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THE DEVELOPMENT OF ANTISERA TO PROSTAGLANDINS B_2 AND $F_{2\alpha}$ AND THEIR ANALYSIS USING SOLID-PHASE AND DOUBLE ANTIBODY RADIOIMMUNOASSAY METHODS

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1 A method for the production of highly substituted prostaglandin-bovine serum albumin conjugates has been developed.

2 Antisera to prostaglandins B_2 and $F_{2\alpha}$ were raised in rabbits immunized with prostaglandin-bovine serum albumin conjugates.

3 The antisera were assessed for specificity and sensitivity by the double antibody radioimmunoassay method and after they were covalently linked to powdered cellulose to form a 'solid-phase' system.

4 Solid phase radioimmunoassays were developed using conventional shaking and in the presence of sucrose which obviates the need for continuous mixing of the incubates.

Introduction

There have been numerous reports on the development of antisera to prostaglandins of the B, F, A and E series, and the use of these antisera for radioimmunoassay of their respective prostaglandins (Caldwell, Burstein, Brock & Speroff, 1971; Jaffe, Smith, Newton & Parker, 1971; Levine, Gutierrez, Cernosek & van Vunakis, 1971; Stylos & Rivetz, 1972; Yu & Burke, 1972; Zusman, Caldwell, Speroff & Behrman, 1972; Jaffe, Behrman & Parker, 1973). Radioimmunoassays have been developed using the double antibody (Levine *et al.*, 1971; Stylos & Rivetz, 1972; Yu & Burke, 1972), dextran coated charcoal (Caldwell *et al.*, 1971; Zusman *et al.*, 1972; Jaffe *et al.*, 1973), and ammonium sulphate precipitation (Jaffe *et al.*, 1971) methods to separate the free from antibody-bound prostaglandin.

We now describe the production of antisera to prostaglandin B_2 and prostaglandin $F_{2\alpha}$ and their analysis by both solid phase and double antibody radioimmunoassay methods. Solid phase radioimmunoassay which is based upon the use of antibodies that have been covalently linked to cellulose is a new technique for prostaglandin estimation.

Antisera were raised in rabbits immunized with prostaglandin-bovine serum albumin conjugates. A method was developed for producing highly substituted conjugates.

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Methods

Production of antibodies

Conjugate formation Prostaglandins B_2 and $F_{2\alpha}$ were conjugated to bovine serum albumin (BSA) by the mixed anhydride method described for oestrone (Erlanger, Borek, Beiser & Lieberman, 1959).

Preparation of prostaglandin $F_{2\alpha}$-bovine serum albumin conjugate Prostaglandin $F_{2\alpha}$ (7 mg, 0.02 mM) and [9-$^3H_2$]-prostaglandin $F_{2\alpha}$ (49,389 d/min) were transferred in methanol to a 20 ml glass vial. The methanol was evaporated in a

stream of N_2 and the residue dried in a vacuum desiccator. Dioxan (0.5 ml), tri-*n*-butylamine (9.5 μl, 0.04 mM, Kodak Ltd.) and isobutylchloroformate (2.1 μl, 0.02 mM, Kodak Ltd.) were then added to the prostaglandin. This solution was cooled to 0° C and stirred by the addition of a small magnetic stirrer (0.5 x 0.1 mm) for 30 minutes. This procedure esterified the mixed anhydride.

The prostaglandin solution was then added to a 20 ml glass vial containing BSA (23 mg, 0.38 μM; Armour Pharmaceutical Co. Ltd.) dissolved in water (0.6 ml) and 1 N NaOH (23 μl). Aliquots of dioxan (a total of 0.5 ml) were used to rinse out any remaining prostaglandin from the original reaction vessel into the vial containing the solution of BSA. This latter solution was stirred continuously for 4 h and kept cool by surrounding it with ice-cold water. During this period, the prostaglandin $F_{2\alpha}$-BSA conjugation occurred. The solution was then transferred to Visking dialysis tubing (30-32) and dialysed against running tap water for 48 h in order to remove unconjugated prostaglandin. After dialysis, the solution was centrifuged for 20 min at 40° C and 15,000 *g*. The supernatant was freeze-dried to yield the prostaglandin $F_{2\alpha}$-BSA conjugate.

In order to determine the number of moles of prostaglandin $F_{2\alpha}$ bound to 1 molecule of BSA, two small amounts (0.3-0.7 mg) of conjugate were weighed accurately and transferred to separate scintillation vials. The conjugates were digested by warming with redistilled water (0.2 ml) and Nuclear Chicago Scintillant solubilizer (2 ml). Toluene scintillant (10 ml) was added to each solution and they were monitored for radioactivity in a Nuclear Chicago liquid scintillation counter. Once the number of disintegrations per minute (d/min) in a given weight of prostaglandin conjugate was known, the number of moles of prostaglandin $F_{2\alpha}$ bound to one mole of BSA could be calculated since the weight of prostaglandin $F_{2\alpha}$ and the number of d/min of [$^3H_2$]-prostaglandin $F_{2\alpha}$ originally used were known.

In a second system used later, the amounts of BSA, water and 1 N NaOH in the second reaction vessel were halved.

Prostaglandin B_2-BSA conjugates were prepared by identical methods to those used for prostaglandin $F_{2\alpha}$.

Injection regimen

Twelve adult male New Zealand white rabbits (approximately 2 kg) were used. Six were injected with prostaglandin $F_{2\alpha}$ conjugate and six with B_2 conjugate. The back of each animal was shaved and under sterile conditions it was injected intradermally, at approximately 50 sites, with a total of 2 ml of an emulsion containing prostaglandin conjugate (100 μg) in Freund's complete adjuvant. Pertussis vaccine (0.5 ml) was then injected subcutaneously to prime the animal's immune responses (Vaitukaitis, Robbins, Nieschlag & Ross, 1971). Boost injections of conjugate, emulsified in Freund's incomplete adjuvant, were administered subcutaneously at four sites (suprascapular and femoral) 26 weeks later and subsequently at 6-weekly intervals. The first and second boost injections contained 100 μg of prostaglandin conjugate and later injections contained 1 mg of conjugate.

Blood collection

Rabbits were bled at 4-weekly intervals until the first boost and thereafter at 6-weekly intervals, one week after each subsequent boost. During blood collections, the rabbit was restrained in a box, xylene was applied to one ear to increase the blood flow, soft yellow paraffin wax was applied to both surfaces of the ear in the region to be incised and, under sterile conditions, a stab incision was made in the marginal vein. Blood dripped from the vein over the surface of the soft paraffin wax and into a large glass centrifuge tube. At each bleed 50 ml of blood was collected from every rabbit.

The blood was allowed to clot at room temperature then left at 4° C overnight. The serum (containing some cells) was collected and kept at 4° C. The clot was warmed to room temperature and additional serum was removed. The serum was centrifuged at 1000 *g* for 15 to 20 min to remove a small cellular deposit, then decomplemented by incubation at 56° C for 30 min and finally cooled to room temperature. The volume of serum was measured and sodium azide (0.1 ml of a 10 mg/ml solution per 10 ml of serum) was added to it. It was then dispensed in 2 ml volumes in sterile McCartney bottles (5 ml) and stored at −20° C until use.

Reagents for radioimmunoassay

Double antibody radioimmunoassay method A diluent with the following composition was used; 0.05 M tris-hydrochloride buffer (pH 8), sodium azide (0.1 g/l) and gelatine (1 g/l). Tritiated prostaglandin $F_{2\alpha}$ (35.6 ng/μCi; New England Nuclear Company), [$^3H_2$]-prostaglandin $F_{2\alpha}$ (2.2 ng/μCi) and [$^3H_2$]-prostaglandin B_2 (2.5 ng/μCi; Amersham) were dissolved in methanol and stored in siliconized vials at −20° C. Before use, the methanol was evaporated in a stream of N_2 and

the residue dissolved in diluent to give the following concentrations: 3 ng/ml of 35.6 ng/μCi [$^3H_2$]-prostaglandin $F_{2\alpha}$ (i.e. 150 pg/50 μl), 0.2 or 0.6 ng/ml of 2.2 ng/μCi [$^3H_2$]-prostaglandin $F_{2\alpha}$ (i.e. 10 or 30 pg, respectively/50 μl) and 0.2 or 0.4 ng/ml of 2.5 ng/μCi [$^3H_2$]-prostaglandin B_2 (i.e. 10 or 20 pg, respectively/50 μl). These are referred to below as the tracer solutions.

Donkey anti-rabbit serum (Wellcome Reagents Ltd.) was stored at -20° C in 25 ml aliquots until use. After thawing it was kept at 4° C for periods of approximately 4 weeks during use. A stock solution was prepared by diluting 3 ml of serum to 10 ml with diluent. Normal rabbit serum was obtained from non-immunized donors by the techniques already described and a stock solution was prepared by diluting the serum 1 in 53 with diluent. The methods for determining the concentrations of normal rabbit serum and donkey anti-rabbit serum required for a radioimmunoassay have been described by Hunter (1973).

A scintillant with the following composition was used for both the double antibody and solid phase radioimmunoassay methods: naphthalene, 75 g; PPO (2,5, diphenyloxazole), 7 g; DMPOPOP (1,4-di-(2-(4 methyl-5-phenyloxazolyl)-benzene), 0.6 g; 2-ethoxyethanol 600 ml, toluene 1 litre. A volume of 13 ml of this scintillant absorbs 0.6 ml of an aqueous solution. Counting efficiency is then approximately 30%.

Solid phase radioimmunoassay method Two diluents were employed in the solid phase radioimmunoassay method. The first, a diluent identical to that described for the double antibody method (referred to subsequently as diluent), and the second, a sucrose diluent (always referred to as sucrose diluent). The sucrose diluent was composed of 0.05 M tris-hydrochloride buffer (pH 8), sodium azide (0.1 g/l), gelatine (1 g/l) and sucrose 200 g/l). Tritiated prostaglandin $F_{2\alpha}$ (2.2 ng/μCi) and [$^3H_2$]-prostaglandin B_2 (2.5 ng/μCi) were used. Solutions in diluent of 0.6 ng/ml of [$^3H_2$]-prostaglandin $F_{2\alpha}$ (30 pg/50 μl) and 0.4 ng/ml of [$^3H_2$]-prostaglandin B_2 (20 pg/50 μl) were made for radioimmunoassay.

An antiserum-cellulose complex as used by Wide (1969) and prepared according to the method of Bolton, Dighe & Hunter (1974), was used for the solid phase radioimmunoassays. A 1 in 100 dilution of the antiserum in the antiserum-cellulose complex was prepared as a stock solution and stored at 4°C. This dilution was made using diluent with a sodium azide concentration of 20 g/litre.

Dilution curves

Double antibody radioimmunoassay method The serum (0.1 ml) from each injected rabbit was diluted with diluent to give a 1 in 100 stock solution. This solution was used to prepare a series of 2-fold dilutions (1 ml each), ranging from 1 in 1000 up to 1 in 256,000. Tracer solution (50 μl) was then added to each dilution and the solutions were mixed and incubated for 1 h at room temperature (18-20° C). After incubation, normal rabbit serum (50 μl of a 1 in 53 dilution) was added to each solution and after mixing and standing for 10 min to equilibrate, donkey anti-rabbit serum (50 μl of a 3 in 10 dilution) was added to all tubes. (At a serum dilution of 1 in 1270, the rabbit gamma globulin present required a 1 in 80 dilution of the donkey anti-rabbit serum for adequate precipitation). The contents of the tubes were mixed and incubated for 16 h at 4° C. After incubation, the samples were centrifuged at 4° C, 1200 *g* for 45 min, then 0.6 ml of the supernatant was removed from each tube and added to a vial containing 13 ml of scintillant. The vials were monitored for radioactivity in a Nuclear Chicago liquid scintillation counter, using the external standards channels/ratio method and counting each vial for 4 or 10 minutes. Each dilution curve also included 4 blanks (non-specific binding control tubes) and 4 counting standards. The blanks contained 1 ml of diluent, 50 μl of tracer solution, 50 μl of diluted normal rabbit serum and 50 μl of diluted donkey anti-rabbit serum. The counting standards contained 1 ml of diluent and 50 μl of tracer solution. Counts from the liquid scintillation counter were recorded on a paper tape which was fed into a PDP8 computer programmed to calculate disintegrations per minute (d/min), counting efficiency and percentage binding of the tracer by the antiserum. The latter parameter was calculated by the following equation:

$$\text{Percentage bound} = 100 - \frac{(\text{d/min} \times 100)}{\text{average counting standard d/min}}$$

If the non-specific binding, as shown by the blanks, exceeded 10% in any experiment, the experiment was repeated.

A dilution curve (percentage binding of the tracer by the antiserum against dilution of antiserum) was drawn for each antiserum and the 'titre' was read as that dilution which bound 60% of the tracer in the absence of non-radioactive standard prostaglandin.

Solid phase radioimmunoassay method The solid phase shaking method was employed for dilution curves. A series of 2-fold dilutions in 1 ml of diluent, ranging from 1 in 1000 up to 1 in 512,000

was prepared in duplicate from the stock solution (1 in 100) of the prostaglandin antiserum-cellulose complex. This stock solution was stirred continuously, with a magnetic stirrer, while aliquots were removed from it for the preparation of antiserum dilutions. Tracer solution (50 μl) was added to each dilution of antiserum. These solutions were mixed then shaken without stoppering on a horizontal agitator for 2 h at room temperature (18-20° C). After incubation the samples were centrifuged at 4° C, 1200 *g* for 15 min then 0.6 ml of the supernatant was removed from each tube and added to a vial containing 13 ml of scintillant. The vials were monitored for radioactivity (using 4 min counts) and the percentage binding of the tracer by the antiserum was calculated as in the double antibody dilution curve method. A standard curve was plotted for each antiserum and the 'titre' was determined.

Each dilution curve also included 4 counting standards which contained 1 ml of diluent and 50 μl of tracer solution.

Standard curves

Double antibody radioimmunoassay method Standard curves were obtained only for antisera with high 'titres'. A dilution of the antiserum was prepared so that when 50 μl was added to the radioimmunoassay tube (10 x 75 mm) in which the antibody reaction took place (a total volume of 1.2 ml) the final dilution was equal to the 'titre'.

Tubes containing standard prostaglandin solutions (1 ml) were set up in duplicate (Table 1). In addition, zero standards, blanks and counting standards were set up in quadruplicate, each containing 1 ml of diluent. Tracer solution (50 μl) was added to each tube and the solutions were mixed and equilibrated for 10 minutes. Diluted antiserum (50 μl) was then added to every tube, except the blanks and counting standards, and after mixing, the samples were incubated at room temperature (18-20° C) for 1 hour. After incubation, the procedure for a standard curve was identical to that performed for a dilution curve.

A standard curve (percentage binding of the tracer by the antiserum against weight of prostaglandin) was drawn for each antiserum. The detection limit of each radioimmunoassay was defined loosely and for comparative purposes only as the amount of standard prostaglandin required to give a 10% fall in binding from the zero standard under these closely defined conditions. Binding of approximately 60% was obtained with the zero standards.

Solid phase radioimmunoassay method Standard curves were obtained for prostaglandin $F_{2\alpha}$ antiserum (rabbit number 6, 3rd boost) and for prostaglandin B_2 antiserum (rabbit number 7, 1st boost). Both the shaking and the sucrose methods for solid phase radioimmunoassay were performed for prostaglandin $F_{2\alpha}$ antiserum but the shaking method only was performed for prostaglandin B_2 antiserum. A dilution of the stock solution of the prostaglandin antiserum-cellulose complex was prepared so that when 0.5 ml was added to each radioimmunoassay tube (15 x 75 mm) in which the antibody reaction took place (a total volume

Table 1 Amounts (ng) of prostaglandin B_2 (PGB_2) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) used as standards for double antibody and solid phase radioimmunoassay

Double antibody standards			*Solid phase standards*	
$PGF_{2\alpha}$ (35.6 ng/μCi)	*$PGF_{2\alpha}$ (2.2 ng/μCi)*	*PGB_2 (2.5 ng/μCi)*	*$PGF_{2\alpha}$ (2.2 ng/μCi)*	*PGB_2 (2.5 ng/μCi)*
0.15	0.01		0.01	
0.45	0.02		0.02	
1.05	0.03		0.03	
2.25	0.07		0.05	
4.65	0.15		0.07	
9.45	0.31		0.1	
19.05	0.63		0.15	
	1.27		0.3	
	2.55		0.6	
			1.2	

In the double antibody method these amounts were added to each radioimmunoassay tube in 1 ml of diluent. In the solid phase method double these amounts were added to each assay tube in 0.5 ml of diluent and they were diluted by the addition of the antiserum-cellulose complex in 0.5 ml of diluent. The specific activity of the tracer used is in brackets.

of 1.05 ml), the final dilution was equal to the 'titre'.

Tubes containing standard prostaglandin solutions (0.5 ml) were set up in duplicate (Table 1). In addition, zero standards and blanks (sucrose method only) containing 0.5 ml of diluent, and counting standards containing 1 ml of diluent, were set up in quadruplicate. Tracer solution (50 μl) was added to each tube and the solutions were mixed and left to equilibrate for 10 min at room temperature (18-20° C). The procedure for the shaking and the sucrose methods now differed slightly.

Shaking method Diluted antiserum, 0.5 ml in diluent, was added to every tube except the counting standards and, after mixing, the samples were shaken at room temperature (18-20° C) for 2 hours. (No blanks were included in this method as they would have been identical to the counting standards.

Sucrose method Diluted antiserum, 0.5 ml in sucrose diluent, was added to every tube, except the blanks and counting standards. Sucrose diluent (0.5 ml) was added to the blanks. After mixing, the samples were left to incubate without shaking at room temperature (18-20° C) for 3 hours.

After incubation using the shaking or the sucrose methods, all the samples were centrifuged and sampled according to the methods described for the solid phase dilution curves.

Standard curves were plotted, as in the double antibody radioimmunoassay method, and the sensitivity of each assay was determined.

Optimum time for incubation of antibody with antigen

Double antibody radioimmunoassay method Four sets of two zero standards were prepared as for a standard curve. Incubation periods of 1, 2, 4 and 24 h were then left before adding the diluted normal rabbit serum and the diluted donkey anti-rabbit serum.

Solid phase radioimmunoassay method Four sets of two zero standards were prepared as for a standard curve, using the sucrose (prostaglandin $F_{2\alpha}$ only) and shaking (prostaglandin $F_{2\alpha}$ and B_2) methods. The samples were incubated for periods of 0.5, 1, 2, 3 and 4 h before centrifuging.

Effect of varying tracer concentrations

Double antibody radioimmunoassay method A standard curve was set up in which 10 pg of tracer was added to all the samples and a tracer curve in which four sets of two tubes containing 20, 40, 80 and 160 pg of tracer in a total of 1 ml of diluent was prepared. The procedure for adding antiserum, normal rabbit serum and donkey anti-rabbit serum was then followed, as for a standard curve.

Graphs of percentage binding of the tracer by the antiserum against weight (pg) of prostaglandin were then plotted for the standard and the tracer samples. Since 10 pg of tracer was added to all the samples in the standard curve, the tracer samples (containing a total of 20, 40, 80 and 160 pg) were equivalent to 10 + 10, 10 + 30, 10 + 70 and 10 + 150 pg of tracer prostaglandin. Thus, in order to compare the tracer samples with the standards, a graph was plotted in which the percentage of tracer bound was plotted against tracer prostaglandin amounts of 10, 30, 70 and 150 pg (see Figure 4).

Solid phase radioimmunoassay method Standard and tracer curves for prostaglandins B_2 and $F_{2\alpha}$ were prepared using the same principles as for the double antibody radioimmunoassay method. The standard curves were obtained using 20 pg of tracer prostaglandin B_2 and 30 pg of tracer prostaglandin $F_{2\alpha}$. The shaking and sucrose solid phase radioimmunoassay methods were used for prostaglandin $F_{2\alpha}$ and the shaking method only was used for prostaglandin B_2.

Specificity studies

Double antibody and solid phase methods A standard curve for the prostaglandin to which the antiserum was raised was set up. In parallel, standard curves for different prostaglandins were prepared, starting with a prostaglandin concentration of 0.1 ng/ml and making four-fold dilutions. All dilutions were set up in duplicate.

The percentage cross reactivity of the prostaglandin B_2 or $F_{2\alpha}$ antisera with another prostaglandin (e.g. prostaglandin A_2) was determined by finding the concentration of prostaglandin A_2 which corresponded with a 10% fall from the binding obtained with the zero standard. The following calculation was then applied:

e.g. percentage cross reactivity of prostaglandin B_2 (PGB_2) antiserum with prostaglandin A_2 (PGA_2) =

$$\frac{\text{Concentration of } PGB_2 \text{ at 10\% fall from binding of zero standard}}{\text{Concentration of } PGA_2 \text{ at 10\% fall from binding of zero standard}} \times 100$$

Results

Antisera to prostaglandins B_2 and $F_{2\alpha}$ were raised in rabbits immunized with prostaglandin-bovine serum albumin conjugates. The 'titres' of all the antisera were determined using the double antibody radioimmunoassay method, and standard curves, optimum incubation times, tracer studies and specificity studies were performed for antisera with high 'titres'.

As a result of these studies prostaglandin $F_{2\alpha}$ antiserum from rabbit number 6 (3rd boost) and prostaglandin B_2 antiserum from rabbit number 7 (1st boost) (Table 2) were selected for the routine estimation of prostaglandins $F_{2\alpha}$ and B_2, by the double antibody radioimmunoassay method and for the development of a solid phase radioimmunoassay method.

Solid phase radioimmunoassay was performed using both the shaking and sucrose methods. The shaking method was developed for both prostaglandin B_2 and $F_{2\alpha}$ antisera but the sucrose method has, as yet, only been developed for the prostaglandin $F_{2\alpha}$ antiserum. Standard curves, optimum incubation times, tracer and specificity studies were performed for the prostaglandin B_2 and $F_{2\alpha}$ antisera using the solid phase radioimmunoassay methods.

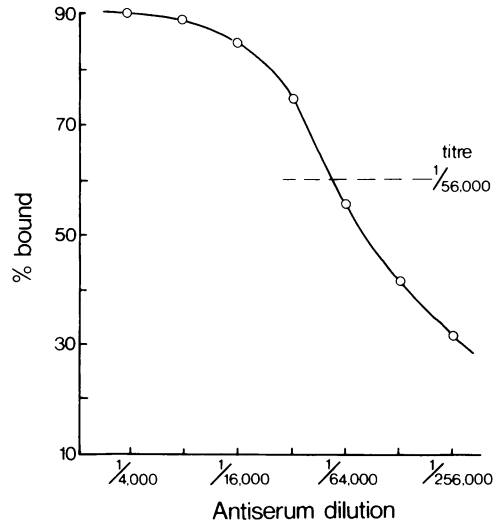

Figure 1 Dilution curve for prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) antiserum, obtained from rabbit number 6 (3rd boost). Double antibody radioimmunoassay method used with 10 pg of [$^3H_2$]-prostaglandin $F_{2\alpha}$ (2.2 ng/μCi).

Conjugate formation

The first conjugation procedure yielded conjugates with between 20-37 moles of prostaglandin $F_{2\alpha}$ and 18-26 moles of B_2 bound to one mole of BSA.

The second system resulted in 50 moles of prostaglandin $F_{2\alpha}$ and 36 moles of prostaglandin B_2 being bound to one mole of BSA. These very highly substituted conjugates were particularly effective in increasing the antibody 'titre' in injected rabbits.

Testing of antisera

Dilution curves for double antibody radioimmunoassay method These were obtained for all the antisera and their 'titres' were determined. In

Table 2 The highest antiserum 'titres' produced by each rabbit

Prostaglandin	*Rabbit number*	*'Titre'*	*Boost number*
$F_{2\alpha}$	1	1 in 51,200	5
	2	Died	–
	3	1 in 70,400	4
	4	1 in 108,800	4,5.
	5	1 in 20,800	5
	6	1 in 76,800	5
B_2	7	1 in 35,200	5
	8	1 in 17,600	4,5.
	9	1 in 13,600	3
	10	Died	–
	11	1 in 54,400	6
	12	Died	–

The 'titres' were obtained from dilution curves performed using the double antibody radioimmunoassay method and 20 pg of [$^3H_2$]-prostaglandin B_2 (2.5 ng/μCi) or 10 pg of [$^3H_2$]-prostaglandin $F_{2\alpha}$ (2.2 ng/μCi). Rabbits 1-6 were injected with prostaglandin $F_{2\alpha}$ conjugate and rabbits 7-12 were injected with B_2 conjugate.

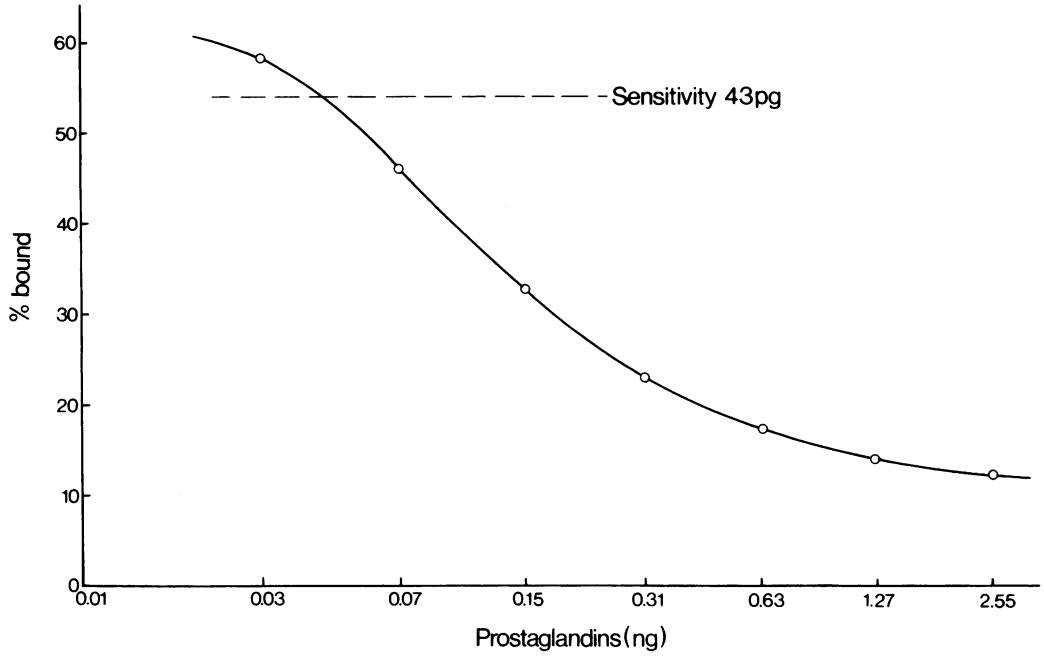

Figure 2 Standard curve for prostaglandin B_2 (PGB_2) using the double antibody radioimmunoassay method. Antiserum obtained from rabbit number 7 (1st boost); 20 pg of [$^3H_2$]-prostaglandin B_2 (2.5 ng/μCi) used; 'titre' of antiserum 1 in 24,800. An average binding of 64% was obtained from the 4 zero standards.

rabbits injected with prostaglandins B_2 and $F_{2\alpha}$ conjugates, antisera with 'titres' as high as 1 in 54,400 (20 pg tracer) and 1 in 108,800 (10 pg tracer), respectively, were obtained (Table 2).

A dilution curve for prostaglandin $F_{2\alpha}$ antiserum, obtained from rabbit number 6 (3rd boost), is illustrated in Figure 1.

Dilution curves for solid phase radioimmunoassay method These were obtained for prostaglandin B_2 antiserum from rabbit number 7 (1st boost) and for prostaglandin $F_{2\alpha}$ antiserum from rabbit number 6 (3rd boost). The shaking method for solid phase radioimmunoassay was used.

The 'titres' of these antisera (as determined by double antibody radioimmunoassay) were reduced using the solid phase radioimmunoassay method (Table 3).

Standard curves for double antibody and solid phase radioimmunoassay methods Standard curves were prepared (using the double antibody radioimmunoassay method) for all antisera with high 'titres'. Standard curves for prostaglandins B_2 and $F_{2\alpha}$ are illustrated in Figures 2 and 3. The detection limit of each radioimmunoassay was determined from the standard curve and these limits for prostaglandins B_2 and $F_{2\alpha}$, using the

Table 3 The 'titres' of two antisera, as determined by solid phase and double antibody radioimmunoassay (RIA) methods

Prostaglandin	*Antiserum used*	*Tracer used*	*'Titre' Double antibody RIA*	*'Titre' Solid phase RIA (Shaking Method)*
$F_{2\alpha}$	Rabbit number 6 (3rd boost)	30 pg (2.2 ng/μCi)	1 in 38,400	1 in 8,800
B_2	Rabbit number 7 (1st boost)	20 pg (2.5 ng/μCi)	1 in 24,800	1 in 9,600

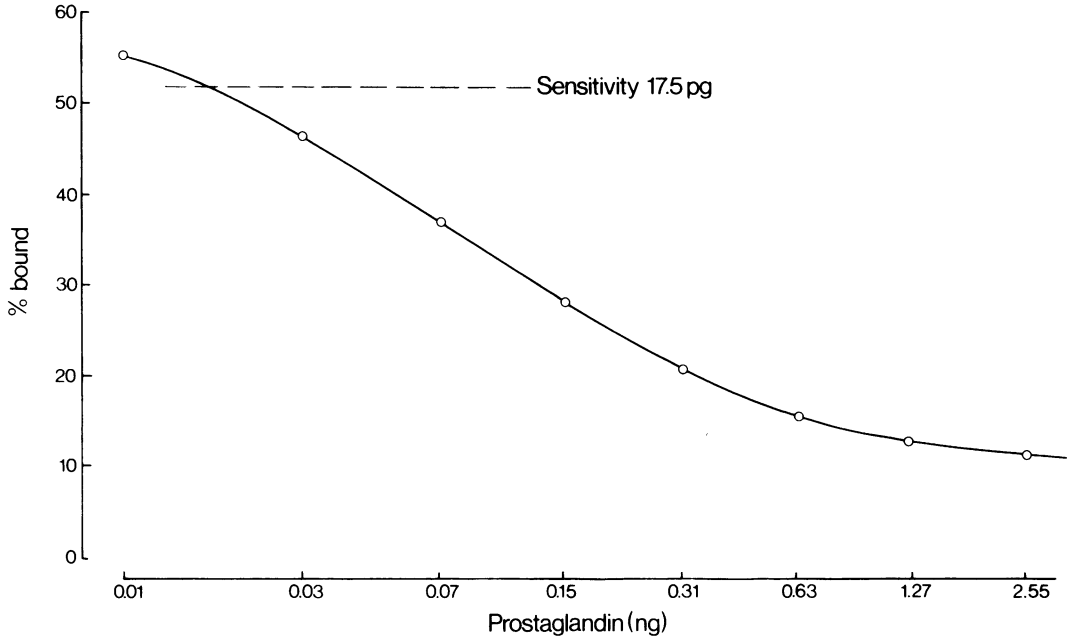

Figure 3 Standard curve for prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) using the double antibody radioimmunoassay method. Antiserum obtained from rabbit number 6 (3rd boost); 10 pg of [$^3H_2$]-prostaglandin $F_{2\alpha}$ (2.2 ng/μCi) used; 'titre' of antiserum 1 in 64,000. An average binding of 61.5% was obtained from the 4 zero standards.

double antibody and solid phase radioimmunoassay methods, are illustrated in Table 4. There was little change in the detection limit of the radioimmunoassay for prostaglandin B_2, whether the double antibody or solid phase methods were used, but the detection limit of the assay for prostaglandin $F_{2\alpha}$ decreased when the solid phase (sucrose and shaking) methods were used (Table 4).

Optimum time for incubation of antibody with antigen

The effect of changing incubation time on the efficiency of the antibody-antigen reaction, is illustrated in Table 5. As a result of these experiments, a one hour incubation period was chosen for all radioimmunoassays for prostaglandins B_2 and $F_{2\alpha}$ using the double antibody

Table 4 The effect of the double antibody and solid phase radioimmunoassay methods on the sensitivities of assays for prostaglandin B_2 (PGB_2) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)

			Sensitivity (pg) of each radioimmunoassay		
		Tracer used	*Double antibody*	*Solid phase method*	
PG	*Antiserum used*	*(Specific activity)*	*method*	*Shaking*	*Sucrose*
B_2	Rabbit number 7 (1st boost)	20 pg (2.5 ng/μCi)	43 s.e. = ± 1.25 n = 8	43 s.e. = ± 2.78 n = 13	
$F_{2\alpha}$	Rabbit number 6 (3rd boost)	10 pg (2.2 ng/μCi)	17.5		
		150 pg (35.6 ng/μCi)	100 s.e. = ± 6.18 n = 5		
		30 pg (2.2 ng/μCi)	27 s.e. = ± 1.54 n = 5	51 s.e. = ± 6.22 n = 7	60 s.e. = ± 3.68 n = 13

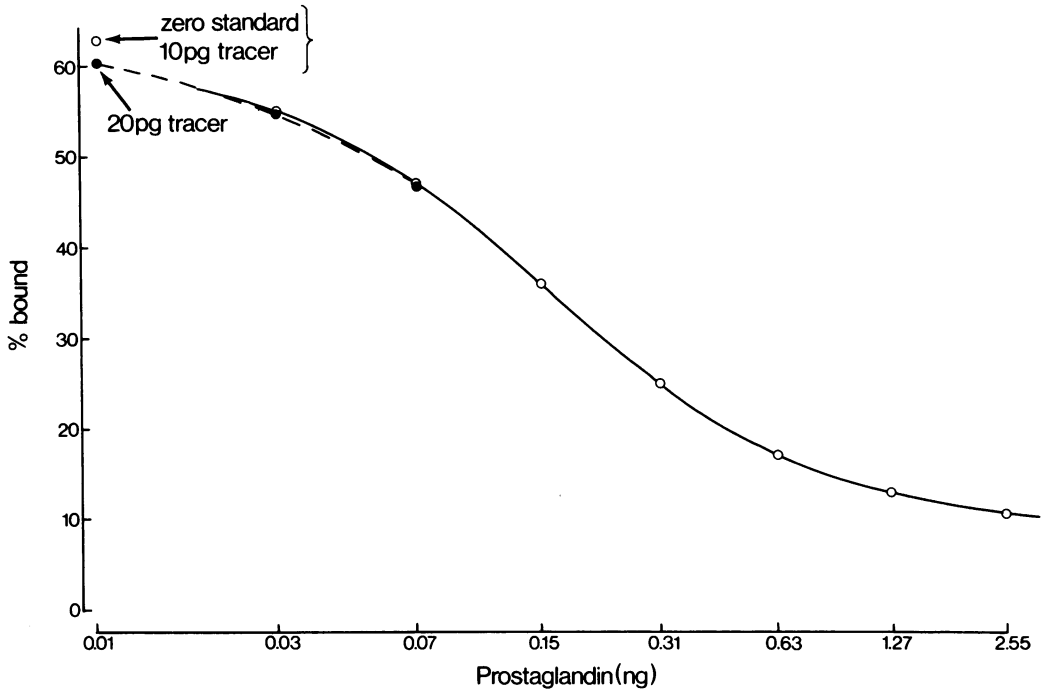

Figure 4 Comparison of standard (o—o) and tracer (•---•) curves for prostaglandin B_2 (PGB_2) using the double antibody radioimmunoassay method. Antiserum obtained from rabbit number 7 (1st boost), 10 pg of [$^3H_2$]-prostaglandin B_2 (2.5 ng/μCi) used in standard curve.

Table 5 Effect of changing incubation time (h) on the development of the antibody-antigen reaction

RIA Method	*PG*	*Antiserum from rabbit*	*Tracer (Specific activity)*	*Incubation time (h)* 0.5	1	2	3	4	24
	$F_{2\alpha}$	Number 6 (3rd boost)	150 pg (35.6 ng/μCi)		67.6	69.1		69.6	68.8
Double antibody	$F_{2\alpha}$	Number 6 (3rd boost)	10 pg (2.2 ng/μCi)		63.7	65.8		66.8	65.8
	B_2	Number 7 (1st boost)	20 pg (2.5 ng/μCi)		64.3	66.1		66.9	65.9
Solid phase									
Shaking method	$F_{2\alpha}$	Number 6 (3rd boost)	30 pg (2.2 ng/μCi)	47.4	55.0	58.5	57.8		
Sucrose method	$F_{2\alpha}$	Number 6 (3rd boost)	30 pg (2.2 ng/μCi)		56.5	57.0	60.1	60.1	
Shaking method	B_2	Number 7 (1st boost)	20 pg (2.5 ng/μCi)	59.1	62.5	66.0	64.1		

The results, which are the average of duplicate zero standards, show the percentage of tracer prostaglandin bound to antiserum after various incubation times.

method. When the solid phase radioimmunoassay methods were employed for prostaglandins B_2 and $F_{2\alpha}$ estimation, incubation periods of 2 and 3 h were used for the shaking and sucrose methods, respectively.

Effect of varying tracer concentrations

Double antibody radioimmunoassay method The effect of varying tracer concentrations of prostaglandin B_2 is shown in Figure 4. Since the standard and tracer curves are almost superimposable the mass of prostaglandin in the tracer and cold prostaglandin samples is almost identical and as expected the antiserum is unable to distinguish between labelled and unlabelled prostaglandin B_2.

In Figure 4, it can be seen that the difference in binding between using 10 and 20 pg of tracer prostaglandin B_2 was negligible. In all further studies, 20 pg of tracer prostaglandin B_2 was therefore used as it allowed a shorter counting time. After similar experiments with tracer prostaglandin $F_{2\alpha}$, 2.2 and 35.6 ng/μCi, 10 and 150 pg, respectively of the two tracer preparations were used for the double antibody radioimmunoassay method.

Solid phase radioimmunoassay method As a result of varying tracer concentrations using the solid phase radioimmunoassay methods, 20 pg of tracer prostaglandin B_2 (2.5 ng/μCi) and 30 pg of tracer prostaglandin $F_{2\alpha}$ (2.2 ng/μCi) were used routinely.

Specificity studies

The percentage cross reactivity of prostaglandin $F_{2\alpha}$ antiserum from rabbit number 6 (3rd boost) and prostaglandin B_2 antiserum from rabbit number 7 (1st boost) with various prostaglandins is shown in Table 6. Apart from 100% cross-reaction with prostaglandin $F_{1\alpha}$, the prostaglandin $F_{2\alpha}$ antiserum was fairly specific. The 40% cross reactivity with prostaglandin D_2 is significant. The prostaglandin B_2 antiserum exhibited 16-18% cross reaction with prostaglandin B_1 but otherwise it was reasonably specific (Table 6). There was little difference in the specificity of the prostaglandin $F_{2\alpha}$ antiserum whether used by the solid phase or double antibody radioimmunoassay method, but the specificity of the prostaglandin B_2 antiserum appeared to improve when it was used in the solid phase assay method (Table 6).

Some of the specificity studies (double antibody method) for prostaglandin B_2 antiserum from rabbit number 7 (1st boost) are illustrated in Figure 5.

Discussion

Antisera to prostaglandins B_2 and $F_{2\alpha}$ were raised

Table 6 Percentage cross reactivity of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) antiserum from rabbit number 6 (3rd boost) and prostaglandin B_2 (PGB_2) antiserum from rabbit number 7 (1st boost), with other prostaglandins. The mass and specific activity of the tracer prostaglandin used and the 'titre' of the antisera are tabulated.

	Double antibody radioimmunoassay method				*Solid phase radioimmunoassay method*	
					Sucrose	*Shaking*
PG tested	*Tracer PG*				*Tracer PG*	
		$F_{2\alpha}$		B_2	$F_{2\alpha}$	B_2
	10 pg	*30 pg*	*150 pg*	*20 pg*	*30 pg*	*20 pg*
	(2.2 ng/μCi)		*(35.6 ng/μCi)*	*(2.5 ng/μCi)*	*(2.2 ng/μCi)*	*(2.5 ng/μCi)*
	Titre	*Titre*	*Titre*	*Titre*	*Titre*	*Titre*
	1 in 64,000	*1 in 38,000*	*1 in 11,200*	*1 in 24,800*	*1 in 8,800*	*1 in 9,600*
E_2	2.5	1.21	0.7	7.0	1.5	0.6
E_1	0.4	0.52	0.5	2.0	3.3	1.3
A_2	0.1	<0.056	0.003	10.0	<0.04	4.7
A_1				6.0		1.7
E_2				9.0		
B_2	0.0017	0.0016	0.1		0.008	
B_1				18.0		15.9
$F_{2\alpha}$				0.004		0.018
$F_{2\beta}$	0.3	0.189	0.3		0.4	
$F_{1\alpha}$	120.0	100.0	73.0		92.1	
15 oxo $F_{2\alpha}$	0.2	2.6	0.2		3.25	
dihydro B_1				2.0		4.4
D_2					40.0	

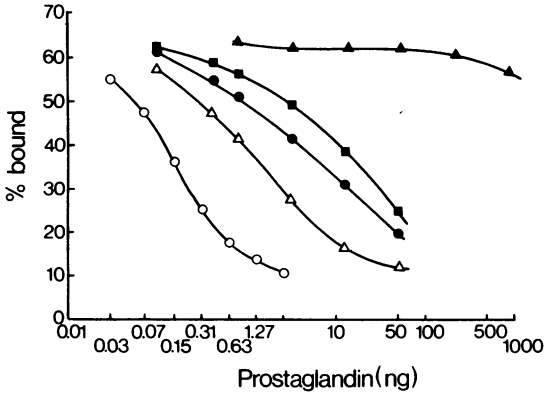

Figure 5 Specificity studies for prostaglandin B_2 (PGB_2) antiserum, obtained from rabbit number 7 (1st boost). The double antibody radioimmunoassay method was used with 20 pg of [$^3H_2$]-prostaglandin B_2 (2.5 ng/μCi); antiserum 'titre' 1 in 24,800. Binding of 63% was obtained with the zero standards (an average of 4 readings). (▲) $F_{2\alpha}$; (■) Dihydro B_1; (●) A_1; (△) B_1; (○) B_2.

in rabbits immunized with prostaglandin-bovine serum albumin conjugates. The antisera were analysed by both the solid phase and double antibody radioimmunoassay methods.

Solid phase radioimmunoassay, in which the prostaglandin antiserum is complexed to cellulose, is a new immunological technique for prostaglandin estimation. In this paper, two solid phase methods (shaking and sucrose) are discussed. Practically, the sucrose method is the simpler of the two since it involves the use of a sucrose diluent which acts as a support medium for the cellulose bound antibody. The contents of the assay tubes therefore do not require shaking.

Solid phase radioimmunoassay has proved to be a highly sensitive and reproducible method for prostaglandin estimation. It has several advantages over the double antibody (Levine *et al.*, 1971; Stylos & Rivetz, 1972; Yu & Burke, 1972), dextran-coated charcoal (Caldwell *et al.*, 1971; Zusman *et al.*, 1972; Jaffe *et al.*, 1973) and ammonium sulphate precipitation (Jaffe *et al.*, 1971) methods which have previously been used for separating free prostaglandin from that bound to antiserum. Its main advantage is that it requires the addition of only two solutions (antiserum and tracer) to each sample, and is, therefore, extremely quick and suitable for handling large numbers of samples. After the addition of antiserum and tracer, only a short incubation period is required before the samples are centrifuged and the supernatant monitored for radioactivity. These procedures contrast sharply with the double antibody method which requires the addition of four solutions to each sample and two incubation periods, one of which lasts overnight. The solid phase method is manipulatively simple and suffers neither from a tendency to high non-specific binding, which is the case with ammonium sulphate, nor from the need for careful timing, as is the case with charcoal.

The solid phase and double antibody methods described in this paper were capable of detecting concentrations as low as 10 pg/ml of prostaglandins B_2 and $F_{2\alpha}$. Apart from the high cross reactivity of the prostaglandin $F_{2\alpha}$ antiserum with $F_{1\alpha}$, and of prostaglandin B_2 antiserum with B_1, the particular antisera used in these assays had low cross reactivities with other prostaglandins tested. The high cross reactivity with prostaglandin D_2 may be a serious limitation in situations where this prostaglandin occurs naturally.

We consider that the solid phase radioimmunoassay method described here has many advantages for workers in the prostaglandin field who wish to measure small quantities of prostaglandins F_α and B in larger numbers of samples. The problems encountered in applying these methods to biological samples and to the estimation of prostaglandins of the E and A series are the subject of a subsequent paper.

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