Observations on the Use of Solid-Phase-Coupled Antibodies in the Radioimmunoassay of Human Placental Lactogen

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A detailed comparative assessment was made of the use of solid-phase-coupled antibodies in radioimmunoassay, by using an assay for human placental lactogen as a model system. The major advantages of the solid-phase technique are: (1) in common with the use of a second antibody, it is universally applicable; (2) separation can be carried out rapidly; (3) in contrast with some other techniques, the separation of antibody-bound and free antigen is virtually complete. The disadvantages when compared with other procedures are: (1) a considerable proportion of the antibody may be lost during the initial coupling reaction; (2) the tubes must be continuously mixed during incubation, and much effort is expended in removing and replacing the caps; (3) there is a decrease in the apparent affinity constant of the antibody after coupling, which is reflected in a lower sensitivity of the assay system. It is concluded that solid-phase antibodies are of greatest value in those systems in which the supply of antiserum is abundant, and in which the achievement of high sensitivity is not a requirement.

The use of antibodies coupled to a solid phase is, in common with the double-antibody technique, a reliable and universally applicable method for the separation of bound from free antigen in a radioimmunoassay. A number of different solid phases have been described, including cross-linked dextran (Wide & Porath, 1966), agarose (Hart, 1972), styrene derivatives (Catt *et al.*, 1966), polystyrene tubes (Catt *et al.*, 1970), and paper discs (Ceska & Lundkvist, 1972). In addition, several techniques have been described for coupling of antibody, including diazotization (Catt *et al.*, 1966), physical adsorption (Catt *et al.*, 1970), and the use of isothiocyanates (Wide & Porath, 1966) and cyanogen halides (Wide, 1969).

Few detailed comparisons have been made between the operation of solid-phase and soluble systems in a radioimmunoassay. The present study was designed to compare the two systems in a radioimmunoassay for human placental lactogen.

Materials and Methods

Materials

Human placental lactogen. Highly purified human placental lactogen was obtained from the Division of Biological Standards, Medical Research Council, London N.W.3, U.K., in capillary tubes containing 30 μ g of peptide (code number 71/169), and was used for iodination. Further supplies were obtained from

Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A., and were used for standardization and immunization. Stock standard solutions were prepared by dissolving placental lactogen in assay diluent at a concentration of 100 μ g/ml, dividing into batches of 0.5 ml and storing at -20°C .

Superfine Sephadex G-25, Sepharose 4B and Sepharose 6B. These were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden.

Microcrystalline cellulose (Merck Darmstadt, Germany). This was supplied by Anderman and Co. Ltd., London S.E.1, U.K.

Cyanogen bromide. This was obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K., and stored in a desiccator at room temperature.

Diluent. Diluent for the iodination and chromatography was 0.06 M-barbitone buffer, pH 8.6, containing 0.5 mg of human serum albumin (AB Kabi, Stockholm, Sweden)/ml. The diluent for assays was 0.01 M-sodium phosphate buffer, pH 7.4, containing 4 mg of human serum albumin/ml, 0.1% (v/v) Tween 20 (Koch-Light Laboratories Ltd.) and 0.1% (w/v) NaN_3 .

Production and assessment of antiserum

Four female New Zealand White rabbits were given an initial injection, and then two booster injections 6 and 8 weeks later, each comprising 0.5 mg of placental lactogen prepared as a water-in-oil emulsion by vigorous mixing of 2 vol. of complete

Freund's adjuvant (Difco Laboratories, Detroit, Mich., U.S.A.), with 1 vol. of aqueous peptide solution. A sample (1 ml) of this emulsion was injected subcutaneously in the neck. Blood was obtained from an ear vein 10–14 days after each boost. All sera, tested by antiserum dilution and standard curves, bound 50% of 0.8 ng of ^{125}I -labelled placental lactogen at final dilutions ranging from 1:50000 to 1:500000. One was chosen for subsequent studies.

A crude γ -globulin fraction was prepared by adding 1.8 g of Na_2SO_4 (anhydrous) to 10 ml of serum, mixing for 30 min, centrifuging at 1875g for 10 min and washing the precipitate twice with 10 ml of 18% (w/v) Na_2SO_4 . The precipitate was then dissolved in 10 ml of 0.9% NaCl and stored in batches at -20°C . The titre of the γ -globulin fraction was compared with that of the original antiserum by antiserum dilution and standard curves.

Preparation of ^{125}I -labelled placental lactogen

Placental lactogen was iodinated by the technique described by Greenwood *et al.* (1963), as modified by Letchworth *et al.* (1971). The reaction mixture was poured into a tube containing 5 ml of iodination diluent, thoroughly mixed and then applied to a column ($4\text{cm}^2 \times 35\text{cm}$) of Sephadex G-75, which was eluted with iodination diluent. Fractions (5 ml) were collected and counted for radioactivity in a 5 cm (2 in) well-type crystal and scaler (IDL 7000, Nuclear Enterprises Ltd., Beenham, Berks., U.K.), and the most radioactive fractions of the first peak were pooled and assessed by chromatoelectrophoresis and binding in antibody excess. The mean yield of ten consecutive iodinations, calculated as described by Landon *et al.* (1967), was 79%, ranging from 66 to 89%. The pooled fractions were divided into 0.5 ml batches and stored at -20°C .

Activation of solid phase

(1) *Superfine Sephadex G-25 and microcrystalline cellulose.* Superfine Sephadex G-25 is a cross-linked dextran gel in bead form, with a fractionation range for globular proteins of 1000–5000 molecular weight. The method was a modification of that described by Wide (1969). Sephadex (1 g) was swollen in 20 ml of distilled water for 15 min and then mixed for 3 min with a fresh solution of 1 g of CNBr in 20 ml of distilled water. Microcrystalline cellulose was treated identically but without preswelling. Then 5 ml of 0.1 M-NaOH was added dropwise, with constant stirring, the pH being maintained between 10 and 10.5. The suspension was transferred to a beaker containing 40 ml of 0.01 M- NaHCO_3 (to neutralize free CNBr). Acetone (240 ml) was added slowly, the mixture placed in a Buchner funnel fitted

with a grade 4 Scinta glass filter (Hoslab, London E.C.1, U.K.), washed with 500 ml of 75% (v/v) acetone in water and then 500 ml of acetone, and finally dried under suction for 1 h.

(2) *Sepharose 4B and Sepharose 6B.* The Sepharoses are agarose gels in bead form. Sepharose 4B and Sepharose 6B have protein fractionation ranges of 1×10^5 – 20×10^6 and 1×10^5 – 4×10^6 molecular weight respectively.

The procedure followed that described by Hart (1972). Sepharose (1 g) was washed with several volumes of distilled water, resuspended in water (twice bed volume) and a fresh solution of 4 g of CNBr in 35 ml of water was added slowly, followed by 0.1 M-NaOH added dropwise, the pH being maintained between 10 and 10.5 for 10 min. The suspension was transferred to a Buchner funnel with a grade 4 Scinta glass filter, washed with 500 ml of ice-cold water and 500 ml of 0.1 M- NaHCO_3 , and finally collected as a suspension in NaHCO_3 to give a volume of 50 ml. The Sepharose was not allowed to dry at any stage until the activation was complete.

The N content of each batch of activated material was measured by the micro-Kjeldahl method, and contributed 2.8, 0.5, 2.7 and 3.6% to the total weights of activated Superfine Sephadex G-25, microcrystalline cellulose, Sepharose 4B and Sepharose 6B respectively. All activated materials were stored at -20°C .

Coupling of γ -globulin to activated solid phase

The activated solid phase (100 mg) was suspended in 5 ml of 0.1 M- NaHCO_3 containing $100\mu\text{l}$ of γ -globulin solution (approx. 10 mg/ml, as determined by the biuret method) and mixed by vertical rotation (25 rev./min) for 24 h at room temperature or 68 h at 4°C .

Each immunosorbent was washed once with 10 ml of 1.0 M-ethanolamine, pH 8.0, with continuous mixing for 2 h, and then twice with 10 ml of 0.5 M- NaHCO_3 , twice with 5 ml of 0.2 M-sodium acetate buffer, pH 4.0, and twice with 10 ml of assay diluent, with 30 min continuous mixing for each wash. Antibody activity was measured in all supernatants and wash fluids by means of antiserum dilution curves. The final preparations were stored as a 5 mg/ml suspension in assay diluent, either at 4°C for immediate use, or at -20°C for storage.

There was no significant difference in the binding of ^{125}I -labelled placental lactogen by immunosorbents coupled for 24 h at room temperature, and those coupled for 68 h at 4°C .

Construction of antiserum dilution curves

For non-solid-phase assays, doubling dilutions were set up in $200\mu\text{l}$ of assay diluent, followed by 1 ml

of diluent containing approx. 0.8 ng of ^{125}I -labelled placental lactogen. Control tubes, containing no antiserum, were included with every assay. Tubes were incubated at room temperature, or at 4°C , before separation of antibody-bound from free peptide by addition of second antibody (donkey anti-rabbit precipitating antiserum; Wellcome Research Laboratories, Beckenham, Kent, U.K.) at a final dilution of 1:60, and normal rabbit serum at a final dilution of 1:600, followed by overnight incubation at 4°C , centrifugation at 1875g for 30 min and aspiration of supernatants. For solid-phase assays, the procedure was exactly the same as that described above. Duplicate dilution curves were also set up, where (a) $50\mu\text{g}$ of unlabelled placental lactogen was added to each tube and (b) immunosorbent was replaced by solid-phase-linked γ -globulin derived from normal rabbit serum. Dilution curves for each immunosorbent were included in which $100\mu\text{l}$ of diluent was replaced by horse serum (Wellcome Research Laboratories) or human male plasma. Tubes were capped and vertically rotated at 25 rev./min for times from 1 to 48 h at room temperature, or at 4°C , before separation of antibody-bound from free peptide. This was carried out by brief centrifugation before removal of caps, centrifugation at 1875g for a further 5 min, aspiration of supernatants leaving a constant volume (about $100\mu\text{l}$) in each tube, and one wash with 1 ml of assay diluent.

Construction of standard curves

For non-solid-phase assays, doubling dilutions of placental lactogen were set up in $100\mu\text{l}$ of assay diluent, followed by addition of $900\mu\text{l}$ of diluent containing approx. 0.8 ng of ^{125}I -labelled placental lactogen, and $200\mu\text{l}$ of diluent containing an appropriate dilution of antibody. Control tubes were set up containing either no unlabelled placental lactogen (zero standard), or no antiserum (diluent blanks), or an excess of antiserum. All tubes were incubated for 24 h at 4°C before separation of bound from free antigen.

For solid-phase assays, the procedure was as described above, but substituting an appropriate quantity of immunosorbent in each tube in place of soluble antibody. Control tubes were set up containing either no unlabelled placental lactogen (zero standard), or an excess of unlabelled placental lactogen ($50\mu\text{g}$), or a solid phase coupled to γ -globulin derived from normal rabbit serum. With the Sepharoses and microcrystalline cellulose, tubes containing excess of immunosorbent were also included. This was not possible with Superfine Sephadex owing to low binding capacity. All tubes were capped and vertically rotated at 25 rev./min for 24 h at 4°C before separation of antibody-bound from free hormone.

For comparison of solid-phase assays with non-solid-phase assays, standard curves in the different systems were set up at an identical bound-to-free ratio in the zero standard.

Assessment of 'free' ^{125}I -labelled placental lactogen

Antiserum dilution curves were constructed by using Sepharose 6B and the tubes were incubated at room temperature or 4°C for 1 and 24 h. Supernatant (0.5 ml) was then removed from each tube and treated with second antibody as described above. In a similar experiment, dilution curves were obtained by incubation of the tubes at 4°C or room temperature for 72 h; 0.5 ml of supernatant was removed and mixed with an excess of antiserum (at a final dilution of 1:8500), incubated for 1 h at room temperature and then treated with second antibody. Controls included dilutions of Sepharose conjugated to non-immune rabbit serum γ -globulin, and tubes containing ^{125}I -labelled placental lactogen in diluent only.

Calculation of results

The following formula was used for calculation of results:

$$B = \frac{A_p - D}{A_{\text{max.}} - D} \times 100$$

where B is the true percentage of ^{125}I -labelled placental lactogen bound to antibody, A_p is the total radioactivity (c.p.m.) in the precipitate, D is the radioactivity precipitated in the diluent blank (in practice, not greater than 4%) and $A_{\text{max.}}$ is the radioactivity precipitated in tubes containing an excess of antiserum or immunosorbent.

For the calculation of affinity constants, the results from standard curves were plotted as the ratio of antibody-bound to free hormone against the concentration of bound antigen expressed in mol/litre (Scatchard, 1949). For the antiserum studied, the function was non-linear, indicating heterogeneity of the antibody population. The affinity constant was therefore calculated as the initial slope of the plot, at the greatest values of the bound-to-free ratio.

The sensitivity of the standard curves was expressed as the precision of measurement of a zero quantity (Ekins, 1971) and precision was defined as twice the standard deviation of 30 replicate estimates of the zero standard.

Results

Assessment of antiserum

Fig. 1 shows the dilution curve, standard curve and Scatchard plot of the antiserum chosen for

Fig. 1. Dilution curves (a), standard curves (b) and Scatchard plots (c) of antiserum (●) and derived γ -globulin (■) before attachment to solid phase

For dilution curves, increasing amounts of antiserum were incubated with 0.8 ng of ^{125}I -labelled placental lactogen, and for standard curves, increasing amounts of unlabelled placental lactogen were incubated with 0.8 ng of ^{125}I -labelled placental lactogen and antiserum at a final dilution of 1:360 000 in a total incubation volume of 1.2 ml for 24 h at 4°C. Second antibody and normal rabbit serum were then added at final dilutions of 1:60 and 1:600 respectively, followed by overnight incubation at 4°C, centrifugation at 1875g for 30 min, and aspiration of supernatant. ^{125}I -labelled placental lactogen bound per tube was expressed as a percentage of that bound in antibody excess with no unlabelled placental lactogen present and was plotted against final dilution of antibody solution (a) or unlabelled placental lactogen present (b). Bound-to-free ratio of placental lactogen was plotted against bound placental lactogen expressed in mol/litre (c).

Table 1. Recovery of antibody activity on different solid phases

For details see the text. The Table shows the amount of antibody coupled to immunosorbent.

Solid phase	% of initial antibody preparation apparently coupled	Antibody activity of solid phase, as % of that apparently coupled	Net recovery of antibody activity, as % of that of the original preparation
Superfine Sephadex G-25	27	1.3	0.35
Microcrystalline cellulose	7	26.0	1.8
Sepharose 4B	100	23.0	23.0
Sepharose 6B	100	30.0	30.0

attachment to solid phase, and of the γ -globulin fraction obtained from this antiserum. The apparent affinity constants of the antiserum and γ -globulin, at the same final dilution, were 0.5×10^{11} and 0.7×10^{11} litre/mol respectively. Sensitivity was approx. 2 ng/ml for both.

Amount of antibody coupled to immunosorbent

Table 1 shows the percentage of γ -globulin (100 μl) apparently coupled to each solid phase, and the percentage of that coupled which retained its ability to bind ^{125}I -labelled placental lactogen. In terms of the amount of active antibody recovered from the original γ -globulin preparation, the Sepharoses were

clearly superior to cellulose, and much superior to Superfine Sephadex.

Effect of time and temperature on the binding of ^{125}I -labelled placental lactogen by immunosorbents

At room temperature, the non-solid-phase system reached equilibrium more rapidly than the solid-phase systems (Figs. 2a and 2b). At 4°C, all systems required at least 24 h to reach equilibrium (Figs. 2c and 2d). For all systems the apparent titre was greater at 4°C than at room temperature. At room temperature, the solid-phase systems (in particular the Sepharoses) showed deterioration in the binding of ^{125}I -labelled placental lactogen over a 28 h period;

Fig. 2. Dilution curves of original γ -globulin (a) and γ -globulin attached to Sepharose 6B (b) incubated at room temperature, and also of original γ -globulin (c) and γ -globulin covalently attached to Sepharose 6B (d) incubated at 4°C

For non-solid-phase assays increasing amounts of γ -globulin were incubated with 0.8 ng of ^{125}I -labelled placental lactogen in a total volume of 1.2 ml, for 1 h (■), 4 h (●) and 24 h (▲), before addition of second antibody and normal rabbit serum at final dilutions of 1:60 and 1:600 respectively, overnight incubation at 4°C , centrifugation at 1875g for 30 min and aspiration of supernatants. For solid-phase assays, increasing amounts of immunosorbent were incubated by vertical rotation with 0.8 ng of ^{125}I -labelled placental lactogen, in a total volume of 1.2 ml, for 1 h (■), 4 h (●), and 24 h (▲), before separation by centrifugation and one wash with 1 ml of diluent. ^{125}I -labelled placental lactogen bound per tube was expressed as a percentage of the total added to each tube, and plotted against final dilution of γ -globulin, or μg of immunosorbent per tube.

this was less apparent at 4°C , and also when the amount of human serum albumin in the buffer was decreased from 4 to 0.5 mg/ml.

Omission of Tween 20 from the buffer significantly retarded attainment of equilibrium.

Binding of ^{125}I -labelled placental lactogen bound in immunosorbent excess was consistently higher than that bound in soluble-antibody excess, separated by second antibody. This suggests that the latter procedure, even after careful optimization, did not completely precipitate the antigen-antibody complex.

Tubes containing solid phase coupled to γ -globulin from a non-immunized rabbit retained less than 4% of ^{125}I -labelled placental lactogen, on separation as did tubes containing immunosorbent in the presence of excess of unlabelled placental lactogen.

Comparison of the apparent affinity constants of soluble and insoluble antibodies

The apparent affinity constant of the soluble γ -globulin at dilutions which bound between 35

Table 2. *Percentage of radioactivity in the supernatant bound by an excess of antibody after incubation*

For details see the text.								
	¹²⁵ I-labelled placental lactogen in diluent, no mixing	¹²⁵ I-labelled placental lactogen in diluent, continuous mixing	¹²⁵ I-labelled placental lactogen + non-immune globulin coupled to Sepharose 4B			¹²⁵ I-labelled placental lactogen + anti-placental lactogen coupled to Sepharose 4B		
Sepharose 4B (μ g)	—	—	50	3	0.2	50	3	0.2
Percentage bound	79	58	23	28	28	10	24	39

and 66% of ¹²⁵I-labelled placental lactogen ranged from 0.5×10^{11} to 1×10^{11} litre/mol. The corresponding constants for the solid phases ranged from 0.1×10^{11} to 0.3×10^{11} litre/mol. This was reflected in lower sensitivity, the least amount detected being two to three times greater than with the soluble system.

Precision

There was no difference in precision between the two types of assay.

Effect of human male plasma and horse serum on the solid-phase assay

The addition of 100 μ l of human male plasma to each tube of a solid-phase assay had no significant effect on the results. The addition of a similar quantity of horse serum had a variable effect according to the batch used; in some cases the percentage of ¹²⁵I-labelled placental lactogen bound in the zero standard was decreased, in other cases it was increased by as much as 8%.

Assessment of 'free' ¹²⁵I-labelled placental lactogen

Addition of second antibody to the supernatants of solid-phase antiserum dilution curves precipitated no significant amount of radioactivity. Thus there was no evidence for the release of antibody from the solid phase with the formation of soluble antigen-antibody complexes. The proportion of radioactivity in the supernatant that could be precipitated by an excess of antiserum fell considerably after 72h incubation with Sepharose (Table 2), indicating deterioration of ¹²⁵I-labelled placental lactogen in the presence of Sepharose.

Discussion

The aim of the present studies was to compare a number of solid phases for their behaviour as immunosorbents, and to compare the properties of soluble and insoluble antibodies to placental lactogen. All four materials could be covalently linked to

antibodies. However, as shown by Boegman & Crumpton (1970) with myoglobin, the total amount of protein bound, and the proportion that was active in the bound form, showed considerable variation between the different solid phases. This can be attributed to three factors: (1) the number of activated sites on the material, as judged by their N content; this, in turn, may be a function of particle size; (2) the accessibility of the activated sites to γ -globulin molecules during the coupling procedure, (3) the accessibility of the coupled γ -globulin molecules to antigen. The first factor is illustrated by the results with microcrystalline cellulose: the number of activated sites was considerably less than with the other materials, and this was reflected by the small proportion of the initial γ -globulin that was coupled (Table 1). In the case of this material, the relatively small number of activated sites might be a function of the size of the particles, and it is conceivable that other preparations would have revealed greater uptake of antibody. The second factor is illustrated by comparison of the results with the Sephadex and the Sepharoses: although the number of activated sites on the Sephadex was equivalent to that of the Sepharoses, the amount of antibody bound was considerably less, suggesting that many of the activated sites were inaccessible to γ -globulin molecules. This observation is compatible with the fact that the protein exclusion limit of the Sephadex G-25 is very much less than that of the Sepharoses. The third and final factor, accessibility of coupled antibody to antigen, is illustrated by the results with Sephadex; the proportion of the coupled antibody that retained immunological activity was considerably less than with the other materials (Table 1). One explanation for this might be an alteration in the structure of the Sephadex under the conditions of the coupling procedure, allowing entry of molecules into the gel structure, which would not be possible under the conditions of the assay.

Even under optimum conditions, some 70–80% of the original antibody activity was lost after coupling to a solid phase. This can be attributed in part to steric hindrance, and in part to non-optimum coupling of the γ -globulin molecule

through residues that formed part of the antibody-combining sites. The solid phases required more time to reach equilibrium at room temperature than the soluble system, as noted by Wide *et al.* (1973). This may be due to the need for the antigen molecules to diffuse further in order to react with discrete packages of antibody, when compared with the ready availability of soluble antibody. In the present studies, the percentage of ^{125}I -labelled placental lactogen bound in antibody excess was consistently greater with the solid-phase system than with the second antibody system. Other authors (e.g. Wide *et al.*, 1973) have also commented on the efficiency of solid-phase techniques in achieving a complete separation of antibody-bound and free antigen. However, in the presence of solid-phase antibodies, and in particular the Sepharoses, there was a deterioration in the immunoreactivity of ^{125}I -labelled placental lactogen. The reason for this is unknown. One possibility is that it represents progressive elution of the antibody from the immunosorbent, subsequent to contact with antigen, with the formation of soluble antigen-antibody complexes which, after separation, remain in the free phase. A phenomenon of this type has been observed with deoxycorticosterone linked to agarose (Ludens *et al.*, 1972). However, the experiments designed to examine this question yielded negative results, inasmuch as the 'free' fraction appeared to contain no radioactivity precipitable by second antibody. The point is particularly critical, since leaching of antibody could be responsible for the observed decrease in the apparent affinity constant of the insoluble antibodies, and hence the sensitivity of the resulting antibody. Thus, if the release of antibody from solid phase is associated with antigen binding, then the antibodies of highest affinity will be most affected by the process.

The decrease in the apparent affinity constant of an antibody after coupling to a solid phase has not previously been documented. However, Arends (1971) noted that a solid-phase system for human chorionic gonadotrophin had a lower sensitivity when compared with the same antibody in solution, as did Hart (1972) with sheep and goat prolactin; these findings are compatible with a decreased apparent affinity constant. The present experiments demonstrate a clear difference between solid-phase and soluble antibodies to placental lactogen, for which the most likely explanation is a change in the basic kinetics of the antibody-antigen reaction. The latter obeys the Law of Mass Action, which assumes that the various components are freely available throughout the system. However, with a solid phase, the antibody is present in discrete 'packets' and thus occupies a volume very much smaller than that of the antigen. A full theoretical analysis of this type of system, on the lines

laid down by Ekins (1971) and Feldman & Rodbard (1971), would be of great interest.

The loss of sensitivity consequent on the decrease in affinity constant can be partly overcome by adjusting the antibody concentration to decrease the binding of labelled hormone in the zero standard (c_0). Thus Wide *et al.* (1973), as a routine, operate solid-phase gonadotrophin assays with a c_0 of 10–15%, in contrast with the 50–60% normally used with a soluble system. A decrease in c_0 will decrease the slope of the standard curve and, inevitably, the precision of estimates. However, for many purposes, such as the measurement of placental lactogen, high sensitivity is not a requirement, and the solid-phase system can then be operated at a higher c_0 , with an increase in both slope and precision. In addition, the high efficiency of the separation with solid-phase antibodies will yield a further advantage over other techniques in those situations where sensitivity is not critical.

Of the solid phases studied here, the Sepharoses with their high efficiency and capacity have many advantages for use in radioimmunoassay and affinity chromatography. Further, the limits of their capacity were not reached in the present experiments, and can be enhanced at least an order of magnitude by increasing the amount of antibody available for coupling. The disadvantages of the Sepharoses are their instability in the dried state before activation, and the inaccuracies involved in handling the viscous suspensions in which they are supplied.

In common with the use of second antibody, solid-phase systems are universally applicable for the separation of bound and free hormone. Their particular advantages over the second-antibody method are speed and the efficiency of the separation. Their disadvantages are the loss of antibody during the initial coupling reaction, the need for continuous mixing during incubation, involving considerable manual effort in capping and uncapping of tubes, and, at least with human placental lactogen, human chorionic gonadotrophin and prolactin, a decrease in the apparent affinity constant and of the sensitivity of the assay.

It may be concluded that the use of solid-phase antibodies offers many advantages in the systems in which the supply of antiserum is abundant, and in which achievement of high sensitivity is not a primary requirement of the assay.

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