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Binding of prostaglandin to human serum albumin

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The interaction of tritiated prostaglandin E_1 (^{3}H-PGE_1) with various constituents of human blood was examined by equilibrium dialysis and incubation experiments. Plasma bound ^{3}H-PGE_1 weakly, apparently due to an interaction with serum albumin. Blood cells and plasma globulins did not interact with PG. When albumin and other proteins were precipitated by ethanol, as a preliminary stage of extracting PG from plasma, the recoveries of added PGs were poor. Addition of acid and non-precipitating amounts of ethanol (40–50%) to the plasma allowed quantitative extraction of added PGE, F, and A compounds into chloroform.

Examination of the possible roles and the fate of circulating prostaglandins (PGs) requires their quantitative extraction from blood. The method for extracting PGs from tissues or seminal plasma with 70–90% ethanol (Samuelsson, 1963, 1964) or acetone (Ambache, 1963; Daniels, Hinman & others, 1967) gives poor and inconsistent recoveries when applied to blood. Since blood does not inactivate PGs (Änggård & Samuelsson, 1964; Ferreira & Vane, 1967), and PGs can be quantitatively recovered from aqueous-ethanol or acetone solutions, the low recoveries appear to be due to binding by blood constituents. The equilibrium dialysis experiments below indicate that ^{3}H-PGE_1 becomes bound to serum albumin, but that quantitative extraction from blood is possible if the initial precipitation of albumin by ethanol or acetone is avoided.

MATERIALS AND METHODS

Equilibrium dialysis was carried out with ^{3}H-PGE_1 and samples of (a) fresh human blood cells washed with cold saline; (b) freeze-dried human serum albumin (Blood Products Laboratory, Lister Institute, Elstree) containing approximately 12% α_1, α_2 and β_1 globulins and 4·0 μ-mol of fatty acid per g protein; (c) fresh and stored human blood plasma; (d) human gamma globulin (A. B. Kabi, Stockholm); (e) human serum albumin (Bohringwerke, A.G., Germany), electrophoretically homogeneous and containing approximately 2·5 mol of fatty acid per mol of albumin (measured by the method of Spector, Steinberg & Tanaka, 1965), and (f) human plasma globulin fractions [fractions II, III and IV, prepared by Cohn's method six (Cohn, Oncley & others, 1944), washed with distilled water and shown electrophoretically to contain no albumin]. PGE_1, E_2, $F_{1\alpha}$ and $F_{2\alpha}$ were gifts from J. E. Pike, Upjohn Ltd., Kalamazoo. ^{3}H-PGE_1 (5,6 ^{3}H-PGE_1, 37×10^6 d/min mg, stored in methanol at 4°) containing 1 to 3% ^{3}H-PGA_1, was a gift from D. A. van Dorp, Unilever Research, Vlaardingen. ^{3}H-PGA_1 was prepared from ^{3}H-PGE_1 by treatment with glacial acetic acid (Pike, Kupiecki & Weeks, 1967).

Dialysis was performed in a duplicate series of 30 ml glass-stoppered tubes with bags made from pre-soaked (Warner & Weber, 1953) "24/32" cellulose tubing; 5 ml of a blood cell suspension (in Krebs solution) or of a protein solution (in isotonic phosphate buffer, pH 7·5, or physiological saline) were put into a dialysis bag immersed in 5 ml of the salt solution. Equal amounts of ^{3}H-PGE$_1$ were included in the contents of one bag and in the fluid outside a duplicate bag as paired controls. In some experiments with serum albumin, ethanol or formic acid or both were included in the dialysis system. The tubes were shaken while equilibrating at 37° for 18–36 h. Aliquots (0·5 ml) from inside and outside the bags were counted on a Beckman-LS-200 scintillation counter in 12 ml toluene–methanol (55 : 45) solutions containing butyl-PBD (0·7%) and PBBO (0·05%) phosphors (Kowalski, Anliker & Schmid, 1967). Counting efficiency was 20 to 25% and correction for quenching was made by use of the automatic external standard channels ratio method and a quench-calibration curve constructed for the scintillation mixture. Equilibration was assumed to be complete when the distribution of tritium inside and outside the paired bags was the same. With very dilute protein solutions and with blood cells, 18 h was sufficient for equilibration; higher concentrations of protein required a longer incubation period (24–36 h).

The addition of phosphate-buffered albumin solution from inside the bags to the scintillation fluid, resulted in the gradual formation of a small amount of precipitate. This did not appear to alter the counting efficiency since the sum of inside and outside counts remained equivalent to the total activity added in the system. Moreover, the same counting efficiency was found with aliquots containing various amounts of albumin (0·5–5·0 mg/0·5 ml) but containing the same amount of ^{3}H-PGE$_1$. Aliquots from inside and outside the bags were also assayed biologically for prostaglandin activity on the rat fundus preparation (Vane, 1957) in Krebs solution at 37° bubbled with 5% carbon dioxide in oxygen. The contractions of the tissue were recorded on a kymograph by an isotonic frontal writing lever.

RESULTS

Interaction of ^{3}H-PGE$_1$ *with human blood*

When ^{3}H-PGE$_1$ (0·33 to 1·0 μg initially in a 5 ml compartment) was dialysed against 5 ml human blood cells (1·3 to 5 $\times$ 10^9 red cells and 10^6 white cells per ml) the radioactivity and biological activity were distributed equally inside and outside the bags, indicating that no binding had occurred (4 experiments). Aliquots from inside the bags containing blood cells could not be counted so that the calculations were based on a comparison of aliquots taken outside the bags with ^{3}H-PGE$_1$ standards. The total biological activity of ^{3}H-PGE$_1$ and the activity ratio (the ratio of radioactivity: biological activity) were the same before and after dialysis, indicating that the cells had not inactivated PGE$_1$. Furthermore, the biological activities of unlabelled PGE$_1$, E$_2$, F$_{1\alpha}$ and F$_{2\alpha}$ (0·1, and 0·05, 25 and 14 μg/ml respectively) on the rat fundus were unaltered by incubation with blood cells (5 $\times$ 10^9 red cells and 10^6 white cells per ml) at 37° in Krebs solution, physiological saline, or in saline containing 0·1% (w/v) dextrose. These findings agree with the conclusions of other workers that blood cells do not inactivate the PGs (Änggård & Samuelsson, 1964; Ferreira & Vane, 1967), and indicate also that blood cells do not release PGE or PGF compounds.

In contrast, equilibrium dialysis showed that ^{3}H-PGE$_1$ did bind to plasma (5 experiments in which 2 to 9 μg ^{3}H-PGE$_1$ was dialysed against 3·2 to 48% (v/v) plasma

in 0·15M phosphate buffer, pH 7·3) since increasing concentrations of plasma decreased the concentration of unbound PG at equilibrium (36 h incubation). Equilibrium dialysis of ^{3}H-PGE$_1$ with solutions of freeze-dried albumin (8 to 75 mg/5 ml. i.e. similar concentrations to the albumin concentration in the plasma solutions),

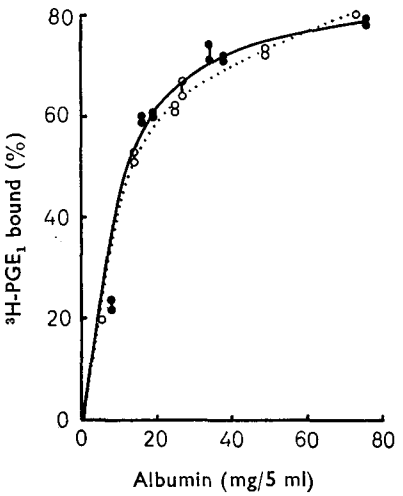

FIG. 1. Binding of ^{3}H-PGE$_1$ by 'freeze-dried' human serum albumin and by human plasma. Equilibrium dialysis of 9 μg ^{3}H-PGE$_1$ against human blood plasma (diluted to between 3·2 and 48% in 0·15M phosphate buffer, pH 7·3) and against human serum albumin (7·6 to 76 mg in 5 ml). Both protein preparations were first dialysed against phosphate buffer. Two points joined by a vertical line (● for albumin and ○ for plasma) show the two results for the paired dialysis tubes.

Table 1. *Comparison of ^{3}H-PGE$_1$ binding by solutions of human blood plasma and freeze-dried serum albumin.* A total of 9 μg ^{3}H-PGE$_1$ ($2{\cdot}54 \times 10^{-8}$ mol total) was dialysed against plasma diluted with 0·15M phosphate buffer, pH 7·3, (3·2 to 48% plasma containing 5·5 to 73 mg albumin within the 5 ml compartment) and against serum albumin (7·6 to 76 mg/5 ml). Both protein preparations were dialysed against 2 changes of phosphate buffer before dilution. The 'initial PG/Alb' is the molar ratio of ^{3}H-PGE$_1$ to albumin included in the 10 ml dialysis system. 'PG % bound' is given separately where ^{3}H-PGE$_1$ was added to (A) the inside and (B) the outside compartment. r is the mol PG bound per mol of albumin used (average of the pair) and C is the molar concentration of unbound ^{3}H-PGE$_1$/litre (average of pair).

Plasma %	Albumin mg/5 ml	Specific activity d/min mg $\times 10^{-5}$	Initial PG/Alb	PG % bound A	PG % bound B	r $\times 10^2$	r/C $\times 10^{-4}$
	38	0·75	0·044	72	71	3·1	4·3
	19	0·75	0·087	61	60	5·3	5·2
	7·6	0·75	0·44	22	24	5·0	2·5
16	27	0·75	0·061	64	67	4·0	2·4
8	14	0·75	0·122	51	53	6·4	5·2
3·2	5·5	0·75	0·301	20	20	6·0	2·9
	76	7·5	0·021	79	78	1·7	3·1
	34	7·5	0·049	71	74	3·6	5·1
	16	7·5	0·100	60	59	6·0	5·7
48	73	7·5	0·023	80	80	2·7	5·3
32	49	7·5	0·034	73	72	2·4	3·5
16	25	7·5	0·066	62	61	4·0	4·1

indicated that the percentage binding of PG by the plasma dilutions was close to that expected from their albumin contents (Fig. 1 and Table 1). Both give results which are similar to those found with purified albumin (compare Tables 1 and 2).

^{3}H-PGE$_1$ (0·33 to 1·0 μg, initially in one 5 ml compartment) was not bound either to human gamma globulin (0·33 to 1·7 $\times$ 10^{-7} mol in 5 ml; 4 experiments) during equilibrium dialysis or to Cohn plasma globulin fractions II, III and IV (6 to 12 mg in 5 ml; 3 experiments).

The responses of the rat isolated fundus strip to PGE$_1$, E$_2$, F$_{1\alpha}$ and E$_{2\alpha}$ were unchanged by the addition of fresh human plasma or of albumin to the bathing fluid so that the binding to the plasma constituent was weak and freely reversible.

The total biological activity and the activity-ratio of added ^{3}H-PGE$_1$ were unaltered after incubation or dialysis with human plasma or albumin as shown in the following two ways:

(1) Portions of a ^{3}H-PGE$_1$ solution (0·05 to 0·2 ml of 40 μg PGE$_1$ and 7·5 μg ^{3}H-PGE$_1$ per ml) were incubated at 37° for 4 h with samples (4 ml) of fresh human blood plasma which were then extracted into chloroform (Unger, Stamford & Bennett, 1971). Aliquots of each extract were counted and assayed for biological activity. The recoveries of isotope and of biological activity were 86 to 91% and 86 to 92% respectively (4 determinations) so that the activity-ratio after extraction (1300 to 1386 counts/min μg PGE$_1$ activity) was approximately equal to that of the original sample (1301 to 1311 counts/min μg PGE$_1$ activity).

(2) Portions containing ^{3}H-PGE$_1$ from outside and from inside dialysis bags containing plasma solutions, were extracted after equilibrium dialysis (18–24 h at 37°). Seventy to 96% of the biological activity and 75 to 98% of the radioactivity (6 samples) were recovered in the extracts and their activity-ratio was approximately the same as that in the original sample of PG. These experiments exclude the possibility of a measurable exchange of tritium label on ^{3}H-PGE$_1$ with the hydrogen of water or of a plasma constituent, and indicate little destruction of biological activity of PGE$_1$ during the incubation with plasma and subsequent extraction. They do not agree with the apparently rapid conversion of ^{3}H-PGE$_1$ to a less-polar metabolite by a blood plasma enzyme (McDonald-Gibson, McDonald-Gibson & Greaves, 1972). The validity of the conclusions of these authors rests largely upon the precision of thin-layer chromatographic separation of plasma extracts but this cannot be assessed from their abstract.

Interaction of ^{3}H-PGE$_1$ with purified serum albumin

Binding of PG by purified serum albumin was examined (4 experiments) using very dilute solutions of albumin (7·69 $\times$ 10^{-8} mol, i.e. 5 mg in 5 ml) and ^{3}H-PGE$_1$ diluted with unlabelled PGE$_1$ (5·45 and 27·3 $\times$ 10^5 d/min per mg). This produced a high initial molar ratio of PG to albumin which enabled an approximation of the binding properties to be made. The albumin preparation contained between 2 and 3 mol of unesterified fatty acid per mol albumin so that experiments were necessary to see whether this affected the affinity of the albumin for PG. Parallel equilibration studies (3 experiments) were therefore made using albumin which had been 'defatted' with charcoal (Chen, 1967) and contained less than 0·3 mol unesterified fatty acid per mol albumin.

The percent of PG bound to albumin in each dialysis bag was computed from the difference of the quench-corrected counts inside and outside the bag. The molar

ratio of bound PG to albumin (r) and the free, unbound PG concentration (C = mol PG per litre) were calculated (Table 2). A plot of r/C against r according to the expression r/c = Kn–Kr (Klotz, 1953) enables a graphic estimation of the maximum association constant (vertical intercept = Kn where K is the association constant and n is the number of binding sites on each albumin molecule), and the number of binding sites (n; horizontal intercept). As shown in Fig. 2, the maximum association (Kn)

Table 2. *Binding of ^{3}H-PGE$_1$ to purified human serum albumin and to 'defatted' albumin.* ^{3}H-PGE$_1$ (4·8 to 242 μg = 1·4 to 68·5 × 10^{-8} mol) was dialysed against electrophoretically homogeneous albumin (5 μg/5 ml = 7·69 × 10^{-8}mol total) and against the same amount of 'defatted 'albumin. 'PG % bound' is given separately where ^{3}H-PGE$_1$ was added to (A) the inside and (B) the outside compartment. r is the mol PG bound per mol of albumin used (average of pair) and C is the molar concentration of unbound ^{3}H-PGE$_1$ per litre (average of pair).

PG (Mol × 10^8)	Specific activity d/min/mg PG × 10^{-5}	PG % bound A	PG % bound B	r	C PG (Mol/litre) × 10^6	r/C × 10^{-4}
(albumin)						
68·5	5·45	14	13	1·2	59·4	2·0
51·4	5·45	18	16	1·1	42·4	2·6
34·3	5·45	19	18	0·80	28·2	2·8
17·1	5·45	20	20	0·45	13·8	3·2
6·85	27·3	25	26	0·23	5·1	4·5
3·43	27·3	28	27	0·12	2·5	4·8
1·37	27·3	29	31	0·05	0·96	5·7
'defatted' albumin)						
68·5	5·45	18	17	1·5	56·4	2·7
51·4	5·45	20	21	1·3	41·0	3·2
34·3	5·45	25	23	1·0	26·1	3·9
17·1	5·45	28	27	0·58	12·4	4·7
6·85	27·3	30	31	0·26	4·8	5·4
3·43	27·3	33	32	0·14	2·3	6·0
1·37	27·3	36	34	0·06	0·94	6·7

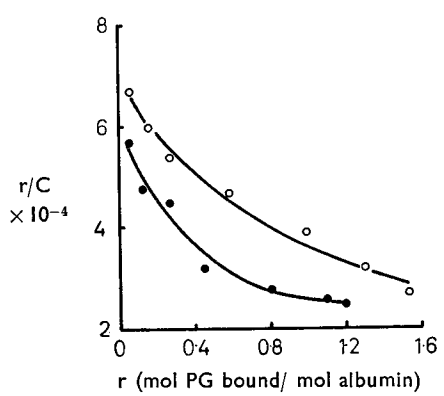

FIG. 2. Binding of ^{3}H-PGE$_1$ by purified human serum albumin and by "defatted" human serum albumin. ^{3}H-PGE$_1$ (1·4 to 68·5 × 10^{-8} mol) was dialysed against electrophoretically homogeneous albumin (●—●; 5 mg (7·69 × 10^{-8} mol) in 5 ml) and against "defatted" albumin (○—○, 5 mg in 5 ml). r is mol PG bound per mol albumin. Each point represents the average of the paired controls. The vertical intercept equals 'Kn', the product of the association constant and the number of binding sites on albumin.

is about 70 000 for defatted albumin and 61 000 for the untreated albumin. Neither curve is linear, however, indicating some heterogeneity of binding. Either curve (Fig. 2) might be resolved into two components—one corresponding to binding of PG to 1 or 2 primary binding sites per albumin molecule (K = 70 000 or 35 000 respectively) and the other to binding of PG to sites whose number and association constant cannot be assessed from the present data. In any case the primary association constant of PGE_1 with serum albumin does not appear to exceed 70 000. This was confirmed in other equilibrium experiments using lower initial molar proportions of ^{3}H-PGE_1 to albumin ($4{\cdot}2 \times 10^{-3}$ to 4·75) and indicates a rather weak interaction.

Nevertheless, although the binding affinity of albumin for PG is weaker than for related free fatty acids (see Discussion) more than 99% of any PG circulating in blood, would be expected to be associated with albumin (up to levels of 500 μg/ml).

The interaction of PG with albumin thus appears to be the likely cause of the poor recoveries from blood in procedures where albumin is first precipitated. This was substantiated by ethanol extractions (method of Samuelsson, 1963) of PGE_1 and E_2 (100 ng per g protein) added to solutions of freeze-dried human serum albumin in which the recoveries were only 0–14%. ^{3}H-PGE_1 was likewise bound to albumin precipitated from 0·15M phosphate buffer (pH 7·3) containing 70% ethanol. Methods of preventing the PG-albumin interaction were therefore explored, and the most effective procedure is described below.

Prevention of prostaglandin-albumin interaction

Binding of ^{3}H-PGE_1 to albumin was much reduced by the prior acidification of the albumin solution with formic acid (1% v/v; pH approximately 3) or by the addition of non-precipitating amounts of ethanol (5–15% v/v) to the aqueous solution (4 experiments). Binding of ^{3}H-PGE_1 was entirely absent with albumin in 40% v/v ethanol containing 1% v/v formic acid (^{3}H-PGE_1 0·015 to 1·0 μg; albumin $2{\cdot}4 \times 10^{-7}$ mol in 5 ml; Fig. 3). Although other proteins were precipitated, albumin (from 0·5 to 4·0% tested) remains soluble in acidified 40–50% ethanol for several hours at room temperature, and the now completely unbound PG may be extracted quantitatively into chloroform. ^{3}H-PGE_1 and ^{3}H-PGA_1 was extracted in this way with 94–98% recovery of the radioactivity added to human plasma (Table 3). The recovery was confirmed by chromatography on silicic acid (Samuelsson, 1963; Mallinkrodt Silic Ar-CC-4, 100–200 mesh, eluting with 30–100% ethyl acetate in benzene) followed by chromatography on silica gel thin-layer plates (A1 solvent, Gréen & Samuelsson, 1964) on which the extracted PGs exhibited the same R_F values as the respective standards. The extraction of $PGF_{2\alpha}$ was also confirmed by chromatography (Table 3).

These results form the basis of the following method (tested in 52 experiments) of extracting PGE, F and A compounds added to human blood and for measuring the concentration of circulating prostaglandin-like activity. Plasma in one volume of physiological saline and two volumes of ethanol is extracted with 40–60° light petroleum. The PG compounds are then extracted from the aqueous phase into chloroform after acidification to pH 3·0 to 3·5 with formic acid (Unger & others, 1971).

DISCUSSION

Since PGs do not interact with blood cells, the extraction and estimation can be made using plasma, thus obviating the handling of large quantities of cellular material.

Table 3. *Recovery of PGs (a) after extraction from human blood plasma and (b) after further purification on silicic acid columns.* "Recovery in extract" is the % of the total PG added to plasma. "Recovery from column" is the % of extracted PG loaded on to the column.

PG	Amount added		Recovery in extract (%)		Recovery from column (%)	
	μg/ml	Total	Radio-activity	Biological activity	Radio-activity	Biological activity
^{3}H-PGE$_1$ ..	0·75	15·0	94	—	90	91
	0·27	2·7	96	—	102	70
	0·27	2·7	98	—	101	95
	6·0	60·0	98	—	92	—
^{3}H-PGA$_1$ ..	0·063	0·63	98	—	98	—
	0·063	0·63	98	—	97	—
PGF$_{2\alpha}$ ..	1·43	14·3	—	95	—	103
	1·43	14·3	—	95	—	97
	1·43	14·3	—	94	—	86

The absence of binding of PG by human gamma globulin should facilitate the immunoassay of PGs, since there will be no need to differentiate non-specific binding of PG to gamma globulin from the specific interaction with antibody.

PGs bind to serum albumin more weakly than saturated and other unsaturated fatty acids of various chain lengths. Using two-phase equilibration of C_{14}–C_{18} fatty acids between aqueous albumin and n-heptane, Goodman (1958) found the protein has at least three classes of binding sites. The first could accommodate 2 mol of ligand ($K_1 = 1 \times 10^8$ for oleate), the second 5 mol ($K_2 = 4 \times 10^6$), and the third about 20 mol ($K_3 = 1 \times 10^3$). Spector, Kathryn & Fletcher (1969) obtained similar results with charcoal-treated albumin.

In the present experiments, the association of PG with albumin (K = 70 000) and the molar ratio of bound ^{3}H-PGE$_1$ to albumin (r not more than 2) were considerably less than reported for other fatty acids, Although higher r values (and therefore a

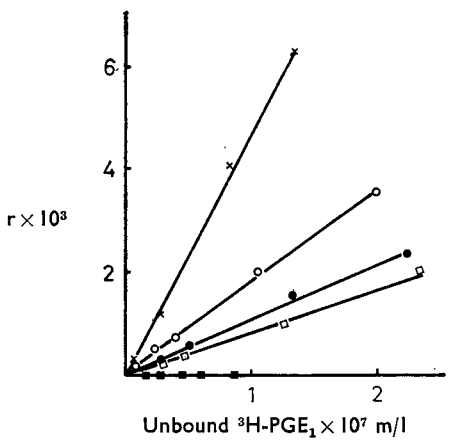

FIG. 3. Inhibition of binding of ^{3}H-PGE$_1$ (0·042 to 2·8 × 10^{-9} mol; 0·015 to 1·0 μg), to serum albumin (2·4 × 10^{-7} mol) by ethanol and formic acid. The ratio r of mol of bound PG per mol of albumin (vertical axis) plotted against the concentration of unbound PG (mol/litre, horizontal axis) after equilibration, is reduced with either ethanol or formic acid alone and is zero with both formic acid and 40% ethanol: ×, albumin; ○ albumin + 15% ethanol; ●, albumin + 1% formic acid; □, albumen + 15% ethanol and 1% formic acid; ■, albumin + 40% ethanol and 1% formic acid (■ taken from a different experiment in which 0·1–0·3 μg ^{3}H-PGE$_1$ was used).

greater number of theoretical binding sites) could probably have been demonstrated by using higher ratios of PG to albumin (see Fig. 2) the maximum association constant for the PG albumin interaction would not be affected and any secondary binding sites would have an extremely low affinity for PG,

This weaker affinity of albumin for PG than for similar unsaturated fatty acids (Goodman, 1958) probably results in part from the greater hydrophilic nature of the PG molecule and from its more compact shape. The present results do not permit definition of the physicochemical nature of the PG/albumin association with certainty. However, the primary binding energy is most likely hydrophobic; ethanol (5 to 40% v/v) or non-precipitating amounts of methanol (5 to 15%) reduced both the association constant and the bound molar ratio of PG to albumin,

The reason why PG is lost during an ethanol precipitation stage of extraction from blood may be that the PG is not completely disassociated from the albumin and becomes physically trapped in the precipitate. Acidification causes an increase in the molecular volume of albumin (Yang, 1961) leading, in the presence of aqueous ethanol, to an effective perturbation or displacement of associated PG. Thus, the partition between chloroform and an acidified aqueous ethanolic solution of plasma, in which albumin is not initially precipitated, allows quantitative extraction of PGs from plasma.

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