Solid-Phase Radioimmunoassay of Human Growth Hormone

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In the technique of radioimmunoassay, antigen is measured by its effect on the binding of a small quantity of radioactive tracer antigen to a fixed quantity of specific antibody. It follows that all methods of radioimmunoassay must include some procedure to separate antibody-bound tracer from free tracer after completion of the immune reaction. To facilitate this separation, we have investigated the use of specific antibody coupled covalently to an insoluble polymer (Catt, Niall & Tregear, 1966). The most obvious advantage that should accrue from the use of such a 'solid-phase-antibody' for radioimmunoassay is simplicity of separation of 'bound' and 'free' tracer, which is achieved merely by washing the solid phase. It should also be possible to avoid the problem of tracer 'damage' (Berson, Yalow, Glick & Roth, 1964) that occurs during the incubation of tracer antigen in the presence of serum proteins. Serum, if present in the original incubation mixture, may be removed by washing the solid-phase-antibody after reaction with the unknown quantity of antigen, before addition of the tracer antigen.

Finally, the use of such a washing step before addition of the tracer antigen should give a new dimension to the technique of 'disequilibrium' (Samols & Bilkus, 1964). By thus avoiding dilution of the tracer with excess of unlabelled antigen, the number of radioactive antigen molecules combining with the unoccupied binding sites on the solid-phase-antibody must be considerably increased. This should lead to increased accuracy when estimating very small quantities of unlabelled antigen, as the effective specific activity of the tracer is much higher than when added to the system in the presence of the unlabelled antigen to be measured. The testing of a solid-phase-antibody system for the radioimmunoassay of human growth hormone showed that these advantages were, in fact, available. It further appeared that a characteristic of this system was the elimination of the reversible nature of the classical antigen-antibody reaction.

The solid-phase materials that were examined for suitability included diazotized polystyrene and PAB-cellulose,* and a substituted graft copolymer

of styrene and polytetrafluoroethylene. In preliminary experiments, it was found that diazotized PAB-cellulose, although easy to prepare, showed considerable non-specific adsorption of tracer antigen, and was slow to settle after the washing steps. Diazotized polystyrene showed less non-specific adsorption, and enabled a standard curve to be obtained over the range 1–10 µg. of human growth hormone, but did not settle rapidly after washing.

A much more useful solid-phase material, developed to overcome these deficiencies, was the isothiocyanate derivative of the graft copolymer of styrene and polytetrafluoroethylene (Imperial Chemical Industries of Australia and New Zealand Ltd., 1966). This material, which provides the advantages of high density and uniform particle size, also possesses surface-reactive substituents suitable for conjugation with protein molecules. It can be stored in the dry state without deterioration, and is readily coupled to antibody simply by shaking in an alkaline buffer.

To prepare this solid phase, the copolymer of styrene and polytetrafluoroethylene (Fluon G4) was nitrated and reduced to form poly(tetrafluoroethylene-g-aminostyrene) powder. The amino groups were then converted into isothiocyanate by treatment with CCl₂. Infrared spectroscopy was used to follow the successive modification of these groups on the aromatic rings of the polymer surface.

The human growth hormone, used to prepare high-titre antiserum by repeated immunization of rabbits, was prepared by a modification of the technique of Raben (1957). This growth hormone, after further purification by gel filtration, was also used for the preparation of tracer by labelling with ¹²⁵I or ¹³¹I to a specific activity of 100–200 µCi/µg. by a modification of the method of Hunter & Greenwood (1962). The growth hormone used for assay standards was generously given by Professor A. E. Wilhelmi, as batch HS705A.

All dilutions were performed with a solution of 0.5% bovine serum albumin in 0.15 M-NaCl solution, 0.01 M with respect to phosphate buffer, pH 7.4.

The solid-phase-antibody was prepared by shaking the poly(tetrafluoroethylene-g-isothiocyanato-styrene) powder with hyperimmune serum or

* Abbreviation: PAB-cellulose, *p*-aminobenzylcellulose.

γ -globulin in bicarbonate buffer, pH 9.6. Usually, 4.0g. of the isothiocyanate graft copolymer was shaken with 0.1ml. of antiserum, 0.9ml. of normal rabbit serum and 9.0ml. of bicarbonate buffer. After 16hr. at room temperature, the solid phase was washed and incubated with diluent solution for several hours, then finally incubated overnight with human serum diluted 1:5 with 0.15M-NaCl. The serum used for this step was obtained from a resting male volunteer after ingestion of 50g. of glucose in water.

The radioimmunoassay procedure was commenced by preparing uniform 10mg. portions of solid-phase-antibody in 20ml. counting vials. This was achieved by dispensing with an automatic syringe, from a rapidly stirred suspension of the solid-phase-antibody in 0.15M-NaCl. The supernatant was then aspirated and replaced by 0.5ml. of diluent solution. Samples of plasma, or human growth hormone standards in horse serum, were then added to each vial to give a final volume of 1.0ml. After incubation for 24–48hr. at 37°, the solid phase was washed once with distilled water,

then 70000–100000 counts/min. of tracer human growth hormone in 0.5ml. of diluent was added. After further incubation for 12–24hr. at 37°, the solid phase was washed twice with tap water, and then counted in a well-type automatic γ -radiation counter.

Extensive use of this method for the estimation of human growth hormone has shown clearly that this solid-phase system allows the radioimmunoassay procedure to be greatly simplified and abbreviated. Further, the sensitivity of the assay appears to be potentially capable of considerable improvement. The sensitivity of the method for plasma growth hormone estimation was always 0.4 $\mu\text{g.}/\text{ml.}$ or less, frequently under 0.1 $\mu\text{g.}/\text{ml.}$, and in the most sensitive assay to date, 0.02 $\mu\text{g.}/\text{ml.}$ (Fig. 1). Thus further refinement of this procedure should enable accurate determination of antigen, present in body fluids at concentration below 1 $\mu\text{g.}/\text{ml.}$, to be readily performed. Other procedures of radioimmunoassay that can detect down to 0.01 $\mu\text{g.}$ of growth hormone/ml. in the incubation mixture have employed plasma dilutions of 1:10 to 1:20 to minimize the problem of tracer damage. Thus the lower level for detection of human growth hormone in plasma by such methods is 0.1 $\mu\text{g.}/\text{ml.}$

Experiments were performed to examine the degree of reversibility of the antigen-antibody reaction on the solid phase. It would have been very convenient to add solid-phase-antibody, to which tracer antigen had been combined, to the unknown antigen solution to measure the latter by the appearance of radioactivity in solution. However, when 50 $\mu\text{g.}$ of human growth hormone was added to the system 60min. after the commencement of incubation of solid-phase-antibody with tracer growth hormone, no decrease in the degree of specific binding of the tracer was observed after further incubation for 90min. Further, the incubation of solid-phase-antibody, which had been allowed to bind tracer human growth hormone, for 16hr. with 1000 $\mu\text{g.}$ of growth hormone (representing a millionfold excess of unlabelled antigen) did not result in displacement of labelled growth hormone from the solid phase. The anchoring of the antibody molecule to a solid phase leads to a situation in which specific binding of antigen becomes an irreversible process, i.e.:

instead of:

This lack of reversibility shortens the time required to achieve optimum binding of antigen to antibody. Thus irreversibility more than outweighs the physical disadvantages of a system in

Fig. 1. Standard curve for human growth hormone. Amounts of human growth hormone in the range 10–100 $\mu\text{g.}$ were incubated for 48hr. in the presence of solid-phase-antibody. After washing, ^{131}I -labelled human growth hormone was added for a further 24hr. incubation. Radioactivity remaining on the solid phase after further washing is plotted against values for human growth hormone.

which the contact between antigen and antibody molecules is much less likely to occur than when both are in solution.

The potential usefulness of the solid-phase radioimmunoassay for simplified determination of antigen in the micromicrogram range is shown by these experiments with human growth hormone as antigen. The technical aspect of this method involves no more than the addition of standard and unknown solutions to small quantities of the solid-phase-antibody, followed by a series of washing steps before and after the addition of tracer. The entire procedure can be performed in a vial suitable for counting in a well-type γ -radiation counter. The present method of assay has been further simplified by the preparation of the antibody-coated solid phase in the form of thin wafers of uniform size and surface area, one of which can simply be dropped into the assay vial with each portion of unknown or standard solution, and antigen-antibody systems other than human

growth hormone have also been investigated by this method (K. Catt, H. D. Niall & G. W. Tregear, unpublished work).

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The Isolation of *O*- β -D-Galactopyranosyl-(1 $\rightarrow$ 4)-D-galactose: a Product of the Partial Acid Hydrolysis of the Galactan of White-Lupin (*Lupinus albus*) Seeds

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Investigations in the last few years have shown that many mammalian tissues contain a range of neutral glycolipids as minor components of the total tissue lipid. They have been characterized as ceramide monohexosides, dihexosides, trihexosides and *N*-acetylhexosaminyltrihexosides with the carbohydrate moiety linked to the ceramide through the reducing group of either glucose or, more rarely, galactose. Some glycolipids were shown to be haptens (Rapport, Graf, Skipski & Alonzo, 1959; Joffe, Rapport & Graf, 1963) and the differences in immunological specificities were due to differences in the carbohydrate structures in the molecules. A ceramide dihexoside, cytolipin H, a lipid hapten found originally in human epidermoid carcinoma grown in conditioned rats

(Rapport *et al.* 1959) but now known to occur in a variety of normal and tumour tissues, has been completely identified by chemical synthesis (Shapiro & Rachaman, 1964) as 1-*O*- β -lactosyl-ceramide.

Recent evidence indicates that several ceramide dihexosides from natural sources contained not only ceramide lactoside but also a ceramide digalactoside, and methylation studies by Makita & Yamakawa (1965) show that the structure of a ceramide digalactoside in human kidney was galactosyl-(1 $\rightarrow$ 4)-galactosyl-(1 $\rightarrow$ 1)-ceramide. The pure ceramide dihexoside has not yet been isolated from natural sources.

Because of the close association of ceramide digalactoside with cytolipin H in mammalian