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Development of a non-extracted 'two-site' immunoradiometric assay for corticotropin utilizing extreme amino- and carboxy-terminally directed antibodies

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The development of a 'two-site' immunoradiometric assay (i.r.m.a.) for the direct estimation of human corticotropin-(1–39)-peptide in plasma is described. The assay is based on the simultaneous addition of ^{125}I-labelled sheep anti-(*N*-terminal corticotropin) IgG (immunoglobulin G) antibodies and rabbit anti-(*C*-terminal corticotropin) antiserum to standards and unknowns (0.5ml) followed by 18h incubation. The use of solid-phase reagents was avoided in order to minimize non-specific effects and the time required for reactants to reach equilibrium. Instead, the separation of corticotropin-bound from free labelled antibody is achieved by the addition of sheep anti-(rabbit IgG) antiserum, which precipitates bound labelled antibody by complex-formation with rabbit anti-corticotropin antibodies, which are also hormone-bound. Several ^{125}I-labelled sheep anti-(*N*-terminal corticotropin) IgG preparations were assessed in the i.r.m.a. Although each was derived from antisera raised to a thyroglobulin conjugate of synthetic corticotropin-(1–24)-peptide (Synacthen), purification of immunoglobulins before iodination by selective immunoadsorption resulted in preparations with distinct specificities which demonstrated marked differences in binding to intact human corticotropin-(1–39)-peptide. These preparations are compared in combination with two rabbit anti-(*C*-terminal corticotropin) antisera. A 'two-site' assay based on the use of ^{125}I-labelled sheep anti-[corticotropin-(2–16)-peptide] IgG and rabbit anti-[corticotropin-(34–39)-peptide] antiserum was optimized, since steric inhibition of antibody binding was avoided with this combination and because the measurement of only intact human corticotropin-(1–39)-peptide and not fragments was assured by the use of terminal antibodies. This i.r.m.a. is characterized by rapid equilibration of reactants, a wide 'operating range' (the precision of dose estimates was <4% over the range 30–2200pg/ml) and high sensitivity [8pg of corticotropin/ml (95% confidence interval 3.7–12.0) (4pg minimal detectable mass) can be detected directly in plasma].

The determination of corticotropin in biological fluids is of importance in the assessment of hypothalamo–pituitary–adrenal function and the management of its disorders (Krieger & Ganong, 1977; Rees & Lowry, 1979). The 39-amino-acid peptide hormone has been measured by a variety of biological (Sayers *et al.*, 1948; Beaven *et al.*, 1964; Chayen *et al.*, 1971) and radioreceptor (Wolfsen *et al.*, 1972) assays, but it is the corticotropin radioimmunoassay (r.i.a.; Yalow *et al.*, 1964; Landon & Greenwood, 1968; Donald, 1968) which has been of the greatest use to the clinician. Nonetheless, corticotropin r.i.a.'s are not without problems. The estimation of basal circulating levels of the hormone (20–60pg/ml) by direct r.i.a. (Berson & Yalow, 1968) requires an antibody of very high affinity, and such antibodies are not readily available. Therefore, the vast majority of corticotropin r.i.a.'s employ antibodies of only moderate affinity and utilize a preliminary extraction step involving adsorption to glass powder

Abbreviations used: i.r.m.a., immunoradiometric assay; r.i.a.('s), radioimmunoassay(s); IgG, immunoglobulin G.

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followed by elution and evaporation of solvent, to concentrate analyte from relatively large volumes of sample plasma in order to obtain the necessary assay sensitivity. However, sample extraction is a laborious procedure and subject to manipulative error and variable recoveries. A second problem has been the lack of specificity of corticotropin r.i.a.'s. Thus the cross-reaction of precursor molecules, fragments resulting from peptidase activity on the intact molecule, and other peptides with sequence homology in the r.i.a., has led to dissociation between estimates obtained by bioassay and immunoassay and difficulty in interpreting the latter (Imura *et al.*, 1965; Fleischer *et al.*, 1966). Plasma peptidases present in the corticotropin fraction after extraction may also interfere directly in the r.i.a. by degrading the radioiodinated corticotropin during the long incubation periods which are characteristic of the assay. The need for a corticotropin immunoassay which overcomes the limitations of the r.i.a. has prompted us to develop an immunoradiometric assay (i.r.m.a.) for the hormone. There is increasing evidence that such assays, which are based on the use of radioisotopically labelled specific antibodies, have several practical advantages over labelled-antigen techniques. Thus they are potentially more sensitive and specific and are quicker and easier to perform (Hunter & Budd, 1981; Al-Shawi *et al.*, 1981). The potential of labelled-antibody immunoassays for the measurement of corticotropin has long been recognized (Addison & Hales, 1971), but the method developed by those workers failed to demonstrate the advantages expected of it. This may be because the assay was of the conventional or 'one-site' variety and therefore subject to the limitations of that system. The assay that we have developed is of the 'two-site' variety (Wide, 1971; Addison & Hales, 1971), except that it avoids the use of solid-phase reagents in order to reduce non-specific effects and the time required for reacants to reach equilibrium.

We have previously reported the development of such a 'liquid-phase' i.r.m.a. for human prolactin (Hodgkinson *et al.*, 1984) and have discussed the limitations, imposed on the performance characteristics of the assay, of using antibodies (labelled and unlabelled) which compete for binding sites on the analyte molecule. As such problems would be aggravated in assays for smaller analytes such as corticotropin, we believe that the problem would have to be overcome in order to establish a highly sensitive, clinically useful, assay for the hormone which avoids the need for sample extraction. Consequently, we have raised antibodies to extreme terminal fragments of corticotropin in order to minimize competition. This approach also has other advantages. The use of *N*- and *C*-terminally directed antibodies greatly increases assay specificity by ensuring the measurement of only the intact analyte molecule and not degradation fragments. Furthermore, addition of specific antibodies in excess ensures the rapid sequestration of analyte, removing it from the influence of plasma peptidases. The present paper discusses the development and initial validation studies of the assay.

Materials and methods

Materials

Highly purified (200 i.u./mg) human corticotropin-(1–39)-peptide for use as standard was prepared in this laboratory by the method of Scott & Lowry (1974). Synthetic corticotropin-(1–24)-peptide (Synacthen), corticotropin-(17–39)-peptide, [D-Ser^{1},Lys^{17,18}]corticotropin-(1–18)-octadecapeptide amide and corticotropin-(11–39)-peptide were gifts from Ciba–Geigy Ltd., Horsham, Sussex, U.K. Corticotropin-(34–39)-peptide was purchased from Bachem, Torrance, CA 90505, U.S.A. Glutaraldehyde, thyroglobulin, phenylmethanesulphonyl fluoride, *p*-hydroxymercuribenzoate, *N*-ethylmaleimide, EDTA, bacitracin and thiomersal [ethyl-(2-mercaptobenzoato-sulphur)mercury sodium salt] were purchased from Sigma. A sheep anti-(rabbit IgG)-precipitating antiserum and normal sheep serum were purchased from ILS Ltd., London E.C.1, U.K. Horse serum 3 (non-inactivated) and normal rabbit serum were obtained from Wellcome Reagents, London S.E.13, U.K. Iodogen (1,3,4,6-tetrachloro-3α,6α-diphenylglycouril) was obtained from Pierce and Warriner, Chester, U.K., and acetonitrile (h.p.l.c. grade) from Rathburn Chemicals, Walkerburn, Peeblesshire, Scotland, U.K.; Sephacryl S-300 and CNBr-activated Sepharose 4B were from Pharmacia; human serum albumin from the Blood Products Laboratory, Department of Health and Social Security, Elstree, Herts., U.K.; and Freund's complete and incomplete adjuvant from Difco Laboratories (Detroit, MI, U.S.A.). All other reagents were AnalaR grade and purchased from BDH.

Methods

Immunogens. Preparation of immunogens was achieved by the glutaraldehyde conjugation of peptide to thyroglobulin. Peptide (1–2 mg) and thyroglobulin (2–3 mg) were dissolved in 0.1 M-$NaHCO_3$ (0.5 ml). Glutaraldehyde [10 μl of an aq. 25% (v/v) solution] was added to the peptide/thyroglobulin solution and incubated for 30 min in the dark before the addition of glycine (0.5 M, 100 μl). The conjugates were stored at −20°C.

Antiserum production. Antisera were raised in

two sheep (code names PS1 and PS2) by immunizations at 1 month intervals with conjugated Synacthen [150 μg of corticotropin-(1–24)-peptide] emulsified in Freund's complete adjuvant for the primary injection (conjugate/adjuvant, 1:3, v/v) and subsequently with incomplete adjuvant. The animals were bled 10–14 days after each immunization and antisera assessed by the preparation of antiserum dilution and r.i.a. dose–response curves. Antisera were raised in rabbits by immunizations at 1 month intervals with conjugated corticotropin-(34–39) (Apro; 100 μg of peptide) as described above. The rabbit anti-[corticotropin-(17–39)-peptide] (CII) antiserum was a gift from Professor J. Edwardson, MRC Neuroendocrinology Unit, Western General Infirmary, Newcastle upon Tyne, U.K.

Preparation of immunoadsorbents. Immunoadsorbents were prepared by the coupling of peptides to CNBr-activated Sepharose-4B according to the procedure outlined by the manufacturers. Thus peptide [8 mg of either corticotropin-(1–24)-peptide or [D-Ser^{1},Lys^{17,18}]corticotropin-(1–18)-peptide amide or corticotropin-(11–39)-peptide] dissolved in 0.1 M-$NaHCO_3$ buffer, pH 8.3, containing NaCl (0.5 M) was added to CNBr-activated Sepharose-4B (1 g) that had previously been swollen and washed with copious amounts of 1 mM-HCl. Coupling was allowed to proceed overnight at 4°C with gentle mixing. Uptake of peptide was never less than 98%. The solid phase was then washed to remove uncoupled peptide, and the blocking of remaining active sites was achieved by mixing with β-alanine (0.2 M) in the above buffer. Immunoadsorbents were stored at 4°C in 0.15 M-NaCl containing thiomersal (0.01%, w/v) as preservative and washed before use with 0.1 M-formic acid containing acetonitrile (20%, v/v) and then with 0.01 M-Tris containing 0.15 M-NaCl until the pH returned to neutral.

Purification of specific immunoglobulin. Specific immunoglobulin was purified from the sheep antisera by a modification of the method of Hodgkinson & Lowry (1982). Antiserum (10 ml) was made 1.9 M with respect to $(NH_4)_2SO_4$ and mixed at room temperature (15 min) before centrifugation (3000 g, 10 min). The supernatant was discarded and the precipitate resolubilized in 0.15 M-NaCl (10 ml) before reprecipitation as described above and resolubilization in 0.01 M-$NaHCO_3$ containing 0.15 M-NaCl and thiomersal (0.01%; 8 ml). Immunoadsorption was carried out on the resolubilized immunoglobulin fraction by mixing gently overnight with Sepharose-coupled peptide (2 ml) at 4°C. The immunoadsorbent was transferred to a glass column (0.7 cm × 10 cm) and washed with 0.01 M-$NaHCO_3$ containing 0.15 M-NaCl and thiomersal (0.01%; 10 ml) and with the same buffer containing acetonitrile (20%, v/v). Adsorbed immunoglobulin was eluted from insolubilized peptide by stepwise reduction of pH with the following buffers: 0.05 M-ammonium acetate containing acetonitrile (20%, v/v) (5 ml), pH 6.5; 0.0675 M-ammonium acetate buffer, pH 5 (0.05 M-ammonium acetate/0.017 M-acetic acid), containing acetonitrile (20%, v/v) (5 ml); and 0.155 M-ammonium acetate/formate buffer, pH 3.7 (0.05 M-ammonium acetate/0.105 M-formic acid), containing acetonitrile (20%, v/v) (5 ml). Fractions (1 ml) were collected into NH_4HCO_3 (1 M, 200 μl), and the acetonitrile removed by placing eluant fractions under a stream of nitrogen. The bulk of specific immunoglobulin (1.6–1.8 mg, >70%) from each of the sheep antisera was eluted at pH 3.7 in 2 × 1 ml fractions and was identified by the preparation of antiserum dilution curves and by u.v. spectrophotometry at 280 nm (for IgG, $A_{280,\,1\,cm}^{1\,mg/ml} = 1.4$). The immunoglobulin was stored at −20°C in 80 μg (0.5 nmol, 80 μl) samples for radioiodination.

Sheep anti-corticotropin immunoglobulin preparation. The highly purified immunoglobulin required for radioiodination was prepared from the two sheep antisera (PS1 and PS2), which were raised against the synthetic corticotropin-(1–24)-peptide (Synacthen) conjugate by using the general procedure outlined above. However, a range of immunoadsorbents were employed, which resulted in preparations with distinct specificities. Thus preparation PS1a was immunopurified on insolubilized corticotropin-(1–24)-peptide, and PS1b was immunopurified on insolubilized [D-Ser^{1},Lys^{17,18}]-corticotropin-(1–18)-peptide amide. Antiserum PS2 did not bind to insolubilized [D-Ser^{1},Lys^{17,18}]-corticotropin-(1–18)-peptide amide. Instead, PS2a was prepared by immunopurification on insolubilized corticotropin-(11–39)-peptide.

Radioiodination of sheep anti-corticotropin immunoglobulin fractions. Radioiodination was achieved by a modification of the Iodogen method (Salacinski *et al.*, 1981). Iodogen (100 μl of a 92.5 μM solution in dichloromethane) was added to a polypropylene vial [no. 690; Sarstedt (U.K.) Ltd., Beaumont Leys, Leicester, U.K.] and the solvent evaporated. To the vial, 0.5 M-phosphate buffer, pH 7.4 (20 μl), sheep anti-corticotropin IgG (80 μg, 0.5 nmol) and $Na^{125}I$ (10 μl, 1.0 mCi, 0.5 nmol; The Radiochemical Centre, Amersham, Bucks., U.K.) were added in order. The iodination was allowed to continue for 10 min before transferring the vial contents to 0.05 M-phosphate buffer, pH 7.4 (250 μl) for 5 min, followed by addition of carrier sheep serum (250 μl). The incorporation of iodine into IgG ranged from 75% to 90%, resulting in preparations with specific radioactivities between 9.4 and 11.2 μCi/μg. The mixture was applied to a column

(1.0cm × 90cm) of Sephacryl S-300 previously equilibrated with 0.05M-phosphate buffer, pH 7.4, containing bovine serum albumin (0.5%) and thiomersal (0.01%), and was eluted at $3\,ml\cdot h^{-1}$. Fractions (1.5 ml) were collected. A single major peak of radioactivity was eluted in the position expected of sheep anti-corticotropin IgG ($K_{av.} = 0.26$). To the pooled peak, mannitol (1.0%, w/v) and normal sheep serum (10%, v/v) were added before freeze-drying and storage at −20°C.

The immunoreactivities of radioiodinated sheep anti-corticotropin IgG preparations were assessed by their ability to reassociate with an excess of the immunoadsorbent on which they were purified. PS1a, PS1b and PS2a each demonstrated in excess of 90% binding to their respective immunoadsorbents. Non-specific binding of labelled antibody to Sepharose 4B was less than 3%. Binding of ^{125}I-labelled sheep anti-corticotropin IgG preparations had declined to approx. 50% after 2 months storage.

Standards. Corticotropin standards were prepared by adding a known weight of highly purified human corticotropin-(1–39)-peptide to horse serum which had previously been treated to eliminate peptidase activity. The treatment included two successive freezing and thawing cycles, heating the horse serum to 54°C for 30min, then, after cooling, addition of phenylmethanesulphonyl fluoride, *p*-hydroxymercuribenzoate, *N*-ethylmaleimide and EDTA, each to a final concentration of 1 mM and addition of bacitracin and thiomersal (both 0.01%). The horse serum was then mixed for 2h at room temperature and the pH adjusted to 7.4 by the dropwise addition of phosphoric acid (2M) before filtration through a 0.45 μm-pore-size filter. Whereas a loss of immunoreactive corticotropin of 35% was observed in the untreated horse serum after 3h incubation at 37°C, this was decreased to less than 5% in treated serum. A range of corticotropin standards from 5 to 4000pg/ml were prepared in treated horse serum and stored at −20°C in portions for assay.

I.r.m.a. development. The buffer for all assay experiments was 0.128M-phosphate, pH 7.4 (0.104M-Na_2HPO_4/0.024M-NaH_2PO_4) containing poly(ethylene glycol) 6000 (2%, w/v), human serum albumin (0.5%, w/v), mannitol (1.0%, w/v), normal rabbit serum (1.0%, v/v), normal sheep serum (2.0%, v/v), thiomersal (0.01%) and phenylmethanesulphonyl fluoride (0.2mM).

The corticotropin i.r.m.a. avoids the use of solid-phase reagents. This was achieved by using specific antibodies raised in two different species. The radiolabelled *N*-terminal corticotropin antibody preparations were of sheep origin and the *C*-terminal-specific corticotropin antisera were raised in rabbits. The separation of corticotropin-bound from free labelled antibody could then be achieved by addition of a sheep anti-(rabbit IgG) antiserum, which precipitates bound labelled antibody by complex-formation with rabbit anti-corticotropin antibodies, which are also corticotropin-bound. The amount of sheep anti-(rabbit IgG) antiserum [200 μl of a 1:20 (v/v) solution in 0.064M-phosphate buffer, pH 7.4] required for the complete precipitation of rabbit anti-corticotropin antiserum in the presence of normal rabbit serum (1.0%, v/v) as carrier had previously been established by a well-proven procedure (Buckler, 1971). The general procedure for the development of the assay has previously been outlined in full (Hodgkinson *et al.*, 1984), with one modification, which is as follows. The amount of rabbit anti-corticotropin antiserum required to bind all of the corticotropin in the standard of highest concentration (1000pg/ml, 0.5ml of standard) was established by using a system analogous to an r.i.a. antiserum dilution curve. In this system, samples (0.5ml) of horse serum containing corticotropin (1000pg/ml) and ^{125}I-corticotropin (20000c.p.m.) were incubated with increasing dilutions of rabbit anti-corticotropin antisera in assay buffer (0.2ml, 1:100–1:1000) for 24h and separated by the addition of sheep anti-(rabbit IgG) antiserum as described above. The dilution of rabbit anti-corticotropin antiserum chosen for use in the i.r.m.a. was the greatest dilution to achieve complete precipitation of the ^{125}I-corticotropin, that is, the greatest dilution of antibody that completely bound the added corticotropin. Both the rabbit anti-[corticotropin-(34–39)-peptide] antiserum (Apro) and the rabbit anti-[corticotropin-(17–39)-peptide] antiserum (CII) bound the corticotropin (500pg) at a dilution of 1:500.

Assay procedure. The corticotropin i.r.m.a. was of the 'simultaneous addition' variety. Standard curves were prepared by the addition of a reagent mixture (0.2ml) containing ^{125}I-labelled sheep anti-corticotropin IgG (50000c.p.m./0.2ml of either PS1a, PS1b or PS2a) and rabbit anti-corticotropin antiserum {1:500; either anti-[corticotropin-(34–39)-peptide] (Apro) or anti-[corticotropin-(17–39)-peptide (CII)} in assay buffer to corticotropin standards (0–1000pg/ml) and unknown plasma (0.5ml), followed by mixing and incubation overnight at room temperature. Separation of corticotropin-bound from free labelled antibody was achieved by precipitation of the bound complex using a sheep anti-(rabbit IgG) antiserum as described above. After addition of the precipitating antiserum the tubes were mixed, incubated (0.5h, room temperature) and centrifuged (3000***g***, 0.5h, 4°C) after the addition of 1ml of poly(ethylene glycol) solution (2.0%, w/v, in 0.05M-phosphate). The soluble free fraction was aspirated to waste and precipitated radioactivity

was determined in an Innotron Hydragamma 16 counter.

Results and discussion

The highly purified immunoglobulin required for radioiodination was prepared from the two sheep antisera (PS1 and PS2) which were raised against the corticotropin-(1–24)-peptide (Synacthen) conjugate as described above. However, a range of immunoadsorbents were employed, which resulted in preparations with distinct specificities (Fig. 1). Thus preparation PS1a was immunopurified on insolubilized corticotropin-(1–24)-peptide and may therefore contain immunoglobulins directed against determinants on any part of the Synacthen molecule. Preparation PS1b was immunopurified on insolubilized [D-Ser[1],Lys[17,18]]-corticotropin-(1–18)-peptide amide and is specific for corticotropin-(2–16)-peptide; antibodies directed against the residue-16–24 region of corticotropin having been excluded. Antiserum PS2 did not bind to insolubilized [D-Ser[1],Lys[17,18]]-corticotropin-(1–18)-peptide amide. Instead, PS2a was prepared by immunopurification on insolubilized corticotropin-(11–39)-peptide and must therefore be specific for the residue-17–24 region of the molecule.

Standard curves prepared by using each of the ^{125}I-labelled sheep anti-corticotropin IgG preparations in combination with each of the rabbit anti-corticotropin antisera can be seen in Fig. 2. The curves demonstrated large differences in dose response, which relate to the specificities of labelled and unlabelled antibodies. Thus the standard curve prepared by using labelled sheep antibody PS1a and rabbit anti-[corticotropin-(34–39)-peptide] antiserum (Apro) (Fig. 2, □) demonstrated only a 2.7-fold change in bound radioactivity over the range of standards and only 15.1% binding of labelled antibody at a corticotropin concentration of 1000pg/ml. In contrast, the standard curve prepared by using PS1b and Apro (Fig. 2, ■) demonstrated a 17.2-fold change in binding across the range of standards and 52.0% of the labelled anti-

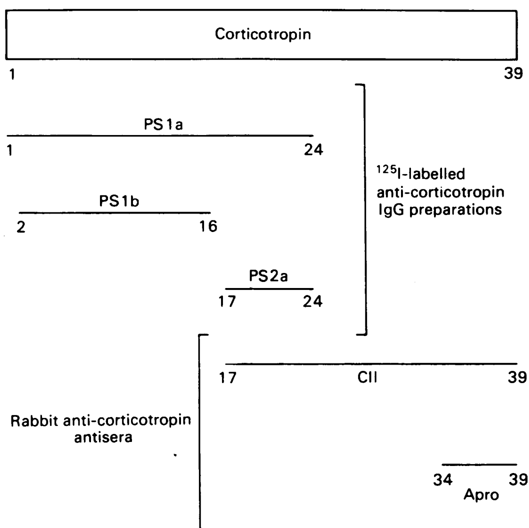

Fig. 1. *Specificities of antibody preparations used in the corticotropin i.r.m.a.*

Sheep antisera were raised against a conjugate of corticotropin-(1–24)-peptide (Synacthen). The anti-corticotropin immunoglobulin was purified before radioiodination by selective immunoadsorption, resulting in preparations with the specificities shown. Rabbit anti-corticotropin antisera were raised to conjugates of the synthetic *C*-terminal sequences shown.

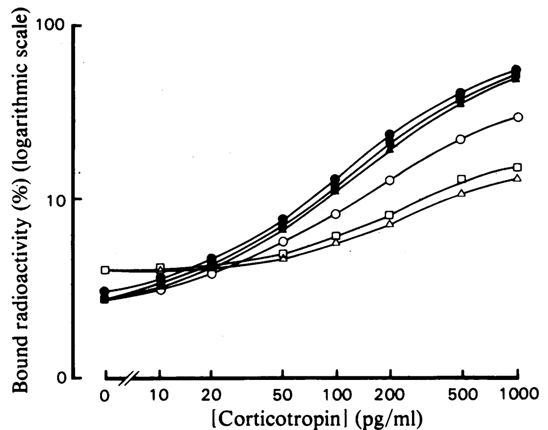

Fig. 2. *Corticotropin 'two-site' i.r.m.a. standard curves prepared by using each of the ^{125}I-labelled sheep anti-corticotropin IgG preparations in combination with each of the rabbit anti-corticotropin antisera*

Standard curves were prepared by the addition of a reagent mixture (0.2ml) containing ^{125}I-labelled sheep anti-corticotropin IgG and rabbit anti-corticotropin antiserum to corticotropin standards (0.5ml) in horse serum, followed by 18h incubation. The separation of corticotropin-bound from free labelled antibody was achieved by precipitation of the bound complex using a sheep anti-(rabbit IgG) precipitating antiserum. The antibody preparations used in the respective curves are as follows: □, ^{125}I-labelled sheep anti-corticotropin-(1–24)-peptide] IgG (PS1a) and rabbit anti-[corticotropin-(34–39)-peptide] (Apro); △, PS1a and rabbit anti-[corticotropin-(17–39)-peptide] (CII); ■, ^{125}I-labelled sheep anti-[corticotropin-(2–16)-peptide] IgG (PS1b) and Apro; ●, PS1b and CII; ▲, ^{125}I-labelled sheep anti-[corticotropin-(17–24)-peptide] IgG (PS2a) and Apro; ○, PS2a and CII.

body was bound at 1000 pg of corticotropin/ml. As the ^{125}I-labelled sheep anti-corticotropin IgG preparations (PS1a and PS1b) were derived from the same sheep anti-[corticotropin-(1–24)-peptide] antiserum (PS1) and demonstrated similar immunoreactivities, as assessed by reassociation with the immunoadsorbent on which they were purified, the most likely explanation for the low binding encountered with PS1a is the presence of radioiodinated antibodies specific for determinants that are unavailable in the intact corticotropin-(1–39)-peptide molecule. Whereas preparation PS1a may contain such antibodies directed against the *C*-terminus of Synacthen [synthetic corticotropin-(1–24)-peptide], preparation PS1b should not, since antibodies directed against determinants in the region of corticotropin-(17–24)-peptide, including the free *C*-terminus of Synacthen, were excluded by purifying the immunoglobulin on [D-Ser^1,Lys^{17,18}]corticotropin-(1–18)-peptide amide. A greater proportion of the radioiodinated immunoglobulin in preparation PS1b could therefore bind to the analyte molecule. *C*-Terminally directed sheep anti-[corticotropin-(1–24)-peptide] antibodies were similarly excluded from preparation PS2a. This antiserum (PS2) did not bind to [D-Ser^1,Lys^{17,18}]corticotropin-(1–18)-peptide amide and was subsequently purified on insolubilized corticotropin-(11–39)-peptide. Preparation PS2a must therefore be specific for the mid-portion of corticotropin (residues 17–24), and antibodies directed against the free *C*-terminus of Synacthen were thereby excluded. A standard curve prepared by using ^{125}I-labelled sheep anti-[corticotropin-(17–24)-peptide] IgG (PS2a) and rabbit anti-[corticotropin-(34–39)-peptide] antiserum (Apro) can be seen in Fig. 2(▲) and is characterized by high binding of labelled antibody (51% at 1000 pg of corticotropin/ml) and a large change in bound radioactivity (17.0-fold) across the range of standards. These data suggest that the exclusion of antibodies, directed against determinants which are absent in the intact analyte molecule, from antisera raised to analyte fragments is a necessary prerequisite to establishing 'two-site' i.r.m.a.'s and that this can be achieved by selective immunoadsorption.

The need to minimize the steric inhibition of antibody binding resulting from the use of labelled and unlabelled antibodies which are specific for the same or adjacent immunodeterminants is also illustrated in Fig. 2. Whereas the standard curve prepared by using the labelled antibody preparation PS2a and rabbit anti-[corticotropin-(34–39)-peptide] antiserum (Apro) (Fig. 2, ▲) demonstrated high binding (51% at 1000 pg of corticotropin/ml), a second standard curve prepared by using PS2a and the rabbit anti-[corticotropin-(17–39)-peptide] (CII) antiserum did not (Fig. 2, ○). Only 31% of the labelled antibody was bound at 1000 pg of corticotropin/ml. The inhibition of binding was in this case probably due to competition between the labelled and unlabelled antibodies for common immunodeterminants on the corticotropin molecule, since the actual specificity of the labelled antibody preparation [PS2a; corticotropin-(17–24)-peptide] is entirely contained within the sequence 17–39 against which the rabbit antiserum was raised (Fig. 1). It is surprising, therefore, that the inhibition of binding of the labelled antibody was not greater, but this may be due to a predominance of antibodies in the rabbit antiserum which are directed against determinants further towards the *C*-terminus of corticotropin. Competition between labelled and unlabelled antibodies for binding sites on the analyte molecule was also observed in a standard curve prepared by using the labelled antibody preparation PS1a with the CII antiserum (Fig. 2, △) when compared with a curve prepared by using the same labelled antibody preparation (PS1a) and the rabbit anti-[corticotropin-(34–39)-peptide] (Apro) antiserum (Fig. 2, □). But

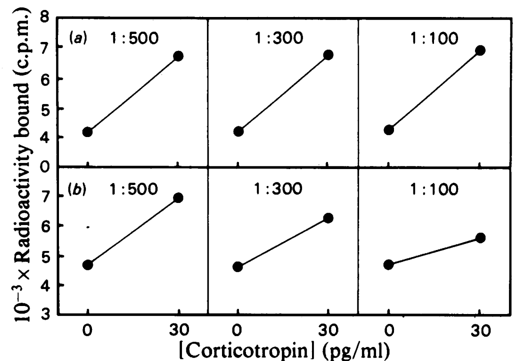

Fig. 3. *Influence of rabbit anti-corticotropin antiserum dilution on dose response*

The dose response of the corticotropin i.r.m.a. resulting from the use of antibodies with distinct specificities {^{125}I-labelled sheep anti-[corticotropin-(2–16)-peptide IgG and rabbit anti-[corticotropin-(34–39)-peptide] antiserum} (*a*) is compared with the dose response of a corticotropin i.r.m.a. (*b*) in which antibodies share common immunodeterminants on the corticotropin molecule {^{125}I-labelled sheep anti-[corticotropin-(17–24)-peptide] IgG and rabbit anti-[corticotropin-(17–39)-peptide] antiserum}. Dose response was assessed at a range of dilutions (1:500, 1:300 and 1:100) of the rabbit anti-corticotropin antisera by the addition of reagent mixtures (0.2 ml) containing labelled and unlabelled antibodies to corticotropin standards (0.5 ml, 0 and 30 pg/ml), followed by overnight incubation and separation of bound and free labelled antibody as described in the text.

there was no evidence of competition in standard curves prepared by using the labelled antibody preparation PS1b in combination with either the Apro (Fig. 2, ■) or CII (Fig. 2, ●) rabbit antisera. This would be expected, since this labelled antibody and these unlabelled antibodies have no immunodeterminants in common. The absence of competition between these antibodies was confirmed by comparing the 'simultaneous addition' standard curves with curves prepared by delaying the addition of rabbit antiserum (results not shown), that is, by preincubating the labelled antibody with corticotropin. Neither the 'delayed addition' standard curve prepared by using Apro rabbit antiserum nor the 'delayed addition' standard curve prepared by using the CII antiserum showed any improvement in dose response over respective 'simultaneous addition' standard curves. In standard curves in which competition was demonstrated, the concentration of rabbit antiserum had a pronounced effect on dose response (Fig. 3*b*). In an experiment in which reagent mixtures (0.2ml) containing ^{125}I-labelled sheep anti-[corticotropin-(17–24)-peptide] IgG (50000c.p.m./200μl; PS2a) and rabbit anti-[corticotropin-(17–39)-peptide] antiserum (CII) at a range of dilutions (1:500, 1:300 and 1:100) in assay buffer were added to corticotropin standards (0 and 30pg/ml; 0.5ml) and incubated overnight (room temperature), a progressive inhibition of dose response was observed with increasing concentrations of rabbit antiserum. This further illustrates the need to avoid the use of labelled and unlabelled antibodies which compete for binding sites on the analyte molecule, since the phenomena must limit the sensitivity of resultant assays. However, the progressive inhibition of dose response with increasing concentration of rabbit antiserum was not observed in a similar experiment (Fig. 3*a*) using ^{125}I-labelled sheep anti-[corticotropin-(2–16)-peptide] IgG (preparation PS1b) and the rabbit anti-[corticotropin-(34–39)-peptide] (Apro) antiserum. This was expected, since the labelled and unlabelled antibodies are specific for distinct regions of the analyte molecule and consequently the 'two-site' i.r.m.a. using these reagents was chosen for further validation. A standard curve was prepared by the addition of a reagent mixture (0.2ml) containing ^{125}I-labelled sheep-[corticotropin-(2–16)-peptide] IgG (PS1b; 50000c.p.m./200μl) and rabbit anti-[corticotropin-(34–39)-peptide] antiserum (Apro; 1:500) in assay buffer to corticotropin standards (0–1000pg/ml; 0.5ml), followed by mixing and incubation (overnight at room temperature). In addition to the standard curve, progressive dilutions of plasma samples from patients with corticotropin-dependent Cushing's syndrome and Nelson's syndrome were included in the assay, together with dilutions of purified corticotropin-(1–24)-peptide and CLIP (Corticotropin-Like-Intermediate lobe Peptide) (1μg/ml) and the plasmas (09:00–11:00h) of 25 normal volunteers (Fig. 4). The sensitivity of the i.r.m.a. (the lowest detectable dose of corticotropin) was 8pg/ml (4pg; 95% confidence interval 1.8–6.0) as judged by a 2.5-S.D. increase in duplicate radioactivity counts from counts at zero corticotropin. This compares favourably with detection limits quoted by other investigators for corticotropin r.i.a.'s employing preliminary extraction of analyte. The normal volunteers (25–65pg of corticotropin/ml) were clearly delineated from zero and in substantial agreement with other estimates of normal corticotropin levels by r.i.a. (Landon & Greenwood, 1968) and by bioassay (Holdaway *et al.*, 1973). The estimates of corticotropin in the plasma of patients with Nelson's syndrome and Cushings' disease were both elevated and the immunoreactivity was diluted in parallel with the standard curve.

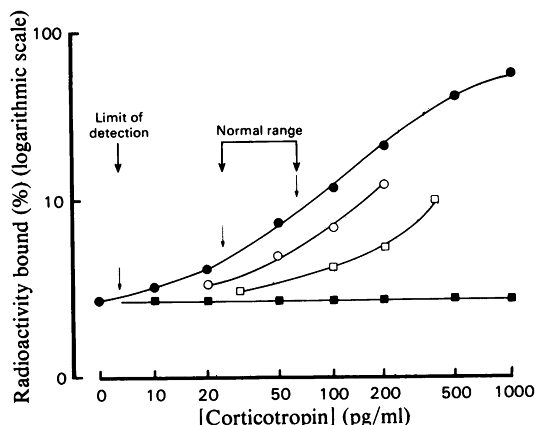

Fig. 4. *Validation of the 'two-site' corticotropin i.r.m.a.* A standard curve (●) was prepared by the addition of a reagent mixture (0.2ml) containing ^{125}I-labelled sheep anti-[corticotropin-(2–16)-peptide] IgG (PS1b) and rabbit anti-[corticotropin-(34–39)-peptide] antiserum (Apro) to corticotropin standards (0–1000pg/ml, 0.5ml), followed by overnight incubation. The separation of corticotropin-bound from free labelled antibody was achieved by precipitation of bound complex using a sheep anti-(rabbit IgG) antiserum followed by centrifugation and aspiration of the soluble free fraction to waste. In addition to the standard curve, arbitrary dilutions of plasmas from patients with Nelson's syndrome (○) and corticotropin-dependent Cushing's syndrome (□) are included, together with dilutions of 1μg/ml samples of corticotropin-(1–24)-peptide and CLIP (■). Also included in the assay are the plasmas of 25 normal volunteers collected between 09:00 and 11:00h. The range of normal values and the limit of detection of the assay are shown.

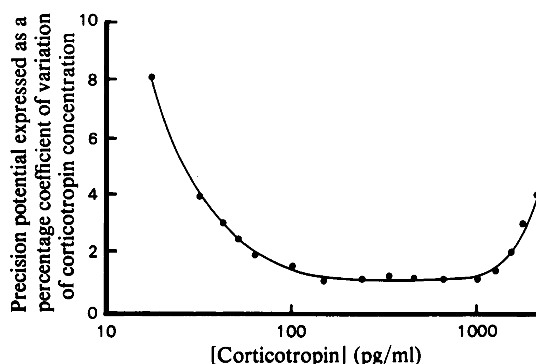

Fig. 5. *Precision profile of the corticotropin i.r.m.a.* Data for the calculation of the precision profile were obtained from a standard curve as in Fig. 4 and from the assay of 25 unknown plasmas.

In addition to avoiding competition, the use of labelled and unlabelled antibodies which are directed against opposite ends of the analyte molecule also has the advantage of enabling a substantial increase in assay specificity. Whereas corticotropin fragments may cross-react in the r.i.a., the detection of corticotropin immunoreactivity in the 'two-site' i.r.m.a. for the hormone requires the presence of both the corticotropin 2–16 sequence and, in combination, the corticotropin 34–39 sequence. The *N*-terminal sequence is required for the binding of the labelled antibody and the *C*-terminal antibody is required to ensure the precipitation of the bound labelled antibody. Thus only the intact biologically active corticotropin molecule may be measured in this system, and the divergence of results obtained by bioassay and radioimmunoassay in the past (Fleisher *et al.*, 1966) can be overcome. This is also illustrated in Fig. 4. No cross-reaction of the corticotropin fragments 1–24 and CLIP was observed in the 'two-site' i.r.m.a. at concentrations 1000 times higher than that of the highest corticotropin standard.

A precision profile (Ekins, 1976) of the corticotropin i.r.m.a. can be seen in Fig. 5. The data were obtained from a 'simultaneous addition' assay utilizing the ^{125}I-labelled sheep anti-[corticotropin-(2–16)-peptide] IgG (PS1b) antibody preparation and the rabbit anti-[corticotropin-(34–39)-peptide] antiserum (Apro) and included nine standards (0–2000pg of corticotropin/ml) and 25 unknown plasmas, which were assayed in duplicate. The precision is based upon adjacent duplicates and was calculated by the method of Raab & McKenzie (1981) in which the response–error relationship is calculated as an intermediate step in the generation of the precision profile. The assay demonstrates high precision and a wide 'operating range'; the precision potential of dose estimates is less than 4% over the range 30–2000pg/ml, less than 2% over the range 60–1600pg/ml and 1% between 150 and 1000pg/ml. Furthermore, the precision potential of dose estimates is still less than 7% at 20pg/ml, and this indicates that the assay may be able to distinguish between normal values of the hormone and the subnormal levels which may be encountered in disorders of the hypothalamo–pituitary–adrenal axis. The high precision and wide 'operating range' of the assay results from the use of antibody in excess and because the assay is of the 'simultaneous addition' variety. Thus reagent excess reduces the need for precise pipetting of antibodies, and addition of labelled and unlabelled antibodies as a single reagent mixture increases the convenience of the method by eliminating a second pipetting step and with it a further source of random assay error. Meaningful estimates of subnormal corticotropin levels using the i.r.m.a. are dependent on the stability of the baseline response. The susceptibility of the assay to non-specific interference was therefore assessed as follows. Two sets of 12 duplicate tubes were labelled. To the first set was added horse serum (0.5ml) treated as described above, and to the second set 12 unknown plasmas (0.5ml) from individual subjects. The only preparation of plasma samples before assay was mild centrifugation to remove fibrin clots. Baseline response due to plasma constituents and not to endogenous corticotropin was assessed by the addition of a reagent mixture (0.2ml) containing labelled antibody (preparation PS1b) and a rabbit antiserum (1:500) in assay buffer which was raised not to corticotropin but to prolactin. Corticotropin therefore could not react in this system and variation in the baseline response would be due to interference. The tubes were incubated overnight and the separation of 'bound' and free labelled antibody was achieved by the addition of a sheep anti-(rabbit IgG) precipitating antiserum as described in the text. The mean precipitated radioactivity in the horse serum group was 2649c.p.m. and the coefficient of variation (c.v.) 6.2%. There was no significant difference between the mean for this group and the mean precipitated radioactivity (2599c.p.m.) of the sample plasma group. This indicates that interference in the assay from sample plasma constituents was no greater than from the horse serum base for the standards. Furthermore, the c.v. for the unknown plasma group (6.1%) was similar to that of the horse serum group, indicating that the individual sample plasmas had little influence on the stability of the baseline. It is concluded that sample plasmas are unlikely to interfere in the determination of corticotropin with the i.r.m.a.

In conclusion, we have developed a liquid-phase 'two-site' i.r.m.a. for corticotropin that does not

require sample extraction. The assay is simple to perform, requiring only one pipetting to initiate and one to complete. The assay is highly sensitive, precise, has a wide operating range, and may be completed in 24h. These are desirable characteristics for a clinical corticotropin assay.

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