Reviews on Immunoassay Technology

Volume 1

Edited by

S. B. Pal

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Preface

Immunology is a rapidly developing scientific discipline, as a result of which many new techniques are now available. On this basis it was decided to publish this series of monographs which we have called *Reviews on Immunoassay Technology*, which is targeted mainly at immunologists and other laboratory workers. The first volume contains 10 chapters by 28 authors with wide experience in immunoassay, and it is hoped that readers will find the information presented interesting and thought provoking.

Much credit goes to The Macmillan Press Ltd for making the necessary arrangements for the publication of this series. I should also like to take this opportunity of thanking Dr. D. Donaldson, MRCP, FRCPath, Consultant Chemical Pathologist, East Surrey Hospital, Redhill, Surrey, UK, for his helpful suggestions during the preparation of this volume, and Mrs. M. R. Lingard-Pal for her assistance as an honorary editorial secretary.

Ulm, 1987

S. B. P.

Contents

1. Apolipoprotein Quantitation by ELISA: Technical Aspects and Clinical Applications

J. B. BURY AND M. Y. ROSSENEU

INTRODUCTION

The structure of the major apolipoproteins and their specific functions in lipoprotein metabolism have been extensively studied (Jackson *et al.*, 1976; Mahley *et al.*, 1984; Eisenberg, 1984; Scanu *et al.*, 1982). The quantitation of apolipoproteins by chromatographic means is cumbersome and bioassays hardly exist. Therefore, one has to rely on the differential quantitation of specific apolipoproteins by immunological techniques (Lippel, 1983). Several immunoassays have already been described (Lippel, 1983; Steinberg *et al.*, 1983; Rosseneu *et al.*, 1983a), among which are radial immunodiffusion (RID), electroimmunodiffusion (EID), immunonephelometric assay (INA) and radioimmunoassay (RIA).

Applicability of the immunodiffusion technique to apolipoprotein quantitation is limited by differences in the diffusion constants between the purified apolipoproteins and the various lipoprotein classes, thereby obscuring the assay standardization. In addition, these assays have a limited sensitivity compared with RIA, require large amounts of antisera and are not easily automated. In contrast, the INA, based on the proportionality between the antigen concentration and intensity of light scattered by the insoluble antigen-antibody complex, is fast, precise, simple and easily automated. However, the apolipoproteins belong to several lipoprotein classes of different size and with different kinetics of antigen-antibody complex formation. Therefore, application of this technique is restricted to endpoint laser nephelometry of apolipoproteins present in normolipidaemic plasma. The accuracy of the INAs decreases with increasing turbidity of hypertriglyceridaemic samples, owing to high blanks. Therefore, these samples have to be pretreated with detergents, lipoprotein lipase or organic solvents (Bury *et al.*, 1985; Rosseneu *et al.*, 1981a).

The most sensitive technique for apolipoprotein measurement is RIA (Blum, 1983). Most assays are, however, time consuming and suffer from the major drawbacks associated with the use of radioisotopes, i.e. the high cost and short

shelf-life of reagents, the expensive gamma counters and the biohazards which attend the preparation, use and disposal of isotope-labelled reagents.

Therefore, alternative analytical techniques have been developed, whereby the radioisotope was replaced by an enzyme (Scharpé *et al.*, 1978; Oellerich, 1984), a fluorochrome (Hemmilä, 1985) or another indicator (Schall and Tenoso, 1981). Since the introduction in 1971 of the enzyme immunoassay (EIA) by Avrameas and Guilbert (1971), Engvall and Perlmann (1971) and Van Weemen and Schuurs (1971), multiple variations of this technique have been developed, which can be classified among two main assay types: homogeneous and heterogeneous assays. When the activity of the enzyme is influenced by the formation of an antigen–antibody complex, the EIA is called homogeneous. This technique has been applied mainly to the quantitation of small molecules such as drugs (Scharpé *et al.*, 1978) and does not require separation of free and bound enzyme. In the heterogeneous assays, however, where the enzyme activity is not influenced by the formation of the antigen–antibody complex, bound and unbound enzyme conjugate have to be separated. In the classical EIA (Avrameas and Guilbert, 1971; Engvall and Perlmann, 1971; Van Weemen and Schuurs, 1971), the antigen or the antibody is attached to an insoluble carrier (solid phase), enabling easy separation of free and bound enzyme. This technique is called the enzyme-linked immunosorbent assay (ELISA) technique, and it can be applied as well to competitive as to non-competitive-assays. In view of the limited stability of apolipoproteins in solution, the competitive ELISA techniques, requiring an antigen–enzyme complex or an antigen-coated solid phase, are of limited use for apolipoprotein quantitation.

Therefore, we chose to develop a sandwich ELISA, using immunoglobulins for both the coating of the solid phase and the preparation of the enzyme conjugate. The principle of the assay is presented in figure 1. After coating with monospecific antibodies (I) and washing, the solid phase is incubated with the test samples containing the antigen for assay (II). The solid phase is then washed, incubated with the monospecific enzyme-labelled antibodies (III) and washed again. The amount of bound enzyme, which is proportional to the amount of antigen in the test solution, is assayed colorimetrically (IV).

ISOLATION OF APOLIPOPROTEINS

The four major lipoprotein classes were obtained by sequential ultracentrifugal flotation from hypertriglyceridaemic plasma for chylomicrons and very low density lipoproteins (VLDL) and from normolipaemic plasma for low density lipoproteins (LDL) and high density lipoproteins (HDL) (Mills *et al.*, 1984). All fractions were subsequently delipidated with ether:ethanol (Mills *et al.*, 1984). The apolipoproteins A-I and A-II were isolated from apo HDL by ion exchange chromatography on diethylaminoethyl cellulose (DEAE cellulose) (Blaton *et al.*, 1977). Water-soluble apo B was prepared from LDL (d = 1.030–1.050 g/ml) as

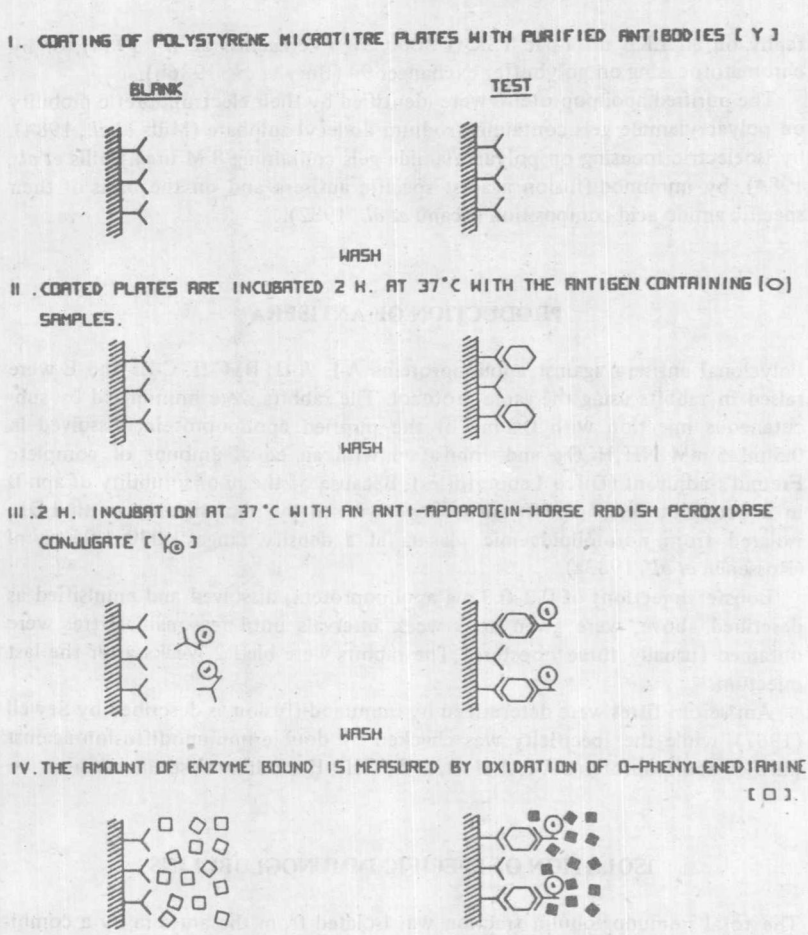

Figure 1 Principle of the sandwich ELISA for apolipoprotein quantitation.

described by Cardin *et al.* (1982). Apo VLDL was fractionated by gel filtration on a Sephacryl S_{200} column (Bury *et al.*, 1985a), yielding three major fractions containing apo B, apo E and apo C. Apo C-III_0, apo C-III_1 and apo C-III_2 were isolated from the apo C-containing fraction by ion exchange chromatography on DEAE cellulose (Bury *et al.*, 1985a), while apo C-II was isolated from the same fraction by chromatofocusing on polybuffer exchanger 94 (Bury *et al.*, 1986a). The apo E-containing fraction of apo VLDL was further purified either by high performance liquid chromatography (HPLC), using size exclusion chromatog-

raphy on an LKB ultra-pac TSK-G 3000 SW (Vercaemst *et al.*, 1984), or by chromatofocusing on polybuffer exchanger 94 (Bury *et al.*, 1986b).

The purified apolipoproteins were identified by their electrophoretic mobility on polyacrylamide gels containing sodium dodecyl sulphate (Mills *et al.*, 1984), by isoelectric focusing on polyacrylamide gels containing 8 M urea (Mills *et al.*, 1984), by immunodiffusion against specific antisera and on the basis of their specific amino acid composition (Scanu *et al.*, 1982).

PRODUCTION OF ANTISERA

Polyclonal antisera against apolipoproteins A-I, A-II, B, C-II, C-III and E were raised in rabbits using the same protocol. The rabbits were immunized by subcutaneous injection with 0.5 mg of the purified apolipoprotein, dissolved in 0.5 ml 5 mM NH_4HCO_3 and emulsified with an equal amount of complete Freund's adjuvant (Difco Laboratories). Because of the poor solubility of apo B in aqueous buffers, apo B antisera were raised by immunization with LDL, isolated from normolipidaemic plasma at a density range 1.030–1.050 g/ml (Rosseneu *et al.*, 1983a).

Boosfer injections of 0.2–0.3 mg apolipoprotein, dissolved and emulsified as described above, were given at 3 week intervals until reasonable titres were obtained (usually three boosters). The rabbits were bled 2 weeks after the last injection.

Antiserum titres were determined by immunodiffusion as described by Sewell (1967), while the specificity was checked by double immunodiffusion against purified apolipoproteins A-I, A-II, B, C-II, C-III, E and human serum albumin.

ISOLATION OF SPECIFIC IMMUNOGLOBULINS

The total immunoglobulin fraction was isolated from the antisera by a combination of ammonium sulphate precipitation and anion exchange chromatography on DEAE cellulose (Johnstone and Thorpe, 1982). Affinity-purified antibodies were prepared by immunosorbent chromatography. Affinity chromatography columns were prepared by covalent linkage of 0.5–10 mg of the purified apolipoprotein, dissolved in 5 ml 0.1 M $NaHCO_3$ buffer, pH 8.3, with 0.5 M NaCl, to 3–15 ml CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden) suspended in the same buffer (Pharmacia, 1979). The yield of coupling, determined from the absorbance at 280 nm of the protein solution before and after incubation with the gel, was $> 90\%$ for all apolipoproteins. The residual coupling sites of the CNBr-activated Sepharose were blocked by incubation with 0.2 M glycine-HCl buffer, pH 8.0. An apo B-Sepharose matrix was prepared by covalent linkage of LDL (d = 1.030–1.050 g/ml) as a source of apo B (Rosseneu *et al.*, 1983a).

The apolipoprotein-Sepharose gel was incubated for 90 min at room temperature with 3–10 ml of the corresponding antisera and poured into a small column (2 cm × 10 cm). The gel was eluted at a rate of 35 ml/h with a 10 mM sodium phosphate buffer, pH 7.4, containing 0.15 M NaCl, until absorbance of the eluate at 280 nm had reached baseline levels. The immunoglobulins, linked to the Sepharose matrix, were eluted with 20 ml of 0.2 M glycine-HCl buffer, pH 2.6, and collected into 5 ml of 1 M K_2HPO_4. After concentration on a YM 30 Diaflo membrane (Amicon Co., Danvers, MA 01923), the purified immunoglobulins were dialysed against 10 mM sodium carbonate buffer, pH 9.5, and used for the preparation of an antibody-enzyme conjugate (this page). If the immunoglobulins were used for solid phase coating (page 6), the eluate was dialysed against 10 mM sodium phosphate buffer, pH 7.4, containing 0.15 M NaCl and 1 g/l NaN_3, filtered through a 0.45 μm pore-size filter (Millipore Corp., Bedford, MA 01730), and stored at 4°C.

PREPARATION OF THE ENZYME–ANTIBODY CONJUGATE

Several enzymes, including alkaline phosphatase and horse radish peroxidase (HRPO), have been proposed as suitable labels for EIA (Scharpé *et al.*, 1978; Oellerich, 1984). In view of its excellent properties and its easy conjugation with immunoglobulins (Scharpé *et al.*, 1978; Voller *et al.*, 1979), HRPO (E.C. 1.11.1.7) was selected for conjugate preparation. The antibody-enzyme conjugate was prepared by a modification of the periodate coupling procedure described by Nakane and Kawaoi (1974). HRPO is a glycoprotein whose reducing hydroxyl groups can be oxidized by sodium periodate to form the corresponding aldehyde. The free primary alkylamino groups of the immunoglobulins can form a Schiff base with the HRPO aldehyde, thereby covalently linking the two proteins.

Five milligrams HRPO (grade I, RZ > 3.0, Boehringer Mannheim, FRG) was dissolved in 2 ml of 0.3 M $NaHCO_3$ and gently mixed with 100 μl of a 0.1 g/l ethanolic solution of 2,4-dinitro-monofluorobenzene, in order to block the free amino groups of the enzyme. After 2 h incubation at room temperature, 1 ml of 80 mM $NaIO_4$ solution was added. The reaction was stopped after 1 h by the addition of 0.2 ml of glycerol. The incubation mixture was extensively dialysed at 4°C against 10 mM sodium carbonate-bicarbonate buffer, pH 9.5.

One millilitre of a 10 g/l solution of affinity-purified immunoglobulins was dialysed against the same carbonate-bicarbonate buffer and subsequently incubated with the peroxidase aldehyde for 3 h at room temperature. The incubation mixture was dialysed against 0.1 M sodium phosphate buffer, pH 7.4, and stored in 100 μl aliquots without further reduction of the Schiff base or fractionation of free and bound enzyme. After the addition of an equal mount of glycerol, storage was carried out, either at −20°C for conjugates used daily, or at −70°C for longer storage periods. The conjugates, stored at −70°C were stable for at least two years without significant loss of immunologic or enzymatic activity.

Both total immunoglobulins and affinity-purified immunoglobulins are suitable for the preparation of an enzyme-antibody conjugate. However, the low specific activity of the total immunoglobulin fraction requires higher concentrations of conjugated enzyme to obtain a specific response. As a consequence, the non-specific binding is significantly increased. Therefore, the enzyme-antibody conjugates used in the apolipoprotein immunoassays were prepared with affinity-purified immunoglobulins.

COATING, WASH AND ASSAY BUFFERS

Coating of the solid phase is usually performed in alkaline solutions (Voller *et al.*, 1979; Wood and Gadow, 1983). Similar results were, however, obtained with a 10 mM sodium phosphate buffer, pH 7.4, containing 0.15 M NaCl, which was further used throughout the whole assay procedure, for coating, for washing, for antigen or conjugate dilution, and for incubation. In addition, the coating buffer contained 1 g/l NaN_3 as a preservative, whilst the assay buffer contained bovine serum albumin (1 g/l) and the wash buffer contained Tween 20 (0.5 ml/l) to reduce non-specific binding (page 9).

COATING OF THE SOLID PHASE

Although several carriers such as grains of glass, silicone, cellulose or various kinds of plastic materials have been proposed for the solid phase, polystyrene beads and microtitre plates have found a wide application owing to their practical use and reproducible coating properties.

The wells of polystyrene microtitre plates were coated with the specific antibodies by passive adsorption from 110 µl coating buffer containing 1–3 µg immunoglobulins. The plates were sealed with sealing tape (Dynatech, Alexandria, VA 22234) to reduce evaporation and incubated for 3 h at 37°C, followed by overnight incubation at 4°C. In view of possible batch-dependent edge effects (Kricka *et al.*, 1980), the outer rows of the microtitre plates were excluded. The coated plates, stored at 4°C in the presence of the immunoglobulin solution, were stable for three to six months without any significant loss of immunoreactivity or assay precision.

Before use, the microtitre plates were washed three times with wash buffer and incubated for 1 h at room temperature with 150 µl assay buffer, containing bovine serum albumin to block the residual binding sites. After washing with the wash buffer, the plates were ready for use.

Polystyrene beads with a diameter of 6.5 mm (Seroa, Monaco) were washed with distilled water and coated by immersion in a solution of specific immunoglobulins (10–30 µg/ml coating buffer) and incubated for 3 h at 37°C. As des-

cribed for the microtitre plates, the residual binding sites were blocked with albumin.

When affinity-purified antibodies were used for coating, the excess immunoglobulins were recycled as they could be used for at least five consecutive coatings.

As the assay precision of the ELISA techniques is mainly determined by reproducibility of the coating (McCullough and Parkinson, 1984b), several polystyrene supports were tested for their use as a solid phase in the apolipoprotein assay: polystyrene beads (Seroa), Greiner and Dynatech microtitre plates, Dynatech MicroELISA plates and Nunc Immunoplates, number I. Although high reproducibility was obtained with polystyrene beads (mean intra-assay coefficient of variation (CV) 4.4%, $n = 10$), the ELISA performed on the microtitre plates, using the same assay conditions (coating concentration, conjugate dilution, incubation time and temperature; pages 8–10), was about 10 times more sensitive (figure 2). In addition, polystyrene beads are not well suited for scaling-up procedures, owing to their lack of practical use and high cost compared with microtitre plates. Among the microtitre plates, the Micro-ELISA plates M 129B (Dynatech) were selected because of their high and reproducible coating.

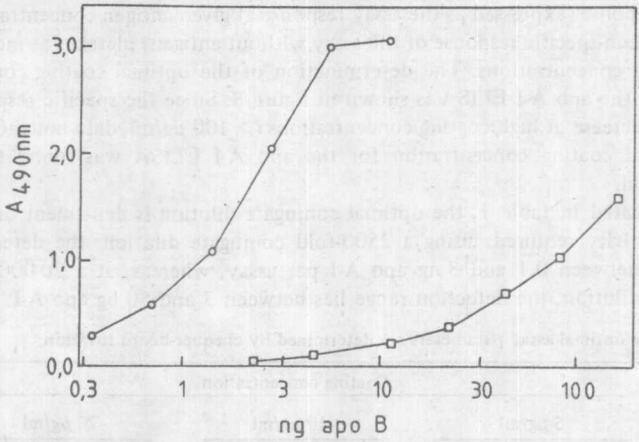

Figure 2 Comparison of the apo B ELISA performed on polystyrene microtitre plates (○) and on polystyrene beads (□) using the same assay conditions.

The selection of either total immunoglobulins or affinity-purified antibodies for solid phase coating was dependent on the apolipoprotein to be assayed. For example, a total immunoglobulin fraction was satisfactory for the apo B ELISA, whereas the use of affinity-purified antibodies was essential in most other apolipoprotein assays in order to minimize non-specific binding. As such factors

are probably dependent on the titre and the affinity of the polyclonal antisera raised, and may differ from one batch to another, it is advisable to use either affinity-purified antibodies or well-characterized monoclonal or oligoclonal antibodies for coating and for the preparation of the enzyme-antibody conjugate.

OPTIMIZING ASSAY CONDITIONS

The optimal coating concentration, conjugate dilution and minimal antigen concentration can be determined by a single chequer-board titration (Voller *et al.*, 1979). For this purpose, the horizontal rows of microtitre plates were coated with increasing antibody concentrations, ranging from 0 to 100 μg immunoglobulins/ml coating buffer. The assay was performed with antigen amounts between 0 and 100 ng, using several conjugate dilutions (1000- to 20 000-fold) in the vertical rows of the microtitre plates. The incubations with antigen and conjugate were performed at 37°C for 2 h. If a slow antigen-antibody formation is to be expected, the incubations can be extended overnight.

Some of the results obtained by chequer-board titration for the apo A-I ELISA are summarized in table 1. They indicate that the non-specific response increases with increasing coating and conjugate concentrations. However, the specific response (expressed as the assay response at given antigen concentrations minus the non-specific response of the assay without antigen) plateaus at increasing coating concentrations. The determination of the optimal coating concentration for the apo A-I ELISA is shown in figure 3. Since the specific response tends to decrease at high coating concentrations (> 100 μg/ml, data not shown), the optimal coating concentration for the apo A-I ELISA was found to be 15–20 μg/ml.

As indicated in table 1, the optimal conjugate dilution is dependent on the assay sensitivity required: using a 2500-fold conjugate dilution, the detection range lies between 0.1 and 3 ng apo A-I per assay, whereas, at a 20 000-fold conjugate dilution, the detection range lies between 3 and 50 ng apo A-I. High

Table 1 The optimal assay parameters are determined by chequer-board titration

Conjugate dilution	Coating concentration								
	5 μg/ml			10 μg/ml			20 μg/ml		
	0 ng	1 ng	10 ng	0 ng	1 ng	10 ng	0 ng	1 ng	10 ng
1000	0.81	2.28	>3.2	1.23	2.29	>3.2	1.41	3.81	>3.2
2500	0.29	1.22	>3.2	0.40	1.64	>3.2	0.53	1.82	>3.2
5000	0.16	0.67	2.98	0.16	0.86	3.13	0.22	1.03	3.16
10000	0.02	0.26	1.04	0.05	0.40	2.14	0.08	0.48	2.36
20000	0.02	0.14	0.55	0.02	0.18	0.87	0.03	0.19	0.98

The data represent absorption measurements at 490 nm, compared with a blank containing 100 μl substrate and 100 μl 2.5 M H_2SO_4. The test solutions contained 0, 1 or 10 ng of antigen as indicated.

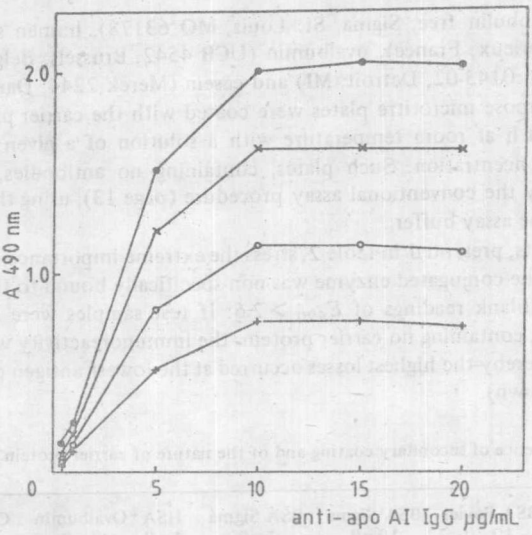

Figure 3 Influence of the coating concentration on the specific response of various amounts of antigen: +, 2.5 ng apo A-I; ○, 5 ng apo A-I; X, 10 ng apo A-I; ●, 20 ng apo A-I. The assay was performed using the standard assay procedure. (Reproduced with permission from Bury and Rosseneu (1985a).)

conjugate dilutions result, however, in poor slopes of the calibration curves. Therefore, the best combination of a high sensitivity and a low zero-dose response was obtained by using a 7500-fold conjugate dilution, whereby the assay could quantify 0.5–15 ng apo A-I.

INHIBITION OF NON-SPECIFIC BINDING

As a consequence of their hydrophobic character, significant losses of apolipoproteins, as a result of adsorption onto plastic recipients and pipettes, might occur during the manipulation of dilute solutions (Holmquist, 1982a). Such losses should, therefore, be minimized by the presence of a carrier protein in all buffers used for sample dilution and during assay incubations (Livesey and Donald, 1982; Samaké *et al.*, 1983; McCullough and Parkinson, 1984a). In addition, non-specific binding of the antigen and of the enzyme-antibody conjugate to the solid phase should be reduced by blocking any residual binding sites with a non-interfering protein. Widely used albumin preparations might contain some contaminating apolipoproteins (Deckelbaum *et al.*, 1980), requiring the comparison of several carrier proteins such as bovine serum albumin (Poviet grade, Organon Technika, Boxtel, Holland), bovine serum albumin (A-7030 fatty

acid and globulin free, Sigma, St. Louis, MO 63178), human serum albumin (Institut Merieux, France), ovalbumin (UCB 4542, Brussels, Belgium), gelatine (Difco-Bacto 0143-02, Detroit, MI) and casein (Merck 2244, Darmstadt, FRG). For this purpose microtitre plates were coated with the carrier protein by incubation for 1 h at room temperature with a solution of a given protein at the specified concentration. Such plates, containing no antibodies, were further processed by the conventional assay procedure (page 13), using the same carrier protein in the assay buffer.

The results, presented in table 2, stress the extreme importance of a secondary coating, as the conjugated enzyme was non-specifically bound to the solid phase, resulting in blank readings of $E_{490} > 2.5$. If test samples were diluted in the assay buffer, containing no carrier protein, the immunoreactivity was reduced by 10–50%, whereby the highest losses occurred at the lowest antigen concentrations (data not shown).

Table 2 Influence of secondary coating and of the nature of carrier protein on non-specific plates binding

Non-coated plates	BSA Poviet 10 g/l	BSA Sigma 10 g/l	BSA Sigma 1 g/l	HSA 1 g/l	Ovalbumin 1 g/l	Casein 1 g/l	Gelatine 1 g/l
2634	423	11	38	658	276	23	687

Tests were performed in microtitre plates, coated with the carrier protein only, using the standard assay procedure for the apo E ELISA, with assay buffers containing the concentrations specified for each protein. Data represent the $E_{490\,nm}$ readings for tests, performed in the absence of antigen. BSA, bovine serum albumin; HSA, human serum albumin.

As shown in table 2 non-specific binding was inhibited by using casein or a fatty acid- and globulin-free bovine serum albumin preparation (Sigma A-7030). Bovine serum albumin, at a concentration of 1 g/l, was selected as carrier protein in the assay buffers and for the secondary coating of the solid phase, especially in view of its solubility properties.

Non-specific protein–protein interactions were inhibited by washing and soaking the incubated microtitre plates in wash buffer containing Tween 20 (0.5 ml/l). The use of this non-ionic detergent reduced the zero-dose response values by 30–70%, while the intra- and inter-assay coefficients of variation, determined for the apo A-I ELISA, concomitantly decreased from 7.5% to 3.9% and from 12.1% to 7.8% respectively. The combination of a secondary coating with albumin and the use of Tween 20 in the wash buffer decreased the intra-assay coefficient of variation for the apo C-III ELISA from 13.5% to 3.8%.

KINETICS OF THE ANTIGEN–ANTIBODY REACTION

The temperature-dependent kinetics of the antigen–antibody reaction were investigated by performing the assay (page 13), with incubation periods of either 30 min, 1 h, 2 h or 4 h at room temperature, 37°C or 45°C, or of 4 h at