PLASMA PROTEIN BINDING

OF

VITAMIN B_{12}

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THESIS

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BY

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****

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I hereby give my consent for one copy of my thesis submitted for the degree of Doctor of Medicine, entitled "Plasma Protein Binding of Vitamin B_{12}" to be deposited in the University Library, understanding that it will be available for both loan and photo-copying.

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CONTENTS

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A thesis is a record of original work, and this thesis has been certified as such. It is also true that, in matters of subject selection, guidance, encouragement and support, the author of a thesis is indebted to a large number of people and organizations, who contributed in some way towards the completion of the work.

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CERTIFICATION OF ORIGINALITY.

.In accordance with the Regulations set down, I hereby declare that this thesis is my own composition, and that the work described in this thesis is original work, carried out by me, initially in the Clinical Research Unit of the Royal Prince Alfred Hospital, Camperdown, New South Wales, and the Department of Medicine, the University of Sydney, between January 1962 and January 1964, and at the Red Cross Blood Transfusion Service, Adelaide, South Australia, since March, 1964. The acknowledgements which precede this Certification, and the list of publications at the end of the thesis (Appendix B), indicates the extent to which I am indebted to other persons for assistance in this work. The material contained in this thesis has not been submitted for any other degree.

It is also required (Regulation 4b) that "the candidate shall indicate wherein he considers the thesis to advance medical knowledge or practice". This has been set out in the Introduction (Chapter 1) under the heading "Significance of Results".

CHAPTER 1

INTRODUCTION

INTRODUCTION

The introduction of radioactive isotopes into investigative medicine less than two decades ago has aided greatly in the understanding of the processes of absorption, utilization, turnover and excretion of many biological substances; the advances in the knowledge of the mechanisms of normal and abnormal haemopoiesis have been at least as substantial as those in other disciplines. Several isotopes are available for labelling erythrocytes, leukocytes and platelets, and three important haemopoietic building blocks, namely, iron, cyanocobalamin and folate, are available in one or more radioactive forms.

The radioactive isotopes used in the investigation of vitamin B_{12} metabolism fulfil several of the important criteria laid down for biological acceptability; the radioactive cobalt atom is an integral part of the molecule, and not an extraneous "tag", and labelling is therefore specific. However, for a number of reasons, investigations of vitamin B_{12} metabolism have not proceeded as far as comparable studies of iron metabolism. These reasons include:

i) the vitamin is physiologically active in relatively low concentrations;

ii) the radioactive isotopes generally used have specific activity so low as to make it difficult, if not impossible, to carry out investigations

with truly physiological quantities;

iii) cyanocobalamin has a number of biologically active analogues, and it is still not certain in which form or forms the vitamin exists in man;

iv) whereas iron is transported in plasma by a specific binding protein, transferrin, there are at least two specific in vivo binding proteins for vitamin B_{12}, and the interrelation between these two specific binders, which is still not clarified, may be altered in disease;

v) whereas iron is bound in vitro by transferrin only, vitamin B_{12} is bound by a number of electrophoretically separable protein moieties.

Purpose of the Present Study

In general terms, a study of the plasma protein binding of vitamin B_{12} has relevance in at least three areas; first, plasma protein binding of vitamin B_{12}, especially in vitro, is a facet of the metabolism of the vitamin which is not fully understood; second, there are important aspects of protein chemistry involved; and third, an understanding of the abnormality of protein binding of vitamin B_{12} seen in myeloid leukaemia may well throw some light on the understanding of this disorder.

The several specific, and interrelated, aims of the present study are:-

i) to evaluate critically the dialysis method of measurement of vitamin B_{12} binding capacity in vitro, and to evolve a standardized method which takes into account those factors which produce variation in binding;

ii) to investigate, by different methods of protein separation, the in vitro binding of vitamin B_{12} in normals, and in those situations where vitamin B_{12} binding is abnormal, such as chronic myeloid leukaemia;

iii) to assess the effect of leukocytes on vitamin B_{12} binding by plasma proteins;

iv) to investigate the vitamin B_{12} binding properties of isolated protein fractions.

Significance of Results.

It is considered that the following conclusions may be drawn from the results of the material presented and discussed in this thesis, and that these conclusions are original observations:-

1. That a method of measurement of in vitro vitamin B_{12} binding capacity of plasma proteins has been described which takes into consideration a number of variable factors not previously assessed in other methods;

2. That in vitro binding of vitamin B_{12} to an abnormal protein in myeloid leukaemia occurs preferentially, and not as an overflow phenomenon following upon saturation of normal binding proteins;
3. That separated α_1 acid glycoprotein will bind significantly more vitamin B_{12} added in vitro than all other protein fractions;
4. That increased in vitro binding can occur in patients with myeloid leukaemia at a time when the leukocyte count is normal, as a result of therapy. It has been considered previously that the increased capacity for binding vitamin B_{12} was seen characteristically in patients with untreated myeloid leukaemia, and that this increased binding capacity fell towards normal when treatment was instituted to lower the total leukocyte count and eliminate primitive forms from the peripheral circulation.

In addition, evidence is presented which adds to, and extends, previous knowledge in this field. It has been shown that:-

- Increased binding of vitamin B_{12} to plasma proteins takes place in patients with myeloid leukaemia;

- The abnormal vitamin B_{12} binding protein in myeloid leukaemia has many of the characteristics of α_1 acid glycoprotein, or a component of this protein complex;
- Normal granulocytes, or the breakdown products of normal granulocytes, may influence plasma protein binding of vitamin B_{12} added in vitro.

Format of the Thesis.

The format of this thesis is a conventional one; three features only require comment.

i) In the light of recent statements concerning thesis references (Witts, 1967), the references in this thesis are given in full.

ii) Two case histories are appended in detail (Appendix A) because of the importance of findings in these two patients to certain of the material contained in the thesis.

iii) In addition to the two papers attached, several other papers based on this material have been presented at meetings of the Australian Society for Medical Research, the Haematology Society of Australia, and at the XIth Congress of the International Society of Haematology, Sydney, 1966; a list of these papers is attached at Appendix C. A further list of publications of material contained in this thesis

is attached at Appendix B, including a number of abstracts of papers referred to in Appendix C.

Conventions

i) The references for this thesis are styled in the manner laid down in the World Medical Association's publication, "World Medical Periodicals", 3rd edition, 1961.

ii) The following conventions of terminology have been observed throughout the thesis:

(a) except where otherwise specifically indicated, the term "Vitamin B_{12}" is understood to refer to cyanocobalamin. In general, the terminology used by authors whose work is referred to in the literature review has been maintained in this respect.

(b) the term "myeloid leukaemia" has been used throughout the work, and this term is used to cover other synonymous (or nearly synonymous) terms used by various authors, including granulocytic leukaemia and myelocytic leukaemia.

(c) the units of measurement of vitamin B_{12} used are in general, those used by the authors

referred to in the literature review; thus, in some parts, the terms nanogram (ng.) and picogram (pg.) are used, and in other parts millimicrograms (mμg.) and micromicrograms (μμg.) are used, according to the style of the authors.

(d) the only abbreviations used in this thesis are those referring to bacteria such as Lactobacillus leichmanii (abbreviated as L. leichmanii) and Euglena gracilis (abbreviated as E. gracilis) and those referring to certain chemicals in which the commonly used name is a shortened form of the correct chemical name, e.g. DEAE cellulose is used for diethylaminoethyl-cellulose, CM cellulose is used for carboxymethyl-cellulose, and the term "tris" is used for tris (hydroxymethyl) aminomethane.

CHAPTER 2

REVIEW OF THE LITERATURE

REVIEW OF THE LITERATURE

2.1 Historical Aspects.

Pernicious anaemia has been recognised as a disease entity for more than one hundred years, and for more than half that time it was truly pernicious, and in most instances lethal, until it was found that raw liver would produce relief of the symptoms and signs of the megaloblastic anaemia and neuropathy (Minot and Murphy, 1926).

Castle described, in his classical experiments (Castle and Townsend, 1929), the effectiveness of meat and normal gastric juice in achieving a remission in patients with pernicious anaemia, compared with the ineffectiveness of meat alone, and gastric juice alone. He proposed that pernicious anaemia was due to a lack of gastric secretion, and that an intrinsic (or gastric) factor was required to combine with the extrinsic (food) factor to make the active principle available. This concept has been modified only to the extent that the extrinsic factor is now known to be vitamin B_{12}, and that it is considered that intrinsic factor mediates the intestinal absorption of vitamin B_{12} without altering its chemical structure.

More than twenty years intervened between the

discovery of the effectiveness of raw liver, and the eventual isolation of the active principle by two groups, working independently in the United States and Great Britain (Rickes et alii, 1948; Smith and Parker, 1948). The initial clinical studies (West, 1948) showed that the isolated material was effective in eliciting a therapeutic response in pernicious anaemia patients.

Although the chemical structure of vitamin B_{12} has been determined by a number of workers since its initial isolation (Smith, 1965), it has not yet been possible to synthesize the vitamin. Smith (1965) has reviewed in detail these aspects of vitamin B_{12}.

The earliest studies on the binding of vitamin B_{12} by plasma proteins came from Pitney, Beard and van Loon (1954). Subsequent developments in this field, the subject of this thesis, are discussed under the appropriate headings in later sections of the review.

2.2 Chemistry of Vitamin B_{12}*

2.2.1 Structure.

Vitamin B_{12}* is a red crystalline compound, which was

*When used in this way, the term "vitamin B_{12}" is taken to mean cyanocobalamin, unless otherwise specified.

first isolated in 1948 (Rickes et alii, 1948; Smith and Parker, 1948). It is a complex porphyrin derivative containing a tervalent cobalt atom, and linked to a nucleotide. Nomenclature of the several forms of vitamin B_{12} has been based on the nature of the nucleotide group; cobalamin (5, 6 dimethylbenzimidazolylcobamide) is the term reserved for those compounds having the 5, 6 dimethylbenzimidazole base. The formula agreed upon for vitamin B_{12} (Documenta Geigy, 1962; Smith, 1965) is $C_{63}\ H_{88}\ O_{14}\ N_{14}\ P\ Co$, giving a molecular weight of 1355.42; a suggested structure based upon existing knowledge is shown at Figure 1.

Cyanocobalamin has absorption maxima in water at 2790Å, 3610Å and 5500Å; hydroxocobalamin has corresponding maxima at 2730Å, 3510Å and 5250Å.

Certain analogues of cyanocobalamin and their clinical and laboratory aspects are discussed below.

Stahlberg (1964), in a preliminary report, suggested, on the basis of growth studies, that the dominant form of vitamin B_{12} in circulating plasma was the so-called "fourth factor", in all probability methylcobalamin.

The chemistry of cobalt per se within the mammalian body has been reviewed extensively by Carlberger (1961).

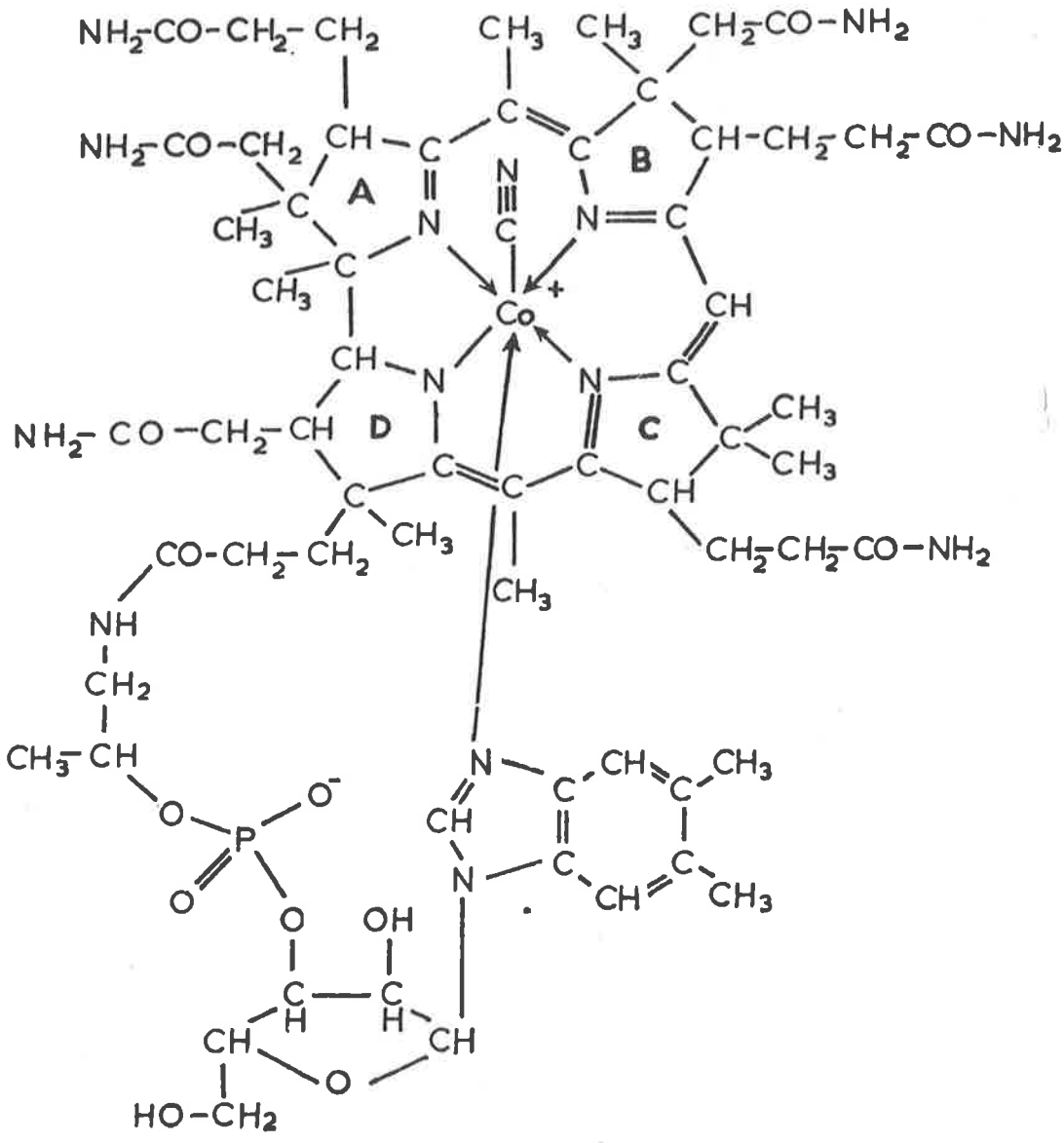

Figure 1. Structural formula of cyanocobalamin.

From Lester Smith (1965).

TABLE I.

DERIVATIVES OF COBALAMIN
(after Lester Smith, 1965)

ORIGINAL NOMENCLATURE	ION OR MOLECULE CO-ORDINATED	SEMI-SYSTEMATIC NAME
Vitamin B_{12}	CN^-	Cyanocobalamin
Vitamin B_{12a}	OH^- (alkaline solution)	Hydroxocobalamin
Vitamin B_{12b}	H_2O (acid solution)	Aquocobalamin
Vitamin B_{12c}	ONO^-	Nitritocobalamin
	SCN	Thiocyanatocobalamin
Ammonia Cobalichrome	NH_3	
Histidine Cobalichrome	Histidine	

2.2.2 Derivatives and Analogues of Vitamin B_{12}

Several derivatives of vitamin B_{12} are known; these are compounds in which the major part of the molecule is retained; a list of those recognised at present is at Table I (After Smith, 1965). Of these, only cyanocobalamin and hydroxocobalamin have been extensively studied in man (see below).

Vitamin B_{12} occurs in nature as a family of closely related chemical compounds; a number of analogues of vitamin B_{12}, both naturally-occurring and synthetic, have been recognised (Smith, 1965). Their relevance to this thesis concerns differences observed in serum binding of cyanocobalamin after the saturation of binding sites with vitamin B_{12} analogues (Meyer et alii, 1963, 1965) and the implication of these findings on the mechanism of protein binding. Detail of the analogues of vitamin B_{12} is given by Lester Smith (1965) and Rosenblum (1965).

2.2.3 Relationship of Structure to Clinical Effectiveness.

There is now substantial evidence for the clinical superiority of the cobalamin derivative hydroxocobalamin over cyanocobalamin. Jerzy Glass and his colleagues (Glass, Lee and Hardy, 1961; Glass, Skeggs, et alii, 1961) showed that a single intramuscular dose of hydroxocobalamin

gave higher blood levels than similar doses of cyanocobalamin at intervals of from 5 hours to 2-4 weeks. Similar findings were obtained when hydroxocobalamin was compared with a cyanocobalamin-zinc tannate complex, a slow release preparation; the only real advantage of the cyanocobalamin-zinc tannate complex is the relative infrequency of injection compared with cyanocobalamin (Gough et alii, 1964). As would be expected, the loss of hydroxocobalamin in urine was much less than the urinary loss following similar injections of cyanocobalamin and cyanocobalamin-zinc tannate complex. Labelled hydroxocobalamin was shown to be more slowly absorbed from the site of injection, and was stored in liver and muscle to an equal or greater extent than cyanocobalamin. The evidence of Adams and Kennedy (1965) also supported the finding of reduced urinary output of hydroxocobalamin; they suggested that its advantages over cyanocobalamin as a maintenance agent were only marginal.

Glass, Lee et alii, (1963) showed that, after large weekly injections of hydroxocobalamin, blood levels were obtained which were higher, and longer sustained, than those observed with cyanocobalamin. Later work from this group extended these findings, and confirmed the

efficiency of hydroxocobalamin (Glass and Lee, 1966; Glass et alii, 1966).

A comparison was made by Hertz et alii (1964) between the retention of intramuscular cyanocobalamin and intramuscular hydroxocobalamin - the levels at 24 hours were 20% cyanocobalamin retained, and 75% hydroxocobalamin retained. The reasons offered for this finding are discussed below (2.2.4).

2.2.4 Relationship of Structure and Protein Binding.

Meyer (Meyer et alii, 1963) investigated the vitamin B_{12} binding capacity of various analogues of vitamin B_{12} at differing levels of added vitamin B_{12}, in accordance with the methods previously described (Meyer et alii, 1957). They measured the binding capacity of serum for radioactive hydroxocobalamin, and also assessed the effect of prior addition of non-radioactive analogues on the subsequent serum binding of radioactive cyanocobalamin. It was found that the pattern of binding obtained with hydroxocobalamin showed less tendency to be flattened after the initial rapid phase of binding, and that there was increased binding compared with cyanocobalamin, especially in the second phase. Analogues were found to compete for binding sites on the serum proteins to the same degree as cyanocobalamin.

It was concluded that when changes were made in the $CO-NH_2$ groups on either side of the B pyrrol ring, blocking of phase I binding did not occur, suggesting that these $CO-NH_2$ groups participate in phase I binding. The cobalt atom itself is thought to be responsible for phase II binding. The Co-CN bond appears to dissociate in aqueous solution, leaving the cobalt atom free to combine with protein receptors.

Hertz et alii (1964) used a series of dialysis experiments to show that hydroxocobalamin passed more slowly through a semi-permeable membrane than did cyanocobalamin, and that hydroxocobalamin was bound to serum proteins in greater quantities than cyanocobalamin. These two findings are put forward as an explanation of the decreased urinary loss and increased retention of injected hydroxocobalamin (2.2.3 above).

In a later study, Meyer et alii (1965) compared the plasma protein saturating or blocking effect of various analogues of cyanocobalamin. They found that hydroxocobalamin was the most potent analogue in promoting rapid plasma disappearance of injected radioactive cyanocobalamin; the aquanalide analogue had an intermediate effect, and the

others tested had no effect. This may be explained by an increased avidity of binding of hydroxocobalamin to serum proteins in vitro (Meyer et alii, 1965; Hertz et alii, 1964), and the longer retention for hydroxocobalamin at sites of injection (Glass et alii, 1961).

2.2.5 Radioactive Vitamin B_{12}

Smith (1965) records the attempts made to obtain radioactive vitamin B_{12}. Carbon^{14} was used but was found to be too labile for use in biological investigations. Tritium labelling of vitamin B_{12}, although not as specific as radioactive cobalt labelling, has been used to study by autoradiography, the intracellular incorporation of vitamin B_{12} into bone marrow and liver cells (Taglioretti et alii, 1963).

High specific activity radioactive cobalt labelling has been achieved by fermentation in the presence of radioactive cobalt in a medium lacking non-radioactive cobalt. Extensive use has been made of the isotopes of vitamin B_{12}, as is apparent from later aspects of this review. Mollin (1959) and Pribilla (1961) have summarised most of the clinical applications of radioactive vitamin B_{12} in blood disorders and in digestive disturbances.

2.3 Metabolism of Vitamin B_{12}

As yet, the biochemistry of vitamin B_{12} is not clearly understood. The metabolically active form of the vitamin is a coenzyme (coenzyme B_{12}) in which the cyano-group of cyanocobalamin is replaced by adenine nucleoside (Smith, 1965). Lack of vitamin B_{12} produces inhibition of methionine synthesis, as a result of which the formation of essential amino acids and proteins is decreased. Further, vitamin B_{12} deficiency will block the synthesis of ribonucleic acid. These disturbances of protein metabolism are most marked in those cells, such as haemopoietic and nervous system cells, which have high protein turnover and mitotic frequency (Documenta Geigy, 1962; Smith, 1965).

While the extent of the studies of vitamin B_{12} metabolism in man, and in many other living organisms in which it is relevant, is very considerable, this review will deal only with those aspects of metabolism which are relevant to the main content of this thesis.

2.3.1 Absorption.

The classic experiments of Booth and Mollin (Booth et alii, 1957; Booth and Mollin, 1959) showed first in rats, and then in man, that the principal site of vitamin

B_{12} absorption was the lower portion of the small intestine. They also postulated the presence of a specific vitamin B_{12} receptor system.

Ronnov-Jensen and Hansen (1965), using the techniques of intestinal intubation with polyethylene glycol as a marker, confirmed that most vitamin B_{12} was absorbed in the ileum, but showed also that there appeared to be, in some instances, absorption at a much higher level within the small intestine.

In work with guinea pigs, Cooper et alii (1962) showed that intact guinea pig intestine will absorb cyanocobalamin bound to human intrinsic factor; two fractions were identified - fraction SE, which could be removed by EDTA, and fraction E, which remained unaltered. They stressed also the importance of calcium ions in absorption of vitamin B_{12}, a point of emphasis which was also made by Gräsbeck et alii (1959), who showed that an oral chelate such as EDTA would reduce the urinary radioactivity of normal subjects undergoing Schilling tests, and that in some patients with steatorrhoea, the failure to absorb vitamin B_{12} may be due to lack of calcium ions. Herbert's group (Retief et alii, (1967) have also stressed the importance of

calcium in vitamin B_{12} metabolism by demonstrating that the delivery of vitamin B_{12} from serum proteins to reticulocytes is calcium dependent.

It has been suggested that prednisone may increase the absorption of vitamin B_{12} from the gastrointestinal tract by stimulating gastric production of intrinsic factor (Kristensen and Friis, 1960). Weinstein and Watkin (1960) studied the absorption of ingested radioactive vitamin B_{12} in patients with haematological disorders. They could demonstrate no correlation between the vitamin B_{12} level of serum and the amount absorbed, a finding which confirmed the work of Heinrich and Erdmann-Oehlecker (1956b) and Halstead et alii (1956). As corollaries, Weinstein and Watkin (1960) suggested that the serum vitamin B_{12} concentration does not control the amount of vitamin B_{12} absorbed; and further, that the increase of plasma concentration of unsaturated vitamin B_{12} binding proteins did not influence the amount of vitamin B_{12} absorbed, since the binding capacity of plasma from chronic myeloid leukaemic patients was markedly increased (Pitney et alii, 1954; Weinstein et alii, 1959). They concluded that increased gastrointestinal absorption of vitamin B_{12} could not be

implicated in the abnormalities of serum vitamin B_{12} and vitamin B_{12} binding capacity seen in myeloid leukaemia.

Doscherholmen and Hagen (1959) used Co^{60}-labelled vitamin B_{12} to allow them to give, orally, physiological doses of vitamin B_{12}. They provided confirmation of the pattern of absorption described by Booth and Mollin (1956), in which a peak of radioactivity was achieved 8-12 hours after ingestion. This peak could be distinguished by them from the "early-rise" curves observed by other authors using massive (i.e. non-physiological) dosages. They inferred from these data that there were two different modes of absorption.

Herbert has recently summarised the data on the nutritional requirements for vitamin B_{12} (Herbert, 1966); the suggested normal minimum daily requirement for an adult is of the order of 0.1 µg.

Although it is recognised that unphysiological doses may be absorbed from a number of other mucosal surfaces, including the upper gastrointestinal tract (Chalmers and Shinton, 1958), the nasal membranes (Monto and Rebuck, 1954; Israels and Shubert, 1954), the alveolar surfaces of the lungs (Shinton and Singh, 1967), and the rectum (Ungley, 1955), none of these modes of absorption has any real therapeutic significance.

2.3.2 Plasma Clearance - Normal and Abnormal.

(Although it is recognised that the terms "plasma clearance" and "plasma disappearance" are not synonymous, they are used in the literature to describe the same phenomenon; therefore, although this section is headed "Plasma Clearance" the two terms are used interchangeably, according to the usage of the authors concerned.)

Most of the early investigations directed towards tracing the fate of injected radioactive vitamin B_{12} are open to the criticism that decidedly unphysiological quantities of vitamin B_{12} had to be used so that subsequent radioactivity measurements could be performed with a reasonable degree of accuracy.

Mollin et alii (1956), after administering the smallest dose of ^{56}Co-labelled vitamin B_{12} compatible with accurate measurement, found that the rate of plasma clearance was slowest in patients with chronic myeloid leukaemia, and in those with pernicious anaemia in severe relapse. They considered that there was a correlation between the rate of plasma clearance and the degree of serum unsaturation, since the slower clearance appeared to be proportional to the vitamin B_{12} binding capacity.

A larger dose of 4 μG., which exceeded the binding capacity of normal serum but which was within the range of total binding capacity of chronic myeloid leukaemic serum, was used by Miller et alii (1957). They demonstrated that there was a clear-cut differentiation between normals and patients with chronic myeloid leukaemia, and postulated a biochemical abnormality in the plasma of such patients.

Hall (1960) gave 0.043 μG. of high specific activity Co^{58}-vitamin B_{12}, and noted an initial rapid removal of more than 80% in one hour; this was taken up by the tissues and not excreted. In vitro serum or plasma binding prior to administration did not affect the rate of disappearance of the radioactive vitamin. An increase in dosage of up to 2×10^3 did not significantly alter the fraction removed per unit time.

It was also shown by Ritz and Meyer (1960) that the plasma disappearance of intravenously injected radioactive vitamin B_{12} was delayed in patients with myeloid leukaemia. A large loading dose of crystalline vitamin B_{12} given to these leukaemic patients corrected the delayed clearance to a more rapid, normal clearance, possibly because the non-radioactive vitamin B_{12} injected saturated the plasma

receptors, and thus enhanced the tissue binding of the radioactive vitamin.

Abnormally slow plasma clearance curves were described by Brody et alii (1960) in patients with chronic myeloid leukaemia and in patients with deficiency of vitamin B_{12}. They ascribed this phenomenon in the former group to the increased binding capacity of plasma proteins, and in the latter, to the lack of a hypothetical plasma "B_{12}-transferase".

Hall (1964) later studied the long-term excretion of Co^{57}-labelled vitamin B_{12} in eight normal subjects. He found that there was rapid plasma disappearance in a complex manner until the end of the second month, and a subsequent linear loss from the plasma, with the average $T\frac{1}{2}$ being 690 days.

The finding of delayed plasma disappearance of radioactive vitamin B_{12} in myeloproliferative disorders, notably chronic myeloid leukaemia, was discussed by Hall (1961), who considered that the findings indicated an abnormality of vitamin B_{12} metabolism common to the diseases of the myeloproliferative group.

Meyer (Meyer et alii, 1965) investigated the plasma disappearance of radioactive cyanocobalamin after the

administration of vitamin B_{12} analogues (see Section 2.2.4 above), and demonstrated rapid plasma disappearance following the prior administration of hydroxocobalamin.

The complexity of the mechanisms involved in plasma clearance of injected vitamin B_{12} was apparent to Hall and Finkler (1962) when they postulated that vitamin B_{12} was transported in plasma by more than one binding protein, and that the different disappearance rates found with plasma fractions suggested different functions in the transport of vitamin B_{12} by various proteins. This postulate was a significant one in determining the direction of their further studies (Hall and Finkler, 1963, 1964, 1965, 1966a, 1966b).

Hall and his co-workers (Hall, Kulona and Oka, 1962) studied the plasma disappearance of intravenously injected radioactive vitamin B_{12} in vitamin B_{12} deficiency states. They found that plasma disappearance of vitamin B_{12} was delayed in patients with untreated pernicious anaemia, but was normal in patients whose vitamin B_{12} deficiency had been corrected by either long-term treatment, or a large dose of vitamin B_{12} prior to the investigation. A similar abnormality was demonstrated in patients whose vitamin B_{12} deficiency was on the basis of tapeworm

(Diphyllobothrium latum) infestation; however, tapeworm carriers without overt vitamin B_{12} deficiency showed normal plasma disappearance of vitamin B_{12}. They considered that the delayed disappearance was related to a tissue or plasma defect which was not permanent. It was not possible to determine from this study, which is at variance with the findings of Brody et alii (1960), whether this phenomenon of slow disappearance in vitamin B_{12} deficiency was due to an increase in vitamin B_{12} binding, an abnormally firm binding, a defect in tissue extraction of circulating vitamin B_{12}, or a shift of vitamin B_{12} to the plasma from the tissues.

Reizenstein et alii (1963) used a double isotope technique to compare the fate of injected and absorbed vitamin B_{12}. They found that, in the four patients studied, the plasma clearance of absorbed vitamin B_{12} was more rapid than that of intravenously administered vitamin B_{12}, even though the rate of intake was greater after intravenous injection. Their cautious conclusions were that the behaviour of oral and parenteral vitamin B_{12} cannot be compared during the initial period after administration, and that the more rapid clearance of oral vitamin B_{12} is due to factors other than the

differences in rates of ingress and distribution resulting from differing modes of administration. Hall (1964) has also commented that plasma vitamin B_{12} is never in equilibrium with the total body vitamin B_{12}, but appears to be higher than expected if it were to be regarded as an accurate reflection of the body's vitamin B_{12} stores.

2.3.3 Storage.

The complexity of the relationship between circulating vitamin B_{12} and body vitamin B_{12} stores has been referred to above (Reizenstein et alii, 1963; Hall, 1964). Most of the body's vitamin B_{12} is stored in the liver following plasma clearance; this applies in the normal state and in myeloid leukaemia (Meyer et alii, 1964).

Reizenstein (1959a) has argued that there are possibly three compartments within the body for vitamin B_{12} storage, with each compartment having a different turnover rate.

An important observation in this context is that of Weinstein and Watkin (1960), who calculated that a shift of 3% of the liver vitamin B_{12} to the plasma compartment would be required to increase the serum concentration tenfold. This mechanism, if it exists, could be involved

in the explanation of increased serum vitamin B_{12} levels in myeloid leukaemia and related diseases.

The major drawback to these and other studies of vitamin B_{12} storage is the difficulty in tracing labelled vitamin B_{12} once it is present in one or other of the tissue compartments.

2.3.4 Excretion.

Vitamin B_{12} when excreted appears in urine and faeces, and use has been made of these excretory routes in assessment of vitamin B_{12} uptake (Heinle et alii, 1952; Schilling, 1953). Watkin et alii (1961) claimed that at plasma levels from 0.3-1.5 mμg./ml. urinary excretion of vitamin B_{12} was a function of the degree of binding of the vitamin B_{12} by plasma proteins; when higher levels were induced (12-26 mμg./ml.) urinary excretion of vitamin B_{12} was found to be proportional to the plasma level of vitamin B_{12} and to the glomerular filtration rate. Nelp et alii (1964) attempted to use this finding to measure the glomerular filtration rate in a simplified manner.

Hall (1964), in long term studies, showed that urinary excretion was higher shortly after the intake of vitamin B_{12} than subsequently, regardless of the route of intake. Faecal excretion was not uniform until at least a month after the intravenous injection of radioactive

vitamin B_{12}. Urinary excretion during the period of uniform loss was equal to 0.028% of the retained dose per diem, and faecal excretion represented 0.031% of the retained dose per diem. A comparison of urinary Co^{57}-vitamin B_{12} and the total vitamin B_{12} measured microbiologically suggested that only 10% of the urinary vitamin B_{12} was excreted in an active form. The total daily excretion from Hall's subjects was 0.66-2.1 ug., with a mean of 1.3 ug.

Weinstein and Watkin (196) were unable to implicate impairment of renal excretion as the cause of the abnormally high vitamin B_{12} levels in serum of patients with chronic myeloid leukaemia. Reizenstein et alii (1964) showed, using radioactive vitamin B_{12}, that high levels of vitamin B_{12} excretion, averaging two to five times normal, occurred in chronic myeloid leukaemia, although they were cautious in drawing inferences, in view of the known non-equilibrium between labelled and unlabelled vitamin B_{12}.

Miller and Sullivan (1959c) described the presence of a vitamin B_{12} binding substance in the urine of patients with chronic myeloid leukaemia; Kallee et alii (1966) showed high values for the vitamin B_{12} binding

capacity of urinary glycoprotein in chronic myeloid leukaemia, using immunological techniques (Karypidis and Kallee, 1965).

2.3.5 Vitamin B_{12} and Iron.

There appears to be a relationship between these two haematopoietic agents, although it is by no means a clearly defined one. Cox et alii (1959) published the results of their studies on 13 patients with iron deficiency and normoblastic anaemia, associated with a low serum vitamin B_{12} level, which returned to normal in all patients when they received iron therapy; the interpretation of these findings was that the vitamin B_{12} deficiency had been corrected when vitamin B_{12} absorption was corrected by the administration of iron. Cook and Valberg (1965) could not find evidence of vitamin B_{12} deficiency, nor of mechanisms likely to lead to vitamin B_{12} deficiency, in iron deficient subjects in whom gastric acid secretion and gastric biopsy were normal. They felt that when vitamin B_{12} deficiency occurs in iron deficient subjects, it does so as a result of gastric atrophy.

In rats, Biggs et alii (1962) found that iron deficient rats excreted significantly higher amounts of ingested vitamin B_{12} than did iron-supplemented controls;

impairment of intrinsic factor production was suggested by them as the underlying mechanism.

2.3.6 Vitamin B_{12} and Folic Acid.

The relationship between these two haematinic principles is an important and detailed one, and has been reviewed recently by Arnstein (Arnstein, 1965). Comment is made here on only those aspects relevant to the thesis subject.

Hoogstraten and his colleagues (Hoogstraten et alii, 1965) determined serum folate and serum vitamin B_{12} levels in over 200 patients with malignant blood diseases; low folate levels were found in patients with active disease, whereas most patients in remission or with mild disease had normal values. This correlation did not appear with vitamin B_{12} levels. The folate level in patients with chronic myeloid leukaemia was inversely proportional to the total white cell count.

The folic acid levels and urinary excretion of FIGLU (formiminoglutamic acid) was studied by Rose (1966) in patients with leukaemia or lymphoma. He found folic acid deficiency to be a common complication of these diseases; some patients showed the bone marrow changes of megaloblastic erythroid hypoplasia. Cooper and

Lowenstein (1966) studied patients with low serum folate levels in an attempt to correlate the serum vitamin B_{12} and folate levels with megaloblastic bone marrow changes. Their findings indicated that a patient with a low red cell folate level or low serum vitamin B_{12} level in the presence of a serum folate level of less than 4.1 µµg./ml. would always show megaloblastic or macrogranulocytic changes in the bone marrow.

2.3.7 Assessment of Vitamin B_{12} Metabolism.

A summary may well be made at this stage of the various methods of assessment of vitamin B_{12} metabolism, covering some aspects already mentioned and, in some instances, anticipating later detail.

In investigative medicine, the following methods of assessment may be used:-

i) Serum vitamin B_{12} levels (2.4.1, 2.4.2 below)

ii) Binding capacity for vitamin B_{12} (2.6 below)

iii) Plasma clearance (2.3.2 above)

iv) Absorption Studies. These may involve any, or all, of the following:

a) faecal counting (Heinle et alii, 1952)

b) urinary excretion (Schilling, 1953)

c) hepatic uptake (Glass et alii, 1954)

d) hepatic uptake - double label (Weisberg and Glass, 1966)

e) plasma levels (Doscherholmen and Hagen, 1957; Hall, 1964; Forshaw and Harwood, 1966).

These techniques have been reviewed by Mollin (Mollin et alii, 1957; Mollin, 1959), Pribilla (1961), and Lester Smith (Smith, 1965), amongst others.

2.4 Vitamin B_{12} Levels.

2.4.1 Methods of Measurement.

The number of methods currently in use for the measurement of vitamin B_{12} in blood, other body fluids and cells, underlines the biological truism which states that if there are many methods in use for making a biological measurement, then none of those methods is of itself entirely satisfactory.

2.4.1.1 Microbiological Methods. Microbiological assay is based on the observation that certain organisms are directly dependent upon vitamin B_{12} for their growth, and an assessment of the vitamin B_{12} available while these organisms are growing under controlled conditions makes it possible to assay the amount of vitamin B_{12} present in the growth medium. Lactobacillus leichmannii

was the first microbiological assay devised for vitamin B_{12}; the difficulties which arise with this particular method, common to all microbiological methods, lie in the extraction of the vitamin from the study sample in the form suitable for assay.

Ross (1952) introduced the refined Euglena gracilis method of determining vitamin B_{12} levels in body fluids, following on the initial work of Hutner and his colleagues (1949), who discovered that E. gracilis, a relatively workable organism, required vitamin B_{12} for its growth. The assay is extremely sensitive (Wagner and Folkers, 1963), but has several drawbacks; it is a slow method, it requires special equipment, and it is not specific for cyanocobalamin, since the organism will respond to other analogues of vitamin B_{12}. There are also, inevitably, variations observed when free and bound vitamin B_{12} are determined by this and other methods (Hoff-Jørgensen and Worm-Petersen, 1962).

Papain digestion (Hall and Allen, 1964) produced a two-fold increase in measured normal vitamin B_{12} levels, using E. gracilis as the test organism, and this increase was shown not to be due to stimulation of growth of the micro-organism by non-specific factors. The yield from vitamin B_{12}-deficient subjects was not increased, and it

was considered by these workers that papain digestion would increase the differential between normal and abnormal values, and give additional assistance in the diagnosis of vitamin B_{12}-deficient states. The mode of action of papain in producing this effect is uncertain.

2.4.1.2 Isotope Dilution Techniques. The basis of these methods is saturation analysis, an extension of techniques used in fermentation chemistry (Smith, 1965). The sample to be measured is first treated to convert all activity to free cyanocobalamin and a measured trace amount of high activity radioactive vitamin B_{12} is then added, together with sufficient standardized binding protein to bind part of the total vitamin B_{12}. Free and bound vitamin B_{12} are then separated and after counting, results are read from a standard curve. This method was introduced by Barakat and Ekins (1961, 1963) who utilized human plasma as the protein, and dialysis as the separation technique. Rothenberg (1961, 1963) used intrinsic factor as the binding protein, and precipitation techniques for separating bound vitamin B_{12}. He claimed as advantages for this technique its sensitivity, its rapidity, and the ease of obtaining suitable equipment

for its performance. He points out (Rothenberg, 1961) that this method is dependent upon an accurately assayed commercial preparation, since this is used at its stated value for preparing the standard curve.

Other recent variations of this technique include the use of a DEAE cellulose column for separation (Frenkel et alii, 1966) and the introduction by Hall (Hall, 1966) of cyanide to convert all vitamin B_{12} in the processed plasma to cyanocobalamin, thus removing the possibility of increased protein binding of vitamin B_{12} in the hydroxocobalamin form (Hertz et alii, 1964; Meyer et alii, 1965) which would possibly give falsely low results.

2.4.1.3 Charcoal Techniques. When charcoal is precoated with a large molecule, such as haemoglobin, it is capable of absorbing only free vitamin B_{12}, whereas uncoated charcoal is able to absorb both bound and unbound vitamin B_{12}. Use has been made of this property of charcoal in measuring vitamin B_{12} content of serum (Miller, 1957), and more recently, by Herbert's group, for the assay of intrinsic factor, serum and gastric juice unsaturated vitamin B_{12} binding capacity, serum antibodies against intrinsic factor (Gottlieb et alii, 1965), serum

vitamin B_{12} levels (Lau et alii, 1965), and erythrocyte vitamin B_{12} levels (Kelly and Herbert, 1967). The results obtained with the method of Lau et alii (1965), which combined isotope dilution with charcoal coating, were comparable with those obtained by other means, and the advantages of greater simplicity, rapidity, reproducibility and extensive range were claimed. However, it is apparent that there have been difficulties in other laboratories in setting up this technique and in achieving reproducibility (Herbert et alii, 1966).

2.4.1.4 Comparisons of Methods of Measurement. Few definitive studies have compared and contrasted the various methods outlined above. Raven et alii (1966) compared the isotope dilution charcoal method (Lau et alii, 1965) with L. leichmanii assays and found a satisfactory correlation between them, concluding that the radioactive method compared favourably with the microbiological method as far as reproducibility was concerned.

Hoff-Jørgensen and Worm-Petersen (1962) compared E. gracilis, L. leichmanii and wild escherichia coli for the measurement of serum binding capacity for vitamin B_{12}, and found different, but consistent results (see 2.6.1 below for further discussion). These workers stressed that there was no correlation between the original serum

concentration of, and the binding capacity for, vitamin B_{12}.

2.4.2 Serum Vitamin B_{12} Concentration.

2.4.2.1 Normal Levels. The normal range of serum levels of vitamin B_{12} is from 200 pg./ml. to 1000 pg./ml., depending on the method used, and the particular study concerned (see references 2.4.2.2 below). Fleming reported (Fleming, 1966) that in normal Nigerians, the serum vitamin B_{12} level may be as high as 2000 pg./ml., possibly because of an increase in the serum binding capacity for vitamin B_{12}. Herbert et alii (1965) drew attention to the fact that chlorpromazine will suppress the growth of E. gracilis, and may lead to falsely low levels in patients receiving this drug.

2.4.2.2 Elevation of Serum Vitamin B_{12} Concentration. Following the initial description of elevated vitamin B_{12} levels in myeloid leukaemia (Beard et alii, 1954a, 1954b), several papers were published which confirmed and extended these observations. Mollin and Ross (1955) found that the mean serum vitamin B_{12} level was greater than normal in a group of patients with chronic myeloid leukaemia, sub-acute myeloid leukaemia, polycythaemia rubra vera, myelosclerosis and chronic non-myeloid leukocytosis; the highest levels

were found in chronic myeloid leukaemia, and all these were considerably greater than normal; almost all the vitamin B_{12} was in the bound form. No increase in serum vitamin B_{12} levels was noted in patients with an acute undifferentiated leukaemia. It was noted that the elevated serum vitamin B_{12} level decreased when the granulocyte count was reduced with radiation therapy.

Microbiological methods were also used by Erdmann-Oehlecker and Heinrich (1956) in their studies, in which they described increases in serum vitamin B_{12} levels of from two-fold to fifty-fold in patients with myeloid leukaemia. They could determine no relationship between serum vitamin B_{12} levels and the leukocyte counts, nor the degree of leukocyte maturation, although in remission, the vitamin B_{12} level fell. Elevated serum vitamin B_{12} levels were found by them in some patients with acute leukaemia and polycythaemia vera.

Rachmilewitz et alii (1957) confirmed the finding of elevated serum vitamin B_{12} levels in chronic myeloid leukaemia; they found further that these levels were elevated in myelosclerosis, but not in polycythaemia with marked leukocytosis. On the basis of these observations, they suggested that the determination of the serum

vitamin B_{12} concentration may be of value in the differentiation of various types of leukaemia and other myeloproliferative disorders. Using L. leichmanii as the assay procedure, Mendelsohn and Watkin (1958) showed that most of their patients with myeloid leukaemia, both acute and chronic, had elevated serum vitamin B_{12} levels; one-third of their patients with chronic lymphatic leukaemia had elevated serum vitamin B_{12} levels. The only other clinical situations in which they found high serum vitamin B_{12} levels were in patients who had gross liver damage due to metastatic malignant disease of a non-leukaemic type.

In addition to the demonstration of increased total serum vitamin B_{12} levels in myeloid leukaemia, Raccuglia and Sacks (1957) found that there was an increase in the free vitamin B_{12} serum level in acute and chronic lymphatic leukaemia, unlike the aforementioned authors. Banerjee et alii (1960) observed that in chronic myeloid leukaemia, acute lymphatic leukaemia and acute myeloid leukaemia, the mean value for free serum vitamin B_{12} exceeded the normal free level; in the first two clinical groups, the free serum vitamin B_{12} level did not account for more than 20% of the total serum vitamin B_{12}, while

in the latter group, the free serum vitamin B_{12} was one-third of the total serum vitamin B_{12}.

An attempt was made by Stahlberg et alii (1963) to utilize serum vitamin B_{12} determinations in association with cytochemical investigations to classify morphologically those patients with an undifferentiated acute leukaemia. While it was found that a high serum vitamin B_{12} level, a diffuse PAS reaction, and a low neutrophil alkaline phosphatase score suggested the myeloid form of leukaemia, and that a normal serum vitamin B_{12} level, absence of staining on the PAS reaction, and high neutrophil alkaline phosphatase score suggested the lymphatic variety, it was concluded that these three investigations were of nc value in those patients in whom no morphological classification could be made on the basis of the marrow appearances alone.

An investigation of the metabolism of vitamin B_{12} in twenty patients with polycythaemia vera (Delamore, 1961) showed that the mean serum vitamin B_{12} level in these patients was greater than normal.

In the series of Hoogstraten et alii (1962), no correlation could be identified between serum vitamin B_{12} levels and the degree of activity and severity of the

malignant blood disease, although a correlation was observed between these aspects of disease and folate levels. They also found an elevation of serum vitamin B_{12} in myeloid leukaemia.

Britt and Rose (1966) cited in a case report six previous recorded instances of the chance occurrence of chronic myeloid leukaemia in conjunction with pernicious anaemia. The patient who formed the basis of their report had a normal circulating level of vitamin B_{12} when other clinical evidence of vitamin B_{12} deficiency was present.

Britt and Rose suggested that an increase of serum vitamin B_{12} binding α_1 globulin in their patient had resulted in rapid depletion of tissue stores, which in turn led to megaloblastic erythropoiesis. (The evidence of these present studies suggests that one other factor, namely, the post-gastrectomy state, may contribute to demonstrable vitamin B_{12} deficiency in a patient with chronic myeloid leukaemia; it is well known that vitamin B_{12} deficiency is a common occurrence in patients after gastrectomy (Deller and Witts, 1962).) It was also suggested by Britt and Rose that the vitamin B_{12} present in excess in chronic myeloid leukaemia was not available for normal haematopoietic metabolism.

Disorder of liver function has been advanced by some as a possible explanation of the high serum vitamin B_{12} level seen in certain leukaemias; it is certainly recognised that liver disease may be associated with an increased level of serum vitamin B_{12} (Jones et alii, 1957; Rachmilewitz et alii, 1956); it has been suggested that this increase was due to an increase in the serum binding capacity of plasma proteins as a result of abnormalities in the proteins caused by the underlying liver disease; others have failed to confirm this postulate (Stevenson and Beard, 1959).

In patients with malignant disease in which hepatic metastases have occurred, increased serum vitamin B_{12} levels were demonstrated by Rachmilewitz' group (Grossowicz et alii, 1957), but normal levels were present in patients with malignant disease in which there were no hepatic metastases; they suggested that this finding in patients with hepatic metastases was due to release of stored vitamin B_{12} from damaged liver cells. Mendelsohn and Watkin (1958) also found elevated levels of vitamin B_{12} in patients with malignant disease and hepatic metastases.

A further relevant paper has recently been published from Booth and Mollin (Neale et alii, 1966), who made use of serum vitamin B_{12} levels to differentiate intra-hepatic sepsis from extra-hepatic sepsis. Intra-hepatic sepsis was associated with a raised serum vitamin B_{12} level, and clear differentiation from extra-hepatic sepsis was possible with this laboratory investigation, which could not be achieved with other standard liver function tests. Although they considered the possibility that the raised serum vitamin B_{12} levels may have been due to increased granulopoiesis, the fact that they were able to demonstrate a markedly elevated serum vitamin B_{12} concentration when the white cell count was normal suggested strongly that the increase was due to liver cell breakdown.

Grasbeck (1960) summarised the state of knowledge at that time by concluding that the high levels of serum vitamin B_{12} observed in blood dyscrasias and liver disease were probably secondary phenomena, resulting from the underlying pathological processes, rather than features of the mechanisms causing the disease. There is little reason to modify this proposition at the present time.

2.4.3 Vitamin B_{12} Content of Erythrocytes.

It has been asserted that the erythrocyte levels

of vitamin B_{12} may be a more faithful reflection of tissue stores than the serum level in diseases in which the serum level is abnormal (Kelly and Herbert, 1967), on the grounds that vitamin B_{12} enters mainly the reticulocytes, and not mature erythrocytes (Retief et alii, 1967b). Kelly and Herbert found that the level of vitamin B_{12} in the erythrocytes of normal subjects varied from 30% to 90% of the serum levels. Biggs et alii (1964) showed that the vitamin B_{12} content of red cells was normal in patients with chronic myeloid leukaemia, even when the plasma values for vitamin B_{12} were raised. In chronic lymphatic leukaemia and acute leukaemia, they found that red cell levels were usually normal.

In an earlier study, Baker et alii (1957) found that although cellular (erythrocyte and leukocyte presumably) vitamin B_{12} levels were reduced in pernicious anaemia, they were relatively higher in comparison with plasma vitamin B_{12} levels, than comparable normal values. They suggested firstly, that vitamin B_{12} was available for the initiation of erythrocyte formation, but not for normal maturation, and secondly, if this postulate were correct, that the plasma levels of vitamin B_{12} in

pernicious anaemia reflected tissue stores more faithfully than the cellular levels.

2.4.4 Vitamin B_{12} Content of Leukocytes.

Mollin and Ross (1955) found that the vitamin B_{12} concentration of leukocytes was low in comparison with the concentration in other body tissues; it was also suggested that the vitamin B_{12} content of chronic myeloid leukaemic leukocytes was less than that of chronic lymphatic leukaemic leukocytes.

Leukocytes obtained from leukocyte-rich plasma were homogenized prior to measurement of their vitamin B_{12} content in the study by Thomas and Anderson (1956). They were able to demonstrate no difference between the vitamin B_{12} concentration of leukocytes from leukaemic patients having normal serum vitamin B_{12} levels, and the vitamin B_{12} concentration of leukocytes from leukaemic patients with high serum vitamin B_{12} concentrations. Their series of patients was small (ten in all) and no detail of total or differential leukocyte counts was given. These studies were extended later to a larger series of patients (Kidd and Thomas, 1962). Mean values for total leukocyte levels of vitamin B_{12} in this series were low in chronic myeloid leukaemia, normal in chronic lymphatic

leukaemia, and elevated in acute leukaemia. The total leukocyte vitamin B_{12} level tended to be high when there was a high proportion of primitive cells present. There was no demonstrable relationship between the serum and leukocyte vitamin B_{12} concentrations, nor between the cellular vitamin B_{12} concentration and the peripheral leukocyte count. No indication of the therapeutic response could be obtained from the initial total leukocyte vitamin B_{12} level. They reached the conclusion that the low level of total vitamin B_{12} in leukocytes of chronic myeloid leukaemia did not support the hypothesis that the high serum vitamin B_{12} levels are due to release of the vitamin from the leukaemic cells; neither did it disprove it.

2.5 Vitamin B_{12} and Intrinsic Factor.

2.5.1 Intrinsic Factor.

It was only three years after the discovery of the active liver extract that Castle postulated the existence of the intrinsic and extrinsic factors (Castle and Townsend, 1929), and little alteration has been necessary to Castle's original hypothesis to allow it to remain in harmony with the present state of knowledge. However, although the molecular detail of the extrinsic factor has

been known for some years, a great deal still remains to be discovered about the chemical nature and detailed mode of action of the intrinsic factor. The literature on intrinsic factor has been extensively reviewed by Glass (1962, 1963, 1965).

It is generally accepted (Glass, 1965) that human gastric intrinsic factor has a molecular weight of above 40,000, has the ability to bind preferentially large quantities of vitamin B_{12} and its analogues, and is resistant to papain and trypsin digestion, storage, freeze drying at a neutral pH, and alkalinization up to pH 10.0.

Latner, who introduced the use of paper electrophoresis for gastric juice separation (Latner et alii, 1953), found that the majority of vitamin B_{12} binding activity was present in protein peaks other than those in which intrinsic factor activity was found. Berlin et alii (1959) reached similar conclusions regarding certain commercially available intrinsic factor concentrates, although they were later (Berlin et alii, 1961) able to show a correlation between the vitamin B_{12} binding material and intrinsic factor activity in hog intrinsic factor concentrates obtained from their own processing. Barlow and Frederick (1959) also showed,

by combining autoradiography with horizontal paper electrophoresis, that the major protein components of hog intrinsic factor concentrates were not the same as the major vitamin B_{12} binding components. Latner later (1958) identified three highly purified extracts of intrinsic factor, having molecular weights of 5,000, 15,000 and 40,000, which were apparently homogenous mucoproteins; the two with smaller molecular weights had lower vitamin B_{12} binding activity than did the heavier component. Much of the more recent work, such as that of Chosy and Schilling (1963) has made use of recently developed techniques, including ion exchange chromatography, gel filtration and electrophoresis, to attempt to separate the active principle of intrinsic factor.

Glass (Glass et alii, 1957) has suggested for some years that what is termed intrinsic factor may well represent several different proteins from gastric mucosal extracts, having a common prosthetic group and thus possessing intrinsic factor activity; they may well arise from a common precursor. Glass more recently (1963, 1965) has speculated that intrinsic factor may not even be a direct secretory product of gastric glands, but may be

formed during the process of activation of proteolytic enzymes in the stomach.

These concepts of protein complexity and lability, and their relationship to the binding of vitamin B_{12} have relevance in the consideration of vitamin B_{12} binding to serum proteins.

2.5.2 Vitamin B_{12} Binding Capacity of Gastric Juice.

Gräsbeck (1956), in his own studies of the vitamin B_{12} binding properties of human gastric juice, has reviewed the prior literature on the subject. He used techniques of starch gel electrophoresis and dialysis in his method, and showed initially that the binding capacity of gastric juice was four times that of saliva from the same person. Gräsbeck considered that saliva, which had one vitamin B_{12} binder, could be excluded as an important contributing factor to the gastric juice binders of vitamin B_{12}. He found two vitamin B_{12} binders in gastric juice; one, a slowly migrating one, was heat labile, and had a strong affinity for vitamin B_{12}, while the other, a more rapidly migrating one, was more heat stable, and dissociation from bound vitamin B_{12} increased with time.

Pepsin digestion slowly diminished the vitamin B_{12} binding power of gastric juice, while acid incubation

augmented the faster migrating vitamin B_{12} binding peak. Gräsbeck concluded that his studies supported the view that the vitamin B_{12} binding principle and intrinsic factor were identical. Berlin et alii (1961) reached the same conclusions regarding their hog intrinsic factor concentrate, although they had previously found differing activities in other intrinsic factor preparations (Berlin et alii, 1959).

Later studies from this group (Gräsbeck et alii, 1962; Simons, 1964; Simons et alii, 1964) have indicated that there may be three binders of vitamin B_{12} in gastric juice, of which one, "R", appeared to be common to saliva, serum and leukocytes, and did not possess intrinsic factor activity. The other two binders "S" and "I" (which is possibly a digestion product of "S"), separated by ion exchange cellulose, cross-linked dextrans and Pevikon electrophoresis, had intrinsic factor activity. The molecular weight of "S" was estimated at 93,000.

Gullberg (1960) reviewed the evidence for the association of intrinsic factor with vitamin B_{12} binding (Gräsbeck, 1956; Castle, 1959), together with the evidence for vitamin B_{12} binding by substances without intrinsic factor activity (Gregory and Holdsworth, 1957),

and for the low vitamin B_{12} binding capacity of substances with high intrinsic factor activity (Latner, 1958). In this study, Gullberg measured the vitamin B_{12} binding capacity of gastric juice (Reizenstein, 1959b) which was incubated with Co^{60}-labelled vitamin B_{12}, dialysed, subjected to paper electrophoresis and subsequent autoradiography. Two vitamin B_{12} binders were found, one of which, a slower anodically migrating protein, was always present in normal gastric juice, and in the gastric juice of patients with histamine fast achlorhydria and a normal Schilling test, but which was invariably absent in the gastric juice of patients with pernicious anaemia. In some patients with achlorhydria and a normal Schilling test, a slower vitamin B_{12} binder was identified following parasympathetic stimulation. Gullberg believed that the slower moving vitamin B_{12} binder was related to intrinsic factor, and found it difficult to reconcile these findings with the view of Grasbeck, that this was a breakdown product of peptic digestion, since the binder was invariably present in the achlorhydric state, and since peptic digestion did not produce any significant differences in the pattern of vitamin B_{12} binding.

Glass' group (Uchina et alii, 1964) measured the vitamin B_{12} binding capacity of human gastric juice, and performed separation by paper electrophoresis. The gastric juice from two patients with pernicious anaemia bound significantly less vitamin B_{12} than normal. The main vitamin B_{12} binding areas were identified on the anodic side of the electrophoretic pattern, and were designated primary and secondary. There was no correlation between the binding of vitamin B_{12} by the primary and secondary binders, and the total concentration of protein or carbohydrate in the binding area of the electrophoretogram. In the anacidic gastric juice of patients with pernicious anaemia, and in the gastric juice of patients with histamine-fast anacidity without pernicious anaemia, the primary binder was low in content, or absent, and the secondary area of binding was always absent; however, a tertiary area of intermediate mobility was noted in these instances.

Herbert and his colleagues (Gottlieb et alii, 1965) applied their technique of charcoal separation and isotope dilution to the measurement of the unsaturated vitamin B_{12} binding capacity of gastric juice. They have more recently applied the technique of DEAE cellulose chromat-

ography (Retief et alii, 1967a) to the separation of radioactive vitamin B_{12} labelled gastric juice, and found that there were two peaks of vitamin B_{12} binding material, one of which contained all intrinsic factor vitamin B_{12} binder (as well as some non-intrinsic factor vitamin B_{12} binder), and the other consisting of non-intrinsic factor binders. Gastric juice from patients with pernicious anaemia produced two peaks of vitamin B_{12} binding material, both of which lacked intrinsic factor activity. Although they admitted that the technique used does not yield individual, discrete proteins, they felt that the patterns observed tended to confirm the similarity between gastric juice non-intrinsic factor vitamin B_{12} binder and the salivary vitamin B_{12} binder; they also felt that the principal beta binder of serum may not be the same binder as the beta binder of saliva and gastric juice.

It has been demonstrated by Nyberg et alii, (1961) that the tape-worm Diphyllobothrium latum was capable of splitting the complex formed between vitamin B_{12} and its binding protein in human gastric juice, in such a manner that the bound vitamin was liberated in a dialysable form. They considered that the parasite

contained an enzyme which was capable of liberating the bound vitamin B_{12} as such, and which may have prevented the vitamin B_{12} from being bound to the protein with which it was normally associated in gastric juice.

Abels et alii (1963) have made use of the vitamin B_{12} binding capacity of gastric juice as a means of assaying the level of circulating antibody against intrinsic factor. Using dialysis techniques and radioactive vitamin B_{12}, they showed that eleven patients with anti-intrinsic factor antibody in their sera had diminished vitamin B_{12} binding capacity of their gastric juice, whereas gastric juice from twelve patients whose sera were inactive in respect of anti-intrinsic factor antibody did not have a decrease in the binding capacity for vitamin B_{12}.

2.6 <u>In Vitro Vitamin B_{12} Binding Capacity of Plasma Proteins</u>.

It is again necessary in this section to include under the one general heading material relating to two theoretically dissimilar situations, namely, serum protein binding, and plasma protein binding. Later results reported in this work show that there is no difference, from the point of view of vitamin B_{12} binding, between

serum and plasma, and for this reason studies on serum binding and plasma binding have been included in this one category.

Meyer (1965) has recently reviewed this subject in detail.

2.6.1 In Vitro Vitamin B_{12} Binding Capacity of Plasma Proteins - Methods of Determination.

2.6.1.1 General. Many biological substances are bound to plasma proteins, and frequently, this binding is highly specific, both for the protein and the ligand. Among the instances cited in Murphy's review (Murphy, 1964) of the application of protein binding to assay procedures are antigen/antibody complexes, certain hormones and their specific binding globulins (such as thyroxin), vitamin B_{12} and its globulin(s), and metals such as iron and copper, and their specific binding proteins transferrin and caeruloplasmin.

Many different methods have been described for measurement of vitamin B_{12} binding capacity; some rely on the radioactive forms of the vitamin for its identification in the particular technique involved, and others use microbiological methods of measurement. The techniques used include dialysis, charcoal separation,

TABLE II.

A COMPARISON OF VARIOUS DIALYSIS METHODS FOR IN VITRO MEASUREMENT OF SERUM VITAMIN B_{12} BINDING CAPACITY - I. MATERIALS

Authors	Detection	Amount of Vitamin B_{12} Added (ng./ml.)	Incubation
Bertcher and Meyer, 1958	Co^{60}	1-500	2 Hrs., Room temp.
Meyer et alii, 1957, 1961	Co^{60}	1-100	2 Hrs., Room temp.
Bertcher et alii, 1960	Co^{60}	1-125	2 Hrs., Room temp.
Meyer et alii, 1963	Co^{57}	1-100	2 Hrs., Room temp.
Miller, 1958	(E. gracilis)		
Miller and Sullivan, 1958	Co^{60}	Excess	1 Hr., 22°C
Hoff-Jørgensen and Worm-Petersen, 1962	Various	Excess	Room temp.
Rosenthal and Austin, 1962	Co^{60}	Two- to three-fold excess	½ Hr., Room temp.
Rosenthal et alii, 1965	Co^{60}	Excess	?
Heller et alii, 1964	L. liechmannii	Excess	4 Hrs., 4°C
Present Study	Co^{58}	10-100	1 Hr., 37°C

TABLE III.

A COMPARISON OF VARIOUS DIALYSIS METHODS FOR IN VITRO MEASUREMENT OF SERUM VITAMIN B_{12} BINDING CAPACITY - II. CONDITIONS OF DIALYSIS

Authors	Duration (Hrs.)	Temperature	Buffer
Bertcher and Meyer, 1958	48	Cold	Running tap water
Meyer et alii, 1957, 1961	48	Cold	Running tap water
Bertcher et alii, 1960	48	Cold	Running tap water
Meyer et alii, 1963	48	Cold	Running tap water
Miller, 1958		(4°C)	
Miller and Sullivan, 1958	24, 48	22°C	Phosphate
Hoff-Jørgensen and Worm-Petersen, 1962	9	Room temp.	Saline
Rosenthal and Austin, 1962	72	4°C	Phosphate
Rosenthal et alii, 1965	Overnight	4°C	Acetate
Heller et alii, 1964	48	4°C	Saline
Present Study	96	4°C	Tris phosphate

and various protein fractionation methods based on paper electrophoresis, and various gel and cellulose column separation methods.

2.6.1.2 Dialysis Techniques. These techniques require the addition of an excess of vitamin B_{12} to the test sample; following a period of incubation, dialysis is carried out to remove the excess, unbound vitamin from the test sample. It is then assumed that all vitamin B_{12} remaining and detectable, by either microbiological assay or radioactive counting, within the dialysis bag is bound to protein. In reviewing the various methods below, it will be apparent immediately that there are marked variations in the detail of these techniques, and in the physical conditions of the in vitro experiments. For ease of reference, these methods have been summarized in Tables II and III. An initial part of this present investigation was to assess the significance of the large number of variables in the dialysis methods before any comparison could be attempted between values obtained by any two different methods.

Meyer and his co-workers (Bertcher and Meyer, 1957; Meyer et alii, 1957; Bertcher et alii, 1960; Meyer et alii, 1961) added aliquots of from 1.0 to 500 mμg. of

radioactive (Co^{60}) vitamin B_{12} to their 1 ml. serum samples, and incubated the material in a Visking bag at room temperature for two hours; they then dialysed this material for 48 hours against cold running tap water. The commonly used amounts of added vitamin B_{12} were 1, 2.5, 5, 10, 25, 50, and 100 mμg./ml. The radioactivity of the bag and its contents was measured after the bag had been dissolved in concentrated sulphuric acid; thus the radioactivity after dialysis, representing bound vitamin B_{12}, included that bound to the bag, and to the precipitate which presumably (it is not mentioned) was formed during dialysis. This group felt that continuing dialysis for 72 or 96 hours did not decrease the residual activity further than the 48 hour period used. They also found that dialysis against normal saline, 6% dextran in normal saline, and normal human plasma gave similar results to dialysis conducted against tap water. Meyer et alii used the same basic technique, with a change of isotope to Co^{57}-cyanocobalamin, in their studies of the serum binding effects of vitamin B_{12} analogues (Meyer et alii, 1963). Miller and Sullivan (Miller, 1958) used both isotopic and microbiological methods in their early studies. They diluted serum with

Co^{60} vitamin B_{12} and phosphate buffer and placed it in Visking dialysis bags, allowing an hour for incubation at 22°C. Dialysis of the 9 ml. sample was carried out against 250 ml. phosphate buffer, pH 7.3, at 22°C, initially for a period of 24 hours, and later, for 48 hours, although no explanation was given for this change in length of dialysis time. They found that similar binding results were obtained whether dialysis was performed in the cold (4°C) or at room temperature (22°C); the binding to the dialysis bag was higher at 22°C (4-8%) than at 4°C (1-2%). They also stated that there was no difference produced in binding when other solutions were used in place of the phosphate buffer for dialysis; those tested included distilled water, dextran, barbital buffer, and normal saline.

The comparative study of Hoff-Jørgensen and Worm-Petersen (1962) is discussed in detail below (2.6.1.6); they performed their dialysis procedure against saline for nine hours at room temperature.

A study of the unsaturated vitamin B_{12} binding capacity of various animal sera was carried out by Rosenthal and Austin (1962) using dialysis techniques. They incubated the 0.3 ml. samples with Co^{60}-vitamin B_{12}

in two- to three-fold excess for 30 minutes at room temperature. The dialysis bags were placed in 50 ml. tubes of buffer and refrigerated at 4°C in the dark for 72 hours; buffer was replaced at regular intervals. Heller et alii (1964) measured vitamin B_{12} binding capacity by the L. leichmanii method, after incubation of the sample with excess vitamin B_{12} for four hours at 4°C, and dialysis at 4°C for 48 hours against sodium chloride.

2.6.1.3 Microbiological Techniques. At least two groups have used microbiological techniques to measure the total binding capacity of vitamin B_{12}, without dialysis. Rachmilewitz et alii (1957) measured the binding of free and bound vitamin B_{12} after excess crystalline vitamin B_{12} had been incubated with serum for 24 hours at 4°C.

Banerjee et alii (1960) used microbiological techniques to measure the in vitro vitamin B_{12} binding capacity in 51 subjects; they added 5.000 µug. vitamin B_{12} to 1 ml. serum, and measured the vitamin B_{12} content of the mixture following incubation for 2 hours at 37°C. They considered the results obtained for the bound form to represent the binding capacity of the serum.

2.6.1.4 Charcoal Techniques. In her studies of vitamin B_{12} binding in pernicious anaemia, Christine Lawrence (1966a, 1966b) used the charcoal separation technique of Miller (1957) for measuring vitamin B_{12} binding capacity. An excess of radioactive (Co^{57}) vitamin B_{12} was added to the test serum, and after incubation at 37°C for 30 minutes, removal of the unbound vitamin B_{12} was effected by centrifugation with charcoal. Gottlieb et alii (1965) also used the techniques of charcoal assay for determination of serum unsaturated vitamin B_{12} binding capacity.

2.6.1.5 Protein Fractionation Techniques. Several groups, beginning with Beard and Pitney (Pitney et alii, 1954) relied on the different electrophoretic mobilities of bound and unbound vitamin B_{12} for the separation and measurement of vitamin B_{12} binding capacity of blood proteins. Pitney et alii (1954) used paper electrophoresis after the addition of excess crystalline vitamin B_{12} to determine the differential binding of the vitamin to electrophoretically separable protein fractions. Hift (1964) used zinc sulphate and sodium hydroxide precipitation to separate free and bound vitamin B_{12}, and measured the capacity for vitamin B_{12} binding with

radioactive isotopes. The incubation time chosen was 20 minutes at 37°C, although it was stated that the binding reaction was complete at five minutes, over a range of temperature from 0°C to 40°C. Ion exchange cellulose columns were utilised by Gabuzda et alii (1965) to differentiate free vitamin B_{12} from protein bound vitamin B_{12}, after prior incubation with an excess of vitamin B_{12} and dialysis against the starting buffer used for column chromatography. They selected E. gracilis for assay of vitamin B_{12} in their column fractions.

In their in vitro studies, Hom et alii (1966) added approximately 0.2 ng. of Co^{57}-vitamin B_{12} per millilitre to normal and chronic myeloid leukaemic sera, which were then applied to DEAE Sephadex, CM Sephadex and Sephadex G200 columns, while approximately 0.4 ng. of Co^{57}-vitamin B_{12} per millilitre of serum was added for paper electrophoresis. Thus, the binding capacity in most instances was unlikely to be exceeded; this creates further difficulties in comparison of these results with others in which saturation of the binding capacity of plasma proteins for vitamin B_{12} was sought.

2.6.1.6 Comparison of Methods. Hoff-Jørgensen and Worm-Petersen (1962) made a comparison of four methods of assaying serum binding of vitamin B_{12}. Different results were obtained using dialysis, L. leichmanii, E. gracilis, and uptake by wild E. coli; however, the differences were consistent. It was also shown that there was no correlation in any of the four methods between the binding capacity for vitamin B_{12} and the original serum concentration. The dialysis method and the wild type E. coli gave good agreement on binding; the vitamin B_{12} unavailable for L. leichmanii increased almost in proportion to the total vitamin B_{12} concentration, while that unavailable for E. gracilis was almost constant. These authors proposed that the serum vitamin B_{12} binding capacity should be defined as that amount of vitamin B_{12} per millilitre of serum which is non-dialysable at an initial concentration, such that more than one-quarter, and less than one-half of the total vitamin B_{12} remains undialysed.

2.6.2 In Vitro Vitamin B_{12} Binding Capacity of Plasma Proteins - Values.

Because of the wide range of methods employed, and the lack of consistent agreement within these procedures,

it is of little worth to compare absolute values obtained; it is of considerable value, however, to compare and correlate the patterns of binding determined by various means in both normal and abnormal clinical situations.

2.6.2.1 Normal Patterns. Bertcher and Meyer (1957) showed that as the amount of vitamin B_{12} added was increased, the fraction which was bound fell, while the absolute amount bound rose. Their results showed higher binding than those determined by microbiological assay; that this increased binding was not due to ionic cobalt resulting from vitamin B_{12} breakdown was shown by very different results obtained by them with $Co^{60}Cl_2$ in comparison with Co^{60}-vitamin B_{12}. They suggested that the vitamin B_{12} available for microbiological assay may not represent the total vitamin bound to serum proteins; the work of Hoff-Jørgensen and Worm-Petersen (1962) has confirmed this. They further postulated that the primary binder of vitamin B_{12} was quickly saturated at normal levels, and that non-specific binding took place to other protein fractions at higher concentrations.

These same workers demonstrated, from these and later studies (Meyer et alii, 1957; Bertcher et alii, 1960), that the binding of vitamin B_{12} to human serum

proteins in vitro occurs in two phases, an initial phase which is constant for normals, but which is increased in most patients with myeloid leukaemia and in some patients with polycythaemia vera (Meyer et alii, 1957; Meyer et alii, 1961); in the second phase of binding, there was less avidity for the vitamin, but this avidity was equal in health and disease. They showed (Bertcher et alii, 1960) that it was reasonable to assume that radioactive vitamin B_{12} and "cold" vitamin B_{12} possessed the same properties of serum binding. The firmness of the binding process was demonstrated by adding larger quantities of non-radioactive vitamin B_{12} to protein previously bound to radioactive vitamin B_{12}, without altering the original binding relationship. They found that in vitro binding commenced within 20-40 seconds, and after two hours the bond was not able to be disturbed.

Miller (1958) considered that, over a four-fold range of added vitamin B_{12}, binding was only slightly increased. He suggested from this study, in which isotopic and microbiological methods of measurement were used, that a larger amount of the radioactive vitamin B_{12} bound by normal serum was measured as free vitamin B_{12}

by the E. gracilis method. This may also be a factor contributing to the high free levels of vitamin B_{12} which were demonstrated by Banerjee and his colleagues (Banerjee et alii, 1960).

The unsaturated binding capacity for vitamin B_{12} of sera from differing species measured by Rosenthal and Austin (1962) ranged from 0.29 ng./ml. in the alligator to 6956 ng./ml. in the chinook salmon. Although there were exceptions to the general rule, it was apparent that the vitamin B_{12} unsaturated binding capacity of serum was higher in animals with nucleated erythrocytes than in those with erythrocytes which had no nuclei.

2.6.2.2 Abnormal Patterns. Mollin and Ross (1955), using microbiological techniques, reported that in their leukaemic patients in whom they were able to demonstrate elevated serum vitamin B_{12} levels, most of the vitamin was in the bound form, and was associated with an increased capacity for binding vitamin B_{12}. These findings were confirmed by Rachmilewitz et alii (1957) and by Raccuglia and Sacks (1957).

The initial series from Meyer and his colleagues (Meyer et alii, 1957) contained 14 patients with chronic myeloid leukaemia, of whom 10 had unequivocal elevation

of vitamin B_{12} binding capacity, 2 had borderline levels and 2 were within the normal range. Using the 25 mug./ml. level of binding as a reference point, they found fair correlation between the binding capacity for vitamin B_{12} and the leukocyte count, and a less impressive correlation with the absolute myeloblast count. Increased vitamin B_{12} binding capacity was also demonstrated in some patients with acute myeloid leukaemia, and in some with other blood diseases, including polycythaemia vera. In their later, larger series (Meyer et alii, 1961) these findings were confirmed and extended. The increase in vitamin B_{12} binding in patients with myeloid leukaemia was again shown to occur in the first phase of binding. No relationship was apparent between the vitamin B_{12} binding capacity and increased seromucoid levels seen in certain neoplastic diseases. They considered that the slow fall in vitamin B_{12} serum levels and serum binding capacity after the commencement of therapy could represent persistence of the previously elaborated binding substance. They mentioned three possible reasons for the elevated vitamin B_{12} binding capacity in myeloproliferative disorders, between which they were not able to differentiate; these were, first, that the increase may result from an increase

in the number of binding sites on specific protein molecules, second, that an abnormal binding substance elaborated as part of the disease process may be responsible, and third, that leukocytes or their breakdown products may be responsible for the increased vitamin B_{12} binding.

Miller (1958) also showed that, in chronic myeloid leukaemia, the unsaturated vitamin B_{12} binding capacity and the total vitamin B_{12} binding capacity were both elevated. Speculations were also advanced concerning the several possible explanations for this phenomenon. Further aspects of this study relating to the proteins responsible for binding vitamin B_{12} are discussed below.

Although other workers (Beard et alii, 1954a; Mollin and Ross 1955; Rachmilewitz et alii, 1957) considered that most, if not all of the increased vitamin B_{12} in serum of leukaemic patients was in the bound form, Banerjee et alii (1960) found that the free vitamin B_{12} level was increased in patients with leukaemia. In chronic myeloid, acute myeloid and acute lymphatic leukaemia, the mean value of free vitamin B_{12} was greater than normal, the acute myeloid form having the highest relative increase. A possible explanation for this

finding has been mentioned above (2.6.2.1) in discussing the work of Miller (1958).

The results obtained by Heller et alii (1964) were similar to those of Meyer's group mentioned above; increased binding occurred with increasing amounts of vitamin B_{12} added, and a biphasic curve was observed, suggesting that there are two (or more) binders of vitamin B_{12}. Heller et alii regarded the point of intersection of the primary and secondary slopes as the "unsaturated binding capacity", and the result obtained by adding this figure to the measured serum level of vitamin B_{12}, they referred to as the total binding capacity. This group found that the unsaturated binding capacity in patients with vitamin B_{12} deficiency was not significantly different from normal, but, because of the initial low serum level, the total binding capacity for vitamin B_{12} was diminished, probably due to decreased tissue binding. They postulated that vitamin B_{12} stimulates the synthesis of its own binding protein in tissues as well as in plasma. Herbert's group (Gottlieb et alii, 1966) have recently reported confirmation of the reduced vitamin B_{12} binding capacity in untreated patients with pernicious anaemia.

2.7 Vitamin B_{12} Binding Proteins.

2.7.1 Protein Alterations in Disease.

It is relevant to refer only to those alterations of plasma protein in disease states which have some relevance to the immediate subject of this study.

It has been known for some years that the mucoprotein content of plasma of patients with malignant disease is higher than normal (Winzler et alii, 1948). However, no patients with leukaemia were included in the group studied. Rundles et alii (1954), using the Tiselius technique, examined the serum protein patterns of over 200 leukaemic patients, and although a relative hypoalbuminaemia and increase in globulins was demonstrable in most of the seriously ill patients, these were considered to be non-specific effects. More recently, Hyde and Garb (1965) assessed the incidence of the occurrence of C-reactive protein in patients with leukaemia; it was detected in 69% of patients with acute myeloid leukaemia, 50% of patients with chronic myeloid leukaemia, and in 32% of those with chronic lymphatic leukaemia. They drew attention to the possible relevance of this finding to the search for leukaemic-specific antigens.

Raccuglia and Sacks (1957) stated that, in their patients with chronic myeloid leukaemia in whom increased vitamin B_{12} binding capacity was demonstrated, there were no gross abnormalities detectable in the serum proteins by paper electrophoretic separation.

In a study of the interrelations of human serum protein fractions, Brackenridge (1964) showed that there was a highly significant (p less than 0.001) correlation coefficient between albumin and α_1 globulin levels in patients with malignant disease, which was not present in healthy persons, pregnant women, and in cirrhotics. He considered that albumin may be broken down in certain malignant diseases to material possessing α_1 globulin mobility.

Easton and his colleagues (Easton et alii, 1962) measured the levels of orosomucoid and another α_1 acid glycoprotein in normals and in abnormal states. They observed a rise in both proteins following surgical procedures; the α_1 acid glycoprotein rose to a peak within 2-3 days and fell again to normal over a similar period, while the orosomucoid fraction rose more slowly over 4-7 days and returned to normal 10-14 days later. Estimation of these two proteins in 24 patients with a

variety of diseases (but not myeloid leukaemia) showed that they may vary independently of each other.

Occasional plasma protein abnormalities have been described in patients with vitamin B_{12} deficiency. Neill and Weaver (1958) studied by paper electrophoresis the plasma proteins of 30 pernicious anaemia patients and found, in the untreated patients, reduction of the total protein and of certain components, especially albumin; alpha and beta globulin levels were also decreased. The fact that the total protein level and the level of protein fractions returned to normal after treatment suggested to these authors that vitamin B_{12} exerts an influence on protein synthesis. Support for these findings came from van Dommelen et alii (1963), who added reversible hypogammaglobulinaemia to the other abnormalities described by Neill and Weaver.

2.7.2 Vitamin B_{12} Binding Proteins - Techniques of Separation and Identification.

Since the development of new and refined techniques of protein separation has played an important part in the increase of knowledge of the vitamin B_{12} binding protein, it is pertinent to mention briefly the methods available, and those chosen by various authors, since these

undoubtedly have an effect on the results obtained and their interpretation. The techniques of protein separation which have been used include paper, starch gel, and starch block electrophoresis, and various forms of column chromatography. The theory and practice of these methods of protein separation are outside the scope of this present discussion; they are well covered in recent reviews (Peterson and Sober, 1960; Tombs et alii, 1961; Tiselius et alii, 1963; Kekwick, 1966).

In their initial studies using DEAE cellulose columns and gradient elution, Fahey et alii (1958) identified an alpha globulin as a vitamin B_{12} binder by means of L. leichmannii assay, confirming the earlier observations of Pitney, Beard and van Loon (1954) and of Heinrich and Erdmann-Oehlecker (1956a), who used the techniques of paper electrophoresis together with microbiological assay. Mendelsohn et alii (1958) used similar techniques of column chromatography to extend these observations.

Others have approached the problem by using precipitation techniques, alone and in combination with other methods, to separate and identify the vitamin B_{12} binding proteins. Weinstein et alii (1959) used a

perchloric acid-phosphotungstic acid technique, together with the method of Weimer et alii (1950) for separation of the mucoprotein fraction MP-1 in their approach to the problem.

Miller and Sullivan (Miller, 1958; Miller and Sullivan, 1959b, 1961) used firstly paper electrophoresis, then protein precipitation methods (Winzler et alii, 1948; Lever et alii, 1951) and later starch block electrophoresis in their work on the vitamin B_{12} binding protein. Hall and his group have also used several methods of fractionation in their several studies; these have included Geon block electrophoresis (Hall and Finkler, 1962), column chromatography and the immunodiffusion technique of Ouchterlony (Hall and Finkler, 1963), and more recently, DEAE cellulose and CM cellulose ion exchange chromatography (Hall and Finkler, 1965) in their separation of the vitamin B_{12} binding proteins.

Other recent variations of these techniques have included that of Hom et alii (1966), who used DEAE cellulose, CM cellulose, Sephadex G200 columns, and ammonium sulphate precipitation, and that of Retief et alii (1967a), who utilised a variation of the DEAE cellulose column for their work.

2.7.3 Vitamin B_{12} Binding Proteins.

Following upon their descriptions of abnormal serum concentrations of vitamin B_{12} in patients with leukaemia (Beard et alii, 1954a, 1954b), Pitney, Beard and van Loon, (1954) published their observations on vitamin B_{12} serum binding, using E. gracilis to measure vitamin B_{12} and paper electrophoresis to separate the relevant protein fractions. They found that in normals, alpha globulin was the primary source of the bound vitamin. In some instances, vitamin B_{12} was also found in association with albumin and beta globulin; this they felt was due to imperfections in the method. No constant relationship could be demonstrated between the binding to α_1 globulin and α_2 globulin. When vitamin B_{12} was added in vitro, the major part was located with alpha globulin. A similar approach was adopted by Heinrich and Erdmann-Oehlecker (1956a), who found that in health, 52% of the vitamin B_{12} was bound to the α_1 globulin, 21% to the α_2 globulin, 16% to albumin, 7% to the β globulin, and 4% to the gamma globulin. In chronic myeloid leukaemia, the binding to the α_1 and α_2 globulins was increased, although no essential quantitative changes were demonstrable in the concentrations of the serum

protein fractions per se. Not only was there an absolute increase in total binding, but proportionately more was bound to the α_1 globulin fraction; in chronic myeloid leukaemia, the vitamin B_{12} binding to α_1 globulin was increased nineteen-fold, and to albumin eleven-fold, in comparison with normal. Approximately three-quarters of the vitamin B_{12} was bound to the α_1 globulin in the sera of myeloid leukaemic patients, and the remainder was equally distributed between the α_2 globulin and albumin. Although the total binding fell after treatment, the distribution did not alter. In a further study (Heinrich and Erdmann-Oehlecker, 1956b), it was demonstrated that injected vitamin B_{12} was strongly bound by the three major binding protein fractions, and retained within the body. These observations were confirmed by Fahey et alii (1958) using DEAE cellulose column chromatography and gradient elution; they found that the vitamin B_{12} was associated with an alpha globulin, and in an extension of this study (Mendelsohn et alii, 1958), using paper electrophoresis and polyvinyl blocks to achieve further separation, they confirmed that the binding protein was an alpha globulin. The increased vitamin B_{12} levels

in the serum of patients with chronic myeloid leukaemia they considered could be attributed to an increased concentration of this protein, which also accounted, in their view, for the increased vitamin B_{12} binding capacity. They considered it possible that the increased vitamin B_{12} binding capacity could be due to an increased number of binding sites on the otherwise normal vitamin B_{12} binding protein. They also calculated the probable total vitamin B_{12} binding protein content of serum (on the basis of one molecule of vitamin B_{12} to one molecule of binding protein of molecular weight approximately 150,000) as 0.11 µg./ml., a level at which direct measurement of this protein is not feasible.

After considering the possibility of leukaemic cell destruction and hepatic damage as sources of the vitamin B_{12} binding protein, they remained uncertain whether the demonstrable metabolic anomaly of this protein was directly related to the myelocytic disorder, or whether it represented a separate manifestation of the disease process.

Miller (Miller, 1958) initially approached the differentiation of the vitamin B_{12} binding protein using paper electrophoretic techniques, by which he demonstrated

that the bound vitamin B_{12} in normal serum was present principally in association with the α_1 and α_2 globulins; when vitamin B_{12} was added to normal serum in vitro, it was bound to all five protein fractions, but chiefly to the β and α_2 globulins. It was suggested that this may have been due to saturation of the α_1 globulin, or to an in vitro avidity of the beta globulin which was not present in vivo. Most added vitamin B_{12} appeared in myeloid leukaemic sera in association with the α_1 and α_2 globulins. It was suggested that the increased serum vitamin B_{12} concentration in myeloid leukaemia may be due to firmer protein binding than normal (for which no comparable turnover rates were available) or to excess vitamin B_{12} entering the circulating serum (possibly from leukaemic leukocytes) without an altered turnover rate. Their final and presumably alternative speculations concerning the binding substance were, first, that this was a normal protein present in increased quantities in patients with myeloid leukaemia; second, that vitamin B_{12} binding took place to altered proteins occurring as a result of the disease process, and third, that there were present, as a result of disease, abnormal tissue proteins foreign to normal serum.

The next report from this group (Miller and Sullivan, 1958, 1959 a) dealt with the nature of the vitamin B_{12} binding substances in normal and chronic myeloid leukaemic sera, and the physicochemical characteristics of the binding reaction. It was found that added radioactive vitamin B_{12} was rapidly bound, and once bound, did not exchange with carrier vitamin B_{12}; this occurred with both normal and chronic myeloid leukaemic sera. Normal serum binding of vitamin B_{12} was shown to be unaffected by pH change to 4.5, but a marked decline in binding was demonstrated at pH 3.6; at this latter pH, binding by chronic myeloid leukaemic sera was unaffected. Pre-heating of the tested serum decreased the vitamin B_{12} binding capacity in both normal and chronic myeloid leukaemic sera, although the effect was more marked in normals. Vitamin B_{12} binding by the seromucoid fraction accounted for 13% of the total binding in normals, while in chronic myeloid leukaemia, it represented 61% of the total binding.

These authors undertook further electrophoretic studies of the vitamin B_{12} binding protein (Miller and Sullivan, 1959b), this time using starch gel electro-

phoresis at pH 4.5. Electrophoretic distribution at pH greater than 4.5 of protein, sialic acid and endogenous vitamin B_{12} and bound added radioactive vitamin B_{12} in chronic myeloid leukaemic seromucoid (Winzler et alii, 1948) was similar to normal. When electrophoresis was conducted at pH 4.5, differences were observed between the amount of bound added radioactive vitamin B_{12} in normal sera and in sera from patients with chronic myeloid leukaemia. They suggested that these findings may indicate that the vitamin B_{12} binding protein in the normal state is saturated, and that further binding takes place to non-acidic proteins. Vitamin B_{12} binding protein was found by Miller and Sullivan to have properties similar to an acidic α_1 glycoprotein and an α_2 glycoprotein from Cohn fraction VI (Schmid, 1953, 1956). They produced calculations which suggested that, because the vitamin B_{12} binding protein is such a minute constituent of the total seromucoid fraction, an increase of one hundred-fold in its concentration would only give an increase of 0.2% of the normal concentration of seromucoid. It was therefore considered likely that observed elevations of seromucoid concentration in chronic myeloid leukaemia

were due to constituents of the seromucoid fraction other than the vitamin B_{12} binding protein.

Similar findings and conclusions were proposed in a later electrophoretic study (Miller and Sullivan, 1961) on the vitamin B_{12} binding protein of man and other vertebrate species. Corroborative investigations were reported by Weinstein et alii (1959), who found that, although an increase in mucoprotein fraction MP-1 was considered by some (Weimer et alii, 1950) to be responsible for the increase in α_1 globulin in patients with malignant disease, it was not the major binding protein for vitamin B_{12} in normals or in patients with chronic myeloid leukaemia. These workers showed that the vitamin B_{12} binding protein in the sera of normals and patients with chronic myeloid leukaemia appeared in the seromucoid fraction, and that the bulk of radioactive vitamin B_{12} (representing Co^{58}- vitamin B_{12} and not Co^{58}) appeared with the globulin fraction on precipitation by the perchloric acid-phosphotungstic acid method.

The starch block electrophoretic technique of Miller and Sullivan (1959b) was used by Hall and Finkler (1962) when they set out to determine whether injected

radioactive vitamin B_{12} was bound by the native plasma binding protein immediately, or if not, when, and whether the process was abnormal in vitamin B_{12} deficient states. They observed that injected vitamin B_{12} was delayed in its disappearance, that it was bound to more than one plasma fraction and further, that disappearance from each fraction took place at different rates, so that the plasma binding pattern changed with time. Greater amounts of added vitamin B_{12} were bound in vitamin B_{12} deficient patients to the fraction with the slower rate of loss. They also considered that their data disproved the possibility that non-specific binding may have taken place, even though the binding capacity of the vitamin B_{12} transport system was not exceeded. Their evidence suggested that vitamin B_{12} is transported in plasma by more than one binding protein, and that the differing disappearance rates suggested differing functions in vitamin B_{12} transport by different proteins.

This suggestion was supported in their next publication (Hall and Finkler, 1963), in which, by means of DEAE cellulose gradient chromatography and CM cellulose chromatography, they were able clearly to show the presence of a second binding substance, participating at

an early stage of transport, clearly separable from α_1 globulin; the two binders were termed transcobalamin I and II. They were shortly (Hall and Finkler, 1964) able to demonstrate that there were marked differences in transport of vitamin B_{12} by these two plasma protein components in chronic myeloid leukaemia. Normally, most absorbed vitamin B_{12} was taken up by transcobalamin II, and rapidly lost within the first 24 hours; transcobalamin I, an α_1 globulin, accounted for the binding of most of the endogenous vitamin. In chronic myeloid leukaemia, they found that almost all radioactive vitamin B_{12} was taken up by transcobalamin I, a complete reversal of the normal pattern. A slowly achieved plasma peak (24 hours vs. 8 hours for normals) was followed by a slow loss. The various possible reasons for this phenomenon were put forward, including: i) a possible increase in transcobalamin I ii) a possible decrease in transcobalamin II iii) a change which alters the binding capacity of either (or both) iv) a chemical change in plasma altering the union of vitamin B_{12} and the transcobalamins.

Further study (Hall and Finkler, 1965) of transcobalamin II by labelling, column chromatography

and isolation (by chromatographic and precipitation techniques) showed this protein to have the mobility of a beta globulin, and it appeared to have some intrinsic factor activity in the Schilling test, although no data presented suggested that it was circulating intrinsic factor.

They considered that their findings as applied to in vitro studies of vitamin B_{12} binding (Hall and Finkler, 1966a) supported the concept that the primary binders, including transcobalamin I and II, took up added vitamin B_{12} preferentially below the level of 1 ng./ml.; above this level, the secondary binders assumed importance, although at lower concentrations they were weak binders.

Hall and Finkler (1966b) also studied the vitamin B_{12} binding capacities of transcobalamin I and II in leukaemia. In patients with chronic myeloid leukaemia in relapse, the binding capacity of transcobalamin II was very much diminished, or absent, while that of transcobalamin I was very much increased; this finding was less marked in acute myeloid leukaemia. In patients with pernicious anaemia, there was diminished or absent binding to transcobalamin II, but the binding capacity

of transcobalamin I was not altered.

These findings received support from the work of Retief et alii (1967a), who used a simplified DEAE column to achieve separation of the main vitamin B_{12} binders of body fluids, including saliva, gastric juice and serum, into two fractions, one containing the alpha globulin binder, the other the beta globulin binder. Their elution pattern showed a beta globulin:alpha globulin ratio of 4:1 in normal serum, 2:1 in pernicious anaemia serum, and 1:3 in chronic myeloid leukaemia serum. They noted, inter alia, that protein peaks and peaks of radioactivity did not correspond, which was not expected in view of the "trace" nature of these vitamin B_{12} binding proteins. These workers also suggested that granulocytes may be the source of beta and alpha globulin binders, and that the explanation for the marked change in pattern evidenced in chronic myeloid leukaemia may be that myeloid leukaemic granulocytes produced more alpha globulin vitamin B_{12} binder and less beta globulin vitamin B_{12} binder than normal granulocytes.

Following upon studies of in vivo plasma binding of vitamin B_{12} (Meyer et alii, 1965) it was suggested that the measured in vivo binding capacity for vitamin B_{12}

represented to a large extent the vitamin B_{12} carrying capacity of transcobalamin II (Schiffer et alii, 1966), since further cyanocobalamin could be added in vitro at the point of in vivo saturation; this additional vitamin B_{12} was thought to be bound to other plasma binding proteins.

The work of Heller et alii (1964) in which they described the location of added radioactive vitamin B_{12} in the beta globulin fraction, which was apparently transferred to the α_1 fraction when protein precipitation was performed, may have some relevance to the dynamics of the interrelationship of the vitamin B_{12} binders. They also made calculations which show that the total binding capacity of normal serum for vitamin B_{12} is approximately 2.5 ng./ml., a very small fraction of the total seromucoid of approximately 600 µg./ml.

Gabuzda et alii (1965) found that native serum vitamin B_{12} binding protein was eluted from the DEAE column used at pH 5.7, while vitamin B_{12} added in vivo or in vitro was bound to a different protein, which was eluted earlier in the chromatogram, at pH 7.4. Occasional inconsistent peaks were seen elsewhere, and the use of a large excess of vitamin B_{12} was found to produce binding of only a small amount of the added

vitamin B_{12}. The unbound excess appeared unabsorbed at the first peak, along with gamma globulin.

They concluded that, in contrast to normal sera, sera from patients with chronic myeloid leukaemia appeared to bind large amounts of vitamin B_{12}, chiefly to the serum protein to which the native vitamin B_{12}, already at an increased level, was bound; an increased amount of the vitamin B_{12} binding protein, as well as a greater degree of absolute unsaturation would account for this finding.

Column chromatographic and high voltage electrophoresis studies by Lindstrand et alii (1963), using undialysed Co^{57} labelled vitamin B_{12}, suggested that the radioactivity present in the gamma fraction represented free radioactive vitamin B_{12}, while the radioactivity present with the beta and alpha globulins represented protein-bound vitamin B_{12} added in vitro.

Hom et alii (1966) investigated the fractionation characteristics of transcobalamin I and II after a large intravenous injection of Co^{57} vitamin B_{12} in a control patient. Using DEAE cellulose, CM cellulose and Sephadex G100 column chromatography, and ammonium sulphate precipitation, they derived molecular weights

of 121,000 for transcobalamin I and 36,000 for transcobalamin II. Their in vitro studies showed that the bulk of the added radioactive vitamin B_{12} appeared with transcobalamin II on the column, with less than 20% appearing with transcobalamin I. With chronic myeloid leukaemic serum, the position was reversed, and of the added vitamin B_{12} which was protein-bound (90%), 73% appeared with transcobalamin I and 17% with transcobalamin II. On paper electrophoresis, protein-bound Co^{57} vitamin B_{12} appeared in normal sera distributed between α_2 globulin and beta globulin, and in chronic myeloid leukaemic sera, with α_1 globulin. They concluded that transcobalamin I was the principal vitamin B_{12} binder in chronic myeloid leukaemic sera in vitro, and transcobalamin II its normal counterpart.

Hardwicke and Jones (1966) considered that the α_1 globulin which appears to bind the endogenous vitamin B_{12} had electrophoretic properties of orosomucoid (obtained by ammonium sulphate precipitation), but it was separable from orosomucoid on DEAE cellulose columns, and did not react with specific anti α_1 globulin antisera. They demonstrated that radioactive vitamin B_{12} added in vitro was bound by a number of proteins, especially by one

with properties similar to an α_1 acid glycoprotein but again, no reaction was observed with a specific anti α_1 acid glycoprotein antiserum. They suggested that specific antisera against vitamin B_{12} binding proteins would be invaluable in the identification and investigation of the vitamin B_{12} abnormality in myeloproliferative disorders.

In a preliminary communication which is somewhat contradictory of the general body of opinion, Ochs et alii (1965) considered that the albumin fraction obtained by paper electrophoresis contained the principal vitamin B_{12} binder when radioactive vitamin B_{12} was added to serum from normals, and from patients with pernicious anaemia, and macroglobulinaemia with high serum vitamin B_{12} levels. They considered that the binding shown to beta globulins when larger amounts of vitamin B_{12} were added represented an overflow mechanism, with binding to secondary protein sites.

Rosenthal et alii (1964, 1965) have endeavoured to resolve the problem of identification of multiple vitamin B_{12} binding components of serum in a number of species, including the sheep, horse, pig, cow, rabbit, guinea pig, rat, cat and dog, as well as in man. They identified,

on CM cellulose chromatography, two binding components, which they designated A and B; A appeared early in association with a large protein peak, and B appeared later in the elution pattern. The A and B binders were present in all sera except chicken sera, which lacked the B binder. The B binder accounted for 5%-30% of the total vitamin B_{12} binding substance in all sera other than normal humans, dogs, and sheep, in which it accounted for approximately half. A minor peak C was found only in rabbits with chronic myeloid leukaemia. In human serum, when increasing amounts of radioactive vitamin B_{12} were added in vitro, the percentage bound to component B decreased. In human chronic myeloid leukaemic sera, only 20% of the binding activity was found in component B. Component A was found to be an acidic fraction, which did not bind to CM cellulose, while component B was dialysable, suggesting it had low molecular weight.

In her studies of plasma protein binding in pernicious anaemia, Christine Lawrence (1966a, 1966b) found that, on paper and polyvinyl chloride electrophoresis, almost all added vitamin B_{12} was associated with beta globulins when normals, normal pregnant

women, and B_{12} deficient pregnant women were tested. In pernicious anaemia sera, the α_1 globulin was responsible for binding significant amounts of the added vitamin B_{12}; it was also proposed that in some instances, there was a deficiency of the beta globulin binder in pernicious anaemia.

The work of the Finnish group (Gräsbeck et alii, 1962; Simons, 1964; Simons et alii, 1964; Simons and Weber, 1966) suggested that saliva and leukocytes contained a vitamin B_{12} binding protein, similar to the vitamin B_{12} binder "R" of human gastric juice (Gräsbeck et alii, 1962); this binder, unlike the other two binders, "S" and its possible digestion product "I", did not possess intrinsic factor activity. They also considered (Simons et alii, 1964) that this binder "R" was present in serum, but in a much lower concentration than in gastric juice and saliva.

2.7.4 Leukocytes and Vitamin B_{12} Binding.

The vitamin B_{12} content of leukocytes has been referred to above (2.4.4.). The possible relationship between white cells and their breakdown products, and vitamin B_{12} binding, especially as an explanation of the increased binding seen in myeloid leukaemia, has

interested several groups (Thomas and Anderson, 1956; Meyer et alii, 1962; Retief et alii, 1967b) since the report of Mollin and Ross (1955) that white cell breakdown products were capable of binding vitamin B_{12}.

Meyer et alii (1962) showed that mature neutrophils had the highest vitamin B_{12} binding capacity, while other white blood cells did not possess this property to any degree. They also found that the vitamin B_{12} binding capacity of mature leukocytes collected from patients with chronic myeloid leukaemia and polycythaemia vera was higher when these cells were subjected to ultrasonic disintegration than when the cells were intact. They considered that disintegration products from mature neutrophilic leukocytes contributed to the increased vitamin B_{12} binding capacity of serum in myeloproliferative disorders.

A Scandanavian group (Simons, 1964; Simons and Weber, 1966) found, by using techniques of dialysis, DEAE cellulose column chromatography, CM cellulose column chromatography, autoradiography and immunoelectrophoresis, that leukocytes possessed a vitamin B_{12} binding protein. They then used C14-leucine to label leukocyte-rich plasma; granulocytes from this preparation were found to possess

more vitamin B_{12} binding protein than lymphocytes; this protein had the same separation characteristics and molecular weight as that isolated in the first set of experiments. A concentrated sample of the leukocyte vitamin B_{12} binding protein gave precipitation bands against anti-human leukocyte, anti-gastric juice, and anti-saliva sera, suggesting that it was similar to the gastric non-intrinsic factor vitamin B_{12} binder "R" (Gräsbeck et alii, 1962).

A preliminary note from Herbert's group (Gottlieb et alii, 1966) reports that, despite the frequent occurrence of leukopaenia in pernicious anaemia, in which the unsaturated vitamin B_{12} binding capacity was lowered, the unsaturated vitamin B_{12} binding capacity was reduced only slightly in non-vitamin B_{12} deficient leukopaenic states - they suggested that a possible increase in the total leukocyte pool may compensate for the reduced leukocyte turnover. This concept may also have relevance to the relationship of white cells and the elevated vitamin B_{12} levels of various leukaemic states.

Retief et alii (1967b) have linked their findings of alpha and beta globulin binders with the aforementioned

work of Simons (Simons, 1964; Simons and Weber, 1966) and have suggested that the leukocytes may be the source of these vitamin B_{12} binding proteins, and that the increase seen in the levels of the alpha globulin binding protein in chronic myeloid leukaemic sera may be due to the myeloid leukaemic granulocyte giving rise to more of the vitamin B_{12} binding alpha globulin, and less of the vitamin B_{12} binding beta globulin than is normally delivered into the serum by normal granulocytes.

CHAPTER 3

MATERIALS AND METHODS

MATERIALS AND METHODS

3.1 Vitamin B_{12} Binding Capacity.

The technique used for this procedure was one of exhaustive dialysis, and is illustrated diagrammatically in Figure 2. Venous blood was collected into sterile containers, using either heparin or acid-citrate-dextrose as the anticoagulant; plasma was obtained by centrifugation. Serum was obtained by allowing blood to clot in a sterile container, and serum was removed after centrifugation. All plasma and serum samples not processed immediately were stored at -20°C. until required. Cobalt^{58}-labelled cyanocobalamin was obtained from the Radiochemical Centre, Amersham, England, through the Commonwealth X-Ray and Radium Laboratory, Melbourne. The material used in the early experiments was CR_1P cyanocobalamin (^{58}Co) BP, in sealed glass ampoules, each ampoule containing 1.1 µg. of the freeze dried vitamin, with a specific activity of not less than 1 µc. per µg. In a number of later experiments, CR_2P cyanocobalamin (^{58}Co) BP was used; this was a high specific activity aqueous solution

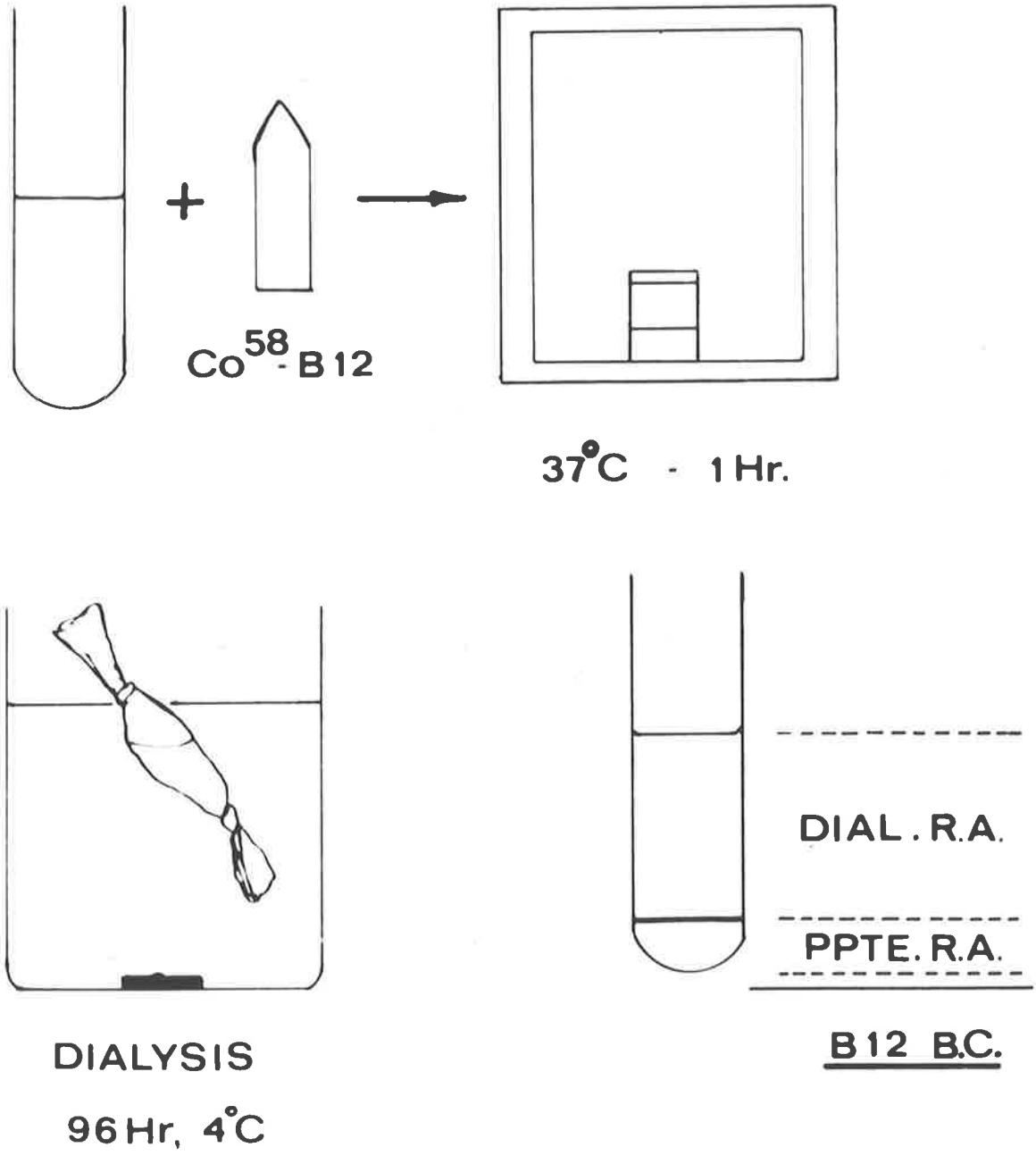

Figure 2. Diagrammatic outline of the dialysis method used in this study for measurement of the vitamin B_{12} binding capacity.

containing 0.9% benzyl alcohol, having a specific activity of 10-300 µc. per µg. The specific activity of the particular material used has been indicated in the relevant experiments; it was usually between 75 and 100 µc. per µg. The standard radioactive B_{12} was dissolved in sterile physiological saline before use.

Aseptic precautions were observed in handling the material used in these experiments, and they were conducted at 4°C. to minimise the possibility of bacterial growth.

The sample was mixed with the appropriate vitamin B_{12} solution, and after incubation at 37°C. for one hour, a known volume of from 2 ml. to 5 ml. of labelled plasma was placed in Visking dialysis sacking, and dialysis was performed at 4°C. in a large beaker with mechanical stirring, against 0.01M tris phosphate buffer, pH 6.5. In a number of experiments both tap water and distilled water were used, without significant differences being noted. The buffer most commonly used was tris [tris (hydroxymethyl) aminomethane] phosphate buffer, 0.01M, pH 6.50. This buffer was chosen because the dialysed plasma obtained was thus equilibrated with

the DEAE cellulose columns used for column chromatography. Following dialysis, with at least two changes of buffer, for a minimum of 96 hours, the dialysant was removed from the sac, and centrifuged to remove the precipitate which formed during dialysis.

Aliquots of the original undialysed labelled material, the dialysant, and the precipitate were counted in a well-type scintillation counter. In the initial experiments, this was performed in a Nuclear Chicago well-type scintillation counter, with a 2" sodium iodide crystal; later experiments were conducted with a Philips scintillation counter with a 1" sodium iodide crystal. Counts were performed for sufficient time to give an error of less than 5% in each count. In a number of the early experiments, the total radioactivity of each wash, together with the radioactivity attached to the sac was counted, in order to account for all radioactivity present at the commencement of dialysis. This was done, since previous experiments by other workers had not attempted to account for all initial radioactivity. The mean yield with this method was in excess of 95%. A number of variants of this basic method were studied, and these have been discussed under

the section "Validation of the Methods Used" (4.1).

3.2 Column Chromatography.

3.2.1 General.

Glass columns of 300 mm. length and 10 mm. internal diameter were used, some of which had a coarse sintered glass plug at the lower end, while others had a glass wool plug. It was found that a more rapid rate of flow was obtained using appropriately packed glass wool. A measured amount (usually 1.8 gm.) of Selectacel DEAE40 (Carl Schleicher and Schuell, Keene, N.H.) was suspended in 50 to 60 ml. 2M NaCl in 0.01M tris phosphate buffer, pH 6.5, and after careful stirring to break up any aggregated particles, the DEAE was poured into the glass column and allowed to pack under gravity. The relatively high specific gravity of the solution (approximately 1.100) made it unnecessary to remove any fines. Equilibration of the column was performed using 200 ml. of 2M NaCl in 0.01M tris phosphate buffer, pH 6.5, and this was followed by 300 ml. 0.01M tris phosphate buffer, pH 6.5. The column was then ready for use with plasma or sera which had been dialysed against this latter buffer.

3.2.2 Column Chromatography of Plasma Samples.

A measured quantity (usually between 0.5 and 1.0ml.)

of the plasma or serum under study was placed on the column, and the following buffer programme was followed, to achieve step-wise elution:

0.01M tris phosphate, pH 6.5

0.05M tris phosphate, pH 6.1

0.05M tris phosphate, pH 5.0

0.20M tris phosphate, pH 4.0

The various fractions were collected in 5 ml. or 6 ml. lots, using initially an LKB automatic fraction collector, and later, a Paton Industries refrigerated fraction collector, utilising either meniscus sensing or electrode sensing to obtain samples of equal volume.

3.2.3 Measurements.

Optical Density of the fractions collected was measured in a Unicam SP500 spectrophotometer at 2800Å, or in a Beckman DU spectrophotometer, at the same wave length. An automatic flow cell was used with the Unicam machine.

Radioactivity of the samples was measured, in those earlier studies in which all tubes were counted, in a Packard well-type scintillation counter. In other studies a Philips well-type counter, employing a 1" sodium iodide crystal was used or a Nuclear Chicago

well-type counter, with a 2" sodium iodide crystal. All samples were counted for sufficient time to give an error of less than 5% in each count.

3.3 Separation of Leukocytes.

The method used was basically that of Rabinowitz (1964, 1965), and it is illustrated diagrammatically in Figure 3. The blood from which white cells were later separated was collected into heparinised plastic packs, and sedimentation was allowed to take place by gravity, aided by the addition of 6% dextran in saline, in the proportion of 1 ml. dextran in saline to 5 ml. blood. Smaller quantities of blood were collected into sterile glass containers, mixing 10 volumes of blood (approximately 20 ml.) with one volume of acid-citrate-dextrose mixture and one volume of 6% dextran in saline (Beal, Read and Turvey, 1967). This mixture was likewise allowed to sediment by gravity.

Leukocyte-rich plasma was then removed and spun at 150 g for 10 minutes at 4°C. The supernatant plasma was removed, and the cell button which remained was washed. If red cell contamination was present, washing with hypotonic saline was used to lyse these cells and to clear the cell button. The white cell button was

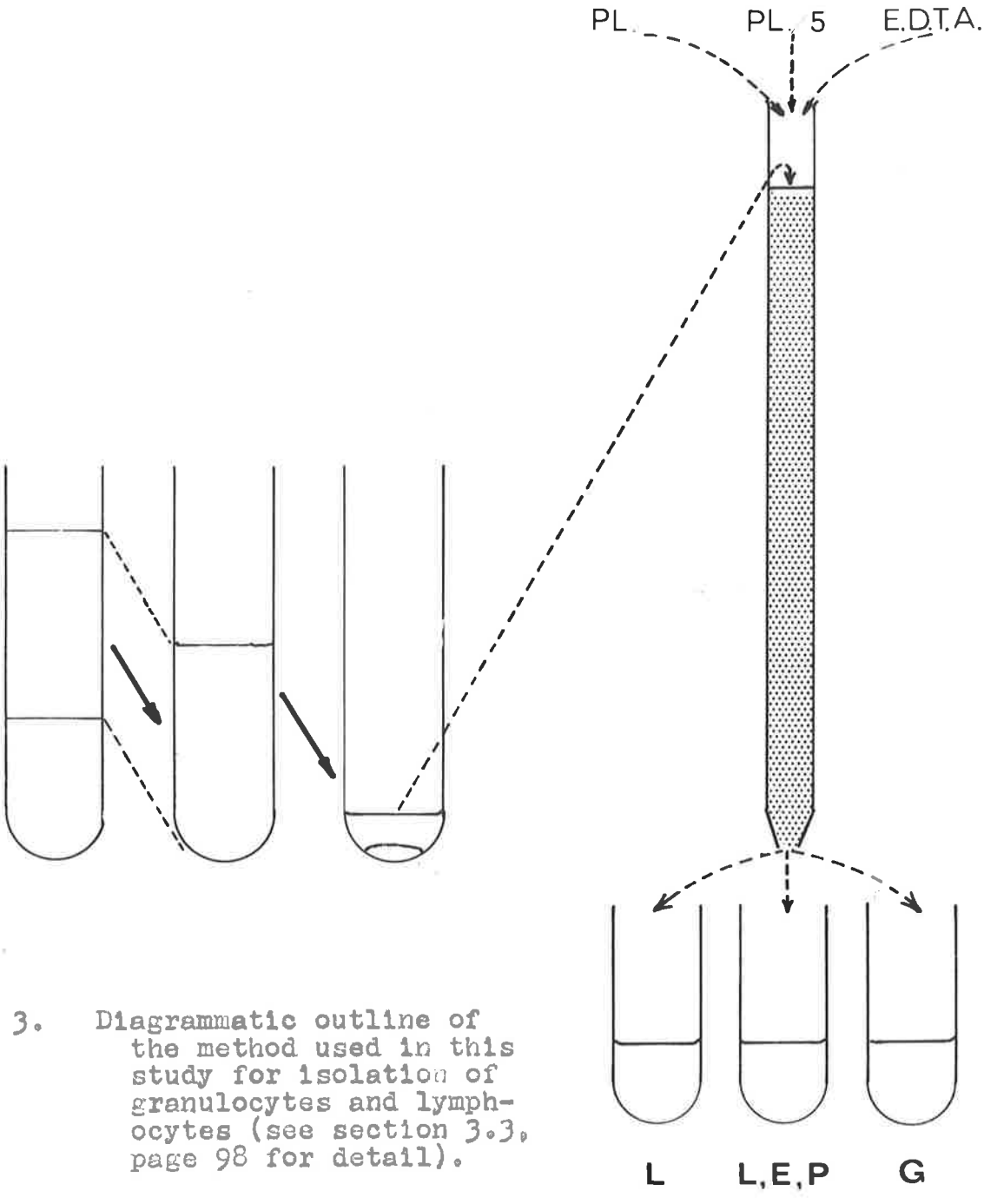

Figure 3. Diagrammatic outline of the method used in this study for isolation of granulocytes and lymphocytes (see section 3.3, page 98 for detail).

resuspended in a small volume of its own plasma, and placed on a 160 mm. x 10 mm. glass bead column, using beads of approximately 100 mesh, which were somewhat smaller than those recommended by Rabinowitz, who used glass beads of 0.02 cm. in diameter.

The total column with the material upon it was incubated at 37°C for half an hour, and supernatant plasma from the original samples, also warmed to 37°C, was placed on the column, producing the first eluates, which were then spun to reduce the volume in which the cell button was present. These first eluates were almost entirely lymphocytes, and a yield of above 95% lymphocytes was obtained in most instances. The next solution placed on the column was a 1 in 5 dilution of warmed plasma in saline, which removed the remaining lymphocytes, erythrocytes, and platelets from the column. The final eluates were obtained by passing buffered EDTA solution through the column. These eluates contained 97%-98% mature granulocytes. Again, the volume of these eluates was reduced by centrifugation and removal of the supernatant. Cell counts were made on the final cell suspension in all instances, and slides were made and stained to ascertain the efficiency of separation.

An aliquot of 0.5 ml. of a suspension of the white cell series under study, either lymphocytes or granulocytes, was added to the pre-dialysis incubation mixture referred to above; an identical volume of saline was added to a duplicate system to act as a control. (Because of the processing involved, the addition of 0.5 ml. plasma to the system would not have yielded a strictly comparable blank.)

Cell counts on the suspensions obtained have varied from experiment to experiment, but have been between 5,000 and 12,000 per cu. mm. for lymphocytes, and slightly less for granulocytes. In the experiments described below, the number of cells used in the lymphocyte and granulocyte experiments was similar. The addition of 0.5 ml. of cell suspension to the dialysis mixture resulted in the addition of between 2.5 and 6.0 x 10^6 cells.

3.4 Validation of Methods Used.

3.4.1 Vitamin B_{12} Binding Capacity.

Because of the widely differing methods used throughout the literature, and the results produced by these methods which are at times in conflict with each other, it was necessary during the initial stages of this

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investigation to assess a number of variable factors involved in the dialysis method of measurement of vitamin B_{12} binding capacity. The following variants of the basic method set out above were studied:

i) Plasma binding and serum binding.

Plasma and serum for the study were obtained from blood collected from the same subject at the same time. Plasma and whole blood taken at the same time from a patient with chronic myeloid leukaemia were used to investigate any differences between the vitamin B_{12} binding capacity of plasma and that of whole blood.

ii) Concentration of added vitamin B_{12}.

Aliquots of normal, and chronic myeloid leukaemic plasma were incubated with 1 ng. per ml., 10 ng. per ml., and 100 ng. per ml. concentrations of radioactive vitamin B_{12}, and dialysed under identical conditions.

iii) Duration of incubation.

Three aliquots of normal plasma were incubated at room temperature (22°C) for two hours, six hours, and twenty-four hours, and were then dialysed under similar conditions for similar lengths of time.

iv) Temperature during incubation.

Two aliquots of normal plasma were incubated at

15°C and 37°C, and were then dialysed for similar lengths of time.

v) Duration of dialysis.

The effect of the duration of dialysis was studied by measuring the radioactivity present in the wash buffers at each change of buffer. In some instances, this consisted of measurement of radioactivity at 48 hours and at the end of dialysis, while in another instance, six buffer changes were made over a 162 hour dialysis, with measurement of the buffer radioactivity at each change.

vi) Temperature during dialysis.

Aliquots of the same normal labelled plasma were dialysed in the cold (4°C) and at room temperature (20°C).

vii) Volume of dialysis buffer.

Two aliquots of the same normal labelled plasma were dialysed, one in a 250 ml. beaker against tap water, and the other against running tap water.

viii) Effect of differing buffers.

Aliquots of the same labelled plasma were dialysed against normal saline, tris phosphate buffer (0.01M, pH 6.5), and tap water, pH 6.75.

ix) Effect of papain on binding.

Three aliquots of plasma were prepared in the

standard manner; to the first, 1 ml. of a 2% solution of papain was added prior to incubation; to the second, the same quantity of papain was added after incubation; to the third which represented the experimental blank, normal saline was added after incubation.

x) Integrity of binding.

In an experiment designed to assess the firmness of the bond between vitamin B_{12} and its binding proteins, three aliquots in duplicate were taken at the completion of a standard 96 hour dialysis; the first pair of dialysants was then further dialysed against 500 ml. of 0.01M tris phosphate buffer, pH 6.5, to which was added 2,000 µg. of crystalline vitamin B_{12} ("Cytamen", obtained from Glaxo Allenbury); the second pair of dialysants was similarly treated, except that 2,000 µg. of hydroxocobalamin ("Neocytamen", Glaxo Allenbury) was added to the buffer; the third pair of dialysants, set up as controls, was dialysed against the buffer without added vitamin B_{12}. Dialysis was continued for a further twenty-four hours.

3.4.2 Column Chromatography.

The standard column chromatogram obtained with both normal and pathological sera, dialysed or undialysed,

consisted of four principal peaks, which have been designated I, II, III-IV, and V; peak Ia is a minor peak. Peaks I and Ia were obtained by elution with the commencing buffer immediately after the material was placed upon the column, and peaks II, III-IV, and V followed each consecutive stepwise change in buffer of increasing molarity and increasingly acid pH.

The location of various protein fractions on the column chromatogram has been determined by the use of several human blood protein fractions obtained from the Commonwealth Serum Laboratories, Melbourne. These have included 5% albumin solution in Hartmann's buffer, immune serum globulins (gamma globulin), Cohn fraction IV-7 (transferrin), Cohn fraction III-1, and α_1 acid glycoprotein (Lever et alii, 1951; Schmid, 1950, 1953, 1955, 1956).

CHAPTER 4

RESULTS

VALIDATION OF METHODS USED

RESULTS

VALIDATION OF METHODS USED

4.1 Vitamin B_{12} Binding Capacity.

4.1.1 Plasma Binding and Serum Binding.

In the definitive experiment, plasma from a normal subject bound 1.15 ng./ml., at the 10 ng./ml. level of added radioactive vitamin B_{12}, while serum from the same subject bound 1.17 ng./ml.; this difference is not significant. Throughout the whole series ofexperiments reported, no differences which could be considered significant have been obtained when serum and plasma have been used in parallel; this applies to normal subjects, and to patients with chronic myeloid leukaemia and other blood disorders. Although this has not been discussed specifically by other authors, it is evident from the literature review that the terms "serum protein binding of vitamin B_{12}" and "plasma protein binding of vitamin B_{12}" are synonymous to many who have written on this subject; for example, Rosenthal et alii (1964, 1965) have studied and discussed serum and plasma binding capacities interchangeably in their work.

Since the only distinction between serum and plasma is that serum lacks the fibrinogen which is contained in plasma, any difference between serum binding of vitamin B_{12} and plasma binding of vitamin B_{12} would relate to fibrinogen. It has been demonstrated in a later section of this study (5.4) that fibrinogen does not bind significant amounts of added radioactive vitamin B_{12}. It is therefore considered reasonable to assume that there is no difference between in vitro binding of vitamin B_{12} to serum and plasma.

A difference was observed between the amount of vitamin B_{12} bound to whole blood and the amount bound to plasma taken at the same time from the same patient; at the 10 ng./ml. level, plasma binding was measured at 32.4% (3.24 ng./ml.), while binding to the whole blood sample was 46.2% (4.62 ng./ml.). Possible explanations for this finding are discussed later.

Yield. In all early experiments, when the radioactivity of the buffers was measured, as well as the radioactivity of the dialysant, the precipitate and the sac, a yield was calculated, and expressed as a percentage of the expected yield. In all experiments in which this

TABLE IV.

EFFECT OF DIFFERING CONCENTRATIONS OF Co^{58}-B_{12} ON BINDING

Co^{58}-B_{12} ADDED (ng./ml.)	PERCENTAGE BOUND	AMOUNT BOUND (ng./ml.)
NORMAL		
1.0	65.8	0.658
10.0	19.8	1.98
100	10.09	10.09
C.M.L.		
10.0	51.7	5.17
100	23.2	23.2

TABLE V.

EFFECT OF DURATION OF INCUBATION ON Co^{58}-B_{12} BINDING AT 21°C

DURATION (Hr.)	NORMAL PLASMA	
	PERCENTAGE BOUND	AMOUNT BOUND (ng./ml.)
2	15.3	2.19
6	18.2	2.86
24	20.5	3.22

was measured, the observed yield was within ± 10% of the expected figure, with a mean value of 98.5%. Variations were naturally smaller in the experiments using added vitamin B_{12} at the 100 ng./ml. level than in those performed at the 10 ng./ml. level.

4.1.2 Concentration of Added Vitamin B_{12}.

The effect of differing concentration of Co^{58}-vitamin B_{12} added to normal and chronic myeloid leukaemic plasma is illustrated in Table IV. Although the percentage of radioactive vitamin B_{12} bound to the plasma proteins decreases as the concentration of vitamin B_{12} added prior to incubation increases, the absolute amount bound increases. This applies in both normal subjects and in patients with chronic myeloid leukaemia. At the 1 ng./ml. level, the chronic myeloid leukaemic plasma shown in Table IV bound all detectable radioactivity.

4.1.3 Duration of Incubation.

The effect of the duration of incubation on the binding of radioactive vitamin B_{12} to plasma protein at room temperature is shown in Table V. The amount of radioactive vitamin B_{12} bound increases with the duration of incubation, the increase between the 2 hour figure and the 24 hour figure being 47%. A duplicate of the 24 hour

TABLE VI.

EFFECT OF DURATION OF DIALYSIS ON BINDING OF Co^{58}-B_{12}

PLASMA	DURATION (Hr.)	PERCENTAGE OF TOTAL RADIOACTIVITY REMOVED IN 48 Hr.	PERCENTAGE OF TOTAL RADIOACTIVITY REMOVED AFTER 48 Hr.
Normal	96	78.2	3.5
C.M.L.	122	48.0	5.9
Normal	144	50.1	7.1
C.M.L.	144	38.2	11.5

sample was refrigerated for a further 24 hours, and subjected to dialysis. The binding to this sample was 20.1%, which is not different from the 24 hour figure. This suggests that the binding may be maximal at 24 hours, or possibly, that cooling reduces the rate of continuing binding to negligible levels after 24 hours.

4.1.4 Temperature During Incubation.

No significant difference was observed between two samples of normal plasma, one of which was incubated at room temperature (15°C) and bound 2.80 ng./ml.; the other was incubated at 37°C, and bound 2.67 ng./ml. The initial amount added was in both instances 10 ng./ml., and dialysis was performed for 99 hours against 0.01M tris phosphate buffer, pH 6.5, at 4°C.

4.1.5 Duration of Dialysis.

Table VI sets out four randomly selected dialysis experiments, two using plasma from normal subjects, and two using plasma from patients with myeloid leukaemia; these results are typical of those obtained throughout this study. In these two sets of experiments, the amount of radioactivity removed during the first 48 hours was measured, as was the amount of radioactivity removed between 48 hours and the end of dialysis, which was

TABLE VII.

EFFECT OF PROLONGED DIALYSIS ON BINDING OF Co^{58}-B_{12} (NORMAL PLASMA)

WASH (No.)	DURATION Hr.	TOTAL TIME ELAPSED (Hr.)	PERCENTAGE OF TOTAL RADIO-ACTIVITY REMOVED	PERCENTAGE OF RADIOACTIVITY REMOVED BY DIALYSIS
1	18	18	47.4	61.2
2	25	43	17.4	22.5
3	21	64	6.7	8.7
4	48	112	4.3	5.6
5	28	140	1.2	1.6
6	22	162	0.4	0.5

never less than 96 hours. It is seen that the radioactivity removed between 48 hours and the end of dialysis ranges from 3.5% to 11.5% of the total initial radioactivity, representing 4.3% to 23.1% of the radioactivity actually removed during dialysis. Six buffer changes were made in the experiment shown in Table VII, which illustrates the effect of prolonged dialysis on the binding of radioactive vitamin B_{12}. The percentage of total radioactivity removed is indicated, representing the amount of radioactivity measured in the buffer against which the plasma was dialysed. Again it is apparent that significant radioactivity is removed by dialysis after the first 48 hours. From the results of these experiments, and others similar to them, it was concluded that a 96 hour dialysis would remove 95% or more of the dialysable vitamin B_{12} present.

4.1.6 Temperature During Dialysis.

No difference was observed between the two samples which were dialysed at differing temperatures against tris phosphate buffer. The aliquot of normal radioactive vitamin B_{12} labelled plasma dialysed at 4°C bound 2.75 ng./ml. of the radioactive vitamin B_{12}, while the aliquot dialysed at 20°C bound 2.80 ng/ml. At the higher

TABLE VIII.

EFFECT OF DIALYSIS AGAINST TAP WATER AT ROOM TEMPERATURE AND AT $4^{o}C$

	ROOM TEMPERATURE ($22^{o}C$)	COLD ($4^{o}C$)
A. AT 100 ng./ml. LEVEL.		
Dialysant Binding	10.4%	15.7%
Precipitate Binding	17.6%	1.4%
TOTAL	28.0%	17.1%
B. AT 10 ng./ml. LEVEL.		
Dialysant Binding	13.4%	22.2%
Precipitate Binding	24.4%	1.8%
TOTAL	37.8%	24.0%

Each set of figures represents the mean of two duplicates.

temperature, more radioactivity was associated with the sac (5.4%) than at the lower temperature (3.1%).

However, differences were observed when dialysis was performed against tap water at room temperature and in the cold (Table VIII). In those experiments conducted at room temperature a much heavier precipitate was formed which accounted for the majority of the measured increase in binding. Similar results were not observed with distilled water, and it is likely that the heavy salt content of Adelaide tap water (which, for normal domestic use, requires anion exchange columns for "softening") may have produced the heavier precipitate as a result of its insoluble salts.

4.1.7 Volume of Dialysis Buffer.

The amount of radioactivity removed from an aliquot of radioactive vitamin B_{12} labelled plasma dialysed in a 250 ml. beaker (64.6%) was not significantly different from the amount removed by dialysis of an identical aliquot against tap water running at approximately 1 L. per minute throughout the dialysis (64.0%).

4.1.8 Effect of Differing Buffers.

The results of dialysis against normal saline, tris phosphate buffer, and tap water are shown in Table IX.

TABLE IX.

EFFECT OF DIFFERING BUFFERS ON BINDING OF Co^{58}-B_{12}

(C.M.L. PLASMA)

BUFFER	NORMAL SALINE (A)	TRIS PHOSPHATE BUFFER pH 6.5 (B)	TAP WATER (C)
Percentage of radioactivity in dialysant	70.1	44.8	50.2
Percentage of radioactivity in precipitate	1.2	18.3	17.7
Percentage of radioactivity bound to sacking	3.1	5.8	4.4
Total radioactivity	74.4	68.9	72.3

Although the total amount of radioactivity accounted for in the dialysant, the precipitate, and the sacking is not significantly different, there are marked differences between the distribution of the radioactivity between the dialysant, precipitate, and sacking. This difference is most marked in the experiment in which dialysis was conducted against normal saline; a higher percentage of the radioactivity was detected in the dialysant, and less radioactivity was found in the precipitate. The figures obtained for dialysis against tris phosphate buffer and tap water are similar. The significance of these findings is discussed in section 4.1.9 below.

4.1.9 Precipitate.

It is apparent (Table IX) that the precipitate formed during dialysis against tris phosphate buffer and tap water contains more radioactivity than does the smaller, almost negligible precipitate which formed during dialysis against normal saline. That this radioactivity is bound to the precipitated protein, and is not due to trapped plasma, was shown in the following way:-

The precipitates B and C, similar in volume and radioactivity, were broken up and resuspended,

precipitate B in normal saline, and precipitate C in tap water. After agitation and further centrifugation, precipitate B was almost completely redissolved, and most of the radioactivity was present in the supernatant. In precipitate C, taken up with tap water, more than 90% of the initial radioactivity was present in the second precipitate. Totalling the radioactivity of the dialysed plasma, the precipitate and the sacking in each instance, a similar figure for total binding was obtained in these three experiments. Starch gel electrophoresis of the redissolved precipitate B showed that it consisted mostly of gamma globulin, with some beta globulin present.

4.1.10 Effect of Papain on Binding.

The addition of papain to the mixture of plasma and radioactive vitamin B_{12} did not produce any differences in the amount bound, irrespective of whether the papain was added before or after incubation. At the 10 ng./ml. level, the plasma aliquot to which papain was added prior to incubation bound 2.30 ng./ml.;

that to which papain was added after normal incubation bound 2.32 ng./ml., and the blank (plasma to which no papain was added) bound 2.12 ng./ml.

4.1.11 Integrity of Binding.

In the experiments in which dialysis was continued beyond 96 hours against buffer containing cyanocobalamin and hydroxocobalamin, no decrease in binding was observed between the aliquot dialysed against the vitamin B_{12} and that dialysed against buffer without added vitamin B_{12}. In addition, no radioactivity was detected in the buffers which contained vitamin B_{12} in either of the two forms used.

4.2 Identification and Localization of Known Protein Fractions.

The column chromatograms obtained when known protein fractions were subjected to the same elution procedure as whole plasma are shown in Figures 4-8. Gamma globulin (Figure 4) appears principally at peak I, with much smaller components at peaks Ia, II and V. Cohn fraction III-1, which is a globulin fraction containing the majority of antibodies, including the blood group antigens, showed (Figure 5) a similar pattern to the immune serum globulins, with the principal component being at peak I. Albumin

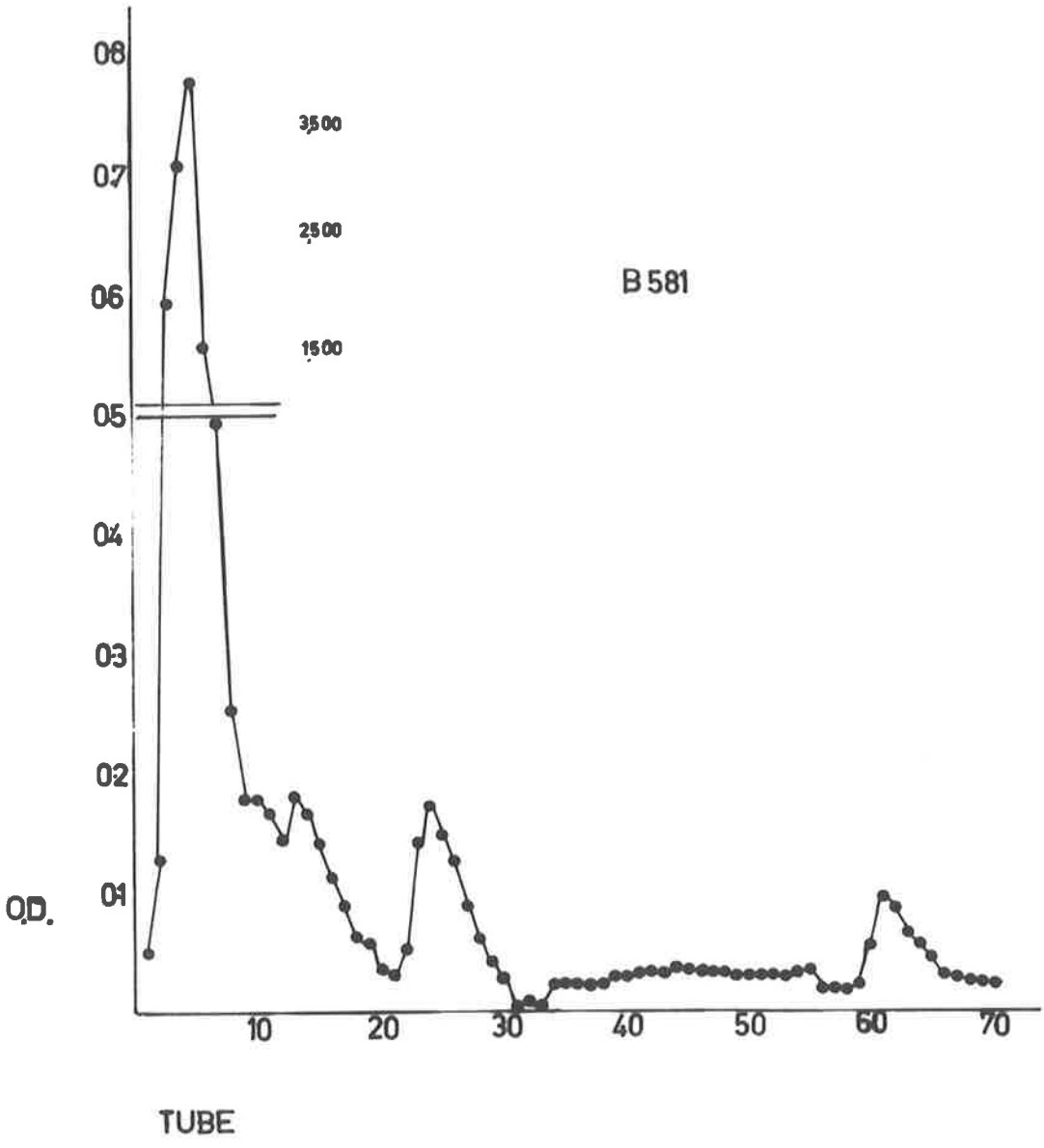

Figure 4. Column chromatogram of 1.0 ml. undialysed immune serum globulin (gamma globulin). Changes of eluent were made at tubes 20, 33 and 57.

B581.

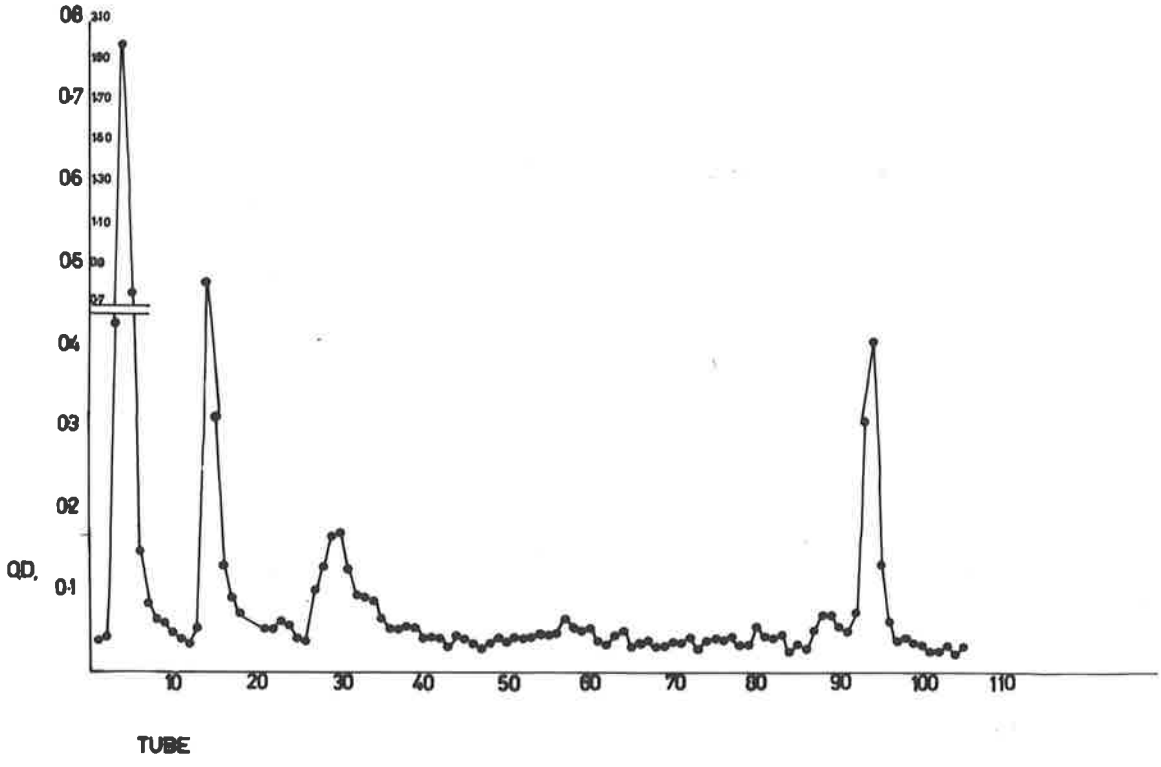

Figure 5. Column chromatogram of 2.0 ml. Cohn fraction III-1 (2% solution).

Changes of eluent were made at tubes 24, 45, and 84.

B503.

(5% solution in Hartmann's buffer) (Figure 6) appears principally at peak III-IV, with a minor component at peak V. Transferrin, Cohn fraction IV-7, a beta globulin (Figure 7), appears predominantly in peak II, with smaller, less marked component peaks at I, Ia, III-IV, and V. The α_1 acid glycoprotein appeared at peak V exclusively (Figure 8). In general terms, the distribution of these protein fractions is not dissimilar to the patterns obtained by Fahey et alii (1958), using gradient elution on a DEAE cellulose column.

Duplicate samples of dialysates and plasma protein fractions run at different times showed virtually identical patterns, indicating that the method gives reproducible results.

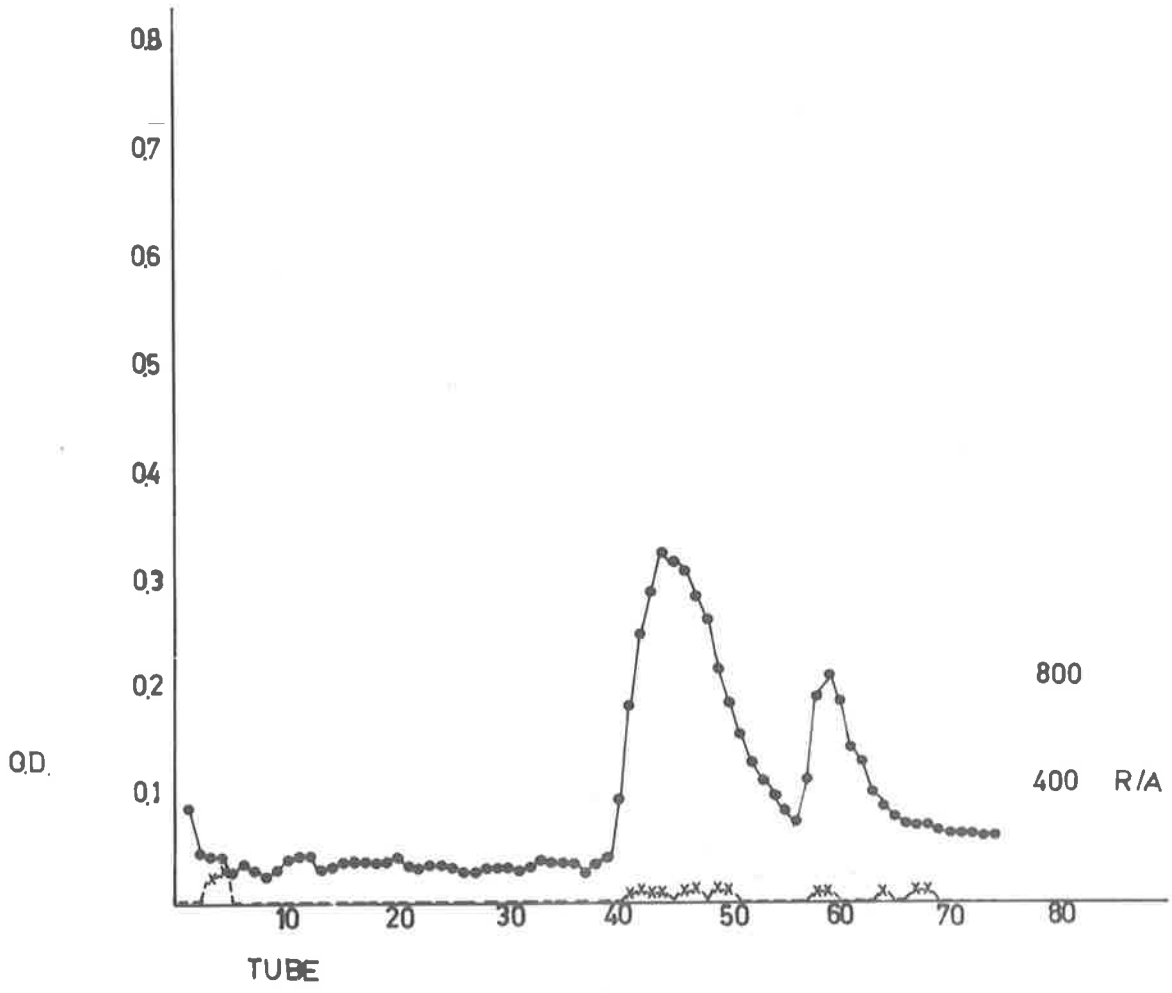

Figure 6. Column chromatogram of 1.0 ml. dialysed albumin (5% in Hartmann's solution).

Changes of eluent were made at tubes 12, 27 and 55.

B664.

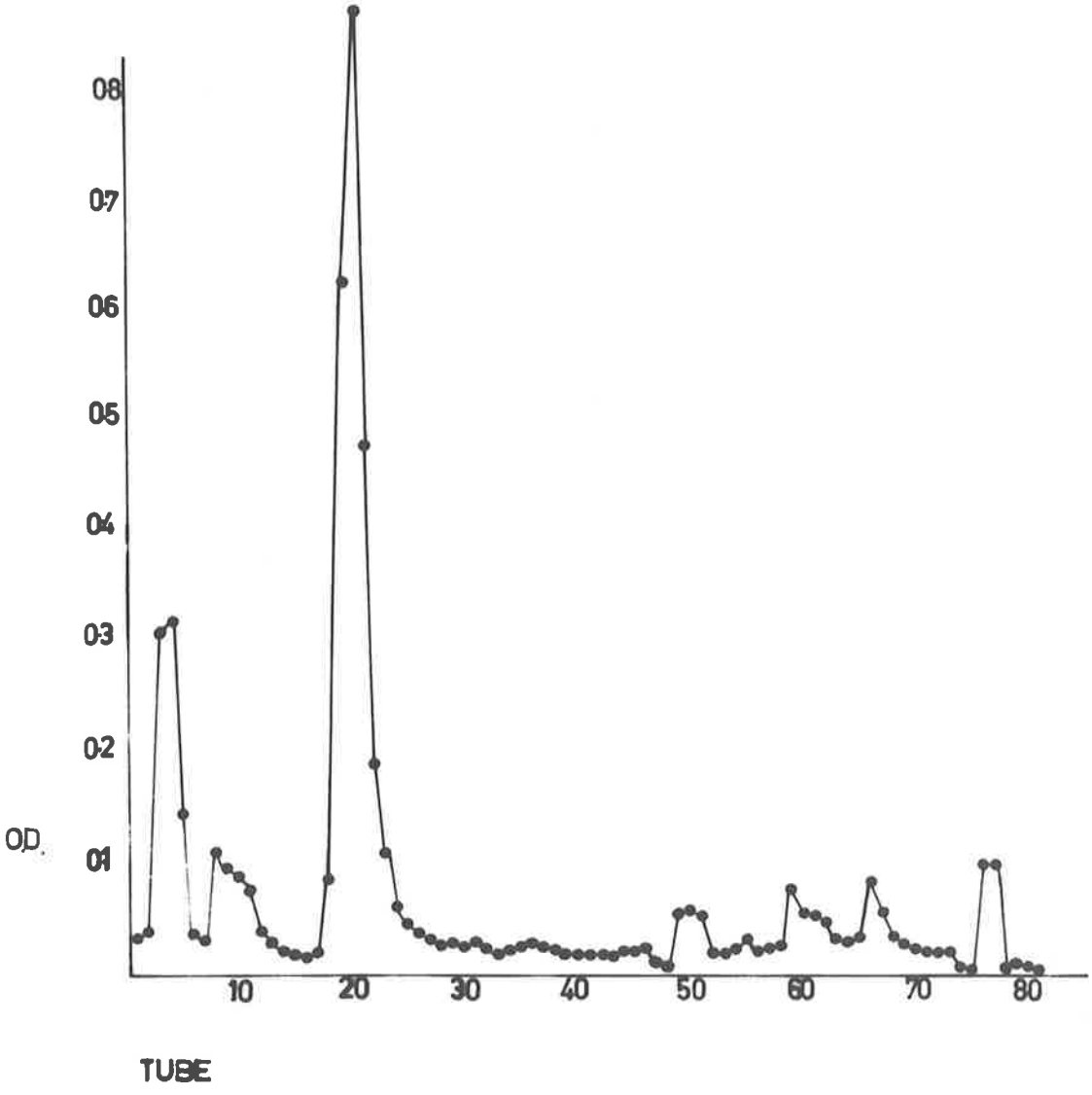

Figure 7. Column chromatogram of 1.0 ml. undialysed transferrin (1% solution).

Changes of eluent were made at tubes 15, 45 and 57.

B501.

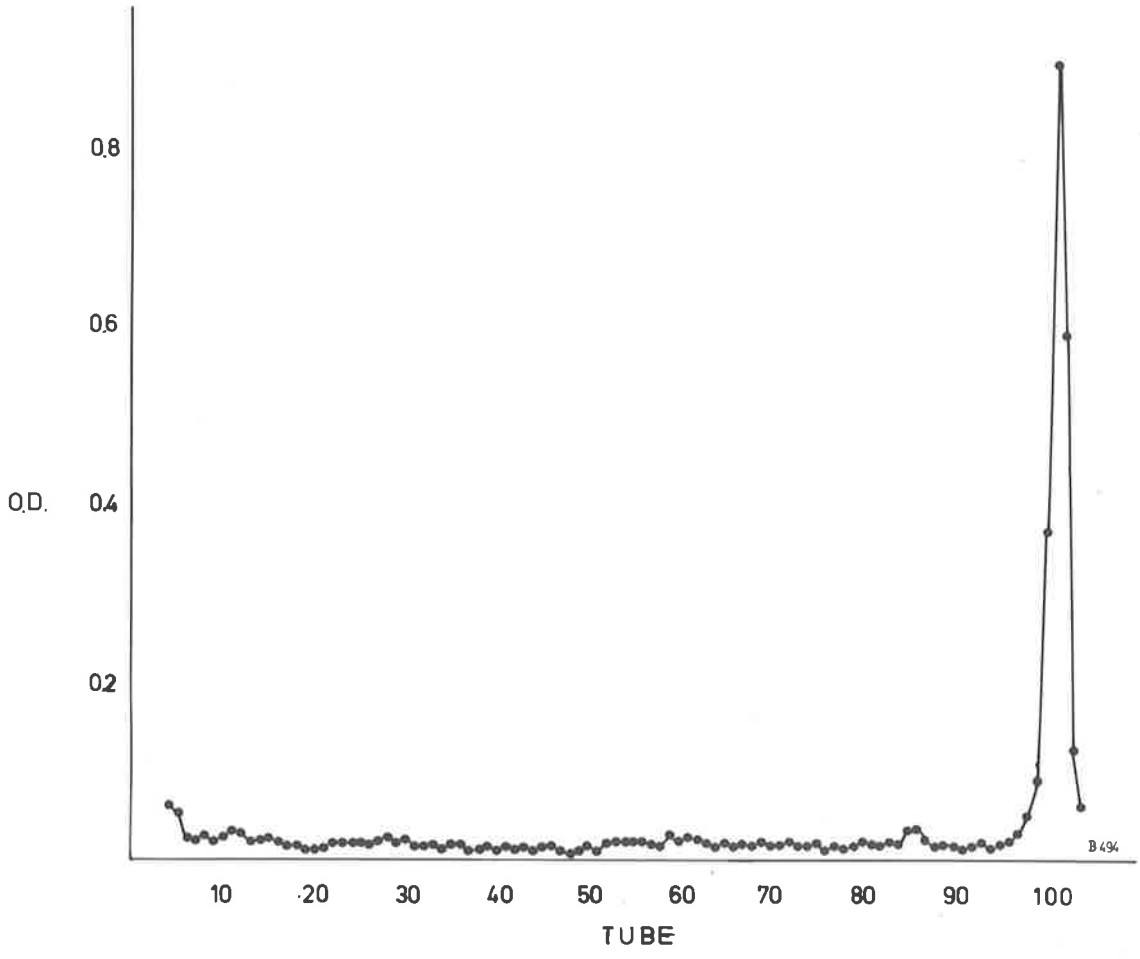

Figure 8. Column chromatogram of 1.0 ml. dialysed α_1 acid glycoprotein (B494).
Changes of eluent were made at tubes 48, 67 and 94.

CHAPTER 5

RESULTS

GENERAL

RESULTS

GENERAL

5.1 Vitamin B_{12} Binding Capacity.

5.1.1 Normal Values.

The normal range for the vitamin B_{12} binding capacity was obtained by measurements at the 10 ng./ml. level and the 100 ng./ml. level, using samples from normal subjects, the majority of whom were blood donors. The mean value for plasma vitamin B_{12} binding capacity in 28 normal subjects was 32.4% ± 2.3% at the 10 ng./ml. level, that is, 3.24 ng./ml. ± 0.23 ng/ml. (1 S.D.). Ninety-five percent of normal values would be expected to fall in the range 27.8% - 37.0% (2.78 - 3.70 ng./ml.) at the 10 ng./ml. level. The mean amount bound to the precipitate was 7.1%, representing 22% of the mean amount of radioactive vitamin B_{12} bound after a 96 hour dialysis.

The mean value for plasma vitamin B_{12} binding capacity in 22 normal subjects was 17.2% ± 2.0% at the 100 ng./ml. level, that is 17.2 ng./ml. ± 2.0 ng./ml. (1 S.D.). Ninety-five percent of normal values would be expected to fall in the range 13.2% - 21.2% (13.2 - 21.2 ng./ml.) at the 100 ng./ml. level. The mean amount

TABLE X.

MYELOID LEUKAEMIA - VITAMIN B_{12} BINDING CAPACITY.
(10 ng./ml. level)

SERIES NUMBER		SEX AND AGE	BINDING CAPACITY (Percentage)	MORPHOLOGICAL CLASSIFICATION
50	MAL	M27	35.3	Chronic
51	RYA	F46	36.1	Chronic
52	SMI	F52	54.8	Chronic
54	FAL	M63	32.4	Chronic
56	COW	M57	39.5	Chronic
57	LAN	M70	39.4	Chronic
63	SME (1963)	F63	73.6	Chronic
73	GOW	M28	51.7	Chronic
75	BIG	F38	92.5	Chronic
77	WIT	M48	67.0	Acute
82	XER	M23	66.1	Chronic
87	SME (1961)	F61	80.8	Chronic
98	SCH	M59	67.7	Chronic
542C	CAN	M48	92.0	Acute
550	LOW	M19	45.9	Acute
579A	COR	F49	*100	Chronic
579C	KUM	M21	63.0	Acute
619A	PER	M52	24.4	Acute

*See text for discussion of this result.

bound to the precipitate was 2.4%, representing 13.9% of the mean amount of radioactive vitamin B_{12} bound after a 96 hour dialysis.

5.1.2 Values in Patients with Haematological Disorders.

5.1.2.1 Myeloid Leukaemia. Results for vitamin B_{12} binding capacity at the 10 ng./ml. level obtained with plasma from 18 patients with myeloid leukaemia are set out in Table X, and results for binding at the 100 ng./ml. level in a further 18 patients with myeloid leukaemia are set out in Table XI; there are six patients common to both groups. The mean value for vitamin B_{12} binding capacity in myeloid leukaemia at the 10 ng./ml. level is 59.0% ± 18.9% (1 S.D.), and there is no difference between the mean value for the six patients with acute myeloid leukaemia (58.5%) and the mean for the group as a whole. These results are set out diagrammatically in Figure 9, which shows them in association with the normal range (mean ± 2 S.D.) for comparison. There is a wide scatter of results; three patients with chronic myeloid leukaemia had levels which fall just within the normal range, and one with acute myeloid leukaemia had a value which was unusually low. The remainder were outside the normal range.

Figure 9. Vitamin B_{12} binding capacity of myeloid leukaemic plasma at the 10 ng./ml. level; normal range (mean ± 2 S.D.) shown as broken line.

Δ = acute myeloid leukaemia
● = chronic myeloid leukaemia

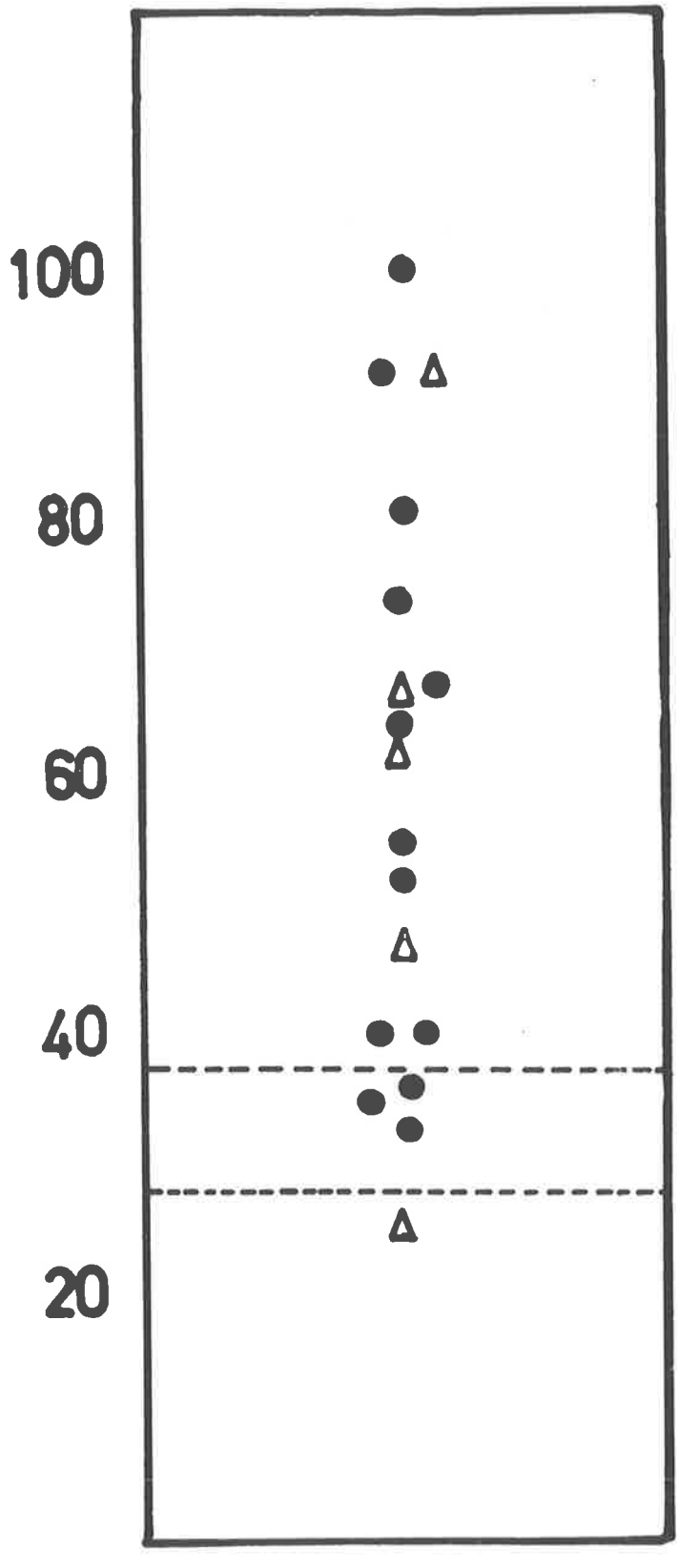
100
80
60
40
20

TABLE XI.

MYELOID LEUKAEMIA - VITAMIN B_{12} BINDING CAPACITY.

(100 ng./ml. level)

SERIES NUMBER		SEX AND AGE	BINDING CAPACITY (Percentage)	MORPHOLOGICAL CLASSIFICATION
74	GOW	M28	23.2	Chronic
121	CAR	F14	22.0	Chronic
122	MUL	F20	37.8	Chronic
129	COW	F60	22.3	Chronic
203	LEW	M28	18.2	Acute
204	ECK	M62	23.7	Chronic
429	GOL	M38	24.5	Acute
430	LEV	M22	26.0	Chronic
431	SME (1965)	F65	21.4	Chronic
461	HAR	M19	12.1	Acute
489A	WRI	F28	22.7	Chronic
495A	LIN	M26	17.6	Acute
500	BAR	F47	17.2	Acute
542A	CAM	M48	23.7	Acute
544	COO	M47	37.9	Chronic
550	LOW	M19	23.2	Acute
579B	COR	F49	35.7	Chronic
579D	KUM	M21	11.3	Acute

The mean value for vitamin B_{12} binding capacity at the 100 ng./ml. level obtained with plasma from 18 patients with myeloid leukaemia is 23.4% ± 5.1% (1 S.D.). Here, however, there are differences between the values obtained for the acute myeloid leukaemia patients and the chronic myeloid leukaemia patients. The mean vitamin B_{12} binding capacity at the 100 ng./ml. level for the 8 patients with acute myeloid leukaemia is 18.5% ± 4.0% (1 S.D.), which is not significantly different from the normal range; the corresponding figure for the 10 patients with chronic myeloid leukaemia is 27.3% ± 5.9% (1 S.D.) which is different from both the normal range and the values obtained in the acute myeloid leukaemic group. Application of "Student's" t test (Bradford Hill, 1966) gives a value in this latter group of $0.02 > p > 0.05$ ($t = 2.32$, $n = 16$). These results are set out diagrammatically in Figure 10, which shows them in association with the normal range for this level of binding (mean ± 2 S.D.). All values for patients with chronic myeloid leukaemia are above the normal range, while there is scattering of the values obtained in patients with acute myeloid leukaemia; three values fall within the normal range, two fall just below it and three are above the normal range.

Figure 10. Vitamin B_{12} binding capacity of myeloid leukaemia plasma at the 100 ng./ml. level; normal range (mean $\pm$ 2 S.D.) shown as broken line.

Δ = acute myeloid leukaemia
● = chronic myeloid leukaemia

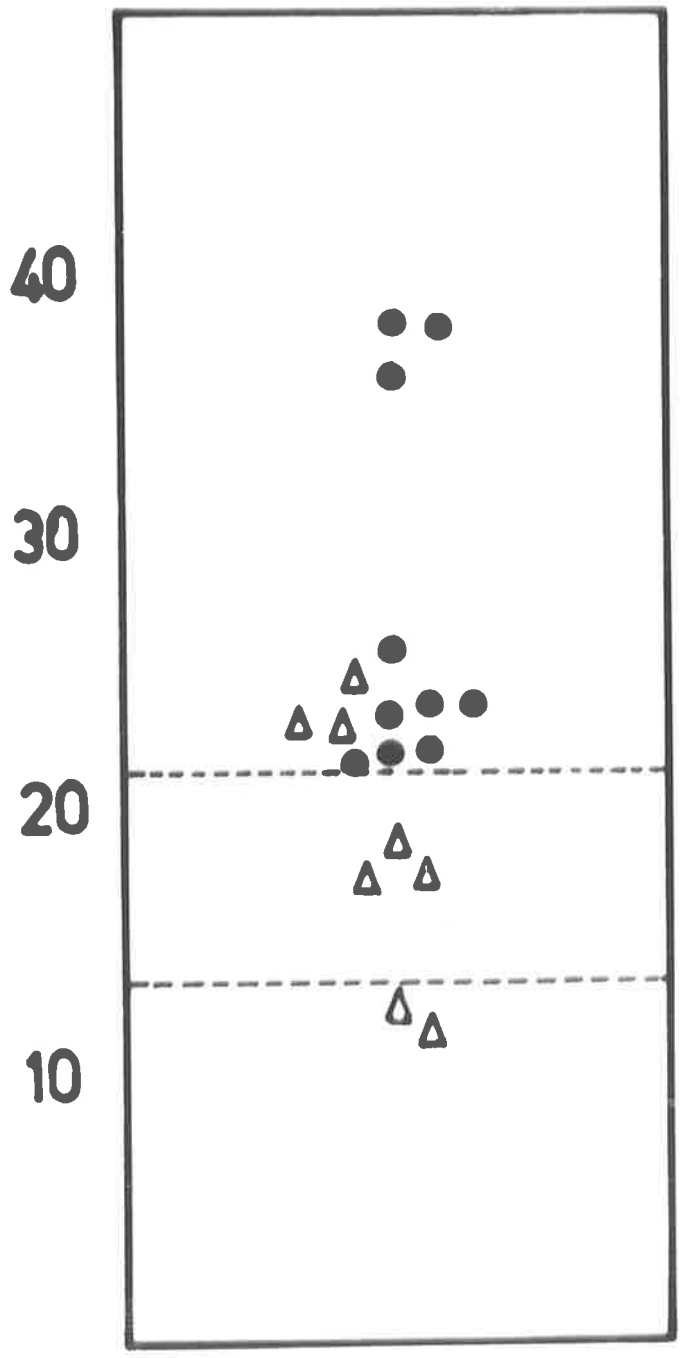
40
30
20
10

TABLE XII.

SERIAL VITAMIN B_{12} BINDING CAPACITY ESTIMATIONS - PATIENT COR.

(PERCENTAGE BOUND)

DATE	Concentration of Radioactive Vitamin B_{12} Added (ng./ml.)					
	2**	10	25	50	75	100
28.1.67	-		-	-	81.9 (15.1)*	
1.2.67	-	-	-	-	-	35.4
3.2.67	-	-	-	-	-	46.7
4.2.67	-	100†	85.7 (19.8)*	50.3 (15.7)*	50.4 (15.0)*	35.7
18.2.67	76.4	-	-	-	-	-
22.2.67	97.2	-	-	-	21.0	23.5
3.3.67	61.3	70.8	-	-	-	13.8

** High Specific Activity Co^{58}-Vitamin B_{12}

† See text for discussion of this value.

* Normal control value.

Serial determinations of vitamin B_{12} binding capacity were possible in the patient COR, a forty-nine year old woman with chronic myeloid leukaemia (Appendix AII); these are set out in Table XII. It will be seen that the binding values at the 100 ng./ml. level fell over a period of six weeks from an elevated level to a normal level, although the binding values at the 2 ng./ml. level and at the 10 ng./ml. level at this latter time remained abnormally high. The initial binding figure recorded for this patient at the 10 ng./ml. level, 100%, requires further comment. The test sample on this occasion not only bound all radioactive vitamin B_{12} added, but bound additional radioactive vitamin B_{12} which had been dialysed into the buffer out of the control sample which was being dialysed in the same vessel at the same time. The recorded value was "189.9%"; the validity of this result was confirmed by reproducing the conditions of the experiment in the following manner:

> 1 ml. of unlabelled plasma collected on the same day was placed in dialysis sacking, and dialysed against 100 ml. 0.01M tris phosphate buffer, pH 6.5, to which was added 100 ng. radioactive vitamin B_{12}. The binding capacity measured at the end of the

otherwise standard dialysis procedure was 35.7% (that is, 35.7 ng./ml.), a value identical with that found by measuring, in the standard manner, the binding capacity of this sample at this level of added radioactive vitamin B_{12} (Table XI). The measured yield from this experiment was 87.4% of the calculated value. Although the conditions of the experiments were not identical, since the radioactive vitamin B_{12} was not incubated with the plasma sample prior to dialysis, it is certain that the binding capacity of this plasma sample was greater than 10 ng./ml., and that the unusual binding capacity measured at the 10 ng./ml. level was a valid observation. The figure obtained for binding at the 25 ng./ml. level (85.7%) on a sample collected at the same time also confirms this initial observation.

It has not been possible to relate the vitamin B_{12} binding capacity (at any level) to the total white cell count nor any component of it, either on a linear, semi-logarithmic or logarithmic basis. The only associated finding which in chronic myeloid leukaemia appears to have relevance is that of basophilia, a finding unusual

TABLE XIII.

VITAMIN B_{12} BINDING CAPACITY AND BASOPHIL COUNTS IN MYELOID LEUKAEMIA

(Binding at 10 ng./ml. Level)

PATIENT		VITAMIN B_{12} BINDING CAPACITY. (Percentage)	WHITE CELL COUNT 10^3/cu.mm.	BASOPHILS	
				Percentage Count	Absolute Count (per cu.mm.)
FAL		32.4	80	2	1600
SME	1961	80.8	161	11	17710
	1963	73.6	27	10	2700
BIG		92.5	252	5	12600
XER		66.1	240	4	9600
SCH		67.7	30	12	3600
COR		100	9.1	20	1820

TABLE XIV.

RELATIONSHIP OF VITAMIN B_{12} BINDING CAPACITY, BASOPHILIA, AND ANTI-Fy^a TITRES IN A PATIENT WITH CHRONIC MYELOID LEUKAEMIA (COR)

DATE	VITAMIN B_{12} BINDING CAPACITY. (100 ng./ml. Level) Percentage	ANTI-Fy^a TITRE	WHITE CELL COUNT (10^3/cu.mm.)	BASOPHILS (per cu.mm.)
1.2.67	35.4	1/2048	9.2	1288
3.2.67	46.7	-	-	-
6.2.67	35.7	-	9.1	1820
10.2.67	-	-	13.6	2856
17.2.67	23.5	1/2048	7.9	1738
22.2.67	-	-	7.0	1330
24.2.67	-	1/1024	4.0	200
4.3.67	13.8	1/256	2.2	110

in the acute form of myeloid leukaemia. Although it was not possible to correlate accurately the vitamin B_{12} binding capacity with the basophil count, either absolute or relative, it was nevertheless apparent in several instances that the abnormal vitamin B_{12} binding capacity was demonstrable at the same time as basophilia, and that both tended to return to normal with effective treatment at approximately the same time. The results obtained on several patients in this regard have been set out in Table XIII, and serial results, relating the vitamin B_{12} binding capacity to the absolute and relative basophil count in the patient COR are set out in Table XIV; this same information is set out diagrammatically in Figure 11.

5.1.2.2 Other Forms of Leukaemia. Determinations of vitamin B_{12} binding capacity were made in four patients with lymphatic leukaemia, either chronic or acute. In all four instances, the levels obtained were within the normal range, both at the 10 ng./ml. level and the 100 mg./ml. level.

5.1.2.3 Dysproteinaemias. Five patients with various forms of dysproteinaemia were studied. In three of these, abnormally high values were obtained. These were found in a patient with macroglobulinaemia (22.1% binding at the

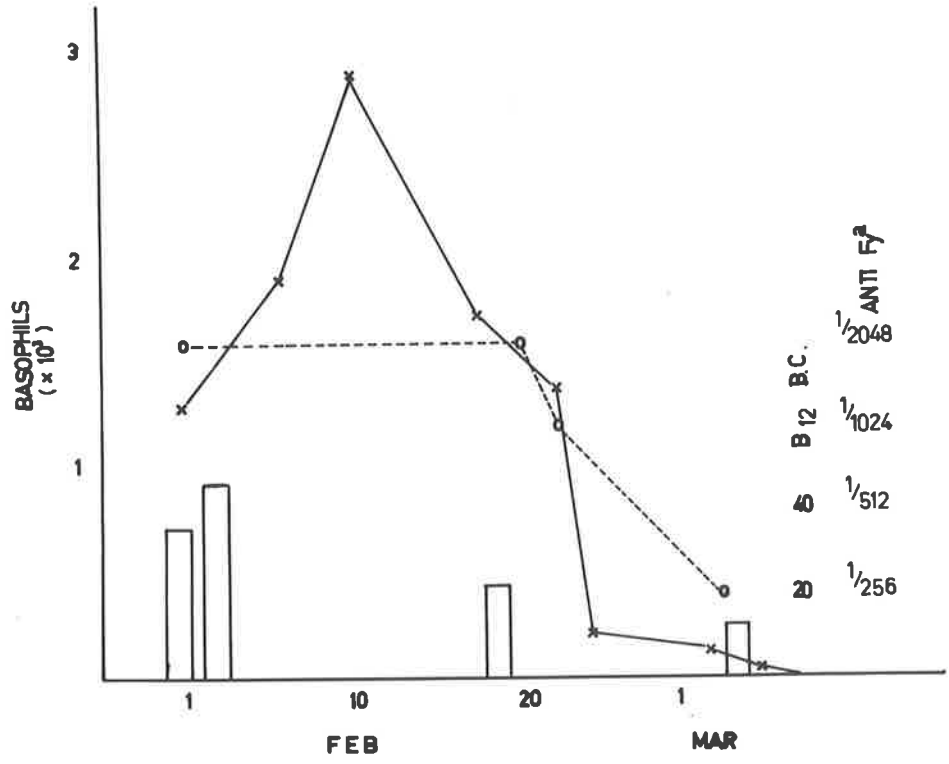

Figure 11. Data from patient COR showing absolute basophil counts (unbroken line), anti-Fy^a titre (broken line) and vitamin B_{12} binding capacity at the 100 ng./ml. level (open columns).

100 ng./ml. level), in a patient with severe rheumatoid arthritis and a markedly elevated serum gamma globulin level (41.4% binding at the 10 ng./ml. level), and in a patient with multiple myeloma (42.0% binding at the 10 ng./ml. level). In each of these three instances, the increased binding was associated with the precipitated proteins, and the amount bound to the dialysant was within normal limits. As each of these patients had a gross increase in the circulating level of a high molecular weight plasma protein at the time of study, these results are not surprising.

5.1.2.4 Isologous Antibodies. Because an abnormally high vitamin B_{12} binding capacity was demonstrated in the plasma of patient COR (Appendix AII) at the same time as an unusually high titre of anti-Fy^a antibody, the vitamin B_{12} binding capacity of the plasma of several other patients with circulating isologous antibodies was measured. Three patients were studied; the first, with an anti-D antibody present to a titre of $^1/2048$, bound 24.4% of the radioactive vitamin B_{12} added at the 10 ng./ml. level, and 13.7% of the radioactive vitamin B_{12} added at the 75 ng./ml. level; these results are normal. The second patient had made an anti-Kell antibody, and his plasma bound 14.8% of the radioactive vitamin B_{12} added at the 75 ng./ml. level;

this is also a normal result. Similarly, normal results were obtained from another patient with an anti-Fy^a antibody present to a titre of $^1/128$, whose plasma bound 17.6% of the radioactive vitamin B_{12} (high specific activity) added at the 2 ng./ml. level, and 26.0% of the standard radioactive vitamin B_{12} added at the 10 ng./ml. level.

5.1.2.5 Vitamin B_{12} Deficiency. Vitamin B_{12} binding capacity was measured at the 10 ng./ml. level on plasma samples from six patients with vitamin B_{12} deficiency, with serum vitamin B_{12} levels ranging from 0-100 pg./ml. The mean binding capacity was 45.3% ± 4.8% (1 S.D.), that is, 4.53 ± 0.48 ng./ml., and the range was 40.2% - 54.9%. The plasma sample which gave the 40.2% result at the 10 ng./ml. level was also investigated at the 100 ng./ml. level, and the vitamin B_{12} binding capacity was 17.1%, which is a normal result.

Subsequently, the vitamin B_{12} binding capacity of the serum from 18 patients with vitamin B_{12} deficiency was measured at the 100 ng./ml. level. The serum vitamin B_{12} levels were between 0 and 137 pg./ml., the mean being 37 pg./ml. The mean vitamin B_{12} binding capacity was 15.2% ± 1.8 (1 S.D.), that is, 15.2 ± 1.8 ng./ml., which

TABLE XV.

VITAMIN B_{12} BINDING CAPACITY IN VITAMIN B_{12} DEFICIENCY.

(At 100 ng./ml. Level)

PATIENT	SEX AND AGE	DIAGNOSIS	SERUM B_{12} pg./ml.	BINDING CAPACITY. Percentage
BLA	M62	Megaloblastic Anaemia	24	16.4
BRO	F69	" "	41	15.8
DRI	M66	Post-gastrectomy	121	17.2
DUN	F73	Pernicious Anaemia	24	17.0
HAS	F86	" "	109	15.1
KNI	M72	" "	Less than 20	17.3
LAN	F62	" "	66	15.7
LIN	F77	" "	Less than 20	19.2
ROU	F73	" "	Less than 20	15.4
STO	M67	Megaloblastic Anaemia	24	14.5
BUR		" "	41	17.1
WIL	M74	" "	22	16.6
WIS	M55	" "	86	13.4
MacD	F51	" "	116	14.7
ROS	F67	Post-gastrectomy	137	9.5
HAR	M76	Pernicious Anaemia	45	12.2
SHE	M74	" "	83	13.0
TON	M76	Post-gastrectomy	85	12.2

is not significantly different from normal at the 100 ng./ml. level. Although there was a detectable trend (Table XV) towards an association between lower serum vitamin B_{12} levels, and higher vitamin B_{12} binding capacities, this was not sufficiently marked for statistical significance to be proven.

5.1.3 Values in Pregnancy.

The plasma vitamin B_{12} binding capacity was measured at the 10 ng./ml. level in 24 pregnant women and at the 100 ng./ml. level in a further 14 pregnant women. The mean value at the 10 ng./ml. level was 23.9% ± 2.8%(1 S.D.), that is, 2.39 ± 0.28 ng./ml., which is slightly lower than the normal range, and the mean value at the 100 ng./ml. level was 16.9% ± 1.5% (1 S.D.), that is, 16.9 ± 1.5 ng./ml.; this is not different from normal.

5.1.4 Resumé.

Gross elevations of the plasma vitamin B_{12} binding capacity were demonstrable in the majority of patients with chronic myeloid leukaemia at the 10 ng./ml. level, and at the 100 ng./ml. level. In patients with acute myeloid leukaemia, the vitamin B_{12} binding capacity at the 10 ng./ml. level was consistently elevated, and the mean value was similar to that for patients with chronic myeloid leukaemia;

however, at the 100 ng./ml. level, the mean value for vitamin B_{12} binding capacity of acute leukaemic plasma differed significantly from the mean value for chronic myeloid leukaemic plasma, and was not significantly different from the normal mean value.

Attention has been drawn to the possible relationship between basophilia and the elevated vitamin B_{12} binding capacity in chronic myeloid leukaemia patients.

In lymphatic leukaemia, the vitamin B_{12} binding capacity did not differ from normal.

An increase in the binding of vitamin B_{12} to the plasma proteins of patients with dysproteinaemias was noted in three of five patients studied; the increase in each instance was in the precipitated portion of the dialysed labelled protein.

The vitamin B_{12} binding capacity of plasma from patients with isologous antibodies did not differ from normal.

The mean plasma vitamin B_{12} binding capacity at the 10 ng./ml. level for six patients with vitamin B_{12} deficiency was above normal, but the mean plasma vitamin B_{12} binding capacity at the 100 ng./ml. level in a further 18 vitamin B_{12} deficient patients was not different from the normal mean value at this level.

The mean plasma vitamin B_{12} binding capacity at the 10 ng./ml. level in 24 pregnant women was somewhat lower than normal, but the mean plasma vitamin B_{12} binding capacity at the 100 ng./ml. level in a further 14 pregnant women did not differ significantly from the normal mean.

5.2 Column Chromatography.

The column chromatograms to be described below have been carried out in accordance with the methods set out earlier in this study. Optical density is shown on the left-hand side of each figure, and radioactivity, in counts per minute above background, appears on the right-hand side of each figure. The positions on the chromatogram at which the elution buffers were changed have been indicated as part of the legend for each figure. Although it would be possible by calculation, allowing for decay and the volume of material placed on the column, to measure the vitamin B_{12} binding capacity, using the data from dialysed and undialysed samples, this has not been undertaken, since each dialysed sample placed on the column has had a binding capacity measurement performed in the more direct standard manner prior to column chromatography. For this reason, the columns have been assessed as qualitative rather than quantitative patterns of distribution of the labelled vitamin

B_{12} throughout the plasma proteins.

5.2.1 Normal Plasma.

The column chromatogram obtained when 1 ml. of dialysed plasma, labelled with radioactive vitamin B_{12} at the 100 ng./ml. level was eluted is shown in Figure 12. Four major peaks, I, II, III-IV and V were noted. The radioactivity was located principally at the III-IV peak, with other detectable amounts at peaks I and V. This figure also shows, for reference purposes, the effect of the step-wise elution on the pH values in each tube of the column.

The pattern exhibited by vitamin B_{12} unassociated with plasma protein, when subjected to the standard column chromatographic procedure, is shown in Figures 13 and 14; Figure 13 shows the optical density at 280 Å and at 550 Å of 1,000 µg. of cyanocobalamin placed upon the column and subjected to the usual elution procedure. The single initial peak indicates that the material is not bound to the column, but proceeds through with the first buffer used, which is identical with the column equilibrating buffer. Similar results were obtained (Figure 14) when 3 ml. of saline containing 0.3 µg. of radioactive vitamin B_{12} was placed on the standard column; all radioactivity

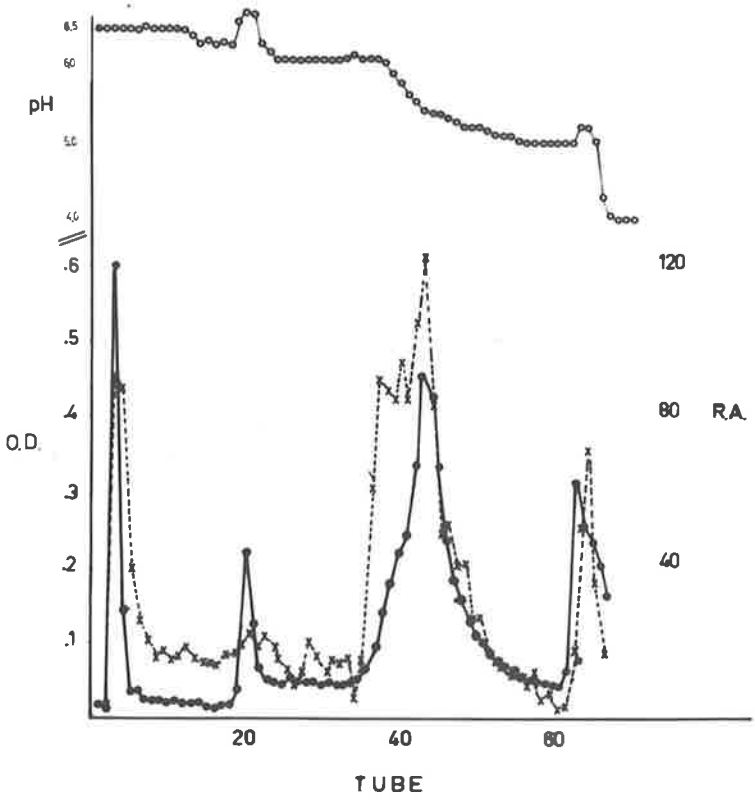

Figure 12. Column chromatogram of dialysed radioactive vitamin B_{12} labelled normal serum (1 ml.). Changes in eluent were made at tubes 17, 33 and 63.

●——● = optical density at 280 mμ.
x----x = radioactivity in counts per minute above background
o——o = pH

B113

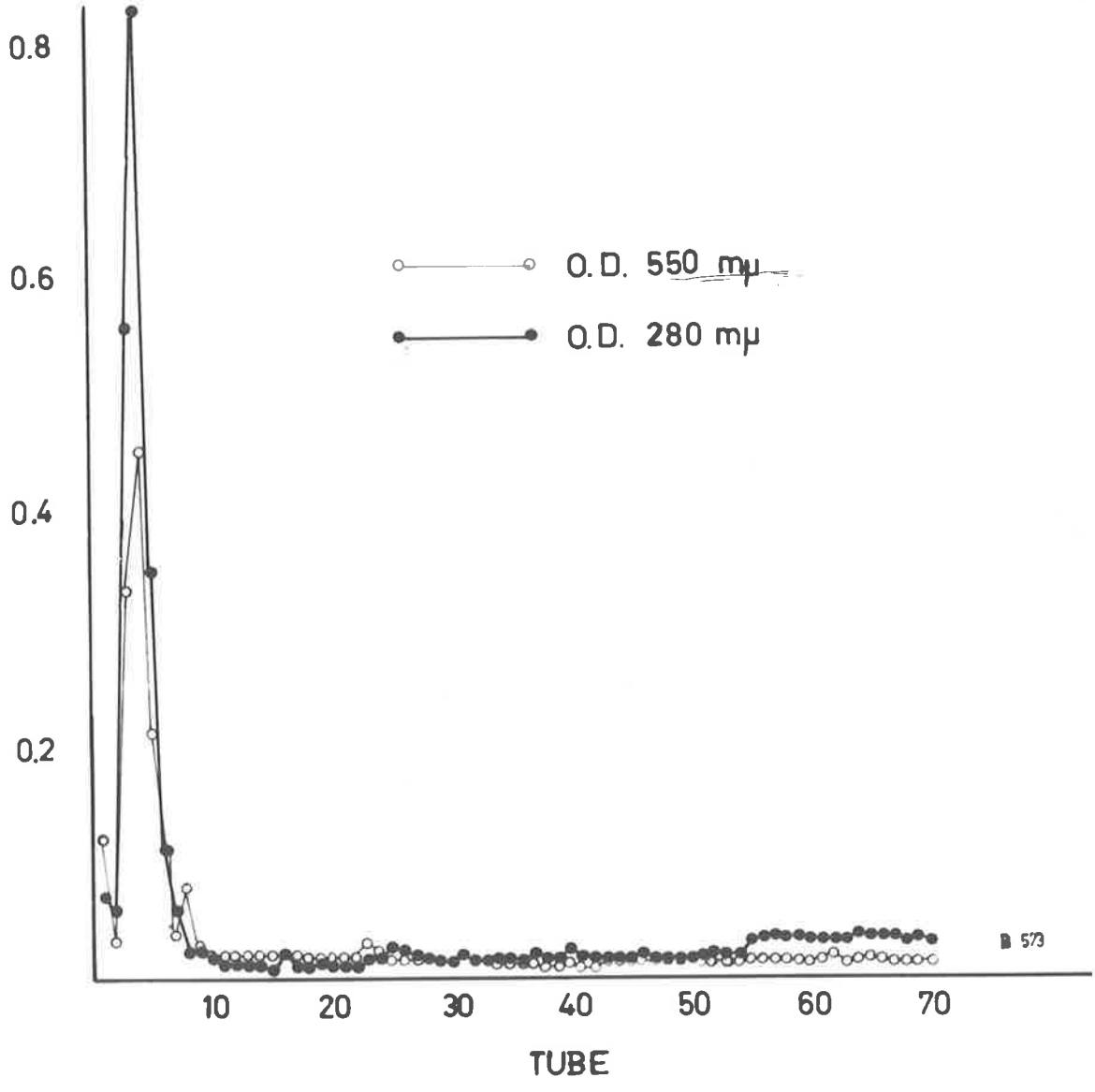

Figure 13. Column chromatogram of cyanocobalamin (1000 μg.).

Changes of eluent were made at tubes 21, 37 and 52.

B573.

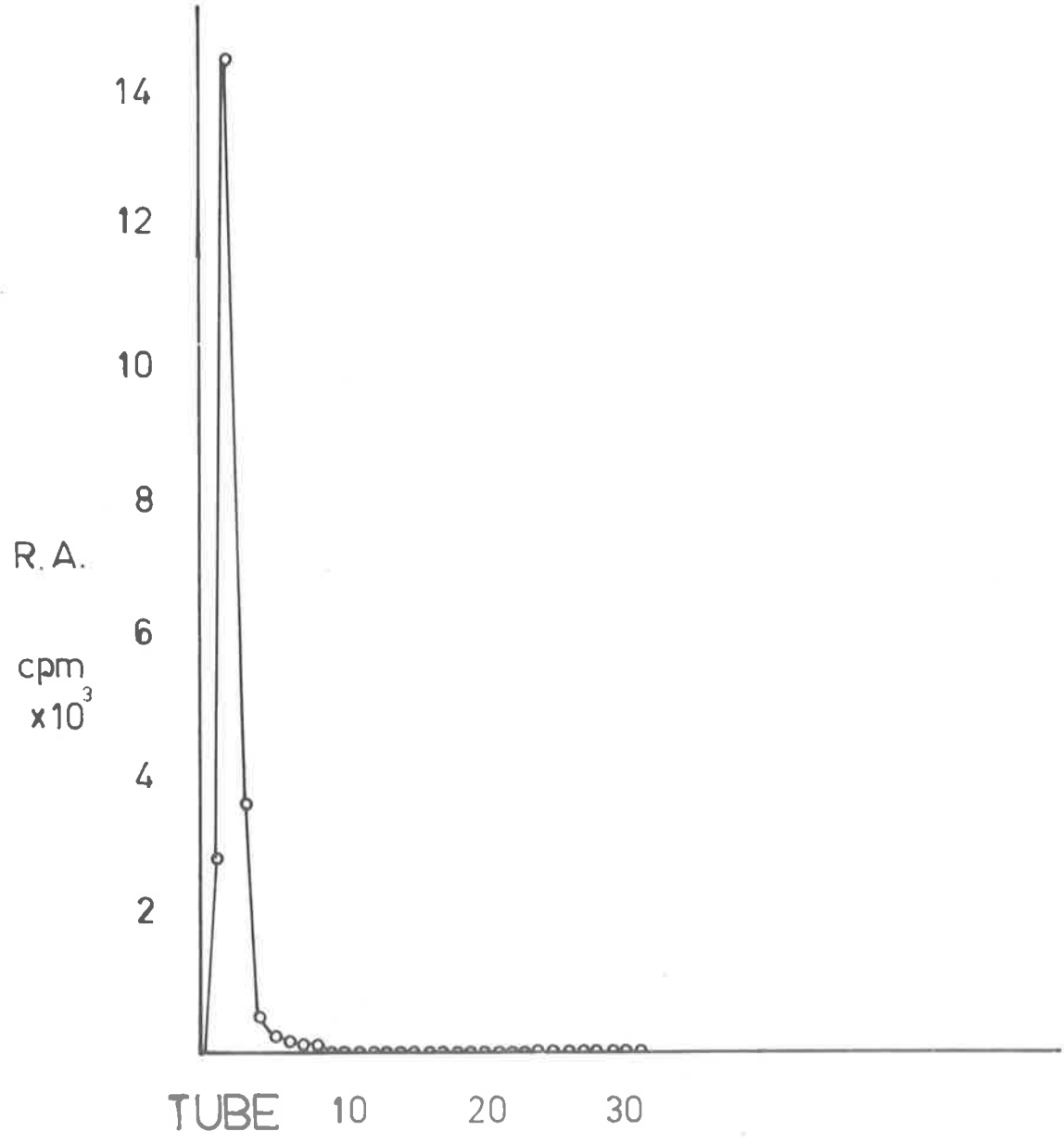

Figure 14. Column chromatogram (radioactivity in counts per minute above background only) of 0.3 µg. radioactive vitamin B_{12}.

No eluent change; yield was greater than 92%.

appeared at the first peak, and the radioactivity detected in the eluent accounted for above 92% of that placed on the column.

5.2.2 Normal Plasma - High Specific Activity Radioactive Vitamin B_{12}.

The pattern obtained with undialysed normal plasma, labelled with high specific radioactivity vitamin B_{12} at the 2 ng./ml. level is shown in Figure 15. It will be noted that most of the radioactivity is associated with the initial peak; approximately one-tenth the amount of radioactivity is associated with peak II, and a smaller, detectable amount of radioactivity is present at peak V. The chromatogram obtained from this plasma after dialysis is shown in Figure 16. Here, little radioactivity is seen at peak I, and most of the radioactive material is found at peaks V and II, the amount at peak II being approximately half that at peak V. It is therefore concluded that the majority of the material seen in peak I at Figure 15 represents radioactive vitamin B_{12} which was not bound to plasma proteins, and which proceeded through the column in the manner shown in Figure 14 above. It will be noted that there is more radioactivity at peak V of the dialysed sample than at peak V of the undialysed standard; similar

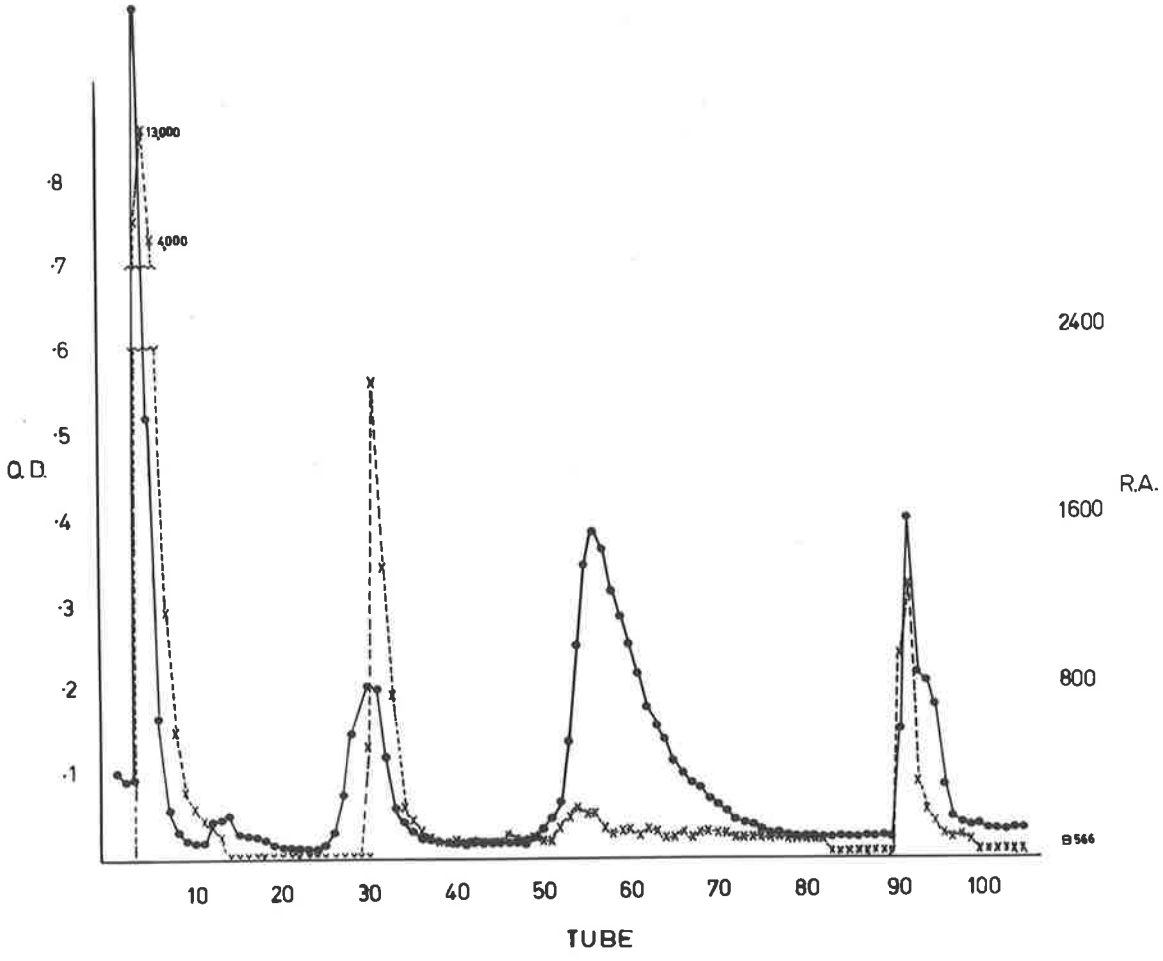

Figure 15. Column chromatogram of 0.75 ml. normal undialysed plasma labelled with high specific activity radioactive vitamin B_{12} at the 2 ng./ml. level.

Changes of eluent were made at tubes 26, 42 and 90.

●——● = optical density at 280 mμ.
X-----X = radioactivity in counts per minute above background.

B566

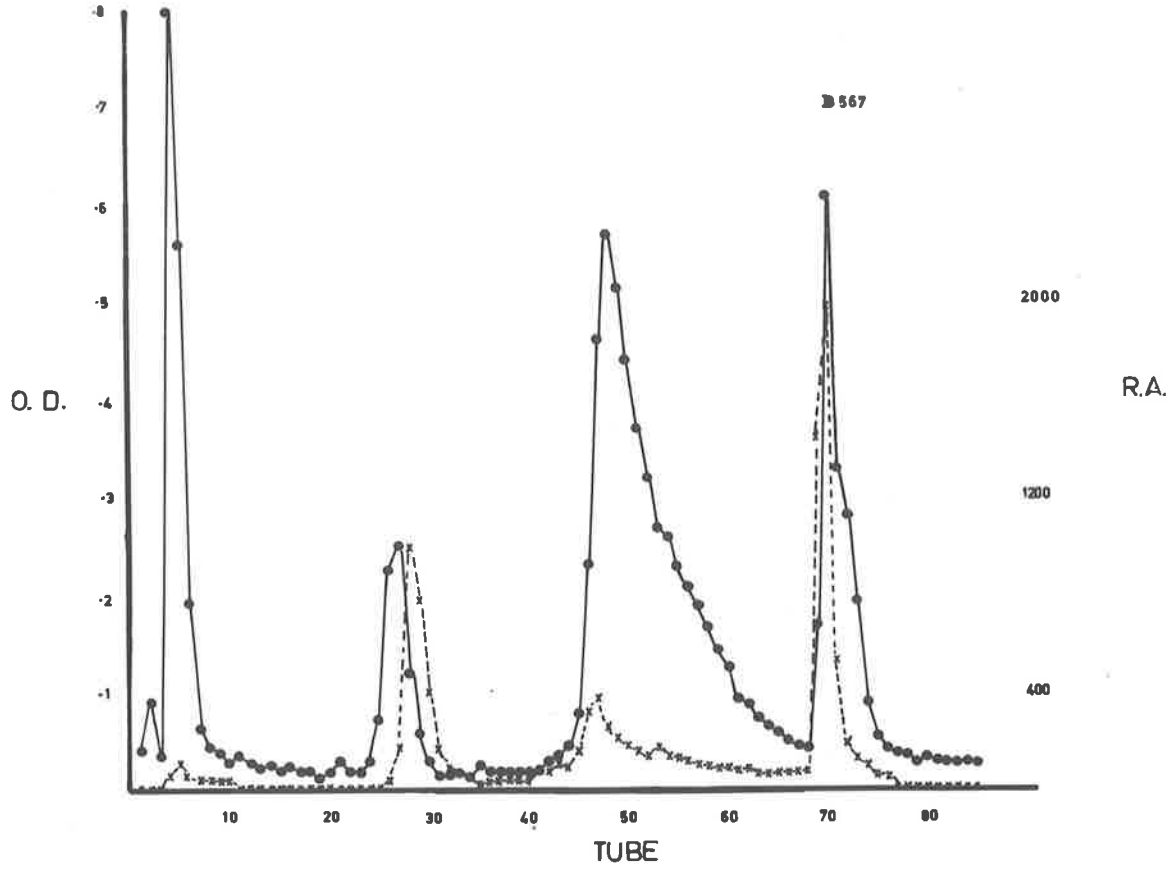

Figure 16. Column chromatogram of 1.0 ml. normal dialysed plasma labelled with high specific activity radioactive vitamin B_{12} at the 2 ng./ml. level.
Changes of eluent were made at tubes 21, 34 and 67.

●——● = optical density at 280 mμ.
x---x = radioactivity in counts per minute above background.

B567.

volumes of plasma were placed on each column, and the two columns were conducted four days apart, so that radioactive decay alone will not account for this difference. It is also observed that the material at peak II is less on the dialysed specimen than on the undialysed specimen. Since peak II is known to be the peak at which most of the beta globulin appears, and since peak V is known to be the peak at which most of the α_1 globulin appears, it is possible that at the end of the period of dialysis, a transfer of vitamin B_{12} from the beta to α_1 globulins has taken place in vitro in much the same manner as takes place in vivo. Attention is also drawn to the fact that in both instances, the peak of radioactivity associated with peak II is slightly behind the peak of optical density; this finding will be discussed later.

5.2.3. Abnormal Plasma. The pattern obtained when 1 ml. of dialysed radioactive vitamin B_{12} labelled plasma from a patient with chronic myeloid leukaemia was placed on the standard column is shown in Figure 17. The plasma used was obtained from patient GOW (Table X), and had been bound at the 10 ng./ml. level; the binding at this level was 51.7%, that is, 5.17 ng./ml. Most of the radioactive material was detected at peak V, with smaller quantities

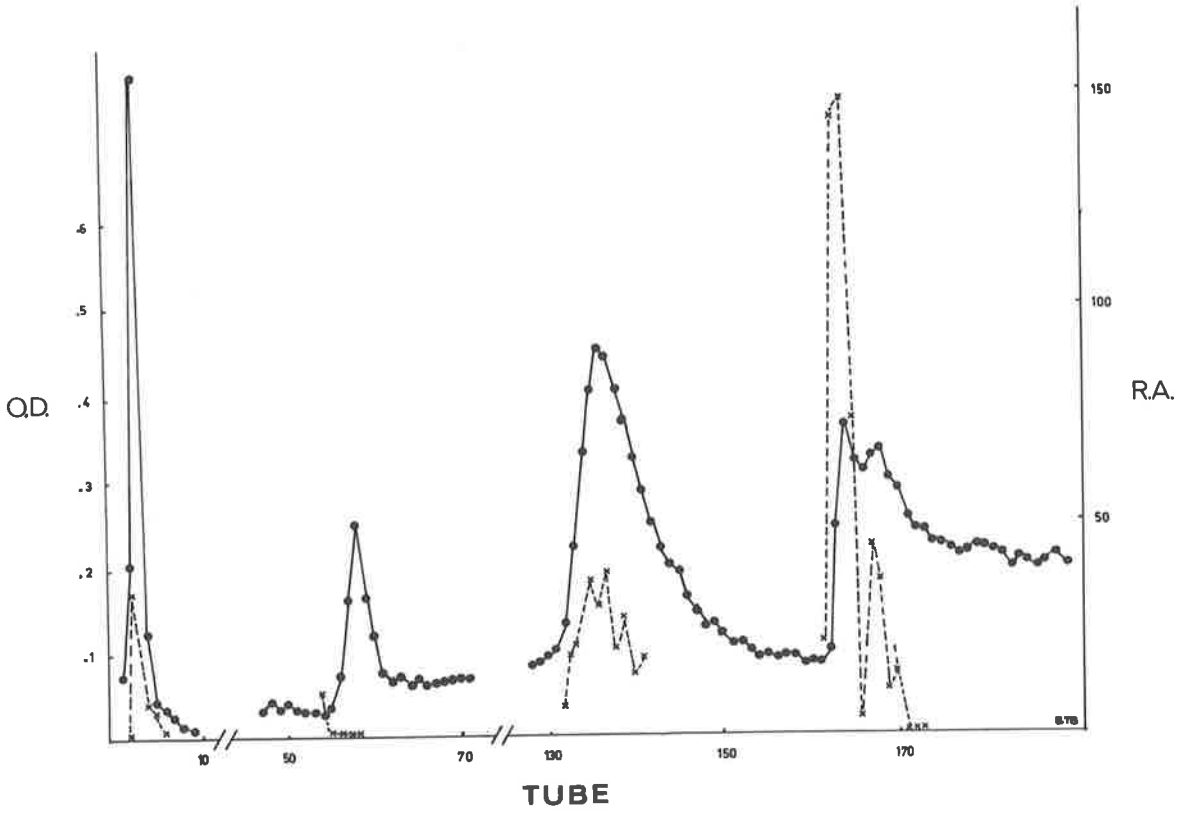

Figure 17. Column chromatogram of 0.5 ml. dialysed chronic myeloid leukaemia plasma labelled with radioactive vitamin B_{12} at the 10 ng./ml. level (patient GOW, B73).

Changes of eluent were made at tubes 50, 130 and 160.

●——● = optical density at 280 mμ.
x– – –x = radioactivity in counts per minute above background.

B78

being found at peaks I and III-IV.

The chromatography pattern obtained from dialysed labelled plasma from another patient with chronic myeloid leukaemia (Patient MUL, Table XI) is shown at Figure 18; binding was performed at the 100 ng./ml. level. Although peaks of radioactivity were observed in association with column peaks I, II, III-IV and V, most of the radioactive material was located at peak V; the two points at the top of this peak represent 5408 counts above background.

Figure 19 is the column chromatogram of dialysed radioactive vitamin B_{12} labelled serum from a 19 year old female patient, who had a serum vitamin B_{12} level of 60 pg./ml., and vitamin B_{12} binding capacity of 20.4 ng./ml. at 100 ng./ml. level. Her primary disease was considered to be idiopathic steatorrhoea. It will be noted that the complex peak III-IV was of an unusual shape, and was associated with considerable amounts of the radioactive vitamin B_{12}, in two separate areas. Since this is the peak at which albumin normally appears, and as the patient was hypoalbuminaemic, the abnormality in the protein pattern is probably explained on this basis; however, the pattern of binding of the radioactive vitamin B_{12} is quite different from normal. Similar patterns have not been found in other patients with

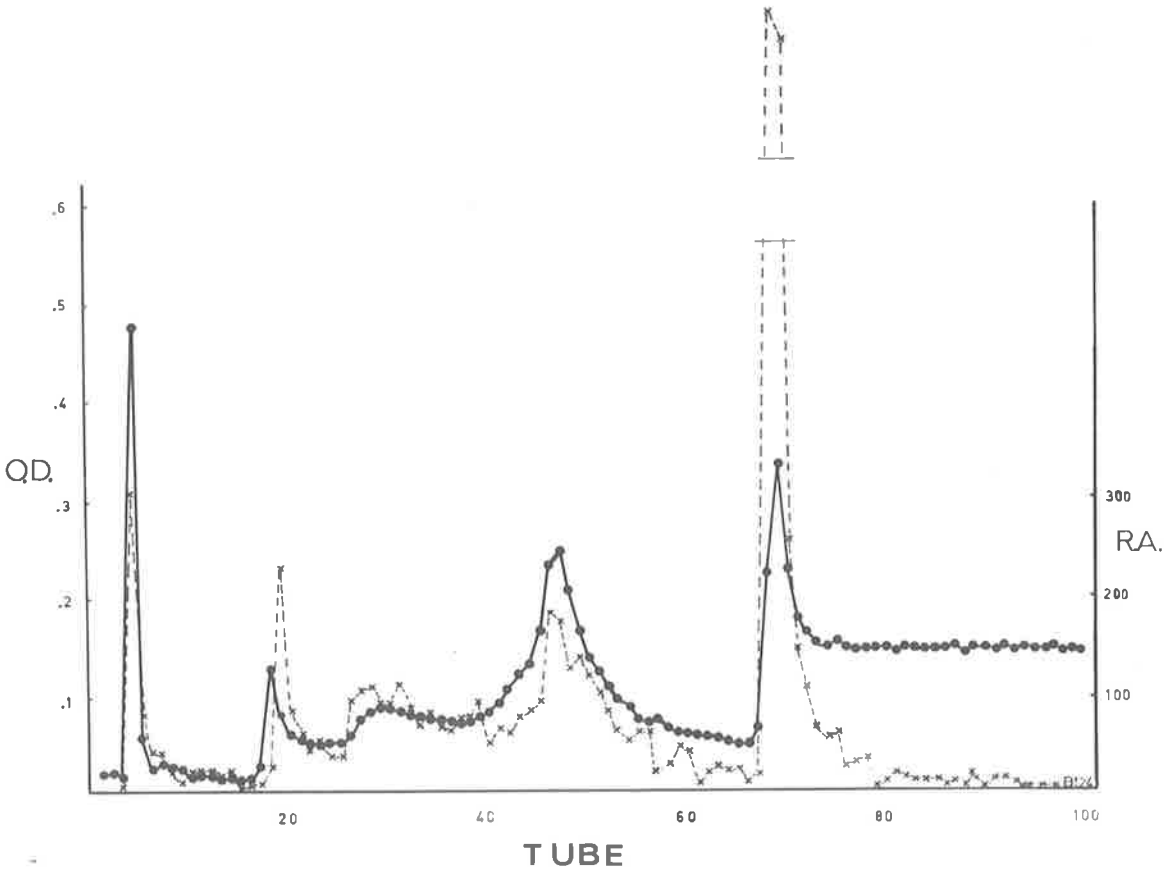

Figure 18. Column chromatogram of 1.0 ml. dialysed chronic myeloid leukaemia plasma labelled with radioactive vitamin B_{12} at the 100 ng./ml. level (patient MUL, B122).

Changes of eluent were made at tubes 16, 34 and 64. Radioactivity at peak V was in excess of 5000 cpm.

●——● = optical density at 280 mμ.
x---x = radioactivity in counts per minute above background.

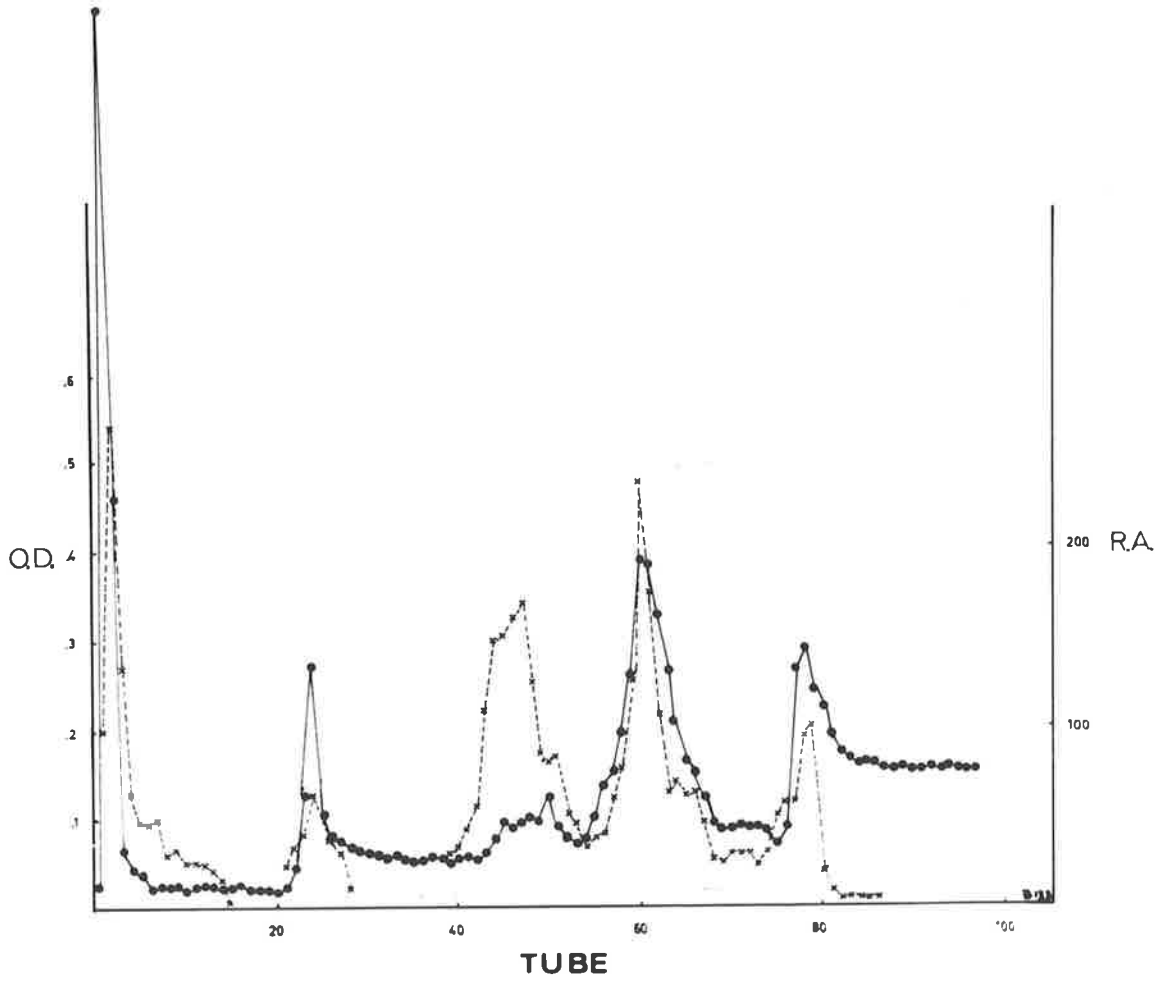

Figure 19. Column chromatogram of 1.0 ml. dialysed plasma labelled with radioactive vitamin B_{12}. Sample from patient with idiopathic steatorrhoea and vitamin B_{12} deficiency.

Changes of eluent were made at tubes 20, 50 and 77.

●——● = optical density at 280 mμ.
x----x = radioactivity in counts per minute above background.

vitamin B_{12} deficiency unrelated to idiopathic steatorrhoea, and it is considered likely that this particular pattern may be a result of protein deficiency as well as a reflection of vitamin B_{12} deficiency.

5.2.4 Abnormal Plasma - High Specific Activity Radioactive Vitamin B_{12}.

All studies in this particular section were performed with high specific activity Co^{58}-vitamin B_{12} (approximately 90 µc./µg.), and binding was performed in each instance at the 2 ng./ml. level. The pattern obtained from undialysed radioactive vitamin B_{12} labelled plasma from a patient with chronic myeloid leukaemia, at a time when this plasma showed no detectable bound vitamin B_{12} (patient SME, sample taken 6.5.65) is shown at Figure 20. In contrast to the normal binding pattern seen at this level (Figure 15), almost one-third of the radioactivity prior to dialysis was associated with peak V. The pattern obtained following dialysis of this particular undialysed specimen is shown at Figure 21, and almost all detectable radioactivity was associated with peak V; there was some radioactivity in association with peak II and peak III-IV, but this was less than 5% of the radioactivity represented on this column chromatogram.

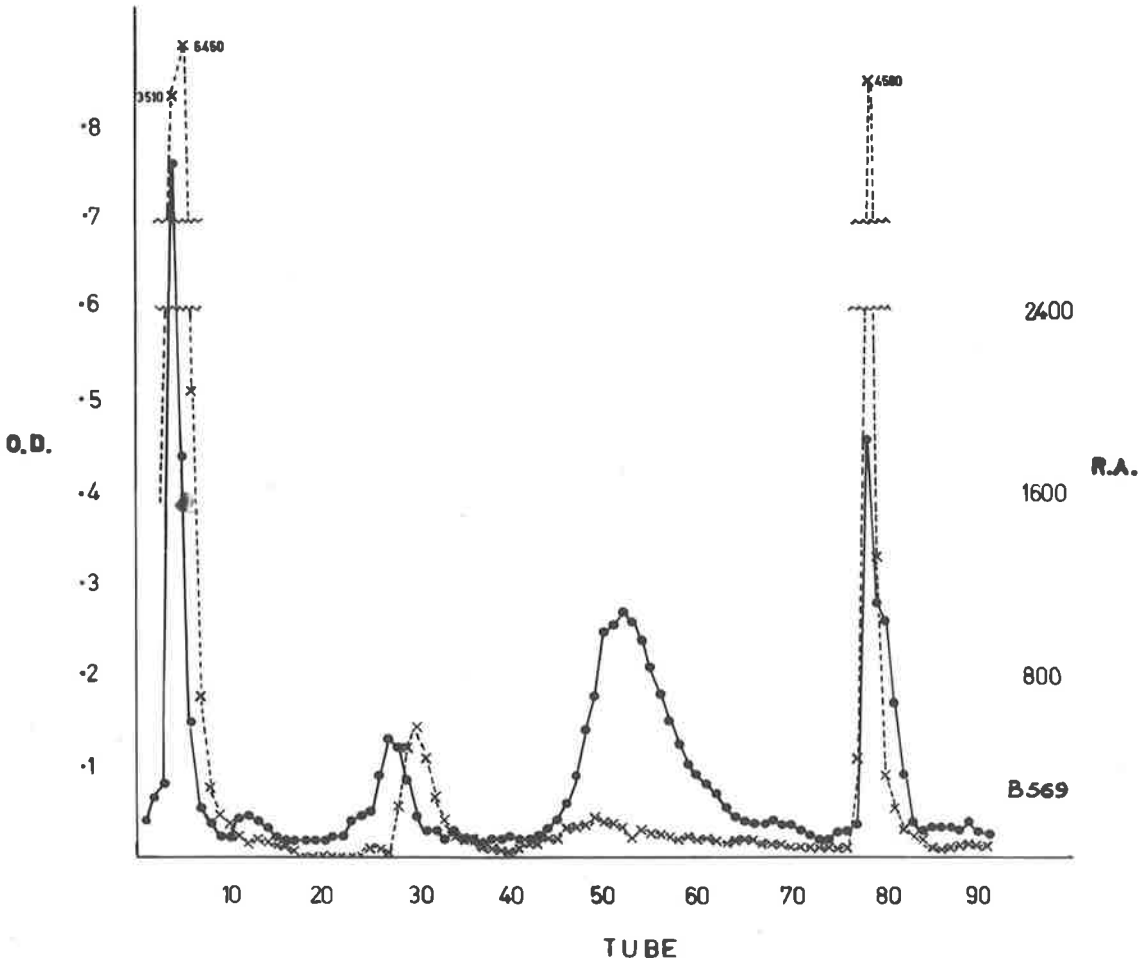

Figure 20. Column chromatogram of 1.0 ml. undialysed chronic myeloid leukaemia plasma labelled with radioactive vitamin B_{12} at the 2 ng./ml. level (high s.a.); Patient SME.

Changes of eluent were made at tubes 23, 36 and 75.

●——● = optical density at 280 mμ.
x---x = radioactivity in counts per minute above background.

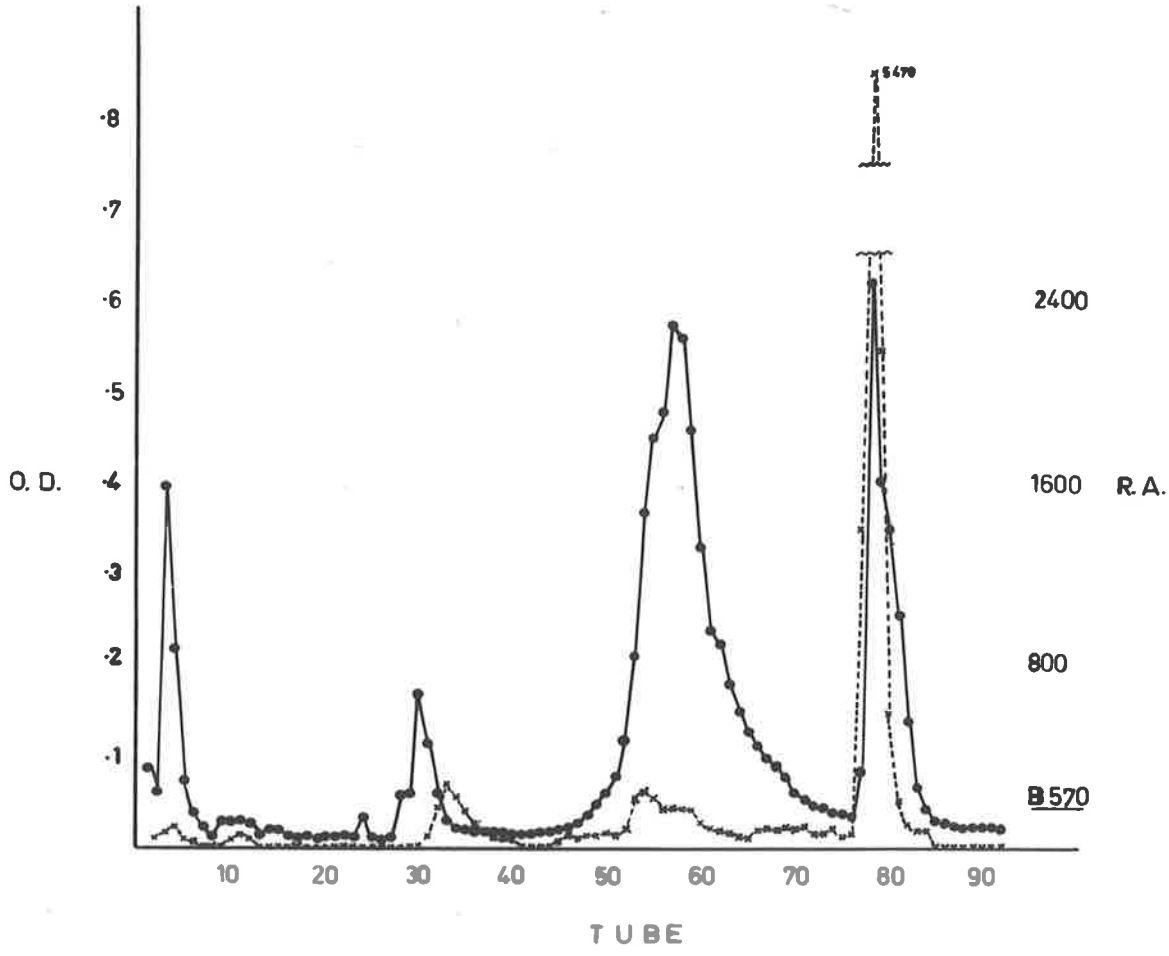

Figure 21. Column chromatogram of 1.0 ml. dialysed chronic myeloid leukaemia plasma labelled with high specific activity radioactive vitamin B_{12} at the 2 ng./ml. level; patient SME.

Changes of eluent were made at tubes 25, 37 and 75.

●——● = optical density at 280 mμ.
x----x = radioactivity in counts per minute above background.

B570.

A column chromatogram of an undialysed radioactive vitamin B_{12} labelled specimen of plasma from patient COR (18.2.67) is shown at Figure 22. The most striking feature of this particular chromatogram is that all radioactivity, with the exception of a small peak in association with peak II, was located in association with peak V. It is therefore probable that all radioactive vitamin B_{12} was bound to protein when the material was placed on the column. The dialysant of this particular sample, which was chromatographed some weeks before the standard, is shown at Figure 23. Similar results are shown at Figures 24 and 25 for further samples of plasma from patient COR. Again, almost all detectable radioactivity was located at peak V.

Two later chromatograms of this patient's plasma are shown at Figures 26 and 27; Figure 26 shows the results of column chromatography of a dialysed radioactive vitamin B_{12} labelled specimen of plasma collected on 3.3.67, and Figure 27 shows the pattern obtained from column chromatography of a dialysed radioactive vitamin B_{12} labelled specimen obtained on 28.4.67. It will be noted that in both instances, significant amounts of radioactivity were located at peak II, in addition to the larger amount of

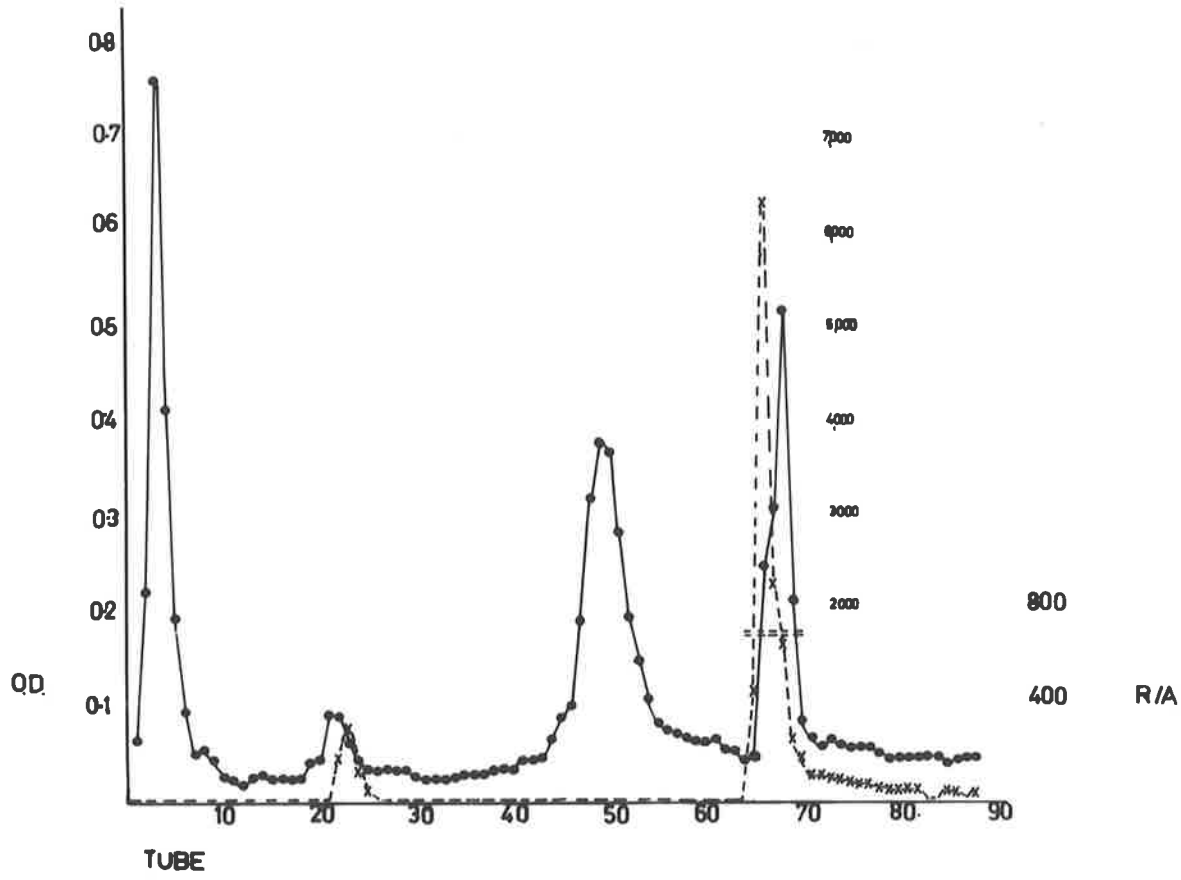

Figure 22. Column chromatogram of 1.0 ml. undialysed chronic myeloid leukaemia plasma labelled with high specific activity radioactive vitamin B_{12} at the 2 ng./ml. level; patient COH; 18.2.67.

Changes of eluent were made at tubes 17, 34 and 64.

●——● = optical density at 280 mμ.
x---x = radioactivity in counts per minute above background.

B621.

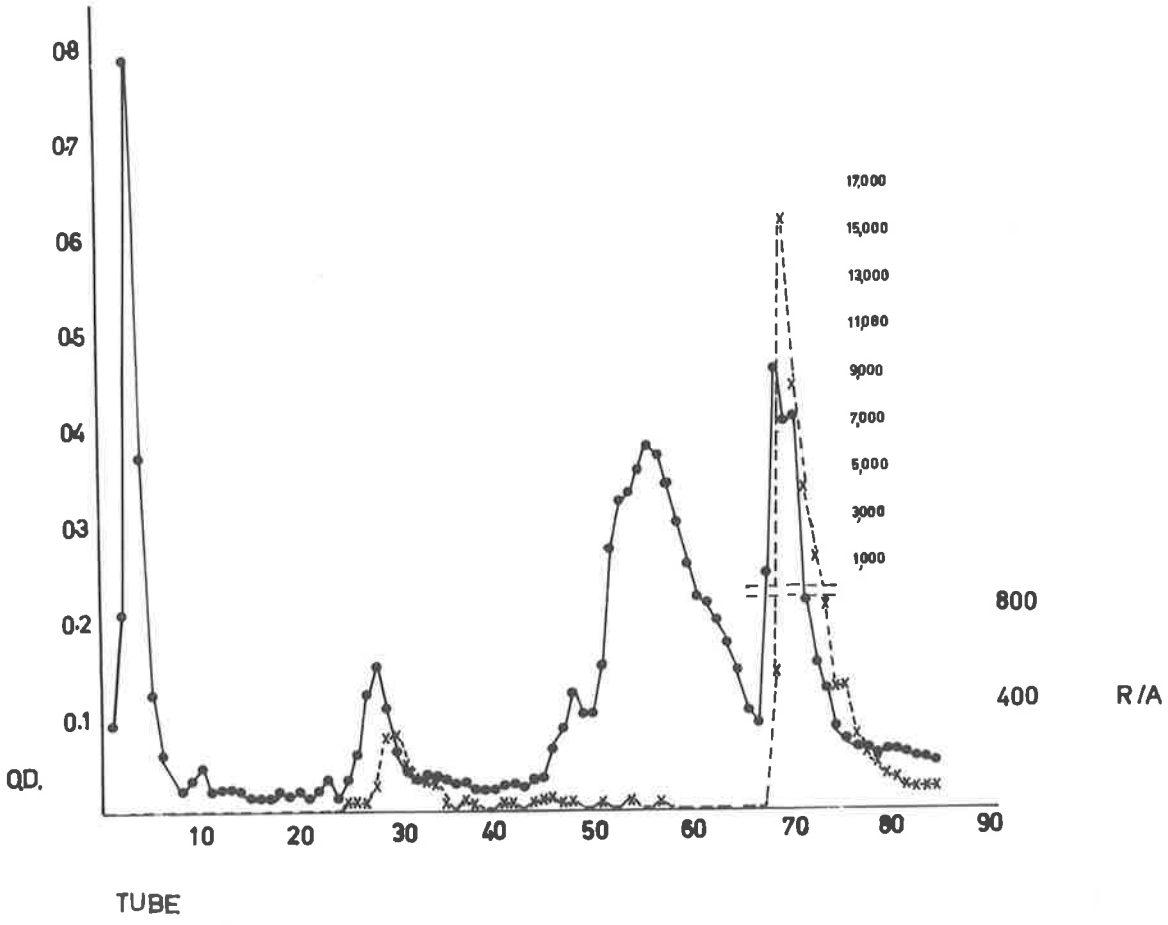

Figure 23. Column chromatogram of 1.0 ml. dialysed chronic myeloid leukaemia plasma labelled with high specific activity radioactive vitamin B_{12} at the 2 ng./ml. level; patient COR, 18.2.67.

Changes of eluent were made at tubes 22, 38 and 67.

●——● = optical density at 280 mμ.
x----x = radioactivity in counts per minute above background.

B615.

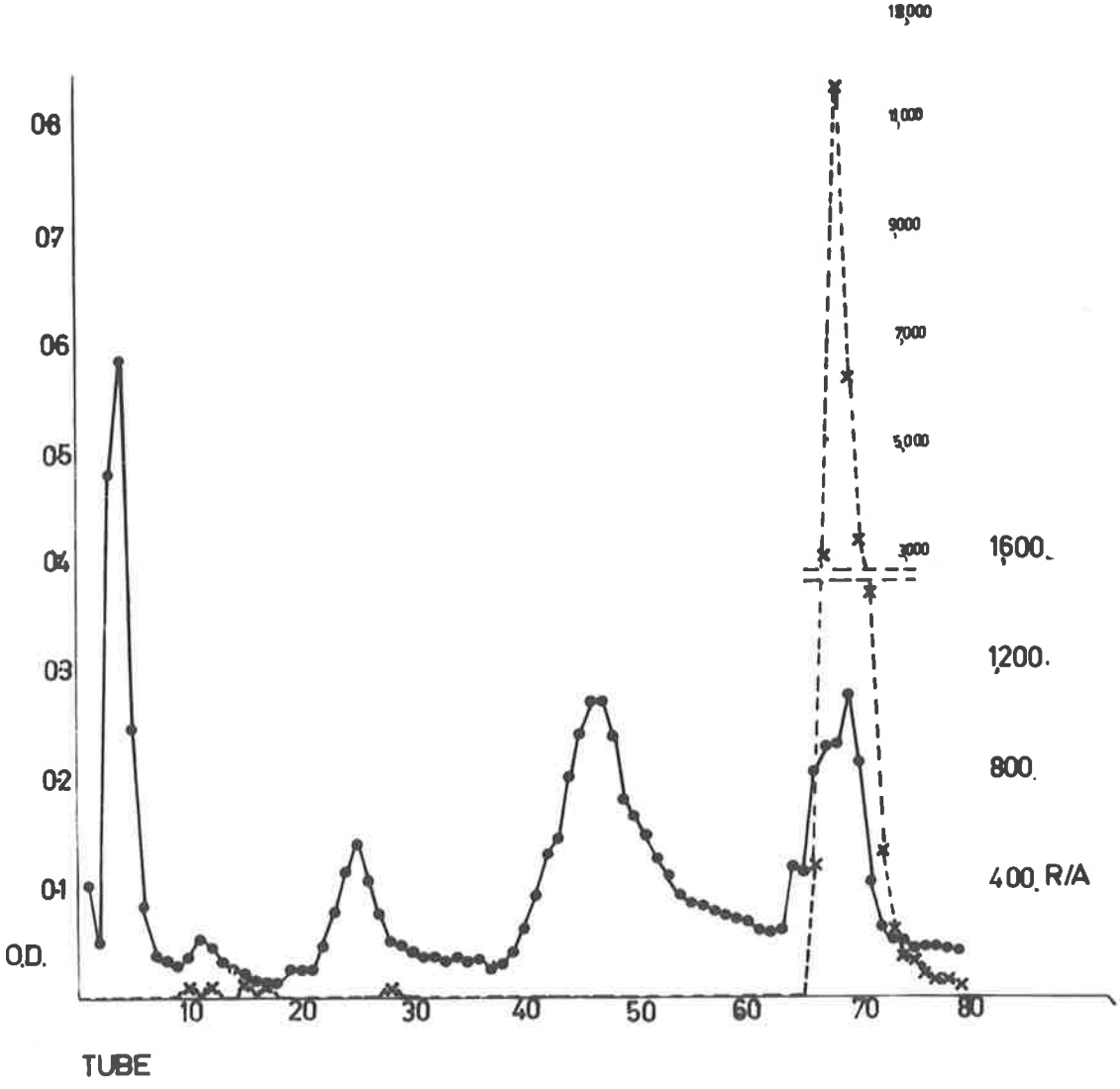

Figure 24. Column chromatogram of 0.75 ml. undialysed chronic myeloid leukaemia plasma labelled with high specific activity radioactive vitamin B_{12} at the 2 ng./ml. level; patient COR, 22.2.67.

Changes of eluent were made at tubes 19, 30 and 63.

●——● = optical density at 280 mμ.
x- - -x = radioactivity in counts per minute above background.

B628.

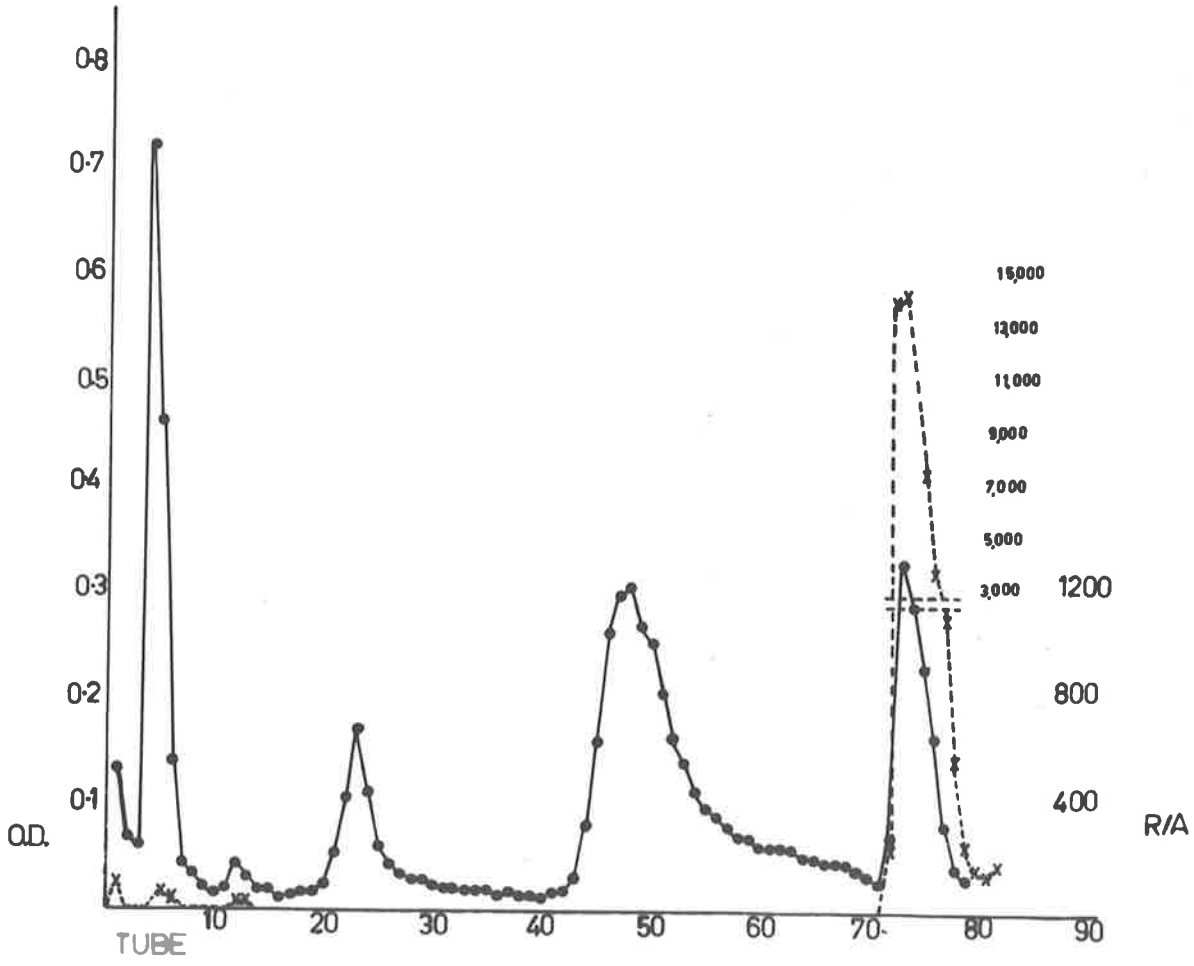

Figure 25. Column chromatogram of 1.0 ml. dialysed chronic myeloid leukaemia plasma labelled with high specific activity radioactive vitamin B_{12} at the 2 ng./ml. level; patient COR, 22.2.67.

Changes of eluent were made at tubes 18, 33 and 70.

●——● = optical density at 280 mμ.
x-----x = radioactivity in counts per minute above background.

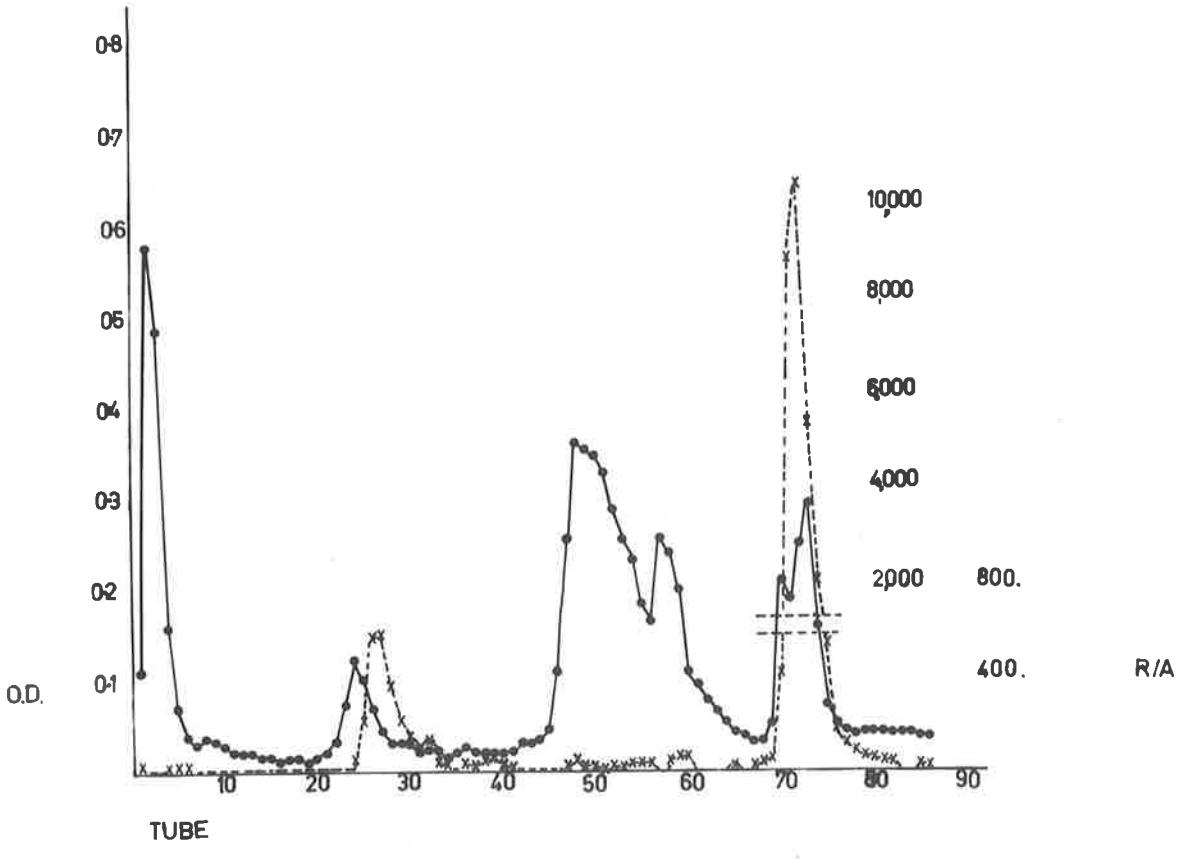

Figure 26. Column chromatogram of 1.0 ml. dialysed chronic myeloid leukaemia plasma labelled with high specific activity radioactive vitamin B_{12} at the 2 ng./ml. level; patient COR, 3.3.67.

Changes of eluent were made at tubes 17, 32 and 67.

●——● = optical density at 280 mμ.
x-----x = radioactivity in counts per minute above background.

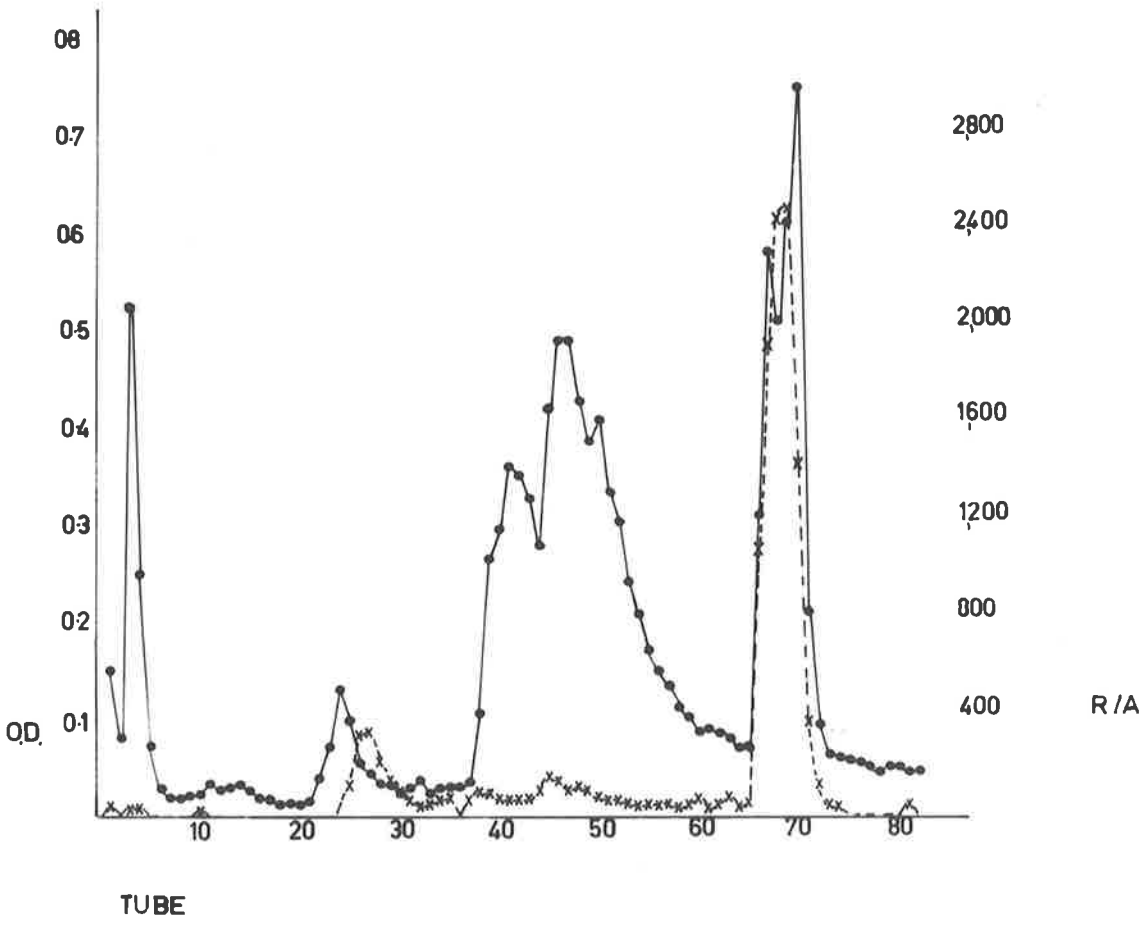

Figure 27. Column chromatogram of 1.0 ml. dialysed chronic myeloid leukaemia plasma labelled with high specific activity radioactive vitamin B_{12} at the 2 ng./ml. level; patient COR, 28.4.67.

Changes of eluent were made at tubes 18, 29 and 63.

●——● = optical density at 280 mμ.
x-----x = radioactivity in counts per minute above background.

radioactivity at peak V; reference to Table XIV will indicate that the vitamin B_{12} binding capacity of this patient's plasma was falling from its previously high levels when these two particular specimens were collected and studied.

5.2.5 Preferential Nature of Binding in Myeloid Leukaemia.

In addition to the findings set out above, it was possible to study plasma from the patient SME at a time when her myeloid leukaemia was still active, when her vitamin B_{12} binding capacity was still elevated, and when there was no detectable vitamin B_{12} in her serum (See Appendix AI). Figure 28 shows the results of column chromatography of dialysed radioactive vitamin B_{12} labelled plasma, which had been bound at the 100 ng./ml. level. All radioactivity detected was located at peak V, and this accounted for more than 95% of the radioactivity placed on the column at the beginning of the elution process.

The evidence presented above in Figures 22 and 23 is also strongly indicative of the fact that there was, in the sera of these patients with chronic myeloid leukaemia, a protein which, with the system of column chromatography used for this study, moved with peak V; this protein had

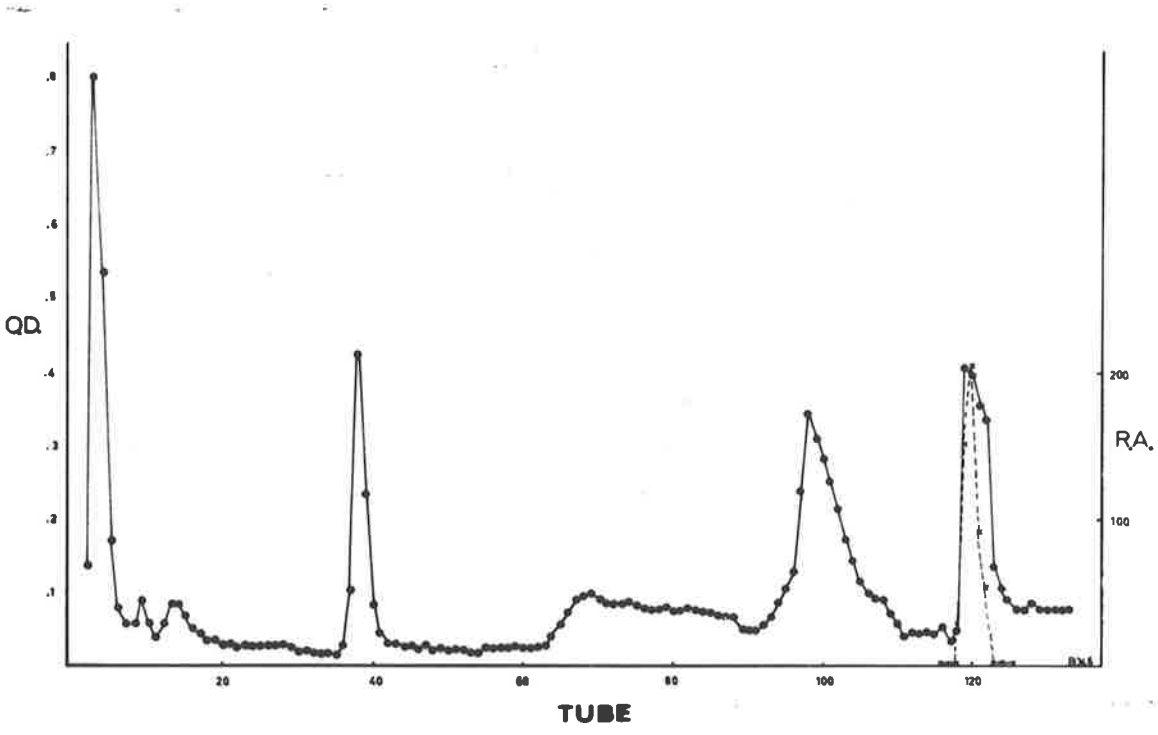

Figure 28. Column chromatogram of 1.0 ml. dialysed chronic myeloid leukaemia plasma labelled with radioactive vitamin B_{12} at the 100 ng./ml. level; patient SME, 28.4.65.

Changes of eluent were made at tubes 34, 84 and 116.

●——● = optical density at 280 mμ.
x----x = radioactivity in counts per minute above background.

B346.

the property of binding vitamin B_{12} in preference to other proteins.

Figure 29 shows the results obtained from column chromatography of a dialysed labelled specimen of α_1 acid glycoprotein. All radioactivity was associated with the only peak of optical density, which was located at peak V on the standard chromatogram.

5.2.6 Relationship Between Vitamin B_{12} Binding Protein and Isologous Antibodies.

Reference has been made previously to the temporal association of the abnormally high vitamin B_{12} binding capacity in patient COR with an abnormally high anti-Fy^a titre, which apparently arose as the result of blood transfusion. The binding capacities measured in three patients with isologous antibodies were not different from normal. Although the column chromatography technique results in the dilution of the 1 ml. starting sample to a final volume of approximately 500 ml., it was possible, because of the high titre of the anti-Fy^a antibody, to identify this antibody in the chromatogram shown at Figure 23; anti-Fy^a activity was found to a titre of 1 in 4 by the Coombs test in tube 3 of this column, which represented the highest point of peak I. No anti-Fy^a

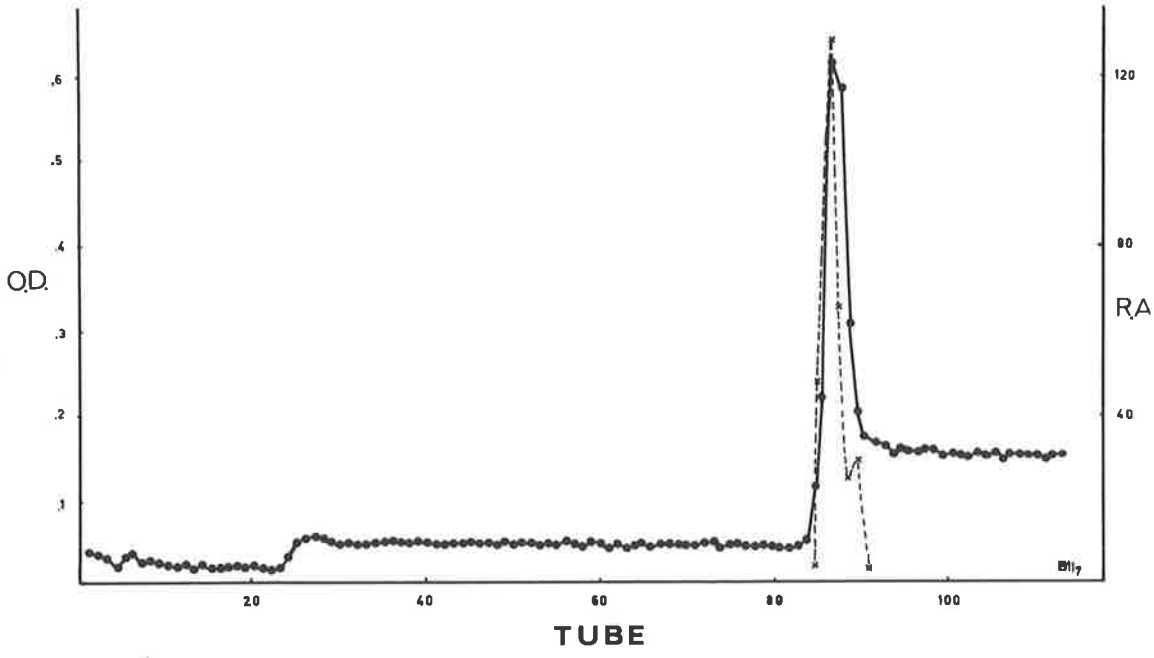

Figure 29. Column chromatogram of 1.0 ml. dialysed α_1 acid glycoprotein labelled with radioactive vitamin B_{12} at the 10 ng./ml. level.

Changes of eluent were made at tubes 20, 46 and 83.

●——● = optical density at 280 mμ.
x----x = radioactivity in counts per minute above background.

B117.

activity was detectable in other peaks of the chromatogram. The location of Cohn fraction III-1, almost entirely at peak I (Figure 5) is further corroborative evidence for this finding, since this particular Cohn fraction is that which contains the majority of antibodies, both autologous and isologous.

5.2.7 Resumé. Column chromatography of plasma from patients with chronic myeloid leukaemia has shown marked differences from the normal patterns obtained, in both the dialysed and undialysed specimens, and at low and high levels of binding.

Avidity of vitamin B_{12} binding by a protein associated with peak V of the chromatogram has been demonstrated in several patients with demonstrably high in vitro vitamin B_{12} binding capacity. These patterns are consistently found in patients with chronic myeloid leukaemia who have an increased vitamin B_{12} binding capacity. In addition, in one patient, it was shown, at a time when there was no detectable vitamin B_{12} in the plasma, that all radioactivity was detected after binding of added radioactive vitamin B_{12} at peak V.

In the normal pattern, a change in distribution between the proportion of radioactivity detectable in peaks

II and V has been likened to the known in vivo transfer from the beta binder (transcobalamin II) to the alpha binder (transcobalamin I).

An abnormal pattern has been demonstrated in one patient with vitamin B_{12} deficiency, which was present in association with idiopathic steatorrhoea; it is not certain whether this finding was related to hypoproteinaemia, or an abnormality of vitamin B_{12} binding, or a combination of both.

It has been demonstrated in a patient who had an abnormally high vitamin B_{12} binding capacity at the same time as a high antibody titre was observed, that the high vitamin B_{12} binding capacity was not associated with the abnormal antibody in her plasma, since the peak at which most radioactivity was located was not that with which the antibody was associated.

5.3 Effect of Leukocytes on Plasma Protein Binding of Vitamin B_{12}.

Leukocytes were harvested from freshly collected anticoagulated blood and the lymphocytes and granulocytes separated in the manner described above. The results obtained when vitamin B_{12} binding capacity was determined for the lymphocyte-rich plasma from five normal blood

TABLE XVI.

PLASMA PROTEIN BINDING OF Co^{58} - VITAMIN B_{12}
(at 10 ng./ml. Level)

I. CONTROL (N. Saline)

SAMPLE	PERCENTAGE BOUND TO DIALYSATE.	PERCENTAGE BOUND TO PRECIPITATE.	TOTAL BINDING PERCENTAGE	TOTAL BINDING (ng./ml.)	PERCENTAGE INCREASE
A	17.0	3.8	20.8	2.08	
B	20.0	6.5	26.5	2.65	
C	18.0	4.4	22.4	2.24	
D	18.0	4.5	22.5	2.25	
E	20.0	4.7	24.7	2.47	

II. LYMPHOCYTES ADDED.

A	20.0	2.9	22.9	2.29	10.1
B	20.0	6.3	26.3	2.63	- 0.8
C	21.0	3.8	24.8	2.48	10.7
D	22.0	4.4	26.4	2.64	17.3
E	21.5	5.2	26.7	2.67	8.1

B528

TABLE XVII

PLASMA PROTEIN BINDING OF Co^{58}-VITAMIN B_{12}

(at 10 ng./ml. level)

I. CONTROL (N. Saline)

SAMPLE	% BOUND TO DIALYSATE.	% BOUND TO PRECIPITATE.	TOTAL BINDING Percentage	TOTAL BINDING (ng./ml.)	PERCENTAGE INCREASE
A	31.0	4.5	35.5	3.55	
B	29.0	4.1	33.1	3.31	
C	29.0	5.0	34.0	3.40	

II. GRANULOCYTES ADDED

A	31.0	5.7	36.7	3.67	3.5
B	47.0	8.1	55.1	5.51	66.5
C	51.0	10.2	61.2	6.12	80.0

donors are set out in Table XVI, together with the control figures; binding was performed at the 10 ng./ml. level, and the lymphocyte-rich suspensions contained less than 5% leukocytes other than lymphcotyes. There is no significant difference between the results obtained with lymphocyte-rich plasma in comparison with the results of control specimens.

In Table XVII, the results of three experiments are set out, in which the vitamin B_{12} binding capacity of granulocyte-rich plasma from three normal blood donors was measured, and compared with values obtained for the control samples which did not contain granulocytes. Binding was performed at the 10 ng./ml. level and the leukocytes in granulocyte-rich plasma were more than 90% granulocytes. A marked increase in the vitamin B_{12} binding capacity was observed in two of the three samples, (B,C) and the levels of binding were in the abnormal range, unlike the control values. No significant alteration in binding was noted in the third sample tested (A).

Similar experiments have been performed with similar results; that is, lymphocyte-rich plasma did not bind significantly more added vitamin B_{12} than the control plasma, and granulocyte-rich plasma bound more added

TABLE XVIII

VITAMIN B_{12} BINDING TO SEPARATED PLASMA PROTEIN FRACTIONS

Percentages

FRACTION		LEVEL OF ADDED VITAMIN B_{12} (ng./ml.) 10	25	50	75	100
Gamma 1, 2	Dial.	6.4	3.1	2.70	0.58	1.66
	Ppte.	0.3	0.75	0.86	0.41	0.25
	Total	6.7	3.85	3.56	0.99	1.91
Gamma 3	Dial.	3.8	0.73	1.28	1.96	1.44
	Ppte.	0.9	0.56	0.94	1.04	0.35
	Total	4.7	1.29	2.22	3.00	1.79
α_1 acid glycoprotein	Dial.	53.0	86.0	54.0	46.0	45.0
	Ppte.	2.9	3.5	3.5	2.73	1.5
	Total	55.9	89.5	57.5	48.73	46.5
α_2 glyco-protein	Dial.	8.5	3.3	3.2	2.77	1.68
	Ppte.	2.0	0.76	1.18	0.73	0.25
	Total	10.5	4.06	4.38	3.50	1.93

TABLE XIX

VITAMIN B_{12} BINDING TO SEPARATED PLASMA PROTEIN FRACTIONS

Percentages

FRACTION		LEVEL OF ADDED VITAMIN B_{12} (ng./ml.) 10	25	50	75	100
Crystalline transferrin	Dial.	12.8	6.15	4.10	5.00	2.85
	Ppte.	0.9	0.48	2.0	1.66	0.15
	Total	13.7	6.63	6.10	6.66	3.00
Fibrinogen	Dial.	12.2	6.05	5.8	4.5	5.65
	Ppte.	7.6	0.89	2.0	2.04	2.0
	Total	19.8	6.94	7.8	6.54	7.65
Fraction III-I	Dial.	-	-	3.02	1.26	-
	Ppte.	-	-	2.36	1.03	-
	Total			5.38	2.29	

vitamin B_{12} than lymphocyte-rich plasma and the control plasma, although this did not occur in every instance.

5.4 Vitamin B_{12} Binding to Separated Plasma Protein Fractions.

This set of experiments was designed to measure and compare the in vitro vitamin B_{12} binding capacity of a number of isolated protein fractions. The vitamin B_{12} binding capacity was measured in the standard manner, as described above, on the following plasma protein fractions obtained from the Commonwealth Serum Laboratories, Melbourne:-

Fibrinogen	(Cohn fraction I - 4)
Gamma Globulin	(Cohn fraction II - 1, 2)
Gamma Globulin	(Cohn fraction II - 3)
Crystalline Transferrin	(Cohn fraction IV-7.2)
α_2 glycoprotein	(Cohn fraction VI - 1a)
α_1 acid glycoprotein	(Cohn fraction VI - 1b)

The fractions were received in a lyophilized form, and were dissolved in sterile physiological saline to give a 2% concentration (that is, 200 mg./10 ml.). The data obtained in the experiments are shown in Tables XVIII to XXI; Tables XVIII and XIX show the percentage of the added radioactive vitamin B_{12} bound to dialysate and

TABLE XX

VITAMIN B_{12} BINDING TO SEPARATED PLASMA PROTEIN FRACTIONS

ABSOLUTE AMOUNTS

(ng./ml.)

FRACTION		LEVEL OF ADDED VITAMIN B_{12} (ng./ml.)				
		10	25	50	75	100
Gamma 1, 2	Dial.	0.64	0.77	1.35	0.44	1.66
	Ppte.	0.03	0.19	0.43	0.31	0.25
	Total	0.67	0.96	1.78	0.75	1.91
Gamma 3	Dial.	0.38	0.18	0.64	1.47	1.44
	Ppte.	0.09	0.14	0.47	0.78	0.35
	Total	0.47	0.32	1.11	2.25	1.79
α_1 acid glycoprotein	Dial.	5.30	21.5	27.0	34.5	45.0
	Ppte.	0.29	0.87	1.75	2.07	1.5
	Total	5.59	22.37	28.75	36.57	46.5
α_2 glyco-protein	Dial.	0.85	0.83	1.60	2.07	1.68
	Ppte.	0.20	0.19	0.59	0.55	0.25
	Total	1.05	1.02	2.19	2.62	1.93

TABLE XXI

VITAMIN B_{12} BINDING TO SEPARATED PLASMA PROTEIN FRACTIONS.

ABSOLUTE AMOUNTS

(ng./ml.)

FRACTION		LEVEL OF ADDED VITAMIN B_{12} (ng./ml.) 10	25	50	75	100
Crystalline transferrin	Dial.	1.28	1.54	2.05	3.75	2.85
	Ppte.	0.09	0.12	1.0	1.25	0.15
	Total	1.37	1.66	3.05	5.00	3.00
Fibrinogen	Dial.	1.22	1.52	2.9	3.39	5.65
	Ppte.	0.76	0.22	1.0	1.53	2.0
	Total	1.98	1.74	3.9	4.92	7.65
Fraction III-I	Dial.	-	-	1.51	0.950	-
	Ppte.	-	-	1.18	0.770	-
				2.69	1.720	

precipitate at the 10, 25, 50, 75 and 100 ng./ml. levels, and Tables XX and XXI contain the same data calculated as absolute amounts of the radioactive vitamin bound. The data from Tables XVIII and XIX are shown diagrammatically at Figure 30 and the data from Tables XX and XXI are similarly shown at Figure 31.

It is apparent that the α_1 acid glycoprotein bound much more added vitamin B_{12} at all levels than any of the other protein fractions. It is also apparent that, whereas the other protein fractions appeared to have reached an equilibrium state (referred to variously as secondary binding or non-specific binding) at or before the 100 ng./ml. level, the binding to α_1 acid glycoprotein does not appear to have reached this secondary binding phase. Up to the limits of this series of experiments, that is, 100 ng./ml., there was a continuing increase in the absolute amount bound, unlike the other protein fractions used, suggesting that the primary binding capacity for vitamin B_{12} of this protein fraction (or one of its components) had not been reached at the upper levels of the amounts added.

Similar results, not shown, have been obtained using, under slightly different experimental conditions, albumin, Cohn fraction III-1 (a beta globulin fraction) and Cohn fraction III-0.

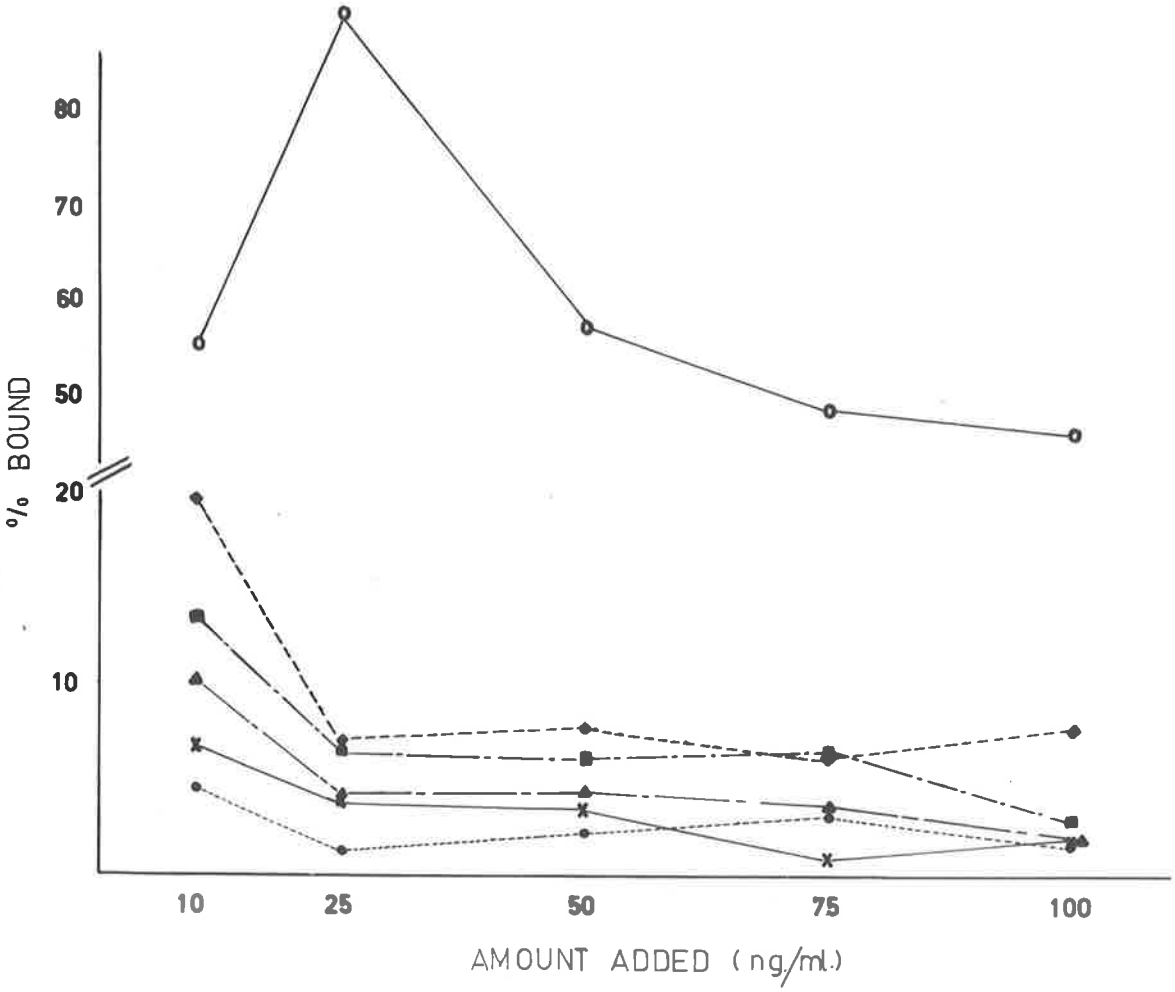

Figure 30. Vitamin B_{12} binding capacity of separated protein fractions, showing the percentage bound at different levels of added radioactive vitamin B_{12}.

◆----◆ = Fibrinogen
x——x = Gamma 1, 2
●·····● = Gamma 3
■--■ = Transferrin
o——o = α_1 acid glycoprotein
▲--▲ = α_2 glycoprotein

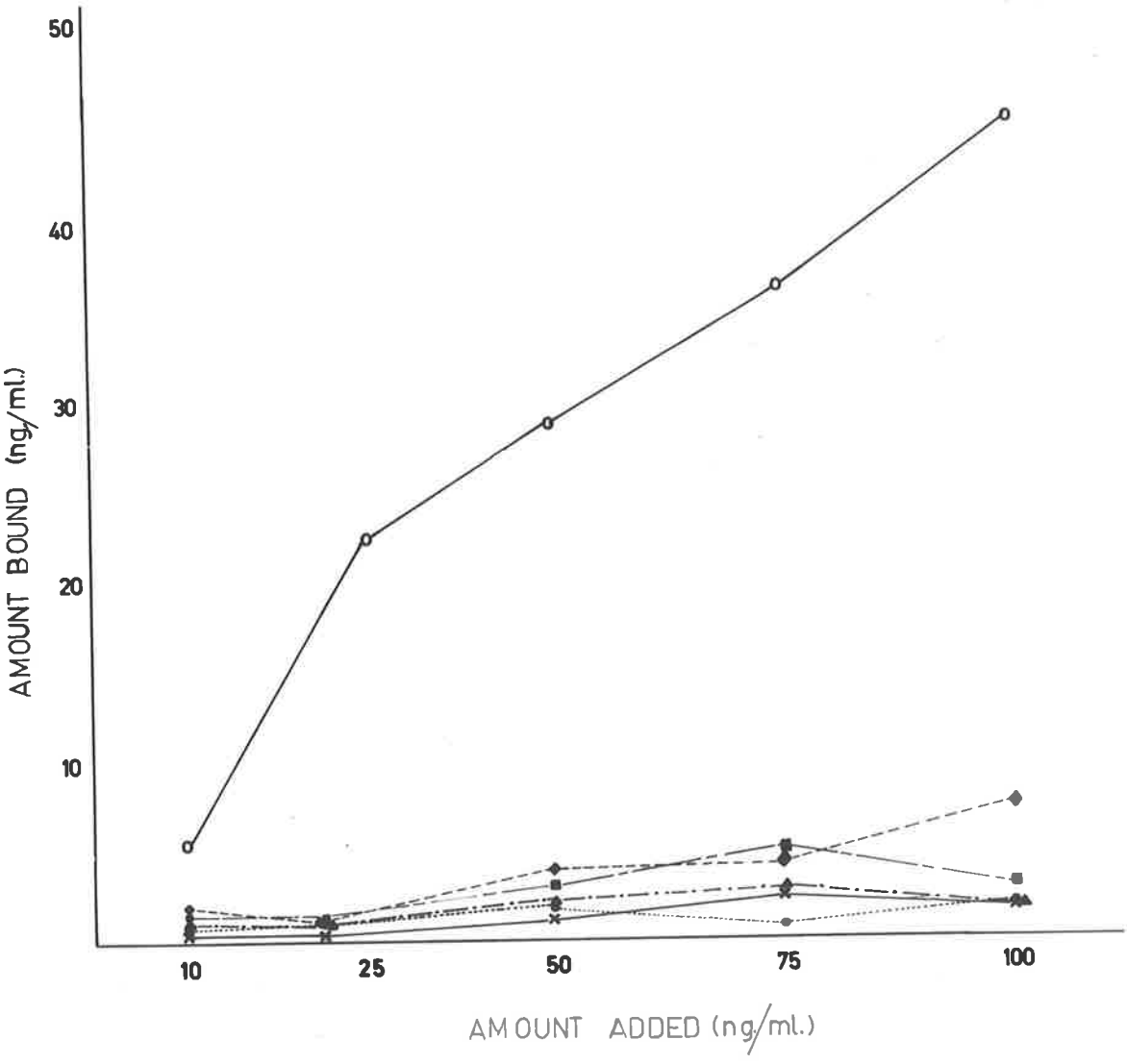

Figure 31. Vitamin B_{12} binding capacity of separated protein fractions, showing the absolute amount bound at different levels of added radioactive vitamin B_{12}.

◆----◆ = Fibrinogen
■---■ = Transferrin
×——× = Gamma 1, 2
o——o = α_1 acid glycoprotein
●-----● = Gamma 3
▲---▲ = α_2 glycoprotein.

5.5 Other Methods of Protein Separation.

In this section, for purposes of completeness and later discussion, the results of protein separation by paper electrophoresis and starch gel electrophoresis are recorded; the small volumes of plasma which can be utilized in these techniques, and the low levels of radioactivity which result, together with the lack of discrete separation, made it impracticable to continue using these techniques.

Paper electrophoretic separation was performed on a dialysed labelled plasma sample from a patient with chronic myeloid leukaemia (patient GOW, Table X); binding had been performed at the 10 ng./ml. level. The paper strips were divided and radioactivity counted in a well-type scintillation counter. The majority of the radioactivity (82%) was associated with the albumin fraction; the remainder of the radioactivity was associated evenly between the α_1 globulin (5%), and α_2 globulin (7%) and the gamma globulin (6%); no radioactivity was detected in association with the beta globulin fraction. The α_1 acid glycoprotein, when studied by this electrophoretic system, was located within the boundaries of the albumin portion of the electrophoretogram.

Similar patterns were obtained when dialysed labelled plasma samples were subjected to starch gel electrophoresis;

most radioactivity (45-60%) was associated with the albumin area of the electrophoretogram; again, the α_1 acid glycoprotein had a mobility which brought it within the albumin area of the separated whole plasma.

The small number of counts of radioactivity obtained for each protein segment did not permit the error of each count to be reduced below 20% in either method.

CHAPTER 6

DISCUSSION

DISCUSSION.

It is now well recognised that there are vitamin B_{12} binding proteins present in a number of biological fluids, including gastric juice, plasma, saliva, and there are also apparently vitamin B_{12} binding proteins in white cells (Simons, 1964; Simons and Weber, 1966) and cerebrospinal fluid (Meyer et alii, 1959). There are apparently three vitamin B_{12} binders in gastric juice (Gräsbeck et alii, 1962; Simons et alii, 1964); not all of these binders have intrinsic factor activity, although it was originally thought that the vitamin B_{12} binder of gastric juice and intrinsic factor may have been identical (Gräsbeck et alii, 1959). At least one of the gastric juice vitamin B_{12} binders, that designated "R" by Gräsbeck, is thought to be similar to, if not identical with, the vitamin B_{12} binding substance of other body fluids; the "R" binder in gastric juice does not have intrinsic factor activity.

The patterns of in vivo plasma binding of vitamin B_{12} in man appear to be fairly well characterized, although at the present stage of our knowledge, the specific protein moieties which are responsible for binding vitamin B_{12} have not been identified nor isolated from the larger protein

complexes. Although the specific vitamin B_{12} binders have been termed transcobalamin I and II by Hall and Finkler (1964), and α and β binders by Herbert and his colleagues (Retief et alii, 1967a), it is certain from the calculations made by Heller et alii (1964) and others referred to above (Miller and Sullivan, 1959b; Mendelsohn et alii, 1958) that the vitamin B_{12} binder is likely to be only a very small component of these particular protein aggregates. Vitamin B_{12} which is injected is bound to transcobalamin II, the beta globulin binder, and is rapidly lost from this over the next 24 hours to the alpha binder, or transcobalamin I, which accounts for most of the endogenous vitamin B_{12} present in the circulation. Variations in the ratios of these proteins, or possibly, in the binding capacities of these proteins, occur in certain disease states, including myeloid leukaemia and pernicious anaemia (Hall and Finkler, 1966b; Retief et alii, 1967a; Lawrence, 1966b).

The patterns obtained for in vitro plasma binding of vitamin B_{12} are not nearly as clearly defined as the patterns of in vivo binding, and there are a number of reasons for this. In addition to the problems of relatively low specific activity, and of the lack of

success (as yet) in isolating the definitive binders which the in vitro technique shares with the in vivo technique, there are methodological problems connected with the in vitro techniques which are basically difficulties inherent in the methods used for separation. In all forms of electrophoretic separation, the division of the proteins must be regarded as artificial, and, particularly with paper electrophoresis, the number of separated protein fractions which can be obtained is less than with other techniques. A further difficulty arises from the minute amounts of material which can be successfully used in certain of the more elegant separation techniques, such as immunoelectrophoresis and acrylamide gel electrophoresis. For these reasons, column chromatography was the method of choice for these studies.

6.1 Vitamin B_{12} Binding Capacity.

6.1.1 Technical Aspects.

There is no difference, from the results presented previously, between serum binding and plasma binding of vitamin B_{12}. This absence of difference applies, whether the material studied was from normals, or from patients with myeloid leukaemia or vitamin B_{12} deficiency. It is apparent from other work on this subject that no

distinction has been drawn by other authors between plasma and serum. Certain authors have used serum (Meyer et alii, 1957; Fahey et alii, 1958), and other workers have used plasma (Weinstein et alii, 1959); some have used plasma and serum without distinguishing between them in their experiments (Rosenthal et alii, 1964, 1965), while others have changed from one to the other during the course of their studies; Miller and Sullivan (1959a) used serum in some of their experiments, and plasma in others (Miller and Sullivan, 1961). It would appear, therefore, although it may be more precise to adhere to one form, and one term or the other, that from the practical point of view, serum binding and plasma binding of vitamin B_{12} represent the same phenomenon in the same degree.

The storage of plasma for lengthy periods of time at -20°C does not appear to alter the vitamin B_{12} binding capacity. Several results obtained in patient SME were checked at intervals of up to 4 years, and the duplicate results obtained were well within the normal standard deviation of the experimental method. It was apparent, however, that if plasma was thawed and frozen repeatedly, the precipitate which formed during dialysis was increased, and the amount of radioactive vitamin B_{12} bound to it was

increased. Thus, if a control specimen is to be used at regular intervals, it should be divided into aliquots which are then not likely to be subjected to repeated freezing and thawing.

Increased binding was demonstrated when whole blood was compared with the plasma from the same patient. This increase is probably accounted for by binding to specific vitamin B_{12} binders released from the blood cells during the period of dialysis, together with non-specific binding which took place to the large amount of additional protein present in the dialysis mixture.

Although the yield was not measured in the more recent experiments, in the initial experiments in which the method of measurement of binding capacity was evaluated, attempts were made to account for all radioactivity by measuring not only the amount of radioactivity bound to the dialysant and to the precipitate, but also the radioactivity associated with the sac and present in the buffer. Sufficient counts were performed to allow the error for each individual count to be less than 5%, and in the majority of the experiments, the yield was between 90% and 110% of the radioactivity present at the commencement of the experiment. Naturally, with the experiments conducted at 100 ng./ml.

level of added vitamin B_{12}, the number of counts obtained was higher, the order of accuracy greater, and therefore the calculated yield was closer to 100%.

The described technique has also been used to assess the binding of radioactive iron to transferrin, and in these experiments, all radioactivity was found associated with the dialysant when the test sample used possessed latent iron binding capacity in excess of the amount of radioactive iron added. In experiments conducted on plasma from patients with haemochromatosis, in whom the iron binding capacity was fully saturated, all the radioactive iron was present in the buffer solutions used for dialysis; the yield here again approximated 100% in each instance.

Although a number of different temperatures have been used for incubation in the various methods described, the binding of radioactive vitamin B_{12} to plasma does not appear to be affected by the temperature at which incubation is performed. Hift (1964), who incubated his material at 37°C. for 20 minutes, considered that the serum binding of cyanocobalamin was complete within five minutes over a range of 0-40°C.

The <u>duration</u> of incubation has been shown in this present study to affect the amount of radioactive vitamin

B_{12} which is bound to plasma proteins, and appears to be an important factor which may affect the comparison of results from different series. The exact nature of the increased binding which occurs when vitamin B_{12} is incubated for 24 hours, rather than for a shorter period, is not certain; it may represent increased binding to protein fractions in the same proportions as the binding which follows shorter periods of incubation, or there may be a differing distribution throughout the plasma fractions. Since there was no increase in binding when the 24 hour sample was refrigerated for a further 24 hours and then dialysed, it is likely that the effect is a non-specific one, which occurs as a result of the material being left at room temperature for a period of 24 hours. A relatively short period of incubation is adequate, and this may be performed conveniently at either room temperature or 37°C., provided that incubation does not take place for very long at the higher temperature, in view of the effect of heat on protein structure; Hift (1964) pointed out that excessive heating of protein during its manipulation may alter protein sufficiently to provide more binding sites, and possibly give an artificially high figure.

It has been shown on a number of previous occasions

(Meyer et alii, 1957, 1961; Heller et alii, 1964; Meyer, 1965), as well as in the present study, that the amount of radioactive vitamin B_{12} bound to plasma following incubation and dialysis is related to the concentration of the radioactive vitamin added prior to incubation. As the concentration of radioactive vitamin B_{12} is increased, the absolute amount bound increases, although the percentage bound is less. This increase in binding does not bear a linear relationship to the increase in concentration, and has led to the suggestion of primary and secondary binding (summarized by Meyer, 1965).

When dialysed labelled plasma is applied to the DEAE cellulose column, there does not appear to be any significant difference in the distribution of the bound radioactive vitamin B_{12} between the protein fractions, whether the experiments have been carried out at 10 ng./ml. or at higher concentrations, although differences have been demonstrated in the distribution of bound radioactive vitamin B_{12} at different concentrations by precipitation techniques (Weinstein et alii, 1959).

The increase in the binding of radioactive vitamin B_{12} to plasma from patients with chronic myeloid leukaemia (Beard et alii, 1954a; Mollin and Ross, 1955; Raccuglia and Sacks, 1957; Rachmilewitz et alii, 1957; Meyer et alii,

1957, 1961; Miller, 1958; Mendelsohn and Watkin, 1958; Hoogstraten et alii, 1962) has again been observed in these studies, and is discussed below.

It has been shown from these studies that the duration of the dialysis procedure is a significant factor in determining the value reported for plasma binding of radioactive vitamin B_{12}, since significant quantities of radioactivity were shown to be removed by the dialysis process up to 96 hours from the time of commencement of dialysis. The periods chosen by other authors for their dialysis procedures have varied from 9 hours (Hoff-Jørgensen and Worm-Petersen, 1962) to 72 hours (Rosenthal and Austin, 1962), while certain authors used differing dialysis times in different studies (Miller, 1958; Miller and Sullivan, 1961). It has been stated by these latter authors (Miller and Sullivan, 1959a) that when serum whose binding capacity was saturated with added radioactive vitamin B_{12} was required, their serum-vitamin B_{12} mixture was dialysed against large volumes of phosphate buffer for 96 hours, although others did not observe any difference in binding at 72 or 96 hours compared with the 48 hour period (Bertcher and Meyer, 1957). Thus, comparison of results from one study to another becomes difficult, if not

impossible, when it is realised that the samples dialysed for shorter periods will have reported higher binding capacities than similar samples dialysed for longer periods. It is also apparent from this present study that dialysis for a short period does not necessarily remove a proportional amount of the total dialysable vitamin present in a designated period of time. Thus, unless adequate dialysis (that is, at least 96 hours) has been performed, no two sets of results are strictly comparable. Although it is recognised that a 96 hour laboratory procedure is a somewhat laborious one from the point of view of time, it would nevertheless appear more sensible to measure all vitamin B_{12} binding capacities at a known end point, that is, the point of complete removal of unbound vitamin B_{12}.

The temperature at which dialysis is performed does not appear to be critical, except as far as the amount of radioactivity which is bound to the sac is concerned. The above results (4.1.6) are in agreement with the results of Miller and Sullivan (1958), who found that the only difference in binding conducted at 4°C. and at 22°C. was in the amount of radioactivity bound to the dialysis sac, which, in their experiments, was 4-8% at the higher temperature, and 1-2% in the cold.

A difference was observed when dialysis was performed against tap water at room temperature and in the cold (Table VIII). A much heavier precipitate was formed in these experiments, and it is considered likely, as similar results did not occur with distilled water, that the effect observed was due to the precipitation of proteins by the heavy salt content of Adelaide tap water, which for normal domestic use requires anion exchange columns for "softening". No differences were observed between tap water dialysis at room temperature and in the cold when these experiments were performed in Sydney, against Sydney tap water, and this would appear to be further confirmatory evidence for the explanation offered.

Since the amount of radioactivity bound to the dialysis sacking when dialysis was performed at 4°C. represented a consistent 1-3% of the total radioactivity added, in the majority of the experiments conducted this has not been measured, and the binding capacity reported represents the sum of the measured binding of radioactive vitamin B_{12} to the dialysant and the amount of radioactivity bound to the precipitate which formed during dialysis.

Bertcher and Meyer (1957) found similar results to those obtained following dialysis against tap water when dialysis was performed against saline, 6% dextran in saline and human plasma. Miller (1958) suggested that there was no difference between dialysis carried out against phosphate buffer, and dialysis carried out against water, dextran, barbital and saline. This present study has shown that there is an observed difference between the dialysis results obtained with certain buffers, related chiefly to the precipitate which forms during dialysis against tris phosphate buffer and tap water, and which contains significant amounts of bound radioactivity; this precipitate is minimal when dialysis is carried out against normal saline. Fahey et alii, (1958) noted that after dialysis had been carried out for a minimum of 6 hours at 4°C. against 0.01M phosphate buffer, pH 8, a white, flocculent precipitate, accounting for 0.5-3% of the total serum protein had been formed and was able to be separated by centrifugation. Some methods of measurement of bound radioactivity (Meyer et alii, 1957, 1961) require the dissolution of the dialysis sac and its contents in concentrated sulphuric acid. The results reported in their

studies as the amount bound to plasma would presumably have included the amount bound to the precipitate, as well as the amount bound to the sac; the readings obtained will therefore be somewhat higher by comparison with other methods. This may also account for the similarity of binding values obtained against a variety of buffers (Bertcher and Meyer, 1957).

It is considered that the precipitate does not represent denaturation, but precipitation of high molecular weight protein molecules (beta and gamma globulins) during dialysis against a solution which is not isotonic. It was noted when dialysis was carried out against an isotonic saline solution, that the amount of the precipitate which formed was much smaller than when dialysis was performed against non-isotonic solutions.

Although it has been shown by Reizenstein (1959b) that digestive enzymes have some effect on the binding of vitamin B_{12} in vivo, there was, in these present studies, no evidence to suggest that papain was capable of modifying in any degree the effect of binding of vitamin B_{12} to plasma proteins in vitro; this applied, whether the papain was added before or after incubation. Hall and Allen (1964) have reported that papain digestion

produced an increase in the amount of vitamin B_{12} present in normals which was available to E. gracilis assay. This probably reflects an inherent variation in the method (Hoff-Jørgensen and Worm-Petersen, 1962), since it was shown that microbiological methods may not measure all vitamin B_{12} present.

It was apparent from these studies that the bond which forms in vitro between vitamin B_{12} and plasma proteins was not easily broken. After dialysis, it was not possible to remove any of the radioactive vitamin B_{12} bound to plasma proteins by dialysing the sac and its contents against a solution containing large amounts of non-radioactive vitamin B_{12}; both cyanocobalamin and hydroxocobalamin gave similar results.

6.1.2 Results.

The normal range demonstrated was not greatly different from that of Meyer and his co-workers (Meyer et alii, 1957, 1961; Meyer, 1965). It is also apparent that the normal range of the present series is not an exceptionally wide one, since one standard deviation, at both the 10 ng./ml. and 100 ng./ml. levels of binding, represents approximately 10% of the mean value for normals.

There have been marked differences from normal observed in the binding capacity of plasma from patients with myeloid leukaemia (Mollin and Ross, 1955; Meyer et alii, 1957, 1961; Meyer, 1965). Although in most instances, serum vitamin B_{12} levels were not done in this present study, it has been shown by others that there is little or no correlation between the serum vitamin B_{12} level and the vitamin B_{12} binding capacity (Erdmann-Oehlecker and Heinrich, 1956; Hoff-Jørgensen and Worm-Petersen, 1962). A closer correlation has been suggested between the white cell count and the degree of binding (Mollin and Ross, 1955; Meyer et alii, 1967) but, except in the most general terms, this finding has not been confirmed in other studies, or in this present study. It has been noted in the patient COR that the abnormally high vitamin B_{12} binding capacity of her plasma (the highest seen in any patient in this study) was measured at a time when her white cell count was normal, with very few primitive forms present, after twelve months of adequate therapy with "Myleran". Thus, it is hard to correlate vitamin B_{12} binding capacity to the total white cell count, except to suggest that in the patient who has untreated

chronic myeloid leukaemia with a grossly elevated white cell count, it is likely that a high vitamin B_{12} binding capacity will be demonstrated.

A closer relationship, not previously commented upon, has been observed from this present study between the vitamin B_{12} binding capacity and basophils, which are a common finding in chronic myeloid leukaemia, and which are usually more numerous in myeloid leukaemia than in any other disease, often accounting for 3-20% of the total white cell count in untreated patients (Wintrobe, 1961). Although no direct relationship was demonstrable between the total basophil count and the vitamin B_{12} binding capacity, nor between the percentage which the basophils represented of the total white cell count and the vitamin B_{12} binding capacity, it was nevertheless found that, for most patients in this present series, the vitamin B_{12} binding capacity was elevated when there were basophils present in the peripheral blood film, and that when the patient responded to treatment, the basophils were no longer detected in the peripheral blood and the vitamin B_{12} binding capacity returned to within the normal range. It could well be, however, that both of these findings are unrelated facets of untreated chronic myeloid leukaemia

which are the result of similar stimuli, and which respond similarly to the same form of therapy. The exact nature of the function of basophils remains uncertain; it has been suggested that they may represent in man a function-less evolutionary rest. It is also recognised that these cells may have a relationship to the mast cells of connective tissue, and it is known that they have a high histamine content, and that the metachromatic granules contain heparin (Wintrobe, 1961). Although the hypothesis that the increased amounts of vitamin B_{12} binding protein arise in relation to increased numbers of basophils in chronic myeloid leukaemia is not able to be proven, there is no doubt that the increase in vitamin B_{12} binding capacity and the increase in basophils appear to be causally related, either to one another, or to common stimuli arising as part of the disease process.

It has been found in the chronic myeloid leukaemic patients studied in this series, that most had increased binding levels at the 10 ng./ml. level and 100 ng./ml. level. The same did not apply to patients with acute leukaemia, irrespective of their peripheral white cell count at the time of study. A significant difference was demonstrated between the mean values for vitamin B_{12}

binding at the 100 ng./ml. level in patients with acute leukaemia, and patients with chronic myeloid leukaemia. The levels obtained in patients with acute myeloid leukaemia did not differ significantly from normal, whereas those of the patients with chronic myeloid leukaemia differed significantly from normal. The reason for this could be that patients with acute myeloid leukaemia, although their plasma may bind increased amounts of vitamin B_{12} at the 10 ng./ml. level, do not elaborate sufficiently large amounts of the vitamin B_{12} binding protein (or proteins) to produce abnormal levels of binding at the 100 ng./ml. level. If it is accepted that mature granulocytes contribute significantly to the vitamin B_{12} binding phenomenon, then this explanation is strengthened, since in most of the acute leukaemic patients, there was a predominance of primitive cells, whereas in chronic myeloid leukaemia, there may often be a large number of morphologically mature white cells present.

Although the measurement of vitamin B_{12} binding capacity at several levels (for example, 10 ng./ml. and 100 ng./ml.) may provide valuable corroborative information in the diagnosis of leukaemia, such

investigations are not likely to prove of positive value on their own; none of the patients in this present study was diagnosed as having myeloid leukaemia solely on the basis of the vitamin B_{12} serum levels or binding studies, although it was possible on the basis of normal vitamin B_{12} binding values in two patients to suggest that the underlying disorder was a non-leukaemic disease producing a leuko-erythroblastic blood picture, and on these grounds specific cytotoxic treatment was withheld and was later proven not to be indicated. Stahlberg et alii (1963) found that when the diagnosis of a specific morphological form of leukaemia was made on the basis of an elevated serum vitamin B_{12} level in conjunction with cytochemical studies, this same diagnosis was also possible by study of the marrow smears; where a clear-cut morphological diagnosis was not possible, these other investigations were of little or no diagnostic assistance.

The vitamin B_{12} binding capacity was shown to be increased in three patients with dysproteinaemias of the five studied. In each instance, the observed total increase was due to increased binding to the precipitate. As all five major protein fractions are capable of binding

vitamin B_{12} (Miller, 1958), it is not surprising to find, in patients who have gross increases in gamma globulin levels, that this particular protein (the principal constituent of the dialysis precipitate) accounts for an increased total binding. It is unlikely that the amount bound per unit of protein is greater than normal, and the phenomenon is considered to be quantitative, and not qualitative.

The vitamin B_{12} binding capacity in vitamin B_{12} deficient states was shown to be increased at the 10 ng./ml. level, but not at the 100 ng./ml. level. It is again possible that the 10 ng./ml. level reflects more closely the situation in which vitamin B_{12} binding sites of a primary nature are available, and that binding at the 100 ng./ml. level accounts for more non-specific binding than occurs at lower levels. This finding is not in conflict with the results of other workers, including those of Lawrence (1966a, 1966b). It is possible that, at the 10 ng./ml. level, the increased binding represents attachment to sites to which vitamin B_{12} was normally bound in the in vitro method, together with binding to sites not occupied in the vitamin B_{12} deficient state, but which would be unavailable for binding in normal plasma, with normal amounts of vitamin B_{12} already present.

There was no evidence from column chromatographic studies, in all but one instance (Figure 19) that there was any abnormality of vitamin B_{12} binding protein in vitamin B_{12} deficiency. There was no evidence to suggest that there is an elaboration of a vitamin B_{12} binding protein in vitamin B_{12} deficient states which is comparable with the elaboration of iron binding protein in iron deficient states.

A slight decrease was noted in vitamin B_{12} binding capacity at the 10 ng./ml. level in pregnant women. The explanation for this is not immediately apparent, since the plasma protein levels during pregnancy are usually within the normal range, and the binding capacity for other biological substances, such as iodine, is increased in pregnancy, particularly during the second trimester (Dr. W. Roman, personal communication).

Attention has been drawn to the marked species variation in vitamin B_{12} binding capacity described by Rosenthal and Austin (1962); because of this, and the species variation in plasma proteins which precludes comparative conclusions, it is highly unlikely that a suitable laboratory animal could be obtained for use as an experimental counterpart to the clinical studies in

man. For example, the vitamin B_{12} binding capacity of a "B_{12} binding globulin concentrate" produced from the Commonwealth Serum Laboratories was found to be minimal when tested in the standard manner; it was later found that this was processed from human plasma, and prepared according to a method for concentrating the plasma binders of vitamin B_{12} in cattle. The absence of demonstrable binding in the human "concentrate" is not surprising when it is known that the principal vitamin B_{12} binding protein in cattle is albumin, a negligible binder in humans.

6.2 Protein Binding of Vitamin B_{12}.

The concepts of primary and secondary binding, and the variations in binding produced by analogues of vitamin B_{12} have been discussed in the review of the literature above. Meyer's results (Meyer et alii, 1963) suggested that the initial phase of vitamin B_{12} binding occurs to the B pyrrol ring, through its $CO\text{-}NH_2$ groups, and that "phase II" binding appears to occur at the Co-OH linkage, which presumably dissociates in aqueous solution, making this additional binding site available.

6.2.1 Technical Aspects.

The technique of column chromatography was chosen

for the study of the distribution of protein bound vitamin B_{12}, since, in this way, larger amounts of the labelled material could be separated than was possible with the methods of paper and starch gel electrophoresis. Further difficulties may arise with paper and starch gel electrophoresis, because of the limited degree of separation possible with these particular methods (See 5.5 above); the most important limitation was that the α_1 acid glycoprotein was not separable from albumin, which may explain the apparent increase in binding to albumin in some studies (Ochs et alii, 1965).

The column chromatography technique selected was one which did not require packing under nitrogen pressure; adequate packing was achieved by the use of a 2M NaCl solution, which, because of its high specific gravity (S.G. = approx. 1.100), allowed packing to occur without the need for external pressure. This solution was cleared from the column with adequate volumes of the starting buffer before use. The location on the column of the protein fractions has been set out in section 4.2.1, and is illustrated in Figures 4-8.

The initial pH of 6.5 was chosen, since, when dialysis was carried out between pH 8.0 and pH 6.5, and

followed by column chromatography commencing at pH 8.0, no significant protein peaks, other than any excess of protein, appeared before pH 6.5. It is also apposite to note that the pH 4.5 suggested as the critical pH for the alpha globulin binder of vitamin B_{12} by Miller and Sullivan (1959a) has been confirmed in this present study, since the significant peak for abnormal binding, peak V, appears in this step-wise system after pH 5.0 has been replaced by pH 4.0.

The effect of the step-wise change on pH has been indicated in Figure 12. It will be observed that when there was a change in molarity, as well as a change in pH, there was an initial upward swing, away from the ultimate direction of the pH change, before the rapid drop to the new pH level. When there was no change in molarity, and pH only was altered, there was a slower, more gradual fall to the new pH level. There are two possible explanations for this phenomenon, the first and more likely being that, when molarity and pH were changed, the column took up the excess of phosphate, and became transiently more alkaline. Alternatively, the finding may have been due to properties of the proteins themselves; this is, however, considered unlikely, since the same

pattern of pH change has been demonstrated in experiments such as that shown in Figure 29, in which the same transient rise was evidenced, at a time when no protein was removed from the column, at the change between 0.01M tris phosphate buffer, pH 6.5 to 0.05M tris phosphate buffer, pH 6.1; this change occurred at tube 20.

The effect of dialysis upon the pattern of protein peaks obtained was most marked in relation to peak I; this is not surprising, when it is remembered that the precipitate which formed during dialysis, (the principal difference between dialysed and undialysed protein placed on the column) consisted of gamma globulin with some beta globulin; the reduction in the column component was principally at peak I, where most gamma globulin is located (Figure 4).

It has been demonstrated (Figures 13 and 14) that vitamin B_{12}, whether it were radioactive or non-radioactive, did not adhere to the column used in the present study, unless it was bound to plasma proteins. All detectable radioactivity placed on the column appeared in peak I, as did all optical density, when unbound vitamin B_{12} was placed on the column alone. Thus, in columns of both

normal and abnormal sera, it is considered that any radioactivity present in peak I in the undialysed sample represented principally radioactive vitamin B_{12} which was not protein-bound, and which would ultimately be removed by dialysis; it is emphasised that the undialysed specimen contained both bound and unbound radioactive vitamin B_{12}, which was added in excess as part of the experimental procedure. The normal patterns of protein-bound vitamin B_{12} distribution throughout the column differ slightly from one another, but have in common the fact that the vitamin is distributed throughout the column, and no one particular peak predominates. This is in keeping with previously observed findings obtained from other forms of protein separation, in which all five major protein components were shown to bind added vitamin B_{12} in vitro (Pitney et alii, 1954; Heinrich and Erdmann-Oehlecker, 1956a).

6.2.2 Results.

Quite different patterns of binding from normal were observed when dialysed radioactive vitamin B_{12} labelled plasma from patients with chronic myeloid leukaemia was placed on the column. The principal peak of radioactivity was shown to be peak V, and this accounted for the majority

of material placed on the column. This has been demonstrated in the two instances in the early studies (Figures 17 and 18) in which radioactive vitamin B_{12} was added at the 10 ng./ml. level and 100 ng./ml. level respectively. In the latter instance, the ratio of the amount of radioactive material located at peak V to the amount of radioactive material located in the other peaks is much higher than at the 10 ng./ml. level. It should also be noted that plasma vitamin B_{12} binding capacity of the patient whose column chromatogram is shown in Figure 18 was 37.8% at the 100 ng./ml. level, the highest level recorded in this series up to that time; only one patient (COR, Appendix AII) has shown higher levels since.

In the studies which were performed using high specific activity radioactive vitamin B_{12}, in which the amount added was smaller (2 ng./ml.), patterns different from normal were again obtained with plasma from patients with chronic myeloid leukaemia. In the normal (Figure 15), undialysed labelled plasma showed the highest peak of radioactivity in peak I; there was also radioactivity detected in peak II and in peak V. After dialysis, there was little radioactivity in peak I, since this represents

in the pre-dialysis sample the unbound radioactive vitamin B_{12}, and most radioactive material was located in peaks II and V.

It has been postulated that the relative increase in radioactivity in peak V after dialysis, compared with the relative decrease in radioactivity in peak II after dialysis, may be analogous to the situation in vivo, in which vitamin B_{12} is transferred from beta to alpha globulin binders. Alternatively, the dialysis procedure may produce alterations of certain of the vitamin B_{12} binding proteins, so that they may exhibit altered mobility on the column chromatogram; this, however, seems unlikely, since there is no demonstrable change in the optical density ratios between undialysed and dialysed specimens, normal and abnormal.

The patterns obtained from myeloid leukaemia plasma labelled with high specific activity radioactive vitamin B_{12} showed that, even in the undialysed specimen, significant quantities of radioactive vitamin B_{12} appeared at peak V; in patient SME, this amounted to one-third of the total, and in patient COR, it amounted to almost all the material added. This same pattern was observed following dialysis. Attention has also been drawn to the

fact that, in patient COR, as the vitamin B_{12} binding capacity fell, relatively more radioactivity appeared at peak II, the beta peak. This is referred to again below.

It has been previously argued that vitamin B_{12} binding in myeloid leukaemia to proteins of the α_1 globulin group takes place as an overflow phenomenon, following saturation of the normal binding sites (Miller and Sullivan, 1959b; Weinstein et alii, 1959). However, the findings of these studies indicate that binding in chronic myeloid leuakemia occurred to a protein with the mobility of an α_1 acid glycoprotein in a preferential manner, rather than as an overflow phenomenon; the studies of binding to isolated protein fractions also support this contention (See 6.2.3 below). In patient SME at a time when there was no vitamin B_{12} detectable in her serum (Figure 28), all plasma protein bound radioactivity detected, representing 96% of the radioactivity initially placed upon the column, was present at peak V. This finding has been confirmed by two facets of the study of patient COR; first, it is noted in the column chromatogram (Figure 23) that, in the undialysed sample, the majority, if not all, of the

radioactive material appeared at peak V when high specific radioactive vitamin B_{12} was used. Secondly, the fact that the beta binding protein (peak II) in patient COR appeared to account for an increasing amount of bound vitamin B_{12} as the patient's vitamin B_{12} binding capacity fell, may also be interpreted as evidence for the preferential nature of binding by the α_1 globulin in chronic myeloid leukaemia; it is argued that, as less of the abnormal binding protein was produced, reflected by a fall in total binding capacity and in the relative amount of radioactivity present at peak V, more was available for binding by the beta (peak II) binding protein. As well as an increase in either the α_1 globulin, or one of its components, or in its binding capacity, it may well be that in chronic myeloid leukaemia there is an absence, or a deficiency of the beta globulin binder, or possibly, a loss of its normal binding capacity. It is not possible to exclude this concept by the results of this present study.

Comment is also required concerning the relationship of the optical density in peak II to the radioactivity in peak II. It will be noted that the peak of radioactivity is consistently one to two tubes behind the peak of optical density, and this relationship applies for normal

and abnormal plasma, at all levels of binding. Reports have been published concerning isotope effects on ion exchange chromatography (Gottschling and Freese, 1962); in this cited report, it was shown, in experiments designed to measure the incorporation of tritium-labelled 2-aminopurine into DNA, that the radioactivity ran slightly ahead of the optical density in an otherwise well separated peak. It was considered that this was due to a difference of electrical charge between tritiated and non-tritiated aminopurine in this particular case. However, in the present study, it is considered that the radioactivity findings represent the location on the column chromatogram of the beta globulin binder of vitamin B_{12}, which is likely to be one very small component of the entire beta globulin peak. It has been calculated by various workers (Heller et alii, 1964; Miller and Sullivan, 1959b; Mendelsohn et alii, 1958) that the amount of vitamin B_{12} binding protein is very small, both in normal and in abnormal states; it is therefore likely that the beta globulin binder is a small component of peak II on the present chromatogram, and its location is more faithfully reflected by the peak of radioactivity than by the larger, less specific peak of optical density.

6.2.3 Identification of Vitamin B_{12} Binding Protein.

Emphasis has been laid upon the fact that it is difficult, if not impossible, in the state of our present technical knowledge, to isolate the very small components of the total plasma proteins which account for binding of vitamin B_{12}. Although it has previously been suggested that seromucoid, or certain of its constituents were responsible for vitamin B_{12} binding (Miller and Sullivan, 1959b), this result has been called into question at various times (Mendelsohn et alii, 1958; Weinstein et alii, 1959; Hardwicke and Jones, 1966).

The findings of this present study have indicated that the abnormal binding of vitamin B_{12} seen in chronic myeloid leukaemia was consistently associated with an abnormally high peak of radioactivity upon the standard column chromatogram used. This peak was that peak at which the α_1 acid glycoprotein (labelled with radioactive vitamin B_{12} or unlabelled) appeared when subjected to the usual column procedure (Figures 8 and 29). It has also been shown that this particular protein fraction was capable of binding very large amounts of added vitamin B_{12}, in excess of any other separated protein fraction tested. It is therefore considered that evidence has

been presented from two separate directions to show that the α_1 acid glycoprotein, or one or more of its constituents, is the protein responsible for binding abnormal amounts of vitamin B_{12} in the myeloid leukaemic state.

6.3 Relationship of Granulocytes to Vitamin B_{12} Binding.

For some time, the possibility of leukaemic cell destruction as a source of the vitamin B_{12} binding protein has been considered (Fahey et alii, 1958; Mendelsohn et alii, 1958). Mollin and Ross (1955) found that the vitamin B_{12} concentration of leukocytes was lower than the concentration in other body tissues, and that the content of chronic myeloid leukaemic leukocytes was diminished. Thomas and Anderson (1956), and later, Kidd and Thomas (1962), studied leukocyte vitamin B_{12} levels in chronic myeloid leukaemia, and found that they were lower than those in normals, but that in acute leukaemia, the vitamin B_{12} level of leukocytes was elevated. They were unable to demonstrate any relationship between serum and leukocyte vitamin B_{12} concentrations. They considered that the low level of total vitamin B_{12} bound by the leukocytes of chronic myeloid leukaemic patients did not support the hypothesis that elevated serum vitamin B_{12} levels were

due to release of this vitamin from the leukaemic cell; they also agreed that their studies did not disprove this. Meyer et alii (1962) showed that mature neutrophils had the highest vitamin B_{12} binding capacity of all leukocytes studied; other leukocytes did not possess this property to any marked degree. They also showed that leukocytes from patients with chronic myeloid leukaemia had a higher vitamin B_{12} binding capacity when disintegrated than when the cells were intact. They considered that the disintegration product from mature neutrophils contributed to the increased vitamin B_{12} binding capacity of serum in myeloproliferative disease. It was not until 1964 that the presence of a vitamin B_{12} binding protein in white cells was demonstrated (Simons, 1964; Simons and Weber, 1966). It has been postulated recently (Retief et alii, 1967a) that leukocytes may be the source of alpha and beta vitamin B_{12} binding proteins, and that the myeloid leukaemic granulocyte may give rise to more of the alpha vitamin B_{12} binding globulin and less of the beta vitamin B_{12} binder than is delivered into the normal serum by normal granulocytes.

The findings of this present study confirm that intact granulocytes from some persons, but not all, are

capable of binding considerably increased amounts of vitamin B_{12}. This is the first study in which separated lymphocytes and granulocytes have been investigated in parallel; it has been shown that granulocytes and not lymphocytes possess the property of vitamin B_{12} binding. It is not yet certain whether this vitamin B_{12} binding protein was released from within the white cells during these experiments, or was present on their surfaces, or whether the vitamin B_{12} binding protein of granulocytes is a breakdown product of the normal granulocyte which binds vitamin B_{12} non-specifically.

6.4 Hypotheses.

There are a number of findings concerning vitamin B_{12} metabolism in myeloid leukaemia which require explanation, and it is pertinent at this stage to consider these, and the possible explanations which have been offered, and to attempt to evaluate these in the light of our present knowledge, and in the light of the findings of this present study. The following facts are generally accepted:

1. In myeloid leukaemia, increased serum vitamin B_{12} levels are common;

2. Increased in vitro and in vivo binding capacity of the plasma proteins occurs in myeloid leukaemia;

3. There is a delayed plasma clearance of vitamin B_{12} injected intravenously or ingested orally in patients with myeloid leukaemia;

4. Gastrointestinal absorption of vitamin B_{12} is not increased in these patients;

5. There is not impaired renal excretion of vitamin B_{12} in these patients;

6. There is an apparent variation in the proportion of transcobalamin I and II (or the alpha and beta globulin binders), in the plasma of patients with chronic myeloid leukaemia.

The following explanations have been offered for some or all of the above demonstrable phenomena, and the evidence for these has been discussed previously.

* Weinstein et alii (1959) have shown that gastrointestinal absorption of vitamin B_{12} was not increased in patients with chronic myeloid leukaemia.

* Similarly, these same workers have shown that the renal absorption was not increased in chronic myeloid leukaemia; thus, neither of these explanations may be invoked to explain the increased serum levels and increased in vitro binding of vitamin B_{12}.

* It has been suggested by Weinstein et alii (1959)

that a shift in the body stores of only 3% of the total vitamin B_{12} content of the body would be sufficient to raise the plasma level by ten times the normal value; although this is an attractive theory, there is no evidence for this at present, and in fact, it would be most difficult, in the light of Reizenstein's work (Reizenstein, 1959b), to calculate this with any certainty.

* It was suggested that the binding of vitamin B_{12} by plasma proteins in myeloid leukaemia might be more firm than binding in the normal state (Miller, 1958); this does not appear to have been demonstrated. A defect in vitamin B_{12} binding was postulated some years ago (Horrigan and Heinle, 1952), as an explanation of refractory anaemia, but this does not appear to be different from the binding abnormalities demonstrable in vitamin B_{12} deficiency (Lawrence, 1966a, 1966b).

* It has been suggested that the phenomenon of increased binding may be due to an increase in normal proteins present in chronic myeloid leukaemia; because of the difficulty in detecting such subtle alterations as discussed above, this is not yet proven or disproven; no gross abnormality has yet been demonstrated in these patients (see pp68-70).

* Several authors, including Miller (1958), have suggested that altered proteins resulting from the disease process may be responsible for binding excess amounts of vitamin B_{12}, and that it is possible that there may be an increased number of binding sites on the same total quantity of protein; this also remains unproven.

* An abnormal tissue protein foreign to normal serum has also been invoked as a possible explanation for increased binding capacity in myeloid leukaemia. The possible sources again include white cells, and/or their breakdown products, and an increase in gamma globulins (which occurs in the analogous situation of leukaemia in fowls (Lagerlöf and Sundelin, 1963)). Although isologous antibodies represent an abnormal tissue protein foreign to normal serum, plasma containing isologous antibodies does not bind increased amounts of radioactive vitamin B_{12}.

* The possibility that the excess of vitamin B_{12} is released as a result of liver damage has been invoked, but this does not appear to be likely, since it is recognised that increased levels of vitamin B_{12} are seen only in patients with malignant disease when hepatic metastases

are present (Grossowicz et alii, 1957; Mendelsohn and Watkin, 1958).

* Heller et alii (1964) suggested that vitamin B_{12} itself may be the stimulus to the production of its own binding protein; the source of the increased circulating vitamin B_{12} in myeloid leukaemia still remains to be explained in this hypothesis.

* A further suggestion, which arises from the present studies, is that an increase in vitamin B_{12} binding protein may result from an increased stimulus to the production of this protein, which stimulus may be the basophils or granulocytes, or the breakdown products of either of these; it is possible that both the abnormally high levels of basophils and the increased vitamin B_{12} binding capacity may occur as the results of a common, and as yet unrecognised, stimulus.

* Britt and Rose (1966) have discussed the unusual finding of normal vitamin B_{12} levels in patients with pernicious anaemia who have also had, concurrently, chronic myeloid leukaemia; they suggested that the circulating vitamin B_{12} was not available for normal metabolism in this situation. The patient SME of this study represents the equally unusual and converse

occurrence of a patient with chronic myeloid leukaemia, who had no detectable vitamin B_{12} in her serum, as the end result of prolonged failure of absorption by a gastric remnant. Although preferential binding to a protein with the chromatographic characteristics of α_1 acid glycoprotein was demonstrated, it would nevertheless appear that all vitamin B_{12} was available for metabolism in this patient.

It is not possible to investigate retrospectively the natural history of the development of such a clinical situation, and it is possible only to speculate whether the vitamin B_{12} abnormality associated with leukaemia delayed the onset of the deficiency state, or whether, if large amounts of vitamin B_{12} were mobilised from the tissue stores as part of the leukaemic process, the manifestation of deficiency was hastened by the leukaemic process.

CHAPTER 7

GENERAL SUMMARY AND CONCLUSIONS

GENERAL SUMMARY AND CONCLUSIONS

Although it has been recognised for some years that vitamin B_{12} is bound to the plasma proteins during its passage through the body's metabolic pathways, a great deal remains to be discovered concerning a number of the dynamic aspects of this transport; the details awaiting resolution include the manner of binding, transport and release of the vitamin by plasma proteins, the isolation and identification of these carrier proteins, and the basis of the abnormalities of vitamin B_{12} transport in such diseases as myeloid leukaemia.

The general aims of the present studies have been first, to evaluate critically the dialysis method of measurement of in vitro vitamin B_{12} binding capacity and the factors influencing it, and second, to assess by various methods of protein separation the significance of observed alterations in the vitamin B_{12} binding capacity, particularly the elevations seen in the plasma of patients with chronic myeloid leukaemia.

The specific aims of these studies, and their potential value in the understanding of both vitamin B_{12} metabolism and the myeloid leukaemic disorder have already been given in detail (Chapter 1, pages 2-3).

The review of the literature has covered certain aspects of the chemistry and biochemistry of vitamin B_{12} relevant to the broad theme of the study, including the structure of cyanocobalamin and the relationship of structure to protein binding, and the absorption, plasma clearance, storage and excretion of the vitamin in man. The methods of measurement of vitamin B_{12} levels in biological material have been reviewed, together with the results, both normal and abnormal, obtained with these methods.

Intrinsic factor, its relationship to vitamin B_{12} and the vitamin B_{12} binding capacity of the gastric juice proteins have been discussed in detail because of their relevance to the subject of the study; the uncertainty surrounding the structure and function of the gastric juice binders of vitamin B_{12}, and the probable lability of these proteins, highlights similar difficulties inherent in the study of protein binding of the vitamin in plasma.

The literature review concludes with a detailed survey of the methods used for determination of the binding capacity of vitamin B_{12}, the results obtained with these methods, and the explanations offered for

the abnormal binding demonstrable in certain disease states, notably myeloid leukaemia.

Most described methods for measurement of vitamin B_{12} binding capacity have their own defined conditions, differing from one method to the next; it was therefore an essential part of this present study to assess which physicochemical factors influenced the end result obtained, and to evolve a method which took into consideration these variables.

The method described in this study requires, after incubation of the test sample with excess radioactive vitamin B_{12}, a ninety-six hour dialysis against a standard buffer in the cold, with mechanical stirring and regular changes of buffer.

The concentration of the radioactive vitamin B_{12} added prior to incubation, the duration of incubation, the duration of dialysis, and the solutions used for dialysis have been shown to be significant variables in the method; these variable factors require standardization to ensure the reproducability of the method. Attention has been drawn to the precipitate which is formed during dialysis, and the nature and binding properties of this precipitate have been commented upon. Other variable

aspects of the method did not produce variation in the end results, although to ensure reproducability, these aspects have also been standardized.

The described method of measurement of vitamin B_{12} binding capacity was used to determine the normal range, and to study several groups of patients in which variation from the normal might have been expected.

Gross elevation of the vitamin B_{12} binding capacity was demonstrated in patients with chronic myeloid leukaemia at the 10 ng./ml. level and at the 100 ng./ml. level of added vitamin B_{12}. In patients with acute myeloid leukaemia, the mean vitamin B_{12} binding capacity at the 10 ng./ml. level was consistently elevated, and was not different from that of the chronic myeloid leukaemic group; at the 100 ng./ml. level, however, the mean value for the acute leukaemic group was not different from normal, and the difference between the means for the acute and chronic myeloid leukaemic groups was statistically significant. Attention has been drawn to the possible relationship between basophilia and the elevated vitamin B_{12} binding capacity in myeloid leukaemia.

The vitamin B_{12} binding capacity was not different from normal in patients with lymphatic leukaemia, patients with isologous antibodies and in pregnant women.

An increase in vitamin B_{12} binding capacity was observed in three patients with dysproteinaemias; the increase in each instance was in the binding to the precipitated portion of the protein.

An increased mean vitamin B_{12} binding capacity was demonstrated at the 10 ng./ml. level in patients with vitamin B_{12} deficiency, but the mean vitamin B_{12} binding capacity at the 100 ng./ml. level was normal; the possible reasons for this finding have been discussed.

Column chromatography of radioactive vitamin B_{12} labelled plasma from patients with myeloid leukaemia revealed marked and consistent variations from the normal pattern, at all levels of binding.

Increased binding was consistently associated with a column chromatogram peak (V) at which radioactive vitamin B_{12} labelled, and unlabelled, α_1 acid glycoprotein appeared; this separated protein fraction was shown to be capable of binding much more added vitamin B_{12} at all levels than the other protein fractions studied.

In the plasma of a myeloid leukaemia patient with no detectable circulating vitamin B_{12}, all vitamin B_{12} added and bound was located at this significant peak V.

Granulocyte-rich plasma was shown to be capable of binding more added vitamin B_{12} than lymphocyte-rich plasma and leukocyte-poor control plasma. The possible implications of this finding upon the binding of vitamin B_{12} in normal and disease states has been discussed.

The significance of these results, and their considered originality, has already been stated on pages 3-5.

Hypotheses.

It is considered, from the results of this study, that the demonstrable increase in vitamin B_{12} binding capacity of myeloid leukaemia plasma is found in association with a protein which has the chromatographic mobility of an α_1 acid glycoprotein, which is itself in the separated form capable of binding large amounts of added vitamin B_{12}. This critical binding protein may be the α_1 acid glycoprotein itself or one of its components. Binding to this protein in vitro takes place preferentially, and not as an overflow phenomenon following saturation of the normal binding sites.

In the present state of our knowledge, it is not

possible to state whether this protein is present in increased quantities in chronic myeloid leukaemia, or whether the increased binding represents a qualitative change in the protein; similarly, the relationship of this binding protein to the beta globulin binder of vitamin B_{12}, and the factors which induce change (and possibly interchange) in the levels of these proteins and their binding capacities in certain disease states remain to be defined.

It is also considered that normal granulocytes (or possibly their breakdown products) play a significant part in plasma protein binding of vitamin B_{12} in vitro, and that the increased binding of vitamin B_{12} in myeloid leukaemia may be due to the presence of increased numbers of mature granulocytes (or their breakdown products), or possibly, the basophilic granulocytes.

APPENDICES

APPENDIX A.

CASE HISTORIES.

I. SME.

This patient was admitted first to the Clinical Research Unit of the Royal Prince Alfred Hospital, in July 1961, when she was aged 62. Her only relevant medical history was a partial gastrectomy and gastroenterostomy in 1952 for active gastric ulcer. At the time of her initial admission in 1961, she had complained of symptoms of weakness, breathlessness on exertion and spontaneous bruising for twelve months. Apart from a psoriatic rash on the back of her hands, and a palpable spleen, the physical examination was otherwise negative.

Her admission haemoglobin (See Table XXII) was 7.9 gm./100 ml., and the total white cell count was 161,000/cu.mm., with 35% primitive forms, 44% neutrophils and 11% basophils. The platelet count was 1,452,000/cu.mm. Marrow aspiration was performed, and the findings were typical of chronic myeloid leukaemia. She was transfused, and treatment with busulphan

TABLE XXII

HAEMATOLOGICAL DATA - SME.

	HAEMOGLOBIN (gm./100ml.)	WHITE CELL COUNT (x 10^3/cu.mm.)	PLATELET COUNT (x 10^3/cu.mm.)	SERUM VITAMIN B_{12} (µµg/ml.) TOTAL	FREE
July, 1961	7.9	161	1,452	2,864	80
Jan., 1963	10.6	27	2,200	1,530	0
April, 1965	12.9	37.5	715	0	0
May, 1965*	-	-	-	1,440	Most

*After 2,000 µg. vitamin B_{12} intramuscular injection.

("Myleran") was commenced, at a dosage of 4 mg. daily. After two weeks, and little clinical response, the dosage of "Myleran" was increased to 6 mg. daily, and a response was induced. She was discharged well, one month after her initial admission, on a maintenance dose of 4 mg. "Myleran" daily.

She was readmitted to the Clinical Research Unit in January, 1963, having taken "Myleran" from the time of her initial admission until December, 1962. Her complaints were, at this time, of intermittent epigastric pain, thought to be associated with a non-functioning gall bladder. Her platelet count at the time was high, and for this reason, she was readmitted for further treatment. Investigation showed a haemoglobin value of 10.6 gm./100 ml., a total white cell count of 27,000/cu.mm., of which 7% were primitive forms, 63% neutrophils, 10% lymphocytes, 4% monocytes, 6% eosinophils and 10% basophils; the platelet count was 2,200,000/cu.mm. The total serum vitamin B_{12} level done at this time was 1530 µµg/ml. Sternal bone marrow aspiration yielded a grossly hypercellular smear, with an M/E ratio 25:1. The findings were consistent with the original diagnosis of chronic myeloid leukaemia. She was given iron, because of overt

iron deficiency, and because of difficulties in supervision (she lived in the country) it was decided to treat her with P_{32}; 2.5 mc. were given in February, 1963. She was discharged on maintenance doses of iron.

Later in 1963, and in 1964, small doses of "Myleran", of the order of 2 mg. daily, or every second day, were given. She was readmitted to the Clinical Research Unit in April, 1965 (at a time when the "Myleran" therapy had been temporarily suspended) with the principal presenting complaints of abdominal pain and weight loss. At the time of admission her haemoglobin was 12.9 gm./100 ml., and the erythrocytes showed slight central pallor. The white cell count was 37,500/cu.mm. and the platelet count 715,000/cu.mm. On examination sternal tenderness and epigastric tenderness were elicited. The patient was obviously ill and febrile, but no source of infection could be found. A further sternal marrow biopsy showed a picture consistent with chronic myeloid leukaemia, and because of this, treatment was recommenced on 4 mg. of "Myleran" per day, which induced a steady fall in the white cell count to 7,500/ cu.mm., and in the platelet count to 485,000/cu.mm.

Although abdominal pain persisted, a barium meal showed a normally functioning portion of stomach, following partial gastrectomy and gastroenterostomy.

Further investigations at this time showed the serum iron to be 37 µg./100 ml., the serum folic acid 2.9 µg./ml., and there was no demonstrable vitamin B_{12} present in her serum. The Schilling test without carbachol resulted in 6% excretion, and with carbachol, 8.8% excretion was observed.

The ulcer-type pain disappeared on antacid treatment, and she was given a large intravenous dose of iron, and 1,000 µg. of vitamin B_{12} intramuscularly twice weekly. It was at this stage that she volunteered that the numbness in her feet, present for some months, disappeared after the first injection of vitamin B_{12}. Her response to treatment was good, and she left hospital in May 1965.

She died at home in the country from a cerebro-vascular accident later in 1965.

TABLE XXIII

HAEMATOLOGICAL DATA - COR; 1966.

DATE -	28/1	4/2	3/5	28/6	26/7	27/9	22/11
Hb. (gm./100ml.)	8.9	8.6	12.7	14.1	13.0	12.8	12.0
WCC ($x10^3$/ cu.mm)	294	106	18.5	6.0	6.5	6.4	7.5
Platelets ($x10^3$/cu.mm)	370	430	235	200	210	Norm.	280
Neutrophils (%)	55	49	68	58	55	65	81
Eosinophils (%)	1	2	3	-	-	1	-
Basophils (%)	9	15	2	-	2	-	3
Lymphocytes (%)	4	-	15	40	35	31	13
Monocytes (%)	9	1	9	2	8	3	3
Myeloblasts (%)	5	5	-	-	-	-	-
Promyelocytes (%)	5	3	-	-	-	-	-
Myelocytes (%)	5	5	3	-	-	-	-
Metamyelocytes	7	20	-	-	-	-	-
Nucleated Red Cells	5	4	Occ.	-	-	-	-

II. COR.

This married woman was 49 years of age when she presented to the Queen Elizabeth Hospital, Woodville, in January, 1966, complaining of weight loss, an abdominal mass, and general malaise, which had been present for several months. On examination, she was found to have an enlarged spleen, approximately 10 cm. below the left costal margin; there were no other abnormal clinical findings. The initial blood count is shown at Table XXIII. The initial bone marrow showed a myeloid:erythroid ratio of 90:1, and the findings were consistent with the diagnosis of chronic myeloid leukaemia. She received blood transfusion, and treatment was commenced with "Myleran", 2 mg. three times daily. She was discharged after 10 days in hospital on "Myleran", 2 mg. twice daily. When seen in August, 1966, she was well, and her spleen was no longer palpable.

In late January, 1967, she was readmitted with symptoms of anaemia, and was found on examination to be extremely pale. The spleen was once more palpable, 2 cm. below the left costal margin. "Myleran" was continued in the same dosage which had been maintained for the full year, and blood transfusion was given. A blood transfusion

TABLE XXIV.

HAEMATOLOGICAL DATA - COR; 1967A.

DATE -	31/1	1/2	6/2	10/2	17/2	22/2	24/2	4/3	6/3
Hb. (gm./100ml.)	4.4	5.2	9.4	11.2	6.4	6.6	9.7	8.0	8.6
WCC ($x10^3$/cu.mm.)	7.2	9.2	9.1	13.6	7.9	7.0	4.0	2.2	4.4
Platelets ($x10^3$/cu.mm.)	225	220	196	200	237	184	210	200	195
Neutrophils (%)	66	62	53	53	61	66	67	84	76
Eosinophils (%)	3	-	1	-	-	-	-	-	1
Basophils (%)	13	14	20	21	22	19	5	5	2
Lymphocytes (%)	10	9	5	3	10	7	24	5	11
Monocytes (%)	1	-	4	2	-	1	4	6	6
Myeloblasts (%)	2	-	-	1	1	-	-	-	-
Promyelocytes (%)	2	3	-	-	-	-	-	-	1
Myelocytes (%)	2	6	4	7	4	-	-	-	1
Metamyelocytes (%)	1	6	13	13	2	7	-	-	2
Nucleated Red Cells (%)	-	-	-	-	-	-	-	-	-

reaction occurred, and on investigation it was found that the patient had developed an anti-Fy^a antibody which was present to a titre of 1/2064, with extreme avidity. She was discharged in early February, but was readmitted on 18th February, 1967, again anaemic. She was transfused, and "Myleran" was continued at the same dosage.

She was again admitted on 3rd March, 1967, only one week later with further symptoms of anaemia. Her weakness had become progressive, and her haemoglobin level had fallen to 4.0 gm./100 ml. Once again she was transfused with appropriately matched blood, and corticosteroids were commenced, since it was considered that a haemolytic component was contributing to her rapid anaemia. Prednisone was begun on 3rd March, 1967, in a dosage of 15 mg. four times daily; "Myleran" was continued until 8th March, 1967, when it was stopped.

Because of the apparent autoimmune haemolytic anaemia which this patient had developed at this time, azathioprine ("Imuran") was begun in the dosage of 50 mg. twice daily. Further transfusion was given, and, following some improvement in the haemolytic process, she was discharged on 24th March, 1967, on prednisone 10 mg. three times daily, and azathioprine 50 mg. twice daily.

TABLE XXV.

HAEMATOLOGICAL DATA - COR; 1967B.

DATE -	4/4	18/4		20/4	21/4	24/4	25/4	28/4
Hb. (gm./100 ml.)	3.0	11.2	O	13.9	14.4	12.7	10.4	6.1
WCC ($x10^3$/cu.mm.)	3.4	3.9	P	7.4	16.0	11.0	18.1	23.0
Platelets ($x10^3$/cu.mm.)	280	120	E	310	305	710	780	1,200
Neutrophils (%)	91	83	R	93	95	89	91	88
Eosinophils (%)	-	-	A	-	-	-	-	-
Basophils (%)	3	4	T	-	-	-	1	4
Lymphocytes (%)	5	8	I	6	1	9	8	3
Monocytes (%)	1	5	O	1	4	2	-	-
Myeloblasts (%)	-	-	N	-	-	-	-	-
Promyelocytes (%)	-	-		-	-	-	-	4
Myelocytes (%)	-	-		-	-	-	-	-
Metamyelocytes (%)	-	-		-	-	-	-	1
Nucleated Red Cells (%)	-	-		-	-	-	-	-

A further admission was necessary on 4th April, 1967, because of anaemia, and after transfusion with 9 units of blood, the dosage of "Imuran" was increased to 100 mg. twice daily. A serum vitamin B_{12} level done on 11th April, 1967, by the E. gracilis method, was 1700 pg./ml.

A further admission was necessary on 16th April, 1967, because of symptoms of profound anaemia; she was again transfused. In view of the red cell survival studies, which showed a half-life of 8 days (normal 28-32 days) and marked splenic uptake, splenectomy was considered to be indicated for the relief of this patient's haemolytic process. This operation was performed on 19th April, 1967, and the trans-thoracic route was used. Initially, the patient's progress was satisfactory, and her haemoglobin value remained normal for one week. In addition to the Howell-Jolly bodies which were present immediately after splenectomy, the blood film showed unusually large and oddly-shaped platelets.

Commencing with the blood film on 24th April, 1967, the red cells began to show marked spherocytosis. The film made on 27th April, 1967, showed that 40% of the red cells were spherocytic, and that made the following day

showed 60-70% of all red cells in the peripheral blood to be spherocytic. From 25th April, 1967, the patient's haemoglobin value began to fall rapidly, and she died four days later. Since the exacerbation of her illness in January, 1967, she had received 58 pints of blood.

The Coombs test on 18th April, 1967, was positive to a dilution of 1:20,480. After operation, it had fallen to 1:5120 on 24th April, 1967.

Histological Reports.

The spleen removed at operation on 19th April, 1967, weighed 1140 gm. Although the splenic architecture was preserved, massive haemosiderosis was noted, and much blood was present in the sinusoids. There was no histological evidence of leukaemia or extra-medullary haemopoiesis.

The post-mortem findings were of a thrombosed splenic vein (post-splenectomy), pulmonary oedema, early renal tubular necrosis, and chronic myeloid leukaemia. The bone marrow showed diffuse primitive myeloid cells, and numerous megakaryocytes. The bony architecture of the marrow was eroded and distorted by the leukaemic material.

APPENDIX B

PUBLICATIONS.

The following publications contain material from this thesis:

1. Studies on the plasma protein binding of radioactive vitamin B_{12}. I. Factors influencing in vitro binding.
   J. Lab.clin. Med., 63, 1964, 969.

2. Preferential in vitro binding of radioactive vitamin B_{12} by an abnormal serum protein in chronic myeloid leukaemia.
   Nature (Lond.), 213, 1967, 81.

The following abstracts refer to certain of the material in the above two papers, but also record other aspects of the material of this thesis:

3. Physical factors influencing in vitro plasma binding of radioactive vitamin B_{12}.
   Med. Research, 1, 1963, 55.

4. Column chromatography of plasma labelled with Co^{58}-vitamin B_{12}. (R.W. Beal and B.G. Firkin)
   Med. Research, 1, 1963, 56.

5. Serum binding of Co^{58}-vitamin B_{12}: Further studies of abnormal binding in disease states.
   Med. Research. 1. 1965. 151.

6. Studies of abnormal serum binding of Co^{58}-vitamin B_{12} in disease states.
   XIth Congress. Internat. Soc. Haematol., Sydney. 1966. p. 226.

7. Serum protein binding of Co^{58}-vitamin B_{12}: Relationship of granulocytes to increased binding. (R.W. Beal and W.M.F. Read).
   Med. Research. 2. 1966. 30.

The following papers have been submitted for publication:

Studies on the plasma protein binding of radioactive vitamin B_{12}: III. Binding capacity in disease states.

Studies on the plasma protein binding of radioactive vitamin B_{12}: IV. Column chromatography of normal and abnormal plasma.

Studies on the plasma protein binding of radioactive vitamin B_{12}: V. Relationship of granulocytes to binding capacity.

Studies on the plasma protein binding of radioactive vitamin B_{12}: VI. Binding capacity of separation protein fractions.

APPENDIX C

PAPERS GIVEN TO SCIENTIFIC SOCIETIES.

The following papers, dealing with aspects of the material of this thesis have been presented to meetings of the undermentioned scientific societies:

Physical Factors Influencing In Vitro Binding of Radioactive Vitamin B_{12}.
Haematology Society of Australia; Melbourne, August, 1963.

Column Chromatography of Plasma Labelled with Co^{58}-Vitamin B_{12}.
Australian Society for Medical Research; Adelaide, October, 1963.

Studies of Abnormal Serum Binding of Co^{58}-Vitamin B_{12} in Disease States.
XIth Congress of the International Society of Haematology; Sydney, August, 1966.

Serum Protein Binding of Co^{58}-Vitamin B_{12}: Relationship of Granulocytes to Increased Binding.
Australian Society for Medical Research; Adelaide, December, 1966.

Plasma Factors in Vitamin B_{12} Binding.
Haematology Society of Australia; Canberra, May, 1967. (Contribution to symposium on "Advances in Laboratory Techniques").

In Vitro Binding of Co^{58}-Vitamin B_{12} to Separated Protein Fractions.
Australian Society for Medical Research; Adelaide, June, 1967.

APPENDIX D

EXPERIMENTAL CORRELATION.

Throughout the series of experimental studies reported in this thesis, a sequential numbering system has been used for record purposes. This Appendix relates the information contained in the text, tables and figures with the appropriate experimental reference.

SECTION		EXPERIMENT
4.1.1		B37 B54, 55
4.1.2	Table IV	B46-9, B73-4
4.1.3	Table V	B40-44
4.1.4		B107
4.1.5	Table VI Table VII	B40, 51, 79, 80. B22
4.1.6	Table VIII	B323 B262-3, 266-7
4.1.7		B34, 36
4.1.8	Table IX	B96-9
4.1.9		B100
4.1.10		B401-3
4.1.11		B64
4.1.2	Figure 4 Figure 5	B581 B503

SECTION		EXPERIMENT
4.1.2	Figure 6	B664
	Figure 7	B501
	Figure 8	B494
5.1.1		Various experiments
5.1.2.1	Table X	As shown in Table
	Table XI	As shown in Table
	Table XII	B579, 588, 597, 599, 601, 606, 611, 614, 630, 631.
5.1.2.2		B398
5.1.2.3		B76, 379-81
5.1.2.4		B612, 616, 617
5.1.2.5		B142-5
	Table XV	B535, 537, 538
5.1.2.6		B464, 470, 474, 477, 479, 486.
5.2.1	Figure 12	B113
	Figure 13	B573
	Figure 14	B208
5.2.2	Figure 15	B566
	Figure 16	B567
5.2.3	Figure 17	B78
	Figure 18	B124
	Figure 19	B133
5.2.4	Figure 20	B569
	Figure 21	B570
	Figure 22	B621
	Figure 23	B615
	Figure 24	B628
	Figure 25	B618
	Figure 26	B620
	Figure 27	B632

SECTION		EXPERIMENT
5.2.5	Figure 28 Figure 29	B346 B117
5.3	Table XVI Table XVII	B528 B519
5.4	Tables XVIII-XXI Figures 30, 31	B623-7
5.5		B134
6.1.2		B28, 61, 120

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Studies on the plasma protein binding of radioactive vitamin B_{12}

I. Factors influencing in vitro binding

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The present study has been designed to evaluate the variable factors in the dialysis method of measuring the binding of radioactive vitamin B_{12} to plasma. The concentration of the radioactive vitamin B_{12} added, the duration of dialysis, and the duration of incubation have been shown to influence the binding of the radioactive vitamin B_{12} to plasma. The significance of the precipitate formed during dialysis has also been assessed. Standardization of method with particular reference to the significant variables described here is suggested, so that results from different series may be compared.

Elevations of the serum vitamin B_{12} level are common in patients with chronic myeloid leukemia[1, 2] and are also seen in certain acute leukemias and myeloproliferative disorders. The possible use of this estimation in differential diagnosis has been discussed.[3] Furthermore, the in vitro binding of vitamin B_{12} to serum or plasma proteins is also greatly increased in chronic myeloid leukemia.[4, 5] The electrophoretic distribution of the bound protein in these patients is said to be different from normal,[5] although other authors using different techniques have not found a difference.[6, 7]

During the initial stages of an investigation of the plasma B_{12} binding proteins,[8] it was necessary to assess some of the variable factors in the method

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used for measurement of in vitro binding of the radioactive vitamin to plasma, because of the widely differing methods used and conflicting results found in the literature.[4-6, 9-13]

The basic method adopted by most workers for measurement of in vitro binding of radioactive vitamin B_{12} to plasma consists of incubation of radioactive vitamin B_{12} with plasma or serum, after which dialysis is performed to remove excess radiovitamin not bound during incubation. It is then assumed that the radioactivity remaining within the dialysis sacking represents vitamin B_{12} which has been bound to the plasma proteins.

The following variable factors in the basic method have been studied:

1. whether plasma and serum give differing results;
2. the effect of the concentrations of radioactive vitamin B_{12} on the amount of radioactive vitamin bound;
3. the effect of the temperature of incubation on the amount of radioactive vitamin bound;
4. the effect of the duration of incubation on the amount of radioactive vitamin bound;
5. the influence of the duration of dialysis on the amount bound;
6. the effect of the temperature at which dialysis was performed on the amount of radioactive vitamin bound;
7. the effect of varying volumes of dialysis buffer on the binding;
8. the influence of differing buffers upon dialysis, and
9. the significance of the precipitate formed during dialysis.

Materials and methods

The standard technique for this study required the collection of venous blood into a sterile, heparinized container and the separation of plasma by centrifugation. Cobalt^{58}-labeled vitamin B_{12}* was then added in known amount, generally with 1 volume of the radiovitamin mixed with 9 volumes of plasma to give a final concentration of 10 mμg per milliliter, except where otherwise specified. The Cobalt^{58}-vitamin B_{12} contained 1 μc/1 μg, and was dissolved in sterile physiologic saline. After incubation at 37° C. for 1 hour unless otherwise indicated, a known volume of 2 ml. to 5 ml. (usually 4 ml.) labeled plasma was placed in Visking dialysis sack, and dialysis was performed, usually at 4° C. in a 250 ml. beaker, with mechanical stirring against 150 to 200 ml. buffer. The buffer most commonly used was Tris (trihydroxymethylaminomethane) phosphate buffer, 0.01M, pH 6.5. This buffer was chosen because the dialyzed plasma obtained was thus equilibrated with DEAE columns used for column chromatography.[8] Following dialysis with changes of wash for a minimum of 96 hours, as much of the dialysant as possible was taken from the sac and centrifuged to remove the precipitate which formed during dialysis.

Aliquots of the original labeled plasma, the dialysant, and each wash, together with the whole of each precipitate and each sac, were counted in a Nuclear-Chicago well-type scintillation counter (2 in. sodium iodide crystal) for sufficient time to give an error of less than 5 per cent in each count. Thus an attempt, not made in other studies, was introduced to account for all the radioactivity present at the commencement of dialysis. The mean yield with this method in a series of 58 dialyses was 97.6 per cent ± 4.0 per cent (2 S.D.).

The following variants of this basic method were studied:

1. *Plasma and serum binding.* Plasma and serum were obtained from blood collected from the same subject at the same time.

*Obtained from Amersham, England, through the Commonwealth X-ray and Radium Laboratory, Melbourne, Australia.

2. *Concentration.* Aliquots of normal and chronic myeloid leukemic plasma were incubated with 1 mμg per milliliter, 10 mμg per milliliter, and 100 mμg per milliliter concentrations of the radioactive vitamin B_{12}, and dialyzed under identical conditions.

3. *Temperature during incubation.* Two aliquots of normal plasma were incubated at room temperature (21° C.) for 2 hours, 6 hours, and 24 hours, and were then dialyzed under similar conditions for similar lengths of time.

4. *Duration of dialysis.* The effect of the duration of dialysis was studied by measuring the radioactivity present in the wash buffers at each change. In some instances this consisted of measurement of radioactivity at 48 hours and at the end of dialysis, while in another instance, 6 buffer changes and measurements were made over a 162 hour dialysis. Aseptic precautions were observed in handling the material used in this and the other experiments, and they were conducted at 4° C. to minimize the possibility of bacterial growth.

5. *Temperature during dialysis.* Aliquots of the same normal labeled plasma were dialyzed in the cold (4° C.) and at room temperature (20° C.).

6. *Volume of dialysis buffer.* Two aliquots of the same normal labeled plasma were dialyzed, one in a 250 ml. beaker and the other against running tap water.

7. *Effect of differing buffers.* Aliquots of the same labeled plasma were dialyzed against normal saline, Tris phosphate buffer (0.01M, pH 6.5) and tap water, pH 6.75.

Results

Plasma and serum binding. At the 10 mμg per milliliter level, plasma from a normal subject bound 1.15 mμg per milliliter, while serum from the same subject bound 1.17 mμg per milliliter. This difference is not significant. Similar findings have been obtained with other normal subjects and from patients with chronic myeloid leukemia.

Table I. Effect of differing concentrations of Co^{58}-B_{12}

Co^{58}-B_{12} added (mμg/ml.)	*Per cent bound*	*Amount bound (mμg/ml.)*
Normal		
1.0	65.8	0.658
10.0	19.8	1.98
100	10.09	10.09
C. M. L.		
10.0	51.7	5.17
100	23.2	23.2

Concentration (Table I). Although the percentage of radioactive vitamin B_{12} bound to plasma decreases as the concentration added prior to incubation increases, the absolute amount bound increases. This applies both to normal subjects and to patients with chronic myeloid leukemia. At the 1 mμg per milliliter level, the chronic myeloid leukemic plasma shown in Table I bound all detectable radioactivity. The results of a series of binding experiments on myeloid leukemic plasma (Table II) confirm previous workers' observations concerning increased levels of binding.

Temperature during incubation. No significant difference was observed between two samples of normal plasma incubated at room temperature (15° C.,

2.80 mμg per milliliter) and 37° C. (2.67 mμg per milliliter), following dialysis for 99 hours against 0.01 Tris phosphate buffer, pH 6.5 at 4° C.

Duration of incubation (Table III). At room temperature, the amount of radioactive vitamin B_{12} bound increases with the duration of incubation, the increase between 2 hours and 24 hours being 47 per cent on the 2 hour figure.

Table II. Binding capacity of myeloid leukemic plasma

	Number	*Range (mμg/ml.)*	*Mean (mμg/ml.)*	*Normal range (mμg/ml.)*
Binding at 10 mμg/ml.	12*	3.03- 8.60	5.25	1.07- 3.14
Binding at 100 mμg/ml.	5†	15.6 -34.6	21.9	10.07-14.15

*Includes 3 patients with acute myeloid leukemia.
†Includes 2 patients with acute myeloid leukemia.

Table III. Effect of duration of incubation on Co^{58}-B_{12} binding at 21° C

Duration (Hr.)	Normal plasma	
	Percentage bound	*Amount bound (mμg/ml.)*
2	15.3	2.19
6	18.2	2.86
24	20.5	3.22

Table IV. Effect of duration of dialysis on binding of Co^{58}-B_{12}

Plasma	*Duration (Hr.)*	*Per cent of total radioactivity removed in 48 hr.*	*Per cent of total radioactivity removed after 48 hr.*
Normal	96	78.2	3.5
C. M. L.	122	48.0	5.9
Normal	144	50.1	7.1
C. M. L.	144	38.2	11.5

Table V. Effect of prolonged dialysis on binding of Co^{58}-B_{12} (normal plasma)

Wash (No.)	*Duration (Hr.)*	*Total time elapsed (Hr.)*	*Per cent of total radioactivity removed*	*Per cent of radioactivity removed by dialysis*
1	18	18	47.4	61.2
2	25	43	17.4	22.5
3	21	64	6.7	8.7
4	48	112	4.3	5.6
5	28	140	1.2	1.6
6	22	162	0.4	0.5

Duration of dialysis (Tables IV and V). Table IV sets out 4 randomly selected dialysis experiments, two from normal subjects and two from patients with myeloid leukemia. These results are typical of the whole series. The radioactivity removed after 48 hours of dialysis ranges from 3.5 per cent to 11.5 per cent of the total initial radioactivity, representing 4.3 per cent to 23.1 per cent of the radioactivity removed by dialysis. Table V shows the radioactivity removed at varying periods of a 162 hour dialysis, and again it is seen that significant radioactivity is removed after the first 48 hours.

From the results of this and other experiments, it is concluded that a 96 hour dialysis will remove 95 per cent or more of the dialyzable vitamin present.

Temperature during dialysis. No difference was observed between the two samples which were dialyzed at differing temperatures. The aliquot of normal labeled plasma dialyzed at 4° C. bound 2.75 mμg per milliliter of the radioactive vitamin B_{12}, while the aliquot dialyzed at 20° C. bound 2.80 mμg per milliliter.

Volume of dialysis buffer. The amount of radioactivity removed from an aliquot of labeled plasma dialyzed in a 250 ml. beaker (64.6 per cent) was not significantly different from the amount removed by dialysis of an identical aliquot for the same time (72 hours) against tap water running at approximately 1 L. per minute (64.0 per cent).

Table VI. Effect of differing buffers on binding of Co^{58}-B_{12}

	C. M. L. plasma		
Buffer	*Normal saline (A)*	*Tris phosphate buffer pH 6.5 (B)*	*Tap water (C)*
Per cent of radioactivity in dialysant	70.1	44.8	50.2
Per cent of radioactivity in precipitate	1.2	18.3	17.7
Per cent of radioactivity bound to sacking	3.1	5.8	4.4
Total radioactivity	74.4	68.9	72.3

Effect of differing buffers (Table VI). The results of this experiment show that more radioactivity is bound to plasma when dialysis is performed against normal saline than when it is done against Tris phosphate buffer or tap water. It is also apparent that the precipitate formed during dialysis against Tris phosphate buffer and tap water contains more radioactivity than does the smaller, almost negligible precipitate which forms during dialysis against normal saline. That this radioactivity is bound to the precipitated protein and is not due to trapped plasma was shown in the following way: The precipitates B and C, similar in volume and radioactivity, were broken up and resuspended, precipitate B in normal saline, and precipitate C in tap water. After further centrifugation, precipitate B almost completely redissolved, and most of the radioactivity was in the supernatant. In precipitate C, taken up with tap

water, more than 90 per cent of the initial radioactivity was present in the second precipitate. Totaling the radioactivity of the dialyzed plasma, the precipitate and the sac in each instance, a similar figure for total binding was obtained in these three experiments.

Starch gel electrophoresis of the redissolved precipitate B showed that it consisted mostly of gamma globulin, with some beta globulin present.

Discussion

In the literature on binding of radioactive vitamin B_{12} to plasma proteins, the technical procedures used have varied greatly, and this present study was planned to evaluate the effects of some of the variants.

Differences between serum and plasma. Certain authors[4, 5, 9, 10] have used serum, and others[6, 11, 12] have used plasma, with some overlap and variation within the various studies. No difference has been shown between serum and plasma binding of radioactive vitamin B_{12} in this present study.

Concentration effects. It has been shown on a number of occasions[4, 6] as well as in the present study that the amount of radioactive vitamin B_{12} bound to plasma is related to the concentration of the radiovitamin added prior to incubation. As the concentration of the radiovitamin increases, the amount bound increases, although the percentage bound is less. This increase in binding does not bear a linear relationship to the increase in concentration.[4] When dialyzed plasma is applied to a DEAE column, there does not appear to be any difference in the distribution of the bound radiovitamin between the protein fractions in the low concentration (10 mμg per milliliter) and high concentration (100 mμg per milliliter) experiments,[8] although differences have been demonstrated in distribution of bound radioactive vitamin B_{12} at different concentrations by precipitation techniques.[6]

The recognized increase in binding of radioactive vitamin B_{12} to plasma seen in chronic myeloid leukemia[4, 5] has again been confirmed in this study. The increase in binding related to increased concentration is also apparent in plasma from leukemic subjects.

Temperature during incubation. Varying temperatures of incubation, ranging from room temperature[4, 5, 9] to 30° C.,[12] have been used by other authors. No significant difference has been observed in this study between the binding of radioactive vitamin B_{12} by plasma with incubation at 15° C. and 37° C.

Duration of incubation. The duration of incubation has been shown to affect the amount of radioactive vitamin B_{12} bound to the plasma proteins and is an important factor affecting the comparison of results from different series. The exact nature of the increased binding has not been determined. It may represent increased binding in the same proportions as the binding following shorter periods of incubation, or there could be a differing distribution throughout the plasma fractions.

Duration of dialysis. It has been shown that the duration of dialysis is a significant factor in determining the value reported for plasma binding of radioactive vitamin B_{12}, since radiovitamin in significant quantities is removed by dialysis up to 96 hours. Other authors have chosen differing dialysis periods

from 6[9] to 72 hours,[6] and some authors use differing incubation times in different studies.[5, 11] Comparison of these results from one paper to another becomes difficult, if not impossible, when it is realized that the samples dialyzed for shorter periods will have reported higher binding capacities than similar samples dialyzed for longer periods. It is also apparent from this study that dialysis for a short period does not necessarily remove a proportional amount of the total dialyzable vitamin in a designated period of time. Thus, unless adequate dialysis (i.e., at least 96 hours) has been performed, no two sets of results are strictly comparable.

The precipitate. Although it has been reported that no difference exists between dialysis against phosphate buffer and dialysis against water, dextran, barbital, and saline,[5] the present study has shown that the precipitate which forms in dialysis against Tris phosphate buffer and tap water contains significant amounts of bound radioactivity. Some authors have noted the presence of a precipitate in their work,[9] while others do not mention it.[10] It is felt that the precipitate probably does not represent denaturation of the binding protein, but the precipitation of high molecular weight protein molecules (β-and γ-globulins) during dialysis against a solution which is not isotonic.

In another paper,[14] the bound radioactivity is counted by dissolving the dialysis sac and its contents in concentrated sulfuric acid. The result reported as the amount bound to plasma in these experiments will include the amount bound to the precipitate, as well as the amount bound to the sac, and the reading will therefore be high in comparison with other studies.

A suggested standard system for studying the in vitro binding of radioactive vitamin B_{12} to plasma would therefore involve the dialysis (for a minimum of 96 hours) of plasma, incubated with radioactive vitamin B_{12} at 37° C. for 1 hour, against a selected buffer. At the completion of dialysis, the radioactivity present in dialysant, precipitate and sac, together with the undialyzed standard, would then be counted, and the amount of binding calculated as a percentage and as an absolute amount.

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