Radioimmunoassay for medical diagnosis and research

by R.A. Dudley and B. Vavrejn*

Health and disease in living systems depend on the dynamic interplay of thousands of biochemical substances. Most of these are complex molecules, containing dozens to thousands of atoms. Many are very delicate, and some are distinguished from others only by the type or position of a few atoms. These molecules occur in mixtures, in concentrations ranging from parts per hundred to parts per billion or trillion.

The task of measuring the concentration of such biochemical substances (assaying) might appear hopeless. Obviously it is not, since living systems accomplish it all the time on their own constituents. First attemps to measure biological substances were therefore *bioassays* – for example, to determine whether a female is pregnant: inject a frog with a sample of her urine and observe its response. While much was learned from such methods, they are limited in scope and accuracy, and are enormously tedious. Twenty-five years ago the new method *radioimmunoassay* (RIA) was devised; it also, in part, mimics life processes, but now in a test tube. This method, with numerous variants, has so revolutionized the study of hormones and other substances that Rosalyn Yalow, a physicist engaged in nuclear medicine, was awarded the 1977 Nobel Prize for medicine in honour of her part in its discovery.

The key requirements for a useful assay system are specificity (the ability to distinguish unambiguously between very similar molecules) and sensitivity. In RIA, these requirements are served by using, as the distinctive "chemical" reagent, an antibody to the substance (ligand) being assayed. Antibodies are the protein molecules synthesized by animals to inactivate foreign biological substances that have intruded into them. For example, if a rabbit is injected with human insulin, it synthesizes an antibody to react with and neutralize this foreign molecule. Necessarily, however, the antibody must be so discriminating that it does not react with any of the rabbit's own molecules, even its own insulin: it can serve as a highly *specific* reagent. Furthermore, the antibody is created to defend the animal against even microscopic amounts of foreign substance: it can thus serve also as a reagent providing high *sensitivity*.

The principle of RIA is very simple – this is one Nobel Prize that the thoughtful layman can comprehend. Suppose a test tube is prepared containing antibody that is known (from calibration data) to be capable of binding 1 nanogram (1 billionth of a gram) of insulin. Suppose an unknown amount of insulin (containing more than 1 ng) is added. One nanogram will be bound, and this bound fraction can be separated out by relatively simple means. How can the remaining unbound fraction be measured? Not by weighing – it is too small, and is accompanied by myriads of contaminants weighing millions of times as much. The problem is solved by *tracer* techniques. Before the original unknown sample is mixed with the antibody, a trace amount (such as 0.1 nanogram) of radioactive insulin is added. (Insulin labelled with ^{125}I would serve.) The mass (and therefore radioactivity) of this tracer will be apportioned between the bound and unbound fractions just as is the mass of non-radioactive insulin being assayed. If the radioactivity in the unbound fraction is measured to be ten times as much as in the bound fraction, then the unbound insulin must be 10×1 nanogram $= 10$ nanograms, and the total (unbound plus bound) must be $10 + 1 = 11$ nanograms. The assay of the unknown sample of insulin has thus been achieved. It is evident that the success of this approach depends on the detectability of very small masses of tracer, a property that radionuclide labels confer. Of course, to do any real assay properly requires attention to numerous theoretical and empirical details.

Many variants of this technique have been devised. The "binder" may be not an antibody but some other specific biological molecule: e.g. a "binding protein" (used in the body to transport particular substances), or cell receptors (used by cells to selectively extract their required nutrients from the soup that bathes them). Other labels besides radionuclides have been developed: e.g. enzymes, fluorescent substances. The label may be attached alternatively to the binder rather than to the ligand.

* Mr Dudley is Head, Medical Applications Section, in the Agency's Division of Life Sciences. Mr Vavrejn is a staff member of the Medical Applications Section.

Altogether, these many related procedures, now based on a vast technological lore, represent one of the most dramatically expanding areas of medical diagnosis and research. The International Atomic Energy Agency therefore convened a symposium* to review recent progress, with emphasis on methodology and on its adaptation and application in developing countries.

Assay techniques

A recent breakthrough in technique is the production and use of monoclonal antibodies. Heretofore, antibodies were "raised", as needed, in animals, and were inevitably very heterogeneous. Recent developments in genetic engineering unrelated to RIA have allowed the production of antibodies from cultures of individual cells. Each culture, which can be endlessly propagated, produces one unique antibody. By screening many such cultures, favourable antibodies can be identified. In the future such antibodies may be sold much like other chemical reagents: ideally matched to the particular task, free of significant contaminants, standard in performance, and unlimited in supply. Examples described at the symposium showed improved sensitivity, working range, and consistency using techniques based upon radioactively labelled monoclonal antibodies.

The tracer substance, be it labelled ligand or binder, must meet numerous stringent criteria, and in many assays it is the limiting feature in the achievement of high quality. It thus remains the focus of intensive study. ^{125}I is the ideal radionuclide from the point of view of convenience of measurement. However, in many substances – especially steroids – it represents a foreign atom in the molecule and thus prevents the tracer from acting identically to the substance it is supposed to trace. The problem is even more severe if the label is not a single atom but a large molecule, as in the case of enzymes and other non-radionuclide labels. At least for ^{125}I, the labelling conditions required to yield acceptable tracer performance are becoming more clear. For small molecules, rules have been developed as to where and how the ^{125}I atom should be attached to the molecule. Acceptable performance of certain ^{125}I-labelled steroids, as compared with chemically unaltered ^{3}H-labelled steroids, was reported, and the use of the ^{125}I label for this important class of ligand may become widespread. For protein ligands, procedures for labelling satisfactorily without disrupting the molecule are also reaching maturity, as are procedures for purification of the labelled product (especially with high-pressure liquid chromatography).

For many reasons, attempts are being made to replace radionuclides with other labels, and considerable success has already been achieved. It is hoped that this search will lead to cheaper analytical instruments and to reagents with a longer shelf-life, improved sensitivity, and increased convenience and speed. For fundamental reasons, much higher sensitivity could in principle be achieved by certain non-radionuclide labels, and probably more rapid sample throughput. Several symposium reports concerned these developments. Enzyme labels are now widely used, and their sensitivity may be comparable with radionuclide labels; however, doubts persist as to whether their performance can be robust in the face of interfering substances and variable analytical conditions. Some investigators predict future reliance on chemiluminescent labels or on delayed fluorescence labels incorporating rare-earth compounds; progress, but no breakthroughs, was reported for these techniques.

Methods for separation of the reacted ligand-binder product from the incubation mixture continue to receive much study, as the stage of separation is often responsible for much of the imprecision and operator time in assays. Several symposium reports described new or modified solid-phase materials, i.e. particles to which one of the components (binder or ligand) is attached (preferably covalently) in order to permit convenient mechanical separation by gravity, centrifuge, or magnet. This interest confirms the trend toward dominance of solid-phase separation methods.

A different type of methodological innovation is represented by the recent development of techniques for analysis of "free" hormones. Many hormones present in the blood are found predominantly bound to specific carrier proteins. It is believed that the physiological action of such hormones is caused only by the small fraction of unbound hormone which exists in equilibrium with the larger fraction of carrier-bound hormone. Hence the free hormone level alone, if it could be measured, might provide more relevant diagnostic information than the total hormone level. The theory of alternative assay methods for this purpose is now reasonably clear. Practical implementation of some of these methods has been achieved, and their diagnostic usefulness has been experimentally confirmed, but further research is needed to establish the scope and role of these techniques.

Quality assurance

The assessment and improvement of the quality of measurements is an essential part of any analytical programme. Quality assurance is especially important in the case of RIA and related procedures which are vulnerable to many disturbances flowing either from incompletely standardized analytical techniques or from irregularities in the reagents. *Internal* quality control describes the procedures carried out within a laboratory to assure assay reliability, while *external* quality assessment refers to monitoring by an external organization through distribution of test samples.

* International symposium on radioimmunoassay and related procedures in medicine, held from 21–25 June 1982 in Vienna, Austria.

One aspect of internal quality control is the critical analysis of measurement results, whether by computer (as in many laboratories) or by hand. One speaker at the symposium recommended that every part of such analyses should be performed by computer in order to extract the most reliable information, but other opinions were more skeptical of the utility of computers. This diversity of outlook probably relates to the quality of the computer programs: whether locally designed or commercially supplied, programs used in most laboratories today are poor. It was stressed that a good computer program must not only relieve the analyst of the burden of repetitive error-prone manual calculations, but in addition must assess the reliability of the results. To construct such a program is a major task, requiring perhaps one man-year of informed programming. Rapidly falling costs of computers will eventually permit all laboratories to exploit the best professionally designed programs, which should then replace all other methods of critical analysis.

The goal of internal quality control, namely, consistently high assay quality, cannot, however, be reached merely by contemplating the results. Active intervention of the analyst is required to determine the cause of unreliability and to eliminate it by altering analytical methods. The assay design must be tested to establish its validity in the face of potential interferences, and data on quality control specimens and other indices of assay integrity must be acquired in each assay batch to assess routine performance. As a key objective, laboratory discipline and organization must be focused on quality.

In a typical external quality assessment, a central laboratory prepares and distributes identical suites of specimens to many laboratories, and analyses the reported results with regard to comparability and stability. If possible, the "true" ligand concentration should be independently established; otherwise the median result may be tentatively used as an approximation of ligand concentration. In any case, variability within and between laboratories can be displayed objectively. Some quality assessment schemes incorporate additional tests, such as vulnerability to possibly interfering substances or recovery of added ligand. All programmes open up opportunities for education of participating laboratories. In a panel discussion, 10 external quality assessment schemes were described. Two of these – one sponsored by the IAEA on thyroid-related hormones as measured by some 80 laboratories, and one by WHO on reproductive hormones as measured by about 120 laboratories – were world-wide in scope.

Quality control, both internal and external, is universally applauded but widely neglected. The number of such projects displayed at this symposium suggests that the importance of the issue is being taken progressively more seriously.

Analysis and application

The number of different ligands for which RIA or a related procedure constitutes a useful analytical technique continues to expand, and many established techniques are being improved so as to make their applications more practical. Such advances with respect to several particular substances were reported. For example, assays can now measure: beta-endorphin (a "natural opiate") in cerebrospinal fluid – a substance that relates to pain; secretin (a hormone controlling pancreatic secretion of gastric enzymes) in plasma; clomipramine and amitriptyline (drugs for treatment of depression) in plasma during their therapeutic use; Suriclone (a tranquilizer) in plasma during therapeutic use; and anabolic steroids (drugs sometimes used by athletes) in plasma.

Special attention was given to applications of potential importance in developing countries, whose medical problems may be quite different from those being investigated in the developed countries where most RIA techniques have been designed. It is hoped that RIA or related procedures may eventually replace current methods in the study of many infectious and parasitic diseases. In the study of hepatitis B, this has already happened.

Three fields of application are important: diagnosis, epidemiology, and research. In diagnosis, the objective is to identify the presence of a particular disease by detecting products (antigens) shed by the agent (e.g. virus) currently present in the patient. It is now believed that almost all disease agents, from viruses to worms, shed microscopic amounts of antigens. This belief opens the need for research into the quantities and unique qualities of a vast number of antigens. If sensitivity and specificity are adequate, RIA and related procedures may permit rapid and reliable diagnosis.

If the disease agents shed antigens, the body will respond by generating antibodies to combat the agents. These antibodies – because they themselves are complex biological molecules – can also be assayed by RIA or related procedures. However, since antibody levels may persist long after the disease has been cured, they are unreliable evidence of current infection even though they demonstrate that the patient had the disease at some time and, depending on the disease, may now carry immunity to its. Since determination of the fraction of the population that at one time or another has had the disease, and the fraction that may carry immunity, is central to epidemiology, RIA and related procedures have a role to play in this discipline. In fact, antibody levels tend to be higher and hence more readily measurable than antigen levels, and therefore epidemiology has to date advanced more rapidly than diagnosis. Research, of course, underlies all progress in diagnosis and epidemiology; it will represent the most important, even if not the most frequent, application of these techniques.

Tuberculosis can serve as a model for diagnosis of infectious diseases, and here two papers reported progress. When sputum samples are cultured in the laboratory, leading to multiplication of the tubercle bacilli (TB) and their attendant antigens, RIA can detect TB sooner than other methods. In sputum samples and in other body fluids reflecting foci of TB (e.g. cerebrospinal fluid in tubercular meningitis), antigens may soon be detectable. Thus RIA (or probably some related procedure more suited to field use) may become the method of choice for diagnosing many forms of tuberculosis.

Diagnosis of various parasitic diseases by analysis of specific antigens is also becoming possible, with progress reported in malaria, Chagas disease, amoebiasis, schistosomiasis, and certain nematode diseases. Measurement of antibodies also gives some useful evidence as to current infection for several such diseases.

Finally, in most of the above mentioned diseases, epidemiological investigations have already been conducted using RIA or related procedures.

In current research, the identification, characterization, and production of specific antigens for these diseases is the most urgent task.

Practice and pitfalls in developing countries

RIA and related procedures are already practised in developing countries, and are likely in the future to play an even more important role. Introduction of the methodology into this environment, for which the Agency's help is sought and given, presents numerous problems that were reviewed at the symposium. Before an analytical service can be established, the authorities must be convinced that public support is justified. Therefore, its relevance to something more than abstruse Western medicine must be demonstrated.

If RIA and related procedures are chosen as medical priorities, personnel must be suitably educated. Technicians should be trained in a relevant laboratory environment near home, and academic specialists, where appropriate, in more advanced laboratories abroad. Equipment must be selected by criteria of workload and maintenance requirements. Concerning equipment problems, deficiencies are frequently organizational and psychological rather than technical; for these reasons, the availability of spare parts, test equipment, and expertise must be assured according to a rational plan. Consumable supplies are difficult to prepare or obtain, and foreign exchange for their purchase is always in short supply. It may consequently be necessary to substitute "in-house" reagents for commercial assay kits. Major assistance in supply of reagents has also been organized by "sister laboratories" and by international agencies such as WHO and IAEA; similar regional co-operation should also be a goal. To combat specific diseases afflicting more than one developing country, regional collaboration in research would also be rewarding. Techniques, after adaptation to local needs and conditions, must be conscientiously subjected to quality control.

The future of tracer techniques in medicine

Undoubtedly RIA and related procedures will grow in reliability and applicability. Among significant developments in technique, it is probable that non-radionuclide tracers will gradually come to dominate. Breakthroughs in the use of these techniques for infectious and parasitic diseases are likely to raise greatly their relevance in developing countries. There are many opportunities for increasing the efficiency of such techniques in this environment, especially through improving local organization and regional co-operation.