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CHARACTERIZATION AND LOCALIZATION OF PROSTAGLANDIN E_1 RECEPTORS IN RAT LIVER PLASMA MEMBRANES

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SUMMARY

Methodology for measurement and characterization of prostaglandin binding to membranes has been developed. The binding assay was used to study the presence of prostaglandin receptors in highly purified cell fractions derived from rat liver. High affinity binding receptors which have a saturation value of 1.0 pmole/mg protein and a dissociation constant of 1.2 nM were found exclusively in the plasma membrane. High affinity receptors were not found in cell fractions containing nuclei, rough microsomes, Golgi complex or mitochondria. The binding by other prostaglandins was competitive with prostaglandin E_1. Competitive binding studies were used to obtain dissociation constants for prostaglandins $F_{1\alpha}$, $F_{2\alpha}$, B_1, B_2, A_1, A_2, and 15-keto prostaglandin E_2 which were 1100, 100, 300, 180, 16, 16 and 700 nM, respectively. Eicosa-5,8,11,19-tetraynoic acid, an inhibitor of prostaglandin synthesis did not bind appreciably to the prostaglandin E receptor, whereas two prostaglandin analogues which have high physiological activity compete effectively with prostaglandin E_1 for the receptor. Thus, the binding receptor for the E-type prostaglandins is highly specific both with respect to cell localization as well as the type of substrate. Numerical routines for the fitting of the data and a procedure for the determination of the specific activity of the labelled prostaglandin are provided.

INTRODUCTION

Prostaglandins have been implicated in the regulation of a wide variety of physiologically important activities including the tone of blood vessels [1,2], lipolysis [3], the secretion of gastric acid [4] and reproduction [5]. In animals and man the breakdown of prostaglandins is extremely rapid [6] and the basal levels found in tissues are often less than 1 μg/g [7]. When ^{3}H-labelled prostaglandin E_1 is injected into a mouse, it is rapidly removed from circulation and the label is primarily found in liver, kidney and uterus [8,9]. The accumulation of prostaglandin E_1 in these organs suggests that they possess high affinity binding sites to sequester the prostaglandin from the very low concentrations in the serum.

Prostaglandins are known to raise the level of cyclic AMP in many cells and tissues including platelets [10], ovary [11], lung [12] and spleen [12]. Prostaglandins

Abbreviations: HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethane-sulfonic acid; PIPES, piperazine-*N*-*N'*-bis(2-ethane-sulphonic acid).

are also known to antagonize the epinephrine stimulated elevation of cyclic AMP levels in adipocytes [12,13]. Stimulation of adenyl cyclase activity by prostaglandins has been reported in membranes isolated from diverse sources including platelets [14], thyroid [15], ovary [16] and heart [17].

Possible sites of action of prostaglandins are on the cell's internal and plasma membranes. Plasma membrane sites might be expected to mediate the uptake of prostaglandins from the blood and to be involved with prostaglandin stimulation of adenyl cyclase, an enzyme which is thought to be associated with the plasma membrane [18]. Although binding of various prostaglandins to membrane preparations from rat epididymal fat pads [19] and from rat forestomach [20] have been reported, no systematic study on the binding characteristics of subcellular membranous organelles has been reported. We investigated the binding of ^{3}H-labelled prostaglandin E_1 to plasma membranes, rough and smooth endoplasmic reticulum, Golgi apparatus, mitochondria and nuclei derived from rat liver. High affinity binding sites were found only in the plasma membrane fraction. The binding sites were shown to have high specificity for E type prostaglandins. A preliminary report has been presented [21].

MATERIALS AND METHODS

PIPES (Piperazine-*N*-*N*'-bis[2-ethane-sulfonic acid]) and HEPES (*N*-2-hydroxyethylpiperazine-*N*'-2-ethane-sulfonic acid) were obtained from Sigma Chemical Co. (St. Louis, Mo.) and Calbiochem. (San Diego, Calif.), respectively. Non-radioactive prostaglandins were generously donated by the Upjohn Co. (Kalamazoo, Mich.). ^{3}H-Labelled prostaglandin E_1, (50 Ci/mmole) was supplied by New England Nuclear (Boston, Mass.). The methyl esters of prostaglandin E_1 and ^{3}H-labelled prostaglandin E_1 were prepared by addition of excess diazomethane to methanolic solutions of the respective acids [22]. The reaction was carried out at room temperature for 10 min and was followed by evaporation of volatile components under a stream of nitrogen. Stock solutions of all prostaglandins were stored at 0 °C in ethanolic solutions. Crystalline bovine serum albumin was obtained from Armour Pharmaceutical Co. (Chicago, Ill.) and sucrose, special enzyme grade was from Schwartz–Mann. All other reagents were reagent grade.

Protein was estimated by the method of Lowry et al. [23] using bovine serum albumin as a standard. Phosphorus was determined by a modification of the method of Chen et al. [24,25]. Scintillation counting was performed in 4.5 ml of a cocktail consisting of 1 l toluene + 5 g PPO + 0.5 g POPOP + 333 ml Triton X-100. Counting efficiency for ^{3}H was 38–40%.

Rotenone insensitive NADH cytochrome *c* reductase, succinate cytochrome *c* reductase and Mg^{2+}-stimulated ATPase were performed as previously described [26].

Large scale preparation of rat liver plasma membrane

The livers from 50 decapitated rats (Holtzman strain, Sprague–Dawley, approx. 250 g males, fed ad libitum) were collected in ice-cold 0.25 M sucrose. They were blotted dry, weighed (approx. 500 g) and 4 vol. of 0.25 M sucrose–10 mM HEPES buffer (pH 7.5) was added. A homogenate was prepared using a 50 ml Potter–Elvehjem type homogenizer with the pestle rotating at 1200 rev./min. Two to three up–down strokes were made first using a Teflon pestle of 0.974 inch diameter and then a pestle

of 0.988 inch diameter in a glass homogenizer of 1.000 inch inner diameter. The homogenate was filtered through 110-mesh Nitex bolting cloth, diluted to a total volume of 2500 ml with sucrose–HEPES buffer and centrifuged at 4000 rev./min for 15 min in two JA-14 rotors in Beckman J-21 centrifuges cooled to 2 °C. The supernatants were decanted and the pellets were combined and resuspended with 62.5% sucrose–10 mM HEPES–1 mM $MgCl_2$ pH 7.5 using a Dounce homogenizer with a loose pestle (Type A). The sucrose concentration was adjusted, using a Bausch and Lomb refractometer, to 47 ±0.1% sucrose by addition of sucrose–HEPES–$MgCl_2$ buffer. The final volume was approx. 900 ml. Two Spinco Ti 15 zonal rotors were each loaded at 4000 rev./min with 50 ml of 0.25 M sucrose–10 mM HEPES–1 mM EDTA followed by a linear gradient formed with 450 ml each of 34% sucrose–10 mM HEPES–1 mM EDTA (pH 7.5) and 43.4% sucrose–10 mM HEPES–1 mM EDTA (pH 7.5). The gradient was followed by 250 ml of 43.4% sucrose–10 mM HEPES–1 mM EDTA pH 7.5. One-half of the resuspended pellet in 450 ml of 47% sucrose was then added to each rotor followed by a 50% sucrose cushion as necessary to fill the rotor. The rotors were brought to 32000 rev./min and centrifuged for 60 min. The speed was then reduced to 4000 rev./min and 30-ml fractions were collected from the inner core by pumping 55% sucrose in from the outer edge. The plasma membrane enriched fraction, which banded broadly in the gradient between 38% and 40% sucrose, was diluted with 2 vol. of cold deionized water and was centrifuged at 7000 rev./min for 10 min in a JA-14 rotor. The upper white layers of the pellets were collected with the aid of a fine stirring rod and gentle swirling and resuspended in sucrose–HEPES–EDTA buffer with hand homogenization using a Potter–Elvehjem homogenizer with a 0.988 inch pestle. The lower brown pellet which remained packed was discarded. The resuspended material was centrifuged 7000 rev./min for 10 min in a J-14 rotor and the upper white layers resuspended in sucrose–HEPES–EDTA buffer. This 7000 rev./min wash was repeated a total of 6 times. The combined upper white layers were resuspended in a final volume of approx. 30 ml of 0.25 M sucrose, frozen and stored in liquid nitrogen. The yield of the plasma membrane fraction was 300–500 mg protein. All operations were carried out at 0–4 °C.

Early preparations were made with minor variations in the procedure using 100-g batches of liver and gradient centrifugation in a Spinco Ti 14 zonal rotor.

Other subcellular fractions derived from rat liver including Golgi complex, mitochondria, nuclei, rough and smooth microsomes were isolated as described previously [27].

Prostaglandin binding assay

To each 2-ml cellulose nitrate centrifuge tube (5/16 × 1–15/16 inches, Beckman 303369) was added 25 μl of 0.3 M NaCl–20 mM PIPES buffer, pH 6.25, 15 μl water, 1 μl ^{3}H-labelled prostaglandin E_1 (approx. 50 Ci/mmole) in ethanol containing from 0–100 pg nonradioactive prostaglandin E_1 and finally 15 μl of a membrane suspension of about 10 mg protein/ml. In the isotope dilution assay, it is vital that the membrane suspension be added last in any measurement involving competition of two substances for the binding site since bound prostaglandin does not rapidly equilibrate off the membrane. Binding assays were routinely carried out with 6 levels of prostaglandin E_1 and in duplicate. The contents of the tubes were mixed by vortexing for 5 s and incubated at 37 °C for 60 min. They were then chilled on ice, and centrifuged 10000

rev./min for 5 min in a Spinco 50 Ti rotor using plastic adapters. 40 μl of supernatant fluid was sampled for scintillation counting. Total radioactivity was determined using control tubes to which 15 μl water was substituted for the membrane suspension and incubation, centrifugation and sampling were carried out as before.

To determine the dissociation constant, K_s, and the binding capacity, E_T, the binding data is plotted by the method of Scatchard. The bound/free ratio is a directly measured quantity while the level of bound prostaglandin is obtained by multiplying the fraction of the radioactivity which is bound by the total amount of prostaglandin in the incubation mixture. The total amount of prostaglandin has an appreciable (approx. 10 pg) contribution from the ^{3}H-labelled prostaglandin E_1 used. A successive approximation method for determining K_s, E_T and the specific activity is described below.

Determination of specific activity

In assay using a significant concentration of labelled isotope knowledge of the specific activity of the isotope is necessary to obtain linear Scatchard regressions at high ratios of bound/free. We determine the specific activity of each lot of ^{3}H-labelled prostaglandin E_1 by measuring the changes in the bound/free ratio as we increase the total radioactivity in the assay medium. If S_T is the amount of prostaglandin E_1 in 1 μl of a solution of ^{3}H-labelled prostaglandin E_1, then it is easy to show that when μl_i of the ^{3}H-labelled prostaglandin E_1 solution is incubated with a constant amount of plasma membrane, characterized by a dissociation constant of K_s and a saturation binding capacity of E_T, the binding varies as:

$$\left[\left(1 + \frac{S}{ES}\right)\right]\frac{1}{\mu l_i} = \frac{S_T}{E_T} + \frac{K_s}{E_T}\left(1 + \frac{ES}{S}\right)\frac{1}{\mu l_i} \qquad (1)$$

where S and E are the concentrations of unbound prostaglandin and receptor, respectively, and S/ES is the ratio of free/bound prostaglandin. Fig. 1 shows experimental data plotted according to this equation. The slope and intercept are equal to K_s/E_T and S_T/E_T, respectively.

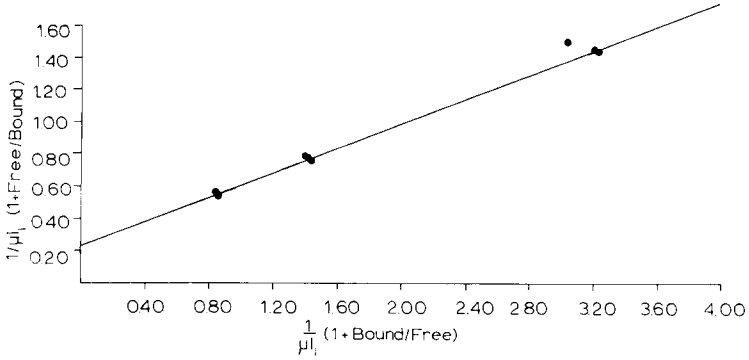


Fig. 1. Determination of specific activity of ^{3}H-labelled prostaglandin E_1 by isotope dilution. Plasma membrane, 0.24 mg protein, was incubated in a final volume 68 μl containing 0.15 M NaCl–10 mM PIPES pH 6.5 and either 1 μl ^{3}H-labelled prostaglandin E_1 in ethanol + 2 μl ethanol, 2 μl ^{3}H-labelled prostaglandin E_1 in ethanol + 1 μl ethanol or 3 μl ^{3}H-labelled prostaglandin E_1 in ethanol. Bound prostaglandin E_1 was determined by sedimenting the membranes and counting an aliquot of the supernatant.

An iterative procedure can be used to determine S_T in which one assumes a value for the specific activity of the isotope and uses this value in determining E_T and K_s from a Scatchard plot of a set of binding data. These numbers are used to compute S_T from Eqn 1 and this value is used to recompute the binding parameters. This procedure usually converges in 2–3 passes. It is our experience that this calibration of the specific activity of the highly radioactive commercial ^{3}H-labelled prostaglandin E_1 is required for careful binding studies.

RESULTS

The plasma membrane fraction produced by the zonal procedure is at least 80% pure as judged from the enzymic activities indicative of mitochondrial and microsomal contamination (Table I).

TABLE I

CHARACTERIZATION OF PLASMA MEMBRANES PREPARED BY LARGE SCALE PREPARATION

The enzymic activities are expressed as μmoles/min per mg protein measured at 32 °C. NADH Cytochrome *c* reductase was assayed in the presence of rotenone as an index of contamination by endoplasmic reticulum. The specific activity of rat liver smooth microsomes is approx. 2.0 [27]. The ratio of the measured specific activity in the cell fraction divided by 2.0 multiplied by 100 will give an estimate of the percentage contamination with smooth microsomes. This will be a maximal value, since mitochondrial contamination will also contribute in part to this activity. Purified mitochondria have a specific activity of approx. 0.14 (Fleischer, S. and Kervina, M., unpublished) which is largely referable to activity localized in the outer mitochondrial membrane [28]. Mitochondrial contamination may be estimated from the succinate cytochrome *c* reductase activity. Purified rat liver mitochondria have a rate of approximately 0.37. However, the electron microscopy of the cell fraction reveals that the mitochondrial activity in the plasma membrane fraction must be largely referable to vesicles rather than to intact mitochondria. Only 35% of the protein of rat liver mitochondria is referable to submitochondrial vesicles; the remainder is soluble upon disruption of the mitochondria (Fleischer, S., unpublished). Therefore, 1.05 rather than 0.37 should be used as the specific activity of succinate cytochrome *c* reductase to estimate the contamination by rat liver submitochondrial vesicles. K_s and E_T refer to the dissociation constant and the saturation value for prostaglandin E_1 high affinity receptors, respectively, as determined by Scatchard plot analysis (cf. Fig. 4).

Expt No.	Bound phosphorus (μg P/mg protein)	Yield (mg/g)	Enzymic activity			K_s (pM)	E_T (pmoles/mg protein)
			ATPase	NADH cytochrome *c* reductase	Succinate cytochrome *c* reductase		
1		0.78	1.04	0.283	0.046	1.2	1.0
2	21.5	1.1	1.08	0.261	0.065	2.2	1.2
3	26.3		1.46	0.161	0.069	2.2	0.78
4		0.59	0.75	0.223	0.072	1.0	0.78

The binding assay is normally carried out in 0.15 M NaCl buffered with 10 mM PIPES at pH 6.25 at 37 °C. Similar binding is also observed in physiological salt solutions while some decrease in binding is found in 0.25 M sucrose.

The time course of binding is shown in Fig. 2. At 70 min after the addition of

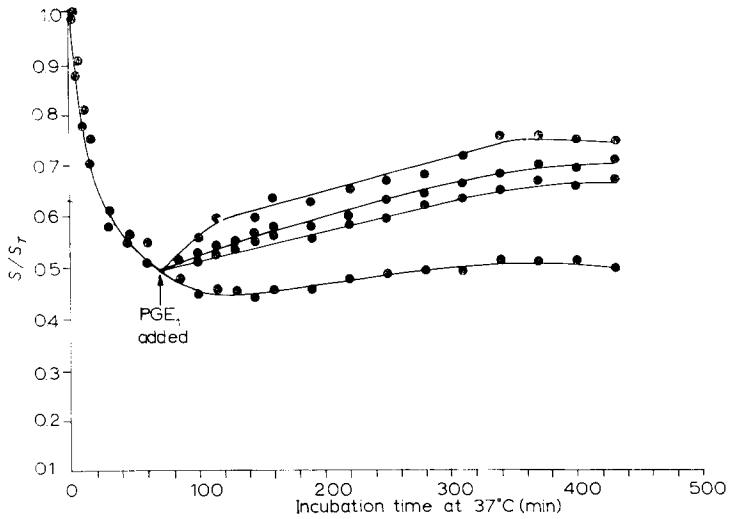


Fig. 2. Uptake and release kinetics of prostaglandin E_1 binding. Plasma membrane, 2.1 mg/ml, was incubated with ^{3}H-labelled prostaglandin E_1, 180 pg/ml in 0.15 M NaCl–10 mM PIPES pH 6.5 at 37 °C. At the indicated times, 65-μl samples were taken, centrifuged and the supernatants counted to determine free ^{3}H-labelled prostaglandin E_1 as in Materials and Methods. After 70 min nonradioactive prostaglandin E_1 (PGE_1) was added to identical samples prepared as above and an equal volume of ethanol was added to the control tube. The final concentrations of nonradioactive prostaglandin E_1 added were, from bottom curve to top, 0, 0.38 ng/ml, 1.17 ng/ml and 3.60 ng/ml. Aliquots, 65 μl, from each tube were taken after different time intervals, centrifuged and analyzed (cf. Materials and Methods). Control tubes, kept on ice, were used to determine the total radioactivity. The ratio S/S_T, is the fraction of the total prostaglandin which is free in solution.

radioactive prostaglandin E_1 to the plasma membrane suspension various amounts of nonradioactive prostaglandin E_1 were added. By 5 h after the addition of unlabelled prostaglandin E_1 the binding of radioactive prostaglandin had re-equilibrated to final values which reflect the total amount of prostaglandin E_1 in the incubation mixture. The small increase in free ^{3}H-labelled prostaglandin E_1 found after several hours of incubation with no addition of unlabelled prostaglandin E_1 may represent equilibration of a small amount of prostaglandin E_1 which is on the membrane as isolated with the added radioactivity. If the binding assay is carried out at 2 °C, the reaction is quite slow with a halftime of about 72 h (data not shown).

To gain a quantitative understanding of prostaglandin E_1 to rat liver plasma membranes, binding studies were performed with varying levels of unlabelled prostaglandin E_1 added to a constant amount of radioactive prostaglandin E_1. Figs 3 and 4 present this data plotted by the method of Scatchard. In Fig. 3 can be seen evidence indicating binding sites of high and medium affinity, while Fig. 4 shows evidence for medium and very low affinity binding sites. In this experiment the high affinity site had a dissociation constant of $1.04 \cdot 10^{-9}$ M and saturation value of 0.56 pmoles/mg protein while the medium affinity site had an apparent dissociation constant of $25 \cdot 10^{-9}$ M and a saturation value of 2.9 pmoles/mg. The variation in K_s and E_T values measured on a number of different plasma membrane preparations is shown in Table I.

It is estimated that the protein content of liver is about 120 mg/g wet weight and that plasma membrane accounts for about 2.5% of the cellular protein [29]. Assuming

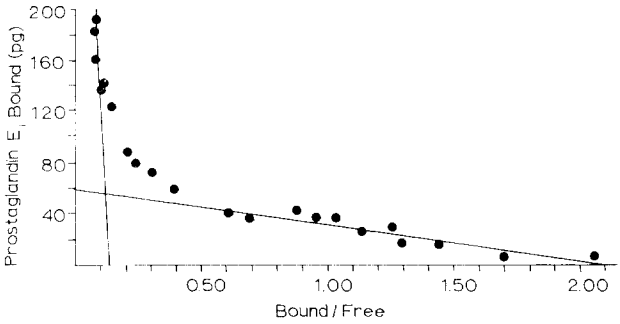


Fig. 3. High and medium affinity prostaglandin E_1 binding sites. Plasma membrane, 0.3 mg protein, was incubated for 90 min at 37 °C with 75 μl 0.15 M NaCl–10 mM PIPES (pH 6.5) containing 6 pg ^{3}H-labelled prostaglandin E_1, and 0–2500 pg PGE_1. Membranes were sedimented, the supernatants sampled and radioactivity determined as described in Materials and Methods.

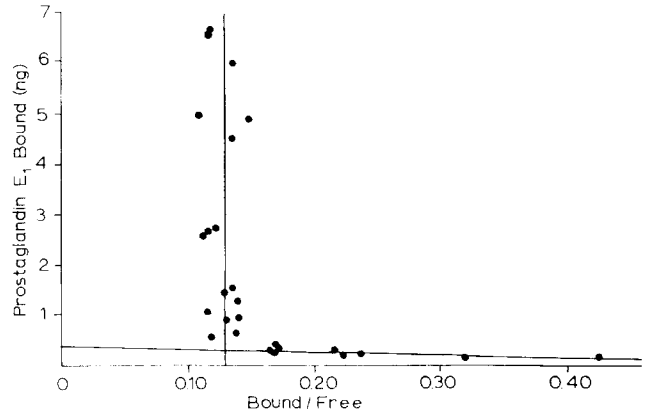


Fig. 4. Medium and low affinity prostaglandin E_1 binding sites. Plasma membrane, 0.38 mg proteins, was incubated for 90 min at 37 °C with 75 μl 0.15 M NaCl–10 mM PIPES pH 6.5 containing 6 pg ^{3}H-labelled prostaglandin E_1 and 500–62500 pg PGE_1. Membranes were sedimented, the supernatants sampled and radioactivity determined as described in Materials and Methods.

a value of $169 \cdot 10^6$ cells/g [30] implies that there are about 6000–12000 high affinity prostaglandin E receptors per liver cell. Liver preparations of rough endoplasmic reticulum, smooth endoplasmic reticulum, Golgi apparatus, mitochondria and nuclei show no evidence of high affinity binding sites, although nonspecific binding is found in varying amounts in the various preparations (see Fig. 5). The slight sloping towards the plasma membrane's line seen in the Scatchard plot of the Golgi membrane is consistent with the 10% contamination of this fraction by plasma membrane.

The binding of the methyl ester of prostaglandin E_1 was measured as for the free acid. Scatchard analysis of the binding of ^{3}H-labelled methylester of prostaglandin E_1 in the presence of varying levels of the unlabelled methyl ester, shows a dissociation constant of $1.3 \cdot 10^{-9}$ M and a saturation value of 0.63 pmole/mg protein for the high affinity binding site in striking similarity to the binding data obtained with free acid.

The pH dependence for the binding of prostaglandin E_1 and the methyl ester of prostaglandin E_1 to the plasma membranes is shown in Fig. 7. The binding is pH

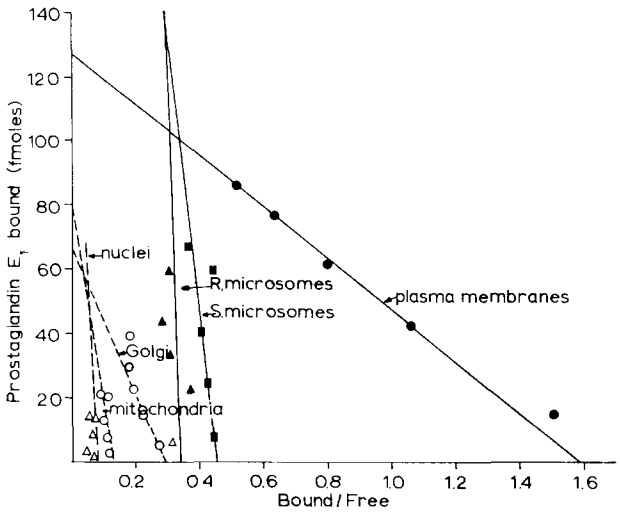
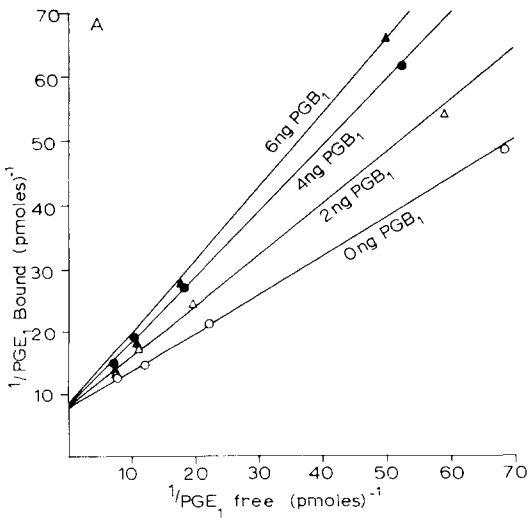


Fig. 5. Binding of prostaglandin E_1 to rat liver cell fractions. Each cell fraction, 0.15 mg protein, was added to a total volume of 55.8 μl of 0.15 M NaCl–10 mM PIPES pH 6.5 containing 1200 cpm (15 pg) ^{3}H-labelled prostaglandin E_1 and assayed for prostaglandin E_1 binding as described in Materials and Methods.

dependent and decreases monotonically from pH 6.0 to 8.5; the figure shows the binding only to 7.5. The binding of prostaglandin E_1 and its methyl ester without added carrier (upper two curves) and in the presence of added carrier (lower two curves) is referable mainly to high and medium affinity binding, respectively. The binding of the methyl ester of prostaglandin E_1 to the membrane is similar to that of the prostaglandin E_1 for both the high and medium affinity binding. Since the methyl ester is devoid of an ionizable group it would appear that the effect of pH on the



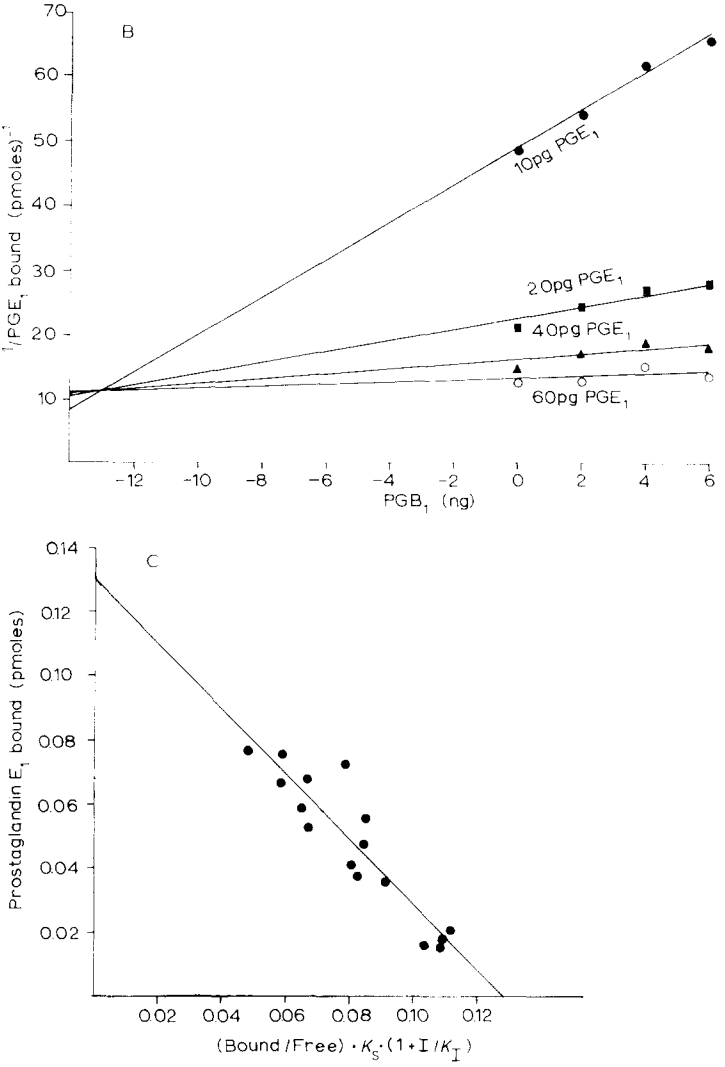


Fig. 6. Competitive binding of prostaglandin E_1 (PGE_1) and prostaglandin B_1 (PGB_1) to rat liver plasma membrane. Plasma membrane, 0.15 mg protein, was incubated with the combinations of 10, 20, 40, 60 pg PGE_1 and 0, 2, 4, 6 ng PGB_1 in a volume of 55.2 μl. Binding was measured as described in Materials and Methods. A. Lineweaver–Burke plot. A dissociation constant for prostaglandin B_1 of $305 \cdot 10^{-9}$ M was obtained. B. Dixon plot. A dissociation constant for prostaglandin B_1 of $665 \cdot 10^{-9}$ was obtained. C. Least squares fit of competition data to Eqn 1 of Appendix. A dissociation constant for prostaglandin B_1 of $280 \cdot 10^{-9}$ M was obtained. The dissociation constant for prostaglandin E_1 binding to plasma membrane was found to be $1.3 \cdot 10^{-9}$ M. The number of high affinity binding sites was found to be 0.92 pmoles/mg protein of plasma membrane.

binding of prostaglandin E_1 to the membrane is referable to the effect of pH on the membrane.

Addition of several different unlabelled prostaglandins decreases the binding of ^{3}H-labelled prostaglandins E_1 to differing degrees. Analysis of the inhibition

shows that it is of a simple competitive nature (Fig. 6A) and dissociation constants for various prostaglandins were obtained from least square fits of the competitive binding equation (Eqn 1 of Appendix) to data obtained at four substrate levels and at least three inhibitor levels, cf. Appendix. A representative plot of the converged least squares fit is shown in Fig. 6C. Results are summarized in Table II. A major metabolite of prostaglandin E_2, 15-keto prostaglandin E_2 binds about 800 times more weakly than E-type prostaglandin.

TABLE II

PROSTAGLANDIN BINDING BY RAT LIVER PLASMA MEMBRANE

Prostaglandin (Type)	Dissociation constant (nM)	
E_1	1.2 ± 0.2*	
Methyl ester of prostaglandin E_1	1.3	
E_2	1.5	
$F_{1\alpha}$	1250	1000
$F_{2\alpha}$	---	96
B_1	342	279
B_2	200	137
A_1	16	
A_2	16	
15-Keto E_2	840	554

* The value for prostaglandin E_1 is the mean of 5 determinations performed on each of 2 plasma membrane preparations and is ± 1 standard deviation.

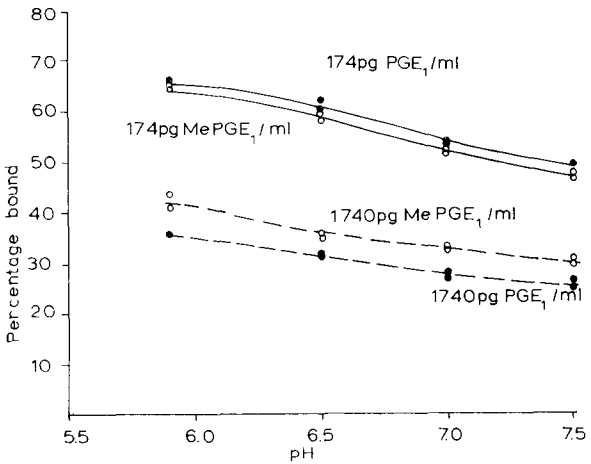


Fig. 7. Variation of the binding of prostaglandin E_1 (PGE_1) and the methyl ester of prostaglandin E_1 ($MePGE_1$) with pH. Plasma membrane, 0.3 mg, was incubated in 60 μl of an aqueous solution which contained 0.15 M NaCl–10 mM PIPES at the indicated pH values and 2 μl ethanol containing 1600 cpm of either; (1) 10.4 pg of ^{3}H-labelled prostaglandin E_1; (2) 110.4 pg of ^{3}H-labelled prostaglandin E_1; (3) 10.4 pg of the methyl ester of ^{3}H-labelled prostaglandin E_1; or (4) 110.4 pg of the methyl ester of prostaglandin E_1. Samples were incubated at 37 °C for 90 min and then centrifuged and sampled for scintillation counting as described in Materials and Methods.

Various prostaglandin derivatives are known to possess high physiological activity [31,32]. In binding assays carried out at 250 pg/ml ^{3}H-labelled prostaglandin E_1, 1.7 ng/ml of 16,16-dimethyl prostaglandin E_2, or 15-*S*,15-methyl prostaglandin E_2 competed as well as 1.7 ng/ml of prostaglandin E_1 for the binding sites. That is to say, these analogues bind approximately as effectively as the natural prostaglandin E. Under similar conditions 1 μg/ml of arachidonic acid, a precursor of prostaglandin E_2, did not detectably inhibit binding. One of the 7-oxaprostaglandins, prostaglandin EC-I-148 is known to antagonize prostaglandin stimulation of smooth muscle [33]. It did not influence the binding of 250 pg/ml prostaglandin E_1 at levels up to 17 μg/ml of the antagonist. Under similar conditions neither 2 μg/ml of eicosa-5,8,11,19-tetraynoic acid, an inhibitor of prostaglandin synthesis [34] nor 2 μg/ml of SC19220, an inhibitor of prostaglandin induced smooth muscle contraction [35] detectably inhibited the binding of the radioactive PGE_1 to the plasma membrane's high affinity binding sites.

To check on possible metabolic modification of the added prostaglandin E_1 ^{3}H-labelled prostaglandin E_1 was bound to plasma membranes for 90 min as described in Materials and Methods. The membranes were sedimented. The pellets were adjusted to pH 3 with HCl and extracted 3 times with 20 vol. of chloroform. The chloroform extracts were combined, evaporated to dryness, and the extract was redissolved in ethyl acetate and spotted on a thin-layer chromatography plate. Development with the AII system of Green and Samuelsson [36] showed only one radioactive band which co-chromatographed with authentic prostaglandin E_1. This indicates that the prostaglandin, even though bound to plasma membrane, is not metabolized under the conditions of the assay.

DISCUSSION

An isotope dilution procedure is described for the measurement of prostaglandin binding receptors on plasma membranes. The number of receptors as well as the affinity of binding (dissociation constant) can be obtained using Scatchard plot analysis.

The Scatchard analysis assumes that the measurement is made at equilibrium. To test this assumption, the time course study in Fig. 2 was performed. The addition of unlabelled prostaglandin E_1 at 70 min caused displacement of the previously bound ^{3}H-labelled prostaglandin E_1 with apparent re-equilibration by 6 h after the "chase". Quantitative treatment of the forward and reverse kinetics is hampered by the presence of both the high and medium affinity sites. In this case, the initial rates of binding and release cannot be simply related to the forward and reverse rate constants. Our data show no evidence of an "irreversible" binding of the prostaglandin to the receptor, rather, after the addition of unlabelled prostaglandin E_1 to the membranes containing previously bound ^{3}H-labeled prostaglandin E_1, the radioactive prostaglandin re-equilibrated to a level which was determined by the total prostaglandin E_1 concentration in the incubation mixture and the binding parameters of the membrane. The graded nature of the equilibrium levels argues against release of the bound prostaglandin being caused by degradative metabolism of the prostaglandin on the binding site of the membrane.

In enzyme kinetic analysis, the assumption is made that only an insignificant fraction of the substrate is bound to the enzyme so that the amount of free substrate

plus the amount of substrate converted to product is essentially equal to the amount of substrate added. In the binding assay the amount of substrate added is adjusted so that a significant portion is bound; hence the level of free substrate does not equal the amount added. This leads to differences in the analysis of competitive inhibition of binding and competitive inhibition of enzymic rates. For instance, in enzyme kinetics one may analyze inhibition studies in two equivalent fashions. In one, the inverse of the rate is plotted against the inverse of the substrate concentration (Lineweaver–Burke plot) for several values of added inhibitor. Alternatively, one may plot the inverse of the rate against the inhibitor concentration (Dixon plot) for various, fixed levels of substrate. Since the concentration of free substrate does not depend on the addition of the inhibitor, the methods give equally good values for the substrate and inhibition constants. In competitive binding studies as increasing amounts of inhibitor are added, the concentration of free substrate changes appreciably (or else there is no measurement) so that Dixon plots cannot be validly drawn. In the case of a weakly bound inhibitor one may validly use a Lineweaver–Burke double reciprocal analysis since the inhibitor does not appreciably change concentration as the extent of substrate binding varies. However, in the case of a substrate and inhibitor of similarly high affinities as found with prostaglandin E_1 versus prostaglandin E_2, a less direct analysis must be used, see Appendix. Large errors in the determination of the inhibition constants may be made if the analysis is not correctly pursued. As seen in Fig. 6, the Dixon plot overestimates the dissociation constant for prostaglandin B_2 by approximately a factor of 2.

Direct demonstration of E-type prostaglandin binding sites has been recently reported by Kuehl and Humes [19] in a membrane fraction from rat adipocytes. Rat liver plasma membrane shows about three times the binding affinity and about twice the total number of binding sites per mg protein. This similarity is notable in that the fat cell membranes were not purified plasma membranes and are derived from a different tissue. This is reminiscent of the similarity in insulin receptors found in liver and fat cell membranes as shown by Cuatrecasas [37]. While the fat cell preparation showed about equal affinities for prostaglandin $F_{1\alpha}$ and prostaglandin $F_{2\alpha}$ the liver membrane preparation binds prostaglandin $F_{2\alpha}$ about 10 times better than it binds prostaglandin F_1r.

The excellent binding of the methyl ester of prostaglandin E_1 demonstrates that the ionization of the prostaglandin's carboxyl group is not needed for the binding to the membrane site. The possibility that the prostaglandin's pK is shifted to higher pH by the negative surface change given to the plasma membrane by its sialic acid residues was raised by the reduction of binding seen with increasing pH. To test this possibility a comparison of the pH dependence of prostaglandin E_1 binding and that of its methyl ester was performed. As can be seen in Fig. 7, both the binding of prostaglandin E_1 and its methyl ester show a similar decrease with increasing pH. This implies that the pH dependence observed must reflect changes in the affinity of the binding site rather than changes in the charge of the substrate.

The isotope dilution procedure for measuring prostaglandin receptors can also be exploited as a sensitive, specific assay for E-type prostaglandins. The procedure consists of preparing a lipid extract containing prostaglandin which can, if necessary, be purified by LH-20 chromatography (Frolich, J., personal communication). Aliquots of the unknown sample are incubated with ^{3}H-labelled prostaglandin E_1 and rat liver

plasma membranes of known binding characteristics. The ratio of bound to free radioactivity found in the unknown samples then allows computation of the amount of prostaglandin E in the unknown. The ease of separation of bound and free by centrifugation and the high specificity of the membrane binding site are considerable advantages to existing antibody assays for E-type prostaglandins [38]. In collaboration with J. C. Frolich, the membrane binding assay for prostaglandin E_1 has already been applied to the measurement of levels of urinary prostaglandins [39].

This procedure for assaying prostaglandin requires the availability of plasma membranes as a reagent. The large scale procedure described here allows the preparation of approx. 400 mg quantities of plasma membrane which is enough membrane for 1000 binding determinations. The procedure is designed to prepare large quantities of plasma membrane for routine laboratory assay rather than plasma membrane of highest purity.

The use of the isotope dilution procedure and competition analysis for characterizing prostaglandin E binding to membrane receptors and the availability of plasma membranes as a source of prostaglandin receptors offers a simple and rapid in vitro assay to screen for drugs with potential prostaglandin hormonal influence. We find 16,16-dimethyl prostaglandin E_2 and 15-*S*,15-methyl prostaglandin E_2, two drugs with potent prostaglandin hormonal activity, bind very strongly to the prostaglandin receptors. Their mode of action would thus appear to be directly on the prostaglandin receptor.

The binding parameters measured on over twenty different rat liver plasma membrane preparations varied between 1.0–2.2 nM/mg protein for K_s and 0.50–1.2 pmoles/mg protein for E_T, respectively. This variation seems to be due to differences in the plasma membranes prepared at different times. It may well be that such differences may be referable to the physiological state of the animal since repeat analyses on a single preparation give superior reproducibility. We find that repeated freezing in liquid nitrogen and thawing at 37 °C of the plasma membranes does not result in measurable changes in the binding parameters.

The method described in this study to analyze competitive binding data is a simple, general approach for the characterization of receptors. It is for this reason that we have discussed it in greater detail in the Appendix. We have already included a procedure for the determination of the specific radioactivity of the tracer ligand (Fig. 1). We feel that these procedures will be useful in a variety of applications such as radioimmunoassay and other specific protein binding assays.

APPENDIX

Although the treatment of competitive binding is formally identical to that of Michaelis–Menten kinetics, some care must be taken due to the non-negligible amount of bound substrate and its variation in competitive binding studies. For ease of reference we will state some well-known results. Let the competitive binding be described by two equilibria.

$$E + S \rightleftharpoons ES;\ K_s = \frac{[E]\,[S]}{[ES]} = \frac{E \cdot S}{ES}$$

$$E + I \rightleftharpoons EI;\ K_I = \frac{[E]\,[I]}{[EI]} = \frac{E \cdot I}{EI}$$

and three conservation equations

$$E_T = E + ES + EI$$

$$S_T = S + ES$$

$$I_T = I + EI$$

where E_T, S_T, and I_T represent the total concentration of binding site, substrate and inhibitor respectively and E, S, I represent the free binding site, substrate and inhibitor concentrations. This leads to the following equation of competitive binding:

$$ES = E_T - K_s(1 + I/K_I)\frac{ES}{S} \tag{1}$$

rearranging:

$$\frac{ES}{K_s} + \frac{ES}{S} = \frac{E_T}{K_s} - \frac{I}{K_I}\left(\frac{ES}{S}\right)$$

Now although I is not a measured quantity it can be computed from the measured quantities ES and ES/S and binding parameters E_T and K_s.

$$I = I_T - E_T + K_s\left(\frac{ES}{S}\right) + ES$$

Our procedure in the case of tight binding of both substrate and inhibitor is to compute the values of I for various combinations of S_T and I_T and to plot $ES/K_s + ES/S$ versus $I(ES/S)$. This gives a straight line with slope $-1/K_I$ and intercept E_T/K_s which provides a check on the procedure. This method was used to determine the competition between prostaglandin E_1 and prostaglandin E_2.

In the case of competitive binding with a weakly bound inhibitor we assume that $I = I_T$ and then determine the coefficients in Eqn 1 which minimize the least square error of the plot of ES versus ES/S. Assume that the binding study was done with n levels of substrate and m levels of inhibitor. Let the concentration of bound substrate found with the ith substrate and jth inhibitor level be defined as

$$Y_{ij} = (ES)_{ij}$$

while the ratio of bound to free substrate under these conditions is defined to be

$$X_{ij} = \left(\frac{ES}{S}\right)_{ij}$$

For given values of E_T, K_s and K_I the squared error of the fit, Δ^2, is given by

$$\Delta^2 = \sum_{i=1}^{n}\sum_{j=1}^{m}\left\{Y_{ij} - E_T + K_s\left(1 + \frac{I_j}{K_I}\right)X_{ij}\right\}^2$$

Minimizing their error with respect to K_I, E_T and K_s implies that the least square error estimates for these parameters are

$$<K_I> = \left(\sum_{i=j}^{n}\sum_{j=1}^{m} X_{ij}^2 K_s I_j^2\right) \Big/ \left(\sum_{i=1}^{n}\sum_{j=1}^{m} X_{ij} I_j (E_T - Y_{ij} - X_{ij} K_s)\right) \tag{2}$$

$$<E_T> = \frac{1}{nm} \sum_{i=1}^{n} \sum_{j=1}^{m} (Y_{ij} + X_{ij} K_s (1 + I_j/K_I)) \quad (3)$$

$$<K_s> = \left[\sum_{i=1}^{n} \sum_{j=1}^{m} X_{ij} (1 + I_j/K_I) (E_T - Y_{ij}) \right] \Big/ \left[\sum_{i=1}^{n} \sum_{j=1}^{m} X_{ij}^2 (1 + I_j/K_I)^2 \right] \quad (4)$$

Since we saw no easy solution to these non-linear coupled equations and since we had good estimates of K_s and E_T from previous binding assays, we choose to do an iterative procedure. We assumed values of K_s and E_T and computed the least square error estimate for $<K_I>$. We then used this value for K_I and our previous estimate of K_s and computed $<E_T>$. We then used this value of E_T and our derived value of $<K_I>$ and computed $<K_s>$. We then iterated the procedure to self-consistency in about 50 cycles.

Typical experimental data are shown in Figs 6A, B, C. The competitive data are plotted according to Lineweaver–Burke, Dixon and Eqn 1. The Dixon plot gives $K_I = 665 \cdot 10^{-9}$ M. The Lineweaver–Burke plots give an average value of $305 \cdot 10^{-9}$ M while Eqn 1 gives $280 \cdot 10^{-9}$ M. The value derived from the Dixon plot is in considerable error due to changes in the free substrate concentration as the inhibitor binds, as mentioned in Discussion.

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REFERENCES

1 Horton, E. W. and Main, I. H. M. (1963) Br. J. Pharmacol. 21, 182–189
2 Nakano, J., Cole, B. and McCurdy, J. R. (1967) J. Pharmacol. Exp. Ther. 156, 538–547
3 Steinberg, D., Vaughan, M., Nestle, P. and Bergström, S. (1963) Biochem. Pharmacol. 12, 764–766
4 Robert A. (1968) Prostaglandin Symposium Worchester Found. for Experimental Biology (Ramwell, P. W. and Shaw, J. E., eds), pp. 47–54, Wiley-Interscience, New York
5 The Prostaglandins (1972) (Karim, S. M. M., ed.), Wiley-Interscience, Great Britain
6 Granström, E. (1967) Prog. Biochem. Pharmacol. 3, 89–93
7 Christ, E. J. and Nugteren, D. H. (1970) Biochim. Biophys. Acta 218, 296–307
8 Bergström, S., Carlson, L. A. and Weeks, J. R. (1968) Pharmacol. Rev. 20, 1–48
9 Hansson, E. and Samuelsson, B. (1965) Biochim. Biophys. Acta 106, 379–385
10 Robison, G. A., Arnold, A. and Hartmann, R. C. (1969) Pharm. Res. Commun. 1, 325–332
11 Kuehl, F. A., Humes, J. L., Tarnoff, J., Cirillo, V. J. and Ham, E. A. (1970) Science 169, 883–885
12 Butcher, R. W. and Baird, C. E. (1968) J. Biol. Chem. 243, 1713–1717
13 Butcher, R. W. and Baird, C. E. (1970) Proc. 4th Int. Congr. Pharmacol. 4, 42–48
14 Butcher, R. W., Pike, J. E. and Sutherland, E. W. (1967) Prostaglandins (Bergström, S. and Samuelsson, B., eds), pp. 133–138, Wiley- Interscience, New York

15 Kaneko, T., Zor, U. and Field, J. B. (1969) Science 163, 1062–1063
16 Marsh, J. M. (1970) FEBS Lett. 7, 283–286
17 Sobel, B. E. and Robison, A. (1969) Circulation 40, Suppl. III, 189
18 Davoren, P. R. and Sutherland, E. W. (1963) J. Biol. Chem. 238, 3016–3023
19 Kuehl, F. A. and Humes, J. L. (1972) Proc. Natl. Acad. Sci. U.S. 69, 480–484
20 Miller, O. V. and Magee, W. E. (1973) Adv. Biosci, 9, 83–89
21 Smigel, M. and Fleischer, S. (1973) Fed. Proc. 32, 459, Abstr. 1298
22 Sweetman, B. J., Frolich, J. C. and Watson, J. T. (1973) Prostaglandins 3, 75–87
23 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
24 Chen, Jr, P. S., Toribara, T. Y. and Warmer, H. (1956) Anal. Chem. 28, 1756–1758
25 Rouser, G. and Fleischer, S. (1967) Methods in Enzymology (Estabrook, R. N. and Pullman, M. E., eds), Vol. 10, pp. 385–406
26 Fleischer, S. and Fleischer, B. (1967) Methods in Enzymology (Estabrook, R. N. and Pullman, M. E., eds), Vol. 10, pp. 406–433
27 Van Golde, L. M. G., Fleischer, B. and Fleischer, S. (1971) Biochim. Biophys. Acta 249, 318–330
28 Sottocasa, G. L., Kuylenstierna, B., Ernster, L. and Bergstand, A. (1967) J. Cell Biol. 32, 415–438
29 Fleischer, B. and Fleischer, S. (1969) Biochim. Biophys. Acta 183, 265–275
30 Weibel, E. R., Staubli, W., Gnagi, H. R. and Hess, F. A. (1969) J. Cell Biol. 42, 68–112
31 Karim, S. M. M., Sharma, S. D., Filshie, G. M., Salmon, J. A. and Ganesan, P. Adaikan (1973) Adv. Biosci. 9, 811–830
32 Robert, A. and Magerlein, B. J. (1973) Adv. Biosci. 9, 247–253
33 Fried, J., Lin, C., Mehra, M., Kao, W. and Dalven, P. (1971) in Prostaglandins (Ramwell, P. and Shaw, J., eds), Ann. N.Y. Acad. Sci. 180, 38–63
34 Lands, W. E. M., Letellier, P. R., Rome, L. H. and Vanderhoek, J. Y. (1973) Ad. Biosci. 9, 15–28
35 Sanner, J. H., Mueller, R. A. and Schulze, R. H. (1973) Adv. Biosci. 9, 139–148
36 Green, K. and Samuelsson, B. (1969) J. Lipid Res. 5, 117–120
37 Cuatrecasas, P. (1972) J. Biol. Chem. 297, 1980-1991
38 Jaffe, B. M. (1973) J. Clin. Invest. 52, 398–405
39 Frolich, J. C., Wilson, T. W., Sweetman, B. J., Smigel, M., Carr, K., Watson, J. T. and Oates, J. A. (1973) Fed. Proc. 32, 788. Abstr. 3227