QUALITATIVE AND QUANTITATIVE STUDIES OF THE RADIOACTIVE MATERIAL IN URINE AFTER PARENTERAL RADIOACTIVE COBALAMINS.

BY

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PREFACE

It is customary to divide knowledge into many spheres, and to subdivide each of these further. Such divisions are useful, but the lines of demarcation are often only arbitrary. While these divisions are especially useful in purely academic spheres, there are circumstances in which they can not, and should not, be made. Such circumstances arise in medical research work where chemistry, biochemistry and clinical practice often become so interdependent that it is impossible to separate them without destroying the whole scheme of research.

In such a situation the work described in this thesis was undertaken, and completed. The work is thus a peculiar mixture of various spheres and if it serves no other purpose it shows the danger of keeping each sphere of knowledge rigidly separate from the others.

ACKNOWLEDGMENTS

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INTRODUCTION

Terminology

A brief review of the Chemistry, Distribution, Isolation, Commercial Production, Metabolic Functions, and Therapeutic and Diagnostic uses of Vitamin B_{12}.

Outline of Present Studies.

TERMINOLOGY

As there is still some confusion regarding terminology, the following conventions will be adopted in this thesis. The term vitamin B_{12} will be used as a general name for cobalamins and like compounds, and x-cobalamin will be used to describe specific cobalamins, where x is the sixth complexing group on the central cobalt atom. The term coenzyme B_{12} will be used to refer to that coenzyme containing the base 5:6 - dimethyl benzimidazole. This terminology is in agreement with that suggested by the Nomenclature Commission (Smith 1957).

To specify radioactive cobalamins, the name of the compound will be prefixed by ^{57}Co or ^{58}Co, the radioactive atom being cobalt and the number denoting its atomic weight.

CHEMISTRY

Cobalamins are large organic molecules, with molecular weights about 1350, containing cobalt in the trivalent state. This cobalt has six positions available for coordination by ligands, five of which are occupied by nitrogens from the organic part of the molecule. The cobalamin structure, as elucidated by Hodgkin (1957), is shown in Figure 1. The remaining sixth place on the cobalt can be taken by various ligands, such as the cyanide ion (CN^-), the hydroxide ion (OH^-), or ammonia, giving rise to different cobalamins. In every other respect the molecule is unchanged (Rosenblum 1965) and any differences in colour (and therefore in absorption spectra), basicity and acidity, or partition coefficients, are due to the ligand occupying this sixth position on the central cobalt atom (Hill et al 1962). The studies described in this thesis are concerned with some of these.

One possible ligand is the cyanide ion, giving cyanocobalamin. Cyanide complexes very strongly with cobalt in the trivalent state, and, therefore, the cyanocobalamin molecule is one of the most stable cobalamin molecules. However, cyanocobalamin is readily photolyzed in aqueous solution, and the cyanide split from the molecule. This change is reversed on keeping the solution in the dark (Veer et al 1950). A product of photolysis is hydroxocobalamin, in which the sixth place on the central cobalt is taken by the hydroxide ion. In protonating solvents, hydroxocobalamin exists as aquocobalamin, the complexing group being OH_2^+ (Smith et al 1962). The hydroxocobalamin molecule is less

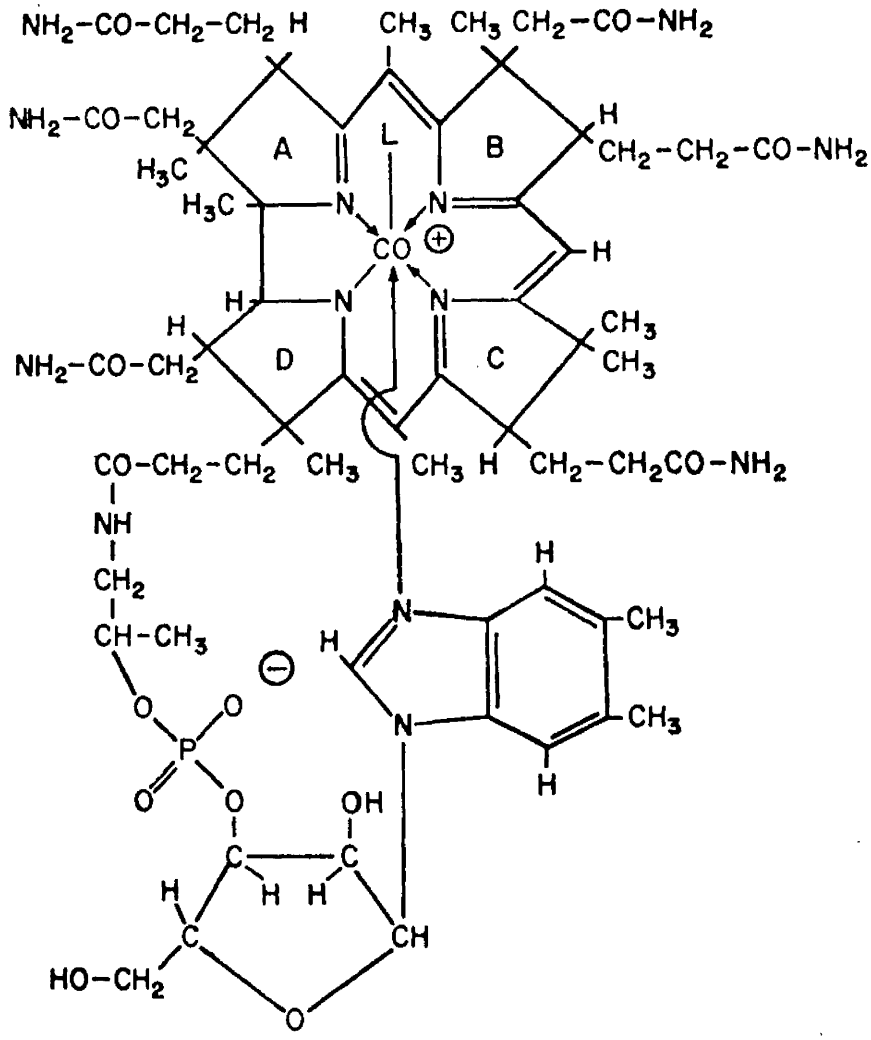

Fig. 1:- Structure of the Cobalamin Molecule.

stable, and, therefore, more easily degraded, than is the cyanocobalamin molecule. It, and all other cobalamins, are readily converted to cyanocobalamin by aqueous inorganic cyanide (Rosenblum 1965).

Cyanocobalamin is a very slightly basic compound but its basicity is so slight that it is generally considered to be a neutral compound and only in acidic solutions does it behave as a base (Brink et al 1949). Hydroxocobalamin is more strongly basic, due to the hydroxide ligand.

Several cobalamins in which the sixth ligand is an organic moiety, have recently been isolated (Barker et al 1960); Lindstrand 1964). One of these organic complexes is 5'- deoxyadenosyl cobalamin, known as coenzyme B_{12} (Figure 2). Another, rather simpler, organic complex is methylcobalamin (Figure 3). Both these compounds are light sensitive, being readily photolysed to hydroxocobalamin. Both are only very slightly basic compounds.

Cobalamins are readily hydrolysed by dilute acids and alkalis. The products of hydrolysis vary according to the conditions used (Smith 1955), but on mild acid hydrolysis the products are acidic compounds called "red acids" (Armitage et al 1953). In these, some of the side chains of the pyrrole rings of the cobalamin molecule have been hydrolysed from $-CONH_2$ to $-COOH$.

Partly because of these factors - the ease with which the

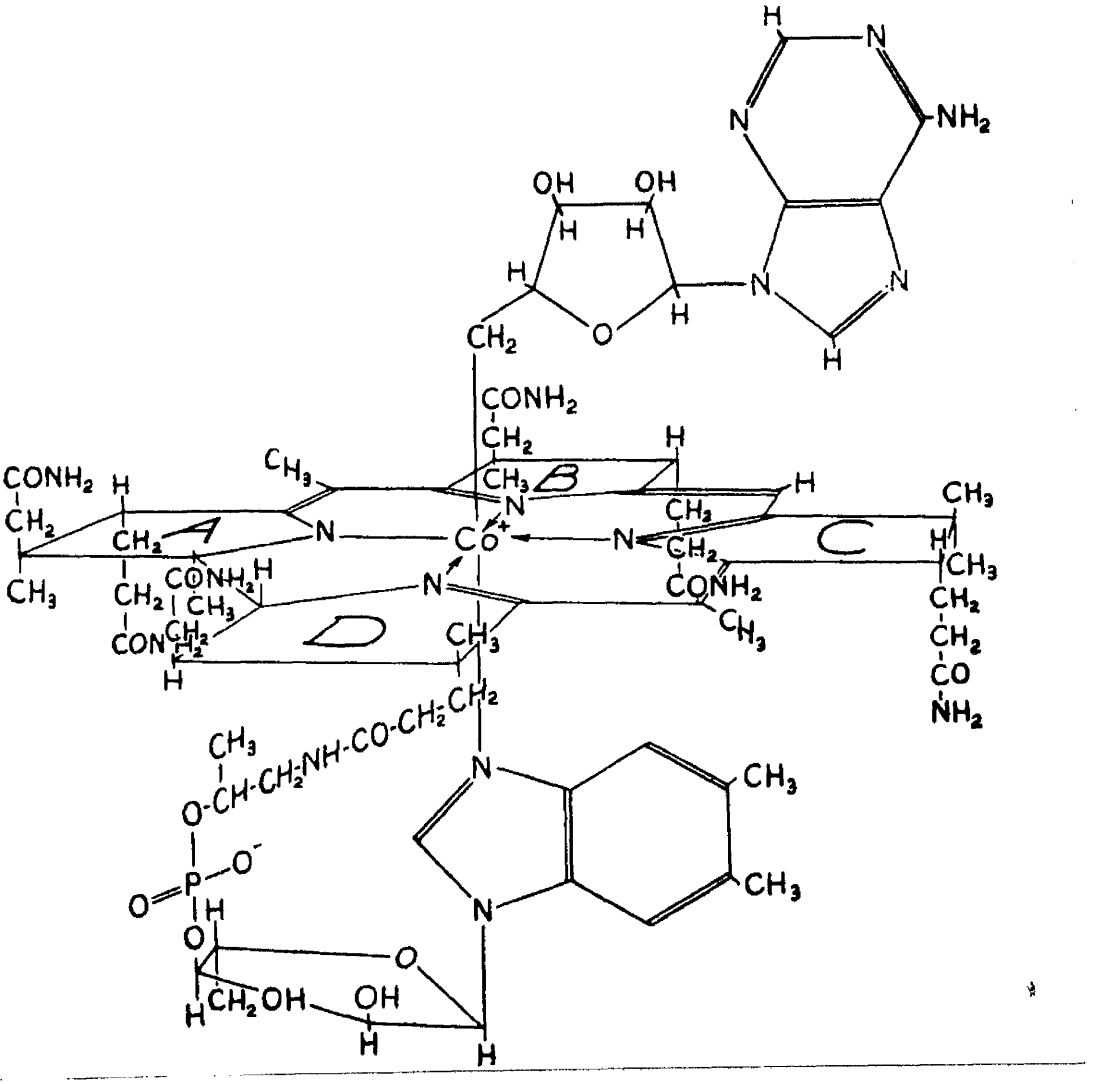

Fig. 2:- Structure of 5:6 - Dimethyl Benzimidazole Cobamide Coenzyme (Coenzyme B_{12}).

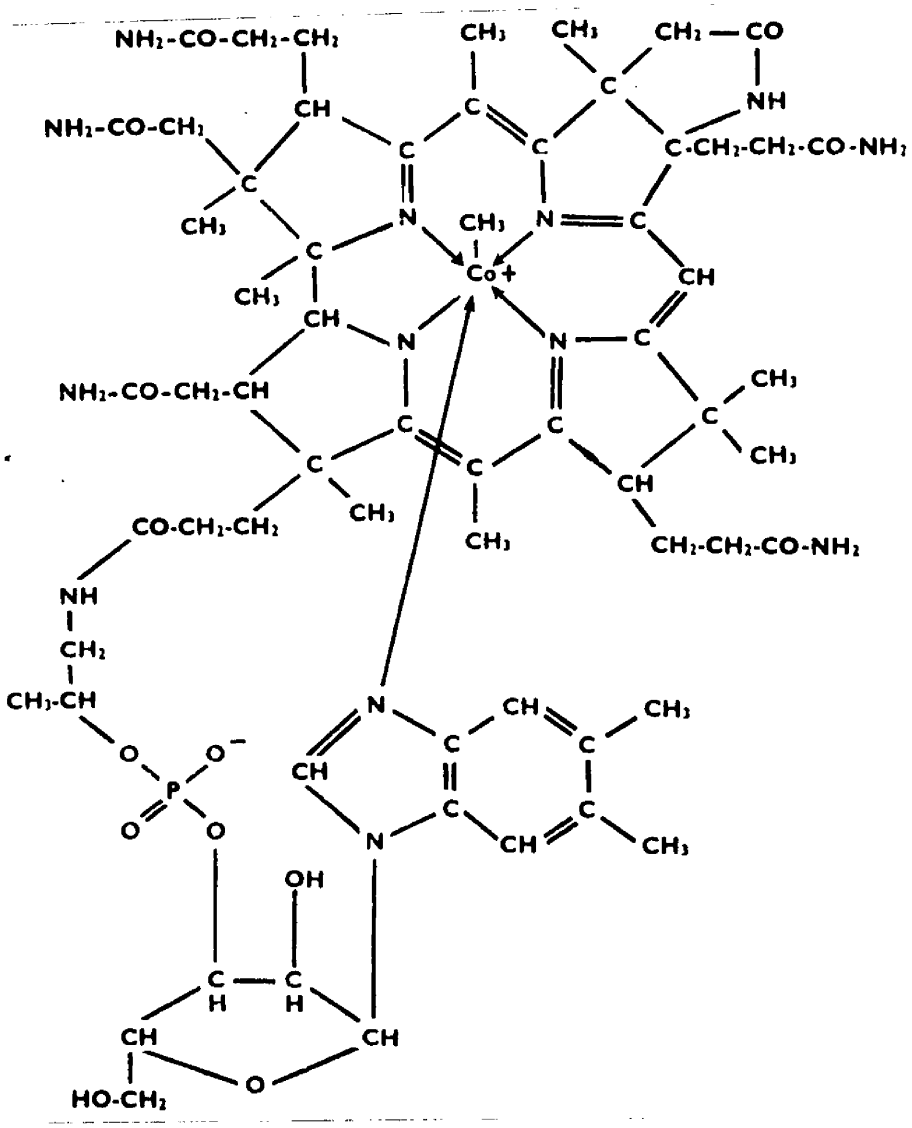

Fig. 3:- Structure of Methylcobalamin.

cobalamins are photolysed and hydrolysed, and the multiplicity of possible forms due to complexing on the cobalt by different ligands - the chemistry and biochemistry of the cobalamins have posed many problems. Many of these problems have been solved, but some still remain.

DISTRIBUTION

It is known that vitamin B_{12} is synthesized by microorganisms (Halbrook et al 1950). This is believed to be the origin of all the vitamin, regardless of distribution. In general, animal tissues and animal products are the richest natural sources of the vitamin (Schweigert et al 1951), but sewage sludge also contains appreciable amounts (Bernhauer and Friedrich 1954). Vegetable matter usually contains very little vitamin B_{12} (Robbins et al 1950), but in the experience of the author and her colleagues, the roots and nodules of certain leguminous plants appear to contain appreciable amounts of the vitamin as measured by microbiological assay (Bond et al 1965).

In man, the highest concentration is to be found in liver (Swendseid et al 1957. Ross & Mollin 1957. Nelson & Doctor 1958). Faeces contain an appreciable amount (Girdwood 1950), possibly due to bacterial action in the large bowel, and possibly due to unabsorbed vitamin B_{12} in the diet. Urine normally contains little vitamin B_{12}, but it is the main vehicle of excretion following large parenteral doses of the vitamin (Chow et al 1950).

As the highest concentrations of vitamin B_{12} are found in animal liver and in certain fermentation broths (Hallbrook et al (1950), these were the most important starting materials for isolation of the vitamin.

ISOLATION

The first work which showed that liver contained material, or materials, which played a part in red cell production was done by Whipple and his colleagues (1920) on dogs. This work was followed by the demonstration of the efficacy of liver therapy in patients with pernicious anaemia by Minot and Murphy (1926). Soon Castle & Townsend (1929) put forward their theory of "extrinsic factor" and "intrinsic factor" which acted together to produce "liver factor". This "liver factor" was considered to be the anti-pernicious anaemia factor. Meat was found to be a source of the "extrinsic factor", now known to be vitamin B_{12}, and a quest for this in pure form was started. Ox-liver was most frequently used as the starting material and Cohn and his associates (1928) pioneered a method for fractionation of liver using varying concentrations of ethanol. Their fractionation methods were adopted and adapted by other workers and some of the extracts obtained from liver were found to have vitamin B_{12} activity.

Further progress in the purification of these extracts was slow for a variety of reasons. Although the starting material, liver, had the highest concentration of all tissues, it is now clear that the

concentration of vitamin B_{12} in liver is only of the order of a microgram per gram of tissue, or less (Shenoy & Ramasarma 1954). Early workers, moreover, did not have a satisfactory "in vitro" method of estimating the amount of vitamin (the activity) in the extracts they obtained, as the only known methods of estimation were clinical tests on patients with pernicious anaemia and growth tests on animals. A relevant microbiological assay was first devised by Shorb (1947) using Lactobacillus lactis Dorner. This technique was further developed using other organisms (Skeggs et al 1950: Ross 1950), and these assay methods remain the only reliable way of estimating vitamin B_{12} in the concentrations found in natural materials. Another technique which had not been developed when the earlier workers were attempting to isolate vitamin B_{12} was chromatography. It is still not practicable to isolate the vitamin in pure form without chromatography.

Because of these, and other, difficulties, only industrial laboratories possessed the necessary resources to process the large amounts of starting material required for isolation of the vitamin. Consequently, most of the further fruitful work was done in the laboratories of Glaxo Ltd. in the United Kingdom, and of Merck & Co. Inc., in America. Eventually, Rickes and his colleagues, of Merck & Co. Inc., announced the isolation of a red crystalline compound which they named vitamin B_{12}. (Rickes et al 1948a). A few weeks later, Smith and his colleagues, of Glaxo Ltd., announced the independent isolation of the same substance from liver (Smith & Parker 1948) Soon after these two

original isolations, another British team, at British Drug Houses, also isolated vitamin B_{12} (Ellis et al 1949).

Details of the procedures used by Rickes and his colleagues have never been published, but are under patents. From these patents it seems clear that adsorption of methanolic solutions on alumina played an important part in the isolation. The Glaxo team have published their isolation methods in detail (Fantes et al 1949). They used chromatography on charcoal, alumina, and silica, and a summary of their technique has been given by Smith (1960). In their procedure, the vitamin was crystallized from acetone in the final stages, and this is still the usual solvent for crystallization.

The final yield of the vitamin was very low, being only a few per cent of the activity of the original liver. The reason for this is now known to be that the vitamin exists in bound forms in liver. As this bound vitamin would not have been released by the techniques used, much of it would have been coprecipitated with the protein to which it was bound.

Later isolations, especially that of Wijmenga of Organon N.V. in Holland (Wijmenga 1950), used inorganic cyanide. This gave a very much higher yield, but converted other forms of cobalamin into cyanocobalamin. This was not at first realised, and when the structure was finally elucidated by X-ray crystallography (Hodgkin 1957)

it was assumed that vitamin B_{12} existed "in vivo" as cyanocobalamin. However, another cobalamin had been observed in biological materials (Smith 1948), most likely hydroxocobalamin, but the significance of this was not fully appreciated at the time.

Little further work was done on the isolation of cobalamins from natural sources until Parker and his fellow workers (Weissbach et al 1959: Barker et al 1960a: Barker et al 1960b) announced the isolation and crystallization of several B_{12}-coenzymes. These compounds are extremely light sensitive and on photolysis are all rapidly converted to hydroxocobalamin, which itself is readily converted to cyanocobalamin, by cyanide. Barker and his colleagues (Barker et al 1960b) isolated coenzyme B_{12} from cultures of Clostridium tetanomorphum under very gentle and careful conditions, most stages being done at +4°C in the dark. This group used alcohol precipitation, liquid/liquid extraction, and ion-exchange chromatography on Dowex resins. Coenzyme B_{12} has also been isolated from rabbit liver (Weissbach et al 1959), and Barker and his colleagues have put forward evidence that it is the natural cobalamin in tissues (Barker 1962). Earlier workers did not isolate this material because they did not, or rather, could not take the precautions necessary to preserve such a labile molecule.

More recently, Lindstrand and Stahlberg (1963) have isolated from human plasma a "fourth factor", now known to be methylcobalamin (Lindstrand 1964). This is a light sensitive compound, being rapidly photolysed to hydroxocobalamin. It was isolated in the dark using

alcohol precipitation, liquid/liquid extraction, and ion-exchange chromatography. Liver has been shown to contain methylcobalamin, and it has also been isolated from certain cell cultures (Lindstrand 1964). As a result of this recent work, it seems possible that cyanocobalamin and hydroxocobalamin are artifacts and that the vitamin exists in the storage form mainly as the coenzyme, and in the transport form mainly as methylcobalamin. However, it has been shown that cyanocobalamin (West & Reisner 1949), hydroxocobalamin (Schilling et al 1951), and coenzyme B_{12} (Sullivan & Herbert 1965) are haemopoetically active suggesting that there are systems in the body for interconversion of cobalamins, akin to those described in rats by Uchino et al (1965). It is conceivable that other forms of the vitamin exist, but have not yet been isolated from natural materials.

COMMERCIAL PRODUCTION

Many microorganisms synthesise vitamin B_{12}, provided the media in which they are grown contain inorganic cobalt (Ledingham 1953). This was a promising source for the commercial production of vitamin B_{12}, and the conditions for maximum yield of the vitamin from the microorganisms were soon discovered for each organism which promised to be of value. The organism now most commonly used is Streptomyces griseus. This organism was used for the commercial production of streptomycin, and vitamin B_{12} could be obtained as a by-product (Rickes et al 1948b). However, it was found to be more economical to obtain the streptomycin and the vitamin B_{12} from separate cultures, each grown under the optimum

conditions for the desired product. When required, the cells are harvested and the vitamin extracted from them. For production of vitamin B_{12} containing radioactive cobalt, the Streptomyces is grown in a medium containing radioactive inorganic cobalt, and this is incorporated into the vitamin by the microorganisms. The radioactive vitamin is extracted from the microorganism in the following way (Hailes, personal communication 1964). The vitamin is extracted from the cells by heating to 95°C in the presence of $Na_2 SO_3$ and KCN, at pH3. The use of cyanide increases the yield of the vitamin, possibly by assisting its release from protein, and certainly by converting other cobalamins into the most stable cyanocobalamin. Digestion with buffer is followed by a liquid/liquid extraction, ion-exchange chromatography and paper chromatography. The pure material, radioactive cyanocobalamin, is crystallized from aqueous acetone. If hydroxocobalamin is required, it is obtained by photolysis of the cyanocobalamin, paper chromatography, and crystallization from aqueous acetone.

Both the United Kingdom Atomic Energy Authority and Merck, Sharp and Dohme Ltd. supply radioactive cyanocobalamin and hydroxocobalamin. Cyanocobalamin for therapeutic purposes is supplied mainly by Glaxo Ltd. under the trade name of Cytamen, and hydroxocobalamin under the trade name of Neocytamen. Merck, Sharp and Dohme Ltd. also produce hydroxocobalamin under the trade name Rediso1-H.

METABOLIC FUNCTIONS

Since the isolation of vitamin B_{12} there has been considerable speculation about its metabolic functions. With the isolation of coenzyme forms, many opinions have had to be revised in the light of the known functions of these coenzymes. Three such functions have been established for coenzyme B_{12} in isolated systems. These are the isomerization of glutamic acid to methylaspartic acid (Barker et al 1958), the isomerization of methylmalonyl coenzyme A to succinyl coenzyme A (Wood et al 1964), and the isomerization of 1:2-diols to aldehydes (Abeles and Lee 1964). These functions of coenzyme B_{12} have recently been reviewed by Arnstein (1965), by Weissbach and Dickerman (1965), and by Wagner (1966). Only one of these functions, the isomerization of methylmalonyl coenzyme A to succinyl coenzyme A, has been shown to be of significance in man. In vitamin B_{12} deficient subjects there is an elevated urinary excretion of methylmalonate which rapidly returns to normal levels on parenteral treatment with vitamin B_{12} (Cox & White 1962).

Vitamin B_{12}, possibly in its coenzyme form, is also involved in folate metabolism but the precise mechanism is not fully understood. This aspect of the subject has recently been reviewed by Buchanan (1964), and by Herbert (1965).

THERAPEUTIC AND DIAGNOSTIC APPLICATIONS

In clinical medicine, both cyanocobalamin and hydroxocobalamin have been used, with or without good reason, for the treatment of a variety of diseases. However, the main clinical application is in the treatment of vitamin B_{12} deficient states, of which the most common is pernicious anaemia. In treatment of deficiency states, parenteral administration is usually preferred. It is accepted that treatment with cyanocobalamin (West & Reisoner 1949) hydroxocobalamin (Schilling et al 1951) or coenzyme B_{12} (Sullivan & Herbert 1965) in adequate amounts, corrects all the haematological and biochemical abnormalities and halts the progress of neuropathy.

Radioactive cyanocobalamin is an important tool in diagnostic practice, and the tests of absorption of radioactive cyanocobalamin have replaced time-consuming balance studies involving microbiological assay of faecal collections. Three tests are in common use:-

1. The faecal excretion test (Heinle et al 1952) which involves the collection of faeces for up to 10 days after an oral dose of radioactive cyanocobalamin. The radioactivity in the faeces represents unabsorbed cobalamin.

2. The urinary excretion test (Schilling 1953) which involves the administration of an oral dose of radioactive cyanocobalamin and the simultaneous parenteral administration of a large amount of nonradio-

-active cyanocobalamin. This parenteral dose "flushes out" in the urine a proportion of the absorbed radioactivity. The amount of radioactivity in a 24 hour collection gives a measure of the amount of cobalamin absorbed.

3. The hepatic uptake test (Glass et al 1955), in which the accumulation of absorbed radioactivity in the liver is counted by surface counting about 10 days after an oral dose of radioactive cyanocobalamin.

In tests 1 and 2, accurate collection of faeces or urine is essential, but such collections are not required in test 3.

OUTLINE OF PRESENT STUDIES

The work described in this thesis is a qualitative and quantitative study of the radioactive material found in urine after parenteral radioactive cyanocobalamin and hydroxocobalamin. It can be conveniently divided into six sections.

Section 1: The quantitative excretion of radioactive material in urine was studied after parenteral radioactive cobalamin in various groups of subjects.

Section 2: Experimental investigations relevant to the isolation of the radioactive material found in urine were undertaken, and the techniques used in Section 3 developed.

Section 3: The nature of the radioactive material found in urine was investigated using the techniques developed in Section 2.

Section 4: Further experimental investigations relevant to the isolation of the radioactive material were undertaken, and techniques for characterization were developed.

Section 5: An application of the techniques which had been developed in Sections 2 and 4, for isolation of the radioactive material, and applications of the techniques developed for characterization were undertaken.

Section 6: The quantitative excretion of radioactive material in urine after parenteral radioactive cobalamins was compared to the quantitative excretion of microbiologically active material in urine.

Some of the work described has been published but is presented here in greater detail. Reprints of published work are appended to the thesis, together with a list of published work not directly connected to this thesis.

SECTION 1

Studies on the Quantitative Urinary Excretion of Radioactive Material After Parenteral ^{57}Co Hydroxocobalamin and ^{58}Co Cyanocobalamin

It has been reported that less hydroxocobalamin is excreted in urine after parenteral administration, than cyanocobalamin after an equivalent dose (Killander & Schilling 1961: Glass 1961a: Heinrich & Gabbe 1961). It was felt that investigations could be carried further and observations on the quantitative loss of radioactive material in urine after parenteral administration of radioactive cobalamin in different groups of subjects are reported in this section.

MATERIALS AND METHODS

CLINICAL MATERIALS

The excretion of radioactive material after parenteral ^{57}Co hydroxocobalamin was studied in the following groups of subjects.

Group 1: This group consisted of 26 subjects who were hospital in-patients with no known derangement of cobalamin metabolism, nor hepatic nor renal disease. None were anaemic, and all had normal serum vitamin B_{12} levels. This was regarded as a group of normals.

Group 2: This was a group of 10 anaemic (Hb. < 10g%) subjects who were not vitamin B_{12} deficient, all having normal serum vitamin B_{12} levels. In 3 the anaemia was associated with reticulosis, in 3 it was due to iron deficiency, and in the remaining 4 it was due to

myelofibrosis, hyperplasia of the marrow, chronic myeloid leukemia, and folic acid deficiency, respectively.

Group 3: This group was composed of 10 subjects all of whom, prior to treatment, were anaemic (Hb. < 10g%), had serum vitamin B_{12} levels less than 60 uug/ml, and had megaloblastic marrows. 9 had Addisonian pernicious anaemia, and the remaining subject a post-gastrectomy megaloblastic anaemia. The diagnosis was confirmed by appropriate tests performed later. Initial studies were made on this group while they were anaemic and vitamin B_{12} deficient. They were then treated with 6-10 injections of cyanocobalamin, 1000 ug, intramuscularly, and placed on maintenance therapy, usually 1000 ug cyanocobalamin intramuscularly each month. After an interval, which ranged from 3-17 months after normal blood values had been regained, and at least 5 days after any maintenance therapy had been given, further studies were conducted.

The excretion of ^{58}Co cyanocobalamin was studied in the following groups of subjects.

Group 4: This group consisted of 26 subjects, who were hospital in-patients. In every way these subjects were comparable to those in Group 1.

Group 5: This group was composed of the same subjects as Group 3,

and these studies with ^{58}Co cyanocobalamin were conducted when they were in full haematologic remission, being non-anaemic and non-vitamin B_{12} deficient.

This gave a total of six groups of results, four relating to hydroxocobalamin, and two to cyanocobalamin.

MATERIALS FOR INJECTION

Hydroxocobalamin

Ampoules of non-radioactive hydroxocobalamin were obtained from Merck, Sharp and Dohme Ltd. under the trade name Redisol-H. Each ampoule contained 1000 ug/ml of a sterile aqueous solution of hydroxocobalamin. Radioactive hydroxocobalamin, containing ^{57}cobalt, was obtained, through Merck, Sharp and Dohme Ltd., from the Stonewall Plant, U.S.A., in sterile aqueous solution. This compound was obtained at a concentration of 3.31 uc/1.103 ug/3 ml. It was diluted aseptically with sterile distilled water to give a final solution containing 0.0473 uc/ml. The resulting solution was stored in the dark in sterile rubber capped dark glass Clinbrit bottles at +4^{o}C when not in use.

When required for injection, 4 ml ^{57}Co hydroxocobalamin solution was mixed aseptically with 1000 ug Redisol-H, giving a final solution for injection containing 0.19 uc/1000 ug/5 ml. This was given intramuscularly. The purity of representative samples was checked as

described in Section 3, and also by electrophoresis and partition chromatography, as described in Section 5.

Cyanocobalamin

Ampoules of non-radioactive cyanocobalamin were obtained as commercially available Cytamen (1000 ug/ml) from Glaxo Ltd. Radioactive cyanocobalamin was obtained in vials from the Radiochemical Centre, Amersham, as freeze-dried, non-sterile ^{58}Co cyanocobalamin. Each vial contained 1.1 uc/1.1 ug. A solution was made up in sterile distilled water into sterile rubber capped dark glass Clinbrit bottles, to give a final solution containing 0.1 uc/0.1 ug/ml. This was sterilized by autoclaving at 15 lb/sq.in. for 15 minutes, then stored at +4°C in the dark when not in use.

When required for injection, 4 ml ^{58}Co cyanocobalamin was mixed aseptically with 1000 ug Cytamen. The resulting solution, containing 0.4 uc/1000 ug/5 ml, was given intramuscularly to subjects. The purity of representative samples was checked as described in Section 3 and also by electrophoresis and partition chromatography, as described in Section 5.

URINE COLLECTIONS

From each subject, a pre-injection collection of urine was made to ensure that there was no measurable radioactivity present.

Patients studied with ^{57}Co hydroxocobalamin were given 0.19 uc/1000 ug/5 ml parenterally at a convenient time, and all the urine passed in the subsequent 72 hours collected in 24 hour periods in dark glass bottles. Patients studied with ^{58}Co cyanocobalamin were given 0.4 uc/1000 ug/5 ml parenterally at any suitable time and all the urine passed in the subsequent 24 hours collected.

MEASUREMENT OF RADIOACTIVITY

Each urine collection was shaken, its total volume measured, and a 450 ml aliquot transferred to a clean 500 ml capacity polythene bottle, with a screw cap. If the volume of the total urine collection was less than 450 ml, then the total collection was used and made to 450 ml with water. This aliquot was counted by "end on" counting, using an I.D.L. type 663 scintillation counter, with a sodium iodide thallium activated crystal and a photomultiplier tube. The crystal dimensions were 5.5 cm diameter and 6.9 cm deep. The crystal and photomultiplier tube were shielded by 10.0 cm lead. The crystal had a well of diameter 2.75 cm and depth 5.5 cm, in which small volumes (0-20 ml) could be counted by the "all in" method. The crystal and shielding are shown in Figure 4. For urine collections the "end on" method was used because of the large volumes involved. All samples were counted until at least 5,000 counts or, more usually, 10,000 counts had been recorded, to reduce the counting error to less

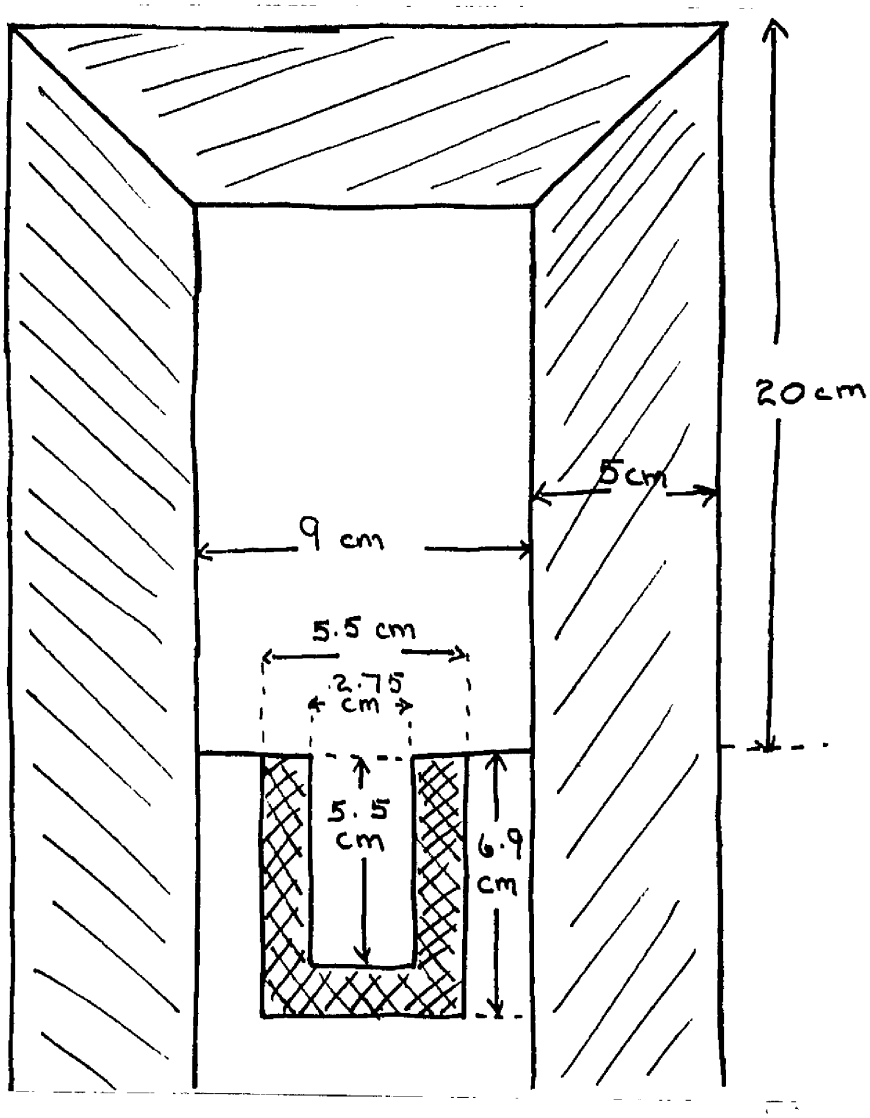

Fig. 4:- Cross-Section of Counting Chamber showing Crystal and Head Shielding.

than ± 2%.

Standards contained the same amount of radioactivity as that used in the injection dose. These were 0.19 uc ^{57}Co hydroxocobalamin, or 0.4 uc ^{58}Co cyanocobalamin, made up to 450 ml with water. The total amount of radioactivity in each urine collection was calculated and, by comparison with the appropriate standard, expressed as a percentage of the given dose.

OTHER METHODS

In each subject, the serum vitamin B_{12} level was measured by the author using a microbiological assay, as described in detail in Section 5.

Originally, all glassware was washed in chromic acid, but latterly Diversey Pyroneg was used, and found to be equally effective. Glassware was steeped in cleaning fluid for one hour, rinsed thoroughly in tap water, and finally rinsed in glass distilled water.

The water used was glass distilled in our own laboratory, using a high capacity all-glass still supplied by Baird and Tatlock Ltd.. For making up sterile aqueous solutions bottles of sterile glass distilled water, prepared by the Fluid Infusion Laboratories were used.

RESULTS AND DISCUSSION

The representative samples of the solutions used for injection were found to be more than 70% pure for both cobalamins, as described in Section 3 and Section 5.

No measurable radioactivity was found in any of the pre-injection collections. From Tables 1-5, and Figures 5-8, it can be seen that a considerable proportion of the injected radioactivity is excreted in the urine. In the case of ^{58}Co cyanocobalamin, this excretion is complete within 24 hours of injection for 1000 ug doses (Adams 1962), but in the case of ^{57}Co hydroxocobalamin, for 1000 ug doses, it may not be complete until 72 hours after injection (Tables 1 & 2). Thus, the figures quoted in Tables 3-5 and those used in constructing the diagrams are those for the 24 hour collection period for cyanocobalamin, and for the 72 hour collection period for hydroxocobalamin. All the results were analyzed by an analysis of variance (Table 5).

A comparison of the percentage of the dose of radioactivity excreted after parenteral ^{57}Co hydroxocobalamin and ^{58}Co cyanocobalamin in 1000 ug doses in normals is shown in Figure 5. It is clear that less radioactivity is excreted after hydroxocobalamin (57.1%) than after cyanocobalamin (70.7%). The percentage of the dose of radio-activity excreted in normals and in initially vitamin B_{12} deficient anaemic subjects when they were in remission, is compared after

	COLLECTION PERIOD		
No.	1-24 hours	24-48 hours	48-72 hours
1	62.5	3.4	<2.5
2	45.9	2.9	<3.5
3	51.9	3.5	<2.2
4	52.9	<4.7	<5.4
5	40.5	7.1	<5.0
6	30.1	5.5	3.6
7	61.5	2.1	0
8	59.6	4.0	0
9	51.9	4.2	<2.1
10	60.2	1.9	0

TABLE 1 Percentage of the Dose of Radioactivity Excreted in Urine by Normals in the Periods 1-24, 24-48 and 48-72 Hours following Parenteral Administration of 1000 ug/0.19 uc ^{57}Co Hydroxocobalamin. A Random Selection of Results.

	COLLECTION PERIOD		
No.	1-24 hours	24-48 hours	48-72 hours
1	52.2	2.5	0
2	30.0	11.7	5.4
3	35.2	4.1	<1.3
4	53.3	1.9	1.6
5	43.5	0	-
6	46.1	0	-
7	34.1	3.5	0
8	29.2	4.8	-
9	51.6	1.5	0
10	26.1	2.1	0

TABLE 2 Percentage of the Dose of Radioactivity Excreted in Urine by Anaemic Subjects in the Periods 1-24, 24-48, and 48-72 Hours following Parenteral Administration of 1000 ug/0.19 uc ^{57}Co Hydroxocobalamin: A Random Selection of Results.

GROUP No.	SUBJECTS	NUMBER	MEAN % DOSE EXCRETED	STANDARD DEVIATION
1	Normals	26	57.1	9.21
2	Anaemic, non-B_{12} Deficient	10	43.8	9.12
3	Anaemic, B_{12} Deficient (Megaloblastic)	10	43.5	12.16
4	3 in Remission	10	56.9	12.62

TABLE 3 Grouping of Patients and Basic Results for Percentage Urinary Excretion after Parenteral Administration of 1000 ug/0.19 uc ^{57}Co Hydroxocobalamin.

GROUP No.	SUBJECTS	NUMBER	MEAN % DOSE EXCRETED	STANDARD DEVIATION
5	Normals	26	70.7	6.36
6	3 in Remission	10	71.0	9.65

TABLE 4 Grouping of Patients and Basic Results for Percentage Urinary Excretion after Parenteral Administration of 1000 ug/0.4 uc ^{58}Co Cyanocobalamin.

	Parenteral cobalamin	Mean % dose excreted	Normal subjects	Normal subjects	Miscellaneous anemia subjects (non B_{12}-deficient)	Megaloblastic (B_{12}-deficient)		
						In relapse	In remission	In remission
			CN B_{12}	OH B_{12}	OH B_{12}	OH B_{12}	OH B_{12}	CN B_{12}
			70.7	57.1	43.8	43.5	56.9	71.0
Normal subjects	CN B_{12}	70.7		Significant $P < 0.001$	Significant $P < 0.001$	Significant $P < 0.001$	Significant $P < 0.001$	Not significant $P > 0.05$
Normal subjects	OH B_{12}	57.1	Significant $P < 0.001$		Significant $P < 0.001$	Significant $P < 0.001$	Not significant $P > 0.05$	Significant $P < 0.001$
Miscellaneous anemia subjects (non-B_{12}-deficient)	OH B_{12}	43.8	Significant $P < 0.001$	Significant $P < 0.001$		Not significant $P > 0.05$	Significant $P < 0.01$	Significant $P < 0.001$
Megaloblastic, in relapse (B_{12}-deficient)	OH B_{12}	43.5	Significant $P < 0.001$	Significant $P < 0.001$	Not significant $P > 0.05$		Significant $P < 0.01$	Significant $P < 0.001$
Megaloblastic, in remission	OH B_{12}	56.9	Significant $P < 0.001$	Not significant $P > 0.05$	Significant $P < 0.01$	Significant $P < 0.01$		Significant $P < 0.01$
Megaloblastic, in remission	CN B_{12}	71.0	Not significant $P > 0.05$	Significant $P < 0.001$	Significant $P < 0.001$	Significant $P < 0.001$	Significant $P < 0.01$	

TABLE 5 Results of Statistical Analyses and Significance of Difference of Mean Values.

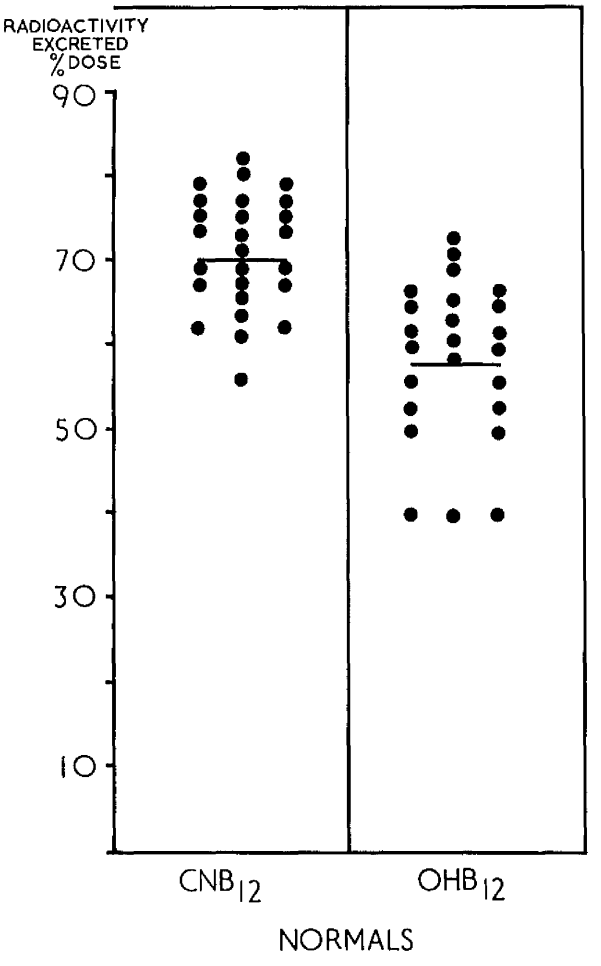

Fig. 5:- Comparison of Percentage Excretion of Radioactivity after 1000 ug ^{57}Co Hydroxocobalamin and after 1000 ug ^{58}Co Cyanocobalamin intramuscularly.

cyanocobalamin in Figure 6, and after hydroxocobalamin in Figure 8. It can be seen that these groups show the same behaviour, for both the cobalamins studied. A comparison of the percentage of the dose of radioactivity excreted in initially vitamin B_{12} deficient subjects in remission, who, as shown, apparently behave as normals, is shown in Figure 7, where it is again seen that less radioactivity is excreted after hydroxocobalamin (56.9%) than after cyanocobalamin (71.0%).

This greater retention, or lower excretion, of radioactive material after parenteral hydroxocobalamin, as compared to that after cyanocobalamin, is possibly due in part to the greater binding capacity of plasma proteins and of tissue for hydroxocobalamin (Bauriedel et al 1956: Skeggs et al 1960). Another factor which may be involved is the slower absorption of hydroxocobalamin, due to its stronger binding to tissues, inferred from the fact that urinary excretion is greater after intravenous than after intramuscular injection (Milhaud 1961), and proven by direct measurement of radioactivity at the injection site at intervals after injection (Killander & Schilling 1961: Glass et al 1961b). These results, viz, the lower excretion of radioactive material after parenteral ^{57}Co hydroxocobalamin than after parenteral ^{58}Co cyanocobalamin, are in agreement with those obtained by other workers (Glass et al 1961a: Killander & Schilling 1961: Heinrich & Gabbe 1961).

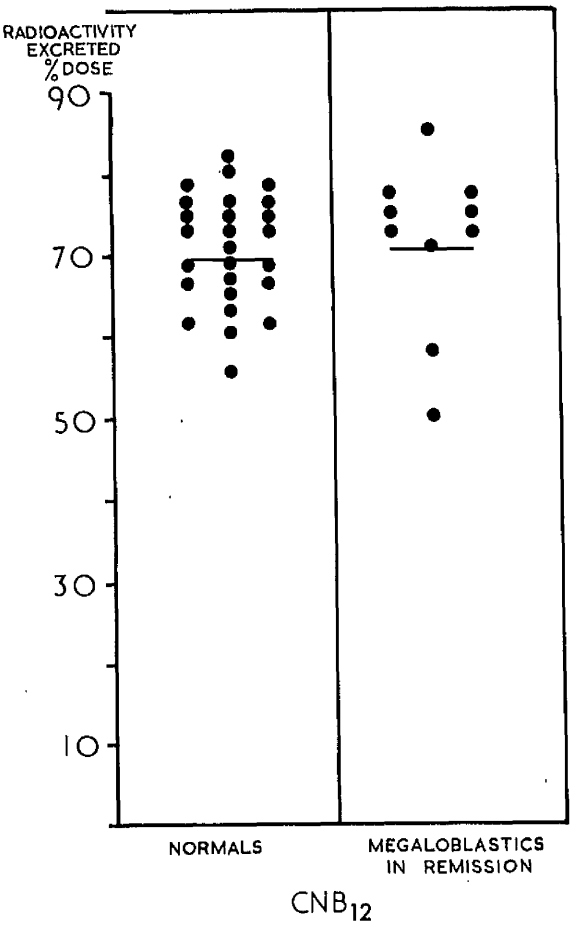

Fig. 6:- Comparison of Percentage Excretion of Radioactivity after 1000 μg ^{58}Co Cyanocobalamin, intramuscularly, in Normals and Megaloblastics in Remission.

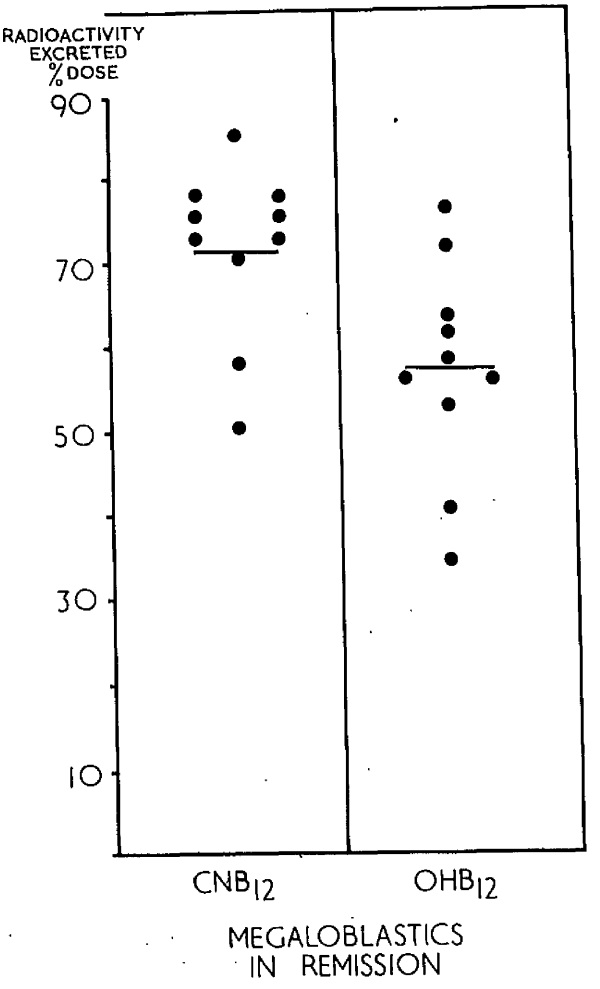

Fig. 7:- Comparison of Percentage Excretion of Radioactivity after 1000 ug ^{57}Co Hydroxocobalamin and after 1000 ug ^{58}Co Cyanocobalamin, intramuscularly, in Megaloblastics in Remission.

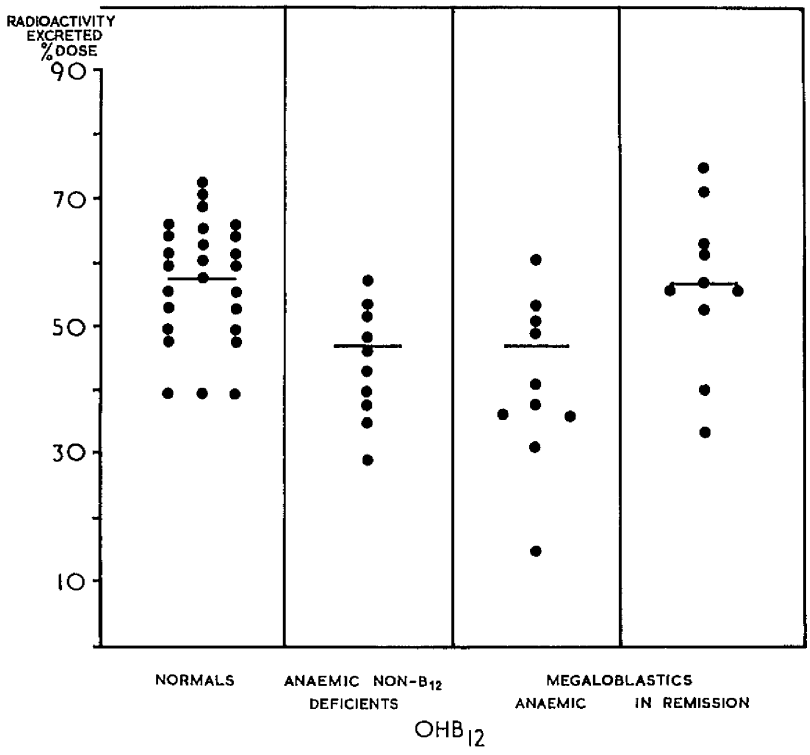

Fig. 8:- Comparison of Percentage Excretion of Radioactivity after 1000 ug ^{57}Co Hydroxocobalamin, intramuscularly, in Various Groups of Patients.

Comparing the percentage excretion of ^{57}Co hydroxocobalamin in the various groups studied the most outstanding feature is the lower excretion (43.7%) in the anaemic subjects, both vitamin B_{12} deficient and non-deficient, as compared to the excretion (57.0%) in the non-anaemic subjects, both vitamin B_{12} deficient and non-deficient (Figure 8). This lower excretion, and hence greater retention, is not connected to the vitamin B_{12} status of the subject as it occurs in both the vitamin B_{12} deficient and non-deficient anaemic subjects. This would suggest that the greater retention is caused by the anaemia 'per se'. This difference is not found with cyanocobalamin where the percentage excretion is not significantly different between normal and anaemic vitamin B_{12} deficient subjects in the dose range 40-1000 ug (Mollin & Ross 1953), and among normal, anaemic non-vitamin B_{12} deficient subjects and anaemic non-vitamin B_{12} deficient subjects with doses of 100 ug and 1000 ug (Adams 1962).

The reason for this greater retention of hydroxocobalamin in anaemic subjects is not clear, but it may be due to a number of factors. Greater binding of hydroxocobalamin by plasma proteins might be thought to be involved as it is generally accepted that plasma volume increases in anaemia (Whitby & Britton 1963) although there is some doubt about this point (Tasker 1959). However, assuming that the binding capacity of plasma for hydroxo-

-cobalamin is 3000 uug/ml (Appendix 1), then a normal plasma volume (5 litres) would bind 15 ug. In anaemic subjects, the plasma volume may be increased to 7 litres, giving a binding capacity of 21 ug. Such a difference of 6 ug would be insufficient to account for the observed difference in the amounts of radioactivity excreted. Greater and stronger binding by tissues such as liver where the bulk of the injected cobalamins are deposited (Glass et al 1954) may also be a factor. Another factor which may be involved is the effect of anaemia on renal function. It has been shown that renal function may be affected by chronic anaemia (Bradley & Bradley 1947), but as the precise mechanism for hydroxocobalamin excretion by the kidney is not known, it is not possible to say whether this impaired function is a significant factor in this lower excretion of hydroxocobalamin. There are other factors which could be involved and much work has yet to be done on this aspect of cobalamin excretion. The only issue pursued by the author was an investigation of the relative binding capacities of plasma and serum for hydroxocobalamin and cyanocobalamin. This is described in Appendix 1.

In this work it has been assumed, as it has been by other workers, that the amount of radioactivity excreted is a measure of the amount of cobalamin excreted. This assumption appears to be valid for cyanocobalamin (Smith 1952: McLean & Bloch 1954), but it has not tested for hydroxocobalamin. In subsequent sections the validity of

this assumption is tested for both cyanocobalamin and hydroxocobalamin.

SECTION 2

Experimental Investigations and Development of Techniques for Isolation of Radioactive Materials in Urine after Parenteral Radioactive Cobalamins.

Introduction

Preliminary Work

Phenol Purification

Ether/Acetone/Water Displacement

Ion-Exchange Chromatography

INTRODUCTION

In Section 1, there have been described certain quantitative investigations into the radioactive material found in urine after parenteral ^{57}Co hydroxocobalamin and ^{58}Co cyanocobalamin. It was felt that a qualitative investigation into the nature of the radioactive material would be of value. This section describes investigations into methods of isolation of the radioactive materials and the development of techniques for isolation which are applicable to urine. This section describes work which is not easily compartmented into the usual headings, and so the investigations undertaken will be described in the order in which they were tackled.

PRELIMINARY WORK

Certain preliminary investigations were made to determine the possibility of isolating and characterizing the radioactive materials in urine. For much of this preliminary work solutions of ^{57}Co hydroxocobalamin in pools of urine were used since most of the work described in Section 1 had been done on hydroxocobalamin and because pools of urine were easy to obtain. Enough non-radioactive hydroxocobalamin was added to the urine sample to give a visible pink colour.

As urine is the chief excretory vehicle for biochemical by-

-products it is a very complex solution. It was obviously necessary to remove as much unwanted matter from the urine as was possible. Ion-exchange resins had been used to remove impurities from biological materials (Heftmann 1961), and it was felt that investigations into the possibilities of ion-exchange chromatography would be of value. Ion-exchange columns had also been recommended for use in separation of cobalamins (Pawelkiewicz 1962: Smith et al 1962).

However, it was not at first realized how sensitive ion-exchange resins were to the presence of inorganic ions in a solution. This is particularly true of resins with a very small capacity, such as the ion-exchange celluloses. The first resin used in an attempt at purification was carboxymethyl cellulose (CMC) supplied by Whatman as CM 70 floc.. It is a cation exchanger, capacity 0.7 meq/ml, and was used as a column 12.0 cm long by 1.5 cm. diameter, at a flow rate of 1.8 ml/minute. A sample of urine with added ^{57}Co hydroxocobalamin was passed through a column of CMC, prepared by washing with 0.1 N HCl followed by distilled water. There was no retention of radioactivity on the column. Next a combination of columns was tried, using a high capacity resin, Amberlite IRC 50, made by Bio-Rad, and CMC. The Amberlite resin was packed into columns (20 ml) straight from its package and washed with water. Another sample of urine with added ^{57}Co hydroxocobalamin was passed through a column of the Amberlite to remove some of the extraneous material

in the urine, then passed through the CMC column. It was thought that the high capacity resin would remove impurities from the urine so that there would be some retention of materials on the CMC. Again, however, there was no retention of radioactive, or other, materials on the CMC, though the Amberlite column did seem to remove some non-radioactive material from the urine.

As hydroxocobalamin, in a solution in distilled water, was found to be retained by CMC, it was realized that the presence of other materials, especially of inorganic ions, in urine, prevented retention of hydroxocobalamin on the CMC. This was confirmed by attempting to chromatograph on CMC aqueous solutions of hydroxocobalamin, in sufficient concentration to give a visible colour. To these solutions NaCl, NaOH, NH_4Cl, and combinations of these salts eg NaCl+ NH_4Cl, had been added. In no case was the hydroxocobalamin retained on the column. It was obviously necessary to remove these interfering ions, and possibly also other contaminants, from the solution of urine and radioactive material before any isolations on columns of ion-exchange resins could be attempted.

Attention was therefore directed to ways in which these contaminants could be removed, and the possibility of extraction of the radioactive material into organic solvents was considered. Various modifications of liquid/liquid extraction, and other methods of preparation and purification of cobalamins have been reviewed by Pawelkiewicz (1962), and Barker and his fellow workers used liquid/

/liquid extraction with various organic solvents to extract cobalamins from tissues and organisms (Barker et al 1960a: 1960b). It was thought that some type of liquid/liquid extraction might be applicable to the present problem of removing the radioactive material from contaminants, but such an extraction would only be applicable to the radioactive material in urine after parenteral radioactive cobalamin if the material was in an organic form, and did not exist as inorganic cobalt. There was evidence that this was so for cyanocobalamin (Smith 1952: McLean & Bloch 1954), but there was no information concerning hydroxocobalamin. However, such an extraction was attempted, in the hope that a useful separation would be achieved.

PHENOL PURIFICATION

In initial studies, samples of urine with added ^{57}Co hydroxocobalamin, in sufficient concentration to give a visible colour, were used. As phenol is a common organic solvent, its use in extraction was investigated. About 10 ml phenol, liquified by heating, was added to a sample of urine, containing added ^{57}Co hydroxocobalamin, in a separating funnel. After shaking, and allowing a time lapse of up to eight hours, it was found that only a small degree of separation into layers took place. The reason for this was that the specific gravity of the liquified phenol was

not sufficiently greater than that of water to give a good and rapid separation of layers. Also, the phenol formed a solution with a considerable amount of the aqueous layer and took this and the materials present in it into the small volume of phenolic layer which was obtained.

It was decided to liquify the phenol with some inert organic solvent of high density in which cobalamins were not soluble before addition to the solution of urine and hydroxocobalamin. The organic solvent would be required to be soluble in phenol, but not in water. This would increase the density of the organic phase and cause a more rapid separation of layers. However, only a small amount of this inert liquid would have to be used, or it would reduce the solubility of the cobalamins in phenol. A solution of equal volumes of phenol and carbon tetrachloride was tried, but it was found that the amount of radioactivity taken into the phenol/carbon tetrachloride layer was very low. The relative concentrations of phenol and carbon tetrachloride were varied, but the amount of radioactivity extracted into the organic phase could not be increased beyond 20-30% of the original radioactivity. A solution of one volume cresol to two volumes carbon tetrachloride was also tried, but this too gave a very poor extraction efficiency.

The next inert solvent to be tried in solution with phenol was chloroform. Again solutions of varying proportions of phenol/

/chloroform were tried, and it was eventually found that the optimum concentration was a 1/1 by volume solution of these. This gave an extraction efficiency of about 80%.

The basis for this extraction is, of course, the preferentially solubility of cobalamins in phenol as compared to water. Cobalamins are soluble in both of these, i.e. in water and in phenol, but they also form a complex with phenol (Mervyn 1963), and this greatly increases their solubility in this solvent. The inert solvent was present in comparatively small amounts, and did not appreciably reduce the solubilities of cobalamins in phenol.

The exact procedure finally used was as follows:-

To the urine with added cobalamin in a separating funnel there was added 1/10 of its volume of phenol/chloroform (1/1 by volume). The funnel was capped and inverted several times. The layers were allowed to separate and the lower phenolic layer was drained into a beaker. The upper aqueous layer was further extracted in a similar way in the separating funnel another two times, and the phenolic layers pooled. The aqueous layer was discarded.

The phenolic layer was washed three times with distilled water to remove aqueous matter still present. This was done by adding 1/10 of its volume of water to the phenolic solution, and

separating the layers, either by using a separating funnel or by centrifuging. The latter was found to be the more rapid and the more efficient. The phenolic layer was washed three times in this way. If it were not washed, there were many inorganic ions still present, and these had an adverse effect on subsequent procedures. The final washed phenolic solution was then subjected to the next stage of the extraction procedure.

This extraction was first tried on urine samples to which ^{57}Co hydroxocobalamin had been added in sufficient concentration to give a visible pink colour. When the procedure had been worked out, and when it could be performed in good yield, the extraction was tried on urine collected after parenteral ^{57}Co hydroxocobalamin. It was found that 60-80% of the radioactive material in the urine could be extracted into phenolic solution. To the urine there had been added, as carrier, enough non-radioactive hydroxocobalamin to give a visible colour. This was to prevent loss of the small amount of radioactive material, presumed to be cobalamin, present, by adsorption on to glassware. However, the extraction procedure was also tried on urine collected after parenteral ^{57}Co hydroxocobalamin, without the addition of carrier. Virtually the same results were obtained with and without carrier. This extraction was similarly tried on urine collected after ^{58}Co cyanocobalamin with non-radioactive cyanocobalamin added as carrier. Again the same results were obtained with or without carrier, and

about 80% of the radioactivity could be extracted into the phenolic solution. The fact that the radioactive material in urine collected after parenteral cobalamins could be extracted into phenol is evidence for its cobalamin-like character.

ETHER/ACETONE/WATER DISPLACEMENT

The phenolic solution obtained from the extraction procedure contained about 80% of the radioactivity present in the original sample, but this was now concentrated into a volume of about 1/3 that of the original solution. As it is much more convenient to work in aqueous solution than in phenolic solution, it was decided to attempt to displace the radioactive material from the phenol into distilled water. This was tried using the method described by Barker and his colleagues (Barker et al 1960a: 1960b). The basis for the method lies in the fact that diethyl ether breaks up the complex which has been formed between the phenol and the cobalamins, so decreasing the solubility of cobalamins in phenol. Also, phenol and diethyl ether are mutually soluble, whilst ether and water are immiscible, and thus the phenol and ether formed a solution. Cobalamins are insoluble in ether, so the formation of an ether/phenol solution further decreased the solubility of cobalamins in the organic ether/phenol phase.

The exact methodology adopted was as follows:-

To the phenolic solution, in a separating funnel, there was added three times its volume of diethyl ether, one time its volume acetone, and a small amount of water. The resulting mixture was shaken up, and the layers allowed to separate. The lower aqueous phase was drained into a beaker. To the ether/acetone solution, still in the separating funnel, there was added a little more water (1/10 of the volume of the original phenol solution), the mixture again shaken up and allowed to separate into layers. As before, the lower aqueous was drained into a beaker. The top layer was further treated with small amounts of water until all the radioactive material had been displaced into the aqueous phase, as determined by radiometric measurements. The aqueous solutions thus obtained were pooled and washed twice in a separating funnel with 1/10 of their total volume of ether/acetone (3/1 by volume) to remove any residual phenol.

Occasionally, at this stage, a white cloudy precipitate had formed in the aqueous extract. The extract was centrifuged and this precipitate was counted to ensure no radioactive material was present in it, then was discarded. On no occasion was the precipitate found to contain any radioactivity.

This extraction and displacement resulted in an aqueous solution of the radioactive material, in 60-80% yield, at a concentration of

ten times that in the original solution in urine. The possibilities of this technique were explored using samples of urine to which ^{57}Co hydroxocobalamin had been added. When satisfactory results, in terms of high extraction rates, were achieved on these samples, urine collected from patients after parenteral ^{57}Co hydroxocobalamin or ^{58}Co cyanocobalamin were subjected to the extraction technique. These samples, either with or without carrier, also gave satisfactory results in terms of extraction rate, even though the urine in these was generally more concentrated.

The aqueous solution obtained after extraction, was thought to be almost entirely free of inorganic ions, as was later proved to be the case, but contained some organic material from urine, particularly some yellow pigments, and these, too, had been concentrated. These pigments were to prove the source of a considerable amount of difficulty in subsequent procedures.

ION-EXCHANGE CHROMATOGRAPHY

Ion-exchange resins were used in an attempt to separate and concentrate the radioactive material or materials present in the aqueous extracts obtained by the two liquid/liquid extractions. Throughout the use of the columns of ion-exchange material, the position of the cobalamins was localized by colour, if possible, or by measurement of the radioactivity in the eluates, or by both these

methods.

To find out which ion-exchange resins were most suitable, solutions of cyanocobalamin or of hydroxocobalamin in distilled water were passed through columns of various ion-exchange materials at different pH values. In every case, a column of about 20 ml was used. Specifications of the resins used, and details of their preparation and use are given.

Zeo-Karb 215/-/H+ form (Permutit Co. Ltd.)

This is a phenolic resin with the functional groups -OH and $-SO_3H$ and is a strongly acid cation exchanger. 20 ml of the resin was steeped in approximately 2N HCl for an hour to convert it to the H+ form. The resin was then transferred to the 100 ml burette with a wad of glass wool at the foot which served as a container. It was back-washed for a few hours at a slow flow rate using tap water, until the effluent was neutral, then allowed to settle and topped with more glass wool. One time the bed-volume (20 ml) of 1N HCl was passed downwards through the column, followed by several times the bed-volume of distilled water. A solution of hydroxocobalamin in distilled water was passed through the column of resin and was retained by it, but could not be eluted by 10% NaCl, 2N HCl, 10% NaCl + 0.1N NaOH, mixed in equal amounts, nor by 10% $CaCl_2$. A solution of cyanocobalamin in distilled water was passed through another column of this resin, prepared in the same way, and the cyanocobalamin was

retained on the column. This could not be eluted by the same eluents.

Neither hydroxocobalamin nor cyanocobalamin could be eluted from Zeo-Karb 215, so this resin was not suitable for separation of these cobalamins.

Zeo-Karb 225/-/H+ form (Permutit Co. Ltd.)

This resin is a cross-linked polystyrene with an $-SO_3H$ functional group, and is a strongly acid cation exchanger. The resin was treated in the same way as Zeo-Karb 215. When it had been converted into its H+ form and washed free of excess regenerant, solutions of cyanocobalamin or hydroxocobalamin in distilled water were passed downwards through columns of resin. Again both cobalamins were retained by the resin, but neither could be eluted, not even by 2N HCl.

As neither cobalamin could be eluted from Zeo-Karb 225, it was not suitable for separation of these cobalamins.

Dowex 50W/-/H+ form (Bio-Rad)

This resin is a strongly acid cation exchanger of a styrene type in which the functional group is $-SO_3H$. The material used was Analytical Grade 50W (AG 50) with a cross-linking of 8 (x8) and particle size 200-400 mesh. It is supplied by the manufacturers

in the Na+ form, but was changed to the H+ form by soaking in 2N HCl for a few hours. The resin column was prepared as described for Zeo-Karb 215.

Solutions of cyanocobalamin or hydroxocobalamin in distilled water were passed downwards through columns of this resin in its H+ form. Both cobalamins were retained firmly by the resin. However, neither cobalamin could be eluted by 10% NH_4Cl + 0.1N NaOH in equal volumes, 10% NH_4Cl alone, 10% NaCl, nor by 2N HCl, so this resin was not suitable for the separation of these cobalamins.

Dowex 1/-/OH- form (Bio-Rad)

This is a strongly basic anion exchanger with a quaternary ammonium functional group. The material used was Analytical Grade 1 (AG 1) with a cross-linking of 8 (x 8) and particle size 100-200 mesh. It is supplied by the manufacturers in the Cl- form, and as it was required in the OH- form it had to be converted to this before use.

This resin was prepared by steeping it in alkali (2N NaOH) for an hour, transferring to a 100 ml burette with a wad of glass wool at the foot, and back-washing at a slow flow rate with tap water until the effluent was neutral. It was allowed to settle, and topped with glass wool, then fully converted to the OH- form using 1N NaOH. It was, finally, thoroughly washed with distilled

water to remove excess regenerant.

Solutions of hydroxocobalamin or cyanocobalamin in distilled water were passed downwards through columns (20 ml) of this resin, but neither of these were retained by the resin, and so this resin was not suitable for separation of these cobalamins.

Dowex 1/-/Cl- form (Bio-Rad)

The use of this resin in the Cl- form to purify extracts obtained from urine samples by the double liquid/liquid extraction described earlier was explored.

The resin in the Cl- form was prepared simply by washing the resin (20 ml) several times with distilled water then transferring it to the 100 ml burette used as a container. The resin was allowed to settle under gravity and topped with glass wool. The extracts being purified were passed downwards through the column of resin, at a flow rate of approximately 1 ml/minute. No radioactivity was retained by the resin, but some of the yellow pigments were removed from the extracts. As this resin has a very high affinity for Cl-, in this form it retains only very strongly anionic compounds, which these yellow pigments appeared to be. They could be seen as a dark yellow band at the top of the column of resin. No attempt was made to elute these pigments, and the resin in the column was discarded after use.

Amberlite IRC 50/-/H+ form (Rohm and Hass Co.)

This is a weakly acidic cation exchanger, made of cross-linked methacrylic acid, with -COOH as the functional group. The resin was prepared in the same way as the other acidic cation exchangers and packed into columns of 20 ml bed-volume.

Solutions of hydroxocobalamin or cyanocobalamin in distilled water were passed downwards through columns of this resin, but neither hydroxocobalamin nor cyanocobalamin were retained to any great extent on this material, and thus this resin was unsuitable for the separation of these cobalamins.

Amberlite IR 4/-/OH- form (Rohm and Hass Co.)

This resin is a phenolic type resin with functional groups -OH and nuclear amino groups. It is a weakly basic anion exchanger.

The resin was prepared in the same way as the other basic resins and packed into columns, of 20 ml bed-volume. Solutions of cyanocobalamin or hydroxocobalamin in distilled water were passed through this column and neither were retained. This resin, therefore, was unsuitable for separation of these cobalamins.

Carboxymethyl Cellulose (CMC) (Whatman)

This is supplied as Cm 70 floc.. It is a cation exchanger with functional group -COOH, and a capacity of 0.7 meq/ml resin. It was used at a flow rate of 1.8 ml/minute, in a column (20 ml)

of 1.5 cm diameter by 12.0 cm long.

When this material was first obtained, conditions for its use were not clear and several preliminary studies were made to establish optimum conditions for its use in separating hydroxocobalamin and cyanocobalamin. For this work, solutions of ^{57}Co hydroxocobalamin or ^{58}Co cyanocobalamin in distilled water were used, except where stated otherwise. Solutions were sufficiently concentrated to have a visible colour.

Attempts to Define the Optimum Conditions for the Use of CMC

The cellulose was prepared by making a slurry of the white flocculent powder with 0.5N HCl, adding water, and decanting the supernatant after the powder had been allowed to settle. More water was added and again decanted after the powder had settled. This was repeated several times. Ethyl alcohol was then added, and decanted, several times. The cellulose was then suspended in water and transferred to a 100 ml burette plugged with glass wool used to support the column of cellulose. It was then allowed to settle and topped with more glass wool. About 20 ml 0.1N HCl (one bed-volume) was allowed to drip through, followed by distilled water until the effluent was neutral. As much conflicting advice on the use of ion-exchange cellulose had been received, it was decided to investigate various methods of using it. In particular, the question of buffering the cellulose

was investigated.

Buffered to pH 4.0

The CMC column, prepared as described, was treated with 20 ml buffer of pH 4.0 (made from BDH buffer tablets). The column was not washed through with water. Hydroxocobalamin in distilled water was passed through and could be seen retained at the top of the column in a diffuse band. This was readily eluted by the same buffer, 1% NaCl, and by 0.1N HCl. Cyanocobalamin in distilled water was not retained on another column of CMC prepared in the same way.

Buffered to pH 4.6

A column of CMC was buffered to pH 4.6 with acetate buffer (7.5 ml 2N NaOH + 2 ml glacial HA made to 450 ml with water) and the column was not washed free of buffer. A solution of hydroxocobalamin in distilled water was passed through, and some of the hydroxocobalamin was retained in a diffuse band. This was readily eluted by the same buffer, 1% NaCl, and by 0.1N HCl. Cyanocobalamin in distilled water was passed through another column of CMC prepared in the same way, and was not retained.

Buffered to pH 6.5

The column of CMC was prepared as before, but before use, 20 ml phosphate buffer of pH 6.5 (26.05 g $NaH_2PO_4.2H_2O$ + 15.5 g $Na_2HPO_4.2H_2O$

in 500 ml water) was passed through the column and the column was not washed with water. Hydroxocobalamin in distilled water was then passed through and some, but not all, was retained in a diffuse band at the top of the column. This could be eluted with the same buffer of pH 6.5. A solution of cyanocobalamin was not retained on a column of CMC prepared in the same way.

Buffered to pH 9.02

The column of cellulose was buffered by passing through the column 20 ml of a buffer of pH 9.02 (made from BDH buffer tablets). The column was not washed with water. A solution of hydroxocobalamin in distilled water was passed through but there was no retention of hydroxocobalamin. Similarly, a solution of cyanocobalamin in distilled water was passed through a column of CMC at pH 9.02, but again there was no retention.

Fully H+ form - non-buffered

A column of CMC was prepared as described. After the passage of 20 ml 0.1N HCl through the column to make sure the cellulose was in its fully H+ form, it was washed thoroughly with distilled water to remove excess regenerant. A solution of hydroxocobalamin in distilled water was passed through and the hydroxocobalamin was retained firmly on the column. A solution of cyanocobalamin in

distilled water was passed through a column of CMC in its H+ form, and it was not retained. Hydroxocobalamin could be eluted using 1% NaCl or 0.1N HCl. The use of 0.1N HCl was preferred because this eluted the retained material and converted the column to the H+ form at the same time, so that, after washing with distilled water, the column was ready for re-use.

As this was the simplest way to use the CMC, and as it gave good results with solutions of cyanocobalamin and hydroxocobalamin in distilled water, both separately and mixed, it was decided to use it in this way.

A purer batch of CMC was later obtained directly from the manufacturers. The previous material was old stock which had been in the laboratory for some time. This purer material required only suspension in water and transferrence to columns before use. However, it was always found to be advisable to treat the column of cellulose with 20 ml 0.1N HCl, to make sure it was fully in the H+ form. If this were not done, the cellulose was likely to be contaminated with atmospheric ions, which would hinder the retention of hydroxocobalamin. The cellulose was finally washed with distilled water, to remove excess regenerant.

Diethylaminoethyl Cellulose (DEAEC) (Whatman)

This cellulose is supplied as DE 40 (DE 11). It is an anion

exchanger with a low capacity of 0.5 meq/ml. It was used as a column (20 ml) 12.0 cm long by 1.5 cm diameter, at a flow rate of 1 ml/minute. It was used only in its OH- form.

A column of this material was prepared simply by suspending the powder in water and transferring it to the 100 ml burette. The cellulose was allowed to settle under gravity and care was taken not to pack the material too firmly as this reduced the flow rate. The column of cellulose was topped with a plug of glass wool. Before use, the column was washed with 0.1 N NaOH (20 ml), to make sure it was fully in the OH- form. It was then washed thoroughly with water to remove excess regenerant.

Neither hydroxocobalamin nor cyanocobalamin were retained on this column. However, its use in removing anionic impurities from hydroxocobalamin had been reported (Smith et al 1962) and it was thought that it might be of use in purifying the extracts from urine. Accordingly, extracts were passed through columns of DEAEC in its OH- form, and it was found that it retained most of the yellow and orange pigments in the urine extract, as distinct bands. It also retained a certain proportion of the radioactive material and this proved to be of use, as will be described later. Most of the material held on the DEAEC could be eluted using 0.1N NaOH, but some of the pigments could only be eluted using 0.5N NaOH. The NaOH both eluted material held on the cellulose and regenerated it to the OH- form. After washing with distilled

water to remove excess NaOH the column of cellulose was ready for re-use. On certain occasions, the material held on the DEAEC column was eluted by 1% NaCl. The column then had to be regenerated with NaOH and washed with water before it could be re-used.

Conclusion from Studies on Ion-exchange Chromatography

In view of the results of these investigations it was decided to pass the extract from urine ,obtained by the extraction/concentration technique, through Dowex 1, in its Cl- form to remove pigments, which were strong anions. This resin did not remove any radioactive material. The effluent from this column was passed through DEAEC in its OH- form to remove other anions, and finally through CMC to remove cations. The use of DEAEC and CMC thus enabled the radioactive material to be divided into three fractions. Anionic material was retained on the DEAEC and could be eluted by 0.1N NaOH. Basic material was retained on the CMC and could be eluted using 0.1N HCl. Neutral material was not retained on either of these, but was present in the final solution obtained after the extract had been passed through all the columns.

SUMMARY

From the results of the investigations described in this section,

and in accordance with the observations made, a method of isolation and separation of the radioactive materials in urine after parenteral radioactive cobalamine was determined. The radioactive material could be extracted from urine into phenol/chloroform, and displaced back into water by ether/ acetone. The resulting aqueous extract contained about 80% of the original radioactive material. Impurities could be removed by Dowex 1 and the radioactive material could be separated into three fractions on DEAE and CM.

An application of this double liquid/liquid extraction, followed by ion-exchange chromatography of the extracts, is described in Section 3.

SECTION 3

Quantitative Analyses of Radioactive Material Found in Urine after Parenteral ^{57}Co Hydroxocobalamin and ^{58}Co Cyanocobalamin.

It has been common practise to use radioactive cobalamins to measure the urinary loss after parenteral dosing. This appears to be valid when radioactive cyanocobalamin is used, as there is some proof that the radioactive material found in urine is cyanocobalamin. (Smith 1952: McLean & Bloch 1954). In the case of radioactive hydroxocobalamin, however, there is no such evidence. Moreover, the work done previously on cyanocobalamin would not now be considered to be conclusive as the standards used for comparison were probably not pure. It was, therefore, decided to reinvestigate this point and also to investigate the nature of the radioactive material in urine after parenteral radioactive hydroxocobalamin. No work on this latter point has been reported. The techniques developed in Section 2 were used for this work.

MATERIALS AND METHODS.

Both "in vivo" and "in vitro" studies were performed. In the former, the material being investigated was the radioactive material found in urine after parenteral ^{57}Co hydroxocobalamin or ^{58}Co cyanocobalamin. In the latter, the material investigated was that which was in urine after the addition of ^{57}Co hydroxocobalamin to pools of urine.

"In vivo" Studies.

Doses of cyanocobalamin and of hydroxocobalamin were prepared

as described in Section 1. The dose of ^{57}Co hydroxocobalamin was 0.24 uc/1000 ug/6 ml, and of ^{58}Co cyanocobalamin was 0.5 uc/1000 ug/6 ml. All the subjects used in this study were ward in-patients, who had no known derangement of cobalamin metabolism, nor renal nor hepatic disease. Subjects were given parenteral radioactive cobalamin at 10 p.m. and all urine passed in the subsequent 10 hours collected in dark glass bottles. In certain experiments, these bottles had been sterilized by autoclaving at 15 lb/sq.in. for 15 minutes, and in these experiments urine was passed into the bottles.

"In vitro" Studies.

Urine pools were made by pooling overnight urine collections from various apparently normal staff and family members. To these were added appropriate amounts of ^{57}Co hydroxocobalamin to give much the same concentration of radioactive material as was thought to be in the urine collected after parenteral ^{57}Co hydroxocobalamin. It has been shown in Section 1 that about 50% of the injected dose of radioactivity is excreted and it was observed that about 500 ml urine was passed overnight. Thus 0.12 uc/500 ug was added to each 500 ml of pooled urine.

Analytical Procedure.

This was as described in Section 2, and consisted essentially of the following steps:-

1. Liquid/liquid extraction using phenol/chloroform (1/1 by volume),

to extract the radioactive material in the urine into phenolic solution.

2. Back-displacement of the radioactive material into distilled water using ether/acetone (3/1 by volume).

3. Ion-exchange chromatography of the extracts thus obtained on Dowex 1 in its Cl- form, DEAE and CMC.

In the work described in this section, non-radioactive cobalamin was added as carrier to the urine before extraction. This carrier was added to prevent the radioactive material, probably cobalamin, present in very small amounts, being lost by adsorption on to glassware, and to enable the position of the cobalamins to be localized visually throughout the experimental work. The addition of carrier did not appear to make any difference to the results obtained. After parenteral ^{57}Co hydroxocobalamin, 5 mg crystalline non-radioactive hydroxocobalamin, obtained from Dr. Mervyn, of Glaxo Ltd., was added to each urine collection. 5 mg. of this material was also added to each 500 ml pooled urine to which ^{57}Co hydroxocobalamin had already been added. After parenteral ^{58}Co cyanocobalamin, 5 mg lyophilized non-radioactive cyanocobalamin, obtained from Dr. Mervyn of Glaxo Ltd. as a gift, was added to each urine collection.

Measurement of Radioactivity

Radioactivity was measured using the apparatus already described.

However, as small volumes were being dealt with, 10 ml aliquots were counted in the well of the crystal. This enabled small amounts of radioactivity to be counted with greater accuracy. Each sample being counted was thoroughly mixed and its total volume measured. 10 ml was taken from it by means of a pipette and the amount of radioactivity in this determined. The container in which it was counted was a polythene bucket with an external diameter slightly smaller than that of the well. These buckets were of standard size and so introduced no additional counting errors. By calculation, the total amount of radioactivity in the samples was determined.

The radioactivity of the original urine collection was taken as 100% and the radioactivity of the extract, obtained by the double liquid/liquid extraction, compared to this to give the percentage efficiency of the extraction. The radioactivity of the extract was then taken as 100% and the radioactivity of the fractions separated by ion-exchange chromatography compared to it to give the relative percentage of each of these.

Other Methods

All glassware was steeped for at least an hour in either chromic acid or Diversey Pyroneg then rinsed six times in tap water and finally rinsed in distilled water. Chemical reagents were Analar where possible. Water, from a high capacity all-glass still, supplied by Baird and Tatlock Ltd., was generally

used, but occasionally Elgostat water was used. Sterile distilled water for making the solutions for injection was obtained from the Fluid Infusion Laboratories.

RESULTS AND DISCUSSION

Analyses of Injection Solutions

Hydroxocobalamin

By ion-exchange chromatography, representative injection doses (0.24 uc/1000 ug/6 ml) of ^{57}Co hydroxocobalamin were separated into three fractions. This was done on two separate occasions. Representative injection doses (0.24 uc/1000 ug/6 ml) were subjected to the extraction/concentration procedure before ion-exchange chromatography to make sure that this technique had no detrimental effect on hydroxocobalamin. This was done by adding an injection dose (0.24 uc/1000 ug/6 ml) to 1 litre water and subjecting the resulting solution to the extraction/concentration procedure, then separating the three fractions by ion-exchange chromatography. The relative proportions of the three fractions in the injection dose, before and after extraction/concentration, are shown in Table 6, and these show that the dose contains more than 70% basic cobalamin and that the extraction/concentration technique causes no apparent damage to this cobalamin. The basic cobalamin is, presumably, hydroxocobalamin.

Cyanocobalamin

Similarly, before any work was done using cyanocobalamin, a representative dose (0.5 uc/1000 ug/6 ml) of ^{58}Co cyanocobalamin was

SAMPLE	PROPORTION OF CONSTITUENTS %		
	NEUTRAL	BASIC	ANIONIC
Before Extraction	10	79	9
	12	74	11
After Extraction	7	80	15

TABLE 6 Results of Analyses of Representative Samples of 1000 ug/0.19 uc ^{57}Co Hydroxocobalamin Before and After Extraction/Concentration.

separated into three fractions by ion-exchange chromatography. Another representative dose was added to 1 litre water, subjected to the extraction/concentration procedure, then separated into three components by ion-exchange chromatography. This latter was done on three separate occasions. Results are shown in Table 7 and these show that the injection solution contains more than 70% neutral cobalamin, presumably cyano-cobalamin, and that the extraction/concentration procedure caused no apparent change.

Radioactive Material in Urine after Parenteral ^{57}Co Hydroxocobalamin - "in vivo" Studies

A. Non-Sterile Collections of Urine

Seven subjects were given parenteral ^{57}Co hydroxocobalamin (0.24 uc/1000 ug/5 ml) and the radioactive material found in the urine in the subsequent 10 hours separated into three fractions. The results are shown in Table 8 where it can be seen that in all cases less than half of the radioactive material is in a basic form (35.2%), the remainder being in an anionic form (36.3%), and a neutral form (23.9%). However, these urine collections were made without regard to sterility and it was possible that bacterial infection could have caused some change in the radioactive material.

SAMPLE	PROPORTION OF CONSTITUENTS %		
	NEUTRAL	BASIC	ANIONIC
Before Extraction	72	13	11
After Extraction	71	12	12
	72	14	14
	84	8	9

TABLE 7 Results of Analyses of Representative Samples of 1000 ug/0.4 uc ^{58}Co Cyanocobalamin Before and After Extraction/Concentration.

CASE No.	EFFICIENCY of RECOVERY %	PROPORTION OF CONSTITUENTS %		
		NEUTRAL	BASIC	ANIONIC
1	80	26.5	30.5	42.0
2	82	35.0	27.0	38.0
3	81	15.0	39.0	45.0
4	92	17.5	32.0	48.0
5	77	22.0	42.0	29.6
6	74	22.6	42.3	27.3
7	75	29.5	33.5	38.2
MEAN	80.1	23.9	35.2	38.3

TABLE 8 Results of Analyses of Extracts of Urine Collected after Parenteral 1000 ug/0.24 uc ^{57}Co Hydroxocobalamin in Normal Subjects.

B. Sterile Collections of Urine

To gauge this possibility, two subjects were given parenteral ^{57}Co hydroxocobalamin (0.24 uc/1000 ug/6 ml) and urine collected directly into sterile bottles. Each collection was subjected to the extraction/concentration technique as soon as possible after the collection period (10 hours) had finished. The results from these two subjects shown in Table 9, are comparable to those obtained when urine had been collected without regard to sterility. The radioactive material in urine was found to be composed of approximately 41% basic, 38% anionic and 19% neutral components.

However, this conversion of hydroxocobalamin to other compounds could have taken place either in the body or in the urine. In an attempt to obtain information on this problem the effect of urine on ^{57}Co hydroxocobalamin was studied, "in vitro".

Radioactive Material in Urine after the Addition of ^{57}Co Hydroxocobalamin and Incubation - "in vitro" Studies

A. At Room Temperature (17°C)

The effect of urine on hydroxocobalamin "in vitro" at room temperature was investigated by adding ^{57}Co hydroxocobalamin (0.12 uc/500 ug/3 ml) to 500 ml pooled urine. After mixing,

CASE No.	EFFICIENCY of RECOVERY %	PROPORTION OF CONSTITUENTS %		
		NEUTRAL	BASIC	ANIONIC
8	82	10.0	47.0	40.5
9	52	28.0	36.0	35.0

TABLE 9 Results of Analyses of Extracts of Urine Collected With Regard to Sterility after Parenteral 1000 ug/0.24 uc ^{57}Co Hydroxocobalamin in Normal Subjects.

the resulting solution was incubated at room temperature (17°C). Aliquots were taken immediately after mixing and at convenient intervals. Each aliquot was subjected to the extraction/concentration procedure and separated into three fractions by ion-exchange chromatography. This was done on three different pools of urine and the results are shown in Table 10. From these results it is clear that urine has an effect on hydroxocobalamin and that it occurs immediately after mixing, little change being observed with time.

B. At Body Temperature (37°C)

However, urine spends quite a considerable time in the bladder at 37°C and it is possible that a temperature of 37°C would increase the amount of conversion of hydroxocobalamin with time. With this in mind, the effect of a pool of urine (500 ml) on ^{57}Co hydroxocobalamin (0.12 uc/500 ug) was investigated. After addition of the hydroxocobalamin to the urine, the mixture was shaken and incubated at 37°C. Aliquots were taken at 3 and 24 hours of incubation, subjected to the extraction/concentration technique and separated into three fractions using ion-exchange chromatography. The results, shown in Table 11, suggest that conversion has been carried further at 37°C.

SAMPLE	TIME AFTER ADDING ^{57}Co HYDROXO-COBALAMIN HRS.	EFFICIENCY of RECOVERY %	PROPORTION OF CONSTITUENTS %		
			NEUTRAL	BASIC	ANIONIC
Pool 1	0	85	6	67	20
	24	62	12	67	17
Pool 2	0	76	14	60	14
	24	76	25	46	20
Pool 3	0	73	14	60	21
	3	60	14	59	20
	24	60	20	58	20

TABLE 10 Results of Analyses of Extracts of 500 ml Pools of Urine with Added 500 ug/0.12 uc ^{57}Co Hydroxocobalamin: The Mixtures were incubated at 17°C.

TIME AFTER ADDING ^{57}Co HYDROXO-COBALAMIN HRS.	EFFICIENCY of RECOVERY %	PROPORTION OF CONSTITUENTS %		
		NEUTRAL	BASIC	ANIONIC
3	63	27	52	17
24	63	35	49	17

TABLE 11 Results of Analyses of Extracts of a 500 ml Pool of Urine with Added 500 ug/0.12 uc ^{57}Co Hydroxocobalamin: The Mixture was incubated at 37°C.

From these "in vitro" studies it is clear that urine itself has an effect on hydroxocobalamin converting some of it to neutral and some to anionic material.

Summary of Results - ^{57}Co Hydroxocobalamin

The facts arising from this work are that after parenteral ^{57}Co hydroxocobalamin the radioactive material found in urine exists as approximately 35% basic fraction, 24% neutral fraction, and 35% anionic fraction. It is likely that each of these fractions is a cobalamin as they are all extractable into phenol and can be displaced from this by ether. This conversion of hydroxocobalamin to other cobalamins is not caused wholly by urine after it has been voided, nor does it seem to be caused by bacteria.

Discussion - ^{57}Co Hydroxocobalamin

As no other work has been published to date on this subject and as the mechanism of clearance of hydroxocobalamin from the blood stream by the kidney is not known, it is not possible to form clear ideas about the location and mechanism of the observed conversion of hydroxocobalamin to other forms. However, the fact that some degree of conversion was caused by urine itself would suggest that conversion may take place after the hydroxocobalamin has left the blood stream. It is possible that it is a purely chemical reaction, not affecting the cobalamin molecule, but affecting

only the ligand on the central cobalt atom. The nature of the three fractions obtained, basic, anionic and neutral has not been ascertained, but it seems likely that the basic material is hydroxocobalamin and the neutral cyanocobalamin.

The nature of the anionic material is quite unknown, but it could be either a degradation product, e.g. a red acid, or an anionic complex of the cobalamin molecule.

Radioactive Material in Urine after Parenteral ^{58}Co Cyanocobalamin - "in vivo" Studies

Two subjects were given parenteral ^{58}Co cyanocobalamin (0.5 mc/1000 ug/6 ml), and the results of analyses of the urine collections (10 hours) after extraction/concentration and ion-exchange chromatography shown in Table 12. From these, it is clear that cyanocobalamin is excreted as more than 70% neutral cobalamin.

Discussion - ^{58}Co Cyanocobalamin

It is known that cyanocobalamin is cleared purely by glomerular filtration (Watkin et al 1961; Nolp et al 1963), and from the results obtained it appears to be excreted mainly as a neutral cobalamin. It seems likely that this is cyano-cobalamin, but this has not been conclusively proved merely by using column chromatography. If it is cyanocobalamin, then

CASE No.	EFFICIENCY of RECOVERY %	PROPORTION OF CONSTITUENTS %		
		NEUTRAL	BASIC	ANIONIC
10	87	79.5	5.5	6.5
11	87	83.5	6.5	8.0
		81.5	6.0	7.7

TABLE 12 Results of Analyses of Extracts of Urine Collected after Parenteral 1000 ug/0.5 uc ^{58}Co Cyanocobalamin in Normal Subjects.

the results obtained are in agreement with those of other workers (Smith 1952; McLean & Bloch 1954).

SUMMARY AND CONCLUSION

It has been shown that after parenteral administration of ^{57}Co hydroxocobalamin (0.24 uc/1000 ug/6 ml), the radioactive material found in the urine in the first ten hours after injection can be separated into three components using the techniques developed and described in Section 2. These components are a basic, an anionic and a neutral cobalamin, found in the relative proportions of approximately 35%, 35%, and 25%, respectively. Evidence was found which suggested that this change may be caused in part by urine itself.

It has also been shown that after parenteral ^{58}Co cyanocobalamin (0.5 uc/1000 ug/6 ml) the radioactive material found in the urine in the first ten hours after injection is almost wholly in the form of a neutral cobalamin.

The chemical nature of these various components was not further investigated at this stage as techniques for characterization of cobalamins were not sufficiently defined. Investigations into possible techniques for isolation and characterization are described in the next section.

SECTION 4.

Further Experimental Investigations and Development of Techniques for Isolation and Characterization of Cobalamins.

The nature of the three components of the radioactive material in urine after parenteral radioactive cobalamins in "in vitro" and "in vivo" urine samples was still unknown. These fractions had been isolated by the techniques developed in Section 2, but they had not been completely characterised. They did show cobalamin-like character in that they were extractable into phenol, and they were identified as basic, neutral and anionic components. Work on the characterization of these fractions was undertaken, and investigations into possible methods of further isolation and characterisation are described below.

As in Section 2, the work described here is not conveniently divided into the conventional headings, but will be described in the order in which it was done.

In the work described in Section 3, carrier had been added to each urine collection to facilitate separation of the radioactive material from the other materials present in urine. This was satisfactory for quantitative work, as it had been found that the addition of carrier did not alter the relative proportions of the radioactive materials present. It was not, however, satisfactory for qualitative work as, in this, the nature of the actual radioactive materials found in urine was under investigation.

As it has been established that about one half of the dose is excreted after parenteral administration of radioactive cobalamin

(see Section 3), this would give a concentration of approximately 500 ug/500 ml overnight urine. After extraction/concentration this was possibly only 350 ug/50 ml. As this was insufficient to work on satisfactorily, especially as this amount is the sum of three components, it was obviously necessary to pool the extracts obtained from several "in vivo" or "in vitro" urine samples though each of these was dealt with separately.

For "in vivo" samples, administration of radioactive cobalamin was made as described, and urine collected overnight (10 hours). No carrier was added and the urine collection subjected to the extraction/concentration technique. Extracts from several urine collections were then pooled and separated into components by ion-exchange chromatography. This was repeated several times and pools of the three fractions were built up. There did not appear to be any reason not to pool the urine extracts, nor to pool the separate fractions obtained. Similarly, "in vitro" samples, with added ^{57}Co hydroxocobalamin, were made and subjected to the extraction/concentration and ion-exchange techniques without added carrier. Again, pools were built up by pooling the extracts before column chromatography, and by pooling the separate fractions obtained from several ion-exchange chromatography runs. Much of the preliminary work was done on "in vitro" samples and the fractions obtained from them, as these were very much easier to obtain.

The pooling of several batches of radioactive material excreted in urine increased not only the amount, but also the volume of the final fraction which was being investigated. Except where stated otherwise, each fraction, from "in vivo" or "in vitro" urine samples, was subjected to the same investigations.

CONCENTRATION BY EVAPORATION

The fractions were concentrated using a rotary evaporator, made by Wright Scientific Co.. This apparatus made it possible to evaporate them to dryness using very little heat, so reducing the risk of destruction of the material. The material deposited on the flask was resuspended in a few ml distilled water.

However, the three fractions thus obtained still contained yellow contaminants and attempts were made to remove some of this colouration by further purification.

CHARCOAL TREATMENT

Adsorption of cobalamins on to charcoal was used in the original attempts to isolate vitamin B_{12} and has remained a useful method for removing contaminants. Basically, the method involves adsorption of materials on to charcoal and differential elution from it. The use of charcoal for purification of cobalamins has been reviewed by Pawelkiewicz (1962).

For these investigations, fractions obtained from the "in vitro"

(^{57}Co hydroxocobalamin) urine samples were used. The solution of radioactive materials, after evaporation, and resuspension in a few ml distilled water, was treated with activated charcoal (as 110). This charcoal was obtained as a gift from Dr. Mervyn of Glaxo Ltd., who had found it suitable for purposes similar to those to be described here. A knife edge of charcoal was added to the solution in a test-tube and the tube stoppered and shaken. The tube was centrifuged at 2000 rpm for 10 minutes, and the supernatant decanted. It was found, by measurement of radioactivity, that the charcoal had taken up all of the radioactivity, and hence all of the cobalamin-like compounds. The charcoal also seemed to take up most of the pigments still remaining as contaminants, as the supernatant was clear and colourless. The charcoal was washed with distilled water, and the radioactivity remained firmly attached to the charcoal, as shown by the absence of radioactivity in the washings. The pigments also remained attached to the charcoal as judged by the lack of colour in the washings.

A. Elution with Aqueous Acetone.

Elution of the radioactive material from charcoal was tried using solutions of acetone and water (Ford & Porter 1953). Solutions of varying proportions of acetone and water were tried, but in each case 10 ml of the solution was added to the charcoal in the test-tube, and the tube stoppered, shaken and centrifuged. The supernatant was decanted and counted. The amount of radioactivity in the supernatant

was compared to that in the original solution. The recovery rates for various acetone concentrations are shown in Table 13. 60% aqueous acetone gave the highest recovery rate, but this was only 50-60%, and it could not be increased by increasing the volume of the eluting solution. The resulting aqueous acetone solution was again evaporated to dryness, and the material deposited on the flask resuspended in a few ml distilled water. Little improvement had been made on the purity of the material as the yellow pigments appeared to have been taken up by the charcoal, and eluted from it along with the radioactive material by the aqueous acetone.

It was thought that a preliminary treatment of the urine sample, before extraction/concentration, might remove some of the impurities and also make the extraction/concentration and subsequent procedures more easy and more efficient. Such a preliminary treatment was tried on "in vitro"(^{57}Co hydroxocobalamin)urine samples. The urine sample was shaken with charcoal (ss 110) and centrifuged at 2000 rpm for 10 minutes. The supernatant was decanted and counted, and it seemed that all the radioactivity had been adsorbed on to the charcoal. This was verified by counting the charcoal. It was then washed several times with distilled water and elution attempted using 60% aqueous acetone. Again it was not possible to get a recovery of more than 50-60% of the radioactivity. This could not be increased by using different concentrations of acetone, nor by

ELUTING SOLVENT % ACETONE	% ELUTED
100	0
80	36
60	60
40	40

TABLE 13 Elution of ^{57}Co Hydroxocobalamin from Charcoal by Aqueous Acetone.

using a larger volume of eluent. As this recovery was too poor for the first stage of purification this method was discarded. However, it was felt that the use of charcoal could be adapted to give good results. The uptake of radioactive material by charcoal was very high and so this presented no problems. The problem was to find something which eluted the radioactivity in good yield, but did not interfere with subsequent steps.

B. Elution with Phenol/Chloroform

It was decided to try as eluent the solution used for the original extraction of urine, i.e. phenol/chloroform (1/1 by volume), as it has been established that cobalamins are soluble in this solution. Accordingly, an "in vitro" (^{57}Co hydroxocobalamin) urine sample was shaken with charcoal and centrifuged at 2000 rpm for 10 minutes. The charcoal was washed with distilled water to remove inorganic ions, which had also been adsorbed on to the charcoal. It was then washed with phenol/chloroform several times so that the final volume of phenol/chloroform was approximately 1/5 that of the original urine volume (about 100 ml). It was found that the radioactivity was eluted from the charcoal in 60% yield. Increasing the volume of the eluant did not increase the amount of radioactive material eluted. This yield was acceptable because this method dispensed with the stage of phenol extraction of the radioactive material from urine which would be done in only 80% yield. The

phenol/chloroform solution required to be washed only once with water before being subjected to the ether/acetone/water displacement. The reason for this was that the water washing of the charcoal had already removed most of the inorganic contaminants. This modified method gave a final efficiency of just less than 60%. The extracts obtained in this way were passed through ion-exchange columns in the same way as those obtained by the first method. Each extract again yielded three fractions in the same relative percentages.

Charcoal treatment and elution with phenol/chloroform had, however, removed more of the yellow pigments than the original extraction/concentration had. There were still some yellow pigments remaining, especially in the anionic fraction eluted from the DEAE, and it was feared that these would interfere with subsequent identification of the compound. The other two fractions, basic and neutral, had very much less contaminating material present and the possibility of identifying these seemed much better.

C. Preliminary Alkali Washing of Charcoal before Elution with Phenol/Chloroform

One other attempt was made to obtain a purer extract on elution from the charcoal. Charcoal containing radioactive material was obtained by shaking a 500 ml "in vitro" (^{57}Co hydroxocobalamin) urine sample with charcoal, centrifuging and decanting the super-

natant. After washing the charcoal once with water it was washed once with 25 ml 1N NaOH, in an attempt to remove more of the yellow pigments. This was partially successful in that pigments were eluted by the alkali, as could be seen by the colour of the eluate. After washing the charcoal with alkali, it was again washed with water, using 150 ml, then the radioactive material was eluted with phenol/chloroform. The extract thus obtained was subjected to ion-exchange chromatography, as before. This method gave a purer extract, again separable into three components in the same relative percentages as before. These fractions were also more purified than those from previous extractions, but there were still some impurities present, especially in the anionic fraction.

The amount obtained of each of the three fractions was very small, but there was a visible colour in each fraction. The neutral and basic fractions were pink, but the anionic was orange, probably due to contamination.

The fractions from both "in vitro" (hydroxocobalamin) samples and "in vivo" (hydroxocobalamin and cyanocobalamin) samples, obtained by any of the methods described in this Section or in Section 2, were subjected to various experimental observations in an attempt to characterize them. Some investigations required evaporation to dryness and resuspension in a few ml distilled water,

but for others the fractions were studied in more dilute solution such as that in which they were obtained from the ion-exchange chromatography.

PREPARATION OF STANDARDS

As the materials under investigation were thought to be cobalamins, standards of known cobalamins were prepared. These standards, of cyanocobalamin and hydroxocobalamin, were subjected to the same investigations as the unknown materials, for comparison purposes. They were made by adding Cytamen '1000' (1000 ug cyanocobalamin) or Redisol-H (1000 ug hydroxocobalamin) to 400 ml tap water and treating the resulting solutions by the first described extraction/concentration technique. Each extract obtained in this way was run through DEAE and CMC columns. The hydroxocobalamin, being basic was retained on the CMC column, from which it was eluted, as was the basic fraction from urine, by 0.1M HCl. The solution thus obtained was evaporated to small volume using the rotary evaporator, and used as hydroxocobalamin standard. Cyanocobalamin, being neutral, was not retained on either column, but was collected as an effluent from the CMC column. This solution was evaporated to a few ml using the rotary evaporator and used as standard cyanocobalamin.

SPECTROPHOTOMETRY

The spectra of the unknown compounds and of the standards were recorded, in comparatively dilute solutions, using an Optika (U.K.) Spectrophotometer, for the range 220-520 mu. This covers most of the infra-red and visible ranges. This photometer is the property of the University of Strathclyde Pharmacology Department, and permission to use it was kindly granted to the author by Professor Stenlake.

The spectra of the standards could be recorded easily but some difficulty was encountered in recording the spectra of the unknown compounds. In the infra-red range, there seemed, in each case, to be some substance absorbing very strongly, as shown in an example of a spectrum in Figure 9. The interfering substance was thought to be possibly either phenol or NaOH. To test if it was phenol, the solution whose spectrum was being recorded was extracted using hexane, and the spectrum of the hexane recorded. (see Figure 10). If phenol had been present, there should now have appeared three peaks at about 280 mu. These peaks did show up, but, by their height, it was judged that there was insufficient phenol present to interfere substantially with the spectrum of the compound being investigated. Thus the substance causing interference seemed likely to be NaOH, though the origin of this was not clear, except in the case of the anionic

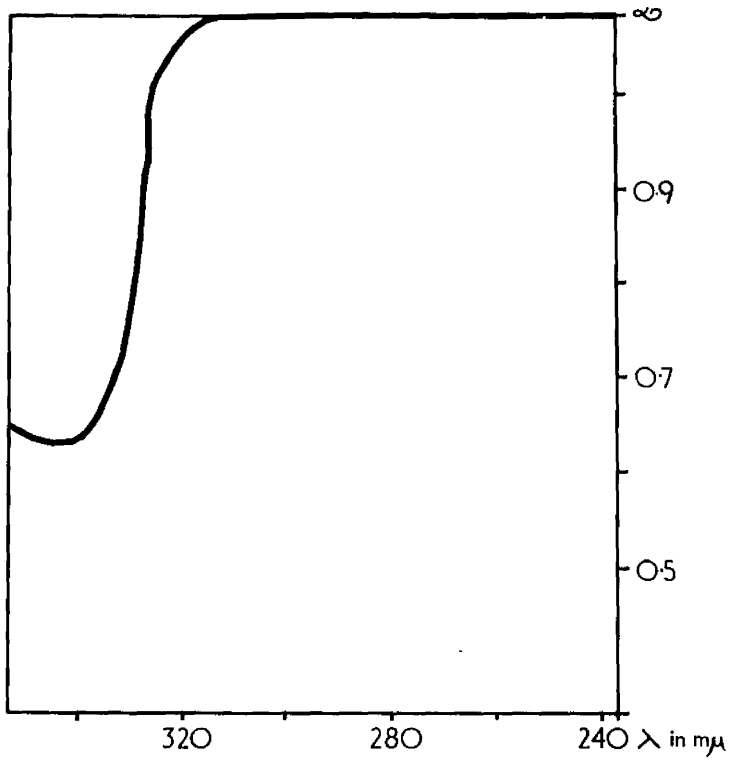

Fig. 9:- Sceptrum of a Representative Fraction to show strong absorption below 320 mu.

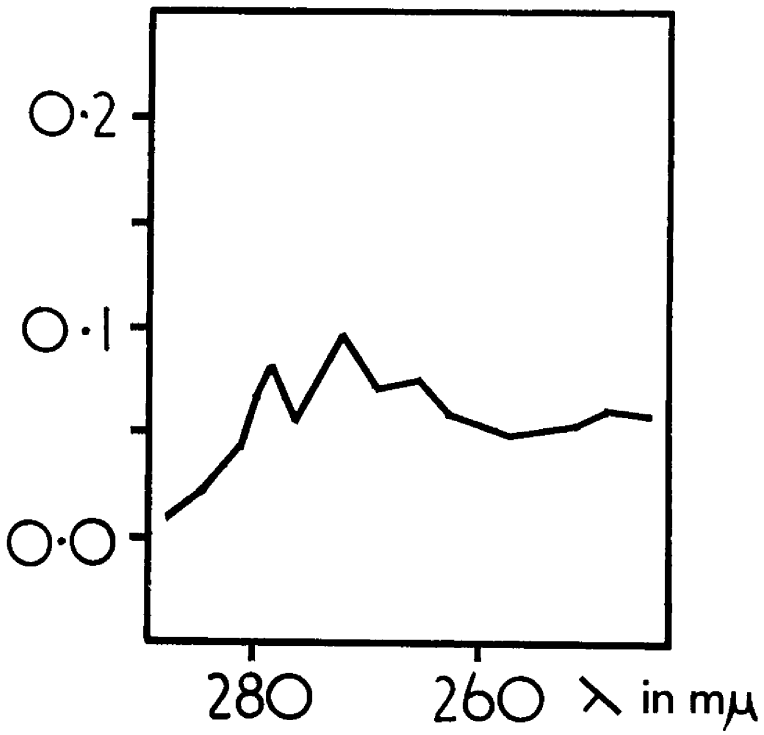

Fig. 20:- Spectrum of Hexane Extract.

material which was eluted from the DEAEC by 0.1N NaOH. However, this interference was still present in the solution containing the anionic material even when it had been eluted from the DEAEC using 1% NaCl. The solution under investigation was diluted in an attempt to dilute out this interference, but so much dilution was required that the spectrum of the compound itself was lost.

The spectra of the unknown compounds could be obtained for the visible range, but this was not of very much help in identifying the material. (Hill et al 1962). An example of a spectrum in the visible region is shown in Figure 11.

PARTITION CHROMATOGRAPHY

Partition chromatography was tried using columns of Whatman Cellulose Powder (Standard Grade). Columns of 20 ml were used and were prepared simply by suspending the powder in distilled water and transferring it to the 100 ml burette which served to contain the cellulose. It was allowed to settle under gravity and topped with a plug of glass wool. It was now ready for use, and the solution being chromatographed was applied gently, after evaporation to a small volume, to the top of the column, by allowing it to run down the sides of the burette. However, there was no retention of any of the constituents of the solution

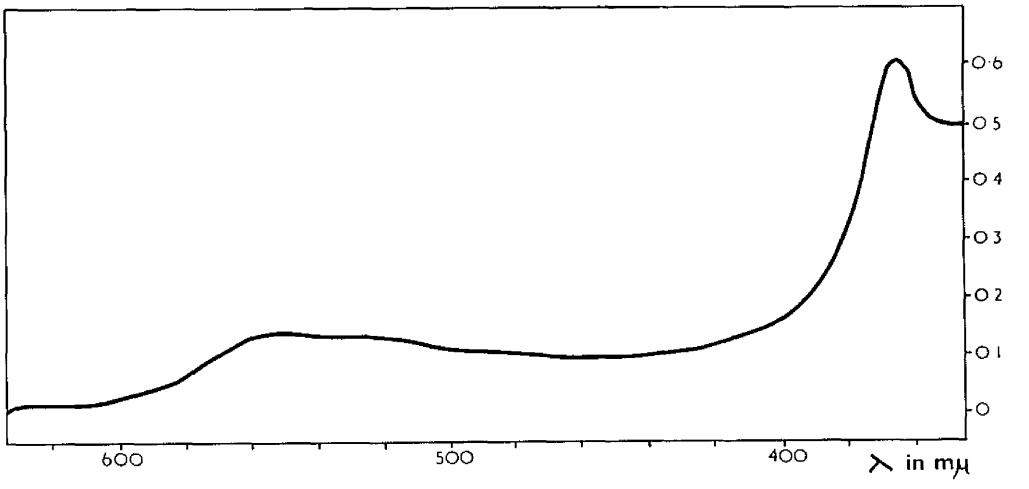

Fig. 11:- Spectrum in the Visible Range of a Representative Fraction.

on the column. No retention was observed with any of the fractions under investigation, nor with the standards, so no partition studies could be carried out on cellulose. Similar studies were tried on columns of Whatman No. 1 Filter paper. Commercially available paper clippings were steeped in enough distilled water to thoroughly soak them, then homogenized in a Kenwood mixer until a homogeneous mass had been obtained. This was packed into a column in a 100 ml burette, and again partition chromatographic studies were attempted using concentrated solutions of the different fractions. No greater retention was observed on this material.

Eventually the standard procedure of partition chromatography on paper (Whatman No. 1) was tried, and this promised success. Ascending chromatography was used, because of the simplicity of the necessary apparatus, and chromatograms were run for up to eight hours. The apparatus used initially was "home-made", consisting of a 3 litre beaker, with a sheet of polythene across the top, held down under the rim of the beaker with an elastic band. The paper was suspended on an orange stick cut to size and attached to the sides of the beaker with sellotape. This makeshift apparatus gave satisfactory results, allowing strips of paper up to 12 cm long to be used. Towards the end of the work, square tanks suitable for partition chromatography were

obtained, with plate-glass lids and metal frames for solvent and paper. For use in these tanks, Whatman make a special size of No. 1 paper, with holes already cut for the frame. No better results were obtained with this apparatus.

Partition chromatography on paper was tried using the more dilute solutions obtained directly from the ion-exchange columns, and trying to localize the unknown materials by autoradiography on Kodak Films, as the spots were not visible. The amount of radioactivity present was found to be too low to have an effect on the films after two weeks, and waiting longer was not considered practicable. The solutions of the various fractions were then concentrated sufficiently to give visible spots on paper. Partition chromatography on paper was done using these concentrated solutions, with water as the stationary phase. Two developing solvents were used with success, though others were tried without success. The two successful solvent systems were sec-butanol/glacial acetic acid/water in the ratio 100/3/50 by volume, and 90% sec-butanol saturated with water, with 10% sec-butanol added. Acetone was also tried as a developing solvent a few times, but with little success. Other solvents systems were also tried but none gave a successful separation of the standards, cyanocobalamin and hydroxocobalamin, prepared as described. The solvents tried unsuccessfully were 99% acetone/1%

glacial HA: H_2O saturated with phenol: 25% Amyl acetate/75% ethyl alcohol: 50% phenol/50% chloroform: 50% Amyl acetate/50% ethyl alcohol. As this technique did not in every case give well-defined spots partition chromatography was also tried using the two sec-butanol solvents, but adding 2 ml 0.1 M versene solution (ethylenediamine tetracetic acid) to 153 ml of the former, and 2 ml 0.1 M versene solution to 100 ml of the latter. The versene complexed inorganic ions in the paper and so prevented them from causing tailing of the spots. In every run, standards, prepared as described, were included with the unknowns.

ELECTROPHORESIS

The apparatus necessary for this was obtained from Shandon Ltd., and consisted of a polythene electrophoresis tank with a glass lid, which could be connected, through a Power Pack, to the mains. With this Power Pack, the voltage and current could be controlled and could be set at either constant voltage or constant current. In the work described here constant voltage was used so that the voltage did not alter during the course of the electrophoresis. This gave more consistent results. The tank had a polythene bridge, slotted at the ends, across which paper could be fitted.

Electrophoresis was tried using two buffers, 3% glacial acetic

acid in water, giving a pH of 3, and a phosphate buffer ($Na_2HPO_4.2H_2O$ - 15.5 g, $NaH_2PO_4.2H_2O$ - 26.05 g in 500 ml water) giving a pH of 6.4. It was tried first on comparatively dilute solutions of the material under investigation, and localization of the spots attempted using autoradiography, as was tried with partition chromatography. Again no satisfactory results were obtained as there was not enough radioactivity present to have an effect upon the photographic film, at least in the time available. Electrophoresis was then tried on more concentrated solutions of the compounds under investigation so that the spots on the paper could be localized visually. This proved to be successful.

Electrophoreses were generally run at 300 volts for up to five hours. Again, one or both of the standards were run along with the unknowns.

SUMMARY

Several methods of extraction and concentration of the radioactive material excreted in urine after parenteral radioactive cobalamins have been investigated and described. Techniques for characterization of the components of the radioactive material, separated by these methods, have also been investigated. It was

found that the most satisfactory methods for characterization were electrophoresis at pH3 and pH6.4, and partition chromatography on paper in sec-butanol/acetic acid/water (100/3/50 by volume) and 90% sec-butanol/water. For these characterization techniques, concentrated solutions of the unknown components were used, so that the materials could be localized visually.

An application of these methods and techniques is described in the next section.

SECTION 5

Qualitative Analyses of the Radioactive Material Found in Urine After Parenteral Radioactive Cobalamins

In the last section, there have been described various methods for isolation and characterization of the components of the radioactive material in urine, whose presence was shown in Section 3. These methods were applied to the various fractions, from both "in vitro" (hydroxocobalamin) and "in vivo" (hydroxocobalamin and cyanocobalamin) samples, in an attempt to establish their nature. This gave a possible total of 9 substances to be identified, 3 being basic, 3 neutral and 3 anionic. Each of these was investigated in the same way.

MATERIALS AND METHODS

The materials and methods used for injections and urine collections were as previously described.

"In vivo" Studies

Following either parenteral ^{57}Co hydroxocobalamin (0.24 uc/1000 ug/6 ml) or ^{58}Co cyanocobalamin (0.4 uc/1000 ug/6 ml) in normal patients, urine was collected and extracted by the first described method of extraction/concentration. For urine after cyanocobalamin, this proved to be satisfactory, and the extracts so obtained were passed through Dowex 1, DEAEC and CMC to give three fractions. As most of the radioactive material after cyanocobalamin was neutral, this was the only fraction investigated further. After hydroxocobalamin, this extraction/concentration procedure did not give sufficiently pure extracts, so these urine collections were extracted by the charcoal/

/alkali method. Again, using ion-exchange columns, three fractions were collected. Each of these was further investigated. When it was considered that enough of these four fractions, one after cyanocobalamin and three after hydroxocobalamin, had been collected by pooling, they were each evaporated to dryness and resuspended in a small volume of distilled water. The nature of these fractions was then investigated using electrophoresis and chromatography.

"In vitro" Studies

0.24 uc/500 ug ^{57}Co hydroxocobalamin was added to each 500 ml pooled overnight urine. The solutions were thoroughly mixed then extracted by the first described method of extraction/concentration. If the extracts thus obtained were not sufficiently free from contaminants, other urine pools were treated by the charcoal/alkali method, to give a purer extract. The extracts obtained from either of these methods were passed through columns of Dowex 1, DEAEC and CMC and separated into three fractions. Pools of these fractions were built up and when it was considered that sufficient material had been collected, the fractions were evaporated to dryness and resuspended in a small volume of distilled water. The nature of the three components thus obtained was investigated using electrophoresis and partition chromatography.

Standards, prepared as described, were used for comparison

with the unknown materials in the techniques used for identification.

RESULTS AND DISCUSSION

Standards

To make sure that the extraction/concentration techniques did not alter the electrophoretic and chromatographic behaviour of the standard cobalamins, electrophoresis and chromatography were done on these before and after extraction/concentration. Results of electrophoresis and chromatography are shown in Figures 12, 13, and 14.

Cyanocobalamin - "In vivo" Studies

This was the most obvious investigation to tackle first as most of the radioactive material present in the urine collected after parenteral ^{58}Co cyanocobalamin was neutral cobalamin (see Section 3). This was the only fraction after cyanocobalamin investigated, as the amounts of the other two were very small. As cyanocobalamin is neutral, it seemed likely that this neutral excretion product was cyanocobalamin. This was shown to be the case using electrophoresis at pH 3 and pH 6.4, and partition chromatography in the two sec-butanol solvent systems. The results are shown in Figures 15 to 18, where it can be seen that the neutral material shows the same behaviour as the cyanocobalamin standard.

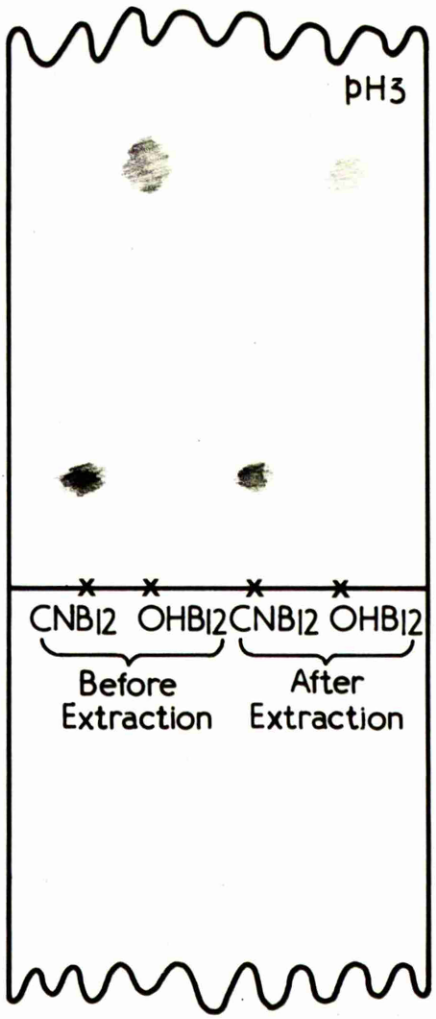

Fig. 12:- Electrophoresis in Acetate Buffer of Samples of Standards before and after extraction.

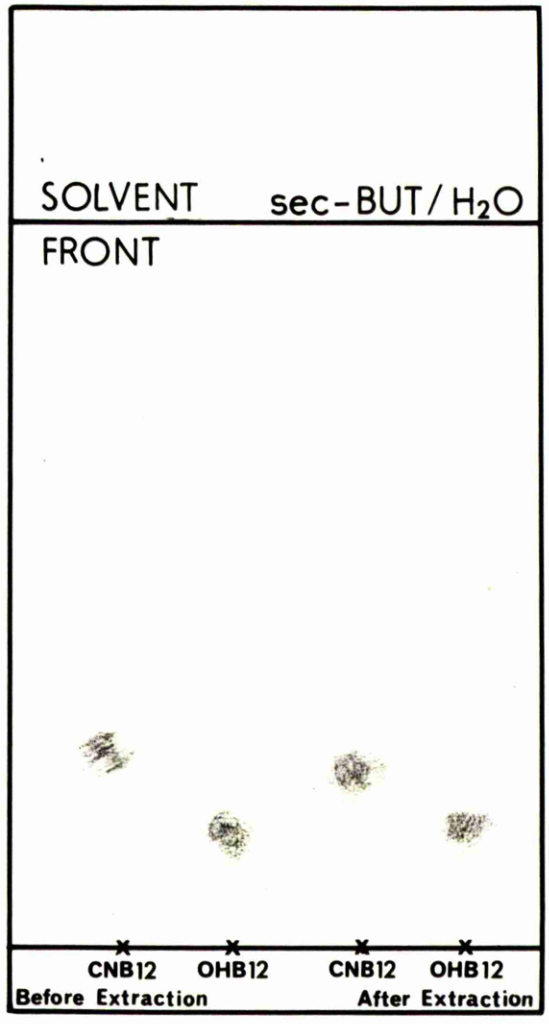

Fig. 13:- Partition Chromatography of Samples of Standards before and after extraction.

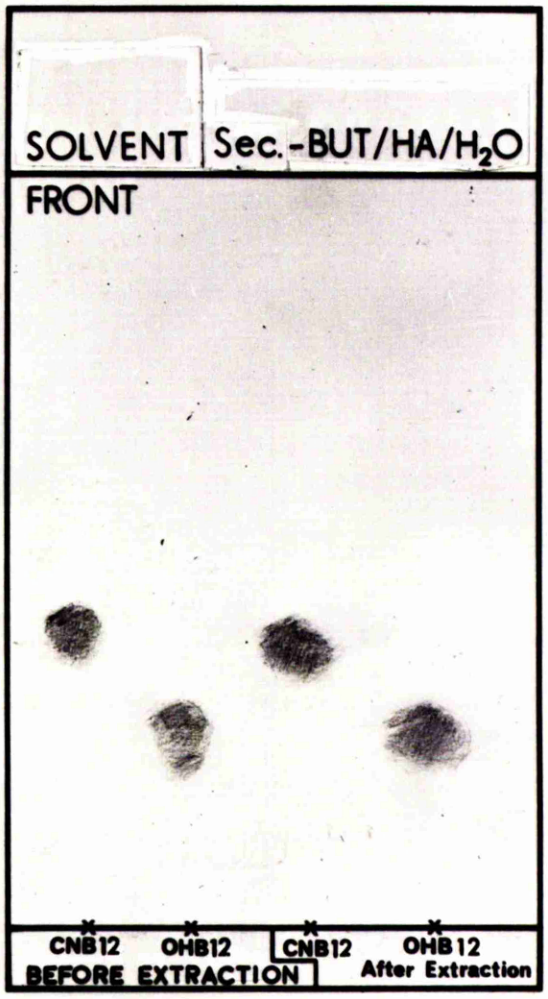

Fig. 14:- Partition Chromatography of Samples of Standards before and after extraction.

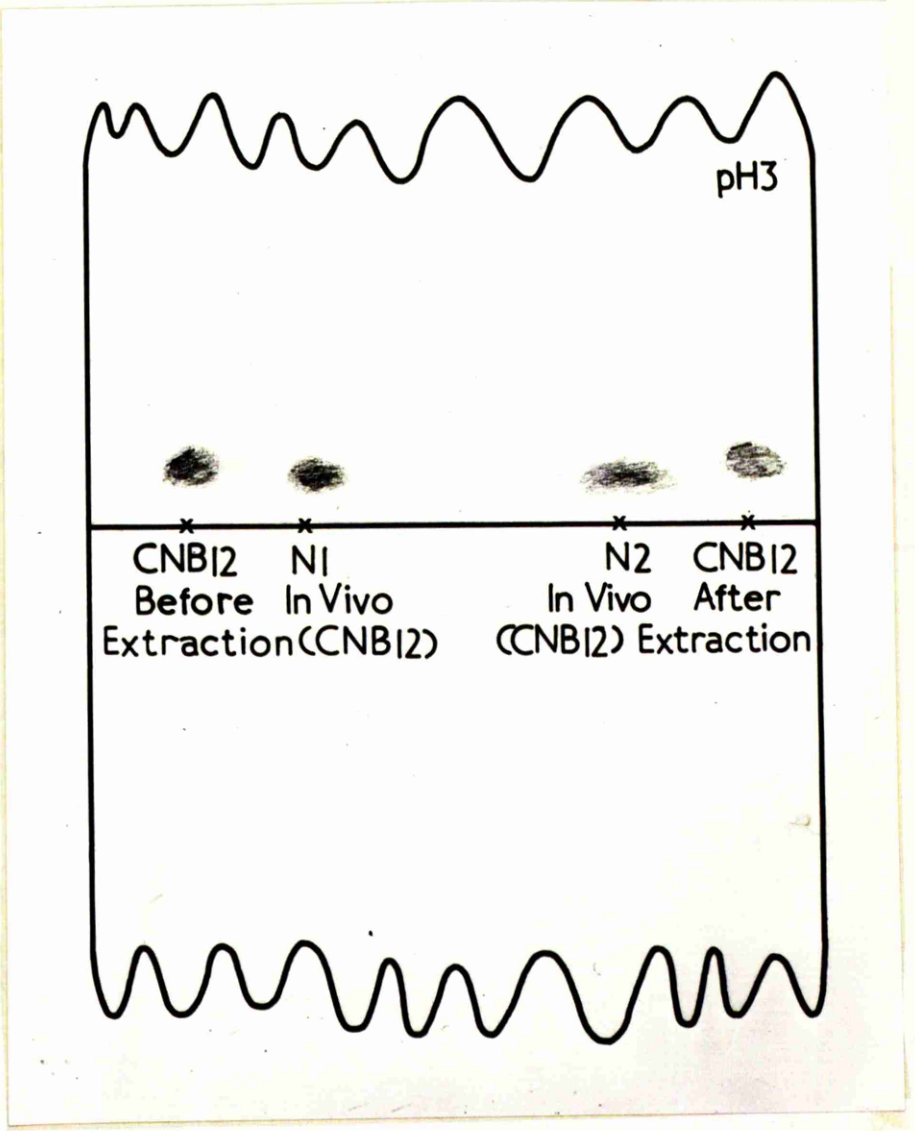

Fig. 15:- Electrophoresis in Acetate Buffer of Cyanocobalamin and Neutral Fraction, from "in vivo" Cyanocobalamin Studies.

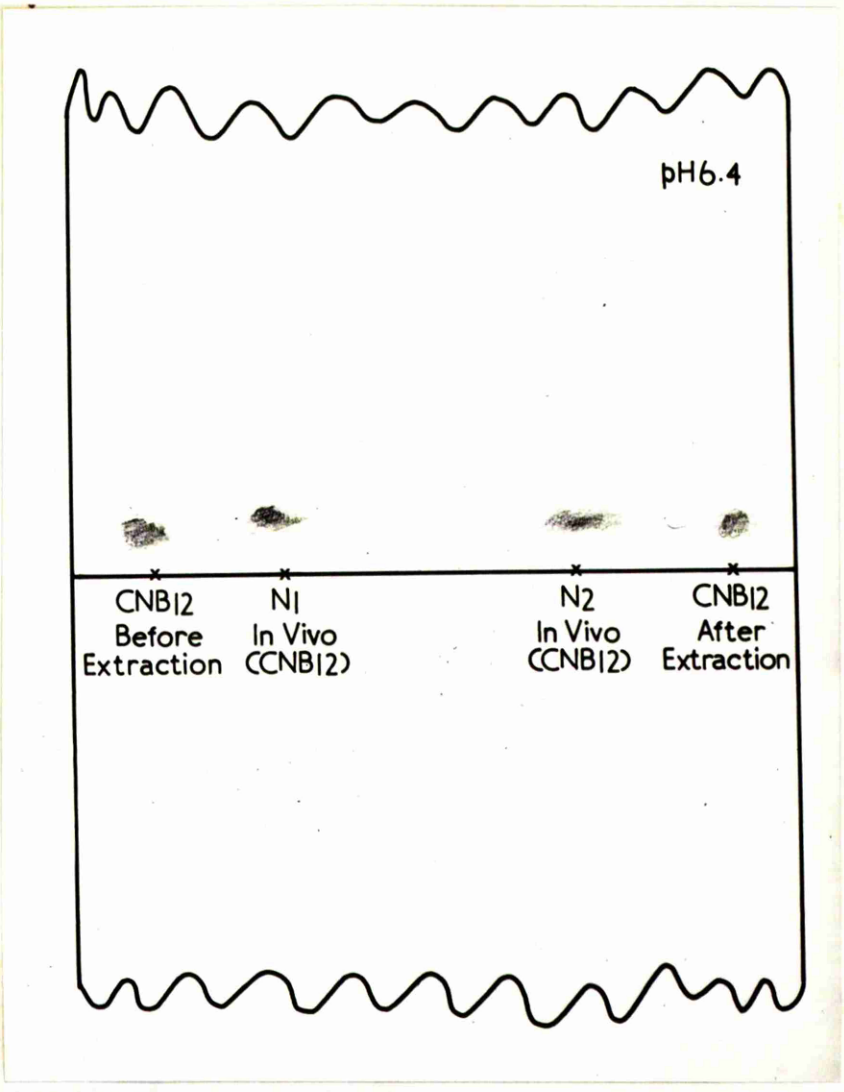

Fig. 16:- Electrophoresis in Phosphate Buffer of Cyanocobalamin and Neutral Fraction, from "in vivo" Cyanocobalamin Studies.

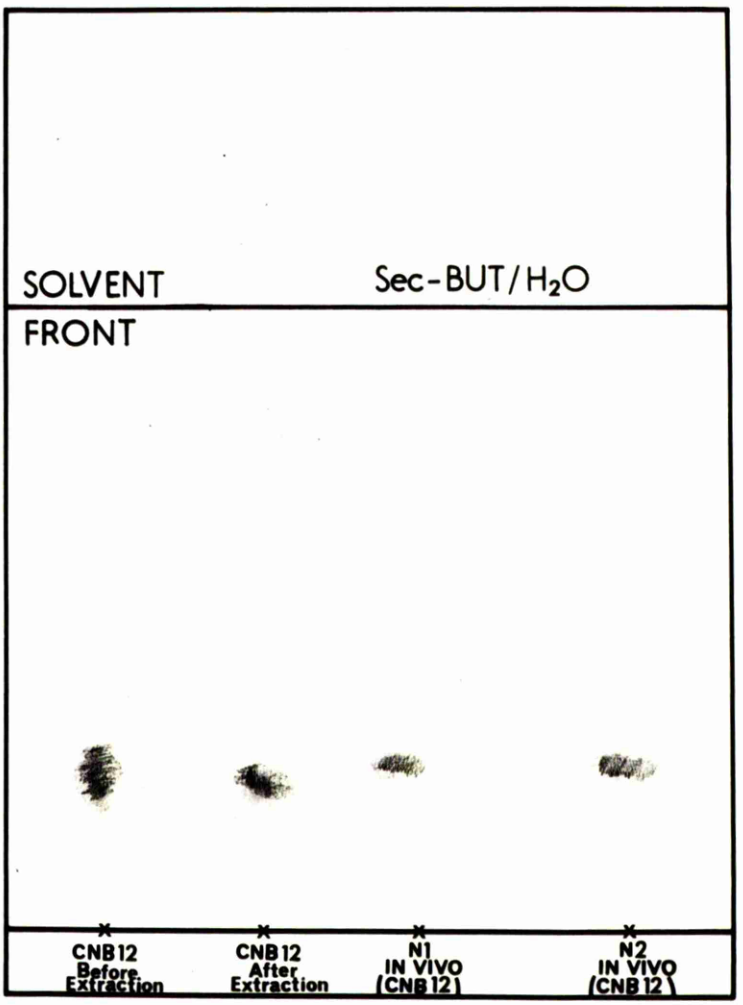

Fig. 17:- Partition Chromatography of Cyanocobalamin and Neutral Fraction, from "in vivo" Cyanocobalamin Studies.

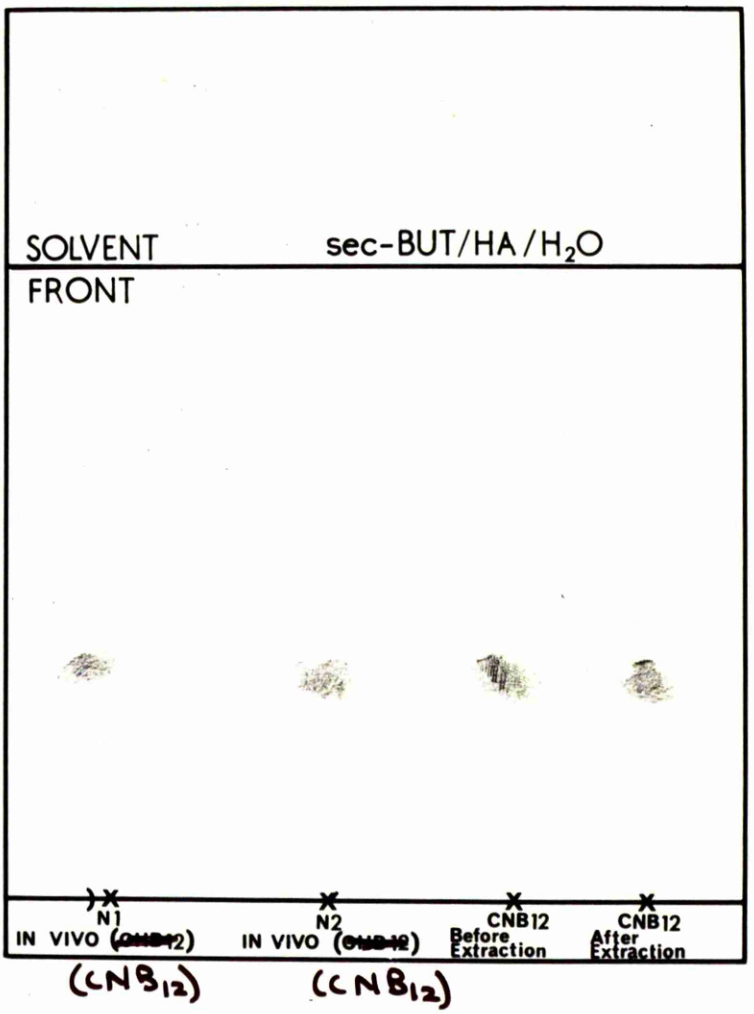

Fig. 18:- Partition Chromatography of Cyanocobalamin and Neutral Fraction, from "in vivo" ^{57}Cyanocobalamin Studies.

Cyanocobalamin - Discussion

Other workers had already put forward the suggestion that this was so, and took some steps to prove the point (Smith 1952: McLean & Bloch 1954), but due to the present state of increased knowledge their results would not now be accepted as valid. The work described here confirms their work and adds to it, by showing that, so far as can be judged at the present date, the neutral radioactive material found in the urine after parenteral ^{58}Co cyanocobalamin behaves as cyanocobalamin. It is conceivable that as knowledge of cobalamin chemistry and biochemistry increases the proofs put forward here will be inconclusive, but at present they are sufficient.

Hydroxocobalamin - "In vivo" and "in vitro" Studies

Identification of the fractions found in urine after ^{57}Co hydroxocobalamin, given by parenteral administration, was rather more difficult. This was due to the smaller amount of materials involved, because of the three fractions obtained, and to the presence of urinary pigments which were retained on and eluted from the ion-exchange columns along with the radioactive material.

First of all, the possibility of the anionic material in particular being a degradation product was investigated. This was done by giving a normal subject ^{57}Co hydroxocobalamin (0.24 uc/1000 ug/

/6 ml) parenterally at 10 p.m. and collecting urine until 8 a.m. To this there was added 2 ml 0.25% potassium cyanide solution. The resulting solution was mixed and allowed to remain at room temperature (17°C) for thirty minutes. It was then subjected to the extraction/concentration procedure, and chromatographed on Dowex 1, DEAEC, and CMC. The results, shown in Table 14, show that all the radioactive material had been converted to a neutral cobalamin, presumably cyanocobalamin. These results excluded the possibility that the anionic material was a degradation product, e.g. a red acid, a form to which the cobalamin molecule is readily degraded, and whose nature is described in the Introduction. The anionic material must, therefore, be an anionic complex in which the ligand occupying the sixth position on the cobalt atom is anionic, so making the whole molecule anionic. With inorganic cyanide this ligand is replaced by CN-, which complexes strongly with trivalent cobalt, to give the neutral cobalamin, cyanocobalamin. In the same way the basic cobalamin obtained in urine, must contain a basic group on the central cobalt, and this again has been removed by KCN and replaced by CN-, to give the neutral cobalamin, cyanocobalamin.

A. Hydroxocobalamin - "In vitro" Studies

As it has been shown that adding ^{57}Co hydroxocobalamin to urine

SAMPLE	EFFICIENCY of EXTRACTION %	PROPORTION OF CONSTITUENTS %		
		NEUTRAL	BASIC	ANIONIC
Urine After Parenteral ^{57}Co Hydroxocobalamin +KCN Solution	76	79	8	15

TABLE 14 Result of Analysis of an Extract of Urine after Parenteral 1000 ug/0.24 uc ^{57}Co Hydroxocobalamin: Potassium Cyanide was added to the Urine Collection Prior to Extraction.

"in vitro" gives rise to three fractions with the same column characteristics as those fractions obtained from the "in vivo" studies using ^{57}Co hydroxocobalamin (see Section 3), it was decided to try, first of all, to characterize conclusively the fractions from the "in vitro" studies. The main reason for this decision was the comparative ease with which pools of urine could be obtained as compared to the problem of finding suitable subjects for injection.

Accordingly, the nature of the three fractions, obtained by the extraction/concentration technique, was investigated by the methods described and the results are shown in Figures 19 to 24 for the neutral and basic fractions. The results show that the basic fraction behaves as hydroxocobalamin, and the neutral fraction as cyanocobalamin. No satisfactory results could be obtained from the anionic fraction, and the other methods of purification of the urine pools using charcoal/alkali did not lead to any better results. A sample of the chromatography results obtained are shown in Figure 25, where it can be seen that the spot of the anionic material shows a great deal of tailing and streaking. This was quite unsatisfactory and no conclusions could be drawn from it. A further attempt at purification was made by cutting out that part of the paper containing the red portion of the anionic material after partition chromatography in sec-butanol, and eluting from the paper

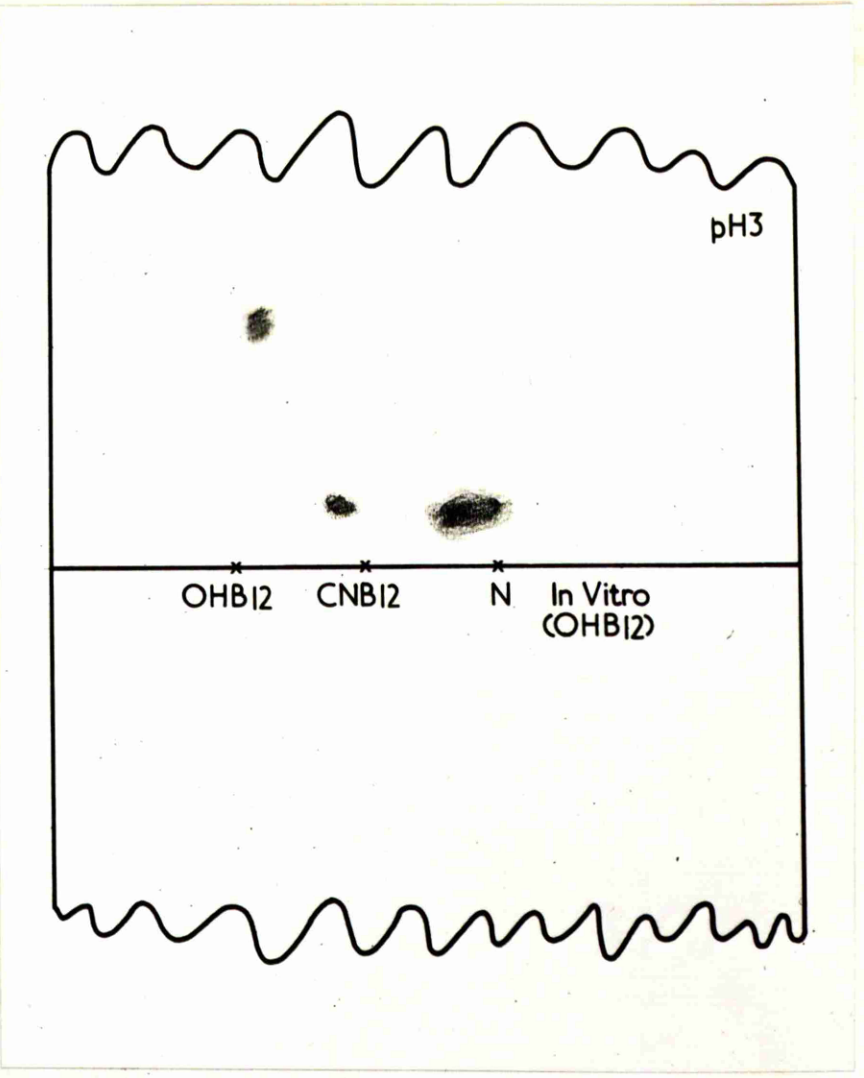

Fig. 19:- Electrophoresis in Acetate Buffer of Standards and Neutral Fraction, from "in vitro" Hydroxocobalamin Studies.

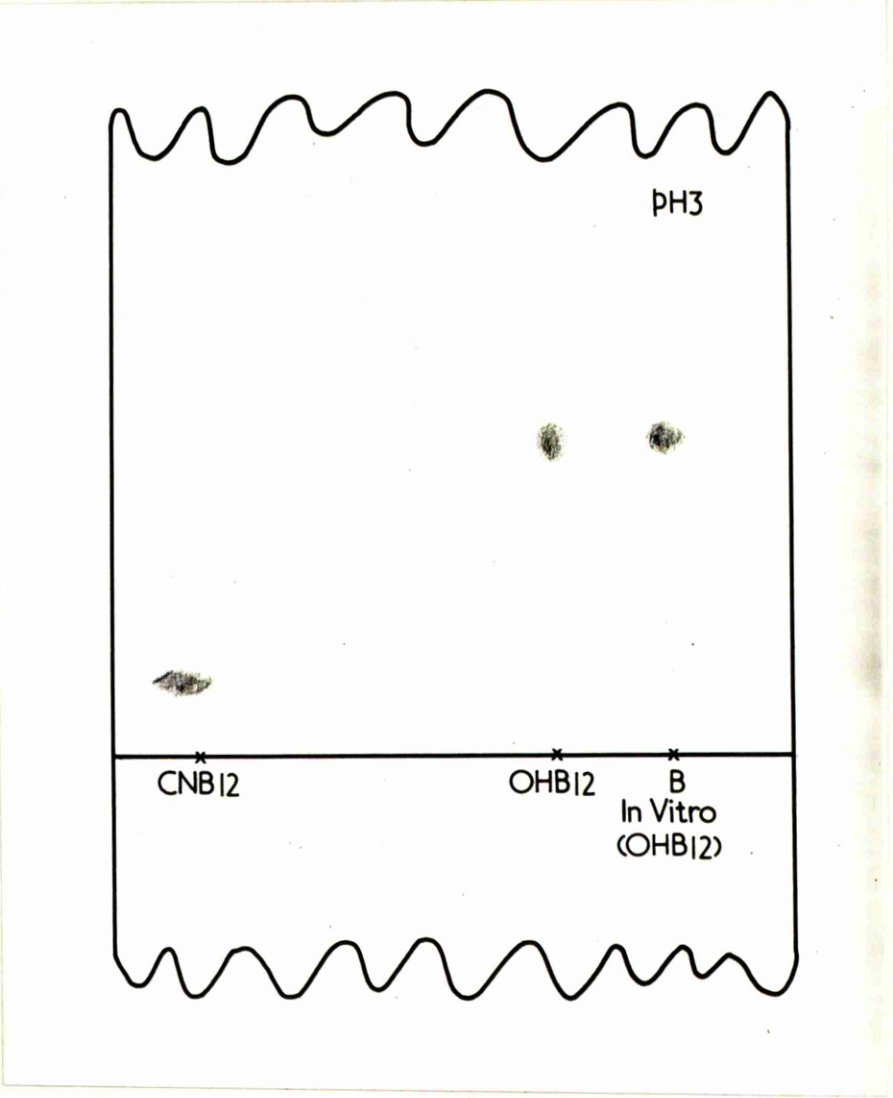

Fig. 20:- Electrophoresis in Acetate Buffer of Standards and Basic Fraction from "in vitro" Hydroxocobalamin Studies.

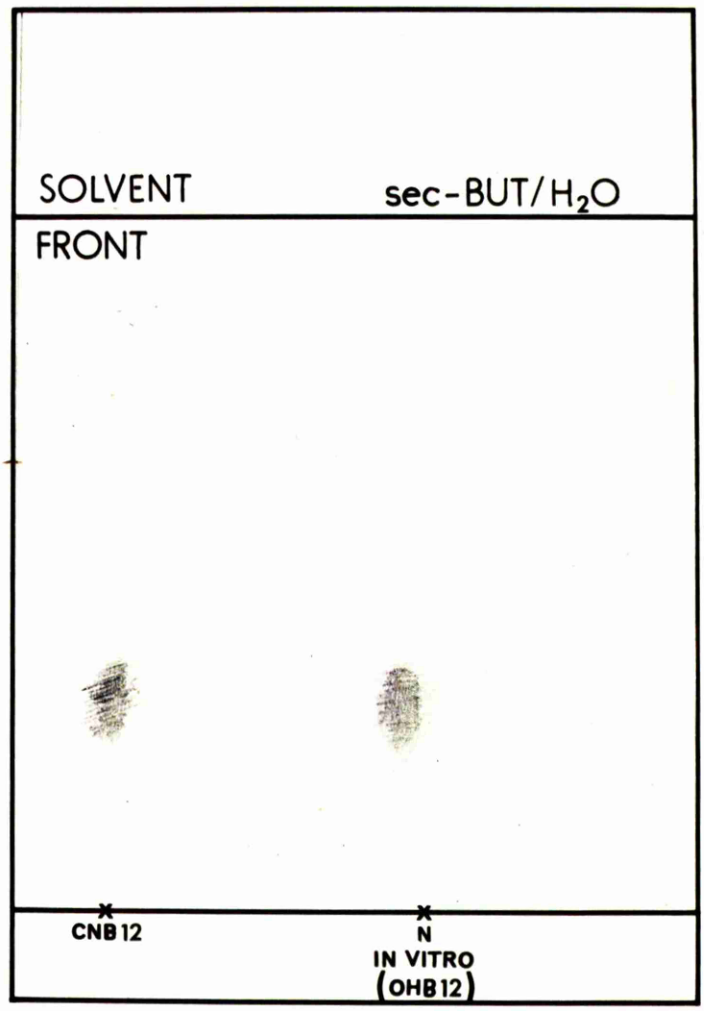

Fig. 21:- Partition Chromatography of Cyanocobalamin and Neutral Fraction from "in vitro" Hydroxocobalamin Studies.

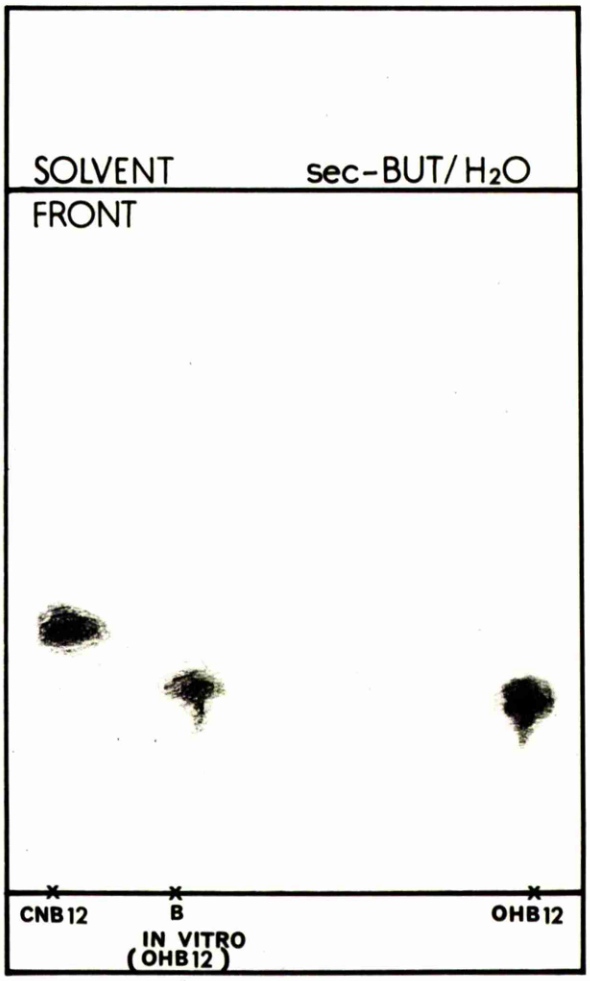

Fig. 22:- Partition Chromatography of Standards and Basic Fraction from "in vitro" Hydroxocobalamin Studies.

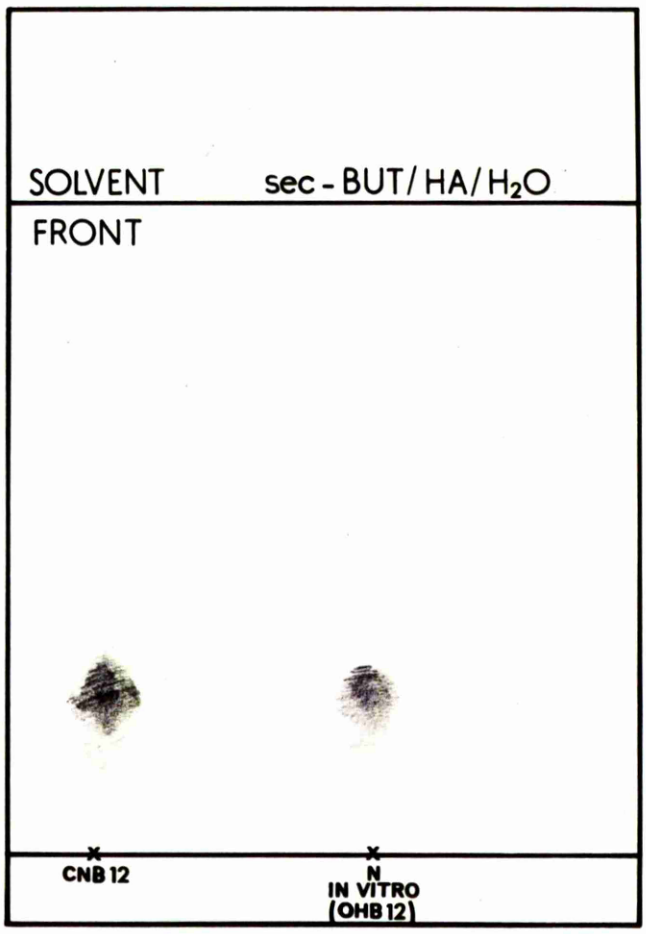

Fig. 23:- Partition Chromatography of Cyanocobalamin and Neutral Fraction from "in vitro" Hydroxocobalamin Studies.

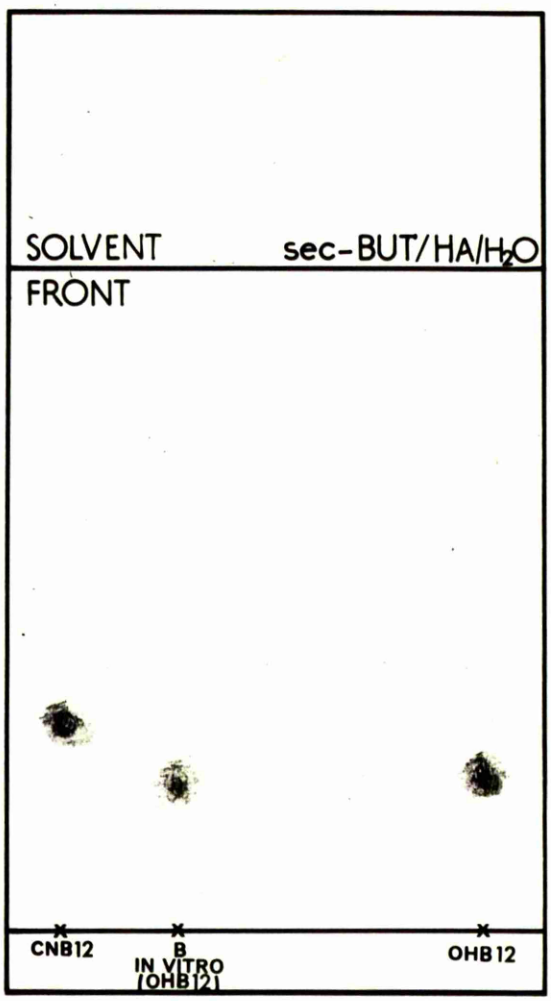

Fig. 24:- Partition Chromatography of Standards and Basic Fraction from "in vitro" Hydroxocobalamin Studies.

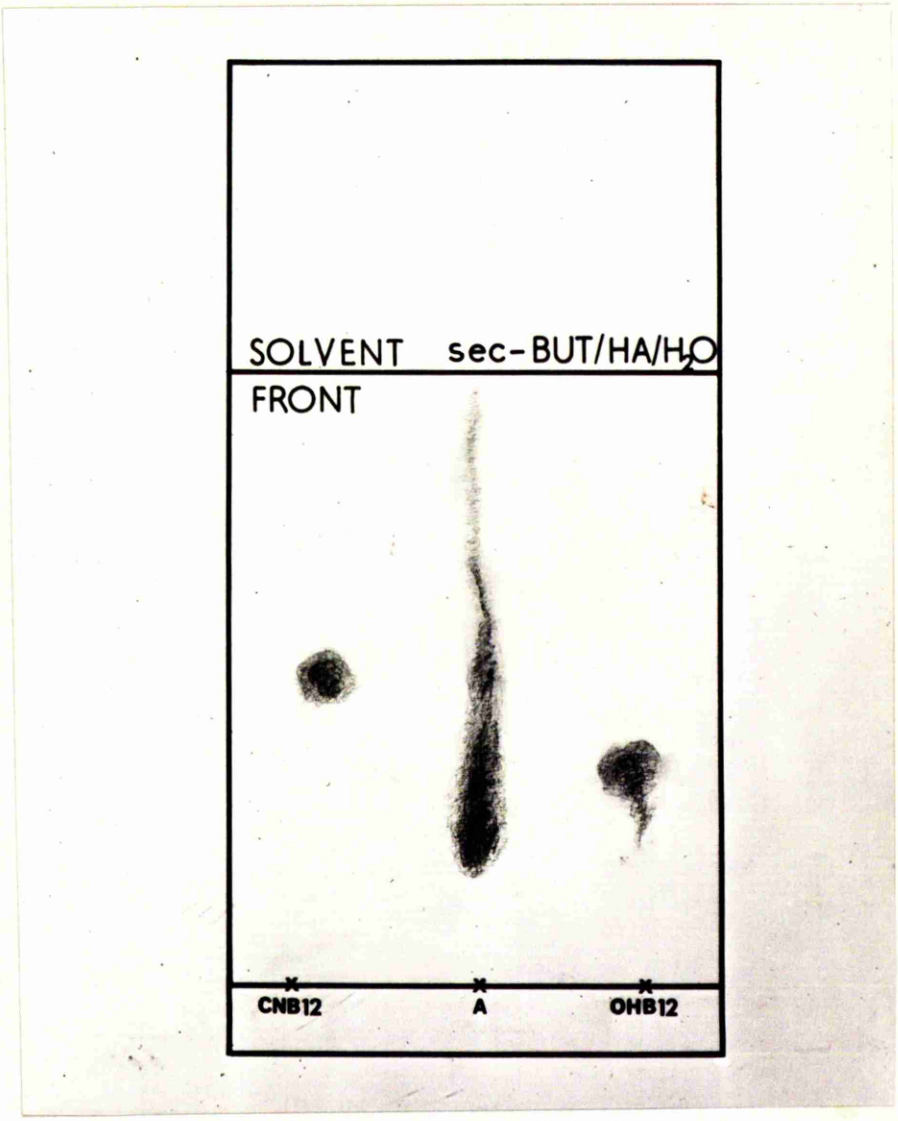

Fig. 25:- Partition Chromatography of Standards and Anionic Fraction.

using distilled water. The resulting solution was again evaporated to a small volume and partition chromatography on paper repeated, but still no satisfactory clear-cut spots were obtained. The difficulties here were due to the presence of urinary pigments, which were very difficult to remove, and also, of course, to the small amount of the material under investigation.

B. Hydroxocobalamin - "In vivo" Studies

The "in vivo" characterization proved even more difficult, and the only fraction for which satisfactory evidence was obtained was the neutral material. This had been obtained by the extraction/concentration procedure and ion-exchange chromatography, and it was shown to be cyanocobalamin. Partition chromatograms are shown in Figures 26 & 27. Consistent and clear-cut results could not be obtained for either the basic material, nor for the anionic complex, and fractions obtained by the charcoal/alkali method did not give any better results. The further the purification methods were carried the less material was there to work on and it was eventually decided to stop at this stage.

One point worth noting was that occasionally the hydroxocobalamin standard, instead of giving a well-defined spot on partition chromatography scarcely moving from the origin, gave two spots.

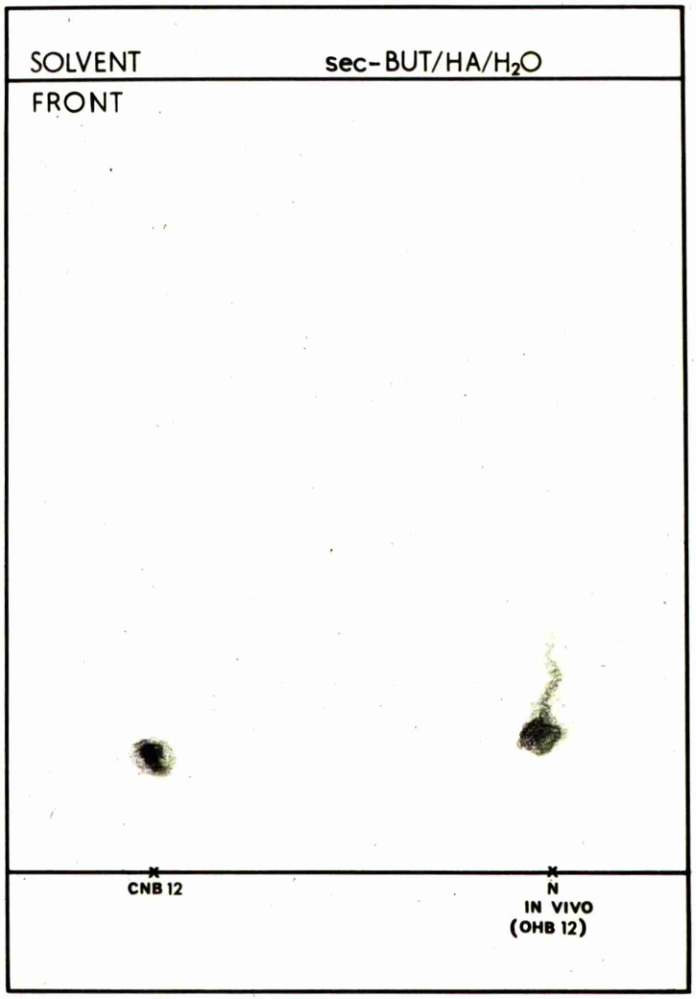

Fig. 26:- Partition Chromatography of Cyanocobalamin and Neutral Fraction from "in vivo" Hydroxocobalamin Studies.

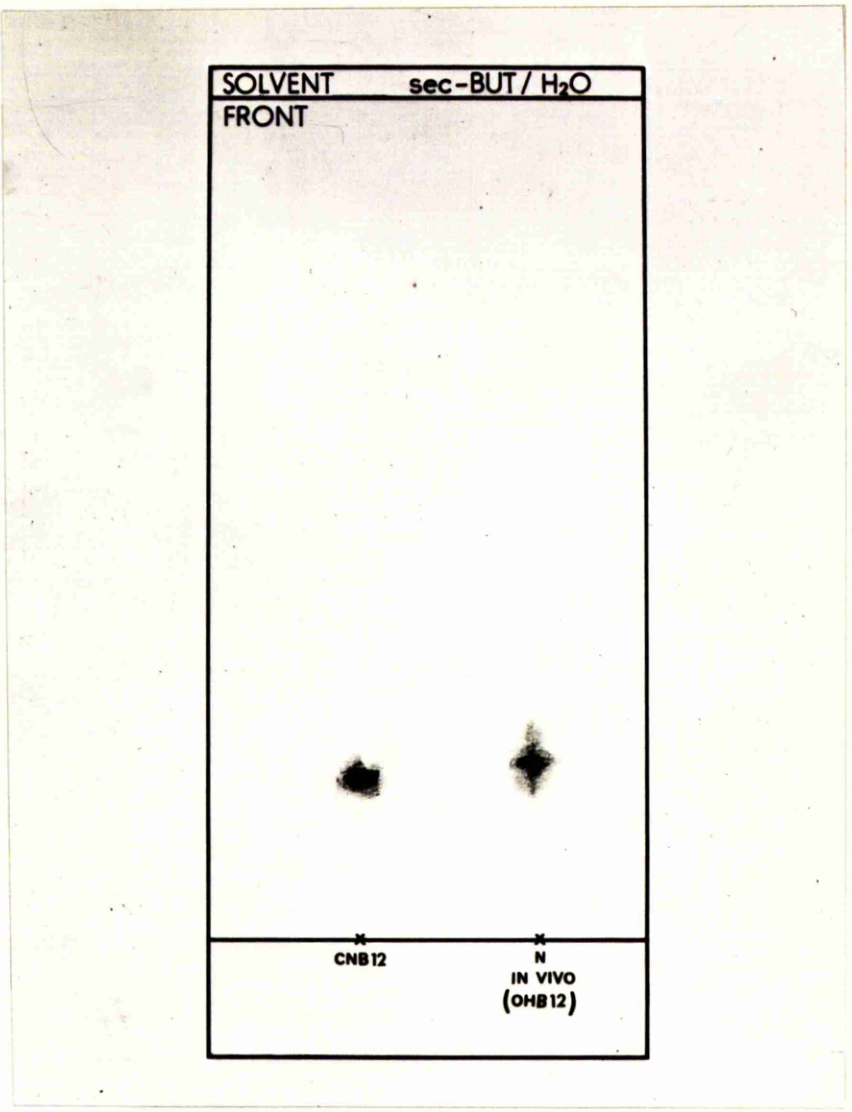

Fig. 27:- Partition Chromatography of Cyanocobalamin and Neutral Fraction from "in vivo" Hydroxocobalamin Studies.

One of these had an R_f of about 0.7, while the other was diffuse with an R_f of about 0.3. An example of this is shown in Figure 28. The reason for this could not be pinpointed, but it most likely lies in the inherent instability of hydroxocobalamin to contaminants, possibly in the atmosphere. Although this change in partition chromatography characteristics was observed, the behaviour of the solution on the CMC was the same, i.e. it was retained strongly by it. The "in vivo" basic fraction from urine also occasionally showed this same behaviour. On the ion-exchange chromatography, the basic fraction was retained on CMC, but on partition chromatography two spots were observed, one moving with an R_f of about 0.3 and the other with an R_f of 0.7 in both sec-butanol systems. An example is shown in Figure 29. This could possibly be taken as evidence that the basic "in vivo" fraction is hydroxocobalamin, but it is not conclusive.

Hydroxocobalamin - Discussion

No previous results concerning the nature of the radioactive material excreted in urine after parenteral hydroxocobalamin have been published, so the findings described here cannot be compared to those of other workers. It has been shown that these fractions possibly arise from contact of hydroxocobalamin with urine (see Section 3), and the nature of one of these fractions has now been shown to be cyanocobalamin. This may have arisen from contact

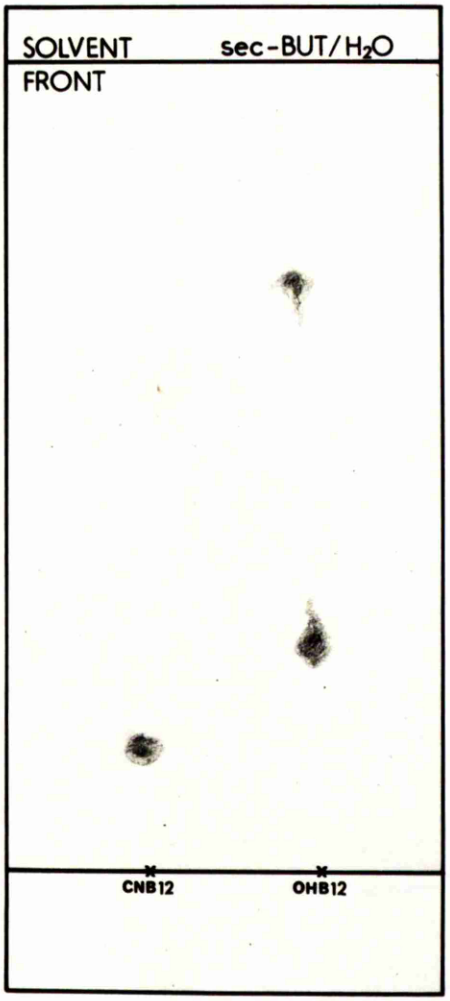

Fig. 28:- Partition Chromatography showing Anomalous Behaviour of Hydroxocobalamin.

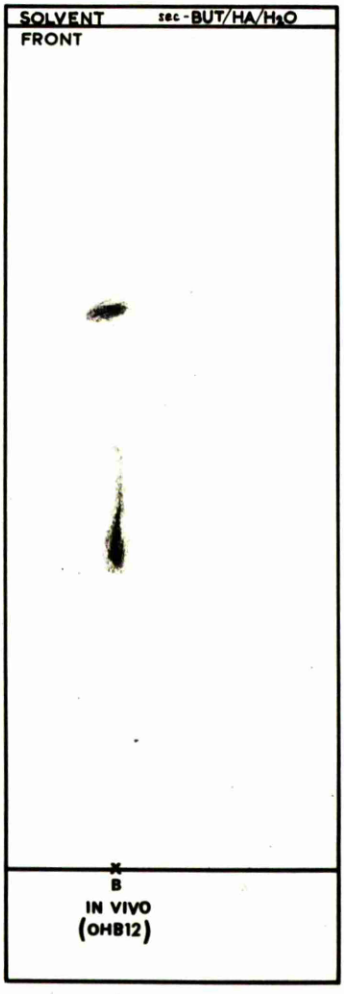

Fig. 29:- Partition Chromatography showing Anomalous Behaviour of the Basic Fraction from "in vivo" Hydroxocobalamin Studies.

with the small amount of cyanide which is excreted in urine, as only a very minute amount of cyanide would be required to give rise to the small amount of cyanocobalamin isolated. Evidence has been put forward that the basic material is unchanged hydroxocobalamin, and it has been shown that the anionic material is a complex and not a degradation product, so lending weight to the suggestion that the observed compounds in urine are caused by the action of urine on hydroxocobalamin.

SUMMARY AND CONCLUSION

It has been shown that after parenteral administration of ^{58}Co cyanocobalamin, the radioactive material found in urine is in the form of cyanocobalamin, and that after parenteral ^{57}Co hydroxocobalamin, the radioactive material found in urine is a mixture of cyanocobalamin, a basic cobalamin, for which there is some evidence that it is hydroxocobalamin, and an anionic complex. On addition of ^{57}Co hydroxocobalamin to urine "in vitro", in comparable amounts, there is formed a mixture of cyanocobalamin, hydroxocobalamin, and an anionic complex. In all cases, the anionic material was uncharacterized, but was shown to be an anionic complex of the cobalamin molecule, and not a "red acid" degradation product.

These results are in agreement with those of other workers for cyanocobalamin (Smith 1952: McLean & Bloch 1954), but no previous

investigations appear to have been undertaken for hydroxocobalamin.

SECTION 6

Comparison of the Amounts of Radioactive Material and Microbiologically Active Material in Urine After Parenteral ^{57}Co Hydroxocobalamin

The investigations into the nature of the radioactive material in urine after parenteral radioactive cobalamins began from an enquiry as to whether the assumption that the amount of radioactive material excreted could be taken as a measure of the amount of cobalamin excreted was valid.

The work so far described has confirmed that the radioactive material in urine is cyanocobalamin. However, it has not shown that the amount of radioactive material and the amount of cyanocobalamin are equal. Similarly, for hydroxocobalamin, it has so far been shown that the radioactive material present in urine can be divided into three cobalamins, but it has not been shown that the amount of radioactive material and the amount of cobalamin excreted are equal. The work described in this section was designed to find out whether these amounts, after both cyanocobalamin and hydroxocobalamin, were equal, and thus whether the amount of radioactive material excreted in urine is a measure of the amount of cobalamin excreted in urine.

This was done by comparing the amount of radioactive material in the urine with the amount of microbiologically active material, as measured by Euglena gracilis strain z.

MATERIALS AND METHODS

CLINICAL MATERIAL

Group 1: This group consisted of 20 ward in-patients with a variety of diseases, none of which were known to be associated with deranged cobalamin metabolism. All had normal serum vitamin B_{12} levels. This was considered to be a group of normals. Each patient was given a single parenteral dose of ^{57}Co hydroxocobalamin.

Group 2: This group contained 3 patients. Each had a macrocytic anaemia, megaloblastic marrow, histamine-fast achlorhydria and low serum vitamin B_{12} level on admission. At a later date they were all shown to have Addisonian pernicious anaemia. 1 patient received a single intramuscular injection and the other 2 patients were given repeated parenteral injections at 72-hourly intervals. 1 was given 10 injections and the other 12. This was considered to be a group of controls.

INJECTION DOSE

A sterile solution of ^{57}Co hydroxocobalamin was used, as described in Section 2. 2 ml (0.0946 uc) ^{57}Co hydroxocobalamin was mixed aseptically with 1 ml Redisol-H (1000 ug/ml), giving an injection dose of 1000 ug/0.0946 uc/3 ml.

URINE COLLECTIONS.

All urine was collected into clean dark glass bottles. From

each patient a pre-injection urine collection was made. Each was then given parenteral ^{57}Co hydroxocobalamin and urine collected for the subsequent 72 hours in 24-hourly periods. Those subjects in Group 2 who received more than one injection were treated at 72-hour intervals and urine collections made until 72 hours after the last injection. Pools of urine from normal subjects were made by pooling 24-hour collections from various staff and family members.

MEASUREMENT OF RADIOACTIVITY

The amount of radioactivity was measured as described in Section 1, using "end-on" counting of 450 ml aliquots of each urine collection. The total amount of radioactivity present in each urine collection was expressed as a percentage of the injection dose.

WATER AND GLASS-WARE

Glass-distilled water, made in our own laboratory, was used. For microbiological assay work, sterile distilled water was used. Glass-ware was steeped in Diversey 'pyroneg' or chromic acid for at least one hour, then rinsed six times in tap water, and finally rinsed once in distilled water. Sterilization and care of glass-ware used for the microbiological assay is described later.

MICROBIOLOGICAL ASSAY

This was used to estimate the amount of vitamin B_{12} present in serum from subjects used in all the investigations described in this thesis and also to estimate the amount in the urine collections. To ensure accuracy, samples were assayed in duplicate in at least two different assay batches.

Samples

Serum

Blood was collected by venipuncture into sterile universal containers. The blood was allowed to clot, then centrifuged, and the serum transferred to sterile bijou bottles (5 ml). These were stored at $-20^{o}C$ until required for assay.

Urine

Collections were made as described, and were diluted with sterile distilled water immediately the collection period had finished. Necessary dilutions after injection of cobalamin, were as shown below:-

(a) pre-injection - no dilution necessary

(b) 1-24 hours - dilute 1:1000 or 1:100

(c) 24-48 hours - dilute 1:100 or 1:10

(d) 48-72 hours - dilute 1:10 or not at all.

Medium

The medium used at first was that described by Hutner et al (1956) and its composition is shown in Table 15. In later work, commercially available Difco Euglena B_{12} Dehydrated Medium, of the same composition, was used. No differences in assay results were observed using the home-made and commercial media.

Large volumes (10 litres) were made up at a time using sterile distilled water. Single strength medium was dispensed in 500 ml aliquots in sterile MRC blood bottles sealed by a rubber bung and metal screw cap. Double strength medium was dispensed in 15 ml aliquots in sterile universal containers. After dispensing, the medium was sterilized by autoclaving at 15 lb/sq.in. for 20 minutes.

Stock Cultures

Stock cultures of Euglena were maintained in single strength medium, containing 0.05% tryptone and 20-40 uug cyanocobalamin/ml. These were sub-cultured every two weeks, after washing with single strength medium.

Procedure

This was slightly different from that described by Ross (1952); Hutner et al (1956) and by Ross et al (1957) in that single strength

TABLE 15 A

CONSTITUENTS OF EUGLENA B_{12} GROWTH MEDIUM

Amounts for 5 litres double strength Medium
(or 10 litres single strength)

KH_2PO_4	3.0 g
$MgSO_4.7H_2O$	4.0 g
L-Glutamic acid	30.0 g
$CaCO_3$	0.8 g
Sucrose	150.0 g
dl Aspartic acid	20.0 g
dl Malic acid	10.0 g
Glycine	25.0 g
Ammonium succinate	6.0 g
* Triturate	0.6 g
+ Metal Mix	0.22 g

* Triturate

Thiamine HCl	1.0 g
Sucrose	99.0 g

+ Metal Mix	See Table 15 B

TABLE 15 B

Dry Metal Mix

$FeSO_4 (NH_4)_2 SO_4 6H_2O$	14.0 g
$ZnSO_4 7H_2O$	4.40 g
$MnSO_4 H_2O$	1.55 g
$CuSO_4 5H_2O$	0.31 g
$CoSO_4 7H_2O$	0.48 g
H_3BO_3	0.57 g
$(NH_4)_6 Mo O_{24} 4H_2O$	0.64 g
$Na_2VO_4 16H_2O$	0.0925 g

medium was used throughout.

Solutions for assay containing final concentrations of 0.0, 1.25, 2.5, 5.0, 10.0, 15.0, 25.0 and 50.0 uug cyanocobalamin were prepared by adding suitable amounts of solutions of known cyanocobalamin content, made up in single strength medium, to appropriate amounts of single strength medium to give a total volume of 4 ml.

The cyanocobalamin standard solutions were made from commercially available cyanocobalamin, Cytamen, diluted in single strength medium to give solutions containing 20 uug/ml and 100 uug/ml. The solutions for assay were made by adding 0.25, 0.5, 1.0, and 2.0 ml of the solution containing 20 uug/ml, to 3.75, 3.5, 3.0, and 2.0 ml single strength medium to give standards containing 0 to 10.0 uug cyanocobalamin. Similarly for standards containing 10.0 to 50.0 uug cyanocobalamin, 0.4, 0.6, 1.0, and 2.0 ml of the solution containing 100 uug/ml were added to 3.6, 3.4, 3.0, and 2.0 ml single strength medium. These gave the required final concentrations.

Samples for assay were prepared by adding 0.1 or 0.2 ml of the test solution to 3.9 or 3.8 ml single strength medium, which was dispensed by an automatic burette. The dilution of the test solution was thus 1:40 or 1:20. Samples were assayed in duplicate in aluminium capped Pyrex rimless glass test tubes ($6''x\frac{5}{8}''$). After

preparation as described above, all solutions were heated at 100°C in a water bath for 15 minutes. After cooling they were inoculated and incubated.

Inoculum

This was prepared from 2-week-old subcultures. They were washed 3 times with single strength medium and resuspended in a suitable volume of this before use. One drop of this suspension was added to each tube by means of a sterile Pasteur pipette.

Incubation

The incubation period was 5 days in a perspex water bath (see Figure 30). The bath was illuminated from below by two 30 watt fluorescent strip lights. An even temperature of 28.5°C was maintained by two 150 watt "Queensborough" aquarium heaters connected by a Sunvic type 102/4 relay to a mercury contact thermometer. A "Handilab" stirrer was used to keep the water circulating.

Readings

These were made in a Unicam SP 300 GP colourimeter. The values for the unknown samples were calculated from a curve constructed from the values obtained from the standards. Optical cells with a light path of 2.5 mm were used to allow readings to be made without diluting.

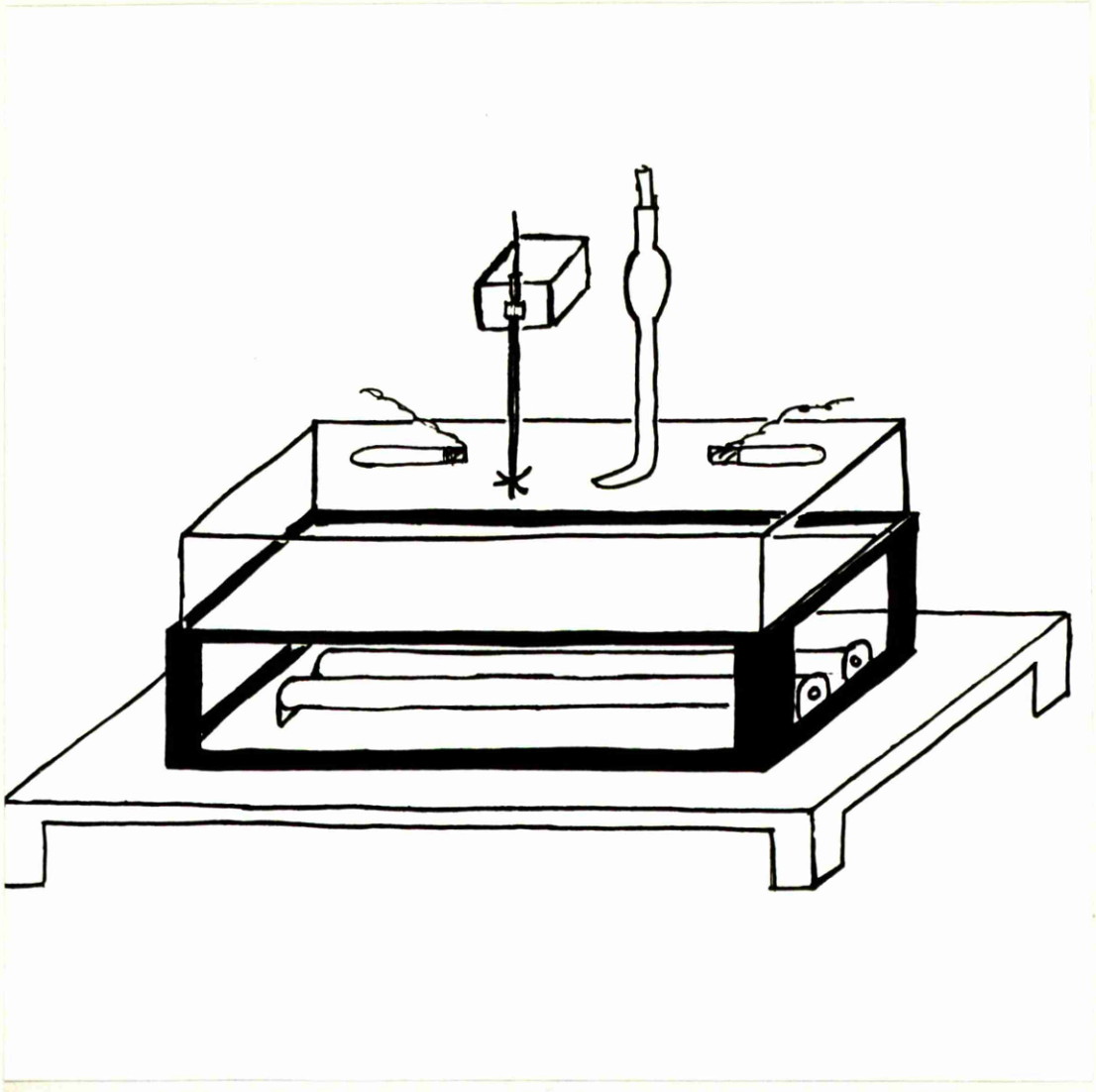

Fig. 30:- Diagram of Microbiological Assay Tank.

Preparation of Glass-ware and of Metal Caps

All glass-ware used for microbiological assay was kept strictly for this purpose. After use it was steeped in Diversey 'Pyroneg' or chromic acid for at least one hour, scrubbed and rinsed at least six times with tap water. It was then dried. The test tubes were capped and all the glass-ware sterilized by steam heat.

The metal caps were boiled three times in nine changes of tap water. They, too, were dried then put on the test tubes before sterilizing.

RESULTS

All results were tested by statistical analysis and a comparison of the amount of radioactive material, as a percentage of the dose, with the amount of microbiologically active material is shown graphically in Figures 31, 32, and 33. The analyses show a highly significant correlation between these amounts. Results are shown, following parenteral hydroxocobalamin, for the periods 1-24 and 24-48 hours only, as not enough subjects excreted significant amounts of radioactive material in the period 48-72 hours to give sufficient data for analysis. The agreement between the amount of radioactive material and the amount of microbiologically

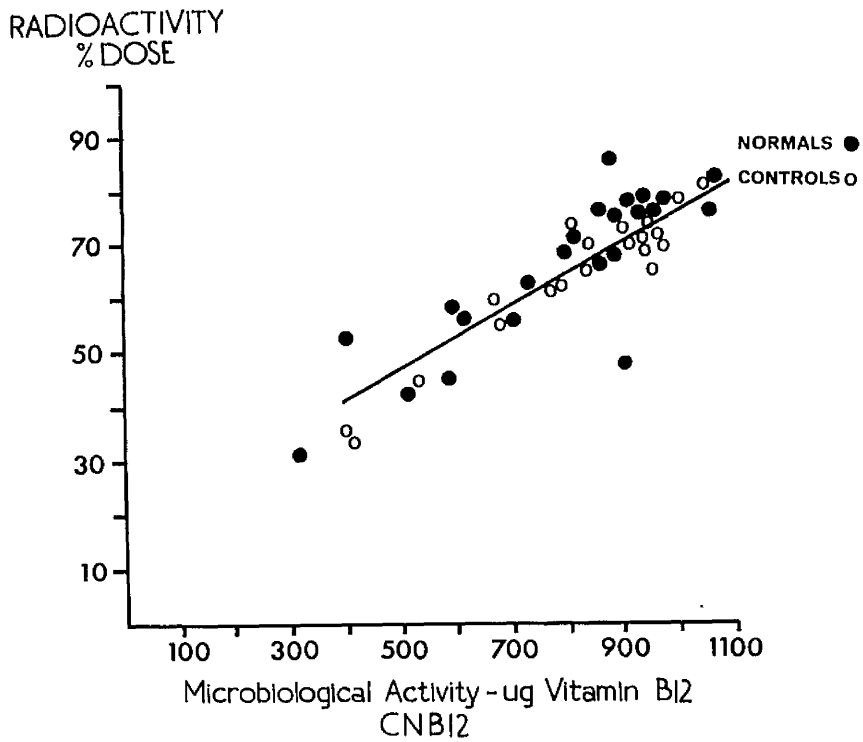

Fig. 31:- Scattergram of Results obtained from the 1-24 hr collections of Urine after injection of 1140 ug ^{58}Co Cyanocobalamin. The regression line for the controls is shown. (J.F.A.)

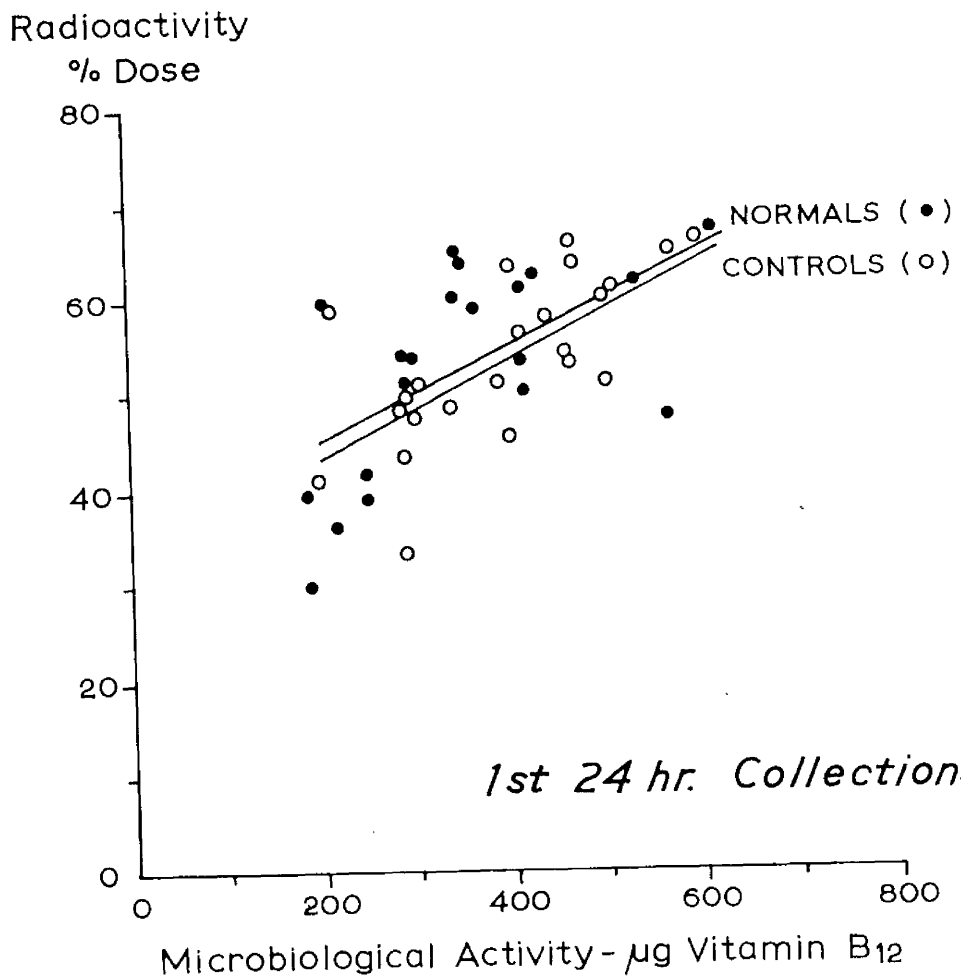

Fig. 32:- Scattergram of Results obtained from the 0-24 hr collections of Urine after injection of 1000 µg ^{57}Co Hydroxocobalamin in Normals and Controls. The Regression Lines for both groups are shown.

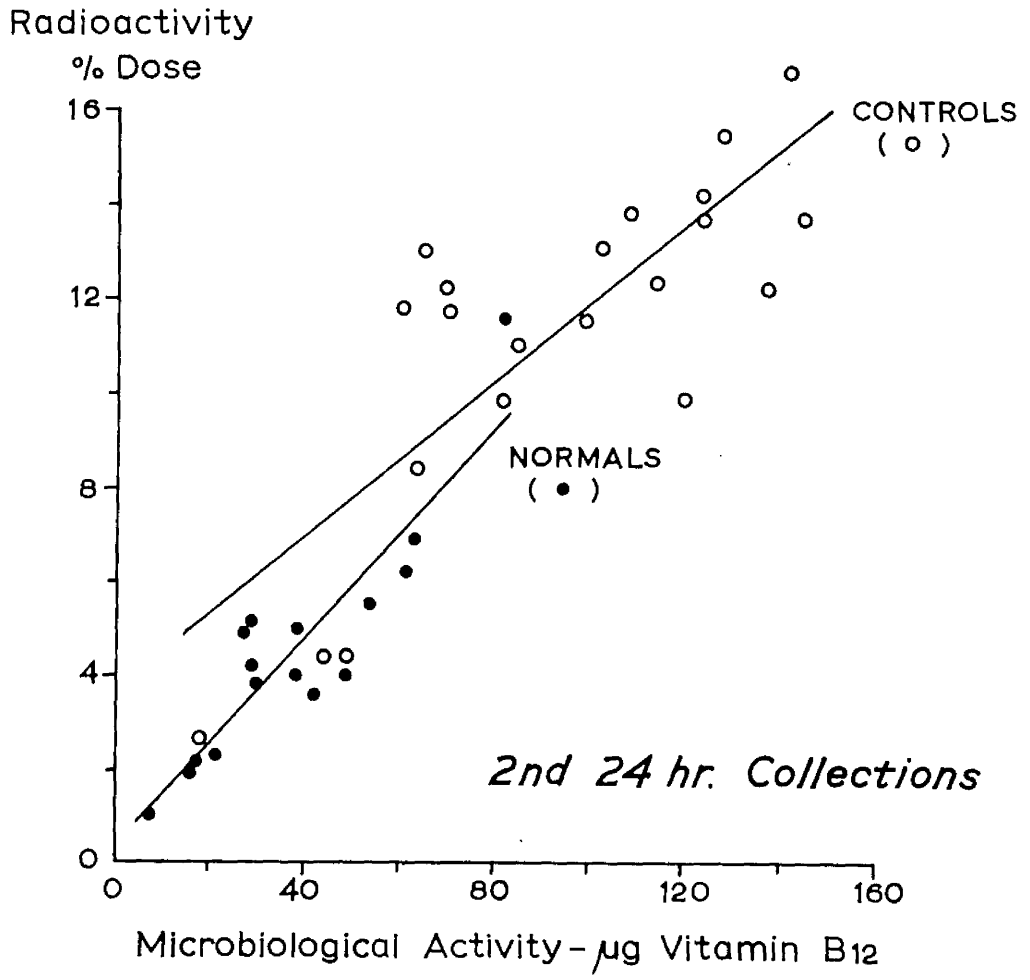

Fig. 33:- Scattergram of Results obtained from the 24-48 hr collections of Urine after injection of 1000 ug ^{57}Co Hydroxocobalamin in Normals and Controls. The regression lines for both groups are shown.

active material is good after both hydroxocobalamin and cyanocobalamin. The latter, done by Adams (1962) in this laboratory, is quoted for completeness. After parenteral hydroxocobalamin, in normals (non-vitamin B_{12} deficient) and controls (vitamin B_{12} deficient) the regression lines do not differ significantly in either the period 1-24 or 24-48 hours.

However, the implications of these results can only be accepted if certain conditions are fulfilled. These conditions are that the three fractions obtained after hydroxocobalamin (see Section 3) have the same microbiological activity as cyanocobalamin, and that urine, a very complex solution, has no effect on the growth of Euglena. Certain further investigations were undertaken to find out if these conditions were fulfilled.

FURTHER EXPERIMENTAL INVESTIGATIONS

The first point investigated was the activity of various cobalamins for Euglena, and some of this is described in detail in Appendix 2, where the relevant findings are that cyanocobalamin and hydroxocobalamin have the same microbiological activity. This point was further investigated using a batch of ^{57}Co hydroxocobalamin, 0.1 uc/0.01 ug/ml, which was analyzed and found to contain 3.5% neutral compound, 63% basic compound, and 30% anionic compound. The neutral material was probably cyanocobalamin and

the basic material hydroxocobalamin. The original solution of this material was diluted with sterile distilled water and assayed using Euglena, on three different occasions. The mean assay result was 0.009 ug/ml in terms of cyanocobalamin. The concentration of the solution was quoted to be 0.01 ug/ml and this would suggest that a solution of mixed cobalamins - probably cyanocobalamin, hydroxocobalamin, and anionic material with the same ion-exchange behaviour as that found in urine, has the same microbiological activity for Euglena as pure cyanocobalamin. As it has already been established that hydroxocobalamin and cyanocobalamin have the same microbiological activity, these results would imply that the anionic material also has the same microbiological activity as cyanocobalamin.

The next investigation concerned the effect of urine on the growth of Euglena. This was investigated by adding 500 ug hydroxocobalamin to 1 litre pooled urine. An aliquot of this solution was diluted with sterile distilled water and assayed on three different occasions. The mean assay result was 0.5 ug/ml, the same as the stated original concentration. These investigations showed that urine had no effect on Euglena growth, as it has already been shown that the three components formed on the addition of hydroxocobalamin to urine (see Section 3) all have the same microbiological activity as cyanocobalamin.

SUMMARY AND DISCUSSION

As the necessary conditions were fulfilled the implications of the results obtained for the comparison of the amount of radioactive material and the amount of microbiologically active material could be taken as valid. The significant correlation between these values, in both the first and second 24 hours after injection can therefore be taken as evidence that the amount of radioactive material excreted is a true measure of the cobalamin excreted.

The similarity of the slope of the regression line obtained from the vitamin B_{12} deficient and non-deficient subjects would suggest that the injected cobalamin did not equilibrate with body stores to a significant degree. Had there been a significant degree of equilibration then the amount of radioactive material would have represented only a fraction of the amount of microbiologically active material.

One reason for lack of equilibration may possibly be that excretion of cobalamin is so rapid that there is not sufficient time for absorption into tissues to occur, but this explanation is excluded by consideration of observations using body counting techniques (Glass 1954: Glass 1959: Glass et al 1955) which show that absorption of material into tissues occurs very rapidly. Another possible reason is the fact that body stores are protein

bound and hence not free to equilibrate (Wijmenga et al 1954), and this explanation is very much more feasible. Yet another reason may be the fact that body stores are probably in the coenzyme form (Weissbach et al 1959). These last two reasons are probably in themselves sufficient to explain the observed lack of equilibration.

However, whatever the reason for lack of equilibration it means that, as has been done for many years, the radioactive material excreted can be taken as a measure of the cobalamin excreted, after both hydroxocobalamin and cyanocobalamin. Practically, this is of great value as it means the measurement of urinary loss of cobalamin can be done by measurement of radioactivity in the urine instead of tedious microbiological assay. This has, of course, been done up till now, but without proof of the validity of the assumption made. Such proof has now been put forward.

APPENDIX 1

Plasma and Serum Binding of Cyanocobalamin and Hydroxocobalamin

The methodology used in this investigation was that described by Gottlieb et al (1965). It consists essentially of separating protein bound cobalamin from non-bound cobalamin by albumin-coated charcoal. The non-bound cobalamin is adsorbed on to the charcoal, and can be readily removed from solution.

MATERIALS AND METHODS

^{57}Co hydroxocobalamin and ^{57}Co cyanocobalamin were obtained freeze-dried from The Radiochemical Centre, with an activity of 10 uc/ 4.21 ug. Each of these was diluted in distilled water to a concentration of 0.01 uc/4,210 uug/ml. All water used in this work was glass-distilled in our own laboratory.

The solution of albumin-coated charcoal was made by mixing 10 g 'Norit A' charcoal in 200 ml water, with 6.67 ml 30% bovine serum albumin made to 200 ml with water.

The serum and plasma were obtained from apparently normal staff members aged 18-42 years, with a mean age of 30 years. Blood was taken by venipuncture using a sterile syringe. Serum was collected in clean sterile universal containers, and plasma collected in sterile containers to which a known amount of solid anticoagulant had been

added. The anticoagulants used are detailed in Table 16. The plasma or serum was separated from the cells and this was used in the investigation. It was kept at -20°C when not in use.

The amount of radioactivity in the solutions was measured using the apparatus already described. As all the solutions concerned had a volume of less than 10 ml the "all in" method was used. Standards containing 0.01 uc/4210 uug were used, with a total volume of 6 ml.

Procedure

To 2 ml saline (0.9%) in a polythene tube, 1 ml serum or plasma was added, followed by 1 ml (4210 uug/0.01 uc) ^{57}Co hydroxocobalamin or cyanocobalamin. The mixture was shaken, then 2 ml albumin-coated charcoal added. The mixture was again shaken, then centrifuged at 2000 rpm for 5 minutes, and the supernatant (6 ml) decanted and counted. The amount of radioactivity in the supernatant was compared to that in the standard, and by calculation the amount of cobalamin bound by the serum or plasma was found. Each sample was done in duplicate and the mean result taken.

RESULTS

The results for the various anticoagulants are shown for each

Sodium Oxolate	16 mg
Sodium Citrate	60 mg
Ammonium Oxalate	12 mg
Potassium Oxalate	8 mg
Heparin	40 mg

TABLE 16 Solid Anticoagulants

(Amounts for 10 ml Blood)

cobalamin in Tables 17 and 18. The mean values are also given, along with the ratio of the mean binding capacity for hydroxocobalamin to that for cyanocobalamin. The reproducibility of the method is demonstrated in Table 19 where the results of several estimations on three different pools of serum are given.

DISCUSSION

The most striking fact from these results is that plasma and serum have a much greater binding capacity for hydroxocobalamin than for cyanocobalamin. The mean result is that it is 1.7 times greater.

In Table 19 it can be seen that the method of estimation of binding capacity gives reproducible results.

CYANOCOBALAMIN

	AMMONIUM & POTASSIUM OXALATE	SODIUM OXALATE	SODIUM CITRATE	HEPARIN	SERUM
1	3368	1083	1143	1928	1743
2	1488	1924	1233	2180	1639
3	2210	1353	1190	1879	1368
4	1849	1458	1443	2105	1428
5	2661	2180	1579	2330	1759
6	2015	1974	1143	1699	1218
7	1549 1609	1549	1368	1413	1113
8	2661	1504	1669	1940	1564
9	1549	1203	1098	1624	1443
10	1368	1188	1083	1259	1218
MEAN	2030	1542	1250	1836	1449

TABLE 17 Binding capacity of Serum and Plasma in uug/ml.

HYDROXYCOBALAMIN

	AMMONIUM & POTASSIUM OXALATE	SODIUM OXALATE	SODIUM CITRATE	HEPARIN	SERUM
1	3732	2237	2416	3779	3217
2	2679	3337	2619	3732	3397
3	3421	2081	2583	3397	2344
4	2918	2392	2787	3197	2583
5	3516	3409	2799	3671	2942
6	2775	2894	2332	3241	2440
7	2415 2380	3241	2607	2894	2021
8	3540	2346	3014	3421	2559
9	2607	2485	2512	3193	2918
10	2835	2454	2392	2811	2272
MEAN	2933	2589	2606	3334	2659
RATIO OH/CN	1.47	1.57	2.09	1.81	1.85

TABLE 18 . Binding capacity of Serum and Plasma in uug/ml.

HYDROXOCOBALAMIN BINDING

	P.1	P.2	P.3
12/10/66	2870	2476	2344
5/10/66	2668	2574	2691
10/10/66	2700	2500	2230
10/10/66	2575	2270	1660
MEAN	2703	2455	2231

TABLE 19 A

CYANOCOBALAMIN BINDING

	P.1	P.2	P.3
5/10/66	1420	1635	1340
11/10/66	1383	1519	917
10/10/66	1310	1520	1310
10/10/66	1613	1722	1408
MEAN	1432	1599	1241
RATIO OH/CN	1.888	1.535	1.798

TABLE 19 B

TABLES 19 A & B Consistency of Results on Repeated Assays.

APPENDIX 2

Microbiological Assay of Different Cobalamins

The usual standards for the microbiological assay using Euglena gracilis strain z, are made from commercially available cyanocobalamin. During the course of the investigations described in this thesis the question of the microbiological activity of different cobalamins arose. Many cobalamins and similar compounds were obtained as a gift from Dr. Mervyn of Glaxo Ltd.. The method of assay used was as described in Section 6. The results are described below and shown in tabular form in Table 20. The nature of each compound is also described.

1. Cyanocobalamin

The nature of this compound has already been described (p.3) This was used as the standard, with an arbitrary activity of 100, and all others compared to it.

2. Monocarboxylic Acid of Cyanocobalamin

This is one of the "red acids", and consists of the cyanocobalamin molecule, with the cyanide group present, but with one of the side chains on one of the pyrrole rings altered from $-CONH_2$, an amide group, to $-COOH$, a carboxylic acid group. On assay, a solution of this compound containing 0.2 ug/ml had the same activity as a solution of

MATERIAL	ACTIVITY
CYANOCOBALAMIN	100 (Standard)
Monocarboxylic Acid of CYANOCOBALAMIN	0
Dicarboxylic Acid of CYANOCOBALAMIN	0
HYDROXOCOBALAMIN	100
Monocarboxylic Acid of HYDROXOCOBALAMIN	0
FACTOR B	0
METHYLCOBALAMIN	66
NITRITOCOBALAMIN	66
SULPHITOCOBALAMIN	100
Co-ENZYME B_{12}	50

TABLE 20:- Microbiological Activity of Different Cobalamins, using Euglena Gracilis Strain Z.

100 uug/ml cyanocobalamin. This means that this compound has an activity for Euglena of only 0.2. This is probably due to some cyanocobalamin present as an impurity.

3. Dicarboxylic Acid of Cyanocobalamin

This again is a "red acid" of cyanocobalamin, but in this two of the amide side chains $-CONH_2$, have been changed to the carboxylic acid grouping -COOH, giving a dicarboxylic acid. A 0.2 ug/ml solution of this compound had no growth-promoting activity in the microbiological assay, giving an activity of 0.

4. Hydroxocobalamin

The nature of this compound has already been described (p.3) A standard curve of this material was the same as that given by cyanocobalamin, and is shown in Figure 34. Thus this material has an activity of 100.

5. Monocarboxylic Acid of Hydroxocobalamin

This is a monoacid of hydroxocobalamin in which one $-CONH_2$ group is replaced by a -COOH. A 0.2 ug/ml solution of this had no growth-promoting activity for Euglena, thus this had an activity of 0.

6. Factor B

This compound, shown in Figure 35, is cyanocobalamin without the nucleotide. A standard curve was attempted using this compound,

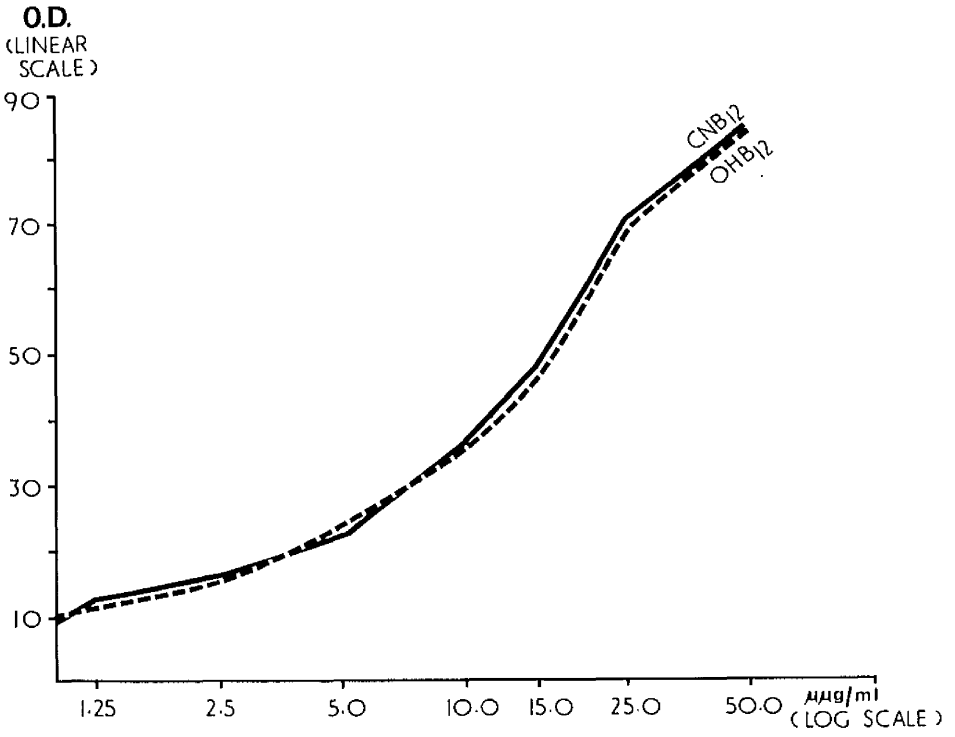

Fig. 34:- Assay Curves of Cyanocobalamin and Hydroxocobalamin.

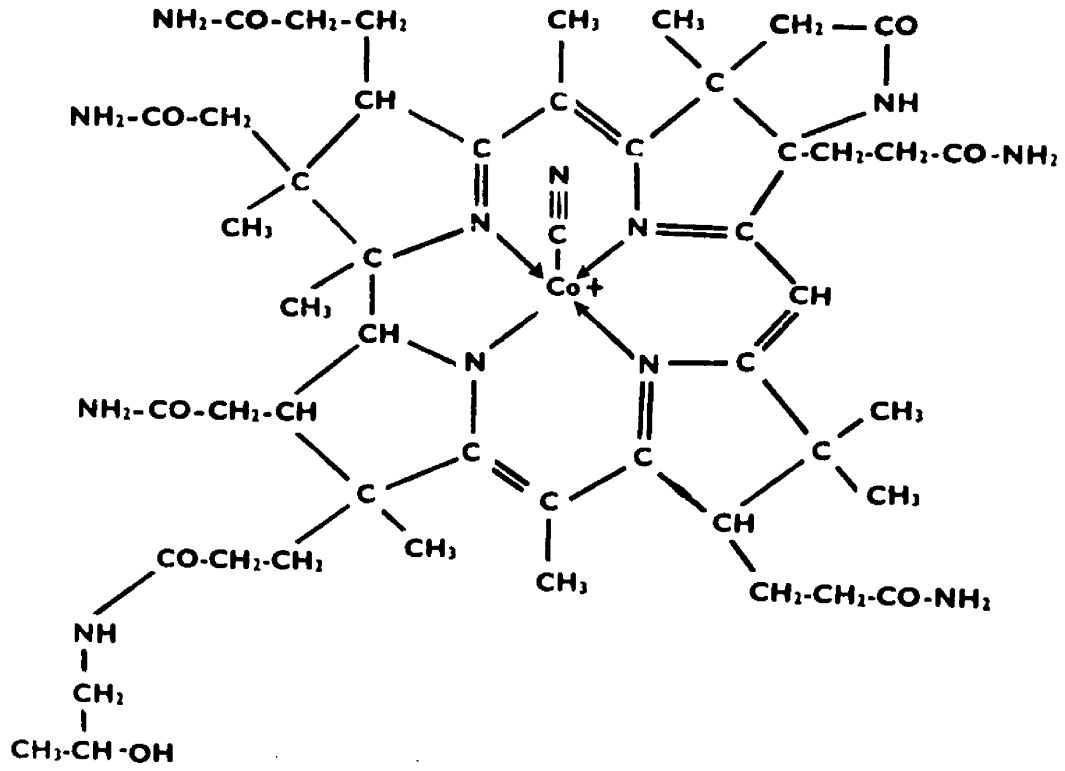

Fig. 35:- Structure of Factor B.

but no growth was observed. A 10 ug/ml solution of this compound had the same activity as a 1150 uug/ml solution of cyanocobalamin. This is an activity of only 0.1 and is possibly due to a small amount of contaminant cyanocobalamin. This result is in agreement with those already published (Coates & Kon 1957).

7. Methylcobalamin

This compound, already described (p. 5) , is light sensitive, being photolyzed to hydroxocobalamin. A standard curve was grown using this compound, and it was found to give values of about 2/3 those given by cyanocobalamin (activity of 67). The assay curves are shown in Figure 36. On assay of solutions of methylcobalamin, a solution of 824 uug/ml, made up in the dark, gave a mean value, from two assays, of 503 uug/ml, while a solution of 412 uug/ml gave a mean value, from two assays, of 250 uug/ml. These results give an activity of 61.

After exposure to light, the solution containing 824 uug/ml was again assayed on two occasions, and gave a mean value of 620 uug/ml, an activity of 75.

8. Nitritocobalamin

This compound contains the cobalamin molecule in which the sixth position on the central cobalt is occupied by the nitrite

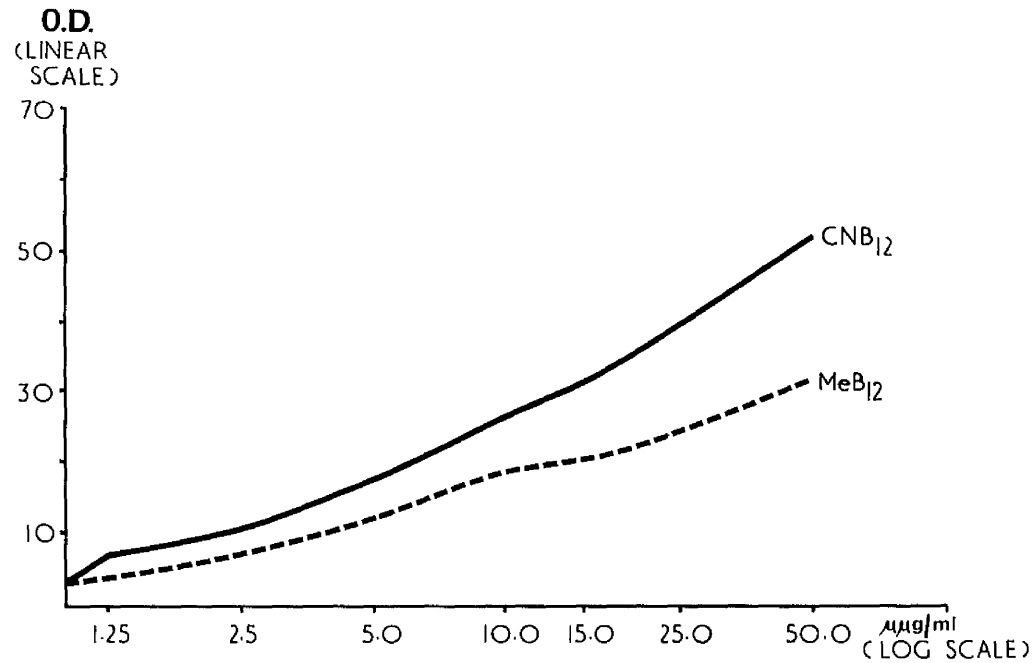

Fig. 36:- Assay Curves of Cyanocobalamin and Methylcobalamin.

group, $-NO_2$. This compound, like methylcobalamin, is light sensitive. A standard curve of this compound gave values about 2/3 of those given by cyanocobalamin (activity of 67). The assay curves are shown in Figure 37. On assay of solutions of this compound, made up in the dark, a solution of 808 uug/ml gave a mean value, from two assays, of 483 uug/ml. A solution of 404 uug/ml gave a mean value, from two assays, of 323 uug/ml. These results give an activity of 70.

After exposure to light, these solutions were again assayed on two occasions, and gave results of 503 uug/ml and 350 uug/ml, respectively. These results give an activity of 74.

9. Sulphitocobalamin

This compound was made by mixing hydroxocobalamin with Na_2SO_3. Sulphitocobalamin contains the cobalamin molecule with the sixth ligand space occupied by $-SO_3$. Standard solutions of this compound were made by making a solution of known amount hydroxocobalamin, and adding an equivalent amount of inorganic sulphite. The resulting solutions were diluted to the appropriate concentrations for assay standards, then assayed. The standard curve (Figure 38) was the same as that for cyanocobalamin, showing that the two compounds have an equal activity (100) for Euglena.

10. Coenzyme B_{12}

The nature of this compound has already been described (p. 5)

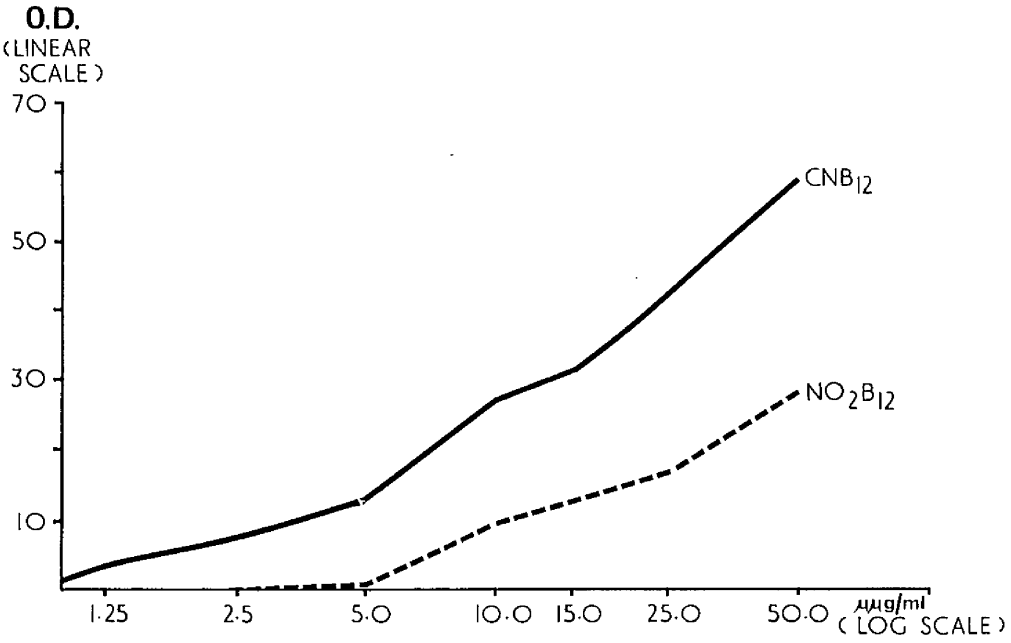

Fig. 37:- Assay Curves of Cyanocobalamin and Nitritocobalamin.

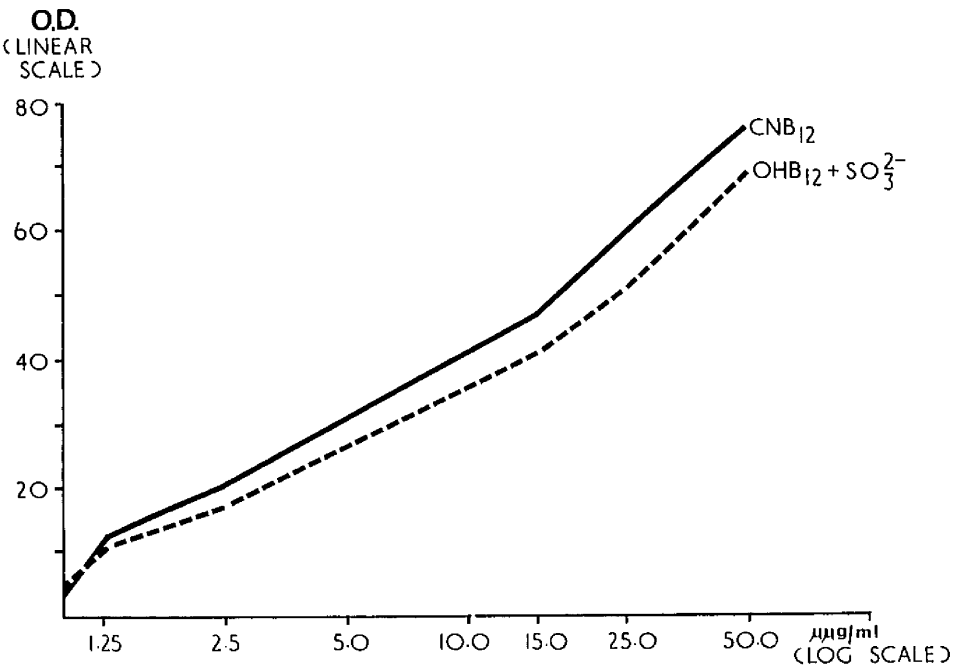

Fig. 38:- Assay Curves of Cyanocobalamin and Hydroxocobalamin + Sulphite.

As it is light sensitive, all manipulations prior to assay were performed in the dark. A standard curve (Figure 39) was found to give 1/2 the values given by cyanocobalamin (activity 50).

Conclusion

Cyanocobalamin, hydroxocobalamin and sulphitocobalamin were found to have an equal activity (100) for Euglena. Nitrito-cobalamin and methylcobalamin assayed at about 2/3 value (activity 70) and coenzyme B_{12} at 1/2 (activity 50). The three "red acids" tested, and Factor B, did not have any microbiological activity (activity 0).

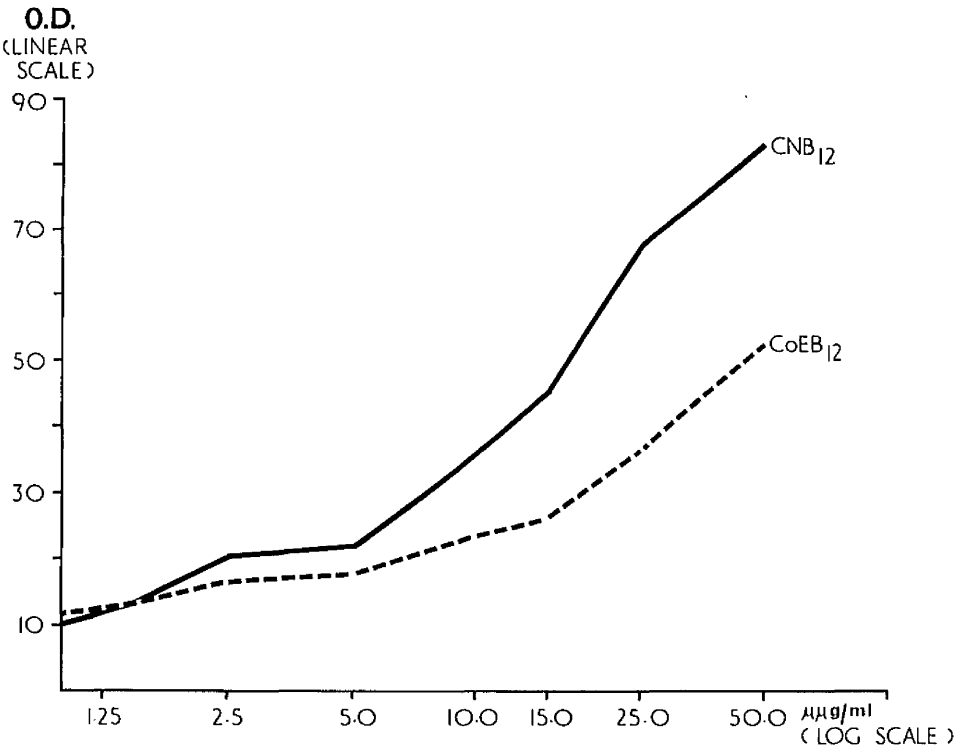

Fig. 39:- Assay Curves of Coenzyme B_{12} and Cyanocobalamin.

SUMMARY

This thesis describes a qualitative and quantitative study of the materials found in urine after parenteral cyanocobalamin and hydroxocobalamin. The investigations developed in different directions, and, for convenience, each aspect is reported in a self-contained section, although the various aspects are inter-dependent.

1. The amount of radioactive material in urine was investigated for periods of up to 72 hours after parenteral administrations of cobalamins in various groups of subjects. It was found that in comparable groups the percentage of the dose excreted after 1000 ug hydroxocobalamin was significantly less than that after 1000 ug cyanocobalamin. It was also found that the amount of radioactive material in urine after hydroxocobalamin was significantly less in anaemic subjects, regardless of their vitamin B_{12} status, than in non-anaemic subjects.

2. Experimental investigations relating to methods of isolation of cobalamins were undertaken to enable a quantitative study of the materials in urine to be carried out. Techniques applicable to urine were developed for the isolation and separation of cobalamins. They consisted of two liquid/liquid extractions followed by ion-exchange chromatography.

3. These methods were applied to overnight collections of urine, from normal subjects, after parenteral cobalamins. It was found that the radioactive material could be divided into three fractions - basic, anionic and neutral. After cyanocobalamin, the bulk of the radioactive material was neutral, but after hydroxocobalamin, in both sterile and non-sterile collections, was composed of 35% basic, 35% anionic and 25% neutral.

4. "In vitro" studies on hydroxocobalamin in urine were conducted. These showed that urine alters a proportion of the hydroxocobalamin producing anionic and neutral material.

5. The chemical nature of the three components was investigated by incubation of urine, collected after parenteral hydroxocobalamin, with KCN at 17°C. All the radioactive material then behaved as a neutral cobalamin, presumably cyanocobalamin, showing that the three fractions contained the intact cobalamin molecule.

6. The different radioactive materials in urine were characterized by the use of standard techniques modified for the purpose. In every case, the neutral material was shown to be cyanocobalamin and the anionic uncharacterized. The basic material from "in vitro" hydroxocobalamin studies was shown to be hydroxocobalamin, and there was some evidence that the basic material from "in vivo" studies was

also hydroxocobalamin.

7. The amounts of radioactive material and microbiologically active material in urine after parenteral hydroxocobalamin were compared in vitamin B_{12} deficient and non-deficient subjects. These amounts correlated significantly in both groups. Urine had no effect on the growth of Euglena and the various cobalamins in urine all had the same microbiological activity for Euglena gracilis as cyanocobalamin. The results imply that there is no equilibration between body stores and injected cobalamin, and also indicate that the amount of radioactive material excreted in urine can be taken as a measure of the amount of cobalamin excreted.

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QUALITATIVE AND QUANTITATIVE STUDIES OF THE RADIOACTIVE MATERIAL IN URINE AFTER PARENTERAL RADIOACTIVE COBALAMINS.

by

ELIZABETH HOLLAND KENNEDY, B.Sc. (Glas.)

A Thesis Submitted for the

Degree of Ph. D. in the Faculty of Medicine.

SYNOPSIS

This thesis describes a qualitative and quantitative study of the materials found in urine after parenteral cyanocobalamin and hydroxocobalamin. The investigations developed in different directions, and, for convenience, each aspect is reported in a self-contained section, although the various aspects are inter-dependent.

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