THE ROLE OF TRANSCOBALAMINS IN VITAMIN B12 METABOLISM

BY

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S U M M A R Y

A study of the clearance of a tracer amount of radioactive vitamin B12 from serum was carried out, so that information on the daily dietary requirement of vitamin B12 could be obtained by applying the occupancy principle to the data. This study was then extended in order to follow the clearance of vitamin B12 from the individual binding proteins. Information on the whole body content of the tracer was used to estimate the total body B12 stores.

In addition to these kinetic studies the distribution of vitamin B12 added to serum in vitro was also examined. The ratio of added B12 bound to each protein was noted in different clinical conditions as well as in serum samples obtained from hospital personnel. An investigation was carried out as to whether the mass of added vitamin B12 affected the percentage bound by each protein. It was found that the results were in no way affected. A brief study of the forms of vitamin B12 carried by each transcobalamin was also made but no specificity was observed.

During the course of the investigations it was observed that vitamin B12 was retained on a column of Sephadex G - 200 under conditions which had not been previously described. This binding was studied in detail but was found to be of importance

only in special circumstances. The work was concluded with a comparison of the results obtained with Sephadex G - 200 and with other separation materials.

A B B R E V I A T I O N S

The terms cobalamin and vitamin B12 (B12) are used when the native vitamin B12 is being discussed and when its chemical form is either not known or is of no particular relevance to the study. Radioactive vitamin B12 is the term used to indicate the use of a radioactive isotope. This isotope is generally ^{57} Co and the labelled vitamin is referred to as ^{57} Co cyanocobalamin (when the vitamin is in the cyano-form) or abbreviated to ^{57} Co B12.

The abbreviation 'tris' is used for 2 - amino - 2 - (hydroxymethyl) propane -1, 3 -diol (tris).

In the tables the heading B12 ng/l refers to the serum vitamin B12 level in nanograms per litre. The heading folate refers for the most part to the serum folate in nanograms per litre but on certain occasions to the whole blood folate or red cell folate. The unsaturated serum vitamin B12 binding capacity in nanograms per litre is referred to as UBBC ng/l and the amount of added radioactive cyanocobalamin bound to specific carrier proteins is expressed as a percentage bound on each fraction. The fractions are abbreviated to TCO (transcobalamin 0), TCI or alternatively TCI + III (transcobalamins I + III) and TCII (transcobalamin II). Where details of the capacity to absorb vitamin B12 are given, the value given under the column headed B12 absorption alone refers to the percentage of a one microgram dose of radioactive cyanocobalamin retained

in the body two weeks after administration, as measured by whole body monitoring. The value under the heading B12 absorption plus IF (intrinsic factor) refers to the percentage retained in the body using the same technique when the dose of radioactive cyanocobalamin is given with a source of intrinsic factor. On occasions the capacity to absorb vitamin B12 was measured by the Schilling Test using a one microgram dose of radioactive cyanocobalamin and these values are marked with an asterisk. The heading marrow refers to the state of erythropoiesis and normo is an abbreviation for normoblastic. The abbreviation MAC refers to the maximal acid output usually in response to pentagastrin and the values are given as milliequivalents in the hour after stimulation. The abbreviation PFA refers to pentagastrin fast achlorhydria. Where the actual output was not measured but the pH fell below 3 the column simply refers to acid being present. In table 23 the abbreviation alk phos is used for alkaline phosphatase, the value being in international units per millilitre, and the abbreviations AST and ALT are for aspartate transaminase and alanine transaminase respectively, both values being in international units per litre. The abbreviations m for month and y for year are used to indicate the duration of time of treatment in the appropriate tables.

I N T R O D U C T I O N

THE DEVELOPMENT OF KNOWLEDGE OF THE PLASMA

TRANSPORT OF VITAMIN B12

This chapter is devoted to an historical review of the literature on transcobalamin studies. The review is an attempt to provide the background to the work which will be described in later chapters. In this introductory section a great deal of emphasis will be laid on the different separation methods used by different groups of workers, as the accumulation of knowledge has been dependent on, and often confused by, this variety of methods. The review also attempts to clarify how the current concepts of the transport of vitamin B12 in plasma in man have evolved.

It is common in biological systems for specific proteins to be involved in the transport of essential molecules. The first evidence that vitamin B12 was bound to protein came when Ross (1) reported that heat treatment of serum was necessary to release vitamin B12 for microbiological assay. Paper electrophoresis of serum followed by microbiological assay of sections of the strips, showed that the endogenous vitamin B12 in serum was located in the alpha globulin fraction only. This observation led Pitney et al (2) to postulate the existence of a specific

serum B12 carrier protein. They observed that this carrier protein had only a limited capacity to bind vitamin B12. Later studies by Mendelsohn et al (3) confirmed these findings. Miller and Sullivan (4) showed that although the native vitamin B12 was bound to an alpha globulin protein, any radioactive vitamin B12 which was added to the serum was predominantly associated with another fraction. They separated the serum by starch gel electrophoresis and found that the native vitamin B12 migrated to the anode but most of the added ^{60}Co labelled B12 migrated to the cathode. This radioactive vitamin B12 was detected in spite of dialysis, which suggested that it was associated with another protein. It was concluded from these experiments that at the levels of vitamin B12 present in normal serum, the B12 binding protein was almost saturated and that the addition of further vitamin resulted in the excess being bound to other serum proteins.

It is worth stating at this point that whereas the work carried out by Ross, by Pitney et al, and by Mendelsohn et al depended on microbiological assay to detect vitamin B12 later studies made use of radioactive forms of the vitamin. Three forms of radioactive cyanocobalamin were available: ^{57}Co B12 and the less frequently used ^{58}Co B12 and ^{60}Co B12. One or more of these isotopes could be introduced either in vivo (by oral or parenteral administration) or it could be

added to a sample of serum or plasma in vitro : The distribution of the radioactivity in the sample was used to indicate the presence of the added vitamin B12.

The first indication that more than one specific vitamin B12 binding protein existed in plasma came from in vivo studies by Hall and Finkler (5). They observed that vitamin B12 was bound by more than one portion of a plasma sample which had been fractionated with ammonium sulphate. The radioactive vitamin B12 was cleared from the different plasma fractions at different rates and this was attributed to the presence of more than one vitamin B12 binding protein. Hall and Finkler (6) separated a second B12 binding protein from the carrier located in the alpha-1 globulin region on a diethylaminoethyl-cellulose (DEAE-cellulose) anion exchange column. They noted that this second binding protein, which they christened the 110 component, bound the major part of any vitamin B12 added to the serum in vitro. The other protein which they named the 210 component and which corresponded to the alpha globulin binder described earlier, bound only a small amount of added B12. In a later study by Hall and Finkler (7) the 210 component was renamed transcobalamin 1 (TCI) and the 110 component renamed transcobalamin II (TCII) and this is the terminology which has been generally adopted.

The separation of the transcobalamins was studied by Hom et al (8) using a variety of techniques. They found that two

vitamin B12 binding proteins could be separated from each other on carboxymethyl - Sephadex (CM - Sephadex), on diethylaminoethyl - Sephadex (DEAE-Sephadex), by Sephadex G-200 gel filtration and by ammonium sulphate $\left((NH_4)_2\ SO_4\right)$ precipitation in addition to the DEAE - cellulose technique already described by Hall and Finkler (6). The G-200 separation also gave information on the molecular weights of the two proteins, these being estimated at approximately 121,000 (TCI) and 36,000 (TCII). Each analytical method yielded two fractions in which radioactive B12 was detected. It was assumed that the two fractions yielded by one method corresponded to the two yielded by another and that they were due to the complete separation of two vitamin B12 binding proteins. This assumption confused the issue for some time.

Studies by Lawrence (9) using zone electrophoresis showed that vitamin B12 appeared to be bound by three fractions in serum : an alpha-1 globulin and a beta globulin (which correspond to TCI and TCII respectively) and also by a binder with alpha-2 globulin mobility. The presence of a third vitamin B12 binding protein in serum was also postulated by Hall and Finkler (10) when they added Co^{57} B12 to serum in vitro and noted that the second radioactivity peak which eluted from a DEAE-cellulose column did not appear to be homogeneous.

In retrospect this observation was probably due to an artefact produced by the separation technique, possibly related to the presence of heparin, for the later work (11) has shown that the second radioactivity peak eluted from DEAE-cellulose contains only one vitamin B12 binding protein. The demonstration by Hom (12) that TC11 could form complexes and bind to G - 200 at low ionic strength further complicated the picture as it became apparent that this new binding protein could be a polymer of TC11. Gizis et al (13) also demonstrated a third binder when they separated the transcobalamins on DEAE-cellulose and then checked the two fractions for homogeneity on Sephadex G - 200. The 'TC11' fraction eluted from G - 200 as a homogeneous peak (corresponding to a protein with a molecular weight of 40,000) but the TC1 fraction obtained from DEAE gave two radioactivity peaks on G - 200 : the major one at a large molecular weight and a smaller binder which accounted for almost 33% of the eluted radioactivity. They attributed this to TC11 contamination.

Similar work was undertaken by Lawrence (11) when she compared the two binders separated by DEAE-cellulose with the two separated on G - 200. Normal serum was shown to contain three binders : a high molecular weight alpha - 1 globulin (TC1) and a high and a low molecular weight beta globulin (the new binder and TC11 respectively). The high molecular weight beta globulin binder appears to correspond to the protein

which Lawrence had previously (9) classified as an alpha - 2 globulin binder. She demonstrated the presence of three binders by removing the low molecular weight protein (TC11) from the serum by absorption on to uncoated charcoal and separating the remaining fraction on a DEAE-cellulose column. Two peaks of vitamin B12 binding protein were still eluted from the column although the first peak was smaller than in comparable experiments when uncoated charcoal absorption was not used.

The position was finally clarified by Bloomfield and Scott (14) who confirmed the presence of a third binder as described earlier by Lawrence. They fractionated a serum sample on a DEAE-cellulose column and found 92% of the added radioactivity eluted with TC11 and 8% eluted with TC1 whereas G - 200 separation of another sample of the same serum showed that 74% of the added activity eluted with TC11 and 26% eluted with TC1. The discrepancy between the results obtained from the two different methods was attributed to the presence of a third binder which eluted with TC11 from DEAE-cellulose and with TC1 from Sephadex G - 200. The third binder, which they named TC111, was found in fifty normal serum samples and was therefore not a pathological binder.

The properties of the third binder have been studied by several groups. Bloomfield and Scott (15) showed that in the absence of TC11, TC111 had a greater affinity for B12 than TC1,

and that although it eluted from DEAE-cellulose with TC11, by varying the conditions it was shown to be less firmly held on the ion-exchange resin. In an in vivo study, Chanarin et al (16) examined the possibility that a protein which they called binder 111 (and which appears to correspond to TC111) was a polymer of TC11. They separated the three transcobalamins on a DEAE-cellulose column followed by G - 200 fractionation of the TC11 + binder 111 peak, and showed that all three binders took up cyanocobalamin from the gut simultaneously, in proportions depending on their unsaturated B12 binding capacities at the time. They were 'unable to comment on the nature of the third binder' and on whether or not it was a polymer of TC11. Carmel (17) showed that the third binder, which is present in small amounts in normal serum, is responsible for the elevated unsaturated B12 binding capacity (UBBC) observed in the serum of patients with leukocytosis. He compared the binder with one found previously only in the serum of patients with polycythaemia rubra vera (described by Hall and Finkler (18) and suggested that the two were identical.

Immunological studies showed that the third binder was precipitated by anti-sera reacting with TC1 (17, 19). England et al showed by immunodiffusion that both TC1 and binder 111 gave reactions of identity with antiserum raised to salivary R binder, and from that deduced that TC1 and binder 111 were both circulating R binders and therefore closely related. The term R binder had

been introduced by Grasbeck et al (21) to describe the non-intrinsic factor vitamin B12 binding protein found in gastric juice. The name was derived from the characteristic rapid mobility of the protein compared with the mobility of intrinsic factor. (Intrinsic factor is the vitamin B12 binding protein concerned specifically with absorption from the gut.) More recently the use of the term R binder has been extended to describe all the vitamin B12 binding proteins which have rapid electrophoretic mobility, regardless of the body fluid in which they are found, thus covering all the specific B12 binding proteins apart from TC11 and intrinsic factor.

Stenman has recently used isoelectric focusing to separate the transcobalamins from each other (22, 23). He renamed the R binders 'cobalophilin' in order to avoid the implication of rapid mobility, as the R binders obtained from different body fluids have electrophoretic mobilities varying from alpha to beta (23). Stenman characterised the cobalophilin from different sources (22, 23) and found it to be a "microheterogeneous mixture" consisting of five or more isoproteins with isoelectric points (pI) between 2.3 and 5.0. He found that the cobalophilin from different cells and fluids consisted mainly of the same isoproteins in different proportions. The isoproteins were divided into two populations, one pI 2.3 - 4.2 and the other pI 4.0 - 5.0. TC11 had a pI value of around 6 and therefore did not overlap with the cobalophilin isoproteins. It was

suggested (22) that these variations in the patterns of isoproteins present could account for the differenct electrophoretic mobilities ascribed to the R binders, and for the description of TC1 as an alpha - 1 globulin and TC111 as having alpha - 2 and beta mobility.

Additional vitamin B12 binding proteins have been observed in the serum of patients with clinical abnormalities. One such binder, the PV binder, was found by Hall and Finkler (18, 24) in the blood of eight patients with polycythaemia vera in relapse. The protein reacted with anti - TC1 and anti-salivary R anti-sera but differed from TC1 in its electrophoretic mobility. On the basis on Stenman's classification the observation can be attributed to a distribution of the cobalophilin isoproteins different from that found in normal serum. The foetal binder described by Kumento (25) as eluting from Sephadex G - 200 with TC1 but having a different electrophoretic mobility is presumably also due to a variation in the cobalophilin isoprotein pattern.

Affinity chromatography has been used to isolate two vitamin B12 binding proteins from serum. The separation of TC11 in a homogeneous form resulted in a two million fold purification relative to human plasma (26). The plasma content of TC11 has been estimated to be 25 µg TC11/litre of plasma (27) with a binding capacity of almost 1000 ng B12/litre of plasma (28). The isolation of pure TC11 allowed its properties to be studied (26). The molecular weight as determined by sedimentation equilibrium ultracentrifugation was 53,900 and by Sephadex G - 150 gel filtration was 60,000. Sodium dodecyl sulphate polyacrylamide gel

electrophoresis showed that two peptides were present. From this it was inferred that the protein was a dimer and that it consisted of one subunit with a molecular weight of 25,000 and another with a molecular weight of 38,000. Pure TC11 was able to bind 28.6 µg B12/mg of protein and this binding of vitamin B12 by TC11 shifted the peak of B12 absorption from 361 nm to 364 nm.

A vitamin B12 binding protein was also isolated from the granulocytes of patients with chronic granulocytic leukaemia, using affinity chromatography as the sole purification technique (29). A homogeneous protein was obtained from 9,800 fold purification, and its molecular weight was found to be 56,000 as determined by sedimentation equilibrium ultracentrifugation and 58,200 as determined by amino acid and carbohydrate analysis. The protein had a high carbohydrate content (33%) which accounts for the high molecular weight values (121,000 to 138,000) which have been reported for the R binders when separated by gel filtration. The granulocyte vitamin B12 binding protein had a single cobalamin binding site and bound 34.9 µg vitamin B12/mg of protein. No shift in the vitamin B12 absorption peak was observed on its binding to the R protein.

Immunological studies using crude or partially purified proteins have shown that the vitamin B12 binding proteins found in human body fluids can be divided into three distinct groups : intrinsic factor, TC11, and the R binders (TC1, TC111, PV binder, foetal binder, the granulocyte binder, salivary binder

and others). No cross-reactivity has been found between the three groups (30). Variations in the sialic acid content of the R proteins are probably responsible for the heterogeneity observed by Stenman during isoelectric focusing, since treatment with sialidase appears to reduce this heterogeneity.

In studies using serum labelled with ^{57} Co B12 added in vitro, radioactivity was eluted from a Sephadex G - 200 column in fractions other than those corresponding to the vitamin B12 binding proteins already mentioned. When an excess of ^{57} Co B12 was added, four peaks of radioactivity were detected (28). The first peak, named TCO by Hom and Ahluwalia was eluted at the same time as the first protein peak. This protein was consistently found in G - 200 fractionation as a vitamin B12 binder over the range of 75 to 20,760 ng B12 added/litre of serum. Although TCO accounted for up to 5.2% of the unsaturated B12 binding capacity the protein has never been studied in any detail. One explanation for its occurence suggested by Hom and Ahluwalia was that TCO is a complex of TCII, similar to the complex formed in low ionic strength buffer. The second and third peaks of radioactivity noted by Hom and Ahluwalia (28) corresponded to TCI and TCIII as discussed above. The fourth peak of radioactivity eluted from the column was attributed to "non-protein bound ^{57} Co B12 and ^{57} Co not associated with vitamin B12". This free ^{57} Co was ascribed to radiochemical impurities in the isotope preparation and not to disintegration during gel

filtration. In a similar study Hall and Finkler (10) added large amounts of vitamin B12 to serum in vitro and observed that the excess B12 did not remain free but was bound by proteins other that TC1 and TC11. These binders were considered to be secondary or non-specific binders. A secondary binder was also described by Gizis et al (31) as eluting from DEAE-cellulose with 0.1M sodium phosphate buffer. The binder was only found when large amounts of vitamin B12 were added to the serum (at least 300 ng B12/litre of serum) and the percentage of the activity which it bound was increased as the mass of vitamin B12 added to the serum was increased. It would appear that this secondary binding is very 'loose' and that although it is able to survive DEAE-cellulose fractionation the binding does not withstand gel filtration.

It is evident from the number of artefacts of separation which have been described that care is needed in interpreting any results which appear to suggest that a new vitamin B12 binding protein is present. TC11 in particular appears to be a very labile protein, complexing with itself and binding to Sephadex G - 200 in the presence of low ionic strength buffers (12) and also forming complexes with heparin. Heparin is a strong cation exchanger and therefore it is able to bind TC11 which has a strong affinity for the cation exchange resin carboxymethyl-cellulose. Heparin also affects the elution of the vitamin B12 binding proteins from DEAE-cellulose : TC11 and binder 111

are no longer eluted with phosphate buffer but with 1M NaCl solution i.e at the same time as TC1 (20). It is possible that it was this effect of heparin which was observed by Hall and Finkler (10) when they described the heterogeneity of the TC1 peak eluted from DEAE-cellulose. In vitro studies (20) have also shown that the properties of TC11 and binder 111 are altered following G - 200 separation; the eluted binders have a decreased affinity for vitamin B12, and TC1 and binder 111 can no longer be separated on DEAE-cellulose. Ethylenediaminetetra-acetic acid (EDTA) also affects the properties of the transcobalamins. In the presence of EDTA more ^{57}Co B12 eluted at the peak corresponding to TC11 on Sephadex G - 200 than when no EDTA was present. The explanation which was proposed for this observation (32) was that TC11 is present in serum as a complex held together by bivalent cationic bridging. In the presence of EDTA the cations are unavailable and TC11 does not associate.

Information on the role of the transcobalamins in the transport of vitamin B12 in the bloodstream has come from studies following the administration of radioactive B12 either orally or parenterally. Hall and Finkler (5) observed that there was a difference in the rate of clearance of the ^{57}Co cyanocobalamin from the fractions corresponding to the two transcobalamins. They gave ^{57}Co B12 to volunteers, both orally and intraveneously and observed that the vitamin B12 was initially selectively bound to TC11 (7). The radioactivity was rapidly cleared from TC11 and

re-appeared bound to TC1, in amounts which increased for the first twenty-four hours. This rapid clearance of TC11 bound B12 was also observed by Hom (33) when he injected a TC11 - ^{57}Co B_{12} complex into volunteers and showed that only 32.1% of the dose was still present at 10 minutes post-injection. He detected ^{57}Co B_{12} bound to TC1 at 4 - 8 hours, and by twenty four hours observed that all the radioactivity was bound to TC1.

A different theory for the roles of the transcobalamins was proposed by Chanarin et al (16) when they attempted to elucidate a role for binder 111 in the transport of vitamin B12. They observed that an oral dose of ^{57}Co B_{12} was bound by all three transcobalamins simultaneously and suggested that this binding was in proportion to the unsaturated B12 binding capacities of the proteins at that time. In a further study by the same group (20), ^{57}Co B_{12} was observed to be still bound to TC11 at 8 - 10 days after oral administration. The theories which have been suggested for the roles of the transcobalamins in B12 transport are discussed in greater detail in later chapters.

Information about vitamin B12 transport proteins has also come from studies of patients with an inherited deficiency of one of the binders. These cases are extremely rare, however, only one family has been described as having members with a TC1 deficiency (34) and only two cases of families with TC11 deficiencies have been reported (35, 36).

The only significant clinical abnormality observed in either of the two brothers who had the alpha - 1 globulin (TC1) defect was a low serum vitamin B12 level. Separation of serum samples by starch gel electrophoresis and by rapid DEAE-cellulose anion exchange chromatography showed that there was virtually no TC1 present. Leukocyte extracts and saliva samples from both subjects also yielded virtually no vitamin B12 binding capacity. It must be emphasised that although the brothers appeared to lack all R binders there was no evidence of any metabolic B12 deficiency and the ability of tissues to store B12 was not impaired.

In contrast to the clinical normality of these two brothers the cases of TC11 deficiency which have been described show very marked symptoms of severe vitamin B12 deficiency, in spite of normal serum B12 levels. In both of the families described complete remission was achieved by the administration of large amounts of vitamin B12 to the affected infants. The withdrawal of therapy led to a rapid relapse. The separation by DEAE-cellulose chromatography and polyacrylamide gel electrophoresis of serum samples from one family (35) and by polyacrylamide gel electrophoresis of serum from the other (36) showed that there was no TC11 present. In the case of the latter family this was confirmed by experiments with anti - TC11 antiserum. The response to massive amounts of vitamin B12 could be due to the existence of secondary or non-specific B12 binders, to diffusion, or as has

been proposed by Hitzig et al (36) to the appearance of a new B12 binder with alpha - 2 electrophoretic mobility similar to TC111 and foetal binder. The infant described by Hitzig et al was unable to synthesise immunoglobulins prior to B12 therapy but this defect of the immune system was corrected after treatment began. The significance of this finding is not yet known.

Although the two groups of serum B12 binding proteins have been studied in some detail the terminology used still gives rise to some confusion. In addition to the nomenclature which has been described, other workers have given the transcobalamins alternative names e.g. TCS, TCL, SBP and LBP as suggested by Gullberg (37, 38). In the work to be described it is proposed to retain the term TC11 and to use TC1 + 111 to describe the R binders present in serum. The TCC binder has been considered separately in the in vitro experiments as there is no information yet on whether it is a TC11 complex or should be regarded as an R binder.

	TC1 + 111	TC11
Hall and Finkler (6)	210 component	110 component
Hall and Finkler (7)	TC1	TC11
Grasbeck et al (21)	R binders	
Stenman (22)	cobalophilin	
Gullberg (37)	TCL	TCS
Gullberg (38)	LBP	SBP

Table 1 Transcobalamin Nomenclature

	TC1	TC111	TC11
	α_1	$\alpha_2\beta$	β
zone electrophoresis pH 8.6 (9,11)	1M NaCl	phosphate buffer	phosphate buffer
DEAE-cellulose elution (14)		TC1 & 111	TC11
G-200 elution (8)		mol wt 121,000	mol wt 36,000
CM-Sephadex elution (8)		early	late
CM-cellulose elution (27)		early	late
iso electric point (23)		2.3 - 5.0	6
starch block electrophoresis pH 4.5 (27)		anodal	cathodal
adsorption by uncoated charcoal (11)		no	yes
role in B12 transport (27)		binds endogenous B12	binds newly absorbed B12
$(NH_4)_2SO_4$ precipitation (8)		2.5 - 3.5 M	1.5 - 2.3 M
carbohydrate content		33% (29)	none (26)
molecular weight		around 55,000 (29)	around 55,000 (26)
no of subunits		1(29)	2(26)
no of binding sites		1(29)	1(26)
amount of B12 bound (µg B12/mg protein)		34.9 (29)	28.6 (26)
B12 absorption peak shift on binding		No(29)	Yes(26)
site of synthesis		granulocytes (39,42)	liver (41)
reaction with			
i) anti R (30)		+	-
ii) anti TC1 (30)		+	-
iii) anti TC11 (30)		-	+
promotes uptake of B12			
by i) Hela cells (27)		no	yes
ii) reticulocytes (41)		no	yes
plasma content (µg protein/litre) (27)		60	25
total binding capacity (ng B12/litre plasma) (27)		700 - 800	986
approx. % saturation (27)		50%	2%

Table 2 : showing differences in the properties of the transcobalamins.

C H A P T E R 1

THE APPLICATION OF THE OCCUPANCY PRINCIPLE TO IN VIVO STUDIES ON THE METABOLISM OF VITAMIN B12.

SECTION 1

THE OCCUPANCY PRINCIPLE

The occupancy principle provides a means of relating the flow of a substance into a system with its capacity and occupancy. The foundation of the theory was described by Bergner (43 - 47) and it was propounded in a form applicable to the analysis of radionuclide kinetic data by Orr and Gillespie (48). The principle has been utilised in studies of thyroxine secretion (49), iron metabolism (50), calcium absorption (51), thyroid iodine content estimation (52) and drug dosage studies (53 - 55), but it has never been applied to the analysis of the data obtained using radioactive vitamin B12.

The occupancy principle is the main analytical method used in the studies described in this chapter and therefore it is appropriate to restate the essentials of the principle with particular reference to the metabolism of vitamin B12.

The application of the occupancy principle to kinetic studies of vitamin B12 metabolism requires certain assumptions to be made. Firstly it is assumed that the radioactive vitamin B12 (in this case ^{57}Co cyancobalamin) is a true tracer, that is, it is treated throughout the system in the same way as the non-radioactive vitamin B12 which has come from the diet. Cyanocobalamin is not a naturally occurring form of the vitamin, either in food (56) or in human tissue (57) but there is evidence

that it is rapidly converted to biological forms (57). A second requirement for the application of the occupancy principle is that the system is in a steady state, that is, the entry flow to any compartment is balanced by the exit flow and thus the amount of substance in any compartment is constant. It follows from this that the flow through the system is also constant. Small variations in the input flow, represented by variations in the dietary intake, can however be neglected because the body turnover time for vitamin B12 is long. The final requirement for the application of the occupancy principle is that the tracer material is introduced into the flow which is to be measured.

The salient features of vitamin B12 metabolism are represented schematically in figure 1 (a). The dietary intake is represented as F_T which, since the system is in a steady state, is equal to the sum of the faecal excretion E_F and the urinary excretion E_U. Only a part (F_A) of the total flow is absorbed and this constitutes the entry flow into plasma. The remainder, E_D, is excreted directly as a component of the faecal excretion E_F. Of the amount which is excreted into bile, a part (F_R) is reabsorbed, and the remainder (E_R) contributes to the total faecal excretion E_F.

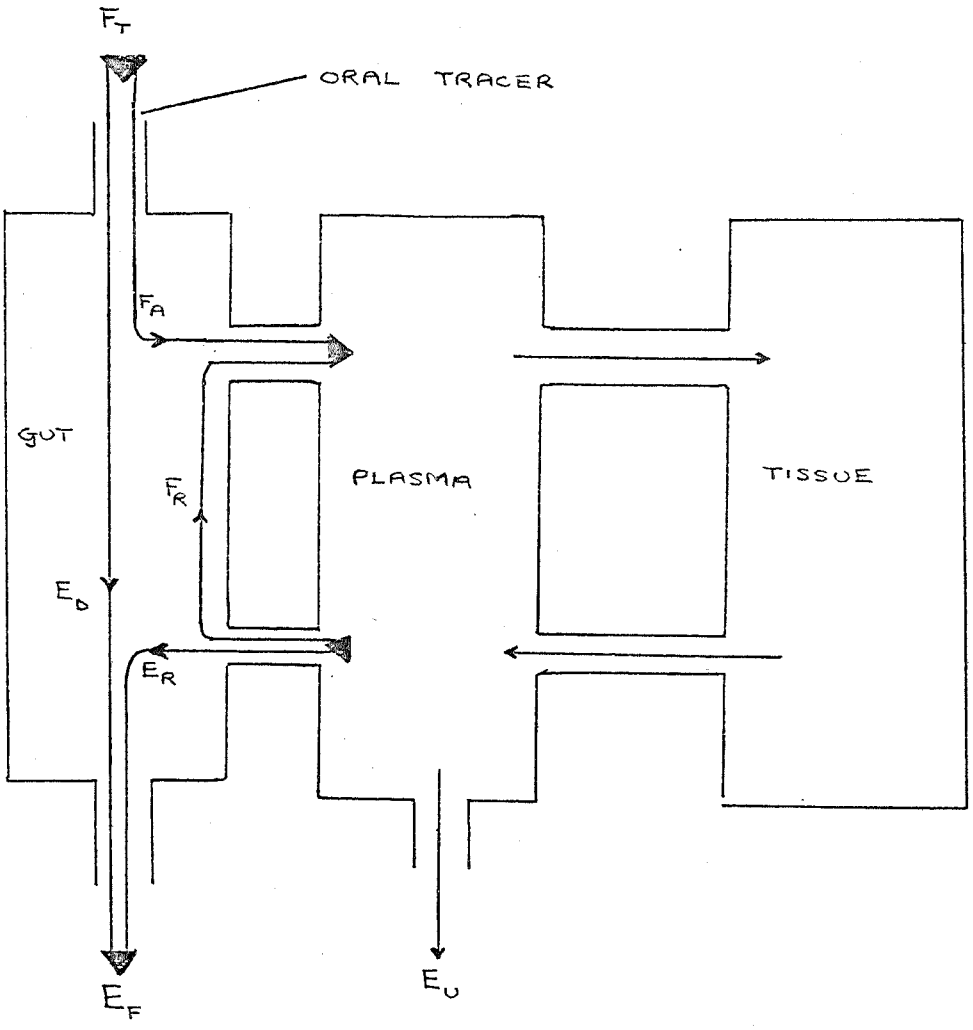


Figure 1a) : A schematic diagram of the essential features of vitamin B12 absorption.

The Derivation of the Occupancy Principle

It is assumed that a bolus of radioactive tracer is introduced instantaneously into the input flow to a system at time t = 0. At time t later, a fraction fi of the tracer will be in a particular part i of the system. The value of fi at any particular time t is denoted as fi (t).

At the time of the injection there is obviously none of the tracer in part i. The fraction rises to a maximum, different for each part of the system, as the tracer is carried into each part by the flow of stable material. The fraction then falls to zero as all the tracer is carried out of that part. It is assumed that the tracer is treated in exactly the same way as the stable material. Clearly, by the time all the tracer has been removed from the part, all the stable material which entered the system before the injection of the tracer will also have been carried out of the part in question. In other words, the total content of stable material in that part, called its capacity, is made up of material which entered the system after the tracer injection. Since the input flow, F, is constant, in any short time δt an amount $F\delta t$ of material enters the system. Tracer and stable material behave in the same way so an amount $F\delta t.fi\,(t)$ of this material will be present in part i at time t later. The total amount of stable material in part i is, therefore, made up of all those elementary contributions from

the time of injection of the tracer until the time when tracer activity in the part i has fallen to zero. To be strictly correct this latter time is represented as infinity. Thus,

capacity $C_i = F \int_0^\infty fi\,(t)\,dt.$

The latter integral is termed the occupancy of part i and is symbolised by θi. It is simply the area under the activity/time curve for the part in question where activity at each time is measured as a fraction of the total injected activity.

The extension to the real situation where the tracer is not injected instantaneously is now straightforward. The rate of administration of the tracer is not important, as the input may be regarded as a series of instantaneous doses. Each of these elementary doses will have the same activity/time curve in that part of the system of interest, but displaced in time from the others. However, it is only the area under the total activity/time curve that is required. This is equal to the sum of the areas under the elementary activity/time curves, whatever their relative displacement in time. The derivation above is thus valid whatever the time scale of administration of the tracer. Different rates of administration will produce activity/time curves of different shapes but of the same area.

Thus, the occupancy principle is obtained : $(\text{capacity})_i$ equals flow x $(\text{occupancy})_i$ for any part i of the system. By making serial activity measurements for any part and determining

the amount of stable material in that part, the total flow F through the system may be deduced from the equation:

$$\text{flow} = \frac{(\text{capacity})_i}{(\text{occupancy})_i}$$

Thus, by measuring the serum B12 level and by making serial activity measurements following oral ^{57}Co B12 administration an estimate of the daily dietary intake of vitamin B12 may be obtained. Furthermore, since the flow is a constant of the system the ratio of capacity to occupancy for any part is the same, $\frac{C_A}{\theta_A} = \frac{C_B}{\theta_B} = \frac{C_C}{\theta_C}$ etc. If the ratio of capacity to occupancy for plasma is found as above and whole body occupancy is deduced from serial whole body activity measurements, the whole body capacity of B12 may be estimated.

The occupancy principle may be used to deduce either the daily dietary intake of B12 or the absorbed flow into plasma, depending on whether the tracer is given orally or intravenously. If the tracer is given orally then it is introduced into a flow which is coming entirely from the outside and which includes an unabsorbed component E_D as well as the absorbed flow F_A. On the other hand, intravenous administration of the tracer is equivalent to introducing it into the entry flow into plasma since there is rapid mixing and all points of introduction into plasma are equivalent. The flow that is measured is then the dietary absorption F_A only and the recirculation is not included. This point is made clearer by redrawing the relevant part of

the schematic diagram as in Figure 1 (b). The recirculation flow F_R is separated from the dietary absorption flow F_A which is the only flow entirely from outside this particular system. The recirculation is now seen as something totally within the system being considered. As such it will not be revealed by application of the occupancy principle to the system in Figure 1 (b) following intravenous administration of the tracer.

It was assumed that the injected tracer mixes rapidly with the stable B12 and is treated in the same way as B12 newly absorbed from the gut. It therefore follows that the area under a plasma activity/time curve for unit activity entering the plasma will be the same, whether it enters directly by injection or indirectly from the gut. Since the activity/time curves of all units of activity entering the plasma are additive the total area under a measured plasma activity/time curve would be proportional to the total activity entering plasma. In the case of intravenous injection of tracer 100% of the activity enters the plasma, whereas with oral administration only the absorbed fraction enters the plasma. The ratio between the plasma occupancies in the two cases is therefore equal to the fractional intestinal absorption of vitamin B12. The capacity of the plasma does not change and therefore it is possible to calculate the two different flows : the total dietary intake of B12 and the absorption flow of dietary B12.

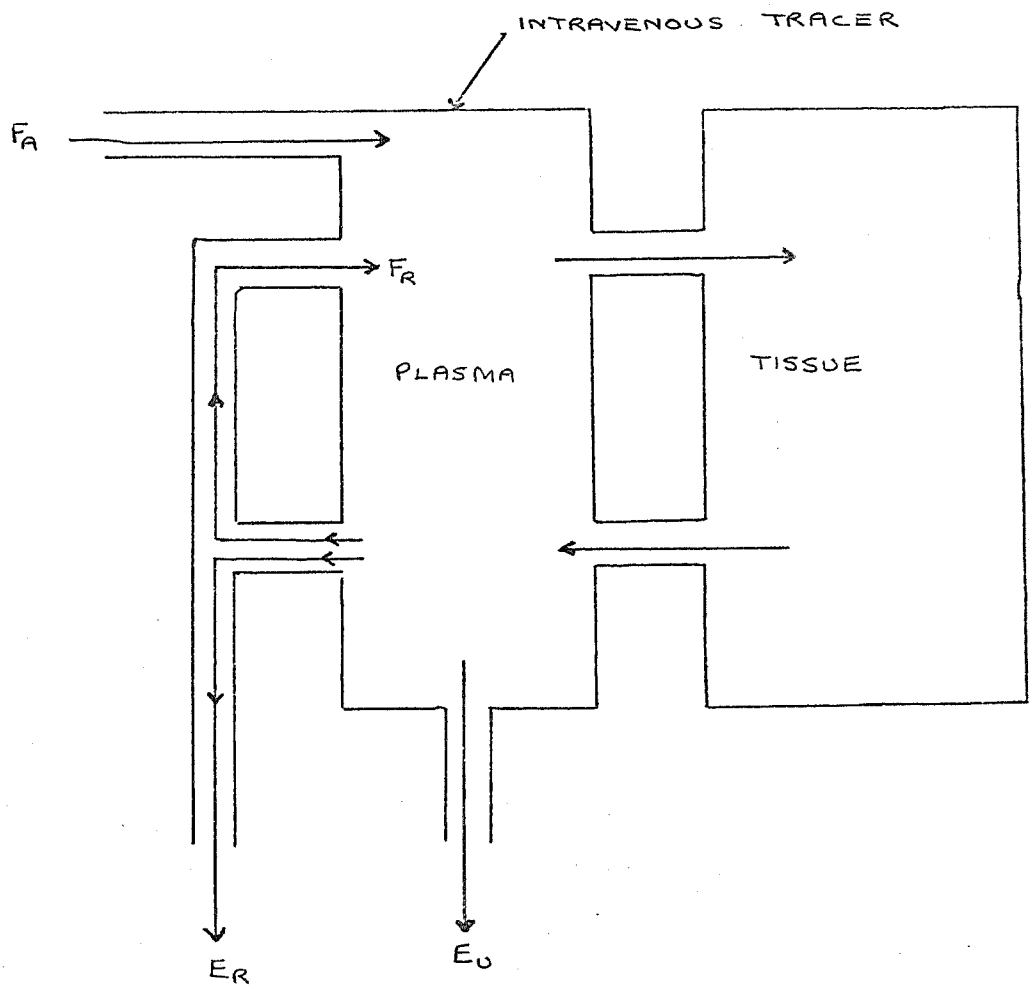


Figure 1b) : A schematic diagram of the essential features of vitamin B12 absorption.

The occupancy principle has a major advantage over compartmental analysis (58) as it does not assume that the tracer is uniformly distributed within pools. This is often not the case and the pools are not necessarily physically equivalent to any particular organs. In the case of the occupancy principle the measurements are made on defined parts of the system and therefore any calculatioms made refer unambiguously to those parts. The disadvantage of the occupancy principle which it shares with all forms of exponential analysis, is that measurements have to be made until the activity in the part of interest is effectively zero. In the case of vitamin B12 with its long biological half-life these measurements can be unduly protracted. However, after a long time has elapsed, and when the tracer has fully equilibrated with the stable material, the activity/time curve becomes a single exponential $Ae^{-\alpha t}$ with α being the fractional daily loss of stable material. The area to infinity under this tail is equal to $\frac{A}{\alpha}$ which is added to the directly computed area under the earlier part of the activity/time curve.

SECTION 2

THE APPLICATION OF THE OCCUPANCY PRINCIPLE TO THE PLASMA TRANSPORT OF VITAMIN B12

Introduction

It will be clear, from what has been said in the previous section, that the occupancy principle can be used to obtain a measurement of the plasma flow of vitamin B12. The term 'flow' is not one which is commonly found in the literature on vitamin B12 metabolism but it may be equated with the terms 'need' and 'requirement' which have been used to denote the mass of vitamin B12 normally absorbed from the diet by a healthy subject. Estimates of this need have been obtained before, but there is poor agreement between the results obtained by the different methods. The study which is reported in this section was undertaken in order to obtain estimates of the daily need from data analysed by the occupancy principle.

MATERIALS AND METHODS

Patient Details

Nineteen subjects took part in this study. They were given ^{57} Co B12 and their plasma activity was measured at various times thereafter. Nine of them had been treated for vitamin B12 deficiency for periods ranging from five months to seventeen years. The nine had all originally presented with symptoms of a macrocytic anaemia, megaloblastic erythropoiesis and a low serum vitamin B12 level and all but one (case 5 who had previously undergone total gastrectomy) h ad a histamine or pentagastrin fast achlorhydria. None of these patients could absorb orally administered radioactive cyanocobalamin normally but all did so when the dose was given with a source of intrinsic factor. For the sake of brevity this group of patients is subsequently referred to as the PA (pernicious anaemia) group. No patient in this group received vitamin B12 therapy in the month before the study, or, with one exception (case 3), during the study. A group of ten patients who presented with various conditions, and who made up a control group, were also studied. Details of both groups of subjects are given in tables 3 and 4.

The purpose and nature of the study was explained to all the patients and their verbal and written consent was obtained.

Case No	Age	Sex	Clinical State	Hb	B12	Dose and route of $^{57}Co\ B_{12}$.
1	76	M	PA. Treated 5 m	13.9	1825	0.09 µg 15.0 µCi IV.
2	68	M	" " 9 m	14.7	380	0.08 " 15.0 " "
3	67	M	" " 11 m	14.9	200	0.12 " 15.0 " "
4	69	M	" " 14 y	14.4	240	0.09 " 15.0 " "
5	65	F	" " 17 y	12.2	520	0.12 " 15.0 " "
6	57	F	PA. " 6 y	13.9	120	0.10 " 15.0 " oral (+IF)
7	66	M	" " 7 y	14.2	-	0.09 " 15.0 " " "
8	67	F	" " 9 y	12.6	1000	0.09 " 15.0 " " "
9	72	M	" " 12 y	14.5	250	0.09 " 15.0 " " "

Table3 : Showing details of 'Pernicious Anaemia' group of patients.

Case No	Age	Sex	Clinical State	Hb g/dl	B12 ng/l	folate ng/l	Dose and route of $^{57}Co\ B_{12}$.
10	80	F	CVA. Carcinoma colon	13.8	400	-	0.09 µg 15.0 µCi oral
11	65	F	Myocardial infarction	13.3	360	5.0	0.09 " 15.0 " "
12	88	F	Peripheral vascular disease Myelofibrosis	9.8	280	3.2	0.10 " 15.0 " "
13	81	F	Duodenal ulcer	14.7	750	3.5	0.10 " 15.0 " "
14	52	F	Secondary carcinomatosis	9.2	250	2.6	0.08 " 15.0 " IV
15	67	F	Pancreatitis	12.7	250	-	0.08 " 15.0 " "
16	58	F	Renal Calculi.Pyelonephritis	10.8	300	20.0	0.12 " 15.0 " "
17	72	F	Ulcerative colitis	11.5	260	-	0.10 " 15.0 " "
18	63	F	Myocardial infarction Treated folate deficiency	11.0	140	>20.0	0.12 " 15.0 " "
19	59	F	Chronic renal disease	9.9	400	6.0	0.11 " 15.0 " "

Table: 4 Showing details of the 'Control' group of patients.

Radioactive B12 preparations

The ^{57}Co B12 dose was 15 μCi ^{57}Co cyanocobalamin, containing 0.08 to 0.12 μg of vitamin B12. This dose was administered either orally or intravenously. The radioactive preparations were made up from standard solutions (specific activity approximately 150 $\mu Ci/\mu g$) obtained from The Radiochemical Centre, Amersham. The intravenous dose was sterilised by Millipore filtration and given in a volume of 5 ml . The oral dose was made up to 100 ml . with water.

Sample collection

20 ml samples of blood were obtained by venepuncture at intervals up to a year after the administration of the radioactive vitamin B12. The blood was collected into clean, but not sterile, glass tubes and was centrifuged within three hours of collection to separate the serum. 5 ml . aliquots of the serum were stored in plastic tubes at -20°C.

Serum vitamin B12 levels

Serum B12 levels were determined by a microbiological assay using Lactobacillus leichmannii performed by the staff of the Department of Haematology, Southern General Hospital, Glasgow. A standard of known vitamin B12 content was assayed with each batch.

Radioactivity measurement

Many of the samples contained very little radioactivity, so the following method was used to maximise the significance of the counting results. Each serum sample (volume 1 ml) was alternated with a background sample of local tap water (volume 1 ml). At the beginning of each batch of alternating serum samples and tap water samples a standard was counted. This standard contained either 0.02% or 0.05% of the dose administered to the patient and was obtained from the same batch of isotope. The radioactivity was measured in a refrigerated two channel gamma scintillation spectrometer (Packard model No. 3002) with a sodium iodide well-type crystal. Both channels were set to detect the ^{57}Co photopeak, using a narrow window in order to minimise the background activity. A counting period of 5 minutes was chosen and the batch was re-cycled until each sample had been counted about ten times. The results were printed on a teletype and punched on paper tape. In this way each sample and background was counted for about 50 minutes in total. Any errors due to changes in counter efficiency or background level could be eliminated by using the background values on either side of the sample.

The results were analysed from the paper tape by a Varian 620/L - 100 mini-computer. Statistical analysis of the approximately 150 5-minute background counts in each run showed that

on every occasion the actual standard deviation was very closely equal to the square root of the background count, and that individual values were normally distributed about the mean. No systematic variation in the background was detected and so the mean value was used for calculation throughout each run.

The appropriate 5-minute counts for each serum sample and for the standard were summed and the relevant total background was derived as the product of the mean background and the number of times each sample was counted. This total background value is subject to statistical variation with a standard deviation equal to the square root of the number of counts. Since the probability of the background differing from the mean by two standard deviations is 1 in 20, any total sample count which exceeded the total background by less than this amount was discarded as being totally indistinguishable from it.

These procedures were carried out for both the counting channels, as they had slightly different counting efficiencies. The two measurements of sample activity obtained however are not statistically independent since, on the whole, the same detector pulses are being counted by each channel. The only differences are in the slightly different settings and in the statistical fluctuations in the counting windows. To make sensible use of the slightly different results from the two channels the

average value of the two final results for each sample was obtained. This final value was expressed as a percentage of the administered dose per litre of serum.

Results

It was apparent from a plot of the serum results against time of log/linear graph paper (for examples see Figure 2) that the ^{57}Co activity from day 1 to the end of the study (about 300 days in the cases observed for the longest time) could in general be described by a triple exponential function.

$$A(t) = A_1 \exp(-\alpha_1 t) + A_2 \exp(-\alpha_2 t) + A_3 \exp(-\alpha_3 t)$$

The method of deriving the parameters varied slightly with the quality of the actual data but for most cases the results from about day 14 onwards were fitted to a double exponential curve. The value of this function was calculated and the result was subtracted from the observed value at each of the earlier times of measurement. These difference values were then fitted to a single exponential curve, describing the early phase of plasma activity

Both the single exponential and the double exponential fitting routines used an iterative method to obtain a least squares fit of the function to the actual data values rather than the less accurate method of fitting the exponents to the logarithms of the data values. The fitting routines began by deriving crude estimates of the required parameters by fitting the logarithms of the early and late values to single

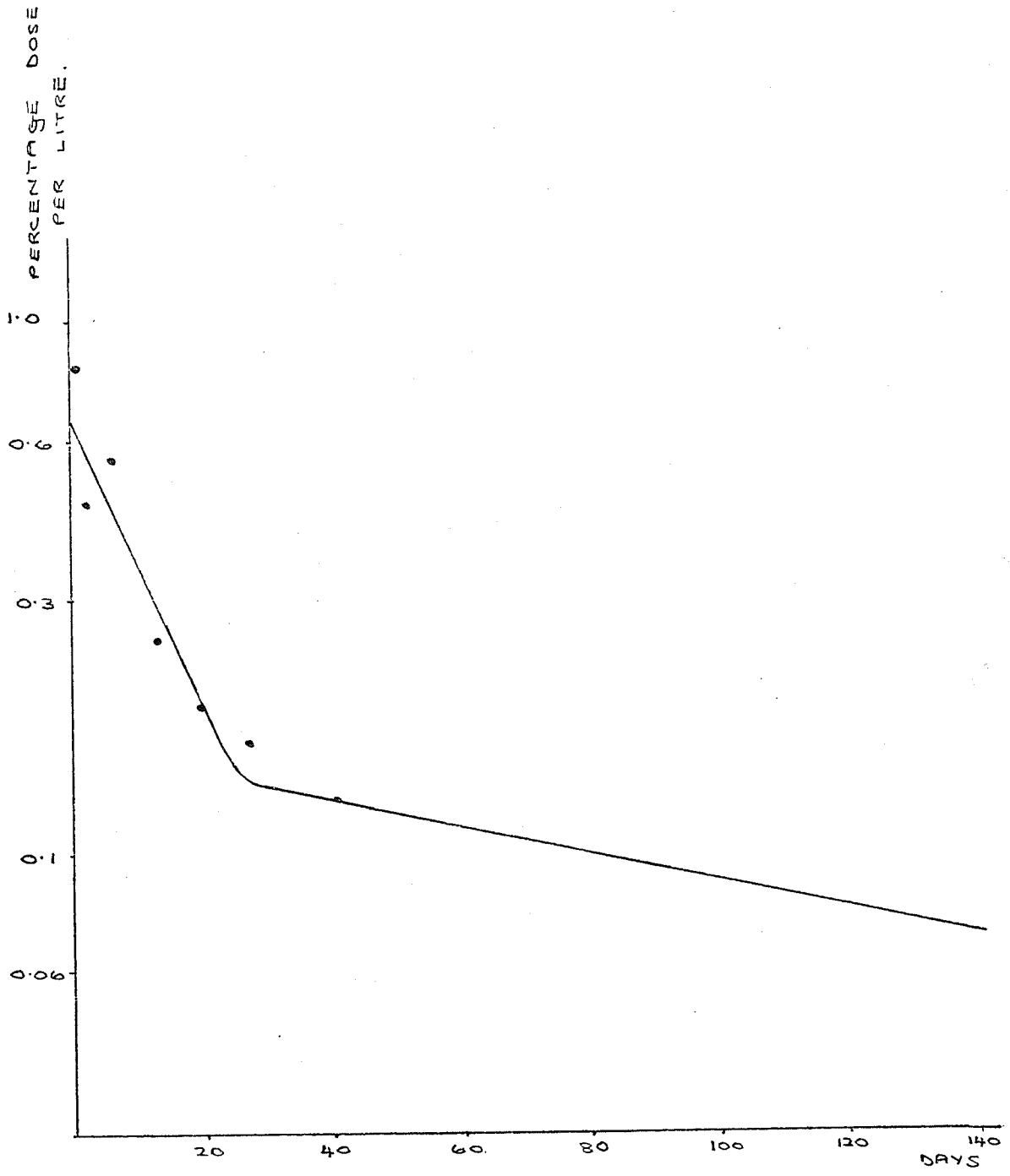


Figure 2a) : Total plasma activity/time curve

Example of the fitted function for case 13.

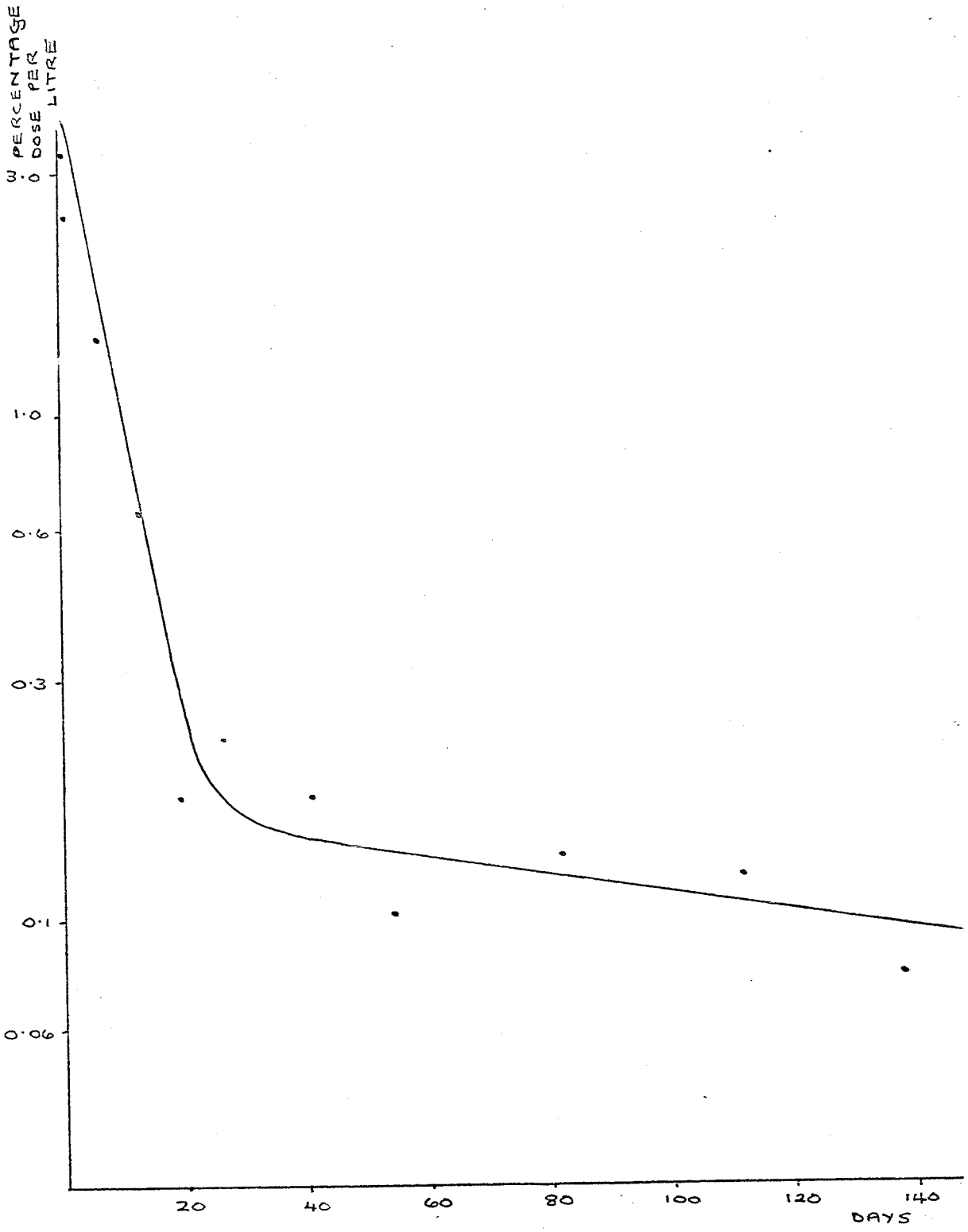


Figure 2b) : Total plasma activity/time curve.

Example of the fitted function for case 17.

exponentials. With these values for the parameters, the sum of the squares of the differences between each observed value and that calculated from the equation at each time was found. By taking derivatives the initial values of the parameters were all changed by a small amount in such a direction as to give the greatest reduction in this sum of squares ("path of steepest descent.") The sum of squares was recalculated for the new values of the parameters and the process was iterated until a further change of the parameters did not reduce the sum of squares significantly. The final values were printed out as the "least squares" values.

The values of the parameters describing the total plasma activity curves for 18 patients are given in table 5, together with the length of time over which the results used in their derivation were obtained. The area under each activity/time curve (the occupancy) may be calculated from the parameters of the triple (or in some cases double) exponential parameters, viz:

$$\text{Area} = A_1/\alpha_1 + A_2/\alpha_2 + A_3/\alpha_3$$

In stating this equality it is assumed that the third exponential component represents the final rate of decline of the plasma activity to negligible values. The values for the plasma occupancy for each patient (except for those with too short a study period) are given in table 5.

Case No	Study Time (days)	A 1 (%/1)	α_1 (d^{-1})	half-life (d)	A 2 (%/1)	α_2	half-life (d)	A 3 (%/1)	α_3	half-life (d)	Occupancy
1	1 - 28	1.3	.096	7.2							
2	1 - 7	1.02	.12	5.9							
3	1 - 325	1.26	.32	2.2	1.49	.061	11.4	.034	.0033	211	.389
4	1 - 238	.97	.41	1.7	.63	.090	7.7	.084	.0012	587	.800
5	2 - 321	.54	.24	2.9	.720	.063	11.20	.033	.0023	307	.278
6	2 - 325	.31	1.10	.63	.26	.091	7.6	.028	.0027	260	.138
7	3 - 168	10.01	.25	2.8	.76	.012	57				
8	2 - 213	7.6	.38	1.82	.38	.058	12	.068	.0019	361	.622
9	3 - 84	.26	.26	2.7	.17	.012	58				
10	3 - 84	.55	.22	3.2				.23	.0022	321	
12	1 - 84	.56	.21	3.3	.40	.028	25				
13	1 - 154	.65	.11	6.2				.16	.0058	120	.321
14	1 - 49	1.85	.13	5.1	.31	.018	38				
15	21 - 28	2.82	.41	1.7	1.12	.068	10.2				
16	1 - 262	.55	.33	2.1	.62	.069	9.9	.037	.0023	297	.263
17	1 - 140	3.69	.16	4.3				.17	.0044	157	.621
18	1 - 67	.81	.50	1.4	1.11	.072	9.7	losing weight			
19	1 - 18	2.5	.96	.72	1.20	.048	14.4				

Table 5: Showing results for in vivo plasma clearance studies

	A_1	half life	A_2	half life	A_3	half life	occupancy
all patients	2.07	3.10	.70	21	.094	291	.429
SEM	0.63	0.45	.11	5	.025	45	.080
all oral	2.85	2.95	.39	32	.12	266	.360
SEM	1.56	0.65	.10	11	.05	53	.141
all IV	1.57	3.20	.90	14	.072	312	.470
SEM	0.31	0.63	.14	4	.026	74	.104
oral control	0.59	4.23	.40	25	.20	221	.321
SEM	0.03	0.98	v		.035	101	
oral PA	4.55	1.99	.39	34	.048	311	.380
SEM	2.5	0.50	.13	14	.020	51	
IV control	2.04	2.55	.87	16	.10	227	.442
SEM	0.49	0.71	.17	5	.07	70	
IV PA	1.01	3.98	.94	10	.05	368	.489
SEM	0.14	1.09	.28	1	.017	113	

Table 6 : showing the average values of the parameters describing total plasma activity curves.
(Half-life is expressed in days and A as % per litre).

An estimate of the daily flow of vitamin B12 was derived from the data from the subjects who had a fairly constant serum vitamin B12 level. This estimate was obtained from the plasma occupancies which are related to flow by the equation shown earlier :

$$\text{Flow} = \frac{\text{capacity}_{\text{plasma}}}{\text{occupancy}_{\text{plasma}}}$$

DISCUSSION

1. The plasma turnover of vitamin B12

Previous descriptions of the disappearance of intravenously administered vitamin B12 suggested that there was a rapid initial phase followed by a slower rate of turnover (59). Kinetic analysis of experiments using tracer amounts of radioactive vitamin B12 (60) suggested that the biological half-life of B12 in plasma was about 6 days and this was confirmed as 5.14 days by Adams (61).

These results suggest that only the first two phases of the activity/time curve were observed. This is not surprising for it was only in those subjects who were studied for more than twenty weeks that a third exponential could be fitted to the data points. The half-lives observed in the first and second phases of the function were 0.63 - 7.2 days (mean = 3.1 days) and 7.6 to 58 days (mean = 20.9 days). Mollin et al (59) observed a delayed clearance of vitamin B12 from the plasma of subjects with untreated pernicious anaemia. This difference was not apparent in the present study because the patients in the PA group had all received treatment prior to the study.

2. The flow of vitamin B12

Estimates of the dietary intake of vitamin B12 gave a measure of its flow into the body but not into plasma, because only a proportion of the ingested B12 is absorbed. These estimates have been obtained from assays of meals and of items of diet and have ranged from 0.7 µg per day (the minimum value found by Heeley and Moyer (62)) to a maximum value of 31.6 µg per day reported by Chung et al (63). The actual value was considered to be dependent on the cost of the diet, protein content and nature of the proteins present. The values quoted for fatty foods such as eggs and milk may be underestimated because of the low recoveries of B12 from extraction prior to assay (56). An estimate of the average daily dietary absorption of vitamin B12 has also been obtained from kinetic analysis (58) and a value of 1.22 µg was suggested for people living on a Western diet.

Estimates of the flow of vitamin B12 have also been obtained from studies of the daily loss. The amount of B12 excreted could reasonably be taken to reflect the daily need, as this is the amount of B12 which must be replaced to maintain body stores. Three methods have been used to estimate the losses by excretion: kinetic analysis, whole body monitoring and a theoretical calculation based on the length of time required to deplete the

body stores after total gastrectomy (64). The loss of radioactive vitamin B12 has been monitored over lengthy periods of time by two groups of workers (65, 66). On the assumption of equilibration the loss was found to correspond to 0.1% to 0.2% of body stores per day. Translation of this percentage into an absolute value requires some knowledge of the total body store of vitamin B12 but assuming a value of 2.5 mg. then the daily loss is of the order of 2.5 to 5.0 µg per day. The estimated loss by kinetic analysis (58) was 1.2 µg per day. Grasbeck (64) assumed that the loss of B12 after total gastrectomy could be described by a single exponential function. He observed that the mean time for development of megaloblastic anaemia was four years after total gastrectomy and assumed that this was due to depletion of the body stores to about 10% of the normal. He calculated that this loss was at a rate of 0.15 to 0.3% per day which is equal to 6 - 12 µg per day assuming body stores of 4 mg.

The daily requirement for vitamin B12 has also been estimated by Sullivan and Herbert (67). They studied the amount of parenteral vitamin B12 required to induce a remission in patients with pernicious anaemia. A value of 0.1 µg per day was suggested but objection has been made to this figure on the grounds that if the daily need is related to the body stores of B12, as suggested by Adams and Boddy (65), then the requirement for B12 will be lower in a patient with pernicious anaemia in

relapse than in a normal subject.

Even making allowances for the fact that only a proportion of the amount of vitamin B12 which is ingested is absorbed, there are obvious discrepancies between the estimates for dietary intake and for the daily loss (or amount required for balance.) The results obtained from the present study, although limited to seven of the subjects, range from 0.3 µg per day to 2.7 µg per day (see table 7). The mean value of the daily dietary flow (1.43 µg) as one would expect, is higher than the mean value for the absorption flow (0.84 µg per day). The ratio of these two figures suggests that on average approximately 59% of the daily intake of B12 is absorbed. This value accords well with what is known of the fractional absorption of cyanocobalamin at a dose of 1 µg (quoted by Chanarin (68) at 56%).

Case Number	Route	Serum Occupancy (θ)	Serum Capacity (C)	Flow (μg/day)
3	IV	.389	186	0.5
4	IV	.800	216	0.3
5	IV	.278	468	1.7
6	oral	.138	149	1.1
8	oral	.622	1700	2.7
13	oral	.321	175	0.5
16	IV	.263	347	1.3
17	IV	.621	256	0.4

Table 7 : The estimated flows of vitamin B12.

SECTION 3

THE APPLICATION OF THE OCCUPANCY PRINCIPLE TO STUDIES ON THE TRANSPORT OF VITAMIN B12 BY ITS SERUM BINDING PROTEINS

Introduction

Although there is agreement that the transcobalamins are specific carrier proteins for vitamin B12 there is some doubt about the precise role of each transcobalamin in the metabolism of vitamin B12. In an attempt to resolve this point the studies described in the previous chapter were extended to yield information about the distribution of tracer B12 on the transcobalamins.

Hall and Finkler (7) have produced evidence that vitamin B12 absorbed from the small bowel is transported selectively on TC11 and that the biological half-life of the TC11 - B12 complex is relatively short. This theory is supported by studies by Hom (33) in which he injected a TC11 - ^{57}Co B12 complex and noted that all the radioactivity had been transferred to TC1 by 24 hours. Chanarin et al (16) and England et al (20) on the other hand believe that the vitamin B12 absorbed from the gut is bound by the transcobalamins in proportions depending

on their unsaturated B12 binding capacities at the time of absorption, and that TC11 does not have a specific function in this transport.

Serum samples from the subjects described in the previous section were fractionated on Sephadex G - 200 in order to study the distribution of ^{57}Co B12 on the transcobalamins. The percentage of the original activity bound by each fraction of the serum was plotted against the time after administration of the dose. The biological half-life of the TC1 + 111 - ^{57}Co B12 and of the TC11 - ^{57}Co B12 complexes could be obtained from the respective activity/time curves. It was hoped that from an analysis of the data by the occupancy principle an estimate of the distribution of stable vitamin B12 could be obtained. The occupancies for TC1 + 111 bound B12 and the TC11 - B12 fraction were obtained by integrating the respective activity/time curves and, from the ratio of the occupancies, the ratio of the capacities was calculated (since the flow is constant.) If it is assumed that all the vitamin B12 present in the serum is bound to either TC1 + 111 or TC11 then, since the total serum B12 is known, the absolute amounts of stable B12 on each protein can be ascertained.

MATERIALS & METHODS

Subject details.

As described in section 2.

Sample preparation

The serum samples were allowed to thaw at room temperature prior to fractionation. Visible insoluble material was observed in some of the samples and these were filtered in order to prevent the column membrane from becoming clogged. This filtration was through a disc cut from cellulose acetate electrophoresis paper supported on a sintered glass base (Millipore Corporation) attached to a water vacuum pump. The samples from one subject (case 11) had visible lipaemia and would not filter through the cellulose acetate disc. In order to remove the fat the samples were centrifuged at 2,000 x g for twenty minutes and the serum was removed from below the surface layer of fat.

Sephadex G - 200 chromatography

12g of Sephadex G - 200 (Pharmacia Fine Chemicals AB) was allowed to swell for three days in approximately 500 ml. of tris-HCl buffer pH 8.0 containing 1M NaCl being decanted once in order to remove the 'fines'. Most of the buffer was poured off, leaving a thick slurry of gel which was deaerated with a water vacuum pump at a pressure of approximately 2.7 kPa. The degassed slurry was poured into a chromatographic column

(Pharmacia K26/70) to give a gel column with a bed volume (V_t) of approximately 300 ml and a bed height of about 560 mm.

4 ml serum samples were separated on the column by upward elution with degassed tris-HCl buffer at a flow rate of 12 ml/hour maintained by a peristaltic pump (LKB 12000 Varioperpex pump). In an attempt to prevent bacterial growth in the column the buffer was sterilised by boiling before use, and 0.02% (W/V) of sodium azide was added to it. Samples were collected as a continuous process, in an Ultrorac fraction collector (LKB), each sample representing 30 minutes' elution from the column. The optical density was continuously monitored at 280 nm using a Uvicord II (LKB) linked to a chopper bar recorder (LKB). Although the radioactivity was recovered in the first 300 ml of buffer collected (the bed volume) a further 300 ml of buffer were eluted before another sample was added to the column.

The serum samples from one patient were all run on the same column whenever this was possible. The column was calibrated with an in vitro labelled sample of serum from the same patient before the in vivo samples were separated. The samples from each subject, were fractionated in order of increasing radioactivity (i.e. the late samples were separated first) in an attempt to minimise any effects of radioactive contamination accumulating on the column.

Radioactivity measurement

The 6 ml fractions eluted from the column were counted in the automatic gamma scintillation spectrometer described in section 2, for a convenient length of time (usually 20 minutes per sample) and a graph of the number of counts against the fraction number was made. From this graph (for examples see figures 3 - 7) the dip between the two radioactivity peaks was identified and the two lowest values were allocated, one to each peak. The fractions were pooled in threes, outwardly from this point, until all the tubes containing radioactivity had been incorporated. Occasionally it was impossible to decide where the true minimum values lay. When this happened the peak values were compared with those on previous separations from that subject (including the in vitro run) and the position of the minima were obtained from a sample whose peak corresponded with that from the problem sample. The results were calculated from these pooled fractions (volume 18 mls) which were counted for 40 to 100 minutes depending on the activity present in the samples. The samples from the first patients studied were counted for one period but later samples were measured as multiples of twenty minute counting times, in order to minimise any changes in the background. Only statistical variations in the background were observed however, as described in the studies reported in the previous section.

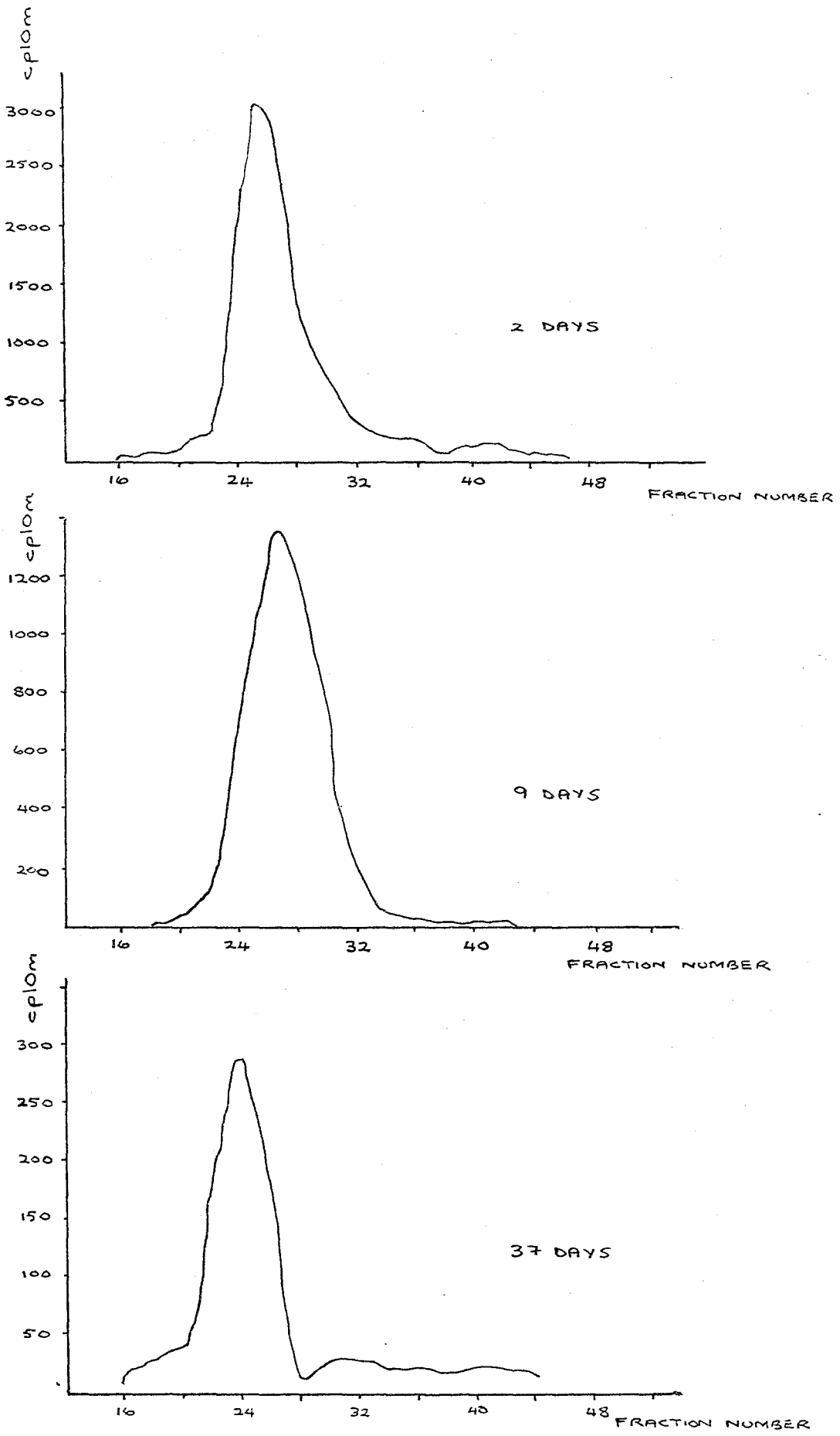


Figure 3 : Sephadex G - 200 gel filtration of samples from case 3.

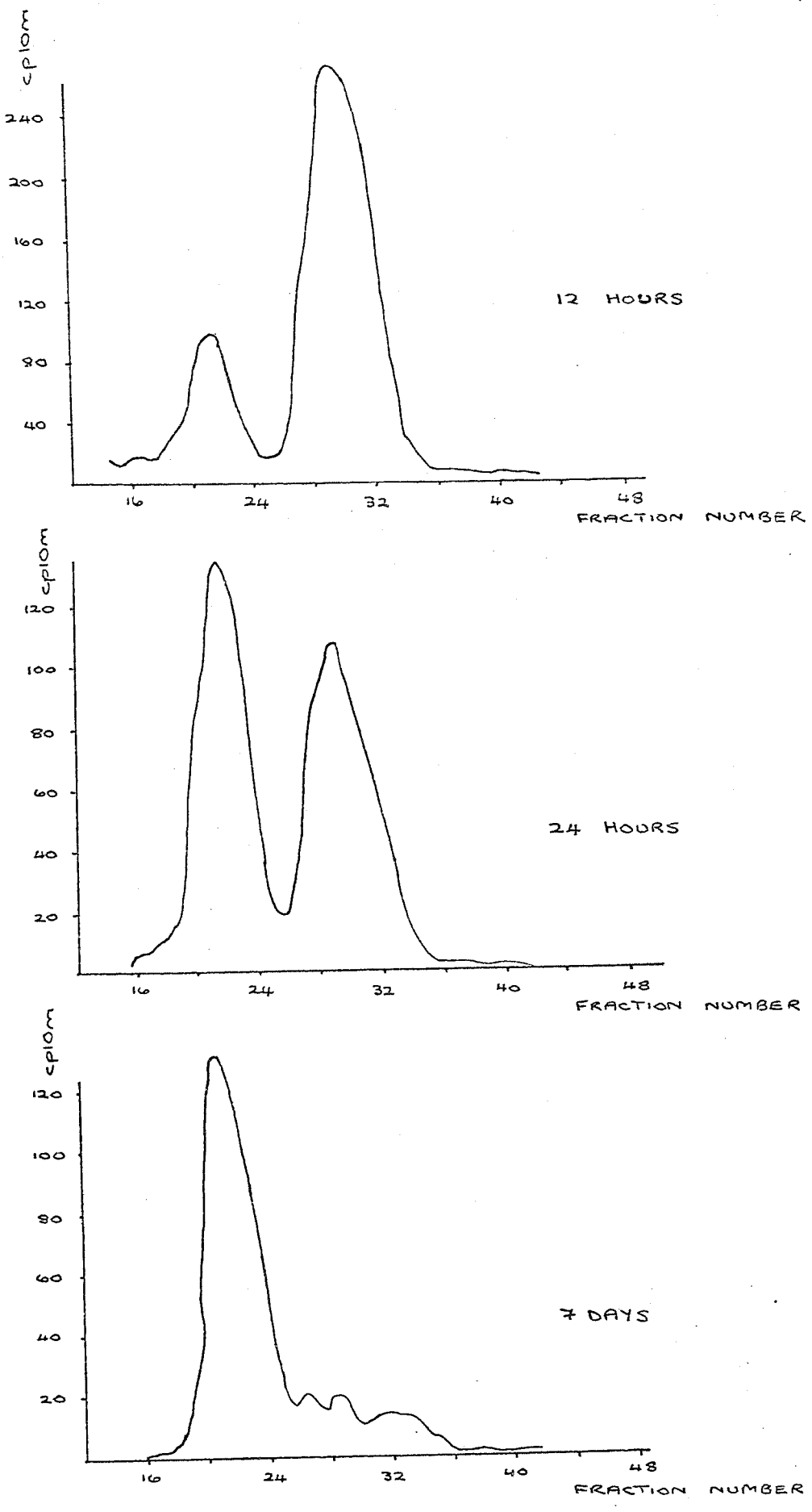


Figure 4 : Sephadex G - 200 gel filtration of samples from case 13.

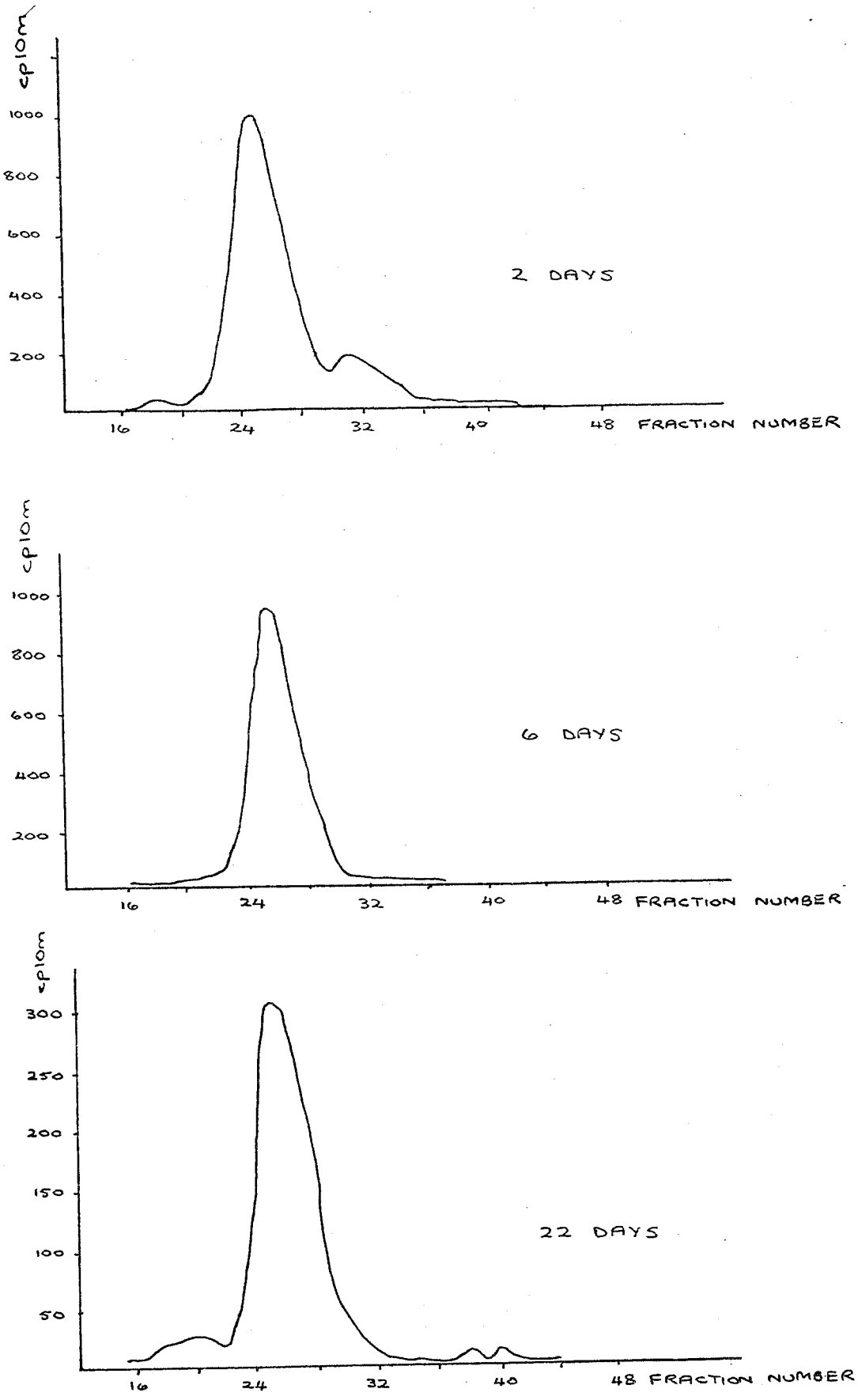


Figure 5 : Sephadex G - 200 gel filtration of samples from case 16

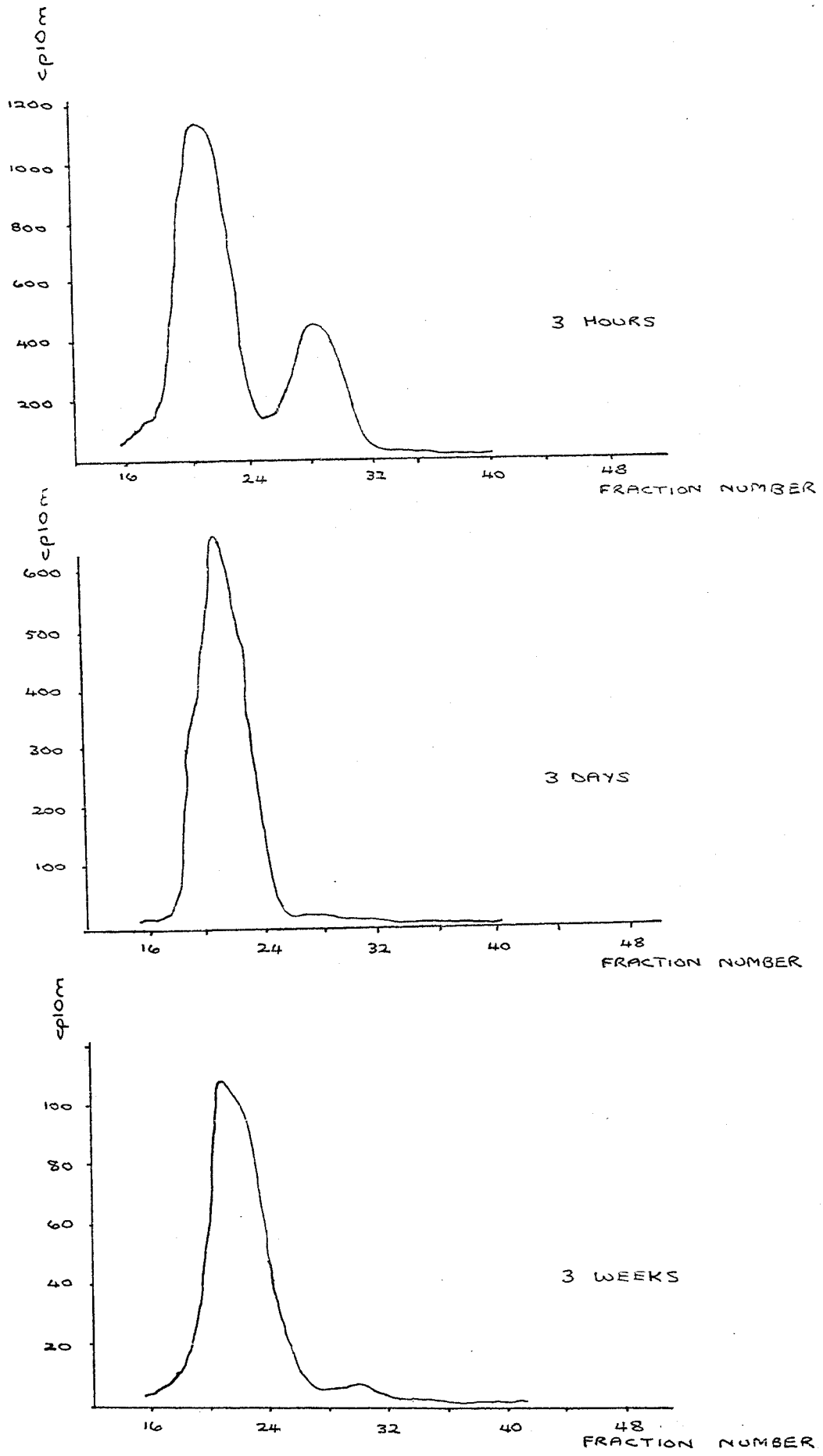


Figure 6 : Sephadex G - 200 gel filtration of samples from case 17.

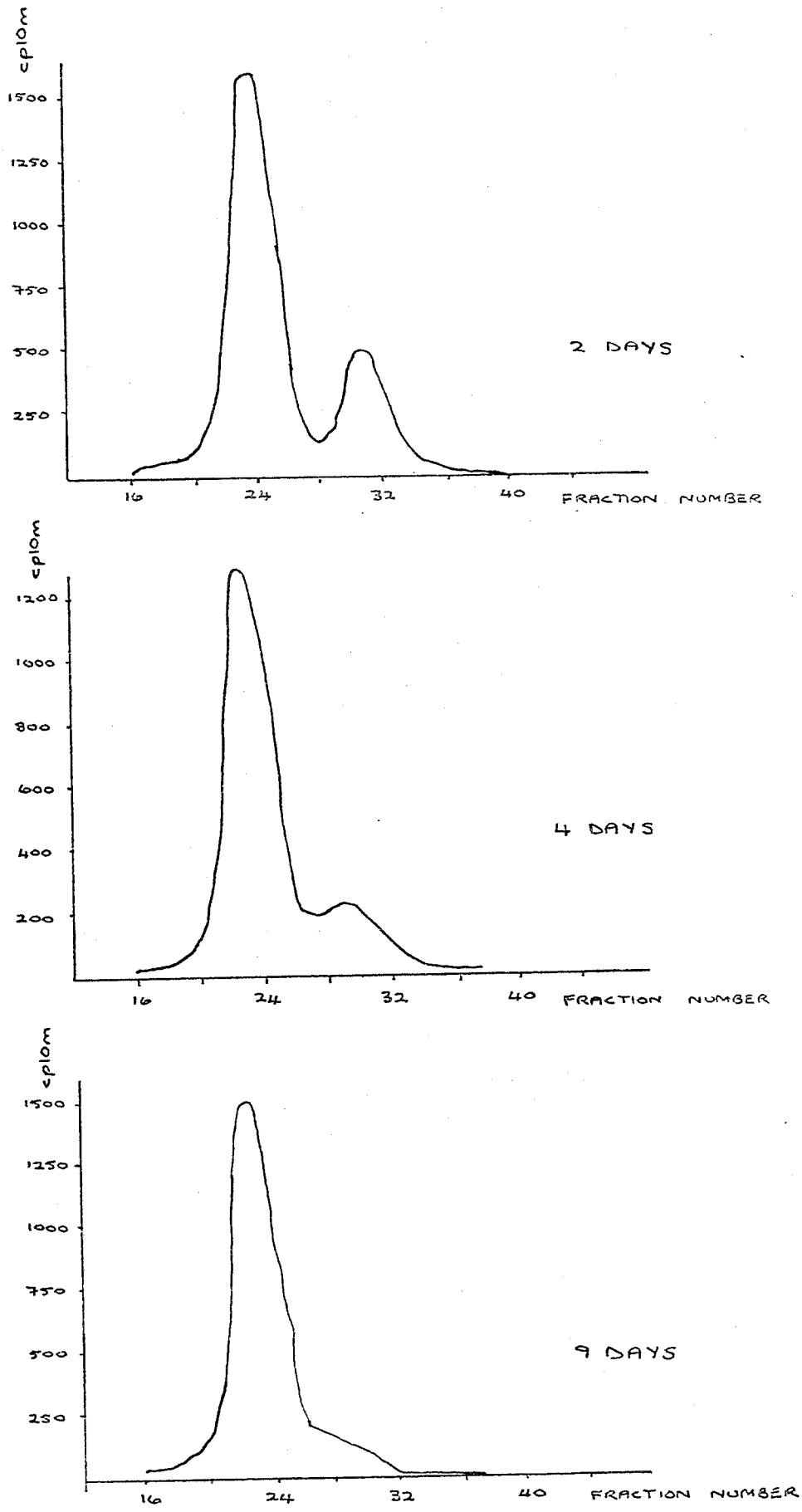


Figure 7 : Sephadex G - 200 gel filtration of samples from case 19.

A standard of either 0.02% or 0.05% of the dose which had been administered to the subject was counted at the same time and under the same conditions as the pooled fractions. An accurate estimate of the background radiation was obtained from frequent measurements during each batch of samples and this was subtracted from the number of counts obtained from the sample. The remaining activity, due to the sample, was expressed as a percentage of the administered dose per litre of serum. Only those values which were greater than 0.01% of the original dose/litre of serum were included in the results. After the samples from some subjects had been studied the protocol was altered slightly to include the measurement of the radioactivity present in the 4 ml serum sample prior to fractionation. The inclusion of this step meant that the percentage recovery from the column could be estimated. Any results from samples which indicated a recovery of over 120% were discarded in case this was due to contamination from the column. Relatively few samples were discarded for this reason, normally only the first one or two samples fractionated after the in vitro separation. The recovery for the remaining samples was in the range 80 - 120%.

Experiments on rates of recovery with samples labelled in vitro (and thus with more radioactivity present) have shown that the normal recoveries from a column are 90 - 100%. It was observed that in the vivo samples with a recovery of over 120% the increased radioactivity was predominantly associated with the TCll fraction, and not spread equally between the binders.

Results and Discussion

1. The distribution of eluted radioactive B12

Table 8 shows the percentage of the eluted radioactivity which was bound to TC11 at a given time after administration of the tracer. There is a marked variation in the results obtained from individual subjects, for example, the percentage of activity bound to TC11 at 3 days post-injection varies from 4% (case 17) to 40.4% (case 1).

The data presented in table 8 could be used to support either of two contradictory theories, depending on which subjects are selected. The results from cases 3 and 18 could be used to back up the view of Hall and Finkler (7) and Hom (33) for at 24 hours after injection the radioactivity bound to TC11 was only 2.5% and 8.6% respectively. If on the other hand the results from cases 1 and 10 are presented, there is good support for the theory that TC11 might have a role in late transport. This theory was originally proposed by Benson et al (69) to explain the observation of TC11 bound B12 in subjects who had been deprived of recent dietary B12 and in patients who had undergone total gastrectomy.

It must be emphasised at this point, that with the exception of case 7 who is discussed later, serum samples from all of the patients studied in this section bound in vitro added ^{57}Co B12 to both TC1 + 111 and to TC11. The binding patterns

	Case Number					
Time after injection	1	2	4	14	15	17
½ hour	79.3	79.7	77.9	72.0	62.6	34.2
1 hour	72.8	71.2	72.2	64.1	53.5	30.4
3 hour	64.7	44.9	62.8	49.1	38.8	21.3
5 hour	58.7	38.9	55.0	38.9	31.5	20.9
7 hour		36.1			24.8	
8 hour	57.1		50.0			
12 hour				26.1		
1 day	46.8	21.0	36.7	18.7	15.1	9.2
2 day				10.7		
3 day	40.4	16.4	18.4	9.7	8.3	4.0
6 day				10.6		
1 week	42.4	14.3	23.9		4.0	2.1
2 week					2.1	0
3 week						0
4 week		10.6			5.2	

Table 8a) : showing the percentage of eluted activity bound by TCII.

	Case Number				
Time after dose	9	10	11	12	13
4 hour	98.5	92.5	96.2	65.0	87.7
6 hour	91.5	89.1	93.5		
8 hour	91.2		89.7	86.2	89.2
10 hour		90.2	89.9		
12 hour			84.8	67.2	79.3
24 hour	69.7	67.5		35.0	46.0
3 day	32.9	49.6		11.1	25.1
5 day		28.9			
1 week	19.3	29.0	18.2	10.2	20.3
2 week		24.1		14.8	
3 week				11.8	

Table 8b) : showing the percentage of eluted activity bound by TCII.

	Case Number						
Time after dose	3	5	6	8	16	18	19
1 day	2.5		67.9	41.8	50.9	8.6	
2 day	2.3		39.3	39.3	14.0	6.0	26.1
3 day	3.0		25.1	46.7	9.5	5.3	16.4
4 day	5.7		17.1		6.4	4.2	14.2
5 day					1.8		16.8
6 day				39.7	3.7		
7 day			10.7		3.2	3.7	11.3
8 day				41.0			12.4
9 day	1.8					3.8	7.1
10 day							13.7
11 day		2.0					
12 day	2.7						
14 day							11.4
15 day			9.0				
16 day	3.4	5.4					
22 day			7.6				
23 day		2.5					
29 day		6.7	7.8				
37 day	0.4						

Table 8c) : showing the percentage of eluted activity bound to TCII.

observed for cases 3 and 18 were not due to the absence of TC11.

Support for the theory that TC11 has a role in the transport of B12 apart from that described as 'post-absorption' has come from studies by Hall (70). He observed TC11 bound B12 in the plasma of a terminal cancer patient 51 days after an oral dose of 1.12 µg/229 µCi of ^{57}Co B12. At 4 hours 95% of the eluted radioactivity was bound to TC11, falling to 22% at day 7. The fraction bound by TC11 remained more or less constant until week 4 and then it increased slightly. This late phase of TC11 transport was detected in only a few of the subjects studied in this series possibly because the radioactivity present in the serum was very difficult to measure accurately at this late stage. The studies by England et al (20) also detected TC11 bound B12 at relatively late times : at 8 - 10 days after an oral dose 18% of the recovered radioactivity was bound to TC11 in the serum from one subject and 30% in the serum from the other.

2. The half-life of TCI + III - B12 and of TCII - B12

The percentage of the original dose per litre of serum bound by the relevant fraction of the serum was plotted against time on log/linear graph paper and a double or triple exponential function was fitted to the data by a least squares iterative procedure as described in section 2. Examples of the fitted functions are shown in figures 8 and 9. Results were obtained from only eighteen of the nineteen subjects because the serum from case number 7 could not be completely fractionated on Sephadex G - 200. A fresh serum sample was obtained from the subject and ^{57}Co B12 was added in vitro but it was still impossible to separate the radioactivity into two components. Figure 10 shows the distribution of radioactivity in a fractionated sample from case 7 compared with the type of separation normally observed.

The results from the fractionated serum samples for the eighteen patients are shown in table 9.

Oral administration : The results for TCll bound B12 from most subjects were best fitted by a double exponential function. The first component of the function had a rapid turnover ($t_{½}$ = 0.23 - 0.67 days) and this was followed by a second phase ($t_{½}$ = 2.6 - 3 days in all except one subject). In two of the subjects (cases 6 and 12) there was a final section with a very long half-life (6.3 and 27.4 days respectively). A measure of the TCl and lll bound activity could be obtained only for

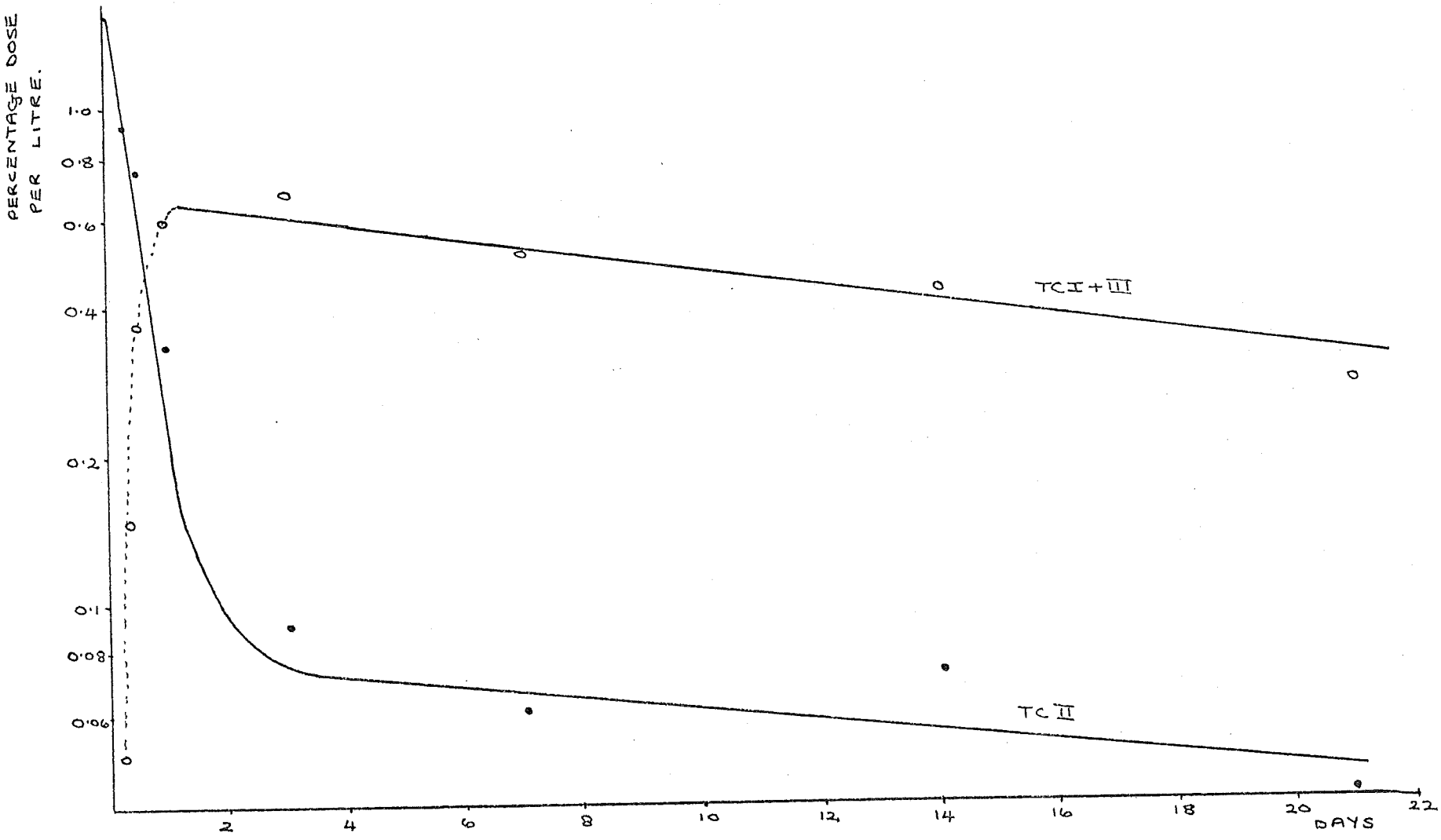


Figure 8: Fitted activity/time curves from case 12.

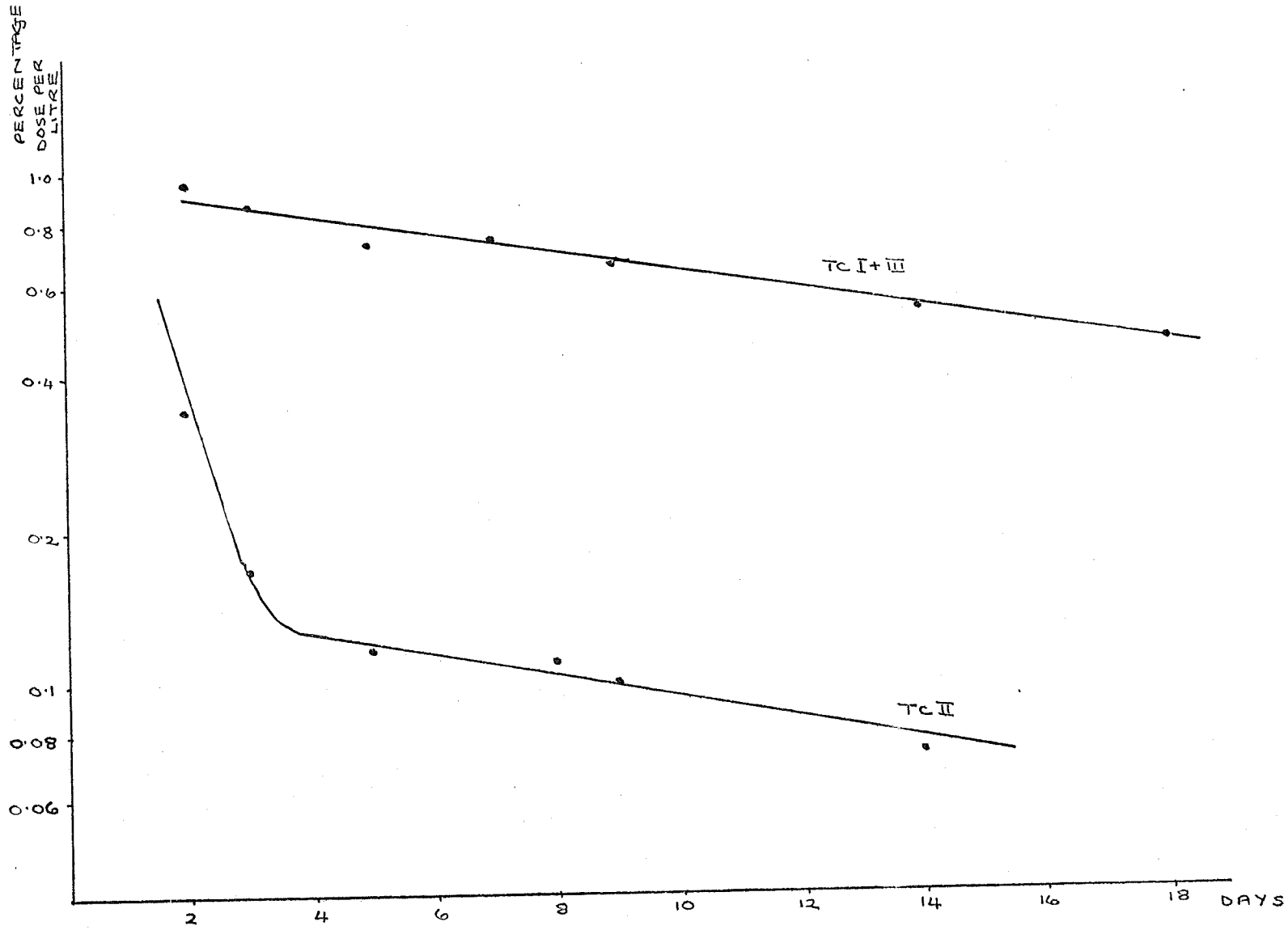
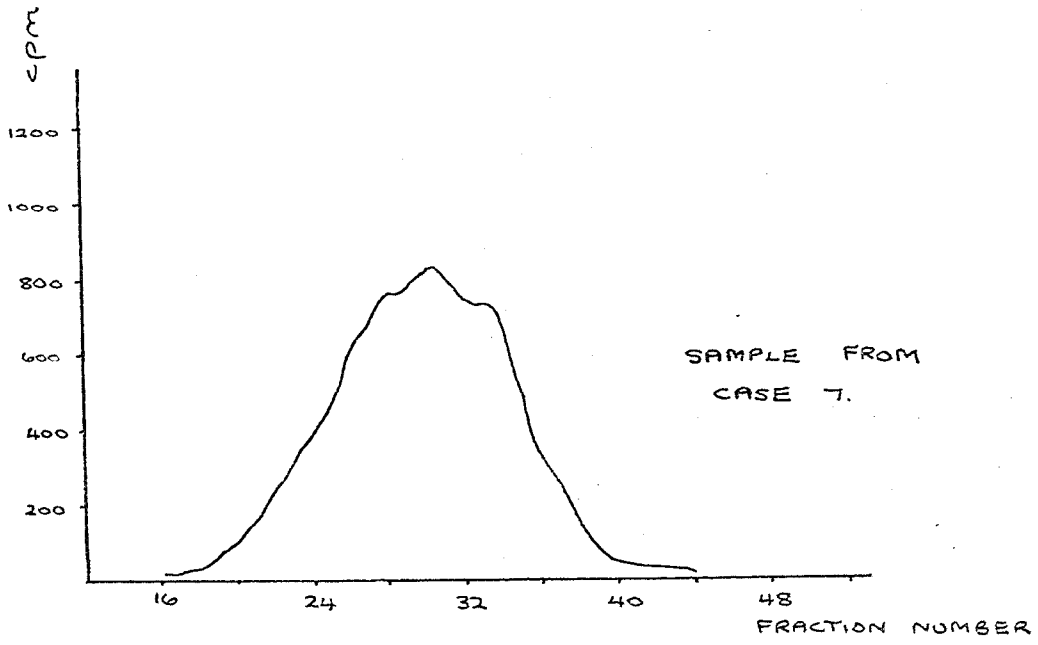


Figure 9 : Fitted activity/time curves from case 19.



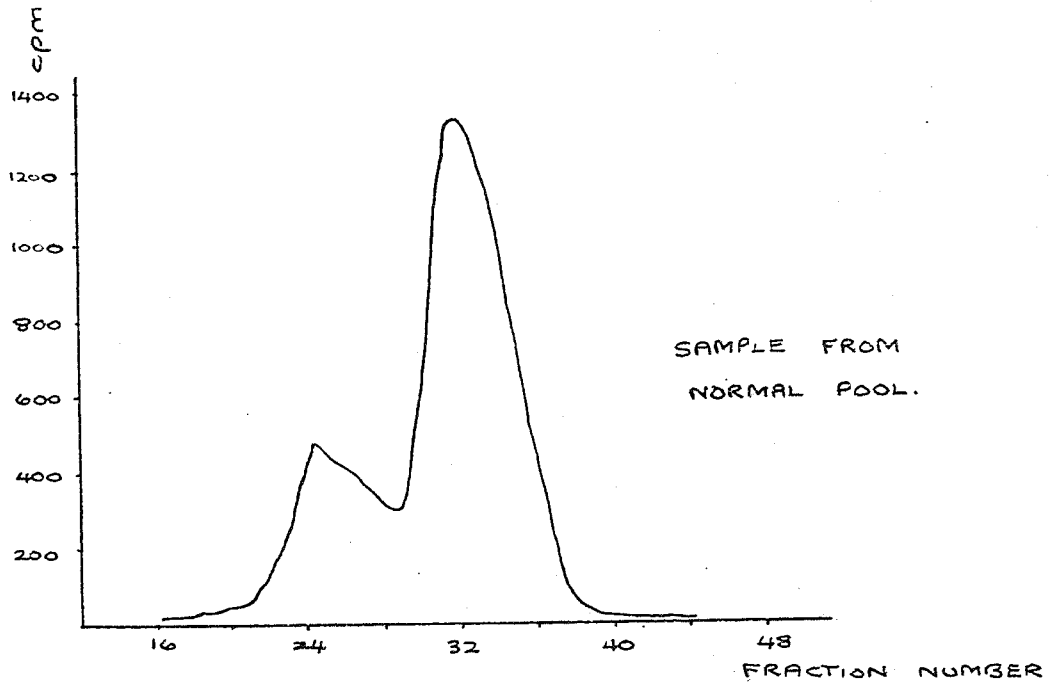


Figure 10 : A comparison of the G - 200 separation of a serum sample from case 7 and of one from a normal pool.

Case No	TC11 bound activity: length of study	A_0 (%)	half life 0 (d)	A_1	half life 1 (d)	A_2	half life 2 (d)	A_3	half life 3 (d)	TC1 & 111 bound activity: length of study	A_1	half life 1 (d)	A_2	half life 2 (d)
1	7	5.4	0.024	2.09	.67	-	-	-	-	7	0.70	.40	.65	16.7
2	28	5.2	.025	1.27	.46	-	-	-	-	28	.75	.24	1.1	5.9
3	51	-	-	-	-	-	-	0.06	8.2	58	1.27	2.4	1.43	13.1
4	7	3.5	.038	1.45	.71	-	-	-	-	14	0.58	2.0	0.51	22.9
5	-	-	-	-	-	-	-	-	-	95	-	-	0.86	7
6	29	-	-	1.0	.58	-	-	0.04	6.3	116	-	-	0.19	8.8
8	73	-	-	4.0	.67	1.98	2.9	-	-	73	-	-	0.62	12.5
9	7	-	-	1.5	.46	0.18	3.0	-	-	14	-	-	0.26	8.5
10	21	-	-	4.1	.24	.60	2.6	-	-	21	-	-	0.35	13.6
11	12	-	-	3.5	.24	0.93	2.8	-	-	-	-	-	-	-
12	21	-	-	1.6	.40	-	-	0.08	27.4	28	-	-	0.68	19.5
13	7	-	-	4.6	.23	0.17	1.5	-	-	-	-	-	-	-
14	35	10.3	.025	1.85	.46	-	-	-	-	35	1.4	0.04	2.03	7.3
15	28	8.4	.021	2.3	.42	-	-	-	-	28	1.0	0.96	2.7	7.8
16	7	-	-	4.2	.30	0.19	1.9	-	-	53	-	-	0.92	7.0
17	28	4.1	0.018	1.27	0.5	-	-	-	-	28	3.0	0.05	3.4	5.2
18	39	-	-	0.21	.96	-	-	0.04	17.7	39	-	-	1.42	18
19	18	-	-	5.34	.42	-	-	0.15	14.6	18	-	-	0.97	17.1

Table 9 : Showing fractionated results of in vivo plasma clearance studies.

5 of the 7 subjects, and in each case a single exponential function was fitted to the data points. The half-lives ranged from 8.5 days to 19.5 days and were slightly shorter in the PA group (mean value 9.9 days) than in the controls (mean value 16.5 days).

Intravenous administration : Eleven of the eighteen subjects came into this category. The TCll - B12 was initially cleared very rapidly from the plasma ($t_{1/2}$ = 37 minutes), a phase which was not observed in the oral group due to the slower rate of entry of the tracer into the plasma. In the oral patients the maximum plasma activity was reached at 10 - 12 hours after intake and so the gradual accumulation of ^{57}Co B12 obscured the phase of rapid clearance. This rapid phase was followed by a second phase, corresponding to the clearance observed in the oral group ($t_{1/2}$ = 0.3 - 0.96 days). In a few of the subjects another exponential could be fitted to the data points to give the final part of the function. The TCl + lll - B12 activity/time curve also showed an initial rapid phase similar to that described for TCll - B12. This was followed by a second section with a long half-life ($t_{1/2}$ = 5.2 to 22.9 days). There did not appear to be any significant difference between the results observed in the PA group and in the control group.

Table 10 gives the mean values for the half-lives of the different groups with the results expressed as $\pm$ S.E.M.

The first estimate of the half-life of TCll - B12 came from Hall and Finkler (7) in a study of the clearance of an

	TC11				TC1		
	t_o (d)	t_1 (d)	t_2 (d)	t_3 (d)	t_o (d)	t_1 (d)	t_2 (d)
All	.025	.48	2.4	14.8	0.18	1.8	11.9
Patient	± .007	± .2	± 0.6	± 8.4	± 0.17	± 0.7	± 5.5
All	-	.4	2.5	16.8			12.6
Oral		± .18	± 0.6	±14.9			± 4.5
All	.025	.54	-	13.5			11.7
I.V.	± .007	± .2		± 4.8			± 6.1
Oral	-	.28	2.3	-			16.5
Normal		± .08	± 0.7				± 4.2
Oral	-	.57	2.9	-			9.9
P.A.		± .10	± 0.3				± 2.2
I.V.	.022	.51	-	-			10.4
Normal	±.0038	± .23					± 5.6
I.V.	.029	.61	-	-			13.1
P.A.	±.0079	± .13	-	-			± 7.0

Table 10: showing average half-lives for clearance of B_{12} from the transcobalamins.

injected TC11 - ^{57}Co B12 complex. The TC11 - B12 complex disappeared completely after 6 - 24 hours and had a half-life in the region of 1 hour. Further studies however showed that the initial clearance of TC11 - B12 was even more rapid, for half of an injected dose of TC11 - B12 left the plasma in under 5 minutes (71). In a similar study Hom (33) injected TC11 - ^{57}Co B12 and found its half-life to be 1½ hours, and observed a half-life of 9.3 days for injected TC1 - B12. Results obtained by England et al (20) gave much higher values : the half-life of TC11 - B12 was measured in three subjects and found to be 8 hours in one, 27 hours in another and 28 hours in the third. England et al attributed the short half-life observed by previous workers to the partial damage of the TC11 - ^{57}Co B12 complex prior to reinjection. It must be pointed out however that the previous studies with intravenous administration were making observations on the initial rapid phase. England et al gave the ^{57}Co B12 orally and therefore could not expect to detect the phase of rapid turnover. The apparent loss of all the ^{57}Co B12 from TC11 by about 24 hours (7) was probably due only to a failure to detect the low levels of activity present.

The results reported by Hom (33) for the half-life of the TC1 - ^{57}Co B12 complex correspond very closely with the values observed in the current studies. Although Hom described the complex as TC1 - B12 it was fractionated on Sephadex G - 200 and

is therefore directly comparable with the TC1 + TC111 binder discussed here. England et al (20) on the other hand studied the clearance of TC1 - B12 and TC111 - B12 separately and obtains values of 1.9 - 2.2 days and 2.5 - 3 days respectively. Although no direct comparison can be made it is evident that it was the middle part of the function which was observed. This had a mean half-life value of 1.8 days in the present study.

3. The distribution of endogenous B12 on the serum binding proteins

The activity/time curves were plotted for the TC1 + 111 bound B12 and for the TC11 bound B12 and the respective functions were integrated to obtain the plasma occupancies from the data from each patient. Since the total flow of vitamin B12 into the system is constant the ratio of the capacity to occupancy of any part is equal to the same ratio in any other part:

$$F = \frac{C_1}{\theta_1} = \frac{C_2}{\theta_2} = \frac{C_{1+2}}{\theta_{1+2}}$$

If it is assumed that the B12 present in serum is all bound either to TC1 + 111 or to TC11 then, since both the total capacity (serum B12 level) and the individual occupancies for both fractions are known, the absolute amount of B12 present on either binder can be calculated:

$$C_1 = \frac{\theta_1}{\theta_{1+2}} \times C_{1+2}$$

The absolute amount of B12 bound to TC1 + 111 was obtained and this value was also expressed as a percentage of the total serum B12. The results (table 11) show the wide range of values

Case Number	$\theta_{TCl + 111}$	θ_{TCll}	Serum B12 (ng/l)	$C_{TCl + 111}$ (ng/l)	% Bound to TCl + 111
1	.161	.022	-	-	-
2	.096	.010	322	291	90.4%
3	.314	.007	186	182	97.8%
4	.185	.017	216	198	91.7%
5	.087	-	463	-	-
6	.024	.012	149	99	66.4%
8	.112	.122	1700	815	47.9%
9	.032	.018	182	117	64.3%
10	.069	.037	448	292	65.2%
11	-	.050	417	-	-
12	.191	.041	254	209	82.3 %
13	-	.019	175	-	-
14	.218	.016	231	215	93.1%
15	.318	.017	287	273	95.1%
16	.093	.023	347	277	79.8%
17	.257	.010	256	246	96.1%
18	.369	.013	124	120	96.8%
19	.239	.064	650	513	78.9%

Table 11 : An estimate of the amount of native B12 bound to TCl + 111.

obtained, indicating the variation between individual subjects. The percoentage of the endogenous B12 which was bound to TCll varied from 2.2% (case 3) to 52.1% (case 8) with a mean value of 18.2%. A high serum B12 level was noted in case 8 and it is possible that an overload on TCl + lll caused an overspill on to TCll. It has been previously estimated that TCl has the capacity to bind only 700 - 800 ng B12/litre of serum (27) and this is consistent with the amount of B12 which is bound in case 8 (815 ng/l).

The distribution of native vitamin B12 has been studied by Hall (27). He observed that TCl had a potential capacity of 70C - 800 ng R12/litre but that it was normally only half saturated, i.e. carrying 350 - 400 ng/l. TCll on the other hand has a capacity of 986 ng B12/litre (28) but normally less than 2% of the available TCll is used at any given time during absorption. Thus about 200 ng B12/litre of plasma is carried by TCll and 350 - 400 ng/l by TCl + lll. Alternatively this could be expressed as 4.8 - 5.4% of the total B12 bound to TCll.

THE APPLICATION OF THE OCCUPANCY PRINCIPLE TO THE ESTIMATION OF WHOLE BODY B12

In the studies described in section 2 it was appreciated that an estimate of the whole body stores of vitamin B12 could be made with data obtained by whole body monitoring followed by analysis with the occupancy principle. In view of the very wide range of values estimated for total body B12 by various techniques, it was thought to be of interest to pursue this point.

It has already been shown that the ratio of capacity to occupancy for any part of a system is equal to the flow of mother substance through the system. This flow into the system is the same no matter which part of the system is being used for the measurements of capacity and occupancy. The equation

$$F = \frac{C_{plasma}}{\theta_{plasma}} = \frac{C_{whole\ body}}{\theta_{whole\ body}}$$

can therefore be re-arranged to give

$$\text{capacity}_{\text{whole body}} = \frac{\text{occupancy}_{\text{whole body}}}{\text{occupancy}_{\text{plasma}}} \times \text{plasma B12}$$

This expression can be used for estimating whole body B12 content regardless of whether the tracer is given orally or intravenously. The reduction of the whole body occupancy in the case of oral administration is by the same factor as the reduction of plasma

occupancy because both are due to the unabsorbed fraction. Thus the ratio of the occupancies is not altered.

An estimate of the body stores of B12 was also obtained from the unfractionated plasma data. If it is assumed that the system has equilibrated then a measure of the total body B12 can be obtained. from the plasma content of B12.

Materials and methods

Whole body measurements of radioactive vitamin B12 were made on four of the subjects described in section 2 : cases 5, 6, 8, and 16. Details of their clinical states and of the doses of radioactive tracer which they received are given in tables 3 and 4. The method used for calculating the plasma occupancies is described in section 2 as is the method for measuring serum B12 levels.

Whole body monitoring

At frequent intervals after the administration of the ^{57}Co B12 whole body B12 measurements were made in a shielded room whole body monitor. The radiation was monitored by six 6" diameter x 4" thick sodium iodide scintillation detectors. These detectors were arranged in a circle round the patient's body and attached to a vertical framework which was driven slowly from one end of the subject to the other. The amount of radioactivity present was summed throughout the scan which took 5 minutes. Appropriate corrections were made for background radiation and for natural body radioactivity.

The use of the whole body monitor allows a direct measure of the body radioactivity to be made. It gives accurate results even at lengthy intervals after the administration of the isotope. Initially the amount of radioactivity remaining in the body can be derived from a measure of the losses in excretion but

within a few days these losses become so small that accurate measurements can be made only by serial whole body monitoring.

Results

1. from whole body monitoring

The results for whole body B12 as determined by the application of the occupancy principle to the data obtained by whole body monitoring are given in table 12. The study continued for over 300 days in all four cases. The measurements of the whole body retention fall very closely (figure 11) on a single exponential curve:

$$A(t) = A_o \exp(-\alpha t)$$

The occupancy is taken as the integral of this function to infinity : A_o/α. The fact that A_o is not equal to 100% for the intravenous cases is probably due to rapid unphysiological loss of about 10% of the injected tracer B12. This has the effect of reducing the whole body and plasma occupancies by about 10% but it does not affect their ratio.

2. from assuming equilibration of tracer and body stores

The results obtained in section 2 can also be used to estimate the total body content of B12. On the assumption that the tracer B12 has equilibrated with the endogenous B12 the data can be used to calculate the total body B12 on the isotope dilution principle. If 0.1% of the tracer is present in one litre of

Case Number	A_o	α	Half life	$\theta_{W.B}$	θ_p	Plasma B12	body B12
	(%)	d^{-1}	d	d	d	ng/l	mg.
5	89.7	.00109	637	825	.278	468	1.4
6	77.3	.00108	640	715	.138	149	0.77
8	69.0	.00130	535	533	.622	1700	1.5
16	94.5	.00112	619	844	.263	347	1.1

Table 12 : showing estimated whole body B12 content from data obtained by whole body monitoring.

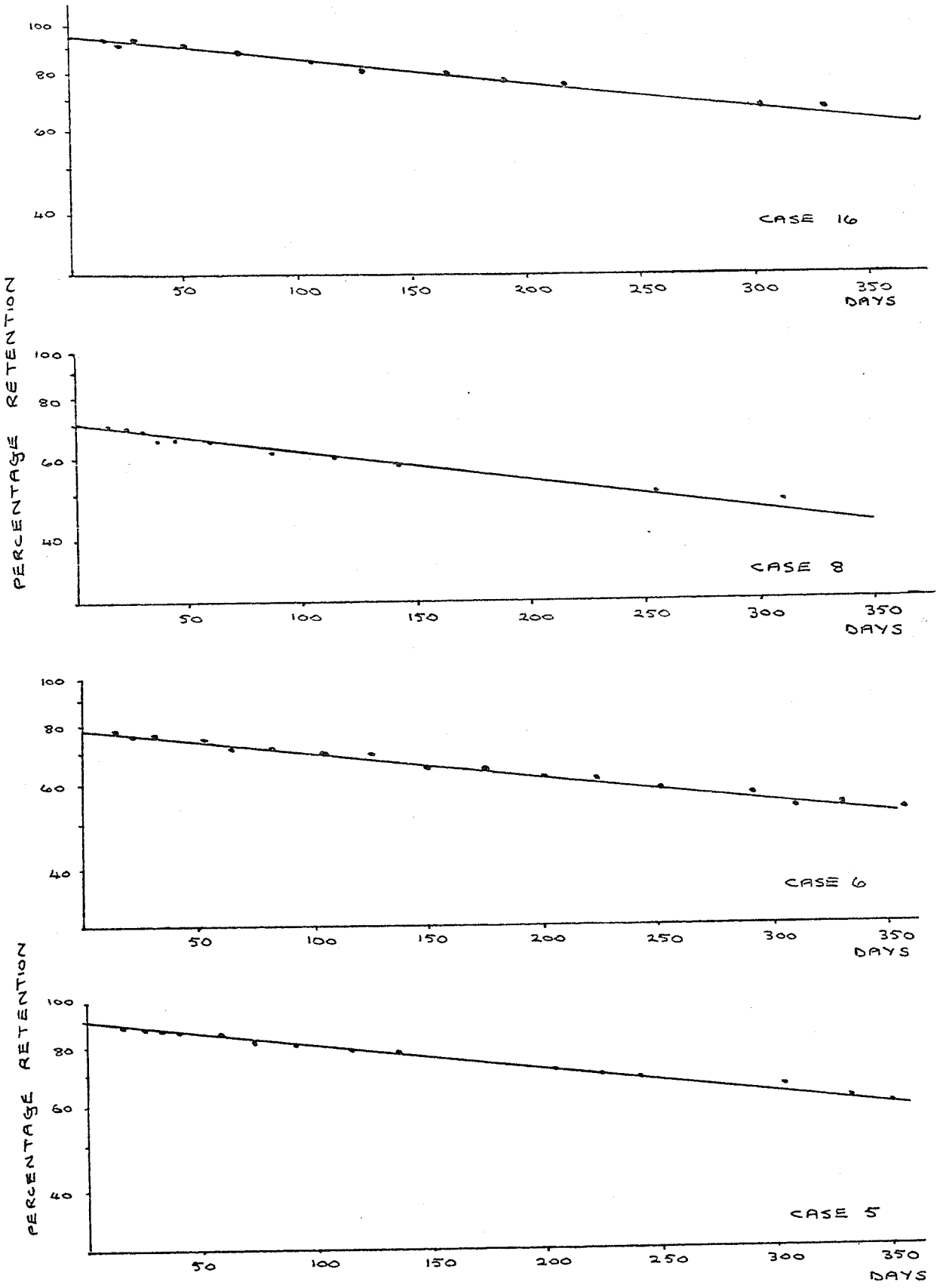


Figure 11 : The whole body exponential functions obtained by whole body monitoring of cases 5, 6, 8 and 16.

serum at day 50 and the B12 level is 250 ng/litre of serum then the total body stores can be estimated as

$$250 \quad x \quad \frac{100}{0.1} \text{ ng} \quad \text{i.e} \quad 0.25 \text{ mg.}$$

The value of the tracer present at any given time was obtained from the activity/time curve. The results obtained are shown in table 13. A more accurate estimate can be obtained from data derived from the four subjects who were used for the whole body monitor studies. The percentage of ^{57}Co B12 retained is available for these subjects and this can be used in the calculation instead of assuming 100% retention . The amended results for these four are given in table 14.

	Case Number						
Days after tracer given	3	4	5	6	8	9	11
50	0.194	0.251	0.851	0.552	2.073	0.2	0.181
100	0.664	0.292	2.035	0.687	3.091	0.364	0.379
130							0.587
140						0.569	
150	0.886	0.318	2.305	0.784	3.469		
200	1.094	0.327	2.376	0.903	3.778		
240		0.343					
250	1.329		2.463	1.014			
300	1.431		2.6	1.164			
320	1.55						

	Case Number						
Days after tracer given	12	13	14	15	16	17	18
50	0.273	0.146	0.193	0.125	0.655	0.183	0.151
80			0.33				
100	1.104	0.199		0.191	1.197	0.233	0.2
135							0.177
150	4.379	0.269		0.287	1.388	0.291	
160		0.282					
180	10.583					0.332	
200				0.435	1.509		
250					1.693		
260					1.735		

Table 13 : showing estimated values of whole body stores (in mg.) assuming equilibration of tracer B12 and endogenous B12.

Case Number	Equilibration if 100% retained	Equilibration (corrected)	Whole body monitoring
5	2.6	2.3	1.4
6	1.2	0.90	0.77
8	3.8	2.6	1.5
16	1.7	1.6	1.1

Table 14 : showing a comparison of the estimated total body B12 obtained by different methods of analysis.
(values in mg)

DISCUSSION

Estimates of the whole body stores of vitamin B12 have been largely based on the microbiological assay of tissues obtained post mortem from patients : three varying estimates were obtained. These were 2.689 mg., range 1.634 - 3.475 mg (72), 3.9 mg., range 0.79 - 11.1 mg. (73) and 5 mg., range 3.48 - 10.95 mg. (74). A combined isotope dilution and microbiological assay (75) gave values of 2.221 mg. (range 0.953 - 4.304 mg.).

Only two estimates of total B12 stores have been made from data from living subjects. The first was a study by Reizenstein et al (58) in which an average value of 3.03 mg. was obtained by kinetic analysis of values for whole body retention, faecal excretion and plasma clearance after parenteral administration of radioactive vitamin B12. A second estimate of total body B12 was calculated for eighteen patients by giving a tracer dose of radioactive cyanocobalamin and measuring the radioactivity and microbiological activity in liver biopsies obtained at laparotomy. This study by Adams et al (76) was based on the isotope dilution principle and assumed that equilibrium had occurred by the time of the liver biopsy (6 - 29 days later.) Values ranging from 0.96 mg. to 5.984 mg (mean 2.528 mg) were obtained.

The estimated values for whole body B12 content obtained by whole body monitoring with analysis by the occupancy principle are lower than most of the values obtained by the methods

described above. They range from 0.77 mg. to 1.5 mg, with a mean value of 1.2 mg. Three of the four subjects monitored were from the PA group (cases 5, 6 and 8) and therefore were not strictly speaking in a steady state since their input flow of vitamin B12 would be lower than their outflow. The body stores of B12 will be gradually depleted in these subjects. It is of interest to note that although the lowest estimate is from a PA the two highest values were also obtained from PA patients. The estimated stores in all four patients were lower than the results obtained by assuming equilibration from the data from the same four subjects.

The results shown in table 13 suggest that equilibration cannot occur until at least 150 to 200 days have elapsed after administration. The steady increase in the estimate obtained from the data from any given patient is due to the fact that the amount of tracer in the serum initially is higher than it would have been if equilibration had occurred.

CHAPTER 2

IN VITRO STUDIES ON THE BINDING OF VITAMIN B12 BY THE TRANSCOBALAMINS

SECTION 1

THE ADDITION OF VITAMIN B12 TO SERUM

The total vitamin B12 content of an aliquot of serum can be determined by microbiological or radio assay. The distribution of this endogenous B12 on carrier proteins is more difficult to evaluate. It can be done by separation of the serum on G - 200 followed by an assay of the B12 content of the fractions but this method is too laborious for routine use and the small amount of B12 on TC11 adds to the technical problems. The addition of a mass of radioactive B12 to serum, followed by fractionation to identify the amount of ^{57} Co B12 bound by each carrier protein gives information only on the distribution of the added B12 and says nothing about the distribution of endogenous B12. It was thought that if smaller amounts of B12 were added and if this added B12 were selectively bound to say TC11 then this could be of practical importance in determining the amount of endogenous B12 on the fractions and would also give information on the relative affinities of the transcobalamins for added B12. Accordingly an investigation of the distribution of B12 added to serum in vitro was initiated.

Materials and Methods

20 ml blood samples were obtained by venepuncture from ten hospital patients, none of whom were on vitamin B12 therapy. The blood was collected into clean but not sterile glass tubes, allowed to clot and centrifuged to separate the serum. The serum was pooled and 4 ml. aliquots were stored at -20°C.

Each serum sample was thawed at room temperature prior to the addition of radioactice vitamin B12. The radioactivity was measured in a thallium activated sodium iodide crystal (IDL type 663), 55 mm diameter and 69 mm deep, shielded by 100 mm of lead. The crystal was linked to a counter ratemeter MS 310 (J and P Engineering (Reading) Ltd). The sample was counted for one minute then applied to a column of Sephadex G - 200.

Four radioactive vitamin B12 solutions were prepared from a standard solution of ^{57}Co cyancobalamin (specific activity 150 μCi/μg) obtained from the Radiochemical Centre, Amersham. Additional vitamin B12 was furnished from the diluted contents of an ampoule of 1 mg/ml cyancobalamin BP obtained from Glaxo Ltd., Greenford, England. The following amounts of radioactive B12 solution were added to 4 ml. of serum to give the required concentration:-

1.	25	ng/l	0.1 ml of 1 ng/0.2 μCi/ml solution
2.	50	"	0.2 ml "
3.	100	"	0.4 ml "
4.	62·5	"	0.1 ml of 2.5 ng/0.05 μCi/ml solution
5.	125	"	0.2 ml "
6.	250	"	0.4 ml "
7.	500	"	0.2 ml of 10 ng/0.2 μCi/ml solution
8.	750	"	0.3 ml "
9.	1000	"	0.4 ml "
10.	5000	"	0.2 ml of 100 ng/0.25 μCi/ml solution

The number of solutions prepared was kept to a minimum since the potential errors in dilution were thought to be greater than the errors due to the variation in sample volume.
The same Sephadex G - 200 column was used for each separation.

The column eluant was collected as previously described and the fractions were counted for 1 - 5 minutes (depending on the activity present) in a Packard well-type gamma scintillation spectrometer. A standard of known radioactive B12 content was counted in both the auto gamma counter and the counter ratemeter. A correction factor was obtained which allowed a direct comparison of the results obtained from the two instruments. The recovery of radioactive B12 from the column could be calculated although the added and eluted samples were counted in different instruments.

Results and Discussion

The results shown in tables 15 and 16 demonstrate that the mass of added vitamin B12 does not alter the pattern of its distribution between the carrier proteins. The percentage of eluted activity bound to TC11 in the samples from pool 1 was 77.3% ± 2.2%. This is similar to results which have been obtained from repeated fractionation of aliquots of a serum pool to which a constant amount of vitamin B12 was added, the mean and the standard deviation being respectively 81.2% and 2.1%. The mean and standard deviation were calculated for the data shown in table 16 and the results were : pool 2 72.7% ± 2.1%; pool 3 68.0% ± 2.8%; pool 4 73.6% ± 6.1%; pool 5 60.2% ± 3.7% and pool 6 74.7% ± 4.3%.

The unsaturated B12 binding capacities of three of the serum pools could be estimated from the recoveries obtained when a large mass of vitamin B12 was added. In the case of pool 1 only 28.6% of the added radioactivity was eluted with the transcobalamins when 5000 ng B12 was added/litre of serum. This is equivalent to a UBBC of 1430 ng/l. Recoveries of 34.7% and 16.4% were obtained when 5000 ng/l and 10,000 ng/l were added to pool 5. The UBBC for pool 5 is therefore 1688 ng/l. When 5000 ng of B12/litre was added to pool 6 a recovery of 32.3% was observed which corresponds to a UBBC of 1615 ng/l.

The remaining vitamin B12 was not eluted as a peak of radioactivity (as described by Hom and Ahluwalia (28)). Instead

ng B12/litre of serum	TCO	TCl + lll	TCll	Estimated Recovery
25	0	22.4%	77.6%	99.5%
50	0	21.9%	78.1%	91.4%
62.5	3.1%	24.1%	72.8%	111.3%
100	0.6%	21.9%	77.5%	77.5%
125	0	22.7%	77.3%	88.5%
250	0	24.0%	76.0%	83.7%
500	0	22.8%	77.2%	88.2%
750	0.7%	22.9%	76.4%	93.8%
1000	0.8%	20.8%	78.4%	89.5%
5000	1.6%	17.0%	81.4%	28.6%

Table 15 : Showing the effect of the mass of vitamin B12 added to samples of pool 1 on its distribution between the transcobalamins.

Similar results were obtained in experiments with 5 other serum pools (see table 16).

	Pool Number				
Mass of B12 Added	2	3	4	5	6
ng/litre					
25	70.4			55.6	71.7
50	74.0				
84			68.2		
168		70.4	66.2		
250	71.5	65.1		58.7	72.9
500		70.4	75.3		
1000	74.8	66.1			
2000		61.3	78.2		
3000			79.9		
5000				63.6	79.6
10,000				62.7	

Table 16 : showing the percentage of added B12 bound to TCII and its variation with the mass of B12 added.

some of the 'lost' activity eluted gradually from the column and the remainder became associated with the gel. This apparent binding of free B12 to Sephadex G - 200 is discussed in the next section.

SECTION 2

OBSERVATIONS ON SOME TECHNICAL ASPECTS OF THE SEPARATION OF FREE AND TRANSCOBALAMIN BOUND B12 BY SEPHADEX G - 200 GEL FILTRATION.

The literature on the use of Sephadex G - 200 to separate free and transcobalamin bound radioactive vitamin B12 suggests that when an excess of ^{57} Co B12 is present it is eluted as a clearly defined peak which because of its smaller molecular size comes later than the protein bound B12 (28). All the radioactivity which is introduced into the column in the form of ^{57} Co B12 elutes either in the transcobalamin bound or free vitamin B12 peaks.

Practical experience however has not supported this view. In the first place the free vitamin B12 peak frequently was not observed. Secondly rates of recovery of 200 - 300% were occasionally observed when an in vivo labelled sample was fractionated on a column in which the preceding sample had been labelled with ^{57} Co B12 added in vitro and which therefore contained substantially more radioactivity. The excess or additional radioactivity was eluted with TCII to a much greater extent than with TCI but it was not confined to TCII.

These observations suggested that there was retention of free vitamin B12 on the Sephadex columns and possibly also that

there was binding of TC11 - B12 to the Sephadex. This latter phenomenon had been observed by Hom (12) and attributed to the use of low ionic strength buffers. This explanation does not account for the binding observed in the present studies nor does it account for the retention of unbound B12 on the column. Further investigations into the problem were therefore carried out.

Materials, methods and results

1. Demonstration of the retention of B12 on columns.

The retention of vitamin B12 on Sephadex columns was demonstrated initially by the recovery rates observed. It was consistently shown that if an in vitro labelled serum sample containing 0.02 μCi of ^{57}Co B12 was fractionated on a column which was used next for an in vivo labelled sample containing approximately 0.001 μCi of ^{57}Co B12, then recoveries of 200 - 300% could be obtained from the in vivo sample. This effect could not be shown when two samples of approximately equal radioactivity were used because the resulting increase in recovery would be only about 5%.

The fact that vitamin B12 was being retained on columns could also be shown by the identification of radioactivity in the gel. It was possible to locate this radioactivity by measuring the column in the whole body monitor and also by dividing the gel into aliquots and counting in the auto gamma counter.

A column which had reached the end of its useful life and was being discarded was divided into approximately 8 ml fractions. As far as was possible the fractions were obtained without mixing because it was hoped that it would be possible to locate the position of the radioactive B12. The column fractions were counted for two minutes each in a Packard auto-gamma scintillation spectrometer. The radioactivity was

predominantly associated with the bottom 30 - 35 ml of gel and with the nylon support membrane at the bottom of the column. Some radioactivity was found throughout the column and there was a substantial amount associated with the top membrane. The total radioactivity present was equivalent to 0.046 μCi. This treatment was repeated for a second column and 0.049 μCi ^{57}Co B12 was found distributed in a similar way.

It had been observed that after the columns had been in use for 2 - 3 weeks bacterial growth appeared on the lower nylon membrane. This growth was in spite of the use of 0.02% ($^{W}/_{V}$) of sodium azide in every batch of buffer. In an attempt to see if it was the presence of the bacteria which was responsible for the retention of ^{57}Co B12 on the gel an entirely fresh column was prepared. A sample of radioactive vitamin B12 solution (10 ng B12/0.025 μCi) was added to the column with buffer and blue dextran solution. Only 25% of the added radioactivity was recovered in the fractions corresponding to the elution volume of vitamin B12. The residual radioactivity in the column was measured by counting it in the whole body monitor. Radioactivity corresponding to 0.003 μCi (± 10%) was detected although 0.019 μCi (75% of 0.025 μCi) was expected. Most of the radioactivity must have eluted gradually with the buffer which was run through the column between samples.

2. A study of methods of removing B12 from Sephadex.

This study arose from the observation that serum could pick up B12 in transit through a column. Although most of the radioactivity had been removed from the column described above, it was decided to apply a sample of serum to which no radioactivity had been added in order to see if it could pick up ^{57}Co B12 from the column. Normal human serum did not bind any of the remaining radioactivity present. The experiment was repeated with a preparation of hog intrinsic factor because it has a greater affinity for vitamin B12 than that of human serum. A very small amount of radioactivity was bound (0.0004 μCi) by the hog intrinsic factor. A sample of chick serum obtained from the Immunology Department, Western Infirmary, Glasgow was finally added to the column. Only slightly more radioactivity (0.0005 μCi) was bound by the chick serum although it contains a very avid vitamin B12 binder.

Discussion

The binding of ^{57} Co B12 to Sephadex is an important practical point which if disregarded can give rise to misleading results. Having said that it is apparent that only a small amount of this B12 can be removed from the Sephadex by serum in transit. This is therefore a problem only when the serum contains very little radioactive B12. There appears to be some ^{57} Co B12 which is held on to the gel very firmly and it is not removed even by an avid binder like chick serum binding protein.

There is no evidence that the gel is able to remove ^{57} Co B12 which is bound to the transcobalamins for the results obtained from a serum pool are consistent regardless of whether the sample is fractionated early or late in the life of a column.

SECTION 3

THE FORMS OF VITAMIN B12 BOUND BY THE TRANSCOBALAMINS

Different forms of vitamin B12 have been reported in the plasma of healthy subjects. Methylcobalamin is the predominant form (60 - 80%) but adenosylcobalamin and hydroxocobalamin are also present and cyanocobalamin is occasionally observed (57). As there have been no reports of the distribution of these forms on the transcobalamins, the present studies were undertaken to examine the possibility that some specificity of binding takes place.

Materials and methods

It is known that some of the forms of vitamin B12 are light sensitive (57) and in consequence all the procedures described in this section were carried out in the dark.

A fresh column of Sephadex G - 200 was set up in a dark room illuminated by two Ilford 'safelights', and the column was calibrated with a 4 ml. sample of serum to which ^{57}Co cyanocobalamin had been added. The elution volumes of the two transcobalamins were noted. Buffer was run through the column for several days in order to remove as much of the cyanocobalamin as possible.

Blood samples were obtained from four subjects by venepuncture with a foil-covered syringe. The blood was transferred to glass tubes also wrapped with foil and centrifuged after clotting. The serum was removed in the dark room and aliquots were stored at -20°C in foiled plastic tubes.

4 ml samples of serum were applied in darkness to the column and the eluant was collected in 30 minute fractions as described before. These fractions were pooled to give a TCl + lll sample and a TCll sample, and the samples were stored in foil wrapped containers at -20°C.

Identification of the forms of vitamin B12

This part of the work was carried out by Farquharson and full details of the method are given elsewhere (77). Briefly, the eluant was dialysed overnight to remove the salt which was present at high concentration in the buffer. The vitamin B12 was extracted by refluxing for 30 minutes with ethanol. The coagulated protein produced was removed by filtration and the cobalamin was extracted with phenol/chloroform (1 : 1 v/v). The organic phase was then shaken with diethyl ether/distilled water/acetone (4 : 2 : 1 v/v) to extract the cobalamin into the aqueous phase. This aqueous phase was rotary evaporated to a small volume.

The cobalamins present in this extract were separated by thin-layer chromatography. The concentrations present are so low however that visualisation of the separated forms is by bio autography using a vitamin B12 dependent E. coli mutant and a tetrazolium growth indicator in an agar overlay. The growth zones of the organism give a measure of the forms of vitamin B12 present.

The extraction and bio autographic procedures were carried out for three samples from each of four subjects. In each case a sample of unfractionated serum was studied in addition to the fractions corresponding to TC1 + 111 - B12 and to TC11 - B12.

Results and discussion

The results obtained from each subject show that all the forms of vitamin B12 observed in the serum are present in both the TC1 + 111 and TC11 extracts. In all subjects methylcobalamin and adenosylcobalamin were observed. Hydroxocobalamin and cyanocobalamin were found in samples from two and three of the cases respectively. Full details are given in table 17.

There is no evidence in this study to support the view that one form of vitamin B12 is specifically carried on one transcobalamin. The observation of cyanocobalamin in samples from three of the patients was initially thought to be due to the calibration run on the column (which used ^{57} Co cyanocobalamin). Cyanocobalamin was also observed however in the samples of unfractionated serum which were studied and it must therefore be considered to be genuinely present.

Form of Cobalamin Present	1		2		3		4	
	TC1 & 111	TC11	TC1 & 111	TC11	TC1 & 111	TC11	TC1 & 111	TC11
Methyl	+	+	+	+	+	+	+	+
Cyano	+	+	-	+	-	-	-	+
Adenosyl	+	+	+	+	+	+	+	+
Hydroxo	+	-	-	-	-	+	-	-

Table 17: showing the forms of B_{12} present on the transcobalamins.

SECTION 4

A STUDY OF THE TRANSCOBALAMIN BINDING PATTERN IN 100 SUBJECTS

Little is known about the way in which vitamin B12 added to serum in vitro is distributed on the transcobalamins other than the fact that when one is absent the B12 is bound by those which are present. For this reason a study of the patterns of the binding of added ^{57}Co B12 was initiated. Serum samples were obtained from control subjects and also from patients suffering from a variety of diseases.

In addition to the study of the distribution of added B12 the amount of B12 bound was also considered. A comparison of the unsaturated B12 binding capacity (UBBC) obtained from two different methods was made.

Materials and Methods

Serum samples were obtained from 100 subjects. In order to facilitate analysis and presentation of the results they have been grouped accordingly to the clinical state of the donor. The nine groups and the relevant clinical details of each subject are given here.

Group 1 : Pernicious anaemia

Cases 1 - 16 in this group of nineteen all presented with megaloblastic erythropoiesis and all had a pentagastrin fast achlorhydria. Three of the patients were not anaemic on presentation : case 3 presented with sub-acute combined

degeneration, case 5 with peripheral neuritis and case 13, during investigations for ill health, was observed to have macrocytosis. All patients had low serum vitamin B12 levels on presentation except case 10 who had ingested large amounts of liver in the three days prior to admission to hospital. On admission his serum B12 level was 200 ng/l but erythropoiesis was still clearly megaloblastic. Haematinic therapy was withheld and over the next few days a reticulocytosis was observed and one week after admission the erythropoiesis was found to be normoblastic.

Included in this group of patients are two (cases 17 and 18) who had previously undergone total gastrectomy and who, like cases 1 - 16 were unable to absorb radioactive vitamin B12 unless it were given with a source of intrinsic factor. One of these patients (case 17) had received parenteral vitamin B12 since the operation and the other (case 18) since the development of a megaloblastic anaemia five years after the operation. Case 19 had a defect of intrinsic factor mediated absorption of vitamin B12 which differed in important respects from that which is found in pernicious anaemia but which permits her entry into this group. She presented at the age of 26 with a megaloblastic anaemia and a low serum B12 level. Treatment with parenteral vitamin B12, folic acid and vitamin C induced a haematological response but when treatment was withdrawn it was noted that her serum B12 level fell to borderline values. Although acid and intrinsic factor were

present in her gastric juice, absorption tests with radio-active vitamin B12 showed sub-normal absorption when the dose was given alone but normal absorption when the dose was given with a source of human or hog intrinsic factor.

Group 2 : latent vitamin B12 deficiency

All twelve patients in this group had normal haemoglobin levels but evidence of latent vitamin B12 deficiency in the form of a low or borderline serum vitamin B12 level and mal-absorption of vitamin B12. The condition was regarded as latent because cytological evidence of vitamin B12 deficiency was either absent or not sought. In ten of the patients haematinic therapy had not been given before the samples were obtained, the two exceptions being cases 30 and 31 in whom parenteral vitamin B12 therapy had been initiated. The patients came under observation for a variety of reasons. Those with diabetes mellitus had been found to malabsorb vitamin B12 in the course of another study and their impaired capacity for absorption had been shown to be unrelated to hypoglycaemic drug therapy. The two patients with malabsorption (cases 24 and 25) had come under observation because of weight loss and both had other evidence of malabsorption and had partial villous atrophy on a small bowel biopsy. The patient with gastritis (case 26) presented with paraesthesiae of the limbs. The patients who had previously undergone partial gastrectomy came under observation for a variety of reasons and their

serum B12 level was estimated as part of the general examination. Case 31 presented originally with thyrotoxicosis and during routine immunological survey was found to have parietal cell antibodies. This observation led to gastroscopy and gastric biopsy which showed an atrophic gastric mucosa.

Group 3 : Low serum vitamin B12 level - normal capacity to absorb vitamin B12.

The nine patients in this group all had a normal capacity to absorb vitamin B12 but all, at some time, had a low or borderline serum vitamin B12 level on more than one occasion. The serum vitamin B12 levels were estimated in cases 32 and 33 because the clinical history suggested malnutrition; in case 34 because the presenting symptom raised the possibility of vitamin B12 deficiency and in case 35 because a macrocytosis was noted on routine peripheral blood film. Cases 36 and 37 both had anaemia without distinguishing features but accompanied by a very high E.S.R. and in both patients serum B12 levels of 80 - 140 ng/l were observed over a period of several months. In case 38 the serum vitamin B12 level was determined because of the history of gastrectomy and in cases 39 and 40 the serum vitamin B12 level had been observed to remain below 100 ng/l six months after parturition.

One patient in group 4 (case 46) and two patients in group 5 (cases 49 and 51) also had the common features of this group but because they presented with a megaloblastic anaemia they have been included in other groups.

Group 4 : Megaloblastic anaemia due to folate deficiency

The seven patients in this group all presented with megaloblastic erythropoiesis and low serum folate levels and all were subsequently found to have a normal capacity to absorb radioactive vitamin B12. One patient (case 43) was not anaemic on presentation and the haematological investigations were precipitated by the presence of a high mean corpuscular red cell volume. In case 41, who three years later was re-admitted to hospital with a recurrence of megaloblastic anaemia, the aetiological factors were considered to be malnutrition and alcoholism. Polycythaemia secondary to gross pulmonary disease was considered to be an aetiological factor in case 43 and myelofibrosis in case 45. It could not be clearly established in the remaining cases what the cause of the deficiency was. The possible aetiological significance of a carcinoma of the rectum which had been excised five years previously in case 46 was also not clear. As already noted case 46 also comes into the category of a patient with a borderline or low serum vitamin B12 level but a normal capacity to absorb B12.

Group 5 : Miscellaneous megaloblastic anaemias

The seven patients in this group all presented with a megaloblastic anaemia but in no case has the aetiology of the anaemia been defined with acceptable precision. Case 54 presented with a megaloblastic anaemia apparantly due to folic acid deficiency, but refused consent for various investigations. She was discharged from hospital on oral folic acid and did not re-attend until 18 months later by which time she was grossly myxoedematous. Although not anaemic at that time her serum vitamin B12 level was found to be low and her capacity to absorb vitamin B12 was also low. Case 48 was a blind, ill-nourished, verminous woman with obvious local secondary carcinoma of the vulva. On admission there was evidence to suggest vitamin B12 deficiency but she had a normal capacity to absorb vitamin B12. Treatment with vitamin B12, folate and vitamin C produced some benefit but she was still anaemic when she died four months later. Case 50 had gross rheumatoid arthritis, refused investigations, and has attended hospital on only one occasion in the two years since she had in-patient treatment. Case 52 had an anaemia which was considered to be megaloblastic but which responded incompletely to vitamin B12, folic acid and vitamin C.

Case 53 had presented with a megaloblastic anaemia and clinical features suggestive of Marfans Syndrome. The diagnosis of cystathionine synthetase deficiency was established but the aetiology of megaloblastic anaemia was never satisfactorily defined. Case 51 was noted to have a mild anaemia while convalescent from a myocardial infarction and the evidence suggested folate deficiency. After one month of treatment with folic acid however, the serum B12 level remained at a borderline value in spite of the normal capacity to absorb vitamin B12 and the patient can therefore also be classified in group 4. A similar situation occured in case 49 who was found to have a megaloblastic anaemia after a haematemesis. Although the patient was treated with folic acid for a lengthy period of time, the serum vitamin B12 level remained persistently in the region of 80 ng/l in spite of a normal capacity to absorb vitamin B12.

Group 6 : Jaundice and liver disease

This group of eleven patients was studied because of the known relationship between liver function and vitamin B12. Cases 55 - 60 had presented with obstructive jaundice and the final diagnosis was established at laparotomy or post-mortem. Cases 61 - 65 had parenchymal liver disease and the diagnosis was establised by conventional techniques including liver biopsy in every patient. Samples were taken at different

times in three patients with no treatment in the intervening period, in case 65. Steroid therapy had been instituted and then withdrawn in case 64 and a variety of medicaments had been given in case 61.

Group 7 : The control subjects

Serum from this group of fifteen subjects was examined as a control group. All were hospital staff and were apparently in good health. The majority of subjects were young and female.

Group 8 : Tobacco amblyopia and Lebers disease

Serum samples from six cases of tobacco amblyopia and two cases of Lebers disease were made available by Professor W.S.Foulds, Tennent Institute of Opthalmology, Western Infirmary, Glasgow. The diagnostic criteria were determined by the responsible clinician.

Group 9 : Patients with miscellaneous diseases

Details of the diagnosis made for each of the twelve patients included in this group are given in table 26.

Sample collection

10 - 20 ml samples of blood were obtained by venepuncture and the blood was collected into clean but not sterile glass tubes. The samples were allowed to clot at room temperature and were centrifuged within 3 hours of collection to separate the serum. The serum was stored at -20°C and as far as is known the samples were thawed only prior to the studies reported in this section.

Radioactive vitamin B12 solutions

To each 4 ml sample of thawed serum an aliquot (0.1 ml) of standard radioactive solution was added. This solution contained 100 ng of B12 per ml and also 0.25μCi of ^{57}Co B12 per ml and will be expressed as 100 ng/0.25μCi/ml. Earlier work was carried out using a 20 ng B12/ml solution of cyanocobalamin with between 0.1 and 0.5 μCi/ml. 0.2 ml of this solution was added to each 4 ml serum sample. These standard solutions were prepared from ^{57}Co B12 specific activity 210 μCi/μg obtained from Philips - Duphar BV Holland and from ^{57}Co B12 specific activity 150 μCi/μg obtained from The Radiochemical Centre, Amersham. The additional cyanocobalamin in the standard solutions was added by the dilution of the contents of an ampoule of 1 mg/ml cyanocobalamin BP obtained from Glaxo Ltd., Greenford, England.

Serum vitamin B12 levels

These were determined by Lactobacillus leichmanni assay performed by the staff of the Department of Haematology, Southern General Hospital, Glasgow.

Unsaturated vitamin B12 binding capacity

The unsaturated vitamin B12 binding capacity (UBBC) of the serum samples was measured by the method of Gottlieb et al (78) with minor modifications. An excess of ^{57}Co B12 was added to the samples : 0.1 ml of 10 ng/0.025 μCi/ml ^{57}Co B12 was added to 0.2 ml of serum. The sample was left for a few minutes whereupon the unbound vitamin B12 was removed from the serum by absorption on to 2% (w/v) albumin-coated charcoal. A 30% solution of bovine albumin from Armour Pharmaceutical Co. Ltd., Eastbourne, England was used with Norit 'A' charcoal from the Amend Drug and Chemical Company Inc., New York. A fresh charcoal suspension was prepared for every batch of UBBC determinations. 0.5 ml of charcoal suspension was added to the serum and the mixture was shaken at room temperature for 5 minutes and then counted for radioactivity for 1 minute (in a sodium iodide crystal linked to a counter ratemeter MS 310). The charcoal was removed by centrifugation at 1000 g for 15 minutes. The supernatant, containing the serum bound B12, was counted for

1 minute. The UBBC was calculated as shown:

$$\text{UBBC (ng/l)} = \frac{\text{cpm (serum bound)}}{\text{cpm (total added)}} \times \text{pg B12 added per ml of serum}$$

Fractionation of serum samples

The samples were separated on a column of Sephadex G - 200 as described in Chapter 1, section 3. An excess of vitamin B12 (2500 ng/litre of serum) was added to many of the samples. The amount of this added B12 which was bound by a sample could be calculated from the percentage of the added activity which eluted from the column bound to the transcobalamins. A second estimate of the UBBC could be obtained for many of the samples in this way.

Radioactivity measurement

The samples were counted in a Packard well-type auto gamma scintillation spectrometer as described earlier. A standard solution of ^{57}Co cyanocobalamin was counted with the samples and again prior to fractionation when the 4 ml serum sample + ^{57}Co B12 was counted in the sodium iodide crystal linked to the scaler ratemeter. Thus the results could be expressed as a percentage of the added activity.

RESULTS

The results of the studies in which vitamin B12 was added to serum and its distribution on the transcobalamins was established are presented in tables 18 - 26. The relevant clinical data are also incorporated in these tables. The distribution of the results in the control subjects (group 7) is shown in table 24.

The serum samples from the subjects in group 3 were taken because of a history of low serum B12 levels. The level of B12 measured from the sample at the time of study was sometimes higher than the level which had been determined previously. This increased value may be a true reflection of the B12 content or it may be due to bacterial contamination of the sample.

The estimates of unsaturated B12 binding capacity obtained by charcoal absorption are also shown in tables 18 - 26. These estimates were plotted against the estimates obtained by G - 200 and the distribution of values is shown in figure 12. A linear correlation was found with the regression equation $y = 130.5 + 0.94x$. The correlation coefficient (r) was 0.83 which is significant at the $p = 0.01$ level ($n = 88$).

The serum B12 levels obtained in the control subjects almost all fell within the 'normal' range of 160 - 700 ng/l. The two exceptions to this were the samples obtained from the two laboratory workers who routinely use cyanocobalamin in their investigations!

Case No	Age	Sex	Pretreatment Hb g/dl	folate ng/l	B12 ng/l	UBBC ng/l	TC0 %	TC1 %	TC2 %	Post Treatment Time	Hb g/dl	folate ng/l	B12 ng/l	UBBC ng/l	TC0 %	TC1 %	TC2 %
1	75	F	10.1	12.8	60	1295	3.9	30.9	65.2	-	-	-	-	-	-	-	-
2	62	M	10.5	10.8	30	1307	5.1	20.3	74.6	-	-	-	-	-	-	-	-
3	52	M	12.7	20.0	30	1195	2.8	31.9	65.3	1 m	13.9	-	1650	520	4.7	10.5	84.8
4	74	F	5.0	9.2	20	1084	1.3	6.6	92.1	5 m	14.6	-	1250	833	0.7	24.1	75.2
5	68	M	15.7	7.6	70	1413	0	22.0	78.0	1 y	16.1	-	1250	1303	4.1	10.8	85.1
6	52	F	5.7	6.0	30	1564	1.6	14.2	84.2	1y2m	14.7	4.0	750	2045	1.9	14.3	83.8
7	59	F	6.4	3.4	20	1692	4.1	14.7	81.2	2y6m	18.9	-	2400	4510	0	42.3	57.7
8	78	M	7.4	4.0	40	1038	2.4	31.3	66.3	2y6m	15.0	-	3800	1319	5.3	11.1	83.6
9	58	M	6.7	4.2	80	-	-	-	-	1 y	13.2	-	200	3200	1.3	42.5	56.2
10	65	M	8.7	9.0	200	-	-	-	-	3 y	14.8	-	600	1469	3.7	17.3	79.0

Table 8 Showing results from patients with established vitamin B_{12} deficiency states (continued overleaf).

Case No	Age	Sex	Pretreatment Hb g/dl	folate ng/l	B12 ng/l	UBBC ng/l	TCO %	TC1 %	TC2 %	Time	Post treatment Hb g/dl	folate ng/l	B12 ng/l	UBBC ng/l	TCO %	TC1 %	TC2 %
11	68	F	9.0	8.5	25	-	-	-	-	6 y	12.1	-	1000	1917	5.6	6.5	87.9
12	68	F	9.0	8.5	25	-	-	-	-	6 y	13.9	-	120	-	2.4	20.6	77.0
13	67	F	13.3	9.6	80	-	-	-	-	7 y	12.6	-	1000	4750	0	31.3	68.7
14	62	M	6.0	7.6	30	-	-	-	-	8 y	14.9	-	480	1256	7.1	11.3	81.6
15	59	M	8.7	-	40	-	-	-	-	8 y	15.4	-	1500	4086	2.2	7.6	90.2
										10 y	14.8	-	3700	4669	1.4	5.4	93.2
16	74	F	7.4	-	60	-	-	-	-	10 y	14.9	-	360	1382	3.8	22.8	73.4
17	63	F	14.0	-	-	-	-	-	-	12 y	14.2	5.2	750	1758	5.5	22.8	71.7
18	65	F	10.6	80	40	-	-	-	-	17 y	11.7	-	520	1344	1.9	11.5	86.6
19	26	F	3.7	-	50	-	-	-	-	2y9m	14.8	3.2	290	1070	1.0	6.1	92.9
										4y8m	13.6	3.8	100	1196	0	6.5	93.5
										5y7m	13.6	-	100	1187	1.5	9.9	88.6

Table 8: Showing results from patients with established vitamin B_{12} deficiency states.

Case No	Age	Sex	Diagnosis	Hb g/dl	folate ng/l	B12 absorption alone	B12 absorption +IF	marrow	MAO meq	B12 ng/l	UBBC ng/l	TC0 %	TC1 %	TC2 %
20	63	M	Diabetes	15.1	4.8	5.0	47.0	normo	PFA	120	1545	5.8	18.4	75.8
21	61	F	"	14.1	16.0	3.0	39.1	normo	PFA	95	1629	3.6	20.2	76.2
22	79	F	"	13.1	12.0	4.4	36.5	normo	PFA	70	1397	5.1	25.6	69.3
23	75	F	Glossitis B_{12} 100:150	12.8	4.0	17.5	40.5	normo	PFA	-	-	1.3	20.9	77.8
			6 m later	13.2	-	-	-	-	-	-	-	1.0	23.9	75.1
24	42	F	Enteropathy	11.9	2.6	7.0*	2.3*	normo	-	140	1420	0.6	14.8	84.6
25	69	M	"	14.1	3.0	19.2	41.0	normo	15.2	120	1927	1.1	14.4	84.5
26	65	M	Gastritis B_{12} 80:140	13.7	7.6	26.6	42.5	normo	0.2	220	1583	3.5	24.3	72.2
27	64	F	Post Gastrectomy 6y	14.0	5.2	14.0	9.0	-	-	110	1635	3.3	19.5	77.2
28	56	M	" 16 y B_{12} 80:100	14.0	7.2	12.6	42.6	-	-	160	1444	3.0	37.3	59.7
29	66	M	" 20 y	13.2	5.8	18.0	54.0	normo	-	60	1606	4.1	13.4	82.5
30	55	M	" 14 y	16.4	-	10.5	-	-	-	200	1490	2.2	33.8	64.0
31	34	F	Thyrotoxicosis B_{12} 120.	11.5	-	16.4	39.7	-	PFA	1300	1093	5.4	12.3	82.3

Table 9: Showing results in patients with latent vitamin B_{12} deficiency (Group 2).

* Denotes Schilling Test result.

Case No	Age	Sex	Diagnosis	Hb	folate	B12 absorption alone	+IF	marrow	MAO	B12	UBBC	TC0	TC1	TC2
				g/dl	ng/l				meq	ng/l	ng/l	%	%	%
32	79	F	Malnourished	11.2	>20.0	37.0	48.0	-	-	140	1345	4.6	7.3	88.1
			1 m later	11.8	-	-	-	-	-	140	1304	6.3	9.5	84.2
33	81	F	Malnourished B_{12} 120:150	11.4	3.6	14.6*	-	-	-	230	1452	0	26.2	73.8
34	74	F	Glossitis B_{12} 110:100	14.7	2.4	39.0	28.0	normo	PFA	210	1188	4.3	24.2	71.5
35	75	F	CVA B_{12} 100:100	12.9	5.6	35.0	42.0	-	-	160	1620	2.6	14.8	82.6
36	69	F	Anaemia ESR 100 B_{12} 130:140	10.2	7.4	72.0	56.0	normo	-	180	1477	3.3	9.1	87.6
37	66	F	Anaemia ESR 100 B_{12} 80:160	12.9	-	67.0	28.0	normo	acid+	155	-	1.6	5.9	92.5
			2 y 6 m later	12.5	3.0	-	-	-	-	100	1285	1.9	6.7	91.4
			2 y 10 m later	12.5	-	-	-	-	-	-	-	5.5	8.0	86.5
38	49	M	Post gastrectomy 18 y B_{12} 100:110	11.6	4.0	37.0	48.0	normo	-	160	1147	8.5	9.2	82.3
39	41	F	Post partum B_{12} 80:60	12.3	8.0	30.0	22.0	-	-	180	-	0.8	33.5	65.7
40	51	F	Post partum B_{12} 100											
			7 y later	12.9	3.8	19.0*	-	-	acid+	170	-	1.7	5.7	92.6
			7 y 3 m later	12.2	-	-	-	normo	-	-	-	2.6	6.4	91.0
			8 y later	12.4	3.8	-	-	-	-	120	-	1.4	7.6	91.0
			10 y later	12.8	-	44.0	43.0	normo	-	190	1124	3.7	6.6	89.7

Table 20 Showing results: Patients with low serum vitamin B_{12} levels but having a normal capacity to absorb vitamin B_{12} (Group 3).

* Denotes Schilling Test results.

			Pretreatment							Post treatment							
Case No	Age	Sex	Hb g/dl	folate ng/l	B12 ng/l	UBBC ng/l	TCO %	TC1 %	TC2 %	Time	Hb g/dl	folate ng/l	B12 ng/l	UBBC ng/l	TCO %	TC1 %	TC2 %
41	29	F	6.5	1.8	270	1243	0	42.8	57.2	-	-	-	-	-	-	-	-
42	28	M	10.4	1.6	140	1059	5.4	10.1	84.5	1 m	12.6	>20.0	220	937	5.2	14.3	80.5
43	58	F	17.9	2.2	480	1882	2.1	38.7	59.2	3 m	17.5	-	220	1774	3.7	23.7	72.6
44	68	F	5.6	2.2	190	2439	1.9	9.9	88.2	4 m	13.7	-	750	1504	3.9	10.2	85.9
45	68	F	5.9	2.2	240	1392	1.8	10.7	87.5	7 m	11.0	-	220	1646	2.1	24.7	73.2
46	63	F	8.5	1.2	80	-	-	-	-	5 y	12.2	>20.0	320	-	1.8	19.8	78.4
										6y6m	11.3	>20.0	130	1184	3.6	12.8	83.6
47	42	F	4.3	0.8	130	987	6.1	9.4	84.5	10 m	15.4	3.4	-	-	1.6	13.5	84.9
										1 y	13.5	2.4	380	-	1.9	25.3	72.8
										1y1m	12.5	6.0	-	-	0	62.5	37.5
										1y6m	12.4	1.8	280	1068	0.7	21.2	78.1
										1y7m	15.4	-	200	1018	0.9	16.2	82.9
										2y7m	13.7	8.4	300	1679	4.0	22.4	73.6

Table21 : Showing results of patients presenting with megaloblastic anaemia due to folate deficiency (Group 4).

			Pretreatment							Post treatment							
Case No	Age	Sex	Hb g/dl	folate ng/l	B12 ng/l	UBBC ng/l	TCO %	TC1 %	TC2 %	Time	Hb g/dl	folate ng/l	B12 ng/l	UBBC ng/l	TCO %	TC1 %	TC2 %
48	74	F	4.9	5.8	80	1297	0	27.8	72.2	1 m	6.5	5.2	700	1144	3.9	13.7	82.4
										2 m	8.5	10.2	3700	375	1.8	11.5	86.7
49	41	M	11.6	0.6	60	1104	0.7	22.6	76.7	9 m	16.3	>20.0	80	1464	1.7	22.1	76.2
50	63	F	6.5	3.4	160	1248	2.8	9.0	88.2	1 y	13.00	>20.0	300	1072	0	24.0	76.0
51	64	F	10.4	1.4	90	-	-	-	-	1 m	11.4	-	140	2176	0	24.3	75.7
52	7	M	10.6	-	-	-	-	-	-		7.2	2.4	>1000	-	0	26.5	73.5
53	43	M	7.6	55.0*	70	-	-	-	-	6 m	-	-	257		0.9	7.4	91.7
										8 m	-	-	160	1362	3.4	8.6	88.0
54	70	F	7.2	30.0*	400	-	-	-	-	1y6m	13.7	>20.0	90	2210	2.6	21.5	75.9

Table 22 : Showing results from patients presenting with megaloblastic anaemia of unclassified origin (Group 5).

* Denotes Whole Blood Folate

Case No	Age	Sex	Diagnosis	Bilirubin μ mol/l	Alk Phos iu/ml	Ast iu/l	Alt iu/l	Hb g/dl	B12 ng/l	UBBC ng/l	TC0 %	TC1 %	TC2 %
55	86	F	Carcinoma pancreas	308	220	22	11	10.5	>1000	2047	2.6	17.0	80.4
56	60	F	" "	291	237	140	88	11.7	>1000	1282	6.0	9.8	84.2
57	83	F	" "	492	983	90	60	10.0	400	1950	1.9	20.0	78.1
58	70	F	" "	362	248	67	60	14.0	440	1068	4.3	16.9	78.8
59	87	F	" "	243	362	39	20	10.5	>1000	3601	0.8	59.1	40.1
60	62	F	Gallstones	103	327	77	236	15.5	270	1857	3.4	4.7	91.9
61	37	F	Biliary cirrhosis	147	372	440	425	11.3	600	1303	3.2	4.6	92.2
			" 1 year later	446	508	296	112	12.8	640	1642	4.5	10.3	85.2
62	66	F	Macronodular cirrhosis	65	78	72	47	11.7	750	1294	2.7	7.8	89.5
63	25	F	Chronic persistant hepatitis	9	78	28	40	13.9	-	-	3.1	20.3	76.6
64	30	F	Active hepatitis -										
			at onset	85	83	68	90	12.2	860	1565	3.4	8.8	87.8
			2 y later	9	50	21	25	13.0	130	1308	5.5	14.9	79.6
65	53	F	Cirrhosis: Portocaval shunt 4 y	19	70	29	21	14.2	500	1435	2.6	9.2	88.2
			6 y	22	95	32	23	12.7	400	892	7.3	6.9	85.8

Table 23: Showing results in patients with jaundice and liver disease (Group 6).

Case No	Age	Sex	B12 ng/l	UBBC ng/l	TC0 %	TC1 %	TC2 %
66	47	F	440	1168	4.7	10.2	85.1
67	24	M	860	890	10.8	11.2	78.0
68	50	F	500	1352	3.5	11.7	84.8
69	22	F	270	1232	8.7	12.2	79.1
70	24	F	660	1229	6.9	8.9	84.2
71	24	M	300	974	4.8	10.9	84.3
72	40	F	440	1088	3.7	9.9	86.4
73	20	F	500	1145	4.4	12.8	82.8
74	20	F	160	1404	5.3	14.0	80.7
75	20	F	160	1686	3.4	24.6	72.0
76	51	F	250	1793	3.6	16.6	79.8
77	20	F	600	1090	6.6	20.2	73.2
78	23	F	330	1366	6.1	11.9	82.0
79	23	F	170	1103	8.4	17.9	73.7
80	23	F	710	833	3.7	11.1	85.2

Table 24 : Showing results in control subjects.

Case No	Age	Sex	Diagnosis	B12	UBBC	TC0	TC1	TC2
81	50	M	Tobacco amblyopia. Alcoholic	250	-	1.3	3.8	94.9
82	52	F	" " .	130	1760	3.4	34.9	61.7
83	66	M	" " .	260	1472	4.7	17.7	77.6
84	72	M	" " .	190	1939	2.5	17.4	80.1
85	52	F	" " .	200	1171	3.3	8.7	88.0
86	68	M	" " .	430	1819	4.0	25.2	70.8
87	13	M	Lebers disease	150	1234	4.8	9.6	85.6
88	13	M	" "	800	1135	2.5	6.1	91.4

Table 25 Showing results in patients with toxic amblyopia and Lebers disease (Group 8).

Case No	Age	Sex	Diagnosis	B12 ng/l	UBBC ng/l	TC0 %	TC1 %	TC2 %
89	46	F	Acute myeloid leukaemia	550	1240	2.1	10.5	87.4
90	71	F	Chronic lymphatic leukaemia	-	-	0.9	13.9	85.2
			7 w later	-	-	0.6	31.7	67.7
91	35	F	Drug overdose	190	1684	4.1	16.8	79.1
92	39	F	Enteropathy. On folate and diet	190	1164	4.0	14.8	81.2
93	58	F	Chronic renal disease	300	2181	2.0	11.5	86.5
94	70	M	Cerebrovascular accident	400	1422	4.2	13.9	81.9
95	68	F	Rheumatoid arthritis	150	1128	2.9	8,2	88.9
96	8m	M	Anaemia ? type	-	-	2.8	15.4	81.8
97	47	F	Hypertension	530	2028	4.2	23.8	72.0
98	80	F	Peripheral neuritis	170	1304	3.9	6.4	89.7
99	59	M	" "	160	1169	0	28.2	71.8
			6 m later	200	1857	0	36.9	63.1
100	5	F	Daughter of Case 19	660	1948	5.0	8.8	86.2

Table 26 showing results in patients with miscellaneous disease (Group 9).

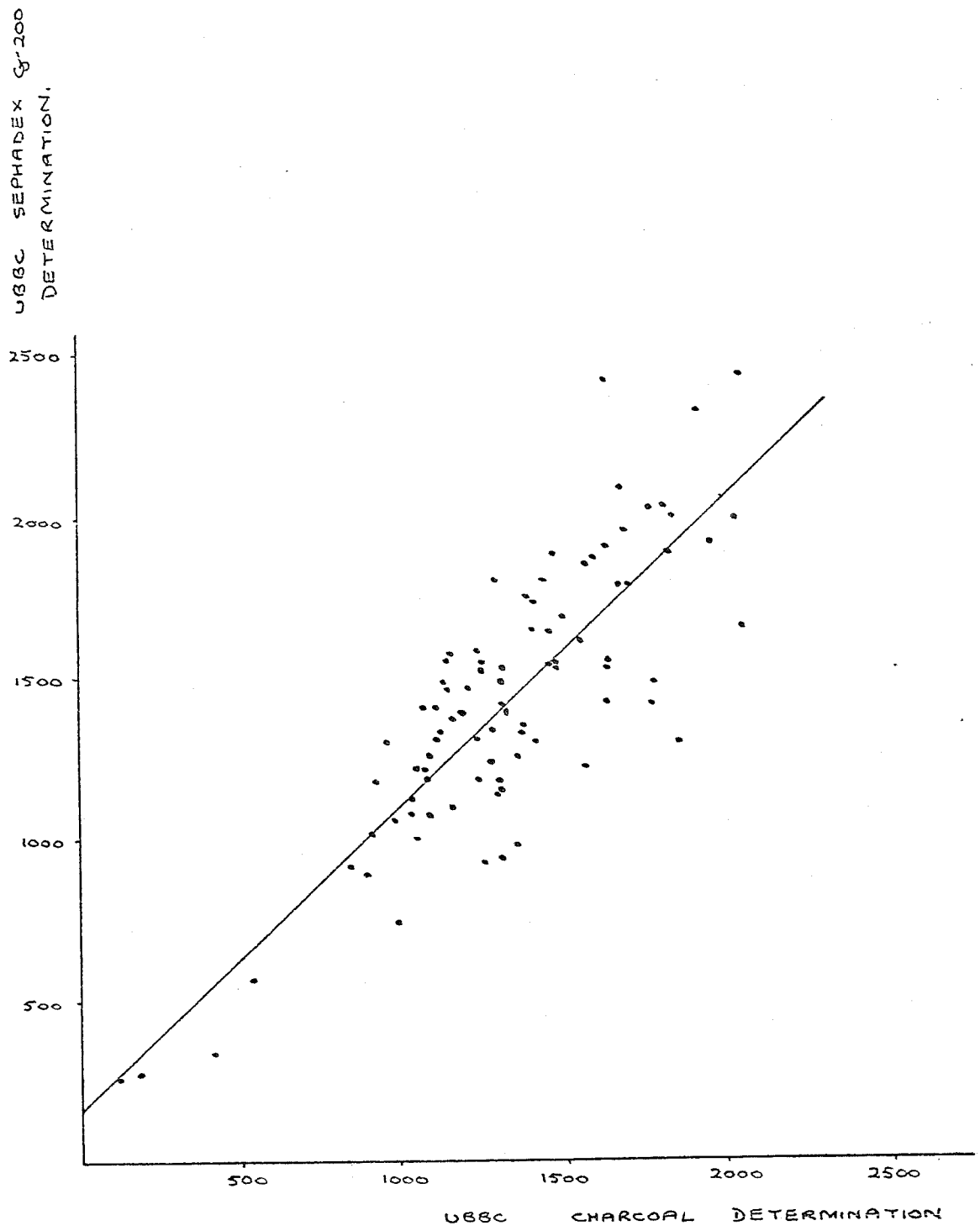


Figure 12 : A comparison of the unsaturated B12 binding capacity of serum samples as estimated by albumin-coated charcoal absorption and by Sephadex G - 200.

DISCUSSION

The initial problem which is presented by these results is to decide what can reasonably be described as normal. The distribution of samples in the control group may or may not be Gaussian. If it is assumed that they can be described by a Gaussian distribution then the normal range of values could be taken to be mean ± 2S.D. Over 95% of a normal population are included in this range

This would give normal values of:-

TCC	1.2% - 10.0%
TCI + III	4.8 - 22.4%
TCII	71.4 - 90.2

Only two of the forty-five values obtained in the control group fall outwith these ranges. The TCC peak was not consistently observed and therefore the lower limit of the range was not applied. The only sample which bound more than 10% to TCC was found in the control group. It was found that forty-two samples (of a total of 137) fell outwith this 'normal' range. Thirteen of these bound more than 90.2% of added activity to TCII and twenty-nine bound either less than 71.4% to TCII or more than 22.4% to TCI + III or both. The twenty-nine samples were reduced to the eleven 'most abnormal' i.e those which bound more than 30% to TCI + III. These samples are listed in table 27 as are

	TCI + III	TCII	group
47	62.5%	37.5%	4
59	59.1%	40.1%	6
9	42.5%	56.2%	1
7	42.3%	57.7%	1
28	37.3%	59.7%	2
99	36.9%	63.1%	9
82	34.9%	61.7%	8
30	33.8%	64.0%	2
39	33.5%	65.7%	3
90	31.7%	67.7%	9
13	31.3%	68.7%	1

Table 27 a) : showing results from patients who bound more than 30% of added ^{57} Co B12 to TCI + III.

	TCI + III	TCII	group
81	3.8%	94.9%	8
19	6.5%	93.5%	1
15	5.4%	93.2%	1
19	6.1%	92.9%	1
40	5.7%	92.6%	3
37	5.9%	92.5%	3
61	4.6%	92.2%	6
60	4.7%	91.9%	6
53	7.4%	91.7%	5
88	6.1%	91.4%	8
37	6.7%	91.4%	3
40	6.4%	91.0%	3
40	7.6%	91.0%	3

Table 27 b) : showing results from patients who bound more than 90.2% of added ^{57} Co B12 to TCII.

the samples which bound more than 90.2% to TCII.

The mean percentage bound to each protein was calculated for the patients in each group, as was the standard deviation. The results did not appear to differ significantly from those obtained in the control group (see table 28).

The significance of these abnormal results, if indeed they are abnormal, is not known. They are not related to age or sex nor are they confined to, nor even predominant in, any one clinical group. The possibility that they might correlate with serum vitamin B12 level or with the unsaturated B12 binding capacity was also examined and eliminated. No correlation was found between low serum B12 level and the percentage of activity bound by TCI + III. The samples obtained from patients in group 3 were examined as a possible source of TCI + III deficiencies. The features which were characteristic of the group (low serum B12 but normal absorption) appeared to suggest either a dietary deficiency of B12 or a TCI deficiency. No such transcobalamin deficiency was observed however inasmuch as all the samples bound some added B12 to TCI+III.

It seems reasonable to conclude that measurements of the distribution of added B12 on the transcobalamins have no practical value at the present time, apart from the cases of transcobalamin

Group Number	No. of samples	TCO		TC I+III		TC II	
		mean	SD	mean	SD	mean	SD
1 - pre	8	2.7	1.7	21.5	9.4	75.8	9.9
1 - post	20	2.7	2.1	16.7	11.4	80.6	10.8
2	13	3.1	1.7	21.4	7.6	75.5	7.6
3	15	3.3	2.3	12.0	8.7	84.7	8.3
4 - pre	6	2.9	2.4	20.3	15.9	76.8	14.5
4 - post	12	2.5	1.6	22.2	13.7	75.3	12.9
5 - pre	3	1.2	1.5	19.8	9.7	79.0	8.3
5 - post	9	1.6	1.5	17.7	7.4	80.7	6.7
6	14	3.7	1.7	15.0	13.8	81.3	12.9
7	15	5.6	2.2	13.6	4.4	80.8	4.7
8	8	3.3	1.2	15.4	10.6	81.3	11.1
9	14	2.6	1.7	17.2	9.4	80.2	8.4
	137						

Table 28 : showing a comparison of the mean values of the percentage of added B12 bound by samples from subjects in each group.

deficiency. It is possible that if details of the mass of transcobalamin present and of the distribution of endogenous B12 on the transcobalamins were known then the results might be more meaningful.

A comparison of the methods for separating free and bound vitamin B12 was described in a study by Adams and McEwan (79). They did not examine serum but reported that for human gastric juice, bile and saliva there was no significant difference between the results obtained with gel filtration (G - 10) and with coated charcoal. In contrast to this a poor correlation ($r = 0.48$) was found by Hom and Ahluwalia (28) for UBBC values as measured by Sephadex G - 200 and coated charcoal in a sample of fifty subjects.

C H A P T E R 3

THE APPLICATION OF ALTERNATIVE PROTEIN SEPARATION TECHNIQUES TO STUDIES ON THE TRANSCOBALAMINS

Introduction

Sephadex G - 200 was used for the separation of the major cobalamin binding proteins throughout the studies described in the preceding chapters. It was chosen initially because it appeared to be the method used most frequently by previous workers and therefore a direct comparison could be made with their findings. Gel filtration with Sephadex G - 200 is a slow technique (each separation takes two days) but the results show a high degree of consistency.

Alternative fractionation materials have been proposed recently for the separation of the transcobalamins. This chapter describes some of the results obtained by these methods. Direct comparisons with the results obtained using Sephadex G - 200 have been made.

DEAE - cellulose chromatography

It has been reported that TCl can be separated from TCll and TClll using DEAE - cellulose (14). Experiments were therefore carried out with a view to using DEAE in conjunction with Sephadex G - 200 in order to obtain results for all three proteins.

Materials and methods

A 1 ml. sample of serum was applied to a column (Pharmacia K9/15) containing DEAE - cellulose anion exchange resin (Whatman DE23, Whatman Biochemicals, Maidstone, Kent). The resin had been pre-treated in accordance with the manufacturer's instructions. The serum sample had been labelled with ^{57}Co B12 and the excess B12 removed with albumin-coated charcoal as described before. The serum was eluted from the ion-exchange resin with approximately 30 ml. of 0.06 M phosphate buffer followed by 30 ml. of 1M NaCl. These fractions were counted for radioactivity in a sodium iodide well crystal connected to a counter ratemeter MS 310.

The buffer eluant + approximately 30 ml. of distilled water were concentrated to 4 ml. by ultrafiltration with an Amicon cell (model 52) at a pressure of 83 kPa supplied from a nitrogen cylinder (British Oxygen Company). The sample was filtered through a UM - 05 membrane which retains all molecules > 500 A.M.U. The filtrate was checked and found

to contain no radioactivity. The salt eluant from the DEAE - cellulose column was concentrated in a similar fashion.

The 4 ml. samples obtained were applied (separately) to the Sephadex G - 200 column described earlier. The fractions which eluted were each counted for 10 minutes in the Packard automatic gamma counter.

Results

84.9% of the radioactivity which was recovered from the DEAE cellulose was eluted from the column with buffer and therefore corresponded to TCII and TCIII according to Bloomfield and Scott (14). The remaining 15.1% eluted with molar saline and was therefore TCI. When these two fractions were run on Sephadex G - 200 they both contained approximately 34% high molecular weight binder and 66% low molecular weight binder (actual values for low molecular weight were buffer eluant 66.1%, salt eluant 66.5%).

Three samples of serum from the same normal pool were obtained and the experiment was repeated. The percentage of recovered activity which was eluted with buffer was 84.5%, 83.7% and 82.1%. These values are similar to those reported by Bloomfield and Scott.

The buffer and salt eluants from the first two samples were also separated on G - 200, and the distribution of ^{57}Co B12 was:

	TCO	TCI + III	TCII
buffer eluant (1)	9.8%	25.4%	64.8%
" " (2)	16.4%	53.6%	30.0%
salt eluant (1)	1.8%	32.4%	65.8%
" " (2)	4.8%	29.4%	65.8%

At this point the experiments were discontinued because the DEAE - cellulose fractionation did not appear to separate TCI from TCII and TCIII. It would have been useful to have studied the reverse separation : fractionation of the TCI + III peak from G - 200 and also the TCII peak from G - 200 on DEAE - cellulose. This was not possible however due to the presence of 1M NaCl in the buffer used for gel filtration.

Discussion

Experiments of this nature have also been carried out by Gizis et al (31). They fractionated the DEAE - cellulose buffer eluant on Sephadex G - 200 and observed only one peak (at a molecular weight of 36,000). The diagram which illustrates this observation does not however show a clean sharp peak at this point and it could possibly be interpreted as containing more than one B12 binder. They also separated the TCI peak (salt eluant from DEAE cellulose) and observed two peaks of radioactivity. The first (approximately 67% of the eluted

^{57}Co B12) corresponded to the elution volume of TCI and the second (33%) to TCII.

Similar studies have also been carried out by Bloomfield and Scott (14). They demonstrated that the buffer eluant from DEAE - cellulose contained two proteins (TCII and TCIII) by virtue of their different elution volumes on Sephadex G - 200. There were however no results to show that the salt eluant from DEAE was homogeneous on Sephadex.

At the time of the experiments reported in this section the failure to show that the salt eluant from DEAE contained only one protein was attributed to lack of familiarity in handling the ion-exchange material. Consistent results were however obtained from the simple elution of the sample from DEAE. It is possible that the properties of the proteins are altered in some way following ion-exchange chromatography. This view is supported by the observation of England et al (20) that the transcobalamins are very labile proteins.

Ultrafiltration

A variety of membrane sizes are available for the Amicon ultrafiltration cell and it was decided to see if any of these would be suitable for the separation of the transcobalamins. The molecular weights of the B12 binding proteins which have been reported by different groups of workers have varied slightly but values of 36,000 (TCII) and 121,000 (TCI + III) seem to be reasonable estimates.

Materials and methods

These experiments were carried out using 0.5 ml. of serum labelled with ^{57} Co B12. The unbound vitamin B12 was removed by absorption on to albumin-coated charcoal as described earlier. The serum was placed in the cell (model 52, Amicon Corporation, Lexington, Mass.) and the volume was made up to approximately 50 ml. with infusion saline (0.15M NaCl). The cell was attached to a cylinder of nitrogen (British Oxygen Company) and a pressure of 83 kPa was applied. After the volume had been reduced to around 5 ml. the cell was topped up with saline solution. This was repeated until a total volume of 150 ml. was eluted There are two membrane sizes available which were thought to be suitable for this study : the XM - 50 which retains molecules >50,000 A.M.U and the XM - 100 which retains those with a molecular weight greater than 100,000.

Results

The first experiments were carried out with the XM - 100 membrane. A comparison of these results with G - 200 fractionation showed that not all of the TCII bound radioactivity was passing through. Even less of the TCII - ^{57} Co B12 was filtered in the experiment with the XM - 50 membrane. The results obtained by Amicon cell ultrafiltration were neither consistent nor comparable with those from Sephadex G - 200 (see table 29)

Serum number	Amicon retained (TCI + III)	Amicon filtrate (TCII)	Sephadex TCI + III	Sephadex TCII
1	68.0%	32.0%	26.0%	74.0%
2	38.2%	61.8%	13.8%	86.2%
3a)	80.5%	19.5%	25.0%	75.0%
3b)	64.7%	32.5%	"	"
4a)	32.5%	67.5%	39.3%	60.7%
4b)	45.3%	54.7%	"	"
5a)	22.2%	77.8%	12.6%	87.4%
5b)	28.8%	71.2%	"	"
5c)	29.9%	70.1%	"	"
5d)	35.1%	64.9%	"	"
5e)	56.0%	44.0%	"	"

Table 29 showing the differences in the separation results obtained from Sephadex G - 200 and ultrafiltration.

All samples were concentrated at a pressure of 83 kPa except 4b) which was at a pressure of 166 kPa. The ultrafiltration was through an XM - 100 membrane in each case.

Five different serum samples were used, replicate samples are indicated as a), b), c) etc.

The most disconcerting aspect of the results was the rate at which the filtered radioactivity was recovered. An example of this is shown in a sample of serum from case 4 when the 67.5% of the radioactivity was made up of the following amounts (each referring to a volume of approximately 18 ml.)

Filtrate	
1	17.0%
2	12.7%
3	10.6%
4	5.2%
5	7.1%
6	6.6%
7	3.6%
8	3.9%

A further 20 ml. of saline was added and when filtered it contained 0.8% of the total radioactivity.

Discussion

The use of affinity chromatography to produce pure proteins has allowed more accurate estimates of the molecular weights of the two transcobalamins (26, 29) to be made. It now appears that the molecular weights of both binders are in the range of 50,000 - 60,000. Previous reports of 121,000 for TCI + III were due to a high carbohydrate content which is responsible for its altered elution from Sephadex G - 200. The value obtained for TCII appears to be the result for only one of its peptide chains, as there is now evidence (26) that the molecule is a dimer.

In the light of this new information there is no reason to expect that the proteins can be separated by ultrafiltration.

Ammonium Sulphate precipitation

The polyfractionation of serum by protein precipitation with ammonium sulphate, $(NH_4)_2SO_4$, was first reported by Efferse (80). This method has been adapted and reported as suitable for the separation of the vitamin B12 binding proteins by two separate groups (81, 82). Although differing slightly in the details the two methods described are very similar.

Materials and Methods

Method 1 (Carmel)

A simple one-step separation using 0.5 ml serum, 1.4 ml 0.4M K_2HPO_4 and excess ^{57} Co B12. The unbound B12 was removed by adding this mixture to a tube containing a pellet of 1 : 20 haemoglobin-coated charcoal (prepared by centrifuging 1 ml of coated charcoal suspension and sucking off the supernatant.) The serum bound B12 (in the charcoal treated supernatant) was added to a tube containing 4 ml. of 3M $(NH_4)_2SO_4$ and mixed by inversion. The mixture was incubated for 30 minutes at room temperature followed by centrifugation for 15 minutes at 3,000 x g. The TCII bound B12 was precipitated under these conditions but the TCI + III bound B12 remained in the supernatant.

Method 2 (Begley & Hall)

2 ml of serum was labelled with excess ^{57} Co B12, incubated at 37°C for 20 minutes, and dialysed overnight against 0.04M sodium phosphate containing 0.85% NaCl (final pH 7.4) to remove free B12. Half the sample was mixed with 0.1 ml of I.)M sodium acetate (pH 7.8) and brought to final volume of 4 ml with distilled H_2O. 1.19g of $(NH_4)_2$ SO_4 was added and dissolved in small amounts. The sample stood 17 - 18 hours at 4°C before centrifugation at 17,300 x g for 30 minutes at 4°C. The TCII - B12 was precipitated by this process and TCI + III bound B12 was left in the supernatant.

Serum samples from ten subjects were used in the experiments described here. An aliquot of serum from each had previously been separated on Sephadex G - 200 which meant that a comparison of results was possible. The samples were chosen from patients who appeared to have an abnormal binding pattern.

The procedure described by Carmel was followed exactly, apart from the substitution of albumin-coated charcoal for haemoglobin-coated charcoal. Four samples were prepared for each subjects : to two of them 2M $(NH_4)_2$ SO_4 was added and to the remaining two 3M $(NH_4)_2$ SO_4. There is ambiguity in the paper about which of the two concentrations of ammonium sulphate solution was used.

The method described by Begley and Hall was followed with the removal of free B12 by albumin-coated charcoal (1 ml) instead of dialysis. Duplicate samples were also used in this method.

Results

The results (the mean value of duplicate samples) are given in table 30. The precipitation with 2M ammonium sulphate solution did not give results comparable with G - 200 : very little protein was precipitated at this strength and these results are not included in the table. A good correlation was obtained between G - 200 and 3M ammonium sulphate precipitation ($r = 0.94$, $n = 10$) and also between the Begley and Hall method and G - 200 gel filtration ($r = 0.91$, $n = 10$). There was also a good correlation between the results obtained from the two charcoal methods ($r = 0.98$, $n = 10$). These results are significant at the $p = 0.01$ level.

Discussion

The two methods described are basically very similar : the main differences are in the buffer used, the length of time allowed for precipitation and in the speed of entrifugation. The pellet obtained is firmer after high speed centrifugation and this makes it easier to remove the supernatant without disturbing the precipitated proteins. The effective concentration of ammonium sulphate in the final solution is 2M

Case no.	Carmel ammonium sulphate precipitation	Begley & Hall ammonium sulphate precipitation	G - 200
4	93.5%	87.0%	92.1%
4	83.2%	80.6%	75.2%
8	70.2%	67.3%	60.3%
19	92.6%	82.2%	93.5%
28	68.1%	63.1%	59.7%
39	83.6%	78.9%	65.7%
41	67.4%	63.8%	57.2%
44	93.2%	83.9%	88.2%
61	94.2%	90.3%	92.2%
65	94.2%	86.8%	88.2%

Table 30 : showing percentage of added activity bound by TCII as determined by three different methods.

in the Carmel method and 2.25M in the technique described by Begley and Hall. At a concentration of 1.5 - 2.3M TCII is precipitated whereas to obtain precipitation of TCI + III one must use a 2.5 to 3.5M ammonium sulphate solution (8).

Either of the two methods described here would be suitable for a rapid routine separation of added B12 into TCI + III bound B12 and TCII bound B12. The method described by Begley and Hall is slightly more laborious but gives results which compare well with those obtained by G - 200. The results obtained from the Carmel method are in nine of the ten cases higher than those obtained by G - 200 and in all ten cases higher than those from the Begley and Hall Method Although a very good correlation was obtained between the values obtained by the Carmel method a t test of the differences between these results and the others showed that there was a significant difference between the values. This is due to the increased magnitude of the results obtained by the Carmel method. There was no significant difference at the $p = 0.01$ level between the results obtained with Sephadex G - 200 and with the Begley and Hall precipitation technique.

Although the method described by Begley and Hall (82) gives results which are closer to those obtained with G - 200 than was found using the Carmel method (81) this study was

only a small, preliminary investigation. It would be necessary to use a larger sample in order to make meaningful comparisons of the methods.

In addition to the three separation methods described, the B12 binding proteins have been fractionated by conventional methods of protein separation e.g. polyacrylamide gel electrophoresis as well as by some more unusual methods. Included in the latter category are:

i) the use of zirconyl phosphate gel to selectively bind TCII (83)

ii) the absorption of TCII on a microfine precipitate of silica (CuSo G32) (84).

iii) the separation of the proteins on charged cellulose filters : trapping TCII on cellulose-nitrate filters and TCI on DE-81 filter discs. (85).

It is possible that one of these separation methods would also be useful for rapid diagnostic purposes. Whether this information would be of value clinically is perhaps another matter, for the spread of results observed in the preceding chapter might indicate that information on the distribution of added B12 would be of little practical value.

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