Immunoassays for the 80s

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Preface

Analyses for naturally occurring biological substances or administered materials have been with us for many years. These were usually based on the physical or chemical characteristics of the substances to be measured. However in recent years there has been an explosion of interest in analytical methods which made use of the high specificity and sensitivity of immunological reactions. These methods can be very simple in terms of technical procedures and can usually be performed on minute samples of biological fluids – factors which have ensured their ready acceptance in most laboratories.

Recently there have been numerous meetings on technical aspects of particular immunoassays and on their application in specific diseases. We felt however that the time was ripe for an 'overview' of the whole field. To this end a conference on 'Immunoassays for the 80s' was held at the Zoological Society of London in 1980, and this book is largely based on that meeting. Both the immunoassay techniques and their numerous applications were discussed and are dealt with at length in this volume.

The editors wish to thank all the contributors for their chapters and to acknowledge the debt they owe to Jean Ryan (NLCM) without whose organization and assistance this volume would not have been completed.

A.V., D.B., A.B.

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Part I Immunoassay Techniques

1 Historical Perspectives

P. G. H. GELL

Immunological methods have been used for analysis ever since the demonstration of 'optimal proportions' in the early 1920s, at a time when playing with such things was still a 'hobby for gentlemen'. One may say nevertheless that quantitative immunology dated from this discovery, which led to a crucially important clinical advance, namely the definition of 'units' of toxins and antitoxins. This made possible rational immunotherapy and immunoprophylaxis in diphtheria and other diseases. Based on this understanding, *in vivo* tests using groups of animals allowed estimations down to the microgram level of either reagent; and the tests were good in that the activities measured (toxicity of toxin, protective power of antisera) were those relevant to clinical use. Developments in the 1930s gave a clearer picture of the nature of determinant groups of epitopes. The immunogenicity of chemically combined haptens was described and determinant groups were shown to have molecular sizes of around 200–1000 daltons. After the development of isotopic labelling the complete set-up was available for very precise immunoassays of high, medium or low molecular weight substances by competition methods. The very great advances of the 1950s in knowledge of antibody structure and cellular immunology were only marginally relevant to immunoassays. In view of the passage of 30 years it is disappointing that the assay of small molecules, such as drugs and neurotransmitters, has not developed more rapidly. However methods for estimating peptide and other hormones of intermediate molecular weight have proceeded in step with their discovery.

A real advance in convenience and sensitivity has been gained in recent years by the use of enzyme-labelled, instead of radio labelled, reagents. This allows one to use simple colorimeters rather than gamma-counters, which are not always reliable. It is possible that a current major advance is in the use of monoclonal antibodies in immunoassays. This means that we can obtain antibodies of uniform avidity and affinity directed at a single definable

epitope. Although these properties may appear at first to be just a nuisance, compared to the blunderbuss activities of conventional antisera, there is little doubt that in spite of the formidable difficulties in defining the precise specificity of a monoclonal antibody derived from a hybridoma, and the influence upon experimental conditions entailed by the uniform and possibly not very high avidity, the improved precision of such antibodies cannot fail to contribute to improvement of test systems.

2
Merits and Disadvantages of Different Labels and Methods of Immunoassay

R. EKINS

INTRODUCTION

Antibodies comprise molecules of biological origin generally possessing a very high degree of structural specificity; this renders them especially suitable for use as 'specific reagents' in assays designed for the measurement of biological substances such as hormones, vitamins, viral and tumour antigens, etc. particularly those of large molecular size and complex composition. Nevertheless antibodies constitute only one of several classes of biological compound endowed with a high capacity for molecular recognition, sharing this property with – for example – hormone 'receptors' located within or on the surface of target cells, specific 'transport' proteins, enzymes, etc. Each of these classes of 'binding substance' can be exploited, for assay purposes, in techniques virtually identical in concept and in experimental detail with 'immunoassay'. In short, from the standpoint of the assayist, antibodies are distinguished from other specific 'binding proteins' chiefly by virtue of their origin and mode of production; otherwise there is little to distinguish 'immunoassay' methods from a wide group of analogous techniques relying on essentially identical analytical principles.

Thus, in this presentation I propose to use the term 'immunoassay' as representing an analytical method relying on the use of an antibody as the 'specific reagent'* whilst recognizing that many of the concepts herein

* The term 'immunoassay' has also frequently been used to describe assays in which an antibody represents the analyte irrespective of whether or not an antibody is used as the 'specific reagent' in the procedure. Although hallowed by tradition, this use of the term departs from the general convention, whereby the prefix applied to the word 'assay' is descriptive of the nature of the assay system rather than that of the analyte, e.g. 'bioassay'.

discussed have a wider applicability than to those assays which are based on antibodies *per se*.

BASIC PRINCIPLES OF IMMUNOASSAY

All assays fundamentally rely on interaction between the analyte and a 'specific' analytical reagent. The term 'specific' as employed here is, of course, a relative one; virtually no reagent is absolutely specific in the sense that it will react solely with a single analyte of unique molecular composition or structure. Although the specificity of an assay system often relies heavily on the specificity of the 'analytical reagent' used in the basic analytical reaction, additional specificity may be imparted to a system relying on an analytical reagent of low specificity by extraction and purification of the analyte prior to its exposure to the 'reagent' (e.g. by chromatographic techniques).

The term 'analytical reagent' as used above is intended to embrace both physical reagents (e.g. ultraviolet light, electron beams) and chemical reagents (e.g. specific binding proteins). Moreover it is intended to refer essentially to the reagent which, by interaction with the analyte, enables the amount of the latter to be quantified (i.e. it is not intended to refer to substances such as solvents, or to other reagents which are used, for example, in connection with preliminary extraction or purification procedures).

Immunoassay systems (i.e. those relying on antibody as the specific analytical reagent) may be subdivided into two main classes distinguished by their respective reliance on (a) observation of the reagent (antibody) and (b) observation of the analyte, following interaction between the two, as the basis of the analytical measurement. For reasons discussed below, it is usual (though not obligatory) that the design of assays of Type I is based on the use of an *excess* (usually large) of antibody over analyte; conversely assays of Type II rely on the use of an amount of antibody *less than* the amount of analyte in the system (i.e. a 'saturable' amount of antibody). Expressed in their simplest form, the concepts underlying the two forms of assay may be expressed thus:

Type I (excess antibody)

$$\text{analyte} + \text{antibody} \longrightarrow \underline{\text{analyte antibody complex} + \text{residual antibody}}$$

(Analytical measurement here depends on observation of the distribution of *antibody* between the complex and the residual moiety following reaction.)

Type II (excess analyte)

$$\text{analyte} + \text{antibody} \longrightarrow \underline{\text{analyte antibody complex} + \text{residual analyte}}$$

(Analytical measurement here depends on observation of the distribution of *analyte* between the complex and the residual fraction following reaction.)

These two forms of assay inevitably share many methodological features; nevertheless the fundamental difference in the concepts on which they rely manifests itself, *inter alia*, in the manner in which assays of each type should be 'designed' and in their relative sensitivity and specificity characteristics. This difference arises essentially from the differing impact of the Law of Mass Action on systems of each type.

With regard to the ultimate sensitivities attainable by the two approaches, it is evident that, considering an assay of Type I, however little analyte may be present in a test sample, an amount of antibody may be introduced into the assay system sufficient to ensure that *some* of the antibody will combine with analyte to form the complex in a given time-interval, however short this may be. This follows from consideration of the rate of formation of complex which is given by:

$$\text{rate of complex formation} = k_1[\text{An}][\text{Ab}]$$

where k_1 = associative rate constant
and [An] and [Ab] are the analyte and antibody concentrations respectively.

Indeed, the existence of even a single molecule of analyte can, in principle, be revealed by introducing a sufficiency of antibody into the system to ensure that the analyte molecule will react to form the antibody–analyte complex. These considerations reveal that

(1) the ultimate sensitivity of an assay system of Type I is one molecule of the analyte; and
(2) that maximal sensitivity in such a system is attained using an amount of Ab approaching infinity.

In contrast, systems of Type II essentially depend on the notion of 'saturation' of antibody-binding sites by analyte. Although this notion represents something of an over-simplification, it may readily be shown that maximal sensitivity of an assay system in this category is achieved when the antibody concentration approaches zero[1].

However, it also follows that the measurement of very small concentrations of analyte necessarily demands the use of very low concentrations of antibody; this in turn implies exceedingly low rates of analyte–antibody complex formation, and a correspondingly low concentration of complex in the mixture following attainment of thermodynamic equilibrium.

The upshot of these considerations is that it may be shown that the ultimate sensitivity of a 'Type II' or 'saturation assay' system is governed by the equilibrium constant (K) of the reaction between analyte and antibody, and is

given by ε/K where ε is the relative error in the experimental estimate of the amount (or fraction) of analyte in the complex[2]. In practice, since the equilibrium constants characterizing even the most avid antigen–antibody reactions seldom surpasses 10^{12} l/mol, and since the experimental errors incurred in assay systems of this type never, in practice, fall below 1% the maximal sensitivity theoretically attainable in a Type II system using conventionally prepared antibodies is of the order of 1×10^{-14} mol/l, i.e. approximately 10^7 molecules/ml. In practice assays of this type have never achieved sensitivities significantly superior to this.

SPECIFICITY

Non-specificity of an immunoassay system can arise as a result of two prime effects:

(1) 'cross-reaction' of substances structurally resembling the analyte;
(2) effects of ions and other substances on the kinetics of the analyte–antibody reaction.

(Other causes of assay non-specificity also exist: they are of less fundamental importance and will not be considered in the present discussion.)

The effects of each of these two sources of non-specificity are different in assays of either type and are best considered separately.

'Cross-reaction'

Type I assays

The notion of 'cross-reaction' rests on the proposition that an antibody is capable of reaction with two molecules sharing a common, or closely similar, antigenic determinant. Assuming that a single 'monoclonal' antibody population is present in large excess *vis-à-vis* two cross-reacting antigens, it is evident that the number of antibodies forming complexes with each of the two antigens will be broadly proportional to the respective numbers of antigen molecules present, irrespective of the respective energies of reaction between the two antigens and the antibody. In short, the two antigens will appear 'equipotent' in this type of system.

In addition to true 'cross-reactivity', the question of antibody heterogeneity must also be considered. Assuming an antiserum to comprise a mixed population of antibodies endowed with differing 'structural specificities', and assuming that any antibody purification procedures have not succeeded in isolating a single 'species' of antibody, then it is plausible that certain antibodies in the mixture will react with antigens other than the antigen of interest. Such substances will likewise appear equipotent in the assay system.

Type II assays

In this type of assay system – as a result of the reliance on amounts of antibody less than, or comparable to, the amount of analyte present – the effect of cross-reacting antigens is considerably more complex. In essence, the potency of a cross-reactant is a complex function of the 'occupancy' of antibody by the analyte or – alternatively stated – of the fractional binding by antibody of the analyte. In the simplest circumstances, in which a single species of antibody is present, the effect on the assay system of a cross-reactant may be expressed thus[3]:

$$\text{Relative potency (of cross-reactant)} = b + \left(f \frac{K_c}{K^*} \right)$$

where b = fraction of analyte bound to antibody
f = fraction of analyte 'free'
K^*, K_c = equilibrium constants of analyte *v.* cross-reactant respectively.

The implication of this equation is that, in circumstances in which the analyte is entirely antibody-bound following reaction, the cross-reactant will be equipotent; conversely, in circumstances in which the analyte is entirely free, then the cross-reactant will display a relative potency given by the ratio of the two equilibrium constants. (These represent extremes which, of course, are never normally attained in a practical assay system.)

The situation is naturally greatly complicated by the presence in antisera of heterogeneous antibody-binding sites possessing differing 'cross-reactivities' characterized by varying 'avidity ratios' (K_c/K^*) and by the relative concentrations of antibodies falling within each class, etc. A particular point of dissimilarity from assays falling into Type I is that the existence of antibodies in the mixture reacting with antigens other than the analyte, and entirely non-reactive with the analyte, have no influence on the system, i.e. such antigens have a zero relative potency in Type II assays as compared with a relative potency of unity in Type I assays.

The outcome of these general considerations is that, in a Type I system, much greater reliance is implicitly placed on the 'purity' of the specific antibody employed. Moreover all cross-reactants tend to display equal potencies in such a system. In contrast, in Type II assays, homogeneity of antibody is less important; indeed it is conventional to set up such assays using antiserum *per se* rather than an isolated and purified antibody. Because the majority of 'cross-reactants' are likely to be less avidly bound than the analyte by the principal specific antibody present, a Type II system is inherently more specific than one of Type I, albeit the additional measures of antibody purification which are frequently adopted in the development of Type I systems usually preclude a straightforward comparison.