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REVIEW ARTICLES

Immunological Techniques in Diabetes Research*

The Minkowski Award Lecture delivered on September 16, 1971 before the European Association for the Study of Diabetes at Southampton, England

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Summary. Solid phase biochemical systems have many technical advantages. The lecture reviews a number of ways in which solid phase immunological methods may be used in diabetes research. Solid phase antibodies are very convenient for use in radioimmunoassay. The immunoradiometric assay involves the isolation of antibodies onto solid phase antigen, the iodination of the antibodies whilst combined to the solid phase antigen, the elution of the iodinated antibodies and finally their use as a reagent for the measurement of antigen. The two site assay utilises solid phase antibodies to extract and concentrate antigen from solutions such as plasma. The extracted antigen may then be assayed whilst combined with solid phase antigen if a second site on the antigen is available for reaction with I^{125} — labelled antibody. High specific activity, purified labelled antibodies may also be used for antigen localization, antibody estimation and labelling cell membranes. Cell membrane purification using solid phase antibodies is considered as a further development of this methodology.

Key words. Solid phase radioimmunoassay; immunoradiometric assay; labelled antibody; two site assay; antigen localisation; antibody estimation; cell membranes.

In 1967 the Journal of the American Medical Association published an Editorial on Minkowski entitled "Oskar Minkowski Designer of Experimental Diabetes" and described the emphasis which Minkowski placed on technique. Mettler (1947) in discussing Minkowski's achievement in producing experimental diabetes makes a special note that the paper reporting the results contained one of the first descriptions of experimental operative procedure in which comments were included upon bacteriology and operative asepsis. In 1964 Shapiro and Strauss pointed out that Minkowski's technique for the study of left atrial pulse waves in mitral stenosis was years ahead of other work on this problem and furthermore was (in 1962) being re-introduced as a simpler substitute for left heart catheterisation.

I believe therefore that it is not inappropriate to the memory of Minkowski to devote this lecture to a consideration of techniques. There is a very obvious connection between immunological techniques in diabetes research and Minkowski. In 1955 Moloney and Coval described a technique for the production of experimental diabetes which remains the most elegant successor to Minkowski's total pancreatectomy. They showed that the injection into rats of an antiserum to insulin produced immediately and specifically an insulin deficiency leading to the diabetic syndrome. This observation in addition to providing the final proof of the mechanism of Minkowski's diabetes also provided the strongest validation of immunoassay as a means of measuring plasma insulin concentrations. Of course we hardly ever hear the faintest echoes now of the sometimes bitter arguments about validity that accompanied the introduction by Yalow and Berson (1959) of their radioimmunoassay and we all acknowledge their tremendous contribution which provides us with techniques for the measurement of virtually everything from insulin to cyclic AMP. The role of diabetic patients themselves in this development of immunological techniques should not be forgotten. Yalow and Berson's radioimmunoassay was a direct outcome of experiments on the half life of radioactive insulin in insulin treated diabetics (Berson *et al.*, 1956). The subsequent use of radioimmunoassay to show the presence of insulin-like material with a molecular weight greater than insulin in extracts of islets provided one of the crucial pieces of evidence in the discovery of proinsulin by Steiner (Steiner *et al.*, 1969). This in turn led to the discovery of many insulin-like contaminants in commercial insulin with the subsequent demonstration by Schlichtkrull (1971) that the removal of these contaminants reduced or abolished the antigenicity of therapeutic insulin. From this we now hope that diabetics themselves will receive direct benefit.

My interest in immunological techniques began in 1960 when I was working as a research student in Professor Young's Department in Cambridge under

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the supervision of Dr. Philip Randle. In attempting to overcome some difficulties in insulin radioimmunoassay we devised a method which involved the use of an insoluble derivative of an insulin antibody (Hales and Randle, 1963). This was the first solid phase radioimmunoassay method and took advantage of the manipulative simplicity which is introduced when one of the reactants in a system is insoluble. This allows its rapid and complete removal from the reaction (Fig. 1). Other solid phase radioimmunoassay techniques have subsequently been developed and provide highly, convenient methods (Catt, 1969). Solid phase reagents have been in use in other systems for many years but they continue to provide some of the most important modern developments in techniques in enzymology (Cuatrecasas and Anfinsen, 1971) and chemical protein synthesis (Stewart and Young, 1969). The general theme which I wish to develop in this lecture is that of the present and future exploitation of solid phase immunological systems in diabetes research.

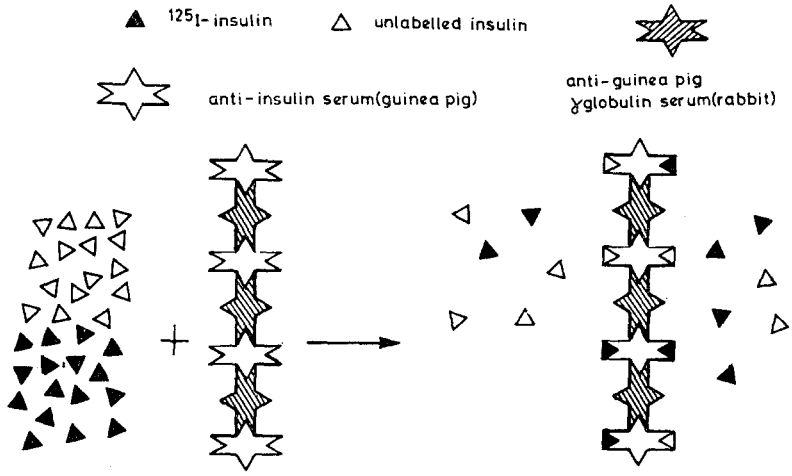

Fig. 1. Diagram of a solid phase radioimmunoassay of insulin using an antiserum to insulin raised in the guinea pig and which had been preprecipitated with an antiserum to guinea pig γ globulin raised in the rabbit

The Immunoradiometric Assay

In 1964 L.E.M. Miles and I reviewed immunoassay techniques to decide how immunoassay's might be related to generally established assay procedures and to determine whether the maximum potential (in methodological terms) had been realised. We came to the conclusion that it had not.

Radioimmunoassays may not achieve the maximum potential sensitivity because:

1. not all the unknown is reacted with antibody as a result of competition with the iodinated derivative (Fig. 2).
2. the unknown hormone may react with antibodies with which the iodinated hormone, because of the iodination, may not react. This means that antibodies directed against the site of iodination may sequester unknown but not iodinated antigen.
3. the antibody concentration is reduced to very low levels in order to obtain competition between unknown and iodinated antigen. This means that the equilibrium of the reaction between antibody and antigen may limit the amount of derivative formed.
4. they do not offer any potential for the introduction of cycling steps which would increase the yield of derivative from unknown (Miles and Hales, 1968a).

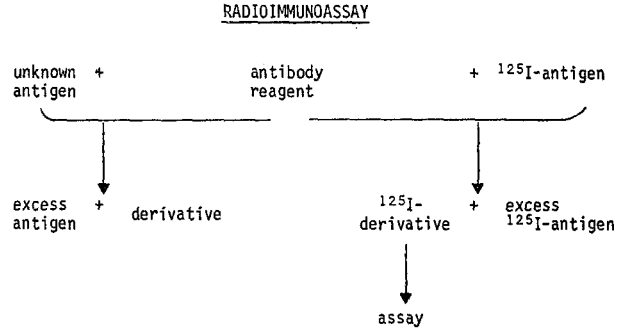

Fig. 2. General scheme of radioimmunoassay to show the indirect nature of the technique

Another important consideration which affects sensitivity is the background "noise" against which changes are measured. It has been found that radioimmunoassays achieve greatest sensitivity when half or less of the total radioactive antigen is bound to antibody in the absence of unlabelled antigen. This means that the background radioactivity against which any change is detected must be 50% of the total counts added, and therefore only relatively large changes are detectable.

In theory, immunoassay techniques would be considerably improved by the use of a relatively straightforward procedure in which labelled antibodies

were used as a reagent to produce a radioactive derivative (Fig. 3).

This would:

a) produce a stable, uniform radioactive product since antibody molecules are relatively stable and uniform molecules.

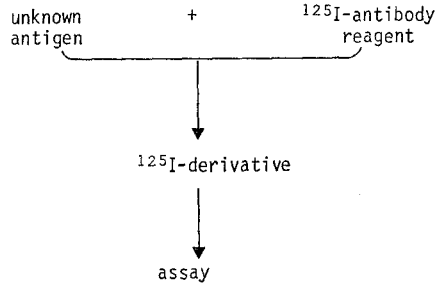

Fig. 3. General scheme of the immunoradiometric assay

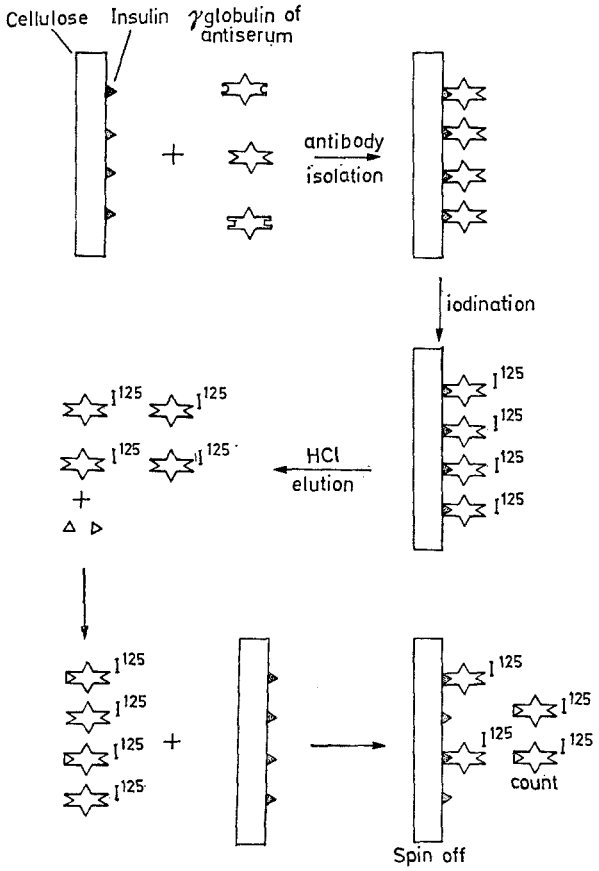

Fig. 4. Diagram of the procedure for the isolation, iodination and use of antibodies as iodinated reagents for the assay of antigen

b) avoid iodinating and therefore damaging antigen.

c) use excess antibody thus favouring the reaction in terms of production of derivative.

d) operate against a low background since in the absence of antigen no labelled derivative would be produced.

A technique was devised which allowed the use of labelled antibodies in highly sensitive immunoassays (Miles and Hales, 1968b). This is summarised diagramatically in Fig. 4.

The procedure involves: isolation of specific antibodies on an immunoadsorbent; iodination of the antibodies whilst combined to the immunoadsorbent; elution of the iodinated antibodies and finally their use as reagent for the measurement of antigen. The details of the individual steps and the rationale of the precedure have been reviewed elsewhere (Miles and Hales, 1970; Addison *et al.*, 1970; Addison and Hales, 1971a).

Immunoadsorbent. The method of Gurvich *et al.* (1961) has been selected because of the resulting immunoadsorbents non-protein matrix, multiple orientation of coupled antigen, high capacity, low non-specific protein adsorption and high yield of active antibody.

Iodination. The introduction of iodine into antibody whilst the latter is bound to the solid phase has the advantage of providing protection of at least one of the binding sites and easy removal of unincorporated iodine, iodination reagents and damaged protein. Iodinated antibodies produced either by iodine monochloride (McFarlane, 1958) or chloramine-T (Greenwood, Hunter and Glover, 1963) iodination have proved satisfactory and stable. Levels of iodine up to 8—10 atoms I per molecule of antibody appear to be tolerated.

Elution. A stepwise reduction in the pH of the eluting fluid achieves a separation of the bound antibodies according to their affinity for antigen. Antibodies are damaged by exposure to low pH and in practice therefore an optimum pH exists for the isolation of antibodies. This optimum pH usually lies in the range 2—3. The precise pH optimum is determined for each antiserum.

Storage of labelled antibody. After iodination and elution, the iodinated antibodies are stored recombined with fresh immunoadsorbent. Antibodies stored in this way may be satisfactory for at least six months.

Sensitivity. At a level of 1 atom of I^{125} per antibody molecule it may be calculated that 2×10^{-17} moles of insulin should be detectable (Miles and Hales, 1970). This sensitivity (0.2 pg) has been achieved in practice with an insulin assay. Whilst such extreme sensitivity is not required for plasma insulin determinations it could be significant in the investigation of insulin release in vitro. A rat islet of Langerhans contains approximately 5000 β cells and 100 ng insulin. Each β cell therefore contains approximately 20 pg insulin. An assay of the sensitivity described would therefore allow for the detection of as little as 1% of the insulin in a single cell. In other words it would now appear to be feasible to study insulin secretion from individual β cells.

The immunoradiometric assay would appear to have an additional advantage in the measurement of high molecular weight antigens. Such antigens bind

several molecules of antibody per molecule of antigen. For example we have found that ferritin binds 10—20 molecules of antibody per molecule of ferritin. This means that by assaying with labelled antibodies a complex is produced with 10—20 times the specific activity of that for a monovalent antigen. This advantage is not utilised in competition assays such as radioimmunoassay where the specific activity of the antigen limits the sensitivity obtainable. We have found that even relatively lightly labelled antibodies to ferritin (averaging < 1 atom I per molecule) yielded an assay which was in molar terms comparable in sensitivity to that of the most sensitive insulin assay (Addison *et al.*, 1972).

Affinity. Clearly if one has low affinity antibodies one is limited with respect to sensitivity irrespective of the assay technique employed. In theory one would expect, in the immunoradiometric assay procedure, that exchange would occur between the derivative formed and the immunoadsorbent. In practice, we have observed this to occur in a low affinity ACTH assay system (Addison and Hales, 1971a).

Specificity. Specificity remains a problem in immunoassay procedures. As we learn more of the structural characteristics of different hormones we find that more and more of them have close similarities and it becomes increasingly difficult to produce descriminating assays. The problem of determining insulin in the presence of proinsulin is but one example of a series of problems which is repeated in the similarities between pancreatic glucagon and gut glucagon-like activity, ACTH and MSH, FSH and LH and hormones and their immediate degradation products.

The immunoradiometric procedure can be used to improve specificity. Once the behaviour of the total antibody population has been characterised the cross-reacting antibodies can be quantitatively adsorbed using immunoadsorbents (Miles, 1971). Interference by antibodies which have a low affinity for a cross-reacting antigen may be eliminated by manipulation of the time of incubation with immunoadsorbent during the final separation stage in the assay (Miles, 1971; Addison and Hales, 1971a). Low affinity complexes are likely to dissociate more rapidly and hence to exchange from the cross-reacting antigen onto the immunoadsorbent.

Two site or "sandwich" assay

G.M. Addison and I have continued to extend the application of the immunoradiometric technique in response to two outstanding assay difficulties: the need for extreme and still further assay sensitivity and the problem of fragments of molecules which are immunologically apparently indistinguishable from intact molecules.

In radioimmunoassay increased sensitivity has been achieved by a preliminary extraction procedure followed by concentration of antigen. It seemed to us that the ideal extraction procedure could be produced by a solid phase antibody preparation. Using this material a highly specific extraction of antigen onto antibody could be achieved and at the same time the antigen could be concentrated on to the surface of this solid phase system. The antigen could then be detected and quantitated by reaction with labelled antibody (Fig. 5).

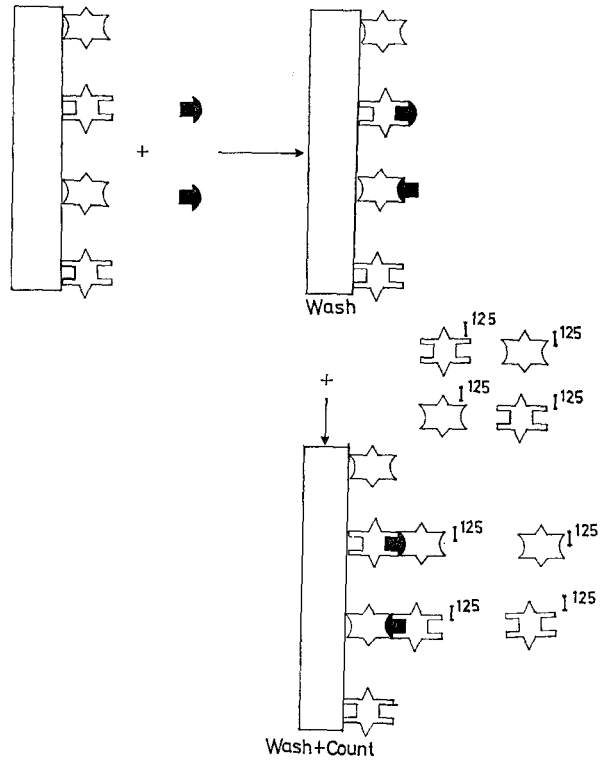

Fig. 5. Diagram of the two site or "sandwich" assay technique

This procedure would be very sensitive as a result of

1. the concentration step
2. the very low background against which changes would be measured. We have found that the non-specific uptake of radioactive antibodies can be reduced to 1% of the total counts added.

In addition the extraction procedure can be continued to use several samples and hence large volumes of plasma. The protocol of the procedure we have devised is shown in Table 1. The method has been applied to the assay of human growth hormone (Addison and Hales, 1971b; Addison and Hales, 1971c). Using recycling we have detected as little as 10 pg HGH/ml.

A further possible advantage of this type of procedure occurred to us and is illustrated diagramatically in Fig. 6. The simultaneous recognition by the "two site" technique of two different parts of a molecule

Table 1. *Protocol of 'two site' assay*

1. *Extraction*

Antibody-paper (0.5 × 0.5 cm) + Unknown antigen (0.5 ml)

Serial extraction — 3—72 h 4°

Antigen-antibody paper

2. *Wash*

3 times in buffer

3. *Reaction with labelled antibody*

Antigen-antibody paper + ^{125}I-Antibody

3—96 h 4°

^{125}I-Antibody-antigen-antibody paper + Excess ^{125}I-Antibody

4. *Wash*

4 Times in buffer

5. *Count paper*

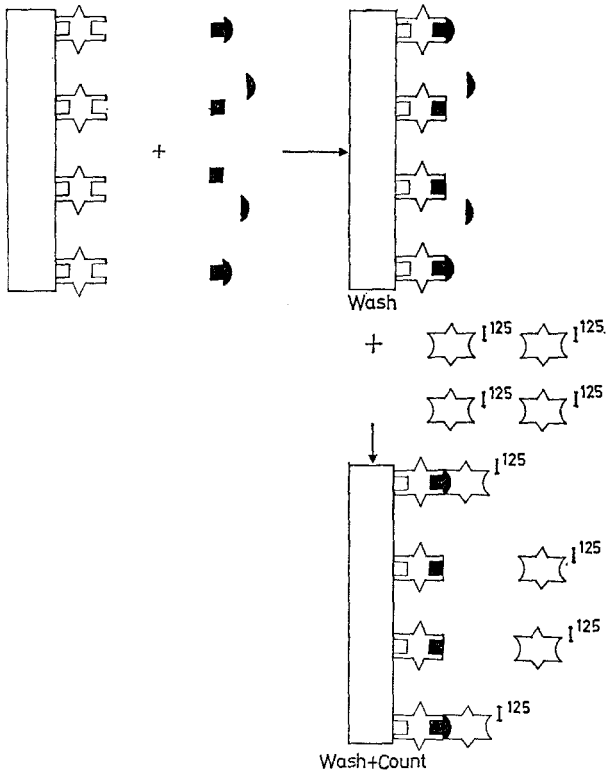

Fig. 6. Diagram of the two site assay technique in the presence of cross-reacting fragments of antigen

would considerably improve assay specificity. By using two antibodies one against insulin and the other against the C-peptide one could produce a technique for assaying proinsulin in the presence of insulin and the C-peptide. That these ideas are not entirely fanciful has been rather nicely demonstrated by very recent work which Drs. Woodhead, Addison and O'Riordan have undertaken in collaboration with Dr. Potts' group at the Massachusetts General Hospital (O'Riordan *et al.*, 1972). Using bovine parathyroid hormone and fragments of the molecule these workers have attempted to work with antibodies either directed against the N- or the C- terminal ends of the molecule. In so doing they produced an assay which would specifically detect intact bovine parathyroid hormone in the presence of the N-terminal 1—29 and C-terminal 53—84 amino acid sequences (Fig. 7).

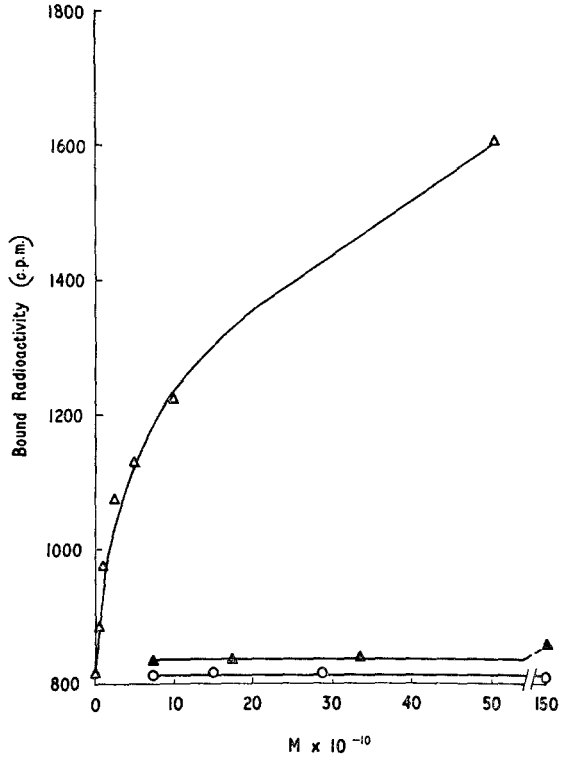

Fig. 7. Two site assay of bovine parathyroid hormone (△——△) in the presence of the N-terminal (1—29) (▲——▲) and C-terminal (53—84) (0——0) fragments.

Antigen Localisation

The use of labelled antibodies (fluorescein, iodine or enzyme conjugated) to localise antigens histologically is far from new. However most of these procedures do not in fact employ labelled antibodies but rather labelled antisera or a labelled IgG fraction of serum in which only a very small proportion of the labelled molecules are actually the active antibodies themselves. It would seem that our procedure of using an initial purification of the antibody onto immunoadsorbent would allow highly specific labelling of antibodies which could then be eluted into non-labelled carrier protein with a consequent increase in the specificity of the labelled reagent. G.M. Addison, L. Herman and I have used antibodies so labelled in the ultrastructural localisation of insulin in isolated islets of Langerhans. We intend to extend the procedure

to antibody-enzyme conjugates to explore other aspects of antibody labelling.

Antibody Estimation

The immunoradiometric assay procedure has been used to produce a sensitive assay for IgG (Addison and Hales, 1971a). Since many antibodies are IgG molecules this assay could provide a highly sensitive assay for antibodies. In practice we have used it for the measurement of antibody uptake onto immunoadsorbent in preference to a standard protein assay since it is approximately 100 times more sensitive (Addison and Hales, 1971a).

Antibodies to Cell membranes

The IgG assay may be used to assay the uptake of antibodies by cell-membranes. T.P. Luzio and I have investigated this procedure as a means of monitoring the preparation of purified fat cell plasma membrane. In order to achieve this we required an antibody specific for the cell membrane and which could be detected by the use of a ^{125}I-labelled antibody to the IgG bound to the cell membrane (Fig. 8).

Immunisation of rabbits with rat fat cells was undertaken. This was considered likely to lead to the production of antibodies to many cell constituents including cell membranes other than the plasma membrane. It was found that antisera to rat fat cells cross reacted with both rat red cells and liver cells (Fig. 9). Similarly antisera raised in rabbits against rat red cells or liver cells cross reacted with rat fat cells (Fig. 9). Antisera raised against red cells were unlikely to contain antibodies to cell membranes other than

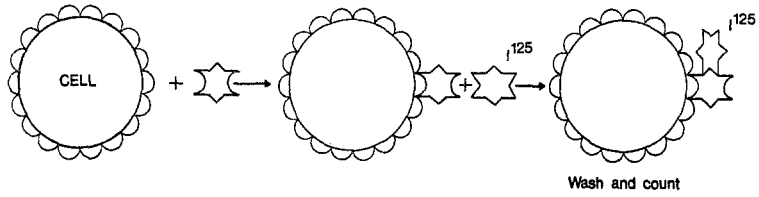

Fig. 8. Diagram showing the use of iodinated antibodies to IgG in the labelling of cell membranes. ☆ IgG antibody against cell membrane; ☆ I^{125} iodinated anti- (IgG) antibody

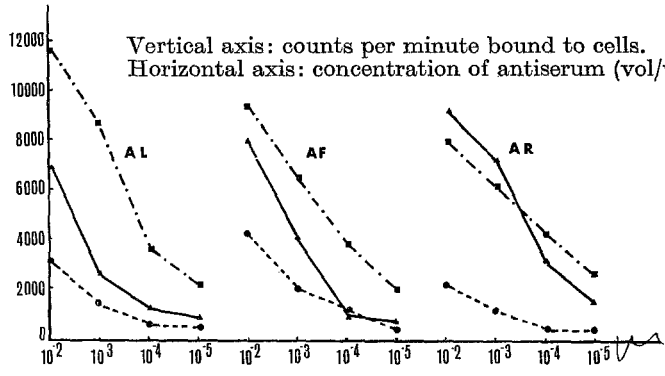

Fig. 9. Cross reactivity of rabbit anti- (rat cell) antisera with rat red cells ▲——▲, liver cells ■-·-·-■ and fat cells ●---● studied using iodinated donkey anti-(rabbit serum) antibodies. AL, rabbit anti-(rat liver cell) antiserum; AF, rabbit anti-(rat fat cell) antiserum; AR, rabbit anti-(rat red cell) antiserum

The range of this technique has been extended to the detection and quantitation of autoantibodies. Using sections of human tissue it has been shown that there is close agreement between antibody detection using labelled anti-Human IgG antibodies and the fluorescein techniques for the detection of autoantibodies. The use of iodinated antibodies in this procedure offers potentially higher sensitivity and specificity and is more amenable to automation (Hales, 1972).

the plasma membrane due to the absence of red cell intracellular organelles. It appeared therefore that the cross-reaction of antisera to the red cell membrane with the fat cell membrane could be used as a more specific means to label the fat cell membrane. This procedure appears to be valid since there is in a fat cell homogenate a reasonably close approximation between the basal and fluoride stimulated adenyl cyclase activity of different subcellular fractions and the fractions content of antibodies to red cells.

Cell membrane purification. An insoluble derivative of an antiserum to the cell plasma membrane might be capable of reacting with antigens on cell-membrane fragments in a tissue homogenate and hence of purifying this fraction. J.P. Luzio and I have conjugated antibodies to red cell membranes to cellulose for use as

an immunoadsorbent to isolate fat cell plasma membrane. Whilst some success has been achieved with this procedure it would appear that it is more the consequence of a non-specific binding of membranes to cellulose rather than to the conjugated antibodies. We are continuing to explore this approach using supporting material with a lower non-specific membrane binding capacity. It seems clear to us that this technique may be potentially rewarding in the further study of cell membrane structure and function, topics of vital importance in future research into diabetes.

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