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Report on the Discussion of the First Session

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DR. PRESSMAN: In connection with the emphasis on radioimmunoassay at this meeting I would like to make two comments: The first concerns methodology. The methodology which has been described and used here is primarily that using radiolabeled antigens. A large literature also exists concerning the application of radiolabeled antibodies which have certain unique properties and which have been extremely useful in radioimmunoassays. My second comment is in connection with the history of the radioimmunoassay method. The history extends back to Dr. Wormall (1), who just about 25 years ago in England reported the use of radio sulphur- and radio phosphorus-labeled antigens in radioimmunochemical studies. Then in 1948 several reports appeared on the use of radiolabeled antibodies by Butement (2) in England and by our laboratory at Caltech (5) and on the use of radiolabeled antigens by Warren and Dixon (6) and by Eisen and Keston (3). This year, therefore, I think really is more or less the 25th anniversary of the radioimmunoassay method.

DR. YALOW: Thank you, Dr. Pressman, for your comments. When you speak of the applications, in what systems were they measuring their antigens and their antibodies? Were they using them primarily for detection? Also, can you give us some quantitative aspects of some of their work?

DR. PRESSMAN: I did not really come prepared to present a paper. I came here to listen and learn. But Wormall *et al.* (1) used phosphorus-labeled ovovitellin which they prepared biologically, and they used sulphur mustard-labeled ovalbumin. They used the radioactive isotope as a tracer to determine quantitatively the amount of precipitate formed, thus creating a quantitative analytical method. Eisen and Keston (3) used iodine-labeled ovalbumin in a reaction with antiovalbumin, and again they used competition with ovalbumin. These methods are really quite similar to those which are being used here on a competitive method. On the basis of our work and the almost simultaneous report of Butement (2), preliminary studies were performed with radiolabeled antigens to establish the methods we used, and then we used radioiodinated antibody to detect the presence of particular antigens in tissues. These methods have since been used in a competitive basis type experiment. So there is a large background here.

DR. YALOW: Dr. Pressman's comments are quite interesting. If we are to introduce a historical note, we should perhaps designate this as the 42nd anniversary of what may be the original paper on the use of isotopic indicators for chemical analysis with the dilution principle by the creative and imaginative Nobel Laureate, Professor Hevesy (4). There has since followed a long and interesting record of the application of the dilution method (with isotopic markers, radioactive or non-radioactive or with non-isotopic markers such as dyes *etc.*) for the purpose of evaluating quantitatively the volume of a system, the mass of a particular unlabeled substance, the rate of a chemical reaction *etc.* In the classical dilution principle the assumptions necessary for validation of the measurements require: 1) that labeled and unlabeled substances behave identically; 2) that they are uniformly mixed in the system to be analyzed; and 3) that both labeled and unlabeled substances in an aliquot of the mixture be quantitatively determined. In the papers described by Dr. Pressman, labeled antigen or labeled antibody are employed

for the quantitative analysis of the antigen-antibody reaction with the classical dilution principle, *i.e.*, the labeled marker is used as a valid tracer for the unlabeled substance. However, the assumptions on which competitive assay (of which radioimmunoassay is a special case) are based are clearly different: 1) there is *no* requirement that labeled and unlabeled substances be identical or behave identically in the system employed; 2) there is *no* requirement for uniform mixing of labeled and unlabeled material—in fact, some assays are designed specifically to avoid uniform mixing; 3) there is a requirement for identical behavior of standards and unknowns in competitive inhibition of binding of labeled substances, but *no* quantitative measurement of the unlabeled substances need be made in the sample selected from the system. Thus, it is evident that the fundamental principles of the dilution method and competitive assay differ, although, of course, since both employ marker molecules they are sometimes confused.

DR. SPECTOR: Dr. Gorden, you have mentioned that the receptors, like the antibodies, possess a number of affinity constants, so that there are high affinity receptors as well as low affinity receptors. I wonder how you differentiate non-specific binding onto this membrane from the specific low-affinity constant receptor.

DR. GORDEN: We define non-specific binding as that binding that is not displaced by a "very large" concentration of hormone. In our studies this quantity is defined as 50 μg of insulin. What we call non-specific thus is that which is completely outside of the concentration range of the different affinities under discussion. Our first high affinity binding site in the lymphocyte, *e.g.*, covers the concentration range of 0.1 to 1.0 ng, and the second one covers a range of 10 to 50 or 100 ng. Anything beyond that range we do not really consider. You are quite right in saying that there may be another component or even several other components, in these varying affinities. We have merely defined our affinities over a fixed concentration rate.

DR. ERLANGER: I was curious as to whether anybody knows anything about the structure of big insulin.

DR. YALOW: I assume you are referring to "big-big" insulin and not to proinsulin, whose structure is known. We know absolutely nothing about the structure of the "big-big" insulin. It was present in high concentration only in the plasma of one patient sent to us by Dr. J. Páv of Universita Karlova, Praha, Czechoslovakia. Unfortunately the patient died and did not come to autopsy. I recently received a letter from Dr. F. Melani of the University of Chicago who is again working in the insulin culture system and feels he has detected "big-big" insulin as the pre-precurser of the insulin family. These are unpublished experiments of his; therefore, I cannot comment on them.

DR. ERLANGER: The only thing that I can determine from Dr. Roth's slide is that there is only one free carboxyl and one free amino group in the proinsulin. In other words the proinsulin has the bridge between one amino and one carboxyl, but there is still another amino and another carboxyl free.

DR. YALOW: That is right. If it is a precursor of proinsulin it would likely be connected to one side or the other, probably to the N-terminal side because there is no free lysine or arginine at the other end. Rather interesting has been our inability, with controlled tryptic digestion, to get to the proinsulin stage, whereas we can convert the big adrenocorticotrophic hormone (ACTH) to little ACTH in spite of the five or six lysines or arginines in the middle of the ACTH molecule; this suggests that in this conversion the site for tryptic digestion is more readily available. We have been unable with controlled tryptic digestion to demonstrate the conversion of the "big-big" type through proinsulin to insulin. We are hoping that with Dr. Melani's pulse chaser type experiment this will be possible.

DR. HABER: Dr. Yalow, for many years, I have been intrigued and very troubled by your observation of two insulins having the

same amino acid sequence but having a different immunoreactivity. Now you have proposed an interesting solution to this dilemma, namely, the metastable conformation induced by the proinsulin difference in sequence or structure. Can you approach this directly now (failing the synthesis experiment which you propose) simply by attempting to fully unfold the three-dimensional structure of these insulins in strong denaturants without breaking their disulfide bridges, and by allowing them to refold and see whether they come to the same conformation as judged immunochemically?

DR. YALOW: With all the work that has been done with insulin synthesis, it would be much simpler to take the natural dog or pig insulin, break the disulfide bonds, and recombine them. I am hoping that Dr. P. G. Katsoyannis of Mount Sinai Hospital, New York or someone else will provide us with this material for testing. It seems now that there is good reason for its happening, except that the synthetic approach should provide knowledge as to which is the ground state.

DR. ERLANGER: There is a good chance that you are right in your suggestion that the structures might be the same and the three-dimensional conformations different. In the history of the synthesis of insulin one of the big problems was to put the two chains together properly. In Anfinsen's work on ribonuclease, he got practically 100% yields by allowing it to reassociate, whereas Katsoyannis had a great deal of trouble getting a decent yield when he tried to reassociate insulin. The answer to this was furnished by the discovery that the proinsulin, if it could be disassociated, would reassociate properly. But insulin was in a metastable state and its conformation had been determined while it was proinsulin. I think that this is good evidence that your beliefs are well founded.

DR. GRISHNA: Why is only about 3% of the radioactive insulin or growth hormone bound to the lymphocyte receptors?

DR. GORDEN: In general, the binding to receptors of a whole variety of types is not as great as it is in binding to antibodies. Therefore, most people who have worked with these systems do not work at the level of 50% binding or at a bound to free ratio of 1. However, the binding is primarily a function of the cell concentration; one can increase the binding essentially in the same way as you increase binding to antibodies—by increasing the concentration of the receptor. More recently we have done just that; we have been able to decrease the concentration of the labeled hormone and increase the concentration of the cells. There are certain physical limitations in terms of the amount of the material one is working with, such as a sludging effect which create technical problems, thus limiting the increased cell concentration beyond a certain amount. We are, therefore, working in the range of approximately 10% binding, displacing down to less than ½%. This is a more typical range. The slides I have shown represented somewhat lower binding because we were using low cell concentrations.

DR. GRISHNA: What is the background in your receptor system with heat-denatured lymphocytes?

DR. GORDEN: If the cells die or presumably if you denature them, there is binding, but the binding is non-displaceable. We refer to the non-displaceable binding as non-specific. We do not know the nature of that binding.

DR. GRISHNA: Why is the slope of your displacement so shallow by comparison to other receptor-binding proteins, such as cyclic AMP to its binding protein. Thus, in your system half-displacement of the radioactivity from its receptor requires an increase of an order of magnitude rather than merely a doubling of concentration.

DR. YALOW: Essentially your question relates to the equilibrium constant for the binding of the hormones to the receptor sites. As was pointed out by Dr. Gorden it is now clear that in all of these receptor site assays, the equilibrium constants are generally somewhat less, and sometimes very much less, than the equilibrium constants that we had purposely selected for immunoassay from

the various antisera which are available. Not infrequently the equilibrium constants for the receptor sites are in range of 10^7 to 10^9 for different classes of binding sites, whereas for the antisera not infrequently selected for radioimmunoassay where we need high sensitivity, the equilibrium constants will range anywhere from 10^9 up. I think this difference accounts for the relatively low sensitivity of some of these assay systems, and is one of the disadvantages of the receptor site assay as compared to the immunoassay. On the other hand there is the suggested advantage for the receptor assays that they measure a quality related to biological activity, although I think they will eventually find that there is not always a 1 to 1 correspondence between assay results and biological effect.

DR. GUSTON: Dr. Dyrenfurth, it was interesting to see that intravenous Premarin led to an increase in the release of LH. I was curious whether or not this increase was sufficient to cause ovulation.

DR. VANDE WIELE: The same slide depicting the LH showed levels of progesterone indicating that there was no ovulation. For ovulation you need two things: firstly, a sufficient amount of LH which was present here; and secondly a ripe follicle, which was not present.

DR. FARRELL: What is the metabolic role of insulin in the central nervous system?

DR. GORDEN: There is some evidence now that insulin does influence glucose transport in nerve. This formerly was thought not to be true. Generally it was thought that the nervous system was an insulin-independent tissue. There has been some evidence in the past year that there might possibly be insulin receptors in nerves. It is probably fair to say that while nerve tissue may not have the same kind of sensitivity to insulin as muscle or adipose tissue, it probably is an insulin-responsive tissue.

DR. YALOW: Probably, nowadays, in order to show a definitive role of insulin in a particular system, one should find a receptor in that system. Can I ask Dr. Gorden if he is thinking of other receptor site systems, *e.g.*, for insulin?

DR. GORDEN: We could probably expand the list of tissues that have been studied. Besides the obvious ones such as muscle, fat, and lymphocytes, specific hormone receptors have been demonstrated in fibroblasts, placenta, nervous tissue, granulocytes, red blood cells, and other blood cells. Some work has been done with bone, although I am not aware that specific receptor sites have been demonstrated. Of course, when dealing with insulin one is dealing with a hormone which has profound effects on a host of tissues. It is probably a much more important and universal hormone than the much vaunted pituitary "conductor" of the endocrine symphony.

DR. LEFKOWITZ: In speaking of receptors and binding sites it is useful to point out that receptors conceptually have at least two major functions. The first is the binding function; the second is the more distal function of activating some biological process through adenyl cyclase activation, through a change in sodium ion permeability, or through other unknown changes. It is certainly conceivable that the binding apparatus, *e.g.*, for insulin, might be present in a tissue but that the subsequent apparatus might be not functional. One can conceptually picture a tissue which would have an appropriate binding site but in terms of ultimate biological response would not be responsive.

DR. GORDEN: I would certainly agree.

DR. YALOW: This was the reason I indicated that the 1 to 1 correspondence between certain types of biological activity in receptor site assays will not continue to be found. This dissociation between binding to the receptor and subsequent activation is likely to occur with analogues or in other systems.

DR. GROSSMAN: My question stems entirely from ignorance. It has been said that the use of receptors may be biologically more sound, if not perhaps as refined. Does the binding of a hormone to a tissue or tissue membrane, or part of the tissue or part of the

cell, imply necessarily some biological activity by that hormone on that tissue?

DR. GORDEN: The point made just now by Dr. Lefkowitz is a perfectly valid one. For instance, we have worked with purified factions of liver membrane, and we cannot demonstrate a biological effect of the binding. On the other hand, the pioneering work by Dr. Lefkowitz with ACTH showed that ACTH binding parallels adenyl cyclase activation. The work by Rodbell with glucagon is another example. With lymphocytes, there are a variety of effects that have been demonstrated although none of them are very dramatic. They are certainly not of the same magnitude seen in other systems.

Goldfine has shown recently that in thymic lymphocytes from rat one can show a parallelism between the binding of insulin and the transport of amino isobutyric acid. Here is an example in which we can pick out a specific effect and show correlation. On the other hand, insulin may have other more chronic effects. When we speak of effects, we usually speak of acute effects that can be measured. We do not often measure chronic effects. A very interesting observation is the demonstration that insulin can replace serum in a variety of cells in cultures. It has been shown with fibroblasts. Dr. Gavin has shown, with his cultured lymphocytes, that those cells with binding sites for insulin can be maintained in culture in a medium of very low serum concentration by replacement of the serum with insulin, whereas lymphocytes without insulin binding sites cannot survive in culture without serum, irrespective of the amounts of insulin added. I do not know exactly what we are measuring in the cell culture system. Presumably we are measuring some kind of longer term biological effect. Therefore we are left with the question of: a) the proposal of Dr. Lefkowitz, "Is it possible to have binding sites that are not coupled to a metabolic event?"; or b) is it possible that the coupling is due to some mechanism other than an acute effect that we commonly measure, such as the transport of something or the activation of an enzyme? We really do not know.

DR. PRESSMAN: In connection with biological binding sites it is important to realize that something is not going to be biologically active unless it does bind. Conceivably it could bind without having the effect. But in order to have the effect we can assume that it must bind. In connection with the effects in cultured lymphocyte cells we are now at a stage in the study of lymphocytes at which we have recognized a tremendous number of different kinds of lymphocytes, all with different properties. It is very interesting to hear of the differentiation made between lymphocytes which do bind insulin and those which do not bind insulin. I wonder whether you have any further information on which types of cells or lines of cultured cells do the binding and if they have other properties which are associated with the binding of insulin?

DR. GORDEN: The cells which have been used most extensively were obtained from patients with chronic myelogenous leukemia. These are lymphocytes and they have been grown in established cultured lines. They are thought to be predominantly B lymphocytes. On the other hand, a whole variety of lymphocytes have been screened. A group of lymphocytes which came from patients with Burkitt's lymphoma did not appear to bind insulin. Beyond that we do not really understand exactly what the differences are at the present time. I cannot answer the question of whether with circulating cells there might be differences between B and T lymphocytes and whether there might be shifts in these that account for the alterations that we see. We think we are seeing alterations in metabolic effects. Maybe that is because we are endocrinologists and not immunologists. The other evidence that would support our view is that the deficiencies we see in insulin-resistant states, the decrease in binding, and the abnormalities, seem to correct towards normal by manipulations that improve insulin sensitivity. In the obese mice of the genetic and of the nongenetic kind or in obese man, both the liver receptor and the adipose tissue receptor in

the mice and the lymphocyte receptor in man, improve towards normal with caloric restrictions and weight loss. Those are situations in which we can routinely demonstrate improvement in glucose tolerance, decrease in endogenous hyperinsulinemia, and greater responsiveness to exogenously administered insulin. I suppose the alternative is possible, namely that we are switching B and T lymphocytes. We have not found methods to give such a quantitative distinction between the two so that it could be added as an additional factor.

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