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STUDIES ON THE ISOLATION AND PROPERTIES OF HUMAN AND HOG INTRINSIC FACTOR

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Ph.D THESIS

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ABBREVIATIONS

I.F.	-	Intrinsic Factor
B_{12}	-	Vitamin B_{12}, where the form is not being specified.
[^{57}Co] cyanocobalamin	-	cyanocobalamin radioactively labelled with the isotope ^{57}Cobalt.
[^{125}I] I.F.	-	intrinsic factor labelled with the isotope ^{125}Iodine
I.F.-B_{12}	-	the complex of vitamin B_{12} bound to intrinsic factor.
I.F.-[^{57}Co] cyanocobalamin	-	the complex of cyanocobalamin labelled with [^{57}Co] bound to intrinsic factor
Ab	-	Antibody

SUMMARY

The main aim of this research work was to isolate pure preparations of both human and hog Intrinsic Factor and examine some of their physico-chemical and immunological properties.

The method chosen to isolate pure I.F. was affinity chromatography, in particular the method used by Allen and co-workers in America. Their method was studied and modified, mainly by the use of lower molarity guanidine-HCl to elute the affinity chromatography column, thus providing a method of separating I.F. from non-I.F. binders.

The results gained in the present work by amino acid analysis and gel filtration were similar to those gained by Allen except that the carbohydrate composition of the hog I.F. did not contain any galactosomine and there was no appearance of any dimerised or oligomerised form of I.F. The molecular weight for both human and hog I.F. was approximately 60,000 and the other physico-chemical properties were similar for both preparations.

The two were not identical immunologically, since the Type II antibody, which was raised to human I.F, would not react with hog I.F.

Both human and hog I.F. preparations were successfully labelled with [^{125}I] using the Chloramine T method and this preparation was used to compare the properties of I.F. with and without the addition of B_{12}.

Methods for possible elucidation of I.F. mediated B_{12} absorption from the intestine are suggested using a peroxidase linked to specific I.F. antibody staining technique and also a specific radioimmunoassay for very small quantities of I.F.

A routine clinical radioassay for B_{12} is also described.

INTRODUCTION

The initial project undertaken during the course of study was an examination of methods of measuring vitamin B_{12} (B_{12}) in biological material by radioassay.

Measurements of B_{12} for clinical use by microbiological assay were introduced by Ross in 1952 (1) and have proved to be of value in clinical and academic practice. In 1961, Barakat and Ekins introduced a radioassay (2) which appeared to have some assets over microbiological techniques especially in the shorter amount of time required. Although over ten years had elapsed since this first description of a radioassay method, it is clear from the multiplicity of techniques which have emerged and from the comparative studies of radioassay and microbiological results (3 - 15) that no single method has gained uniform acceptance. The general principles of all radioassay methods for estimating B_{12} are essentially the same -

1. Extraction of B_{12} in the test sample from its binding proteins.

2. Addition of a substance with a finite, reproducable B_{12} binding capacity for use in the competition between the added hot and endogenous cold B_{12}.

3. Separation of free from bound B_{12}.

From a study of the various methods employed in radioassay work it was obvious that the choice of a B_{12} binder was a central issue. With this in mind, a project was initiated to look at possible B_{12} binders and develop a reliable radioimmuno-assay for B_{12}. The possible sources of binder studied were commercial hog intrinsic factor (I.F.), human serum, rabbit serum, chicken serum and charcoal granules encapsulated in dialysis membrane. It was found that chick serum contained a great deal of B_{12} binding protein and an assay system was developed using dilute chick serum with albumin coated charcoal for separation of free and bound B_{12}. This assay was being compared to the existing microbiological assay in use in the Southern General Hospital, Glasgow and a commercial radioassay kit when a paper was published (9) describing an essentially similar assay. At much the same time a series of papers appeared on the isolation of B_{12} binders by the relatively new technique of affinity chromatography by Allen and co-workers in America (16-20). These papers appeared to provide a relatively simple method of obtaining a pure B_{12} binder.

It was therefore, decided to abandon further study of the radioassay and to concentrate on the isolation of pure B_{12} binders.

While this alteration of course limited, to a certain extent, the amount of time available for study, it was felt that work on the isolation of pure B_{12} binders would be of value not only to confirm the original work of Allen, but also to examine the properties of pure vitamin B_{12} binders in vivo and in vitro with a view to

resolving conflicting results found by other workers using impure or partially purified preparations.

The term 'binder' is used in the literature on B_{12} to denote substances which complex with B_{12}. The specific binders of B_{12} can be divided into two groups, the so called R-binders (the R being derived from the more Rapid electrophoretic mobility of these materials) which do not appear to have a clearly defined biological function and the specific binders whose role has been discovered. One of the specific binders has the somewhat intriguing name of "Intrinsic Factor" (I.F.) which has a role in the absorption of B_{12} in the intestine and the others are classified as the Transcobalamins [TCI, TCII, & TCIII] which have a role in the intravascular transport of B_{12} (21).

The transcobalamins are present in blood in such very small quantities that it was not possible to obtain sufficient serum to make their isolation in sufficient quantity to study their properties a practical possibility. It was, therefore, decided to concentrate our effort in isolating I.F. from human gastric juice and from commercial freeze-dried dessicated hog gastric mucosa.

The existance of I.F. was postulated by Castle in 1928 (22). He and several others had observed that beef muscle fed to patients with pernicious anaemia did not induce haematological response unless it was preincubated with normal human gastric juice or given along with normal human gastric juice. The term "intrinsic factor" was

used for the substance in normal human gastric juice which, Castle postulated, combined with an "extrinsic factor" in beef muscle to form a "haemopoetic factor" which was absorbed and alleviated the disease.

When B_{12} was isolated in 1948, almost simultaneously by Lester Smith with Glaxo (23) in Britain and Folkers with Merck (24) in America, it was recognised that B_{12} exhibited the properties of "extrinsic factor". It was observed, however, that B_{12} given parenterally could induce a haematological remission in pernicious anaemia. This fact, together with the demonstration that radio-active cyanocobalamin could not be absorbed by patients with pernicious anaemia except when given with a source of I.F. led to the modification of Castle's theory that the function of I.F. was to mediate the absorption of B_{12}. Vitamin B_{12} appears to be the only dietary requirement among animals which is solely produced by microorganism e.g. streptomyces griseus, which may explain why a unique mechanism has evolved for its absorption requiring I.F.

Work on the isolation of I.F. and its structure has proceeded only slowly for a variety of reasons. Initially the only method of measuring I.F. activity was by biological effect as judged by a reticylocyte count in patients with pernicious anaemia in relapse and it was not until 1965 that Gottlieb et al (25) introduced a type of radio assay method for the detection and quantification of I.F.

While certain properties of I.F., notably its thermal lability, had been observed by Castle (26, 27), the progress in the characterisation and isolation of I.F. was disappointingly slow for many years, this being largely related to the technical methods available.

In 1952, Glass and his group isolated a glycoprotein fraction from human gastric juice (28) which exhibited the properties of I.F. but required daily dosage of 100 mg of their fraction plus 20 μg B_{12} to produce a haematopoeitic response. With the advent of techniques such as continuous electrophoresis, ion exchange chromatography, gel filtration, sequential ammonium sulphate precipitation and ultra-centrifugation, progress became more rapid. However, most of the purification procedures described are tedious and contain many stages e.g. the seven-stage purification achieved by Grasbeck et al in 1966 (29) used a starting pool of 40 litres of gastric juice from 276 patients but only recovered about 20% of the original I.F. activity, and some of this [complex 1] was thought to be partly degraded.

This and the other work was reviewed by Glass in 1963 in his substantial monograph (30) which contained over 1400 references. This review was updated in 1974 in a volume which gives a further 700 references (31), thus giving some indication of the volume of work done on this substance.

Until recently, probably the most pure preparations isolated, apart from Grasbeck's, would be those of Chosy and Schilling (32)

using ion exchange chromatography, gel filtration and ultrafiltration on human gastric juice and Ellenbogen and Highley (33) on hog pyloric mucosa. They used a multi-step procedure which included ammonium sulphate precipitation ion exchange chromatography, proteolytic digestion of the products and further column chromatography. Both these preparations were biologically active at doses of less than 0.5mg.

In 1970, Grasbeck and co-workers suggested that B_{12} analogues could be co-valently bound to cellulose to yield an adsorbent for B_{12}-binding proteins (34) and in 1971 a paper was published by Olesen et al (35) in which described a method for coupling a hydroxocobalamin-albumin conjugate to bromoacetyl cellulose.

What appeared to be a dramatic advance came in 1972 with the publications of Allen et al (16 - 18) describing the isolation of I.F. by another modification of this technique of Affinity Chromatography. This group used the monocarboxylic derivative of cyanocobalamin bound to 3, 3' diamino-dipropylamine substituted Sepharose-4B via a 1-ethyl 3-(3 dimethylaminopropyl)-carbodiimide bridge as their affinity adsorbent. The vitamin B_{12} binding proteins were eluted from the column by partially denaturating them with 7.5M guanidine-HCl and then renaturating them by dialysing off the guanidine against water.

The particular attractions of this technique are that it has relatively few steps and the yields of apparently pure I.F. are higher than with previous methods.

In essence, the major part of this thesis is a report of a study of the isolation of human and hog intrinsic factor using the above method of affinity chromatography. Also the pure proteins isolated have been studied with a view to characterising some of their physical, chemical and immunological properties. This work is, by necessity, of a preliminary nature, but some possible uses of pure I.F. have been explored and it is hoped will form a basis for further work.

CHAPTER I

RADIO-ASSAY FOR VITAMIN B_{12}

INTRODUCTION

The assay described here is a simplified, reproducible and economic isotope dilution assay for B_{12} quantification in biological material arrived after testing various methods of releasing B_{12} from its natural binding proteins, possible B_{12} binders for use in the competition between labelled and unlabelled B_{12} and methods of separating free from bound B_{12}.

The final assay developed is described here because a substantial amount of time at the beginning of the research period was expended on it and also because it was used during subsequent research as a method for determining B_{12} levels in various preparations. However, since this assay is not being presented as the major outcome of the research, the results of the work on possible methods and materials for the assay, which were later discarded as less suitable than those employed finally, are not presented here.

Chick serum was finally chosen as the binder in this assay because of certain properties it was found to possess. It had a very high unbound binding capacity for B_{12} which meant it could be used highly diluted. Its binding capacity was linear over the range of physiological B_{12} values and was more stable than other binders to environment, e.g. pH, ionic strength, presence of extraneous

material and storage conditions. Chick serum binding has been found to be associated with a single binder (36) which may account for the linearity of its binding properties. Comparison of chick serum to some other B_{12}-binding agents has also been described by Newmark et al in 1973 (8).

MATERIALS AND METHODS

High specific activity [^{57}Co] cyanocobalamin (approx. 150 μCi/μg) supplied from Radiochemical Centre, Amersham, was diluted with a solution of unlabelled crystalline cyanocobalamin (Glaxo Ltd.) in 0.2M acetate-cyanide buffer pH 4.3 to a concentration of 1 ng/ml. The specific activity was adjusted so that 50 μl of this solution gave approximately 10,000 c.p.m.

Acetate-cyanide buffer was prepared from 700 ml of 0.2M acetic acid, 300 ml of 0.2M sodium acetate, 50 mg potassium cyanide and 29.2g Sodium Chloride. The same buffer was used both for extraction and assay, and it proved to be stable for at least two weeks at 4°C.

Cyanocobalamin standards were prepared from crystalline cyanocobalamin (Glaxo Ltd.) diluted in the dark in acetate cyanide buffer to 1600 pg/ml.

Pooled chick serum was supplied from the Immunology Department Western Infirmary Glasgow. It was diluted 1 in 350 in 0.15M saline solution and frozen in 1 ml aliquots at -20°C, under which conditions

it was stable for at least 6 months.

The albumin coated charcoal used was a suspension of neutralised Norit I activated charcoal (Sigma Chemical Company) in Bovine serum albumin fraction V solution from B.D.H. Ltd. Normally 1g charcoal and 0.2g albumin were mixed together in 40ml distilled water and allowed to incubate at room temperature for at least 30 minutes before use. This suspension was stable for up to 1 week at 4°C.

PREPARATION OF TEST SAMPLE

0.5ml of the unknown test sample was added to 2.5ml acetate-cyanide buffer in a 15cm x 1.5cm pyrex glass test tube fitted with a metal cap. The mixture was boiled for 20 minutes in a water bath at 100°C. It was then shaken to facilitate protein separation and cooled to approximately 25°C quickly in running tap water. The tube was then centrifuged at 3000g for 15 minutes to separate the denatured protein, and 2 x 1ml samples of the clear supernatant removed into duplicate 10cm x 1.5cm glass test tubes.

PREPARATION OF STANDARD CURVE

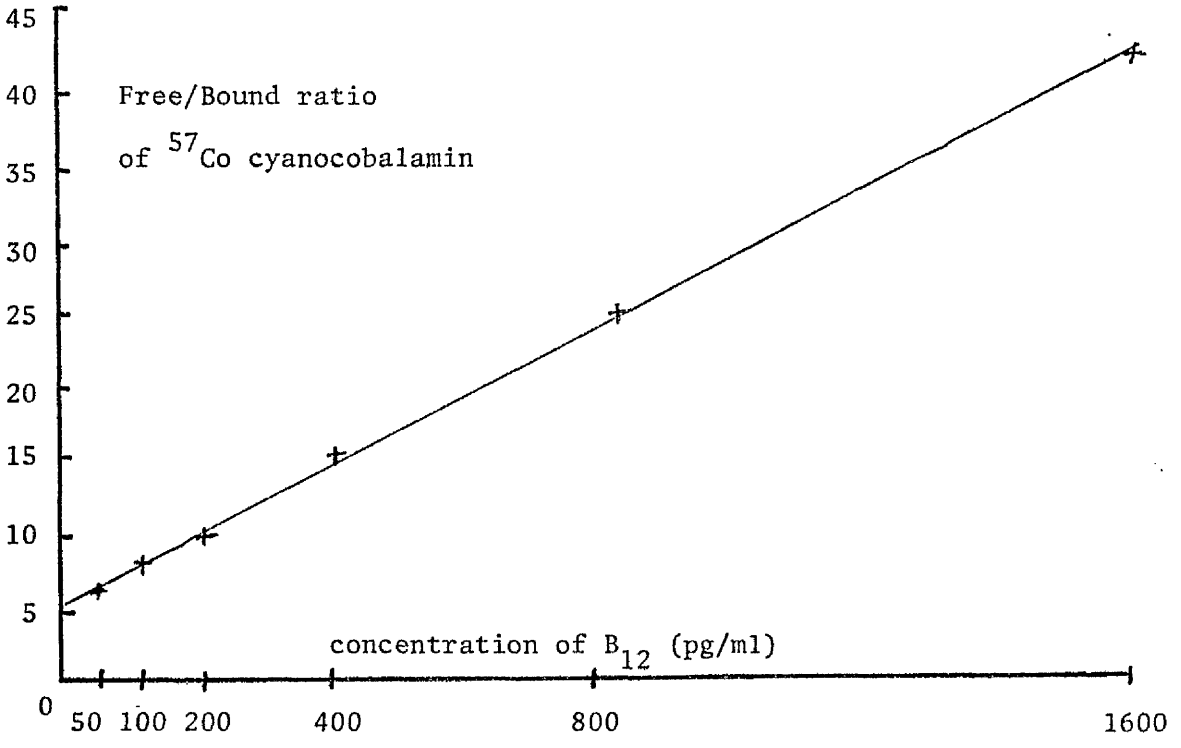
A serial dilution of the previously prepared 1600 pg/ml unlabelled cyanocobalamin was set up using acetate-cyanide buffer

to give seven tubes containing respectively 0.5ml of the concentrations 1600, 800, 400, 200, 100, 50 and 0 pg/ml cyanocobalamin, the last tube containing only 0.5ml acetate cyanide buffer. To each of these tubes was added 2.5ml buffer, they were shaken and 2 x 1ml aliquots from each were transferred into duplicate glass tubes labelled 1600 - 0. These tubes were then treated in the same way as the unknown test samples.

ASSAY PROCEDURE

50µl of [^{57}Co] cyanocobalamin solution previously prepared was added to each of the unknown sample tubes and to the standard curve tubes and mixed. This volume contained 50 pg labelled cyanocobalamin which was the amount found to give the most sensitive results. A suitable quantity of the diluted chick serum was allowed to defreeze at room temperature and 50 µl of this solution was added to each tube. Two background control tubes were also set up, which did not receive any chick binder. The tubes were incubated at room temperature for 15 minutes before adding 1 ml of the charcoal suspension to each tube, mixing and incubating for a further 10 minutes. The tubes were then centrifuged at 3000g for 10 minutes and 1 ml of the clear supernatant removed from each tube into a clean labelled glass test tube and counted for radioactivity in a well type gamma scintillation counter using a J & P Engineering MS310 Counter/Ratemeter. This separation of free from

GRAPH I - Typical standard curve of Free/Bound ratio of [^{57}Co] against a serial dilution of competing unlabelled cyanocobalamin using Chick Serum as binding agent.



(b) Unknown Samples

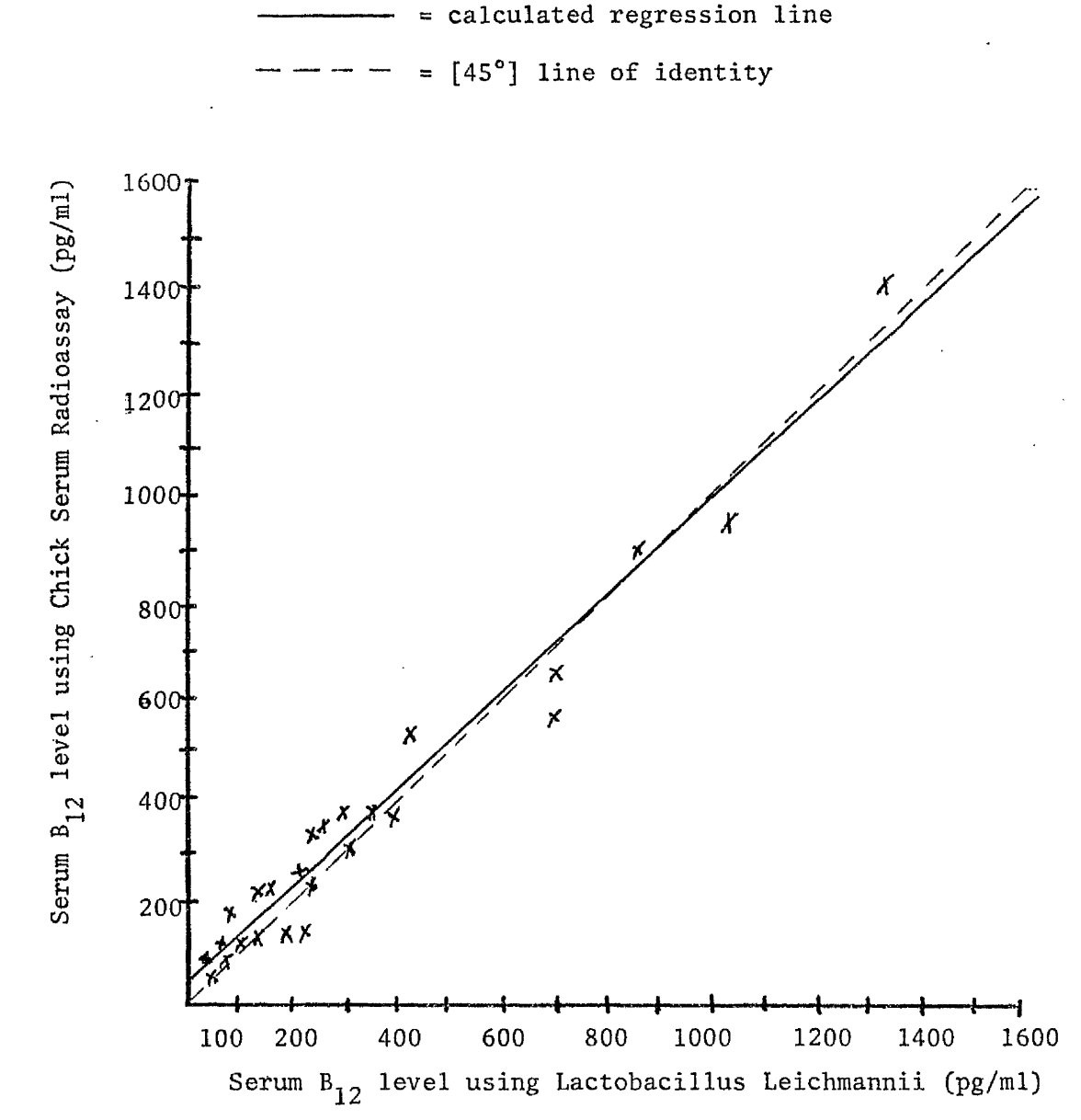
The counts in 1 ml of the supernatants of the unknown samples were treated in the same way and by application of the Free/Bound ratio calculated for each sample to the standard curve, a direct reading of the concentration of B_{12} in the sample in pg/ml was found. Any sample with too high a concentration for this range was suitably diluted in acetate cyanide buffer and assayed again.

COMPARISON OF CHICK SERUM ASSAY WITH MICROBIOLOGICAL ASSAY AND WITH COMMERCIALLY AVAILABLE RADIO-ASSAY KIT.

A number of patient sera, covering the range of physiological B_{12} values were obtained from the Haematology Department, Southern General Hospital, Glasgow, where a sample from them had already been subjected to a microbiological assay using Lactobacillus Leichmannii (37, 38) and a result obtained. Such sera were assayed at various stages of the development of the present assay, as a check on the comparability of results from the two methods. When the assay was in its final form, as described above, it was used to assay a number of sera which were also assayed by the microbiological technique. The results from the chick serum radioassay were not previously known to the people carrying out the microbiological assay and vice versa.

The correlation in a range of 25 samples assayed by both methods as shown in Fig. 1 was very good.

FIG. I - Correlation between the results of serum B_{12} levels obtained by both radioisotope dilution assay using chick serum as binder and microbiological assay using Lactobacillus Leichmannii.



The calculated regression line for the two methods has the formula $y = 0.9627x + 18.3$ with an r value of 0.9506 ($p < 0.01$).

This means, as is also shown in Fig. 1, that the calculated regression line is very close to the [45°] line of identity, thus the normal range of results for both methods is effectively identical (150 - 1400 pg). No untreated Pernicious Anaemia patient tested with chick serum radioassay had a B_{12} level higher than 130 pg/ml and most were considerably lower.

In all, over 100 sera were tested using this chick serum radioassay. The diagnoses for these patients included - pernicious anaemia, folate deficiency, abnormal intestinal absorption, post gastrectomy, diabetic, treated pernicious anaemia. Serum was also tested from healthy volunteers.

Some of the sera were also tested with the Phadebas B_{12} Test Kit supplied by Pharmacia. The calculated regression line for the correlation between these results and the Chick serum radioassay had the formula $y = 0.9806x + 102$, with an r value of 0.9739 ($p < 0.01$). Thus the kit gave a very good correlation with the developed assay but with a higher normal range of values, i.e. above 250 pg B_{12}/ml serum.

REPRODUCABILITY OF CHICK SERUM RADIOASSAY

Samples from the same serum pool were assayed each time an assay was being performed over a period of time. When 20 results

had been obtained they were analysed statistically and found to have a mean value of 257 pg/ml and a standard deviation of $\pm$ 14.7pg/ml. This is a coefficient of variance of 5.7 ($p < 0.05$).

Also from the values found for duplicate samples for over 150 assays the correlation coefficient was found to be 0.9975 ($p < 0.01$).

DISCUSSION

Although in samples which were assayed with and without the centrifugation step to remove denatured protein, there was not found to be any significant difference ($p < 0.05$), it was decided to include the step because it allowed the withdrawal of 2 x 1 ml duplicate samples of clear supernatant which more closely resembled the media of the standard curve tubes. It was also felt that it reduced the risk of any spontaneous partial renaturation of any peptide strands which might have some non-specific B_{12}-binding activity (39). This risk was also lessened by cooling the denatured serum quickly down from 100°C to room temperature using cold running tap water.

Although chick serum does not exhibit its highest possible binding capacity at pH 4.3, this pH was chosen for the following reasons. The assay was substantially simplified, since extraction and assay and standard curve were all carried out in the same buffer. This buffer had sufficient buffering capacity to maintain its pH

during extraction.

Also it was found that at higher pH levels, the results gained from the assay were consistently higher than those gained from the same sample using the microbiological assay. Whereas at pH 4.3 the results were very similar. In the past, most radio-isotope dilution assays have tended to give higher results than microbiological assays (9, 10, 11, 15) and this may be one reason why, although radioassays are much faster to carry out than microbiological assay, they still have failed to be generally used clinically. This may be due to people being unwilling to adjust to a new range of normal and abnormal values.

Thus it was decided to adjust the assay conditions to yield results which were in the same range as the microbiological results as far as possible.

The ionic strength chosen (0.5M) was sufficiently high to minimalise any possible differences between the standard curve samples and the test samples. It was found that results were more reproduceable when cyanide was added to the buffer (13).

The radioassay was much more sensitive than the microbiological assay, and in assays carried out with known concentrations of cyanocobalamin it could distinguish 10 pg/ml differences in concentrations and could also be used at concentrations below 50 pg cyanocobalamin/ml.

Although only a few sera were assayed using the Phadebas Kit, it was found to give satisfactory results which were comparable to those found by Raven & Robson (11) and Frenkel et al (12) with the same kit. However, the B_{12} binder used in this assay, which is an impure I.F. preparation bound to Sephadex (40,41) was found to be very delicate and could easily be destroyed e.g. by overvigorous mixing. The standard curve obtained for this assay was not linear, which was perhaps a function of the non-homogeneity of the binder, and the treatment of results was more complicated.

Also this assay suffered, to a certain extent, from the flaw of other radioassays mentioned above, in that the range of results gained was higher than that generally accepted, gained from microbiological assays.

It was also calculated that if both the chick serum radioassay and the commercial radioassay were in general use in a clinical situation, it would cost approximately 200 times more to assay one sample by using the commercial kit.

Finally, sufficient assays on sera from patients with any one specific category of illness were not performed. Therefore, it was not possible to ascertain whether this assay gave anomalous results with any specific group.

CHAPTER II

THE ISOLATION OF HOG AND HUMAN INTRINSIC FACTOR USING AFFINITY CHROMATOGRAPHY

INTRODUCTION

The technique of affinity chromatography for proteins depends on the unique properties of a given protein to bind specifically to given ligands and for this binding to be reversible under different conditions. The method requires that the ligand can be covalently bound to some insoluble matrix over which the biological fluid which contains the protein of interest can be passed. This protein should bind to the "affinity adsorbent" while all the other miscellaneous biological material can be washed through. Then, when only the protein of interest is left behind, the physico-chemical conditions are changed in such a way as to cause the release of this protein from its ligand and thus it can be collected in a pure form.

The technique is very appealing for its apparent simplicity and its use of the inherent biological properties of a given material to secure its own purification without subjecting it to many mechanical separation procedures.

Much of the pioneer work on affinity chromatography for proteins was done by Cuatrecasas and his co-workers (42) in the late 1960's.

He recognised that cross-linked dextran (Sephadex) exhibited properties which would make it an efficient insoluble matrix on to which the biological ligand could be bound i.e. it was stable mechanically and chemically, had good flow properties, was free from non-specific adsorption effects with proteins and formed gel particles of uniform spherical size. He also saw that for maximum biological interaction between ligand and substrate, the ligand must be far enough away from the support backbone to prevent steric hindrance.

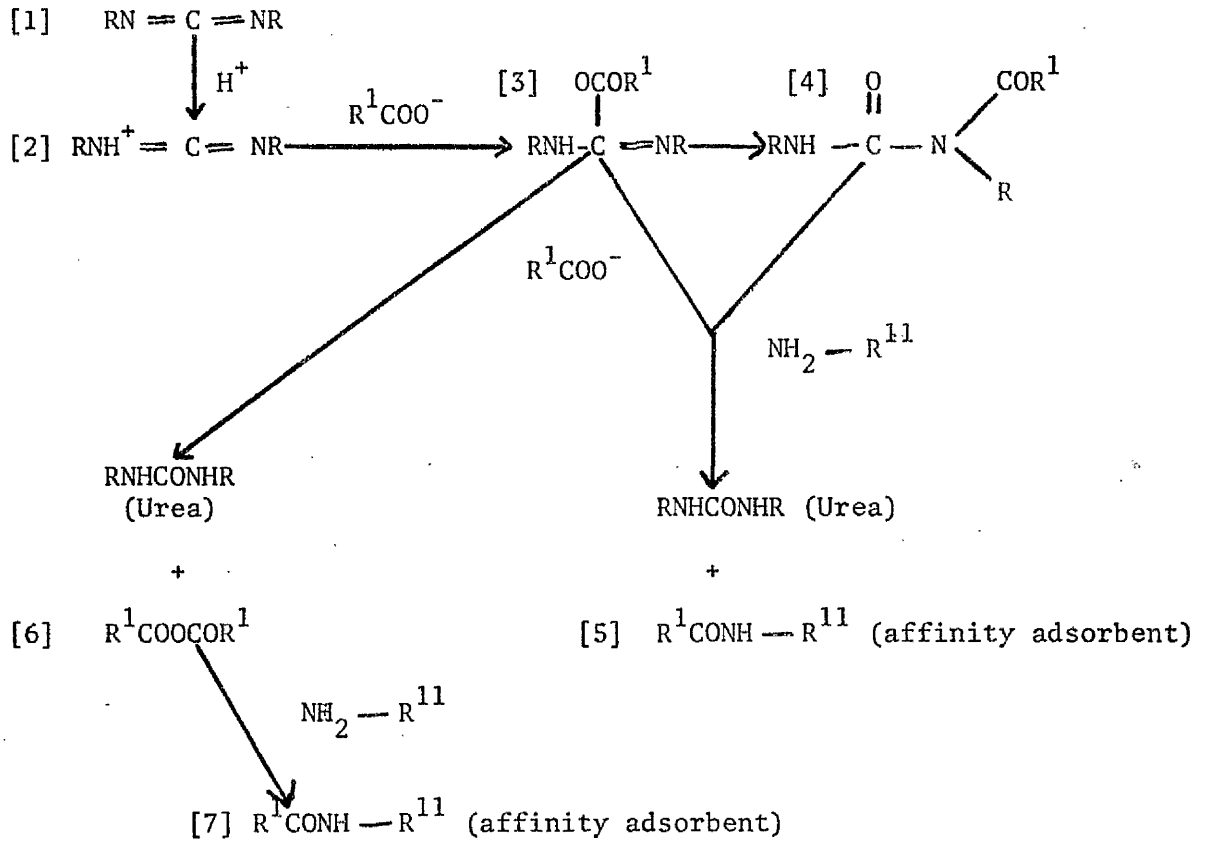
Thus he developed several methods for either attaching a long hydrocarbon chain or "bridge" to the ligand before attachment to the insoluble matrix or else attaching the "bridge" to the matrix before bonding to the ligand.

In the case of I.F., the ligand which has to be bound to the insoluble matrix is cyanocobalamin, or in fact a monocarboxylic acid derivative of cyanocobalamin. It is bound via a 3, 3' diamino-dipropylamine bridge to cyanogen bromide activated Sepharose 4B by an amide bond formed in the presence of a carbodiimide, according to the method of Allen et al (16). This method of preparing the affinity adsorbent was followed almost exactly except Spermidine was substituted as the bridging chain when 3, 3' diamino-dipropylamine was not available and commercially prepared cyanogen bromide activated Sepharose 4B (Pharmacia) was used so as to allow the emission of the activation step. The exact product of this activation is not known but it has been hypothesised (43, 44) that the cyanogen bromide

reacts with the hydroxyl groups of Sepharose to form cyclic and acyclic imidocarbonates which are then available to react with the amino groups of the ligand or bridging chain covalently.

In the method used here the 3, 3' diamino-dipropylamine is first attached to the CNBr-activated Sepharose and then the mono-carboxylic acid derivative of cyanocobalamin is joined to the bridging chains. The reaction pathway for the amide bond formation is shown in Fig. 2.

FIG. 2 - Brief Pathway of amide bond formation between mono-carboxylic acid cyanocobalamin and the 3, 3' diaminodipropylamine substituted Sepharose.



RN = C = NR is $CH_3 - CH_2 - N = C = N - (CH_2)_3 - N^+(CH_3)_2 - HCl$

[1] = 1 — ethyl — 3 (3 dimethylaminopropyl) carbodiimide — HCl

R^1 = monobasic cyanocobalamin

$R^{11} = -(CH_2)_4\ NH(CH_2)_4\ NH-$ Schematic Sepharose

3, 3' diaminodipropylamine bridging group.

MECHANISM

The initial step in the formation of the amide bond between the monobasic cyanocobalamin and the bridging chain bound to the Sepharose is the protonation of the 1-ethyl-3-(3 dimethylamino-propyl) carbodiimide-HCl [1] to yield [2]. This intermediate product is attacked by the cyanocobalamin monocarboxylic anion to produce the O-acylurea [3] which then rearranges intramolecularly to form the N-acylurea [4]. [3] and [4] react with the terminal amino group of the 3, 3'-diamino-dipropylamine chain attached to the Sepharose to form an amide bond with the substituted Sepharose [5] plus urea. [3] can then react with another cyanocobalamin monocarboxylic anion to yield urea plus the acid anlydride [6] which can then react with more terminal amino groups of the bridging chain to form more amide bonds and thus the cyanocobalamin is covalently bound, via the hydrocarbon bridge, to the inert support matrix.

In effect, after the hydrolysis forming the amide bond, native cyanocobalamin was formed covalently linked through an eight carbon chain to the activated Sepharose.

In the isolation of I.F. using this affinity adsorbent, there were some modifications made to Allen's method. These will be discussed later.

The major disadvantage of using affinity chromatography to isolate I.F. is that the biological fluids which contain I.F. also contain B_{12}-binding proteins which do not exhibit I.F. activity, (non-I.F. or R-binding proteins). In the isolation of human I.F. from gastric juice, particular care was taken to select for processing only samples which had $>$ 85% I.F. activity in their B_{12} binding. However, since commercial freeze dried hog gastric mucosa, which was the source of hog I.F. , contained $>$ 60% non-I.F. binders, some other means of separating I.F. from non-I.F. binders was necessary. Again there was introduced a modification to the Allen technique, which in fact proved to be a simplification. This too, will be discussed later.

SECTION I - PREPARATION OF AFFINITY LIGAND AND ISOLATION OF HUMAN AND HOG I.F.

MATERIALS AND METHODS

Crystalline Cyanocobalamin	was obtained from	Glaxo Limited
Cyanogen-Bromide activated Sepharose 4B	"	Pharmacia Limited Uppsala, Sweden
Q.A.E. Sephadex A-25	"	" " "
Sephadex G100	"	" " "
Sephadex G200	"	" " "
3, 3' Diaminodipropylamine	"	Eastman-Kodak Ltd. Rochester, New York
1-Ethyl-3-(3 dimethylaminopropyl)carbodiimide - HCl	from	Phase Separations Ltd.
[^{57}Co] vitamin B_{12} (specific activity approx. 150 uCi/ug)	"	Radiochemical Centre, Amersham.
Biorex AG1 - X8 (acetate form)	"	Biorad Ltd.
Guanidine Hydrochloride - Ultra Pure	"	Mann Research Laboratories Orangeburg, New York.,
	"	B.D.H. Ltd. and
	"	Serva, Heidelberg.

Human Gastric Juice was obtained from		Gastro-Intestinal Centre, Southern General Hospital, Glasgow. Surgical Unit Glasgow Royal Infirmary Western General Hospital, Edinburgh. University Dept. of Surgery, Royal Infirmary, Manchester. University Dept. of Surgery Sheffield.
Bovine Serum Albumin, Fraction V	from	B.D.H. Ltd.
All materials for buffers	"	" "
Activated Neutralised Charcoal	"	Sigma Chemical Company St. Louis
Freeze dried Hog Intrinsic Factor concentrate Batches WC2650 & TC2750	"	Armour Pharmaceuticals Ltd.
Folin and Ciocalteus Phenol reagent	from	B.D.H. Ltd.

MEASUREMENT OF RADIOACTIVITY

Unless otherwise stated, activities were measured in a well-type scintillation counter I.D.L Type 663 with a thallium activated sodium iodide crystal 5.5 cm diameter and 6.9 cm deep shielded by 10 cm lead.

The crystal was connected to a J & P Engineering MS 310 Counter/Ratemeter.

MEASUREMENT OF pH

The pH level of buffers etc. was measured using an E.I.L. Model 23A direct reading pH meter with a glass electrode.

MEASUREMENT OF TOTAL PROTEIN CONCENTRATIONS

All total protein concentrations were done by a modification of the Lowry method (45) using Folin and Ciocalteus phenol reagent and reading the absorbance of the samples at 540nm against a blank on a Unicam SP600 spectrophotometer. A standard curve was set up using bovine serum albumin.

PREPARATION OF MONOCARBOXYLIC ACID DERIVATIVE OF CYANOCOBALAMIN

Five grams of crystalline cyanocobalamin and 1 μCi [^{57}Co], 0.0067 μg vitamin B_{12} were added to 500 mls of 0.4N HCl and stirred at room temperature in the dark for approximately 3 days, to remove ammonia from the propionamide side chains on the corrin ring and yield the various mono-, di- and tribasic acid derivatives.

The products of this mild acid hydrolysis were applied to a column (60cm x 2.5cm) of Bio-Re x AG1-x8 (acetate form) 100-200 mesh, which had been previously washed with 2 litres of 0.1M Sodium Acetate followed by 4 litres of distilled water. The column was eluted with 0.025M acetic acid and collection of eluant commenced when the red material reached the bottom. The bulk of the red material was recovered in a volume of 800 mls and this proved to contain 95% of the added hydrolysate, as judged by radioactivity. A sample of the eluant failed to cause any precipitation of silver nitrate, showing that all free chloride ions had been removed. The eluant was then taken to dryness in a rotary evaporator at 60°C, redissolved in 300 mls distilled water and the operation repeated. Finally the red material was redissolved again in 300 mls distilled water and 50 mls pyridine added. The pH of the solution was adjusted to 9 with concentrated NH_4 OH and then subjected to ion exchange chromatography on a column (60 cm x 2.5 cm) of QAE-Sephadex A-25 (chloride form), previously washed with 2 litres of 0.2M pyridine, at room temperature. The hydrolysate was applied to the top of the column and washed in with 600 mls of 0.4M pyridine.

The column was then eluted with a linear acetate gradient at a flow rate of 50 mls per hour, the mixing chamber containing 1 litre of 0.4M pyridine, and the reservoir 1 litre of 0.4M pyridine plus 0.32M acetic acid. The column was further eluted with 1 litre of 0.4M pyridine plus 0.32M acetic acid.

The elution could be followed visually since the red material separated into well defined bands. Each major band was collected separately and evaporated to dryness in a rotary evaporator at 40°C. Each was redissolved twice in 100 mls distilled water and re-evaporated at 50°C and 60°C consecutively. Each was finally redissolved in as small a volume of distilled water as possible (a few ml.), transferred to small beakers of known weight, and evaporated to dryness in a dessicator under vacuum at room temperature.

Ascending paper chromatography of each of the products was carried out on Whatman 3mm Chromatography paper using N-Butyl alcohol + Propan-2-ol + Water, 2 : 2 : 1 and 0.04% w/v Potassium Cyanide, as solvent. It was allowed to run in the dark for 24 hours at room temperature then dried. Crystalline cyanocobalamin and crystalline monocarboxylic acid derivative obtained from Glaxo Ltd. were used as standards. The monocarboxylic B_{12} (100 mg) was a gift from Dr. Bilkus, Glaxo Research Laboratories.

PREPARATION OF THE AFFINITY ADSORBENT

Ten grams of Cn-Br activated Sepharose-4B were swollen and washed in 1 litre 0.01M HCl, pH 2-3. The Sepharose was then washed in a Büchner sinta-glass funnel with 1 litre 0.1M Na HCO_3, pH-10 under vacuum at room temperature, transferred to a flask, allowed to settle and the excess supernatant removed. Thirty ml. of 2M 3, 3' diaminpdipropylamine - HCl, pH 10 were added to the flask and the mixture stirred continuously with a paddle stirrer for 18 hours at room temperature. The substituted Sepharose was poured back into a Büchner funnel and washed successively under vacuum with 200 ml distilled water, 500 ml 0.025M glycine - NaOH, pH-10 containing 0.1M NaCl and 2 litres of water. After this the Sepharose was re-transferred to a flask, allowed to settle and the supernatant removed. To this was added 0.5g of Mono-carboxylic acid derivative of vitamin B_{12} dissolved in 35 mls distilled water and the pH raised to 5.6 with 0.2M NaOH. The mixture was gently stirred continuously with a paddle stirrer and 150 mg 1-ethyl-3-(3 diethylaminopropyl) carbodiimide dissolved in 3 ml water added slowly in 0.5 ml aliquots. After stirring continuously at room temperature in the dark for a further 18 hours, the affinity adsorbent was finally re-transferred to a Büchner funnel and washed successively with 300 ml water, 600 ml 0.1M Glycine-NaOH pH-10 (thus blocking off any bridging chains not filled with vitamin B_{12}), 300 ml water and 300 ml 0.1M phosphate buffer pH-7.5. An average of 10% of the monocarboxylic acid B_{12} added to the Sepharose becomes

incorporated, giving it a red colour. This amount of affinity adsorbent was enough to pour two columns and each column could be used approximately six times. The adsorbent could be stored in o.1M phosphate buffer pH 7.5 at 4°C (foiled to keep out any light) for several months with only slight release of vitamin B_{12}. Sodium azide 0.02% w/v was used during storage to prevent bacterial growth.

PREPARATION OF ALBUMIN COATED CHARCOAL

One gram of neutralised activated charcoal was mixed with 0.2g Bovine serum albumin fraction V and 40 ml distilled water in a flask. The suspension was mixed with a magnetic stirring bar slowly at room temperature for at least 30 minutes before use, and could be stored at 4°C for up to 7 days without impairment of function. After this time it deteriorated and could absorb protein bound B_{12} as well as free B_{12}, rendering it useless as a separation technique for these two. While being pipetted out during use, the suspension was continuously stirred.

ASSAY OF INTRINSIC FACTOR - IN VITRO

The "in vitro" assay of I.F. binding activity of any sample was done using albumin coated charcoal to separate free from bound vitamin B_{12}. A duplicate series of glass test tubes was set up

at room temperature and numbered as shown in Table I. This method was a modification of the technique of Gottlieb et al (25)

TABLE I - Protocol for "in vitro" assay of I.F. activity

	Tube Number	Unknown Test Material (ml)	Human I.F.Ab Serum (ml)	0.1M phosphate buffer pH 7.5 (ml)	[^{57}Co] vit.B12 7.5 ng/ml (ml)	Albumin coated Charcoal (ml)
Total Binding Capacity of Unknown	1 & 1'	0.1	-	0.1	1.0	0.8
Binding Capacity of Ab.Serum	2 & 2'	-	0.1	0.1	1.0	0.8
Non I.F Binding capacity of Unknown	3 & 3'	0.1	0.1	-	1.0	0.8
Background Control	4 & 4'	-	-	0.2	1.0	0.8
Standard	5 & 5'	-	-	1.0	1.0	-

The relative amounts of Unknown sample, antibody serum and buffer were added to each tube as shown. They were mixed and allowed to incubate for 5 minutes before adding 1 ml of a solution of 7.5 ng/ml [^{57}Co] B_{12} in 0.1M phosphate buffer pH 7.5 to each tube, shaking and incubating for a further 10 minutes. The [^{57}Co] B_{12} solution is

is diluted with cold B_{12} so that each 1 ml aliquot contains approximately 10,000 c.p.m.

The albumin coated charcoal suspension was then added to each tube, except 5 and 5', to adsorb any vitamin B_{12} not bound to protein. After 15 minutes incubation, all the tubes were spun at 10,000 g for 10 minutes and had 1 ml of their clear supernatant removed and counted in a well type gamma counter for 1 minute.

Tubes 1 & 1' gave a measure of the total binding capacity of the unknown sample. Tubes 2 & 2' were controls which gave the number of counts bound by the antibody serum. In tubes 3 & 3' any I.F. activity was knocked out by the I.F. antibody serum, therefore, they gave a measure of the non-I.F. binding capacity of the unknown sample. Tubes 4 & 4' contained no protein binder therefore, the supernatant should not contain any bound counts. The 1 ml supernatant from these tubes thus gave a background count which was subtracted from the counts given by the other tubes before further calculation. Tubes 5 & 5' were the standards which gave the total c.p.m. given by 7.5 ng [^{57}Co] vitamin B_{12}. The average of the counts contained in the duplicate tubes was used in the calculations.

Thus the I.F. binding activity of the sample was given by the calculation :-

$$X = \frac{\text{(Tubes 1 c.p.m.- Tubes 3 cpm + Tubes 2 cpm)}}{\text{Tubes 5 c.p.m.}} \times 10 \times 7.5\text{ng } B_{12}/\text{ml sample}$$

The total binding activity is given by :-

$$Y = \frac{\text{(Tubes 1 c.p.m.)}}{\text{Tubes 5 c.p.m.}} \times 10 \times 7.5 \text{ ng } B_{12}\text{/ml sample}$$

Therefore, the percentage of I.F. binding activity in the sample is given by :-

$$\frac{X}{Y} \times 100\%$$

$$= \frac{\text{(Tubes 1 cpm - Tubes 3 cpm + Tubes 2 cpm)}}{\text{Tubes 1 c.p.m.}} \times 100\%$$

Any sample which had enough activity to bind all the counts available was diluted in 0.1M phosphate buffer pH 7.5 and assayed again.

COLLECTION AND PREPARATION OF HUMAN GASTRIC JUICE

Gastric juice was collected on ice by naso-gastric suction from patients undergoing routine Maximal Acid Output tests for diagnostic purposes. The gastric secretion was stimulated by intramuscular injection of Pentagastrin in a dose of 6 μg/Kg body weight.

The juice was filtered through glass wool to remove excess mucus, cell debris and residual food particles. The pH was then raised to 10 by adding 5N NaOH and stirred continuously for 15 minutes to destroy peptic activity. Then the pH was adjusted to 7 with 1N HCl and assayed for Intrinsic Factor and Non-Intrinsic Factor binding activity. Only samples with greater than 85% I.F. activity were selected. These selected samples were filtered through sinta-glass Büchner funnels, porosity 3, to reduce viscosity and then either immediately subjected to affinity chromatography or stored at -20°C in approximately 400 ml aliquots. Sodium azide 0.02% w/v was added to the samples and to all buffers to help prevent bacterial growth.

PREPARATION OF HOG I.F. CONCENTRATE

For each affinity chromatography run, 10g of commercial, freeze dried, dessicated hog gastric mucosa was reconstituted in 500 ml 0.1M phosphate buffer pH 7.5. These components were stirred

together continuously for 60 minutes at room temperature. The preparation was then centrifuged at 10,000 g for 15 minutes and the supernatant removed, filtered through a sinta-glass Büchner funnel, porosity 3, and immediately applied to an affinity chromatography column.

ISOLATION OF HUMAN INTRINSIC FACTOR

Samples of human gastric juice, collected and assayed as described above, which contained > 85% I.F. activity were diluted 1 : 1 in 0.1M phosphate buffer pH 7.5 to reduce their viscosity and filtered through a sinta-glass funnel immediately prior to application to the affinity chromatography column.

The column of vitamin B_{12} Sepharose was first washed with 1 litre of o.1M Glycine Na-OH pH-10 and then with 1 litre of 0.1M phosphate buffer pH 7.5 at room temperature. The gastric juice was pumped up the column from bottom to top at a flow rate of approximately 50 ml per hour. It was pumped up the column so that gravity counteracted, to some extent, the packing effect of the relatively viscous gastric juice. The amount of gastric juice which could be passed through the column was limited by the fact that at room temperature the gastric juice preparation was a good growth medium for bacteria and so despite reasonable precautions being taken, the effective maximum time that gastric juice could be run through any column was 48 hours. This meant that the

maximum amount of gastric juice preparation which could be processed on any one column was 2.5 litres. The pumping speed could not be increased since it caused the column to pack tightly and thus inhibit the flow. A similar effect occurred when less buffer was used to dilute the sample, since, although this decreased the volume to be processed, the resultant increase in viscosity slowed down the flow and more than counteracted any possible benefit. The samples were usually in approximately 400 ml aliquots, stored at -20°C. These were thawed in a 37°C water bath, filtered, diluted and kept at 4°C until required for the column, when they were allowed to reach room temperature. The column was not kept at 4°C while in use because although the lower temperature would have slowed down bacterial growth, it also increased the viscosity of the gastric juice and slowed the flow through the column. Apart from this practical consideration, the column was deliberately run at room temperature since this temperature is closer to physiological conditions.

After the gastric juice had been passed through the column, it was washed with 200 ml 0.1M glycine - NaOH pH-10, 200 ml 0.1M phosphate buffer pH 7.5 and then eluted with 5.0M Guanidine hydrochloride in 0.1M phosphate buffer pH-7.5. After the void volume of eluant had passed from the column, collection proceeded. Approximately 90% of the recovered Intrinsic Factor was found in the first 30 ml of eluant after the void volume. This fraction was filtered through Whatman No. 3 filter paper to remove any Sepharose finings which were eluted by the high density of the

Guanidine HCl, placed in neprophane dialysis tubing and dialysed against 15 litres distilled water at 4°C for 72 hours with water changes at 24 and 48 hours. The final preparation was assayed for I.F. : non-I.F. activity and stored in 1 ml aliquots at -20°C.

ISOLATION OF HOG INTRINSIC FACTOR

The basic procedure was identical to above, using 500 ml of the Hog I.F. preparation, previously described, instead of human gastric juice. This isolation was faster because of higher initial I.F. concentration and thus lower total volume of the sample. Risk of infection and loss of protein by denaturation, therefore, was reduced.

ASSAY OF INTRINSIC FACTOR ACTIVITY - (IN VIVO)

The biological activity of preparations of intrinsic factor was assessed by measurements of the effect on the absorption of cyanocobalamin by patients with pernicious anaemia. A double tracer technique was used and the amounts of radioactivity retained in the body two weeks after oral dosing was taken as a measure of the cyanocobalamin absorbed. On the first day of the test the patient ingested 1.0 μg, 0.5 μCi [^{58}Co] cyanocobalamin in 100 ml water and 24 hours later 1.0 μg, 0.5 μCi [^{57}Co]

cyanocobalamin with the intrinsic factor preparation made up to 100 ml in water. The patients fasted for twelve hours before and three hours after each oral dose. Whole body radioactivity was measured in a multidetector system housed in a steel room before and after each oral dose and finally two weeks after the dose. Counting rates were corrected for background radioactivity, natural body radioactivity, radioactivity decay and where required for the contribution of the[^{58}Co] to the counting rate in the [^{57}Co] energy band.

Experience with this technique and monitoring system has shown that patients with pernicious anaemia rarely absorb more than 20% and never more than 28% of a 1 μg test dose. Normal subjects absorb an average of 50.6% of the dose (standard deviation 14.7) (46).

RESULTS

MONOCARBOXYLIC ACID DERIVATIVE OF CYANOCOBALAMIN

Each major band of red material recovered from the ion exchange column was taken to dryness, weighed and had its radioactivity measured. A typical set of results and the yield from each band, calculated from the dry weight are shown in Table 2.

TABLE 2 - Yield of hydrolysis products from cyanocobalamin

Source	dry weight (g)	c.p.m.	Yield %
Acid Hydrolysate	5	1,014,817	100
Biorex Eluate	-	968,673	95 (from c.p.m.)
Q.A.E. 1st Band	2.22	459,178	44.4
Q.A.E. 2nd Band	1.57	319,217	31.4
Q.A.E. 3rd Band	0.63	123,427	12.6
Q.A.E. 4th Band	0.1	21,381	2

Aqueous solutions (10mg/ml) of each of the major bands, cyanocobalamin and monocarboxylic acid cyanocobalamin were prepared and 0.2 ml from each was spotted onto the Whatmann No. 3 paper and ascending paper chromatography performed. The first band contained the unconverted cyanocobalamin and also various components which gave purple spots and were not investigated further. The second band gave an identical Rf value to the

monocarboxylic standard. The total material contained in this band weighed 1.57g and thus the yield of monocarboxylic acid recovered was 31.4% of the original cyanocobalamin. Band 3 and band 4 were assumed to contain di- and tri-carboxylic acids respectively (47). There remained some minor bands of faint orange and pink colour on the Q.A.E. Sephadex column after the fourth major band had been eluted. These were discarded.

PREPARATION OF AFFINITY ADSORBENT

When the protocol described for the preparation of the affinity adsorbent was strictly adhered to, good yields of covalently bound cyanocobalamin were achieved (approximately 10% incorporation, as judged by radioactivity). However, if the pH levels quoted for the reactions were deviated from the yield fell off drastically. Also when spermidine was used in place of 3, 3 diaminodipropylamine as the bridging chain, the yield was only about 2% incorporation. There was no apparent reason for this since the only difference in structure between the two compounds is that spermidine has one less CH_2 group in its hydrocarbon chain.

ASSAY OF INTRINSIC FACTOR ACTIVITY - (IN VITRO)

The assay of a typical sample yields results as shown in Table 3. The reproducability of the assay was measured by storing

a particular sample of neutralised gastric juice in 1 ml aliquots in plastic tubes at -20°C. One of these 1 ml aliquots was thawed and assayed every time an assay was being carried out until 25 results had been obtained. The coefficient of variance gained from these results was 6.8.

TABLE 3 - Results of "in vitro" assay for I.F. activity.

Tube	Description	c.p.m. in 1 ml supernatant	Average c.p.m — Background
1	Unknown Sample	4029	3980
1'		4163	
2	Antibody Serum	239	112
2'		217	
3	Unknown Sample + Antibody Serum	565	456
3'		579	
4	Background	120	-
4'		112	-
5	Standard	5763	5706
5'		5882	

$$\text{Total binding of Sample} = \frac{\text{Tubes 1 cpc}}{\text{Tubes 5 cpm}} \times 75 \text{ ng } B_{12}\text{/ml sample}$$

$$= \frac{3980}{5706} \times 75 \text{ ng } B_{12}\text{/ml sample}$$

$$= \underline{\underline{52.2 \text{ ng } B_{12}\text{/ml sample}}}$$

$$\text{I.F. binding of Sample} = \frac{\text{Tubes 1 cpm - tubes 3 cpm + tubes 2 cpm}}{\text{Tubes 5 cpm}} \times 75$$

$$= \frac{3980 - 456 + 112}{5706} \times 75 \text{ ng } B_{12}\text{/ml sample}$$

$$= \underline{\underline{48 \text{ ng } B_{12}\text{/ml sample}}}$$

$$\% \text{ I.F.} = \frac{48}{52.2} \times 100\% = \underline{\underline{92\%}}$$

COLLECTION AND PREPARATION OF HUMAN GASTRIC JUICE

After preparation and assay for I.F. activity, only samples which contained > 50 ng/ml B_{12} binding capacity which was > 85% I.F. mediated were further processed. Over the period of study approximately 40% of the samples collected were suitable, the others either having too low a concentration of I.F. or too high a proportion of non I.F. activity. Samples which could not be processed immediately, and were therefore stored at -20°C, showed no appreciable loss of I.F. activity over a 9 month period.

TABLE 4 - Typical results of Affinity Chromatography on human gastric juice and reconstituted hog gastric mucosa

	Volume (ml)	Total Protein (mg.)	B_{12} Binding Activity (µg B_{12} bound)	I.F. Specific Activity (µg B12 bound/mg protein)	Purification factor	Yield of I.F. (%)
Human Gastric Juice	2,500	6,740	115.8 (85% I.F)	0.0146	-	-
Final Preparation	35	3.1	56 (94% I.F.)	16.9	x1163	52
Hog Gastric Mucosa	500	3,170	400 (35% I.F)	0.044	-	-
Final Preparation	40	2.7	61.2 (96% I.F)	21.7	x493	42

As can be seen from the results in Table 4, affinity chromatography is a very powerful tool in the purification of Intrinsic Factor. However, although all the non B_{12} binding proteins passed easily through the column uninhibited, the B_{12} binding proteins which do not exhibit I.F. activity presented a major problem.

With human gastric juice the problem was partly overcome by only selecting juices with low non-I.F. content. This was not possible with the commercial hog preparation which contained approximately 65% non-I.F. binding activity. Initially the procedures for eluting the column were performed very closely to those described by Allen et al (18) using 7.5M Guanidine-HCl ph 7.5 and under these conditions there was no improvement in the ratio of I.F. : non-I.F. recovered. However, this procedure was later modified by using lower concentrations of Guanidine-HCl at different pH's and it was found that at a 5 Molar concentration at pH 7.5, predominantly I.F. was eluted from the column leaving the non-I.F. binders behind. Since the reported association constants for I.F. and non-I.F. binding to cyanocobalamin ($1.5 \times 10^{10} M^{-1}$) are not significantly different (17, 18 & 48), it may be that the higher carbohydrate content of non-I.F., approximately double that of I.F. at 35.5% of the molecular weight, rendered the non-I.F. proteins less susceptible to denaturation under these conditions and thus they were not eluted from the column.

Assuming a molecular weight for I.F. of approximately 60,000 the final binding activity of the Human I.F. preparation should have been given in a total protein weight of only 80% of that found. The other 20% was probably accounted for by I.F. which failed to renature properly during dialysis giving a non-functional protein or even pieces of polypeptide. Some evidence for this was found using gel filtration (shown later).

Since this small amount of non-functional protein was probably a function of the denaturation/renaturation part of the purification, it could not be removed by further application of the purification procedure. In experiments where it was attempted to process more than 2,500 ml gastric juice on one run, the percentage of non-functional protein recovered rose drastically.

The total yield of I.F. was approximately 50% for both human and hog I.F. The initial gastric juice and hog mucosa preparations, tested after chromatography, contained $< 2\%$ of their initial binding activity. Therefore, the loss of I.F. is not due to non-binding in the column. Some protein would denature spontaneously and be lost during the washing procedure and some would fail to be denatured during the Guanidine elution and thus stay on the column.

After the 5M Guanidine elution, the columns were further eluted with 7.5M Guanidine to wash out the non-I.F. proteins before further affinity chromatography was carried out on them.

Before it was discovered that I.F. and non-I.F. could be separated by eluting the column with a lower concentration of guanidine-HCl, the method of "Selective affinity chromatography" as used by Allen et al.(18) was investigated. This method used the monocarboxylic derivative of cyanocobalamin, with the nucleotide portion removed, bound to Sepharose-4B as the affinity adsorbent. It depended on the property of non-I.F. binders to bind to this

derivative of cyanocobalamin which lacked the nucleotide portion much more avidly than I.F. However, it was found that the increase in processing and the repeated denaturation/renaturation involved caused such a decrease in yield as to make this procedure impractical. The only difference in the method used was to run the columns at room temperature instead of 4°C.

It was found that after any given column had been used more than approximately six times, pink material started to appear in the guanidine-HCl eluant. This material proved, under light microscopy, to be small pieces of the affinity adsorbent showing that some of the Sepharose-4B was disintegrating through usage. The pink material could be removed by filtration of the guanidine-HCl eluant through Whatman No. 3 filter paper. However, when this disintegration of the Sepharose started, the efficiency of the columns decreased and they were discarded.

ASSAY OF I.F. ACTIVITY - (IN VIVO)

Some I.F. preparations which gave positive "in vitro" binding results were tested for biological activity in volunteers who were known to have pernicious anaemia. A dose of 30 µg I.F. preparation was used, as judged by the I.F. binding capacity "in vitro" of the given sample.

The results of the assay are given in Table 5. Each patient

received an I.F. preparation from a different batch.

TABLE 5 - Retention of [^{57}Co] cyanocobalamin in Pernicious Anaemia patients using purified I.F. preparation.

	% cyanocobalamin dose absorbed		
Patient	Dose given alone 0.5 μCi ^{58}Co	Dose 0.5 μCi ^{57}Co + 30 μg Human I.F.	Dose 0.5 μCi ^{57}Co + 30 μg Hog I.F.
A	2	-	70% (100)
B	1.5	21 (31)	
C	1	23 (34)	
D	5		61 (90)
E	7		50 (74)

The increases in the amount of cyanocobalamin absorbed is lower for the human I.F. preparation than the hog preparation. The vitamin B_{12} content of the various preparations was assayed using a microbiological assay. For the hog I.F. preparations used the level of vitamin B_{12} was less than 200 pg/dose whereas the I.F. used had a level of more than 5,000pg/dose. This endogenous vitamin B_{12} could have diluted the test dose of ^{57}Co cyanocobalamin and thus reduced the amount of radioactivity absorbed. Patient C also underwent the same procedure using 50 mg of commercial hog

I.F. (which is the amount used routinely to test vitamin B_{12} absorption) in place of the purified preparation. This test gave an absorption of 25% which is not significantly different from the result with purified I.F. Therefore, either this patient had some cause for malabsorption other than a lack of I.F. production, or he did not fast for the prescribed period before and after ingestion of the test dose.

The values in brackets in Table 5 represent corrected absorption percentages. On a theoretical basis, if the molecular weight of I.F. is approximately 60,000 and that of cyanocobalamin in 1360, then 30 μg of I.F. has a maximum B_{12} binding capacity of 0.68 μg. Thus the maximum possible absorption of B_{12} was 0.68 μg or 68% of the 1 μg test dose, assuming a stochiometric 1 : 1 relationship between I.F. and B_{12} and that each I.F. molecule only acts once (49, 50). Thus the values in brackets have been corrected on the basis that 68% absorption represents 100% of the total possible absorption.

It would have been desirable to carry out more absorption tests with human I.F., but suitable patients were not available in time. Patient B was also given a 1 μg test dose of [^{57}Co] cyanocobalamin with 60 μghuman I.F., but this still failed to increase further the amount of dose retained.

DISCUSSION

Once the monocarboxylic acid derivative of cyanocobalamin had been prepared and purified it was a relatively simple procedure to bind it to the substituted Sepharose. The addition of the bridging chain to the Sepharose was also simplified by using the commercially available Sepharose-4B which had already been activated with cyanogen bromide, thus allowing this potentially noxious reaction to be missed out. The preparation could now probably be simplified further by employing a recent product of Pharmacia Ltd., which already has a 6 carbon bridging chain attached to it (AH-Sepharose 4B). This product was not tested. It is thus not possible to comment on whether the shorter hydrocarbon chain would affect the steric hindrance and thus the yield of I.F. bound or not. The low yields obtained by Oleson et al (35) using hydroxocobalamin covalently bound to albumin could possible be explained on the basis of steric hindrance caused by the albumin.

After the adsorbent had been used several times, the Sepharose tended to break up and finings of Sepharose with cyanocobalamin attached appeared in the guanidine-HCl eluant. This pink material was recognisable as damaged Sepharose under low power light microscopy. Although the bulk of this material could be removed by filtration, its appearance was invariably accompanied by decreased I.F. yields and increased cyanocobalamin concentration in the final preparation. This breakdown of the matrix was probably the result

of bacterial attack despite the use of sodium azide. The B_{12} appearing in the eluant was primarily cyanocobalamin and not the monocarboxylic derivative which proves that it had been bound to the terminal amino group of the bridging chain and that the bond between this amine and the hydrocarbon chain must have been broken to release native cyanocobalamin.

The criteria for being cyanocobalamin was that it supported growth of Lactobacillus Leichmanii in the microbiological assay and thus could not have been the monocarboxylic derivative which is not a metabolite of this organism. Otherwise, the adsorbent seemed fairly stable.

The main difference between Allen's method and the ones employed in this study were :-

(i) Temperature. Allen carried out all his isolations at 4°C whereas room temperature was used for this report.

One reason for this was that cold room facilities were not available. However, this could have been overcome by running the column in a fridge, but when this was tried it was found that the decreased temperature increased the viscosity of the gastric juice or hog preparation and inhibited flow. Allen also carried

out some of this I.F. characterisation on experiments at 4°C and it was felt that it was possible that artefactual results might occur at this temperature. Thus it was decided to carry out all procedures at room temperature which is closer to physiological conditions. The major drawback of this decision undoubtedly being the increase in bacterial activity. One exception to this rule was the dialysis of the guanidine-HCl eluant against water which was carried out at 4°C. It was found that higher yields of I.F. were gained at this temperature, possibly due to the slower and perhaps more accurate renaturation of the protein.

(ii) Allen used a gravity feed for his column but it was found that a high enough flow rate (50 ml/hr) could not be achieved by this method. Thus it was necessary to pump the fluid through this column. It must be assumed that Allen's preparation somehow decreased the viscosity of the sample.

(iii) Allen used a process of "selective affinity chromatography" to separate I.F. from non-I.F. hog B_{12} binding proteins. However, this method did not prove to be practical in the present study. On employing the technique, it was found that the affinity adsorbent lacking the nucleotide portion of the cobalamin molecule, failed to effectively

resolve the I.F. and non-I.F. binders and also that the additional processing of the material caused drastic reduction in the final yield of protein.

By adjusting the molarity of the guanidine-HCl used to elute the protein, it was discovered that when using 5M guanidine-HCl instead of 7.5M a high proportion of I.F. was eluted, leaving the non-I.F. bound to the column. This was a considerable simplification on Allen's technique and appeared to be effective. As was stated in the results section, the relative affinities of hog I.F. and non-I.F. for B_{12} are not significantly different, therefore it must be assumed that hog non-I.F. has a more stable structure than I.F. under these elution conditions and that it is not denatured sufficiently to break its bond with the affinity adsorbent. The most significant difference so far reported (18) is the higher carbohydrate content of non-I.F. This may protect it to a certain extent from the unfolding action of the guanidine-HCl.

When the 5M concentration was used to elute human I.F., it was also found that the proportion of non-I.F. binders was reduced.

There was the added advantage that 5M guanidine-HCl was obviously not quite such a drastic denaturant as 7.5 Molar.

In general the specific activity of the final preparations of I.F. (μg B_{12} bound/mg protein) were slightly lower than those found by Allen, usually in the range 16-25 μg B_{12} bound/mg protein. This presumably depended on the quality of the starting material and the efficiency of the affinity chromatography column at the time. However, the highest yield of I.F. recovered was approximately 55% whereas Allen claims yields of over 80%. No explanation for this was found except that perhaps the higher temperature caused faster spontaneous denaturation of the I.F. or that the 5M guanidine-HCl failed to eluate all the I.F. from the column. However, when a given column was subsequently eluted with 7.5M guanidine-HCl, no appreciable further I.F. activity was recovered.

Even allowing for this reduced yield, this method still showed a marked improvement on results gained using more conventional multi-stage purification systems. A feature of previous purification methods was the saturation of the B_{12} binders with radioactive Cobalt label, thus allowing the purification to be followed by monitoring the behaviour of the radioactivity (21, 51). This yields a product which is an I.F.-B_{12} complex and is thus not suitable for some experimentation. Although this B_{12} could be removed using guanidine-HCl (see later), this would further decrease the overall yield.

The glycoprotein recovered by this method could be shown

to be immunological identical to I.F. in that its B_{12} binding was $>$ 95% destroyed by serum from pernicious anaemia patients which contained I.F. antibodies and also it was biologically active at a 30 μg dose level. The results for the biological activity of human I.F. were not as convincing as for hog I.F. and would have been desirable to have carried out more trials.

From the total protein measurements and the specific activity calculations it is clear that the substance isolated is a very pure preparation of I.F. and the impurities are probably partially denatured I.F.

This method does not require any very elaborate or expensive equipment and so could be universally used to purify B_{12} binding proteins.

CHAPTER III

PHYSICO-CHEMICAL PROPERTIES OF PURE HUMAN & HOG INTRINSIC FACTOR

Although there have been reports (31, 52) on the properties of Intrinsic Factor, many of these have been conflicting and very often in the literature the phrase appears "this work needs to be repeated on a pure I.F. preparation".

Neither the time nor the facilities were available to cover all or even most of the properties of pure I.F. in this study. The results which were obtained appeared to be reproducable among the different batches of I.F. prepared and except where specifically stated, there was no significant difference found between the behaviour of the human and the hog preparations.

Parallel results for each individual result obtained were to be found scattered in the literature, but the range of results quoted in the past is so extensive that it was difficult to make meaningful comparisons, especially where previous work was carried out on impure or partially pure preparations.

B_{12} BINDING CAPACITY OF I.F. AND CRITERIA OF PURITY

The total B_{12}-binding capacity of the final I.F. preparation was found by serially diluting a sample from the preparation and subjecting the various dilutions to the "in vitro" I.F. activity assay.

The purity of a given sample was judged by the percentage of the B_{12}-binding activity which was blocked by the addition of serum

from pernicious anaemia patients containing I.F. antibodies and also the specific activity of the sample found by dividing the total B_{12} binding capacity of the sample in micrograms by the total protein contained in the sample in milligrams.

At a later stage in the study, after antibodies to pure I.F. preparations had been raised in rabbits (see Chapter 4), these antibodies were used to check the percentage of I.F. in a given preparation in preference to the pernicious anaemia patient serum.

Some of the less pure I.F. preparations which were made displayed properties which might have given rise to the conflicting results found in the past.

SECTION I

DETERMINATION OF MOLECULAR WEIGHT OF I.F. BY GEL FILTRATION ON SEPHADEX G-200

A sample of 1 ml, 100 μg I.F. preparation in 0.1M Tris-acetate buffer was pre-incubated at room temperature with excess [^{57}Co] cyanocobalamin for 30 minutes, then the excess cyanocobalamin was removed using 1 ml albumin coated charcoal and centrifugation. The clear supernatant was counted for total activity, adjusted to 4 ml with 0.1M tris-acetate buffer pH 7.8 containing 1M NaCl and applied to a Pharmacia column 55 cm x 2.5 cm containing Sephadex G-200. The sample was pumped up the column in the same Tris-acetate buffer by an LKB peristaltic pump at a flow rate of 12 ml/hr. The column eluant was passed through an LKB Uvicord II ultraviolet absorptiometer which passed a continual reading of the U.V. absorption at 260 nm to an LKB multichannel pen recorder. The eluant was collected in 6 ml fractions on an LKB Ultrorac automatic fraction-collector.

The samples were labelled and counted for ^{57}Co activity on a Packard Autogamma Scintillation Spectrometer for 2 minutes each.

Dextran Blue was used to find the void volume of the column and pure commercial protein preparations used to calibrate it. These proteins had the following molecular weights :-

myoglobin (B.D.H.) - 16,000; pepsin (Sigma) - 34,000; bovine serum albumin (B.D.H.) - 66,000; haemoglobin (B.D.H.) - 65,000; aldolase (Sigma) - 150,000; phosphorylase A (Sigma) - 360,000.

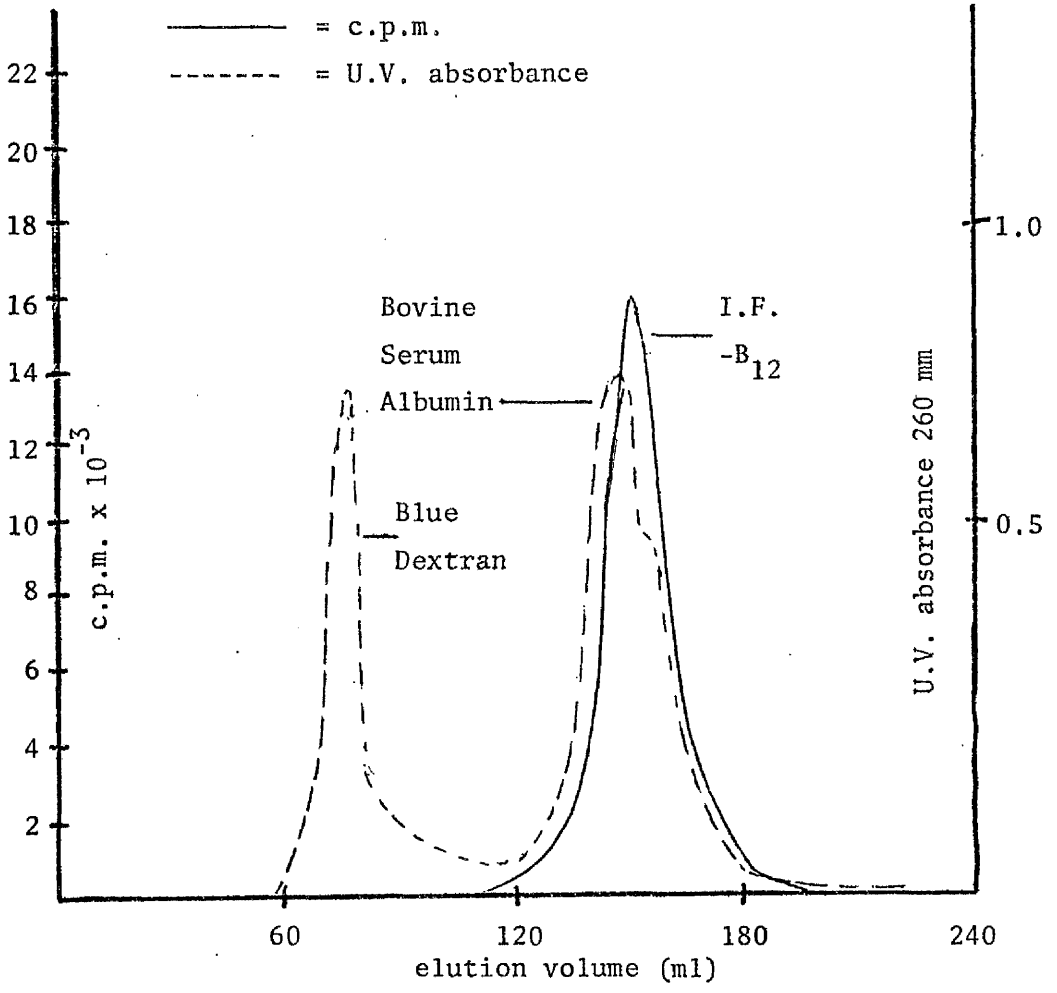
The experiment was carried out under these conditions for both Human and Hog I.F. and was also carried out in a similar manner with Hog I.F. except that the I.F. was pre-incubated with the [^{57}Co] cyanocobalamin at 4^{o}C for 24 hours.

Also 100 µg of pure hog U.F. which had been labelled with ^{125}I (description later) was applied to the G-200 column. This was carried out to check the molecular weight of I.F. alone, not complexed to cyanocobalamin.

The U.V. absorption at 260 nm of the fractions was read on a Unicam S.P. 1800 spectrophotometer against a blank of elution buffer.

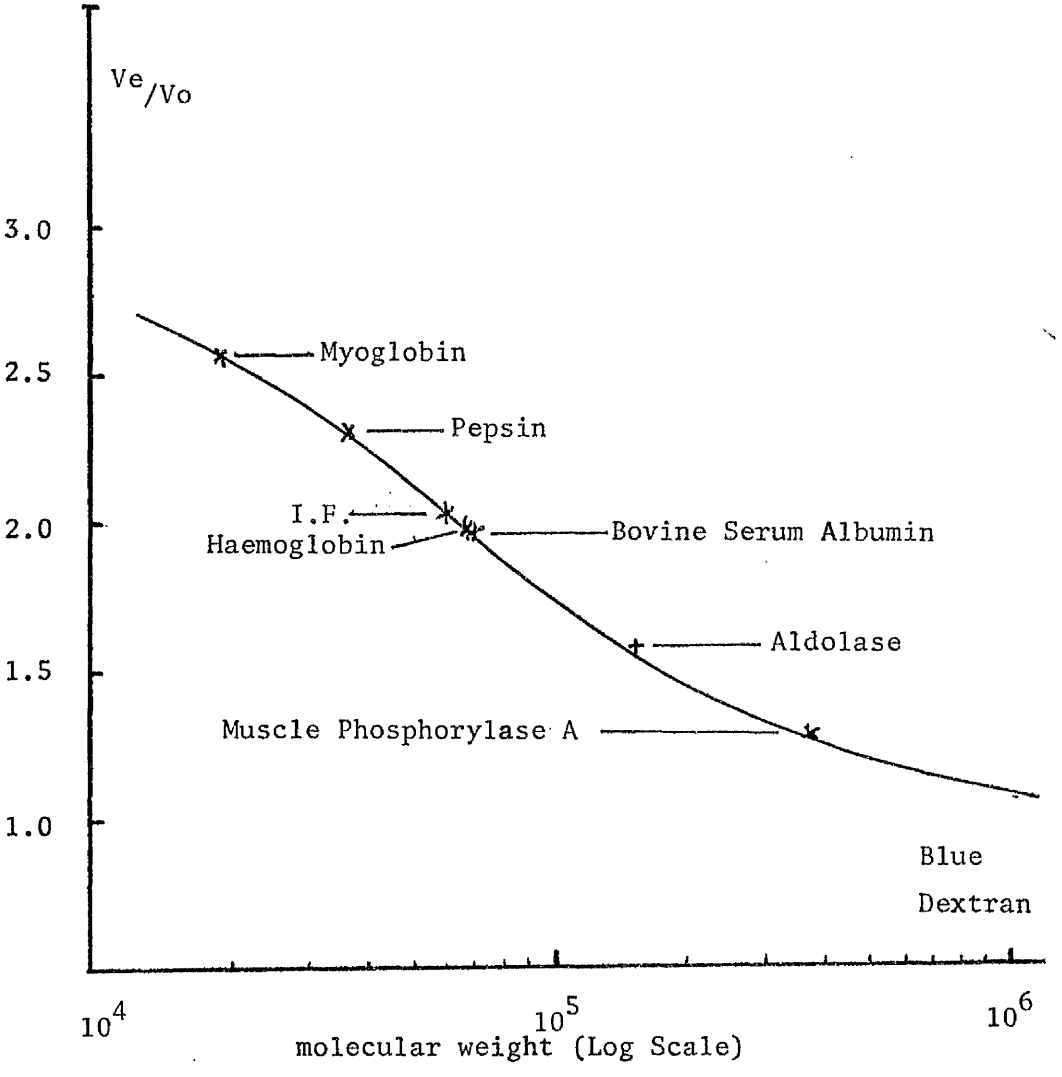
RESULTS

When a high purity sample of I.F. (% I.F. > 95%, specific activity > 25 ug B_{12} bound/mg protein) was saturated with [^{57}Co] cyanocobalamin and applied to a Sephadex G-200 column as described, a single binding peak of [^{57}Co] was found as shown in Graph 2.



GRAPH 2 - Elution pattern of Blue Dextran and bovine serum albumin as judged by U.V. absorbance and of I.F.-B_{12} complex as judged by radioactivity.

This same column was calibrated with pure proteins of known molecular weight. The void volume was taken to be the elution volume of Blue Dextran (Vo) and the elution volume of each of the other proteins was taken as the volume of eluant up to the centre of the absorbance peak for each individual protein (Ve). The ratio $^{Ve}/Vo$ was plotted against the log of the accepted molecular weight of each of the proteins to yield Graph 3.



GRAPH 3 - Graph of relationship of Molecular Weight to elution volume on 2.5 cm x 55 cm Sephadex G-200 column

Taking the results of ten runs through the column of pure hog and human I.F.-B_{12} complex, a value of $^{Ve}/Vo$ 2.04 with a standard deviation of $\pm$ 0.03 was found which, when applied to graph 3 gives a molecular weight of 56,000 $\pm$ 2,000. The samples of I.F. applied to the column were adjusted to contain 100 μg total protein. They had an average binding capacity of 25 μg B_{12} bound/mg protein. Therefore each sample contained 2.5 μg B_{12} which is a ratio of weights of 100 : 2.5 = 40 : 1. This means that one molecule of I.F. binds one molecule of B_{12} (molecular weight = 1360) then the theoretical molecular weight of I.F. is 54,000. This suggests that the experimental molecular weight found must be that of an I.F. monomeric form.

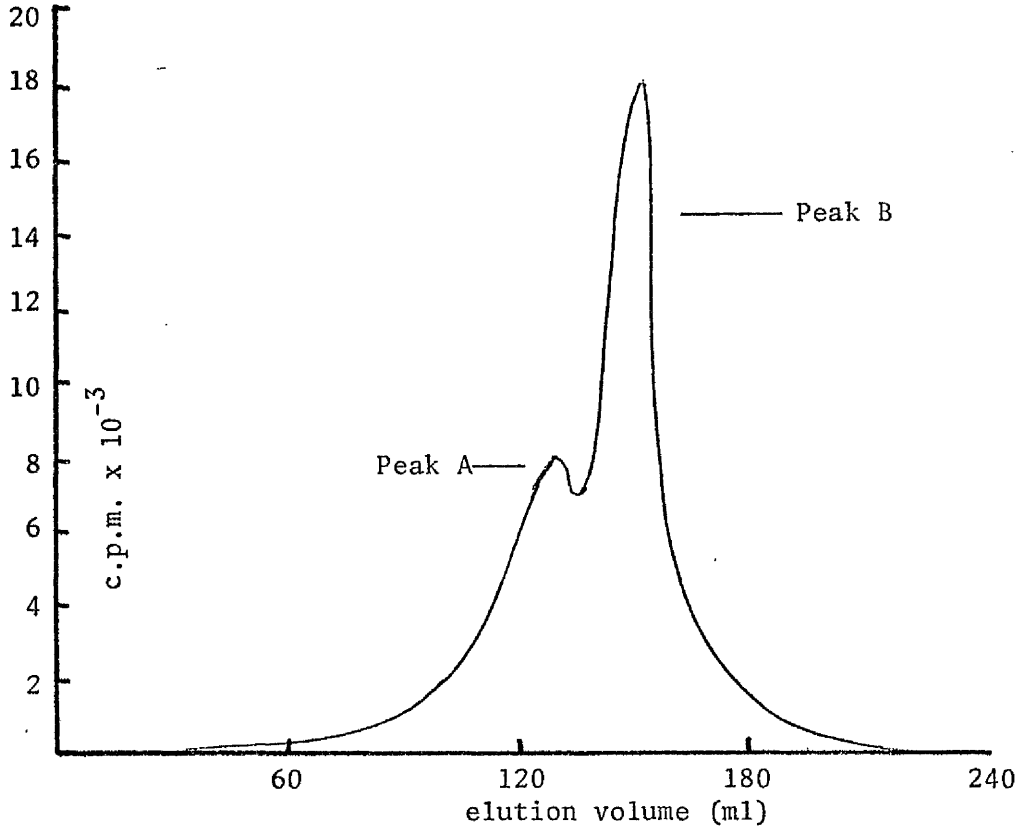
Both hog I.F. and human I.F. gave the same molecular weight when bound to cyanocobalamin.

Some samples of pure hog I.F. were allowed to incubate with excess cyanocobalamin for 24 hours at 4°C before gel filtration, but the elution pattern of the bound radioactivity was not changed. There was no evidence of oligomer formation which would have introduced a component with higher molecular weight.

When some pure hog I.F. had been successfully labelled with [^{125}I] (see Chapter 5), 100 μg of the preparation was column chromatographed in the absence of cyanocobalamin. This was repeated twice and each time the $^{Ve}/Vo$ value was slightly lower

than the mean of the value for the I.F.-B_{12} complex, suggesting a higher molecular weight (approximately 64,000). This seems unlikely but could be explained on the basis of change of configuration of the molecule i.e. if the I.F. were more loosely coiled when not bound to B_{12}. This would be in agreement with the results of Gräsbeck in 1967 (53) and Hippe in 1970 (54) who found that the Stoke's radius of the I.F. molecule decreased by 4Å when cyanocobalamin bound to it.

When some I.F. preparations which had lower percentages of I.F. activity were labelled with [^{57}Co] cyanocobalamin and applied to the column, a typical elution pattern with two converging peaks was found as shown in Graph 4.



GRAPH 4 - Elution pattern of an I.F. preparation which had lower I.F. specific activity

Peak B in Graph 4 had a $^{Ve}/Vo$ value of 2.05 which gives it a molecular weight of 56,000. Peak A has a $^{Ve}/Vo$ value of 1.72 which gives it a molecular weight of approximately 100,000.

The fractions from the centre of peak A were pooled as were the fractions from the centre of peak B. The volume of each pool was reduced to approximately 2 ml by ultrafiltration using an Amicon PM-10 membrane. They were then run against I.F. antibody on Ouchterlony plates (for protocol - see Chapter 4).

The sample from peak B precipitated with the antibody, whereas the sample from peak A did not.

This suggests that peak B probably contained I.F. whereas peak A probably did not, but was a contamination caused by non-I.F. binders being eluted from the affinity chromatography column. It is possible, however, that peak A was a dimerised form of I.F. and that the antigenic sites were altered by this process, but that seems unlikely.

SECTION 2

ULTRAFILTRATION

An Amicon Ultrafiltration cell, using membranes of differing retentivity was used in various experiments.

A PM-10 membrane which retains molecules > 10,000 M.W. was used to concentrate samples of I.F. This membrane was also used to alter the type of buffer medium in which the I.F. sample was contained if this was necessary in any operation, e.g. any I.F. sample being subjected to gel filtration was transferred to 0.1M Tris-acetate buffer pH 7.8 containing 1M NaCl.

An XM-50 membrane which retains molecules > 50,000 M.W. was used to remove any "protein debris" i.e. pieces of polypeptide which were the result of the denaturation during the elution of the column. This type of membrane was also used in an experiment to remove any cyanocobalamin bound to the I.F. molecules as a result of hydrolysis of cyanocobalamin from the affinity adsorbent. The experiment was carried out by adding 6.6 ng, 0.1 μCi [^{57}Co] cyanocobalamin to 30 ml of the guanidine-HCl eluant and incubating for 30 minutes before placing the eluant in the ultrafiltration cell and applying a positive pressure of 350 KPa Nitrogen. The cell was stirred continuously until the volume of the sample had been reduced to approximately 2 ml. The pressure was released and

a further 30 ml of 7.5M guanidine-HCl added and the pressure re-applied. This operation was repeated four times and all the filtrate was collected and counted for radioactivity. After ultrafiltration the sample was removed from the cell and dialysed against water as was described in Chapter 2.

A rough approximation of the molecular weight of I.F.-[^{57}Co] cyanocobalamin complex was attempted by using a range of membrane retentivities consecutively on the same sample. The filtrate from each membrane was tested for [^{57}Co] activity then the retained sample was restored to its original volume with fresh buffer and applied to the membrane of the next highest retentivity. Used membranes were also counted for any bound activity.

If activity appeared in the filtrate from a given membrane then albumin coated charcoal was added to the filtrate, incubated for 15 minutes and centrifuged at 10,000g for 15 minutes to remove any free [^{57}Co] cyanocobalamin. If the activity was left in the supernatant then it had been shown to be bound to protein. PM-10, XM-50, XM-100 and XM-300 membranes were used giving ranges of $>$ 10,000; $>$ 50,000; $>$ 100,000 and $>$ 300,000 molecular weight retentivity respectively.

RESULTS

When the final I.F. preparations were assayed for B_{12} content it was found that they contained low amounts of endogenous B_{12}. These values varied from preparation to preparation but tended to be higher for human than for hog I.F. and also increased with usage of any given column. The range was from 2 pg B_{12}/μg I.F. - 1.5 ng B_{12}/μg I.F. Since 1 μg I.F. would be theoretically capable of binding approximately 23 ng of B_{12}, this range represented 8.7×10^{-3}% - 6.5% of the total B_{12} binding capacity of the I.F.

Thus the preparations with B_{12} content in the upper part of the range would contain a significant proportion of I.F. molecules complexed to B_{12}.

Where it was required to have an I.F. preparation free from B_{12} repeated ultrafiltration with an Amicon XM-50 membrane and 7.5M guanidine-HCl was used as described.

The efficiency of this procedure and the effect on the I.F. preparation was checked by repeating the experiment on samples of I.F. with 50 ng cyanocobalamin containing 0.1 μCi [^{57}Co] cyanocobalamin added.

TABLE 6 - The effect of successive ultrafiltrations in 7.5M guanidine-HCl in 0.1M phosphate buffer pH 7.5 through an Amicon XM-50 membrane at 350 KPa positive pressure with continual stirring on the bound B_{12} content of I.F. and the total binding capacity of I.F.

	% of total [^{57}Co] activity	% of total [^{57}Co] activity after coated charcoal treatment	Total Cyanocobalamin binding capacity (ng B_{12} bound)
Total [^{57}Co] activity added to I.F.	100	98	212
Activity in 1st Filtrate	35.9	0	0
Activity in 2nd Filtrate	33.2	0.5	0
Activity in 3rd Filtrate	19.0	0.5	0
Activity in 4th Filtrate	2.9	0	0
Activity stuck to XM-50 membrane	1.3	-	-
Activity retained inside cell	7.6	7.5	161

Thus, as can be seen from Table 6, although the exposure to guanidine-HCl and ultrafiltration procedure could remove over 90% of the B_{12} from the I.F. preparation, the total B_{12} binding capacity had been reduced by approximately 25% . This was probably due to

irreversible denaturation caused by the guanidine-HCl concentration.

The percentage of B_{12} which was retained in the cell after four successive ultrafiltrations was only slightly reduced by further repetitions of the procedure. Thus even in its partially denatured form, the I.F. retains some B_{12} binding activity.

As can be seen from the percentage of activity left in the various fractions in Table 6, after treatment with albumin coated charcoal the [^{57}Co] activity inside the cell was not affected and thus protein bound, whereas the [^{57}Co] activity which appeared in the filtration was removed by the charcoal and was thus not protein bound but free B_{12}.

The results from the experiment to find the approximate molecular weight range of I.F.-B_{12} complex are shown in Table 7.

TABLE 7 - Percentage of [^{57}Co] cyanocobalamin-I.F. complex passing through a range of Amicon ultrafiltration membranes in 0.1M phosphate buffer pH 7.5 with positive pressure and continual stirring.

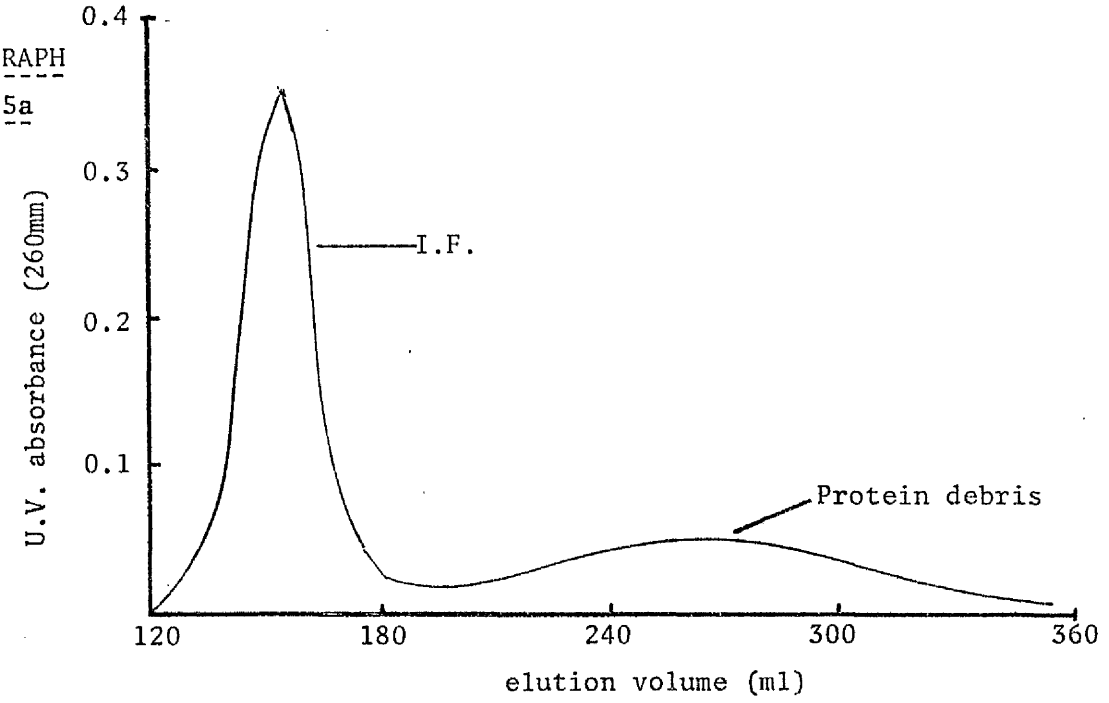
	M.W. Retentivity	% total bound [^{57}Co] activity appearing in filtrate	% filtrate activity removed by charcoal
PM-10	10^4	0.42	99
XM-50	5×10^4	0.68	97
XM-100	10^5	81	2
XM-300	3×10^5	96	1.5

Table 7 shows that 81% of the I.F.-B_{12} complex passed straight through an XM-100 membrane while none passed through an XM-50 membrane. In fact, when the XM-100 membrane had a further 30 ml of phosphate buffer passed through it, a further 18% of the total [^{57}Co] activity appeared in the filter. Therefore, in effect, 99% of the total [^{57}Co] activity, which was shown to be protein bound by the albumin coated charcoal technique, could pass through the XM-100 membrane.

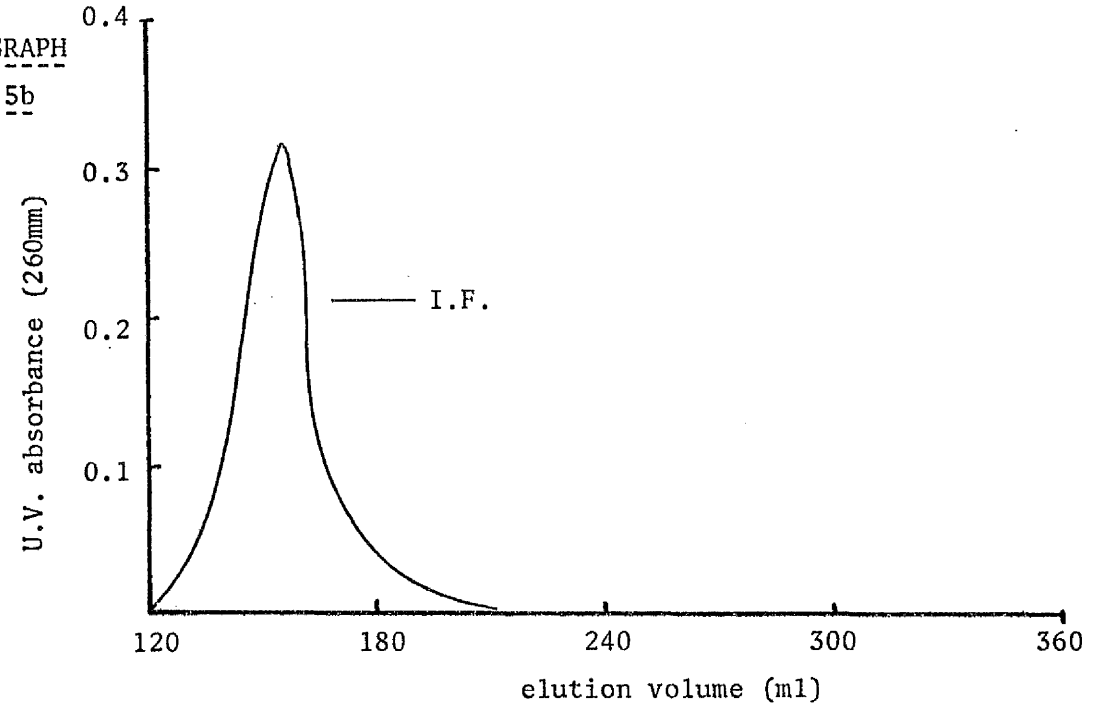
Thus the molecular weight of the I.F.-B_{12} complex must lie in the range 50,000-100,000.

I.F. preparations which had lower specific activities ($<$20 ug B_{12} bound/mg protein) and gave elution patterns similar to that shown in Graph 5a were ultrafiltered using an XM-50 membrane and a further sample subjected to gel filtration on the G-200 column (Graph 5b).

GRAPH 5a



GRAPH 5b



GRAPH 5 - Elution pattern of a sample of an I.F. preparation with a specific activity $< 20\ \mu g\ B_{12}$ bound/mg protein on a Sephadex G-200 column.

(a) before ultrafiltration on an Amicon XM-50 membrane

(b) after ultrafiltration on an Amicon XM-50 membrane.

As can be seen from comparison of Graphs 5a and 5b, the contaminating material could pass through the XM-50 membrane thus effectively purifying the I.F. preparation and increasing its specific activity.

This material was presumed to be denatured protein pieces caused by the guanidine-HCl. It had some B_{12} binding activity which could not be blocked by I.F. antibody.

SECTION 3

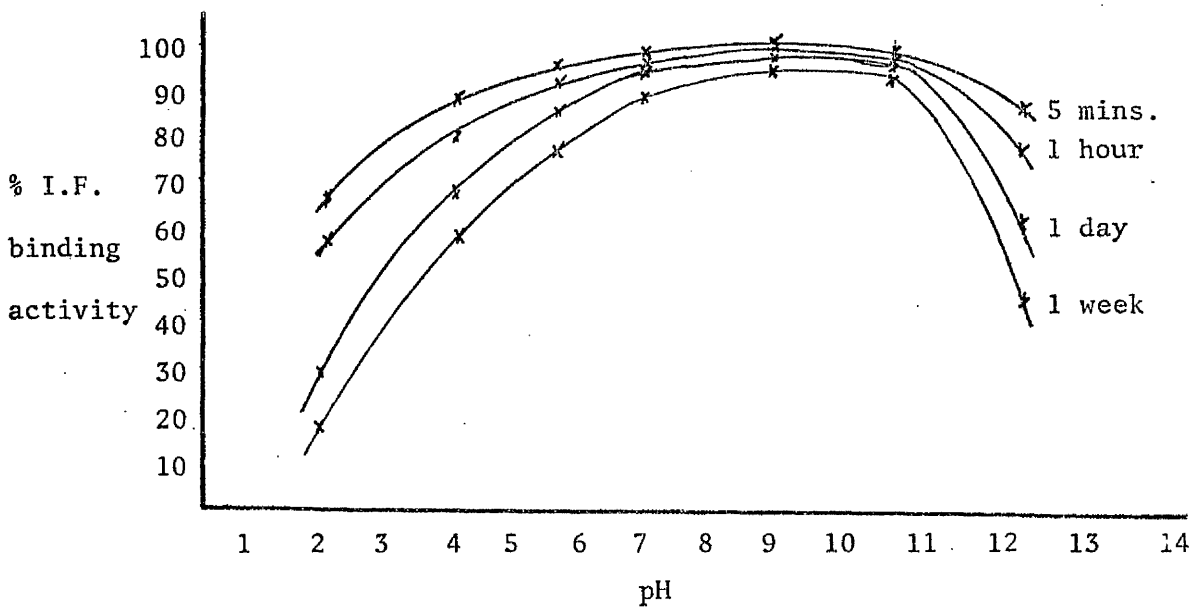
STABILITY OF I.F. OVER A PERIOD OF TIME AT DIFFERENT pH

An aqueous sample of 2 ml, 100 µg I.F. was incubated with 5 µg 0.1 µCi [^{57}Co] cyanocobalamin for 30 minutes. The excess cyanocobalamin was removed with albumin coated charcoal and centrifugation for 15 minutes at 10,000g. The supernatant was removed and 0.1 ml aliquots injected into each of a series of test tubes set up in duplicate over a range of pH. Each tube contained 1.5 ml distilled water which had been previously adjusted to a certain pH value using either HCl or Na-OH. The pH values used were:- 2; 4; 5.5; 7; 9; 10.5 and 12.5. The timing of the experiment commenced when the 0.1 ml I.F. was added to each tube. Samples of 0.1 ml were taken and added to 1 ml albumin coated charcoal at 5 minutes, 20 minutes, 2 hours, 24 hours, 2 days and 7 days. These samples were spun at 10,000g for 15 minutes and 0.5 ml of the clear supernatant removed and counted for[^{57}Co] activity. Any decrease in the amount of counts present was taken to show denaturation of I.F.

This experiment was also carried out in a similar manner, except that the I.F. was incubated at the various pH's without the presence of cyanocobalamin. When the samples were taken at the specified time intervals, a standard "in vitro" assay of I.F. binding activity was carried out on them as described in Chapter 2.

RESULTS

Both hog I.F. and human I.F. behaved very similarly when subjected to a range of pH values at room temperature over a period of time up to seven days. They were both stable between pH 6.5 - 10.5 and at pH 10.5, the I.F. retained almost 100% activity even after seven days. The results are shown in Graph 6.

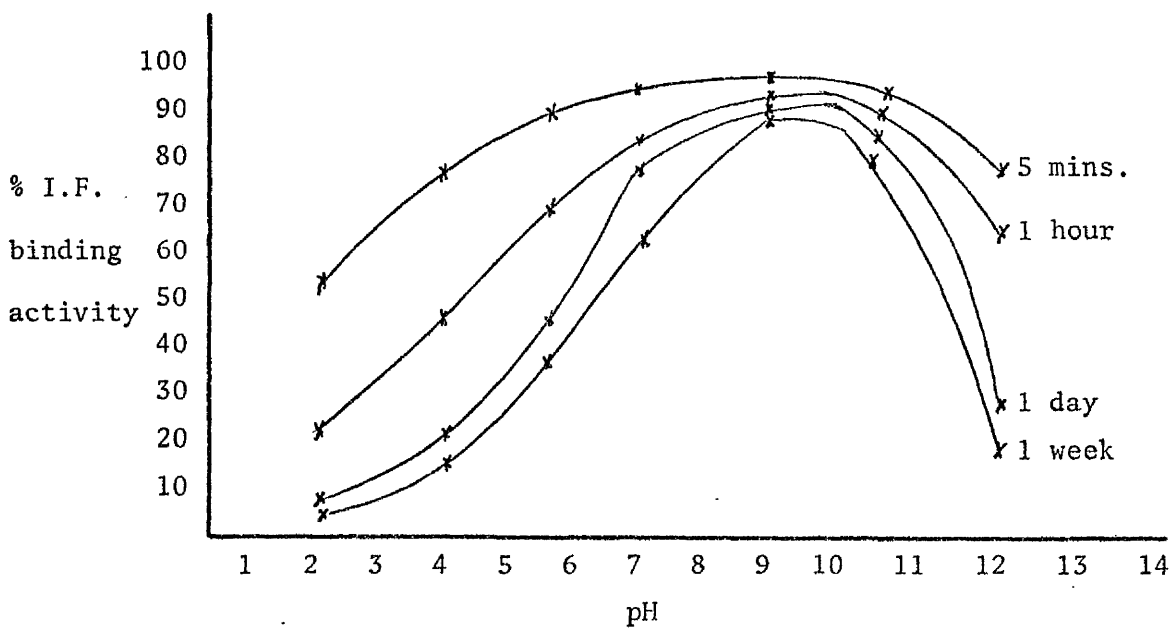


GRAPH 6 - The effect of pH on stability of the I.F.-B_{12} complex at room temperature over a period of time.

This graph shows that in the absence of proteolytic enzyme, I.F. is very stable between pH 6 and pH 10.5. Below pH 4 and above pH 12 the cyanocobalamin binding capacity falls off quite rapidly. The results shown in Graph 1 are the average of two

sets of experiments using both human and hog I.F.

When a similar experiment was carried out but with the I.F. being incubated at the various pH's without cyanocobalamin, the results were slightly different. Whereas in the above experiment the physical state of the I.F. was judged by the amount of labelled cyanocobalamin still protein bound, in this experiment the state of the I.F. was judged by the amount of labelled cyanocobalamin still capable of being bound after the incubation time. The results are shown in Graph 7, where 100% I.F. binding was taken as the amount of labelled cyanocobalamin a sample of the I.F. preparation at pH 9 could bind at time zero.



GRAPH 7 - The effect of pH on the stability of I.F. in the absence of B_{12} at room temperature over a period of time.

A comparison of Graph 6 and Graph 7 show that I.F. appears to be more stable to pH when it is complexed with cyanocobalamin. Any difference in the percentage activity on the two shorter time intervals may be due to the slightly different method required to assess a B_{12}-binding capacity. However, over the two longer time intervals, the shape of the binding curve changes, suggesting that this is a genuine effect.

SECTION 4

REVERSIBILITY OF THE pH INACTIVATION OF I.F. BINDING ACTIVITY

In this experiment samples of I.F. were incubated at 37°C at the extremes of the pH range of the previous experiment, namely 2, 4, 10.5 and 12.5. The samples were incubated at body temperature for 30 minutes which was judged to be a reasonable time which I.F. might be subjected to acid pH in the normal stomach. After this time, two 0.1 ml samples were removed from the incubation tubes, one of which was taken to pH 7.5 by the addition of 0.5 ml 0.1M phosphate buffer, the other had 0.5 ml of distilled water added. Both samples were then immediately subjected to an "in vitro" assay of I.F. activity.

RESULTS

The experiment was carried out on the same concentrations of I.F. as used in the time experiments above. The I.F. was not complexed to cyanocobalamin for two reasons :

(i) to allow the "in vitro" I.F. assay to be carried out on the samples.

(ii) To observe how the I.F. would behave under physiological conditions, i.e. leaving the acid environment of the stomach to the near neutral environment of the intestines when it was not complexed to B_{12} and in the absence of proteolytic enzymes.

The results are shown in Table 8.

TABLE 8 - Percentage of total B_{12}-binding activity of I.F. after pH exposure at 37°C for 30 minutes, with and without restoration of neutral pH before assay.

pH	B_{12} binding capacity of Human & Hog I.F. (%)	
	assayed in distilled water	phosphate buffer pH 7.5
2	15	82
4	41	89
10.5	89	98
12.5	33	65

All the samples showed a marked improvement in B_{12} binding activity when they were returned to pH 7.5 before assay. However, the high alkaline pH (12.5) appeared to have a more permanent denaturing influence than the extreme acid pH.

SECTION 5

RATE OF EXCHANGE OF FREE CYANOCOBALAMIN WITH I.F. BOUND CYANOCOBALAMIN

Pure I.F. (100 µg) was pre-incubated at 37°C with excess [^{57}Co] cyanocobalamin (10 µg) of known specific activity in 5 ml 0.1M phosphate buffer pH 7.5. After 15 minutes the excess cyanocobalamin was removed using 5 ml albumin coated charcoal and centrifugation.

Two 3 ml aliquots of the clear supernatant were removed and counted for radioactivity. This result gave a measure of the cyanocobalamin bound to the protein. An equal quantity of unlabelled cyanocobalamin was added to each tube in a volume of 1 ml and both tubes were placed in a water bath at 37°C. Samples of 0.1 ml were removed from both tubes at 30 minute intervals up to 8 hours, then one sample at 16 hours and one sample at 24 hours. When each sample was taken it was immediately added to 1 ml albumin coated charcoal, incubated at room temperature for 10 minutes and centrifuged at 10,000g for 15 minutes. The clear supernatant was counted for radioactivity.

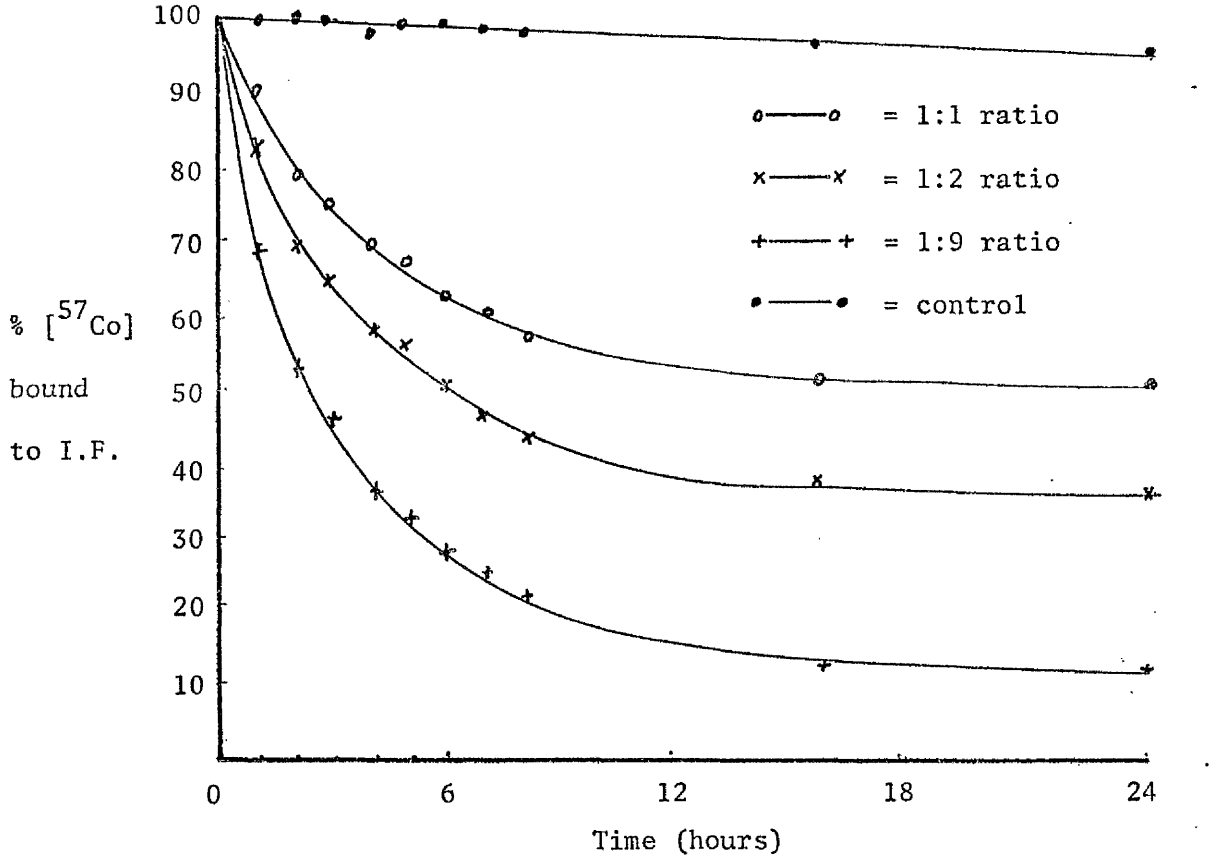
The experiment was then repeated using double the amount of unlabelled cyanocobalamin and then again using nine times as much unlabelled cyanocobalamin as labelled cyanocobalamin bound to the I.F. The results of the counts found in the supernatants of the

timed samples were expressed as percentages of the total number of counts bound to the I.F. at time zero.

A control tube was also set up which also contained 3 ml of the [^{57}Co] labelled I.F. preparation, but to which 1 ml of buffer had been added which did not contain any unlabelled cyanocobalamin. Samples were taken from this tube at the same time as the other tubes and treated in the same way. This control was to check for denaturation of the I.F. causing artefactual decreases in the labelled cyanocobalamin found in the supernatant after charcoal treatment.

RESULTS

When the results of the competition between the increasing amounts of unlabelled free cyanocobalamin and the fixed amount of [^{57}Co] labelled cyanocobalamin bound to I.F. were compared on the same graph, (Graph 8) it was shown that there was a continual exchange between free and bound cyanocobalamin and that the rate of the exchange was dependant on the concentration of free cyanocobalamin present in the reaction mixture.



GRAPH 8 - Comparison of the rates of exchange of free and I.F. bound cyanocobalamin with increasing concentrations of free cyanocobalamin.

Since it is known that I.F. will achieve its maximum binding capacity of cyanocobalamin in the presence of excess cyanocobalamin in approximately 20 seconds (52), the rate limiting step for the exchange of free and bound cyanocobalamin must be the release of the bound form from the I.F. molecule. The maximum rate for this step had not been reached at the 9-fold excess of free cyanocobalamin, where if the initial reaction rate was extrapolated, there would

be 100% turnover of cyanocobalamin bound to I.F. in less than 2 hours.

The theoretical equilibrium level between labelled and unlabelled, for all three concentrations of unlabelled, had been over 80% achieved by 8 hours of incubation and nearly 100% by 16 hours.

The amount of [^{57}Co] bound to I.F. in the control tubes had only fallen a few percent by 24 hours. Therefore, the disappearance of bound radioactivity from the supernatant of the samples was not due to denaturation of the I.F.

SECTION 6

AMINO-ACID AND CARBOHYDRATE ANALYSIS

A sample of pure hog I.F. prepared by this method of affinity chromatography was subjected to amino-acid and carbohydrate analysis by Dr. G. Leaf, Department of Biochemistry, University of Glasgow.

A total of 120 µg of protein (as judged by the Lowry method) was hydrolysed -

(a) with 2.0 ml 6M HCl at 115°C for 16 hours

(b) with 0.5 ml 3M methane sulphonic acid at 110°C for 16 hours in an evacuated sealed tube. Dissolved air was removed by repeated freezing and thawing under vacuum.

The analyses were performed on a Jeol 105C amino acid analyser using the conditions described by Benson et al (55) except that the temperature was maintained at 70°C throughout.

RESULTS

The results of the amino acid analysis using the two different conditions of hydrolysis are shown in Table 9.

TABLE 9 - Amino-acid and carbohydrate analysis of 120 µg pure hog I.F. using two different conditions for hydrolyses

a) with 2.0 ml of 6M HCl at 115°C/16 hours

b) with 0.5 ml 3M methane sulphonic acid at 110°C/16 hours

AMINO-ACID	NANOMOLES	
	(a)	(b)
Lysine	63.5	57.0
Histidine	20.4	20.2
Arginine	51.8	49.0
Aspartic Acid	97.0	106.1
Theonine	66.3 #	64.3 #
Serine	67.4	72.5 #
Glutamic Acid	106.0	106.9
Proline	78.5	71.3
Glycine	85.1	83.5
Alanine	76.1	85.2
Cystine	8.1	9.7
Valine	54.3	39.5 *
Methionine	16.3	19.4
Leucine	92.3	87.4
iso-Leucine	55.2	37.2 *
Phenylalanine	33.7	31.8
Tyrosine	29.6	30.1
Carbohydrates		
Glucosamine	-	33.8 #
Galactosamine	-	-

\# corrected for destruction during hydrolysis
* recoveries expected to be low for this type of hydrolysate.

The specific activity of the hog I.F. was 30 ng B_{12} binding/ mg protein.

Therefore, if we assume approximately 90% yield for the amino acid hydrolysis, then 108 µg of hog I.F. could bind 2.38 nanomoles of B_{12}. By dividing the individual quantities of each amino acid shown in Table 9, a value is given for the number of nanomoles of amino acid present in the protein when it binds 1 nanomole of B_{12}. Since it has already been established that 1 molecule of I.F. binds 1 molecule of B_{12}, the figures found are numerically equal to the number of residues of each amino acid per mole of bound B_{12}. These figures are shown in Table 10 and compared with the result of Allen et al (18) for the same protein.

TABLE 10 - Comparison of the amino acid and carbohydrate composition of the hog I.F. purified in the present study and that of Allen et al.

AMINO ACID	Residues per mole of bound B_{12}	
	Present Study Results #	Allen et al
Lysine	24	17
Histidine	8	9
Arginine	21	12
Aspartic Acid	45	50
Theonine	27	37
Serine	31	36
Glutamic Acid	45	48
Proline	30	31
Glycine	35	25
Alanine	36	32
Cystine	8	9
Valine	23	26
Methionine	8	10
Leucine	37	43
iso-Leucine	23	25
Phenylalanine	13	14
Tyrosine	13	9
Carbohydrates		
Glucosamine	14	12
Galactosamine	-	8

These results are calculated from the results in Table 9b except for Valine and iso-leucine whose results were thought to be low for this hydrolysate, and the values for them shown in list (a) were used.

The amino acid results are fairly comparable between the two sets of results. The values for Lysine, arginine and glycine are rather higher than those found by Allen et al and the Threonine value is somewhat lower.

The more striking difference was the failure to demonstrate the presence of galactosamine at all in the present hog I.F. preparation.

Gas liquid chromatography was not performed on the hog I.F., therefore no value was found for the Fucose, Galactose or mannose concentrations. Similarly, no value was found for Tryptophan or Sialic Acid.

The molecular weight of the residues shown in Table 10 is 56,230 which compares favourably with the molecular weight of the corresponding Allen results (56,776).

If a correcting factor is added for the missing results, based on the results found by Allen, then the molecular weight of hog I.F. including amino acid and carbohydrate would be approximately 64,500.

DISCUSSION

The molecular weight of both human and hog I.F. was found to be approximately the same both by ultrafiltration and gel filtration. The ultrafiltration put the value between the limits of 50,000 and 100,000 but was not capable of any further resolution. Using gel filtration on Sephadex G200 the value of 56,000 $\pm$ 2,000 was found for I.F.-cyanocobalamin complex and approximately 64,000 for I.F. alone. This discrepancy probably means that the I.F. molecule changes configuration slightly when bound to B_{12}, taking up a more tightly spherical shape. This is consistent with the concept of Gräsbeck (53) that the B_{12} molecule fits into a pit in the I.F. molecule.

These molecular weight values are in reasonable agreement with the value found from amino acid analysis and agree with the values found by Allen et al for the monomeric forms (17,18). This group did find a lower molecular weight - 44,200, for human I.F. by amino-acid analysis although the gel filtration value was 66,000.

At no time in the present study was evidence for a higher molecular weight form of I.F. found, i.e. a dimer or oligomer, either using ultrafiltration or gel filtration. These forms have been found by Allen et al and by Highley et al (56) who claim dimers of I.F. form slowly in the presence of high concentrations of I.F., but even when the present preparations were allowed to incubate for 24 hours with excess B_{12}, no dimers were found. This is

probably a much longer time than I.F. will exist for in a physiological environment. Therefore dimers can have no useful function.

Higher molecular weight B_{12} binding protein was found in some less pure preparations, but it did not have the immunological identity of I.F., since it would not form precipitin lines with I.F. antibody on Ouchterlony plates.

The other possibility exists that the lower molarity of the guanidine-HCl used to elute the affinity chromatography columns in the present work, only elutes a species of I.F. with an incomplete complement of carbohydrate. This decreased carbohydrate content making this I.F. more susceptible to partial denaturation thus elution. This would explain the failure to detect any galactosamine during carbohydrate analysis. If this were so, it may mean that the carbohydrate part of the glycoprotein is responsible for oligomer formation. It is quite feasible that the carbohydrate content of the individual I.F. molecules varies quite considerably since this part of the molecule is added enzymically after the protein part has been synthesised. This might also explain the microheterogeneity of I.F. preparations on isoelectric focusing (57,58) which showed up to seven separate components in the work done by Gräsbeck.

Some workers have found the molecular weight of human I.F. to be over 100,000 (59, 60) but this can only be explained on the basis of the present work if their preparations contained a high

proportion of non-I.F. binders or if they did possess an oligomerised form.

Ultrafiltration was found to be a useful tool for increasing the specific activity of the final preparations and for removing "protein debris". Some of the smaller molecular weight material, presumed to be fragments of I.F., contained residual B_{12} binding activity but this could not be blocked by I.F. antibody.

Both the pure I.F. preparations were very stable in the absence of proteolytic enzymes over a period of time between pH 6 and 10.5. Below pH 5 and above pH 11.5 their B_{12} binding ability fell off quite rapidly. A comparison of Graph 6 and Graph 7 suggests that the I.F. is more stable to pH when bound to cyanocobalamin than when not.

The mode of action of I.F. in vivo would require that it be capable of functioning over a range of pH values. It is produced into an acid environment which facilitates the release of B_{12} from foodstuffs allowing the I.F. to become complexed with it whence it passes into a neutral environment in the intestines where it has its maximum binding capacity. This increase in B_{12} binding capacity on moving from acid to neutral environment is demonstrated in Table 8.

These results show that over the short term the pH inactivation of I.F. is not caused by denaturation but merely that the extreme

acid pH environment is not conducive to B_{12} binding. This suggests that the amino acids at the B_{12} binding site have an optimum pH which is slightly basic. The reduction in cyanocobalamin binding ability caused by extremely basic pH tended to be of a more permanent nature, probably because the I.F. molecule has not evolved to exist in a very basic environment.

These results are in accordance with those found by Goldberg et al (61) and Shum et al (62) except no evidence was found for the increase in B_{12} binding ability found below pH 3 by the latter group for hog I.F. This group used commercial hog I.F. which probably contained at least 65% non-I.F. This increase in binding below pH 3 was probably due to this non-I.F. component.

The experiment, examining the turnover of cyanocobalamin on I.F. showed that in the presence of excess free cyanocobalamin, the equilibrium level of exchange between the initially bound and initially free cyanocobalamin was over 80% achieved after 8 hours. Extrapolation of the initial turnover cyanocobalamin when a 9-fold excess of free cyanocobalamin was present showed 100% turnover of cyanocobalamin in less than 2 hours.

These rates appear to be about double that found by Donaldson and Katz (63) but again this is probably due to the fact that they used gastric juice as the source of I.F.

This result highlights the importance of fasting before and after a Schilling Test or Whole Body cyanocobalamin retention test.

A table of vitamin B_{12} content in foodstuffs shown by Chanarin (64) suggest that 10 µg B_{12} could easily be ingested.

This amount could exchange with at least 50% of the labelled cyanocobalamin on the I.F. before absorption, giving a falsely low result.

The comparison of the results of the amino acid analysis with those of Allen et al (18) shows that the protein isolated was most likely the same.

The difference in the carbohydrate composition is difficult to explain except in terms of the differences between the two methods of isolation, i.e. temperature and molarity of guanidine-HCl used.

It is disappointing that these results were not closer, because it is probable that the different properties of I.F. found by different groups of workers cannot be resolved until a universally accepted method of preparation of pure I.F. is established.

CHAPTER IV

IMMUNOLOGICAL PROPERTIES OF HUMAN AND HOG I.F. AND HUMAN AND HOG I.F.-CYANOCOBALAMIN COMPLEX

INTRODUCTION

In order to find if there were any immunologically recognisable differences between the structure of pure human I.F. and pure hog I.F., antibodies to each were raised in rabbits. It was hoped to raise pure Type I or blocking antibodies to pure human I.F. and pure hog I.F. and examine the cross specificity between them to the heterologous I.F. and also to heterologous and autologous pure I.F.-cyanocobalamin complex.

Impure commercial hog I.F. preparations have long been used orally in the treatment of pernicious anaemia. It is known that patients undergoing long term treatment with hog I.F. develop a refractoriness to it (65) and that this state is accompanied by the appearance of antibodies specific to hog I.F. in the patient's serum. These antibodies do not block the binding of B_{12} to I.F. (66) nor do they affect the absorption of B_{12} administered orally with human I.F. Therefore, it seems likely that antigenic determinants of such antibodies are not on the B_{12} binding site of the hog I.F. molecule and that these antibodies are species specific. Such antibodies have been called Type II or binding antibodies and are in contrast to Type I antibodies which were thought not to be species specific (67), although some workers could not demonstrate this cross specificity, (68, 69). This was probably due to the high proportion of non-I.F. B_{12} binders contaminating the hog I.F. preparations used, which could be as high as 90% (70).

It was also hoped to use these pure antibodies in the "in vitro" assay for I.F. described in Chapter II instead of serum from pernicious anaemia patients. Furthermore, it was hoped that such an antibody might be used to develop a more specific immunoassay for I.F. (see Chapter V).

A specific antibody for I.F. could perhaps be used to trace more accurately the metabolism of I.F. in mammals. Some preliminary work was carried out with the antibody produced to pure human I.F. by Dr. I. Brown in the Department of Pathology, Western Infirmary, Glasgow, using a peroxidase staining technique.

SECTION I

IMMUNISATION OF RABBITS

Intrinsic factor preparations, isolated as described above, were used to immunise New Zealand White female rabbits. Blood was taken from the rabbits before immunisation commenced, allowed to clot overnight at 4°C, spun at 5,000g for 15 minutes and the serum removed and stored at -20°C in 1 ml aliquots for use as controls in later immunoprecipitation experiments. Three rabbits received Human I.F. and four received Hog I.F. Each dose contained approximately 75 µg I.F. as measured by Vitamin B_{12} binding activity, in aqueous solution in a 2 ml emulsion with Freunds adjuvant in a 1 : 1 ratio. The components were emulsified by repeated passage through a fine guage syringe needle. The dose was administered by one x 0.5 ml intramuscular injection into each hind quarter and several approximately 0.1 ml subcutaneous injections into the rabbit's sides, back and neck. The initial two doses were mixed with Freund's incomplete adjuvant and subsequent doses repeated at 14 day intervals, with Freund's complete adjuvant. Blood was collected by nicking a marginal ear vein after the fourth dose and tested for antibody activity. After the titre of antibody had risen to a reasonable level, the time between booster doses of antigen was lengthened and the amount of antigen reduced to approximately 30 µg/dose.

DETECTION OF ANTIBODIES IN RABBIT SERUM

The initial test for presence of antibody in Rabbit Serum was to utilize a modification of the procedure used to measure I.F. activity "in vitro" described in Chapter II, in which the I.F. preparation used as antigen was substituted for the test material and the rabbit serum as source of antibody. The uptake of B_{12} by the rabbit antibody serum (Tubes 2) was more important in this test since the B_{12} binding capacity of rabbit serum was much higher than human serum, and thus would cause an erroneous result if not taken into consideration. If this test proved positive for a given rabbit, further quantification and characterisation of the antibody was carried out.

QUANTIFICATION OF I.F. ANTIBODY IN RABBIT SERUM

In order to assay the titre of I.F. antibody present in a given sample of rabbit serum, a modification of the method of Ardeman & Chanarin (71) was used.

Two sets of duplicate glass test tubes were set up and labelled. One set received 50 µl each of normal rabbit serum and the other set received 50 µl each of rabbit serum containing I.F. antibody. Then increasing amounts of the I.F. preparations, which were used as antigen to raise the particular antibody being assayed, were serially added to both sets of test tubes. The

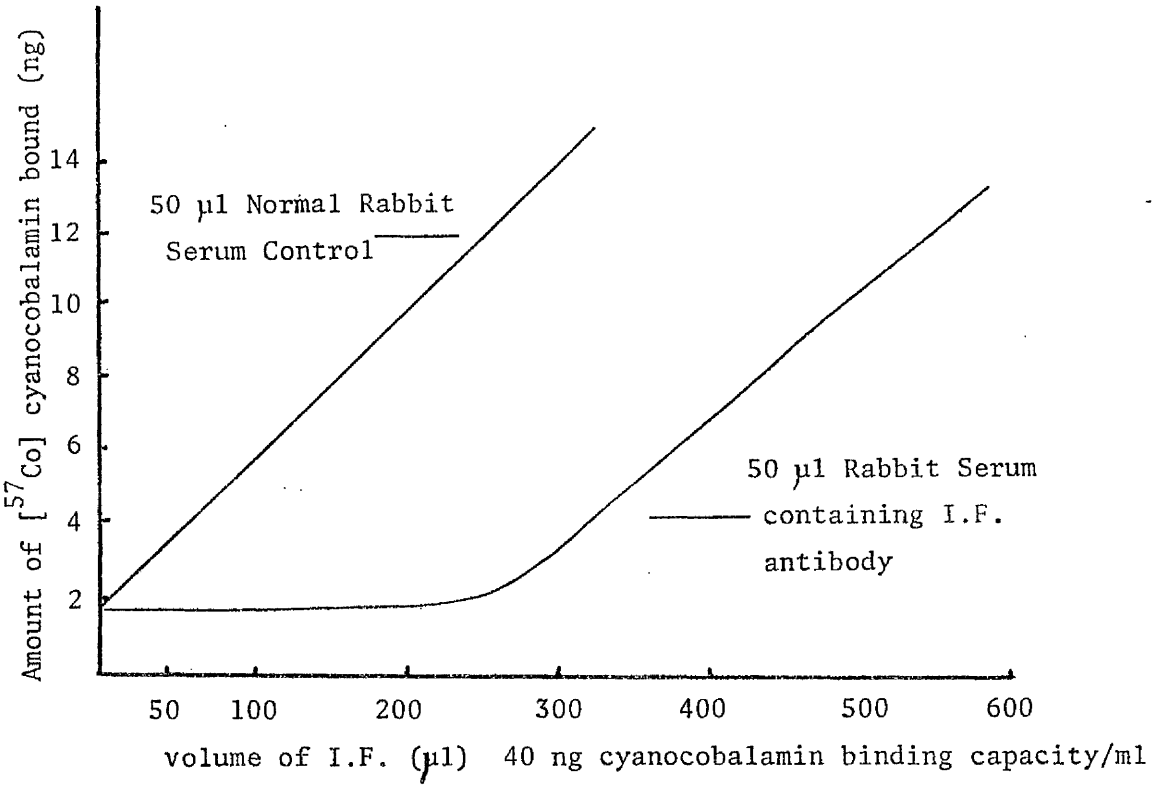
I.F. preparation had previously been diluted to contain 40 ng cyanocobalamin binding capacity per ml, and the amounts added to the tubes were 50, 100, 200, 250, 300, 350, 450 µl etc. respectively. Normally 600 µl (24 ng cyanocobalamin binding capacity) was enough to use up all the antibody in a given 50 µl sample of serum. The volumes in all the tubes were equalised with 0.1M phosphate buffer pH 7.5.

After 5 minutes incubation at room temperature with occasional shaking, 30 ng [^{57}Co] cyanocobalamin, containing approximately 10,000 c.p.m. was added to each tube in a volume of 0.5 ml.

The tubes were incubated for a further 10 minutes, then the free [^{57}Co] cyanocobalamin was separated using albumin coated charcoal and centrifugation as described previously. One ml aliquots of the clear supernatant were counted for protein bound [^{57}Co] activity and by calculation the amount of I.F. binding to each tube was found and expressed in ng units.

RESULTS

The titre of antibody found after two months of immunisation as described above, in the serum of a rabbit innoculated with pure hog I.F. is shown in Graph 9.



GRAPH 9 - Amount of [^{57}Co] cyanocobalamin bound by increasing amounts of hog I.F. in the presence of either 50 µl normal rabbit serum or 50 µl rabbit serum containing antibody to hog I.F.

From Graph 9 it can be seen that in the absence of I.F. the 50 µl of rabbit serum could still bind 1.7 ng of cyanocobalamin. Therefore, this serum has a cyanocobalamin binding capacity of 34 ng/ml.

The hog I.F. used was previously diluted to contain 40 ng cyanocobalamin binding capacity per ml. It is, therefore, not surprising that when incubated with the fixed amount of normal rabbit serum the I.F. bound cyanocobalamin found in the supernatant

after treatment with albumin coated charcoal increased by approximately 2 ng with every 50 µl increase in the amount of hog I.F. added.

With the serum containing the antibody, the amount of I.F. bound [^{57}Co] cyanocobalamin did not start to increase until 270 µl of I.F. preparation had been added. This corresponds to the blocking of 10.8 ng cyanocobalamin binding by the hog I.F. by 50 µl serum.

Therefore, the titre of the antibody could be expressed as 216 units of antibody per ml, since 1 ml of serum would block the binding sites of an amount of hog I.F. which would have bound 216 ng of cyanocobalamin in the absence of the antibody.

SECTION 2

CHARACTERISATION OF INTRINSIC FACTOR ANTIBODIES

Ouchterlony Plates were prepared as follows:-

Agar containing 1.7% w/v "Oxoid" purified agar and 0.1% w/v sodium azide in 0.9% w/v saline was melted in an oven at 120°C. Twenty ml of this agar was pipetted into each disposable petri dish used and allowed to set at room temperature. After setting wells were cut in the agar using cork borers at a distance of approximately 12mm (6mm diameter for antigen well and 10mm for serum well). The agar plugs were removed with a pasteur pipette using suction. Various patterns were used for the wells, depending on the antibody/antigen reaction being studied. The plates were kept at room temperature after the wells had been filled and were observed every 24 hours for signs of precipitation. If the titre of any sample under test was low, its well was refilled after the first aliquot had diffused into the agar.

RESULTS

After several days incubation, single precipitation lines appeared between the rabbit anti-human I.F. serum and both pure human I.F. and pure hog I.F., also a faint accompanying line

appeared between this antibody and the human I.F.-cyanocobalamin complex.

Single precipitation lines appeared between the rabbit anti-hog I.F. antibody and both the pure hog I.F. and pure human I.F. but no lines appeared between this antibody and either the hog I.F.-cyanocobalamin complex or the human I.F.-cyanocobalamin complex.

This suggested that there was cross specificity between the human and hog Type I antibodies raised to the pure I.F. preparations.

It also suggested that the rabbit anti-human I.F. antibody serum might contain more than one antibody.

Either of the antibody sera was capable of blocking the I.F. mediated B_{12} binding capacity of normal human gastric juice on commercial hog I.F. preparations, but good precipitin lines could not be shown with either of these antigens, probably due to the low I.F. concentration.

REACTION OF EXPERIMENTALLY PRODUCED ANTIGEN TO HETEROLOGOUS AND AUTOLOGOUS I.F. AND I.F.-B_{12} COMPLEX IN SOLUTION

In order to explore further the cross reactions of the antibody raised to pure human I.F. and the one raised to pure hog I.F., it

was necessary to set up a series of test tubes containing all the possible combinations and permutations of the materials available. These were :- rabbit antiserum against human I.F. , antiserum against hog I.F., pure human I.F. and pure hog I.F., both of which could be complexed with either unlabelled or [^{57}Co] labelled cyanocobalamin and pure human I.F. and pure hog I.F. labelled with [^{125}I] (protocol, Ch.5).

Briefly, 0.1 ml of either antiserum, diluted to contain 5 ng units of antibody, was preincubated for 30 minutes at room temperature with a 50-fold excess of either the heterologous or autologous I .F. or I.F.-unlabelled cyanocobalamin complexed in a volume of 1 ml in 0.1M phosphate buffer pH 7.5 in duplicate conical glass test tubes. Control tubes were also set up which contained 0.1 ml of normal rabbit serum, collected before immunisation commenced. Then to each tube was added 0.1 ml, 5 ng units of heterologous or autologous I.F. labelled either by having complexed to [^{57}Co] cyanocobalamin or with [^{125}I].

The permutations and combinations used are shown in Figs. 3, 4 and 5.

After a further 30 minutes incubation at room temperature with occasional shaking, 0.1 ml of a 1 in 40 dilution in saline of donkey anti-rabbit precipitating serum (Wellcome Reagents Ltd.) was added to each tube, shaken, and left overnight at 4°C.

FIGURE 3 - CONTROL SERA

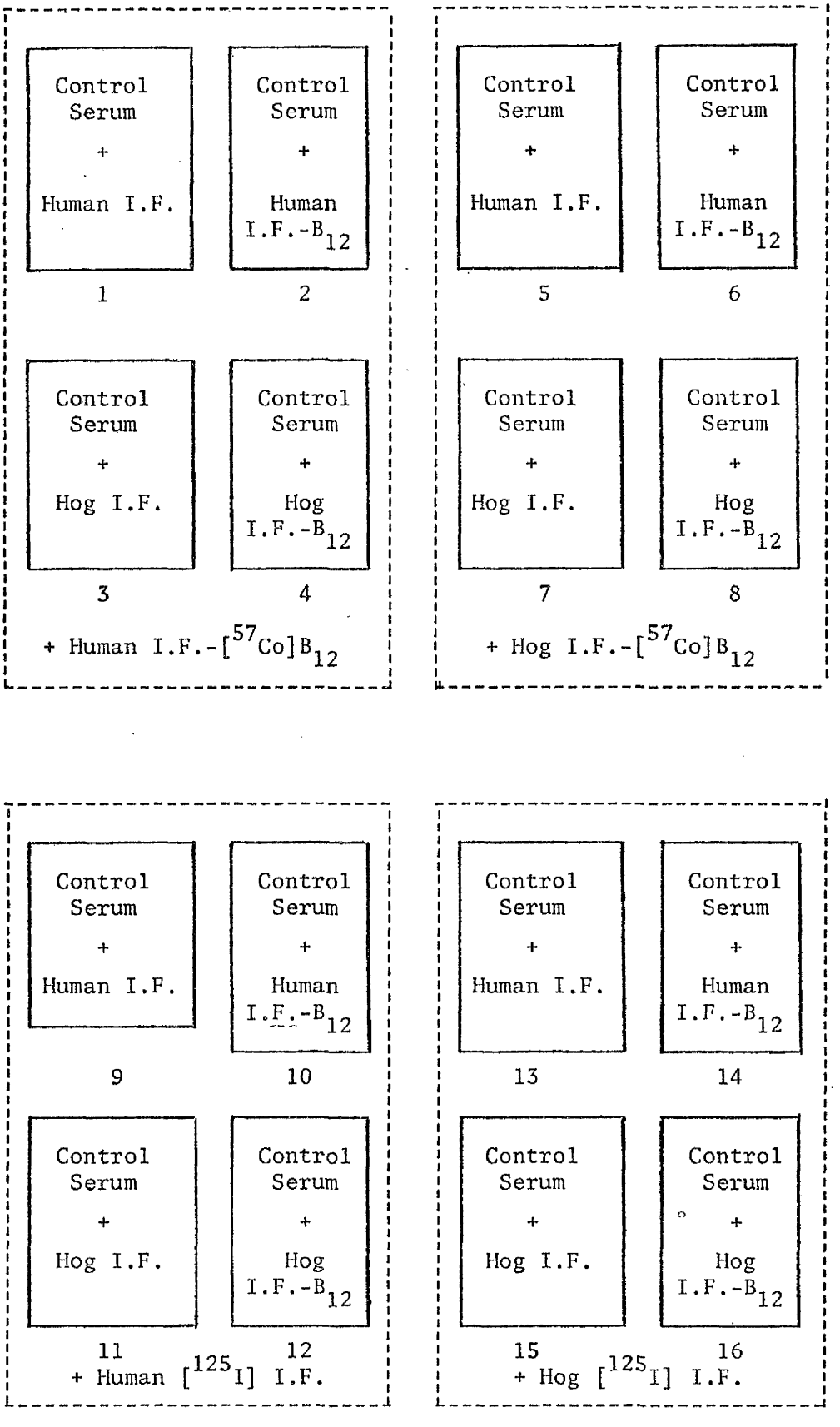


FIGURE 4 - HUMAN I.F. ANTIBODY

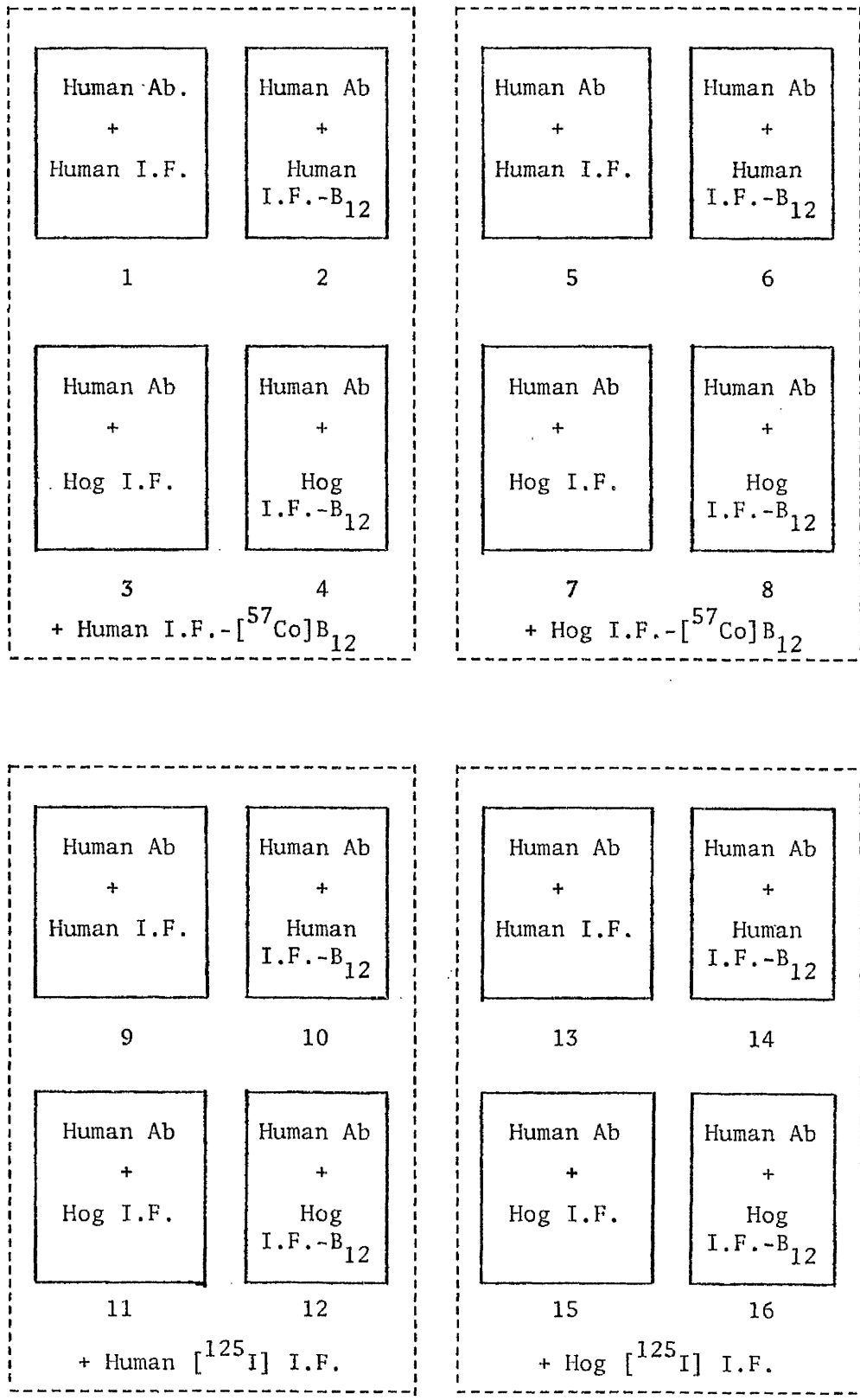
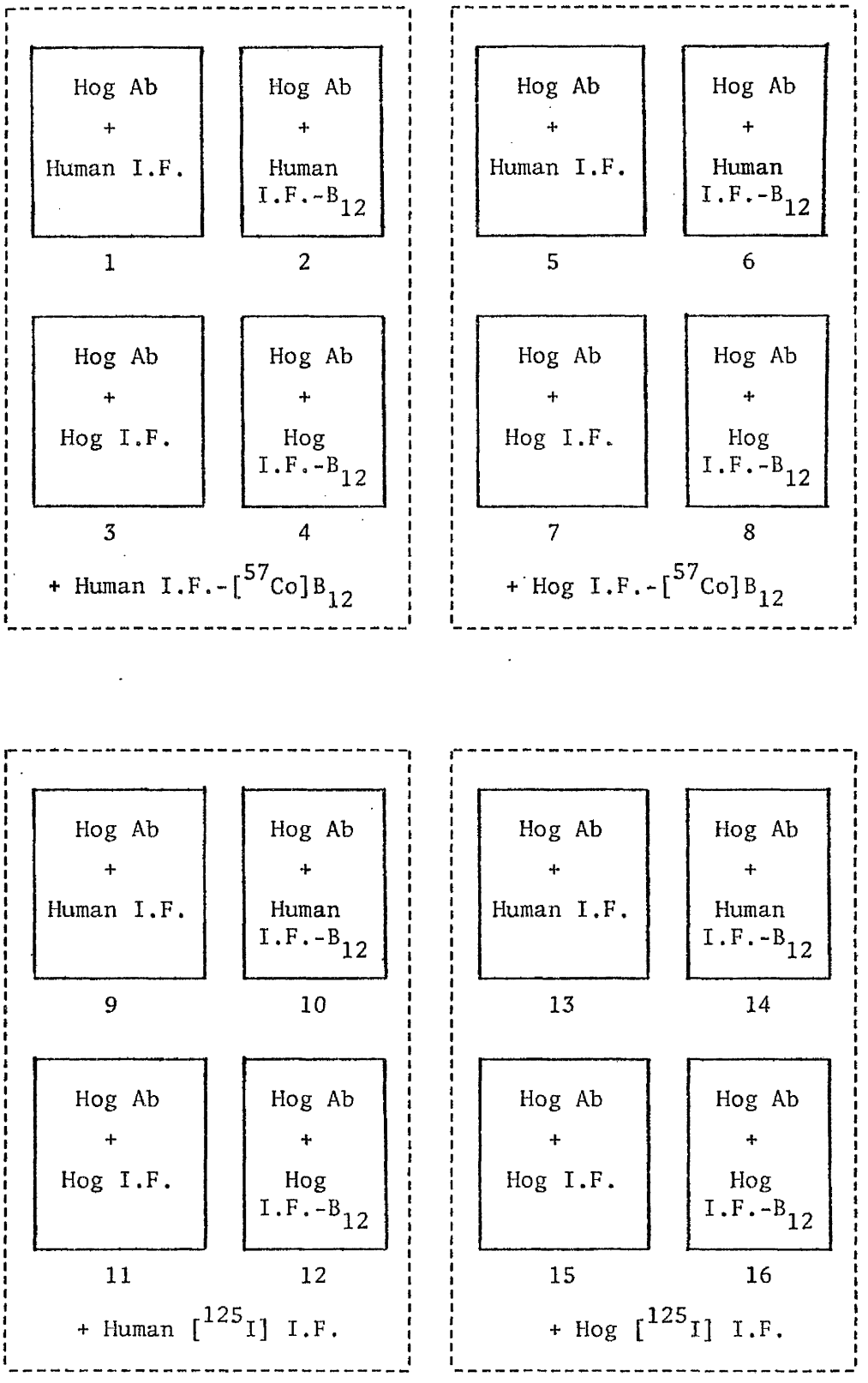


FIGURE 5 - HOG I.F. ANTIBODY



The tubes were then centrifuged at 10,000g for 15 minutes. The supernatants were sucked off with a pasteur pipette and discarded. The tubes were allowed to drain upside down onto paper towels for 15 minutes then the pellets were counted in the well-type gamma-radiation counter. The KeV and E.H.T. settings of the J & P Engineering counter/rate meter were adjusted for maximum efficiency on [^{57}Co] or [^{125}I] standards as required.

RESULTS

The results shown in Table 9 are the average of the duplicate tubes over two runs of the experiment. The numbers refer to the numbers of the tubes shown in Figs. 3, 4 and 5.

TABLE 11 - The percentage added I.F. activity [^{57}Co] or [^{125}I] precipitated out by the donkey anti-rabbit precipitating serum after preincubation of the antibody with excess heterologous or autologous I.F. or I.F.-B_{12} complex.

TUBE No.	PERCENTAGE ACTIVITY IN PELLET (Tubes 1-8 are [^{57}Co] activity: tubes 9-16 are [^{125}] activity)		
	Normal Rabbit Serum Control (Fig. 3)	Rabbit serum antibody against human I.F. (Fig. 4)	Rabbit serum antibody against hog I.F. (Fig. 5)
1	4.2	6.2	2.7
2	3.7	3.7	5.2
3	4.1	29.8	3.6
4	2.7	32.4	2.8
5	3.5	2.4	4.4
6	4.2	4.7	7.2
7	4.1	6.3	5.4
8	7.2	3.7	6.1
9	1.9	2.5	8.2
10	3.6	76.7	78.2
11	4.3	7.5	3.9
12	8.7	69.8	81.3
13	4.4	3.4	6.7
14	3.2	63.2	72.2
15	3.1	5.9	4.9
16	2.5	67.4	74.3

As might be expected, the pellets from tubes detailed in Fig.3 all contained less than 10% of the added I.F. activity since there was no specific antibody in the control serum to bind with any of

the pure I.F. preparations.

In the pellets from the tubes detailed in Fig. 4 which contained the antibody serum raised to pure human I.F. about 30% of the added activity was found in Tubes 3 and 4. This suggested that there was an antibody to Human I.F.-B_{12} complex present. Few counts appeared in pellet 2 which backed up this proposal, since the excess of human I.F.-unlabelled B_{12} present would use up such an antibody. Furthermore, few counts appeared in pellets from Tubes 5, 7 and 8 which might suggest that this antibody is species specific.

In the human I.F. antibody tubes to which [^{125}I] labelled I.F. not complexed to cyanocobalamin was added, about 70% of the activity appeared in Tubes 10 and 12. This suggested that the rabbit antiserum to human I.F. contained a second antibody which was not blocked by I.F.-cyanocobalamin complex. This antibody was not species specific since it bound to both human and hog [^{125}I] I.F. as shown by the approximately 60% binding in Tubes 14 and 16. This percentage was slightly lower than Tubes 10 and 12 which was probably due to the rabbit anti-human I.F. Type II antibody not binding to the hog [^{125}I] labelled I.F.

The I.F. antibody appears from these results to be present in approximately double the concentration of the I.F.-B_{12} complex antibody. The appearance of two antibodies could have been

caused by a minute amount of human I.F.-B_{12} complex contamination in the human I.F. preparation used to immunise the rabbits.

The pellets from the tubes detailed in Fig. 5 only contained a high percentage of added activity in Tubes 10, 12, 14 and 16 which suggests that only one antibody was present.

This antibody raised against pure hog I.F. does not appear to be species specific since it binds to both human and hog [^{125}I] labelled I.F. but it was not blocked by either of the I.F.-cyanocobalamin complexes.

SECTION 3

The Use of the rabbit anti-pure human I.F. antibody conjugated to peroxidase for staining of sections of human stomach.

The rabbit anti-human I.F. antibody was used in a modification of the technique described by Petts and Roit (72) to stain sections of human stomach.

1. First, fixed sections were washed with 0.5% hydrogen peroxide in methanol to block the endogenous peroxidase activity e.g. from red blood cells, eosinophils.

2. The collegen tended to be stained, but this was toned down by preincubating the section with swine normal serum diluted 1 in 5 in Tris-saline.

3. The section was then washed and incubated with the rabbit anti-human I.F. antibody diluted 1 in 250 with the tris-saline.

4. The section was again washed and swine anti-rabbit protein antibody added.

5. The excess swine anti-rabbit protein antibody was removed and Peroxidase conjugated to rabbit anti-peroxidase antibody applied to the section. This antibody was bound to the

rabbit-anti-human I.F. antibody by the swine anti-rabbit protein antibody.

6. Finally, the section was washed and 3, 3' diaminobenzidine tetrahydrochloride was applied, giving rise to the colour reaction.

The principle of this colour reaction is :-

The electron donor is oxidised at the very site of action of the enzyme, (peroxidase, linked to the rabbit anti-human I.F. antibody) and not at a distance from it.

Diaminobenxidine, when donating electrons to the hydrogen peroxide via the peroxidase, apparently forms an oxidative intermediate (see Fig. 6) that rapidly polmerises to an amorphous insoluble brown deposit which can easily be seen under light microscopy.

Controls were set up using normal rabbit serum instead of the rabbit anti-human I.F. serum, and they showed no specific staining of any cells.

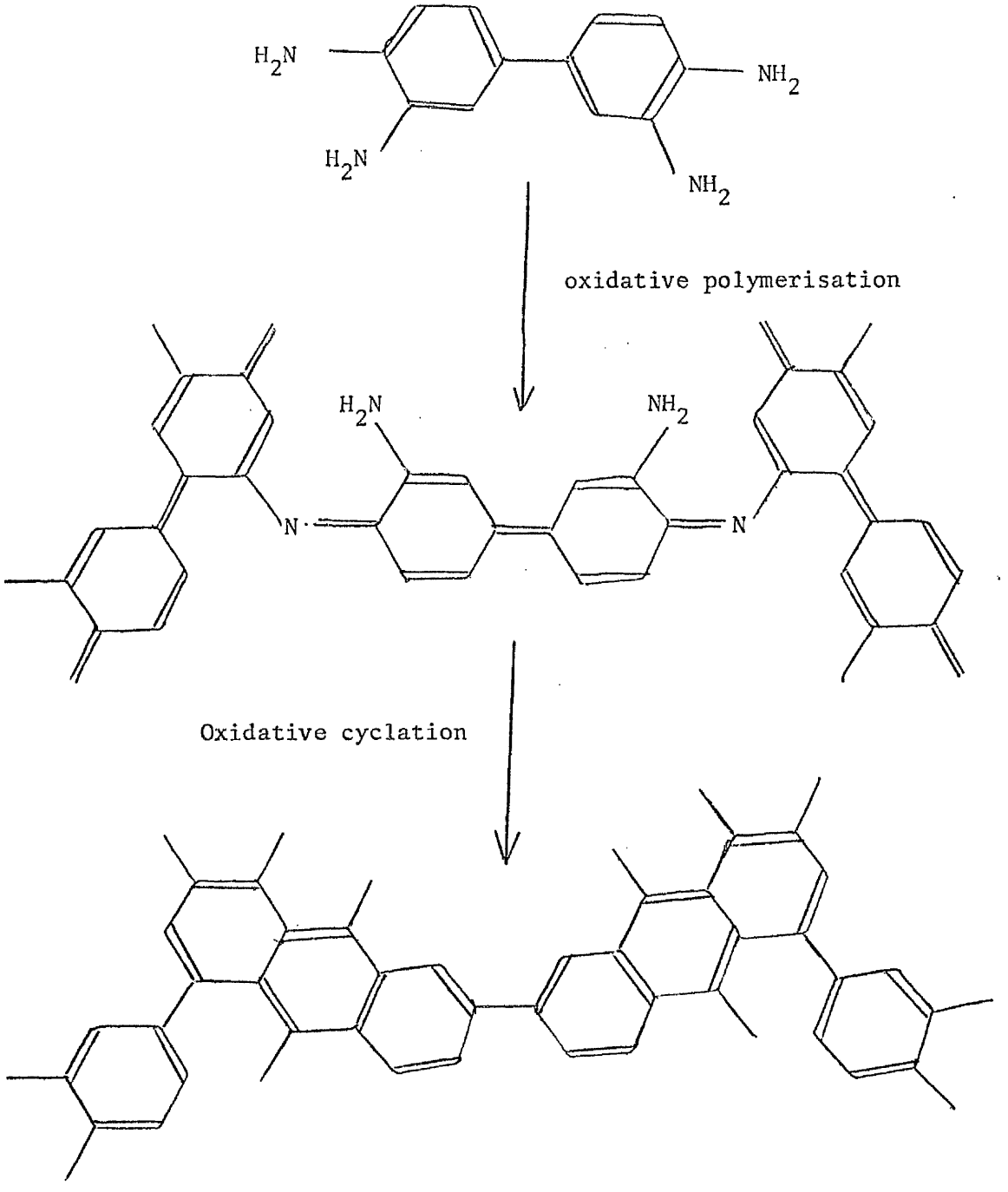


FIGURE 6 - Polymerisation of the diaminobenzidine to form the insoluble brown deposit visible under light microscopy

RESULTS

This technique has proved to be very effective in the preliminary work done so far. The examples shown in Illustrations 1 and 2 are comparisons of sections of stomach mucosa under 100-fold magnification stained (Illus. I) using a conventional periodic acid Schiff-toluidine blue-tartrazine method, and (Illus. 2) using the antibody linked peroxidase reaction using the diaminobenzidine stain.

The parietal cells, which are the site of production of I.F. are much more clearly defined in Illustration 2.

Illustrations 3 and 4 show a higher magnification (x 500) comparison of the two techniques.

The black staining with the immunoperoxidase reaction in Illustration 4 shows the presence of intrinsic factor within the parietal cells.

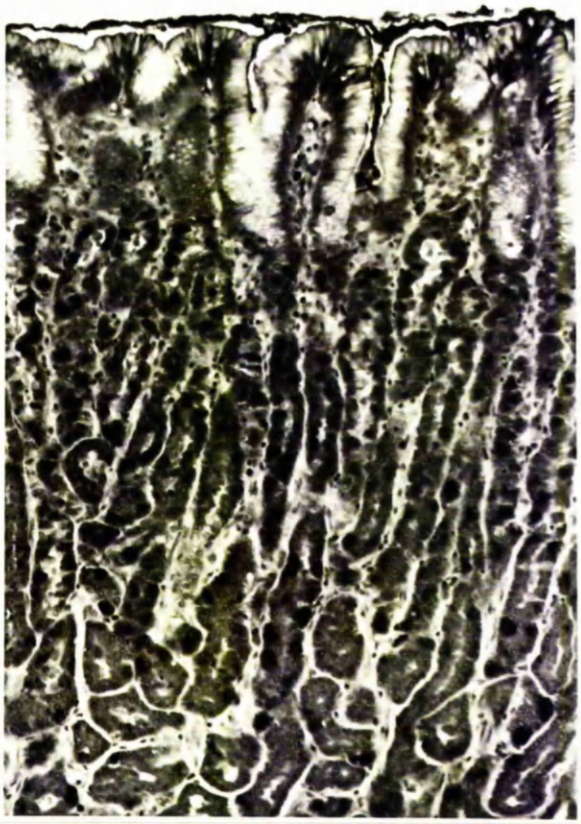
On some sections of stomach from patients with gastritis, where the mucosa had become atrophied and under conventional staining had the appearance more of intestinal mucosa, the I.F. antibody linked peroxidase showed up the parietal cells and made the identification of the sections much easier.

ILLUSTRATION I

Gastric mucosa

Specimen from patient with duodenal ulcer showing parietal cell hyperplasia. Section is stained with periodic acid Schiff-Toluidine Blue-Tartrazine in which the parietal cells stain yellow with the Tartrazine, and are the light grey cells in the photomicrograph. (x 100)

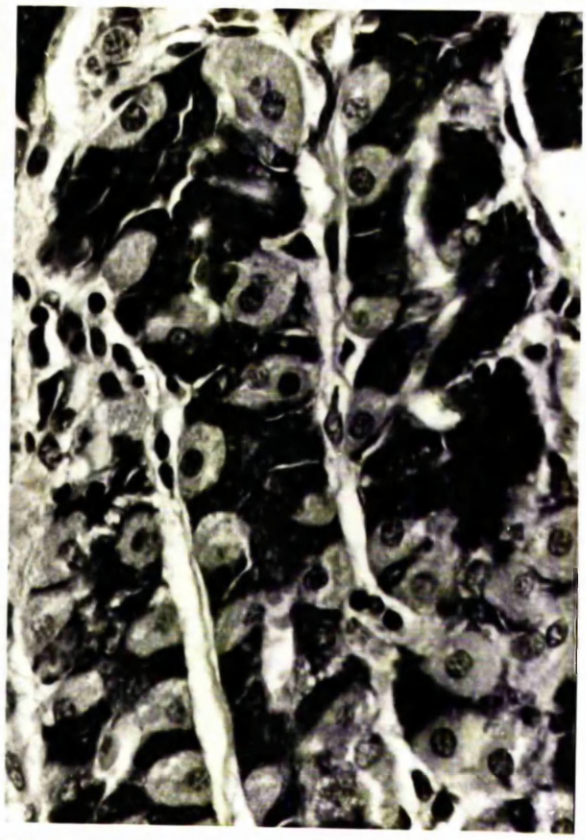
ILLUSTRATION 2



Gastric mucosa

treated with Rabbit Anti-human I.F. antibody, the immunological reaction being visualised by the Immuno-peroxidase technique (Peroxidase-antiperoxidase reaction) and stained with the diaminobenzidine (DAB) reaction. A positive reaction is indicated by an intense black colouration of the cells. The positive cells are all histologically parietal cells.
(PAP-DAB, Mayers Haematoxylon Counterstain x 100)

ILLUSTRATION 3



Gastric Mucosa -

Higher magnification of Gastric Tubular Glands showing typical parietal cells among peptic cells.

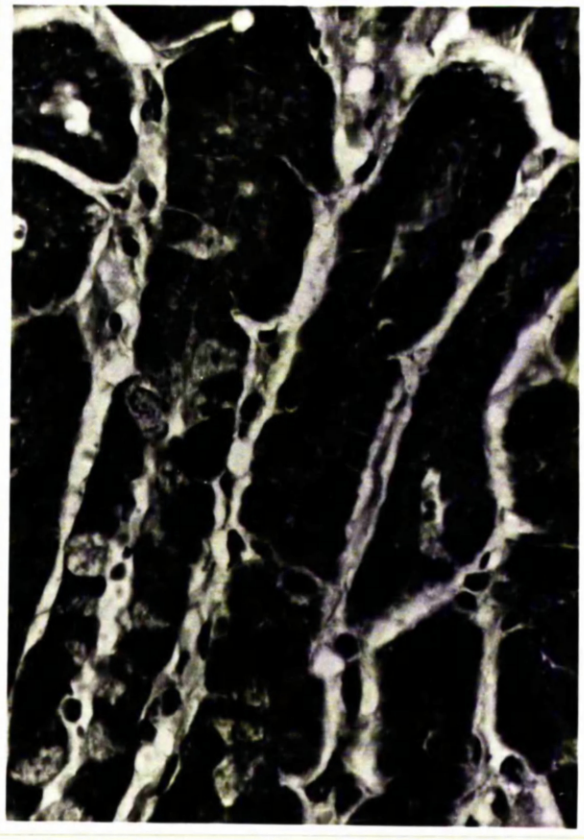
(PAS-TB-TART x 500

ILLUSTRATION 4

Gastric mucosa-

Higher magnification of gastric glands showing cells with the characteristics of parietal cells staining black with the immuno-peroxidase reaction, indicating the presence of intrinsic factor within the cells.

(PAP-DAB, Mayers Haematoxylin Counterstain X 500



An interesting development was that when the I.F. antibody-peroxidase staining technique was carried out on sections from liver and kidney tumours, some of the tumours had cells which appeared darkly stained, perhaps suggesting that the primary tumour came from the stomach.

DISCUSSION

None of the rabbits which were immunised with either of the I.F. preparations failed to produce any antibodies although the titres varied. Thus, the I.F. preparation was very antigenic.

The results of both the Ouchterlony plates and the reaction of the antibody and antigen in the presence of excess heterologous and autologous I.F. suggest that in fact, two antibodies corresponding to Type I and Type II antibodies appeared in the rabbits immunised with the human I.F. preparation. This was probably due to the presence of some cyanocobalamin in the human I.F. preparation, which was not intentional, but in fact, provided a more interesting result, since unlike the Type I antibodies, this antibody showed a marked species specificity and could not be blocked by pure hog I.F.-cyanocobalamin complex or pure hog I.F. This antibody was blocked by pure human I.F. not complexed to cyanocobalamin, therefore, the cyanocobalamin was not one of its antigenic determinants.

The fact that neither the human nor the hog anti-human I.F. Type I antibody was blocked by excess of either autologous or heterologous I.F.-cyanocobalamin complex, suggests that their antigenic determinants are on or near the cyanocobalamin binding site of the I.F. molecule. This binding site portion of the molecule must be very similar for both sepcies hence the cross specificity of the Type I antibody.

However, the molecules are not immunologically identical, since the Type II antibody, which has determinants remote from the cyanocobalamin binding site is species specific. These findings are in agreement with those of Gullberg (73, 74, 75).

One of the persisting uncertainties in B_{12} metabolism is whether the B_{12} is carried into the ileal enterocyte still bound to I.F. or whether the delay found in the appearance of B_{12} in portal blood (76) is caused by the time taken to be split from I.F. It being well known that B_{12} is not absorbed in the absence of I.F. (except if given in vast excess), is the role of I.F. merely to transport the B_{12} through the intestine and bind it to specific receptor sites, after which its role is ended? There is much evidence that this is certainly the initial role of I.F. using tissue homogenates (77) and everted sacs of animal intestines (78,79) etc., but it has not yet been resolved definitely if its role ends there.

In 1964, Herbert et al (80) suggested that B_{12} was split from I.F. during absorption but did not present any evidence to support this. Cooper & Castle (81) claimed to have found an I.F. releasing factor in homogenates of small bowel, but it has now almost certainly been shown that this releasing factor was free B_{12} itself, interchanging with the I.F. bound B_{12}.

More recently Katz and Cooper (82) isolated a soluble receptor from human intestine, but they have not shown whether this receptor mediates the absorption of I.F.-B_{12} or only B_{12}.

The fact that patients treated with oral hog I.F. develop antibodies towards hog I.F. seems to suggest that I.F. is absorbed along with the B_{12}. The refractoriness to treatment with oral hog I.F. associated with these antibodies is not evident when human I.F. is given orally. It is possible that only part of the I.F. molecule is absorbed, but most likely part to be absorbed would be the B_{12} binding site area. The Type I I.F. antibodies raised in the present study displayed no species specificity but the antibody found in patients treated with hog I.F. does not affect the absorbance of B_{12} when it was given orally with human I.F.

Therefore, this antibody does show species specificity as did the Type II antibody which was raised in the present study and whose antigenic determinants were remote from the B_{12} binding site.

Therefore, it seems likely that at least most of the I.F. molecule is absorbed.

Recent work by Rothenberg et al (83) using differential centrifugation on guinea pig ileal homogenates, the animals being killed after [^{57}Co] labelled human I.F. had been incubated in their ileal loops, found a large percentage of the radioactivity was localised in the mitochondial fraction. This finding had been made before (84) but the more important result was that 57% to 78% of this activity could not be removed by coated charcoal and that 54% to 69% of the activity could be precipitated along with anti-human I.F. at 15% Sodium Sulphate concentration indicating that a high percentage of the B_{12} inside the enterocyte is localised at the mitochondrial membrane and is bound to a large molecule with the immunological identity of I.F.

The work of Peters & Hoffbrand (84) again using differential centrifugation, supports that B_{12} is localised in the mitochondial fraction during absorption, and in fact present results showing that a high proportion of the B_{12} is associated with the inner rather than the outer mitochondial membrane although they have no firm explanation for this.

Perhaps the enterocyte mitochondia are the site for the exchange of B_{12} from I.F. to its transcobalamin to which recently absorbed B_{12} is attached in portal blood, (85). If so, what then is the eventual fate of I.F.?

The evidence presented by Ardeman et al (86) that I.F. is not absorbed, is based mainly on the observations that patients with

pernicious anaemia who have serum antibodies to I.F. still absorb orally administered B_{12} bound to I.F., but the results presented here show that the activity of the Type I antibody was not blocked by I.F.-B_{12} complex, since the antigenic determinants are probably on or near the B_{12} binding site of the I.F. molecule. Therefore, I.F.-B_{12} absorption would not be affected by these antibodies.

These results obtained by Rothenberg (83) seem to be close to finding a solution to the question, but this work was still carried out using human gastric juice as the source of I.F.

The work could be repeated and expanded using the pure I.F. preparations and pure antibodies made possible by the use of affinity chromatography. Also with a pure I.F. preparation, it is possible to attach a label directly to the protein (See Chapter V), in order that the fate of the I.F. molecule after it has split from B_{12} could be followed.

Further the pure I.F. antibody linked peroxidase staining technique offers much scope for further localisation of I.F. sites of action. At the sub-cellular level it could be used at much higher magnification (electron microscopy) on sections from the pellets formed with differential centrifugation on tissue homogenates.

CHAPTER V

RADIOACTIVE LABELLING OF HUMAN AND HOG INTRINSIC FACTOR USING ^{125}IODINE

Until recently, the most common way of tracing the whereabouts of I.F., or for that matter, any B_{12}-binding protein, was to form a complex with radioactive [^{57}Co]. Much insight and information about I.F. metabolism has been gained using this technique, but it has a severe limitation.

The binding between I.F. and B_{12} is a chemical reaction which has an equilibrium constant, which can be affected by the concentrations of either component.

$$\text{I.F.} + B_{12} \rightleftharpoons \text{I.F.-}B_{12} \text{ complex}$$

Therefore, in the presence of free unlabelled B_{12}, the labelled B_{12} will effectively be diluted off the I.F. present. This means there is always an element of doubt about whether the I.F.-[^{57}Co]B_{12} complex is being monitored or free [^{57}Co]B_{12}.

Similarly, with the mechanism of B_{12} absorption, it is difficult to trace the fate of the I.F., since the complex may be split at the brush border of the distal ileum.

For these reasons, and also with a view to developing a more rigid "in vitro" assay for I.F., it was decided to try to incorporate a radioactive label directly into the pure I.F. preparation isolated in the present work.

SECTION I

PRODUCTION OF [^{125}I] LABELLED I.F.

METHOD

The only isotope studied for the purpose of labelling I.F. was [^{125}I]. The method employed was a modification of that of Greenwood et al (87) as described by MacKenzie et al (88).

These workers estimated the quantity of Chloramine T which would have the least destructive effect on the [^{60}Co] B_{12} binding activity of I.F. They used human I.F. purified by the method of Chosy & Schilling (32).

For the present study, a range of chloramine T concentrations, suggested by MacKenzies results, were tried to give maximum incorporation of label.

The best yields were found using the following protocol -

50 µg of either the human or hog I.F. preparation in 0.5 ml 0.1M phosphate buffer pH 7.5 was placed in a conical plastic disposable tube behind a 10 cm thick lead shield. 0.5 mCi Na^{125}I (Radiochemical Centre, Amersham) in a volume of 10 µl was added using an automatic pipette with a disposable tip. Then 30 µg Chloramine T (B.D.H.) was added in a volume of 15 µl and the plastic tube was

vortex mixed and allowed to incubate at room temperature for 10 minutes with occasional shaking.

The reaction was stopped by the addition of 120 µg sodium metabisulphite in a volume of 100 µl. The tube was vortex mixed again, and the contents applied to the top of a 10 cm x 0.5 cm Sephadex G-10 column to separate the free from bound [^{125}I], except for a 10 µl sample which was subjected to high voltage paper electrophoresis to find % incorporation of the label. This electrophoresis was carried out on 24 cm x 2 cm Whatmann No.1 paper strips, using 0.05M barbitone buffer pH 8.6 at a voltage of 500v for 50 minutes at room temperature.

The column was eluted with 0.1M phosphate buffer and 1 ml aliquots of the eluant were collected and counted for [^{125}I] activity.

The fractions containing the protein bound [^{125}I] were pooled, and another sample was subjected to high voltage electrophoresis to check the effectiveness of the column separation.

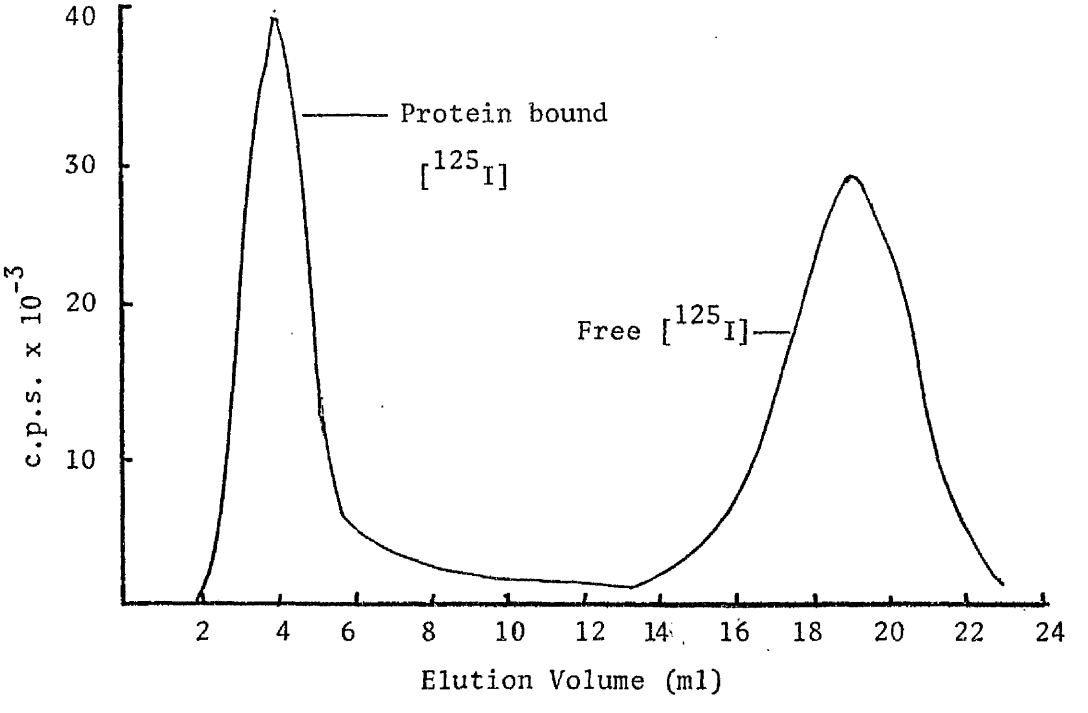
The paper strips were previously marked in 1 cm lengths from the origin at the Cathode.

When the electrophoresis was complete, the strips were dried in a drying cupboard at 37°C overnight, then cut into 1 cm long segments and each segment counted for [^{125}I] activity.

RESULTS

SEPARATION OF FREE FROM BOUND [^{125}I]

The 1 ml aliquots which were collected from the G-10 column were counted for [^{125}I] activity and the results are plotted in Graph 10).



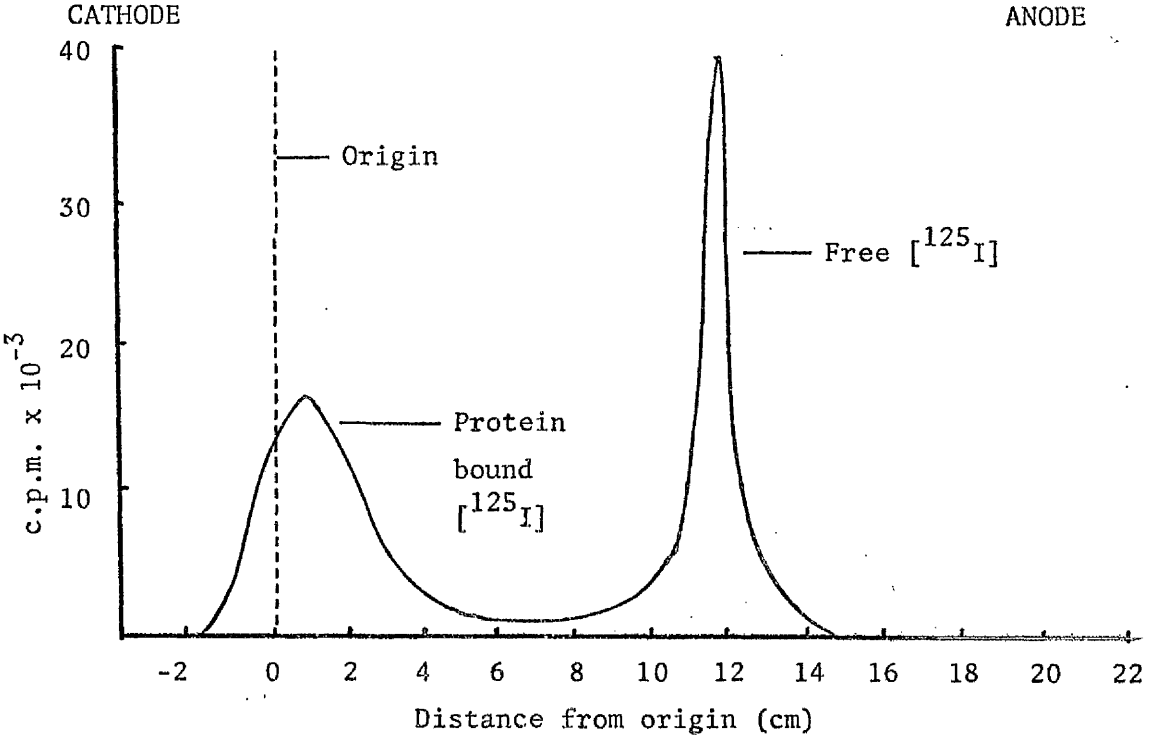
GRAPH 10 - Elution pattern of [^{125}I] labelled hog I.F. reaction mixture from Sephadex G-10 column

Graph 10 shows the elution pattern found when 50 µg pure hog I.F. was subjected to the [^{125}I] labelling process. The protein bound [^{125}I] was excluded from the column and appeared in a peak

between volume 3 ml and 6 ml. The free iodine did not appear until 19 ml had flowed through the column.

EFFICIENCY OF INCORPORATION

To find the percentage incorporation of the label in the protein, a 10 µl sample from the above reaction mixture was subjected to paper electrophoresis. After the paper strips had been run, dried and cut into 1 cm lengths, each segment was counted for [^{125}I] activity. The results are shown in Graph 11.



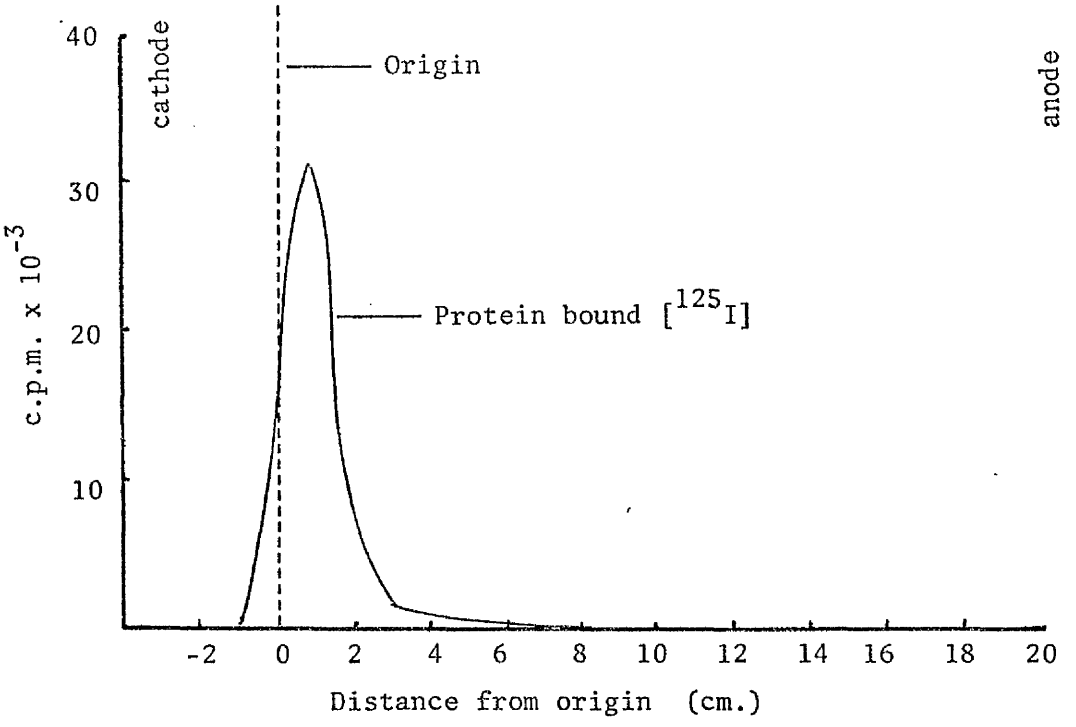
GRAPH 11 - Electrophoretic Distribution of [^{125}I] activity of 10 ul labelling reaction mixture run in 0.05M barbitone buffer at 500v for 50 minutes.

As can be seen from Graph 11, the protein bound [^{125}I] had remained close to the origin whereas the free [^{125}I] has migrated 12 cm towards the anode.

By adding of all the counts found in the protein peak and dividing by the total no. of counts, it was found that 43% of the [^{125}I] label had been incorporated in the I.F.

PURITY OF [^{125}I]-I.F. PRODUCT

A 10 µl sample was taken from the pool of the fractions from the G-10 column containing the [^{125}I] I.F. This was also subjected to high voltage paper electrophoresis as described above and the results are shown in Graph 12.



GRAPH 12 - Efficiency of separation of free from bound [^{125}I] on Sephadex G-10 column

From addition of all the counts found in the protein peak in Graph 12, it was found that the [^{125}I] eluted with the I.F. from the G-10 column was 97% bound.

SECTION 2.

PROPERTIES OF [^{125}I] LABELLED I.F.

It was necessary to check that the identity of the pure I.F. preparation had not been altered by the [^{125}I] labelling process.

Some of the results for this have already appeared in previous Chapters.

The [^{125}I] I.F. was subjected to gel filtration on a Sephadex G-200 column as described in Chapter III. The [^{125}I] activity was eluted in a single peak associated with a protein with a molecular weight very similar to I.F.-[^{57}Co] cyanocobalamin complex.

The B_{12} binding activity of the [^{125}I] I.F. complex was tested by the "in vitro" I.F. assay described in Chapter II. This procedure was complicated slightly by the overflow of the [^{125}I] activity into the [^{57}Co] energy band. This problem was eliminated by increasing the specific activity of the [^{57}Co] cyanocobalamin used and by introducing a 1 mm thick sheet of aluminium between the crystal of the counter and the sample. The aluminium screened out the [^{125}I] emissions but allowed the [^{57}Co] activity through to the crystal.

It was found that approximately 85% of the B_{12} binding activity of the I.F. was retained after the iodination.

A sample of [^{125}I] I.F.-[^{57}Co] cyanocobalamin was also applied to the Sephadex G-200 column and the two activity peaks coincided in the elution pattern.

In Chapter IV, the [^{125}I] I.F. was used to examine the cross-specificity of the human and hog I.F. antibodies. This experiment also showed that the [^{125}I] I.F. retains the immunological identity of I.F.

Thus the [^{125}I] I.F. was judged not to have been significantly altered by the process of iodination, using the method described, although an "in vivo" absorption test was not carried out with it because of its high activity (approximately 50 μCi/ug).

The rate of inactivation of the I.F. caused by the activity of the [^{125}I] was fairly high. On storage at -20°C about 50% of the B_{12} binding capacity of the I.F. was lost after 14 days, and at room temperature 50% binding was lost after four days. Lower activity samples could be made to reduce this inactivation.

SECTION 3

RADIOIMMUNOASSAY FOR INTRINSIC FACTOR

With an isotope labelled species of intrinsic factor and a pure antibody to it, it seemed logical to try to use these in an assay system for I.F. This assay would be specific for I.F. and would not recognise non-I.F. protein or even I.F. bound to B_{12}. Thus it could be used to probe tissue homogenates, portal blood etc. for the existance of absorbed I.F. molecules, perhaps after the transfer of the B_{12} from I.F. to the transcobalamins, if this happens within the cell.

There was not time to try the above experiment, but a preliminary assay was developed which appeared to work satisfactorily .

The rabbit anti-hog I.F. antibody was used as the fixed binder. It was diluted in normal saline to contain 5 ng units of antibody per ml.

A series of test tubes were set up and 5 ng units of [^{125}I] I.F. in a volume of 0.25 ml was added to each (approximately 0.2 µg).

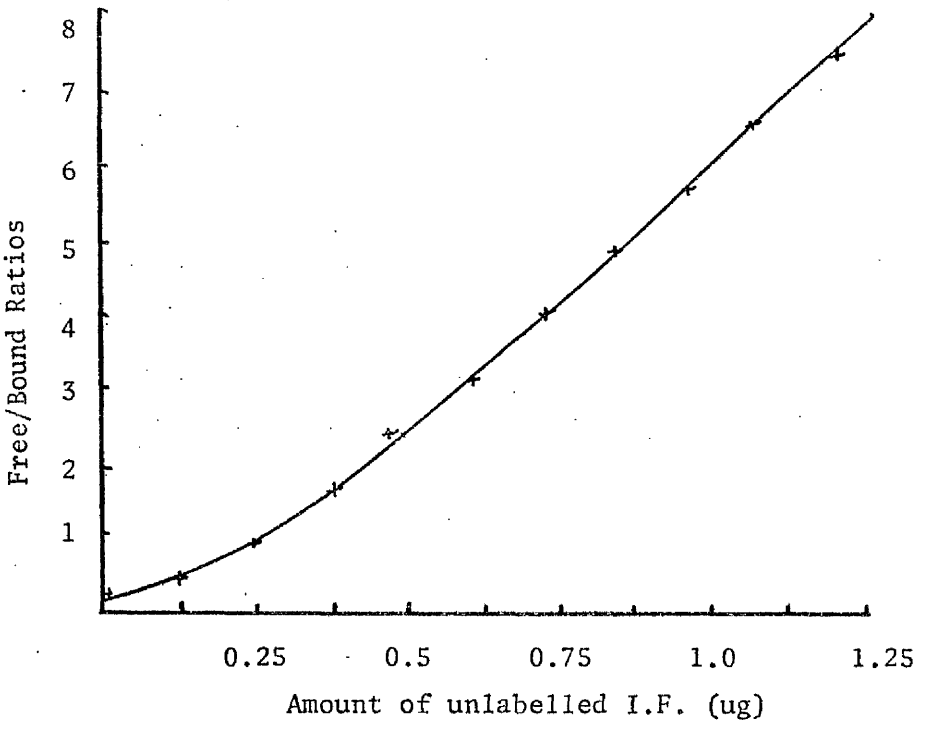
A serial dilution of unlabelled hog I.F. was prepared containing 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5 and 5 µg I.F. each and 0.25 ml from each was added to the appropriate test tube and mixed. Then 0.5 ml of the antibody preparation was added to each tube, mixed and

incubated at room temperature for 10 minutes. After this time 0.1 ml of Donkey anti-rabbit precipitating protein, (Wellcome Labs.) 1 in 50 dilution in saline, was added plus 0.2 ml of normal rabbit serum. This rabbit serum was merely to increase the size of the final pellet.

The tubes were incubated at 4°C overnight and then centrifuged at 10,000g for 15 minutes.

The supernatants were removed by pasteur pipette and discarded and the pellets were counted for [^{125}I] activity.

The results are shown in Graph 13. The activity found in the pellet was subtracted from the total activity added to find the % Free and % Bound activity. The activity in the pellet was then expressed as a $^{\text{Free}}$/Bound ratio.



GRAPH 13 - Standard Curve for radioimmunoassay of I.F.

The work done on this is of a very preliminary nature, although the results seem to indicate that a very sensitive assay for I.F. could be developed.

This assay could be speeded up by using ammonium sulphate precipitation to separate the free and antibody bound I.F. instead of anti-rabbit precipitating serum, but this was not explored.

DISCUSSION

Radioactive iodine was the only isotope label investigated but it is not the ideal label for I.F. One reason for this is that if the I.F. molecule broke up and released the iodine, when [^{125}I] I.F. was being used to test for I.F. absorption in the intestine, the free iodine would be absorbed, thus giving inconclusive results.

Further [^{125}I]I.F.-[^{57}Co] cyanocobalamin complex is not useful for whole body monitor studies, because the [^{125}I] emission is too weak to be effectively measured at a particular location in the body.

More useful results could be obtained using ^{51}Chromium label but this isotope was not available at the time. The actual incorporation of [^{51}Cr] should be no more difficult than [^{125}I].

The [^{51}Cr] could be more easily traced using a Whole Body Monitor and also would not be absorbed if released from the I.F.

Yamaguchi et al (89) used [^{51}Cr] to label I.F. in 1970 to measure the intestinal absorption of I.F. They found only trace amounts of the label absorbed.

They used a stepwise column chromatography technique to isolate human I.F. from 200 ml of gastric juice. This must have yielded a very small quantity of pure I.F. indeed. They also used a commercial hog I.F. preparation which probably contained a high proportion of non-I.F. B_{12}-binders.

Many of the "in vitro" properties of pure I.F. could be studied using [^{125}I] incorporation as opposed to relying on labelled B_{12} which is only transiently bound to I.F.

The conditions used for the iodination were the mildest possible consistent with a reasonable level of incorporation of label. The activity of the Iodine used was probably too high since it caused fairly rapid destruction of the I.F. binding capacity at room temperature, but this could easily be lowered. The experiments with the [^{125}I] labelled I.F. suggested that the molecular structure had not been affected by the iodination process. Free iodine was not released from the fresh [^{125}I] I.F. preparation.

The specific radioimmunoassay for I.F. has the potential to be made very sensitive and could possibly measure down to picogram quantities of I.F. This sensitive a tool could be very useful in elucidating the metabolism of I.F. mediated B_{12} absorption, perhaps in conjunction with differential ultracentrifugation on tissue homogenates.

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