Short Title of Thesis:

BIOLOGICALLY ACTIVE SUBSTANCES FROM OAK GALL EXTRACTS

Ph.D. Experimental Medicine

David Chu

Isolation of Biologically Active Substances from Oak Galls exerting Antihistaminic and Anti-Inflammatory Activity.

Abstract

The work presented in this thesis describes a method for the isolation of an antihistamine-like substance (KC-18) from the Hungarian oak gall extracts. Data obtained concerning the structure of KC-18 strongly suggested that it is an ester of piperonylic acid. In animal experiments, KC-18 was found to antagonize the actions of histamine in a dose-related manner when tested by the aerosol and capillary permeability methods. KC-18 also exerted anti-anaphylactic activity in actively sensitized guinea pigs. In addition, KC-18 inhibited histamine-induced hypotension and gastric acid secretion, but it could not antagonize the histamine-induced contraction of the isolated guinea pig ileum.

The investigations also resulted in obtaining a highly purified oak gall extract which exerted potent anti-inflammatory activity in rats when tested by the carrageenan-induced paw edema test. The pretreatment of rats with this purified oak gall extract also partially protected the animals from the subsequent development of polyarthritis induced by the injection of mycobacterial adjuvant.

ISOLATION OF BIOLOGICALLY ACTIVE SUBSTANCES FROM OAK GALLS EXERTING ANTIHISTAMINIC AND ANTI-INFLAMMATORY ACTIVITY.

- by -

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THESIS

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CHAPTER I. HISTORICAL REVIEW

PART 1. IMMEDIATE HYPERSENSITIVITY

Section A. Present Concept of the Anaphylactic Reaction

The first fundamental contribution to the field of anaphylaxis originated from the observations of Richet (1898) and Richet and Portier (1902). They noted that the serum of eels or extracts of actinaria given intravenously to dogs were somewhat toxic on the first administration but caused sudden death whenever they were injected, even in a much smaller dose, into the same animals for the second time. Richet called this unexpected reaction "anaphylaxis" as opposed to the prophylactic reaction that he had anticipated.

In 1903, Arthus observed that when small doses of horse serum were injected subcutaneously into rabbits at weekly intervals, the latter doses in such a series were followed by local areas of inflammation, which were extensive and severe, frequently progressing to necrosis. This peculiar reaction of sensitized rabbits to subcutaneous treatment with the specific protein is frequently referred to as the "Arthus Phenomenon". In 1904, Theobald Smith (Lewis, 1908) observed that guinea pigs,treated repeatedly with small amounts of horse serum,frequently died suddenly after a

later injection. For this sudden death he had no explanation, but two years later, in 1906, Otto published his work on "Das Theobald Smithsche Phänomen der Serumüberempfindlichkeit", in which he described what we now recognize as clear cut anaphylaxis.

In 1906, Rosenau and Anderson described the first of their beautifully planned experiments, published between 1906 - 1910, in which they worked out many of the fundamental features of anaphylaxis.

In 1910, Meltzer recognized that the lungs of patients who died of asthma looked much the same as the lungs of guinea pigs following a lethal anaphylactic shock. Schloss, in 1912, published his classical description of a patient sensitive to eggs, almonds and oatmeal. He also observed positive skin reactions with the extracts of the offending substances. Thus, the phenomenon of immediate hypersensitivity in man was correlated with the phenomenon of experimental anaphylaxis in animals.

From 1910 on, research on anaphylaxis rapidly extended into many different phases of the problem. Numerous theories have been put forward to explain this phenomenon. The two most important theories, formulated nearly at the same time, will be discussed here. They are the so called "humoral" or "anaphylactoxin theory", put forward in 1910 by Friedberger and the "cellular theory" brought

to life following the observations of Schultz in 1910 and of Dale in 1912.

The "humoral" theory was based on the work of Friedberger (1910). He found that when normal guinea pig serum was incubated with washed, preformed immune precipitate, a toxic substance was formed in the serum, which, if injected into guinea pigs, elicited a typical anaphylactic reaction. This finding led him to think that this toxic substance "anaphylatoxin" was formed in the guinea pig serum through the action of a proteolytic enzyme, activated by the antigen-antibody complex. However, besides the antigen-antibody complexes, it was found that a whole host of substances , e.g. agar, kaolin, dextran, etc., were also capable of producing anaphylatoxin when incubated with normal guinea pig serum (Keysser and Wasserman, 1911; Bordet, 1913). It was then generally agreed that the presence of immune precipitate or certain other substances mobilize non-specific proteolytic enzymes to cause the cleavage of normal serum protein to produce anaphylatoxin (Jobling et al., 1915; Bronfenbrenner, 1915). This theory requires the presence of serum factors for the formation of anaphylatoxin. The "humoral" theory, however, was virtually abandoned for about 30 years, when Dale and Kellaway (1922) demonstrated that while the addition of anaphylatoxin could only induce a weak and irregular contraction of the isolated guinea pig uterus, the addition of minute quantities of specific antigen to the isolated uterus of

a sensitized guinea pig, which had been thoroughly washed and was definitely free of any serum factor, brought about a strong contraction.

Interest in the "humoral" theory was revived after Hahn and Oberdorf (1950) reported that anaphylatoxin most probably acts through histamine release, since its effects were inhibited by synthetic antihistamines. This report was confirmed by Rocha e Silva (1951) who found that anaphylatoxin releases large quantities of histamine from isolated perfused guinea pig lungs. Giertz et al. (1961) reported that the intravenous injection of anaphylatoxin into guinea pigs resulted in an increased plasma histamine level and the animals frequently died. They also observed that as they increased the dose of anaphylatoxin injected into these animals, the plasma histamine level and incidence of mortality correspondingly increased. On the other hand, they did not find any increase in plasma histamine level of the same species during a lethal anaphylactic shock. From these findings they concluded that during anaphylatoxin shock in guinea pigs, extrapulmonary histamine was probably responsible for the reaction. Recent work by Lepow et al. (1968) and Muller-Eberhard (1968) have provided strong evidence to show that anaphylatoxins are derived from the complement system and are believed to represent small split products of C'3 (anaphylatoxin I) and C'5 (anaphylatoxin II).

In contrast to the "humoral" theory, which states that the anaphy-

lactic reaction starts with the union of antigen and antibody in the serum and that the antigen-antibody complex thus formed results in the formation of anaphylatoxin, the "cellular" theory postulates that the union of antigen and antibody takes place on the cell surface and that the combination of the antigen with its antibody induces the release of "histamine-like substances" which leads to the anaphylactic or allergic syndromes. This theory was proposed by Schultz (1910) and Dale (1912), based on the studies of the anaphylactic response of the guinea pig plain or smooth muscle. Dale reported that the addition of a specific antigen elicited a strong contraction from the sensitized guinea pig uterus, which was washed free of cells and serum. Earlier experiments by Dale and Laidlaw (1910) had shown that the anaphylactic symptoms and those produced by parenterally administered histamine were strikingly similar. From these results, Dale concluded that the most important basic mechanism of anaphylaxis is the combination of a specific antigen to a cell-bound antibody and that this antigen-antibody combination causes the release of "histamine-like substances" which leads to anaphylactic symptoms.

Today, Dale's original concept seems to be basically still valid and all available evidence indicates that the combination of an antigen with its cell-bound antibody initiates a series of tissue damaging reactions. At present, four types of tissue damaging hypersensitivity reactions are recognized. Type I reaction is ini-

tiated by the allergen (antigen) reacting with tissue cells that have already been passively sensitized by antibody or reagin, produced elsewhere in the body, and leading to the release of pharmacologically active substances. In the Type II reaction, the antibody reacts with an antigen present in or attached to a cell-membrane - for example, as occurs in mismatched blood transfusions. Type III (Arthus) reactions are initiated by antigen reacting in the tissue space with precipitating antibody complement, the resulting microprecipitates, after ingestion by phagocytic cells and liberating lysosomes, causing damage to cells and blood vessels - for example, serum sickness. Type IV reactions involve a reaction between antigen and sensitized cells - for example, the tuberculin response,(Turner-Warwick, 1969). In this review, we shall discuss the Type I reaction only.

In the Type I reaction, the most frequently implicated chemical mediator is histamine. When the "cellular" theory of anaphylaxis was gaining widespread acceptance, evidence was accumulated to show that histamine is present in normal mammalian tissues (Best et al., 1927; Dale and Dudley, 1929). Furthermore, the release of histamine has been observed in both in vivo and in vitro anaphylactic reactions following antigenic challenge (Gebauer-Fuelnegg and Dragstedt, 1932; Bartosch et al., 1932; Code, 1939; Katz and Cohen, 1941; Scroggie and Jaques, 1949). Though these data firmly established that the release of histamine is intimately asso-

ciated with anaphylactic and allergic symptoms, it was soon discovered that the "cellular" theory, in its original form, which suggested that histamine alone was responsible for the anaphylactic symptoms, was not entirely correct. For example, it was found that besides histamine other biologically active substances, with similar pharmacological activity, were also released during anaphylaxis (Frey et al., 1933; Schild, 1936; Trethewie, 1939). Furthermore, the histamine theory could not explain the experimental findings of Rocha e Silva (1942), who reported that the administration of a specific antigen in rabbit produced a decrease in blood pressure, while the injection of histamine increased it. In addition, the failure of antihistamines to completely inhibit anaphylaxis (Mota and Vugman, 1956; Malkiel and Hargis, 1952) further suggests that other mediators also participate.

While findings in experimental anaphylaxis were confirming the involvement of other biologically active substances besides histamine, similar results were obtained as well with regard to human anaphylactic reactions. Schild (1951), who used the isolated human bronchial muscle preparation, obtained from an asthmatic patient, found that a very low dose (10^{-9} gm/ml) of the antihistaminic agent mepyramine, markedly reduced the contractions of the bronchial muscle elicited by histamine. The allergen-induced contractions, on the other hand, were only slightly inhibited, even with a much higher dose of antihistamine (10^{-5} gm/ml.) The ob-

servations that the exposure of human asthmatic lung tissue in vitro, to specific allergen, resulted in the release of both histamine and SRS-A further indicated the participation of these chemical mediators in certain forms of anaphylactic bronchospasms in man (Brocklehurst, 1960; 1962).

Since the first prerequisite for the development of an anaphylactic reaction is that the antibody should be adsorbed onto the surface of the target cells, understanding the nature of the anaphylactic antibody was considered important in the elucidation of the mechanism of the anaphylactic reaction.

In man, evidence for the nature of the anaphylactic antibodies (also referred to as reaginic antibodies, skin sensitizing antibodies, Prausnitz-Kustner antibodies or P-K antibodies) was first provided by Perlemutter et al (1966; 1966a) who reported that these antibodies do not belong to any of the known classes of immunoglobulins (IgG, IgA, IgM and IgD). At the same time, Ishizaka and co-workers (1966; 1966a) reported direct evidence that the anaphylactic antibodies in man belong to a new immunoglobulin class - IgE.

It has since been shown that the immunoglobulin in sera of atopic (allergic) individuals capable of mediating the P-K reactions (or the PCA reactions in experimental animals) in man and monkey skin

belong to the IgE class (Ishizaka et al., 1966b; 1967). The capacity of atopic sera to mediate this skin reaction, due to interaction of cell-bound IgE antibody with specific antigen, had been correlated with their ability to passively sensitize human peripheral leukocytes for the antigen-induced release of histamine (Levy and Osler, 1966). In 1970, Ishizaka et al. (1970) were able to demonstrate that both direct and reversed-type anaphylactic release of histamine and SRS-A from primate lung are mediated by an immunoglobulin of the IgE class.

The release of two different mediators by a single class of homologous immunoglobulin has precedence also in other species. In the guinea pig, homologous, heat stable, 7Sγ1 antibodies are capable of preparing perfused, chopped guinea pig lung slices for the antigen-induced release of both histamine and SRS-A (Stechshulte et al., 1967). In the rat, homologous, heat-stable IgGa antibodies mediate the antigen-induced intraperitoneal release of both histamine and SRS-A (Morse et al., 1968; 1969).

Although in man the exact role of histamine and SRS-A in allergic reactions or extrinsic asthma has not yet been clearly established, the association of these conditions with positive skin test, mediated by reaginic antibodies, presumably of the IgE class, has long been appreciated. The findings that the IgE antibodies prepare primate lung tissue for both the direct and reversed-type

anaphylactic release of these two mediators and the apparent sensitivity of the human bronchiole to both histamine and SRS-A, afford suggestive evidence for their participation.

Although, on the cellular level, the exact mechanism of the hypersensitivity reaction, caused by the antigen-antibody combination, remains to be elucidated, it is generally agreed that anaphylaxis occurs when the cell-bound antibody reacts with the antigen. The most widely accepted theory today states that the antigen-antibody complexes formed on the cell surfaces cause some structural changes to the portion of the antibody which is in intimate contact with the cell surface. These alterations or "toxic configurations" activate a chain of energy dependent enzymatic reactions which culminate in the release of histamine and other biogenic amines (Stanworth, 1970).

In the following sections, the possible roles of those chemical mediators (histamine, 5-HT, SRS-A, and kinins), which may contribute to the development of anaphylactic and allergic syndromes,will be briefly considered.

Section B. Role of Chemical Mediators in Anaphylaxis and Allergy.

a) Histamine

Histamine was first suspected to play an important role in the de-

velopment of anaphylactic reactions when Dale and Laidlaw (1910) reported that the symptoms of anaphylactic shock could be mimicked by administering histamine to guinea pigs. Subsequently, a "histamine-like principle" was extracted from urticarial wheals (Lewis and Grant, 1924; Hare, 1926). Furthermore, Lewis (1927) was able to demonstrate that the urticarial wheals formed on the skin of allergic individuals following the intradermal injection of a specific allergen were similar to those seen after the intradermal injection of histamine. In 1927, Best et al. (1927) reported the presence of histamine in normal tissues (liver and spleen) and their findings were confirmed by Harris (1927) and Thorpe (1929).

The first conclusive evidence which proved that histamine is liberated during anaphylaxis was presented by Gebauer-Fuelnegg and Dragstedt (1932). They found that the histamine level was markedly increased during anaphylaxis. Their results were confirmed by Bartosch et al. (1932), who identified histamine in the perfusate of the shocked lungs of guinea pigs as the substance responsible for the bronchoconstriction manifested in guinea pig anaphylaxis.

Since then, it has been shown by many workers that histamine plays an important role in anaphylaxis (Rose, 1940; Austen and Brocklehurst, 1960; Akscasu and West, 1960) in many mammalian species with the exception of rat and mice. In man, besides

histamine, other chemical mediators (Rose, 1950; Schild, 1951; Lecomte, 1956; van Arsdel et al., 1958; Middleton and Sherman, 1961) most probably also contribute to the development of allergic reactions.

The evidence of histamine participation in anaphylaxis stimulated great interest in the possible elucidation of the mechanism of histamine release. This aspect of the investigation was greatly facilitated when Riley and West (1953) established the anatomical locale of histamine store. They were able to show that the treatment of rats with compound 48/80 resulted in a marked decrease in the mast cell population, which was accompanied by a corresponding fall in histamine content. This finding was confirmed and extended by Mota (1957), who observed that when mast cells were exposed to antigen-antibody reaction in the rat, there was an increase in the plasma histamine level. Other investigators have shown that, besides the antigen-antibody reaction, the release of histamine from mast cells could be accomplished by many non-specific substances, e.g., proteolytic enzymes, snake venom, surface active agents, dextran, etc. (Haining, 1955; Halpern and Briot, 1953).

Since Riley and West (1953) reported the relationship between mast cells and histamine, mast cells have continued to be the most commonly used model in the effort to resolve the mechanism of histamine

release in anaphylaxis and allergy. Although the exact mechanism of histamine release remains to be elucidated, a number of hypotheses have been put forward to explain the release of histamine. At present, the two most widely accepted theories were put forward by Mongar and Schild and Unas et al. According to Mongar and Schild (1955, 1957a, b), in the sensitized guinea pig tissues, histamine is released through an energy requiring enzymatic process following antigenic challenge, since histamine release can be inhibited by anoxia, iodoacetic acid and other metabolic inhibitors. The fact that the process is heat labile further supports their contention that the release of histamine is mediated through an enzymatic pathway. A modification of the Mongar and Schild theory was suggested by Austen and Brocklehurst (1961a) when they reported that the release of histamine in guinea pigs required the presence of a chymotrypsin-like enzyme. Supporting evidence for the Mongar and Schild theory recently came from Ranadive and Cochrane (1971) who were able to show that histamine release is temperature sensitive and inhibited by treating mast cells with iodoacetic acid or 2,4-dinitrophenol in glucose free medium. Furthermore, the release of histamine by the "band 2 protein" (specific histamine liberator from rabbit neutrophil) is blocked by diisopropyl (DFP). On the basis of these observations, they concluded that the release of histamine is energy dependent, mediated through a certain labile enzyme system, and that esterases are involved in the release.

Another theory, formulated by Hogberg and Uvnas (1958), stated that the combination of antigen with its antibody activates a normally inactive lytic enzyme located on the mast cell surface. This activated enzyme splits the phospholipid membrane into fatty acid and lysolecithin. Lysolecithin, being strongly lytic, causes cellular damage which leads to the release of histamine.

The recent work of Rothschild (1970) has helped to resolve part of the contradictory data so far obtained. He demonstrated that compound 48/80 releases histamine by two processes. The first is dependent on cell metabolism involving mast cell granule (histamine-containing) secretion. The second is independent of cell metabolism and seems to consist of simple exchange reaction between compound 48/80 and histamine. These findings have been confirmed by Frisk-Holmberg and Stradberg (1971) who reported that while histamine released by turbocurarine is an active process dependent on intact energy metabolism, the release by chlorpromazine is exerted through a membrane permeability change and is unaffected by metabolic blocker. Further confirmation came from the electron microscopic study by Bloom and Chakravarty (1970) who were able to demonstrate that the anaphylactic release of histamine is associated with morphological changes of the mast cells and that these changes were characterized by vacuolation, granule alteration and granule extrusion. In addition, they reported that the ultrastructural findings were consistent with the dual mechanism

of histamine release.

With the information obtained on the release of histamine by antigen-antibody complexes, the release mechanism can be summarized as follows (Figure 1): As a result of the combination of the cell-fixed antibody with the antigen, antigen-antibody complexes will be formed on the surface of the cells. The reaction of the antigen with the antibodies causes structural changes on part of the antibody molecule, which in turn initiates a series of biochemical reactions leading to the release of mediators. In the case of the mast cells, the antigen-antibody causes the extrusion of histamine-containing basophilic granules. The process of the granule extrusion is dependent on energy, cations and temperature. The basophilic granules contain histamine complexed to heparin. Histamine is then released from the granules by an ion-exchange process (probably between histamine and Na^{+} ions).

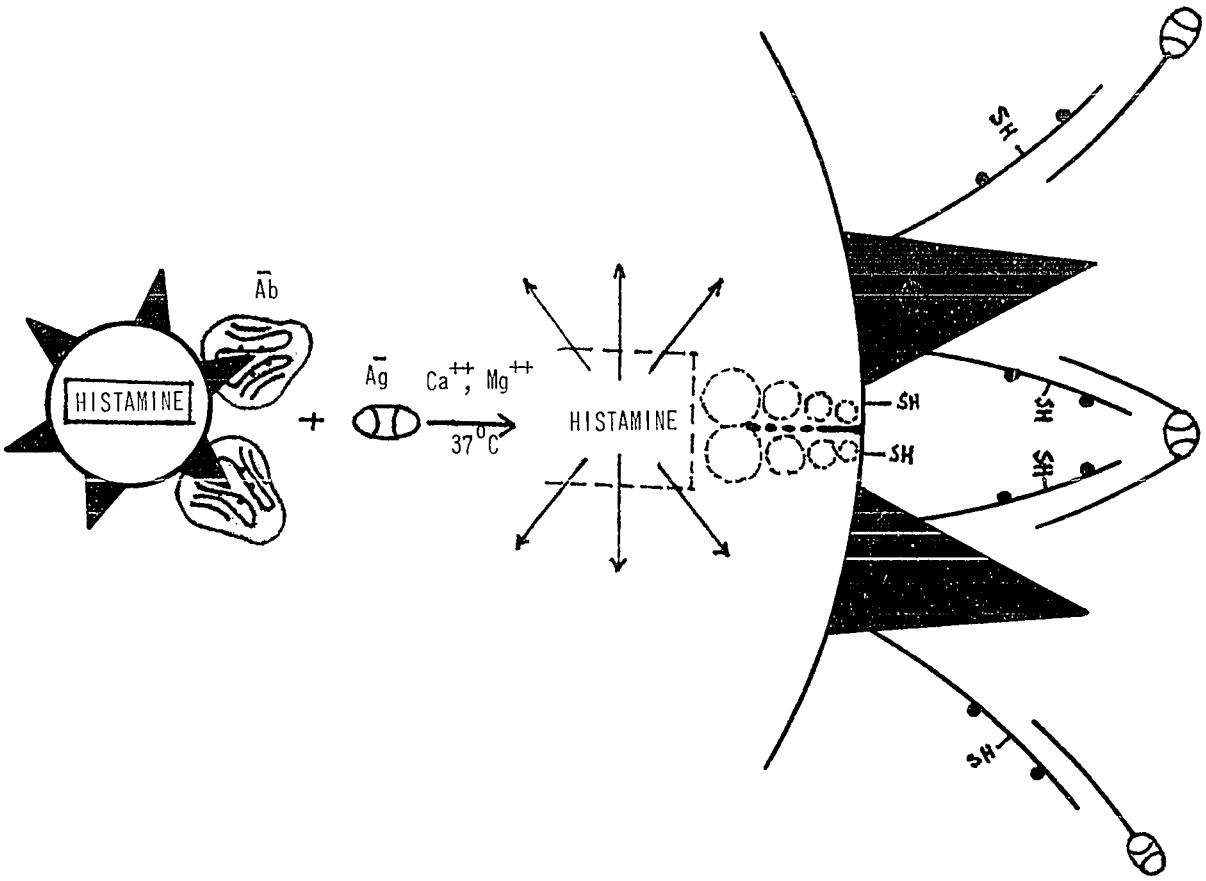


Figure 1

Schematic representation of allosteric transition of cell-bound anaphylactic antibody following combination with specific antigen or allergen (Stanworth, 1969).

b) 5-Hydroxytryptamine

In 1940, Erspamer reported the presence of an unidentified smooth muscle stimulant in mammalian tissue extracts. The active principle was later isolated by Rapport et al. (1948) and identified as 5-hydroxytryptamine (5-HT). It is now known that 5-HT is formed in the body from tryptophan by hydroxylation and decarboxylation (Clark et al., 1955).

The first evidence that 5-HT might be involved in anaphylaxis was reported by Humphrey and Jaques (1955) who found that the addition of a specific antigen to rabbit platelets suspended in heparinized serum, which contained the antibody, resulted in the release of histamine and 5-HT from the platelets. However, no 5-HT was detected in the perfusate of sensitized guinea pig lungs (Fink and Gardner, 1956; Brocklehurst, 1958). Similarly, during anaphylaxis in dogs the liver 5-HT level did not change nor was the liver capable of releasing 5-HT *in vitro* following the addition of a specific antigen (Akscasu and West, 1960). Although a raised plasma 5-HT level was observed during anaphylaxis in rabbit (Waalkes and Coburn, 1959), depletion of 5-HT by reserpine administration could not modify the intensity of the shock (Fisher and Lecomte, 1956), In the mast cells of rat and mouse, Benditt et al. (1955) found not only histamine but also 5-HT in significant quantities. However, there is no direct evidence that 5-HT plays

an important role in the rat anaphylactic reaction (Austen and Humphrey, 1963). On the other hand, depletion of serotonin by reserpine and methyl DOPA markedly suppressed the mouse anaphylactic shock (Fox et al., 1958; Gershon and Ross, 1962). The work of Halpern et al. (1963) showed that 5-HT plays a major role in the development of the passive cutaneous anaphylactic reaction in the mouse. It has been shown by Movat et al. (1962) and Provost-Danon et al. (1966) that 5-HT is released almost simultaneously with histamine from the rat and mouse mast cells. There is also some evidence that they compete for the same binding site in the peritoneal rat mast cells (Cabut and Haegermark, 1966). According to Carlsson and Ritzen (1969), antigen-antibody complexes act on the rat mast cells to liberate 5-HT without concurrent release of histamine-containing granules.

Although Jansson (1970) has shown that while certain substances, such as chlorpromazine and mepyramine, release 5-HT from mast cells by a non-energy dependent pathway, other substances such as reserpine mediate the release through an energy dependent pathway. In the platelets, the release mechanism is, according to Okuda and Nemerson (1971), simpler in that the platelets concentrate 5-HT by an active energy requiring process which counterbalances the loss of the amine from the platelets by passive diffusion.

There are no experimental data which would implicate the participa-

tion of 5-HT in the anaphylactic reaction in man. The isolated human bronchiole is resistant to 5-HT (Brocklehurst, 1958). Herxheimer (1955) has shown that inhalation by aerosol has no effect in normal man and the broncho-constrictor action of the aerosol in some asthmatics (Herxheimer, 1955) merely reflects the general hyperactivity of the asthmatic bronchiole (Austen, 1971). Furthermore, the human lung contains virtually no 5-HT and the mast cells of man, like those of most other species, are devoid of this amine (Austen, 1971).

At present, it may be concluded that 5-HT, in species other than the mouse and rat, does not play an important role in the development of the anaphylactic symptoms.

c) Slow reacting substance of anaphylaxis (SRS-A)

The existence of SRS-A was first reported by Feldberg and Kellaway (1938). They demonstrated that when the guinea pig lung was perfused with cobra venom, a substance was released which elicited a slow and prolonged contraction of the isolated guinea pig ileum. A similar substance(s) was found to be released together with histamine from shocked guinea pig lungs (Kellaway and Trethwie, 1940). The presence of SRS-A was unequivocably confirmed by Brocklehurst (1953) who obtained the characteristic response on the isolated guinea pig ileum in spite of the administration of a potent anti-

histaminic into the bath prior to the addition of the perfusate of the anaphylactic guinea pig lung. Later, Brocklehurst (1960) reported that while a large quantity of SRS-A was released in the lungs of the guinea pig during anaphylaxis, only a trace amount was present in unshocked tissues. He thus concluded that SRS-A was both formed and released during the antigen-antibody reaction and that the lung tissues were the main site of SRS-A formation. In 1963, Berry et al. (1963) demonstrated that SRS-A,given intravenously, induced bronchoconstriction in guinea pigs. However, according to Orange and Austen (1969), SRS-A does not appear to be the most important mediator in the guinea pig, even though it may contribute to the anaphylaxis of this species.

Herxheimer and Stresemann (1963) reported that asthmatic patients exposed to SRS-A aerosol, developed a mild and prolonged bronchoconstriction which was not observed in normal healthy subjects. More recently, Sheard et al (1967) and Parish (1967) described the antigenic-induced release of SRS-A from normal human tissues. The prolonged contraction of the human bronchial smooth muscle <u>in vitro</u> produced by SRS-A underlines the possible involvement of SRS-A in human asthma and anaphylaxis. Of particular relevance is the recent finding of Ishizaka et al. (1969) that the IgE antibodies present in human atopic sera are capable of sensitizing monkey lung tissue <u>in vitro</u> for the subsequent immunological release of both histamine and SRS-A when challenged by the specific antigen.

Although the exact mechanism of the release of SRS-A remains to be elucidated, the available evidence indicates that the release mechanism is species dependent. In the rat, for instance, pre-treatment of the animals with extract of cobra venom (naja naja), which markedly depletes the animal's C'3, was accompanied by an inability of the tissues to release SRS-A (Nelson, 1966; Muller-Eberhard, 1967). On the other hand, a slow reacting material was released by the guinea pig lung tissue following the treatment with extract of cobra venom (Middleton and Phillips, 1964). Macmorine et al. (1969) reported that in rats, a slow reacting substance was released from the neutrophils following the phagocytosis of antigen-antibody complexes in vitro.

It has been shown that while in man and other primates, a single heat labile immunoglobulin IgE is involved in the release of SRS-A and histamine, it appears at present that in guinea pig (Stechschulte et al., 1967) and rats (Morse et al., 1968), a heat-stable immunoglobulin mediates the release of SRS-A. Orange et al. (1970) has also established that the release of histamine and SRS-A mediated by rat IgE is dependent on the participation of mast cells but it does not require the presence of circulating polymorphonuclear leukocytes or intact complement system - thus differing from the PMN leukocyte and complement dependent pathway to SRS-A release mediated by IgGa antibodies of hyper-immune serum. Preliminary studies by Ishizaka et al. (1970), using the monkey lung fragments

incubation technique, have revealed that agents leading to an accumulation of cyclic AMP inhibit the ability of human IgE to mediate the release of histamine and SRS-A. These findings were confirmed by Koopman et al. (1970) who further demonstrated that the administration of dibutyryl cyclic AMP inhibits SRS-A release in a dose response fashion.

d) Kinins

Kinins are a group of biologically active polypeptides which originate mostly from the plasma proteins. Their characteristic pharmacological properties may be briefly summerized as follows: They i) produce hypotension; ii) increase capillary permeability; iii) produce pain when applied to the blister base of the human skin; and iv) are spasmogenic for most isolated smooth muscle preparations.

Frey and associates (1928 - 1933) first reported the presence of a hypotensive substance in the extracts of urine and pancreas. They were able to show that these extracts, injected intravenously into dogs, elicited a fall in blood pressure. They called the active principle "kallikrein". Later, it was indicated by the experiments of Rocha e Silva (1949) that kallikrein is actually an enzyme which by itself is not a hypotensive agent but reacts with the plasma globulin to release a hypotensive polypeptide (named "Brady-

kinin" by him). Bradykinin was isolated in 1960 and its molecular configuration elucidated (Elliot et al., 1960). In the following year, the original observation of Rocha e Silva was further confirmed by Werle (1961) who found that when kallikrein was incubated with globulin, a hypotensive substance (Kallidin) was released. The molecular configuration of kallidin (Werle, 1961) was found to be almost identical with that of bradykinin (Elliot et al., 1960). Furthermore, the activity profile of kallidin was also strikingly similar to that of bradykinin (Holdstock et al., 1957; Pierce and Webster, 1961).

The possible involvement of kinins in anaphylaxis was first indicated by Beraldo (1950) who drew attention to the fact that a bradykinin-like substance was released into the blood of the dog during anaphylactic shock. His observations were confirmed by Brocklehurst and Lahiri (1962) who reported the presence of significant amounts of kinins in the blood of shocked guinea pig, rat and rabbit. Furthermore, they also found that the perfusate of shocked guinea pig lung contained no detectable amount of bradykinin but that it was formed when the perfusate was incubated with plasma pseudoglobulin. Diniz and Cavalho (1963) reported that in rabbits, after systemic anaphylaxis, the plasma substrate for bradykinin formation was depleted. Brocklehurst and Zeitlin (1967) observed that the release of free kinins led to a sudden fall in plasma kininogen levels. According to Abe et al. (1967) the plasma kinin level was

significantly increased in most patients with severe bronchial asthma when compared to normal, healthy subjects. Brocklehurst and Lahiri (1962; 1963) and Jonasson and Becker (1966) have demonstrated the appearance of a kallikrein-like material following the induction of anaphylaxis in blood-free guinea pig lung preparation. According to the data obtained by Collier and James (1967), kinins definitely contribute to the early phase of anaphylaxis in the guinea pig.

The role of plasma kinins in human allergic reactions is not clear. In recent years, naturally occuring vasoactive polypeptides have been suspected to play some role in a variety of pathological conditions (Back, 1966; Erdos, 1966; Kellermeyer and Graham, 1968). There is mounting evidence to suggest the participation of kinins in allergic conditions (Cirstea et al., 1965; Back et al., 1968; Konopa and Tchorzewski, 1968). Bradykinin aerosol was shown to cause bronchoconstriction in asthmatics but not in normal subjects (Herxheimer and Stresemann, 1961; Veronier and Panzani, 1968). Recently, Dolovich et al. (1970) reported that nasal secretion, obtained from allergic subjects immediately following antigenic challenge, usually contained a smooth muscle stimulating substance which has been identified as a polypeptide.

At present, there seems to be little doubt that kinins are formed during anaphylaxis. However, the exact mechanism of their release

and the extent to which they contribute to anaphylaxis remains to be solved.

Section C. Antagonists of Chemical Mediators in Anaphylaxis and Allergy.

All accumulated evidence supports the generally accepted assumption that histamine and other chemical mediators with similar biological activity play a vital part in the development of anaphylactic and allergic manifestations. The realization of this fact prompted many investigators to search for drugs which either prevent the release of these biologically active substances or antagonize their action on smooth muscles and blood vessels.

The most important agents or classes of agents investigated and used in combating allergic diseases to date are:

a. Non-Specific antagonists, e.g. glucocorticoids.
b. Physiological antagonists, e.g. catecholamines.
c. Pharmacological antagonists, e.g. synthetic antihistamines.

This review will only discuss the last group of these agents in detail.

a. Non-Specific Antagonists (Glucocorticoids)

It is well established that symptoms of immediate hypersensitivity reactions in man such as hay fever, atopic asthma, and serum sickness respond dramatically to glucocorticoid therapy (Bordley et al., 1949; Randolph et al., 1950; Rose, 1954). It is not surprising, therefore, that many studies were done on the possible effects of the glucocorticoids on the pharmacological actions of chemical mediators released by antigen-antibody reactions.

A sound basis for the possible involvement of the adrenal steroids in histamine dependent allergic responses was suggested by the observations that, i) adrenalectomy leads to a considerable increase in tissue histamine (Rose and Browne, 1941), ii) adrenalectomy increases manifold the toxicity of histamine in rats and mice (Halpern et al., 1952). Although Oskov (1949) claimed that ACTH protected guinea pigs from passive anaphylaxis, Leger et al. (1948), Feinberg and Malkiel (1952), Halpern (1956) and many others could not confirm Oskov's finding. It is generally accepted that ACTH or corticosteroids are not effective in active or passive anaphylaxis and they do not antagonize the pharmacological action of histamine.

Although, according to most investigators, glucocorticoids do not exert any significant antagonizing effect on the actions of chemical mediators in acute experiments, they can, however, influence

both antibody and histamine biosynthesis in chronic experiments. It has been found that glucocorticoids can i) depress antibody formation (Germuth et al., 1951; Bjorneboe et al., 1951), ii) depress the formation of histamine from histidine (Halpern et al., 1953; Schayer et al., 1955), and iii) deplete tissue histamine content in the guinea pigs (Kovacs, 1965; Hicks, 1965). It should be noted, however, that these results were obtained when steroids were given in very high doses for several days. Thus, the dramatic therapeutic effect seen in man within 24 hours after the administration of glucocorticoids remains an unsolved problem. In spite of its initial dramatic therapeutic effect, there are several inherent disadvantages which make long term therapy with glucocorticoids dangerous.

b. Physiological Antagonists (Epinephrine and Related Substances)

The sympathomimetics have a long and intimate association with allergy. They can be considered as physiological antagonists of histamine, i.e. they produce effects opposite to those evoked by histamine on the same organ system, but they do not act on the same receptors as histamine. For example, histamine stimulates the smooth muscles of the alimentary tract and bronchioles, dilates capillaries and produces a fall in blood pressure. In contrast to these histamine-induced effects, epinephrine relaxes the smooth muscles of the bronchioles and the gut, and elevates blood pressure. According to Loew (1947), these actions represent "the antithesis

of those produced by histamine".

It is thus not surprising, that epinephrine and its congeners have been the mainstay in the symptomatic treatment of allergic diseases. However, despite the immediate therapeutic advantages of epinephrine and its congeners in combating hypersensitivity reactions, their short duration of action and the tendency of patients to develop tachyphylaxis after repeated administration, limit their usefulness.

c. Pharmacological Antagonists (Synthetic Antihistamines)

As evidence accumulated to show that histamine plays a major role in the development of anaphylactic and allergic symptoms, an intensive search for a histamine antagonist was launched leading to the discovery of the first synthetic antihistamine in 1933 by Bovet and Fourneau. They reported that one of a series of amines (designated by them as 929F or 2-isopropyl-5-methylphenoxyethyldiethylamine) protected guinea pigs against several lethal doses of histamine, antagonized histamine-induced bronchoconstriction and more importantly, lessened the symptoms of anaphylactic shock. Although 929F was found to be too toxic at the therapeutic level (Bovet and Staub, 1937), the original observations of Bovet and Fourneau (1933) nevertheless opened the way for the preparation of better and less toxic analogs from which synthetic antihistamines, with acceptable therapeutic index, soon emerged.

The first therapeutically important synthetic antihistamines were antergan (Halpern, 1942) and neo-antergan (Bovet et al., 1944). In the next few years, a large number of antihistamines with basically the same pharmacological actions were synthesized (e.g. phernergan by Halpern, 1947; pyribenzamine by Huttrer et al., 1946; histadyl by Weston, 1947; chlorothen by Clapp et al., 1947; etc.).

Antihistamines act specifically by competing with histamine for the same receptor sites (Ariens, 1954) and this relationship is referred to as "competitive inhibition" (Rocha e Silva, 1959). The chemical structures of antihistamines have a certain similarity to that of histamine, and according to our present knowledge, antihistamines counteract the action of histamine by forming a reversible union with a common receptor site, without eliciting intrinsic actions of their own (Ariens, 1954). Furthermore, they do not inactivate histamine chemically, either in vivo or in vitro, and they do not interfere with the release of histamine caused by either antigen-antibody reaction or by histamine liberating agents (Wilhelm, 1961). The antihistamines, in general, antagonize in varying degrees most but not all pharmacological actions of histamine. The methods chosen to assess the potency of potential antihistamines are based on their ability to antagonize the following basic pharmacologic actions of histamine:

Action on Smooth Muscles

Histamine exerts a highly characteristic effect on smooth muscles.

According to Dale and Laidlaw (1910), histamine stimulates smooth muscles. The sensitivity of the smooth muscles to histamine depends on the organ and the species from which they originate. Among the most sensitive are the smooth muscles of the uterus and bronchioles of certain species, especially the rodents, and to a lesser degree, the intestinal smooth muscles. Antihistamines can antagonize the actions of histamine on these smooth muscles both in vitro and in vivo. Thus, the evaluation of potential antihistamines can be assayed on such smooth muscle preparations as ileum, uterus and the bronchial chain of guinea pigs. Potent antihistamines can abolish the effects of histamine on the isolated guinea pig ileum preparation in a concentration as low as 10^{-9} g/ml.

Histamine administered to guinea pigs in the form of an aerosol is frequently used as an in vivo method to evaluate the potency of antihistamines on smooth muscle preparations. In untreated guinea pigs, histamine aerosol causes strong bronchoconstriction, manifested by a series of characteristic symptoms, i.e. cough, dyspnea, falling, lying and death. The aerosol technique was originally described by Alexander et al. (1926) and Kallos and Pagel (1937) to study the development of anaphylactic shock in guinea pigs. In 1942, Halpern (1942) modified the original aerosol technique to evaluate potential antihistaminic compounds. The most potent antihistamine can inhibit the lethal effects of histamine aerosol at dose levels as low as 0.1 - 5 mg/kg body weight.

Histamine injected intravenously in doses of 0.1 - 0.4 mg/kg into guinea pigs, exerts effects very similar to those provoked by histamine aerosol. The protective effect of antihistamines against the lethal effects of histamine aerosol and intravenously injected histamine was thought to be highly specific, brought about directly by competitive antagonism of these agents on the smooth muscle receptors of the bronchi. Recently, however, Lish et al. (1966) re-examined and compared the specificity of histamine aerosol and intravenously injected histamine shock techniques. They found that while the histamine aerosol technique was quite specific, prevention of intravenously injected histamine shock by antihistamine could not be entirely explained by competitive inhibition. They further observed that the shock induced by intravenously injected histamine led to the release of epinephrine which potentiated the action of antihistamines. This potentiating effect was evident when the action of the liberated epinephrine was inhibited by a beta-adrenergic blocker such as propranolol, since a much higher dose of antihistamine was needed to antagonize the shock.

Histamine exerts a profound effect on the capillaries. The injection of a small amount of histamine (1 - 10 ug) intracutaneously to man or experimental animals, brings about the following classical response (the triple response): i) vasodilatation resulting in a prompt reddening of the area; ii) increased capillary permeability leading to edema or the formation of a wheal; and iii) a bright

crimson "flare" surrounding the wheal, which is probably due to peripheral sensory nerve involvement. This effect can easily be demonstrated by injecting a dye (which binds to plasma protein) intravenously into the experimental animals just before the intradermal administration of histamine. Thus, when 0.1 - 1.0 ug histamine is injected intradermally, the permeability of the capillaries is increased, permitting the leakage of the plasma protein-bound dye into the extravascular space which causes the blueing of the affected area. This technique is used to evaluate the capacity of potential antihistamines to antagonize the vascular effects of histamine.

The effects of histamine on the capillary is less influenced by antihistamines than its effects on the smooth muscles. Nevertheless, potent antihistamines, such as phenergan and neo-antergan, can diminish or completely inhibit the triple response.

Action on the Blood Pressure

Histamine, injected in small doses (1 - 5 ug), produces a fall in blood pressure in most species. If a large dose of histamine (1 - 10 mg) is administered intravenously, the "triphasic" response may be observed, where the initial hypotension is followed by an attempt to return to normotension which then is followed by another drastic fall in blood pressure and this may in turn lead to an irreversible shock and death.

Most antihistamines have a relatively weak effect in antagonizing the action of histamine on circulation. Very small doses (4 ug) of histamine should be injected into the dog in order to have any appreciable reduction of the hypotensive effect after treatment of the animal with large doses of antihistamines (Rosenthal and Minard, 1939). Even with potent antihistamines, such as neo-antergan, only partial inhibition of the hypotensive effects of small doses of histamine (10 - 50 ug) can be observed (Bovert and Walthert, 1944).

Action on Gastric Secretion

Popieski (1920) was first to demonstrate that histamine is a powerful stimulant of gastric secretion. In the 40 years that followed, it was established that histamine is a potent stimulator of gastric secretion, although the exact mechanism of gastric secretion mediated through histamine is yet to be clarified (Code, 1965). The success of antihistamine in antagonizing the effects of histamine on the capillaries and the smooth muscles prompted investigators to explore the activity of these agents against histamine-induced gastric acid secretion. As early as 1941, Loew and Chickering (1941) reported that compound 929F was not able to influence the histamine-induced gastric acid hypersecretion. This report was confirmed by Hallenbeck (1943) who tested compound 1571F in dogs with denervated Heidenhain gastric pouches. Today it is well established that even the most potent antihistamines are completely inactive against

histamine-induced gastric acid hypersecretion (Douglas, 1965).

The lack of activity of antihistamines against histamine-induced gastric acid hypersecretion may indicate that the receptors for histamine in the effector cells of the glandular tissues are different from those present in smooth muscle structures and capillaries (Ash and Schild, 1966).

Antihistamines and Allergy

It appears that antihistamines are most effective in the exudative types of allergy such as pollinosis and urticaria. In seasonal hay fever, antihistamines relieve the sneezing, rhinorrhea, and the itching of the eyes, nose and throat - except where the pollen counts are extremely high. Although the results in perennial vasomotor rhinitis are less gratifying, about 50% of the patients respond favourably to antihistamine therapy.

In certain allergic dermatoses, especially in acute urticaria, antihistamines have been of great value. To a lesser degree, they are also useful in chronic urticaria, angioedema, atopic and contact dermatitis. In serum sickness, only the urticarial and edematous lesions respond to antihistamines.

Although antihistamines are very useful as therapeutic agents,

nevertheless, they possess a number of undesirable side effects which tend to lessen their therapeutic value. For example, almost all antihistamines at therapeutic doses elicit undesirable side-effects, such as sedation and reduced co-ordination. Furthermore, they are ineffective if other mediators are also released and this is exemplified by bronchial asthma, where antihistamines have been dismally ineffectual.

Thus, although the role of antihistamines as effective combatants against allergy is far from being ideal, they nevertheless offer some very significant relief to patients suffering from certain allergic symptoms.

d. Naturally occurring Antihistamine-like substances

1. Antihistamine-like Activity of Mammalian Tissues and Urine Extracts

It was first reported by Kovacs (1950) and Kovacs and Kovacs-Juhasz (1951) that guinea pigs treated with the extracts of rabbit leucocyte suspensions were protected from the lethal effect of a histamine aerosol. Furthermore, the degree of protection bore a direct relationship to the total number of eosinophils present in the suspensions. Extracts of eosinophil rich human leucocytes also demonstrated similar results. These early reports on antihistamine-

like activity of leucocyte suspensions were confirmed by Vercauteren and Peeters (1952) and Vercauteren (1953) who used extracts prepared from the granules of horse eosinophils. In 1960, Archer (1960) reported that the watery extracts prepared from horse eosinophil suspensions, which were relatively free from other blood elements, inhibited the histamine-induced local edema formation on horse skin and also counteracted the effects of 5-HT and bradykinin. According to Feldberg and Kovacs (1960) the isolated ileum obtained from guinea pigs pretreated with the extracts of the buffy coat layer was found to be 100 - 1000 times less sensitive to histamine than that obtained from normal untreated animals. Furthermore, guinea pigs injected intraperitoneally with the extracts from the buffy coat layer were found to be protected from the lethal effect of a histamine aerosol. Recently, Lee (1969) found that guinea pigs having eosinophilia were protected from the lethal effects of histamine aerosol.

The chemical structure of the active principle(s) present in the extracts of the eosinophils is not as yet known. Vercauteren (1953) suggested that the observed activity was in part attributable to the presence of a free arginine molecule on the "histonlike protein". On the other hand, a highly purified extract of bovine eosinophils indicated that the active principle(s) could be a steroid-like substance (Kovacs and Kovacs-Juhasz, 1955).

The mechanism by which the effects of histamine is antagonized by

the extracts of eosinophils has not yet been elucidated. It is now known that histamine is not inactivated when incubated with the aqueous extract of the eosinophils. However, histamine is inactivated when a small amount of hydrogen peroxide is added to the eosinophil extract-histamine mixture (Archer, 1963). The fact that hydrogen peroxide itself could, to some extent, inactivate histamine in the presence of heavy metal ions, led Archer (1963) to postulate the involvement of a potent peroxidase enzyme within the eosinophil. An intriguing aspect of the histamine-eosinophil interaction is that on exposure to eosinophils, histamine itself is changed and thus eliminates the possibility of competitive inhibition and promotes the possibility of enzymatic degradation. According to Lee (1969), the development of eosinophilia may be a defence mechanism in the event of subsequent histamine release.

The presence of an antihistamine-like substance(s) in the eosinophils indicated that the same or similar substance(s) might also be present in other tissues. In 1951, Karady et al. (1951) reported that the extracts of different animal organs, such as the guinea pig liver, lung and spleen, and human urine possessed antihistamine-like activity. Similarly, Francis and Melville (1958) demonstrated that the extracts of human and dog gingival tissues diminished the histamine-induced contractions of the isolated guinea pig ileum preparation. Work on the antihistamine-like activity of the human urine was extended by Kovacs and Melville (1962) who found that the ex-

tracts, besides being inhibitory to histamine, also antagonized 5-HT. When the extracts were given either orally or intraperitoneally to guinea pigs, they were protected against the lethal effect of a 0.2 % histamine aerosol. Furthermore, they showed that histamine or bradykinin-induced capillary permeability was prevented or markedly reduced by the extract (Kovacs and Melville, 1963). In the meantime, Francis et al. (1962, 1963) and Kovacs et al. (1963) showed that the extracts of human colon, gingiva, stomach, lung and breast also exhibited antihistamine-like activity. In 1963, Pelletier (1963) prepared extracts from 14 different human organs and reported that all the extracts exerted antihistamine-like activity. Furthermore, he demonstrated that the extractable antihistamine-like activity in the tumorous part was significantly higher than in the non-tumorous part of the same organ. This finding was confirmed and extended by Stotland (1966). She also reported that in the guinea pig, the concentration of the extractable antihistamine-like principle(s) was not associated with the tissue level of histamine or other amines. In 1966, Kovacs and Voith (1966) demonstrated that the partially purified extracts of human or horse urine, which exerted antihistamine-like activity, also antagonized the histamine-induced acid hypersecretion in the guinea pig. Furthermore, the extracts, administered either orally or intraperitoneally into rats, prevented or strongly reduced the development of ulcer induced either by the Shay or restraint (stress) method. Recently, Pelletier and Desjardin (1971) and Pelletier (1971) reported that

an increased amount of antihistaminic substance is excreted in the urine of patients suffering from chronic and acute infection and mastocytosis. Furthermore, they found that patients having peripheral blood eosinophilia did not excrete higher amounts of antihistaminic substance. In their opinion, the antihistaminic substance could be linked to stress and play a role in the defence of the body.

2. Antihistamine-like Activity of Plant Tumour Extracts

i) Plant Tumours: Plant tumours or galls are hypertrophies of the plant tissues caused by insects, mites, bacteria, fungi and possibly other organisms. Wasps, for example, are instrumental in the production of oak galls, while crown-gall tumour is induced by bacteria. The exact mechanism by which the galls are produced is not known. In the case of the oak galls, the wasps deposite their eggs on the branches and the fluid or chemicals in the eggs may contribute to the formation of the galls which envelope the eggs. Another type of plant tumour is the crown-gall. Naturally occurring crown-galls are frequently found on the stem of the tomato plant, tobacco and sunflower. The tumour is considered malignant, since it produces metastases and frequently kills the host. In 1907, Smith and Townsend first demonstrated that crown-gall tumour was induced by a bacterium, agrobacterium tumefaciens. They produced the galls by puncture innoculation of agrobacterium tumefaciens

on the paris daisy, tobacco, tomato, root of sugar beet and the root of peach tree.

ii) Antihistamine-like Activity in the Crown-Gall Extract: It was first reported in 1952 that the extract of the crown-gall infected tomato plants, when injected intraperitoneally into guinea pigs, protected the animals from the lethal effects of histamine aerosol (Kovacs et al., 1952). This finding was subsequently confirmed by Broome et al. (1962) who showed that a single injection of the partially purified extracts of crown-galls brought about a protection which lasted for several days or weeks. In 1964, two groups of investigators (Kovacs et al., 1964; Calam and Callow, 1964), working independently of each other, reported the isolation of a crystalline substance with antihistamine-like activity from the crown-gall infected tomato stalks. Calam and Callow (1964) identified their isolated crystalline substance as tomatine, while Kovacs et al. (1964) reported that their crystalline substance possessed chemical properties closely resembling tomatine.

iii) Tomatine: The report of Gottlieb (1943) that the expressed juice of the tomato plant retarded the growth of Fusarium oxysporum f. lycopersici, an organism which causes the wilting of the tomato plant, drew considerable interest on the nature of the active substance. Irving et al. (1945, 1946) observed that the active principle, found in the extract of the tomato plant, was a potent fungi-

static agent with some ability to inhibit the growth of certain bacteria, e.g., staphylococcus aureus, bacillus subtilis, etc. and they named this active principle "tomatine". These findings were confirmed by Ma and Fontaine (1948) who demonstrated that the growth of candida albicans was strongly inhibited by the extracts of tomato plants. In the same year, Fontaine et al. (1948) and Kuhn and Low (1948), isolated tomatine from the tomato plant and found that tomatine possessed potent fungistatic and mild bacteriostatic properties.

Chemically, tomatine has been identified as a steroid glycoside alkaloid consisting of two molecules of glucose, one molecule of galactose, one molecule of xylose and the aglycone tomatidine (Kuhn et al., 1950; Ma and Fontaine, 1950; Fontaine et al., 1951; Sato et al., 1951).

Very little is known about the pharmacological effects of tomatine. Some preliminary studies on its pharmacologic and toxic properties were carried out by Wilson et al. (1961) and their findings may be summarized as follows: Tomatine was not toxic orally, except in very high doses. When given intravenously to rats and rabbits, it caused a sudden and short-lived hypotension with no cardiac effect and the rate and depth of respiration were significantly increased. In rats, the blood pressure effect was largely eliminated or reversed by vagotomy. Hemolysis was observed both <u>in vivo</u> and <u>in vitro</u>.

In 1964, Calam and Callow (1964) demonstrated that guinea pigs treated with tomatine isolated from the normal tomato plants, were protected from the lethal effects of histamine aerosol. However, when tomatine was tested in vitro it did not inhibit the histamine-induced contractions of the isolated guinea pig ileum. On the other hand, Kovacs et al. (1964) found that the commercial tomatine inhibited the histamine-induced (as well as bradykinin-, 5-HT-, and acetylcholine-induced) contractions of the isolated guinea pig ileum in a concentration as low as 3×10^{-6} gm/ml, but showed a relatively weak protection of the guinea pigs against the lethal effects of histamine aerosol. Furthermore, they also reported that the crystalline substance isolated from the crown-gall infected tomato plant not only inhibited histamine in vitro experiments, but also protected the animals from the lethal effects of a histamine aerosol (Kovacs et al., 1964).

The antihistamine-like property of tomatine as presented by the above data was somewhat contradictory, however, the situation was amended when a substance very similar to tomatine was isolated from the crown-gall infected tomato plants (Wakkary, 1968) and this active substance was called "gomatine". According to Chu (1969), the antihistaminic activity of tomatine is probably due to the contamination by gomatine which has antihistaminic activity.

Recently, Filderman and Kovacs (1970) reported that tomatine is an

effective anti-inflammatory compound. They observed that tomatine, given to rats intraperitoneally or orally, brought about a significant, dose-dependent inhibition in

1) carrageenan-induced paw edema,
2) granulation tissue formation induced by implantation of carrageenan impregnated cotton pellets,
3) leakage of protein-bound dye into the peritoneal cavity induced by the intraperitoneal injection of dilute acetic acid.

iv) Antihistamine-like Activity in the Oak Gall Extract: The knowledge that severe burns lead to symptoms strikingly similar to those seen after the intravenous injection of histamine into guinea pigs led Barsoum and Gaddum (1936) to study the changes in blood histamine level in patients with extensive burns. They found that a sharp rise of blood histamine coincided with the period when secondary shock was likely to develop. Rose and Browne (1940) confirmed this finding and Dekanski (1945) reported that a marked increase in the blood histamine of mice was observed 10 minutes following the induction of extensive burns.

Tannic acid was introduced by Davidson (1925) and had been successfully used for many years in the treatment of burns. Thus it seemed possible that the therapeutic effect of tannic acid was in some way brought about by the neutralization of the released histamine

or that this mechanism was, at least in part, responsible for its therapeutic effect. To test this hypothesis, the effect of tannic acid was investigated against a lethal histamine aerosol in the guinea pigs (Gyure and Kovacs, 1949). It was demonstrated that the intraperitoneal injection of 20 - 40 mg/kg body weight of commercial tannic acid gave a definite protection against a lethal histamine aerosol when the injected guinea pig was tested 1 - 2 hours later. On the strength of this experimental observation, it was thought that a purer preparation of commercial tannic acid could confer a longer protective effect. On further purification of the commercial tannic acid, however, the antihistamine-like activity decreased correspondingly and no antihistamine-like activity was observed with chemically pure tannic acid preparation. It seemed possible, therefore, that the antihistamine-like effect of the commercial tannic acid, which in Hungary is prepared from oak galls, was most likely due to some impurities derived from the oak galls. In 1950, Kovacs and Szabadi (1950) reported that the simple ethanolic extract of oak galls contained 10 times more of the active substance(s) than comparable amounts of commercial tannic acid. In addition, guinea pigs treated with the extract were protected from the lethal effects of histamine for 18 - 20 hours. In the following year, it was found that galls from other plants, e.g. rose, poplar and willow, also possessed antihistamine-like activity (Kovacs et al., 1951). In 1952, Kovacs et al. (1952) described a method by which tannin-free extracts of oak galls could be obtained.

Feldberg and Kovacs (1960) were able to confirm the antihistamine-like activity of the oak gall extracts by showing that the ethanolic extracts of the oak galls protected guinea pigs from the lethal effect of the histamine aerosol and that the protection lasted 24 hours or longer. They were also able to demonstrate that the antihistamine-like activity did not result from the tannic acid present in the extracts, because tannic acid injected intraperitoneally into guinea pigs did not protect the animals, while the ethanolic extract of oak galls, from which tannic acid had been removed by lead hydroxide precipitation showed protection.

In 1962, Broome et al. (1962) described a method to obtain a tannin-free and stable extract of the oak galls and further confirmed the antihistamine-like activity of the oak gall extract. These findings were confirmed by Berry et al. (1962) who also reported that the extract was able to antagonize the bronchoconstrictive effect of 5-HT in the guinea pig. In 1966, Calam (1966) studied two kinds of oak gall extracts and confirmed the antihistamine-like activity in them. He also observed that severe toxic symptoms were manifested by the treated animals and thus concluded that the observed antihistamine-like activity was non-specific. However, it should be noted that the extracts Calam used were of a very crude nature and that no attempt was made to remove the large quantity of tannic acid present in these extracts. Recently, a highly purified tannin-free oak gall extract was obtained through the use of column chroma-

tography (Chu, 1969). The antihistamine-like activity of the purified extract, originally demonstrated with the histamine aerosol technique, was substantiated by the ability of the extract to inhibit histamine-induced capillary permeability and significantly reduce the intensity of systemic anaphylactic shock. Furthermore, its inability to inhibit bradykinin-induced capillary permeability favours the possibility of a specific histamine antagonism.

CHAPTER I. HISTORICAL REVIEW

PART 2. THE INFLAMMATORY PROCESS

Section A. Present Concept of the Inflammatory Process

Inflammation may be defined as the local reactive alterations in the tissues following injury or irritation in an attempt to dispose the stimulus, localize the reaction and initiate repairs. Any harmful stimulus (Glenn et al., 1968),such as microbial invasion, chemical or physical irritants, antigen-antibody reaction, etc., can elicit an inflammatory response. Irrespective of the causative agent, the ensueing sequence of events is similar (Houck, 1968).

The inflammatory reaction may be conveniently divided into three phases (Spector, 1963), namely

i) increased vascular permeability, with resulting edema and swelling

ii) leucocyte migration or cellular infiltration and

iii) cellular maturation with fibroblast proliferation and the synthesis of new connective tissue for tissue repair.

However, since inflammation is a dynamic process it is not possible to define sharply the point at which one phase ends and the

next begins. A somewhat oversimplified diagrammatic presentation of some of the factors thought to possibly play a role in the pathogenesis of inflammation is shown in Figure 1.

When tissue injury is initiated, the probable sequence of events may be briefly summarized as follows: The stimulating effect of the irritant on the vessel walls produces a momentary contraction of the blood vessels which is probably mediated through axon reflexes (Zweifach, 1965). This, however, is rapidly replaced by dilatation of the exposed vessels and local hyperaemia. Fleisch and Domenjoz (194o) believed that this dilatation is mediated by ATP (adenosine triphosphate). In spite of the vasodilatation, there is only a transitory increase in the rate of blood flow which gives way to a slowing of the current and ultimately to stagnation and stasis (Boyd, 1961). Swelling and irregularity of the vascular endothelial linings (Boyd, 1961) as well as an increase in the viscosity of the blood (Anderson, 1960) lead to sludging of the red blood cells (Knisely et al., 1947). The slowing of the blood flow in small vessels also permits the leucocytes to leave the axial stream and pave or marginate along the inner vessel walls, an event preceding their diapedesis (Anderson, 1960). There is an increase in the passage of fluid, largely plasma, (Boyd, 1961), outward from the vessels due to an elevation of capillary pressure and increased capillary permeability (Zweifach, 1965).

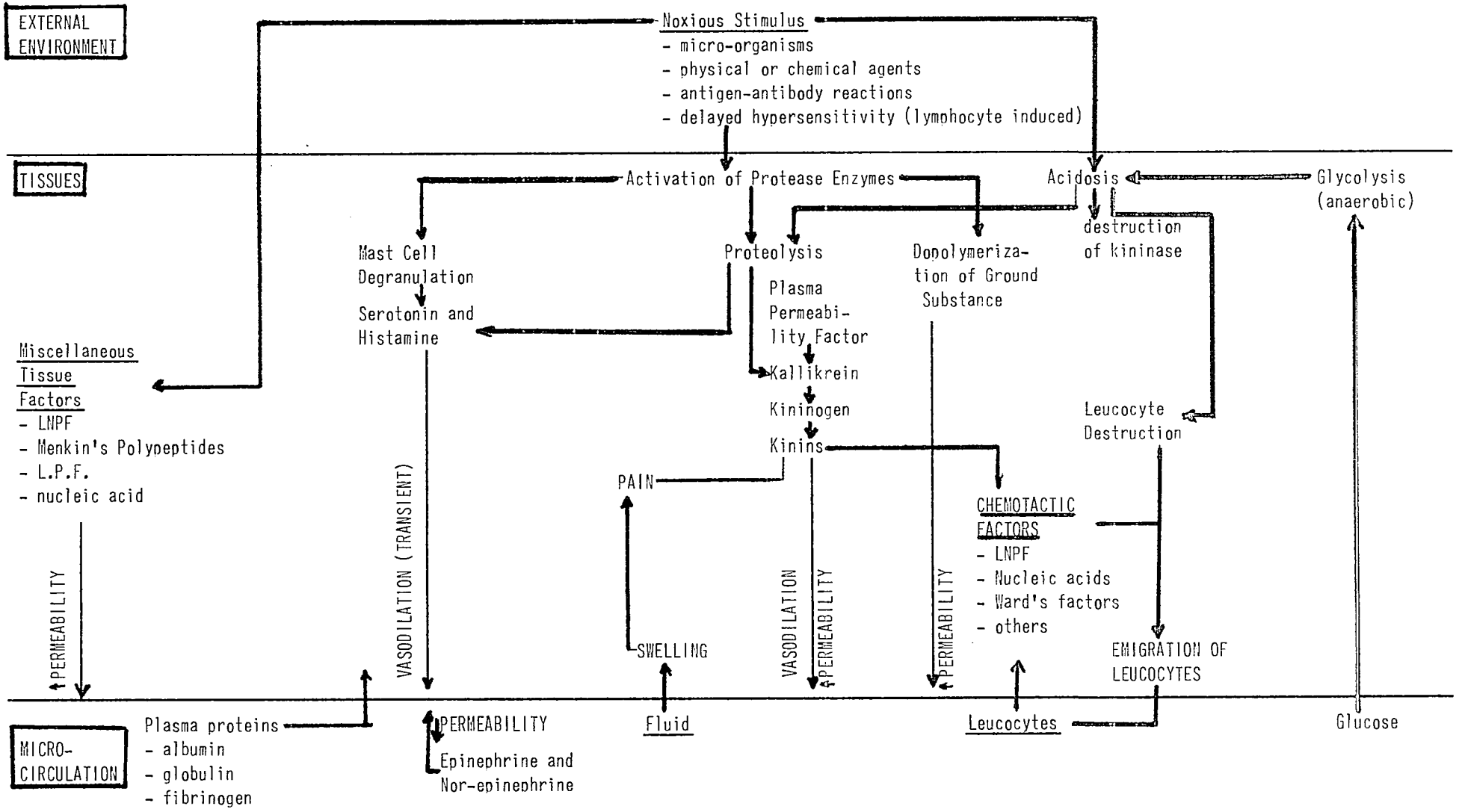


Figure 1 A conceptual Schema of the Inflammatory Process

Kulka (1965) has shown that venular dilatation, the first event of experimental inflammation, is rapidly followed by progressive microcirculatory impairment, which he conceptualizes as "the final common pathway" of inflammatory tissue damage (Figure 2).

The possibility that the inflammatory reaction described above could be initiated by some mediators formed in the inflamed tissues was first proposed by Ebbecke in 1923 when he suggested that an agent was produced in the stimulated epithelium, which led to dilatation of capillaries and smaller arterioles as it diffused through the tissues. This hypothesis seems to be basically a correct one and still valid today. Current speculation (Houck, 1968) is that as a consequence of an injury, intracellular enzymes or chemical mediators are released into the extracellular compartment of the tissue, and there they attack substrates in the ground substance. The products of this enzyme-substrate reaction then act upon the microcirculation of the tissue to produce the pathological changes we recognize.

Apart from the breakdown products of ground substance, some physiochemical alterations in inflammation, i.e. the development of local acidosis with its consequent disturbance of electrolyte equilibrium, are also important. Frunder (1953) has demonstrated that a primary local acidosis (pH 6.8 - 6.0) occurs a few seconds after injury and acts as an inducing agent for the inflammatory

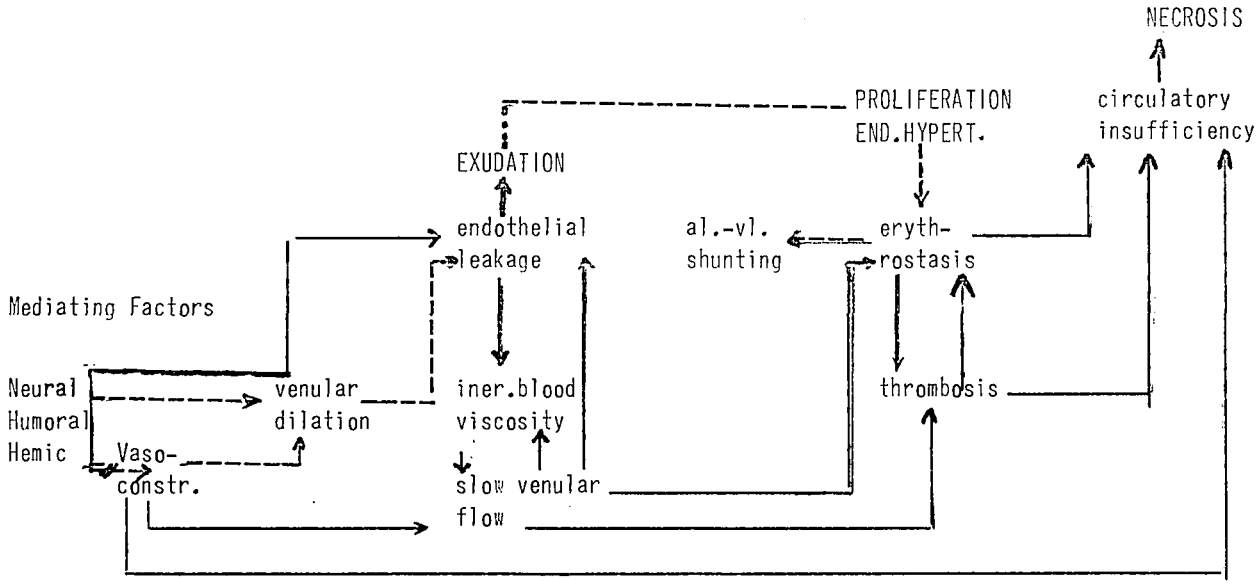


Figure 2

A tentative schema of microcirculatory changes occurring in the inflamation.

The mediating factors lead to direct and indirect effects on venules and capillaries. The central role of endothelial leakage is emphasized but is qualified by the inclusion of diverse intra- and extravascular factors augmenting leakage and leading to local tissue anoxia and necrosis. The possible importance of a number of vicious cycles is shown. The net result is that the small vessels can no longer do their job of providing essential metabolites to the dependent tissues (Kulka, 1964).

reaction, which, according to him, was followed by a secondary acidosis. The primary acidosis was attributed to glycogenolysis and the secondary acidosis to an increase in anaerobic glycolysis. Proteolysis is enhanced by an accumulation of lactate, a final product of glycolysis (Rubel, 1936).

The alteration of pH in the inflammatory focus may also enhance the enzymatic depolarization of the macromolecular ground substance by an as yet unknown mechanism. The degradation of mucopolysaccharides then leads to the appearance of biologically active metabolites which could play a role in the inflammatory reaction (Linder, 1957).

Besides these breakdown products, biologically active substances, e.g. histamine, kinins, etc., are at present considered to be mainly responsible for the altered vascular permeability changes seen at the site of inflammation. The roles played by these chemical mediators in mediating vascular changes during inflammation will be considered in the next section.

Apart from the chemical mediators, there is an increase in the degradation of nucleic acids and in the appearance of energy yielding compounds, e.g. ATP during the early stages of inflammation. The significance of these processes in the inflammatory response, however, has as yet not been fully evaluated (Domenjoz,

1966). Menkin (1960) has isolated a series of polypeptides (e.g. leucotaxine, exudine, pyrexine, etc.) whose chemical identities and specific biological roles are unknown, although he considered them to be inflammation inducing agents.

Willoughby et al. (1962) have shown that a potent factor capable of increasing vascular permeability to plasma proteins is present in lymph node cells (LNPF). Although similar activity has been shown to exist in extracts of cells from other types of tissues, lymph-node cells are the richest source of this factor (Walters and Willoughby, 1965; Willoughby, 1966). There is evidence that this lymph-node factor could mediate the vascular changes of delayed hypersensitivity reaction (Willoughby and Spector, 1964). Besides the lymph-node permeability factor or LNPF, another permeability factor has been extracted from normal lymphocytes, called the lymphocyte permeability factor or LPF (Spector, 1963).

An increase in both mechanical and osmotic pressures, which occurs very early in the inflamed area, has been shown by Shade (1923; 1935) to be associated with the enzymatic depolarization of the macromolecular mucopolysaccharides of the ground substance, resulting in a greater capacity for hydration. Edema formation is thought to be ascribed to both increased vascular permeability and hydration of the ground substance.

During the development of an acute inflammatory lesion, both poly-

morphonuclear and mononuclear cells migrate together from the onset of the lesion (Paz and Spector, 1962). In the very early stages, there is a predominance of polymorphonuclear cells. By about the second day, the polymorphonuclear cells are replaced by an increasing proportion of monocytes, which, according to Lack (1968) can transform into histiocytes and macrophages. However, there is evidence to indicate that small lymphocytes are the source of macrophages (Rebuck and Crowley, 1955; Sierachi and Rebuck, 1960).

The migration of the white blood cells through the vessel walls are often followed by the red blood cells. The exact mechanism of leucocyte infiltration into the site of injury remains to be elucidated. However, it is probable that stimuli known as chemotactic factors are responsible for the cellular migration. Both negative and positive chemotactic factors are known to exist (Boyd, 1961).

Positive attraction could possibly be attributed to the kinins (Horton, 1963) and to a lesser extent histamine (Spector and Willoughby, 1964). The local acidosis injures leucocytes, which leads to the release of lysosomes and intracellular enzymes (Lack, 1968). Leucocyte destruction in the vicinity of a blood vessel may provoke the emigration of more leucocytes (Lack, 1968). Chemotactic effect was also one of the prime effects of Menkin's leuco-

taxine (Anderson, 1960). The LNPF of Spector and Willoughby (1964) stimulates an extensive migration of leucocytes as well as the disposition of a fibrinoid-like material.

Ward (1968) has shown that during antigen-antibody reaction, the activated trimolar complex of C5, 6 and 7 and a plasmin split fragment of C3 are also capable of acting as chemotactic factors. The net effect of these and perhaps other chemotactic factors is to increase cellular infiltration into the inflamed site.

The accumulation of the inflammatory exudate, consisting of both cells and fluid, is partially dependent on the vascular effects. The fluid part of the exudate, consisting largely of blood plasma, coagulates and forms a network of fibrin. The inflamed area tends to be walled off by the fibrinous material and by thrombotic occlusion of the lymphatics draining the area.

The damaged tissue may also activate autolysis of the tissue components by releasing hydrolytic enzymes normally present within the tissue cells. Intracellular proteases can digest inhibitors of collagenolysis so that collagen breakdown ensues. According to Houck (1968), some of the peptides produced by collagenolysis could induce platelets aggregation. Other factors such as collagen, micro-organisms and the vasoactive amines can also cause platelets to aggregate (Packham et al., 1968).

The relationship between platelet aggregation and blood coagulation is complex. When platelets are aggregated by ADP, which can be released by intravascular stimuli from platelets and other cells, the platelet phospholipoprotein at the membrane is exposed and accelerates the clotting reaction (Mustard et al., 1964; Castaldi et al., 1965; Hardisty and Huttor 1966; Marcus, 1966). This phospholipoprotein is involved in interactions between factors IX and VIII and between factors X and V (Hember and Kahn, 1967; Esnouf, 1968). The release of platelet factor 3, resulting from platelet aggregation, may actually activate the Hageman factor which, in turn, may act on factor XI (PTA) and these acting on factor IX (PTC) may induce the intrinsic coagulation process (Bertelli, 1968). Thrombin, generated in the area of platelet aggregates not only converts fibrinogen to fibrin but also can cause a further release of platelet constituents in the same manner as collagen and antigen-antibody complexes (Holmsen et al., 1969; Mustard and Packham; 1970). The role of the fibrinolytic system, which may also be activated by Hageman factor (Packham et al., 1968), in the inflammatory reaction is not yet clearly defined. Nevertheless, Barnhart (1971) recently proposed the following sequence of events:

i) Fibrinogen and fibrin coating of antigen-antibody complexes with subsequent local fibrinolysis could lead to the release of fibrinogen degradation product D (FDP-D) and FDP-E.

ii) The FDP-D, as a potent chemotactic agent, attracts granulocytes.

iii) The FDP-E, as a chemical mediator, increases vascular permeability.

Platelet aggregation and fibrin polymerization lead to blockade of the microcirculation and ischemia of the wound area. This in turn produces anoxia and acidosis which can culminate in necrosis.

Concurrent with this phase of inflammation the initial stages of the reparative phase are set into motion. Soluble precursors of collagen and mucopolysaccharides appear in the exudate and there is a proliferation of fibroblastic cells and endothelial cells which grow into all areas of the exudate. The re-opening of pre-existing capillaries as well as the formation of new capillaries and lymphatics establish an intricate vascular network. The necrotic material, thrombus, and exudate are replaced with granulation tissue. Eventually, collagen fibers develop and the blood vessels decrease in size and number and ultimately disappear. Finally, the connective tissue condenses and shrinks and the normal tissue homeostasis is re-established.

Section B. Role of Chemical Mediators in Inflammation

Introduction

Despite the great volume of work that has been conducted on the elucidation of the mechanism of inflammation, the only mediator which has been proven to play a role in the inflammatory reaction is histamine. In this review, both the role of histamine and some of the other mediators in inflammation will be discussed on the basis of the following criteria (Spector and Willoughby, 1968):

i) The substance should be demonstrably present during the inflammatory reaction and absent when the reaction subsides.

ii) The substance should possess properties which qualify it as a mediator of inflammation.

iii) Inhibition of the substance by specific antagonists should lead to a diminution of that aspect of the inflammation for which the substance is assumed to be responsible.

iv) Depletion of the tissues of the suspected mediator prior to the injurious stimulus should similarly suppress that part of the inflammatory reaction for which the substance is assumed to be responsible.

a. Histamine

Ever since Lewis (1927) postulated the release of an "H substance" during inflammation, histamine has been suspected to play a role in the inflammatory process.

In the last two decades, it has been adequately shown that histamine is liberated consequent to tissue injury (Schachter and Talesnick, 1952; Halpern and Briot, 1952; Hahn and Wellmann, 1952). Furthermore, histamine also appeared to be important for the initiation of the vascular reaction and for edema formation (Meier, 1959; Wilhelm, 1962).

Several groups of investigators have postulated that histamine is most probably involved in the early phase of the inflammatory reaction (Halpern, 1953; Rocha e Silva, 1953; Ungar, 1953). This conception found strong support when histamine was identified in the early inflammatory exudates (Spector and Willoughby, 1957) and in the lymph draining of the injured limb (Edery and Lewis, 1963). The early vascular permeability following such mild stimuli as thermal burns (Spector and Willoughby, 1959; Wilhelm and Mason, 1960), the intrapleural administration of turpentine (Spector and Willoughby, 1959) or the intracutaneous injection of specific antigen (Hayashi et al., 1964) is largely attributable to the release of histamine.

In their inhibition studies, Stern and associates (Stern and Milin, 1956; Stern and Nikulin, 1957) reported that the granuloma pouch of rats treated with azulenes, an antagonist of compound 48/80, not only showed less necrosis and exudation but also was thinner and healed considerably better than untreated rats. These observations were confirmed by Ferluga et al. (1963). Furthermore, Spector and Willoughby (1959) reported that pretreatment with antihistaminics or histamine depletion induced by compound 48/80 inhibits the increase of capillary permeability in turpentine oil pleurisy of guinea pigs without suppressing subsequent exudate formation. More recently, Hurley and Spector (1965) demonstrated that in turpentine-induced rat pleurisy, carbon particle leakage is first observed in the venules and then in the capillaries at the initial stages of inflammation. Moreover, when they treated the rats with the antihistamine mepyramine, the carbon particle leakage in the venules was inhibited. This observation lends strong support to the possible involvement of histamine in the early phase of inflammation.

Today, all available data indicate that histamine appears to act as the initial pharmacological mediator of the vascular changes associated with the inflammatory response (Spector and Willoughby, 1968). Its role, however, is transient and during the development of the inflammatory response, the vessels again become or remain freely permeable after the effect of histamine has worn off. It is

necessary, therefore, to seek other mediators and mechanisms to explain the maintained increase in vascular permeability.

b. Serotonin or 5-Hydroxytryptamine (5-HT)

The evidence implicating 5-HT as a mediator of inflammation is much less convincing than that supporting the role of histamine. Moreover, the biological significance of 5-HT seems to be species-dependent. For example, the mast cells of rats are very rich in 5-HT (Benditt et al., 1955) while this is not the case with the other species.

It is well known that 5-HT is released in tissue injury in the rat (Bhattachrya and Lewis, 1956; Parratt and West, 1957; Halpern et al., 1959; Morsdorf, 1959). According to Ratnoff (1969) 5-HT is particularly important in the pathogenesis of inflammation in the rat, in which it increases vascular permeability, contracts some smooth muscles (Brocklehurst, 1958), induces a modest migration of leucocytes to the site of its injection (Spector and Willoughby, 1964) and enhances the inflammatory response around subcutaneously implanted polyvinyl sponge (Scherbel, 1961). Raised levels of 5-HT have been found in the pleural exudates of rats following intrapleural injection of turpentine and the 5-HT content of the exudate was found to increase after the release of histamine (Spector and Willoughby, 1968). Similarly, they were

also able to show that on the second day after irradiation, the 5-HT content of the intestine falls to a low level, suggesting a maximal release of this amine. It appears then that 5-HT is released during inflammation and that its release is subsequent to the liberation of histamine.

However, Stern and Gmaz-Nikulin (1960) reported that in rat tissue the depletion of 5-HT by reserpine did not modify the intensity of the inflammatory response. On the other hand, Spector and Willoughby (1968) observed that if the rat tissues were depleted of their tissue 5-HT and histamine by repeated administration of compound 48/80 the exudate formation was delayed longer than when animals were treated with an antihistamine alone. These results might suggest that the additional delay in the onset of inflammation was due, at least in part, to 5-HT depletion. Some confusion may exist due to results indicating that 5-HT antagonists can suppress certain inflammatory test models to a much higher degree than in other species. On inspection, however, it appears that these inflammatory test models usually involve the edema of rat hind paw and that the inflammatory stimulus is the injection of certain large molecular substances, such as egg white and dextran. The tissue of the rat hind paw is exceptionally rich in 5-HT and the stimuli are renowned for their ability to release this amine from the tissues. In such artificial conditions, where the importance of 5-HT is exaggerated, 5-HT antagonists may seem strikingly active.

In the skin of man there is very little 5-HT (Lewis, 1958) and it is very doubtful if 5-HT plays any role in the pathogenesis of inflammation in man.

In view of the somewhat shaky evidence, it could be suggested that in the rat, 5-HT acts as one of the mediators of inflammation and is released as the effect of histamine is beginning to wane. However, there seems little doubt that even in the rat and mouse 5-HT is probably less important than histamine - with the possible exception of cutaneous anaphylaxis in these rodents.

c. Kinins

The simplistic view that histamine is the sole mediator of inflammation was sharply challenged by Menkin (1937) who attributed the vascular changes to the formation of a thermostable agent which he named leukotaxine. Leukotaxine was thought to be a polypeptide (Menkin, 1938; 1938a) of intracellular origin (Menkin, 1956). Although the role of leukotaxine is very questionable, Menkin's fresh concept concerning polypeptides as possible mediators in the inflammatory processes opened the road for the kinins.

Hilton and Lewis (1957) stated that bradykinin has all the characteristics of a "mediator of inflammation". Important features of its actions are vasodilatation, increase of permeability (Holdstock

et al., 1957), pain producing effect (Armstrong et al., 1957; Keele, 1960), chemotaxis and stimulation of leucocyte migration (Lewis, 1962). Following thermal injury, Rocha e Silva and Rosenthal (1961) could identify histamine, 5-HT and bradykinin in the perfusate of the traumatized rat skin. However, when only a mild thermal injury was induced by heating at 44-45°C, there was no liberation of histamine or 5-HT from rat skin; the "thermic edema" appearing under these conditions can be ascribed to the liberation of bradykinin (Rocha e Silva, 1962; 1963). In experiments with lymph draining from tissues subjected to thermal injury and other forms of tissue injuries, Edery and Lewis (1962) were able to demonstrate a marked increase in the kinin-forming enzyme activity. Kinins have been demonstrated in experimental (Spector and Willoughby, 1962) and clinical (Armstrong, 1957) pleural exudates; thermal burn blister fluids (Armstrong, 1953; 1957); rheumatoid (Armstrong, 1957) and gouty (Melmon, 1967) synovial fluids and nasal secretions in hay fever (Dolovich, 1968).

Substances which interfere with the release of bradykinin from its inactive precursor in plasma, such as hexadimethrine bromide (Armstrong and Stewart, 1962), markedly inhibit the development of heat edema in the rat's paw (Garcia Leme, Schapoval and Rocha e Silva, 1967) or yeast-induced edema (Kellet, 1965). Depletion of the "labile poole of bradykininogen", caused by chronic treatment with sulphated polysaccharides, also significantly reduced the

intensity of heat edema (Garcia Leme, Schapoval, Rocha e Silva, 1967). Similarly, soya-bean trypsin inhibitor, which blocks the release of bradykinin from its inactive precursor, was found to be effective in counteracting experimental inflammatory reaction (Kaller, Hoffmeister and Kroneberg, 1966).

The role of polypeptides as possible mediators in inflammation seems to be further supported by the generally accepted observations that the formation of kinins can be mediated through several major pathways. For example, kinins may be formed when blood clots (Spector and Willoughby, 1968). Margolis (see Spector and Willoughby, 1968) proposed that activated Hageman factor reacts with a plasma component to bring about the formation of kinins. The kinin-forming system is also activated by plasmin (Beraldo, 1950; Lewis, 1958; Webster and Pierce, 1960). Proteolytic enzymes including plasmin, trypsin and kallikrein form kinins by acting on the α-2 globulin substrates (Erdos, 1966). Other enzymes, such as PF/dil (permeability factor/dilute), may be formed from inactive precursors in the plasma and they in turn may activate kallikrein. Furthermore, acidosis present in injured tissues may alone be sufficient to activate the circulating kallikrein (Domenjoz, 1966). Recently, Garcia Leme et al. (1970) reported that in the thermal injury of the rat paw, heating elicits a process leading to plasma extravasation into the subcutaneous tissues where it provokes the release

of bradykinin. On the basis of this observation, they proposed that irritative stimuli could initiate a process leading to minute local plasma extravasation into the subcutaneous space, where the bradykinin would be formed from an inactive precursor (bradykininogen) in amounts large enough to maintain the vascular alteration responsible for the development of edema. Once formed, the process could also lead to the release of other endogenous substances, such as histamine, which would reinforce the vascular changes.

Presently available data (Arturson, 1969) indicate that an increase in the permeability of venules, induced by histamine, permits the plasma kallikreinogen, kininogen, globulin, etc., as well as such enzymes as kallikrein and kinases to enter the extravascular spaces. Some of the released proteases may act directly in a kallikrein-like manner or they may activate kallikrein, which releases kinins from the kininogens (an α-2 globulin normally found in plasma). The free kinins increase the vascular permeability, causing further increases in the concentration of plasma, kallikreinogen, kininogen, etc., thus initiating a vicious circle. The released proteases also give rise to peptide breakdown products which are believed to be chemotactic to granulocytes and leukocytes. This leukocyte migration may also participate in the formation of kinins (Zachariae et al., 1967). Investigations into the enzyme activity of the polymorphonuclear leukocytes, obtained from

inflammatory exudates, indicates the presence of several types of kinin-forming enzymes in these cells (Greenbaum et al., 1968).

Thus, at present, the fact that kinins are involved in inflammation appears to be quite well documented (Lewis and Winsey, 1970; Northover and Northover, 1971), but the exact extent to which they contribute to tissue injury remains to be elucidated.

d. Prostaglandins

Prostaglandins are a group of at least 13 chemically related long chain fatty acids which are derived from a 20-carbon parent compound, prostanoic acid. They are in most mammalian tissues and are highly active pharmacologically, though the pattern of activity varies greatly between different members of the group. Relationships between the prostaglandins and a wide range of physiological processes have now been described, including fertility, parturition, behavior, nervous reflex activity, intestinal motility and the responses of target organs to hormones. It is now being realized that they may also have important pathological functions.

One of the more striking pharmacological effect of prostaglandins is their ability, in low concentrations, to produce vasodilatation (Crunkhorn and Willis, 1971). Prostaglandins E_1 and E_2 are most active in this respect, the intradermal injection of nanogram

amounts being followed by extensive flare lasting several hours (Sondergaard and Greaves, 1971). That this experimental response has its clinical counterparts has been shown by Williams et al. (1968) and Sandler et al. (1968). They found increased concentrations of prostaglandins in blood and tumour tissue, in medullary carcinoma of the thyroid and carcinoid tumours of the intestine, both of which are associated with flushing.

Of potentially greater significance is the rapidly strenthening evidence that prostaglandins are mediators of inflammation. They have been recovered from inflammatory exudates in a variety of experimental inflammatory reactions, including delayed inflammation due to carrageenan in the rat (Willis, 1969), burnt tissues in the dog (Angaard et al., 1970) and blister fluid in man (Angaard et al., 1970), and a substance with prostaglandin-like activity has been recovered from turpentine-induced pleural inflammatory exudate in the rat (Giroud and Willoughby, 1970).

Direct evidence of prostaglandin activity in man is naturally of special importance. Greaves and his colleagues (1971) used a continuous skin-perfusion technique to demonstrate the increased concentrations of prostaglandins in the interstitial fluid of inflamed human-skin. Delayed cutaneous inflammatory reactions, due to allergic contact dermatitis, were obtained by applying patch tests to the skin of allergic volunteers. The

presence of prostaglandins in the perfusate was confirmed by solvent partition thin-layer chromatography and bioassay. The prostaglandins recovered from the inflamed skin consisted of a mixture of E_1, E_2, $F_{1\alpha}$, and $F_{2\alpha}$. It is also of interest that histamine was found in some of these perfusates, since prostaglandin E_1 is known to behave as a histamine liberator (Sondergaard and Greaves, 1971). By contrast, perfusates from healthy skin and from skin undergoing short-lived wealing reactions of different types contained little or no prostaglandin activity.

The source of prostaglandins in inflammation is uncertain, but it is likely that a biosynthetic reaction between cell-membrane phospholipid and membrane-bound phospholipase A is involved. These observations should stimulate a search for prostaglandins in inflammatory reactions to a wide range of stimuli. But the results will need cautious interpretations, because, as Greaves et al. (1971) point out, finding prostaglandins in inflamed tissues is not proof that they have any role as mediators. Measurements are required relating the concentrations in the tissues to the evolution and regression of vascular changes in the inflammatory reaction. Probably final proof will have to await the availability of specific antagonists to the prostaglandin group of compounds.

Approaching the same problem in a different way Vane and his colleagues (Vane, 1971; Smith and Willis, 1971; Ferreira et al.,

1971) have reported recent experiments which shed new light on the mode of action of some anti-inflammatory drugs, including aspirin, indomethacin and salicylate. They observed that guinea pig lung tissue and human platelets contain prostaglandin-forming enzymes, which, when incubated with arachidonic acid substrate, synthesize prostaglandins. In both these systems, indomethacin and aspirin inhibit the biosynthesis of prostaglandin at concentrations of 0.1 - 1.0 ug/ml respectively. Salicylate was about 10 times less active than aspirin. Similar results were obtained in platelets from volunteers to whom aspirin or indomethacin had previously been administered. By contrast, hydrocortisone did not affect prostaglandin synthesis. Vane and his coworkers also showed that the inhibitory effect was not confined to tissue homogenates or isolated cell systems, since indomethacin and aspirin also abolished prostaglandin synthesis and release from the isolated perfused, intact spleen of the dog. It is to be hoped that this promising work will be extended to a wider range of human tissues and that attempts will be made to analyse the mode of action of indomethacin and aspirin in this situation. Clearly, if prostaglandins mediate inflammation, drugs of the aspirin and indomethacin type may owe their anti-inflammatory properties at least in part to their inhibitory activity on the biosynthesis of prostaglandins.

Section C. The Role of Lysosomes in Inflammation

De Duve and associates (1955, 1959) observed that several distinct acid hydrolytic enzymes in the rat liver homogenates exhibited activity only when they were released from their cytoplasmic particles into the extracellular compartment. The theory proposed by de Duve that acid hydrolases or "lysosomes" (de Duve et al., 1955) exist in an inactive form within the confines of a membraneous sac and are activated upon release (de Duve, 1964) has gained general acceptance.

There are now at least 12 acid hydrolases known to exist in lysosomes of various cell-types (Lewis, 1965). According to Koenig (1962), the lysosomal enzymes are actually bound to solid complexes with acidic glycolipids.

The history of the pathogenesis of inflammation has long favoured the hypothesis that proteolysis is a basic mechanism in inflammatory and cellular injury (Jobling and Peterson, 1914). In more recent years, it has been suggested that the activation or release of proteases into tissues is a primary step in inflammation (Ungar, 1956; Rocha e Silva, 1956; Hayashi et al., 1960, 1962). The advent of the lysosome concept then provided a hypothetical mechanism for the activation of pre-existing proteases.

On the basis of de Duve's (1959) findings that cathepsins are

present in various tissues, Dingle (1961) found that the addition of vitamine A into lysosomes suspensions resulted in the release of soluble cathepsin, which produces rapid degradation of the embryonic cartilage matrix in vitro. Cohen and Hirsch (1960 a,b) demonstrated that the specific granules of PMN leukocytes are similar to lysosomes in their enzymatic composition and general properties. The role of these granules in the digestion of engulfed material was elucidated by phase and electron microscopic observations together with chemical studies during phagocytosis: As soon as a phagocytic vacuole was formed around an ingested particle, the granules proceeded to fuse with the membrane and empty their content into the vacuole. Thus, the degranulation of leukocytes, which characterizes phagocytosis (Robineau and Frederick, 1955; Hirsch and Cohen, 1960), represents the transfer mechanism of the hydrolases from the lysosomes to the surface of engulfed material.

According to Weissmann and Dukor (1970), whatever the role of humoral mediators in acute inflammation, structural injury to tissues (as opposed to vasodilation, pain and swelling) cannot proceed without hydrolytic degradation of extracellular and intracellular macromolecules. Lysosomes appear suitably equipped for this role. Substances found in lysosomes of leucocytes or other cells have now been shown capable of degrading the following materials relevant to tissue injury: collagen, elastin, protein

polysaccharides of cartilage, intact cartilage, hyaluronate, nucleic acids, etc. (Weissmann and Dukor, 1970).

It is possible that these enzymes, under the appropriate stimuli, may produce damaging effects on the same cell or other surrounding tissue components. The administration of streptolysin, which has a selective lytic action of the granules, may result in the autolysis of the cells. Considerable evidence has accumulated to implicate the involvement of lysosomes in the Schwartzman and Arthus reactions in that both are prevented by administering agents which cause polymorphonuclear leukopenia (Becker, 1948; Stetson and Good, 1951; Humphrey, 1955). In 1959, Cochrane et al. (1959) reported that transient leukopenia does not affect the immunological mechanism during the Arthus reaction, however, no haemorrhage or tissue necrosis takes place until the appearance of leukocytes. Furthermore, Daems and Oort (1962) demonstrated the degranulation of leukocytes during the Arthus reaction with the electron microscope. Recently, it has been shown that the intravenous injection of a suspension of leukocyte granules in rabbit brings about a state remarkably similar to the Schwartzman reaction (Thomas, 1964). Furthermore, the Schwartzman-like reaction, produced by the leukocyte granules, was inhibited by pretreating the animals with cortisone, which is known to stabilize the lysosomal membrane. Supporting evidence, indicating the active participation of the leukocyte granules in necrotizing lesions,

also came from the studies of Halpern (1964).

Although the exact mechanism is not known, three principal hypotheses have been forwarded to explain the release of enzymes from leukocytes following their uptake of bulky foreign materials. The first is based on the original observations of Metchnikoff (1905), which implies that loss of integrity of the lysosomal membrane is accompanied by the loss of integrity of the plasma membrane. This cytolytic process should be followed by the release of unrelated cytoplasmic substances into the extracellular compartment. However, this was not found to be the case. Dingle (1968) suggested that endocytosis affects the membrane of the primary lysosomes to such an extent that they fuse with similarly perturbed portions of the plasma membrane, thereby resulting in the release of lysosomal enzymes directly to the outside of the cell, without the passage of the enzymes through an aqueous phase of the cytoplasm. In this manner, large amounts of enzymes may be secreted without cytoplasmic damage or concurrently with non-lysosomal enzymes. On the other hand, Weissmann et al. (1969) hypothesize that cells which phagocytize bulky foreign materials "regurgitate" lysosomal enzymes from phagocytic vacuoles which are connected to enzyme-rich lysosomes at their internal borders and external borders to the outside of the cells by incompletely fused endocytic channels. Data consistent with this hypothesis were reported by Karnovsky (1962) and Zucker-Franklin and Hirsch

(1964). Recently, Weissmann et al. (1971) confirmed that the phagocytosis of indigestible materials causes the selective extrusion of lysosomal enzymes from macrophages and PMN leukocytes. Furthermore, they found that compounds which increased the intracellular concentrations of cyclic - AMP appeared to inhibit the release of acid hydrolases, after particle ingestion, without significantly inhibiting particle uptake.

The lysosomes of the polymorphonuclear leukocytes are probable mediators of inflammation since the phlogistic agents, released in vitro by the phagocyting leukocytes of certain species, when tested in the same species, induced vascular changes of inflammation by a mechanism other than the release of vasoactive amines (Movat et al., 1969). Furthermore, Beckman et al. (1971) found an increase of acid phosphatase in the synovial fluid of rheumatoid arthritic patients and that the activity of acid phosphatase was directly proportional to the number of white cells in the fluid. The possible role of lysosomes in inflammation is further strengthened by the fact that cortisone, in conditions with lysosomal dysfunction, can favourably modify the outcome of tissue injury by enhancing the stability of the lysosomal membrane of leukocytes.

Section D. Anti-Inflammatory Compounds

a) Experimental Approaches to the Study of Inflammation

The most common purpose in setting up models of inflammation is the search for and comparison of anti-inflammatory drugs (Whitehouse, 1965). Potentially useful anti-inflammatory compounds are most frequently evaluated *in vivo*, on the basis of their ability to inhibit or modify the course of experimentally induced acute or subacute inflammatory reactions in small animals. However, a number of *in vitro* methods have also been designed for the testing of potential anti-inflammatory compounds against a variety of parameters of inflammation (Domenjoz, 1960; Stone et al., 1961; Winder et al., 1962; Winter and Porter, 1957; Winter et al., 1963; Weiner and Piliero, 1970). In the present review both experimental approaches will be briefly discussed.

1. In Vitro Models

Essentially all processes of living tissue, including inflamed tissue, are in the last analysis physical-chemical. The following concepts represent currently fashionable attempts to design simple *in vitro* evaluations for the testing of anti-inflammatory activity.

Since phosphorylation is critical to many energy dependent reactions

of living tissue, interference with it has been suggested as a mechanism for anti-inflammatory activity (Skidmore and Whitehouse, 1965; Whitehouse, 1967; 1968; Yamasaki, 1967). At present, the general consessus is that the hypothesis needs to be developed much more specifically before in vitro evaluation of drug effects on phosphorylation can be fruitfully employed in the search for anti-inflammatory activity (Goldstein et al., 1968). Since inflammation involves enzymatic and metabolic processes, which are often dependent on natural polyvalent metal complexes, it is reasonable to expect that inflammation will be influenced by agents which compete or otherwise interfere with metal binding by these natural ligands (Gerber, 1966). At least one anti-inflammatory compound was developed through the in vitro study of its copper chelating capacity (Wiesel, 1966). Hydrocortisone itself may owe some of its activity to its ability to bind with copper. On the other hand, extremely potent chelating agents such as EDTA are not clinically useful anti-inflammatory compounds.

Many inflammatory diseases, particularly arthritis, are accompanied by detectable changes in plasma or serum proteins which are reflected in a number of in vitro tests, such as erythrocyte sedimentation rate and turbidity of plasma or serum upon heating (Glenn, 1966; Glenn and Kooyers, 1966; Mizushima, 1964; 1965; Mizushima and Nakagawa, 1966; Piliero and Colombo, 1967; 1969). The courses of these diseases are often mirrored in these blood protein para-

meters. Essentially all clinically effective steroid and non-steroid broad spectrum anti-inflammatory compounds are able to bind to serum proteins and influence the turbidity of heated serum (Piliero and Colombo, 1967). At present, however, it seems unlikely that these effective anti-inflammatory compounds act by virtue of their binding to serum protein, although their capacity to bind in this manner may represent a trait reflecting their action at some tissue site.

Empirical studies of enzyme activity in human synovial fluid (Kerby and Taylor, 1967) provoked interest in the enzymatic approach of inflammation. ϵ- amino-caproic acid and aprotinin, potent inhibitors of proteolytic activity, can control experimental protease-induced inflammation (Bertelli, 1968), but have generally been unsuccessful as clinical anti-arthritic agents. Nevertheless, several <u>in vitro</u> enzymatic assays are used to study and screen potential anti-inflammatory compounds (de Duve, 1964; Lorber, 1968; Weissmann, 1967; Weissmann and Thomas, 1963).

According to cinematographic evidences lysosomes "explode" in areas involved in the inflammatory process, releasing numerous acidic hydrolases and proteases which play a role in inflammation. Consequently, factors which influence the stability of the lysosomal membrane (de Duve, 1964; Weissmann, 1964; 1965; 1967; Weissmann, Becker and Thomas, 1974) may be expected to influence the inflammatory reaction. A variety of techniques have been developed to study

this membrane "stability" in vitro (de Duve, 1964; de Duve et al., 1955; Dingle, 1961; Janoff et al., 1962; Sawant et al., 1964; Tannaka and Yoshio, 1968). It is now known that many anti-inflammatory compounds have the capacity to inhibit the release of various hydrolases (Dumphy and Udupa, 1955; Ennis et al., 1968; Lorber, 1968; Oronsky et al., 1969). However, it must be stressed that there is no one-to-one relationship between in vitro effects on lysosomes and clinical anti-inflammatory activity (Brown and Schwartz, 1969).Furthermore, it cannot be assumed that all proteolytic activity involved in inflammation is of lysosomal origin (Eisen and Gross, 1965).

In summary, the generally accepted view seems to be that none of the presently available in vitro techniques is sufficiently specific and reliable for the evaluation of useful anti-inflammatory compounds.

2. In Vivo Models

In most in vivo models, the degree of inflammation is quantified by measuring one or more facets of the inflammatory reaction, i.e., local swelling (volume or weight), temperature, redness, pain, etc. Acute non-specific models are frequently used to screen and compare anti- inflammatory activity. Quantitative differences may appear depending on the species of animal used, time factors and

other features of the test design. Qualitative differences may also occur, depending on the nature of the observed parameters. Thus, pure analgesics may be positive in pain-dependent test and not in test measuring swelling. Drugs which systemically alter fluid balance may influence local swelling without being at all anti-inflammatory. There are also less well understood differences in the way classes of anti-inflammatory compounds differ in different models. Potent anti-inflammatory steroids do not influence ultraviolet-induced erythema in guinea pig skin, while many non-steroid anti-inflammatory compounds are consistently effective in this model.

Experimental inflammation may be classified in terms of the most prominent symptoms produced and some of the more frequently used models will be briefly discussed on their ability to induce edema, erythema, granuloma tissue and experimental arthritis.

i) Locally Induced Edema

New methods of testing for anti-inflammatory activity or modification of existing methods have appeared with increasing frequency within the past few years. In these tests most attention has been paid to those changes which lead to a loss of fluid or leakage of protein.

The various substances used to produce local edema work by diff-

erent mechanisms and give varying responses to drugs (Winter, 1965). Increases in amino acid concentration in edemic fluid following the injection of various irritants are reported to follow different time courses (Kalbhen, 1963). The composition of edema fluid after the topical application of xylene has been reported to change with time (Ascheim, 1964; Langgard et al., 1964; Szporny et al., 1964). The severity of the swelling of the rat paw with respect to time varies with the various edema-producing agents (Winter, 1965).

Several workers have proposed that the leakage of fluid and of plasma proteins are two separate mechanisms (Cotran and Majno, 1964; Gözsy and Kato, 1956); for example, Brown and Robson (1964) observed a differentiation in the effect of diverse anti-inflammatory drugs on the colouring, due to accumulation of injected dye in the inflamed ear of the mouse and the inhibition of swelling in the same ear.

Studies of the extravasation of protein during local inflammation have usually involved the labelling of plasma proteins (Vogin et al., 1962; Witte et al., 1961). A more common method of labelling plasma proteins is to inject an animal intravenously with a dye which specifically binds protein (e.g., trypan blue). Accumulation of protein-bound dye in a locally inflamed area is used as an estimate of the intensity of the inflammatory reaction. This may be estimated subjectively (Northover and Subramanian, 1961; 1962) or by

extracting the dye and quantitate it colorimetrically (Judah and Willoughby, 1962). The increase in permeability may also be quantified by producing an intraperitoneal irritation in animals previously injected with a dye and the amount of dye later recovered from the peritoneal cavity is taken as a measure of "peritoneal permeability" (Northover and Verghese, 1962; Northover, 1963; 1963a). However, it was subsequently found that the method was insensitive to steroids and not able to differentiate the relative potencies of different anti-inflammatory agents (Winter, 1966).

Many methods have been reported in an attempt to measure the experimentally induced edema of the rat (Domenjoz, 1953; Winder et al., 1957; Buttle et al., 1957; Hillebrecht, 1954). The method of Ungar et al. (1959), involving the weighing of the skin after the intradermal injection of "inflammatory-producing substances" has not been widely adopted, possibly because the foot edema of the rat can be more easily and accurately measured. At present, the induction of edema in the rat paw and the measurement of its severity by the plethysmographic method (Winter et al., 1962) became one of the most widely used tests because of its simplicity.

However, the reliability of the foot edema method has suffered because some investigators tend to draw conclusions from results obtained after the intraperitoneal administration of toxic doses of anti-inflammatory agents despite the fact that nonspecific inhibi-

tion of foot edema had been reported when irritating substances were intraperitoneally injected (Benzi and Frigo, 1964; Buch and Wagner-Jauregg, 1960; 1962).

Dextran, egg white and fomalin have been widely used to induce edema in rat paws. The edema produced by these agents can be inhibited by anti-inflammatory steroids, antihistamines and antiserotonin compounds (Domenjoz, 1953; Halpern and Briot, 1950; Kato and Gözsy, 1961) but not by phenylbutazone, indomethacin or flufenamic acid (Lorenz, 1961; Stucki and Thompson, 1958; Winder et al., 1957; Winter, 1965). Since the last three compounds are unquestionably active anti-inflammatory and antirheumatic agents, the phlogistic agents listed above may be considered unsuitable for the screening of anti-inflammatory compounds (Lorenz, 1961; Winter, 1965).

At present, the most widely used phlogistic agent is carrageenan, a polysaccharide derived from Chondrus, an Irish sea moss. The use of carrageenan to induce rat paw edema was introduced by Winter et al. (1962),and its edema-inducing activity was confirmed by others (Arrigoni-Martelli and Conti, 1964, 1964a; Niemegeers et al., 1964). It was shown too that all clinically effective antirheumatic drugs also possess anti-carrageenan activity (Niemegeers et al., 1964). Carrageenan-induced edema is inhibited equally well by steroid and non-steroid anti-inflammatory agents (Benitz and Hall, 1963; Winter et al., 1962).

Recently, Di Rosa and Sorrentino (1968; 1970) have shown both by the inhibition of plasma kallikrein with aprotinin and by depletion of plasma kininogen with cellulose sulphate that kinins are released in the inflammatory response to carrageenan in the rat. Fekete and Kürti, (1970) have reported evidence of 5-HT involvement in carrageenan-induced rat paw edema. They found that methylsergide - an antagonist of 5-HT - suppressed the carrageenan edema. These observations were confirmed by Crunkhorn and Meacock (1971) who also reported that kinins and 5-HT are most important in the first four hours of the carrageenan-induced edema. In 1968 Coppi and Bornadi demonstrated that there is a considerable increase in acid phosphatase (a lysosomal enzyme) in the soft tissues of the rat 24 hours after the injection of carrageenan. In addition, prostaglandins E_2 and $F_{2\alpha}$ have also been identified in the edema fluid induced by carrageenan (Willis, 1971). These findings were recently confirmed by Anderson et al. (1971) who identified both lysosomal enzymes (β-glucuronidase and acid phosphatase) and PGE_2 in the edemic fluid induced by carrageenan.

ii) Erythema

Another manifestation of inflammation is the development of erythema, which has also been widely used for drug testing. Tetrahydrofurfuryl nicotinate, when applied onto the skin, produces erythema both in man and guinea pig. In man, this erythema is highly sensitive to

asprin, but apparently not reliably so to other known antirheumatic drugs (Adams and Cobb, 1963); in guinea pigs, it responds to sodium salicylate and phenylbutazone (Haining, 1963). In most of the studies erythema was brought about by using ultraviolet light as the irritant. Introduced by Schikoor (1932) and applied to guinea pigs by Wilhelmi (1949; 1960), this method has been studied by a number of investigators (Adams, 1960; Adams et al., 1963; Adams and Cobb, 1958; Brock et al., 1954; Kadatz, 1957; Winder et al., 1958). Winder et al. (1958) made a particularly thorough analysis of the method and achieved results with doses of phenylbutazone comparable to those effective in foot edema induced by kaolin or carrageenan. There are, however, some inconsistencies in this method. Generally, anti-inflammatory agents do not prevent the appearance of erythema, they merely delay it (Winter, 1966). Recent data (Winder et al., 1962; 1963) indicate that the assay may be markedly affected by feeding or fasting the animals. Certain metabolic enzyme inhibitors showed an apparent correlation between their ability to inhibit erythema, glycolysis and oxidation (Görög and Szporny,1964). Salicylates, which are potent inhibitors of several enzymes important in intermediary metabolism (Smith, 1963; Whitehouse, 1965), are also active against ultraviolet-induced erythema (Winder, 1958).

iii) Granuloma

Inhibition of granuloma tissue formation has also been widely used

for anti-inflammatory testing. Granuloma growth may be stimulated by the subcutaneous injection of an irritant, such as carrageenan or turpentine (Atkinson et al., 1962; Trnavky et al., 1962), by the well known granuloma pouch technique (Robert and Nezamis, 1957; Selye, 1953), or by the implantation of a cotton pellet (Meier et al., 1950). The granuloma pouch, used mainly for the testing of steroids, has also been applied to non-steroids (Boris and Stevenson, 1965; Laskin and Kolodny, 1965; Zileli et al., 1962).

Technical differences in the performance of the cotton pellet test in various laboratories (Alexander and Bush, 1960; Tarnaka et al., 1960; Winder et al., 1962; Winter et al., 1963) have led to diverse figures but, in general, there is agreement that both steroids and non-steroids are capable of inhibiting granuloma tissue formation (Winter, 1966).

A modification of the granuloma test, introduced by Rudas (1960), consists of removing a circle of skin from the back of a rat, preventing regrowth of skin over the wound by a plastic ring, and weighing the granulation tissue covering the wound after a week. The formation of granulation tissue can be inhibited by steroidal and non-steroidal anti-inflammatory drugs (Lindner and Rudas, 1961; Rudas, 1960; Jørgensen, 1962; Garn et al., 1963).

The inhibitory effect of anti-inflammatory drugs upon granuloma tissue formation has not received a satisfactory explanation in

biochemical terms; indeed, the cotton pellet has almost been ignored by those studying the biochemistry of granuloma tissue formation (Winter, 1966). Some differences between steroids and non-steroids in their biochemical effects upon granuloma, induced by turpentine or by cotton pellet soaked with carrageenan, have been described (Trnavsky et al., 1962; Trnavsky and Trnavska, 1964; Trnavsky and Trnavska, 1965). No biochemical changes were seen to be induced by either steroids or non-steroids in granulation tissues produced by the Rudas method (Jørgensen, 1962; Rudas, 1963; Winter, 1966); the only change was in the weight of the granuloma.

iv) Experimental Arthritis

Various infective, chemical, hormonal, immunological or physical agents (Gardner, 1960) have been injected into the joint of small animals in order to produce arthritis bearing some resemblance to human rheumatoid arthritis. The experimental model which has attracted the most attention is the adjuvant arthritis in rats (Winter, 1966). The injection of killed mycobacteria or a wax fraction derived from mycobacteria suspended in mineral oil into the plantar surface of the foot or intradermally into the tail produces a local swelling or primary inflammation initially. The adjuvant arthritis begins after a delay of 10 to 14 days, characterized by painful inflammation of the joints of one or all of the feet and other inflammatory lesions on ears, tails or lungs. These lesions

begin to abate after about 30 days (Newbould, 1963). Several steroidal and non-steroidal anti-inflammatory agents have been reported to be effective in decreasing the severity of this experimental arthritis (Newbould, 1963; Glenn, 1966; Winter and Nuss, 1966; Winder et al., 1969).

The long lasting polyarthritis induced in rats by the injection of mycobacterial adjuvant is now used in many laboratories (Glenn, 1966; Graeme et al., 1966; Kapusta and Mendelson, 1969; Newbould, 1963; Pearson and Wood, 1959; Winder et al., 1969; Winter and Nuss, 1966). It has several features in common with human arthritis (Currey and Ziff, 1968; Katz and Piliero, 1969) including the histopathology of the joints (Burstein and Waksman, 1964; Flenn, 1966) and the lack of a direct correlation in time of circulating antibody with joint lesions (Weiner and Piliero, 1970).

Adjuvant disease is believed to be due to a form of delayed hypersensitivity reaction to the mycobacterial antigen(s) because: 1) the induction of tolerance to mycobacterial antigen in the neonatal period can inhibit subsequent production of the disease (Flax and Waksman, 1963; Gery and Waksman, 1967); 2) there is a characteristic 10 - 14 days delay or latent period between induction and the onset of arthritis (Pearson and Wood, 1959); 3) the disease can be passively transferred between highly inbred rats by means of intact lymphoid cells (Waksman and Wennersten, 1963; Pearson and Wood, 1964); and 4) anti-rat lymphocyte serum inhibits the appearance of

arthritis (Currey and Ziff, 1966; 1968).

b) Compounds with Anti-Inflammatory Activity

Although the ideal anti-inflammatory drug would suppress the reactions of both the early and late phases of inflammation as well as prevent further tissue damage, no such drug exists today. In this section, some of the drugs which have proven to be useful in the treatment of the rheumatic diseases will be discussed, paying particular attention to their possible mechanism(s) of action in the inflammatory process.

1. Steroids

The anti-inflammatory activity of steroids was first described by Hench et al. (1949). They are among the most potent anti-inflammatory agents known and are extensively used in the treatment of patients with rheumatoid arthritis, although their use and therapeutic value have become somewhat controversial. According to Stones and Slocumb (1968) there is insufficient evidence to demonstrate that these drugs stop or significantly alter the natural course of the underlying disease. In spite of the uncertainty, they are still widely used because of their palliative effect in suppressing the symptoms due to joint inflammation.

The effects of steroids on the early stages of inflammation seem

to be rather weak and doubtful. Though cortisone and its analogues are capable of preventing the first event in inflammatory reactions, namely venular dilatation (Glenn et al., 1968), it is a generally accepted fact that corticosteroids do not influence the pharmacological actions of histamine, however, they have been shown to depress the transformation of histidine to histamine (Halpern et al., 1953; Schayer et al., 1955) and to deplete the tissue histamine content in guinea pigs (Kovacs, 1965; Hicks, 1965). Melmon and Cline (Cline and Melmon, 1966; Melmon and Cline, 1967) have suggested that steroids may exert their anti-inflammatory action partly by inhibiting the activation and activity of kinin-forming enzymes. However, recent evidence provided by Eisen, Greenbaum and Lewis (1968) seems to indicate that the anti-inflammatory action of steroids cannot be explained by the inhibition of kinin formation. Furthermore, cortisol is neither an inhibitor of esterases, proteases, nor is it a vasodilator (Houck and Jacob, 1964).

Experimentally, the most prominent effects of steroids can be seen in the later stages of the inflammatory process. Hydrocortisone diminishes the number and motility of polymorphonuclear leukocytes entering the inflamed area (Dougherty and Berliner, 1959; Dougherty, Berliner and Berliner, 1966). It inhibits platelets aggregation as well as the release of platelet constituents by binding to the platelets and interfering with their adherence to surfaces (Packham, Nishizawa and Mustard, 1968). In necrotic wounds, characterized

primarily by a profound loss of collagen, cortisone administration inhibited the expected accumulation of glyciprotein within the injured area (Anderson, 1964). Dougherty and associates (1959; 1966) have found that hydrocortisone protects fibroblasts from destruction in an inflamed area of connective tissue by causing them to round up. This protective effect persists even after the hydrocortisone has been metabolized.

Although the intimate mechanisms by which steroids exert their anti-inflammatory activity are not known, they seem to act through different mechanisms than non-steroids (Whitehouse, 1965). The local anti-inflammatory properties of steroids correlate well with their ability to inhibit the oxidation of NADH by the electron transport chain of animal mitochondria (Anderson, 1964) and to stimulate mitochondrial ATP-ase activity, thus inhibiting energy yielding oxidative reactions (Whitehouse, 1965). Whitehouse suggests that their systemic anti-inflammatory activity may be the sum of two factors, namely their intrinsic ability to inhibit NADH oxidation (which hydrocortisone does poorly - Glenn et al., 1963) and their ability to reach the site of inflammation without being inactivated. Indeed, it has been shown, for example, that at the site of inflammation, induced in mice by the local injection of egg white, there is a non-specific concentration of exogenously administered hydrocortisone (Dougherty et al., 1958).

Weissmann and Dingle (1961) on the other hand, proposed that the

ability of corticosteroids to stabilize lysosomes is the basis of their anti-inflammatory activity. They showed that the exposure of rat liver lysosomes to ultraviolet light caused the release of cathepsin and other lysosomal enzymes; pretreatment of the rats with cortisone resulted in a significant decrease in the fragility of the lysosomes to ultraviolet light. Furthermore, Weissmann and Thomas (1963) found that hydrocortisone, added to lysosomes in vitro, will block the lytic action of vitamin A. These early observations were confirmed by Seeman (1968) who also showed that the protective effect or stabilization of the lysosomal membrane is lost when steroids were given in high concentration. Recently, Lewis et al. (1970) reported that although most steroids stabilize lysosomes at pharmacological concentrations, they lyse them at high concentrations. Furthermore, they found that in high concentrations, steroids cause the denaturation of albumin. They, therefore, suggested that the lytic effect of the anti-inflammatory steroids could be due to a change in membrane configuration by the process of denaturation while their stabilizing effect is probably due to steroid-lipid interaction. Hempel et al. (1970), however, failed to demonstrate a protective effect when isolated rabbit leukocyte lysosomes were stressed by detergent in the presence of hydrocortisone. Furthermore, Gotjamanos (1970) reported that cortisone has the ability to profoundly impair the phagocytic capacity of fixed macrophages.

The present concensus of corticosteroids as anti-inflammatory agents

is that there is no evidence that the antirheumatic properties of these steroids actively suppress the disease. Moreover, at the therapeutic dose level, they invariably produce one or more serious side-effects which by themselves are deletrious to the patients. In addition, at sufficiently high dose, they are capable of suppressing the immunological apparatus, thus removing one of the most important host defence mechanism.

2. Non-Steroids

i). Salicylates

Salicylates are among the oldest known remedies. They still occupy a very important place in modern therapeutics. The most commonly used preparation is acetylsalicylic acid (A.S.A. or aspirin). Following absorption, 50 - 80 % of the salicylate is plasma bound, although it is believed that only the free salicylate ion can exert therapeutic effect (Wood, 1963). The findings of Wilhelmi and Pulver (1955) and Wilhelmi et al. (1959) have shown that the concentration of salicylate is no higher in the inflamed area or exudate than found in the normal tissues.

It is generally believed that the anti-inflammatory activity of the salicylates is related to their ability to uncouple oxidative phosphorylation (Whitehouse, 1965), to affect various enzymatic processes and to suppress vascular activity (Wood, 1963). Acetylsali-

cylic acid delays the appearance of erythema produced by ultraviolet light and thurfyl nicotinate in humans (Adams and Cobb, 1963). Swyer (1948) observed that salicylates could inhibit histamine-induced increase of capillary permeability. Wood (1963) noted that salicylates, injected locally, specifically inhibited the increase in capillary permeability induced by the injection of kallikrein or rat serum globulin permeability factor. Collier and associates (1959; 1960) have shown that small doses of A.S.A. inhibited bradykinin-induced bronchoconstriction in guinea pigs.

Despite the seeming diversity of the effects of A.S.A., its therapeutic actions can be classified as "anti-defensive" (Collier, 1963; 1969) in that it inhibits the development of excessive defensive reactions in the body, such as fever, pain and inflammation. Some of the noxious actions of histamine, kinins, SRS-A, prostaglandin $F_{2\alpha}$, ATP and acetylcholine, such as erythema, edema, bronchoconstriction or a fall in blood pressure were reported to be influenced by aspirin (Collier and Shortley, 1960; Berry and Collier, 1964; Vergaftig et al., 1969; Jaques, 1959; Collier et al., 1966; 1968). However, the antagonism of the effects of these substances by aspirin differs from a specific receptor blockade of the conventional type in several ways. First, the pattern of selectivity varies. Thus, in guinea pigs, aspirin antagonizes bronchoconstriction <u>in vivo</u> induced by bradykinin (Collier and Shortley, 1960; 1963), arachidonic acid (Berry, 1966)

and SRS-A (Berry and Collier, 1964), but not that induced by acetylcholine or histamine. Secondly, aspirin blockade is confined to certain species and organs. For example, aspirin antagonizes bradykinin-induced bronchoconstriction in guinea pigs but not in rats or rabbits (Bhoola et al.,1962). Furthermore, while it antagonizes the development of bronchoconstriction induced by the intravenous injection of bradykinin, it is inactive when bradykinin is applied topically onto the pleural surface. From the foregoing data, Collier (1969) proposed that "aspirin and like acting drugs block a route leading to and from the specific receptors for the agonists rather than blocking those receptors themselves".

Another possible mechanism of action for some of the effects of anti-inflammatory acids such as A.S.A. was postulated by Piper and Vane (1969) who found that lungs could release a previously undetected substance which, because of its action, was named "rabbit aorta contracting substance" or RCS. RCS, released by the antigen-antibody reaction, bradykinin and SRS-A, causes bronchoconstriction. They further reported that both the release of RCS and the associated bronchoconstrictive effect were antagonized by aspirin-like drugs. Although the structure of RCS is not known, it was postulated that RCS is probably a precursor of prostaglandins. The possibility arises therefore, that aspirin inhibits the enzyme(s) which generates prosta-

glandins. This hypothesis was tested and confirmed by Vane (1971) who found that aspirin and indomethacin strongly inhibited the synthesis of $PGF_{2\alpha}$ and PGE_2. These findings were quickly confirmed by Smith and Willis (1971) who observed that aspirin and indomethacin inhibited the production of prostaglandin in human platelets. Further endorsement came from Ferreira et al. (1971) who demonstrated that aspirin and indomethacin inhibited the biosynthesis of prostaglandins from the spleen. Prostaglandins have been identified in exudates during the second phase of carrageenan-induced inflammation in rats (Willis, 1969), in the inflamed skin of patients with allergic contact eczema (Greaves et al., 1971) and in erythema in man (Macmillan, 1968; Juhlin and Michaelsson, 1969). If prostaglandins indeed mediate the inflammatory reaction, the clinical effectiveness of aspirin could be then explained by the inhibition of prostaglandins production.

ii). Phenylbutazone

Phenylbutazone was first synthesized by Stenzl (1950) but it was not until 1952 that the first reports of its clinical efficacy appeared (Whitehouse, 1965). Phenylbutazone has proven to be an effective analgesic and anti-inflammatory drug, with a special value in the treatment of ankylosing spondylitis, gout and acute bursitis. It appears to be less effective in rheumatoid

arthritis, although, in some rheumatoid patients, it provides better results than salicylates (Smyth, 1968). An interesting finding with phenylbutazone is that the anti-inflammatory effect exerted by one of its metabolites, oxyphenbutazone, is comparable to that of the mother compound (Yu et al., 1958).

Unlike salicylates, the concentration of phenylbutazone is significantly higher in the inflamed area than in normal tissues (Wilhelmi and Pulver, 1955; Wilhelmi et al., 1959). Wallenfels and Sund (1959) reported that the drug is transported by serum protein, bound in a complex to the zinc atoms of serum albumin. At normal physiological pH almost all phenylbutazone is in the bound form, but with the development of inflammatory acidosis, the complex is dissociated and free phenylbutazone appears mainly in a neutral undissociated form (Wallenfels and Sund, 1957).

Phenylbutazone has been found to be active in a variety of experimental inflammation. Haining (1963) reported that it actively suppresses thurfyl nicotinate erythema and UV erythema in guinea pigs. Phenylbutazone has also been reported to inhibit the increase of peritoneal capillary permeability following a mild stimulus (Northover, 1963). Adams and Cobb (1967) noted that a much higher dose of phenylbutazone is required to produce a protective effect in rat paw edema than to reduce erythema. They also found a quantitative difference in the responsiveness of various types of edemas

to the drug. Their observations confirmed the work of Domenjoz and Morsdorf (1964) who reported that phenylbutazone inhibited trypsin-induced edema while Stucki and Thompson (1958) found that phenylbutazone could not modify the edema induced by the intraperitoneal administration of dextran.

Phenylbutazone has been shown to protect guinea pigs from the lethal effects of histamine (Domenjoz, 1952; 1953). Later, Collier et al. (1959) showed that phenylbutazone, like the salicylates, could antagonize the bronchoconstrictive effect of bradykinin in guinea pigs. Their results were confirmed by Lecomte and Troquet (1960) who demonstrated that phenylbutazone inhibited the increase in capillary permeability induced by intradermally injected bradykinin. Lecomte (1960) also reported that phenylbutazone suppressed the development of hypotension and altered vascular permeability following the challenge of a sensitized rabbit by the specific antigen. However, pain produced by the injection of bradykinin into guinea pig, dog and man was not modified by phenylbutazone (Frunder, 1953). According to Lewis (1964), phenylbutazone neither antagonizes the action of plasma kinins nor interferes with the kinin-forming system.

Although phenylbutazone does not influence the accumulation or distribution of leukocytes, it suppresses their phagocytic activity (Adams and Cobb, 1967). Trnavsky et al. (1964) noted that phenyl-

butazone suppressed the early reaction in turpentine-induced granuloma formation. According to these authors, it does not interfere with the synthesis of collagen, but there is some evidence that it increases the maturation process of collagen. On the basis of these data, they concluded that phenylbutazone probably acts as an anti-permeability, an anti-exudative but not as an anti-proliferative agent. On the other hand, Heilmayer et al. (1953) and Haberland (1959; 1960) were of the opinion that phenylbutazone inhibited the growth of fibroblast cultures. Furthermore, Bostrom et al (1964) also showed that phenylbutazone retarded the anabolic processes of granulation tissue formation by inhibiting the incorporation of sulphur into the mucopolysaccharides of the cartilage. Phenylbutazone can inhibit platelet aggregation and the subsequent release of its nucleotides, permeability factors and 5-HT by binding to the platelets and interfering with their adherence to surfaces (Packham, 1968). Phenylbutazone is also a potent uncoupler of oxidative phosphorylation (Adams and Cobbs, 1968; Whitehouse and Haslam, 1962). In addition, phenylbutazone appears to retard the degradation of anti-inflammatory steroids which may represent an aspect of its mechanism of action (Domenjoz, 1966).

iii) Indomethacin

Indomethacin is an indole acetic acid derivative which was first introduced for clinical trials as a non-steroid antirheumatic agent

in 1961 (Lockie and Norcross, 1966). Its ability to reduce fever, joint inflammation and joint swelling is superior to that of salicylates but not as dramatic as that of the anti-inflammatory steroids (Lockie and Norcross, 1966). Like phenylbutazone, 90 % of indomethacin is bound to the plasma protein and exerts its anti-inflammatory action in the free form (Healy, 1967).

The ability of indomethacin to inhibit experimental edema is dependent on the phlogen used. For example, it is able to inhibit or suppress the formation of edema induced by carrageenan, mustard (Adams and Cobb, 1967) and trypsin (Domenjoz and Morsdorf, 1964) but not that produced by egg albumin, fomalin, yeast and 5-HT (Adams and Cobb, 1967). Healy (1967) has also shown that indomethacin is able to uncouple oxidative phosphorylation, however, it is not known if this effect contributes to its anti-inflammatory activity. Recently, Vane et al. (1971) proposed that the anti-inflammatory activity of indomethacin may be explained by its ability to inhibit the biosynthesis of prostaglandins. The similarity in the mode of action between indomethacin and aspirin has been shown by Van Arman et al. (1970). However, they believe that the anti-inflammatory activity of these non-steroidal agents lies in their ability to prevent the neutrophils from releasing enzymes following their migration to the inflamed area.

From the therapeutic standpoint, indomethacin is very similar to

phenylbutazone; it is very effective in treating ankylosis spondylitis and acute gouty arthritis (Healy, 1967). However, it often requires months of treatment to achieve any therapeutic effects (Smyth, 1968).

iv) Gold Salts

Elemental gold has been employed for centuries to relieve itching. Modern application of gold in therapeutics began with the observations of Koch (1890) who reported that the tubercle bacillus is adversely affected by a low concentration of gold salts. In 1929, Forestier reported the efficacy of gold salts in the treatment of arthritis. Today, gold salts are used strictly in the treatment of rheumatoid arthritis.

In spite of its long therapeutic history, its mechanism of action is largely unknown. Persellin et al. (1967) suggested that they affect the immunological response of the patient. Lawrence (1961), on the other hand, felt that gold compounds act locally on the inflamed tissues by inhibiting the enzymes which participate in the inflammatory reaction. Sodium aurothiomalate has been shown to decrease the permeability of rabbit synovial membrane (Sharp, 1963) and reduce leukocyte infiltration or granulation tissue formation in rats (Sawyer et al., 1964), but not to affect the exudate formation (Saxena, 1960). Adams and Cobb (1960a) reported that in rats

chronically treated with gold preparations, the gold is bound to the tail collagen. Similarly, gold has been found in the collagen of patients treated with gold salts and this gold-collagen complex increases ultrastructural linkages, making the collagen structure more stable. Gold salts have been shown to inhibit lysosomal enzymes after their release (Ennis et al., 1968; Paltemaa, 1968). According to Strunk and Ziff (1970), in rats treated with gold thiomalate, electron-dense deposits were observed in the lysosomes of the glomerular epithelial cells - indicating the passage through the capillary wall. Recently, McArthur et al. (1971) were able to demonstrate that gold compounds displace L-tryptophane from the serum proteins to which they are bound.

At present, gold salts are widely used in the treatment of rheumatoid arthritis. It has recently been shown that in patients with rheumatoid arthritis, the therapeutic effects of gold administration can be correlated to the plasma gold level (Krusius et al., 1970; Rokkanen et al., 1971). In spite of the relatively high incidence of side-effects, gold salts are able to check further tissue deterioration due to rheumatoid arthritis and they have never been known to worsen the disease (Hill, 1968).

v) Chloroquine

The anti-malarial drug, chloroquine (a 4-aminoquinoline), has also

been found to be a useful antirheumatic agent. Following administration, chloroquine is detected in the nuclear and lysosomal cellular constituents of leukocytes (Zvaifler, 1964) and in complex with nucleic acids (Cohen and Yielding, 1965). According to Weissmann (1964), chloroquine also stabilizes the lysosomal membrane. Other pharmacological actions of chloroquine include the suppression of lymphocyte responsiveness (Hurvitz and Hirschorn, 1965), interference with protein metabolism (Gerber, 1965), chemotaxis of leukocytes (Ward, 1966) and mucopolysaccharide metabolism (Whitehouse, 1963). It has been shown that chloroquine reduces the contraction of the isolated rat uterus induced by acetylcholine, 5-hydroxytryptamine, angiotension, vassopressin, oxytocin and barium chloride (Garcia et al., 1968). Recently, Garcia et al. (1971) also reported that chloroquine has an eserine-like action by preventing the destruction of acetylcholine by cholinesterase. Unlike other non-steroid anti-inflammatory compounds, chloroquine does not uncouple oxidative phosphorylation (Anderson, 1965). Although chloroquine is very rapidly absorbed, it is very slowly metabolized (Goth, 1961). Whitehouse and Bostrom (1962), using an <u>in vitro</u> system, showed that there is a long time lag between the administration of chloroquine and the onset of action. It is interesting to note that in spite of its clinical efficacy, chloroquine does not show any anti-inflammatory activity in UV-induced erythema, rat paw edema test (Adams and Cobb, 1967) and bacterial-induced polyarthritis (Newbould, 1963).

The mechanism of action of chloroquine is not known. Aviado et al. (1970) reported that chloroquine alters neither the anaphylactic response nor the content of histamine, 5-HT and catecholamines. According to Whitehouse (1965), chloroquine is an antirheumatic agent which, because of its basic nature, could fail to arrive at the proper activation site until the base-binding site of all other tissues are occupied. It has also been suggested that it is not chloroquine but one of its metabolite which actually is the active substance.

Whatever is the mechanism of chloroquine's activity, it is a useful antirheumatic agent, but the toxicity of chloroquine, most frequently manifested in retinal damage, (Gleiser et al., 1969; Lee et al., 1971) has somewhat limited its therapeutic use.

vi) Mefenamic and Flufenamic Acids

These are N-substituted anthranilic acids which have recently been introduced as antirheumatic agents. Cass and Frederick (1963) reported that mefenamic acid is 2 - 3 times more potent than ASA as an analgesic.

Both mefenamic and flufenamic acids have been shown to be more potent than ASA as uncoupler of oxidative phosphorylation (Whitehouse and Haslam, 1962). Furthermore, these authors have obtained

evidence from their in vitro studies to show that mefenamic and flufenamic acids are potent inhibitors of cartilage mucopolysaccharide biosynthesis. In 1964, Northover found that both mefenamic and flufenamic acids are capable of inhibiting vascular permeability in the mouse peritoneal cavity. It has also been reported that flufenamic acid antagonizes bradykinin-induced bronchoconstriction in guinea pigs (Stresemann, 1963). Furthermore, he observed that patients with chronic bronchial asthma treated with flufenamic acid showed a significant improvement in their vital capacity. In the carrageenan edema test, Winter (1964) noted that flufenamic acid is about 4 times more potent than mefenamic acid. Both acids are known to possess antigranulation effect in the rat cotton pellet test (Adams and Cobb, 1967). Recently, Chignell and Starkweather (1970) reported that flufenamic acid may share the same binding site as ASA in the human serum albumin. However, when flufenamic acid is administered after ASA, the binding site for flufenamic acid is changed, indicating that these binding sites have greater affinity for ASA.

These are relatively new anti-inflammatory compounds and, consequently, they have not been widely used. Nevertheless, they seem to have definite therapeutic possibilities.

3. Naturally Occurring Substances

There have been numerous substances of natural origin which have

shown to exert anti-inflammatory activity in experimental inflammation. For example, materials extracted from granuloma pouches and shrubs have been shown to inhibit formalin edema in rat paw, while bioflavinoids extracted from citrus fruits were found to inhibit granuloma formation (Winter, 1966). Cirelli (1962) reported that a proteolytic enzyme isolated from pineapples was effective in a variety of acute inflammatory conditions. Filderman and Kovacs (1970) have demonstrated that tomatine, a steroid alkaloid glycoside (Fontaine et al., 1948), significantly inhibited carrageenan-induced paw edema, carrageenan impregnated cotton pellets and granuloma tissue formation. Anti-inflammatory activity has also been claimed for many other substances (Winter, 1966). Of these, very few are useful clinically, even on the basis of very limited therapeutical application. However, two of the more outstanding compounds will be briefly discussed below.

i) Glycyrrhetinic Acid

Glycyrrhetinic acid, which at one time enjoyed some popularity as an antirheumatic compound, is the aglycone of glycyrrhizic acid found in the licorice root. It bears some structural resemblance to cortisone. Although its anti-inflammatory activity in animals is well documented (Finney and Tarnoky, 1960), clinical trials have not yet substantiated its experimental antirheumatic efficacy (Whitehouse, 1965). The compound does uncouple oxidative

phosphorylation (White, 1962; Lee and Whitehouse, 1964) but, because it is so rapidly excreted, plasma levels of therapeutic significance are not obtained (Whitehouse, 1965) and anti-inflammatory activity does not seem to be manifested in the inflamed tissues.

ii) Dimethyl Sulphoxide (D.M.S.O.)

Dimethyl sulphoxide or DMSO is prepared from lignin in wood. According to the reports of Rosenbaum and Jacob (1964) and Rosenbaum et al. (1965), DMSO has remarkable therapeutic properties when applied topically to relieve swelling, stiffness and pain in a variety of inflammatory conditions such as musculoskeletal trauma, bursitis, osteoarthritis and rheumatoid arthritis. DMSO enhances the absorption of some drugs by the skin and has no permanent effects on the horny layer of the skin (Kligman, 1965). According to Kligman (1965) it is a potent histamine liberator and a potent cutaneous vasodilator. DMSO has been reported to exert therapeutic effect on the collagen of the dermis in the treatment of several dermatosis (Engel, 1967; Frommhold et al., 1967). Recently, Middleton et al. (1970) observed that DMSO swells collagen in the dermis and decreases the tensile strength of both normal and burned dog skin. According to Nuss and Winter (1965), DMSO has anti-inflammatory activity when tested in the bacterial adjuvant induced polyarthritis in rats. Although the drug is relatively non-toxic, its present use is limited to clinical research.

CHAPTER II. GENERAL METHODS

Section A. Method of Purification and Isolation of the Active Principles

Reagents and Solvents

All solvents and reagents were of analytical reagent grade: anhydrous methyl alcohol (Fisher Scientific), Chloroform (Fisher Scientific), ether absolute (Anachemia), anhydrous ethyl acetate (A & C American Chemicals) and Nitrogen gas (Union Carbide of Canada).

Apparatus

A grinding mill (Quacker City Mill) was used to grind the galls to a fine powder. A rotating evaporator (Buchler Instruments), connected to a Duo-Seal vacuum pump (The Welch Scientific) through a dry ice trap, was employed for all drying procedures.

a. Preparation of the Crude Extract

The Hungarian oak galls (Fig. 1) were collected into wooden crates in the autumn months and shipped to Montreal (Agrimpex Hungarian

Trading Co., Budapest, Hungary) in refrigerated compartments. On arrival, the galls were stored in commercial cold storage at -4°C until used.

The Hungarian oak galls (Andricus quercus tozae), which were blown due to the escape of the insects, and those that were mouldy were discarded. The selected galls were ground to a fine powder and extracted with chloroform: methanol (2:1 v/v), at 10 ml/gm powder, in a stoppered round bottom flask. The mixture, saturated with nitrogen gas, was stored for 16 hours and filtered through a

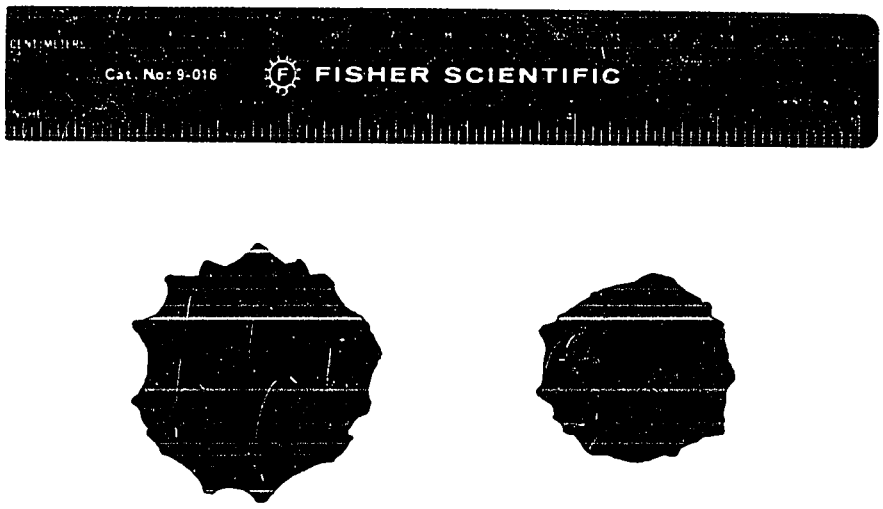


Figure 1

HUNGARIAN OAK GALLS (ANDRICUS QUERCUS TOZAE)

Buchner funnel using Whatman No. 3 filter paper. The filtrate was taken to dryness with a bath temperature of 40° C. The residue was extracted three times with anhydrous ethyl acetate (50 ml/100 gm oak powder) and filtered through a Whatman No. 3 filter paper in a Buchner funnel. The filtrate was thoroughly dried and then washed twice with ether absolute (20 ml/100 gm oak gall powder) in order to eliminate the chlorophyll. The ether insoluble residue was thoroughly dried.

b. Silica Gel Filtration

i). Material

The resin consisted of medium size (Max. 10 - 40 microns) silica gel G according to Stahl (E. Merck AG., Darmstadt, Germany). A chromatographic column (Scientific Glass Blowing Reg'd, Montreal) with dimensions of 4.0 x 60.0 cm with a ground glass socket joint at the top and a teflon stopcock at the bottom was used throughout the silica gel filtration experiments. The filter of the column was made of sintered glass - coarse. The top of the column was connected to a one liter capacity separatory funnel which served as reservoir by means of a ground glass ball joint.

ii) Procedures

The "fines" of silica gel, suspended in anhydrous ethyl acetate,

were removed by suction and the gel resuspended in ethyl acetate. This procedure was repeated until the slurry became relatively free of fine particles. The slurry was poured into the chromatographic column and allowed to be packed under 5 lb/sq.in. of nitrogen pressure. The packed chromatographic column was then allowed to equilibrate over a period of three hours. Following equilibration, the eluent was allowed to run out until the eluent level coincided with the surface of the packed silica gel. A saturated solution of the sample to be chromatographed was prepared in anhydrous ethyl acetate and applied onto the silica gel dropwise, without disturbing the surface of the packed gel and allowed to penetrate the gel under nitrogen pressure. The same volume of the eluent was similarly applied to wash-in the sample and eluted. The column was filled with the eluent connected to the reservoir and the chromatogram developed at the same pressure as that employed for equilibration. Small samples were taken at regular intervals to test for the appearance of tannic acid by mixing the samples with the ferric chloride solution. The presence of tannic acid was indicated by the formation of a blue colour complex. The tannin-free effluent was collected in a glass beaker, thoroughly dried in vacuo with a bath temperature of 40^{o} C and extracted twice with methanol:ethyl acetat:water (4:1:2, v/v) and centrifuged at 2ooo RPM for 10 minutes. The supernatant was dried and extracted thrice with chloroform. The chloroform soluble filtrate was thoroughly dried.

c) Sephadex LH-20 Column Chromatography

i) Material and Apparatus

The resin consisted of Sephadex LH-20 of size 25 - 100 u (Pharmacia, Montreal). Analytical grade anhydrous methyl alcohol (Fisher Scientific) was employed without further purification. A chromatographic column with dimensions of 2.5 x 80.0 cm was provided with a coarse glass filter disc, a water jacket and a teflon stopcock (Scientific Glass Blowing Reg'd). The column was connected to a two liter capacity separatory funnel by means of a ground glass ball joint. The Buchler refrigerated fraction collector (Buchler Instruments), placed directly under the column, was employed to collect the effluent fractions which were monitored by a UA-2 UV analyzer (Isco Instrumentation Specialties Inc.) at 280 mu.

ii) Procedure

Sephadex LH-20 was suspended in anhydrous methanol and allowed to swell for two hours. The gel was "de-fined" as described for silica gel (page 110). The slurry was poured into the column and allowed to pack by gravity. The reservoir containing anhydrous methanol was connected to the Sephadex LH-20 column which was eluted by the methanol at a fixed flowrate until two liters of the effluent were collected. The equilibration was done at 4°C. Following equilibra-

tion, a saturated solution of the sample dissolved in methanol was applied dropwise without disturbing the gel surface. The sample volume (15 ml) was allowed to diffuse into the gel and was washed-in three times using equal volume of methanol. The chromatogram was developed with anhydrous methanol and the effluents were collected in 10 ml/tube directly into the refrigerated fraction collector at 4°C. The effluents were continuously monitored by the UA-2 at 280 mu.

d. Isolation of the Antihistamine - KC-18

i) Material and Apparatus

The preparation of the sephadex LH-20 column and the silica gel G column were as described on page 110 and 112 respectively. Redistilled petroleum ether (boiling point range 30 - 60°C) was supplied by A & C American Chemicals.

ii) Procedure

The sample was applied onto the sephadex LH-20 column (4.8 x 20.0 cm) as previously described on page 112 and eluted stepwise with methanol:water (1:1, v/v) and methanol:water (2:1, v/v) respectively. The effluents were monitored by the UA-2 ultraviolet analyser (Instrumentation Specialties Co., Inc.) at 280 mu and collected

at 2 ml/min. in aliquots of 20 ml/tube. The selected fractions (based on the optical densities) were thoroughly dried. The dried extract was extracted twice with a mixture of petroleum ether:ethyl acetate (20:1, v/v) using 20 ml for each extraction and centrifuged at 2000 rpm for 10 minutes. The soluble fraction was collected and dried. The dry extract was then redissolved in the petroleum ether: ethyl acetate (20:1, v/v) mixture and applied onto a silica gel G column (0.8 x 10 cm). It was eluted with the petroleum ether:ethyl acetate mixture (20:1, v/v) under 5 lb/sq.in. of nitrogen pressure. The effluents, monitored by the UA-2 ultraviolet analyser at 280 mu, were collected at 2 ml/min. in aliquots of 5 ml/tube.

e. Purification of the Anti-Inflammatory Substance

i) Materials

The resins and chromatographic columns employed were identical to those described on page 110. Monitoring of the active fraction was based on the retention volume of the effluents. Chloroform and hexane were supplied by Fisher Scientific.

ii) Procedure

The gel was defined as described on page 110 and equilibrated with chloroform. The standard crude extract was suspended in

20 ml chloroform and was applied dropwise into the silica gel column (1.0 x 25.0 cm). The column was eluted under 5 lb/in^2 of nitrogen pressure with the following solvent systems: chloroform; chloroform:methanol (9:1, v/v); chloroform:methanol (7:3, v/v); chloroform:methanol (1:1, v/v); and chloroform: methanol (3:7, v/v). For each solvent system, 500 ml of the effluents were collected into a round bottom flask as a single fraction and the fractions were thoroughly dried. The active fraction thus obtained was used for all subsequent tests.

Section B. Monitoring of Chromatographic Effluents.

a. Continuous UV Analysis

i) Apparatus

The UA-2 ultraviolet analyser, with a dual beam optical unit, (Instrumentation Specialties Co. Inc.) was connected to the out-flowing end of the chromatographic column (CanLab) and the fraction collector (LKB Radi Rac) by a teflon tubing.

ii) Procedure

The selected operational wavelength for the analysis of the eff-luents was 280 mu with an OD range of 2.5. The effluents were

collected in aliquots of 20 ml/tube.

b) Volume Retention

Several preliminary chromatograms were developed to determine the region of effluents which contain the active compound. In these experiments, the flow rate, dead volume and the column dimension remained constant, while the volume of the fractions varied.

Section C. Thin Layer Chromatography (TLC)

i) Materials and Apparatus

For qualitative TLC, sheets precoated with Silica Gel (Przybylowicz et al., 1965) were used. The silica gel TLC sheets (chromatogram) were purchased from Eastman Kodak Co., N.Y. The apparatus for developing the chromatograms was obtained from Fisher Scientific, Montreal and consisted of metallic racks, solvent troughs and sandwhich type developing chambers.

Chloroform and hexane (Fisher Scientific) were of spectrograde and were employed without redistillation. The developed chromatograms were visualized with iodine (Mallinkrodt Chemical Works).

ii) Procedure

A light pencil line was drawn parallel (about 2 cm) to the edge of

the precoated sheet (20 x 20 cm), Pencil cross marks were made 2 cm from the edge and 2 cm apart. The sample, dissolved in chloroform, was applied on each cross mark by means of a micropipet (Lang-Levy) using a hair dryer to evaporate the chloroform. The area occupied by each sample was less than 5 mm in diameter. The sheet was placed in the developing chamber, which was then transferred into the solvent trough. The developing solvent was poured into the trough and the chromatogram was developed until the solvent front had travelled approximately 17 cm. The developing chamber was then removed from the solvent trough, the chromatogram taken out and the solvent front marked immediately and visualized by either I_2 vapour, UV light or sprayed with 87 % O-H_3PO_4 and heated at 65°C for 5 minutes.

Section D. Instrumental Analyses.

a. Infra-Red Spectrophotometry

IR analyses were carried out in a Perkin-Elmer 257 infra-red spectrophotometer.

b. Ultra-Violet Spectrophotometry

UV analyses were done in an Unicam SP 800 ultraviolet-visible spectrophotometer.

c. Nuclear Magnetic Resonance

The nuclear magnetic resonance spectra of the samples were determined in a Varian A - 60 or T-60 NMR spectrometer.

d. Mass Spectrometry

All mass spectra were recorded on an AEI MS902 mass spectrometer. The operating conditions were: 70-eV electron energy, resolution of 1000 and 8 KV accelerating voltage.

e. Cellulose Acetate Electrophoresis

The cellulose acetate electrophoretic mobilities of samples were determined with a Beckman Duostat. The buffer used was the Beckman B-2 Buffer, which contains (per liter) 2.76 gm diethyl barbituric acid and 15.4 gm sodium diethyl barbiturate.

Section E. Base Hydrolysis

i) Materials

NaOH pellets were obtained from Anachemia and Concentrated HCl were supplied by A & C American Chemicals.

ii) Procedure

The compound was dissolved in 0.1 N NaOH at a concentration of

1 mg/ml and allowed to reflux at 50°C for 2 hours. The solution was then cooled and extracted three times with ether (10 ml/mg compound). The pH of the aqueous fraction was adjusted to 4.5 by adding 0.1 N HCl and the acidic solution was extracted immediately three times with ether (10 ml/mg compound). The ether soluble fraction was thoroughly dried.

Section F. Methods For Testing The Biological Activity

a. In Vitro Method - The Isolated Guinea Pig Ileum Preparation

i) Chemicals

Histamine dihydrochloride (Fisher Scientific) of high purity was used. Unless otherwise stated, the concentration of histamine solution was calculated as base. Sodium bicarbonate, sodium chloride, dextrose, calcium chloride, magnesium chloride, potassium chloride and atropine sulphate (Fisher Scientific) were of reagent grade.

ii) Apparatus

A constant temperature bath (Palmer Co.,), made of plexiglass with metal reinforcement of the following dimensions 20 x 23 x 30 cm, was employed for the *in vitro* testing. The water bath was provided with a thermostat, a heating element, an electric stirrer, a thermometer, an organ bath and a coiled polyethylene tubing.

The 10 ml organ bath was supported by a glass out flow tube which

led to a rubber drainage tube. The inlet tube of the organ bath was connected to the Tyrode reservoir by the coiled polyethylene tubing and a ground glass stopcock, which served to regulate the flow of the Tyrode solution. The oxygen inlet tube, provided a means for attaching the ileal strip within the organ bath. The other end of the ileum was fixed to a hook which was attached to a thread. The thread was in turn fixed to the writing lever, located directly above the organ bath. The magnification of the writing lever was 5:1. When the organ bath fluid was renewed (during washing), the lever may be held at the fulcrum by a cable releaser. A kymograph (Palmer Co.) with variable speed was used for recording.

iii) Preparation of the Tyrode Solution

10 liters of Tyrode solution were prepared by dissolving the following:

10 gm $NaHCO_3$,	20 ml of 10% $CaCl_2$ solution,
80 gm NaCl,	5 ml of 40% $MgCl_2$ solution,
10 gm dextrose,	20 ml of 10% KCl solution,
7 ampules atropine sulfate (0.4 mg/ampule)	10 ml of 5% NaH_2PO_4

The atropine was incorporated into the Tyrode solution for all the *in vitro* assays.

iv) Preparation of Standard Histamine Solution

A 100 ug/ml stock solution of histamine was prepared by dissolving 16.6 mg histamine dihydrochloride into 100 ml distilled water. This solution remained stable for several months when kept at 4°C. The standard solution was prepared by diluting 1 ml stock solution to 100 ml with Tyrode. Freshly prepared standard solution of histamine was used for each assay.

v) The Isolated Guinea Pig Ileum Preparation

A guinea pig of either sex, fed with normal diet and weighing between 250 - 300 gm, was killed by a blow on the head. The abdomen was immediately exposed and a piece of ileum, approximately 20 cm long, near the caecum was removed and transferred to a Petri dish containing 50 ml Tyrode. The inside and the outside of the ileum was thoroughly washed with Tyrode. The end of the ileal strip nearest to the caecum was marked with a 21 gauge hypodermic needle. As this section is the most sensitive to histamine, a length of 5 - 6 cm of the ileal strip was cut out. Care was taken to avoid handling the ileal strip, and the section which was not in use was kept immersed in Tyrode solution at room temperature and was discarded after 6 hours. Each end of the 5 - 6 cm ileal strip was attached to a hook in a manner that permitted the lumen to remain open at both ends. The entire procedure was carried out with the

ileal strip immersed in Tyrode. The preparation was then transferred to a 10 ml organ bath, the temperature of which had previously been adjusted to 34 ± 1°C. A mixture of 95% O_2 and 5% CO_2 was bubbled through the oxygen inlet tube. The pressure of the mixture was regulated to about 2 bubbles/sec. and maintained at this rate throughout the experiment. The writing lever, balanced with plasticine, was adjusted to give a magnification of five times. The load of the tissue was approximately 350 mg.

vi) <u>The Testing of Antihistaminic Activity</u>

The sensitivity of the ileal strip preparation was tested as follows: The kymograph was switched on and a fixed amount of the standard histamine solution, usually 0.05 ugm, was added into the organ bath and left in contact with the ileal strip for 20 seconds. The kymograph was then switched off; the organ bath was drained and refilled with fresh Tyrode. Thirty seconds before the next administration of 0.05 ugm standard histamine solution, the kymograph was switched on and remained "on" until the 20 seconds contact time of histamine with the ileal strip had elapsed. The organ bath was then emptied and refilled with Tyrode. The administration of 0.05 ugm histamine solution was repeated until the maximum response of the ileum was elicited at this dose level. The optimal response was reached when the ileal contraction recorded on the smoke drum was about 5 cm high.

When the sensitivity of the ileum was established, the antihistaminic activity of the compound was tested as follows: First, the solvent used for dissolving the compound and then the compound in solution were added in different doses to the organ bath, immediately after the replacement of the histamine containing Tyrode with fresh Tyrode. The test solution remained in contact with the ileum for 2 minutes, after which, addition of the standard histamine solution was repeated at 3 minutes intervals until the contraction was back to normal.

b. In Vivo Methods

i) Histamine Aerosol

Material and Apparatus: Normal albino or multicoloured guinea pigs of the short hair variety (Canadian Breeding Labs),of either sex, weighing between 250 - 300 gm were used throughout the experiments. Delivery of the animals was made a few days prior to the experiments and they were allowed free access to water and normal guinea pig chow.

The wooden test chamber (30 x 30 x 60 cm) was provided with a sliding door on top and two glass walls (front and back), to permit observation of the animals during the test. One of the two holes of the chamber was used for ventilation while the other was

used as an inlet of the aerosol into the chamber. The histamine dihydrochloride solution (0.15% histamine base) was nebulized with the Jouan histamine aerosol apparatus, which has a capacity of 20 ml/hr. The aerosol particles produced varied from 1 - 3 microns.

Procedure: A freshly prepared 0.15% histamine solution was poured into the nebulizer. The test chamber was then saturated with histamine aerosol prior to placing the animals into the chamber in order to obtain uniform testing conditions. A treated and a control guinea pig were placed into the test chamber and the time was immediately noted by means of a stop-clock. The test was scored on the basis of four distinct stages of physical symptoms, which marked the development of progressive respiratory deterioration and these are the following:

1) Coughing, dyspnea and gasping;
2) Swaying, falling and convulsive struggling;
3) Lying on the floor of the chamber;
4) Cessation of respiration - death.

The time course of each of these stages was recorded and stage 4 was taken as the end point of the experiment. The maximum exposure time of the guinea pigs to the histamine aerosol was set arbitrarily at 20 minutes.

ii) Capillary Permeability

The abdominal area of the guinea pigs was carefully shaved with an

Oster electric clipper 24 hours prior to the experiment. The animals designated as "treated" were given the drug intraperitoneally and the controls were given the corresponding volume of vehicle. 5 hours later, 30 mg/kg pontamine sky blue in normal saline was injected intracardiacally with a 23 gauge needle, which was immediately followed by administering 0.01 ml and 0.05 ml of histamine (dissolved in saline) intradermally on the shaved abdominal area. Thirty minutes later, the animals were sacrificed by a blow on the head. The skin on the shaved area was carefully removed, the soft tissues of the inner side cleaned and the areas of blueing for both the treated and the controls were measured. This measurement was based on the product of the two largest diameters of the affected area.

iii) The Blood Pressure Experiment

Material and Apparatus: Normal cats of either sex weighing between 1 - 2 kg were used. Sodium pentobarbitone was obtained from May & Baker Ltd. (England). The blood pressure was monitored by a 23 AC Statham pressure transducer (Statham Laboratories Inc.) connected to a Model 7 Grass polygraph (Grass Instruments, Inc.).

Procedure: The cat was anaesthetized with 35 mg/kg of sodium pentobarbitone administered intraperitoneally. The animal was secured firmly on the operating table and the neck was shaved.

Using a No. 10 blade, a midline incision was made to isolate the trachea and a tracheal tube was inserted through a transverse incision. Then, the carotid artery was isolated and a PE-190 cannular was inserted - retrograde - through a small incision and the cannular was connected to the pressure transducer. A 1 - 2 in. incision was made at the ventral surface of the shoulder and the brachial vein was isolated. Through a small incision, a PE - 190 cannular was inserted and firmly secured. The administration of all drugs/vehicles was made through the brachial vein unless otherwise specified.

iv) Carrageenan Edema Test

Plethysmographic Apparatus for Recording Volume Changes in the Rat Paw. This volumetric method, first described by Harris and Spencer (1962), was employed with slight modifications. The Apparatus (Figure 2) consists of a 3 ml microburette subtending a cylindrical reservoir (3.0 x 10.0 cm) at the base of which is connected a side tube (0.3 x 10.0 cm). The base of the microburette is connected to a 5 ml glass syringe by means of a thick wall rubber tubing. The syringe and the lower part of the microburette are filled with mercury while the reservoir and the upper part of the microburette are filled with 1% aqueous Triton X-100 (to ensure the complete wetting of the paw). There are two reference lines; (A) a horizontal line on the side tube located 1.5 cm from the top, (B) a horizontal line on the reservoir

located 5.0 cm from the top. The horizontal reference line (B) of the reservoir is cut by the perpendicular line (C). The mercury level is adjusted to "zero" in the microburette with a screw adjustment. The level of the fluid is adjusted to line (A) with a Pasteur pipette. The animal is lightly anaesthetized to ensure complete limb flaccidity.

The paw to be measured is completely immersed into a beaker containing 1% aqueous Triton X-100 to ensure complete wetting. The paw is removed and allowed to drain onto a gauze pad for a few seconds. It is then inserted into the reservoir in such a manner that the tip of the foremost toe nail comes to the intersection of lines B and C while the heel rests against the wall of the reservoir. (This procedure permits the measuring of the paw volume to be carried out with minimal fluctuation). Insertion of the paw results in the rising of the fluid to level D (Figure 2) which is returned to A by means of the screw adjustment. The new level of the mercury meniscus in the microburette now gives a direct reading of the paw volume.

The apparatus is re-standardized following each measurement and the results are reproduced to ± 0.02 ml.

Procedure: The drug or vehicle was given intraperitoneally to male wistar rats (Canadian Breeding Labs., Montreal) weighing

between 150 - 175 gm. Four hours later, 0.05 ml of 1% carrageenan was injected into the planar surface of the left hind paw, with the animal under light ether anaesthesia. The paw volume was measured immediately thereafter and again three hours later using the plethysmograph described above. The anti-inflammatory activity of the extract was expressed as:

$$\% \text{ Inhibition} = 100 - \frac{V_{ft} - V_{ot}}{V_{fc} - V_{oc}} \times 100$$

V_{ft} = Final Paw Volume of Treated Rat

V_{ot} = Initial Paw Volume of Treated Rat

V_{fc} = Final Paw Volume of Control Rat

V_{oc} = Initial Paw Volume of Control Rat

v) Induction of Polyarthritis in rats by Mycobacterial Adjuvant (according to Stoerk et al., 1954; Pearson, 1956, 1959)

Material: Male Lewis rats (Canadian Breeding Labs., Montreal) weighing between 150 - 160 gm were used. Mycobacterium butyricum were obtained from Difco Laboratories and suspended in a light mineral oil (Shell) at a concentration of 6 mg/ml.

Procedure: All rats were fed on purina rat chow and water ad libitum. The migratory arthritis was induced by injecting 0.1 ml of M. butyricum suspension into the planar surface of the left hind

paw. All drugs/vehicles were administered through the intraperitoneal route. Paw edema was determined by the above described plethysmograph.

The administration of the extract or vehicle started three (3) days before the induction of arthritis and continued for 16 days post-induction. The inflammation induced by the mycobacterial adjuvant was scored on the basis of a) edema of the hind paws and b) joint swelling of all four paws (Kapusta and Mendelson, 1969; Rosenthale, 1970). Edema of the hind paws was determined daily,for 25 days after the induction of the adjuvant arthritis,by the previously described plethysmographic method (see page 126). Joint swelling, expressed as arthritic score, was obtained by assigning one (1) point to definite swelling in the following joints: radiocarpal, tibiotarsal, and proximal interphalangeal. All metacarpophalangeal and metatarsophalangeal joints of each extremity were considered as single joint and, therefore, were given one point for definite swelling. Thus, the maximum obtainable arthritic score per paw is 11 points. Since the joints of the paw injected with the mycobacterial adjuvant have a greater tendency to develop arthritis, a separate arthritic score was obtained for the injected paw. No distinction was made between the severity of the joint swelling.

PLETHYSMOGRAPHIC APPARATUS FOR RECORDING VOLUME CHANGES IN THE RAT PAW

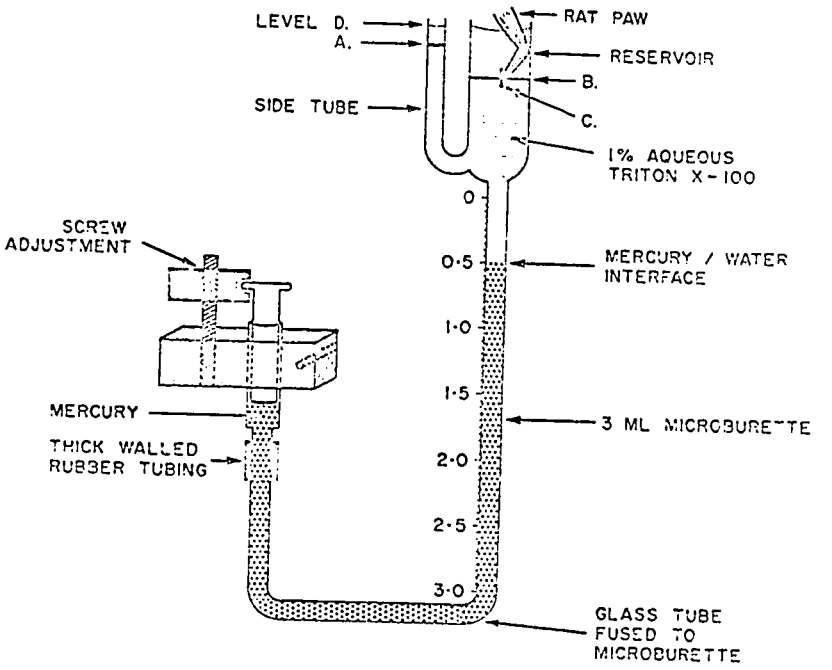


Figure 2

vi) Anaphylactic Shock - Herxheimer Microshock Technique

Male or female guinea pigs (Canadian Breeding Labs.) weighing between 200 - 225 gm were sensitized with 10 mg/kg crystalline egg ovalbumine (Pentex Inc.). The antigen was prepared by dissolving the egg ovalbumine in normal saline in a concentration of 10 mg/ml and administered to the animals intraperitoneally twice on two consecutive days. Three weeks after the second injection, the guinea pigs were injected intraperitoneally with oak gall extracts or vehicle.

Five hours later, a freshly prepared 2% egg ovalbumine solution was poured into the nebulizer of the aerosol apparatus (for details see page 123). The test chamber was saturated with the egg ovalbumine aerosol prior to placing the animals into the chamber in order to obtain uniform testing conditions and then, a treated and a control guinea pig were placed into the test chamber and the time was immediately noted by means of a stop-clock. The exact time when dyspnea developed in the animals, which is considered to be the first sign of anaphylactic shock, was taken as the end point of the experiment. The maximum time of exposure was arbitrarily set at 10 minutes.

vii) Continuous Recording of Gastric Acid Secretion in the Rats. (according to Ghosh and Schild, 1958).

Material and Apparatus: Normal male hooded rats (Canadian Breeding

Labs., Montreal) weighing between 270 - 330 gm were used throughout these experiments. Histamine dihydrochloride (Fisher Scientific) of high purity was used and the concentration was calculated as base unless otherwise specified. The recording system used to monitor the acid secretion consisted of a polystaltic pump (Buchler Instruments), a glass electrode (Fisher Scientific) connected to a type PHM 28 pH meter (Radiometer, Copenhagen) and a Derva-rite II recorder (Texas Instruments, Inc.).

Preparation of the Citrate Phosphate Buffer: The stock solution was prepared as follows: 24 gm citric acid and 15.6 gm sodium monophosphate were dissolved in one liter distilled water. The solution was kept at $4^{o}C$ until used. The working solution was prepared as follows: 2.05 ml 1 N sodium hydroxide was added to 5.0 ml stock buffer solution and the mixture was diluted to one liter with distilled water. The buffer solution so prepared had a pH of 6.5.

Operative Procedure: The rat, having been fasted overnight, was anaesthetized with 800 mg/kg of 20% ethyl urethane solution, administered intraperitoneally. The trachea was exposed and cannulated. A 35 cm long polyethylene tubing (No. 10 Rusch, West Germany) was passed into the stomach through the esophagus and tied to the esophagus at the neck - excluding the vagus. The jugular vein was then exposed and cannulated with a polyethylene tubing (PE 50, Ingram and Bell) bevelled at the tip. The abdomen was opened through

a midline incision and the s tomach was carefully exposed. A small incision was made at the fundus and at the pyloro-duodenal junction. A 5 cm long PE 320 polyethylene tubing (Ingram and Bell) was used to cannulate the stomach at the pyloro-duodenal junction - being introduced through the fundic incision and held at the pyloro-duodenal junction by firmly tying a ligature around the pylorus. Care was exercised not to include any blood vessels within the ligature. The pyloric cannular was led outside the animal through a small incision made at the right latero-ventral aspect of the abdomen - this permitted the stomach to remain in its natural position. The abdomen was closed with interrupted sutures. The animal was then placed at a 30^{o} incline on its right side. The stomach was thoroughly washed with saline through the esophageal tubing.

Continuous Recording of Acid Secretion: The stomach was perfused continuously with the citrate phosphate buffer and the perfusate passed over a glass electrode which recorded the pH of the perfusate. The perfusate from an unstimulated stomach had a pH range of 6.0 - 6.6.

Gastric Perfusion System: Figure 3 shows the gastric perfusion system. The perfusing fluid (the citrate phosphate buffer), kept in a reservoir, was delivered to the animal through a polyethylene tubing. The perfusion rate was controlled by passing the polyethylene tubing through a variable speed polystaltic pump. From the

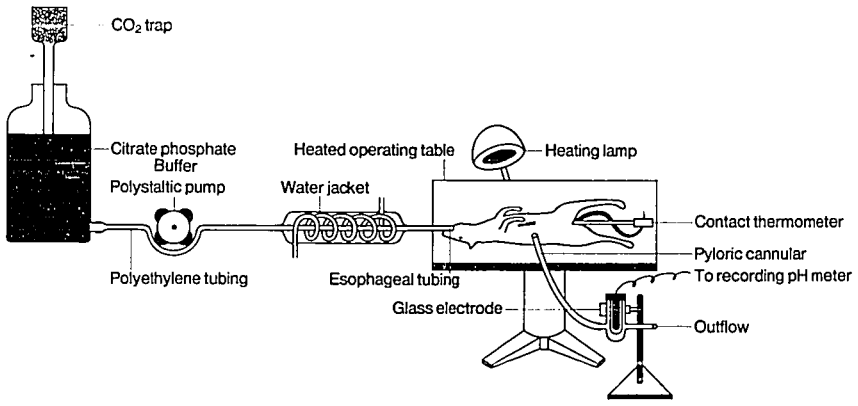


Figure 3.

Gastric perfusion system for a continued recording of gastric acid secretion.

polystaltic pump, the polyethylene tubing entered a water jacket where the temperature was thermostatically controlled at 37°C. The emerging polyethylene tubing was then connected to the esophageal tubing. The polystaltic pump was adjusted to perfuse the rat stomach at a constant rate of 1 ml/min. The pyloric cannular was connected to the recording glass electrode by means of a short polyethylene tubing. The glass electrode was connected to the pH meter which, in turn, was connected to the recorder equiped with an ink writer.

Testing of the Purified Oak Gall Extract: Optimal conditions for an assay were obtained when the perfusate emerging from the pylorus had an initial pH of 6.0 - 6.5.

The control histamine response was obtained by administering intravenously 400 - 600 ugm of histamine in a concentration of 2 mg/ml which stimulated the gastric acid secretion. When the pH of the perfusate returned to the baseline, the purified oak gall extract, dissolved in ethanol in concentrations of 120 - 360 mg/ml, or vehicle was injected intravenously into the rat. One hour later,a second dose of histamine (400 - 600 ugm) was again given and the histamine administration was continued at the same dose level (400 - 600 ugm) every hour until the pattern of the gastric secretion returned to that of the control. The activity of KC-18 against the histamine-induced gastric secretion was expressed as the diff-

erence between the total acid output due to histamine alone (control) and that due to histamine after treatment with KC-18. The total acid output was determined by calculating the area under the pH curve plotted against time (Rosenoer and Schild, 1962). Hence,

$$\% \text{ inhibition} = 100 - \frac{\text{Smallest area of treated}}{\text{Area of control}} \times 100$$

viii) Statistical Analysis of Data

Means and standard errors were computed by standard methods. The significance of differences between means were estimated by the Student's t Test (Hoel, 1960).

CHAPTER III. RESULTS

Section A. The Standard Crude Extract

a. Preparation of Standard Crude Extract

The procedure used in obtaining the standard crude extract, with slight modifications, is the same as that described by Chu (1969).

600 gm of freshly ground oak gall powder were mixed thoroughly with a mixture of 6 liters of chloroform:methanol (2:1, v/v). The gall powder-solvent mixture was kept at room temperature for 16 hours and filtered. The filtrate was thoroughly dried. The dried chloroform:methanol extract (dry weight ranged from 500 - 2500mg/ 100 gm oak gall powder) was extracted with 300 ml ethyl acetate three times and the pooled filtrate was taken to dryness. The average dry weight of the ethyl acetate soluble fraction was 600 mg/100 gm oak gall powder. In order to remove the chlorophyll, the dry ethyl acetate extract was washed with ether absolute (20 ml/ 100 gm oak gall powder) twice at room temperature and the ether insoluble fraction was thoroughly dried. The average dry weight of the ether insoluble fraction was 480 mg/100 gm oak gall powder. The dry chlorophyll-free extract was dissolved in 100 ml ethyl acetate and applied onto a silica gel G column (4.0 x 60.0 cm). The column was eluted with ethyl acetate at a flow rate of 10 ml/ min. The effluent was collected as a single fraction until the

appearance of traces of tannic acid (determined by the $FeCl_3$ reaction) then the collection was terminated. The tannin-free effluent (approximately 1.2 liters) was thoroughly dried. The yield of six different batches of tannin-free crude extract is listed in Table I. The dried tannin-free extract was extracted twice with 70 ml methanol:water:ethyl acetate (4:2:1, v/v) and centrifuged for 10 minutes at 2000 rpm. The supernatants were pooled and taken to dryness. The average dry weight was found to be 32 mg/100 gm oak gall powder. The dry extract was dissolved in 15 ml methanol and applied onto a sephadex LH-20 column (2.5 x 80.0 cm). The column was eluted with methanol at a flow rate of 2 ml/min. at 4°C. The effluents were collected in 10 ml aliquots and analysed by the DU-spectrophotometer at 280 mu (Figure 1).

According to the optical densities the effluents were pooled into six fractions and each fraction was bioassayed for antihistaminic and anti-inflammatory activities.

Histamine Aerosol Test: An aliquot of each of the dried fractions was dissolved in ethyl alcohol and the solutions were injected intraperitoneally into guinea pigs (2 animals). The control animals (12 guinea pigs) were simultaneously injected with the corresponding amount of the vehicle. Five hours following the treatment, each treated guinea pig and its corresponding control were placed into the aerosol chamber and exposed to a 0.15% histamine aerosol. The

Table I

Oak Gall Powder (in gm)	Yield of the tannin-free Crude Extract mg.	%
600	260	0.043
600	310	0.052
600	464	0.077
600	482	0.080
600	445	0.074
600	242	0.041
Mean ± SE	407 ± 39.7	0.062 ± 0.0072

Table I compares the yields of the tannin-free crude extracts obtained from six batches of Hungarian oak galls. The yields, ranging from 0.041 to 0.080 % (w/w), are representative of all the tannin-free crude extracts obtained in these studies.

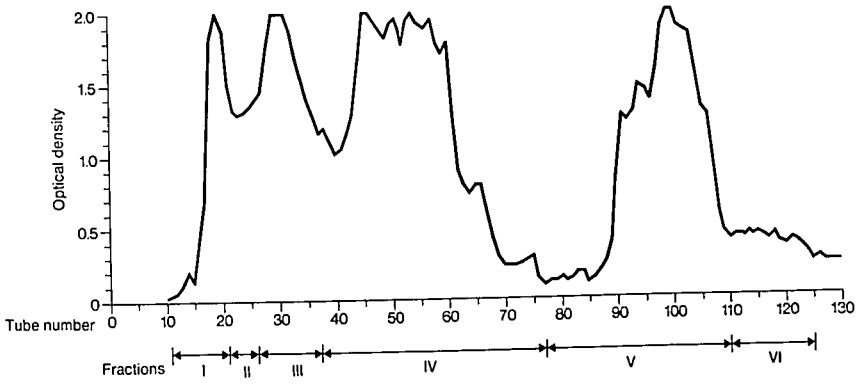


Figure 1.

The fractionation of the tannin-free methanol:water:ethyl acetate (4:2:1, v/v soluble oak gall extract by sephadex LH-20 column chromatography. The chromatogram was developed at $4^{o}C$ with methanol at a flow rate of 2 ml/min. The effluents were collected in 130 tubes of 10 ml portions and pooled into six fractions according to the optical densities determined by the DU-spectrophotometer at 280 mu.

results obtained from five experiments are summarized in Table II. Of the six fractions tested, fractions I, II and III were found to be active. Statistical analyses of the results showed that the mean survival time of the guinea pigs treated with fractions I, II and III was significantly longer than that of the control animals.

Carrageenan Edema Test: Similarly, an aliquot of each of the six fractions was dissolved in ethyl alcohol and injected intraperitoneally into male wistar rats (5 animals). The control animals (5 rats) were given the corresponding volume of the vehicle. Four hours later, 0.05 ml of a 1% carrageenan solution was injected into the plantar surface of the left hind paw of each rat. Immediately thereafter and again three hours later, the volume of the injected paw was measured plethysmographically under light ether anesthesia.

The results obtained from five experiments are summarized in Table III. Among the six fractions tested, only rats treated with fraction VI did not show a significant development of edema in the carrageenan treated paw.

In a second series of experiments, groups of rats (5 rats/group) received fraction VI in doses of 9, 18 and 36 mg/kg and were tested as described above.

Table II

Dose mg/kg	Fractions	Number of Guinea Pigs	Mean Survival Time (in min.)	Signif-icance (p)
Vehicle	--	60	6:20	---
25.0	I	10	17:15	< 0.001
4.0	II	10	19:10	< 0.001
3.2	III	10	16:45	< 0.001
16.0	IV	10	7:00	NS
10.0	V	10	5:55	NS
24.0	VI	10	6:30	NS

Antihistamine-like activity of the sephadex LH-20 column chromatographic fractions of the Hungarian oak galls. The extracts/vehicle were injected intraperitoneally into guinea pigs and the animals were exposed to 0.15% histamine aerosol five hours later.

Table III

Dose mg/kg.	Fractions	Number of rats	Initial mean paw volume (in ml)	Final mean paw volume (in ml)	% Inhibition
--	control	25	1.20	1.56	
30	I	25	1.19	1.61	--
6	II	25	1.18	1.47	20
48	III	25	1.23	1.53	17
24	IV	25	1.21	1.60	--
15	V	25	1.17	1.50	9
36	VI	25	1.21	1.23	94

Anti-inflammatory activity of sephadex LH-20 column chromatographic fractions of the Hungarian oak galls. The extract/vehicle was injected intraperitoneally into male wistar rats. Four hours later 0.05 ml of a 1% carrageenan solution was injected into the plantar surface of the animals. Immediately thereafter and again three hours later the volume of the injected paw was measured pletismographically under light ether anesthesia.

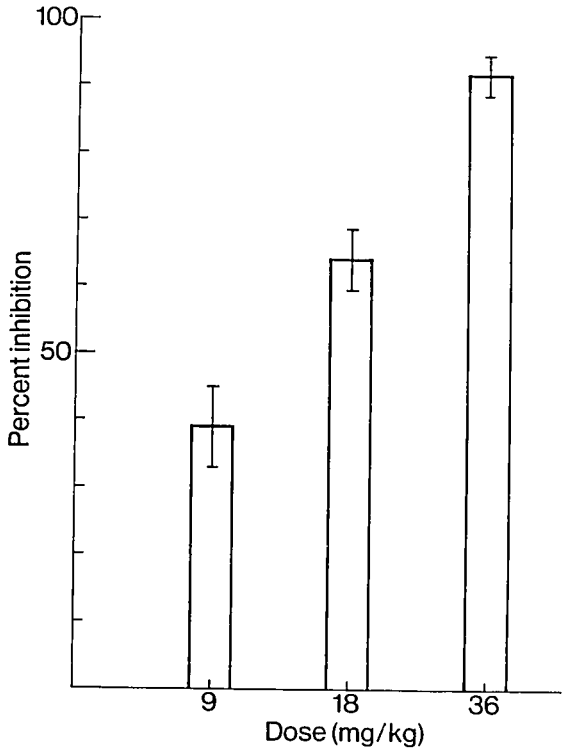


Figure 2

The anti-inflammatory activity of fraction VI of the sephadex LH-20 column chromatography of the oak gall extracts against carrageenan-induced rat paw edema. The extract was injected in three different doses and each dose represents the mean value of 25 animals.

Table IV

Oak Gall Powder (in gm)	Fraction I		Frac. II & III		Fraction VI	
	mg	%	mg	%	mg	%
600	59	0.009	42	0.007	70	0.012
600	65	0.011	45	0.008	82	0.014
600	40	0.007	30	0.005	64	0.011
600	48	0.008	28	0.005	44	0.007
600	36	0.006	36	0.006	72	0.012
Mean	49.6	0.0082	36.2	0.0062	66.4	0.011
± SE	5.5	0.0009	3.3	0.0006	6.3	0.001

Table IV compares the yields of sephadex LH-20 column chromatographic fractions I, II, III and VI obtained from five batches of Hungarian oak galls. These yields are representative of all these four fractions obtained in these studies.

The results obtained in five experiments are summarized in Figure 2. It can be seen that fraction VI exerted a dose-related anti-inflammatory activity against carrageenan-induced rat paw edema. The yields of the active fractions from five representative experiments are listed in Table IV.

b. Discussion

The initial step in the extraction procedure, with very slight modifications, was essentially the same as that reported by Broome et al. (1962) and most of the procedures employed for the preparation of the standard crude extract were identical to those described by Chu (1969).

In the present studies, greater quantity of starting material was employed than that previously reported. Instead of using only 300 gm oak gall powder, 600 gm of powder were used to prepare the standard crude extract. The amount of solvents used for the extraction was increased proportionately and the resulting extract showed an increase in the amount of active principle proportional to the amount of starting material. The doubling of the quantity of oak gall powder increased the efficiency of the production of the standard crude extract which is a point of some significance in view of the unstable nature of the active extract. However, in spite of the advantage of increasing the quantity of starting material, it

was not possible to increase the quantity beyond double the original amount.

The extraction of the chloroform:methanol soluble fraction with ethyl acetate was carried out as described by Chu (1969) with the exception of increasing the volume of ethyl acetate from 150 ml to 300 ml. The mean dry weight of the ethyl acetate soluble fraction was comparable to that previously obtained by Chu (1969).

In using silica gel G for column chromatography, it was necessary to use pressure (nitrogen) to obtain the optimum flow rate of 10 ml/min. because the flow rate due to hydrostatic pressure alone was too slow and hence time consuming and inefficient. Only a single fraction was collected as it was not possible to locate the active "peak" by the optical densities, measured by the DU-spectrophotometer or the Coleman Jr. spectrophotometer. However, it was quite easy to detect the presence of tannic acid by using ferric chloride which forms a coloured complex with tannins in microgram quantities. Since the primary objective was to separate the active principle from tannic acid, the silica gel column chromatographic procedure (Chu, 1969) proved to be highly efficient and reproduceable in achieving this goal.

When the dry tannin-free fraction was extracted with methanol: water:ethyl acetate (4:2:1, v/v) and centrifuged at 2000 rpm, the

clear supernatant consistently contained the active principle. This step proved to be extremely important in preparing the extract for subsequent sephadex LH-20 column chromatography because it effectively removed the pigments which otherwise made the sephadex LH-20 column chromatographic procedure difficult to reproduce.

The bioassay of the sephadex LH-20 column chromatographic fractions showed that anti-histamine-like activity was exerted by three fractions. In view of this rather broad distribution of the active principle, several attempts were made to improve its resolution, however, none of the methods tried appeared to be satisfactory. For instance, the use of less polar solvents seemed to increase the elution of other impurities, which coincided with the chromatographic area that contained the active principle. This is particularly true in the case of those preparations which contained a relatively high yield of chlorophyll-like pigments. On the other hand, the increase in the polarity of solvents also greatly increased the elution time without significantly increasing the resolution of the active principle. Thus, while the active principle was eluted over a wide chromatographic area, the chromatographic pattern, according to the optical densities at 280 mu, was very constant and, consequently, made the column chromatographic procedure highly reproduceable. The variability of the dry weight obtained from fractions I, II and III reflected not so much on the yield of the active substance as on the accompanying impurities.

Since there was no method by which these three active fractions could be separated it was assumed, as a working hypothesis, that the antihistaminic activity found in the three fractions was most probably the effect of a single active compound rather than those of three distinct compounds. Instead of combining the three active fractions, it might have been possible to use only fraction III which, according to the dry weight determinations, represented the fraction with the least amount of contaminants. In electing to do this, however, more than 60 % of the active principle would have been lost. Furthermore, since the amount of the active principle required for continuous in vivo assays is large compared to the yield, it would have rendered further purification practically impossible. Accordingly, the three active fractions were pooled into a single fraction and used as starting material in further purification procedures.

Apart from the presence of an antihistamine-like principle(s) (fractions I, II and III) in the purified oak gall extract, potent anti-inflammatory activity was also detected in a fraction of the standard crude extract (fraction VI) which occurred at a chromatographically different region from the fractions containing the antihistamine-like principle(s). The anti-inflammatory activity of fraction VI, as evaluated by the carrageenan edema test, was shown to be dose-dependent. In order to assess the anti-inflammatory activity of this fraction more fully, it (fraction VI) was subjected to further purification.

Section B. Isolation, Identification and Pharmacological Actions of the Antihistaminic Principle - KC-18.

I. Isolation of the Antihistaminic Principle - KC-18

a) Method of Isolation

The dry standard crude antihistaminic extracts were pooled together and dissolved in methanol:water (1:1, v/v) in a concentration of 20 mg/ml. The mixture was applied onto a sephadex LH-20 column (4.8 x 20.0 cm) and eluted with methanol:water (1:1, v/v). Following the collection of 1100 ml of the effluents, the eluant was changed to methanol:water (2:1, v/v) and the effluents were collected in 10 ml/tube aliquots at a flow rate of 2 ml/min. The optical densities of the effluents were continuously monitored by a UV analyser at 280 mu and on the basis of their optical densities, they were divided into 13 fractions (Figure 3) and each fraction was then taken to dryness.

An aliquot of each of the dried fractions was dissolved in ethanol at concentrations of 0.8 - 14.0 mg/ml and injected intraperitoneally into guinea pigs at doses of 0.8 - 14.0 mg/kg of body weight. The control animals were similarly injected with the same amount of the vehicle. Each guinea pig together with its control was exposed to 0.15 % histamine aerosol five hours after the treatment with the extract. Of the 13 fractions tested, only fraction XIII

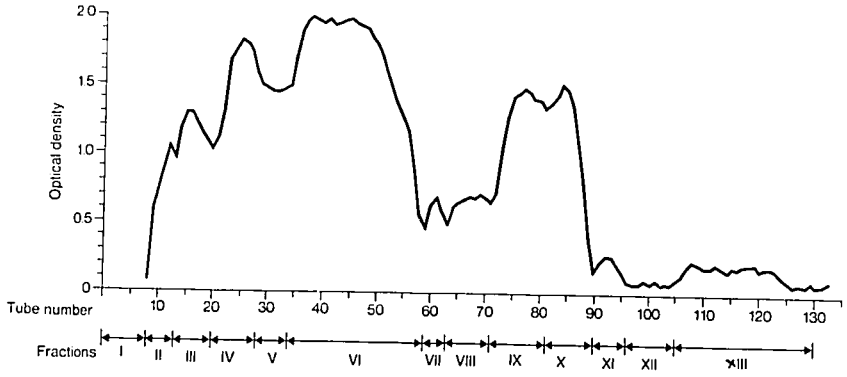


Figure 3

The fractionation of the standard crude extract containing the antihistaminic principle on the sephadex LH-20 column. The chromatogram was eluted stepwise with methanol:water (1:1, v/v) and (2:1, v/v) respectively at a flow rate of 2 ml/min. The effluents were monitored at 280 mu and collected in 130 tubes of 10 ml/aliquot. The effluents were divided into 13 fractions according to the optical densities.

exerted antihistaminic activity. The average yield of fraction XIII is shown in table V.

The results of 12 experiments in which guinea pigs (22 animals) were treated with fraction XIII are shown in figure 4. It can be seen that the mean survival time of the treated guinea pigs is significantly greater than that of the control animals (24 guinea pigs).

The dried fraction XIII was extracted three times with 20 ml petroleum ether:ethyl acetate (20:1, v/v) mixture at room temperature. After each extraction, the mixture was centrifuged at 2000 rpm for 10 minutes. The pooled supernatants and the precipitate were thoroughly dried. The average yields of both the supernatant and precipitate are listed in table VI.

An aliquot of the dried supernatant and the precipitate was dissolved in ethanol at a concentration of 5.4 mg/ml and 9 mg/ml respectively. The solutions were injected intraperitoneally into guinea pigs in doses of 5.4 mg/kg and 9.0 mg/kg of body weight. The control guinea pigs received the equivalent volume of the vehicle. Five hours following the treatment, each treated guinea pig with its corresponding control was exposed to 0.15% histamine aerosol.

The results of six experiments in which guinea pigs were treated

Table V

Amount of Oak Gall Powder (in gm)	Fraction XIII	
	mg	%
600	21.0	0.0036
600	17.4	0.0029
600	15.6	0.0026
600	26.4	0.0044
600	24.6	0.0041
600	30.0	0.0050
600	12.6	0.0021
Mean ± SE	21.6 ± 2.4	0.0036 ± 0.0004

Table V compares the yields of the sephadex LH-20 column chromatographic fraction XIII obtained from seven batches of the Hungarian oak galls. The yields are representative of all the fraction XIII obtained in these studies.

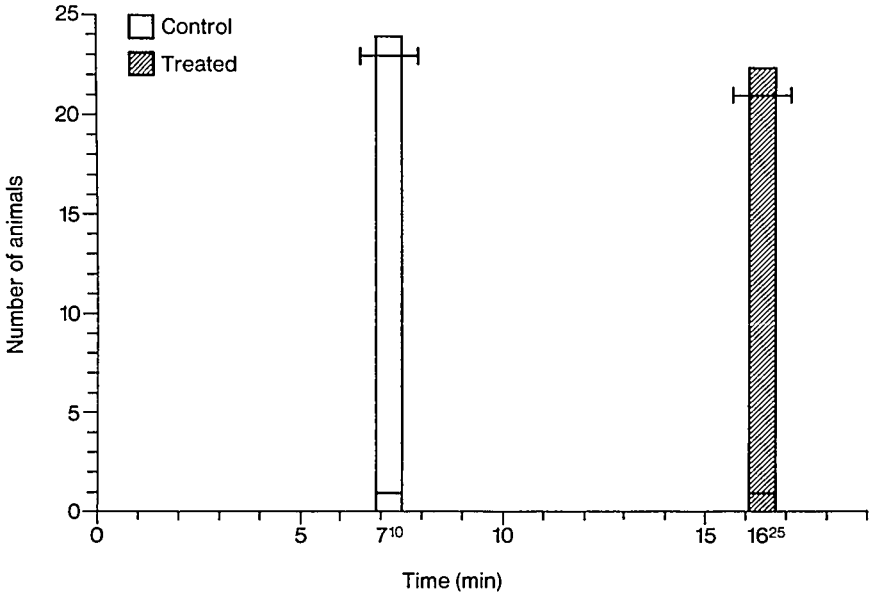


Figure 4

The protective effect of the sephadex LH-20 column chromatographic fraction XIII against the action of a 0.15 % histamine aerosol. The extract or vehicle was injected intraperitoneally into guinea pigs and the animals were exposed to the aerosol five hours later. The abscissa measures the time (in min.) of the cessation of respiration while the ordinate indicates the number of guinea pigs.

Table VI

Amount of Oak Gall Powder (in gm)	Petroleum ether:Ethyl Acetate			
	Supernatant		Precipitate	
	mg	%	mg	%
600	13.5	0.0023	8.1	0.0014
600	10.9	0.0018	6.5	0.0010
600	9.7	0.0016	5.9	0.0009
600	12.3	0.0020	14.1	0.0025
600	13.1	0.0022	11.5	0.0019
600	26.9	0.0045	6.7	0.0011
600	8.1	0.0014	4.5	0.0008
Mean ± SE	13.5 ± 2.3	0.0023 ± 0.0004	8.24 ± 1.3	0.0014 ± 0.0002

Table VI compares the yields of the petroleum ether:ethyl acetate (20:1, v/v) soluble and insoluble fractions of the sephadex LH-20 column chromatographic fraction XIII obtained from seven batches of Hungarian oak galls.

with the supernatant and the precipitate clearly show that while the mean survival time of the animals treated with the precipitate was not significantly different from that of their corresponding controls, guinea pigs treated with the supernatant survived significantly longer than their controls (figure 5).

The dried supernatant was dissolved in 2 ml petroleum ether:ethyl acetate (20:1, v/v) and applied onto a silica gel G column (0.8 x 20.0 cm). It was eluted with petroleum ether:ethyl acetate (20:1, v/v) at a flow rate of 2 ml/min. and the effluents were collected in 2 ml/tube aliquots. The effluents were continuously monitored at 280 mu and according to the optical densities they were divided into four fractions.

An aliquot of each of the dried fractions was dissolved in ethanol in concentrations of 2 - 6 mg/ml. The solutions were injected intraperitoneally into guinea pigs in doses of 2 - 6 mg/kg of body weight. The control guinea pigs received the corresponding volume of the vehicle. Five hours following the treatment, each treated guinea pig and its corresponding control were exposed to a 0.15 % histamine aerosol. Of the four fractions tested, only fraction I exerted antihistaminic activity.

Table VII shows the average yield of fraction I, while the results of 15 experiments in which guinea pigs (28 animals) were treated

with 4 mg/kg of fraction I are shown in figure 6. The difference in the mean survival time between the control and the treated animals clearly shows that fraction I exerted a potent antihistamine-like activity ($p < 0.001$).

Fraction I, obtained from the silica gel G column, was analysed by thin layer chromatography using the following solvent systems: a) methanol:water (11:1, v/v), b) methanol:water (11:5, v/v) and c) methanol:water (11:8, v/v). 100 ug of a freshly prepared 0.1 % chloroform solution of Fraction I were applied onto a 20.0 x 20.0 cm silica gel G thin layer chromatographic plate by means of a micropipette. The TLC plate was then transferred into the developing tank and allowed to run for 18 cm. The plate was removed from the developing tank, air-dried and visualized by UV lamp, iodine vapour and 87 % O-H_3PO_4.

The results of three experiments (from three different batches of oak gall preparations) clearly demonstrate that fraction I, obtained from the silica gel G column, consisted of a single spot only. A representative experiment is shown in figure 7. Hereafter, this chemically pure substance will be referred to as KC-18 in the text.

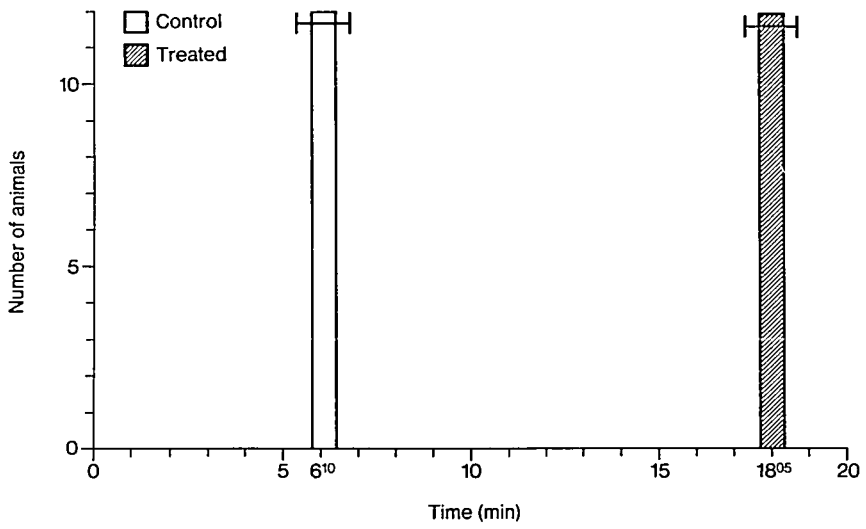


Figure 5

The protection produced by the petroleum ether:ethyl acetate (20:1, v/v) soluble part of the sephadex LH-20 column chromatographic fraction XIII of the Hungarian oak galls. The extract/vehicle was injected intraperitoneally into guinea pigs and the animals were exposed to a 0.15 % histamine aerosol five hours later. The abscissa measures the time (in minutes) of the cessation of respiration of the animals while the ordinate indicates the number of animals.

Table VII

Amount of Oak Gall Powder (in gm)	KC-18	
	mg	%
600	3.3	0.00055
600	2.5	0.00041
600	2.2	0.00037
600	4.1	0.00069
600	3.9	0.00065
600	3.4	0.00057
600	2.6	0.00043
Mean ± S.E.	3.1 ± 0.3	0.00052 ± 0.00005

Table VII compares the yields of the silica gel G column chromatographic Fraction I obtained from seven batches of Hungarian oak galls. The yields are representative of all the fraction I obtained in these studies.

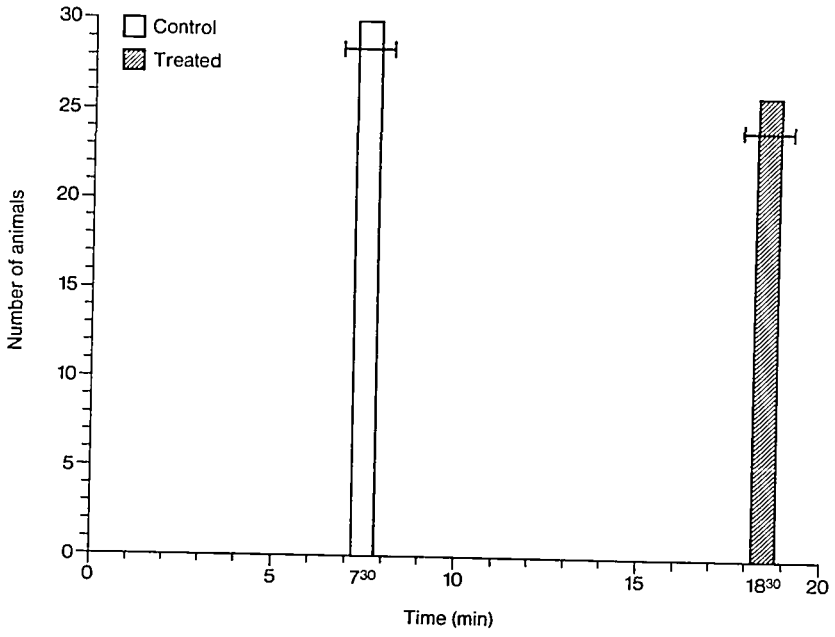


Figure 6

The protection produced by the silica gel G column chromatographic fraction I against the action of a 0.15 % histamine aerosol. The extract/vehicle was injected intraperitoneally into guinea pigs and the animals were exposed to the aerosol five hours later. The abscissa measures the time (in minutes) of the cessation of respiration of the animals while the ordinate indicates the number of animals.

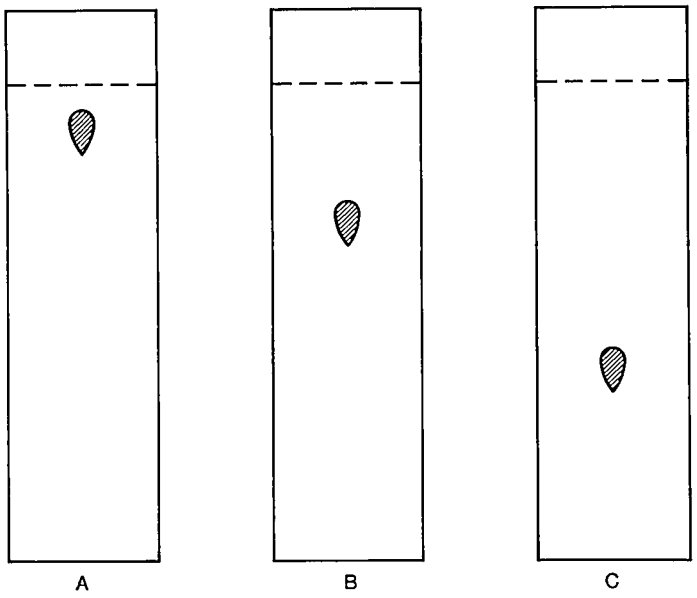


Figure 7

Thin layer chromatography of fraction I obtained from the silica gel G column. The chromatograms were developed with (A) methanol: water (11:1, v/v) (B) methanol:water (11:5, v/v) and (C) methanol: water (11:8, v/v), and visualized by UV lamp, iodine vapour and 87 % O-H_3PO_4 spray.

b. Discussion

Sephadex LH-20 was selected for the stepwise elution of the pooled fractions I, II and III because of the virtual absence of adsorption of the active principle(s) onto the gel. However, the fraction containing the active principle(s) was found to occupy a relatively wide chromatographic area. Several attempts were made to improve the resolution of the active principle(s) by the use of different developing systems and various chromatographic resins. However, none of the systems attempted improved the resolution appreciably. Neutral alumina, for instance, was abandoned because the active substance adsorbed so strongly onto the particles that it remained bound in spite of highly polar eluants. Fractionation of the pooled fractions on silica gel column, developed by methanol, increased the resolution of the active principle(s). Unfortunately, silica gel has the disadvantage of being partially soluble in methanol which would complicate subsequent separation. Cellex N-1 (a non-ionic cellulose) has little adsorptive capacity but was also found to produce very poor separation.

The advantage of starting the elution of the pooled fractions I, II and III on the sephadex LH-20 column with the more polar solvent was that most of the contaminants were removed with the earlier effluents and when the eluents were changed to methanol:water (2:1, v/v), the active fraction was obtained in a highly purified form.

It is interesting to note that the active principle could not be extracted by non-polar solvents before the removal of the polar compounds by the preceding sephadex LH-20 column chromatographic procedure. Following its elution by methanol:water (2:1, v/v) the active principle(s) became freely soluble in all non-polar solvents. The reason for this unusual solubility behaviour is not clear. It is, however, possible that the active principle was in some way bound to the polar compound.

Since fraction I not only exerted a potent antihistamine-like activity but was also eluted as a sharp and uniform peak, it was decided to examine its chemical purity. The presence of a single spot on the thin layer plates, developed with multiple solvent systems, strongly suggested that fraction I contained only one compound. Apart from the multiple solvent systems, highly non-specific agents were employed for visualization - thus ascertaining the detection of all organic materials.

As in the preparation of the standard crude extract, the localization of the antihistamine-like principle during the purification and isolation procedures was accomplished by the histamine aerosol technique. Besides the absence of toxicity, fraction I also showed an increase in specific activity - indicating that the antihistamine-like activity was most probably a specific activity.

II. Structure Analysis

Since the application of the method described in the previous section enabled us to prepare the antihistamine-like principle (KC-18) in chemically pure form quite routinely, it was of interest to make an effort to identify the structure of this naturally occurring antihistamine-like compound.

General Description: KC-18 is a semi-solid and a colourless substance with a slight pungent odour. It is very soluble in all lipid solvents from chloroform to hexane and insoluble in water. The semi-solid nature of the substance did not permit the melting point determination. From each preparation, a portion of the chemically pure sample was tested for biological activity and then, in order to determine the chemical structure, the rest of the sample was analysed by one or more of the following methods:

a) Analysis of KC-18

1. Infrared Spectrophotometric Analysis

The IR spectra of KC-18 were obtained from a Perkin-Elmer 257 IR spectrophotometer at medium scan speed. A typical IR spectrum is shown in Figure 8, where KC-18 was analysed in the film form. The most significant peak is probably that at 1710 cm^{-1} which indicates the presence of carbonyl group possibly in association with an

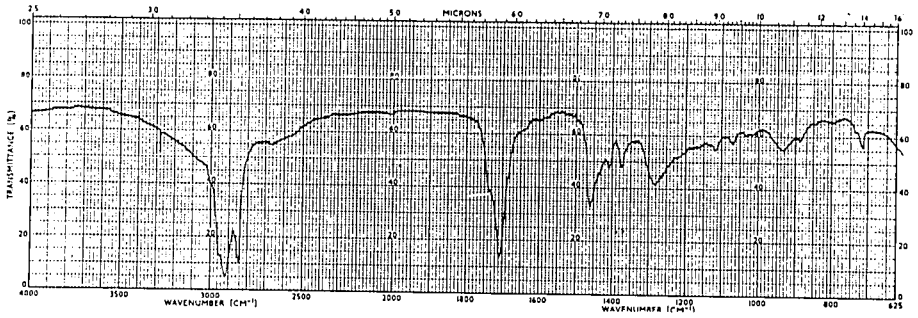


Figure 8

A typical IR spectrum of KC-18 (film).

ester. The peak at 2920 cm^{-1} indicates the presence of methyl group and this is confirmed by the peak at 1460 cm^{-1}. The presence of $-CH_2-$ is shown by the peak at 2850 cm^{-1}. The analysis of these data indicated the possibility of KC-18 being an ester with an aliphatic side chain:

$$R - \overset{\overset{O}{\|}}{C} - O - (CH_2)_x - CH_3$$

2. Ultraviolet Spectrophotometric Analysis

The UV spectrophotometric analysis of KC-18 was performed in a Unicam SP800 UV spectrophotometer. A typical UV spectrum of KC-18, dissolved in ethanol, is shown in Figure 9. A λ_{max} was observed at 258 mu and a shoulder at 285 mu. The extinction coefficients calculated for these peaks were 0.70×10^3 and 0.43×10^3 respectively. These data were compared to those obtained from an authentic sample of piperonylic acid dissolved in methanol. Piperonylic acid yielded a λ_{max} at 260 mu and 295 mu with the calculated extinction coefficients of 7.0×10^3 and 6.3×10^3 respectively. The extinction coefficients were calculated by the expression

$$E^{1\%}_{1cm} = \frac{A}{Cl}$$

where A is the optical density, C the concentration of the substance (gm/100ml), and l is the pathlength of the sample cell. The molecular weight of KC-18 was assumed to be 279. The λ_{max} of KC-18

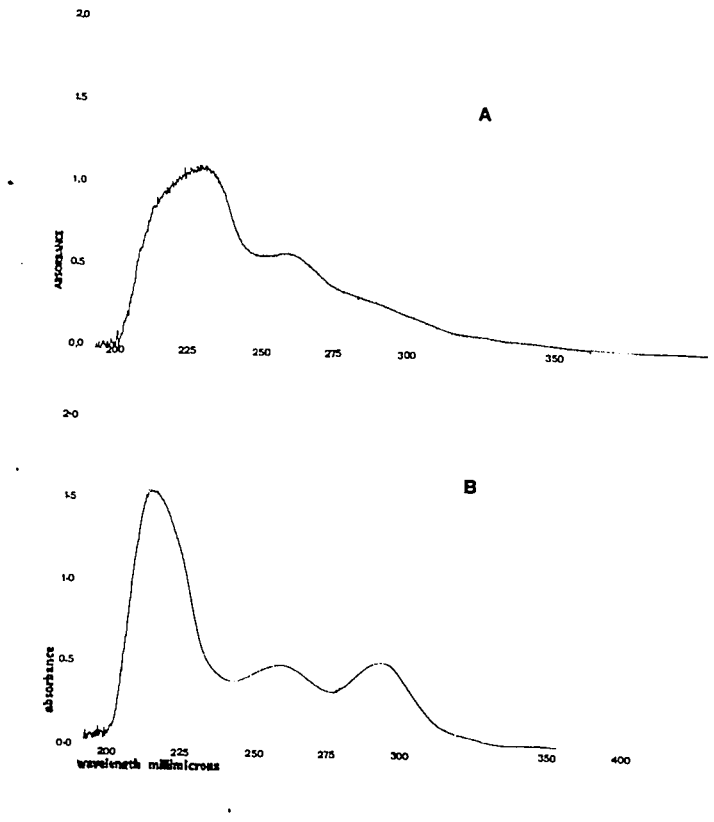


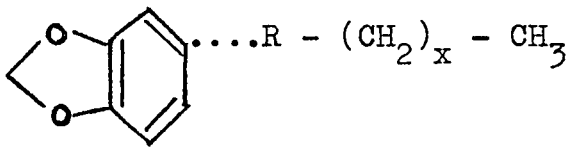
Figure 9

The UV spectrum of KC-18 (A) compared to that of an authentic sample of piperonylic acid (B).

indicated the presence of C=O in conjugation with an aromatic group.

3. Nuclear Magnetic Resonance Spectrometric Analysis

The NMR spectral analysis of KC-18 was performed in a Varians A-60 (60 MHz) NMR spectrometer. The substance was dissolved in chloroform-d for the analysis. A typical analysis is shown in figure 10. The aromatic hydrogens appeared as multiplet at 7.0 - 7.8 ppm and the methylenedioxy hydrogen gives a singlet at 5.8 ppm. Furthermore, the presence of hydrocarbon hydrogen was indicated at 1.3 ppm for $-CH_3$ and 1.7 ppm for $-CH_2-$. These results were compared to those obtained from an authentic sample of piperonylic acid (in chloroform-d) which showed multiplet at 6.6 - 7.5 ppm for the aromatic hydrogens and a singlet at 5.9 ppm for the methylenedioxy. The NMR spectral results thus confirmed the presence of aromatic group and aliphatic chain and also showed the presence of a methylenedioxy group.



4. Mass Spectrometric Analysis

The mass spectrometric analysis of KC-18 was carried out in an AEl MS902 mass spectrometer with a source temperature of 90°C. The operating conditions were a 70-ev electron energy, resolution of

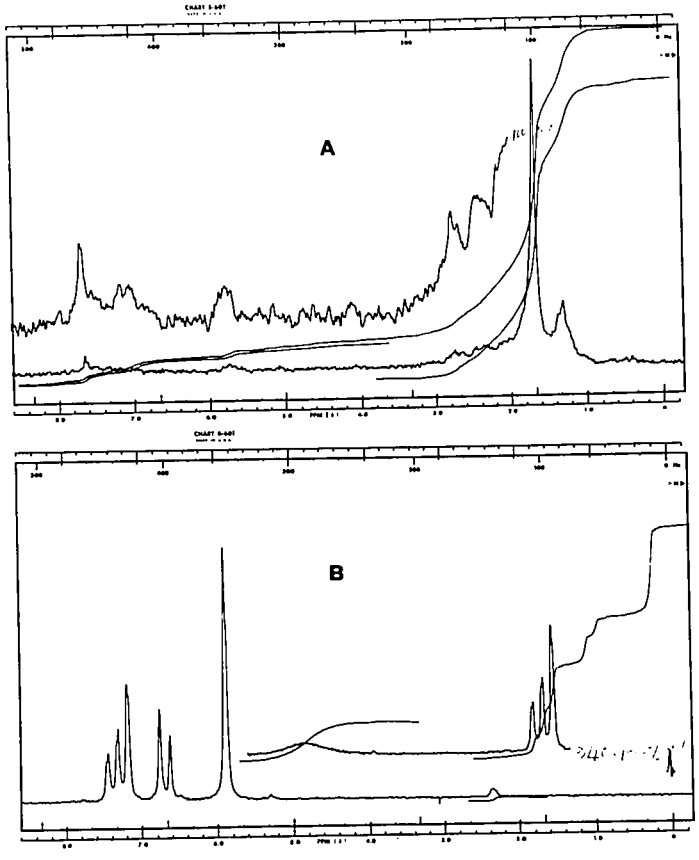
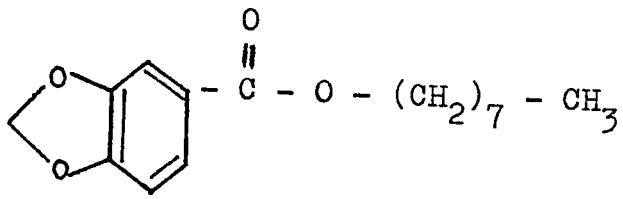


Figure 10

The NMR spectrum of KC-18 (A) compared to that of an authentic sample of piperonylic acid (B).

1000 and a 8 Kv accelerating voltage. A typical low resolution mass spectrum is illustrated in Figure 11 and it shows three peaks in the high mass region, viz. m/e = 149, 167 and 279. High resolution mass measurement was carried out for the 149 peak and was found to have a m/e = 149.0234. Assuming the 279 peak to be the parent compound, the probable molecular weight of KC-18 is 279 with a molecular formula of $C_{16}H_{21}O_4$. The fragmentation pattern was consistent with the data obtained from the other spectral analyses and on the basis of these data, KC-18 was proposed to be an ester of piperonylic acid:



In order to confirm this possibility, KC-18 was subjected to base hydrolysis and the hydrolysate thus obtained was compared to an authentic sample of piperonylic acid.

b) Base Hydrolysis

2.0 mg KC-18 were dissolved in 0.1N sodium hydroxide at a concentration of 1 mg/ml and the solution was allowed to reflux at 50°C for two hours with constant stirring. It was cooled to room temperature and extracted three times with 20 ml ether to remove the non-acidic product. The pH of the aqueous fraction was brought down to 4.5

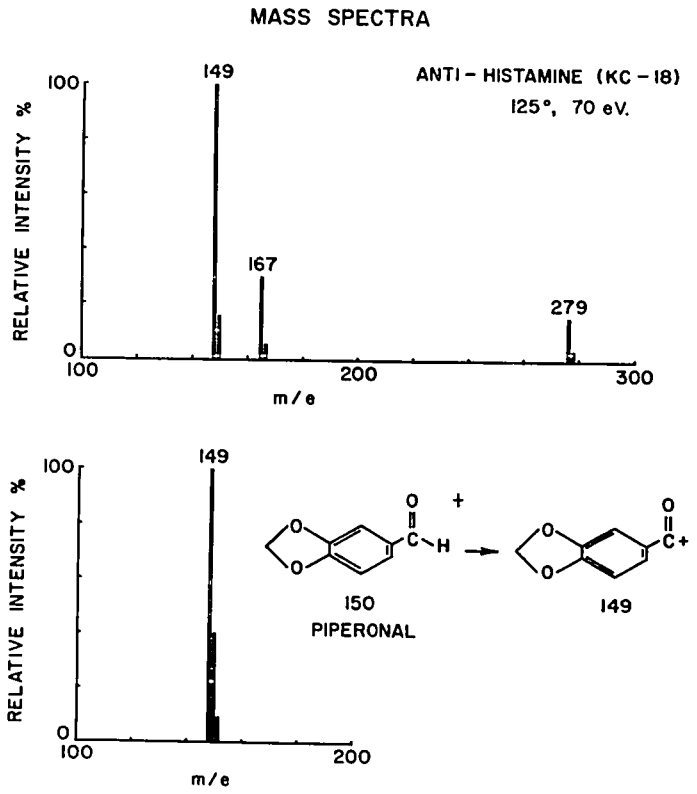


Figure 11

Typical low resolution mass spectra of KC-18 (top) and piperonal (bottom).

by the addition of 0.1N hydrochloric acid. The acidic moiety of KC-18 was immediately extracted three times with 20 ml ether. The ether soluble fraction was carefully dried with the vacuum pump and analysed.

c) Analysis of Hydrolysate.

1. Mass Spectrometric Analysis

The mass spectrum of the acidic component of KC-18 was obtained in an AEI MS902 mass spectrometer with a source temperature and operating conditions as previously described. A typical mass spectrum of the acidic component of KC-18 (Figure 12) showed that it is identical to that of an authentic sample of piperonylic acid but not to that of the isomeric 2,3 -methylenedioxy-benzoic acid.

2. Thin Layer Chromatography (TLC)

The TLC of the acidic component of KC-18 was done with precoated silica gel plates and developed with a) chloroform, b) ethyl acetate: chloroform (4:1, v/v) and c) ethyl acetate:chloroform (8:1, v/v). Together with the acidic component of KC-18 were two reference samples: i) authentic and untreated piperonylic acid and ii) authentic piperonylic acid after undergoing identical hydrolytic treatment.

The results, as shown in Figure 13, indicate that the acidic moiety

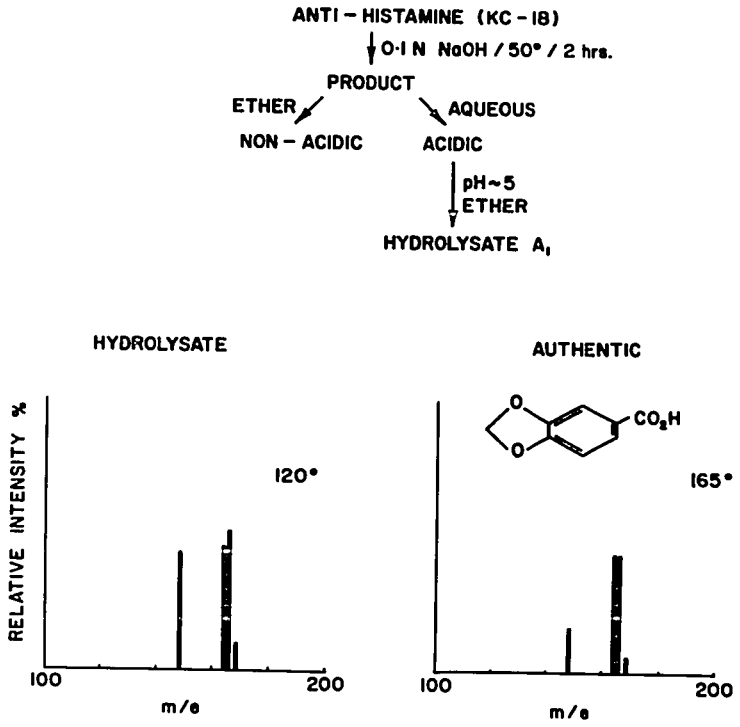


Figure 12

A typical mass spectrum of the acidic component of KC-18 (A) compared to that of an authentic sample of piperonylic acid (B).

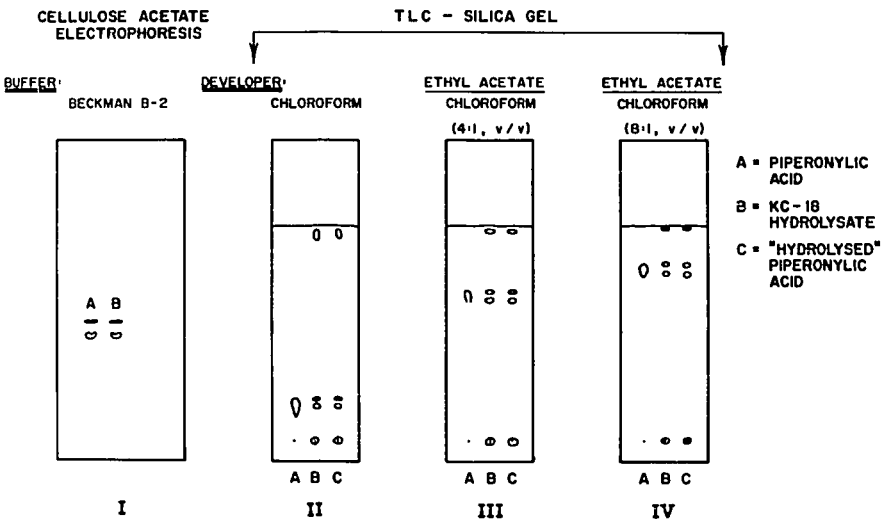


Figure 13

(I) The electrophoretic behavior of the acidic component of KC-18 compared to that of piperonylic acid.

(II-IV) Thin layer chromatograms of the acidic component of KC-18 with both the treated and the untreated piperonylic acid as references (see text for details).

of KC-18 has the same TLC behavior as that of an authentic sample of piperonylic acid.

3. Cellulose Acetate Electrophoretic Analysis

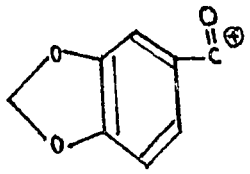
The electrophoretic behavior of the acidic component of KC-18 was also analysed on the cellulose acetate strip by a Beckman Duostat. 0.5 mg of the acidic component of KC-18 were dissolved in a drop of 0.1N sodium hydroxide and applied onto a cellulose acetate strip. Piperonylic acid was similarly prepared, in the same concentration, and applied as the reference compound. The cellulose acetate strip was then run at 0.50 volt in 0.15 milli-amp. for 20 minutes. The results obtained showed that the electrophoretic mobility of the acidic component of KC-18 is identical to that of an authentic sample of piperonylic acid and only a single spot was observed. Figure 13 is a typical example of these experiments.

d. Discussion

In view of the very low yields of KC-18 from individual batches of the oak gall extracts, the preliminary investigations on the structure of KC-18 had to be performed almost exclusively by spectral analysis - especially by the mass spectrometer and the infrared spectrophotometer.

The results obtained from the mass spectral analysis of KC-18 raised

the possibility that KC-18 might be an ester of piperonylic acid, since it is well known that the most favourable fragmentation pathway for piperonal is the cleavage alpha to the carbonyl to give



which has a m/e of 149. This is also true for the ester of piperonylic acid (Budjikieweiz et al., 1967). Furthermore, the infrared spectra of KC-18 showed the presence of a carbonyl group in association with an ester ($V_{C=O}$ = 1710 cm^{-1}). In addition, the UV and NMR spectra of KC-18, although far from ideal, gave values comparable to those of piperonylic acid.

The assumption that KC-18 might be an ester of piperonylic acid was further investigated by submitting KC-18 to base hydrolysis. The rationale for using base hydrolysis was that if KC-18 indeed contained piperonylic acid, a base hydrolytic treatment should cleave the acidic moiety from its ester and the hydrolysate could then be compared with an authentic sample of piperonylic acid - thus proving or disproving the assumption. Since the results of the mass spectral analyses of the two compounds showed that they were almost but not completely identical, the use of other methods was required to establish the identity of the hydrolysate of KC-18. The application of the hydrolysate with an authentic sample of piperonylic acid as the reference compound, on TLC plates in diff-

erent developing systems, was employed since identical R_f values in several solvent systems, according to generally accepted standards, can only be obtained when the substances applied onto the TLC plates are chemically identical. Our results showed that the R_f values of the hydrolysate in three different solvent systems were indeed identical to that of the authentic sample of piperonylic acid after being submitted to identical hydrolytic treatment. The slight separation on the TLC plates may be due to small amounts of sodium ions which were carried over into the ether solution during extraction and subsequently gave rise to "ghost" spots for the acid. In addition, the electrophoretic behavior of the hydrolysate being identical in all respects to an authentic sample of piperonylic acid provided further convincing evidence that the hydrolysate of KC-18 was identical to piperonylic acid.

On the basis of these data, we concluded that the acid component on the hydrolysis of KC-18 is piperonylic acid. Consequently, KC-18 itself is most probably an ester of piperonylic acid. Although the data obtained from the spectral analyses indicated that the side-chain of KC-18 is most likely to be an 8-carbon aliphatic alcohol, the exact structure of this component of KC-18 remains to be elucidated.

III. Pharmacology

a). In Vivo Tests

1. Histamine Aerosol Test

A 0.2%, 0.4% and 0.6% ethanolic solution of KC-18 was injected intraperitoneally into 10 guinea pigs in a dose of 2 mg/kg, into 11 guinea pigs in a dose of 4 mg/kg and into 5 guinea pigs in a dose of 6 mg/kg. 26 control guinea pigs received the corresponding volume of the vehicle. Five hours later, a treated and a control guinea pig were placed into the test chamber and exposed to 0.15% histamine aerosol.

The results of the experiment, shown in figure 14, indicate that while KC-18 in a dose of 2 mg/kg did not modify the survival time of the animals, the administration of 4 mg/kg and 6 mg/kg of KC-18 brought about a significant ($p < 0.01$) dose-related protection against the lethal effects of histamine aerosol.

2. Capillary Permeability Test

A 0.25%, 0.40% and 0.60% ethanolic solution of KC-18 was injected intraperitoneally into 4 albino guinea pigs in a dose of 2.5 mg/kg, into 5 albino guinea pigs in a dose of 4 mg/kg and into 6 albino guinea pigs in a dose of 6 mg/kg. 12 control guinea pigs received

the corresponding volume of the vehicle. Five hours later, the guinea pigs were given 30 mg/kg of pontamine sky blue intracardiacally, immediately followed by the intradermal injection of 0.5 ug histamine (concentration of histamine was 10 ug/ml) at the previously shaven abdominal area. 30 minutes later, the animals were sacrificed, the skin removed and the area of blueing measured.

The results obtained from treating guinea pigs at three dose levels of KC-18 are shown in Table VIII. It can be seen that KC-18 exerted a significant ($p < 0.01$) dose-related inhibitory effect against the histamine-induced capillary permeability.

3. Micro-Anaphylactic Shock Test.

A 0.4% ethanolic solution of KC-18 was injected intraperitoneally into 20 sensitized guinea pigs at a dose of 4 mg/kg. 20 control guinea pigs were given the corresponding volume of the vehicle. Five hours later, one treated and one control animal were placed into the test chamber and exposed to a 2 % egg ovalbumin aerosol for a maximal period of 10 minutes.

Figure 15 shows that while in the control group dyspnea developed at an average time of 2 minutes, no dyspnea was observed in any of the treated animals during the 10 minutes exposure time.

4. Histamine-Induced Hypotension Test

A 0.16 %, 0.32 % and 0.64 % ethanolic solution of KC-18 was injected intraperitoneally into 5 cats at a dose of 4 mg/kg, into 5 cats at a dose of 8 mg/kg and into 3 cats at a dose of 16 mg/kg. One hour later, the animals were given histamine solutions (prepared in concentrations of 2 ug/ml, 10 ug/ml and 20 ug/ml) intravenously in doses of 0.1 ug/kg, 0.5 ug/kg and 1.0 ug/kg respectively and, thereafter, the same doses of histamine solutions were administered intravenously every 30 minutes.

A typical example of the results obtained, as shown in figure 16, demonstrate that KC-18 administered in a dose of 4 mg/kg did not modify the effect of intravenously administered histamine. However, KC-18 given in dose of 8 mg/kg and 16 mg/kg exerted a significant ($p < 0.02$) and dose-related (30 % and 52%) inhibition against the histamine-induced hypotension.

5. Histamine-Induced Acid Gastric Hypersecretion Test

A 24 % and 40 % ethanolic solution of KC-18 was injected intravenously into 5 male hooded rats in a dose of 72 mg/kg and into 5 animals in a dose of 120 mg/kg one hour after the intravenous administration of 1.8 mg/kg histamine solution (freshly prepared in a concentration of 1 mg/ml).

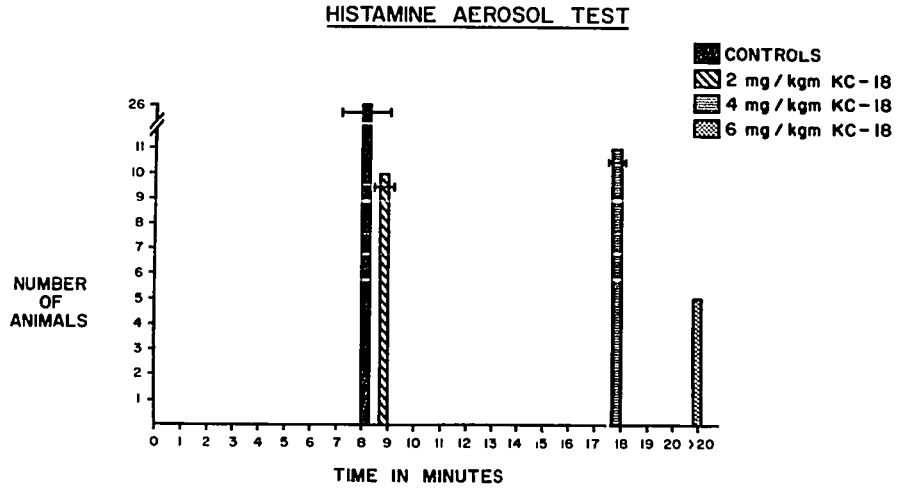


Figure 14

The mean survival time of guinea pigs exposed to a 0.15 % histamine aerosol five hours after the intraperitoneal injection of KC-18 in doses of 2 mg/kg, 4 mg/kg and 6 mg/kg or vehicle. The abscissa measures the mean survival time (in minutes), while the ordinate indicates the number of animals in each group.

Table VIII

CAPILLARY PERMEABILITY TEST

NUMBER OF ANIMALS	DOSE OF KC - 18 (mg / kgm)	AVERAGE AREA (mm^2)	PROBABILITY
12	-	359	-
4	2·5	219	< 0·001
5	4·0	107	< 0·02
6	6·0	11	< 0·001

Area of blueing in the guinea pig skin as a measure of the increase in capillary permeability induced by the intradermal administration of 0.5 ug histamine immediately after the intracardiac injection of 30 mg/kg of pontamine sky blue into the guinea pigs, which were pretreated with 2.5 mg/kg, 4 mg/kg and 6 mg/kg or vehicle five hours before.

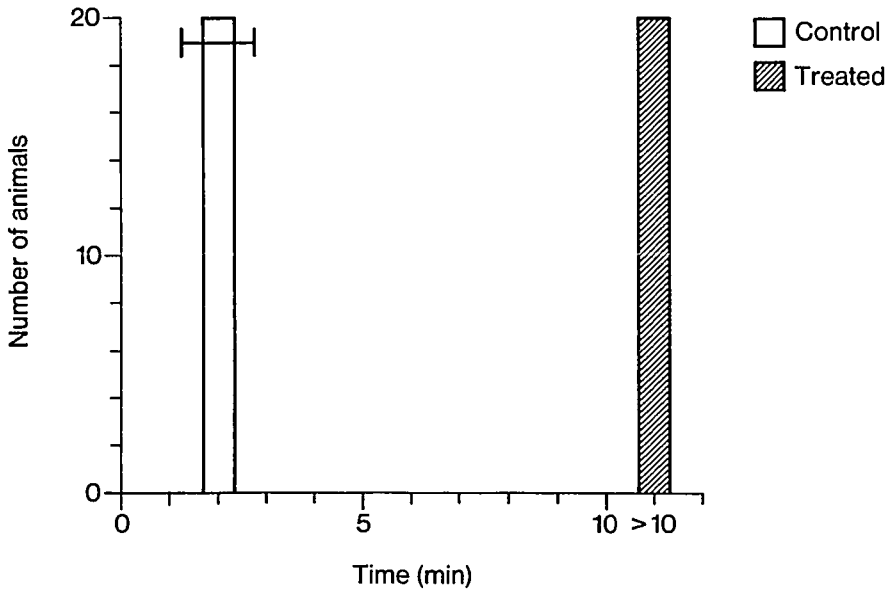


Figure 15

The mean dyspnea time of sensitized guinea pigs exposed to a 2 % egg ovalbumin (the challenging antigen) five hours after the intraperitoneal injection of KC-18 in a dose of 4 mg/kg or vehicle. The abscissa measures the mean dyspneic time (in minutes) while the ordinate indicates the number of animals in each group.

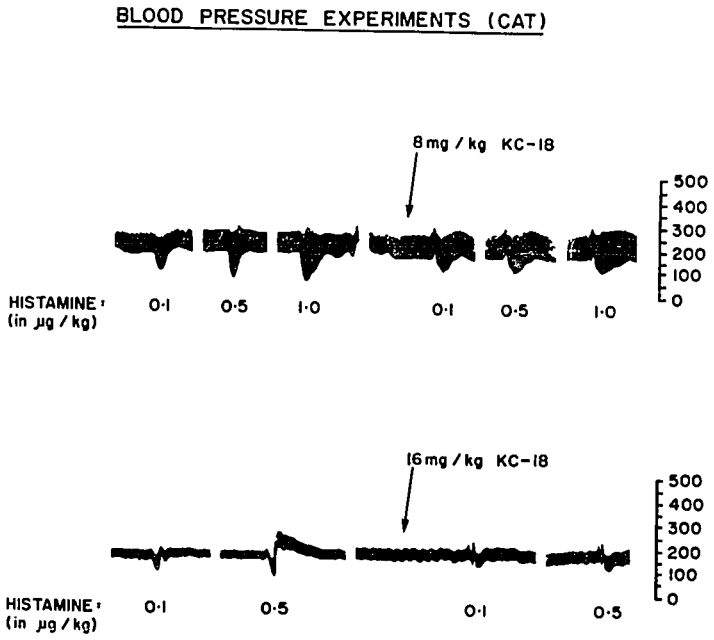


Figure 16

The hypotensive action of histamine administered intravenously into cats in doses of 0.1 ug/kg, 0.5 ug/kg and 1.0 ug/kg one hour after the intraperitoneal injection of 8 mg/kg and 16 mg/kg KC-18. The same doses of histamine were similarly given at 30 minutes intervals till the end of the experiment.

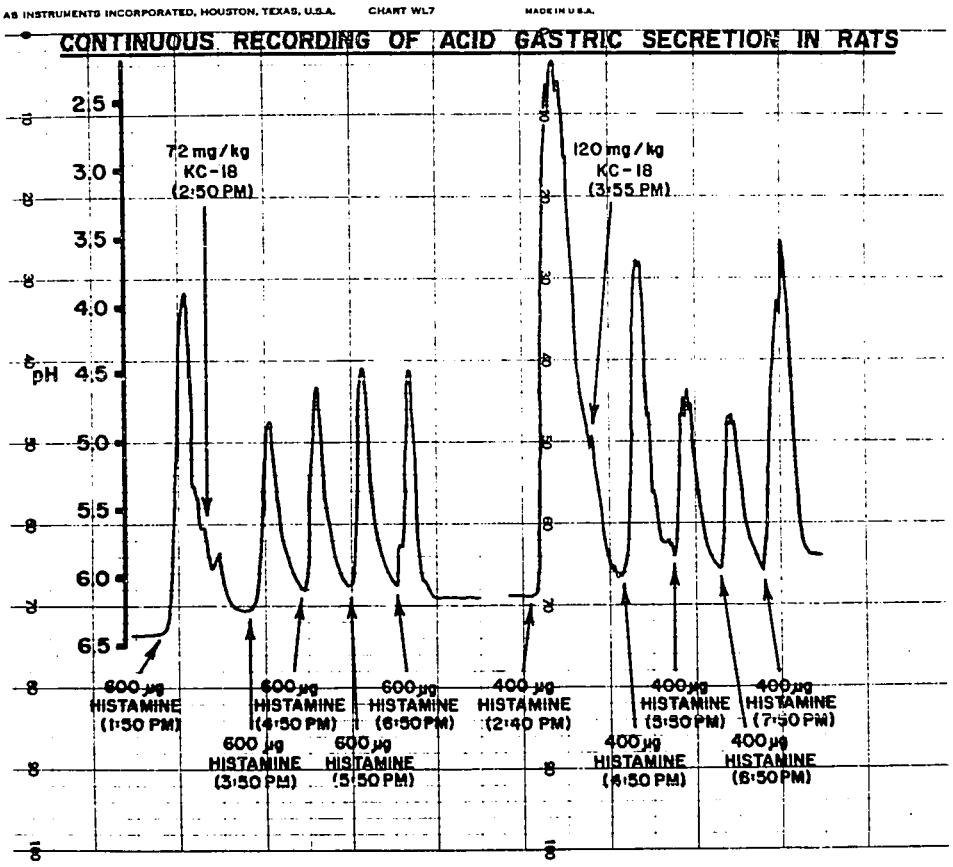


Figure 17

pH of the perfusion fluid as a measure of the histamine-induced gastric acid secretion in male hooded rats one hour prior to and at one hour intervals following the intravenous administration of KC-18 in doses of 72 mg/kg (A) and 120 mg/kg (B).

The results obtained, shown in Figure 17, demonstrate that KC-18 in doses of 72 mg/kg and 120 mg/kg exerted a significant ($p < 0.01$) and dose-related (56.7 % and 81.4 %) inhibition against the histamine-induced gastric acid hypersecretion. This inhibitory effect of KC-18 persisted for several hours and,although, histamine was injected intravenously into the rat at the same dose repeatedly at one hour intervals, the acid stimulatory effect of histamine was still partially inhibited even after 4 - 5 hours.

b. In Vitro Test

1. The Isolated Guinea Pig Ileum Preparation

KC-18 in 0.25 % ethanolic solution was added to the preparation in doses varying from 10 to 100 ug/ml.

The results obtained from four experiments showed that KC-18, in concentrations as high as 100 ug/ml,did not significantly inhibit the histamine-induced contractions of the guinea pig ileum.

c. Discussion

The effects of a histamine aerosol in normal untreated guinea pigs have been well documented by various investigators (Broome et al., 1963; Calam and Callow, 1964; Wakkary, 1968; Chu, 1969). The symptoms brought about by the aerosolized histamine leading to broncho-

constriction and asphyxial death develop in guinea pigs in a fairly uniform sequence, that is: cough, dyspnea, swaying, lying and death. Any one of these symptoms may be taken as the end point of the experiment (Halpern, 1942; Loew et al., 1945; Herxheimer, 1951; Broome et al., 1962; Herxheimer and Stresemann, 1963; Calam and Callow, 1964; Wakkary, 1968; Chu, 1969). Most workers believe the development of dyspnea to be the most constant symptom and used it as the end point of the experiments. According to Halpern (1942) and Lish et al. (1966) if guinea pigs exposed to histamine aerosol were removed from the test chamber at the onset of coughing or dyspnea, these same animals may be re-exposed 2 - 4 hours later without any significant changes in the predyspneic interval. On the other hand, Wakkary (1968) noted that the re-exposure of guinea pigs to histamine aerosol within 2 - 4 hours produced a significant change in the predyspneic interval in a large percentage of the animals. In view of the foregoing contradiction, the method of Broome et al. (1962), which uses the cessation of respiration as the end point of the experiment, was selected as a routine testing procedure.

The determination of changes in capillary permeability is another common method used to evaluate the specificity and potency of potential antihistamine. Bain et al. (1948) reported that there is an inverse linear relationship between the logarithms of the dose of antihistamine and the area of flare induced by histamine.

It is well known, however, that only the most potent antihistamines can antagonize the increase in capillary permeability induced by histamine (Celice and Durel, 1942; Parrot and Lefebvre, 1943; Rocha e Silva, 1955).

The use of systemic anaphylactic shock to evaluate antihistamines originated from Staub and Bovet (1937) and it has since been commonly used (Halpern, 1942; Mayer et al., 1945; Feinberg et al., 1950:, Wakkary, 1968; Chu, 1969). It is well established that a potent antihistamine can prevent death of sensitized guinea pigs from anaphylactic shock and this fact serves to indicate that histamine plays an important role in the development of anaphylactic symptoms in the guinea pig (Nordine and Seigler, 1964; Kabats and Mayer, 1961). However, fatal anaphylactic shock can be produced by the reaction between widely varying amounts of antigen and antibody which does not allow quantitation. Furthermore, difficulties in interpreting results occur if the animals are not uniformly sensitized. Kabat and Landow (1942) have shown that it is possible to produce mild anaphylactic shock by reducing the amount of antigen used. In 1952, Herxheimer developed the microshock technique, which consists of the production of a non-lethal anaphylactic reaction of a definite intensity. He showed that the preconvulsive time of sensitized guinea pigs, exposed to aerosolized antigen, is very constant. This microshock technique has been successfully used for the quantitative evaluation of a number of anti-anaphylactic

agents (Armitage et al., 1952; Herxheimer and Stresemann, 1963).

Inhibition of hypotensive effect of small doses of intravenously administered histamine in cats and dogs has also been utilized for the evaluation of potential antihistamines (Broadbent and Bain, 1964). Potent antihistamines can inhibit histamine-induced hypotension by antagonizing both the vasoconstrictor and more importantly, the vasodilator effects of histamine (Labelle and Tislow, 1955; Roth and Govier, 1958; Douglas, 1970). Although this method is not very sensitive in differentiating the potency of antihistamines (Loew et al., 1947; Rocha e Silva, 1955) it can, nevertheless, be used to obtain information on the activity profile of a potential antihistamine.

Since Ghosh and Schild introduced the perfused rat stomach method in 1955 for measuring the acid output in response to various stimuli, the original method has been modified by a number of investigators. Several workers measured the acid output by titrating regular 10 - 30 minutes samples, either by hand or using an automatic titrator (Lai, 1964; Barrett, 1966; Pissidis and Clark, 1967; Moore et al., 1967). This technique requires careful timing and is laborious. Others have used a flow electrode to record pH changes in a dilute buffer (Ghosh and Schild, 1958; Rosenoer and Schild, 1962; Thompson and Sircus, 1967). Recently, Smith et al. (1970) reported another method, based on the reperfusion of a measured quantity of

fluid through the rat stomach, using the cumulative pH changes as an index of acid secreted. In spite of some drawbacks of the method, which is based on the continuous measurement of pH change (Ghosh and Schild, 1958) where the differential record obtained necessitates the cumbersome measurement of the area in order to compute the amount of acid secreted in response to stimulus/antagonist, we found that it is satisfactory for the detection of an inhibitory substance.

The dose-dependent activity of KC-18 in antagonizing the actions of histamine was clearly demonstrated. For example, while the administration of 2 mg/kg of KC-18 did not modify the survival time of the guinea pigs in a histamine aerosol as compared to non-treated controls, KC-18 given in a dose of 6 mg/kg completely protected the animals against a fatal outcome during the 20 minutes exposure time. Similarly, results obtained from the histamine-induced capillary permeability test and the histamine-induced hypotension experiments also demonstrated the dose-dependent histamine antagonizing activity of KC-18.

Although KC-18 was tested only in a single dose in the micro-anaphylactic shock experiments, the results clearly showed its anti-anaphylactic activity. All these results appear to confirm the previous observations that the antihistamine-like activity of the extracts of the Hungarian oak galls was brought about by a single

substance (Kovacs and Szabadi, 1950; Kovacs et al., 1952; Feldberg and Kovacs, 1960; Broome et al., 1962; Chu, 1969). Furthermore, as it was previously shown (Chu, 1969), KC-18 had no in vitro activity in the isolated guinea pig ileum preparation.

Almost everybody who investigated the actions of the numerous compounds classified as "antihistamines" concluded that these compounds can antagonize all but the gastric secretory response of histamine. This lack of action has been demonstrated both in the human and various animal species (Davenport and Chavre, 1950; Dutta and Tamhane, 1953; Howat and Schofield, 1954; Masoni et al., 1957; Ragins et al., 1958; Kohout et al., 1960). Not only do the antihistamines fail to inhibit gastric secretion, but it has been observed repeatedly that these compounds actually augment the basal secretion or intensify the histamine response in man (Reitter, 1952), dog (Paton and Schachter, 1951), and cat (Howat and Schofield, 1954). Recently Ash and Schild (1966) provided the plausible explanation for this lack of activity by showing that the histamine receptors in the stomach (H_2 receptors) differ from those of the bronchiole smooth muscles or the vascular smooth muscles (H_1 receptors).

To our knowledge, KC-18 is the first substance known to possess the ability of antagonizing the actions of histamine on both H_1 and H_2 receptors. As is the case with its action on H_1 receptors,

there is some suggestive evidence that the anti-gastric activity of KC-18 may be attributable to an active metabolite since it takes 1-2 hours for the inhibitory effect to reach optimal level after its administration by the intravenous route. It may also be stated that the inhibitory activity is not likely to be the result of toxicity since the histamine-induced secretion always tends to return towards normal level following repeated histamine administration.

Although the mechanism of action of KC-18 has not yet been elucidated, it is interesting to note that in spite of its lipid-like nature which, theoretically, should enable it to be easily absorbed follwing its intraperitoneal administration, KC-18 has been found to have a long latent period (5 hours) before reaching its peak activity in all the tests with the exception of its antagonistic activity against histamine-induced gastric acid secretion. Furthermore, attempts to demonstrate <u>in vitro</u> activity against histamine-induced smooth muscle contraction repeatedly failed, even when very high doses were used. This conspicuous absence of <u>in vitro</u> activity would also seem to indicate that the observed <u>in vivo</u> activity most probably cannot be attributed to a direct action on the smooth muscles. Thus, its mechanism of action may likely differ from that of the conventional antihistamines, especially since it also inhibits histamine-induced gastric acid secretion. A further difference in its mode of action as compared to the conventional anti-histamines is the fact that KC-18 does not appear to exert

any central nervous system depressant action since it has been previously shown that the purified oak gall extracts do not inhibit passive cutaneous anaphylaxis (Chu, 1969), though it has not been tested, it is likely that the chemically pure substance would similarly be lacking in this activity. Thus, it seems reasonable to assume that the anti-anaphylactic activity of KC-18 is not due to an immuno-suppressant effect. The fact that it takes 5 hours for KC-18 to reach optimal activity and the absence of *in vitro* activity may indicate that KC-18 per se is not the active principle and that the *in vivo* activity could be attributable to an active metabolite.

Without testing KC-18 against all the known chemical mediators, the specificity of the action of KC-18 is not known. However, the data obtained indicate that KC-18 is most probably a specific antagonist of histamine. For example, it has been shown that purified oak gall extracts did not influence the bradykinin-induced capillary permeability (Chu, 1969). Furthermore, in tests where different doses of KC-18 were administered, the activity has been consistently dose-dependent. In addition, since no toxic symptoms, such as irritation of the abdominal area (site of injection), diarrhea, ruffing of fur or any other signs of toxicity were observed in the treated animals, the possibility of a non-specific activity due to toxicity of KC-18 seems to be highly unlikely.

Section C. Purification and Pharmacology of the Anti-Inflammatory Principle(s)

I) Method of Purification

About 130 mg of the dry standard crude extract exerting anti-inflammatory activity (fraction VI - see page 141) were suspended in 20 ml chloroform and applied onto a silica gel G column (1.0 x 25.0 cm). The column was eluted under nitrogen pressure (5 lb/in^2) with 500 ml of each of the following solvent mixtures: chloroform; chloroform:methanol (9:1, v/v); chloroform:methanol (7:3, v/v); chloroform:methanol (1:1, v/v) and chloroform:methanol (3:7, v/v). For each solvent mixture, 500 ml of the effluents were collected as a single fraction in a round bottom flask and thoroughly dried.

An aliquot of each of the dried fractions was dissolved in ethanol in the following concentrations: fraction I - 0.71 %; fraction II - 0.42 %; fraction III - 0.13 %; fraction IV - 0.59 % and fraction V - 0.81 % and the solutions were injected intraperitoneally into five groups of male wistar rats (10 rats/group) in doses of 8.5 mg/kg; 5 mg/kg; 15.6 mg/kg; 7.0 mg/kg and 9.6 mg/kg respectively. The control animals (10 rats) were similarly injected with the corresponding volume of the vehicle. Four hours after treatment, 0.05 ml of a 1% carrageenan solution was injected into the plantar surface of the left hind paw of each animal. Immediately

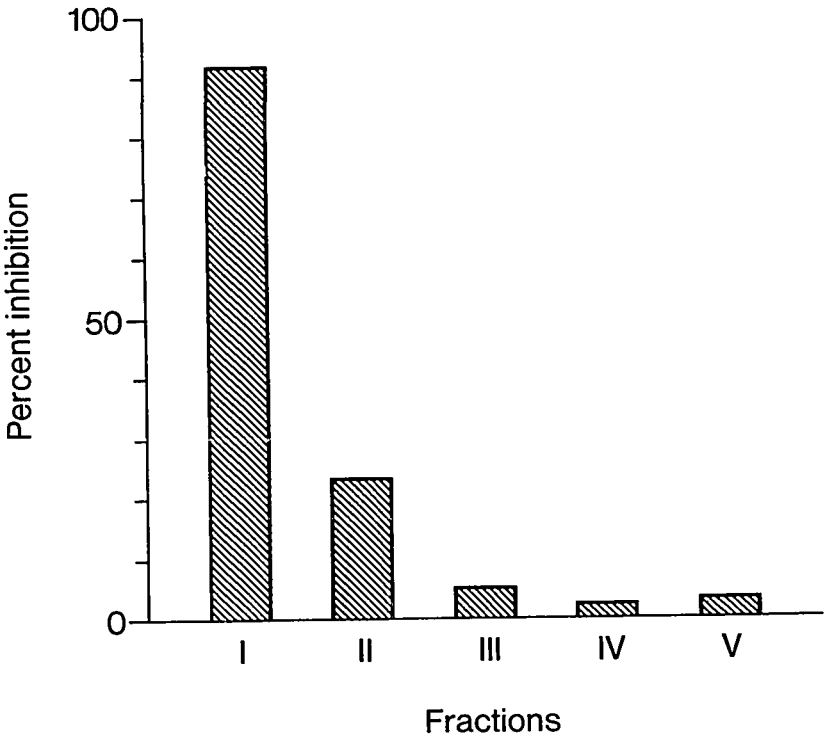


Figure 18

The effect of the silica gel G column chromatographic fractions (I - V) of the Hungarian oak gall extracts against carrageenan-induced edema in the rat. The fractions or vehicle were injected intraperitoneally into groups of male wistar rats (20 animals/group). Four hours later, 0.05 ml of a 1% carrageenan solution was injected into the plantar surface of the left hind paw of the animals. Immediately thereafter and again three hours later, the volume of the injected paw was measured under light ether anaesthesia. The abscissa indicates the fraction number while the ordinate measures the percent inhibition.

Table IX

Oak Gall Powder (in gm)	Fraction I	
	mg.	%
600	16.0	0.0025
600	9.0	0.0015
600	11.6	0.0019
600	15.4	0.0026
600	19.2	0.0032
Mean ± S.E.	14.24 ± 1.8	0.0023 ± 0.0003

Table IX compares the yields of the silica gel G chromatographic fraction I obtained from five different batches of oak gall extracts. The yields are representative of all the fraction I obtained in these studies.

thereafter and again three hours later, the volume of the injected paw was measured plethysmographically under light ether anaesthesia.

The results obtained from five experiments are shown in figure 18. It can be seen that the carrageenan-induced paw edema was only inhibited in animals treated with fraction I. The yield of fraction I obtained from five different batches of oak gall extracts, which is representative of all experiments, is shown in Table IX.

a. Discussion

The screening for possible pharmacological actions other than the antihistamine-like effect showed that one fraction of the standard crude extract of the Hungarian oak gall extracts consistently exerted a potent anti-inflammatory effect when tested against the carrageenan-induced paw edema test. This fraction containing the anti-inflammatory principle(s) was further purified by stepwise elution in the silica gel G column.

For each fraction, a large volume of the effluents was collected in order to prevent the loss of the active material into the next fraction through overlapping. The individual fractions were separated on the basis of a fixed volume of effluents since the active principle(s) was either UV transparent or the concentration was too low for detection by the conventional UV analyser. This

rather unusual method, however, proved to be satisfactory since the dry weight of the fractions obtained from several batches of the oak gall preparations was found to be fairly uniform (Table IX), and the purity of the extract increased about five-folds without any loss of activity.

The assay of the five fractions obtained from the silica gel G column consistently showed that only fraction I exerted anti-inflammatory activity. At present, it seems very unlikely that the anti-inflammatory activity exerted by fraction I was due to the toxic effects of the extracts since no overt symptoms were revealed by careful examination of the animals following the administration of the purified fraction. The lack of any toxic signs together with the increase in specific activity of the purified oak gall extracts could fairly safely be taken as an indication that the observed effect was brought about by a biologically active substance(s) present in the extract. That this substance is chemically different from the antihistamine-like principle has been clearly indicated by the lack of antihistamine-like activity of the fraction containing the anti-inflammatory principle(s) and, correspondingly, the lack of anti-inflammatory activity of the chemically pure antihistamine - KC-18. In addition, the physical properties of the anti-inflammatory substance(s) also differ from those of KC-18. Thus, it seems likely that the anti-inflammatory principle(s) chemically is not identical with that of the antihistamine, how-

ever, it might be that the two substances are chemically related. Final proof of this possibility awaits the isolation and identification of the anti-inflammatory principle.

II) Pharmacology

a) Carrageenan-Induced Rat Paw Edema Test

Fraction I, in 0.35 % and 0.71 % ethanolic solutions, was injected intraperitoneally into groups of male Wistar rats (20 animals/group) in doses of 4.3 mg/kg and 8.5 mg/kg respectively, while 20 control rats received the corresponding volume of the vehicle. Four hours following treatment, 0.05 ml of a 1 % carrageenan solution was injected into the plantar surface of the left hind paw of each animal. Immediately thereafter and again three hours later, the volume of the injected paw was measured plethysmographically under light ether anaesthesia.

The results obtained from five experiments are shown in Figure 19. It can be seen that the anti-inflammatory activity of the oak gall extract is dose-dependent since, when injected in a dose of 4.3 mg/kg it exerted a 53 % inhibition, while given in a dose of 8.5 mg/kg it brought about a 94 % inhibition of the carrageenan-induced rat paw edema.

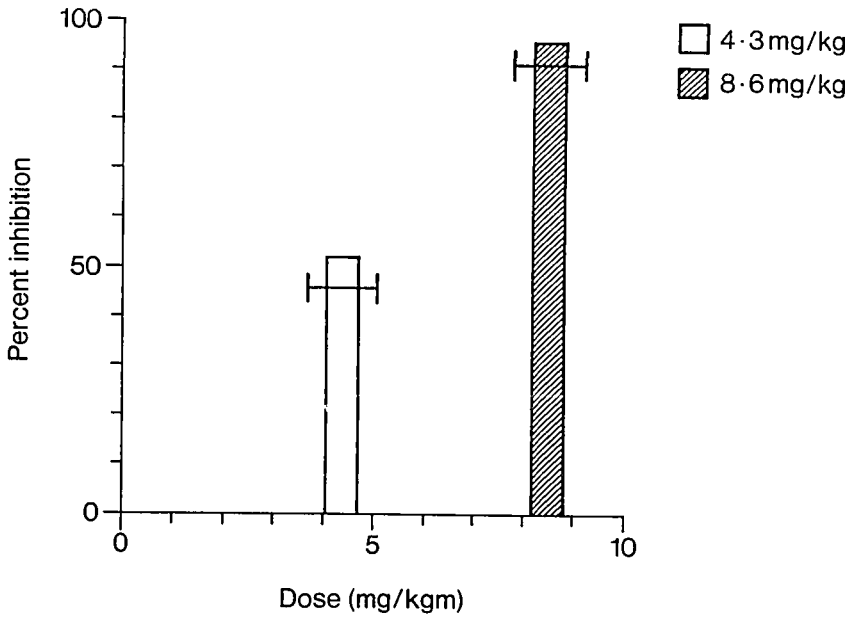


Figure 19

The effect of the silica gel G column chromatographic fraction I of the Hungarian oak gall extracts against carrageenan-induced paw edema in rats. Fraction I or vehicle was injected intraperitoneally into groups of male Wistar rats (20 rats/group). Four hours later, 0.05 ml of a 1 % carrageenan solution was injected into the plantar surface of the left hind paw of the animals. Immediately thereafter and again three hours later, the volume of the injected paw was measured under light ether anaesthesia. The abscissa indicates the dose of fraction I, while the ordinate measures the percent inhibition.

b) Mycobacterial Adjuvant Induced Polyarthritis

Fraction I, in 0.71 % and 1.07 % aqueous solutions, was injected intraperitoneally into groups of male Lewis rats (20 animals/group) in doses of 8.5 mg/kg and 12.8 mg/kg respectively. The control animals (40 rats) received the corresponding volume of the vehicle. The administration of the extract or vehicle started on day -3 and continued daily till day 16. On day 0, 0.1 ml of a 0.6 % M. butyricum suspended in light mineral oil was injected into the plantar surface of the left hind paw of each animal. The paw volume of the animals was determined daily by the plethysmographic method till day 25, while the degree of joint inflammation of the animals, expressed as arthritic score, was determined every second day from day 11 till day 25.

The results obtained in four series of experiments are shown in figures 20 - 25. Figure 20 shows the photograph of the left hind paws of two rats taken 21 days after the mycobacterial adjuvant was injected into the plantar surface. The paw of the left belonged to the rat treated with 12.8 mg/kg extract as described above while the paw on the right of the photograph belonged to the arthritic control which received the corresponding volume of the vehicle.

Figures 21 and 22 show the difference in paw swelling from day 0 to day 25 between the animals treated at two dose levels of the

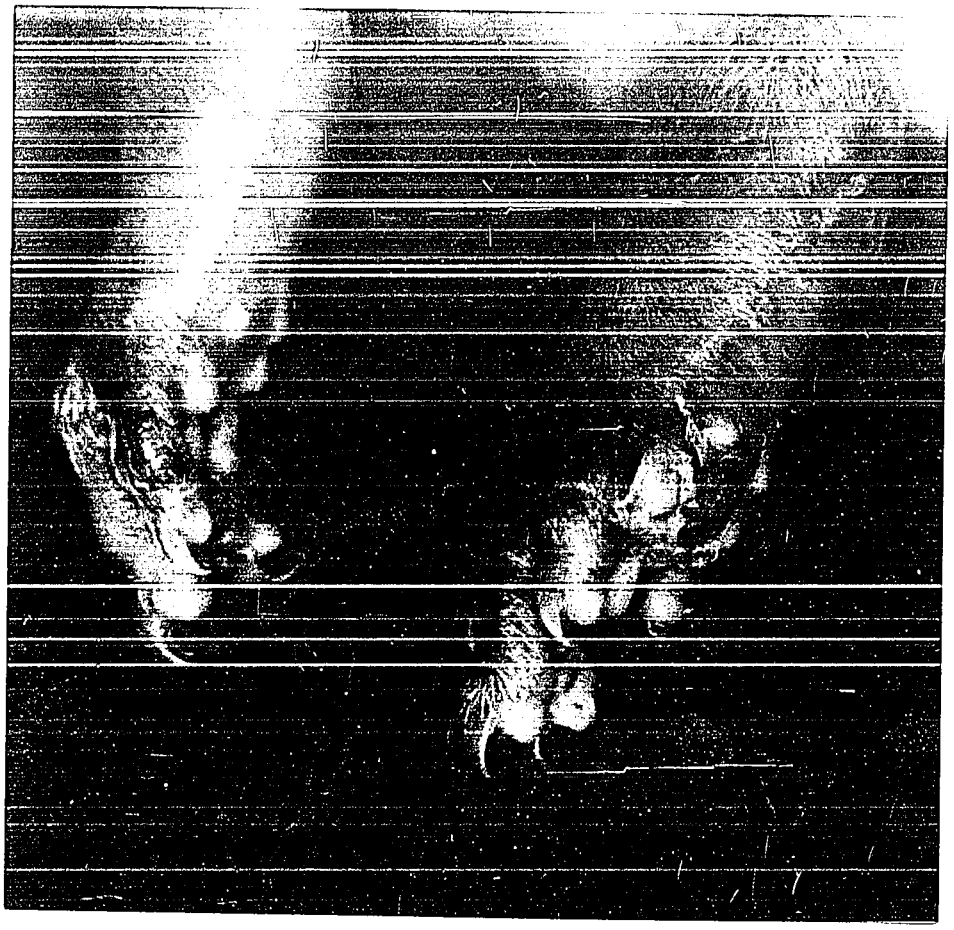


Figure 20

The photograph shows the left hind paws of two rats 21 days after the mycobacterial adjuvant was injected into the plantar surface. The paw on the left of the photograph belonged to a rat treated with 12.8 mg/kg oak gall extract 3 days before and 16 days after the injection of the adjuvant, while the paw on the right belonged to a rat (arthritic control) treated with the corresponding volume of the vehicle.

extracts (8.5 mg/kg and 12.8 mg/kg) and the animals treated with the corresponding volumes of the vehicle (arthritic controls). For the left hind paws, the degree of inhibition obtained on days 1, 5, 10, 15, 20 and 22 (arbitrarily chosen) in animals treated with 8.5 mg/kg extracts was 23.6 % (ns), 26.3 % (ns), 41.7 % ($p < 0.04$), 14.0 % (ns), 72.8 % ($p < 0.01$), and 31.5 % ($p < 0.05$), while that obtained in animals treated with 12.8 mg/kg extracts was 97.0 % ($p < 0.001$), 85.4 % ($p < 0.01$), 68.6 % ($p < 0.01$), 50.2 % ($p < 0.03$), 70.5 % ($p < 0.01$) and 55.0 % ($p < 0.03$). Similarly, for the right hind paws, the degree of inhibition of paw swelling obtained on days 18, 20, 22 and 24 (arbitrarily chosen) in animals treated with 8.5 mg/kg extracts was 50.0 % ($p < 0.03$), 74.2 % ($p < 0.01$), 86.6 % ($p < 0.01$) and 0 %, while that obtained in animals treated with 12.8 mg/kg extracts was 64 % ($p < 0.02$), 85.7 % ($p < 0.01$), 91.6 % ($p < 0.01$).

These results show that the purified oak gall extracts exerted a significant and dose-dependent suppression of the paw swelling induced by the mycobacterial adjuvant till day 22. There was, however, no longer any difference in the paw swelling between the treated animals and the arthritic controls by day 25.

Figures 23 and 24 show the difference in joint swelling from day 0 (day of adjuvant injection) to day 25 between the animals treated at two dose levels of extracts (8.5 mg/kg and 12.8 mg/kg) and

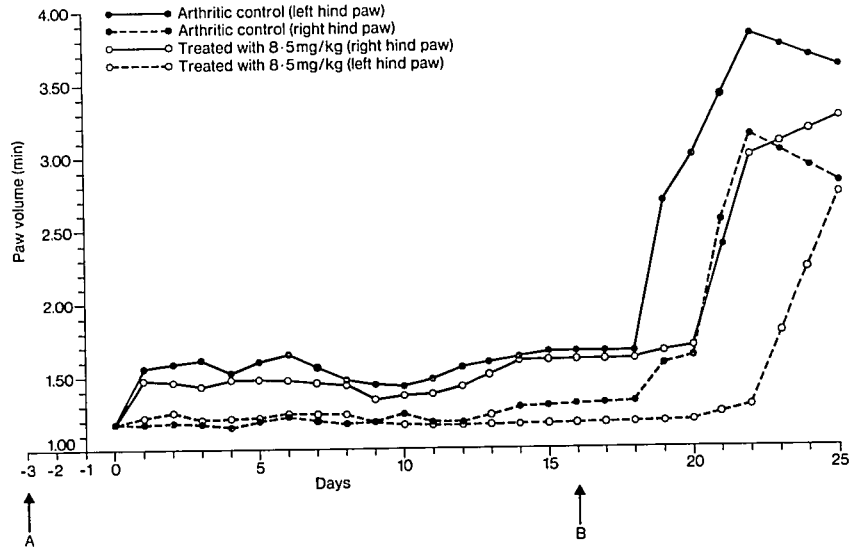


Figure 21

The effect of the purified oak gall extracts against the mycobacterial adjuvant induced paw swelling in male Lewis rats. The extract, in a dose of 8.5 mg/kg, was injected intraperitoneally daily into the animals from day -3 (A) to day 16 (B), while the control rats (arthritic controls) received the corresponding volume of the vehicle. On day 0, 0.1 ml of a 0.6 % M. butyricum suspended in light mineral oil, was injected into the plantar surface of the left hind paw of each animal. The volumes of both hind paws were determined daily by the plethysmographic method. The abscissa indicates the duration of the experiment (in days) while the ordinate measures the mean volumes (in ml) of the hind paws.

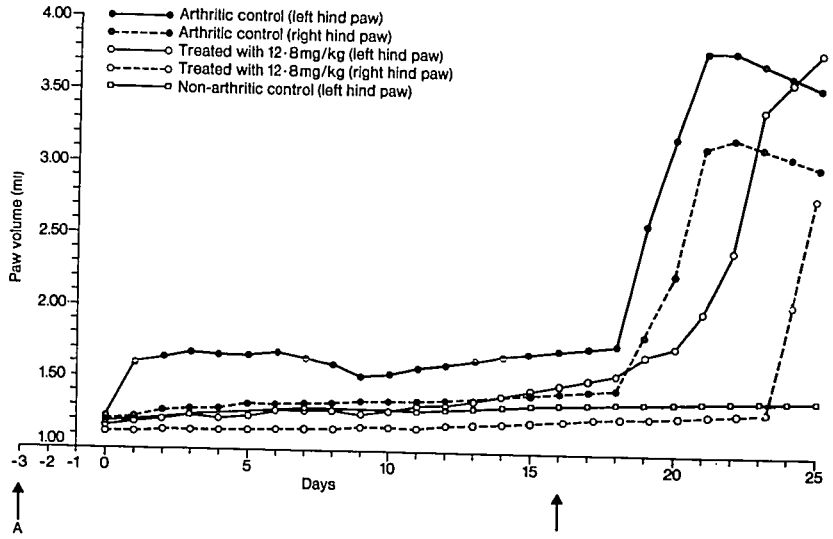


Figure 22

The effect of the purified oak gall extracts against the mycobacterial adjuvant induced paw swelling in male Lewis rats. The extract, in a dose of 12.8 mg/kg, was injected intraperitoneally daily into the animals from day -3 (A) to day 16 (B), while the control animals (arthritic controls) received the corresponding volume of the vehicle. On day 0, 0.1 ml of a 0.6 % M. butyricum suspended in light mineral oil was injected into the plantar surface of the left hind paw of each animal. The volumes of both hind paws were determined daily by the plethysmographic method. The abscissa indicates the duration of the experiment (in days) while the ordinate measures the mean volumes (in ml) of the hind paws.

animals treated with the corresponding volumes of the vehicle (arthritic controls). For the left hind paws, the percent inhibition obtained on days 11, 14, 18, 20, 22 and 25 (arbitrarily chosen) in animals treated with 8.5 mg/kg extracts was 100 % ($p < 0.01$), 85.3 % ($p < 0.01$), 66.3 % ($p < 0.01$), 66.6 % ($p < 0.02$), 20.8 % (ns) and 0 %, while that obtained in animals treated with 12.8 mg/kg extracts was 100 % ($p < 0.01$), 93.0 % ($p < 0.01$), 75.7 % ($p < 0.01$), 70.6 % ($p < 0.01$), 40.4 % ($p < 0.05$) and 0 %. Similarly, for the three other paws, the percent inhibition obtained on days 11, 14, 18, 20, 22 and 25 in animals treated with 8.5 mg/kg extracts was 100 % ($p < 0.01$), 83.3 % ($p < 0.01$), 84.2 % ($p < 0.01$), 68.4 % ($p < 0.01$), 33.3 % ($p < 0.05$) and 0 %, while that obtained in animals treated with 12.8 mg/kg extracts was 100 % ($p < 0.01$), 100 % ($p < 0.01$), 71.0 % ($p < 0.01$), 78.6 % ($p < 0.01$), 72.6 % ($p < 0.01$) and 12 % (ns).

The results show that the purified oak gall extracts exerted a significant and dose-dependent effect against joint swelling induced by the mycobacterial adjuvant. The protective effect rapidly disappeared between day 23 and day 24 and by day 25 there was no longer any significant difference between the joint swelling of the arthritic controls and those of the animals treated with the extracts.

The rates of weight gain of the treated groups and their corresponding controls (arthritic) as compared to those of the normal non-arthritic animals are shown in figure 25. While the weight gaining

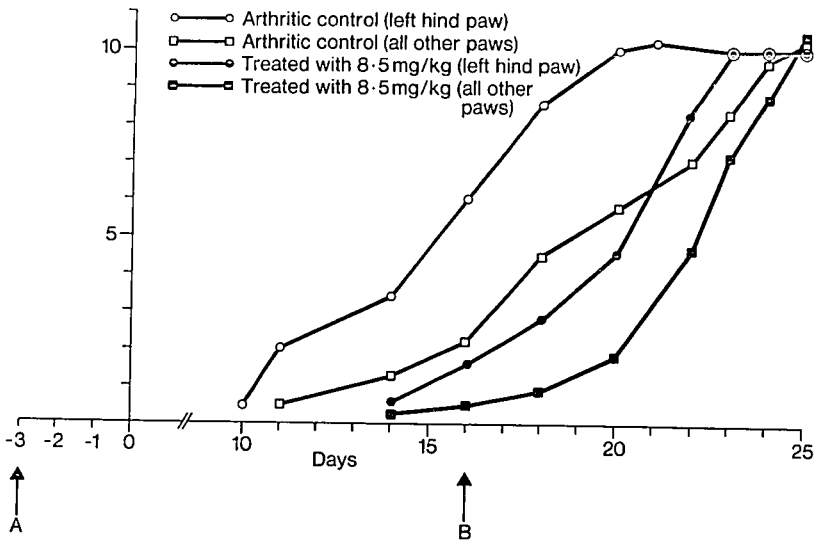


Figure 23

The effect of the purified oak gall extracts against the mycobacterial adjuvant induced joint swelling in male Lewis rats. The extract, in a dose of 8.5 mg/kg, was injected intraperitoneally daily into the animals from day -3 (A) to day 16 (B), while the control animals (arthritic controls) received the corresponding volume of the vehicle. On day 0, 0.1 ml of a 0.6 % M. butyricum suspended in light mineral oil, was injected into the plantar surface of the left hind paw of each animal. The joint swelling was determined (expressed as arthritic score) on day 10, 11, 14 and at least every second day thereafter till day 25. The abscissa indicates the duration of the experiment (in days) while the ordinate measures the mean joint swelling (arthritic score).

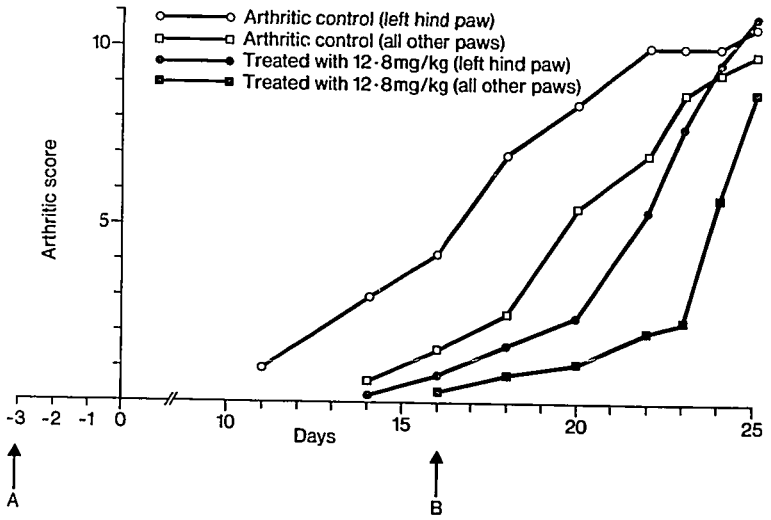


Figure 24

The effect of the purified oak gall extracts against the mycobacterial adjuvant induced joint swelling in male Lewis rats. The extract, in a dose of 12.8 mg/kg,was injected intraperitoneally daily into the animals from day -3 (A) to day 16 (B), while the control animals (arthritic controls) received the corresponding volume of the vehicle. On day 0, 0.1 ml of a 0.6 % M. butyricum suspended in light mineral oil, was injected into the plantar surface of the left hind paw of each animal. The joint swelling (expressed as arthritic score) was determined on day 10, 11, 14 and at least every second day thereafter till day 25. The abscissa indicates the duration of the experiment (in days) while the ordinate measures the mean joint swelling (arthritic score).

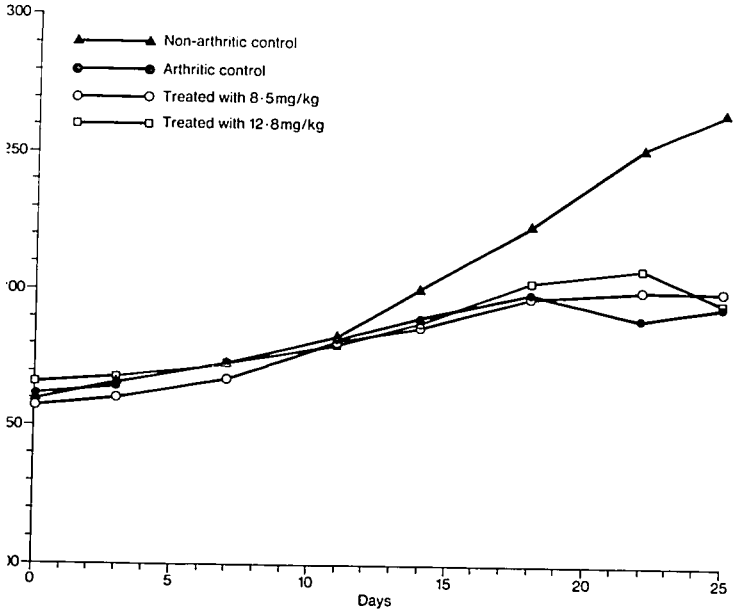


Figure 25

The effect of the purified oak gall extracts/vehicle on the rate of weight gain in arthritic rats as compared to that of the normal non-arthritic rats. The abscissa indicates the duration of the experiment (in days) while the ordinate measures the mean weight (in grams).

rates between the treated groups and the arthritic controls are comparable, they were significantly ($p < 0.05$) lower than those of the non-arthritic controls from day 11 (post induction) till the end of the experiment (day 25).

c) Discussion

The carrageenan-induced rat paw edema test was first described by Winter, Risley and Nuss (1962; 1963) and Winter (1965) as a useful method in the search for anti-inflammatory drugs. Since then, this test has been widely adopted as a reliable experimental tool for the evalution of potential anti-inflammatory agents (Shanahan, 1968; Yamamoto et al., 1969; Yongdale et al., 1969; Filderman and Kovacs, 1969; Swingle et al., 1970). Carrageenan is preferred by most investigators over several time honoured phlogens because of the edema produced which is less influenced by non-specific factors such as vasodilatation, ganglion blockade or diuresis (Garanttini et al., 1965) and because the activity of the anti-inflammatory agents in this test seem to correlate well with their clinical anti-inflammatory activity (Kampmann and Frey, 1966).

The other test used in our studies was the mycobacterial adjuvant induced polyarthritis in rats. This method was developed by Stoerk et al. (1954) and Pearson (1956; 1959). These authors reported that in rats, an intradermal injection of an emulsion containing various

tissues incorporated in Freund's adjuvant (a mixture of dead mycobacteria and liquid paraffin) produced inflamed lesions in areas of the body remote from the site of injection after a delay of 10 to 15 days. Subsequent work showed that the inclusion of animal tissues was unnecessary (Pearson and Wood, 1959) and that the arthritis could be produced by injecting a suspension of dead mycobacteria in either mineral or vegetable oil (Ward and Jones, 1962).

The polyarthritis induced by the intradermal injection of the mycobacterial adjuvant is widely used today as an experimental model of chronic inflammation for the evaluation of potential anti-inflammatory agents (Newbould, 1963; Glenn, 1964; Graeme et al., 1966; Piliero et al., 1966; Kapusta and Mendelson, 1967; 1969). This experimental arthritis has several features in common with human arthritis (Currey and Ziff, 1968; Katz and Piliero, 1969), including the histopathology of the joints (Pearson and Wood, 1959; Burstein and Waksman, 1964; Glenn, 1964; Piliero et al., 1966) and the lack of a direct correlation in the time of circulation antibody with joint lesion (Weiner and Piliero, 1970). Effective clinical antirheumatic agents such as paramethasone, salicylates, phenylbutazone and indomethacin have been reported to decrease the severity of the mycobacterial adjuvant induced polyarthritis in the rats (Ward et al., 1964; Phelps and McCarthy, 1967; Butler et al., 1969; Piliero and Colombo, 1969).

The results obtained by treating rats with the highly purified oak gall extracts showed that these extracts exerted a dose-dependent and in higher doses almost 100 % inhibition against the carrageenan-induced paw edema. The inhibitory effect was found to be much more pronounced than that of other anti-inflammatory agents previously reported (Yamamoto et al., 1968; Jahn and Adrian, 1969; Silvestrini et al., 1969; Di Rosa and Sorrentino, 1970; Filderman and Kovacs, 1969). To exclude factors which might contribute in bringing about this unusually high activity, all treated animals were carefully observed for toxic symptoms. The possible contributary factors taken into account were a) the loss of systemic fluid through abdominal irritation and diarrhea due to the intraperitoneal administration of the oak gall extracts, b) hypothermia due to the impairment of the central regulatory mechanism and c) fluctuation of room temperature (Green et al., 1971). Our studies showed that the intraperitoneal administration of the oak gall extract did not cause gastro-intestinal distress, diarrhea or hypothermia. The animals were kept in a temperature regulated animal house to ensure the uniform production of carrageenan-induced edema. Although we have made no attempts to rule out the possibility of diuresis induced by the extract, the fact that the animals suffered from no loss of weight after the final paw measurement (compared to before the experiment), minimized the effects of diuresis - if at all present - as a major contributory factor of non-specific activity.

A rather unusual feature of these experiments is that the purified oak gall extract exerted significant anti-inflammatory activity only when it was given at least three hours prior to the induction of the carrageenan edema. No inhibition was evident when carrageenan was injected less than three hours following the intraperitoneal injection of the oak gall extracts. On the other hand, the anti-inflammatory activity increased proportionally when the time interval between treatment with extract and induction of edema with carrageenan was increased up to four hours, after which time no further increase was observed.

From the data so far obtained, it is not possible to elucidate the mode of action of the active principle. However, in view of the recent work of Crunckhorn and Meacock (1971), who suggested that kinins and 5-HT play a role as major mediators in the development of early edema induced by carrageenan, it would be logical to assume that the active principle exerted its anti-inflammatory activity, at least in part, by antagonizing either or both kinin and 5-HT activity. This assumption, however, is most unlikely since Chu (1969) has shown that the partially purified oak gall extract exerted no activity against bradykinin-induced increase in capillary permeability. Nevertheless, the rather long latent period raises another interesting possibility - namely, that the substance <u>per se</u> is inactive and that the observed anti-inflammatory effect is attributable to an unidentified active metabolite.

The isogenic Lewis rats (male) have been reported to be particularly susceptible to adjuvant arthritis (Rosenthale, 1970; Watnick, Personal Commun.) and hence they were the animal of choice in our studies. The mycobacterial adjuvant was injected into the foot pad rather than the tail because it was also of interest to examine the possible activity of the oak gall extract against the primary inflammatory reaction i.e., paw edema, produced by the injection of the adjuvant.

The dose dependent anti-inflammatory activity of the oak gall extracts was clearly demonstrated also in the polyarthritic rat model. In preliminary experiments with this model, we noted that when the oak gall extract was given to the rats on the same day as the induction of the polyarthritis, no inhibition was observed either in the paw edema or joint inflammation. Neither was the extract active when it was given to the rats after the induction of the adjuvant arthritis. After experimenting with various treatment schedules, we found that the animals must be pretreated with the extracts at least three days prior to the induction of the adjuvant arthritis before any anti-inflammatory and antirheumatic effects were observed. The protective action exerted by the extract virtually disappeared 6 days after the treatment had ended.

The dose dependent suppression of joint inflammation exerted by the oak gall extracts follows a similar course as seen in its inhibitory

action against the edema formation. Our studies made no attempts to differentiate the differences in the severity of joint inflammation because, as the intensity of the paw edema increased, it became almost impossible to give meaningful grading of the degree of joint swelling. Furthermore, since the severity of the joint swelling is probably directly related to the number of joints involved, we feel that a difference in the number of joints involved is a sufficient index of the anti-arthritic activity of the extracts.

As in the case of the carrageenan-induced edema test, the treated animals in the mycobacterial adjuvant test were also carefully observed for possible toxic symptoms, especially since they were treated with fairly high doses for a period of 20 days. Apart from the lack of toxic signs, the non-toxicity of the extracts is reflected also by the comparable weight gain rates between the treated and the arthritic control groups. Furthermore, the fact that when the treatment began on the same day as the injection of the mycobacterial adjuvant, no inhibition either of the paw edema or joint inflammation was seen, further supports our contention that the inhibitory activity was not due to a non-specific toxic effect. The data obtained do not form sufficient groundwork for speculation into its mode of action against the adjuvant arthritis, since the pathology of the induced arthritis itself remains to be elucidated.

CHAPTER IV

GENERAL DISCUSSION

The results of the present studies not only confirm previous observations showing that the extracts of the Hungarian oak galls exert antihistamine-like activity (Kovacs and Szabadi, 1950; Kovacs et al., 1952; Feldberg and Kovacs, 1960; Broome et al., 1962; Calam, 1966; Chu, 1969), but it also reports a method for the isolation of the active principle responsible for the antihistamine-like activity of these oak gall extracts.

In the past 20 years, several methods were reported for the preparation of the crude extracts from different species of galls. Kovacs and Szabadi (1950) reported that an extract with antihistamine-like activity was obtained when Hungarian oak gall powder was extracted with ethanol. In the following year, it was found that similar extracts, prepared from galls of rose, poplar and willow also exerted an antihistamine-like activity (Kovacs et al., 1951). In 1960, Feldberg and Kovacs confirmed the activity of the oak gall extract, but also noted that the potency of different batches of the extracts varied widely, especially when old, stored galls were used. They attributed this variability in activity to the entry of air through fine holes produced by the escaping wasps, since it was presumed that oxygen inactivates the antihistamine-like principle.

According to Berry et al. (1962), the crude alcoholic extracts of the oak galls not only protected the animals from the effects of histamine but also from that of serotonin (5-HT). In the same year, Broome et al. (1962) obtained a stable extract of the Hungarian oak galls, using chloroform:methanol (2:1, v/v) mixture for extraction. Calam (1966), who confirmed the antihistamine-like activity of the oak gall extract prepared either by ethanol or by the chloroform: methanol method, also reported that the guinea pigs receiving the oak gall extract showed toxic symptoms and the toxicity of the extract appeared to vary according to the extracting solvents. He thus concluded that the observed antihistamine-like activity of the Hungarian oak gall extract was probably a non-specific effect, produced by the interactions between a toxic substance(s) (probably tannin or other polyphenolic compounds) in the extract and the animal tissues. Moreover, he suggested that the protective effect of the oak gall extract against histamine and 5-HT, as reported by Berry et al. (1962), further supported his assumption of a non-specific antihistamine-like activity. However, he did not make any attempt to explain the finding of the same authors, who showed that the extract was ineffective against acetylcholine induced bronchoconstriction (Berry et al., 1962). Furthermore, Calam did not take into account previous experimental findings that the tannin-free extract retained its antihistamine-like activity with an increased specific activity and a concommitant decrease in toxicity (Kovacs et al., 1952; Feldberg and Kovacs, 1960; Broome et al., 1962). In

1969, Chu reported a method for the preparation of a highly purified tannin-free extract of the Hungarian oak gall which not only exerted potent antihistamine-like activity but also showed a marked lack of toxicity.

Although Chu's (1969) report proved that the observed antihistamine-like activity of the Hungarian oak gall extract was not attributable to the toxic effects of tannins, it was still remotely possible that the activity could be due to non-specific activity. It was thus important for us to resolve the question of the specificity of the observed antihistamine-like activity of the Hungarian oak gall through the isolation of the active principle. In order to isolate the active principle, it was logical and important to first find a method by which a stable and active crude extract could regularly be obtained. Our results showed that the method of Broome et al. (1962), subjected to slight modifications by Chu (1969), was highly reproduceable.

The toxic effects of tannic acid present in the crude extract of the oak galls were recognized by early workers (Kovacs and Szabadi, 1950). Three methods have been developed to obtain tannin-free extract of the oak galls. In 1952, Kovacs et al. removed the tannic acid by precipitation with lead hydroxide and the antihistamine-like activity of the tannin-free extract was confirmed by Feldberg and Kovacs (1960). Broome et al. (1962) worked out a method to ob-

tain a stable tannin-free extract of the oak galls by re-extracting the dry chloroform:methanol residue with chloroform. The tannin-free extract so obtained retained its antihistamine-like activity. In 1969, Chu reported a highly reproduceable method by which a very stable and highly active tannin-free extract of the Hungarian oak galls was obtained. He re-extracted the dry chloroform:methanol residue with ethyl acetate and then subjected the ethyl acetate soluble fraction to silica gel G column chromatography. The effluent, containing the active principle, was completely free of tannic acid. Although the first two methods (Kovacs et al., 1952; Broome et al., 1962) yielded tannin-free extracts, they were, however, found to be labile, making further purification almost impossible. The method reported by Chu (1969) was not only highly reproduceable, but the resulting tannin-free extract was also very stable. For these reasons, the method developed by Chu (1969) was adopted as part of the initial purification procedure.

According to Chu (1969), when the methanolic solution of the tannin-free extract was applied onto a sephadex LH-20 column, the active principle was eluted as two non-consecutive fractions (fraction I and fraction IV). However, we showed that if the tannin-free extract was first extracted with methanol:water:ethyl acetate (4:2:1, v/v), and then the methanolic solution of the methanol:water:ethyl acetate soluble fraction applied onto the sephadex LH-20 column, the active principle was eluted into fractions I, II, III. Although we failed

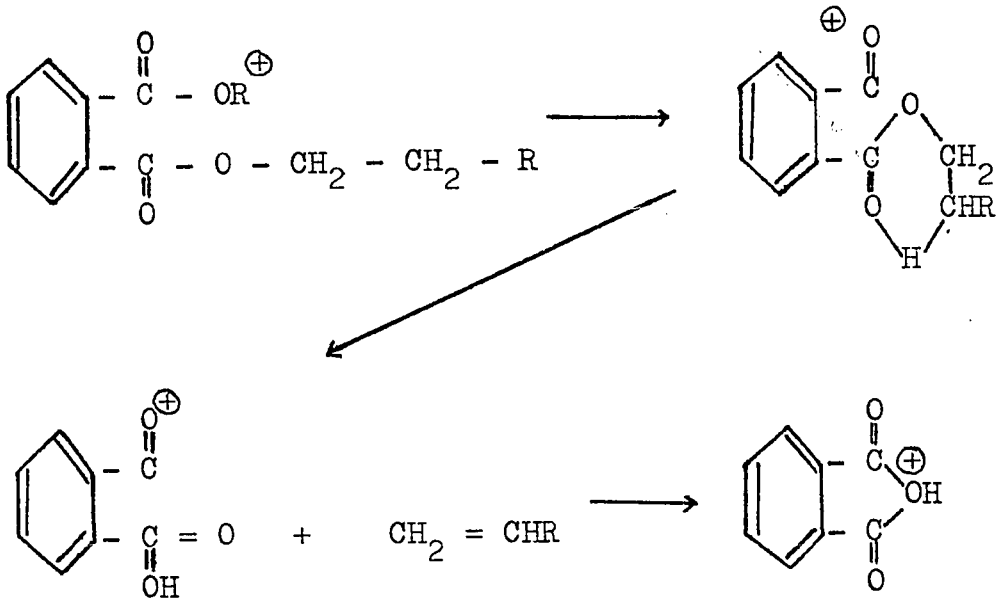
to elute the active principle into a single fraction (as defined by the optical density), the chromatographic pattern was very constant and highly reproduceable. Furthermore, the yield represented a significant increase in the purity of the active fraction.

Sephadex LH-20 was used again for the step-wise column chromatographic elution of the active fraction because it is milder than alumina or silica gel and it gave a better separation than cellex N-1 cellulose. Silica gel G, on the other hand was preferred over the others for the elution of KC-18 from the petroleum ether:ethyl acetate soluble fraction since it gave, by far, the best separation.

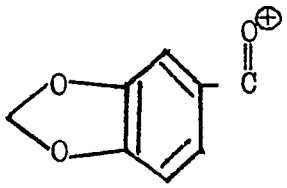
Our attempts to recrystallize KC-18 obtained from the silica gel G column failed, primarily because of its semi-solid nature and the extremely low yield of the substance obtained from individual batches. Admittedly, one could presumably collect a sufficient amount of KC-18 by pooling the yields from several experiments, however, we noted that it lost its biological activity within a few days, even when kept refrigerated at -15° C. The loss of activity, indicating structural changes, would defeat the purpose of pooling KC-18. Furthermore, owing to the semi-solid nature of the substance, no melting point determination was performed.

According to the mass spectral analysis, the most prominent fragment of KC-18 is the peak with a mass of 149. Interpretation of mass 149

favours it as a fragment rather than the molecular ion itself since mass 149 is characteristic for all esters of phthalic acids higher than the dimethylester (Silverstein and Bassler, 1963), piperonal and the esters of piperonylic acid (Budjikiewiez et al., 1967). While mass 149 is formed from the esters of phthalic acid (higher than the dimethylester) by the following sequence:



mass 149 derived from piperonal or the ester of piperonylic acid is formed by the cleavage α to the carbonyl to give

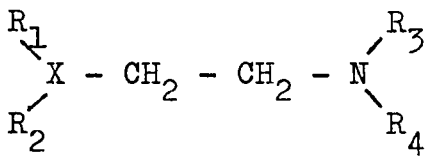


it is unlikely that the mass 149 is a derivative of phthalic acid since the IR spectrum did not indicate the presence of two carbonyl

ions. Furthermore, the NMR spectrum showed the presence of a methylenedioxy moiety.

Using the assumption that KC-18 might be an ester of piperonylic acid as a working hypothesis, we carried out the base hydrolysis of KC-18. The resulting hydrolysate was further analysed by the mass spectrometer, thin layer chromatography and electrophoresis. An authentic sample of piperonylic acid was used in all cases as reference sample. The results obtained on the basis of these three parameters showed that the hydrolysate is identical to the authentic sample of piperonylic acid. We, therefore, concluded with some confidence that the acid component on the hydrolysis of KC-18 is piperonylic and, consequently, KC-18 itself is most probably an ester of piperonylic acid. The structure of the alcoholic moiety remains to be elucidated.

The relationship between the chemical structure and antihistaminic activity of the synthetic antihistamines are highly complex. Nevertheless, most antihistamines have the following structural formula:



This core, a substituted ethylamine, is also present in histamine.

However, comparisons of the physical properties (solubility, ionization constants, and surface tension) of 16 established antihistamines with their antihistaminic, local anaesthetic, irritant and toxic effects failed to reveal any direct correlation between their physical and pharmacological properties (Roth and Tabachnick, 1965). Thus,it is not surprising that KC-18, having a core other than the substituted ethylamine, has shown antihistamine-like activity - at least in animal experiments.

The results of the present studies clearly showed that KC-18, isolated from the oak galls, when tested by a number of bioassay methods, e.g., histamine aerosol test, capillary permeability test, Herxheimer's micro-anaphylactic shock technique, histamine-induced hypotension and histamine-induced gastric acid secretion, can antagonize the actions of histamine. The results also indicated that the profile of activity of KC-18 differs from that of the conventional antihistamines. Apart from confirming previous reports that the extracts of the Hungarian oak galls exerted an antihistamine-like activity (Kovacs and Szabadi, 1950; Kovacs et al., 1952; Feldberg and Kovacs, 1960; Broome et al., 1962; Calam, 1966; Chu, 1969), the results also conclusively proved that the antihistamine-like activity is not attributable to some non-specific toxic effect but may be ascribed to a single substance (KC-18) which most probably specifically antagonizes the different actions of histamine.

A number of investigators have shown that synthetic antihistamines possess anti-anaphylactic activity. For example, Mota and Dias Da Silva (1960) were able to demonstrate the anti-anaphylactic activity of synthetic antihistamines in in vitro experiments in terms of the percent inhibition of mast cell disruption. But due to the very high doses used, the protective effect could be non-specific. Most antihistamines showed anti-anaphylactic activity when evaluated by the Herxheimer's microshock technique (Armitage et al., 1952; Herxheimer and Stresemann, 1963). Here again, the dose required for significant protection is at least 10 folds higher than that required to antagonize the effects of histamine induced bronchoconstriction. Thus, the enormous dose difference, i.e., dose blocking effects of histamine : dose inhibiting the development of anaphylactic reaction, of synthetic antihistamines make it doubtful if the synthetic antihistamines can be classed as having any specific anti-anaphylactic property at the therapeutic dose levels. KC-18, on the other hand, could be classed as both antihistaminic and anti-anaphylactic agent on the basis of its equipotency against the effects of histamine and the micro-anaphylactic shock.

In spite of the potent antihistaminic activity exerted by KC-18 in in vivo tests, it completely failed to inhibit the histamine-induced contraction of the isolated guinea pig ileum, even when tested in very high doses. On the other hand, the potent inhibitory effect of the conventional antihistamines in this in vitro test is one of the

most common and universal pharmacological action of this group of compounds. However, the lack of an in vitro effect does not disqualify KC-18 as an antihistamine - it merely reflects a difference in biological actions as a consequence of the difference in structure between KC-18 and the synthetic antihistamines. In fact, several clinically effective histamine antagonists, e.g., the corticosteroids and disodium cromoglycate, do not exert either in vivo or in vitro antihistaminic activity. Thus, while drugs such as corticosteroids and disodium cromoglycate cannot be classified as antihistamines, their effectiveness in the symptomatic treatment of allergic diseases indicates that agents whose profile of activity in animal experiments does not parallel those of the synthetic antihistamines, can still be very important in clinical practice.

While almost all synthetic antihistamines possess, to a varying extent, central nervous system depressant activity at therapeutic doses, the administration of KC-18 to animals did not cause any depression of the central nervous system. It could be argued that the lack of central nervous depressant activity may be due to the inability of the compound to penetrate the blood-brain barrier. However, its lipid-like property makes it very unlikely that the lack of a central nervous action is due to the blood-brain barrier.

It is generally recognized that synthetic antihistamines are totally inactive in antagonizing the histamine-induced gastric acid secre-

tion (Paton and Schachter, 1951; Kay, 1953; Ragins et al., 1958; Kouhout et al., 1960; Kahlson et al., 1968). Although there have been claims that synthetic antihistamines inhibited histamine-induced gastric acid secretion (Wood, 1950; Kay and Forrest, 1956; Janowitz and Hollander, 1957), these observations have been attributed by others to mucosal injury due to the topical application of high concentrations of antihistamines (Ragins et al., 1958; Blair and Forrest, 1960). According to Ash and Schild (1966), the ineffectiveness of synthetic antihistamines in antagonizing histamine-induced gastric acid secretion can simply be explained by the difference in the types of histamine receptors found in the smooth muscle on the one hand and the stomach on the other.

A further important departure of KC-18 from the activity profile of the synthetic antihistamines is the fact that besides antagonizing, <u>in vivo</u>, the effects of histamine on the smooth muscle receptors (H_1 histamine receptors) it can also block the action of histamine on the gastric H_2 histamine receptors. The inhibition of the histamine-induced gastric acid hypersecretion cannot be attributed to toxic effects since , after repeated histamine administration, the response of the preparation always returned to almost the normal control levels - indicating a gradual loss of the inhibitory effect.

Apart from the fact that the antihistaminic activity of KC-18 is

not attributable to non-specific toxic reactions, the mode of its action is open to speculations. On the assumption that the lack of *in vitro* activity of KC-18 against histamine-induced contraction of the isolated guinea pig ileum is not a question of solubility, it could be interpreted as that KC-18 itself does not act as a direct antagonist of histamine on the smooth muscle. Therefore, the observed *in vivo* activity could be ascribed to an active metabolite acting perhaps competitively with histamine at the receptor sites. Admittedly, the fact that KC-18 is capable of inhibiting the actions of histamine on the smooth muscle and on gastric acid secretory glands makes it difficult to explain the action of KC-18 by the theory of competitive antagonism. However, while the optimal protective action of KC-18 against the action of histamine on smooth muscles e.g., histamine-induced bronchoconstriction can be seen only five hours after its parenteral administration, the activity of KC-18 directed against histamine-induced gastric acid hypersecretion reaches optimal activity in two hours after its intravenous administration. Thus, it is possible that the inhibitory activity of KC-18 against histamine-induced smooth muscle contraction is due to an active metabolite while the antagonistic action against histamine-induced gastric acid secretion is due to KC-18 itself. Owing to limited data the mode of action of the anti-anaphylactic activity of KC-18 remains highly speculative. However, Chu (1969) has shown that the activity is not directed against the antigen-antibody reaction since there was no activity against an increase in capillary

permeability induced by passive cutaneous anaphylactic reaction. The multiplicity of the antihistaminic activity of KC-18, i.e., the antagonism of the effects of histamine in vivo in the smooth muscles, histamine-induced gastric acid secretion and anaphylaxis, emphasizes the basic structural differences between that of KC-18 and those of the conventional antihistamines. Furthermore, when the above discussed pharmacological effects of KC-18 are considered together with its lack of toxic or central nervous system depressant effects, the possibility of its usefulness as a therapeutic agent in the treatment of allergic diseases can seriously be considered.

As the general screening test revealed, the Hungarian oak gall extracts also contain, besides the antihistaminic principle, a substance(s) which exerts potent anti-inflammatory activity. The active principle(s), located in fraction VI of the standard crude extract, was further purified by using stepwise elution in the silica gel G column. Although rather large effluent fractions were collected from the silica gel G column, it nevertheless yielded a highly purified active fraction.

Even though direct comparison cannot be made between the chemical structure of KC-18 and that of the anti-inflammatory principle(s), the data obtained from our studies indicate that they are most probably chemically different. For example, fractions containing the antihistamine and the anti-inflammatory principle(s) were eluted

from different ends of the sephadex LH-20 chromatogram. More importantly, the anti-inflammatory fraction did not exert any anti-histaminic activity and neither did KC-18 exert any anti-inflammatory activity.

Although only two bioassay techniques were used for the evaluation of the anti-inflammatory activity of the purified oak gall extracts they are, nevertheless, considered to be the most commonly used and the most reliable methods (Green et al., 1971). Besides, they are more specific than most other frequently used tests, e.g., dextran-induced or egg white induced edema (Green et al., 1971). Of greater significance, most investigators found that the activity of the anti-inflammatory agents in these two techniques (carrageenan-induced paw edema and the mycobacterial adjuvant induced polyarthritis) correlate well with their clinical anti-inflammatory activity (Glenn, 1964; Garanttini et al., 1965; Kampmann and Frey, 1966; Piliero et al., 1966; Currey and Ziff, 1968; Piliero et al., 1970).

The results obtained with the carrageenan edema test showed that the purified oak gall extracts exerted a potent dose-related inhibitory activity. In view of the unusually high degree of anti-inflammatory activity, the test animals were carefully examined and found to be free from toxic signs and other non-specific factors which may contribute to the inhibitory activity. Thus, the observed activity is most probably the result of a substance(s) present in the oak gall extracts.

Furthermore, a dose-dependent inhibitory activity of the oak gall extracts was seen against both edema and joint swelling in the polyarthritis rat model as well. Since the weight gaining rate of the treated animals was comparable to that of the arthritic controls, we concluded that the suppression of the mycobacterial adjuvant induced polyarthritis in rats cannot be ascribed to non-specific toxic reactions. In addition, the fact that the activity is not only dose-dependent but also dependent on the time of administration in relation to the induction of arthritis, further negates the possibility of non-specific activity.

The anti-inflammatory activity of the purified oak gall extract, as evaluated by the carrageenan edema test, showed that it is very potent compared to established clinical anti-inflammatory agents. For example, phenylbutazone, indomethacin and aspirin injected intraperitoneally at the following doses: 120 mg/kg, 4.5 mg/kg and 240 mg/kg brought about inhibitions varying from 50 - 80 % (Green et al., 1971). The oak gall extracts, on the other hand, consistently showed more than 90 % inhibition when administered in a dose of 8.6 mg/kg. However, the efficacy of the oak gall extract in the polyarthritis rat model was found to be considerably less than that of phenylbutazone, flufenamic acid and mefenamic acid since the inhibitory effect exerted by the oak gall extracts was completely lost six days after the treatment ended while that of phenylbutazone, flufenamic and mefenamic acids was found to be still present 75 days

after the treatment had stopped (Winder et al., 1969). During the treatment period, the anti-inflammatory activity of 12.8 mg/kg of oak gall extract is comparable to that obtained with 1 mg/kg indomethacin, 30 mg/kg phenylbutazone and 70 mg/kg aspirin (Ward and Cloud, 1966).

At present, very little can be deduced from the data obtained about the possible mode of action of the anti-inflammatory principle(s) present in the oak gall extracts, apart from the fact that this effect cannot be attributed to some non-specific actions. According to Crunkhorn and Meacock (1971), bradykinin and 5-HT play an important role in the development of carrageenan-induced paw edema in the rat. However, previous studies by Chu (1969) showed that the oak gall extracts do not antagonize the pharmacological actions of bradykinin. It is thus possible, although this hypothesis was not investigated in the present studies, that the purified oak extracts inhibit the action of 5-HT, which is especially important in the development of paw edema in rats. This possibility finds some support from the work of Berry et al. (1962) who reported that the oak gall extracts can antagonize the bronchoconstriction induced by 5-HT. Another possible explanation is - based on the findings that there is a three hours delay in the onset of action of the extracts - that the anti-inflammatory effect was not exerted by the active principle(s) in the extracts but by a metabolite(s).

SUMMARY

1. The previously reported antihistamine-like activity of partially purified oak gall extracts has been confirmed.

2. The partially purified extracts were further purified by ethyl acetate extraction. The ethyl acetate soluble fraction was then applied onto a silica gel G column and a tannin-free fraction, exerting an antihistamine-like activity, was obtained.

3. The tannin-free fraction was applied onto a sephadex LH-20 column and the effluents were divided into 6 fractions according to the optical density at 280 mu. The results of the bioassays showed that fractions I, II and III exerted antihistamine-like activity while fraction VI exerted an anti-inflammatory activity.

4. The fractions exerting antihistamine-like activity were applied onto another sephadex LH-20 column and the effluents were divided into 13 fractions according to the optical density at 280 mu. The results of the bioassays showed that only fraction XIII exerted an antihistamine-like activity.

5. This active fraction was extracted with petroleum ether:ethyl acetate and the soluble fraction was applied onto a silica gel G column. The effluents were divided into 5 fractions according

to the optical density at 280 mu. The results of the bioassays showed that only one fraction (fraction I) exerted antihistamine-like activity.

6. This active fraction was analysed by thin layer chromatography in three solvent systems and found to contain one substance.

7. This chemically pure antihistamine-like substance or KC-18 is a semi-solid and colourless substance with a slight pungent odour. It is very soluble in lipid solvents from chloroform to hexane and insoluble in water.

8. Base hydrolysis of KC-18 and the application of the acid component into mass spectrometer, TLC and cellulose acetate electrophoresis showed that it is identical with an authentic sample of piperonylic acid.

9. Further studies on the structure of KC-18, performed with IR spectrophotometer, UV spectrophotometer, NMR spectrometer and mass spectrometer, showed that it has a molecular weight of 279 and that it is probably an ester of piperonylic acid.

10. The bronchoconstrictive effect of histamine was significantly inhibited in a dose-related manner in guinea pigs treated intraperitoneally with 4 mg/kg and 6 mg/kg five hours prior to the

exposure to a 0.15 % histamine aerosol.

11. KC-18 administered intraperitoneally in doses of 2.5 mg/kg, 4 mg/kg and 6 mg/kg into guinea pigs, exerted a significant and dose-related inhibition of the increase in capillary permeability induced by histamine, intradermally injected, four hours after the administration of KC-18.

12. The development of anaphylactic shock in actively sensitized guinea pigs was prevented by the intraperitoneal administration of 4 mg/kg KC-18 five hours prior to the antigenic challenge.

13. The hypotensive effect of intravenously administered histamine (0.1 ug/kg, 0.5 ug/kg and 1.0 ug/kg) was significantly inhibited in a dose-related manner in anaesthetized cats, which received 8 mg/kg and 16 mg/kg of KC-18 intraperitoneally five hours prior to the histamine injections.

14. KC-18 administered intravenously to anaesthetized rats in doses of 72 mg/kg and 120 mg/kg significantly inhibited, in a dose-related manner, the gastric acid stimulatory effect of intravenously administered histamine in the perfused rat stomach preparation (Ghosh and Schild, 1958).

15. The histamine-induced contraction of the isolated guinea pig

ileum was not modified by KC-18 when added to the preparation in doses as high as 100 ug/ml.

16. Fraction VI of the standard crude extract, exerting an anti-inflammatory activity, was further purified by applying it onto a silica gel G column. The purified fraction I, administered intraperitoneally in doses of 4.3 mg/kg and 8.5 mg/kg four hours prior to the subcutaneous injection of 0.05 ml of carrageenan, exerted a significant and dose-related inhibition against the carrageenan-induced paw edema in male Wistar rats.

17. KC-18, given intraperitoneally daily to male Lewis rats in doses of 8.5 mg/kg and 12.8 mg/kg for 20 days starting with the treatment three days prior to the single subcutaneous injection of 0.1 ml of the mycobacterial adjuvant into the plantar surface of the left hind paws, brought about a dose-related protection against the development of paw edema and joint swelling.

CLAIMS TO ORIGINALITY

The antihistamine-like activity of the Hungarian oak gall extracts has previously been established.

The work presented in this thesis on the isolation, identification and pharmacology of the antihistamine-like principle extended the research into areas which have not previously been investigated. Furthermore, this thesis also reports the presence of a potent anti-inflammatory principle(s) in the oak gall extract, an observation which has not been previously reported. The present studies established:

1) a method for the isolation of an antihistamine-like principle (KC-18) from the Hungarian oak gall extracts;
2) that this pure antihistamine-like substance (KC-18) is most probably an ester of piperonylic acid;
3) that when KC-18 is injected into experimental animals (guinea pigs and cats), it antagonizes the bronchoconstricting and hypotensive actions of histamine and it also inhibits the increase in capillary permeability induced by histamine in a dose-related manner;
4) that KC-18 is capable of protecting guinea pigs against the development of an anaphylactic shock, but it does not inhibit the histamine-induced contraction of the isolated

guinea pig ileum;

5) that KC-18, unlike any known synthetic antihistamine, can antagonize the histamine-induced gastric acid hypersecretion;

6) a method to obtain purified oak gall extracts which exert a potent anti-inflammatory activity when tested against the carrageenan-induced paw edema in rats;

7) that the severity of the arthritis brought about by the injection of mycobacterial adjuvant can be significantly reduced in rats by the daily injection of the purified oak gall extracts.

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