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A Native Cobalamin–Polypeptide Complex from Liver: Isolation and Characterization

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In mammalian liver vitamin B_{12} and related substances occur mainly as weakly bound protein or polypeptide complexes. These bound forms should probably be regarded as the biologically functional units rather than the free cyanocobalamin (Smith, 1958). Very little information, however, is as yet available about the nature of these complexes.

In a preliminary note from this Laboratory, the isolation of a cobalamin polypeptide from a liver concentrate (Organon WBC) was described (Hedbom, 1955). Because further supplies of this particular raw material were not available, we have now developed a method for obtaining a similar concentrate from fresh ox liver. From this it has been possible to isolate a cobalamin polypeptide, with essentially the properties previously described, in quantities which enable us to perform a more detailed examination.

This paper deals mainly with the isolation procedure and characterization of the cobalamin polypeptide.

EXPERIMENTAL AND RESULTS

Isolation of the cobalamin–polypeptide complex

No method of assay which is specific for the vitamin B_{12} conjugates in question is yet known. Therefore, the isolation could be guided only by determination of the total vitamin B_{12} content.

In the starting material and the first fractions from the isolation procedure, the vitamin B_{12} activity was detected and estimated by microbiological assay. Various extraction methods were tested in order to find the optimum conditions for the liberation of cyanocobalamin from the samples without destruction, and different micro-organisms requiring vitamin B_{12} were used and compared for the estimation. A modification of Burkholder's (1951) *Escherichia coli* tube method was found to be satisfactory for the purpose.

In more concentrated fractions the vitamin B_{12} content was estimated by absorption spectrophotometry, or by cobalt determination, as a check on the microbiological determination.

Microbiological assay

Preparation of samples. The vitamin B_{12} was released from the protein moiety by papain digestion. A sample (1 g.) was suspended in 10 ml. of 0·1 M-sodium acetate buffer, pH 5·0, containing 50 mg. of papain (E. Merck, Darmstadt) and a trace of cyanide. The mixture was incubated for 3 hr. at 40° and subsequently diluted with water to a total volume of 50 ml. After filtration and appropriate dilution, samples of the solution were assayed for vitamin B_{12} activity as described below.

Test organism. This was *Escherichia coli* mutant 113–3 (Davis & Mingioli, 1950). Stock cultures were stored and the inoculae prepared as described by Burkholder (1951).

Basal medium. The medium (in fivefold concentration) contained, in 200 ml. of water: K_2HPO_4 7·0 g., KH_2PO_4 3·0 g., trisodium citrate 0·5 g., $MgSO_4,7H_2O$ 0·1 g., $(NH_4)_2SO_4$ 1·0 g., glucose 10·0 g., thiomalic acid 0·1 g., L-asparagine 4·0 g., L-arginine 0·1 g., L-glutamic acid 0·1 g., glycine 0·1 g., L-histidine 0·1 g., L-proline 0·1 g., DL-tryptophan 0·1 g.

The pH was measured with a Beckman glass-electrode pH meter, and adjusted, if necessary, to 7·0, by addition of KOH. In each tube 1 ml. of medium was used.

Vitamin standard. This was a water solution containing 0·200 μmg. of cyanocobalamin/ml., prepared fresh before each assay from a stock solution containing 2 μg. of cyanocobalamin (on a colorimetric basis) and 2 mg. of KCN/ml. The standard solution was added in duplicates to assay tubes in the volumes 0, 0·2, 0·5, 1·0, 1·5, 2·0 and 4·0 ml. per

tube, i.e. over a range of 0·04–0·8 μmg. of vitamin B_{12} per tube.

Procedure. The volume of samples or standards in each tube was made up to 4 ml. with water and 1 ml. of medium was added, making the total volume of liquid 5 ml. The tubes were sterilized by autoclaving for 5 min. at 112°, subsequently cooled and incubated at 30° for 20 hr. The tubes were steamed for 10 min., cooled and the growth was estimated by measuring the turbidity at a wavelength of 650 mμ with a Hilger Spekker absorptiometer type H 760.

A standard curve showing growth measured as turbidity at different vitamin B_{12} levels is given in Fig. 1.

Chemical determinations

Spectrophotometric measurements. Absorption spectra were obtained with a Beckman model DU spectrophotometer. Silica cells of 1 cm. light path and 4 ml. capacity were used for routine measurements and cells of 5 cm. light path for accurate determinations. The cobalamin content of concentrated fractions was estimated from the absorption maximum in the region 350–360 mμ, assuming an extinction coefficient $E^{1\%}_{1\,cm.}$ 207 for pure cyanocobalamin.

Cobalt analyses. The material was dried to constant weight *in vacuo* over phosphorus pentoxide at 56°. After ignition of the samples in a platinum crucible, the cobalt was determined by a colorimetric procedure with nitroso-R-salt according to Shipmen, Foti & Simon (1955).

Isolation methods

For the isolation of the cobalamin–peptide complex the following procedure, with minor modifications, gave satisfactory results in five preparations. When more information has been collected about the properties of the complex it may be possible to work out a more efficient isolation method.

Step 1: preparation of the crude extract. Fresh ox liver was obtained from newly slaughtered animals. The liver (about 20 kg.) was cleaned, chilled and ground in a mincing machine at the slaughter house. The pH was adjusted to 8 by slow addition of 0·1 N-NaOH to inhibit autolysis.

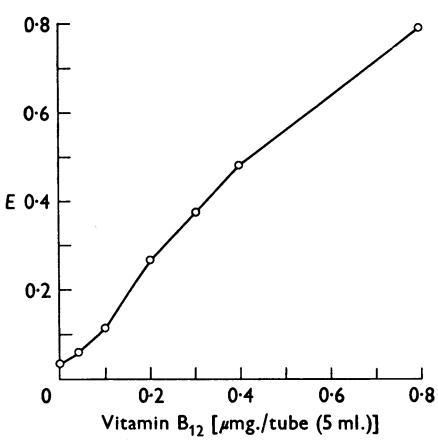

Fig. 1. Response of *E. coli* 113:3 to vitamin B_{12}. Growth (turbidity), measured as extinction at 650 mμ, is plotted against vitamin B_{12} concentration.

After addition of 2 l. of water/kg. of tissue, batches of the mixture were homogenized in a 6 l.-Turmix Blendor (type MV) at a temperature of 2–4° and squeezed through cheese-cloth.

The solution was acidified to pH 4·5 with N-H_2SO_4, and the precipitate formed was removed by centrifuging at 2500 *g* in the stainless-steel cups of a preparative Stock centrifuge (total capacity 7·8 l.). The supernatant was concentrated to about 5 l. under reduced pressure at 40° and again centrifuged. These operations were carried out in the shortest possible time.

Step 2: fractionation with ammonium sulphate. The extract was then fractionated at room temperature (18–20°) between 50 and 80 % $(NH_4)_2SO_4$ saturation by the addition of solid $(NH_4)_2SO_4$ (1565 + 1070 g.) in two steps, each followed by centrifuging. The precipitate, formed at 80 % and containing the bulk of active material, was then treated with ethanol–ether (1:4) to remove lipid material and redissolved in 1 l. of water.

Step 3: phenol extraction. The solution from step 2 was extracted with phenol in a separating funnel. The phenol phase was repeatedly washed with water and the vitamin B_{12}-containing material transferred into the aqueous phase by addition of ether to the system. After evaporation of the ether, the active material was precipitated by addition of cold acetone to 80 % (v/v) concentration. The precipitate was redissolved in 500 ml. of water.

Step 4: calcium phosphate treatment. The solution was filtered through a calcium phosphate column (60 cm. × 3 cm.). The absorbent was prepared by suspending 100 g. of cellulose powder (Munktell) in 1000 ml. of $CaCl_2,6H_2O$ solution (110 g./l.). With stirring, 150 ml. of $Na_3PO_4,12H_2O$ solution (125 g./l.) was then added. The column was washed with 0·05 M-sodium phosphate, pH 5·0. Some inactive brown-and-yellow impurities were strongly adsorbed on the column and thus removed. After concentration and precipitation with acetone, the active material was dissolved in 100 ml. of water.

Step 5: adsorption chromatography on alumina. The solution was allowed to pass through an adsorption column (60 cm. × 1 cm.) packed with activated alumina (Merck; 100–200 mesh size). The active material appeared as a number of poorly separated fast-migrating red-and-pink zones and some strongly adsorbed red or brown zones. Some yellow impurities were also visible. The fast-migrating zones were collected in an automatic fraction collector, pooled, concentrated to 100 ml. and used for further purification.

Step 6: butanol extraction of free cobalamins. The coloured solution from step 5 was saturated with $(NH_4)_2SO_4$ and extracted with butanol according to Ellis, Petrow & Snook (1949) and Fantes, Page, Parker & Smith (1949). The precipitate formed during this procedure was dissolved in water, and the solution was again saturated with $(NH_4)_2SO_4$ and extracted under the same conditions. The extraction was continued with fresh butanol until no more coloured material could be removed. The residual active material was taken up from the aqueous $(NH_4)_2SO_4$ phase by extraction with phenol. It was then transferred to the water phase by addition of ether as in step 3. After concentration and precipitation with acetone, a cobalamin–peptide concentrate resembling Wijmenga's fraction (Organon WBC) was obtained (Hedbom, 1955). The free cobalamins were found in the butanol extract.

Step 7: partition chromatography. A saturated aqueous solution of the concentrate from step 6 was applied to the top of a chromatography column (60 cm. × 1 cm.) packed with Munktell's cellulose powder and developed butanol saturated with water. The red or pink zones were collected and examined by spectroscopy. Fractions with absorption maxima at 350 mμ were concentrated and, if necessary, re-chromatographed.

Step 8: final purification by zone electrophoresis. The main fraction from step 7, with absorption maxima at 350 and 523 mμ, was then further purified by the electrophoretic method of Flodin & Porath (1954), Porath (1956). A column (50 cm. × 3·5 cm.) filled with Munktell's cellulose powder in a volatile buffer solution (0·1 M-pyridine–0·1 M-acetic acid, pH 4·5) was used for the experiment. The substance was first displaced to 10 cm. below the top of the column, then a current of 40 mA was passed through it for 24 hr. Under these conditions three different cobalamin-containing compounds were separated. After freeze-drying, the main fraction was obtained in solid form as a pure cobalamin–peptide complex.

Results

A summary of the procedure and the results for preparation no. 3 are reported in Table 1.

This preparation, chosen as a typical one, gave 5·7 mg. of the pure cobalamin–peptide complex with a vitamin B_{12} activity (for *E. coli*) of 14 900 (μg. of vitamin B_{12}/g.) compared with an initial activity of 1·12 (μg. of vitamin B_{12}/g.) for the minced liver tissue. This represents a purification of 13 300-fold. The final yield was 3·8 % calculated on the same basis. It must be remembered, however, that the cobalamin peptide is responsible for only part of the total activity in the starting material; thus the recovery was in fact even greater.

Some general properties of the cobalamin–polypeptide complex

The isolated cobalamin peptide is a pink amorphous powder, readily soluble in water and dilute salt solutions. It is precipitated at high salt concentrations and is almost insoluble in ether and acetone. The vitamin B_{12} complex is stable for weeks in the pH range between 2 and 8; examination showed that no step in the isolation procedure could bring about any detectable destruction.

From qualitative tests, the complex seems to contain a peptide or protein part free from detectable carbohydrates and amino sugars. (Protein reactions: biuret +; ninhydrin −?; xanthoproteic reaction +; Millon +; Pauli +; Adamkiewicz–Hopkins −; Sakaguchi −. Carbohydrate reactions: Molisch −?; Seliwanoff −; Ehrlich −; anthrone −.)

The criteria for protein purity will be reported in a separate paper on the physicochemical properties of the complex. The following facts may be used for a preliminary assessment of purity.

The substance behaved as a single component when examined by paper electrophoresis at pH 4·0 (acetate buffer), pH 6·5 (phosphate buffer) and pH 8·6 (veronal–acetate buffer) as well as by partition chromatography on paper with four different solvent systems [butanol–acetic acid–water (4:1:5); pyridine–*iso*amyl alcohol–water (1:1:2); phenol–water (4:1); butanol–aq. 3 % NH_3 solution (3:1)]. The absorption spectrum and solubility properties remained unaltered during these experiments.

Amino acid composition. The complex (1·06 mg.) was dissolved in 1 ml. of HCl–water (1:1) and hydrolysed in a sealed Pyrex tube at 102° for 24 hr. After evaporation to dryness the residue was examined for amino acids by two-dimensional ascending paper chromatography with methanol–water–pyridine (40:10:2) in the first and butanol–methyl ethyl ketone–water–diethylamine (20:20:10:2) in the second direction as described by Sisakyan, Bezinger, Garkavi & Kivman (1954). The following amino acids were detected: glycine, alanine, threonine, valine, leucine, isoleucine, aspartic acid, glutamic acid, tyrosine, histidine, cysteine, proline and lysine.

To check this result, one-dimensional descending chromatograms were made with butanol–acetic acid–water (5:1:4). The chromatograms were sprayed with reagents specific for certain amino acids. In this way the presence of histidine was

Table 1. *Isolation of the cobalamin–peptide complex from ox liver*

Step no.	Volume (ml.)	Weight (mg.)	Vitamin B_{12} activity (μg./ml.)	Total vitamin B_{12} activity (μg.)	Recovery (%)
0. Minced liver (20 kg.)	50 000	—	0·448	22 400	100
1. Crude extract	5 000	—	2·10	10 500	47
2. $(NH_4)_2SO_4$ precipitation	1 000	—	8·95	8 950	40
3. Phenol extraction	500	—	17·6	8 800	39
4. Calcium phosphate chromatography	100	—	71·5	7 150	32
5. Alumina chromatography	100	—	59·7	5 970	27
6. Butanol extraction	—	127·8	—	1 250	5·6
7. Partition chromatography	—	12·5	—	1 210	5·4
8. Column electrophoresis	—	5·7	—	850	3·8

confirmed with the Pauli reagent (Sanger & Tuppy, 1951), tyrosine with α-nitroso-β-naphthol (Acher & Crocker, 1952), proline with isatin (Acher, Fromageot & Jutisz, 1950) and cysteine with the platinic chloride–KI reagent (Toennies & Kolb, 1951).

Cobalt content and minimal molecular weight. Cobalt analyses on four different preparations gave results of 0·632, 0·653, 0·647 and 0·651 % of cobalt, corresponding respectively to minimal molecular weights of 9326, 9026, 9110 and 9053 (mean value 9129).

Absorption spectrum. The complex was examined in aqueous solution (0·15 mg./ml.) for absorption in the visible and ultraviolet regions with a Beckman spectrophotometer model DU. The absorption curve (Fig. 2) shows pronounced peaks at 274 and 350 mμ and broad maxima at 500 and 523 mμ. For comparison the absorption spectrum of cyanocobalamin is also shown.

Crude preparations of the complex have a broad maximum at 350–360 mμ. After the extraction of free cobalamins in step 6, this maximum shifts toward lower wavelengths and becomes sharper.

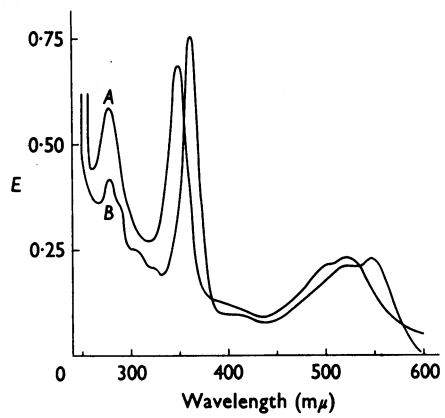

Fig. 2. Absorption spectrum (*A*) of the cobalamin–polypeptide complex (0·15 mg./ml. in aqueous solution of pH 7·0) compared with that of an equimolar solution of cyanocobalamin (*B*).

After the final column electrophoresis it remains constant at 350 mμ.

The absorption at 274 mμ is mainly due to the peptide part, whereas those at 350 and 523 mμ are due to the cobalamin. The spectrum differs from that of vitamin B_{12} itself, both in the position and the relative height of the peaks. It is thus impossible to get an accurate estimation of the vitamin B_{12} content of the complex from the absorption at any particular wavelength.

Effect of cyanide on the absorption spectrum. Addition of trace amounts of cyanide to a neutral or slightly acid solution of the complex is followed by a bathochromic shift of the peaks at 350 and 523 mμ. The result is a spectrum of cyanocobalamin type with characteristic absorption at 361 and 550 mμ, with extinction coefficients ($E^{1\%}_{1\,cm.}$) of 30·5 and 9·35. These extinction values may constitute a more reliable basis for the calculation of the cobalamin content by comparison with a cyanocobalamin standard (see Table 2). A cobalamin (molecular weight 1329) content of 14·68 % corresponds to a minimal molecular weight of 9053, in fairly good agreement with the value of 9129 from the cobalt content.

Infrared absorption. The infrared-absorption spectra of the peptide complex and of cyanocobalamin have been determined and compared. A Perkin–Elmer model 21 spectrophotometer equipped with sodium chloride prism was used for the absorption measurements in the region 2–15 μ. The substances were examined as solid-pressed potassium bromide plates according to the method described by Schiedt & Reinwein (1952). The absorption curves are shown in Fig. 3. The two curves show trivial differences in the peptide-bond regions but also a more interesting one, namely the absence of the characteristic cyanide absorption at the 4·0–4·5 μ region in the spectrum of the complex.

Sedimentation coefficient. A 1·0 % (w/v) solution of the cobalamin peptide in phosphate buffer, pH 6·83, *I* 0·2, sedimented with a single boundary in a prolonged sedimentation run (6 hr.) at 260 000 *g* in Svedberg's analytical ultracentrifuge.

Table 2. *Calculation of the cobalamin content of the complex by comparison of the absorption spectrum at different wavelengths with a cyanocobalamin standard*

Cobalamin peptide sample		Cyanocobalamin standard		Calculated cobalamin content
mμ	$E^{1\%}_{1\,cm.}$	mμ	$E^{1\%}_{1\,cm.}$	(%)
361	14·5	361	207	7·00
350	26·8	361	207	12·90
550	5·72	550	64	8·94
523	8·90	550	64	13·90
After addition of cyanide				
361	30·5	361	207	14·75
550	9·35	550	64	14·6(

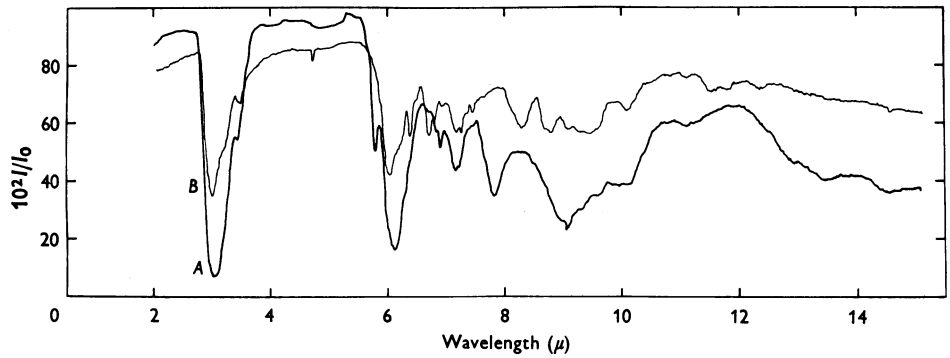

Fig. 3. Infrared spectrogram of the cobalamin–polypeptide complex (*A*) compared with that of cyanocobalamin (*B*).

The result corrected to a temperature of 20° in water gave a sedimentation coefficient $S_{20,w}$ of 1·0s.

Diffusion coefficient. The diffusion coefficient was preliminarily estimated with the porous-disk method essentially according to Northrop & Anson (1929). The experiments were performed with samples which still showed minor impurities in the ultracentrifuge. The samples were dissolved in a phosphate buffer, pH 6·80, *I* 0·2. The cell was calibrated by means of 0·1 M-KCl solution of known diffusion coefficient (Lamm, 1937).

The diffusion coefficient thus obtained and corrected to water at 20° had a value $D_{20,}$ of $11 \cdot 10^{-7}$ cm.^{2} sec.$^{-1}$.

Molecular weight. From the determined values for the sedimentation and diffusion coefficients, assuming a partial specific volume ($\bar{V}$) of 0·73, the molecular weight was calculated to be 8200 (Svedberg & Pedersen, 1940). As $S_{20,w}$ has not been extrapolated to zero concentration, D_{20} was determined by a somewhat inaccurate method and $\bar{V}$ estimated only by comparison with similar substances, this value is only approximate. However, it indicated that the true molecular weight is equal to the previously determined minimal weight of about 9100.

DISCUSSION

It seems plausible that the absorption, transport, storage and biological function of vitamin B_{12}, other cobalamins and vitamin B_{12} analogues are accomplished by the formation of protein or peptide complexes. The existence of such entities is fairly well established, and the difficulties encountered in the early attempts to isolate vitamin B_{12}, as summarized by Subbarow, Hastings & Elkin (1945), may partly be explained by such a circumstance. Lester Smith and his co-workers (Fantes *et al.* 1949) observed some protein-containing active fractions during their isolation of the anti-pernicious-anaemia factor from liver. Treatment with proteolytic enzymes made a further purification possible. Scheid & Schweigert (1950) reported that hot water could extract bound forms of vitamin B_{12} and make them available for micro-organisms. Wijmenga, Veer & Lens (1950–51) found treatment with cyanide to have the same liberating effect, whilst converting other cobalamins into the more stable cyanocobalamin. Hausmann (1949) and Hausmann & Mulli (1951, 1952*a*, *b*) extracted heat-stable pigments from ox liver and other sources, and assumed the vitamin B_{12} activity of these preparations to be due to cobalamin peptides.

Pitney, Beard & Van Loon (1955) investigated liver homogenates by paper electrophoresis. Under their conditions (electrophoresis of 1·0 and 0·9 M-sodium chloride homogenates of human-liver tissue on Whatman no. 3MM paper with veronal buffer of *I* 0·10 and 0·05 at pH 8·6), the vitamin B_{12} activity (assayed with *Euglena gracilis*) was found to migrate towards the anode. From these experiments they considered it evident that vitamin B_{12} in liver tissue exists in some type of combination with protein. They also stated that the specific protein in liver responsible for the 'binding' of vitamin B_{12} has an electrophoretic mobility similar to that of serum β-globulin.

Our fractionation studies have confirmed most of these results. We found vitamin B_{12} activity, however, in several fractions. Differences in spectra and availability for micro-organisms that could not be explained as denaturation or degradation effects indicated the existence of more than one type of complex. As it seems possible to isolate not only peptide complexes but also some high-molecular-weight vitamin B_{12} compounds, it may be more relevant to discuss questions of denaturation in connexion with further studies of such compounds. It will be enough to point out that the mild isolation procedure applied in the present experiments can hardly cause any appreciable alteration of the primary structure; breaking

of covalent bonds seems to be unlikely under the conditions employed.

The most plausible idea about the isolated vitamin B_{12} peptide seems to be that it represents a structural unit, occurring also in the whole liver cell either as such or possibly as a part of a larger arrangement formed, for instance, by protein–protein interaction. Its biological role is still an open question; it may constitute a storage form of vitamin B_{12} or take part in some metabolic system.

The absorption spectrum of the cobalamin peptide shows a peak at 350 mμ with a marked shift to 361 mμ on addition of cyanide. This suggests a cobalichrome type of connexion between the cobalamin and the peptide part (Cooley *et al.* 1951), a view also supported by the absence of cyanide absorption in the infrared spectrum of the complex.

SUMMARY

1. A cobalamin–polypeptide complex has been isolated from ox liver by a technique involving essentially physical methods. The complex was obtained as a pink amorphous powder in a yield of 3·8 %, representing a purification of more than 13 000-fold.

2. The substance behaved as a single component when examined by chromatography and electrophoresis on paper. After acid hydrolysis the following amino acids were identified: glycine, alanine, threonine, valine, leucine, isoleucine, aspartic acid, glutamic acid, tyrosine, histidine, cysteine, proline and lysine.

3. The absorption spectrum has characteristic peaks at 274, 350 and 523 mμ. After addition of cyanide the absorption changes to a cyanocobalamin type of spectrum with maxima at 274, 361 and 550 mμ. The infrared spectrum of the complex shows no absorption in the 4·5 μ region. This indicates the absence of cyanide.

4. Calculations of the cobalamin content from the absorption spectrum at different wavelengths gave somewhat divergent results. After addition of cyanide more consistent results of 14·7 % were obtained.

5. The molecular weight, as determined by cobalt analyses and estimated from sedimentation and diffusion data, was found to be about 9100.

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