A study of possible chemical mediators of a hypersensitivity reaction in teleost fishes.

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A STUDY OF POSSIBLE CHEMICAL MEDIATORS OF A HYPERSENSITIVITY REACTION IN TELEOST FISHES

by

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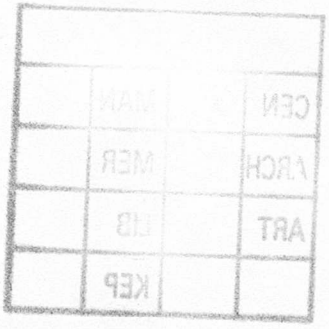

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ABSTRACT

Alan A Anderson. A study of possible chemical mediators of a hypersensitivity reaction in teleost fishes.

The manifestations of anaphylaxis vary considerably in different species. One common factor appears to be the release of pharmacological mediators, potent biologically-active substances which have a profound effect on vascular and bronchial smooth muscle. A cutaneous anaphylactic reaction in teleost fish has only recently been described and there is little information about the causal mechanisms. As a consequence, this thesis is primarily concerned with the pharmacological mediators of the response and their involvement in the overall reaction mechanism.

As a model of cutaneous anaphylaxis, chopped plaice skin was incubated with a fish Ringer saline in the presence of a fungal challenging agent, Epidermophyton floccosum. A mammalian smooth muscle contracting material was found to be released, reaching a maximum after sixty minutes incubation. Initial extraction and purification suggested the presence of a prostaglandin-like substance. Identity was confirmed as prostaglandin E_2 by mass spectrometric analysis. In addition, using a continuous perfusion technique in anaesthetized plaice, nanogram quantities of a prostaglandin-like material were recovered in subcutaneous perfusates of skin challenged with E.floccosum. The ability of the skin to synthesize prostaglandin E_2 was demonstrated by the incubation of an enzymic preparation from plaice skin with exogenous arachidonic acid. Although indomethacin abolished prostaglandin release from the skin in vitro, it did not inhibit the cutaneous reaction in whole plaice, indicating that other mediators must be involved in addition to prostaglandin. Evidence is presented that histamine, 5-HT and bradykinin are not available in significant quantities in the plaice.

Transfer of the skin reaction to flounders was observed after the injection of plaice serum and also of C-reactive protein, a factor purified from plaice serum. The implications with respect to the overall reaction mechanism in plaice are discussed in relation to the phylogenetic development of the animal kingdom.

CHAPTER ONE

INTRODUCTION

1.1 GENERAL PERSPECTIVE

The four cardinal signs of inflammation, rubor (redness), tumor (swelling), calor (heat) and dolor (pain) were first documented by Celsius in the first century A.D. This remains as the classical description of the visible manifestations of the localised response to tissue damage whereby a complex series of interrelated cellular and vascular mechanisms allow the penetration of plasma proteins and of phagocytes from the microcirculation into the damaged area. As a result, fragments of dead tissue and invasive pathogens can be destroyed and the process of tissue repair can commence.

Both the cause of inflammation and the overall mechanism of the response can vary and depend upon the nature of the injurious agent (pathogen, heat, trauma), the site of injury and on the state of the animal. However, one common feature in each case is the release of potent, biologically active substances of low molecular weight which exert their effect on the microcirculation so that the vascular components can penetrate to the damaged area of the tissue. Of the various signals and mechanisms involved in the inflammatory response, perhaps the most challenging and relevant to many problems of human and veterinary medicine is that observed for specific antigens. The overall mechanism involves the antibody system and sensitised lymphocytes.

It is in this general context that this investigation is concerned with an immune hypersensitivity reaction in teleost fish. Hypersensitivity has only recently been described in the lower vertebrates and there is practically no information about the mechanisms involved. For this reason, this thesis is particularly concerned with the chemical mediators of the response in teleost fish as a first step in the study of the overall mechanism. This general perspective is now followed by a more detailed outline of the extensive knowledge of the inflammatory response, which has been derived mainly from the study of higher vertebrates including man and his domestic animals.

1.2 INFLAMMATION

Inflammation has been described as the response of living tissue to injurious stimuli (Spector & Willoughby, 1968) and is probably a basic defence reaction. The inflammatory response provides a mechanism for the elimination of the foreign agent and the subsequent repair of damaged tissue. Naturally, it is often viewed as being of benefit to the host. However, the inflammatory reaction can occasionally be the cause of severe disease states such as gout and rheumatoid arthritis.

In mammals, the inflammatory response is critically dependent upon intact blood vessels and the circulating cells and fluids within these channels. The portion of the vasculature involved is called the microcirculation or the terminal vascular bed, which consists of a terminal arteriole, a thoroughfare channel, a network of true capillaries and a venule. The blood-tissue barrier is made up of an endothelial lining resting on a basement membrane, the latter completely or incompletely surrounded by smooth muscle cells. Endothelial cells interlock, forming so-called intercellular junctions.

The vascular events in inflammation usually start with a transient arteriolar constriction. Chemical mediators such as histamine are released as a result of injury and cause a localised dilatation of capillaries and venules. This effect is superimposed upon the arteriolar dilatation stimulated by an antidromic reflex in the region of the damaged area and results in erythema due to blood pooling. The endogenous mediators also increase vascular permeability which leads to vascular leakage and the formation of œdema. Whereas erythema involves pooling of blood in arterioles and venules, œdema results from events occurring mainly in the venules (Majno, Ryan, Gabbiani, Hirschel, Irle & Joris, 1972). The endothelial cells contract, adjacent cells separate from one another and gaps are created at the intercellular junctions (Majno, Shea & Leventhal, 1969; Joris, Majno & Ryan, 1972). Cells and fluid constituents cross the endothelium by passing through these gaps. The underlying basement membrane then presents a seemingly continuous barrier to transport. The mechanism by which cellular passage is accomplished is poorly understood but it has been suggested that the basement membrane opens like a curtain at the same sites where endothelial gaps are formed and gives passage to cells and particles (Bohm, 1977).

The leakage of plasma from the vessels can play an important role in defence mechanisms. The plasma can dilute bacterial toxins and carries antibodies and certain factors which attract blood cells to the damaged area, cells which are actively phagocytic for micro-organisms and for other foreign material. The lymphatic system is also stimulated to effect drainage from the injured area.

One characteristic of the inflammatory response is its relative lack of specificity. Thus the same or closely related responses are observed following physical or chemical damage, bacterial or viral injury or immunological reactions. The common factor is probably the release of the pharmacologically-active agents which produce the classical symptoms.

1.3 HYPERSENSITIVITY IN MAMMALS

The introduction of a specific antigen into a host is followed by the induction of an immune response. Further contact with the same antigen leads to a more vigorous response, which can have either a protective function or can cause tissue damaging reactions : the former has been termed immunity and the latter hypersensitivity.

Immunopathological events mediating tissue injury have been divided by the scheme of Coombs and Gell (1968) into four types : type I or anaphylactic, type II or cytotoxic, type III or immune complex-mediated and type IV or cell-mediated hypersensitivity. Types I, II and III are immediate-type reactions and involve an interaction of specific antigen with humoral antibody. Type I reactions only occur if the antibody is fixed to cells. This sensitivity can be passively transferred by the serum of the sensitised host. The reactions are usually only observed in vascularized tissues and depend largely upon changes in the microvasculature. In contrast,type IV or delayed hypersensitivity requires the prolonged contact of antigen with lymphocyte-bound receptors and reactivity cannot be transferred with serum antibody from a sensitised to a non-sensitised individual. One prime example is contact dermatitis. Cellular sensitivity can be demonstrated in tissue cultures and the reaction can be elicited in non-vascular tissues.

The most rapid hypersensitivity reaction of the immediate type is known as anaphylaxis (type 1 hypersensitivity). This is characterised by an explosive response occurring within minutes of giving the challenging dose and can be systemic or localised, depending upon the mode of application of the antigen : intravenous injection will lead to shock phenomena and intradermal injection to the development of the inflammatory reactions.

The manifestations of sytemic anaphylaxis vary in different species, in which the "shock" organs which are affected are different. A few minutes after a guinea-pig has been injected intravenously with the second challenging dose of antigen, the animal will show signs of distress. It will sneeze, cough, gasp for breath, give convulsive kicks and in severe cases will collapse and die. In less sensitive animals, death may not occur for thirty minutes or more. Asphyxia is the immediate cause of death, primarily due to bronchial oedema and bronchoconstriction. In contrast, the most important pathological result of anaphylaxis in the dog is hepatic engorgement, probably due to constriction of hepatic veins. Anaphylactic death occurs relatively infrequently in the rabbit following injection of specific antigen but when it does happen, it usually takes place very rapidly. Autopsy reveals that congestive heart failure is the main cause of death. In man, generalised anaphylaxis exhibits many symptoms, including itching, erythema, vomiting and respiratory distress. In severe cases, laryngeal oedema and vascular collapse may result in death. The pathological changes following anaphylactic shock in various animals, of which this is merely a synopsis, has been described in detail by Seegal (1935).

Arthus (1903) was the first to observe that the subcutaneous injection of a non-toxic foreign protein in an appropriately sensitised animal will produce haemmorrhage, oedema and finally, over the course of about two days, necrosis, with the formation of an abscess at the site of inoculation. The so-called "Arthus" phenomenon has also been observed in lung (Opie, 1924), testicle (Long & Seyfarth, 1924), the joints (Landouzy, Gougerot & Salin, 1910) and the peritoneum (Longcope, 1923). A more "immediate" type of skin anaphylaxis is known as the wheal and flare inflammatory reaction. It is observed following the intradermal injection of a minute amount of specific antigen into a sensitised animal and does not result in necrosis, but clears up completely within a few hours. In animals, the cutaneous reaction is similar to that in man but

is less readily visible. The local increase in vascular permeability may be demonstrated using an albumin-bound dye, such as Evans blue (Ramsdell, 1928) which leaks out of vessels at the reaction site and stains the tissues. Localised anaphylaxis can also be passively transferred between animals and is termed passive cutaneous anaphylaxis (Ovary, 1964). Antibody is injected intradermally and, after a latent period, antigen, along with Evans blue dye is given parenterally. Concentration of the dye is observed within a few minutes. The human equivalent is termed the Prausnitz-Kustner reaction (Prausnitz & Kustner, 1921) and allows a completely localised transfer. Serum from a sensitised individual is injected intradermally in a recipient. After a latent period of one to two days, antigen is injected into the same site and a localised wheal and flare reaction occurs in minutes. The latent period is required for the antibody to fix to the reactive cells. The fixing of anaphylactic antibodies can be demonstrated by adding antigen to a bath containing the isolated ileum or uterus of a sensitised guinea-pig. An abrupt smooth muscle contraction is then observed (Schultz, 1910; Dale, 1913).

The pathological symptoms of anaphylaxis seem to be surprisingly different in different species. It is apparent however, that contraction of smooth muscle and increased vascular permeability can account for most of these symptoms (Seegal, 1935). The difference observed may be due, in part, to variations in the quantity of smooth muscle present in these organs and also to the intrinsic differences in the degree of sensitivity which organs may possess in different species. Variation in the distribution of pharmacologically active mediators, sensitive vessels and smooth muscle will also be important.

1.4 ANTIBODIES IN MAMMALS

The first information regarding the chemical structure of antibodies was provided by Tiselius in 1937. He demonstrated that the fraction of serum proteins known as the γ -globulins, which migrated most slowly during electrophoresis, contained most of the serum antibodies. It is now apparent that the so-called immunoglobulins are protein molecules which share many antigenic, structural and biological similarities but differ in their primary amino acid sequence ; this enables their function as antibodies to be highly specific.

In man, five major structural types or classes are known to exist; immunoglobulin G (abbreviated to Ig G), IgM, IgA, IgD and IgE. The tissue-fixing antibodies which react with antigen to produce immunological reactions (reaginic or homocytotropic antibodies) have proved difficult to identify owing to the extremely low levels in the serum. However, in recent years, considerable functional evidence has accumulated to suggest that IgE is critical in acute hypersensitivity reactions in humans (Kay & Austen, 1971; Orange, Kaliner & Austen, 1971). There is, in addition, a clinical association between elevated circulating levels of IgE and allergic asthma (Johansson, 1967). Moreover, histological studies have shown that IgE is selectively bound to mast cells and basophils as detected by electron microscopy (Sullivan, Grimley & Metzger, 1971) and autoradiography (Ishizaka, Tomioka & Ishizaka, 1970). Although the most effective homocytotropic antibodies belong to the IgE class, it is clear that in some experimental animals, IgG antibodies can also act as reagins (Stechschulte, Orange & Austen, 1970). The extent of their contribution to the allergic state in humans is not clear.

A variety of immunological mechanisms may be operative in immediate-type reactions, but most have in common a mediation pathway which involves the release of pharmacologically-active substances from target cells, which leads to the observed changes in the target organs. The main cell reservoirs of the vasoactive amines, mast cells and basophils, have been extensively studied. Many early investigators used perfused or chopped, sensitised lungs (Brocklehurst, 1960) and measured the release of active substances following antigen challenge. More recently, the use of isolated preparations of peritoneal mast cells or blood leukocytes containing basophils has provided a more defined system. Thus, many investigators have observed the release of mediators from mast cells and basophils coated with IgE antibody following reaction with antigen or anti-IgE antibody (Ishizaka et al., 1970; Kay & Austen, 1971; Kulczycki, Isersky & Metzger, 1974). It seems clear that the cells become coated with antibodies of the IgE class bound specifically to sites on their cell surface. Cross-linking of the bound antibody with specific antigen induces a membrane signal which leads to degranulation of the cell (Roitt, 1977a). The result is the appearance intracellularly of unstored mediators and the external secretion of both preformed and newly-generated mediators (Austen, Lewis, Stechschulte, Wasserman, Leid & Goetzl, 1974; Austen, Lewis, Wasserman & Goetzl, 1975).

1.5 MEDIATORS OF HYPERSENSITIVITY REACTIONS IN MAMMALS

Over the last sixty years, a number of chemical agents have emerged as potential mediators of immediate hypersensitivity. These include: histamine, 5-hydroxytryptamine, polypeptides, anaphylatoxins slow-reacting-substance of anaphylaxis and prostaglandins. Other substances which may be released during anaphylaxis include platelet activating factor, several chemotactic factors and catecholamines.

The observation of Dale & Laidlaw in 1919 that histamine is capable of imitating the anaphylactic reaction of smooth muscle *in vitro* and, to a certain degree, on intravenous injection *in vivo*, brought the first evidence of a possible participation of histamine in these processes. Barger & Dale (1911) and Abel & Kubota (1919) extracted histamine from tissues and paved the way for later proof that histamine exists in a preformed state but loosely-bound and therefore in an inactive form (Best, Dale, Dudley & Thorpe, 1927; Harris, 1927; Lewis & Harmer, 1927). Riley (1959) has shown that there is a consistent correlation between histamine content and mast cell numbers in a variety of tissues. Moreover, mast cell degranulation is usually followed by a release of histamine. Other cells in which histamine is present include platelets (Humphrey & Jaques, 1954) and basophils (Graham, Lowry, Wheelwright, Lenz & Parish, 1955).

Direct proof for the release of histamine in systemic anaphylaxis was demonstrated in 1932 (Bartosch, Feldberg & Nagel, 1932; Dragstedt & Gebauer-Fuelnegg, 1932) and has since been confirmed by many authors (Daly, Peat & Schild, 1935; Code, 1939; Katz, 1940). There is also some evidence that histamine is released at the site of cutaneous anaphylaxis (Katz, 1942; Søndergaard & Greaves, 1971), although considerably less is known about the mechanisms involved.

Some doubt arose as to whether histamine release could explain all the manifestations of anaphylaxis. In the guinea-pig, there is a very close similarity between the symptoms following histamine injection and anaphylactic shock and in this animal it is probably the principal mediator. However, exogenous histamine cannot, as a rule, mimic the manifestations of anaphylaxis (Kellaway, 1930; Schild, 1936 a & b) and anti-histamines are not as effective against anaphylactic reactions as against added histamine (Schild, Hawkins, Mongar & Herxheimer, 1951; Hawkins & Rosa, 1956).

It has been suggested that this is merely a concentration phenomenon (Schild, 1956; Paton, 1957). An alternative explanation for this discrepancy is that it is due to the release of other active substances which are superimposed upon histamine release.

5 - hydroxytryptamine (5-HT), like histamine, is an endogenous biogenic amine which has potent effects on small blood vessels and smooth muscles in certain mammalian species. Rat and mouse mast cells, but not those of many other species, are rich in 5-HT (Keller, 1957; Parratt & West, 1957; Day & Green, 1959; West, 1959). Moreover, this amine is stored in the same cytoplasmic granules that contain histamine (Austen, 1971). 5-HT is also found in basophils (Aborg, Novotny & Uvnas, 1967) and in most mammals, platelets act as reservoirs of 5-HT. In 1955, Humphrey & Jaques observed that, in the course of an antigen-antibody reaction in vitro, 5-HT, as well as histamine, is released from rabbit platelets. There is also both direct and indirect evidence supporting the hypothesis that 5-HT may participate in anaphylactic reactions in rats and mice (Sanyal & West, 1958; Tokuda & Weiser, 1961; Gershon & Ross, 1962). In other species, its role in these reactions is probably of little importance.

In 1930, it was reported that injection of a pancreatic secretion results in a fall of blood pressure in dogs (Frey, Kraut & Schultz, 1930). The active principle, kallikrein, was an intracellular protease with trypsin-like activity, which on addition to blood, forms a smooth muscle stimulating agent (Werle, Gotze & Keppler, 1937). Subsequently, it was demonstrated that a vasodilating polypeptide, bradykinin, is formed by the action of trypsin or snake venoms on plasma proteins (Rocha e Silva, Beraldo & Rosenfield, 1949). There is good evidence that plasma kinins are important for the development of anaphylactic symptoms. The initial observations of Beraldo (1950) that a bradykinin-like substance was present in the blood of dogs following anaphylactic or peptone shock were confirmed and extended by Brocklehurst & Lahiri (1962, 1963). Furthermore, Diniz & Carvalho (1963) showed that the levels of substrate for bradykinin in plasma is markedly decreased during this reaction. Several workers have provided evidence that, in the course of antigen-antibody reactions, activation of proteolytic enzymes occurs (Ungar, 1947; Ungar, Yamura, Isola & Kobrin, 1961). In support of this are the observations that administration of trypsin (Rocha

& Silva, 1941) and chymotrypsin (Tagnon, Weinglass & Goodpastor, 1945) is followed by a shock-like syndrome. The substance occupying a key position in the kinin-forming system is Hageman factor, which is also critical in the intrinsic coagulation and fibrinolytic systems and in the activation of complement. (Margolis, 1963; Ogston, Ogston, Ratnoff & Forbes, 1969). Hageman factor has been implicated in the activation of prekallikrein to kallikrein (Davies, Holman & Lowe, 1967) which in turn liberates kinin from kininogen. However, there is some doubt as to whether bradykinin is a primary mediator of the anaphylactic response since the kinin system cannot be activated directly by antigen-antibody reactions (Lichtenstein, 1972). Nevertheless its involvement as a result of indirect activation during the allergic response should not be discounted.

More than seventy years ago, the term "anaphylatoxin" was introduced to indicate a substance formed during an immunological reaction and which could mimic many of the features of anaphylaxis (Friedberger, 1910). Later, it was demonstrated that anaphylatoxins are small peptides formed from the complement components, C3 and C5 (Lepow, 1957) and that their effects depend upon their ability to release histamine (Mota, 1959). Until the discovery of histamine, these substances were thought to play a major role in the genesis of the immune response. However, there is compelling evidence to suggest that type 1 reactions are not complement-dependent, since they are mediated by non-complement-fixing antibodies (Bloch, Kourilsky, Ovary & Benacerraf, 1963; Ovary, Benacerraf & Bloch, 1963). An involvement of complement might be explained by the fact that Hageman factor, which controls coagulation, fibrinolysis and kinin generation, can also trigger the classic complement activation pathway (Kretschmer, 1954). Moreover, there is an alternative or properdin pathway of complement activation, which generates a C3 convertase independent of C1, C4 and C2 and therefore of the antigen-antibody reaction (Kretschmer, 1954).

The term slow-reacting substance was introduced by Feldberg and Kellaway (1938) to describe the in vitro smooth muscle activity of material obtained during perfusion of guinea-pig lung with cobra venom. Anaphylactic shock in the same tissue was then shown to release a substance with similar smooth muscle activity (Kellaway & Trethewie, 1940).

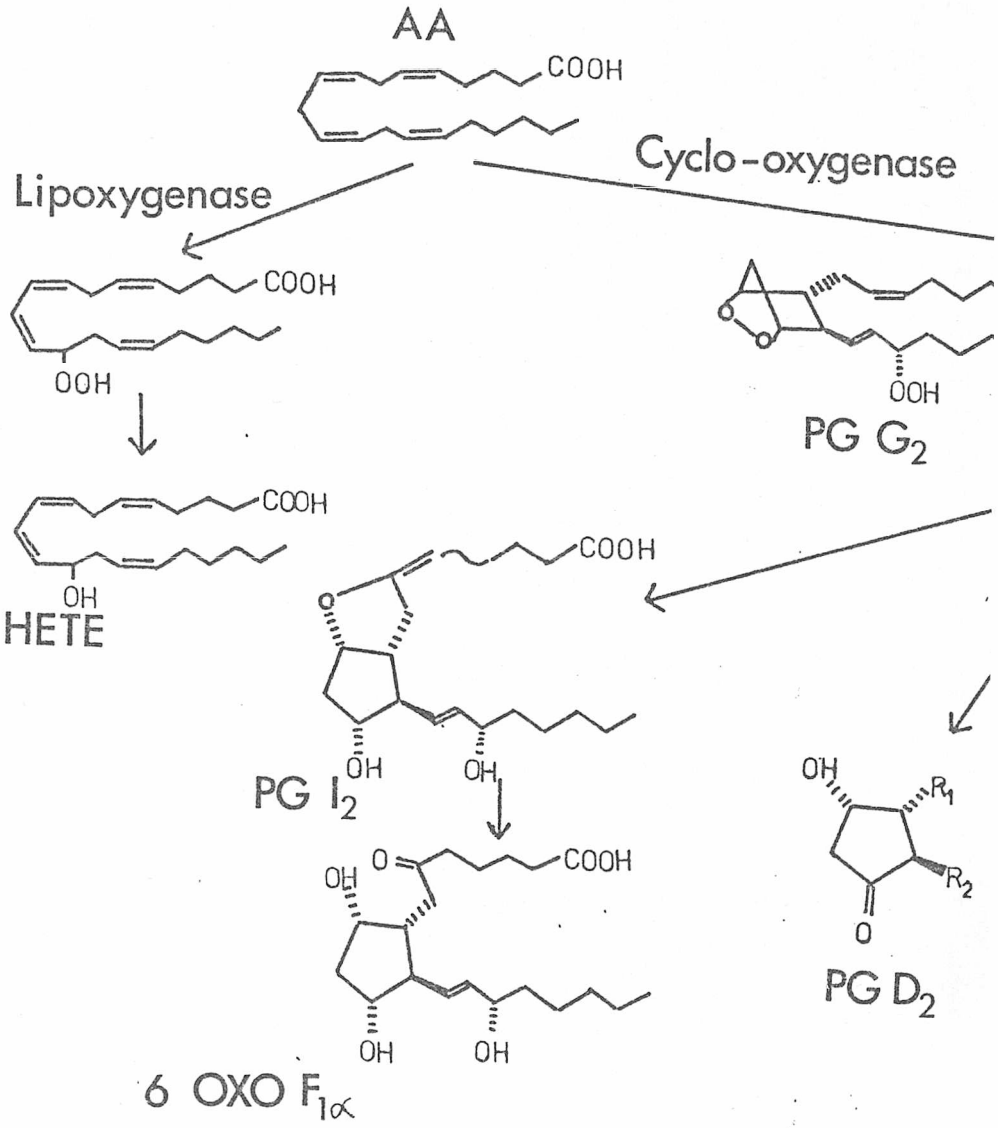
Slow-reacting substance of anaphylaxis (SRS-A), generated from tissue by an anaphylactic reaction, was first identified as being functionally distinct from histamine by the use of histamine antagonists (Brocklehurst, 1953; 1960). Later studies described unique physicochemical properties of this material (Chakravarty, 1960; Brocklehurst, 1962; Orange, Murphy, Karnovsky & Austen, 1973). SRS-A was recognised as a low molecular weight, acidic lipid with a free sulphate group which gives a characteristic contraction of isolated smooth muscle, properties which clearly differentiated it from other substances which are active on smooth muscle. Subsequently SRS-A was released in vivo into the peritoneal cavity of the hyperimmunized rat by intraperitoneal challenge (Orange, Valentine & Austen, 1968a) and following systemic anaphylaxis into the plasma of the sensitised guinea-pig (Stechschulte, Orange & Austen, 1973). It was demonstrated that IgG antibody could sensitise guinea-pig lung fragments for release of histamine and SRS-A (Stechschulte, Austen & Bloch, 1967), whereas similar studies with human lung specifically identified IgE antibody as the responsible immunoglobulin for SRS-A release. Mast cells were implicated as a source of SRS-A because their removal from rat peritoneal cavity prevented release (Orange, Stechschulte & Austen, 1970). Moreover, SRS-A has been detected after antigen challenge in the supernatent of an IgE - sensitised basophil-rich fraction of human leukocytes (Grant & Lichtenstein, 1974) and in a cell suspension containing mast cells derived from monkey lung (Ishizaka, Ishizaka, Orange & Austen, 1970). A number of workers have reported that SRS-A, unlike histamine, which is recognised as a preformed mediator of anaphylaxis, is present in tissues only in a precursor form from which the active form must be generated immunologically (Brocklehurst 1962; Austen & Orange, 1975). However, human lung homogenates have been found to contain a preformed slow-reacting-substance which was indistinguishable from SRS-A (Turnbull, Jones & Kay, 1976). The authors suggest that there may be two distinct intracellular pools of slow-reacting-substance and that following anaphylaxis there is a conversion of the preformed mediator to an active form.

There is much evidence to suggest therefore that SRS-A may be an important component of anaphylactic reactions in certain species, involving bronchial smooth muscle contraction and increased vascular permeability. In man, SRS-A may be partly responsible for the prolonged bronchospasm seen in asthma and could account for the inability of

antihistamines to alleviate the reaction completely.

Since the original discovery of prostaglandins (Goldblatt, 1933; Von Euler, 1934), many investigators have reported their synthesis or release in almost all mammalian tissues studied (Bergstrom, Carlson & Weeks, 1968). Prostaglandins are not stored within any subcellular compartment but are formed at the time they are released (Anggard, Bohman, Griffin, Larsson & Maunsbach, 1972). They are formed by the enzymic oxygenation of certain polyunsaturated fatty acids, which form part of the structure of most cell membranes. Most processes which disturb membrane function or alter its structure activate the hydrolysis of arachidonic acid from phospholipids (Gilmore, Vane & Wyllie, 1969), the rate-determining step in prostaglandin biosynthesis (Samuelsson, 1970). Thus, phospholipase - A_2, which is able to cleave bound fatty acid, seems to play a central role in prostaglandin formation (Kunze & Vogt, 1971). The fatty acid precursors are converted to prostaglandins by the action of cyclo-oxygenase (Samuelsson, 1976), an enzyme complex previously known as prostaglandin synthetase, which is located in the microsomal fraction of cells. The action of this enzyme was found to be inhibited by aspirin-like drugs (Vane, 1971). In the first instance, oxygen is incorporated into the substrate molecule (principally arachidonic acid), resulting in the formation of the cyclic endoperoxides, prostaglandins G_2 and H_2. In addition to possessing considerable biological activity, (Hamberg, Svensson & Samuelsson, 1974; Hamberg, Hedqvist, Strandberg, Svensson & Samuelsson, 1975), they also occupy a central position as intermediates for the synthesis of prostaglandins. The endoperoxides are enzymatically converted either to prostaglandins of the E, F or D series or to non-prostaglandin derivatives such as the thromboxanes (Hamberg, Svensson & Samuelsson, 1975) (Fig. 1). Recently, Moncada and co-workers have shown that when prostaglandins G_2 or H_2 act on the walls of blood vessels, prostacyclin (prostaglandin I_2) is formed enzymatically (Moncada, Gryglewski, Bunting & Vane, 1976). The stable metabolite of prostacyclin is 6-oxo-$F_{1\alpha}$ prostaglandin. Although many cells can produce endoperoxides, it would appear that they differ radically in the manner by which they further metabolise them (Hamberg et al.,1975). As a result, each tissue will produce different types and amounts of prostaglandin. Since the prostaglandins have differing (and often antagonistic) actions, the ultimate response of an organ to their synthesis can represent a series of complex interactions.

Fig. 1 Metabolic pathways of arachidonic acid (AA). The main pathways discussed in this investigation are the formation of the endoperoxides, PGG_2 and PGH_2 and the subsequent three pathways to thromboxanes (TxA_2 and TxB_2), to stable prostaglandins (PGE_2, $PGF_{2\alpha}$ and PGD_2) and to prostacyclin (PGI_2 and its metabolite, 6-oxo-$PGF_{1\alpha}$). The pathways via lipoxygenase to HETE and via cyclo-oxygenase to malondialdehyde and a hydroxy acid (not included) are not discussed.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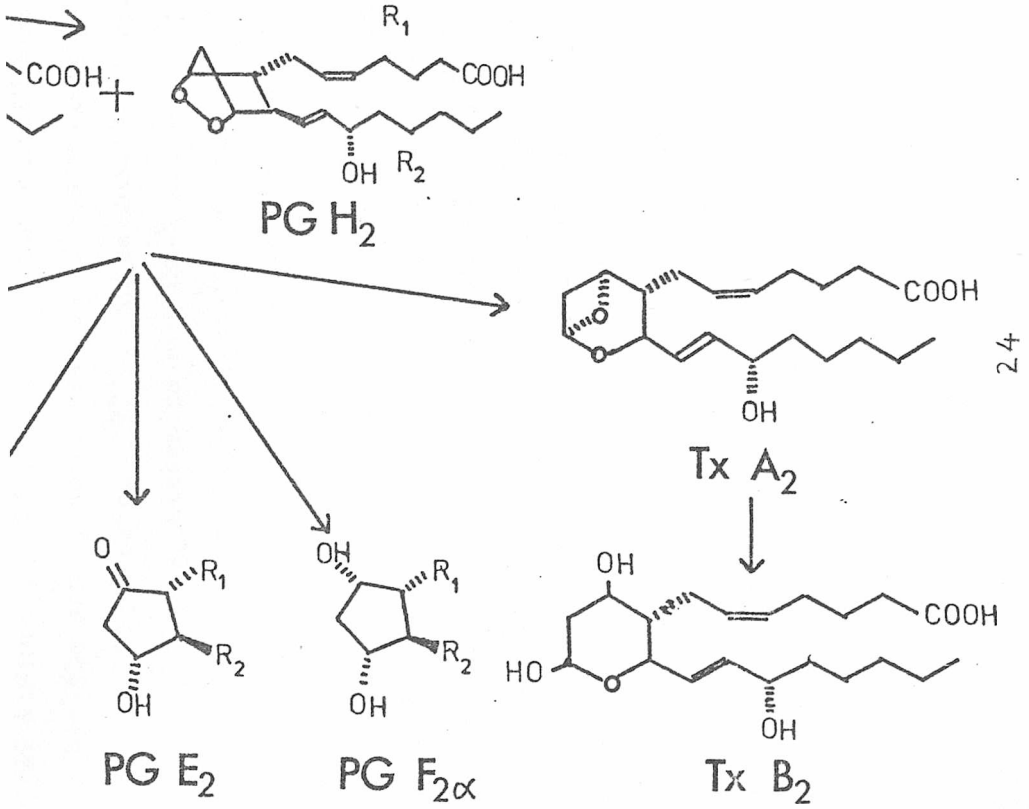
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HO
COOH
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When anaphylaxis occurs in isolated, perfused, guinea-pig lungs or human chopped lung tissue, prostaglandins of the E and F series are released into the perfusate (Piper & Vane, 1969; Piper & Walker, 1973). Prostaglandins are also released into the circulation of guinea-pigs during anaphylactic shock in vivo (Ruff, Allouche & Dray, 1976). However, since the F-prostaglandins cause bronchoconstriction while the E's produce bronchodilatation (Sweatman & Collier, 1968), it is difficult to envisage a role for these prostaglandins during anaphylaxis in the lung. In 1969, Piper and Vane found that an unstable material which contracted the rabbit aorta (rabbit aorta contracting substance, RCS) was released from sensitised guinea-pig lungs after perfusion with antigen. RCS has been identified as a mixture of prostaglandin endoperoxides and thromboxane A_2 (Svensson, Hamberg & Samuelsson, 1975; Bunting, Moncada & Vane, 1976). The possibility became clear that the endoperoxides and not the parent prostaglandins themselves might be the active components. However, it has been observed that non-steroidal anti-inflammatory drugs greatly decrease the levels of prostaglandins released from sensitised guinea-pig lungs during antigen challenge while at the same time increasing the concentration of SRS-A and histamine release (Liebig, Bernauer & Peskar, 1975; Engineer, Piper & Sirois, 1976; Engineer, Niederhauser, Piper & Sirois, 1977). Moreover prostaglandins E_1, E_2 and $F_{2\alpha}$ can inhibit the anaphylactic release of histamine and SRS-A (Tauber, Kaliner, Stechschulte & Austen, 1973). These results suggest that, at least in lung anaphylaxis, histamine and SRS-A are more important mediators than the prostaglandins. Prostaglandins are more likely to have a role in the modulation of release and of responses of other mediators.

Prostaglandin release has been detected in many kinds of inflammation including allergic eczema and irritant dermatitis in man (Greaves, Sondergaard & McDonald-Gibson, 1971; Sondergaard & Greaves, 1974). Also, administration of prostaglandin E, but not $F_{2\alpha}$ will reproduce the erythema associated with inflammation (Solomon, Juhlin & Kirschenbaum, 1968; Juhlin & Michaelsson, 1969). Oedema, the second component, is poorly mimicked by E_1 itself, but synergism was noted when E-prostaglandins were added in conjunction with bradykinin. This added effect was interpreted as a potentiating role for the prostaglandins. (Moncada, Ferreira & Vane, 1973; Williams & Morley, 1973).

Although administered prostaglandins fail to mimic inflammatory processes fully, a parallel has been demonstrated between the ability of aspirin-like drugs to inhibit prostaglandin synthesis and anti-inflammatory activity (Takeguchi & Sih, 1972; Tomlinson, Ringold, Qureschi & Forchielli, 1972). Kuehl and co-workers have investigated the possibility that instead of the primary prostaglandins themselves (E and F series), other oxygenation products of arachidonic acid, subject to inhibition by aspirin-like drugs, are important mediators of inflammation (Kuehl, Humes, Egan, Ham, Beveridge & Van Arman, 1977). Their results suggest that the prostaglandin endoperoxide, G_2 has a pivotal role in acute inflammation. It would seem that prostaglandins are better candidates as mediators of allergic inflammation in skin than of anaphylaxis in the lung.

Recently, some controversy has arisen over whether or not SRS-A is a product of arachidonic acid metabolism. Dawson and Tomlinson (1974) have shown that labelled arachidonic acid produces labelled prostaglandins during an anaphylactic reaction in isolated lung, whereas SRS-A produced concurrently is unlabelled. Further studies with indomethacin, which increased the yield of SRS-A, whilst strongly inhibiting prostaglandin synthesis (Walker, 1973; Dawson & Tomlinson, 1974), indicated that SRS-A is not derived from arachidonic acid. However, there is now very strong evidence to suggest that the converse is true, that SRS-A is a product of arachidonic acid metabolism (Bach, Brashler & Gorman, 1977; Jakschik, Falkenhein & Parker, 1977). These investigators were using single cell preparations, as opposed to whole lung, which is, of course a complex tissue. The unique chromatographic and spectrometric properties of SRS-A suggest nevertheless, that it is not a close structural analogue of other known metabolites of arachidonic acid (Morris, Taylor, Piper, Sirois & Tippins, 1978). Although SRS-A may be generated via the cyclo-oxygenase pathway, it is probably formed through the lipoxygenase pathway (Jakschik et al, 1977), by which arachidonic acid can be transformed into a hydroxy acid (Hamberg & Samuelsson, 1974; Nugteren, 1975).

Molecules possessing the ability to induce release of 5-HT from platelets have been identified in anaphylactic exudates generated in IgE - dependent reactions from human lung tissue as well as from peripheral blood cell suspensions in a variety of species (Cochrane,

Revak, Aikin & Wuepper, 1972; Bogart & Stechschulte, 1974). Platelet activating factor, which is not present prior to activation, can induceplatelet rosettes around rabbit basophils (Benveniste, Henson & Cochrane, 1972) and platelet aggregation (Benveniste, Kamoun & Polonsky, 1975) as well as causing platelet mediator release.

Another substance released from lungs during anaphylaxis is called the eosinophil chemotactic factor of anaphylaxis (ECF-A) (Kay & Austen, 1971; Kay, Stechschulte & Austen, 1971). The kinetics of ECF-A release parallel those of histamine (Wasserman, Goetzl & Austen, 1974) and led to the finding that ECF-A is preformed in tissues rich in mast cells and in isolated mast cells, where it is associated with the granules (Wasserman et al.,1974). Eosinophils are thus attracted to the site of mast cell degranulation. This cell probably has a number of functions in allergic tissue reactions. Thus, eosinophil-derived prostaglandins may inhibit further histamine release (Hubscher, 1975 a & b) and eosinophil histaminase (Zeiger & Colten, 1974) and arylsulphatase (Wasserman, Goetzl & Austen, 1975) inactivate histamine and SRS-A respectively. While ECF-A is not a mediator in the pharmacological sense, it explains one aspect of the allergic response in vivo.

A chemotactic factor for neutrophils (NCF-A) has also been recognised (Austen, Wasserman & Goetzl, 1976; Lewis, Goetzl, Wasserman, Valone, Rubin & Austen, 1975) but its action in vivo is unknown. It is released concurrently with ECF-A and histamine.

Piper, Collier and Vane (1967) directly demonstrated the release of catecholamines into the circulation of guinea-pigs during anaphylaxis. In addition,the mediators which cause bronchoconstriction also led to catecholamine release. Some thirty years earlier, it was observed that adrenaline inhibited the release of histamine from actively sensitised guinea-pig lung (Schild, 1936a) and suppressed the reagin-dependent wheal and flare reaction in human skin (Tuft & Brodsky, 1936). The mechanism of action of the catecholamines is more complex than simply a physiological antagonism. In 1968, Lichtenstein and Margolis demonstrated that β -adrenoceptor agonists and methylxanthines could inhibit the IgE - mediated release of histamine from human peripheral leukocytes and simultaneously increase the tissue concentrations of cyclic 3', 5' - adenosine monophosphate (cyclic AMP).

β-adrenocepter stimulants induce cyclic AMP formation by stimulating the enzyme, adenylate cyclase (Robinson, Butcher & Sutherland, 1967).

Increased intracellular cyclic AMP can also inhibit histamine and SRS-A release (Austen, 1974). All the evidence supports the hypothesis that the cyclase system, as a second messenger, mediates the anti-allergic actions of sympathomimetic agents.

In 1960, Spector & Willoughby suggested that catecholamines may indirectly contribute to the vascular permeability associated with tissue injury. The authors proposed that the presence of adrenaline and noradrenaline could lead to reduced vascular permeability and that their local inactivation by monoamine oxidase to enhanced vascular permeability. In support of this hypothesis, reduced levels of catecholamines and elevated monoamine oxidase activity have been found in injured skin (Möller, 1962; Raekallio, 1963).

1.6 MECHANISMS OF MEDIATOR RELEASE

Studies using a variety of *in vitro* models of acute hypersensitivity reactions have led to the possibility that the immunological release of chemical mediators from cells or sensitised target tissues involves a mechanism analogous to a secretory rather than a cytotoxic reaction (Austen & Becker, 1968). Among the evidence was the observation that a variety of metabolic inhibitors blocked the reaction (Becker & Henson, 1973), which suggested that mediator release was an energy-requiring process. The morphological changes observed in the target cells also supported this view. (Mota & Vugman, 1956; Hastie, 1971). Cross-linking of bound antibody with specific antigen initiates a sequence of biochemical events which has not been clearly elucidated, but includes activation of a cellular serine esterase, calcium uptake, changes in cyclic nucleotide levels and an energy-dependent exocytosis involving microtubule function (Austen, 1973; Lichtenstein, 1975).

It seems clear that the cyclic AMP system is intimately involved in the control of mediator release. Cyclic AMP inhibits release (Austen, 1974), while its counterpart cyclic GMP (cyclic 3', 5' - guanosine monophosphate) augments release (Kaliner, Orange & Austen, 1972).

It is now obvious that there is in operation a series of feedback control mechanisms by endogenous substances acting via this system. The effects of released catecholamines have already been mentioned. Histamine, also, acts on two sets of receptors, termed H_1 and H_2 (Black, Duncan, Durant, Ganellin & Parsons, 1972). Activation of H_2 receptors is associated with elevated cyclic AMP levels and therefore may provide a regulation mechanism of its own release (Lichtenstein, 1973). However, activation of H_1 receptors is now thought to be associated with increased cyclic GMP levels in lung (Stoner, Manganiello & Vaughan, 1974). In fact chlorpheniramine, an H_1 antagonist, has shown beneficial effects in some asthmatics (Karlin, 1975). Despite the well recognised direct effects of prostaglandins on smooth muscle, they may be more important in hypersensitivity reactions in a regulatory capacity. Thus, prostaglandin E_1 inhibits histamine release from human leukocytes in parallel with an increase in cyclic AMP (Lichtenstein & Barnardo, 1971). In most tissues, prostaglandins E_1 and E_2 stimulate adenylate cyclase (Kuehl, 1974) while prostaglandin $F_{2\alpha}$ stimulates cyclic GMP production (Dunham, Haddox & Goldberg, 1974). Cholinergic activation of lung (Stoner, Manganiello & Vaughan 1973) and heart (George, Polson, O'Toole & Goldberg, 1970) can selectively increase cyclic GMP levels. Moreover, Kaliner and co-workers (1972) observed that acetylcholine, at very low concentrations, enhanced immunological mediator release from human lung, as in fact did bromocyclic GMP. It may be that a relationship exists between the nervous system and mediator release. An antidromic reflex in injured skin may lead to mast cell degranulation (Kiernan, 1972) and histological studies indicated a proximity between mast cells and nerves in some cases (Olsson, 1968).

These feedback mechanisms provide the cell with an important means for intrinsic modulation of hypersensitivity reactions. Another balance seems to exist for the control of the kinin, complement, clotting and fibrinolytic systems. The kinetics of these enzyme cascades have been analysed by MacFarlane (1969). Numerous positive and negative feedback mechanisms are evident inside each individual system and also across systems. Thus, after an immunological reaction, we can envisage changes brought about by all of these systems.

In conclusion, it would seem that anaphylactic reactions should be regarded as multi-mediated phenomena, in which different mediators are released to play their roles in contracting smooth muscle, increasing vascular permeability or attracting cells. The relative contribution of any one will depend on its ability to produce symptoms of anaphylaxis, whether by a direct action or by an indirect effect on other mediators.

1.7 HYPERSENSITIVITY IN LOWER VERTEBRATES

Despite the surge of interest in comparative immunology over the last few years very little attention has been paid to the phylogeny of hypersensitivity reactions. There have been a few attempts to demonstrate anaphylaxis in fish but it is not yet clear whether this phenomenon is even exhibited by lower vertebrates.

Dreyer and King (1948) reported in various teleost fish a behavioural pattern, which they claimed to be symptomatic of anaphylaxis, following a shocking dose of horse serum or egg albumin. This reaction was only observed after appropriate sensitisation and was modified by the action of adrenaline. Unfortunately, Lukyanenko (1967) could not repeat their results as the "anaphylactic-type" reactions observed by Dreyer and King (1948) were observed after injections of saline. Furthermore, a number of other investigators have failed to elicit anaphylactic responses in teleosts (Arloing & Langeron, 1922; Hodgins, Weiser & Ridgway, 1967; Clem & Leslie, 1969; Harris, 1973).

All vertebrates are capable of producing immunological responses to appropriate antigenic stimuli (Good & Papermaster, 1964; Grey, 1969). Unlike higher vertebrates, teleosts only appear to have one immunoglobulin class, IgM (Clem & Leslie, 1969). IgM antibodies are extremely efficient agglutinating and cytolytic agents and are likely to be important in the event of bacterial infection (Roitt, 1977b). Frogs and toads, members of the amphibian group, have been induced to produce IgG in addition to IgM immunoglobulins (Marchalonis & Edelman, 1966; Lykakis, 1969). Thus, IgM would appear to precede IgG in the phylogeny of the immune response in vertebrates; this indicates the primitive position of teleosts in this respect.

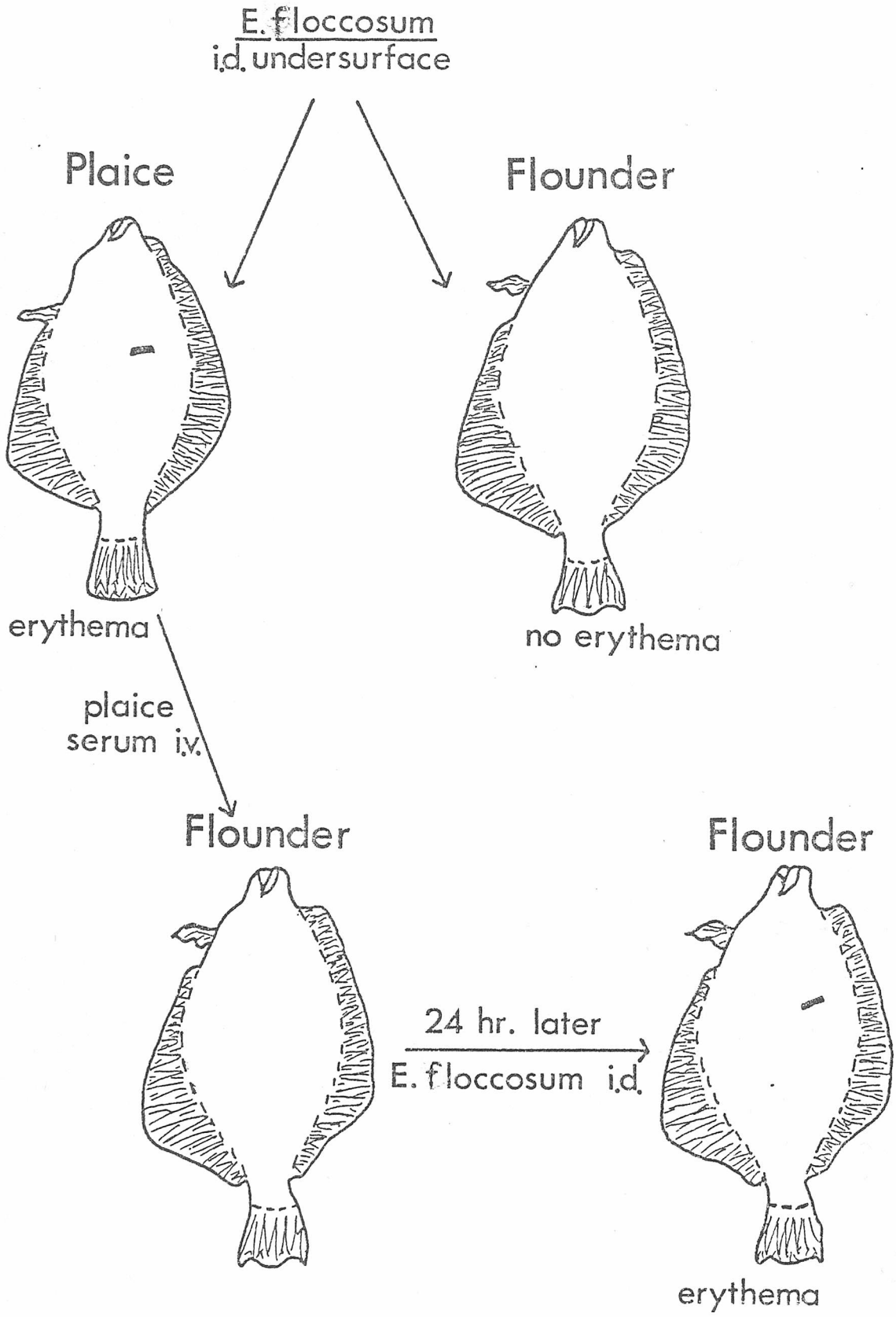


Fig 2 Demonstration of transfer of immediate skin reaction to flounders after intravenous injection of plaice serum (from T.C. Fletcher).

In 1974, Fletcher and Baldo observed that the intradermal injection of certain fungal extracts caused immediate-type skin reactions in some unsensitised teleost flatfish. Extracts of the fungi (Longbottom & Pepys, 1964) produced erythema-like reactions in plaice, Pleuronectes platessa (L.), turbot, Scophthalmus maximus (L.), brill, Scophthalmus rhombus (L.) and dab, Limanda limanda (L.). Interestingly, skin reactions could not be observed to any of the fungal extracts in flounders, Platichthys flesus (L.). It was found, however, that the skin reaction could be transferred to the flounder, twenty-four hours after the intravenous injection of fresh plaice serum (Fig 2). The transferrable factor(s) were destroyed by heating the plaice serum for four hours at 56°C. Passive transfer of the skin reaction was also shown: intradermal injection of plaice serum and saline was followed three hours later with intravenous Evans blue and fungal extract. Bluing was only observed at the site of serum injection. These experiments show that the skin reaction is similar to immediate, type 1, hypersensitivity reactions, described in mammals (Coombs & Gell, 1968).

The skin reaction was specific for fungal extracts which had a component which will precipitate with C-reactive protein (CRP). The common factor was subsequently found to be C-substance (Baldo, Fletcher & Pepys, 1977), a species-specific antigen of Diplococcus pneumoniae discovered nearly 50 years ago (Tillett, Goebel & Avery, 1930). Moreover, CRP-like precipitins were also found in normal plaice serum (Baldo & Fletcher, 1973). The flounder was the only fish examined in which precipitins were not detected. The flounder, too, was the only fish which did not display skin reactions to fungal extracts. These observations led to the suggestion that the skin reactions are mediated through the CRP-like constituent of plaice serum (Fletcher & Baldo, 1974).

CRP is an acute phase protein found in the sera of many vertebrates in a number of pathological conditions (Tillett & Francis, 1930; Koj, 1974). In fact, during reactions of inflammation and tissue destruction, the concentration of CRP in the human circulation has been reported to be elevated as much as one thousand fold (Claus, Osmand & Gewurz, 1976). Recent evidence suggests that CRP may have an important role in the response of higher vertebrates to inflammatory or injurious processes.

Thus, CRP can interact with the complement system (Volanakis & Kaplan, 1974; Mortensen, Osmand, Lint & Gewurz, 1976; Claus, Siegel, Petras, Osmand & Gewurz, 1977) and has profound effects on the lymphoid system (Mortensen, Braun & Gewurz, 1977) and on platelet function (Fiedel & Gewurz, 1976). There was only one brief report of CRP-like proteins in lower vertebrates (Watson, Paulissen & Yen-Watson, 1968). However, the findings of Baldo and Fletcher (1973) suggest that CRP-like proteins are normal components of plaice serum and do not only appear as a result of inflammation or trauma as in higher vertebrates.

1.8 PURPOSE OF THE PRESENT INVESTIGATION

The object of the present investigation is to identify the individual pharmacological mediators responsible for the immediate hypersensitivity reaction observed in the skin of plaice by Fletcher and Baldo (1974). Although the mediators implicated in hypersensitivity responses in mammals have been extensively studied, very little is known of such mediators in poikilotherms. A preliminary study indicated that the skin reaction in the plaice can be inhibited *in vivo* by disodium cromoglycate and diethylcarbamazine citrate (Baldo & Fletcher, 1975), compounds known to inhibit some anaphylactic responses in mammals (Cox, 1967; Orange, Valentine & Austen, 1968b). Erythema-type reactions were also produced by the intradermal injection of high concentrations of the histamine liberator, compound 48/80, and by histamine and 5-HT.

1.9 A RATIONALE FOR THE RECOVERY AND IDENTIFICATION OF INFLAMMATORY MEDIATORS

Greaves and Kobza Black (1976) have discussed the problem of recovering and identifying the mediators of inflammatory skin diseases in humans. The authors suggest that there are two types of experiment that can be performed in order to indicate the relative contribution of different substances to the pathogenesis of an inflammatory reaction. Firstly, direct experiments may be conducted in which an inflammatory exudate is collected. This can

then be tested for pharmacological activity and assayed either biologically or chemically. These experiments are extremely useful, but limited, in that no information is provided about the role of active material *in vivo*. Secondly, indirect experiments can be performed. This involves the use of specific pharmacological antagonists such as promethazine or methysergide to reveal some of the actions due to histamine and 5-HT or inhibitors of synthesis such as aspirin or indomethacin to reveal prostaglandins. Agents like compound 48/80 (Paton, 1951) can also be used to cause depletion of a material stored in tissues. The usefulness of this approach depends obviously, on the specificity of the inhibitors, a feature which is often not possible: promethazine, for example, has anti-5-HT activity in addition to being an antihistamine. Nevertheless, this approach has the advantage that inhibition of the response indicates the involvement of that substance as a mediator. A combination of direct and indirect experiments is probably the best way to approach the problem.

A number of methods have been developed for the collection of inflammatory exudate from skin. Probably the simplest one is that described by Kuritzky and Goodfriend (1974) in which suspensions of sensitised monkey skin were incubated *in vitro* with a physiological saline and challenge was effected simply by addition of antigen to the sample. After incubation, supernatants were assayed for activity. The obvious disadvantage is that this is an artificial method. However, immunological release of histamine and SRS-A has been demonstrated using this technique (Goodfriend & Luhovyj, 1968; Kuritzky & Goodfriend, 1974).

A continuous, *in vivo* perfusion technique has been described as a method of studying the release of pharmacologically-active material from human skin (Winkelman, 1966; Greaves & Søndergaard, 1970). In this way, histamine was recovered directly following cutaneous anaphylaxis (Søndergaard & Greaves, 1971) and prostaglandins from the skin of patients with allergic contact eczema (Greaves *et al.*, 1971). The value of this technique is limited by its relative insensitivity and by the fact that it is only semi-quantitative (Greaves & Søndergaard, 1971). However, it does make it possible to obtain samples for analysis directly from the undersurface of the skin during experimental conditions.

The most recent development of Greaves and co-workers is a technique whereby a series of suction blisters (Kiistala, 1972) are raised at the site of inflammation in skin due to dermal-epidermal separation. The fluid content of these blisters can be withdrawn and used as a source of inflammatory exudate (Kobza Black, Greaves, Hansby & Plummer, 1976). The production of a concentrated sample indicates the value of this method.

Most of the substances known to act as mediators also have a profound effect on smooth muscle. Hence, inflammatory exudate should firstly be tested for smooth muscle activity in vitro. A variety of different isolated tissues should be used, which should vary in their relative sensitivity to prospective mediator substances. Thus, the rat stomach strip is very sensitive to 5-HT and E-series prostaglandins but is relatively insensitive to histamine, whereas the guinea pig ileum is sensitive to all three (Brocklehurst, 1973). The superfusion technique introduced by Gaddum (1953) enables much smaller amounts of drug to be analysed and was adapted by Vane (1964) so that up to six tissues could be arranged in a cascade and superfused with the same bathing fluid. This method has the great advantage of giving instant results since exudate can be assayed immediately on a number of tissues. The responses of selected tissues will show the presence of any of the usual smooth muscle stimulating substances in the inflammatory exudate. Further confirmation can be obtained by the use of specific antagonists. Final identification will usually depend on extraction and the use of biochemical and/or chemical techniques.

In general, bioassay is the most appropriate method for the identification of prospective mediators, since it provides the only means of measuring biological activity. Nanogram quantities may be detected and parallel assay on a number of tissues (Chang & Gaddum, 1933) can confirm the identity of purified material obtained during experiment by comparison with authentic samples. However, crude, mixed samples will often be used and only active material will be revealed. The advantage of immediate assay is that it will also reveal dynamic changes in mediator levels; without bioassay, rabbit aorta contracting substance probably would not have been discovered (Piper & Vane, 1969) or suggested as an intermediate in the biosynthesis of prostaglandins (Gryglewski & Vane, 1971).

In contrast, chemical assay often involves a complicated extraction procedure and is often not as sensitive. On the other hand, it is unlikely to fail in mid-experiment. If a mediator is extremely labile in vivo, it may only be possible to implicate its involvement by showing elevated levels of a particular metabolite in the sample. Metabolites usually cannot be assayed biologically but may be assayed chemically.

Histamine can be assayed by either biological or chemical assay. Very small amounts (1ng/ml) can be detected by bioassay on the guinea-pig ileum and identity can be confirmed by the use of mepyramine, a specific antagonist. The fluorimetric method (Shore, Burkhalter & Cohn, 1959) is much less sensitive, involves a lengthy extraction procedure and is rather impractical for small samples. 5-HT is usually assayed by fluorimetric methods (Maikel, Cox, Saillant & Miller, 1968) but a sensitive bioassay method has been described (Vane, 1957). The confirmation of identity is effected by the use of the antagonist, methysergide. The principle metabolite of 5-HT, 5 - hydroxyindoleacetic acid cannot be assayed biologically and the fluorimetric method must be used. Ferreira & Vane (1967) have developed a superfusion method for the detection and estimation of bradykinin-like peptides.

Perhaps the easiest way to show that the active material is a polypeptide is to show that activity is destroyed following incubation with chymotrypsin (Brocklehurst, 1973). If a mixture of kinins is suspected in the sample, they should be separated prior to assay (Babel, Stella & Prado, 1968). SRS-A can only be assayed biologically and this is accomplished using the terminal guinea-pig ileum (Chakravarty, 1959; Brocklehurst, 1962). A specific antagonist of SRS-A, FPL 55712 has been described (Augstein, Farmer, Lee, Sheard & Tattersall, 1973). Moreover, SRS-A contains a free sulphate group which is essential for biological activity. Incubation with arylsulphatase will lead to loss of activity. Prostaglandins, as a group, can be readily distinguished from other substances active on smooth muscle - they are acidic lipids and therefore can be easily extracted from aqueous solution. However, they are a large and ever-increasing group of compounds. Assay techniques must therefore have a high degree of specificity and this often necessitates the use of physical techniques (Hensby, 1977).

Horton (1976) has critically assessed the relative value of different methods of prostaglandin measurement. A combination of techniques will probably be required.

The screening procedure described should allow the identification of substances which have been recognised as mediators in other systems. Any activity remaining after these substances have been removed can then be characterised. However, it is still possible to miss the presence of previously unknown mediator substances which are inactive on the smooth muscle assay tissues. It would be better to test also for activity on a model of the vascular bed itself, i.e. the perfused rabbit ear or the perfused hindquarters of the rat. These methods, whilst lacking pharmacological specificity, may reveal activity which cannot be ascribed to the "classical" mediators.

Before a substance can be accepted as a mediator of a biological response, four main criteria should be satisfied (Lewis, 1977):

(1) The substance should be detectable during or immediately following the application of the stimulus.

(2) A mechanism of synthesis of the substance should be present.

(3) The substance should produce effects which mimic the pathophysiological response.

(4) A specific antagonist should affect the response in the same way as it influences the action of the proposed mediator.

In an effort to identify the pharmacological mediators of the inflammatory reaction in plaice skin, a combination of *in vivo* and *in vitro* methods were employed to prepare an inflammatory exudate from the skin. Exudate was screened for biological activity using the techniques discussed in section 1.9. The identification of any active material then allowed the design of specific experiments to try to satisfy the second, third and fourth criteria outlined above. If, however, a number of mediators are involved and their functions overlap, simply removing one of them may not significantly alter the response and the fourth criterion will break down.

CHAPTER TWO

METHODS AND MATERIALS

2.1 SOLUTIONS & DRUGS

Isolated smooth muscle preparations were in contact with a physiological saline (Krebs & Heinseleit, 1932) of the following composition (mM): Na Cl 118.2, K Cl 4.75, Ca Cl_2 2.54, KH_2 PO_4 1.19, Mg SO_4 1.19, Na HCO_3 25.0, glucose 11.2. This "Krebs" saline was maintained at 37°C and gassed with a mixture of 95% O_2 and 5% CO_2.

Skin from plaice and flounders was incubated with and perfused with a specific Ringer saline (Cobb, Fox & Santer, 1973). The composition was (mM) : Na Cl 140.5, K Cl 5.2, Mg Cl_2 1.1, Ca Cl_2 4.9, Na H CO_3 2.4, Na H_2 PO_4 1.8, glucose 5.6.

The rabbit ear preparation was perfused with a Ringer saline of the following composition (mM) : Na Cl 153.8, K Cl 5.6, Ca Cl_2 2.2, Na HCO_3 5.6, glucose 5.6.

The following drugs were used : acetylcholine chloride (BDH); (-)-adrenaline (base) (Koch Light); aspirin (BDH); atropine sulphate (Koch Light); bradykinin triacetate (Sigma); carrageenin (Sigma); chymotrypsin (Worthington Biochemical Corporation); Compound 48/80 (Sigma); corticosterone (Sigma); diethylcarbamazine citrate (Wellcome); disodium cromoglycate (Fisons); 5,8,11,14,17 - eicosapentaenoic acid (Serva Feinbiochemica); 5,8,11,14 - eicosatetraenoic acid (grade 1) (Sigma);8,11,14 - eicosatrienoic acid (P-L Biochemicals); flurbiprofen (Boots Company); FPL 55712, 7 - (3-(4-acetyl-3-hydroxy-2-propylphenoxy)-2-hydroxypropoxy)-4-oxo-8-propyl-4H-1-benzopyran-2-carboxylic acid sodium salt (Fisons); glutathione (reduced) (Sigma); histamine acid phosphate (BDH); hydrocortisone (Sigma); 5-hydroxytryptamine creatinine sulphate (BDH); (-)-hyoscine hydrobromide (BDH); indomethacin (Merck, Sharp & Dohme); mepyramine maleate (May & Baker); methysergide bimaleate (Sandoz); metiamide (Smith,Kline & French); MS 222 (Sandoz); L-noradrenaline bitartrate (Koch Light); phenoxybenzamine hydrochloride (Smith, Kline & French); phentolamine hydrochloride (Ciba); promethazine hydrochloride (May & Baker); propranolol hydrochloride (Smith, Kline & French); prostaglandins A_1, B_1, B_2, D_2, E_1, E_2, E_3, $F_{1\alpha}$; $F_{2\alpha}$ and U-46619 (Upjohn).

Culture media of the fungus, Epidermophyton floccosum, obtained from Greer Laboratories was dialysed for at least 24 hours against distilled water, filtered and then lyophilized prior to use.

C - reactive protein and P - component from plaice serum were prepared (Pepys, Dash, Fletcher, Richardson, Munn & Feinstein, 1978) and gifted by Dr. Pepys (Royal Postgraduate Medical School, London). Lumpsucker CRP was prepared by Dr Fletcher (Fletcher, White & Baldo, 1977).

2.2 FISH

The fish used in this study were seine-netted in shallow water off the Aberdeenshire coast and transferred to aerated seawater tanks at the Institute of Marine Biochemistry, Aberdeen where they were maintained at 11 - 14°C. Fish were examined between 24 hours and 6 months after capture. Plaice are readily available throughout the year and were used principally in this investigation. A small number of flounders which were not always readily available were also used. Both male and female fish were employed varying in age from 1 - 10 years. For certain experiments, plaice were transferred to the School of Pharmacy in 10 litre plastic tanks, where they were kept for a maximum of 8 hours.

Intradermal injections were made in saline on the non-pigmented undersurface of these fish, where skin reactions could be observed easily. Generally, 0.05 ml was injected using a 27 gauge needle. Intravenous injections and blood collection was effected via the caudal vein. Drugs were also administered by intraperitoneal injection.

2.3 EXPERIMENTS WITH PLAICE SKIN in vitro

2.3 i) Preparation of skin

The skin was removed from freshly-killed plaice which had shown a positive skin reaction following the intradermal injection of the fungal extract, E.floccosum. Both upper (pigmented) and lower (non-pigmented) skin was dissected completely free from underlying muscle, finely chopped and washed thoroughly. It was blotted dry, weighed and then incubated at 18°C with 2 volumes (1 g/2 ml) of plaice Ringer saline in the presence and absence of E. floccosum (in a final concentration of 1 mg/ml), hereafter referred to as challenged and

non-challenged skin respectively. Aliquots of the incubation mixtures were withdrawn at timed intervals for biological assay (Fig 3). In some cases, skin was challenged with compound 48/80 (1 mg/ml) instead of E. floccosum. Skin from flounders was treated in exactly the same manner.

The effect of drugs on the release of active material from plaice skin was tested in two ways: either by injecting the fish 24 hours prior to killing and in vitro incubation or by adding the drug to the reaction mixture immediately before incubation. All experiments included controls in which the challenging agent was incubated with Ringer saline alone.

To enable the identification of active material, large-scale incubations were performed and a standard sample prepared for analysis.

2.3 ii) Isolated tissues

Samples of incubation media were tested for activity on smooth muscle using the following isolated preparations: stomach strip (Vane, 1957), duodenum (Horton, 1959) and colon (Regoli & Vane, 1964) of the rat, trachea (Anderson & Lees, 1976) and ileum of the guinea-pig, chick rectum (Mann & West, 1950), gerbil colon (Ambache, Kavanagh & Whiting, 1965) and rabbit aorta (Piper & Vane, 1969). The organs were taken from animals which had just been killed and the preparations either mounted in 20 ml organ baths in Krebs saline or arranged in banks of 3 tissues and superfused with a stream of oxygenated Krebs saline, delivered at a constant rate by a roller pump (Watson Marlow). Isometric contractions were detected by strain gauge transducers (UF1 ± 2 ozs) and displayed on a Devices recorder. Where appropriate, specific antagonists were included in the bathing solution.

2.3 iii) Bioassay

In a preliminary attempt to identify the unknown material, parallel quantitative assays (Chang & Gaddum, 1933) were performed using three different smooth muscle preparations : rat colon, rat stomach strip and gerbil colon.

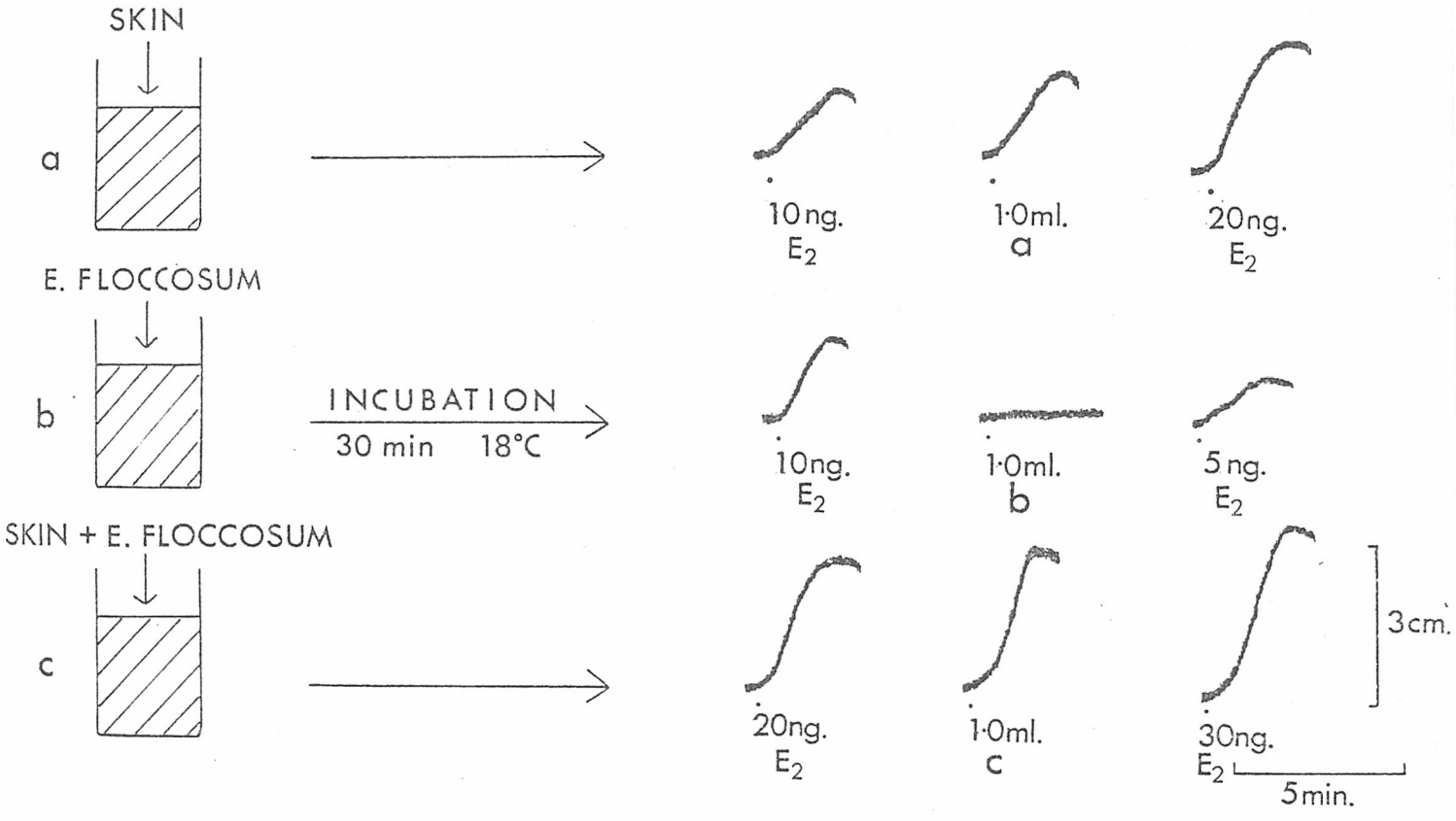


Fig 3 Incubation of chopped plaice skin with Ringer saline in the presence and absence of the fungal extract, E. floccosum. Following incubation, samples were withdrawn and tested for activity on a number of isolated smooth muscle preparations. Here, the rat stomach strip was used and responses to 1 ml samples were bracketed between responses to standard E_2. E floccosum incubated alone acted as a control and did not change the smooth muscle tone.

These vary in their relative sensitivity to authentic prostaglandin standards. A standard sample of active material was prepared (see page 40) and was partially purified by extraction and preparative thin-layer chromatography (TLC) (see section 2.3 iv). Comparison of unknown with standards was made at 3 dose levels and the tests performed in a random sequence to offset interference between one dose and the next. This enabled the results to be treated mathematically to establish the accuracy of the assay (Schild, 1942). Assay values were compared for each standard and should be the same if the substance under test and the authentic material are identical.

In subsequent experiments, active material was assayed by the bracketing technique (Brocklehurst, 1973) against authentic prostaglandin E_2 on the rat stomach strip (Fig. 3) in Krebs saline containing hyoscine hydrobromide (10^{-7}g/ml), methysergide bimaleate (2×10^{-7}g/ml), mepyramine maleate (10^{-7}g/ml), phenoxybenzamine hydrochloride (10^{-7}g/ml), propranolol hydrochloride (2×10^{-6}g/ml) and indomethacin (10^{-6}g/ml) (Gilmore, Vane & Wyllie, 1968, as modified by Bennett, Stamford & Unger, 1973). This drug combination increases the sensitivity and selectivity of the assay.

2.3 iv) Extraction and characterisation of prostaglandin-like material

A solvent partition method as described by Hubscher (1975b) was employed. Incubation of plaice skin was terminated by the addition of two volumes of ethanol. An equal volume of petroleum ether was added and the mixture inverted. After removal of the petroleum ether, each sample was acidified to pH3 and extracted 3 times with chloroform (4 volumes). The pooled chloroform layers were evaporated to dryness under reduced vacuum. Preparative TLC was carried out on silica gel G (0.25 μm) coated glass plates using 4 different solvent systems (Table 1). Methanolic solutions of prostaglandin standards and skin extracts were applied to the plates and developed in the solvent mixture. Standards were visualised by spraying with 10% phosphomolybdic acid in ethanol followed by heating at 120°C. Zones (1 cm) of silica gel were then scraped off the plate and eluted with a mixture of chloroform/methanol (1:1). Eluates were dried under a stream of nitrogen, the residue re-suspended in Krebs saline and tested for contractile activity on the rat stomach strip.

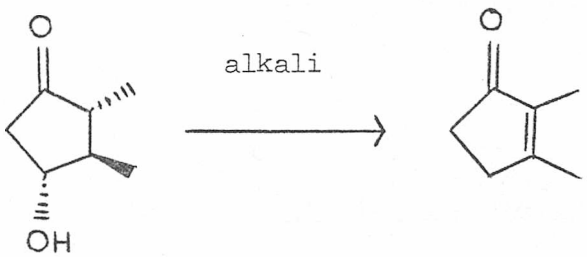
Composition		Adsorbant
Chloroform-methanol-acetic acid-water	(90:10:1:0.75)	Silica Gel G
Ethyl acetate - formic acid	(400:5)	Silica Gel G
Chloroform-ethyl acetate-ethanol-acetic acid	(20:20:4:1)	Silica Gel G
Benzene-chloroform-butanol-ethanol	(4:10:5:1)	Silica Gel G
Chloroform-methanol-acetic acid	(18:3:1)	Silica Gel G - $AgNO_3$ (10:1)
Ethylacetate-ethanol-acetic acid	(100:1:1)	Silica Gel G - $AgNO_3$ (10:1)

Table 1 Solvent systems used in preparative thin-layer chromatography

Using two further solvent systems (Table 1), silver nitrate was incorporated into the plates (Barrett, Dallas & Padley, 1962). This enables separation of the individual members of the E-series prostaglandins. As before, zones of silica gel were extracted and the dried eluates re-suspended. However, before bioassay could be performed, samples had to be re-extracted from aqueous solution into chloroform to remove the interfering silver ions. Prostaglandin-like activity was expressed as equivalents of authentic E_2.

2.3 v) Spectrometry

Ultraviolet spectra of skin extracts, partially purified by TLC, were recorded on a Unicam SP 1800 by a method based on that of Yoshimoto, Ito & Tomita (1970). An aliquot of the prostaglandin-like material was diluted to 2 ml with methanol. This was divided into two samples, to one of which was added 1 ml of 1N KOH in methanol and to the other, 1 ml of methanol. The increase in absorption at 278 nm was continuously recorded until a plateau was reached after about five minutes at room temperature. The spectrum was then recorded. The total increase in absorption at 278 nm after the addition of alkali was calculated on the basis of the known molar extinction coefficient of prostaglandin compounds of the B series (an increase of absorbance of 1.00 is equivalent to 13.13 µg/ml ofprostaglandin B). This method provides a useful quantitative measurement of E-series prostaglandins, which are converted to the respective B compounds by alkaline hydrolysis:



PG E PG B

For mass spectrometric analysis, samples of incubation media from challenged and non-challenged skin were acidified to pH3 and extracted twice with equal volumes of ethyl acetate. Following evaporation of the combined ethyl acetate extracts, the residue was subjected to preparative TLC using the F VI solvent system of Andersen (1969).

The zones of silica gel corresponding to authentic E_2 were eluted with methanol and the eluates were evaporated to dryness. Samples were further purified by chromatography using a column packed with Lipidex 1000 (Hensby, 1977). The derivatives used were the methyl ester, methyloxime, trimethylsilyl ethers. Samples were analysed using a Finnigan 3200 gas chromatograph-mass spectrometer and compared with authentic E_2 derivatives.

2.3 vi) Measurement of dye leakage in rats

The method was based on that of Chahl & Chahl (1976). Female rats (Sprague Dawley, 100 - 200g) were anaesthetized by the intraperitoneal injection of sodium pentobarbitone (60 mg/kg). The jugular vein was cannulated and Evans blue dye injected intravenously (2.5 ml/kg of a 2% solution in saline). Intradermal injections of solutions under test, in 0.1 ml volumes, were then made into the abdominal skin, which had been previously shaved. All solutions were diluted in 0.9% saline and a control injection of saline was made in each animal. In order to minimize any variation in the results due to different sites of injection, these were varied in different animals. Animals were killed 20 minutes after the last injection. The abdominal skin was removed and the blue areas cut finely and placed into a mixture of sodium sulphate and acetone (3:7) (Harrada, Takeuchi, Fukao & Katagiri, 1971). 24 hours later, the samples were centrifuged and the absorbance of the supernatants measured at 620 nm using an Evans Spectrometer. The amount of dye present was read directly off a calibration graph (μg/ml dye vs. absorbance) and expressed as μg/ml of dye.

2.3 vii) Preparation of acidic and basic skin extracts

Incubation media from plaice skin challenged with E. floccosum in vitro was tested for its ability to induce increased permeability in rat skin in the following way. After incubation for 45 minutes, the skin supernatant was adjusted to either pH3 or pH 11 and then extracted with 4 volumes of ethyl acetate to produce respectively an acidic and a basic extract. The combined organic layers were evaporated to dryness under reduced vacuum and the residues re-suspended in a small volume of 0.9% saline for estimation of dye leakage. Extracts were tested initially

for smooth muscle activity *in vitro*. The acidic extract was assayed in equivalents of prostaglandin E_2. Dye leakage was measured to acidic and basic extracts alone and in combination with standard histamine solutions (50, 100, 200 and 400 μ M).

2.4 DIRECT SKIN PERFUSION *in vivo*

2.4 i) Perfusion

Plaice were chosen if they showed positive skin reactions to *E. floccosum*. They were anaesthetized by the addition of the anaesthetic MS 222 to the seawater. A concentration of 1 part in 20,000 was found to be the most satisfactory. After about 15 minutes, the fish were transferred to a small dish containing aerated seawater for ease of handling. The technique of subcutaneous perfusion was based on that described by Greaves & Søndergaard (1970). Two needles (length 4 cm, internal diameter 1 mm) were inserted immediately subdermally in parallel about 2 cm apart in opposite directions using the non-pigmented undersurface of the plaice. Both needles had 4 holes perforated along the shaft. Ringer saline, at 18°C, was infused through one needle and recovered through the other. Continuous perfusion and withdrawl of Ringer saline was facilitated by the use of a peristaltic pump (Fig. 4). Initially, the infusion rate was varied but was eventually fixed at 1 ml/min and kept constant during the period of collection. Using these conditions, the recovery of perfusate collected was in the range 30 - 70% and did not vary significantly during an experiment. When recovery was less than 50%, however, the experiment was disregarded. Visible oedema was seen to develop within the first few minutes of perfusion.

In initial experiments, the perfusate was superfused directly over the assay organs in an analogous manner to the "blood bathed organ" technique employed by Vane (1964) to assay the release of substances into the circulation. Challenge was made by injection of *E. floccosum* or compound 48/80 either into the infusion cannula (i.e. subcutaneously) or intradermally over the area of perfusion.

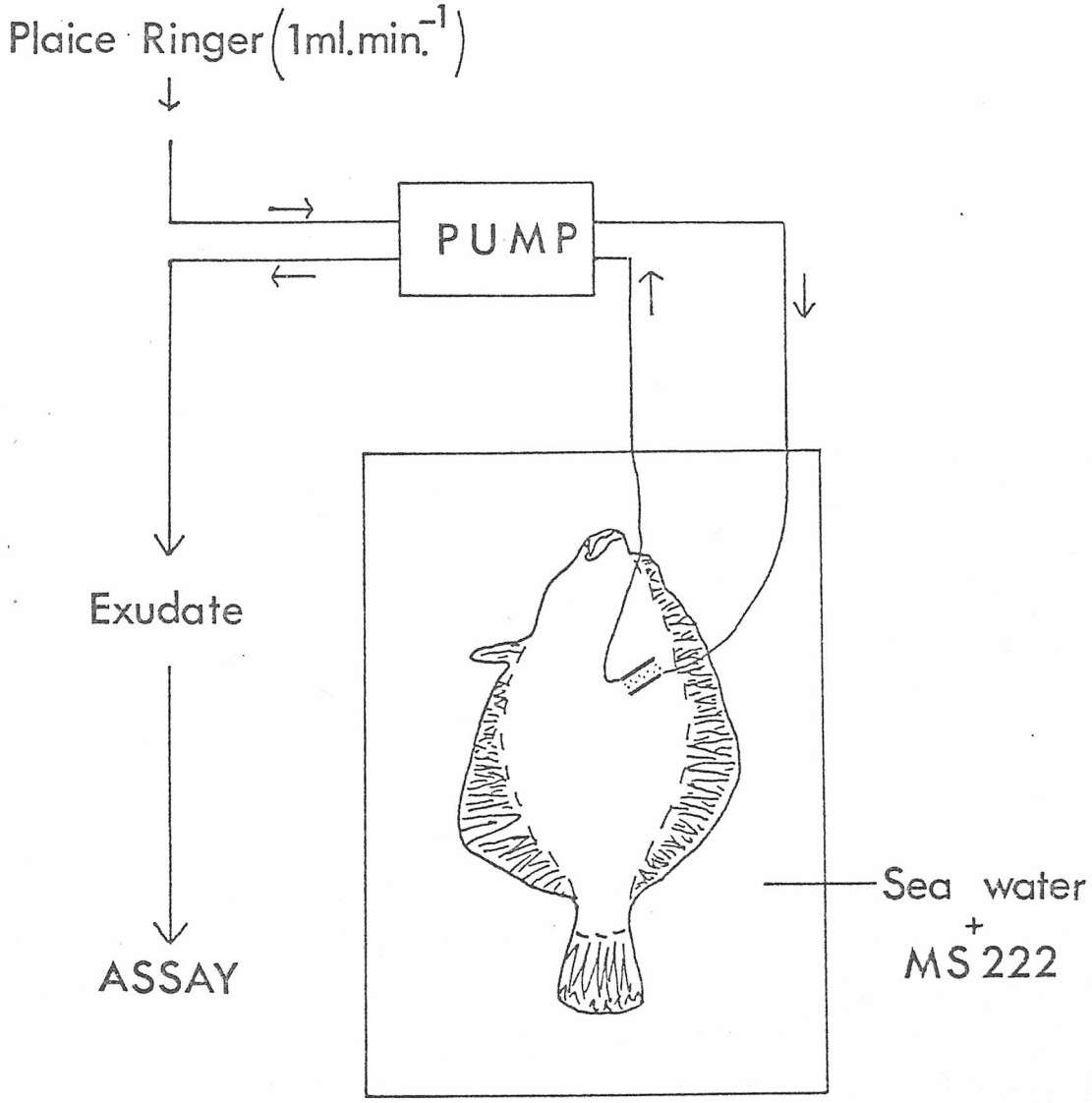


Fig 4 Schematic representation of skin perfusion in anaesthetized plaice. Ringer was continuously perfused and recovered subcutaneously by means of a peristaltic pump. The dotted area indicates the area of perfusion.

In later experiments, Ringer saline was not perfused directly over the tissues, but was recovered into siliconised glass tubes in an ice bath. After the perfusion was established, the initial 10 minutes collection was discarded. Challenge was then made intradermally over the area of perfusion and samples collected over the next hour. Aliquots were then removed and tested for smooth muscle activity. All blood stained samples were rejected.

2.4 ii) Assay

Perfusates were tested for smooth muscle stimulating activity using a combination of the following isolated preparations : guinea-pig ileum, rat stomach strip, rat colon and rabbit aorta. The tissues were set up in banks of 2 and superfused with Krebs saline, initially at a rate of 5 ml/min. Mineral oil was then added to the bath as described by Ferreira & Souza Costa (1976) (Fig. 5). This enables the perfusion rate to be dropped to 0.1 - 0.2 ml/min and allows a consequent increase in sensitivity. Isometric contractions were recorded and displayed on a Devices recorder. The aorta and mesenteric arteries of the cod and the ileum of the plaice were also tested as prospective assay tissues. Plaice Ringer saline was then substituted for Krebs saline as the superfusing solution. As a model of skin vasculature, the perfused rabbit ear preparation was used (Burn, 1952). The central artery of the ear from a heparinized rabbit was cannulated and perfused with Ringer saline. A suitable rate of outflow was obtained and was measured by means of a drop counter triggered to a rate meter. The effect of samples of plaice skin perfusate was observed on the perfusion rate through the ear. A decreased rate indicated vasoconstriction and an increased rate, vasodilatation.

Before assays could be performed, prostaglandin-like activity had to be concentrated by extraction. Samples were acidified to pH 3 and extracted twice with equal volumes of ethyl acetate. After evaporation of solvent, the residue was re-suspended in Krebs saline and bioassayed by the bracketing technique on the rat colon or the rat stomach strip in equivalents of prostaglandin E_2.

Preparative TLC was performed on the combined perfusates of 4 experiments as described earlier (see page 43). As before, each zone was assayed in equivalents of prostaglandin E_2.

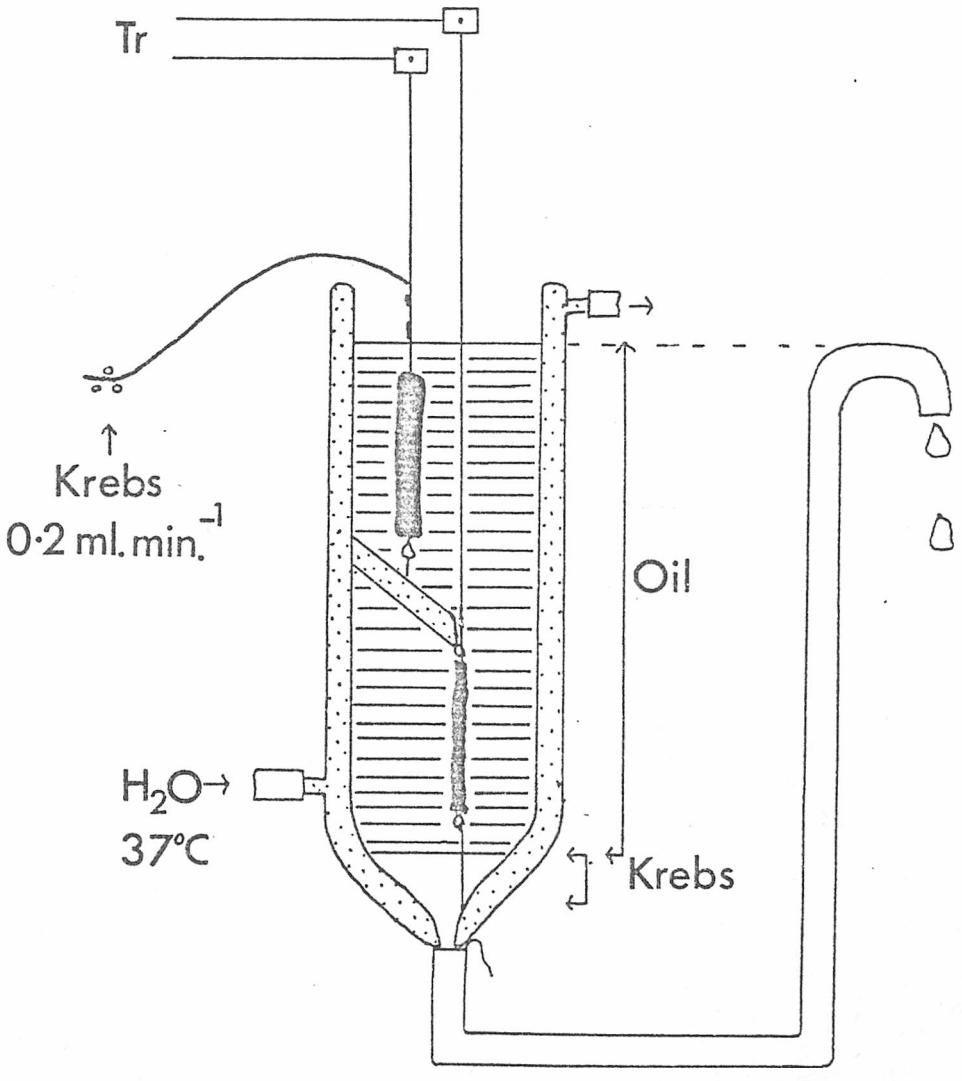


Fig 5 Diagrammatic representation of the oil-bathed superfusion technique. Isolated smooth muscle preparations were immersed in mineral oil in banks of two and superfused in series by dropping Krebs saline on a thread which connected the tissue to the recorder. Test substances were injected (20 - 100μl) into the capillary tubing linking the reservoir of Krebs saline to the pump. The level of superfusion fluid was kept constant with a syphon whose tip was positioned at the beginning of the experiment.

Quantitative GC-MS analysis was carried out on a number of samples. Each sample consisted of the combined perfusates of 4 experiments and was prepared for analysis as described previously (see page45). Due to the very small amounts of prostaglandin-like material present, complete spectra could not be obtained. Assay was performed by focussing the mass spectrometer to collect ions of m/e 295. The increase in intensity at the appropriate GC retention time was proportional to the absolute amount of prostaglandin derivative injected.

2.4 iii) Recovery experiments

To give an indication of the sensitivity of the subcutaneous perfusion technique, known amounts of certain agents (prospective mediator substances) were injected subcutaneously into perfused plaice skin. 4 fish in each group were injected with 10 µg of histamine, bradykinin, 5-HT or prostaglandin E_2. The perfusate was collected in successive 15 minute fractions for 60 minutes after which time they were bioassayed for residual activity in organ baths using the following isolated preparations. Histamine was assayed on the guinea-pig ileum and 5-HT on the rat stomach strip. Specific activity was confirmed in each case using the antagonists mepyramine and methysergide respectively. Bradykinin was assayed on the guinea-pig ileum in the presence of mepyramine (10^{-7}g/ml). Identity was confirmed by showing loss of activity following incubation with chymotrypsin (0.25 mg/ml at pH7) for 30 minutes. Prostaglandin E_2 was assayed on the rat stomach strip in the presence of methysergide (2×10^{-7}g/ml). Partition behavior confirmed the lipidic nature of the material. Bracketing assays were performed and activity expressed in terms of the active base.

2.5 ATTEMPTED FORMATION OF SUCTION BLISTERS

In an attempt to raise suction blisters in plaice skin as a source of inflammatory exudate, pressure was applied to the skin of anaesthetized plaice with a metal suction cup (internal diameter 2 cm). This was less versatile than the cup utilised by Kiistala (1972) in which the local skin temperature could be varied. Nevertheless, pressures

of up to 700 mm below atmospheric pressure could be applied to the skin.

2.6 DETERMINATION OF FATTY ACID COMPOSITION OF PLAICE SKIN

Finely-chopped plaice skin (10g) was transferred to a mortar containing liquid nitrogen and ground finely with a pestle. Lipids were then extracted by the method of Bligh and Dyer (1959) as modified by Allen, Good, Davis, Chisum and Fowler (1966). Skin was homogenized with a chloroform/methanol mixture in such proportions that a miscible system was formed with the water in the tissue. Further addition of chloroform and water separated the homogenate in two layers, the chloroform layer containing the lipids and the methanol layer containing the non-lipids. The lipid extract was evaporated to dryness and the weight of the residue determined. Aliquots were transesterified with methanolic hydrochloric acid for gas chromatographic (GC) analysis of methyl esters (Farquhar, 1962). GC was performed on a Pye 104 gas chromatograph equipped with a flame ionization detector. Two different glass columns (1.5 m x 4 mm) were used to separate the fatty acids : 10% EGSS - X on Gas Chrom Q and 10% EGSS - Y on DCMS - treated P (Field Instrument Co Ltd.). Both columns were operated at 200°C. Individual peaks were identified by comparison with the retention times of reference fatty acids. The problem of "chain-length overlap", found with most polar liquid phases (Ackman, 1967) was overcome by chromatographing the same sample on both columns and comparing the results since the extent of the overlap is different for each column. The relative proportion of each fatty acid was found by calculating the product of peak height and the retention time at half peak width and expressed as a percentage of the total.

2.7 SUBCELLULAR LOCALISATION OF PROSTAGLANDIN BIOSYNTHESIS

2.7 i) Subcellular fractionation

Skin from freshly-killed plaice was chopped finely and washed thoroughly in buffer (Tris HCl, 0.05M, pH8) to remove mucus. It was

then blotted dry, weighed and homogenized in 4 volumes of ice cold Tris buffer using a Polytron homogenizer (type PCU-2) and the homogenate filtered using a plastic sieve. The nuclear pellet was obtained by centrifugation of the filtrate at 800g for 15 minutes at 4°C. The supernatant fluid, after removal of the pellet was centrifuged at 8,000 g for 30 minutes to give the mitochondrial pellet. The supernatant fraction from this centrifugation was subjected to further centrifugation at 79,000g for 3.5 hours, yielding a microsomal pellet. The final supernatant fraction and the lyophilised pellets were stored at -20°C prior to use. The protein content of each fraction was assayed by the method of Lowry, Rosebrough, Farr and Randall (1951). The microsomal marker enzyme, glucose - 6 - phosphatase was estimated by measuring the amount of inorganic phosphate formed on incubation of each fraction with glucose - 6 - phosphate (Baginski, Foa & Zak, 1974).

2.7 ii) Purification of substrate fatty acids

3 substrate fatty acids were used : 8, 11, 14 - eicosatrienoic acid (20:3 w6)*, 5, 8, 11, 14 - eicosatetraenoic acid (arachidonic acid)(20 : 4 w6) and 5, 8, 11, 14, 17 - eicosapentaenoic acid (20 : 5 w3). They were purified by high performance liquid chromatography (H P L C). A stainless steel column (100 mm long, 4.6 mm internal diameter) packed with silica gel (5 µm, Partisil) was employed. The pressure was maintained at 400 psi and dichloromethane was the solvent used. Fatty acids (1 mg) were detected by measuring the absorbance at 254 nm U-V. Samples were collected for each major peak. The purified fatty acids were stored in ethanol at -20°C prior to use in a concentration of 10 mg/ml. The concentration was determined by quantitative conversion of a small aliquot to a conjugated diene hydroperoxide by the action of soy bean lipoxidase (MacGee, 1965).

* The notation, n : x w y is commonly used for designating particular fatty acids : n represents the number of carbon atoms in the fatty acid chain, x is the number of ethylenic double bonds and y denotes the number of carbon atoms from the centre of the double bond farthest removed from the carboxylic group (indicating the position of this double bond in the fatty acid chain) to the w end of the molecule.

2.7 iii) Conversion of fatty acids into prostaglandins

Initially, the incubation conditions were varied to find the optimum conditions for the synthesis of prostaglandins. In these experiments, the effects of different cofactors and incubation temperatures were studied on the synthesis of prostaglandins from arachidonic acid. Thereafter, the reaction mixture contained the following : fatty acid (0.33M), reduced glutathione (5 mM), adrenaline (5 mM), particulate enzyme fraction (20 mg) and Tris HCl buffer (0.05M) to a final volume of 2 ml. As a control, incubations were performed in the absence of substrate. The effects of unsaturated fatty acids and anti-inflammatory agents were determined by addition of the various compounds in 0.01 ml ethanol to each incubation mixture as indicated in the text; 0.01 ml ethanol without the compounds was added to the control vial containing active enzyme fraction. At this concentration no inhibitory effect of ethanol on the conversion of arachidonic acid into prostaglandin E_2 was observed. Incubation was carried out at 18°C for 60 minutes with aeration. The mixture was acidified to pH3 and extracted twice with 2 ml of ethyl acetate. The organic layers were combined and evaporated to dryness under a stream of nitrogen. Preparative TLC was performed, with authentic prostaglandins as markers, on plastic-backed plates coated with silica gel G (0.25 μm) using the FVI solvent system. Following visualisation of standards, zones of silica gel were removed and after elution were assayed for smooth muscle activity on the rat stomach strip. For quantitation of E - series prostaglandins, only the zones corresponding to authentic E_2 were taken. Eluted samples were divided and assayed biologically (on the rat stomach strip and the rat colon) and by ultra-violet absorption spectrometry (Yoshimoto et al., 1970) in parallel. Assay values by each technique were then compared and the results expressed as the percentage conversion of substrate.

Final identification of individual prostaglandins was established by comparison of mass spectra with those of authentic standards. Samples were prepared as described earlier (see page 45).

2.8 DETERMINATION OF SKIN HISTAMINE LEVELS

2.8 i) Extraction and fluorimetric assay

Skin from freshly-killed plaice was finely chopped and then ground to a powder in a mortar containing liquid nitrogen. Powdered skin was soaked in 0.4N perchloric acid (2 volumes) for 15 minutes and the histamine in the supernatent extracted by the method of Shore et al., (1959). The acidic extracts were then made alkaline with 5 N sodium hydroxide and the histamine extracted into n-butanol. Following re-extraction into aqueous solution, histamine was condensed with o - phthalaldehyde (Sigma) in strongly alkaline solution to yield a fluorescent product which was stabilised by the addition of hydrochloric acid. The fluorescence at 450 nm resulting from activation at 360 nm was measured in a Baird Atomic fluorimeter. Concentrations as low as 5 ng/ml can be assayed (Shore et al., 1959). Identity was confirmed by comparison of activation and excitation spectra with those of authentic histamine. Internal standards were included to allow for losses during the extraction procedure by adding known amounts to perchloric acid and carrying out the extraction as before. Recoveries of 40 - 50% were observed. In some experiments, other tissues of the plaice were analysed for their histamine content. The extraction procedure in these cases was identical to that for the skin.

2.8 ii) Effect of agents on skin histamine levels

Acute Experiments

24 plaice, which showed a positive skin reaction to E. floccosum were injected subcutaneously with either E. floccosum or compound 48/80 (1 mg/ml in 0.9% saline) over an area of approximately 10 cm^2. One hour later, the fish were killed. Samples of skin (1g) from the injected areas and from distant areas (which had been injected with saline) were removed and extracted for histamine content. Levels of histamine from injected and non-injected skin were compared by paired t test.

Chronic experiments

3 groups of 4 plaice, of approximately 300g, were injected intra-peritoneally with compound 48/80 (1 mg) once daily for 3,8 or 15 days. After this period, the fish were killed and samples of skin, subcutaneous muscle, mesentery and ileum were removed and the histamine content of these tissues estimated. For each group, control histamine levels were measured on skin from an equal number of untreated plaice. Differences between treated and untreated groups were compared by unpaired t test. Plaice were skin tested with E. floccosum at the beginning and the end of treatment.

2.9 FLUORIMETRIC DETERMINATION OF SKIN 5-HT LEVELS

Skin samples from 10 freshly-killed plaice were powdered in liquid nitrogen and soaked in 2 volumes of 0.4 N perchloric acid for 15 minutes. The supernatent was extracted and assayed for 5-HT by the method of Maickel et al., (1968). This involved extraction at alkaline pH into n - butanol and then at acid pH back into the aqueous phase. 5 - HT was then reacted with o - phthalaldehyde under conditions of heat and strong acid to produce a complex which fluoresced at 470 nm as a result of excitation at 360 nm. The lower limit of detection by this assay is about 10 ng. Recoveries of at least 50% were obtained by the extraction procedure.

2.10 PREPARATION OF A METHANOLIC EXTRACT OF PLAICE SKIN

The method was based on that employed for frog skin by Anastasi, Erspamer & Bertaccini (1965). Plaice skin (20 - 75g) was extracted with 4 parts of methanol (w/v) for 48 hours and then for 24 hours with 3 parts of 80% methanol. In one experiment, 33g of frog skin was extracted in parallel for comparison. Combined filtrates were stored at 4°C. The methanol extract was evaporated to about 50 ml at 40 - 45°C and the remaining liquid shaken repeatedly with petroleum ether to remove fats, before being evaporated to dryness. The residue was re-suspended in 25 ml of 95% ethanol in a warm water bath and stored overnight at 4°C.

After discarding the precipitate formed during storage, the liquid was passed through a column of 30g alkaline alumina (BDH). Elution of the column was performed by the addition of successive 50 ml volumes of 95, 85, 70, 60, 50, 40, 30 and 20% ethanol and water. Fractions of 50 ml were collected and evaporated to dryness. Each fraction was then bioassayed for its content of 5-HT and bradykinin-like peptides. 5-HT was measured by bracketing assay on the rat stomach strip and identity confirmed with the specific antagonist, methysergide. Bradykinin-like activity was estimated by parallel bracketing assay on the rat duodenum and the guinea-pig ileum against standard bradykinin. The presence of a peptide was confirmed by the loss of activity after incubation with chymotrypsin (0.25 mg/ml at pH 7 for 30 min.).

In four separate experiments, fractions of the plaice skin extract eluted from the column were tested for their ability to produce dye leakage in the skin of rats injected with Evans blue dye. The method used was that of Chahl & Chahl (1976) as described earlier (see page46).

2.11 PREPARATION OF A CELL-FREE SUPERNATANT OF PLAICE SKIN

Finely-chopped skin was homogenized with 2 volumes (w/v) of ice-cold Tris HCl buffer (0.05M, pH 8) using a Polytron homogenizer. The homogenate was filtered and the filtrate centrifuged at 79,000 g for 3.5 hours at 4°C. The supernatant fraction was tested as a source of metabolizing enzymes (Yamamoto, Francis & Greaves, 1976) by incubating 1 ml aliquots with a known amount (100 ng) of histamine, 5-HT, bradykinin or prostaglandin E_2 at 18°C in glass stoppered tubes. After incubation, samples were immediately bioassayed for smooth muscle activity. The assay systems used were the same as those described for the recovery experiments (see page 51). Loss of activity was expressed as a percentage and was calculated from the formula X-Y/X x 100, where X = concentration of agent in the positive control in which the agent was added to the enzyme preparation and then immediately assayed; Y = concentration of agent after incubation.

2.12 STATISTICAL ANALYSES

Differences in histamine and prostaglandin levels were compared by either paired or unpaired t test and were taken to be significant when $p \leqslant 0.05$, where p indicates the probability of 2 points being similar. Limits of error have been expressed as standard errors of the mean.

Dose-response relationships and the time-course experiments were analysed by stepwise polynomial regression analysis to obtain the equation of best fit for the data. The correlation between the two variables was shown numerically by calculating a correlation coefficient to describe the position of the line relative to the observations. A perfect correlation would be $r = 1$ and no correlation would be $r = 0$. An analysis of variance was performed to indicate whether there was a significant correlation in each case. When it was suspected that differences between animals contributed to the variation, an allowance was made for this by the introduction of dummy variables (Snedecor & Cochrane, 1967).

2.13 INDIRECT EXPERIMENTS

The skin reaction in plaice skin was quantitated by the adoption of a direct, 4 tier, visual scoring system (0, +, ++, +++ in a grade of increasing intensity). Thus, the ability of prospective mediator substances to mimic the skin reaction to E. floccosum could be investigated. A number of experiments were also performed to see if some compounds which are effective in preventing the symptoms of immediate hypersensitivity reactions in higher vertebrates could inhibit the skin reaction on intradermal challenge with E. floccosum. In acute experiments, plaice were skin tested before and immediately after an intravenous injection of drug. They were also skin tested repeatedly over the following 60 minutes. In chronic experiments,plaice were pretreated with an intraperitoneal injection of drug once daily for 5 days. Again, fish were skin tested with E. floccosum before and after treatment.

CHAPTER THREE

RESULTS

The results section of this investigation has been arranged principally in chronological order to give an indication of the manner in which the problem was approached. Thus, in certain instances, the results of one section directly influenced the design and execution of the subsequent experiments.

3.1 CUTANEOUS ANAPHYLAXIS IN WHOLE PLAICE

The intradermal injection of *E. floccosum* was found to cause an immediate, erythema-type reaction in the skin of plaice as manifested by a red mark on the non-pigmented undersurface of these fish (Fig. 6). The response developed within 5 minutes of injection and did not fade but persisted for several hours. The erythema-like response was not accompanied by obvious swelling. No skin reactions were observed when plaice were injected with 0.9% saline. These results confirm the earlier observations of Fletcher & Baldo (1974).

As a result of the skin testing of several hundreds of plaice over the 3-year period during which the investigation was carried out, it became obvious that the intensity of the skin reactions varied considerably at different times of the year. In general, the reactions were more pronounced in the early months of the year than in the summer and autumn. Indeed, from June to September, 1976, skin reactions were very poor. Although a controlled study was not carried out, the levels of CRP - like precipitins to *E. floccosum* in plaice serum (Baldo & Fletcher, 1973) were also lower than normal during this period and there appears to be a direct relationship between the intensity of the skin reaction and the level of circulating CRP. It is possible that there is an annual cycle of CRP levels as reported during the breeding season for male and female lumpsuckers (Fletcher *et al.*, 1977).

3.2 INCUBATION OF PLAICE SKIN *in vitro* AS A MODEL OF CUTANEOUS ANAPHYLAXIS

In an attempt to collect an inflammatory exudate from plaice skin, an *in vitro* method based on that described by Kuritzky & Goodfriend (1974) (see page 40) was employed as the initial step in the identification of the mediators responsible for the anaphylactic reaction in whole plaice.

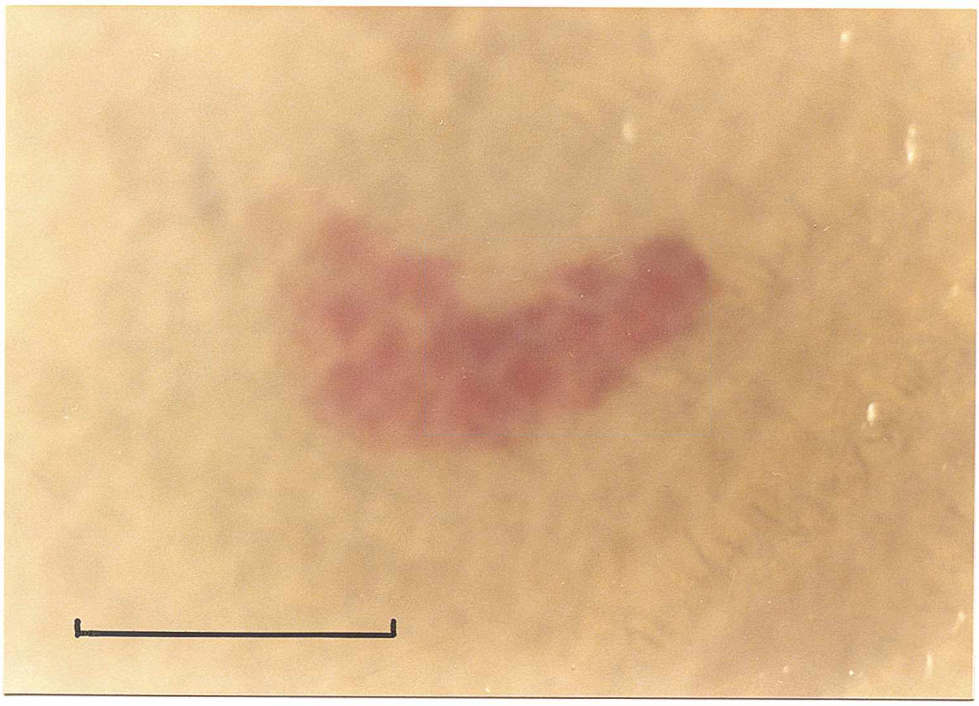


Fig 6 Immediate skin reaction observed on the undersurface of plaice 5 minutes after the intradermal injection of E. floccosum (0.05 ml of a 1 mg/ml solution in saline). The bar is equivalent to 0.5cm.

3.2 i) Release of smooth muscle contracting material from plaice skin

Incubation media from challenged and non-challenged skin contracted all the tissues which were examined, with the exception of the rat duodenum which relaxed, and the rabbit aorta which did not respond. Fig. 7 illustrates the effect of aliquots of incubation media on 3 superfused tissues, in this case the guinea-pig ileum, rat stomach strip and rabbit aorta. Whereas the challenging agent, E. floccosum did not itself change the smooth muscle tone of the tissues, responses to challenged skin samples were found to be larger than those to non-challenged samples. The assay of media of skin, incubated with compound 48/80 (1 mg/ml), was negated by the observation that this agent had a direct effect on the assay tissues. However, responses to samples of incubation media of skin challenged with 0.1 mg/ml 48/80 were not greater than those to non-challenged samples.

The rat stomach strip gave the most reproducible and dose-dependent responses and was therefore used to assay the activity from plaice skin in subsequent experiments (Fig. 3). The contractile responses to active material persisted even in the presence of antagonists of acetylcholine, histamine, 5-HT and SRS-A. In addition, incubation with chymotrypsin, which destroyed activity due to bradykinin, did not reduce the response to the unknown factor in the solution.

3.2 ii) Identification of the unknown material

By following the biologically active material in all steps of the extraction procedure, it was demonstrated that smooth muscle stimulating activity could be extracted from an acid aqueous phase into chloroform, from there into a buffered saline (pH 7) and finally back into chloroform again at pH 3. This behaviour is consistent with the properties of an organic acid, such as a fatty acid or a prostaglandin. After extraction of this material, the aqueous phase did not contain any detectable residual smooth muscle activity.

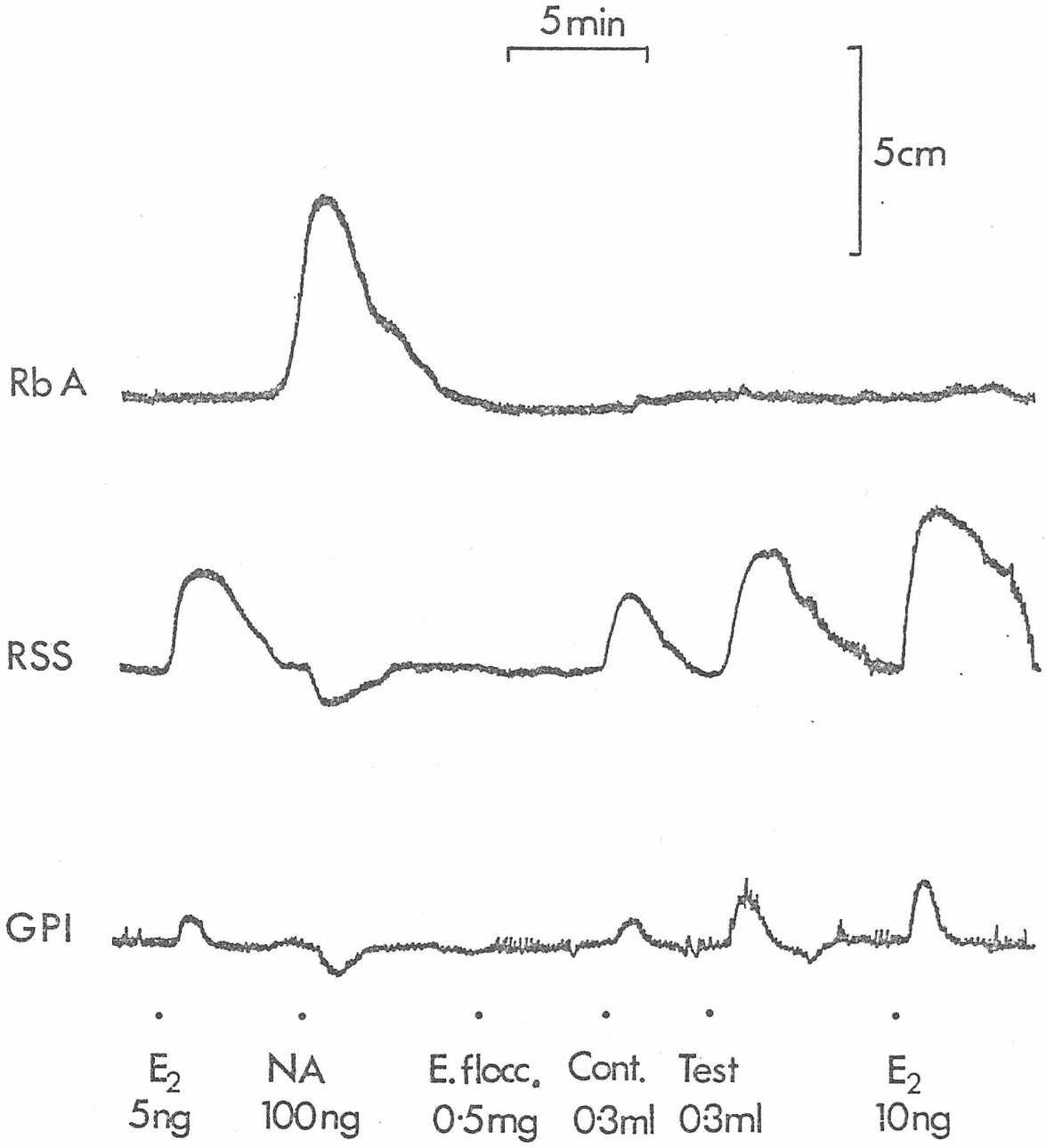


Fig 7 Effect of skin incubation media on the smooth muscle tone of 3 isolated preparations. A rabbit aortic strip (Rb A), a rat stomach strip (RSS) and a guinea-pig ileum (GPI) were superfused with Krebs saline at 37°C at a rate of 5 ml/min. Aliquots of challenged skin incubations (Test) were found to give larger contractions of the RSS and the GPI than samples of non-challenged incubations (Cont.). The challenging agent, E. floccosum had no direct effect on the tone of the preparations. The RbA only responded to noradrenaline (NA). E_2, prostaglandin E_2; Time, 5 min; vertical scale, 5 cm.

Preliminary identification of the active material was obtained by the employment of preparative TLC. Using 4 different solvent systems (Table 1), the partially-purified material co-chromatographed almost exclusively with the E - series prostaglandins. Fig. 8 illustrates one of these experiments. Furthermore, re-chromatography using 2 additional solvent systems on thin-layer plates impregnated with silver nitrate showed that the prostaglandin-like material migrated in the Rf range corresponding closely with the distance travelled by standard E_2 but not with E_1 or E_3. The results of one experiment are shown in Fig. 9.

An aliquot of active material, purified by TLC showed an ultra-violet absorption with a maximum at 278 nm, after treatment with alkali, suggesting that the unknown contains a prostaglandin of the E series (see page 45). Moreover, the smooth muscle contracting activity of the unknown material was destroyed by alkaline hydrolysis: this behaviour is also consistent with the presence of an E-series prostaglandin (Bennett, Stamford & Stockley, 1977). However, this method cannot discriminate between prostaglandins E_1, E_2 and E_3 or other susceptible prostaglandins.

Table 2 shows the results of quantitative assays of the prostaglandin-like material performed in parallel. When the unknown was assayed in terms of authentic E_2, consistent potencies were obtained on all 3 tissues. In contrast, the potency varied considerably when assayed as E_1, E_3 $F_{1\alpha}$ or $F_{2\alpha}$. A summary of the analyses of variance of these assays (Table 3) shows that the linear regression was significant in all cases. Potency ratios calculated by graphical means agreed reasonably well with the mathematically calculated values but did not allow any indication of the accuracy of the assay.

More conclusive evidence for the identity of the prostaglandin-like material was obtained by mass spectrometric analysis. The presence of prostaglandin E_2 in the challenged skin extract was confirmed by comparison of the mass spectrum with that of a standard preparation of E_2 (Fig. 10). Unfortunately, there was insufficient material present in an extract of non-challenged skin to obtain a satisfactory spectrum.

Fig 8 Preparative thin-layer chromatography of prostaglandin-like material from plaice skin. The 3 histograms represent the migration of smooth muscle contracting material released from challenged skin, from non-challenged (control) skin and of a mixture of standard prostaglandins $F_{2\alpha}$ (1.5 μg), E_1 and E_2 (0.5 μg each). Zones of silica gel were scraped off the plates and following elution were assayed for biological activity on the rat stomach strip. The solvent system was ethyl acetate-formic acid (400:5).

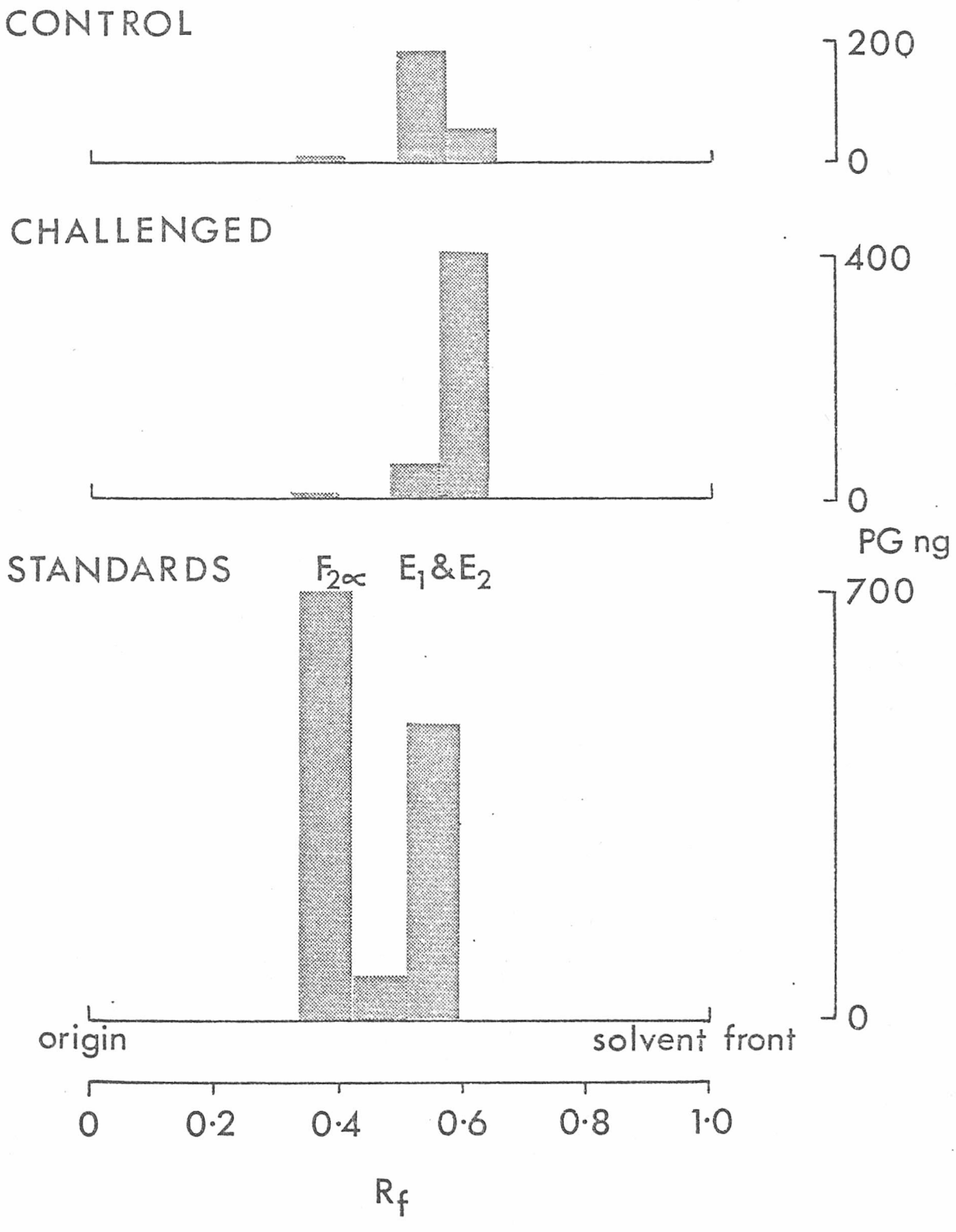
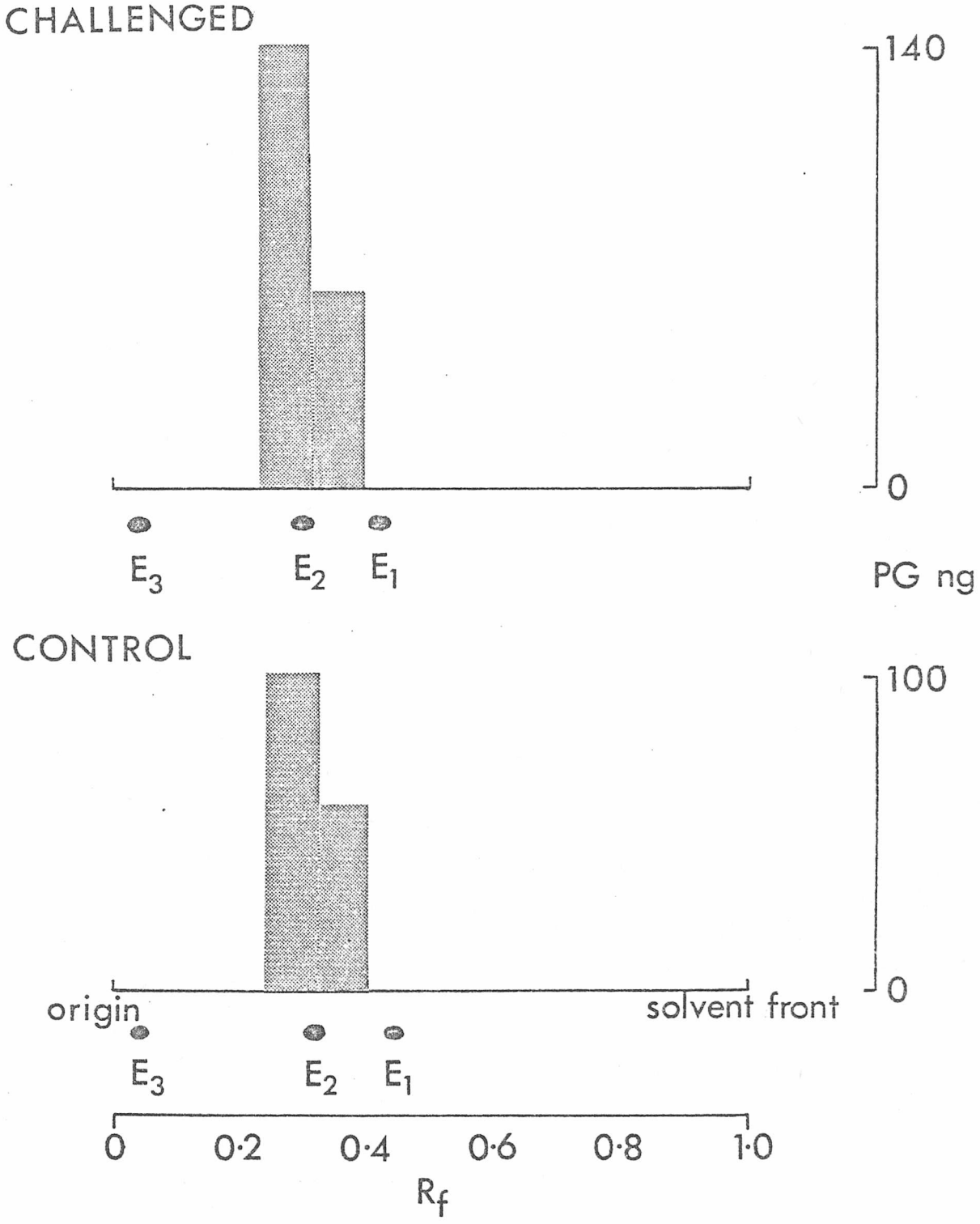

CONTROL
200
0
CHALLENGED
400
0
PG ng
STANDARDS
$F_{2\alpha}$
E_1&E_2
700
0
origin
solvent front
0
0·2
0·4
0·6
0·8
1·0
R_f

Fig 9 Argentation thin-layer chromatography of prostaglandin-like material from plaice skin. The 2 histograms represent the migration of smooth muscle contracting material released from challenged and from non-challenged (control) skin. The distance travelled by standard prostaglandins E_1, E_2 and E_3 is indicated under each histogram. The solvent system was ethyl acetate-ethanol-acetic acid (100:1:1).


CHALLENGED
140
0
E3
E2
E1
PG ng
CONTROL
100
0
origin
solvent front
E3
E2
E1
0
0·2
0·4
0·6
0·8
1·0
Rf

Standard	Tissue*	Concentration of unknown (ng/ml)	Fiducial Limits
E_1	R S S	446	408 - 489
	C R	269	235 - 311
	R C	343	255 - 484
E_2	R S S	208	189 - 229
	C R	197	184 - 212
	R C	185	154 - 221
E_3	R S S	782	658 - 941
	C R	1065	1008 - 1124
	R C	3220	2711 - 3904
$F_{1\alpha}$	R S S	1648	1498 - 1806
	C R	6425	5721 - 7282
	R C	913	821 - 1019
$F_{2\alpha}$	R S S	882	741 - 1065
	C R	1355	1160 - 1614
	R C	815	680 - 1001

Table 2 3+3 parallel assays using prostaglandin standards E_1, E_2, E_3, $F_{1\alpha}$ and $F_{2\alpha}$

* R S S, rat stomach strip
C R, chick rectum
R C, rat colon

See methods, page 41.

Standard Assay tissue*	E_1 RSS	E_1 CR	E_1 RC	E_2 RSS	E_2 CR	E_2 RC	E_3 RSS	E_3 CR	E_3 RC
Linear Regression	+	+	+	+	+	+	+	+	+
Deviation from Parallelism	-	-	-	-	-	-	-	-	+
Deviation from Linearity	-	-	-	-	+	-	-	-	-
Deviation from Curvature	-	-	-	-	-	-	-	-	-
Between Preparations	+	+	+	-	-	-	+	+	+
Between Blocks	+	+	+	+	+	+	+	+	-

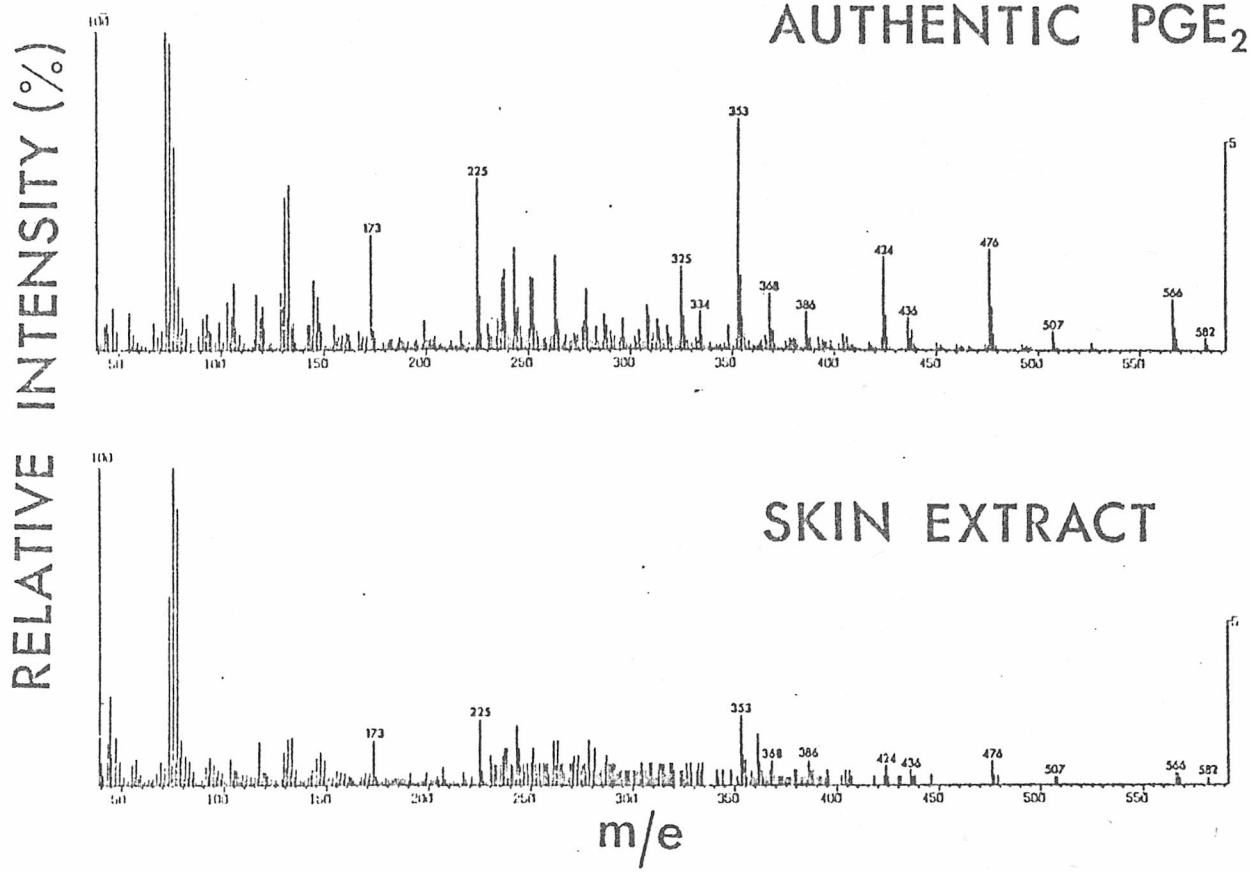
Table 3 Summary of analyses of variance of 3 + 3 assays

\+ indicates a significant difference ,

\- a non-significant difference at the 5% probability level

* RSS, rat stomach strip
CR, chick rectum
RC, rat colon

Fig 10 Mass spectra of the prostaglandin E-like material extracted from incubation media of challenged plaice skin and of authentic prostaglandin E_2. The samples were run as the methyl ester, methyloxime, trimethyl silyl ethers. Principal peaks characteristic of E_2 are indicated by horizontal numbering (see page 46).


AUTHENTIC PGE2
SKIN EXTRACT
RELATIVE INTENSITY (%)
m/e
100
50
100
150
200
250
300
350
400
450
500
550
173
225
325
334
353
368
386
424
435
476
507
566
582

3.2 iii) Time-course of release of prostaglandin-like material

Release of prostaglandin-like material from skin challenged with E. floccosum and from non-challenged skin was studied over a period of 120 minutes (Fig. 11). Release of active material from challenged skin was significantly greater than that from non-challenged skin from 20 - 80 minutes incubation and was maximal (50 ng/g) after about 60 minutes. Application of regression analysis showed that in mathematical terms, the pattern of non-challenged release was best explained by a linear equation, whereas the pattern of release from challenged skin was more complex and was better explained by a cubic equation. This strongly indicates a different mechanism of release. Fig. 12 shows the same results plotted as the difference in prostaglandin levels (between challenged and non-challenged output) released over 120 minutes and illustrates the effect of challenge on the release of smooth muscle stimulating material without interference from non-challenged release.

In much the same way as the intensity of the skin reactions in whole plaice varied during the investigation, the release of prostaglandin -like material from plaice skin in vitro was also inconsistent. During the latter part of 1976, there was no significant difference between prostaglandin release from challenged and from non-challenged skin, although prostaglandin-like material was still released from skin incubated in the absence of challenging agent. By the beginning of 1977, however, there was once again a significant elevation of prostaglandin release from skin challenged with E. floccosum, correlating well with the improved skin reactions. Therefore, in subsequent experiments, plaice were always skin tested with E. floccosum in vivo prior to incubation of skin in vitro.

The effect of compound 48/80 on the release of smooth-muscle contracting material from skin was also observed. Due to the direct action of 48/80 on smooth muscle, samples were extracted into chloroform at pH 3 prior to assay. As with E. floccosum, 48/80 (1 mg/ml) caused a significant potentiation of the release of prostaglandin-like material from skin after 60 minutes incubation as compared with the control (Table 4). There was no difference, however when lower concentrations of 48/80 were employed.

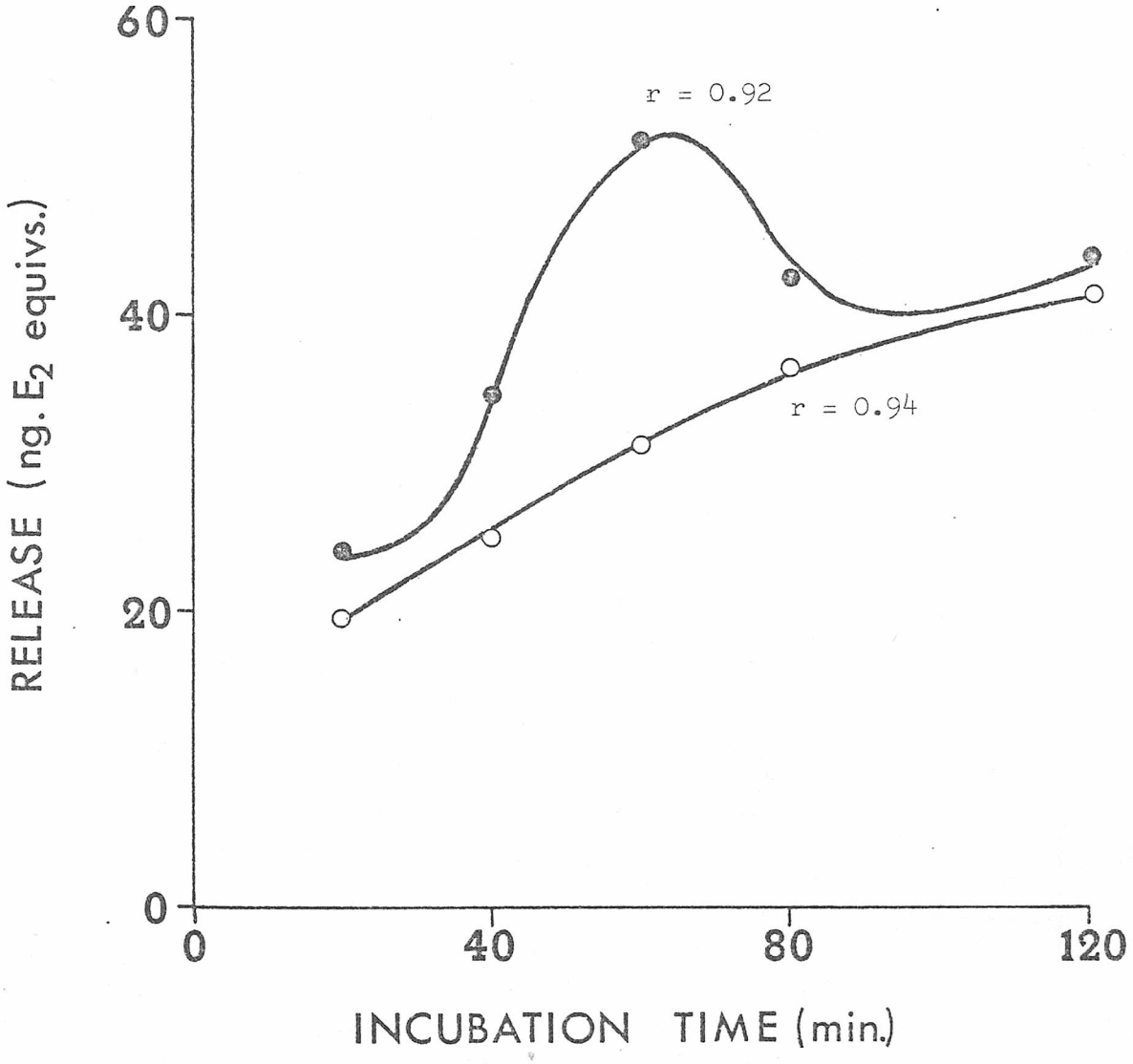


Fig 11 Time course of release of prostaglandin-like material from challenged (● – ●) and from non-challenged skin (O - O). Each point represents the mean of 6 observations of the amount of prostaglandin released from plaice skin (1g) expressed as nanograms of E_2 equivalents. The line of best fit for the data, estimated by regression analysis is indicated by the correlation coefficient, r.

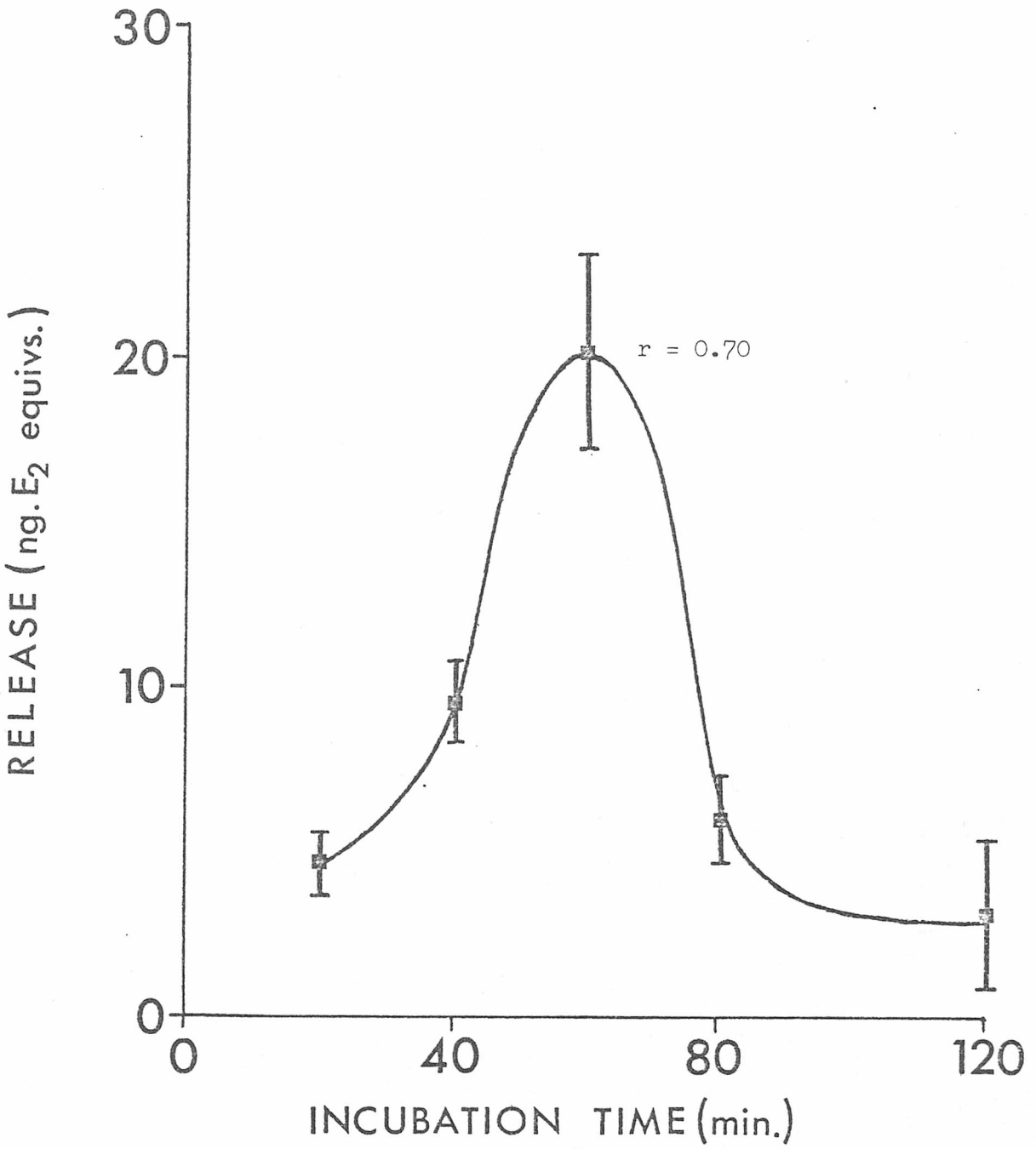


Fig 12 Time course of release of prostaglandin-like material from plaice skin (expressed as the difference between challenged and non-challenged release). Each point is the mean ± s.e. mean of 6 observations. The line which best fits the data is estimated by the correlation coefficient, r. Release of material was maximum after about 60 minutes.

Concentration of 48/80 (mg/ml)	Prostaglandin release (ng/g E_2 equivalents)		Statistical analysis	
	Challenged skin	Non-challenged skin	t	P
0.01	21 ± 2.0	22 ± 3.2	0.18	> 0.05
0.1	21 ± 1.5	21 ± 1.2	0.17	> 0.05
1.0	30 ± 2.2	19 ± 0.9	4.66	< 0.01

Table 4 Effect of compound 48/80 on the release of prostaglandin-like material from plaice skin in vitro after 60 minutes incubation

Results are the mean with s.e. mean of 3 observations. P values indicate the difference between challenged and non-challenged release.

3.2 iv) Effect of drugs on the release of prostaglandin-like material from plaice skin

Indomethacin

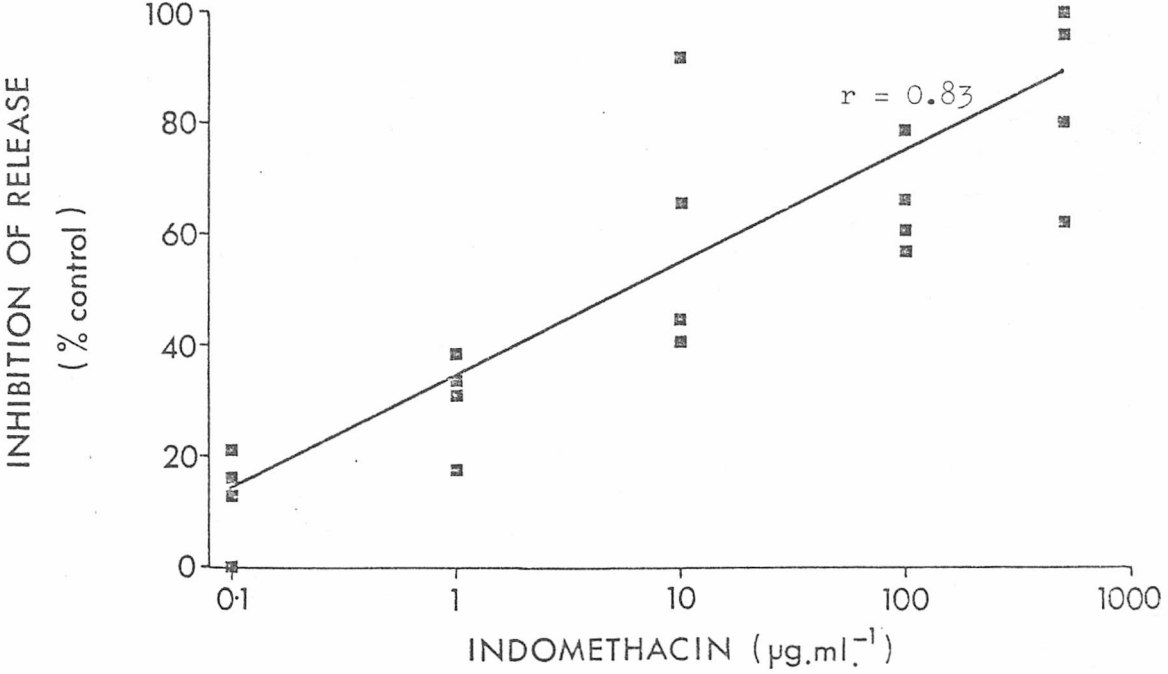
(a) In vitro

Indomethacin (0.1 - 500 µg/ml), added to skin samples just prior to incubation *in vitro* caused an inhibition of release of prostaglandin-like material from both challenged and non-challenged skin after 60 minutes incubation (Fig 13 a). Non-challenged release was reduced (by a maximum of 50%) but not completely abolished, possibly because the inhibitor was added to the incubation media after the skin had been chopped, that is after the initial stimulus for release. In contrast, prostaglandin release stimulated by challenge with *E. floccosum* (the difference between challenged and non-challenged release) was totally inhibited by indomethacin (Fig 13b) with an ID_{50} of about 5 µg/ml.

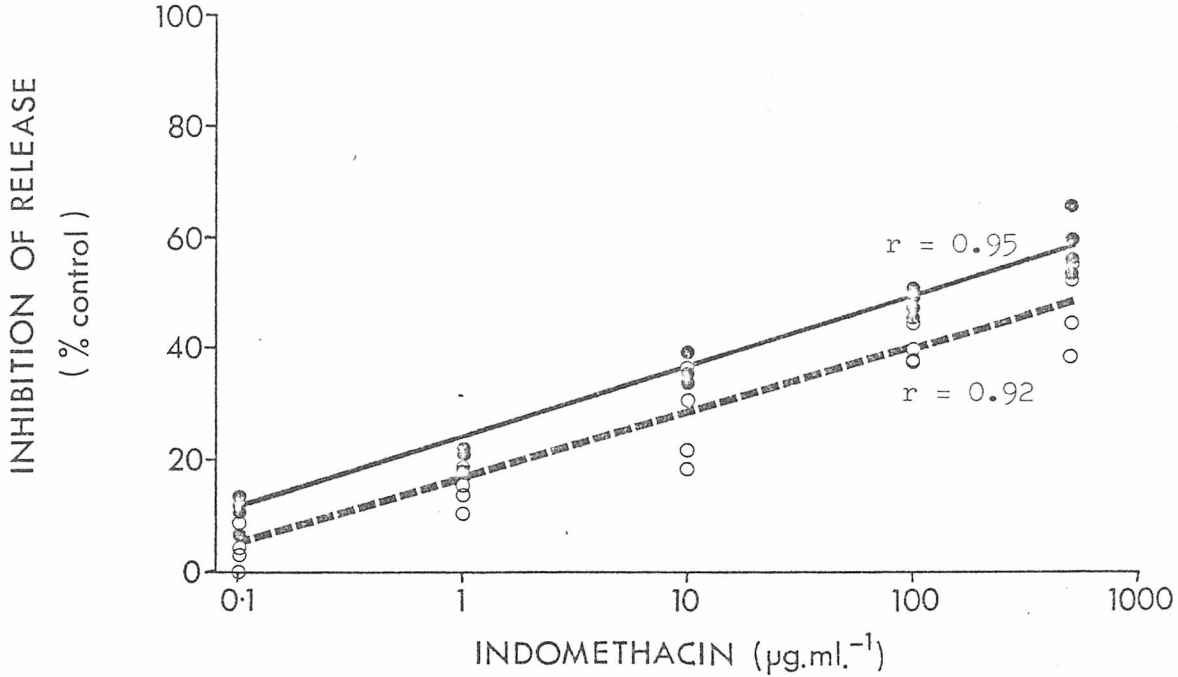
(b) Ex vivo

Indomethacin (0.1 - 1000 µg/kg), given to plaice by intraperitoneal injection 24 hours before killing, produced a dose-dependent inhibition of release of active material from both challenged and non-challenged skin, tested after incubation for 60 minutes *in vitro* (Fig 14a). The fact that prostaglandin release was completely abolished can be explained by the presence of inhibitor drug in the skin prior to the application of the stimulus for synthesis. However, inhibition of challenged release was significantly greater than inhibition of non-challenged release. To illustrate this point, as before the effect of indomethacin on the net prostaglandin release induced by challenge (the difference between challenged and non-challenged release) was plotted graphically (Fig 14b). An ID 50 of about 4µg/kg was observed. Whilst inhibiting prostaglandin release *in vitro*, indomethacin did not inhibit the skin reactions induced *in vivo* by *E. floccosum* in these fish immediately prior to killing.

Fig 13 a) Inhibition by indomethacin of release of prostaglandin-like material from challenged (● — ●) and non-challenged (o — o) plaice skin. Indomethacin was added to skin incubation samples immediately prior to incubation in vitro. b) Inhibition by indomethacin of prostaglandin release stimulated by challenge with E. floccosum (that is the difference between challenged and non-challenged release in a). In a) and b), individual observations (4) for each concentration of indomethacin are shown. The straight lines which best fit the data were drawn; the correlation is indicated by the coefficient, r.

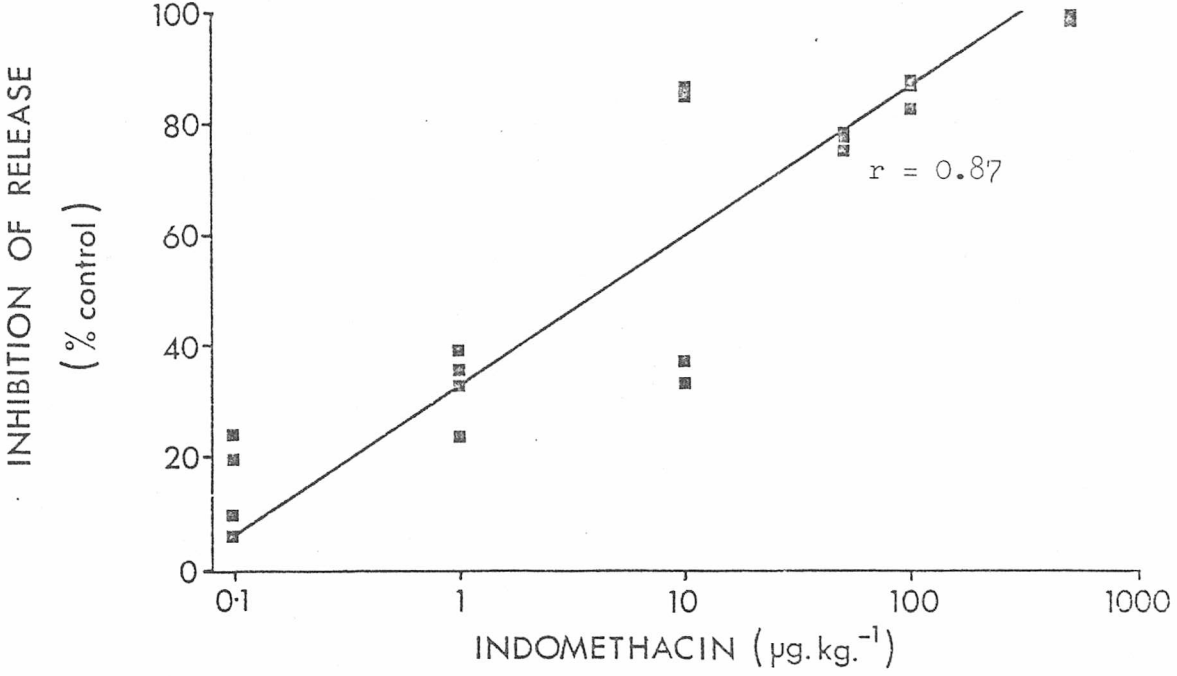

INHIBITION OF RELEASE
(% control)
100
80
60
40
20
0
r = 0.83
0·1
1
10
100
1000
INDOMETHACIN (μg.ml.$^{-1}$)

b)

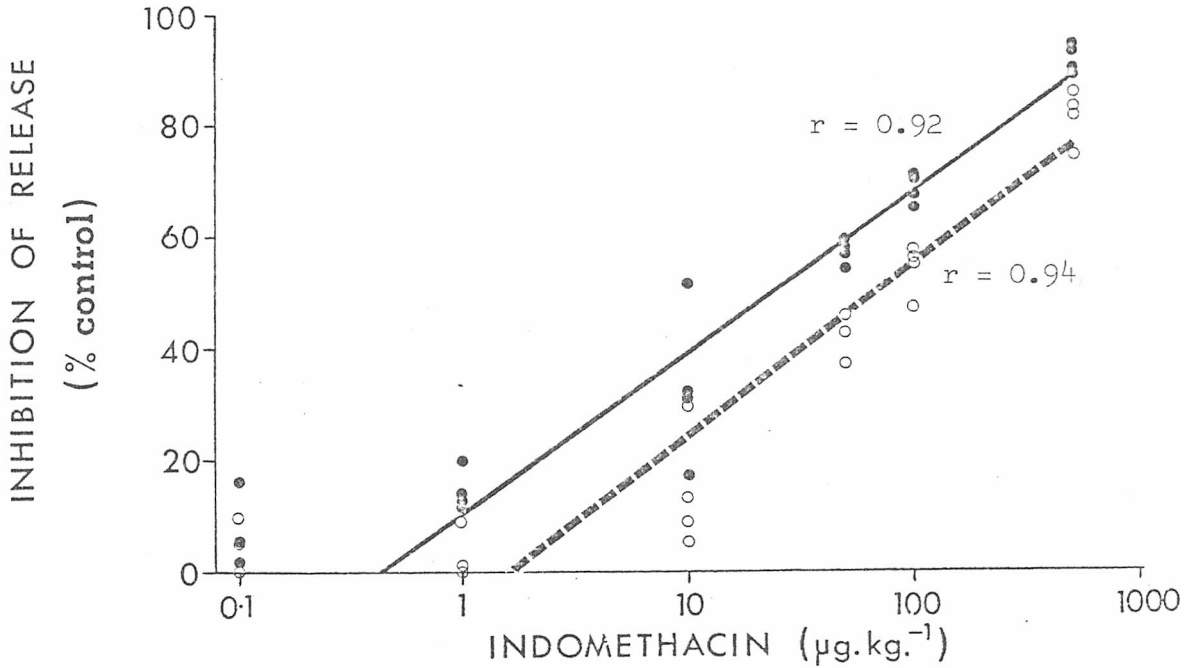

INHIBITION OF RELEASE
(% control)
100
80
60
40
20
0
0·1
1
10
100
1000
r = 0.95
r = 0.92
INDOMETHACIN (μg.ml.$^{-1}$)

a)

Fig 14 a) Inhibition by indomethacin of release of prostaglandin-like material from challenged (● — ●) and non-challenged (o — o) plaice skin. Indomethacin was given to plaice by intraperitoneal injection 24 hours prior to killing and incubation of chopped skin in vitro. b) Inhibition by indomethacin of prostaglandin release stimulated by challenge with E. floccosum (that is the difference between challenged and non-challenged release in a). In a) and b), individual observations (4) for each dose of indomethacin are shown. The straight lines which best fit the data were drawn: the correlation is indicated by the coefficient, r.


INHIBITION OF RELEASE
(% control)
100
80
60
40
20
0
0·1
1
10
100
1000
INDOMETHACIN (μg.kg.$^{-1}$)
r = 0.87

b)


INHIBITION OF RELEASE
(% control)
100
80
60
40
20
0
0·1
1
10
100
1000
INDOMETHACIN (µg.kg.⁻¹)
r = 0.92
r = 0.94

a)

Disodium cromoglycate (DSCG)

Fig 15 shows the effect of DSCG (160 mg/kg), given to plaice by intravenous injection 10 minutes before killing, on prostaglandin release from challenged and non-challenged skin, on incubation *in vitro*. There was a significant reduction in challenged release ($p < 0.01$) after 30 and 60 minutes incubation but no inhibition of non-challenged release. In all 5 plaice, DSCG caused a complete inhibition of the skin reaction to *E. floccosum*, the effect being maximum 10 minutes after injection. In a dose of 80 mg/kg, DSCG did not completely inhibit the skin reaction *in vivo* and did not reduce prostaglandin release *in vitro* from either challenged or non-challenged skin.

Hydrocortisone

The effect of hydrocortisone (1 mg/ml) on prostaglandin release *in vitro* was studied in experiments on skin from 5 plaice. Added to skin samples before incubation, hydrocortisone did not affect prostaglandin release from either challenged or non-challenged skin after 60 minutes incubation.

3.2 v) Effect of skin extracts on dye leakage in rats

Extracts of incubation media from plaice skin challenged *in vitro* with *E. floccosum* were also tested for their ability to influence capillary permeability in rat skin. Fig. 16 shows the dye leakage responses to prostaglandin E_2 and acidic extract (assayed previously against authentic E_2) as performed in parallel. Both extract and E_2 caused a dye leakage but this was not dose-dependent. There was no significant difference between the responses at all the concentrations used. The effect was also observed of a low dose of acidic extract on the dye leakage response to standard histamine (Fig. 17). Histamine (50 - 400 μM) caused a poor dye leakage which also was not dose-dependent. The responses to histamine were significantly potentiated by the presence of acidic extract (0.19 μM in E_2 equivalents). However, this effect could be wholly attributable to prostaglandin E_2, since an equivalent concentration of authentic E_2 was also found to potentiate dye leakage responses to histamine.

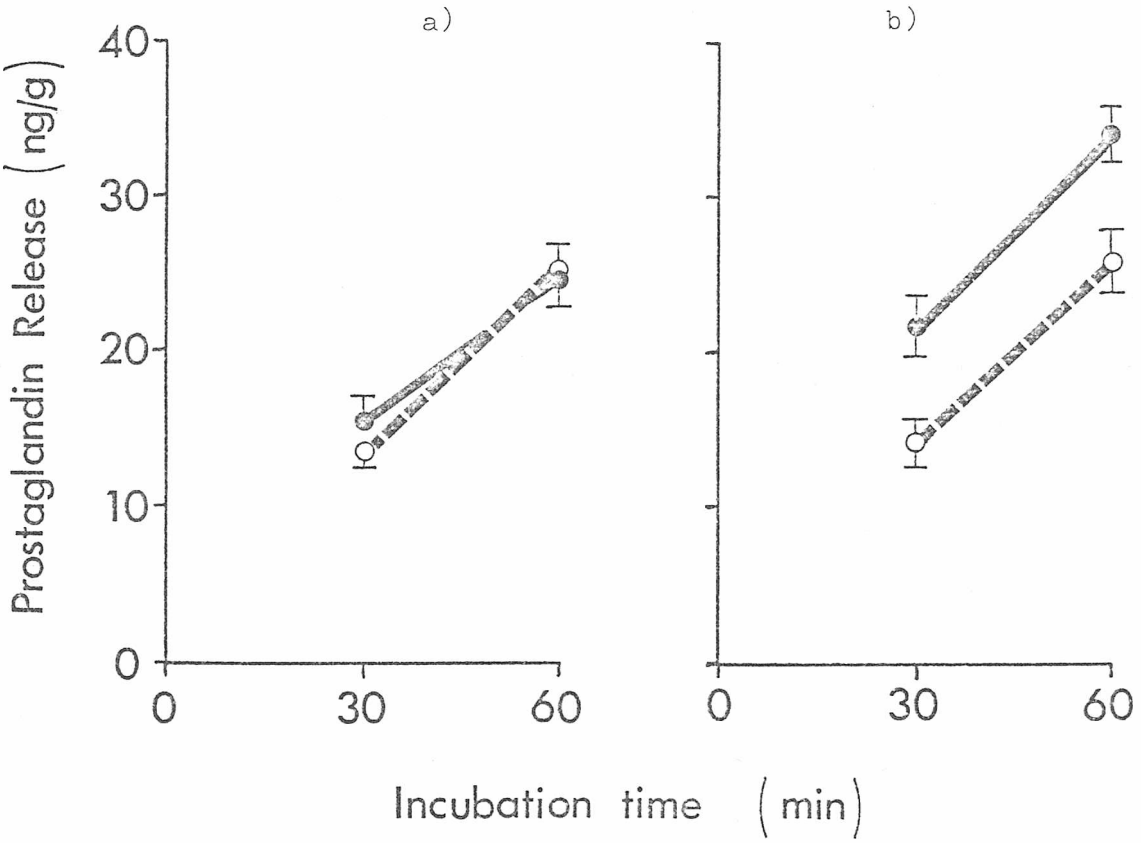


Fig 15 Effect of disodium cromoglycate (DSCG) (160 mg/kg) on the release of prostaglandin-like material from non-challenged (a) and challenged (b) plaice skin. DSCG was given to plaice by intravenous injection 10 minutes prior to killing and incubation of skin in vitro. Closed circles represent skin from untreated plaice and open circles, skin from fish injected with DSCG. There was a significant reduction of challenged release but no change in non-challenged release. Each point is the mean with s.e. mean of 5 observations.

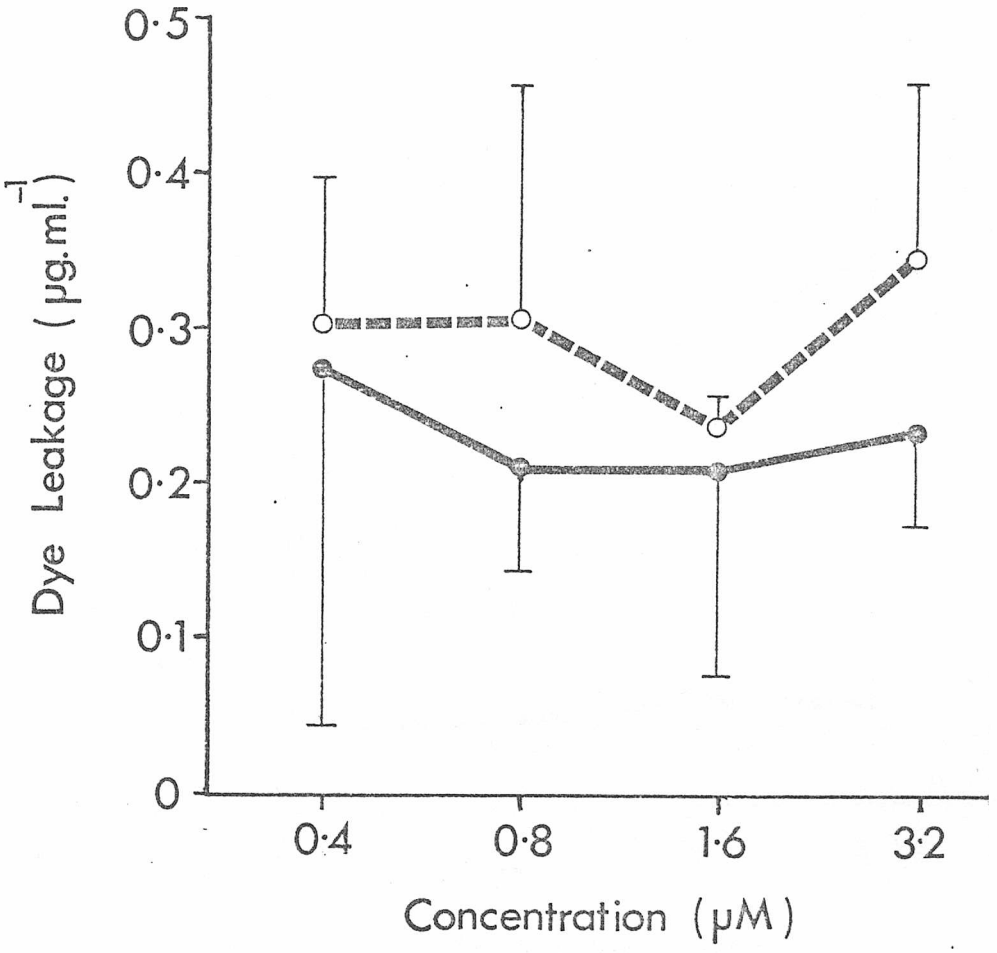


Fig 16 Dye leakage responses to prostaglandin E_2 (o—o) and an extract of incubation media from challenged plaice skin (assayed against authentic E_2) (●—●) in rat skin. Each point is the mean with s.e. mean of 4 observations. There was no significant difference between the responses at any concentration.

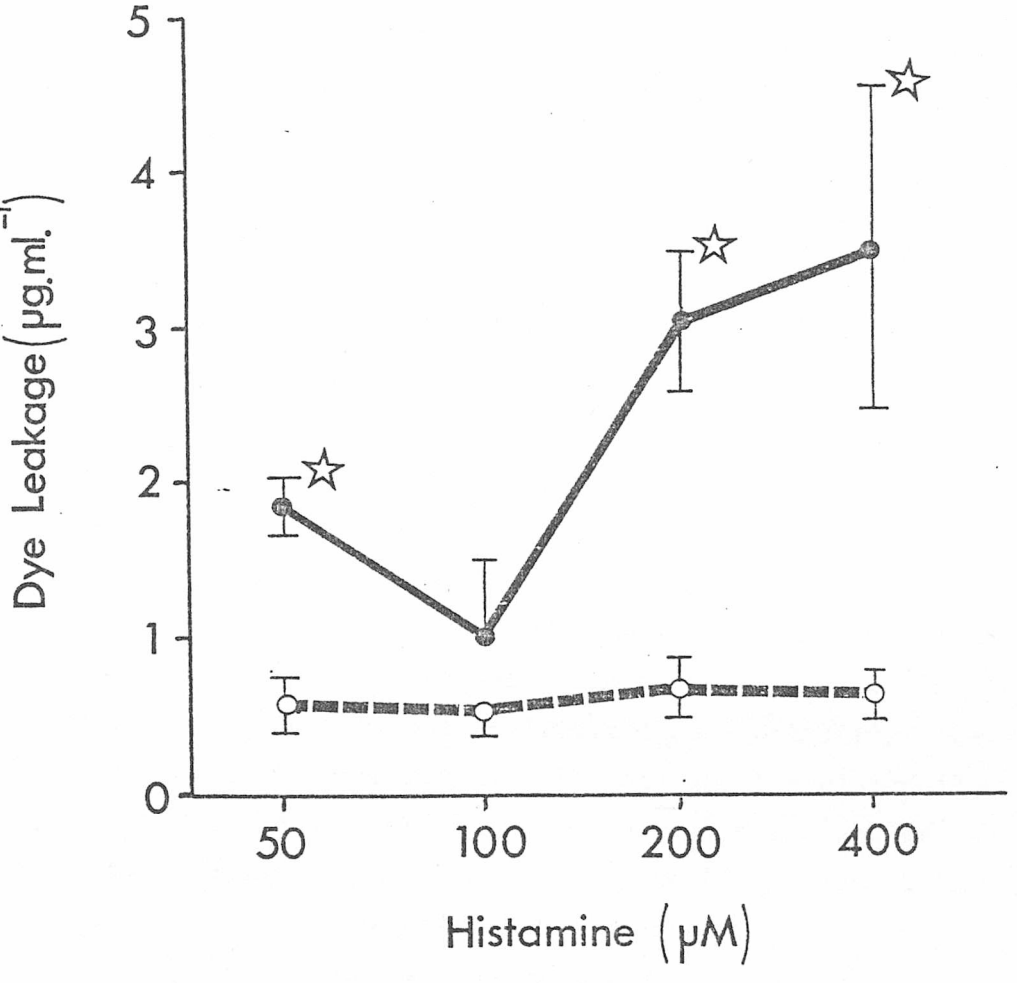


Fig 17 Effect of an acidic extract of plaice skin incubation media (0.19 µM in equivalents of standard E_2) on the dye leakage responses to histamine in rat skin. Control responses are shown as open circles and responses in the presence of extract as closed circles. Each point is the mean with s.e. mean of 5 observations. A star indicates a significant difference ($p < 0.05$) at that concentration.

The concentrated basic extract did not change the smooth muscle tone of the guinea-pig ileum or the rat stomach strip. In 5 rats, it did not cause a detectable leakage of dye into the skin. Moreover, there was no increase in the responses to standard histamine when given in combination with basic extract.

3.3. DIRECT PERFUSION OF PLAICE SKIN *in vivo* AS A MODEL OF CUTANEOUS ANAPHYLAXIS

A continuous perfusion technique was used as an alternative method to provide an inflammatory exudate of plaice skin for pharmacological analysis.

3.3 i) Sensitivity of assay tissues

The very high sensitivity of the laminar flow superfusion technique claimed by Ferreira & Souza Costa (1976) could not be reproduced. Nevertheless, preliminary experiments showed that the oil-bathed organs could detect a minimum of 0.1 ng prostaglandin E_2 (rat stomach strip), 0.2 ng 5-HT (rat stomach strip), 0.2 ng histamine (guinea-pig ileum), 1 ng bradykinin (guinea-pig ileum) and 5 ng noradrenaline (rabbit aorta). The mesenteric artery of the cod was found to respond to a threshold of 10 ng noradrenaline and 5-HT but to little else. Similarly, the cod aorta only responded to noradrenaline (10 ng) and the plaice ileum to 5-HT (10 ng). The perfused rabbit ear preparation was not as sensitive as some of these assay tissues. Noradrenaline (5 ng) and histamine (10 ng) caused a constriction of the ear (reduced perfusion rate) whereas prostaglandin E_2 (10 ng) and acetylcholine (10 µg) produced a dilatation (increased perfusion rate). In each case, values are minimum effective doses.

3.3 ii) Analysis of skin perfusates

Perfusion was carried out in 85 anaesthetized plaice for 15 - 60 minutes. In initial experiments, the perfusates from 28 plaice were superfused directly over the assay tissues. After an initial change in baseline, the isolated organs responded normally to standards when the superfusing solution was changed from Krebs to plaice Ringer saline.

Of the 12 plaice challenged subcutaneously with compound 48/80 (1 mg) and E. floccosum (1 mg), smooth-muscle-stimulating activity could only be detected in the perfusate from 1 fish, as detected by the rat stomach strip. In a further 3 experiments, challenge with E. floccosum was made by infusion into the cannula over a period of 2 minutes. No activity was detected in the perfusates. Challenge by intradermal injection over the perfusion area was performed in 13 plaice. In 2 of these experiments, contractions of the rat stomach strip were observed shortly following challenge both with compound 48/80 (1 mg) and E. floccosum (1 mg) and, in one instance a contraction of the rabbit aorta was produced. Gentle scoring of the skin over the perfused area did not result in any change in the smooth muscle tone of the assay tissues. Thus, 25 of the 28 fish did not release any substance which altered smooth muscle activity.

Subsequently, perfusates were collected in fractions prior to assay. As before, recovery of smooth muscle activity was not reproducible with only occasional contractions of the rat stomach strip and rabbit aorta being observed. The guinea-pig ileum, the rat colon and the tissues of the cod and plaice did not respond at all to skin perfusate. However, the collection of relatively large volumes of perfusate (30 - 50 ml) enabled extraction to be performed to concentrate any activity. In 8 plaice, perfusates were collected in 15 minute samples for 60 minutes after intradermal challenge with 1 mg of E. floccosum. The samples were then extracted with ethyl acetate at pH 3 and the organic phase reconstituted with Krebs saline and assayed against prostaglandin E_2 using the rat colon and the rat stomach strip. Table 5 shows that activity was confined to the first 15 minute collection period and contained an average of 3.2 ng E_2 equivalents. Extracts of perfusates from 4 plaice "challenged" with 0.9% saline as a control did not contain detectable activity ($<$ 1 ng E_2). Preparative TLC was then performed on the combined extracts of 4 perfusates of plaice skin challenged with E. floccosum. Using the F VI solvent system, activity was found to co-chromatograph exclusively with the E-series prostaglandins (Fig 18). Similar extraction of perfusates at pH 10 did not reveal the presence of any further pharmacologically-active material.

Quantitative GC-MS analysis of prostaglandin-like activity was carried out on the ethyl acetate extracts of the combined perfusates from groups of 4 plaice challenged with E. floccosum, compound 48/80, carrageenin, silver nitrate, a non-specific irritant in rat skin (Chahl & Chahl, 1976) and saline. The results are shown in Table 6.

Plaice no.	Prostaglandin released (ng E_2 equivalents) RSS	RC
1	4.8	4.7
2	3.5	3.9
3	4.0	5.0
4	3.0	3.8
5	2.8	3.5
6	ND	ND
7	2.3	2.3
8	3.5	4.0
Mean ± s.e. mean	3.0 ± 0.5	3.4 ± 0.6

Table 5 Release of postaglandin-like material from perfused plaice skin challenged with E. floccosum (1 mg)

Values are of prostaglandin equivalents in perfusates of the skin of 8 plaice after challenge in the 15 minute collection period following challenge. Assay was performed on the rat stomach strip (RSS) and rat colon (RC) in parallel. Prostaglandin- like material could not be detected (ND) in perfusates collected 15 - 60 minutes after challenge.

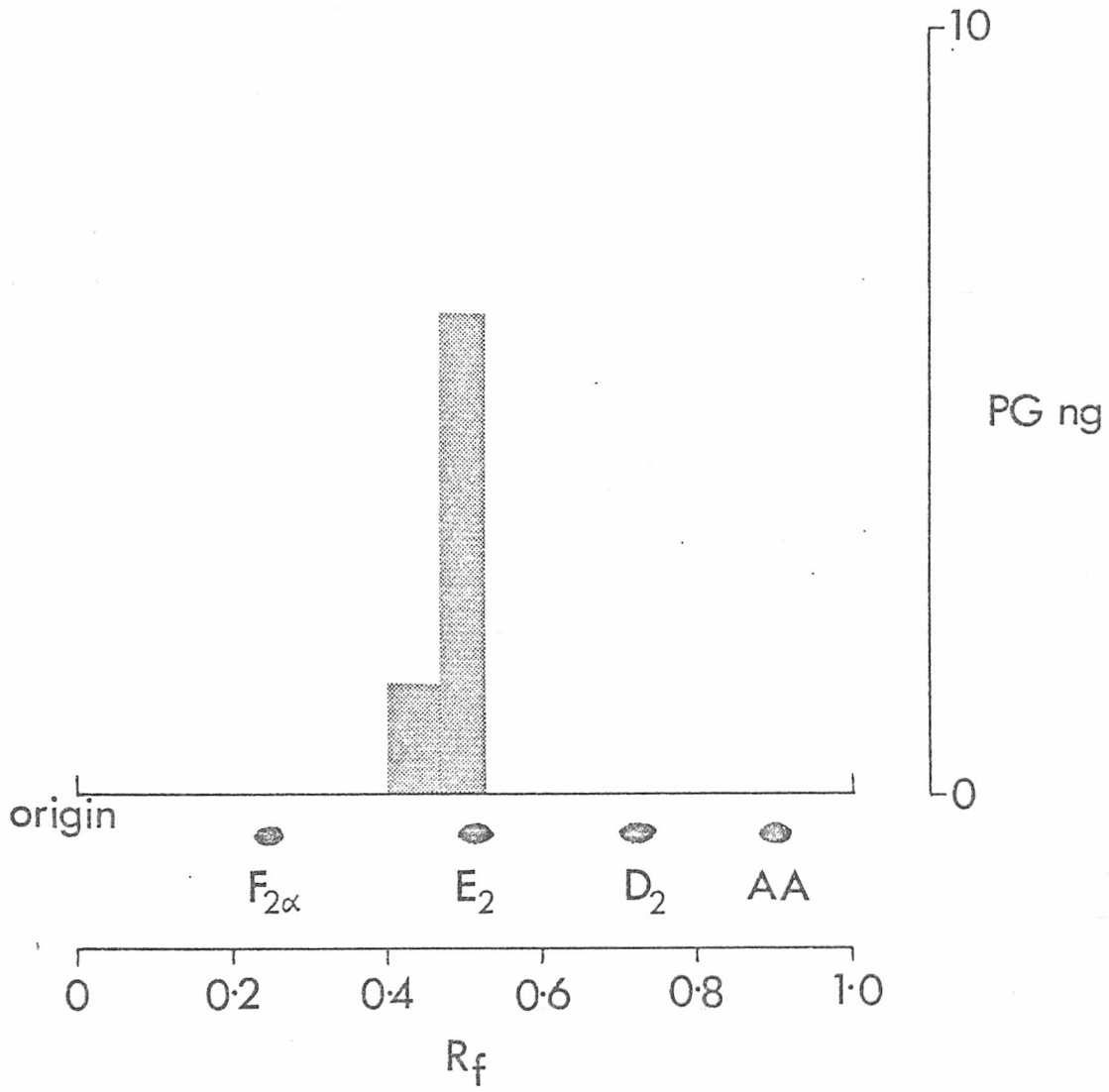


Fig 18 Preparative thin-layer chromatography of prostaglandin-like material in an extract of perfusates of plaice skin. The extract was made from the combined perfusates of 4 experiments in which plaice skin was challenged with E. floccosum. The histogram represents the migration of smooth muscle contracting material in the solvent system, ethyl acetate - acetone - acetic acid (90 : 10 : 1). The distance travelled by standard prostaglandins $F_{2\alpha}$, E_2 and D_2 and arachidonic acid (AA) is indicated under the histogram.

Challenging agent	Dose	Prostaglandin release (ng E_2 equivs)
saline	-	ND
silver nitrate	1 mg	ND
carrageenin	1 mg	ND
E. floccosum	1 mg	11
Compound 48/80	1 mg	13

Table 6 Quantitative GC-MS analysis of perfusates of skin challenged with different agents

Each value represents the prostaglandin content in equivalents of authentic E_2 of perfusates combined from 4 experiments. ND indicates that prostaglandin-like activity was not detected.

Prostaglandin activity was only detected in perfusates of plaice skin challenged with E. floccosum and compound 48/80.

Skin perfusates from 8 plaice were tested directly for activity on blood vessels using the perfused rabbit ear. 5 plaice were challenged intradermally with compound 48/80 (200 µg) and the perfusate collected in successive 2 minute fractions. In 3 of these fish, the initial collection after challenge caused a marked dilatation of the ear, manifested by an increased flow rate. After standing at room temperature for 10 minutes this activity had declined and within 30 minutes had almost disappeared, although the sensitivity of the ear to the dilatating action of the authentic prostaglandin E_2 had not changed (Fig 19). Subsequent collections of perfusate dilated the ear to a lesser extent and after 6 minutes were ineffective. The active material was shown to be prostaglandin-like, since it could be extracted into ethyl acetate at pH 3 but it only produced a small contraction of the rat stomach strip and had no effect on the rabbit aorta. Direct injection of compound 48/80 (50 µg) into the perfused ear caused, if anything a slight constriction (reduced flow rate). Neither intradermal injection of saline nor gentle scoring of the skin caused the release of similar active material in these fish.

Of the 3 plaice challenged intradermally with E. floccosum (1 mg), only the perfusate from one fish dilated the rabbit ear. This dilating activity, which was detected up to 4 minutes after challenge disappeared after standing at room temperature for 30 minutes. As for the active substance released by compound 48/80, this active material was prostaglandin-like. In 4 of the 8 fish studied, dilating activity was not detected. Unfortunately, skin reactions were usually not observed in these experiments. This may be due to the fact that the skin was distended as a result of the perfusing Ringer saline.

3.3 iii) Recovery experiments

The recovery of prospective mediator substances administered into perfused plaice skin by subcutaneous injection was studied in groups of 4 fish. The results are shown in Tables 7 to 10 and show that whereas about 20% of the bradykinin and 5-HT injected into the skin was recovered in the perfusate over the 60 minute collection period, this figure dropped to only 6% for histamine and prostaglandin E_2.

Fig 19 Experimental record of the effect of perfusates of plaice skin on the perfused rabbit ear preparation obtained in a single experiment. Authentic noradrenaline (NA) and prostaglandin E_2 produced respectively a constriction (decreased perfusion rate) and a dilatation (increased perfusion rate). At X, 0.1 ml of the initial perfusate collected after intradermal challenge of plaice skin with compound 48/80 (200 µg) was tested : a dilatation was observed. This injection was repeated after 5, 15 and 30 minutes incubation at room temperature with a subsequent loss of activity. The sensitivity to E_2 had not changed. Direct application of compound 48/80 caused, if anything a constriction.

← constriction
dilatation →
NA
5ng
E2
10ng
48/80
50µg
X
X
+5
X
E2
+15
5ng
X
E2
+30
10ng
5 min

Total histamine (ng)

Duration of perfusion (min) 0-15	15-30	30-45	45-60	Recovery (%)
567	142	ND	ND	7.1
396	190	50	ND	6.4
363	113	35	ND	5.1
288	98	ND	ND	3.9
			Mean ± s.e. mean	5.6 ± 0.7

Table 7 Recovery of 10 μg histamine injected subcutaneously into perfused plaice skin

The results of 4 experiments are shown. ND indicates that histamine was not detected.

Total prostaglandin E_2 (ng)

Duration of perfusion (min) 0-15	15-30	30-45	45-60	Recovery (%)
360	120	ND	ND	4.8
480	184	50	ND	7.1
500	120	40	ND	6.6
320	130	ND	ND	4.5
			Mean ± s.e. mean	5.8 ± 0.8

Table 8 Recovery of 10 μg prostaglandin E_2 injected subcutaneously into perfused plaice skin.

The results of 4 experiments are shown. ND indicates that E_2 was not detected.

Total 5-HT (ng)				
Duration of perfusion (min)				Recovery
0-15	15-30	30-45	45-60	(%)
1560	683	180	ND	24.5
1265	413	38	ND	17.2
1440	645	ND	ND	20.9
1060	700	102	ND	18.6
			Mean ± s.e. mean	20.3 ± 1.6

Table 9 Recovery of 10 μg 5-HT injected subcutaneously into perfused plaice skin

The results of 4 experiments are shown. ND indicates that 5-HT was not detected.

Total bradykinin (ng)				
Duration of perfusion (min)				Recovery
0-15	15-30	30-45	45-60	(%)
1520	580	120	ND	22.2
1240	620	ND	ND	18.6
1690	600	58	ND	23.5
1280	780	50	ND	21.1
			Mean ± s.e. mean	21.4 ± 1.0

Table 10 Recovery of 10 μg bradykinin injected subcutaneously into perfused plaice skin

The results of 4 experiments are shown. ND indicates that bradykinin was not detected.

Therefore, this technique is only likely to detect the presence of relatively large amounts of histamine and E_2 released from plaice skin.

3.4 ATTEMPTED FORMATION OF SUCTION BLISTERS ON PLAICE SKIN

An effort was made to raise suction blisters at the site of inflammation in plaice skin as a source of a more concentrated inflammatory exudate for biological assay. In 10 plaice, pressures of up to 700 mm below atmospheric pressure were applied to plaice skin after challenged with E. floccosum or compound 48/80. However, no blistering was observed at any time in these experiments even after application of maximum pressure for 2 hours.

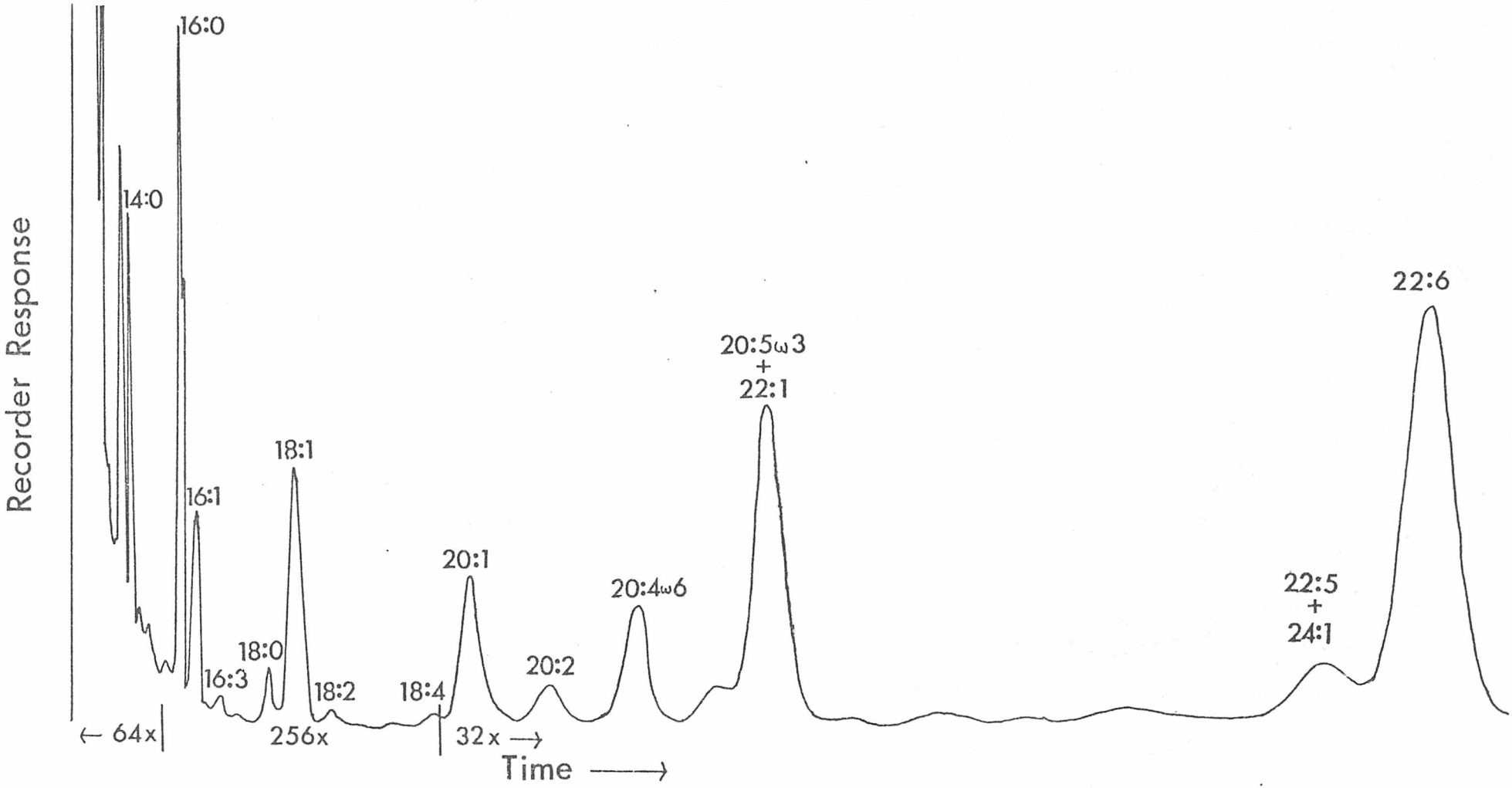
3.5 FATTY ACID COMPOSITION OF PLAICE SKIN

In 2 experiments, 10g of plaice skin yielded 200 mg of lipid on extraction, that is 20 mg/g of skin. The results from a representative GC analysis are shown in Fig 20. Table 11 summarizes the overall fatty acid composition of the skin. There was good agreement between the results obtained with the 2 columns. The fatty acids which were major components were C 16 : 0, C 18 : 1 and C 22 : 6, contributing about 60% of total. Of the prostaglandin precursors, C 20 : 4 w6 fatty acid (E_2 precursor) was present in a concentration of 2.8%. In contrast, the concentration of C 20 : 3 w6 fatty acid was very low but the precursor of prostaglandin E_3, C20 : 5 w 3 contributed about 9% of total.

3.6 PROSTAGLANDIN SYNTHESIS BY A SUBCELLULAR FRACTION OF PLAICE SKIN

It has been found that although E_2 was the only prostaglandin identified in incubations of plaice skin, arachidonic acid was not the major prostaglandin precursor in the skin, as expected. In an effort to resolve this anomaly, a study was undertaken of the conversion of the synthetic precursor fatty acids into prostaglandins by a particulate enzymic fraction of plaice skin.

Fig 20 Gas chromatogram of the fatty acid methyl esters in a lipid extract of plaice skin. The glass column was packed with 10% EG SS-Y organosilicone copolymer on DCMS-treated P. Temperature, 200°C; flame ionization detector. Attenuations as marked.


Recorder Response
16:0
14:0
16:1
18:1
18:0
16:3
18:2
18:4
20:1
20:2
20:4ω6
20:5ω3
+
22:1
22:5
+
24:1
22:6
← 64x
256x
32x →
Time ⟶

Fatty acid	% of total fatty acids	
	Column 1 (EGSS-X)	Column 2 (EGSS-Y)
14:0	2.2	2.0
16:0	18.5	16.2
16:1	8.4	8.0
18:0	4.5	4.0
18:1	20.0	19.8
18:2	1.1	1.6
18:3	0.6	1.2
18:4	1.6	1.8
20:1	2.5	2.3
20:3 w 6	0.2	trace
20:4 w 6	2.8	2.9
20:5 w 3	8.7	9.0
22.4	1.2	0.9
22.5	3.2	3.9
22:6	23.2	23.8
Others	1.9	2.6

Table 11 Fatty acid composition of plaice skin

The methyl esters were analysed by gas chromatography on 2 phases, 10% EGSS-Y and 10% EGSS-X (see methods, page 52)

3.6 i) Purification of precursor fatty acids

Preliminary experiments using unpurified fatty acid precursors showed that there was a very low conversion of substrate into prostaglandins. Thereafter, fatty acids were purified prior to use. Of the fatty acids applied to the HPLC column, about 70% were recovered as pure material. Representative chromatograms are illustrated in Fig. 21. There seemed to be a lot of contaminating material present, considering the manufacturer's claim of at least 97% purity. However, most of this is probably due to conjugated material with a high molar extinction coefficient.

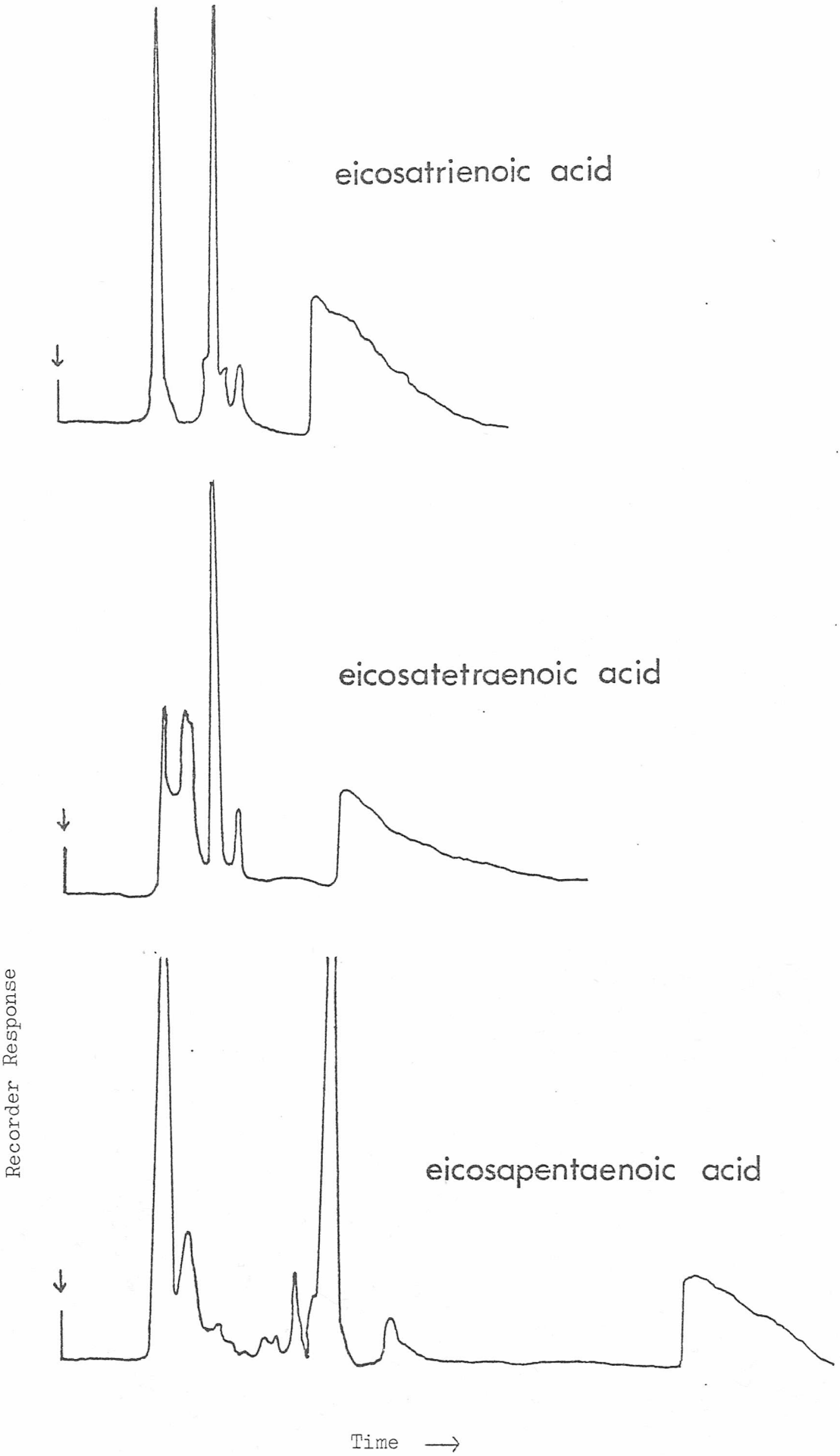
3.6 ii) Subcellular distribution of synthesizing enzyme

The ability of different subcellular fractions of plaice skin to synthesize prostaglandins from synthetic arachidonic acid was measured by both bioassay and ultra-violet absorption spectrometry (the latter will only detect the presence of E - prostaglandins after hydrolysis). Table 12 shows that the bulk of the prostaglandin synthetase activity was located in the fraction of plaice skin that corresponds to the microsomal fraction of mammalian cells since a substantial part (66%) of the glucose - 6 - phosphatase activity (a microsomal marker enzyme) was also located in that fraction. The high values of conversion detected by absorption spectrometry for the other 3 fractions are probably artefacts since the absorbing material did not show a maximum at 278 nm.

3.6 iii) Incubation with arachidonic acid

Preparative TLC using the F VI solvent system showed that the smooth muscle contracting material synthesized from arachidonic acid migrated in the Rf range corresponding very closely with the distance travelled by the E - series prostaglandins. Moreover, alkaline hydrolysis yielded a chromophore which showed a maximum absorbance at 278 nm UV. At the same time, smooth muscle activity was destroyed by this treatment.

Fig 21 High pressure liquid chromatography profile of 3 fatty acids : The stainless steel column was packed with silica gel (5μm); pressure, 400 p.s.i; solvent, dichloromethane; detection, absorbance at 245 nm U-V. The arrow marks the point of injection. Samples were collected for each major peak. The purified fatty acids were found to have a longer retention time than the bulk of the impurities present.


eicosatrienoic acid
eicosatetraenoic acid
eicosapentaenoic acid
Recorder Response
Time ⟶

Fraction	Prostaglandin synthesis (% conversion of arachidonic acid)		G-6-pase
	Bioassay	Ultraviolet Spectrometry	(n moles phosphate/mg/ 15 min)
Nuclear	0.08	0.41	12.4
Mitochondrial	0.04	0.37	15.5
Microsomal	2.04	2.18	64.0
Supernatant	0.03	0.37	5.2

Table 12 Distribution of prostaglandin synthetase and glucose-6-phosphatase in subcellular fractions of plaice skin

The synthesis of prostaglandin E_2 from arachidonic acid was assayed by bioassay on the rat stomach strip and by ultraviolet absorption spectrometry (after conversion to B_2) in parallel. Conversion was expressed as a percentage of substrate added.

In the absence of cofactors, formation of prostaglandin-like material could barely be detected. Glutathione (5 mM) was found to stimulate prostaglandin synthesis and adrenaline (5 mM), although ineffective on its own, greatly potentiated prostaglandin synthesis when incubated in combination with glutathione.

The prostaglandin synthetase activity did not seem to be greatly affected by temperature and in 2 experiments did not vary significantly from 15 - 35°C. Below 10°C, activity was suppressed but not totally inhibited.

Table 13 shows the effect of indomethacin and hydrocortisone on the synthesis of prostaglandin E_2 in a single experiment. Drug was pre-incubated with enzyme for 5 minutes before addition of substrate. Indomethacin caused a dose-dependent inhibition of synthesis whereas hydrocortisone was ineffective even in a concentration of 10 mM.

3.6 iv) Relative conversion of fatty acids

The relative conversion of the 3 purified fatty acids into prostaglandins was studied under identical conditions of incubation and extraction. Preparative TLC in conjunction with bioassay showed that all smooth muscle activity migrated with the E-series prostaglandins. Therefore, ultraviolet absorption spectrometry was used to assay for these compounds in parallel with bioassay. The results of 4 experiments are shown in Table 14. The highest conversion to prostaglandins was observed with arachidonic acid as substrate. Eicosatrienoic acid was also converted but to a lesser extent and eicosapentaenoic acid, the most abundant of precursors in plaice skin, gave a very low yield of prostaglandin. The formation of prostaglandins from the different fatty acids was checked by TLC. After development, spots were made visible by spraying with phosphomolybdic acid and heating at 120°C and the prostaglandins were located by comparison with reference compounds. GC-MS analysis of large-scale incubations was performed to identify the products. The mass spectra illustrated in Figs 22 to 24 show that prostaglandin E_1 was synthesized from eicosatrienoic acid, E_2 from arachidonic acid and E_3 from eicosapentaenoic acid.

Drug	Concentration (mM)	Conversion of substrate(%)	Inhibition (%)
None	-	2.32	-
Indomethacin	0.2	0.79	65.9
	1.0	0	100
Hydrocortisone	1.0	2.51	0
	10.0	2.40	0

Table 13 Effect of anti-inflammatory drugs on synthesis of prostaglandin E_2 by plaice skin microsomes

The conversion of arachidonic acid into E_2 was assayed by ultraviolet absorption spectrometry (after conversion to B_2). Conversion was expressed as a percentage of substrate added. In each case drug was pre-incubated with enzyme before addition of substrate.

Substrate	% Conversion of substrate		
	Bioassay		Ultraviolet spectrometry
	RSS	RC	
20:3w6	0.86±0.04	0.92±0.04	0.91±0.03
20:4w6	1.99±0.06	2.01±0.07	1.95±0.04
20:5w3	0.29±0.04	0.27±0.01	0.28±0.04

Table 14 Relative conversion of purified substrate fatty acids into E-series prostaglandins by a microsomal enzyme fraction of plaice skin

The formation of E-series prostaglandins was assayed in parallel by ultraviolet absorption spectrometry (after conversion to the respective B-series prostaglandins) and bioassay on the rat stomach strip (RSS) and rat colon (RC). Conversion was expressed as a percentage of substrate added. Each value represents the mean with s.e. mean of 4 observations.

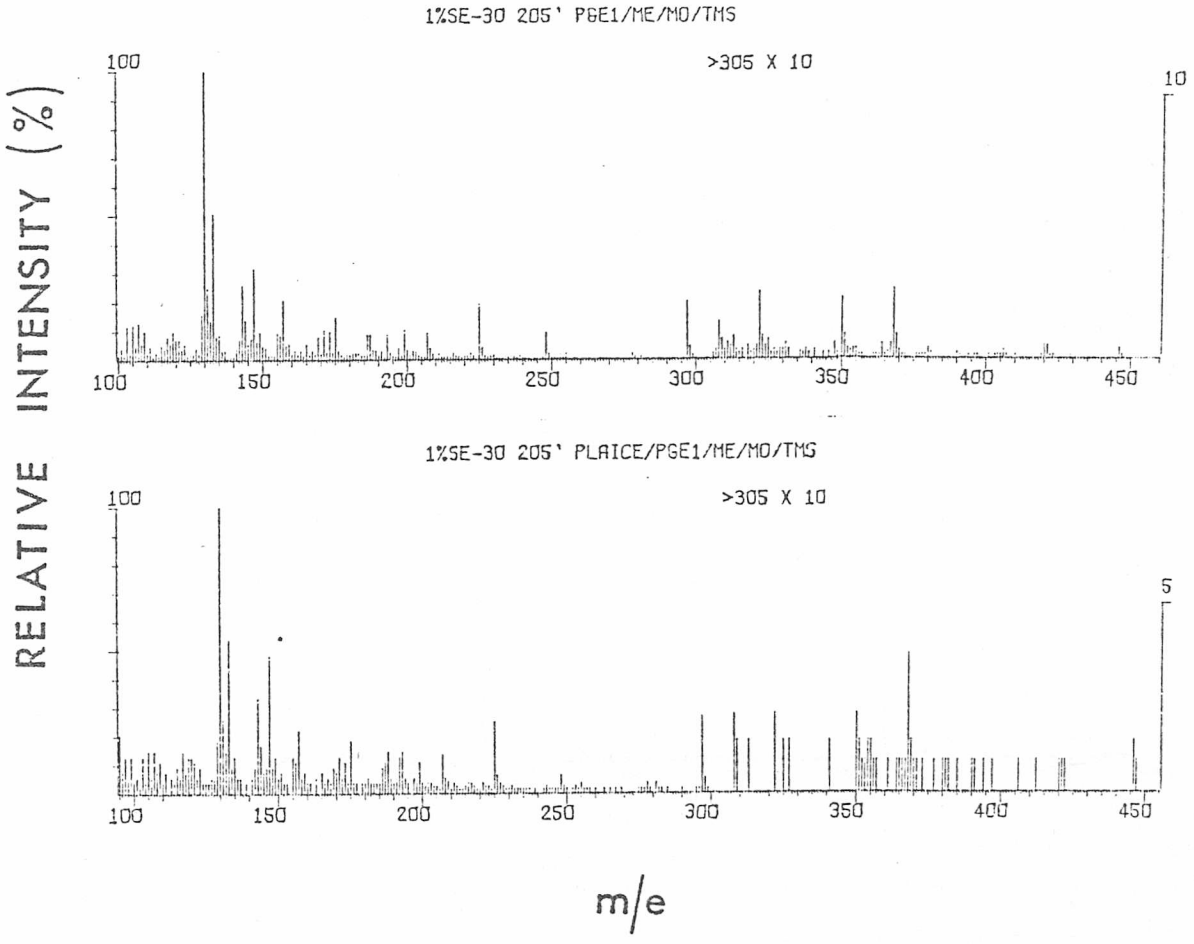


Fig 22 Mass spectra of the prostaglandin E-like material formed by incubation of a microsomal preparation of plaice skin with eicosatrienoic acid (bottom) and of authentic E_1 (top). The samples were run as the methyl ester, methyloxime, trimethylsilyl ethers.

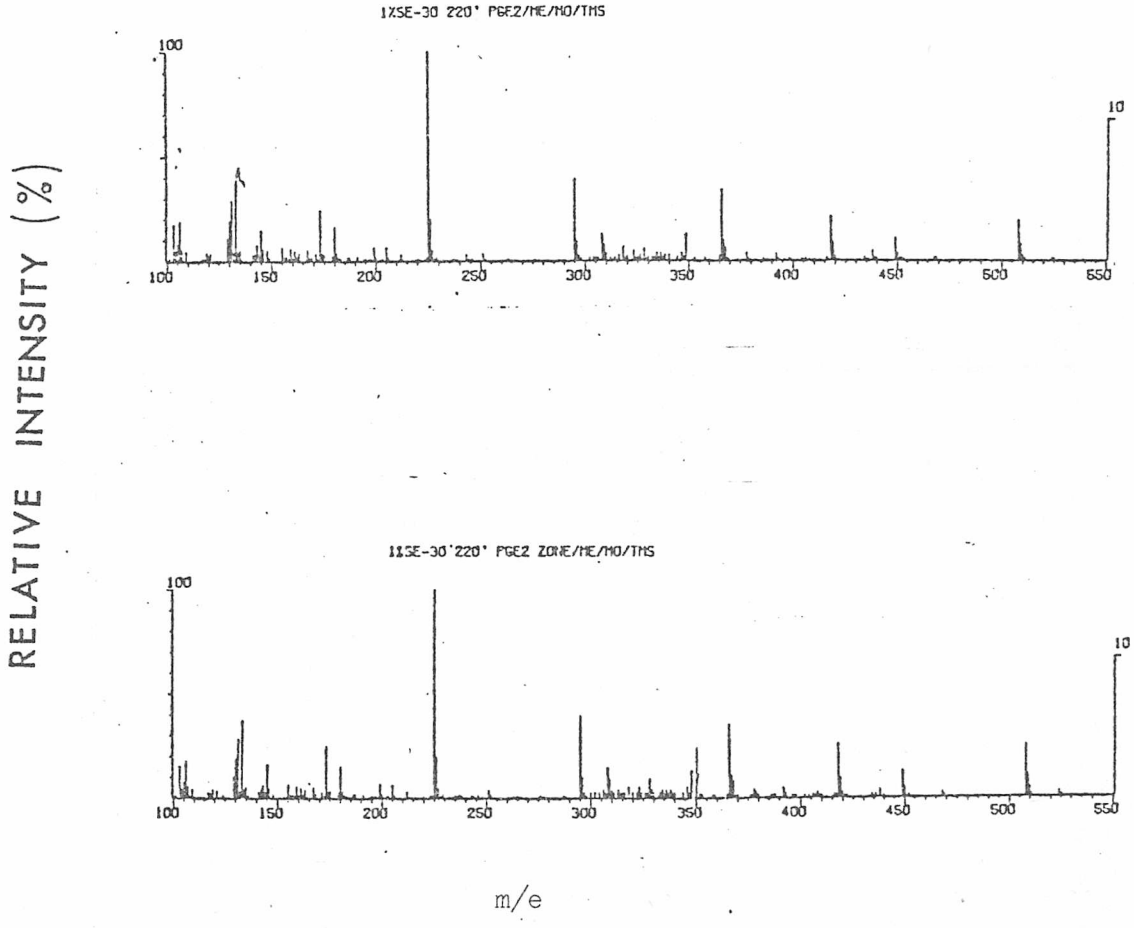


Fig 23 Mass spectra of the prostaglandin E-like material formed by incubation of a microsomal preparation of plaice skin with arachidonic acid (bottom) and of authentic E_2 (top). The samples were run as the methyl ester, methyloxime, trimethylsilyl ethers.

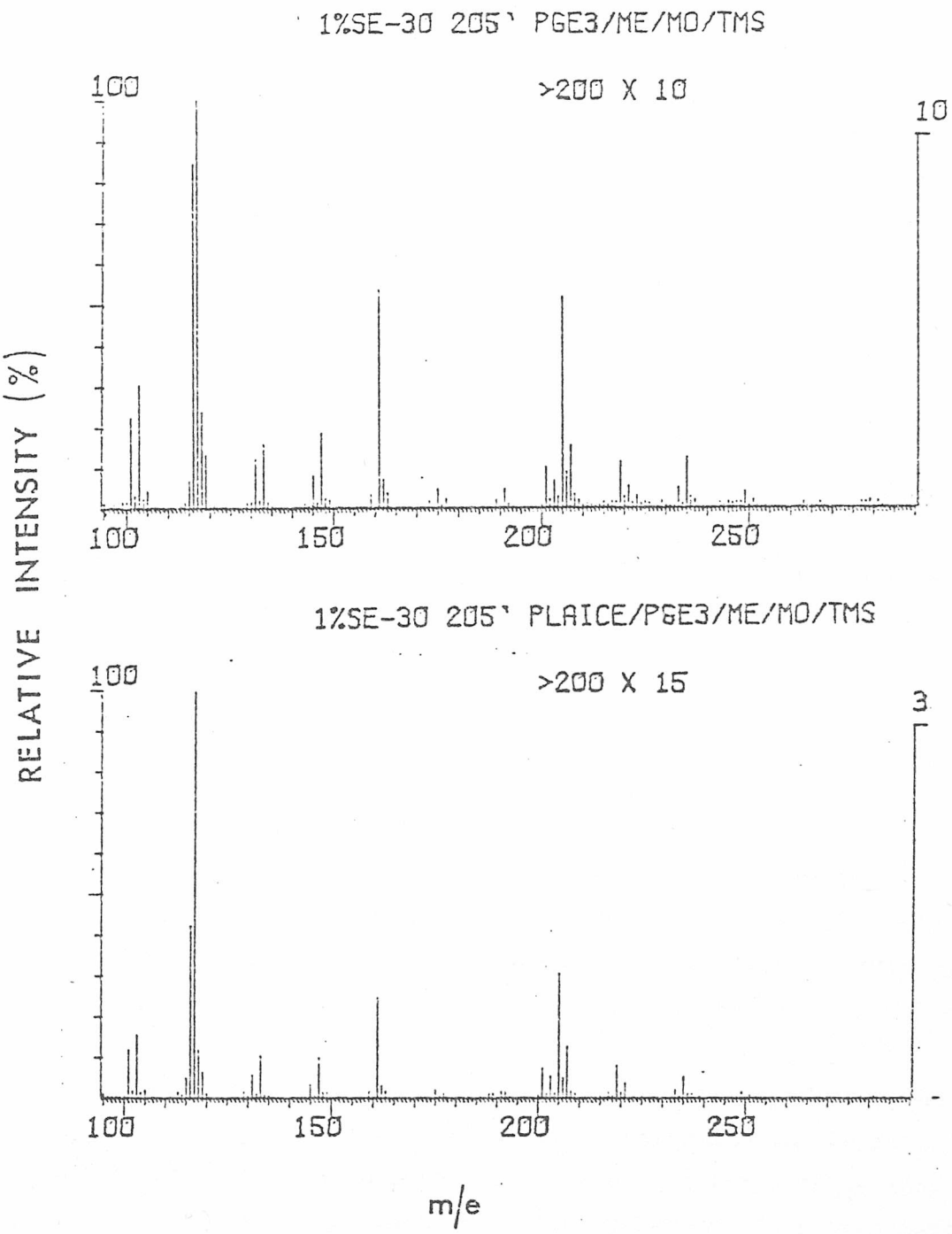


Fig 24 Mass spectra of the prostaglandin E-like material formed by incubation of a microsomal preparation of plaice skin with eicosapentaenoic acid (bottom) and of authentic prostaglandin E_3 (top). The samples were run as the methylester, methyloxime, trimethylsilyl ethers.

The effect of eicosapentaenoic acid on the synthesis of prostaglandin E_2 (measured as B_2 by absorption spectrometry) from arachidonic acid is shown in Table 15. Eicosapentaenoic acid, preincubated with enzyme for 5 minutes before addition of arachidonic acid, caused a dose-dependent inhibition of the production of E_2. However, this detection method does not discriminate between the formation of E_2 and E_3 and part of the chromophore estimated is probably due to E_3 and not E_2. Thus, the inhibition of E_2 production has probably been underestimated.

3.7 LEVELS OF BIOGENIC AMINES IN PLAICE SKIN

3.7 i) Histamine

Initial experiments suggested that the skin contained relatively low levels of histamine ($\simeq 0.2\mu$ g/g). In order to observe whether other tissues of the plaice contained appreciable quantities of histamine, a comparative study was carried out on subcutaneous muscle, mesenteric tissue, ileum and skin, tissues which contain appreciable quantities of histamine in mammals. Samples of these tissues, extracted from skin and condensed with o-phthalaldehyde formed a fluorophore with identical activation and fluorescence spectra to that of authentic histamine treated in the same way (Fig 25). Table 16 shows that the skin contained over twice as much histamine as subcutaneous muscle, about the same as samples of ileum and roughly half that of mesenteric tissue.

Table 17 shows the effect of subcutaneous injections of compound 48/80 and E. floccosum on the concentration of histamine in the skin at the area of injection as compared with areas injected with saline. There was no significant reduction in histamine levels after treatment.

The effect of the chronic treatment with the histamine liberator, compound 48/80 on tissue histamine levels was also observed. After intraperitoneal injection of compound 48/80 the fish did not show any obvious signs of distress. Moreover, there was no mortality during the period of treatment, emphasizing the low toxicity of this compound in plaice. The results, shown in Table 18, indicate that there was no significant reduction in the histamine content of plaice skin even after

Eicosapentaenoic acid (mM)	Inhibition of E_2 synthesis (%)
0.16	15.4 ± 0.1
0.33	26.1 ± 4.7
0.66	56.6 ± 5.9

Table 15 Effect of eicosapentaenoic acid on the synthesis of prostaglandin E_2 from arachidonic acid

Eicosapentaenoic acid was preincubated with enzyme for 5 minutes before addition of arachidonic acid (0.33 mM). Synthesis of E_2 was assayed by ultraviolet absorption spectrometry (after conversion to B_2). Each value is the mean with s.e. mean of 2 observations. Inhibition was expressed as a percentage of conversion to E_2 in the absence of inhibitory fatty acid.

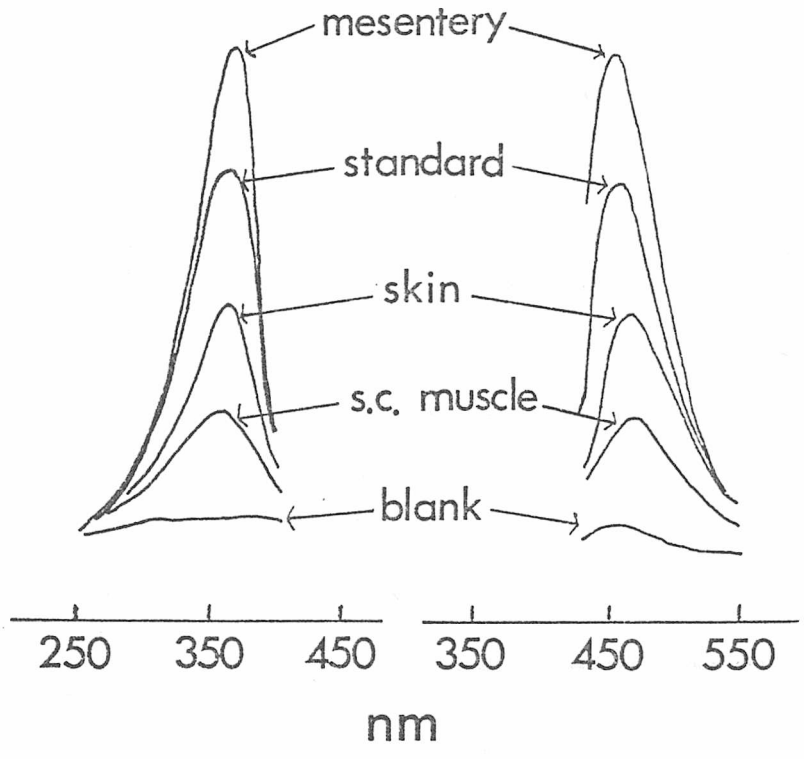


Fig 25 Activation (left) and fluorescence (right) spectra of the fluorophore resulting from OPT treatment of authentic histamine and apparent histamine from various plaice tissues.

Tissue	n	Histamine content (ng/g wet weight)
Skin	25	233 ± 8
Subcutaneous muscle	16	93 ± 3
Mesentery	10	420 ± 17
Ileum	8	259 ± 34

Table 16 Histamine content of different tissues of the plaice

Values are of mean with s.e. mean. Numbers listed under n refer to the number of fish sampled in each case.

Agent	Concentration (mg/ml)	Skin histamine (ng/g) Challenged area	Control area	Statistical analysis t	P
48/80	10	228 ± 13	219 ± 12	0.99	> 0.05
E. floccosum	10	221 ± 12	219 ± 15	0.28	> 0.05

Table 17 Effect of subcutaneous injection with compound 48/80 and E.floccosum on skin histamine levels measured 60 mins. after treatment

Each value is the mean with s.e. mean of 12 observations. Histamine levels in challenged and control areas were measured in all fish and were compared by paired t test.

Days pretreatment	Histamine content (ng/g)		Statistical analysis	
	Treated	Control	t	P
3	191 ± 8	198 ± 4	0.85	> 0.05
8	217 ± 9	227 ± 6	0.92	> 0.05
15	276 ± 9	282 ± 6	0.59	> 0.05

Table 18 Effect of chronic treatment with compound 48/80 on skin histamine levels

Each value is the mean with s.e. mean of 4 observations. For each group, control histamine levels were measured in skin from an equal number of untreated plaice. Levels in control and treated fish were compared by unpaired t test.

15 days treatment. Similarly, there was no change in the histamine content of mesenteric, subcutaneous or ileal tissue after this period. Skin reactions to E. floccosum were observed after treatment in all fish.

3.7 ii) 5-HT

Skin samples from 10 plaice did not contain detectable amounts of 5-HT as measured by spectrofluorimetric assay. The limit of detection, allowing for losses during extraction was about 20 ng 5-HT per gram of skin.

3.8 ASSAY OF METHANOLIC EXTRACTS OF PLAICE SKIN

3.8 i) Levels of bradykinin and 5-HT in extracts

Methanolic extracts of the skin of frogs (Rana temporaria) have been found to contain large quantities of 5-HT and a bradykinin-like peptide (Anastasi et al., 1965). Using the same method, methanolic extracts of plaice skin were assayed for the presence of potential mediator substances.

In one experiment, methanolic extracts of plaice and frog skin were subjected to column chromatography on alkaline alumina in parallel. The recovery of bradykinin-like polypeptide and 5-HT in the eluate fractions of both columns are shown in Table 19. The values obtained for frog skin agree very closely with those found by Anastasi and co-workers (1965). However, the corresponding fractions for plaice skin extract revealed no 5-HT and only very low levels of bradykinin-like material. In a further 4 experiments, this was found to be equivalent to 65 $\pm$ 5 ng (mean $\pm$ s.e. mean) per gram of tissue as assayed against authentic bradykinin. The polypeptide-like nature of these combined eluates was confirmed by the loss of activity following incubation with chymotrypsin.

Eluates (% ethanol)	Bradykinin-like polypeptide (μg/g)		5-HT (μg/g)	
	Frog	Plaice	Frog	Plaice
95	ND	ND	2.7	ND
85	ND	ND	84.8	ND
70	1.3	ND	2.2	ND
60	56.0	0.05	ND	ND
50	44.8	0.03	ND	ND
40	3.3	ND	ND	ND
30	1.4	ND	ND	ND
20	ND	ND	ND	ND

Table 19 Chromatography of methanol extracts of frog and plaice skin on a column of alkaline alumina

Methanolic extracts were passed through alkaline alumina columns in parallel. Recoveries of bradykinin-like polypeptide and 5-HT (in μg/g fresh tissue) in the eluate fractions obtained with descending concentrations of ethanol are shown. ND indicates that activity was not detectable.

3.8 ii) Effects of extracts on vascular leakage in rats

The individual fractions eluted from the alumina column as described above were also tested for their ability to produce dye leakage in rats (a measure of vascular permeability). It was found that 2 relatively polar fractions (20% and 30% ethanol) caused a considerable leakage of dye, whereas the more non-polar fractions did not (Fig. 26). Moreover the active fractions did not have a direct effect on the smooth muscle of the rat stomach strip or the guinea-pig ileum.

The original methanolic extract of skin was shown to contain negligible amounts of prostaglandin-like material following extraction into ethyl acetate at pH 3.

3.9 PLAICE SKIN SUPERNATANT AS A SOURCE OF ENZYMES

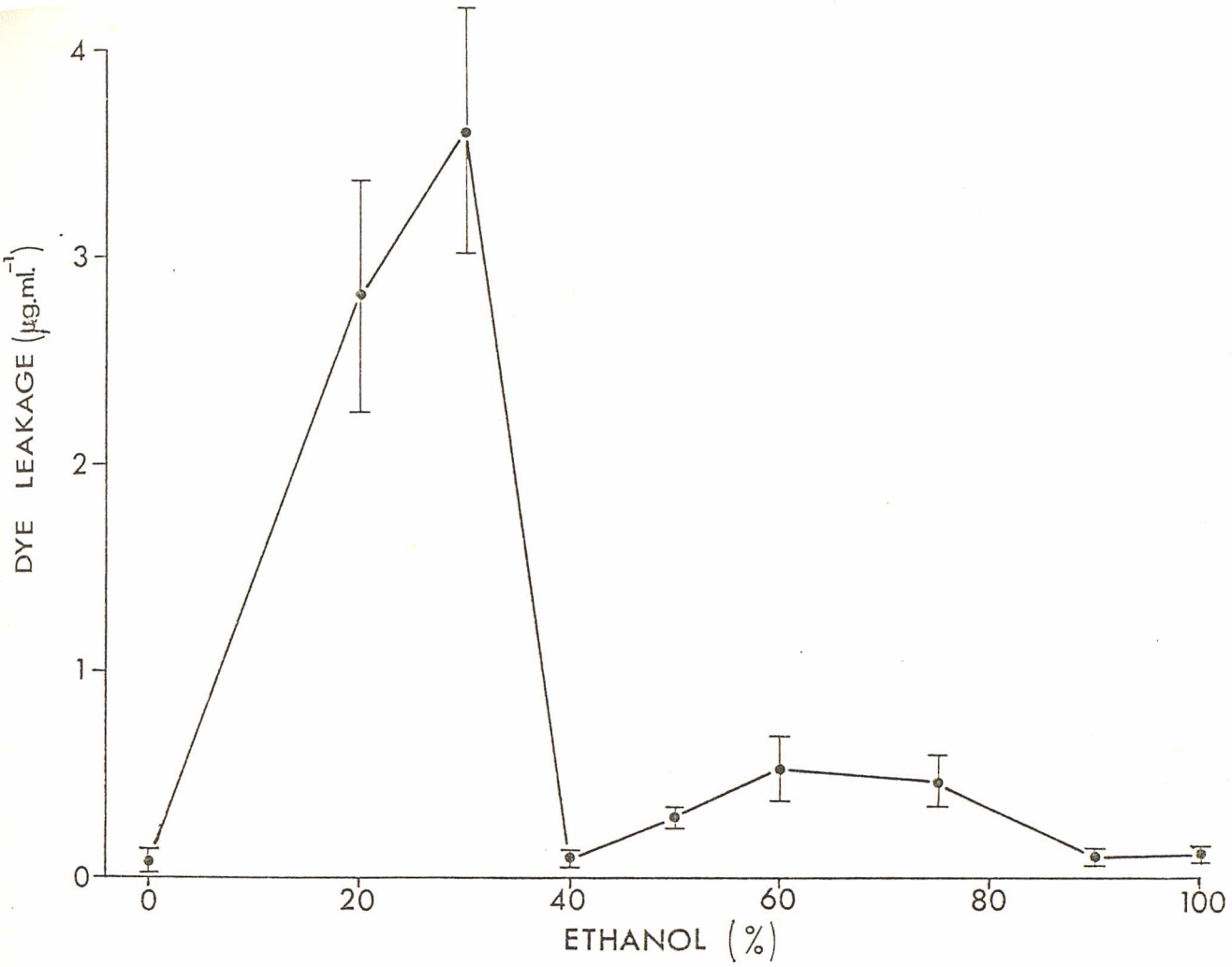
The ability of plaice skin to metabolise 4 possible mediators, histamine, bradykinin, 5-HT and prostaglandin E_2 was tested by incubating aliquots of those substances with a cell-free fraction prepared from homogenates of plaice skin. The results are shown in Fig. 27. Whereas histamine, bradykinin and 5-HT were only degraded by about 20% after 60 minutes incubation, over 50% of the prostaglandin E_2 activity had been lost after this time. In contrast, control samples in which the supernatant was replaced by buffered saline showed negligible loss of activity after incubation. The supernatant itself did not have a direct action on smooth muscle.

3.10 INDIRECT EXPERIMENTS IN WHOLE PLAICE

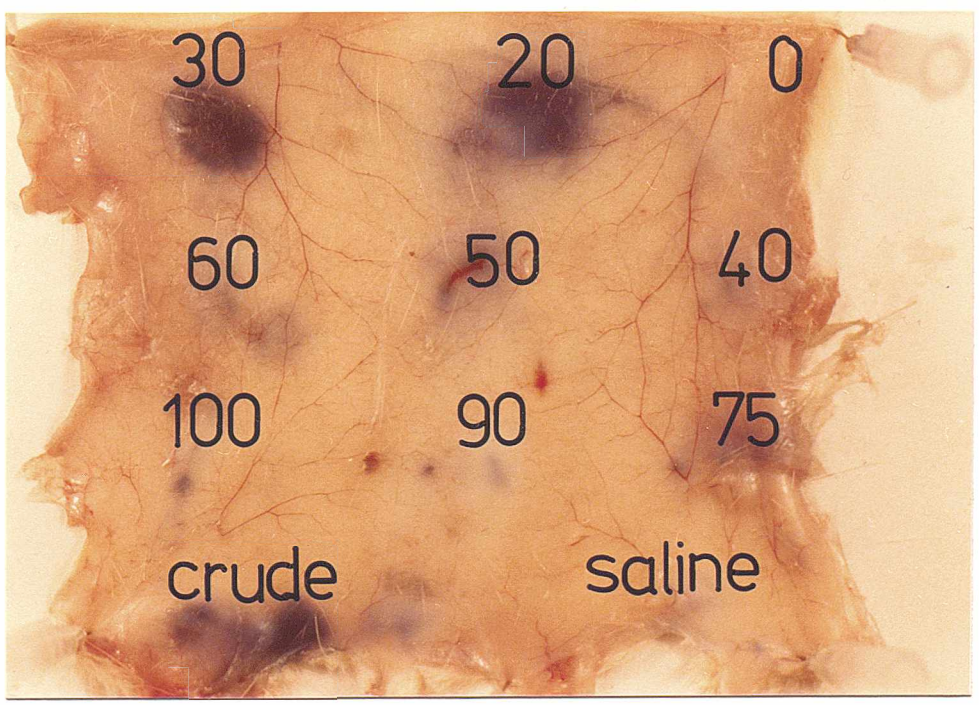
3.10 i) Ability of vasoactive agents to produce skin reactions in plaice

In a series of experiments, a number of prospective mediator substances were tested for their ability to mimic the skin reaction produced by E. floccosum. The effects of different concentrations of

Fig 26 a) Chromatography on alumina column of a crude methanol extract of plaice skin. The ability of individual eluate fractions to produce dye leakage in the skin was tested in rats which had received an intravenous injection of Evans blue dye 15 minutes prior to sacrifice. The blue areas of the skin were extracted and assayed as described on page 46 . Each point is the mean with s.e. mean of 4 separate experiments. Ordinate : dye leakage (μg/ml), abscissa : percentage ethanol. b) Dye leakage responses in rat skin produced by eluate fractions of a crude methanol extract of plaice skin (as in a)) in a single experiment. 0.1 ml samples were injected intradermally. Numbers indicate the eluate fraction (%) injected at each point. 'Crude' refers to unpurified extract. The bulk of the activity was found in the 20% and 30% ethanol eluates.


DYE LEAKAGE (µg.ml⁻¹)
4
3
2
1
0
0
20
40
60
80
100
ETHANOL (%)

a)


30
20
0
60
50
40
100
90
75
crude
saline

b)

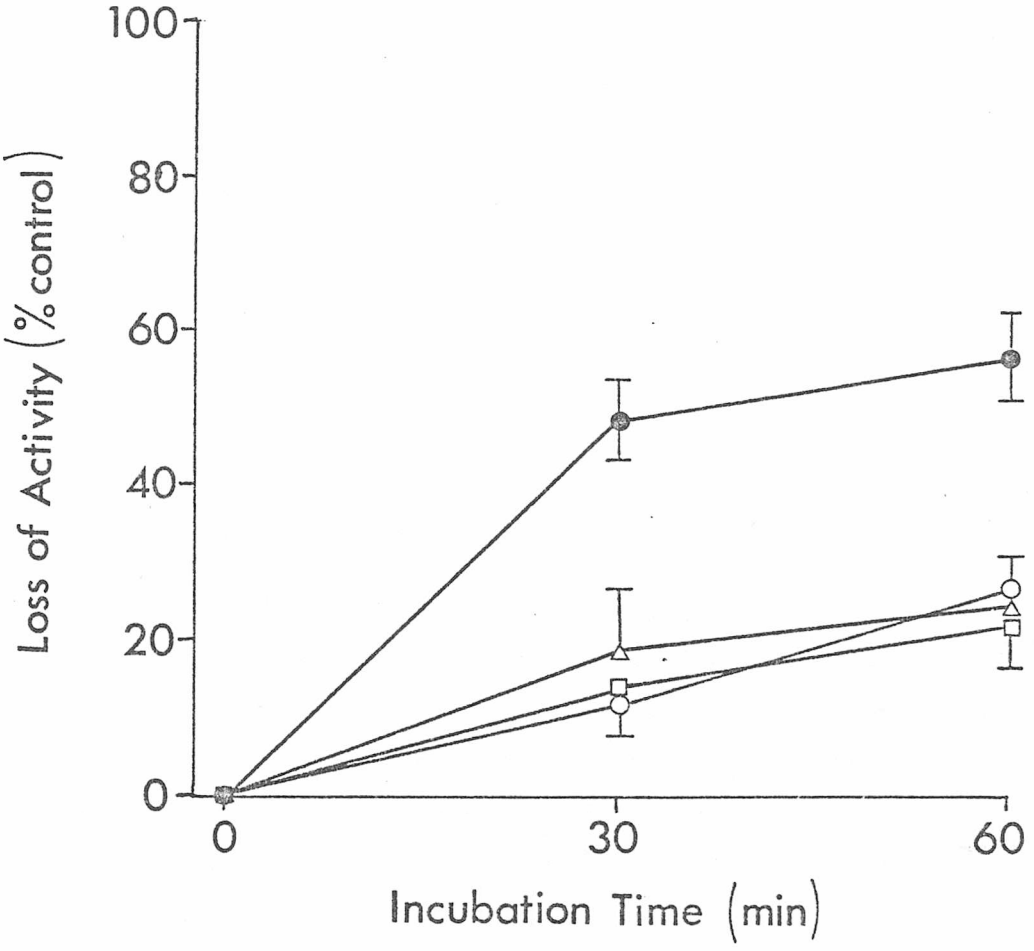


Fig 27 Ability of a cell-free homogenate of plaice skin to degrade 4 prospective mediator substances, histamine (△—△), bradykinin (○—○), 5-HT (□—□) and prostaglandin E_2 (●—●). 100 ng of material was incubated with the skin fraction (prepared as described on page 57). After incubation, samples were bioassayed for smooth muscle activity. The loss of activity was expressed as a percentage. Each point is the mean with s.e. mean of 4 observations.

each substance was observed in a minimum of 4 plaice. 0.05 ml volumes were injected intradermally in each case. Table 20 summarises the results obtained. The skin reactions produced by different substances appeared to be identical with those observed following injection of E. floccosum. An example of a skin reaction to E. floccosum is shown in Fig. 6. Compound 48/80 produced a similar response in a concentration of 1 mg/ml, but 0.1 mg/ml was only partially effective (Fig 28). Histamine and 5-HT could also mimic the skin reaction, when the concentration was raised to 10 mg/ml but bradykinin was only partially effective (Fig 29). In contrast, prostaglandins of the E-series, especially E_2 produced skin reactions in much lower concentrations, as shown for E_2 in Fig. 30. An endoperoxide analogue, U46619, only produced a faint reaction in a concentration of 10 µg/ml.

3.10 ii) Effect of inhibitor substances on cutaneous anaphylaxis in plaice

A variety of drugs were tested for their ability to inhibit the skin reaction by E. floccosum. Of the drugs given acutely (Table 21), only disodium cromoglycate completely inhibited the reaction. Although a very large concentration was employed, the fish showed no signs of distress. In the 7 plaice tested, the inhibitory effect was maximum 10 to 30 minutes following injection. Adrenaline, diethylcarbamazine, FPL 55712, hydrocortisone, indomethacin and metiamide did not appear to reduce the skin reaction within 60 minutes of injection. While promethazine failed to inhibit the skin reaction induced by E. floccosum, skin reactions to histamine (10 mg/ml) were inhibited in 5 of the 7 plaice tested within 30 minutes of injection of antagonist. All 7 fish died within 60 minutes of injection.

The effect of chronic dosing with inhibitor substances on the skin reaction to E. floccosum is shown in Table 22. No inhibitory effect was observed in any fish. In a further experiment, indomethacin treatment was continued for 15 days. However, this also failed to inhibit the reaction. Chronic pretreatment of plaice with compound 48/80, as reported earlier (see page 56) did not reduce the intensity of the skin reaction.

Agent	INTENSITY OF SKIN REACTION Concentration					
	0.1 µg/ml	1µg/ml	10 µg/ml	100µg/ml	1 mg/ml	10 mg/ml
E. floccosum			0	0	++	+++
Compound 48/80			0	+	++	
histamine			0	0	0	++
5-HT			0	0	0	++
bradykinin			0	0	+	
prostaglandin E_1	0	0	++			
prostaglandin E_2	0	+	+++			
U.46619		0	+			

Table 20 Ability of different substances to produce a skin reaction in plaice

The effect of each agent was tested in a minimum of 4 fish. A 4-tier visual scoring system was used (see page 58).

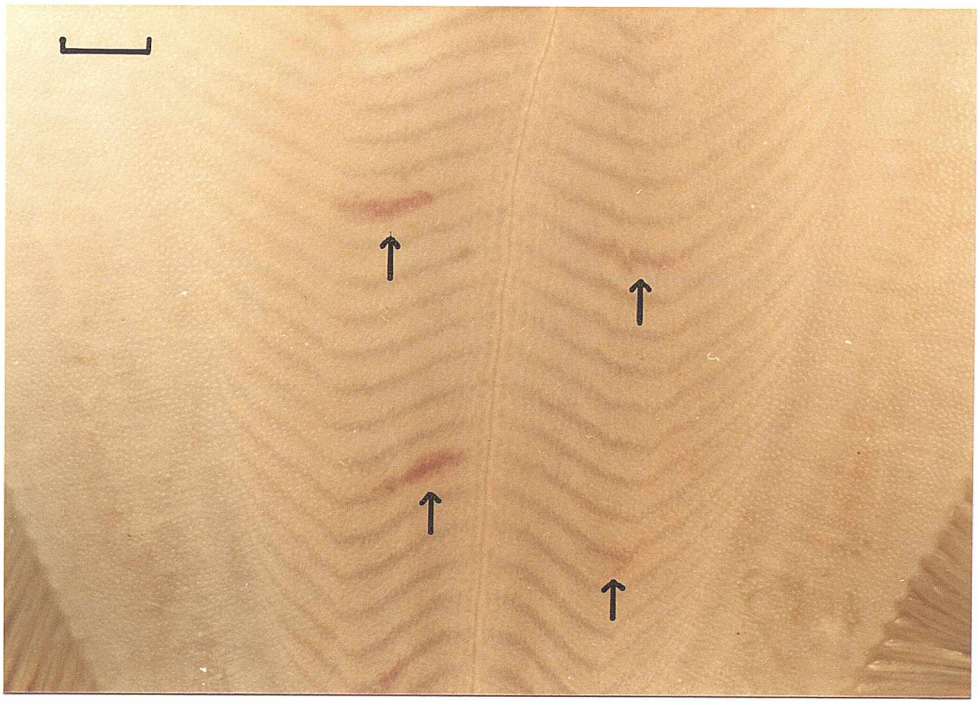
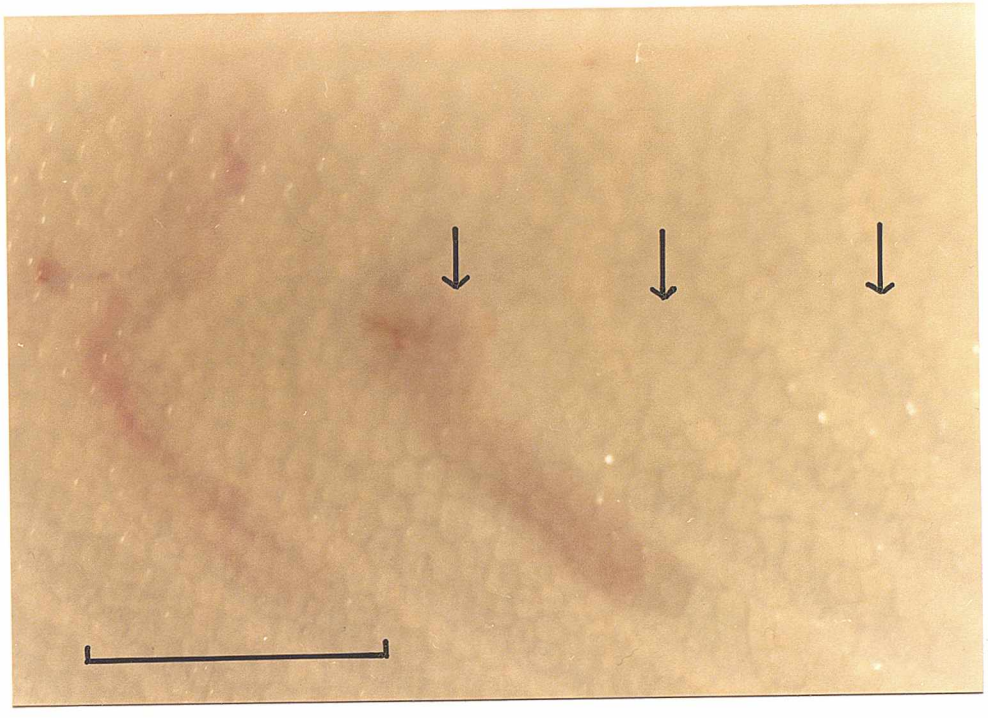
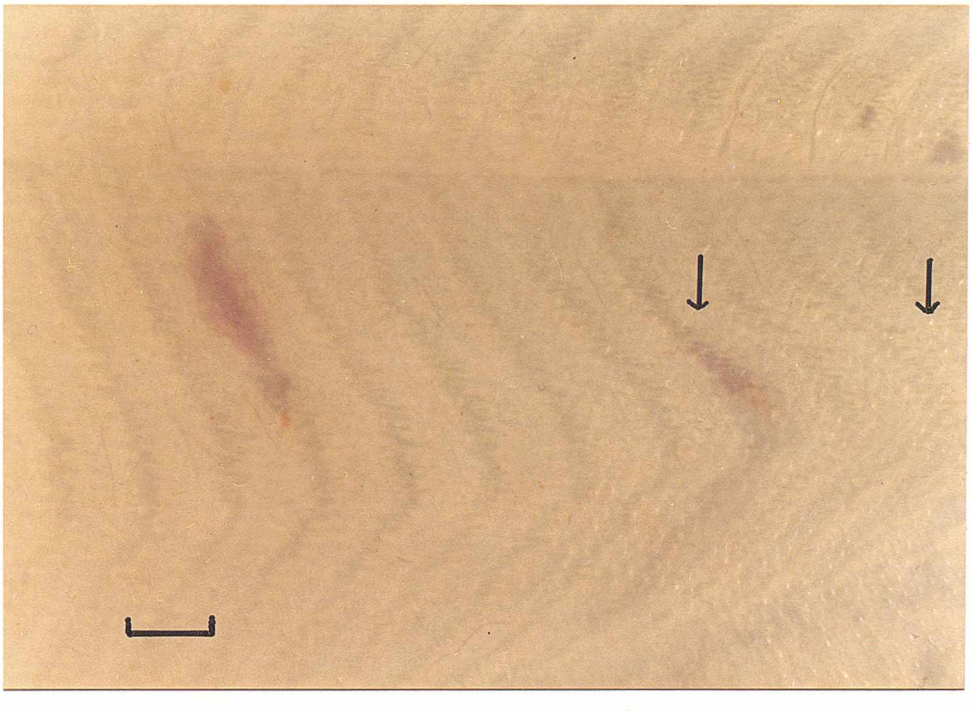


Fig 28 Immediate skin reactions observed on the undersurface of plaice 5 minutes after the intradermal injection of compound 48/80 (top) and E. floccosum (bottom). On the left side of the fish, 1 mg/ml was injected and on the right 0.1 mg/ml in both cases. 0.05 ml volumes were injected at the arrows. The bar is equivalent to 1 cm.

Fig 29 a) Immediate skin reactions observed on the undersurface of plaice 5 minutes after the intradermal injection of 1 mg/ml E. floccosum (far left) and histamine (left to right), 10, 1 and 0.1 mg/ml at the arrows. b) Immediate skin reactions observed on the undersurface of plaice 5 minutes after the intradermal injection of 1 mg/ml E. floccosum (far left) and bradykinin (left to right), 1 and 0.1 mg/ml at the arrows. In a) and b), 0.05 ml volumes were injected. The bar is equivalent to 1 cm.



a)



b)

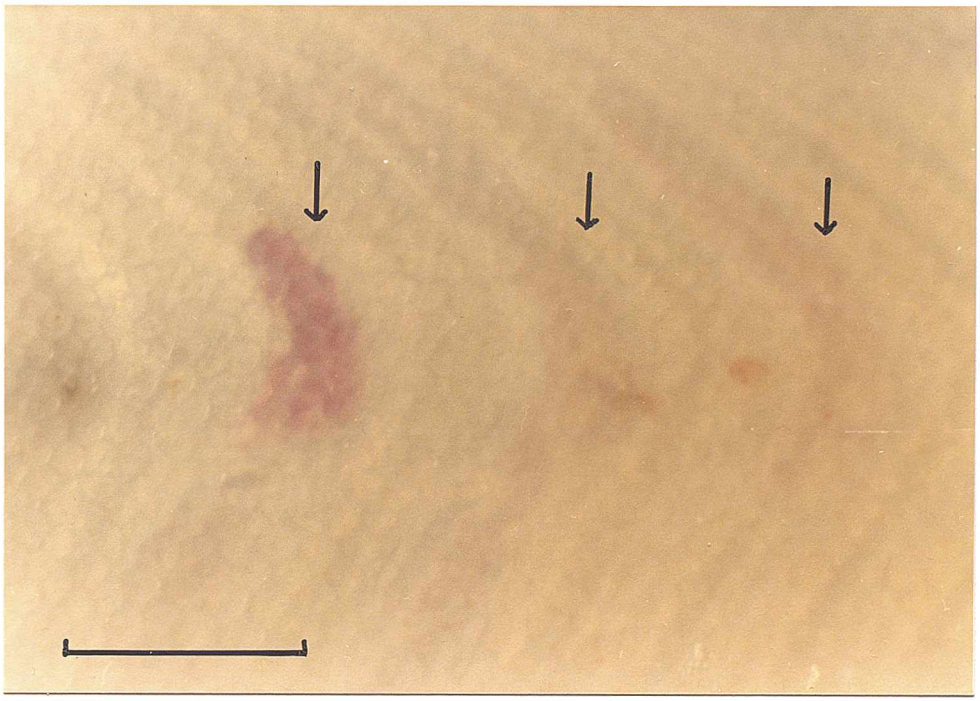


Fig 30 Immediate skin reactions observed on the undersurface of plaice 5 minutes after the intradermal injection of 1 mg/ml E. floccosum (far left) and prostaglandin E_2 (left to right), 10, 1 and 0.1 μg/ml at the arrows. 0.05 ml volumes were injected. The bar is equivalent to 1 cm.

Drug	Dose (mg/kg i.v.)	n	Effect
indomethacin	50	7	-
disodium cromogycate	160	7	Inhibition (10-30 mins. after injection).
diethylcarbamazine citrate	100	6	-
hydrocortisone	10	5	-
FPL 55712	5	5	-
promethazine hydrochloride	20	7	-
metiamide	10	5	-
adrenaline	10	6	-

Table 21 Ability of different drugs to inhibit the skin reaction in plaice

Drugs were given by intravenous injection. Plaice were skin tested with E. floccosum before injection of drug and then repeatedly over a period of 60 minutes after injection. Numbers listed under n refer to the number of experiments performed in each case. — indicates no reduction in the intensity of the skin reaction (i.e. no inhibition).

Drug	Dose (mg/kg i.p.)	n	Effect
aspirin	25	6	-
flurbiprofen	25	6	-
indomethacin	25	8	-
hydrocortisone	5	5	-
corticosterone	5	5	-
atropine	3	5	-
promethazine	4	5	-
methysergide	3	5	-
propranolol + phentolamine	3	5	-

Table 22 Effect of chronic treatment of plaice with inhibitor substances on the skin reaction

Drugs were given by intraperitoneal injection once daily for 5 days. Plaice were skin tested with E. floccosum before and after treatment. Numbers listed under n refer to the number of experiments performed in each case. – indicates no reduction in the intensity of the skin reaction.

3.11 EXPERIMENTS IN FLOUNDERS

These experiments were carried out in conjunction with Dr. T. C. Fletcher.

3.11 i) Cutaneous anaphylaxis in whole flounders

Skin reactions to E. floccosum were not observed in 30 untreated flounders tested over a 2 year period. However, the challenging agent did produce skin reactions in 6 flounders, 24 hours after the injection of plaice serum (2 ml i.v.). This result confirms the earlier observation of Fletcher and Baldo (1974) that the cutaneous response observed in plaice can be transferred to the flounder by a factor (or factors) in plaice serum and also that the reaction is similar to the type 1 hypersensitivity reactions described in higher vertebrates.

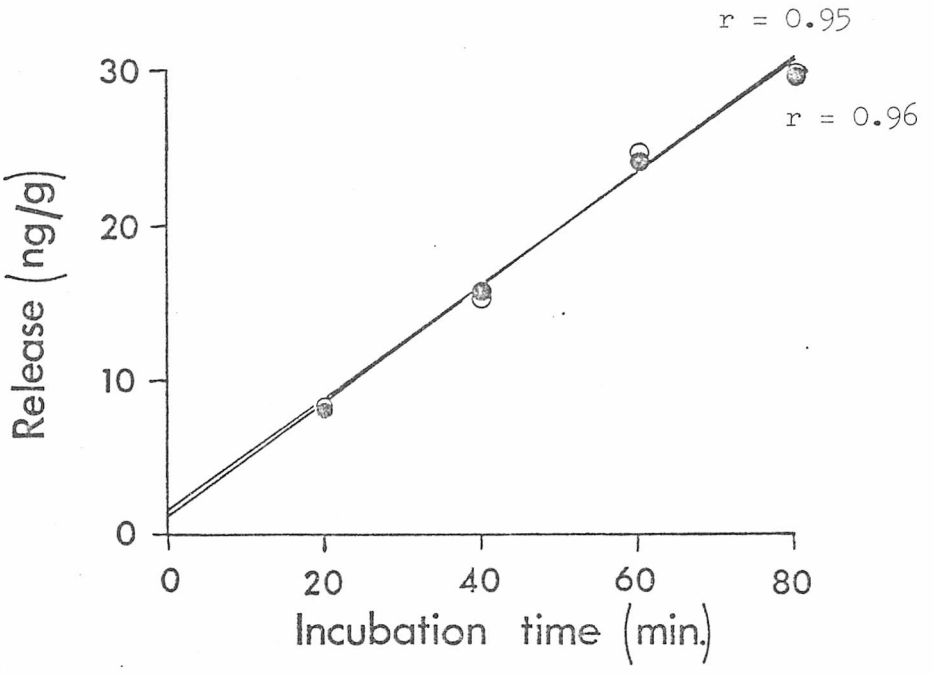
In an attempt to identify the tissue-fixing factors involved in this hypersensitivity reaction, 2 factors, purified from plaice serum were tested in the same manner as serum to see if transfer of the reaction could be produced. 2 groups of 6 flounders were injected with either plaice CRP (400 µg/kg i.v.) or plaice P-component (660 µg/kg i.v.). Twenty-four hours later, the fish injected with CRP all showed skin reactions to E. floccosum whereas those injected with P-component did not. In a further 4 experiments, the skin reaction was not produced in flounders after injection of pure CRP, prepared from another teleost, the lumpsucker, and given in amounts up to 20 mg/kg i.v.

3.11 ii) Release of smooth muscle contracting material from flounder skin in vitro

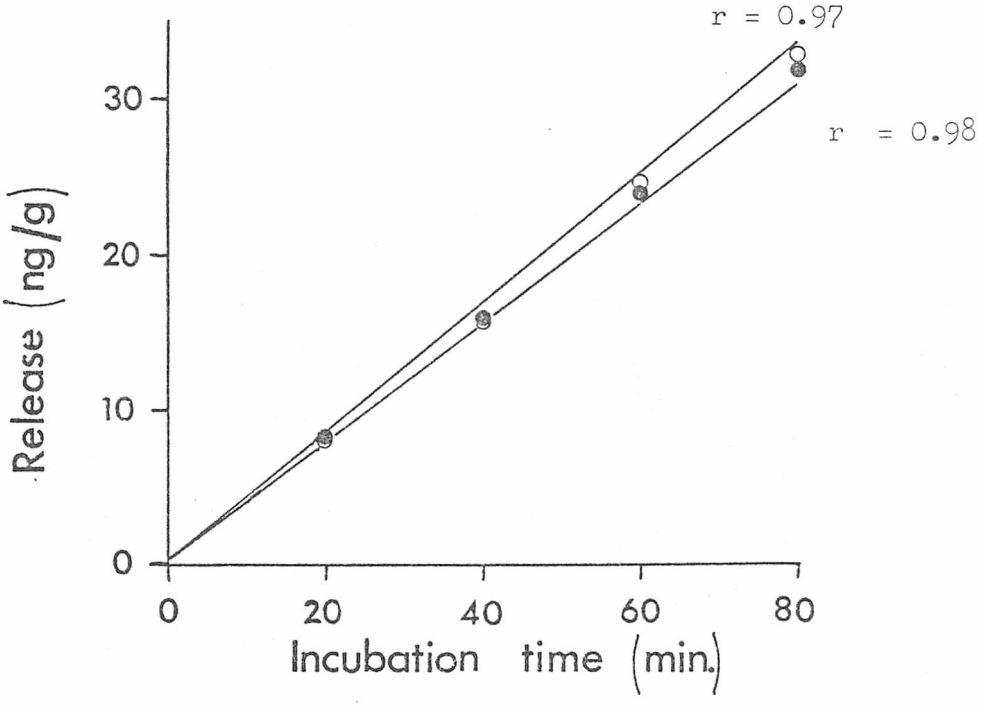
Incubation of chopped flounder skin with Ringer saline revealed that a smooth muscle contracting substance was released over a period of 60 minutes. However, challenge with E. floccosum in vitro did not potentiate the release. As in the plaice, the partition behaviour of the active material suggested the presence of a prostaglandin-like substance. Moreover, this activity co-chromatographed with the E - series prostaglandins using preparative TLC.

The time-course of release of prostaglandin-like material was studied in 6 untreated flounders and in groups of flounders injected with plaice serum, plaice CRP and plaice P-component. Fig 31a shows the release as assayed against authentic E_2 from untreated flounders. Regression analysis of the data showed that there was no significant difference between challenged and non-challenged skin incubated for 20 - 80 minutes and that the pattern of release from flounder skin was best explained by a linear equation (96% of the variation explained in both responses). A similar result was obtained for flounders injected with P-component (Fig 31b). In contrast, prostaglandin release from flounder skin challenged in vitro with E.floccosum was significantly greater than release from non-challenged skin in these fish injected 24 hours previously with plaice serum or with plaice CRP (Fig 32). The patterns of prostaglandin release over the 80 minute incubation period were also significantly different; in both cases, non-challenged release was best fitted mathematically by a linear equation whereas challenged release was more complex, being fitted by a quadratic equation. This is highly indicative of a different mechanism of release from the skin on challenge, as was suggested for unsensitised plaice skin. The results are summarised in Table 23.

Fig 31 a) Time course of release of prostaglandin-like material from challenged (● – ●) and from non-challenged (○ – ○) skin from untreated flounders. b) Time course of release of prostaglandin-like material from challenged (● – ●) and from non-challenged (○ – ○) skin from flounders which had been injected with P-component (660 μg/Kg iv), 24 hours prior to killing. In a) and b), each point is the mean with s.e. mean of 6 observations of the amount of prostaglandin released from flounder skin (lg) expressed as ng E_2 equivalents, as assayed on the rat stomach strip. The line of best fit for the data, estimated by regression analysis is indicated by the correlation coefficient, r.

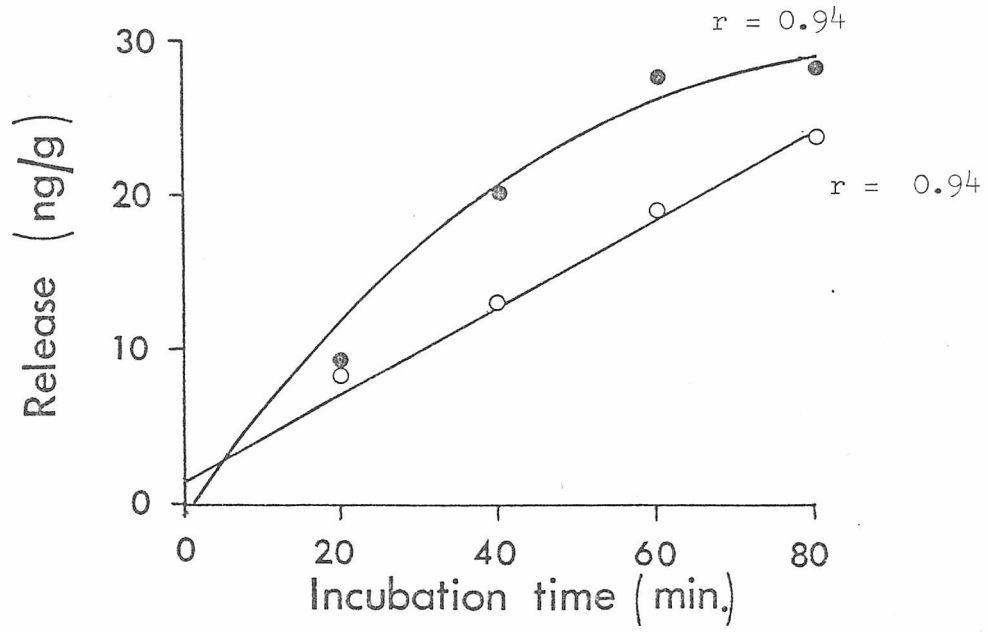

r = 0.95
r = 0.96
30
20
10
0
Release (ng/g)
0
20
40
60
80
Incubation time (min.)

a)

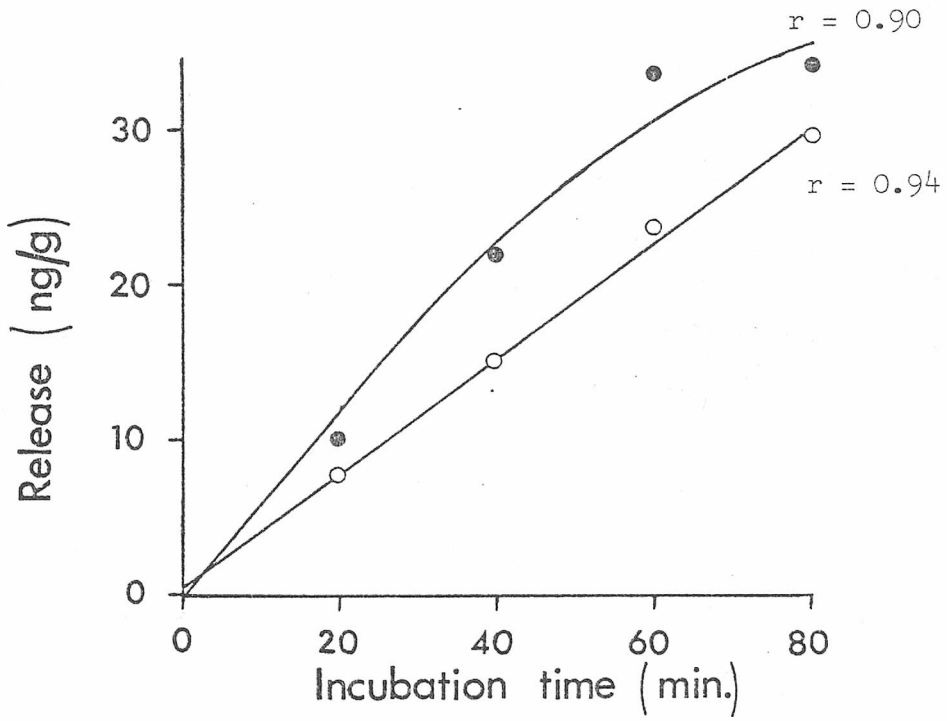

r = 0.97
r = 0.98
30
20
10
0
Release (ng/g)
0
20
40
60
80
Incubation time (min.)

b)

Fig 32 a) Time course of release of prostaglandin-like material from challenged (● – ●) and from non-challenged (○ – ○) skin from flounders which had been injected with plaice serum (2 ml i.v.), 24 hours prior to killing. b) Time course of release of prostaglandin-like material from challenged (● — ●) and from non-challenged (○ – ○) skin from flounders which had been injected with CRP (400 μg/Kg i.v.), 24 hours prior to killing. In a) and b), each point is the mean with s.e. mean of 6 observations of the amount of prostaglandin released from flounder skin (1g) expressed as ng E_2 equivalents as assayed on the rat stomach strip. The line of best fit for the date is indicated by the correlation coefficient, r.


r = 0.94
r = 0.94
Release (ng/g)
30
20
10
0
0
20
40
60
80
Incubation time (min.)

a)


r = 0.90
r = 0.94
Release (ng/g)
30
20
10
0
0
20
40
60
80
Incubation time (min.)

b)

Treatment	Skin Test	Pattern of prostaglandin release		P
		Non-challenged	Challenged	
none	-	linear	linear	> 0.05
Plaice P-component	-	linear	linear	> 0.05
plaice serum	+	linear	quadratic	< 0.05
plaice CRP	+	linear	quadratic	< 0.05

Table 23 Summary of experiments with flounders

The effect of injection of flounders with plaice serum factors on prostaglandin release from flounder skin *in vitro*. The pattern of release from challenged and non-challenged skin for each treatment is expressed in mathematical form by the type of equation which best fits the release of prostaglandin over the 80 minute incubation period. P values show the difference between challenged and non-challenged release in each case. - indicates no response to the intradermal injection of *E. floccosum*; + indicates an immediate skin reaction.

CHAPTER FOUR

DISCUSSION

4.1 THE NATURE OF AN INFLAMMATORY REACTION IN THE SKIN

4.1 i) In mammals

Although very little is known about the inflammatory mechanism in lower vertebrates, in mammals the picture is more clear. This subject was reviewed in the introduction to the thesis but the essential facts relevant to my results are reproduced here for convenience.

It is known that, following injury there is an alteration in the flow of blood through the vascular bed. Vasodilatation and hyperaemia are usually followed by a slowing of the blood, giving rise to redness or erythema, the first cardinal sign of inflammation. When the injury is severe, blood flow in the dilated vessels may stop altogether, resulting in stasis; thrombosis and haemorrhage may also develop. The second sign of inflammation, swelling or oedema, is the result of plasma exudation caused by an increase in vascular permeability. The ultrastructural feature which seems to account for these permeability alterations is the formation of gaps between endothelial cells (see page 13).

Substances which are known to cause an increased permeability in skin vasculature include histamine, bradykinin, 5 - HT, SRS-A and prostaglandins of the E - series (Kaley & Weiner, 1971; Lewis, Wells & Eyre, 1972; Willoughby, 1973; Burka & Eyre, 1977), the relative effectiveness of each one varying in different species. Although large doses of prostaglandins cause substantial oedema (Kaley & Weiner, 1971), other studies have suggested that the major effect of small quantities of E - series prostaglandins is probably to potentiate the oedema-producing capacity of other mediators (Moncada et al., 1973; Williams & Morley, 1973). Using a technique which allows the simultaneous measurement of local plasma exudation and blood flow changes in rabbit skin (Williams, 1976), it has been shown that the intradermal injection of E-prostaglandins produced a large increase in blood flow with little vascular permeability (Williams, 1976; Williams & Peck, 1977). In contrast, bradykinin and histamine were considerably more potent at increasing vascular permeability than they were at increasing blood flow. Addition of a small quantity of prostaglandin to a fixed dose of histamine resulted in an increased blood flow and a marked potentiation of vascular permeability implying that vasodilatation might be responsible for the increased permeability (Williams, 1976). It was suggested that vascular changes

in inflammatory reactions are dependent upon the release of two types of chemical mediators, those important for their permeability-increasing properties and those which are principally vasodilators (Williams, 1977).

4.1 ii) In fish

It has become apparent that the circulatory system in fish is quite different from that of terrestial vertebrates. A single ventricle pumps blood successively through the small vessels of the gills and peripheral vessels before it is returned to the heart. The systemic pressure will therefore be higher in the blood vessels of the gills than in systemic capillaries. In general, the hearts of fish generate less than half the pressure of those of mammals (Satchell, 1971). Since a proportion of this pressure is lost across the gill circulation, capillary and venous pressures must be very low indeed. However, unlike in land animals, the blood vessels of fish are not subjected to the hydrostatic pressures produced by gravity. There is evidence that fish capillaries are very permeable to proteins and therefore that the colloid osmotic pressure is much lower than would be expected from the concentration of protein in the blood (Hargens, Millard & Johansen, 1974). Otherwise, it is likely that an imbalance would arise.

There have been few attempts to study the microvasculature of lower vertebrates during injury. The vascular bed of frogs seems to respond in a similar manner to that of mammals during local injury, that is with vasodilatation and increased permeability of capillaries and venules to plasma proteins. This response cannot, however be mimicked by histamine (Krogh, 1921; Grant & Duckett-Jones, 1929). Unfortunately, there is no information available for fish. The "immediate-type" skin reaction described in plaice (Fletcher & Baldo, 1974) was manifested simply as a red mark on the undersurface of the skin (Fig. 6). There was no obvious swelling or oedema as is usually associated with an increased vascular permeability. Nevertheless, some vascular leakage may occur, since challenge of plaice injected with Evans blue led to dye leakage in the skin at the site of injection (Fletcher & Baldo, 1974). However, leakage was not sufficient to enable extraction and quantitation of the dye as is possible with rat skin (Chahl & Chahl, 1976). It is possible that in the plaice the permeability component is of lesser importance in the manifestation of tissue injury than in higher vertebrates.

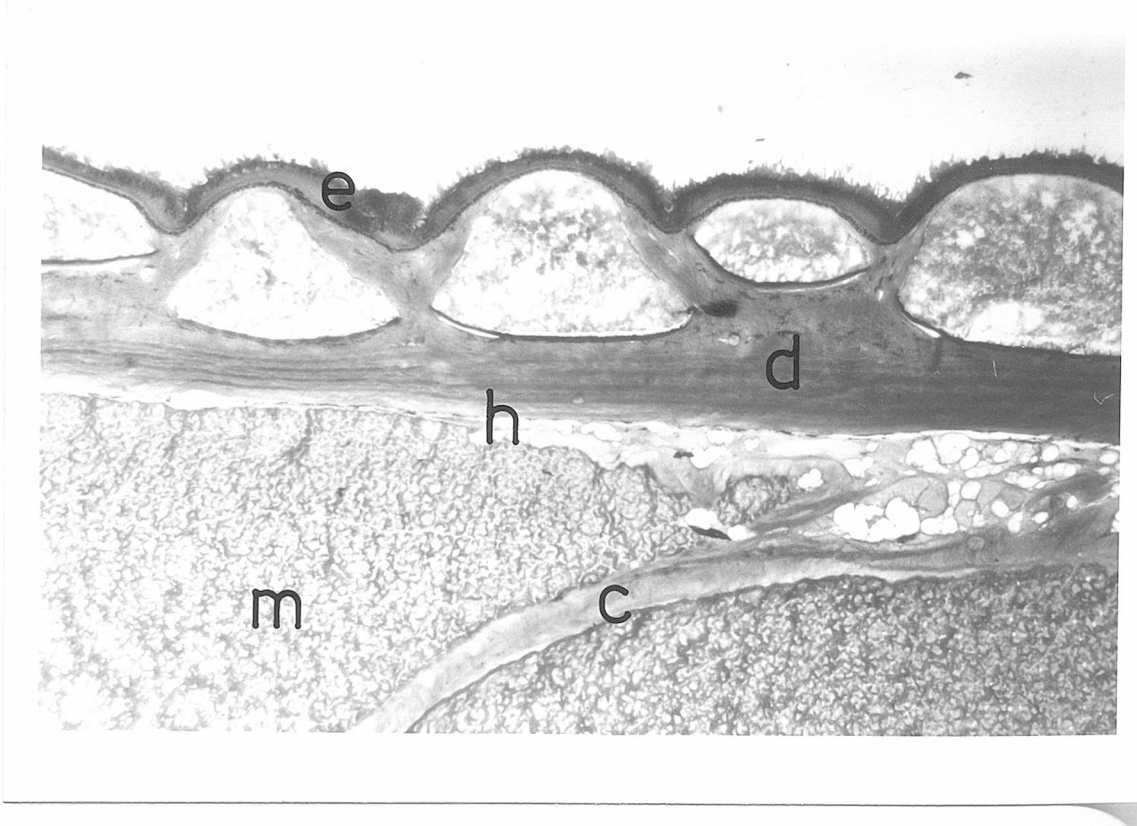


Fig 33 General structure of plaice skin : e = epidermis; d = dermis; h = hypodermis; m = muscle; c = connective tissue. Magnification X25. The skin was stained with alcian blue/acid fuchsin.

The "erythema-like" response may be due to dilatation of the vascular bed though it seems likely that this is accompanied by slowing or even standstill of the blood. The development of stasis might explain the fact that the response is not short-lived but can last for several hours after challenge. It was interesting then, that prostaglandins of the E-series could produce a similarly long lasting "erythema" (Fig. 30), as indeed they can in mammalian skin (Ferreira, 1972).

It has been suggested than an E-series prostaglandin is a mediator of delayed inflammation in human skin but that histamine is more important in the mediation of cutaneous hypersensitivity reactions characterised by the acute wheal and flare response (Greaves, 1974). The results of the present investigation indicate that histamine is not involved as a mediator of the cutaneous hypersensitivity reaction in plaice but that prostaglandins have a greater potential as the active agents responsible for the development of the inflammatory reaction.

4.2 THE STRUCTURE OF PLAICE SKIN

The skin of the plaice can easily be divided into three layers: epidermis, dermis and hypodermis (Fig. 33). It is, in fact, a highly differentiated mucus membrane with a dual function as an external protective covering and as an osmotic barrier (Roberts, Young & Milne, 1971). Teleost epidermis does not usually contain keratin (Burgess, 1956) and the main structural component is the highly fibrous Malphigian cell (Roberts et al., 1971). The outer epidermis contains numerous mucus secreting goblet cells which respond by hypersecretion when fish are frightened (Murray & Fletcher, 1976). The dermis is separated from the epidermis by a basement membrane beneath which the scale and scale pocket lie and the poorly developed hypodermis.

Mast cells, which probably play an important role in the mediation of the vascular phase of inflammation in mammals, have also been demonstrated in the dermis of plaice skin (Roberts et al., 1971). However, the cells described as mast cells in fish have been identified merely on the grounds that they have a connective tissue habitat and possess cytoplasmic granules which are basophilic and metachromatic. Thus, there is no functional evidence for identifying these cells in fish. In fact, mast cells are reported to be more numerous in many

tissues of lower vertebrates than in any other tissue in the animal kingdom. (Michels, 1938). More recently, it has become evident that fish mast cells contain little or no histamine or 5-HT (Reite, 1965; Takaya 1968). Indeed, there is some controversy as to whether compound 48/80 can degranulate the mast cells of lower vertebrates. Reite (1965) found that it was effective in Rana temporaria but even large doses of this agent did not produce any distinct morphological changes in the mast cells of Rana catesbeiana (Takaya, Fujita & Endo, 1967). The involvement of these cells as target cells in lower vertebrates must therefore be regarded as doubtful.

Roberts and co-workers also found considerable numbers of previously undescribed cells occupying a basal position in the plaice epidermis. These were called eosinophilic granular cells (EGC) despite being morphologically distinct from the blood eosinophils of goldfish and of mammals. Although the function of EGC remains obscure, the authors suggested that these cells might be implicated in the secretion of mucus or in the transport of serum components across the membrane. Of particular relevance to the present investigation is an experiment performed by Fletcher and Murray (personal communication) in which they observed the position of the EGC following an intradermal injection of compound 48/80. The cells were found to have become active and were observed migrating towards the exterior of the epidermis (Fig. 34). This suggests that these cells might be involved in the inflammatory reaction in plaice skin, possibly as target cells acting as a store of mediators or alternatively as cells containing enzymes contributing to the control of released mediator substances.

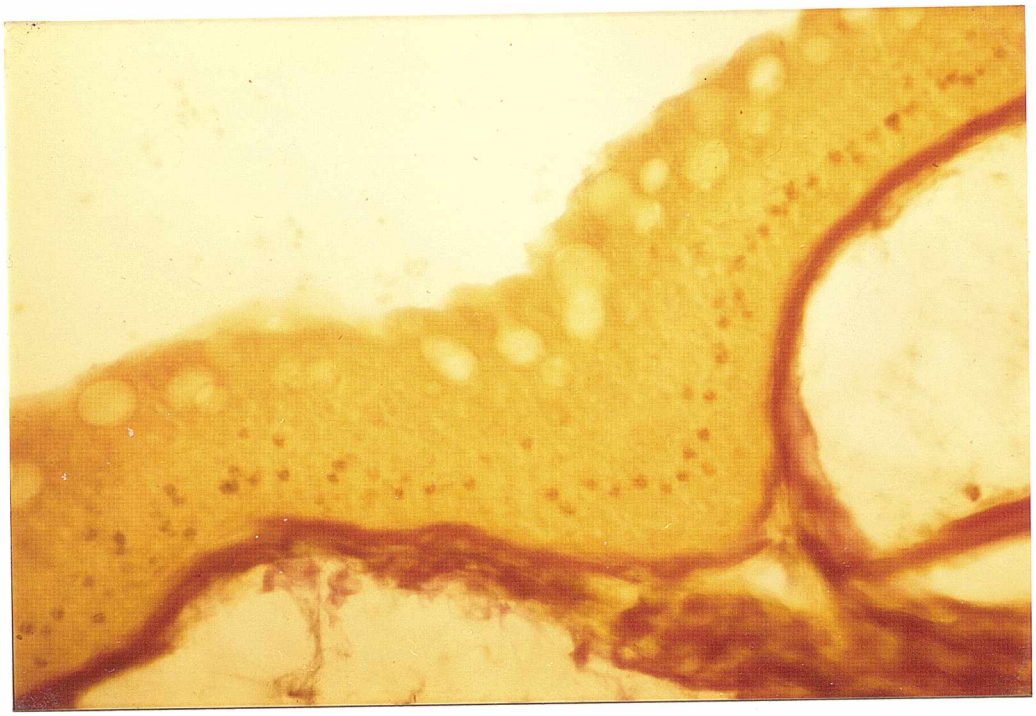
4.3 EVIDENCE THAT A PROSTAGLANDIN IS RESPONSIBLE FOR THE SKIN REACTION IN PLAICE

4.3 i) Direct evidence : release from model systems

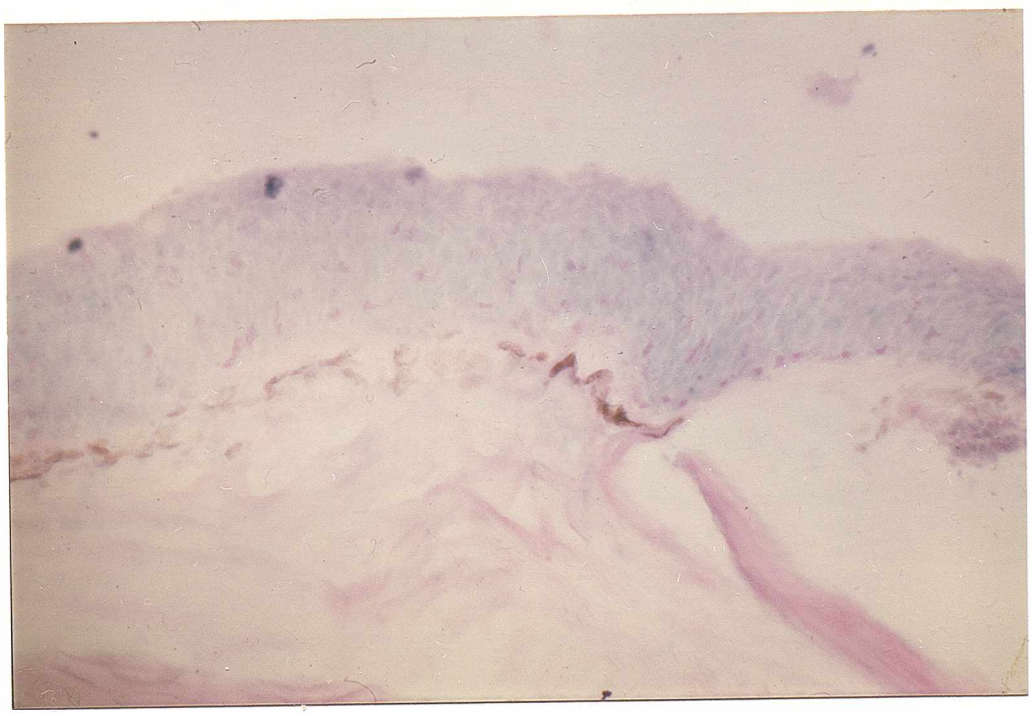
The detection of prostaglandin-like material in incubations of plaice skin in vitro provided the first indication that these substances might be acting as mediators. Subsequently, an effort was made to isolate, identify and quantitate the prostaglandins(s) produced. Incubation media from challenged and non-challenged skin was shown to contain prostaglandin E_2, identified by bioassay, thin-layer chromatography

Fig 34 Effect of compound 48/80 on the position of eosinophilic granular cells (EGC) in the epidermis of plaice skin. a) The normal position of the EGC, at the base of the epidermis (series of black dots in a line). Skin was stained with Chlorazol black. b) Position of EGC after the injection of compound 48/80 (1 mg). The cells can be seen to be migrating towards the exterior of the epidermis (visible as a series of purple dots). Skin was stained with Giemsa stain (rapid). In a) and b), skin was fixed with formaldehyde (10%) for 2 hours, then sectioned in cryostat before staining.

Photographs are shown by kind permission of C.K. Murray and T.C. Fletcher.



a)



b)

and ultraviolet absorption spectrometry. Unequivocal evidence for the release of E_2 from challenged skin was obtained by mass-spectrometric analysis. Although an intensive effort was made to isolate and identify any additional prostanoic acids, no other known naturally-occurring prostaglandins were detected.

The fact that very little prostaglandin-like material could be extracted from the skin with methanol (see page 118) indicates that events which induce prostaglandin release from skin must also stimulate their synthesis. The linear pattern of release of prostaglandin E_2 from skin incubated with Ringer saline alone (Fig. 11) may reflect a simple leakage of prostaglandin out of the skin as a result of an explosive synthesis stimulated by the removal and chopping process. The increased prostaglandin release from skin incubated with the challenging agent, E. floccosum implicates a further synthesis of prostaglandin on challenge, which when superimposed on the simple basal release leads to the observed more complex pattern of release (Fig. 12). The concentration of a mediator present at any time depends upon a balance between its formation and its inactivation. Prior to 60 minutes incubation, synthesis would seem to outweigh metabolism. After this time however, there is a fall in prostaglandin output probably due to diminished synthesis accompanied by enzymic degradation of released prostaglandin. There is some evidence that there is an inactivation mechanism for prostaglandin E_2 in plaice skin, since a microsomal supernatant reduced the smooth muscle activity of authentic E_2 by 60%. The metabolites of the E-series prostaglandins also possess some smooth muscle activity (Anggard & Samuelsson, 1966), so that this figure may reflect an underestimation of the metabolism of E_2.

The in vivo perfusion experiments provided further evidence for prostaglandin release by plaice skin. Perfusates from skin challenged with E. floccosum and compound 48/80 contained nanogram quantities of prostaglandin-like activity. The response showed some specificity, since prostaglandins could not be detected when skin was injected with saline, carrageenin or silver nitrate (a chemical irritant) or after mechanical agitation. Even when perfusates were combined and condensed, only very small quantities of the active material were available for analysis so that absolute identification was not possible. Activity co-chromatographed with the E-series prostaglandins using TLC and a prostaglandin of the 2-series was positively identified by GC-MS analysis. These results indicate the presence of E_2 but these effects could possibly be due to

other metabolites of arachidonic acid such as 6-oxo - PG $F_{1\alpha}$ or thromboxane B_2.

The principal criticism of the perfusion method employed is that any material which is released by the skin is immediately diluted in the perfusing solution. The recovery of agents in the perfusate depends on several factors which are difficult to control. The Ringer saline seemed to form a subcutaneous pool, which would cause dilution of the pharmacological activity in proportion to the volume of the pool and any enzymes present in the overlying skin could bring about degradation of released mediators. Other variables which may be important include uptake by lymphatic and blood vasculatures. The recovery of known quantities of authentic E_2 confirmed that this method is only likely to detect the presence of relatively large amounts of this substance.

Perhaps the most interesting results were obtained using the perfused rabbit ear preparation as a model of skin vasculature (Fig. 19). The labile, dilating material detected in four experiments was prostaglandin-like in as much as it could be extracted from aqueous solution at pH 4 into ethyl acetate but was not due to E_2, since it was labile in aqueous solution, whereas authentic E_2 was not. The presence of prostaglandin endoperoxides should be considered since they are unstable in aqueous media and spontaneously decompose (with a half-life of approximately five minutes) to a mixture of prostaglandins E_2 and D_2 in mammals (Fig. 1). Indeed, the appearance of smooth muscle contracting material after twenty minutes incubation in aqueous solution coupled with a concomitant loss of dilating activity suggests that the labile substance might have been an unstable intermediate in the synthesis of prostaglandin E_2 by plaice skin, such as an endoperoxide. The fact that a contraction of the rabbit aorta (which can be used to assay the endoperoxides) was not consistently observed may be explained by the apparently low sensitivity of this tissue. Thromboxane A_2 on the other hand has been reported to be at least thirty times more potent than the endoperoxides in inducing a contraction of the aorta (Bunting, et al., 1976) and so their presence should have been more evident. It is possible that the rabbit ear preparation is particularly sensitive to the endoperoxides. Certainly, nanogram quantities of prostaglandin G_2 have been found to produce a protracted phase of vasodilatation in the vessels of the hamster cheek pouch, another model of the microvasculature (Lewis, Westwick & Williams, 1977).

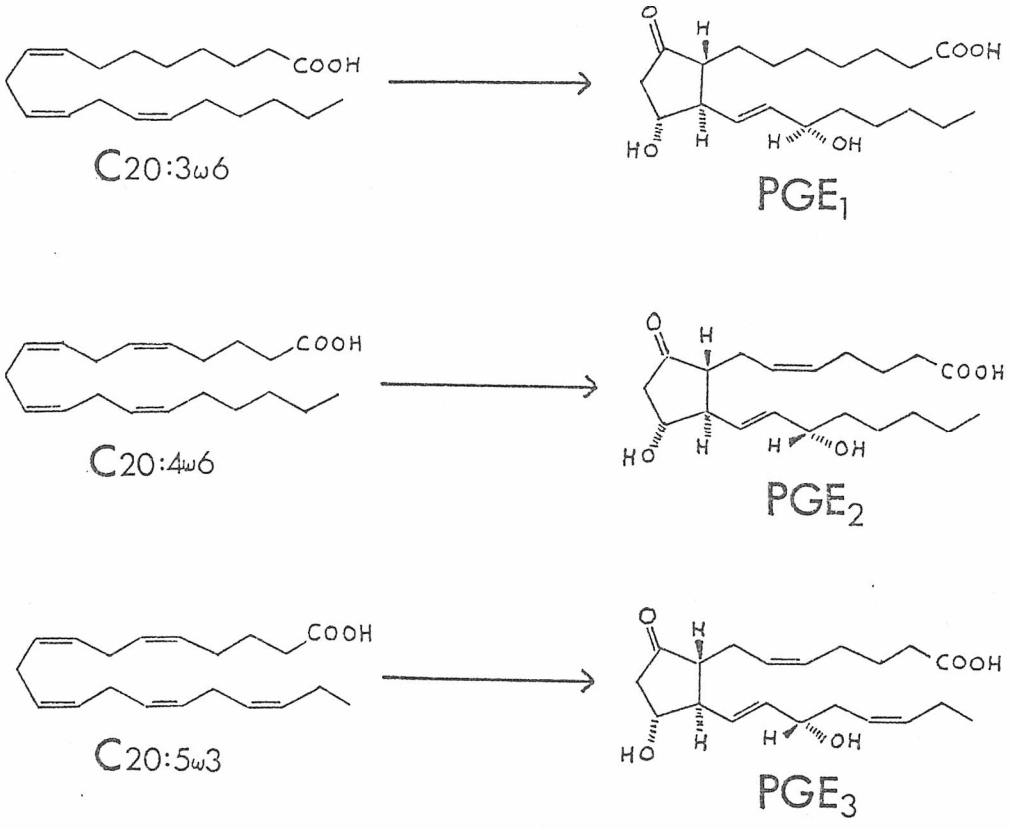


Fig 35 Synthesis of E-series prostaglandins from their respective precursor fatty acids.

The results of these last experiments might explain the observation that the immediate skin reaction in whole plaice developed within five minutes, whereas the release of prostaglandin E_2 from skin challenged in vitro did not reach a maximum until after sixty minutes incubation. It is possible that prostaglandin E_2 is only a metabolite of the primary mediator, which could be prostaglandin G_2 or H_2 as suggested by Kuehl and co-workers (see page 26). Although an endoperoxide might be released by the skin within five minutes of challenge to initiate the inflammatory reaction, there would, without doubt be a finite period required for degradation into prostaglandin E_2 to take place. The finding that E_2 is pro-inflammatory, in that it can produce a long-lasting skin reaction like E. floccosum suggests that E_2 may play a vital role in the maintenance of the skin reaction. The lack of availability of authentic prostaglandins G_2 or H_2 prevented the testing of this hypothesis.

4.3 ii) Fatty acids and prostaglandin biosynthesis

Prostaglandins of the 1, 2 and 3 series are synthesized from the 20 - carbon (C20) unsaturated acids eicosatrienoic acid (20 : 3 w 6), eicosatetraenoic acid (20 : 4 w 6) and eicosapentaenoic acid (20 : 5 w 3) respectively (Fig 35) (Bergstrom, Danielsson, Klenberg & Samuelsson, 1964; Bergstrom, Danielsson & Samuelsson, 1964; Van Dorp, Beerthuis, Nugteren & Vonkeman, 1964). In the present experiments, prostaglandins E_1 and E_3 were not detected. It has been suggested that the amount of each prostaglandin is related to the composition of the precursor fatty acids (Ogata & Nomura, 1975). Therefore, it is interesting that the major C20 acid in plaice skin was not 20 : 4 w 6 as in human skin (Jonsson & Anggard, 1972) but 20 : 5 w 3, the precursor of E_3 (table 11), since E_2 was the only prostaglandin found to be released by plaice skin. This indicates some biochemical specificity with regard to the biosynthesis or distribution of prostaglandins in plaice skin. The anomaly was resolved by comparing the three prostaglandin precursors as substrates for one and the same particulate enzyme fraction from plaice skin (Table 14). Although 20 : 3 w 6 was converted to E_1 in this system, the very low level of this fatty acid in the skin accounts for the absence of E_1 in skin incubations. There was only a low conversion of 20 : 5 w 3 into E_3 suggesting that its importance as a precursor of prostaglandins in plaice skin is minimal. The fact that most conversion was observed with 20 : 4 w 6 as precursor explains the predominance of E_2 in skin exudates. In a similar study using ram seminal vesicle microsomes, Struijk, Beerthuis & Van Dorp (1967) showed that

whereas E_1 and E_2 were converted at a high rate from their respective precursor fatty acids, E_3 was formed at a much lower rate. However, a possible regulatory role for this fatty acid in prostaglandin synthesis by plaice skin was indicated by the observation that E_2 synthesis from 20 : 4 w 6 was inhibited by 20 : 5 w 3 (Table 15). Other endogenous fatty acids may also be important in this inhibitory capacity.

The prostaglandin synthetase enzymes from different tissues have been found to have different synthesizing capacities. Plaice skin microsomes converted arachidonic acid into E_2 in a yield of only 2 percent. Similar studies in human (Ziboh, 1973) and mouse (Wilkinson & Walsh, 1977) skin indicated a 5 percent conversion of substrate, but even these enzymes are about ten times less active than the enzyme from ram seminal vesicles (Struijk et al., 1967). In 1972, Christ and Van Dorp demonstrated prostaglandin biosynthesis in tissue homogenates of a number of fish. The activities found were generally lower than those in mammalian tissues but conversions of up to 10 percent were found. Moreover, analysis of the fatty acid composition of the gills of some fish revealed significant amounts of 20 : 5 w 3, the C_{20} fatty acid predominant in plaice skin. In fact, the lipids of marine fish have been found to contain high proportions of unsaturated fatty acids of the w 3 series (Ackman, 1964), unlike mammals in which the w 6 series predominates. The function of these fatty acids has long been linked with the cell membrane. If it is assumed, then that the chain length and the extent of unsaturation of the fatty acids in the membrane contribute to the physical characteristics of the membrane, these properties might be expected to vary between fish and land animals, although the significance of this difference remains obscure.

4.3 iii) Use of exogenous substances

One of the criteria which a potential mediator substance must satisfy is that it should duplicate, on injection, some of the features of the inflammatory response (see page 37). In an extension of the experiments of Baldo and Fletcher (1975), it was found that histamine, 5 - HT and bradykinin, three substances believed to act as chemical mediators of the microvascular injury response in mammals (Wilhelm, 1962; Rocha e Silva, 1966), were capable of mimicking the skin reaction produced by E. floccosum in plaice, but only in very high concentrations (Table 20). In contrast, prostaglandins E_1 and E_2 could produce a similar response in considerably lower concentrations

suggesting that they are more important than the "classical" mediators in this tissue. Unfortunately, samples of an authentic prostaglandin endoperoxide were not available for testing, although an endoperoxide analogue was not active.

There are a number of problems associated with the use of exogenous "mediator" substances. Exogenous agents may be readily degraded *in vivo* before being able to reach their site of action. In addition, these substances could activate other receptor sites to produce actions different from locally synthesized mediators. Moreover, biosynthesis at the site of action would produce high local concentrations of material that would be very difficult to mimic by exogenous application. It would appear, therefore, that the ability of prostaglandin E_2 to mimic the inflammatory response does not necessarily indicate that the endogenous process following the stimulus is actually due to a prostaglandin.

4.3 iv) Use of inhibitors

The discovery that aspirin-like drugs can inhibit the prostaglandin synthetase enzyme (Vane, 1971) has provided an invaluable tool to determine the importance of intrinsic prostaglandin biosynthesis. Indomethacin, which inhibited prostaglandin synthesis in all species studied (Vane, 1974) also inhibited the synthesis of prostaglandin E_2 from arachidonic acid by plaice skin microsomes. Moreover, there was a similar inhibition of prostaglandin release from chopped plaice skin, although it was shown that challenged release was inhibited to a greater degree than non-challenged release (Fig 14). The latter result suggests that either there are two types of enzyme synthesizing prostaglandin E_2 in plaice skin or that the enzyme is compartmentalised in some way. In fact, Smith and Lands (1972) have found two types of cyclooxygenase activity in the sheep vesicular gland, a slow cyclooxygenase activity (E b) providing the basal level of prostaglandins and a rapid activity (E a) appearing only when stimulated. If a similar mechanism is in operation in plaice skin, the results indicate that the E_a activity is more susceptible to indomethacin than the E b activity. In addition, the observation that disodium cromoglycate caused a selective inhibition of prostaglandin release from challenged skin *in vitro* (Fig. 15) might suggest that this compound is specifically inhibiting the E a activity.

Hydrocortisone, unlike indomethacin, did not inhibit prostaglandin synthesis from arachidonic acid by plaice skin microsomes. Similarly, corticosteroids have been shown to have little or no effect on prostaglandin synthesis from microsomal preparations of other tissues (Flower, Gryglewski, Herbaczynska - Cedro & Vane, 1972), although they can inhibit prostaglandin synthesis in rat skin homogenates (Greaves & McDonald - Gibson, 1972). In fact, corticosteroids have been reported to inhibit prostaglandin production and release in a number of mammalian tissues (Lewis & Piper, 1975; Floman & Zor, 1976). The drugs are thought to prevent the activation of the hydrolytic enzyme, phospholipase A_2 (Blackwell, Flower, Nijkamp & Vane, 1978; Brain, Lewis & Whittle, 1977), which initiates the release of substrate fatty acids from the phospholipid matrix of the cell. However, there was no inhibition of prostaglandin release from plaice skin in the presence of hydrocortisone (1 mg/ml). In addition, hydrocortisone and corticosterone failed to have any inhibitory action on the skin reaction in whole plaice. It may be that the phospholipase in plaice skin is resistant to hydrocortisone or that another enzyme is responsible for the release of the free fatty acid. Unfortunately there have not been any attempts to study the mechanism of prostaglandin synthesis in the lower vertebrates to assist any explanation.

Whereas indomethacin, in a dose of 1 mg/kg maximally inhibited prostaglandin release from plaice skin in vitro, even 50 mg/kg did not inhibit the skin reaction produced by E. floccosum in vivo. In addition, there did not appear to be any delay in the onset or change in the duration or intensity of the erythema-type reaction. Moreover, chronic treatment with indomethacin and also with aspirin and flurbiprofen did not have any inhibitory effect. The inability to quantitate the intensity of the skin reaction did mean that a partial blockade of the response could have been overlooked. Certainly, any inhibitory activity would have been strongly indicative that a prostaglandin was behaving as a mediator; however, the converse does not apply. If a number of mediator substances are involved in the pathogenesis of the skin reaction, removal of the effects of one of them may not significantly reduce the lesion observed. Only when the effects of one mediator really predominate can blockade of its synthesis or action be fully appreciated. Thus, Ferreira and Vane (1974) demonstrated a strong correlation between the inhibition of prostaglandin biosynthesis in vivo and suppression of the signs and symptoms of inflammation. The inability of indomethacin to inhibit the skin reaction in plaice does suggest that prostaglandins are not involved as primary mediators

and that other mediators must be implicated. A similar conclusion was drawn by Greaves and co-workers who observed that indomethacin could not consistently suppress the erythema produced by ultra-violet irradiation in human skin (Greaves, Hensby, Plummer & Warin, 1977).

Disodium cromoglycate (DSCG) was the only drug tested which completely inhibited the skin reaction, although the dose required was very large (160 mg/kg). In mammals, DSCG can inhibit the immunological release of mediators from a number of tissues (Assem & Mongar, 1970; Kusner, Dubnick & Herzig, 1973) but it does not seem to interfere either with the antigen-antibody reaction or with the responses of target organs to mediators (Cox, 1967; Assem & Mongar, 1970). DSCG can, however, inhibit mast cell degranulation (Orr, Hall, Gwilliam & Cox, 1971; Taylor, Francis, Sheldon & Roitt, 1974), possibly by preventing the entry of calcium into the cell induced by the antigen-antibody reaction (Foreman, Mongar & Hallett, 1977). The fact that mast cells do not seem to play a role as target cells in lower vertebrates (see page 141) might explain the high doses of DSCG required to inhibit the skin reaction but gives no indication as to the mode of action in plaice skin. In mammals, DSCG can inhibit cyclic AMP phosphodiesterase in vitro (Roy & Warren, 1974) suggesting that it may exert its effect by elevating cyclic AMP levels in the cell. However, DSCG is inactive against the prostaglandin synthetase enzyme (Flower, 1974), suggesting that the inhibition of prostaglandin release from plaice skin might be indirect, that is secondary to the principal inhibitory effect. DSCG does prevent prostaglandin release during anaphylaxis in human lung tissue but only at doses which completely inhibit the release of histamine and SRS-A (Piper & Walker, 1973). Nevertheless, the inhibition of prostaglandin E_2 release in parallel with the blockade of the skin reaction does imply a mediator role for prostaglandins in plaice skin.

4.3 (v) Role of prostaglandins in plaice

The prostaglandin synthesizing system has been found both in the higher animals and in lower animals such as mussels and lobsters (Christ & Van Dorp, 1972). An indication of the fundamental properties of prostaglandin synthesis was revealed in a study of spawning in molluscs (Morse, Duncan, Hooker & Morse, 1977). Addition of hydrogen peroxide to seawater was found to cause spawning in abalones. This effect was blocked

by aspirin, suggesting that prostaglandin synthesis was required for spawning to occur. Although plants can produce arachidonic acid (Nichols & Appleby, 1969), there is no indication that such a system occurs in the plant kingdom. This may indicate that prostaglandins are involved in the regulation of elementary membrane functions for which the plant has no need. Christ and Van Dorp (1972) have speculated that the regulation of ion and water transport is such a function, in view of the high prostaglandin synthetase activity found in the gills of fish, mussels and lobsters, in mammalian lung and renal medullary tissue and in frog bladder. In frog skin, a reduction in the osmolality of the bathing solution was found to cause an increase in prostaglandin release, accompanied by a concomitant increase in cyclic AMP levels within the skin (Hall, O'Regan & Quigley, 1977). It was suggested that physiological changes in frog skin permeability are regulated by altered rates of prostaglandin biosynthesis (Haylor & Lote, 1976; Hall et al., 1977). In an analogous situation, it is possible that endogenous prostaglandins could be important regulators of the microcirculation of both higher and lower vertebrates. Certainly, in mammals, prostaglandins can modulate the action of other mediators on the vasculature (see page138). However, whereas in man prostaglandins cannot, on their own, mimic all the visible signs of inflammation (in particular, oedema and erythema) prostaglandins E_1 and E_2 caused a skin reaction in plaice which was indistinguishable from that produced by E. floccosum. This suggests that in lower vertebrates, prostaglandins may function not merely as modulators but also as mediators.

4.4. EVIDENCE THAT SOME CLASSICAL MEDIATORS ARE NOT INVOLVED IN THE SKIN REACTION IN PLAICE

4.4. (i) Histamine

The assay systems employed in this study failed to detect the presence of histamine either in skin incubations in vitro or in perfusates of the skin of whole plaice. It is possible that very small quantities of liberated histamine escaped detection or that there was a rapid inactivation or uptake mechanism present in the skin. The low recovery of authentic histamine from perfusion experiments (Table 7) suggested that this might be the case although an enzymic preparation of plaice skin did not greatly

reduce histaminic activity (Fig. 27). Moreover, only high concentrations of histamine were capable of mimicking the skin reaction produced by E. floccosum, which was also found to be resistant to treatment with H_1 and H_2 receptor antagonists (Tables 21 and 22).

The histamine content of plaice tissues was extremely low (0.2 µg/g in skin) when compared with those of higher vertebrates : rat skin, for example contains 20 to 40 µg/g of histamine (Parratt & West, 1956). In addition plaice skin histamine could not be depleted by treatment with compound 48/80, an agent believed to act by releasing histamine from tissue mast cells (Riley & West, 1955). It is perhaps significant that while this compound is active in mammals in a concentration of 1 µg/ml (Paton 1951; Riley & West, 1955), it could only cause a reaction in plaice skin when the concentration was raised one hundred-fold; indeed it is possible that this may reflect a direct action on the microcirculation. Although it is well established that the major portion of histamine in mammals is contained in mast cells (Riley, 1959), the general validity of this association in vertebrates has been questioned. Reite (1965) measured the tissue levels of histamine in nineteen different species of animals ranging from fish to mammals. The very low levels detected in lower vertebrates coupled with the finding of numerous mast cells in every tissue of lower vertebrate studied, strongly indicated that their mast cells are devoid of histamine. This conclusion was reinforced by Takaya and co-workers (Takaya et al., 1967; Takaya, 1968). It would be logical to assume, therefore, that there are important differences in the mechanisms of microcirculatory responses between higher vertebrates with substantial mast cell histamine and lower vertebrates with little or no histamine in their mast cells.

4.4. (ii) 5-HT

As with histamine, there was no evidence to suggest that plaice skin released 5-HT when provoked by challenge with either E. floccosum or with compound 48/80. or indeed that the skin had any mechanism which could degrade this amine (Table 9 and Fig 27). If it is present in plaice skin, it occurs at levels which could not be detected by fluorimetric assay (see page116) or by bioassay (Table 19). Nevertheless, the isolated organs of cod and plaice which were tested as assay tissues were stimulated strongly by 5-HT. It is probable though, that this action is not a direct

one, since, in the stomach of the plaice, 5-HT contracts the tissue by releasing acetylcholine from cholinergic neurones (Edwards, 1972; Grove, O'Neill & Spillett, 1974).

4.4 (iii) Bradykinin and related peptides

In mammals, one of the most striking properties of bradykinin is its capacity to increase vascular permeability (di Rosa & Willoughby, 1971), although there is little direct evidence to implicate bradykinin as a mediator in mammalian skin. The evidence against an involvement of bradykinin in the skin reaction in plaice stemmed from the lack of polypeptide activity in skin incubations and perfusates either before or after challenge. In addition, plaice skin did not seem to be able to metabolise authentic bradykinin, nor did it show a skin reaction to this agent, except in the relatively high concentration of 1 mg/ml.

In a study of plasma kinins throughout a wide range of vertebrates, Dunn & Perks (1975) concluded that fish and amphibians do not contain a kallikrein-kinin system comparable to that of mammals (see page 19). Fish plasma did not liberate kinin-like activity following incubation with trypsin or glass, and bradykinin and trypsin did not affect fish blood pressure. However, some years earlier, it had been reported that the skin of the frog, Rana temporaria contained large amounts of bradykinin, the same polypeptide originating in the blood of mammals by the action of kallikrein enzymes (Erspamer, Bertaccini & Cei, 1962; Anastasi et al., 1965). This material was only loosely-bound since it could be freed by treatment with methanol. Fischer and Albert (1971) also found a bradykinin-like peptide in methanol extracts of lamprey skin. More recently, four kinds of bradykinin analogues were identified in the skin of Rana rugosa (Yasuhara, Ishikawa, Nakajima, Araki & Tachibana, 1979). In the present study, methanolic extracts of plaice skin were analysed in parallel with frog skin extracts but did not contain comparable amounts of kinin-like material, as assayed against authentic bradykinin (Table 19), suggesting that it is probably not an important inflammatory component in plaice skin. Moreover, this fraction, purified on an alkaline alumina column did not cause a significant dye leakage in rat skin (Fig 26).

Methanolic extracts of plaice skin did contain a factor which could cause dye leakage in rats (Fig 26) but it was inactive on smooth muscle preparations. At present, however, there is no evidence to suggest that

such a factor is involved as a mediator of the skin reaction in plaice. Extracts of incubation media challenged with E. floccosum did not cause dye leakage in rat skin. Further characterisation of the active material will be necessary before any conclusions can be made as to a role (if any) in the microvasculature of plaice skin. There are a number of reports describing permeability factors which have been extracted from mammalian skin (Inderbitzin, 1964; Lazarus & Barrett, 1974; Levine, Hatcher & Lazarus, 1976; Yamamoto & Kambara, 1978) but as yet no role has been attached to these factors in pathophysiological events. However, a new type of amphibian skin peptide has recently been described which acts on rat peritoneal mast cells to liberate granules and histamine from these cells (Yasuhara, Ishikawa & Nakajima, 1979).

4.4 (iv) Other agents

The pharmacological screening did not reveal any vasoactive material in inflammatory exudates of plaice skin which was not of lipidic nature. The presence of SRS-A, which, like the prostaglandins is a hydrophilic lipid, was made improbable since FPL 55712 did not inhibit the response to the smooth muscle contracting material released from plaice skin by E. floccosum in concentrations known to antagonise responses to mammalian SRS-A (Augstein et al., 1973). Furthermore, FPL 55712 could not inhibit the skin reaction produced by E. floccosum even in a dose of 5 mg/kg. Diethylcarbamazine citrate, an antifilarial chemotheraputic agent which can suppress SRS-A release from mammalian lung (Orange et al., 1968b; Ishizaka, Ishizaka, Orange & Austen, 1971), was equally ineffective on the immediate response in plaice skin, although this last observation is not in accord with the findings of Baldo and Fletcher (1975) that this substance was an effective in vivo inhibitor in plaice. The reason for this inconsistency is unknown. SRS-A has been demonstrated in primate skin (Kuritzky & Goodfriend, 1974) but there are no reports of SRS-A in lower vertebrates and it seems unlikely that it is involved as a mediator in plaice skin.

Within the fishes can be seen the evolution of the autonomic nervous system and the emergence of a double innervation of many organs. However, arteries are supplied by both constrictor adrenergic and constrictor cholinergic nerves in fish, whereas in mammals the latter seem to have taken on an inhibitory (dilator) role (Burnstock, 1969). Veins do not appear to have an adrenergic supply at all in fish and in general, direct nervous

control has been developed to a much less sophisticated degree than in mammals. As if to compensate for this fact, the influence of circulating catecholamines seems to play a more significant role in lower vertebrates. Indeed, it is likely that control of vascular resistance in fish is maintained by circulating catecholamines released from locally distributed chromaffin cells (Randall & Stevens, 1967). In this light, it was found that an intravenous injection of adrenaline was unable to inhibit the skin reaction produced by E. floccosum in plaice skin, contrasting with its ability to suppress the classical wheal and flare response in human skin (Tuft & Brodsky, 1936). The latter anti-inflammatory effect is probably mediated by β - receptors, since β - adrenoceptor agonists inhibit mediator release from human lung fragments (Wasserman, Goetzl, Kaliner & Austen, 1974). On the other hand, α - adrenoceptor agonists can augment the release of mediators (Kaliner et al., 1972). It is interesting then, that in lower vertebrates there are no catecholamine receptors that can mediate inhibition in large arteries, all the receptors being of the α - type (Burnstock, 1969). It may be that, in these primitive vertebrates, catecholamines are pro-inflammatory by virtue of this predominance of adrenoceptors. This hypothesis was not supported by the observation that α or β - adrenoceptor blocking drugs did not inhibit the immediate skin reaction in plaice. Atropine, a muscarinic blocking drug was similarly ineffective in plaice skin. Nevertheless, there may still be a link between the nervous system and mediator release as has been already suggested for mammals (see page 29).

4.5 ROLE OF C-REACTIVE PROTEIN IN INFLAMMATION AND MEDIATOR RELEASE IN TELEOST FISH

The nature of the skin-fixing serum factors responsible for the immediate skin reaction in plaice has yet to be established. Of the fish examined by Fletcher and Baldo (1974), only the flounder failed to show a skin reaction on challenge with E. floccosum and only the flounder lacked CRP-like serum precipitins to the C-substance constituent of this fungal extract. Flounder serum contains only trace amounts of CRP (< 2 µg/ml) compared with plaice serum (55 µg/ml) (Pepys et al., 1978). In the present study, confirmation was obtained of the ability of plaice serum to transfer the skin reaction to flounders. Furthermore, an immediate skin reaction was observed in flounders following the intravenous injection of CRP, prepared from plaice serum. A closely-related serum protein, P-component

(Osmand, Friedenson, Gewurz, Painter, Hofmann & Shelton, 1977) was not similarly effective. These observations provide strong support for the original suggestion that the CRP-like constituents of plaice serum are intimately involved in the genesis of the skin reaction (Fletcher & Baldo, 1974).

It is clear that at low temperatures, antibody production is often restricted (Good & Papermaster, 1964; Thoenes & Hildemann, 1970). If it is considered that the natural environment of flatfish is usually at a temperature below 10°C, the development of specific antibodies is probably a slow process and other molecules might provide the first line of defence. As yet, there is no evidence to show that teleosts have more than one IgM - like immunoglobulin class (see page 30) or possess antibodies comparable to the reaginic antibodies described in higher vertebrates.

CRP shares many functional features with the immunoglobulins (Fiedel & Gewurz , 1976). However, they differ in their sites of synthesis and in the stimuli required for their release (Osmand et al., 1977; Fiedel & Gewurz, 1976). Human CRP shows extreme specificity for phosphorylcholine, the determinant on E. floccosum peptido-polysaccharide which is responsible for the reaction with plaice serum (Baldo et al., 1977). In addition, phosphorylcholine has been shown to be an important structural component in animal cell membranes and bacterial cell walls (Tomasz,1967; Briles & Tomasz, 1973). The possibility therefore exists than non-antibody precipitins form part of the fishes'humoral defences against invasion by micro-organisms and parasites (Baldo & Fletcher, 1973).

There was good correlation between the transfer of the skin reaction to flounders and prostaglandin release from flounder skin challenged in vitro. Thus, challenge with E. floccosum stimulated prostaglandin release from the skin of flounders which had been injected with plaice serum or CRP but did not affect prostaglandin release from the skin of untreated flounders or those fish injected with P - component (Figs 31 and 32). These results provide further evidence that prostaglandins are involved as mediators in plaice skin, although it is possible that this stimulus is non-specific and that prostaglandin release is observed merely as a result of membrane distortion or secondary to the release of other mediators. Nevertheless, the reaction of the C-substance component of E. floccosum with CRP appears to stimulate prostaglandin synthesis in some way. It has been mentioned that among its many actions, CRP can

activate complement through the classical pathway (Volanakis & Kaplan, 1974). If the combination of C-substance with CRP can lead to an activation of complement, the activated components and cleavage products would almost certainly be capable of mediating tissue injury, either directly or indirectly. Type - 1 hypersensitivity reactions in mammals are probably not complement dependent : at least, reaginic antibodies do not have the ability to fix complement (see page 20). However, this is not the case with CRP and it is suggested that the complement system may be critical to the hypersensitivity reaction in plaice skin.

4.6 GENERAL CONCLUSIONS

This investigation has indicated that plaice are relatively low in the phylogenetic development of the animal kingdom. Thus, the appearance of histamine and 5-HT in the mast cell and a kinin-generation system in the serum is at a phylogenetically higher level than that of the prostaglandin synthetase system. The results show that prostaglandins (of the E-series) are potent inflammatory agents in plaice skin and indicate that they might be of correspondingly greater importance as regulators of the micro-circulation in lower vertebrates. Furthermore, fish do not have a complex antibody defence mechanism as do mammals, apparently possessing only one class of immunoglobulin. It is possible that CRP fulfils part of the fishes' humoral defence system since it is present in much higher concentrations (55 µg/ml plaice serum) than in the sera of higher vertebrates (Pepys et al., 1978). With the evolution of antibodies, perhaps the requirement for CRP declined.

It has been postulated that the combination of C-substance with the CRP - like component of plaice serum can lead to the activation of the complement system. It would be interesting, therefore to observe the effect of treating plaice with cobra venom factor, a protein which can cause depletion of serum complement in vivo (Cochrane, Muller-Eberhard & Aikin, 1970). If complement is critically involved, the skin reaction to E. floccosum should be completely inhibited.

One possibility is that the variation in the intensity of the skin reactions observed during the study was related to the absolute level of CRP - like precipitins in plaice serum. It would certainly be worthwhile to pursue this concept. If it is possible to raise an antiserum to the

precipitin in plaice serum, this would provide a method of quantitating the level of precipitin at any time and would enable a long term monitoring study to be carried out.

The flounder provided an invaluable model with which to study the mechanism of the immediate skin reaction in flatfish. Transfer of the skin reaction to flounders was accomplished following active sensitisation with plaice serum and CRP with a resultant increase in prostaglandin release from skin challenged *in vitro*. Flounder skin has not yet been sensitised *in vitro*. It will be interesting to observe if there is a similar result after passive sensitisation of skin with plaice serum or CRP to complement the *in vivo* transfer experiments.

Although there was no evidence for the release of any inflammatory mediator substance from plaice skin other than a prostaglandin, this does not necessarily mean that other agents were not released. Small quantities of released mediators may have escaped detection by the assay methods employed, especially those substances which are not very active on smooth muscle. In addition, the detection system favoured the prostaglandins, which are easily extracted to concentrate any activity. Nevertheless, the results with these model systems indicated that prostaglandin E_2 release was predominant.

If a mediator has a short half-life in aqueous solution (i.e. thromboxane A_2) it may only be possible to infer its release by measuring the biologically inactive (or less active) metabolite. Thus, it is possible that, apart from prostaglandin E_2, other products of arachidonic acid metabolism were also present in inflammatory exudates but escaped detection due to their inability to contract smooth muscle. Ideally, a radiochemical assay would have been employed (Flower, Cheung & Cushman, 1973) at least in the prostaglandin synthetase experiments (see page 96). The use of labelled precursor fatty acids would have allowed the measurement of all the products of metabolism, independent of smooth muscle activity. Unfortunately, this technique was not available.

The inability of indomethacin to inhibit the *in vivo* skin reaction in plaice showed that other mediators must be involved in addition to a prostaglandin. Some evidence was presented that plaice skin contains a factor which can cause dye leakage in rats. If time had been available, an attempt would have been made to purify and identify this material and to

ascertain whether it is a potential mediator substance. This would be an appropriate starting point for a further study.

It is suggested that any further investigation should concentrate on the development of alternative methods to provide an inflammatory exudate from plaice skin. One possibility arises from a study by Ellis and de Souza (1974), in which they cannulated the neural lymphatic duct in plaice under anaesthesia. It may be feasible to recover lymph draining the skin of plaice in a similar manner as a source of inflammatory mediators.

It remains possible that platelets are an important source of mediators in plaice, especially if stasis develops during the course of the skin reaction. It would be very interesting, therefore to study platelet aggregation and the release of any pharmacologically active material during aggregation.

It may be necessary to look for an alternative model system with which to study mediator release. The collection of peritoneal fluid could provide a source of inflammatory cells, which, after challenge would be analysed for smooth muscle activity. In addition, the whole concept of systemic anaphylaxis warrants investigation. The effect of intravenous challenge with fungal extracts could be widespread and might well facilitate the identification of released mediators.

In conclusion, the mechanism involved in the immediate skin reaction in plaice would seem to be at variance with the mechanisms thought to be in operation in cutaneous hypersensitivity reactions in man and the higher vertebrates. This is especially so with regard to the individual pharmacological mediators responsible for the expression of the reaction. Histamine, bradykinin and SRS-A are considered to be primary mediators in higher vertebrates whereas the prostaglandins are probably more important as modulators of their effects. This does not seem to be the case in lower animals and in particular in fish, in which the former mediators do not seem to be present. On the other hand it is suggested that prostaglandins are involved not merely as modulators but as mediators in their own right.

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APPENDIX I - POSTGRADUATE COURSES

Appropriate systematic courses were not available. As a substitute, a programme of guided reading and tutorials was undertaken.

Details of Guided Reading

1) Statistics and experimental design

a) Cox, D.R.	Planning of experiments
b) Pearce, S.C.	Biological statistics
c) Freund	Elementary statistics
d) Draper and Smith	Applied regression analysis
e) Rickers and Todd	Statistics

2) Immunology

a) Roitt, I.	Essential immunology
b) Humphrey, J.H. and White R.G.	Immunology for students of medicine.
c) Kabat, E.A.	Structural concepts in Immunology and Immunochemistry.
d) Weissman, G.	Mediators of inflammation.

During the first year, two hours reading and one hour tutorial were allocated for each topic. In subsequent years, statistical studies were concentrated on the application of some computer packages to specific problems. These studies were satisfactorily completed.

In addition, the relevant research literature has been studied, as evidenced by the "Bibliography" section of this thesis.

APPENDIX II - ATTENDANCE AT SCIENTIFIC MEETINGS

Additional advanced study included attendance at and participation in the following scientific meetings:

British Pharmacological Society in July, 1976 (Dundee), January 1977 (London), July 1977 (Newcastle upon Tyne) and September, 1979 (Leeds).

7th International Congress of Pharmacology in July, 1978 (Paris)

Scottish Pharmaceutical Sciences Group and Scottish Dermatological Society in October, 1979 (Dundee).

Symposium on "Inflammation and the Initiation of Immunity" in April, 1977, (London).

APPENDIX III - COMMUNICATIONS AND PUBLICATIONS

Summer meeting of the British Pharmacological Society, July, 1977, Newcastle upon Tyne. The presence of prostaglandin-like substance in the skin of the plaice, Pleuronectes platessa, L., A.A. Anderson, Thelma C. Fletcher and G.M. Smith. Abstract - Br. J. Pharmac. (1977), 61, 140 P.

Autumn meeting of the Scottish Pharmaceutical Sciences Group and Scottish Dermatological Society, October, 1979, Dundee. A study of possible chemical mediators of cutaneous anaphylaxis in teleost fish, A.A. Anderson, Thelma C. Fletcher and G.M. Smith.

Poster demonstration at the 7th International Congress of Pharmacology July, 1978, Paris. Prostaglandin release from the skin of plaice. A.A. Anderson, Thelma C. Fletcher and G.M. Smith.

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Br. J. Pharmac. (1979), **66**, 547–552

THE RELEASE OF PROSTAGLANDIN E_2 FROM THE SKIN OF THE PLAICE, *Pleuronectes platessa L.*

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1 A fungal extract which produces a cutaneous hypersensitivity reaction in the plaice, *Pleuronectes platessa L.*, was incubated *in vitro* with the skin of this teleost fish. Samples of incubation media were assayed for smooth muscle stimulating activity.

2 Prostaglandin E_2 was identified by bioassay, thin-layer chromatography, ultraviolet absorption spectroscopy and gas chromatography–mass spectrometry. Release from challenged skin was maximum after 60 min incubation.

3 Analysis of the fatty acid composition of plaice skin showed that although arachidonic acid was present (3% of total fatty acids), the precursor of prostaglandin E_3, eicosapentaenoic acid contributed 9% of total.

4 Indomethacin (50 mg/kg i.p.) did not inhibit the erythema induced by the fungal extract, whilst a dose of 1 mg/kg maximally inhibited prostaglandin release from skin on incubation *in vitro*.

5 It is concluded that prostaglandins do not have an exclusive role in the mediation of the hypersensitivity reaction.

Introduction

An immediate erythematous response has been observed in the skin of the plaice (*Pleuronectes platessa*), a marine teleost fish, following the intradermal injection of certain fungal extracts (Fletcher & Baldo, 1974). A similar response was not observed in the closely related flounder (*Platichthys flesus*) unless it received an intravenous injection of plaice serum, at least 24 h before fungal challenge. The evidence for systemic anaphylaxis in fish is controversial (Dreyer & King, 1948; Clem & Leslie, 1969) but the skin reaction was considered to be a hypersensitivity response, since it was transferrable to the flounder with plaice serum containing precipitins to the fungal extracts. The flounder normally contained no detectable precipitins and the common factor in the fungal extracts eliciting the response was C-substance (Baldo, Fletcher & Pepys, 1977).

The mediators implicated in hypersensitivity responses in mammals have been extensively studied but little is known of such mediators in poikilotherms. The skin reaction in the plaice can be inhibited *in vivo* by disodium cromoglycate (Baldo & Fletcher, 1975) and preliminary work using an *in vitro* system has identified prostaglandin-like substances released from challenged plaice skin (Anderson, Fletcher & Smith, 1977). The present work extends the findings on the *in vitro* release of mediators and the characterization of the prostaglandin involved.

Methods

Preparation of skin

Skin from freshly-killed normal plaice was chopped, washed and incubated at 18°C with 2 volumes (1 g/2 ml) of a specific plaice ringer (Cobb, Fox & Santer, 1973) in the presence and absence of the fungal extract, *Epidermophyton floccosum* (1 mg/ml) prepared as described by Baldo *et al.* (1977), hereafter referred to as challenged and non-challenged skin respectively. Samples of the incubation mixtures were withdrawn at timed intervals for biological assay.

For the identification of active material, large-scale incubations were performed and a standard extract prepared.

Extraction and characterization of prostaglandin-like material

Incubation was terminated by the addition of 2 volumes of ethanol. Petroleum ether was added and

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the mixture inverted. After removal of the petroleum ether, each sample was acidified to pH 3 to 3.5 with formic acid and extracted three times with 4 volumes of chloroform. The pooled chloroform layers were dried under a stream of nitrogen at 30°C. Preparative thin-layer chromatography (t.l.c.) was carried out on silica gel G (0.25 μm) coated glass plates using 4 solvent systems (Table 1). Methanolic solutions of prostaglandin standards and skin extracts were applied to the plates and developed in solvent mixture. Following visualization of standards, zones (1 cm) of silica gel were scraped off the plate and eluted with a mixture of chloroform/methanol (1:1). Eluates were dried under nitrogen, the residue resuspended in Krebs solution and tested for contractile activity on the rat fundus strip.

The use of 2 further solvent systems (Table 1) with silver nitrate impregnated plates enabled separation of the individual members of the E series prostaglandins. As above, zones of silica were extracted and the eluates tested for contractile activity. Amounts of prostaglandin-like material were expressed as prostaglandin E_2 equivalents.

Bioassay

Assay of smooth muscle stimulating activity was performed on the following isolated preparations: rat stomach strip, chick rectum, gerbil colon, rat colon and guinea-pig ileum. Preparations were mounted in organ baths in Krebs solution (composition (mM): NaCl 118.2, KCl 4.75, $CaCl_2$ 2.54, KH_2PO_4 1.19, $MgSO_4$ 1.19, $NaHCO_3$ 25.0, glucose 11.2) at 37°C ± 0.5°C gassed with a mixture of 95% O_2 and 5% CO_2. Isometric contractions were detected by strain gauge transducers and displayed on a Devices Recorder.

For the identification of the active material, 3 + 3 quantitative assays were performed in parallel using a randomized block design on 3 different smooth muscle preparations which vary in their relative sensitivity to authentic prostaglandin standards. An analysis of variance was performed on the results of the parallel assays to obtain potency ratios and fiducial limits.

In subsequent experiments, active material was assayed by the bracketing technique against prostaglandin E_2 on rat fundus strips (Vane, 1957) in Krebs solution containing a drug combination which increases the sensitivity and selectivity of the assay (Gilmore, Vane & Wyllie, 1968, as modified by Bennett, Stamford & Unger, 1973). Differences in prostaglandin levels were analysed by paired *t* test.

The following drugs were used: bradykinin triacetate (Sigma), α-chymotrypsin (Worthington Biochemical Corporation); FPL 55712, 7-[3-(4-acetyl-3-hydroxy-2-propylphenoxy)-2-hydroxypropoxy]-4-oxo-8-propyl-4H-1-benzopyran-2-carboxylic acid sodium salt (Fisons); (−)-hyoscine hydrobromide (BDH); indomethacin (Merck, Sharpe & Dohme), methysergide bimaleate (Sandoz), mepyramine maleate (May & Baker), phenoxybenzamine hydrochloride (Smith Kline and French); propranolol hydrochloride (ICI); prostaglandins E_1, E_2, E_3, $F_{1\alpha}$ and $F_{2\alpha}$ (Upjohn).

Spectrometry

Ultraviolet spectra of partially purified skin extract prepared as above were recorded on a Unicam SP 1800 by the method of Yoshimoto, Ito & Tomita (1970). For mass spectrometric analysis, samples of incubation media from challenged and non-challenged skin were acidified to pH 3 and extracted twice with equal volumes of ethyl acetate. Following evaporation of the combined ethyl acetate extracts, the residue was subjected to preparative t.l.c. using the solvent system, ethyl acetate–acetone–acetic acid (90:10:1) (Andersen, 1969). The zones of silica gel corresponding to authentic E_2 were eluted with methanol. The eluates were evaporated under vacuum and the residue derivatized for analysis using a Finnigan 3200 gas chromatograph–mass spectrometer. The samples were run as the methyl ester, methyloxime,

Table 1 Solvent systems used in preparative thin-layer chromatography of prostaglandin-like material from plaice skin

Composition	*Absorbant*
Chloroform–methanol–acetic acid–water (90:10:1:0.75)	Silica Gel G
Ethyl acetate–formic acid (400:5)	Silica Gel G
Chloroform–ethyl acetate–ethanol–acetic acid (20:20:4:1)	Silica Gel G
Benzene–chloroform–butanol–ethanol (4:10:5:1)	Silica Gel G
Chloroform–methanol–acetic acid (18:3:1)	Silica Gel G–$AgNO_3$ (10:1)
Ethyl acetate–ethanol–acetic acid (100:1:1)	Silica Gel G–$AgNO_3$ (10:1)

trimethylsilyl ether and compared with the corresponding authentic E_2 derivatives.

Fatty acid composition

Finely-chopped skin (10 g) was transferred to a mortar containing liquid nitrogen and ground finely with a pestle. Lipids were extracted by the method of Bligh & Dyer (1959) as modified by Allen, Good, Davis, Chisum & Fowler (1966). Samples of lipid extract were transesterified with methanolic hydrochloric acid for gas chromatographic analysis (g.l.c.) of methyl esters (Farquhar, 1962). A Pye 104 gas chromatograph equipped with a flame ionization detector was used for g.l.c. Two different glass columns (1.5 m × 4 mm) were used to separate the fatty acids: EGSS-X on gas chrom. Q and 10% EGSS-Y on DMCS-treated P (Field Instrument Company Ltd.). Both columns were operated at 200°C. The relative proportion of each fatty acid was found by calculating the product of peak height and the retention time at half-peak width and expressed as a percentage of the total.

Results

Incubation media from challenged and non-challenged skin contracted all the tissues studied. The fungal extract of *E. floccosum*, incubated with Ringer alone, did not change the smooth muscle tone. The rat stomach strip gave the most reproducible and dose-dependent responses and therefore was used to assay the active principle from skin in subsequent experiments. The contractile response to skin extract persisted in the presence of atropine, mepyramine, methysergide and FPL 55712 which antagonize the effects of acetylcholine, histamine, 5-hydroxytryptamine and slow-reacting substance of anaphylaxis (Augstein, Farmer, Lee, Sheard & Tattersall, 1973) respectively. In addition, incubation with chymotrypsin, which destroyed activity due to bradykinin, did not reduce the response to the skin extract.

Table 2 3 + 3 parallel assays using prostaglandin standards E_1, E_2, E_3, $F_{1\alpha}$ and $F_{2\alpha}$

	Concentration of unknown in terms of standard (ng/ml)				
*Tissue**	E_1	E_2	E_3	$F_{1\alpha}$	$F_{2\alpha}$
RSS	446	208	782	1648	882
CR	269	197	1065	6425	1355
RC	343	185	3220	913	815

* RSS: rat stomach strip, CR: chick rectum, RC: rat colon.

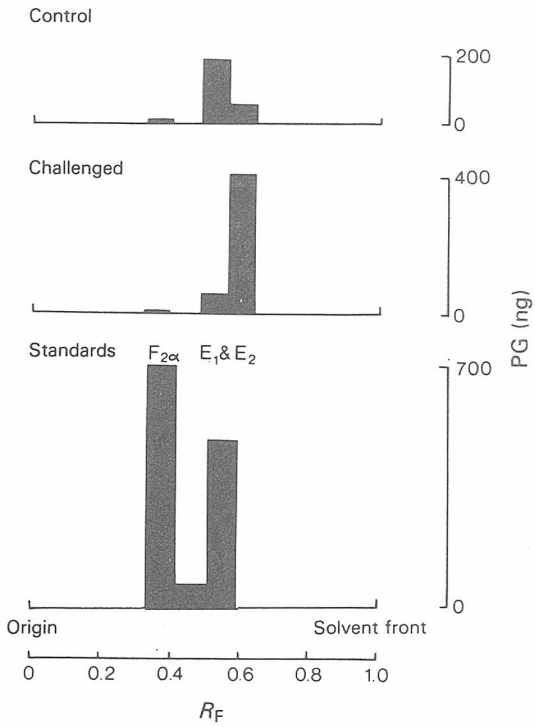

Figure 1 Thin-layer chromatography of prostaglandin-like material from plaice skin using the solvent system ethyl acetate–formic acid (400:5). The 3 histograms represent migration of smooth muscle contracting material released from challenged skin, from non-challenged skin and from a mixture of standard prostaglandins $F_{2\alpha}$ (1.5 μg), E_1 and E_2 (0.5 μg each).

Identification of prostaglandin E_2

The activity could be partitioned from an acid aqueous phase into chloroform, from there into Krebs solution (pH 7) and finally back into chloroform at pH 3. This behaviour is consistent with the properties of a prostaglandin.

Preparative t.l.c. using 4 different solvent systems indicated that the active material co-chromatographed almost exclusively with the E-series prostaglandins (Figure 1). An aliquot of the skin extract, treated with alkali showed an absorption maximum of 278 nm in methanol. Rechromatography using 2 further solvent systems on thin-layer plates impregnated with silver nitrate showed that the prostaglandin-like material migrated in the R_F range corresponding closely with the distance travelled by standard E_2 but not with E_1 or E_3 (Figure 2).

Table 2 shows the results of quantitative assays of the prostaglandin-like material performed in parallel. When the unknown was assayed in terms of standard

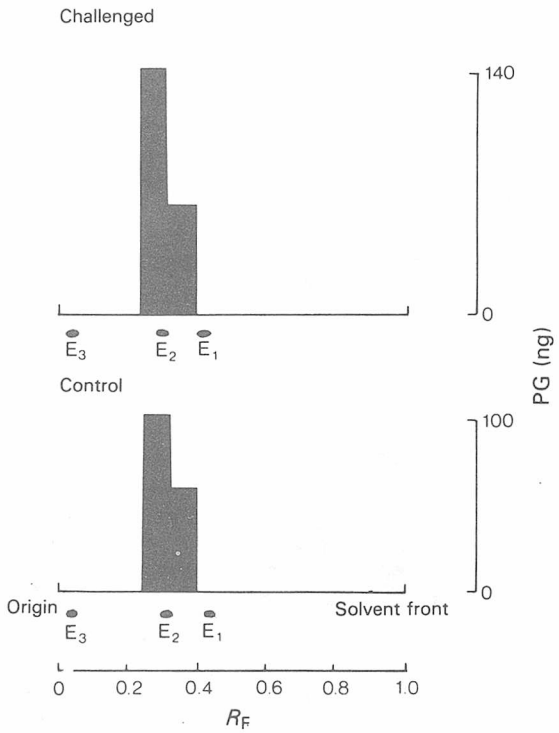

Figure 2 Argentation thin-layer chromatography of prostaglandin-like material from plaice skin using the solvent system ethyl acetate–ethanol–acetic acid (100:1:1). The 2 histograms represent migration of smooth muscle contracting material released from challenged and from non-challenged skin. The distance travelled by standard E_1, E_2 and E_3 is indicated under each chromatogram.

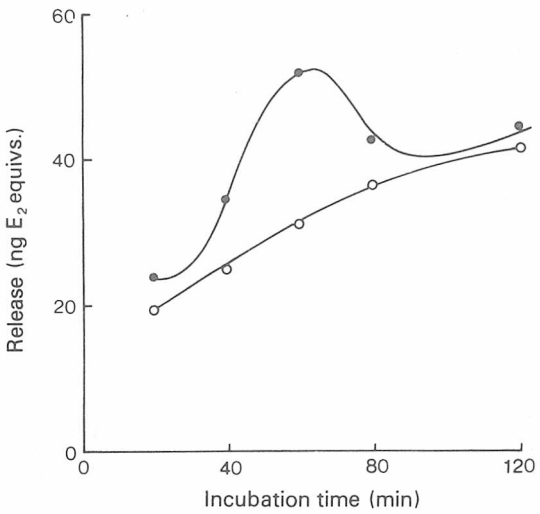

Figure 3 Time course of release of prostaglandin-like material from challenged (●) and from non-challenged plaice skin (○). Each point represents the mean of 6 observations of the amount of prostaglandin-like material released from plaice skin (1 g) expressed as ng PGE_2 equivalents.

E_2, consistent potencies were obtained on the three tissues. In contrast, the potency varied considerably when assayed as E_1, E_3, $F_{1\alpha}$ or $F_{2\alpha}$. The presence of E_2 in the challenged skin extract was confirmed by comparison with the mass spectra of a standard preparation of E_2. There was insufficient material present in the non-challenged skin extract to obtain a satisfactory spectrum.

Time course of release from place skin

Release of prostaglandin-like material from challenged and non-challenged skin was studied over a period of 120 min (Figure 3). Release of active material from challenged skin was significantly ($P < 0.05$) greater than that from non-challenged skin from 20 to 80 min incubation and was maximum (50 ng/g) after about 60 min.

Effect of indomethacin on prostaglandin release

The effect of indomethacin was studied on *in vitro*

Table 3 Fatty acid composition of plaice skin

Fatty acid	*% of total fatty acids*	
	Column 1 (EGSS-Y)	*Column 2 (EGSS-X)*
E_1 Precursor (20:3 ω6)	Trace	0.2
E_2 Precursor (20:4 ω6)	2.9	2.8
E_3 Precursor (20:5 ω3)	9.0	8.7
Others	88.1	88.3

The methyl esters were analysed by gas chromatography on two phases, 10% EGSS-Y and 10% EGSS-X (methods: fatty acid composition).

release of prostaglandin-like material from skin after 60 min incubation. Indomethacin (1 to 1000 μg/kg), given to plaice by intraperitoneal injection 2 h before killing, produced a 10 to 90% inhibition of release of active material from both challenged and non-challenged skin. While indomethacin maximally inhibited *in vitro* release of prostaglandin-like material in a dose of 1 mg/kg, even 50 mg/kg did not reduce the erythema induced by *E. floccosum in vivo*.

Fatty acid composition

The fatty acid composition of the skin of the plaice is summarized in Table 3. There was good agreement between the results of the two columns. The fatty acids which were major components were C16:0, C18:1 and C22:6, contributing about 60% of total. The precursor of prostaglandin E_2, namely C20:4 ω6 fatty acid was present in a concentration of 2.8%. The level of the C20:3 ω6 fatty acid was very low but the precursor of prostaglandin E_3, C20:5 ω3 contributed about 9% of total.

Discussion

This investigation has provided evidence that one active substance is released from plaice skin following challenge *in vitro* with extracts of fungus which will produce an immediate erythema in the skin of teleost fish (Fletcher & Baldo, 1974). Incubation media from challenged and from non-challenged skin were shown to contain prostaglandin E_2, identified by bioassay, thin-layer chromatography and ultraviolet absorption spectrometry. Unequivocal evidence for the release of prostaglandin E_2 from challenged skin was obtained by mass-spectrometric analysis.

Although prostaglandin E_2 was released from skin incubated with Ringer solution alone, the linear pattern of release suggests a simple leakage of prostaglandin out of skin as a result of a sudden synthesis stimulated by the removal and chopping process. The increased prostaglandin output from skin incubated in the presence of *E. floccosum* indicates a further synthesis of prostaglandin on challenge which, when superimposed on the simple basal release, leads to the observed more complex pattern of release. The fall in prostaglandin release after 60-min incubation is probably due to diminished synthesis, possibly accompanied by enzymatic degradation of released prostaglandin.

Work in progress using a skin perfusion technique similar to that employed by Greaves & Søndergaard (1970) indicates that acidic lipid activity is present in perfusates of plaice skin challenged intradermally with *E. floccosum*, whereas no activity has been observed in perfusates of non-challenged skin. *E. floccosum* therefore appears to stimulate prostaglandin synthesis.

Prostaglandins of the 1, 2 or 3 series are synthesized from the C_{20} unsaturated fatty acids, eicosatrienoic acid (20: 3 ω6), eicosatetraenoic acid (20:4 ω6) and eicosapentaenoic acid (20:5 ω3) respectively. In these experiments we were unable to detect prostaglandin E_1 or E_3. The absence of E_1 is probably due to the fact that there is a low concentration of 20:3 ω6 in plaice skin. It is interesting, however, that 20:5 ω3 is abundant in the skin. Using a particulate enzyme fraction from sheep vesicular glands, Struijk, Beerthuis & Van Dorp (1967) showed that whereas E_1 and E_2 are converted at a high rate from their respective precursor fatty acids, E_3 is formed at a much lower rate from 20:5 ω3. It would be worthwhile, therefore, to study the comparative aspects of prostaglandin biosynthesis in more detail, preferably using an enzyme fraction of plaice skin.

Prostaglandins are synthesized or released by almost all mammalian tissues studied. In lower animals, Christ & Van Dorp (1972) demonstrated prostaglandin biosynthesis from tissue homogenates of a number of fish and Ogata & Nomura (1975) identified prostaglandin E_2 from the gastrointestinal tract of the shark. Hall, O'Regan & Quigley (1977) have indicated that prostaglandins could have an important role in regulating osmolal changes in frog skin; indeed their ubiquitous occurrence has led to the suggestion that prostaglandins may be involved in the regulation of fundamental biological processes (Christ & Van Dorp, 1972). In humans, the intradermal injection of prostaglandin E_1 causes increased vascular permeability and erythema (Søndergaard & Greaves, 1971; Crunkhorn & Willis, 1971a, b) and increased concentrations of prostaglandins have been found in skin inflamed as a result of various injuries (Anggard, Arthurson & Jonsson, 1970; Greaves, Søndergaard & McDonald-Gibson, 1971; Søndergaard, Greaves & Jorgenson, 1974). However, since prostaglandins seem to be synthesized whenever cell membranes are distorted (Gilmore, Vane & Wyllie, 1969), it is difficult to ascertain whether they are formed primarily to mediate the inflammation or as a result of tissue injury caused by other mediators.

Challenge of plaice skin *in vitro* with fungal extracts caused an increased release of prostaglandin E_2 in the incubation mixtures, whereas in preliminary experiments a similar challenge of flounder skin did not lead to an increased release. This correlates well with the observation that intradermal injection of fungal extracts caused an immediate erythema in the skin of plaice but not of flounders and suggests that prostaglandins may therefore have some role in the mediation of the skin reaction. However, the inability of indomethacin, which inhibited prostaglandin synthesis in plaice skin *in vitro*, to suppress the erythema

induced by fungal extracts *in vivo* suggests that prostaglandins may not be the only mediators of the reaction.

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