Radioimmuno-methods for Hormonal Determination in Blood

By N. Hales, Cambridge

The first major attempt to employ insulin antibodies in a sensitive assay for insulin was that of *Stavitsky* and *Arquilla* (1953). They reported that the inhibition by insulin of the haemagglutination of insulin-coated red cells mixed with an insulin antiserum could be used to measure fractions of a microgram of insulin. However, this system, like many assays employing haemagglutination inhibition proved to be insufficiently sensitive and too subject to non-specific interfering factors for its use in the assay of insulin in plasma (Ciba Foundation Colloquia on Endocrinology, 1962).

Yalow and *Berson* (1960) from studies of the binding of (^{131}I)-labelled insulin by plasma from human diabetics treated with insulin developed an immunological assay for insulin. This assay depended upon the ability of (^{131}I)-insulin and unlabelled insulin to compete for binding to insulin antibodies in a dilute antiserum to insulin. It was shown that the use of the isotopically labelled hormone and an insulin antiserum provided an assay of great sensitivity and specificity. They devised empirically a system in which a mixture of standard or unknown solutions of insulin with tracer amounts of very high specific activity (^{131}I)-insulin was made. This mixture was allowed to react with a dilute insulin antiserum for 4 days. The soluble insulin-(anti-insulin) serum complex was then separated from insulin free in solution by chromato-electrophoresis. Uncombined insulin when present in the low concentrations employed remained stuck at the origin of the electrophoretogram whilst antibody-bound insulin migrated with the γ-globulins. These two fractions of insulin were known as the "free" and the "bound" respectively. The radioactivity corresponding to each fraction was assayed by scanning the paper for radioactivity and measuring the area under the two peaks of activity planimetrically. The ratio of the "bound" to "free" radioactivity could then be calculated and was found to be a function of

added unlabelled insulin. A standard curve of this ratio plotted against the concentration of unlabelled insulin was constructed and used to calculate the concentration of insulin in unknown solutions which were substituted for the standard insulin solutions in other mixtures.

One of the disadvantages of the method as described originally was the need to use 80 mc of ^{131}I in order to prepare insulin of a high enough specific activity. This lead us to undertake a detailed investigation of the principles and conditions of the method in an attempt to produce an assay which would involve the use of less radioactivity and be completed in a shorter time.

It was considered that the basic principle underlying this method was that of isotope dilution. A fixed amount of (^{131}I)-insulin was added to a pool of unlabelled insulin of unknown size. By reaction with a limited amount of an insulin antiserum an aliquot of the pool was purified and quantitated. The specific activity of this aliquot should be a function of the size of the pool. Due to difficulties in transferring a known sample of the reaction mixture to electrophoresis paper in the Yalow and Berson system, it was necessary to calculate the "bound" to "free" ratio of radioactivity before a standard curve could be constructed. However, if either of the fractions could be recovered in a reproducible fashion in both standard and unknown determinations it should suffice to determine the radioactivity of either of the two fractions alone. Theoretically it would appear to be more satisfactory to determine the radioactivity bound to the specific antiserum. In this way it should be possible to carry out the assay in the presence of labelled substances which do not react with antibody.

In order that the principle of isotope dilution be followed it is necessary that labelled and unlabelled insulin should behave identically with respect to binding by insulin antibodies and that a fixed amount of total insulin should be bound by a given concentration of antiserum at all concentrations of standard and unknown insulin solutions within an individual assay.

The ideal type of labelled insulin would therefore be one which was internally labelled and thus possessed an amino acid sequence which was identical with that of the insulin which it was proposed to estimate. Furthermore, the antiserum used in the assay should be prepared against that same species of insulin. It was already known that antibodies to ox insulin could have a lower affinity for human insulin. Therefore in order to measure insulin in human plasma it was decided to prepare an antiserum to human insulin. Human insulin was also required as a standard preparation.

250

Antisera to human insulin were prepared in guinea pigs by the method of *Robinson* and *Wright* (1961). Antisera were stored at —10°. For the assay a potent antiserum obtained from one guinea pig on one occasion only was diluted with phosphate buffer containing bovine plasma albumin and merthiolate as a preservative.

Preparation of a high specific activity radioactively labelled insulin

The ideal radioactive insulin preparation would be one in which some or all of the normal atomic constituents of the molecule were replaced by radioactive isotopes. However, making certain assumptions one can calculate that the minimum specific activity required in order to obtain adequate sensitivity is approximately 3 mc/mg. Even if the highest specific activity ^{14}C-labelled amino acids commercially available were incorporated into the molecule without dilution this would not provide an adequate specific activity. Alternative methods involving tritiation of the intact molecule have not as yet provided a technique for internal labelling of protein molecules at high specific activity. The sole possibility therefore remained of labelling insulin by its covalent combination with a high specific activity substituent. This inevitably implies alteration of the structure of the molecule. It was decided to explore the possible methods of substitution with the aim of producing the minimum of change in the behaviour of the molecule. *MacFarlane* (1963) has shown that proteins may be iodinated with ^{131}I so that on injection into rabbits their disappearance curves were identical with ^{14}C-labelled samples of the same proteins. *Springell* (1962) in an extension of this work, explored the conditions for and the results of the iodination of insulin with iodine monochloride. He discovered a number of features of the procedure which suggested that it might be the method of choice for reproducibly preparing undamaged, lightly iodinated insulin.

First, iodine monochloride iodination can be carried out at low levels of iodination under acid conditions without loss of efficiency of incorporation. Advantage can therefore be taken of the greater stability of insulin at low pH.

Secondly, when only one atom of iodine per molecule of insulin is incorporated at acid pH the "A" chain of insulin is exclusively iodinated.

Thirdly, iodination of histidine does not appear to any appreciable extent when insulin is iodinated at pH 1.

Springell found some loss of biological activity when insulin was iodinated at 1 atom/molecule at pH 1. However, since the ability to combine with antiserum was required for immunoassay the loss of biological activity need not be important.

251

The biological activity was not tested since the accuracy of the assays available was very low and since any loss would be very difficult to interpret in terms of the likely scatter of insulin derivatives produced by iodination. The sample would be expected to contain at least three species – di-iodinated, mono-iodinated and non-iodinated insulin.

Using iodine monochloride iodination to give a final iodine content of 0.5–1 atom/molecule of insulin the characteristics of the preparation was as follows:

Trichloracetic acid precipitation was found to leave 1–3 % of the total radioactivity in the supernatant.

Chromatography in 2° butanol-acetic acid (*Fenton* 1959) was found to give a band of radioactivity which had the same mobility as that of added carrier (non-iodinated) insulin. A preparation was then made which contained twice as much iodine and was found to exhibit two bands of radioactivity following chromatography. One band migrated with the marker non-iodinated insulin and the other more rapidly.

Electrophoresis of a trace of labelled insulin mixed with carrier unlabelled-insulin was carried out in 8 M urea and the radioactivity shown to migrate with the band of protein. The preparation containing twice as much iodine again showed two bands of radioactivity one of which moved more rapidly than the marker insulin.

In both these preparations the slower band of radioactivity migrated at the front edge of the band of protein suggesting that even the lightly iodinated insulin migrated a little faster than the unlabelled insulin. Such an effect is compatible with the known effects of the incorporation of one atom of iodine into tyrosine. The dissociation constant of the hydroxyl group is reduced by this incorporation.

The maximum percentage of radioactivity bound to insulin antibodies was determined for each preparation of labelled insulin. The mean of these recoveries was 92 %. This recovery could be almost completely abolished if a high concentration of unlabelled insulin was present during the reaction of labelled insulin with insulin antiserum.

It was found that preparations of labelled insulin could be stored at –10° C for 3–5 weeks. The loss in radioactivity bound to antiserum after this storage could be entirely accounted for by the half-life of the isotope.

The above results, therefore, indicated that iodination with iodine monochloride to give (^{131}I) iodine incorporation in the region of 25 % and a specific activity of 5–25 mc/mg gave a satisfactory product for use in the immunoassay of insulin.

Separation of the insulin-(anti-insulin) complex from non-antibody bound insulin

The insulin-(anti-insulin) complex formed when insulin is reacted with an insulin antiserum prepared in guinea pigs and both components are present in very low concentration remains in solution. Therefore in order to determine the radioactivity of the insulin-antibody complex it is necessary to devise a means of separating it from insulin free in solution. Chromatoelectrophoresis as used by *Yalow* and *Berson* suffers from the disadvantages that it requires a very high specific activity labelled insulin, is difficult to carry out quantitatively and requires a large amount of apparatus and cold-room space.

Skom and *Talmage* (1958) showed that when the serum of insulin treated diabetics was allowed to react with ^{131}I-insulin and the γ-globulins were precipitated by an anti-human γ-globulin serum prepared in rabbits labelled insulin bound to antiserum γ-globulin was also precipitated. This suggested the possibility that an antiserum to guinea pig γ-globulin prepared in rabbits might be used to precipitate the insulin-anti-insulin complex formed when labelled insulin was reacted with an anti-insulin serum prepared in guinea pigs.

The reaction of the labelled insulin with anti-insulin serum followed by the addition of anti-guinea pig γ-globulin serum was carried out in 5×1 cm glass tubes. The reaction was interrupted by withdrawing the contents of the reaction vessel and filtering them through a cellulose acetate membrane held in a filter-holder. The reaction vessel was washed out twice and the washings filtered through the same membrane. The membrane was dried for 1 h at 75° C and assayed for radioactivity in a Nuclear Chicago Gas-Flow Counter.

It was found that some retention of radioactivity by the membrane occurred even in the absence of insulin antiserum. This was thought to be due to non-specific adsorption of labelled insulin by the filter membrane since it could be reduced by washing the membranes with a solution of bovine plasma albumin. A procedure of soaking the filter membranes in 4 g% albumin solution followed by washing twice with a 2 g% albumin solution was therefore adopted.

Definition of the optimum conditions for the immunoassay of insulin, concentration of labelled insulin

Reduction of the concentration of labelled insulin increases the sensitivity of the assay. Two practical considerations limit the extent to which it is desirable to reduce this concentration. Enough radioactivity

must be retained to allow the recording of a reasonable total of disintegrations thereby providing satisfactory accuracy for the estimation of the radioactivity. Secondly, reducing the concentration of the labelled insulin slows the rate of reaction between it and insulin antiserum and therefore increases the time taken to carry out the assay. In order to carry out the assay in the shortest time it is necessary to use the highest concentration of labelled insulin which provides adequate sensitivity.

The optimum concentration of anti-insulin serum to be used in the assay was found to be a function of the concentration of labelled insulin. To define this concentration an anti-insulin serum dilution curve was constructed. Labelled insulin 250 $\mu\mu$g in 0.1 ml was allowed to react with increasing dilutions of anti-insulin serum and the complex formed precipitated by the addition of 0.1 ml of increasing dilutions of rabbit anti-guinea pig γ-globulin serum. The total length of reaction was 22 h.

The percentage of the total radioactivity bound to the anti-serum falls with dilution of the anti-serum. The concentration of anti-insulin serum which removed approximately half the total radioactivity was selected for the assay of insulin. This concentration was taken to ensure that the insulin antiserum remained saturated with insulin at all concentrations of insulin used in the assay. In this way it was hoped to fulfil the conditions mentioned earlier that the insulin antiserum should bind the same total amount of insulin at all concentrations of insulin used.

Concentration of anti-(guinea pig γ-globulin) serum

This precipitation system like other antibody precipitation systems exhibits the so-called pro-zone phenomenon (*Kabat* and *Mayer* 1961). Precipitation is inhibited when either of the reactants is present in great excess over the other. It is therefore necessary by serial dilution to determine the optimal concentration of precipitating antiserum to be used with the concentration of anti-insulin serum selected.

Since human plasma contains a high concentration of γ-globulin any cross reaction between this and the antiserum to guinea pig γ-globulin could interfere with complete precipitation of guinea pig γ-globulin in the assay of insulin in human plasma. Even a cross reaction of the order of 1000-fold less than of guinea pig γ-globulin might be expected to interfere. Experiments were therefore carried out to determine whether human γ-globulin cross-reacted with the rabbit anti-(guinea pig γ-globulin) serum. It was found that under conditions in which a very dilute prepitating antiserum was used in the assay human plasma had an apparently high insulin content and that this could not be reduced by dilution

254

in a manner which paralleled the effect of dilution of an equivalent concentration of pure insulin. However, if higher concentrations of precipitating antiserum were used human plasma showed a much lower insulin content and this could be satisfactorily reduced by dilution. A similar effect was noted when pure human γ-globulin was substituted for plasma. It was also found that the complex between labelled insulin and guinea pig anti-insulin serum could be precipitated by an anti-(human γ-globulin) serum prepared in rabbits. Precipitation of human γ-globulin and human plasma by rabbit anti-(guinea pig γ-globulin) serum was observed by the Oucherlony-plate technique (*Kabet* and *Mayer* 1961).

The observation that the reaction between labelled insulin and insulin antiserum continued even after precipitation of the latter with anti-γ-globulin serum suggested the possibility of performing the precipitation reaction before the addition of labelled insulin and unlabelled insulin. It was hoped that the completion of precipitation prior to the addition of the cross-reacting human γ-globulin would prevent the interference of the latter with complete recovery of guinea pig γ-globulin. It was found that when guinea pig anti-(insulin) serum and rabbit anti-(guinea pig γ-globulin) serum were mixed and allowed to react for 24 h complete recovery of labelled insulin could be achieved (by its addition for a subsequent 22 h) even in the presence of undiluted normal guinea pig serum. This procedure was therefore adopted for the assay of insulin in plasma as a more satisfactory alternative to the use of high concentrations of precipitating antiserum.

Experiments were then carried out to determine whether the results obtained with the technique corresponded to those predictable from the theory of isotope dilution. From the latter one may predict that a linear relationship should be observed between the ratio of the radioactivity bound to antiserum when no unlabelled insulin is present (designated Co) to the radioactivity bound to antiserum when a given concentration of unlabelled insulin is present (designated Ci) and the concentration of unlabelled insulin. In the first experiment a system comprising labelled and unlabelled ox insulin and anti-(ox insulin) serum was used. A linear relationship was observed and as predicted by theory the line intersects the abscissa at a point numerically equal to the concentration of labelled insulin present in the system. The same result was obtained when a system comprising unlabelled human insulin, labelled ox insulin and anti-human insulin serum was tested.

In an attempt to increase the sensitivity of the assay obtained at a given concentration of labelled insulin the effect of adding unlabelled

insulin to the antiserum before the addition of labelled insulin was investigated. It was found that both increased sensitivity and a steeper standard curve resulted from this procedure. Therefore for the assay of insulin in plasma the following sequence of reactions is now employed.

Firstly, anti-human insulin serum is reacted with anti-guinea pig γ-globulin serum at $4°$ C for 24 h. Standard and unknown solutions containing human insulin are then added and a further period of 6 h reaction at $4°$ C is carried out. Finally, labelled ox insulin is added and allowed to react for 17 h at $4°$ C. The contents of the reaction vessels are then filtered and the precipitate assayed for radioactivity. Determinations of standard insulin preparations are performed in quadruplicate and unknowns in duplicate.

More recently it has been found that the sequence may be interrupted after the first 24 h and the contents of the reaction vessels freeze-dried. This allows the precipitated antisera to be stored in a desiccator at $4°$ C until required. One may thereby eliminate the initial delay in carrying out the assay whilst waiting for precipitation to occur. A further advantage of this procedure is that it allows the preparation of a large amount of pooled antiserum thereby increasing the standardization of the procedure.

We have tested the ability of antisera to human, ox and pig insulins to detect and assay pure human insulin. It has been found that, of the antisera tested, one of the antisera to human insulin was by far the most sensitive. However since different antisera to human insulin vary greatly in the sensitivity with which they detect human insulin this apparent species difference could be accounted for by animal variation. It is of interest to note that the best antiserum to human insulin was obtained from an animal which had been subjected to a 7 month period of monthly injections of human insulin. It is therefore possible that antisera of higher affinity may result from prolonged innoculation. Therefore in spite of the theoretical objection that the danger of producing antisera to contaminating proteins is increased, possible advantages of prolonged innoculation should not be overlooked.

The two antisera to human insulin which we have tested do not discriminate between human, ox and pig insulin. This allows ox insulin to be used as a standard in the determination of human insulin. It is not known whether this is a general property of antisera to human insulin. The antisera to ox and pig insulin which we have tested have always discriminated between human and the homologous insulin. It is therefore always necessary when introducing an antiserum for the assay of a non-homologous species of insulin to determine the extent to which the heterologous insulin cross-reacts. We have shown that an antiserum

to human insulin cross-reacts more weakly with pure rat insulin and twenty times more weakly with pure guinea pig insulin.

Using the procedure outlined it has proved possible to measure insulin in plasma from normal fasting individuals. Human insulin added to human plasma may be recovered quantitatively. One may plot the standard curve as a log-dose response curve. If one assumes that the middle portion of the standard curve is a straight line, an index of precision may be calculated. This is given by the standard deviations of the points about the regression line divided by the slope of the regression line. The index of precision calculated in this manner was 0.062.

The assay has been found to give reproducible results when the same sample of plasma is assayed on different occasions. The standard error of the mean of two estimations of eleven samples was estimated. The mean of these standard errors was found to be 2.4 μUnits. This represented an average reproducibility within a standard error of $\pm 5\%$.

In conclusion, I should like to summarize the advantages of radio-immunological methods when compared with bioassays of plasma insulin concentration.

1. *Specificity*. Immunologically detectable insulin disappears from plasma following pancreatectomy or alloxan administration. This is not true of many bioassays.

2. *Reproducibility from laboratory to laboratory*. The results of the determination of the concentration of insulin in normal plasma (both fasting and after the administration of glucose) obtained by different immunoassays in different laboratories agree closely. Bioassays are notoriously unreliable in this respect.

3. *Sensitivity*. Immunoassays are capable of detecting insulin in 0.1 ml or less of plasma from normal fasting individuals. Bioassays require relatively large volumes of plasma.

4. *Statistical evaluation*. Parallel and linear dose response curves for insulin and plasma are readily obtainable with immunoassays. This is not true of bioassays. The accuracy of immunoassays is much greater than that of bioassays.

5. *Rapidity and number of estimations*. With the modifications described, assays may be carried out within 24 h. Whilst this is also true of most bioassays many more determinations may be performed immunologically. With the method described it is possible to assay 50–100 samples in duplicate daily.

Therefore the sole justification for the use of bioassays in the determination of plasma insulin is the idea that there may exist in plasma forms of insulin which are biologically but not immunologically active. I would

suggest that this belief stems from the fact that some bioassays are not specific for insulin. That this is so is strongly suggested by the variation in the results obtained as a result of variations in the type of bioassay, the laboratory which performs the bioassays and the dilution of plasma which is used. In spite of innumerable attempts, it has not been possible to increase the amount of insulin measured by immunoassay following chemical or physical manipulation of isolated plasma.

Summary

The principles underlying procedures used for the radio-immunoassay of insulin have been discussed. Methods for the radio-immunoassay of insulin in plasma which behave according to the theory of isotope dilution have been described. A mixture of insulin and (^{131}I)-insulin was allowed to react with an antiserum to insulin raised in guinea pigs. The complex formed was precipitated by a rabbit anti-(guinea pig γ-globulin) serum. Modification of the procedure avoids the interference of heterologous γ-globulins in the precipitation of the insulin-(anti-insulin) complex and increases the sensitivity of the technique.

Radio-immunoassays of insulin have many advantages over sensitive bioassays when used for the determination of the concentration of insulin in blood.

Zusammenfassung

Der Autor diskutiert die hauptsächlichsten Grundlagen der radio-immunologischen Bestimmungen des Insulins. Die beschriebenen Methoden der radio-immunologischen Insulinbestimmung im Plasma gehorchen den Gesetzen der Theorie der Isotopendilution. Eine Mischung von Insulin mit I^{131} markiertem Insulin wurde mit einem im Meerschweinchen entwickelten Insulin-Antiserum zur Präzipitation gebracht und der daraus entstandene Komplex hierauf mit einem Meerschweinchen-Gammaglobulin enthaltenden Kaninchen-Antiserum präzipitiert. Durch eine kleine Abwandlung des Vorgehens kann die Interferenz mit heterologen Gammaglobulinen bei der Präzipitation des Insulin (Anti-Insulin)-Komplexes verhindert und die Sensibilität der Technik erhöht werden.

Bei der Bestimmung der Insulinkonzentration im Blut zeigt die radio-immunologische Methode gegenüber der empfindlichen biologischen mancherlei Vorteile.

Résumé

L'auteur discute des principes qui sont à la base des méthodes utilisées dans la détermination radio-immunologique de l'insuline. Les méthodes

de détermination radio-immunologique de l'insuline qui sont décrites obéissent aux lois de la dilution isotopique. Un mélange d'insuline et de I¹³¹-insuline a été mis en présence d'un antisérum à l'insuline développé sur le cobaye. Le complexe qui s'est formé, a été ensuite précipité avec un sérum de lapin antigamma-globuline de cobaye. En modifiant un peu la technique, l'on évite une interférence avec des gamma-globulines hétérologues, lors de la précipitation du complexe insuline- anti-insuline, et l'on obtient une sensibilité toujours plus grande de la technique.

La détermination radio-immunologique de l'insuline présente de grands avantages sur la méthode biologique, lorsqu'il s'agit de trouver les concentrations d'insuline dans le sang.

Riassunto

L'autore descrive gli elementi principali della determinazione radio-immunologica dell'insulina. I metodi di determinazione radio-immunologica dell'insulina che vengono descritti ubbidiscono alle leggi della diluizione isotopica. Una miscela d'insulina e d'insulina-I¹³¹ fu messa a contatto di un antisiero ricavato dal porcellino d'India. Il complesso formatosi fu precipitato con un antisiero di coniglio, attivo contro la gammaglobulina del porcellino d'India. Modificando di poco la tecnica, si può evitare una interferenza con le gammaglobuline eterologe durante la precipitazione del complesso insulina- anti-insulina, aumentando così la sensibilità della reazione. Il metodo di determinazione radio-immunologico dell'insulina presenta grandi vantaggi rispetto a quello biologico, quando si tratta di trovare concentrazioni d'insulina nel sangue.

Ciba Foundation Colloquia on Endocrinology, Vol. 14. Ed. *G. E. W. Wolstenholme* and *M. P. Cameron*. J. & A. Churchill Ltd., London 1962. – *Fenton E. L.:* Biochem. J. *71*, 507 (1959). – *Kabat F. A.* and *Mayer M. M.:* Experimental Immunochemistry. Charles C. Thomas, Springfield Ill. 1961. – *MacFarlane A. S.:* J. clin. Invest. *42*, 346 (1963). – *Robinson B. H. B.* and *Wright P. H.:* J. Physiol. (Lond.) *155*, 302 (1961). – *Skon J. H.* and *Talmage P. W.:* J. clin. Invest. *37*, 783 (1958). – *Springell P. H.:* Biochem. biophys. Acta (Amst.) *63*, 136 (1962). – *Stavitsky A. B.* and *Arquilla E. R.:* Fed. Proc. *12*, 461 (1953). – *Yalow R. S.* and *Berson S. A.:* J. clin. Invest. *39*, 1157 (1960).

Discussion

A. E. Renold (Genève): I should like to point out that Dr. *Hales*, who is a very good friend of mine, has perhaps been somewhat less than fair in his comparison of the meaning of immunoassays and bioassays for insulin. It is easy to ridicule insulin bioassays by indicating the enormous scatter of values obtained in different laboratories and by different assays. However, anyone of these values may be no less incorrect in *absolute* terms than the immunoassay values, since in all instances, immunoassays included, we use as a standard a pancreatic extract, insulin, which we think of as a

hormone. However, the circulating hormonal activity to be equated with insulin may well exhibit structural differences from the purified pancreatic protein. So far it is certainly true that nobody knows the structure of the circulating hormone insulin!

Because of this it would seem to me that we must continue to consider bioassay data as well as immunoassay data in the evaluation of any problem concerned with serum insulin. This does not mean that everything that is measured as insulin-like activity is physiologically significant, but it does mean that we must keep an open mind as to the possible complexities of plasma insulin or insulins.

B. Halpern (Paris): Je voudrais demander aux conférenciers quelle est l'explication du phénomène biologique très curieux et notamment de l'absence de l'hyperglycémie chez le cobaye porteur d'anticorps anti-insulines expérimentalement induits. Ces animaux, dont les sérums sont susceptibles de neutraliser l'insuline de diverses expèces animales, y compris leur propre insuline *in vitro*, ne présentent aucun signe de diabète. Faut-il admettre qu'il y a une différence essentielle entre l'insuline circulante et l'insuline intrapancréatique?

N. Hales (Cambridge): Whilst I agree with Prof. *Renold*'s generalisation I think that we should keep quite clear two points of discussion: 1. Is the structure of insulin in plasma different from that isolated from pancreas? 2. Are there two forms of insulin in plasma?