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GENERAL DISCUSSION

Cooke: I'd like to ask a general question regarding the relative capacity of the g.l.c., the protein-binding and the radioimmunoassay methods for testosterone. Dr. Collins, you said your capacity is 12 samples in 3 days, which is rather surprising because radioimmunoassay is supposed to be very much faster than the other methods. Are these single determinations on one plasma sample?

Collins: In our radioimmunological methods, we extract each plasma sample and then remove aliquots in triplicate for equilibration with antiserum. The mean value of [^{3}H]-testosterone bound is used to obtain the reading from the standard curve. It is possible to shorten the assay by sacrificing a little in resolution and specificity, for example, by equilibration with antiserum for shorter periods of time and by omitting the chromatographic step. At the moment we are trying to shorten the method by using a gamma emitter in place of [^{3}H]-testosterone.

Grant: May I point out that we seem to be leapfrogging methods. It is interesting to hear Dr. van der Molen comparing gas chromatography with competitive protein binding, but one might ask what are we *doing* with these methods? For instance, Dr. Grenier suggested that some of his methods might be used to study sub-fertility in patients. You can use oestrogen determinations for monitoring foeto-placental well-being, or for studying sub-fertility. Two groups in Glasgow, in my department and in another laboratory, have auto-analyser procedures which give the clinicians all they need to know about oestrogen measurements in urine for sub-fertility studies, at a rate of at least 20 specimens an hour. The other group claims to be measuring non-pregnancy urinary oestrogens by auto-analysers at many specimens per h. I think these methods are needed for research, but please let us be clear, what we are developing the methods for. Let us hope that all this time we are spending is going to be useful.

Van der Molen: I agree completely with Dr. Grant that apart from the clinical problems our research creates too many samples for our present capacity.

Dr. Ekins, when may we, in practice, expect to profit from the theoretical discussions which you have given on precision, accuracy, etc.? I think that too many of us are still estimating what we consider the accuracy and precision of methods by running multiple samples and replicate analyses on the same samples, etc., and just by trial and error we hope to gain our information about accuracy, precision, etc It is a figure which normally, I think, does not go lower than 5–10% for these analyses. I wonder whether you have any comment as to how we can take more advantage of the theoretical background than we are doing at the moment and whether you expect that such theoretical considerations could increase the efficiency of techniques such as protein binding and immunoassay as compared with their present performance.

Ekins: First of all, the fundamental feature about a radioimmunoassay 'theory' is that it should be conceptually correct, in contrast to the precepts which underlie the approach of many experimentalists in the field and which are basically erroneous. In other words, when people set up their assays and base their assay design on the slopes of response curves alone and so forth, they are doing something that is conceptually wrong and which can lead them into more experimental effort and

yield poorer results than is necessary. So in practice I firmly believe that theoretical analysis can be helpful; I am thinking very much in this context of the measurement of cyclic AMP. We have recently developed an assay for this compound using a specific binding protein found in adrenal tissue. Two publications, including that from our own group, have appeared in the last year. A second group (which is very well versed in the isolation and use of this protein) have likewise devised an assay based upon it, though their system appeared to be some 20 or 30 times less sensitive than our own which was set up strictly on the basis of the theoretical concepts that I have been describing today. Now I think it would be true to say that this particular group was relatively new to protein-binding or radioimmunoassays, so perhaps it is a little unfair to compare the sensitivities we have respectively achieved using a theoretically rigorous as opposed to a relatively intuitive approach. When an experienced assayist sets up his method, he implicitly must do what we have attempted to formalize in our analysis, and slowly, in the course of trial and error, he will eventually attain the sensitivities and precision that we can predict that he should obtain on the basis of our theoretical models. In short, perhaps the major advantage of this kind of approach is that one is able to set up optimal assay systems very much more quickly than might otherwise be the case

Rodbard: Dr. Ekins has just answered the question with regard to assay design. I'd like to attempt to answer it from the point of view of data processing. We've previously reported a model for predicting 95% confidence limits for unknowns, so that, without running samples in duplicate or triplicate, we can still obtain 95% confidence limits for unknowns, by analysis of the scatter of the points on the standard curve around the least-squares regression line. Thus, in our laboratory we routinely run samples in a single determination, and obtain the confidence limits for that sample from our computer output. Then we take a small number of samples, (preferably a random sample of about 5–10% of the tubes in the assay), and run them in duplicate, and in several assays. In this way we obtain an empirical estimate of both the within-assay and the between-assay variance. Then we check that the empirically observed precision agrees with the theoretically predicted precision, and I'm happy to say, it usually does. Of course if there were a discrepancy, we would certainly take the empirically observed precision as our estimate. Accordingly, I think that we are now at a point where we can apply the theoretical models and obtain a considerable saving in terms of time, money and number of tubes per assay. (Rodbard, D.: In *Principles of Competitive Protein Binding Assays*, (Edited by Odell, W. D. and Daughaday W.), Lippincott, Phila., (1970) Chap. 8.)

Ekins: I'd just like to add a little to my answer to Dr. van der Molen's question: that is, that one of the advantages of these theoretical models is that one is enabled to predict, for example, how precision will increase or decrease in response to a change in a particular assay parameter such as the counting time expended for each sample tube. Very often it can be demonstrated that a reduction in counting time by a factor of two or seven of ten will have a surprisingly small effect on assay sensitivity or the precision of one's measurements. This kind of information can obviously be of great practical benefit in the circumstances in which many of us find ourselves, with great pressure on our counting pressure on our counting resources. It really can be quite advantageous to use one counter in an optimal way rather than to buy an extra ten counters.

Van der Molen: I just want to ask Dr. Ekins and Dr. Rodbard, whether they have

come across any application yet which does not conform to their theoretical predictions? The same considerations applied 5–10 years ago when the theoretical precision and accuracy of what you might achieve with regard to chromatographic tracings, etc. was well known. I have seen very few biological samples which conform exactly to the theory, apart from the ones where you work almost with pure steroids, because of the very high concentrations. With respect to protein binding or immunoassay I would like to know therefore if you think that the theory will always agree with the actual practice in analyzing unknown samples from biological sources? So as not to misinterpret my question, I do not doubt the value of the theory, but how does it hold in actual practice?

Ekins: All I can say is that the theory is as good as the model. In short, if there are unknown, unsuspected, steroids in our samples, or there exist other contaminents which affect the assay system without our knowledge, then our model is inadequate, and the predictions which it yields will be wrong, since the very fact of a deviation from the predicted result can lead the experimenter to search for factors which would otherwise pass unnoticed. In practice, though, we find that our theoretical predictions regarding experimental design correspond well with experimental findings.

Rodbard: Fortunately, we do obtain very good agreement most of the time. I would certainly acknowledge that there are situations where there are discrepancies. The reason we do obtain such good agreement is that we have a sufficient number of empirically observed constants built into the models, such as the coefficient of variation for pipetting errors, and the coefficients of variation for misclassification errors, (errors in the separation of bound and free). These constants are estimated on the basis of empirical data: if these parameters are accurate, then the results of the theory should be correct.

Exley: Whilst I agree with all these discussions on the theoretical side, and since Dr. Grant asked the important question, why are we hopping from method to method, I think we need to just say a word about the practical side. On the practical side we would like to see something simple like measuring glucose with a dipstick. In solid radioimmunoassay you coat your antibodies on the side of a little bit of polystyrene stick. You can then dip it in the solution for assay and then you can count it directly, and I think we're slowly coming towards the day when things can be computerized, and therefore when a good simple method is produced, there will be no necessity to hop from method to method. Unfortunately, competitive protein binding has suffered from the fact that not only were the affinity constants a little lower than those of some of the antibodies, but also the specificity was an inherent property of the binding site, and therefore we couldn't model or tailor it in the same way as we can antibodies. So at the present moment, the radioimmunoassay approach offers the best promise of being able to do something very simple very quickly. I believe the assays at the moment are in a stage of transition, and that is why we are hopping from method to method.

Kellie: I'd like to come back to something that Dr. Rodbard said earlier in the morning which is bothering me a little. He suggested that the radioimmunoassay could be carried out under three sets of conditions: one in which the radioactive ligand was mixed with the non-radioactive ligand and then incubated with the receptor to equilibrium; secondly, that one could incubate the receptor with the non-radioactive ligand and at a later stage add the radioactive ligand; and thirdly, that one could incubate the receptor with the radioactive ligand and add

the non-radioactive ligand later on. My difficulty is that it seems to imply that the radioimmunoassay can be carried out under non-equilibrium conditions, and that it may be better to do it under non-equilibrium conditions. Now there are at least two factors involved here: one is the question of equilibrium in a chemical sense, and the other of equilibrium in the isotope sense; and all the radioimmunoassay methods operate on the basis of the distribution of the radioactive ligand. I wonder if Dr. Rodbard could extend and explain precisely what is happening under these conditions.

Rodbard: A mathematical analysis of the kinetics of radioimmunoassays is given by Rodbard D., Ruder H. J., Vaitukaitis J., and Jacobs H. S., in *J. Clin. Endocr.* **33** (1971) 343. We find, as others have found empirically, that one can obtain a valid radioimmunoassay under non-equilibrium conditions. Under this set of circumstances, the bound/total ratio for the labelled ligand and the bound/total ratio for the unlabelled ligand may differ as a function of time. We have obtained computer simulations (utilizing both analog and digital computers) to describe the exact concentrations of all chemical species present, as a function of time. This model can also describe the situation when the labelled and unlabelled ligand have different affinity constants and/or different rate constants. We have also found excellent agreement between the "kinetic" theory and experimental results.

Munck: Do you in fact get true equilibrium under these conditions? Do you get the same results whether you start from one end or the other?

Rodbard: Yes, as reaction time becomes large or "infinite", one obtains the same final equilibrium results. In practice, the rate of approach to equilibrium differs considerably. When the labelled and the unlabelled ligands are added simultaneously, you approach equilibrium most rapidly. On the other hand, if you use delayed addition of the labelled ligand, it takes much longer to get to equilibrium, especially in the high-dose region. It is this differential effect which results in an increase in the slope of the dose-response curve. This will result in improved sensitivity and precision if the scatter around the curve remains constant. However, when you are working under non-equilibrium conditions, any minor variation in the duration or temperature of the reaction may significantly increase the scatter around the dose response curve. Accordingly, the improvement in sensitivity that is actually observed using delayed addition of tracer is less than one would expect by looking at the slope alone. Also, certain receptor proteins, such as uterine cytosol receptor, are stabilized by the steroid. In these cases, it may be self-defeating to utilize delayed addition of tracer.

Ekins: We have been interested in this particular subject for some years, and would have liked, had we had time, to have done the same analysis as David Rodbard and his collaborators have now done. I'd like to ask him; what are the optimal durations to yield maximum assay precision? Has Dr. Rodbard developed this aspect of the analysis at the present time or has he been solely concerned with effects on the response curve?

Rodbard: I believe it is advantageous to permit the "first" reaction, (unlabelled ligand and antibody) to go to completion, i.e. to equilibrium, followed by addition of labelled ligand. Of course, one never reaches perfect equilibrium, so that one has to settle for approximately 90–95%, equilibrium for the first reaction. At the present time, we do not have an optimization programme for the purpose of selecting the duration of the first and the second reaction. However, we can do simula-

tion studies, and these enable us to select the appropriate conditions. In practice there is very often a third incubation, i.e. an incubation with charcoal, florisil, etc. for separation of bound and free. Thus, if one is interested in minimizing the total duration of the assay, then it would be necessary to optimize the duration of all three of these incubations simultaneously. We have the necessary equations which enable us to do this. However, before this can be applied in practice we must obtain the rate constants for each of the reactions.

Munck: I would like to ask a general question which I think is in many peoples' minds: not everybody has a computer available, and not everyone who has a computer knows how to programme it for this kind of work. Is the theoretical analysis that you have developed available in such a way that people who have neither the computer nor the programmes, can use it? Can you put it into the form of, say, curves, so that somebody starting out could pick the best assay conditions from a curve, or use some fairly simple cookbook procedure?

Ekins: There's a very simple procedure that we have published elsewhere (Ekins R. P. and Newman G. B., *2nd Karolinska Symposium, Acta Endocr.* **64** Suppl. 147 (1970) 11.) for optimizing for best sensitivity, which is the practical requirement in many situations. One often wishes to make one's assay as sensitive as possible, even when one is faced with the measurement of relatively high concentrations of hormone. When radioimmunoassaying a steroid, for example, it is probably better practice to make the assay extremely sensitive, and to dilute the extract until it falls within the range of the assay as set up. This may have the effect of diluting out some of the non-specific factors that we have been discussing. Now, as I have said, there is a very simple cookbook procedure whereby anyone without a computer can design an assay to make it as sensitive as possible. The problem arises when one wants to measure a large hormone concentration. Then the equations become too complicated to be solved analytically. For example, the relationship between the error and the response metameter (which David Rodbard would predict, and we would look at experimentally) plays a large part in the determination of the optimal reagent mixture, and I can think of no practical way of selecting this other than by using computer methods. But the computer programs are relatively simple and straightforward – David Rodbard and ourselves have both got them for distribution – and they can be tagged onto a computer analysis of assay results of the kind that David Rodbard has previously described to give an automatic prediction of how to set up subsequent assays. In practice, the experimenter is required to do little other than state the target hormone concentration, the present specific activity of his tracer and so forth. I agree that it is a disadvantage that one cannot do these things without a computer, but quite a small machine will suffice, and access to time sharing facilities is becoming more widespread.

Rodbard: With the introduction of time-shared computer services, all you need is a telephone and a teletype terminal; this will enable you to communicate with a variety of commercially-available computer services which are now essentially ubiquitous. Also, virtually every medical school and most hospitals have adequate computer facilities. It isn't necessary for everyone to do their own programming, since there are programmes available to anyone who wishes to use them (in any event, *our* programmes are available). Computer processing of radioimmunoassay results provides speed, reliability, accuracy, and economy. It is much more economical to have a computer calculate your results from radioimmunoassay using least-squares methods, than it is to read the values from a curve by eye.

De Moor: In order to stay in the cookbook tradition, may I ask you if somebody could comment on the relation between sensitivity of the assay and affinity of the antibody you use, and—the second question—how to get antibodies as monocolonal, as homogeneous as possible?

Exley: Well, one of the problems is of separating out the various immunologic molecular species which are present in antisera. Some attempts are now being made to purify these by affinity chromatography. This is a difficulty procedure with the particular antisera we are using for the simple reason that once the antigen binds with the antibody we cannot separate it. This leads me to the second point, which is that although the specificity of the antisera can be tailored by chemical means, the actual maximum affinity constant you can get out of antisera can only be that of the species which contains the highest affinity constant, and you will probably be throwing a lot of your molecular species away.

Grant: I think at this stage it's worth saying in answer to Dr. de Moor, that the Medical Research Council in London has set up an office which will eventually be able to distribute tested antisera, and I'm sure the National Institute of Health will do the same.

De Moor: I was also thinking about how long you have to immunize your rabbits.

Fazekas, (A.T.A.): Why do you immunize a rabbit, and why not rat or other animal species?

Ekins: Personally, I would like to know the answer to this question because I am ignorant whether one species is more immunorespective than the other to certain classes of compound. At one stage we spent a lot of time trying to raise antibodies against the thyroid hormones T_3 and T_4, and we were initially very successful with one rabbit. Subsequently, we tried for about a year with many, many other rabbits, and were met with complete failure. Ultimately we acquired a small flock of six sheep and produced anti-thyroid hormones in five of them almost immediately. Whether in fact we were inadvertently using a very slightly different antigen or a more optimal schedule, I don't know because as so frequently happens this was not a particularly well controlled experiment. Nevertheless our observation was that sheep were much more responsive than rabbits, and I wonder whether this was entirely fortuitous?