The Basis of Radioimmunoassay

The Scientific Basis of Medicine

With Professor John Landon, St Bartholomew's Hospital Medical College.
Introduced by Dr Ian Gilliland.

University of London Audio-Visual Centre, 1972.
Made for British Postgraduate Medical Federation.

Directed by David Sharp.
Produced by Peter Bowen.

Black-and-white
Duration: 00:38:01:24

00:00:00:00

<Opening titles>

<Dr Gilliland to camera>

Professor John Landon is Professor of Chemical Pathology of the University of London at St Bartholomew's Hospital Medical School. Prior to this he worked at St Mary's Hospital, Paddington. He has made some notable contributions to the development of radioimmunoassays and this is the subject of the following discourse. Professor Landon.

<Professor Landon, seated, to camera. Two charts are displayed on the wall to his right>

Radioimmunoassay is the most powerful analytical tool, at present, available to medical science. With a specificity beyond the dreams of avarice, sorry, a sensitivity

beyond the dreams of avarice and a specificity, which is almost amoral in this promiscuous era, explains why I have two lectures. The second of these will relate to the applications of radioimmunoassay, whereas in the first of these, armed with a knitting needle as a pointer and a clipboard, I have to explain its basis.

The first radioimmunoassay for insulin was introduced by Yalow and Berson in 1960. At the same time, Professor Ekins from the Middlesex Hospital was introducing assays based on the same principle for vitamin B12 and thyroxine, and pointed out that radioimmunoassay was only one of a number of related techniques differing only in the binding protein used. This can best be summarised in the next slide.

<Landon narrates over table listing binding proteins for various assays>

Radioimmunoassay uses as its binding protein a specific antibody. In a much more recently introduced modification, immunoradiometric assay, an antibody is used as the binding protein and this is also labelled. For competitive protein binding assays, circulating proteins, such as transcortin or thyroxine-binding globulin, are the binding materials. In receptor assays, proteins are extracted from the tissue where the hormone has its biological effect and these are used. Finally, enzymes can be used in a type of assay referred to as the radioenzymatic assay.

<Landon to camera>

The period since 1960, that's a period of 12 years, I suppose could be divided into 4 periods each of 3 years. The first 3 years was a period of success. Radioimmunoassays were introduced for a series of large peptide hormones like insulin, growth hormone, human placental lactogen. The classical immunologists who said that it was impossible were shown to be wrong, and bioassayists were put to rout. The second period was the phase of arrogance, where radioimmunoassayists, cheered on by their early successes, evolved a series of assays for the smaller peptides and published the results, all of which were manifestly impossible if life was to exist in the animal. This was the period where they ceased or ignored all other developments and all other knowledge.

The third period was a period of realisation, where they realised that bioassays and classical immunology had a lot to offer. And this final 3 years has seen the diversification of the technique out of endocrinology into many other fields, such as oncology and haematology.

As a clinical chemist, I would like to deal with this subject in the broader context of clinical chemistry and of the other analytical procedures which are available. Clinical chemistry departments have for a long time been facing a staggering increase in workload as is shown in the first chart.

00:04:57:09

<Landon narrates briefly over chart showing workload in thousands of test per annum, and then to camera>

Thus, in our own department, after a slow start, work since about 1968 increases in logarithmic proportions and is now exceeding 1 million tests a year. This has been possible by the introduction of automated procedures, and undoubtedly those of the continuous flow type introduced by Technicon have kept our heads above water. This next film sequence shows one of the latest of the continuous flow systems, the SMA 1260.

<Landon narrates over brief film footage of Technicon Autoanalyzer and then photograph of PDP-11 system, interspersed with talk to camera>

It's referred to as 12 because on each plasma it does 12 determinations, and the 60 refers to the number of samples dealt with in an hour. These, of course, have now been superseded by things like the Vickers which will do 20 determinations per sample at a rate of 300 an hour.

This type of automated equipment, together with computer facilities, as illustrated in the next picture, which shows the laboratory PDP-11, and which enables workload,

quality control and many other things to be dealt with, plus the use of quality control systems, such as that introduced by Birmingham, means that the common workload can now be dealt without undue difficulty and leaves time for the increase of the range of determinations that we can offer and which are required, for example, by the endocrinologist. Certainly, chemical means can be used, some of the steroids; and automated procedures have been evolved for things like pregnancy oestrogens and for things like protein-bound iodine determinations as shown on the next film sequence.

<Landon narrates over film showing a protein-bound iodine machine in operation in a laboratory>

This sequence shows the classical protein-bound iodine machine, where samples are mixed with strong acid and then passed, at very high temperatures, down a glass spiral. As they pass down the spiral, the protein is denatured and the iodine is freed. The iodine is then used to catalyse a colour reaction, which is carried out in an ordinary Technicon unit, and the amount of iodine can be read from comparison with standards.

<Landon to camera and then over film showing various mass spectrometers in a laboratory>

Now, these chemical techniques have got a limitation because many hormones are in such low concentrations that they're beyond the sensitivity of chemical techniques. Other techniques have been of use, such as gas-liquid chromatography, and when gas-liquid chromatography is combined with mass spectroscopy, a mass spectrometer as shown on the next slide, you can get a very valuable tool. This shows the simple mass spectrometer, whereas we move on to the other machine in this laboratory, which is a high resolution mass spectrometer fed by a gas-liquid chromatograph, which allows you to put in, for example, freeze-dried ovaries and to determine, without any further purification, the oestrogen content.

<Landon to camera>

Now, this equipment is much too expensive and much too complex for the routine laboratory, and also the numbers of samples that can be dealt with are too few and it is not applicable to peptide hormones. For peptide hormones, therefore, one in the past had to use bioassays – assays based on the biological activity of the material you were measuring.

<Landon narrates over photographs of assay apparatus, interspersed with talk to camera>

One of these assays is shown on the next picture. This is the classical assay for measuring plasma ADH levels, antidiuretic hormone levels, using the alcohol-anaesthetised rat. What one does is to inject the material and determine the decrease in urine flow and the concomitant increase in conductivity of the urine.

A much more recent bioassay, by far the most sensitive assay ever introduced, came from Dr Cheyenne and this is illustrated in the next two pictures. Here thin sections are cut in a cryostat and are kept in a non-proliferative culture. The hormone is added and the redox, changes in redox, are measured. These are measured as shown in the next picture in a reflecting densitometer. Now, this sort of assay is of quite astronomic sensitivity. It can measure 1 femtogram of ACTH. And there is promise that it can go down into the attogram level. And we will be talking about these units somewhat later.

00:10:33:02

Having said that, we must say, I think, that the bioassays must remain the reference technique for hormones. They are not, however, without their disadvantages. For example, there‘s one memorable bioassay for oxytocin that, when you actually determine how much oxytocin is present, it works out at one third of a molecule. And from this, I think, one can assume that it is a response of a non-specific type. The nicest bioassay is one involving a frog sitting in a glass of water and you add the prolactin-containing material, and the level of assay is when the frog jumps out. So, I

think it can be seen that there was a need for other techniques as well and it is here that radioimmunoassay has come in.

Now, the first problem I have is to try to explain the basis of radioimmunoassay. There are many ways of doing this, but I thought I would try a technique based on the law of mass action.

<Landon narrates over slide>

<Slide>

Determining the % total Ag present in the bound (B) fraction at equilibrium.

[Ag] + [Ab] ↔ [Ag : Ab]
(Free fraction) (Bound fraction)

If an antigen is reacted with an antibody and the reaction allowed to come to equilibrium, at equilibrium a part of the antigen will remain in the free fraction and a part bound to the antibody is termed the bound fraction. At any instance in time, some of the free antigen and antibody are combining at a rate, K1, and some of the bound complex is dissociating at a rate, K2. In radioimmunoassay, what is necessary to do is to determine the percentage of the total antigen present, in the tube, which is present in the bound fraction. You could see from this equation that if we keep the amount of antibody constant, then the amount of antigen which is present in the bound fraction will be inversely proportional to the total amount of antigen there. That is to say, if we have a vast amount of antigen, relative to that constant amount of antibody, very little will be bound. In contrast, if we have a very small amount of antigen, then the majority of it will be bound.

<Landon to camera, then stands and walks to charts on display wall. Narrates over chart showing results of a radioimmunoassay for triiodothyronine>

Now, if we go to a practical example of, let us say, setting up a radioimmunoassay for triiodothyronine, what do we do? Well, first, one will have immunised the animal and we will have to see what level of antibodies its sera contains. So we take tubes and we set up doubling dilutions of the rabbit sera, and to each tube we add a constant amount of labelled triiodothyronine. Now, you can see that when we have a lot of serum, the majority of the labelled triiodothyronine will be bound. If we have a very great dilution then very little will be bound. What we do is to choose a concentration of antibody which will be about 50 to 60% of our constant amount of tracer. Then we can mix in a tube the constant amount of antibody and the constant amount of tracer, and the only thing that varies is the amount of unlabelled triiodothyronine we are using as standard. If we have a very large amount of unlabelled T3, then it will occupy most of the binding sites. And, therefore, less of the labelled T3 will be bound. If we have little unlabelled T3 then more of the labelled T3 will be bound.

00:14:51:07

<Landon returns to seat, narrates to camera and then over slide summarising requirements for a radioimmunoassay>

Now, if we return, we might consider now, what are the requirements needed for a radioimmunoassay? In the next slide I have summarised the general requirements. We are going to need certain reagents. We must have facilities for performing the assays. We must have facilities for conjugating haptens to proteins if we are dealing with things which are not themselves immunogenic. We must have facilities for labelling the materials, that is to say, shielded rooms where we can carry out radioiodination. And we must have animal facilities where we can perform immunisation schedules and raise our antisera. We must also have some equipment, which we will deal with in a few minutes.

<Landon to camera and then over slide listing types of reagents used in radioimmunoassay>

Now, dealing first with the reagents. These are again listed on the next slide. It is absolutely essential to have highly purified antigen for the standard. Now, this may either be a natural antigen, for example, human growth hormone extracted from pituitaries and highly purified, or a synthetic material, such as synthetic oxytocin; or in the case of a drug, something like digoxin. Then, we must have labelled antigen. We must also have some specific antisera. You'll have seen from what I said earlier that we must have a technique for separating the anti-bound and the free antigen. And in certain assays, where the concentrations of the material to be assayed are very low, we must be able to extract the material and to concentrate the antigen.

<Landon briefly to camera and then over slide listing difficulties of radioimmunoassay>

Now, if we could have the next slide, this emphasises that the major factor in radioimmunoassay is the specific antisera. Now, for hormones, like growth hormone or human placental lactogen, there is very little problem in the assay because the materials are easily available and they are immunogenic and it is very easy to raise antisera. In other examples, for example, human parathyroid hormone, difficulty is experienced because of the paucity of the immunogen. With others, such as steroids or drugs, these are not themselves immunogenic and therefore must be conjugated covalently to larger protein molecules to become immunogens. Then we have certain small peptides, like antidiuretic hormone and glucagon, which are poor immunogens and, in addition, have very low circulating levels. Since sensitivity depends predominantly on the antibody, and yet we have just said that these are poor immunogens, one can see that this may cause tremendous problems. Finally, we may have problems with materials which are present in quite good supply and which are immunogenic, but which share certain antigenic determinants. This explains the problems with TSH, LH, FSH and HCG, which have the common alpha subunit, and so that if you develop antibodies against that, then you will get cross-reaction from the other material.

<Landon to camera and then over photograph of counters used in radioimmunoassay>

Now, as I said before, one would also need certain items of equipment and, as shown in the next picture, one of the most expensive of these are counters. And you can see here, in fact, a back of three counters. And remembering that each costs the price of a Jensen car, you can realise that this is an expensive step.

<Landon to camera and then over photograph of a radioimmunoassay>

Then, where we have to deal with very large numbers, we have to introduce automation. And the next picture shows the first automated, or semi-automated, radioimmunoassay, which we evolved based on a piece of equipment called the Analmatic, which enables the very accurate addition of small volumes of a number of reagents and, in this way, avoids the tedium of adding them manually.

<Landon to camera>

Now, it is apparent that not every lab can have the facility for raising its own antisera or carrying out its own radioiodinations. And for this reason, the pharmaceutical industry and other firms are providing diagnostic kits which contain all the reagents. And it is also for this reason that places like the National Biological Standards Institute will provide standards and antisera for use.

00:19:55:77

Now, I think that we can next move on to the advantages of radioimmunoassay.

<Landon narrates over slide listing advantages of radioimmunoassay>

These are summarised on this slide. The first one, which we've touched on briefly is sensitivity and this is the result of using a radioisotope. The detectors for isotopes are so very sensitive that this allows sensitivity in the method. The specificity is a product of all antigen-antibody reactions. One can think of the antigen as a Yale key and the antibody as a Yale lock which only it will fit. These types of assay are also much

more precise than bioassays because they avoid individual animal variation, and they have a tremendous application so, as I've said, in our second talk we will be dealing with how this is diversifying into many, many different fields.

Next, the cost per assay sample can be reduced to a very small sum, for example, less than 10p if you can set up an automated assay and if the throughput of samples is sufficiently great. And finally, and of key importance in clinical chemistry is that this type of technique is ideally suitable for automation.

<Landon to camera and then over slide defining units of weight>

Now I'll just take the first two of these in more detail. Let us consider, first of all, the sensitivity and I think to do this, I had better just remind you of the units of weight. You'll remember that a gram of water is the weight of 1 millilitre of water and that in every gram there are 1000 milligrams, and that one can fairly easily see a milligram. Now, 1000 micrograms are 1 milligram; 1000 nanograms make 1 microgram; 1000 picograms, 1 nanogram; and 1000 femtograms make 1 picogram. You'll have heard earlier that we were referring also to attograms and there are 1000 attograms per femtogram.

<Landon to camera, then stands and walks to charts on display wall. Narrates over chart showing results of a radioimmunoassay for oxytocin >

Now, if we now move over and consider a particular assay; this is a standard assay for oxytocin. Here we are presenting the percentage of the labelled oxytocin that is bound, and here the amount of unlabelled oxytocin present in the standard curves. And you can see that the presence of 2 or 4 or 8 picograms is sufficient to cause a definite inhibition of the binding of the labelled material. Now, it's remarkable when one thinks that a picogram is to a gram as, for example, a millimetre is to the distance to the moon, or a farthing is to our gross national debt. Indeed, when we get down to the level of femtograms and picograms, it is easier to think in terms of numbers of molecules. For example, 1 femtogram of growth hormone is 32 000 molecules.

<Landon narrates over chart illustrating radioimmunoassay for deoxycorticosterone. Chart is superimposed, in turn, with molecular structures of deoxycorticosterone, progesterone, corticosterone>

Now, a second advantage, which we might just mention, is specificity. This line here illustrates a radioimmunoassay set up for deoxycorticosterone, where labelled deoxycorticosterone and unlabelled standard deoxycorticosterone are incubated with antiserum directed against deoxycorticosterone. And one can see that one gets a very marked degree of inhibition of binding with quite small amounts like 64 picograms of deoxycorticosterone. This assay can, in fact, now measure quite competently 1 picogram. Now, if we now take progesterone, which differs only in the absence of one hydroxyl group, it can be seen that if this is incubated with the labelled deoxycorticosterone and the antibody against deoxycorticosterone, it requires 60 or more times as much progesterone to cause inhibition.

If we move to the next example, which is corticosterone, which contains an additional hydroxyl group, which might be regarded as a piece of solder in the part of the key, then it no longer really fits the lock and it requires some 200 times as much. If we add two additional hydroxyl groups, as we have in cortisol, then you no longer get parallel inhibition and you need some 2000 times as much to get any inhibition of binding at all.

00:25:23:12

<Landon to camera and then over slide listing disadvantages of radioimmunoassay, and then back to camera>

I think we should also mention, of course, that radioimmunoassay has certain disadvantages, which I have summarised on the next slide. It is complex and repetitive. There are certain delays in obtaining results. It employs radioisotopes with their slight danger. There are certainly high costs of introduction and development, for example, in buying the counters. And, perhaps, most important, results obtained

depend on the antisera used and are liable to specific inhibition or liable to non-specific inhibition. Dealing perhaps with the latter, we can say that anything which interferes with the binding of the labelled material by the antisera will be measured as the hormone or antigen being assayed. For example, a very high concentration of urea or of sodium chloride will interfere with such binding, and if one does not make corrections or avoid this, then you may think you have hormone present when you do not.

Now similarly, a radioimmunoassay, the antisera does not bind with the whole of the antigen but only with part – termed the antigenic determinant. In the case of a peptide, this may only be some 3 to 8 amino acids long. So if one has fragments containing that antigenic determinant, or if another peptide contains the identical sequence, then this will be measured in the assay. This explains why it is possible to get dissociation between bio- and immuno-activity if one uses a bioassay and a radioimmunoassay. Perhaps, I could give as an example of this and as an example of why antisera against the same hormone may give different results, some experiments performed with antisera directed against arginine-vasopressin.

<Landon narrates over chart listing structural differences of various hormones>

So, if we now go on to this chart here, we see here the structure of arginine-vasopressin, which is the antidiuretic hormone of man. Here, we have lysine-vasopressin, which differs only in the amino acid residue in postion-8. Here, we have phenylalanine lysine-vasopressin, here, ornithine-vasopressin. This is oxytocin, which differs only, again, in postion-3 and in postion-8. And finally, the material arginine-vasotocin, which differs only in postion-3. So you'll see that most of the changes in the molecule which we're seeing here are at position-8, but that in others involve either position-3 or position-2.

<Landon narrates over charts of immunoassays, interspersed with talk to camera>

Now, if we move now to a consideration of two antisera raised in two different rabbits and determine the degree to which these various vasopressin analogues cause inhibition. You can see here that arginine-vasopressin, lysine-vasopressin, phenylalanine-vasopressin and ornithine-vasopressin all cause inhibition. This tells us, therefore, that the antigenic determinant, against which that particular antisera is directed, does not involve postion-7 of the molecule. Nor can it involve position-2 because here we have a phenylalanine in position-2. However, it must involve postion-3 because oxytocin, and in particular arginine-vasopressin, differs only in postion-3. So we could say that the antigenic determinant to that particular antisera is positioned between 3 and 6.

If we take, however, another antisera, this is a rabbit – the p actually stands for piddles because so powerful were the antisera that they neutralised its own antidiuretic hormone and therefore it developed diabetes insipidus, and therefore you picked it up only at your own risk. But we see here that basically only arginine-vasopressin inhibits. Lysine-vasopressin does not, that is to say, that antigenic determinant must have been directed against part of the molecule which included position-7.

00:30:40:14

Now, if we applied these, as we have done, these two antisera to the measurement of urinary vasopressin, the results obtained with this antisera are 3 times or more those obtained with this <*indicates Rp*>. This is because fragments with any loss of the C-terminal end won't be measured by this assay, but they will by this assay.

In fact, the one which measures most fragments of urine is, in fact, the more valuable. Now, you might think that when we hear of all these disadvantages of the danger of non-specific inhibition due to osmolality, or differences in pH, or we hear to specific inhibition due to fragments or to other peptides, which contain the same antigenic determinant, that this would occasion great problems. In fact, this is very seldom the case, so that if we take this next slide of a plasma assay of arginine-vasopressin, <*next chart*> we see this was an experiment involving a sheep which

was bled; with the drop in blood pressure, one sees a rise in plasma arginine-vasopressin and that the rise of immunoreactivity parallels that of bioactivity. Indeed, what one finds is that non-specific inhibition very seldom occurs, except in urine, and that providing care is taken, the results of bioassay and of radioimmunoassay will be the same for pituitary or organ extracts, the same for blood or nearly the same, although they may frequently vary with urine with these metabolites. Now, it finally remains to consider the likely developments in the future with radioimmunoassay. And I have summarised some of these on the next slide.

<Landon narrates over slide listing possible future developments in immunoassay techniques>

Not mentioned is that, under political pressure, the organisation of the radioimmunoassay services in this country are being improved so that the clinicians, irrespective of where they work, can have access to these valuable assays in the management of their patients. Now, this will obviously lead to a large increase in workload and I think one can be certain that this will be met, as has the routine analytical workload, by improved automation and data handling. Already the Analmatic type of approach has been improved on by machines like the Micromedic, and now fully automated equipment is becoming available, such as that developed by Dr Bagshawe.

At the same time, an ever increasing range of kits will be evolved, with the quality control of the reagents being guaranteed by the manufacturers, which will supply to the smaller lab all the reagents they require to carry out these complex assays. I think, equally certain, new assays will be developed at an ever increasing rate and we will see the further diversification of this technique into new disciplines, such as microbiology and even more so into oncology. We will also, I think, see improved analytical techniques, thus probably, in time, those compounds which are at present labelled with tritium or carbon-14 will have gamma emitters introduced because of the much greater ease of counting. And finally, probably, radioisotopes will be done away with and the antigens will be labelled with phage[?] or with enzymes. Only one

thing is certain, however, and that is that at some time in the future, a new and still more sensitive and still more specific technique will replace radioimmunoassay.

<Landon to camera>

Now, in conclusion, I thought it might be quite a good idea to show you, first of all, a short film sequence of how it all started.

<Landon narrates over brief film footage of chromato-electrofluoretic system and then to camera>

This shows the chromato-electrofluoretic system that the late Saul Berson and his colleagues employed in 1956, when they were studying the antibody production in patients with diabetes treated with insulin. What they did was to apply radioactive insulin, which had been incubated with sera, to a strip of paper and then under chromatographic and electrofluoretic forces, the free insulin remained adsorbed to the paper at the origin, whereas any which had become bound by antibody migrated towards the centre. It's really astonishing to think that from this small beginning there most now be several thousand laboratories, throughout the world, involved in this type of assay, such as our own seen in this next sequence.

<Landon narrates over brief film footage of laboratory with scientists at work on assays >

Now any lab of moderate size can perform 40 or so assays with really quite simple equipment, providing they have the counters available. Mind you, as the discerning physician and psychiatrist will have appreciated, basically, radioimmunoassays are a form of occupational therapy designed to solve the unemployment problems of the graduates of this country.

<End credits>