A STUDY OF THE RABBIT EYE TEST SYSTEM TO DETERMINE THE ACTIVITY OF ACIDIC NON-STEROIDAL ANTI-INFLAMMATORY AGENTS

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A STUDY OF THE RABBIT EYE TEST SYSTEM TO DETERMINE THE ACTIVITY OF ACIDIC NON-STEROIDAL ANTI-INFLAMMATORY AGENTS

INTRODUCTION:

"Inflammation *per se*, has been defined sufficiently to permit a rational approach to the search for drugs that modify this process, but satisfactory animal models for most rheumatoid diseases are not available". (Swingle 1974)

In the search for new meaningful procedures for the detection and evaluation of anti-inflammatory drugs, the rabbit eye as a test system was studied.

In designing a laboratory test system to detect new acidic non-steroidal anti-inflammatory (ANSAI) compounds, it is necessary to define which component in the inflammatory process is to be suppressed. This is necessary as there does not appear to be a non-steroidal anti-inflammatory agent capable of suppressing all the components of both the acute and chronic inflammatory responses. (Spector & Willoughby 1968).

In the rabbit-eye-test system, the exudation phase is the event in the inflammatory process which is modified, while in the rat paw oedema method of assay, the inhibition of swelling (oedema) is the parameter measured.

REASON FOR CHOOSING THE RABBIT EYE AS A TEST SYSTEM:

It is known that prostaglandin-like substances are present in ocular tissues (Ambache & Brummer 1968) and low

concentrations thereof administered either into the anterior chamber or onto the cornea cause changes to occur, that are associated with ocular inflammation. (Beitch & Eakins 1969), (Eakins 1970), (Kelly & Starr 1971), (Starr 1971), (Bethel & Eakins 1972)

This observation led to the suggestion that substances which inhibit the action or synthesis of prostaglandins might be of value in counteracting ocular inflammation. (Eakins et al., 1972). It was shown by Bhattacherjee & Eakins (1974) that indomethacin produces an in vitro inhibition of the prostaglandin synthetase system in ocular tissues. Later, Bhattacherjee (1975) in an in vivo study, demonstrated the suppressive effects of indomethacin on the inflammatory response in the rabbit eye brought about by Shigella endotoxin. Arachidonic acid, the precursor of prostaglandins, was subsequently used by Bhattacherjee & Eakins (1975) to produce an inflammatory response in the rabbit eye. This inflammatory response was shown to be inhibited by pretreatment with non-steroidal anti-inflammatory compounds.

Straughan (1975), using arachidonic acid to induce the inflammatory response, tested the anti-inflammatory effects of a number of ANSAI compounds in this manner.

It is the purpose of the present investigation to examine the possibility of using the rabbit eye test system for the detection and evaluation of acidic non-steroidal anti-inflammatory agents, by comparing it with the classical rat-paw method of assay (Winter Risley and Nuss 1962).

CHAPTER 1

1.1 THE INFLAMMATORY REACTION

Acute inflammation has been regarded as a mechanism serving to protect the individual from a wide range of hazards. However, it is sometimes difficult to accept the concept of crippling inflammatory diseases as being part of such life-preserving mechanisms.

1.2 DESCRIPTION AND DEFINITION

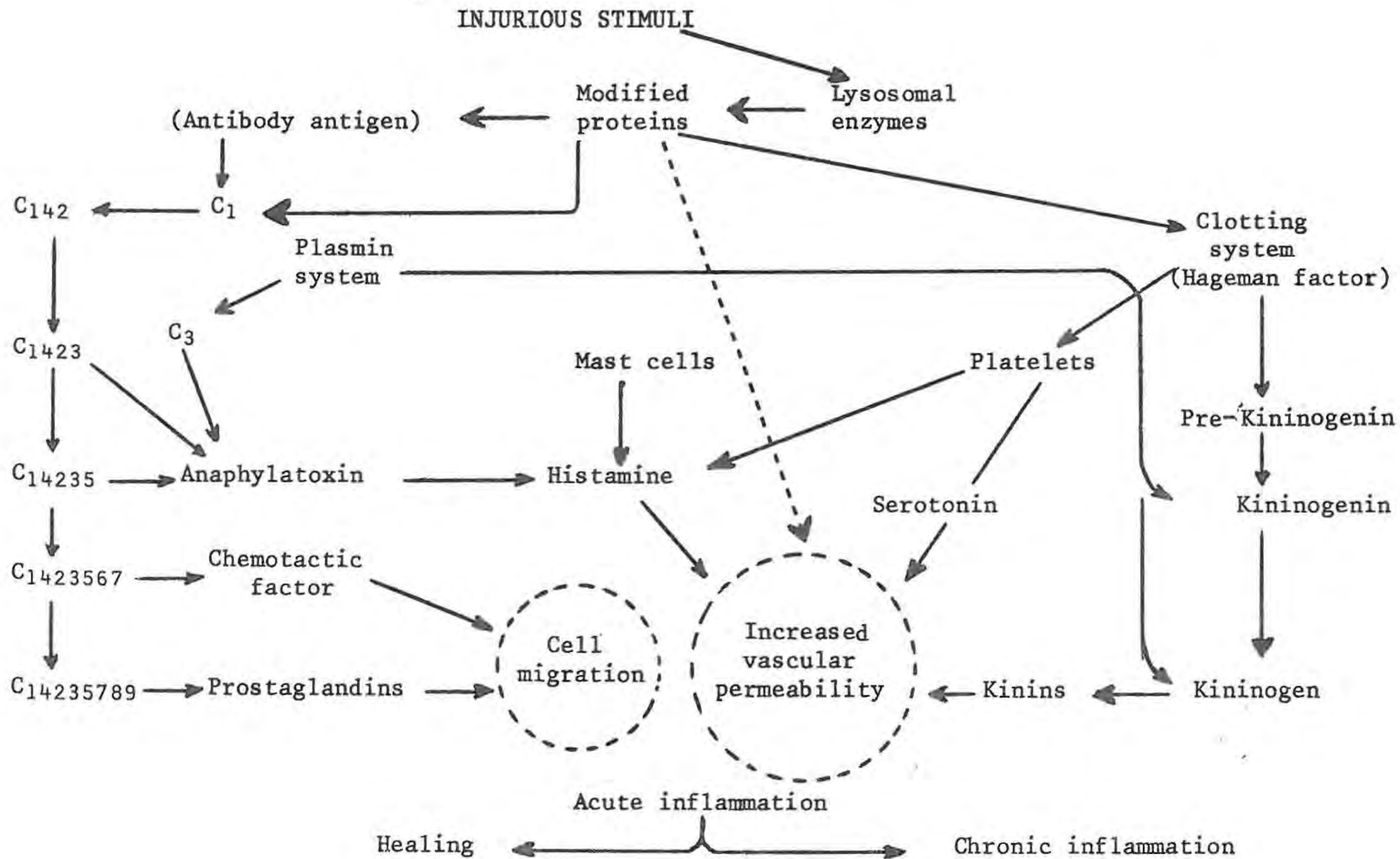
The inflammatory process is considered by Hicks (1975) to be a protective function, designed to minimise or eliminate the cause of damage and, subsequently, to remove damaged tissue and lead to its repair.

Walter and Israel (1974) proposed the following definition: "Inflammation is the reaction of the vascular and supporting elements of a tissue to injury, and results in the formation of a protein-rich exudate, provided the injury has not been so severe as to destroy the area".

1.3 CARDINAL SIGNS

The five cardinal signs of acute inflammation are: heat (calor); redness (rubor); pain (dolor); swelling (tumour); and loss of function. These signs and symptoms reveal changes in the vascular supply to the area and are brought about by a number of local hormonal mediators.

FIG.1. OPERATIONAL SCHEME OF THE MULTI-MEDIATED INFLAMMATORY REACTION



(Rocha e Silva and Garcia and Leme 1972)

Walter and Israel subdivide the acute inflammatory reaction as follows:

1.3.1 THE VASCULAR RESPONSE

The changes that take place in blood vessels are the most obvious signs of acute inflammation.

(i) Changes in the calibre of blood vessels

There is an initial constriction in response to trauma, causing blanching of the area, followed by a persistent vasodilation. The vasodilation results in an increased blood flow to the affected area, and a marked increase in the redness ("rubor") of the area occurs. This rapid spread of erythema is called a "flare". The "calor", or heat, associated with inflammation is seen in parts near the body surface - skin, face, ears and fingers. It is not a feature of internal inflammation. Cohnheim (1889).

(ii) Changes in the vessel wall and flow of blood

The arteriolar dilation causes an increase in blood velocity followed by a slowing of the stream and finally stasis. The main factor in the production of stasis is that the increase in the vascular permeability causes fluid to be lost from the blood which in turn becomes more viscid. In addition, the cross-section in the effective vascular bed increases hence the pressure decreases and leucocyte adherence takes place, a phenomenon which is highly characteristic of damage to blood vessels. The leucocytes line the endothelium, the phenomenon being known as margination of the white cells or pavementing of the endothelium. The endothelium itself undergoes change and a swelling of the endothelium cells can be seen.

1.3.2 EXUDATION AND SWELLING

(i) The fluid exudate

Acute inflammation is characterised by the formation of an exudate. There are possibly four mechanisms involved in the production of the fluid exudate:

(a) Increased vascular permeability to proteins;
(b) Increased capillary blood pressure;
(c) A breakdown of large-molecule tissue proteins;
(d) Increased fluidity of the tissue ground substance.

The increased vascular permeability is considered to be the crucial factor in the formation of the inflammatory exudate.

The result is a substantial exudation of plasma-like fluid into the surrounding tissue interstices which produces swelling and oedema. As the exudate fluid accumulates, pressure within the tissue in= creases and may reduce the rate of blood flow resulting in cyanosis of the inflamed area. Sevitt (1958) and Miles (1966) state that, in experimentally produced acute inflammation, the exudation of fluid occurs in two phases. Vinegar et al (1969) consider carrageenan-induced rat paw oedema to be a biphasic event. (See Section 2,4,3 page 19 Volume/Time Curve).

(ii) The Cellular Exudate

The increased permeability of the vascular wall results not only in proteins passing through, but also in a migration of leucocytes into the damaged area. In the first stage, mostly polymorphonuclear leucocytes migrate (neutrophils). Later monocytes migrate in increasing numbers into the area of inflammation, become phagocytic and are called macrophages. The polymorphonuclear cells provide enzymes for the destruction of cellular material. Higgs and Youlten (1972) have shown that rabbit polymorphonuclear leucocytes produce a Prosta= glandin E-like material thought to be prstaglandin E_1.

Lymphocytes are considered not to be generally involved in acute inflammation.

1.3.3 CHEMOTAXIS

Hicks has pointed out that in addition to vasodilation and exudation, inflammation creates conditions that are conducive to a migration of leucocytes and tissue macrophages into the damaged area. Leucotaxine, a mixture of kinins, has been associated with chemotaxis. Also the components of the complement system have chemotactic properties leading to cellular migration into the inflamed area.

1.3.4 THE CHEMICAL MEDIATORS OF INFLAMMATION

The first agents at the site of a local injury are local hormones, which are formed or released *in situ*. Whitehouse (1974) divides the chemical mediators of inflammation into two groups:

(i) The acids: prostaglandins, heparin, the adenylates (cyclic AMP, ADP) and lysolecithins.

(ii) The bases: histamine, serotonin, the kinins, the catecholamines.

Walter and Israel listed eight groups of substances which act as chemical mediators:

(i) Amines
   - (a) histamine
   - (b) 5-hydroxytryptamine (5-HT)

(ii) The kinins

(iii) Kinin-forming enzymes
   - (a) kallikrein
   - (b) the globulin permeability factor
   - (c) plasmin

(iv) Biologically active products of the complement system.

(v) Biologically active components of polymorpho= nuclear cells.

(vi) Prostaglandins.

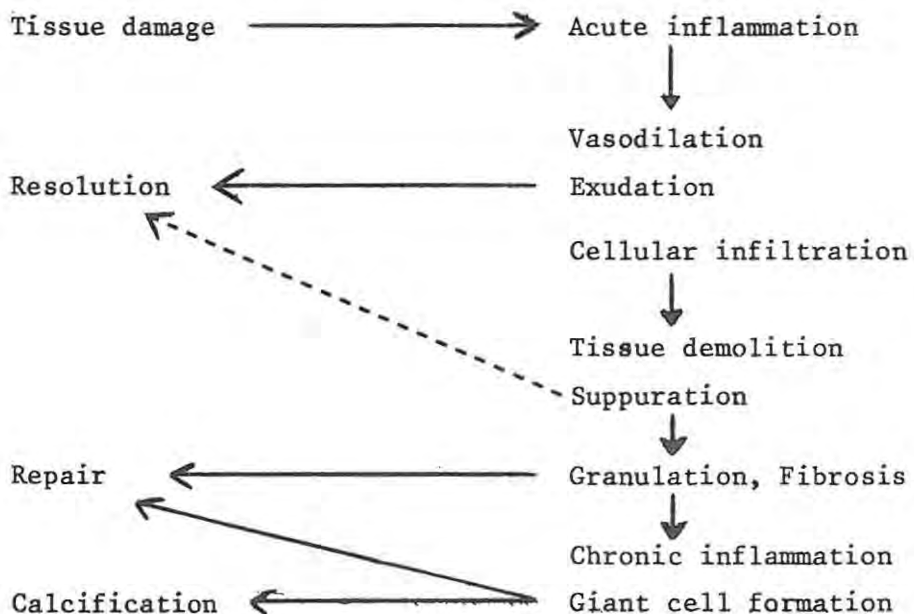
(vii) Nucleic acid and breakdown products.

(viii) Others, as yet unknown.

These mediators appear to serve primarily as "alert mechanisms" rather than functioning as true effectors of tissue injury. Their effect is transitory, either because of rapid dilution, removal in the lymph or inactivation by enzyme systems.

If, at any stage, the cause of inflammation is eliminated and no severe tissue damage is present, the tissue reverts rapidly to its previous normal state. If severe damage exists, repair mechanisms come into play.

1.3.5 STAGES IN THE DEVELOPMENT OF INFLAMMATION



From: Hicks, R., "Inflammation and Repair in Damaged Tissue" Brit. Pharmacy Journal September 6, 1975.

1.3.6 INTRA-OCULAR INFLAMMATION

The uveal tract, consisting of iris, ciliary body and choroid, forms the vascular and muscular coat of the eye and as such bears the burden of all intra-ocular inflammation. Any of its three components may be affected and the neighbouring tissues are always involved in the inflammatory response and seldom escape the effect of the damage that results. (Trevor-Roper 1962). The characteristic changes known to occur when the rabbit eye responds to irritation, namely miosis, vasodilation, an increase in capillary permeability and a sustained rise in I.O.P., are thought to result from the release of prostaglandin E_2 and prostaglandin $F_{2}\alpha$, into the aqueous humour. Ambache et al (1965)

The sequence of events in uveitis is thought to be: (Higgs and Youlten, 1972).

(i) release of prostaglandins into aqueous humour from the iris,

(ii) invasion of leucocytes, in part resulting from the leucotactic effects of the prostaglandins present in the aqueous humour,

(iii) further prostaglandin release from the leucocytes.

Injection of prostaglandin into rabbit eyes has been shown by Waitzman and King (1967) to produce a sustained rise in intra-ocular pressure. There is also a significant increase in the protein content of aqueous humour indicating an increase in the permeability of the blood-aqueous barrier. Anatomically, the blood-aqueous barrier consists of: (Trevor-Roper)

(i) the anterior iris surface;

(ii) the ciliary epithelium;

(iii) the retina overlying the anterior choroidal vessels;

(iv) the capillary walls of the retinal vessels in the posterior half of the retina.

The increase in the protein content of aqueous humour and the congested appearance of blood vessels in the region of the iris suggested to Waitzman and King that both vasodilation and increased capillary permeability play a significant role in the response of the I.O.P. to prostaglandins in the rabbit eye.

The similarities between the observed effects of the prostaglandin in the eye and those produced by irritation and trauma are clear, showing that prostaglandins can mimic the effects of acute ocular irritation. (Eakins, 1974) The prostaglandins bring about local vasodilation and increased permeability resulting in exudation of fluid into the aqueous compartment of the eye from the intra-ocular blood vessels. This breakdown in the blood-aqueous barrier results in a sustained rise in intra-ocular pressure. (Beitch & Eakins 1969).

1.3.7 INTRA-OCULAR PRESSURE

Normal I.O.P. is an expression of the balance between the inflow and outflow of aqueous humour and any sustained alteration in I.O.P. reflects a change in one or both of these parameters. The aqueous humour is driven out of the anterior chamber largely by hydrostatic pressure, and its principal outlet being through the spongy mass of trabecular tissue in the angle of the anterior chamber and into the circular canal of Schlemm.

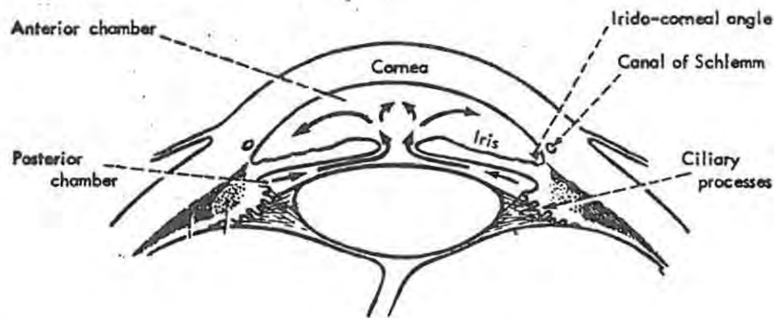


FIG.2. Structures involved in the formation and drainage of aqueous humour. The arrows indicate the circulatory pathway taken by the aqueous humour.

In animals other than primates, there is no canal of Schlemm (although aqueous veins are sometimes found) and the aqueous humour drains directly into a diffuse meshwork of veins in the ciliary plexus and thence through the sclera into the episcleral plexis (Trevor-Roper 1962).

1.3.8 PHYSIOLOGICAL VARIATIONS IN I.O.P.

Variations can occur throughout the day depending on factors such as:

(i) changes in secretory activity of the ciliary body;

(ii) respiration and arterial pulse. Pressures may fluctuate over a range of 5 mm Hg in the course of one inhalation and 1-2 mm Hg with each heart-beat;

(iii) the time of day - on waking in the morning, the I.O.P. can be 3-4 mm Hg greater than at 6 p.m. in the evening;

(iv) dilation of the pupil - This may cause an increase in I.O.P. (Trevor-Roper 1962).

The above possible physiological variations were effectively countered in the experimental work in the following manner:

(i) a number of I.O.P. readings were taken on each rabbit and a reproducible reading established to ensure that respiration and the arterial pulse were not causing a fluctuation of pressure;

(ii) the time of day used for experimental work was standardised, i.e. the experiments were performed in the morning;

(iii) the rabbits were anaesthetized before the I.O.P. readings were taken.

CHAPTER 2

2.1 REVIEW OF METHODS FOR THE DETECTION AND EVALUATION OF NEW ACIDIC NON-STEROIDAL ANTI-INFLAMMATORY AGENTS

In seeking to evaluate the worth of the rabbit eye as a test system, it was necessary first to review existing methods of evaluation. Methods of assay which have achieved popularity have aspects in common:

(i) they are basically simple to perform;

(ii) they are economical;

(iii) they can be used to select drugs known to afford some benefit in the clinical management of rheumatoid disease (Swingle 1974).

2.2 SCREENING PROCEDURES

Screening procedures can be grouped into four categories of assays according to the criterion for activity.

2.2.1 METHOD (1)

INTERFERENCE with the manifestation of one of the cardinal signs of inflammation, e.g.

(i) Inhibition of swelling. (This parameter is used in the classical rat-paw-oedema method of assay).

(ii) Inhibition of heat.

(iii) Inhibition of redness.

(iv) Inhibition of pain.

(v) Inhibition of loss of function.

2.2.2 METHOD (2)

MODIFICATION of one of the events occurring during the inflammatory process.

(i) Inhibition of inflammatory exudation. (This parameter is used in the rabbit-eye test system).

(ii) Inhibition of inflammatory isolation. (The isolation of an acutely inflamed area is aided by the precipitation of fibrin which obstructs lymphatic and microvascular flow. Early isolation of the area is thought to be desir= able because it allows time for phagocytic cells to assemble at the site of inflammation). (Menkin 1940).

(iii) Inhibition of inflammatory granulation.

2.2.3 METHOD (3)

POSSESSION of a property that has been associated with a class of drugs known to be anti-inflammatory. The property may be used as a basis for detecting a class of anti-inflammatory compounds. Many of these assay methods for the acidic non-steroidal anti-inflammatory drugs are conducted in vitro and rely on some type of interaction of the drug with protein.

(i) Properties of non-steroidal anti-inflammatory drugs. They are:

(a) the uncoupling of oxidative phosphorylation;
(b) the protection of protein in solution from being denatured by heat;
(c) the stabilization of erythrocytes and inhibition of heat-induced haemolysis of canine erythrocytes;

(d) the acceleration of the sulphydryl exchange reaction;

(e) the stabilization of lysosomes (reports on this action of non-steroidal anti-inflammatory drugs have been conflicting (Hichens 1974);

(f) the promotion of dissolution of fibrin clots;

(g) the inhibition of platelet aggregation;

(h) the promotion of excretion of uric acid.

(ii) Properties of steroidal anti-inflammatory drugs. They are:

(a) the ability to cause a reduction in weight of the lymphoid tissues of rodents;

(b) the ability to inhibit replication of cells derived from connective tissue using cell culture techniques.

2.2.4 METHOD (4)

MODIFICATION of syndromes purported to be animal systems used as models for human rheumatoid diseases.

(i) Modification of experimental arthritis;

(ii) Modification of experimental gout;

(iii) Modification of spontaneous disease.

2.3 AN "IDEAL" SCREENING PROGRAMME

Even though drugs such as aspirin, cortisone and phenylbutazone and gold preparations were discovered without the use of a screening programme, successful programmes have been designed.

2.3.1 ACUTE INFLAMMATORY MODELS

(i) Rat-paw oedema method;

(ii) Ultraviolet-light-induced erythema of guinea-pig skin. This represents a second model of acute inflammation in a second species.

2.3.2 CHRONIC-INFLAMMATORY MODELS

(i) Evaluation of the effect of the compound on a chronic inflammatory condition, e.g. adjuvant-induced arthritis of the rat;

(ii) Effect of the compound on the proliferative phase of an inflammatory response, e.g. the cotton-pellet-induced granuloma in rats.

2.3.3 ASSESSMENT OF RELEVANT ANCILLARY ACTIVITIES

An assessment of the analgesic, antipyretic and uricosuric ability of the compound is made.

2.3.4 ESSENTIAL FEATURES OF A TEST SYSTEM

Whitehouse (1974) suggested the following as being essential features of a test system.

(i) It should be relevant to human disease;

(ii) It should be adequate for the type of compound to be tested;

(iii) It should permit clear interpretation;

(iv) It should avoid biological complication, i.e. avoid the need to use animals with an unusual pattern of drug metabolism;

(v) It should be practical, i.e.

(a) require modest skills and facilities,
(b) not need prolonged induction time (with reference to studies involving disabling joint conditions),
(c) allow economical use of the compound being evaluated.

2.4 THE TWO ASSAY PROCEDURES USED IN THIS STUDY

2.4.1 THE RAT-PAW-OEDEMA METHOD

Swingle (1974) has stated that probably the best single method to evaluate a new drug substance for anti-inflammatory activity is the rat-paw-oedema method of assay. Di-Rosa & Willoughby (1971) suggest that, because the standard non-steroidal drugs inhibit leucocyte emigration and the "prostaglandin phase" of oedema induced by carrageenan injection, this assay can be successfully used to search for effective anti-inflammatory compounds. This classical method of assay, to which the discovery of indomethacin is attributed, was therefore chosen for comparison with the rabbit-eye test system.

2.4.2 METHODOLOGY

The assay as originally described by Winter, Risley and Nuss (1962), and as conducted in this study, was carried out as follows:

(i) the drug to be administered was injected intraperitoneally at -60 minutes into Wistar rats of either sex;

(ii) at zero time, 0,05 cm^3 of a 1% suspension of carrageenan was injected into the plantar tissue of one hind paw of the Wistar rat, and the size of the paw determined at this time;

(iii) at hourly intervals for the next three hours after the injection of carrageenan suspension, the magnitude of swelling was determined by the method of volume displacement of the hind paw.

2.4.3 RESULTS

The findings were expressed as % inhibition of the oedema.

$$\% \text{ inhibition} = 100\left[1 - \frac{Vt}{Vc}\right]$$

where Vt and Vc are increases in the volume of the carrageenan-injected paws of the drug-treated and control groups respectively. Winder *et al* (1957) have pointed out that there is no apparent right or left "footedness" in rats and that paw volume (non-oedematous) to body-weight ratio is constant among rats. Arrigoni-Martelli & Conti (1964) found that the strain, the sex and the body weight of the rats are unimportant variables in the assay. Vinegar *et al* (1969) showed that it is the second phase of the oedematous response, occurring after injection of carrageenan, that is sensitive to most anti-inflammatory drugs. This second-phase inhibition by ANSAI compounds was also noticed by this investigator and was the phase used to measure the percentage inhibition of oedema occurring. The existence of two phases of swelling probably explains the failure of standard anti-

inflammatory drugs to effect complete inhibition of oedema.

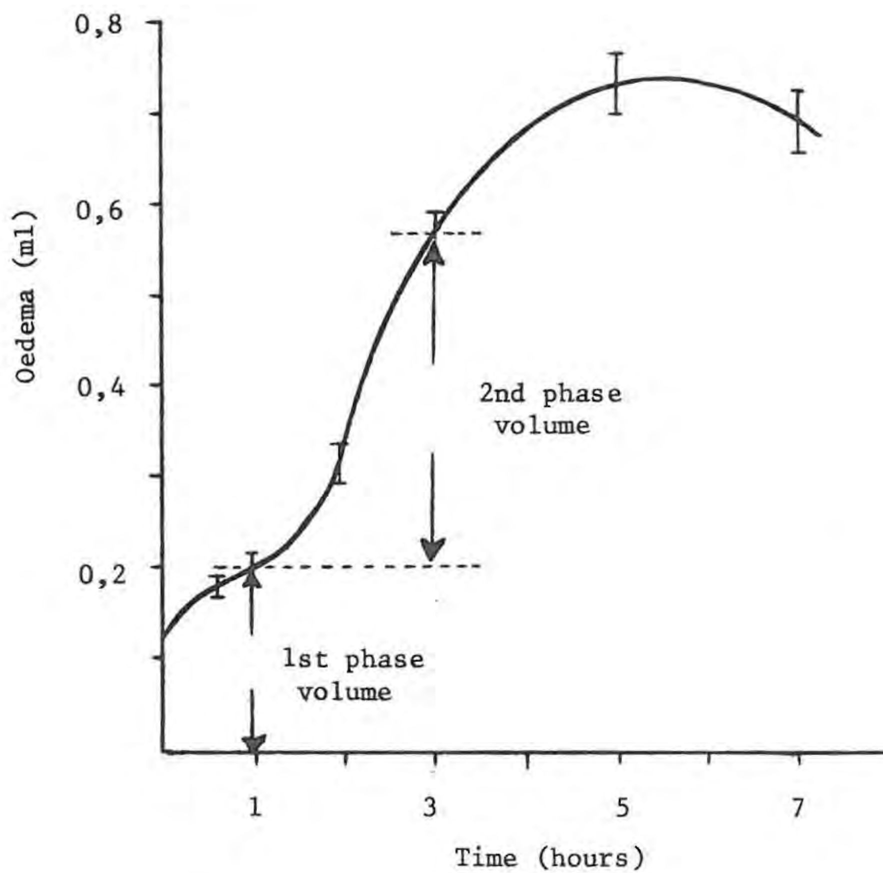


FIG.3. Volume-time curve of developing oedema in rats injected in the right footpad with 0,50 mg of carrageenan (Vinegar et al 1969)

2.5 DISADVANTAGES OF THE RAT-PAW METHOD OF OEDEMA

(i) Spector & Willoughby (1968) have pointed out that the rat hind paw method suffers from certain disadvantages as a test system due to the excessive concentration of serotonin which may be released and give an erroneous impression of the importance of these amines in inflammation.

(ii) It has been shown by Doherty & Robinson (1975) that the early phase of the reaction resulting from an injection of carrageenan is due to the trauma of the injection process, since it is seen also after injection of the saline alone. The early phase of the reaction is independent of the quantity of carrageenan injected and is not seen following intrapleural injection.

2.6 THE RABBIT-EYE-TEST SYSTEM AS A METHOD OF ASSAY

It is well established that certain chemical and mechanical injuries to the blood-aqueous barrier are frequently followed by the formation of a protein rich aqueous humour and an increase in intra-ocular pressure. It seems likely that, in both cases, the rate of aqueous formation is increased and that capillaries of the anterior uvea and of the ciliary epithelium become more permeable to protein.

The inhibition of the acute inflammatory response in the eye of the rabbit, by ANSAI compounds caused by instillation of the prostaglandin precursor, arachidonic acid (sodium salt), was regarded as being the result of a modification of ONE of the events occurring during the inflammatory process.

2.6.1 METHOD

The method consisted of:

(i) measuring the resting I.O.P. of anaesthetized albino rabbits, one eye of the rabbit being used;

(ii) instilling sodium arachidonate, which, when converted to PGE_2, produces vasodilation, increased vascular permeability and cellular migration;

(iii) measuring the I.O.P. at 15, 30 and 60 minutes after the instillation of sodium arachidonate;

(iv) aspirating the eye three hours after inflammation was induced, approximately 0,2 cm^3 being aspirated;

(v) determining the protein content in the aspirated aqueous humour of the drug treated rabbits and the control rabbits.

2.6.2 RESULTS

The findings were expressed as % inhibition in the elevation of I.O.P.

$$\% \text{ inhibition of I.O.P.} = 100 \left[1 - \frac{Pt}{Pc}\right]$$

where Pt and Pc are increases in the I.O.P. of the drug treated and control groups of rabbits respectively.

2.6.3 AQUEOUS PROTEIN

A relative comparison was made of the concentration of protein present in the aqueous humour of the test

rabbits and that of the protein concentration in the aqueous humour of the control rabbits.

The effectiveness of the ANSAI compound evaluated in this test system is directly proportional to the ability of the compound to inhibit elevation of both the intra-ocular pressure and the aqueous humour protein content.

2.7 SUMMARY

Current methods of evaluating non-steroidal anti-inflammatory drugs have been scrutinised and four categories of assays presented. An "ideal" screening programme, as suggested by Swingle, has been presented.

The assay procedures used in this study, viz. the rat-paw-oedema method and the rabbit eye method have been described.

CHAPTER 3

3.1 INFLAMMATORY AGENTS

3.2 CARRAGEENAN AS AN INFLAMMAGEN

Carrageenan is a mixture of polysaccharides composed of sulphated galactose units. It is derived from Irish sea moss. (*Chondrus crispus*). (Smith *et al* 1955). Its use as an oedemogen was introduced by Winter *et al* (1962) who, after testing a variety of oedema-producing materials, concluded that it was the phlogistic agent of choice for testing anti-inflammatory drugs.

Not all samples of carrageenan are equally effective in eliciting an inflammatory response. Effective preparations contain λ-type galactan (McCandless, 1962) (McCandless *et al* 1964). A certain structural integrity of the polysaccharide is essential for oedemogenic activity and a degree of heat denaturation sufficient to cause the release of esterified sulphuric acid from the substance, results in a partial loss of activity. (Vinegar *et al* 1969).

It would appear that there are distinct phases in the development of carrageenan-induced oedema.

The initial phase occurring during the first hour is due to the release of histamine and 5-hydroxytryptamine. Thereafter, an increased vascular permeability is maintained up to 2½ hours by kinin release. During the following 3½ hours the mediator appears to be one of the prostaglandins, which gives rise to a phase of accelerated swelling. This prostaglandin-mediated

phase appears to be closely associated with a migration of leucocytes into the inflamed area. (Di-Rosa & Willoughby 1971). All the mediators involved appear to be dependent upon an intact complement system for activation and release. (Giroud & Willoughby 1970). Carrageenan activates plasmin (Serafini, Cessi & Cessi 1963) as well as the Hageman factor. (Schwartz & Keller=meyer 1969). In addition, carrageenan is also able to release a kinin-like substance from plasma heated at 56°C for three hours. (Di-Rosa & Sorrentino 1968).

The apparent importance of leucocyte exudation for a full development of an oedema induced by carrageenan was recognized by Vinegar _et al_ (1971) and van Arman _et al_ (1971).

These investigators were able to correlate with the amount of oedema present, either the number of PMN's that emigrated into the carrageenan injected paw or the peripheral-leucocyte count. Di Rosa & Willoughby have suggested that an inter-relationship exists between leucocyte emigration and prostaglandin activation in the latter phase of carrageenan-induced oedema and postulate that the predictive value of the assay resides in the involvement of leucocytes in the response. Swingle (1974) states that both mononuclear and polymorphonuclear cells are involved in the acute-inflammatory response to the injection of carrageenan.

Garrattini _et al_ (1965) compared a number of oedemas of the rat paw and concluded that the response induced by carrageenan was least affected by non-specific

influences, viz., ambient temperature, humidity, diuresis, drug-induced hypothermia or adrenalectomy. Swingle supports the view that the carrageenan test has proved to be a reliable method for detecting anti-inflammatory substances while Wiseman (1974) points out that the potency ratios determined in the carrageenan-induced rat-paw-oedema test, beats rank order correlation to clinical doses of the anti-inflammatory drugs examined.

3.3 ARACHIDONIC ACID AS AN INFLAMMAGEN

To induce an inflammatory response in the eye of the rabbit, a one-percent solution of arachidonic acid, in the sodium salt, was used. Arachidonic acid is the most abundant precursor fatty acid of prosta= glandins found in the body of mammals giving rise to prostaglandin E_2 (PGE_2), and prostaglandin $F_2\alpha$ (PGF_2).

3.3.1 HISTORY

The name "prostaglandin" was introduced by von Euler in 1935 to describe vasodepressor-smooth-muscle-stimulating substances in human semen and in semen and vesicular glands of animals. The belief at the time that these substances were secreted by the prostate gave rise to the name of "prostaglandin". It serves as the generic name for a family of closely related lipids whose basic structure, prostanoic acid, was first hypothesized by Bergström et al (1963). More than a dozen compounds have been recognized as members of the principal classes of prostaglandins. The principal, naturally occurring prostaglandins are classified according to the functional groups attached to the five-membered ring of prostanoic acid.

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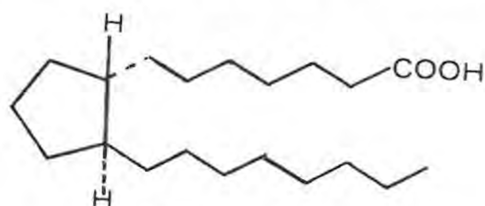
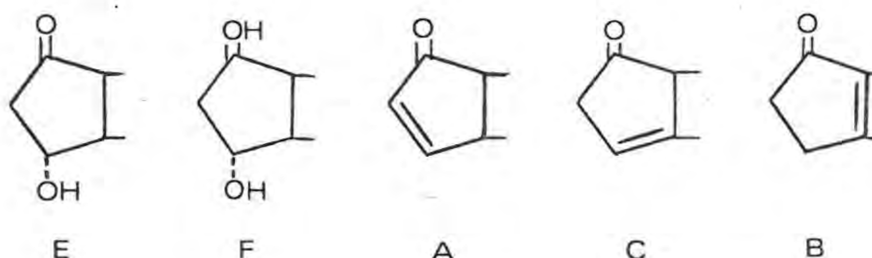


Prostanoic acid

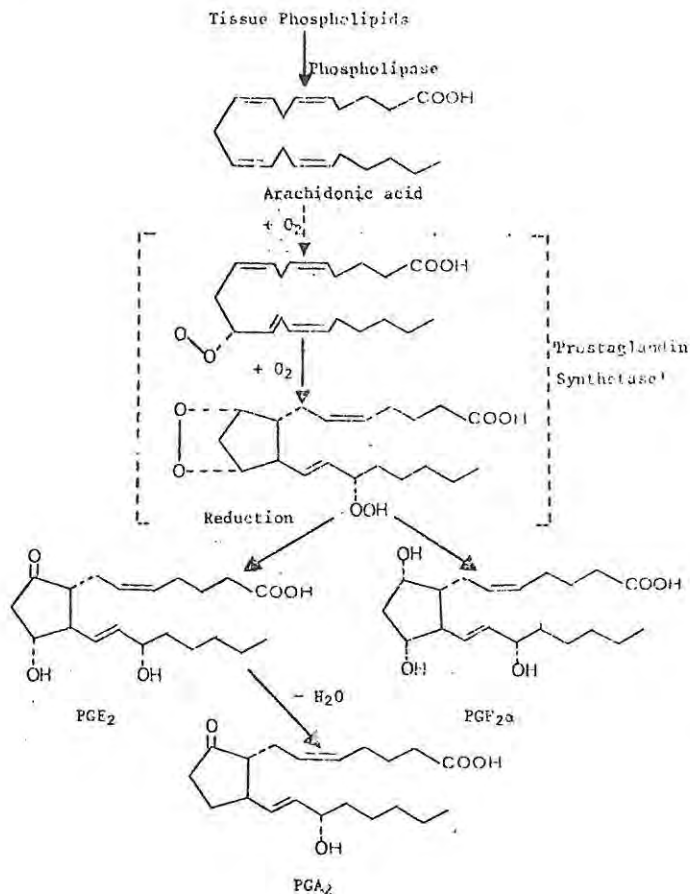


The letter designations E and F are given to prosta= glandins possessing a ketonic group and an hydroxyl group at C-9, respectively. Both prostaglandin E (PGE) and prostaglandin F (PGF) possess a hydroxyl group at C-11 as well. For biosynthetic reasons the E and F prostaglandins are considered to be "parent" prostaglandins. Numerical subscripts, e.g. PGE_1, indicate the number of double bonds con= tained in the aliphatic chain segment of the prosta= glandins. The "α" and "β" subscripts for the F-prostaglandins designate whether the hydroxyl group at C-9 is on the same side of the molecule as the aliphatic side chain containing the carboxylic acid group, or if it is on the opposite side. The naturally occurring F prostaglandins all have the "α" configuration, e.g. $PGF_2\alpha$.

3.3.2 BIOSYNTHESIS

Prostaglandins are biosynthesized from poly-unsaturated essential fatty acids by a series of complex reactions. The current understanding of the details of prostaglandin synthesis is based on work done by Klenberg and Samuelsson (1965), and Hamberg and Samuelsson (1967).
The basic substrate requirements for the enzyme system of prostaglandin synthesis are 20-carbon fatty acids with multiple sites of unsaturation, molecular oxygen, and co-factors that can act as reducing agents.

FIG. 4 SUMMARY OF THE BIOSYNTHETIC PATHWAY OF PROSTAGLANDINS



Evidence for this pathway was recently presented by Hamberg & Samuelson (1973) by the isolation of an internal hydroperoxide of arachidonic acid. Phospholi= pase activity is believed to be a rate-limiting factor in the control of prostaglandin formation.

3.3.3 MECHANISM OF ACTION OF PROSTAGLANDINS

Prostaglandins are present in virtually all tissues of the body and they have been shown to produce a wide variety of responses. They influence intracellular responses to many hormones, local blood flow and ion movements across membranes and thus, as Russell et al (1975) points out, it is difficult to attribute their actions to one mechanism.

General support is given to the concept that E & F prostaglandins exert their affects by acting locally within a tissue or organ after an appropriate stimulus to that organ. It is noted that in the inflammatory response in the carrageenan-injected rat paw, the activation and release of mediators involved, including prostaglandin, appear to be dependent upon the complement system to trigger it off. Prostaglandins act intra= cellularly and Steinberg et al (1964) and Kuehl (1974) have studied the interaction of prostaglandins with the cyclic-AMP system, and shown that there is a possible relationship between the two, as PGE_1 was shown to block the action of lipolytic agents (which act by increasing concentrations of cyclic-AMP) in fat cells.

Another mechanism of action involves the autonomic nervous system. Autonomic-nerve stimulation releases endogenous prostaglandins in amounts sufficient to exert an inhibitory

action on transmitter release at nerve endings. (Russell et al) The observation that prostaglandin biosynthesis can be virtually abolished by drugs, such as aspirin or indomethacin in non-lethal doses, might suggest that, whatever the role of prostaglandins, their formation cannot be essential for the continued existence of the organism.

3.3.4 THE ROLE OF PROSTAGLANDINS IN OCULAR INFLAMMATION

There is evidence to suggest that prostaglandins are involved in acute ocular inflammation. (Ambache et al 1965) (Christ & van Dorp 1972). Eakins (1974) has shown similarities between the observed effects of prostaglandins on the eye, and those produced by irritation and trauma, indicating that prostaglandins can reproduce the effects of acute ocular irritation.

The use of the prostaglandin precursor arachidonic acid, resulted in an ocular response in the rabbit which can most likely be attributed to the presence of prosta= glandin in the eye. Bhattachejee & Eakins (1975) found that PG-like activity (16 ± 7 $\mu g/cm^3$) could be detected in the aqueous humour, thirty minutes after instillation of sodium arachidonate. They also point out that, although elevated levels of prostaglandin-like activity were found in the aqueous humour following topical application of sodium arachidonate, it was not clear whether the conversion occurred externally in the conjunctiva, with the resulting prostaglandins passing into the aqueous, or internally in the iris/ciliary body, since both of these tissues are able to convert arachidonic acid to prostaglandins.

Beitch & Eakins (1969) found that prostaglandins could produce many of the signs of ocular inflammation, including vasodilation, increased vascular permeability and a raised I.O.P. They suggest that local ocular vasodilation and increased vascular permeability were important factors in raising the I.O.P.

Beitch & Eakins also observed that intracameral injections of as little as 10 ng of PGE and PGF resulted in a significant increase in the protein content of the aqueous humour, a phenomenon indicat= ing an increase in the permeability of the blood-aqueous barrier. They found good correlation between the concentration of protein in the aqueous humour and the sustained increase in I.O.P. produced by either prostaglandin. These findings on the protein content of aqueous humour, together with the congested appearance of blood vessels in the region of the iris, suggest that both vasodilation and increased capillary permeability play a significant role in the response of I.O.P. to prostaglandins in the rabbit.

In the sequence of events in developing uveitis, prostaglandins were implicated as follows:

(i) prostaglandins were released into the aqueous humour from the iris;

(ii) an invasion of leucocytes occurred and resulted possibly from the leucotatic effect of the prostaglandins present in the aqueous humour;

(iii) a further prostaglandin release from the leucocytes occurred, Higgs and Youlten (1972) and Vogt (1974)

It has been stated by Cochrane & Janoff (1974) that the invasion of an inflamed area by polymorphonuclear and mononuclear cells is an important factor in prolonging inflammation. As soon as leucocytes have accumulated and deteriorated, a vicious circle may develop due to the release of lysosomal enzymes, which can degrade macromolecules and liberate mediators including prostaglandins as well as chemotactic factors. Kaley and Weiner (1971) support the view that prosta= glandins are chemotactically active and may act as mediators in leucocyte migration. However, McCall and Youlten (1973) found that only PGE_1 possesses chemotactic properties for the polymorphonuclear leucocytes.

3.3.5 PROSTAGLANDIN EFFECT ON INTRA-OCULAR PRESSURE

The available evidence suggests that the increased inflow of aqueous humour noticed in the rabbit eye after prostaglandin formation is related more to permeability changes of the blood aqueous barrier than to increased secretory activity. Starr (1971) demonstrated that prostaglandins could raise intra-ocular pressure in rabbits even when given by way of intravenous injection. Masuda (1972) showed that topical application of PGE_1 to the rabbit eye increased the inflow, but not the outflow of aqueous humour. The lack of effect of prostaglandins on the outflow has also been reported by Chiang & Thomas (1972).

3.3.6 THE ROLE OF PROSTAGLANDINS IN THE CARRAGEENAN-INDUCED INFLAMMATORY RESPONSE IN THE RAT PAW

There is evidence to support the view that prostaglandins are involved in the carrageenan-induced inflammatory response in the rat paw.

Flower et al (1972) and Vane (1971) demonstrated a close relationship between inhibition of prostaglandin synthetase and the anti-inflammatory action of a number of drugs used in rheumatology with indomethacin, a prostaglandin synthetase-inhibitor, showing a particularly strong action on rat-paw-oedema.

Di Rosa and Willougby (1971) have implicated prostaglandin-like substances in carrageenan-induced rat-paw-oedema and believe that the second accelerating phase of swelling is due to a prostaglandin. This is the phase which is blocked by acidic non-steroidal anti-inflammatory drugs.

Willis (1969) found an E type prostaglandin in the rat-paw exudate, during this secondary phase of inflammation. Smith et al (1974) used a specific prostaglandin antagonist, dibenzoxazepine hydrazide (SC 19220), which reduced the development of the carrageenan-induced paw swelling significantly, a response showing that the prostaglandin system is concerned in the development of carrageenan-induced paw oedema.

Results from this present study, in which four prosta= glandin synthetase inhibitors were used, showed inhibition of the secondary phase of inflammation. These results indicate that prostaglandins are involved. The release of prostaglandins appear to be closely associated with the migration of leucocytes into the inflamed site.

3.4 SUMMARY

Carrageenan as a phlogistic agent and its suitability as an inflammogen in the rat-paw-oedema method of assay, has been discussed.

A short review has been given on arachidonic acid, a precursor of the prostaglandins, and the history, bio= synthesis and mechanism of action of the prostaglandins

presented. Russell et al (1975)

The role of the prostaglandins, with particular reference to ocular inflammation, has been reviewed with reference to the effect of prostaglandins on intra-ocular pressure.

The carrageenan-induced inflammatory response in the rat-paw has been examined, and the possible involve= ment of prostaglandins in the secondary phase of swelling, has been considered.

CHAPTER 4

4.1 CHEMISTRY OF ACIDIC NON-STEROIDAL ANTI-INFLAMMATORY AGENTS

4.1.1 DEFINITION

Scherrer (1974), has defined a classical non-steroidal anti-inflammatory agent as a compound that is active in the carrageenan and adjuvant-arthritis assays; is antipyretic; and is active in the ultra-violet-erythema assay. This definition includes the salicylates and aspirin-like compounds.

4.1.2 RECEPTOR SITE

A hypothetical receptor outline was proposed by Scherrer et al (1964), for classical anti-inflammatory agents.

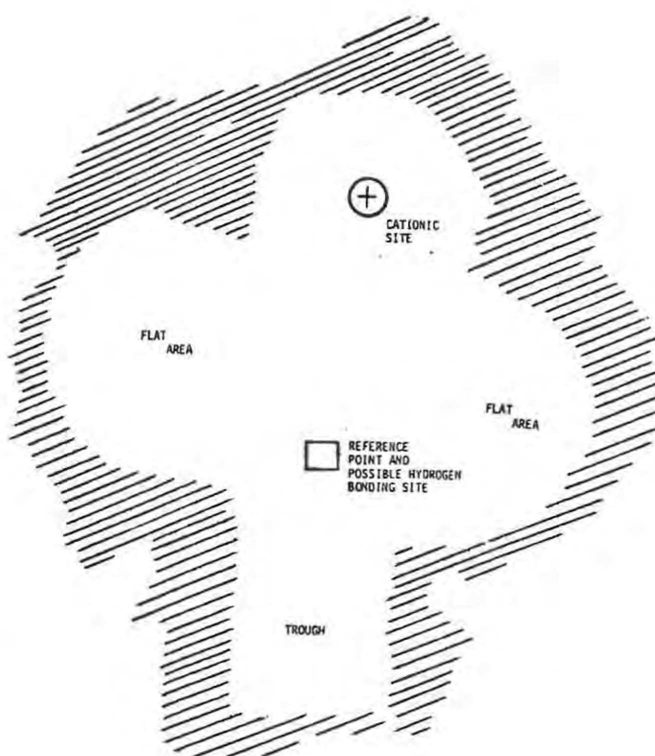


Fig.5. The same receptor design is a model for prostaglandin synthetase. The reference point underlies the N and carbonyl carbon, respectively, of the fenamic acids and indomethacin.

Two features of the receptor were a cationic site and a trough to accept a twisted ring. Shen (1965) has suggested a similar receptor topography based on the analysis of structure-activity relationships and the preferred configuration in the indomethacin-compound series.

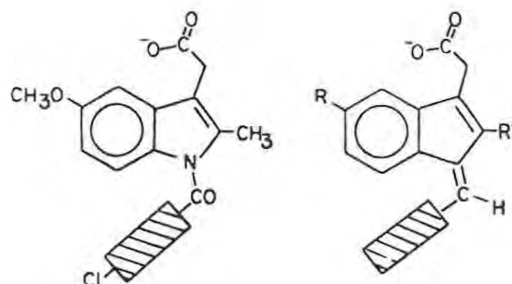


Fig. 6. The configuration of indomethacin as proposed to fit a hypothetical antiinflammatory receptor (Shen, 1965). This receptor has the main features indicated in Fig. but with a different orientation for the trough.

4.1.3 ACIDIC NON-STEROIDAL ANTI-INFLAMMATORY AGENTS AND PREFORMED PROSTAGLANDINS

Scherrer (1974) has shown that prostaglandin E_2 closely resembles classical non-steroidal anti-inflammatory agents as shown:

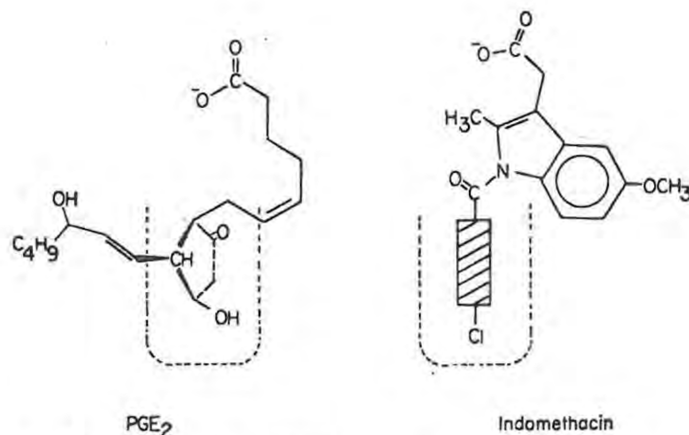


Fig. 7. A possible conformation of PGE_2 at the prostaglandin synthetase antiinflamatory receptor (absolute configuration) compared with indomethacin, a potent inhibitor.

Just a acetylcholine and histamine have multiple receptors, Scherrer proposes that wherever the classical anti-inflammatory agents act as potent inhibitors of the action of a preformed prostaglandin, this action is more likely to occur at a receptor at which the prostaglandin has a conformation similar to that in Fig.7. Vane (1971) has shown that indomethacin tested in vitro inhibited the synthesis of prostaglandins. If the inhibition resulted from competition with arachi= donic acid for the active site of the enzyme, it could explain why the anti-inflammatory substances contain an acidic group.

4.1.4 INHIBITION OF PROSTAGLANDIN SYNTHESIS

Non-steroidal anti-inflammatory agents have been shown to inhibit the biosynthesis of prostaglandins in a variety of tissues. (Vane 1971), (Smith and Willis 1971) and (Fereira et al 1971). This action may account for their therapeutic activity (Ferreira and Vane 1973). It has been suggested by Eakins (1974) that the importance of prostaglandins in certain forms of ocular inflammation suggests that drugs that inhibit either the actions or synthesis of prostaglandins may prove useful as ocular anti-inflammatory agents. Samuelsson et al (1975) state that arachidonic acid can be converted to prostaglandin E_2 and prostaglandin $F_2\alpha$.

Ham et al (1972) have shown that prostaglandin production from arachidonic acid can be inhibited in vitro by a variety of anti-inflammatory agents and Gryglewski (1975) reported that non-steroidal anti-inflammatory drugs inhibit microsomal prostaglandin synthetase. Smith and Willis (1971) have stated that indomethacin, aspirin and salicylic acid, but not hydrocortisone, inhibited the thrombin-induced

production of prostaglandin by human platelets. Tomlinson et al (1972) recovered the arachidonic acid unchanged after inhibition by anti-inflammatory drugs utilizing a bull seminal vesicle enzyme system and naproxen. It has been postulated by Flower et al (1972) that the high level of activity and specificity of many acidic non-steroidal anti-inflammatory compounds in inhibiting prostaglandin synthetase, could explain the mode of action of these agents in vivo. This observation has been supported and the ability of the ANSAI agents to inhibit prostaglandin synthetase, is thought to underlie the therapeutic anti-inflammatory activity of this class of drug. Stone et al (1974).

4.1.5 PROSTAGLANDIN-SYNTHETASE INHIBITORS

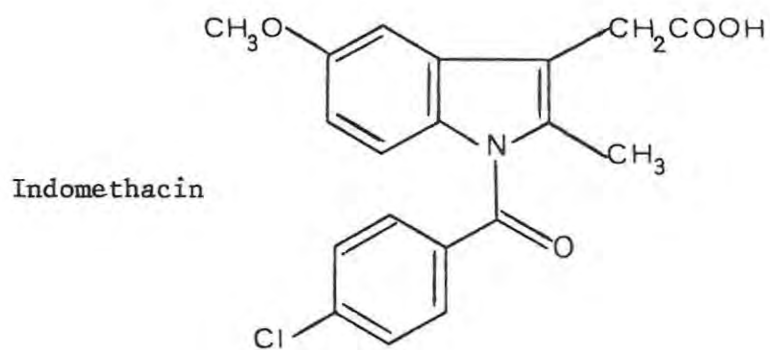
Prostaglandins are believed to be responsible for many of the clinical signs in acute anterior uveitis. Therefore, substances which antagonize their actions or synthesis, should be of value in controlling ocular inflammation. Rodger et al (1973).

Thus, in the rabbit-eye test system, sodium arachidonate was instilled into the rabbit eye, prostaglandin formation was brought about and an inflammatory response noticed, i.e. an increase in intra-ocular pressure and an increase in the protein content of aqueous humour resulted.

The author used four prostaglandin-synthetase inhibitors, viz., indomethacin, diclofenac sodium, flufenamic acid and sulindac, to inhibit the formation of prostaglandins.

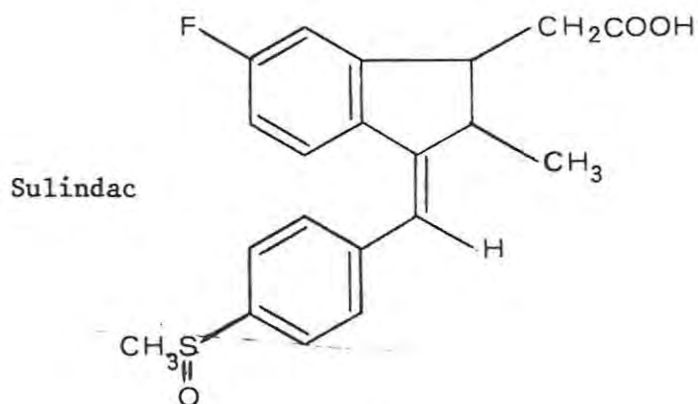
4.1.6 CHEMICAL STRUCTURES OF THE ACIDIC NON-STEROIDAL ANTI-INFLAMMATORY COMPOUNDS EVALUATED

Indomethacin



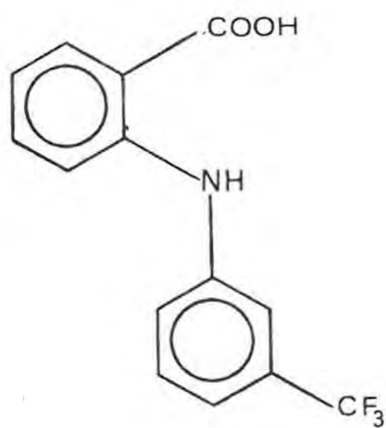
I

Sulindac



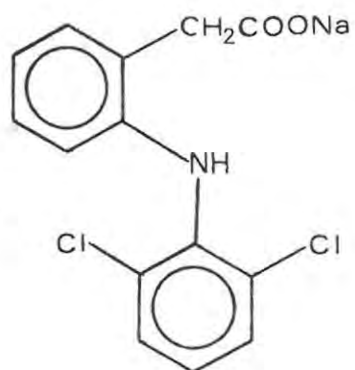
II

Flufenamic acid



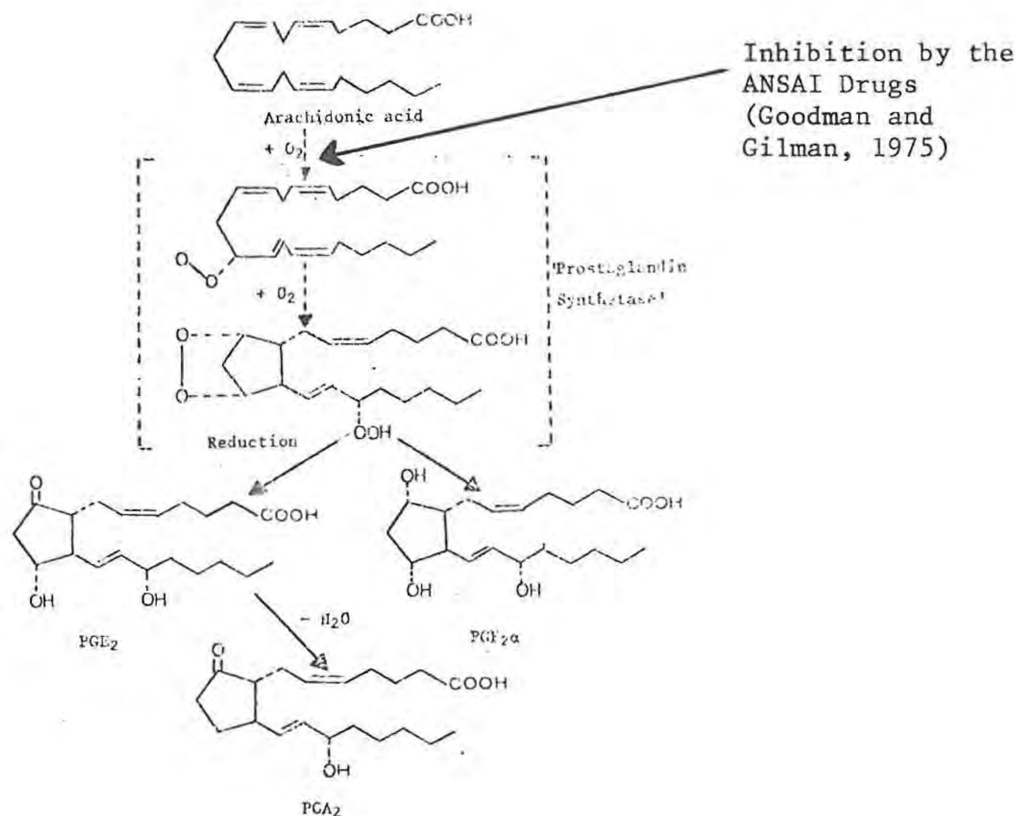
III

Diclofenac sodium



IV

FIG.8. MECHANISM OF ACTION OF THE ANSAI AGENTS SHOWING THE INHIBITION OF THE SYNTHESIS OF PGE_2 AND $PGF_{2\alpha}$



4.2 INDOMETHACIN (See Structure I)

Indomethacin, an indole acetic acid derivative, was shown by Winter et al (1963) to exhibit prominent anti-inflammatory and antipyretic properties in experimental animals. Ham et al (1972) have reported that kinetic studies indicate that the conversion of arachidonic acid to prostaglandin E_2 is competitively inhibited by indomethacin. According to Ariens E.J., (1971), this means "that a relationship in chemical structure may be expected between the natural substrate-product and the anti-inflammatory agent". See Fig Vane showed that indomethacin, tested in vitro, inhibited the synthesis of prostaglandins and Ku et al (1975) states that indomethacin acts as a competitive and irreversible inhibitor of prostaglandin synthetase.

Spector and Willoughby (1968) believe that indomethacin also interferes with the kinin-forming system. It was shown by Ford-Hutchinson et al (1975) that indomethacin inhibited the migration of both polymorphonuclear cells and mononuclear cells into the exudates of implanted sponges.

4.3 DICLOFENAC SODIUM O-(2,6-DICHLOROANILINO) PHENYLACETIC ACID SODIUM SALT (VOLTAREN[R]) (See Structure IV)

The active substance contained in Voltaren is the sodium salt of N-phenylamino-phenylacetic acid (Krupp et al 1975).

It is equal in oedema inhibiting ability, to indomethacin in the carrageenan-oedema assay and such correlation has also been shown by use of the rabbit-eye-test system. Diclofenac sodium is also reported to have similar activity to indomethacin in adjuvant arthritis. Krupp et al (1972).

Piper and Vane (1969) and Moncada et al (1973) state that the pharmacological effects of diclofenac sodium in their work, were consistent with a mechanism of action involving inhibition of prostaglandin synthesis, the mechanism that appears to explain the biological effects of non-steroidal anti-inflammatory drugs. This concept is supported by Ku et al (1975), who have shown that diclofenac sodium in low concentration inhibited prostaglandin synthetase, markedly interferring with the synthesis of prostaglandin E_2 from arachidonic acid. This effect on the biosynthesis of prostaglandins, coupled with the ability of diclofenac to accumulate at sites of inflammation (Früh et al 1975), is considered a factor contributing to the high level of potency of diclofenac.

The low K_1 (inhibition constant) of diclofenac sodium is an indication that this substance is among the most potent compounds within the non-steroidal anti-inflammatory drugs.

Ku et al (1975). Results obtained with diclofenac sodium in the rabbit-eye-test system support this observation. Diclofenac sodium was administered in doses of one mg per Kg, the results comparing extremely well with those obtained with drugs administered in far higher doses.

4.4 FLUFENAMIC ACID (See Structure III)

The N-arylanthranilic acids, used in man have been given the generic name of "fenamic acids". They were first described as an anti-inflammatory agent in a series of reports from Parke-Davis by Winder et al (1963).

The mechanism of action of flufenamic acid is believed to be that of an inhibition of prostaglandin synthesis. Inhibition has been shown to occur in *in vitro* experiments in which meclofenamic acid is active in inhibiting prosta= glandin synthetase. (Flower et al 1972). Meclofenamic acid was found to have an ED_{50} value of 0,03 $\mu g/cm^3$ to inhibit prostaglandin synthetase. The value for flufenamic acid is 0,7 $\mu g/cm^3$. (Scherrer 1974).

The ability of flufenamic acid to counter the effects of the prostaglandin-induced inflammatory response in the rabbit-eye-test system compared favourably with the compounds evaluated. This compound showed a marked effect in inhibiting increase of both the intra-ocular pressure and of the aqueous protein level at a dose of 10 mg per kg.

4.5 SULINDAC, MSD (See Structure II)

Sulindac, like indomethacin, is an indene-type compound, with a structure similar to indomethacin

Sulindac inhibits prostaglandin synthesis and the action thus resembles that of indomethacin. The ability of Sulindac to inhibit the prostaglandin-induced inflammatory

response in the rabbit-eye-test system, was noticed.

4.6 SUMMARY

A classical non-steroidal anti-inflammatory agent has been defined and a hypothetical receptor proposed (Scherrer et al 1964) (Shen 1965). A possible conformation of prostaglandin E_2 has been compared with that of indomethacin. The inhibition of prostaglandin synthesis has been studied and four prostaglandin synthetase inhibitors were discussed. The ANSAI com= pounds studied were indomethacin, diclofenac sodium, flufenamic acid and sulindac.

CHAPTER 5

5.1 EXPERIMENTATION : AIMS & PRELIMINARY CONSIDERATIONS

In order to determine the inhibition of the increase of intra-ocular pressure and the protein content of the aqueous humour of the eye, it was necessary to perfect techniques for:

(i) measuring the intra-ocular pressure (I.O.P.) of the rabbit eye;

(ii) aspirating the eye of the anaesthetized rabbit.

These techniques initially presented more problems than the rat paw oedema method of assay where Winter Risley and Nuss (1962), and subsequently many other investigators, have published widely.

5.2 INFLAMMATORY RESPONSE

Various methods of producing an inflammatory response were studied and turpentine oil was used to significantly increase the I.O.P. as well as the rat paw volume. However, it was discarded as a suitable screening substance because of its low sensitivity and specificity. (Swingle 1974).

Instead, the prostaglandin precursor, arachidonic acid, was chosen as the agent to induce the inflammatory response in the rabbit eye and carrageenan was chosen as a suitable phlogistic agent to induce acute inflammation in the rat paw.

5.3 MEASUREMENT OF INTRA-OCULAR PRESSURE

At first measurement of I.O.P. was recorded by means of a hand-operated Schiotz tonometer, but later by means of the Mackay-Marg tonometer, which supplies a print-out record of the pressure recorded.

5.4 PROTEIN DETERMINATION

The modified Biuret method was used in initial determinations, but was found not to be sensitive enough. However, the turbidimetric method of protein determinations, used in C.S.F. investigations, proved to be suitable.

5.5 MEASUREMENT OF RAT PAW VOLUME

A method adopted by Winter *et al* (1962) for measurement of paw volume makes use of a 25 mm diameter glass cylinder containing mercury and connected *via* a Statham pressure transducer to a galvanometer. Immersion of an object of 1 ml volume into the mercury produced a deflection of 35 scale divisions on the galvanometer. Hall and Hallet (1975) have made use of a method based on Archimedes' principle. The experimental set-up provides a relatively large surface area of mercury and thus provides ease of access as well as the possibility of accurate alignment of the paw. This method, which is described under 5,19,2 was adopted by this investigator.

5.6 METHOD OF ANAESTHETIZATION

Initially, anaesthesia was induced by inserting a small vein set into the main drainage vein of the ear and then anaesthesia maintained at a uniform level by administration

of the anaesthetic as required. This approach proved to be a time-consuming operation and subcutaneous administration of the anaesthetic was therefore adopted. (Straughan, 1975)

5.7 EXPERIMENTAL ANIMALS

Albino rabbits, with a body mass of two to three kg, were found to be suitable and were used in the rabbit-eye-test system. It was noticed by the writer that younger, lighter rabbits tended to present difficulties for the measuring of the I.O.P., for their eyes are not as well developed as older, more mature rabbits. This difficulty was especially prominent when oedema of the eye developed as a result of the instillation of sodium arachidonate. Rabbits of both sexes were used, as the sex did not appear to affect the reponse.

Wistar rats were also used, having an average mass of 200 g. Weight or sex did not appear to affect the response. (Arrigoni-Martelli, and Conti, 1964).

5.8 EXPERIMENTAL - MATERIALS AND METHOD

5.8.1 RABBIT EYE : THE TEST SYSTEM

Albino rabbits of either sex, weighing approximately 2 to 3 kg, were used; eight rabbits for each determination. This was the maximum number that it was possible to manipulate at once. The drug used to evaluate the rabbit-eye-test system was injected intravenously into four rabbits into the marginal vein of the ear at -60 minute time. At -30 minutes the drug was instilled into the rabbits' left eye.

The control rabbits received sterile normal saline by the same route. A dose of 2,5 ml of Alfathesin[R] per kg, injected subcutaneously, induced a level of anaesthesia sufficient to allow measurement of the intra-ocular pressure. This light anaesthesia persisted usually for more than an hour after which time anaesthesia could be maintained by a further subcutaneous injection of two to three ml of Alfathesin[R]. The rabbits tolerated the anaesthetic very well provided the anaesthetic was administered at a slow rate. Pentobarbitone was also tried as an anaesthetic, but the response of the rabbits was variable and a number of animals were lost.

Once a uniform level of anaesthesia had been attained, the resting intra-ocular pressure was measured. The probe which converted the I.O.P. into an electrical signal, was brought into contact with the cornea. The ensuing electrical signal was amplified and recorded by means of a hot stylus onto heat-sensitive tonopaper.

Once the resting I.O.P. had been recorded, sodium arachidonate solution was instilled into the left eye at time 0, and the intra-ocular pressure was measured after 15, 30 and 60 minutes.

5.8.2 ASPIRATION OF THE EYE

Three hours after inflammation had been induced, the anterior chamber of the eye was aspirated by the use of a 26 gauge needle. The operation was carried out as follows:

Alfathesin was administered intravenously to re-anaesthetize the rabbit, and once a suitable depth of anaesthesia had been attained, the eye was immobilized by means of opthalmic forceps. (See page 92, P6). The needle was then carefully inserted into the anterior chamber of the eye, horizontally to the iris, and the aqueous humour removed. (Approximately 0,2 ml was obtained from each eye). The aqueous humour from the four test animals was pooled and a protein determination carried out. The aqueous humour of the control eyes was similarly treated. A 0,4% solution of oxybuprocainium was instilled into the eye to cause local anaesthesia, but the author found that the local anaesthesia produced was insufficient to allow aspiration to be carried out and general anaesthesia was necessary.

5.9 PREPARATION OF THE MATERIALS

5.9.1 Sodium arachidonate was obtained from the Sigma Chemical Company in ampoules containing 50 mg, and a 1% aqueous solution was prepared with distilled water. The aqueous solution of sodium arachidonate was stored in a frozen state and kept its potency for at least one week in this form. 100 µl was instilled into the rabbit eye.

Initially 5% arachidonic acid was dissolved in soya bean oil which, due to its anti-oxidant properties, was able to keep the arachidonic acid from oxidation. (Straughan, 1975). The most effective application form of arachidonic acid was as the sodium salt and a one-percent solution yielded a reproducible sub-maximal response. (Bhattacherjee and Eakins, 1975).

5.9.2 Indomethacin (Merck, Sharp and Dohme). Four mg per kg was administered to the test rabbits. The 0,25% indomethacin solution was prepared by adding equimolar quantities of Na_2CO_3 to an aqueous suspension of indomethacin. Using a 25 gauge needle, the solution was injected intravenously into the marginal ear vein through a 0,2 µm filter (Acrodisc - Gelman) to render the solution sterile.

5.9.3 Flufenamic Acid (Parke-Davis). A 1% solution was prepared with triethanolamine as a solubilizer. 10 mg per kg body weight was administered intravenously.

5.9.4 Diclofenac Sodium (Ciba-Geigy). A 0,2% aqueous solution of sodium diclofenac was prepared and administered intra= venously. 1 mg per kg body weight was administered.

5.9.5 Sulindac (Merck, Sharp and Dohme). The dose administered was 3 mg per kg body weight. An aqueous 0,2% solution was prepared by the addition of an equimolar quantity of Na_2CO_3. 3 mg per kg was administered intravenously.

The above four drug solutions were also used for topical administration into the rabbit eye. The drug being evaluated was instilled 30 minutes before instillation of the sodium arachidonate.

5.10 THE ANAESTHETIC

Alfathesin^R which is well tolerated in cats, also produced a level of anaesthesia in the rabbit suitable for allowing I.O.P. measurements to be taken and for the eye to be aspirated.

Alfathesin^R is a steroid anaesthetic for intravenous use with both induction and recovery from anaesthesia occurring rapidly. The active ingredients of this preparation are two pregnanedione derivatives, alphaxalone (3α-hydroxy-5α-pregnane-11,20 dione) and alphadolone acetate (21-acetoxy

3α-hydroxy-5α-pregnane-11,20 dione). The latter has about one half the anaesthetic activity of the former and has been included to improve the solubility of alphaxalone. Alfathesin[R] has a therapeutic index of about 30 in mice as compared to 6,9 in the case of thiopentone sodium. Each ml of Alfathesin[R] contains 9 mg of alphaxalone and 3 mg of alphadolone acetate.

5.11 THE MACKAY-MARG TONOMETER

The tonometer consists of two basic parts: the probe, which converts the intra-ocular pressure into an electrical signal, and the amplifier-recorder unit, which aplifies and records the output signal from the probe. The probe shell is made of stainless steel and at the centre of the tip of the nose-piece (which is pressed against the cornea through a rubber membrane during measurement) consists of a ceramic actuating rod, whose sub-microscopic movements are detected and amplified to produce a tonogram. (See page 92 P3). Measurements are derived from intra-ocular pressure acting through the flattened cornea against the actuating rod causing minute movements.

5.12 AMPLIFIER-RECORDER

(See Page 92 P5). The amplifier recorder supplies a very weak high-frequency alternating current to the transducer in the probe. This current, modified according to the pressure on the ceramic actuating rod, is amplified several hundred times in the probe. It then returns to the amplifier for further amplification. The recorder now converts this amplified current to a permanent record by means of a hot stylus on a heat-sensitive chart paper.

5.13 CALIBRATION OF TONOMETER

It is necessary to check the calibration of the instrument before taking I.O.P. readings. This is done by setting the instrument at x2; each small division on the tonopaper then is equivalent to 2 mm Hg pressure on the probe face. The probe is then held vertically between thumb and fore= finger with the tip of the probe pointing upwards. The probe cable is held in the other hand and, using it as a lever, the probe is inverted smoothly and quickly so that the tip is pointing straight down. The thumb and forefinger holding the probe act as a pivot. After a five-second pause, the probe is pivoted again so that the tip points straight up and is held in this position for five seconds. Repetition of this procedure produces a series of step changes which are compared with the Proper Calibration Trace supplied with the tonometer.

5.14 PAPER SPEED

The paper speed of 5 mm per second was used in recording the intra-ocular pressure. A speed of 25 mm per second reduces paper consumption, but produces compressed tonograms. Economy was sacrificed for clarity.

5.15 TONOMETER SCALE

Normally the I.O.P. was recorded at a setting of x2, but in cases where the I.O.P. increased to high levels, the x4 setting was used. Each scale division on the tonopaper then represented 4 mm Hg.

5.16 PROBE APPLICATION (See page 92, P4).

The probe was brought into contact with the cornea with a feather-light tapping motion, the probe having been positioned about 2 to 4 mm away from the cornea. The motion of touching is done in very short strokes with the tip of the probe perpendicular to the middle of the corneal surface.

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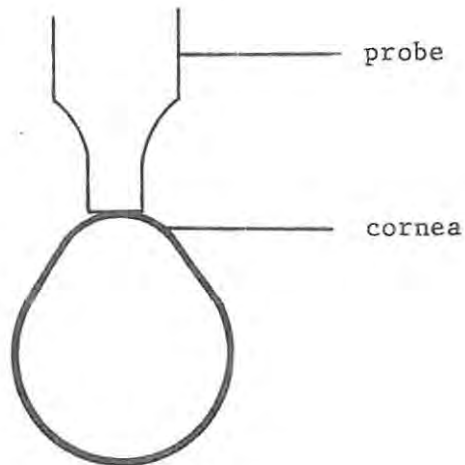
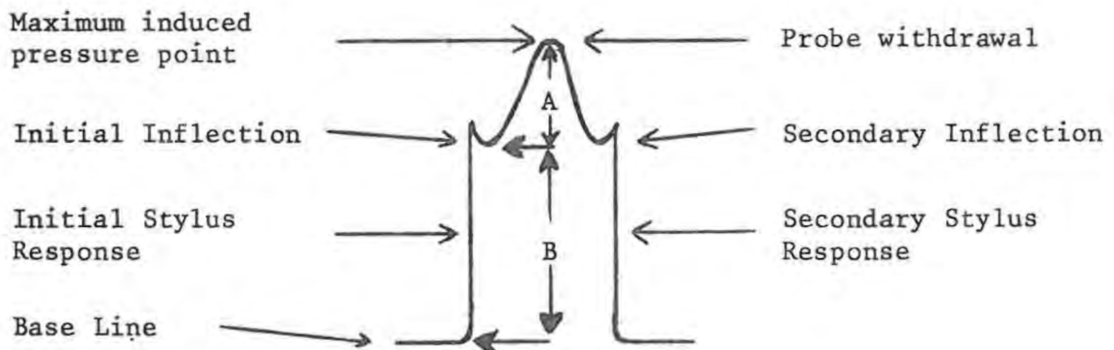
CORRECT TECHNIQUE



This procedure ensured that the sensing rod was the first portion to touch, a pre-requisite for producing a good tonogram.

5.17 ANALYSIS OF THE TONOGRAM

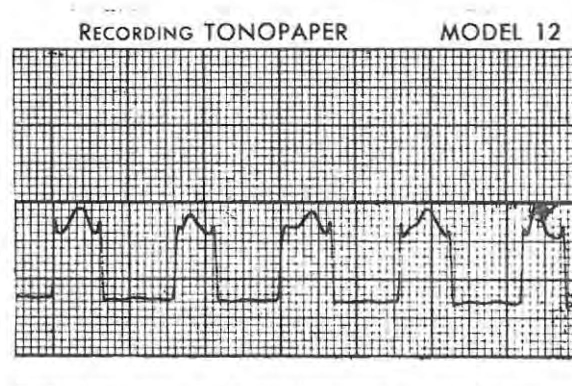
An ideal tonogram contains seven main components, the most important one being the initial inflection, which provides the actual measurement of the intra-ocular pressure.



THE SEVEN COMPONENTS OF A TONOGRAM

Line A shows the amount of induced pressure due to flattening of the cornea. This induced pressure occurs after the measurement of the intra-ocular pressure and is caused by changing the volume of the eye by flattening it beyond the area where the pressure reading is taken.

Line B represents the actual measurement of the intra-ocular pressure. This measurement is not affected by the induced pressure change.



A TONOGRAM

(Each division is equivalent to 2mm Hg)

5.18 PROTEIN-DETERMINATION PROCEDURE

A turbidimetric method using Sulphosalicylic acid was employed for determining the protein content of the aqueous humour.

5.18.1 METHOD

0,1 ml of the pooled aqueous humour sample was made up to 1 ml with distilled water. 1 ml of a 5% - sulphosalicylic acid solution was then added and the solution allowed to stand for five minutes.

A Leitz colorimeter fitted with a 640 red filter was employed to obtain readings and the protein content of the aqueous humour was calculated in g per 100 ml.

5.19 THE RAT PAW OEDEMA METHOD OF ASSAY

5.19.1 METHOD

Wistar rats of either sex and weighing approximately 200 g were used for this assay. The four ANSAI compounds used, i.e. indomethacin, diclofenac sodium, flufenamic acid and sulindac, were solubilized as under 5.9 Each drug was injected intraperitoneally into the rat one hour (-60 minutes) before injection of the carrageenan into the rat paw.

The dosage used was:			
	Indomethacin	-	4 mg per kg
	Flufenamic acid	-	10 mg per kg
	Diclofenac sodium	-	1 mg per kg
	Sulindac	-	3 mg per kg

The control rats received an intraperitoneal injection of sterile normal saline.

Thiopentone sodium was used to provide a light anaesthesia so as to allow easy handling of the rats. The anaesthetic

was administered both to test and control rats thus eliminating possible variation in swelling, which might have been attributed to the anaesthetic.

The suggested human dose of thiopentone sodium is 40 mg per kg. The average mass of the rat was taken to be 200 g, and the dose administered was 8 mg. This made the rats sufficiently docile for the purposes of manipulation.

The anaesthetic started to take effect after approxi= mately ten to fifteen minutes and usually lasted for about two hours.

At zero time, i.e. one hour after administering the ANSAI compound, the phlogistic agent, carrageenan, was injected into the right hind paw. (See page 92, P1). Carrageenan, an extract of chondrus was prepared as a 1% suspension in sterile 0,9% NaCl water solution.

A few drops of 95% alcohol were employed as a wetting agent to wet and disperse the carrageenan before the sterile normal saline was added. Thorough mixing was necessary to ensure a uniform suspension. A volume of 0,05 ml was injected through a 26-gauge needle into the plantar tissue of the right hind paw. Immediately thereafter, the volume of the injected foot was measured by immersing the foot into the beaker of mercury exactly to an ink mark on the skin just beyond the lateral malleolus of the hind paw. Swelling of the paw reached a peak in 3 - 5 hours, but for routine drug testing, the increase in foot volume three hours after the phlogistic agent was injected was adopted as the measure of the effect. (Winter, Risley and Nuss, 1962). The increase in foot volume between one and three hours after the injection of carrageenan was adopted as the measure of

the effect by this investigator.

5.19.2 METHOD OF MEASURING FOOT VOLUME (See page 92, P2).

The procedure developed by Hall and Hallet (1975) is based on Archimedes' principle. A beaker of mercury (50 ml beaker containing approximately 35 ml of mercury) is mounted on a two decimal top-loading digital balance. (Sauter SM 1000). The increase in weight registered by the balance was read after insertion of the animals paw into the mercury. The weight of the volume of mercury displaced by the swollen paw is proportional to the volume of the paw. When necessary, it was possible to tare rapidly between readings (two operators were necessary).

The paw volumes were measured hourly for three hours after the injection of carrageenan. The data were expressed as the percentage inhibition of the oedema formation.

$$\% \text{ inhibition} = 100 \left[1 - \frac{Vt}{Vc} \right]$$

where Vt and Vc are the increases in volume of the carrageenan-injected paws of the drug-treated and the control groups respectively. In this investigation, the second-phase volume of the swelling was determined by calculating the increase in paw size that occurred between hour 1 and hour 3 after the injection of carrageenan. The ANSAI compounds were evaluated against the response in the second phase of oedema, the phase which was blocked by the class of drug used in this study.

Batches of 24 rats appeared to be a suitable working arrangement. Eight rats were used as a control and eight rats for each drug. A total of 236 readings

of paw volumes were taken in the evaluation of the four ANSAI compounds used.

5.20 SUMMARY

(i) A description of the experimental procedures used for the determination of the two parameters, viz. intra-ocular pressure and the protein content of aqueous humour of the eye of the anaesthetized albino rabbit, have been given. The methods in solubilizing the four ANSAI compounds have been discussed. The anaesthetic used and the method of producing anaesthesia has been explained. The Mackay-Marg tonometer has been discussed and the interpretation of a tonogram studied. The technique necessary to obtain legible tonograms was presented. The aspiration of aqueous humour has been described and the method of protein determination given.

(ii) The experimental detail of rat paw oedema method of assay has been described. Sedation of the rats with thiopentone sodium has been discussed. Hall and Hallet's method for measurement of paw volume was also presented.

CHAPTER 6

R E S U L T S

The Student's T Significance Test was the method of statistical analysis used.

TABLES		1 - 8
GRAPHS	Figures	IX - XX

Table I

INHIBITORY EFFECTS OF INDOMETHACIN ON THE ACTION OF TOPICALLY INSTILLED SODIUM ARACHIDONATE

Pretreatment	Resting I.O.P. mm Hg (x_1)	Intra-ocular pressure recorded at 15, 30 and 60 minute intervals after instillation of 1% Sodium Arachidonate			Increase in Pressure mm Hg (x_2-x_1)	% Increase In I.O.P.	Aqueous Protein[3] g %	Percentage Inhibition of Intra-ocular Pressure[2] $= 100\left[1 - \frac{Pt}{Pc}\right]$
		15 min	30 min	60 min (x_2)				
CONTROL RABBITS (8)[1]								
Sterile normal saline	20,62 ±4,2[4]	55,6 ±13,1	42,6 ±7,4	35,62 ±5,0	15,0 ±5,9	72,7%	2,0	
TEST RABBITS (8)								$= 100\left[1 - \frac{5,2}{72,7}\right]$
Indomethacin 4 mg per kg	19,6 ±3,4	25,5 ±8,1	27,0 ±7,0	20,6 ±2,4	1,0 ±1,7	5,2%	0,4	= 92,9%

[1] Number of animals used in parenthesis

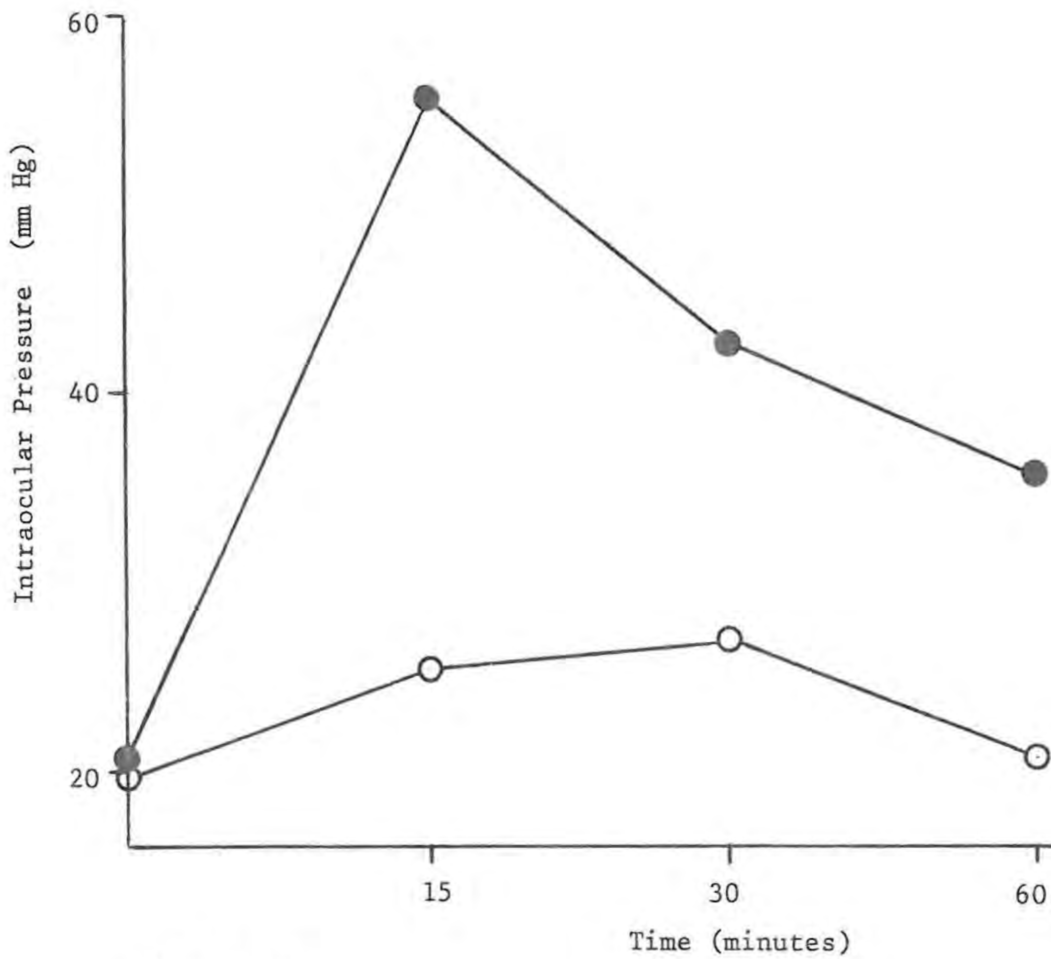
[2] Pt and Pc are % increases in the I.O.P. of the drug treated and control groups of rabbits respectively.

[3] Pooled samples were used to estimate protein.

[4] Standard deviation.

($P < 0,005$)

FIG.9. INTRAOCULAR PRESSURE READINGS IN EIGHT TEST RABBITS PRETREATED WITH INDOMETHACIN ○—○ AND IN EIGHT CONTROL RABBITS ●—●



(P < 0,005)

TABLE II

INHIBITORY EFFECTS OF DICLOFENAC SODIUM ON THE ACTION OF TOPICALLY INSTILLED SODIUM ARACHIDONATE

Pretreatment	Resting I.O.P. mm Hg (x_1)	Intraocular Pressure recorded at 15, 30 and 60 minute intervals after instillation of 1% Sodium Arachidonate			Increase in Pressure mm Hg ($x_2 - x_1$)	% Increase in I.O.P.	Aqueous Protein[3] g %	Percentage Inhibition of Intraocular Pressure[2] $= 100\left[1 - \frac{Pt}{Pc}\right]$
		15 min	30 min	60 min (x_2)				
CONTROL RABBITS (4)[1]								
Sterile normal saline	18,75 ±3,9[4]	39,5 ±5,0	39,75 ±2,9	34,5 ±7.2	15,75 ±7,5	84%	2,0	
TEST RABBITS (4)								$= 100\left[1 - \frac{8}{84}\right]$
Diclofenac Sodium 1 mg per kg.	21,75 ±2,9	32,0 ±7,3	29,75 ±7,2	23,5 ±4,1	1,75 ±3,1	8%	1,0	= 90,4%

[1] Number of animals used in parenthesis

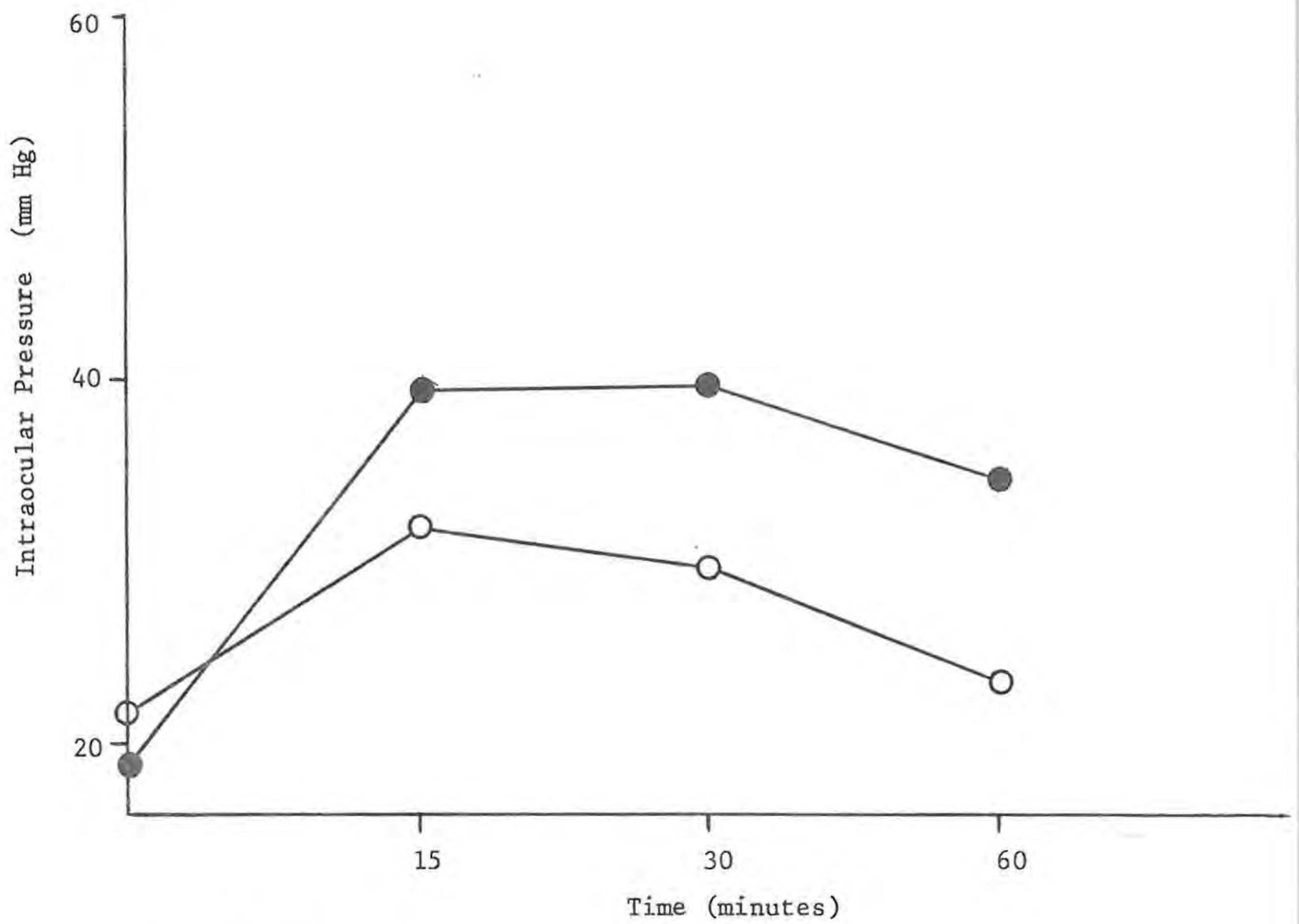
[2] Pt and Pc are % increases in the I.O.P. of the drug treated and control groups of rabbits respectively

[3] Pooled samples were used to estimate protein

[4] Standard deviation.

($P < 0,01$)

FIG.10. INTRAOCULAR PRESSURE READINGS IN FOUR TEST RABBITS PRETREATED WITH DICLOFENAC SODIUM ○—○ AND IN FOUR CONTROL RABBITS ●—●



($P < 0,01$)

TABLE III

INHIBITORY EFFECTS OF FLUFENAMIC ACID ON THE ACTION OF TOPICALLY INSTILLED SODIUM ARACHIDONATE

Pretreatment	Resting I.O.P. mm Hg (x_1)	Intraocular Pressure recorded at 15, 30 and 60 minute intervals after instillation of 1% Sodium Arachidonate			Increase in Pressure mm Hg (x_2-x_1)	% Increase in I.O.P.	Aqueous Protein[3] g %	Percentage Inhibition of Intraocular Pressure[2] $= 100\left[1 - \frac{Pt}{Pc}\right]$
		15 min	30 min	60 min (x_2)				
CONTROL RABBITS (8)[1] Sterile normal saline	23,0 ±6,8[4]	50,8 ±9,4	48,5 ±8,4	37,0 ±5,5	14,0 ±5,2	60,9%	1,64	$= 100\left[1 - \frac{6,7}{60,9}\right]$ = 89%
TEST RABBITS (8) Flufenamic acid 10 mg per kg.	30,0 ±8,6	38,0 ±10,9	41,0 ±10,4	32,0 ±6,3	2,0 ±8,4	6,7%	0,61	

[1] Number of animals in parenthesis.

[2] Pt and Pc are % increases in the I.O.P. of the drug treated and control groups of rabbits respectively.

[3] Pooled samples were used to estimate protein.

[4] Standard deviation.

($P < 0,005$)

FIG.11. INTRAOCULAR PRESSURE READINGS IN EIGHT TEST RABBITS PRETREATED WITH FLUFENAMIC ACID ○—○ AND IN EIGHT CONTROL RABBITS ●—●

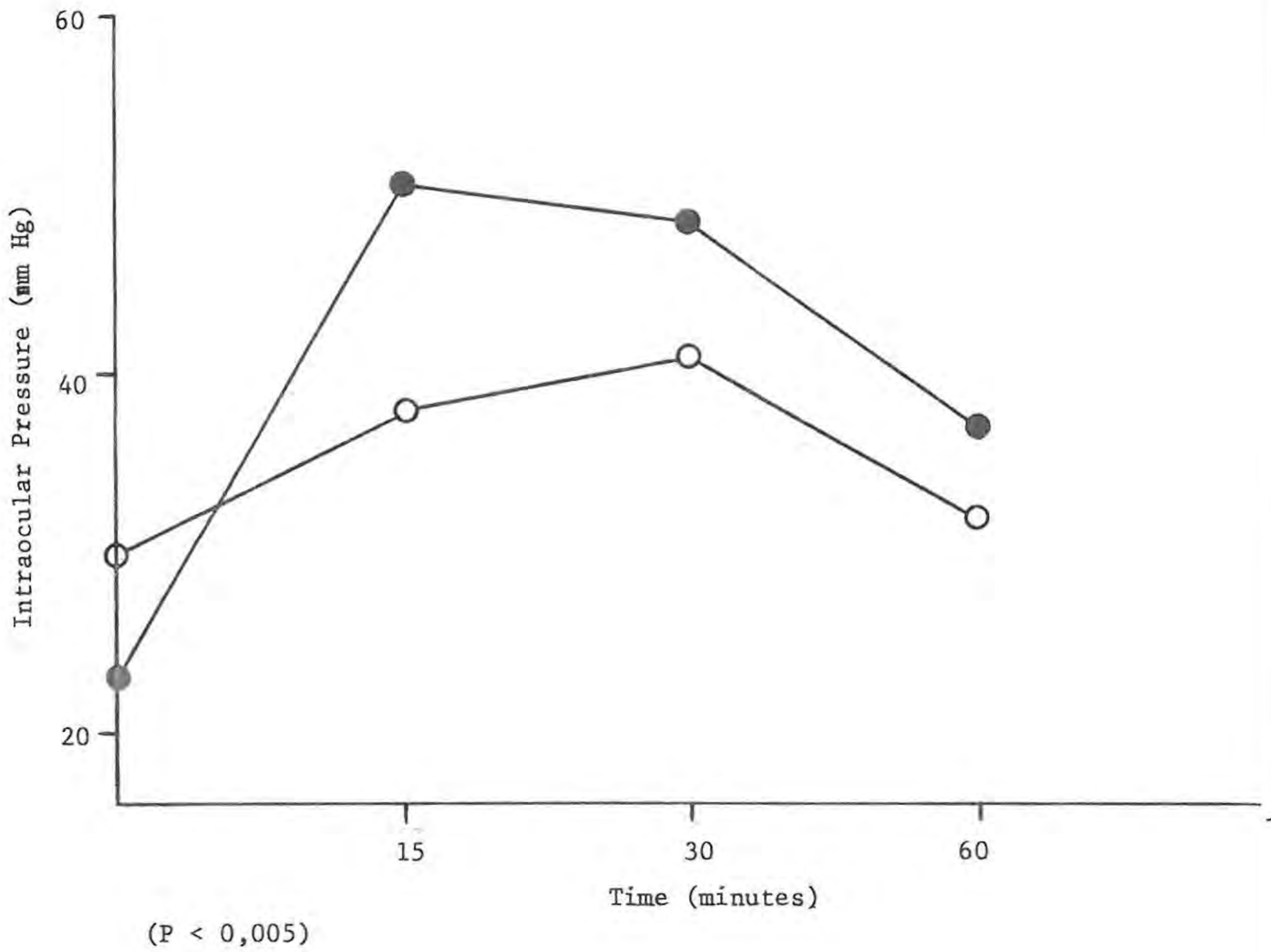


TABLE IV

INHIBITORY EFFECTS OF SULINDAC ON THE ACTION OF TOPICALLY INSTILLED SODIUM ARACHIDONATE

Pretreatment	Resting I.O.P. mm Hg (x_1)	Intraocular Pressure recorded at 15, 30 and 60 minute intervals after instillation of 1% Sodium Arachidonate			Increase in Pressure mm Hg (x_2-x_1)	% Increase in I.O.P.	Aqueous Protein g % [3]	Percentage Inhibition of Intra-ocular Pressure [2] $= 100\left[1 - \frac{Pt}{Pc}\right]$
		15 min	30min	60 min (x_2)				
CONTROL RABBITS (4)[1] Sterile normal saline	22,0 ±2,3[4]	49,5 ±6,4	50,0 ±7,7	41,5 ±6,0	19,5 ±5,7	88,6%	2,7	
TEST RABBITS (4) Sulindac 3 mg per kg	23,3 ±2,1	34,8 ±7,4	39,0 ±4,8	27,5 ±6,8	4,2 ±5,1	18,3%	1,0	$= 100\left[1 - \frac{18,3}{88,6}\right]$ $= 79,4\%$

[1] Number of animals used in parenthesis.

[2] Pt and Pc are % increases in the I.O.P. of the drug treated and control groups of rabbits respectively.

[3] Pooled samples were used to estimate protein.

[4] Standard deviation.

($P < 0,005$)

FIG.12. INTRAOCULAR PRESSURE READINGS IN FOUR TEST RABBITS PRETREATED WITH SULINDAC ○—○ AND IN FOUR CONTROL RABBITS ●—●

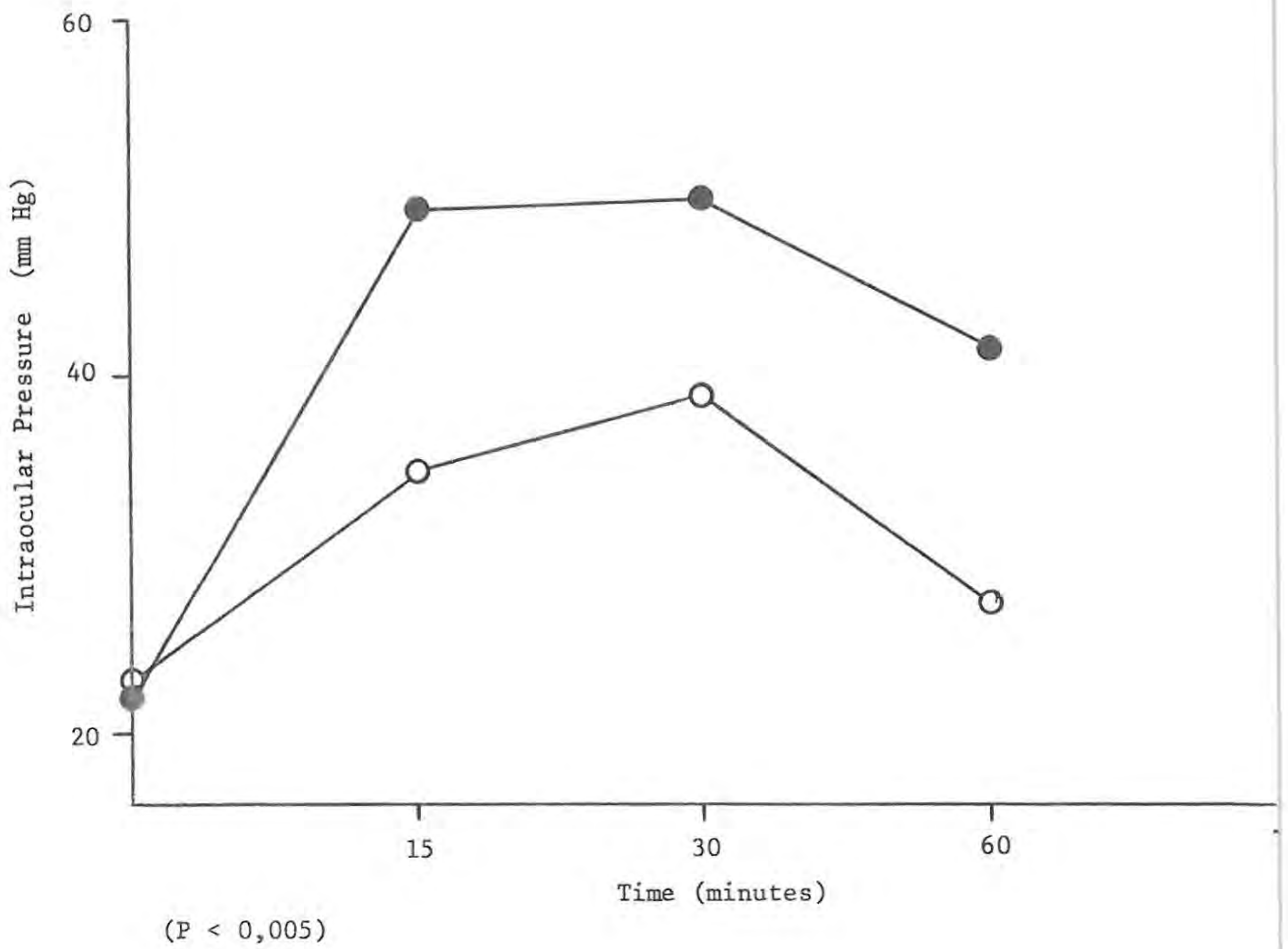


TABLE V

INHIBITION OF CARRAGEENAN - INDUCED OEDEMA IN THE RAT PAW BY INDOMETHACIN

Pretreatment	Paw Mass at Injection Time	Paw Mass at 1 Hour (x_1)	Paw Mass at 2 hours	Paw Mass at 3 hours (x_2)	Increase in Mass ($x_2 - x_1$) [3]	% In= crease	Percentage Inhibition [2] $= 100\left[1 - \frac{mt}{mc}\right]$
CONTROL RATS (11) [1]							
Sterile normal saline	19,97g ±2,3 [4]	21,15g ±2,5	21,76g ±2,6	22,5g ±3,1	1,35g ±1,0	6,38%	
TEST RATS (12)							$= 100\left[1 - \frac{,47}{6,38}\right]$ = 92,6%
Indomethacin 4 mg per kg.	18,13g ±1,2	19,28g ±2,0	19,36g ±2,2	19,37g ±2,0	,09g ±0.2	,47%	

[1] Number of animals used in parenthesis.

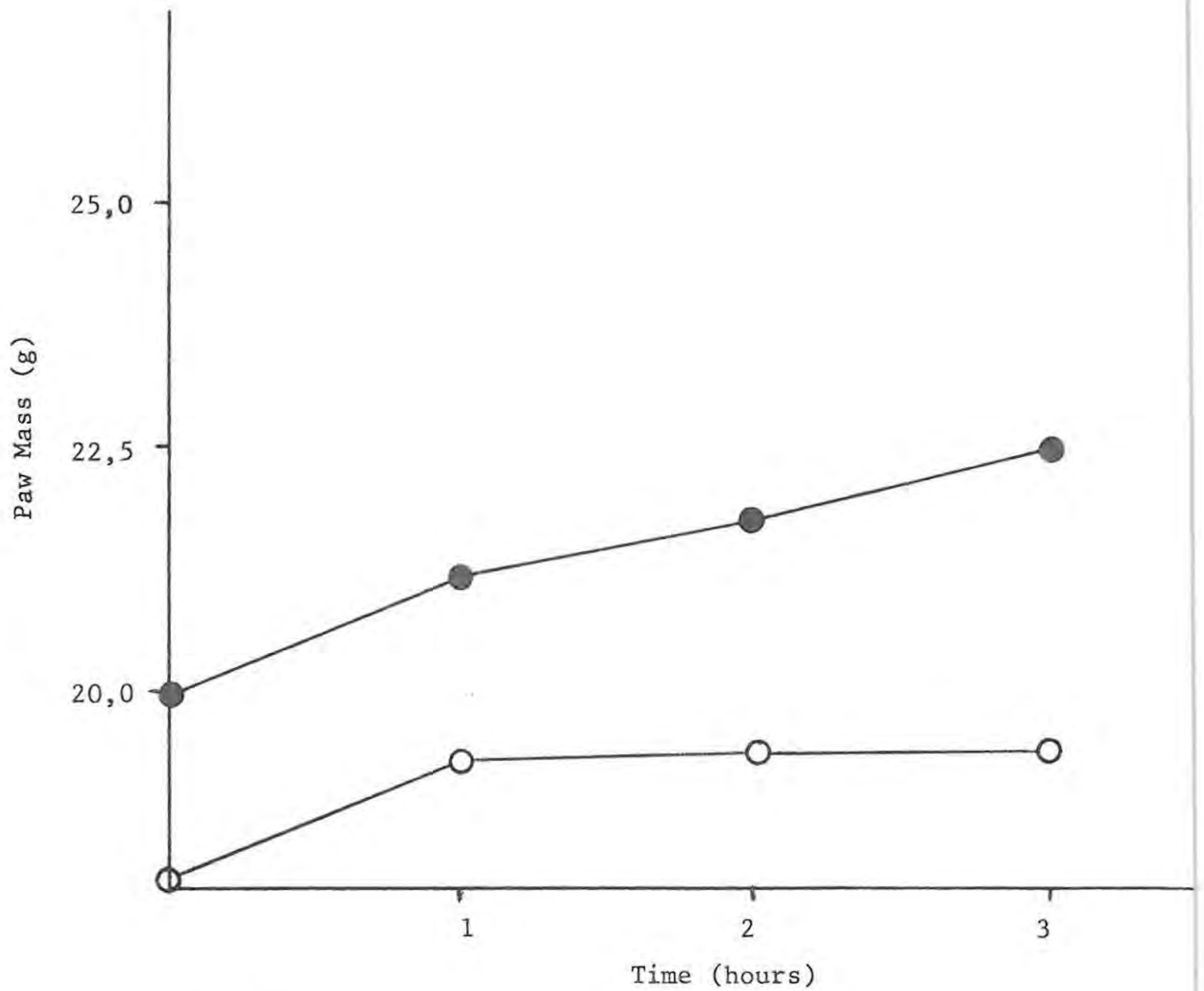
[2] Mt and Mc are % increases in paw mass of the drug treated and control groups of rats respectively, (increase in mass is taken as being proportional to an increase in volume).

[3] The increase in paw mass is measured between one hour and three hours.

[4] Standard deviation.

($P < 0,005$)

FIG.13. INCREASE IN PAW MASS IN TWELVE TEST RATS PRETREATED WITH INDOMETHACIN 4mg PER kg O—O AND IN ELEVEN CONTROL RATS ●—●



(P < 0,005)

TABLE VI

INHIBITION OF CARRAGEENAN - INDUCED OEDEMA IN THE RAT PAW BY DICLOFENAC SODIUM

Pretreatment	Paw Mass at Injection time	Paw Mass at 1 hour (x_1)	Paw Mass at 2 hours	Paw Mass at 3 hours (x_2)	Increase in mass ($x_2 - x_1$)[3]	% In= crease	Percentage Inhibition[2] $= 100\left[1 - \frac{mt}{mc}\right]$
CONTROL RATS (8)[1] Sterile normal saline	20,19g ±3,2[4]	22,57g ±3,2	24,3g ±3,4	26,33g ±4,0	3,76g±1,3	16,66%	
TEST RATS (8) Diclofenac Sodium 1 mg per kg.	18,97g ±1,8	20,97g ±1,6	21,2g ±1,6	21,5g ±1,6	0,53g±0,7	2,53%	$= 100\left[1 - \frac{2,53}{16,66}\right]$ = 84,8%

[1] Number of animals used in parenthesis.

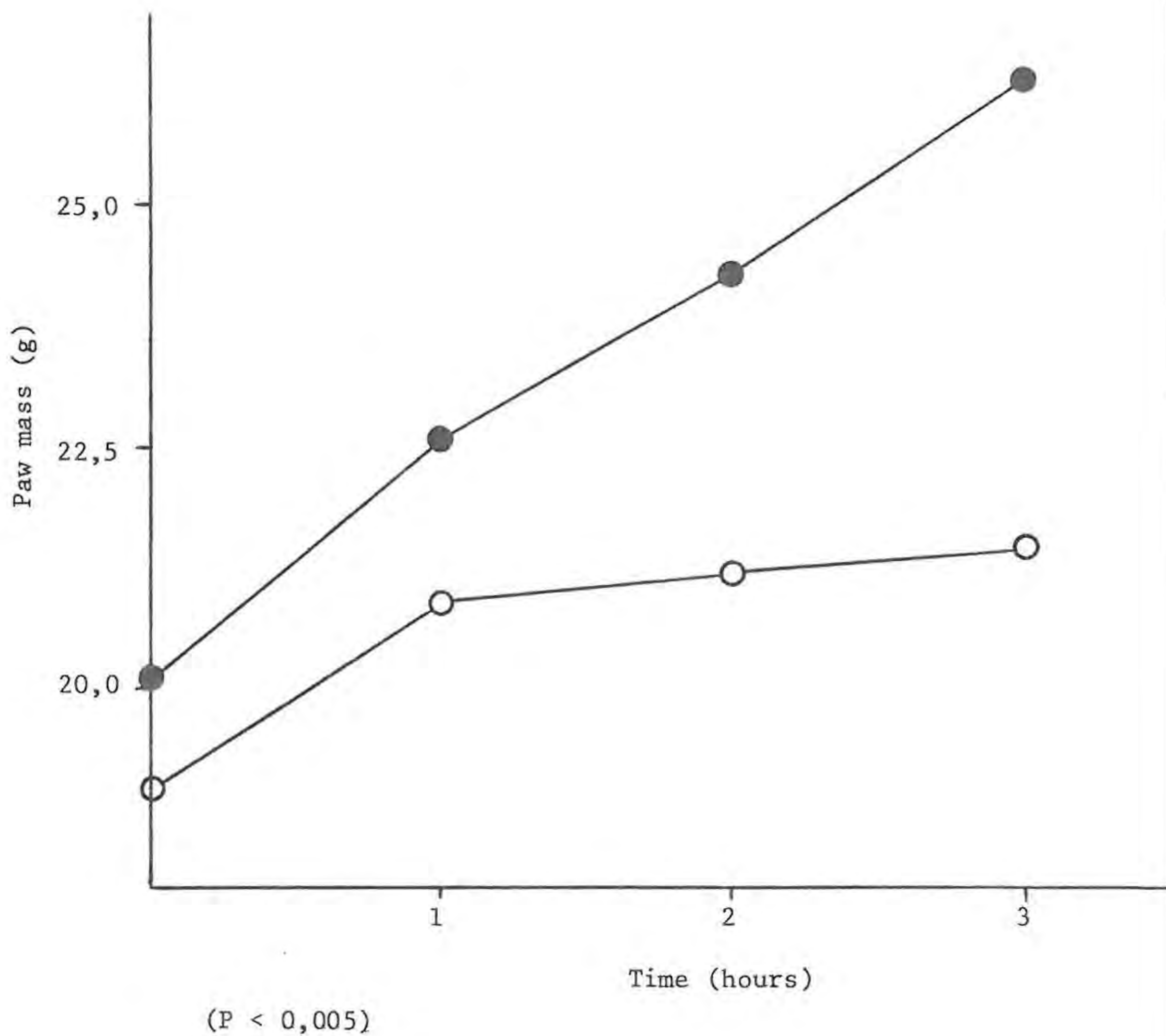
[2] Mt and Mc are % increases in paw mass of the drug treated and control groups of rats respectively, (increase in mass is taken as being proportional to an increase in volume).

[3] The increase in paw mass is measured between one hour and three hours.

[4] Standard deviation.

($P < 0,005$)

FIG.14. INCREASE IN PAW MASS IN EIGHT TEST RATS PRE-TREATED WITH DICLOPHENAC SODIUM 1mg PER kg ○—○ AND IN EIGHT CONTROL RATS ●—●



(P < 0,005)

TABLE VII

INHIBITION OF CARRAGEENAN - INDUCED OEDEMA IN THE RAT PAW BY FLUFENAMIC ACID

Pretreatment	Paw Mass at Injection Time	Paw Mass at 1 hour (x_1)	Paw Mass at 2 hours	Paw Mass at 3 hours (x_2)	Increase[3] in mass (x_2-x_1)	% In=crease	Percentage Inhibition[2] $= 100\left[1 - \frac{mt}{mc}\right]$
CONTROL RATS (11)[1]							
Sterile Normal Saline	19,97g ±2,3[4]	21,15g ±2,5	21,76g ±2,6	22,5g ±3,1	1,35 ±1,0	6,38%	
TEST RATS (12)							$= 100\left[1 - \frac{0,72}{6,38}\right]$ = 88,7%
Flufenamic Acid 10 mg per kg.	18,82g±1,5	19,43g ±1,6	19,64 ±1,6	19,57g ±1,5	0,15 ±0,3	0,72%	

1 Number of animals used in parenthesis

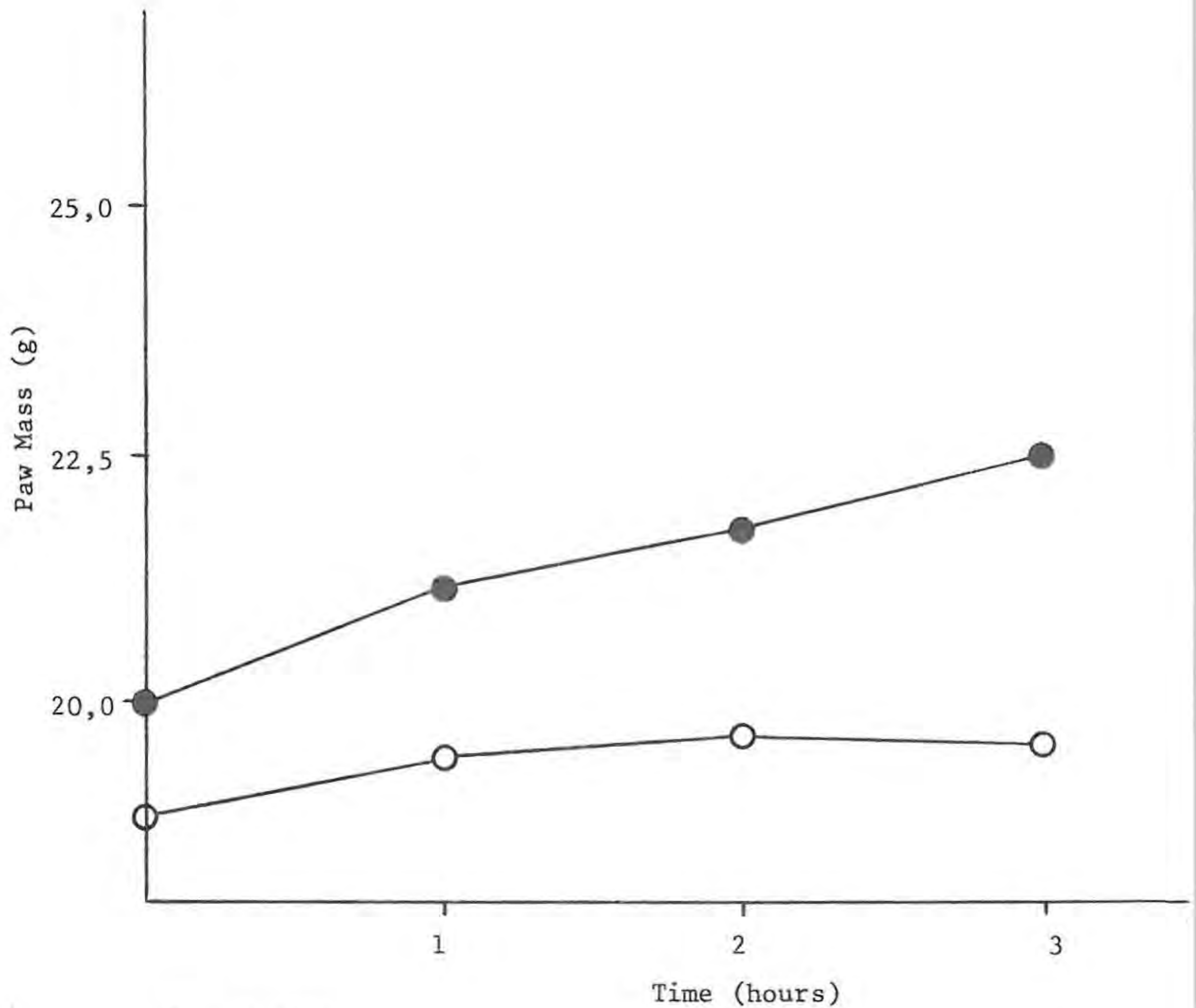
2 Mt and Mc are % increases in paw mass of the drug treated and control groups of rats respectively (increase in mass is taken as being proportional to an increase in volume)

3 The increase in paw mass is measured between one hour and three hours

4 Standard deviation.

($P < 0,005$)

FIG.15. INCREASE IN PAW MASS IN TWELVE TEST RATS PRETREATED WITH FLUFENAMIC ACID 10mg PER kg. O—O AND IN ELEVEN CONTROL RATS ●—●



(P < 0,005)

TABLE VIII

INHIBITION OF CARRAGEENAN - INDUCED OEDEMA IN THE RAT PAW BY SULINDAC

Pretreatment	Paw Mass at Injection time	Paw Mass at 1 hour (x_1)	Paw Mass at 2 hours	Paw Mass at 3 hours (x_2)	Increase in mass ($x_2 - x_1$)[3]	% In=crease	Percentage Inhibition[2] $= 100\left[1 - \frac{mt}{mc}\right]$
CONTROL RATS (8)[1] Sterile normal saline	20,19g ±3,2[4]	22,57g ±3,2	24,3g ±3,4	26,33g ±4,0	3,76g ±1,3	16,66%	$= 100\left[1 - \frac{4,97}{16,66}\right]$
TEST RATS (8) Sulindac 3 mg per kg.	19,48g ±1,5	21,72g ±2,2	22,6g ±2,9	22,80g ±3,3	1,08g ±1,6	4,97%	= 70%

[1] Number of animals used in parenthesis.

[2] Mt and Mc are % increases in paw mass of the drug treated and control groups of rats respectively, (increase in mass is taken as being proportional to an increase in volume).

[3] The increase in paw mass is measured between one hour and three hours.

[4] Standard deviation.

($P < 0,005$)

FIG.16. INCREASE IN PAW MASS IN EIGHT TEST RATS PRETREATED WITH SULINDAC 3mg PER kg ○—○ AND IN EIGHT CONTROL RATS ●—●

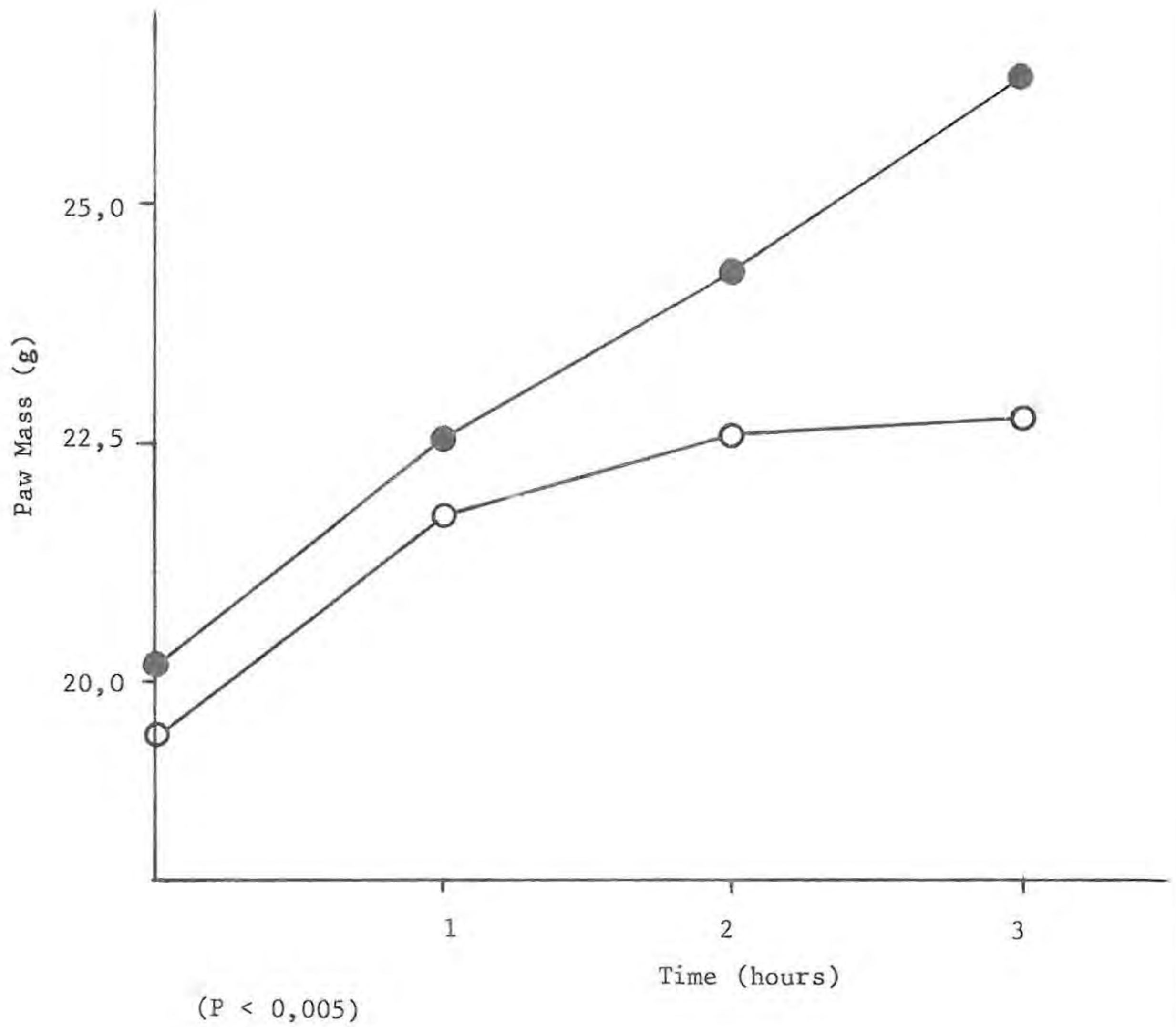
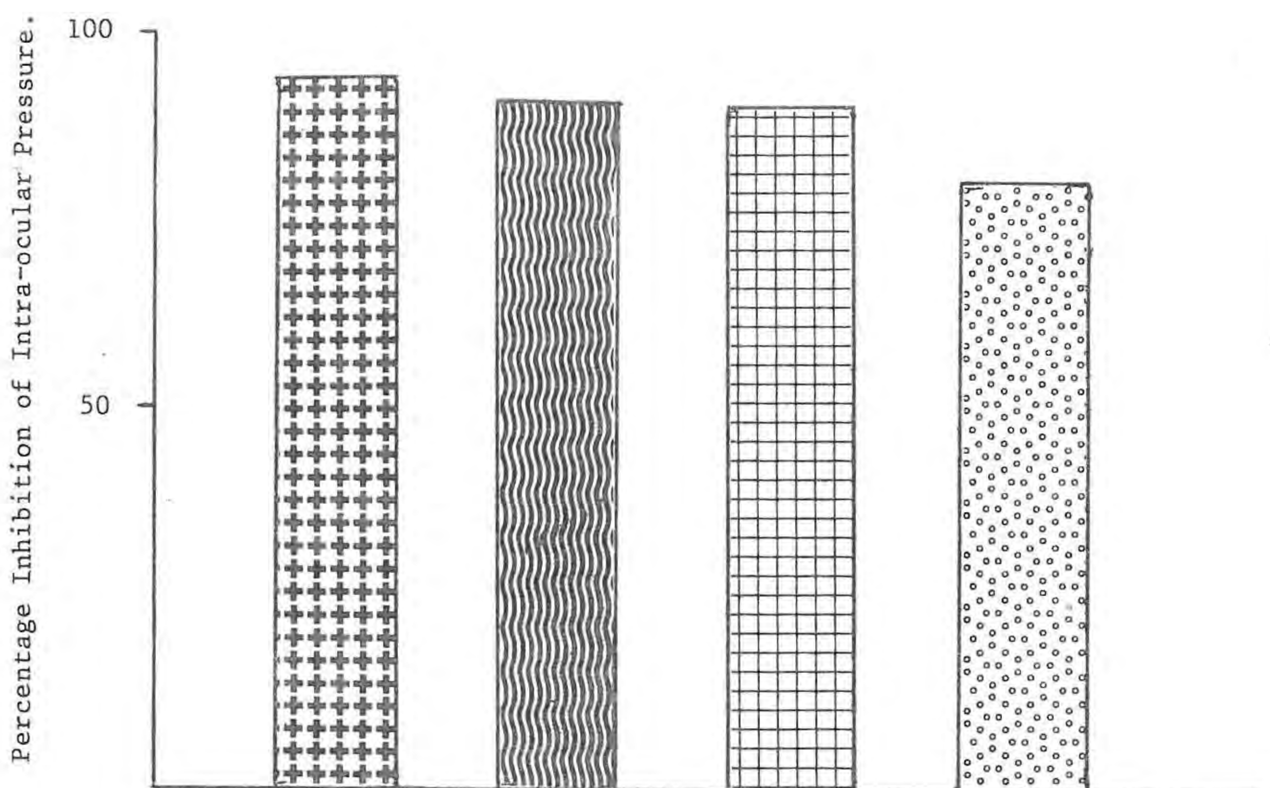
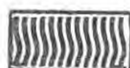


FIG.17. PERCENTAGE INHIBITION OF INTRA-OCULAR PRESSURE AFTER PRETREATMENT WITH ANSAI COMPOUNDS



Indomethacin
4 mg per kg.

Flufenamic acid
10 mg per kg.


Diclofenac sodium
1 mg per kg.

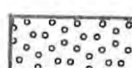
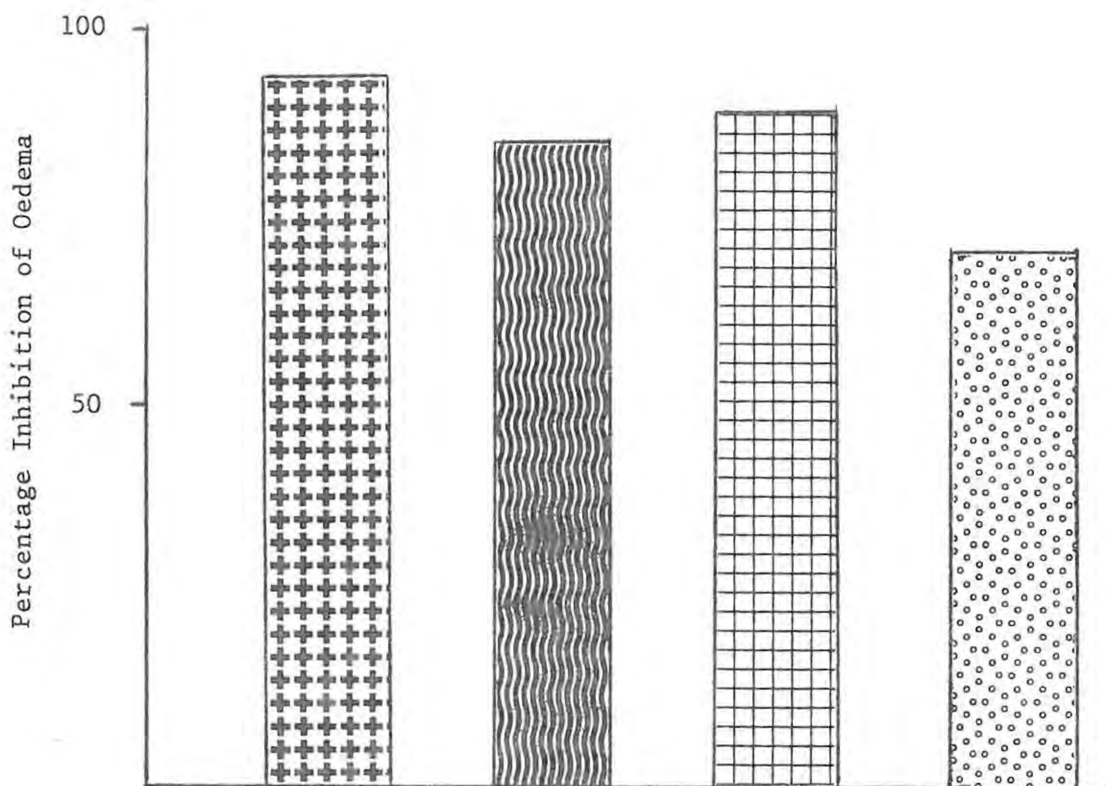
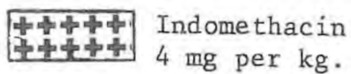
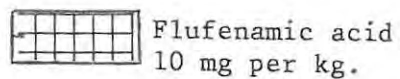
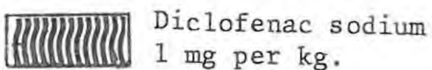

Sulindac
3 mg per kg.

FIG.18. PERCENTAGE INHIBITION OF CARRAGEENAN-INDUCED OEDEMA IN THE RAT PAW AFTER PRETREATMENT WITH ANSAI COMPOUNDS









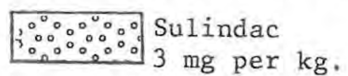
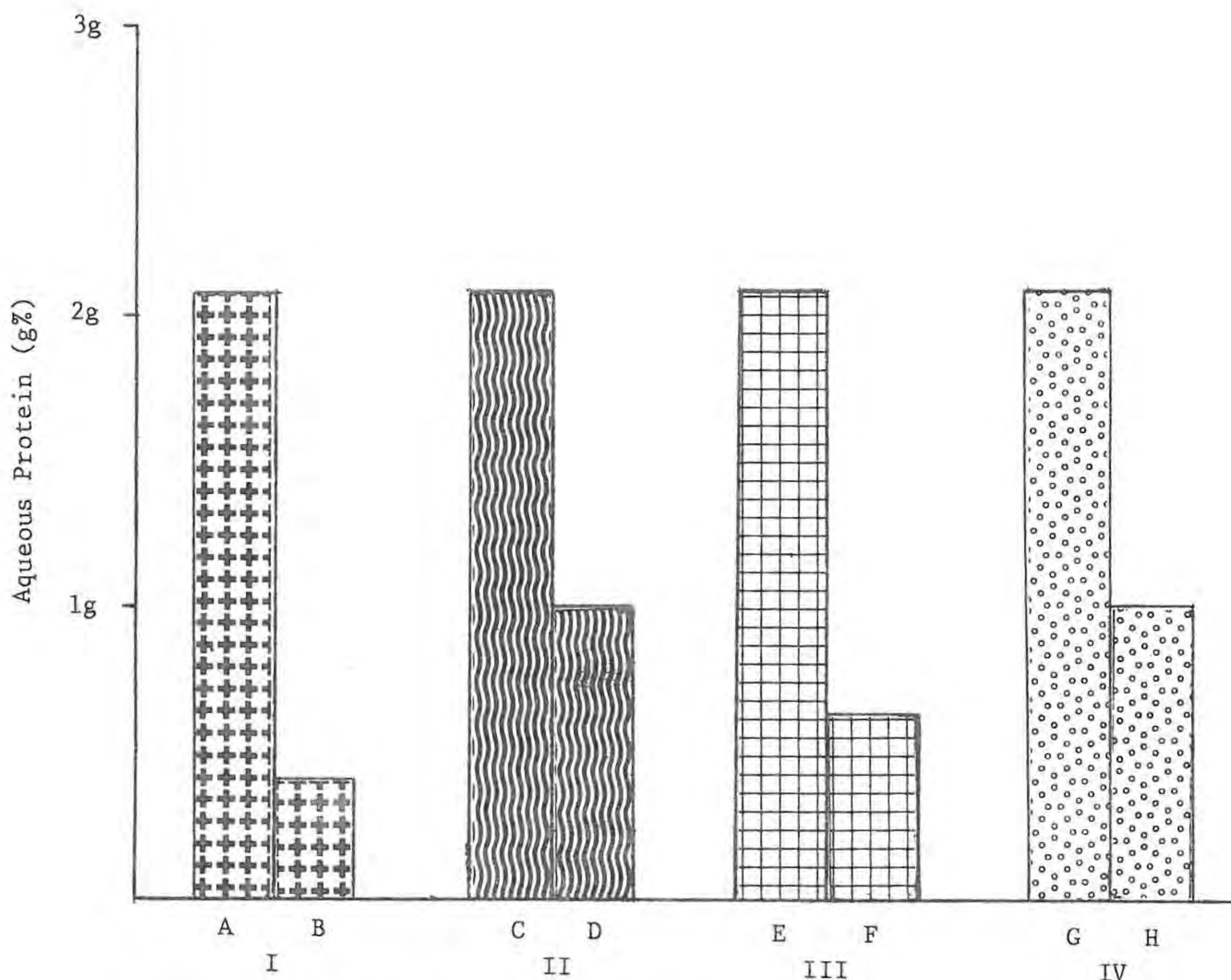


FIG.19. A COMPARISON OF THE PROTEIN CONTENT OF THE AQUEOUS HUMOUR AFTER PRETREATMENT WITH ANSAI COMPOUNDS



CONTROL RABBITS (24)[1]: Groups A, C, E and G

PRETREATED RABBITS : Groups B, D, F and H

I Rabbits in group B (8) were pretreated with Indomethacin 4 mg per kg.

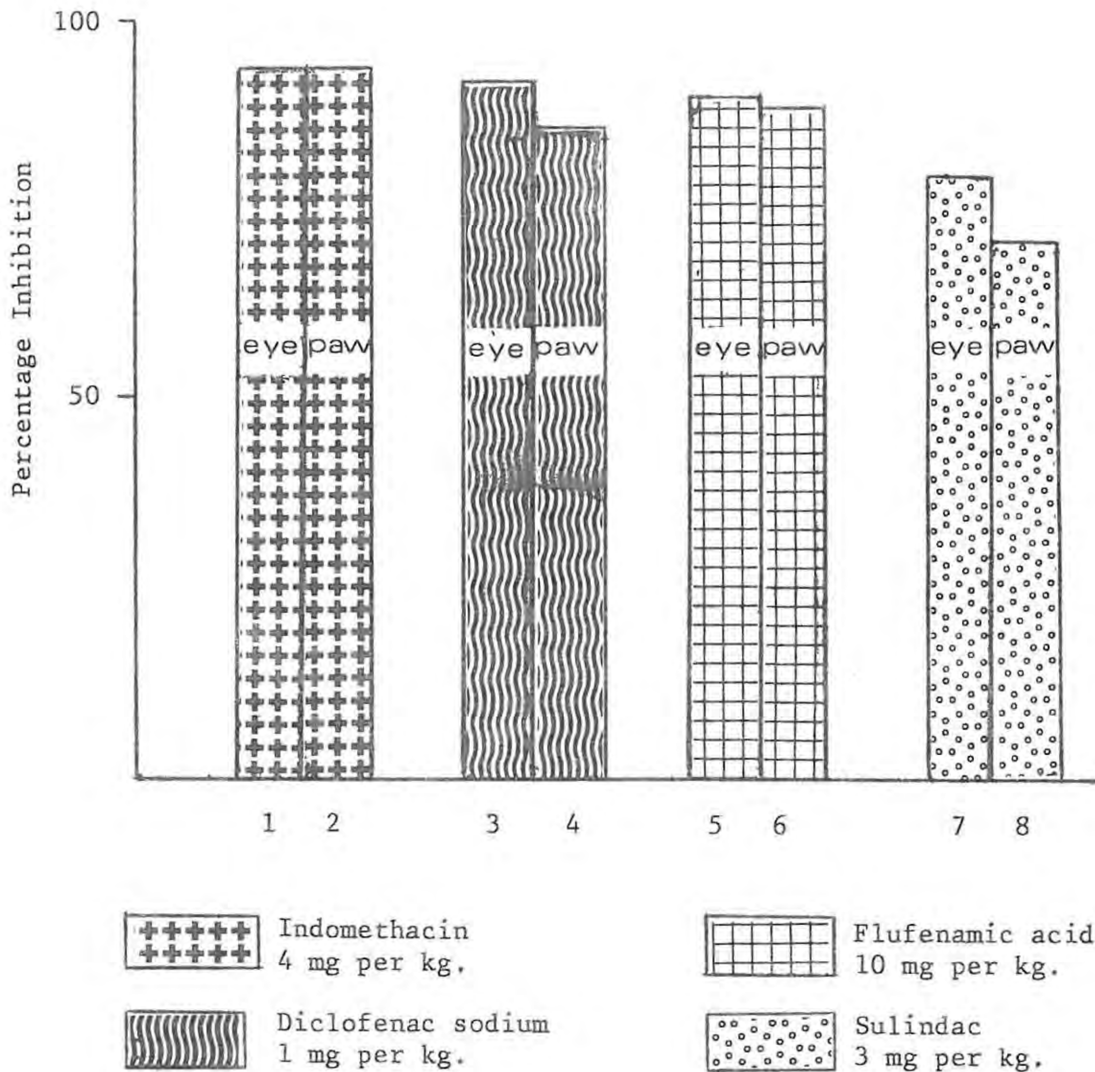
II Rabbits in group D (4) were pretreated with Diclofenac sodium 1 mg per kg.

III Rabbits in group F (8) were pretreated with Flufenamic acid 10 mg per kg.

IV Rabbits in group H (4) were pretreated with Sulindac 3 mg per kg.

[1] Number of rabbits used in parenthesis.

FIG.20. COMPARISON OF THE TWO TEST SYSTEMS



COLUMNS 1,3,5 and 7 : Percentage inhibition of the elevation of intra-ocular pressure in the rabbit

COLUMNS 2,4,6 and 8 : Percentage inhibition of oedema in the rat paw.

CONCLUSION

The four ANSAI compounds showed a similar degree of activity in both the rabbit eye test system and the rat paw oedema methods of determining anti-inflammatory action. Indomethacin was equally effective in both systems, while the other three compounds viz. diclofenac sodium, flufenamic acid and sulindac, had a slightly greater effect on the rabbit eye test system. (See Fig 20 Pg 78 i.e. comparison of results).

Results obtained suggest that the rabbit eye test system has a number of advantages over the rat paw oedema method as an *in vivo* test system for the determination of anti-inflammatory activity of acidic non-steroidal anti-inflammatory (ANSAI) compounds:

(i) in the rabbit-eye-test system the responses to the inflammagen are large allowing an accurate assessment of effectiveness of the compound being evaluated;

(ii) two parameters can be measured, viz. I.O.P. and the protein content of aqueous humour, compared to one, viz. the increase in volume in the rat paw;

(iii) there is no injection trauma. An advantage of the rabbit eye test system is that the inflammatory agent is instilled into the eye whereas the carrageenan has to be injected into the rat paw;

(iv) the rabbit eye test system is more specific. The mode of action of ANSAI compounds probably involves the inhibition of the synthesis of prostaglandins. By using sodium arachidonate, the prostaglandin precursor, substances which inhibit the biosynthesis of the prostaglandins can be evaluated for anti-inflammatory effect.

The rabbit eye test system deserves serious consideration as a model for the production of an acute inflammation when designing a screening programme for the detection of acidic non-steroidal anti-inflammatory compounds.

SUMMARY

A study of acute inflammation, with particular reference to acute ocular inflammation, has been undertaken and the histopathology of acute ocular inflammation, caused by sodium arachidonate, has been examined.

Current methods of evaluating non-steroidal anti-inflammatory drugs have been scrutinised and four categories of assays presented. An "ideal" screening programme, as suggested by Swingle, has been presented.

The assay procedures used in this study, viz. the rat-paw-oedema method and the rabbit eye method have been described.

Carrageenan as a phlogistic agent and its suitability as an inflammagen in the rat-paw-oedema method of assay, has been discussed.

A short review has been given on arachidonic acid, a precursor of the prostaglandins, and the history, biosynthesis and mechanism of action of the prostaglandins presented. Russell _et al_ (1975)

The role of the prostaglandins, with particular reference to ocular inflammation, has been reviewed with reference to the effect of prostaglandins on intra-ocular pressure.

The carrageenan-induced inflammatory response in the rat-paw has been examined, and the possible involvement

of prostaglandins in the secondary phase of swelling, has been considered.

A classical non-steroidal anti-inflammatory agent has been defined and a hypothetical receptor proposed (Scherrer et al 1964) (Shen 1965). A possible conformation of prostaglandin E_2 has been compared with that of indomethacin. The inhibition of prostaglandin synthesis has been studied and four prostaglandin synthetase inhibitors were discussed. The acidic non-steroidal anti-inflammatory (ANSAI) compounds studied were indomethacin, diclofenac sodium, flufenamic acid and sulindac.

A description of the experimental procedures used for the determination of the two parameters, viz. intra-ocular pressure and the protein content of aqueous humour of the eye of the anaesthetized albino rabbit, have been given. The methods in solubilizing the four ANSAI compounds have been discussed. The anaesthetic used and the method of producing anaesthesia has been explained. The Mackay-Marg tonometer has been discussed and the interpretation of a tonogram studied. The technique necessary to obtain legible tonograms was presented. The aspiration of aqueous humour has been described and the method of protein determination given.

The experimental detail of rat paw oedema method of assay has been described. Sedation of the rats with thiopentone sodium has been discussed. Hall and Hallet's method for measurement of paw volume was also presented.

References

A

Ambache, N., Brummer, H.C., A simple chemical procedure for distinguishing E from F prostaglandins with application to tissue extracts. Brit. J. Pharmacol. Chemotherapy, 33, 162-170 (1968).

Ambache, N., Kavanagh, L., Whiting, J. Effect of Mechanical Stimulation on rabbits' eyes, release of active substance in anterior chamber perforates. J. Physiol. 176, 378-408 (1965).

Ariens, E.J. In: "Drug Design" (E.J. Ariens, Ed.) Vol I. New York. Academic Press. 1971 p. 162-193.

Arrigoni-Martelli, E., and Conti, I. Farmaco, Ed. Prat. 19, 134 (1964)

B

Beitch, B.R. Eakins, K.E. The effects of prostaglandins on the I.O.P. of the rabbit. Br. J. Pharmacol. 37, 158-167. (1969).

Bergström, S., Ryhage, R., Samuelsson, B., and Sjovall, J. Prostaglandins and Related factors, 15. The Structures of Prostaglandins E_1, $F_1\alpha$, and $F_1\beta$. J. Biol. Chem. 238, 3555-3564 (1963)

Bethel, R.A. and Eakins, K.E. The mechanism of the antagonsim of experimentally induced ocular hypertension by polyphoretin phosphate. Exp. Eye Res. 13, 83-91 (1972).

Bhattacherjee, P. Release of prostaglandin-like substances by Shigella endotoxin and its inhibition by non-steroidal anti-inflammatory compounds. Br. J. Pharmac., 54, 489-494 (1975).

Bhattacherjee, P. and Eakins, K.E. Inhibition of the ocular effects of sodium arachidonate by anti-inflammatory compounds. Prostaglandins Vol. 9 No. 2 pg 177 (1975).

Bhattacherjee, P. and Eakins, K.E. Inhibition of the prosta= glandin synthetase systems in ocular tissues by indomethacin. Brit. J. Pharmacol. 50 : 227, (1974)

C

Chiang, T.S., and Thomas, R.P., Invest. Opthalmol. 11, 169-176 (1972)

Christ, E.J. and van Dorp, D.A., Biochem. Biophys. Acta 270 537-545 (1972).

Cochrane, C.G. and Janoff, A. In the inflammatory process III London Academic Press (1974) p. 86-155.

Cohnheim, J. (1889). In: "Lectures on General Pathology", p. 248 London : New Sydenham Society. (This is a translation of the 2nd edition of Cohnheim's book published in 1882).

D

Di-Rosa, M., and Sorrentino, L. The mechanism of the inflammatory effect of carrageenan. Eur. J. Pharmac. 4, 340 (1968).

Di-Rosa, M., Willoughby, D.A. Screens for anti-inflammatory drugs. Letters to the editor, J. Pharm. Pharmac, 23, 297 (1971).

Doherty, D.S. and Robinson, B.V. Inflammatory Response to carrageenan - Communication J. Pharm. and Pharmac, 27, 701 (1975).

E

Eakins, K.E. Increased intra-ocular pressure produced by prostaglandins E_1 and E_2 in the cat eye. Exp. Eye Res. 10, 87-92 (1970).

Eakins, K.E. Prostaglandins and Prostaglandin synthetase Inhibitor : Actions in Ocular Disease. In Prostaglandin Synthetase Inhibitors Ed. by H.J. Robinson and J.R. Vane. Raven Press New York (1974).

Eakins, K.E., Whitelocke, R.A.F., Bennett, A and Martenet, A.C. Prostaglandin-like activity in ocular inflammation. Brit. Med. J. 3, 452-453 (1972).

F

Ferreira, S.H., Moncada, S and Vane, J.R. Indomethacin and aspirin abolish prostaglandin release from spleen. Nature New Biology, 231 : 237 (1971).

Ferreira, S.H. and Vane, J.R. Inhibition of prostaglandin biosynthesis - an explanation of the therapeutic effects of non-steroid anti-inflammatory agents. In : Seminaire Inserm Prostaglandins, p. 345-357 Paris 1973

Flower, R., Gryglewski, R., Herbaczyńska-Cedro, K., and Vane, J.R. Nature (London) New Biol. 238 104 (1972).

Ford-Hutchinson et al - J. Pharm. and Pharmacol. 27, 106-112 (1975).

Früh, F., Faigle, J.W., Krupp, P.J., Menassé, R., Schmid, K. p. 17. Personal communication with Ciba-Geigy S.A. Literature on Diclofenac sodium (1975).

G

Garrattini, S., Jori, A., Bernardi, D., Carrara, C., Paglialunga, S. and Segre, D. Int. Symp. Non-steroidal anti-inflammatory drugs, Proc., p. 151 (1965).

Giroud, J.P. and Willoughby, D.A. Ibid., 101, 241 (1970).

Goodman, L.S. and Gilman, A. The Pharmacological Basis of Therapeutics - 5th Edition N.Y. MacMillan 1975 p. 643.

Gryglewski - Copernicus Academy - PG and PG synthesis inhibitors in inflammation. In report by Prof. W.P. Leary on 6th International Congress of Pharmacology (1975).

H

Hall, J.M., Hallett, C. A simple precise method for measuring rodent paw volume. Communications J. Pharm. Pharmac. 27, 623 (1975).

Ham, E.A., Cirillo, V.J., Zanetti, M., Shen, T.Y. and Kuehl, F.A. In : "Prostaglandins in Cellular Biology" (P.W. Ramwell and B.B. Phariss eds.) p. 345-352 Plenum New York (1972).

Hamberg, M., and Samuelsson, B. On the mechanism of the biosynthesis of prostaglandin E_1 and $F_1\alpha$. J. Biol. Chem. 242, 5336-5343 (1967).

Hamberg, M. and Samuelsson, B. Detection and isolation of an endoperoxide intermediate in prostaglandin synthesis Proc. Natn. Acad. Sci. U.S.A. 70, 899-903 (1973).

Hichens In Vitro properties. Anti-inflammatory agents. Chemistry and Pharmacology Vol. II. Scherrer and Whitehouse Academic Press 1974 p. 285.

Hicks, R. Inflammation and Repair in Damaged Tissue. Brit. Pharmaceutical Journal Sept. 6, 1975.

Higgs, G.A., and Youlten, L.J.F. Prostaglandin production by rabbit peritoneal polymorphonuclear leucocytes in vitro Br. J. Pharmac. 44, 330 P. (1972).

K

Kaley, G and Weiner, R., 1971 Ibid. 234 114-115 (1971).

Kelly, R.G.M., and Starr, M.S. Effects of prostaglandins and a prostaglandin antagonist on intra-ocular pressure and protein in the monkey eye. Canad. J. Ophthal, 6, 205-210 (1971).

Klenberg, D., and Samuelsson, B. The biosynthesis of prostaglandin E_1 studied with specificially ^3H-labelled 8, 11, 14-eicosatrienoic acids. Acta Chem. Scand. 19, 534-535 (1965).

Krupp, P.J., Jacques, R., Menassé, R., Wilhelmi, G. and Ziel, R. Int. Congr. Pharmacol. 5th. Abstract No. 782 (1972).

Krupp, P.J., Menassé, R. and R. Ziel. Chemistry and Pharmacology of Diclofenac (Literature supplied by Ciba Geigy Labs.) 1975.

Kuehl, F.A. Jr. Prostaglandins, cyclic nucleotides and cell function. Prostaglandins 5, 325-340 (1974).

Ku, E.C., Wasvary, J.M., Cash, W.D. Diclofenac sodium (GP 45840 Voltaren) A potent inhibitor of prostaglandin synthetase Biochemical Pharmacology Vol. 24 Printed in Great Britain Pergamon Press1975 : 641-643.

M

Masuda, K., Nippin Ganka Gakkai Zasshi 76 : 664-668 (1972).

McCall, E., and Youlten, L.J.F. J. Physiol. London 234, 98-100 (1973).

McCandless, E.L. Fed. Proc. Fed. Amer. Soc. Exp. Biol. 21, 166 (1962).

McCandless, E.L. and Lehoczky-Mona, J. Growth 28, 143 (1964)

Menkin, V. "Dynamics of Inflammation" : MacMillan, New York (1940).

Miles, A.A. In : "Wound healing" C. Illingsworth London Churchill 1966 3p

Moncada, S., Ferreira, S.H., Vane J.R. Nature London, 246, 217 (1973).

P

Piper, P.J., Vane J.R. Nature, London, 223 29 (1969).

R

Rocha e Silva M., and Garcia Leme, J. "Chemical Mediators of the acute inflammatory reaction". Pergamon Press. Inc. 1972 : 199-229.

Rodger, A.F., Whitelock, M.B., Eakins, K.E. Vascular changes in the anterior uvea of the rabbit produced by prostaglandins - Arch. Ophthalmol, 89 495-499 (1973).

Russell, P.T., Eberle, A.J., Cheng, H.C. The Prostaglandins in Clinical Medicine. A developing role for the Clinical Chemist. J. Clinical Chemistry 21/6, 653-666 (1975).

S

Samuelsson et al, Role of PG endoperoxides in human platelets From Report on 6th Int. Congress of Pharmacology 1975 (Report by Prof. W.P. Leary).

Scherrer, R.A. Chemistry of anti-inflammatory agents in : "Anti-inflammatory Agents - Chemistry and Pharmacology". Vol. I eds. Scherrer and Whitehouse. N.Y. Academic Press (1974) 33-56.

Scherrer, R.A., Winder, C.U., and Short, F.W. Nat. Med. Symp. Amer. Chem. Soc. Div. Med. Chem. Abstract 11a-11i (1964).

Schwartz, H.J. and Kellermeyer, R.W. Carrageenan and delayed hypersensitivity. Activation of Hageman factor by carrageenan and its possible significance. Proc. Soc. Exp. Biol. Med., 132, 1021 (1969).

Serafini-Cessi, F. and Cessi, C. Chromatographic separation of an inhibitor reversibly associated with fibrinolysin in the serum of the guinea pig. Experientia 19, 357 (1963).

Sevitt, S. Journal of Pathology and Bacteriology, 75, 27 (1958).

Shen, T.Y. (1965) Int. Symp. Non-steroidal Anti-inflammatory Drugs Proc. p. 13-20 (1964)

Smith, M.J., Ford-Hutchinson, A.W., Elliott, P.N.C., Bolam, J.P. J. Pharm. and Pharmac., 26, 692-698 (1974).

Smith, D.B., O'Neill, A.N. and Perlin, A.S. Can. J. Chem. 33, 1352 (1955)

Smith, J.B. and Willis, A.L. Aspirin selectively inhibits prostaglandin production in human platelets. Nature New Biology, 231 p. 235 (1971).

Spector, W.G. and Willoughby, D.A. In : "The Pharmacology of Inflammation" London - The English University Press Ltd. (1968).

Starr, M.S. Exp. Eye Res. 11 : 161-177 (1971)

Steinberg, D., Vaughan, M., Nestel, P.J. et al. Effects of prostaglandins on hormone-induced mobilization of free fatty acids. J. Clin. Invest. 43, 1533-1540 (1964).

Stone, C.A., van Arman, C.G., Peck, H.M., Minsker, D.H., Ham, E.A. Pharmacologic and Toxicologic actions of PG synthetase inhibitors. Potential role of PG synthetase blockade. In PG synthetase Inhibitors. H.J. Robinson and J.R. Vane. New York. Raven Press. (1974).

Straughan, J.L. University of Stellenbosch - Faculty of Medicine Personal correspondence (1975).

Swingle, K.F. Evaluation for anti-inflammatory activity. In : Anti-inflammatory agents - Chemistry and Pharmacology. Scherrer and Whitehouse Vol. II N.Y. Academic Press 1974, 34-63.

T

Tomlinson, R.V., Ringold, H.J., Qureshi, M.C. and Forchielli, E. Biochem. Biophys. Res. Commun. 46, 552 (1972).

Trevor-Roper, P.D. In : "Ophthalmology"- a text book for diploma students - 2nd edition, London : Lloyd-Luke (Medical Books) Ltd. 4,7,8,91,93,95 and 535.(1962).

V

van Arman, C.G., Risley, E.A. and Kling, P.J. (1971) Pharmacologist 13, 284 (1971).

Vane, J.R. Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs, Nature (London) New Biol. 231, 232 (1971).

Vinegar, R., Macklin, A., Traux, J.F., and Selph, J.L. Pharmacologist, 13, 284 (1971).

Vinegar, R., Schreiber, W., and Hugo, R. J. Pharmacol. Exp. Ther. 166, 96 (1969).

Vogt, W. 1974 Pharmac. Review 26, 125-169 (1974)

W

Waitzman, M.B. and King, C.D. Prostaglandin influences on I.O.P. and pupil size. Am. J. Physiol. 212 329-334 (1967)

Walter and Israel : "General Pathology" - 4th Ed. 1974 69-83

Willis, A,L. In : Prostaglandins, Peptides and Amines ed. by Mantegazza, P, and Horton, E.W. London.Academic Press (1969)

Winder, C.V., Wax, J, and Been, M.A. Arch, Int. Pharmacodyn. Ther. 112, 174 (1957)

Winder, C.V., Wax, J., Serrano, B., Jones, E.M. and McPhee, M.L. Arthritis Rheum. 6, 36 (1963)

Winter, C.A., Risley, E.A. and Nuss, G.W. Proc. Soc. Exp. Biol. Med. 111, 544 (1962)

Winter, C.A., Risley E.A., and Nuss, G.W. Anti-inflammatory and antipyretic activities of indomethacin. J. Pharmac. Exp., Ther. 141, 369-376 (1963).

Whitehouse, M.W., "Regulation of Inflammation and the immune response". In : Anti-inflammatory agents - Chemistry and Pharmacology - Scherrer and Whitehouse N.Y. Academic Press 1974 p. 4 and 22

Wiseman, E.H. Drug Kinetics of anti-inflammatory agents. In : Anti-inflammatory Agents - Chemistry and Pharmacology Vol. II. Scherrer and Whitehouse N.Y. Academic Press 1974 p.235.

APPENDIX I

INDEX TO PHOTOGRAPHS

P 1 Injecting carrageenan into the plantar area of the rat-paw.

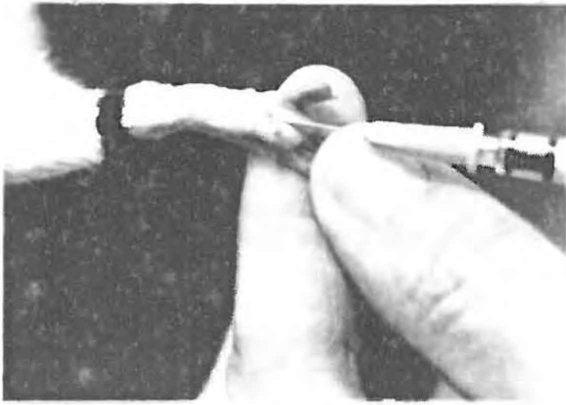
P 2 Measuring paw mass.

P 3 Probe used to measure intra-ocular pressure.

P 4 Measurement of intra-ocular pressure Probe protected by a rubber membrane.

P 5 Mackay-Marg tonometer showing a tonogram.

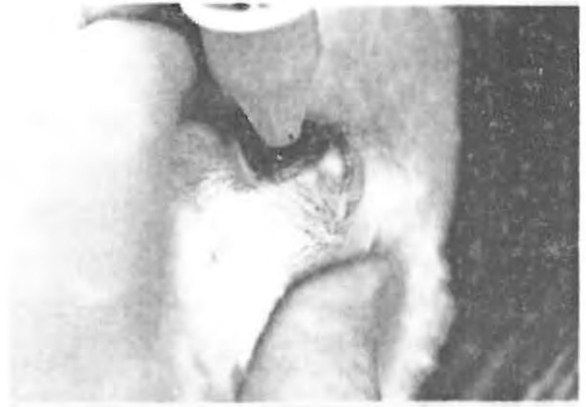
P 6 Aspirating the rabbit eye.



P1

P2

P3



P4

P5

P6

APPENDIX II

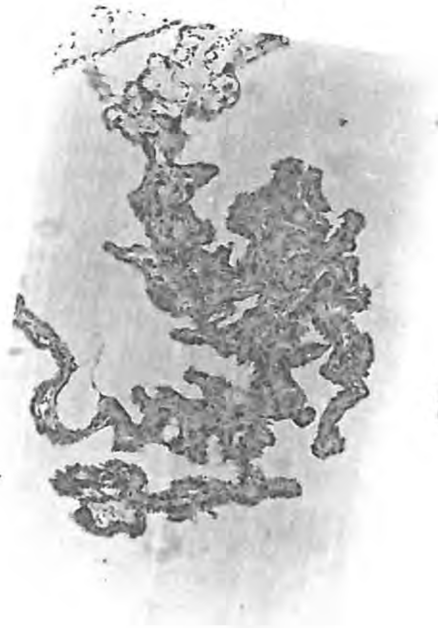
HISTOLOGICAL OBSERVATIONS OF THE SODIUM ARACHIDONATE-INDUCED INFLAMMATORY RESPONSE IN THE RABBIT EYE

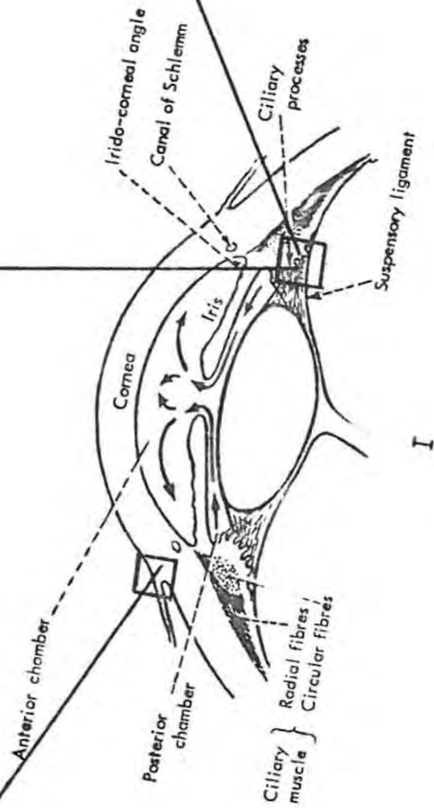
I   Diagram of anterior section of the eye showing the areas (□) which were examined microscopically.

II   Region of aqueous veins with polymorphonuclear leucocyte infiltration.

III   Normal Tissue - Before the induction of inflammation (low magnification).

IV   Capillary dilation, congestion and interstitial oedema after the administration of sodium arachidonate (high magnification).





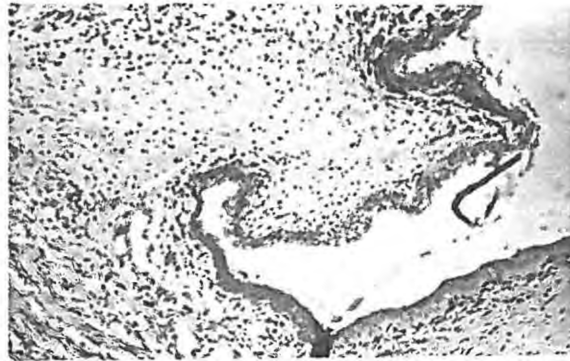
Irido-corneal angle
Canal of Schlemm
Ciliary processes
Suspensory ligament
Cornea
Iris
Anterior chamber
Posterior chamber
Ciliary muscle
Radial fibres
Circular fibres
I
II
III
IV

APPENDIX III

HISTOLOGICAL OBSERVATIONS CONTINUED:

P 1 A section of the angle of conjunctival reflection is shown before the administration of sodium arachidonate.

P 2
3
4 Show magnified sections of the angle of the conjunctival reflection after the administration of sodium arachidonate. The inflammatory response has occurred, and interstitial oedema, polymorphonuclear infiltration, vascular dilatation and congestion can be seen.



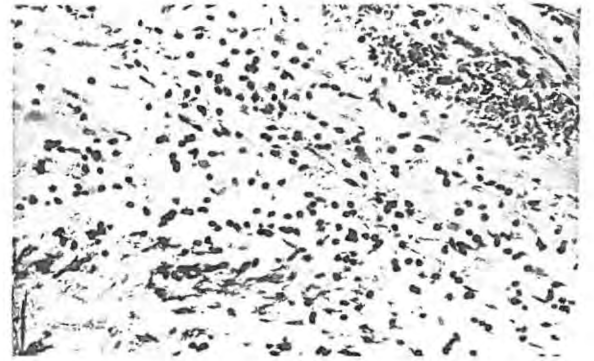
P 3

(Magnification x 250)

Sodium Arachidonate is instilled causing an inflammatory response

P 2

(Magnification x 250)



P 4

(Magnification x 600)

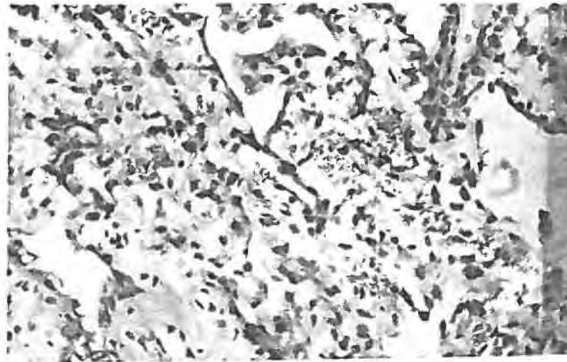
P 1

Angle of Conjunctival Reflection (before induction of inflammation)

APPENDIX IV

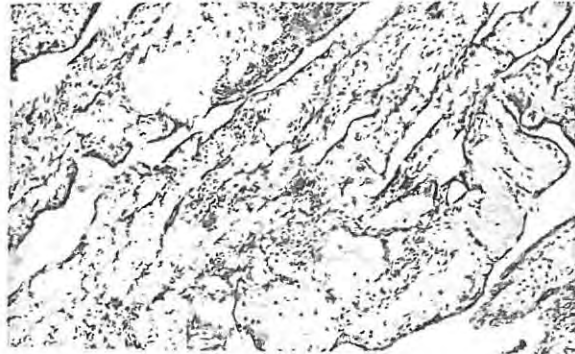
HISTOLOGICAL OBSERVATIONS CONTINUED:

P 1	A section of the Ciliary Body is shown before the administration of sodium arachidonate. The nuclei of the endothelial cells, nuclei of the supporting mesenchymal cells and a few polymorphonuclear cells can be seen.
P 2 3 4	These show magnified sections of the Ciliary Body after the administration of sodium arachidonate. The inflammatory process has occurred and vascular dilatation, vascular engorgement and interstitial oedema can be seen.



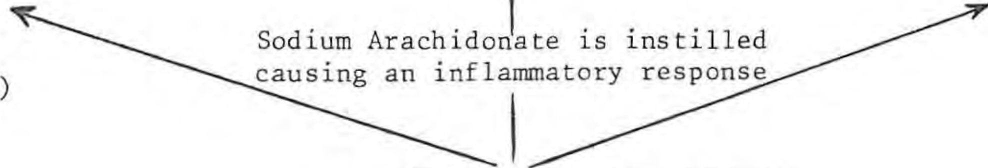
P 3

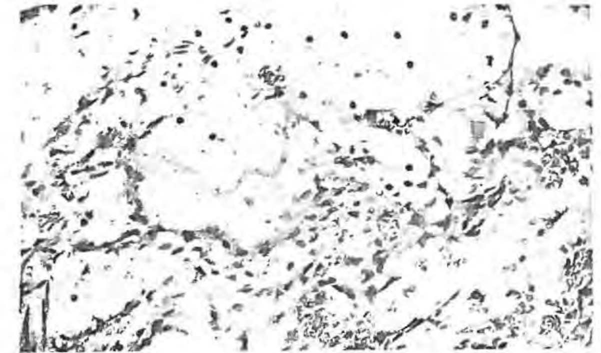
(Magnification x 250)



P 2

(Magnification x 100)





P 4

(Magnification x 400)

P 1

Ciliary Body (before the induction of inflammation)