Observations on the Forms of Vitamin B_{12} in the Body and Metabolic Inter-relationships between Vitamin B_{12} and Cyanide

John Christopher LINNELL, B.Sc

Department of Experimental Chemical Pathology
Westminster Medical School
London

A THESIS submitted for the Degree of DOCTOR OF PHILOSOPHY in the Faculty of Science of the University of London

1974

BIBL.
LONDIN.
UNIV

BEST COPY

AVAILABLE

Variable print quality

The effect of Differentiation on a Particle is very remarkable, the first Differential being frequently of a greater value than the original Particle

Lewis Carroll

CONTENTS

page

List of Tables vii

List of Figures xi

Acknowledgements xv

Publications xvi

1 SUMMARY 1

2 INTRODUCTION 6

3 DEVELOPMENT of METHODS

3.1 Radioisotopic assay for total vitamin B_{12} 19

3.2 Extraction of plasma cobalamins

Introduction 28

Procedure 29

Results 31

3.3 Chromatography

Introduction 35

Procedure 36

Results 39

Mixed-layer chromatography 41

Two-dimensional chromatography 44

3.4 Bioautography

Introduction 45

Procedure 46

Results 48

Influence of inoculum age on bioautographic response 50

3.5 Chromatogram quantitation

Zone elution 54

Elution of indicator from the bioautogram 55

Densitometric scanning 56

Results 56

page

4 METHODS

4.1 Collection and preparation of samples
Plasma, erythrocytes and leucocytes 62
Bone marrow 63
Liver and other tissues 64

4.2 Radioisotopic assay of total B_{12}
Equipment and apparatus 65
Principle 67
Procedure: preparation of standards 68
extraction of plasma or serum 68
extraction of biopsies/ autopsy tissues 69
Analysis 69
Spectrometric calibration 70

4.3 Chromato-bioautographic estimation of cobalamins
Preparation of standards:
equipment and materials 71
principle 72
procedure 72
Extraction of cobalamins:
equipment and materials 73
procedure 75
Chromatography:
equipment and materials 78
preparation of plates 79
one-dimensional chromatography 80
two-dimensional chromatography 81
Bioautography:
equipment and materials 82
procedure 83
Densitometric quantitation:
principle 85
procedure 85

4.4 Other estimations 87

4.5 Validation of radioisotopic assay
Variation between batches 88
Variation within a batch 90
Efficiency of the extraction process 90
Recovery of cobalamins added to serum 93
Comparison of results with those obtained by other methods 93

page

4.6 Validation of chromato-bioautographic method
Extraction losses 97
Overall recovery 98
Replication 99
Quantitation of bioautograms 101

5 NORMAL VALUES

5.1 Total serum B_{12} 102
5.2 Plasma cobalamins 104
5.3 Leucocyte cobalamins 107
5.4 Erythrocyte cobalamins 109
5.5 Cobalamins in bone marrow 111
5.6 Cobalamins in liver, kidney, spleen and other tissues 113
5.7 Cobalamins in pregnancy and lactation 116
5.8 Discussion 119

6 PATHOLOGICAL RESULTS

6.1 Cobalamins in B_{12} deficiency
Untreated pernicious anaemia 122
Other causes of B_{12} deficiency 126
Discussion 127
6.2 Cobalamins in folate deficiency 129
6.3 Cobalamins in leukaemias and liver disease 130
6.4 Cobalamins in ophthalmological and neurological disease 132
Plasma and erythrocyte cobalamins in European patients with visual failure 132
Plasma and liver cobalamins in Nigerian patients with tropical ataxic neuropathy 134
Discussion 136
6.5 Effects of oral and parenteral administration of cobalamins 139
6.6 Inborn errors of B_{12} metabolism 141
Plasma and erythrocyte cobalamins 142
Effect of parenteral administration of cobalamins 145
Tissue cobalamins 147
Cobalamins in cultured fibroblasts 149
Discussion 149

page

7 APPENDIX

7.1 Studies of B_{12} deficiency and cyanide toxicity in the baboon 152

Animals 152
General experimental plan 153
Cobalamin studies:
Plasma total B_{12} 155
Plasma cobalamins 158
Erythrocyte cobalamins 161
Urinary excretion of methylmalonic acid 162

7.2 Studies of inter-relationships between cyanide and vitamin B_{12} 163

7.3 Effects of smoking on metabolism and excretion of vitamin B_{12}
(Reprinted from British Medical Journal, Volume 2, pages 215-216 (1968) 170

7.4 Absorption of vitamin B_{12} in the guinea-pig III The forms of vitamin B_{12} in ileal mucosa and portal plasma in the fasting state and during absorption of cyanocobalamin
(Reprinted from British Journal of Haematology, Volume 20, pages 299-305 (1971) 176

BIBLIOGRAPHY 188

LIST of TABLES

		page
3.1 - 1	Influence of different charcoal adsorbents on the total B_{12} assay	27
3.3 - 1	Preparation of chromatographic adsorbents	37f*
3.3 - 2	Solvent systems investigated	38f
3.3 - 3	R_f values of aqueous cobalamin markers in 143 chromatography systems	40f
3.3 - 4	Adsorbent systems investigated by gradient TLC	41f
3.4 - 1	Composition of the bioautography assay medium	46f
3.4 - 2	Growth response of the E.coli mutant after various changes in the conditions of pre-inoculation culture	52f
4.3 - 1	Culture-maintenance medium	83f
4.5 - 1	Within-a-batch variation in total B_{12} assay	91f
4.5 - 2	Efficiency of the total B_{12} extraction procedure	92
4.5 - 3	Recovery of total B_{12} following addition of cobalamins to serum	93f
4.5 - 4	Comparison of total serum B_{12} results obtained in 7 laboratories	96
4.6 - 1	Efficiency of the alcoholic extraction of cobalamins	98f
4.6 - 2	Recovery of cobalamins added to plasma	99f
4.6 - 3	Estimation of cobalamins: variation within a batch	100f

* facing page

		page
4.6 – 4	Estimation of cobalamins: variation between batches	100
5.2 – 1	Plasma cobalamins in normal subjects and hospital controls	105f
5.2 – 2	Plasma cobalamins in control subjects: summary of results	106
5.3 – 1	Leucocyte cobalamins in normal subjects and hospital controls	108
5.4 – 1	Erythrocyte cobalamins in normal subjects and hospital controls	110
5.5 – 1	Cobalamins in normal bone marrow	112
5.5 – 2	Cobalamins in lower cell layer from marrow aspirates	112
5.6 – 1	Cobalamins in liver, kidney, spleen, brain and pituitary	114f
5.6 – 2	Cobalamins in bile and cerebrospinal fluid	114
5.7 – 1	Plasma cobalamins in normal women of child-bearing age	117
5.7 – 2	Plasma cobalamins in healthy pregnant women at or approaching delivery	117
5.7 – 3	Plasma and milk cobalamins during lactation	118
6.1 – 1	Plasma cobalamins in patients with untreated pernicious anaemia	123f
6.1 – 2	Blood and bone-marrow cobalamins in control subjects and patients with pernicious anaemia	124
6.1 – 3	Plasma cobalamins in Veganism	127f
6.1 – 4	Plasma cobalamins in gastrointestinal disease	127f
6.2 – 1	Plasma cobalamins in folate deficiency	129

		page
6.3 - 1	Plasma cobalamins in leukaemia and liver disease	131
6.4 - 1	Plasma and erythrocyte cobalamins in ophthalmological disease	133
6.4 - 2	Cobalamins in ophthalmological disease: statistical comparison of results	135
6.4 - 3	Plasma cobalamins in tropical ataxic neuropathy	136f
6.4 - 4	Liver cobalamins in tropical ataxic neuropathy	138f
6.5 - 1	Changes in plasma cobalamins following cobalamin administration	140
6.6 - 1	Inborn errors of B_{12} metabolism: details of patients and studies carried out	142f
6.6 - 2	Plasma cobalamins in patients and control subjects	143
6.6 - 3	Erythrocyte cobalamins in 2 patients	144f
6.6 - 4	Plasma cobalamins in Patient MM following Ado-B_{12} administration	144f
6.6 - 5	Plasma and leucocyte cobalamins in Patient JR following OH-B_{12} administration	145f
6.6 - 6	Plasma and leucocyte cobalamins in Patient JR after Ado-B_{12} and OH-B_{12} administration	146
6.6 - 7	Tissue cobalamins in Patient MM and control children	147f
6.6 - 8	Tissue cobalamins in patients and control subjects	148f
7.1 - 1	Composition of low-B_{12} baboon diet	153f
7.1 - 2	Regime of baboons in each group	154

		page
7.1 – 3	Plasma cobalamins in five groups of baboons at intervals during the experiment	156f
7.1 – 4	Plasma cobalamins at 35 months: statistical comparison of groups	156
7.2 – 1	Effect of added cyanide, thiocyanate and $CN-B_{12}$ on extractability of B_{12} from plasma	167f
7.2 – 2	Extractability of $Ado-B_{12}$, $Me-B_{12}$, $CN-B_{12}$ and $OH-B_{12}$ from plasma	167f
7.2 – 3	Effect of $CN-B_{12}$ *in vivo* on total B_{12} extractability	168f

TABLES 5.7-1, 5.7-2 and 5.7-3 are reproduced by permission of the Editor and publishers of the Journal of Clinical Pathology

TABLES 5.2-2, 5.6-1, 5.6-2, 6.1-1, 6.1-2, 6.1-3, 6.1-4, 6.2-1, 6.3-1, 6.4-3, 6.4-4, 6.5-1 and 6.6-7 are reproduced by permission of the Editor and publishers of Clinical Science and Molecular Medicine

page

LIST of FIGURES

3.1 - I	Adaptor for centrifuging assay tubes	24f*
3.1 - II	Standard curves obtained with different binding agents	25f
3.2 - I	Early separation of plasma cobalamins from a healthy smoker	30f
3.3 - I	In search of a chromatography system: preliminary results with 33 systems	39f
3.3 - II	Separation of visible loadings of cobalamin markers on a thin layer gradient of cellulose and silica gel in solvent 20	42f
3.3 - III	Separation of visible loadings of cobalamin markers in solvent 20	43f
3.3 - IV	Separation of visible loadings of cobalamin markers in solvents 14, 21 and 22	43
3.4 - I	Reduction of the tetrazolium growth-indicator to triphenyl formazan	50f
3.4 - II	Influence of pre-inoculation culture conditions on growth response of E. coli to cobalamins	53f
4.1 - I	Technique used in separating leucocytes from whole blood	63f
4.2 - I	Adaptor for centrifuging assay tubes	69f
4.2 - II	The radioisotopic assay: standard curve	70f
4.3 - I	Absorption spectrum of cyanocobalamin	72f
4.3 - II	Preparation of a thin-layer plate for two-dimensional chromatography.	81f
4.3 - III	Construction of bioautography plate	81f

* facing page

		page
4.3 - IV	Two-dimensional chromatography and bioautography of cobalamin markers	85f
4.3 - V	Standard curves for the chromato-bioautographic estimation of cobalamins	86f
4.5 - I	Repeated estimates for the total B_{12} concentration of pooled serum in 100 assays	89
4.5 - II	Estimated error within an assay at various total B_{12} concentrations	90f
4.5 - III	Comparison of results by the isotopic assay and by Euglena gracilis	94f
4.5 - IV	Comparison of results by the isotopic assay and by L. leichmannii	95f
4.6 - I	Comparison of plasma Me-B_{12} results by visual assessment of bioautograms and by photometric scanning	101f
5.1 - I	Serum total B_{12} results in 100 healthy adults	103
5.2 - I	Separation of plasma cobalamins in a healthy subject	106f
5.6 - I	Separation of cobalamins in an autopsy control sample of liver	115f
5.6 - II	Separation of cobalamins in an autopsy control sample of spleen	115
5.7 - I	Plasma and milk cobalamins in a healthy mother	117f
5.7 - II	Appearance of CN-B_{12} in breast milk following administration of CN-B_{12}	117f
5.7 - III	Plasma cobalamins in maternal and foetal blood at delivery	118f
6.1 - I	Plasma and erythrocyte cobalamins in normal subjects and cases of pernicious anaemia	124f

		page
6.1 – II	Leucocyte and bone marrow cobalamins in normal subjects and cases of pernicious anaemia	125f
6.1 – III	Results in pernicious anaemia as a percentage of those in normal subjects	126f
6.1 – IV	Separation of plasma cobalamins in a case of untreated pernicious anaemia	128f
6.4 – I	Plasma and erythrocyte cobalamins in European patients with visual failure and control subjects	133f
6.4 – II	Separation of plasma and erythrocyte cobalamins in a case of optic atrophy	134f
6.4 – III	Plasma cobalamins in Nigerian patients with tropical ataxic neuropathy and control subjects	135f
6.4 – IV	Separation of plasma cobalamins in a case of tropical ataxic neuropathy	137f
6.6 – I	Separation of plasma cobalamins in a patient (MM) with an inborn error of B_{12} metabolism	143f
6.6 – II	Changes in plasma and leucocyte Me-B_{12} and excretion of methylmalonic acid and homocystine in a patient (JR) following administration of OH-B_{12}	146f
6.6 – III	Uptake of OH-B_{12} and conversion to Ado-B_{12} and Me-B_{12} by cultured fibroblasts	149f
6.6 – IV	Separation of cobalamins in fibroblasts from a patient (MR) with an inborn error of B_{12} metabolism	150f
6.6 – V	Scheme of probable steps leading to synthesis of Ado-B_{12} and Me-B_{12}	151

		page
7.1 - I	Changes in plasma total B_{12} of a colony of baboons during the course of the experiment	155f
7.1 - II	Changes in plasma Me-B_{12} with time in B_{12}-supplemented animals	159f
7.1 - III	Changes in plasma Me-B_{12} with time in B_{12}-deficient animals	159
7.1 - IV	Variation in the proportion of Me-B_{12} in the plasma during the experiment	160f
7.1 - V	Variation in the proportion of CN-B_{12} in the plasma during the experiment	160
7.1 - VI	Erythrocyte cobalamins in each group of animals after 25, 30 and 35 months	161f
7.1 - VII	Relationship between plasma total B_{12} and methylmalonic acid excretion in baboons	162f
7.2 - I	Relationship between plasma CN-B_{12} and total B_{12} extractability with and without cyanide	169

FIGURES 4.2 - II, 4.5 - IV, 5.7 - I, 5.7 - II and 5.7 - III are reproduced by permission of the Editor and publishers of the Journal of Clinical Pathology

FIGURES 4.3-V, 5.2-I, 5.6-I, 5.6-II, 6.1 - IV and 6.6 - V are reproduced by permission of the Editor and publishers of Clinical Science and Molecular Medicine

ACKNOWLEDGEMENTS

I must thank Professor N.F. Maclagan and Professor David Matthews for the provision of all Departmental facilities, and Professor Matthews for his helpful encouragement throughout this work. I sincerely thank Mr Hassan A.A. Hussein and Miss Heather Mackenzie for their expert technical assistance and acknowledge with gratitude the help given in certain aspects of the study by Miss I.J. Wise, Mrs G.K. Bannerjee, Mr J. Morgan and Mr P.W. Pramod.

I am very conscious of the debt I owe to a number of people, including Professor Victor Hoffbrand, Dr John Wilson, Dr Harvey Mudd, Mr Ian Craft, Dr Tim Peters and Professor Ben Osuntokun, for providing samples and for helpful discussion; Dr Len Mervyn for reference compounds, and Mr S. Vincent for the culture organism. Mrs C. Angelo kindly typed many of the original drafts. I must thank Mrs Elizabeth Gurney for her interest in the study from its inception and in particular for her design and production of the final typescript.

I acknowledge with gratitude the support so generously given by the Wellcome Trust, without which this investigation would not have been possible. Certain expenses incurred in the foetal studies were defrayed by Glaxo Laboratories, and are gratefully acknowledged.

PUBLICATIONS

MATTHEWS, D.M., GUNASEGARAM, R. and LINNELL, J.C. (1967) Radiosiotopic assay of serum B_{12} using serum as binding agent. Proceedings of Seventh International Congress of Biochemistry, Tokyo, Vol.4, p.830

MATTHEWS, D.M., GUNASEGARAM, R. and LINNELL, J.C. (1967) Results with radioisotopic assay of serum B_{12} using serum binding agent. J. clin. Path., 20, 683-686

LINNELL, J.C., SMITH, A.D.M., SMITH, C.L.,WILSON, J. and MATTHEWS, D.M. (1968) Effects of smoking on metabolism and excretion of vitamin B_{12}. Brit. med. J., 2, 215-216

LINNELL, J.C., MACKENZIE, H.M. and MATTHEWS, D.M., (1969) Normal values for individual plasma cobalamins. J. clin. Path., 22, 506

LINNELL, J.C., MACKENZIE, H.M., WILSON, J. and MATTHEWS, D.M. (1969) Patterns of plasma cobalamins in control subjects and in cases of vitamin B_{12} deficiency. J. clin. Path., 22, 545-550

LINNELL, J.C., WILSON, J. and MATTHEWS, D.M. (1969) Estimation of cyanocobalamin and other plasma cobalamins in hereditary optic atrophies and other ophthalmological disorders. Clin. Sci., 37, 878

LINNELL, J.C., HOFFBRAND, A.V., PETERS, T.J. and MATTHEWS, D.M. (1969) Estimation of individual plasma B_{12} compounds in normal subjects and patients with deranged B_{12} metabolism. J. clin. Path., 22, 742

HOFFBRAND, A.V., LINNELL, J.C., MATTHEWS, D.M. and PETERS, T.J. (1970) The forms of vitamin B_{12} in the ileal enterocyte of the guinea-pig during fasting and absorption of cyanocobalamin. J. Physiol., 208, 66-67 P

LINNELL, J.C., HUSSEIN, H.A-A. and MATTHEWS, D.M. (1970) A two-dimensional chromato-bioautographic method for complete separation of individual plasma cobalamins. J. clin. Path., 23, 820-821

LINNELL, J.C., HOFFBRAND, A.V., PETERS, T.J. and MATTHEWS, D.M. (1971) Chromatographic and bioautographic estimation of plasma cobalamins in various disturbances of vitamin B_{12} metabolism. Clin. Sci., 40, 1-16

WILSON, J., LINNELL, J.C. and MATTHEWS, D.M. (1971) Plasma cobalamins in neuro-ophthalmological diseases. Lancet, 1, 259-261

PETERS, T.J., LINNELL, J.C., MATTHEWS, D.M. and HOFFBRAND, A.V. (1971) Absorption of vitamin B_{12} in the guinea-pig III The forms of vitamin B_{12} in ileal mucosa and portal plasma in the fasting state and during absorption of cyanocobalamin. Brit. J. Haemat., 20, 299-305

CRAFT, I.L., MATTHEWS, D.M. and LINNELL, J.C. (1971) Cobalamins in human pregnancy and lactation. J. clin. Path., 24, 449-455

MATTHEWS, D.M. and LINNELL, J.C. (1971) Investigations of B_{12} metabolism using chromatography and bioautography of individual cobalamins. Glaxo Symposium on The Cobalamins Ed. Arnstein, H.R.V. and Wrighton, R.J. Churchill Livingstone, Edinburgh and London. pp 23-33

LINNELL, J.C. (1972) Identification and quantitation of cobalamins in plasma and tissues. Proceedings of Eighth International Congress on Clinical Chemistry, Copenhagen. Scand. J. clin. lab. Invest., 29, Supplementum 126, pp 6.6 - 6.8 (by invitation)

LINNELL, J.C., HOFFBRAND, A.V., HUSSEIN, H.A-A., MATTHEWS, D.M. and WISE, I.J. (1973) Distribution of coenzyme and other forms of vitamin B_{12} within the body. Clin. Sci. molec. Med., 45, 15P

LINNELL, J.C., HOFFBRAND, A.V., HUSSEIN, H.A-A., WISE, I.J. and MATTHEWS, D.M. (1974) Tissue distribution of coenzyme and other forms of vitamin B_{12} in control subjects and patients with pernicious anaemia. Clin. Sci. molec. Med., 46, 163-172

OSUNTOKUN, B.O., MATTHEWS, D.M., HUSSEIN, H.A-A., WISE, I.J. and LINNELL, J.C. (1974) Plasma and hepatic cobalamins in tropical ataxic neuropathy. Clin. Sci. molec. Med., 46, 563-567

DILLON, M.J., ENGLAND, J.M., GOMPERTZ, D., GOODEY, P.A., GRANT, D.B., HUSSEIN, H.A-A., LINNELL, J.C., MATTHEWS, D.M., MUDD, S.H., NEWNS, G.H., SEAKINS, J.W.T., UHLENDORF, B.W. and WISE, I.J. (1974) Mental retardation, megaloblastic anaemia, methylmalonic aciduria and abnormal homocysteine metabolism due to an error in B_{12} metabolism. Clin. Sci. molec. Med., 46 - in press

VESEY, C.J., COLE, P.V., LINNELL, J.C. and WILSON, J. (1974) Some metabolic effects of sodium nitroprusside in man. Brit. med. J. - in press

COOKSLEY, W.G.E., ENGLAND, J.M., LINNELL, J.C. and TAVILL, A.S. (1974) Hepatic vitamin B_{12} metabolism and synthesis of transcobalamin II. Gut - in press

LINNELL, J.C. (1974) The Fate of B_{12} in vivo. Chapter in Biochemistry and Biochemical Pathology, Wiley-Interscience, New York - in press

SUMMARY 1

THIS THESIS describes the results of investigations into the coenzyme and other forms of vitamin B_{12} in human and animal tissues. Cobalamin studies have been carried out in normal subjects, hospital controls, and patients with evidence of inborn or secondary disturbance in B_{12} metabolism; inter-relationships between vitamin B_{12} and cyanide metabolism have been studied in healthy smokers, ophthalmological and neurological patients, and an experimental colony of baboons; and absorption and inter-conversion of cobalamins have been studied in normal subjects, Vegans, patients with pernicious anaemia, and two experimental animal species.

As an introduction, the historical background to the present knowledge of vitamin B_{12} is briefly reviewed and previous work and ideas recalled, some of which suggested the need for the present study.

Before investigation of plasma and tissue cobalamins could be attempted, suitable methods had to be developed. This preliminary work is described in Section 3, with an account of the search for the best means of quantitating the results. The

various procedures established for the routine measurement of cobalamin levels, and data on their reliability in performance, are set out in Section 4.

In normal plasma and tissues (Section 5), three forms of vitamin B_{12} - methylcobalamin, deoxyadenosylcobalamin and hydroxocobalamin - were invariably found; the originally isolated form, cyanocobalamin, being frequently detectable in small amounts in body fluids and blood cells, but very seldom in tissues from any organ studied. Within the wide normal B_{12} range, consistent patterns emerged: methylcobalamin predominated in plasma and milk; deoxyadenosylcobalamin in all cellular tissues and the few samples of bile and cerebrospinal fluid obtainable for investigation.

Cobalamin findings in a number of pathological conditions are reported in Section 6. In patients with B_{12} deficiency (from whatever cause) subnormal total B_{12} levels in the plasma were associated with disturbance of the plasma cobalamin pattern - methylcobalamin was disproportionately reduced and cyanocobalamin frequently increased - whereas, with correspondingly low total B_{12} values, the normal cobalamin pattern was sustained throughout the haemopoietic system (Section 6.1). Folate deficiency (Section 6.2) had no consistent effect on the plasma cobalamin pattern although an occasional slight abnormality was

found in the presence of total B_{12} levels within normal limits.

In a study of patients with leukaemia and liver disease (Section 6.3), leukaemic patients with normal or exceptionally high plasma total B_{12} levels (blood B_{12} binding capacity being increased) the plasma cobalamin pattern was found to be unchanged; in the rare cases where plasma total B_{12} levels were subnormal, the cobalamin findings were typical for B_{12} deficiency. In the patients with liver disease, deoxyadenosylcobalamin was sometimes disproportionately raised - probably through reciprocal loss from the liver.

Certain ophthalmological and neurological disorders in which plasma thiocyanate levels were known to be elevated were investigated (Section 6.4). In Leber's disease and other optic atrophies, plasma cyanocobalamin, deoxyadenosylcobalamin and hydroxocobalamin were increased, whereas in the erythrocytes, methylcobalamin was increased - suggesting a redistribution of the cobalamins between plasma and erythrocytes. In tropical ataxic neuropathy, plasma cyanocobalamin, deoxyadenosylcobalamin and hydroxocobalamin were significantly higher than in control subjects - the raised cyanocobalamin levels probably resulting from prolonged dietary exposure to cyanide.

Results of plasma cobalamin estimations before and after

the administration of cyanocobalamin or methylcobalamin are given in Section 6.5. After the oral or parenteral administration of cyanocobalamin, plasma cyanocobalamin increased, and there was evidence of some conversion to methylcobalamin; after parenteral administration of methylcobalamin, this compound increased but levels of the other cobalamins were unchanged.

Section 6.6 describes investigations carried out in seven children with inborn errors of B_{12} metabolism. One child, who died at the age of seven, was studied extensively and found to have a defect either in cellular uptake of B_{12} or in a metabolic pathway leading to the formation of a common precursor of methylcobalamin and deoxyadenosylcobalamin; analyses showed that while plasma total B_{12} was normal or high, the plasma cobalamin distribution was grossly disturbed; erythrocytes taken during life and organs sampled at autopsy had greatly reduced concentrations of all four cobalamins. Absorption studies in two other children suggested that part of an administered cobalamin dose was converted to other forms of the vitamin. Studies of fibroblasts cultured from skin biopsies in six children showed that in those with an error of B_{12} metabolism, in comparison with controls, less B_{12} was taken up from the culture medium and less hydroxocobalamin converted to the coenzyme forms.

Four further reports are given in the Appendix (Section 7). Inter-relationships between B_{12} deficiency and cyanide toxicity were investigated over a period of three years in the baboon (Section 7.1), cyanide in relation to B_{12} metabolism in general was studied and the implications discussed (Section 7.2), and the effects of smoking on B_{12} metabolism and excretion described (Section 7.3). A study in the guinea pig (Section 7.4) showed that oral administration of cyanocobalamin resulted in some conversion of cyanocobalamin to deoxyadenosylcobalamin and hydroxocobalamin during intestinal absorption (although the greater part was absorbed unchanged).

"At length the huge engines were installed, the last holding bolt was screwed down, the final verdict of 'everything shipshape' pronounced, and early in the morning of Saturday, March 31st, 1838, the Great Western slipped her moorings at Blackwall and started under steam for Bristol"*

Victorian England witnessed many such events as the pattern of life changed to fit the new industrial era. Inventions proliferated and medical discoveries also, that would touch the lives of many in the years to follow.

About this time attention was being drawn to a disease, which, perhaps owing to the growing population in many towns, was seen by the observant physician more frequently. This was a form of anaemia, but one without apparent cause, in which the patient's good general nutrition and even obesity presented a marked contrast to his lassitude and habitual exhaustion. In 1855, Thomas Addison clearly described the symptoms of this condition and applied to it the term 'idiopathic anaemia' in order, as he explained, 'to distinguish it from cases in which there exists more or less evidence of some of the usual causes or concomitants of the anaemic state'.

---

* Isambard Kingdom Brunel L T C Rolt Longmans London, 1957

Addison's awareness of this disease had grown over a period of years, first having been awakened, perhaps, by reports of fatal cases of anaemia described some thirty years earlier (e.g. Andral, 1823; Combe, 1824). In a lecture delivered in 1860 at the Long Island College Hospital, New York, while discussing the case histories of several patients suffering from idiopathic anaemia, Austin Flint made the inspired observation, 'I suspect in these cases, although not unduly distinct to the naked eye, there exists degenerative disease of the glandular tubuli of the stomach'. Another ten years were to pass before confirmation of this was obtained.

In 1870, at The London Hospital, Samuel Fenwick examined post-mortem the stomach from a patient whom he considered to have had Addison's idiopathic anaemia. Microscopy of the mucosa revealed that 'the glandular structure was in a state of atrophy', while acidified scrapings of the mucosa 'failed to reduce the weight of a cube of hard boiled egg-white during incubation for nine hours at blood heat'. Evidence of a lack of peptic activity post-mortem was followed by the first demonstration, by Cahn and von Mehring in 1886, of an absence of hydrochloric acid in the gastric juice during life, although it was not for another twenty or thirty years that this finding gained universal acceptance as pathognomonic of pernicious anaemia.

It was Biermer of Zurich who in 1871 first used the term 'progressiver pernicioser Anamie', anglicized five years later to 'pernicious anaemia' by the editor of a British journal. So apposite was this name, with its dire prognostic implications, that it gained immediate acceptance, and was soon in use on both sides of the Atlantic. Paradoxically, a recent analysis (Huser, 1966) of Biermer's clinical data has revealed that the diagnosis of pernicious anaemia was probably unfounded in all but one of his fifteen reported cases.

The formation of blood cells in the bone marrow was first described, by Neumann and Bizzozero independently, in 1868 (see Wintrobe, 1967) and the first blood count in what may have been a case of pernicious anaemia recorded by Sørensen in 1874 (his patient having 0.47 million red cells/mm^3). Osler and Gardner (1877) measured the size of the red cells and were probably the first to observe macrocytes in the blood of an anaemic patient; the clinical findings in their case included numbness of the fingers, hands and forearms, and the post-mortem findings, atrophy of the gastric mucosa. Three years later, in 1880, using his newly-developed staining techniques, Paul Ehrlich demonstrated in bone marrow the abnormal cells he named 'megaloblasts', and so a turning point was reached in the study of pernicious anaemia as it was realized that a diagnosis could be made with greater certainty by laboratory investigation than at the bedside.

The importance of Ehrlich's brilliant contributions would be hard to overstress, even to-day. Richard Cabot, for example, writing on pernicious anaemia in 1908, said, 'Since Ehrlich, no-one has materially added to our knowledge of the disease' - an opinion probably incontrovertible for almost another t.:enty years. During this period, pernicious anaemia remained inevitably fatal - while physicians prescribed bone marrow, fresh air or arsenic in the faint hope of effecting some improvement in their patient's condition.

Before about 1920, the influence of diet on the progress of the disease was controversial. Habershon (1863) wrote, 'Many patients at an early stage completely recover under the influence of bracing air and a nutrient and stimulating diet'. Others, such as Fraser (1894), prescribed fresh red marrow and reported beneficial results. Cabot, fourteen years later, wrote, ' .... ingestion of bone-marrow and countless other remedies have been shown by the test of time to be useless, and, '.... there is no evidence that any special diet has any influence upon the course of the disease'.

Diets had in fact been prescribed in the treatment of the anaemias for a great many years (Menghini, for example, in 1746, had suggested that iron-rich foods would increase the iron content

of the blood), and interest in the dietary treatment of pernicious anaemia was re-awakened by the work of Gibson and Howard in 1923 and Whipple and Robbins in 1925, leading to the larger-scale study by Minot and Murphy in 1926. In this, forty-five patients were put on a special diet which included 120g to 240g lightly cooked liver and at least 120g lean meat daily, with vegetables, fruit and cereal. The improvement in all patients was dramatic, continuous and quite unlike the temporary remissions sometimes occurring spontaneously - and so began the search for the 'anti-anaemic principle' which was to last for twenty years.

If Minot and Murphy's findings suggested that pernicious anaemia might be the result of simple dietary deficiency, the work of Castle which he began to publish three years later showed that a substance which he called 'intrinsic factor', secreted in the normal stomach, was a necessary prerequisite for the intestinal absorption of 'extrinsic factor', the 'anti-anaemic principle' in liver and other meat, and that failure to secrete 'intrinsic factor' was the basic cause of pernicious anaemia.

Laboratory studies directed towards the isolation of 'extrinsic factor' made only slow progress for a number of years. This was partly due to the lack of a suitable assay (laboriously, use was made of patients with pernicious anaemia in relapse) and

partly because the chemical nature of the substance and its tissue concentration were quite unknown. Liver extracts had become available for clinical use during the 1930s, but isolation of the vitamin itself seemingly awaited the publication of the revolutionary chromatographic methods proposed by Martin and Synge (1941) for the separation of amino acids; these methods proved an ideal tool in the final purification of vitamin B_{12}. The isolation of crystalline cyanocobalamin in 1948 was in fact achieved quite independently and almost simultaneously by Lester Smith in England and by Rickes et al. in America (Smith and Parker, 1948; Rickes et al., 1948). Following metabolic studies in Clostridium tetanomorphum, the first coenzyme form of the vitamin was isolated some ten years later (Barker et al., 1958) as a light-sensitive orange-yellow compound, which was later found to include adenosine as the nucleotide. Further work showed that this organism could be made to synthesize other B_{12} coenzymes, one of which (with 5,6-dimethylbenzimidazole as nucleotide) was identical to the major form later found in the liver of a number of mammals, including man (Toohey and Barker, 1961). A second mammalian coenzyme form has since been isolated from liver (Lindstrand, 1964) and shown to be the major cobalamin in normal human plasma (Lindstrand and Ståhlberg, 1963; Ståhlberg, 1964).

Possible inter-relationships between vitamin B_{12} and cyanide metabolism

In B_{12} deficiency, although haematological changes are usually the most conspicuous, it has long been known that clinical signs of abnormal neurological function may occur and, rarely, may be the presenting feature. In the older literature, references to neurological sequelae were common, particularly when cases of advanced pernicious anaemia were described. Thus Osler and Gardner (1877) mentioned numbness of the patient's extremities and Cabot (1908) observed that of the 1,200 cases of pernicious anaemia included in his survey, the majority progressed to subacute combined degeneration of the cord.

Impaired vision may be related to B_{12} deficiency, although, where present, other evidence of neurological involvement is usually found. The visual impairment may be due to haemorrhage in the macular region, or to a retrobulbar neuritis. Retinal haemorrhage is more common when the red cell count is below 1.5 million per mm^2 (Chanarin, 1969). Unlike the sex distribution of uncomplicated pernicious anaemia, there is a marked male preponderance in patients who develop retrobulbar neuritis; Hamilton, Ellis and Sheets (1959) recorded 29 cases, of whom 26 were men. This is very similar to the incidence in tobacco

amblyopia; Traquair (1930) found only 7 women afflicted in a total of 1,525 cases. The similarity of the sex distribution in these two conditions might suggest either a common aetiology or at least a possible relationship between retrobulbar neuritis, tobacco amblyopia, and the metabolism of vitamin B_{12} and cyanide.

During the late nineteenth century a major point of contention concerned the cause of 'toxic amblyopia', as it was then known. Some maintained that tobacco alone was responsible for the symptoms (Nettleship, 1887; Eales, 1887; Marcus Gunn, 1887), but Connor (1890) described an amblyopia symptomatically identical with that attributed to tobacco in alcoholic patients who smoked little or not at all. De Schweinitz (1896) and others claimed that it was impossible to distinguish the symptoms of one from the other. Even to-day, these issues are still of lively interest and remain largely unsettled. It is generally accepted that a nutritional deficiency is involved, and some authors (for example, Victor, 1963) contend that this is the primary cause of tobacco amblyopia, since other amblyopias of acknowledged toxic origin have different manifestations and lead rapidly to complete blindness. The view is at variance with the observations of other workers (Foulds et al., 1969; Wilson and Matthews, 1970) but the exact role of cyanide in the pathogenesis of visual failure has yet to be elucidated.

In West Africa, the West Indies, and in certain other countries where the incidence of various neuropathies is high, the staple diet for much of the population is 'cassava', or 'gari', prepared from the tuberous root of Manihot utilissima, a plant which flourishes in the poorest soil. Both sweet and bitter strains are cultivated, the latter giving the larger crop. An appreciable quantity of linamarin, a cyanogenic glucoside hydrolysable to free cyanide, is contained in the tuber, the concentration being highest in the outer cortical layer. The dried cortex from the bitter cassava root will yield up to 245 mg HCN per 100 g (Montgomery, 1969). Moore, in 1934, suggested a causal relationship between cassava eating and amblyopia, and two years later, Clark (1936) suggested cyanide might be the toxic constituent. According to Montgomery (1969), the poisonous properties of cassava were first recorded by Clusius in 1605, and certainly the comments of other travellers, including Charles Darwin (1832), indicate that this knowledge was widespread. Recent findings suggest however that by no means all the cyanide is removed during the preparation of the food. Osuntokun et al. (1968) have shown that patients subsisting largely on a cassava diet have elevated plasma thiocyanate levels and a greatly reduced plasma concentration of the sulphur-containing amino acids cysteine and methionine; plasma B_{12} levels remain

normal or are above normal. Osuntokun's patients all presented with ataxic neuropathy, a condition in which optic atrophy, bilateral perceptive deafness, and sensory changes associated with posterior column conduction loss, are clearly demonstrable. In an earlier study of neurological disease, Monokosso and Wilson (1966) also reported raised levels of thiocyanate in the plasma of their patients, with normal B_{12} concentrations.

Studies on the possible influence of cyanide and cyanide metabclism on the course of various demyelinating diseaseswere stimulated by an hypothesis proposed by A.D.M. Smith in 1961 — that, as a route of cyanide detoxication, the reaction:

$$CN\text{-}B_{12} \underset{\text{cyanide}}{\overset{\text{light}}{\rightleftharpoons}} OH\text{-}B_{12}$$

might compete with the rhodanese-catalysed reaction:

$$CN^- \xrightarrow[\text{S-amino acid}]{\text{rhodanese}} SCN^-$$

first described by Lang (1933)

From animal experiments, it was known that vitamin B_{12} is involved in the detoxication of cyanide, although thiocyanate is the major excretion product (Boxer and Rickards, 1952). Hydroxocobalamin has been shown to have impressive antidotal

properties in acute cyanide poisoning (Mushett et al., 1952) whereas cyanocobalamin has not (Beaven, 1950), and in 1954, Duke-Elder reported encouraging results with vitamin B_{12} in the treatment of tobacco amblyopia. Similar findings were published in 1958 by Heaton et al., and were later attributed to the appreciable content of hydroxocobalamin in commercially-available 'Cytamen' at that time (Baxter et al., 1953; Smith and Duckett, 1965).

The form or forms in which vitamin B_{12} occurred in the body were at this time unknown, although the presence of deoxy-adenosylcobalamin had recently been demonstrated in liver (Toohey and Barker, 1961). Microbiological assays of serum B_{12} using Lactobacillus leichmannii gave much higher values when samples were extracted with added cyanide than when cyanide was omitted from the buffer (Boger et al., 1955; Spray, 1955; Girdwood, 1960). The values obtained with added cyanide represented total serum B_{12} while low values obtained in the absence of cyanide were associated with loss of the vitamin in the protein precipitate (Matthews, 1961; 1962). Smith (1961) stated that the difference between the values obtained with and without added cyanide was 'insignificant' in heavy pipe smokers, and attributed this to the effects of the high cyanide content of tobacco smoke (Osborne et al., 1956; Johnstone and Plimmer, 1959; Surgeon-General of the United States, 1964); he

maintained that the assay value obtained without added cyanide represented serum cyanocobalamin, while the difference between this and the value obtained with added cyanide represented serum hydroxocobalamin. Following the first separation of cobalamins in human plasma (Lindstrand and Ståhlberg, 1963) and the discovery of methylcobalamin in liver (Lindstrand, 1964), an attempt was made to confirm Smith's hypothesis by identifying the plasma cobalamins in normal healthy smokers. Lindstrand, Wilson and Matthews (1966) found that cyanocobalamin could be detected in the blood of only a minority of the smokers, and when detected was present in only relatively small amounts.

Although Smith's original hypothesis was thus precluded, it nevertheless appeared likely, from the considerable body of evidence then accumulating (for example, Smith and Duckett, 1965; Wilson and Langman, 1966; Chisholm et al., 1967; Cima et al., 1967; Foulds et al., 1968; Torres, Smith and Oxnard, 1969), that some inter-relationship existed in the metabolism of cyanide, thiocyanate and vitamin B_{12}.

The PRESENT INVESTIGATION

Very little is known about the distribution of the various forms of vitamin B_{12} in the body and how this distribution may be altered in disease (Chanarin, 1969). Deoxyadenosylcobalamin has been identified and estimated in human liver (Toohey and Barker, 1961; Morrow et al , 1969), and is apparently the main form of the vitamin in this organ. Lindstrand and Ståhlberg (1963) were the first to demonstrate the presence of at least two different cobalamins in human plasma: methylcobalamin - the major component - and a second compound, which was probably either deoxyadenosylcobalamin or hydroxocobalamin (Lindstrand, 1964; Ståhlberg, 1967). Plasma cobalamins have also been separated in cases of chronic myeloid and other leukaemias - by chromatography (Ståhlberg, 1964) and by electrophoresis (Myasishcheva et al., 1968)

The AIM of the INVESTIGATION was:

To develop a method for the estimation of cobalamins sufficiently sensitive to require only small amounts of blood or other tissues

To study the distribution of cobalamins in blood and other tissues both in health and disease; hopefully to shed some further light on the functions of cobalamin coenzymes in man

To obtain evidence of any inter-relationship between the metabolism of vitamin B_{12} and that of cyanide

DEVELOPMENT of METHODS 3

3.1 RADIOISOTOPIC ASSAY for TOTAL VITAMIN B_{12}

The basic principle of radioactive isotope dilution analysis is a simple one (Gorsuch, 1968) and depends on the fact that when a radioactive tracer is mixed with the corresponding unlabelled compound, the specific activity of the compound will be reduced. If this reduction in specific activity can be measured, the concentration of the diluting material may be calculated. Normally this requires that the compound be purified and weighed, although the mass may be determined spectrophotometrically or by other means. However, when the amount of purified compound recovered is less than about a microgram, the accuracy of the estimation declines sharply.

Isotope-dilution methods of greater sensitivity have been developed which do not suffer from this failing, and have variously been called 'saturation analysis' (Ekins, 1960), 'sub-stoichiometric dilution analysis' (Johannesson, 1961) or 'radio-immunoassay' (Yalow and Berson, 1960), although the principle is similar in each case. In recent years, these methods have been applied to the

estimation of many substances of physiological importance (Ekins, 1960; Yalow and Berson, 1960; Barakat and Ekins, 1961; Murphy et al., 1963; Midgley et al., 1969; Abraham, 1969). The concentration of most of these compounds in tissue fluids is very low, and in consequence high demands are made of any assay system. Where applicable, microbiological methods afford very high sensitivity and have traditionally been accepted as the sine qua non for the estimation of vitamin B_{12}. The more recently developed radioisotopic methods can however provide comparable sensitivity and do offer some advantages, particularly in terms of the overall time taken for an assay, the elimination of much glassware washing and in the freedom from the interference sometimes caused by antibiotics present in the sample.

The basic requirements of any radioisotopic assay are: a labelled compound of high specific activity chemically identical to that being estimated, a high affinity binding agent, and a means of separating bound and free compound once equilibrium is reached. Various means may be used to fulfil these requirements in vitamin B_{12} assay, and methods have been described using as binding agent either serum (Barakat and Ekins, 1961, 1963; Ekins and Sgherzi, 1965; Frenkel, Keller and McCall, 1966; Tibling, 1968) or intrinsic

factor (Rothenberg, 1961, 1963; Lau, Gottlieb, Wasserman and Herbert, 1965; Raven, Walker and Barkhan, 1966; Friedner, Josephson and Levin, 1968) and membrane dialysis, charcoal or diethyl aminoethyl cellulose for the separation of bound and free fractions. Some of these methods have proved troublesome to set up, to have unexpected technical snags, and to give erratic and low results (e.g. Brafield, 1966; Hall, 1966; Raven and Barkhan, 1966). All methods make use of ^{57}Co cyanocobalamin as isotope. Cyanocobalamin is the most stable form of the vitamin and can be prepared labelled with ^{57}Co at high specific activity (up to approximately 300 $\mu Ci/\mu g$) without self-degradation. The labelled compound is stable in dilute aqueous solution for many months, and since the radiochemical half-life is relatively long (270 days) a single batch provides sufficient material for a large number of assays.

Procedure

The method of Ekins and Sgherzi (1965) was chosen as being relatively straightforward to set up and of demonstrable reliability, at least in the hands of its authors. The method employs normal human serum as binding agent and albumin-coated charcoal to take up free cyanocobalamin once equilibrium has been established. No major changes in the procedure were necessary,

but it was found that at several points in the assay, techniques could be simplified without an attendant loss of precision. In an effort to eliminate the contamination which can arise in an assay of this sensitivity from inadequately-washed glassware, much of the apparatus was made disposable.

Extraction of B_{12} from the sample is an important first step which in the original method is efficiently carried out with acetate buffer pH 4.5 and cyanide at 100°. The recommended procedure was as follows:

> 'Mix serum (1 ml) and buffer (6 ml) in a tube and transfer by Pasteur pipette to a 10 ml ampoule. Fuse the neck to seal it and place the ampoule in boiling water for 20 min; remove and cool. Centrifuge down the protein precipitate then cut open the neck of the ampoule and transfer supernatant by Pasteur pipette to a fine sintered-glass funnel, supported within a conical centrifuge tube. Centrifuge to assist filtration and assay aliquots of the resulting filtrate'.

After some experiment it was found that the technique could be simplified without disadvantage by using plain glass tubes sealed with parafilm and aluminium foil for the extraction, and filtering the extract directly without intermediate steps. Whatman No.1 gave rather too slow a filtration rate, but No.41 was both faster and a stronger paper which satisfactorily retained the

precipitate. A small polythene funnel was used to hold the filter circle, while the filtrate was collected in a 10ml polystyrene tube. All items except the pipettes were sufficiently inexpensive to be used once only and discarded, which not only saved time but, more important, eliminated further possible sources of contamination.

Other modifications were largely concerned with apparatus. At first, Hamilton microsyringes were used to add label (20μl) and binding serum (25μl) to each tube, and proved to be a reliable means of dispensing these small volumes. Care was needed however in filling the syringe to avoid including air bubbles in the barrel which would otherwise reduce the volume of liquid delivered. A piston pipette with a fixed stroke (Eppendorf micro-liter pipette) became available, which was faster to use than the Hamilton and of similar quoted accuracy. This was verified by weighing 10 aliquots of water (20μl) dispensed from the pipette which gave a mean delivery of 20.000 $\pm$ SD 0.278μl (coefficient of variation 1.4%). Satisfactory results were obtained in use and after a trial period, the pipette was included as standard equipment in the assay.

The small glass tubes originally used required checking beforehand to ensure that the polythene stopper fitted well enough

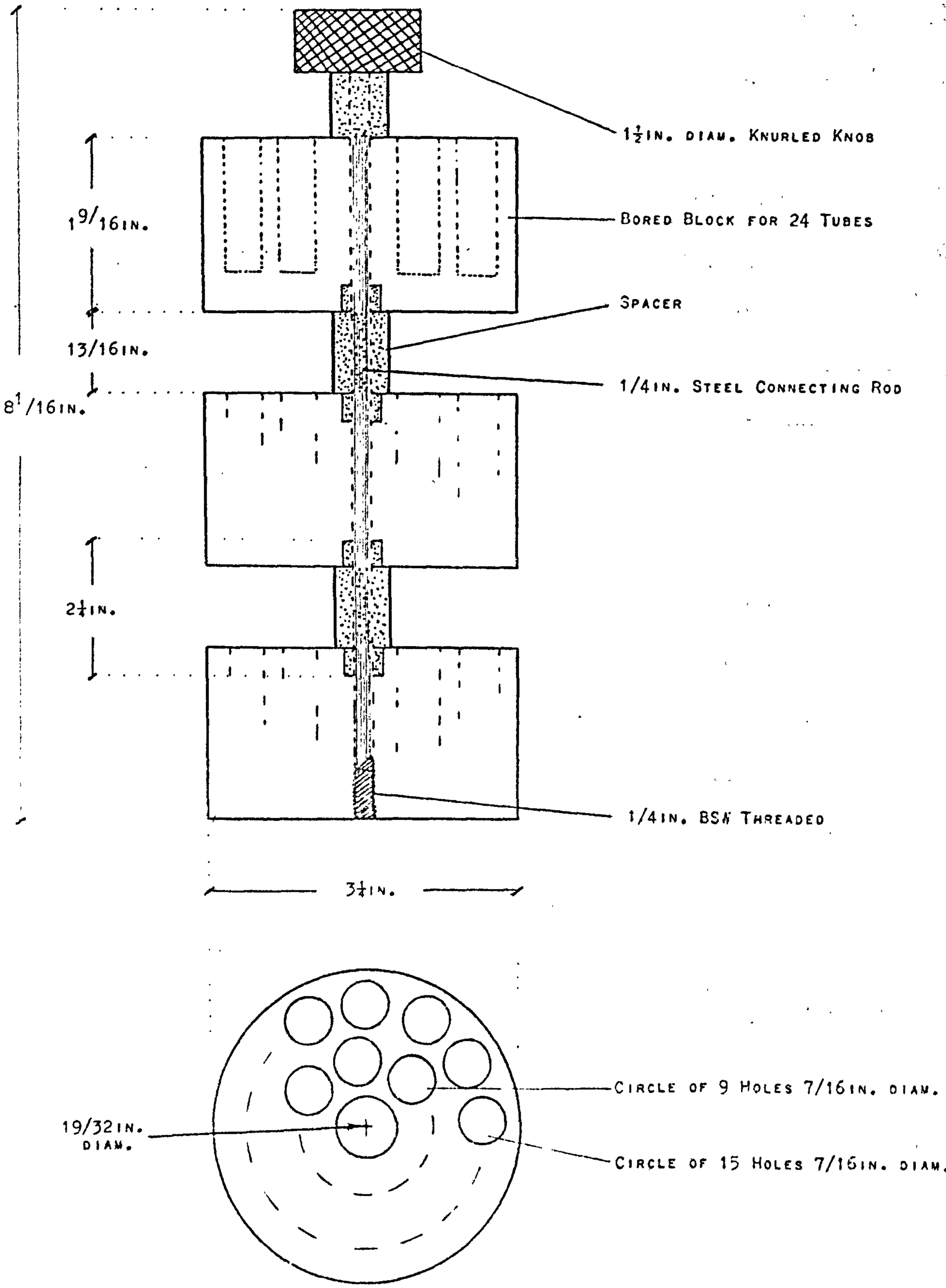


Figure 3.1 – I

Multiple adaptor for carrying assay tubes during centrifugation. The balanced pair of adaptors accommodate 144 assay tubes

to prevent leakage, but not so tightly as to be liable to cause fracture of the tube during the assay (it was commonly found that almost half the tubes inspected failed to pass this test). To remedy the situation a similar-sized polystyrene tube was obtained whose cap could be relied upon to fit well, since both parts were manufactured to close tolerance. The tube appeared to be without disadvantage at any stage in the assay and was adopted forthwith.

To operate the assay satisfactorily, it was necessary that samples and standards should have identical treatment and preferably should be taken through each stage together. This gave no difficulty in the incubation and adsorption stages, but the charcoal had then to be centrifuged down, a few tubes at a time in a small bench centrifuge, as at first no other facility existed. An M.S.E. 'Major' centrifuge fitted with large swing-out buckets became available and a multiple tube adaptor (Fig 3.1 - I) was designed to fit. A pair of these were kindly constructed by the Westminster Hospital Physics Department, turned out of solid duralumin, balanced, polished, and finally anodised to prevent corrosion. Together these would accommodate 144 tubes, sufficient for an assay of 65 samples plus standards. It was found that the slight temperature rise in the centrifuge during

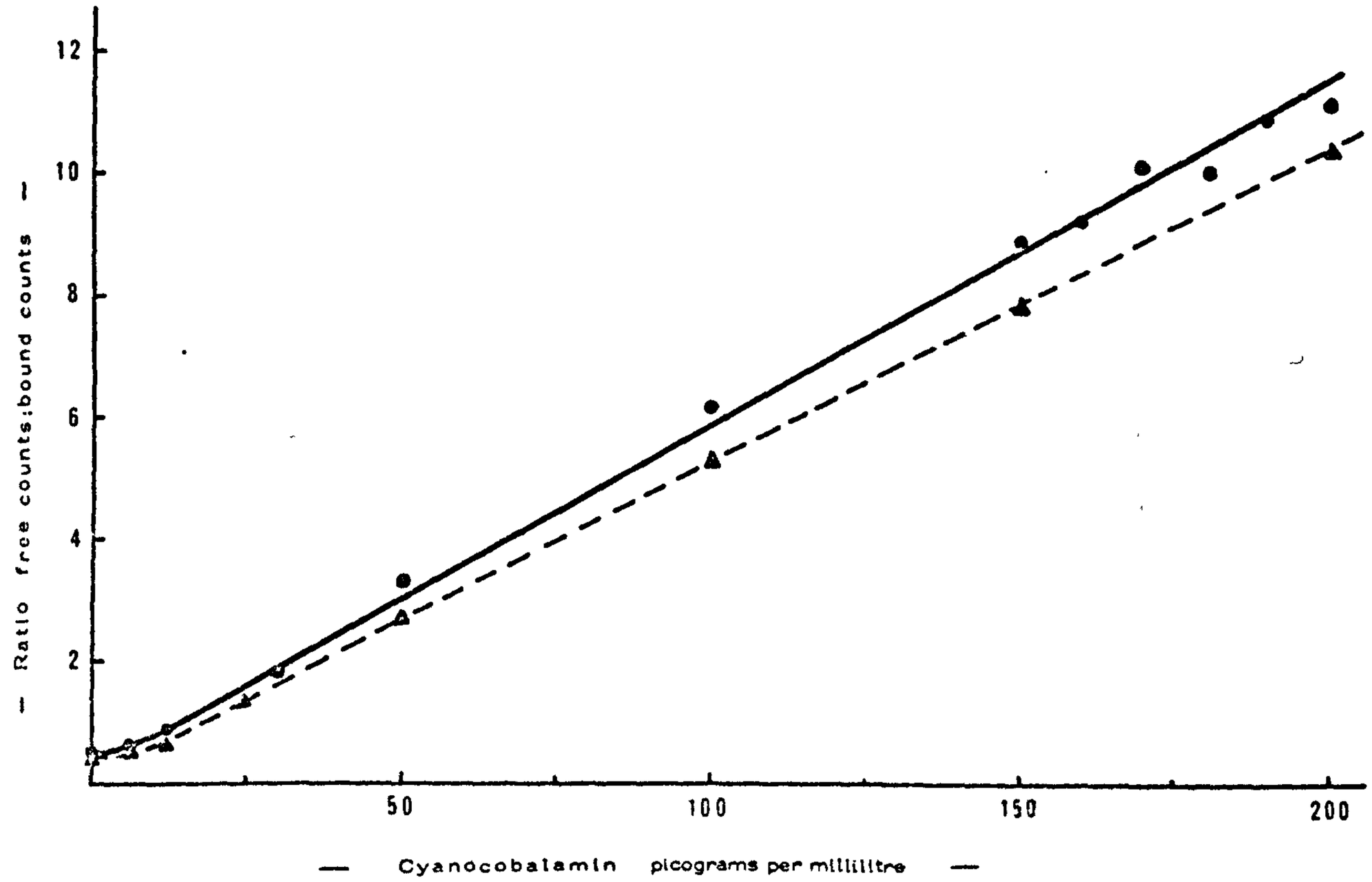


Figure 3.1 - II

Standard curves obtained using different binding agents. — 25μl fresh serum from single donor — 25μl pooled serum reconstituted from freeze-dried powder

use could be offset if the adaptors were cooled at 4° before being loaded with sample tubes.

As pointed out by Ekins and Sgherzi (1965), it was found that the slope of the standard curve, and hence the sensitivity of the assay, could be altered by adjusting the volume of binding serum, a reduction in this volume increasing sensitivity. The usefulness of this facility for increasing sensitivity was limited, however, by a concomitant rise in the F/B ratio of the blank. In practice an adequately wide working range was obtained when a fixed volume of 25µl binder was employed. At first, serum was taken as required from one of several donors, but in order to standardise the source, a comparison was made between one of these and reconstituted freeze-dried serum. The standard curves obtained (Fig 3.1 - II) were closely similar. The freeze dried material was obtained as out-dated stock from the blood transfusion department and was made up with sterile distilled water, dispensed in 2ml aliquots and stored at -15 to -20° until required.

The assay ran uneventfully for several months, when a breakdown occurred which led to the changing of one reagent. The failure arose on an occasion when the ratio, free counts:

bound counts, unexpectedly approximated unity for all standards and samples. Replacing the albumin had no effect but a near normal standard curve was obtained again when an alternative brand of charcoal was used. It was not possible to reactivate the defective charcoal (Norit O L) by heating, and, surprisingly, a replacement batch of the same material performed little better. A third lot was obtained together with a range of other charcoals from different sources and these were compared in an assay of high (200 pg/ml) standards, each charcoal being made up as usual with albumin and buffer. The results (Table 3.1 - 1) showed that three samples (1, 2 and 3) were almost equally effective in producing a wide difference between a 200 pg cyanocobalamin standard and the blank. One of these (BDH activated acid-washed decolourising charcoal) was tested further; found to be reliable and was adopted for standard use in the assay.

ADDENDUM

Since setting up the assay, an automatic γ-scintillation counter (Packard Tricarb) has become available which is of considerable assistance. Normally a batch assayed one day may be counted overnight and the results calculated on the following morning

TABLE 3.1 - 1

EFFECT of USING DIFFERENT CHARCOAL ADSORBENTS on the ASSAY of REPLICATE 200 pg/ml STANDARDS

	CHARCOAL	Cyanocobalamin 200 pg/ml	0 pg/ml	Difference
		(ratio free counts: bound counts)		
1	Activated, acid-washed decolourising (BDH)	13.25	0.85	12.40
2	Norit A (H & W)	13.30	0.90	12.40
3	Norit NK (H & W)	13.10	0.75	12.35
4	Norit SXI (H & W)	12.75	1.25	11.50
5	Norit OL (H & W)	10.00	1.04	8.96
6	Animal, powder, technical (BDH)	2.33	-	-
7	Animal, powder, commercial (H & W)	2.22	-	-
8	*Coconut shell (BDH)	0.13	-	-
9	*MFC 300 mesh (H & W)	0.11	-	-
10	*Sugar (BDH)	0.03	-	-
11	*Wood (H & W)	0.03	-	-

* finely ground before use

DEVELOPMENT of METHODS

3.2 EXTRACTION of PLASMA COBALAMINS

Introduction

The earliest workers (Smith and Parker, 1948; Rickes et al., 1948) devised methods for extracting and concentrating vitamin B_{12} from liver homogenates, but owing to the use of cyanides, included in their methods to improve the vitamin yield, all B_{12} compounds were converted to cyanocobalamin. The natural occurrence of coenzyme forms of the vitamin was first established in bacteria by Barker (Barker et al., 1958), but it was not until three years later that a cobalamin coenzyme (5-deoxyadenosylcobalamin) was isolated from human liver (Toohey and Barker, 1961).

Lindstrand and Ståhlberg (1963) were the first to describe a procedure for separating cobalamins from human plasma. Their extraction method was based on the earlier work of Lester Smith et al., and employed ethanolic deproteinisation of the sample followed by phenol extraction to purify and concentrate the cobalamins. Owing to the requirements of their chromatography system, the volume of plasma needed (20-40 ml) was relatively large, although the total amount of B_{12} involved (perhaps 30 ng) was small.

The aim of the present study was to develop a reliable extraction method which would permit the estimation of plasma cobalamins using blood samples no larger than those routinely taken in a hospital laboratory (10ml).

Procedure

Use was made of the procedure described by Lindstrand and Ståhlberg. Initially the method was scaled down to accept 10 ml plasma, but this required the use of flasks, reflux condensers and separating funnels in handling the necessary volumes of solvents. Since this produced ample cobalamin concentrate for the chromatographic separation, it was possible to reduce the plasma volume considerably and thereby simplify the method. Starting with 7 ml plasma it was found that all solvent extraction could be performed in stoppered test-tubes, thus eliminating the need for separating funnels. Leakage of solvent while shaking the tubes was prevented by replacing the glass stoppers by ones of soft polythene. Conveniently, the lower phase had to be retained at each stage and separation was effected by aspirating the upper layer.

The first plasma cobalamin chromatograms showed zones which were poorly resolved and had a tendency to 'tail', suggesting that salts had been concentrated together with

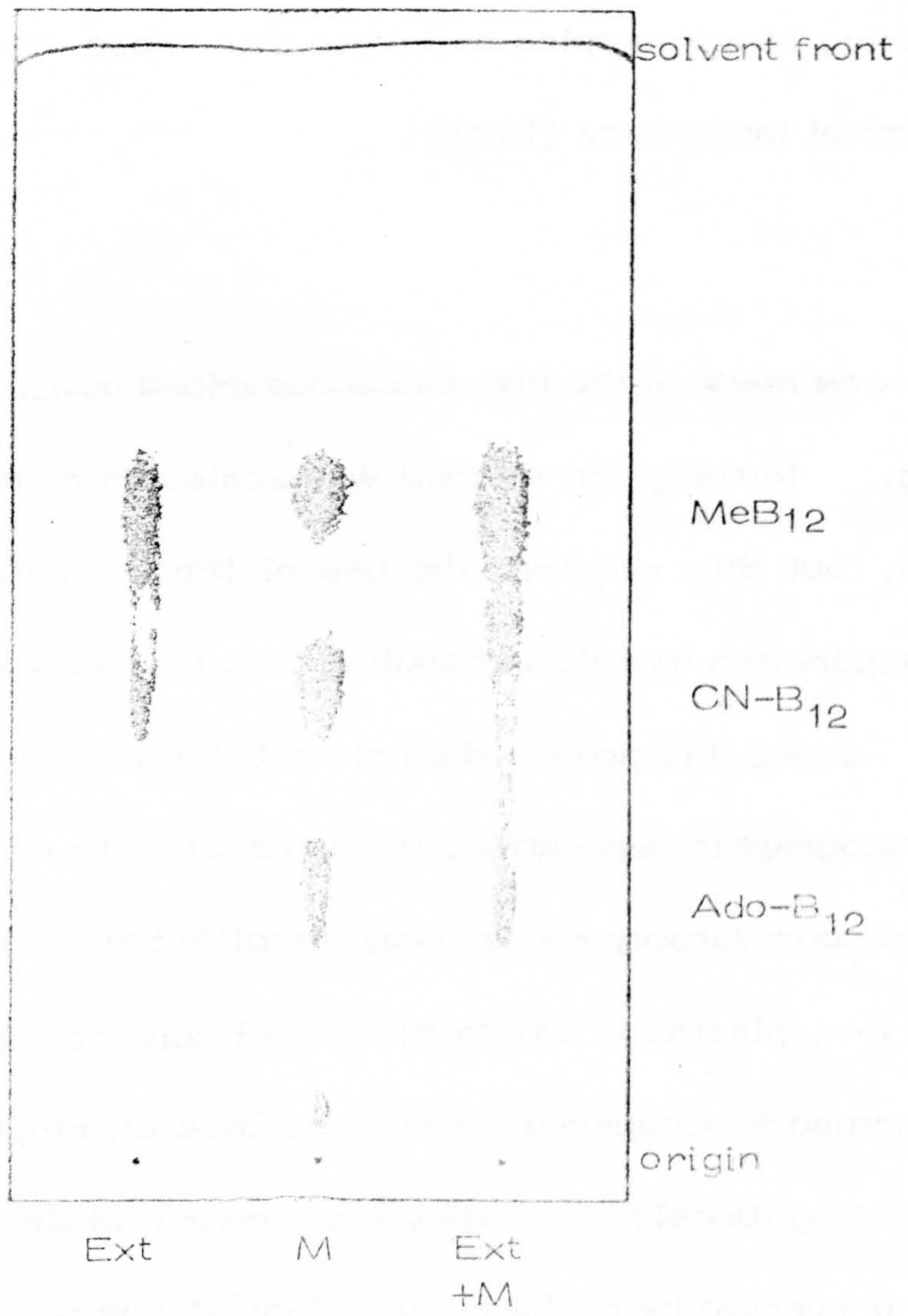


Figure 3.2-I Early separation of plasma cobalamins from a healthy smoker. Streaking was caused by the high salt content of the extract. Ext = Extract of plasma
M = Cobalamin markers

cobalamins in the plasma extract (Fig 3.2 - 1). This was confirmed by Na^+ estimation using flame photometry. Additionally, chromatograms of cobalamin standards made up in saline were run alongside aqueous markers, when the salt effect on the former was clearly apparent. A commercial ion-exchange cell (Baird and Tatlock desalting apparatus) was tested for its suitability in overcoming this problem. Preliminary experiments were carried out with sodium acetate buffer and dilute aqueous cobalamin standards, estimating sodium ion concentrations by flame photometry and cobalamins by chromatography before and after dialysis. Chromatograms were then run on plasma extracts with and without desalting, and the results compared.

One other experiment was carried out in an attempt to purify the plasma extract further before chromatography. A volume of cobalamin concentrate (0.2 ml), the extract from 7 ml plasma, was transferred to 6 cm of dialysis tubing (Visking 8/32") previously softened by immersion in distilled H_2O overnight, the ends tied together and the loop suspended in a 2 ml foil-covered plastic tube. The stoppered tube and its contents were centrifuged at 15,000 x G for 90 min at 5^o and cobalamins in both ultrafiltrate and residue separated chromatographically.

Preliminary recovery experiments were carried out with ^{57}Co cyanocobalamin, using sufficient isotope to keep counting times reasonably short. The labelled cobalamin was equilibrated with normal plasma (170ng $CN\text{-}B_{12}$/ml) for 3hr at 5° before extraction. The protein precipitate and all upper phases were retained and counted together with the final extract. The procedure was repeated using a smaller addition of ^{57}Co cyanocobalamin (1ng/ml) which fell within the likely binding capacity of the plasma. Later on, when the quantitative method was developed, more detailed recovery experiments were carried out with unlabelled cobalamins added to normal and low-B_{12} plasma.

Results

The original method, while adequately producing a cobalamin extract from 20-40ml plasma, was unwieldy in use and could not readily be applied to small samples of blood. To economise both in time and materials, it was felt that the method, involving as it did some two dozen steps for the extraction alone, should be capable of handling batches rather than single samples. The rate-limiting step in the method was solvent evaporation which could take up more than half the total time. This difficulty was alleviated by obtaining a 'spider' multiple-tube or flask adaptor for the rotary evaporator, making possible the

simultaneous evaporation of five samples. The procedure was gradually scaled down from 10 ml plasma, while solvent volumes and tube sizes were adjusted accordingly. With careful handling it was possible to obtain a useable extract from as little as 1 ml plasma, but to allow for the occasional unsuspected low B_{12}, and to leave sufficient extract to duplicate the chromatography where necessary, an initial plasma volume of 2 ml was preferred.

Ultrafiltration of the plasma extract did not materially improve the chromatography, and little difference in the cobalamin separation could be detected between filtrate and unfiltered residue. On the other hand, electrolytic desalting effected a marked improvement in the separation of cobalamins from plasma, yet caused very little if any breakdown or loss. The effect of 40 min electrolytic desalting on the chromatography of dilute aqueous solutions of cobalamin markers was negligible. compounds were made up in pH 4.5 sodium acetate buffer and during dialysis sodium ion concentration fell from 360 mEq/l to 33 mEq/l. Chromatography showed that all compounds had slightly higher R_f values after desalting. A small amount of cyanocobalamin would appear to have been converted to hydroxocobalamin, probably by loss of cyanide during the extended dialysis period. The apparatus was equipped with a milliammeter to indicate current passed by the cell. While desalting a

plasma extract the current would fall from 15-20mA to 5-6mA within the first 5min but subsequently alter little. If a current was passed for 8min, the Na^+ concentration of an extract could be reduced by approximately 130 mEq/l, and this much improved the subsequent cobalamin separation. Since the dialysis time was only one-fifth of that used in the preliminary experiment above, it seemed unlikely that other than a negligible loss or interconversion of cobalamins would arise.

The electro-desalting step as developed was incorporated into the method and used with success in the analysis of a large number of plasma samples. Owing to the small volume of final extract (25 μl), desalting was carried out on the ether-washed aqueous extract of cobalamins recovered from phenol, prior to the final evaporation. This stage was inevitably time-consuming since not more than two samples could be desalted at a time. In addition the cell had to be rigorously washed to avoid inter-sample contamination, and both electrolyte and ion-exchange membranes required replacement at regular intervals. After continued use of the method it was found that adequate chromatographic separation of the cobalamins could still be achieved if, in place of electrolytic desalting, the extract was more extensively washed at the phenol stage. During the early method development, much difficulty

was encountered with the formation of phenol/water emulsions but it was found that if the volume of phenol was reduced (to 5ml), effective washing could still be carried out in a 10ml centrifuge tube, carefully agitated by hand. The phases could be separated by spinning the tube in a bench centrifuge (MSE 'Minor'). For convenience, this procedure was adopted as a later modification of the method.

Measurement of the γ-emission from ^{57}Co cyanocobalamin following its addition to plasma (170ng/ml) and in the final extract, showed that the overall recovery of this compound was 86.5% - 11.3% remaining in the precipitated plasma proteins. Using a smaller amount of labelled cyanocobalamin (1000pg/ml) within the likely binding capacity of the plasma, recovery in the extract was only 55.0%, while loss in the alcoholic precipitate (25.2%) was greater. Details of the recovery experiments carried out with both 'hot' and 'cold' cobalamins are described under Validation of the chromato-bioautographic method.

3.3 CHROMATOGRAPHY

Introduction

As a technique, chromatography has been closely associated with the separation and purification of vitamin B_{12} from the earliest times, and indeed played an important rôle in the original isolation by both American and English teams (Rickes, Brink, Koniuszy, Wood and Folkers, 1948; Smith and Parker, 1948). Column chromatography on silica, alumina, bentonite or charcoal, utilising both partition and adsorption effects, were employed in separating the vitamin from the alcoholic extracts of raw minced liver.

Partition chromatography on paper was later used as a qualitative tool in separating the cobalamin analogues produced by microbial fermentation (Ford and Holdsworth, 1954). This permitted the partial separation of cyanocobalamin, pseudo-vitamin B_{12}, Factor A, Factor B, etc. to be achieved, sufficient to indicate the likely course of a given fermentation.

More recently, paper chromatography has been used by Lindstrand and Ståhlberg (1963) to separate cobalamins from human plasma, enabling them to demonstrate for the first time the presence of methylcobalamin as a major plasma form of the vitamin.

In the present study, it was considered from the outset that thin-layer chromatography would most likely provide the key to obtaining an effective separation of cobalamins from small volumes of plasma. A wide range of adsorbents is available for thin-layer chromatography which allows full advantage to be taken of both partition and adsorption phenomena. Closely related compounds may be separated with good resolution, and compared with paper chromatography the detection sensitivity may be enhanced by virtue of the smaller zones usually obtained. In addition, the handling of TLC plates is many times easier than that of the often necessarily large paper chromatograms which become most delicate to handle after development when they are saturated with solvent and easily damaged.

Procedure

In order to evaluate a large number of possible chromatography systems, a pilot study was set up using microscope slides as TLC plates and small jars as chromatography tanks. Localisation problems were minimised by using visible loadings of an aqueous cobalamin mixture, since it was intended initially only to study the possible separation of compounds.

TABLE 3.3 - 1

PREPARATION of CHROMATOGRAPHIC ADSORBENTS

Layer		Slurry composition	
Silica gel:	neutral acidic basic	10 g silica gel-G (Merck)	+ 20 ml H_2O + 20 ml 0.1 N HCl + 20 ml 0.1 N NaOH
Cellulose:	neutral acidic basic	10 g cellulose CC 41 (Whatman Chromedia)	+ 25 ml H_2O + 25 ml 0.1 N HCl + 25 ml 0.1 N NaOH
Alumina:	neutral acidic basic	10 g alumina-G (Merck)	+ 20 ml H_2O + 20 ml 0.1 N HCl + 20 ml 0.1 N NaOH
Kieselguhr:	neutral acidic basic	10 g Kieselguhr-G (Merck)	+ 20 ml H_2O + 20 ml 0.1 N HCl + 20 ml 0.1 N NaOH
Polyamide:	neutral	8 g + 2 ml ethanol	+ 24 ml H_2O

The adsorbents used were prepared as shown in Table 3.3 - 1. Microscope slides (77 mm x 25.5 mm) were coated with a slurry of adsorbent to a thickness of 0.010", using a micro-TLC spreader, and then air-dried in histological slide-staining dishes fitted with spacers to accommodate ten slides. Solvents were chosen as those likely to separate polar compounds, and included several systems referred to in the older B_{12} literature. After some preliminary experiment, the eleven solvents listed in Table 3.3 - 2 were selected for detailed study. Solvents were made up and approximately 10 ml transferred to each vessel to be used as a developing tank (4oz screw-capped sample jars were found suitable) which were equilibrated in a cupboard in the darkroom. An aqueous mixture of cobalamins was prepared containing approximately 10 µg each of methylcobalamin (Me-B_{12}), 5-deoxyadenosylcobalamin (Ado-B_{12}), cyanocobalamin (CN-B_{12}) and hydroxocobalamin (OH-B_{12}) per µl. One µl was applied to a point 10 mm from the end of each TL plate, and ascending chromatography carried out until the solvent front had risen to within 5 mm of the top of the plate. The chromatogram was then removed from its tank, air-dried, photocopied, and the R_f of each zone measured.

TABLE 3.3 - 2

SOLVENT SYSTEMS INVESTIGATED

Solvent number	Composition
3	n-butanol saturated with water (upper phase)
4	upper phase (from 3) + 15 % w/v phenol
5	sec-butanol saturated with water (upper phase)
12	sec-butanol / tert-butanol / water (43:8.6:48.4) (upper phase)
14	sec-butanol / iso-propanol / water (10:7:10)
15	n-butanol / acetic acid glacial / water (4:1:5) (upper phase)
16	n-butanol / 0.880 ammonia / water (4:1:5) (upper phase)
17	n-butanol / formic acid / water (8:1:10) (upper phase)
18	n-butonal / Dioxan / water (4:1:5) (upper phase)
19	sec-butanol saturated with water (upper phase) / acetic acid (210:2)
20	sec-butanol saturated with water (upper phase) / 0.880 ammonia (300:9.75)

In this way, it was quickly possible to assess the potentialities of some 140 systems in separating the four cobalamins of interest. The most promising adsorbent/solvent combinations were re-tested on larger (5 cm x 20 cm) plates layered with adsorbent to a thickness of 0.025 mm. Finally, the characteristics of several mixed layers were evaluated using gradient-layer plates prepared with a Desaga gradient-TLC spreader. This produced, in effect, a series of narrow plates varying in composition from approximately 5% to 95% of one adsorbent mixed with 95% to 5% of a second adsorbent. One μl spots of the aqueous cobalamin mixture were applied at 1 cm intervals along one edge of the plate (across the gradient), producing the equivalent of 18 separate chromatograms per 20 cm square plate (Fig 3.3 - II). The proportions of adsorbent permitting the best separation of cobalamins were confirmed using homogeneous mixed-layer plates developed with the appropriate solvent.

Following the development of a suitable bioautographic technique, the best chromatography systems were tested using picogram loadings of cobalamins to simulate plasma extracts.

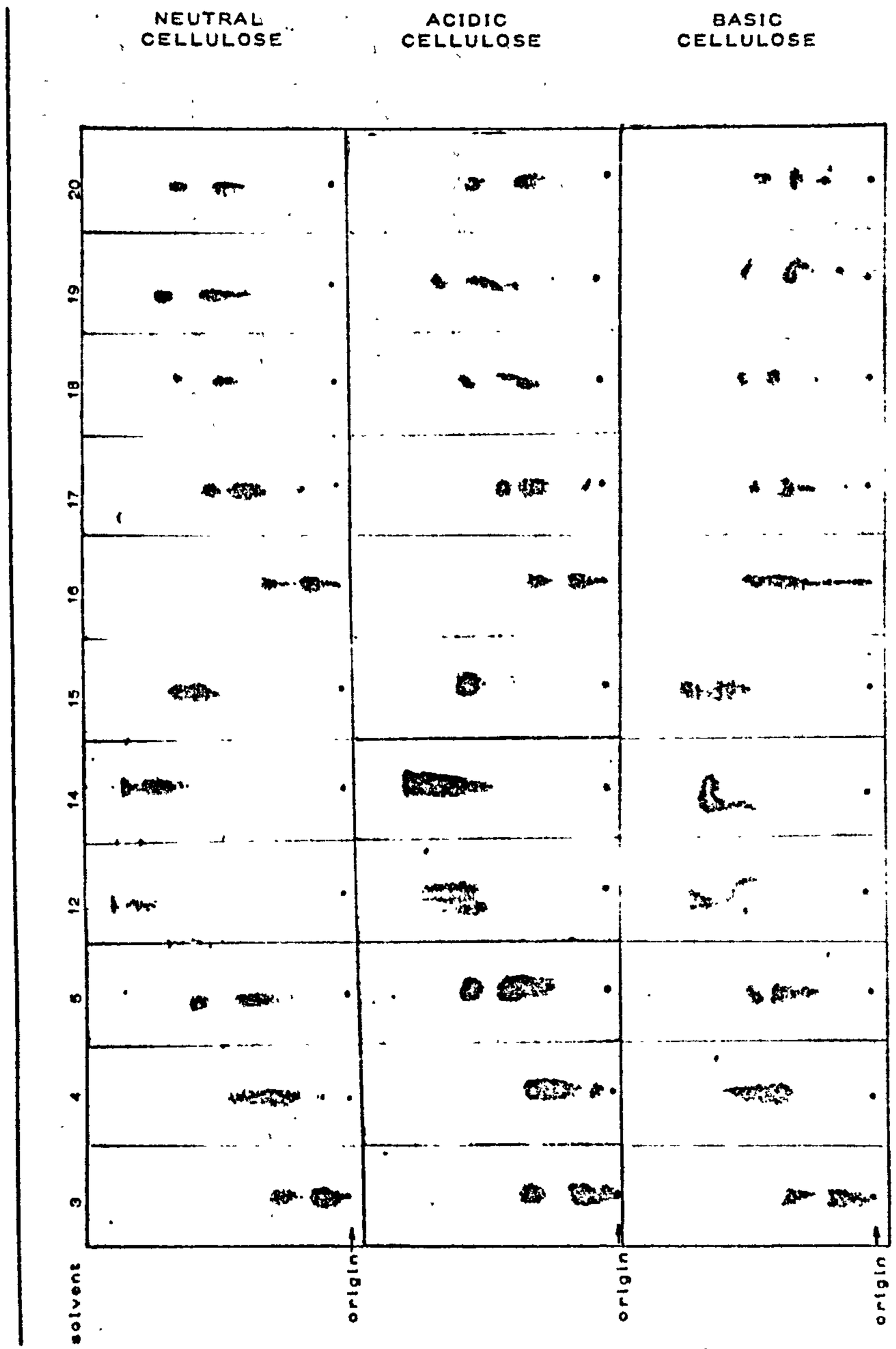


Fig. 3.3 – 1 The search for a suitable chromatography system: Results from screening various combinations of solvent/adsorbent on micro-TLC plates. An aqueous mixture of the four cobalamins was applied as a visible spot at the origin on each plate. Adsorbents and solvent systems are described in Table 3.3-1 and 3.3-2 R_f values are listed in Table 3.3-3

Results

Results from the pilot experiment, using 77 mm x 25·5 mm plates, are recorded in Table 3·3 - 3, and typical chromatograms shown in Fig 3.3 - I. The cobalamins were absorbed strongly to silica gel layers and many solvents failed to move the mixture from the origin. None of the solvent systems separated more than two zones from the mixture, and while solvents 12 and 14 in conjunction with a basic layer produced the highest R_f values, neutral silica gel developed with solvent 20 clearly provided the best resolution and most symmetrical spots.

On cellulose layers, the mobility of all cobalamins was higher, and over half the solvents separated the mixture into two distinct zones. Development of either neutral or acidic cellulose with solvent 17 separated three components, while a similar separation, but of slightly better resolution, was achieved on a basic layer in solvents 18, 19 and 20.

Chromatography on alumina yielded a wide range of R_f values for the mixture in the different solvents, but zones were frequently diffuse and in some cases only streaks appeared. On neutral alumina, the most effective solvent systems were Nos. 3 and 16, where R_f values were low but three zones were just

TABLE 3.3 - 3

SEPARATION of AQUEOUS COBALAMIN MARKERS
on MICRO - T L C PLATES (R_f values x 100)

ADSORBENT	SOLVENT SYSTEM 3	4	5	12	14	15	16	17	18	19	20
Silica gel (neutral)	NS	NS	6,12	6,12	10,20	5	NS	NS	6,12	5,12	12,22
Silica gel (acidic)	NS	NS	NS	15	9,14	10,15	NS	NS	3,12	8,12	12,20
Silica gel (basic)	NS	NS	5,13	25,37	19,34	10	NS	NS	NS	10	12,15
Cellulose (neutral)	9,25	str 0-50	33,59	str 50,92	75,90	63,70	10,30	21,35,50	3,62	50,72	45,65
Cellulose (acidic)	5,14,34	str 9,12-35	str 30-45,55	str 50-70	str 45-80	35,55	10,26	5,75,40	7,52	str 30-55,65	30,52
Cellulose (basic)	10,30	str 30-60	str 30-40,50	str	str	str 50-90	str 0-50	,30,45	,40,50	10,30,50	13,30,45
Alumina (neutral)	12,23,33	15	60,70	64	75,70	65	10,20,30	22,30	50,43	30	60,72
Alumina (acidic)	str 0-40	str 10-35	str 0-3,50	str 30-80	str 0-70	str 0-80	str 10-30,50	str 11-45	35,60	str 40-60	30,52
Alumina (basic)	str 25-45	40	45-70	75	80	68	45	10,30	55	38	55
Kieselguhr (neutral)	82	68,90	90	95	96	100	65,85	90	95	85,90	87
Kieselguhr (acidic)	20,48	5,50,60	32,53	60	55,73	45,55	25,52	17,40,62	35,60	35,59	34,60
Kieselguhr (basic)	44,75	55,65	64,75	65,78	75,88	str 85-100	49,73	40,72	65,80	30,55,80	72,81
Polyamide (neutral)	str 0-100	str 0-50	NS	10,20	NS	str -40	str 0-60	80	str 0-50	str 0-50	NS

NS=No separation str=streak [ ] = best separations

separated. Acidified alumina was less effective, although two reasonably well-defined zones appeared after running in solvents 18 and 20. The cobalamins are known to be unstable at high pH and it was suggested that some conversion of compounds may have occurred on the basic alumina layers since none of the solvents separated more than one zone (with the possible exception of solvent 17, which incorporated formic acid).

The separation of cobalamins attained on Kieselguhr was generally unremarkable and in many solvents the mixture ran as a single spot, albeit of high R_f. On neutral and basic layers two well-separated zones appeared in solvents 4 and 16, and development of basic Kieselguhr with solvent 19 yielded three zones. On acidic layers, 7 of the 11 solvents produced one or two markedly crescent-shaped zones, but in solvent 17, three spots were resolved and symmetry was better.

Polyamide layers normally find application in the separation of non-polar compounds, and were included in the present series only for completeness. The cobalamin mixture was strongly absorbed by the layer and was not separable by any of the 11 solvent systems, with the possible exception of solvent 12 which partly separated two crescent-shaped bands (Table 3.3-3).

TABLE 3.3-4

ADSORBENT SYSTEMS INVESTIGATED

Adsorbent gradient		*Solvent numbers
Silica gel (neutral) →	Cellulose (basic)	5,15,16,17,18,19,20
Silica gel (neutral) →	Alumina (neutral)	5,15,16,18,20
Silica gel (neutral) →	Kieselguhr(neutral)	5,15

* for composition of solvents, see TABLE 3.3-2

No further work with polyamide layers was undertaken, since it seemed unlikely that the separation of such polar compounds could be achieved on this adsorbent.

Using these preliminary results as a guide, combinations of solvent and adsorbent were selected for further evaluation on 5 cm x 20 cm TLC plates, but it was soon apparent that no reliable separation of all four cobalamins was possible with any of the solvents so far investigated.

AT THIS POINT, the alternatives appeared to be:

a) to investigate a further series of solvent systems, perhaps modifying certain of those already tested;
b) to re-test some of the more promising solvents on chromatograms layered with a mixture of adsorbents

The latter course appeared the more attractive one since information regarding R_f values of the cobalamins on single-adsorbent layers had already been obtained.

Mixed-layer chromatography

Mixtures (1 : 1) of 'fast' and 'slow' adsorbents were developed in selected solvents, and from these and the previous results appropriate combinations of solvent and adsorbents were chosen for thin gradient-layer chromatography. Using 10

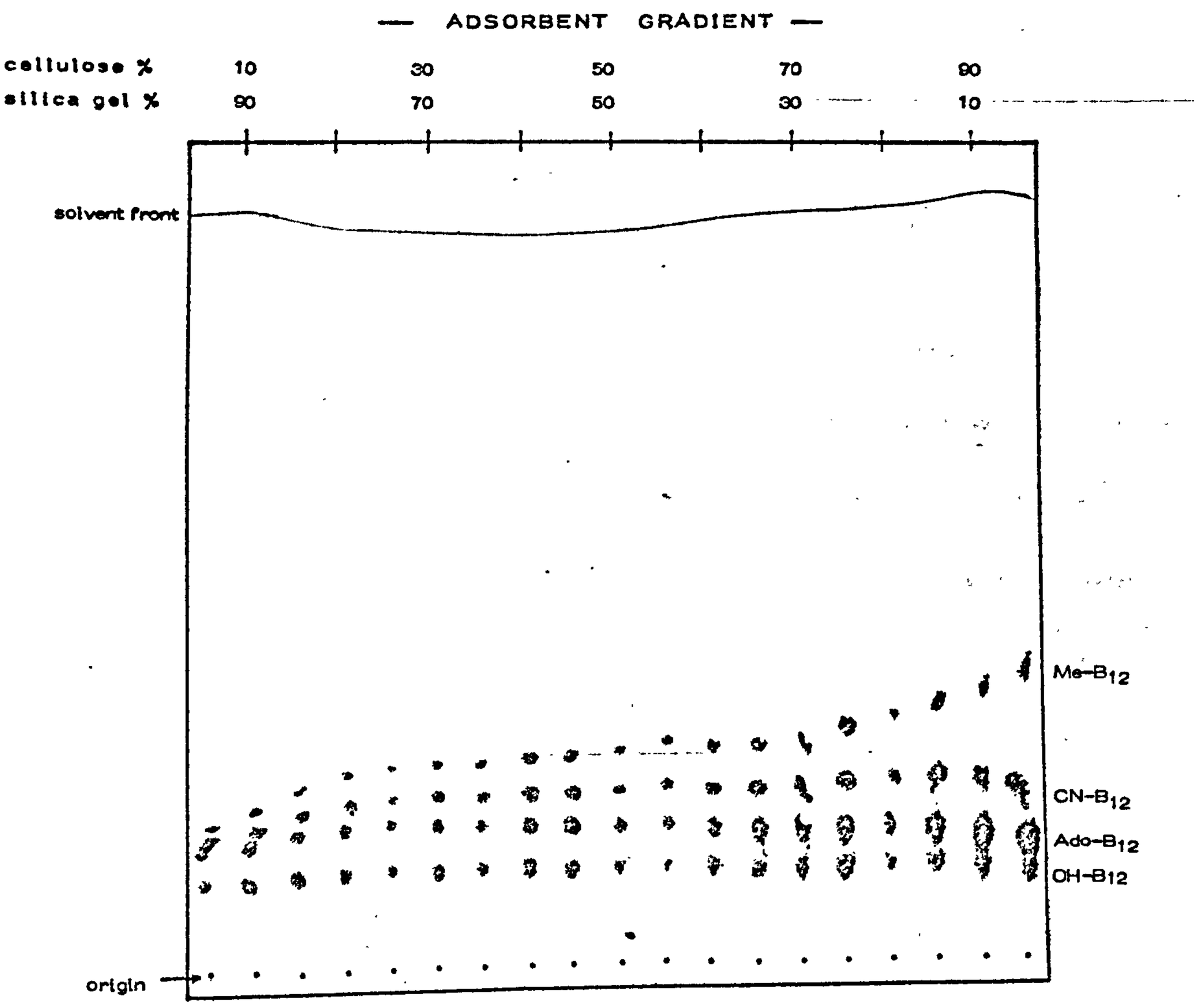


Fig. 3.3 – II Separation of an aqueous cobalamin mixture on a thin gradient layer of cellulose CC41 and silica gel G. Visible spots of the mixture were applied at the origin. The chromatogram was developed in solvent 20

applications of the cobalamin mixture per plate, three adsorbent gradients and seven solvents, the equivalent of a further 140 chromatographic systems were evaluated. Details of the solvents and adsorbent mixtures used are given in Table 3.3 - 4. The best separations were realised on cellulose layers containing between 20% and 30% silica gel and developed with sec.butanol / ammonia (Fig 3.3 -III), although a number of other systems (Fig 3.3 - IV) separated the cobalamin mixture almost as well. Results were confirmed on homogenous mixed-layer plates using both visible and picogram loadings (the latter requiring bioautographic location). Separation of the cobalamins from the first plasma extracts showed only two and sometimes three zones corresponding to Me-B_{12}, Ado-B_{12} and traces of CN-B_{12}, while recovery of cobalamins added to plasma underlined that OH-B_{12} and Ado-B_{12} under such conditions ran with identical R_f (details in Validation of the chromato-bioautographic method). It was clear that the separation of OH-B_{12} and Ado-B_{12} could only be achieved under quite different conditions which might well prove unsuited to the resolution of the other cobalamins.

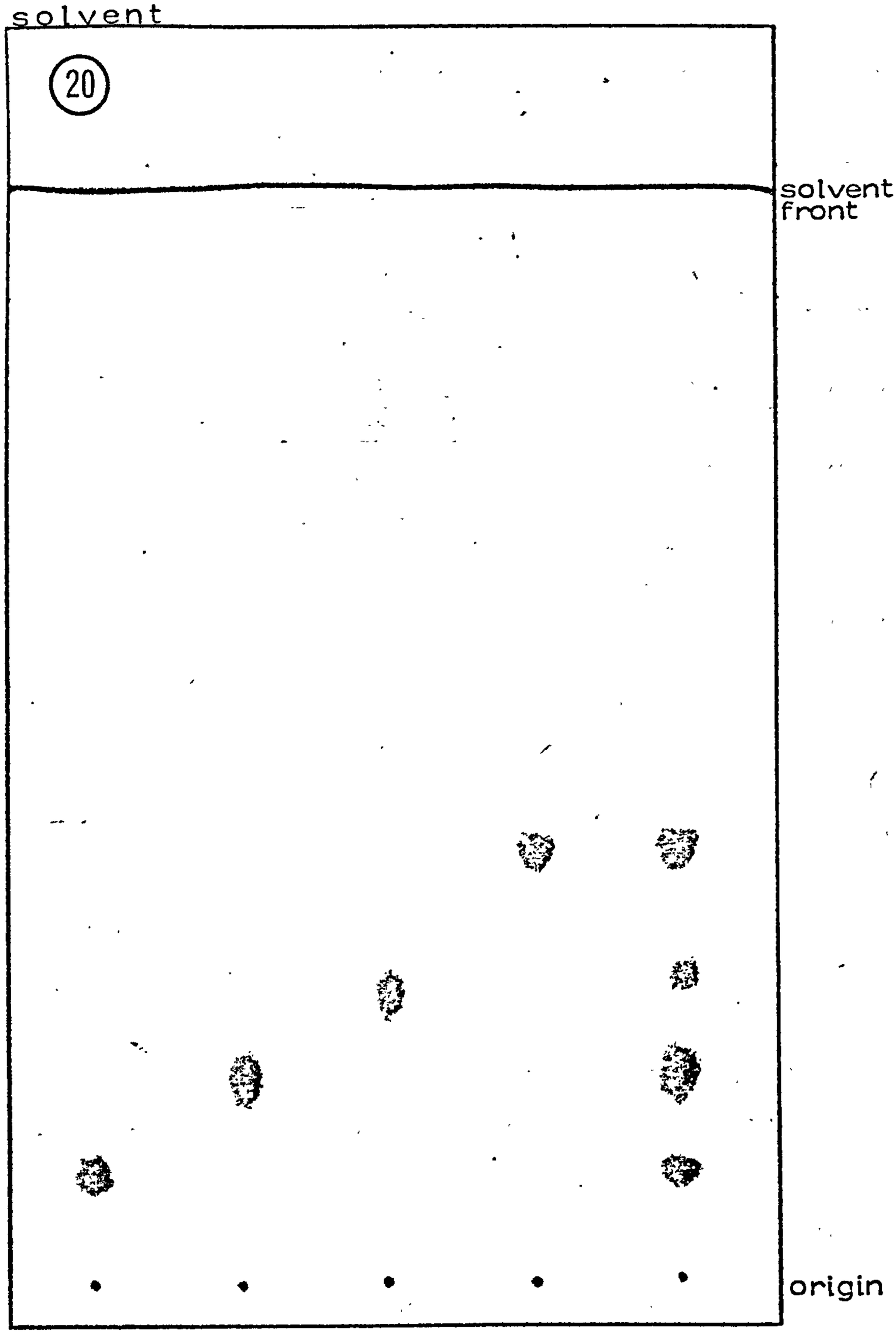


Figure 3.3-III Chromatography in solvent 20 of cobalamin markers applied as visible loadings to a TLC plate coated with a mixture of cellulose CC 41 and silica gel G (3:1)

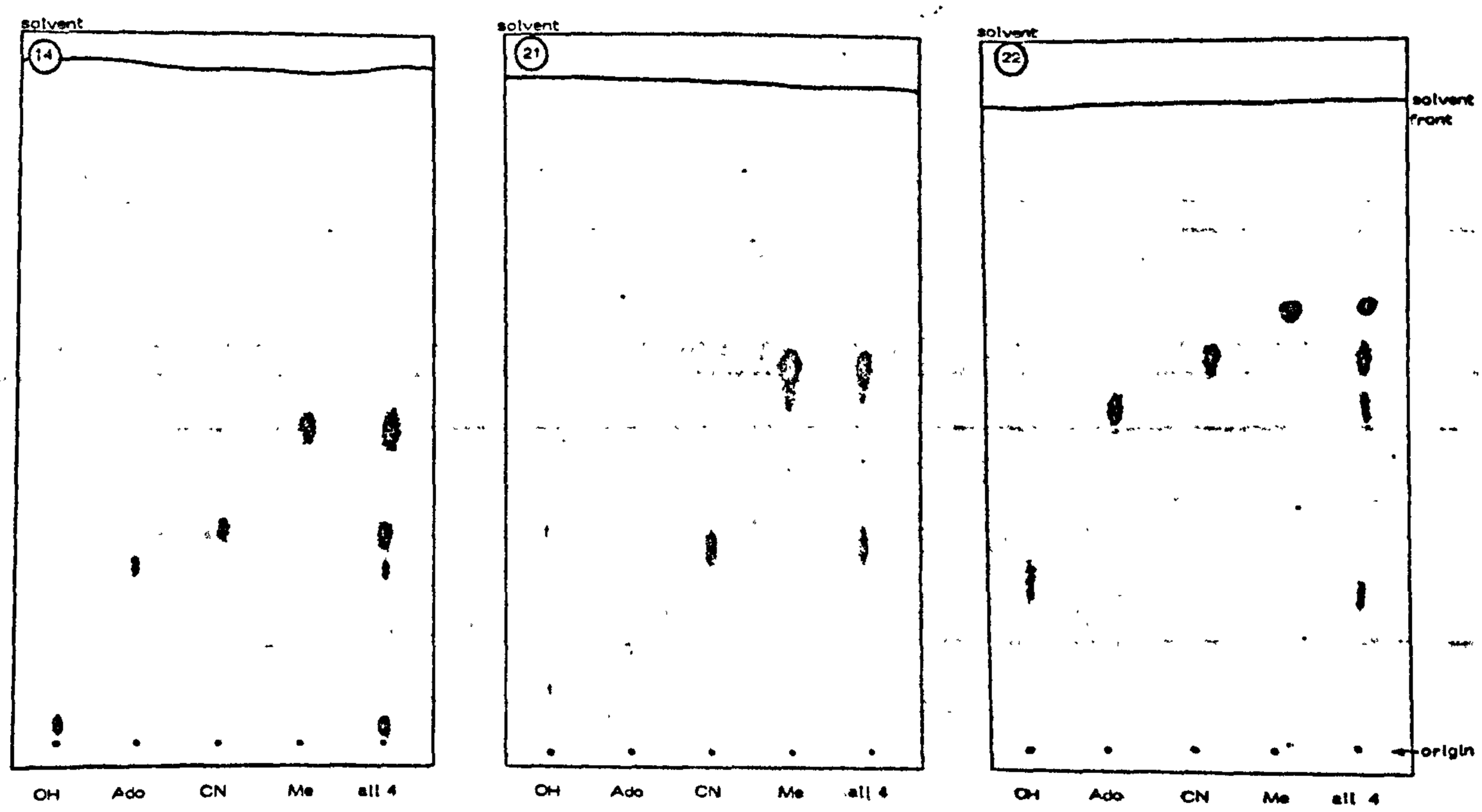


Fig. 3.3 - IV Separation of cobalamin markers at visible loadings on thin layers of cellulose and silica gel (3:1), in solvent systems 14, 21 and 22

Two-dimensional chromatography

$OH-B_{12}$ can be separated in a number of solvent systems (e.g. N/1000 acetic acid or water saturated with benzyl alcohol) from the other cobalamins which remain incompletely resolved. After some experiment it was found that a cobalamin mixture when applied to a cellulose/silica gel (3:1) layer could be completely separated by two-dimensional chromatography if the chromatogram was first developed in sec.butanol/ammonia and then turned at right angles and re-developed in water saturated with benzyl alcohol. Owing to the high mobility of the second solvent, it was necessary to limit the development time most carefully to prevent general diffusion adversely affecting the resolution.

3.4 BIOAUTOGRAPHY

Introduction

The cobalamins are highly coloured compounds ranging from deepest crimson to a brick-orange according to the substituent group on the central cobalt atom, and appear as visible spots on chromatograms when more than about 5 µg is applied. Smaller amounts cannot readily be detected by eye, but some added sensitivity may be gained using the O-tolidine reaction (Stahl, 1965) which gives an intense blue colour with as little as 0.2 µg cobalamin.

The highest detection sensitivity is afforded by isotopic labelling of the cobalamin molecule, and ^{14}C and ^{32}P have been used for this purpose although only low specific activities can be obtained (Shaw and Bessell, 1960). Various isotopes of cobalt may be introduced into the cobalamin molecule during microbial synthesis, and stable compounds incorporating ^{60}Co and ^{58}Co have been produced, but the highest specific activities are obtained using ^{57}Co (see Radioisotopic assay of total B_{12}). By this means less than 1 pg cobalamin may readily be located on a chromatogram by γ-scintillation counting techniques.

TABLE 3.4-1

COMPOSITION of BIOAUTOGRAPHIC ASSAY MEDIUM

Salt mixture	Di-potassium hydrogen phosphate	14.0 g
	Potassium dihydrogen phosphate	6.0 g
	Sodium citrate	1.0 g
	Magnesium sulphate, heptahydrate	0.2 g
	Ammonium sulphate	2.0 g
	Sodium chloride	0.1 g
	Distilled water	to 1.0 l

Ingredients are dissolved separately, mixed, and the solution filtered, before adjusting pH to 7.0 The solution is dispensed into screw-capped bottles (125 ml aliquots), autoclaved at 15 lb/in^2 for 20 min, and stored at room temperature

Double strength agar	Agar (Davis, New Zealand brand)	3.2 g
	Distilled water	115 ml

The agar is soaked in screw-capped bottles for 15 min, autoclaved at 15 lb/in^2 for 20 min, and stored at room temperature

Using non-radioactive cobalamins, the most sensitive means of detection relies on the growth promoted in a B_{12}-dependent micro-organism. As a means of locating cobalamins on a chromatogram, a suitable microbiological culture supported in a solid medium is incubated in contact with the chromatogram, when the resulting growth areas form a bioautographic record of the separate cobalamins. Various organisms have been used and the ideal culture should be equally sensitive to a few pg of each cobalamin and capable of rapid growth. A strain of E.coli fulfils these requirements (Lester Smith, 1965), and detectable growth occurs in contact with 1 pg to 2 pg cobalamin.

Procedure

Use was made of a B_{12}-responsive strain of E. coli (NCIB 9270) developed for the cup-plate assay of this vitamin. It seemed likely that the agar-based medium might equally well serve for bioautographic use, which proved to be the case.

The medium (Table 3.4 - 1) was made up in two parts, agar and salt mixture, separately. In use, glucose was added to the salt solution and mixed with the melted agar before seeding. With paper chromatography the seeded agar may be poured into a flat dish, allowed to cool and the chromatogram

placed on the gel. However, using thin-layer chromatography, a problem arises, since any contact with the surface of the chromatogram may displace the layer. To find the most satisfactory bioautographic method with thin-layer chromatograms the following methods were assessed:

1 The chromatogram, either dry or pre-wetted by sterile saline spray, was inverted on to the agar layer, previously poured on to a glass plate and allowed to cool

2 Warm seeded agar was poured over the chromatogram either dry or after pre-wetting with a saline spray

3 Warm seeded agar was poured over the chromatogram after covering the plate with damp filter paper

4 The chromatogram, covered with damp filter paper, was overlaid with a square of agar gel cut from the cooled layer

5 The agar gel was covered with a square of filter paper, cut to size and the 'sandwich' transferred to the chromatogram, being laid either paper or agar side down

6 The chromatogram was sprayed directly with warm seeded agar sol at approximately 50°

7 The chromatogram was first sprayed with hot sterile agar then coated with a further layer of poured seeded agar

Results

1 Inversion of the chromatogram onto a poured layer of seeded agar gel resulted in the occlusion of many air bubbles which could not be removed either by roller or by direct pressure on the agar surface. Spraying the chromatogram with sterile saline as a preliminary step did not materially assist in the removal of trapped air

2 Pouring the warm agar medium directly onto the chromatogram caused many small air bubbles to become irremovably set in the cooling gel. Pre-wetting the chromatogram was of some help but a few bubbles still became trapped and in addition the TLC layer was liable to become displaced by the flowing sol as the medium was poured on

3 Covering the chromatogram with damp Whatman No 1 paper helped stabilise the layer but a few air bubbles still became trapped, and these were difficult to displace before the gel set

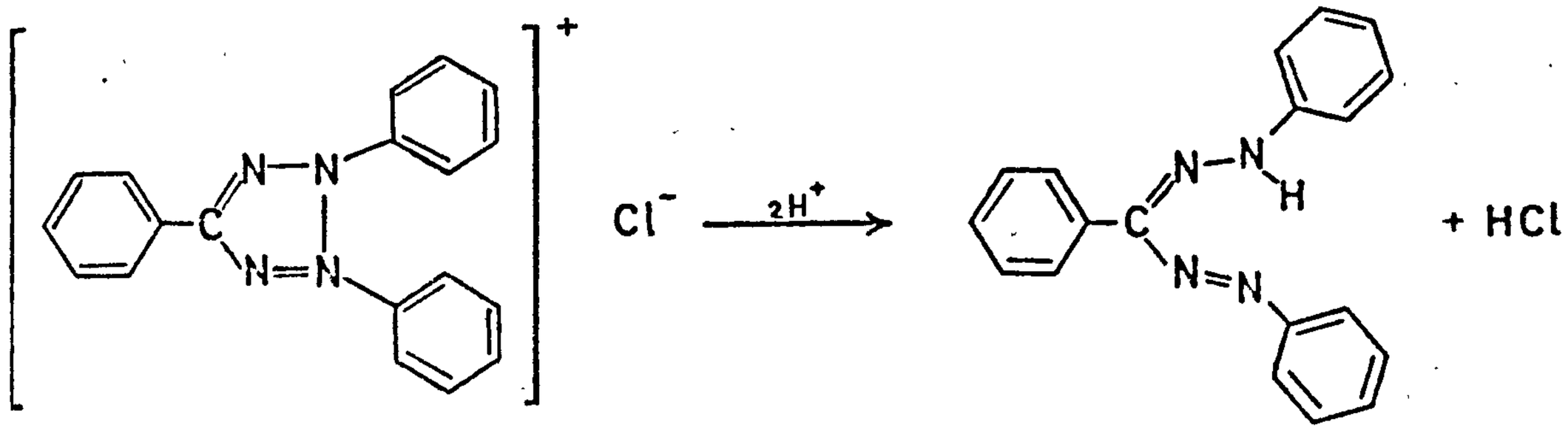
4 Pouring an even layer of agar on a levelled glass plate proved to be quite simple and the few air bubbles formed were readily eliminated with a bunsen flame or sterile needle. Transfer of agar to the chromatogram, however, proved more hazardous, the low tensile strength of the gel often resulting in its tearing in mid-transfer

5 Covering the agar with a square of Whatman paper provided sufficient strength to facilitate the uneventful transfer of gel to the chromatogram. It was found that the 'sandwich' was best laid paper side down. This minimised disturbance of the chromatogram and hence 'blurring' of the bioautogram, while trapped air bubbles could be readily displaced by hand, using a sterile glove to prevent contaminating the bioautogram

6 Attempts to spray a layer of seeded agar onto the chromatogram were doomed to failure since the medium could not be taken to a high enough temperature without killing the inoculum. When temperatures of up to 50^{o} were used, cooling of the medium at the nozzle blocked the spray

7 In order to coat a TLC plate satisfactorily by spray, the sterile agar needed to be maintained at 80^{o} or above, but owing to the temperature drop at the nozzle, frequent blockage still occurred, making it difficult to form an even layer on the chromatogram

The best of the methods tested was without doubt No 5, where the chromatogram was overlayered with an agar/paper sandwich, paper side down. With practice this step could be preformed so that the agar was placed in accurate alignment with the chromatogram (no movement could be permitted once the sandwich had touched the TLC plate).



2:3:5-Triphenyl Tetrazolium Chloride
(Colourless)

Triphenyl Formazan
(Red)

Figure 3.4 - I

Reduction of the tetrazolium growth-indicator to triphenyl formazan

Following overnight incubation, opalescent zones appeared on the bioautogram in positions corresponding to the cobalamins separated by chromatography. An accurate copy technique was needed to record the results but it was found that owing to the poor contrast obtained by direct illumination, normal photocopies were unsatisfactory. The alternative would have been to photograph the plate by edge-illumination, but no facilities for this existed. Bacteriological growth-indicators have been reported (Ford and Holdsworth, 1953) and these generally rely on the chemical reduction of a compound incorporated in the medium to form a coloured product. 2,3,5-triphenyl tetrazolium chloride is a suitable redox indicator as it is converted to the crimson-red compound triphenyl-formazan (Fig 3.4 - I) in contact with a growing micro-organism (Pegram, 1969). Inclusion of a small amount of this indicator in the agar medium gave crimson zones on the bioautogram which could readily be photocopied and also enabled a simple semi-quantitative method to be developed.

Influence of inoculum age on bioautographic response

As experience with the method increased, it was found that bioautographic response could be greatly influenced by the conditions under which the inoculum was grown. To investigate

this more fully, bioautograms of aqueous cobalamins separated chromatographically were prepared using inocula of different ages.

An aqueous mixture of the four cobalamins was applied to ten 20 cm square TLC plates (25 pg and 50 pg to each in quadruplicate) and chromatograms were developed together in a Shandon multiplate tank with sec.butanol/ammonia/water (75 : 2 : 25). E.coli from an agar maintenance slope was sub-cultured on blood agar at 37° for 20 hr. Two colonies were transferred to peptone water (3 ml) and incubated for 4 hr at 37° before being mixed with a further volume (20 ml) peptone water and incubated continuously for 44 hr. Aliquots (2 ml) were removed at intervals to inoculate sequentially ten batches of agar medium. The optical density of each inoculum was measured at 545-635 nm in a Unicam SP 1300 colorimeter and in some cases the viable cell-count was estimated. After seeding, the batch of agar medium was divided between two plates and when cool a rectangle 20 cm x 10 cm was cut from each to cover the duplicate halves of a chromatogram.

The bioautogram was then incubated for 24 hr at 37°, when the zones were scanned by densitometer and an average value calculated from the duplicated cobalamin spots on either side of the bioautogram.

Results

There were marked differences in the sensitivity to cobalamins obtained from the various batches of seeded agar, and densitometric results from the bioautograms correlated poorly with either the optical density or viable cell-count of the original inoculum (Table 3.4 - 2). Since both these parameters for the inoculum were highest after the longest incubation time (44 hr at 37°), it was clear that a factor other than maximal inoculum growth was responsible for the high bioautogram sensitivity. The growth-response of each batch of medium was compared by calculating the difference between the densitometric areas of the 25 pg and 50 pg cobalamin zones. This provided some indication of the slope of the growth-response curve from each bioautogram. The mean differences were plotted against inoculum age, when it was seen that the points lay on a hyperbola (Fig 3.4 - II), with a maximum value corresponding to the inoculum which had been grown for 20 hr at 37° and a further 96 hr at 4°.

The results suggest that the age of the culture is a more important requirement for sensitive bioautography than either the total or viable cell-count of the inoculum. At 37°, rapid cell division continues as long as adequate nutrients are available,

whereas at lower temperatures the time between divisions increases progressively. Although the reason for the greater sensitivity of the older cells is not known, it may be that these become relatively more B_{12}-depleted than those in active division and so react more dramatically when provided with small amounts of cobalamin from the chromatogram. Later experiments showed that the viability of the culture was better maintained over long periods if the peptone water incubation was carried out aerobically in a shaking water-bath, while the growth of contaminants was discouraged if the temperature of all incubations was recuced to 27^{o}.

3.5 CHROMATOGRAM QUANTITATION

Methods developed thus far enabled vitamin B_{12} to be estimated in blood and other tissues, and the constituent cobalamins to be separated. The early cobalamin patterns were assessed from the bioautogram by eye, using a simple score system. The major zone was assigned a value of +++, and the other zones ++,+ or $\pm$, according to their size and density. This at least enabled results to be compared and provided some guide to the proportions of each cobalamin making up the total B_{12}. It was not possible to record small differences with this scheme however, and later it was found that the smallest spots had been somewhat overestimated owing to the non-linear growth response of the culture organism. In the search for a suitable means of quantitating the separated cobalamins, the following methods were investigated:

1 Zone elution

The aim was to elute cobalamins from the chromatogram, convert to cyanocobalamin and estimate the concentration by radioisotopic assay. To determine the positions of the cobalamins on the chromatogram, markers were run at either side of the plasma extract and located

bioautographically using narrow strips of seeded agar. The main adsorbent area was then divided into bands to correspond with the markers and each zone removed from the plate using a small spatula. The adsorbent was collected with the aid of a vacuum pump into the broad end of a pasteur pipette whose tip had first been cut short and plugged with cellulose tissue. Cobalamins were eluted from the column with water or acetate/cyanide buffer, pipetted onto the adsorbent, and forced through with air from a large syringe. At first, labelled cyanocobalamin was used to help decide on the most efficient elution technique. Zones from the chromatograms of plasma extracts were then eluted and assayed by the radioisotopic method.

2 Elution of indicator from the bioautogram

2,3,5-triphenyl tetrazolium chloride, included in the bioautographic medium, is reduced during the growth of the organism to crimson-coloured triphenyl-formazan (Fig 3.4 - I). This is a stable compound, soluble in alcohol and acetone but insoluble in water (Pegram, 1969), although it was found that in dilute aqueous agar a coloured sol was formed. Zones were cut from the bioautogram with a cork-borer and immersed in a small volume of water or ethanol. The agar was dissolved in the water by heating at 100°, but with ethanol, slight warming was sufficient to release the formazan directly into the solution, when the agar dish could be removed. The visible spectrum of the solutions was recorded by spectrophotometer with 1 cm

micro-cuvettes, and using the wavelength of maximum absorption, the extinction of the formazan corresponding to chromatography standards of different cobalamin concentrations was measured.

3 Densitometric scanning

The bioautogram on removal from the chromatogram presents a pattern of coloured zones on a translucent and almost colourless background. If the area and density of each zone were measurable directly, this would provide the basis of a simple quantitative method. The response to the bioautogram zones was therefore measured in a recording densitometer (Joyce-Loebl 'Chromoscan') designed to scan electrophoretic strips, etc., either by transmitted or by reflected light. Zones were recorded as peaks whose area was integrated automatically. After adjusting the instrument for optimum response, bioautograms of samples and cobalamin standards were scanned and compared.

RESULTS

1 Zone elution

Hot acetate/cyanide buffer pH 4.5 was the most effective eluant tested and at temperatures of $70^{o}-80^{o}$ a small volume of buffer would elute 90% to 100% of the aqueous cobalamin from a small column. Likewise, after chromatography of 100pg - 120pg aqueous ^{57}Co-labelled cyano- or hydroxocobalamin, 92% to 102%

was recovered in a 2ml eluate of the respective zone. With lower chromatogram loadings (10pg-20pg), 73% to 83% of the cobalamin was eluted. Experiments with plasma were less successful; when extracts from normal plasma containing 200pg - 500pg cobalamin were applied as a band to chromatography plates, eluates of the separated zones were found to contain collectively no more than 10% to 15% of the plasma total B_{12}. It has previously been shown that OH-B_{12} binds firmly to silica (Dony and Conter, 1956). Using plasma equilibrated with ^{57}Co cyanocobalamin before extraction, it was possible to show that much of the cobalamin separated on the chromatogram remained bound to the adsorbent and was not eluted. With a different solvent, efficient elution may have been possible but as this would have involved further steps in returning the cobalamin to water or acetate/cyanide buffer in order to complete the assay, the extra development of this already lengthy procedure seemed unwarranted and the method was abandoned.

2 Elution of indicator from the bioautogram

Macerating the bioautogram zone in hot water did not yield a solution suitable for colorimetric use. Apart from the inevitable dilution of colour, the sensitivity of the method was impaired by the opalescent background of the agar itself. In practice, there was a

further complication since the agar set at or below 35°, hence all tubes and cuvettes had to be kept warm. Extraction with warm ethanol was more satisfactory; at 35°, the formazan was readily extracted from the agar, which could then be removed from the tube with forceps, leaving a clear alcoholic solution. This had a visible spectrum with peak absorption at 475nm. Zones corresponding to a series of cobalamin standards were cut from the bioautogram with a cork borer 1 cm in diameter. Each was dissolved in warm ethanol (1 ml) and the OD of the formazan solution read in 1 cm microcuvettes at 475nm. When plotted against concentration, these gave a satisfactory standard curve down to 10pg cobalamin.

Although this might have proved a satisfactory means of quantitation, it had the disadvantage of adding another stage to the procedure. If the zones could be estimated without recourse to dye dilution, the method might be shortened and the bioautogram perhaps preserved for future reference.

3 Densitometric scanning

This quickly showed promise of being a technique likely to furnish a satisfactory method for quantitating bioautograms; optical densities of the zones could be measured directly without

elution, and samples compared with standards. Using a Joyce-Loebl 'Chromoscan', the best operating conditions were found in the reflectance mode with a mid green filter (No.5-044) in the light beam. A slit 10mm x 2mm just covered the largest zone while resolving adjacent peaks with a valley less than 10% of the peak height. Peaks were integrated automatically. To confirm the linearity of this operation, counts were recorded over 5 cm of chart and plotted against the distance the pen had been pre-set from its zero. A straight line was obtained for pen deflections between 0.5 cm and 13.5 cm. Theoretically, there should have been an advantage in using the instrument as a transmission densitometer, but there was no increase in sensitivity in this position and it proved more difficult to set a constant baseline than when using it as a reflectance instrument.

Bioautograms from known concentration aqueous cobalamins were scanned by the densitometer, and graphs of peak area against concentration were prepared. The response of the instrument proved satisfactory down to 10 pg, but below this, peaks were small and very variable, due in part to inertia in the mechanically-driven recorder system. Later on, another densitometer (Gelman 'Gelscan') became available and was used with success for some months. This was a transmission instrument

of high sensitivity capable of recording the smallest zone detectable on the bioautogram by eye. Peak areas were plotted by a second recorder pen driven by a disc integrator.

With both the 'Chromoscan' and 'Gelscan', bioautograms had to be cut into strips to be accepted by the sample holder, so when, later still, a densitometer became available which not only had the merits of the other instruments but would also accommodate a complete bioautogram without cutting, this was adopted as an ideal instrument for the method (Kipp and Zonen recording and integrating transmission densitometer). Highest sensitivity was achieved with this instrument's linear amplifier, when zones corresponding to 1 pg - 2 pg cobalamins were capable of estimation. Peaks were recorded on a 10-inch-wide chart and integrated automatically by digital counter. Plasma concentrations of each cobalamin could then be calculated using factors derived from the volume of extract applied to the chromatogram and the extraction efficiency:

$$\text{ZONE COBALAMIN} \times \frac{V(E)}{V(TLC)} \times \frac{100}{E} \times \frac{1}{V(pl)} = \text{plasma concentration (pg/ml)}$$

where V(E) = total volume of extract (µl)
V(TLC) = volume applied to chromatogram (µl)
V(pl) = volume of plasma extracted (µl)
E = extraction efficiency (%)

Considerable errors were likely to be introduced in using this formula since much depended (a) on the accurate measurement of the small volume (1 μl to 5 μl) applied to the chromatogram, and (b) on the extraction efficiency, for which no individual value was normally available. A simple scheme was therefore devised which did not require exact knowledge either of the chromatogram loading or of the actual extraction efficiency. The amount of cobalamin in each zone was expressed as a percentage of the amount in all zones separated from the sample, and these percentages, with the plasma total B_{12} as estimated by radioisotopic assay, used to calculate the sample concentration of each cobalamin:

$$\frac{\text{Zone cobalamin (pg)} \times 100}{\text{Total cobalamins all zones}} \times \text{Total } B_{12} \text{ (pg/ml)}^* = \text{Cobalamin (pg/ml)}^*$$

---

* or pg/mg as applicable

METHODS 4

4.1 COLLECTION and PREPARATION of SAMPLES

Owing to the light sensitivity of cobalamin coenzymes, precautions were taken as described below to minimise the exposure of all samples to white light.

Plasma Using a syringe covered with aluminium foil, venous blood (10 ml) was taken and ejected into a foil-covered heparin tube. After mixing, the tube and contents were centrifuged for 10 min at 2,000 x G, and the plasma removed to a clean tube by the light of a red photographic 'safelight'. The sample was then foil-wrapped and stored at -20^{o} until the analysis.

Erythrocytes Blood was collected as for plasma and the cells centrifuged down. Plasma and buffy coat were removed and the packed erythrocytes, without further treatment, frozen in a foil-covered tube.

Leucocytes Freshly drawn blood (10 ml) was mixed in the darkroom with 'Dextraven-110' (Fisons), 10 ml, in a 20 ml disposable syringe. The syringe was left in a vertical position with tip uppermost for 45 min, when sedimentation of the red

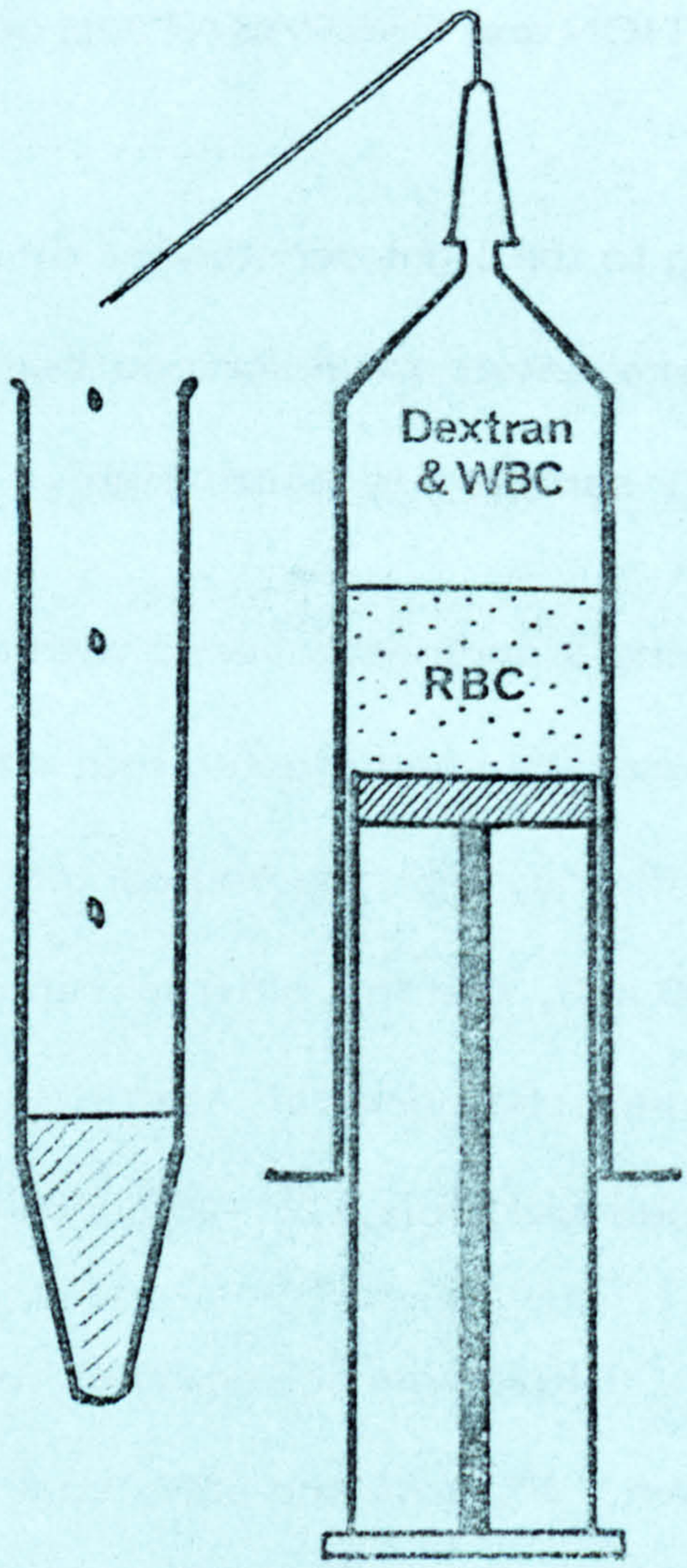


Figure 4.1 - I Technique used in separating leucocytes from whole blood

cells was complete. A 21 G x 40 mm hypodermic needle was bent through an angle of approximately 130° and carefully fitted to the syringe. The upper dextran + WBC layer was then expelled into a 15 ml conical centrifuge tube placed beneath the needle tip (Fig 4.1 - I). The cells were centrifuged down (10 min at 1,500 x G), the dextran was aspirated, and the pellet resuspended in distilled water (1 ml) to lyse the residual erythrocytes. After 30 sec, saline (10 ml) was added to the tube and the leucocytes were again centrifuged down. The saline was aspirated from the tube and the walls wiped with paper tissue to leave the cell pellet as dry as possible. The foil-wrapped tube and its contents were then weighed, and the cells lysed by the addition of a known volume of water (3 ml - 5 ml). Thus prepared, the sample was transferred to a clean foil-covered tube and frozen until required. The centrifuge tube was finally dried and reweighed with its foil wrapping in order to calculate the weight of the leucocytes.

Bone marrow A specimen taken by marrow needle from either the iliac crest or sternal cavity was ejected into a foil-covered heparin tube. The marrow was transferred by Pasteur pipette to an haematocrit tube and centrifuged (10 min at 2,000 x G). The fat and plasma layers were removed with a Pasteur pipette. The marrow cells, which appeared by red light paler than the

underlying erythrocytes, were carefully removed and washed into a 15ml conical centrifuge tube with saline. The lower red-cell layer was likewise transferred to a second conical tube. The cells were then centrifuged down, and the saline was aspirated. The walls of each tube were dried with paper tissue, leaving behind the moist cell pellet. The foil-wrapped tube was weighed and the cells lysed in water (3ml - 5ml according to sample). The specimens were finally transferred to clean tubes labelled 'marrow' and 'red marrow', foil-protected, and stored at -20^{o} until required. Each centrifuge tube after drying was weighed together with its foil wrapper and the cell weights calculated.

Liver and other tissues Biopsy specimens were received in foil-covered tubes. The tube was weighed before and after removal of the sample to a Potter-Elvehjem homogeniser. The biopsy was homogenised in water (5ml) by red light and the homogenate transferred to a clean tube, foil-wrapped, and frozen until analysis.

With fresh autopsy specimens, a small piece of tissue (approximately 100mg) was cut by red light from the centre of the sample. This was weighed after wrapping in foil, homogenised in water (10ml) and the resulting homogenate stored frozen in a foil-covered tube.

4.2 RADIOISOTOPIC ASSAY of TOTAL B_{12}

Equipment and apparatus

1 Automatic gamma-scintillation spectrometer (Packard)

2 Centrifuge (MSE Major)

3 Specially-adapted centrifuge buckets (Fig 4.2 - 1)

4 Rotary mixer (Matburn Limited)

5 'Repette' syringe, 2 ml (Jencons Limited)

6 'Hamilton' microsyringe, 25 µl, with Chaney adaptor (V.A. Howe and Company)

7 'Eppendorf' micropipette, 20 µl (V.A. Howe and Company)

8 Bulb pipettes 1,2,3,4,5,6,8 and 10 ml (E-Mil gold line)

9 Rimless bacteriological test tubes 152 x 16.5 mm (A 1 Laboratory Supplies)

10 Crystal polystyrene stoppered tubes 2 ml and 10 ml (Tek-lab Limited)

11 Polystyrene spectrometer tubes 152 x 15.5 mm (Packard)

12 'Kenutuf' polythene filter funnels 385 mm diameter (A 1 Laboratory Supplies)

13 Whatman No. 41 filter circles 7 cm (Reeve Angel)

14 Batswing burner

15 Capillary pipettes (drawn in batswing flame from 12.7 cm Harshaw pipettes) (A 1 Laboratory Supplies)

16 Parafilm (Gallenkamp)

17 Aluminium foil (British Aluminium Company)

18 Fine sand, acid washed (British Drug Houses)

19 Red rubber tube racks (Esco Rubber Limited)

20 Small glass pestle and mortar (Gallenkamp)

Reagents

1 ^{57}Co cyanocobalamin (Radiochemical Centre, Amersham) supplied at a specific activity of not less than 100 µCi/µg; stored at -20°

Working solution: prepare solution containing 1,000 pg/ml in acetate/cyanide buffer; store at -20°. Make freshly at monthly intervals

2 Cyanocobalamin (supplied standardised by Glaxo)

Stock standard: prepare a standard containing 1.0 µg by dilution of ampouled cyanocobalamin in acetate/cyanide buffer; store in plastic tubes as approximately 1.5 ml aliquots at -20°

Working standard: (1,000 pg/ml) Make up 1 ml stock standard to 1,000 ml with water. Prepare afresh before each assay

3 Binding agent

Reconstitute freeze-dried human serum with sterile water and store in 2ml aliquots at -20°. On the day of assay, mix 2 ml serum with 0.6 ml aqueous potassium cyanide (40 mg KCN in 100 ml water, stored at 4°)

Reconstitute fresh freeze-dried plasma at three-monthly intervals

4 Acetate/cyanide buffer

Dissolve 12.0 g sodium hydroxide (BDH Analar) in approx. 200 ml water, and mix with 45.0 ml glacial acetic acid (BDH Analar) and 40 mg potassium cyanide (BDH Analar). Make up to 1 litre and adjust pH to 4.5; store at 4°.

Replace at monthly intervals

5 Saline buffer

Prepare freshly for each assay by mixing 5 ml normal saline (0.85 % NaCl) with 30 ml acetate/cyanide buffer

6 Coated-charcoal suspension

To 7.5 ml acetate/cyanide buffer add 2.5 ml 20 % bovine serum albumin (Stayne Laboratories) and 500 mg activated charcoal (BDH - acid washed, decolorising) and mix well.

Prepare freshly before each assay

Principle

A series of cyanocobalamin standards is prepared and a small constant amount of labelled cyanocobalamin added to each, followed by a constant volume of normal human serum as binding agent. The amount of binding agent used is such that in the lowest standard most of the vitamin will be bound and in the highest most will remain free. After incubation to allow binding to take place, bound and free fractions are separated by adding coated charcoal which adsorbs the free. The radioactivity in each fraction is then determined. A standard curve may be constructed in a number of ways, but for the greatest accuracy at low B_{12} levels, it has been recommended (Ekins, 1965 that the concentration of the standards should be plotted against the ratio Free counts : Bound counts. Estimates of the ratio are most accurate when this approaches unity, corresponding to a low B_{12} concentration.

In estimating the total B_{12}, the various cobalamins present in the plasma or tissue sample are first converted to cyanocobalamin by heating with a cyanide-containing buffer. An aliquot of the extract is treated in the same way as the standards, and its cyanocobalamin concentration determined by reference to the standard curve.

Procedure

Preparation of standards: seven glass test tubes are set out and labelled 1 to 7. 8 ml saline buffer is pipetted into Tube 1, and 3 ml into each of the others. Working standard, 2 ml, is added to Tube 1, and after mixing, an aliquot, 3 ml, is transferred to Tube 2. The contents are mixed and an aliquot, 3 ml, likewise transferred to Tube 3, and so on to Tube 6 (i.e., by doubling dilutions). The resulting standards contain: 200 pg, 100 pg, 50 pg, 25 pg, 12.5 pg and 6.25 pg cyanocobalamin/ml. Tube 7 is the blank.

Extraction of plasma or serum: sample, 1 ml, and acetate/cyanide buffer, 6 ml, are mixed in a glass test tube, sealed with parafilm and foil, and heated in a boiling water bath for 20 min. The tube is removed and when cool the contents are filtered on No.41 Whatman paper, the filtrate being collected in a 10 ml plastic tube. The extract is stored at -20^{o} unless the assay is to be carried out directly.

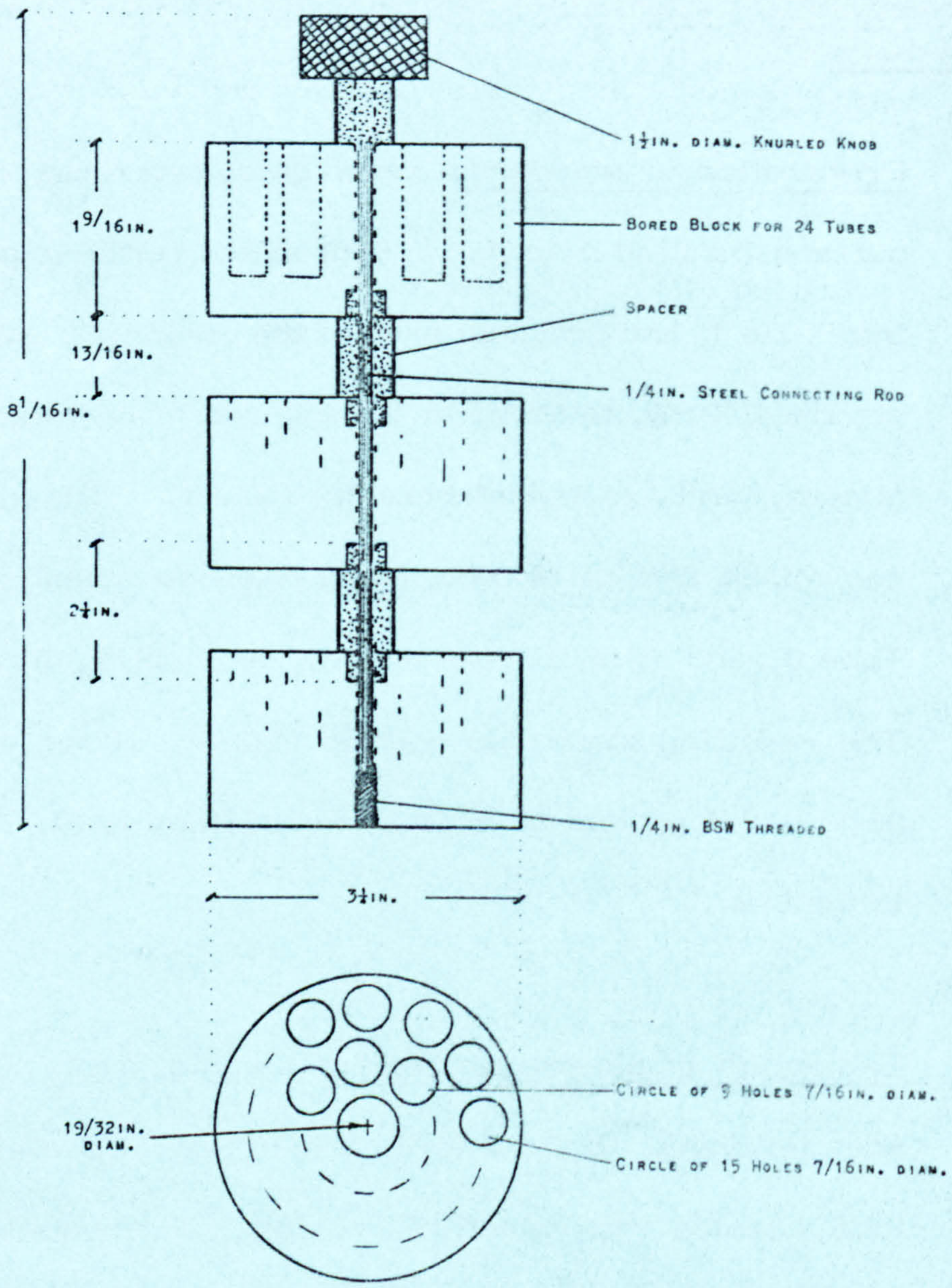


Figure 4.2 - 1

Multiple adaptor for carrying assay tubes during centrifugation. The balanced pair of adaptors accommodate 144 2 ml tubes

Extraction of liver biopsies / autopsy tissues: the weighed tissue is mixed with water, 4 ml, and homogenised in a Potter-Elvejhem homogeniser. An aliquot, 1 ml, of the suspension is mixed with acetate/cyanide buffer, 6 ml, and extracted as for plasma. The filtered extract is diluted with buffer to obtain a B_{12} concentration likely to be below 1,400 pg/ml.

Analysis: 2 ml plastic tubes are set out and labelled in duplicate both for standards and samples. ^{57}Co cyanocobalamin working solution, 20 µl, is added to each by Eppendorf micropipette, followed by standard extract, 1 ml, and binding serum, 25 µl. Tubes are capped and the contents mixed thoroughly before being left 1 hr at room temperature and a further hour at 4° to allow time for binding to take place. Albumin-coated charcoal, 100 µl, is then added by 'Repette' syringe to separate free B_{12} from bound B_{12}, and the tubes rotated for 30 min on a 'Matburn' mixer (25 - 30 r p m) in a 4° refrigerator. The charcoal is centrifuged down for 15 min at 3,000 r p m, using special tube carriers (Fig 4.2 - I) and the supernatant carefully transferred to clean 2 ml tubes by means of fine-tipped Pasteur pipettes. The supernatant containing bound B_{12}, and the charcoal containing the free, are separately counted in a well-type auto-gamma-scintillation spectrometer and

RADIO-ISOTOPIC ASSAY

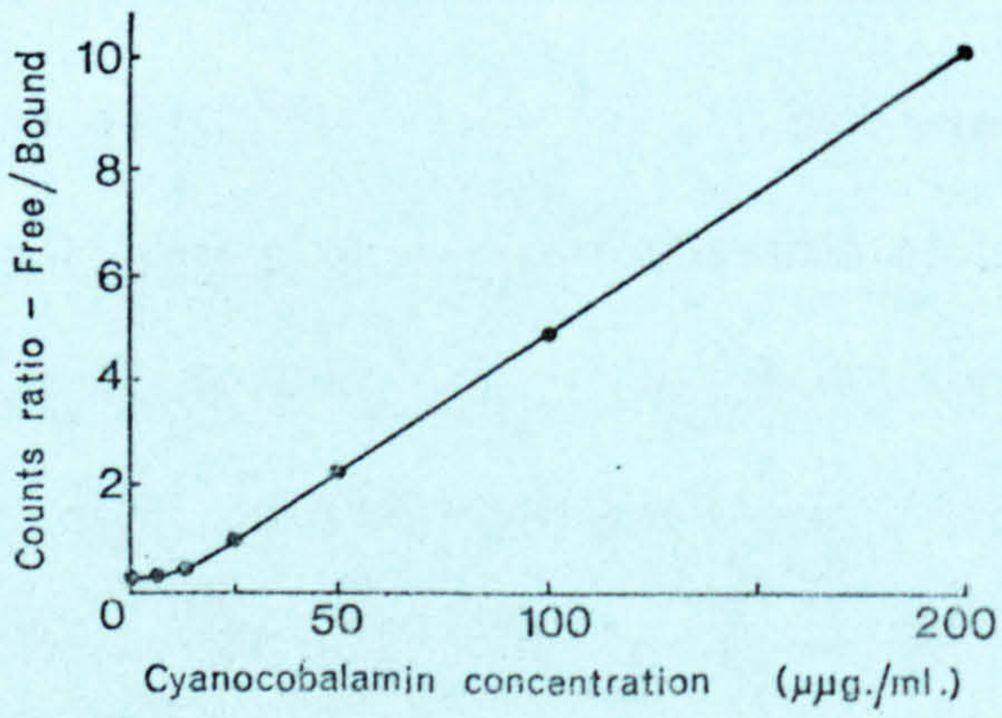


Fig. 4.2 - II A standard curve

the ratio Free counts: Bound counts is calculated. A standard curve is constructed (Fig 4.2 - II) and the total B_{12} concentration of the extract is read off. Plasma or serum B_{12} (pg/ml) is calculated from the extract concentration x 7. Total B_{12} in liver or other organs is calculated from the formula:

$$\frac{\text{Extract concentration} \times 7 \times \text{other dilution factors}}{\text{Tissue weight (mg)} \times 1{,}000} \text{ ng/g}$$

Spectrometer calibration: ^{57}Co is a gamma-emitter with its major peak at 122 Kev. Optimal counting conditions are obtained when the spectrometer is adjusted to receive energies between 102 and 142 Kev (i.e., with a 4% window). While setting up the instrument, a high activity ^{57}Co source is used, and the window, centred on 122 Kev, is closed to 2% in order to improve resolution. The photomultiplier voltage is then adjusted until the count-rate is at a maximum. The peak is re-checked at monthly intervals.

4.3 CHROMATO-BIOAUTOGRAPHIC ESTIMATION of COBALAMINS

Preparation of standards

Equipment and materials

1 Dark laboratory, remote from main laboratory area, equipped with red photographic 'safelight'

2 Spectrophotometer (SP 500)

3 Water bath, 100°, steam heated (Gallenkamp)

4 Micropipettes, 100, 200, 500 and 1,000 µl (Eppendorf: V.A. Howe)

5 Graduated pipette, 10 ml, Grade B (E-Mil)

6 Volumetric flasks, 50 and 100 ml, Grade A (E-Mil, green line)

7 Rimless bacteriological test tubes, 152 x 16.5 mm (A1 Laboratory Supplies)

8 Plastic tubes, 2 ml, stoppered (Teklab Medical Laboratories)

9 Acetate/cyanide buffer - see Section 4.2

10 Crystalline cobalamins: Me-B_{12}, CN-B_{12}, Ado-B_{12} and OH-B_{12} (Glaxo Laboratories)

11 Standardised CN-B_{12}, 100µg/1 ml ampoule (Glaxo Laboratories 'Cytamen')

12 Parafilm (Gallenkamp)

13 Aluminium foil (British Aluminium Company)

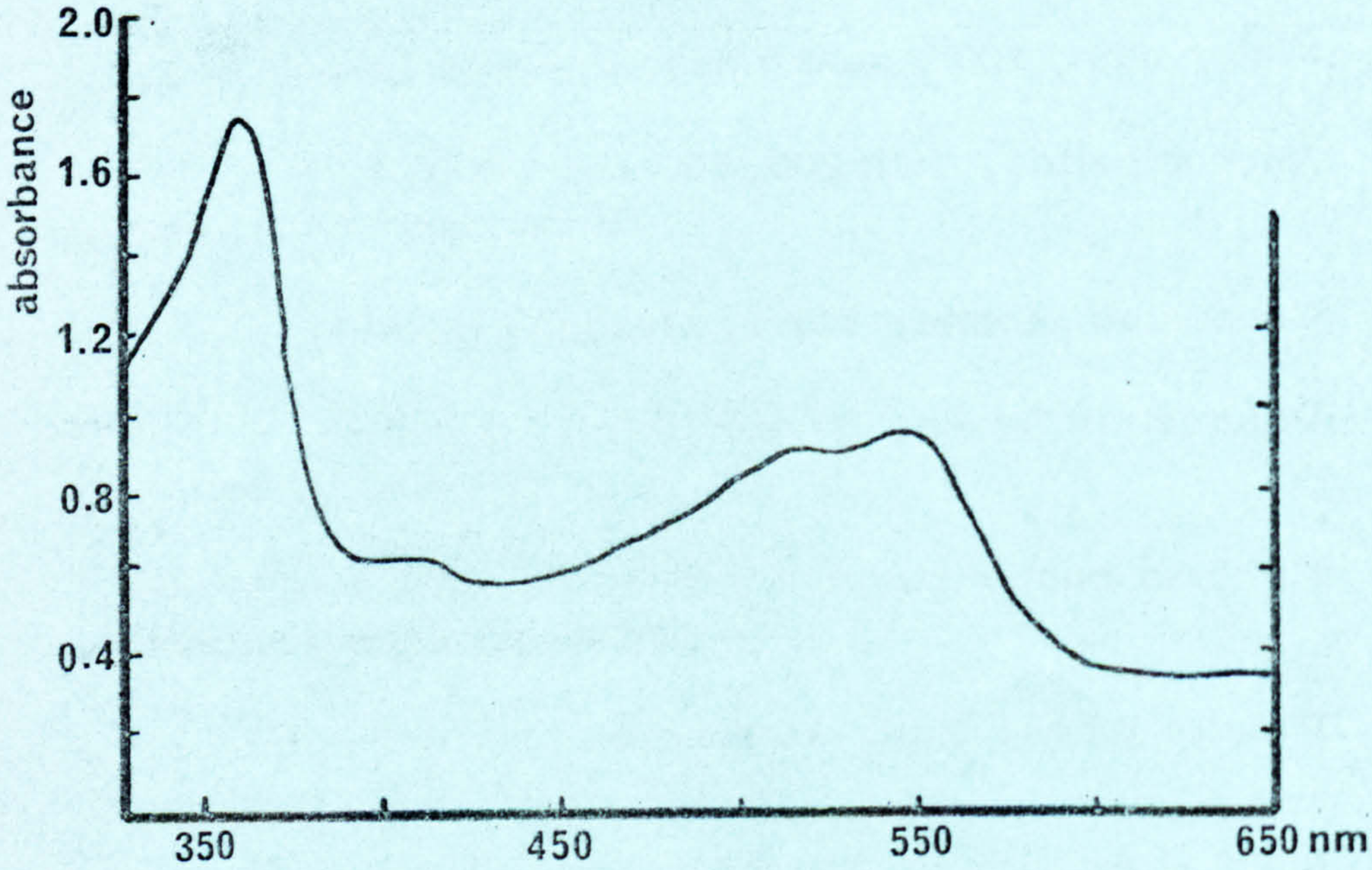


Figure 4.3 - I Absorption spectrum of CN-B_{12}

Principle

CN-B_{12} absorbs strongly at 361 nm (Fig 4.3 - I) and use is made of this in estimating concentrations down to approximately 5 µg/ml. An aqueous cobalamin solution of approximately known concentration is made up from purified crystalline compound. An aliquot is taken, converted to cyanocobalamin and its extinction at 361 nm compared with that of known cyanocobalamin standards. The concentration of the original solution can then be calculated.

Procedure

Crystalline cobalamin (approximately 200 µg), sufficient to cover the tip of a micro-spatula, is placed in a 2 ml plastic tube by the light of a red darkroom lamp and dissolved in approximately 1.5 ml water. An aliquot (0.5 ml) is taken by micropipette into a glass boiling-tube, pH 4.5 acetate/cyanide buffer (0.5 ml) is added and the tube sealed with Parafilm and foil. The solution is exposed to bright daylight for 1 hr then heated for 20 min in a 100° water bath. After cooling, dilutions are made and their extinction measured at 361 nm in a 1 cm cuvette. The procedure is repeated with each of the four cobalamins, Me-B_{12}, CN-B_{12}, Ado-B_{12} and OH-B_{12}.

An ampouled CN-B_{12} standard (100µg/ml) is diluted with an equal volume of pH 4.5 acetate/cyanide buffer, and serial dilutions prepared in water. The extinction of each is measured at 361 nm in a 1 cm cell, and a standard cerve constructed. From this, the concentrations of the unknown cobalamin solutions may be read and the original concentrations calculated. The standards are then diluted by red light to 200 ng/ml, and equal volumes of each mixed to furnish the 50 pg chromatography standard (50 pg each compound/µl). A 1:1 dilution of this is used to provide the 25pg standard (25 pg each cobalamin/µl). Multiple aliquots (approximately 1 ml) of each are dispensed into Auto-Analyzer cups which are capped individually foil-wrapped and stored at -20°.

To prevent subsequent contamination of bioautograms, all concentrated cobalamins, and especially dry compounds, are handled in a darkroom remote from the main working area.

Extraction of cobalamins

Equipment and materials

1 Dark laboratory equipped with red photographic 'safelight', water and vacuum supplies

2 Rotary evaporator with 'spider' adaptor and five B 24 spigots, lifting jack and thermostatically controlled water bath (Buchi Rotavapor R, Orme Scientific, Manchester)

3 Bench centrifuge with fixed angle head for 10 ml and 50 ml tubes (M S E 'Minor')

4 Rimless bacteriological test tubes, 152 x 16.5 mm (A 1 Laboratory Supplies)

5 130 mm square wire-mesh basket divided to accommodate 25 tubes

6 Parafilm (Gallenkamp)

7 Aluminium foil (British Aluminium Company)

8 Buchner funnels with 30 mm diameter sinter porosity 3 (Baird and Tatlock)

9 Graduated centrifuge tubes, 10 ml, with C 14 polythene stoppers (E-Mil)

10 Centrifuge tubes, 50 ml (Quickfit and Quartz)

11 Polythene stoppers for above (E-Mil)

12 Florentine flasks, 100 ml, with B 24 socket (Quickfit and Quartz)

13 Cork rings, 7 cm diameter (Scientific Supplies)

14 Vacuum fitting: B 24 cone and socket with side arm (Quickfit and Quartz)

15 Rubber sleeve for Buchner funnel (Esco Rubber Company)

16 Heavy duty 500 ml conical flask fitted with B 24 air inlet tube (Quickfit and Quartz)

17 B 24 conical flask, 250 ml, and tilt measure, 5 ml (Quickfit and Quartz)

18 Measuring cylinder, 25 ml, Grade B (E-Mil)

19 Pasteur capillary pipettes, 150 mm (Harshaw Chemicals)

20 Graduated microsyringe, 50 µl
(Hamilton Company, California)

21 AutoAnalyzer sample cups with caps
(Technicon Limited)

22 Graduated pipettes, 2, 5 and 10 ml, Grade B (E-Mil)

23 Ceramic anti-bumping granules
(British Drug Houses)

24 Ethanol, absolute (Burroughs Limited)

25 Phenol mixture (500 g Analar BDH Phenol + 125 ml water)

26 Acetone, Analar (British Drug Houses)

27 Diethyl ether, peroxide-free (May and Baker)

28 'Decon 75' anionic detergent
(Medical and Pharmaceutical Developments)

29 Crushed ice, as required, in 500 ml polythene beaker

30 Glass-distilled water

All glassware is cleaned by soaking for 24 hr in 2% Decon followed by thorough rinsing in tap and distilled water. Sintered glass funnels are in addition rinsed with distilled water at the pump. The rimless bacteriological tubes and Pasteur pipettes are disposable, and used once only.

Procedure

Ethanol (8 ml) and plasma (2 ml) are measured into a 152 x 16.5 mm rimless glass tube and the mouth sealed with Parafilm and aluminium foil. The tube, supported in a wire basket, is placed in the water bath at 70° for 15 min then cooled in crushed

ice. The alcoholic extract is filtered under reduced pressure into a 100ml Florentine flask and both tube and sinter washed with ethanol to be collected with the filtrate. After the addition of a few anti-bumping granules, the flask is attached to a spigot on the rotary evaporator and the system evacuated. Initially, the bath temperature is adjusted to 30 ^{o} and the flask rotated at approximately 60 rpm, but after the first few minutes, when boiling has become less vigorous, the temperature is gradually increased to 40^{o} and the remaining alcohol evaporated smoothly. The flask is then detached, the socket degreased and ether (approximately 20ml) added to the aqueous residue. A polythene stopper is used to close the flask, which is shaken briefly, the pressure released, then shaken again for 1 min. On standing, two phases separate and the upper, containing lipid material, is removed by aspiration. The remaining aqueous layer is taken almost to dryness on the evaporator, the residue redissolved in a little water (2 x 0.5 ml) and transferred to a 10ml graduated tube by Pasteur pipette. Phenol (5ml) is added by tilt measure and the cobalamins extracted, shaking the tube for 1 min but taking care to avoid forming an emulsion. Phases are separated by low speed (1,000 rpm) centrifugation, and the upper aqueous layer removed. The phenol is gently agitated with water (3 x 1 ml aliquots) to remove salts, and after

centrifuging, the upper aqueous phase is aspirated. The phenol is transferred to a 50ml centrifuge tube, acetone (5ml) and ether (15ml) are added and the cobalamins extracted into water (3ml). The tube is centrifuged briefly at 1,000 rpm and the upper organic phase aspirated. The aqueous layer is washed with ether (15ml) to remove traces of phenol and the upper layer rejected. The final extract is taken to dryness on the rotary evaporator while increasing the bath temperature from 35° to 40°. Water (25 µl) is added by microsyringe and the tube revolved slowly in the hand to collect and redissolve the film of extract. A fine-drawn Pasteur pipette is used to transfer the extract to an Auto-Analyzer cup which is finally capped, foil-wrapped and then stored frozen unless chromatography it to be carried out immediately.

The rate-limiting step in the method as developed is the rotary evaporation of solvents, which in consequence dictates the batch size. Five extracts may be evaporated together and a total of 10 samples processed in the course of a working day.

Chromatography

Equipment and materials

1 Thin-layer spreader and template (Desaga, Heidelberg)

2 Glass plates, 20 x 20 cm and 20 x 5 cm (Camlab Limited)

3 Aluminium rack for T L C plates (Camlab Limited)

4 Forced-draught drying oven (Gallenkamp)

5 Hair dryer and stand (Morphy-Richards)

6 Chromatography tanks (Shandon S/P 'Chromatank')

7 Graduated cylinder, 100 ml, Grade B (E-Mil) and Gelman ITLC chamber

8 Polypropylene beakers, 50 ml, with lids (Clay-Adams Inc., New York)

9 Mounted needle (Gallenkamp)

10 Capillary pipettes, 1 µl (Drummond 'Microcap')

11 Perspex sample-applicator guide (Camlab Limited)

12 Silica gel G (Merck T L C materials)

13 Cellulose CC 41 (Whatman 'Chromedia')

14 Butan-2-ol laboratory reagent (British Drug Houses)

15 Ammonia solution S G 0.880, Analar (Hopkin and Williams)

16 Benzyl alcohol, laboratory reagent (May and Baker)

17 White absorbent bench-covering paper

Preparation of plates

Five 20 cm square plates are aligned on the template with a 5 cm x 20 cm plate at either end. Adequate adhesion of plates to the template is assured by the use of a few drops of water. The exposed surface of the plates is wiped with water-dampened, then alcohol-treated, tissue before setting the spreader in place on the left hand 5 cm-wide plate, the outlet slot adjusted to 0.25 mm but closed. Cellulose powder (15 g) and silica gel (5 g) are weighed together into a 50 ml plastic beaker and 45 ml water added. A lid is pressed into place and the beaker shaken vigorously for a few seconds completely to suspend all particles without clumping. The slurry is rotated slowly for approximately 30 sec to allow air bubbles to disperse and then poured into the spreader. The outlet slot is opened and the device promptly slid to the opposite end of the template, leaving a row of coated plates in its wake. The edges of the plates are tapped sharply a few times to bring the 'fines' to the surface and give the layer a more even finish. The plates are allowed to air-dry in situ before loading them into a carrier and transferring to a 110° oven where they are stood vertically for 30 min. Rack and plates are removed to a closed box for storage until required.

1 One-dimensional chromatography

Developing solvent is prepared by shaking together sec-butanol (75 ml), water (25 ml) and 0.880 ammonia (2 ml). The resulting single-phased mixture is poured into a glass chromatography tank, lined with absorbent banch-paper to improve the vapour-saturation, and left sealed in a darkroom cupboard for at least 4hr before use.

A section of darkroom work-surface is covered with bench-paper and a thin-layer plate, previously checked for surface defect, is laid on top. Using template and mounted needle, 12 points are set out, 1.5 cm apart, in a line 2 cm from one edge of the plate. Details of sample and loading are recorded in pencil on the bench-paper. Standards (25 pg and 50 pg of each cobalamin) and extracts (1 μl and 4 μl) are applied by 1 μl capillary pipette, drying each spot with the hair dryer before applying the next. To avoid inter-sample contamination, capillaries are used for one sample only, then discarded. When loaded, the plate is stood upright in the equilibrated tank, and ascending chromatography allowed to proceed until the solvent front has risen to within 2 cm to 3 cm of the top (3½hr at 22°). Two plates are readily accommodated in the tank which provides space for 10 samples (2 loadings of each) plus standards. The chromatograms are then removed, the position of the solvent front marked, and the layer dried with the aid of a hair dryer. This and all subsequent steps may be carried out in white light.

2 Two-dimensional chromatography

Two solvents are employed, the first (sec-butanol / ammonia) is the same as that used for unidirectional TLC, while the second (water / benzyl alcohol) is prepared as follows:

> Benzyl alcohol (10 ml) and water (90 ml) are shaken in a stoppered funnel. After separation, part of the upper phase (60 ml) is transferred to the ITLC tank. The remainder is used to impregnate the fibre pad housed in the upper compartment to help maintain saturation of the atmosphere

Two points 17 cm apart are marked on a TLC plate, 2 cm from one edge. A strip of adsorbent 3 mm to 5 mm wide across the width of the plate is removed, using a micro-spatula and an edge of the template, thus dividing the chromatogram in two (Fig 4.3-II). A sample and standard, or two samples, are applied by red light and developed together by ascending chromatography in the first solvent. The plate is then removed and dried by red light. The second separation is carried out at right angles to the first, by ascending chromatography in water / benzyl alcohol (10 min at 22°). Development is critical since resolution is quickly lost once the solvent front has reached the central division and the zones diffuse outward. The chromatogram is removed and dried, then turned through 180° before repeating the process to complete separation of the cobalamins on the second half of the plate.

Bioautography

Equipment and materials

1 Autoclave

2 Incubator (Laboratory oven, Griffin-Grundy)

3 Glass plates, 25 x 25 cm (Window glass, 32 oz)

4 Perspex frame, 25 x 25 cm
(constructed from 12.5 mm square rod)

5 Spirit level, 15 cm

6 Bunsen burner (Gallenkamp)

7 Plastic Petri dishes (Sterilin, Richmond Surrey)

8 Glass universal bottles, screw-capped

9 Flat-sided medicine bottles, 100 g

10 Thermometer, -10^{o} to $+110^{o}$ C (E-Mil, gold line)

11 Platinum wire loop (Johnson-Matthey)

12 Mounted needle (Gallenkamp)

13 Whatman No1 paper, 20 x 20 cm (Reeve-Angel)

14 Vinyl examination gloves (Travenol 'Triflex')

15 Escherichia coli strain NCIB 9270, freeze-dried
(Glaxo Laboratories)

16 Bioautographic assay medium - salt mixture and
double strength agar (see Table 3.4 - 1)

17 Culture maintenance medium and peptone water
(see Table 4.3 - 1)

18 Glucose (British Drug Houses)

19 2,3,5-triphenyl tetrazolium chloride (British Drug Houses)

20 Sterile distilled water

Procedure

E. coli NCIB strain 9270 is obtained in ampoules freeze-dried, and stored at 4° as the master culture. To discourage contaminants, incubations are carried out at 27°, unless otherwise indicated.

The contents of an ampoule are transferred to peptone water (2 ml) and incubated for 18 hr. Three slopes of maintenance medium are seeded from the suspension obtained and again grown for 18 hr. Colonies from the surface of one slope are re-suspended in peptone water (100 ml) in a 10 oz screw-capped bottle and incubated for 7 hr in a shaking water bath, keeping the bottle horizontal to encourage aerobic growth.

The resulting suspension is stored in a 4° refrigerator as the working inoculum, and aliquots (2 ml) removed as required to seed the agar bioautography medium. The remaining slopes are stored at 4° and used to inoculate the next batch of peptone water and further slopes two weeks later. To ensure that a pure strain is maintained, the organism is plated out on a Petri dish of

maintenance medium at three-monthly intervals. A single colony is selected for culture in peptone water and then used to inoculate fresh maintenance slopes.

Double strength agar (approximately 100 ml) is melted in a boiling water bath while tetrazolium (0.03 g) and glucose (0.6 g) are dissolved in a bottle of salt mixture (approximately 100 ml). The melted agar is mixed with the salt mixture and cooled to 43° in tap water. Working inoculum (2 ml) is added, and the medium, after gentle but thorough mixing, poured onto a levelled bioautography plate, previously flamed after wiping with ethanol. Air bubbles are immediately pricked by sterile needle. When cool, the layer is covered with a 20 cm square of No 1 Whatman paper and cut round with sterile spatula. The agar and paper square is lifted, using gloved hands to prevent contamination, and laid paper-side down on the dried chromatogram. Air bubbles trapped between the layers are displaced by sliding a finger from side to side across the gel surface, and the 'sandwich' enclosed by perspex-framed glass plates (Fig 4.3 -III). The bioautogram is incubated at 27° for 18 hr and an additional period (approximately 4 hr) at 37°. This incubation procedure increases the sensitivity of the method (down to approximately 5 pg cobalamin), while ensuring an almost colourless background. Crimson zones of



Figure 4.3 - IV

Separation of cobalamin markers (50 pg each)

The crimson coloured zones appear on the bioautogram during incubation in contact with the chromatogram

formazan appear on the bioautogram as the tetrazolium in the medium is reduced by growing organisms in those areas in contact with the cobalamins separated on the chromatogram.

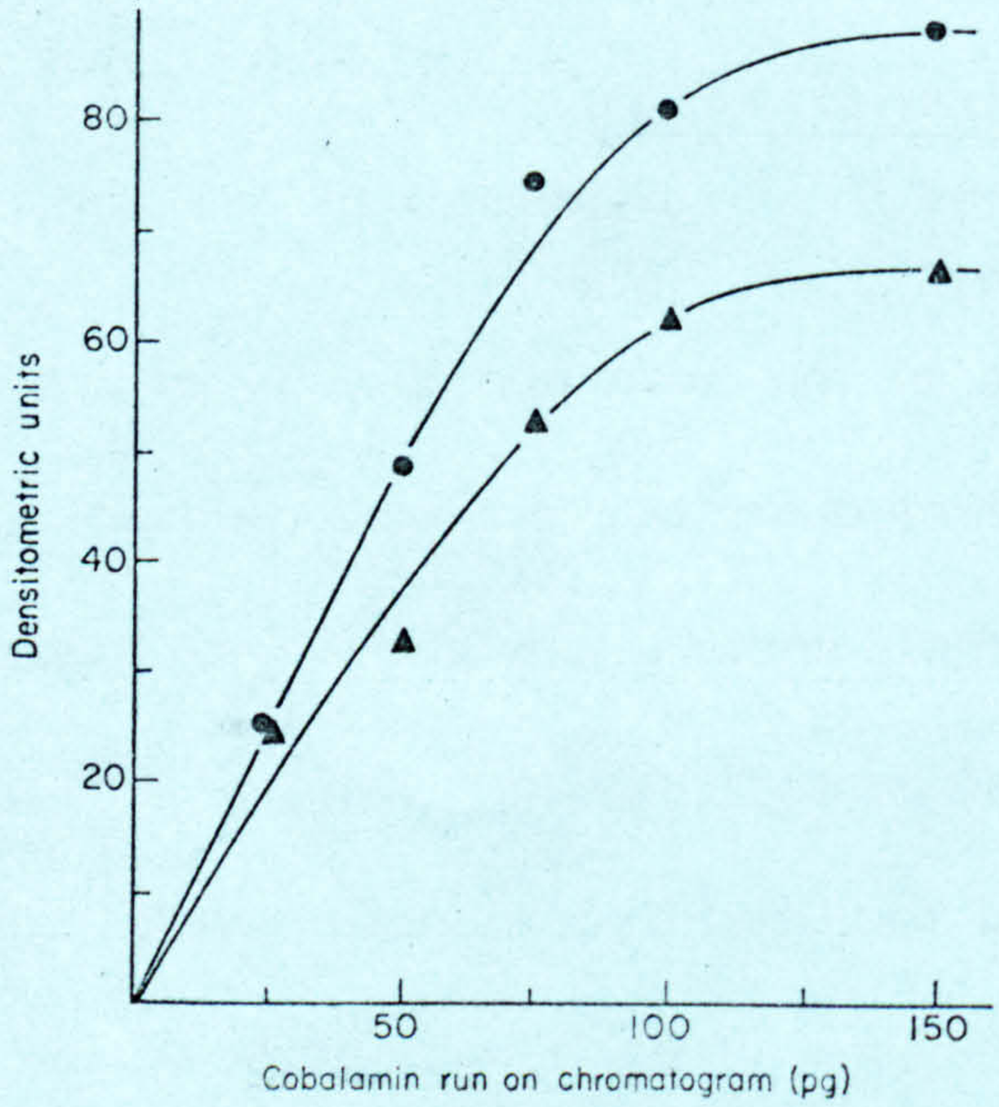
Densitometric quantitation

Principle

Use is made of the area and density of the bioautogram zones to provide a semi-quantitative assay method. The light transmission of the sample spots is compared with that of standards in a recording and integrating densitometer. The proportion of each zone is estimated and used in conjunction with the total B_{12} to calculate the concentrations of each cobalamin in the original plasma or tissue sample.

Procedure

The bioautogram is separated from the underlying chromatographic adsorbent and laid on a clean glass plate, previously moistened with a little water to facilitate the removal of air bubbles. The plate is adjusted to the diameter of the largest spot. Then, using a clear area of gel background and with the instrument switched to 'transmission', the zero and 100% limits are adjusted to correspond with the ends of the chart recorder scale. The integrator is balanced to give zero counts at 100% transmission (i.e., gel background).



Standard curves for deoxyadenosylcobalamin (●) and methylcobalamin (▲). The growth responses to cyanocobalamin and hydroxocobalamin, which are not shown, are usually indistinguishable from that to deoxyadenosylcobalamin.

Figure 4.3 - V Quantitation of the bioautogram

Bioautograms of known cobalamin standards are scanned at 20 mm/min, and the zones recorded as peaks whose areas are integrated automatically in arbitrary densitometric units. Standard curves are drawn for each cobalamin. Bioautograms of plasma or tissue extract are similarly scanned and the amounts of cobalamin in each spot read from the standard curve (Fig 4.3 - V). Results are expressed as a percentage, and the concentrations calculated using the percentage of each cobalamin on the chromatogram and the total B_{12} estimated by radioisotopic assay.

4.4 OTHER ESTIMATIONS

Serum folate was estimated microbiologically by the method of Waters and Mollin (1961). Urine thiocyanate was determined spectrophotometrically (Wilson and Matthews, 1966), and the excretion of methylmalonic acid, before and after valine (10 g) loading, was estimated in twenty-four-hour urine specimens by the method of Dreyfus and Dube (1967). Protein was estimated by the method of Lowry, Rosebrough, Farr and Randall (1951), using bovine serum albumin (Armour) as standard

Where, as in the guinea pig experiments, a microbiological method was required for the estimation of total vitamin B_{12}, the method of Anderson (1965) using Euglena gracilis 'z' strain was employed

4.5 VALIDATION of RADIOISOTOPIC ASSAY

Variation between batches

To provide an adequate quantity of serum for repeated assays, a pool was made up using samples surplus to the needs of the routine laboratory. After mixing, the serum was centrifuged to separate it from debris, and small volumes (approximately 2 ml) were then dispensed into plastic tubes and stored at -15^{o} until required. One tube was used for each assay, an aliquot of the serum being included with the other samples in that batch. Using values obtained for the total B_{12} of the serum pool on different days, the coefficient of variation was calculated.

Estimates of the serum B_{12} (Fig 4.5 - I) described an approximately normal distribution with a mean of 619 pg/ml, and standard error of 7.1 pg/ml (n = 100). The 95 % range was 495 pg - 744 pg/ml with a coefficient of variation of 11.5 %. This compares favourably with the variation recorded in microbiological assays: e.g., by E. gracilis, 13.5 % (Killander, 1957) and 17.5 % (Shinton, 1959); by L. leichmannii, 13.8 % (Spray, 1962) and 9.4 % (Matthews, 1962). The present results were collected over about three years and spanned the various small changes inevitable over such a period, as, for example, isotope and other reagents were

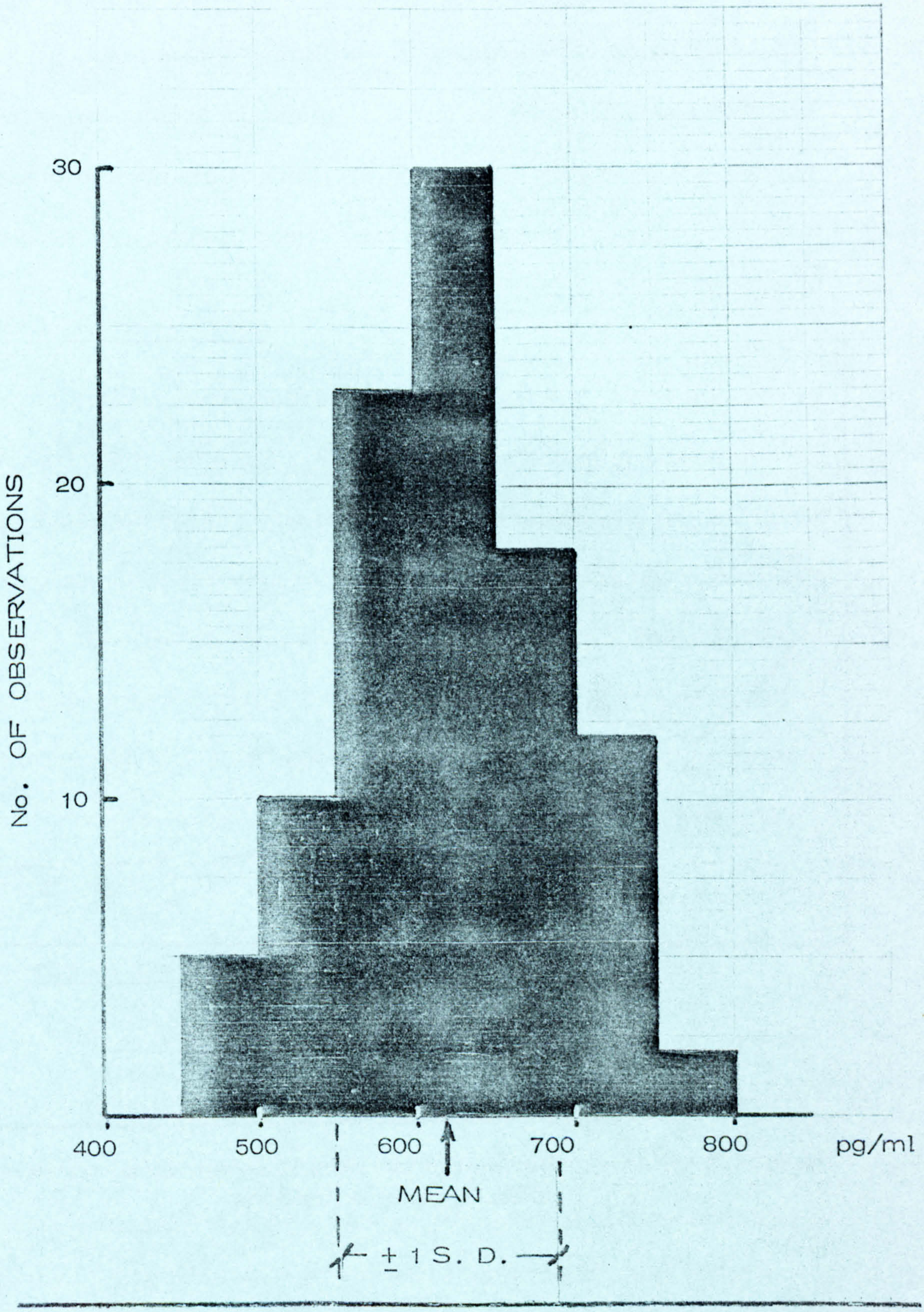


Figure 4.5 - I Estimates obtained for the total B_{12} concentrations of pooled serum in 100 assays

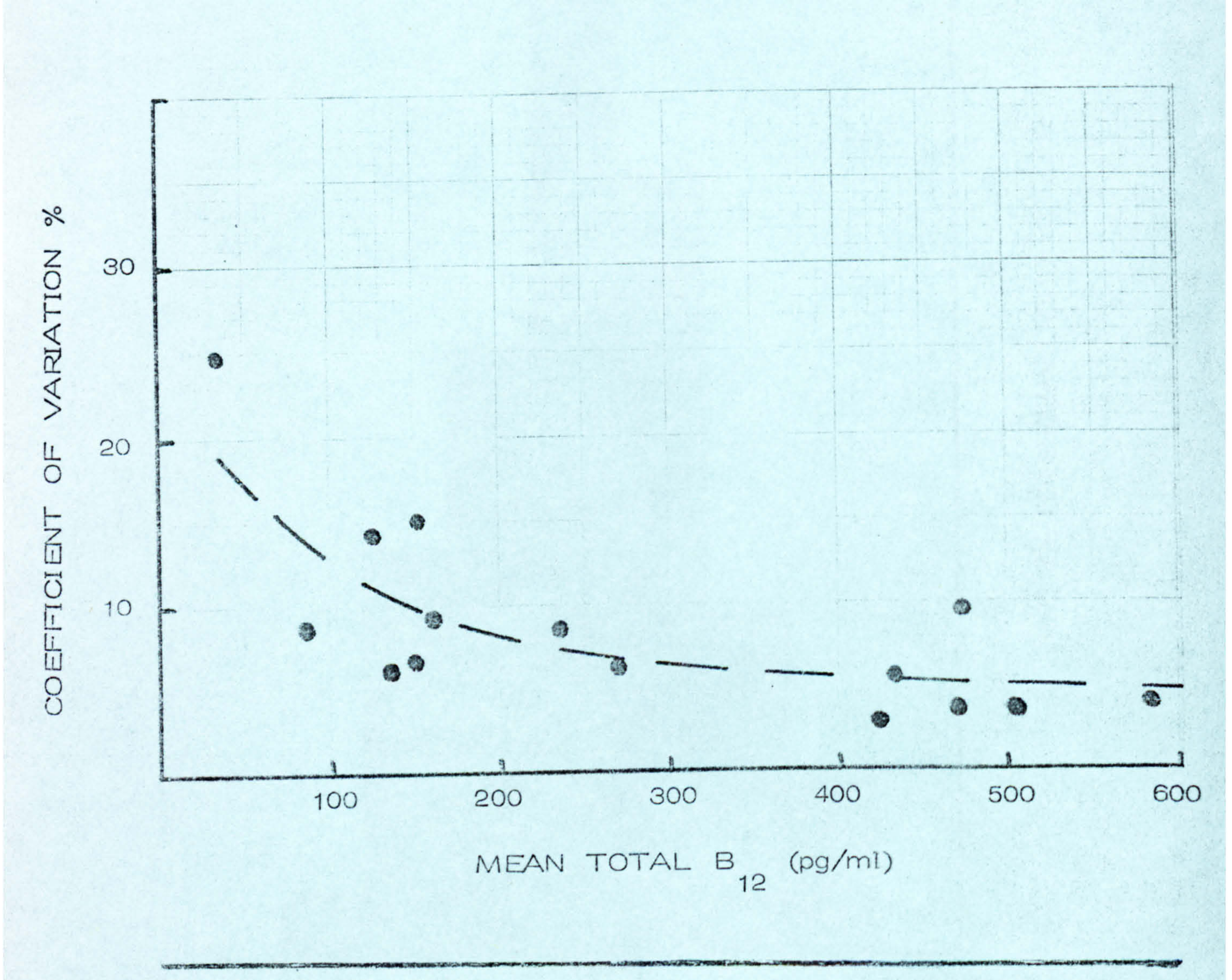


Figure 4.5 - II Estimated error within an assay at various total B_{12} concentrations

used up and replaced, and the counting conditions altered with the use of an automatic γ-scintillation spectrometer in place of the manually-operated γ-counter. None of these changes had any obvious effect on the results, although each may have contributed slightly to the overall coefficient of variation.

Variation within a batch

Fifteen sera, both normal and low-B_{12}, were assayed in replicate on the same day. The mean coefficient of variation was 8.5% (Table 4.5 - 1). The percentage variation was not the same at every concentration but was related inversely to the serum B_{12} (Fig 4.5 - II). Hence between 300 pg and 600 pg/ml, the coefficient of variation approximated to 5% but rose to nearly 10% for a serum level of 150 pg/ml. At lower concentrations, scatter increased and the results suggest that for samples containing less than 100 pg/ml B_{12}, errors of 10% to 20% are likely.

Efficiency of the extraction process

^{57}Co CN-B_{12} or ^{57}Co OH-B_{12} was added to normal serum at a level within the likely binding capacity. Samples were extracted in pH 4.5 acetate buffer with or without added cyanide (potassium cyanide, 40 mg/l) using the standard procedure. Aliquots of the filtered extract were counted in a well-type γ-scintillation detector and the counts compared with those from

TABLE 4.5 - 1

VARIATION within an ASSAY BATCH

(1) Plasma sample	(2) Total B_{12} (pg/ml) Mean ± SD	Coefficient of variation (%)
1	587 ± 23.3	4.0
2	506 ± 18.2	3.6
3	475 ± 45.8	9.6
4	471 ± 15.8	3.3
5	432 ± 23.2	5.4
6	425 ± 12.7	3.0
7	270 ± 16.5	6.1
8	236 ± 19.8	8.3
9	160 ± 14.6	9.1
10	151 ± 22.6	15.0
11	149 ± 10.0	6.7
12	137 ± 8.3	6.1
13	126 ± 17.7	14.0
14	86 ± 7.6	8.8
15	35 ± 8.6	24.5
	Mean	8.5

(1) Each sample was assayed in a separate batch
(2) Values represent the mean of 6 replicates

an equal volume of untreated serum. The extraction efficiency was then calculated.

The results (Table 4.5 - 2) show that extraction of either compound was adequate as long as cyanide was included in the assay buffer. In the absence of cyanide, $CN-B_{12}$ was better extracted than was $OH-B_{12}$, but even this incurred a loss of 16% cobalamin.

Although the value of added cyanide in this connexion is now well known, the extraction of vitamin B_{12} for assay purposes was originally carried out with a plain acid buffer, and not only were estimates of serum B_{12} too low, since a proportion of the cobalamins remained attached to the precipitated proteins, but results from different laboratories varied widely. Since 1962, when attention was focussed on this point (Matthews, 1962), the use of a cyanide-containing buffer in serum B_{12} evaluation has become widespread, and the results with different procedures more uniform. Methods using E. gracilis are exceptional but valid results are nevertheless obtained without a cyanide-containing buffer.

TABLE 4.5 - 2

EFFICIENCY of the EXTRACTION PROCESS

^{57}Co-labelled cobalamin added (500 pg/ml serum)	$CN\text{-}B_{12}$	$CN\text{-}B_{12}$	$OH\text{-}B_{12}$	$OH\text{-}B_{12}$
Acetate buffer (pH 4.5)	without cyanide	(1) with cyanide	without cyanide	with cyanide
(2) Percentage recovery (mean ± SD)	84.2 (± 1.5)	93.5 (± 0.4)	66.9 (± 3.9)	94.1 (± 0.6)
Number of estimations	20	10	18	10

(1) Potassium cyanide concentration 40 mg/litre of buffer

(2) Calculated from initial radioactivity of sample and final radioactivity of serum extract

TABLE 4.5 - 3

RECOVERY of COBALAMINS ADDED to SERUM

^{57}Co-labelled cobalamin added to serum*		Total B_{12}		Cobalamin recovered	Percentage recovery	
Form	Amount	Endogenous	Final			
	picograms per millilitre					
$CN-B_{12}$	500	196	588	392	78.5	
	500	196	630	434	87.0	
	500	196	595	399	80.0	
	500	196	686	490	98.0	
	500	196	693	497	99.5	
	500	196	599	403	80.5	
	500	196 ± 5.9	631.8 ± 47.0	435.8 ± 47.0	87.3 ± 9.4	MEAN ± SD
$Me-B_{12}$	500	35	476	441	88.0	
	500	35	420	385	77.0	
	500	35	417	382	76.0	
	500	35	413	378	75.5	
	500	35	434	399	79.5	
	500	35	431	396	79.0	
	500	35	434	399	80.0	
	500	35	441	406	81.0	
	500	35	455	420	84.0	
	500	35	462	427	85.5	
	500	35	392	357	71.5	
	500	35	462	427	85.5	
	500	35 ± 10.8	436.4 ± 24.2	401.4 ± 24.2	80.2 ± 4.9	MEAN ± SD

* After addition of the cobalamin the serum was left for 2 hr at room temperature and a further 2 hr at 4° before assay

Recovery of cobalamins added to serum

CN-B_{12} and Me-B_{12} (500 pg/ml) were added to separate aliquots of serum, and total B_{12} was estimated. The CN-B_{12} was diluted from an ampoule of known concentration, but Me-B_{12} was made up beforehand from a sample of crystalline compound and standardised spectrophotometrically. For this a portion (1 vol) was diluted with acetate/cyanide buffer (6 vol) and heated in a 100° bath for 20 min, then cooled and diluted with water. The extinction at 361 nm was compared with that of a series of known CN-B_{12} standards and the concentration of the sample calculated. A further portion was then diluted as necessary and added as a small volume to the serum to give a final concentration of 500 pg Me-B_{12}/ml.

The results (Table 4.5-3) show that the mean recovery of Me-B_{12} (80.2% ± SD 4.9) was slightly lower than that of CN-B_{12} (87.3% ± SD 9.4), $t = 2.12$; $P = 0.05$.

Comparison of results with those obtained by other methods

Samples were exchanged with other laboratories routinely estimating total vitamin B_{12}, and the results compared. Ten sera were assayed by the present method and by six other laboratories using either a microbiological assay based on

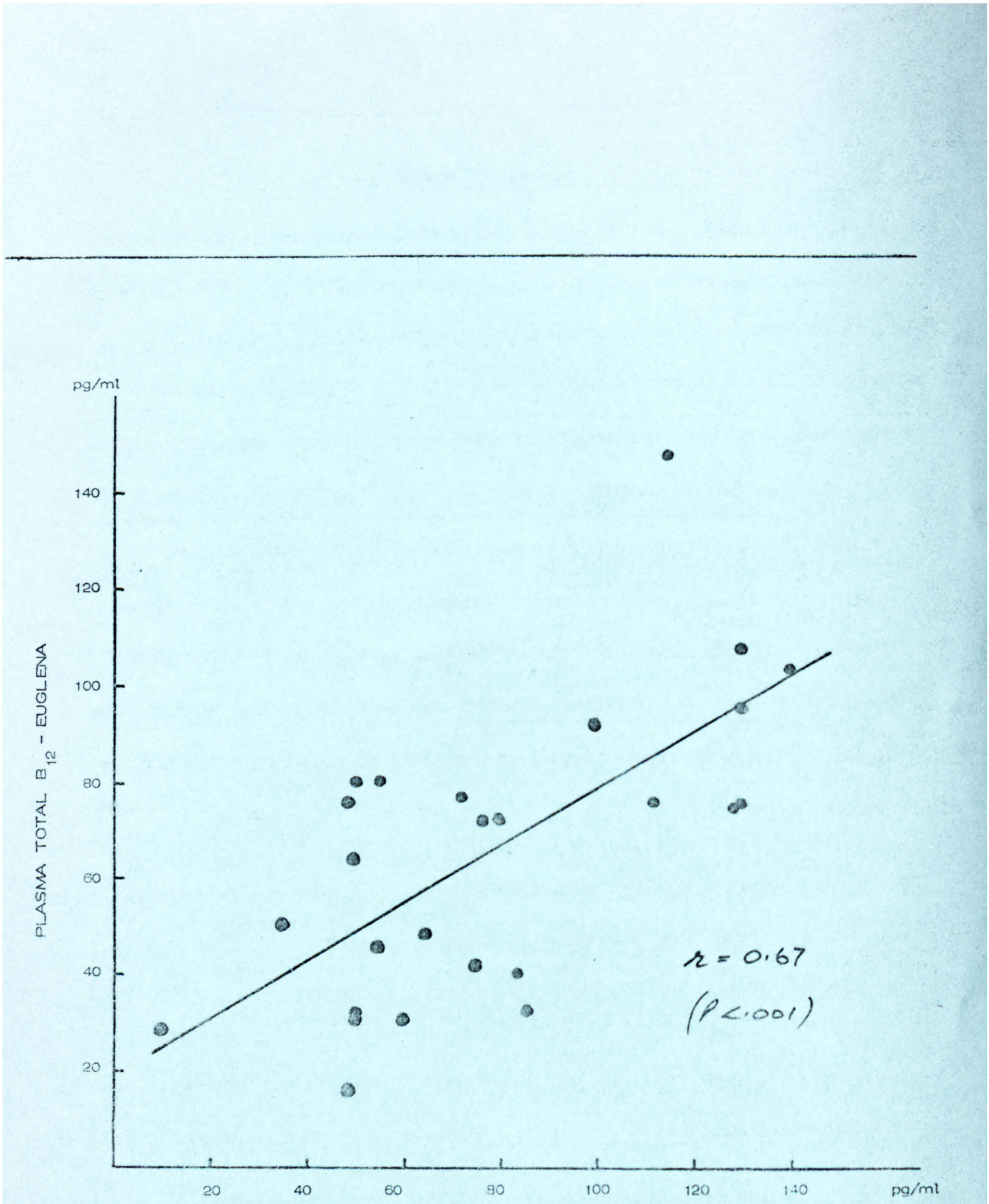
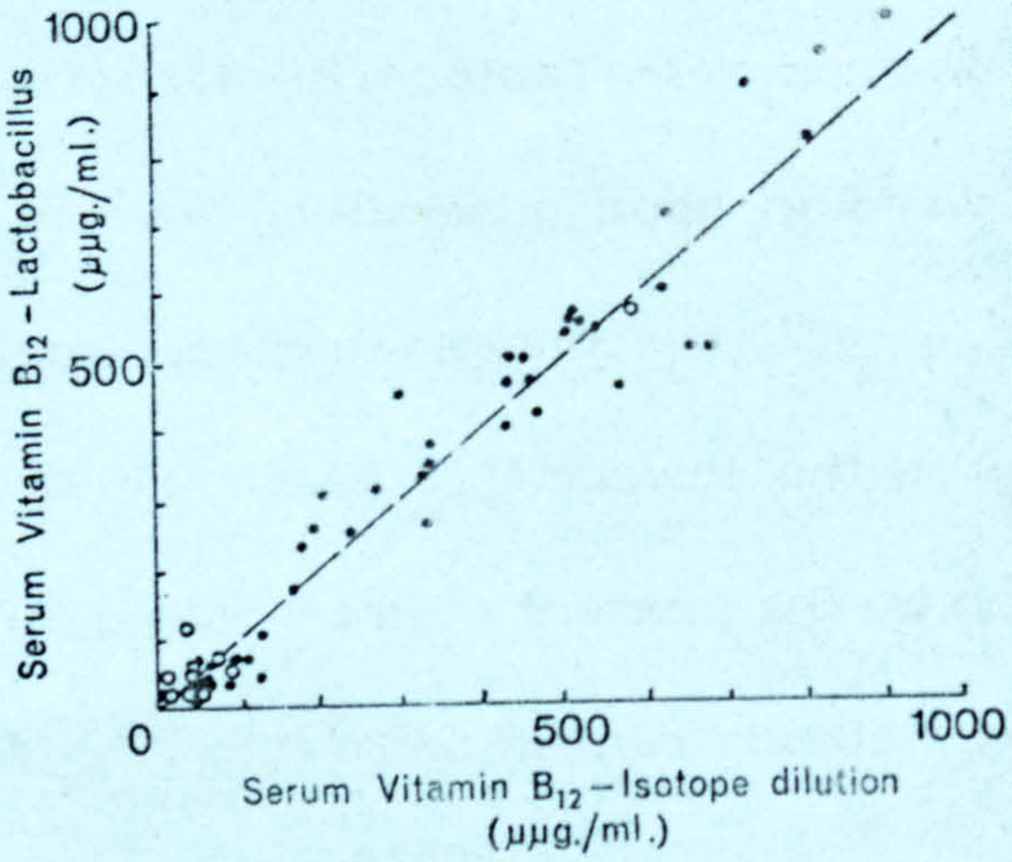


Fig. 4.5 – III Comparison of results by the Euglena assay with those by radio-isotopic assay

Lactobacillus leichmannii (Spray, 1962) or Euglena gracilis, 'z' strain (Hutner, Bach and Ross, 1956) or a radioisotopic method with intrinsic factor and coated charcoal (Lau et al., 1965). Most of the laboratories estimated each sample on three occasions and the results are shown in Table 4.5-4. Values obtained by the present method were in good general agreement with those by the other methods. Correspondence between methods was poorest for the serum with the lowest B_{12} level (No.4) where the highest value was given by the present method (53 pg/ml $\pm$ 4.9) while values by the other methods ranged from 0 to 32 pg/ml.

Duplicate estimations on 26 low-B_{12} sera from vegans and cases of pernicious anaemia were carried out by the present method and by E. gracilis assay (the latter were kindly undertaken by Dr A.V. Hoffbrand and Mr J. Morgan at the Royal Postgraduate Medical School, London). A comparison of the results obtained by these two methods (Fig 4.5 - III) gave a correlation coefficient of 0.67 ($P < 0.001$). The regression line showed a slight skew as it passed from a mid-point between the axes at 45 pg/ml to a point where 100 pg/ml on the 'Euglena' axis corresponded to 136 pg/ml on the 'radio-isotopic' axis. This suggested a slight increase in variation between the two methods from about 70 pg/ml up to 140 pg/ml, although no indication of such a discrepancy was seen



The broken line represents the relationship that would be obtained if isotope and microbiological results were identical.

● *comparison with Dr. Spray's laboratory.*

○ *comparison with assays performed in this and other laboratories.*

Figure 4.5 - IV Comparison of results by the Isotopic assay and by the L. leichmannii assay.

between the estimations described above, carried out in six other laboratories. Some comment has been made (Raven, Robson, Walker and Barkhan, 1969) on systematic differences between microbiological and radioisotopic results in the range 100 pg to 200 pg/ml but no entirely satisfactory explanation has so far been put forward to account for them.

Duplicate estimations on 53 sera from healthy subjects and from hospital patients, including cases of pernicious anaemia, were performed by the present method and by a microbiological method using Lactobacillus leichmannii (the greater part of the microbiological assays were kindly carried out by Dr G.H. Spray at the Radcliffe Infirmary, Oxford, using a method previously described; Spray, 1955). The results (Fig 4.5 - IV) corresponded well. There was no significant difference between the mean values obtained in these sera by the isotopic assay (311 pg/ml; SE 26) and the L. leichmannii assay (320 pg/ml; SE 37). The lower limit of the normal range was 150 pg/ml by the microbiological method and 185 pg/ml by the present method; all values found to be subnormal by the one were subnormal by the other.

TABLE 4.5 - 4

COMPARISON of TOTAL SERUM B_{12} RESULTS from SEVEN LABORATORIES

Laboratory	Sample number 1	2	3	4	5	6	7	8	9	10
	TOTAL SERUM B_{12} (pg/ml) - mean ± SD									
A	210 ± 10	1070 ± 10	483 ± 7	20 ± 5.0	564 ± 14	302 ± 25	370 ± 5	86 ± 1.7	116 ± 2.0	480 ± 15
B	253 ± 32	1275 ± 150	547 ± 29	24 ± 13	623 ± 15	306 ± 15	294 ± 13	95 ± 5.0	156 ± 5.8	465 ± 21
C	238 ± 45	1060	575	-	600	370	-	190	250	-
D	192 ± 10	753 ± 50	382 ± 23	32 ± 7.8	440 ± 17	208 ± 11	220 ± 10	86 ± 5.7	118 ± 12	350 ± 28
E	273 ± 1.5	1195 ± 33	623 ± 23	19 ± 0.6	640 ± 57	355 ± 14	339 ± 19	120 ± 8.3	143 ± 15	566 ± 19
F	160	750	357	0*	307	297	266	268	183	308
G (present method)	242 ± 7.0	1042 ± 104	580 ± 146	53 ± 4.9	555 ± 83	280 ± 32	340 ± 52	126 ± 37	181 ± 30	451 ± 57
Mean	224 ± 39	1021 ± 201	507 ± 103	28 ± 17	533 ± 119	303 ± 53	305 ± 56	139 ± 67	164 ± 46	437 ± 93

* 31 pg/ml by Euglena gracilis method

METHODS employed in OTHER LABORATORIES

A B C Lactobacillus leichmannii, based on Mollin and Ross (1952) and Matthews (1962)

D E Euglena gracilis, based on Hutner, Bach and Ross (1956)

F Radioisotopic with IF binder, Lau, Gottlieb, Wasserman and Herbert (1965)

4.6 VALIDATION of CHROMATO-BIOAUTOGRAPHIC METHOD

To assess the reliability of the chromato-bioautographic method, recovery experiments were carried out using plasma to which cobalamins had been added. Losses attributable to the extraction itself were estimated separately with ^{57}Co CN-B_{12}. Day to day variation was monitored by comparing the results from duplicate samples in the same batch and from those in different batches.

Extraction losses

To estimate the losses incurred in the solvent extraction process, preliminary experiments were carried out with pooled serum to which ^{57}Co CN-B_{12} had been added. The additions were made at five concentrations, including several within the likely binding capacity of serum.

The results (Table 4.6 - 1) show that from the highest concentration (170,000 pg/ml), ^{57}Co CN-B_{12} was well extracted. At lower concentrations (between 50 pg and 5,000 pg/ml) a greater proportion of the cobalamin was lost in the alcoholic precipitate. Mainly as a result of this, only about half the initial activity was recoverable in the extract.

TABLE 4.6-1

EFFICIENCY of the EXTRACTION PROCESS

^{57}Co CN-B_{12} added to pooled human serum	Initial activity	Loss in protein precipitate		Recovered in final extract	
(pg/ml)	(cps)	(cps)	(%)	(cps)	(%)
170,000	2,647	300	11.3	2,293	86.6
5,000	913	270	29.6	421	46.1
1,000	290	72.7	25.1	138	47.6
500	93.0	24.9	26.8	44.6	48.0
50	10.4	3.3	31.7	4.7	45.2

Overall recovery

Aqueous solutions of each cobalamin were standardised spectrophotometrically as described in Section 4.3. Plasma from a normal volunteer was separated in the darkroom, and divided into 2ml aliquots. A known amount of cobalamin was added to each aliquot and an equal volume of water to the blank. Time was allowed for binding (at least 2hr at 4^{o}) after which the samples were extracted and cobalamins estimated as described in Section 4.3. The overall recovery of each compound was calculated by expressing the result as a percentage of the amount of cobalamin added initially. The later availability of guinea pig plasma from animals which had received small (10ng) oral doses of ^{57}Co CN-B_{12} allowed further recovery experiments to be carried out with very low levels of cobalamin. Since the levels were below those detectable by the usual bioautographic method, the sample extracts after application to the chromatogram were overspotted with cobalamin markers. Following chromatography and bioautography the growth zones were excised and transferred to a γ-scintillation spectrometer. The cobalamin recovery was calculated from the activity in the excised zones and that of the original plasma.

TABLE 4.6 - 2

RECOVERY of COBALAMINS ADDED to PLASMA of NORMAL SUBJECTS and GUINEA PIGS

	Cobalamin added	Cobalamins detected				Percentage recovery	
		Me-B_{12}	CN-B_{12}	Ado-B_{12}	OH-B_{12}		
	- picograms per millilitre -						
Normal subjects	Ado-B_{12} 900	0	0	- 600 -		67	
	900	0	0	- 330 -		37	
	900	0	0	- 370 -		41	
	900	0	0	- 400 -		45	
	900	0	0	- 620 -		69	
	900	10	0	- 520 -		58	
	900	0	0	- 500 -		55	
	900	0	0	- 580 -		64	
	900	0	0	- 580 -		64	
	250	0	0	96	13	43	
	100	0	0	50	4	54	
						54.3 ± 3.4	Mean SE
	Me-B_{12} 1,000	630	0	0		63	
	1,000	650	0	0		65	
	1,000	400	0	0		40	
	1,000	370	0	0		37	
	1,000	300	0	0		30	
	250	100	0	0		40	
	100	46	0	0		46	
						45.9 ± 5.0	Mean SE
	OH-B_{12} 1,000	0	0	0	390	39	
	1,000	0	0	0	310	31	
	1,000	0	0	0	350	35	
						35.0 ± 2.3	Mean SE
	CN-B_{12} 250	0	100	0	0	40	
	100	0	50	0	0	50	
Guinea pigs *	CN-B_{12} 5.0	0	3.1	0	0	62	
	4.0	0	1.9	0	0	47	
	2.0	0	1.1	0	0	54	
						50.6 ± 3.7	Mean SE

*^{57}Co CN-B_{12} added. Recovery calculated from the sum of the counts in the excised chromatogram zones

The results (Table 4.6 - 2) show that when small amounts of Me-B_{12}, Ado-B_{12}, OH-B_{12} or CN-B_{12} were added to normal plasma, the average recovery of each cobalamin ranged from 35% to 54%. A comparison of these results with the values obtained from experiments on the extraction process alone reveals that the major loss occurs at the deproteinisation stage. Since the losses of each compound are approximately equal this should not detract from the usefulness of the method as long as the total cobalamin concentration is estimated by another method (e.g. radioisotopic assay).

Replication

Twenty samples were estimated in duplicate on the same day and again on a different day. The results showing variation within a batch are given in Table 4.6 - 3; mean differences (100% to 105% were very similar for each cobalamin. The results showing variation between batches are given in Table 4.6 - 4; here the mean differences were only slightly greater (92% to 103%).

TABLE 4.6 - 3

VARIATION within a BATCH

DUPLICATE ESTIMATIONS of COBALAMINS in PLASMA and OTHER TISSUES

Sample number	TOTAL B_{12}	First estimate			Second estimate			Difference (second as percentage of first)		
		Me-B_{12}	CN-B_{12}	Ado/OH-B_{12}	Me-B_{12}	CN-B_{12}	Ado/OH-B_{12}	Me-B_{12}	CN-B_{12}	Ado/OH-B_{12}
		picograms per millilitre								
1	50	15	0	35	14.5	0	35.5	96.7	100	101.4
2	15	2.9	0	12.1	3.2	0	11.8	110.3	100	97.5
3	59	9	0	50	9	0	50	100	100	100
4	215	43	0	172	47	0	168	109.3	100	97.7
5	600	123	0	477	129	0	471	104.9	100	98.7
6	16,000	2,240	1,120	12,640	2,560	1,440	12,000	114.3	128.6	94.9
7	200	116	14	70	102	10	88	87.9	71.4	125.7
8	30	1.7	6.2	22.1	1.8	4.8	23.4	105.9	77.4	105.9
9	135	4.8	6.2	124	5.2	7.8	122	108.3	125.8	98.4
10	515	206	0	309	237	0	278	115.0	100	90.0
11	685	185	0	500	219	7	459	118.4	–	91.8
12	600	420	18	162	366	36	198	87.1	200	122.2
13	575	351	6	218	305	0	270	86.9	0	123.9
14	2,265	657	0	1,608	498	0	1,767	75.8	100	109.9
15	440	220	26	194	260	18	162	118.2	69.2	83.5
16	420	122	13	285	97	4	319	79.5	30.8	111.9
17	105	13	18	74	16	29	60	123.1	161.1	81.1
18	185	7	44	134	15	48	122	214.3	109.1	91.0
19	3,500	210	840	2,450	70	980	2,450	33.3	116.7	100
20	435	309	0	126	335	0	100	108.4	100	79.4
						MEAN		104.9	99.5	100.2
						Coefficient of variation		3.2%	4.3%	1.3%

TABLE 4.6 - 4

VARIATION between BATCHES
DUPLICATE ESTIMATIONS of COBALAMINS in PLASMA and OTHER TISSUES

Sample number	TOTAL B_{12}	First estimate			Second estimate			Difference (second as percentage of first)		
		Me-B_{12}	CN-B_{12}	Ado/OH-B_{12}	Me-B_{12}	CN-B_{12}	Ado/OH-B_{12}	Me-B_{12}	CN-B_{12}	Ado/OH-B_{12}
		picograms per millilitre								
1	50	15	0	35	12	0	38	80.0	100	108.5
2	15	2.9	0	12.1	1.7	0	13.3	58.6	100	109.9
3	215	43	0	172	26	0	189	60.4	100	109.9
4	720	115	0	605	144	0	576	125.2	100	95.2
5	7,740	774	0	6,966	851	0	6,889	109.9	100	98.9
6	2,270	295	1·4	1,861	431	0	1,839	146.1	0	98.8
7	5,515	607	331	4,577	938	0	4,577	156.5	0	100
8	425	230	46	149	285	21	119	123.9	45.6	79.9
9	500	340	0	160	225	0	275	66.2	100	171.9
10	170	71	43	56	79	37	54	111.2	86.0	96.4
11	30	1.7	6.2	22.1	1.7	6.3	22	100	101.6	99.5
12	700	630	0	70	686	0	14	108.9	100	20.0
13	50	14.5	0	35.5	12	0	38	82.8	100	107.0
14	1,230	689	86	455	640	172	418	92.9	200	91.9
15	250	163	67	20	153	67	30	93.9	100	150.0
16	1,130	565	0	565	622	11	497	110.1	-	87.9
17	14	2.9	0	11.1	1.5	0	12.5	51.7	100	112.6
18	215	47	0	168	26	0	189	55.3	100	112.5
19	25	7.5	0.8	16.7	6.7	0	18.3	89.3	0	109.6
20	30	1.8	4.8	23.4	1.7	6.0	22.3	94.4	125.0	95.3
					MEAN			95.8	92.1	102.8
					Coefficient of variation			3.0 %	4.8 %	2.8 %

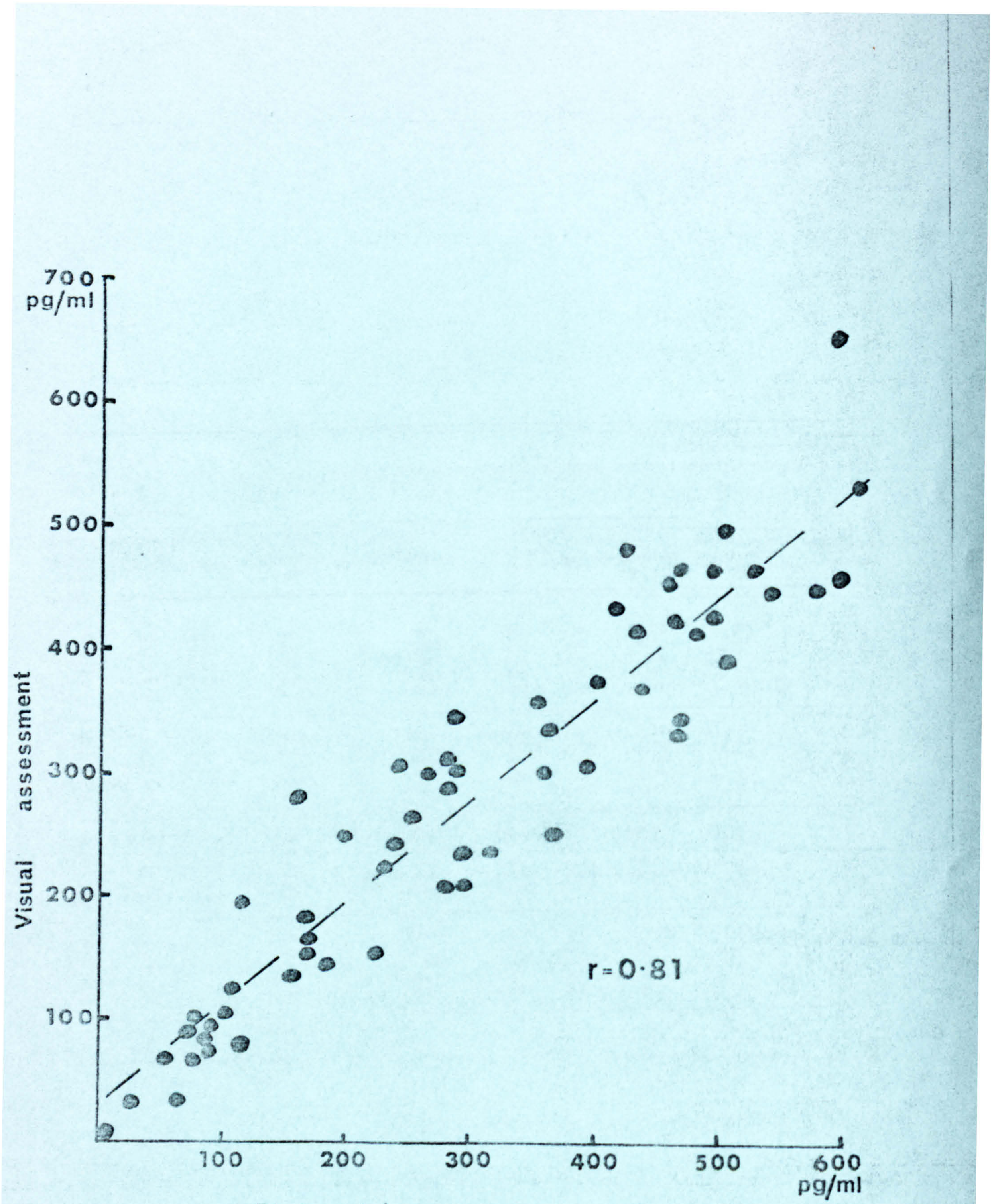


Figure 4.6 - I Comparison of plasma Me-B_{12} results by visual assessment of bioautograms and by photometric scanning. r = correlation coefficient

Quantitation of bioautograms: comparison of photometric scanning and visual assessment

Before the photometric method was devised, bioautograms were assessed visually as described in Section 3.5, and a number of results were obtained in this way. After the photometric method had become established, it was possible to estimate some samples by each method in order to assess the validity of the earlier results.

A comparison of the values for Me-B_{12} obtained by each of these methods in 61 plasma samples is shown in Fig 4.6 - I. The correlation coefficient is 0.81 ($P < 0.001$) and the calculated regression line passes through a mid point at 200 pg/ml - indicating a good general agreement, although as the cobalamin concentration increases, visual assessment slightly but progressively underestimates the value.

* The following results were obtained in healthy members of the hospital and laboratory staff - 'normal subjects' - and patients in whom there was no reason to suspect vitamin B_{12} or folate deficiency or any disturbance of vitamin B_{12} metabolism - 'hospital controls'

5.1 TOTAL SERUM B_{12}

In 100 normal subjects (58 men and 42 women aged 19 to 66 years), total serum B_{12} ranged from 180 pg to 935 pg/ml - mean 482 pg/ml, SD 171, SE 17.2. The 98% range was 184 pg to 919 pg/ml. The distribution (Fig 5.1 - I) was slightly skewed about the mean, more values falling in the lower quartile (34) than in the upper quartile (23). The later inclusion of a further 73 normal subjects and hospital controls altered the above values but little: hence in 173 normal subjects and hospital controls the mean total serum B_{12} was 477 pg/ml, SE 20.7, and the 98% range was 185 pg to 935 pg/ml.

The normal results obtained with this assay are very similar to those given by standard microbiological methods. Using Lactobacillus leichmannii, Spray and Witts (1958) reported a mean of 450 pg/ml (range 150 pg to 1,000 pg/ml); Matthews (1962) reported 480 pg/ml (range 120 pg to 1,150 pg/ml); using Euglena gracilis 'z' strain, Anderson (1964) reported 472 pg/ml (range 165 pg to 925 pg/ml). In a more recent and larger group of control subjects, Spray (1966) found a mean of 421 pg/ml with a range of 140 pg to 1,200 pg/ml. (See also Section 4.5)

* All samples were from European subjects

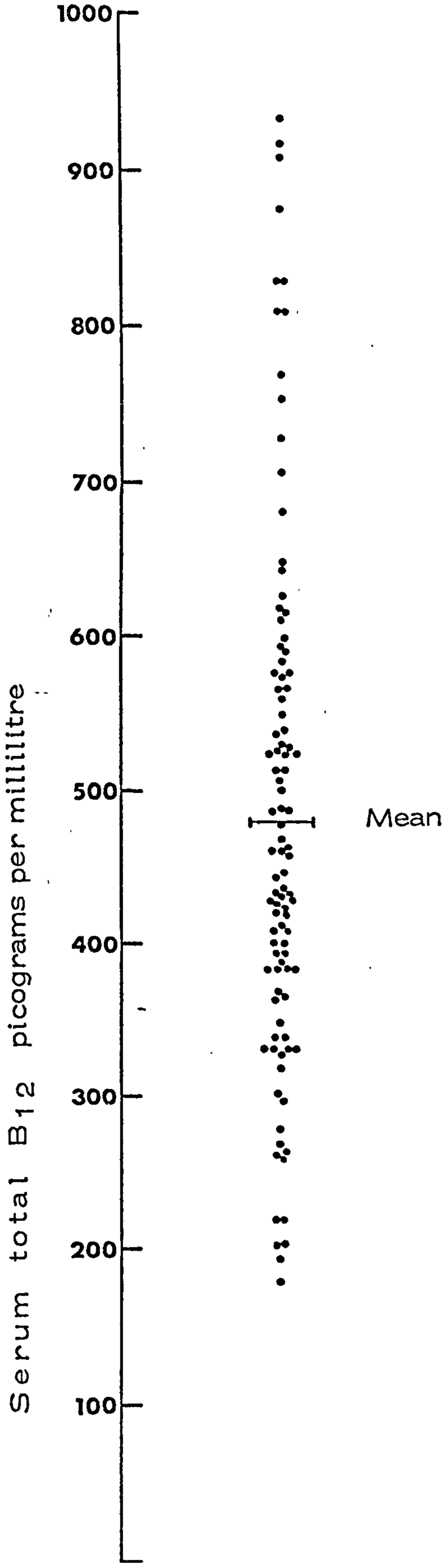


Figure 5.1 - I Values for serum total B_{12} in 100 healthy adults

5.2 PLASMA COBALAMINS

When applied to plasma, the technique normally shows two main growth zones. Zone 1, predominant in healthy subjects, has an R_f corresponding to that of Me-B_{12}. Zone 2 has an R_f corresponding to that of Ado-B_{12}. In the solvent system routinely used, low concentrations of Ado-B_{12} are not adequately separated from OH-B_{12}. The two-dimensional chromatographic technique which completely separates OH-B_{12} from the other cobalamins shows that Zone 2 represents a mixture of Ado-B_{12} and OH-B_{12}, and analysis by this technique of samples from 7 healthy subjects showed that these contained almost three times as much Ado-B_{12} (mean 22.6 %) as OH-B_{12}. In a minority of samples, a third zone appears, with the R_f of CN-B_{12}. The possible relationship between the presence of CN-B_{12} and smoking has previously been studied, and it has been shown that this compound is not confined to smokers (Lindstrand et al., 1966; Linnell et al., 1969).

Results obtained from the plasma of 28 normal subjects are shown in Table 5.2 - 1. Mean values in a number of other samples taken earlier in the study are shown in Table 5.2 - 2, and results obtained by photometric scanning of bioautograms compared with those obtained by visual assessment.

TABLE 5.2 - 1

PLASMA COBALAMINS in NORMAL SUBJECTS and HOSPITAL CONTROLS

		Sex	Age	Smoking habits	TOTAL B_{12}	$Me-B_{12}$	$CN-B_{12}$	$Ado/OH-B_{12}$	Ratio $Me-B_{12}$: $Ado/OH-B_{12}$	Percentage $CN-B_{12}$
			(yr)	*(cig/oz)	picograms per millilitre					
Normal subjects	1	M	35	0	625	437	0	188	2.3	0
	2	M	35	0	350	294	0	56	5.2	0
	3	M	27	0	345	190	0	155	1.2	0
	4	M	38	0	1,085	597	0	488	1.2	0
	5	M	43	40 cig	595	255	0	240	1.5	0
	6	F	52	15 cig	490	314	3	173	1.8	0.6
	7	M	46	30 cig	765	594	3	168	3.5	0.4
	8	M	55	25-30 cig	385	292	0	93	3.1	0
	9	M	31	25 cig	510	398	0	112	3.5	0
	10	F	46	10 cig	570	467	0	103	4.5	0
	11	M	38	20-25 cig	560	465	0	95	4.9	0
	12	M	48	20-25 cig	400	270	0	130	2.1	0
	13	M	52	3½ oz	350	294	0	56	5.4	0
	14	M	55	3 oz	250	157	3	90	1.8	1.2
	15	M	64	6 oz	575	282	46	247	1.1	8
	16	M	63	3½ oz	335	171	3	161	1.1	0.9
	17	F	57	10-15 cig	755	460	0	295	1.6	0
	18	M	43	20 cig	555	480	0	75	6.4	0
	19	F	19	25 cig	660	505	0	155	5.3	0
	20	M	22	25 cig	275	170	0	105	1.6	0
	21	M	52	3½ oz	550	443	3	102	4.3	0.9
	22	M	62	25-35 cig	575	418	3	154	2.7	0.5
	23	M	43	20-25 cig	800	616	24	160	3.8	3.0
Hospital controls	24	F	16	0	550	435	0	115	3.8	0
	25	F	31	20 cig	405	247	0	158	1.6	0
	26	F	55	10 cig	370	230	0	140	1.6	0
	27	M	55	15-20 cig	425	342	0	83	3.0	0
	28	M	55	15-20 cig	510	270	0	240	1.1	0
				MEAN	522	384	3.2	155	2.9	0.5
				SE	± 33.8	± 24.7	± 1.9	± 16.4	± 0.3	± 0.3
				Range	(250 - 1,085)	(157 - 615)	(0 - 46)	(58 - 295)	(1.1 - 6.4)	(0 - 8)

*cigarettes per day/
oz tobacco per week

In comparing pathological values, the following have been taken as normal for plasma cobalamins:

	picograms per ml		
Total B_{12}	185	to	935
Me-B_{12}	130	to	600
CN-B_{12}	0	to	50
Ado/OH-B_{12}	60	to	310
Me-B_{12}: Ado/OH-B_{12} ratio	1:1	to	5.5:1

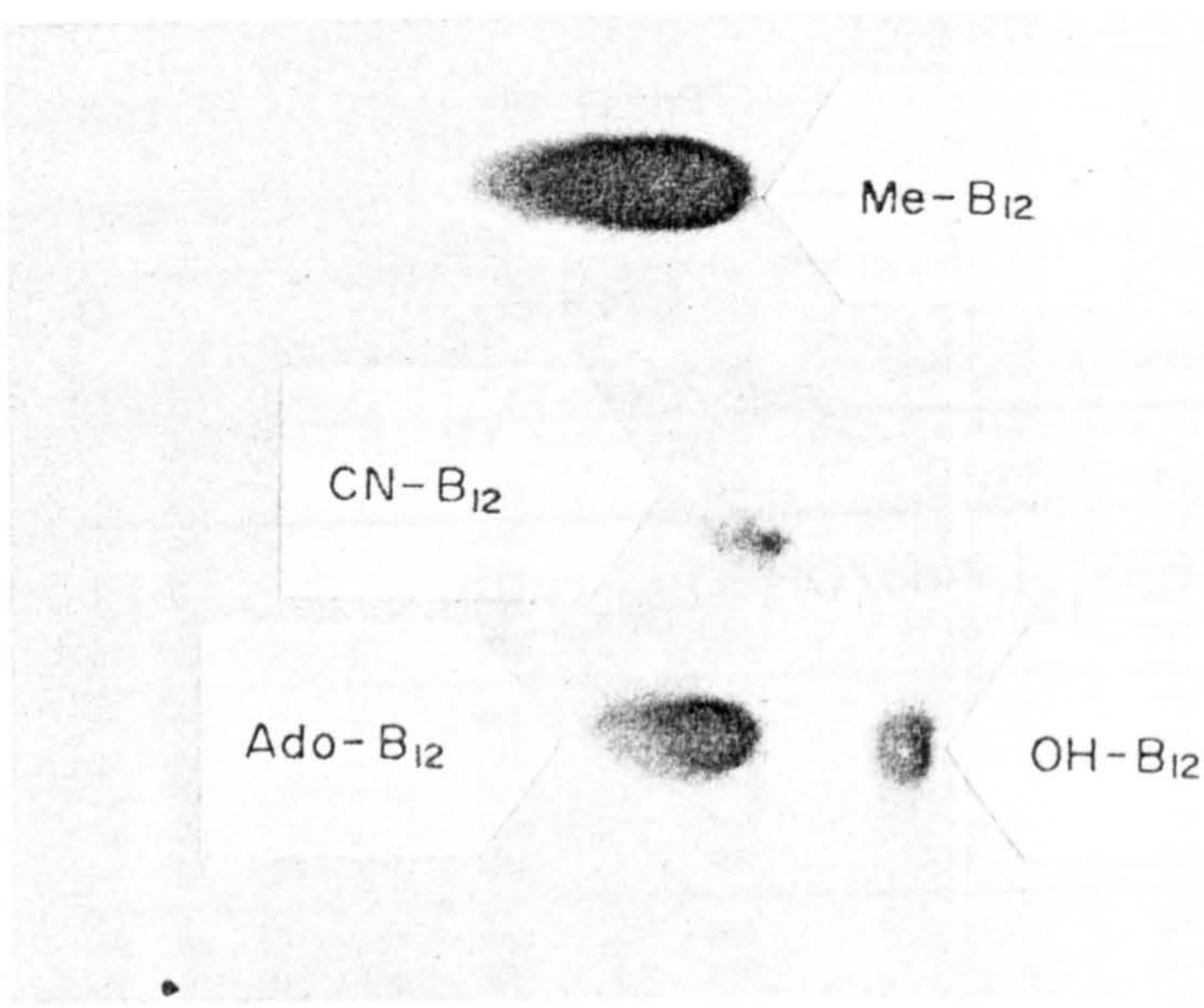


Figure 5.2 - I Separation of plasma cobalamins in a healthy subject

TABLE 5.2 - 2

PLASMA COBALAMINS in NORMAL SUBJECTS and HOSPITAL CONTROLS

SUMMARY of RESULTS OBTAINED by PHOTOMETRIC SCANNING and VISUAL ASSESSMENT of BIOAUTOGRAMS

Method of estimation	Number of subjects	TOTAL B_{12} Mean ± SE (range)	$Me-B_{12}$	$CN-B_{12}$	$Ado/OH-B_{12}$	Ratio Me-B12 Ado/CH-B12	Percentage CN-B12
		picograms per millilitre					
Photometric scanning	28* 23/5	522 ± 34 (250 - 1,085)	364 ± 25 (157 - 616)	3.2 ± 1.8 (0 - 45)	155 ± 18 (36 - 488)	2.9 ± 0.3 (1.1 - 5.4)	0-8
Visual assessment (samples as above)	28 23/5	522 ± 34 (250 - 1,085)	343 ± 24 (150 - 600)	(0 - 45)	178 ± 14 (100 - 434)	2.1 ± 0.2 (1.0 - 3.0)	0-8
Visual assessment	73 39/34	474 ± 25 (220 - 1,085)	298 ± 17 (125 - 651)	(0 - 50)	174 ± 6 (66 - 434)	1.5 ± 0.1 (0.7 - 3.0)	0-8
Control range adopted		185 - 935	130 - 600	0 - 50	60 - 310	1 - 5.5	0-8

* Normal subjects / Hospital controls

Values for individual cobalamins approximate to the 95% range

5.3 LEUCOCYTE COBALAMINS

The results from a series of 16 normal subjects and hospital controls are shown in Table 5.3 - 1. Total leucocyte B_{12} levels (mean 3,994pg/g) were about ten times those in normal plasma. Cobalamin chromatography showed that the greater part of the WBC B_{12} was in the form of Me-B_{12}. In five samples analysed by the two-dimensional technique, Ado-B_{12} was found to be the major cobalamin present.

Me-B_{12} was detected in all the leucocyte samples and accounted for approximately one fifth of the total B_{12}. Although it represented only a minor proportion, the actual Me-B_{12} concentration was invariably higher in white cells than plasma, and in most cases several times higher.

CN-B_{12} was detected as a minor component (mean 4.1%) in 12 of the 16 samples. The concentration of this cobalamin varied widely between samples, being undetectable in some but in others reaching levels comparable with those of the total B_{12} concentration of normal plasma.

TABLE 5.3 - 1

LEUCOCYTE COBALAMINS in NORMAL SUBJECTS and HOSPITAL CONTROLS.

		Total B12	Me-B12	CN-B12	Ado-B12	OH-B12	Ratio Me-B12 Ado/OH-B12	Percentage CN-B12
		(picograms per millilitre)						
Normal subjects	H H	2,260	415	88	925	832	0.23	4
	N W	2,035	558	80	730	667	0.39	4
	G F	1,860	370	35	1,065	390	0.26	2
	I W	6,120	1,022	0	- 5,098 -		0.20	0
	B M	4,215	868	380	1,840	1,127	0.29	9
	G B	2,550	1,002	0	- 1,548 -		0.65	0
	J L	4,060	780	516	- 2,764 -		0.28	13
	J B	3,000	990	180	1,230	600	0.54	6
Hospital controls	O D	2,500	275	100	- 2,125 -		0.13	4
	A W	2,770	165	55	- 2,550 -		0.07	2
	A M	3,580	450	270	- 2,860 -		0.15	8
	A O	8,000	960	480	- 6,560 -		0.15	6
	N P	2,800	190	170	- 1,440 -		0.08	6
	S W	6,365	381	152	- 5,832 -		0.07	2
	R	6,535	1,563	0	- 4,972 -		0.31	0
	M W	5,260	940	0	- 4,320 -		0.22	0
	Mean	3,994	683	157	- 3,092 -		0.25	4.1
	S E	± 475	± 97.5	± 42.5	± 428		± 0.04	± 0.91

5.4 ERYTHROCYTE COBALAMINS

The results obtained in a series of 12 normal subjects and hospital controls are shown in Table 5.4 - 1. Total B_{12} concentrations were approximately half those in normal plasma, and ranged from 125 pg to 425 pg/ml. Cobalamin chromatography showed that while the Ado/OH-B_{12} concentration (mean 159 pg/ml, SE 24) was very similar to that in normal plasma, the Me-B_{12} concentration was much lower (mean 31 pg/ml, SE 4.2), and was generally only 10% to 15% of the Me-B_{12} level in plasma.

CN-B_{12} was detected in 10 of the 12 samples (mean 11 pg/ml, SE 2.3). Although in most samples this formed a higher percentage of the total B_{12} than that in plasma, the actual concentrations in both tissues were very similar.

TABLE 5.4 - 1

ERYTHROCYTE COBALAMINS in NORMAL SUBJECTS and HOSPITAL CONTROLS

			Total B12	Me-B12	CN-B12	Ado-B12	OH-B12	Percentage CN-B12
			(picograms per millilitre)					
Normal subjects	AR	aged 48	150	35	8	- 107 -		5
	SK	22	160	45	5	- 110 -		3
	NG	42	320	34	26	- 260 -		8
	LW	35	125	51	0	- 64 -		0
Hospital controls	JB	aged 30	180	10	10	106	54	5
	OD	43	195	34	27	- 134 -		14
	AW	57	150	21	12	- 117 -		8
	AM	38	160	22	12	- 126 -		8
	AO	37	235	26	12	- 197 -		5
	NP	83	165	11	13	- 141 -		8
	SW	55	155	27	7	- 121 -		5
	HW	15	425	50	0	- 375 -		0
	Mean		202	31	11	- 159 -		5.8
	SE		± 25	± 4.2	± 2.3	± 24		± 1.1

5.5 COBALAMINS in BONE MARROW

The results in 14 subjects found to be haematologically normal are shown in Table 5.5 - 1. Total B_{12} concentrations (mean 12,959 pg/g, range 5,520 pg to 22,590 pg/g) were very similar to those obtained by other workers: Cooke, Cox, Gaddie, Matthews and Meynell (1959) reported a mean of 12,000 pg/ml with a range of 6,900 pg to 19,800 pg/ml.

Cobalamin chromatography showed that the greater part of the marrow B_{12} was in the form of Ado/OH-B_{12}. In three samples separated by the two-dimensional technique, Ado-B_{12} was found to be the major form. Me-B_{12} was detected in all samples and accounted for between 4% and 31% of the total B_{12}. As a concentration (mean 1,701 pg/g) this was two-and-a-half times the Me-B_{12} in normal leucocytes and almost five times the average level in normal plasma. CN-B_{12} was detected as a minor component in 6 of the 14 marrows, but, as was found in leucocytes, the concentration (mean 274 pg/g) varied widely between samples and ranged from 0 to 1,115 pg/g.

Cobalamins were estimated in the red cell layer separated from the marrow aspirate in 7 subjects, although

values for total B_{12} were obtained in only 4. The results (Table 5.5 - 2) show that the concentrations of total B_{12}, Me-B_{12} and Ado/OH-B_{12} were very similar to those in normal leucocytes. Values for CN-B_{12} were lower, and the level (mean 42 pg/g), contributed little to the total B_{12} content of this tissue.

TABLE 5.5 - 1 COBALAMINS in BONE MARROW

		Total B_{12}	Me-B_{12}	CN-B_{12}	Ado/OH-B_{12}	Ratio Me-B12 Ado/OH-B12	Percentage CN-B12
		picograms per millilitre					
Subject	OD	9,500	2,565	950	5,985	0.43	10
	JB	10,500	2,610	525	7,365	0.36	5
	AW	14,400	1,300	0	13,100	0.10	0
	AM	7,430	520	520	6,390	0.08	7
	AO	16,600	1,440	0	14,560	0.10	0
	NP	22,590	2,385	1,115	19,090	0.12	5
	SW	5,520	530	132	4,858	0.11	2
	RR	8,280	654	0	7,626	0.09	0
	GK	14,500	3,150	0	11,350	0.28	0
	K	20,300	4,060	0	16,240	0.25	0
	LB	17,690	1,290	0	16,400	0.08	0
	GN	10,000	920	320	8,760	0.11	3
	LW	11,160	685	0	16,240	0.25	0
	Mean	12,959	1,701	274	10,938	0.17	2.5
	SE	± 1,442	± 318	± 108	± 1,274	± 0.03	± 0.9

TABLE 5.5 - 2 COBALAMINS in LOWER CELL LAYER SEPARATED from MARROW ASPIRATE

		Total B_{12}	Me-B_{12}	CN-B_{12}	Ado/OH-B_{12}	Ratio Me-B12 Ado/OH-B12	Percentage CN-B12
		picograms per millilitre					
Subject	SW	2,175	518	104	1,553	0.33	5
	RR	3,750	600	0	3,150	0.19	0
	LB	5,295	683	64	4,548	0.15	1
	GN	3,010	677	0	2,333	0.29	0
	AB	-				0.39	0
	SG	-				0.38	5
	LR	-				0.58	5
	Mean	3,558	620	42	2,898	0.33	2.3
	SE	± 650	± 30.2	-	± 498	± 0.06	± 1.05

5.6 COBALAMINS in LIVER, KIDNEY, SPLEEN and OTHER TISSUES

Table 5.6 - 1 shows the results in 7 liver biopsies from haematologically normal subjects, and in autopsy samples of liver, kidney, spleen, brain and pituitary. Figs 5.6-I and 5.6-II show the separation of cobalamins in, respectively, a liver biopsy and a control sample of spleen. Values for Ado-B_{12} in liver are in good agreement with values obtained by the diol-dehydrase assay (Cardinale, Dreyfus, Auld and Abeles, 1969). Although Me-B_{12} represented only a small percentage of the total B_{12}, the actual concentration was more than fifty times that in normal plasma. The liver biopsies were found to have proportionately less Me-B_{12} and more OH-B_{12} than the autopsy samples; although the reason for this is uncertain, some photolysis of Me-B_{12} may have occurred in the small biopsies by accidental exposure to white light during sampling.

Ado-B_{12} was the predominant cobalamin in liver, kidney, spleen and brain, and as a <u>proportion</u> of the total B_{12}, values for this cobalamin were remarkably constant in these organs although the actual concentration varied widely; OH-B_{12} followed a similar pattern. In contrast, the proportion of Me-B_{12} varied widely between different organs. The

TABLE 5.6 - 1

COBALAMINS in LIVER, KIDNEY, SPLEEN, BRAIN and PITUITARY

	Tissue	Total B_{12}	$Me-B_{12}$		$Ado-B_{12}$		$CH-B_{12}$		
		ng/g	ng/g	%	ng/g	%	ng/g	%	
BIOPSY	Liver	1,060	11	1.0	635	59.9	414	39.1	
		833	8	1.0	490	58.8	335	40.2	
		500	6	1.2	275	55.0	219	43.8	
		705	7	1.0	423	60.0	275	39.0	
		1,450	16	1.2	943	65.0	489	33.7	
		1,730	25	1.5	1,175	67.9	530	30.6	
		1,060	14	1.3	648	61.1	398	37.6	
		1,048	12.7	1.2	656	61.1	380	37.7	Mean
		± 161	± 2.6	±0.07	± 117	± 1.6	± 42.2	±1.6	SE
AUTOPSY	Liver	670	20	3.0	565	84.3	85	12.7	
		440	23	5.2	243	55.2	174	39.6	
		555	21.5	4.1	404	69.8	130	28.2	Mean
	Kidney	115	31	30.0	55	47.8	29	25.2	
		152	38	23.7	88	56.6	30	19.7	
		134	34	26.9	71	52.2	29	22.5	Mean
	Spleen	70	21	30.0	29	41.4	20	28.6	
		56	25	44.6	25	44.6	6	10.7	
		63	23	37.3	27	43.0	13	19.7	Mean
	Brain	35	9	25.7	20	57.1	6	17.2	
		138	6	4.3	86	62.4	46	33.3	
		69	3	4.3	44	63.8	22	31.9	
		81	6	11.4	50	61.1	25	27.5	Mean
	Pituitary	230	47	21	111	48	72	31	

NO $CN-B_{12}$ was detected in any sample

significance of the proportions of the different cobalamins in the various tissues is not known.

Ado-B_{12} was the predominant form in two samples of bile and cerebrospinal fluid (Table 5.6 - 2); a small proportion of CN-B_{12} was detected in one sample of each.

Aqueous humour was collected from 6 patients in whom there was no evidence of a disturbance of B_{12} metabolism. Analysis of the single pooled sample showed that 75% of the total B_{12} was present as Ado/OH-B_{12} and 25% as CN-B_{12}; no Me-B_{12} was detected.

TABLE 5.6 - 2

COBALAMINS in BILE and CEREBROSPINAL FLUID

		PLASMA Total B_{12}	Sample	Total B_{12}	Me-B_{12}		CN-B_{12}		Ado-B_{12}		OH-B_{12}	
		pg/ml		pg/ml	pg/ml	%	pg/ml	%	pg/ml	%	pg/ml	%
Subject	AH	945	Bile	18,400	700	4	1,235	7	9,255	50	7,210	39
	MK	210	Bile	210	21	10	0	0	154	78	25	12
Subject	BR	280	CSF	18	1.4	8	1.2	7	13.4	74	1.4	8
	SW	150	CSF	20	0.3	1.5	0	0	17.0	85	2.2	11

Samples of BILE were aspirated from the gall bladder removed at cholecystectomy

Samples of CSF were taken for diagnostic purposes:
Subject BR was subsequently found to be normal
Subject SW was diagnosed as an early case of pernicious anaemia

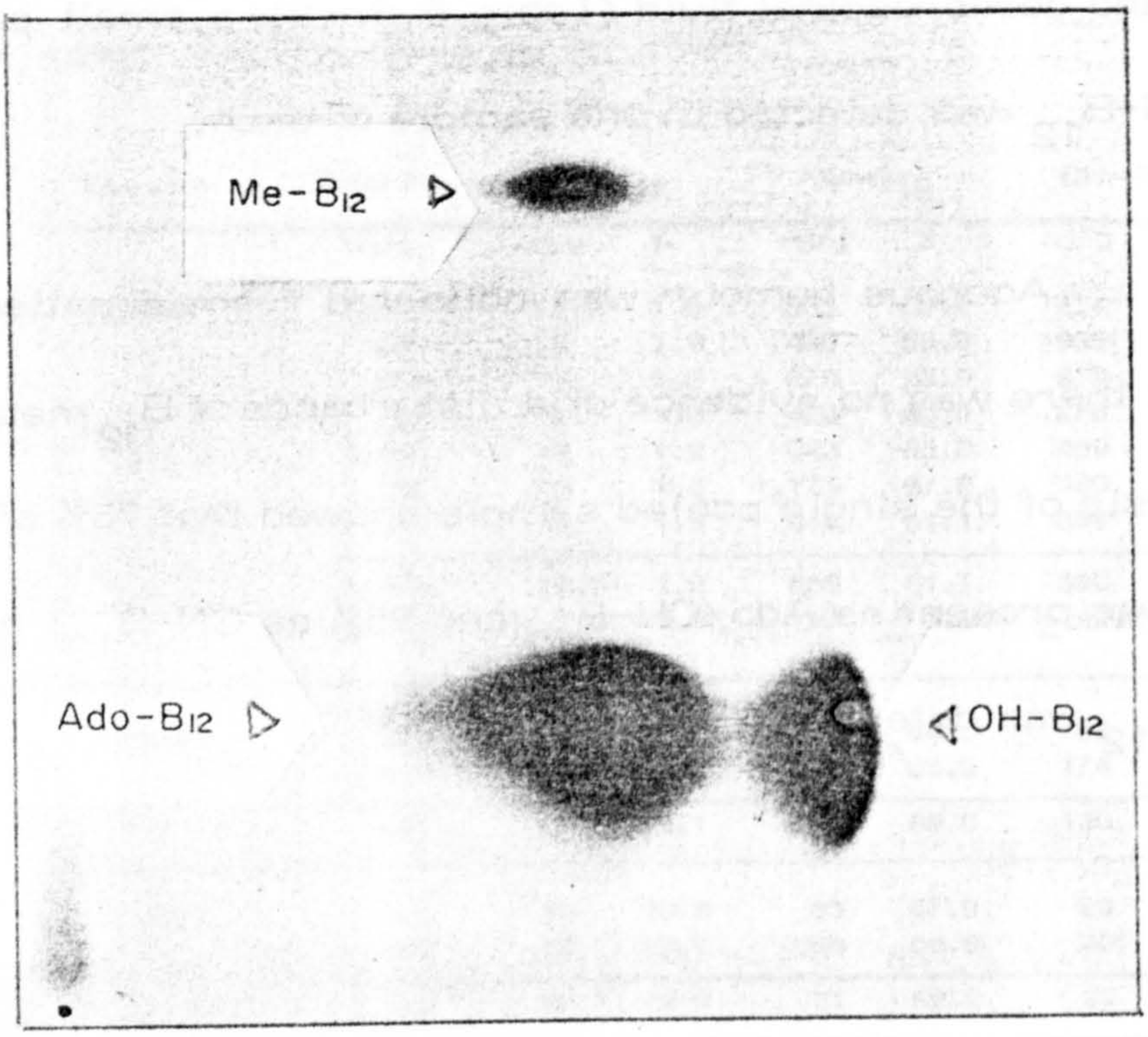


Figure 5.6 - I Separation of cobalamins in a control sample of liver obtained at autopsy

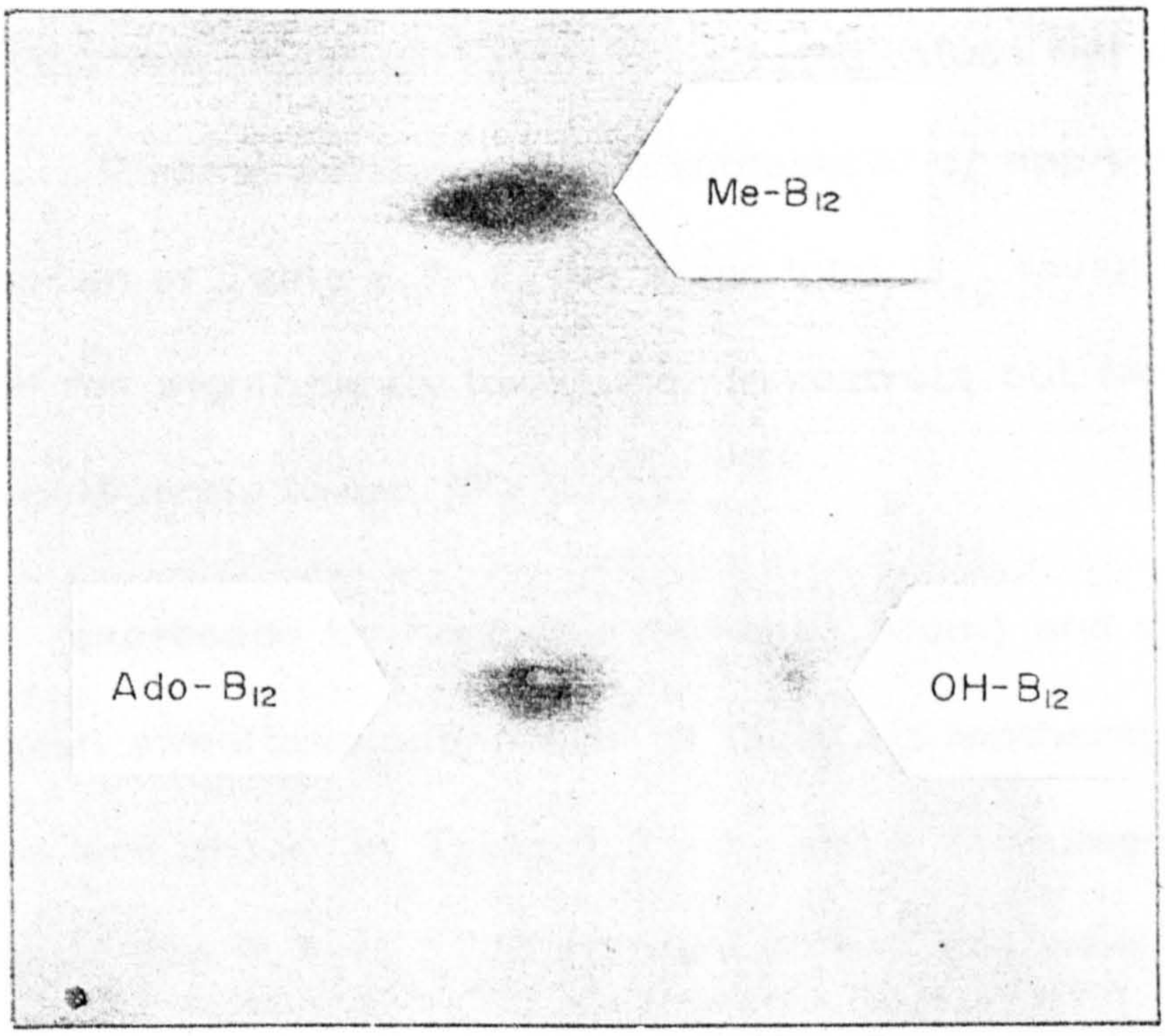


Figure 5.6 - II Separation of cobalamins in a control sample of spleen obtained at autopsy

5.7 COBALAMINS in PREGNANCY and LACTATION

Plasma cobalamins in 12 healthy non-pregnant women of child-bearing age are shown in Table 5.7 - 1; the values fall within normal limits. Plasma cobalamins in 8 subjects at or approaching delivery are shown in Table 5.7-2; the mean total B_{12} levels were slightly though not significantly lower than in controls but Me-B_{12} levels were significantly lower ($P < 0.02$).

Milk (expressed by hand in a darkened room) and venous blood were taken simultaneously from 10 lactating mothers; cobalamin values are shown in Table 5.7 - 3, and a bioautogram from one case at Fig 5.7-I. Significant differences were found in the plasma cobalamins and Me-B_{12}:Ado/OH-B_{12} ratio before and after delivery, and a comparison between the plasma and milk cobalamins in 9 of the 10 subjects revealed that in the milk, Ado/OH-B_{12} was proportionally increased and the difference in the Me-B_{12}:Ado/OH-B_{12} ratio highly significant ($P < 0.001$). Transport into milk of parenterally administered CN-B_{12} in initially different amounts was studied in 2 cases; Fig 5.7-II shows that in both, the CN-B_{12} level 3 hr after administration was considerably higher in the milk than in the plasma.

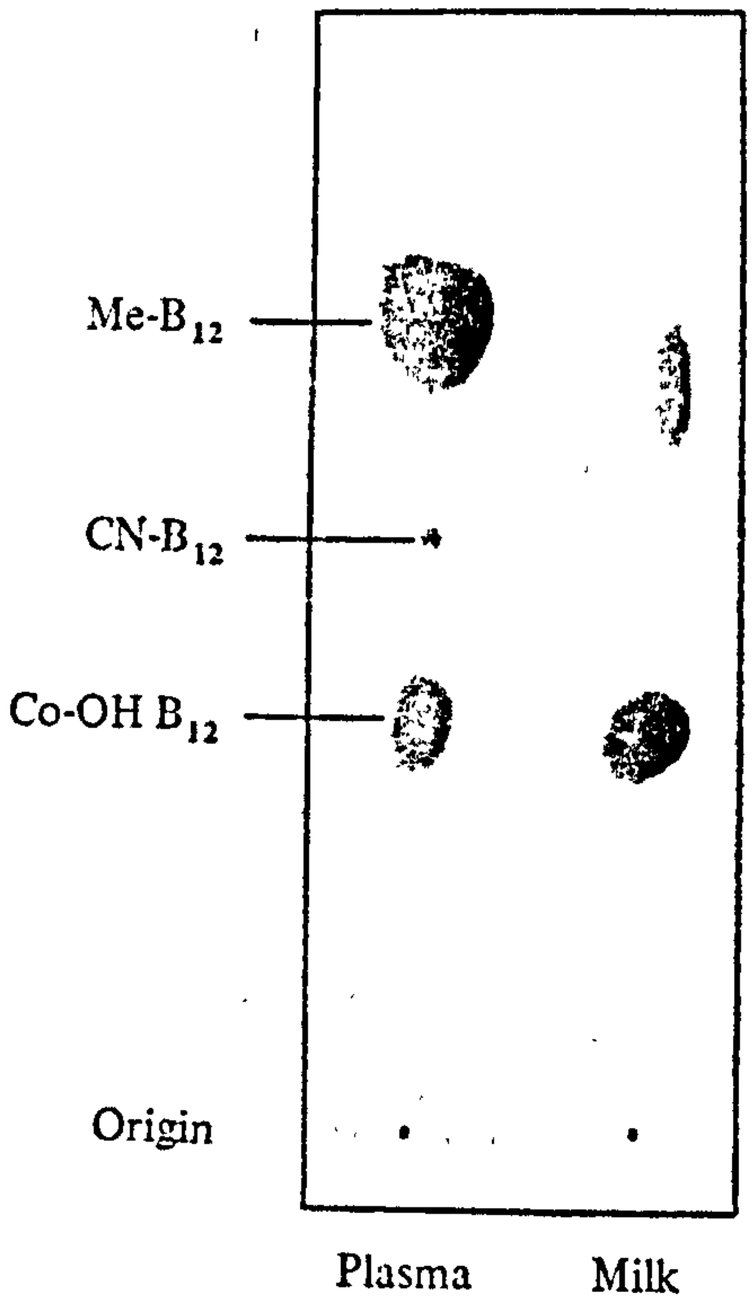


Figure 5.7 - I Plasma and milk cobalamins in a healthy mother

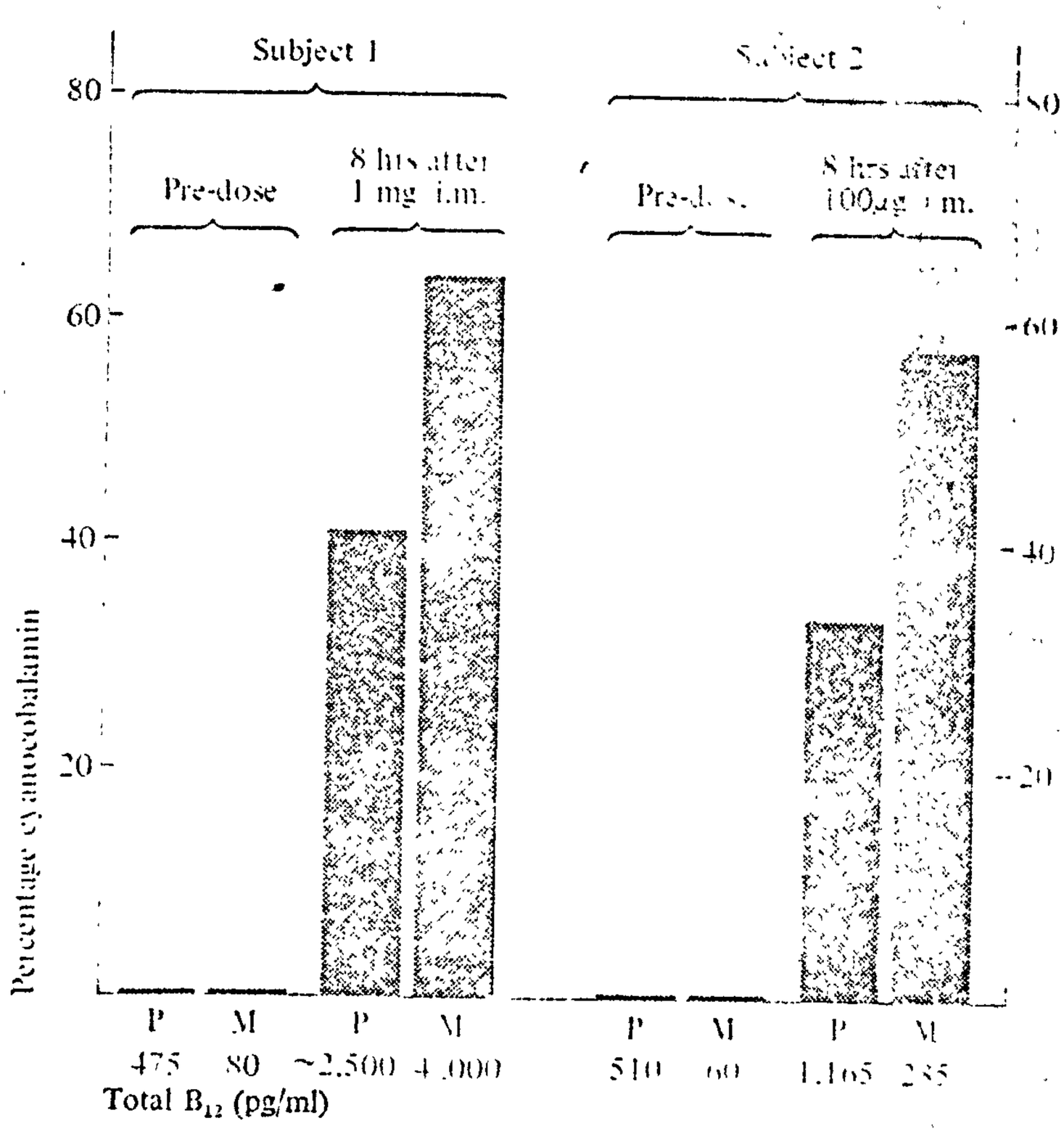


Figure 5.7 - II Appearance of CN-B_{12} in breastmilk following parenteral administration

TABLE 5.7 - 1

PLASMA COBALAMINS in NORMAL WOMEN

of child-bearing age

Subject	*Age*	*Plasma Cobalamins (pg/ml)*[1]				*Ratio*
		Total B_{12}	*MeB_{12}*	*CNB_{12}*	*Co-OHB_{12}*	*MeB_{12}/Co-OHB_{12}*
HM	21	380	250	0	130	1·9
GB	25	450	340	0	110	3·1
AO	26	375	280	0	170	1·6
DM	27	375	250	40	85	2·9
JB	28	265	175	0	90	1·9
EP	26	490	280	0	210	1·3
AW	21	250	160	15	75	2·1
MM	40	320	215	0	105	2·0
DS	31	440	330	0	110	3·0
JG	19	460	305	50	105	2·9
MY	16	550	435	0	115	3·8
JJ	29	600	300	75	225	1·3
Mean (± SE) n = 12	25 8	413 (± 76)	277 (± 22)	15 (± 7)	128 (± 14)	2·3 (± 0 2)

[1]MeB_{12} = methylcobalamin, CNB_{12} = cyanocobalamin, Co-OHB_{12} = deoxyadenosylcobalamin + hydroxocobalamin

TABLE 5.7 - 2

PLASMA COBALAMINS in HEALTHY PREGNANT

WOMEN at or approaching delivery

Subject	*Age*	*Plasma Cobalamins (pg ml)*				*Ratio*
		Total B_{12}	*MeB_{12}*	*CNB_{12}*	*Co-OHB_{12}*	*MeB_{12}/Co-OHB_{12}*
BK	23	220	147	0	73	2·0
CB	25	190	127	0	63	2·0
TG	25	300	225	0	75	3·0
SM	24	210	120	0	90	1·3
DH	30	600	346	39	215	1·6
FA	36	330	195	13	122	1·6
RS	30	355	230	0	125	1·8
GC	18	250	123	17	110	1·1
Mean (± SE) (n = 8)	26 4	307 (± 47)	189 (± 28)	8·6 (± 5 0)	109 (± 17)	1·8 (± 0 1)

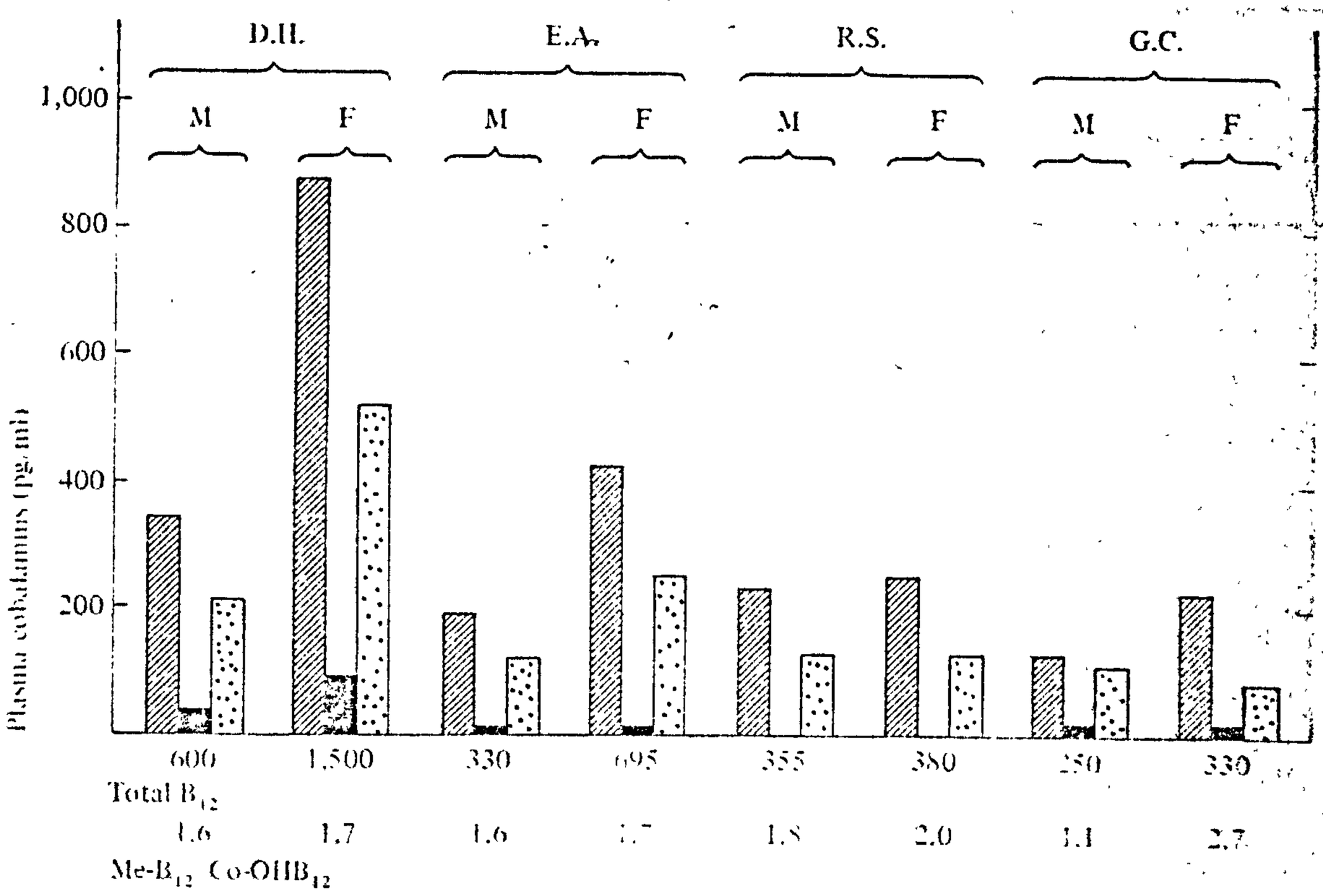


Figure 5.7-III Plasma cobalamins in maternal and foetal blood at delivery

▨ = Me-B_{12} ▩ = CN-B_{12} ⬚ = Ado/OH-B_{12}

In 4 mothers newly delivered of healthy infants, plasma cobalamins were estimated in maternal and cord blood (Fig 5.7-III); the total B_{12} level and percentage of Me-B_{12} were consistently higher in cord blood (in 2 cases, more than 100% higher). Cobalamins in umbilical arterial and venous blood - compared in 3 cases - were essentially similar in pattern.

TABLE 5.7-3

PLASMA and MILK COBALAMINS during LACTATION

Subject	*Age*	*Days after Delivery*	*Plasma Cobalamins (pg/ml)*					*Milk Cobalamins (pg/ml)*				
			Total B_{12}	*MeB_{12}*	*C\\B_{12}*	*Co-OHB_{12}*	*Ratio MeB_{12}/Co-OHB_{12}*	*Total B_{12}*	*MeB_{12}*	*C\\B_{12}*	*Co-OHB_{12}*	*Ratio MeB_{12}/Co-OHB_{12}*
YJ	23	6	905	638	54	213	3·2	1,480	845	0	635	1·4
AA	27	5	405	286	24	95	3·1	530	350	0	180	1·9
ES	18	4	530	398	0	132	3·4	20	8	0	12	0·7
EB	36	4	555	417	0	138	3·2	780	445	0	335	1·3
JC	27	6	365	313	0	52	6·0	320	183	0	137	1·3
PE	24	7	335	250	0	85	3·0	420	240	0	180	1·3
PA	25	4	--	—	--	-	--	1,860	990	125	745	1·3
NC	28	4	330	283	0	47	6·0	500	375	0	125	3·0
JA	25	4	475	407	0	68	6·0	80	46	0	34	1·4
MF	18	8	510	380	0	130	2·9	60	35	0	25	1·4
Mean (± SE)												
	25·1	5·2	490	375	8·7	107	4·1	605	352	12·5	241	1·5
n 10			(±59)	(±39)	(±6·3)	(±18)	(±0·4)	(±185)	(±106)	(±12·5)	(±81)	(±0·2)

5.8 DISCUSSION

Although vitamin B_{12} is essential to all animal life and present in every animal cell, it is not catabolized in vivo and very small amounts are continuously recycled. The adult whole-body B_{12} in man ranges from 1,000 μg to 2,000 μg, and the daily dietary requirement to replace normal excretory losses, from 0.6 μg to 1.2 μg (the 5 μg daily faecal loss resulting principally from colonic floral synthesis of B_{12} not available to the organism as a whole). In physiological conditions, B_{12} is protein-bound - in the gastrointestinal tract to intrinsic factor, in the plasma to the globulin fraction of the circulating proteins, in erythrocytes to haemaglobin, etc. Physiological and pathological changes quite unconnected with B_{12} metabolism (e.g. in diet, urinary output or B_{12} binding capacity) may therefore affect total B_{12} concentrations generally or locally. It might reasonably be expected, however, that the proportions of the individual cobalamins would be little influenced by extraneous factors of this kind and might form a surer basis than total B_{12} levels alone for the study of vitamin B_{12} metabolism in health and disease.

In the course of the present study, consistent cobalamin patterns have emerged. Me-B_{12} predominated in plasma and milk, and Ado-B_{12} in all cellular tissues studied.

In the haemopoietic system in particular the proportions of each cobalamin were remarkably constant. Total B_{12} was highest in the bone marrow (approximately 13,000 pg/ml), and lowest in the erythrocytes (approximately 200 pg/ml), but in all samples, about 50% of the total B_{12} was present in the form of Ado-B_{12}, 25% in the form of OH-B_{12} and 15% in the form of Me-B_{12}; variable small amounts of CN-B_{12} were often found.

In several organs, total B_{12} levels were very much higher than in plasma or even in bone marrow. Ado-B_{12} always predominated, the proportions ranging from 70% in the liver to 45% in the spleen. The proportions of Me-B_{12} varied widely - from 5% in the liver to 37% in the spleen.

It will be apparent that CN-B_{12} is unlikely to play an essential part in human B_{12} metabolism and that its frequent presence in small amounts in body fluids (but very rarely in the tissues) is almost certainly adventitious. Since, however, B_{12} in this form has been administered therapeutically with good effect for many years, it is obviously readily convertible in vivo to metabolically active forms.

Me-B_{12} and Ado-B_{12} are known to have coenzyme functions - which it has been thought more appropriate to describe in Section 6.6.

No known metabolic function has as yet been attributed to OH-B_{12}, and the obvious possibility that its presence in any particular sample may have resulted from photolysis could not of course be overlooked, but the finding that it bears a consistent proportional relationship of 1:2 with Ado-B_{12} would rather suggest some functional connexion between these cobalamins. One explanation might be that OH-B_{12} represents the less stable metabolic intermediates, B_{12a}, B_{12r}, B_{12s}, known to be involved in the interconversion of cobalamins and in the synthesis of the cobalamin coenzymes in the tissues.

PATHOLOGICAL RESULTS 6

6.1 COBALAMINS in B_{12} DEFICIENCY

Untreated pernicious anaemia

Plasma cobalamins in 25 cases of uncomplicated pernicious anaemia and in 4 cases with subacute combined degeneration of the cord are shown in Table 6.1 - 1. Cobalamins in plasma, erythrocytes, leucocytes and bone marrow in a further 12 cases of pernicious anaemia are summarised in Table 6.1 - 2.

Plasma Me-B_{12} in all cases was disproportionately reduced in relation to Ado/OH-B_{12}, so that the ratio Me-B_{12}: Ado/OH-B_{12} was less than 1.0. In just under half of the cases Me-B_{12} was present in the plasma only in traces, or was not detectable at all. Whereas Me-B_{12} was well below the lower limit of normal in all cases, plasma Ado/OH-B_{12}, though frequently subnormal, was just within normal limits in a minority of cases.

An interesting feature of the plasma cobalamin pattern in pernicious anaemia was that 18 of the 41 cases (44%) showed amounts of CN-B_{12} which, although low in absolute terms, made up a substantial proportion (up to 40%) of the total plasma B_{12}. No relationship was observed between this phenomenon and the occurrence of subacute combined degeneration of the cord.

TABLE 6.1 – 1

PLASMA COBALAMINS in UNTREATED PERNICIOUS ANAEMIA

Patient	Age	Sex	Total B_{12} (pg/ml)	Me B_{12} (pg/ml)	CN B_{12} (pg/ml)	Co-OH B_{12} (pg/ml)	Ratio Me/Co-OH B_{12}	CN B_{12} percentage of total	Haemoglobin (g/100 ml)	Serum folate (ng/ml)	Methylmalonate excretion test	
											Pre-valine	Post-valine
Uncomplicated pernicious anaemia												
1	60	M	50	0	6	44	0	12	12·0	13·0	+	+
2	61	M	50	5	0	45	0·2	0	11·2	28·0		
3	66	M	80	29	0	51	0·6	0	11·3	19·0	+	+
4	60	F	100	0	25	75*	0	25	10·0	9·6	+	+
5	77	F	10	0	3	7	0	30	6·0	11·8	+	+
6	71	F	75	0	0	75*	0	0	13·8	21·0		
7	77	F	68	0	0	68*	0	0	8·7	8·0		
8	85	F	64	16	0	48*	0·3	0	10·4	3·0		
9	67	F	36	17	17	52*	0·3	20	6·2	1·2		
10	75	F	50	13	0	37*	0·4	0	7·3	4·2		
11	65	F	30	0	0	30*	0	0	4·9	6·4		
12	65	M	65	26	0	39*	0·7	0	7·8	—		
13	71	F	35	14	0	21*	0·7	0	7·4	—		
14	63	F	75	trace	0	75*	0	0	7·7	4·7		
15	46	F	100	29	29	42*	0·7	29	11·5	8·4		
16	62	F	80	13	27	40*	0·3	34	5·0	2·9		
17	71	F	80	16	16	48*	0·3	20	7·7	>25		
18	34	M	60	0	24	36*	0	40	15·1	—		
19	43	M	35	0	14	21*	0	40	3·8	1·7		
20	74	F	77	trace	trace	75*	0	*ca* 5	11·6	32·0	–	–
21	63	F	130	33	0	97*	0·3	0	18·1	7·6	+	+
22	63	F	86	22	0	64*	0·3	0	7·5	24·0	+	+
23	68	F	49	0	20	29*	0	41	5·9	5·4	+	+
24	49	M	72	0	trace	70*	0	*ca* 5	7·8	28·0	+	+
25	53	F	100	trace	0	100*	0	0	6·1	7·6		
Cases with subacute combined degeneration of the cord												
26	66	M	30	8	2	20	0·4	7	10·0	—		
27	69	F	30	9	9	12*	0·8	30	12·5	—		
28	68	F	50	trace	12	38*	0	24	7·8	11·0		
29	72	M	55	trace	0	55*	0	0	14·9	20·0		
Mean ± SE			64·6 ± 4·8	9·0 ± 1·9	7·2 ± 1·8	48·8 ± 4·3	0·22 ± 0·05	12·5 ± 2·8				
Range			(10–130)	(0–33)	(0–29)	(7–100)	(0–0·8)	(0–41)				

Note: In calculation of statistics 'trace' has been assigned a value of 2.
* Derived from visual score.

There was no correlation between plasma Me-B_{12} and serum folate. Methylmalonate excretion was studied in 9 cases (Table 6.1 - 1) and found to be increased in 4 (Nos 4,21,22,24) in the presence of a plasma Ado/OH-B_{12} just within normal limits, and in 4 (Nos 1,3,5,23) where the plasma Ado/OH-B_{12} was abnormally low (7 pg to 51 pg/ml).

Erythrocyte cobalamins in 9 cases of pernicious anaemia (Table 6.1 - 2; Fig 6.1 - I) showed that although the total B_{12} was reduced, the reduction was by no means as severe as in the plasma, and the ratio Me-B_{12}: Ado/OH-B_{12}, when expressed as a percentage of the values in normal erythrocytes, ranged from 62% to 70%. In contrast, the CN-B_{12} in erythrocytes was raised to over 150% of the normal value.

Leucocyte cobalamins were estimated in 6 cases of pernicious anaemia although values for total B_{12} were obtained in only 3 (Table 6.1 - 2; Fig 6.1 - II). The changes were very similar to those in the erythrocytes. Total B_{12} was reduced, on average, to 65% of normal, but the ratio Me-B_{12}: Ado/OH-B_{12} was not grossly affected. CN-B_{12} was abnormally raised in 4 of the 6 cases.

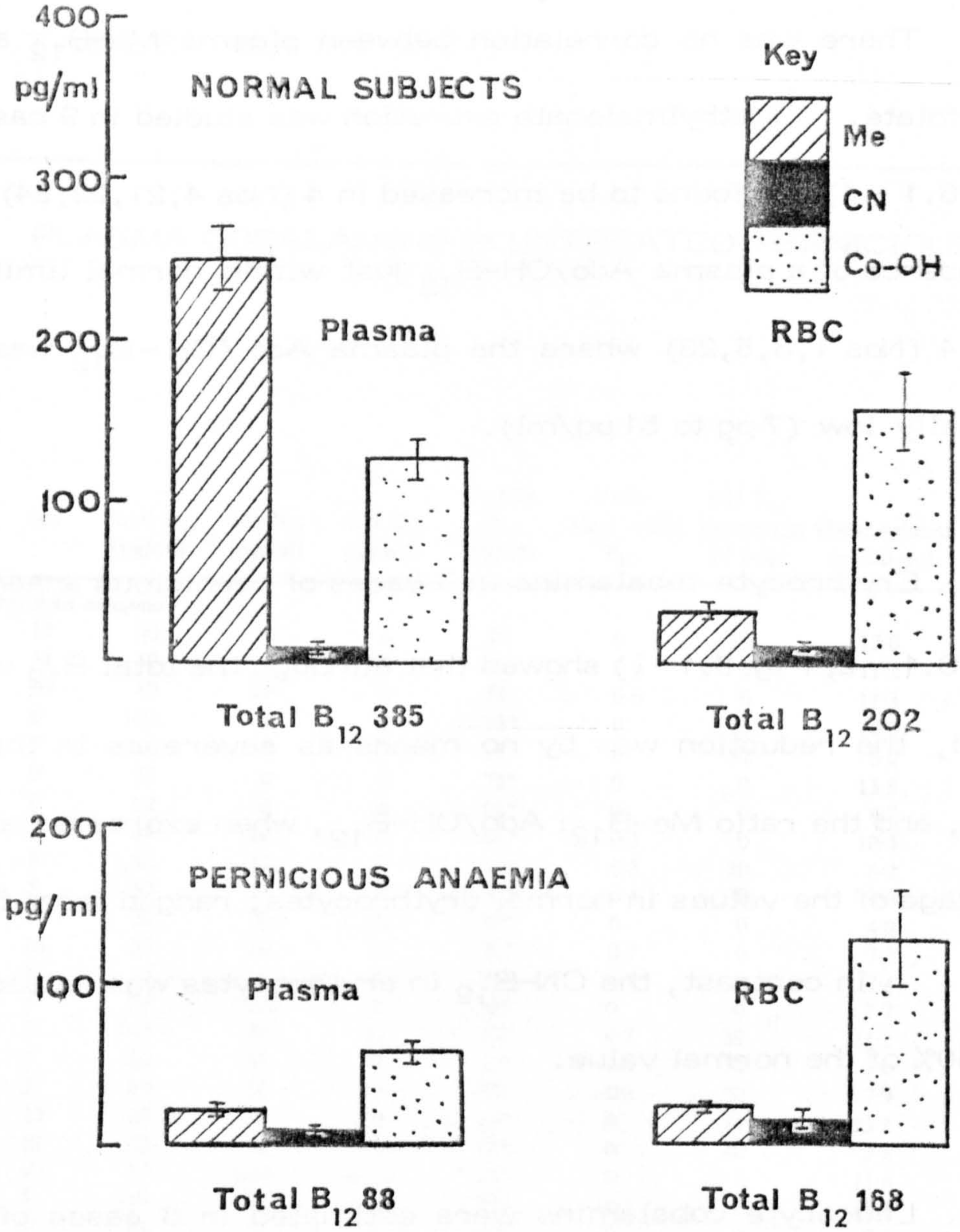


Figure 6.1 – I Plasma and erythrocyte cobalamins in normal subjects and patients with untreated pernicious anaemia

TABLE 6.1 - 2

BLOOD and BONE MARROW COBALAMINS in PATIENTS with PERNICIOUS ANAEMIA and CONTROL SUBJECTS

	No. of samples	Total vitamin B_{12} (pg/ml)	$Me\text{-}B_{12}$ (pg/ml)	(%)	$CN\text{-}B_{12}$ (pg/ml)	(%)	$Ado\text{-}OHB_{12}$ (pg/ml)	(%)
Plasma								
Controls	22	385±31[1]	251±22	65	7·8±1·2	2·0	126±12	33
Pernicious anaemia	12	88±11	22±3·5	25	8·2±2·2	9·3	58±7·6	66
Erythrocytes								
Controls	12	202±25	31±4·2	15	11±2·3	5·5	159±24	79
Pernicious anaemia	9	168±39	23±3·2	14	16±6·1	9·5	129±29	77
		(pg/g)	(pg/g)	(%)	(pg/g)	(%)	(pg/g)	(%)
Leucocytes								
Controls	18	3830±437	657±90	20	139±40	4·1	2978±458	76
Pernicious anaemia	3	2575±1467	518±243	20	243±202	9·4	1813±1364	70
Bone marrow								
Controls	13	12,960±1440	1700±316	13	274±108	2.1	10,940±1275	85
Pernicious anaemia	10	6450±1404	984±462	15	375±139	5·8	5091±1101	79

[1] Mean±SEM.

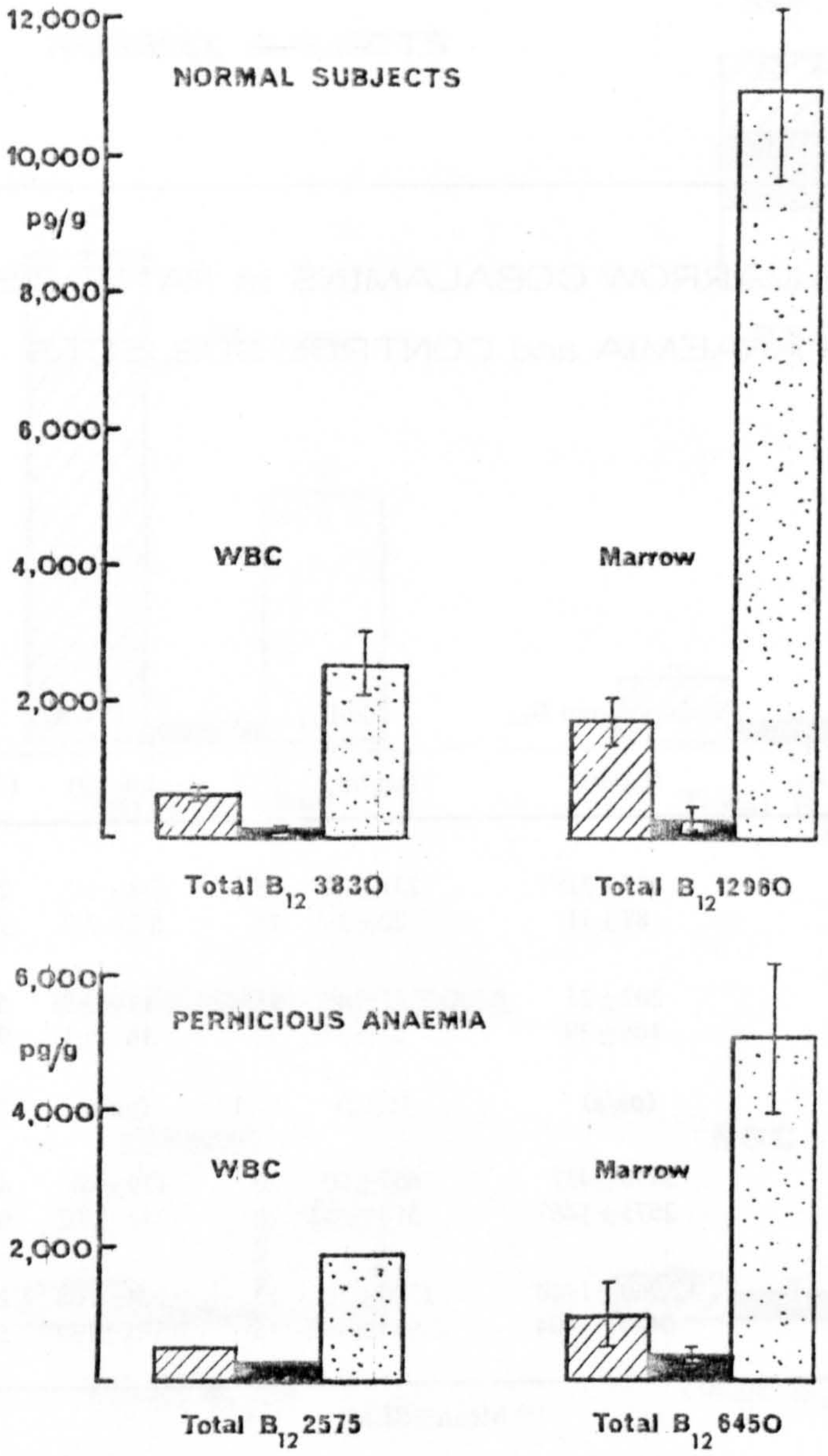


Figure 6.1 - II

Leucocyte and bone marrow cobalamins in normal subjects and patients with untreated pernicious anaemia

The bone marrow cobalamins in 10 cases of pernicious anaemia (Table 6.1-2; Fig 6.1-II) showed changes which were generally similar to those in the red and white blood cells. Total B_{12} was approximately half the value in normal marrow, but the Me-B_{12}: Ado/OH-B_{12} ratio was largely unaffected. CN-B_{12} was detected in 5 of the 10 cases and on average the levels were almost 40% higher than those in the marrow of normal subjects (Fig 6.1 - III).

In 4 cases of pernicious anaemia, cobalamins were also estimated in red cells separated from the marrow. The results (Fig 6.1 - III) showed striking differences both from those found in other tissues in pernicious anaemia and from those in red cells isolated from normal marrow aspirates. The total B_{12} (mean 2,846 pg/g) was less depressed than in any other tissue and only about 20% below the normal average (3,558 pg/g) but the proportions of the constituent cobalamins were considerably disturbed; Me-B_{12} was in all cases disproportionately reduced and the mean value was only 38% of normal, while the Me-B_{12}: Ado/OH-B_{12} ratio was lowered and in some cases this reduction was quite as marked as in the plasma.

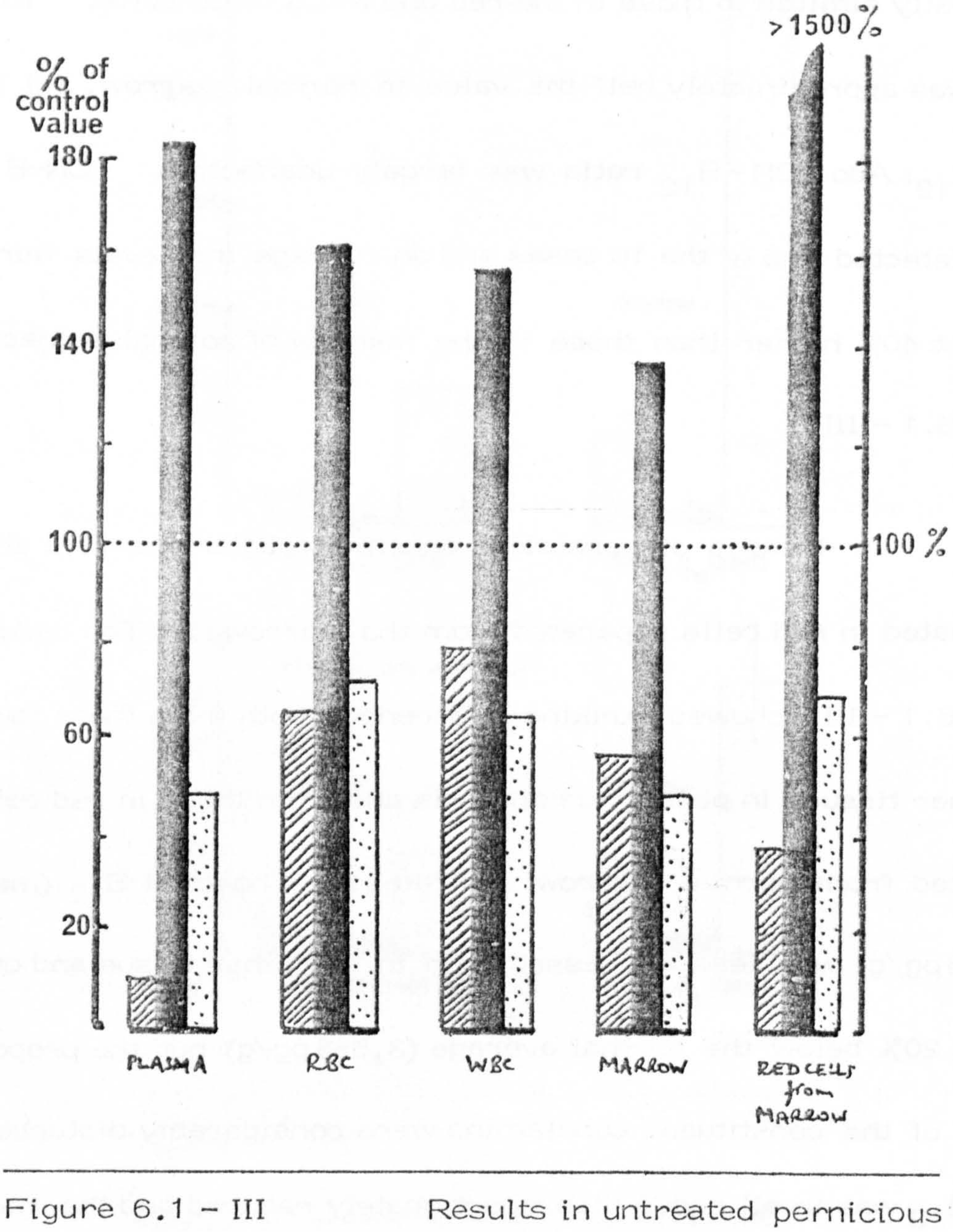


Figure 6.1 - III Results in untreated pernicious anaemia as a percentage of those in normal subjects

▨ = $Me\text{-}B_{12}$ ■ = $CN\text{-}B_{12}$ ⁘ = $Ado/OH\text{-}B_{12}$

Perhaps the most striking finding in the bone marrow erythrocytes in pernicious anaemia was the presence in every sample of CN-B_{12}, amounting in one case to over half the total B_{12}, and in all cases to more than 15%. The mean level of CN-B_{12} was almost three times the value for Me-B_{12}, and no less than fifteen times the average CN-B_{12} found in normal bone marrow erythrocytes. These results suggest that in pernicious anaemia the level of CN-B_{12} in the marrow red cells increases largely at the expense of Me-B_{12}.

Other causes of B_{12} deficiency

Tables 6.1 - 3 and 6.1 - 4 show results in a number of conditions where B_{12} deficiency may occur, though not all the patients were actually deficient.

Of 5 untreated vegans with subnormal plasma total B_{12} levels (Table 6.1-3), 3 showed a pattern of plasma cobalamins indistinguishable from that of pernicious anaemia, but in the other 2, Me-B_{12} was only slightly subnormal and the Me-B_{12}: Ado/OH-B_{12} ratio was within normal limits. In 3 vegans taking B_{12} supplements, all plasma cobalamins were within normal limits.

TABLE 6.1 - 3

PLASMA COBALAMINS in VEGANISM

Subject	Diet	Age	Sex	Total B_{12} (pg/ml)	Me B_{12} (pg/ml)	CN B_{12} (pg/ml)	Co-OH B_{12} (pg/ml)	Ratio Me/Co-OH B_{12}	Haemo-globin (g/100 ml)	Serum folate (ng/ml)	Methylmalonate excretion test	
											Pre-valine	Post-valine
31	B_{12} unsupplemented	38	F	160	107	0	53*	2·0	11·5	8·0		
32	B_{12} unsupplemented	31	F	135	62	0	73	0·9	7·5	3·2	+	+
33	B_{12} unsupplemented	24	F	155	118	0	37	3·2	11·7	0·8	−	+
34	B_{12} unsupplemented	44	F	55	0	0	55	0	13·2	16·6	−	+
35	B_{12} unsupplemented	46	M	100	0	0	100	0	11·0	>25		
36	B_{12} supplemented	33	M	380	190	0	190*	1·0	14·0	>32		
37	B_{12} supplemented	31	F	300	150	trace	150*	1·0	13·6	>16		
38	B_{12} supplemented	64	M	455	273	0	182*	1·5	13·6	14·0		

* Derived from visual score.

TABLE 6.1 - 4

PLASMA COBALAMINS in GASTRO-INTESTINAL DISEASE

Patient	Diagnosis	Age	Sex	Total B_{12} (pg/ml)	Me B_{12} (pg/ml)	CN B_{12} (pg/ml)	Co-OH B_{12} (pg/ml)	Ratio Me/Co-OH B_{12}	Haemo-globin (g/100 ml)	Serum folate (ng/ml)	Methylmalonate excretion test	
											Pre-valine	Post-valine
30	Partial gastrectomy: SACD	61	M	49	12	0	37*	0·3	7·2	19·2	+	+
39	Partial gastrectomy	67	M	112	44	0	68	0·7	10·1	2·4	−	+
40	Partial gastrectomy	54	M	135	81	3	53	1·5	13·5	8·2	−	−
41	Partial gastrectomy	29	M	135	56	0	81	0·7	9·3	7·0	−	−
42	Partial gastrectomy	50	M	155	83	0	72	1·2	11·5	11·0	−	−
43	Partial gastrectomy	56	M	205	84	0	121	0·7	13·3	8·4	−	−
44	Partial gastrectomy	68	F	425	170	trace	255	0·7	11·6	0·7		
45	Partial gastrectomy	63	M	255	102	0	153*	0·7	12·3	>32		
47	Partial gastrectomy	63	M	535	289	0	246	1·2	10·0	14·4		
48	Partial gastrectomy	66	F	265	159	0	106*	1·5	10·0	4·6		
49	Partial gastrectomy	59	F	625	375	0	250	1·5	11·6	17·4		
50	Partial gastrectomy	58	M	150	75	0	75	1·0	4·6	0·8	−	−
51	Atrophic gastritis	49	M	170	82	0	86	0·9	12·8	13·0	−	−
52	Atrophic gastritis	73	M	130	52	0	78	0·7	12·8	5·1	−	−
60	Atrophic gastritis	66	F	190	142	0	48*	3·0	10·4	8·0		
61	Atrophic gastritis	74	F	140	101	0	39	2·6	9·0	6·4		
53	Jejunal diverticulosis	38	M	275	170	22	83	2·0	12·0	30		
54	Jejunal diverticulosis	62	M	310	223	0	87	2·6	13·5	3·8		
55	Jejunal diverticulosis	72	F	265	116	0	149	0·8	14·4	13·1		
56	Jejunal diverticulosis	84	F	50	14	0	36	0·4	8·1	15·0	+	+
57	Jejunal diverticulosis: post-gastrectomy	74	M	185	74	0	112*	0·7	14·2	10·2		
58	Crohn's disease	42	M	150	40	0	110*	0·3	12·6	3·0		
59	Adult coeliac disease	53	M	420	252	0	168	1·5	9·9	0·6		

* Derived from visual score.

Of the patients with a partial gastrectomy or atrophic gastritis (Table 6.1 - 4), 5 out of 9 with subnormal plasma B_{12} levels had a subnormal Me-B_{12}: Ado/OH-B_{12} ratio, the pattern being similar to that in pernicious anaemia. An interesting feature was the occurrence of a subnormal Me-B_{12}: Ado/OH-B_{12} ratio in 3 patients (Nos 43, 44, 45) with total plasma B_{12} levels within normal limits.

In the group with intestinal disease (Table 6.1 - 4), all 3 patients with subnormal or borderline total plasma B_{12} levels had a subnormal Me-B_{12}: Ado/OH-B_{12} ratio, and one (No 55) had a subnormal ratio in the presence of a normal total plasma B_{12}.

Discussion

The present results show that in untreated pernicious anaemia, it is only in plasma that Me-B_{12} is disproportionately reduced. In cellular tissues, the percentage reduction of Me-B_{12}, Ado-B_{12} and OH-B_{12} is fairly similar. In view of the dramatic effects of B_{12} deficiency on the normal functioning of the bone marrow, it is perhaps surprising that in pernicious anaemia, the marrow cobalamin levels are not more severely reduced. B_{12} is taken up preferentially by proliferating

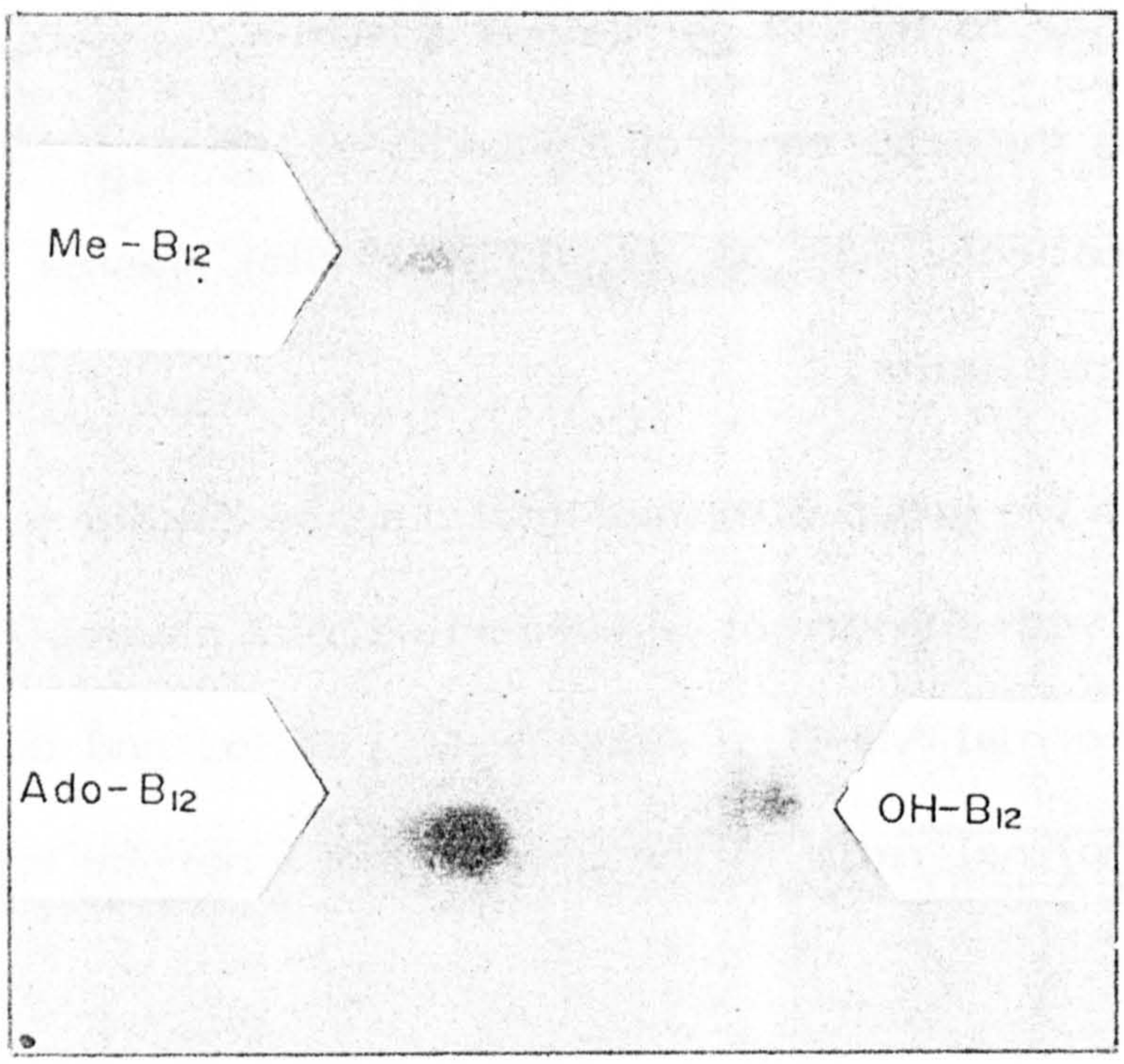


Figure 6.1 - IV

Separation of plasma cobalamins in a case of untreated pernicious anaemia. $Me\text{-}B_{12}$ is disproportionately reduced

primitive cells rather than by mature cells (Schilling and Meyer, 1964; Hoffbrand, Tripp and Das, 1973). The bone marrow in untreated megaloblastic anaemia contains proportionately far more primitive cells than a normal marrow, and it may be that the marrow cells in pernicious anaemia are in fact much more depleted of B_{12} for their stage of development than is apparent by a direct comparison with normal marrow.

6.2 COBALAMINS in FOLATE DEFICIENCY

Table 6.2 – 1 shows results and clinical diagnoses in patients suffering from folate deficiency. A variety of patterns of plasma cobalamins occurred. Some patients (Nos 44 and 71) had a subnormal Me-B_{12}: Ado/OH-B_{12} ratio in the presence of a normal total plasma B_{12}, while in others the proportions of the two major cobalamins were normal. In folate deficiency, in contrast to B_{12} deficiency, there was no consistent change in the proportions of individual cobalamins

TABLE 6.2 – 1

Patient	Diagnosis	Age	Sex	Total B_{12} pg/ml)	Me B_{12} (pg/ml)	CN B_{12} (pg/ml)	Co-OH B_{12} (pg/ml)	Ratio Me/Co-OH B_{12}	Haemo-globin (g/100 ml)	Serum folate (ng/ml)
33	Vegan	24	F	155	118	0	37	3·2	10·8	0·8
78	Nutritional deficiency	56	F	285	196	0	89	2·2	8·3	0·9
79	Nutritional deficiency	65	F	205	110	0	95	1·2	6·9	2·4
80	Nutritional deficiency	77	F	130	65	0	65*	1·0	9·2	0·4
81	Nutritional deficiency	75	F	230	92	0	138	0·7	7·2	0·6
84	Nutritional deficiency	24	M	495	370	0	125	3·0	8·2	0·4
85	Nutritional deficiency	55	F	250	125	0	125*	1·0	6·9	0·6
46	Post-partum megaloblastic anaemia	26	F	500	360	0	140	2·6	12·8	0·8
44	Post-gastrectomy megaloblastic anaemia	68	F	425	170	trace	255*	0·7	16·0	0·7
50	Post-gastrectomy megaloblastic anaemia	58	M	150	105	0	45	2·3	6·5	0·8
59	Adult coeliac disease	53	M	420	252	0	168	1·5	9·9	0·6
82	Carcinoma of stomach	62	M	240	185	0	55	3·4	8·9	0·6
83	Anticonvulsant megaloblastic anaemia	27	M	165	92	0	73	1·3	7·8	1·5
71	Alcoholic hepatic cirrhosis	38	M	480	120	trace	360*	0·3	17·3	2·3

* Derived from visual score.

6.3 COBALAMINS in LEUKAEMIAS and LIVER DISEASE

Table 6.3-1 shows results in patients with leukaemias or liver disease, in some of whom the total plasma B_{12} was pathologically high. Two cases of chronic myeloid leukaemia with elevated total B_{12} (Nos 63 and 64) retained an approximately normal $Me-B_{12}$: $Ado/OH-B_{12}$ ratio; in Case 63, the level of $CN-B_{12}$ was raised although the proportion of this compound was within normal limits. Case 66 (myelomonocytic leukaemia) and Case 67 (erythroleukaemia) had 'borderline' total plasma B_{12} levels and the pattern of individual cobalamins was similar to that in untreated pernicious anaemia.

In liver disease, several patients had normal plasma cobalamins, though one case (No 71) had a subnormal $Me-B_{12}$: $Ado/OH-B_{12}$ ratio in the presence of a normal total B_{12}. Two cases (Nos 69 and 76) had a high total B12 with a disproportionate increase in $Ado/OH-B_{12}$ so that the $Me-B_{12}$: $Ado/OH-B_{12}$ ratio was subnormal.

In five liver biopsy samples (two alcoholic fatty liver, two cirrhotic and one found to be normal) the mean total B_{12} content was 520 ng/g wet weight (range 177 to 1,530 ng/g). In all samples, nearly all the B_{12} was in the form of $Ado/OH-B_{12}$. Two showed traces (10 ng/g) of $Me-B_{12}$; no $CN-B_{12}$ was found.

TABLE 6.3 - 1

PLASMA COBALAMINS in LEUKAEMIA and LIVER DISEASE

Patient	Diagnosis	Age	Sex	Total B_{12} (pg/ml)	Me B_{12} (pg/ml)	CN B_{12} (pg/ml)	Co-OH B_{12} (pg/ml)	Ratio Me/Co-OH B_{12}	Haemo-globin (g/100 ml)	Serum folate (ng/ml)
63	Chronic myeloid leukaemia	50	M	*ca* 2000	*ca* 1600	*ca* 140	*ca* 260	6·1	11·0	—
64	Chronic myeloid leukaemia	58	M	> 3000	> 1800	trace	> 1200*	1·5	7·7	3·6
65	Myelomonocytic leukaemia	57	M	870	678	17	175	3·9	7·0	8·0
66	Myelomonocytic leukaemia	60	F	210	84	0	126*	0·7	5·9	2·1
67	Erythroleukaemia	48	F	185	66	39	80	0·8	6·1	4·4
68	Erythroleukaemia	47	M	385	192	0	193*	1·0	7·9	> 32
69	Active chronic hepatitis	46	F	*ca* 2000	*ca* 960	0	*ca* 1040	0·9	11·4	7·5
70	Cirrhosis, idiopathic	74	M	1140	900	0	240	2·7	13·4	2·6
71	Cirrhosis, alcoholic	38	M	480	120	trace	360*	0·3	18·1	2·3
72	Cirrhosis, alcoholic	70	M	510	255	0	255	1·0	13·2	14·5
73	Cirrhosis, idiopathic	44	F	515	386	0	129*	3·0	13·1	2·4
74	Cirrhosis, alcoholic	58	M	1185	890	0	295	3·0	11·4	6·2
75	Cirrhosis, idiopathic	18	M	> 2000	> 1200	0	> 800*	1·5	11·5	8·8
76	Active chronic hepatitis	26	F	995	458	0	537	0·9	13·3	—

* Derived from visual score.

6.4 COBALAMINS in OPHTHALMOLOGICAL and NEUROLOGICAL DISEASE

Plasma and erythrocyte cobalamins in European patients with visual failure

Plasma and erythrocyte cobalamins were estimated in 12 cases of visual failure, and the results compared with those of normal subjects (Table 6.4-1, Fig 6.4-I). The group included one case of renal retinopathy, 2 of Leber's optic atrophy, one of dominantly-inherited optic atrophy, and 8 of optic atrophy of unknown aetiology. The mean plasma total B_{12} was slightly higher in these patients than in normal subjects although all values were within normal limits, and the mean value for Ado/OH-B_{12} was significantly higher (Table 6.4-2). Plasma Me-B_{12} was slightly increased but the difference between patients and controls was not significant; there was no significant change in the Me-B_{12}: Ado/OH-B_{12} ratio, and no increase in plasma CN-B_{12}.

Erythrocyte total B_{12} concentrations were only slightly higher in these patients than in normal subjects and the difference was not significant but the distribution of the cobalamins differed markedly between the groups. The greatest difference was in Me-B_{12}, the mean level in the patients being more than three

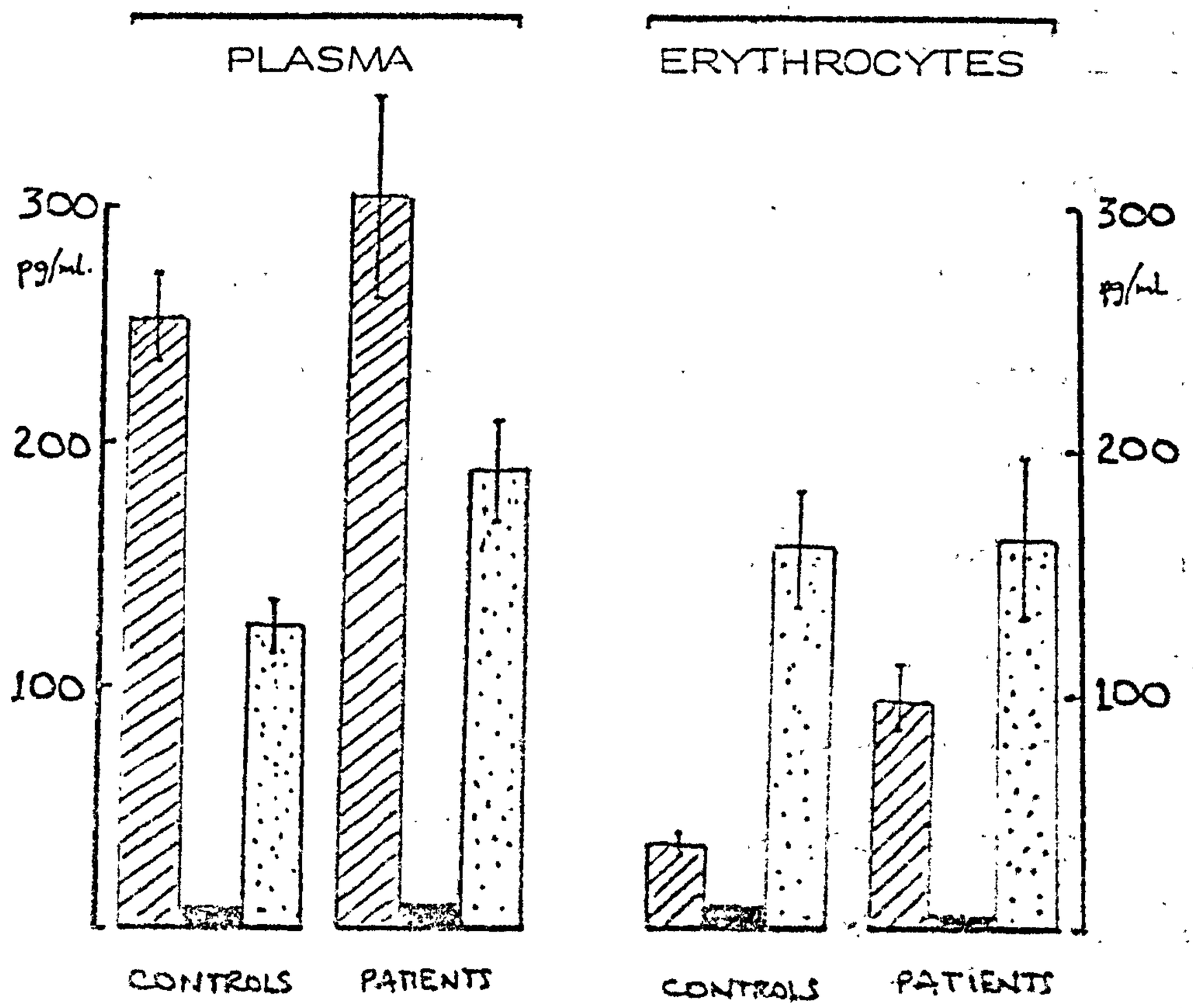


Figure 6.4 - I Plasma and erythrocyte cobalamins in European patients with visual failure and healthy controls

▨ = $Me\text{-}B_{12}$ ▩ = $CN\text{-}B_{12}$ ⊡ = $Ado/OH\text{-}B_{12}$

TABLE 6.4 - 1

PLASMA and ERYTHROCYTE COBALAMINS in OPHTHALMOLOGICAL DISEASE

Patient	Diagnosis	PLASMA (picograms per ml)						ERYTHROCYTES (picograms per ml)						
		Total B12	Me-B12	CN-B12	Ado/OH-B12	Ratio Me-B12 Ado/OH-B12	Percentage CN-B12	Total B12	Me-B12	CN-B12	Ado/OH-B12	Ratio Me-B12 Ado/OH-B12	Percentage CN-B12	
AJ	Optic atrophy of unknown aetiology	535	283	24	228	1.2	4	210	18	8	184	0.1	4	
JJ	Optic atrophy of unknown aetiology	890	653	0	237	2.8	0	215	100	12	103	0.97	5	
AA	Leber's optic atrophy	690	362	0	328	1.1	0	260	119	2	139	0.86	1	
DH	Renal retinopathy	475	204	14	257	0.8	3	195	110	8	77	1.4	4	
GM	Leber's optic atrophy	305	182	0	123	1.5	0	250	120	0	130	0.92	0	
DN	Optic atrophy of unknown aetiology	260	127	0	133	0.9	0	210	90	3	117	0.77	2	
AB	Dominantly-inherited optic atrophy	685	493	7	185	2.7	1	310	200	0	110	1.8	0	
RG	Optic atrophy of unknown aetiology	230	183	3	44	4.2	2	245	98	0	147	0.67	0	
CS	Optic atrophy of unknown aetiology	440	242	22	176	2.5	5	420	109	8	303	0.36	2	
WN	Optic atrophy of unknown aetiology	575	350	5	220	1.6	1	600	125	0	475	0.26	0	
CH	Optic atrophy of unknown aetiology	435	270	14	151	1.8	3	110	55	10	45	1.2	9	
HM	Optic atrophy of unknown aetiology	465	293	0	172	1.7	0	170	23	19	128	0.18	4	
		499	304	7.4	188	1.9	1.6	266	97	5.8	163	0.79	2.6	MEAN
		56	43	2.6	21	0.29	0.51	38	14	1.7	34	0.15	0.79	± SEM
	NORMAL SUBJECTS	385	251	7.9	126	2.1	3.0	202	31	11	159	0.24	6.1	MEAN
	Plasma n = 22 Erythrocytes n = 12	31	22	1.2	12	0.20	0.70	25	4.1	2.2	24	0.06	1.0	± SEM

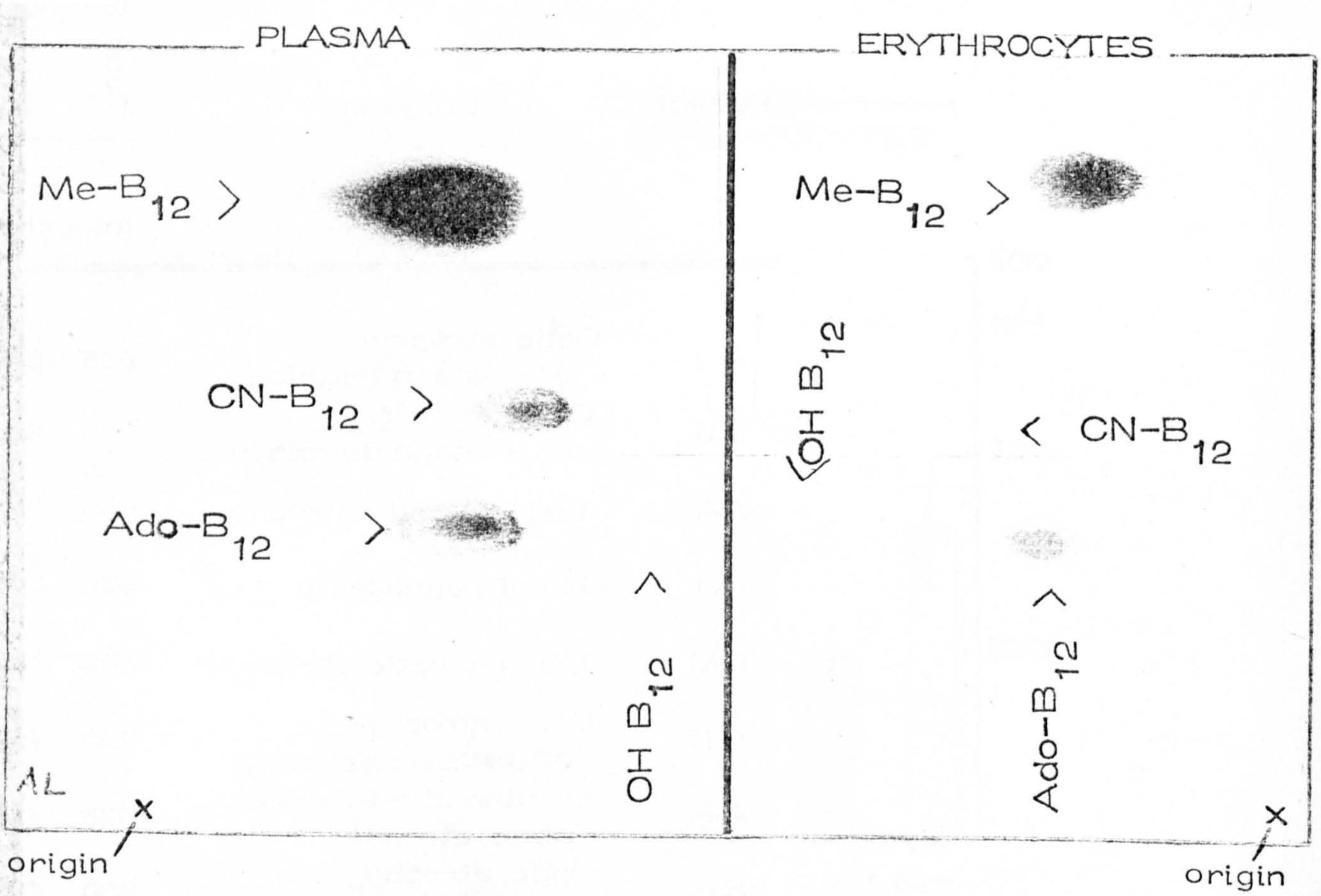


Figure 6.4 - II Separation of plasma and erythrocyte cobalamins in a Portugese boy of 15 suffering from advanced optic atrophy of unestablished aetiology. Both CN-B_{12} in the plasma and Me-B_{12} in the erythrocytes were abnormally elevated

times that in normal subjects. Values for Ado/OH-B_{12} did not differ significantly between the two groups and in only one patient (WN) was the level outside the normal range. In 10 of 12 patients, the raised erythrocyte Me-B_{12} level was reflected in the Me-B_{12}: Ado/OH-B_{12} ratio, the mean value being almost four times that in normal subjects. In contrast, the erythrocyte CN-B_{12} was lower in patients than in controls; although there was only a slight difference in red cell CN-B_{12} concentration between the groups, the proportion of CN-B_{12} in the patients' erythrocytes was less than half that in normal erythrocytes.

Plasma and liver cobalamins in Nigerian patients with tropical ataxic neuropathy

Plasma cobalamins were estimated in 13 patients in whom tropical ataxic neuropathy (TAN) had been diagnosed, and in 15 control subjects who were mainly medical staff and students from the hospital at Ibadan.

Liver cobalamins were estimated in four patients, using tissue from diagnostic biopsies. The biopsies were taken with a de Menghini needle, and the portion retained for cobalamin studies immediately transferred to a light-protected container and stored at -20°C until analysis. Control liver samples were obtained

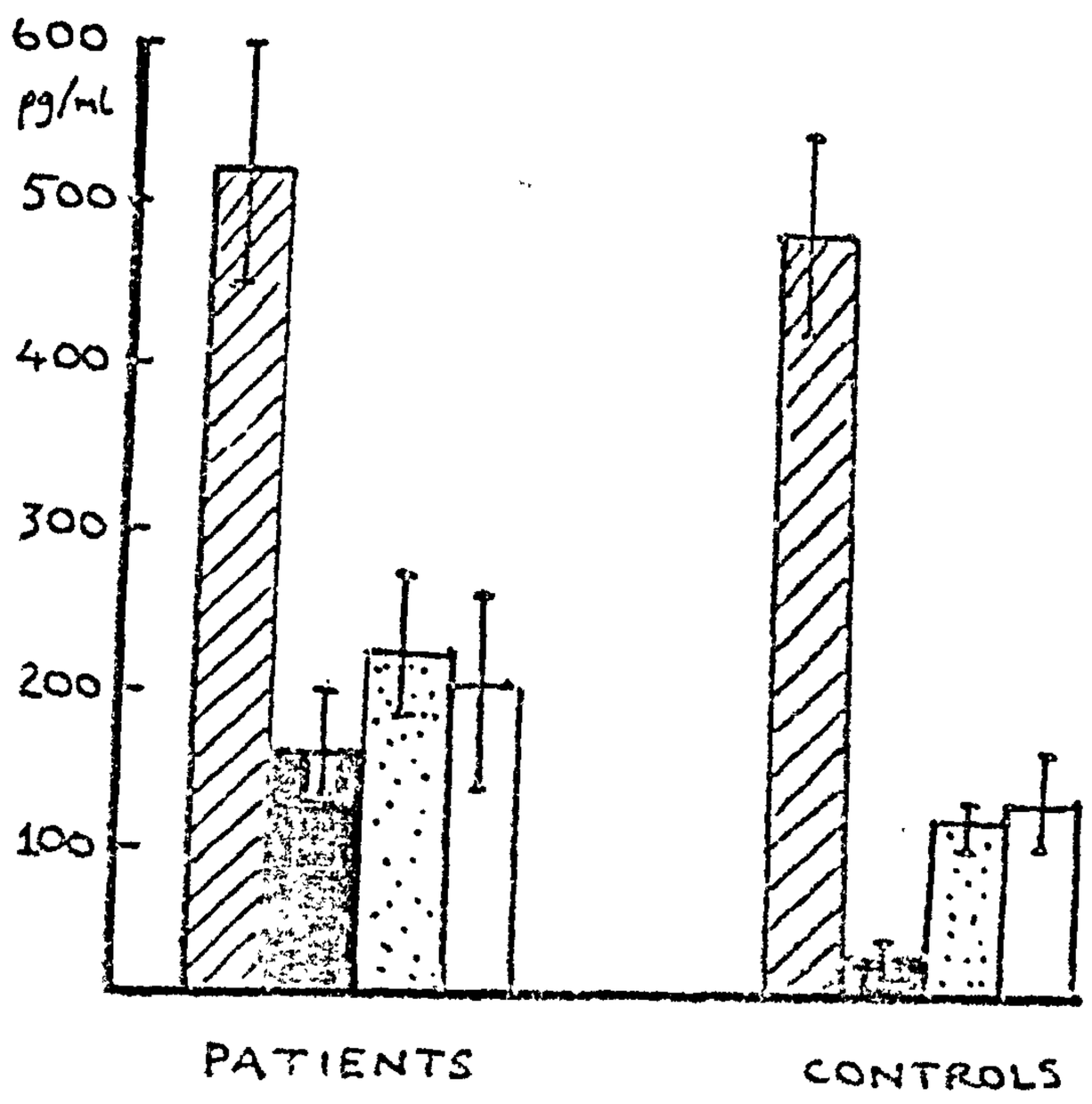


Figure 6.4-III Plasma cobalamins in Nigerian patients with tropical ataxic neuropathy and healthy control subjects

▨ = $Me-B_{12}$ ▩ = $CN-B_{12}$

⊡ = $Ado-B_{12}$ □ = $OH-B_{12}$

TABLE 6.4 - 2

COBALAMINS in OPHTHALMOLOGICAL DISEASE:

[1] Statistical comparison of results from TABLE 6.4-1

PATIENTS vs CONTROLS	Total B_{12}	$Me-B_{12}$	$CN-B_{12}$	Ado/ $OH-B_{12}$	Ratio: $Me-B_{12}$ Ado/$OH-B_{12}$	Percentage $CN-B_{12}$
PLASMA	$t = 1.93$ $P < 0.05$	NS[2]	NS	$t = 2.79$ $P < 0.01$	NS	NS
ERYTHROCYTES	$t = 1.72$ $0.1 > P > 0.03$	$t = 5.98$ $P < 0.001$	$t = 1.97$ $P < 0.05$	NS	$t = 4.44$ $P < 0.001$	$t = 2.64$ $0.02 > P > 0.01$

[1] Using Student's 't' test

[2] No significant difference between groups ($P > 0.1$)

TABLE 6.4 - 3

PLASMA COBALAMINS in TROPICAL ATAXIC NEUROPATHY

	Total B_{12}	$Me-B_{12}$	$CN-B_{12}$	$Ado-B_{12}$	$OH-B_{12}$	Percentage $CN-B_{12}$	
	- picograme per millilitre -						
PATIENTS (n = 13)	1062 ± 199	525 ± 78	159 ± 34	218 ± 40	192 ± 60	13.2 ± 2.5	Mean ± SEM
CONTROLS (n = 15)	709 ± 70	484 ± 62	26 ± 10	112 ± 13	124 ± 13	1.5 ± 0.6	Mean ± SEM
* Statistical comparison of results	t = 1.77 0.1 P 0.05	t = 0.42 P 0.1	t = 3.97 P 0.001	t = 2.69 0.02 P 0.01	t = 1.05 P 0.1	t = 4.87 P 0.001	

* Using Student's 't' test

within twenty-four hours post mortem from four Nigerians who had suffered fatal injuries in motor accidents; these were also stored at -20°C until analysis.

The results (Table 6.4-3, Fig 6.4-II) show that plasma total B_{12} was slightly higher in the patients than in the controls but the difference was not significant. The levels of all cobalamins were raised but only for CN-B_{12} was the increase significant; in 5 of 13 patients, CN-B_{12} represented more than 15% of the plasma total B_{12}.

Analysis of the cobalamins in liver showed that results from patients and controls were very similar (Table 6.4-4). In all samples, Ado-B_{12} predominated and there was approximately half as much OH-B_{12} as Ado-B_{12}. Me-B_{12} concentrations were almost identical in both patients and controls, and no CN-B_{12} was detected in any sample.

Discussion

In a proportion of patients with visual failure of various origins - but particularly in Leber's optic atrophy - the ratio of plasma Me-B_{12}: Ado/OH-B_{12} is reduced while the plasma total B_{12} remains normal or may be slightly raised (Wilson, Linnell

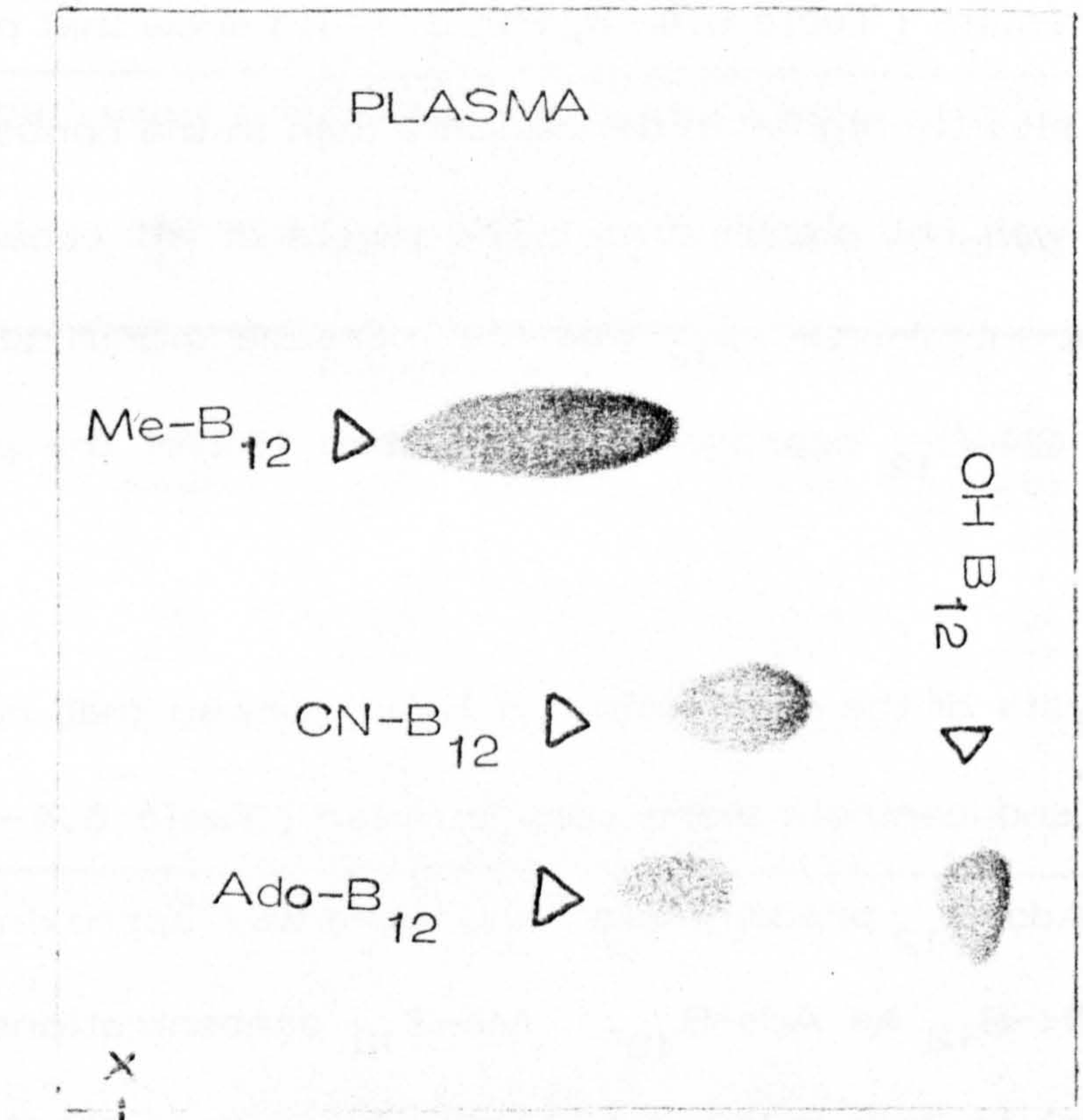


Figure 6.4-IV Separation of plasma cobalamins in a case of tropical ataxic neuropathy. A large proportion of $CN-B_{12}$ is present

and Matthews, 1971; Linnell, 1972). Plasma CN-B_{12} has been found to be increased in certain cases of Leber's optic atrophy, dominantly-inherited optic atrophy, tobacco amblyopia and various tropical neuropathies; the reason for this is not known although it has been suggested that an error in cyanide metabolism may be involved (Matthews and Wilson, 1971). In the present series of European patients with visual failure, CN-B_{12} was not abnormally raised in either plasma or erythrocytes.

In the Nigerian patients with TAN, however, the mean plasma CN-B_{12} concentration was more than six times greater than in control subjects, and as a proportion, accounted for more than 20% of the plasma total B_{12} in almost one third of these patients. No CN-B_{12} was detected in any of the Nigerian liver samples.

It has previously been suggested that in areas where cassava is the staple food, a high dietary intake of cyanide may affect the plasma cobalamin distribution (Matthews and Wilson, 1971). In the present study of patients with TAN, it was not possible separately to assess the effect of diet, but evidence given in the Appendix (Section 7.1) shows that administration of cyanide may increase plasma CN-B_{12} concentrations. It is also possible

TABLE 6.4 - 4

LIVER COBALAMINS in TROPICAL ATAXIC NEUROPATHY

	Total B_{12}	$Me\text{-}B_{12}$	$CN\text{-}B_{12}$	$Ado\text{-}B_{12}$	$OH\text{-}B_{12}$	
	- picograms per millilitre -					
PATIENTS[1] (n = 4)	1580 ± 381	15.5 ± 3.6	0	1078 ± 279	486 ± 114	Mean ± SEM
CONTROLS[2] (n = 4)	1236 ± 224	16.0 ± 3.8	0	797 ± 165	423 ± 56.5	Mean ± SEM

[1] Diagnostic biopsy tissue

[2] Post mortem tissue

that some of the patients may have been given CN-B_{12} in treatment, although the clinical staff were certainly made aware of the need to exclude from the study all patients who may have received any form of B_{12} therapy.

The patients in both groups were found to have a relative increase in plasma Ado-B_{12} or Ado/OH-B_{12} and a corresponding decrease in the Me-B_{12}: Ado/OH-B_{12} ratio. In the Nigerian patients, Ado-B_{12}, and not OH-B_{12}, was abnormally affected, but in the earlier European study, plasma Ado-B_{12} and OH-B_{12} were not separately estimated.

In the ophthalmological study, a wholly different pattern was seen in the erythrocytes. Both Me-B_{12} and the Me-B_{12}: Ado/OH-B_{12} ratio were higher in patients than controls. These results suggest that in visual failure of various origins there may be an associated redistribution of cobalamins between plasma and erythrocytes resulting in a decrease in the Me-B_{12}: Ado/OH-B_{12} ratio in the plasma and an increase in this ratio in erythrocytes. It is possible that this finding, if confirmed, may prove of some diagnostic value.

6.5 EFFECTS of ORAL and PARENTERAL ADMINISTRATION of COBALAMINS

Table 6.5 - 1 shows that an increase in plasma CN-B_{12} occurs after administration of this compound whether by oral or parenteral routes. This increase was detectable after a few days' 'physiological' dosage (1 μg) by mouth (Subjects 32 and 34), showing that such doses are absorbed at least partly unchanged. In Subject 21, the sample may have been taken a little too early for CN-B_{12} to have entered the plasma in appreciable amounts. In Subject 34, a vegan, there was evidence of conversion to Me-B_{12}, which was initially undetectable but appeared in the plasma at increasing concentrations at 5 and 8 days; Ado/OH-B_{12} showed little change.

In two cases of B_{12} deficiency given small doses of Me-B_{12} intramuscularly (Nos 23 and 30), there was a large increase in this compound in the plasma, and the Me-B_{12}: Ado/OH-B_{12} ratio was restored to normal.

TABLE 6.5 - 1

CHANGES in PLASMA COBALAMINS after COBALAMIN ADMINISTRATION by oral and parenteral routes

Subject	Diagnosis	Age	Sex	Compound and dose	Route	Time	Total B_{12} (pg/ml)	Me B_{12} (pg/ml)	CN B_{12} (pg/ml)	Co-OH B_{12} (pg/ml)	Ratio Me/Co-OH B_{12}	CN B_{12} percentage of total
N 29	Normal	18	F	Cyanocobalamin 100 μg	i.m.	Pre-dose	510	306	0	204*	1·5	0
						8 h	1165	260	775	130	2·0	67
N 5	Normal	42	M	Cyanocobalamin 100 μg	i.m.	Pre-dose	595	355	0	240	1·5	0
						24 h	895	546	107	242	2·3	12
N 6	Normal	52	F	Cyanocobalamin 100 μg	oral	Pre-dose	490	314	3	173	1·8	<1
						5 h	475	270	43	162	1·7	9
						24 h	470	268	47	155	1·7	19
P 19	Pernicious anaemia	43	M	Cyanocobalamin 1000 μg	i.m	Pre-dose	35	trace	14	21*	<0·1	40
						3 h	>2000	trace	>1800	>200*	<0·1	*ca* 90
P 86	Cirrhosis of liver	28	F	Cyanocobalamin 1000 μg	i.m.	7 days	>2000	>200	>1300	>500*	0·4	*ca* 65
P 87	Pernicious anaemia	67	F	Cyanocobalamin 4 μg	i.m.	6 days	92	18	56	18*	1·0	61
P 21	Pernicious anaemia	63	F	Cyanocobalamin 1 μg+IF	oral	Pre-dose	77	31	0	46*	0·7	0
						6 h	84	34	0	50*	0·7	0
P 34	Vegan	44	F	Cyanocobalamin 1 μg daily	oral	Pre-dose	55	0	0	55	0	0
						5 days	110	25	3	82	0·3	3
						8 days	115	40	7	68	0·6	6
P 32	Vegan	31	F	Cyanocobalamin 1 μg daily	oral	Pre-dose	135	62	0	73	0·9	0
						7 days	205	82	68	55	1·5	33
P 23	Pernicious anaemia	63	F	Methylcobalamin 1 μg daily	i.m.	Pre-dose	49	0	20	29	0	41
						10 days	175	117	0	58*	2·0	0
P 30	Post-gastrectomy with subacute combined degeneration of the cord	61	M	Methylcobalamin 1 μg daily	i.m	Pre-dose	49	12	0	37*	0·7	0
						14 days	285	250	0	35*	7·2	0
						21 days	320	274	0	46	6·4	0

* Derived from visual score.

6.6 INBORN ERRORS of B_{12} METABOLISM

Urinary excretion of high levels of homocystine (HC) and methylmalonic acid (MMA) has recently been described in several young patients (Mudd, Levy and Abeles, 1969; Levy, Mudd, Schulman, Dreyfus and Abeles, 1970; Goodman, Moe, Hammond, Mudd and Uhlendorf, 1970). In these cases, it has been shown that: (1) a decrease in N^5-methyltetrahydrofolate-homocysteine methyltransferase activity leads to abnormalities in the metabolism of sulphur-containing amino acids - the failure in particular to maintain normal plasma and tissue concentrations of methionine - with homocyst(e)inuria and cystathioninuria; (2) a decrease in methylmalonyl-CoA mutase activity leads to abnormal carbohydrate metabolism and failure to convert propionate to succinate, with consequent methylmalonic aciduria and possible methylmalonic acidaemia.

These are the only two known mammalian enzymes to require B_{12} derivatives, and in the cases in question, evidence suggests that a deficiency of available Me-B_{12} and Ado-B_{12}, rather than of the apo-enzyme proteins, may be the cause of the diminished catalytic activity (Mudd et al., 1969: Goodman et al.,

TABLE 6.6 - 1

INBORN ERRORS of B_{12} METABOLISM:
details of PATIENTS and STUDIES CARRIED OUT

Patient	Age	High urinary excretion of:		COBALAMINS estimated in:		
MM.	5	MMA	HC	Plasma Erythrocytes	Liver Kidney Spleen Brain	Fibroblasts
JR*	14	MMA	HC	Plasma Erythrocytes Leucocytes		Fibroblasts
MR*	2	MMA	HC	Plasma		Fibroblasts
EM	7½ wks	MMA	HC		Liver Kidney	Fibroblasts
KB	4 mths	MMA			Liver Kidney Brain	Fibroblasts
SH	10	MMA		Plasma		
MO	—	MMA				Fibroblasts

* Siblings

All organ cobalamin levels were studied post-mortem; SH and MO were unresponsive to B_{12} and died at the ages indicated above - MM died in the course of the study

1970; Mudd, Levy and Morrow, 1970; Mudd, Uhlendorf, Hinds and Levy, 1970; Mahoney, Rosenberg, Mudd and Uhlendorf, 1971). The most convincing interpretation of these findings would seem to be that a defect in the metabolism or cellular uptake of B_{12} renders the mutant cells unable to accumulate normal concentrations of either Me-B_{12} or Ado-B_{12}.

Support for this hypothesis has now been obtained by the direct estimation of cobalamins in plasma and tissues from 7 children known to excrete MMA and/or HC. In several of these patients, an error in B_{12} metabolism had already been demonstrated or was strongly suspected, but in one case (MM) the findings during the present study provided the first real indication that a disturbance in B_{12} metabolism was involved. Brief details of these children, including the tissues studied, are given in Table 6.6 - 1.

Plasma and erythrocyte cobalamins

Plasma cobalamins were estimated in 4 children aged 2 to 14 years whose urinary excretion of MMA was excessive; 3 of the children, 2 of whom were brothers, were also excreting large amounts of HC. ... For comparison, plasma cobalamins were estimated in 5 control children aged 7 to 14 years with no detectable urinary excretion of MMA or HC, or evident disturbance in B_{12} metabolism.

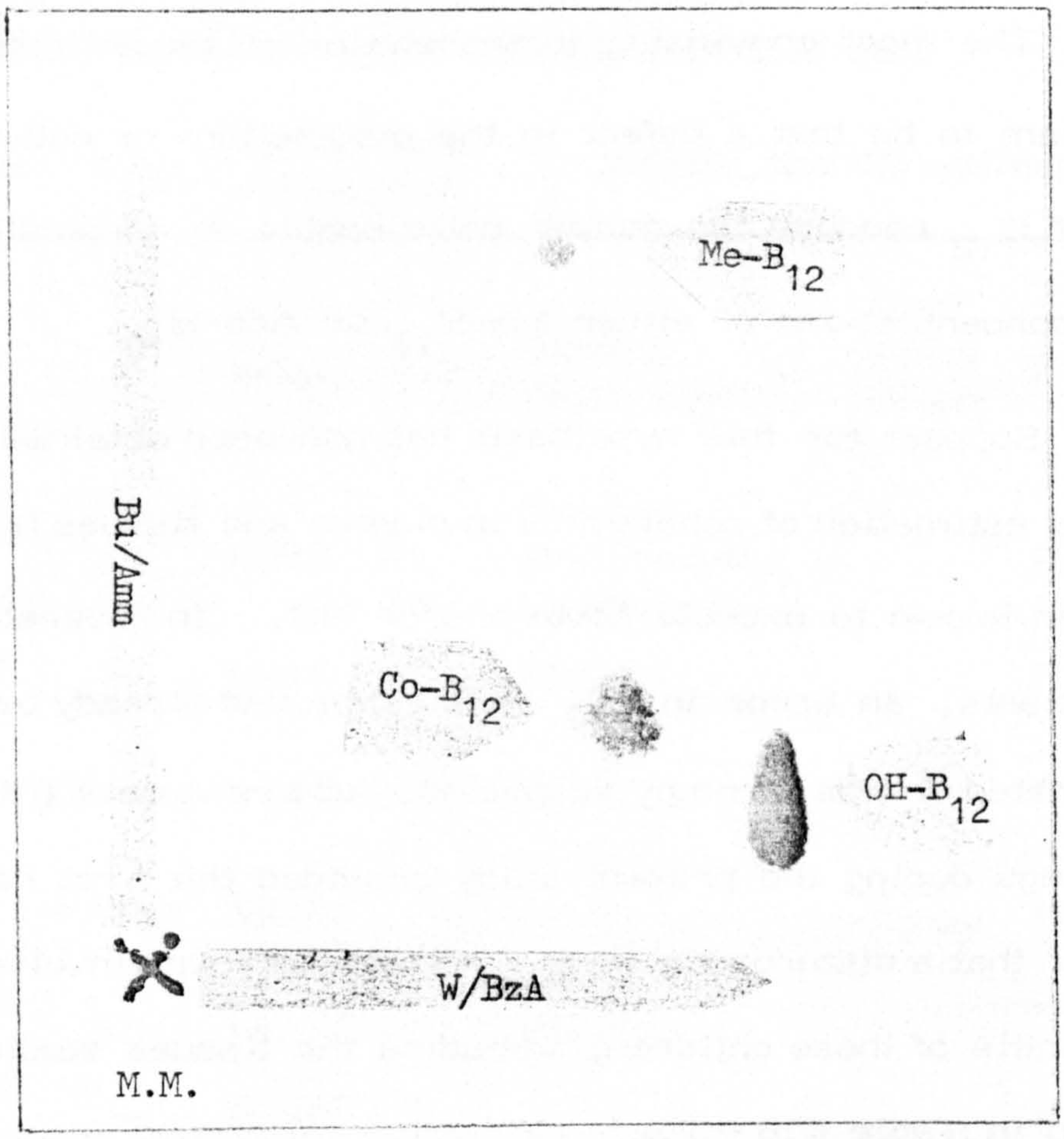


Figure 6.6-I Separation of plasma cobalamins in PATIENT MM. Me-B_{12}, normally the major form of the vitamin, is disproportionately reduced

TABLE 6.6-2

PLASMA COBALAMINS: PATIENTS MM, JR, MR, SH and CONTROL SUBJECTS

	Subject	Age	Abnormal excretion	Total B_{12}	$Me-B_{12}$	$CN-B_{12}$	$Ado-B_{12}$	$OH-B_{12}$	Ado/ $OH-B_{12}$	Ratio: $Me-B_{12}$ / $Ado/OH-B_{12}$
				- picograms per millilitre -						
	MM	5	MMA/HC	1,090	110	0	490	490	980	0.11
	JR	14	MMA/HC	720	130	0	440	150	590	0.22
	MR	2	MMA/HC	610	108	214	129	158	287	0.38
	SH	10	MMA	915	833	0	27	55	82	10.2
Controls	AK	12	-	715	418	0	163	129	292	1.4
	GP	9	-	815	469	0	-	-	348	1.4
	GC	9	-	590	396	0	141	53	194	2.0
	PF	7	-	645	403	0	132	110	242	1.7
	JJ	14	-	890	653	0	-	-	237	2.8
	Mean:	10.2		731	468	0	145	97	262	1.86
	Range:	(7-14)		(590-890)	(396-653)	0	(132-163)	(53-129)	(194-345)	(1.4-2.8)

TABLE 6.6-3

ERYTHROCYTE COBALAMINS:
PATIENTS MM and JR*

Patient	Total B_{12}	Me-B_{12}	CN-B_{12}	Ado/ OH-B_{12}	Ratio: Me-B_{12} Ado/OH-B_{12}
	–	picograms	per	millilitre	–
MM	110	10	5	95	0.11
JR	270	19	17	234	0.08
Normal range:	150-425	10-50	0-27	121-375	0.06 – 0.29

TABLE 6.6-4

PLASMA COBALAMINS after parenteral administration of Ado-B_{12} (25 µg i.m.): PATIENT MM*

Sample taken	Total B_{12}	Me-B_{12}	CN-B_{12}	Ado/ OH-B_{12}	Ratio: Me-B_{12} Ado/OH-B_{12}
	–	picograms	per	millilitre	–
Predose	1,100	110	0	990	0.11
at 4 hr	2,000	600	0	1,400	0.43
at 28 hr	1,125	325	0	800	0.41

* For details of PATIENTS, see TABLE 6.6-1

The results (Table 6.6 - 2) show that in all four of the affected children, although the plasma total B_{12} was normal or slightly elevated, the constituent cobalamins were grossly disturbed. In the 3 patients who excreted both MMA and HC, the plasma Me-B_{12} was less than one third of the lowest level found in normal controls, and in one of the children (MM), Me-B_{12} accounted for only 10% of the plasma total B_{12} (Fig 6.6-I). In contrast, the child excreting MMA but no HC was found to have a high plasma Me-B_{12} which accounted for more than 90% of the plasma total B_{12}, while Ado-B_{12} was below the normal range; in 2 of the other 3 children, the plasma Ado-B_{12} was raised and concentrations were well above those in control subjects. In MR, plasma Ado-B_{12} was within normal limits although the ratio Me-B_{12}: Ado/OH-B_{12}, as in JR and MM, was abnormally low. An additional finding in MR was a raised plasma CN-B_{12} accounting for more than one third of the total B_{12}; the reason for this is not known but CN-B_{12} had not recently been administered.

Analysis of the erythrocytes from one child (MM) showed that the cobalamin concentrations, in contrast to those in the plasma, were abnormally low (Table 6.6 - 3). This provided the first indication that the cellular tissues might be cobalamin-deficient in this child - a possibility that could not have been anticipated from the plasma total B_{12} levels alone. In a

TABLE 6.6-5

PLASMA and LEUCOCYTE COBALAMINS following ADMINISTRATION of $\underline{OH\text{-}B_{12}}$: PATIENT JR*

Dosage $OH\text{-}B_{12}$	Sample taken	PLASMA					LEUCOCYTES				
		Total B_{12}	$Me\text{-}B_{12}$	$CN\text{-}B_{12}$	$Ado\text{-}B_{12}$	$OH\text{-}B_{12}$	Total B_{12}	$Me\text{-}B_{12}$	$CN\text{-}B_{12}$	$Ado\text{-}B_{12}$	$OH\text{-}B_{12}$
µg		- picograms per millilitre -					- picograms per gram -				
500	Predose	720	130	0	442	148	840	140	0	- 700 -	
	Day 2	7,740	800	0	2,650	4,290	1,950	265	30	- 1,655 -	
	Day 8	2,270	360	120	1,130	660	1,815	320	0	805	690
	Day 11	2,245	565	0	1,245	435	1,200	300	0	- 900 -	
	Day 16	2,265	565	0	- 1,700 -						
1,500	Day 9	5,515	745	140	- 4,630 -		1,640	230	100	125	1,185
	Day 16	3,835	575	190	- 3,070 -		1,345	110	140	- 1,095 -	
	Day 23	2,660	260	0	- 2,400 -		985	90	80	- 815 -	

* For details of PATIENT, see TABLE 6.6-1

second child (JR), erythrocyte cobalamins were within normal limits - this child, although excreting high levels of both MMA and HC, is alive and in reasonably good health.

Effect of parenteral administration of cobalamins

Plasma cobalamins were estimated in MM 4 hours and 28 hours after a test dose of Ado-B_{12} (25 µg i.m.). The results (Table 6.6-4) show that plasma Me-B_{12} rose from a predose level of 110 pg to a level of 600 pg/ml 4 hours after the dose suggesting some ability to interconvert these forms; after 28 hours, Me-B_{12} had fallen again to 325 pg/ml but was still within the normal range. During this period, the urinary excretion of MMA, varying between 470 mg and 730 mg/day, was not significantly affected.

Plasma and leucocyte cobalamins were estimated in a second child (JR) following a test dose of OH-B_{12} (500 µg i.m.). The results (Table 6.6-5) show that in both tissues, Me-B_{12}, Ado-B_{12} and OH-B_{12} rose shortly after the dose, suggesting, as in MM, some interconversion. Fig 6.6-II shows that the excretion of both MMA and HC was reduced after the administration of OH-B_{12}; the lowest urinary levels, recorded 7 days after the dose, coinciding with a peak Me-B_{12} level in in the leucocytes. These results suggest a link between the

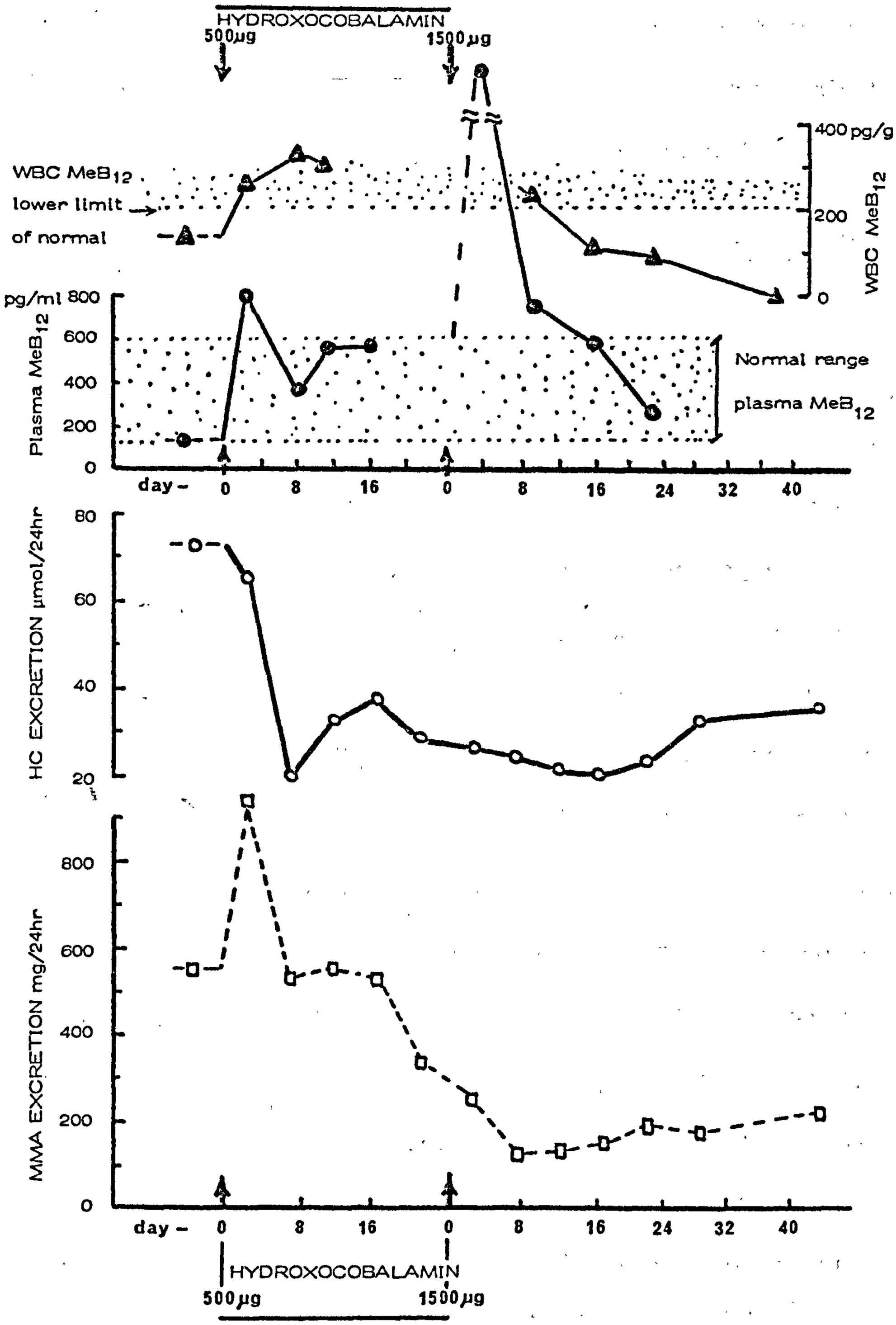


Figure 6.6 – II Changes in plasma and leucocyte Me-B_{12} and excretion of methylmalonate and homocystine in JR following the administration of OH-B_{12} on two occasions

TABLE 6.6 - 6

PLASMA and LEUCOCYTE COBALAMINS following ADMINISTRATION of $OH\text{-}B_{12}$ and $Ado\text{-}B_{12}$: PATIENT JR*

Dosage	Sample taken	PLASMA					LEUCOCYTES				
		Total B_{12}	$Me\text{-}B_{12}$	$CN\text{-}B_{12}$	$Ado\text{-}B_{12}$	$OH\text{-}B_{12}$	Total B_{12}	$Me\text{-}B_{12}$	$CN\text{-}B_{12}$	$Ado\text{-}B_{12}$	$OH\text{-}B_{12}$
		- picograms per millilitre -					- picograms per gram -				
$OH\text{-}B_{12}$ 1.5 mg	Predose	575	200	0	97	278	2,570	820	15	652	1,083
	Day 2	24,600	4,110	4,230	2,460	13,800	5,375	1,800	180	1,700	1,695
	Day 7	3,325	1,010	0	407	1,848	3,170	1,700	0	653	817
	Day 14	2,975	1,435	0	590	950	2,815	–	–	–	–
$Ado\text{-}B_{12}$ 1.5 mg plus 0.6 mg on	Predose	980	47	25	863	45	1,210	38	16	717	439
	Day 6	1,490	60	50	1,240	140	1,490	111	14	600	765
	Day 7										
	Day 13	2,240	482	363	452	943	1,700	185	12	1,368	135
	Day 22	2,120	863	15	752	490	1,690	250	20	870	550

* For details of PATIENT, see TABLE 6.6-1

TABLE 6.6-7

TISSUE COBALAMINS: PATIENT MM* and control subjects

	Tissue	Total B_{12}	$Me-B_{12}$		$CN-B_{12}$		$Ado-B_{12}$		$OH-B_{12}$	
		ng/g	ng/g	%	ng/g	%	ng/g	%	ng/g	%
MM	LIVER	13.6	1.1	8.1	0.2	1.5	6.8	50	5.5	49
Controls (n=6)		834	84	8.8	30	2.9	476	60	245	29
± SEM		± 166	± 34	± 2.9	± 12	± 0.9	± 77	± 5.3	± 62	± 2.6
MM	KIDNEY	19.2	2.3	12	0.2	1.0	4.5	23	12.2	64
Controls (n=5)		229	37	16	1.3	0.7	130	59	60	24
± SEM		± 41	± 8.3	± 2.7	± 0.9	± 0.6	± 16	± 3.5	± 19	± 3.6
MM	SPLEEN	8.3	1.9	23	0	0	3.1	37	3.3	40
Controls (n=3)		32	7.5	25	0.2	0.6	17	53	7.0	21
± SEM		± 3.8	± 2.3	± 8.2	± 0.1	± 0.2	± 2.1	± 2.9	± 4.0	± 11
MM	BRAIN	9.1	0.3	3.3	0.1	1.1	4.7	52	4.0	44
Controls (n=6)		52	6.1	14	0.3	0.6	34	68	11	18
± SEM		± 10	± 1.3	± 3.1	± 0.1	± 0.3	± 6.6	± 1.6	± 4.0	± 4.5

* For details of PATIENT, see TABLE 6.6-1
The ages of the control children ranged from 1 to 15 years

transfer of Me-B_{12} from plasma to tissues and the reduction in excretion of MMA and HC. Similar results were obtained after a higher dose of OH-B_{12} (1,500 µg i.m.).

In the same patient (JR) the results after the administration of Ado-B_{12} were very different. The dose (2,100 µg) was calculated to be equivalent to 1,500 µg OH-B_{12}, but the plasma total B_{12} rose to less than one tenth of the level found immediately following OH-B_{12}. The plasma Me-B_{12} increased slowly to a peak more than two weeks after the test dose, but only once, 6 days after the dose, did the plasma Ado-B_{12} rise above predosage levels. Changes in the leucocyte cobalamins after Ado-B_{12} were broadly similar to those in the plasma. Although these results (Table 6.6 - 6) are difficult to explain, it is possible that Ado-B_{12}, in comparison with OH-B_{12}, may have been less well absorbed from the injection site and far more slowly converted to other forms of the vitamin.

Tissue cobalamins

Table 6.6 - 7 shows the results of analysis of organs obtained at autopsy from MM with control values from children without evidence of B_{12} deficiency or a disturbance in B_{12} metabolism. The major abnormality in the organs from

TABLE 6.6-8

TISSUE COBALAMINS: PATIENTS KB, EM and CONTROL SUBJECTS

Patient	Age at death	Tissue	Total B_{12}	$Me-B_{12}$	$CN-B_{12}$	$Ado-B_{12}$	$OH-B_{12}$
			-	pg/mg wet tissue			-
KB	4 months	Liver	592	1	320	1	270
		Kidney	735	7	471	0	257
		Brain	66	1.2	58.3	0.5	6.0
EM	7½ weeks	Liver	91	4	10	22	55
		Kidney	92	2	4	31	55
Control 1: 3 months		Liver	127	6	5	76	40
Control 2: 4 months		Liver	128	10	4	73	41

All tissues had been stored at -20° for three years prior to analysis

MM was the great reduction in all forms of B_{12}, total B_{12} in the liver being particularly low at less than 2% of control values. There was a moderate increase in the proportion of OH-B_{12} in all tissues, accompanied by a decrease in the proportion of Ado-B_{12}. In the brain, the proportion of Me-B_{12} was markedly depressed.

Table 6.6-8 shows the results obtained in post-mortem tissues from two other children (KB and EM). KB had been found to excrete MMA, but not HC, and died at the age of four months; EM, who died at seven-and-a-half weeks, excreted both MMA and HC and would appear to have had a defect similar to that in MM. All samples, including control liver samples from two other young children, were stored under similar conditions. In all tissues from KB, the cobalamin distribution differed markedly from that in MM, EM or the controls; Ado-B_{12} was almost undetectable but concentrations of other cobalamins were within normal limits - the high levels of CN-B_{12} may have been due to administration of this cobalamin shortly before death. A small amount of CN-B_{12} was also present in tissues from EM and the controls, and might have been formed during the three years' storage of the samples.

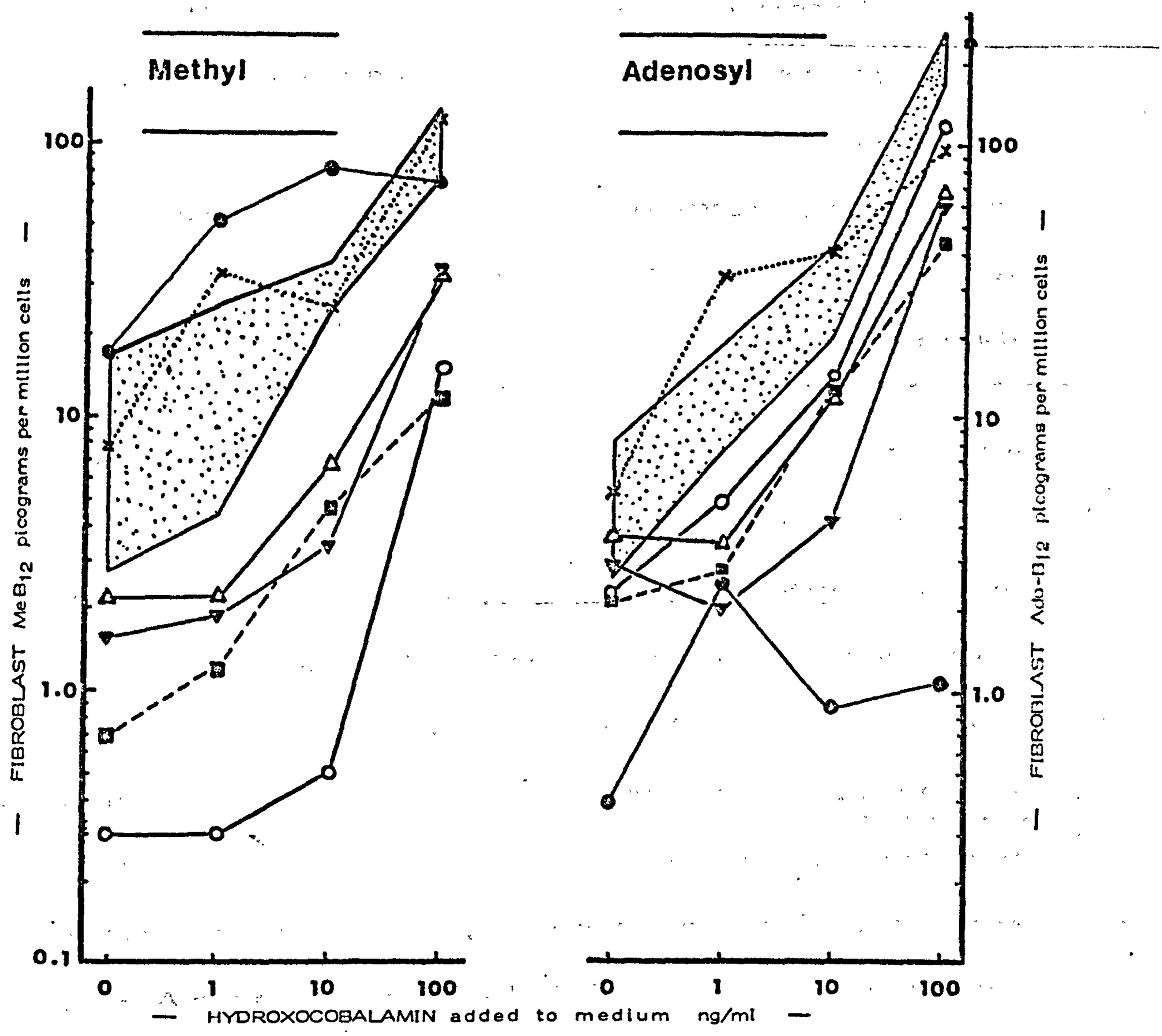


Figure 6.6-III Uptake of OH-B_{12} and conversion to Me-B_{12} and Ado-B_{12} by cultured fibroblasts. Mean values $\pm$ 1SD for control cell lines are plotted as the control range. Patient MO has an apoenzyme defect and synthesis of the coenzymes is virtually normal

Cobalamins in cultured fibroblasts

Fibroblasts were grown from skin biopsies in four children (MM, JR, MR and EM) known to excrete both MMA and HC, two children (KB and MO) who excreted MMA but no HC, and three normal controls; because the number of controls was small, fibroblasts from these children were grown and analysed on two separate occasions.

The results (Fig 6.6 - III) show that in fibroblasts from EM, JR and MR, the cellular concentrations of Ado-B_{12} and Me-B_{12} were considerably lower than in control fibroblasts. Fibroblasts from MO, who had a methylmalonyl CoA mutase apoenzyme defect, differed little from those in controls. In agreement with the analyses of his post-mortem tissues, cells from KB contained high levels of Me-B_{12} while the concentrations of Ado-B_{12} were extremely low.

Discussion

The patient MM has been studied extensively, and results of investigations, including cobalamin and amino acid analyses, enzyme assays and studies of her transcobalamins, are being reported (Dillon, England, Gompertz, Goodey, Grant, Hussein, Linnell, Matthews, Mudd, Newns, Seakins,

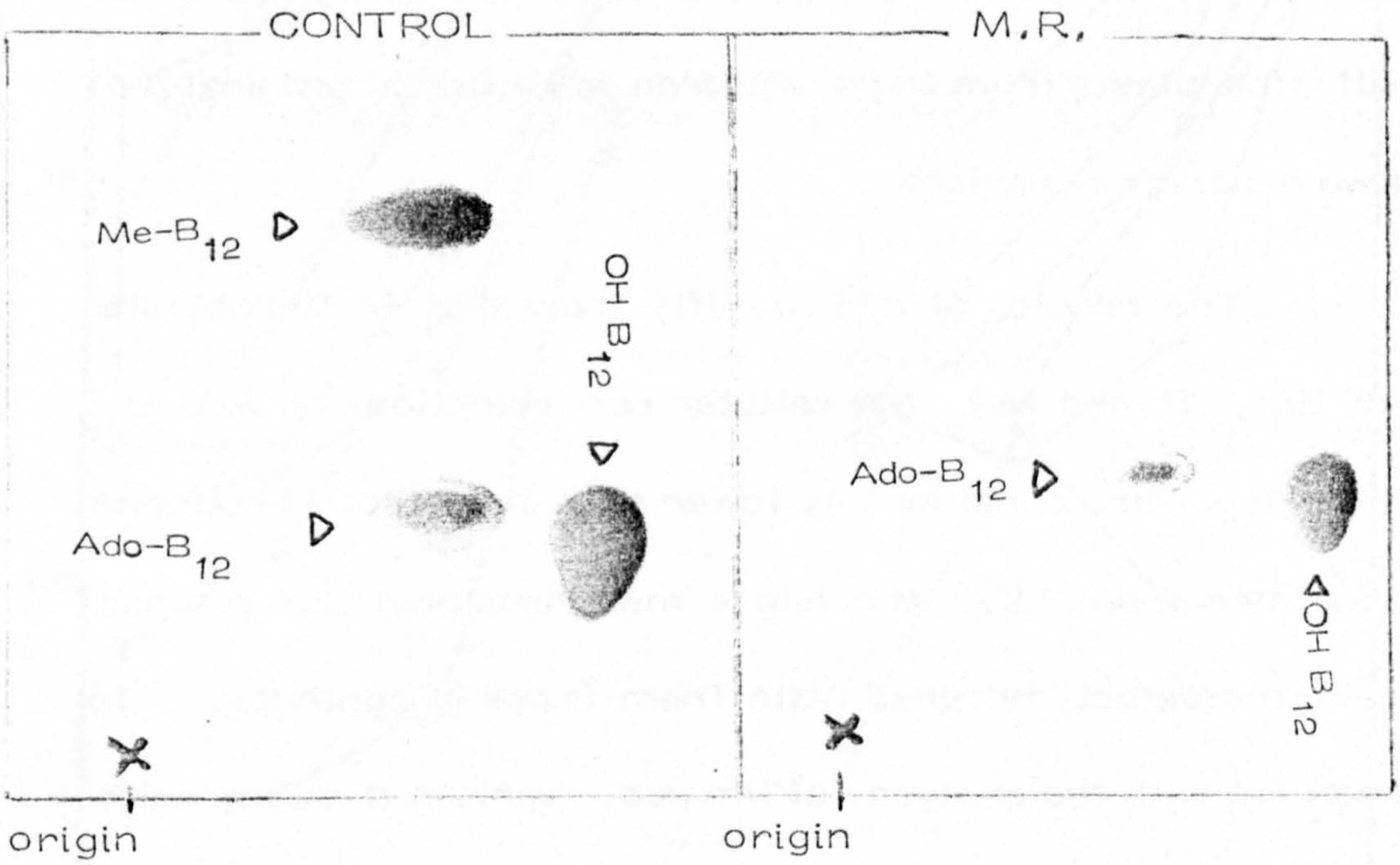


Figure 6.6-IV Cobalamins separated from fibroblasts cultured with OH-B_{12} (10ng/ml medium) from PATIENT MR and from a control child. Cells from the affected child show a disproportionate reduction in both coenzymes though the decrease in Me-B_{12} is particularly marked

Uhlendorf and Wise, 1973). EM was the first child in whom simultaneous methylmalonic aciduria and homocystinuria were detected (Mudd, Levy and Abeles, 1969). Investigations on JR and MR have also been reported (Goodman, Moe, Hammond, Mudd and Uhlendorf, 1970).

The present study shows that inborn errors of B_{12} metabolism are unlikely to be detected through the estimation of plasma total B_{12} alone, since levels are usually normal or elevated. The plasma cobalamin pattern is however grossly disturbed, and in MM, JR and MR, resembled the pattern in B_{12} deficiency. In cellular tissues including fibroblasts, total B_{12} was low in MM, JR, MR and EM, and concentrations of Me-B_{12} and Ado-B_{12} were abnormally depressed.

Although it is not yet possible exactly to define the metabolic error in any of these children, the synthetic pathways for the B_{12} coenzymes have been studied in bacteria (Fig 6.6 - V), and from the collected investigations recently undertaken it seems likely that the defect in MM, JR, MR and EM may be in cellular uptake or reduction of the vitamin as a prelude to alkylation. In KB, the defect appears more likely to be in the terminal alkylation step during which Ado-B_{12} is synthesized.

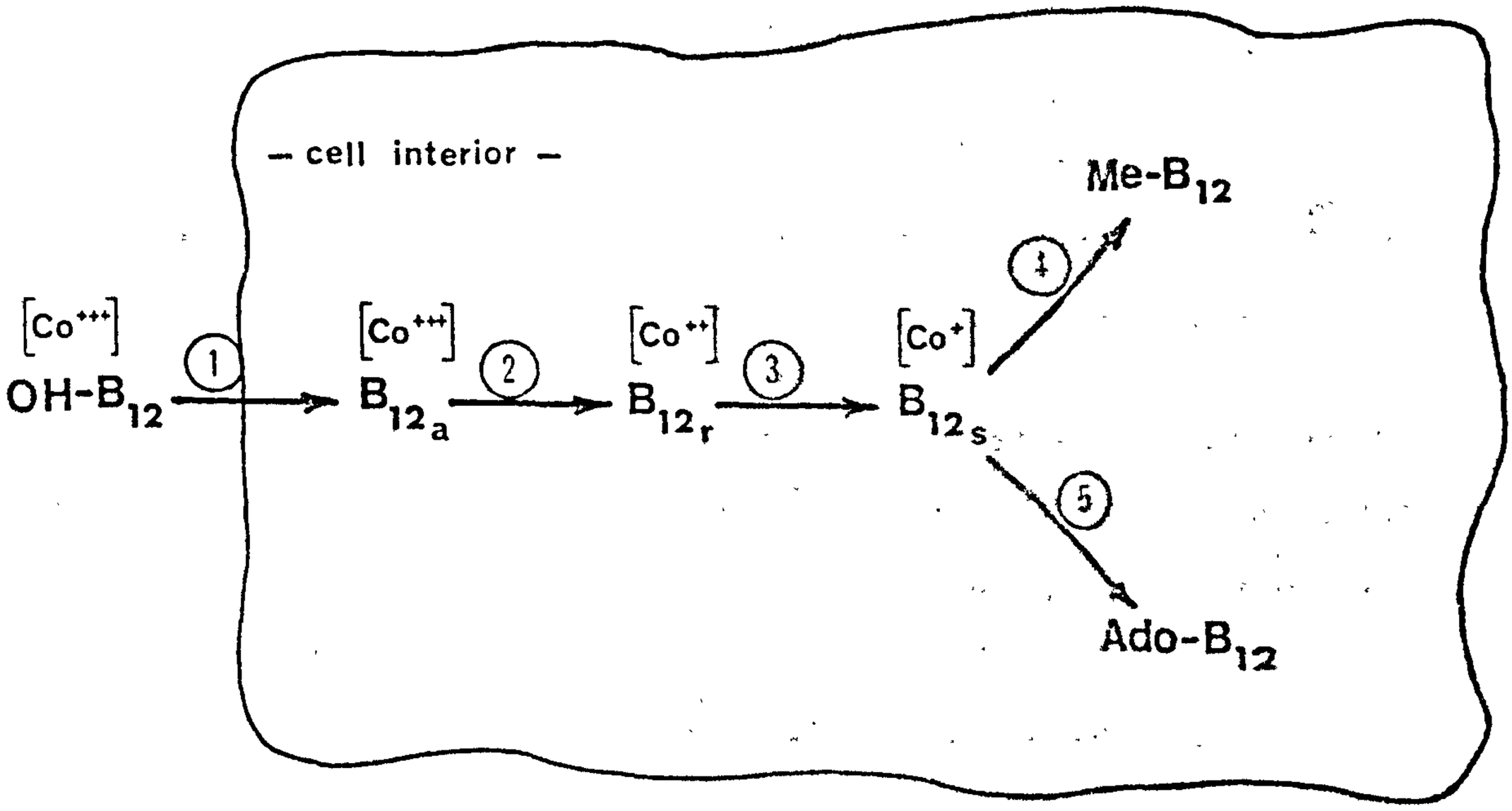


Figure 6.6 - V Scheme of probable steps leading to synthesis of $Me-B_{12}$ and $Ado-B_{12}$. (1) Transport into cell (though uptake of $OH-B_{12}$ is shown, the form or forms in which B_{12} enters cells is uncertain) (2)(3) Reduction steps (4) Synthesis of $Me-B_{12}$ (5) Synthesis of $Ado-B_{12}$

APPENDIX 7

7.1 STUDIES of B_{12} DEFICIENCY and CYANIDE TOXICITY in the BABOON

During the Spring of 1968, this experiment was planned as a long-term study of at least two years' duration in which possible inter-relationships between B_{12} deficiency and neurological and other effects of cyanide and thiocyanate could be investigated.

Neurological degeneration associated with low serum B_{12} levels had been reported in captive monkeys (Oxnard and Smith, 1966) and in this investigation, the baboon was chosen as an experimental animal large enough to provide sufficient blood for all the intended tests but not too inconveniently large to house. It was hoped the results might lead to the better understanding of certain diseases in man.

The experiment was carried out under the general direction of Dr R.F. Crampton at the British Industrial Biological Research Association, and involved individual studies by a number of collaborators - the cobalamin studies are described in this Appendix.

Animals

The colony comprised 36 healthy male baboons, species Papio cynocephalus, initially weighing between 3.1 kg and 5.1 kg (mean 3.9 kg) suggesting that they were about nine months old (Snow, 1967). They were housed individually in cages fitted with wire-mesh floors to minimise coprophagy. Room temperatures were controlled not to fall below 21°C.

TABLE 7.1 - 1

COMPOSITION of LOW-B_{12} BABOON DIET

	Kg
Ground maize	405
Ground wheat	200
Fine millers offal	100
Full-fat soya	100
Soyabean extract	62
Sugar (refined)	50
Molasses	50
Ground limestone	8
Dicalcium phosphate	17.7
Salt mix[1]	2.3
Vitablend supplement[2]	5.0
	1,000

[1] Salt mix

	g
Saccharated ferrous carbonate	65.7
Manganese carbonate	36.5
Copper carbonate	8.7
Potassium iodate	1.5
Cobalt oxide	0.87
Sodium molybdate	0.17
Sodium chloride	to 1.0 Kg

[2] Vitablend supplement

	IU x 10^6/1000Kg
Vitamin A	8.0
Vitamin D_3	2.0
Riboflavin	8.0
Niacin	20.0
Pyridoxine HCl	1.0
Folic acid	1.0
Ascorbic acid	25
Vitamin K	1.0

General experimental plan

All animals were fed the same low-B_{12} diet (Table 7.1 - 1) which was made up in batches every 8 to 10 weeks and assayed for total B_{12} before use; any batch containing more than 4 μg B_{12}/kg was rejected. They were also given fresh fruit daily (one orange, apple or banana, as available).

The animals were assigned to one of five groups, each on a different regime (Table 7.1 -2): Groups I and II received OH-B_{12} (10 μg i.m. weekly); Groups II and V received potassium cyanide (1 mg/kg subcutaneously with adrenalin 5 days per week); Group IV received potassium thiocyanate (40 mg/kg subcutaneously 5 days per week); Group III received only the low-B_{12} diet. Adrenalin was administered with the cyanide to induce vasoconstriction at the injection site and so delay absorption (this was necessary to counter vomiting) and as a control, an equal amount of adrenalin was administered 5 days per week to all other animals.

Since animals in the different groups were not at equal risk, more animals were assigned to the groups receiving cyanide to allow for any interim mortality. Group IV was included to give information on the effects of high levels of thiocyanate - the major detoxication product of cyanide - and the dosage was determined in a preliminary experiment with two baboons as the amount of potassium thiocyanate required to produce a plasma thiocyanate level of 10 μM to 12 μM/ml, the range found in heavy smokers.

Approximately three-and-a-half years after its inception, the experiment was brought to an end by killing the animals.

TABLE 7.1-2

REGIME of ANIMALS in each GROUP
(In addition to low-B_{12} diet and fruit)

Group	Regime	Number of animals
I	OH-B_{12} (10 µg i.m. weekly)	7
II	OH-B_{12} (10 µg i.m. weekly) *Potassium cyanide (1 mg/Kg subcutaneously 5 days/week)	8
III	None	7
IV	Potassium thiocyanate (40 mg/Kg subcutaneously 5 days/week)	4
V	Potassium cyanide (1 mg/Kg subcutaneously 5 days/week)	10

* Prepared in neutral phosphate buffer (2 mg KCN/ml) with adrenalin (0.4 µg/ml) added to retard absorption. An equivalent dose of adrenalin was administered to all other animals, including controls, 5 days/week

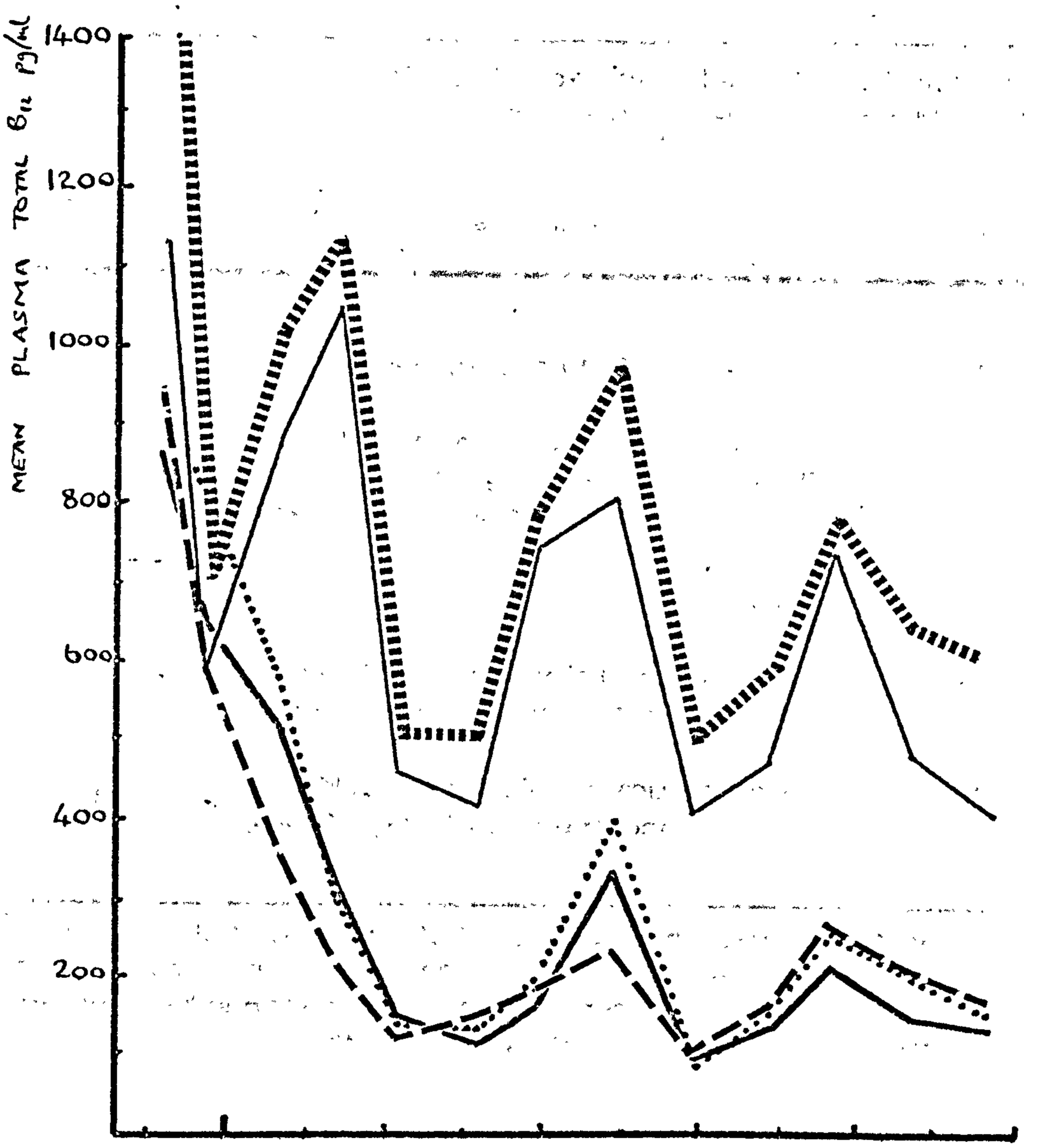


Figure 7.1 - I Mean plasma total B_{12} concentrations for each group of animals at intervals throughout the experiment

—— = GROUP I (B_{12}-supplemented)
▪▪▪▪▪▪ = GROUP II (B_{12}-supplemented plus cyanide)
▬▬ = GROUP III (B_{12}-deficient)
····· = GROUP IV (B_{12}-deficient plus thiocyanate)
— — = GROUP V (B_{12}-deficient plus cyanide)

COBALAMIN STUDIES

The initial plan was to estimate plasma total B_{12} and plasma cobalamins at intervals of a few months. Some way through the experiment, after further method development, it was possible to extend the studies to include erythrocyte cobalamins. The first results were interesting, and plasma and erythrocytes were analysed concurrently on three occasions. At 35 months, twenty-four-hour urine samples were collected after an oral dose of 5 g DL valine per animal, and urinary methylmalonic acid estimated by the method of Gutteridge and Wright (1970).

Plasma total B_{12}

Mean values recorded over the course of the experiment (Fig 7.1 - I) showed that in animals from B_{12}-deficient groups, the plasma total B_{12} declined rapidly at first, and within 9 months had fallen to levels below the lower normal limit in man (160 pg/ml by the radioisotopic method). Thereafter, although considerable fluctuations occurred, plasma levels in these animals remained at about one third of those in the B_{12}-supplemented animals; the lowest levels were recorded at 24 months. Administration of cyanide apparently raised the plasma B_{12}, and on a number of occasions (Table 7.1-3) the mean B_{12} concentration in Groups II and V was significantly higher than that in Groups I and III respectively.

	TOTAL B12 (pg/ml)	Me-B12 (pg/ml)	Me-B12 percentage	CN-B12 (pg/ml)	CN-B12 percentage	Ado/OH-B12 (pg/ml)		TOTAL B12 (pg/ml)	Me-B12 (pg/ml)	Me-B12 percentage	CN-B12 (pg/ml)	CN-B12 percentage	Ado/OH-B12 (pg/ml)		TOTAL B12 (pg/ml)	Me-B12 (pg/ml)	Me-B12 percentage	CN-B12 (pg/ml)	CN-B12 percentage	Ado/OH-B12 (pg/ml)		TOTAL B12 (pg/ml)	Me-B12 (pg/ml)	Me-B12 percentage	CN-B12 (pg/ml)	CN-B12 percentage	Ado/OH-B12 (pg/ml)	
B_{12}-supplemented GROUPS	502	216	42.2	12.7	2.7	273		943							753	150	20.5	4.0	0.5	584		810						
	70	39	2.2	9.8	2.3	32		88							47	29	3.6	4.0	0.5	61		62						
	(7)	(7)		(7)		(7)		(7)							(6)	(4)		(4)		(4)		(6)						Mean SEM (n) I
	640	248	38.8	47	7.1	345		1010	279	29.9	159	17.2	572		793	170	20.7	94	11.6	529		973	42	4.1	308	32.7	603	
	54	29	2.1	16.6	2.3	32		136	87	8.5	51	5.2	148		42	42	4.5	23	2.5	33		63	37	3.4	48	4.8	92	
	(8)	(8)		(8)		(8)		(6)	(6)		(6)		(6)		(7)	(7)		(7)		(7)		(8)	(3)		(3)		(3)	Mean SEM (n) II
	559	123	31.0	3.8	0.7	274		353	95	29.1			218		166	12.0	7.5	12.0	8.5	142		339	2.9	0.9	43.6	12.1	294	
	107	32	5.7	3.8	0.7	54		50	45	10.9			8		16	2.2	1.3	7.3	5.2	22		18	1.8	0.6	15.3	3.9	18	
	(7)	(4)		(4)		(4)		(7)	(2)				(2)		(7)	(6)		(6)		(6)		(7)	(7)		(7)		(7)	Mean SEM (n) III
B_{12}-deficient GROUPS	811	300	43.5	17.5	2.8	355		335	76	25.2			224		199	6.5	2.6	1.3	0.5	191		390	22	6.0	39.3	10.9	298	
	97	90	5.5	2.5	0.9	35		66	17	0.1			49		19	6.5	2.6	1.3	0.5	12		31	11	3.0	14.9	4.2	23	
	(4)	(2)		(2)		(2)		(4)	(2)				(2)		(4)	(4)		(4)		(4)		(4)	(3)		(3)		(3)	Mean SEM (n) IV
	616	222	34.8	106	16.2	307		271	49	18.0	55.1	18.6	175		191	13.2	5.7	34	15.8	185		243	2.5	1.0	46.6	18.7	194	
	52	27	2.4	30	4.7	33		26	9	2.6	8.5	2.2	18		16	3.8	1.8	9.4	5.2	24		11	1.6	0.7	13.3	5.1	13	
	(10)	(9)		(9)		(9)		(10)	(8)		(8)		(8)		(10)	(5)		(5)		(5)		(8)	(8)		(8)		(8)	Mean SEM (n) V
	DECEMBER 1963 to JANUARY 1969 (Month 0)							APRIL to AUGUST 1969 (Months 2 to 6)							JUNE 1970 (Month 16)							OCTOBER 1970 (Month 20)						
B_{12}-supplemented GROUPS	407	95	22.5	48	12.2	263		473	190	38.9	19.0	4.7	264		743	168	23.1	91	13.0	484		490	58	12.0	16.2	3.2	416	
	59	24	2.4	13.0	3.1	39		22	33	5.7	17.8	4.5	14		44	11	2.1	30	4.6	59		25	12.2	2.6	11.5	2.3	27	
	(6)	(6)		(6)		(6)		(6)	(6)		(6)		(6)		(6)	(5)		(6)		(6)		(6)	(6)		(6)		(6)	Mean SEM (n) I
	499	74	15.3	168	28.0	258		590	161	28.5	68.0	10.5	361		781	92	12.0	234	29.2	455		623	82	14.0	71	11.9	470	
	75	18	3.7	63	6.3	24		88	29	4.5	20.6	2.9	59		73	16.4	1.7	45	4.2	43		84	16.7	3.1	28	4.7	82	
	(8)	(8)		(8)		(8)		(6)	(6)		(6)		(6)		(8)	(8)		(8)		(8)		(7)	(7)		(7)		(7)	Mean SEM (n) II
	98	9.7	9.9	22.6	24.0	66		141	23.6	17.7	10.4	6.7	107		212	12.3	5.8	43	20.4	157		152	12.3	8.5	22	13.7	118	
	21	2.0	1.2	5.8	5.9	15		18	2.7	2.7	4.5	2.6	15		9	3.6	1.6	4.6	2.0	9		12	3.4	2.7	6.2	3.5	11	
	(7)	(7)		(7)		(7)		(7)	(7)		(7)		(7)		(7)	(7)		(7)		(7)		(7)	(7)		(7)		(7)	Mean SEM (n) III
B_{12}-deficient GROUPS	95	13	13.3	19.5	20.5	63		152	16.0	7.7	29.7	19.0	106		260	11.3	4.2	59	21.8	190		203	15.8	8.8	42	20.3	146	
	5	7	6.7	3.5	2.5	6		12	5.5	0.2	10.0	6.7	8		35	2.3	0.6	13.3	3.3	22		25	4.8	3.1	11.0	5.2	24	
	(2)	(2)		(2)		(2)		(3)	(3)		(3)		(3)		(4)	(4)		(4)		(4)		(4)	(4)		(4)		(4)	Mean SEM (n) IV
	106	7.9	8.2	23.2	21.3	75		164	10.1	6.8	16.4	10.4	137		275	13.3	5.0	49	17.9	222		204	11.0	5.4	51	22.8	156	
	15	1.3	1.2	3.9	1.8	11		15	2.7	2.1	6.1	3.7	16		24	2.3	1.0	9.5	3.3	26		12	5.3	2.6	9.8	2.4	15	
	(10)	(10)		(10)		(10)		(9)	(9)		(9)		(9)		(9)	(9)		(9)		(9)		(9)	(9)		(9)		(9)	Mean SEM (n) V
	FEBRUARY 1971 (Month 24)							JUNE 1971 (Month 28)							SEPTEMBER 1971 (Month 31)							JANUARY 1972 (Month 35)						

TABLE 7.1 - 3 PLASMA COBALAMINS in FIVE GROUPS of BABOONS at intervals during the experiment

TABLE 7.1 - 4

PLASMA COBALAMINS in BABOONS at 35 MONTHS: Statistical comparison of Groups[1]

GROUPS	$Me\text{-}B_{12}$	$CN\text{-}B_{12}$	Ado/ $OH\text{-}B_{12}$	Ratio: $Me\text{-}B_{12}$ / Ado/$OH\text{-}B_{12}$	Percentage $CN\text{-}B_{12}$
I vs II	t = 1.16 P > 0.1	t = 1.81 0.1 > P > 0.05	-	t = 0.71 P > 0.1	t = 1.66 P > 0.1
II vs V	-	-	-	t = 2.91 P < 0.01	t = 2.07 0.1 > P > 0.05
III vs V	-	t = 2.55 0.05 > P > 0.02	t = 2.04 0.1 > P > 0.05	t = 1.30 P > 0.1	t = 2.14 P < 0.05

[1] Using Student's 't' test

GROUP I B_{12}-supplemented
GROUP II B_{12}-supplemented plus cyanide

GROUP III B_{12}-deficient
GROUP IV B_{12}-deficient plus thiocyanate
GROUP V B_{12}-deficient plus cyanide

A striking feature of the results was the periodic change in plasma total B_{12}. This occurred in all groups, although the effect was most pronounced in the B_{12}-supplemented animals. Levels were lowest during the early months of the year (January to March) but rose during the following months and had doubled some 6 months later. Thereafter, the plasma total B_{12} declined again to a nadir early the following year. This sequence was repeated three times during the experiment, but the autumnal rise was less marked on the third occasion. While considering possible explanations for this finding, the assay variability was scrutinised. Results obtained on human samples during this period were compared for evidence of seasonal variation, but none was found. Control sera were assayed with the baboon samples at intervals during the experiment, and variations of up to 9% of the mean were recorded on occasion, but values were commonly within 5% of the mean, and variation was random. For human plasma and serum, the correspondence between results by the present method and by microbiological methods has been found satisfactory (see Section 4.5). It therefore seems unlikely that the observed seasonal variation in plasma total B_{12} in baboons can be explained on the basis of assay variability.

Plasma cobalamins

When first analysed in October, 1968, the plasma from all animals contained a large proportion of a cobalamin running with the R_f of CN-B_{12}. In view of the high plasma total B_{12} levels at this time, it was considered likely that the animals had received CN-B_{12} before their arrival at BIBRA (although this was repeatedly denied by the suppliers). Three months later the plasma CN-B_{12} had fallen (to mean 7.7%) and in the majority of animals the levels became similar to those for CN-B_{12} in normal human plasma. Ado/OH-B_{12} was now the major plasma form (mean 54.8%) and Me-B_{12} accounted for approximately one third (mean 37.5%) of the total plasma B_{12}. At this point, the animals were divided into groups and the experiment begun.

Plasma cobalamins were estimated in the majority of animals between 2 and 6 months, and again at 13, 16, 20, 24, 28, 31 and 35 months (Tables 7.1-3, 7.1-4). On each occasion, Ado/OH-B_{12} predominated in all animals, but plasma Me-B_{12} levels fluctuated, particularly in the B_{12}-supplemented groups (Fig 7.1 - II). In animals from the B_{12}-deficient groups, Me-B_{12} declined progressively (Fig 7.1 - III) and from 16 to 35 months, the plasma levels remained very low (consistently with the findings in human B_{12} deficiency - in some cases of

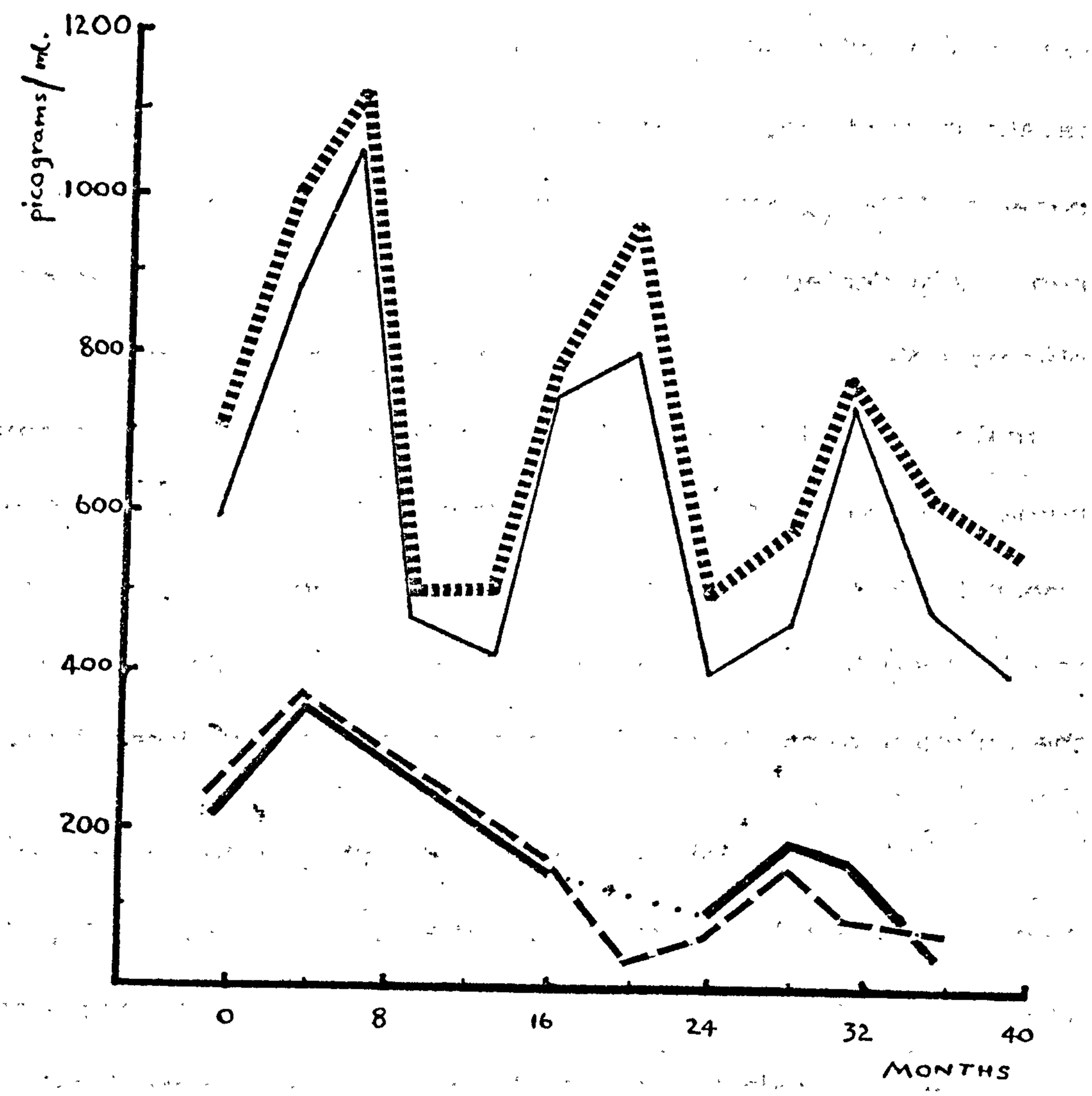


Figure 7.1 – II

Changes in mean plasma Me-B_{12} and total B_{12} in Group I and II animals – B_{12}-supplemented – at intervals during the experiment

– – = Me-B_{12} GROUP I

▬▬ = Me-B_{12} GROUP II

—— = Total B_{12} GROUP I

▪▪▪▪▪ = Total B_{12} GROUP II

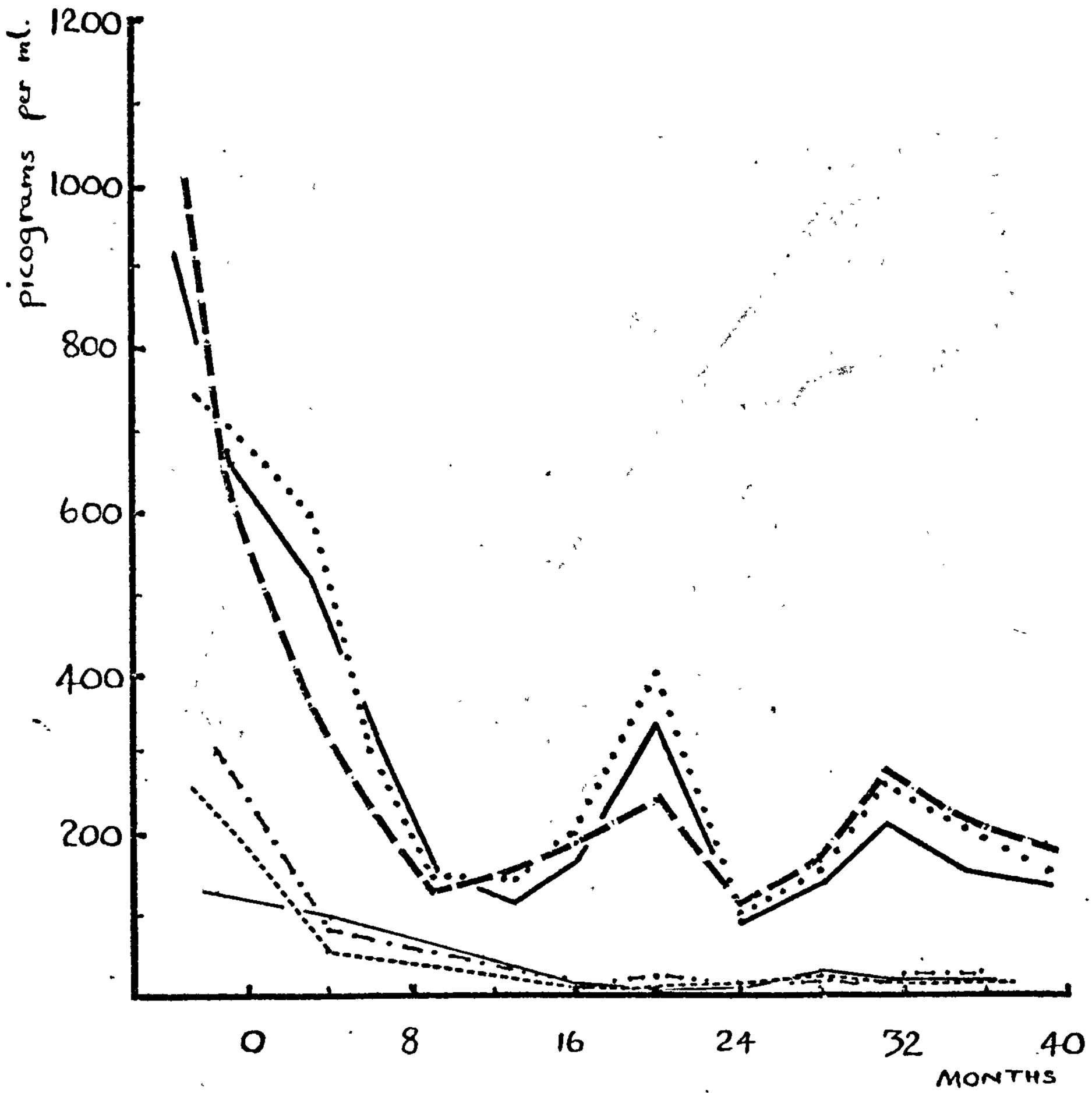


Figure 7.1 - III

Changes in mean plasma Me-B_{12} and total B_{12} in Group III, IV and V animals - B_{12}-deficient - at intervals during the experiment

—— = Me-B_{12} GROUP III
-·- = Me-B_{12} GROUP IV
----- = Me-B_{12} GROUP V

—— = Total B_{12} GROUP III
···· = Total B_{12} GROUP IV
—— = Total B_{12} GROUP V

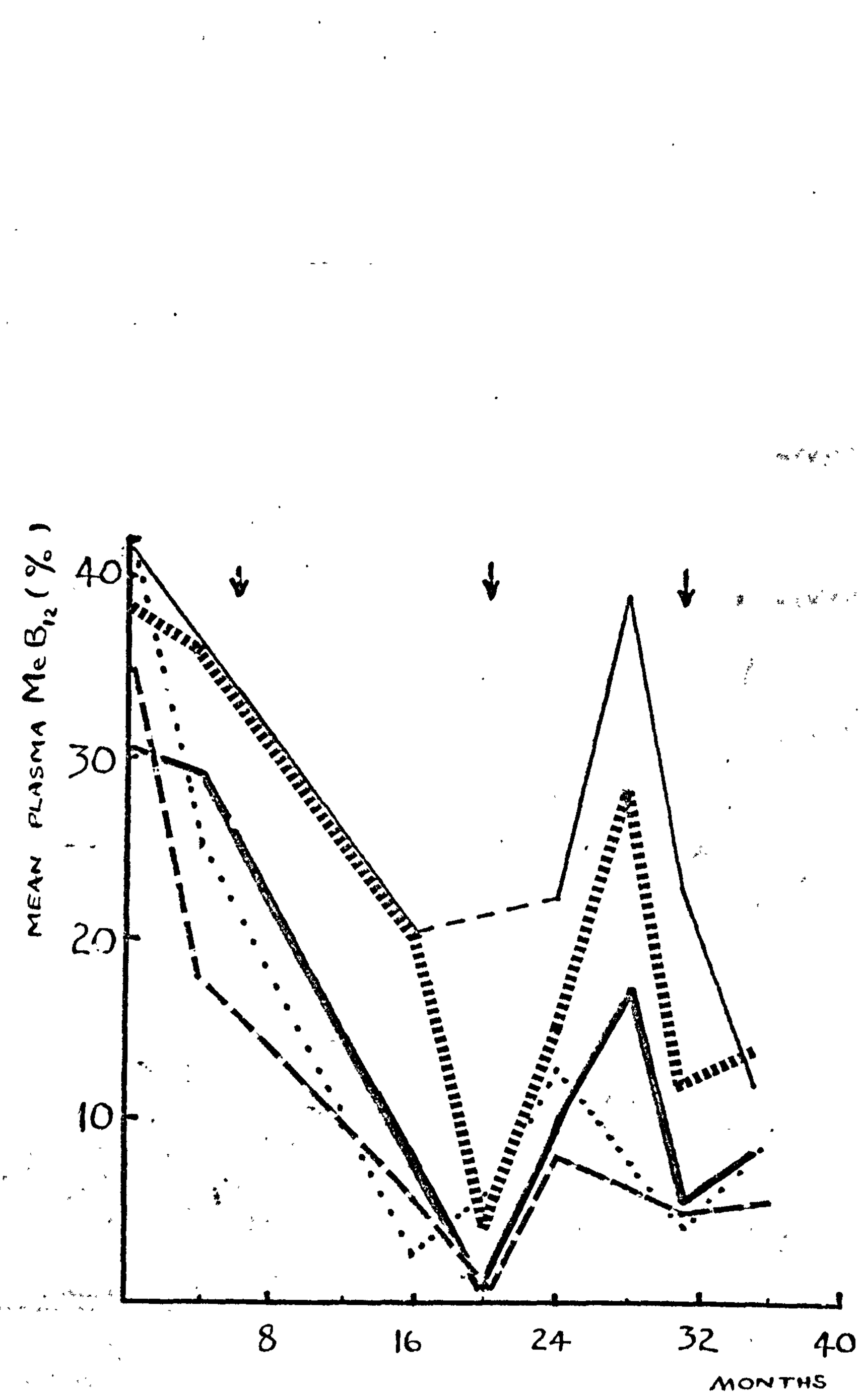


Figure 7.1 – IV

Changes in the proportions of Me-B_{12} in the plasma during the course of the experiment _____

↓ = Peak level of plasma total B_{12}

— = GROUP I
▮▮▮▮▮ = GROUP II
▬▬ = GROUP III
•••• = GROUP IV
— — = GROUP V

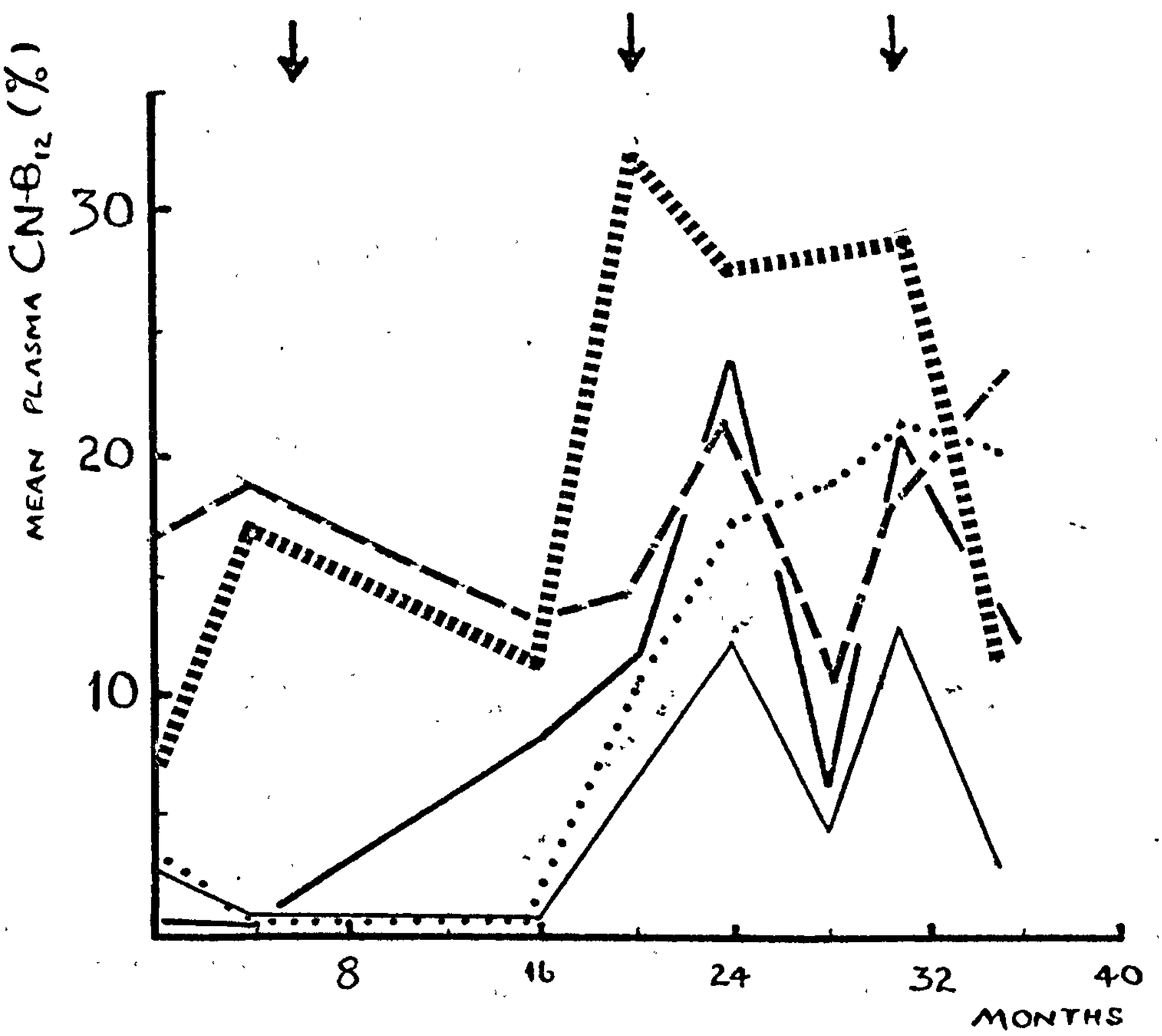


Figure 7.1 - V Changes in the proportions of CN-B_{12} in the plasma during the course of the experiment

↓ = Peak level of plasma total B_{12}

—— = GROUP I
▮▮▮▮ = GROUP II
▬▬ = GROUP III
···· = GROUP IV
—·— = GROUP V

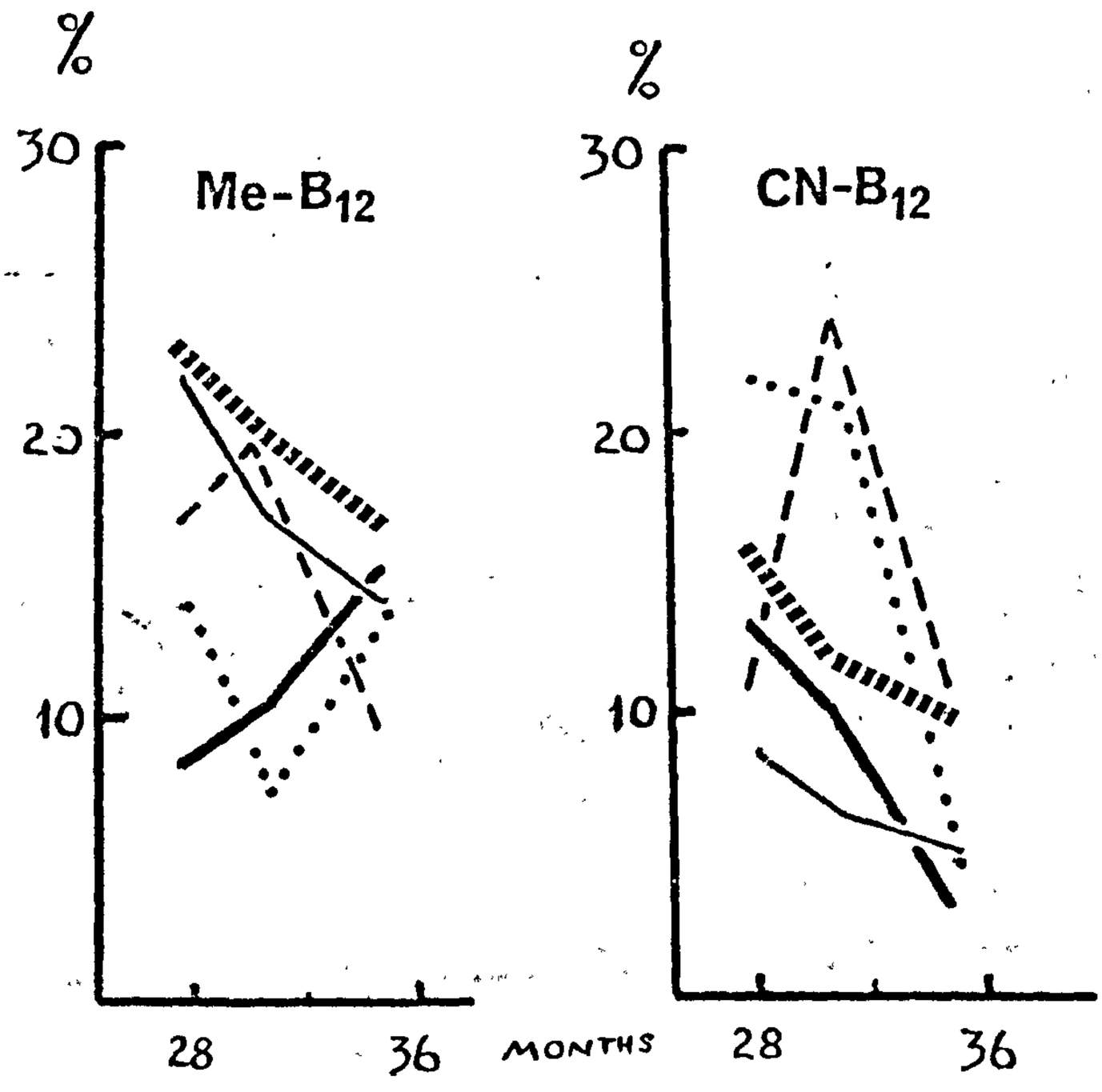


Figure 7.1 – VI Changes in the proportions of $Me\text{-}B_{12}$ and $CN\text{-}B_{12}$ in the erythrocytes

——— = GROUP I
▪▪▪▪▪▪▪▪▪▪ = GROUP II
▬▬▬▬ = GROUP III
••••••• = GROUP IV
– – – – = GROUP V

pernicious anaemia, plasma Me-B_{12} may be virtually undetectable (see Section 6.1). When values for Me-B_{12} were expressed as a proportion of the total B_{12} (Fig 7.1 - IV) a pattern emerged which showed that coinciding with total B_{12} peaks (at 20 and 31 months) the plasma of many animals contained proportionately less Me-B_{12} and more Ado/OH-B_{12} than at other times during the year.

Results for CN-B_{12} when expressed as a percentage of the total B_{12} (Fig 7.1 - V) show that in all groups, the proportion of CN-B_{12} increased after 16 months, although only in the Group IV animals was this increase progressive. The highest proportions of CN-B_{12} were recorded in plasma from the Group II animals. Interestingly, these high values (approximately 30% CN-B_{12}) coincided with the peak total B_{12} levels both at 20 and at 31 months and corresponded in some animals to plasma CN-B_{12} levels of over 300 pg/ml.

Erythrocyte cobalamins

Values for erythrocyte total B_{12} and cobalamins were obtained on samples taken at 28, 31 and 35 months. The results (Fig 7.1 - VI) show a more than twofold difference in total B_{12} between B_{12}-supplemented and B_{12}-deficient groups. In Group II animals, there was a steep decline in total B_{12} of some 200 pg/ml

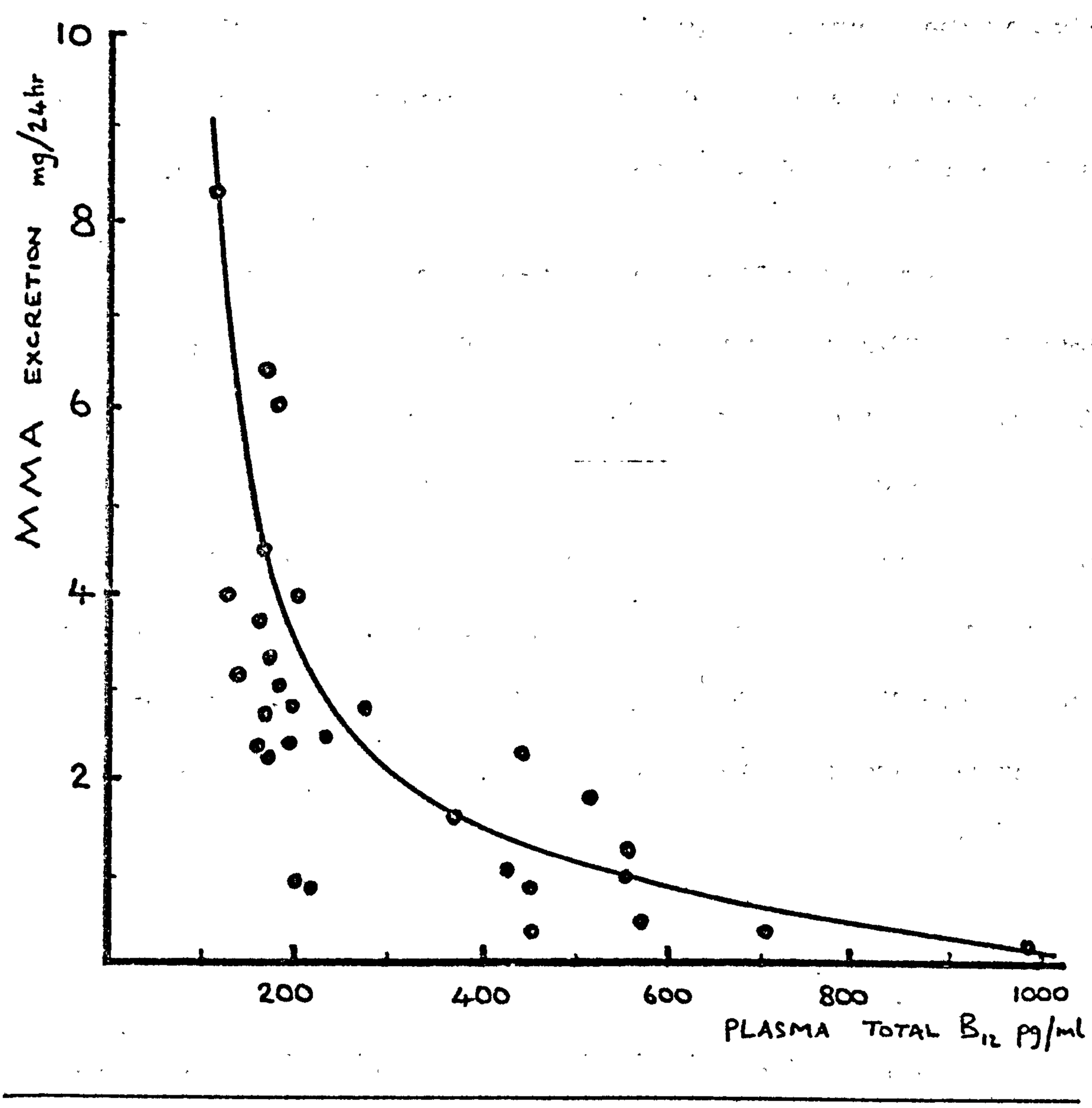


Figure 7.1 - VII

Relationship between plasma total B_{12} and methylmalonate excretion 35 months after the beginning of the experiment

over the seven-month period, but in other groups the changes in level were slight. Alterations in the proportions of Me-B_{12} and CN-B_{12} varied according to the group. In Groups I and II, the proportions of both cobalamins declined but in Groups III and IV, an increase in the percentage of Me-B_{12} was associated with a decrease in that of CN-B_{12}. In the Group V animals, the erythrocytes contained on average 11% of the total B_{12} as CN-B_{12} at 28 and 35 months, while at 31 months, the proportion (24%) had more than doubled. Similar but less pronounced changes were observed in the proportions of Me-B_{12} in the erythrocytes.

Urinary excretion of methylmalonic acid (MMA)

The MMA excretion of each animal was plotted against the plasma total B_{12} at 35 months; the results (Fig 7.1 - VII) show that a relationship existed between these values. Elevated MMA levels were recorded from a number of animals whose plasma total B_{12} fell below 200 pg/ml. These results suggest that had the plasma B_{12} fallen below 100 pg/ml, a very high urinary MMA excretion might have been anticipated.

7.2 STUDIES of INTER-RELATIONSHIPS between CYANIDE and VITAMIN B_{12}

The first indication of a metabolic relationship between cyanide and vitamin B_{12} came from the work of Boxer and Rickards (1952) who showed that administration of ^{14}C-labelled cyanide to dogs led to the appearance of the label in vitamin B_{12} and in the 1-C metabolic pool. Wokes, Badenoch and Sinclair (1955) reported that there was an increased urinary excretion of thiocyanate in dietary deficiency of vitamin B_{12}, and Wokes and Picard (1955) suggested that vitamin B_{12}, in the form of OH-B_{12}, might play an important role in detoxication of cyanide - as an intermediary in an alternative pathway to that leading to thiocyanate formation. Braekkan, Njaa and Utne (1957) showed that prolonged dosage with cyanide could deplete liver B_{12} in rats. Freeman and others (Heaton, McCormick and Freeman, 1958; Freeman and Heaton, (1961) reported that the serum B_{12} was frequently low in tobacco amblyopia, and suggested that B_{12} deficiency might increase the sensitivity to the toxic effects of tobacco smoke, while Wokes (1958) suggested that the toxic factor in tobacco smoke might be cyanide.

In 1961, A.D.M. Smith proposed that the toxic effect of cyanide in tobacco amblyopia might be the result of conversion of $OH\text{-}B_{12}$ to $CN\text{-}B_{12}$. When the L. leichmannii assay for serum B_{12} came into general use, several workers observed that the values for serum B_{12} concentrations were unduly low unless cyanide was added to the acetate buffer with which the serum was heated to extract the vitamin (Spray, 1955; Killander, 1957; Girdwood, 1960). It was originally suggested that the action of cyanide might be to protect the vitamin from destruction, but this was shown to be incorrect by the demonstration (Matthews, 1961; 1962) that the ommision of cyanide from the buffer caused losses of B_{12} in the precipitate of serum proteins, from which the missing B_{12} was fully recoverable. At the time this work was carried out, the forms of B_{12} in the blood were unknown, but it seemed possible that they might be a mixture of $CN\text{-}B_{12}$ and $OH\text{-}B_{12}$. One possible explanation of the effect of cyanide in aiding extraction of the vitamin was that it converted serum $OH\text{-}B_{12}$ to $CN\text{-}B_{12}$, which was more readily extractable from the proteins (Matthews, 1961). If this were the case, the B_{12} extractable without added cyanide would represent $CN\text{-}B_{12}$, and the remainder, extractable only with added cyanide, $OH\text{-}B_{12}$. This possibility was supported by Smith, who observed that the proportion of serum B_{12} extractable without

added cyanide was higher in smokers than in non-smokers. He interpreted this as indicating that the proportion of serum CN-B_{12} was raised in smokers owing to their cyanide intake, and proposed that in cases of tobacco amblyopia, such conversion might be responsible for deficiency of OH-B_{12} (then commonly believed to be the active form of B_{12}) even in the presence of normal total B_{12} levels. Subsequent work has shown that Smith's hypothesis cannot be correct in detail, but it suggested the possible clinical importance of inter-relationships between the metabolism of B_{12} and that of cyanide in man.

Rather more definite evidence suggesting an inter-relationship between smoking, cyanide intake and B_{12} metabolism resulted from an investigation of alleged abnormalities of cyanide and B_{12} metabolism in multiple sclerosis (Fowler and Durbetaki, 1952; Bauer and Heinrich, 1961). The results (Basil, Brown and Matthews, 1965; Matthews, Wilson and Zilkha, 1965) showed no significant abnormalities, but subjects with relatively high plasma cyanide concentrations tended to have relatively low total serum B_{12} concentrations. This relationship was further investigated in a series of healthy smokers and non-smokers (Wilson and Matthews, 1966); it was shown that plasma concentrations of both cyanide and thiocyanate were raised in smokers, and that there was

a positive correlation between them; the negative correlation between plasma cyanide and total serum B_{12} was confirmed and it was shown that there was a small but significant difference in the percentage of B_{12} extractable without added cyanide in smokers (mean 53%) and non-smokers (mean 46%) - the very high percentages (approaching 100%) reported to occur in some smokers (Smith, 1961) were not found.

Later, the application of the technique of Lindstrand and Stahlberg (1963) to a small group of smokers showed that 3 out of 10 had small quantities of $CN\text{-}B_{12}$ in the plasma; in the remaining 7, it was undetectable (Lindstrand, Wilson and Matthews, 1966). As $CN\text{-}B_{12}$ is more readily excreted than other cobalamins (Lester Smith, 1963) urinary excretion was investigated in a further study of B_{12} metabolism in smokers (Linnell, Smith, Smith, Wilson and Matthews, 1968 - Section 7.3).

The general conclusions are that in normal subjects, smokers have raised plasma levels of cyanide and thiocyanate, an increased urinary excretion of thiocyanate and total B_{12}, and a relatively low total serum B_{12} concentration. Although not all smokers have detectable amounts of $CN\text{-}B_{12}$ in the plasma, small amounts probably appear more frequently than in non-smokers.

TABLE 7.2 - 1

EFFECT of ADDED CYANIDE, THIOCYANATE and $CN-B_{12}$ on EXTRACTABILITY of B_{12} from PLASMA

Addition		Percentage extractability
None		45
CN^-	0.2 μM/100 ml*	65
CN^-	2.0 μM/100 ml	95
SCN^-	100 μM/100 ml*	55
$CN-B_{12}$	500 pg/ml* (to low-B_{12} plasma)	95

* These concentrations are about ten times those found in healthy smokers

TABLE 7.2 - 2

EXTRACTABILITY of COBALAMINS

Cobalamin*	Percentage extractability
$OH-B_{12}$	55
$Ado-B_{12}$	60
$Me-B_{12}$	65
$CN-B_{12}$	95

* Added to low-B_{12} plasma at a concentration of 500 pg/ml

Factors promoting increased B_{12} extractability without added cyanide

Experimentally, extractability can be increased in two ways: (1) by adding large concentrations of cyanide or thiocyanate to serum or plasma in vitro, or (2) by increasing the plasma $CN\text{-}B_{12}$ concentration in vitro or in vivo.

The concentrations of cyanide or thiocyanate required to produce an appreciable increase in extractability are very large - thus, an increase of between 10% and 20% in extractability requires a cyanide concentration in plasma of about 0.2 μM/100ml or a thiocyanate concentration of about 100 μM/100ml (Table 7.2-1). These concentrations are approximately ten times greater than those normally found in plasma, so that alterations in cyanide or thiocyanate concentrations in vivo are unlikely to affect extractability by more than a small percentage. The addition of high concentrations (500 pg/ml) of cobalamins other than $CN\text{-}B_{12}$ to low-B_{12} plasma has little effect on extractability, which remains in the region of 50% to 65%. The addition of high concentrations of $CN\text{-}B_{12}$, however, raises extractability to over 90% (Table 7.2 - 2). It is also possible to increase extractability by raising the plasma $CN\text{-}B_{12}$ concentration in vivo (Table 7.2 - 3). For example, in one subject (B) with no detectable $CN\text{-}B_{12}$ in the

TABLE 7.2 - 3

EFFECT of CN-B_{12} *in vivo* on Total B_{12} EXTRACTABILITY

	Total B12	Me-B12	CN-B12	Ado/OH-B12	Percentage extractability
	picograms per millilitre				
SUBJECT A					
Pre-dose	595	355	0	240	67
24 hr after 100 μg CN-B_{12} (i.m.)	895	645	100 (11%)	150	76
SUBJECT B					
Pre-dose	350	322	0	28	65
24 hr after 10 μg CN-B_{12} (i.m.)	645	355	180 (27%)	110	74

Subject A is a smoker (40 cigarettes / day)
Subject B is a smoker

plasma, extractability was 65%, but 1.5 hr after an intramuscular injection of 10 μg CN-B_{12}, bioautographic analysis showed that plasma CN-B_{12} concentration had risen to 180 pg/ml out of a total of 645 pg/ml (27%). This caused an increase in extractability to 74%. Fig 7.2-I shows the relationship between the percentage CN-B_{12} in the plasma, and percentage extractability, in normal subjects and in patients with raised CN-B_{12} concentrations. There is a positive correlation, but it is extremely indefinite and barely significant statistically.

It is clear that the value for percentage extractability is very variable, even in plasma with no detectable CN-B_{12}. There is a tendency for it to increase slightly in the presence of raised plasma cyanide or thiocyanate (accounting perhaps for the small increase in smokers as a group), and large increases in plasma CN-B_{12} do produce definite increases in extractability, but the correlation is so poor that percentage extractability is almost useless as a guide to increases in plasma CN-B_{12} concentration.

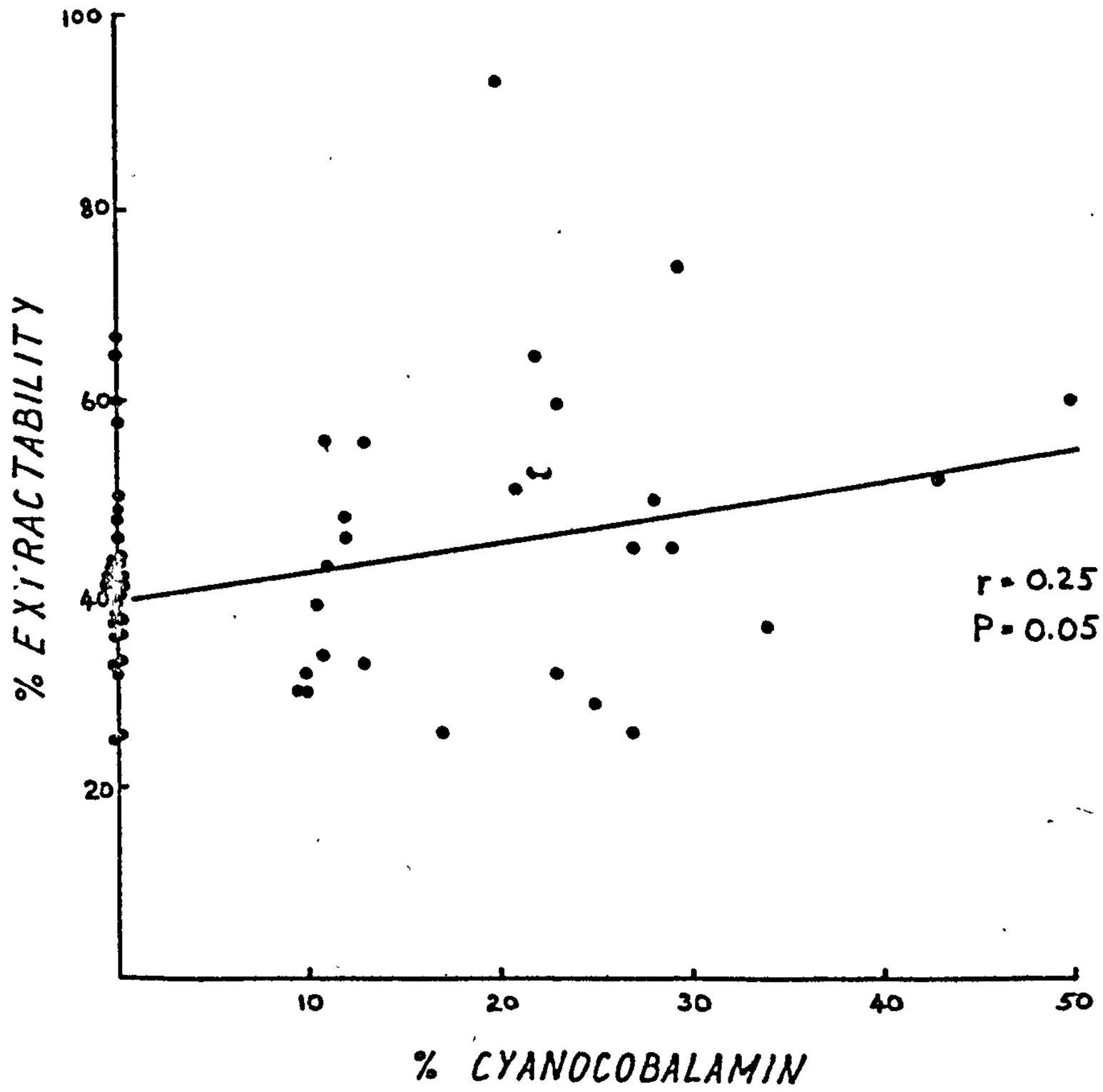


Figure 7.2 - I Relationship between plasma CN-B_{12} and extractability of the total plasma B_{12}

Reprinted from the BRITISH MEDICAL JOURNAL
27 *April* 1968, 2, 215–216

Effects of Smoking on Metabolism and Excretion of Vitamin B_{12}

J. C. LINNELL,* B.SC.
A. D. M. SMITH,† M.B., B.CH., B.SC., M.R.C.P.
C. L. SMITH,† M.B., B.SC., M.R.C.P.
J. WILSON,‡ M.B., B.S., B.SC., PH.D., M.R.C.P.
D. M. MATTHEWS,* M.D., PH.D., M.C.PATH.

Experimental and clinical evidence both suggest that there is some connexion between the metabolism of vitamin B_{12} and that of cyanide, and that smoking, which is associated with a high cyanide intake—for example, Darby and Wilson (1967)—may adversely affect B_{12} metabolism (Boxer and Rickards, 1952 ; Wokes and Picard, 1955 ; Braekkan, Njaa, and Utne, 1957 ; Wokes, 1958 ; Smith, 1961 ; Smith, Duckett, and Waters, 1963 ; Smith, 1964 ; Smith and Duckett, 1965 ; Matthews, Wilson, and Zilkha, 1965 ; Wilson and Matthews, 1966 ; Lindstrand, Wilson, and Matthews, 1966 ; Smith and Foulkes, 1966). The hypothesis that interconnected disturbances of cyanide/B_{12} metabolism may be concerned in the pathogenesis of tobacco amblyopia, the retrobulbar neuritis of pernicious anaemia, Leber's optic atrophy, and certain tropical neurological syndromes apparently associated with a high cyanide intake from cassava (Smith, 1961 ; Wilson, 1965 ; Montgomery, 1965 ; Wilson and Langman, 1966 ; Monekosso and Wilson, 1966 ; Freeman, 1967 ; Chisholm, Bronte-Stewart, and Foulds, 1967) makes it essential to obtain further information about the interrelationships between an increased exogenous cyanide load, cyanide/thiocyanate metabolism, and bodily handling of vitamin B_{12}.

So far the observations apparently providing a direct link between smoking and B_{12} metabolism are few. They include: (1) the observation that there may be a slight or moderate increase in cyanocobalamin in the plasma of some heavy smokers

* Department of Chemical Pathology, Westminster Medical School, London S.W.1.
† Westminster Hospital, London S.W.1.
‡ Medical Research Council Clinical Genetics Research Unit, Institute of Neurology, London W.C.1.

COPYRIGHT © 1968. ALL RIGHTS OF REPRODUCTION OF THIS REPRINT ARE RESERVED IN ALL COUNTRIES OF THE WORLD

279/68

(Lindstrand, Wilson, and Matthews, 1966) ; (2) the finding that a larger proportion of serum B_{12} is extractable in the absence of added cyanide in smokers than in non-smokers, which may be related to (1) ; and (3) the finding of a negative correlation between plasma cyanide concentration and that of total serum B_{12} (Matthews *et al.*, 1965 ; Wilson and Matthews, 1966). This last relationship could be connected with a tendency for chronic administration of cyanide to deplete body B_{12} stores (Braekkan *et al.*, 1957), and the present investigation was made to explore this possibility further.

Methods

Two groups of healthy subjects were studied—18 non-smokers (16 men and 2 women, mean age 27) and 19 smokers (16 men and 3 women, mean age 29). Eighteen of the smokers smoked cigarettes (mean consumption 20 per day) and one smoked a pipe (1 oz. (28 g.) per day). Twenty-four-hour urine collections were made into containers containing 1 g. of neomycin, and aliquots of urine deep-frozen until analysis. Urine thiocyanate was estimated spectrophotometrically (Wilson and Matthews, 1966). Blood was taken for serum B_{12} estimation on the day of the urine collection. Serum and urine B_{12} were estimated by radioisotopic assay (Matthews, Gunasegaram, and Linnell, 1967) after extraction with cyanide-containing buffer.

Results and Discussion

The results showed definite interrelationships between smoking, urine thiocyanate excretion, which is an index of the exogenous cyanide load, and serum and urine B_{12} (Tables I and II ; Fig. 1). The mean thiocyanate excretion in smokers was more than double that in non-smokers. The urine B_{12} excretion was significantly increased in smokers ($P=0.02$). In smokers and non-smokers, taken together, there was a positive correlation between urine thiocyanate excretion and urine B_{12} excretion. There was a negative correlation between thiocyanate

TABLE I.—*Serum B_{12}, Urine B_{12} Excretion, and Urine Thiocyanate Excretion in Smokers and Non-smokers*

Estimation	Smokers	Non-smokers	
Serum B_{12} ($\mu\mu$g./ml.) ..	*444 ± 36·8 (18)	472 ± 26·3 (18)	0·6 > P > 0·5
Urine B_{12} (mμg./24 hours)	81·2 ± 8·7 (16)	60·3 ± 7·9 (16)	P = 0·02†
Urine thiocyanate (μmoles/24 hours)	207·8 ± 15·8 (19)	90·5 ± 7·0 (18)	P ≪ 0·001

* Mean ± S.E.M. (n). † Using $\log_{10}$.

excretion and serum B_{12} concentration. In smokers alone, this latter correlation was more pronounced, and the tendency for high thiocyanate excretion to be associated with relatively low serum B_{12} concentrations was striking (Fig. 2). Extrapolation of the regression line in Fig. 2 suggests that if the urine

TABLE II.—*Correlations Between Serum B_{12}, Urine B_{12} Excretion, and Thiocyanate Excretion in Smokers and Non-smokers*

	Group	r	
Serum B_{12}/thiocyanate excretion	Smokers and non-smokers	−0·42	0·02 > P > 0·01
" " "	Smokers only	−0·57	0·02 > P > 0·01
Urine B_{12}/thiocyanate excretion	Smokers and non-smokers	+0·53	0·01 > P > 0·001
Serum B_{12}/B_{12} excretion	" " "	−0·19	P > 0·9
" " "	Smokers only	−0·24	P > 0·9

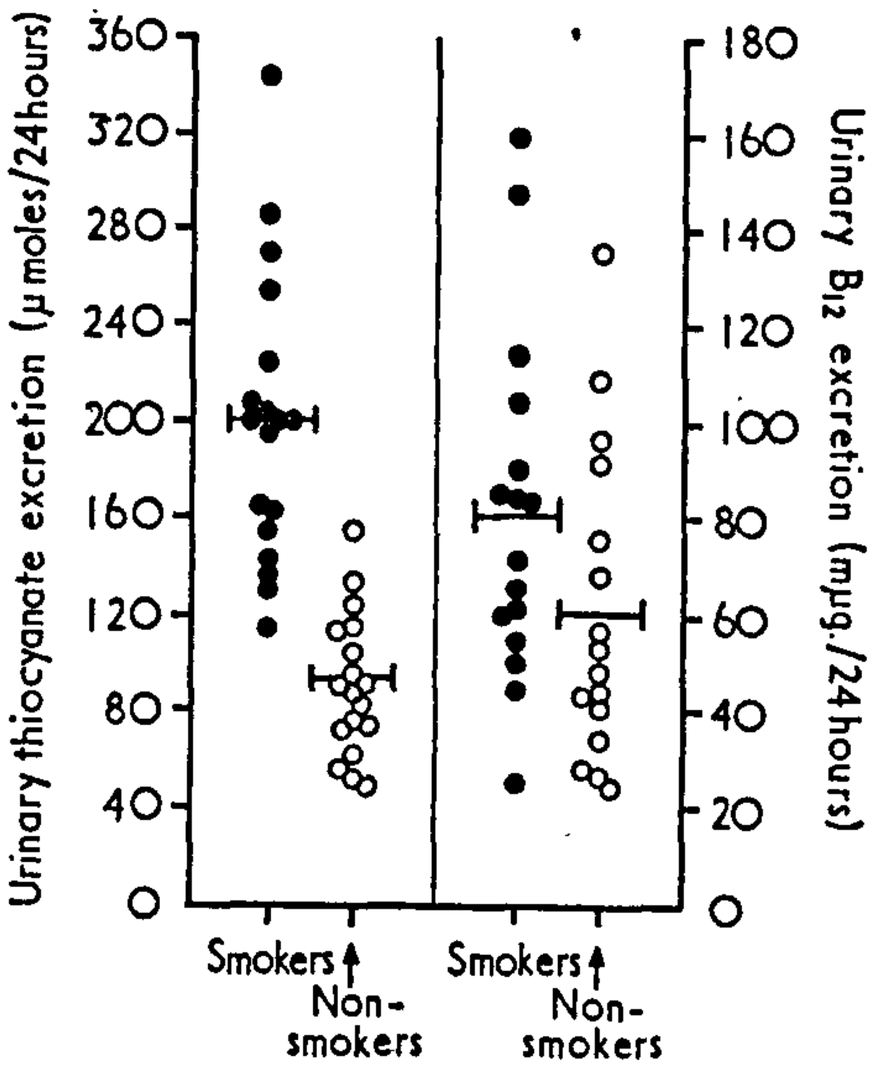


FIG. 1.—Urine thiocyanate excretion and urine B_{12} excretion in smokers and non-smokers. |—| mean value.

thiocyanate excretion reached 300–400 μmoles/24 hours, subnormal serum B_{12} concentrations would be expected.

Summarized, the results show that urine excretion of B_{12} is raised in smokers, and that a high excretion of thiocyanate tends to be associated with an increase in B_{12} excretion and a relatively low serum B_{12} concentration. The association between high thiocyanate excretion and low serum B_{12} concentration, which is especially marked in smokers, recalls that between high serum cyanide concentration and low serum B_{12} (Wilson

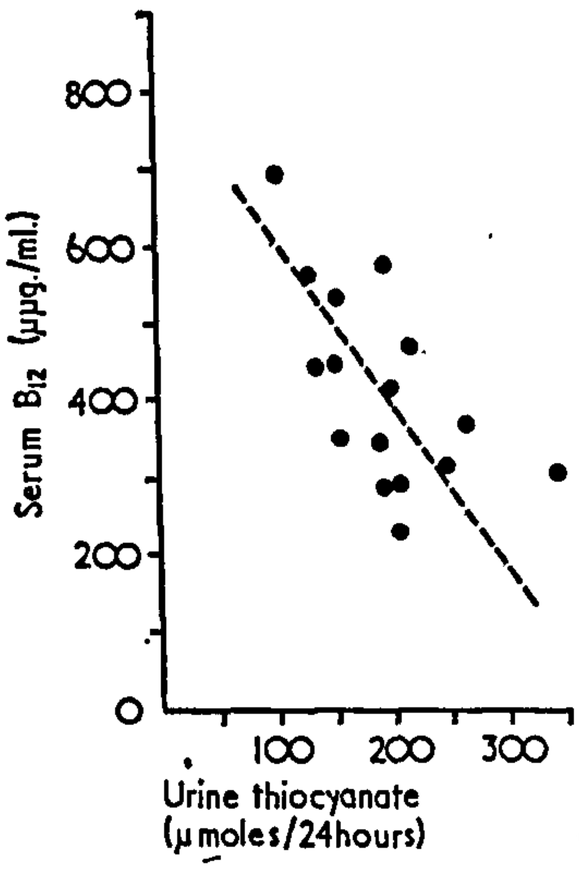


FIG. 2. — Correlation between serum B_{12} and urine thiocyanate excretion in smokers. (The regression line shown bisects the angle between the regression of y on x and that of x on y.)

and Matthews, 1966); these two observations are probably related. More than one hypothesis might be put forward to explain these results. Thus it could be postulated that subjects with relatively low serum B_{12} concentrations have a reduced ability to detoxicate cyanide by pathways involving this vitamin, so that detoxication by the thiocyanate pathway is increased. This would not, however, readily explain the association between high thiocyanate excretion and high excretion of B_{12}.

Alternatively, the low serum B_{12} concentration might reflect B_{12} depletion, possibly resulting from conversion of tissue cobalamins to cyanocobalamin, a form relatively readily excreted by the kidney (Nyberg, Saarni, and Gräsbeck, 1961; Samson, Yeh, and Chow, 1961; Glass, Skeggs, Lee, Jones, and Hardy, 1962; Okuda and Tantengco, 1962). Though chromatography of serum B_{12} in smokers (Lindstrand *et al.*, 1966) showed only small or moderate amounts of cyanocobalamin in some of the group, it did not exclude the possibility that a minor degree of conversion occurred in all. However, the increment in B_{12} excretion associated with smoking (mean 0.021 µg./day, maximum probably about 0.1 µg./day—Fig. 1) is so small in relation to the amount probably absorbed daily (2–5 µg.) and to the liver stores (about 2,000 µg.) that it would seem unlikely that appreciable depletion could be caused in healthy people by this

means. This consideration, together with the very poor correlation between serum B_{12} and urine B_{12} excretion, suggests that some factor other than increased renal excretion of B_{12} must operate to produce the relation between high thiocyanate excretion and low serum B_{12} concentration. It is possible that high plasma cyanide concentrations disturb the equilibrium between serum and urine B_{12}.

At the moment the main significance of this work is that it shows further definite, if unexplained, interrelationships between smoking, cyanide metabolism, and bodily handling of vitamin B_{12}, and gives further support to the idea that high loads of cyanide might produce derangements of B_{12} metabolism. The effects of smoking are slight in healthy subjects, but in patients already in marginal B_{12} balance they might become significant.

Summary

In view of evidence suggesting that there is a connexion between the metabolism of vitamin B_{12} and that of cyanide, and that smoking, which is associated with a high cyanide intake, may disturb the metabolism of B_{12}; serum B_{21}, urine B_{12} excretion, and urine thiocyanate excretion (an index of cyanide detoxication) have been measured in healthy smokers and non-smokers.

The results show that urine B_{12} excretion is raised in smokers and that a high thiocyanate excretion is associated with an increase in B_{12} excretion and a relatively low serum B_{12} concentration. The increase in B_{12} excretion seems insufficient to account for the reduction in serum B_{12} concentration, which may be the result of disturbance of the equilibrium between serum and tissue B_{12} by high plasma cyanide concentrations. The work supports the idea that a high cyanide intake might produce derangements of B_{12} metabolism.

The work was supported by a grant from the Wellcome Trust.

References

Boxer, G. E., and Rickards, J. C. (1952). *Arch. Biochem.*, **39**, 7.

Braekkan, O. R., Njaa, L. R., and Utne, F. (1957). *Acta pharmacol. (Kbh.)*, **13**, 228.

Chisholm, I. A., Bronte-Stewart, J., and Foulds, W. S. (1967). *Lancet*, **2**, 450.

Darby, P. W., and Wilson, J. (1967). *Brit. J. Ophthal.*, **51**, 336.

Freeman, A. G. (1967). *Lancet*, **2**, 422.

Glass, G. B. J., Skeggs, H. R., Lee, D. H., Jones, E. L., and Hardy, W. W. (1962). In *Vitamin B_{12} und Intrinsic Factor*, edited by H. C. Heinrich, p. 673. Stuttgart.

279/68

Lindstrand, K., Wilson, J., and Matthews, D. M. (1966). *Brit. med. J.*, **2**, 988.
Matthews, D. M., Gunasegaram, R., and Linnell, J. C. (1967). *J. clin. Path.*, **20**, 683.
Matthews, D. M., Wilson, J., and Zilkha, K. J. (1965). *J. Neurol. Neurosurg. Psychiat.*, **28**, 426.
Monekosso, G. L., and Wilson, J. (1966). *Lancet*, **1**, 1062.
Montgomery, R. D. (1965). *Amer. J. clin. Nutr.*, **17**, 103.
Nyberg, W., Saarni, M., and Gräsbeck, R. (1961). *Nature (Lond.)*, **189**, 764.
Okuda, K., and Tantengco, V. (1962). *Proc. Soc. exp. Biol. (N.Y.)*, **110**, 396.
Samson, G. D., Yeh, S. D. J., and Chow, B. F. (1961). *Fed. Proc.*, **20**, 451.
Smith, A. D. M. (1961). *Lancet*, **1**, 1001.
Smith, A. D. M. (1964). *Lancet*, **2**, 668.
Smith, A. D. M., and Duckett, S. (1965). *Brit. J. exp. Path.*, **46**, 615.
Smith, A. D. M., Duckett, S., and Waters, A. H. (1963). *Nature (Lond.)*, **200**, 179.
Smith, A. D. M., and Foulkes, M. (1966). *Nature (Lond.)*, **209**, 919.
Wilson, J. (1965). *Clin. Sci.*, **29**, 505.
Wilson, J., and Langman, M. J. S. (1966). *Nature (Lond.)*, **212**, 787.
Wilson, J., and Matthews, D. M. (1966). *Clin. Sci.*, **31**, 1.
Wokes, F. (1958). *Lancet*, **2**, 526.
Wokes, F., and Picard, C. W. (1955). *Amer. J. clin. Nutr.*, **3**, 383.

APPENDIX 7.4

Absorption of Vitamin B_{12} in the Guinea-Pig

III. THE FORMS OF VITAMIN B_{12} IN ILEAL MUCOSA AND PORTAL PLASMA IN THE FASTING STATE AND DURING ABSORPTION OF CYANOCOBALAMIN

T. J. PETERS, J. C. LINNELL, D. M. MATTHEWS AND A. V. HOFFBRAND

Reprinted from
British Journal of Haematology
Vol. 20, No. 3, March 1971

British Journal of Haematology, 1971, 20, 299.

Absorption of Vitamin B_{12} in the Guinea-Pig

III. THE FORMS OF VITAMIN B_{12} IN ILEAL MUCOSA AND PORTAL PLASMA IN THE FASTING STATE AND DURING ABSORPTION OF CYANOCOBALAMIN

T. J. Peters, J. C. Linnell, D. M. Matthews and A. V. Hoffbrand

Medical Research Council Malabsorption Research Group, Department of Medicine and Department of Haematology, Royal Postgraduate Medical School, and Department of Experimental Chemical Pathology, Westminster Medical School

(*Received* 20 *August* 1970; *accepted for publication* 10 *September* 1970)

Summary. The forms of vitamin B_{12} in guinea-pig ileum, liver and portal plasma were studied using a chromatographic-bioautographic technique. 5′ Deoxyadenosylcobalamin was the principal cobalamin in each tissue although significant amounts of methylcobalamin were detected in plasma. An unidentified cobalamin was also found in all three tissues.

During the absorption of labelled cyanocobalamin a proportion of this analogue was converted to 5′ deoxyadenosylcobalamin in the ileum; preliminary experiments suggest that the site of inter-conversion was the mitochondria of the ileal epithelial cells. A significant proportion of the cyanocobalamin was transferred to the portal plasma unchanged however, indicating that the mitochondrial localization of vitamin B_{12} during its absorption, shown in previous studies, is not solely related to the conversion of vitamin B_{12} analogues from one form to another.

Previous studies suggest that the mitochondria of the ileal epithelial cells play a fundamental role in the absorption of vitamin B_{12} by the guinea-pig (Peters & Hoffbrand, 1970). These studies showed that cyanocobalamin enters the mitochondria during its transport through the cell and that it remains in these organelles for approximately 1 hr before the absorbed vitamin B_{12} enters portal plasma. Two physiological vitamin B_{12} analogues, methylcobalamin and 5′ deoxyadenosylcobalamin (adenosylcobalamin), have also been shown to localize to the ileal mitochondria during absorption (Peters *et al*, 1971). The explanation for this localization is unknown but one possibility is that the ileal mitochondria are the site of interconversion of different vitamin B_{12} analogues during their absorption.

The present study was undertaken to determine whether cyanocobalamin is converted to other vitamin B_{12} analogues during absorption and, if so, the intracellular site of this conversion. In addition data is presented on the endogenous forms of vitamin B_{12} in guinea-pig plasma, small intestine and liver. A preliminary communication of this work has appeared (Hoffbrand *et al*, 1970).

METHODS

Analytic procedures. The total vitamin B_{12} content of the different tissues was assayed

microbiologically using the 'z' strain of *Euglena gracilis* (Anderson, 1965). The individual cobalamins were separated and quantitated by chromatography and bioautography (Linnell *et al*, 1969b, c). The cobalamins were extracted from the tissue samples with hot aqueous ethanol (80% v/v) for 20 min at 80°C, separated by thin-layer chromatography and detected bioautographically using a vitamin B_{12}-dependent strain of *Escherichia coli* with a growth indicator dye. The crimson stained zones on the bioautogram were quantitated by densitometric scanning. In experiments using labelled vitamin B_{12}, the crimson zones were excised from the bioautogram and transferred, together with the underlying areas of chromatographic absorbent, to a series of small, stoppered tubes. Radioactivity was measured in a gamma scintillation counter and the radioactivity of each analogue expressed as cpm/mg protein or in the case of plasma samples, cpm/ml plasma.

Protein was estimated by the method of Lowry *et al* (1951) using bovine serum albumin (Armour) as standard.

Experimental procedure. Fasted male guinea-pigs (Hartley strain) weighing between 350 and 450 g were fed approximately 10 ng of high specific activity ^{57}Co-labelled cyanocobalamin (80–120 μCi/μg, Radio Chemical Centre, Amersham). The animals were killed by a blow on the head at ½, 1, 2, 3, 4 and 24 hr post-feeding. The ileal mucosa was homogenized in 10 vols ice-cold 0.15 M NaCl. Ileal mitochondria were also prepared by the method of Parsons *et al* (1966). Aliquots of the homogenate and mitochondria were stored at −20°C until assay. The experimental procedures and cobalamin extractions were all performed in a dark room using a red safelight and the tissue and plasma samples were kept in foil-wrapped containers.

Operative procedure. Two fasted male guinea-pigs underwent splenectomy and had fine polythene cannulae inserted into their portal veins. They were allowed to recover overnight. Two ml samples of portal venous blood were removed before, and up to 8 hr after, the intragastric administration of 10 ng ^{57}Co-labelled cyanocobalamin. The plasma was separated and stored in foil-wrapped heparinized containers at −20°C until assay and chromatography of the cobalamins. The entire experiment was performed in a dark room using a safelight. Full details of the operative technique are described elsewhere (Peters & MacMahon, 1970).

RESULTS

Forms of Vitamin B_{12} in the Fasting Guinea-Pig

Fig 1 compares chromatograms of the cobalamins extracted from ileal mucosa, ileal mitochondria and portal plasma with those of standard markers. Table I shows the total vitamin B_{12} concentration and the concentration of the different cobalamins in plasma, liver, ileal mucosa and ileal mitochondria. Adenosylcobalamin was the principal form detected in ileum, liver and plasma. Only trace amounts of methylcobalamin were found in liver and ileum but larger amounts of this analogue (approximately 6%) were detected in plasma. In addition considerable amounts of an unidentified cobalamin were detected in all tissues studied.

Forms of Labelled Vitamin B_{12} in Ileum during Absorption of Labelled Cyanocobalamin

Fig 2 shows the specific activity of the different vitamin B_{12} analogues in whole ileal

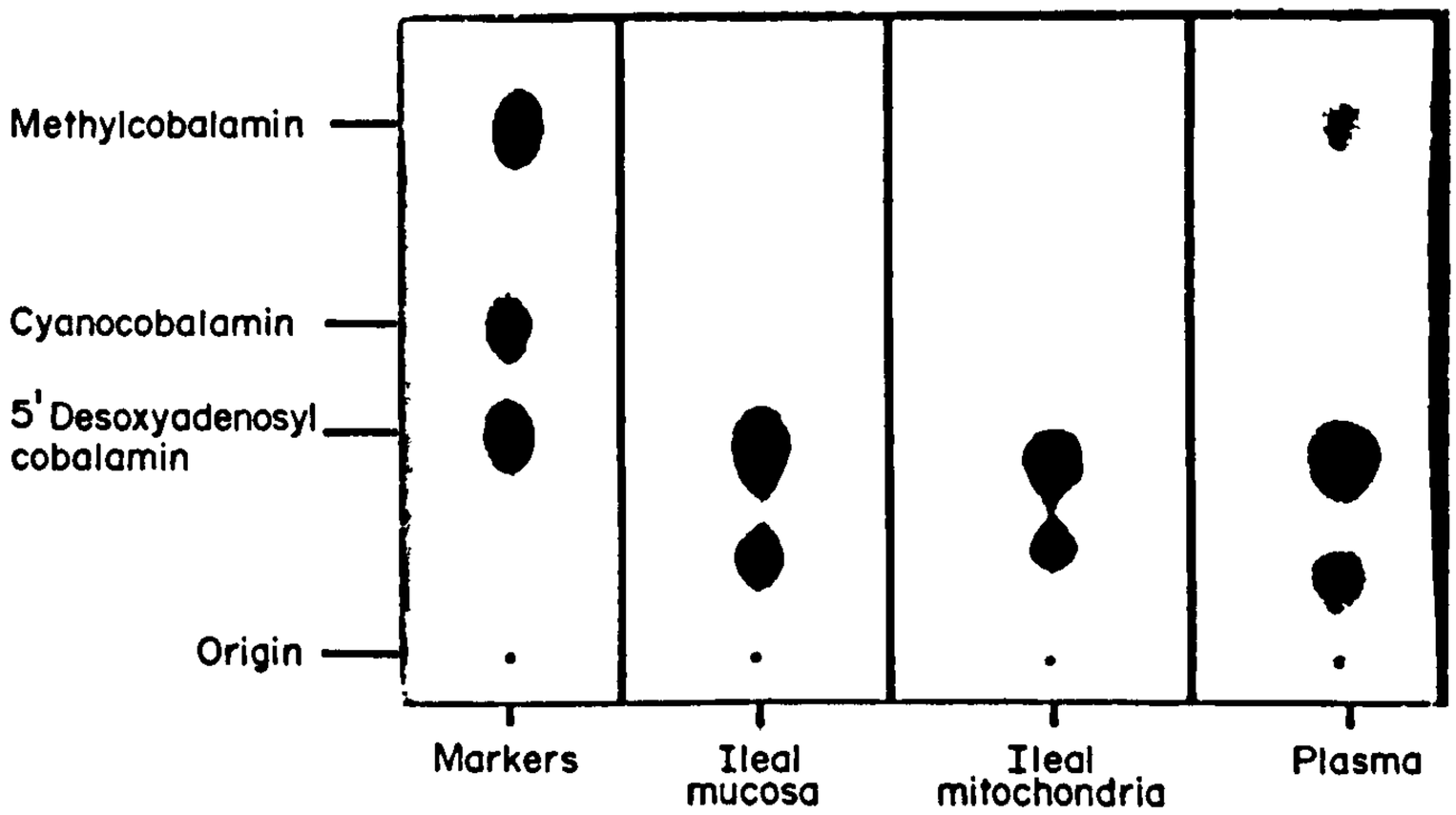


FIG 1. Bioautograms of methylcobalamin, cyanocobalamin, adenosylcobalamin and of the cobalamins extracted from guinea-pig ileum, ileal mitochondria and portal plasma.

TABLE I. The concentrations of the individual cobalamins in guinea-pig liver, ileum, ileal mitochondria and portal plasma

Tissue	*Total vitamin B_{12}* (pg/mg protein)*	*Methyl-cobalamin*	*Cyano-cobalamin*	*Adenosyl-cobalamin (pg/mg protein)*	*Unknown cobalamin*
Ileal mucosa	2080 ± 261 (13)	80	—	1300	700
Ileal mitochondria	5840 ± 180 (4)	—	—	3540	2300
Liver	4910 ± 585 (4)	50	245	2450	2165
Portal plasma	1480 ± 297 (7)	90	—	780	616

* Mean ± SE. Number of observations in parentheses.

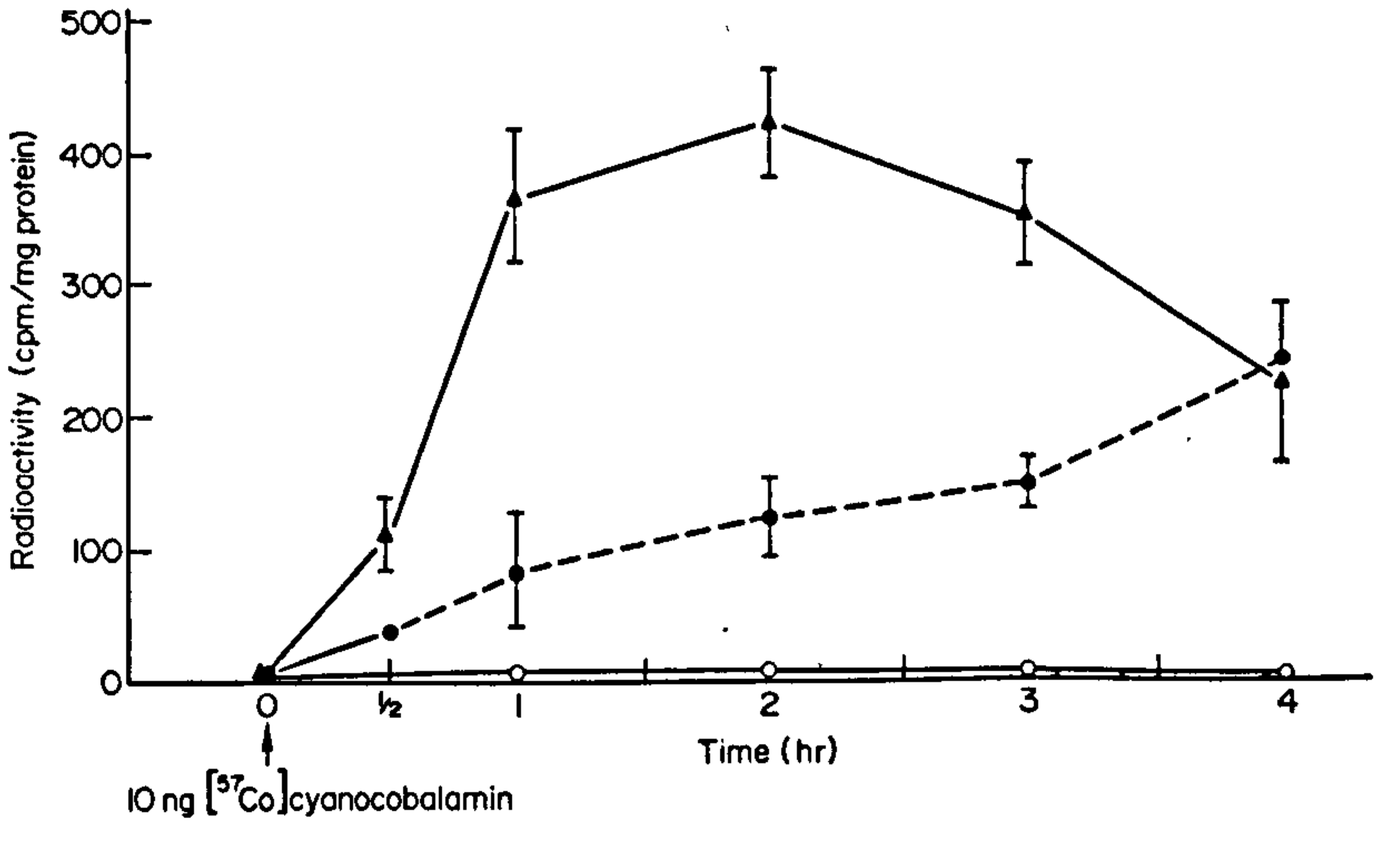


FIG 2. The specific activity of cyanocobalamin (▲), 5'desoxyadenosylcobalamin (●) and methylcobalamin (○) in ileal mucosa at timed intervals after feeding radioactive cyanocobalamin.

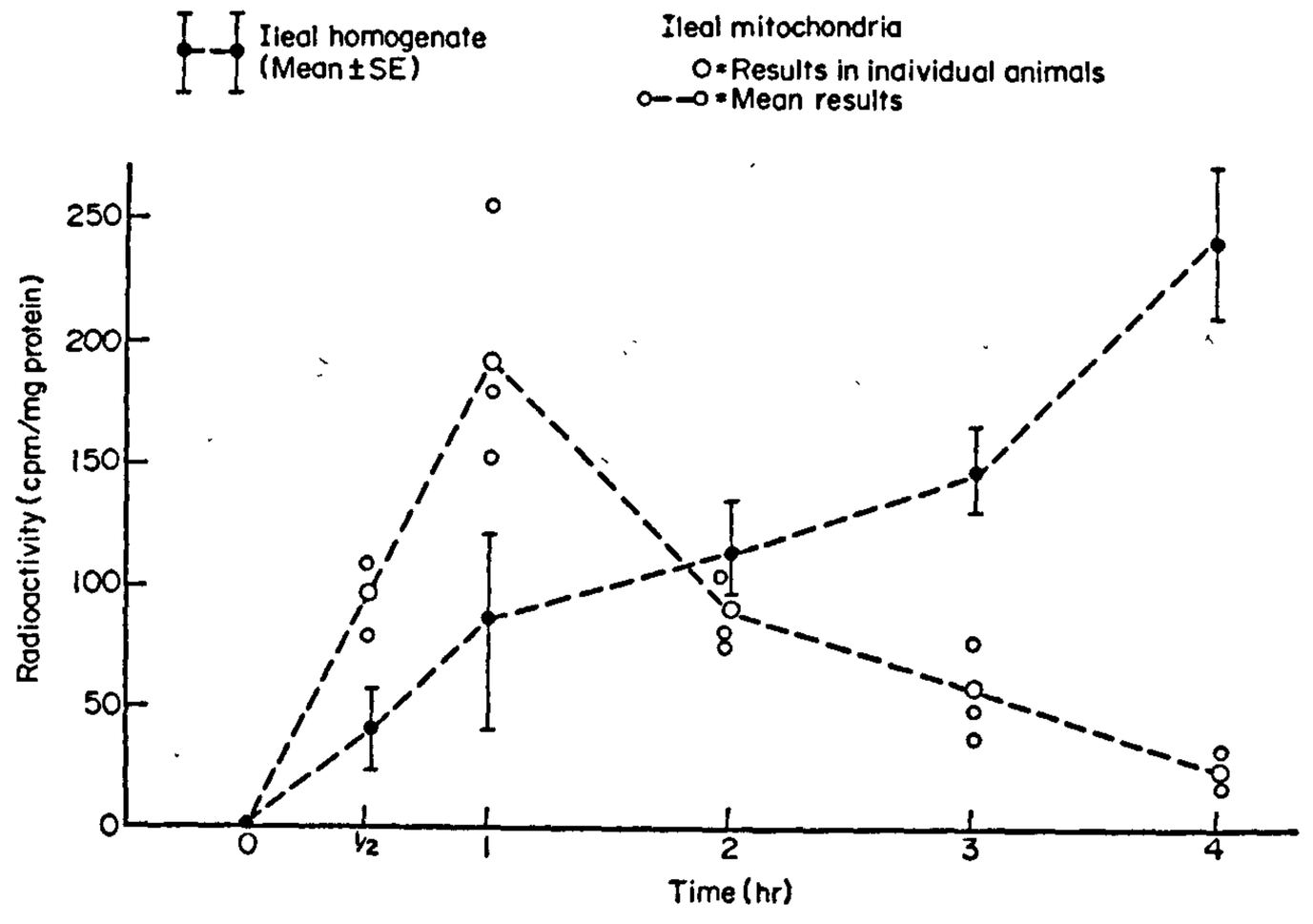


FIG 3. The specific activity of adenosylcobalamin in ileal mucosa and ileal mitochondria at timed intervals after feeding radioactive cyanocobalamin.

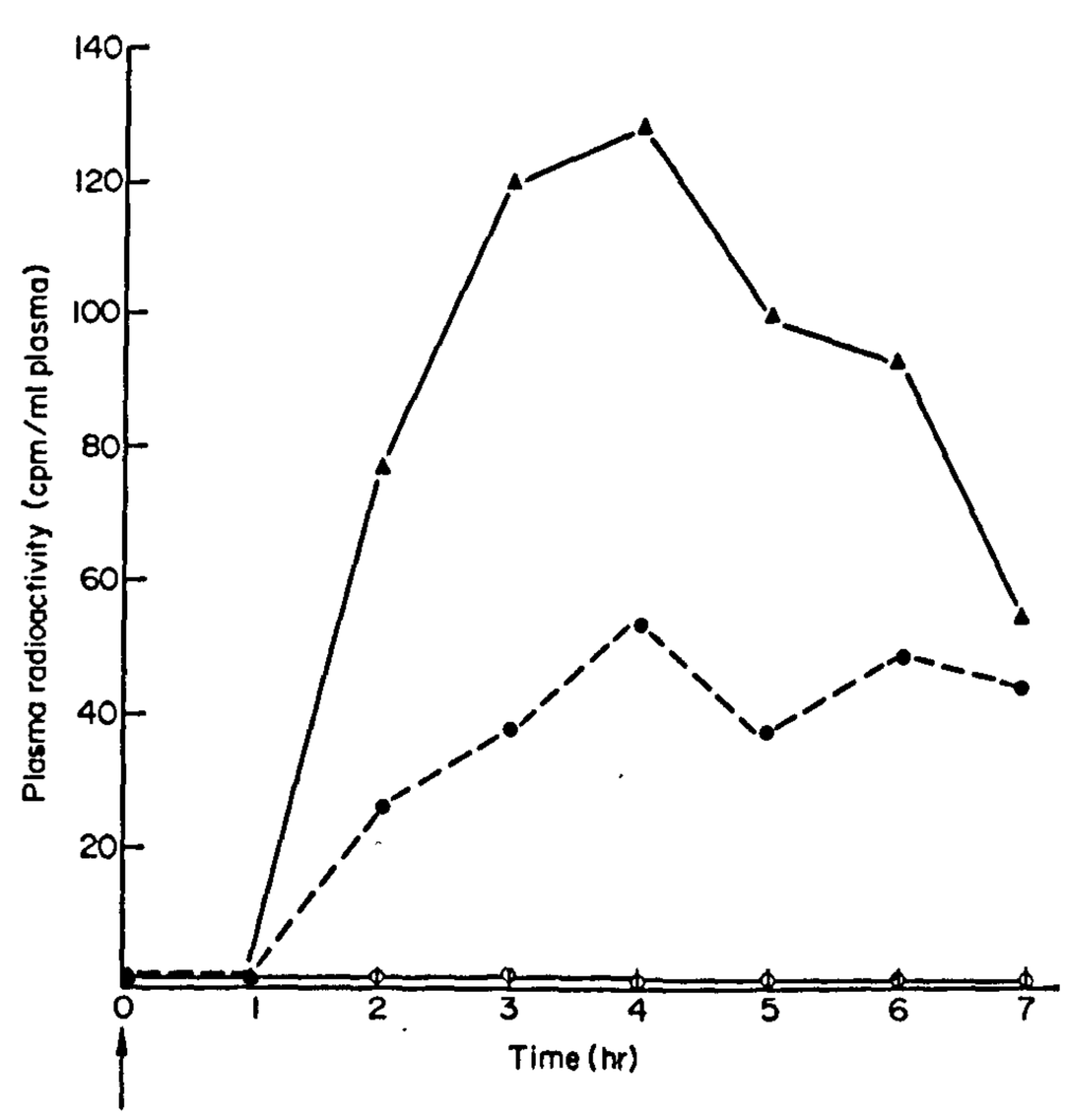


FIG 4. The concentration of labelled cyanocobalamin (▲), 5′ desoxyadenosylcobalamin (●) and methylcobalamin (○) in portal blood at timed intervals after feeding radioactive cyanocobalamin.

mucosa at timed intervals after feeding the labelled cyanocobalamin. Each point is the mean ± SE of duplicate estimations in at least three animals.

No significant radioactivity was detected in either the methylcobalamin or unidentified cobalamin zones at any time during the study. There was, however, a sharp rise in the amount of labelled cyanocobalamin in the mucosa which reached a peak at 1–3 hr and then declined. There was also a progressive rise in radioactive adenosylcobalamin in the mucosa throughout the 4 hr period of study. It is important to note that labelled adenosylcobalamin appeared in the mucosa within 1 hr of feeding the cyanocobalamin, i.e. before any radioactivity had left the mucosa and entered portal blood (Fig 4).

Fig 3 shows the specific activity of labelled adenosylcobalamin in the whole ileal homogenate and in the mitochondrial fraction after feeding labelled cyanocobalamin. Each point is the mean of duplicate estimations in at least two animals. There was a rise in the mitochondrial adenosylcobalamin radioactivity which reached a peak at 2 hr and then declined. In contrast, adenosylcobalamin activity in the whole homogenate increased slowly throughout the experimental period.

Forms of Labelled Vitamin B_{12} in Portal Plasma during the Absorption of Labelled Cyanocobalamin

Fig 4 shows the different radioactive cobalamins in the portal plasma of two guinea-pigs fed 10 ng of ^{57}Co-cyanocobalamin. After the period of mucosal delay, there was a rise in both labelled adenosyl- and labelled cyano-cobalamin in portal plasma. The concentration of labelled adenosylcobalamin increased up to 4 hr and then remained constant throughout the experimental period but the concentration of labelled cyanocobalamin reached a broad plateau between 3 and 5 hr and then declined. No radioactivity was detected in either the methylcobalamin or the unidentified cobalamin zones.

DISCUSSION

These experiments show that the principal form of vitamin B_{12} in guinea-pig ileal mucosa and liver is 5′ deoxyadenosylcobalamin. This is in agreement with observations of other workers on the forms of vitamin B_{12} in mammalian liver (Pawelkiewicz *et al*, 1960; Toohey & Barker, 1961). Significant amounts of methylcobalamin were only detected in plasma and in a much lower proportion than in human plasma (Lindstrand & Stahlberg, 1963; Linnell *et al*, 1969).

The localization of the adenosylcobalamin in the mitochondria is in agreement with the previous report that vitamin B_{12} is found in the highest concentration in the mitochondrial fraction of ileal cells (Peters & Hoffbrand, 1970). It is also consistent with the known mitochondrial localization of methylmalonyl Co-A mutase, the only mammalian enzyme known to require adenosylcobalamin as coenzyme (Gurnani *et al*, 1960; Stadtman *et al*, 1960).

The nature of the unidentified cobalamin is uncertain. The guinea-pig has no foregut and gains its vitamin B_{12} from its food and by coprophagy so the unidentified cobalamin cannot have been made by bacteria in the animal's small intestine. The R_f differs from that of hydroxocobalamin in the chromatography system used here. Several naturally occurring vitamin B_{12} analogues are known but they are generally found only in micro-organisms (Lester Smith, 1965). The low R_f value suggests that the unidentified cobalamin is relatively polar,

FENRYCH, W., PAWELKIEWICZ, J. & MAGAS, S. (1962) Conversion of cyanocobalamin, in vivo, into its coenzyme form in the rabbit. *Bulletin de l'Academie Polonaise des Sciences*, **10**, 117.

GORNA, M. (1965) Enzymatzycne przeksztalcenie witaminung B_{12} io jej forme koenzymatyczna in vitro. *Rocziki Naukowe WSWF W Poznaniv*, **10**, 9.

GURNANI, S., MISTRY, S.P. & JOHNSON, B.C. (1960) Function of vitamin B_{12} in methylmalonate metabolism. I. Effect of a cofactor form of B_{12} on the activity of methylmalonyl-CoA isomerase. *Biochimica et Biophysica Acta*, **38**, 187.

HOFFBRAND, A.V., LINNELL, J.C., MATTHEWS, D.M. & PETERS, T.J. (1970) The forms of vitamin B_{12} in the ileal enterocyte of the guinea-pig during fasting and absorption of cyanocobalamin. (Abstract). *Journal of Physiology*, **208**, 66P.

LINDSTRAND, K. & STÅHLBERG, K.-G. (1963) On vitamin B_{12} forms in human plasma. *Acta Medica Scandinavica*, **174**, 665.

LINNELL, J.C., HOFFBRAND, A.V., PETERS, T.J. & MATTHEWS, D.M. (1969a) Estimation of individual plasma B_{12} compounds in normal subjects and patients with deranged B_{12} metabolism. (Abstract). *Journal of Clinical Pathology*, **22**, 742.

LINNELL, J.C., MACKENZIE, H.M. & MATTHEWS, D.M. (1969b) Normal values for individual plasma cobalamins. (Letter). *Journal of Clinical Pathology*, **22**, 506.

LINNELL, J.C., MACKENZIE, H.M., WILSON, J. & MATTHEWS, D.M. (1969c) Patterns of plasma cobalamins in control subjects and in cases of vitamin B_{12} deficiency. *Journal of Clinical Pathology*, **22**, 545.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. & RANDALL, R.J. (1951) Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry*, **193**, 265.

MAHLER, H.R. & CORDES, E.H. (1966) *Biological Chemistry*, p 224. Harper & Row, New York.

PARSONS, D.F., WILLIAMS, G.R. & CHANCE, B. (1966) Characteristics of isolated and purified preparations of the outer and inner membranes of mitochondria. *Annals of the New York Academy of Sciences*, **137**, 643.

PAWELKIEWICZ, J., BARTOSINSKI, B. & WALERYCH, W. (1960) A new class of derivatives of vitamin B_{12} group: cobalto-corrin compounds. *Bulletin de l'Academie Polonaise des Sciences*, **8**, 123.

PETERKOFSKY, A. & WEISSBACH, H. (1964) Enzymatic synthesis of coenzyme B_{12}. *Annals of the New York Academy of Sciences*, **112**, 622.

PETERS, T.J. & HOFFBRAND, A.V. (1970) Absorption of vitamin B_{12} by the guinea-pig. I. Subcellular localization of vitamin B_{12} in the ileal enterocyte during absorption. *British Journal of Haematology*, **19**, 369.

PETERS, T.J. & MACMAHON, M.T. (1970) The absorption of glycine and glycine oligopeptides by the rat. *Clinical Science*, **39**, 811.

PETERS, T.J., QUINLAN, A. & HOFFBRAND, A.V. (1971) Absorption of vitamin B_{12} by the guinea-pig. II. The subcellular localization of cyano-, methyl- and adenosyl-cobalamins and the effect of fluoroacetate on cyanocobalamin absorption. *British Journal of Haematology*, **20**, 123.

SMITH, E. LESTER (1965) *Vitamin B_{12}*, 3rd edn. Methuen, London.

STADTMAN, E.R., OVERATH, P., EGGERER, H. & LYNEN, F. (1960) The role of biotin and vitamin B_{12} coenzyme in proprionate metabolism. *Biochemical and Biophysical Research Communications*, **2**, 1.

TAKEYAMA, S. & BUCHANAN, J.M. (1961) Enzymatic synthesis of the methyl group of methionine. III. Spectral and electrophoretic studies of the prosthetic group of the B_{12} enzyme. *Journal of Biochemistry (Tokyo)*, **49**, 578.

TOOHEY, J.I. & BARKER, H.A. (1961) Isolation of coenzyme B_{12} from liver. *Journal of Biological Chemistry*, **236**, 560.

VITOLS, E., WALKER, G.A. & HUENNEKENS, F.M. (1966) Enzymatic conversion of vitamin B_{12}s to a cobamide coenzyme, α-(5,6-dimethylbenzimidazolyl deoxyadenosylcobamide) (adenosyl-B_{12}). *Journal of Biological Chemistry*, **241**, 1455.

ALL MISSING
PAGES ARE
BLANK
IN
ORIGINAL

BIBLIOGRAPHY

ABRAHAM, G.E. (1969) Solid-phase radioimmunoassay of estradiol-17β. J. clin. Endocr., 29, 866-870

ADDISON, T. (1855) On the constitutional and local effects of the suprarenal capsules. S. Highley, London

ANDERSON, B.B. (1964) Investigations into the Euglena method for the assay of vitamin B_{12} in serum. J. clin. Path., 17, 14-26

ANDERSON, B.B. (1965) Investigations into the Euglena method of vitamin B_{12}: the results obtained in human serum and liver using an improved method of assay. Ph.D. Thesis, University of London

ANDRAL, C.T. (1823) see CHANARIN, I. (1969) p.2

BARAKAT, R.M. and EKINS, R.P. (1961) Assay of vitamin B_{12} in blood: a simple method. Lancet, 2, 25-26

BARAKAT, R.M. and EKINS, R.P. (1963) An isotope method for the determination of vitamin B_{12} levels in blood. Blood, 21, 70-79

BARKER, H.A., WEISSBACH, H. and SMYTH, R.D. (1958) A coenzyme containing pseudo-vitamin B_{12}. Proc. nat. Acad. Sci. (Wash.), 44, 1093-1097

BAUER, H. and HEINRICH, H.C. (1961) Vitamin B_{12} metabolism in neurological disease. in: Chemical Pathology of the Nervous System Ed. J. Folch-Pi, Pergamon Press, Oxford pp.120-125

BAXTER, N., HORSFORD, J. and WOKES, F. (1953) Cyanocobalamin and hydroxocobalamin in vitamin B_{12} injections. J. Pharm. Pharmacol., 5, 723-736

BEAVEN, G.H., HOLIDAY, E.R., JOHNSON, E.A., ELLIS, B. and PETROW, V. (1950) The mode of linkage of component α in vitamin B_{12}. J. Pharm. Pharmacol., 2, 733-735

BIERMER, A. (1872) Uber eine form von progressiver pernicioser Anamie. Schweiz. Arzteztg., 2, 15

BIZZOZERO (1868) see WINTROBE, M.M. (1967) p.18

BOGER, W.R., WRIGHT, L.O., STRICKLAND, S.C., GYLFE, J.S. and CIMINIRA, J.L. (1955) Vitamin B_{12}: correlation of serum concentration and age. Proc. Soc. exp. Biol. (N.Y.), 89, 375-378

BOXER, G.E. and RICKARDS, J.C. (1952) Studies on the metabolism of the carbon of cyanide and thiocyanate. Arch. Biochem., 33, 7-26

BRAEKKAN, O., NJAA, L.R. and UTNE, F. (1957) The effect on liver reserves of vitamin B_{12}. Acta pharmacol. (Kbh.), 13, 228-232

BRAFIELD, A. (1966) Vitamin B_{12} assay. Lancet, 2, 966

CABOT, R.C. (1908) Pernicious and secondary anaemia, chlorosis and leukaemia. A System of Medicine Ed. W. Osler and T. McCrae, London, Vol.IV, Chapter 10

CAHN, A. and von MEHRING, J. (1886) Die Sauren des gesunden and Kranken Magens. Bt. Arch. Klin. Med., 39, 233-253

CARDINALE, G.J., DREYFUS, P.M., AULD, P. and ABELES, R.H. (1969) Experimental vitamin B_{12} deficiency, its effect on tissue vitamin B_{12} coenzyme levels and the metabolism of methylmalonyl CoA. Arch. Biochem., 131, 92-99

CASTLE, W.B. (1929) Observations on the etiologic relationship of achyla gastrica to pernicious anaemia. I Effect of administration to patients with pernicious anaemia of contents of normal human stomach recovered after ingestion of beef muscle. Amer. J. med. Sci., 178, 748-764

CHANARIN, I. (1969) The Megaloblastic Anaemias. Blackwell, Oxford and Edinburgh, p.580

CHISHOLM, I.A., BRONTE-STEWART, J. and FOULDS, W. S. (1967) Hydroxocobalamin vs. cyanocobalamin in the treatment of tobacco amblyopia. Lancet, 2, 450-451

CHRISTIAN, H.A. (1903) A sketch of the history of the treatment of chlorosis with iron. Med. Lib. & Hist. J., 1, 176

CIMA, L., LEVORATO, C. and MANTOVAN, R. (1967) Enzymatic decyanation of cyanocobalamin in rat tissues. J. Pharm. Pharmacol., 19, 32-40

CLARK, A. (1936) Report on affects of certain poisons contained in food plants of West Africa upon health in native races. J. trop. Med. Hyg., 39, 269-276 and 285-295

CLUSIUS, C. (1605) see MONTGOMERY, R.D. (1969)

COMBE, J.S. (1824) see CHANARIN, I. (1969) p.1

CONNOR, L. (1890) Tobacco amblyopia. J. Amer. med. Ass., 14, 217-225

COOKE, W.T., COX, E.V., GADDIE, R., MATTHEWS, D.M. and MEYNELL, M.J. (1959) The vitamin B_{12}, folic acid and ascorbic acid of bone marrow cells in megaloblastic anaemia of pregnancy. J. Physiol., 149, 36-37 P

DARWIN, C. (1832) The Voyage of the Beagle. Heron Books, London, p.23

De SCHWEINITZ, G.E. (1896) Toxic amblyopias Lea Brothers and Company, Philadelphia

DONY, J. and CONTER, J. (1956) A propos de l'absorption de la vitamine B_{12} par le talc. J. Pharm. Belg., 11, 338-346

DREYFUS, P.M. and DUBE, V.E. (1967) The rapid detection of methylmalonic acid in urine - a sensitive index of vitamin B_{12} deficiency. Clin. chim. Acta, 15, 525-528

DUKE-ELDER, W.S.D. (1954) A Textbook of Ophthalmology Vol. VI. Henry Kimpton, London, p.6838

EALES, H. (1887) Toxic amblyopia. Trans. ophthal. Soc. U.K., 7, 54-61

EHRLICH, P. (1880) Beobachtungen uber einen Fall von pernicioser progressiver Anamie mit Sarcombildung. - Beigrage zur Lehre von der acuten Herzinsufficienz. Verhandl. Gesellsch. charite Arzte. June 10 and Dec 9

EKINS, R.P. (1960) The estimation of thyroxine in human plasma by an electrophoretic technique. Clin. chem. Acta, 5, 453-459

EKINS, R.P. and SGHERZI, A.M. (1965) The microassay of vitamin B_{12} in human plasma by the saturation assay technique. in: Radiochemical Methods of Analysis, Vol. 2, International Atomic Energy Agency, Vienna, pp.239-256

FENWICK, S. (1870) On atrophy of the stomach. Lancet, 2, 78-80

FLINT, A. (1860) A clinical lecture on anaemia (delivered at Long Island College Hospital). Am. med. Times, 1, 181

FORD, J.E. and HOLDSWORTH, E.S. (1953) An improved bioautographic technique. Biochem. J., 53, xxii-xxiii

FORD, J.E. and HOLDSWORTH, E.S. (1954) Biosynthetic relationships between vitamin B_{12}-like factors. Biochem. J., 56, xxxv

FOULDS, W.S., CANT, J.S., CHISHOLM, I.A., BRONTE-STEWART, J. and WILSON, J. (1968) Hydroxocobalamin in the treatment of Leber's hereditary optic atrophy. Lancet, 1, 896-897

FOULDS, W.S., CHISHOLM, I.A., BRONTE-STEWART, J. and WILSON, J. (1969) Cyanide induced optic atrophy. Third Congress of the European Society of Ophthalmology, Amsterdam, 1968. Ed. J. François. S. Karger, Basel

FOWLER, R.C. and DURBETAKI, A.J. (1952) Cyanide and thiocyanate determination in multiple sclerosis and allied demyelinizing disorders. Amer. J. Physiol., 171, 724

FRASER, T.R. (1894) Bone-marrow in the treatment of pernicious anaemia. Brit. med. J., 1, 1172-1174

FREEMAN, A.G. and HEATON, J.M. (1961) The aetiology of retrobulbar neuritis in Addinsonian pernicious anaemia. Lancet, 1, 908

FRENKEL, E.P., KELLER, S. and McCALL, M.S. (1966) Radioisotopic assay of serum vitamin B_{12} with the use of DEAE cellulose. J. Lab. clin. Med., 68, 510-522

FRIEDNER, S., JOSEPHSON, B. and LEVIN, K. (1969) Vitamin B_{12} determination by means of radioisotopic dilution and ultrafiltration. Clin. chim. Acta, 24, 171-179

GIBSON, R.B. and HOWARD, C.P. (1923) Metabolic studies in pernicious anaemia. Arch. intern. Med., 32, 1-16

GIRDWOOD, R.H. (1960) Microbiological methods of assay in clinical medicine with particular reference to the investigation of deficiency of vitamin B_{12} and folic acid. Scot. med. J., 5, 10-15

GOODMAN, S.I., MOE, P.G., HAMMOND, K.B., MUDD, S. H. and UHLENDORF, B.W. (1970) Homocystinuria with methylmalonic aciduria: two cases in a sibship. Biochem. Med., 4, 500-515

GORSUCH, T.T. (1968) Radioactive isotope dilution analysis RCC Review, No.2. The Radiochemical Centre, Amersham

GUNN, M. (1887) Toxic amblyopia. Trans. ophthal. Soc. U.K., 7, 67-71

GUTTERIDGE, J.M.C. and WRIGHT, E.B. (1970) A simple and rapid thin-layer chromatograph technique for the detection of methylmalonic acid in urine. Clin. chim. Acta, 27, 289-291

HABERSHON, S.O. (1863) On idiopathic anaemia. Lancet, 1, 518-519 and 551-553

HALL, C.A. (1966) Vitamin B_{12} assay. Lancet, 2, 862

HAMILTON, H.E., ELLIS, P.P. and SHEETS, R.F. (1959) Visual impairment due to optic neuropathy in pernicious anaemia: report of a case and review of the literature. Blood, 14, 378-385

HEATON, J.M., McCORMICK, A.J.A. and FREEMAN, A.C. (1958) Tobacco amblyopia: a clinical manifestation of vitamin B_{12} deficiency. Lancet, 2, 286-290

HOFFBRAND, A.V., TRIPP, E. and DAS, K.C. (1973) Uptake of vitamin B_{12} by phytohaemagglutinin-transformed lymphocytes. Brit. J. Haemat., 24, 147-156

HUSER, H-J. (1966) A note on Biermer's anaemia. Med. Clin. N. Amer., 50, 1611-1626

HUTNER, S.H., BACH, M.K. and ROSS, G.I.M. (1956) Sugar-containing basal medium for vitamin B_{12} assay with Euglena: application to body fluids. J. Protozool., 3, 101-112

JOHANNESSON, J.K. (1961) Radiochemical determination of microgram amounts of chloride. Analyst, 86, 72

JOHNSTONE, R.A.W. and PLIMMER, J.R. (1959) The chemical constituents of tobacco smoke. Chem. Rev., 59, 885-936

KILLANDER, A. (1957) The assay of vitamin B_{12} in human serum. Acta Soc. Med. upsalien., 62, 39-59

LANG, K. (1933) Die Rhodanbildung im Tierkorpor. Biochem. Z., 259, 243-256

LAU, K.S., GOTTLIEB, C., WASSERMAN, L.R. and HERBERT, V. (1965) Measurement of serum vitamin B_{12} level using radioisotope dilution and coated charcoal. Blood, 26, 202-214

LEVY, H.L., MUDD, S.H., SCHULMAN, J.D., DREYFUS, P.M. and ABELES, R.H. (1970) A derangement in B_{12} metabolism associated with homocystinaemia, cystathioninaemia, hypomethioninaemia, and methylmalonic aciduria. Amer. J. Med., 48, 390-397

LINDSTRAND, K. (1964) Isolation of methylcobalamin from natural source material. Nature (Lond.), 204, 188-189

LINDSTRAND, K. and STAHLBERG, K-G. (1963) On forms of vitamin B_{12} in plasma. Acta med. scand., 174, 665-669

LINDSTRAND, K., WILSON, J. and MATTHEWS, D.M. (1966) Chromatography and microbiological assay of vitamin B_{12} in smokers. Brit. med. J., 2, 988-990

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. and RANDALL, R.J. (1951) Protein measurement with the Folin phenol reagent. J. biol. Chem., 193, 265-275

MAHONEY, M.J., ROSENBERG, L.E., MUDD, S.H. and UHLENDORF, B.W. (1971) Defective metabolism of vitamin B_{12} in fibroblasts from children with methylmalonic aciduria. Biochem. biophys. Res. Commun., 44, 375-381

MARTIN, A.J.P. and SYNGE, R.L.M. (1941) A new form of chromatogram employing two liquid phases. Biochem. J., 35, 1358-1368

MATTHEWS, D.M. (1961) Retrobulbar neuritis in Addisonian pernicious anaemia. Lancet, 1, 1289-1290

MATTHEWS, D.M. (1962) Observations on the estimation of serum vitamin B_{12} using L. leishmannii. Clin. Sci., 22, 101-111

MATTHEWS, D.M. and WILSON, J. (1971) Cobalamins and cyanide metabolism in neurological diseases. in: The Cobalamins: a Glaxo Symposium. Ed. H.R.V. Arnstein and R.J. Wrighton, Churchill Livingstone, Edinburgh and London

MATTHEWS, D.M., WILSON, J. and ZILKHA, K.J. (1965) Cyanide metabolism and vitamin B_{12} in multiple sclerosis. J. Neurol. Neurosurg. Psychiat., 28, 426-428

MENGHINI (1746) see CHRISTIAN, H.A. (1903)

MIDGLEY, A.R., NISWANDER, G.D. and RAM, J.S. (1969) Hapten-radioimmunoassay: A general procedure for the estimation of steroidal and other haptenic substances. Steroids, 13, 731-737

MINOT, G.R. and MURPHY, W.P. (1926) Treatment of pernicious anaemia by a special diet. J. Amer. med. Ass., 87, 470-476

MOLLIN, D.L. and ROSS, G.I.M. (1952) The vitamin B_{12} concentrations of serum and urine of normals and of patients with megaloblastic anaemia and other diseases. J. clin. Path., 5, 129-139

MONOKOSSO, G.L. and WILSON, J. (1966) Plasma thiocyanate and vitamin B_{12} in Nigerian patients with degenerative neurological disease. Lancet, 1, 1062-1064

MONTGOMERY, R.D. (1969) Cyanogens. in: Toxic Constituents of Plant Foodstuffs, Academic Press, N.Y. p.144

MOORE, D.G.F. (1934) Retrobulbar neuritis and partial optic atrophy as sequelae of avitaminosis. Ann. Trop. Med. Parasit., 28, 295-303

MORROW, G. III, BARNESS, L.A., CARDINALE, G.J., ABELES, P.H. and FLAKS, J.G. (1969) Congenital methylmalonic aciduria: enzymatic evidence for two forms of the disease. Proc. nat. Acad. Sci. (Wash.), 63, 191-197

MUDD, S.H., LEVY, H.L. and ABELES, R.H. (1969) A derangement in B_{12} metabolism leading to homocystinemia, cystathioninemia and methylmalonic aciduria. Biochem. biophys. Res. Commun., 35, 121-126

MUDD, S.H., LEVY, H.L. and MORROW, G. III (1970) Deranged B_{12} metabolism: effects on sulphur amino acid metabolism. Biochem. Med., 4, 193-214

MUDD, S.H., UHLENDORF, B.W., HINDS, K.R. and LEVY, H.L. (1970) Deranged B_{12} metabolism: studies of fibroblasts grown in tissue culture. Biochem. Med., 4, 215-239

MURPHY, B.P., ENGELBERG, W. and PATTEE, C.J. (1963) Simple method for the determination of plasma corticosteroids. J. clin. Endocr., 23, 293-300

MUSHETT, C.W., KELLEY, K.L., BOXER, G.E. and RICKARDS, J.C. (1952) Antidotal efficacy of hydroxocobalamin in experimental cyanide poisoning. Proc. Soc. exp. Biol. (N.Y.), 81, 234-237

MYASISHCHEVA, N.V., ARESHKINA, L.Ya, KUTZEVA, L.S. and SKOROBOGATOVA, E.P. (1968) Cobalamin compounds and folic acid derivatives in human blood serum. Vop. med. Khim., 14, 273-277

NETTLESHIP, E. (1887) Toxic amblyopia. Trans. ophthal. Soc. U.K., 7, 36-53

NEUMANN (1868) see WINTROBE, M.M. (1967) p.18

OSBORNE, J.S., ADAMEK, S. and HOBBS, M.E. (1956) Some components of the gas phase of cigarette smoke. Analyt. Chem., 28, 211-215

OSLER, W. and GARDNER, W. (1877) On the changes in marrow in progressive pernicious anaemia. Canad. med. surg. J., 5, 385

OSUNTOKUN, B.O., DUROWOJN, J.E., McFARLANE, H. and WILSON, J. (1968) Plasma amino acids in the Nigerian nutritional ataxic neuropathy. Brit. med. J., 2, 647-649

OXNARD, C.E. and SMITH, W.T. (1966) Neurological degeneration and reduced serum vitamin B_{12} levels in captive monkeys. Nature (Lond.), 210, 507-509

PEGRAM, R.G. (1969) The microbiological use of 2,3,5-triphenyl tetrazolium chloride. J. med. Lab. Technol., 26, 175-198

RAVEN, J.L. and BARKHAN, P. (1966) Vitamin B_{12} assay. Lancet, 2, 1078

RAVEN, J.L., ROBSON, M.B., WALKER, P.L. and BARKHAN, P. (1969) Improved method for measuring vitamin B_{12} in serum using intrinsic factor, ^{57}Co B_{12} and coated charcoal. J. clin. Path., 22, 205-211

RAVEN, J.L., WALKER, P.L. and BARKHAN, P Comparison of the radioisotope dilution-coated charcoal method and a microbiological method. J. clin. Path., 19, 610-613

RICKES, E.L., BRINK, N.G., KONIUSZY, F.R., WOOD, T.R. and FOLKERS, K. (1948) Crystalline vitamin B_{12}. Science N.Y., 107, 396-397

ROTHENBERG, S.P. (1961) Assay of serum vitamin B_{12} concentration using ^{57}Co B_{12} and intrinsic factor. Proc. Soc. exp. Biol. (N.Y.), 108, 45-48

ROTHENBERG, S.P. (1963) Radioassay of serum B_{12} by quantitating the competition between ^{57}Co B_{12} and unlabelled B_{12} for the binding sites of intrinsic factor. J. clin. Invest., 42, 1391-1398

SCHILLING, R.F. and MEYER, O.O. (1964) Incorporation of vitamin B_{12} into immature erythrocytes. Trans. Ass. Amer. Phycns., 77, 79-87

SHAW, W.H.C. and BESSELL, C.J. (1960) The determination of vitamin B_{12}. Analyst, 85, 389-409

SHINTON, N.K. (1959) Total serum vitamin B_{12} concentration in normal adult serum assayed by Euglena gracilis. Clin. Sci., 18, 389-398 .

SMITH, A.D.M. (1961) Retrobulbar neuritis in pernicious anaemia. Lancet, 1, 1001-1002

SMITH, A.D.M. and DUCKETT, S. (1965) Cyanide, vitamin B_{12}, experimental demyelination and tobacco amblyopia. Brit. J. exp. Path., 66, 615-622

SMITH, E. Lester (1965) Vitamin B_{12}. Methuen and Company Limited, London, p.114

SMITH, E. Lester and PARKER, L.F.J. (1948) Purification of anti-pernicious anaemia factor. Biochem. J., 43, viii

SNOW, C.C. (1967) Some observations on the growth and development of the baboon. in: The Baboon in Medical Research, Vol. II Ed. H. Vagtborg, University of Texas, pp. 187-199

SØRENSEN, S.T. (1874) Taellinger af blodlegemer i 3 Tilfalde af excessiv oligocythaemi. Hospitals-Tidende R 2.1 Aargang-513

SPRAY, G.H. (1955) An improved method for the rapid estimation of vitamin B_{12} in serum. Clin. Sci., 14, 661-667

SPRAY, G.H. (1962) The estimation and significance of the level of vitamin B_{12} in serum. Postgrad. med. J., 38, 35-40

SPRAY, G.H. (1966) Personal communication

SPRAY, G.H. and WITTS, L.J. (1958) Results of 3 years' experience with microbiological assay of vitamin B_{12} in serum. Brit. med. J., 1, 295-298

STAHL, E. (1965) Thin layer chromatography Springer-Verlag, Berlin and Heidelberg p.488

STAHLBERG, K-G. (1964) Forms of plasma B_{12} in health and in pernicious anaemia, chronic myeloid leukaemia and acute hepatitis: A preliminary report. Scand. J. haemat., 1, 220-222

STÅHLBERG, K-G. (1967) Studies on methyl-B_{12} in man. Scand. J. haemat. Supplementum No.1

SURGEON-GENERAL, United States (1964) Report on smoking in health. Public Health Service Publication No.1103, U.S. Government Printing Office, Washington, D.C.

TIBLING, G. (1969) A method for determination of vitamin B_{12} in serum by radioassay. Clin. chim. Acta, 23, 209-218

TOOHEY, J.I. and BARKER, H.A. (1961) Isolation of coenzyme B_{12} from liver. J. biol. Chem., 236, 560-563

TORRES, I., SMITH, N.W.T. and OXNARD, C. (1969) Degeneration of the peripheral and central nervous system in vitamin B_{12} deficient monkeys. Experientia (Basel), 25, 273-275

TRAQUAIR, H.M. (1930) Toxic amblyopia including retro-bulbar neuritis. Trans. ophthal. Soc. U.K., 50, 351-385

VICTOR, M. (1963) Tobacco alcohol amblyopia. Arch. Ophthal., 70, 73-78

WATERS, A.H. and MOLLIN, D.L. (1961) Studies and the folic acid activity of human serum. J. clin. Path., 14, 335-344

WHIPPLE, G.H. and ROBSCHEIT-ROBBINS, F.S. (1925) Blood regeneration in severe anaemia II Favorable influence of liver, heart and skeletal muscle in diet. Amer. J. Physiol., 72, 408-418

WILSON, J. and LANGMAN, M.J.S. (1966) Relation of sub-acute combined degeneration of the cord to vitamin B_{12} deficiency. Nature (Lond.), 212, 787-789

WILSON, J. and MATTHEWS, D.M. (1966) Metabolic inter-relationships between cyanide, thiocyanate and vitamin B_{12} in smokers and non-smokers. Clin. Sci., 31, 1-7

WILSON, J. and MATTHEWS, D.M. (1970) Recent research in some optic neuropathies. Trans. ophthal. Soc. U.K., 90, 733-737

WINTROBE, M.M. (1967) Clinical Hematology Sixth edition, Henry Kimpton, London, p.18

WOKES, F. (1958) Tobacco amblyopia. Lancet, 2, 526-527

WOKES, F., BADENOCH, J. and SINCLAIR, H.M. (1955) Human dietary deficiency of vitamin B_{12}. Amer. J. clin. Nutr., 3, 375-382

WOKES, F. and PICARD, C.W. (1955) The role of vitamin B_{12} in human nutrition. Amer. J. clin. Nutr., 3, 383-390

YALOW, R.S. and BERSON, S.A. (1960) Immunoassay of endogenous plasma insulin in man. J. clin. Invest., 39, 1157-1175