ESTIMATION OF VITAMIN B_{12} BY COLORIMETRIC DETERMINATION OF COBALT

MOHAMMAD ABUL HUSSAIN SHARIF

Central Laboratories, Pakistan Council of Scientific and Industrial Research, Karachi

Introduction

Of the various methods that have been suggested for assaying vitamin B_{12}, the microbiological method is the one most frequently used. Another biological method using laboratory animals is also employed, but this is laborious and time consuming. In fact the microbiological method suffers from several drawbacks, among which may be mentioned the variable activities displayed by different naturally occurring forms of vitamin B_{12}. The fact that desoxyribosides and some reducing agents also permit growth of the microbes under the same condition as vitamin B_{12}, introduces another complication on the biological methods of estimation. Several specific chemical and physico-chemical methods for the estimation of the vitamin have therefore been developed. Because of the characteristic ultra-violet absorption spectrum of vitamin B_{12}, the spectrophotometric method was developed early on, and the ratio of the absorbency at wave lengths 360 m μ to that at 548 m μ (theoretical value 3.24) is used in the absence of interfering substances as a measure of the vitamin B_{12} present. The colorimetric methods include (i) the cyanide assay,[2] in which the cyanide from cyano-cobalamin is liberated by aeration of an aqueous solution at pH 5, (ii) the cyanide complex method[3] based on the difference between the visible spectrum of cyano-cobalamin and its di-cyanide complex, this difference being maximal at 582 m μ with E_{1cm} 1%=54 and (iv) the measurement of 5,6-dimethyl-benzimidazole, an hydrolysis product of vitamin B_{12} ,[4] the quantitative liberation of 5,6-dimethylbenzimidazole being attained by the action of 0.1N hydrochloric acid at 120°C. for 4 to 16 hours. Other methods are (a) the countercurrent distribution assay, in which use is made of the distribution of vitamin B_{12} in the water-benzyl alcohol system,[5] subject to the absence of other coloured substances, and (b) the radio-active tracer method depending upon the radioactive tracer dilution principle and using cyanocobalamin with labelled cobalt,[6] which is especially applicable to mixtures containing very small amounts of vitamin B_{12}, where quantitative isolation of the vitamin is not possible.

From the foregoing, it is seen that the chemical methods thus far suggested are somewhat lengthy and are not always applicable to the raw materials that form sources of vitamin B_{12}. There is therefore a need for a chemical method that is short and simple, and is applicable equally well to the different forms of vitamin B_{12} and to the food stuffs and biological materials that contain the vitamin. A method fulfilling these requirements is described below and experimental data on its application are given. An accurate determination of the cobalt content of a sample enables the quantity of the vitamin to be deduced from its formula, $C_{63}H_{88}N_{14}O_{14}CoP$, [7,8] because, apart from the vitamin B12 group, no cobalt-containing organic compounds have yet been found in nature. One gram of vitamin B_{12} contains 0.0435 gm. of cobalt, and therefore, the cobalt content of a sample divided by 0.0435 (*i.e.*, multiplied by 23.0) can be taken as a measure of the vitamin B_{12}.

Experimental

The method was tested with "Cytacon" vitamin B_{12} syrup (Glaxo), and "Campolon" liquid extract of liver (Bayer), and a further test was also made on the quantitative character of the recovery of cobalt from the syrup and the extract.

In each case the cobalt content of the vitamin was determined by the nitroso R-salt (1-nitroso, 2-napthol, 3,6-disodium sulphonate) reagent according to the procedure described by Kidson and Askew[9] and previously used by the author for a survey of the cobalt content of East Pakistan fish.[10].

1. "*Cytacon*" *vitamin* B_{12} *made by Glaxo Laboratories.* This liquid syrup of vitamin B_{12}, meant for oral administration, is stated by the manufacturers to contain 7 micrograms of crystalline vitamin B_{12} per ml. 10 ml. portions of this syrup were taken in a platinum dish and processed as described in references (9) and (10), so as to obtain a cobalt complex of the nitroso R-salt which has a characteristic colour. The solutions were matched with freshly prepared cobalt standard in a Hilger colorimeter, using the complimentary colour at wave length 550 m μ.

This procedure was repeated with 20 ml. and 30 ml. portions of the syrup and the results are shown in Table I.

(2) "*Campolon*" *liver extract manufactured by Bayer.* This injectable extract of liver contains 30

TABLE 1.—TEST OF THE METHOD WITH "CYTACON VITAMIN B_{12} SYRUP.

No. of sample	Volume of syrup taken ml.	Nominal values (micrograms) Cobalt content	Nominal values (micrograms) Vit. B_{12} content	Values found by the suggested method (micrograms) Cobalt	Values found by the suggested method (micrograms) Vit. B_{12}	Error %	Mean error %
1	10	3.044	70	3.25	74.7	6.7	3.2
2	,,	,,	,,	3.0	69.0	1.4	
3	,,	,,	,,	3.0	69.0	1.4	
				Mean=	70.9		
1	20	6.088	140	6.09	140.1	0.0	1.4
2	,,	,,	,,	6.0	138.0	—1.4	
3	,,	,,	,,	6.25	143.0	2.6	
4	,,	,,	,,	6.0	138.0	—1.4	
5	,,	,,	,,	,,	,,	,,	
6	,,	,,	,,	,,	,,	,,	
				Mean=	139.3		
1	30	9.132	210	9.0	207.0	—1.4	1.3
2	,,	,,	,,	9.2	211.6	0.7	
3	,,	,,	,,	9.0	207.0	—1.4	
4	,,	,,	,,	,,	,,	,,	
5	,,	,,	,,	,,	,,	,,	
6	,,	,,	,,	,,	,,	,,	
				Mean=	207.8		

micrograms of vitamin B_{12} per ml. The determinations of cobalt in this sample were carried out by two different processes. First it was determined as in the case of Cytacon syrup, and it was found that the values obtained were consistently higher than the nominal contents. So it was conjectured that iron, which is abundant in liver tissues, was interfering with the cobalt determination. Therefore, the iron was removed by coverting it into ferric chloride and extracting with ether.[11] The amount of cobalt determined from the aqueous extract got after removal of this iron was found to agree to within 3% with the theoretical values, as shown in Table 2.

TABLE 2.—TESTING OF THE METHOD WITH BAYER'S LIVER EXTRACT.

	No. of Sample	Volume of liver extract (ml.)	Nominal values (microgram)		Values found by the suggested method (microgram)		Error %	Mean error %
			Cobalt present	B_{12} present	Cobalt	B_{12}		
Before removal of iron	1	3	3·903	90.0	4.6	105.8	17.5	
	2	,,	,,	,,	4·7	108.1	20.1	19
	3	,,	,,	,,	,,	,,	,,	
	4	,,	,,	,,	,,	,,	,,	
						Mean=107·5		
After removal of iron	1	3	3·903	90.0	4.0	92.0	2.2	
	2	,,	,,	,,	,,	,,	,,	3·3
	3	,,	,,	,,	,,	,,	,,	
	4	,,	,,	,,	,,	,,	,,	
	5	,,	,,	,,	4·25	97·7	8.6	
	6	,,	,,	,,	4.0	92.2	2.2	
						Mean=93.0		

(3) *Cobalt recovery from the syrup and the liver extract.* To study the recovery of cobalt from the syrup and also from the liver extract, some experiments were carried out by adding calculated amounts of cobalt in the form of cobalt chloride solution to both the Cytacon vitamin B_{12} syrup and the injectable liver extract. The samples were digested and ashed as before, and analysed for cobalt contents. The results are presented in Table 3 and indicate nearly 100% recovery.

Discussion

From Table 1, it is observed that the method gives quite satisfactory results with 20 ml. and 30 ml. of vitamin B_{12}, the error being less than 1.5%. With 10 ml. samples, however, the error rises to 3%, which is understandable because we now have a total of only 3.044 microgram of cobalt. For a 2% accuracy of determination, 4 micrograms of cobalt *i.e.*, about 100 micrograms of vitamin B_{12}, are required, which fact is further substantiated by our results in Table 2, in which are presented the analyses of samples of injectable liver extracts prepared by Bayer under the trade name of "*Campolon*." In this case, it was necessary to remove the iron before determining the cobalt content as otherwise the iron interferes seriously and raises the estimated cobalt content by nearly 20%. After the removal of iron, however, the method again gives quite satisfactory results as shown by Table 2. The experimental values, however, still tend to be high by 2-3%, which may be due to incomplete removal of the iron or the presence of traces of copper.

Table 3 shows the recovery of cobalt after addition to the Cytacon B_{12} syrup and to the liver extract. Calculated amounts of cobalt in the form of cobalt chloride solution were added to the samples before analysis and the total cobalt contents were estimated as before. From the table it is evident that, except for two or three cases, the error is always less than 2%. Here again the experimental estimates for the liver extract are seen to be higher by about 4%.

TABLE 3.—RECOVERY OF COBALT FROM THE SAMPLES.

	No. of sample	Volume of samples (ml.)	Cobalt content of the sample (microgram)	Amount of cobalt added (microgram)	Total amount of cobalt present (microgram)	Total amount of cobalt obtained by the method (microgram)	Recovery
Cytocon syrup	1	10	3.044	3.0	6.044	6.0	98.5
	2	,,	,,	,,	,,	,,	,,
	3	,,	,,	,,	,,	,,	,,
	4	,,	,,	,,	,,	,,	,,
	5	,,	,,	,,	,,	,,	,,
	6	,,	,,	,,	,,	5.75	90.2
	7	,,	,,	,,	,,	6.0	98.3
	8	,,	,,	,,	,,	5.75	90.2
							Mean=96.4
Liver extract	1	3	3.913	5.0	8.913	9.25	106.7
	2	,,	,,	,,	,,	9.0	101.7
	3	,,	,,	,,	,,	9.0	101.7
	4	,,	,,	,,	,,	8.75	96.7
	5	,,	,,	,,	,,	8.75	96.7
	6	,,	,,	,,	,,	9.0	101.7
							Mean=100.9

The above experiments show that the method suggested can be used for the estimation of vitamin B_{12} to an accuracy of 2%, provided adequate care is exercised against interfering elements. The method has further been tested with two natural sources of vitamin B_{12}, namely beef liver and shark liver. The results obtained by this chemical method, 253 microgram and 191 microgram, respectively, per 100 g. of fresh tissue, are in satisfactory agreement with the recorded values[12,13] obtained by microbiological methods.

A possible source of error in the method when applied to raw biological materials is that there may occur some cobalt which is not a part of vitamin B_{12}. This extraneous cobalt will also be included in the estimate made by this method, and will thereby produce higher estimates for vitamin B_{12}. However, for such materials we can work out an emperical correction factor by comparison with the value obtained from microbiological assay. This is being investigated further, but inspite of this drawback, the method will, in any case, give us a good approximation to the vitamin B_{12} content of biological materials and should be valuable for laboratories that are not equipped with microbiological techniques. For pure vitamin B_{12} and its preparations the method is entirely satisfactory, being accurate to 2%.

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THE PUPA AND LARVA OF EUBLEMMA SCITULA

S. MAHDIHASSAN

Central Laboratories, Pakistan Council of Scientific and Industrial Research, Karachi

The lac insects of the continent of India and Pakistan are subject to attack by the caterpillars of *Eublemma amabilis*. There are two other species related to this moth, *viz*. *E. coccidiphaga* and *E. scitula* which are also important. On *E. coccidiphaga* an article has already been published.[1] *E. scitula*, is quite a common parasite of most scale insects but usually not of lac. However, with Sind lac it appears to be the most common of the three species. It has emerged from *Lakshadia sindica* found naturally on *Zizyphus jujuba* in the city of Karachi.

It might be imagined that *E. scitula*, already found on other scale insects, could conveniently infect lac on trees growing nearby. However, during the author's considerable experience of similar lac on trees within the municipal limits of cities like Bangalore, Hyderabad Deccan, Bombay, Madras, and New Delhi, no specimen of *E. scitula* was ever found. Karachi, with its species, *Lakshadia sindica*, stands as an exception to this rule. Near Hyderabad Sind, lac is cultivated on *Acacia arabica*, and in such material again, *E. scitula* was found in greater numbers than *E. amabilis*.

The egg of *E. scitula* has been previously illustrated.[1] The larva builds over itself a case with which it moves about well protected. This covering or shield is held by the serrated rows on the dorsal surface of the hind segment seen at the end of the body in Fig. 1. It shows the dorsal view with the posterior end towards the reader. The scale indicates the actual size of the adult caterpillar freed from its covering. Fig. 2 shows the same caterpillar in profile with the posterior end to the left of the reader and the last segment with the serrated portion in profile. Fig. 3 gives the ventral aspect of the same caterpillar ; two pairs of serrated rows enable the caterpillar to clamp fast to the twig or to the encrustation of lac, so much so that it is not easy even for a man to dislodge the caterpillar easily from its place. It is thus impossible for the severest winds to disturb it. In all, there are three pairs of serrated rows serving like pseudo-legs, two pairs on the ventral surface, seen in Fig. 3, and one on the last segment as in Figs. 1 and 2, serving to hold the shield or cover under which it moves like a tortoise. This dorsal pair of serrated rows is shown further enlarged in Fig. 4.

The larva before it pupates builds a tough parchment-like cocoon which is by no means easy to tear. From such an envelope, a pupa was removed which gave Fig. 5 as its dorsal view, Fig. 6 as its ventral, and Fig. 7 as its side-view.