The forms of vitamin B_{12} in foods

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1. The forms of vitamin B_{12} were determined in foods, most of which had been prepared for consumption.
2. Five forms were detected: adenosylcobalamin, hydroxocobalamin, methylcobalamin, cyanocobalamin and sulphitocobalamin. Adenosylcobalamin and hydroxocobalamin were the predominant forms.
3. The intestinal absorption of [^{57}Co]sulphitocobalamin was estimated and found to be lower than that of [^{58}Co]cyanocobalamin.

Soon after the isolation of vitamin B_{12} in the form of cyanocobalamin it was appreciated that there were a large number of chemically related compounds. Some of these were found to be as active as cyanocobalamin in microbiological assay systems and some were found to be as therapeutically active as cyanocobalamin in the treatment of pernicious anaemia. The significance of these compounds in human metabolism, however, remained in doubt for many years, largely because of difficulties in extracting and identifying the form of vitamin B_{12} in human tissues, but it is now clear that deoxyadenosylcobalamin, hydroxocobalamin and methylcobalamin constitute the greater proportion of vitamin B_{12} in human tissues (Toohey & Barker, 1961; Lindstrand, 1964; Ståhlberg, 1964, 1967; Ståhlberg, Radner & Nordén, 1967; Linnell, Mackenzie, Wilson & Matthews, 1969; Morrow, Barness, Cardinale, Abeles & Flaks, 1969; Matthews & Linnell, 1971; Linnell, Hoffbrand, Hussein, Wise & Matthews, 1974).

Man depends on an adequate intake of vitamin B_{12} in order to maintain vitamin B_{12} balance but, while there is information on the amount of vitamin B_{12} in foods (Robinson, 1966; McCance & Widdowson, 1969; Love, 1970; Adams, McEwan & Wilson, 1973), little is known about the forms in which it occurs. This information is necessary, not only to complement the knowledge of vitamin B_{12} in human tissues, but because there is evidence that with crystalline forms of vitamin B_{12} the amount which is absorbed from an oral dose of one form may be significantly different from that which is absorbed from the same dose of another form (Rosenblum, Woodbury, Gilbert, Okuda & Chow, 1955; Rosenblum, Yamamoto, Wood, Woodbury, Okuda & Chow, 1956; Heinrich & Gabbe, 1964; Herbert & Sullivan, 1964; Adams, Ross, Mervyn, Boddy & King, 1971).

We therefore undertook a study of the forms of vitamin B_{12} in foods and, in view of some of the findings, also studied the absorption of sulphitocobalamin by man.

EXPERIMENTAL

Analysis of foods

The majority of items were obtained from the hospital kitchen and the remainder, mostly canned foods, were purchased at local stores. Inedible material was removed from all samples and, where appropriate, oils, sauces and gravies were removed by washing briefly in water. The samples were then weighed and all further procedures carried out in photographic darkroom conditions to avoid photolysis, unless otherwise stated.

The procedures for extraction of vitamin B_{12} from tissues, with purification and concentration in water, followed by separation of the different forms by thin-layer chromatography and their identification by bioautography, were based on those reviewed by Pawelkiewicz (1962) and on those reported by several groups of workers (Lindstrand & Ståhlberg, 1963; Kennedy & Adams, 1965; Linnell *et al.* 1969; Bilkus & Mervyn, 1971); these procedures were, however, modified to allow extraction from foods, to prevent artifactual sulphitocobalamin formation and to allow separation of the different forms of vitamin B_{12} by one-dimensional thin-layer chromatography. Samples of solid (5 g) or liquid (10 ml) material were homogenized in 50 ml ammonia buffer (0·139 M-ammonium hydroxide, 0·05 M-hydrochloric acid), pH 9·6, and the homogenates were stored at $-20°$ in tubes covered with aluminium foil when they were not immediately to be further processed. Stored samples were thawed and further homogenized before the next stage of extraction, i.e. reflux with 100 ml ethanol at 82° for 30 min with constant stirring to precipitate protein. After cooling at 20° the protein was allowed to settle, the supernatant fraction was filtered through Whatman 54 filter paper under reduced pressure (13 kN/m^2) and the filtrate volume was reduced to 20 ml by rotary evaporation at 40° and 2·7 kN/m^2. The concentrated filtrate was then transferred into an equal volume of phenol–chloroform (1:1, v/v) acidified with one drop of 1 mM-hydrochloric acid and shaken vigorously for several minutes to extract the cobalamins into the phenol phase.

After separation of the aqueous and phenol phases, if necessary by centrifugation in tubes covered with Al foil, the aqueous phase was removed and extracted again with phenol. The phenol phases were then pooled and shaken with distilled water (5:1, v/v) to complete the removal of water-soluble salts. After separation of the phenol and aqueous phases, the cobalamins in the phenol phase were extracted back into an aqueous phase by breakdown of solvation by diethyl ether. The phenol phase was run into an organic phase of diethyl ether–acetone–water (4:1:2, by vol.) in a separating funnel and the contents shaken vigorously for 10 min. After separation by gravity, the lower aqueous phase was removed and shaken with half its volume of diethyl ether to remove any dissolved phenol. The aqueous phase was then concentrated to approximately 1 ml by rotary evaporation at 40° and 2·7 kN/m^2 and the concentrated aqueous extract stored at $-20°$ in tubes covered with Al foil.

The forms of vitamin B_{12} in the concentrated aqueous extract were separated by thin-layer chromatography using silica gel sheets (Chromagram 6061; Eastman Chemical International, London WC2), the undiluted extract and 1:4 and 1:16

dilutions of the extract in water being applied in volumes of 2 μl, together with purified standards of cyanocobalamin, hydroxocobalamin, adenosylcobalamin, methylcobalamin (100 pg in 2 μl); the developing solvent was butan-2-ol–*n*-propanol–water–ammonia (7:4:3:1, by vol.) and the development time for the chromatogram was 6·5–7 h.

Visualization and identification of the standards and the extracted forms of vitamin B_{12} on the air-dried chromatogram were done in daylight by bioautography using the vitamin B_{12}-dependent *Escherichia coli* mutant NCIB 9270 and the 'seeded' agar medium described by Linnell *et al.* (1969). The chromatogram was placed on the prepared agar gel, and the chromatogram and gel were inverted onto a metal plate and incubated, with the agar uppermost, at 38° for 16–18 h. The developed chromatogram was examined and the results recorded by scoring as follows: 0, cobalamin absent from the extract; +, cobalamin present in the undiluted extract; ++, cobalamin present in the undiluted extract and in the 1:4-dilution extract; +++, cobalamin present in the undiluted extract and in both the 1:4- and 1:16-dilution extracts.

Twenty-four items of food were studied and duplicate analyses of the same samples were made for all items except one. Two or more different samples of each of nine items were also analysed.

Absorption of cobalamins

The absorption of radioactive cyanocobalamin and sulphitocobalamin was determined in nine subjects using a high-sensitivity whole-body monitor. Consent was obtained from the subjects, who were hospital out-patients under the care of one of the authors. To minimize biological variation a double-tracer technique was used; each subject ingested 1 μg (0·4 μCi) [^{58}Co]cyanocobalamin and after 24 h 1 μg (0·8 μCi) [^{57}Co]sulphitocobalamin. The doses were given with water to a total volume of 100 ml after a 12 h fast, and food was not taken for a further 3 h. Further tests were carried out in two subjects who were known to have impaired ability to absorb vitamin B_{12}; for these tests the test doses were given with 50 mg hog intrinsic-factor concentrate (Armour Pharmaceutical Co. Ltd, Eastbourne, Sussex). Whole-body radioactivity was measured before each dose, 30 min after each dose, and finally at periods ranging from 7 to 35 d after the second dose. Measurements were corrected for background radioactivity, natural radioactivity, radioactive decay, and for the ^{58}Co contribution to the counting rate in the ^{57}Co energy band. [^{58}Co]-cyanocobalamin was obtained from the Radiochemical Centre, Amersham, Bucks. and [^{57}Co]sulphitocobalamin was prepared in light-proof gelatine capsules by Glaxo Research Ltd, Sefton Park, Stoke Poges, Bucks.

RESULTS

Analysis of foods

Five forms of vitamin B_{12}, adenosylcobalamin, methylcobalamin, hydroxocobalamin, sulphitocobalamin and cyanocobalamin, were identified in the twenty-three items of food analysed. The results are given in detail in Table 1. Duplicate extractions

Table 1. *Relative amounts of the different forms of vitamin B_{12} found in twenty-four samples (twenty-three items) of food*

Food and method of preparation	Sample no.	Extract no.	Vitamin B_{12} present* as: Adenosyl-cobalamin	Hydroxo-cobalamin	Cyano-cobalamin	Methyl-cobalamin	Sulphito-cobalamin
Meat and poultry							
Bacon, grilled	1	1	+	+	o	+	o
	1	2	+	+	o	o	o
Beef, brisket, boiled	1	1	++	++	o	o	o
	1	2	++	++	o	o	o
Beef, rump, braised	1	1	+++	++	o	o	o
	1	2	+++	++	o	o	o
Beef, corned, canned (St Michael; Marks & Spencer Ltd, Baker Street, London)	1	1	o	+++	o	o	++
	1	2	o	+++	o	o	++
Beef, spread (Princes; Princes Foods Ltd, Liverpool)	1	1	++	+++	o	++	o
	1	2	+++	+++	o	o	o
Beef, spread (Shippams; Shippams Ltd, East Walls, Chichester)	1	1	++	++	o	+	o
	1	2	++	++	o	+	o
Chicken, casserole	1	1	++	++	o	o	o
	2	1	+++	o	o	+	o
	3	1	+++	++	o	++	o
Gammon, canned (Danoxa; S. Daniels & Co. Ltd, Wilec House, City Road, London)	1	1	+	++	o	o	o
	1	2	o	++	o	o	o
Ham and chicken roll, canned (Cross & Blackwell; Nestlé Ltd, St Georges House, Croydon)	1	1	+	+++	o	o	o
	1	2	++	+++	o	o	o
Lamb, gigot chop, braised	1	1	+	++	o	o	o
	1	2	+	++	o	o	o
	2	1	+	++	o	o	o
Liver, lamb, raw	1	1	+++	+++	o	+	+
	1	2	+++	+++	o	++	o
Oxtail, stewed	1	1	++	++	o	+	o
	1	2	++	++	o	+	+
Tongue, ox, canned (De Haan (Foods) Ltd, Park Lane, Abram, Wigan, Lancs)	1	1	o	+++	o	o	++
	1	2	o	+++	o	o	++
Fish							
Haddock, boiled	1	1	+++	++	+	+	o
	1	2	+++	++	+	+	o
Salmon, canned (John West; John West Foods Ltd, Stanley Street, Liverpool)	1	1	++	+++	o	o	++
	1	2	++	+++	o	o	++
	2	1	++	++	o	++	o
	3	1	+++	++	o	++	o

Table 1 (*cont.*)

Food and method of preparation	Sample no.	Extract no.	Vitamin B_{12} present* as: Adenosyl-cobalamin	Hydroxo-cobalamin	Cyano-cobalamin	Methyl-cobalamin	Sulphito-cobalamin
Salmon, spread (Princes; Princes Foods Ltd)	1	1	++	+++	o	o	o
	1	2	++	+++	o	+	o
	2	1	++	+++	o	+	o
Sardines, canned (Joy to Eat; Joy to Eat Foods Ltd, 50 Mark Lane, London)	1	1	++	+++	o	o	++
	1	2	++	++	o	o	++
	2	1	++	++	o	o	++
			Dairy produce				
Cheese, cheddar	1	1	++	++	o	++	+
	1	2	++	+++	+	++	o
	2	1	++	+++	o	++	o
Cheese, spread (Kraft; Kraft Foods Ltd, Braeview, East Kilbride)	1	1	++	++	o	+++	o
	1	2	++	++	o	+++	o
	2	1	++	o	o	+++	o
Egg, yolk, raw	1	1	++	++	o	++	o
	1	2	+	++	o	+++	+
Egg, white, raw	1	1	+	o	+	o	o
	1	2	+	o	+	o	o
Milk, cows', whole, fresh	1	1	+	++	o	+	o
	1	2	+	++	o	+	o
Milk, cows', pasteurized	1	1	o	++	o	o	o
	1	2	o	++	o	o	o
	2	1	+	++	o	+	o
	3	1	o	++	o	o	o
Milk, cows', evaporated (Carnation; Carnation Foods Ltd, Carnation House, 11 High Road, London)	1	1	++	o	o	++	o
	1	2	++	o	o	++	o
	2	1	+	o	o	++	o
	2	2	++	o	o	++	o

* Detected and identified by thin-layer chromatography and bioautography (for details, see p. 128) and results scored as follows: o, cobalamin absent from extract; +, cobalamin present in undiluted extract; ++, cobalamin present in undiluted extract and in 1:4-dilution extract; +++, cobalamin present in undiluted extract and in both 1:4- and 1:16-dilution extracts.

Table 2. *Intestinal absorption of oral doses* of [^{58}Co]cyanocobalamin and [^{57}Co]sulphitocobalamin in man*

(The vitamin was given with a source of intrinsic factor† in a second test to two subjects with pernicious anaemia (nos. 8 and 9))

				Whole-body retention of:			
				[^{58}Co]cyanocobalamin		[^{57}Co]sulphitocobalamin	
Subject no.	Age (years)	Sex	Clinical condition	Time after dose (d)	Retention (% dose)	Time after dose (d)	Retention (% dose)
1	71	♀	Vertebrobasilar insufficiency	8	68	8	21
				24	61	24	19
2	37	♀	Hypertension	7	51	7	24
				34	39	32	24
3	38	♀	Acne rosacea	7	51	7	35
4	21	♀	Coeliac disease	14	27	14	17
5	70	♀	Treated folate deficiency	14	38	14	13
6	62	♀	Treated folate deficiency	14	65	14	57
7	64	♀	Biliary cirrhosis	14	78	14	56
8	64	♂	Pernicious anaemia	14	22	14	8
				14	64	14	31
9	59	♂	Pernicious anaemia	14	6	14	1
				14	43	24	24

* For details, see p. 129.

† Hog intrinsic-factor concentrate (Armour Pharmaceutical Co. Ltd, Eastbourne, Sussex) (50 mg).

and estimations were made on twenty-four samples (twenty-three items). In fourteen samples there was both qualitative and quantitative agreement, i.e. the same forms of vitamin B_{12} were found in each sample and the score for each form was the same in each sample. Quantitative agreement only was found in eighteen of the twenty-four duplicate samples and qualitative agreement only was found in sixteen. The qualitative disagreements were related to the presence or absence of: methylcobalamin, three samples; sulphitocobalamin, three samples; adenosylcobalamin, one sample; both sulphitocobalamin and cyanocobalamin, one sample.

Absorption studies

The results indicated that the absorption of [^{57}Co]sulphitocobalamin was lower than that of [^{58}Co]cyanocobalamin in all subjects (Table 2). Based on the final whole-body measurement in subject nos. 1–7 the average amount of [^{58}Co]cyanocobalamin absorbed was 51% dose and of [^{57}Co]sulphitocobalamin absorbed was 32% dose. In the two cases of pernicious anaemia (subject nos. 8 and 9) the amount of [^{58}Co]cyanocobalamin and [^{57}Co]sulphitocobalamin absorbed was increased when the cobalamins were given with intrinsic factor.

DISCUSSION

The extraction and identification of the forms of vitamin B_{12} from tissues involve considerable technical problems. The procedures are laborious and time-consuming, and the detection of the extracted cobalamin by bioautography and quantitation by

visual scanning is inevitably imprecise. In these circumstances a qualitative and quantitative agreement of 58% and a qualitative agreement of 67% between duplicate estimations is acceptable, but emphasizes the need for caution in the interpretation of results. The difference between results obtained from different samples of the same foodstuff could be due to genuine differences in the samples, but could also result from photolysis or contamination by sulphite ions. It is not possible from the results in this study to draw any conclusion about the relative importance of these factors, and this problem requires further study.

The most striking result of the study is the extent to which the light-sensitive cobalamins, adenosylcobalamin, methylcobalamin and sulphitocobalamin, were found in foods which had been subjected to various preparative processes including lengthy periods of light-exposure. The persistance of light-sensitive forms is probably due mainly to the fact that only a small part of a solid food is exposed to light, while the greater resistance of these forms to photolysis when they are bound to protein (Pailes & Hogenkamp, 1968; Taylor & Weissbach, 1968) is also important.

Five cobalamins were found in foods and two of these (adenosylcobalamin and hydroxocobalamin) were predominant in terms of frequency of occurrence and of quantity. The presence of hydroxocobalamin was not unexpected as it is the product of photolysis of the light-sensitive cobalamins; it was absent only from evaporated milk, one sample of a cheese spread, and from egg white which contains only a trace amount of vitamin B_{12} (McCance & Widdowson, 1969), and can therefore be regarded as virtually omnipresent in foods. Adenosylcobalamin, which is light-sensitive, was found with unexpected frequency and in unexpected amounts, and was absent only from a canned meat and pasteurized milk which had been exposed to light. In foods in which both hydroxocobalamin and adenosylcobalamin were present, analysis of the results suggests that in general the amount of adenosylcobalamin was the same or greater than the amount of hydroxocobalamin in natural foods, and that there was less adenosylcobalamin than hydroxocobalamin in canned foods, but since the techniques are relatively imprecise, it would probably be more accurate to conclude that when both adenosylcobalamin and hydroxocobalamin are present in food they are there in approximately equal amounts. Methylcobalamin and sulphitocobalamin were detected less frequently than adenosylcobalamin and hydroxocobalamin, but were present in significant amounts in many foods. Methylcobalamin was most prominent in egg yolk and in cheese and cheese products, and the higher proportion of methylcobalamin in cheese when compared to that found in milk suggested that there may have been biosynthesis of methylcobalamin by the bacteria utilized in the manufacture of cheese. Sulphitocobalamin has not been detected previously in biological material, and in our experience can occur readily as an artifact (Farquharson & Adams, unpublished results). In this study precautions were taken to prevent the formation of artifactual sulphitocobalamin, and we regard its occurrence in some foods as genuine; it may be significant that sulphitocobalamin was found only in canned foods. Cyanocobalamin was detected only in three items, haddock, egg white and one sample of cheddar cheese, and then only in small amounts.

From the results it appears that the contribution of cyanocobalamin to the dietary intake of vitamin B_{12} is very low.

When crystalline forms of adenosylcobalamin, methylcobalamin, hydroxocobalamin and cyanocobalamin are ingested by man in doses of 1 μg the amount which is absorbed is greatest when hydroxocobalamin is taken and least when adenosylcobalamin is taken (Adams *et al.* 1973), and from the results given in Table 2 it appears that sulphitocobalamin is as poorly absorbed as adenosylcobalamin at this dose level. With doses of 5 μg the absorption of methylcobalamin is greater than that of hydroxocobalamin and both are significantly better absorbed than adenosylcobalamin (Adams *et al.* 1973). From these results, and from the those given in Table 1, it might be concluded that the amount of vitamin B_{12} which is absorbed from either a mixed or a vegetarian diet will not be as great or as small as the extremes found with the crystalline cobalamins, but will be between these values. This conclusion, however, may be incorrect. First, it assumes that the forms of vitamin B_{12} in foods are as available as they are in crystalline form and, while there is evidence to this effect (Heyssel, Bozian, Darby & Bell, 1966), there are contrary opinions (Doscherholmen, McMahon & Ripley, 1971; Doscherholmen & Swaim, 1973). In this connexion it is relevant to report that with the extraction procedure used in this study the recoveries of radioactive vitamin B_{12} incorporated in human liver in vivo were 74% with raw tissue and 66% with tissue treated at 121° for 20 min, and that recoveries of radioactive cyanocobalamin taken up by hens' eggs in vitro were 49% with raw egg and 39% with eggs treated at 100° for 3 min (Farquharson, 1975). Secondly, the conclusion assumes that the form of vitamin B_{12} ingested in food is the form which is presented to the absorbing site, and this is doubtful. There is evidence that crystalline methylcobalamin in supraphysiological amounts is converted to other cobalamins, at least in the rat intestine (Okuda, Yashima, Kitazaki & Takara, 1973) and the results of in vitro studies suggest that hydroxocobalamin can be converted to sulphitocobalamin in the upper region of the gastrointestinal tract (Farquharson & Adams, unpublished results). These points are relevant to the problem of estimating the capacity to absorb vitamin B_{12}. The current practice of using crystalline radioactive cyanocobalamin is not physiological, but an alternative which is clinically and physiologically acceptable is not obvious. An attractive approach is the use of animal tissues (Heyssel *et al.* 1966) and hens' eggs (Schade & Schilling, 1967; Doscherholmen *et al.* 1971; Doscherholmen & Swaim, 1973) in which radioactive vitamin B_{12} has been incorporated in vivo. With these preparations, however, it remains to be established whether the incorporated vitamin B_{12} corresponds to the naturally occurring forms in the tissues or is simply present in the form in which it was administered. In addition, because of the well-established relationship between the amount of vitamin B_{12} ingested and the amount of vitamin B_{12} absorbed, the amount of vitamin B_{12} in these tissues must be known, and in this connexion it is worth noting that values for the vitamin B_{12} content of fatty foods, e.g. milk and eggs, obtained by radioisotopic or microbiological assays may be underestimates of the actual values (Craft, Matthews & Linnell, 1971; Adams *et al.* 1973).

Particular mention should be made of milk. In early work considerable technical problems were encountered when only a single unknown cobalamin was found. It has been concluded that this cobalamin was sulphitocobalamin, and was an artifactual form resulting from the conversion of hydroxocobalamin which involves not only the presence of sulphite ions but the light- and dark-mediated interrelationships of hydroxocobalamin and sulphitocobalamin (Farquharson & Adams, unpublished results). The results of subsequent studies on fresh light-protected cows' milk confirmed the finding of adenosylcobalamin by Craft *et al.* (1971); some methylcobalamin was also found in one sample. Craft *et al.* (1971) also found a branded milk food to contain mainly hydroxocobalamin, and our finding that hydroxocobalamin is the main constituent in light-exposed pasteurized milk supports their view that light-exposure of milk results in photolysis of the light-sensitive forms of vitamin B_{12} to hydroxocobalamin. The unexpected finding in this study of methylcobalamin and adenosylcobalamin in equal amounts in a preparation of evaporated milk was surprising, but could be explained by the fact that the milk and its derivatives were never exposed to light.

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