Br. J. Pharmac. (1974), 52, 197-203

PROSTAGLANDIN INACTIVATION IN GUINEA-PIG LUNG AND ITS INHIBITION

D.J. CRUTCHLEY & PRISCILLA J. PIPER

Department of Pharmacology, Institute of Basic Medical Sciences,
Royal College of Surgeons of England, Lincoln's Inn Fields, London WC2A 3PN

1 Several compounds were tested for their ability to inhibit prostaglandin metabolism in guinea-pig lungs.

2 In isolated perfused lungs polyphoretin phosphate (PPP) inhibited prostaglandin metabolism being active at concentrations greater than 100 ng/ml and having an ID_{50} of 2.15 μg/ml against prostaglandin E_2 metabolism and 1.55 μg/ml against prostaglandin $F_{2\alpha}$ metabolism.

3 At these doses no antagonism of the actions of prostaglandins was seen.

4 Diphloretin phosphate (DPP) was more active on a weight for weight basis and inhibition of inactivation of prostaglandins was seen at concentrations greater than 10 ng/ml, the ID_{50} being 0.54 μg/ml against both prostaglandins E_2 and $F_{2\alpha}$.

5 At concentrations greater than 5 μg/ml DPP antagonized the actions of prostaglandin $F_{2\alpha}$.

6 The sulphydryl-binders N-ethyl maleimide (NEM) and sodium *p*-chloromercuriphenyl sulphonate (PCMS) also inhibited prostaglandin inactivation by guinea-pig isolated lungs.

7 All four compounds inhibited prostaglandin metabolism by a crude enzyme preparation from guinea-pig lungs.

Introduction

Although stable in blood, prostaglandins E_1, E_2 and $F_{2\alpha}$ are rapidly removed in one circulation through the pulmonary vasculature of cat, dog and rabbit *in vivo* (Ferreira & Vane, 1967; Vane, 1969). Similar removal occurs in guinea-pig isolated perfused lungs and is due to enzymic breakdown (Piper, Vane & Wyllie, 1970). Samuelsson, Granström, Gréen & Hamberg (1971) and Änggård (1971) showed that the most important enzyme responsible for prostaglandin metabolism in guinea-pig lungs is 15-hydroxyprostaglandin dehydrogenase (PGDH), although other enzymes such as the 13,14-reductase also take part.

Since pulmonary removal of the E and F prostaglandins is so efficient, we investigated a wide variety of compounds as potential inhibitors of the inactivation. In order to check that such compounds did in fact inhibit enzymic breakdown we tested them both against prostaglandin inactivation by guinea-pig isolated perfused lungs, and as inhibitors of a crude enzyme preparation from these lungs. A preliminary report of this work was given to the British and German Pharmacological Societies (Crutchley & Piper, 1973).

Methods

Isolated perfused lungs

Lungs were removed from male guinea-pigs (300-400 g) and perfused via the pulmonary artery as described previously (Piper & Vane, 1969). The effluent from the lungs then superfused assay tissues for detection of prostaglandins; these were a rat stomach strip, chick rectum and rat colon. They were made more specific for prostaglandins by the continuous infusion of combined antagonists to acetylcholine, 5-hydroxytryptamine, histamine and catecholamines.

Responses of the assay tissues to prostaglandins were obtained before and during a continuous infusion of a potentital inhibitor, thus establishing the effect, if any, of the inhibitor on the direct actions of the prostaglandins on the assay tissues. Control removal of prostaglandins by the lungs was then obtained with the inhibitor continuously infused over the assay tissues. A dose of prostaglandin was infused into the pulmonary artery and the prostaglandin activity escaping in the effluent was assayed. The inhibitor was then infused continuously into the pulmonary artery and the removal of prostaglandins repeated. In some experiments, the inhibitor was removed and

the time course of recovery, if any, was observed.

Certain of the inhibitors tested had adverse effects on the assay tissues, and when these substances were tested, the following washout technique was used: the lungs were perfused for 1 h with Krebs solution containing the inhibitor, the effluent being discarded. The lungs were then perfused with normal Krebs solution for 15-20 min before the effluent superfused the assay tissues and the pulmonary removal of prostaglandins was measured.

Lungs treated by these methods remained viable for at least 5 h, as shown by the fact that control lungs and lungs treated with inactive compounds still removed 90-98% of administered prostaglandins after this time.

Crude enzyme preparation

Lungs were removed from male guinea-pigs (300-400 g), and after being rinsed in ice-cold buffer, were homogenized with twice their amount (w/v) of buffer (0.1 M Tris-HCl, pH 8.0). The superantant obtained after successive centrifugations at 10,000 *g* for 30 min and 100,000 *g* for 45 min, was dialysed for 12 h at 4°C against 40 volumes of buffer. Aliquots were stored at −22°C until required, but not longer than 7 days. No apparent loss of activity was observed. Protein was estimated by the biuret procedure and found to be 28-39 mg/ml.

Preliminary incubation of enzyme preparation with prostaglandin E_2 showed that 99% of added prostaglandins was destroyed within 5 minutes. For the radioactive experiments, 10 min was chosen as a suitable incubation time. To 5 ml aliquots were added 2 μmol NAD, 14 nmol of unlabelled prostaglandin E_2 and 0.05 μCi of tritium-labelled prostaglandin E_2, 160 Ci/mmol. To other aliquots suitable concentrations of inhibitors were also added. The mixtures were incubated for 10 min at 37°C and the reaction terminated by plunging the tubes into boiling water. Control samples of boiled enzyme with and without [^{3}H]-prostaglandin E_2 were also used.

The tubes were centrifuged; the supernatant was washed twice with equal volumes of petroleum ether, then acidified to pH 3.0 and extracted twice with equal volumes of ethyl acetate. The pooled extracts were evaporated to dryness under reduced pressure. The residue was applied to a Kodak Eastman silica-gel plate and subjected to thin-layer chromotography in the AI or AIII systems (Gréen & Samuelsson, 1964). The plates were dried and cut into 1 cm zones which were added to vials containing 10 ml Diotol liquid scintillator (Herberg, 1960). Radioactivity was measured in a Packard Tri-carb spectrometer, model 3375. Correction for quenching was made by the use of external standards. Percentage of total radioactivity was plotted for each zone from the origin to the solvent front.

The following compounds were used: acetylsalicylic acid, dimercaprol, 2,4-dinitrophenol, disulfiram (B.D.H.); fludrocortisone (Sigma); hyoscine hydrobromide (B.D.H.); ibuprofen (Boots); indomethacin (Merck, Sharp & Dohme); sodium iodoacetate (Koch Light); meclofenamic acid (Parke Davis); mepyramine maleate (Sandoz); 2-mercaptoethanol (Sigma); methysergide hydrogen maleate (Sandoz); β-NAD, N-ethyl maleimide (Sigma); di-4-phloretin phosphate, polyphloretin phosphate (Leo, AB. and Nelson Research & Development Co.); paracetamol, (Winthrop); sodium *p*-chloromercuriphenyl sulphonate (Sigma); phenoxybenzamine hydrochloride (Smith, Kline & French); phenylbutazone (Geigy); (±)-propranolol hydrochloride (I.C.I.); and prostaglandin A_2 (Cambrian Chemicals). Tritium-labelled prostaglandin E_2 (160 Ci/mmole) was obtained from the Radiochemical Centre, Amersham. It was diluted with unlabelled prostaglandin E_2 before use.

Results

Removal of prostaglandins by isolated perfused lungs

The compounds investigated for potential inhibition of prostaglandin inactivation fall into three main groups as shown below.

(1) Polyphloretin phosphate and analogues. Polyphloretin phosphate (PPP) selectively antagonizes the actions of E and F prostaglandins on several isolated smooth-muscle preparations (Eakins, Karim & Miller, 1970; Mathé, Strandberg & Aström, 1971). Antagonism of prostaglandins by PPP *in vivo* has also been reported (Bethel & Eakins, 1971; Mathé, Strandberg & Fredholm, 1972; Villanueva, Hinds, Katz & Eakins, 1972). Marrazzi & Matschinsky (1971; 1972) have shown that it also inhibits PGDH *in vitro*. The action of PPP against the removal of prostaglandins E_2, $F_{2\alpha}$ and $F_{2\beta}$ by isolated perfused lungs was therefore investigated. Since PPP is a polymer of uncertain molecular structure a number of known fractions and analogues of PPP were also tested. Of those investigated, the most active was di-4-phloretin phosphate (DPP).

Both DPP and PPP (0.1-5.0 μg/ml) inhibited the inactivation of prostaglandins E_2, $F_{2\alpha}$ and $F_{2\beta}$ and increased the amount surviving passage through the lungs. (Figure 1). The inhibition of inactivation of prostaglandins E_2 and $F_{2\alpha}$ by DPP

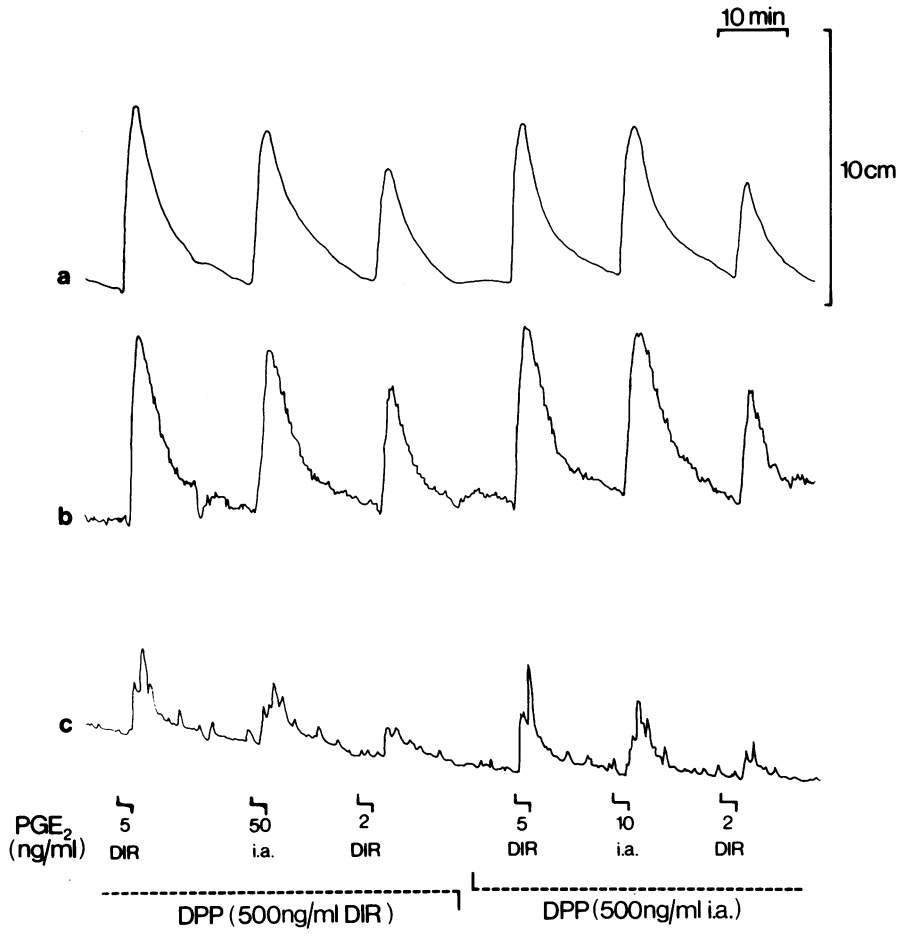

Fig. 1 Inhibition by diphloretin phosphate (DPP) of removal of prostaglandin E_2 by guinea-pig isolated lungs. The effluent from guinea-pig isolated perfused lungs superfused a rat stomach strip (a), a chick rectum (b) and a rat colon (c). The assay tissues were continuously blocked with combined antagonists. In the first panel DPP (500 ng/ml) was infused into the Krebs solution superfusing a, b and c. When prostaglandin E_2 (PGE_2; 50 ng/ml) was infused into the pulmonary artery (i.a.) the contractions of a, b and c caused by prostaglandin activity escaping from the lungs were bracketed by doses of prostaglandin E_2 (2 and 5 ng/ml) given directly to the assay tissues (DIR). In the second panel, DPP (500 ng/ml) was infused continuously i.a. When prostaglandin E_2 10 ng/ml was infused i.a. the prostaglandin activity in the effluent was bracketed by prostaglandin E_2 (2 and 5 ng/ml DIR).

and PPP was compared and the ID_{50}s calculated as shown in Figure 2.

At the doses used, no antagonism of the actions of prostaglandins on the assay tissues was seen except in the case of DPP, which at concentrations of greater than 5 μg/ml caused marked antagonism of prostaglandin $F_{2\alpha}$.

The inhibition of prostaglandin removal by DPP and PPP was reversed by washing the compounds out of the lungs for 30-45 min, after which time the removal had returned to almost normal.

In these experiments DPP was more potent than PPP on a weight basis. The molecular weight of PPP has been assumed to be approximately 15,000 (Eakins, Miller & Karim, 1971: Marrazzi & Matschinsky, 1972), but recent reports from Leo AB show that it may be as low as 2,000, in which case DPP may also be more potent than PPP on a molar basis.

At 5 μg/ml, a concentration which would strongly inhibit prostaglandin inactivation, PPP had no noticeable effect on the removal of

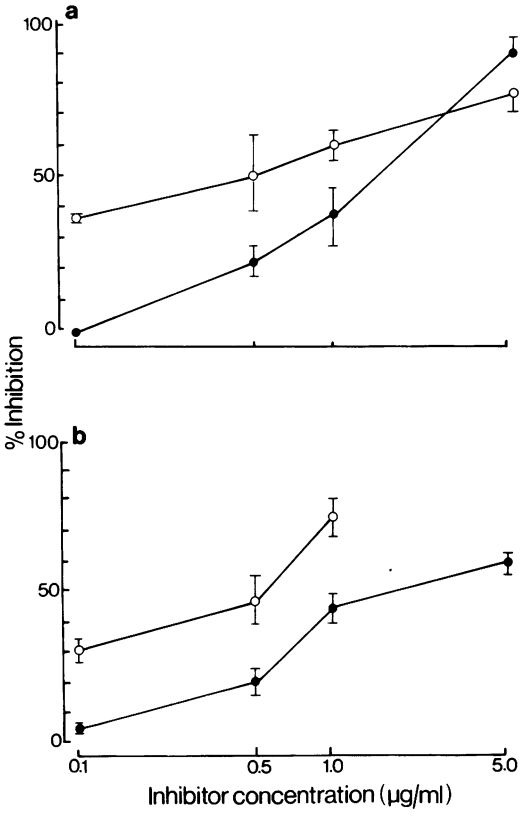

Fig. 2 Inhibition of pulmonary inactivation of prostaglandin E_2 (a) and $F_{2\alpha}$ (b) by diphloretin phosphate (○) and polyphloretin phosphate (●). Each point is the mean of up to six experiments. Vertical bars show s.e. mean.

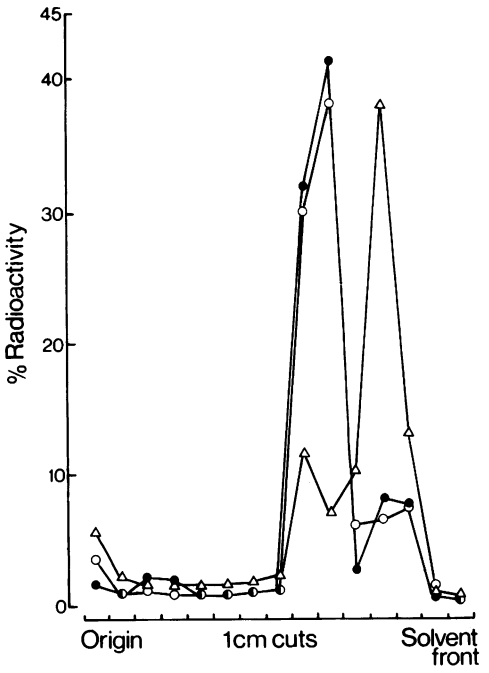

Fig. 3 Thin-layer chromatography of [^{3}H]-prostaglandin E_2 and metabolite in the AI system. Percentage total radioactivity was plotted for each of the 1 cm cuts from the origin to the solvent front of the chromatogram. (○) Enzyme + [^{3}H]-prostaglandin E_2, zero incubation; (△) Enzyme + [^{3}H]-prostaglandin E_2, 10 min incubation; (●) Enzyme + [^{3}H]-prostaglandin E_2 + NEM 50 μg/ml, 10 min incubation. The zero incubation shows a large peak, R_F 0.60, representing unchanged E_2 and small peak R_F 0.80 representing metabolite, with possibly some prostaglandin A_2 formed during extraction. After incubation for 10 min the prostaglandin peak was much reduced and that of metabolite increased. In the presence of NEM this metabolism was almost completely inhibited.

bradykinin or 5-hydroxytryptamine by guinea-pig isolated lungs.

(2) Enzyme inhibitors. A number of substances known to inhibit a variety of NAD-linked dehydrogenases were investigated as inhibitors of the pulmonary inactivation of prostaglandin E_2 (Table 1). These compounds all had adverse effects on the assay tissues and the washout technique described earlier was used for these experiments. Of the compounds tested, the sulphydryl-binders N-ethyl maleimide (NEM) and sodium *p*-chloromercuriphenyl sulphonate (PCMS) were active with ID_{50}s of 8.6 μg/ml (6.9×10^{-5} M) and 249.0 μg/ml (6.0×10^{-4} M) respectively.

There might be a discrepancy between the ID_{50} of NEM and PCMS obtained by the washout technique and the true ID_{50} which would be obtained during infusion of the inhibitor. This would depend on the duration of inhibition by the sulphydryl binders. The magnitude of this possible discrepancy was measured by the method of Alabaster & Bakhle (1972). This allows assay of prostaglandins even during infusion of NEM into the lungs by the simultaneous infusion of 2-mercaptoethanol after the lungs. In both series measurements of inhibition were taken at the same time after the start of treatment with NEM, viz. 75-90 minutes. Very little discrepancy was observed for NEM; the ID_{50} for the washout technique was 8.6 μg/ml (6.9×10^{-5} M); that for direct infusion was 7.4 μg/ml (5.9×10^{-5} M) (18 experiments).

Another sulphydryl-binder, sodium iodoacetate, also showed some activity but only at concentrations greater than 100 μg/ml (5.0×10^{-4} M).

(3) Anti-inflammatory agents. Non-steroidal anti-inflammatory agents such as aspirin and indomethacin inhibit prostaglandin synthesis (Vane, 1971). This probably accounts for several of their actions (Vane, 1972). Gibson, Hodge, Jackson, Katic & Stevens (1972), observed that when high doses of aspirin were given to dogs *in vivo*, removal of prostaglandins in the pulmonary circulation was reduced. In doses many times higher than those needed to inhibit prostaglandin synthesis, some non-steroidal anti-inflammatory agents partially inhibit prostaglandin metabolism by a crude enzyme preparation from the renal cortex of the rabbit (Flower, unpublished observation). A number of these compounds were investigated to see whether they inhibited inactivation of prostaglandins in guinea-pig lungs.

Phenylbutazone (50 μg/ml) and indomethacin (20 μg/ml) showed slight activity, reducing the pulmonary removal of prostaglandin E_2 from 98% to 60% and 80% respectively. Aspirin, paracetamol, ibuprofen and meclofenamic acid were inactive at doses of up to 100 μg/ml.

Metabolism by a crude enzyme preparation

After development of the thin-layer chromatography plates, 1 cm cuts were made from the origin to the solvent front and the radioactivity of each fraction measured by scintillation counting. The percentage of the total radioactivity was plotted for each fraction.

Figure 3 shows a typical result from a plate developed in the AI solvent. When the enzyme was incubated with [^{3}H]-prostaglandin E_2 for zero time, there was a large peak (R_F 0.60) which represents unchanged prostaglandin. This was verified by the application of authentic prostaglandin E_2 to the plate. There was also a smaller peak (R_F 0.80). This may have been due to metabolite, since it often disappeared with prior boiling of the enzyme for 10 minutes. It could also have been due to prostaglandin A_2, 2-3% of which is often formed during extraction. Authentic prostaglandin A_2 gave an R_F 0.85 in this system.

After incubation for 10 min at 37°C the peak with R_F 0.80 was very much larger. This was unlikely to be due mainly to prostaglandin A_2 and so was probably prostaglandin E_2 metabolite(s). This is further suggested by the corresponding reduction in the prostaglandin E_2 peak.

Complete metabolism of prostaglandin E_2 in

Table 1 The effects of some enzyme inhibitors on inactivation of prostaglandin E_2 (PGE_2) by guinea-pig isolated perfused lungs.

Compound	*Dose (M)*	**Mean % of PGE_2 escaping from lungs*	*ID_{50} (M)*
Control	-	2-10%	–
2,4-dinitrophenol	5×10^{-4}	4%	–
Dimercaprol	3×10^{-3}	4%	–
	1.5×10^{-3}	6%	
Disulfiram	6×10^{-6}	6%	–
	4×10^{-6}	11%	
	2×10^{-6}	2%	
Sodium iodoacetate	5×10^{-4}	20%	–
	2.5×10^{-4}	10%	
Sodium *p*-chloromercuriphenyl sulphonate	10^{-3}	90%	6×10^{-4}
	5×10^{-4}	37%	
	10^{-4}	27%	
	5×10^{-5}	25%	
	10^{-5}	15%	
N-ethyl maleimide	2×10^{-4}	100%	7×10^{-5}
	8×10^{-5}	60%	
	4×10^{-5}	38%	
	8×10^{-6}	25%	

* Each value was calculated from up to 3 experiments.

guinea-pig lung leads to two products, 15-keto-dihydro-prostaglandin E_2 and 13,14-dihydro-prostaglandin E_2 (Änggård, Gréen & Samuelsson, 1965), but in these experiments only one metabolite was seen. Attempts were made to clarify this peak by using silver nitrate-impregnated plates in the AIII system (Gréen & Samuelsson, 1964). However, in these experiments, again only two main peaks were seen. These had R_Fs 0.12 and 0.65 and so probably corresponded to unchanged prostaglandin E_2 and 15-keto-dihydro-prostaglandin E_2, which Änggard *et al.* (1965) have shown to have R_Fs 0.14 and 0.65 respectively. As the R_F of 13,14-dihydro-prostaglandin E_2 in this system is 0.32, this metabolite was not detected.

Addition of PPP, DPP, NEM and PCMS before incubation of the enzyme-substrate mixture reduced the formation of metabolite and increased the unchanged prostaglandin E_2 peak. Figure 3 also shows that NEM 50 μg/ml almost completely inhibited the metabolism of prostaglandin E_2; the scan obtained with 10 min incubation with NEM was almost identical to that obtained with zero incubation without NEM. No attempt was made to quantitate the degree of inhibition as this was a crude preparation of prostaglandin dehydrogenase.

Discussion

Since the lung has efficient inactivating mechanisms for a variety of biologically active substances, including E and F prostaglandins, 5-hydroxytryptamine, noradrenaline and bradykinin (Ferreira & Vane, 1967; Thomas & Vane, 1967; Vane, 1969; Piper *et al.*, 1970; Alabaster & Bakhle, 1972) it probably acts as a filter to prevent such substances from reaching the arterial circulation in high concentrations. The inactivation of prostaglandins is especially efficient, approximately 95% of an administered dose disappearing in one passage through the pulmonary circulation. Agents inhibiting this removal would be useful, both because they might provide more information on the mechanism of the inactivation, and as pharmacological tools. The latter may be important because of the efficiency of prostaglandin inactivation; for example, even decreasing the removal from 95% to 90% doubles the amount of prostaglandins surviving passage through the lungs.

PPP is generally accepted as a prostaglandin antagonist, both *in vitro* and *in vivo* (Eakins *et al.*, 1970; Villanueva *et al.*, 1972). We have shown it to be a potent inhibitor of prostaglandin inactivation in guinea-pig lung, a property which would tend to potentiate actions of prostaglandins. However, the doses needed to block metabolism are well below those necessary for direct antagonism. Inhibition of metabolism *in vitro* appears at concentrations of greater than 100 ng/ml, and although widely varying concentrations have been used for *in vitro* prostaglandin antagonism this action does not seem to occur below 2.5 μg/ml (Eakins *et al.*, 1970; Bennett & Posner, 1971). As small changes in removal lead to large changes in the amount of prostaglandins surviving passage through the lungs, it is possible to use PPP to block metabolism of prostaglandins without antagonizing their actions.

The fact that DPP is more potent than PPP in inhibiting prostaglandin metabolism suggests that this property of PPP, like its prostaglandin-inhibitory property, may reside in small molecular weight fractions like monomers and dimers (Eakins, Fex, Fredholm, Hogberg & Veige, 1973).

The removal of prostaglandins from the pulmonary circulation is due to biological inactivation by enzymatic breakdown (Piper *et al.*, 1970). At least three enzymes are involved in prostaglandin metabolism in guinea-pig lung, these being 15-hydroxyprostaglandin dehydrogenase (PGDH), a 13,14-reductase and an enzyme tentatively called 15-ketoprostaglandin reductase (Änggård, 1971). Since the initial and rate-limiting step is catalysed by PGDH, it is probable that substances inhibiting the breakdown of prostaglandins in lungs act by blocking this enzyme. Complete breakdown in guinea-pig lung yields two metabolites, 15-keto-dihydro-prostaglandin E_2 and dihydro-prostaglandin E_2 (Änggård, *et al.*, 1965). Results with our crude enzyme preparation show that only one metabolite peak was obtained. From its behaviour in the AIII system we conclude that it is probably 15-keto-dihydro-prostaglandin E_2 (R_F 0.65). The second metabolite, dihydro-prostaglandin E_2 is usually formed in small amounts and so may not have been detected. However, since there is little information available on 15-ketoprostaglandin reductase, the enzyme responsible for the dihydro-prostaglandin E_2 formation, it is possible that this enzyme is labile and so absent in our preparation.

The phloretins are quite highly ionized at physiological pH and so are unlikely to enter cells readily. This suggests that pulmonary inactivation of prostaglandins takes place in or close to the vasculature. There is evidence that the pulmonary metabolism of kinins, angiotensin I and the adenine nucleotides takes place in the endothelium (Smith & Ryan, 1973). Since PGDH is known to be intracellular, DPP and PPP may act by blocking access or binding of prostaglandins to the cell wall, or transport into the cell.

If inhibition of prostaglandin metabolism could

be achieved *in vivo*, the effects of an intravenous dose of prostaglandins would be expected to be potentiated, thus making their systemic actions clearer. Prostaglandins are given therapeutically as intravenous infusions, and if pulmonary removal were inhibited lower doses could probably be given.

We thank Mr M.A. Palmer for skilled technical assistance. We also wish to thank Dr J.E. Pike of the Upjohn Company, Kalamazoo, U.S.A. for the gift of prostaglandins E_2 and $F_{2\alpha}$; Dr M.E. Rosenthale of Wyeth Laboratories Inc., Philadelphia, U.S.A. for prostaglandin $F_{2\beta}$; both Leo AB, Helsingborg, Sweden and Nelson Research and Development Company, Irvine, U.S.A. for DPP and PPP; and Merck Sharp & Dohme for indomethacin. We also thank the Medical Research Council and the Wellcome Trust for grants.

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(Received March 20, 1974)