STUDIES ON VITAMIN B_{12} AND INTRINSIC FACTOR

FIONA C. McEWAN

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

Summary of Thesis - Studies on Vitamin B_{12} and Intrinsic Factor

The separation of free and bound vitamin B_{12} was studied using different systems. Two forms of vitamin B_{12} and four binders were used and the separation effected by charcoal adsorption, bag dialysis, gel filtration and ultrafiltration. The results suggest that the separation of free and bound vitamin B_{12} is affected by the form of vitamin B_{12}, the nature of the binder and the method of effecting separation.

Measurements of the vitamin B_{12} content of items of diet and meals were made using the microbiological assay with Euglena gracilis z strain. The results are discussed with reference to the various aspects of vitamin B_{12} nutrition in man.

A preliminary study, involving the freezing of gastric juice and fractionation during thawing, suggests that cryoconcentration may have an application in the concentration of intrinsic factor binder from gastric juice.

Four previously published reports concerning the non-specific binding of cyanocobalamin in normal human saliva, total body vitamin B_{12} estimations in the live subject, the activities of various cobalamins for Euglena gracilis, and the clinical value of serum vitamin B_{12} estimation in jaundice, are reprinted in their entirety.

CONTENTS

SUMMARY

1. Methods of separating free and bound vitamin B_{12} are examined using two forms of vitamin B_{12} and four binders. The basic methods were charcoal adsorption, bag dialysis, gel filtration and ultrafiltration and variations on each method were also studied. With one form of vitamin B_{12} and one binder different methods often produced widely differing results and with any one method the results were affected by the form of vitamin B_{12} and the nature of the binder.

2. The vitamin B_{12} content of items of diet and meals were measured using the microbiological assay with Euglena gracilis z strain. The results are discussed with reference to the estimated requirements for vitamin B_{12} as judged by kinetic studies and the limitations to absorption of vitamin B_{12}.

3. A preliminary study, involving the freezing of gastric juice and fractionation during thawing, suggests that cryoconcentration may have an application in the concentration of intrinsic factor binder from gastric juice.

4. Four previously published reports concerning the non-specific binding of cyanocobalamin in normal human saliva, total body vitamin B_{12} estimations in the live subject, the activities of various cobalamins for Euglena gracilis, and the clinical value of serum vitamin B_{12} estimation in jaundice, are reprinted in their entirety.

PREFACE

The work reported in this thesis is presented in four chapters.

The first chapter is a self contained study, dealing with some methods of separating free and bound vitamin B_{12}. Four methods were studied, each in some detail, and an attempt has been made to assess the relative merits and demerits of each method vis a vis the other.

The second chapter, on the vitamin B_{12} content of items of diet and meals, was stimulated by work on the measurement of the total body vitamin B_{12} in the living subject (reported in Section 2 of the fourth chapter) and by kinetic studies carried out in this department.

The third chapter reports on a simple method of concentrating the intrinsic factor in gastric juice. Work on this subject had to be limited by other activities and this report is essentially a preliminary study.

Chapter four includes four previously published reports of joint work carried out in this department.

CHAPTER 1

SEPARATION STUDIES

INTRODUCTION

Vitamin B_{12} rarely occurs naturally in the free form but usually attached or bound to protein. For analytical purposes it is necessary to detach the vitamin and thereafter to separate the free vitamin from the binder and/or any vitamin B_{12}-binder complex. For evaluation of the efficiency of separation procedures it is essential to have techniques which discriminate quantitatively between free and bound vitamin B_{12}. For this purpose charcoal separation, bag dialysis, gel filtration, ultrafiltration, paper chromatography, electrophoresis and microbiological assay have all been used. Separation by microbiological assay is not considered to give meaningful results (Chanarin, 1969) and it is difficult to quantify results obtained by paper chromatography and electrophoresis. Accordingly the study of separation methods was limited to four techniques, charcoal separation, bag dialysis, gel filtration and ultrafiltration.

Chanarin (1969) comments that "In general, dialysis, column chromatography and charcoal adsorption give comparable results. The results of ultrafiltration are similar to those obtained by dialysis." No other consideration about the comparability of results obtained by different methods could be traced and one object of the study was to fill this gap, accumulating enough data to permit statistical evaluation. Accordingly the masses

of reactants, vitamin B_{12} and binder, were kept constant for each method. It was appreciated that what was applicable to one binder and one form of vitamin B_{12} would not necessarily apply to other binders and other forms of vitamin B_{12} and so the scope of the study was widened by using four binders and two forms of vitamin B_{12}.

It is convenient to present the study of each method separately and to evaluate the results obtained by different methods in a final section. It should be stressed here that the study was a practical one and that theoretical considerations relevant to separation methodology were not investigated.

SECTION 1

CHARCOAL STUDIES

INTRODUCTION

Charcoal has been used in separation procedures for many years and was in fact used by Laland & Klem (1936) to adsorb and concentrate the haemopoietically active fraction in crude extracts of liver. With the development of other separation procedures interest in charcoal waned until Miller (1957) used it to separate free and bound vitamin B_{12} in human plasma. A further clinical application came when Ardeman & Chanarin (1963) used charcoal to separate free and gastric juice bound vitamin B_{12} in their intrinsic factor assay. The use of albumin coated charcoal was introduced by Gottlieb et al. (1965) who referred to the separation of free and bound vitamin B_{12} by albumin coated charcoal as "instant dialysis".

Although there is evidence that albumin coating modifies the effect of charcoal (Herbert et al., 1964), there is little information on the effect of different charcoals, whether raw or albumin-coated, and on the effect of variations in the concentrations of any one type of charcoal, whether raw or coated, in relation to the type of binder and the form of vitamin B_{12}.

The primary object of the work reported in this section was to study the effect of variations in the concentration of two charcoals, both raw and albumin-coated, on the separation of free and bound vitamin B_{12} in a standard system, using two forms of vitamin B_{12} (cyanocobalamin and hydroxocobalamin) and

four binders (human gastric juice, hog intrinsic factor concentrate, human bile and human saliva), with the purpose of obtaining information on the importance of these factors. In addition it was intended to accumulate enough data to allow comparison of the results obtained by the various charcoal separations with those obtained by other separation methods.

MATERIALS AND METHODS

The basis of the method is that free vitamin B_{12} is adsorbed by the charcoal. After centrifugation with charcoal the fraction of vitamin B_{12} in the supernatant of the test sample is therefore taken as the fraction of vitamin B_{12} which has been bound to the test binder and is expressed as mμg vitamin B_{12} bound per ml of binder.

General Procedure

To numbered 10 ml plastic tubes were added, sequentially, 2 ml distilled water, 1 ml binder and 1 ml radioactive vitamin B_{12} solution. The tubes were capped, shaken and incubated at room temperature for 15 minutes. 2 ml of the appropriate charcoal suspension were then added to each tube and the contents again mixed and incubated at room temperature for 15 minutes. The tubes were then centrifuged at 2,500 rev./min for 15 minutes and the supernatants decanted. The radioactivity in each supernatant was measured against a suitable standard. Control tubes containing

3 ml distilled water, 1 ml radioactive vitamin B_{12} solution and 2 ml of the appropriate charcoal suspension were included in each batch of samples. All tests were done in duplicate and mean values used for calculations which took account of background radioactivity.

Materials and Reagents

Vitamin B_{12} Radioactive (^{57}Co)cyanocobalamin and (^{57}Co) hydroxocobalamin were obtained from the Radiochemical Centre, Amersham, and working solutions prepared with distilled water to give final concentrations of 100 mμg 0.1 μCi/ml. Such solutions were stored in dark glass bottles at 4°C. As the cobalamins were rarely stored for more than a week, quality control procedures were not used routinely but occasional batches were tested using carboxy methyl cellulose (CMC) and diethylaminoethyl cellulose (DEAEC) columns (Kennedy & Adams, 1965; Kennedy, 1967).

Binders

Four binders were used - human gastric juice (GJ), hog intrinsic factor concentrate (HIF), human bile (BIL) and human saliva (SAL).

The human gastric juice was obtained from patients attending the Gastrointestinal Clinic of the hospital. As each sample of fasting, histamine stimulated or pentagastrin stimulated material was obtained it was neutralised, using phenolphthalein as indicator, by the addition of N/10 sodium hydroxide solution and

then stored at -20°C. When about 10 litres had been collected it was thawed, pooled, filtered through glass wool and stored in aliquots of 20 ml at -20°C until required.

The hog intrinsic factor concentrate was prepared from a preparation of hog gastric mucosa, retailed by Armour Ltd. 100 mg were dissolved in 100 ml distilled water and after standing for 30 minutes at room temperature the mixture was centrifuged at 2,500 rev./min to obtain a clear supernatant which was stored in 10 ml aliquots at -20°C.

Human bile was obtained via T-tubes placed in the common bile ducts of three patients at cholecystectomy five days previously. The collections were pooled, dialysed against tap water at 19°C for 18 hours, and 20 ml aliquots stored at -20°C. (The purpose of dialysis was to render the bile acceptable for in vivo studies, not discussed in this thesis).

Human saliva was collected from staff, centrifuged, pooled and stored at -20°C.

Initially it was not intended to carry out such extensive investigations with the bile and saliva. Therefore, relatively small pools were accumulated and rather than use other material which would not be comparable, investigations with bile and saliva were limited.

In these charcoal studies the use of saliva was limited to cyanocobalamin binding only.

Charcoal

Two types of charcoal - Norit OL activated (Hopkins and Williams) and Norit A neutral (Amend Drug and Chemical Coy. U.S.A.)

the latter gifted by Dr. I.L. MacKenzie of Boston - were used, each in the raw and the albumin-coated states. One batch of each charcoal was used throughout. The charcoals were stored at 4°C and raw suspensions were made with distilled water in concentrations (weight/volume) of 0.5% to 10%. The 10% suspensions of albumin-coated charcoal were prepared by adding 10 grammes of charcoal to 93.3 ml distilled water and 6.6 ml 30% bovine serum albumin solution (Armour Ltd.). Suspensions of lower concentrations were prepared by dilution from the 10% suspension. The charcoal suspensions were stored at 4°C and discarded three weeks after preparation. Because of difficulty in obtaining Norit A (which was used by Gottlieb et al., 1965), the bulk of the work was done with Norit OL which was readily available.

Measurements of Radioactivity

The amount of radioactivity in a sample was measured in a well type scintillation counter, IDL type 663, with a thallium activated sodium iodide crystal 5.5 cm in diameter and 6.9 cm deep, surrounded by 10 cm thick lead shielding. The crystal was connected to an IDL 1700 automatic scaler and measurements were made against standards of the same volume, appropriate corrections being made for background radioactivity.

Statistical Methods

Statistical analyses were carried out using the Mann-Whitney U Test as described by Siegel (1956).

RESULTS

In all about 1,000 results of duplicate studies were obtained and these have been summarised and are presented in Tables 1 - 8 (pages 14 - 21). In each are shown the mean values and standard deviations derived from six duplicate results obtained at different times.

TABLES

Tables 1 - 8 are the main presentations:-

		CONCENTRATIONS OF ADDED CHARCOAL				
		0.5%	1%	2.5%	5%	10%
mμg Vitamin B_{12} Bound Per ml of Binder	GJ	78.9 (16.3)	70.1 (17.9)	50.0 (17.3)	39.0 (16.1)	34.6 (15.7)
	HIF	40.0 (1.5)	39.4 (2.0)	38.2 (1.7)	38.5 (1.0)	36.1 (2.0)
	BIL	76.8 (5.4)	65.1 (5.9)	28.9 (5.4)	7.2 (1.4)	3.3 (1.2)
	SAL	86.7 (8.5)	79.3 (7.9)	66.2 (12.7)	57.6 (14.2)	53.6 (14.3)
Control mμg Vitamin B_{12} not adsorbed		0.9 (0.3)		0.7 (0.1)		1.4 (0.3)

Table 1 Norit OL Charcoal, coated, and Cyanocobalamin
Means and Standard Deviations of six duplicate results for each of the four standard binders and the five charcoal concentrations and for corresponding controls.

		CONCENTRATIONS OF ADDED CHARCOAL				
		0.5%	1%	2.5%	5%	10%
mμg Vitamin B_{12} Bound Per ml of Binder	GJ	88.6 (1.5)	71.4 (4.3)	27.1 (9.4)	6.2 (4.4)	0.6 (0.4)
	HIF	31.6 (5.7)	8.9 (2.3)	1.1 (0.5)	0.5 (0.1)	0.4 (0.2)
	BIL	77.1 (6.1)	61.1 (6.0)	11.7 (5.3)	3.9 (2.5)	0.7 (0.5)
	SAL	84.7 (7.6)	71.6 (12.0)	44.5 (12.9)	28.5 (14.5)	6.4 (3.4)
Control mμg Vitamin B_{12} not-adsorbed		10.1 (4.1)		5.1 (2.1)		0.6 (0.2)

Table 2 Norit OL Charcoal, raw, and Cyanocobalamin
Means and Standard Deviations of six duplicate results for each of the four standard binders and the five charcoal concentrations and for corresponding controls.

		CONCENTRATIONS OF ADDED CHARCOAL		
		0.5%	2.5%	10%
mμg Vitamin B_{12} Bound Per ml of Binder	GJ	77.4 (11.5)	34.1 (7.5)	23.7 (7.7)
	HIF	49.1 (10.2)	45.7 (10.2)	43.6 (9.9)
	BIL	52.9 (6.5)	16.3 (3.7)	5.5 (1.3)
	SAL	78.5 (8.0)	45.0 (8.7)	33.0 (10.4)
Control mμg Vitamin B_{12} not adsorbed		3.3 (2.8)	2.2 (0.3)	1.9 (0.5)

Table 3 Norit A Charcoal, coated, and Cyanocobalamin Means and Standard Deviations of six duplicate results for each of the four standard binders and the three charcoal concentrations and for corresponding controls.

		CONCENTRATIONS OF ADDED CHARCOAL		
		0.5%	2.5%	10%
mμg Vitamin B_{12} Bound Per ml of Binder	GJ	69.8 (8.9)	16.0 (6.8)	0.3 (0.2)
	HIF	34.6 (9.8)	6.0 (1.0)	0.6 (0.2)
	BIL	53.1 (2.8)	7.3 (1.5)	2.7 (0.8)
	SAL	71.2 (6.3)	22.9 (7.3)	1.4 (0.3)
Control mμg Vitamin B_{12} not adsorbed		13.3 (6.9)	2.6 (0.7)	0.6 (0.2)

Table 4 Norit A Charcoal, raw, and Cyanocobalamin
Means and Standard Deviations of six duplicate results for each of the four standard binders and the three charcoal concentrations and for corresponding controls.

		CONCENTRATIONS OF ADDED CHARCOAL				
		0.5%	1%	2.5%	5%	10%
mμg Vitamin B_{12} Bound Per ml of Binder	GJ	75.6 (7.6)	58.6 (9.9)	36.1 (10.1)	27.5 (7.2)	22.6 (6.7)
	HIF	37.2 (1.4)	37.1 (0.8)	36.5 (1.7)	35.3 (1.6)	35.0 (1.1)
	BIL	72.4 (5.8)	60.0 (6.2)	35.0 (6.1)	6.8 (1.9)	3.8 (1.4)
Control mμg Vitamin B_{12} not adsorbed		3.8 (0.3)		3.5 (0.4)		2.2 (0.3)

Table 5 Norit OL Charcoal, coated, and Hydroxocobalamin
Means and Standard Deviations of six duplicate results for each of the three standard binders and the five charcoal concentrations and for corresponding controls.

		CONCENTRATIONS OF ADDED CHARCOAL				
		0.5%	1%	2.5%	5%	10%
mμg Vitamin B_{12} Bound Per ml of Binder	GJ	71.0 (4.5)	43.5 (6.2)	8.6 (5.1)	1.0 (0.8)	0.3 (0.2)
	HIF	34.3 (4.3)	8.8 (1.5)	0.7 (0.3)	0.6 (0.1)	0.3 (0.1)
	BIL	75.4 (7.5)	58.7 (7.9)	15.4 (10.5)	1.7 (1.1)	0.9,(0.4)
Control mμg Vitamin B_{12} not adsorbed		10.8 (2.5)		4.3 (2.0)		0.9,(0.2)

Table 6 Norit OL Charcoal, raw, and Hydroxocobalamin
Means and Standard Deviations of six duplicate results for each of the three standard binders and the five charcoal concentrations and for corresponding controls.

		CONCENTRATIONS OF ADDED CHARCOAL		
		0.5%	2.5%	10%
mμg Vitamin B_{12} Bound Per ml of Binder	GJ	70.9 (4.3)	16.9 (5.3)	6.2 (1.1)
	HIF	23.9 (2.5)	20.0 (1.3)	19.4 (1.1)
	BIL	67.7 (3.7)	20.4 (4.5)	4.6 (1.3)
Control mμg Vitamin B_{12} not adsorbed		2.3 (0.5)	1.9 (0.3)	2.2 (0.4)

Table 7 Norit A Charcoal, coated, and Hydroxocobalamin Means and Standard Deviations of six duplicate results for each of the three standard binders and the three charcoal concentrations and for corresponding controls.

		CONCENTRATIONS OF ADDED CHARCOAL		
		0.5%	2.5%	10%
mμg Vitamin B_{12} Bound Per ml of Binder	GJ	64.4 (5.4)	2.6 (1.0)	0.4 (0.3)
	HIF	16.4 (2.9)	4.6 (0.8)	0.9 (0.2)
	BIL	65.5 (2.7)	6.0 (1.8)	1.7 (0.2)
Control mμg Vitamin B_{12} not adsorbed		13.8 (3.4)	3.0 (0.5)	1.1 (0.3)

Table 8 Norit A Charcoal, raw, and Hydroxocobalamin

Means and Standard Deviations of six duplicate results for each of the three standard binders and the three charcoal concentrations and for corresponding controls.

Because of the mass of these raw results it has been thought best to further present them according to the objects of the study.

Results related to the type of charcoal

With raw charcoals, the relevant results relating to cyanocobalamin are in Tables 2 and 4 (pages 15 and 17) while those relating to hydroxocobalamin are in Tables 6 and 8 (pages 19 and 21)*. These were analysed using the Mann-Whitney U Test and the findings, which are more conveniently presented in tabular than textual form, are set out in Tables 9 and 10 (pages 23 and 24).

	CONCENTRATIONS OF ADDED CHARCOAL		
Binders	0.5%	2.5%	10%
GJ	0.001	0.021	0.197
HIF	0.469	0.001	0.017
HL	0.001	0.120	0.001
SAL	0.008	0.004	0.002
Control	0.120	0.032	0.322

Table 9 Comparison of results obtained with two different types of raw charcoal.

Norit OL Charcoal, raw, - Norit A Charcoal, raw, and Cyanocobalamin.

The results were analysed by the Mann-Whitney U Test and the values in the Table are those for P on a two tail test.

	CONCENTRATIONS OF ADDED CHARCOAL		
Binders	0.5%	2.5%	10%
GJ	0.066	0.001	0.500
HF	0.001	0.001	0.001
BIL	0.013	0.032	0.002
Control	0.066	0.197	0.155

Table 10 Comparison of results obtained with two different types of raw charcoal.

Norit OL Charcoal, raw, - Norit A Charcoal, raw, and Hydroxocobalamin.

The results were analysed by the Mann-Whitney U Test and the values in the Table are those for P on a two tail test.

It is clear from these tables that in the majority of instances (17 out of 27 including control results) the results obtained with one type of charcoal are significantly different ($P < 0.05$) from the results obtained with another type of charcoal and that this occurs with controls, with all binders and with both types of cobalamin.

With albumin-coated charcoals, the relevant results relating to cyanocobalamin are set out in Tables 1 and 3 (pages 14 and 16), with those relating to hydroxocobalamin in Tables 5 and 7 (pages 18 and 20). The analytical findings are set out in Tables 11 and 12 (pages 26 and 24) and again it is clear that in many instances (18 out of 27 including control results) the results obtained with one type of coated charcoal differ significantly ($P < 0.05$) from the results obtained using another type of coated charcoal.

	CONCENTRATIONS OF ADDED CHARCOAL		
Binders	0.5%	2.5%	10%
GJ	0.409	0.090	0.120
HIF	0.032	0.066	0.047
BIL	0.001	0.002	0.008
SAL	0.120	0.008	0.013
Control	0.001	0.001	0.027

Table 11 Comparison of results obtained with two different types of coated charcoal.

Norit GL Charcoal, coated, - Norit A Charcoal, coated, and Cyanocobalamin.

The results were analysed by the Mann-Whitney U Test and the values in the Table are those for P on a two tail test.

	CONCENTRATIONS OF ADDED CHARCOAL		
Binders	0.5%	2.5%	10%
GJ	0.197	0.001	0.001
HIF	0.001	0.001	0.001
BIL	0.120	0.001	0.155
Controls	0.001	0.001	0.469

Table 12 Comparison of results obtained with two different types of coated charcoal.

Norit OL Charcoal, coated, - Norit A Charcoal, coated, and Hydroxocobalamin.

The results were analysed by the Mann-Whitney U Test and the values in the Table are those for P on a two tail test.

In general it can be concluded that the effect of different charcoals varies according to the binder, the form of vitamin B_{12}, the concentration of the charcoals and whether they are used in the raw or albumin-coated states.

Results related to variations in the concentration of charcoals

With raw charcoals the relevant results related to Norit OL are set out in Tables 2 and 6 (pages 15 and 19) and related to Norit A in Tables 4 and 8 (pages 17 and 21). A striking and unexpected feature of the results was the incomplete removal of cyanocobalamin and hydroxocobalamin from aqueous solution (controls) by low concentrations of both Norit OL and Norit A. This alone, however, could not fully account for the overall pattern which is one of a fall in value for bound vitamin B_{12} with increasing concentrations of charcoal, the pattern occurring with both types of charcoal, with all four binders, and with both forms of vitamin B_{12}. This pattern is so striking that statistical evaluation has been considered to be superfluous. It is convenient to mention here the apparent anomalies found with Norit OL, concentration 2.5%, where the values of both hog intrinsic factor bound cyanocobalamin and hog intrinsic factor bound hydroxocobalamin are significantly less than the control values (Tables 2 and 6, pages 15 and 19).

With albumin-coated charcoals the relevant results related to Norit OL are set out in Tables 1 and 5 (pages 14 and 18) and related to Norit A in Tables 3 and 7 (pages 16 and 20). The results are less dramatic than those obtained with raw charcoals but the pattern - a fall in the value for bound vitamin B_{12} with

increasing concentrations of charcoal - is obviously the same in the cases of gastric juice, bile, and saliva bound to cyanocobalamin or hydroxocobalamin, whichever charcoal is used. The results with hog intrinsic factor appear to be an exception but statistical analysis of the results using the Mann-Whitney U Test discloses significant differences between values obtained with different concentrations of charcoals (11 out of 26), showing that this is not the case and that the pattern with hog intrinsic factor, although less obvious, is similar to the others. The results of these statistical analyses are set out in Tables 13 - 16 (pages 30 - 33).

Concentrations of added charcoal	0.5%	1%	2.5%	5%	10%
0.5%		0.409	0.040	0.040	0.002
1%			0.120	0.268	0.021
2.5%				0.409	0.066
5%					0.008
10%					

Table 13 Effect of varying concentrations of coated charcoal on Hog Intrinsic Factor Binding.

Norit OL Charcoal and Cyanocobalamin.

The results were analysed using the Mann-Whitney U Test and the values in the Table are those for P on a two tail test.

Concentrations of added charcoal	0.5%	1%	2.5%	5%	10%
0.5%		0.350	0.294	0.021	0.008
1%			0.220	0.032	0.004
2.5%				0.176	0.066
5%					0.439
10%					

Table 14 Effect of varying concentrations of coated charcoal on Hog Intrinsic Factor Binding.

Norit OL Charcoal and Hydroxocobalamin.

The results were analysed using the Mann-Whitney U Test and the values in the Table are those for P on a two tail test.

Concentrations of added charcoal	0.5%	2.5%	10%
0.5%		0.242	0.242
2.5%			0.268
10%			

Table 15 Effect of varying concentrations of coated charcoal on Hog Intrinsic Factor Binding.

Norit A Charcoal and Cyanocobalamin.

The results were analysed using the Mann-Whitney U Test and the values in the Table are those for P on a two tail test.

Concentrations of added charcoal	0.5%	2.5%	10%
0.5%		0.004	0.001
2.5%			0.197
10%			

Table 16 Effect of varying concentrations of coated charcoal on Hog Intrinsic Factor Binding.

Norit A Charcoal and Hydroxocobalamin.

The results were analysed using the Mann-Whitney U Test and the values in the Table are those for P on a two tail test.

Results related to the effects of albumin coating of charcoals

With Norit OL charcoal the results relevant to the effects of albumin coating related to cyanocobalamin binding are in Tables 1 and 2 (pages 14 and 15) and related to hydroxocobalamin binding in Tables 5 and 6 (pages 18 and 19). With Norit A charcoal, the results relevant to the effects of albumin coating related to cyanocobalamin binding are in Tables 3 and 4 (pages 16 and 17) and related to hydroxocobalamin binding in Tables 7 and 8 (pages 20 and 21).

With both types of charcoal there are two obvious effects of coating. The first is a reduction in control values obtained, especially with the lower concentrations of charcoal both with cyanocobalamin and hydroxocobalamin. The second is an effect which in general results in higher values for bound vitamin B_{12}, whether in the form of cyanocobalamin or hydroxocobalamin, when the charcoal concentrations are high.

The results were subjected to statistical analyses by the Mann-Whitney U test and the findings are presented in Tables 17 - 20 (pages 36 - 39). From these the following conclusions are drawn:-

1) In the controls with the 0.5% concentrations, the albumin coating effects a statistically significant increase in the amount of vitamin B_{12} removed by the charcoal, (i.e. reduces the control value), whether the vitamin B_{12} is cyanocobalamin or hydroxocobalamin and whether the charcoal is Norit OL or Norit A.

2) The effect of albumin coating with both Norit OL and Norit A in concentrations of 2.5% and greater is always to increase, by statistically significant margins, the amount of vitamin B_{12} bound, irrespective of binder or cobalamin.

3) This effect occurs with hog intrinsic factor at all concentrations studied.

	CONCENTRATIONS OF ADDED CHARCOAL				
Binders	0.5%	1%	2.5%	5%	10%
GJ	0.350	0.197	0.047	0.001	0.001
HIF	0.001	0.001	0.001	0.001	0.001
BIL	0.409	0.155	0.001	0.013	0.001
SAL	0.155	0.090	0.008	0.008	0.001
Control	0.001		0.001		0.001

Table 17 Comparison of results obtained with raw and albumin-coated charcoal.

Norit OL Charcoal and Cyanocobalamin.

The results were analysed by the Mann-Whitney U Test and the values in the Table are those for P on a two tail test.

	CONCENTRATIONS OF ADDED CHARCOAL				
Binders	0.5%	1%	2.5%	5%	10%
GJ	0.220	0.004	0.001	0.001	0.001
HHF	0.047	0.001	0.001	0.001	0.001
BIL	0.294	0.350	0.004	0.001	0.001
Control	0.001		0.469		0.001

Table 18 Comparison of results obtained with raw and albumin-coated charcoal.

Norit OL Charcoal and Hydroxocobalamin.

The results were analysed by the Mann-Whitney U Test and the values in the Table are those for P on a two tail test.

	CONCENTRATIONS OF ADDED CHARCOAL		
Binders	0.5%	2.5%	10%
GJ	0.155	0.004	0.001
HIF	0.032	0.001	0.001
BIL	0.350	0.001	0.001
SAL	0.066	0.001	0.001
Control	0.002	0.242	0.001

Table 19 Comparison of results obtained with raw and albumin-coated charcoal.

Norit A Charcoal and Cyanocobalamin

The results were analysed by the Mann-Whitney U Test and the values in the Table are those for P on a two tail test.

	CONCENTRATIONS OF ADDED CHARCOAL		
Binders	0.5%	2.5%	10%
GJ	0.047	0.001	0.001
HIF	0.001	0.001	0.001
BIL	0.155	0.001	0.001
Control	0.001	0.001	0.001

Table 20 Comparison of results obtained with raw and albumin-coated charcoal.

Norit A Charcoal and Hydroxocobalamin.

The results were analysed by the Mann-Whitney U Test and the values in the Table are those for P on a two tail test.

DISCUSSION

The use of charcoal to separate free and bound vitamin B_{12} is a well established stage in measurements of the binding capacity of serum (Miller, 1957; Grossowicz et al., 1962; Rosenthal et al., 1962; Meyer et al., 1963; Kennedy & Adams, 1967), and in the assay of intrinsic factor in human gastric secretion Ardeman and Chanarin, 1963; Gottlieb et al., 1965; Chanarin, 1969). The procedure is, therefore, worthy of study in all its aspects and those selected - the effects of the type and concentration of charcoal, the effects of albumin coating and the effects of the form of vitamin B_{12} - are obviously only a few of many.

Type of Charcoal

Ardeman & Chanarin (1963), who were the first to use charcoal in an intrinsic factor assay, mentioned only that the charcoal they used was acid washed and activated by heating. Gottlieb et al. (1965), who introduced the use of albumin-coated charcoals, used Norit A and stated that they had found other "finely powdered charcoals" to be equally satisfactory. They also stated that activation was unnecessary and that they had found one "coarse" charcoal to be unacceptable. In his recent monograph, Chanarin (1969) does not specify any type of charcoal when describing an intrinsic factor assay procedure and does not mention it in a brief review of the use of charcoal in separation of free and bound vitamin B_{12}.

Workers who have based their techniques for intrinsic factor assay on the methods of Ardeman & Chanarin (1963) and Gottlieb et al. (1965) have sometimes used different charcoals. Irvine (1966)

for example used B.D.H. activated charcoal and in this laboratory we have used Norit OL (Adams et al., 1967; Kennedy & Adams, 1967), partly because of difficulty in obtaining Norit A and partly because of advice that Norit OL was virtually identical to Norit A.

It seems reasonable to conclude from the results that the substitution of one type of charcoal, whether raw or coated, for another in the type of system used, all other factors being constant, may result in a significantly different value for bound vitamin B_{12}. The qualification is important. The results do not support an overall conclusion, and even if they did it would not necessarily be generally applicable. They do, however, show that whether one type of charcoal does or does not have an effect which differs from another depends on the binder, the type of vitamin B_{12} and the concentration as well as the type of charcoal and whether the charcoal is coated or not. The value of the results lies not in the limited comparisons but in the demonstration of the importance of these factors. It would be unrealistic to believe that these are the only factors and this point will be dealt with later.

It would have been satisfactory to find reasons for the observed differences but replies to enquiries about sources, manufacturing processes and physical characteristics of the charcoals have not been helpful.

Concentration of Charcoal

The importance of the concentration of charcoal has not previously been studied in detail and recommendations about this aspect are confusing. Ardeman & Chanarin (1963) used a "knife

edge", about 100 mg and Chanarin (1969) still recommends this. Gottlieb et al. (1965) used 2.5% albumin coated charcoal suspension. More important than the working concentration of charcoal may, in fact, be the ratio of charcoal to vitamin B_{12} in the assay tubes. This may vary considerably in "standard" methods as can be seen in Table 21 (page 43) in which the data have been taken from the intrinsic factor assay protocols.

Author	Vitamin B_{12} : Charcoal ratio in each assay tube µug vitamin B_{12}/mg charcoal
Ardeman & Chanarin (1963)	500
Gottlieb et al. (1965)	150
Rødbro et al. (1965)	200
Irvine (1966)	4,000
Adams et al. (1967)	150
Chanarin (1969)	1,000
Current series	
0.5%	10,000
1%	5,000
2.5%	2,000
5%	1,000
10%	500

Table 21 Variations in ratio of Vitamin B_{12} : Charcoal in Intrinsic Factor Assay Protocols.

It is clear from the results of this study that the concentration of charcoal, particularly raw charcoal, may have a dramatic effect on the results. In the case of raw charcoals this was not unexpected for it has been shown that raw charcoal can adsorb binding proteins (Gregory & Holdsworth, 1959) and can adsorb vitamin B_{12} - intrinsic factor complex from gastric juice (Ardeman & Chanarin, 1963). The magnitude of the changes was, however, unexpected. The effects of changes in concentration of coated charcoals were much less dramatic but were clear cut with most binders and these results will be discussed in relation to the effects of coating.

The results show that the concentration of charcoal, raw or coated, can significantly affect results. That this involves changes in the vitamin B_{12} : charcoal ratio may be an important factor.

Effects of Coating

The coating of charcoal by albumin was introduced by Gottlieb et al. (1965) who regarded the coat as a "molecular sieve" which allows the passage of small molecules, such as vitamin B_{12}, but the barring of large molecules such as bound vitamin B_{12} complexes.

The results of this study leave no doubt that coating modifies the effect of charcoal. The fact that the effect was always statistically significant with charcoal concentrations 2.5% or greater, regardless of binder, type of charcoal or type of vitamin B_{12}, might be regarded as supporting the molecular

sieve concept. That coated charcoals in low concentrations remove more vitamin B_{12}, cyanocobalamin or hydroxocobalamin, from aqueous solutions than uncoated charcoals does not support this concept. Related to this point are the anomalous results for controls and hog intrinsic factor bound vitamin B_{12} when 2.5% Norit OL was used. The control results were greater, and, if this were explicable by the presence of protein in the intrinsic factor solution, would not support the concept. With both coated charcoals in the bile-vitamin B_{12} system, the pattern of results, which shows a steady decrease in the value of bound vitamin B_{12} with increase in the concentration of charcoal, also raises doubts at least in relation to the sieving of free and bound vitamin B_{12}. Finally there is the oddity that although the effect of coating was significant at concentrations of 2.5% and greater, irrespective of type of charcoal, vitamin B_{12} or binder, it was always significant with the hog intrinsic factor - vitamin B_{12} system.

Binder Factor

This last point serves to introduce the "binder factor" in charcoal separation systems. The slopes of the values obtained with the various binders and varying concentrations of charcoal are different and this seems likely to be due to differences in binders or binder-vitamin B_{12} complexes. Little is known about the fundamental process of binding and nothing of the molecular structure of the binding agent in the various binders used in this study. Beyond stressing the fact that the binder

is a factor of importance there is nothing to contribute.

Type of Vitamin B_{12}

It is well established that the binding capacity of one binder for cyanocobalamin may differ from its capacity for hydroxocobalamin (Bauriedel et al., 1956), and this area was not studied in detail. It is worthy of note that, in the control studies, uncoated charcoals removed the same amount of cyanocobalamin and hydroxocobalamin but coated Norit OL charcoal removed more cyanocobalamin than hydroxocobalamin. This is unimportant in systems, such as the intrinsic factor assay system, where the uptake of cyanocobalamin is measured, but systems in which other forms of vitamin B_{12} are being measured would require attention.

It is not possible to tell from the results whether any single result is correct in terms of the separation of free and bound vitamin B_{12}. This question can only be considered in the light of results using other separation techniques and this will be examined later.

A result of this study may be disquiet about the general applicability of charcoal separation of free and bound vitamin B_{12} unless due regard is paid to the importance of the factors discussed. The method has many advantages and its merits and demerits, compared with other separation systems, will be discussed later.

SECTION 2

BAG DIALYSIS

INTRODUCTION

Separation of large and small molecules by dialysis using membranes is a long established and well known procedure which is believed to operate by a sieving mechanism. A recurrence of interest in dialysis has been stimulated by developments in membrane technology and a very wide range of membranes is now available.

It was decided to evaluate dialysis as a method of separating free and bound vitamin B_{12} using five membranes, two forms of vitamin B_{12} (cyanocobalamin and hydroxocobalamin) and the four binders (gastric juice, hog intrinsic factor, bile and saliva) used in the charcoal separation study. The masses of reagents were the same as in the charcoal study.

In order to gain information about the operating time scales, preliminary studies were carried out with aqueous solutions of cyanocobalamin and hydroxocobalamin (i.e. both in the free form).

MATERIALS AND METHODS

General Procedure

Suitable lengths of tubing were soaked in water for 30 minutes and an end of each length was knotted. The test material, always 10 ml, was pipetted into the tubing which was knotted at the free end to enclose the 10 ml of test material and about 5 ml of air.

The sac was then suspended by a thread in a water bath containing 50 litres of tap water which was maintained at 37°C by a heater and thermostat, and constantly agitated by a stirrer. Dialysis was continued for periods of up to 72 hours. The sac was then removed and the radioactivity of the sac and contents counted against a suitable standard as described previously. The sac was then split open and washed with running tap water and the radioactivity adhering to the sac measured. Two values for the vitamin B_{12} retained were therefore calculated - the uncorrected value from the radioactivity of the sac and contents, and the corrected value from the radioactivity of the sac and contents minus that adhering to the sac.

Since theoretically the vitamin B_{12} retained is bound, (the small molecules, including free vitamin B_{12}, having been dialysed out), the results were further expressed as mμg vitamin B_{12} bound per ml of binder.

The masses of reagents used were the same as in the charcoal study i.e. 100 mμg(^{57}Co)cyanocobalamin or(^{57}Co)hydroxocobalamin and 1 ml of the relevant binder.

Dialysis Membranes

Five membranes were studied:-

Cuprophan Cuprophan tubing, 55 mm flat diameter, was donated by Dr. W.M. Muir of the Bioengineering Unit, Strathclyde University. This cellulose film, regenerated from ammoniacal copper hydroxide

solution, has relatively large pore dimensions (20 nm) and an unusually high ultrafiltration rate compared to other cellulose films (Muir et al., 1970).

Nephraphen This cellulose tubing, flat diameter 60 mm, is regenerated from a xanthate derivative and was also donated by Dr. Muir. Like cuprophan it displays an unusually high ultrafiltration rate (Muir et al., 1970) and has relatively large pore dimensions (20 nm).

Visking(2/32") This material, obtained from the Scientific Instrument Centre Ltd., is a seamless regenerated cellulose tubing (viscose process), having a flat diameter of 30 mm. It is stated by the retailers to have an average pore dimension of 2.4 nm.

Nojax 21 This tubing is used in the Hamburger Artificial Kidney and a supply was obtained from Professor A.C. Kennedy of the Royal Infirmary, Glasgow. Technical details of the manufacturing process and specifications were not obtainable. The material used had a flat diameter of 28 mm.

Kalle This cellulose material, also supplied by Dr. Muir, is manufactured in Germany and has a flat diameter of 72 mm. No other details were available.

Preliminary Studies

Preliminary studies were carried out with each membrane to determine whether the rate of loss of free vitamin B_{12} varies with

the form of vitamin B_{12} and with the type of membrane. For this purpose six sacs of each membrane, each sac containing 100 mμg vitamin B_{12} in 10 ml of water, were prepared as described previously and dialysis of all sacs started simultaneously. At suitable intervals one sac was removed and the amount of vitamin B_{12} retained calculated. The six values obtained at various times were plotted as a percentage of the initial vitamin B_{12} against time and, on the assumption that dialysis was proceeding as a first order function, the time at which 50% of the material was lost ($T\frac{1}{2}$) was found from inspection of the plots. The time required until 1% was retained (7 exhaustion) was calculated obtaining λ from $\frac{\log e2}{T\frac{1}{2}}$ and from the function $\frac{Ao}{At} = e^{-\lambda t}$ where Ao = amount of vitamin B_{12} at time zero and At the amount at time t in hours. Each study with each membrane and each form of vitamin B_{12} was performed on two separate occasions.

Separation Studies

Separation studies were performed with the two forms of vitamin B_{12} and the four binders used in the charcoal study. In this procedure the 100 mμg of radioactive vitamin B_{12} and 1 ml of standard binder preparation were made up to a volume of 10 ml with distilled water. Each study was repeated on six separate occasions, the times for dialysis (determined from the preliminary studies) being 24 hours for cuprophan, nephrophan and kalle membranes, 48 hours for nojax 21 membrane and for visking membrane

with cyanocobalamin, and 72 hours for visking membrane when hydroxocobalamin was used.

Because of the shortage of bile and saliva, the investigations with these binders were limited to cyanocobalamin binding only.

Statistical Methods

Statistical analyses were carried out using the Wilcoxon Matched-Pairs Signed-Ranks Test and the Mann-Whitney U Test, both of which are described by Siegel (1956).

RESULTS

Preliminary Studies

The results of the preliminary studies with aqueous solutions of cyanocobalamin and hydroxocobalamin and each of the five membranes are summarised in Table 22 (page 53) and some results are presented graphically in Fig.1 (page 54).

Membrane	Vitamin B_{12}	Observed $T_{\frac{1}{2}}$ (Hours)		Calculated T 1% (Hours)	Dialysis Time Selected (Hours)
Cuprophan	Cyanocobalamin	1.5	1.6	10.6	24
Nephrophan	Cyanocobalamin	1.3	1.5	10	24
Visking	Cyanocobalamin	8.5	12.0	80	48
Nojax 21	Cyanocobalamin	6.0	7.8	52	48
Kalle	Cyanocobalamin	3.4	3.8	25	24
Cuprophan	Hydroxocobalamin	2.4	3.0	20	24
Nephrophan	Hydroxocobalamin	2.0	2.8	18.6	24
Visking	Hydroxocobalamin	10.5	20.5	136	72
Nojax 21	Hydroxocobalamin	9.5	12.5	83	48
Kalle	Hydroxocobalamin	4.4	4.5	30	24

Table 22 Times in hours found for loss of 50% aqueous solutions of cyanocobalamin and hydroxocobalamin with various membranes ($T_{\frac{1}{2}}$). The values for T 1% were calculated from the maximum values for $T_{\frac{1}{2}}$ (see text). The times allowed for separation studies are also shown.

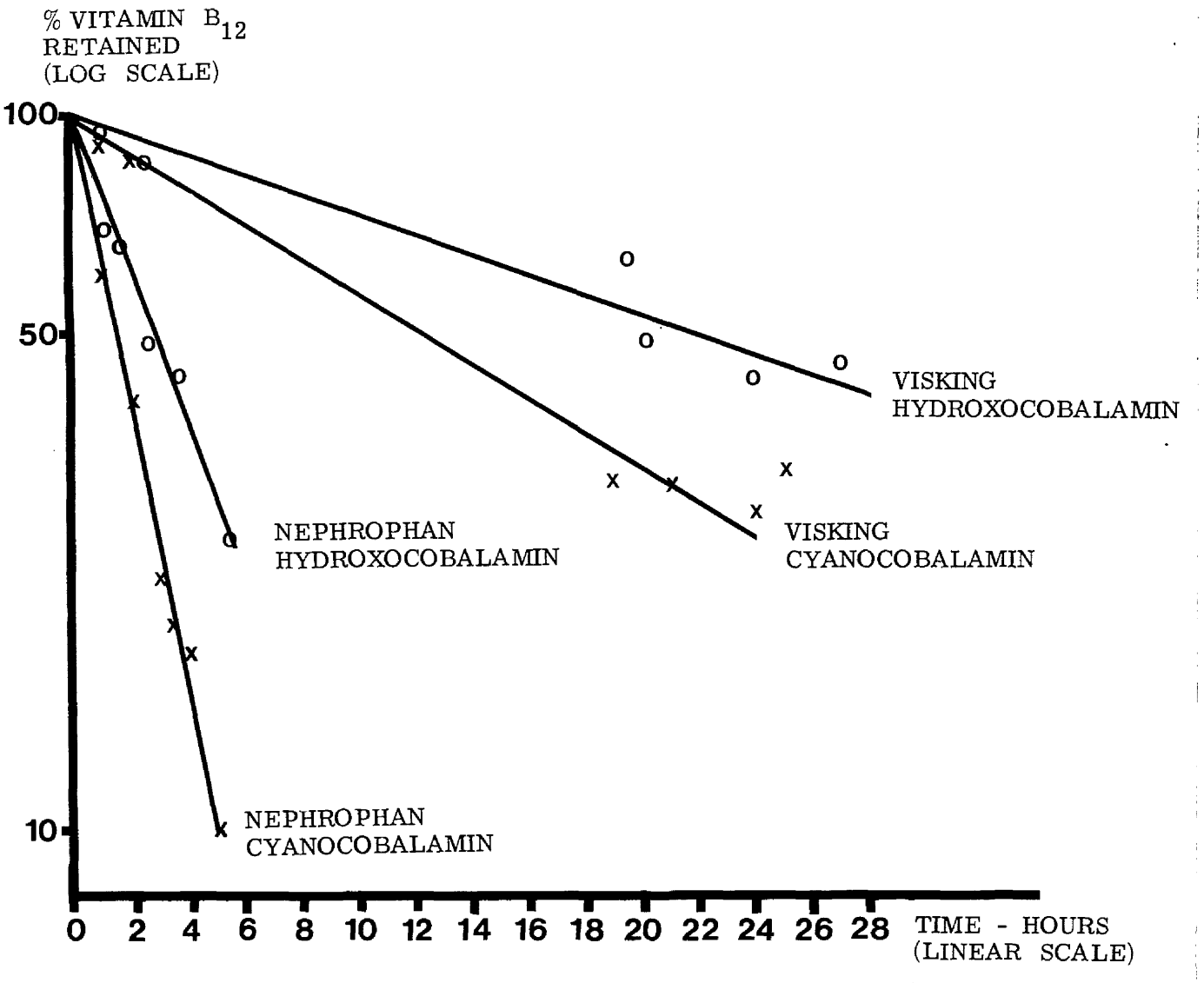


Fig. 1. Some of the results of the preliminary dialysis studies with aqueous solutions of cyanocobalamin and hydroxocobalamin presented graphically.

From these results and calculations it is clear that the time which should be allowed for separation of free and bound vitamin B_{12} by dialysis varies not only from membrane to membrane but also with the type of vitamin B_{12}. Accordingly, taking the results and calculations, and allowing for convenience in laboratory procedures, it was decided to allow dialysis to proceed for 24 hours when using cuprophan, nephrophan and Kalle membranes, for 48 hours when using nojax 21 membrane. 48 hours dialysis time was allowed when using visking membrane and cyanocobalamin and 72 hours when using visking membrane and hydroxocobalamin.

Separation Studies

The results of the separation studies are summarised and presented in Table 23 (page 56) which relates to cyanocobalamin binding and in Table 24 (page 57) which relates to hydroxocobalamin binding. For each membrane, each binder and each form of vitamin B_{12} are shown the mean value and standard deviation for uncorrected and corrected amounts of vitamin B_{12} bound per ml of binder, the values being derived from six separate experiments.

			TYPE OF MEMBRANE				
			Cuprophan	Nephrophan	Visking	Nojax 21	Kalle
mμg Vitamin B_{12} Bound Per ml of Binder	GJ	Uncorrected	28.0 (4.8)	24.4 (5.3)	37.3 (5.0)	35.3 (9.0)	33.9 (7.9)
		Corrected	27.5 (4.6)	20.4 (3.9)	30.9 (7.1)	28.9 (10.2)	25.7 (11.8)
	HIF	Uncorrected	29.3 (1.3)	28.5 (5.1)	34.3 (3.8)	32.5 (2.9)	37.6 (3.1)
		Corrected	28.4 (1.3)	23.5 (5.0)	24.2 (7.2)	22.3 (7.0)	26.8 (3.8)
	BIL	Uncorrected	6.6 (2.0)	7.0 (1.4)	23.4 (3.1)	17.0 (1.9)	23.4 (5.8)
		Corrected	6.1 (1.9)	5.2 (1.2)	14.2 (3.4)	11.6 (1.0)	12.1 (2.7)
	SAL	Uncorrected	52.3 (11.6)	51.1 (13.6)	59.3 (12.4)	56.3 (11.8)	49.2 (17.3)
		Corrected	49.7 (12.1)	46.1 (12.1)	54.6 (13.0)	43.6 (14.0)	37.8 (15.8)

Table 23 Cyanocobalamin

Means and Standard Deviations of six results, uncorrected and corrected, for each of the five membranes and the four standard binders.

			TYPE OF MEMBRANE				
			Cuprophan	Nephrophan	Visking	Nojax 21	Kalle
mμg Vitamin B_{12} Bound Per ml of Binder	GJ	Uncorrected	32.5 (9.2)	32.4 (2.9)	51.3 (4.7)	48.0 (5.0)	50.5 (6.9)
		Corrected	30.7 (8.0)	28.3 (2.8)	35.9 (13.5)	40.6 (5.6)	36.1 (5.0)
	HIF	Uncorrected	39.0 (2.8)	42.1 (1.7)	54.6 (10.8)	45.1 (4.3)	55.8 (5.6)
		Corrected	36.8 (2.9)	33.0 (3.4)	40.8 (14.8)	24.6 (8.7)	39.0 (3.8)

Table 24

Hydroxocobalamin

Means and Standard Deviations of six results, uncorrected and corrected, for each of the five membranes and the two standard binders.

Uncorrected values were compared statistically with the corresponding corrected value using the Wilcoxon Matched-Pairs Signed-Ranks Test. All other values were analysed statistically using the Mann-Whitney U Test. P values obtained are shown in Tables 25 - 30 (pages 59 - 64).

	Cuprophan		Nephrophan		Visking		Nojax 21		Kalle	
	Uncorrected	Corrected	Uncorrected	Corrected	Uncorrected	Corrected	Uncorrected	Corrected	Uncorrected	Corrected
Cuprophan Uncorrected		0.050	0.155	0.013	0.002	0.294	0.120	0.531	0.120	0.409
Cuprophan Corrected			0.155	0.013	0.001	0.294	0.066	0.531	0.090	0.409
Nephrophan Uncorrected				0.050	0.001	0.120	0.008	0.294	0.032	0.294
Nephrophan Corrected					0.001	0.013	0.001	0.120	0.002	0.155
Visking Uncorrected						0.050	0.120	0.090	0.531	0.027
Visking Corrected							0.155	0.409	0.322	0.294
Nojax 21 Uncorrected								0.050	0.469	0.197
Nojax 21 Corrected									0.242	0.350
Kalle Uncorrected										0.050
Kalle Corrected										

Table 25 Cyanocobalamin and Gastric Juice

The results were analysed using the Mann-Whitney U Test and the Wilcoxon Matched-Pairs Signed-Ranks Test and the values in the Table are those for P on a two tail test.

	Cuprophan		Nephrophan		Visking		Nojax 21		Kalle	
	Uncorrected	Corrected	Uncorrected	Corrected	Uncorrected	Corrected	Uncorrected	Corrected	Uncorrected	Corrected
Cuprophan Uncorrected		0.050	0.350	0.138	0.004	0.294	0.008	0.078	0.008	0.120
Cuprophan Corrected			0.197	0.294	0.002	0.242	0.004	0.047	0.002	0.090
Nephrophan Uncorrected				0.050	0.001	0.242	0.001	0.032	0.001	0.090
Nephrophan Corrected					0.001	0.120	0.001	0.032	0.001	0.008
Visking Uncorrected						0.050	0.090	0.021	0.409	0.001
Visking Corrected							0.032	0.409	0.047	0.531
Nojax 21 Uncorrected								0.050	0.220	0.004,
Nojax 21 Corrected									0.066	0.105
Kalle Uncorrected										0.050
Kalle Corrected										

Table 26. Hydroxocobalamin and Gastric Juice.

The results were analysed using the Mann-Whitney U Test and the Wilcoxon Matched-Pairs Signed-Ranks Test and the values in the Table are those for P on a two tail test.

	Cuprophan		Nephrophan		Visking		Nojax 21		Kalle	
	Uncorrected	Corrected	Uncorrected	Corrected	Uncorrected	Corrected	Uncorrected	Corrected	Uncorrected	Corrected
Cuprophan Uncorrected		0.050	0.242	0.002	0.008	0.066	0.027	0.032	0.001	0.197
Cuprophan Corrected			0.120	0.002	0.004	0.120	0.008	0.032	0.001	0.220
Nephrophan Uncorrected				0.050	0.021	0.105	0.047	0.078	0.001	0.242
Nephrophan Corrected					0.001	0.350	0.001	0.294	0.001	0.197
Visking Uncorrected						0.050	0.242	0.002	0.078	0.004
Visking Corrected							0.008	0.220	0.001	0.409
Nojax 21 Uncorrected								0.050	0.013	0.017
Nojax 21 Corrected									0.001	0.120
Kalle Uncorrected										0.050
Kalle Corrected										

Table 27. Cyanocobalamin and Hog Intrinsic Factor.

The results were analysed using the Mann-Whitney U Test and the Wilcoxon Matched-Pairs Signed-Ranks Test and the values in the Table are those for P on a two tail test.

	Cuprophan		Nephrophan		Visking		Nojax 21		Kalle	
	Uncorrected	Corrected	Uncorrected	Corrected	Uncorrected	Corrected	Uncorrected	Corrected	Uncorrected	Corrected
Cuprophan Uncorrected		0.020	0.014	0.017	0.001	0.500	0.011	0.004	0.001	0.446
Cuprophan Corrected			0.001	0.026	0.001	0.223	0.002	0.017	0.001	0.132
Nephrophan Uncorrected				0.050	0.004	0.242	0.066	0.002	0.001	0.066
Nephrophan Corrected					0.001	0.197	0.001	0.032	0.001	0.027
Visking Uncorrected						0.050	0.155	0.001	0.469	0.002
Visking Corrected							0.294	0.021	0.066	0.409
Nojax 21 Uncorrected								0.050	0.002	0.017
Nojax 21 Corrected									0.001	0.008
Kalle Uncorrected										0.050
Kalle Corrected										

Table 28. Hydroxocobalamin and Hog Intrinsic Factor.

The results were analysed using the Mann-Whitney U Test and the Wilcoxon Matched-Pairs Signed-Ranks Test and the values in the Table are those for P on a two tail test.

	Cuprophan		Nephrophan		Visking		Nojax 21		Kalle	
	Uncorrected	Corrected	Uncorrected	Corrected	Uncorrected	Corrected	Uncorrected	Corrected	Uncorrected	Corrected
Cuprophan Uncorrected		0.050	0.242	0.155	0.001	0.002	0.001	0.002	0.001	0.004
Cuprophan Corrected			0.197	0.294	0.001	0.002	0.001	0.001	0.001	0.004
Nephrophan Uncorrected				0.050	0.001	0.004	0.001	0.001	0.001	0.011
Nephrophan Corrected					0.001	0.001	0.001	0.001	0.001	0.001
Visking Uncorrected						0.050	0.001	0.001	0.380	0.001
Visking Corrected							0.013	0.032	0.008	0.066
Nojax 21 Uncorrected								0.050	0.105	0.004
Nojax 21 Corrected									0.001	0.105
Kalle Uncorrected										0.050
Kalle Corrected										

Table 29. Cyanocobalamin and Bile.

The results were analysed using the Mann-Whitney U Test and the Wilcoxon Matched-Pairs Signed-Ranks Test and the values in the Table are those for P on a two tail test.

	Cuprophan		Nephrophan		Visking		Nojax 21		Kalle	
	Uncorrected	Corrected	Uncorrected	Corrected	Uncorrected	Corrected	Uncorrected	Corrected	Uncorrected	Corrected
Cuprophan Uncorrected		0.050	0.409	0.242	0.120	0.409	0.350	0.047	0.531	0.057
Cuprophan Corrected			0.531	0.409	0.090	0.242	0.197	0.197	0.531	0.120
Nephrophan Uncorrected				0.050	0.120	0.242	0.242	0.294	0.469	0.120
Nephrophan Corrected					0.066	0.120	0.120	0.294	0.409	0.242
Visking Uncorrected						0.050	0.350	0.013	0.268	0.021
Visking Corrected							0.409	0.040	0.409	0.066
Nojax 21 Uncorrected								0.050	0.294	0.032
Nojax 21 Corrected									0.380	0.294
Kalle Uncorrected										0.050
Kalle Corrected										

Table 30. Cyanocobalamin and Saliva.

The results were analysed using the Mann-Whitney U Test and the Wilcoxon Matched-Pairs Signed-Ranks Test and the values in the Table are those for P on a two tail test.

DISCUSSION

It is clear from the results of the preliminary studies with aqueous solutions of cyanocobalamin (free cyanocobalamin) that the time elapsing until 50% of the cyanocobalamin had been lost varied from membrane to membrane. The fastest rates of loss were found with cuprophan and nephrophan and the slowest with visking, the other two membranes occupying intermediate positions. It is relevant to note that cuprophan and nephrophan were subsequently reported to have higher ultrafiltration rates than visking (Muir et al., 1970). Similar findings occurred when aqueous solutions of hydroxocobalamin were dialysed.

Within the limitations of the number of results, the values suggest that with each membrane the time taken for 50% loss was greater with hydroxocobalamin than with cyanocobalamin. This point was not further investigated as the purpose of these studies was to provide information about suitable times for dialysing free and bound materials. It might, however, be worthy of study.

The times selected for the separation of free and bound vitamin B_{12} by dialysis should have been adequate for removal of free material, except possibly in the cases of visking with cyanocobalamin and nojax 21 with hydroxocobalamin. In the latter case, it might have been wise to allow 72 hours, but in the case of visking it was felt that a period longer than 72 hours was undesirable particularly in view of the temperature at which dialysis was performed. The temperature (37°C) selected for the procedure can be criticised on several grounds, mainly

because it hastens degradation of binders and dissociation of vitamin B_{12} from the vitamin B_{12} - binder complex. It was felt, however, that the disadvantages would be offset by the increased rate of loss at 37°C compared to the rate of loss at room temperature.

The results show that the type of membrane could affect the results of dialysis separation in a statistically significant manner, depending on the binder and the form of vitamin B_{12} and the choice of corrected or uncorrected values. Again the importance of the "binder factor" and the "vitamin B_{12} factor" previously commented on in the charcoal studies is apparent. Although the corrected value was always less than the corresponding uncorrected value, the magnitude of the differences was remarkably varied, ranging from trivial (a difference of 0.5 mug, i.e. 1.8%, between the mean values with gastric juice bound cyanocobalamin and cuprophan) to considerable (a reduction of nearly 50% with bile bound cyanocobalamin and kalle). There was no clear pattern, relating to magnitude of correction, with binder or type of vitamin B_{12}. The difference between corrected and uncorrected values, however, was least with cuprophan, where the corrected value was never more than 7.6% less than the uncorrected value, and greatest with visking, nojax 21 and kalle, where the corrected values were for the most part (14 out of 18) greater than 20% less than the uncorrected values. The magnitudes of the differences are further displayed in Tables 31 & 32 (pages 67 and 68).

	Cuprophan	Nephrophan	Visking	Nojax 21	Kalle
GJ	1.8	16.4	17.2	18.1	24.2
HIF	3.1	17.5	29.4	31.4	28.7
BIL	7.6	25.7	39.3	31.8	48.3
SAL	5.0	9.8	7.9	22.6	23.2

Table 31 Cyanocobalamin

The values in the Table are the differences between uncorrected and corrected values expressed as a percentage of the uncorrected.

	Cuprophan	Nephrophan	Visking	Nojax 21	Kalle
GJ	5.5	12.7	30.0	15.4	28.5
HIF	5.6	21.6	25.3	45.5	30.1

Table 32 Hydroxocobalamin

The values in the Table are the differences between uncorrected and corrected values expressed as a percentage of the uncorrected.

Despite some very small differences, the corrected values are always significantly less ($P \leq 0.050$) than the corresponding uncorrected values when statistically analysed using the Wilcoxon Matched-Pairs Signed-Ranks Test.

The problems posed by corrected and uncorrected values are well known but little understood from the practical aspect which is, in essence, whether one value or the other is "right" or "wrong". This point will be considered further when the results obtained by dialysis are compared to those obtained by other separation methods.

SECTION 3

GEL FILTRATION

INTRODUCTION

Gel filtration is a relatively new method of fractionating and separating molecules, and a variety of agents, such as modified dextrans, agarose gels and polyacrylamide gels, are now available for this purpose.

The use of gel filtration for the separation of free and bound vitamin B_{12} was first reported by Daisley (1961) and by Kaltei & Glass (1962). The method has been most used in vitamin B_{12} work to effect separation of vitamin B_{12} in the free state from that bound to the plasma carrier proteins, transcobalamins 1 and 2 (Hom et al., 1966; Lawrence, 1966); Hom, 1967).

MATERIALS AND METHODS

Studies were undertaken using the modified dextran Sephadex G-25 fine (Pharmacia Ltd.), with a borosilicate glass column 30 cm long and 1.5 cm in diameter and having suitable end pieces and a nylon bed supporting net (Sephadex K 15/30; Pharmacia Ltd.).

The gel was prepared by hydrating 10 g of Sephadex G-25 fine with 1 litre of distilled water for 3 hours. The bulk of the supernatant was decanted and the slurry transferred to the column, which was clamped in a vertical position as indicated by plumb lines. When a layer of gel a few centimetres thick had formed, the column outlet was opened and water allowed to flow out slowly. More slurry was then added until the column was packed.

Before charging the column the excess eluent (water) above the gel was removed by gentle suction and the outlet opened until the eluent had soaked into the gel. The outlet was closed and the sample was then pipetted carefully onto the gel bed. The outlet was again opened and the effluent collected in 2 ml volumes in graduated vessels. After the sample had entered the gel the column was topped up with water and this was continued throughout. The flow rate of the effluent was 0.5 ml per minute.

The samples for separation were composed of the reagents as used in other separation studies, i.e. 1 ml of binder and 100 mμg (^{57}Co) cobalamin.

The radioactivity in each 2 ml sample of effluent was measured as described previously and the total amount in each peak obtained by addition of individual values. The proportion of bound and free radioactivity was calculated from the values for each peak and the results were further expressed as mμg vitamin B_{12} bound per ml of binder.

RESULTS

The effluent flow rate of 0.5 ml per minute provided good separation of the free and bound fractions in all cases when cyanocobalamin was used. Representative results are shown in graphic forms in Figs. 2 and 3 (pages 73 and 74) in which the first peak is the bound material and the second peak the free material.

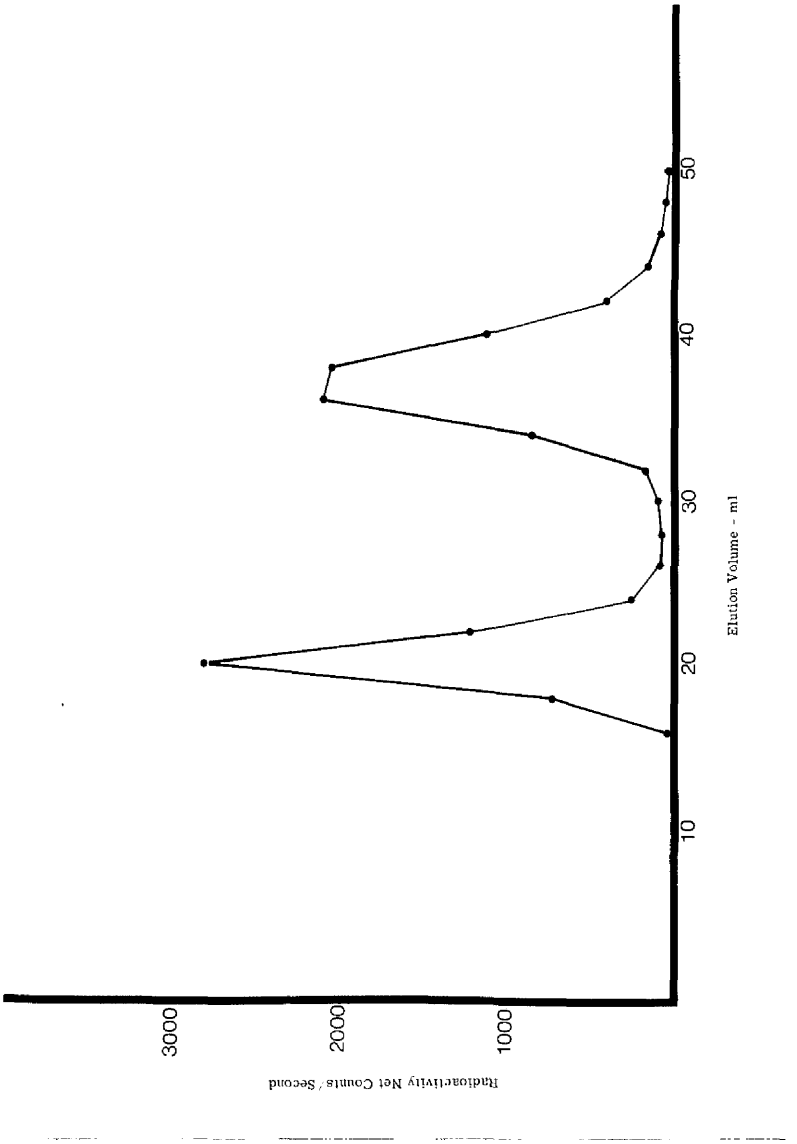


Fig. 2 Sephadex G-25 separation curve of free and Hog Intrinsic Factor bound Cyanocobalamin. The first peak represents the bound fraction and the second peak the free fraction.

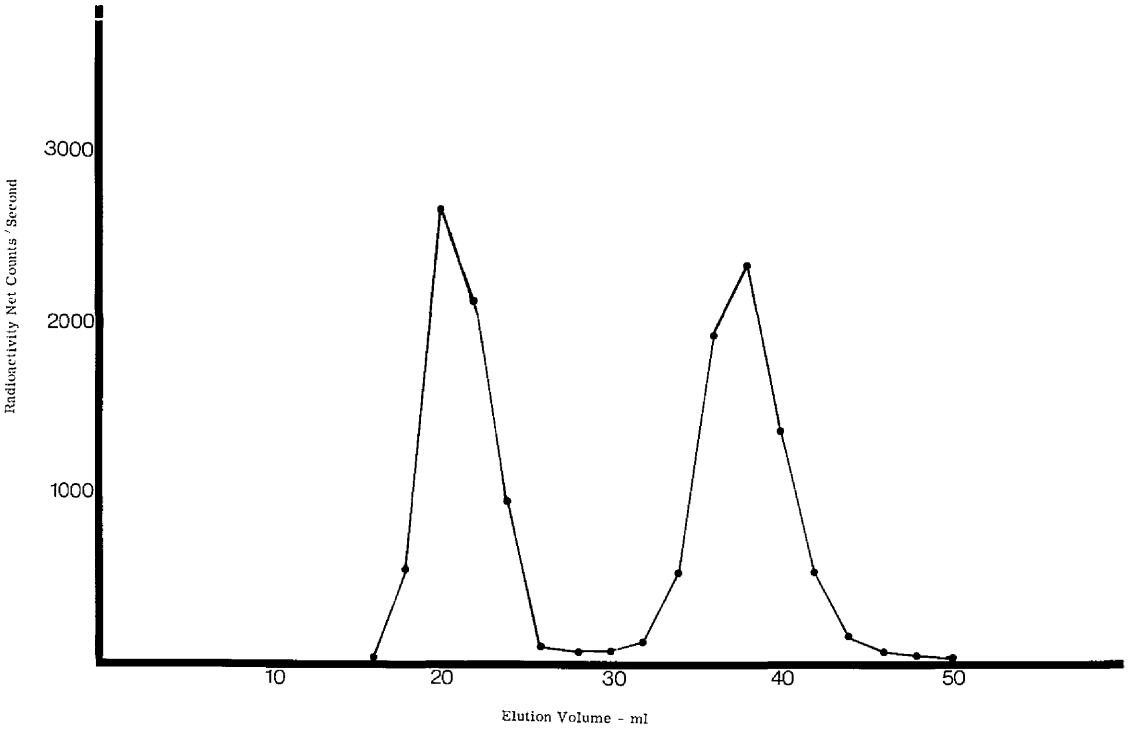


Fig. 3 Sephadex G-25 separation curve of free and Saliva bound Cyanocobalamin. The first peak represents the bound fraction and the second peak the free fraction.

The mean values and standard deviations of six separations of cyanocobalamin binder mixtures are set out in Table 33 (page 74).

Separation of free hydroxocobalamin and hydroxocobalamin bound to the various binders could not be effected as both the free and the bound fractions were retained by the gel.

DISCUSSION

The failure to fractionate free and bound hydroxocobalamin is of interest. Gel filtration is thought to achieve separation by a steric effect (Sephadex Literature), the large molecules failing to penetrate the immediate environment of the cross links between the gel chains and the gel matrix. The small molecules, on the other hand, do so and are therefore eluted after the large molecules. It is known, however, that some substances behave in a way which does not conform to this steric concept. For example adsorption may occur when a hydroxyl ion (Sephadex Literature) on the sample complexes with the polysaccharide framework of the gel and indeed this might explain the anomalies observed with hydroxocobalamin.

It is possible that the phenomenon which renders separation of free and bound hydroxocobalamin by gel filtration impracticable might also seriously interfere with the separation of other free and bound naturally occurring cobalamins such as methylcobalamin and coenzyme B_{12}. In such circumstances there might be a case for

converting the naturally occurring cobalamins to cyanocobalamin by treatment with potassium or sodium cyanide to permit the separation of free and bound compounds.

The phenomenon might also have practical application in the separation of cyanocobalamin from its photolytic product hydroxocobalamin, complementing the use of the cation exchanger carboxy-methyl-cellulose which retains hydroxocobalamin but not cyanocobalamin (Kennedy & Adams, 1965; Kennedy, 1967).

		Sephadex G-25
mμg Vitamin B_{12} Bound Per ml of Binder	GJ	28.0 (3.8)
	HIF	45.3 (5.6)
	BIL	2.4 (0.5)
	SAL	44.3 (1.3)

Table 33 Cyanocobalamin

Means and Standard Deviations of six results for each of the four standard binders.

SECTION 4

ULTRAFILTRATION

INTRODUCTION

Ultrafiltration may be defined as the separation of solute-solute or solute-solvent complexes by the use of semi-permeable membranes and static pressure. The applications of ultrafiltration have broadened by developments in membrane technology and by the introduction of commercially available systems. The effect of two ultrafiltration systems on the separation of free and bound vitamin B_{12} was studied.

MATERIALS AND METHODS

System 1 (Millipore Ultrafiltration)

The ultrafiltration cell used was a commercially available Millipore 25 mm U-F cell, capacity 17 ml, having a polycarbonate barrel and polypropylene end fittings, the membrane being supported on a stainless steel support screen. The membranes used were Millipore Pellicon, type PSED, 25 mm in diameter with an effective area of 3.9 cm^2 and a flow rate (with water) of 0.17 ml/minute at 85 pounds per square inch. After the test solution was placed in the cell, the vacant volume was flushed with nitrogen, the cell connected to a reservoir of compressed nitrogen (British Oxygen Company) and the pressure in the cell raised to and maintained at 85 pounds per square inch.

Preliminary studies were carried out with solutions of radioactive cyanocobalamin and hydroxocobalamin 100 mμg in 5 ml water (free vitamin B_{12} solutions). The first 2 ml of

effluent (ultrafiltrate) were collected, the volume being determined by weighing the collecting vessel before and during ultrafiltration, and the retention of the solute calculated from the formula $R = 100 \left(1 - \frac{Ce}{Co}\right)$, where R is the solute retention as a percentage, Ce the concentration of the solute in the effluent, and Co the concentration in the initial (original) cell charge. It was expected that the value for R with aqueous solutions of free vitamin B_{12} would be zero (Millipore Application Report AR-21 1969). This, however, did not prove to be so (on three occasions the value for R obtained with cyanocobalamin ranged from 13.2% - 37.3% and the value for R obtained with hydroxocobalamin ranged from 50.0% - 66.9%). A wash through method was therefore tried. In this technique, ultrafiltration was continued until flow had ceased, the system was depressurised and 5 ml of water placed in the cell and the system repressurised and ultrafiltration continued until flow had again ceased. With this technique R values approximating to zero were obtained.

Separation studies were carried out with mixtures of radioactive vitamin B_{12}, 100 mμg, and 1 ml of binder (the four standard binders as described previously were again used), the total volume of the test solution being 5 ml. As in the preliminary studies two values for R were obtained with each test. One was obtained using measurements taken during ultrafiltration (formula method) and is subsequently referred to as the uncorrected value. The second was obtained by the wash through method and is subsequently

referred to as the corrected value. Since, in theory, the bound vitamin B_{12} was retained, the results could be further expressed as mμg vitamin B_{12} bound per ml of binder.

System 2 (Vacuum Ultrafiltration)

The apparatus used, shown in Fig. 4 (page 82), was constructed from Quickfit glassware and Visking 8/32" cellulose tubing, ¼ inch flat diameter, and was the same as that described by Gregory (1954) and Gregory & Holdsworth (1955). After the introduction of the test solution a negative pressure of 100 mm of mercury was applied by a vacuum pump and the first 2 ml. of ultrafiltrate, measured by weighing the collecting vessel before and during ultrafiltration, was collected. The value for R (the percentage retention) was calculated from the formula previously described.

Preliminary studies with radioactive cyanocobalamin and hydroxocobalamin, 100 mμg in 5 ml of water (i.e. free vitamin B_{12} solutions) were carried out, and again the values obtained for R were appreciably greater than the expected value of zero. (On three occasions the value for R obtained with cyanocobalamin ranged from 29.9% - 32.2% and with hydroxocobalamin from 27.3% - 38.3%).

Accordingly, it was decided to calculate the results of separation studies of binder-vitamin B_{12} mixtures, firstly by the formula method (uncorrected value) and secondly by correcting this result (corrected value) using the R value obtained from a

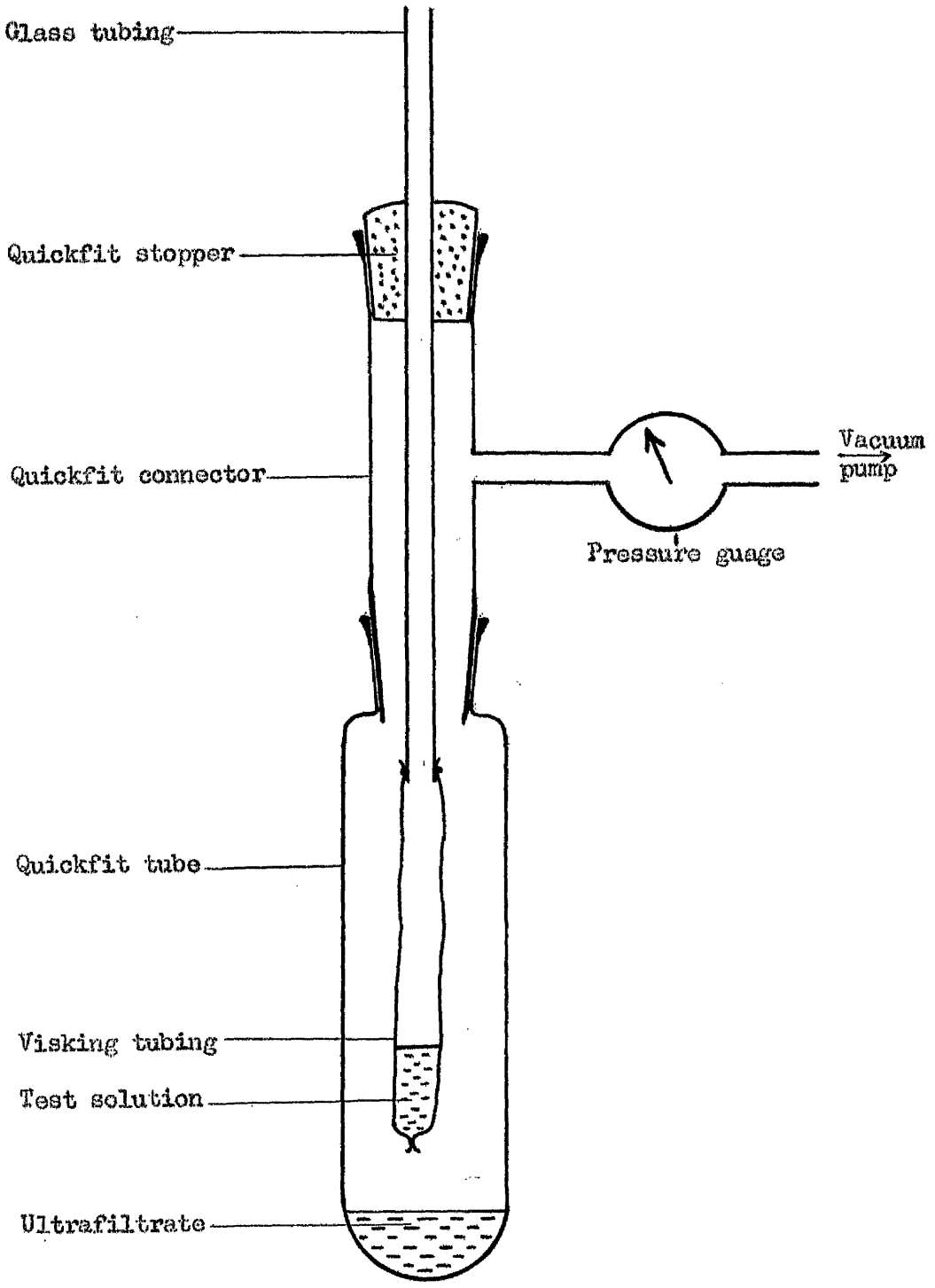


Fig. 4. Ultrafiltration (Vacuum System) Equipment.

control system in which an aqueous solution of vitamin B_{12} (free vitamin B_{12}) was ultrafiltered simultaneously with the binder-vitamin B_{12} mixtures. The correction was accomplished by calculating the percentage of free vitamin B_{12} in the control ultrafiltrate from the control value for R. Since this should, theoretically, have been 100%, the free vitamin B_{12} value obtained from the binder-vitamin B_{12} test could be proportionately adjusted and thus the corrected value for bound vitamin B_{12} was obtained. For example:-

1) R control = 23.5%

   % Free vitamin B_{12} in control ultrafiltrate = 76.5%

2) R test = 74.1%

   Uncorrected value mμg vitamin B_{12} bound = 74.1

   mμg vitamin B_{12} free = 25.9

3) but from control result free vitamin B_{12} represents only 76.5% of expected 100%

4) Corrected (test) mμg vitamin B_{12} free = 33.9

5) Corrected (test) mμg vitamin B_{12} bound = 66.1

As before the results (uncorrected and corrected) were expressed as mμg vitamin B_{12} bound per ml of binder.

With both systems, radioactivity was measured against suitable standards, as previously described, allowances being made for volume changes and background radioactivity.

Also as before, each test was carried out on six separate occasions. However, because of the shortage of bile and saliva the studies with these binders were limited to cyanocobalamin

binding using the Millipore system and were excluded completely from the Vacuum system studies.

Statistical Methods

Statistical analyses were carried out using the Wilcoxon Matched-Pairs Signed-Ranks Test and the Mann-Whitney U Test, both of which are described by Siegel (1956).

RESULTS

The results are presented in Tables 34 - 37 (pages 85 - 88) in which are shown the mean values and standard deviations derived from six results obtained at different times. The results relating to Millipore ultrafiltration are in Tables 34 and 35 (pages 85 and 86) and those relating to Vacuum ultrafiltration in Tables 36 and 37 (pages 87 and 88).

			PSED Membrane
mμg Vitamin B_{12} Bound Per ml of Binder	GJ	Uncorrected	59.7 (10.3)
		Corrected	37.0 (8.9)
	HIF	Uncorrected	66.6 (1.8)
		Corrected	39.1 (2.4)
	BIL	Uncorrected	62.6 (9.2)
		Corrected	5.6 (3.0)
	SAL	Uncorrected	88.5 (2.8)
		Corrected	62.4 (8.0)

Table 34 Millipore Ultrafiltration and Cyanocobalamin

Means and Standard Deviations of six results, uncorrected and corrected, for each of the four standard binders.

			PSED Membrane
mμg Vitamin B_{12} Bound Per ml of Binder	GJ	Uncorrected	84.4 (5.2)
		Corrected	72.5 (10.8)
	HIF	Uncorrected	87.6 (3.3)
		Corrected	73.6 (8.8)

Table 35 Millipore Ultrafiltration and Hydroxocobalamin

Means and Standard Deviations of six results, uncorrected and corrected, for each of the two standard binders.

			Visking Membrane
mμg Vitamin B_{12} Bound Per ml of Binder	GJ	Uncorrected	62.7 (7.6)
		Corrected	51.8 (10.8)
	HIF	Uncorrected	69.3 (7.1)
		Corrected	61.8 (8.1)

Table 36 Vacuum System and Cyanocobalamin

Means and Standard Deviations of six results, uncorrected and corrected, for each of the two standard binders.

			Visking Membrane
mμg Vitamin B_{12} Bound Per ml of Binder	GJ	Uncorrected	43.3 (10.1)
		Corrected	34.9 (10.0)
	HIF	Uncorrected	55.5 (4.4)
		Corrected	48.8 (4.2)

Table 37 Vacuum System and Hydroxocobalamin

Means and Standard Deviations of six results, uncorrected and corrected, for each of the two standard binders.

Uncorrected values were compared statistically with the corresponding corrected value using the Wilcoxon Matched-Pairs Signed-Ranks Test. All other values were analysed statistically using the Mann-Whitney U Test. P values obtained are shown in Tables 38 - 41 (pages 90 - 93).

With bile and saliva the only results obtained are with cyanocobalamin binding and using Millipore ultrafiltration. Thus only uncorrected and corrected values were compared. The Wilcoxon Matched-Pairs Signed-Ranks Test reveals that with both binders the P value is 0.050.

		Millipore		Vacuum	
		Uncorrected	Corrected	Uncorrected	Corrected
Millipore	Uncorrected		0.050	0.001	0.001
	Corrected			0.002	0.001
Vacuum	Uncorrected				0.050
	Corrected				

Table 38 Cyanocobalamin and Castric Juice

The results were analysed using the Mann-Whitney U Test and the Wilcoxon Matched-Pairs Signed-Ranks Test and the values in the Table are those for P on a two tail test.

		Millipore		Vacuum	
		Uncorrected	Corrected	Uncorrected	Corrected
Millipore	Uncorrected		0.050	0.294	0.155
	Corrected			0.001	0.021
Vacuum	Uncorrected				0.050
	Corrected				

Table 39 Hydroxocobalamin and Gastric Juice

The results were analysed using the Mann-Whitney U Test and the Wilcoxon Matched-Pairs Signed-Ranks Test and the values in the Table are those for P on a two tail test.

		Millipore		Vacuum	
		Uncorrected	Corrected	Uncorrected	Corrected
Millipore	Uncorrected		0.050	0.294	0.268
	Corrected			0.001	0.001
Vacuum	Uncorrected				0.050
	Corrected				

Table 40 Cyanocobalamin and Hog Intrinsic Factor

The results were analysed using the Mann-Whitney U Test and the Wilcoxon Matched-Pairs Signed-Ranks Test and the values in the Table are those for P on a two tail test.

		Millipore		Vacuum	
		Uncorrected	Corrected	Uncorrected	Corrected
Millipore	Uncorrected		0.050	0.001	0.001
	Corrected			0.001	0.001
Vacuum	Uncorrected				0.050
	Corrected				

Table 41 Hydroxocobalamin and Hog Intrinsic Factor

The results were analysed using the Mann-Whitney U Test and the Wilcoxon Matched-Pairs Signed-Ranks Test and the values in the Table are those for P on a two tail test.

DISCUSSION

It was perhaps unrealistic to expect that ultrafiltration would present fewer problems than any other separation method but the literature on the subject induced misapprehensions.

The use of ultrafiltration in the separation of free and bound vitamin B_{12} was introduced by Gregory et al., (1952) who, finding no microbiologically assayable material in the ultrafiltrate of milk assumed that the vitamin B_{12} in milk was bound (Gregory et al., 1952; Gregory, 1954). In these and other publications (Gregory & Holdsworth, 1955), there is no mention of control studies with vitamin B_{12} in aqueous solution (free vitamin B_{12}).

The equipment described by Gregory (1954) and used in one of the current studies was crude and it was hoped that the contribution of more technically satisfactory equipment and membranes with known molecular permeability would be advantageous. The membrane used in the Millipore ultrafiltration system (PSED) is stated to have a molecular cut off in the region of 25,000, this being found by studies with substances of known molecular weight. Although it was appreciated that molecular weight alone is not always a reliable guide to membrane performance, it was nevertheless considered probable that the retention of free vitamin B_{12}, with a molecular weight of 1,500, would be zero and this was supported by the manufacturers' data (Millipore Manual, 1969). It is obvious that in the case of vitamin B_{12} and PSED membranes, factors other than molecular weight are at work,

but the nature of these is not clear. One possibility is that the vitamin B_{12} polarises and concentrates on the membrane surface, especially since the system was not agitated. However, the radioactivity on membranes, rinsed after use, was negligible. Discussion with representatives of the manufacturers left the impression that while ultrafiltration studies are usually conducted by the formula method there are occasions when this method is unacceptable, i.e. when results by a wash through method are more meaningful. Although our studies with free vitamin B_{12} appear to support this, it does not necessarily mean that the same applies with separation studies, but it does seem likely.

The statistical analyses (Tables 38 - 41, pages 90 - 93) reveal that method of ultrafiltration, form of binder and vitamin B_{12} and choice of uncorrected or corrected values, affect the results in a statistically significant manner. As with dialysis, the corrected values are always significantly less ($P \leq 0.050$) than the corresponding uncorrected values, irrespective of the magnitude of the difference, when analysed using the Wilcoxon Matched-Pairs Signed-Ranks Test. Of the other comparisons, analysed using the Mann-Whitney U Test, most (12 out of 16) are significant ($P \leq 0.050$).

SECTION 5

SEPARATION EVALUATION

Each of the methods studied - charcoal separation, bag dialysis, gel filtration and ultrafiltration - is a well known separation procedure and all have been used to effect separation of free and bound vitamin B_{12}.

The merits and demerits of each method, vis a vis the other, can be summarised as follows:-

1) Charcoal Separation

This was a very rapid procedure requiring little manipulative skill and only simple reagents. It can be applied to a very large number of samples in a working day. The reproducibility, as judged by the standard deviation expressed as a percentage of the mean, was calculated for each charcoal preparation and each cobalamin and binder and tabulated (Tables 42 - 49, pages 98 - 105).

In general, the values are high and there is a tendency towards a rise with increasing concentrations of charcoal, although this was not invariable. The reproducibility was very good when Norit OL coated charcoal was used to separate hog intrinsic factor bound and free cyanocobalamin, and when Norit OL coated and Norit A coated charcoals were used to separate hog intrinsic factor bound and free hydroxocobalamin. This must be regarded as a point in favour of charcoal separation, at least in some circumstances.

	CONCENTRATIONS OF ADDED CHARCOAL				
	0.5%	1%	2.5%	5%	10%
GJ	20.7	25.5	34.6	41.3	45.4
HIF	3.8	5.1	4.5	2.6	5.5
BIL	7.0	9.1	18.7	19.4	36.4
SAL	9.8	10.0	19.2	24.7	26.7
Control	33.3		14.3		21.4

Table 42 Norit OL Charcoal, coated, and Cyanocobalamin

Reproducibility as judged by the standard deviation expressed as a percentage of the mean.

	CONCENTRATIONS OF ADDED CHARCOAL				
	0.5%	1%	2.5%	5%	10%
GJ	1.7	6.0	34.7	71.0	66.7
HIF	18.0	25.8	45.5	20.0	50.0
BIL	7.9	9.8	45.3	64.1	71.4
SAL	9.0	16.8	29.0	50.9	53.1
Control	40.6		41.2		33.3

Table 43 Norit OL Charcoal, raw, and Cyanocobalamin

Reproducibility as judged by the standard deviation expressed as a percentage of the mean.

	CONCENTRATIONS OF ADDED CHARCOAL		
	0.5%	2.5%	10%
GJ	14.9	22.0	32.5
HIF	20.8	22.3	22.7
BIL	12.3	22.7	23.6
SAL	10.2	19.3	31.5
Control	84.8	13.6	26.3

Table 44 Norit A Charcoal, coated, and Cyanocobalamin

Reproducibility as judged by the standard deviation expressed as a percentage of the mean.

	CONCENTRATIONS OF ADDED CHARCOAL		
	0.5%	2.5%	10%
GJ	12.8	42.5	66.7
HIF	28.3	16.7	33.3
BIL	5.3	20.5	29.6
SAL	8.8	31.9	21.4
Control	51.9	26.9	33.3

Table 45 Norit A Charcoal, raw, and Cyanocobalamin

Reproducibility as judged by the standard deviation expressed as a percentage of the mean.

	CONCENTRATIONS OF ADDED CHARCOAL				
	0.5%	1%	2.5%	5%	10%
GJ	10.1	16.9	28.0	26.2	29.6
HIF	3.8	2.2	4.7	4.5	3.1
BIL	8.0	10.3	17.4	27.9	36.8
Control	7.9		11.4		13.6

Table 46 Norit OL Charcoal, coated, and Hydroxocobalamin

Reproducibility as judged by the standard deviation expressed as a percentage of the mean.

	CONCENTRATIONS OF ADDED CHARCOAL				
	0.5%	1%	2.5%	5%	10%
GJ	6.3	14.3	59.3	80.0	66.7
HIF	12.5	17.0	42.9	16.7	33.3
BIL	9.9	13.5	68.2	64.7	44.4
Control	23.1		46.5		22.2

Table 47 Norit OL Charcoal, raw, and Hydroxocobalamin

Reproducibility as judged by the standard deviation expressed as a percentage of the mean.

	CONCENTRATIONS OF ADDED CHARCOAL		
	0.5%	2.5%	10%
GJ	6.1	31.4	17.7
HIF	10.5	6.5	5.7
BIL	5.5	22.1	28.3
Control	21.7	15.8	18.2

Table 48 Norit A Charcoal, coated, and Hydroxocobalamin

Reproducibility as judged by the standard deviation expressed as a percentage of the mean.

	CONCENTRATIONS OF ADDED CHARCOAL		
	0.5%	2.5%	10%
GJ	8.4	38.5	75.0
HIF	17.7	17.4	22.2
BIL	44.1	30.0	11.8
Control	24.6	16.7	27.3

Table 49 Norit A Charcoal, raw, and Hydroxocobalamin

Reproducibility as judged by the standard deviation expressed as a percentage of the mean.

When used to separate free and bound radioactive cyanocobalamin, as in the assay of intrinsic factor in gastric juice or intrinsic factor antibodies in serum, the cost rises to account for the isotopic reagents and equipment. This however applies to all the separation methods.

2) Bag Dialysis

This technique was time consuming and laborious but requiring modest manipulative skill and only simple equipment. Only a moderate number of samples can be processed daily. Because of the time scale necessary, dissociation of bound vitamin B_{12} seems probable and a source of error. The reproducibility, as judged by the standard deviation expressed as a percentage of the mean, was calculated for each membrane and each cobalamin and binder (uncorrected and corrected). The reproducibilities are tabulated (Tables 50 and 51, pages 107 and 108).

The reproducibility of results using the various membranes varied widely, ranging from 4.0% to 45.9%, with no one membrane giving a low value in every circumstance.

		TYPE OF MEMBRANE				
		Cuprophan	Nephrophan	Visking	Nojax 21	Kalle
GJ	Uncorrected	17.1	21.7	13.4	25.5	23.3
	Corrected	16.7	19.1	23.0	35.3	45.9
HIF	Uncorrected	4.4	17.9	11.1	8.9	8.2
	Corrected	4.6	21.3	29.8	31.4	14.6
BIL	Uncorrected	30.3	20.0	13.2	11.2	24.8
	Corrected	31.1	23.1	23.9	8.6	22.3
SAL	Uncorrected	22.2	26.6	20.9	21.0	35.2
	Corrected	24.3	26.2	23.8	32.1	41.8

Table 50 Cyanocobalamin

Reproducibility as judged by the standard deviation expressed as a percentage of the mean.

		TYPE OF MEMBRANE				
		Cuprophan	Nephrophan	Visking	Nojax 21	Kalle
GJ	Uncorrected	28.3	9.0	9.2	10.4	13.7
	Corrected	26.1	9.9	38.6	13.8	13.9
HIF	Uncorrected	7.2	4.0	19.8	9.5	10.0
	Corrected	7.9	10.3	36.3	35.4	9.7

Table 51 Hydroxocobalamin

Reproducibility as judged by the standard deviation expressed as a percentage of the mean.

3) Gel Filtration

The apparatus for this method is more costly than for the other methods. The technique is very laborious, unless a fraction collector is used, which adds to the cost, and the capacity for dealing with multiple samples is low. Some skill is necessary in the preparation of columns and the introduction of materials into the columns. One considerable limitation, not shared with the other methods, is that separation of free and bound hydroxocobalamin is not practicable, although this might be possible if conversion of hydroxocobalamin to cyanocobalamin were incorporated as a stage. The reproducibility, as judged by the standard deviation expressed as a percentage of the mean, was again calculated (Table 52, page 110).

The reproducibility was remarkably good when Sephadex G-25 was used to separate saliva-bound and free cyanocobalamin but rather poor with the other binders especially bile.

	Sephadex G-25
GJ	13.6
HIF	12.4
BIL	20.8
SAL	2.9

Table 52 Cyanocobalamin

Reproducibility as judged by the standard deviation expressed as a percentage of the mean.

4) Ultrafiltration

The apparatus may be simple or relatively complex if commercially available systems are used and the cost varies proportionately. The manipulative skill required is modest. The speed of the procedure varies, but the capacity for dealing with multiple samples is low. Reproducibility, as judged by the standard deviation, expressed as a percentage of the mean, was calculated for each ultrafiltration method, uncorrected and corrected, and each cobalamin and binder. The results are tabulated (Tables 53 and 54 pages 112 and 113).

Here again the values varied widely, from 2.7% to 53.6%. In all cases the values for corrected results were greater than the corresponding uncorrected values. For the most part, Millipore ultrafiltration was the more impressive, with very low values being obtained in the Millipore separation of free and hog intrinsic factor bound cyanocobalamin (both uncorrected and corrected), of free and saliva bound cyanocobalamin (uncorrected), of free and gastric juice bound hydroxocobalamin (uncorrected), and of free and hog intrinsic factor bound hydroxocobalamin (uncorrected).

		Millipore	Vacuum
GJ	Uncorrected	17.3	12.1
	Corrected	24.1	20.8
HIF	Uncorrected	2.7	10.2
	Corrected	6.1	13.1
BIL	Uncorrected	14.7	NA
	Corrected	53.6	NA
SAL	Uncorrected	3.2	NA
	Corrected	12.8	NA

Table 53 Cyanocobalamin

Reproducibility as judged by the standard deviation expressed as a percentage of the mean. Where no result was obtained NA (Not applicable) has been inserted.

		Millipore	Vacuum
GJ	Uncorrected	6.2	23.3
	Corrected	14.9	28.7
HIF	Uncorrected	3.8	7.9
	Corrected	12.0	8.6

Table 54 Hydroxocobalamin

Reproducibility as judged by the standard deviation expressed as a percentage of the mean.

All these points are fairly clear. What is not clear, however, is how the results obtained by one method compare with those obtained by another method. (The term "accuracy" cannot be used as there is no reason to believe that results obtained by one method are any nearer the truth - if there is such a thing in separation work - than those obtained by another). No record of work designed to evaluate comparability of results can be traced, although Chanarin (1969) states "In general, column chromatography and charcoal adsorption, when properly used give comparable results. The results obtained by ultrafiltration are similar to those obtained by dialysis".

In an attempt to clarify the problem of comparability of results obtained by different methods, all the results reported in the previous sections were collected and studied. Inspection revealed an astonishing range of values and this is best shown graphically in Fig. 5 (page 116), the results being obtained with the separation of free and gastric juice bound cyanocobalamin. Similar wide ranges were found with cyanocobalamin and other binders and also with hydroxocobalamin (Figs. 6 - 11, pages 117 - 122).

The ranges of results are artificially great because of inclusion of all results, including some of which, as mentioned in the appropriate section, must be regarded with reserve. Nevertheless, it was thought wise to include these results, at least in a preliminary analysis. Analyses of results, subdivided by binder and form of vitamin B_{12}, by the Kruskal-Wallis One-Way Analysis of Variance by Ranks (Siegel, 1956) confirmed the

suspicion that significant differences existed. Accordingly, pairs of results were analysed by the Mann-Whitney U Test or the Wilcoxon Matched-Pairs Signed-Ranks Test (Siegel, 1956) and the results are set out in Tables 55 - 61, (pages 123 - 129) in which are given the values of P for each pair of results.

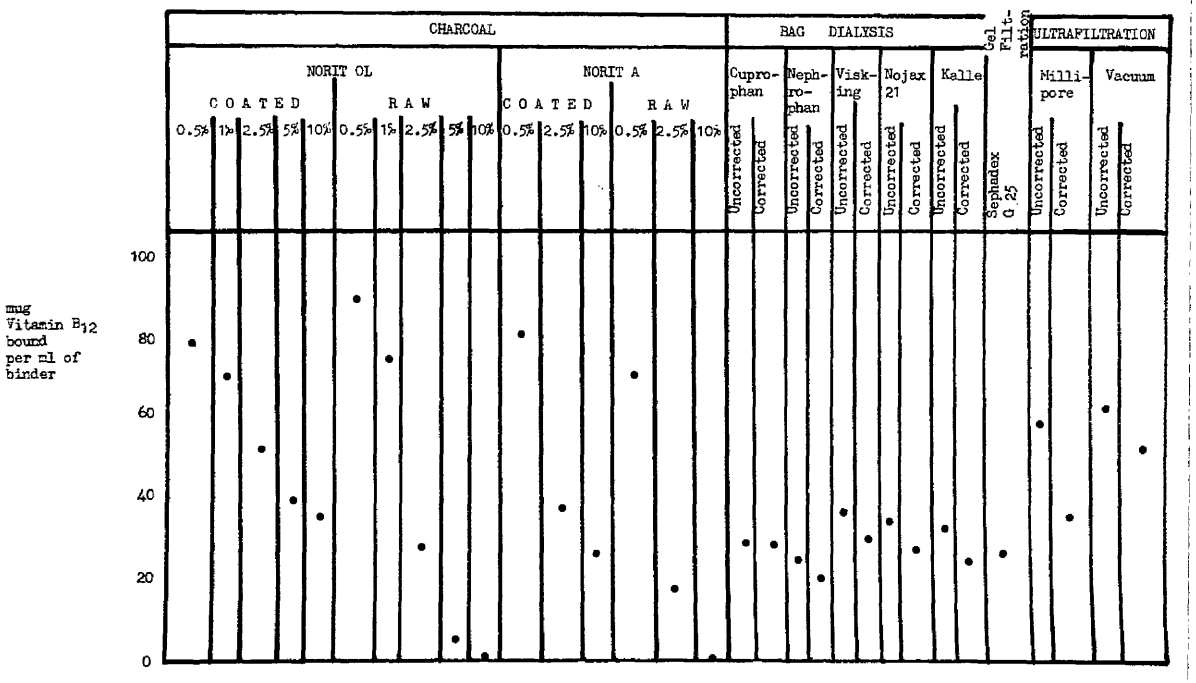


Fig. 5. Gastric Juice and Cyanocobalamin

The results obtained from all the separation methods shown graphically to illustrate the range of results.

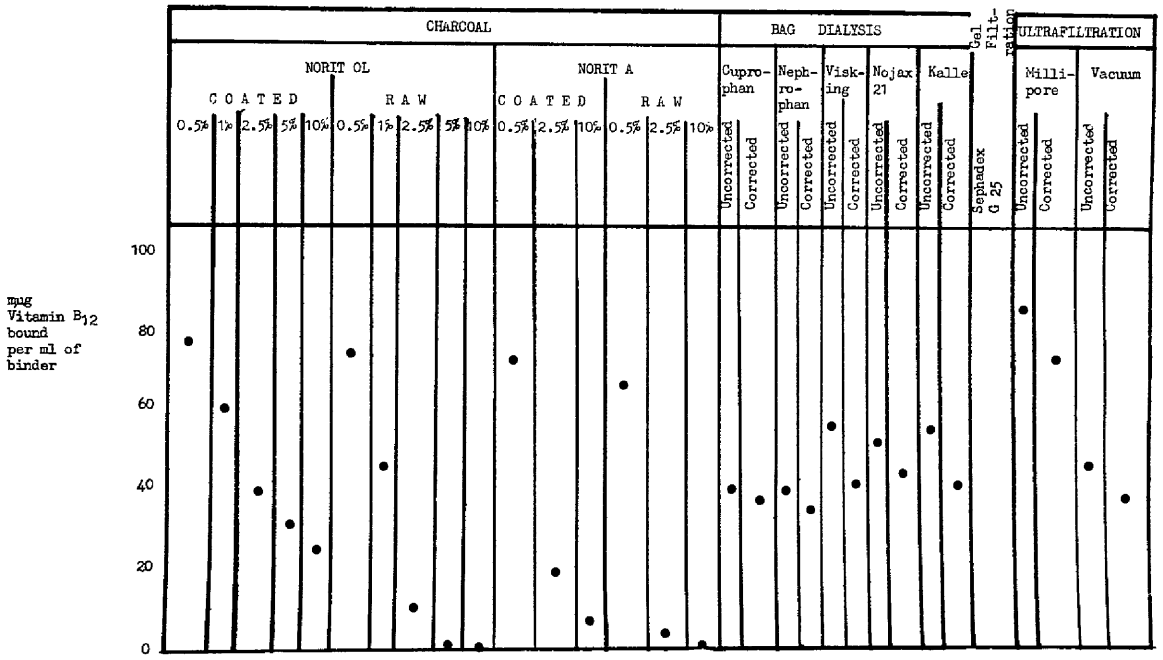


Fig. 6. Gastric Juice and Hydroxocobalamin

The results obtained from all the separation methods shown graphically to illustrate the range of results.

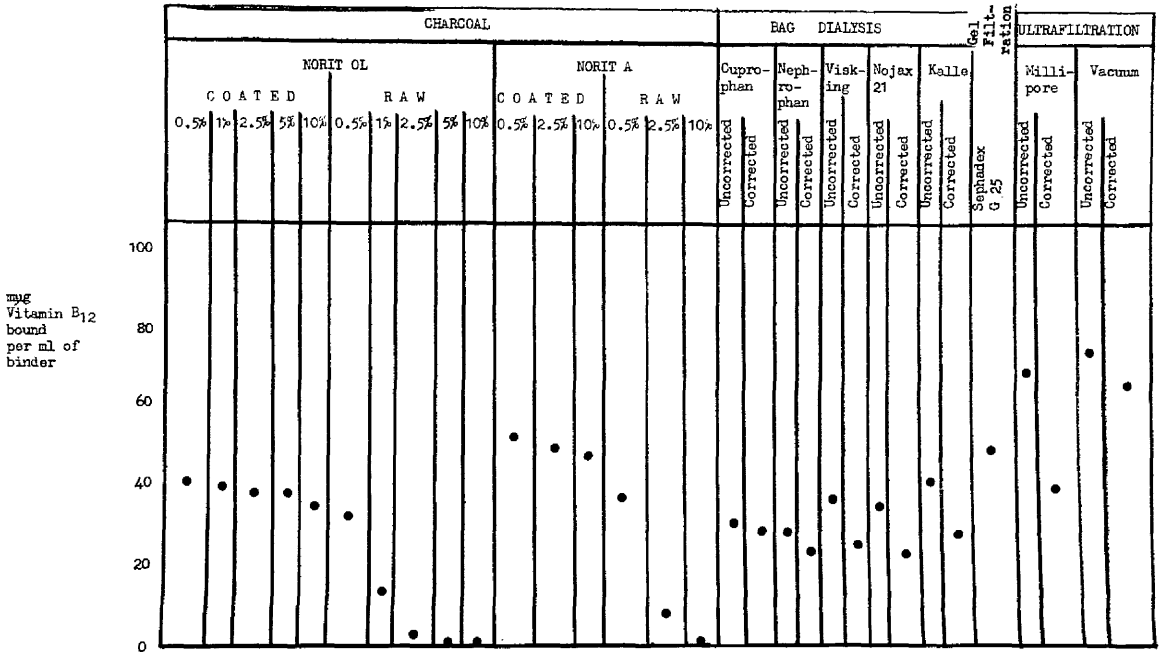


Fig. 7. Hog Intrinsic Factor and Cyanocobalamin.

The results obtained from all the separation methods shown graphically to illustrate the range of results.

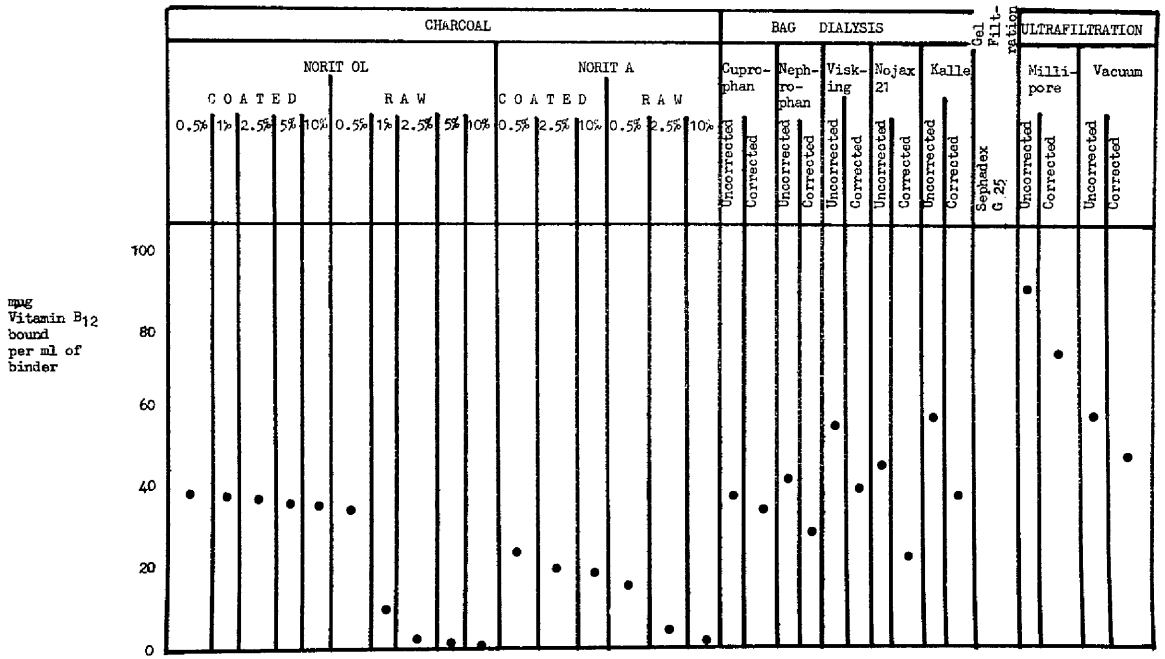


Fig. 8. Hog Intrinsic Factor and Hydroxocobalamin.

The results obtained from all the separation methods shown graphically to illustrate the range of results.

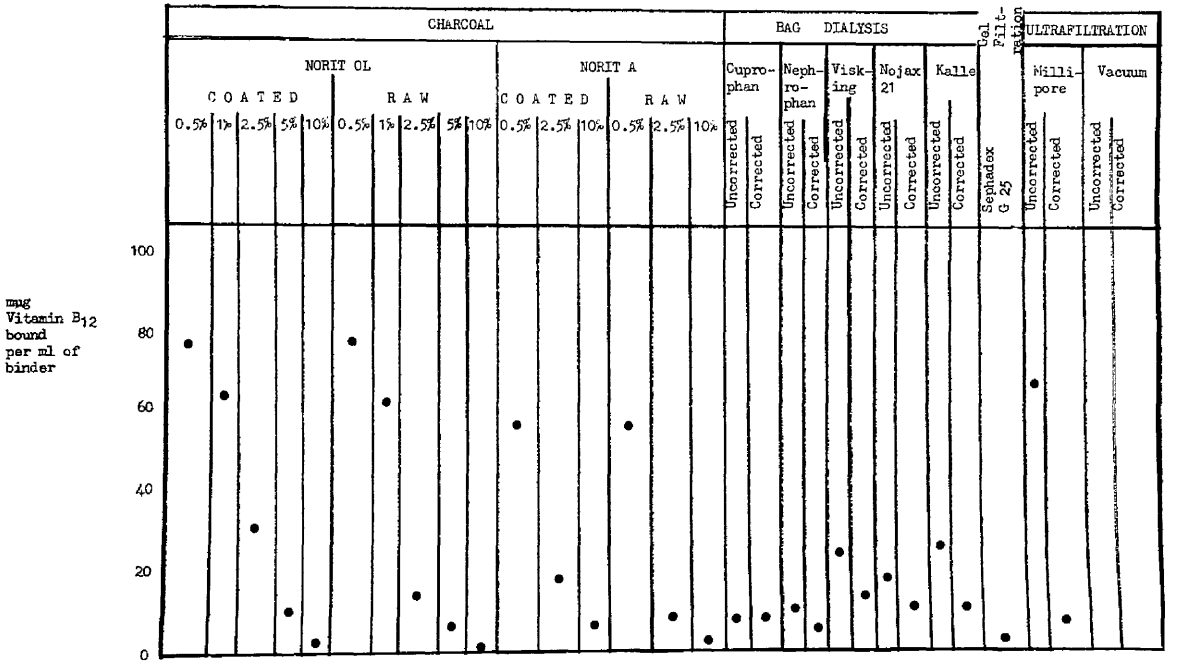


Fig. 9. Bile and Cyanocobalamin.

The results obtained from all the separation methods shown graphically to illustrate the range of results.

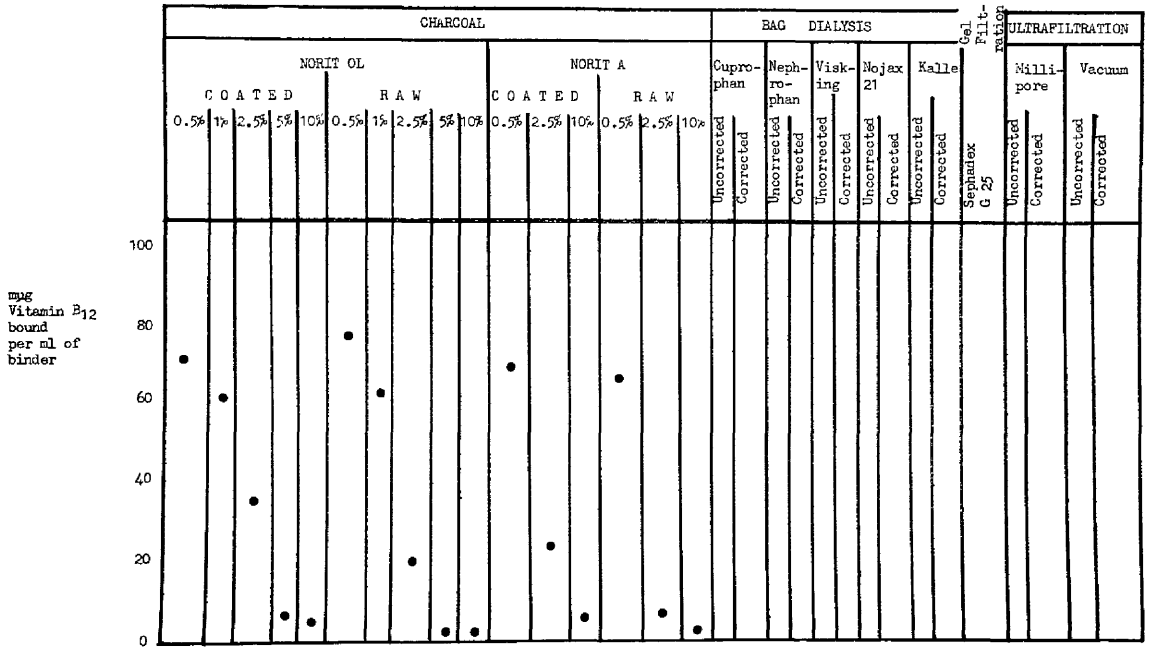


Fig. 10. Bile and Hydroxocobalamin.

The results obtained from all the separation methods shown graphically to illustrate the range of results.

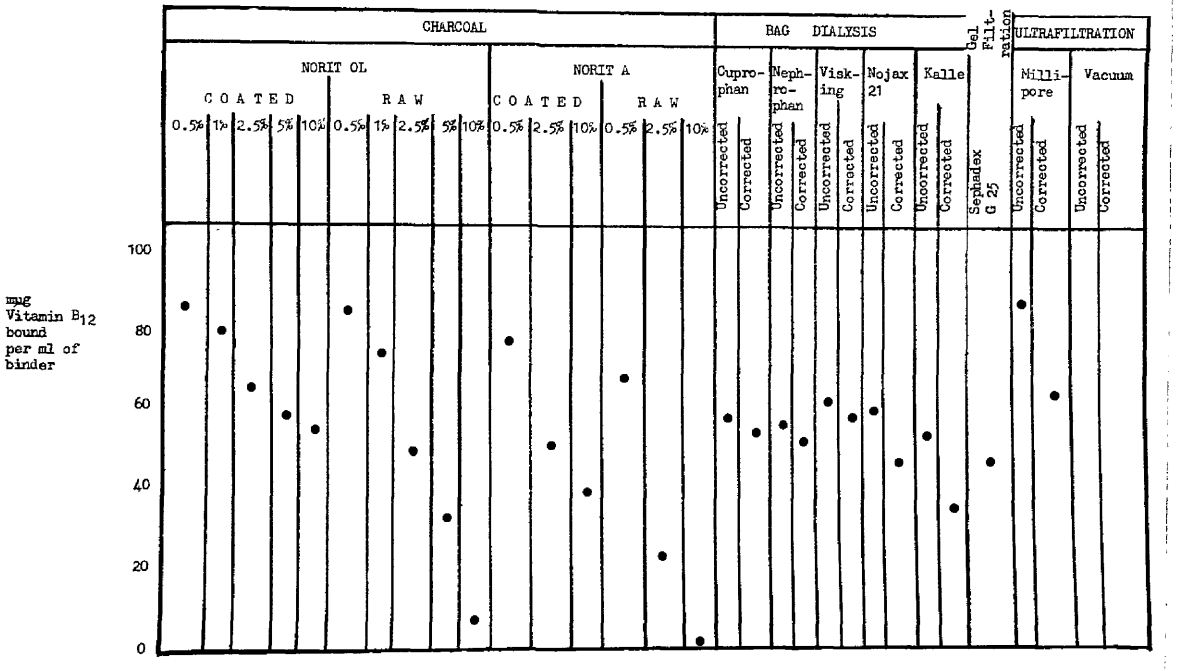
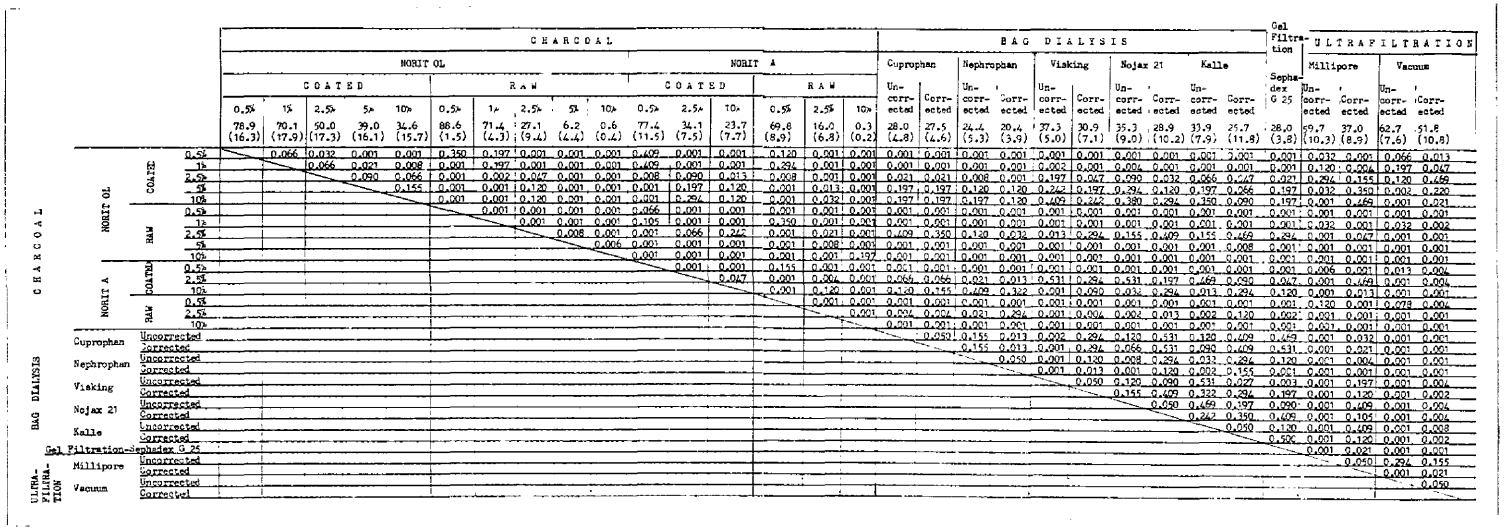


Fig. 11. Saliva and Cyanocobalamin.

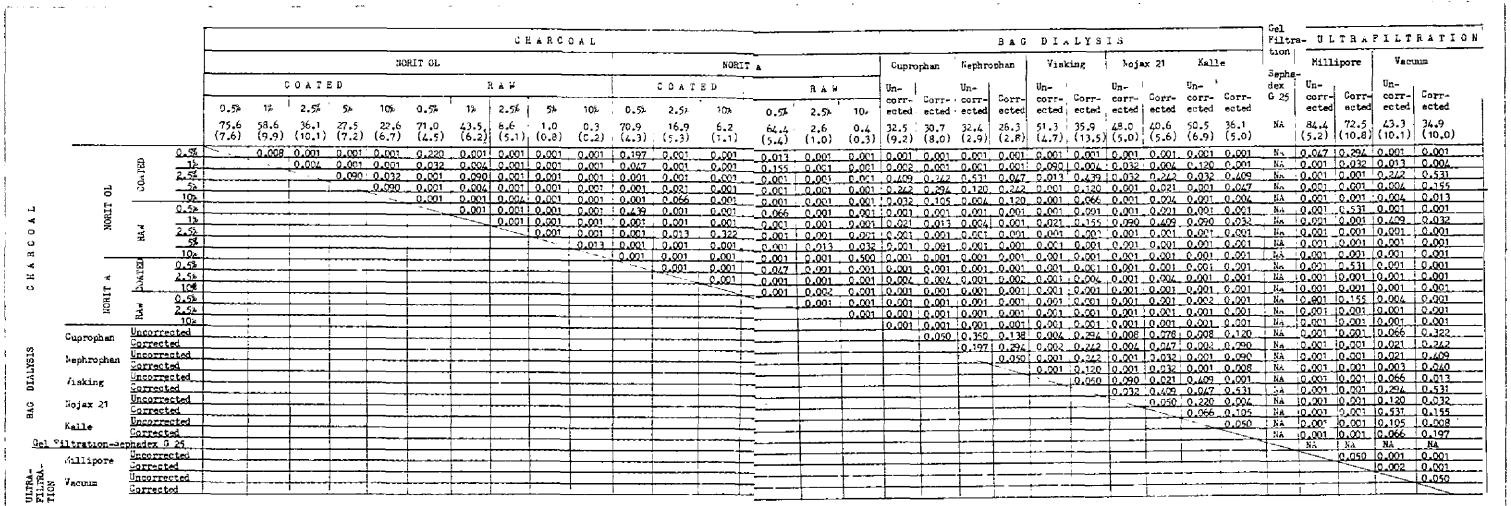
The results obtained from all the separation methods shown graphically to illustrate the range of results.



			CHARCOAL															BAG DIALYSIS										Gel Filtration	ULTRAFILTRATION			
			NORIT OL									NORIT A						Cuprophan		Nephrophan		Visking		Nojax 21		Kalle		Sephadex G 25	Millipore		Vacuum	
			COATED					RAW				COATED			RAW			Uncorrected	Corrected	Uncorrected	Corrected	Uncorrected	Corrected	Uncorrected	Corrected	Uncorrected	Corrected		Uncorrected	Corrected	Uncorrected	Corrected
			0.5%	1%	2.5%	5%	10%	0.5%	1%	2.5%	5%	10%	0.5%	2.5%	10%	0.5%	2.5%	10%															
			78.9 (16.3)	70.1 (17.9)	50.0 (17.3)	39.0 (16.1)	34.6 (15.7)	86.6 (1.5)	71.4 (4.3)	27.1 (9.4)	6.2 (4.4)	0.6 (0.4)	77.4 (11.5)	34.1 (7.5)	23.7 (7.7)	69.8 (8.9)	16.0 (6.8)	0.3 (0.2)	28.0 (4.8)	27.5 (4.6)	24.4 (5.3)	20.4 (3.9)	37.3 (5.0)	30.9 (7.1)	35.3 (9.0)	28.9 (10.2)	33.9 (7.9)	25.7 (11.8)	28.0 (3.8)	59.7 (10.3)	37.0 (8.9)	62.7 (7.6)	51.8 (10.8)
CHARCOAL	NORIT OL	COATED	0.5%		0.066	0.032	0.001	0.001	0.350	0.197	0.001	0.001	0.001	0.409	0.001	0.001	0.120	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.032	0.001	0.066	0.013
			1%			0.066	0.021	0.008	0.001	0.197	0.001	0.001	0.001	0.409	0.001	0.001	0.294	0.001	0.001	0.001	0.001	0.001	0.001	0.002	0.001	0.004	0.001	0.001	0.001	0.001	0.120	0.004	0.197	0.047
			2.5%				0.090	0.066	0.001	0.002	0.047	0.001	0.001	0.008	0.090	0.013	0.008	0.001	0.001	0.021	0.021	0.008	0.001	0.197	0.047	0.090	0.032	0.066	0.047	0.021	0.294	0.155	0.120	0.469
			5%					0.155	0.001	0.001	0.120	0.001	0.001	0.001	0.197	0.120	0.001	0.013	0.001	0.197	0.197	0.120	0.120	0.242	0.197	0.294	0.120	0.197	0.066	0.197	0.032	0.350	0.002	0.220
			10%						0.001	0.001	0.120	0.001	0.001	0.001	0.294	0.120	0.001	0.032	0.001	0.127	0.197	0.197	0.120	0.409	0.242	0.380	0.294	0.350	0.090	0.197	0.001	0.469	0.001	0.021
		RAW	0.5%							0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
			1%								0.001	0.001	0.001	0.105	0.001	0.001	0.350	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.032	0.001	0.032	0.002
			2.5%									0.008	0.001	0.001	0.066	0.242	0.001	0.021	0.001	0.409	0.350	0.120	0.032	0.013	0.294	0.155	0.409	0.155	0.469	0.294	0.001	0.047	0.001	0.001
			5%										0.006	0.001	0.001	0.001	0.001	0.001	0.008	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.008	0.001	0.001	0.001	0.001	0.001	0.001
			10%											0.001	0.001	0.001	0.001	0.197	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
	NORIT A	COATED	0.5%												0.001	0.001	0.001	0.155	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.006	0.001	0.013	0.004
			2.5%												0.047	0.001	0.004	0.001	0.066	0.066	0.021	0.013	0.531	0.294	0.531	0.197	0.469	0.090	0.047	0.001	0.469	0.001	0.004
			10%													0.001	0.120	0.001	0.120	0.155	0.409	0.322	0.001	0.090	0.038	0.294	0.013	0.294	0.120	0.001	0.013	0.001	0.001
		RAW	0.5%														0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.120	0.001	0.078	0.004
			2.5%															0.001	0.004	0.002	0.021	0.294	0.001	0.004	0.002	0.013	0.002	0.120	0.002	0.001	0.001	0.001	0.001
			10%																0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
BAG DIALYSIS	Cuprophan	Uncorrected																																
		Corrected																																
	Nephrophan	Uncorrected																																
		Corrected																																
	Visking	Uncorrected																																
		Corrected																																
	Nojax 21	Uncorrected																																
		Corrected																																
	Kalle	Uncorrected																																
		Corrected																																
Gel Filtration-Sephadex G 25																																		
ULTRAFILTRATION	Millipore	Uncorrected																																
		Corrected																																
	Vacuum	Uncorrected																																
		Corrected																																

Table 55. Gastric Juice and Cyanocobalamin.

All the results were analysed using the Mann-Whitney U Test and the Wilcoxon Matched-Pairs Signed-Ranks Test and the values in the Table are those for P on a two tail test.



				CHARCOAL												BAG DIALYSIS										Gel Filtration	ULTRAFILTRATION			
				NORIT OL							NORIT A					Cuprophan		Nephrophan		Visking		Nojax 21		Kalle			Millipore		Vacuum	
				COATED			RAW				COATED			RAW		Uncorrected	Corrected	Uncorrected	Corrected	Uncorrected	Corrected	Uncorrected	Corrected	Uncorrected	Corrected	Sephadex G 25	Uncorrected	Corrected	Uncorrected	Corrected
				0.5%	1%	2.5%	5%	10%	0.5%	1%	2.5%	5%	10%	0.5%	2.5%	10%	0.5%	2.5%	10%													
				75.6 (7.6)	58.6 (9.9)	36.1 (10.1)	27.5 (7.2)	22.6 (6.7)	71.0 (4.5)	43.5 (6.2)	8.6 (5.1)	1.0 (0.8)	0.3 (0.2)	70.9 (4.3)	16.9 (5.3)	6.2 (1.1)	64.4 (5.4)	2.6 (1.0)	0.4 (0.3)	32.5 (9.2)	30.7 (8.0)	32.4 (2.9)	26.3 (2.8)	51.3 (4.7)	35.9 (13.5)	48.0 (5.0)	40.6 (5.6)	50.5 (6.9)	36.1 (5.0)	NA	84.4 (5.2)	72.5 (10.8)	43.3 (10.1)	34.9 (10.0)
CHARCOAL	NORIT OL	COATED	0.5%		0.008	0.001	0.001	0.001	0.220	0.001	0.001	0.001	0.001	0.197	0.001	0.001	0.013	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	NA	0.047	0.394	0.001	0.001
			1%			0.004	0.001	0.001	0.032	0.004	0.001	0.001	0.001	0.047	0.001	0.001	0.155	0.021	0.001	0.002	0.001	0.001	0.001	0.032	0.004	0.120	0.001	NA	0.001	0.032	0.013	0.004
			2.5%				0.090	0.032	0.001	0.090	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.409	0.342	0.531	0.047	0.013	0.439	0.032	0.242	0.032	0.409	NA	0.001	0.001	0.322	0.531
			5%					0.090	0.001	0.004	0.001	0.001	0.001	0.001	0.021	0.001	0.001	0.001	0.001	0.242	0.294	0.120	0.242	0.001	0.120	0.001	0.021	0.001	0.047	NA	0.001	0.001	0.004	0.155
			10%						0.001	0.001	0.004	0.001	0.001	0.001	0.066	0.001	0.001	0.001	0.001	1.032	0.195	0.004	0.120	0.001	0.066	0.001	0.004	0.001	0.004	NA	0.001	0.001	0.004	0.013
		RAW	0.5%							0.001	0.001	0.001	0.001	0.439	0.001	0.001	0.066	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.091	0.001	0.021	0.001	0.021	NA	0.001	0.531	0.021	0.001
			1%								0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.021	0.013	0.004	0.001	0.021	0.155	0.090	0.409	0.090	0.032	NA	0.001	0.001	0.409	0.032
			2.5%									0.001	0.001	0.001	0.013	0.322	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	NA	0.001	0.001	0.001	0.001
			5%										0.013	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	NA	0.001	0.001	0.001	0.001
			10%											0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	NA	0.001	0.001	0.001	0.001
	NORIT A	COATED	0.5%												0.001	0.001	0.047	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	NA	0.001	0.531	0.001	0.001
			2.5%													0.001	0.001	0.001	0.001	0.001	0.004	0.001	0.002	0.001	0.004	0.001	0.004	0.001	0.001	NA	0.001	0.001	0.001	0.001
			10%														0.001	0.002	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	NA	0.001	0.001	0.001	0.001
		RAW	0.5%															0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.002	0.001	NA	0.001	0.155	0.004	0.001
			2.5%																0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	NA	0.001	0.001	0.001	0.001
			10%																	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	NA	0.001	0.001	0.001	0.001
BAG DIALYSIS	Cuprophan	Uncorrected																		0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	NA	0.001	0.001	0.001	0.001
		Corrected																			0.050	0.156	0.138	0.004	0.394	0.008	0.278	0.008	0.120	NA	0.001	0.001	0.066	0.322
	Nephrophan	Uncorrected																				0.197	0.394	0.003	0.242	0.004	0.047	0.003	0.290	NA	0.001	0.001	0.021	0.242
		Corrected																					0.050	0.001	0.342	0.001	0.032	0.001	0.090	NA	0.001	0.001	0.021	0.409
	Visking	Uncorrected																						0.001	0.120	0.001	0.232	0.001	0.008	NA	0.001	0.001	0.003	0.040
		Corrected																							0.050	0.090	0.021	0.409	0.001	NA	0.001	0.001	0.066	0.013
	Nojax 21	Uncorrected																								0.232	0.409	0.047	0.531	NA	0.001	0.001	0.394	0.531
		Corrected																									0.050	0.220	0.004	NA	0.001	0.001	0.120	0.032
	Kalle	Uncorrected																										0.066	0.105	NA	0.001	0.001	0.537	0.155
		Corrected																											0.050	NA	0.001	0.001	0.105	0.008
Gel Filtration—Sephadex G 25																														NA	0.001	0.001	0.066	0.197
ULTRAFILTRATION	Millipore	Uncorrected																													NA	NA	NA	NA
		Corrected																															0.050	0.001	0.001
	Vacuum	Uncorrected																															0.002	0.001
		Corrected																																0.050

Table 56. Gastric Juice and Hydroxocobalamin.

All the results were analysed using the Mann-Whitney U Test and the Wilcoxón Matched-Pairs Signed-Ranks Test and the values in the Table are those for P on a two tail test. Where no comparison was obtained N.A. (not applicable) has been inserted.

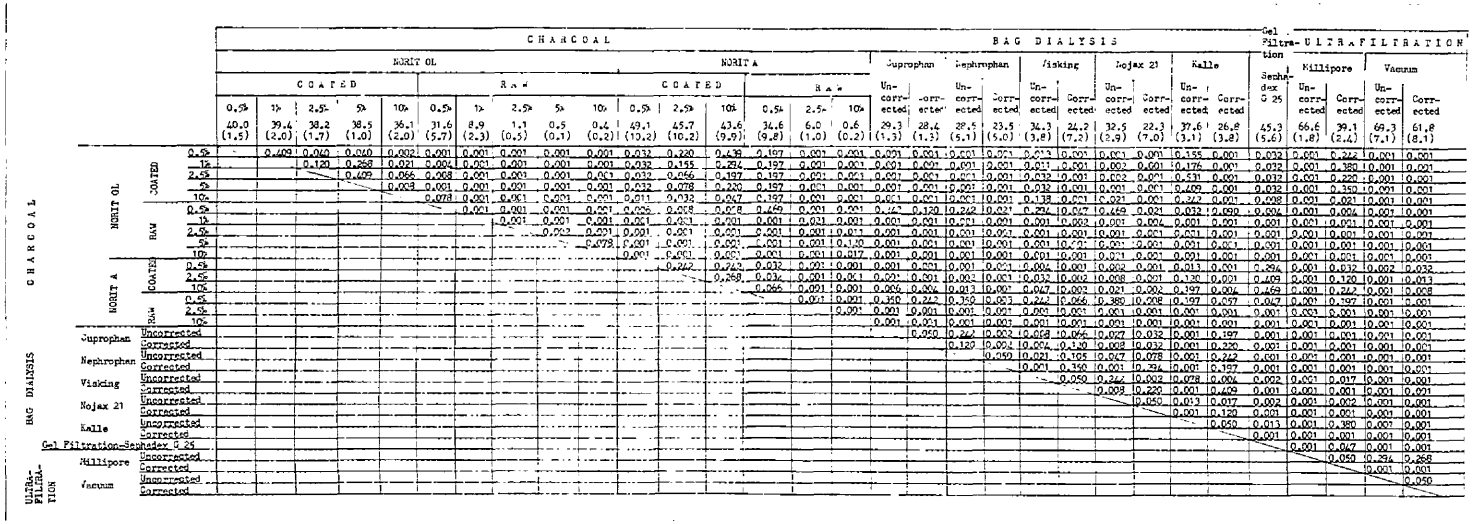
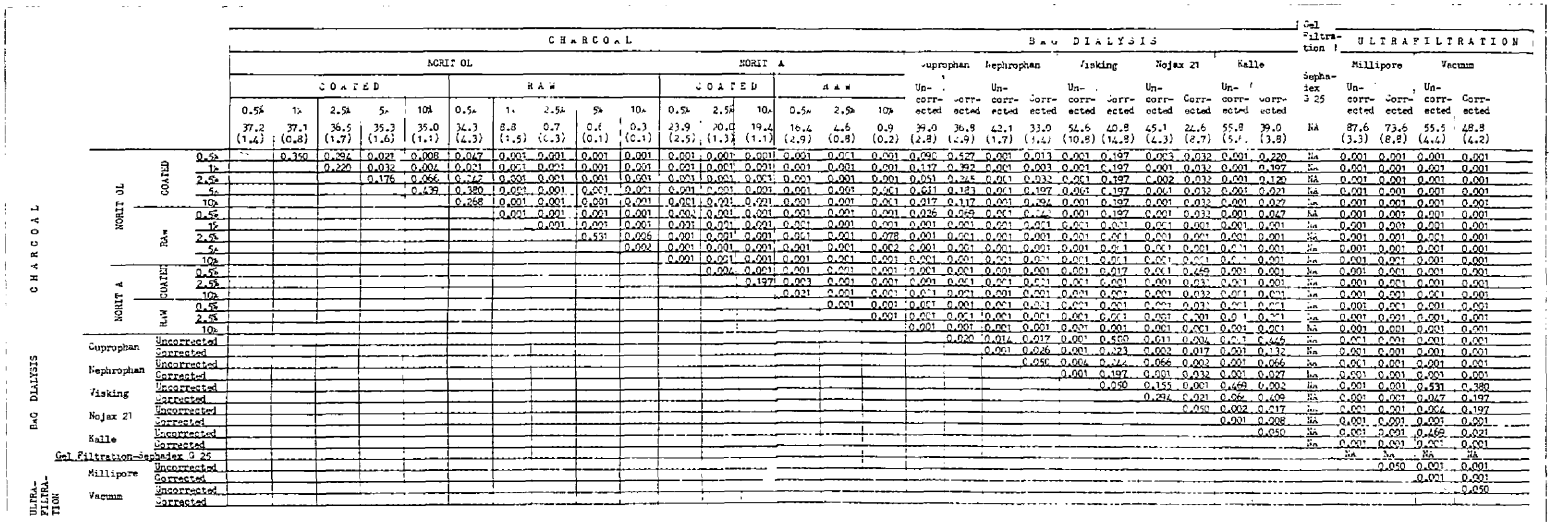


Table 57. Hog Intrinsic Factor and Cyanocobalamin

All the results were analysed using the Mann-Whitney U Test and the Wilcoxon Matched-Pairs Signed-Ranks Test and the values in the Table are those for P on a two tail test.



			CHARCOAL														B&O DIALYSIS										Gel Filtration	ULTRAFILTRATION			
			NORIT OL									NORIT A					Cuprophan		Nephrophan		Visking		Nojax 21		Kalle		Sephadex G 25	Millipore		Vacuum	
			COATED				RAW					COATED			RAW		Uncorrected	Corrected	Uncorrected	Corrected	Uncorrected	Corrected	Uncorrected	Corrected	Uncorrected	Corrected		Uncorrected	Corrected	Uncorrected	Corrected
			0.5%	1%	2.5%	5%	10%	0.5%	1%	2.5%	5%	10%	0.5%	2.5%	10%	0.5%	2.5%	10%														
			37.2 (1.4)	37.1 (0.8)	36.5 (1.7)	35.3 (1.6)	35.0 (1.1)	34.3 (2.3)	8.8 (1.5)	0.7 (0.3)	0.4 (0.1)	0.3 (0.1)	23.9 (2.5)	20.0 (1.3)	19.4 (1.1)	16.4 (2.9)	4.6 (0.8)	0.9 (0.2)	39.0 (2.8)	36.9 (2.9)	42.1 (1.7)	33.0 (1.2)	52.6 (10.8)	40.8 (14.9)	45.1 (2.3)	22.6 (2.7)	55.8 (5.7)	39.0 (3.8)	NA	87.6 (3.3)	73.6 (8.9)	55.5 (4.4)	48.8 (4.2)
CHARCOAL	NORIT OL	COATED	0.5%		0.350	0.294	0.021	0.008	0.047	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.090	0.527	0.001	0.013	0.001	0.197	0.003	0.032	0.001	0.220	NA	0.001	0.001	0.001	0.001
			1%			0.220	0.032	0.004	0.021	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.137	0.392	0.001	0.003	0.001	0.197	0.001	0.032	0.001	0.197	NA	0.001	0.001	0.001	0.001
			2.5%				0.176	0.066	0.242	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.245	0.001	0.032	0.001	0.197	0.002	0.032	0.001	0.120	NA	0.001	0.001	0.001	0.001
			5%					0.439	0.380	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.031	0.183	0.001	0.197	0.001	0.197	0.001	0.032	0.001	0.021	NA	0.001	0.001	0.001	0.001
			10%						0.268	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.017	0.117	0.001	0.294	0.001	0.197	0.001	0.032	0.001	0.029	NA	0.001	0.001	0.001	0.001
		RAW	0.5%							0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.026	0.069	0.001	0.142	0.001	0.197	0.001	0.033	0.001	0.047	NA	0.001	0.001	0.001	0.001
			1%								0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	NA	0.001	0.001	0.001	0.001
			2.5%									0.531	0.001	0.001	0.001	0.001	0.001	0.001	0.028	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	NA	0.001	0.001	0.001	0.001
			5%									0.004	0.001	0.001	0.001	0.001	0.001	0.002	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	NA	0.001	0.001	0.001	0.001
			10%										0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	NA	0.001	0.001	0.001	0.001
	NORIT A	COATED	0.5%											0.004	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.017	0.001	0.249	0.001	0.001	NA	0.001	0.001	0.001	0.001
			2.5%												0.197	0.003	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.03	0.001	0.001	NA	0.001	0.001	0.001	0.001
			10%													0.021	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.032	0.001	0.001	NA	0.001	0.001	0.001	0.001
		RAW	0.5%														0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	NA	0.001	0.001	0.001	0.001
			2.5%														0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.0 1	0.001	0.001	NA	0.001	0.001	0.001	0.001
			10%														0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	NA	0.001	0.001	0.001	0.001
B&O DIALYSIS	Cuprophan	Uncorrected																		0.020	0.014	0.017	0.001	0.550	0.011	0.004	0.017	0.446	NA	0.001	0.001	0.001	0.001
		Corrected																					0.001	0.026	0.001	0.023	0.002	0.017	0.001	0.132	NA	0.001	0.001	0.001	0.001
	Nephrophan	Uncorrected																						0.250	0.004	0.242	0.066	0.003	0.001	0.066	NA	0.001	0.001	0.001	0.001
		Corrected																							0.001	0.197	0.001	0.032	0.001	0.027	NA	0.001	0.001	0.001	0.001
	Visking	Uncorrected																								0.050	0.155	0.001	0.469	0.002	NA	0.001	0.001	0.531	0.380
		Corrected																									0.294	0.021	0.66	0.409	NA	0.001	0.001	0.047	0.197
	Nojax 21	Uncorrected																										0.050	0.002	0.017	NA	0.001	0.001	0.004	0.197
		Corrected																										0.001	0.008	NA	0.001	0.001	0.001	0.001
	Kalle	Uncorrected																											0.050	NA	0.001	0.001	0.469	0.021
		Corrected																												NA	0.001	0.001	0.001	0.001
	Gel Filtration—Sephadex G 25																													NA	NA	NA	NA	NA
ULTRAFILTRATION	Millipore	Uncorrected																												0.050	0.001	0.001		
		Corrected																													0.021	0.001		
	Vacuum	Uncorrected																														0.050		
		Corrected																																

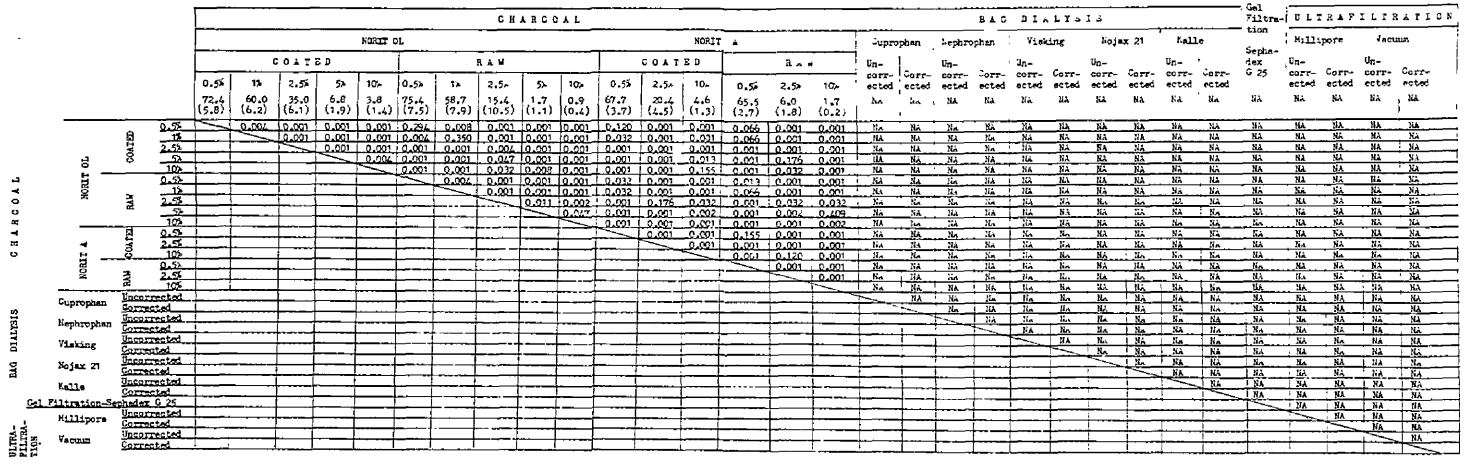
Table 58. Hog Intrinsic Factor and Hydroxocobalamin.

All the results were analysed using the Mann-Whitney U Test and the Wilcoxon Matched-Pairs Signed-Ranks Test and the values in the Table are those for P on a two tail test. Where no comparison was obtained N.A. (not applicable) has been inserted.

	CHARCOAL																BAG DIALYSIS										Gel Filtration	ULTRAFILTRATION			
	NORIT OL										NORIT A						Cuprophan		Nephrophan		Visking		Nojax 21		Kalle		Sephadex G 25	Millipore		Vacuum	
	COATED					RAW					COATED			RAW			Un-corrected	Corrected	Un-corrected	Corrected	Un-corrected	Corrected	Un-corrected	Corrected	Un-corrected	Corrected		Un-corrected	Corrected	Un-corrected	Corrected
	0.5%	1%	2.5%	5%	10%	0.5%	1%	2.5%	5%	10%	0.5%	2.5%	10%	0.5%	2.5%	10%															
	76.8 (5.4)	65.1 (5.9)	28.9 (5.4)	7.2 (1.4)	3.3 (1.2)	77.1 (6.1)	61.1 (6.0)	11.7 (5.3)	3.9 (2.5)	0.7 (0.5)	52.9 (6.5)	16.3 (3.7)	5.5 (1.3)	53.1 (2.8)	7.3 (1.5)	2.7 (0.8)	6.6 (2.0)	6.1 (1.9)	7.0 (1.4)	5.2 (1.2)	23.4 (3.1)	14.2 (3.4)	17.0 (1.9)	11.6 (1.0)	23.4 (5.8)	12.1 (2.7)	2.4 (0.5)	62.6 (9.2)	5.6 (3.0)	NA	NA
CHARCOAL NORIT OL COATED 0.5%		0.004	0.001	0.001	0.001	0.409	0.002	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.008	0.001	NA	NA
1%			0.001	0.001	0.001	0.008	0.155	0.001	0.001	0.001	0.013	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.294	0.001	NA	NA
2.5%				0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.002	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.032	0.001	0.001	0.001	0.090	0.001	0.001	0.001	0.001	NA	NA
5%					0.001	0.001	0.001	0.066	0.013	0.001	0.001	0.001	0.051	0.001	0.469	0.001	0.268	0.120	0.409	0.040	0.001	0.001	0.001	0.001	0.001	0.004	0.001	0.001	0.294	NA	NA
10%						0.001	0.001	0.002	0.531	0.001	0.001	0.001	0.008	0.001	0.001	0.197	0.004	0.004	0.001	0.017	0.001	0.001	0.001	0.001	0.001	0.001	0.066	0.007	0.066	NA	NA
NORIT OL RAW 0.5%							0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.201	0.001	0.006	0.001	NA	NA
1%								0.001	0.001	0.001	0.027	0.001	0.001	0.013	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.201	0.001	0.469	0.001	NA	NA
2.5%									0.004	0.001	0.001	0.090	0.013	0.001	0.120	0.001	0.047	0.032	0.105	0.011	0.001	0.176	0.047	0.350	0.004	0.500	0.001	0.001	0.032	NA	NA
5%										0.002	0.001	0.001	[illegible]	0.001	0.013	0.220	0.021	0.040	0.021	0.066	0.001	0.002	0.001	0.001	0.001	0.002	0.105	0.001	0.155	NA	NA
10%											0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.002	0.001	0.002	NA	NA
NORIT A COATED 0.5%												0.001	0.001	0.350	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.047	0.001	NA	NA
2.5%													0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.008	0.350	0.350	0.004	0.013	0.032	0.001	0.001	0.001	NA	NA
10%														0.001	0.057	0.001	0.120	0.294	0.047	0.409	0.001	0.001	0.001	0.001	0.001	0.002	0.001	0.001	0.469	NA	NA
NORIT A RAW 0.5%															0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.021	0.001	NA	NA
2.5%																0.001	0.268	0.066	0.294	0.032	0.001	0.004	0.001	0.001	0.001	0.013	0.001	0.001	0.268	NA	NA
10%																	0.001	0.001	0.001	0.002	0.001	0.001	0.001	0.001	0.001	0.001	0.350	0.001	0.032	NA	NA
BAG DIALYSIS Cuprophan Uncorrected																		0.050	0.242	0.155	0.001	0.002	0.001	0.002	0.001	0.004	0.001	0.001	0.242	NA	NA
Corrected																			0.197	0.294	0.001	0.002	0.001	0.001	0.001	0.004	0.001	0.001	0.409	NA	NA
Nephrophan Uncorrected																				0.050	0.001	0.004	0.001	0.001	0.001	0.011	0.001	0.001	0.197	NA	NA
Corrected																					0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.294	NA	NA
Visking Uncorrected																						0.050	0.001	0.001	0.350	0.001	0.001	0.001	0.001	NA	NA
Corrected																							0.013	0.032	0.008	0.066	0.001	0.001	0.002	NA	NA
Nojax 21 Uncorrected																								0.050	0.105	0.004	0.003	0.001	0.001	NA	NA
Corrected																									0.001	0.105	0.001	0.001	0.001	NA	NA
Kalle Uncorrected																										0.050	0.001	0.001	0.001	NA	NA
Corrected																											0.001	0.001	0.008	NA	NA
Gel Filtration-Sephadex G 25																												0.001	0.032	NA	NA
ULTRAFILTRATION Millipore Uncorrected																													0.050	NA	NA
Corrected																														NA	NA
Vacuum Uncorrected																															NA
Corrected																															

Table 59. Bile and Cyanocobalamin.

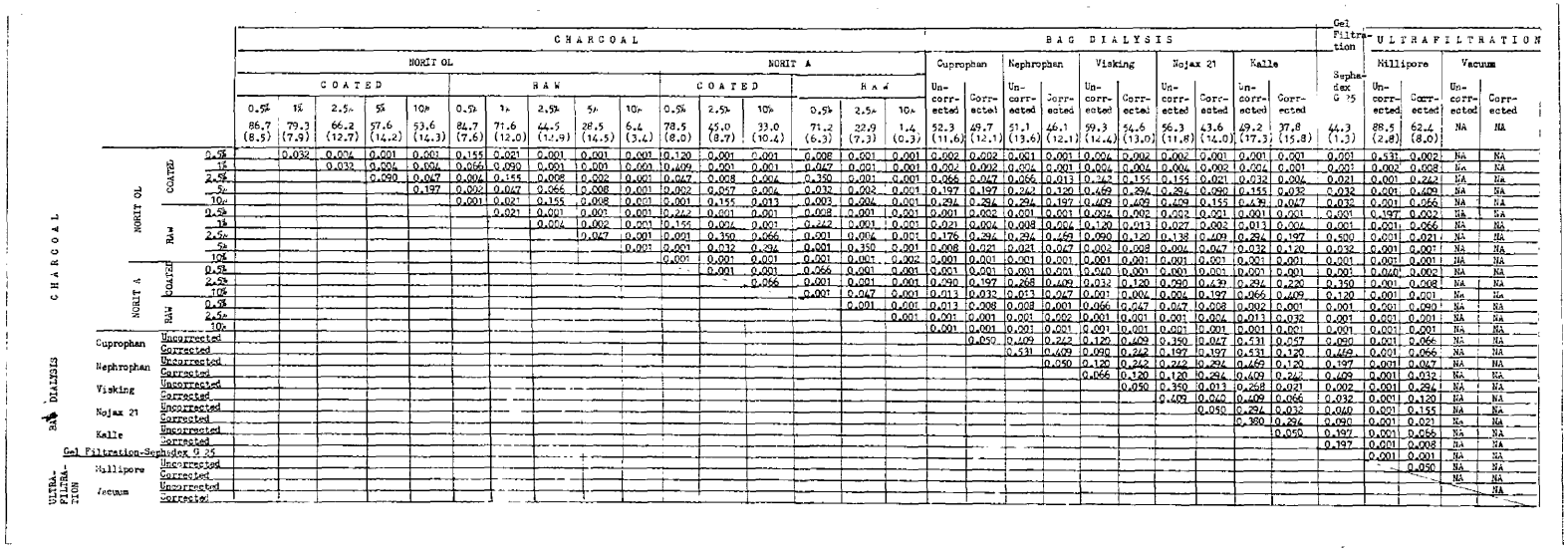
All the results were analysed using the Mann-Whitney U Test and the Wilcoxon Matched-Pairs Signed-Ranks Test and the values in the Table are those for P on a two tail test. Where no comparison was obtained N.A. (not applicable) has been inserted.



			CHARCOAL															BAG DIALYSIS											Gel Filtration	ULTRAFILTRATION			
			NORIT OL								NORIT A							Cuprophan		Nephrophan		Visking		Nojax 21		Kalle			Millipore		Vacuum	
			COATED					RAW				COATED			RAW													Sephadex G 25				
			0.5%	1%	2.5%	5%	10%	0.5%	1%	2.5%	5%	10%	0.5%	2.5%	10%	0.5%	2.5%	10%	Uncorrected	Corrected	Uncorrected	Corrected	Uncorrected	Corrected	Uncorrected	Corrected	Uncorrected	Corrected	NA	Uncorrected	Corrected	Uncorrected	Corrected
			72.4 (5.8)	60.0 (6.2)	35.0 (6.1)	6.8 (1.9)	3.8 (1.4)	75.4 (7.5)	58.7 (7.9)	15.4 (10.5)	1.7 (1.1)	0.9 (0.4)	67.7 (3.7)	20.4 (4.5)	4.6 (1.3)	65.5 (2.7)	6.0 (1.8)	1.7 (0.2)	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
CHARCOAL	NORIT OL	COATED	0.5%		0.004	0.001	0.001	0.001	0.296	0.008	0.001	0.001	0.001	0.120	0.001	0.001	0.066	0.001	0.001	Na	Na	Na	Na	Na	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			1%		0.021	0.001	0.001	0.004	0.350	0.001	0.001	0.001	0.032	0.001	0.031	0.066	0.001	0.001	NA	NA	NA	Na	Na	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			2.5%			0.001	0.001	0.001	0.001	0.004	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	Na	NA	Na	Na	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			5%				0.004	0.001	0.001	0.047	0.001	0.001	0.001	0.001	0.013	0.001	0.175	0.001	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			10%					0.001	0.001	0.032	0.092	0.001	0.001	0.001	0.155	0.001	0.032	0.001	NA	NA	Na	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		RAW	0.5%						0.004	0.001	0.001	0.001	0.032	0.001	0.001	0.013	0.001	0.001	NA	NA	Na	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			1%							0.001	0.001	0.001	0.032	0.001	0.001	0.066	0.001	0.001	NA	NA	Na	Na	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			2.5%								0.011	0.002	0.001	0.175	0.032	0.001	0.032	0.032	Na	Na	Na	Na	NA	NA	Na	Na	NA	NA	NA	NA	NA	NA	NA
			5%									0.027	0.001	0.001	0.002	0.001	0.004	0.459	NA	NA	Na	Na	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			10%										0.001	0.007	0.001	0.001	0.001	0.002	NA	Na	NA	Na	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	NORIT A	COATED	0.5%											0.001	0.001	0.155	0.001	0.001	Na	NA	Na	Na	Na	Na	NA	NA	NA	NA	Na	NA	NA	NA	NA
			2.5%												0.001	0.001	0.001	0.001	Na	Na	Na	Na	Na	Na	NA	NA	NA	NA	Na	NA	NA	NA	NA
			10%													0.061	0.120	0.001	Na	Na	Na	Na	Na	Na	Na	Na	Na	Na	Na	NA	NA	NA	NA
		RAW	0.5%														0.001	0.001	Na	NA	Na	Na	Na	Na	NA	NA	NA	NA	Na	NA	NA	NA	NA
			2.5%															0.001	Na	NA	Na	Na	Na	Na	NA	NA	NA	NA	Na	NA	NA	NA	NA
			10%																Na	NA	NA	Na	Na	Na	Na	Na	Na	Na	Na	NA	NA	NA	NA
BAG DIALYSIS	Cuprophan		Uncorrected																		NA	NA	Na	Na	Na	Na	Na	Na	NA	NA	NA	NA	NA
			Corrected																				Na	Na	Na	Na	Na	Na	NA	NA	NA	NA	NA
	Nephrophan		Uncorrected																						Na	Na	Na	Na	NA	NA	NA	NA	NA
			Corrected																								Na	Na	NA	NA	NA	NA	NA
	Visking		Uncorrected																										NA	NA	NA	NA	NA
			Corrected																												NA	NA	NA
	Nojax 21		Uncorrected																														NA
			Corrected																														NA
	Kalle		Uncorrected																														NA
			Corrected																														NA
Gel Filtration-Sephadex G 25																																	
ULTRA-FILTRATION	Millipore		Uncorrected																														
			Corrected																														
	Vacuum		Uncorrected																														
			Corrected																														

Table 60. Bile and Hydroxocobalamin.

All the results were analysed using the Mann-Whitney U Test and the Wilcoxon Matched-Pairs Signed-Ranks Test and the values in the Table are those for P on a two tail test. Where no comparison was obtained N.A. (not applicable) has been inserted.



			CHARCOAL														BAG DIALYSIS										Gel Filtration	ULTRAFILTRATION			
			NORIT OL									NORIT A					Cuprophan		Nephrophan		Visking		Nojax 21		Kalle			Millipore		Vacuum	
			COATED					RAW				COATED			RAW												Sephadex G 75				
			0.5%	1%	2.5%	5%	10%	0.5%	1%	2.5%	5%	10%	0.5%	2.5%	10%	0.5%	2.5%	10%	Uncorrected	Corrected	Uncorrected	Corrected	Uncorrected	Corrected	Uncorrected	Corrected	Corrected	Uncorrected	Corrected	Uncorrected	Corrected
			86.7 (8.5)	79.3 (7.9)	66.2 (12.7)	57.6 (14.2)	53.6 (14.3)	84.7 (7.6)	71.6 (12.0)	44.5 (12.9)	28.5 (14.5)	6.4 (3.4)	78.5 (8.0)	45.0 (8.7)	33.0 (10.4)	71.2 (6.3)	22.9 (7.3)	1.4 (0.3)	52.3 (11.6)	49.7 (12.1)	51.1 (13.6)	46.1 (12.1)	59.3 (12.4)	54.6 (13.0)	56.3 (11.8)	43.6 (14.0)	49.2 (17.3)	37.8 (15.8)	44.3 (1.3)	88.5 (2.8)	62.4 (8.0)	NA	NA
CHARCOAL	NORIT OL	COATED	0.5%		0.032	0.004	0.001	0.001	0.155	0.021	0.001	0.001	0.001	0.120	0.001	0.001	0.008	0.001	0.001	0.002	0.002	0.001	0.001	0.004	0.002	0.002	0.001	0.001	0.001	0.001	0.531	0.002	NA	NA
			1%			0.032	0.004	0.004	0.066	0.090	0.001	0.001	0.001	0.409	0.001	0.001	0.047	0.001	0.001	0.002	0.002	0.004	0.001	0.004	0.004	0.004	0.002	0.004	0.001	0.001	0.002	0.008	NA	NA
			2.5%				0.090	0.047	0.004	0.155	0.008	0.002	0.001	0.047	0.008	0.004	0.350	0.001	0.001	0.066	0.047	0.066	0.013	0.242	0.155	0.155	0.021	0.032	0.004	0.021	0.001	0.242	NA	NA
			5%					0.197	0.002	0.047	0.066	0.008	0.001	0.002	0.057	0.004	0.032	0.002	0.001	0.197	0.197	0.242	0.120	0.469	0.294	0.294	0.090	0.155	0.032	0.032	0.001	0.409	NA	NA
			10%						0.001	0.021	0.155	0.008	0.001	0.001	0.155	0.013	0.003	0.004	0.001	0.294	0.294	0.294	0.197	0.409	0.409	0.409	0.155	0.439	0.047	0.032	0.001	0.066	NA	NA
		RAW	0.5%							0.021	0.001	0.001	0.222	0.001	0.001	0.008	0.001	0.001	0.001	0.002	0.001	0.001	0.001	0.004	0.002	0.002	0.021	0.001	0.001	0.001	0.197	0.002	NA	NA
			1%								0.004	0.002	0.001	0.156	0.004	0.001	0.242	0.001	0.001	0.021	0.004	0.008	0.004	0.120	0.013	0.027	0.002	0.013	0.004	0.001	0.001	0.066	NA	NA
			2.5%									0.047	0.001	0.001	0.350	0.066	0.001	0.004	0.001	0.176	0.294	0.294	0.469	0.090	0.120	0.138	0.409	0.294	0.197	0.500	0.001	0.021	NA	NA
			5%									0.001	0.001	0.032	0.294	0.001	0.350	0.001	0.008	0.021	0.021	0.047	0.002	0.008	0.004	0.047	0.032	0.120	0.032	0.001	0.007	NA	NA
			10%										0.001	0.001	0.001	0.001	0.001	0.002	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	NA	NA
	NORIT A	COATED	0.5%										0.001	0.001	0.001	0.066	0.001	0.001	0.001	0.001	0.001	0.001	0.040	0.001	0.001	0.001	0.001	0.001	0.001	0.040	0.002	NA	NA
			2.5%											0.066	0.001	0.001	0.001	0.001	0.001	0.090	0.197	0.268	0.409	0.033	0.120	0.090	0.439	0.294	0.220	0.350	0.001	0.008	NA	NA
			10%												0.066	0.001	0.047	0.001	0.013	0.033	0.013	0.047	0.001	0.004	0.024	0.197	0.066	0.409	0.120	0.001	0.001	NA	NA
		RAW	0.5%													0.001	0.001	0.001	0.013	0.008	0.008	0.001	0.066	0.047	0.047	0.008	0.002	0.001	0.001	0.001	0.090	NA	NA
			2.5%															0.001	0.001	0.001	0.002	0.001	0.001	0.001	0.004	0.013	0.032	0.001	0.001	0.001	NA	NA
			10%																0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	NA	NA
BAG DIALYSIS	Cuprophan	Uncorrected																		0.050	0.409	0.242	0.120	0.409	0.350	0.047	0.531	0.057	0.090	0.001	0.066	NA	NA
		Corrected																			0.531	0.409	0.090	0.242	0.197	0.197	0.531	0.120	0.469	0.001	0.066	NA	NA
	Nephrophan	Uncorrected																				0.050	0.120	0.242	0.242	0.294	0.469	0.120	0.197	0.001	0.047	NA	NA
		Corrected																					0.066	0.120	0.120	0.294	0.409	0.242	0.409	0.001	0.032	NA	NA
	Visking	Uncorrected																						0.050	0.350	0.013	0.268	0.021	0.002	0.001	0.294	NA	NA
		Corrected																							0.409	0.040	0.409	0.066	0.032	0.001	0.120	NA	NA
	Nojax 21	Uncorrected																								0.050	0.294	0.032	0.040	0.001	0.155	NA	NA
		Corrected																									0.380	0.294	0.090	0.001	0.021	NA	NA
	Kalle	Uncorrected																										1.050	0.197	0.001	0.066	NA	NA
		Corrected																											0.197	0.001	0.008	NA	NA
Gel Filtration-Sephadex G 75																														0.001	0.001	NA	NA
ULTRAFILTRATION	Millipore	Uncorrected																													0.050	NA	NA
		Corrected																														NA	NA
	Vacuum	Uncorrected																															
		Corrected																															

Table 61. Saliva and Cyanocobalamin.

All the results were analysed using the Mann-Whitney U Test and the Wilcoxon Matched-Pairs Signed-Ranks Test and the values in the Table are those for P on a two tail test. Where no comparison was obtained N.A. (not applicable) has been inserted.

So far, all the results obtained in this study have been included in the analyses. In principle, this seems to be the correct approach, but in practice, the sheer mess of results tends to be somewhat overwhelming and to obscure the issues. As already noted, there are sound reasons for omitting some results. For example, those relating to separation using low concentrations of charcoal in which control studies showed incomplete removal of vitamin B_{12} from aqueous solutions implying that the test results were probably overestimates and also uncorrected ultrafiltration results which were similarly considered as overestimates. In addition, in order to simplify the comparisons, it seems reasonable to omit other results obtained by methods which for one reason or another are not generally used. Into this category come 1) results obtained by bag dialysis using membranes which are not generally available or widely used 2) results obtained with visking membrane which have been corrected as this is not a generally practised procedure 3) results using uncoated charcoals, partly because of the evidence that they can adsorb binders and binder-vitamin B_{12} complexes as well as free vitamin B_{12}, partly because of the striking precipitate pattern found with increasing concentrations of raw charcoals and partly because raw charcoals are not generally used.

Omission of those results limited comparisons to those obtained by two albumin-coated charcoals (Norit OL in concen-

-trations of 2.5%, 5% and 10% and Norit A in concentrations of 2.5% and 10%), bag dialysis using visking tubing, gel filtration using Sephadex G-25, and ultrafiltration using a Millipore membrane and a visking membrane (Vacuum system). These limited comparisons of results, obtained by selected methods, are set out in Tables 62 - 68 (pages 132 - 138).

METHOD			Other selected methods which give comparable (statistically insignificantly different) results.
CHARCOAL COATED	NORIT OL	2.5%	Charcoal coated Norit OL 5% Charcoal coatedNorit OL 10% Charcoal coated Norit A 2.5% Bag Dialysis - Visking uncorrected Ultrafiltration - Millipore corrected Ultrafiltration - Vacuum corrected
		5%	Charcoal coated Norit OL 2.5% Charcoal coated Norit OL 10% Charcoal coated Norit A 2.5% Charcoal coated Norit A 10% Bag Dialysis - Visking uncorrected Gel Filtration - Sephadex G-25 Ultrafiltration - Millipore corrected Ultrafiltration - Vacuum corrected
		10%	Charcoal coated Norit OL 2.5% Charcoal coated Norit OL 5% Charcoal coated Norit A 2.5% Charcoal coated Norit A 10% Bag Dialysis - Visking uncorrected Gel Filtration - Sephadex G-25 Ultrafiltration - Millipore corrected
	NORIT A	2.5%	Charcoal coated Norit OL 2.5% Charcoal coated Norit OL 5% Charcoal coated Norit OL 10% Bag Dialysis - Visking uncorrected Ultrafiltration - Millipore corrected
		10%	Charcoal coated Norit OL 5% Charcoal coated Norit OL 10% Gel Filtration - Sephadex G-25
BAG DIALYSIS	Visking (Uncorrected)		Charcoal coated Norit OL 2.5% Charcoal coated Norit OL 5% Charcoal coated Norit OL 10% Charcoal coated Norit A 2.5% Ultrafiltration - Millipore corrected
GEL FILTRATION	Sephadex G-25		Charcoal coated Norit OL 5% Charcoal coated Norit OL 10% Charcoal coated Norit A 10%
ULTRA-FILTRATION	Millipore (Corrected)		Charcoal coated Norit OL 2.5% Charcoal coated Norit OL 5% Charcoal coated Norit OL 10% Charcoal coated Norit A 2.5% Bag Dialysis - Visking uncorrected
	Vacuum (Corrected)		Charcoal coated Norit OL 2.5% Charcoal coated Norit OL 5%

Table 62. Gastric Juice and Cyanocobalamin Comparison of Selected Methods.

METHOD			Other selected methods which give comparable (statistically insignificantly different) results.
CHARCOAL COATED	NORIT OL	2.5%	Charcoal coated Norit OL 5% Ultrafiltration - Vacuum corrected
		5%	Charcoal coated Norit OL 2.5% Charcoal coated Norit OL 10% Ultrafiltration - Vacuum corrected
		10%	Charcoal coated Norit OL 5% Charcoal coated Norit A 2.5%
	NORIT A	2.5%	Charcoal coated Norit OL 10%
		10%	None
BAG DIALYSIS	Visking (Uncorrected)		None
GEL FILTRATION	Sephadex G-25		N.A.
ULTRAFILTRATION	Millipore (Corrected)		None
	Vacuum (Corrected)		Charcoal coated Norit OL 2.5% Charcoal coated Norit OL 5%

Table 63. Gastric Juice and Hydroxocobalamin Comparison of Selected Methods.

Where no other selected technique was comparable "None" has been inserted. Where no result was obtained N.A. (Not applicable) has been inserted.

METHOD		Other selected methods which give comparable (statistically insignificantly different) results.
CHARCOAL COATED NORIT OL	2.5%	Charcoal coated Norit OL 5% Charcoal coated Norit OL 10% Charcoal coated Norit A 2.5% Charcoal coated Norit A 10% Ultrafiltration - Millipore corrected
	5%	Charcoal coated Norit OL 2.5%. Charcoal coated Norit A 2.5% Charcoal coated Norit A 10% Ultrafiltration - Millipore corrected
	10%	Charcoal coated Norit OL 2.5% Bag Dialysis - Visking uncorrected
CHARCOAL COATED NORIT A	2.5%	Charcoal coated Norit OL 2.5% Charcoal coated Norit OL 5% Charcoal coated Norit A 10% Gel Filtration - Sephadex G-25 Ultrafiltration - Millipore corrected
	10%	Charcoal coated Norit OL 2.5% Charcoal coated Norit OL 5% Charcoal coated Norit A 2.5% Gel Filtration - Sephadex G-25 Ultrafiltration - Millipore corrected
BAG DIALYSIS	Visking (Uncorrected)	Charcoal coated Norit OL 10%
GEL FILTRATION	Sephadex G-25	Charcoal coated Norit A 2.5% Charcoal coated Norit A 10%
ULTRAFILTRATION	Millipore (Corrected)	Charcoal coated Norit OL 2.5% Charcoal coated Norit OL 5% Charcoal coated Norit A 2.5% Charcoal coated Norit A 10%
	Vacuum (Corrected)	None

Table 64. Hog Intrinsic Factor and Cyanocobalamin Comparison of Selected Methods.

Where no other selected technique was comparable "None" has been inserted.

METHOD			Other selected methods which give comparable (statistically insignificantly different) results.
CHARCOAL COATED	NORIT OL	2.5%	Charcoal coated Norit OL 5% Charcoal coated Norit OL 10%
		5%	Charcoal coated Norit OL 2.5% Charcoal coated Norit OL 10%
		10%	Charcoal coated Norit OL 2.5% Charcoal coated Norit OL 5%
	NORIT A	2.5%	Charcoal coated Norit A 10%
		10%	Charcoal coated Norit A 2.5%
BAG DIALYSIS	Visking (Uncorrected)		Ultrafiltration - Vacuum corrected
GEL FILTRATION	Sephadex G-25		N.A.
ULTRAFILTRATION	Millipore (Corrected)		None
	Vacuum (Corrected)		Bag Dialysis - Visking uncorrected

Table 65. Hog Intrinsic Factor and Hydroxocobalamin Comparison of Selected Methods.

Where no other selected technique was comparable "None" has been inserted. Where no result was obtained N.A. (Not applicable) has been inserted.

METHOD			Other selected methods which give comparable (statistically insignificantly different) results.
CHARCOAL COATED	NORIT OL	2.5%	None
		5%	Charcoal coated Norit A 10% Ultrafiltration - Millipore corrected
		10%	Gel Filtration - Sephadex G-25 Ultrafiltration - Millipore corrected
	NORIT A	2.5%	None
		10%	Charcoal coated Norit OL 5% Ultrafiltration - Millipore corrected
BAG DIALYSIS	Visking (Uncorrected)		None
GEL FILTRATION	Sephadex G-25		Charcoal coated Norit OL 10%
ULTRAFILTRATION	Millipore (Corrected)		Charcoal coated Norit OL 5% Charcoal coated Norit OL 10% Charcoal coated Norit A 10%
	Vacuum (Corrected)		N.A.

Table 66. Bile and Cyanocobalamin
Comparison of Selected Methods.

Where no other selected technique was comparable "None" has been inserted. Where no result was obtained N.A. (Not applicable) has been inserted.

METHOD			Other selected methods which give comparable (statistically insignificantly different) results.
CHARCOAL COATED	NORIT OL	2.5%	None
		5%	None
		10%	Charcoal coated Norit A 10%
	NORIT A	2.5%	None
		10%	Charcoal coated Norit OL 10%
BAG DIALYSIS	Visking (Uncorrected)		N.A.
GEL FILTRATION	Sephadex G-25		N.A.
ULTRA-FILTRATION	Millipore (Corrected)		N.A.
	Vacuum (Corrected)		N.A.

Table 67. Bile and Hydroxocobalamin
Comparison of Selected Methods.

Where no other selected technique was comparable "None" has been inserted. Where no result was obtained N.A. (not applicable) has been inserted.

METHOD		Other selected methods which give comparable (statistically insignificantly different) results.
CHARCOAL COATED NORIT OL	2.5%	Charcoal coated Norit OL 5% Bag Dialysis - Visking uncorrected Ultrafiltration - Millipore corrected
	5%	Charcoal coated Norit OL 2.5% Charcoal coated Norit OL 10% Charcoal coated Norit A 2.5% Bag Dialysis - Visking uncorrected Ultrafiltration - Millipore corrected
	10%	Charcoal coated Norit OL 5% Charcoal coated Norit A 2.5% Bag Dialysis - Visking uncorrected Ultrafiltration - Millipore corrected
CHARCOAL COATED NORIT A	2.5%	Charcoal coated Norit OL 5% Charcoal coated Norit OL 10% Charcoal coated Norit A 10% Gel Filtration - Sephadex G-25
	10%	Charcoal coated Norit A 2.5% Gel Filtration - Sephadex G-25
BAG DIALYSIS	Visking (Uncorrected)	Charcoal coated Norit OL 2.5% Charcoal coated Norit OL 5% Charcoal coated Norit OL 10% Ultrafiltration - Millipore corrected
GEL FILTRATION	Sephadex G-25	Charcoal coated Norit A 2.5% Charcoal coated Norit A 10%
ULTRA-FILTRATION	Millipore (Corrected)	Charcoal coated Norit OL 2.5% Charcoal coated Norit OL 5% Charcoal coated Norit OL 10% Bag Dialysis - Visking uncorrected
	Vacuum (Corrected)	N.A.

Table 68. Saliva and Cyanocobalamin
Comparison of Selected Methods.

Where no result was obtained N.A. (not applicable) has been inserted.

From the complete set of results and from the limited comparisons, the following conclusions seem reasonable.

1) Separation methods which give statistically insignificantly different results when applied to one form of vitamin B_{12} and one binder, do not necessarily give statistically insignificantly different results when applied to the same form of vitamin B_{12} and a different binder, or when applied to the same binder and a different form of vitamin B_{12}.

2) Conclusions derived from studies using one separation method, one type of vitamin B_{12}, and one binder are applicable only to that system and extrapolation of results is liable to lead to erroneous conclusions.

3) Generalisations such as those made by Chanarin (1969) about comparability of separation methods are ill advised.

Further evaluation of results is therefore discussed with reference to the binder and form of vitamin B_{12} and to the merits and demerits of each method. In this evaluation, descriptions of the charcoal separation methods will be limited to the type of charcoal and the concentration of the charcoal suspension used, in the interests of brevity and convenience. As was pointed out in the section dealing with charcoal separation studies, it is more satisfactory to express the mass of charcoal added with reference to the mass of vitamin B_{12} in the test solution.

Separation of free and gastric juice bound cyanocobalamin (Table 55, page 123 and Table 62, page 132)

Separation with Norit OL coated charcoal 5% gave results comparable to those obtained by all other methods and Norit OL coated charcoal 10% gave results comparable with those obtained by all other methods except ultrafiltration, vacuum corrected. Because of this and because of the ease, simplicity, speed and capacity to process multiple samples the charcoal separation method with Norit OL coated 5% and 10% appears to be the one of choice in this situation.

Separation of free and gastric juice bound hydroxocobalamin (Table 56, page 124 and Table 63, page 133)

The results show a striking difference from those with the same binder and another form of vitamin B_{12}, emphasising again the importance of the form of vitamin B_{12}. The results with Norit A coated charcoal 10%, with bag dialysis visking (uncorrected) and with Millipore ultrafiltration (corrected), are not comparable to those obtained using any other method, and separation by gel filtration is impracticable. Comparable results were obtained with Norit OL coated charcoal 2.5% and 5% and ultrafiltration vacuum (corrected).

Separation of free and hog intrinsic factor bound cyanocobalamin (Table 57, page 125 and Table 64, page 134)

The results with ultrafiltration vacuum (corrected) are not comparable to those obtained using any other method. The most acceptable methods appear to be Norit OL coated charcoal 2.5%,

Norit A coated charcoal 2.5% and 10%, each of which give comparable results with five other methods. When compared to the results with gastric juice bound and free cyanocobalamin the importance of the binder factor is again illustrated.

Separation of free and hog intrinsic factor bound hydroxocobalamin (Table 58, page 126 and Table 65, page 135)

The comparability of results is extremely poor and gel filtration is impracticable. There may be a case in this situation, therefore, for converting hydroxocobalamin to cyanocobalamin.

Separation of free and bile bound cyanocobalamin (Table 59, page 127 and Table 66, page 136)

Here again comparability of results is poor, Norit OL coated charcoal 2.5%, Norit A coated charcoal 2.5% and bag dialysis visking (uncorrected) not being comparable to any other method. Since results with Norit OL coated charcoal 10% are comparable to those obtained with gel filtration and ultrafiltration Millipore (corrected) it may be considered the most acceptable method.

Separation of free and bile bound hydroxocobalamin (Table 60, page 128 and Table 67, page 134)

Apart from the fact that gel filtration is impracticable all that can be said here is that Norit OL coated charcoal 10% and Norit A coated charcoal 10% give comparable results.

Separation of free and saliva bound cyanocobalamin
(Table 61, page 129 and Table 68, page 138)

Here, comparability is generally good. However, there is no single method that can be regarded as outstandingly representative.

Analysis of the results from the aspect of the binder and the form of vitamin B_{12} brings out some points of interest.

With gastric juice as binder, comparable results were obtained with Norit OL coated charcoal 2.5% and 5% and ultrafiltration vacuum method, whether the vitamin B_{12} form was cyanocobalamin or hydroxocobalamin.

With cyanocobalamin, the results with Millipore ultrafiltration were always comparable to those obtained with Norit OL coated charcoal 5% irrespective of binder. In addition, there was comparability of results with bag dialysis visking (uncorrected) and Norit OL coated charcoal 10% and with gel filtration and Norit A coated charcoal 10%, with gastric juice, hog intrinsic factor, and saliva but not with bile.

With hydroxocobalamin, the main feature is the impracticability of using gel filtration. In general it also appears that comparable results are less common than with cyanocobalamin.

Although the results are perhaps disappointing in that no one separation technique proved superior to the others, the final conclusions do, for the most part, verify predictions made before the work was started. These are:-

1) When applied to the separation of free and bound vitamin B_{12} different methods do not necessarily give the same results.

2) In practice the choice of method probably depends on expediency and circumstances rather than on any scientific point.

3) In any separation method the results are affected by the binder and the form of vitamin B_{12}.

4) Generalisations about the comparability of results obtained by different separation methods such as those by Chanarin (1969) are misleading.

5) To assume anything in separation work and to proceed on this assumption is to invite trouble.

CHAPTER 2

The Vitamin B_{12} Content of Items of Diet and Meals

INTRODUCTION

Information about the daily need for vitamin B_{12} can be obtained in three ways.

Firstly, by treating vitamin B_{12} deficient patients and observing the smallest daily dose of vitamin B_{12} which alleviates the deficiency. An advantage of this method is that the daily dose can be given parenterally enabling factors which limit absorption of vitamin B_{12} from the gut to be disregarded. A flaw in this method is that the smallest amount which alleviates deficiency is not necessarily the same as the amount required to maintain the status quo in normal subjects. If the daily need is related to the body store mass of vitamin B_{12} then the amount which will alleviate deficiency will be small, in proportion to the depleted stores, whereas the normal daily need will be much greater, being in proportion to the stores which are ten or more times greater. For this reason the suggestion by Sullivan & Herbert (1965) that about 0.1 µg is the minimal daily requirement, i.e. the amount which will sustain normality (Herbert, 1968), must be treated with reserve.

The second method is by measurement of the amount of vitamin B_{12} in the diets of normal subjects. The disadvantage of this method is that, because of the limitation of the absorption of vitamin B_{12}, the measurement is of intake and not of amount absorbed or of requirement. Nevertheless information

about dietary intakes is of value and daily intakes of between 1 and 85 µg have been reported (Jollife & Peterman, 1956; Estren et al., 1958; Grasbeck, 1960; Chung et al., 1961).

The third method of estimating daily need is by kinetic studies based on long term whole body turnover measurements of administered radioactive vitamin B_{12}. In the last few years such studies at this hospital and at the Scottish Research Reactor Centre have led to the conclusion that the daily need for vitamin B_{12} is related to the body store mass of vitamin B_{12} and is normally in the range 0.1 - 0.2% of store mass per day (Adams & Boddy, 1968; 1971; Boddy & Adams, 1968). This estimate is in reasonable agreement with results obtained by others using a similar approach (Bozian et al., 1963; Heyssel et al., 1966).

Our own estimates of body vitamin B_{12} store mass in patients who had no evidence of vitamin B_{12} deficiency ranged from about 1,000 to about 6,000 µg (Adams et al., 1970) - (see page 203). With daily turnover rates of 0.1 - 0.2% per day the extreme range of daily needs would be 1 - 12 µg and daily intakes of between 1 and 85 µg reported by Chung et al., (1961) would meet those requirements.

It was appreciated that dietary studies had been carried out abroad, mainly in the U.S.A., but it was thought worthwhile undertaking a study in the population in which the body vitamin B_{12} stores and kinetic studies had been performed. Discussions with dietitians and nutritionists suggested that it would be worthwhile

measuring the vitamin B_{12} content of items of diet and meals rather than calculating the intake by reference to tables and a study was planned with the following objectives.

1) The measurement of the vitamin B_{12} content of items of diet as prepared for consumption, this being done on several samples of each item taken at intervals in order to obtain a range of values for each item.

2) The measurement of the vitamin B_{12} content of meals as prepared for consumption.

3) To compare the vitamin B_{12} content of meals as measured directly, with the content as calculated from values for individual items of the diet.

4) To find out how our estimates of vitamin B_{12} intake compared with those reported previously and how they accorded with estimates of daily need and intake.

In addition, observations were made on the effect of cyanide on the measurement of vitamin B_{12} in foods and on the relative amounts of vitamin B_{12} in the supernatant and deposit of food homogenates.

MATERIALS AND METHODS

The amount of vitamin B_{12} in items and meals was measured by microbiological assay by the method of Hutner et al., (1956) using Euglena gracilis, Z strain, as the test organism.

Items

The majority of items studied were prepared in the hospital kitchen but some, mostly tinned, were obtained commercially and a few were obtained from domestic sources. In general, large amounts were examined, usually between 20 and 100 grammes. The samples were washed under running water to remove gravies if necessary, weighed and if necessary reweighed after removal of bone and obvious fat. The sample was then homogenised with water in a Waring Blendor or M.S.E. Atomix homogeniser to a suitable volume and aliquots stored at -20°C. After thawing and rehomogenising with a Silverson microhomogeniser, each aliquot was assayed in duplicate on at least two occasions. At least one recovery experiment was performed with each aliquot. For this purpose cyanocobalamin was added to the assay tubes so that the concentration of vitamin B_{12} should have been raised by 10 µµg/ml above that due to the sample. The difference between the actual and expected increase was expressed as percentage recovery, i.e. if the observed increase was 7 µµg/ml the recovery was 70%, the expected increase being 10 µµg/ml.

For studies of the relative activities of whole homogenate, supernatant and deposit, several aliquots were prepared. Some were centrifuged and the supernatants and deposits separated and each made up to the volume of the parent sample. The whole homogenate, supernatant sample and deposit sample were assayed simultaneously on at least two occasions.

Whole Meals

All meals studied were replicates of those prepared in the hospital kitchen and served to patients and staff. Each item was measured and remeasured after discarding inedible material and a homogenate prepared as for the items. In addition to the routine assay and recovery study, the effect of added cyanide was studied with ten samples.

Preliminary studies on the effect of adding cyanide to assay tubes containing cyanocobalamin in concentrations of 5 - 50 µµg/ml showed that Euglena growth was not inhibited until 40 µl of M^{-1} sodium cyanide had been added. 40 µl of 4^{-5} sodium cyanide was therefore chosen for the experiments as this was well below the toxic level for Euglena yet adequate to convert all cobalamins present in the sample to cyanocobalamin.

RESULTS

Rehomogenisation

Early results showed a marked fall in activity of many samples when assayed for the second time. This was suspected to be related to the refreezing and rethawing of the sample and was abolished when the sample was rehomogenised with a Silverson microhomogeniser after rethawing. Rehomogenisation was therefore adopted as a routine practice and only results obtained using this procedure are reported.

Effect of Cyanide

The effect of adding cyanide was studied with ten whole meal homogenates, the untreated and cyanide treated samples being assayed simultaneously. In no instance was there any effect on the activity.

Whole Homogenate, Supernatant and Deposit

The relative activities of the supernatants and deposits compared to the parent sample were studied in six items of food and the results are set out in Table 69 (page 152). It was clear from these results that assay of the supernatant alone, which is technically somewhat easier than assay of the whole homogenate, would not give results comparable to assays of the whole homogenate and the matter was not pursued.

Food Items

Values of less than 0.1 µg/100 g solid item or less than 0.1 µg/100 ml liquid item were regarded as negative values and were obtained from at least one sample of each of the following items:-

Apples, apricots, bananas, French beans, baked beans, white bread, brown bread, broth, cabbage, carrots, custard, corn flakes, jam, macaroni, oranges, peas, peaches, pears, porridge, potatoes, pineapple, rice, sago, soups, spaghetti, sprouts, tea, turnip.

Items which had values greater than 0.1 µg/100 g solid item or greater than 0.1 µg/100 ml liquid item are listed in the order

used by McCance & Widdowson (1969). Table 70 (pages 153/154) shows values for meat and poultry, Table 71 (page 155) for fish, Table 72 (page 152) for dairy products and Table 73 (page 154) for miscelleneous items. The method of preparation, the number of samples, the mean value and, in most cases, the standard deviation and the mean percentage recovery of added cyanocobalamin, are shown for each item in the Table.

Item of Food	Whole Homogenate µµg/ml	Supernatant µµg/ml	Deposit µµg/ml
Braised Liver	462	332	106
Fried Sausage	1085	1090	181
Chicken Fricasee	273	119	192
Roast Beef	499	180	303
Boiled Gammon	255	146	116
Fried Haddock	1025	593	510

Table 69 The Activity as µµg/ml of Cyanocobalamin in the whole homogenate, supernatant and deposit of six items of food. The mean value of two assays was taken and all samples were the same volume.

Table 70.

ITEM	No. of Samples	Mean Value & S.D. µg/100 g	Mean Recovery %
Bacon Gammon Boiled	6	0.313 (0.16)	108
Beef Brisket Boiled	6	1.194 (0.10)	108
" Rump Braised	6	0.799 (0.34)	131
" Sirloin Roast	6	1.017 (0.27)	118
" Shoulder Curried	7	1.305 (0.64)	96
" " Stewed	5	1.385 (0.66)	102
" " Minced	6	0.872 (0.45)	135
" Corned Tinned (Fray Bentos)	6	1.741 (0.58)	80
Beefburger	6	1.633 (0.67)	103
Beef Olive, Sausage & Rump Steak Braised	5	1.386 (0.14)	94
Beef Slice	6	0.924 (0.23)	79
Chicken Casserole	5	0.327 (0.12)	113
" Curried	6	0.555 (0.39)	63
" Fricassee	3	0.251	66
" Roast	5	0.550 (0.24)	104
" Breasts Tinned (Ye Olde Oake-Dutch)	6	0.209 (0.08)	86
Ham Baked	6	0.518 (0.14)	112
Ham & Pork Tinned	6	0.551 (0.06)	82
Ham & Chicken Roll " (Crosse & Blackwell)	6	0.894 (0.12)	56
Kidney Sheep Braised	5	79.125 (25.21)	81
" Ox "	3	5.879	59
Lamb Chop Braised	7	1.350 (0.67)	103
" Leg Roast	6	1.633 (0.39)	110
Liver Ox Braised	6	75.850 (19.34)	65
Luncheon Meat Tinned (Ye Olde Oake - Dutch)	7	0.791 (0.20)	75
Meat Roll	3	1.557	100
Mutton Shoulder Boiled	5	1.264 (0.50)	111
" Leg Roast	2	1.058	95

/continued

ITEM	No. of Samples	Mean Value & S.D. µg/100 g	Mean Recovery %
Mutton Pie	2	0.315	90
Pork Chop Braised	2	0.397	118
" Leg Roast	6	0.449 (0.23)	93
Sausages	6	0.668 (0.42)	76
" Beef & Pork Tinned	6	0.400 (0.09)	105
Sausage Roll	2	0.112	98
Spam	6	0.586 (0.22)	91
Sweetbreads (& Sauce) Ox	4	0.940 (0.31)	95
Steak & Kidney Pie	3	3.737	108
Tongue Ox	6	3.301 (1.03)	95
" Lamb Tinned (Cold Dish)	6	3.865 (0.80)	80
Tripe (& Sauce)	4	0.301 (0.38)	67
Turkey Breast Roast	6	2.228 (1.81)	96
Veal Fricassee	3	0.910	105
" Jellied	6	1.435 (0.42)	89
Veal & Ham Pie	2	0.233	96

Table 70. The Vitamin B_{12} content of Items of Meat and Poultry.

Item	No. of Samples	Mean Value & S.D. µg/100 g	Mean Recovery %
Anchovy. Fillets. Tinned (Isabel) (Caravilla, Spain)	6	21.844 (4.21)	84
Brisling. Tinned (Norwegian) (John West)	6	11.006 (1.44)	88
Crab Meat. Tinned (Japanese) (Grand Prix)	6	0.532 (0.18)	102
Cod Fillets - Fried	2	0.853 (	86
Cutlet. Poached	3	0.861	109
Fishcake. Grilled	6	0.687 (0.28)	109
Fish Fingers. Grilled (Birds Eye)	6	0.474 (0.15)	98
Haddock. Fried	6	0.929 (0.19)	87
" Grilled	7	1.178 (0.54)	65
" Poached	11	0.703 (0.22)	93
Herring Fillets. Tinned (John West)	6	8.491 (2.59)	81
Herring. Grilled	5	5.302 (2.22)	107
" Pickled	6	8.010 (1.17)	86
Kipper Fillets. Tinned (John West)	6	10.127 (2.58)	96
" Poached	4	6.549 (2.75)	88
Pilchards. Tinned (John West)	6	11.903 (1.87)	57
Prawns. Tinned (John West)	6	0.546 (0.20)	104
Salmon. Tinned (John West)	6	2.585 (0.60)	75
Sardines. Tinned (John West)	6	12.407 (2.04)	86
Shrimps. Tinned (Sunnan)	6	6.787 (0.15)	97
Tuna. Tinned (John West)	6	4.638 (1.06)	72
Whiting. Fried	3	1.000	83

Table 71 The Vitamin B_{12} content of items of Fish.

Item	No. of Samples	Mean Value & S.D. µg/100 g or µg/100 ml	Mean Recovery %
Cheese - Brie	4	1.292 (0.280)	86
" Cheddar	6	0.772 (0.21)	82
" Cheshire	4	0.894 (0.35)	83
" Danish Blue	6	1.178 (0.29)	90
" Processed	7	0.829 (0.41)	99
Egg - Boiled	6	1.025 (0.60)	81
" Fried	6	0.605 (0.39)	37
" Scrambled	6	0.299 (0.11)	69
Ice Cream	7	0.414 (0.11)	75
Milk - Fresh whole	7	0.648 (0.23)	71
" Pasteurised	10	0.394 (0.09)	95
" Evaporated, Unsweetened (Carnation)	7	0.150 (0.03)	75
Yoghurt	6	0.175 (0.07)	73

Table 72 The Vitamin B_{12} content of Dairy Products.

Item	No. of Samples	Mean Value & S.D. µg/100 g	Mean Recovery %
Black Pudding	4	0.294 (0.004)	68
Haggis	6	1.073 (0.36)	73
Rice Krispies	2	0.134	65
Scotch Egg	6	0.750 (0.28)	79

Table 73 The Vitamin B_{12} content of Miscellaneous Items of Food.

Table 74.

Meal (B_{12} containing items)	Protein content (g)	Calorie Value (kcal)	Vitamin B_{12} (µg)		
			Direct Assay	Calculated Mean	Range
Breakfast					
1) Fried egg: Sheep kidney	21	375	52.000	27.126	9.827-44.490
2) Bacon: Fried egg: Milk	21	529	2.080	0.589	0.251- 1.052
3) Poached cutlet	17	294	1.902	0.689	0.530- 0.809
4) Sausages: Scrambled egg	22	416	1.640	0.974	0.380- 1.569
5) Bacon: Scrambled egg	23	460	0.323	0.262	0.081- 0.444
6) Rice Krispies: Bacon: Milk	15	381	0.419	0.513	0.280- 0.748
7) Rice Krispies: Poached egg: Milk	15	414	1.380	0.856	0.456- 1.310
8) Rice Krispies: Beef Slice: Milk	19	516	1.143	0.921	0.478- 1.362
9) Sausages: Milk	14	986	0.320	0.588	0.306- 1.022
Lunch					
10) Fried Haddock	23	525	0.329	0.659	0.389- 0.929
11) Mince	62	884	0.604	1.308	0.783- 2.577
12) Roast Beef	26	518	7.150	0.620	0.291- 0.949
13) Boiled Beef	30	566	1.750	0.810	0.674- 0.946
14) Fried Haddock	22	462	1.457	0.641	0.379- 0.903
15) Fried Haddock: Ice Cream	24	393	0.960	1.022	0.561- 1.454
16) Roast Beef	31	649	1.360	0.447	0.209- 0.685
17) Braised Liver: Sausages	41	841	77.066	59.370	29.090-93.812
18) Roast Lamb: Ice Cream	21	515	0.939	1.086	0.562- 1.613
19) Sweetbreads	27	595	0.351	0.479	0.163- 0.796
20) Mince: Ice Cream	25	631	1.725	1.370	0.799- 2.627

/continued

Meal (B_{12} containing items)	Protein content (g)	Calorie Value (kcal)	Vitamin B_{12} (µg)		
			Direct Assay	Calculated Mean	Calculated Range
21) Meat Roll	19	502	0.775	0.669	0.396- 1.086
22) Poached Haddock	25	474	1.039	0.478	0.168- 0.777
23) Curried Beef	18	542	1.697	1.057	0.405- 1.707
24) Steak & Kidney Pie	23	484	0.560	4.260	0.546- 8.105
25) Meat Roll	18	616	0.621	0.887	0.526- 1.439
26) Veal Fricassee	37	389	0.575	1.019	0.577- 1.767
27) Lamb Chop	17	507	0.221	0.310	0.104- 0.531
28) Roast Lamb	21	384	1.100	0.996	0.520- 1.472
29) Poached Cutlet	32	622	1.155	0.471	0.176- 0.766
30) Mince Pie	30	489	0.908	0.802	0.480- 1.580
31) Roast Beef	40	921	0.453	0.620	0.291- 0.949
32) Steak Pie	36	857	0.683	0.734	0.530- 1.131
33) Mince: Ice Cream	37	638	0.740	1.588	0.934- 3.072
34) Pickled Herring	16	264	11.030	7.850	5.556-10.143
35) Sweetbreads	33	642	0.888	1.015	0.346- 1.685
36) Mince Pie: Ice Cream	23	552	1.509	0.619	0.371- 1.220
37) Boiled Egg: Milk	21	485	0.908	1.259	0.588- 2.006
Supper					
38) Boiled Chicken: Milk	22	563	0.140	0.444	0.225- 1.042
39) Chicken Croquette	11	199	0.112	0.098	0.026- 0.170
40) Fishcake	9	243	0.507	0.436	0.163- 0.709
41) Mutton Pie	15	397	0.226	0.296	0.234- 0.361
42) Mince	27	317	0.652	0.732	0.438- 1.443
43) Boiled Egg: Cheese: Milk	17	493	0.759	0.430	0.146- 0.784
44) Chicken Fricassee	25	272	0.185	0.226	0.174- 0.259
45) Spam	5	170	0.200	0.205	0.051- 0.359

Table 74. The Vitamin B_{12} content of Whole Meals (direct and calculated). Also shown are the protein content and calorific value for each meal.

Whole Meals

45 whole meals were examined. The vitamin B_{12} containing constituents of each meal and the vitamin B_{12} contents of each meal as found by assay (direct value) and by calculation from values for items (calculated value) are shown in Table 74. (pages 158/59). Also shown in the Table are the protein content and calorific value for each meal. Small volumes of milk such as used in tea are not included and when milk is itemised the volumes were of the order used with cereals and porridge. In the calculations of vitamin B_{12} content of meals from values for items, ranges were used in preference to mean values. The ranges were usually the mean plus or minus two standard deviations. If, however, the number of samples was small or if the distribution was skewed the observed ranges were used. The recoveries of added cyanocobalamin were in keeping with those found in the item study and ranged from 55 - 130% with a mean of 83%.

DISCUSSION

Although information on the vitamin B_{12} content of foodstuffs has accumulated and is contained in the report by McCance & Widdowson (1969) and has been reviewed by Robinson (1966); Chanarin (1969) and Love (1970) the practical value of much of the information on the calculation of the vitamin B_{12} content of diets is limited. In the first place many of the earlier reports were based on methods, such as the rat growth assay, which are now

regarded as less than satisfactory. Secondly little account has been taken of the variation which might be expected in any tissue even when seasonal and geographical factors are excluded. Finally, and most important of all, nearly all reports deal with raw food and although it may be possible to make some allowance for weight changes in cooking (McCance & Widdowson, 1969) it is impossible to allow for loss or destruction of vitamin B_{12} in cooking (Banerjee & Chatterjea, 1963; Heyssel et al., 1966) which may be as much as 27% (Heyssel et al., 1966).

The technical aspects of the study - the necessity for rehomogenisation of samples, the effect of added cyanide and the relative activities of the supernatants and deposits of homogenates were of interest.

That homogenates which had been prepared, frozen, thawed, sampled and refrozen had a marked fall in activity when further thawed and sampled, was unexpected. Although it is a well known phenomenon in relation to the estimation of cortisol in urine and alkaline phosphatase in serum, it does not appear to have been reported previously in relation to vitamin B_{12} assay with Euglena on human serum or urine, or human or rat tissue homogenates. The cause was not identified other than being related to re-freezing and rethawing and it was easily overcome by further homogenisation of the rethawed sample prior to assay.

The lack of effect of added cyanide was not surprising. Although of established value in assays with Lactobacillus leichmannii (Chanarin, 1969) its use in assays with Euglena gracilis is, at best, marginal (Anderson, 1965). In view of

our observations that hydroxocobalamin, methylcobalamin and coenzyme B_{12} have the same growth promoting effect for Euglena when bound to human serum or liver homogenate, although not in aqueous solution, it seemed unlikely that cyanide would have an effect, at least by conversion of other cobalamins to cyano-cobalamin (Adams & McEwan, 1971).

Nor were the results of the assays of whole homogenates, supernatants and deposits surprising. In the absence of proteolytic treatment it seemed unlikely that either the supernatants or deposits would contain all the activity. The results of a limited study (Table 69, page) made it clear that assays of supernatants or deposits could not be substituted for assays of whole homogenates.

From experience of the range of vitamin B_{12} values in normal human tissues it was expected that a similarly wide range would be found in the animal tissues which constituted the vitamin B_{12} containing items of food. This proved to be the case although the range was greater than expected with some items and less than expected with others. Seasonal variations could have been a factor determining the range of values for any one item but as samples of the same item had to be collected at irregular intervals (intervals ranging from a week to six months) it was not possible to study this point. One exception was with pasteurised milk where the results supported the findings of Karlin (1969) of an absence of seasonal variation.

It is doubtful if any worthwhile conclusion can be drawn from the results about the effects of cooking or of different

methods of cooking or of the processes involved in tinning on the vitamin B_{12} content of any one tissue. In part this is because of the necessarily irregular collection of items and in part because of the weight changes in cooking. To examine this aspect with any degree of precision it would be at least desirable to examine the raw tissue and then samples from the same source after cooking. The results for milk however are in accord with others, reviewed and confirmed by Karlin (1969), of a loss of vitamin B_{12} with pasteurisation.

Similarly it would be unwise to compare values for cooked foods shown in Tables 70 - 73 (pages 153 - 157) with those reported previously for raw foods because of weight changes and loss or destruction of vitamin B_{12} in cooking. It is of interest, however, to compare values for items which apparently have similar preparatory processes and some findings are set out in Table 75 (page 164). Those ascribed to Lichtenstein et al., (1961) have been taken from Chanarin (1969) as it proved impossible to obtain the original or a copy. The data reported by Lichtenstein et al., (1961) were not included in that reviewed by McCance & Widdowson (1969). Even this sort of comparison is of limited value when it is appreciated that the term sardine may be applied to nine biologically different fish each having a native habitat thousands of miles from another (Love, 1970).

Comparison of values for the vitamin B_{12} of meals obtained by assay of whole meal homogenates (direct value) with those obtained by calculations based on values for vitamin B_{12} of items

Items of Food	Vitamin B_{12} µg/100 g or µg/100 ml		
	Lichtenstein et al.,(1961)	McCance & Widdowson (1969)	This Series Mean and S.D.
Milk - Raw	-	0.3	0.648 (0.23)
Milk - Pasteurised	0.36	0.3	0.394 (0.09)
Milk - Evaporated	0.132	2.0	0.150 (0.03)
Cheddar	0.99 - 1.17	2.0	0.772 (0.21)
Corned Beef	1.84	-	1.741 (0.58)
Salmon (Tinned)	6.89	2.0	2.585 (0.60)
Sardine (Tinned)	8.34	10.0	12.407 (2.04)
Crab (Tinned)	7.3	0.5	0.532 (0.18)
Tuna (Tinned)	2.8	-	4.638 (1.06)

Table 75 Comparison of Vitamin B_{12} Values of Items of Diet reported in this series with those reported previously.

of food (calculated value) showed agreement in 26 out of 45 meals examined. Without qualification such a result can only be regarded as poor and not one which could be used to support a case that calculation of dietary vitamin B_{12} intake gives results comparable to those obtained by direct measurement.

There are, however, some factors which qualify this conclusion. It could be argued that direct measurement will inevitably tend to give a higher value than the calculated value because of the inclusion of sauces and gravies and such which may be rich in vitamin B_{12} (Heyssel et al., 1966) in whole meal homogenates. It is relevant to note that in the 19 instances in which there was disagreement between direct and calculated values, the direct measurements gave higher values than the calculated in 13 instances, the reverse being the case in 6 instances (Table 76, page 166).

It might also be argued that the terms of the comparison were too limited. The calculated values for meals were obtained using values for items with a range based on the mean plus or minus two standard deviations or, as in a few cases when the distribution of values was skewed or the number of values less than four, on the observed range of values. It seems likely that the agreement between direct and calculated values for means would have been greater if observed ranges had been used throughout. It could also be argued that the magnitude of the disagreements was so small in many cases as to be negligible and certainly if disagreements of less than 0.2 µg are disregarded the agreement

Meal	Number	Number in Agreement	THOSE IN DIS-AGREEMENT					
			Direct > Calculated			Direct < Calculated		
			Meal No.	Direct Value µg	Calculated Value µg	Meal No.	Direct Value µg	Calculated Value µg
Breakfast	9	4	1)	52.000	9.827-44.490			
			2)	2.080	0.251- 1.052			
			3)	1.902	0.530- 0.809			
			4)	1.640	0.380- 1.569			
			7)	1.380	0.456- 1.310			
Lunch	28	16	12)	7.150	0.291- 0.949	10)	0.329	0.389-0.929
			13)	1.750	0.674- 0.946	11)	0.604	0.783-2.577
			14)	1.457	0.379- 0.903	26)	0.575	0.577-1.767
			16)	1.360	0.209- 0.685	33)	0.740	0.934-3.072
			22)	1.039	0.168- 0.777			
			29)	1.155	0.176- 0.766			
			34)	11.030	5.556-10.143			
			36)	1.509	0.371- 1.220			
Supper	8	6				38)	0.140	0.225-1.042
						41)	0.226	0.234-0.361
Totals	45	26 (58%)	13 (29%)			6 (13%)		
If differences of 0.2 µg disregarded		34 (76%)	11 (24%)			0		

Table 76. Vitamin B_{12} values of whole meal homogenates (direct values) compared with calculated values.

rises to a more impressive 76% (34 out of 45).

Although the results seem to form a basis for debate and discussion, it can tentatively be concluded that, from the clinical and nutritional aspect, a reasonable estimate of the vitamin B_{12} content of a meal can be obtained in most cases by calculation from values for individual items. Also calculated estimates are more likely to be underestimated when compared to direct measurements.

In stating this somewhat guarded conclusion it must be emphasised that both direct and calculated values are obtained from measurements by microbiological assay. In spite of recovery values which suggest that, for the most part, the bulk of the vitamin B_{12} in the test substances was available to the organism and that there was no inhibition of growth it would be unwise to assume that the results necessarily reflect the truth. It may be that the values reported here are shown to be erroneous when a new method of measuring vitamin B_{12} becomes available.

The significance of the results in relation to our group's estimates of body vitamin B_{12} store mass and daily need for vitamin B_{12} to maintain stores is not as easily evaluated as might have been hoped or suspected.

At the simplest level, the problem is whether the observed results accord with theoretical values. With a turnover rate of 0.1 - 0.2% of body stores per day and with body stores of 1,000 - 6,000 µg the extreme range of need will thus be 1 - 12 µg daily although in the majority of cases who have stores of less

than 3,000 µg it will be 1 - 6 µg daily. On the suggestion that all the vitamin B_{12} in food is available (Heyssel et al., 1966) and on a rule of thumb basis that about half of the amount ingested is absorbed this need requires an intake of 2 - 24 µg daily to cover all cases and a 2 - 12 µg daily to cover the majority.

The results of the direct measurement of the vitamin B_{12} content of meals can be presented in such a way that they fit in with these theoretical requirements obtained by kinetic studies and body store determinations. It can be shown for instance that the average vitamin B_{12} content of breakfast is 6.8 µg, of lunch 4.2 µg, and of supper 0.35 µg making an averggo daily intake of 11.4 µg. Such a presentation of the facts gives the impression of reasonable agreement between the theoretical requirements and the amount of vitamin B_{12} in diet but on closer inspection this is grossly misleading. In the first place this presentation overlooks the fact that the average values are grossly biased by a minority of results. For instance the average breakfast vitamin B_{12} is 6.8 µg but if one value of 52 µg is excluded it falls to 1.15 µg. Similarly the average lunch vitamin B_{12} is 4.2 µg but if one value of 77 µg is excluded it falls to 1.6 µg and if two others of 11 and 7 µg are also excluded it falls further to 0.9 µg. Thus with the exclusion of only two out of forty five results the average daily dietary vitamin B_{12}, obtained by adding the averages for each meal, falls from 11.3 µg to 3.1 µg and to 2.4 µg if two other results are also excluded.

Excluding very high values simply because they are unusual may not be generally acceptable but there is a further reason for doing so, which is also obscured by simple presentation of averages for all values. This is the limitation to absorption of vitamin B_{12} at between 3 and 5 µg dose level (Glass et al., 1954; Swendseid et al., 1954; Callender & Evans, 1955). Up to this dose level the rule of thumb that about half of what is ingested is absorbed is a reasonable generalisation, but with greater dose levels, this does not appear to be so. Thus with cyanocobalamin about 50% is absorbed from a 1 µg dose but only 20% from a 5 µg dose and 5% from a 25 µg dose (Adams et al., 1971). Because of the block to absorption there might be a case for "correcting" all meal vitamin B_{12} values of greater than 3 µg to 3 µg and if this were done with the presented results the average breakfast vitamin B_{12} would be 1.3 µg, the average lunch vitamin B_{12} 1.15 µg. These and the average supper vitamin B_{12} would give an average daily intake of 2.86 µg.

These results are not only lower than those previously reported for dietary vitamin B_{12} intakes by man but are less than was expected from the kinetic studies and body store determinations. Taking each thrice daily meal to contain 1 µg and assuming absorption of half of this the need for balance would be met if the stores were in the range of 750 - 1,500 µg which is at the very lowest limit reported by us. If all the intake of 3 µg was absorbed then this would maintain balance with stores of 1,500 - 3,000 µg which is within the limits. Thus to equate the results of the kinetic studies, body vitamin B_{12} store determinations

and dietary vitamin B_{12} it is desirable to postulate absorption of more than 50% of the vitamin B_{12} in diet. This could be the case. There is evidence that food stimulates the absorption of cyanocobalamin (Swendseid et al., 1954; Siegel et al., 1961) although this is debatable (Doller et al., 1961; Heyssel et al., 1966). Unpublished results from our group show an increase in absorption of cyanocobalamin (1 µg) from 50% to 63% when pentagastrin was given. If from each 1 µg taken thrice daily a total of 1.9 µg were absorbed then the results would fit in with body stores of 950 - 1,900 µg which is in keeping with most of our body store results and kinetic studies. The calculations do not stretch credulity too far but it must be admitted that Wellington would probably have described them as "close run".

Although the presentation of results does not permit precise comparisons there can be no doubt that the results are more in keeping with the intakes on the "poor" diet (Chung et al., 1961) which ranged from 1.1 - 8.1 µg with a mean of 2.7 µg per day than with the daily intakes of 5 µg (Estren et al., 1958), 6 µg (Jolliffe & Peterman, 1956), 7 µg (Grasbeck, 1960) and means of 16 µg for "low cost" diets and 31 µg for "high cost" diets (Chung et al., 1961). It is not clear why our values should be so low and comparable to those found in diets supplying an average daily intake of 1,000 kilocalories and 32 g protein and which were considered to be "grossly nutritionally inadequate" (Chung et al., 1961). It seems unlikely from the protein content and calorific

values of the meals studied (data calculated by Mrs. Aileen Wilson B.Sc., Chief Dietitian, Southern General Hospital) that they come into this category (Table 77, page 172). The difficulty is compounded by details of the "poor" diet which show that the weekly intake of vitamin B_{12} appears to have been derived from two fried eggs, two helpings of bacon and an unspecified quantity of milk, in two meals.

Little work has been done on the vitamin B_{12} content of foods and diets in the last decade. The results presented here suggest that further work is called for to resolve conflicting opinions on the vitamin B_{12} content of diets and daily intake of vitamin B_{12}.

	CALORIFIC VALUE (kcal)				PROTEIN CONTENT (g)			
	Range	Mean	S.D.	Mean ± 2 S.D.	Range	Mean	S.D.	Mean ± 2 S.D.
Breakfast	294-986	485.7	201.2	83.3-888.1	14-23	18.6	3.4	11.8-25.4
Lunch	264-921	569.5	155.7	258.1-880.9	16-62	27.4	9.8	7.8-47.0
Supper	170-563	331.8	141.0	49.8-613.8	5-27	16.4	7.9	0.6-32.2
Daily Totals	728-2470	1387.0		391.2-2382.8	35-112	62.4		20.2-104.6

Table 77 Summary of the calorific values and protein content of the whole meals.

CHAPTER 3

Cryoconcentration of Intrinsic Factor in Gastric Juice.

A method of concentrating the intrinsic factor in gastric juice which involves little labour and the use of simple equipment would appear to be of interest and value. In this paper some findings, which suggest that cryoconcentration may be such a method, are presented.

MATERIALS AND METHODS

Gastric juice, secreted spontaneously or after pentagastrin stimulation, was obtained from patients with a variety of diseases, undergoing gastric secretory studies. After aspiration, the material was filtered through glass wool and the pH adjusted with decinormal sodium hydroxide solution to pH7, as measured electrometrically. Aliquots of 20 ml from the neutralised individual aspirates were dispensed into cylindrical polystyrene containers with an inside diameter of 2.5 cm and stored at -20°C until assayed between three and ten days later.

Aliquots were thawed on a bench at room temperature, usually about 20°C, and the first half or quarter to thaw was collected separately. The vitamin B_{12} binding capacity of each fraction was measured by the radio-immuno assay method of Gottlieb et al.(1965) the difference between the total binding capacity and the binding capacity after the addition of serum containing antibody to intrinsic factor (i.e. non-specific

binding capacity) being the intrinsic factor binding capacity. The binding capacity of the original was derived from the measurements in each fraction, these having been performed simultaneously. The serum, containing antibody to intrinsic factor, used throughout was from a pool obtained from ten patients with pernicious anaemia. The radioactive vitamin B_{12}, (^{57}Co) cyanocobalamin, was obtained from the Radiochemical Centre, Amersham.

The masses and volumes of reagents were 0.1 ml. of gastric juice, 0.1 ml serum and 7.5 mμg vitamin B_{12} the volume being made up to 3 ml with distilled water. 2 ml 2.5% bovine serum albumin coated Norit OL charcoal (as described previously - page 11) were added to separate free and bound vitamin B_{12}.

Preliminary studies suggested that an increase in the concentration of binders in gastric juice could be achieved by freezing and fractionation during thawing and detailed studies were performed as follows.

1) Observations were made on the effect of fractionating frozen 20 ml volumes of gastric juice into a first thawed fraction of 10 ml and a second thawed fraction of 10 ml. Twenty samples of juice from twenty patients were used.

2) Observations were made on the effect of fractionating 20 ml volumes of gastric juice into a first thawed fraction of 5 ml and a second thawed fraction of 15 ml. Replicates of the samples used in the previous study were investigated.

3) Observations of the reproducibility of results during such fractionations were made using twelve samples each of 20 ml prepared from the same pool of gastric juice used in the separation studies (pages 9%).

RESULTS

Fractionation of 20 ml samples into fractions of 10 ml and 10 ml

The results are summarised and detailed in Table 78 (page 177). In all instances the first thawed fraction had a higher total binding capacity per ml than the parent material. The intrinsic factor binding capacity per ml of the first thawed fraction was greater than that of the parent material in sixteen instances and less in two, there being two samples in which it was absent from both the parent sample and the thawed fractions. The non-specific binding capacity per ml was greater in the first thawed fraction than in the parent material in nineteen instances and less in one.

Plots of total binding capacity per ml of parent material against percentage increase in concentration of total binding capacity per ml in the first thawed (10 ml) fraction and intrinsic factor binding capacity per ml of parent material against percentage increase in concentration of intrinsic factor binding capacity per ml in the first thawed (10 ml) fraction are shown in Figs. 12 and 13 (pages 178 and 179). Not shown in the latter plot are the two percentage decreases (-23.2% and -4.3%).

Sample Number	Original Material (20 ml) mμg Cyanocobalamin Bound/ml			First Thawed Fraction (10 ml) mμg Cyanocobalamin Bound/ml			% Change in Concentration		
	Total	Non-specific	Intrinsic Factor	Total	Non-specific	Intrinsic Factor	Total	Non-specific	Intrinsic Factor
1	58.0	57.9	0	62.3	64.8	0	+ 7.4	+ 11.9	0
2	46.4	14.0	32.4	54.3	20.0	34.3	+16.9	+42.9	+ 5.9
3	34.8	21.7	13.1	44.7	27.2	17.5	+28.4	+25.3	+33.6
4	10.4	10.8	0	14.0	14.2	0	+34.6	+31.5	0
5	28.4	16.5	11.9	40.5	22.9	17.6	+42.6	+38.9	+47.9
6	60.1	38.6	21.5	62.9	36.7	26.2	+ 4.7	- 4.9	+21.9
7	64.2	29.7	34.5	69.6	43.1	26.5	+ 8.4	+45.1	-23.2
8	66.6	18.0	48.6	69.8	23.3	46.5	+ 4.8	+29.4	- 4.3
9	25.4	7.7	17.7	36.0	11.5	24.5	+41.7	+49.4	+38.4
10	17.0	3.5	13.5	27.5	5.5	22.0	+61.8	+57.1	+63.0
11	46.0	36.6	9.4	58.7	47.3	11.4	+27.6	+29.2	+21.3
12	18.6	7.4	11.2	30.5	11.3	19.2	+64.0	+52.7	+71.4
13	11.6	6.3	5.3	19.2	9.6	9.6	+65.5	+52.4	+81.1
14	26.1	15.6	10.5	37.6	21.8	15.8	+44.1	+39.7	+50.5
15	54.4	13.5	40.9	67.9	18.5	49.4	+24.8	+37.0	+20.8
16	48.1	15.3	32.8	60.1	22.3	37.8	+24.9	+45.8	+15.2
17	50.4	20.0	30.4	61.0	30.2	30.8	+21.0	+51.0	+ 1.3
18	38.6	9.7	28.9	53.0	17.0	36.0	+37.3	+75.3	+24.6
19	43.8	6.9	36.8	58.2	9.1	49.1	+32.9	+31.9	+33.4
20	21.6	2.6	19.0	28.6	2.8	25.8	+32.4	+ 7.7	+35.8

Table 78. Summary of the results of the fractionation of 20 ml samples of gastric juice into fractions of 10 ml and 10 ml.

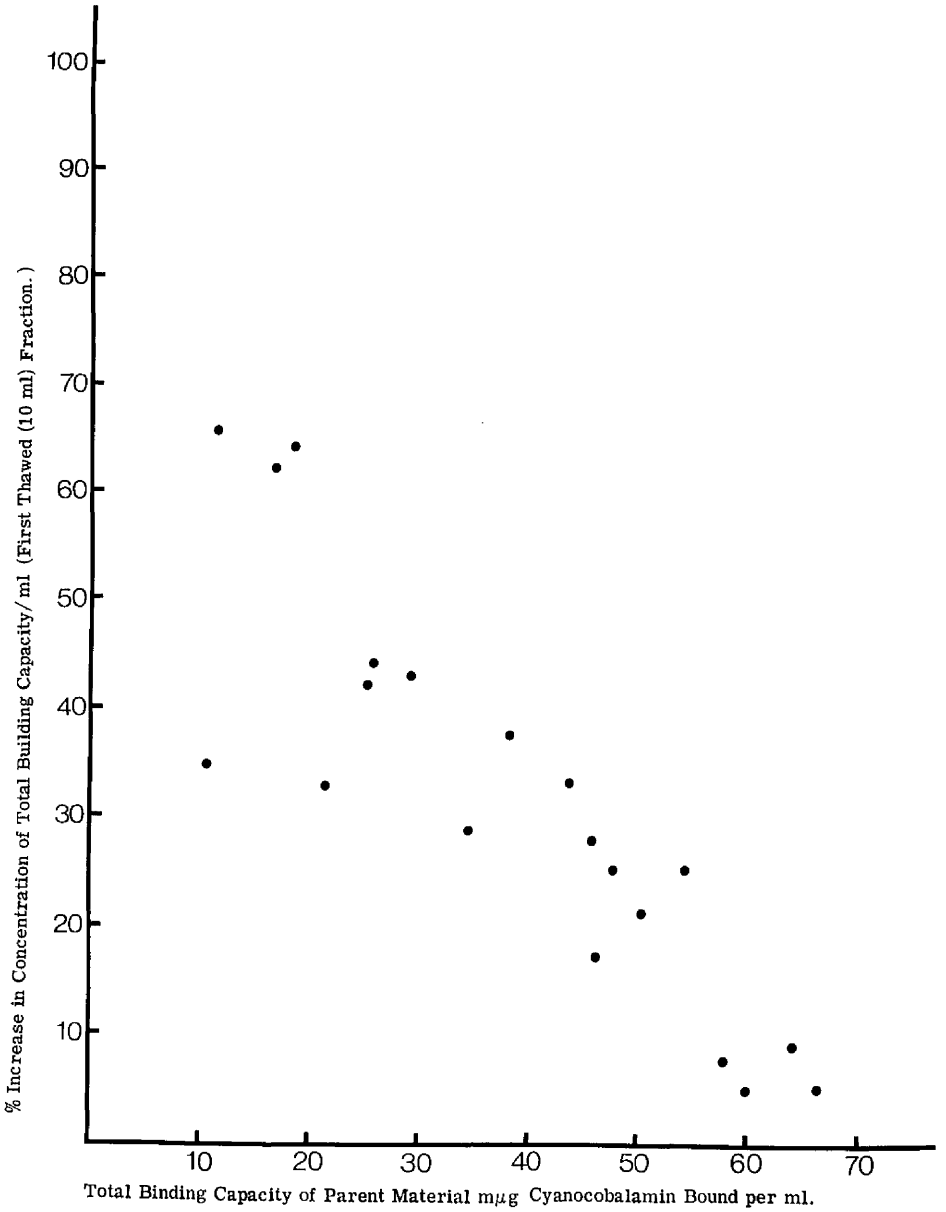


Fig. 12. Plot of total binding capacity per ml of parent material against percentage increase in concentration of total binding capacity per ml in the first thawed (10 ml) fraction.

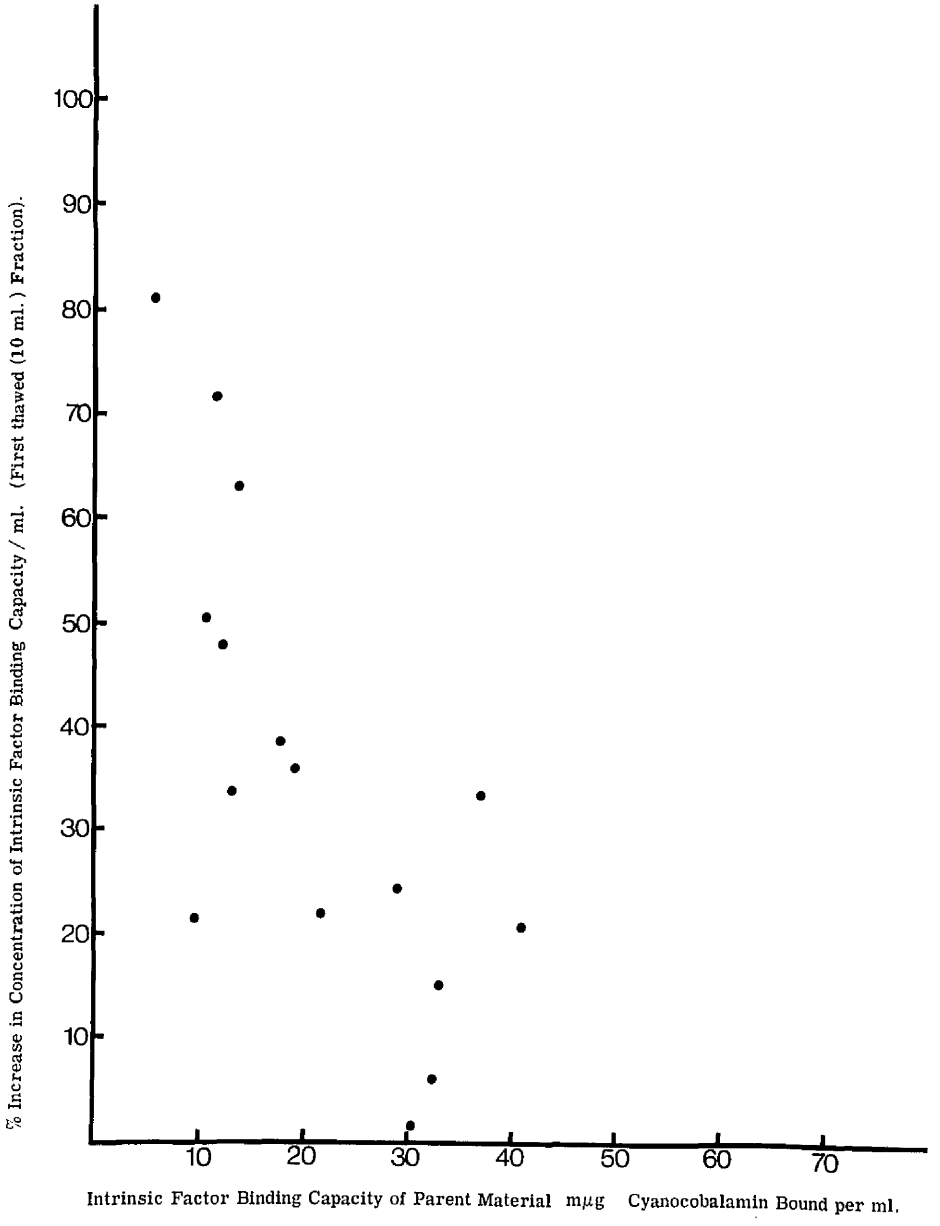


Fig. 13. Plot of Intrinsic Factor binding capacity per ml of parent material against percentage increase in concentration of Intrinsic Factor binding capacity per ml in the first thawed (10 ml) fraction.

Sample Number	Original Material (20 ml) mμg Cyanocobalamin Bound/ml			First Thawed Fraction (5 ml) mμg Cyanocobalamin Bound/ml			% Change in Concentration		
	Total	Non-specific	Intrinsic Factor	Total	Non-specific	Intrinsic Factor	Total	Non-specific	Intrinsic Factor
1	17.5	26.6	0	31.7	46.4	0	+81.1	+74.4	0
2	58.8	30.9	27.9	62.3	41.6	20.7	+ 6.0	+34.6	-25.8
3	30.5	16.0	14.5	36.2	22.7	13.5	+18.7	+41.9	- 6.9
4	4.6	7.8	0	3.4	6.5	0	-26.0	-16.7	0
5	14.0	8.8	5.2	20.0	13.3	6.7	+42.9	+51.1	+28.8
6	29.9	21.6	8.3	34.8	28.6	6.2	+16.4	+32.4	-25.3
7	54.9	31.7	23.2	46.7	25.1	21.6	-14.9	-20.8	- 6.9
8	49.6	15.9	33.7	63.2	4.8	58.4	+27.4	-69.8	+73.3
9	18.9	8.8	10.1	27.8	12.9	14.9	+47.1	+46.6	+47.5
10	11.7	5.2	6.5	23.0	9.6	13.4	+96.6	+84.6	+106.2
11	54.9	38.6	16.3	65.8	54.0	11.8	+19.9	+39.9	-27.6
12	14.2	8.5	5.7	17.1	10.8	6.3	+20.4	+27.1	+10.5
13	9.9	7.3	2.6	26.0	19.3	6.7	+162.6	+164.4	+157.7
14	35.6	19.9	15.7	37.3	21.8	15.5	+ 4.8	+ 9.5	- 1.3
15	60.8	13.2	47.6	67.5	18.0	49.5	+11.0	+36.4	+ 4.0
16	49.4	12.3	37.1	62.2	14.3	47.9	+25.9	+16.3	+29.1
17	55.2	15.6	39.6	55.7	17.6	38.1	+ 0.9	+12.8	- 3.8
18	36.3	9.0	27.3	58.0	14.7	43.3	+59.8	+63.3	+58.6
19	41.4	7.0	34.4	56.7	10.5	46.2	+37.0	+50.0	+34.3
20	22.3	3.7	18.6	39.9	3.4	36.5	+78.9	- 8.1	+96.2

Table 79. Summary of the results of the fractionation of 20 ml samples of gastric juice into fractions of 5 ml (first thawed) and 15 ml.

Fractionation of 20 ml samples into fractions of 5 ml (first thawed) and 15 ml

The results are summarised and detailed in Table 79 (page 180). The total binding capacity per ml was greater in the first thawed fraction than in the parent material in eighteen instances and less in two. The intrinsic factor binding capacity per ml of the first thawed fraction was greater than that in the parent material in eleven instances and less in seven, intrinsic factor being absent from both the parent sample and the thawed fractions in two samples. The non-specific binding capacity per ml of the thawed fraction was greater than in the parent material in sixteen instances and less in four.

Reproducibility

Six 20 ml samples from the same pool of gastric juice were thawed into fractions of 10 ml and 10 ml and another six samples from the same pool into fractions of 5 ml (first thawed) and 15 ml on six separate occasions and assayed. The results are summarised and detailed in Tables 80 and 81 (pages 182 and 183). Mean values and standard deviations were calculated and so the reproducibility, as judged by the standard deviation expressed as a percentage of the mean.

Replicates	Original Material (20 ml) mμg Cyanocobalamin Bound/ml			First Thawed Fraction (10 ml) mμg Cyanocobalamin Bound/ml			% Change in Concentration		
	Total	Non-specific	Intrinsic Factor	Total	Non-specific	Intrinsic Factor	Total	Non-specific	Intrinsic Factor
1	12.7	8.2	5.9	24.5	12.6	11.8	+92.9	+53.7	+100.0
2	18.6	6.8	12.6	34.1	9.0	25.1	+83.3	+32.4	+ 99.2
3	21.2	7.8	13.5	35.9	10.8	25.1	+69.3	+38.5	+ 85.9
4	22.9	9.1	13.8	36.6	10.7	26.0	+59.8	+17.6	+ 88.4
5	19.5	11.7	7.8	33.2	20.4	12.8	+70.2	+74.4	+ 64.1
6	23.3	10.0	13.3	36.2	16.3	20.0	+55.4	+63.0	+ 50.4
Means and S.D.	19.7 (3.9)	8.9 (1.7)	11.2 (3.4)	33.4 (4.6)	13.3 (4.3)	20.1 (6.4)	+69.5	+49.4	+ 79.5
Reproducibility	19.8	19.1	30.4	13.8	32.3	31.8			

Table 80. Summary of the results of the fractionation of six 20 ml samples of the same pool of gastric juice into fractions of 10 ml and 10 ml, the evaluations being carried out on six separate occasions. Means and standard deviations are shown and also the reproducibility as judged by the standard deviation expressed as a percentage of the mean.

Replicates	Original Material (20 ml) mμg Cyanocobalamin Bound/ml			First Thawed Fraction (5 ml) mμg Cyanocobalamin Bound/ml			% Change in Concentration		
	Total	Non-specific	Intrinsic Factor	Total	Non-specific	Intrinsic Factor	Total	Non-specific	Intrinsic Factor
1	16.1	11.3	4.8	47.6	28.3	19.3	+195.7	+150.4	+302.1
2	14.0	12.6	4.0	44.9	28.9	16.0	+220.7	+129.4	+300.0
3	15.2	11.6	4.7	48.5	31.7	18.8	+219.1	+173.3	+300.0
4	23.5	11.2	12.3	45.1	24.7	20.4	+ 91.9	+120.5	+ 67.2
5	23.5	7.1	16.5	41.0	12.0	29.0	+ 74.5	+ 69.0	+ 75.8
6	23.7	8.9	14.7	40.9	16.1	24.8	+ 72.6	+ 80.9	+ 68.7
Means and S.D.	17.6 (4.7)	10.5 (2.0)	9.5 (5.6)	44.7 (3.2)	23.6 (7.7)	21.4 (4.7)	+154.0	+124.7	+125.3
Reproducibility	26.7	19.0	58.9	7.2	32.6	22.0			

Table 81. Summary of the results of the fractionation of six 20 ml samples of the same pool of gastric juice into fractions of 5 ml and 15 ml, the evaluations being carried out on six separate occasions. Means and standard deviations are shown and also the reproducibility as judged by the standard deviation expressed as a percentage of the mean.

DISCUSSION

The procedure of concentrating a constituent in a solution by freezing and collection of fractions during thawing - which we have called cryoconcentration for convenience - is well known. Indeed it is said to have been widely used in an era of prohibition in the U.S.A. to increase the concentration of ethyl alcohol in certain beverages. In spite of this wide general acceptance in the field of applied science, little is known about the change which occurs in the distribution of a solute in a solvent during the change from the liquid to the solid state and any references to the theoretical or practical aspects of this in relation to the vitamin B_{12} binders in gastric juice have not been found.

It is clear from the results that the most common effect of the procedure used, is an increase in the concentration of both intrinsic factor binding capacity per ml and non-specific binding capacity per ml in the first thawed fraction, whether this was 10 ml or 5 ml. It is also clear that in some instances (especially with 5 ml first thawed) the increase in concentration of one binder was disproportionately greater or smaller than the increase or decrease in the concentration of the other. The reasons for this are not clear but analysis of the results as in Table 82 (page 186) may have a relevance.

Whether in thawing and fractionation of 20 ml volumes of gastric juice a better result, in terms of increased

concentration of binders, is obtained when the first thawed fraction is 10 ml or 5 ml is not entirely clear. In general, however, as has been noted, an increase in concentration of binding - total binding capacity, non-specific binding capacity and intrinsic factor binding capacity - was more common when the first thawed fraction was 10 ml. It thus seems likely that for 20 ml volumes fractionation to a first thawed volume of 10 ml is the better procedure.

There is little doubt from the results that a worthwhile increase in the concentration of binders in gastric juice can be achieved by the very simple procedure we have used. However the plots Figs. 12 and 13 (pages 178 and 179) and others not included suggest that the percentage increase is greater with low initial concentrations and less with high initial concentrations - in other words there is a law of diminishing returns. This potential limitation to the practical value of the method requires further study.

This preliminary study has shown that cryoconcentration appears to have application in the concentration of intrinsic factor binder in gastric juice. The full extent of its value and limitations will only be clarified by further study, which should take account of such factors as sample volume and shape, duration of freezing, temperature and possibly also the effects of repeated freezing and thawing in the preparation of pooled samples.

	First Thawed	
	10 ml	5 ml
Rise in concentration of Intrinsic Factor binding capacity and non-specific binding capacity	15	9
Fall in concentration of Intrinsic Factor binding capacity and non-specific binding capacity	0	1
Rise in concentration of Intrinsic Factor binding capacity and fall in concentration of non-specific binding capacity	1	2
Fall in concentration of Intrinsic Factor binding capacity and rise in concentration of non-specific binding capacity	2	6

Table 82. Disproportionality of increase or decrease in concentration of Intrinsic Factor binding capacity compared with that of Non-Specific binding capacity.

CHAPTER 4

Previously Published Reports.

INTRODUCTION

This chapter includes four previously published reports of joint work carried out in this department. Tables and figures have therefore been numbered as published and so are not in sequence with those occurring previously in the thesis. The relevant references, however, are incorporated in the references at the end of the thesis.

SECTION 1

The non-specific binding of Cyanocobalamin in normal human mixed saliva, and in parotid saliva at varying flow rates.

J. Thomson, J.F. Adams, Elizabeth H. Kennedy, D.K. Mason and Fiona C. McEwan.

Department of Medicine, Southern General Hospital, Glasgow.

Published in Clinical Science (1969). 36. 401.

SUMMARY

1. The cyanocobalamin binding capacity of normal human mixed and parotid saliva has been studied using (^{57}Co)cyanocobalamin and albumin-coated charcoal. It is confirmed that this binding is not due to intrinsic factor but to a non-specific binder.

2. A large variation in binding capacity was found in apparently normal persons.

3. No definite pattern was obtained at levels of binding at different times throughout the day, nor from day to day.

4. Levels were studied in parotid saliva at different flow rates. This showed that the resting binding power was usually the highest and any stimulation produced a sharp fall, but thereafter, despite further stimulation, the level remained almost constant.

Human saliva was shown by Castle, Townsend & Heath (1930) to lack intrinsic factor activity by which is now meant the capacity to promote the absorption of vitamin B_{12}. Beerstecher & Altgelt (1951), however, showed that saliva could bind vitamin B_{12}, a feature in common with intrinsic factor, and suggested that the capacity to bind vitamin B_{12} was in some way related to intrinsic factor capacity. It is now clear that this is not the case (Györgyi et al., 1954; Grasbeck, 1956; Jeffries & Sleisenger, 1963; Simons et al., 1964; England, Ashworth & Taylor, 1967) and the term non-specific binder has been used to

describe binders which do not possess intrinsic factor activity.

The binding capacity of saliva for vitamin B_{12} was studied by Beerstecher & Altgelt (1951) using a biological assay and by Bertcher, Meyer & Miller (1958) using a dialysis method. Both groups found a wide range of normality but differed about the variations found in individuals with time. In view of the doubt about the role of salivary binding in the absorption of vitamin B_{12} we reinvestigated these and other aspects of salivary binding.

MATERIALS AND METHODS

Saliva was obtained from healthy medical or paramedical personnel with good dental hygiene and their smoking habits were noted. Mixed saliva was collected after expectoration in polystyrene containers. Parotid saliva was obtained by cannulation as described by Kerr (1961), the salivary flow for each collection period being recorded by the method of Mason, Harden & Alexander (1966) in order to ensure that the pattern of flow was constant and regular. Samples were stored at -20°.

The binding capacity was measured by the method of Gottlieb et al. (1965). The principle of the method is that cyanocobalamin is adsorbed by albumin-coated charcoal whereas cyanocobalamin bound to intrinsic factor or non-specific binders is not adsorbed. The separation of free and bound fractions in a solution can thus be achieved by addition of albumin-coated charcoal and centrifug-

-ation. Binding of cyanocobalamin by intrinsic factor can be prevented by the addition of serum containing antibody to intrinsic factor and a measure of the non-specific binding capacity can be obtained from the binding capacity estimated with and without antibody. The exact method used was as follows.

In a polystyrene tube of capacity 10.0 ml were pipetted sequentially 2.0 ml 0.9% saline, 0.1 ml saliva and 0.1 ml serum containing antibody to intrinsic factor. After mixing by inversion thrice and incubation at room temperature for 10 min, 2.0 ml (^{57}Co)cyanocobalamin (7.5 ng/ml) were added, the contents mixed and incubated for a further 10 min. Albumin-coated charcoal solution 2.0 ml was then added and after further mixing and incubation for 10 min the tubes were centrifuged at approximately 2000 g for 15 min. The supernatant was decanted and counted in a well-type scintillation counter. The values obtained were compared to those obtained from standards containing 4.2 ml saline and 2.0 ml (^{57}Co)cyanocobalamin. The efficacy of the charcoal solution was evaluated by control tubes containing 2.2 ml saline, 2.0 ml (^{57}Co)cyanocobalamin and 2.0 ml albumin-coated charcoal. Estimations were made in duplicate and the mean values expressed as ng cyanocobalamin bound by 1.0 ml saliva.

Materials used

(^{57}Co)cyanocobalamin was obtained from the Radiochemical Centre, Amersham, and was diluted to a concentration of 7.5 ng/ml with distilled water and stored at +4°. The chemical purity was checked by column chromatography using carboxymethyl cellulose and diethyl aminoethyl cellulose (Kennedy & Adams, 1965).

Serum containing antibody blocking the binding of cyanocobalamin and intrinsic factor was obtained from patients with pernicious anaemia the material used being from a pool of serum from six patients. The pooled material, stored at -20°, was effective in reducing the uptake of cyanocobalamin by 0.1 ml pooled neutralized human gastric juice from 40 ng/ml to 15 ng/ml.

Albumin-coated charcoal was prepared by mixing equal volumes of freshly prepared 5% activated charcoal (Norit OL) in distilled water and 1% bovine serum albumin (Armour Ltd) in distilled water. The albumin-coated charcoal solution was stored at +4° and discarded 3 weeks after preparation.

Microbiological assay

Microbiological assays of saliva for vitamin B_{12} were performed by the method of Hutner, Bach & Ross (1956) using commercially available assay medium (Difco Ltd) and Euglena gracilis z strain as the test organism.

The variation in results from a single assay on one occasion and on repeated assays at weekly intervals, the effect of storage on binding capacity and the difference in binding capacity

resulting from centrifugation were studied in two experiments. In both, mixed saliva was collected, one half dispensed in 2 ml aliquots and stored and the other half centrifuged and the supernatant dispensed in 2 ml aliquots and stored. The main studies encompassed the binding capacity of random samples of uncentrifuged mixed saliva, the diurnal, and day-to-day variations in binding capacity of mixed saliva, the capacity of parotid saliva and the effect of stimulation of flow on the binding capacity of parotid saliva.

RESULTS

No microbiological activity was detected in any sample of mixed or parotid saliva.

In no sample of saliva was the binding capacity affected by the addition to the system of serum containing antibody to intrinsic factor binding.

The variation in results for a single assay (intra assay variation) was small (Table 1, page 195). The mean binding capacity of six aliquots of one sample of uncentrifuged mixed saliva assayed together was 39.36 ng/ml and the standard deviation 1.1 ng/ml. The mean binding capacity of six aliquots of the same saliva centrifuged prior to dispensing was 35.88 ng/ml and the standard deviation 0.2 ng/ml. The mean values differ significantly ($P<0.001$).

The variation in results from assays at weekly intervals (inter assay variation) was greater (Table 2, page 195). The mean

TABLE 1. Differences in the non-specific binding capacity of cyanocobalamin by human mixed saliva caused by (a) centrifugation, (b) intra assay variation (aliquots all obtained from one sample of saliva and estimations all performed in the course of one experiment)

Centrifuged saliva		Uncentrifuged saliva	
Estimation	Non-specific binding capacity of B_{12} (ng/ml)	Estimation	Non-specific binding capacity of B_{12} (ng/ml)
1	36·4	1	40·1
2	37·0	2	39·5
3	33·9	3	40·1
4	36·6	4	38·4
5	35·4	5	31·3
6	36·0	6	38·8
Mean	35·88	Mean	39·36
SD	0·2	SD	1·1

TABLE 2. Differences in the non-specific binding capacity of cyanocobalamin by human mixed saliva caused by (a) centrifugation, (b) inter assay variation (aliquots all obtained from one sample of saliva and estimations performed at weekly intervals)

Centrifuged saliva		Uncentrifuged saliva	
Estimation	Non-specific binding capacity of B_{12} (ng/ml)	Estimation	Non-specific binding capacity of B_{12} (ng/ml)
1	50·3	1	51·6
2	53·6	2	46·8
3	45·3	3	41·5
4	38·3	4	43·7
5	45·8	5	55·2
6	47·9	6	47·2
Mean	46·86	Mean	46·00
SD	5·2	SD	3·4

binding capacity of six aliquots of another sample of uncentrifuged mixed saliva was 46.00 ng/ml and the standard deviation 3.4 ng/ml: the mean binding capacity of six aliquots of the same sample centrifuged prior to dispensing was 46.86 ng/ml and the standard deviation 5.2 ng/ml.

No trend to an increase or decrease in binding capacity was found in the results for the same samples assayed at weekly intervals for 6 weeks.

The binding capacity of random samples of uncentrifuged mixed saliva from ten subjects ranged from 33.4 to 101.4 ng/ml, the mean being 56.9 ng/ml (Table 3, page 197). No relation to smoking habits was noted. In five subjects the day-to-day variation in binding capacity of uncentrifuged mixed saliva was evaluated by assaying, in the same bath, samples obtained at the same time each day. The results (Table 4, page 197) show a marked individual variation, the percentage variation for the mean ranging from -7% to +5% in one case to -26% to +24% in another.

The diurnal variation was studied in five subjects on one occasion and in two of these on two occasions. The results (Fig. 1, page 198) again show marked individual variations.

The binding capacity of parotid saliva at various rates of flow was studied in eleven subjects on one occasion and in six of these on three occasions. In the majority of cases the capacity fell with an increase in flow rate and the pattern was reproducible from day to day (Fig. 2, page 199).

TABLE 3. Non-specific binding of cyanocobalamin by random samples of mixed saliva

Subject	Sex	Age (yr)	Smoker	Non-specific binding capacity of B_{12} (ng/ml)
E.H.K.	F	25	No	34·4
F.F.	F	35	No	66·2
M.C.	F	26	Yes	101·4
M.S.	F	25	Yes	75·4
S.S.	F	25	Yes	72·8
J.F.A.	M	40	Yes	43·2
P.McG.	M	28	No	33·4
D.J.McN.	M	29	No	43·4
G.L.	M	25	No	62·8
T.D.	M	26	Yes	36·0

TABLE 4. Non-specific binding of cyanocobalamin by mixed saliva obtained at the same time on consecutive days

Subject	Sex	Age (yr)	Smoker	Non-specific binding capacity of B_{12} (ng/ml)			
				Day 1	Day 2	Day 3	Day 4
T.D.	M	26	Yes	49·6	83·5	73·2	63·1
P.McG.	M	28	No	32·7	40·0	36·5	41·4
L.E.B.	M	24	?	68·5	65·5	61·7	57·4
E.H.K.	F	25	No	40·6	27·3	32·7	35·6
F.F.	F	35	No	48·6	54·9	52·2	—

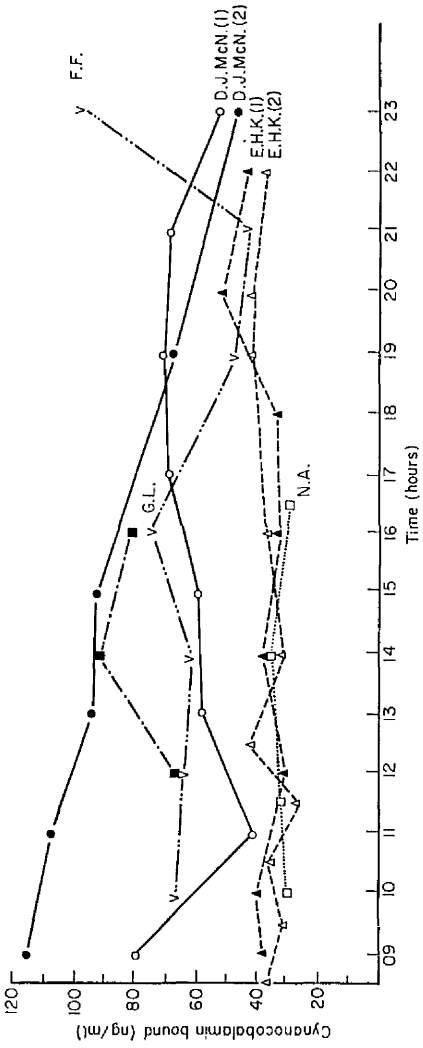


FIG. 1. Variations in the non-specific cyanocobalamin binding power of human mixed saliva throughout the day.

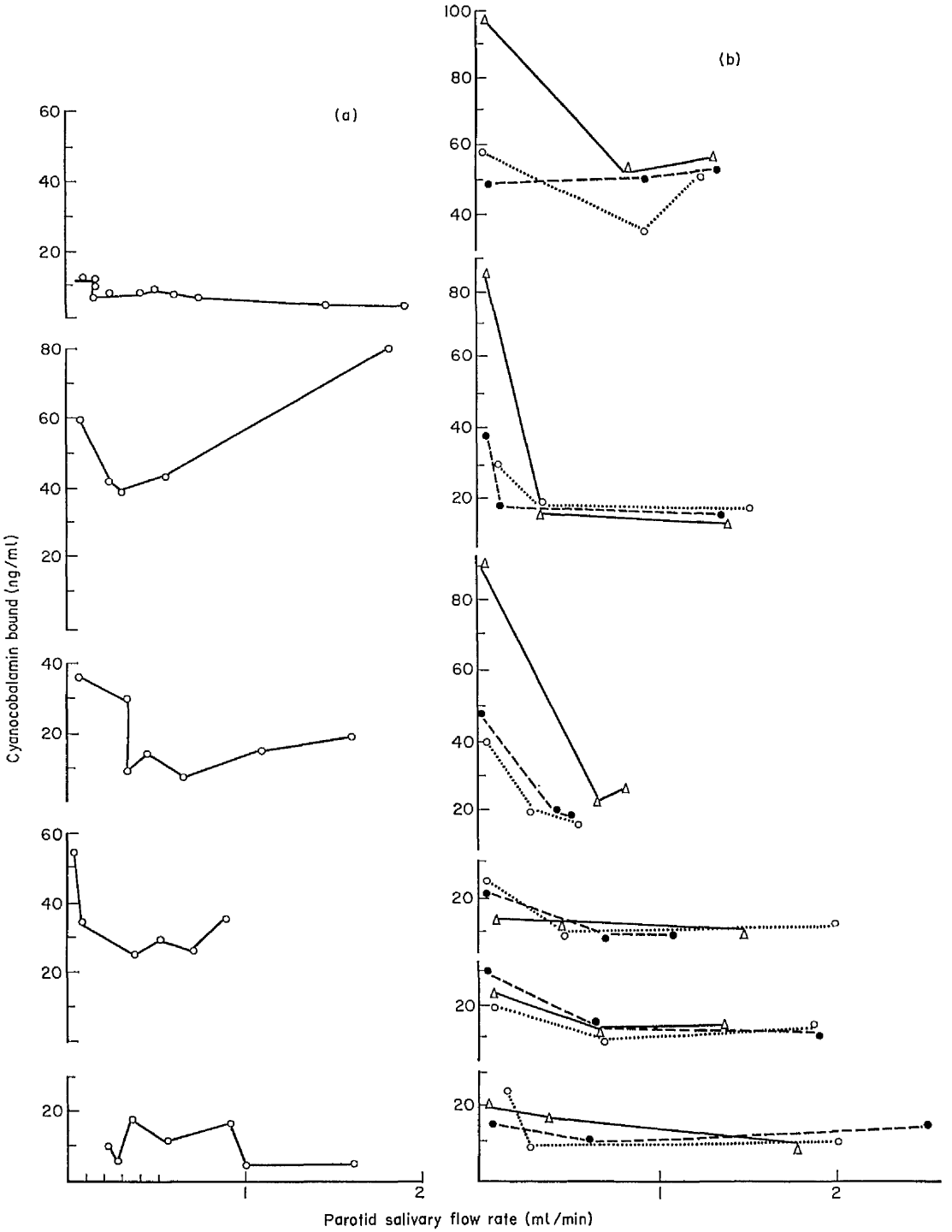


FIG. 2. The non-specific binding of cyanocobalamin by human parotid saliva at different flow rates (a) on one occasion in each of five subjects and (b) on three occasions in each of six subjects.

DISCUSSION

The range of binding capacity of mixed saliva in our subjects is greater than that found by Beerstecher & Altgelt (1951) or by Bertcher et al. (1958) but our mean value corresponds closely to the mean of 55.5 ng/ml found by the former although higher than the 28.5 reported by the latter.

The capacity of mixed saliva is remarkable in relation to the absorption of B_{12}. In theory mixed saliva could bind the free fraction of the B_{12} in food, which amounts to about 20% (Heyssel et al., 1966; Reizenstein, 1959; Adams et al., 1968) and render this fraction unavailable to intrinsic factor. In addition swallowed saliva could compete with intrinsic factor in the stomach for the B_{12} liberated from food in the process of digestion.

The volume of saliva secreted each day ranges up to 1500 ml (Gamble, 1954) and with a mean binding capacity of that found in random samples, this daily output would have a capacity to bind 83.25 µg of B_{12}, amounts which are not only in excess of the daily need which is unlikely to amount to more than 10 µg (Adams & Boddy, 1968; Boddy & Adams, 1968) but are greater than the average amounts usually present in a normal high cost daily diet (31.6 µg B_{12} activity) (Chung et al., 1961).

Several factors may limit this potentially harmful effect of saliva on vitamin B_{12} status. Firstly, a mechanism for the destruction of salivary binder in gastric juice was postulated by Beerstecher & Altgelt (1951) but this, if present, is incomplete

as shown by the recovery of salivary binder from gastric juice by Simons (1964). Secondly, mechanisms by which ingested cobalamins are bound preferentially by intrinsic factor in the presence of non-specific binders, or by which cobalamins bound to non-specific binders are removed and bound by intrinsic factor. We are not aware of unequivocal evidence that in vivo binding of cobalamins by saliva does, or does not, interfere with the uptake of cobalamins by intrinsic factor. However, it is relevant to mention that we have recently observed in a patient with pernicious anaemia that absorption of charcoal bound cyanocobalamin, given immediately before hog intrinsic factor, was only slightly less than when the cobalamin was given with the hog intrinsic factor, suggesting that transfer of at least some cyanocobalamin from charcoal to hog intrinsic factor occurred in vivo. A third factor which might be relevant is the amount of intrinsic factor secreted daily. This is grossly in excess of the amount required to ensure absorption of the daily needs of vitamin B_{12} (Ardeman, Chanarin & Doyle, 1964; Rødbro, Christiansen & Schwartz, 1965; Irvine, 1965, 1966), and it is possible that this excess may be dictated by a need to swamp the competition for cobalamins by non-specific binding. A final factor may be the marked reduction in binding capacity of saliva when parotid salivary flow is stimulated. This response was remarkably reproducible from day to day in subjects tested on repeated occasions. It may be a physiological defence mechanism which has the purpose of minimizing the potentially harmful effect caused by secretion of

large amounts of non-specific binder. The mechanism of this reduction in binding capacity associated with increased flow rate may be simple dilution, but in the absence of a good marker it has not been possible to study this point.

ACKNOWLEDGEMENTS

We are indebted to Professor T. Ferguson Rodger for laboratory facilities in his department and to Miss E. Inglis and Mr. K. Stephen for their help in collecting the parotid saliva. Two of us, J.F.A. and E.H.K., are in receipt of a grant from the Scottish Hospitals Endowment and Research Trust.

SECTION 2

Estimation of the total body vitamin B_{12} in the live subject.

J.F. Adams, H.I. Tenkel and Fiona C. McEwan.

Departments of Medicine and Surgery, Southern General Hospital, Glasgow.

Published in Clinical Science (1970). 39. 107.

SUMMARY

1. Values for total body vitamin B_{12} were calculated for eighteen patients by giving a tracer dose of radioactive cyanocobalamin and measuring the radioactivity and microbiological activity in liver biopsies obtained at laparotomy.

2. The values for total body vitamin B_{12} ranged from 960 to 5984 µg with a mean of 2528 µg. Five values were greater and thirteen less than the mean.

3. Significant correlations were found between the serum vitamin B_{12} and total body vitamin B_{12}, the serum vitamin B_{12} and the hepatic vitamin B_{12} and the hepatic B_{12} and the total body vitamin B_{12}.

INTRODUCTION

Work published to date on the vitamin B_{12} content of the body relies mainly on calculations based on microbiological analysis of tissues obtained post mortem from patients with a wide variety of diseases. Mean values of 2689 µg, 3900 µg and 5000 µg with ranges of 1634 to 3475 µg, 790 to 11,100 µg and 3480 to 10,950 µg have been suggested by Kurlov (1961, 1962), Grasbeck, Nyberg & Reizenstein (1958) and Heinrich (1964) respectively, and Adams (1962) reported a mean of 2221 µg with a range of 953-4304 µg from a combined isotope dilution and microbiological assay procedure.

The only report on the vitamin B_{12} content of the body in the living subject is one by Reizenstein, Ek & Matthews (1966) in which an average value of 3030 µg was obtained by kinetic

analysis of values for whole body retention, faecal excretion and plasma clearance after parenteral radioactive vitamin B_{12}. We are not aware of any other estimates of total body vitamin B_{12} in the living subject and so we report here our experience with a procedure by which approximate values were obtained by relatively simple methods in this situation. Although limited in scope and application we believe it has a place in studies of vitamin B_{12} metabolism in man.

MATERIALS AND METHODS

The basic principle is that of isotope dilution which assumes that, at a finite time after administration, a tracer dose of radioactive material is distributed throughout the body in proportion to the endogenous non-radioactive material. Previous work suggests that equilibration of an intravenously administered dose of 100 ng radioactive cyanocobalamin with body stores of vitamin B_{12} occurs 5 to 10 days after injection and that the loss of radioactive material from the body between the time of injection and equilibration is of the order of 5% (Adams & Boddy, 1968). Thus the total amount of vitamin B_{12} in the body can be calculated by measurement of radioactivity and vitamin B_{12} content of a suitable tissue obtained after equilibration and by allowing for the loss of radioactive material from the body.

On this basis patients referred for elective surgery were appraised of the nature of the study and, after a sample of blood

had been obtained for serum vitamin B_{12} estimation, were given 50 ng 5.0 µCi (^{57}Co)cyanocobalamin in 5.0 ml water intravenously. At operation, which was performed 6-29 days later, a wedge of liver was obtained, usually from the antero-inferior aspect of the left lobe, and haemostasis secured by sutures before the major procedure. The tissue was weighed in a Stanton analytical balance and stored at -20° if necessary before homogenization with water in a Potter Elvehjm or Silverson microhomogenizer to a volume of 20 ml. The activity was measured in a well-type scintillation counter IDL type 663 with a thallium activated sodium iodide crystal, 5.5 cm. diameter and 6.9 cm. deep, shielded by 10.0 cm. lead and connected to an IDL 1700 automatic scaler using three standards each containing 1.0% of the dose in 20 ml water. At least 10,000 counts were obtained from every sample. The homogenate was then further homogenized with water in a Waring Blender to suitable dilutions for microbiological assay by the method of Hutner, Bach & Ross (1956) using Euglena gracilis Z strain as the test organism, and commercially available medium (Difco Laboratories Inc.). The homogenate and serum sample from each patient were assayed together on at least three occasions.

(^{57}Co)cyanocobalamin was obtained from the Radiochemical Centre, Amersham, two batches being used in the study. The material was dissolved in distilled water and dispensed in 5 ml aliquots in dark glass ampoules after sterilization by Millipore filtration. The ampoules were stored at +4°. Before use of a

batch an ampoule was taken at random and the purity of the solution tested by column chromatography using carboxy-methyl-cellulose and diethyl-amino-ethyl-cellulose. The final ampoule in each batch was tested similarly. The rationale of these procedures, described in detail by Kennedy & Adams (1965) & Kennedy (1967) is that cyanocobalamin, being neutral, is not retained by either material whereas hydroxocobalamin, which results from photolysis of cyanocobalamin (Smith, 1965), is retained by the cation exchanger carboxy-methyl-cellulose and the 'red acids', which result from radiochemical decomposition of cyanocobalamin (Smith, 1965), are retained by the anion exchanger diethyl-amino-ethyl-cellulose.

RESULTS

Eighteen patients were studied, the relevant information on each being given with the essential results in Table 3 (page 208). There was no morbidity in the series. There was no evidence, by the methods used, that the (^{57}Co)cyanocobalamin solutions tested had deteriorated during storage.

The amount of radioactivity in the liver biopsies ranged from 0.0535 to 0.3832% of the dose and the microbiological activity expressed as cyanocobalamin ranged from 0.9058 to 7.7263 µg per biopsy and from 0.41 to 1.75 µg/g tissue, mean 1.06 µg/g. The total body vitamin B_{12} values were calculated from the values for radioactivity and vitamin B_{12} in the biopsies assuming retention of 95% of the dose and ranged from 960 to 5984 µg

TABLE 1. Showing details of patients and essential results

Case	Age	Sex	Serum B_{12} (pg/ml)	Disease and operation	Time interval between injection and biopsy (days)	Mass (g)	Liver biopsy activity (% dose)	Assayable B_{12} (μg)	Calculated 'Total Body B_{12}' (μg)
1	20	M	463	Duodenal ulcer—partial gastrectomy	29	3·7210	0·2389	6·1305	2438
2	47	F	—	Gastric ulcer—partial gastrectomy	25	5·4507	0·3832	6·7689	1678
3	59	M	433	Gallstones—cholecystectomy	18	4·1903	0·1979	3·8255	1836
4	67	M	191	Gallstones—cholecystectomy	22	5·0027	0·2184	3·1650	1377
5	74	F	511	Gallstones—cholecystectomy	6	4·4167	0·2867	7·7263	2560
6	64	M	490	Gallstones—cholecystectomy	11	2·0411	0·1017	1·0526	983
7	57	M	444	Duodenal ulcer—gastroenterostomy and vagotomy	15	2·5576	0·1247	2·0680	1575
8	53	M	353	Gallstones—cholecystectomy	14	2·3975	0·0644	1·6156	2383
9	41	F	403	Duodenal ulcer—pyloroplasty and vagotomy	14	3·0041	0·1245	2·5530	1948
10	33	M	540	Duodenal ulcer—pyloroplasty and vagotomy	10	4·1765	0·1878	3·9050	1975
11	46	M	535	Duodenal ulcer—gastroenterostomy and vagotomy	14	2·2592	0·0634	2·1439	3212
12	52	F	617	Cholecystitis—cholecystectomy	15	3·9550	0·1812	4·5601	2391
13	26	M	670	Duodenal ulcer—gastroenterostomy and vagotomy	18	2·7906	0·0715	4·5040	5984
14	54	F	585	Gallstones—cholecystectomy	22	2·4200	0·0874	1·8972	2062
15	43	F	286	Crohn's disease—resection of intestinal stricture	20	2·2201	0·0896	0·9058	960
16	52	M	607	Duodenal ulcer—pyloroplasty and vagotomy	29	2·2922	0·0535	3·3145	5886
17	56	M	333	Gastric ulcer—partial gastrectomy	28	2·8689	0·1029	3·3508	3093
18	37	M	350	Cholecystitis—cholecystectomy	24	2·1527	0·0541	1·8018	3164

with a mean of 2528 µg; five values were greater than the mean and thirteen less than the mean. Serum vitamin B_{12} values were estimated in seventeen subjects (collection of the sample from one patient having been omitted by accident) and ranged from 191 to 670 pg/ml, mean 459 pg/ml.

Significant correlations were found between the values for serum vitamin B_{12} in pg/ml (x) and total body vitamin B_{12} in µg (y) in seventeen patients the regression equation being:

$$y = 6.0634\ x - 207.8946\ (r\ 0.54;\ P<0.05)$$

and between the values for serum vitamin B_{12} in pg/ml (x) and hepatic vitamin B_{12} in µg/g of tissue (y) in seventeen patients the regression equation being:

$$y = 0.001674\ x + 0.2357\ (r\ 0.53;\ P<0.05)$$

and between the hepatic vitamin B_{12} in µg/g tissue (x) and the total body vitamin B_{12} in µg (y) in eighteen patients the regression equation being:

$$y = 2250.4866\ x - 237.0602\ (r\ 0.63;\ P<0.01)$$

DISCUSSION

The validity of the results depends on three premises. First, that the (^{57}Co)cyanocobalamin had equilibrated with the non-radioactive vitamin B_{12} in the body when the liver biopsy was obtained. Second, that the microbiological assay procedure using cyanocobalamin standards measured the liver vitamin B_{12}. Finally that the use of an arbitrary value, in this case 95%, for dose of tracer retained in the body at the time of the liver

biopsy is acceptable for calculation of total body vitamin B_{12} values.

Probably the most contentious of these premises is the first. There is controversy as to whether orally or parenterally administered radioactive vitamin B_{12} does, or does not, equilibrate with the non-radioactive stores in the body. Reizenstein, Matthews & Ek (1964), Reizenstein, Ek & Matthews (1966) and Schiffer, Cohn, Price & Cronkite, (1968) do not consider that equilibration occurs within a finite time and the first two groups of workers link this opinion with the concept of at least three pools in the body. Heinrich (1964) while also favouring a multipool system accepts that equilibration occurs but only after 240-300 days after administration of radioactive vitamin B_{12}. Others have concluded from studies in animals that equilibration occurs more rapidly (Cooperman, Lubby, Teller & Marley, 1960: Grasbeck, Ignatius, Jarnefelt, Linden, Mali & Nyberg, 1961; Newman, O'Brien, Spray & Witts, 1962) and similar conclusions have been reached from studies in men (Bozian, Ferguson, Heyssel, Menoely & Darby, 1963; Heyssel, Bozian, Darby & Bell, 1966; Boddy & Adams, 1968; Adams & Boddy, 1968). With the exception of the time scale suggested by Heinrich (1964) the differences of opinion about the time taken for equilibration are less contentious being related, in part at least, to the mass of the dose given and possibly also to the species studied. In humans given 5000 µg doses parenterally the loss of whole body radioactivity did not occur

at a steady rate, which was taken as evidence of equilibration, for several weeks (Boddy & Adams, 1968), whereas with doses of 100 ng parenterally the rate of loss was steady after 5-10 days (Adams & Boddy, 1968).

We feel that the measurement of liver vitamin B_{12}, which is mainly coenzyme B_{12} (Toohey & Barker, 1961; Stahlberg, Radner & Norden, 1967), by microbiological assay using cyanocobalamin standards is acceptable, our opinions being based on the results of recovery studies with coenzyme B_{12} added to liver homogenates in vitro. The values for vitamin B_{12} are greater than those found by Pitney, Beard & Van Loon (1955) and Jhala & Gadgil (1960) but are comparable to those reported by Blum & Heinrich (1957), Ross & Mollin (1957), Pitney & Onesti (1961), Adams (1962), Joske (1963), Anderson (1965) and Stahlberg et al. (1967) also using Euglena gracilis as the test organism.

The use of an arbitrary value for the proportion of dose retained in the body at the time of liver biopsy was based on data reported by Adams & Boddy (1968). In this study the loss of whole body radioactivity by normal subjects given 100 ng (^{57}Co)cyanocobalamin intravenously was initially rapid but after 5-10 days, by which time about 5% had been lost, settled to a rate of 0.1-0.2% per day. We felt, therefore, that a suitable overall allowance for loss in the time between administration of the smaller dose used in this study and liver biopsy would be 5%. The ideal procedure, of course, would be to use a whole body monitor to obtain a 100% value after administration of the tracer dose and to repeat the measurement on the day of operation

to obtain a value for retained dose. Consideration of the values involved, however, makes it doubtful if the results would be materially affected by such a procedure even given a monitor with the sensitivity and performance required in such a situation. In view of the doubts, however small, which must always attend the use of arbitrary values we regard it as prudent to regard the estimates of total body vitamin B_{12} obtained by this method as approximate values.

Of the correlations between serum, hepatic and total body vitamin B_{12} which we report, two are novel and one, that between serum and hepatic vitamin B_{12}, complements the finding by Anderson (1965) of a correlation between these measurements in a large series of vitamin B_{12} deficient patients and the circumstantial evidence for a relationship adduced by Chanarin (1969) from selected data on vitamin B_{12} deficient and vitamin B_{12} replete subjects studied by Joske (1963), Anderson (1965) and Ståhlberg et al. (1967). In his own results Joske (1963) did not find a correlation probably because, as he states, the results were obtained mainly from patients with parenchymal liver disease which may upset the balance between the serum and hepatic vitamin B_{12}. We think it unwise at present to draw any conclusions from the correlations other than the general consideration that the serum vitamin B_{12} per unit of volume, the hepatic vitamin B_{12} per unit of mass and the total body vitamin B_{12} would appear to be related to each other.

Whether the values for total body vitamin B_{12} we report can be regarded as representative of normality is conjectural. Certainly we have doubts about regarding a patient with Crohn's disease as normal from the point of view of vitamin B_{12} metabolism and the fact that patients with duodenal ulcers have a higher than normal output of intrinsic factor in response to histamine (Rødbro, Christiansen & Schwartz, 1965) might raise doubts about this group. In this connection it may be relevant to note that the mean total body vitamin B_{12} for the seven patients with duodenal ulcers was 3288 µg and for the six patients with gall stones was 1867 µg. These values are significantly different when analysed by the Mann-Whitney 2 tail test ($P = 0.014$) but the mean value for the ulcer patients is heavily loaded by inclusion of two very high results and when these are excluded the significance disappears ($P = 0.53$). Within these limitations and the obvious problems associated with the study of normal subjects we feel that the results at least provide material of relevance and interest in studies of vitamin B_{12} metabolism in man.

ACKNOWLEDGEMENTS

J.F.A. and F.McE. acknowledge with thanks grants from the Scottish Hospital Endowment Research Trust on the advice of the Advisory Committee for Medical Research.

SECTION 3

Activities of various cobalamins for Euglena gracilis with reference to vitamin B_{12} assay with Euglena.

J.F. Adams and Fiona C. McEwan.

Published in The Journal of Clinical Pathology(1971). 24. 15.

SYNOPSIS

Coenzyme B_{12} and methylcobalamin in water are less active in promoting growth of Euglena gracilis Z strain than the same concentrations of cyanocobalamin and hydroxocobalamin which are equally active. When bound to human serum or human liver homogenate, however, the activities of these four cobalamins do not differ significantly with one exception. The results suggest that the Euglena assay using cyanocobalamin standards is not satisfactory for quantitation of coenzyme B_{12} and methylcobalamin in water but acceptable when coenzyme B_{12} and methylcobalamin are bound to serum or liver. Sulphitocobalamin in water is as active as cyanocobalamin and hydroxocobalamin but nitritocobalamin is less active. Factor B, the monocarboxylic acids of cyanocobalamin and hydroxocobalamin, and the dicarboxylic acid of cyanocobalamin in water were inactive.

INTRODUCTION

The measurement of the 'vitamin B_{12}' concentration in tissues by microbiological assay using Euglena gracilis is a widely used procedure. Euglena gracilis variant bacillaris was used by Hutner, Provasoli, Stokstad, Hoffman, Belt, Franklin, and Jukes (1949) and by Ross (1950 and 1952) who described techniques applicable to clinical material and the Z strain was used by Hutner, Bach, and Ross (1956). Previous studies of the growth-promoting effects of cobalamins and other substances for Euglena gracilis, summarized by Smith (1965), preceded the discovery of the roles of

methylcobalamin and coenzyme B_{12} in human metabolism (Toohey and Barker, 1961; Lindstrand and Stålhlberg, 1963; Stålhlberg, Radner, and Norden, 1967), and as the activity of these cobalamins and certain analogues for Euglena gracilis Z strain had not been studied, it seemed desirable to do so.

MATERIALS AND METHODS

In all studies the assay methodology was essentially that of Hutner et al. (1956) using Euglena gracilis Z strain and commercially available medium (Difco Laboratories Inc.). The incubation period was five days at a bath temperature of 29°C and illumination by two 'warm white' 30 watt fluorescent tubes. Samples were assayed in triplicate and the optical density of the cultures was measured in a Unicam SP 300 photometer using an Ilford 204 filter and cells with an optical path of 2.5 mm.

Solutions of cyanocobalamin, hydroxocobalamin, coenzyme B_{12}, methylcobalamin, sulphitocobalamin, nitritocobalamin, factor B, and the 'red acids' - the monocarboxylic acid of cyanocobalamin, the monocarboxylic acid of hydroxocobalamin, and the dicarboxylic acid of cyanocobalamin - were made available in known concentration by Dr. L. Mervyn of Glaxo Ltd. Various batches of cyanocobalamin, hydroxocobalamin, methylcobalamin, and coenzyme B_{12} were used, the concentrations being confirmed by absorption spectrometry with a Unicam SP 800 spectrophotometer. Unless otherwise stated, solutions were stored at +4°C and all manipulations except those immediately preceding microbiological assay were carried out in a dim red light.

Initially all samples were assayed on at least three occasions in final concentrations of 1.25, 2.5, 5, 10, 15, 25, and 50 µµg/ml and the values compared to those obtained with cyanocobalamin at the same final concentrations. In view of the results further studies were undertaken as follows: (1) the effect of increasing concentrations of factor B and the 'red acids'; (2) repeated assays of aqueous solutions of coenzyme B_{12} and methylcobalamin in concentrations of 750 µµg/ml and 375 µµg/ml; and (3) repeated assays of cyanocobalamin, hydroxocobalamin, methylcobalamin, and coenzyme B_{12} in aqueous solution and bound to pools of serum and human liver homogenate. For the last-named study materials were prepared in a dim red light at 17°C by adding 4,000 µµg cobalamin in 1 ml water to 9 ml pooled tissue, and after mixing for 15 min, dispensing in aliquots of 1.25 ml in plastic tubes; dilutions of the same batch of cyanocobalamin used in the preparation of test samples were prepared for use as standard at the same time. Batches of standards, blanks, and test samples were then incubated at 17°C for 12 hours, exposed to daylight for 20 minutes, and then stored at -20°C in the dark until assayed. Each batch was assayed once and then discarded. Assays were performed at intervals of one week. The test samples were assayed at dilutions of 1:20 and the activity was compared to that of the cyanocobalamin standards which, after final dilution at 1.20, had values of 2.5, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, and 60 µµg/ml. The serum pool was prepared from blood obtained from eight healthy subjects and was stored in glassware at -20°C. The liver homogenate pool

was prepared from equal volumes of liver homogenates (1 g per 1,000 ml), the tissues being obtained at necropsy on eight patients who had died of cardiac or cerebrovascular disease and had not received antibiotics.

RESULTS

The activities of aqueous solutions of hydroxocobalamin and sulphitocobalamin were always the same as those of similar concentrations of cyanocobalamin in the range 1.25 to 50 µµg/ml as judged by the optical densities of the cultures. Methylcobalamin, coenzyme B_{12}, and nitritocobalamin were always less active than corresponding concentrations of cyanocobalamin in the range 1.25 to 50 µµg/ml and their activities were about 65% that of cyanocobalamin.

Factor B and the red acids were inactive at low concentrations. At a concentration of 10,000 µµg/ml factor B had an activity equal to cyanocobalamin 10 µµg/ml and the monocarboxylic acid of cyanocobalamin at 100,000 µµg/ml was as active as cyanocobalamin 50 µµg/ml. The monocarboxylic acid of hydroxocobalamin and the dicarboxylic acid of cyanocobalamin were inactive at concentrations up to and including 200,000 µµg/ml.

An aqueous solution of coenzyme B_{12} 750 µµg/ml assayed on six occasions gave a mean value of 483 µµg/ml (standard deviation 81.4) as expressed from the cyanocobalamin standards and a solution of 375 µµg/ml a value of 256 µµg/ml (standard deviation 65.0) the

relative activities being 64% and 68%. Similarly, six assays of methylcobalamin 750 µµg/ml gave a mean value of 544 µµg/ml (standard deviation 123) an activity of 72% and methylcobalamin 375 µµg/ml a mean value of 232 µµg/ml (standard deviation 96) an activity of 62%.

The results for eight assays of cyanocobalamin, hydroxocobalamin, methylcobalamin, and coenzyme B_{12} in aqueous solution and bound to pooled tissues are summarized in Table 4 (page 220) Analysis of the results by the Wilcoxon test (2 tail) showed significant differences in the results from aqueous solutions of cyanocobalamin and coenzyme B_{12} ($P<0.01$), cyanocobalamin and methylcobalamin ($P<0.01$), hydroxocobalamin and coenzyme B_{12} ($P<0.01$), hydroxocobalamin and methylcobalamin ($P<0.01$), and in the results from serum-bound hydroxocobalamin and serum-bound coenzyme B_{12} ($P<0.02$).

DISCUSSION

The most interesting results are those relating to the 'physiological cobalamins' - coenzyme B_{12}, methylcobalamin, hydroxocobalamin and cyanocobalamin. Photolysis of coenzyme B_{12} and methylcobalamin leads to the formation of hydroxocobalamin which was found by Robbins, Hervey, and Stebbins (1950) to be as active as cyanocobalamin for Euglena gracilis var. bacillaris and confirmed for the Z strain in this study. The results of assays of several batches of coenzyme B_{12} and methylcobalamin of known, that is spectrophotometric concentration, were therefore unexpected. Further enquiries were indicated because these

Test Sample		*Mean Values and Standard Deviation of Eight Assays ($\mu\mu$g/ml from cyanocobalamin standards)*
Water (9 ml) + water (1 ml)		2·5
Water (9 ml) + cyanocobalamin	(1 ml – 4,000 $\mu\mu$g)	458 (62·5)
Water (9 ml) + hydroxocobalamin	(1 ml – 4,000 $\mu\mu$g)	392 (65·5)
Water (9 ml) + methylcobalamin	(1 ml – 4,000 $\mu\mu$g)	293 (66·2)
Water (9 ml) + coenzyme B_{12}	(1 ml – 4,000 $\mu\mu$g)	313 (47·4)
Pool serum (9 ml)		
Pool serum + water (1 ml)		357 (64·0)
Pool serum + cyanocobalamin	(1 ml – 4,000 $\mu\mu$g)	527 (89·3)
Pool serum + hydroxocobalamin	(1 ml – 4,000 $\mu\mu$g)	538 (108·6)
Pool serum + methylcobalamin	(1 ml – 4,000 $\mu\mu$g)	507 (111·4)
Pool serum + coenzyme B_{12}	(1 ml – 4,000 $\mu\mu$g)	504 (92·9)
Pool liver homogenate (9 ml)		
Pool liver + water (1 ml)		397 (39.3)
Pool liver + cyanocobalamin	(1 ml – 4,000 $\mu\mu$g)	842 (219·2)
Pool liver + hydroxocobalamin	(1 ml – 4,000 $\mu\mu$g)	720 (78·6)
Pool liver + methylcobalamin	(1 ml – 4,000 $\mu\mu$g)	776 (80·0)
Pool liver + coenzyme B_{12}	(1 ml – 4,000 $\mu\mu$g)	710 (95·9)

Table I *Constitution of test samples and mean values and standard deviation from eight assays*[1]

[1]Separate aliquot of each test sample and appropriate cyanocobalamin standards were assayed on each occasion.

results raised the suspicion that values obtained by Euglena assay of tissues were underestimates. Because of the considerable interassay variation (see Ross, Hutner, and Bach, 1957; Anderson, 1964) repetitive assays were necessary. The results formalized statistically the previous finding that coenzyme B_{12} and methylcobalamin in water have an activity which is significantly inferior to similar concentrations of cyanocobalamin and hydroxocobalamin. In addition it is clear that this inferiority is abolished when the cobalamins are bound to serum or liver homogenate, the sole exception being serum-bound coenzyme and serum-bound hydroxocobalamin. Any explanation of the results must take into account the fact that hydroxocobalamin is a product of photolysis of coenzyme B_{12} and methylcobalamin, and this suggests that other products of photolysis act as inhibitors to the growth of Euglena unless tissues are also present. Treatment of coenzyme B_{12} in water with potassium cyanide, before and after photolysis, by prolonged photolysis in aerobic conditions, and by autoclaving at 15 lb for up to 30 minutes did not affect the activity, and attempts at isolating an inhibitor from solutions of photolysed coenzyme B_{12} were unsuccessful.

From the practical aspect it is clear that quantitation of solutions of coenzyme B_{12} and methylcobalamin in water by Euglena assay using cyanocobalamin or hydroxocobalamin standards will result in a significant underestimate of the true concentration. The absence of significant differences, with one exception, between cobalamins bound to serum or liver homogenate does not necessarily mean, however, that quantitation of serum or liver-bound coenzyme B_{12} and methylcobalamin can be achieved with cyanocobalamin or hydroxo-

-cobalamin standards. Taking the mean values for cyanocobalamin or hydroxocobalamin, pool serum, and pool liver homogenate (Table I, page 220) the recovery rates for cyanocobalamin and hydroxocobalamin added to liver are 98% and 91% respectively which give rise to confidence. With pool serum, however, the recovery rates for cyanocobalamin and hydroxocobalamin are 65% and 72%. Such low rates give rise to doubts about the quantitation of serum-bound vitamin B_{12} but may, however, simply be manifestations of the inhibitory effect of serum on Euglena growth (Anderson, 1964).

The use of the conventional terms for concentration of cobalamins, that is mass of solute in volume of solution, was convenient in this study. On occasion this convention may result in misleading results and it is relevant to point out that calculation of the results in terms of molarity does not affect the conclusions.

There is no evidence that nitritocobalamin, factor B, and the three 'red acids' tested play a part in vitamin B_{12} metabolism in man and the results are of little practical interest. We ascribe the trivial activities of the red acids and factor B as due to traces of cyanocobalamin and the results for factor B accord with those found by Coates and Kon (1957) using Euglena gracilis var. bacillaris. It seems inherently more likely that the lack of B activity of factor B and the 'red acids' tested is due to considerable differences in structure than to the presence of inhibitors.

ACKNOWLEDGEMENTS

We are grateful to Dr. L. Mervyn, to Dr. E.H. Hastings, and to Dr. W.S.T. Thomson, for assistance. We acknowledge with thanks grants from the Secretary of State for Scotland and the Scottish Hospital Endowment Research Trust.

SECTION 4

Clinical value of serum vitamin B_{12} estimation in jaundice.

J.F. Adams, T. Rowan, Elizabeth H. Kennedy, and Fiona C. McEwan.

Published in
Digestion (1971). ~~in Press.~~ 4. 289.

A raised serum vitamin B_{12} level has been reported to be a common finding in liver disease and to be of diagnostic and prognostic value in clinical practice. Provoked by results, which did not accord with many published views and which caused anxiety clinically, we reinvestigated the value of serum B_{12} estimation in diagnosis and prognosis in cases of jaundice seen in hospital practice.

MATERIALS AND METHODS

Sera received in the Biochemistry Department and found to have a bilirubin concentration of 1.0 mg/100 ml or more were further analysed for total protein, albumin, alkaline phosphatase and glutamic oxaloacetic transaminase by appropriate Technicon Autoanalyser methods, zinc sulphate turbidity (Kunkel, 1947), thymol turbidity (Maclagen, 1944), glutamic pyruvate transaminase (Boehringer Kit No. 15956 TGAK) and vitamin B_{12} (Hutner et al., 1956).

The data on each patient was reviewed at least a year after the initial sample was obtained and a final diagnosis was established on the basis of all the available information. Cases of chronic myeloid leukaemia and megaloblastic anaemia were excluded in view of the known abnormalities of vitamin B_{12} metabolism in these conditions and cases in which a final diagnosis could not be established with confidence were also excluded.

RESULTS

A total of 152 sets of results from 100 jaundiced patients were included in the final survey. For convenience, 5 main diagnostic categories were recognised hepatitis (27 cases), obstructive (38 cases), cirrhosis (15 cases), cardiac (14 cases), and miscellaneous (6 cases). The highest observed serum vitamin B_{12} level in each patient is shown by diagnostic category, in Figure 1 (page 227) together with values obtained from 100 hospital in-patients with a variety of diseases excluding renal, hepatic and haematological disorders. Abnormally high values, that is values outside the normal range, were found in 5 cases of hepatitis (18.5%), 6 cases of obstructive jaundice (15.7%), 2 cases of cirrhosis (13.3%) and 3 cases of cardiac jaundice (21.4%). A single result below the lower limit of the normal range was obtained from a patient with obstructive jaundice due to gall stones presenting 5 days post partum.

The hepatitis group included infective hepatitis (11 cases), alcoholic hepatitis (7 cases), chronic active hepatitis (3 cases) and homologous serum jaundice, leptospirosis and drug-induced jaundice (2 cases each). There were 31 cases of uncomplicated neoplastic disease in the obstructive group, the origin of the tumour being pancreas (11 cases), bronchus (8 cases), stomach and unidentified (4 cases each), bile duct, gall bladder, ampulla and breast (1 case each): gall stone alone was found in 3 cases

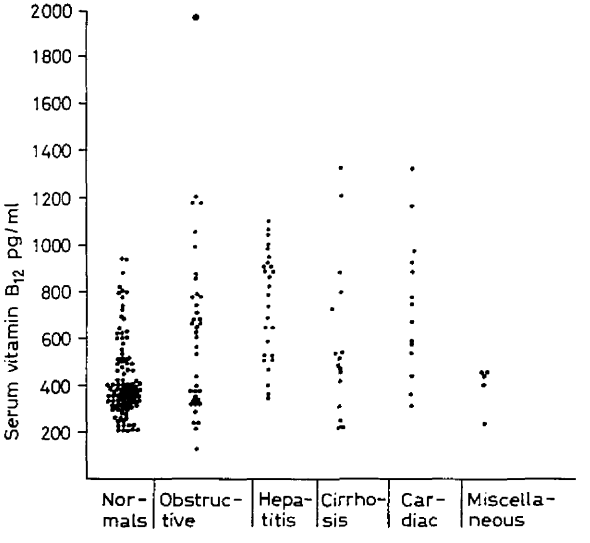


Fig. 1. The highest observed serum vitamin B_{12} level in each patient.

and together with hepatoma, carcinoma of gall bladder, portal cirrhosis and haemolytic anaemia (1 case each). The cirrhosis group consisted of 10 cases of portal cirrhosis and 5 of biliary cirrhosis and the miscellaneous group of haemolytic anaemia (2 cases), Hodgkin's disease, acute leukaemia and chronic lymphatic leukaemia (1 case each).

The cardiac group consisted of patients in chronic congestive cardiac failure due to valvular disease (9 cases), myocardial ischaemia (3 cases) and cor pulmonale (2 cases).

Of the 33 patients with neoplastic disease in the series 29 came to laparotomy and/or necropsy. Of these 29 a primary or secondary growth in the liver was found in 14 and 3 of these had a serum vitamin B_{12} level above the upper limit of the normal range. In 2 cases the primary lesion was in the pancreas and in 1 in the breast. Two other cases in the group of 33 neoplastic cases also had high serum vitamin B_{12} levels, and in both cases the liver was considered to be free of secondary deposits from the primary pancreatic growth at laparotomy; neither case came to necropsy.

Correlations were sought between the serum vitamin B_{12} level and other measurements in the entire series, diagnostic categories and subgroups. The coefficients of correlation reached the 5%-level of significance in the case of the serum vitamin B_{12} level and the serum glutamic oxaloacetic transaminase concentration in patients with infective hepatitis and homologous serum jaundice and the 2%-level of significance in the case of the serum vitamin

B_{12} level and bilirubin concentration in the patients with obstructive jaundice to gall stone, carcinoma of pancreas and carcinoma of the biliary tracts.

DISCUSSION

The results suggest that the distribution of serum vitamin B_{12} values in jaundiced patients differs from normal and shows that abnormally high values were found in roughly equal proportions in the 4 main diagnostic categories. The proportion of abnormally high values both overall and subgroups is smaller than has been reported but this is due, in part, to considerable differences in what is accepted as the upper limit of normality.

The accepted upper limits range from values between 400 and 500 pg/ml (Jones et al., 1957; Rachmilewitz et al., 1956; Rachmilewitz and Eliakim, 1968) through 600-700 pg/ml (Meynell et al., 1957; Stevenson and Beard, 1959) to our own of 940 pg/ml which corresponds closely to that of Neale et al. (1966). This has the result that in portal cirrhosis for example the incidence of abnormally high serum vitamin B_{12} values is about 50%, as judged by the various standards, in the 145 cases studied by Lear et al. (1954), Meynell et al. (1957), Cowling and MacKay (1959), Holdsworth et al. (1964) and Rachmilewitz and Eliakim (1968), whereas only about 20% had values above 940 pg/ml. Similarly,

the very high incidence of abnormally high values in patients with chronic cardiac failure studied by Rachmilewitz et al. (1959) falls to a proportion comparable to that in the present series when judged by the range of normality used in the present series and, conversely, the relatively low incidence of abnormally high values in our cardiac patients rises to a proportion comparable to that found by Rachmilewitz et al. (1959) when judged by their range of normality. It is relevant to assessment of the diagnostic value of the test to point out that a reduction in the upper limit of normality used by us to, say 700 pg/ml to include about 90% of the normal population does not affect the approximately equal proportion of abnormally high values in each main diagnostic category. The problems of delineation of normal and abnormal results, which in practice are further complicated by microbiological assay variation (Ross et al., 1957; Anderson, 1964) do not, however, explain the absence from our results of very high values notably in cases of infective hepatitis. Values between 2,000 and 5,000 pg/ml or even more were found not uncommonly by Cowling and MacKay (1959) and Holdsworth et al. (1964) and frequently by Rachmilewitz and Eliakim (1968). It is known from serial studies in infective hepatitis that the serum vitamin B_{12} level can fall from grosaly abnormal values within a few days (Rachmilewitz et al., 1956; 1958) and that remarkable fluctuations can occur even within hours in carbon tetrachloride poisoned animals (Stein et al., 1956; 1959). Such trends seem more likely

to account for our unusually low incidence of 2 abnormally but modestly, raised serum vitamin B_{12} levels in 11 patients with infective hepatitis and 2 with homologous serum jaundice, than geographical, racial or nutritional factors. Whatever the explanation, two points of practical importance are clear; first that the normal range of values for any test should be defined locally and secondly that an estimate of the frequency of abnormal values should be obtained under similar conditions before the test is applied for diagnostic purposes.

In cases of malignant disease, a raised serum B_{12} level was considered by Grossowicz et al. (1957) to indicate the presence of hepatic metastases. Further experience reported by Cowling and MacKay (1959), Nelson and Doctor (1962), Holdsworth et al. (1964) and Rachmilewitz and Eliakim (1968) has confirmed that this is often the case but that the frequency is not such as to be of diagnostic value. Our results emphasise this point.

In anicteric liver disease there is evidence that a raised serum vitamin B_{12} level is valuable in establishing a diagnosis of intrahepatic infection (Holdsworth et al., 1964; Neale et al., 1966) and there is evidence that serial serum vitamin B_{12} levels may have prognostic value in cases of progressive hepatitis (Holdsworth et al., 1964; Rachmilewitz and Eliakim, 1968). In icteric disease, on the other hand, our results lead us to the conclusion that the test has no value in the management of the individual patient, at least under the conditions in our hospital practice.

ACKNOWLEDGEMENTS

J.F.A., E.H.K. and F.C. McE. acknowledge with thanks a grant from the Secretary of State for Scotland on the advice of the Advisory Committee on Medical Research. We are grateful to Dr. W.S.T. Thomson for his advice and criticism.

REFERENCES

ADAMS, J.F. (1962). In Vitamin B_{12} und Intrinsic Faktor, edited by H.C. Heinrich, p. 397. Enke, Stuttgart.

ADAMS, J.F. & BODDY, K. (1968). J.Lab.clin.Med. 72. 392.

ADAMS, J.F. & BODDY, K. (1971). In The Cobalamins, edited by H.R.V. Arnstein & R. Wrighton. In Press, Churchill, London.

ADAMS, J.F., COX, A.G., KENNEDY, E.H. & THOMSON, J. (1967). Br.med.J. 3. 473.

ADAMS, J.F., KENNEDY, E.H., THOMSON, J. & WILLIAMSON, J. (1968). Br.J.Nutr. 22. 111.

ADAMS, J.F. & McEWAN, F.C. (1971). J.clin.Path. 24. 15.

ADAMS, J.F., ROSS, S.K., MERVYN, L., BODDY, K. & KING, P. (1971). Scand.J.Gastroenterol. 6. 249.

ADAMS, J.F., TANKEL, H.I. & McEWAN, F.C. (1970). Clin.Sci. 39. 107.

ANDERSON, B.B. (1964). J.clin.Path. 17. 14.

ANDERSON, B.B. (1965). Ph.D. Thesis, University of London.

ARDEMAN, S. & CHANARIN, I. (1963). Lancet, 2. 1350.

ARDEMAN, S., CHANARIN, I. & DOYLE, J.C. (1964). Br.med.J. 2. 600.

BANERJEE, D.K. & CHATTERJEA, J.B. (1963). Br.J.Nutr. 17. 385.

BAUMRIEDEL, W.R., PICKEN, J.C. & UNDERKOFLER, L.A. (1956). Proc.Soc.exp.Biol Med. 91. 377.

BEERSTECHER, E., Jr. & ALIGELT, S. (1951). J.biol.Chem. 189. 31.

BERTCHER, R.W., MEYER, L.M. & MILLER, I.F. (1958). Proc.Soc.exp.Biol Med. 77. 513.

BLUM, K.U. & HEINRICH, H.C. (1957). Vitamine und Hormone. 7. 486.

BODDY, K. & ADAMS, J.F. (1968). Am.J.clin.Nutr. 21. 657.

BOZIAN, R.C., FERGUSON, J.L., HEYSSEL, R.M., MENEELY, G.R. & DARBY, W.J. (1963). Am.J.clin.Nutr. 12. 117.

CALLENDER, S.T. & EVANS, J.R. (1955). Clin.Sci. 14. 387.

CASTLE, W.B., TOWNSEND, W.C. & HEATH, C.W. (1930). Am.J.med.Sci. 180. 305.

CHANARIN, I. (1969). The Megaloblastic Anaemias, Blackwell Scientific Publications, Oxford and Edinburgh.

CHUNG, A.S.M., PEARSON, W.N., DARBY, W.J., MILLER, O.N. & GOLDSMITH, G.A. (1961). Am.J.clin.Nutr. 9. 573.

COATES, M.E. & KON, S.K. (1957). In Vitamin B_{12} und Intrinsic Faktor, edited by H.C. Heinrich, p. 72. Enke, Stuttgart.

COOPERMAN, J.M., LUHBY, A.L., TELLER, D.N. & MARLEY, J.F. (1960). J.biol.Chem. 235. 191.

COWLING, D.C. & MacKAY, I.R. (1959). Med.J.Aust. 2. 558.

DAISLEY, K.W. (1961). Nature, Lond. 191. 868.

DELLER, D.J., GERMAR, H. & WITTS, L.J. (1961). Lancet, 1. 574.

ENGLAND, J.M., ASHWORTH, L.A.E. & TAYLOR, K.B. (1967). Q.Jl.exp.Physiol. 52. 357.

ESTREN, S., BRODY, E.A. & WASSERMAN, L.R. (1958). Adv.Int.Med. 9. 11.

GAMBLE, J.L. (1954). Chemical Anatomy, Physiology and Pathology of Extracellular Fluid, 6th edn. Harvard University Press.

GLASS, G.B.J., BOYD, L.J. & STEPHANSON, L. (1954). Proc.Soc.exp.Biol Med. 86. 522.

GOTTLIEB, C.W., LAU, K-S, WASSERMAN, L.R. & HERBERT, V. (1965). Blood, 25. 875.

GRÄSBECK, R. (1956). Acta med.scand. Suppl. 314.

GRÄSBECK, R. (1960). Adv.clin.Chem. 3. 299.

GRÄSBECK, R., IGNATIUS, R., JARNEFELT, J., LINDEN, H., NALL, H & NYBERG, W. (1961). Clinica chim.Acta 6. 56.

GRÄSBECK, R., NYBERG, W. & REIZENSTEIN, P.G. (1958). Proc.Soc.exp.Biol Med. 97. 780.

GREGORY, M.E. (1954). Br.J.Nutr. 8. 340.

GREGORY, M.E., FORD, J.E. & KON, S.K. (1952). Biochem.J. 51. 29.

GREGORY, M.E. & HOLDSWORTH, E.S. (1955). Biochem.J. 59. 329.

GREGORY, M.E. & HOLDSWORTH, E.S. (1959). Biochem.J. 72. 549.

GROSSOWICZ, N., HOCHMAN, A., ARONOVITCH, J., IZAK, G., & RACHMILEWITZ, M. (1957). Lancet, 1. 1116.

GROSSOWICZ, D., SULITZEANU, D. & MERZBACH, D. (1962). Proc.Soc.exp.Biol Med. 109. 604.

GYORGY, P., TOCANTINS, L.M., MILLER, F.R. & WEISS, A. (1954). Trans.Ass.Am.Physns 67. 117.

HEINRICH, H.C. (1964). Sem.Haemat. 1. 199.

HERBERT, V. (1963). Am.J.clin.Nutr. 21. 743.

HERBERT, V., GOT LIEB, C.W., LAU, K-S. & WASSERMAN, L.R. (1964). Lancet, 2. 1017.

HEYSSEL, R.M., BOZIAN, R.C., DARBY, W.J. & BELL, M.C. (1966). Am.J.clin.Nutr. 18. 176.

HOLDSWORTH, C.D., ATKINSON, M., DOSSETT, J.A., & HALL, R. (1964). Gut 5. 601.

HOM, B.L. (1967). Clinica chim.Acta 18. 315.

HOM, B.L., OLESON, H. & LOUS, P. (1966). J.Lab.clin.Med. 68. 958.

HUTNER, S.H., BACH, M.K. & ROSS, G.I.M. (1956). J.Protozool. 3. 101.

HUTNER, S.H., PROVASOLI, L., STOKSTAD, E.L.R., HOFFMAN, C.E., BELT, M., FRANKLIN, A.L. & JUKES, T.H. (1949). Proc.Soc.exp.Biol Med. 70. 116.

IRVINE, W.J. (1965). New Engl.J.Med. 273. 432.

IRVINE, W.J. (1966). Clin.exp.Immunol. 1. 99.

JEFFRIES, G.H. & SLEISENGER, M.H. (1963). J.clin.Invest. 42. 442.

JHALA, C.I. & GADGIL, R.K. (1960). Jour.J.J.Hosp. & Grant Med.Coll. 5. 36.

JOLLIFFE, N. & PETERMAN, R.A. (1956). Quoted by Chung et al. (1961).

JONES, P.N., MILLS, E.H. & CAPPS, R.B. (1957). J.Lab.clin.Med. 49. 910.

JOSKE, R.A. (1963). Gut 4. 231.

KAKEI, M. & GLASS, G.B.J. (1962). Proc.Soc.exp.Biol Med. 111. 270.

KARLIN, R. (1969). Int. 2. Vitaminforsch. 39. 359.

KENNEDY, E.H. (1967). Ph.D. Thesis, University of Glasgow.

KENNEDY, E.H. & ADAMS, J.F. (1965). Clin.Sci. 29. 417.

KENNEDY, E.H. & ADAMS, J.F. (1967). Scand.J.Haemat. 4. 489.

KERR, A.C. (1961). The Physiological Regulation of Salivary Secretions in Man. Pergamon Press, Oxford.

KUNKEL, H.G. (1947). Proc.Soc.exp.Biol Med. 66. 217.

KURLOV, O.V. (1961, 1962). Quoted by Heinrich (1964).

LALAND, P. & KLEM, A. (1936). Acta med.scand. 88. 620.

LAWRENCE, C. (1966). Br.J.Haemat. 12. 569.

LEAR, A.A., HARRIS, J.W., CASTLE, W.B. & FLEMING, E.M. (1954). J.Lab.clin.Med. 44. 715.

LICHTENSTEIN, H., BELOIAN, A. & MURPHY, E.W. (1961). Quoted by Chenarin (1969).

LINDSTRAND, K. & STÅHLBERG, K.G. (1963). Acta med.scand. 174. 665.

LOVE, R.M. (1970). The Chemical Biology of Fishes. Academic Press, London & New York.

McCANCE, R.A. & WIDDOWSON, E.M. (1969). M.R.C.Spec.Rep. No. 297, 3rd edn., H.M.S.O. London.

MACLAGEN, N.F. (1944). Bri.J.exp.Path. 25. 234.

MASON, D.K., HARDEN, R. McG. & ALEXANDER, W.D. (1966). Archs oral Biol. 11. 235.

MEYER, L.M., MULZAC, C.W., MILLER, I.F. & BRENNAN, B.L. (1963). Acta haemat. 29. 229.

MEYNELL, M.J., COOKE, W.T., COX, E.V. & GADDIE, R. (1957). Lancet, 1. 901.

MILLER, O.N. (1957). Archs Biochem.Biophys. 68. 255.

MILLIPORE APPLICATION REPORT AR-21 (1969).

MILLIPORE MANUAL (1969).

MUIR, W.M., GRAY, R.A. & COURTNEY, J.M. (1970).
Med. & biol Engng. 8. 395.

NEALE, G., CAUGHEY, D.E., MOLLIN, D.L. & BOOTH, C.C. (1966).
Br.med.J. 1. 382.

NELSON, R.S. & DOCTOR, V.M. (1962). Gastroenterology 42. 414.

NEWMAN, G.E., O'BRIEN, J.R.P., SPRAY, G.H. & WITTS, L.J. (1962).
In Vitamin B_{12} und Intrinsic Faktor, edited by H.C. Heinrich,
p. 424. Enke, Stuttgart.

PITNEY, W.R., BEARD, M.F. & VAN LOON, E.J. (1955).
J.biol.Chem. 212. 117.

PITNEY, W.R. & ONESTI, P. (1961). Aust.J.exp.Biol.med.Sci. 39. 1.

RACHMILEWITZ, M., ARONOVITCH, J. & GROSSOWICZ, N. (1956).
J.Lab.clin.Med. 48. 339.

RACHMILEWITZ, M., STEIN, Y., ARONOVITCH, J. & GROSSOWICZ, N. (1958).
Archs intern.Med. 101. 1118.

RACHMILEWITZ, M., STEIN, Y., ARONOVITCH, J. & GROSSOWICZ, N. (1959). Archs intern.Med. 104. 406.

RACHMILEWITZ, M. & ELIAKIM, M. (1968). J.med.Sci. 4. 47.

REIZENSTEIN, P.G. (1959). Acta med.scand. 165. 481.

REIZENSTEIN, P.G., MATTHEWS, C.M.E. & Ek, G. (1964). In Proceedings of the 6th International Congress on Nutrition, edited by C.F. Mills & R. Passmore, p.416. Livingstone, Edinburgh.

REIZENSTEIN, P.G., Ek, G. & MATTHEWS, C.M.E. (1966). Physics Med.Biol. 11. 295.

ROBBINS, W.J., HERVEY, A. & STEBBINS, M.E. (1960). Bull.Torrey bot.Club. 77. 423.

ROBINSON, F.A. (1966). The Vitamin Co-factors of Enzyme Systems. Pergamon Press. Oxford.

RØDBRO, P., CHRISTIANSEN, P.M. & SCHWARTZ, M. (1965). Lancet, 2. 1200

ROSENTHAL, H.L., MYERS, P.R. & O'BRIEN, G. (1962). Vitamin B_{12} und Intrinsic Faktor, edited by H.C. Heinrich, p. 410. Enke, Stuttgart.

ROSS, G.I.M. (1950). Nature, Lond. 166. 270.

ROSS, G.I.M. (1952). J.clin.Path. 5. 250.

ROSS, G.I.M., HUTNER, S.H. & BACH, M.K. (1957). In Vitamin B_{12} und Intrinsic Faktor, edited by H.C. Heinrich, p. 305. Enke, Stuttgart.

ROSS, G.I.M. & MOLLIN, D.L. (1957). In Vitamin B_{12} und Intrinsic Faktor, edited by H.C.,Heinrich, p.437. Enke, Stuttgart.

SCHIFFER, L.M., COHN, S.H., PRICE, D.C. & CONKLIE, E.P. (1968). Am.J.clin.Nutr. 21. 665.

SEPHADEX - GEL FILTRATION IN THEORY & PRACTICE. Published by Pharmacia Fine Chemicals, Uppsala. Sweden.

SIEGEL, S. (1956). Nonparametric Statistics for the Behavioral Sciences. McGraw-Hill Book Coy.Inc. New York.

SIEGEL, S., CHOW, B.F., OKUDA, K., CHEN, S. & HANUS, E.J. (1961). Am.J.clin.Nutr. 9. 705.

SIMONS, K. (1964). Soc.Scien.Finn Comm Biol. 27. 5.

SIMONS, K., WEBER, T., STIEHL, M. & GRÄSBECK, R. (1964). Acta med.scand.Suppl. 412. 257.

SMITH, E.L. (1965). Vitamin B_{12}, 3rd edn. Methuen & Co.Ltd., London.

STÅHLBERG, K.G., RADNER, S. & NORDEN, A. (1967). Scand.J.Haemat. 4. 312.

STEIN, O., STEIN, Y., ARONOVITCH, J., GROSSOWICZ, N. & RACHMILEWITZ, M. (1956). Bull.Res.Coun.Israel. 6E. 5.

STEIN, O., STEIN, Y., ARONOVITCH, J., GROSSOWICZ, N. & RACHMILEWITZ, M. (1959). J.Lab.clin.Med. 54. 545.

STEVENSON, T.D. & BAIRD, M.F. (1959). New Engl.J.Med. 260. 206.

SULLIVAN, L.W. & HERBERT, V.(1965). New Engl.J.Med. 272. 340.

SWENDSEID, M.E., GABSTER, M. & HALSTED, J.A. (1954). Proc.Soc.exp.Biol Med. 86. 834.

TOOHEY, J.I. & BARKER, H.A. (1961). J.biol.Chem. 236. 560.

ACKNOWLEDGEMENTS

The work was supported by grants from the Scottish Hospitals Endowment Research Trust on the advice of the Advisory Committee on Medical Research.

I am deeply indebted to Dr. J.F. Adams, Southern General Hospital, Glasgow, for his invaluable guidance and encouragement. Thanks are also due to Professor R.M.S. Smellie, Biochemistry Department, University of Glasgow, Dr. G. Leaf, Biochemistry Department, University of Glasgow, and Dr. W.M. Muir, Department of Bioengineering, Strathclyde University, for technical advice.