Studies with Insulin-binding Antibody

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The investigations to be described in this presentation were initiated originally with the intent of evaluating the turnover rate and disposition of insulin in normal and diabetic subjects. To this end, small quantities of I^{131}-labeled crystalline beef insulin were injected intravenously, and observations were made on the rate of disappearance from the bloodstream.[1] The earliest results were quite provocative in that most diabetic subjects showed a much slower disappearance of labeled insulin than did normal subjects. On occasion, however, normal curves of disappearance were observed in diabetic patients, and closer scrutiny brought to light the fact that insulin disappearance was rapid in all diabetic or nondiabetic subjects never treated with insulin or treated for only a few weeks. In contrast, the labeled insulin showed a prolonged retention in the circulation of patients under insulin therapy for months or years. Thus, differences in the rate of disappearance of insulin were found to be correlated with the past history of insulin treatment and not with the absence or presence of diabetes per se.[1] In confirmation, it was established that insulin disappeared slowly from the circulation of schizophrenic patients who had received extensive insulin coma therapy and rapidly in those never treated with insulin.[1]

In search for an explanation of this phenomenon, it seemed desirable, early in the course of these studies, to examine the electrophoretic behavior of the labeled insulin in the serum of these subjects. Only zone electrophoresis was suitable for this purpose, and paper and starch were employed as the media of choice. The electrophoretic behavior of I^{131}-labeled insulin in plasma is interesting in several respects but not least in the contrast between control plasma and the plasma of insulin-treated subjects. Insulin, in common with many other proteins, possesses the capacity to adsorb on certain inert materials such as paper[1, 2] and glass[3, 4] and, when applied in low concentration to paper strips for electrophoresis or chromatography, remains at the site of application ("origin"). Such adsorption to paper is not inhibited by the presence of normal plasma, and is readily detected when the serum proteins are caused to migrate away from the origin by electrophoresis or chromatography (figure 1). However, a strikingly different picture is observed with insulin in serum or plasma from insulin-treated subjects. In such cases, the labeled insulin is found to have migrated in whole or in part with serum proteins just in advance of the gamma globulins (figure 1), and little or no adsorption to the paper at the origin is observed.[1]

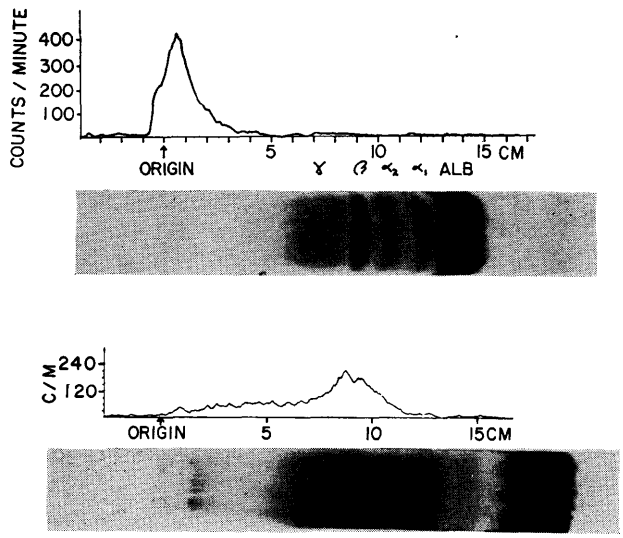

FIG. 1. Paper radio-electrophoretograms-of-insulin-I^{131} in plasma of control subject (upper) and plasma of insulin-treated subject (lower). Site of application along line designated as "origin."

Observations on starch block electrophoresis confirm these differences between insulin-treated and nontreated subjects. Insulin is not adsorbed significantly by starch and, in normal serum, migrates with a mobility almost as great as that of serum albumin, as in free electrophoresis; however, in the sera of insulin-treated patients the insulin is bound to the globulins, and is not free to migrate in the normal fashion.[1]

Insulin binding by serum globulins was also demonstrated by ultracentrifugal fractionation. Since insulin is a much smaller molecule than albumin, it sediments less rapidly than albumin in normal serum, but it sedi-

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ments with the globulins in the sera of insulin-treated subjects.[1]

That the insulin-binding globulin is an acquired antibody, the formation of which is provoked by treatment with insulin, is indicated by its presence in all cases where insulin therapy had been given for three months or longer, and by its absence in all nontreated subjects.[1] Moreover, its appearance was also demonstrated in a newly treated subject who was under observation. Two and a half weeks after the initiation of insulin treatment in this diabetic subject, the injected labeled insulin disappeared rapidly from his bloodstream and, on electrophoresis, was found to be in the free form (figure 2). Repeat studies were performed after four and a half months of continuous insulin therapy and revealed a slow disappearance of the tagged insulin from the plasma; on electrophoretic examination of the serum the labeled insulin was observed to be largely bound to serum globulin (figure 2).

It has been indicated already that the insulin-antibody complex appeared to migrate just in advance of, rather than with, the gamma globulins (figures 1, 2). Further investigations were undertaken with serum fractions, separated with cold ethanol.[5] Antibody was always recovered in the fraction (I + III) containing the beta globulins. In experiments in which the gamma globulin fraction was contaminated with beta globulins, antibody was present in this fraction as well. However, when the gamma globulin fraction (II) was separated from antiserum as a relatively pure fraction, it did not contain the antibody. It was demonstrated that the insulin-antibody complex migrates in the region between the fastest gamma globulins and the slowest beta globulins, which is best described as the inter-gamma-beta zone.[5]

Now, it might be anticipated that the binding of insulin by antibody would have modifying effects both on the tissue response to exogenous insulin and on the action of tissues on insulin. Let us first consider the latter effect. Degradation of insulin by liver homogenates is not interfered with by serum or plasma from normal subjects or from diabetic subjects never treated with insulin.[6] However, the serum of insulin-treated subjects has a marked retarding effect on insulin breakdown, which is well correlated with electrophoretic and chromatographic evidence of insulin binding by antibody. Furthermore, only those serum fractions containing insulin-binding antibody exert an apparent inhibitory effect on liver "insulinase" activity. Thus, while pure gamma globulin separated from antisera is without effect on this activity, fractions containing the inter-gamma-beta proteins prevent the proteolysis of insulin.[6] The sequestration of insulin in the insulin-antibody complex, therefore, serves to protect the hormone from the action of the degrading enzyme in liver. Since the complex dissociates slowly into free insulin and antibody, the insulin is degraded eventually, even in these cases, but at a very much reduced rate.

As indicated previously, the binding of insulin to antibody also leads to retention of insulin within the blood stream and extracellular fluids. This results, on the one hand, in protection of insulin from the insulin-degrading mechanisms of the body, and, on the other, in blockage of the effect of insulin on tissue cells. As the insulin-antibody complex dissociates, some of the insulin

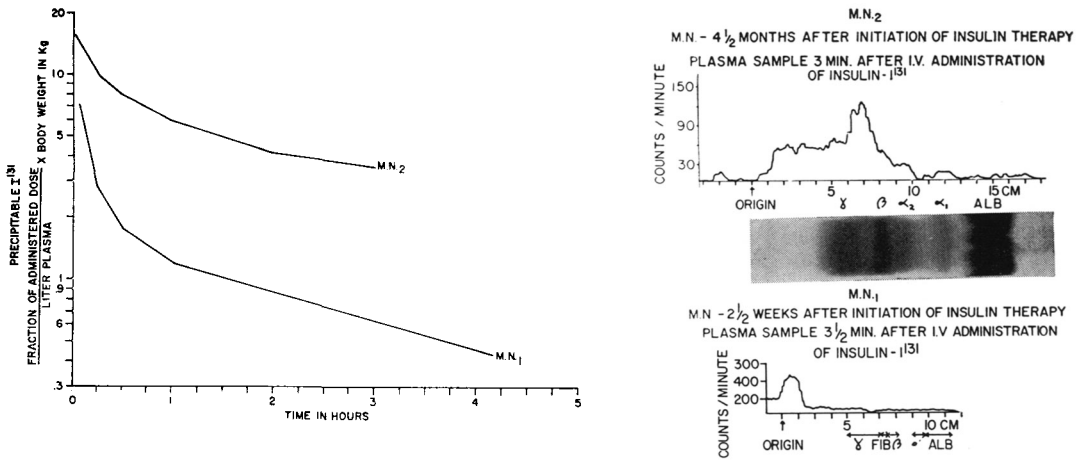

FIG. 2. Left—Concentration of precipitable I^{131} in plasma as a function of time, following intravenous administration of tracer doses of insulin I^{131} to a diabetic subject after 2½ weeks (M.N.$_1$) and 4½ months (M.N.$_2$) of insulin therapy.
Right—Paper radio-electrophoretograms of plasma samples withdrawn about three minutes after injection.

gains access to the cells, and some recombines with antibody. Therefore, it has been of more than academic interest alone to evaluate not only the total insulin-binding capacity of antisera, but also the rate at which insulin and antibody react with each other, and the rate at which the complex dissociates into the two uncombined molecules. By employing electrophoretic and chromatographic technics, these rates have been evaluated in vitro for antisera from insulin-resistant and nonresistant patients. It is of importance, first, to note that, while the insulin-binding capacity of most insulin-treated subjects usually does not exceed about 10 units per liter plasma,[1] the sera of insulin-resistant subjects have been found to be capable of binding from 80 units to more than 500 units per liter plasma.[7, 8] When insulin is administered in amounts which exceed the total binding capacity of the antiserum, the excess is disposed of in the normal fashion. When smaller amounts of insulin are administered, the net effect is determined by the balance between those factors operating to retain insulin within the bloodstream, i.e., the rates of binding to antibody, and those factors permitting its removal, i.e., the rate at which insulin dissociates from the insulin-antibody complex and the rate of passage of free insulin through the capillaries. Binding and dissociation probably also take place in extracellular fluids. Investigation has shown that insulin does not combine with antibody at a single rate, but that one type of insulin-antibody complex is formed rapidly, and that another is formed more slowly. For each type the rate of formation, at any specified insulin concentration, is proportional to the concentration of antibody; thus, in the presence of high antibody titers, as in insulin-resistant patients, the binding of insulin and antibody is so rapid that virtually none of the insulin escapes into the tissues without having first become bound to antibody. In these cases the reaction may be so rapid that it can be studied satisfactorily in vitro only when slowed down considerably. This is easily effected by dilution of the antiserum which decreases the concentration of antibody. Studies in vitro have shown not only that the two types of antibody complex are formed at different rates, but also that they dissociate at different rates, one usually being quite rapid, of the order of 20 per cent to 30 per cent per minute, the other, about 20 per cent to 40 per cent per hour. On mixing of antiserum with insulin, the complex that forms more rapidly also dissociates rapidly. Eventually, after some minutes or hours, depending upon the magnitude of the parameters involved, most of the bound insulin is found in the slowly dissociating complex (figure 3). The relative proportion of rapidly and

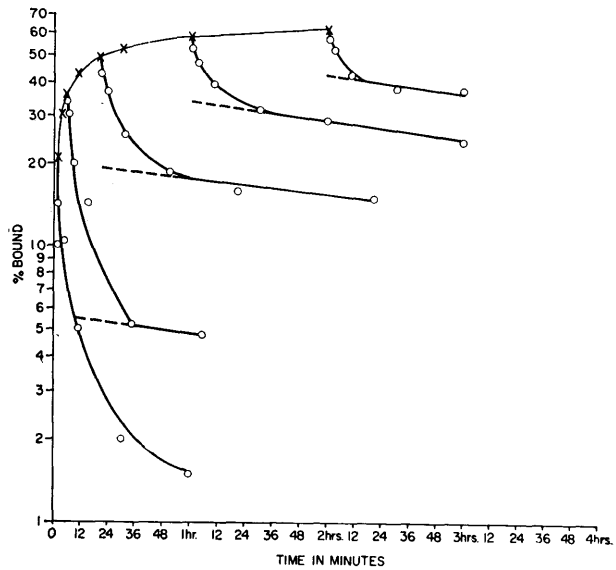

FIG. 3. Per cent of insulin-I^{131} bound to antibody as a function of time following incubation at 37°C. of insulin I^{131} with a 1:16 dilution of antiserum (x — x) and curves of dissociation (o — o) of insulin-antibody complexes, formed after variable periods of association. In the dissociation experiments large amounts of nonradioactive insulin were added to aliquots removed from the incubation mixture in order to inhibit, competitively, the reassociation of radioactive insulin with antibody. It is evident that the rapidly associating complex also dissociates rapidly.

slowly dissociating complexes that exists at equilibrium is unchanged by dilution of the antiserum.[9] This observation and others make it appear likely, on the basis of theoretical considerations of the kinetics of the insulin-antibody reaction, that the different complexes are formed from characteristically different antibody binding sites rather than that they represent different stoicheiometric proportions of insulin and antibody. Since cross reactions between beef and pork insulin are observed[5, 6] it is possible, but not proven, that the different antibody binding sites are beef insulin antibody and pork insulin antibody respectively.

The clinical significance of these findings is chiefly that the immediate reaction to insulin may be lessened markedly because of its capture by circulating antibody, which may result in the picture of apparent insulin insensitivity.[1] In patients with high concentrations of circulating antibody relatively large amounts of insulin are required before the binding capacity of the serum is saturated and the syndrome of striking insulin resistance may become manifest. After a large amount of insulin has been administered in the effort to overcome this resistance, the patient is left with a high concentration of circulating insulin "trapped" in the insulin-antibody

complex. Even if insulin administration is then discontinued, the slow release of insulin from the dissociating complex provides the tissues with a continuous supply of insulin for many hours and occasionally for days. As a consequence of this and of the diminished insulin requirements which attend the lowering of the blood sugar, hypoglycemic reactions may recur repeatedly in the absence of further insulin therapy.[7] This phenomenon is also seen occasionally in schizophrenic patients receiving insulin shock treatment. Although progressively increasing insulin dosage may be required for the induction of coma, there may be considerable difficulty for hours afterward in restoring and maintaining a normal blood sugar level.

While the factors responsible for controlling the rate of antibody formation are not as yet established, it seems reasonable to suspect that the temporary increase in insulin requirements which frequently accompanies acute infection may be due, at least in part, to a nonspecific stimulation of the rate of insulin-antibody production.

Cases of insulin resistance, at least some of which appear to be caused by immune antibodies, not infrequently present challenging clinical problems. There is some evidence that adrenal steroids may be of benefit in these conditions, but the possibility that their action is mediated through an effect on the insulin-binding antibody still requires experimental support with precise quantitative data. Many other questions of academic as well as clinical interest likewise remain unanswered at present, and pose problems for elucidation by future investigations.

SUMMARIO IN INTERLINGUA

Studios con Anticorpore Insulino-Ligante

Es discutite le demonstration del existentia de un anticorpore insulino-ligante in le seros de diabeticos e nondiabeticos tractate con insulina. Le anticorpore migra in le region inter gamma e beta in electrophorese zonal. Le ligation de insulina al anticorpore modifica le responsa del histos a insulina exogene e le action del histos super insulina. Duo typos differente de complexos de insulina e anticorpore ha essite detegite. Il es probabile que le differente typos de complexos es formate ab characteristicamente differente sitos de anticorpore. Le signification clinic de iste constatationes es etiam discutite.

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DISCUSSION

ARNOLD LAZAROW, M.D., PH.D., (*Minneapolis*): I very much enjoyed Dr. Berson's interesting presentation and I would like to ask a question related to the significance of the antibody levels present in the insulin-resistant patients. It is, of course, possible that blood antibodies per se could produce the insulin resistance because of the removal of large quantities of blood insulin. On the other hand it is equally possible that antibodies develop secondarily to the insulin resistance. If, for example, the insulin resistance were the result of a factor other than insulin antibodies, the necessity of administering large amounts of insulin to these patients in order to control the diabetes might be a factor in the production of the high antibody levels. Thus the antibodies to insulin could be a direct consequence of the administration of larger than usual amounts of insulin. The finding of antibody in combination with large amounts of insulin in the blood would then be the secondary result of the insulin resistance rather than its primary cause.

We have considered these two possibilities at great length in the work which Arquilla has carried out on insulin antibodies in diabetes. At the present state of our knowledge we do not have sufficient evidence to differentiate between the two possibilities presented.

ARTHUR R. COLWELL, SR., M.D., (*Chicago*): We have greatly enjoyed the imaginative work of Drs. Berson and Yalow in this field. It may lead to something of fundamental importance, if the question of species specificity in these immunologic reactions can be solved.

We and others have reported cases of striking insulin

resistance in which an immunologic mechanism has been exhibited in an exaggerated fashion. Reversal with steroid therapy has been shown.

I should like to ask Dr. Berson if they have had any experience with tagged human insulin in patients with diabetes who have never received exogenous insulin. If a mechanism such as they have shown could be demonstrated under those conditions, the key to an important etiological factor in so-called idiopathic diabetes would be at hand.

GEORGE E. ANDERSON, M.D., (*Brooklyn*): I should like to ask Dr. Berson whether he and his group have correlated any of this work with evidence of insulin sensitivity or resistance such as we have been finding in our "six-minute test" (DIABETES 3:462-66, 1954).

It seems to me that this simple test should reflect by degree of insensitivity to insulin those patients whose blood on assay would show the antibody combination described. Dr. Berson was able to find a high titer in one of our patients who at the time was unresponsive to insulin by the "six-minute test." She had required 8,000 units of insulin daily. After exposure to cortisone for several days the test reverted to normal sensitivity, insulin dosage being reduced from 600 units (which was her usual dose) to 60 units after three days of the steroid.

JAMES B. FIELD, M.D., (*Bethesda*): I should like to compliment Dr. Berson on this beautiful piece of work. Through the courtesy of Dr. James Moss of Georgetown University Hospital, we recently have had occasion to study a patient with chronic insulin resistance and found that serum from this patient who required approximately 400 units of insulin per day bound 98 per cent of 0.2 units I^{131}-labeled insulin/ml. This patient was then treated with steroids for three days, resulting in a dramatic reduction in insulin requirement to 30 units per day. However, much to our surprise, when we reexamined her serum, after ACTH, 98 per cent of the same dose of insulin was still bound. I should like to know if Dr. Berson has a possible explanation for this finding.

CECIL STRIKER, M.D., (*Cincinnati*): I should like to ask Dr. Berson whether there are any differential patterns of association or disassociation for the various insulins, such as crystalline, PZI, NPH and Lente insulin, that is, in reference to their own protein content?

LEWIS C. PARK, M.D., (*Kew Gardens, New York*): I should like to ask Dr. Berson if he has done any work with acidosis, and what is the mechanism with regard to antibody insulin in relation there?

DR. BERSON: Dr. Lazarow brought up the question that there might be some factor responsible for an increase in the insulin requirements of certain diabetic patients which then provokes the administration of larger amounts of insulin to these patients; they may then develop a higher concentration of circulating antibody. I think that probably in the case of many antigens, the larger the dose of antigen, the more likely the probability that higher concentrations of antibody will result.

The insulin-binding antibody, however, is found uniformly in patients who have received insulin for more than three months or so, even in those who have received relatively small doses of insulin, of the order of 15 to 20 units a day. The fact is that in insulin-resistant patients, we always observe a much higher concentration of antibody than in the usual insulin-treated diabetic. Whether this much larger amount of antibody is due to the fact that these patients have had a larger amount of insulin, I cannot say for sure.

In reference to the question with regard to human insulin, I think that this is a subject that really needs further investigation, i.e., the species specificity of the various insulins. As you know, Sanger has characterized five different species of insulin and has found them to differ only in positions 8, 9 and 10 on the glycyl chain. Moreover, there are only two amino acids for each of these three different positions which have been found in the five different species. There is, therefore, a maximum total of eight possible different mammalian insulins if these findings continue to be borne out in other species.

Whether human insulin resembles that of one or another of the animal species or not, we don't know. However, we must consider the possibility that none of the insulins we have available in crystalline form is the same as the endogenous insulin of that species. There is a suggestion of such a difference, since it has been reported in a recent publication (Maloney, P. J., and Coral, M., *Biochem. J.* 1955, 59:179) that antibodies to pig insulin, produced in guinea pigs, neutralized some crude preparations of guinea pig insulin but not others. Furthermore, a sheep immune to pig insulin was also possibly resistant to crystalline sheep insulin suggesting that the latter differed from endogenous sheep insulin.

It is possible, therefore, that during the extraction of insulin, some change is made in the molecule which makes an antigen out of the insulin. Therefore, it is conceivable that even if we were to obtain crystalline human insulin, antibodies to this insulin might still be provoked in human subjects.

With regard to Dr. Anderson's question about correlation with results on six-minute glucose tolerance tests, we have not done those ourselves. I have little

doubt, however, from our studies in vitro and in vivo on the binding of insulin that many patients who do have antibody will show much less of a response to a small dose of insulin than patients who have never been treated with insulin and have not developed antibody even though they may be diabetic at the time.

With regard to Dr. Field's comments, I have been aware of this patient whom he has studied and who showed approximately the same amount of insulin binding following an apparent break in the insulin resistance. We really cannot answer that satisfactorily, except to say that it may not be quite valid to say that this patient's serum bound the same amount of insulin from observations at a single concentration, especially in the region where about 98 per cent was bound. Actually, within very wide levels of insulin concentration, you may appear to get the same fraction bound, especially at the higher binding ratios, and yet if you were to do this very precisely and follow it out over the entire range of insulin concentrations, you might very well find that the insulin-binding capacity had decreased by 10-fold or even 100-fold.

In regard to the question of NPH insulin, we have not done studies with any insulins other than crystalline beef and crystalline pork insulin. If we used a preparation containing noninsulin-bound tyrosine, we might be labeling the carrier protein, and couldn't be sure whether it was the insulin or this other nonspecific protein which carried the radioactive label.

With regard to the question of acidosis, all I say is that we have received sera from patients who were resistant to insulin, and who were in acidosis at the time that the serum was drawn. These sera show a high concentration of insulin-binding antibody. On the other hand, we find that sera from the same patients, even when no longer in acidosis, show similar findings.

The Future of Enzyme Research

Enzymes are proteins. During recent years we have seen the astounding development of chemical methods, including chromatography, to such power as to permit the determination of long sequences of amino acids in the polypeptide chains of proteins. It is evident, however, that an understanding of the specific properties of proteins cannot be obtained through knowledge of amino-acid sequences alone. It will also be necessary to have knowledge of the way in which the polypeptide chain is folded, and the detailed configuration in space of the side chains. I am confident that the X-ray investigation of crystals of globular proteins will be successful in leading to the complete structure determination—the location of every atom—of the molecules of many proteins long before the next fifty years of enzyme research are over. I am willing to forecast that within ten years there will have been made such a complete structure determination of at least one crystalline globular protein.

It is hard to forecast to how great an extent enzyme research during the next fifty years will lead to progress in the field of medicine. It is now known that a number of diseases are the result of some sort of abnormality involving enzymes, and that perhaps these diseases should be described as molecular diseases, resulting from the synthesis of enzymes of abnormal structure, which are not able to carry out in a proper way the work that the enzyme normally does.

From an article by Linus Pauling, in *Enzymes: Units of Biological Structure and Functions.* Henry Ford Hospital International Symposium, edited by O. H. Gaebler, New York, New York, Academic Press, 1956, p. 180.
