THE PURITY OF VITAMIN B_{12}

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It has been variously reported that hydroxycobalamin, vitamin B_{12b}[1,2] nitrito-cobalamin, vitamin B_{12c}[3] and the thiocyanate analogue[4] all possess hæmatological activity and are all equally effective with vitamin B_{12} in causing remission of the symptoms of Addisonian pernicious anæmia.

It is perhaps unfortunate, therefore, that only cyanocobalamin thus far has received official recognition, for this tends to imply that the therapeutic activity of the substance is in some way associated with the cyanide grouping in the molecule. Apart from this anomaly, if cyanocobalamin only is considered suitable for therapeutic use, then the method of estimation of purity should be specific, within small experimental error, for this particular form of the vitamin. The present method, recommended by the U.S.P. XIV, consists essentially in determining, spectrophotometrically, the extinction coefficient $E_{1\text{ cm.}}^{1\text{ per cent.}}$ at 361 mμ at which wavelength cyanocobalamin possesses its maximum peak. Unfortunately for this recommended procedure, the absorption curve for hydroxycobalamin is similar to that for cyanocobalamin and the maximum absorption peak for this analogue is quite close (351 mμ) to that for the cyano-compound. It follows that quite considerable amounts of the hydroxy compound can be present in commercial samples of vitamin B_{12} without substantially lowering the purity as spectrophotometrically determined, and this has, in fact, been demonstrated in these laboratories. As regards the source of vitamin B_{12b}, the present writer has shown by partition chromatography that this is present in natural fermentation liquors, e.g., of *Streptomyces griseus* to the extent of *ca.* 40 per cent. of the total vitamin B_{12}. Furthermore, hydrolysis of cyanocobalamin to the hydroxy compound can occur at any stage of subsequent processing, especially in the presence of light and heat even if, at one stage, all B_{12}-like substances have been converted to the cyano-type of vitamin. As normally prepared, vitamin B_{12} is crystallised from aqueous organic solvent mixtures, usually water and acetone, but even with repeated crystallisation it is frequently difficult to bring the purity to a high level. This is understandable in view of the non-specific nature of such solvents. Recrystallisation from aqueous solution, however, readily frees cyanocobalamin from the hydroxy analogue and from other water-soluble amorphous impurities and provides the simplest means of preparing the pure compound.

Should confirmation be forthcoming of the reported finding that a pseudo-vitamin B_{12}[5] possessing all the characteristics of cyanocobalamin including microbiological but *not* clinical activity has been produced by fermentation, an even more cogent reason would exist for not placing

absolute reliance on this sole criterion of purity. In the case of pseudo-vitamin B_{12} the only reported difference between it and the authentic vitamin is a difference in partition between organic and aqueous phases —a property which likewise differentiates vitamins B_{12} and B_{12b}. It would appear necessary, then, to apply a secondary test, viz., distribution of the vitamin between water and an organic solvent in order to avoid contamination. A simple and reproducible technique is outlined below for determining the partition coefficient of commercial vitamin B_{12} preparations.

Experimental

Preparation of purified vitamin B_{12}. Approximately 2 g. of vitamin B_{12} crystals prepared by deep fermentation of cultures of *Streptomyces griseus* and of *ca.* 90 per cent. purity was recrystallised from water and, after standing at 5° C. overnight, collected on a Buchner funnel and washed with ice-cold water. It was then sucked dry in air and finally dried *in vacuo* over phosphorus pentoxide. When dry to constant weight a sample was set aside for distribution experiments and also for purity and moisture determinations. The purity of this material was estimated at 97 per cent. on the basis of the spectrophotometric absorption at 361 mμ. The remainder was again subjected to an aqueous recrystallisation procedure and analysis of this twice recrystallised material showed a purity of 99 per cent., i.e., theoretical within the experimental error of the method. Both sets of crystals were then subjected to partition experiments as described below.

Distribution experiments were carried out using specially purified solvents. The method consisted in preparing an aqueous solution of the vitamin of appropriate concentration. In the case of benzyl alcohol, using the samples of recrystallised cyanocobalamin, a concentration of 125 μg./ml. was found to be suitable. Of this solution, 50 ml. was shaken vigorously for 2 minutes with an equal volume of solvent in a 250-ml. separating funnel and the mixture was allowed to stand and separate for 5 minutes. The whole process, shaking and standing, was repeated twice in order to ensure equilibration of the phases, the latter being separated finally by centrifugation. The separated phases were next assayed spectrophotometrically at 361 mμ. against the appropriate blank phases, e.g., water-saturated benzyl alcohol and benzyl alcohol-saturated water. Similar experiments were carried out with hydroxycobalamin but in this case the solutions of purified vitamin were assayed at 351 mμ. The distribution coefficient for vitamin B_{12} between butanol and water was also determined; it was not possible to determine, with accuracy, Cs/Cw for hydroxycobalamin owing to its extremely low value.

Discussion

The results in Table I show that there was little difference in partition coefficient between the two aqueous recrystallised specimens of vitamin B_{12}. The value for this coefficient between benzyl alcohol and water was, for the twice recrystallised cyanocobalamin, 0·78. This value is in good agreement with that (0·79) reported by Anslow *et al.*[6] for the

distribution of vitamin B_{12} between benzyl alcohol and water but does not agree too closely with that (0·84) of Buhs *et al.*[7] In the case of hydroxycobalamin, there is wide discrepancy between the partition coefficient as determined by the latter authors and the value reported in the present paper; this latest value, however, is in good agreement with the qualitative assessment of partition reported by Anslow *et al.*[6]

TABLE I

SOME PARTITION COEFFICIENTS OF CYANO- AND HYDROXYCOBALAMIN

Author	Solvent	Temperature °C.	*p*H	Cs/Cw
Cyanocobalamin (Vitamin B_{12b})—				
Anslow *et al.*[6]	Benzyl alcohol	?	4·0	0·79
Buhs *et al.*[7]	„ „	?	?	0·84
Present study	„ „	21	6·0	0·76*
		„	„	0·78†
	Butanol	„	„	0·045
Hydroxycobalamin (Vitamin B_{12d})—				
Anslow *et al.*[6]	Benzyl alcohol	?	4·0	almost wholly in aqueous phase
Buhs *et al.*[7]	„ „	?	?	0·13
Present study	„ „	22	6·2	0·055

* Corrected mean of 8 determinations on once recrystallised cyanocobalamin.
† Corrected mean of 6 determinations on twice recrystallised cyanocobalamin.

It may seem somewhat academic to draw attention to the possible existence of hydroxycobalamin in pharmaceutical preparations of the vitamin if this "impurity" is equally effective clinically and equally non-toxic. On the other hand, there would appear to be little point in stressing purity determinations at 361 mμ. if substantial amounts of a material possessing a maximum absorption peak at 351 mμ. are thereby allowed to be present. It may, therefore, be considered worthwhile permitting several official forms of cobalamin and laying down purity tests for each form. If, notwithstanding, cyanocobalamin is to become the official form of vitamin B_{12} in the British Pharmacopœia[8] it should be produced by a final aqueous recrystallisation in the absence of organic solvents and the partition coefficient between, say, benzyl alcohol and water should be within prescribed limits. There would seem to be, however, little justification for singling out any one analogue to be the subject of the official monograph. Finally, it is at least arguable that hydroxycobalamin, vitamin B_{12b}, is the truly active natural vitamin.

SUMMARY

1. The anomalous position of the various therapeutically active cobalamins is discussed in relation to their possible inclusion in the British Pharmacopœia. Attention is drawn to the possible presence of other B_{12}-like bodies in commercial samples of cobalamin conforming to the U.S.P. purity standards.

2. The desirability of a final aqueous recrystallisation, or at least of distribution measurements, is stressed.

3. A simple, but specific and reproducible, method for the determination of the partition coefficient of cobalamin between solvent/water systems is described.

4. Values are reported for the distribution of this vitamin between benzyl alcohol/water and butanol/water systems.

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