Some Properties of the Cyanocobalamin–Protein Complex from Sow's Milk, and the Mode of Linkage of Cyanocobalamin with Protein

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The occurrence of a cyanocobalamin-binding protein in milk and the isolation of a cyanocobalamin–protein complex from sow's milk whey is described in the preceding paper (Gregory & Holdsworth, 1955). The first part of this paper describes some chemical and microbiological properties of the complex, and provides evidence that it is a combination of cyanocobalamin with a glycoprotein. The manner in which these two components are linked together has been investigated in two ways. One was to test whether blocking specific groups on the protein decreased its capacity to combine with cyanocobalamin. The second was to modify the cyanocobalamin molecule and test whether it still combined with unaltered protein. The results of these investigations are presented in the second part of this paper.

SOME PROPERTIES OF THE CYANOCOBALAMIN–PROTEIN COMPLEX

Experimental and results

Absorption spectrum. The material, isolated as described in the preceding paper (Gregory & Holdsworth, 1955), was examined in aqueous solution (0·5 mg./ml.) for absorption in the visible and u.v. regions, using a Beckman Spectrophotometer.

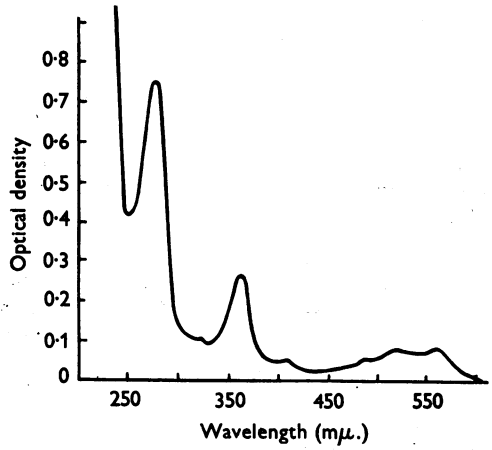

Fig. 1. The absorption spectrum of the cyanocobalamin–protein complex in aqueous solution (0·5 mg./ml.).

The curve (Fig. 1) shows pronounced peaks at 278 and 362 mμ. and inflexions at 410, 520 and 550 mμ., these last four bands arising from absorption due to the cyanocobalamin molecule. The slight shift of the peak from 361 mμ., as in free cyanocobalamin, to 362 mμ. in the complex, was repeatedly observed and is regarded as significant. Considerable excess cyanide ion at pH values up to 11 did not alter the position of the 362 mμ. band of the complex. If it is assumed that combination with protein has not affected the height of the absorption band at 361–362 mμ. of cyanocobalamin ($E^{1\%}_{1\,cm.} = 204$), the optical density of the solution of the complex at 362 mμ., $E^{1\%}_{1\,cm.} = 5{\cdot}7$, can be used to calculate its cyanocobalamin content. From the data obtained 1 mg. of the complex contains 23·6 μg. cyanocobalamin. The absorption band at 278 mμ., $E^{1\%}_{1\,cm.} = 15{\cdot}1$ is almost entirely due to the protein component.

Estimation of tryptophan and tyrosine. The tryptophan and tyrosine content of the complex was measured by the method of Holiday (1936), which compares the optical densities, at 280 and 305 mμ., of the material dissolved in 0·1 N-NaOH. For a solution containing 0·5 mg. complex/ml. of 0·1 N-NaOH, $E_{280} = 1{\cdot}26$ and $E_{305} = 0{\cdot}66$, and from the formulas given by Holiday the tyrosine content was calculated as 17·6% and that of tryptophan as 2·7%.

The value 17·6% is unusually high for the tyrosine content of a protein and a control experiment with a protein known to have a high tyrosine content was done under the same conditions. We found 13% tyrosine in crystalline pepsin (Armour), a figure higher than the generally accepted value of 10% (Calvery, Herriott & Northrop, 1936).

Estimation of total nitrogen. The estimation was done in triplicate with 100 μg. portions of the complex (dried over P_2O_5). The sealed-tube digestion procedure of Grunbaum, Schaffer & Kirk (1952), was used and the ammonia produced estimated by Nessler's reagent. The complex contained 16·1% N.

Amino acid composition. The complex (0·72 mg.) was dissolved in 1 ml. of a 1:1 (v/v) mixture of conc. HCl and glacial acetic acid and hydrolysed in a sealed tube at 102° for 20 hr. After evaporation *in vacuo* over KOH, the residue was dissolved in 0·1 ml. of water and examined for amino acids by two-dimensional chromatography using phenol–water and butanol–acetic acid–water, as the solvent systems. The following amino acids were detected: cystine, aspartic and glutamic acids, arginine, lysine, histidine, serine, glycine, threonine, alanine, tyrosine, valine, methionine, phenylalanine, leucine and isoleucine.

One-dimensional chromatograms were made using butanol–acetic acid–water (5:1:4 by vol.) and sprayed with reagents specific for certain amino acids. The Pauly reagent (Bolling, Sober & Block, 1949) confirmed the presence of histidine, the platinic chloride–KI reagent of Toennies &

Kolb (1951) showed cystine to be present. The Morgan & Elson method for detecting amino sugars, as adapted to paper chromatography by Partridge (1949), showed the presence of a substance with an R_F identical with that of glucosamine or chondrosamine.

Estimation of carbohydrate. A colorimetric method for estimating carbohydrate with anthrone (Morris, 1948) was used, with glucose and glycogen as reference substances. The complex contained 7·3% carbohydrate calculated as glucose. The ribose present in the cyanocobalamin molecule would not make a significant contribution to this figure.

Estimation of hexosamine. The complex (0·5 mg.) was hydrolysed with 3N-HCl at 100° in a sealed tube for 4 hr., and concentrated *in vacuo* over KOH. The residue was dissolved in 1 ml. of water and the hexosamine present estimated by the method of Elson & Morgan (1933). Albumin (0·5 mg.) and albumin (0·5 mg.) with 50 μg. glucosamine were used as negative and positive control mixtures. The complex contained 9% hexosamine calculated as glucosamine.

Microbiological estimation of cyanocobalamin in the complex. A portion of a concentrated solution of the cyanocobalamin–protein complex was diluted with sterile distilled water to contain 0·2 μg. cyanocobalamin/ml. as estimated from the absorption curve (described above). A sample (1 ml.) of this solution, added aseptically to a sterile plugged tube, was digested with 10 mg. papain (British Drug Houses Ltd.) suspended in 1 ml. of sterile 0·1M sodium acetate buffer, pH 4·6, containing a trace of cyanide. A further 1 ml. of the bound cyanocobalamin solution was digested with 2 mg. of crystalline trypsin (Armour) dissolved in 1 ml. of sterile 1% (w/v) $NaHCO_3$ solution containing a trace of calcium. The two mixtures were incubated for 3 hr. at 57°, half the enzyme being added at the beginning and the remainder half-way through the incubation period. The digests were then made to a total volume of 4 ml. with sterile distilled water. A portion of the diluted digest was removed and assayed for cyanocobalamin both unheated and heated with the assay medium. The remainder of the digest was heated for 30 min. in steam, ultrafiltered and the ultrafiltrate assayed for cyanocobalamin. For this experiment the *Escherichia coli* tube assay was used to measure cyanocobalamin as described by Gregory & Holdsworth (1953). The full availability of the cyanocobalamin for *Esch. coli*, after heating the enzyme digest of the complex with the assay medium is apparent from Table 1. The figure, 0·19 μg. of cyanocobalamin/ml. (22·4 μg./mg. substance), agrees with the 0·20 μg./ml., calculated from the optical density of the complex at 362 mμ. Although after heating of the enzyme digests the cyanocobalamin was fully available to the assay organism, only 10% of the vitamin was ultrafiltrable from this heated digest, i.e. it was not free cyanocobalamin that was being measured.

Table 1. *Liberation of cyanocobalamin from the protein complex by enzymic digestion*

The assay organism was *Escherichia coli*.

Treatment	Total cyanocobalamin assayed: Heated (μg./ml.)	Total cyanocobalamin assayed: Unheated (μg./ml.)
None	0·12	None
Papain digestion	0·19	0·04
Tryptic digestion	0·19	0·04

THE MODE OF LINKAGE OF CYANOCOBALAMIN WITH PROTEIN

Experimental

The binding factor concentrate. A protein concentrate was prepared from sow's milk whey by $(NH_4)_2SO_4$ fractionation as described by Gregory & Holdsworth (1953). This protein fraction, capable of binding 100 μg. cyanocobalamin/g., is referred to as SF 8.

Compounds related to cyanocobalamin. The monobasic acid *E*, the corresponding acid without the nucleotide, the tribasic acid, and the mixture of hexa and hepta acids were all fragments of cyanocobalamin prepared by partial hydrolysis, and were kindly supplied by Dr E. Lester Smith (Glaxo Laboratories Ltd.). The preparation of these compounds is described by Armitage *et al.* (1953). The thiocyanatocobalamin was a gift from Dr Wokes (Ovaltine Laboratories Ltd.).

The formaldehyde-treated cyanocobalamin was prepared as follows: 400 μg. cyanocobalamin were dissolved in 9 ml. of 1% (w/v) Na_2CO_3 solution, and 1 ml. of (A.R.) formaldehyde solution (*ca.* 36% (w/v) formaldehyde) was added. After standing 15 hr. at 1° the solution was evaporated to dryness *in vacuo* (temperature less than 37°) and the residue extracted with cold 80% (v/v) ethanol. The substance was purified by chromatography on paper using *sec.*-butanol saturated with water as the solvent. Only one zone, with an R_F slightly greater than that of the original vitamin, was obtained. The material was microbiologically inactive for *Esch. coli* and *Lactobacillus leichmannii* as test organisms.

Acetylation of SF 8. The method for acetylating proteins described by Baddiley, Kekwick & Thain (1952) was used. 2′-Acetylthioethylacetamide (10 mg.) was added to 5 mg. portions of SF 8 dissolved in 0·1M sodium borate buffers at pH 8·0 and 10·0. The air in the flasks containing these solutions as a shallow layer, was displaced by oxygen, the flasks were tightly stoppered and incubated at 37° for 45 hr. The reagent and buffer were then removed by dialysis and the protein solution diluted to a concentration of 1 mg./ml. Crystalline bovine serum albumin (Armour) was acetylated at pH 10 to act as control.

The effect of specific group inhibitors on the combination of SF 8 with cyanocobalamin. When 0·2 μg. cyanocobalamin was added to a solution containing 1 mg. SF 8, half the added vitamin was bound and the rest remained free. The free vitamin was estimated by the ultrafiltration method described in the preceding paper (Gregory & Holdsworth, 1955). The reagents for blocking specific groups on the protein were dissolved in 1–8·5 ml. water and added to 1 mg. SF 8 in 0·5 ml. water as shown in Table 2. The solutions were mixed well and kept at 30° for 1 hr. At the end of this time 0·2 μg. cyanocobalamin was added to each tube and the total volume made to 10 ml. The solutions were then ultrafiltered and the ultrafiltrates assayed for cyanocobalamin using *Lb. leichmannii* as described by Gregory (1954). If the amount of cyanocobalamin in the ultrafiltrate was greater than that obtained from a control containing untreated SF 8 and cyanocobalamin, then the reagent had blocked a group on the protein necessary for its combination with the vitamin. All the inhibiting reagents were tested to ascertain that they had no growth-inhibitory effect on *Lb. leichmannii* at the dilutions present in the assay tubes. The acetylated SF 8 was examined for binding activity by this method and, after saturation with [^{60}Co] cyanocobalamin,

by electrophoresis and autoradiography as described in the preceding paper (Gregory & Holdsworth, 1955).

Combination of the altered cyanocobalamin molecule with SF 8. The combination of the compounds having structures similar to cyanocobalamin with the binding protein (SF 8) was measured by the ultrafiltration method (Gregory & Holdsworth, 1955). The technique varied slightly depending on whether the substance tested possessed microbiological activity.

The monobasic acid *E* (0·2 μg.), or thiocyanatocobalamin (0·2 μg.) were added to 1 mg. SF 8 in distilled water. The volume was made to 10 ml. and the solution ultrafiltered. The ultrafiltrate containing the acid *E*, was diluted to contain 0·2 mμg. of the acid per ml., and that containing the thiocyanatocobalamin to contain 0·04 μmg. per ml. Both were assayed by *Lb. leichmannii* (Gregory, 1954). The concentrations of these two compounds were calculated from growth-response curves given by *Lb. leichmannii* to known amounts of these compounds.

The acid *E*, without the nucleotide, the tribasic acid, the mixture of hexa and hepta acids and the formaldehyde-treated cyanocobalamin were inactive or only slightly active for *Lb. leichmannii*. These compounds, or cobalt sulphate or cobalt mesoporphyrin, were added in 0·1 μg. amounts to 1 mg. SF 8 in distilled water. The mixtures were left at room temperature for 30 min. when 0·2 μg. cyanocobalamin was added. The volumes were made to 10 ml., the solutions ultrafiltered and the ultrafiltrates assayed for free cyanocobalamin. If the various compounds had combined with SF 8 the cyanocobalamin added subsequently would be free and able to pass into the ultrafiltrate. The amount of cyanocobalamin in these ultrafiltrates was compared with that present in the ultrafiltrate from a control experiment using SF 8 and cyanocobalamin. From this comparison it could be deduced whether the binding centres of SF 8 had previously been saturated by the compounds before the introduction of the cyanocobalamin.

Experiment using [^{14}CN]*cobalamin.* Cyanocobalamin in which the cyano group was labelled with ^{14}C, was a gift from Dr E. Lester Smith (Glaxo Laboratories Ltd.). Wet combustion of the material (specific activity approximately 0·2 μc/mg.) and gas counting of the CO_2 produced using a permanent vacuum line technique as described by Glascock (1954) showed that 50 μg. cyanocobalamin contained activity equivalent to $1{\cdot}77 \times 10^4$ counts/min.

A quantity of binding protein sufficient to combine with 1000 μg. cyanocobalamin was taken and mixed in solution with 500 μg. of the [^{14}CN]cobalamin. The mixture was immediately placed in a dialysis bag of cellophan tubing (closed at both ends) and immersed in distilled water. Four portions of 250 ml. water were used for dialysis over a period of 24 hr. To the combined dialysates were added 10 mg. of carrier KCN, the liquid was acidified to pH 3 with H_2SO_4, the HCN removed in a stream of N_2 and trapped in N-NaOH as in the method of estimating cyanide ion described by Boxer & Rickards (1951). The cyanide present in the NaOH was precipitated as AgCN, dried and counted by an end-window counter. After dialysis 500 μg. of unlabelled cyanocobalamin were added to fully saturate the binding factor and the cyanocobalamin–protein complex was purified as described in the preceding paper (Gregory & Holdsworth, 1955). A portion of the complex estimated to contain 50 μg. cyanocobalamin (25 μg. of the [^{14}CN]-cobalamin) by the spectroscopic method described earlier, was converted into CO_2 by wet combustion and the activity of the CO_2 produced was measured by gas counting.

RESULTS

The effect of specific group inhibitors on the combination of SF 8 with cyanocobalamin. The different reagents used to block specific groups on the protein molecule and the effect they had on the combination of SF 8 with cyanocobalamin are shown in Table 2. Of the reagents tested, fluorodinitrobenzene and iodine, at the higher concentrations, completely prevented the protein from combining with the vitamin. The query opposite formaldehyde treatment at pH 11 indicates that no cyanocobalamin could be measured in the ultrafiltrate. It was later found that, under the same experimental conditions, this concentration of formaldehyde at pH 11 (but not at pH 8) completely inactivated cyanocobalamin for *Lb. leichmannii*. In a repeat experiment, the solution of SF 8 and formaldehyde at pH 11 was dialysed to remove the excess formaldehyde before the cyanocobalamin was added. This treatment had no effect on the binding activity of the protein.

Table 2. *Effect of specific group inhibitors on the combination of SF 8 with cyanocobalamin*

The assay organism was *Lb. leichmannii*.

Amount of inhibiting reagent used/mg. SF 8 in 0·5 ml. of water	Effect on the combination of 1 mg. SF 8 with cyanocobalamin
1 ml. of 0·01 M *p*-chloromercuribenzoic acid	None
1 ml. of 0·01 M fluorodinitrobenzene	None
1 ml. of 0·17 M fluorodinitrobenzene	Complete inactivation
1 ml. of 3% formaldehyde at pH 8	None
1 ml. of 3% formaldehyde at pH 11	? (see text)
2 ml. of 0·01 N iodine in 0·05 M KI solution at pH 5·7	None
1 ml. of 0·01 N iodine in 0·01 M KI solution at pH 8·5	25% inactivation
*1 ml. of 0·1 N iodine in 0·1 M KI solution at pH 8·5	55% inactivation
*0·1 ml. of 0·5 N iodine in 0·5 M KI solution at pH 8·5	Complete inactivation
2 ml. of 0·01 M iodoacetic acid	None
8·5 ml. of 0·002 M *o*-iodosobenzoic acid	None
8·5 ml. of 0·002 M phenylmercuric acetate	None

* Dialysed for 6 hr. against running water and distilled water before the addition of cyanocobalamin.

Acetylation of SF 8 at both pH 8 and 10 by the method of Baddiley *et al.* (1952) did not alter its capacity to combine with cyanocobalamin. That the acetylation procedure was effective is apparent from Fig. 2, which shows that when crystalline serum albumin was acetylated, two substances were produced, both having greater electrophoretic mobilities than the original albumin. Furthermore, when acetylated SF 8 was saturated with [^{60}Co] cyanocobalamin, autoradiographs after electrophoresis showed that the cyanocobalamin was bound, and the resulting complex had a greater mobility than that formed from the untreated protein.

Combination of the altered cyanocobalamin molecule with SF 8. The ultrafiltration method of measuring binding activity showed that the monobasic acid *E*, the monobasic acid without the nucleotide, the tribasic acid, thiocyanatocobalamin and formaldehyde-treated cyanocobalamin all combined equally with SF 8. The only compounds tested that did not combine with the protein were the hexa- and hepta-acid decomposition products of cyanocobalamin and cobalt sulphate and cobalt mesoporphyrin.

Experiments with [^{14}CN]*cobalamin.* When cyanocobalamin, labelled with ^{14}C in the cyano group, was added to SF 8 and the mixture dialysed, no radioactivity appeared in the dialysate, indicating that the cyano group was not released during combination between vitamin and protein. Even after the considerable chemical manipulations involved in the isolation of the [^{14}CN]cobalamin–protein complex, the radioactivity in a portion of the material containing 50 μg. cyanocobalamin (25 μg. of the labelled vitamin) was $8{\cdot}55 \times 10^3$ counts/min., i.e. 96 % of the radioactivity of a corresponding amount of the original cyanocobalamin.

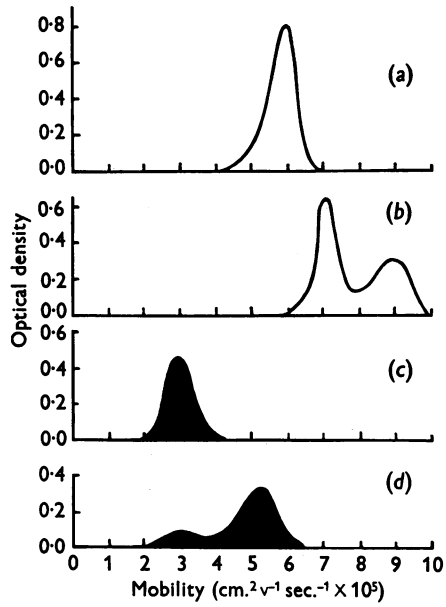

Fig. 2. Electrophoresis in sodium veronal buffer at pH 8·6: (*a*) pattern given by crystalline bovine serum albumin protein; (*b*) pattern given by acetylated bovine serum albumin protein; (*c*) autoradiograph of SF 8 with added [^{60}Co]cyanocobalamin; (*d*) autoradiograph of acetylated SF 8 with added [^{60}Co]cyanocobalamin.

DISCUSSION

The chemical examination of the cyanocobalamin–protein complex showed that the complex contained 16·1 % nitrogen and had an amino acid composition typical of proteins, except for a rather high tyrosine content. Since the anthrone method of Morris (1948) for carbohydrates does not estimate amino sugars, the complex contains 7 % carbohydrate in addition to the 9 % hexosamine. The presence of these sugar residues suggests that the binding substance is a glycoprotein.

The absorption curve clearly indicates the presence of both cyanocobalamin and a protein. The cyanocobalamin content, calculated from the height of the peak at 362 mμ. was 23·6 μg./mg. of complex. The quantity of material isolated was insufficient for application of any of the available methods for determining molecular weights. If it can be assumed that 1 molecule of cyanocobalamin combines with 1 molecule of protein, then the molecular weight can be calculated from the ratio of cyanocobalamin to protein in the complex. A molecular weight of 55 000 can be deduced for the protein from the presence of 23·6 μg. cyanocobalamin (mol.wt. 1300) in 1 mg. of the complex.

When this paper was being prepared for publication, a preliminary note by Wijmenga, Thompson, Stern & O'Connell (1954) reported the isolation of a similar cyanocobalamin–protein complex from pig gastric mucosa. The absorption curve we obtained for our complex was identical with that reported by Wijmenga *et al.* (1954), and yet they only found 12·3 μg. cyanocobalamin/mg. of the complex. From the sedimentation constant they concluded that the molecular weight of their complex was about 100 000.

For a quantitative microbiological estimation of the cyanocobalamin in our complex, we found that a preliminary digestion with papain or trypsin was necessary. Either of these two enzymes converts the microbiologically inactive complex into a form fully active for *Bact. coli* when heated with the basal assay medium, but having only 20 % of this activity when assayed unheated. Furthermore, ultrafiltration of the heated digest showed that not more than 10 % of the total cyanocobalamin had become

ultrafiltrable. Since cyanocobalamin itself can be ultrafiltered quantitatively, it appears possible that the digest contains peptide conjugates of cyanocobalamin.

Without the enzyme digestion, only 60 % of the cyanocobalamin in the complex was available to *Bact. coli* after heat treatment. This fact may explain why Pitney, Beard & Van Loon (1954) were unable to recover, by heat treatment, all of the cyanocobalamin added to human serum.

In a previous paper we showed that the vitamin B_{12}-like compounds, factor *A*, factor *B* and pseudovitamin B_{12} were all bound by a protein concentrate from sow's whey (SF 8) and by an 'intrinsic factor' concentrate, to the same extent as cyanocobalamin (Gregory & Holdsworth, 1953). These vitamin B_{12}-like compounds differ from cyanocobalamin in their nucleotide groups. In factor *A*, the dimethylbenziminazole moiety of cyanocobalamin is replaced by 2-methyladenine (Brown & Smith, 1954) and in pseudovitamin B_{12} it is replaced by adenine (Pfiffner, Dion & Calkins, 1952). Removal of the nucleotide from cyanocobalamin, factor *A* or pseudovitamin B_{12} gives factor *B* (Gant, Smith & Parker, 1954). The nucleotide group, therefore, is not concerned in the combination of the vitamin with the protein.

The experiments reported in this paper have shown further that hydrolysis of the three primary amide groups in the cyanocobalamin molecule does not affect the ability of the vitamin to combine with the protein, but more drastic hydrolysis to the hexa and hepta acids (Armitage *et al.* 1953) gave a substance which no longer combined with the protein.

The experiments with [^{14}CN]cobalamin showed that the protein did not displace the cyano group when it combined with cyanocobalamin, and since thiocyanatocobalamin also combines with the protein, the linkage does not take place through the cyano group. The absorption spectrum of cyanocobalamin has a peak at 361 mμ. which shifts to 367 mμ. when the double cyanide complex is formed in the presence of excess cyanide ions. The absorption spectrum of the cyanocobalamin–protein complex has a peak at 362 mμ. which is unaffected by the addition of excess cyanide ions. This fact suggests that either the protein is linked to the vitamin in the place where the second cyano group normally adds on, or that steric hindrance by the protein prevents the cyanide ion from adding on to the cyanocobalamin in the complex. A more precise determination of the point of attachment of the protein to the vitamin molecule now awaits further information about the 'core' of the cyanocobalamin molecule.

The group on the protein molecule, concerned with its combination with cyanocobalamin, is not a sulphydryl group, since iodosobenzoic acid, iodoacetic acid, iodine acting as an oxidizing agent and *p*-chloromercuribenzoate had no effect on the binding activity of SF 8. Formaldehyde at pH 8 reacts with amino groups on proteins, and at pH 11 it attacks both amino and amido groups (Olcott & Fraenkel-Conrat, 1947). At neither pH did it affect the combination of SF 8 with cyanocobalamin. The reagent, 2′-acetylthioethylacetamide, is reported by Baddiley *et al.* (1952) to acetylate only amino groups on proteins, leaving hydroxyl groups unattacked. The acetylation of SF 8 with this reagent at both pH 8 and 10 had no effect on its combination with cyanocobalamin. However, these findings do not imply that an amino group on the protein is not concerned in its combination with the vitamin, since Baddiley *et al.* (1952) found that at pH 8 the reagent acetylated only one-quarter and at pH 10 one-half of the total free amino groups measured per mole of human serum albumin. If an amino group is concerned, it must be one that is not readily acetylated by this reagent or attacked by formaldehyde.

Fluorodinitrobenzene and iodine, at the higher concentrations used, were the only reagents that prevented the combination of SF 8 with the vitamin. Fluorodinitrobenzene, under the conditions used in these experiments, attacks amino and phenolic groups and to a lesser extent imidazole and sulphydryl groups (Olcott & Fraenkel-Conrat, 1947). Iodine acts as an oxidizing agent at an acid pH, in the presence of a high concentration of KI. With a low concentration and a higher pH, the oxidizing action of iodine is largely suppressed and iodination occurs. Under these conditions, iodination of the protein (presumably in the 3:5 positions of the tyrosyl residues) prevented its combination with the vitamin, and therefore it is possible that a phenolic group on the protein may be concerned in its linkage with cyanocobalamin.

SUMMARY

1. Chemical examination of the cyanocobalamin–protein complex isolated from sow's milk whey has shown that it contains 16·1 % nitrogen, 7 % carbohydrate and 9 % hexosamine. Its amino acid composition is typical of proteins, except for a high tyrosine content. The absorption curve of the complex in aqueous solution has peaks at 278 mμ. (due to protein) and at 362, 410, 520 and 550 mμ. (due to cyanocobalamin).

2. The cyanocobalamin content of the complex is 23·6 μg./mg., calculated from the absorption curve, and 22·4 μg./mg. measured by *Escherichia coli*. Using these figures, and assuming that one molecule of protein combines with one molecule of cyanocobalamin a molecular weight of 55000 has been deduced for the protein.

3. The use of specific reagents to block different groups on the protein molecule, has shown that either a phenolic or an amino group on the protein may be concerned in its combination with the vitamin. The point at which the protein attaches itself to the cyanocobalamin molecule is also discussed.

We are indebted to Dr R. A. Kekwick of the Lister Institute for the gift of 2′-acetylthioethylacetamide and to Dr M. Dixon, F.R.S., of the School of Biochemistry, Cambridge, for the *p*-chloromercuribenzoate. In the experiments using [^{14}CN]cobalamin, the measurements of radioactivity were done by Dr R. F. Glascock, to whom we express our thanks. We also wish to thank Dr S. K. Kon for his interest in this work and valuable help in preparing it for publication. This paper forms part of a Ph.D. thesis presented by one of us (M.E.G.) to the University of Reading.

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An Apparatus for Continuous Electrophoresis on Paper

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The application of electrophoresis in solution with paper as a supporting medium, for separating and detecting substances which have different mobilities in an electric field is now well established. The technique to be described differs from others in that the mixture to be separated is continuously fed into the apparatus and the separated components can be continuously removed, i.e. it can be used as a preparative method. An attempt was made by Philpot (1940) to use electrophoresis in a thin horizontal layer of solution between layers of electrolyte, as a continuous process. Some of the difficulties of this technique were overcome by Svensson & Brattsten (1949), by using a thin vertical layer and powdered glass to prevent mixing during the separation. There was a constant flow of buffer solution down through the glass and out through a series of tubes at the bottom of a narrow Perspex box. If a constant supply of material was applied at one place on the top surface it was carried down by the buffer solution to the outlet immediately below. However, if a potential gradient was applied at the ends of the box the substance was deflected towards one electrode and appeared at some other outlet, depending on its rate of movement in the electrical field. With a mixture of substances of different mobilities a continuous separation could be effected. The apparatus seems to have found very little practical application, probably owing to the difficulty of setting up and also to the poor resolution obtainable. These workers also tried a sheet of filter paper as a supporting medium for the electrolyte but with little success. Grassmann & Hannig (1950) were the first to use paper successfully in this type of apparatus. Platinum wire electrodes were fixed to the sides of the paper and the buffer solution was allowed to drip off at a series of points. Successful continuous separations of amino acid mixtures and proteins were reported. A forerunner of this type of apparatus was that of Haugaard & Kroner (1948), who carried out paper chromatography on a spot of the material to be separated and applied an electric