METHODS IN HEMATOLOGY

THE COBALAMINS

Edited by

CHARLES A. HALL

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Charles A. Hall MD

Director, Nutrition Laboratory for Clinical Assessment and Research,
Veterans Administration Medical Center, Albany, New York, USA

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The Cobalamins

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Preface

This is a book of measurements, an essential process of both science and medicine. In their presentations of techniques, the contributors give concisely a great deal about the metabolism of cobalamin (Cbl, vitamin B_{12}) and the disturbances that produce disease. A reader who seeks a specific method would do well to read beyond it. Basic information that helps to understand a technique may be given, with differing orientations, in several places in the book and cross references are provided. Some processes are common to several methods and here too, cross references are given where appropriate. Each chapter will describe methods in enough detail to permit the reader to perform, understand and apply them, but he will benefit from other parts of the book for additional background information.

The book is divided into sections, each of which brings together a group of techniques used in the study of one phase of Cbl or its metabolism. They are: Form and content of Cbl, Transport, Cell entry and Enzymatic activity. Each section contains an introductory chapter which assists the reader in finding information and provides cross references. It is impossible to predict research needs, but the techniques are there for the investigator to choose from and to combine in order to meet specific objectives. Chapter 12 was written to assist the clinician in applying combinations of the several techniques in patient care.

I wish to express my appreciation to members of my department who helped in the editorial process. Mrs Marie Stoddard performed all of the many secretarial chores. Mr James A. Begley, in addition to preparing his own contribution, read the entire manuscript and provided invaluable advice. Other colleagues examined the description of individual methods and I am indebted to many of them, past as well as present, who developed superior methods, making it possible for me to edit a book of this type. The Medical Media Production Service of the Albany Veterans Administration Medical Center reviewed all of the illustrations and when necessary redrew, photographed or otherwise modified them. I wish also to thank the authors of the individual chapters who all made sacrifices in time and effort in preparation of their contributions. All of them could have written on techniques other than those presented in their chapter. Some, in fact, did exchange contributions to assist in the review process.

1983 C.A.H.

Contributors

Barbara Anderson MD
Department of Haematology, St Bartholomew's Hospital, London, UK

Regula Baumgartner MD
Metabolic Unit, University Childen's Hospital, Basel, Switzerland

William Beck MD
Department of Medicine, Massachusetts General Hospital, Boston, Massachusetts, USA

James A. Begley MS
Nutrition Laboratory for Clinical Assessment and Research, Veterans Administration Medical Center, Albany, New York, USA

I. Chanarin MD, FRCPath
Northwick Park Hospital and Clinical Research Centre, London, UK

Bernard Cooper MD
Department of Hematology, Royal Victoria Hospital, Montreal, Quebec, Canada

Marijke Fráter-Schröder MD
Universitats-Kinderklinik, Zurich, Switzerland

Peter Gimsing MD
Strandøre 13, Copenhagen, Denmark

Ralph Gräsbeck MD
Minerva Foundation, Helsinki, Finland

Charles A. Hall MD
Director, Nutrition Laboratory for Clinical Research and Assessment, Veterans Administration Medical Center, Albany, New York, USA

Ilkka Kouvonen MD
Minerva Foundation, Helsinki, Finland

Michael Muir
Northwick Park Hospital and Clinical Research Centre, London, UK

Ebba Nexø MD
Department of Clinical Chemistry, Bispebjerg Hospial, Copenhagen, Denmark

Nefertiti Sourial MD
St Bartholomew's Hospital, London, UK

S. N. Wickramasinghe MD
Department of Haematology, St Mary's Hospital Medical School, University of London, London, UK

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SECTION 1

THE FORM AND CONTENT OF COBALAMIN

INTRODUCTION

Charles A. Hall

These four chapters describe the identification and measurement, either individually or as total cobalamin (Cbl), of the several chemical forms of Cbl found in biological materials. A measure of Cbl content is often the final step in both clinical evaluations and in research. The opening chapter is a fitting introduction to the book because the form of Cbl has a bearing on almost every measurement in the field whether it be of serum Cbl, binding reactions, entry into cells, or enzymatic reactions. Also presented are the structure of CN-Cbl, Figure 1.1, and a well referenced chronology of events in the study of Cbl, Table 1.1. There follows, Chapters 2–4, an analysis of the assay of the total Cbl component of serum or of other substances and the presentation of three methods.

The forms of Cbl, chapter 1

The form of a Cbl determines its function and the information given here relates closely to the techniques of Chapters 9–11, the measurements of the enzymatic reactions requiring MeCbl or AdoCbl. There are, thus, two complementary approaches to the measure of Cbl as a coenzyme, measurement of the form and measurement of the activity of the holoenzyme requiring that form.

Analogues of Cbl are currently prominent in the literature and it may appear to be an omission that techniques for measuring them are not presented here. There are no distinct methods for measuring analogues. The term 'analogue' as used here implies a corrinoid other than a cobalamin. The designation has no great meaning unless the nature and properties of the substance are known. The techniques devised by biologists to identify corrinoids, presented in Chapter 1, were appropriately those that separated substances found in living systems. But as Figures 1.3, 1.5, and 1.6 show, the number of substances that can be separated and identified is limited. Two substances may chromatograph the same and chromatography of a standard solution of the substance to be identified is essential. Methods useful to the organic chemist often are not sensitive to the small amounts in biological materials. In spite of the growing need to measure analogues native to tissues, the necessary techniques have not been developed. There are some ingenious and valuable methods for revealing the presence or 'footprints' of non-Cbl corrinoids, but they do not identify them.

The form and content of preparations of radioactive Cbl

Often the measure of some phase of Cbl metabolism is achieved through labeled Cbl. For the clinical laboratory the specific activity and Cbl content as stated by the supplier are usually adequate. A further evaluation of content may, however, be necessary in research and two consequences of impurity of CN-Cbl are illustrated in Figures 6.1B and 7.2A. Misinterpretations might have followed had the presence of impurities not been known.

We have established the following routine for the evaluation of every shipment of CN [^{57}Co] Cbl. A volume (a drop if activity is high) of the stock that will give at least 50 000 cts/min is analyzed by SP Sephadex as in Chapter 1, illustrated in Figure 1.5. Purity is checked at monthly intervals until the stock has been consumed. A recent shipment, for example. contained respectively 96.5 per cent, 97.2 per cent, 97.5 per cent, and 97.4 per cent CN [^{57}Co] Cbl over a three month period. The principal contaminent was OH [^{57}Co] Cbl, 1.5–1.6 per cent. The need for this check on purity is evident from the rare shipment that may be contaminated. One shipment, for example, contained 57 per cent CN [^{57}Co] Cbl, 16 per cent HSO_3 [^{57}Co] Cbl and what seemed to be 27 per cent Ado [^{57}Co] Cbl.

For certain measurements a form of labeled Cbl other than CN-Cbl may be required, e.g. the standardization of chromatography of Cbl, Chapter 1. Once converted, the product is also evaluated by SP Sephadex chromatography.

The Cbl content of each new shipment is measured by the *E. gracilis* assay, Chapter 3. The dilution of the Cbl for the assay is determined from the Cbl content as stated by the supplier. The specific activity is then calculated from the measured content of Cbl and the radioactivity.

The content of Cbl, chapters 2–4

Chapter 2 is really two chapters giving first the history and principles of Cbl assays and then the details of what is the best evaluated and probably the most suitable assay for multiple purposes. There follows a thorough discussion of the interpretations of assays of serum Cbl. Included are sections on reference values, the influence of serum binding of Cbl, the physiologic significance of serum Cbl levels and the influence of the forms of the Cbl in serum on the assay. All of this information is equally pertinent to the contents of the next two chapters, 3 and 4, which present two additional assays. Probably all assays now recommended for general use respond to the same substance in serum. Therefore, the significance and interpretation of all assays will be the same. Certain processes such as the extraction of the Cbl from serum, the influence of binding in serum, and the preparation of standards are common to all assays.

Chapter 3 presents a second bioassay which shares many properties of that of Chapter 2. The *Euglena gracilis* assay is considered by some to be the preferred reference assay. Chapter 4 presents two radioisotope dilution assays (RIDA) and some of the factors that might influence a choice are given in Chapter 2. The basic RIDA presented here has unusual versatility in that two different binding agents can be inserted into what is otherwise one assay.

The units designating serum Cbl are not uniform in the three presentations, but conversions are simple. The older expression is in pg/ml, in itself non-standard since the common unit of volume in clinical chemistry has traditionally been 100 ml or dl. Ng/l is currently preferred and does not require a new numerical value since

1 ng = 1 × 10^3 pg and 1 l = 1 × 10^3 ml. Molar units as in pmol/l are useful when dealing with Cbl binding proteins and coenzymes, but create confusion for clinical applications. Cbl levels in these units are, nevertheless, given for cross reference.

Certainly the measure of the Cbl content of serum is and will continue to be the most widely applied measure of the amount of Cbl, but there may also be a need to measure Cbl in other biological materials or as a reagent in stock solutions. The techniques described in Chapters 2–4 were devised for measurement of the Cbl in serum. All are readily adaptable to aqueous solutions of CN-Cbl. They can be applied with caution to cells, tissues, other body fluids or to fractions of those substances. Common problems in adaptation include failure to release all of the Cbl as with milk, interference with an isotope dilution assay by binding proteins from the source as with granulocytes and saliva, and interference from salts used in fractionation. The user must then be certain that (1) the extraction procedure frees all of the Cbl, (2) the final form of the Cbl is one to which the assay responds, (3) there are no residual binding proteins from the source to interfere with the assay, (4) the techniques of preparation do not introduce interfering substances. The potential user for substances other than serum can consult the literature for his specific application of a given assay. If there are no guidelines, he must make his own evaluation.

There is currently much controversy over the choice of assay of serum Cbl and the following chapters provide assistance to the reader. As will be noted in the description of the four assays, attention to detail is essential. In the development of an assay each step must be analyzed and as an assay is performed, each step in the technique must be performed as described. An assay should not be adopted unless the developer of it can show, through reports in the scientific literature, that the technique was meticulously designed and thoroughly tested. Even a modification, commercial or otherwise, of a well-designed assay should not be used unless it can be shown that the modifications do not impair the reliability of the assay. Ideally there follows a long period of use of the assay under field conditions. Ten years were required to uncover the defects of some of the many isotope dilution assays and time will be required before the reliability of the generation now being developed and promoted is known. It is disturbing that assays of Cbl of all types do not perform nearly as well under routine conditions as in reference laboratories.[1]

To be more specific, I would recommend a bioassay for research applications and this would include population surveys. That based on *Euglena*, Chapter 3, may be slightly more specific and somewhat more sensitive than assay with *Lactabacillus leichmannii* (L.l), Chapter 2, but the former requires a special incubator and is slower. Both are much simpler to perform and less subject to interference than is stated by their detractors who in most instances have never used one. I cannot at this time recommend an assay for the average clinical service laboratory; the kind of simplicity demanded for routine work in large volumes has not been achieved. The reliability of the 'simple' assays is unknown and the known assays are too complex to be acceptable. For now the assays should be performed in reference laboratories and the choice should be whatever the specific laboratory can adapt the most readily. Either the L.l bioassay or a well designed and tested isotope dilution will do.

Anyone who was introduced to the field of Cbl assays by the literature of the last four years should be wary of the imprecision of language and the carelessness of thought that he will find there. First the language. Some assays are said to measure 'analogues'

and others measure 'true Cbl'. Cbl assays measure Cbl content and not form. The nature of the corrinoids present can influence the values obtained for content by any assay, but specific nature is not determined by the assay. Some assays are said to measure 'biologically active cobalamins'. Only bioassays can determine this property and only for the one organism used for the assay. Carelessness of thought include the following. Lower values are assumed to be 'truer'. Assays of any sort, including those of Cbl, can give falsely low values. Failure to destroy the Cbl binding proteins of the serum, an easily induced error of technique, will give low values in isotope dilution assays.[2] Evidence is appearing that the new generation of isotope dilution assays can mislead through low values.[3] One encounters the assumption that when two assays give the same values, they are measuring the same thing. It should be obvious that the similarity may be coincidence, both assays may be in error or opposing errors in one may cancel out.[4] Finally there is the simplistic assumption that if the form of corrinoid being assayed is properly addressed, an assay will perform well. Many factors must be considered as given in Chapters 2–4, and in this discussion.

At some time in the future it may become possible to enhance the value of serum assays of Cbl by measuring the amount of specific forms. As discussed in Chapter 1, there is in Cbl deficiency a greater reduction of MeCbl in plasma than of AdoCbl and there are peculiarities of pattern in other disorders. Measurement of the amount carried on a specific plasma carrier such as TC II may be more specific than assay of total serum Cbl. These refinements are not, however, technically ready for large scale use.

REFERENCES

1 Mollin D L, Hoffbrand A V, Ward P G, Lewis S M 1980 Inter-laboratory comparison of serum vitamin B_{12} assay. Journal of Clinical Pathology 33: 243–248
2 Hall C A 1977 The carriers of native vitamin B_{12} in normal human serum. Clinical Science and Molecular Medicine 53: 453–457
3 Schilling R F, Fairbanks V R, Miller R, Schmitt K, Smith M J 1982 Improved vitamin B_{12} assays: A report from the field. Blood 60 Suppl 1: 32a (Abstract 52)
4 Begley J A, Hall C A 1981 Forms of vitamin B_{12} in radioisotope dilution assays. Journal of Clinical Pathology 34: 630–636

1
The forms of cobalamin in biological materials

P. Gimsing E. Nexø

INTRODUCTION

Vitamin B_{12} (CN-Cbl) was isolated and crystallized in the forties.[1,2] The therapeutic effect of the vitamin on Cbl deficiency was established simultaneously,[3] but it was soon settled that the cyanide form was a result of the isolation procedure. Two naturally-occurring, active Cbls — AdoCbl and MeCbl — were isolated and characterized from both microbiological and human material;[4,5] both are light sensitive and are rapidly converted to another Cbl form, OH-Cbl. After the discovery of these Cbl forms numerous studies have been performed to elucidate the biochemistry of the Cbl in human as well as in other organisms, and some of the events are summarized in Table 1.1.

The methods employed for these studies are complex and because of this, analysis of the forms of Cbl has been performed only in a few laboratories. Its main merit from a clinical point of view has, so far, mainly been a help to clarify the defects in patients with rare inborn errors in the conversion of Cbl to the active forms. In recent years simplified methods have been developed and this, in connection with the increased demand for refined analysis of the Cbl, especially in plasma and blood cells, warrants the present chapter. We will summarize our present knowledge concerning Cbls and corrinoids in health and disease and we will describe and discuss methods applicable for the study of these subjects. A detailed account of the function of the different Cbls is outside the scope of the present chapter but will be discussed in Chapters 9 and 10.

DEFINITION OF TERMS

Cbl (Fig. 1.1) consists of a skeleton of corrin with a 5,6-dimethylbenzimidazole group attached to the corrin ring via alfa-D-ribofuranose and to the central cobalt atom as the lower ligand. The upper ligand usually determines the type of Cbl. According to the IUPAC-IUB Commission (1974)[6] and abbreviations indicated,[7] the nomenclature outlined in Table 1.2 will be employed.

The state of oxidation of the cobalt atom is indicated for the reduced forms as Cbl I and Cbl II, formerly known as B_{12s} and B_{12r} respectively. Labeled, Cbl, for example ^{57}Co labeled CN-Cbl, Co-alfa-[alfa-(5,6-dimethylbenzimidazolyl)]-Co-beta-cyano-(^{57}Co)-cobamide is indicated CN[^{57}Co]Cbl.