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A Study of Protein-Binding in the Metabolism of Vitamin B₁₂

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The mechanism by which intrinsic factor from normal human gastric juice promotes the absorption of cyanocobalamin (vitamin B₁₂) in patients with pernicious anaemia is still obscure. In particular there is no agreement about the part played by that substance in the juice which combines with cyanocobalamin and makes it non-diffusible. Substances that combine with cyanocobalamin have been reported in other body fluids and tissues. Rosenthal & Sarett (1952) and Wolff, Karlin & Royer (1952) have shown that human serum contains its vitamin B₁₂ in bound form and that the serum will further bind limited amounts of added cyanocobalamin. With an ultrafiltration technique we showed that milk of several different species contains protein-bound vitamin B₁₂ and we measured the quantity of additional vitamin that the milks would 'bind' (Gregory & Holdsworth, 1955*a*).

Confusion has arisen because different authors use the term 'free vitamin B₁₂' to mean quite different entities (cf. Heathcote & Mooney, 1958). It seems to us that it can be resolved only if authors would restrict the term to vitamin B₁₂ itself, i.e. either cyano- or hydroxo-cobalamin, and

to use the term 'available vitamin B₁₂' when implying that the test material can provide a substance that will support the growth of an assay organism.

Even then it is necessary to define carefully the conditions of test since we have shown that the available vitamin B₁₂ measured depends on the concentration of the test substance and on the organism and method of assay (Gregory & Holdsworth, 1957). Unfortunately there is no completely satisfactory general method for the measurement of 'free vitamin B₁₂'. Gregory & Holdsworth (1957) concluded that the ultrafiltration method was the most reliable technique for measuring the binding capacity of a substance for additional cyanocobalamin. But it must be remembered that this technique will differentiate only those bound forms too large to pass through the membrane. Certain peptide conjugates of the vitamin will pass through Visking tubing although they are obviously bound forms of vitamin B₁₂. Butan-1-ol extracts free vitamin B₁₂ from test material saturated with ammonium sulphate (Ellis, Petrow & Snook, 1949) and also certain small peptide conjugates.

In the experiments reported in this paper we have used the ultrafiltration method to determine whether tissues contain any ultrafiltrable vitamin

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B₁₂ activity, and to measure their ability to combine with added cyanocobalamin. Microbiological, physicochemical and immunological tests on the bound forms are reported which show that the bound forms of vitamin B₁₂ from the same tissues of different species and sometimes from different tissues of the same animal are not identical.

The high cyanocobalamin-binding activity of chick serum enabled us to isolate a pure cyanocobalamin-protein complex from it and to compare it with similar complexes isolated from pig pylorus (Andresen & Skouby, 1956) and from sow's milk (Gregory & Holdsworth, 1955*a, b*).

EXPERIMENTAL AND RESULTS

Materials

Bendogen is a commercial preparation of intrinsic factor from pig pyloric mucosa (G.E.A., Copenhagen). The pure cyanocobalamin-protein complex from Bendogen was a gift from Dr J. G. Andresen (G.E.A., Copenhagen). Its preparation was described by Andresen & Skouby (1956). The vitamin B₁₂-polypeptide was prepared from liver and was a gift from Dr H. G. Wijmenga (Organon Laboratories, Oss, Holland). The preparation of the cyanocobalamin-binding factor from sow's whey was described by Gregory & Holdsworth (1953).

Samples of serum were obtained from venous blood of the cow, goat, pig, rabbit and man. The chick and rat sera were obtained by exsanguination of freshly killed animals.

Extracts of parts of the intestinal tract were prepared as follows: whole pig or rat stomachs, a sheep abomasum or

chick proventriculi were cut open, rinsed briefly with water to remove adhering food, and homogenized in a Waring Blender with acid NaCl soln. (0.9% NaCl in 0.05*N*-HCl). The homogenate was centrifuged and the solid residue re-extracted twice with further portions of acid NaCl soln. The combined supernatant liquids were dialysed against running tap water for 24 hr., filtered and the clear filtrate was freeze-dried. Human gastric juice with intrinsic-factor activity was obtained from Dr D. L. Mollin (Postgraduate Medical School, London).

Livers were collected from six freshly killed vitamin B₁₂-deficient chicks prepared according to the method of Coates, Harrison & Kon (1951). The bulked livers (24 g.) were homogenized with water and made to a total volume of 48 ml. Livers from normal chicks were treated similarly.

[⁶⁰Co]Cyanocobalamin with specific activity 1 μ Ci/mg. was kindly supplied by Glaxo Laboratories Ltd., Greenford, Middlesex.

Measurement of bound forms of vitamin B₁₂ in animal tissues, organs and body fluids

The natural vitamin B₁₂ content of tissues, organs and body fluids of some representative species was measured in tube assays with *Lactobacillus leichmannii* A.T.C.C. 4797 after digestion of the sample with papain as described by Gregory (1954). In our experience treatment with papain is necessary to make the vitamin in these materials fully available to the test organism. The capacity of these tissues and organs to combine with additional cyanocobalamin was measured by an ultrafiltration technique (Gregory & Holdsworth, 1955*a*, 1957). The results are shown in Table 1.

In general, serum can bind additionally about as much vitamin B₁₂ as it originally contains. The one

Table 1. Vitamin B₁₂ content and vitamin B₁₂-binding activity of serum and of tissues of some animals

Test organism: *L. leichmannii* A.T.C.C. 4797; assay conditions were as described in the text. Values for serum are given as μ mg./ml.; those for tissues are as μ mg./g. wet wt. unless otherwise indicated.

	Vitamin B ₁₂ content	Binding capacity for additional cyanocobalamin
Serum		
Cow	2.3	3.5
Goat	3.3	4.4
Chicken	4.3	1 100.0
Man	1.2	2.0
Pig	0.4	4.5
Rabbit	46.0	17.0
Rat	0.8	3.8
Gastrointestinal tract		
Human gastric juice*	0	68
Chicken proventriculus	140	1 700
Chicken duodenum	0	0
Stomach of a newborn pig	0	200
Pig-pylorus extract (Bendogen)†	5	220 000
Rat stomach	70	700
Sheep abomasum (cardiac half)	0	0
Sheep abomasum (pyloric half)	0	100
Liver		
Chicken (normal)	86	117
Chicken (B ₁₂ -deficient)	13	127
Pig (14 days-old)	23	8

* μ mg./ml.

† μ mg./g. of freeze-dried extract.

exception is chick serum, which has a natural vitamin B₁₂ content similar to that of the other species tested, but a binding capacity for additional cyanocobalamin some 300 times this amount. The rabbit serum had the highest vitamin B₁₂ content. Autoclaving the serum samples with alkali destroyed all their growth activity for *L. leichmannii*, showing that deoxyribosides were not interfering with the measurement of vitamin B₁₂ by this organism.

Material with high cyanocobalamin-binding activity was also obtained by extracting certain portions of the gastrointestinal tract of different species. It can be seen from the table that Bendogen prepared from pig pylorus and having intrinsic-factor activity had a high vitamin B₁₂-binding capacity. The vitamin B₁₂-binding factor can even be detected in the stomach of the newborn pig.

The proventriculus (i.e. the glandular stomach) of the chick also has considerable binding activity whereas none could be detected in the duodenum. The pyloric and cardiac halves of the abomasum of a sheep were tested separately and the vitamin B₁₂-binding activity was found in the pyloric half only. Equal weights of liver homogenates from normal and vitamin B₁₂-deficient chicks can combine with about the same amount of additional cyanocobalamin, although on the same weight basis the normal livers have the higher natural vitamin B₁₂ content.

The vitamin B₁₂ present in the chick-, rat- and pig-liver homogenates and in all serum samples tested was not ultrafiltrable. The ultrafiltration method thus confirms that in the liver and serum of the species tested there is no free vitamin B₁₂.

When human gastric juice was mixed with human serum, the total binding activity, measured by the ultrafiltration method, did not exceed the sum of the individual activities of the serum and gastric juice. Thus we were not able to confirm the work of Miller (1957), who reported that gastric juice potentiated the binding capacity of the serum. We applied Miller's charcoal method for measuring binding activity and found that it gave much lower results than the ultrafiltration method, which we attributed to the fact that charcoal could adsorb bound forms of vitamin B₁₂ besides the free vitamin, and confirmed this supposition in experiments with Bendogen combined with [⁶⁰Co]cyanocobalamin.

Examination of the bound forms by electrophoresis

[⁶⁰Co]Cyanocobalamin was added in slight excess over the binding capacity to extracts of pig pylorus (Bendogen), rat stomach and chick proventriculus prepared as described under Materials, and to chick serum, human gastric juice and human serum. Suitable amounts were subjected to paper electrophoresis at various pH values. The position of the proteins was revealed by staining with bromophenol blue and that of cyanocobalamin by radioautography as described previously (Gregory & Holdsworth, 1955a).

As we had previously observed with milk, each tissue gave only one zone on paper electrophoresis that was responsible for the cyanocobalamin-binding activity. Normal human serum was an exception and showed a small second peak of activity in the α_1 -globulin region. Fig. 1 shows the

Fig. 1. Electrophoretic patterns of serum and extracts of intestinal tract. Electrophoresis in sodium veronal buffer, pH 8.6. The filled-in curves represent added [⁶⁰Co]cyanocobalamin measured as the extinction of the radioautographs. Other curves represent protein distribution. Ordinates are arbitrary units of extinction. (a) Normal human serum; (b) chick serum; (c) chick-proventriculus extract; (d) rat-stomach extract; (e) human gastric juice; (f) pig-pylorus extract.

mobilities of some of the complexes at pH 8.6. Under these conditions free cyanocobalamin had zero mobility. It can be seen that the binding component in serum is not one of the well-characterized serum proteins. In extracts of the stomach of the chick, rat and pig, and in human gastric juice, the specific proteins have a mobility of -3.0 to -3.5×10^{-5} cm.²v⁻¹sec.⁻¹ at pH 8.6 in 0.05M-veronal buffer. Although the mobilities were similar at pH 8.6 it was possible to distinguish between some of these materials by examination at other pH values. At pH 6.4 the bound form prepared from chick liver had a higher mobility than that prepared from chick proventriculus. Near the isoelectric point, pH 4.0-5.0, it was possible to show differences in mobility between the bound vitamin B₁₂ in pig-pylorus extract, sow's milk and rat-stomach extract. Chick serum differed from the other materials tested in that the bound form had a high mobility (-4.9×10^{-5} cm.²v⁻¹sec.⁻¹) close to that of α_1 -globulin at pH 8.6.

Examination in plate assays of the growth activities of bound forms of vitamin B₁₂ for Escherichia coli and Lactobacillus leichmannii

In a previous paper we have shown that cyanocobalamin combined with Bendogen had growth activity for *E. coli* when assayed unheated on agar plates (Gregory & Holdsworth, 1957). This led us to test other bound forms of the vitamin.

The *E. coli* plates were prepared as described by Gregory & Holdsworth (1957). The test solutions (0.01 ml.) were

applied to the plates on 6 mm. disks of Whatman no. 4 filter paper. 2:3:5-Triphenyltetrazolium chloride was added to facilitate measurement of the zones of growth (Ford & Holdsworth, 1953). The vitamin B₁₂ activity of the test preparations was calculated both from the measurement of the diameters of the growth zones and by measurement of the amount of red formazan pigment eluted from the growth zones with tetrahydrofuran.

The *L. leichmannii* plate-assay method was essentially the same as the *E. coli* method, except that the medium of Skeggs, Nepple, Valentik, Huff & Wright (1950) was used at pH 6.8 with 2% of agar and 0.1 g. of ascorbic acid (added under aseptic conditions)/150 ml. of medium. 2:3:5-Triphenyltetrazolium chloride was not added. Each plate was inoculated with 4 ml. of a 24 hr. culture of *L. leichmannii* grown in the basal medium supplemented with 0.01 μ mg. of cyanocobalamin/ml. The cyanocobalamin standard was the same as that used for the *E. coli* plates. Incubation was for 18 hr. at 37°. The solutions to be tested contained no ultrafiltrable vitamin B₁₂ activity. The vitamin B₁₂ content of the complexes was determined by the papain-digestion method in tube assays with *L. leichmannii* as the test organism, as described by Gregory (1954).

The growth activities of the different cyanocobalamin complexes tested at various levels of cyanocobalamin content are shown in Table 2. It can be seen that there was no correlation between the cyanocobalamin content of the various bound forms and their growth activities for both test organisms. For example, the human-milk and chick-serum complexes were completely inactive at all levels tested, whereas the preparations from sow's milk, chick proventriculus, rat stomach and pig pylorus all had some growth activity, which

Table 2. Activity of bound forms of cyanocobalamin (expressed as a percentage of that of free cyanocobalamin) for *Lactobacillus leichmannii* and *Escherichia coli* in plate assays

Test preparation (0.01 ml.) was applied to 6 mm. disks.

Source of bound cyanocobalamin	Amount of cyanocobalamin in complex (μ g./ml.)	Activity for <i>L. leichmannii</i> calc. from growth-zone diameter	Activity for <i>E. coli</i>	
			Calc. from growth-zone diameter	Calc. from colour of zone
Sow's milk	1.1	35	40	36
	0.11	44	45	91
Human milk	0.13	1	0	0
Human gastric juice	0.09	106	76	*
	0.009	111	111	*
Chicken serum	1.0	0	0	0
	0.1	0	0	0
Chicken proventriculus	1.0	8	9	10
	0.1	12	19	10
Rat stomach	0.7	36	48	83
	0.07	49	77	165
Liver (polypeptide-vitamin B ₁₂ complex)	0.3	87	123	93
	0.03	100	103	93
Bendogen (100 mg.) + 20 μ g. of vitamin B ₁₂	1.5	0	0	0
	0.15	2	10	11
	0.015	28	63	67

* Not measured.

increased as the test solutions were diluted. Cyanocobalamin added to undiluted human gastric juice was fully available to *L. leichmannii* and 76 % available to *E. coli*. On further tenfold dilution the vitamin was fully available to both organisms. The liver vitamin B₁₂-polypeptide complex was as active as the free vitamin at all levels tested.

Effect of enzymes on bound cyanocobalamin and on binding activity

In order to study whether binding activity could be altered by treatment with enzymes, portions (1 mg.) of the sow's-whey binding factor were digested at 55° for 3 hr. with the following enzymes (preparations from The Armour Laboratories unless otherwise indicated): in buffer of the appropriate pH, 10 mg. of papain (British Drug Houses Ltd.) activated with cyanide, 2 mg. of crystalline trypsin, 2 mg. of chymotrypsin, 2 mg. of carboxypeptidase or 2 mg. of crystalline pepsin. Half of the enzyme was added at the beginning of the experiment and the remainder half-way through the incubation period. After digestion, 0.1 µg. of cyanocobalamin (slightly more than sufficient to saturate the binding activity) was added to each of the digests, which were then brought to a volume of 10 ml. and a portion of each was removed for measurement of available vitamin B₁₂. The remainder of each digest was ultrafiltered and the vitamin B₁₂ activity of the ultrafiltrates measured. Both the digest and ultrafiltrate were assayed without further treatment other than dilution with water. The test organism was *L. leichmannii*. The ability of these same enzymes to release cyanocobalamin from the combination with the binding factor was studied simultaneously in the same way. In this part of the experiment, the cyanocobalamin (0.1 µg.) was added to 1 mg. of the binding factor before treatment with the enzymes as described above.

The results of this experiment are shown in Table 3. When 0.1 µg. of cyanocobalamin was added to the untreated binding factor, only 0.01 µg. appeared in the ultrafiltrate. Digestion of the binding factor with papain, trypsin or pepsin reduced its ability to combine with added cyanocobalamin and from 50 to 60 % of the added vitamin appeared in the ultrafiltrate (see column 4

in Table 3). After digestion with chymotrypsin and carboxypeptidase the binding activity of the sow's-whey factor was also reduced, but to a lesser extent than with the other enzymes. However, when the binding factor was saturated with cyanocobalamin before treatment with the enzymes it was more resistant to their attack. Column 2 in Table 3 shows that there was no increase in the ultrafiltrable cyanocobalamin resulting from enzyme treatments of the bound vitamin. Therefore the enzymes were not able to release free cyanocobalamin from the binding protein, although they were able to increase the microbiological availability of the vitamin in the complex.

In the presence of untreated sow's-whey binding factor, added cyanocobalamin was only 60 % available, whereas cyanocobalamin added to any of the enzyme digests of the binding factor was fully available to *L. leichmannii*. When the enzymes acted on the binding factor saturated with cyanocobalamin, only papain digestion made the vitamin fully available to *L. leichmannii*.

Immunological tests

Cyanocobalamin-protein complex (40 mg.) from pig pylorus adsorbed on alumina (Proom, 1943) was injected intramuscularly into the leg of a 3 kg. rabbit. Blood serum obtained 2 weeks later contained precipitating antibodies, 5 ml. of antiserum being sufficient to precipitate in 3 hr. at room temperature 85 % of a [⁶⁰Co]cyanocobalamin-Bendogen complex containing 5 mg. of Bendogen and 1 µg. of cyanocobalamin. The amount of precipitate, however, appeared to be independent of the presence or absence of the cyanocobalamin.

Rough quantitative tests (ring precipitin) with dilutions of antigen were performed, by using various binding substances which had been saturated with radioactive cyanocobalamin and freed from excess by prolonged dialysis. When quantities of the different materials with equal binding capacities were tested against the antiserum, pig-pylorus and sow's-milk antigens gave closely similar reactions. Other complexes (from chick serum, chick proventriculus, rat stomach and human gastric juice) showed cross-reactions, but only at much higher concentrations.

Table 3. *Effect of enzyme digestion on the binding of 0.1 µg. of cyanocobalamin by 1 mg. of the factor from sow's whey and on the microbiological availability of cyanocobalamin combined with the sow's-whey factor*

Test organism: *L. leichmannii*. Results are the means of two assays.

	Cyanocobalamin (0.1 µg.) added before enzyme digestion		Cyanocobalamin (0.1 µg.) added after enzyme digestion	
	Available cyanocobalamin (µg.)	Ultrafiltrable cyanocobalamin (µg.)	Available cyanocobalamin (µg.)	Ultrafiltrable cyanocobalamin (µg.)
Control (no enzyme)	0.06	0.01	0.06	0.01
Papain	0.11	0.01	0.11	0.05
Trypsin	0.09	0.01	0.11	0.06
Chymotrypsin	0.07	0.01	0.10	0.04
Carboxypeptidase	0.07	0.01	0.11	0.03
Pepsin	0.07	0.01	0.10	0.06

Isolation of a cyanocobalamin-protein complex from serum

A concentrate of the binding substance from chick serum was prepared by adding 1 l. of 11 N-HCl to 5 l. of serum at 4° and centrifuging off the precipitated proteins and immediately dialysing the supernatant fluid against running tap water to remove acid. The volume remaining after dialysis was reduced by freeze-drying to approx. 600 ml.; 1 ml. of this concentrate combined with 6 μ g. of cyanocobalamin.

A volume (300 ml.) of the concentrate was mixed with a solution containing 2.0 mg. of [⁶⁰Co]cyanocobalamin and cooled to 4°. Saturated ammonium sulphate solution at the same temperature was added, with stirring, to 33% saturation. The precipitate was removed by centrifuging and washed with 33% saturated ammonium sulphate solution. Since no radioactivity was detected in the precipitate, it was rejected. A similar precipitate obtained at 50% saturation had only slight radioactivity and was also discarded. Proteins remaining in solution were precipitated by adding solid ammonium sulphate to saturation, again at 4°. The precipitate (containing the radioactivity) was suspended in water and dialysed for 6 hr. against running tap water. The solution (approx. 60 ml.) was refractionated with ammonium sulphate and 66–100% saturation yielded the crude cyanocobalamin complex. After dialysis against distilled water the protein solution (50 ml.) was made 0.025 M with sodium acetate buffer (pH 4.8), and cooled to 1° and propan-2-ol at –12° was added dropwise with stirring. The temperature was gradually lowered to –12° during the fractionation. The various fractions obtained were tested for the presence of cyanocobalamin by dissolving in water and measuring the radioactivity (cf. Gregory & Holdsworth, 1955a). About 90% of the cyanocobalamin protein was precipitated in the fraction containing 35–45% (v/v) of propan-2-ol, and this material, dissolved in 20 ml. of water, was refractionated by similar precipitation with propan-2-ol, at pH 4.0.

The resulting pink protein was dissolved in water and dialysed against changes of distilled water. The solution was cooled to 1° and fractionated with 2% (w/v) acetic acid in propan-2-ol, when the pink material precipitated at a concentration of between 10 and 15% of the acid alcohol. Alternate precipitations at pH 4.8 and with acid propan-2-ol were continued until the ratio of the extinction coefficient at 278 m μ (due mainly to protein) and at 362 m μ (due to cyanocobalamin) reached a steady value. After dialysis against distilled water the complex was obtained as a pink powder by freeze-drying the solution (yield: 24 mg.).

The material migrated as a single pink band, with a mobility close to that of α_1 -globulin, on paper electrophoresis at pH 8.6. It differed from α_1 -globulin in its solubility properties and did not stain on paper with bromophenol blue. In the ultracentrifuge it sedimented as a single symmetrical peak which showed no sign of resolving in 72 min. at 60 000 rev./min. in the Spinco analytical ultracentrifuge. The $S_{0,w}$ for a 0.5% solution was 3.95s. As far as could be ascertained the chemical properties resembled those of the two complexes examined previously, one from sow's milk (Gregory & Holdsworth, 1955b) and one from pig pylorus (Holdsworth & Ottesen, 1955).

The pink protein contained 15 μ g. of vitamin B₁₂/mg., calculated from the absorption at 362 m μ (Gregory & Holdsworth, 1955b), 8.9% of total nitrogen (micro-Kjeldahl) and, after hydrolysis for 4 hr. with 2 N-H₂SO₄, 27% of reducing sugars were found by the method of King & Wootton (1956), and 4.5% of methylpentose (probably fucose) by the method of Dische & Shettles (1948). Thus the complex is a glyco- or muco-protein.

DISCUSSION

Pitney, Beard & Van Loon (1954), Ostrowski, Skarżyński & Zak (1954) and Heinrich & Erdmann-Oehlecker (1956) have separated serum proteins by paper electrophoresis and estimated by microbiological methods the vitamin B₁₂ present in the major protein fractions of the serum. Each group concluded that the vitamin B₁₂ was associated mainly with the α_1 - and α_2 -globulin. This distribution between different fractions can be explained if the vitamin was combined with a minor component whose properties overlap those of the main serum proteins, and since the papers were cut into these main fractions the vitamin activity would be spread over a number of them. The vitamin B₁₂ content and the binding activity of human serum are known to increase in certain diseases such as leukaemia (Heinrich & Erdmann-Oehlecker, 1956). This increase may be associated with the second (minor) binding component of normal human serum since we found that this component was present in a sample of chronic myeloid leukaemic serum to a much greater extent than in normal serum. As shown in Fig. 1, the material in chick serum that combines with additional cyanocobalamin had a mobility between that of α_1 - and α_2 -globulin. Furthermore, the binding protein constituted less than 0.1% of the total protein of serum and could not have been detected by the usual staining procedures used in paper electrophoresis. With chick serum we have added cyanocobalamin to saturate the binding material and isolated the glycoprotein-vitamin complex, which differed from the α_1 - and α_2 -globulin in many of its properties. Thus the binding capacity of serum appears to us to be due to a minor specific glycoprotein and not to any of the main protein components.

The bound forms prepared by adding cyanocobalamin to sow's milk, chick serum and pig-pylorus extract have been obtained in a state approaching purity and the complexes have similar chemical and physical properties. The pig-pyloric complex was obtained in purest form and hence was more fully investigated (Gregory, Holdsworth & Ottesen, 1957; Holdsworth & Ottesen, 1955). More recently the binding substance in Bendogen

has been isolated by chromatography on ion-exchange cellulose columns and shown to consist of two binding materials, only one of which had intrinsic-factor activity (Holdsworth, 1957).

The bound forms of cyanocobalamin differ in their microbiological activity when measured by growth of *L. leichmannii* or *E. coli*. The availability of these complexes to both organisms increased in the following order according to the nature of the binding factor: human milk and chick serum, pig stomach (Bendogen), chick proventriculus, sow's milk, rat serum and human gastric juice. The vitamin B₁₂-polypeptide complex from liver was more available than any of the other bound forms, indicating either that the free vitamin is much more readily released from the peptide moiety or that *L. leichmannii* and *E. coli* can utilize the complex as easily as the free vitamin.

Certain animals lack the ability to absorb cyanocobalamin bound to foreign binding proteins, e.g. the chick will readily absorb the vitamin bound to its own binding factors but has difficulty in absorbing cyanocobalamin bound to the sow's-milk binding factor (Coates *et al.* 1955). This finding also suggests that the digestive enzymes of the test animal have little action on the foreign complex otherwise the vitamin would be released and become available. The experiments *in vitro* reported in this paper confirm that the bound vitamin is little affected by several isolated enzyme systems. The immunological work demonstrates a difference between the binding factors of different species.

A substance that combines with cyanocobalamin is not necessarily intrinsic factor, but it seems likely that intrinsic factor combines with cyanocobalamin. Bishop, Toporek, Nelson & Bethell (1955) used the urinary excretion of ⁶⁰Co as a test for absorption of the labelled vitamin in gastrectomized patients. ⁶⁰Co appeared in the urine only when the labelled vitamin was actually combined with intrinsic factor. The intrinsic-factor preparation from Bendogen, described by Holdsworth (1957), was active (0.5 mg. with 0.5 µg. of radioactive cyanocobalamin) in absorption (Schilling) tests in patients with pernicious anaemia. This preparation would combine with 15 µg. of cyanocobalamin/mg. If this material can be accepted as a pure substance then there seems no doubt that intrinsic factor combines with vitamin B₁₂. With washed-out loops of small intestine in the surviving rat we were able to show that the bound vitamin B₁₂, formed when the vitamin is added to rat-stomach extract, is absorbed two to three times better than the free vitamin (Holdsworth & Coates, 1956). Similarly, the uptake of the vitamin by surviving mucosal cells was increased by rat-stomach extract (Coates & Holdsworth, 1958). Even if the binding substance in rat stomach

extract is not the rat 'intrinsic factor', the bound form was absorbed in greater amount than the free vitamin. If a foreign binding substance was tested in the rat, absorption was depressed below that of the free vitamin (Holdsworth & Coates, 1956), an observation which supports the idea of a species specificity of the binding proteins.

SUMMARY

1. The natural vitamin B₁₂ content and the binding capacity for cyanocobalamin of a number of tissues and body fluids of several animal species have been examined. As judged by electrophoresis, each material contained a specific cyanocobalamin-binding glycoprotein.

2. The cyanocobalamin-protein complex obtained by adding the vitamin to chick serum has been isolated. Its properties were similar to those of the other two complexes (from milk and pig pylorus) we have studied.

3. Although similar in some chemical and physical properties, the complexes from different sources had different availabilities for micro-organisms and for animals and differed immunologically.

4. The evidence for specific binding glycoproteins playing an important role in the absorption and retention of cyanocobalamin in the body is discussed.

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The Alkaloids of Hemlock (*Conium maculatum* L.)

DISTRIBUTION IN RELATION TO THE DEVELOPMENT OF THE FRUIT

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Much of the work published on the formation and role of the alkaloids in the plant has been concerned with the problem of the site of synthesis (whether root or leaf) and the determination of possible precursors by suitable feeding experiments. We have investigated the composition and quantity of the alkaloids of a given plant during a critical stage in its life history and have correlated our findings with physiological age, anatomical changes and histochemical tests. The phase of the development of the flower to the mature fruit was selected for study.

Conium maculatum L. (family Umbelliferae) was chosen because (a) there was already evidence that the quantity of alkaloid varied markedly as the fruit developed, (b) the alkaloids present were relatively simple ones and few in number and (c) the alkaloids are said to be mainly restricted to a special layer of the fruit (the conine layer), which would give scope for anatomical and histochemical studies. Very few members of the Umbelliferae produce alkaloids, the majority producing volatile oils in clearly delimited tissues of the fruit, known as vittae; in *Conium*, vittae occur in the young fruits but almost disappear during maturation.

During the course of our work a paper on the distribution of alkaloids in *Conium maculatum* was published by Cromwell (1956). While Cromwell's work anticipated some of our techniques, and we have made use of some of those which he published, it has not affected the main body of our work.

Some of our findings confirm those of Cromwell but since we have made a more detailed study of a restricted part of the life history of the plant, we can make a more complete report of this aspect.

EXPERIMENTAL

Initially two separate samples of seeds were sown in the School of Pharmacy Medicinal Plants Garden at Myddelton House, Enfield, Middlesex; one sample was derived from plants already growing at Myddelton House and the other was obtained from Chelsea Physic Garden, London. It soon became obvious that these two samples represented two varieties of *C. maculatum* which differed slightly in fruit and leaf morphology. More important still we found that one variety (that from Chelsea) produced little or no methylconiine whereas the other one did. To avoid complication in our work, we decided to use only the Chelsea variety of *C. maculatum*.

Sampling techniques. Careful sampling techniques are essential but previous work on alkaloids in plants has often shown a lack of appreciation of this requisite (Dawson, 1948). As our studies were restricted to the flower and fruit we made a careful study of the distribution of the flowers on the growing plant. The inflorescence is a compound umbel consisting of 10–15 rays, and each ray bears a secondary umbel with 8–18 flowers. It was noticed that all the flowers on a given compound umbel developed at practically the same time but that the stage of development varied from compound umbel to compound umbel on the same plant or even on the same branch. It was therefore decided to use the compound umbel, containing on an average about 150 flowers, as the 'unit'. These compound umbels are first recognized as small greenish masses of