ADVENTURES AND EXCURSIONS IN BIO-ASSAY: THE STEPPING STONES TO PROSTACYCLIN

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by

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Physiology has spawned many biological sciences, amongst them my own field of pharmacology. No man has made a more important contribution to the fields of physiology and pharmacology than Sir Henry Dale (1875-1968, Nobel Laureate in Physiology or Medicine in 1936). Dale had a great influence not only on British pharmacology in general but also on my own scientific endeavours. Indeed, I can put forward a strong case for considering myself as one of Dale's scientific grandchildren. My early days as a pharmacologist were influenced not only by Dale himself but also by his school of colleagues, including Burn, Gaddum and von Euler. It was Burn who taught me the principles and practice of bioassay. Some of Gaddum's first publications were on the development of specific and sensitive methods for biological assay and he maintained a deep interest in this subject for the rest of his life (1). In 1964 he said "the pharmacologist has been a 'jack of all trades' borrowing from physiology, biochemistry, pathology, microbiology and statistics – but he has developed one technique of his own, and that is the technique of bioassay" (2).

Expensive, powerful and sophisticated chemical methods, such as gas chromatography and mass spectrometry, have been developed and perfected for detection and quantification of prostaglandins (PGs) and related substances. One should not forget, however, that starting with the discovery and isolation of prostaglandins by von Euler (3; see also Bergstrom in this volume), biological techniques and bioassay have contributed very substantially to the development of the field. Bioassay has provided crucial information on the role of the lungs in the removal of circulating prostaglandins (4), the participation of prostaglandins in inflammatory reactions (5,6), the contribution of prostaglandins to the autoregulation and maintenance of blood flow to the kidney (7-9), the inhibitory effect of aspirin-like drugs on the biosynthesis of prostaglandins (l0-12), the mediation of pyrogen fever by prostaglandins (13), and the release of rabbit aorta-contracting substance (RCS; now identified as thromboxane A_2, TXA_2) from lungs during anaphylaxis (14, 15). Moreover, in 1976 bioassay made possible the discovery of PGX, now renamed prostacyclin (PGI_2), the latest member of the prostaglandin family (16-19). Indeed, it is

doubtful whether the biological significance of any of the unstable products of arachidonic acid metabolism would have been recognised without bioassay techniques. With extraordinary simplicity and convenience, by its very nature, bioassay distinguishes between the important biologically active compounds and their closely related but biologically unimportant metabolites.

In this review I shall discuss the development of the cascade superfusion bioassay technique and some of the discoveries and concepts which arose from its application, leading up to the discovery and development of prostacyclin. The effects of prostacyclin in man and its clinical assessment (another application of bioassay) will also be discussed.

1. CASCADE SUPERFUSION BIOASSAY

a. Development

Most uses of bioassay involving smooth muscle demand high sensitivity and specificity. These aspects have been achieved first, by limiting the volume of fluid bathing the isolated tissue and second, by using an assay organ sensitive to, and relatively specific for, the test substances under study. Further specificity can be achieved by using a combination of several tissues which present a characteristic pattern of response to the test substance or substances. This takes advantage of the principle of parallel pharmacological assay, regarded by Gaddum (20) as strong evidence for the identity of a compound.

Magnus (21) introduced the idea ofsuspending an isolated portion of smooth muscle in a chamber containing a nutrient fluid and measuring changes in tissue tone. The organ baths used today are modified versions of that used by Dale (22). Gaddum (23) applied the experimental design developed by Finkleman (24) to the assay of minute amounts of biologically active substances. He called his technique "superfusion" in contrast to perfusion. This consisted of bathing an assay tissue with a stream of fluid which was momentarily stopped at the moment of addition of the test substance. Vane (25) introduced the idea of superfusing several tissues in cascade (generally up to six, arranged in two banks) (Fig. 1). Besides being useful for the parallel assay of individually injected samples, this arrangement also allows parallel analysis of the active components present in a fluid stream (most commonly Krebs' solution) taken from the outflow of a perfused organ.

Another innovation introduced by Vane (25) was to use blood as the superfusion medium (the blood-bathed organ technique). The anaesthetised animal is heparinised, and blood is continuously removed at a constant rate of 10-15 ml/min (dogs, cats and rabbits). Lower rates can be used from guinea pigs (26). The blood (either from a vein or from an artery) superfuses the assay tissues and is then returned by gravity to a large vein.

Plainly, when perfusate from an organ or blood from an animal is used for superfusion, substances can reach the assay tissues within a few seconds of generation or release. This element of "instantaneity" is an important aspect of cascade superfusion bioassay in that it detects the biological activity of chemically unstable compounds whose activity would otherwise be lost in an extrac-

tion process. Another important feature of the method is that it gives the maximum opportunity for serendipity. The dynamic nature of the assay also allows the measurement of inactivation of an infused substance across a particular vascular bed. Further modifications of the bioassay technique have been developed by Collier (27), Ferreira and Souza Costa (28) and Gryglewski and colleagues (29).

b. Choice of bioassay tissues

It is usually possible to find a piece of smooth muscle which is particularly sensitive to the hormone under investigation and relatively insensitive to other substances. Indeed, think of any part of the body and you can be sure that the pharmacologist has cut it out, put it into an isolated organ bath, or perfused its vessels in order to study the effects of drugs. For bioassay, segments of the gastro-intestinal tract or spirally cut strips of vascular tissue have mainly been used. Such procedures are the backbone not only of bioassay but also of classical pharmacology.

Figure 2 depicts the reactions of some superfused isolated tissues to various endogenous substances in concentrations likely to be found in circulating blood. It should be remembered that when blood is used as the superfusing medium, some smooth muscle preparations (but not others) exhibit an increased resting tone often lasting for the duration of the experiment. Such increased tone reduces sensitivity to substances which cause contraction but increases sensitivity to those which induce relaxation.

For detection of the classical prostaglandins, the most useful combination of assay tissues is the rat stomach strip, rat colon and chick rectum. For PG endoperoxides and later for prostacyclin, vascular tissues were added such as strips of coeliac or mesenteric artery (30). Strips of bovine coronary artery (31) are especially useful for they contract to PGE_2 but relax to prostacyclin (Fig. 2).

The specificity of a bioassay can be increased still further by the use of antagonists. For instance, contractions of the rat stomach strip induced by 5-hydroxytryptamine can be abolished by a specific antagonist such as methysergide, thereby leaving the preparation more specifically sensitive to the prostaglandins. The rat colon is relaxed by catecholamines but contracted by angiotensin II; when both are present in the superfusion fluid, the catecholamines reduce the contraction produced by angiotensin. This unwanted interference is prevented by blocking the actions of catecholamines with a p-receptor antagonist. When blood is used as the superfusion fluid, the antagonist can be perfused through the closed lumen (Fig. 1) of, say, the rat colon (32) thus localising the blocking agent to the assay tissue and minimising its effects on the whole animal. For a detailed discussion of the limitations of the cascade superfusion bioassay technique, the reader is referred to Vane (33) and Moncada, Ferreira and Vane (34).

c. Measurement of substances by cascade superfusion bioassay

The technique is well suited for measuring substances released into the circulation, such as catecholamines or angiotensin II, and also for determining the fate of substances released or infused into different parts of the circulation.

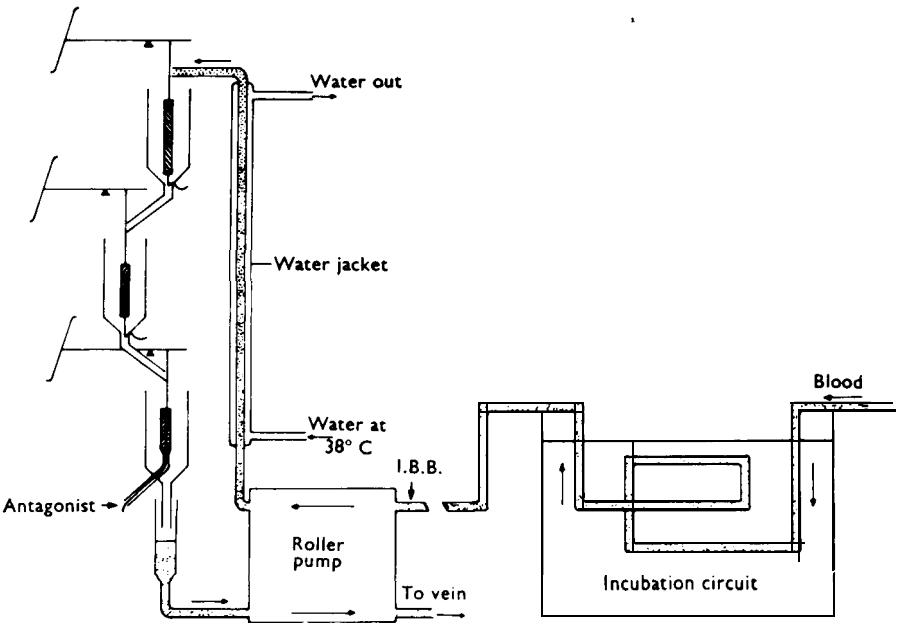

Figure 1. Diagram of the blood-bathed organ technique. Blood is continuously withdrawn from a convenient vessel by a roller pump, kept at 37°C by a water jacket and then allowed to superfuse a series of isolated organs, the longitudinal movements of which are recorded. The blood is then collected in a reservoir and returned to the animal. In some experiments the blood flows through a length of silicone tubing in a water bath (incubating circuit) before superfusing the isolated tissues. Drugs can be applied directly to the isolated tissues by infusions or injections into the bathing blood (I. B. B.) or with a time delay into the incubating circuit (from Vane 1969, reference 33, by permission of The Macmillan Press Ltd.).

i. Release of substances in response to stimuli

Release of catecholamines from the adrenal medulla can be detected and quantitated by use of a rat stomach strip and chick rectum. This technique was used to demonstrate that circulating catecholamines appeared to play little or no part in arterial baroreceptor reflexes (25) and also that catecholamines are released into the circulation during anaphylaxis (35). Of the substances released during the anaphylactic reaction histamine, bradykinin and slow reacting substance in anaphylaxis (SRS-A) will all in turn release adrenaline when injected intravenously, although there may be species differences in the mechanisms of action and in the sensitivity of the adrenal medulla (36-38).

An early use of the blood-bathed organ technique was to show the sequential release of angiotensin II and catecholamines during haemorrhage (39). Release of bradykinin into the blood stream by the intravenous injection of kallikrein or by contact of the blood with glass was easily demonstrated (40) but we consistently failed to demonstrate with this technique the endogenous release of bradykinin by physiological manipulation. However, circulating kinins were demonstrated during hypotension due to haemorrhage in the dog, and the concentrations detected in the bloodstream were sufficient to lower a normal blood pressure (41).

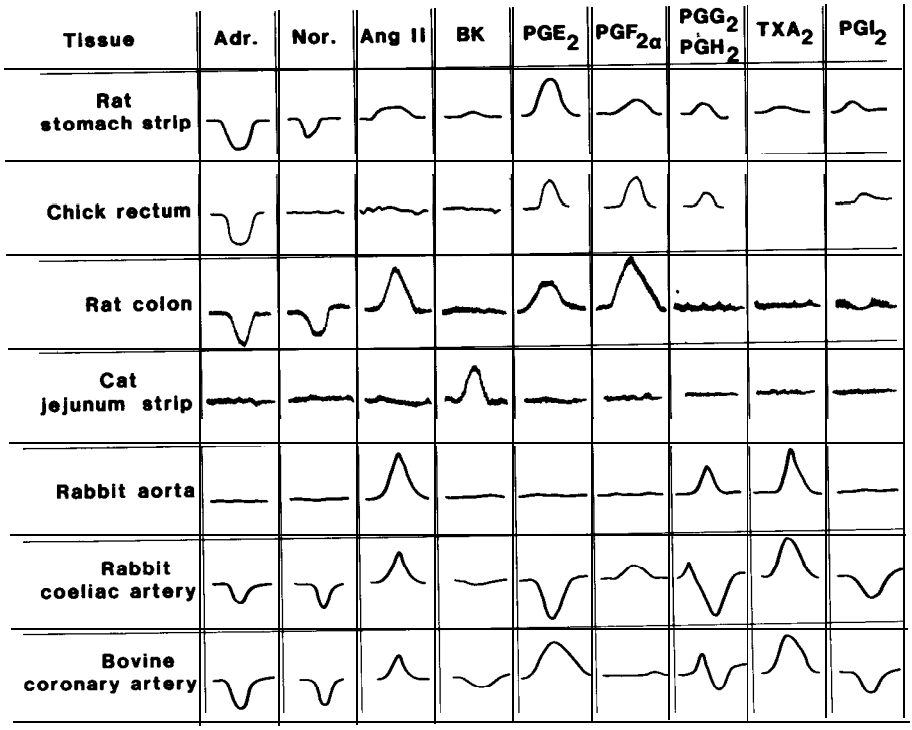

Figure 2. Diagram showing the reactions of some blood-bathed organs to various endogenous substances in concentrations of 0.1-5.0ng/ml. The actions of the catecholamines can be abolished by treatment with suitable blocking agents.

ii. Inactivation of circulating vasoactive substances

By comparing the effects on the blood-bathed assay organs of infusions of a substance over several minutes into a particular vascular bed with the effects of similar infusions given into the effluent of the vascular bed, it is possible to assay the percentage of the substance disappearing in one circulation through that vascular bed (Fig. 3). This technique allowed the demonstration of the inactivation of several vasoactive substances as they passed through vascular beds such as the hind legs, the liver and the lungs.

Angiotensin II, for example, is unaffected by passage through the pulmonary circulation, either *in vivo* or in isolated lungs of all species studied, including rats, dogs, guinea pigs, cats and man (for review see Bakhle and Vane, 42). However, the same substance is substantially inactivated (50-70%) in one passage through peripheral vascular beds such as the liver, the kidneys and the hind legs (43).

We also studied the fate of adrenaline and noradrenaline and found in both cats and dogs that 70-95% of an intra-arterial infusion of adrenaline or noradrenaline disappeared in one passage through the hind quarters. The lungs, however, inactivated up to 30% of an infusion of noradrenaline without interfering with the passage of adrenaline (44, 45).

Bradykinin is fairly rapidly destroyed in blood and has a half-life in the blood stream of cat or dog of about 17 seconds. Ferreira and Vane (46) showed that whereas the liver inactivated about 50%, the lungs inactivated about 80% of the bradykinin infusion.

It was observations such as these that drew our attention to the metabolic and pharmacokinetic function of the pulmonary circulation. The selectivity of the pulmonary inactivation mechanism is strikingly demonstrated by the way in which the lungs inactivate bradykinin but allow other peptides such as eledoisin, substance P, physalaemin, vasopressin and oxytocin to pass through without change (42).

The metabolism of prostaglandins in the pulmonary circulation *in vivo* was first studied by Vane and his colleagues (4, 47) who showed that almost all of an infusion of PGE_1, PGE_2 or $PGF_{2\alpha}$ was inactivated in one passage through the lungs. McGiff et al. (48) confirmed that PGE_1 and PGE_2 were avidly removed by dog lung *in vivo* but further showed that PGA_1 and PGA_2 survived the passage through the lungs without change. Thus, within this very closely related group of substances, the inactivation process can distinguish between the individual members. Interestingly, after the discovery of prostacyclin, we also found (49) that prostacyclin (unlike PGE_2 see Fig. 4) survived passage through the pulmonary circulation without change. In other vascular beds, the inactivation of prostacyclin in a single passage (50-70%) was comparable to that of PGE_2. Thus, the hind quarters, and particularly the liver, removed some of the prostacyclin which reaches those beds.

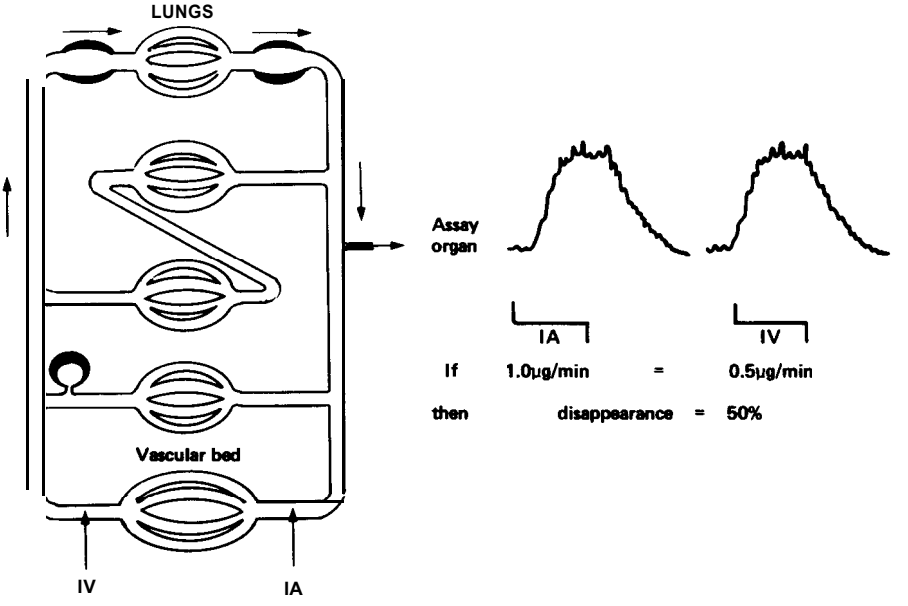

Figure 3. Diagram showing principle of bioassay of the degree of disappearance across a vascular bed. The differences in responses of the blood-bathed organs to infusions made i. a. or i. v. represent the degree of removal of the substance in one passage through the vascular bed.

Overall, the inactivation of prostacyclin in one circulation was about 50% giving a metabolic half life of one circulation time (*c* 30 sec) as compared with the chemical half life of 2-3 min. The inactivation mechanisms for PGE_2 and prostacyclin (50) are similarly dependent on PG 15-hydroxydehydrogenase, (PGDH). However, the disappearance of prostaglandins in the pulmonary circulation depends on two mechanisms, namely uptake and enzyme attack by PGDH, so our results suggested that prostacyclin is not a substrate for the PG uptake mechanism.

From the differential removal of vasoactive hormones by the pulmonary circulation we proposed that they could be divided into at least two types- "local" and "circulating" hormones. The local hormones are those which are largely removed by the lungs and if they have a physiological function, it is probably localised at or near to the site of release. It is intriguing to think that venous blood may be full of potent, as yet unidentified, chemicals released by peripheral vascular beds but removed by the lungs before they can cause effects in the arterial circulation. Interestingly, in 1970, Gryglewski and Vane described the release of an unidentified substance into the venous blood after infusion of isoprenaline into the hind legs of dogs (51). The pattern of activity of this substance on the blood-bathed assay tissues was unlike that of any prostaglandin known at that time, but it can now be clearly identified as prostacyclin.

Circulating hormones are those which pass through the lungs, either unchanged (adrenaline, histamine, vasopressin, prostacyclin) or with an actual increase in activity. One such demonstration of an increase in activity on passage through the pulmonary circulation was associated with the renin-angiotensin system. We showed (52) that, contrary to popular belief, conversion of angiotensin I to angiotensin II did not take place in the bloodstream, but was largely accomplished in the pulmonary circulation. This was demonstrated both *in vivo* (52-54) and *in vitro* (Fig. 5).

iii. Our studies of substances released during anaphylaxis

Piper and Vane (14,55,56) made a series of studies in which they investigated the release of mediators from perfused lungs isolated from sensitised guinea pigs, We found, as expected, that there was a large release of histamine when the lungs were challenged with antigen. As also expected, we found a release of SRS-A. However, we were excited at that time to find the release of three other substances which had not previously been associated with anaphylaxis (Fig. 6). We detected the release of prostaglandin-like substances with our bioassay system and were later able to show by thin layer chromatography that prostaglandins E_2 and $F_{2\alpha}$ were present in the effluent. Even more exciting was the detection of the release of a previously undescribed substance which caused a strong contraction of strips of rabbit aorta and which we called, because of this effect "rabbit aorta contracting substance" or RCS. Two properties of RCS intrigued us. First, it was chemically unstable and if we introduced a delay coil of a few minutes before the lung effluent reached the assay tissues the activity had disappeared, although that of histamine, PGE_2, $PGF_{2\alpha}$ and SRS-A was still present. Second, we found that the release of RCS during anaphylaxis was

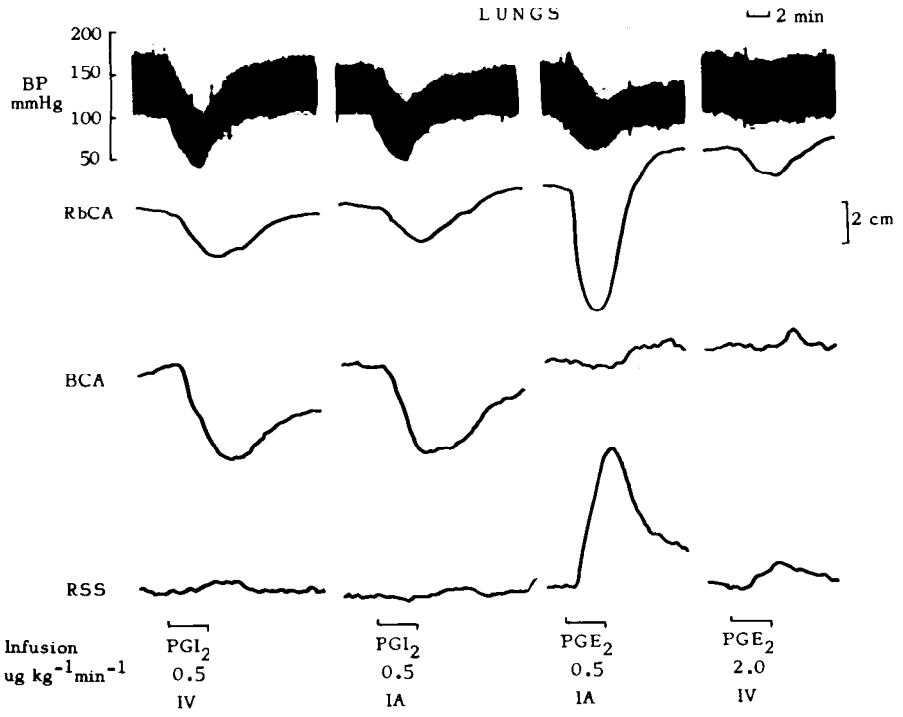

Figure 4. Passage of prostacyclin (PGI_2) and prostaglandin E_2(PGE_2) across the lungs. Spiral strips of rabbit coeliac artery (RbCA), bovine coronary artery (BCA) and rat stomach strip (RSS) were bathed in arterial blood from a dog. $PG1_2$ infused intravenously (i.v.) caused similar effects on the bioassay tissues and blood pressure (BP) as infusion into the root of the aorta (i,a.) indicating that $PG1_2$ did not disappear across the lungs. In contrast, much more than 75% of PGE_2 was inactivated in passage through the pulmonary circulation. (From Dusting, Moncada and Vane, 1978 (reference 49), by permission of The Macmillan Press Ltd.).

selectively prevented by aspirin and other similar compounds. Piper and Vane (14) postulated that RCS may be involved in causing those symptoms which aspirin relieves. Isolated lungs also released prostaglandins into the perfusate when particles (up to 120 microns) were infused into the pulmonary artery (57).

At this time we were also becoming interested in prostaglandin release from other tissues. Mammalian cells of all types disgorge prostaglandins at the slightest provocation, but the tissue content of prostaglandins is very low compared with the release. This is well illustrated in the dog spleen, from which less than 1 μg (or 7 ng per g wet weight) can be extracted; however, the spleen can release up to 10 μg of prostaglandin (assayed as PGE_2) per minute when it is stimulated. Horton and his colleagues (58) were the first to demonstrate that PGE_2 was released into splenic venous blood following splenic nerve stimulation in the dog. We became interested in the characteristics of this release and further showed that the output (PGE_2 and $PGF_{2\alpha}$) was associated with contraction of the spleen, for it could be induced by adrenaline and

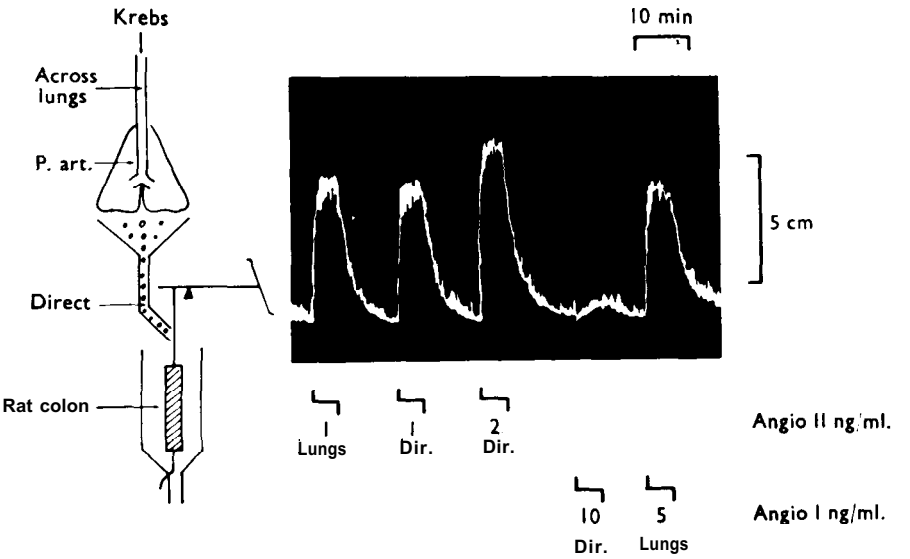

Figure 5. Increased activity of angiotensin I when passed through guinea pig isolated lungs. The diagram on the left shows the experimental procedure. Infusions of angiotensin II (Angio II) through the lungs or direct to the rat colon (Dir.) gave the same response of the rat colon, showing that there was no destruction in the lungs. An infusion of angiotensin I (Angio I) at 10 ng/ml direct to the rat colon gave a minimal response, but when half this concentration (5 ng/ml) was infused through the lungs there was a much greater contraction of the rat colon. Time, 10 min, vertical scale 5 cm. (From Vane, 1969, reference 33, by permission of The Macmillan Press Ltd.).

prevented by α-adrenoreceptor antagonists. Interestingly, as in the lungs, we found that prostaglandins were released by the spleen in response to infusions of particles (59).

2. ASPIRIN AND PROSTAGLANDIN BIOSYNTHESIS

In research there is always a "climate" of experience which acts as a background to important discoveries. I have tried to indicate the "climate" in our laboratory at The Royal College of Surgeons of England around the year 1970. We had a major interest in the release and fate of vasoactive hormones and were pursuing this with especial reference to the lungs. We had discovered RCS as an unstable substance released from lungs during anaphylaxis, and we knew that its release was inhibited by aspirin and other asprini-like drugs. We had become interested in the prostaglandins and had come to the idea that any tissue which was distorted or disturbed or traumatised would release prostaglandins. In this context, it seemed to me that each distension of the lungs might cause a prostaglandin release which could help to adjust the regional pulmonary blood flow. Indeed, the idea that a prostaglandin release might be important in controlling regional blood flow in the lungs had also been suggested by Liljestrand (60). With over-distension, any prostaglandins released might be detected in the arterial blood stream. I started a series of experiments

using the blood-bathed organ technique to test this hypothesis. In anaesthetised dogs it was easy to show that when they were hyperventilated, there was an output into the arterial blood from the pulmonary circulation of an RCS-like substance and of PGE_2 and $PGF_{2\alpha}$ (Vane, unpublished). It was at this time that I was impressed by the effects of an infusion of aspirin into the hyperventilated dog, for not only was the associated hypotension reduced, but there was also a strong inhibition of the prostaglandin release. It was this experiment that led me to the idea (over a weekend) that aspirin might be interfering with prostaglandin biosynthesis. On the Monday morning I said to Sergio Ferreira and Priscilla Piper "I think I know how aspirin works" and set about doing an experiment. Änggård and Samuelsson (61) had described a preparation of guinea pig lungs in which a crude cell-free homogenate was used to convert arachidonic acid into PGE_2 and $PGF_{2\alpha}$. Although I was inexperienced in working with biochemical techniques, for I have always believed in using whole animals or organs whenever possible, I homogenised some guinea pig lungs, spun off the cell debris, divided the supernatant into test-tubes, added arachidonic acid and measured by bioassay the amounts of PGE_2 and $PGF_{2\alpha}$ formed. To some of the tubes was added aspirin, indomethacin or morphine. By the end of that day I was convinced that aspirin and indomethacin (but not morphine) strongly inhibited the formation of prostaglandins from arachidonic acid (see Fig. 7).

During the time that I was confirming and extending this first experiment, Ferreira and Moncada (12) began to study the effects of aspirin and indomethacin on prostaglandin release from the spleen (Fig. 8). Independently of these observations, Smith and Willis were using platelets to measure the effects of aspirin on prostaglandin formation. The results of these three studies were published simultaneously in 1971 (l0-12). Vane (10) developed the hypothesis that this biochemical intervention in prostaglandin formation by the aspirin-like drugs is the basis of their therapeutic action.

An explanation of the therapeutic action of aspirin and its congeners had long been sought in terms of inhibition of a specific enzyme or biological function. Although these drugs inhibited a wide variety of enzymic reactions *in vitro* no convincing relationship could be established between such inhibition and their known anti-inflammatory, antipyretic and analgesic actions. This was largely because of the high concentrations needed for enzyme inhibition. At the time that we discovered that aspirin-like drugs inhibited the biosynthesis of prostaglandins in low concentrations, there was some evidence that the prostaglandins participated in the pathogenesis of inflammation and fever, and this reinforced the suggestion that inhibition of prostaglandin biosynthesis could explain the clinical action of these drugs. In the years which have elapsed since the original observations, a considerable body of evidence has accumulated which supports this hypothesis. Our knowledge about the inflammatory process has also increased, and the way in which prostaglandins participate in this process has been considerably clarified.

Inhibition of prostaglandin biosynthesis is a property peculiar to the aspirin-like drugs, since many otherwise pharmacologically active agents are inactive

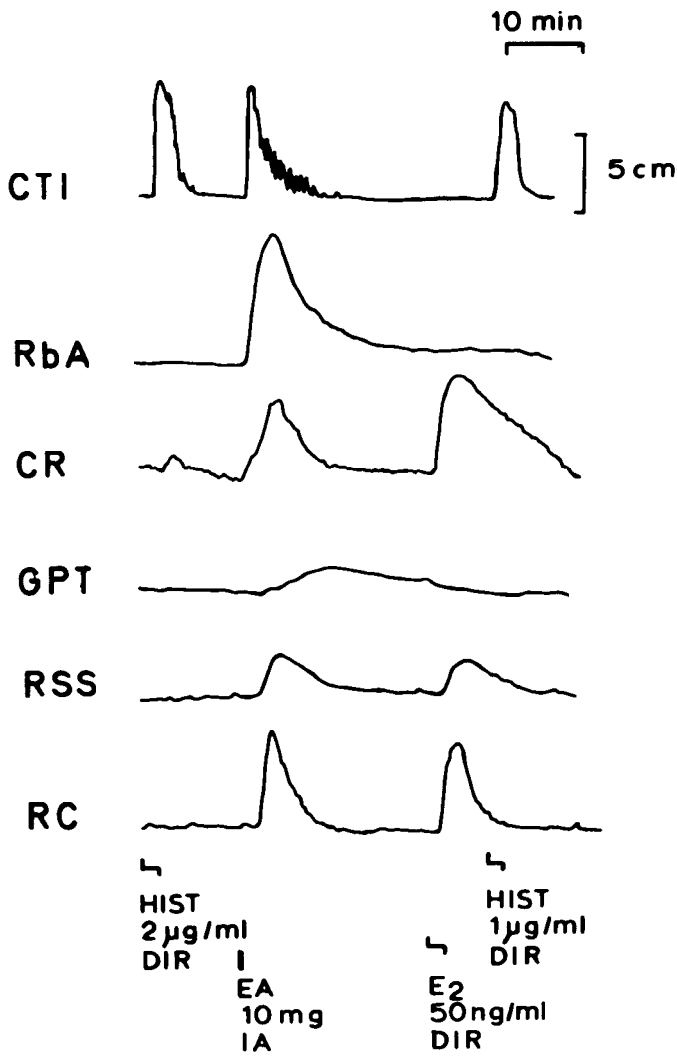

Figure 6. Release of mediators from isolated lungs of sensitised guinea pigs. The lungs were perfused through the pulmonary artery with Krebs' solution and the effluent superfused a cat terminal ileum (CTI), rabbit aorta spiral strip (RbA), chick rectum (CR), guinea pig trachea (GPT), rat stomach strip (RSS) and rat colon (RC). All tissues except CT1 were blocked with antagonists to 5HT, catecholamines and histamine. Infusions of histamine (2 µg and 1 µg/ml DIR) and of prostaglandin E_2 (E_2, 50 ng/ml DIR) directly to the assay tissues demonstrated the selective sensitivity of the assay system. Anaphylaxis was induced in the lungs by injecting ovalbumen intra-arterially (EA 10 mg I.A.). Contractions of CT1 demonstrated release of histamine, RbA release of RCS, GPT release of SRS-A and CR, RSS and RC release of prostaglandins. Time 10 min; vertical scale 5 cm. (Piper and Vane, published in Vane, 1971, reference 163, by permission of The Ciba Foundation).

against this enzyme system, including the opiates, antihistamines, α-and β-adrenoreceptor blocking agents and antagonists of acetylcholine and 5HT. The anti-inflammatory steroids are also inactive against this enzyme although they

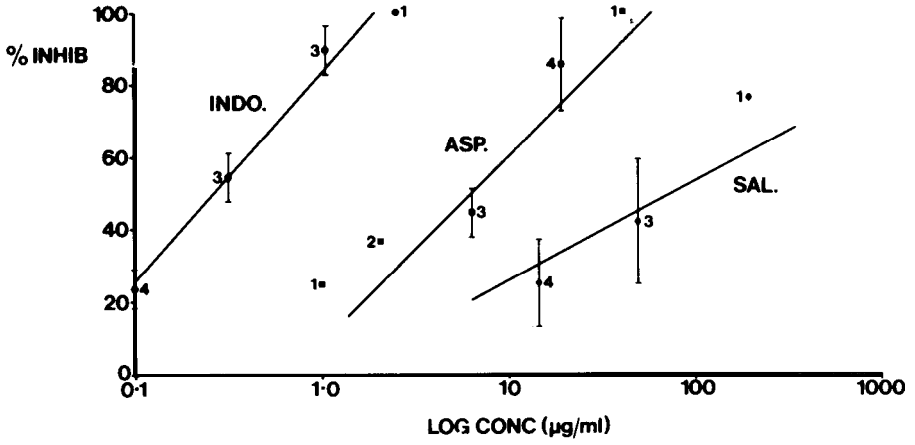

Figure 7. Concentration (µg/ml) of indomethacin (●), aspirin (■) and salicylate (◆) plotted on a log scale against the percentage inhibition of prostaglandin synthesis (assayed as $PGF_{2\alpha}$ on rat colons). The lines are those calculated for best fit. Numbers by the points indicate number of experiments. When three or more estimates were averaged, the standard error of the mean is shown. (From Vane, 1971, reference 10. Reprinted by permission from Nature. Copyright (c) 1971 Macmillan Journals Limited).

can reduce prostaglandin production by inhibition of phospholipase A_2 (for review see Flower, Blackwell, Di Rosa and Parente, 62).

Any hypothesis which purports to explain the action of a drug in terms of an anti-enzyme action must satisfy at least two basic criteria. First, the free concentrations achieved in plasma during therapy must be sufficient to inhibit the enzyme in question. Second, there must be a reasonable correlation between the level of anti-enzyme activity and the therapeutic potency. Clearly, there is abundant evidence to show that both these criteria are satisfied and there is also good evidence that therapeutic dosage reduces prostaglandin biosynthesis in man (for review, see Vane, Flower and Salmon, 63).

3. PROSTACYCLIN

a. The advent of the prostaglandin endoperoxides and the discovery of prostacyclin

The isolation by Samuelsson and others of the prostaglandin endoperoxides in the early 1970's was a major step forward in prostaglandin research (see Samuelsson in this volume). The demonstration that the endoperoxides caused platelet aggregation and that they were transformed in platelets to TXA_2 led us and others to the conclusion that most of the activity associated with RCS was due to TXA_2 (64, 65) (Fig. 9).

From Samuelsson's work we knew that TXA_2 could be released by platelets. We isolated the enzyme from the "microsomal" fraction of platelets and showed by our bioassay techniques that endoperoxides, when incubated with this fraction (even at O°C), were rapidly transformed into TXA_2 which potently contracted rabbit aorta and induced platelet aggregation. This enzyme, which

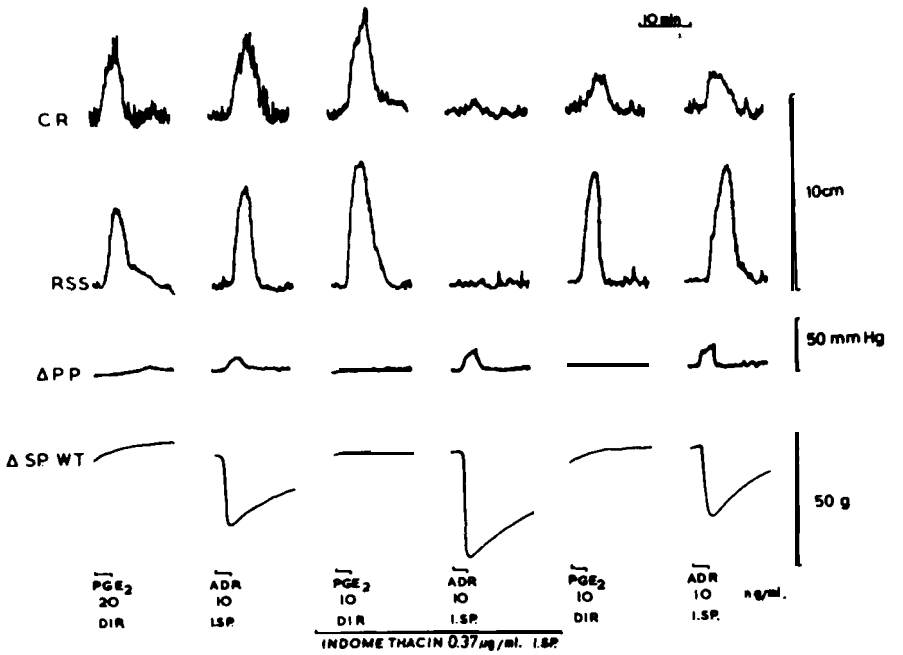

Figure 8. A spleen from a dog was perfused with Krebs'-dextran solution at a rate of 20 ml/min. A continuous sample (10 ml/min) of the splenic outflow, with antagonists to histamine, 5HT and catecholamines added, was used to superfuse the assay tissues. The figure shows the effects of prostaglandins on a chick rectum (CR; top) and a rat stomach strip (RSS). The next two tracings (bottom) show changes in perfusion pressure (PP) and spleen weight (SP.wt.). Except when infused into the spleen indomethacin was added to the splenic outflow to give a concentration of 0.37 μg/ml. The first panel shows contractions of CR and RSS induced by prostaglandin E_2 (20 ng/ml DIR), Next an adrenaline infusion into the spleen (ADR 10 ng/ml 1. SP) induced a rise in perfusion pressure, a fall in spleen weight and an output of prostaglandins equivalent to PGE_2 at about 20 ng/ml. Indomethacin (0.37μg/ml) was then infused into the spleen. During the next 25 min the assay tissues relaxed (not shown) and were then more sensitive to PGE_2 (10 ng/ml DIR). Adrenaline (40 min after start of indomethacin) now caused a greater increase in perfusion pressure, a greater decrease in spleen weight, but no output of prostaglandin. After stopping the indomethacin infusion into the spleen, the reactivity of the assay tissues gradually decreased and the output of prostaglandin induced by adrenaline infusion into the spleen gradually returned. The adrenaline stimulation shown was made 70 min after stopping the indomethacin. (From Ferreira, Moncada and Vane, 1971, reference 12. Reprinted by permission from Nature. Copyright (c) 1971 Macmillan Journals Limited).

we called "thromboxane synthetase" (66, 67) is now an important therapeutic target for the development of compounds with anti-thrombotic potential.

Moncada, Gryglewski and Bunting then began to look at other tissues to determine whether they also could generate TXA_2. To do this, they took microsomal fractions of several different tissues and measured, again with the superfusion cascade bioassay, formation of either the classical stable prostaglandins E_2 and $F_{2\alpha}$, or of TXA_2. It was Moncada's suggestion that we should look into the biosynthetic system of vascular tissue, since vascular endothelium and platelets might share some structural features. Indeed, after several weeks of work we found that microsomal fractions of pig aorta incubated with the

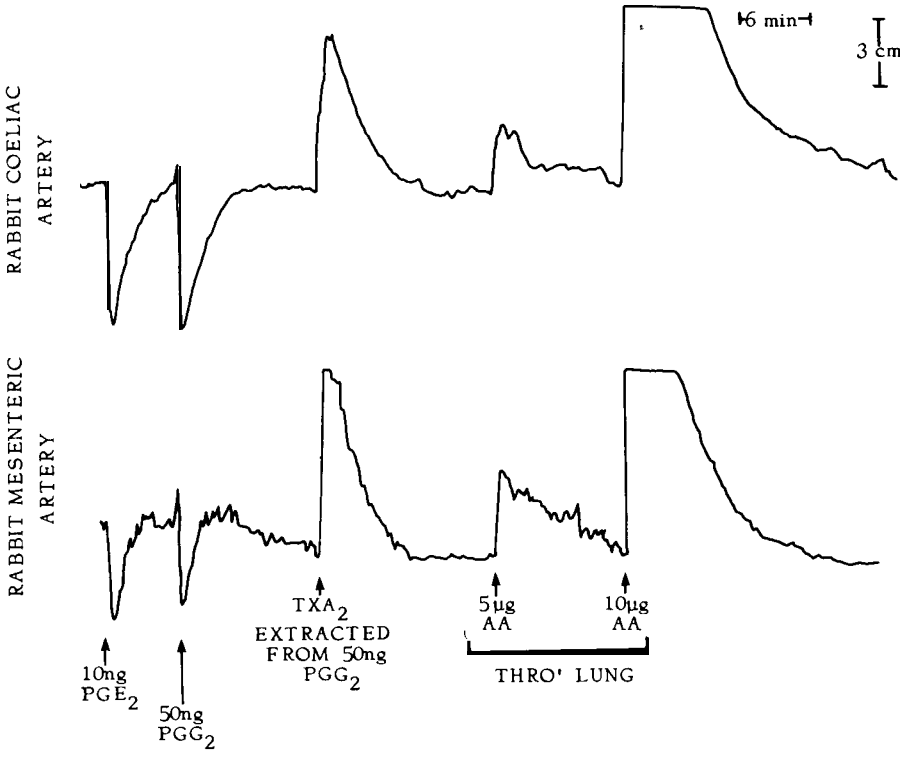

Figure 9. Rabbit coeliac and mesenteric artery strips were superfused with the outflow of a pair of guinea pig lungs perfused with Krebs' solution at 10 ml/min. Prostaglandin E_2 (10 ng) relaxes the two tissues as does PGG_2. Thromboxane A_2 generated from 50 ng PGG_2 produced a contraction. Challenge of the lungs with arachidonic acid (AA 5 and 10 µg) produces a dose-dependent release of TXA_2-like material. (From Moncada and Vane, 1977, reference 65, by permission of Academic Press Inc [New York]).

endoperoxide did not generate classical prostaglandins even though the endoperoxide activity (measured as RCS) disappeared. We eventually came to the conclusion that the endoperoxide was being transformed into an unknown prostaglandin and began to refer to this substance as PGX. By using recently-developed bioassay tissues such as the rabbit coeliac and mesenteric arteries (30) we were able to distinguish between the endoperoxides (which caused a biphasic effect) and PGX (which only relaxed them). Importantly, PGX, in contrast to TXA_2, inhibited the clumping of platelets. Like TXA_2, it was also unstable, with a half life of two minutes at 37°C. Boiling the solution for 15 seconds destroyed all measured activity.

The first paper on PGX was published by Moncada, Gryglewski, Bunting and Vane in Nature in October 1976 (16). Although the structure was then unknown, many of the characteristics of PGX were described, together with some important concepts. PGX was different from the other products of PG endoperoxides and its biological activity on isolated tissues, its instability and its potent anti-aggregatory activity, distinguished it from PGD_2, PGE_2, $PGF_{2\alpha}$,

TXA_2 and TXB_2. PGX relaxed strips of rabbit mesenteric and coeliac arteries, but contracted rat stomach strip, chick rectum, guinea pig' tracheal chain and guinea pig ileum, although its contractile potency on these tissues was less than that of the classical prostaglandins. The rat colon was not contracted by PGX: indeed, spontaneous movement was decreased.

In this first paper, the transformation of the PG endoperoxides by platelets to TXA_2, which caused platelet aggregation and vascular contraction, was contrasted with their transformation by blood vessel microsomes to PGX, which had potent anti-aggregatory properties and relaxed vascular strips. Thus, the concept was suggested that a balance between the amounts of TXA_2 formed by platelets and PGX formed by blood vessel walls might be critical for thrombus formation. Indeed, in the light of the discovery of this anti-thrombotic property associated with arterial walls, we recalled the pre-Lister vitalistic view that in some way the arteries kept the blood fluid.

We also developed the concept that platelets attempting to stick to vessels may release endoperoxides which are then used by the blood vessel wall to generate PGX, thus limiting or preventing further platelet clumping. We also suggested that plaque formation on the arterial wall could hinder access of platelet endoperoxides to the PGX generating system. These important properties and concepts are now well established and developed (for review, see Moncada and Vane, 68).

The structure of PGX was established through a collaborative research programme between scientists at the Upjohn Company in Kalamazoo and The Wellcome Foundation Ltd. in Beckenham (19). This also led to the first chemical syntheses of the substance which was renamed prostacyclin (PGI_2) (19, 69).

There is now a plethora of names for prostacyclin. The chemical name is 5-{(lS,3Z,5R,6R,7R)-7-hydroxy-6-[(lE,3S)-3-hydroxy-1-octenyll-2-oxabicyclo [3.3.0]oct-3-ylidene pentanoic}acid. As a freeze-dried pharmaceutical preparation, the approved name is Epoprostenol and the trade names are Flolan (Wellcome) and Cycle-prostin (Upjohn). To maintain consistency in the scientific literature, the trivial name, prostacyclin, should be used whenever possible.

b. The formation and properties of prostacyclin

Prostacyclin is the main product of arachidonic acid in all vascular tissues so far tested including those of man (Fig. 10). The ability of the large vessel wall to synthesise prostacyclin is greatest at the intimal surface and progressively decreases toward the adventitia (70). Culture of cells from vessel walls also shows that endothelial cells are the most active producers of prostacyclin (71, 72).

Prostacyclin relaxes isolated vascular strips and is a strong hypotensive agent through vasodilation of all vascular beds studied, including the pulmonary and cerebral circulations. (For review, see Moncada and Vane, 73). Several authors have suggested that prostacyclin generation participates in or accounts for functional hyperaemia (74, 75).

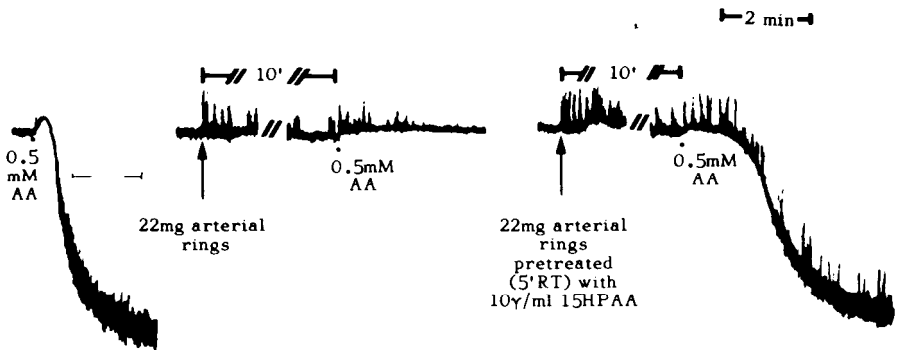

Figure 10. Inhibition of platelet aggregation by rings of human vascular tissue. Cut rings (15-30 mg), when incubated at 37°C in human platelet rich plasma (P.R.P.) for 10 min, inhibited the aggregation produced by 0.5 mmol arachidonic acid (AA). When the rings were pretreated by incubation with 15 hydroperoxyarachidonic acid (15-HPAA) (10 μg/ml) for 5 min at 22°C, and then added to the P.R.P., aggregation was once more observed after addition of 0.5 mmol AA. (From Moncada, Higgs and Vane, 1977, reference 164, by permission of The Lancet).

Prostacyclin is the most potent endogenous inhibitor of platelet aggregation yet discovered. This effect is short-lasting in *vivo,* disappearing within 30 minutes of cessation of intravenous administration. Prostacyclin disperses platelet aggregates *in vitro* (16, 76) and in the circulation of man (77). Moreover, it inhibits thrombus formation in models using the carotid artery of the rabbit (76) and the coronary artery of the dog (78), protects against sudden death (thought to be due to platelet clumping) induced by intravenous arachidonic acid in rabbits (79), and inhibits platelet aggregation in pial venules of the mouse when applied locally (80).

Prostacyclin inhibits platelet aggregation by stimulating adenylate cyclase, leading to an increase in cAMP levels in the platelets (81, 82). In this respect prostacyclin is much more potent than either PGE_1 or PGD_2, and its effect is longer-lasting. In contrast to TXA_2, prostacyclin enhances Ca^{++} sequestration in platelet membranes (83). Moreover, inhibitory effects on platelet phospholipase (84, 85) and platelet cycle-oxygenase (86) have been described. All these effects are related to its ability to increase cAMP in platelets. Prostacyclin, by inhibiting several steps in the activation of the arachidonic acid metabolic cascade, exerts an overall control of platelet aggregability.

Prostacyclin increases cAMP levels in cells other than platelets (for review, see Moncada, 87) raising the possibility that in these cells a balance with the thromboxane system exerts a similar homeostatic control of cell behaviour to that observed in platelets. Thus, the prostacyclin/TXA_2 system may have wider biological significance in cell regulation. An example is that prostacyclin inhibits white cell adherence to the vessel wall (88, 89) to nylon fibres and to endothelial monolayers *in vitro* (90). Prostacyclin increases cAMP in the endothelial cell itself, suggesting a negative feedback control for prostacyclin production by the endothelium (91-93).

One of the functional characteristics of the intact vascular endothelium is its non-reactivity to platelets: clearly, prostacyclin generation could contribute to this thromboresistance. Moreover, prostacyclin inhibits platelet aggregation (platelet-platelet interaction) at much lower concentrations than those needed to inhibit adhesion (platelet-collagen interaction) (94). Thus, prostacyclin may permit platelets to stick to vascular tissue and to interact with it so allowing platelets to participate in the repair of a damaged vessel wall while at the same time preventing or limiting thrombus formation.

c. Prostacyclin and cytoprotection

In addition to its well-known vasodilator and anti-aggregating actions, prostacyclin shares with other prostaglandins a "cytoprotective activity", as yet not clearly defined. This activity has usually been studied on gastric ulcers (95). We have suggested (68, 96) that this third property may be important in explaining certain therapeutic effects of prostacyclin. For instance, in models of myocardial infarction, prostacyclin reduces infarct size (97-99), arrhythmias (l00), oxygen demand (99) and enzyme release from infarcted areas (101). In sheep, prostacyclin protected the lungs against injury induced by endotoxin (102). There was also a beneficial effect in endotoxin shock in the dog (103) and cat (104) where prostacyclin improves splanchnic blood flow and reduces the formation and release of lysosomal hydrolases. The effects of hypoxic damage in the cat isolated perfused liver are also substantially reduced by prostacyclin (105). Canine livers can be preserved *ex vivo* for up to 48 hours and then successfully transplanted using a combination of refrigeration, Sacks' solution and prostacyclin (106).

All these effects could be related to a result obtained recently by Moncada and colleagues (107). The addition to platelets of prostacyclin during their separation from blood and subsequent washing substantially improves their immediate functionality *in vitro.* In addition, whereas platelets normally are functional for about 6 h, when prepared with the addition of prostacyclin they remain functional for more than 72 h (107). This extended viability of platelets *in vitro* is not accompanied by a prolonged increase in levels of cAMP, thus separating the effect from the classical anti-aggregating activity (108). Interestingly, there has been a study demonstrating a dissociation between anti-aggregating and cytoprotective effects of a prostacyclin analogue in a model of acute myocardial ischaemia (109).

All these results suggest that some of the therapeutic effects of prostacyclin might be related to this cytoprotective effect and point to even wider indications for prostacyclin in cell or tissue preservation in *vivo* and *in vitro.*

d. Prostacyclin and atherosclerosis

Lipid peroxides, such as 15-hydroperoxy arachidonic acid (15-HPAA), are potent and selective inhibitors of prostacyclin generation by vessel wall microsomes or by fresh vascular tissue (Fig. 10) (17, 18, 110, 111). There are high concentrations of lipid peroxides in advanced atherosclerotic lesions (112). Lipid peroxidation induced by free radical formation occurs in vitamin E

deficiency, the ageing process and perhaps also in hyperlipidaemia accompanying atherosclerosis (113). Accumulation of lipid peroxides in atheromatous plaques could predispose to thrombus formation by inhibiting generation of prostacyclin by the vessel wall without reducing TXA_2 production by platelets. Moreover, platelet aggregation is induced by 15-HPAA, and this aggregation is not inhibited by adenosine or PGE_1 (114). Human atheromatous plaques do not produce prostacyclin (115, 116). In normal rabbits the production of prostacyclin by the luminal surface of the aorta is abolished by de-endothelialisation and slowly recovers with re-endothelialisation over a period of about 70 days. However, the recovery of prostacyclin formation did not occur in rabbits made moderately hypercholesterolaemic by diet (117). These results suggest that it would be worth exploring whether attempts to reduce lipid peroxide formation by inhibiting peroxidation influence the development of atherosclerosis and arterial thrombosis. Vitamin E acts as an antioxidant and perhaps its empirical use in arterial disease in the past (118-120) had, in fact, a biochemical rationale. For discussion of the implication of prostacyclin and TXA_2 in diseases other than atherosclerosis see Moncada and Vane (68).

e. Clinical applications of prostacyclin

Prostacyclin is available as a stable freeze-dried preparation (Epoprostenol) for administration to man. Intravenous infusion of prostacyclin in healthy volunteers leads to a dose-related inhibition of platelet aggregation, dispersal of circulating platelet aggregates, arteriolar vasodilatation, increases in skin temperature, facial flushing and sometimes headache (87, 121). Infusion of prostacyclin into patients susceptible to migraine or cluster headache induces, in most cases, a headache different from those usually experienced (122).

Extracorporeal circulation of blood brings it into contact with artificial surfaces which cannot generate prostacyclin. In the course of such procedures thrombocytopaenia and loss of platelet haemostatic function occur and make an important contribution to the bleeding problems following charcoal haemoperfusion and prolonged cardiopulmonary bypass in man. Formation of microemboli during cardiopulmonary bypass may also contribute to the cerebral complications which sometimes follow this procedure. Platelet damage and thrombocytopaenia were prevented by prostacyclin both in animal models of extracorporeal circulation (87,121) and in man.

In patients with fulminant hepatic failure undergoing charcoal haemoperfusion (123) prostacyclin infusion prevented the fall in platelet count and elevation of ß-thromboglobulin seen in the control patients. Gimson and his colleagues (124) have made almost 200 charcoal haemoperfusions on a daily basis using prostacyclin for platelet protection in the treatment of 76 patients with fulminant hepatic failure. Remarkable survival rates (65%) were obtained in the 31 patients who had been referred early and in whom the serial haemoperfusions were started whilst the signs of grade III encephalopathy were still apparent (not rousable but may or may not respond to painful stimuli). The authors thought that this was probably the major factor in the improved survival rate, a reflection of the better biocompatibility of the system because

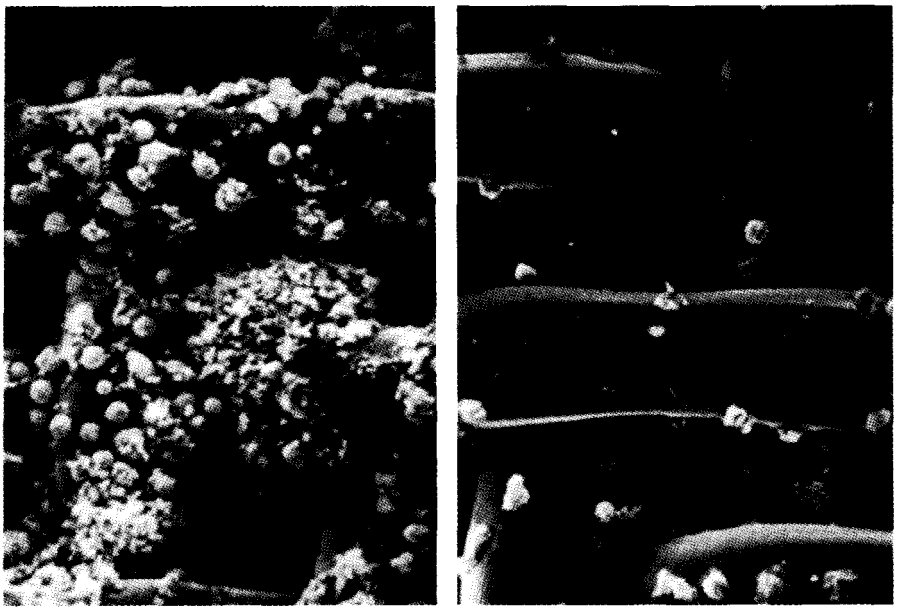

Figure 11. Filters. Electron micrographs of the downstream face of filters taken from the arterial lines of cardio-pulmonary bypass operations in man. The left hand picture (patient receiving placebo) shows formation of platelet aggregates, some clogging the pores (40 ± 5 μ) of the filter. The right hand picture (patient receiving prostacyclin) shows lack of platelet adhesion. The few cells sticking to the filter are leucocytes. (Electron micrographs kindly provided by Dr. N. Read and Mr. P. J. Astbury, Wellcome Research Laboratories).

prostacyclin was used, allowing the patients to be treated at an earlier stage. In the group treated later, with Grade IV encephalopathy already present, 20% survived, so that the overall survival rate from the 76 patients was 38%. These results (especially those treated early) compare favourably with a survival rate of 15% in patients under standard intensive care measures.

Several double blind clinical trials of prostacyclin in cardiopulmonary bypass have been published (125-131). The treatment groups showed a preservation of platelet number and function, with a reduction in the blood loss in the first 18 hours after operation. In the trial by Longmore and colleagues (130) the blood loss was halved. In that by Walker and co-workers (129), filters were used and the formation of platelet aggregates on the filters from the placebo group contrasted strikingly with the lack of platelet adhesion to those from patients treated with prostacyclin (Fig. 11). The heparin-sparing effect of prostacyclin was confirmed and the vasodilator effects were not troublesome; indeed, Nobak and colleagues (131) suggest that these effects may be utilised in controlling intra-bypass hypertension. Clearly, the use of prostacyclin or an analogue should allow improvements in the methodology of extracorporeal circulations.

Therapeutic assessment of prostacyclin is still in its infancy with many trials in progress. The results are, therefore, still preliminary, but nevertheless they point the way to conditions in which prostacyclin therapy may be useful. In

open trials, prostacyclin was of benefit to patients with peripheral vascular disease both through relief of ischaemic pain and improved ulcer healing (132-137). Placebo-controlled blind trials are now in progress, and the results of the first to be analysed (138) are encouraging. In the 13 patients infused intravenously for 4 days with placebo, 3 showed reduction in rest pain at 5 days, 2 at 1 month and 1 at 6 months. After 6 months, 3 had died and 5 others had received surgical intervention. Of those 15 patients who were infused with prostacyclin for 4 days (average 7 ng/kg/min i.v.), at 5 days all had reduction in rest pain. At 1 month, 9 still showed a substantial improvement, which was also evident in 7 patients at 6 months. By this time, two other patients in the group had received surgical intervention and one had died. Zygulska-Mach and colleagues (139) infused prostacyclin into 3 patients with sudden blockage of central retinal veins. Improvement was observed in those two patients who were treated within the first 48 hours.

Prostacyclin also induces long-lasting improvements in patientswith Raynaud's phenomenon. Intravenous infusion of the drug for 72 hours produced striking reductions in the frequency, duration and severity of the disease in 21 of 24 patients. The improvement lasted for a mean of 9-10 weeks, and in 3 patients, subjective improvement was still reported 6 months after the infusion. Pain reliefwas a striking feature presumably associated with the increased blood flow as indicated by increased temperature of the hands and fingers (140). Belch and coworkers (141) have also reported successful treatment in 4 out of 5 patients, and a double-blind clinical trial (142) has now confirmed these results in Raynaud's phenomenon. There was an overall improvement still present at 6 weeks in 6 of 7 patients receiving prostacyclin, but only in 1 of 7 receiving placebo. The prostacyclin patients had a significant fall in the number and duration of attacks over the 6 weeks period post infusion, whereas there was no change in the placebo group.

Gryglewski and his colleagues in Cracow, who first demonstrated the beneficial effects of infusion of prostacyclin in ischaemic disease of the legs, have now obtained dramatic improvements following prostacyclin infusion in 10 patients with ischaemic stroke (143). Patients with transient ischaemic attacks and haemorrhagic stroke were excluded. With prostacyclin treatment there was a reversal of symptoms strikingly sooner in all 10 patients than could have been expected and in 6 patients during the first 6 hour infusion. One patient died 2 weeks later of a second stroke, but the other 9 have maintained return of function for (so far) up to 6 months.

Prostacyclin has been successfully used in a few cases of pulmonary hypertension and is more effective than PGE_1 (144-146). Single case studies have suggested that prostacyclin may be useful in the treatment of patent ductus arteriosus (147) and pre-eclamptic toxaemia (148).

Beneficial effects of intravenous infusion of prostacyclin were obtained in 9 patients with severe congestive heart failure refractory to digitalis and diuretics (149). Mean pulmonary and systemic pressures and vascular resistances were reduced and heart rate, cardiac index and stroke index were all increased during the infusion, with facial flushing as the only side effect.

Bergman and colleagues (150) gave an intravenous infusion of prostacyclin to patients with coronary artery disease with no deleterious effects. Heart rate and cardiac index were increased and mean blood pressure, systemic and pulmonary resistance all fell. Mean atrial pacing time to angina rose from 142 to 241 seconds. They concluded that acute administration of prostacyclin was beneficial in angina, having effects similar to those of the short-acting nitrates. In 5 patients with coronary artery disease, prostacyclin was safely infused directly into diseased coronary arteries (151) and there was a beneficial effect of intravenous prostacyclin infusions in patients with unstable angina (137). However, prostacyclin had no effect on the number, severity and duration of ischaemic episodes in 8 of 9 patients with variant angina, although consistent relief was seen in the ninth patient (152).

A prostacyclin deficiency has been reported in thrombotic thrombocytopaenic purpura (TTP) (153). Infusion of prostacyclin into two patients with TTP did not produce an increase in circulating platelet count (153, 154). However, FitzGerald and colleagues (155) have reported an increase in platelet count and an improvement in the neurological status of one such patient during 18 days of prostacyclin infusion. They were sufficiently encouraged to conclude that the controlled evaluation of prostacyclin in TTP was warranted.

Infusion of prostacyclin protects transplanted kidneys from hyperimmune rejection in dogs (156) and in patients with chronic renal transplant rejection (157).

Clearly, there are many clinical conditions which may respond to prostacyclin treatment and its place (or that of chemically stable analogues) in therapeutics will be defined in the next few years. Some of these conditions are pre-eclamptic toxaemia (158) haemolytic uraemic syndrome (159) peptic ulceration (160), the thrombotic complications associated with transplant rejection (156), the prevention of tumour metastasis (161) and the treatment of pulmonary embolism (162).

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