THE CLINICAL APPLICATION OF HORMONE ASSAY

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FOREWORD

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> " When you can measure what you are speaking about and express it in numbers, you know something about it, but when you cannot measure it, when you cannot express it in numbers, your knowledge is of a meagre and unsatisfactory kind."—LORD KELVIN.

MANY of the most active physicians are rather specially interested in the glands of internal secretion. It is fascinating to think that these small specialised organs should produce such widespread effects and that some of their secretions should be associated with the strongest human emotions. Various dramatic diseases are directly due to derangements of these glands, and most diseases affect them to some extent. Methods of assessing their activities have therefore attracted widespread interest. Direct observations of their effects on the patients themselves can sometimes provide all the information that is needed, but such observations are often not enough, and an army of enthusiastic research workers has therefore sought to devise methods of estimating hormones and their metabolites in the blood and urine.

Various methods of diagnosing pregnancy by biological tests for gonadotrophins in urine were shown to give reliable results without elaborate precautions. It was natural to suppose that similar simple tests for other hormones would also give information of clinical importance but the results have, on the whole, been confusing. Some of the reasons for this have now become apparent. The pregnancy tests are successful because pregnancy causes enormous changes in the hormones in the urine, and quantitative estimates of the amounts present are unnecessary for the diagnosis. For other purposes quantitative results are needed and it is only lately that adequate quantitative methods have been devised.

It was thought at one time that it would be possible to estimate the total activity of various kinds in blood or urine. For example, urine was known to contain several substances which produced œstrogenic effects in small animals. Extracts of urine were therefore injected into mice and the smallest quantity to produce œstrus was called a mouse unit, so that the œstrogenic activity of urine could be estimated in mouse units per litre. There are several reasons why this convenient device gave results which were not reproducible. Since mice were found to vary, the dose for the average mouse was measured. Since one colony of mice gave different results from another and the same colony gave different results on different occasions, standard preparations of œstrogens were used in each

experiment, but even this precaution was not enough. Human urine contains a number of different substances with œstrogenic effects and the ratios of their activities vary enormously according to the exact design of the experiment. By one technique, for example, œstrone is one hundred times as active as œstriol and by another technique the activities of these two substances are about equal. If one of these substances were used as a standard to test urines whose activity was mostly due to the other substance the results in one laboratory might be one hundred times as large as the results in another. Such findings convinced those concerned that the measurement of the total activity of an extract had no real meaning, and that progress could only come from the estimation of the concentration of each of the substances contributing to the effect. This can be achieved by first separating the extract into fractions, each containing one active substance, and then estimating the concentration in each fraction by bio-assay, using a standard preparation of the appropriate substance, but in the case of the œstrogens in urine quicker and more precise results can be obtained by applying colorimetric methods to the fractions. The important point is that it is necessary to estimate the concentration of single substances and not the total activity ; this is true not only of the œstrogens but of all assays.

Chemical methods of assay are replacing biological methods. They are generally quicker and more precise and, if they can be shown to give an estimate of the concentration of a single substance, this estimate is just as valuable as a biological estimate. Some biological methods are still more sensitive than the best chemical methods, but the latter have improved much recently. Some of the simpler hormones can now be estimated in any department of clinical chemistry which has enough trained staff, but each assay involves much work. There is no immediate likelihood that it will be possible to estimate all the polypeptide hormones by simple chemical procedures, but some of them can be estimated by their effects on isolated tissues or tissue slices, and it is probable that the use of whole animals for hormone assays will eventually be abandoned.

Some hormone assays are an aid to diagnosis and a guide to treatment and can be regarded as routine procedures which should be generally available. For example, estimates of human chorionic gonadotrophin may assist in the diagnosis of pregnancy, chorion-epithelioma or hydatidiform mole ; and estimates of sympathomimetic amines may assist in the diagnosis of adrenal medullary tumours. The meaning of estimates of other hormones however is often not immediately obvious, and the purpose of most assays is to advance knowledge rather than to guide the doctor in the treatment of individual cases.

It is still important that researches in this field should be carefully planned with full knowledge of the limitations of the methods used, most of which are laborious and costly, and that the programme

should not be seriously interrupted by demands for isolated observations on interesting cases not connected with the main investigation. It has sometimes happened in the past that clinicians have employed biochemists to estimate hormones by some published method, and that the biochemists have been persuaded to earn their living by using methods in which they have no faith, in researches which seem to them to be badly planned. Such arrangements have been unfruitful.

Attempts have been made to establish central laboratories to carry out hormone assays for the doctors in a district. Such laboratories have at present only a limited use in connection with such things as pregnancy diagnosis. Their scope may widen with the advance of knowledge, but there is a danger that much of the money and effort spent on such laboratories may be wasted on unnecessary assays.

It is probable that all necessary hormone assays will eventually become part of the routine work of departments of clinical chemistry, but in the immediate future real progress is most likely to come from physicians working in the laboratories on problems arising in the wards. Time has justified what Sir Thomas Lewis said about the importance for medicine of the work of such people, to whom he gave the name of clinical scientists. This book provides many examples of the kind of work they can do.

A few years ago most of the best work in clinical endocrinology was being done in the U.S.A. More recently, however, important work on quantitative methods has been done in Great Britain. Dr Loraine is a member of the staff of the Clinical Endocrinology Research Unit (C.E.R.U.) which the Medical Research Council established in Edinburgh in 1946, and it is natural that his book should lay special emphasis on the work of this unit. Most such units have a single full-time director, but C.E.R.U. is directed by a committee consisting of the professors of biochemistry, gynæcology, pharmacology, surgery and therapeutics. This has meant that several departments have collaborated in support of a unit which belongs to them all. The Medical Research Council has allowed the unit to work for long periods on the development of reliable methods, without demanding quick publication. This policy has produced several new methods which are likely to add much to our knowledge of disease in the years to come. This book gives a clear and authoritative account of this work and of the great mass of other work in this field in all parts of the world. No clinical endocrinologist can afford to be without it : all who are interested in the clinical application of hormone assays will find it valuable. It gives a general account of the methods available, without wearisome details, and a full discussion of the ways in which these assays may be of value to those responsible for the care of patients.

PREFACE

THIS book has been written with a threefold purpose: firstly, to review the advantages and limitations of existing hormone assay methods and, where possible, to indicate the most suitable method or group of methods for use in clinical studies ; secondly, to discuss the application of hormone assay procedures to clinical problems and to examine the value of these estimations in the diagnosis, prognosis and treatment of disease in man; and, finally, to suggest the fields of investigation to which assay methods might in the future be profitably applied.

The subject matter deals mainly with pituitary, placental, adrenal and gonadal hormones ; a short chapter on insulin is also included. Consideration has not been given to the parathyroid hormone or to the gastro-intestinal hormones because the methods for the quantitative determination of these substances in body fluids are not yet in a sufficiently advanced state to merit their use in clinical investigations. A section on the thyroid has not been included because it was felt that, although the estimation of the concentration of protein-bound iodine in blood gives a reasonably reliable indication of the activity of this gland in health and disease, assay methods for the thyroid hormones themselves are not at present suitable for clinical application.

The field of hormone assay has been expanding so rapidly in recent years that it is clearly impossible to quote all the relevant literature. An attempt has therefore been made to present an outline only of the work done prior to 1947 and to concentrate more particularly on the literature published between 1947 and 1957.

In writing this book it has been a great privilege for me to express the views which the Edinburgh group as a whole share with regard to the clinical application of hormone assays. One of these views which deserves special mention concerns the question of methodology. We have always believed that, in clinical studies, only the best of the available techniques should be used and that, if unreliable assay methods are employed, the results obtained are liable to be misleading. Fortunately, within recent years, assay methods for many of the hormones have been greatly improved and it is now possible in many cases to employ techniques which satisfy the ' reliability criteria ' discussed in Chapter I.

The Clinical Endocrinology Research Unit has been very fortunate throughout the years in being able to collaborate closely with investigators in various hormone laboratories in Europe, especially with those in Stockholm and Geneva. It is certain that, with the passage of time, this type of international collaboration will increase and should contribute greatly to further progress in clinical and experimental endocrinology. Two examples may serve to illustrate the value of the international approach to the subject. The first of these is the series of ' Colloquia on Endocrinology ' held in London under the auspices of the Ciba Foundation. At these Colloquia it has been possible for research workers from many nations to meet and to discuss common problems in a pleasant and informal atmosphere. The second example is provided by the ' Gonadotrophin Club ' which was formed in 1953, held its first meeting in Geneva in that year and has subsequently met in Birmingham in 1955 and in London in 1957. These meetings have been attended by colleagues from various laboratories in different parts of the world and have enabled those particularly interested in the assay of gonadotrophins to exchange ideas and to plan collaborative research.

I should like to take this opportunity of expressing my thanks to the many people who have helped me in the preparation of this book. I am especially grateful to Professors J. H. Gaddum, John Bruce, D. M. Dunlop, R. J. Kellar and G. F. Marrian—Directors of the Clinical Endocrinology Research Unit, Edinburgh—for their interest, encouragement and co-operation ; the book was written at the suggestion of Professor R. J. Kellar, who felt that such a work might be of value to both clinicians and laboratory workers.

I am much indebted to Dr Rudi Borth (Geneva), Dr Egon Diczfalusy (Stockholm), Dr G. D. Matthew (Edinburgh), Dr J. A. Strong (Edinburgh), and the Directors of the Clinical Endocrinology Research Unit (Edinburgh), who have read large portions of the text and have made many useful suggestions and criticisms. Thanks are also due to various colleagues who have given me helpful advice on specific chapters, namely Dr W. S. Bauld, Montreal (œstrogens), Dr J. B. Brown, Edinburgh (œstrogens), Dr K. Fotherby, Edinburgh (corticosteroids and 17-ketosteroids), Dr K. C. Hooper, Edinburgh (protein hormones), Dr A. Klopper, Edinburgh (progesterone and its metabolites), Dr J. K. Norymberski, Sheffield (corticosteroids), Dr M. Pickford, Edinburgh (antidiuretic hormone and antidiuretic substances) and Dr G. Sayers, Cleveland, U.S.A. (adrenocorticotrophic hormone).

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reported in Chapters II and III could not have been obtained without expert technical assistance in the laboratory, and it gives me great pleasure to acknowledge the help of my technicians, particularly of Miss M. A. Mackay.

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THIS BOOK
IS DEDICATED TO
PROFESSOR
GUY FREDERIC MARRIAN
TO WHOM THE SCIENCE
OF ENDOCRINOLOGY
OWES SO MUCH

CONTENTS

CHAPTER I

General Principles in Hormone Assay

ASSAY methods for hormones and their metabolites can be divided into two groups—*biological methods* and *chemical methods*. Bio-assays are often more sensitive and more specific than chemical assays but the latter are usually more precise, less laborious, less expensive, and much more suitable for routine use in the clinical field. It can be confidently predicted that, in the future, chemical assay procedures will largely replace bio-assays for the quantitative determination of hormones and their metabolites in body fluids.

At the time of writing, biological assays must still be used for the estimation of pituitary gonadotrophins, human chorionic gonadotrophin (HCG), thyrotrophin, adrenocorticotrophic hormone (ACTH), growth hormone, prolactin, posterior pituitary hormones, thyroid hormones, parathyroid hormone and insulin. The chemical determination of these hormones in blood and urine is not possible at the present time. Chemical assay methods should now be used for the determination of œstrogens, progesterone and its metabolites, corticosteroids and 17-ketosteroids. In the case of adrenaline and noradrenaline, chemical methods of estimation have recently become much more reliable and should probably now replace bio-assays in clinical studies.

As indicated in the title this chapter deals mainly with matters of general interest to those concerned with the estimation of hormones for clinical purposes. In order to explain some of the terms which will be used in Chapters II to VIII a section dealing specifically with bio-assay is also included.

ESSENTIAL REQUIREMENTS IN HORMONE ASSAY PROCEDURES

This subject has been reviewed by various workers, including Borth (1952), Marrian (1955), Loraine (1957 *a*)

and Brown *et al.* (1957). It is generally agreed that the efficiency of a given method depends on two main factors—its *reliability* and its *practicability*.

1. 'Reliability Criteria' for Hormone Assays.

According to Borth (1952) there are four such criteria. These are *accuracy*, *precision*, *specificity* and *sensitivity*. Borth (1952) has stated that, in assessing the overall value of a given assay procedure, the four criteria are of equal importance. This conclusion appears to be justified in view of the fact that the criteria may vary greatly in the case of individual methods. Thus a method which shows a high degree of precision may not be highly specific, while a specific, accurate and precise technique may not be sufficiently sensitive for use in a particular set of circumstances.

Throughout this book the term *reliability*, when applied to a method, will be used in a general sense to embrace the four concepts of accuracy, precision, specificity and sensitivity.

(*a*) Accuracy.—This term may be defined as the nearness with which a given analytical result approaches the 'true' result. The accuracy of a quantitative method can be studied by means of 'recovery experiments' in which determinations are made on the material being analysed before and after the addition of known amounts of the substance under investigation. Results are usually expressed in terms of the percentage of the added compound recovered. Accuracy can also be estimated by procedures involving radio-active compounds; it is probable that in the future techniques of this kind will become increasingly popular.

Some of the problems involved in evaluating the accuracy of the various 'steroid' methods have been discussed by Marrian, (1955). Ideally recovery experiments should be conducted by adding to blood or to urine pure steroids in the forms in which they are normally present. Unfortunately this is seldom practicable because the majority of the steroids occur in body fluids in the conjugated form and in most instances these conjugates are very difficult to obtain for investigative purposes. Accordingly, most workers have adopted the expedient of carrying out recovery experiments in which known amounts of the pure unconjugated steroids are added to blood or urine. Experiments of this type, although by no

means perfect in design, may give a reasonably good indication of the losses which occur during the performance of the method.

It is a matter of great difficulty to make a definite statement with respect to the permissible limits of accuracy in a given steroid method. This subject has been recently discussed by Marrian (1955), who suggested that a method might be regarded as satisfactory from the quantitative point of view when the recovery of added pure steroid was 75 per cent. or more. Marrian (1955) has also emphasised the necessity for investigators to determine *in their own laboratory* the accuracy of a given method; this should always be done before the technique is used for investigations in the clinical field.

In hormone assays depending on biological methods, recovery experiments of the type described above are often very laborious and this fact has prevented many workers from employing them as a means of assessing the accuracy of a method. Recently, however, particularly in the case of ACTH and of the pituitary gonadotrophins, data derived from such experiments have been reported in the literature (Sydnor and Sayers, 1952; Loraine, 1957 *b*). It is to be hoped that in the future similar information will be published for the other pituitary hormones.

(*b*) PRECISION.[1]—An estimate of the precision of a chemical assay method can be obtained by carrying out multiple determinations on the same sample of blood or urine or by conducting multiple recovery experiments with the same concentration of the added compound. Precision is usually expressed as the standard deviation of replicate determinations.

As in the case of accuracy, it is very difficult to offer any definite opinion as to what degree of precision is acceptable in a given assay method. Marrian (1955) has suggested that, for steroid methods, a standard deviation of ± 10 per cent. at optimal steroid concentrations is the best that can reasonably be expected while, at sub-optimal concentrations, it may be necessary to be satisfied with a standard deviation of ± 25 to 30 per cent.

[1] It should be emphasised that the terms 'accuracy' and 'precision' when applied to assay methods should not be regarded as synonymous. The term 'precision' refers essentially to the reproducibility of a method and should only be used when repeated estimations have been made on the same sample of blood or urine.

In biological assays the labour involved in conducting replicate determinations is very considerable, and for this reason few investigators have so far attempted to estimate precision in this way. Methods which have been employed for calculating the precision of such assays are described below.

(*c*) SPECIFICITY.—In chemical assay methods specificity can be defined as the determination of one chemical entity to the exclusion of others; in bio-assays the term refers to the determination of one physiological activity to the exclusion of others. Usually the specificity of a method depends on the cumulative evidence that the technique measures what it is supposed to measure and nothing else.

Some of the factors which must be considered in assessing the specificity of a chemical assay method have recently been discussed by Brown *et al.* (1957). These workers attempted to evaluate the specificity of the method developed by Brown (1955) for the estimation of œstriol, œstrone and œstradiol-17β in human urine (see p. 164). They concluded that, for the majority of urines from normal women during the menstrual cycle, normal men and normal post-menopausal women, there was good evidence to support the view that the substances being determined were indeed œstriol, œstrone and œstradiol-17β. This conclusion was based on the following four kinds of evidence: (i) The known high specificity of the Kober reaction on which the final determination in the Brown method depends, (ii) the chromatographic behaviour of the urinary œstrogen fractions on alumina adsorption columns, (iii) the behaviour of these fractions in studies involving countercurrent distribution, and (iv) the comparison of the results obtained by chemical assay on the one hand and by bio-assay on the other.

Marrian (1955), Borth (1956) and others have emphasised that most of the methods used for the final determination of steroids in blood or urine extracts are not of themselves specific. For example, the Zimmermann reaction is not specific for 17-ketosteroids; the production of a yellow colour with sulphuric acid is not specific for 5β-pregnane-3α : 20α-diol (pregnanediol); and the Porter-Silber reaction (see p. 259) is not specific for the 17, 21-dihydroxy-20-keto-corticosteroids. Accordingly, the specificities of methods in which such