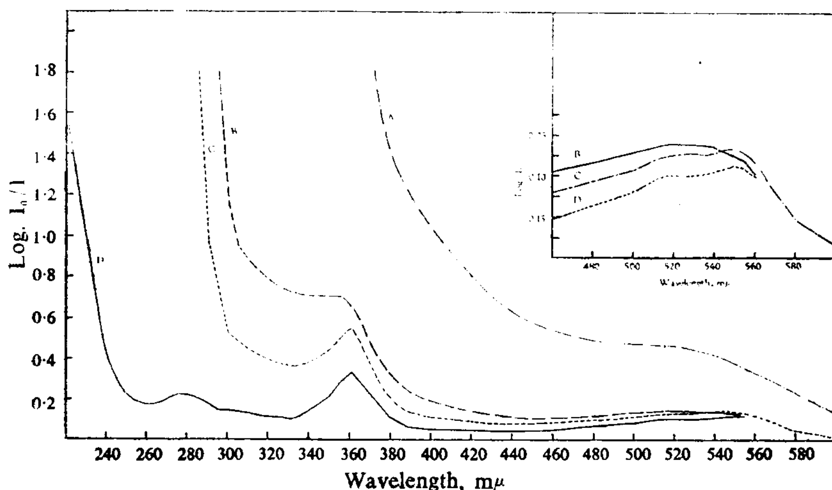

FIG. 1. Absorption spectra of vitamin B_{12} of varying purity. A, containing approximately 0·6 per cent.; B, containing approximately 2 per cent.; C, containing approximately 5 per cent.; D, pure vitamin B_{12}. The curves are adjusted so that they relate to the same total amount of vitamin B_{12} in each case.

Chemical. Although chemical methods of determination of vitamin B_{12} based upon the determination of cobalt are obviously possible, they are of more value in following purification from concentrates than in studies of stability as means are then required to differentiate between cobalt-containing decomposition fragments and unchanged vitamin B_{12}. Assays based on the microchemical determination of cobalt have proved useful in our laboratories in following enrichment during purification. The method used is based upon hydrolysis of the material and determination of the cobalt content of a butyl alcohol extract of the hydrolysate.

Fantes *et al.*[27] have based an assay on the fact, established by Ellis, Petrow and Snook[5], that the acidic fragment formed on acid hydrolysis of vitamin B_{12} can be esterified with methyl alcohol, although octyl alcohol was finally chosen as the esterifying agent. The concentration

of coloured ester formed was then determined photo-electrically. While in our hands the method has proved useful for examination of concentrates relatively rich in vitamin B_{12}, the high colour obtained with a blank determination precludes its use for estimating the vitamin B_{12} content of materials less rich in the vitamin. Furthermore it is insufficiently precise for stability studies.

Biological. As already mentioned, vitamin B_{12} possesses the ability to act as an essential growth factor for several micro-organisms and for chicks. Many microbiological assays have been devised and widely investigated. "Tube assays" have been described, for example, by Shorb and Briggs[28], Caswell[29], Lees and Emery[30], Shaw[31] and Hoffman, Jukes *et al.*[11], the preferred organisms being *L. lactis* Dorner or *L. leichmannii.* Most workers are, however, agreed that these assays are capricious in operation and the results obtained are found to vary from a large number of factors as emphasised by Shorb[28] and by Hartley[32]. The cup-plate method of assay, introduced by Cuthbertson[33], is the most useful for routine operation. In our hands this method based on the technique and medium described by Foster, Lally and Woodruff[34], and using *L. lactis* Dorner A.T.C.C. 10697, has proved to be the most capable of giving repeatable results, although the figures obtained show considerable variations between different laboratories and for some types of preparation, notably concentrates from fermentation broth, the method appears to yield erratic results.

Assays based upon measurement of the growth of chicks appear to show higher results than those obtained in corresponding assays against micro-organisms[19,35].

STABILITY OF VITAMIN B_{12}

It can readily be shown by spectrophotometric examinations that an aqueous solution of vitamin B_{12}, even when adjusted to a reaction in the range of greatest stability, namely, pH 4·0 to 7·0, suffers considerable change in its absorption spectrum within a few days on exposure to sunlight. On the other hand, in our experience, such solutions undergo no change, as shown by absorption spectra, on exposure during a week to diffuse daylight such as is encountered in a normal room or laboratory and may therefore be regarded as stable during the type of exposure occurring in normal handling for analysis, filling or packing.

The relatively low concentrations of vitamin B_{12} used for injection appeared to render it desirable to consider the stability of the pure material in solutions made isotonic with sodium chloride. It was found that no change occurred in the absorption spectra of solutions of pure vitamin B_{12} in concentrations of 10 to 20 microgrammes per ml. in injection of sodium chloride during 12 months' storage in sealed ampoules packed in cardboard boxes and maintained at normal room temperature.

Although it appears to be generally considered that vitamin B_{12} is stable in aqueous solutions at pH 4·0 to 7·0, decomposition occurring in

obtainable by taking the mean of a relatively large number of replicate microbiological assays. The differences between the assays on the original solutions and on those of the solutions after standing for several months are, in our opinion, not significant. On the other hand, the microbiological results for the autoclaved solutions were obtained side by side with those for the original, and in our opinion the slight fall in activity (approximately 10 per cent. at both *p*H 4·0 and *p*H 7·0) is significant, confirmed as it is by a loss detected spectrophotometrically.

TABLE I

STABILITY OF VITAMIN B_{12} AT *p*H 4·0 AND *p*H 7·0

	Spectrophotometric assay ex $E_{361m\mu}$		Microbiological assay cup-plate method	
	Vitamin B_{12} μg./ml.	Loss of Vitamin B_{12} per cent.	Vitamin B_{12} μg./ml.	Loss of Vitamin B_{12} per cent.
Original solution at *p*H 7·0	53·1	—	54·0	—
After standing for 6 months in the dark	52·1	—	52·9	—
After heating for 30 minutes at 115°C.	50·7	4·6	48·4	10·4
Original solution at *p*H 4·0	45·9	—	47·0	—
After standing for 6 months in the dark	46·9	—	45·9	—
After heating for 30 minutes at 115°C.	44·1	4·0	42·6	9·4

The stability results for vitamin B_{12} given in Table I appear to apply only to solutions of the vitamin having an order of purity of about 90 to 95 per cent. Solutions prepared from less pure samples of crystalline material or from solid concentrates containing the vitamin appear to be much less stable, the degree of instability varying with the nature of the impurities present. Table II shows results obtained at *p*H 5·0 with a solution prepared from a sample of vitamin B_{12} approximately 70 per cent. pure. In this case the loss on autoclaving is greater than with pure

TABLE II

STABILITY OF A SAMPLE OF IMPURE VITAMIN B_{12} (CALCULATED EX $E_{1\ cm.}^{1\ per\ cent.}$ 361 Mμ, APPROXIMATELY 70 PER CENT. PURE)

	Spectrophotometric assay ex $E_{361m\mu}$		Microbiological assay cup-plate method	
	Vitamin B_{12} μg./ml.	Loss of Vitamin B_{12} per cent.	Vitamin B_{12} μg./ml.	Loss of Vitamin B_{12} per cent.
Original solution at *p*H 5·0	20·9	—	20·9	—
After heating for 30 minutes at 115°C.	18·1	13·4	11·3	45·9
After heating for a further 30 minutes at 115°C.	16·0	23·4	7·8	62·7

vitamin B_{12}, as shown by the spectrophotometric results, and considerably greater, amounting to about 46 per cent. after 30 minutes' autoclaving, as shown by the microbiological results.

In alkaline solutions vitamin B_{12} suffers rapid decomposition on heating and is much less stable than in slightly acid solutions. Figures 3 and 4 and Tables III and IV show the results obtained at *p*H 9·0 and 12·0. The nature of the decomposition occurring in alkaline solution evidently differs from that in acid solution, in that with loss of activity there occurs loss of the pink colour of the solution.

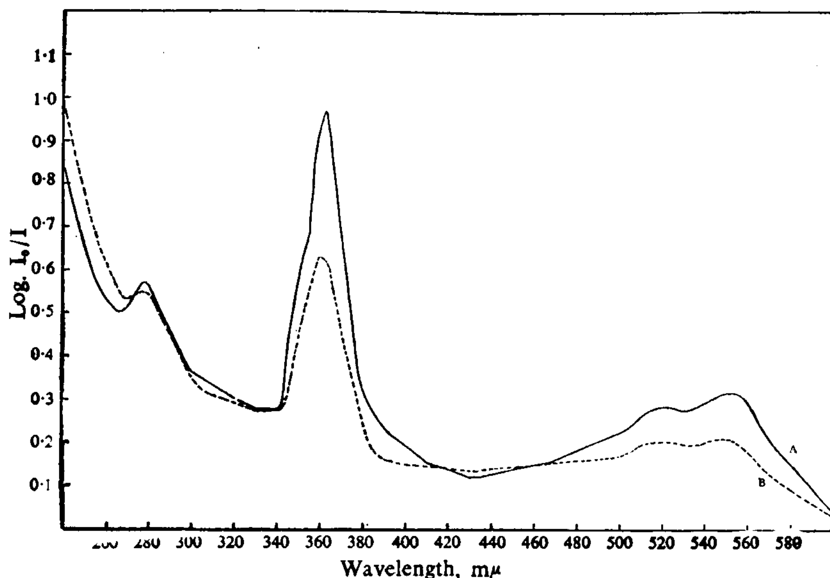

FIG. 3. Absorption spectra of vitamin B_{12}. A, at *p*H 9·0; B, at *p*H 9·0 after heating for 30 minutes at 115°C.

TABLE III

STABILITY OF VITAMIN B_{12} AT *p*H 9·0

	Spectrophotometric assay ex $E_{361 m\mu}$		Microbiological assay cup-plate method	
	Vitamin B_{12} μg./ml.	Loss of Vitamin B_{12} per cent.	Vitamin B_{12} μg./ml.	Loss of Vitamin B_{12} per cent.
Original solution	46·9	—	48·8	—
After standing for 7 days in the dark...	46·9	—	48·8	—
After heating for 30 minutes at 115°C.	30·4	35·2	0·4	99·2

At *p*H 2·0, vitamin B_{12} was less stable than at *p*H 4·0, although the stability is relatively much greater than in an alkaline medium. Figure 5 and Table V show the stability results obtained at *p*H 2·0. Losses found

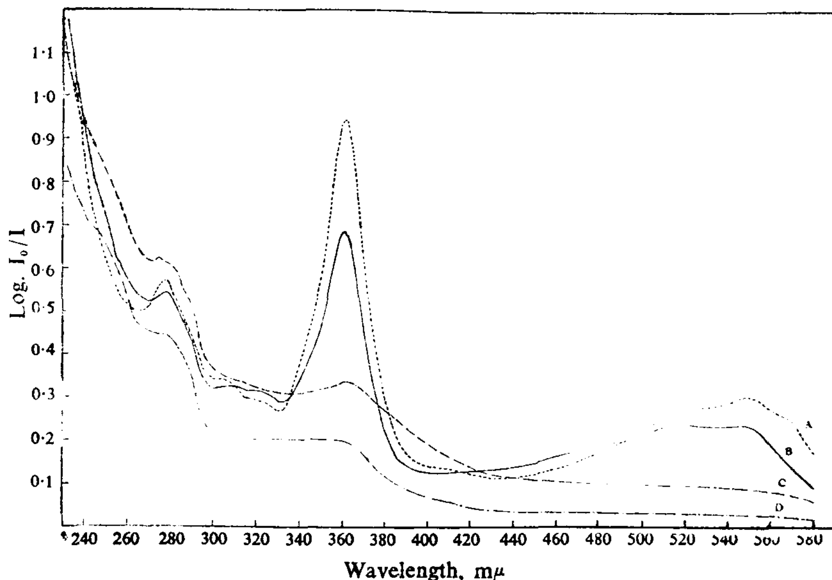

FIG. 4. Absorption spectra of vitamin B_{12}. A, at pH 12·0 determined immediately after adjustment; B, at pH 12·0, after standing in the dark for 24 hours; C, at pH 12·0, after heating for 30 minutes at 115°C.; D, at pH 12·0, after heating for 60 minutes at 115°C.

TABLE IV

STABILITY OF VITAMIN B_{12} AT pH 12·0

	Spectrophotometric assay ex E_{361} mμ		Microbiological assay cup-plate method	
	Vitamin B_{12} μg./ml.	Loss of Vitamin B_{12} per cent.	Vitamin B_{12} μg./ml.	Loss of Vitamin B_{12} per cent.
Original solution	45·7	—	—	—
After standing in the dark for 24 hours	33·1	27·6	2·6	—
After heating for 30 minutes at 115°C.	16·2	64·5	No response	100·0
After heating for 60 minutes at 115°C.	9·7	78·8	No response	100·0

microbiologically were again appreciably greater than spectrophotometric losses. The spectrum obtained after prolonged autoclaving is characteristic of the acid decomposition products obtained; among them being substituted benziminazoles[23,24].

Sterilisation of vitamin B_{12} solutions. In view of the significant, though relatively slight, decomposition of vitamin B_{12} occurring on autoclaving even in the pH range 4·0 to 7·0 and in view of the relatively larger loss under these conditions shown microbiologically, it is our opinion that the use of heat should be avoided in the sterilisation of solutions

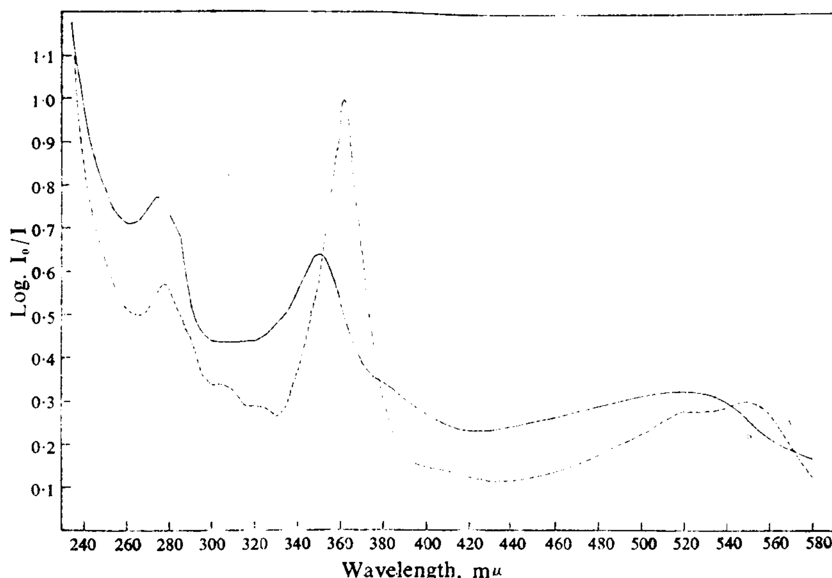

FIG. 5. Absorption spectra of vitamin B_{12}. A. at pH 2·0; B, at pH 2·0 after heating for 6 hours at 115°C.

TABLE V

STABILITY OF VITAMIN B_{12} AT pH 2·0

	Spectrophotometric assay ex $E_{361 m\mu}$		Microbiological assay cup-plate method	
	Vitamin B_{12} μg./ml.	Loss of Vitamin B_{12} per cent.	Vitamin B_{12} μg./ml.	Loss of Vitamin B_{12} per cent.
Original solution	48·3	—	47·4	—
After standing for several days in the dark	48·3	—	47·4	—
After heating for 30 minutes at 115°C.	39·9	17·4	24·3	48·7
After heating for 6 hours at 115°C. ...	30·9*	36·0	No response	100·0

* Maximum at 360 mμ.

of vitamin B_{12} for injection. Although it is recognised that the microbiological assay of vitamin B_{12} is notoriously unreliable, on the basis of many results we consider the loss on autoclaving to be significant.

Stability of vitamin B_{12} in the presence of phenolic bacteriostatics. In view of the conclusion reached that vitamin B_{12} solutions should not be sterilised by autoclaving and the possibility of use in multi-dose containers, the stability of vitamin B_{12} solution in the presence of phenols was accordingly studied. Spectrophotometrically, although the peak at 278 mμ could not be realised owing to the presence of the phenol, there was no change after standing for 2 months in the presence of 0·5 per

cent. of phenol. Table VI shows the microbiological results, which also indicated that there was no loss of potency. Similar results were obtained in the presence of 0·3 per cent. of cresol B.P. Chlorocresol 0·2 per cent. has also been found satisfactory.

TABLE VI

MICROBIOLOGICAL RESULTS OBTAINED FOR VITAMIN B_{12} SOLUTIONS IN THE PRESENCE OF 0·5 PER CENT OF PHENOL

Time	Vitamin B_{12} μg./ml.	Average μg./ml.
1 week ...	13·9	—
	12·8	—
	12·0	12·9
2 weeks...	12·3	—
	12·8	—
	12·2	12·4
6 weeks...	12·2	—
	12·8	—
	12·2	12·4

In the light of the above results we consider that sterilisation of solutions of vitamin B_{12} for injection should be effected by filtration. In cases where the presence of a bacteriostatic is required, 0·5 per cent. of phenol or 0·3 per cent. of cresol B.P. may be used. The *p*H of the final solution should preferably be between 4·5 and 6·5.

Stability to other substances. In addition to its instability in relatively acid or alkaline solution, vitamin B_{12} has been shown to be unstable to hydrogen peroxide[27], sodium bisulphite[36], cysteine hydrochloride, hydroquinone[37] and thioglycollic acid[38]. In general, therefore, in pharmaceutical practice it should be regarded as incompatible with oxidising or reducing agents. For example, vitamin B_{12} is not stable in the presence of ascorbic acid, a solution containing 10 microgrammes of vitamin B_{12} and 1 mg. ascorbic acid per ml. becoming colourless within 24 hours and being then inactive to *L. lactis* Dorner. This result is in conformity with those reported by Gakenheimer and Feller[39].

SUMMARY AND CONCLUSIONS

1. The absorption spectrum is a convenient method of establishing the purity of vitamin B_{12} the maxima at 278, 361 and 548 mμ exhibiting definite ratios for which limits can be fixed. Under these conditions the concentration of vitamin B_{12} in solutions containing substantially pure material can be inferred from determination of the absorption value at 361 mμ.

2. While aqueous solutions of vitamin B_{12} undergo variation in absorption spectra within a few days on exposure to sunlight, such changes do not occur during exposure to the diffuse daylight of a normal room for one week. They can, therefore, be regarded as stable during normal handling for analysis, filling or packing.

3. Aqueous solutions of vitamin B_{12} undergo decomposition, especially on heating, at *p*H 2·0 and more rapidly at *p*H 9·0 and *p*H 12·0.

4. Aqueous solutions of vitamin B_{12} are stable at *p*H 4·0 and 7·0 at normal temperatures, but undergo slight but significant decomposition during autoclaving at 115°C. for 30 minutes. Less pure samples of vitamin B_{12} suffer greater loss on autoclaving in solution. It is, therefore, recommended that the use of heat should be avoided in the sterilisation of solutions of vitamin B_{12} for injection, and that the *p*H of the final solution should preferably be between 4·5 and 6·5.

5. No evidence was found, either spectrophotometrically or microbiologically, of deterioration of pure vitamin B_{12} on storage for 2 months in 0·5 per cent. phenol solution or 0·3 per cent. cresol solution. It is, therefore, recommended that solutions of vitamin B_{12} for injection should be sterilised by filtration, a suitable bacteriostatic being added if necessary.

6. Vitamin B_{12} should be regarded as unstable in relatively acid or alkaline solutions and in the presence of oxidising or reducing substances.

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DISCUSSION

An abstract of the paper was read by Dr. Hartley.

The CHAIRMAN (Mr. A. D. Powell) said that the paper indicated the rapidity with which discoveries were being made; when the abstract was written only vitamins B_{12a}, B_{12b} and B_{12c} were known, but Dr. Hartley now referred to a vitamin B_{12d}. The paper illustrated the great use which could be made of absorption spectrometry, and the authors were to be congratulated on the practical data on the preparation of stable solutions of this vitamin.

DR. G. E. FOSTER (Dartford) asked if the authors had done any work on the adsorption of vitamin B_{12} on the filter during sterilisation.

DR. W. F. J. CUTHBERTSON (Greenford) asked if any significant *p*H changes took place during Seitz filtration and, if so, to what extent, and what steps had been taken to cope with them. Had any stability experiments been done at elevated temperatures corresponding to tropical conditions?

PROFESSOR H. BERRY (London) asked if any decomposition measurements had been made at 100°C. As the vitamin was compatible with 0·2 per cent. of chlorocresol it should lend itself to sterilisation by heating with a bactericide. Did the authors suggest buffering the solution and, if so, what buffer would they use?

DR. F. HARTLEY replied that they had examined the adsorption of vitamin B_{12} on the filter. As the final solution had a concentration of the order of micrograms per ml. it was possible to pass a concentrated solution through the filter and to remove the adsorbed material by washing with a large amount of diluent. The changes in the *p*H values were not significant. They aimed to prepare a solution of *p*H between 4·5 and 6·5 which gave the maximum stability. All the experiments had been carried out at about 25°C. The solution of vitamin B_{12} was made isotonic by means of sodium chloride, and they had not found it necessary to use any special buffer. Difficulties had been reported in investigations on heating at 100°C. with a bactericide due to trace impurities in the phenolic bacteriostatics. As there was some decomposition caused by heat there was probably some decomposition at 100°C. and possibly even at 25°C. The experiments were being continued.