CYANOCOBALAMIN AND HYDROXOCOBALAMIN IN VITAMIN B_{12} INJECTIONS

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Hydroxocobalamin, also termed vitamin B_{12b}, has been isolated from liver[1,2,3] and from *Streptomyces* cultures[3], the two main commercial sources of vitamin B_{12}, in which it apparently may form a considerable proportion of the total vitamin B_{12} potency. Its physiological activity, microbiological and clinical, appears to be the same as that of cyanocobalamin[4]. It is, however, much less stable to ascorbic acid[5], and to certain other biological agents, than cyanocobalamin. Vitamin B_{12} has been officially defined as cyanocobalamin, and characterised by spectrophotometric data which clearly distinguish between the two pure substances. Such data are applied, however, not only to crystalline cyanocobalamin but also to solutions of it used as injections. In such solutions cyanocobalamin can be converted to hydroxocobalamin by exposure to light under suitable conditions[6]. Therefore vitamin B_{12} injections, even though made originally from pure cyanocobalamin, may contain cyanocobalamin and hydroxocobalamin in varying proportions, according to storage conditions.

Our interest in this problem arose during an investigation[7] into the cyanide and thiocyanate metabolism in man, in which injections of vitamin B_{12} were being administered, and it was desired to know how much cyanocobalamin these were providing. A method was needed for the determination of cyanocobalamin, mixed with different proportions of hydroxocobalamin, in the amounts found in vitamin B_{12} injections. Microbiological methods are rather tedious and usually do not differentiate between cyanocobalamin and hydroxocobalamin in such mixtures. Such differentiation can be effected by applying a suitable microbiological method to the mixtures before and after destruction of the hydroxocobalamin with ascorbic acid under suitable conditions[8], but this is not effective in the presence of iron and therefore is not applicable to liver extracts. It has been used by two of us (F.W.N. and S.J.G.F.) in the present investigation as an independent check on a limited number of samples. A less tedious method is based on phasic separation with benzyl alcohol[9], the cyanocobalamin being determined spectrophotometrically after removal of the hydroxocobalamin. This method, however, requires more material than might be available if only one or two ampoules of low potency were being examined. Therefore we made a spectroscopic study[10] of the effect of light on vitamin B_{12}, and the spectrophotometric determination of cyanocobalamin mixed with known proportions of the irradiation product

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containing hydroxocobalamin thus obtained. This provided the basis for the present investigation.

Materials and Methods

Materials

Several hundred vitamin B_{12} ampoules from 66 different batches made by 7 different manufacturers were obtained from hospital pharmacists in 18 centres in the United Kingdom, who kindly provided also details of the storage conditions. Many of the ampoules contained rather more than 1 ml. (e.g., 1·1 to 1·2 ml.), but the results on these are quoted as the content per ml., on the assumption that only 1 ml. would be injected.

Vitamin B_{12} satisfying the U.S.P. XIV requirements was obtained from 3 different batches purchased in the open market, and dried at 105° C. *in vacuo* over phosphorus pentoxide to constant weight before spectrophotometric determinations were made. Similar determinations were made on hydroxocobalamin prepared by illumination of this cyanocobalamin, also on a specimen of crystalline hydroxocobalamin kindly provided by Dr. J. G. Heathcote of the Distillers Co. (Biochemicals) Ltd. and used as received.

Spectrophotometric data were obtained on a Beckman DU photoelectric spectrophotometer, the method of Morton and Stubbs[11] being used for calculation of the actual cyanocobalamin content. A 1 cm. cell was used throughout, as specified in the B.P. 1953. This, however, gave rather low readings with some of the weaker injections. We therefore recommend the procedure of B.P.C. Supplement 1952, which permits the use of cells of different lengths. *p*H measurements were made on a Cambridge portable *p*H meter.

Microbiological assays

(a) *Cyanocobalamin plus hydroxocobalamin*

The method of Cooperman, Drucker and Tabenkin[12] was used. This employs *Lactobacillus lactis* Dorner (ATCC 8000), and the only modification introduced by us lay in the preparation of the medium for inoculum which was prepared according to Hendlin and Soars[13]. Assays were carried out at two levels only (in view of the small amount of material available) but in complete duplicate (different inocula, sets of tubes, etc.). The response was measured in terms of 0·05N sodium hydroxide and smooth curves were drawn with mechanical aid. Results were calculated by direct reference from sample to standard curve by inspection. There is a linear relation between response and logarithm of dose. This was used in one or two instances, but gave results so closely in agreement with those obtained by direct reference that the more lengthy calculation was abandoned.

(b) *Cyanocobalamin only*

Hydroxocobalamin was destroyed by ascorbic acid[8] and residual cyanocobalamin determined microbiologically as before.

In these microbiological assays the dilutions used in first assays on each

sample were all 1 in 10^6. In samples 2 and 3 the content of cyanocobalamin was so much reduced, that less diluted solutions were used in the 2nd assays (see footnote to Table II).

RESULTS

Microbiological and spectrophotometric results for cyanocobalamin plus hydroxocobalamin

The microbiological method was first tested on 3 solutions of known content, prepared from (1) crystalline cyanocobalamin in distilled water, (2) crystalline cyanocobalamin converted to hydroxocobalamin by illumination, (3) mixture of equal volumes of (1) and (2).

TABLE I

MICROBIOLOGICAL AND SPECTROPHOTOMETRIC ASSAYS OF CYANOCOBALAMIN PLUS HYDROXOCOBALAMIN IN VITAMIN B_{12} SOLUTIONS

Sample	Cyanocobalamin + hydroxocobalamin (μg./ml.)			
	Microbiological			
	Mean results in		Final mean	Spectrophotometric*
	1st assay	2nd assay		
Solutions:				
1	105	90	98	90
2	104	86	95	90
3	98	90	94	90
Injections:				
4	12·6	10·9	11·8	20·3
5	19·8	19·6	19·7	26·3
6	18·0	19·0	18·5	30·2
7	23·9	22·3	23·1	21·8
8	22·2	22·3	22·3	23·7
9	17·7	21·0	19·3	26·4
10	25·2	24·9	25·1	31·4
11	16·2	16·7	16·5	17·4

* In calculating these results allowance has been made for $E\,\frac{1\ \text{per cent.}}{1\ \text{cm.}}$ at 361 mμ for hydroxocobalamin being lower than that for cyanocobalamin, as indicated in Figure 1.

The difference in results between the 1st and 2nd assays (Table I) must be ascribed to experimental error. The coefficient of variation observed in these assays was, however, never greater than ±10 per cent. and often much less. The mean microbiological results in these assays ranged from 104 to 109, and averaged 106 as a percentage of the mean spectrophotometric results. The two methods were next compared on samples of 8 different batches of injections. The mean microbiological results ranged from 58 to 106 and averaged 80 per cent. of the mean spectrophotometric results. However, the former had been determined several weeks after the latter, and may have been lower partly because of losses during storage of the diluted injections even although this was in complete darkness in the refrigerator.

Microbiological and spectrophotometric results for cyanocobalamin only

On the above 3 solutions excellent agreement was again obtained between the results by the two methods (see Table II). On the 8 injections

the mean microbiological results were again lower than the mean spectrophotometric results, ranging from 71 to 97 and averaging 86 per cent. of the latter.

Proportion of cyanocobalamin in total cobalamins

Determination of this proportion by the microbiological method gave mean results ranging from 76 to 115 and averaging 95 per cent. of the mean spectrophotometric results (see Table III).

The comparison of the two methods on this limited number of samples having indicated a reasonable degree of agreement, the spectrophotometric method was applied to a much larger number of samples, providing data for a comprehensive survey which would have taken very much longer to complete by microbiological methods.

TABLE II

ASSAYS OF CYANOCOBALAMIN IN VITAMIN B_{12} SOLUTIONS BY MICROBIOLOGICAL METHOD AFTER DESTROYING HYDROXOCOBALAMIN WITH ASCORBIC ACID AND BY SPECTROPHOTOMETRIC METHOD

	Cyanocobalamin μg./ml.			
	Microbiological			
	Mean results in		Final mean	Spectrophotometric
Sample	1st assay	2nd assay		
Solutions:				
1	89	100	94·5	90
2	nil	(*a*) 0·03 (*b*) 0·03	0·03	nil
3	43	49	46	45
Injections:				
4	10·4	10·3	10·4	14·6
5	16·9	18·3	17·6	18·5
6	11·1	12·3	11·7	15·5
7	19·6	20·4	20·0	21·8
8	20·4	21·9	21·2	23·7
9	22·6	21·9	22·3	26·4
10	26·4	27·5	27·0	31·4
11	17·1	15·8	16·5	17·4

Dilutions used in microbiological assay: $1:10^6$ in all cases except
2nd assay Sample 2 (*a*)—1:2500
" " " 2 (*b*)—1:1250
" " " 3 —1:667,000

TABLE III

CYANOCOBALAMIN CONTENT AS PERCENTAGE OF TOTAL COBALAMIN CONTENT* IN SOLUTIONS AND INJECTIONS

Sample	Microbiological	Spectrophotometric	Spectrophotometric as percentage of microbiological
1	97	100	103
2	49	50	102
3	nil	nil	100
4	89	68	76
5	89	71	80
6	63	52	83
7	87	100	115
8	95	100	105
9	115	100	87
10	108	100	93
11	100	100	100
	Mean of all		95·0

* i.e., cyanocobalamin plus hydroxocobalamin as in Table I.

Spectrophotometric assay of vitamin B_{12} injections by official methods

U.S.P. XIV, B.P.C. Supplement 1952 and B.P. 1953 all assay these injections by their extinction at 361 mμ. From the extinction readings is calculated the cyanocobalamin content, using 207 as $E^{1\,\text{per cent.}}_{1\,\text{cm.}}$. However, hydroxocobalamin also produces a definite extinction at 361 mμ (see Fig. 1). Hence the results obtained by these official methods, although purporting to measure only the cyanocobalamin, to a certain degree also measure any hydroxocobalamin which may be present. The extinction at 361 mμ produced by a given concentration of hydroxocobalamin is only about half that given by the same concentration of cyanocobalamin. We have determined the actual cyanocobalamin content in its mixtures with hydroxocobalamin by means of Morton and Stubbs[11] method, taking readings at 355, 361 and 366 mμ for this purpose. Our data indicate that E 355 mμ for cyanocobalamin $= E$ 366 mμ, and 355 mμ is an isosbestic point in such mixtures[10]. The approximate hydrocobalamin content can be calculated by doubling the difference between the cyanocobalamin content, determined as described above, and the official "vitamin B_{12} content" calculated on E 361 mμ.

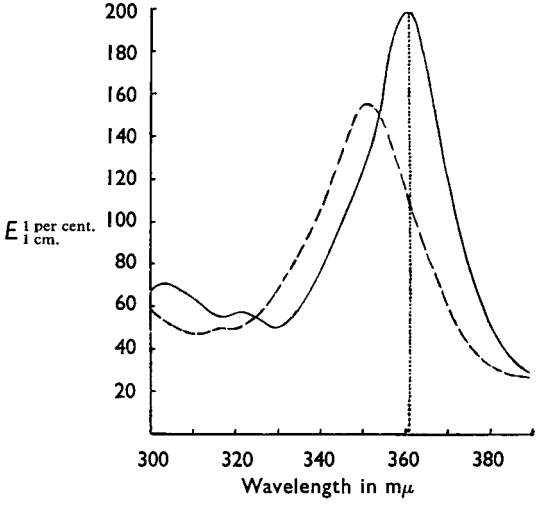

FIG. 1. Absorption curves for cyanocobalamin and hydroxocobalamin. Dotted line indicates wavelength at which extinction is measured in official spectrophotometric assays.

—— Cyanocobalamin.
– – Hydroxocobalamin.

Official identification tests for vitamin B_{12}

U.S.P. XIV specifies a maximum extinction at 361 ± 1 mμ for vitamin B_{12} injections. B.P. 1953 requires extinctions to be taken at 361 mμ for assay of these injections, without specifying that this is a maximum. However, in the B.P. monograph for crystalline vitamin B_{12} the maximum is required to lie between 360 and 362 mμ, giving the same tolerance as in U.S.P. XIV.

Now, in mixtures of cyanocobalamin and hydroxocobalamin, as the proportion of the latter increases the position of the maximum gradually shifts from 361 to 351 mμ. Figure 2 summarises data taken from a number of our experiments[7] on such mixtures, showing that the maximum

does not drop definitely below 360 mμ until the cyanocobalamin content falls to about 75 per cent. of the total cobalamin content.

U.S.P. XIV also specifies a maximum at 278 ± 1 mμ for vitamin B_{12} injections. The presence of bacteriostatics in such injections may interfere with readings at 278 mμ, or even with those at 361 mμ, and B.P. 1953 makes provision for this by stating that if the presence of a bacteriostatic causes an error exceeding 2 per cent. in the assay based on 361 mμ, this assay is not to be used. Presumably readings would then be taken at 548 mμ, as in U.S.P. XIV and B.P.C. Supplement 1952. The tolerances allowed in the actual position of the peak would again permit the presence of a considerable proportion of hydroxocobalamin.

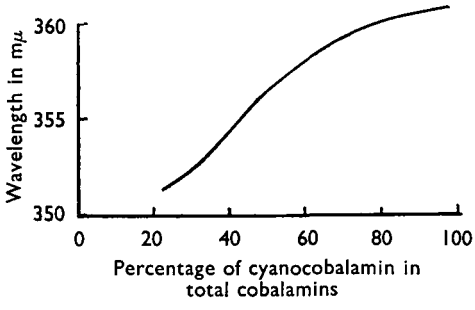

FIG. 2. Shift in 361 mμ max. of cyanocobalamin when mixed with different proportion of hydroxycobalamin.

U.S.P. XIV and B.P.C. Supplement 1952 also give as an identification test for vitamin B_{12} injections a specified range within which the ratio E 361 mμ/E 548 mμ must lie. A similar range is specified in the B.P. 1953 monograph for crystalline vitamin B_{12}. This test, however, does not exclude the presence of hydroxocobalamin in any proportion up to 100 per cent.

Since none of these identification tests satisfactorily excludes hydroxocobalamin, the vitamin B_{12} injections in general use may contain considerable proportions of this cobalamin in the mixture of total cobalamins, although still complying with the official requirements.

"Vitamin B_{12} content" of vitamin B_{12} injections by official methods

These methods purport to measure the content of anhydrous cyanocobalamin but in fact they determine the cyanocobalamin plus about half the hydroxocobalamin. This is the official "vitamin B_{12} content." This content is required in U.S.P. XIV to lie between 90 and 115 per cent., in B.P.C. Supplement 1952 between 90 and 110 per cent., and in B.P. 1953 between 79·5 and 96·5 per cent. of the claim.

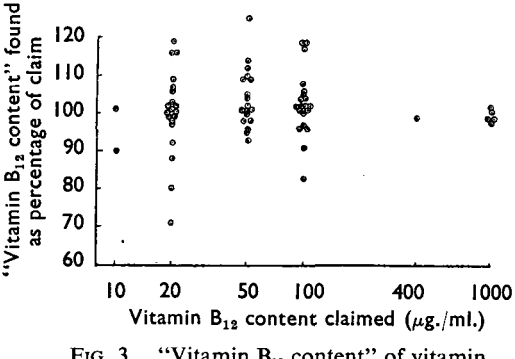

FIG. 3. "Vitamin B_{12} content" of vitamin B_{12} injections as determined by the official spectrophotometric method.

Our results on 66 batches of injections showed their official

"vitamin B_{12} content" to range from 70 to 125 and average 100·3 per cent. of the claim. As will be seen from Figure 3 the variations were similar over the whole range of strengths of the injections, from 10 to 1000 μg./ml. (plotted on logarithmic scale to save space). Hence it seems justifiable to combine all the results in a distribution diagram, as in Figure 4. This shows

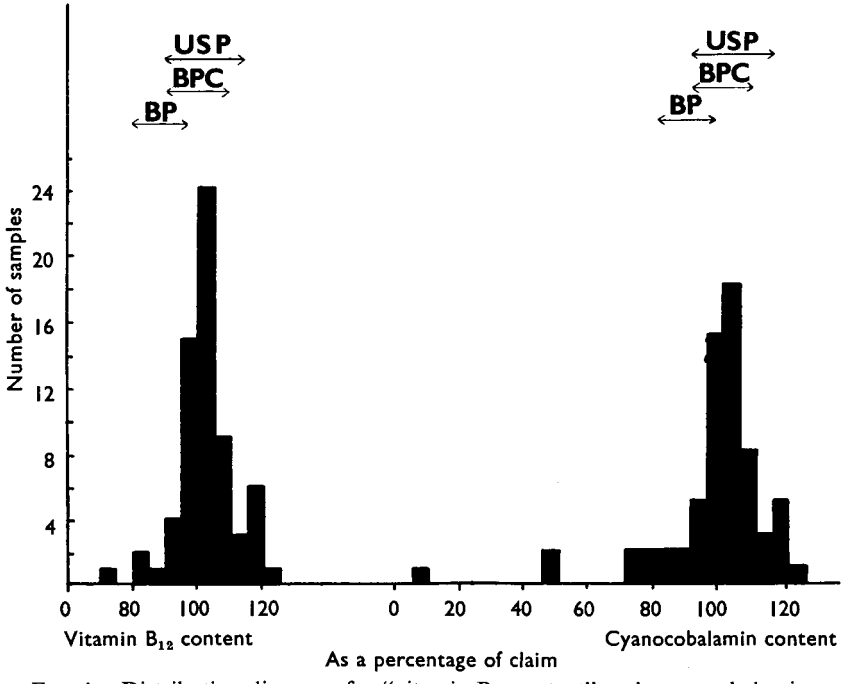

FIG. 4. Distribution diagrams for "vitamin B_{12} content" and cyanocobalamin content of vitamin B_{12} injections, expressed as a percentage of claim. The arrows indicate ranges of this percentage permitted by U.S.P. XIV, B.P.C. supplement 1952 and B.P. 1953.

that 55 out of 66, or 83 per cent. of the batches, satisfied the U.S.P. XIV requirements, and 53 out of 66 or 80 per cent. of the batches, satisfied the requirements of the B.P.C. Supplement 1952. The injections had been manufactured to meet these requirements, and even after storage for some time by far the greater part of them succeeded in doing so.

When the injections were checked against the requirements of B.P. 1953 there was a very different story. Only 11 out of 66 or 17 per cent. of the batches, satisfied these requirements. 54 of the batches, or 82 per cent. gave too high results. Our results in this survey suggest that most of the vitamin B_{12} injections at present in use in this country would not satisfy the Pharmacopœial requirements. In order to avoid wastage of expensive material, it might be advisable to raise the maximum permitted level in B.P. 1953.

Cyanocobalamin content of vitamin B_{12} injections

The true cyanocobalamin content of the injections, determined by

applying the Morton-Stubbs correction to the above figures, showed a rather different distribution, ranging from 9 to 125 per cent. of the claim (see Fig. 4). In 6 batches this content was above 115 per cent. of the claim, thus exceeding the U.S.P. requirements. In 11 batches it was less than 90 per cent. of the claim, and in 49 batches it lay between 90 and 115 per cent. of the claim. For the B.P.C. requirements the distribution was similar, 9 batches being above the requirements, 46 satisfying them and 11 being too low. Only 11 of the injections complied with the B.P. requirements. 48 batches had too high contents and 7 batches too low. In these latter the hydroxocobalamin content was quite considerable, exceeding the corresponding cyanocobalamin content in 3 of the batches.

Comparison of cyanocobalamin and official "vitamin B_{12} content"

Since exposure to light gradually converts cyanocobalamin to hydroxocobalamin[3,14,15] the proportion of cyanocobalamin in the official "vitamin B_{12} content" might be expected to be lower in injections stored for longer periods of time, if they had been exposed to light. In Figure 5 this proportion is plotted against storage time, for each of the batches for which details of storage conditions were available. The results show that whilst the proportion of cyanocobalamin seems to remain constant in some batches, in others it tends to fall during prolonged storage, even though the ampoules were in cardboard boxes not exposed to direct daylight. There was also some indication that, when other factors are equal, the cyanocobalamin content falls rather more rapidly in low potency injections (e.g., 20 μg./ml.) than in high potency injections (e.g., 100 μg./ml. or higher).

Effect of p*H*

Previous workers[6] have found that a *p*H lower than 6 in cyanocobalamin solutions favours its conversion by daylight to hydroxocobalamin. We carried out some experiments on solutions in which the concentrations were similar to those in the above injections, and found that after 1½ hours exposure at *p*H 4 in white glass stoppered test tubes to direct sunlight on the open roof, there was no cyanocobalamin left (see Table IV). *E* 361 mμ fell to little more than half the initial value. A parallel experiment at *p*H 5 again showed complete destruction of the cyanocobalamin, though the reduction in *E* 361 mμ was not quite so great. At *p*H 6·2, 45 per cent. and at *p*H 6·9, 24 per cent. of the cyanocobalamin was destroyed. When the solutions were stored in a dark cupboard or even in diffused light in the laboratory there was no loss of cyanocobalamin. These results

TABLE IV

ACTION OF DAYLIGHT (1½ HOURS) ON VITAMIN B_{12} SOLUTIONS AT DIFFERENT *p*H

	Cobalamin (μg./ml.) after storage in			
	Dark		Direct sunlight	
pH	"Total"	Cyano	"Total"	Cyano
4·0	23·2	23·2	13·2	0
5·0	23·1	23·1	16·2	0
6·2	23·2	23·2	19·8	12·8
6·9	23·2	23·2	21·2	17·7

show that the stability of cyanocobalamin solutions to light can be greatly increased by raising their *p*H from 5 to 7.

No requirements for the *p*H of vitamin B_{12} injections are given in U.S.P. XIV. The B.P.C. Supplement 1952 specifies a *p*H between 4·5 and 6·5 and B.P. 1953 a *p*H between 3·5 and 5·5. None of the injections we examined had a *p*H above 6·3, and the *p*H of most lay between 5 and 6. Hence their *p*H was not a limiting factor.

Protection against light

Ampoules from a batch of vitamin B_{12} injections containing 20·0 μg./ml. by the official method and 18·6 μg./ml. of cyanocobalamin, at *p*H 5·9, were stored under different conditions, and samples examined at intervals. The results, summarised in Table V, showed no loss of cyanocobalamin

TABLE V

EFFECT OF DAYLIGHT ON VITAMIN B_{12} INJECTIONS

	In dark at				In open air in bottle of			
	Room temperature		37° C.		Clear glass		Brown glass	
Days	"Total"	Cyano	"Total"	Cyano	"Total"	Cyano	"Total"	Cyano
0	20·0	18·6	—	—	—	—	—	—
5	20·0	18·6	20·1	18·4	14·1	2·6	15·7	4·4
12	—	—	—	—	10·6	0	—	—

after 5 days in the dark at room temperature, and only 1 per cent. loss in the dark at 37° C. After storage of the ampoules in a white glass bottle in the open air on the roof 86 per cent. of the cyanocobalamin had been lost in 5 days, and 100 per cent. in 12 days. A parallel experiment in which the ampoules were stored in the open air in an amber glass bottle alongside the white glass bottle showed a loss of 76 per cent. of cyanocobalamin in the same 5 days. Thus the amber glass had provided very little protection against the photochemical action of the light.

However, this light was much more intense than the diffused light in our laboratory. Thus, in some 405 μg./ml. ampoules stored for 6 months

TABLE VI

EFFECT OF STORAGE CONDITIONS ON VITAMIN B_{12} INJECTIONS ORIGINALLY CONTAINING 405 μg./ml.

	Contents as percentage of initial content	
	"Total"	Cyano
After 6 months' storage:		
(*a*) in dark at 37° C.	100	100
(*b*) in diffused light at 0° C.	99	99
(*c*) in diffused light at room temperature	98	98

(Dec. to May) in a beaker on a shelf in the laboratory facing north west we found only 2 per cent. loss of cyanocobalamin (see Table VI). There was only 1 per cent. loss in similar ampoules stored in a cardboard box in comparative darkness in the refrigerator, and no loss after storage in the dark at 37° C.

The greater stability towards light of these injections may have been partly due to their high potency. This would fall in line with our general findings on the whole series of injections. We did in fact experience in one batch of ampoules with an initial content of 104 μg./ml. (by official method) a loss of about 10 per cent. of cyanocobalamin after 5 months storage (from August to December) in diffused light in the laboratory, under similar conditions.

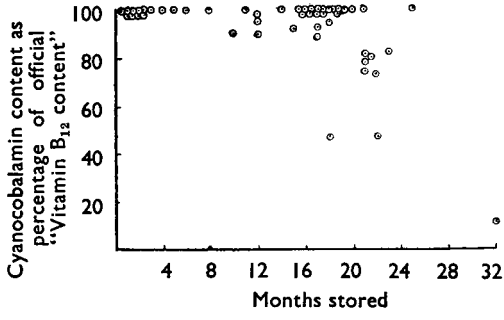

FIG. 5. Effect of storage on cyanocobalamin content (expressed as a percentage of the official "vitamin B_{12} content") of vitamin B_{12} injections.

Reversal effects

It has been stated[6] that if a solution of cyanocobalamin, in which partial conversion to hydroxocobalamin has been produced by the action of light, is then stored in the dark, there is gradual reversion to cyanocobalamin. We tested this effect by taking some of the above 20 μg./ml. injections in which after exposure to direct daylight for 5 days only 24 per cent. of the original cyanocobalamin content remained. On putting them in the dark for another 13 days our assays then showed the cyanocobalamin content to have increased to 96 per cent. of the initial value.

When cyanocobalamin solutions were aerated during illumination to remove the cyanide thus liberated and subsequently stored in the dark, no reversion to cyanocobalamin took place. Thus the reversal effect depends on the hydroxocobalamin which has been formed by the action of the light being able to take up again the cyanide left in the solution, from which it cannot escape because of storage in a sealed container such as an ampoule.

TABLE VII

CYANOCOBALAMIN CONTENT (μg./ml.) OF VITAMIN B_{12} INJECTIONS FROM U.S.A.

Claimed	Found	Found as percentage of claim
30	30·4	101·3
60	60·4	100·7
100	99·3	99·3
1000	1027	102·7
2000	1985	99·3

When this paper was being completed we received from U.S.A. samples of vitamin B_{12} injections in use in that country. Our spectrophotometric assays on these samples showed them all to comply with U.S.P. XIV requirements. No hydroxocobalamin was detected. The cyanocobalamin content ranged from 99·3 to 102·7 and averaged 100·7 per cent. of the claim (see Table VII).

We are indebted to Mr. F. W. Adams, Secretary of the Pharmaceutical Society, for putting us in touch with hospital pharmacists and group pharmacists who kindly supplied numerous samples with details of their storage conditions.

Discussion

Our findings indicate that whilst most vitamin B_{12} injections, as they are used in hospitals in this country, have a cyanocobalamin content lying between 115 and 90 per cent. of the claim, in a certain number of batches this content is well below the claim. Much of this deficiency is probably due to change of cyanocobalamin to hydroxocobalamin, caused by exposure to daylight, even although this has to pass through a cardboard container. The possibility thus arises that patients given vitamin B_{12} injections may sometimes receive appreciable amounts of hydroxocobalamin. We are not aware of any ill effects thus being produced. Pure hydroxocobalamin has been tested clinically and found[16] to have the hæmopoietic activity of cyanocobalamin with no unpleasant side effects. In the course of our investigation into the cyanide and thiocyanate metabolism in man, injections of cyanocobalamin containing up to 50 μg. of hydroxocobalamin have been given without untoward effects.

The present official requirements for vitamin B_{12} injections permit the presence of considerable proportions of hydroxocobalamin. If this is felt to be undesirable, the simplest way of determining the amount present seems to lie in a spectrophotometric method such as we have used. It could provide a more satisfactory check on the efficacy of procedures, especially protection from light, aimed at preventing change of cyanocobalamin to hydroxocobalamin in vitamin B_{12} injections.

Summary

1. A study of 66 different batches of vitamin B_{12} injections made by 7 different manufacturers and obtained from hospital pharmacists in 18 centres in the U.K., has shown that they may contain considerable proportions of hydroxocobalamin, sometimes as much as half of the total cobalamin present.

2. Although vitamin B_{12} is defined in U.S.P. XIV, B.P.C. Supplement 1952 and B.P. 1953 as cyanocobalamin, the official spectrophotometric assays and identification tests for vitamin B_{12} injections do not satisfactorily measure any hydroxocobalamin present, and do not detect it unless it forms at least 25 per cent. of the total cobalamin content.

3. Published methods of determining cyanocobalamin in presence of appreciable proportions of hydroxocobalamin are tedious, and need larger quantities of material than might be available when examining 2 or 3 ampoules of vitamin B_{12} injection. However, one of these methods, based on microbiological assays of vitamin B_{12} activity before and after destruction of hydroxocobalamin with ascorbic acid under given conditions, has been used to check on 11 samples of vitamin B_{12} solutions and injections a spectrophotometric method of determining cyanocobalamin, involving a Morton-Stubbs correction based on extinctions at 355, 361 and 366 mμ. The agreement between results obtained by the two methods was sufficiently satisfactory to justify applying the spectrophotometric method to the 66 different batches.

4. A survey of these has shown that, whilst most vitamin B_{12} injections have a cyanocobalamin content lying between 90 and 110 per cent. of the

claim, in some batches this content is well below the claim. This is probably due to conversion of cyanocobalamin to hydroxocobalamin by the action of light. This conversion takes place readily in the *p*H range of the injections (3·5 to 6·5) and can be prevented by storage in the dark. The cardboard boxes normally used to contain ampoules do not appear to provide sufficient protection. However, when ampoules containing vitamin B_{12} injections in which hydroxocobalamin has been formed by the action of light are subsequently stored in the dark, the hydroxocobalamin may revert to cyanocobalamin.

5. The cyanocobalamin content of most of the vitamin B_{12} injections at present in use in the U.K., when related to the claimed content, appears to be higher than is permitted in B.P. 1953. It is suggested that the present upper limit of 96·5 per cent. of the claim should be increased a little, in order to avoid loss of much valuable material.

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DISCUSSION

The paper was presented by MISS N. BAXTER.

MR. G. SYKES (Nottingham) said he would like to see more evidence for the assertion that cardboard boxes were not light proof. With regard to the method of assay, he wondered whether the authors had used *Lactobacillus leichmannii*, *Bacterium coli* or even the later technique of Ford using an *Ochromonas* culture, because some of the results quoted in Table I were subject to considerable variation. The range of ratios of microbiological assays to spectrophotometric assays appeared to be rather wider than could be normally attributed to microbiological assay errors. The suggestion that it might be due to storage, the microbiological assays having been conducted at different times from the spectrophotometric assays, did not seem to be supported by his impression that cyanocobalamin solutions were much more stable than was indicated in the paper.

MR. D. C. ADAMSON (London) referred to the official identification tests for vitamin B_{12} mentioned in the paper, and said that while he agreed that the ratio of extinctions at 550 and 361 mμ was not very different for hydroxocobalamin and cyanocobalamin, if the wavelengths 278 and 361 mμ were chosen the ratios were of significant difference. The B.P. limits for the latter ratio excluded more than about 10 per cent. of hydroxocobalamin. In his laboratories the position of the maxima and the ratios as determined on the completed solution for injection were taken into consideration. The results quoted for the American samples were somewhat disturbing. The authors inferred that American manufacturers had a more stable form of vitamin B_{12} or that their method of preparation of the solution gave greater protection than did British methods. He wondered whether there was not some other explanation. It would be interesting to know if the American samples were obtained from hospital stocks after some storage period or if they were obtained direct from manufacturers. In the latter case it would manifestly be unfair to compare them with samples taken from hospitals in England. In the early days it was general practice to include a small overage as well as to standardise on the anhydrous cyanocobalamin content. Some of the high results given in the paper might well be due to that. It was well known that light could cause the change from cyanocobalamin to hydroxocobalamin and that the reaction was readily reversible. The authors had not given any experimental evidence that light could affect an ampouled cyanocobalamin solution when protected by a cardboard box. It was difficult to believe that sufficient light could pass through a box to cause even a temporary change in the solution. He had found no evidence of any batch of cyanocobalamin solution being less stable than others. It was well known that cyanocobalmin was fairly readily broken down by both acids and alkalis. Acid conditions were unlikely to develop in an ampouled solution, but it might well be that occasionally the glass ampoules yielded sufficient alkali to account for some of the authors' rather low results.

DR. J. G. HEATHCOTE (Speke) said that whilst there might be some justification for the criticisms which had been levelled at the method described by the authors and possibly at some of the results obtained, it should be pointed out that it was the first serious attempt to obtain a discriminatory method for the determination of cyanocobalamin in the presence of hydroxocobalamin apart from the microbiological method to which reference had been made. He hoped to publish shortly a method for determining various forms of vitamin B_{12}.

MR. H. GRAINGER (London) referred to the storage of vitamin B_{12} injections and said he had found it convenient to obtain solutions of vitamin B_{12} in high concentration and to dilute them as required. It was not clear from the paper whether there was any difference in the rate of change reported by the authors between highly concentrated and dilute solutions.

MR. W. H. C. SHAW (London) said he was particularly interested in the application of the Morton and Stubbs correction for determining

cyanocobalamin in the presence of hydroxocobalamin. That well-known correction made the basic assumption that relative absorption over a range of wavelengths was linear. If hydroxocobalamin were the only absorbing substance other than cyanocobalamin over the wavelength selected, it seemed a reasonable assumption. But if there were any other decomposition products present, it might not be valid. In addition, the Morton and Stubbs correction was not very satisfactory for substances such as cyanocobalamin having absorption curves showing very sharp maxima. Very small errors in the wavelengths, particularly at subsidiary points which were on steep portions of the absorption curve, affected the results considerably. The assays for cyanocobalamin and hydroxocobalamin might have been carried out by utilising the extinction at 361 mμ before and after the addition of cyanide to convert any hydroxocobalamin into cyanocobalamin, and he wondered whether the authors had carried out any assays along those lines.

DR. F. WOKES, in reply, said that the cardboard boxes used were those in which the samples were received. A number were sealed up and therefore it was a fair assumption to make that the samples had been stored in those boxes since manufacture. As some of the ampoules showed losses it was assumed that such losses could occur on storage in cardboard boxes. As a result of subsequent investigation, he had found that in direct sunlight losses in potency of ampoules stored in cardboard boxes were greater than those of ampoules stored in cardboard boxes protected by black photographic paper. The only organism used in the microbiological assays so far had been *Lactobacillus lactis* Dorner. With regard to the question of stability of solutions of different concentrations, the strength of the injections was in the range from 10 to 1000 μg/ml. and no differences had been noted. In tests carried out since the paper was completed he had found that in still higher concentrations vitamin B_{12} was more stable. Some of the ampoules had obviously been given overages to allow for possible losses, and the results were recorded as found. *p*H determinations had been made on most of the injections tested and it had been found that the *p*H lay within the range quoted, so it was not, in his view, a disturbing factor in any of the results. Measurements had been taken at 3 peaks, 278, 361 and 548 mμ. After consideration it was decided to base the findings on 361 mμ first of all because it was sharper and higher than the others, and also because peak 278 mμ was not often available due to the presence of antiseptics in injections. In his view the results in respect of the Morton and Stubbs correction were satisfactory. The American samples were obtained direct from the manufacturers but they were some months old.