Some applications of Radioimmunoassay

The Scientific Basis of Medicine

With Professor John Landon, St Bartholomew's Hospital Medical College.

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<Opening titles>

<Landon to camera>

In my first talk, I discussed rather briefly the basis of radioimmunoassay and the advantages and disadvantages of this and related assay techniques. One of the tragedies to the laboratory worker is that after a time his clinical and other colleagues insist that these techniques should be applied and that the laboratory worker must cease working on the nuances of the method. This talk, therefore, deals with some of the applications of radioimmunoassay. Most people think of radioimmunoassays as being of primary use for the endocrinologist and for the assays being predominantly for the protein and peptide hormones. In fact, this is not so and the major use of this technique will be in other fields as illustrated in the first slide.

<Landon narrates over slides listing uses of radioimmunoassay>

Certainly, at present, most assays are within the province of endocrinology, but we will see how the technique is infiltrating into the clinical biochemistry laboratory and, at a much slower rate, into basic departments of biochemistry. Other branches of pathology, such as haematology, are now using it widely, and we can see already the impact it will have in the pharmaceutical industry and in clinical therapeutics. Finally, there is tremendous promise of the use of this technique in oncology, particularly in the screening of well subjects. If we can now have the next slide: this just summarises, for example, how the pharmaceutical industry are beginning to use radioimmunoassays, first to decrease their need for the rather complex bioassays; second, whereas I said in my first talk of the difficulties of the small laboratory raising its own antisera and labelling its own material, many of the pharmaceutical companies are providing reagents, usually in the form of kits.

Finally, I think it inevitable that radioimmunoassays will be developed for an increasing number of drugs because it is well known that the iatrogenic disease induced by drugs is one of the major problems facing the medical profession at this time. Thus, in the next slide, we see that radioimmunoassays have already been evolved for digoxin, for digitoxin, and for drugs such as barbiturates, morphia and cannabis.

<Landon to camera>

Now, it is impossible to go through all the assays and the applications that are available. On the last count, there were more than 200 radioimmunoassays for different compounds and so rather than do this, I thought I would take examples from our own laboratory and stress at the beginning that none of this work is my own. Now, again, to subdivide it and therefore make the talk more easy to follow, could I have the next slide.

<Landon narrates over slide listing types of hormones and proteins for which radioimmunoassays are used>

I'm going to deal in the first half with the application of radioimmunoassays for the peptide and protein hormones, and then in the second half with assays for other proteins, for the non-protein hormones and for other non-proteins.

<Landon to camera and then walks towards charts on display stand. Refers to and narrates over graphs showing results from various assay studies, interspersed with talk to camera>

Dealing now, then, with radioimmunoassays for the peptide protein hormones and the applications, as illustrated by our own laboratory work. In the beginning, Yalow and Bersen had studied the incidence of antibodies to insulin in patients with diabetes being treated with this. And this illustrates how patients being treated for diabetes insipidus with pitressin, in fact, develop quite high antibody titres against the contaminating neurophysin present in the pitressin. We can see here, for example, that this patient, patient F, had higher antibody titres than did the best rabbit that we have immunised in developing the assay for the material.

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Now, the incidence of antibodies may not be without clinical importance. <*Next graph*>That's if we look at this slide, which was from some work done by Dr de Mowbray, we see that when we give a single intravenous injection of insulin to normal subjects, there is a rapid fall in blood sugar and then a rapid return to the base line by amount 90 minutes. In the insulin treated diabetic, however, almost certainly, because of the antibodies which are circulating and which bind the exogenous insulin, the response is much slower and much more prolonged.

Now, going on from the use of the technique in the measurement of antibody levels, we can consider the use of the technique in the measurement of hormone levels in tissues. <*Next graph*> And this was some work done by Dr Ratcliffe and what has happened is that he, first of all, extracted a large number of control tissues, such as skeletal muscle and liver, and determined the number of micrograms of ACTH per gram of tissue. Then he showed that in the tumours of patients who presented with

ectopic ACTH syndrome, there were very much higher levels, perhaps of most interest, and in contrast to what had been said by others, even control tumours have significantly elevated ACTH levels. Thus, one could conclude that the development of the ACTH ectopic syndrome must depend not only on the synthesis of ACTH but also on the release of this material from tumours. And one assumes that here the tumours were not releasing it, or the ACTH was being very rapidly degradated by proteolytic enzymes before its release.

<*Next graph*> Now, this illustrates how you can use a radioimmunoassay in experiments involving tissue culture, done by Dr Scott, and what has happened is that assays for ACTH have been used in which antibodies have been directed either against the C-terminal, biologically inactive part of the molecule, or against the N-terminal, biologically active part. And one can see that if one cultures rat anterior lobes that you get a rise in the media at a slow rate of ACTH, and that since both C- and N-terminal activity has risen at an equivalent speed, one can presume that this ACTH as we know it, the entire molecule. When, however, the posterior lobes and pars intermedia were cultured, then we see a great disparity. Very little N-terminal activity is seen, whereas there is a very rapid and huge increase in the levels of the C-terminal activity. And this, in fact, led Dr Scott and Dr Radcliffe to the conclusion, which they have since verified, that there is a intermediate lobe peptide which is corticotrophin-like which had not previously been realised.

Now, obviously, the major use of this technique is in its application to blood and, for example, once the assays are available then they can be used to determine physiological control mechanisms. <*Next graph*> This illustrates experiments that I did, some long time ago, with Professor Greenwood, where we were interested in whether growth hormone was under a stress-control mechanism. Professor Greenwood, who had to go to the dentist, had an indwelling needle put into his vein and as he entered the dentist's hall, you can see that stress was causing growth hormone secretion to rise, and despite the fact that all that the dentist did was to look at his teeth – a drill was never used – you can see the astronomic rise in growth hormone levels which didn't fall until he had left the dental surgery. You can also see

that even to me as the person taking the blood samples from Dr Greenwood, this was a traumatic experience when I wasn't even in the dental chair.

So this has allowed many control mechanisms for hormones to be worked out where, for example, there were no known control mechanisms for growth hormone until radioimmunoassay was developed.

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<*Next graph*> Now, this was some work, again, on the physiology of oxytocin performed by Dr Chard in our laboratory, where we were interested, or he was interested, in the role of oxytocin in the initiation of labour. And it was known that in animals the mother raised her circulating oxytocin level, for example, in the sheep or the goat, and that this was associated with increased uterine contractions and with the birth of the child, or in that case – the young animal.

When we came to the human, Dr Chard first found that there were no levels detectable in the maternal circulation. And after that, fortunately, went on to measure the levels in the cord blood from the foetus itself. And one sees here that really quite high levels are detectable in the umbilical artery going to the placenta and much lower levels returning from the placenta to the foetus. And so this is possible evidence that, in the human, the foetus has taken over the control of its own birth.

<*Next graph*> Now again, these illustrate the experiments that can be done using now the recently available releasing hormones, where one can inject intravenously a single injection of 50 micrograms and determine the luteinising hormone and the follicle-stimulating hormone response to this material. And, of course, another factor is that radioimmunoassays are just as applicable to animals, and so Dr Rees had set up an assay for rat ACTH and, again, this can be used to determine the half-life of the rat ACTH following a hypophysectomy.

Now, in addition to applying the assays to tissue, or to tissue culture, and to blood and in determining physiological control mechanisms, obviously, the clinician

requires that they are used for diagnostic purposes. And these, again, can be used, of course, in blood or they can be applied to urine.

<*Next graph*> This, for example, could be regarded as a physiological study, where a normal subject is given a large water load, urine samples are collected at hourly intervals, and the decrease in urinary AVP determined. And one can see that due to the decrease in osmolality, AVP ceases to be secreted by the hypothalamus, in the posterior pituitary, and this is reflected by a decrease in urinary AVP. So that could be our physiological experiment and then, of course, once you know this, you can then apply it to a clinical situation. <*Next graph*> And this was a patient with a bronchial cancer, who had inappropriate ADH secretion and this was shown not by raised urinary AVP levels – because these are, in fact, within the normal range – but by the fact that this autonomous tumour did not decrease its rate of secretion with a decrease in osmolality, and therefore you see urinary AVP levels being relatively unchanged despite the water load, so this could be used in the diagnosis of inappropriate AVP secretion. And these were experiments done by Dr Edwards.

Then, in addition to diagnosis, for clinical purposes the assays can be used for monitoring the effects of therapy. <*Next graph*> And here we see a patient presenting with, again, a bronchial tumour, which was secreting ACTH so that the levels were way above the normal upper range, upper limit of 80, into over two and a half thousand. Having obtained a baseline, one can then give irradiation with cobalt 60 and determine the effect on the tumour by watching the ACTH secretion from the tumour decreasing and, indeed, continue the therapy until the levels have returned to normal, and this is shown here.

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Now, there is no doubt that, from a practical point of view, applications in general endocrinology have been valuable, but the numbers of cases – ectopic ACTH production, or with an inappropriate ADH secretion, or even with acromegaly and diseases like that – are very small. It was important, we thought, therefore to use the technique to measure the hormone in an important situation. Now, when we realise

that in the last few months of intrauterine life and in the few hours of its birth, the human foetus is at a greater risk of morbidity and mortality than at any time for the next 40 or more years, it seemed to us that a concentration on techniques to assess foetoplacental well-being would be well worthwhile.

<*Next graph*> So Dr Chard and Dr Letchworth decided to set up an automated radioimmunoassay for human placental lactogen, which is a protein hormone made purely by the placenta, and once this assay was running and was automated, they were then able to determine the range of values that one got during the latter part of pregnancy, which is related to the mass of the placenta. This was done in a very carefully performed prospective study involving several hundred normal women. Now, once one has obtained this range of normal, it is then possible to use the assay to detect abnormality. So if we could have the slide, this will demonstrate one such study.

<Landon narrates over slide showing results of radioimmunoassay study for HPL in pregnancy>

For example, it was shown that if you took pregnancies which were clinically entirely normal and you then followed the well-being of foetus once it was born, of the neonate, that some of the these children develop neonatal asphyxia or foetal distress. And it's shown in this slide that this could have been predicted despite the fact that the pregnancy was in all other ways normal. In other words, if three serial estimates of human placental lactogen had been below 4 micrograms per ml, then there was a 71% chance that that child would have foetal distress or neonatal asphyxia. In contrast, if three or more serial determinations were more than 5, then the risks were only 4%. And this, therefore, would enable the obstetrician to get the help of his paediatric and other colleagues before the birth so that to be ready for any eventuality.

<Landon to camera>

The second half of this presentation, which has the advantage of having been done after lunch, is dealing with the more important aspects which, to my view, are the applications of radioimmunoassay, that is to say, their use outside endocrinology, although, at the same time, we will be considering some of the hapten endocrine investigations that are now possible.

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Dealing first with non-hormonal peptides and hormones, may we have the next slide?

<Landon narrates over slide listing various plasma proteins for which radioimmunoassays have been set up>

Radioimmunoassays have already been set up for a large number of the plasma proteins and these demonstrate both the specificity and the sensitivity of the technique. For example, a radioimmunoassay for human albumin, it has been shown that you need 50 000 times as much bovine albumin to interfere with the assay. Similarly, a radioimmunoassay for IgA has enabled the demonstration that IgA can be found in cord blood. There are now some 10 radioimmunoassays for enzymes, and this is of great use in clinical chemistry, particularly as they can often distinguish easily between various iso-enzymes and they measure mass rather than activity so that there is no problem from the presence of enzyme inhibitors. The other advantage, of course, of measuring mass is that if you are no good at mathematics, you avoid the problems of enzyme kinetics.

Radioimmunoassays are now, of course, available for the carcinoembryonic antigen and for α-feto-protein. And these serve to show that in the future, tumour-specific antigen assays will become commonplace, and one hopes will eventually enable the screening of well populations to exclude or else to diagnose in its early stages a variety of cancers. Similarly, they can be used in the investigation of clotting mechanisms.

<Landon walks to charts on display board. Refers to chart showing results of radioimmunoassay of IgE and narrates over it>

Now for my own examples from our own lab, I am first going to take a radioimmunoassay of IgE. This can be used, I think, to demonstrate two features: one that a radioimmunoassay can easily give false results if you are not aware of what it measures and, two, that if you have an automated and simple assay capable of large numbers of samples throughput, then you can often come up with new observations. This was work done by Dr Merritt and Mr Jacob, where IgE levels were measured in a normal population and 100 normal subjects, and in 50 patients who had had allergic diseases, such as hay fever or eczema. And you can see, as was well known, that IgE levels are raised in this situation.

Now, for the first time, we extended this into the investigation of IgE levels in cancer on the basis that it would seem unlikely that one had IgE merely to allow you to be allergic. And one sees in this group of some 200 hundred patients with cancer, who had been treated, that the IgE levels were low. Now, that is possibly not surprising in that the treatment may well have impaired the synthesis of IgE. When, however, we deal with these two populations, who had untreated cancer, we find an interesting observation: namely, about half of them have very low levels of IgE, whereas about half of them appear to have astronomically high levels.

Now, in a radioimmunoassay all that is being measured is the inhibition of binding by a labelled material by the antibody, so that if you inhibit binding by, for example, high osmolality or a low pH, this will be measured as the substance that you are trying to measure. What is apparent, we think, is that in early cancer, IgE levels are low. As the cancer advances, it releases into the circulation an inhibitor which stops the binding of IgE onto the cells and, of course, this inhibitor by also stopping the binding of the labelled IgE by the antibody is measured as very high IgE levels so that when these samples were then assayed by a Mancini technique, they were seen, in fact, also to be very low. So this has led to two observations, one that in early and untreated cancer, IgE levels are often low, and that the cancer itself is secreting an inhibitor which may be protecting the cancer against IgE.

<Landon narrates over flow chart of clotting mechanism>

Now, another example, where we have gone, is in the question of clotting. Chemical pathologists are perhaps very aware of the need to help our haematology colleagues. They, you know, look at clotting by shaking things up and using a stopwatch, and really in 1972 this seems remarkable, and so we thought we would look at clotting which is a complex subject, but which, in this particular instance, for example, is being based on proteins. So, for example, fibrinogen, of molecular weight of 300 000, is known under the action of thrombin to be broken into fibrin and fibrinopeptides; and that under plasmin these are broken down to substance X and A, B and C; and that X in turn breaks down to Y plus an end product D, with the molecular weight of 83 000; and that Y in turn breaks down to a further D and to another end product E, with a molecular weight of 55 000.

And this is the classical sort of situation where radioimmunoassays can be set up. And so to set up radioimmunoassays for fibrinogen and, say, for fibrin and perhaps for an early product like A and for a late product like D and E, it is only really the work of about a year. And assays are, in fact, already available for fibrinogen and thrombin and plasmin.

<Landon refers to and narrates over graph of assay for fibrinogen>

And this lower slide shows one of our assays done by Dr Gordon for fibrinogen, which allows the very rapid and accurate determination of fibrinogen levels. Now, one may hope that by setting up of specific assays that one may be able to investigate clotting mechanisms in a variety of situations which it is so obvious is needed in medicine.

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<Landon narrates over slides, interspersed with talk to camera>

Now, if could have the next slide, we can pass over from the other protein assays to the hapten assays. This is to say, assays for materials which do not themselves induce an antibody response and which, therefore, you have to conjugate to a larger material, such as albumin, and so that they then act as a hapten and antibodies are raised against the material that you have added. And radioimmunoassays now are available, for example, for triiodothyronine, for thyroxine and for such releasing hormones as thyrotrophin releasing hormone.

<Slide>

Testosterone
17-β Oestradiol
Progesterone
Aldosterone

And on the next slide, we can see that these have also been applied to a great majority of the steroids. So, for example, in our lab, assays are available for testosterone and aldosterone. And the application of assays for oestradiol and the other oestrogens will, of course, parallel the human placental lactogen work in the assessment of foetoplacental function.

<Landon to camera and then walks towards charts on display stand. Refers to further graphs showing results from various assay studies and narrates over them, interspersed with talk to camera>

Now, taking, for examples, some of these assays: this demonstrates a radioimmunoassay which we have evolved for thyroxine, which we applied to the urine, which had not been done before. And this was, again, work done by Dr Besser and Vivian Chan. And this enables the very easy assay of large numbers of 24-hour urine collections, and we see that in euthyroid men there is virtually no change throughout the month, whereas in euthyroid women there was this rise during menstruation. In the much more common group, perhaps, now of women who are on oral ovulation inhibitors, one sees that there is a much more marked rise during the period that the pill is withdrawn. Now, none of these changes had been observed

before and it is now apparent and other experiments in men have shown that withdrawal of oestrogens is invariably associated with a marked rise in urinary thyroxine, which couldn't be detected if you were to measure the plasma thyroxine. Since it is the plasma thyroxine, some 999 molecules in every 1000 are in the unphysiologically active bound form, whereas the free form, which is reflected in the urine, does show such changes.

<*Next graph*> Another example on this chart is a radioimmunoassay for triiodothyronine, and here which will differentiate quite easily between thyroxine and triiodothyronine despite the fact that there is only the difference of one iodine atom between the two molecules. And this, again, when applied to urine, as you can see, allows a very good differentiation between hypothyroidism, euthyroidism and hyperthyroidism. And it's particularly valuable for the T3 thyrotoxics. You can also see that if you have increased thyroxine-binding globulin levels, which are associated with raised total levels of both T3 and T4 in the plasma, that these are not reflected in the urine, which, of course, is an index of the free fraction. So this and this are exactly in the same range.

<*Next graph*> Now, moving on to an example taken from steroids, one of the great problems that many women face in this country is the problem of hirsutism because whereas in the Mediterranean or in Persia the presence of a large amount of facial hair is a desirable characteristic, in this country it is not. And this was an investigation, which was done by Dr Anderson, measurement of the plasma androgen levels. We can see here that normal men, of course, have very much higher levels than do normal women. This explains why men grow beards. When, of course, we come to the hirsute women, we see that there is no real significant difference between those of the hirsute and those of the normal, so one might say, right then testosterone has nothing to do with the development of hirsutism, but, as Dr Anderson has shown, this would probably be unwise because, again, when we measure total levels, we are measuring the physiologically inactive as well as the physiologically active. So that you can only consider things if you also know what is happening to the binding protein. <*Next graph*> And in this lower slide, what we look at is the levels of sex hormone-binding globulin in normal men and in normal women.

Women have a much higher level of sex hormone-binding globulin; in consequence much more of their testosterone will be in the bound form which will further protect them against the effects of testosterone. Now, in contrast, the hirsute women have lower levels of sex hormone-binding globulin, and a combination of a normal female testosterone level but with a much lower sex hormone-binding level will mean that they have a raised level of the physiologically active free testosterone, which might therefore explain their hirsutism.

00:31:57:02

<Landon narrates over slide>

<Slide>

Drugs – digoxin
– morphia
Vitamins – B12 & D
Cyclic AMP

Now, if we have the next slide, we can just pass now briefly on to a consideration of those assays which are available for non-protein non-hormones. That is to say other haptens. And radioimmunoassays, as we showed earlier, have been evolved for many drugs such as digoxin, morphia, cannabis. Radioimmunoassays and related techniques have been evolved for vitamin B12 and vitamin D. And with the radioimmunoassays of things like cyclic AMP, by first conjugating them to a larger protein, we can see its entry into standard fundamental biochemistry. And these are the sort of assays which one can be certain will increase in use over the next few years.

<Landon refers to charts showing results from assay for digoxin, and narrates over it>

Now, taking as our final example such an assay: here is an assay run in our laboratory by Mr Howard on plasma digoxin. And he and Dr Chamberlain showed

that this could be valuable in what can be a difficult situation of differentiating between a person on digoxin, who one doesn't know if their signs and symptoms are due to too little digoxin, or to too much digoxin and toxicity. So by measuring the plasma levels, one can see that levels of above 3 [ng/ml] invariably indicate toxicity, levels between about 2 and 3 may indicate toxicity, and levels of below 2 virtually exclude toxicity and indicate that they may well be being undertreated, particularly if they are low.

Now, the wide scatter that one sees here, of course, reminds us that a radioimmunoassay for a drug will not be the answer because the only part of the body where there is no value in measuring the drug in some ways is the blood. What we want to know is how it is acting at its site of action. <*Next graph*> And one can see here on this slide, a comparison made between plasma levels and the levels in skeletal muscle, and one can see they're very much higher. And then, when the cardiac surgeons were removing bits of cardiac muscle prior to putting in valves, one could see that the levels in the cardiac muscle could be anywhere between about 30 and some 200 times higher than are the levels in plasma. And this does serve to say that, of course, just the measurement of plasma alone might lead one into troubles.

<Landon to camera, returns to seat>

Well, I think that will serve as the examples of radioimmunoassay. And therefore, one might sum up and say, right, I think, the first talk we emphasised that radioimmunoassay was a highly specific and a highly sensitive analytical tool, and I hope in the second talk to have demonstrated its widespread applicability. And I hope also in the second talk to have shown how likely it is that the technique will now be used in many other spheres outside endocrinology.

As a clinical chemist, of course, one is always hoping to decrease the number of interfaces between the patient and the laboratory. For example, if a patient has hypothyroidism, there are many steps before the laboratory finally helps make the diagnosis, or indeed finally makes the diagnosis, although Papworth would not agree. Thus, a patient has to realise he is ill, or his relatives have to realise he is ill,

the patient then has to see his general practitioner and he has to realise that he is ill, the general practitioner then has to send the patient to the appropriate consultant because it's a very unfortunate occasion where somebody, for example, with hypothyroidism was sent to the wrong clinic at the hospital. The consultant then has to make the diagnosis and only then can he send the blood sample to the lab, which will, of course, in our view, prove the diagnosis. And I think with radioimmunoassays and other analytical techniques, what one can do is remove all interfaces.

Eventually with good screening of the well population, you can remove the patient, the GP and the consultant. And bleeding of the whole population and the sending of the samples straight to the laboratory will allow screening when the patient is still well so that he can take steps to avoid, for example, high lipid patterns, or when a disease in its earliest phase, when he can therefore be treated with much greater chance of success. And it's, perhaps, on this that one would say that the future of medicine lies in the laboratory and not at the bedside. Thank you.

<End credits>