MSc. Experimental Medicine

David Chu

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The antihistamine-like activity of the purified oak gall extract was compared to that of tomatine and gomatine in the guinea pigs. All three substances significantly inhibited histamine-induced bronchoconstriction. Tomatine and gomatine significantly reduced the effects of both histamine and bradykinin, but the purified oak gall extract only antagonized the histaminic effects. Gomatine and the purified oak gall extract but not tomatine, exerted anti-anaphylactic activity. None of the three substances inhibited the PCA reaction.

CHU DAVID CHEE CHON

SHORT TITLE: PURIFICATION OF ANTIHISTAMINE(S)

THE PURIFICATION OF AN ANTIHISTAMINE-LIKE PRINCIPLE(S) OF THE HUNGARIAN OAK GALL AND ITS EFFICACY COMPARED TO TOMATINE AND GOMATINE.

-by-

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THESIS

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TABLE OF CONTENTS

PART I. HISTORICAL REVIEW

Section A. Anaphylaxis and Allergy.

The systematic investigation of the phenomenon of anaphylaxis began with the fundamental observations of Richet (1898) and Richet and Portier (1902). They studied the effects of diverse substances, e.g., eel serum and extracts of actinaria, on experimental animals. In the course of these experiments, they observed that when the eel serum or the extracts of actinaria was given intravenously to dogs, it induced some toxic symptoms. However, if these dogs, which had received one injection previously, were treated with the same extracts or serum, even with a much smaller dose, several weeks later, this second injection invariably resulted in a sudden death of the animals. Richet called this unexpected reaction to the second injection "anaphylaxis".

Rosenau and Anderson (1906), Gay and Southard (1908), and Auer and Lewis (1910) worked out many fundamental features of anaphylaxis using guinea pigs as experimental animals. They noted that the lungs of the guinea pig, which succumbed to the anaphylactic shock, were fully distended - even after they were excised from the thoracic cavity. They concluded that in the guinea pig, the "immobilization of the lungs" was the most characteristic sign of immediate anaphylaxis and that the death of the animal was brought about by the tetanic contraction of the bronchial smooth muscles. In 1906, von Pirquet (1906) recognized that the introduction of foreign substances into human beings may alter their reaction to subsequent applications of the same material. This altered reaction of human beings was named by him "allergie".

However, it was Meltzer (1910) who first noted that the pathological alterations in the lungs of the guinea pigs and the lungs of patients dying of asthma were very similar.

These early observations stimulated great interest, and research on anaphylaxis soon extended into many aspects of the problem. Numerous theories have been put forward to explain this phenomenon and the two most widely accepted were the "humoral" theory and the "cellular" theory of anaphylaxis.

The concept of the "humoral" theory of anaphylaxis was put forth by Friedberger (1910). He observed that the incubation of the normal guinea pig serum with the washed, preformed immune precipitate resulted in the formation of a toxic substance in the serum, since the injection of the supernatant into the guinea pigs evoked a typical anaphylactic shock. Friedberger called this toxic substance "anaphylatoxin". On the basis of these findings, he thought that anaphylactic shock develops because the antigen-antibody complex activates certain proteolytic enzymes in the guinea pig serum which brings about the formation of anaphylatoxin. In the ensuing years, it was found that besides the antigen-antibody complexes, a great many substances, e.g., kaolin, agar, dextran, etc., were also capable of producing anaphylatoxin when incubated with normal guinea pig serum (Keysser and Wasserman, 1911; Bordet, 1913). There was no generally accepted theory to explain the mechanism of anaphylatoxin formation by these inert substances. However, most investigators agreed that an enzymatic process - most probably proteolysis - was involved in the formation of anaphylatoxin. Thus a modified form of the

"humoral" theory was put forward, stating that in the presence of immune precipitate or certain other substances, non-specific proteolytic enzymes are mobilized to cause the cleavage of the normal serum protein to produce anaphylatoxin (Jobling et al., 1915; Bronfenbrenner, 1915). The most important aspect of this theory is the assumption that serum factors are indispensable for the formation of anaphylatoxin and, consequently, anaphylaxis. However, the "humoral" theory was silenced for 30 years when Dale and Kellaway (1922) demonstrated that while anaphylatoxin could induce only a weak and irregular contraction of the isolated guinea pig uterus, the addition of the specific antigen elicited a strong contraction of the sensitized guinea pig uterus. 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 observation was confirmed by Rocha e Silva (1951) who found that anaphylatoxin released a large quantity of histamine from the isolated perfused lungs of the guinea pig. In 1961, Giertz et al. (1961) were able to demonstrate that the increase of plasma histamine level in the guinea pig was directly proportional to the dose of the anaphylatoxin administered, i.e., the higher the dose of anaphylatoxin, the higher is the plasma histamine level and consequently, the higher the mortality rate. The implication that the liberated histamine played a major role in guinea pig death induced by anaphylatoxin was further confirmed by Schmutzler et al. (1963) when they observed that in both anaphylactic and anaphylatoxin shock, similar death rate occurred with equally high plasma histamine concentration. According to the recent work of Lepow et al. (1968), anaphylatoxin is the cleavage product of the complement system, i.e., the C'3 and C'5 components.

At present the general consensus is that anaphylatoxin can be classified as a potent histamine liberator.

The "cellular" theory evolved from the study of the anaphylactic response on the plain muscles of guinea pig by Schultz (1910) and Dale (1912). Dale reported that the isolated uterus of sensitized guinea pigs, freed from all traces of blood and serum, strongly contracted after the addition of the specific antigen. In contrast to the "humoral" theory, the "cellular" theory postulated by Dale, stated that the most important basic mechanism in anaphylaxis is the combination of the antigen with the cell-bound antibody which, through the release of "histamine-like substances", leads to anaphylactic symptoms. The release of histamine-like substances in anaphylaxis proposed by Dale was based on the observation (Dale and Laidlaw, 1910) that the symptoms of anaphylactic shock and those produced by parenterally administered histamine were strikingly similar.

In the subsequent years, the "cellular" theory of anaphylaxis gained widespread acceptance as evidence accumulated to show that histamine is present in normal mammalian tissues (Best et al., 1927; Dale and Dudley, 1929) and that histamine is liberated in anaphylaxis (Gebauer-Fuelnegg and Dragstedt, 1932; Bartosch et al., 1932). Although it was firmly established that the release of histamine was intimately associated with anaphylactic and allergic symptoms, it was soon discovered that the "cellular" theory, which implied that histamine alone was responsible for the anaphylactic symptoms, as originally proposed by Dale, was not entirely correct. For example, it was found that besides histamine, other mediators 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. Further convincing evidence was presented by Schild (1951), who used the isolated human bronchial muscle preparation obtained from an asthmatic patient. He 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 the antihistamine (10^{-5} gm/ml). In the rat, antihistamines (Brocklehurst, 1955, 1960) or histamine depletion with compound 48/80 (Rocha e Silva, 1955) do not modify the cutaneous anaphylactic reaction.

It is now an established fact that not only histamine but several other chemical mediators as well (5-HT, bradykinin, SRS-A) are released in anaphylaxis and allergy. However, histamine is still one of the most important mediators of the anaphylactic and allergic manifestations in most mammalian species. Thus, in the next section, the possible role of histamine and other chemical mediators in anaphylaxis and allergy will be briefly considered.

Section B. Chemical Mediators In Anaphylaxis and Allergy

a. Histamine

The importance of histamine in allergic manifestations was first indicated

by Dale and Laidlaw (1910), who were able to mimic most of the symptoms seen in anaphylactic shock by administering histamine into guinea pigs.

Lewis and Grant (1924) and Hare 1926) were able to extract a "histamine-like principle" from urticarial wheals which elicited contraction of the isolated guinea pig uterus. Lewis (1927) found that the urticarial wheals formed on the skin of the allergic individual following the intradermal injection of a specific allergen were strikingly similar to those seen after the intradermal injection of histamine. Best et al. (1927) demonstrated the presence of histamine in normal tissues (spleen and liver), and their findings were confirmed by Harris (1927) and Thorpe (1928). However, the first and conclusive evidence which proved that histamine is indeed liberated in anaphylaxis was presented by Gebauer-Fuelnegg and Dragstedt (1932). They found that the histamine level was significantly increased during anaphylactic shock in both the blood of the inferior vena cava and the lymph of the thoracic lymph duct of the dog. Further confirmation of these findings came from Bartosch et al. (1932), when they found histamine in the perfusate of the anaphylactic guinea pig lungs and identified it as the substance responsible for the bronchoconstriction manifested in the guinea pig anaphylaxis.

In the 20 years following the important publications of Gebauer-Fuelnegg and Dragstedt (1932) and Bartosch et al. (1932), the role of histamine in anaphylaxis was studied in many mammalian species. Existing data indicate that histamine is probably the most important mediator of anaphylaxis in the guinea pig (Ungar and Parrot, 1936; Austen and Brocklehurst, 1960). Histamine is also an important mediator of anaphylaxis in the dog (Ojers et

al., 1941; Akcasu and West, 1960) and the rabbit (Auer, 1911; Rose, 1940), but not in the rat and mouse. The sensitivity of man to histamine seems to be somewhere between that of the guinea pig and the mouse. That is, in man during hypersensitivity reactions not only histamine but some other chemical mediators, such as SRS-A, are also released (Schild, 1951). It was shown (Katz and Cohen, 1941) that the blood of an allergic individual incubated with a specific allergen in vitro, resulted in an increased histamine content of the supernatant. This report was confirmed by van Arsdel et al. (1958) and Middleton and Sherman (1961). Histamine was also found to be released into the blister fluid of atopic individuals (Katz, 1942). On the other hand, Rose (1941) reported that, with the exception of urticaria, there was a high variability of histamine content in the blood of allergic patients and, furthermore, the histamine level of the femoral blood was not increased significantly during asthmatic attacks (Rose et al., 1950). These observations were confirmed by Guinchi and Serafini (1944), and Hjort et al. (1950) who failed to find elevated histamine levels in the blood of allergic patients. Lecomte (1956) reported that the injection of a small dose of compound 48/80 into man resulted in a three-fold increase of plasma histamine level accompanied by acute clinical allergic symptoms (pruritus urticaria and angioneurotic edema).

The importance of histamine in anaphylaxis and allergy instigated great interest in the possible elucidation of the mechanism of histamine release brought about by the antigen-antibody reaction. This aspect of investigation was greatly facilitated when Riley and West (1953) firmly established that histamine, in most species, was mainly stored in the mast cells. 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 tissue histamine content. This finding was confirmed and extended by Mota (1957) who observed that when the mast cells were damaged by antigen-antibody reaction in the rat, the content of plasma histamine level was increased. Other investigators showed that, besides the antigen-antibody reaction, the release of histamine from the mast cells could be accomplished by many non-specific substances, e.g., proteolytic enzymes, snake venom, surface active agents, dextran, etc. (Rocha e Silva and Essex, 1942; Haining, 1955, 1956; Schachter, 1952; Halpern and Briot, 1953).

The exact mechanism of histamine release during anaphylactic and allergic manifestations has yet to be elucidated. At present, the two most widely accepted theories were put forward by Mongar and Schild and Uvnas et al., According to Mongar and Schild (1955, 1957 a,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 (1961 a) when they reported that the release of histamine in guinea pigs required the presence of a chymotrypsin-like enzyme. 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 mem-

brane into fatty acid and lysolecithin. Lysolecithin, being strongly lytic, causes cellular damage which leads to the release of histamine.

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

The presence of an unidentified smooth muscle contracting substance in extracts prepared from mammalian tissues was first reported by Erspamer (1940). In 1948, Rapport et al. (1948) succeeded in isolating this active principle and identified it as 5-hydroxytryptamine. It is now known that 5-HT is formed in the body from the amino acid tryptophan by hydroxylation and decarboxylation (Clark et al., 1955), and it is present in most tissues.

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 plasma, which contained the antibody, resulted in the release of histamine and 5-HT from the platelets. This observation initiated a number of investigators to study the possible role of 5-HT in anaphylaxis in several species. Fink and Gardner (1956) and Brocklehurst (1958) using the sensitized guinea pig lung preparation could not detect 5-HT in the perfusate of their perfusion experiments. At present, indications are that 5-HT does not play an important role in guinea pig anaphylaxis. Similarly, it is unlikely that 5-HT contributes towards the anaphylaxis in dogs, since the 5-HT level in the liver did not change during anaphylactic shock nor was the liver capable of releasing 5-HT in vitro when the specific antigen was added to it. (Akcasu and West, 1960). Although an increase in the plasma 5-HT level was seen during anaphylactic shock (Waalkes

and Coburn, 1959), the depletion of 5-HT by reserpine administration did not modify the intensity of anaphylactic shock in the rabbit (Fisher and Lecomte, 1956). It is well established that the rats and mice are relatively insensitive to histamine. It was thus assumed that in these species, 5-HT has the same role that histamine has in other species. This assumption was supported by Benditt et al. (1955) who found that the mast cells of both rats and mice contained not only histamine but also 5-HT in fairly large amounts. However, there is no direct evidence that 5-HT plays an important role in the rat anaphylactic reaction (Austen and Humphrey, 1963). In the mouse, 5-HT appears to be a probable candidate as the major chemical mediator during anaphylactic shock, since serotonin depletion by reserpine and methyl DOPA was capable of suppressing the anaphylactic shock (Fox et al., 1958; Gershon and Ross, 1962). The recent 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.

Thus, at present, the role and possible contribution of 5-HT to anaphylactic symptoms in species other than the mouse, remain uncertain.

c. Slow Reacting Substance of Anaphylaxis (SRS-A)

Feldberg and Kellaway (1938) were first to draw attention to the existence of the slow reacting substance. They reported that when the guinea pig lung was perfused with cobra venom, a substance(s) was released which elicited a slow and prolonged contraction of the isolated guinea pig ileum. In 1940, Kellaway and Trethewie (1940) observed that the same or a similar substance

was released together with histamine from the lung of the guinea pig during anaphylactic shock and they referred to this substance as the "slow reacting substance of anaphylaxis" or SRS-A. The presence of SRS-A was unequivocably confirmed by Brocklehurst (1953) who obtained the characteristic response on the isolated guinea pig ileum, even when he added to the preparation a potent antihistamine prior to the addition of the perfusate of the anaphylactic guinea pig lung. Brocklehurst (1960) reported that while a large quantity of SRS-A was released in the lungs of 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 for the formation of SRS-A. In 1963, Berry et al. (1963) demonstrated that SRS-A given intravenously, induced bronchoconstriction in guinea pigs. Asthmatic patients exposed to SRS-A aerosol, developed a mild and prolonged bronchoconstriction which was not observed in normal healthy subjects (Herxheimer and Streseman, 1963). While both the mechanism of the formation, release and the chemical structure of SRS-A remain to be elucidated, the recent work of Movat et al. (1967) and Orange et al. (1967) indicate that the formation and release of SRS-A are closely associated with the polymorphonuclear leucocytes - at least in the rat and the guinea pig.

d. Kinins

Kinins are a group of biologically active polypeptides which originate mostly from the plasma proteins. Their characteristic pharmacological properties may be briefly summarized 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) were the first to show the presence of a hypotensive substance in the extracts of urine and pancreas. They demonstrated that these extracts, when injected intravenously into dogs, elicited a fall in blood pressure, and they called this active principle "kallikrein". In 1949, Rocha e Silva et al. (1949) observed that the incubation of plasma with trypsin produced a substance (which they named bradykinin), the biological activity of which was similar to that of kallikrein. It was then realized that kallikrein is an enzyme which by itself is not a blood pressure depressive agent but reacts with the plasma globulin to release an active hypotensive polypeptide. Werle (1961) found that if kallikrein was incubated with globulin, an active substance (kallidin) was formed. The molecular configuration of kallidin (Werle, 1961) was found to be almost identical with that of bradykinin (Elliot et al., 1960). Furthermore, comparative studies showed that the biological activity of bradykinin and kallidin were also almost identical (Holdstock et al., 1957; and Pierce and Webster, 1961).

The possible involvement of kinins in anaphylaxis was first indicated by Beraldo (1950) who drew attention to the fact that during anaphylactic shock in the dog, a bradykinin-like substance was released into the blood. According to the findings of Brocklehurst and Lahiri (1962), during anaphylaxis, significant amounts of kinins were detectable in the blood of the guinea pig, rat and rabbit. They also reported that the perfusate of the shocked guinea pig lungs contained no detectable amount of bradykinin but bradykinin was formed when the perfusate was incubated with plasma pseudoglobulin. In other

words, the perfusate contained kinin generating enzymes but not kinin. Diniz and Cavalho (1963) found that the plasma substrate for bradykinin formation was depleted after systemic anaphylaxis in rabbit, and Brocklehurst and Zeitlin (1967) observed that the sudden release of free kinin is accompanied by a sudden fall in kininogen level. Abe et al. (1967) showed that the plasma kinins were significantly increased in most patients with severe bronchial asthma when compared to the normal healthy subjects.

Although conclusive evidence is still lacking, nevertheless, present experimental data indicate that bradykinin plays an important role in anaphylaxis and allergy, at least in certain species.

Section C. Histamine Antagonists

All accumulated evidence supports the generally accepted assumption that histamine and other 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. Physiological antagonists, e.g., catecholamines.

b. Pharmacological antagonists. e.g., synthetic antihistamines, histidine decarboxylase inhibitors and histaminase.

c. Non-specific antagonists, e.g., glucocorticoids.

a. 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 act on different receptors than 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 to develop tachyphylaxis limit their usefulness.

b. Pharmacological Antagonists:

1. The Synthetic Antihistamines

Introduction

In 1933, Bovet and Fourneau (1933) reported first that certain phenolic ethers were able to antagonize some pharmacologic actions of histamine. The most active of the series was the classical compound 929F. However, 929F at therapeutically effective dose levels was found to be too toxic (Bovet and Staub, 1937). The original observations of Bovet and Fourneau initiated extensive research for better and less toxic analogs from which synthetic antihistamines with acceptable therapeutic index soon emerged.

The first synthetic antihistamines of therapeutic importance were antergan (Halpern, 1942) and Neo-antergan (Bovet et al., 1944). In the next five years, a great number of synthetic antihistamines with basically the same actions were synthesized and a few examples may be cited as, phenergan (Halpern, 1947); pyribenzamine (Huttrer et al., 1946); histadyl (Weston, 1947); and chlorothen (Clapp et al., 1947).

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 structure 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 in-

activate 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.

2. Histidine Decarboxylase Inhibitors

The existence of an enzyme (histidine decarboxylase) which is capable of converting histidine to histamine was first reported by Werle (1936). As soon as it was established that histamine is a product of the decarboxylation of histidine, it was logically assumed that histamine formation would be prevented or diminished if the activity of histidine decarboxylase was inhibited.

Schayer et al. (1954) observed that when cortisone was given parenterally into rats, it inhibited histamine formation in vivo by inhibiting the activity of histidine decarboxylase. Although Schayer (1956) demonstrated that prednisolone greatly reduced the histidine decarboxylase activity in the rat lung, Kahlson et al. (1963) showed that steroids in general, were relatively weak histidine decarboxylase inhibitors. More recently, Skidmore and Whitehouse (1966) reported that a number of anti-inflammatory drugs (salicylate, ibufenac and phenylbutazone) inhibited histamine formation by competing with pyridoxal phosphate for the co-enzyme binding site,which is believed to be lysyl-ϵ-amino group on the mammalian histidine decarboxylase. This observation confirmed the earlier report that pyridoxal-5-phosphate was necessary for the activity of histidine decarboxylase (Gale, 1953).

In the past six years, several new histidine decarboxylase inhibitors were described (α-methylhistidine by Robinson and Shepherd, 1961; 4-bromo-3-

hydroxybenzyloxyamine or NSD-1055 by Reid and Shepherd,1963; and the hydrazine compounds by Clark, 1963). In man, Sams (1968) found that NSD-1055 therapy alleviated the symptoms of chronic simple urticaria and urticaria pigmentosa.

3. Histaminase

In 1929, Best (1929) reported that some tissue extracts contained a histaminolytic agent(s) called histaminase. It was subsequently proven that histaminase is an enzyme which inactivates histamine (Kapeller-Adler,1949). Rose et al. (1946) observed that the plasma histaminase level in pregnant women was markedly increased - coinciding with a period of clinical remission from allergic manifestations - and, furthermore, when asthmatic conditions persisted during pregnancy, the plasma histaminase level was consistently lower than normal in pregnancy. These observations were confirmed by Janowitz and Grossman (1949), Kullander (1952), and Clark and Tankel (1954), but they also reported that there was no difference in the sensitivity of pregnant and non-pregnant women to injected histamine. These and other observations led some workers to believe that the elevated plasma histaminase level during pregnancy actually was not associated with an increased inactivation of histamine. On the other hand, Lindberg and Torngvist (1960) observed a higher concentration of ^{14}C-histamine in the blood of pregnant women who received the histaminase inhibitor, aminoguanidine. Furthermore, Lindberg, (1963) demonstrated that the intravenous infusion of ^{14}C-histamine resulted in a lower blood concentration of ^{14}C-histamine in pregnant women when compared to non-pregnant women.

The possibility that histaminase might play an important role in hypersensitivity reactions was indicated when Rose and Leger (1952) reported that during anaphylaxis in rabbits, the blood and tissue histamine was significantly reduced, while the serum histaminase was concurrently elevated. In 1957, Angelakos and Loew (1957) reported that histaminase inhibitors potentiated the effects of histamine in rats and mice. The elevation of blood histaminase during anaphylaxis was confirmed by Code et al. (1961) using white rats and by Logan (1961) using guinea pigs. Furthermore, anaphylatoxin shock also increased the plasma histaminase level (Giertz et al., 1964).

In guinea pigs, the liver appears to be the chief source of histaminase (Dave and Sechdev, 1967) and in rats, Waton (1956) had shown that the intestines were the richest source of histaminase. Recent findings indicate that histaminase might be a general term for several enzymes which have the capacity to inactivate histamine. In 1964, Mondovi (1964) reported that the purified pig kidney diamine oxidase possessed histaminase activity. In the same year, Blaschko and Buffoni (1964) identified the pig plasma histaminase as benzylamine oxidase.

As may be evident from the data presented, the role of histaminase in hypersensitivity reactions remains to be clarified.

c. 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 dramati-

cally to glucocorticoid therapy (Bordley et al., 1949; Randolph et al., 1950; and 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 reaction.

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, Benacerraf and Briot, 1952). Although, Oskov (1949) claimed that ACTH protected guinea pigs from passive anaphylaxis, Leger, Leith and Rose (1948), Feinberg and Malkiel (1953) and Halpern (1956) could not confirm Oskov's finding and concluded that ACTH was not effective in either active and passive anaphylaxis or in histamine shock.

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 using very high doses of steroids for several days. Thus, the dramatic therapeutic effect seen in man within 24 hours after the administration of glucocorticoids remains an unresolved problem. In spite of its initial

dramatic therapeutic effect, there are several inherent disadvantages which make long term therapy with glucocorticoids dangerous.

d. 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 anti-histamine-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.

The chemical structure of the active principle(s) present in extracts of the eosinophils is not as yet known. Vercauteren (1953) suggested that the ob-

served 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.

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 extracts, besides being in-

hibitory 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.

e. Antihistamine-like Activity of Plant Tumour Extracts

1. 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 occuring crown-galls are frequently found on the stem of the tomato plant, tobacco and sun-flower. 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.

2. 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 the purified tannic acid preparation. It seemed possible, therefore, that the antihistaminic 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 amount 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.

3. 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 a 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

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of each other, reported the isolation of a crystalline substance with anti-histamine-like activity from the crown-gall infected tomato stalks. Calam and Callow (1964) identified their isolated crystalline substance as tomatine, while Kavacs et al. (1964) reported that their crystalline substance possessed chemical properties closely resembling tomatine.

4. 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 fungistatic 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. Some physical characteristics of tomatine reported by Ma and Fontaine (1948) were as follows: i) Melting point 263 - 7°C.; ii) Insoluble in aquous medium above pH 10 and soluble at pH 4; iii) Minimal molecular weight was 1050. The hydrolysis of tomatine yielded two molecules of glucose, one molecule of galactose, one molecule of xylose and the aglycone tomatidine (Kuhn et al., 1950 and Ma and Fontaine, 1950). The steriodal nature of the aglycone of tomatine was reported by Sato et al., (1951) and Fontaine et al., (1951). In 1963,

the chemical synthesis of tomatidine was accomplished by Schreiber and Adam (1963).

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 is not toxic orally, except in very high doses. No adverse reactions are seen in rats subjected to a 200 day continued feeding test. Tomatine ointments do not irritate or sensitize the skin of guinea pigs. However, when applied topically onto the rabbit eyes, tissue damage was observed and it was reversed on the cessation of treatment. Tomatine administered subcutaneously resulted in the formation of abscesses in rabbit. 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 in vivo and in vitro.

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 a histamine aerosol. Furthermore, they also reported that the crystalline substance isolated from the crown-gall infected tomato plant not only inhibited histamine in 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".

5. Gomatine: Earlier studies have shown that the extracts of plant tumours (Feldberg and Kovacs, 1960; Berry et al., 1962; Broome et al., 1962) exerted a long lasting protection against a subsequent histamine aerosol. In 1964, Kovacs et al. (1964) isolated a tomatine-like substance from the crown-gall infected tomato plants and they found that it exerted a definite antihistamine-like activity both in vivo and in vitro while commercial tomatine only showed a very weak antihistamine-like effect. On the basis of these observations, Wakkary (1968) succeeded in isolating the active principle from the crown-gall infected tomato plant and named the active principle "gomatine". The preliminary pharmacological studies done on the gomatine by Wakkary (1968) may be summarized as follows:

i) Guinea pigs treated intraperitoneally with gomatine were protected from the lethal effects of histamine aerosol.

ii) Contractions of the isolated guinea pig ileum induced by histamine,

bradykinin, 5-HT, SRS-A and barium chloride were effectively inhibited by gomatine.

iii) In guinea pigs, anaphylactic shock brought about by antigen-antibody reaction, was diminished or inhibited by gomatine at a dose level of 20 mg/kg body weight.

Preliminary physical and chemical characterizations of gomatine showed that it resembles tomatine very closely. At present, there are reasons to believe that the antihistamine-like activity of tomatine, isolated from the crown-gall infected tomato plants, was at least in part attributable to the gomatine contamination of the tomatine preparation.

PART II. GENERAL METHODS

Section A. Preparation of Extracts from the Hungarian Oak Galls

Reagents and Solvents

All reagents and solvents were of analytical reagent grade: Anhydrous methanol (American Chemicals), chloroform (American Chemicals), absolute diethylether (Malinckrodt), anhydrous ethyl acetate (American Chemicals), and nitrogen gas (Canadian Liquid Air Ltd.).

Apparatus

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

a. Crude Extract

The Hungarian oak galls (Fig. 1) were obtained through Agrimpex Hungarian Trading Co., Budapest, Hungary, who collected the galls in the autumn months. The galls were then packed into wooden crates and shipped to Montreal in refrigerated compartments. On arrival, the galls were transported to a commercial cold storage, where they were kept in $-4^{\circ}C$ until used.

Hungarian oak galls identified as those of Andricus quercus-tozae were used. The galls 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 in a grinding mill. The powder was 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 Buchner funnel using Whatman No. 3 filter paper. The clear filtrate was taken to dryness with a bath temperature of not higher than 40°C. The residue was extracted three times with ethyl acetate (50 ml/100 gm oak gall powder) and filtered through a Buchner funnel using a Whatman No. 3 filter paper. The filtrate was thoroughly dried and washed twice with ether absolute (20 ml/100 gm oak gall powder). The ether insoluble residue was taken to dryness.

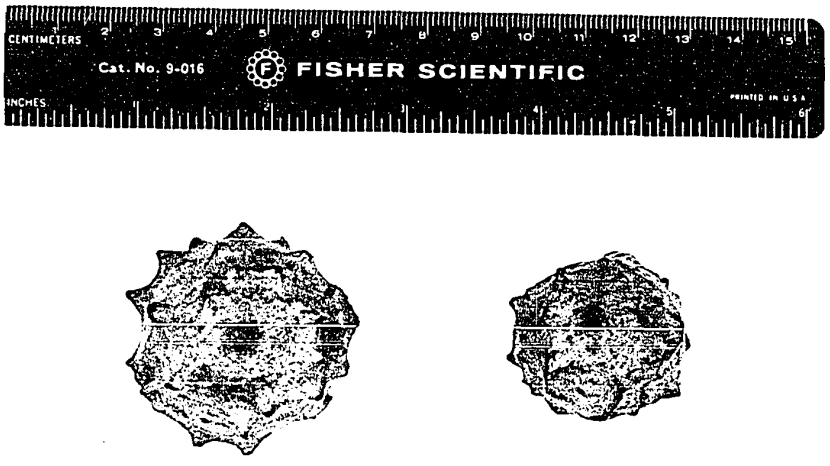


Figure 1

Photograph of Hungarian oak galls (Andricus quercus-tozae).

Section B. Column Chromatography

a. Gel Filtration on Silica Gel G

1. Material

The resin consisted of medium size (max. 10 - 40 μ) silica gel G according to Stahl (E. Merckag, Darmstadt, Germany). A chromatographic column (Scientific Glass Blowing Reg'd, Montreal) of dimensions 4.0 x 60 cm with a ground glass socket joint at the top and a teflon stopcork at the bottom was used throughout all the silica gel G filtration experiements. The filter of the column was made up of glass wool (Corning Glass Works), sea sand (Fisher Scientific) and silicic acid of 200 - 320 mesh (Bio-Rad, California), respectively. The column was connected at the top to a 1 liter capacity separatory funnel which served as reservoir by means of a ground glass ball joint. A saturated solution of ferric chloride was used to detect the presence of tannin.

2. Procedure

Silica gel G was suspended in ethyl acetate. The "fines" were removed by suction and the gel resuspended in the 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 nitrogen pressure (2.5 l/min.). The packed chromatographic column was then allowed to equilibrate over three hours. After equilibration, the eluent was

allowed to run out of the column until its surface coincided with that of the packed silica gel. A saturated solution of the sample to be chromatographed was prepared in the eluent and applied onto the silica gel dropwise, without disturbing the surface of the 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. The effluent was collected in a glass beaker. Small samples were taken at regular intervals for the detection of tannic acid by mixing the samples with the ferric chloride solution. The presence of tannic acid was indicated by the formation of blue colour complex.

b. Sephadex LH-20 Column Chromatography

1. Material and Apparatus

The resin consisted of Sephadex LH-20, of size 25 - 100 μ (Pharmacia, Montreal). Analytical grade anhydrous methyl alcohol (American Chemicals) was employed without further purification.

A chromatographic column of dimension 2.5 x 80 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 2 liter capacity separatory funnel by means of a ground glass ball joint. A Buchler refrigerated fraction collector (Buchler Instruments), placed directly under the column was employed to collect the effluent fractions which were monitored by a DU-spectrophoto-

meter (Beckman).

2. 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 G (Section B, a, 2). The slurry was poured into the column and allowed to pack in the column by gravity. The reservoir containing anhydrous methanol was connected to the column and methanol passed through the resin at a fixed rate (adjusted by the teflon stopcock) until two liters of the effluent were collected. The equilibration was done at 4°C. After the equilibration of the resin, a saturated solution of the sample (prepared in methanol) was applied dropwise without disturbing the surface. The sample volume (15ml) was allowed to diffuse into the gel by gravity and washed-in three times using equal volumes of methanol. The chromatogram was developed with anhydrous methanol and effluent fractions of 10 ml volume were collected directly in the refrigerated fraction collector at 4°C, monitored by the DU-spectrophotometric method (at 280 mμ).

Section C. Monitoring of Column Chromatographic Effluents

a. Ninhydrin Colorimetric Method (Moore and Stein, 1954)

1. Materials.

Reagents and solvents were of analytical reagent grade and were used without further purification: ninhydrin (Fisher Scientific), anhydrous hydrindantin (Brickman and Co.), sodium acetate (Fisher Scientific) and methylcellosolve (Fisher Scientific).

Sodium acetate buffer (4M, pH 5.5) was prepared as follows: 2720 gm $NaOAc.3H_2O$ were added to 2 liters distilled water and the mixture heated in a 100°C water bath until the salt was completely dissolved. The solution was cooled to room temperature. 500 ml glacial acetic acid were added and the volume was made up to 5 liters with distilled water. The final adjustment of the buffer to pH 5.5 was accomplished with sodium hydroxide or acetic acid.

To prepare the ninhydrin reagent, 20 gm ninhydrin and 3 gm hydrindantin were dissolved in 750 ml methylcellosolve (monomethylether of ethyleneglycol). 250 ml of pH 5.5 sodium acetate buffer were added and the resulting solution was immediately transferred to a 1 liter amber glass reservoir. The reservoir, provided with means to permit the storage of the reagent under nitrogen, was, connected to an automatic pipet (Brewer).

2. Procedure

Aliquots of 2% (by volume) of the effluent fractions were transferred to test tubes of the same size and optical transparency and 1 ml of ninhydrin solution was added to each tube by means of the automatic pipet. The tubes were capped, briefly shaken by hand and heated at 100° C for 30 minutes. The tubes were cooled to room temperature and to each tube was added 5 ml of a mixture of ethanol-water (1:1, v/v) using the automatic pipet. The tubes were then thoroughly shaken by a reciprocal shaker for 5 minutes and read in the Coleman Jr. spectrophotometer at 470 mμ. A blank was similarly prepared using the eluting solvent in place of the effluent fractions.

b. Anthrone Colorimetric Method

The method of Morris (1948) for qualitative carbohydrate determination was adapted for monitoring column chromatographic effluent.

1. Materials

Anthrone (Fisher Scientific) and sulphuric acid (Malinckrodt) were of analytical reagent grade and were used without further purification. A 0.1% anthrone solution was prepared by dissolving 1 gm anthrone in 1 liter concentrated sulphuric acid. This solution was used within 24 hours after preparation.

2. Procedure

Aliquots of 2% (by volume) of the effluent fractions were transferred to test

tubes of the same size and optical transparency. 4 ml of the 0.1% anthrone solution were added into each tube. The tubes were shaken by hand, allowed to stand for 30 minutes and read in the Coleman Jr. spectrophotometer at 625 mμ. A blank was similarly prepared using the eluting solvent in place of the effluent fractions.

c. Concentrated Sulphuric Acid Colorimetric Method

The method, described by Metz (1961) for spot detection of thin layer chromatograms, was adapted to monitor column chromatographic effluent.

1. Materials

Sulphuric acid (Malinckrodt) of analytical reagent grade was employed without further purification.

2. Procedure

Aliquots of 2% (by volume) of the effluent fractions were transferred to test tubes of the same size and optical transparency. Into each tube was added 5 ml of concentrated sulphuric acid. The tubes were shaken by hand and read in the Coleman Jr. spectrophotometer at 625 mμ. A blank was similarly prepared using the eluting solvent in place of the effluent fractions.

d. Tryptophan Reaction

1. Materials

Reagents and Solvents were of analytical reagent grade and were used without

further purification: Suphuric acid (Malinckrodt) and Tryptophan (Fisher Scientific).

The concentrated sulphuric acid was diluted to 77% (by volume) with distilled water and this constituted reagent 1. Reagent 2 consisted of a 1% aqueous solution of tryptophan.

2. Procedure

Aliquots of 2% (by volume) of the effluent fractions were transferred to test tubes of the same size and optical transparency and 7.5 ml of reagent 1 were added to each tube by a buret (this procedure was carried out with the tubes containing the effluent fractions placed in a water bath at 10 - 15°C). Then, 1 ml of reagent 2 was added to each tube, shaken by hand and heated for 20 minutes in a water bath at 100°C. The tubes were allowed to cool to room temperature and were read in the Coleman Jr. spectrophotometer at 500 mμ. A blank was similarly prepared with the eluting solvent in place of the effluent fractions.

e. Visible Spectrophotometric Method

For analysis by the visible spectrophotometric method, the tube of the effluent fractions containing the highest optical density was determined by visual estimation in the pigmented region of the effluent fractions. The optical density of this tube of effluent fractions at various wavelengths in the visible region was read in the Coleman Jr. spectrophotometer. The eluting solvent

was used as blank. The wavelength (470 mμ) corresponding to the maximum density was determined from a graph of optical density vs. wavelength. Each of the effluent tubes was read in the Coleman Jr. spectrophotometer at this wavelength.

f. DU - Spectrophotometric Method

To determine the wavelength to be used, the optical density of several tubes of effluent fractions were read at a number of wavelengths not greater than 400 mμ. The eluting solvent was used as the blank. The wavelength (280 mμ) corresponding to the maximum optical density was determined from a graph of optical density vs. wavelength. Each of the effluent tubes was read in the DU-spectrophotometer at this wavelength.

Section D. Thin Layer Chromatography (TLC)

a. Qualitative TLC using Sheet Precoated with Silica Gel (Przybylowicz et al., 1965)

1. Materials and Apparatus

Sheets precoated with silica gel (chromatogram) were purchased from Eastman Kodak Co., NY. The apparatus for developing the chromatograms was obtained from Fisher Scientific, Montreal and consisted of metallic racks, solvent troughs and sandwich type developing chambers.

Anhydrous methanol (American Chemicals) was of analytical reagent grade, and was employed without further purification. Various mixtures of the reagent with water were used as developing solvents.

2. Procedure

A light pencil line was drawn parallel (about 2 cm) to the edge of a precoated sheet (20 x 20 cm). Pencil cross marks were made 2 cm from the edge and 2 cm apart. A methanolic solution of the sample was applied on each mark by means of a micropipet (Lang-Levy) using a hair dryer to evaporate the methanol. 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. The chromatogram was dried

at room temperature. A 47% o-phosphoric acid was sprayed onto the dry and developed chromatogram and heated for 10 minutes at 65° C.

Section E. Preparation of Tomatine and Gomatine from the Crown-Gall Infected Tomato Plants (Wakkary, 1968)

a. Preparation of the Standard Crude Extract

1 kg of the crown-gall infected tomato stalks was cut into small pieces and weighed. The fragments were suspended in sufficient chloroform-ethanol (3:1, v/v) to facilitate grinding in a Waring blendor at top speed for 3 minutes. The mixture was transferred to an Erlenmeyer flask and the volume of the extracting solvent was made up to 300 ml/100 gm tissue. The pulp was thoroughly mixed with the extracting solvent, heated in a 70° C water bath for 5 minutes and filtered through a Buchner funnel with Whatman No. 3 Filter paper. The clear filtrate was dried and taken up in distilled water (15 ml/100 gm tissue). The resulting mixture was transferred to a beaker and adjusted to pH 2 with 12N HCl. The mixture was filtered and the filtrate adjusted to pH 8. It was then extracted three times by 15 ml/100 gm tissue of chloroform-ethanol (6:1, v/v) in a separatory funnel. The organic layers were combined and dried.

b. Isolation of Tomatine from the Standard Crude Extract

The standard crude extract (700 mg) was dissolved in 10 ml anhydrous methanol and applied to a 4 x 100 cm sephadex LH-20 column for column chromatography. The 10 ml effluent fractions, collected directly into a LKB fraction collector, were monitored for tomatine by ninhydrin, anthrone, sulphuric acid and visible spectrophotometry. The tubes indicating the presence of tomatine (fraction I) were pooled and thoroughly dried. The dried residue was dissolved in anhydrous methanol (2 mg/ml) and an equal volume of cholesterol (2 mg/ml) was added. The

two solutions were thoroughly mixed and incubated for 15 minutes in room temperature. 20 ml distilled water was then added to the mixture and thoroughly mixed to ensure complete precipitation of the tomatinide (tomatine-cholesterol complex). The precipitate obtained by centrifuging at 2000 RPM for 15 minutes, was taken to dryness. Excess cholesterol was removed by repeated washing of the precipitate with diethylether and the residue (tomatinide) was dried. The dry tomatinide was dissolved in pyridine (14 mg/ml) and refluxed for one hour in a water bath maintained at 100°C (Schulz and Sander, 1957) to cleave the tomatine-cholesterol complex. On cooling to room temperature, the free tomatine was precipitated out of the solution by adding diethylether until precipitation ceased. The precipitate (tomatine), obtained by centrifuging at 2000 RPM for 15 minutes at room temperature, was washed thoroughly with diethylether to remove all excess cholesterol. The tomatine was dried and dissolved in a minimum volume of hot methanol (60°C) and filtered through a fluted filter paper (Whatman No. 2). The solution was kept at -15°C overnight, centrifuged at 2000 RPM for 15 minutes at -15°C and the mother liquor decanted. The tomatine crystalls were dried in a vacuum dessicator over anhydrous $CaCl_2$. The process of recrystallization was repeated 5 times before the tomatine was used. The average yield of tomatine from 1 kg of tomato stalks was 74 mg.

c. Isolation of Gomatine from the Standard Crude Extract

700 mg of the standard crude extract were processed by the method described for tomatine. Tomatine was removed by cholesterol precipitation (Part II, Section E, b). The tomatine-free supernatant was thoroughly dried and washed repeatedly with diethylether to remove all excess cholesterol. The residue,

a white crystalline-like substance - gomatine - was dried and weighed. Gomatine was used in this form for all the biological studies.

Section F. Methods for testing The Biological Activity of the Extracts

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

1. Pharmacologic Agent

Histamine dihydrochloride (Fisher Scientific) of high purity was used. Unless stated otherwise, the concentration of the histamine dihydrochloride was calculated as base.

2. Apparatus

A constant temperature water 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 stirer, a thermometer, an organ bath and a coiled polyethylene tubing.

The 20 ml organ bath was supported by a glass outflow tube which led to a rubber drainage tube. The inlet tube to the organ bath was connected to the Tyrode reservoir by the coiled polyethylene tube and a ground glass stopcock, which served to regulate the flow of the Tyrode solution. The oxygen inlet tube was suspended above the organ bath. A platinum hook fixed at the end of 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 is attached

to a thread. The thread in turn was fixed to the writing lever, located directly above the organ bath. The magnification of the writing lever was 3:1. 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.

3. Preparation of the Tyrode Solution

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

10 grams $NaHCO_3$	20 ml of 10% $CaCl_2$ solution,
80 grams NaCl,	5 ml of 40% $MgCl_2$ solution,
10 grams dextrose,	20 ml of 10% KCl solution,
7 ampules atropine sulphate (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.

4. Preparation of Standard Histamine Solution

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

5. 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 were thoroughly washed with Tyrode. The end of the ileal strip nearest to the caecum was marked by a gauge No. 21 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 of the ileal strip, and the cleaned section which was not in use was kept immersed in Tyrode solution at room temperature and 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 ileal strip, properly fixed to the hooks, was transferred to the organ bath, the temperature of which had previously been adjusted to $34 \pm 1^{\circ}C$. 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 approximately 2 bubbles/second and maintained at this rate throughout the experiment. The writing lever was balanced with plasticine. The load of tissue was approximately 350 mg.

6. Testing the Antihistamine Activity of the Extracts

Immediately after the preparation was set up, the sensitivity of the ileal strip was first tested as follows: The kymograph was switched on and 0.05 µg of the standard histamine solution was added to the organ bath and left in

contact with the ileal strip for 20 seconds. During this time the contraction of the ileum was recorded. The kymograph was then switched off and the organ bath drained of histamine and Tyrode, and refilled with fresh Tyrode. Thirty seconds before the next administration of 0.05 µg standard histamine solution, the kymograph was switched on and remained "on" until the 20 seconds contact time of histamine with the ileum had elapsed. At this time the organ bath was emptied, washed and refilled with fresh Tyrode. The administration of the 0.05 µg standard histamine solution was repeated at least ten times at intervals of three minutes, until the ileum developed the fullest response it was capable of at this histamine dose level. If the tenth administration of histamine caused no contraction or only a very small one, larger doses of histamine were given. This procedure was repeated until the ileal contraction recorded on the smoked drum was about 5 cm high.

After the sensitivity of the ileum had been established, the antihistaminic activity of the extract was tested as follows: First the solvent employed to prepare the extract solution and then the extract in different doses were added to the organ bath immediately after the replacement of the histamine containing Tyrode with fresh Tyrode. The extract 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 contractions were back to normal.

b. In Vivo Methods

Guinea Pigs

Normal albino or multicoloured guinea pigs of the short hair variety (supplied

by Quebec Breeding Farm and Robidoux) of either sex, weighing between 250 - 300 gm were used throughout the experiments. The animals, received a few days before the experiments, were kept on normal pellet diet (guinea pig chow) with free access to water until used.

1. Histamine Aerosol

The wooden test chamber measured 30 x 30 x 60 cm and 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 on the chamber was used for ventilation while the other was used as inlet of the aerosol into the chamber. The histamine dihydrochloride solution (0.15% histamine base) was nebulized with the Jouan histamine aerosol apparatus which had a capacity of 20 ml/hr. The aerosol particles produced varied from 1 - 3 microns.

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:

i) Coughing, dyspnea and gasping;

ii) Swaying, falling and convulsive struggling;

iii) Lying on the floor of the chamber;

iv) 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 test. The maximum exposure time of the guinea pigs to the histamine aerosol was set arbitrarily at 20 minutes.

2. Anaphylactic Shock

The guinea pigs were sensitized with 10 mg/kg crystalline egg albumin (Pentex Inc.). The freshly prepared sensitizing antigen in a concentration of 10 mg/ml, was administered intraperitoneally twice on two subsequent days. Three weeks after the second sensitizing injection, the guinea pigs were injected intraperitoneally with the extracts and the control animals received the corresponding volume of the vehicle. 3 to 5 hours later (depending on the extracts used), both the treated and the control guinea pigs were given the challenging dose of antigen intracardiacally. The time of challenging was recorded and the guinea pigs were observed for the appearance of the following anaphylactic symptoms:

i) Chewing.
ii) Scratching of nose.
iii) Dypsnea and gasping.
iv) Swaying, falling and convulsive struggling.
v) Lying.
vi) Cessation of respiration - death.

As in the histamine aerosol test, the end point of the experiment was taken at 30 minutes following the antigenic challenge.

3. Capillary Permeability

The abdominal area of the guinea pigs were carefully shaved with an Oster electric clipper 24 hours prior to the experiment. The animals designated as "treated" were given the extracts intraperitoneally and the controls were given the corresponding amount of the vehicle. 3 to 5 hours later, 30 mg/kg pontamine sky blue in saline was injected intracardiacally with a 23 gauge hypodermic needle which was immediately followed by administering 0.01 ml and 0.05 ml saline solution of histamine or bradykinin intradermally on the shaved abdominal area. Thirty minutes later, the animals were sacrificed by a blow on the head. The skin of the shaved area was carefully removed, the soft tissues of the inner side cleaned and the area of blueing for both the treated and the control animals were measured. The area of blueing was measured by the product of the two largest diameters of the affected area.

4. Passive Cutaneous Anaphylaxis (PCA)

The back of the albino guinea pig was shaved with the Oster electric clipper 24 hours before the experiment. The antibody, anti-HSA, was injected intradermally on the shaved area of the back in several dilutions. A four hour interval permitted the antibody to fix itself to the tissues. One group of these guinea pigs was treated with the extracts intraperitoneally 1 hour before (for the oak gall extract) and 1 hour after (for tomatine and gomatine) the intradermal injection of the antibody. The control group was similarly treated with the vehicle. 4 hours after the intradermal injection of the antibody, these animals were challenged by injecting the antigen (2 mg/kg HSA) intra-

cardiacally together with 30 mg/kg pontamine sky blue, and the time was noted. Fifteen minutes later, the animal was sacrificed and the skin was prepared according to the method described in the capillary permeability test.

5. Statistical Analysis of Data

Means and standard errors were calculated by standard procedures. The significance of differences between means was estimated by Student's t test (Hoel, 1960).

Since the maximum time of exposure was arbitrarily limited to 20 minutes in the histamine aerosol experiments, in instances where the animals survived longer than 20 minutes, for the statistical analysis of the data, their survival time was taken as 20 minutes.

PART III. RESULTS

Section A. The Sensitivity of Normal Guinea Pigs to the Histamine Aerosol

To locate the active principle(s) in the different fractions, the histamine aerosol technique was routinely used because of the lack of a reliable chemical method. Consequently, the results of the purification study will be presented in terms of the histamine aerosol results.

It has been shown by several authors using the cessation of respiration as the end point of the experiment that when exposed to a lethal histamine aerosol, 80 - 90% of the normal guinea pigs died between 2 - 10 minutes (Broome et al., 1962; Calam and Callow, 1964; Wakkary, 1968). The reliability of this method - i.e. using the death time of the animal as the end point of the experiment - was further confirmed in the course of the present study in which 815 normal guinea pigs were exposed to 0.15% histamine aerosol. Fig. 2 shows the individual variations in survival time. The ordinate gives the number of animals and the abscissa gives the time in minutes. Each rectangle represents one guinea pig. If respiration ceased between the first and the second minute, the position of the rectangle is between 1 and 2, if between the second and the third minute, it is between 2 and 3, and so on. If the animal survived the 20 minutes period of exposure, the position of the rectangle is after 20.

The percentage of the total number of animals which survived for various length of time was calculated from Fig. 2, and the results so obtained are shown in Table I. Respiration ceased during the first 5 minutes for 20.6%; during the

first 10 minutes for 74.5%; and during the first 15 minutes for 90.8%. Only 3.2% survived the 20 minutes period of exposure. These results are in close agreement with those of Broome et al. (1962), Calam and Callow (1964) and Wakkary (1968).

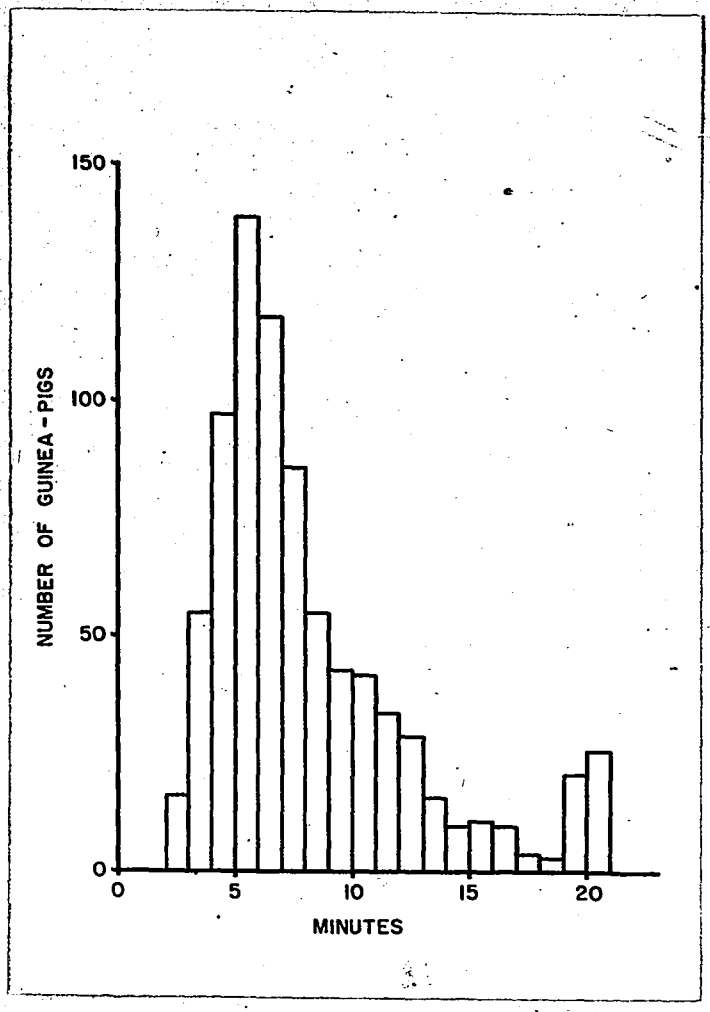


Figure 2

Variations in sensitivity of 815 control guinea pigs exposed to 0.15% histamine aerosol. The abscissa gives the time in minutes from the beginning of the aerosol. The position of the column indicates the time at which the respiration of the animal ceased. The ordinate gives the number of guinea pigs.

TABLE I

Number (%) of Normal Guinea Pigs Expiring After Exposure to 0.15% Histamine Aerosol for Various Times

Time of Respiration Cessation (minutes)	Number (%) of guinea pigs	
	(number)	(%)
0 - 5	168	20.6
5 - 10	441	54.1
10 - 15	131	16.1
15- 20	49	6.0
20	26	3.2

Percentage of Guinea Pigs in which respiration ceased between 0 - 5 minutes, between 5 - 10 minutes, between 10 - 15 minutes, between 15 - 20 minutes and those that survived the 20 minutes period of exposure to 0.15% histamine aerosol.

Section B. Purification of the Antihistamine-like Principle(s) from the Hungarian Oak Galls

The initial step in the extraction procedure, with slight modifications, was essentially the same as that described by Broome et al. (1962). In the early stage of the purification studies, the activity of the extract was calculated and expressed on the basis of the original weight of the oak gall powder. This method of expressing the activity had been frequently used by other investigators (Euler and Gaddum, 1931) and appeared to be most suitable in this stage of the investigation, because the extractable dry weight was highly variable from batch to batch, in spite of identical experimental conditions.

a. Chloroform:methanol Extraction of the Hungarian Oak Galls

300 gm of freshly ground oak gall powder was thoroughly mixed with 3 l. chloroform:methanol (2:1, v/v). The mixture was kept in room temperature for 16 hours and filtered. The clear filtrate was dried (the dry weight of different batches ranged from 500 - 2500 mg/100 gm oak gall powder. An aliquot of the dry residue was taken up in isopropyl myristate and injected intraperitoneally into guinea pigs (each animal received an equivalent of 10 gm oak gall powder or an average of 184 mg extract per animal). Control animals were similarly injected with the same amount of the vehicle. Each treated animal together with its control was exposed to 0.15% histamine aerosol 5 hours after the injection. The results of 15 experiments obtained with 8 different batches of the chloroform:methanol extracts are shown in Fig. 3. In 14 of the 15 experiments, the treated animals survived longer than the controls. The mean sur-

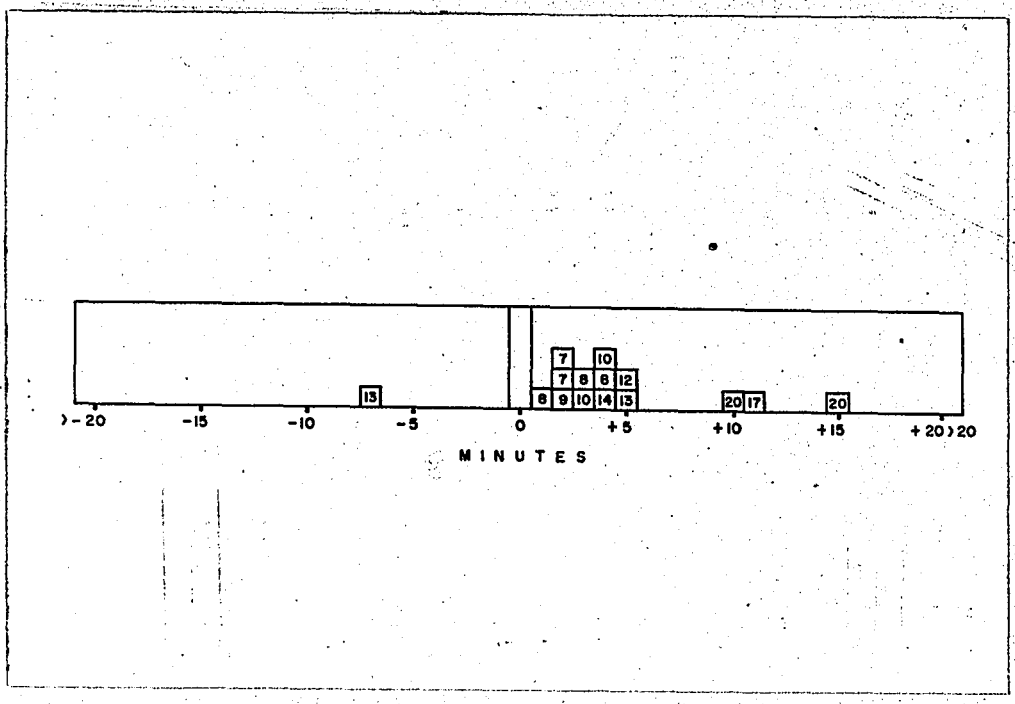


Figure 3

Protection produced by intraperitoneal injection of the chloroform:methanol extract prepared from the Hungarian oak galls in guinea pigs exposed to 0.15% histamine aerosol. In this and the following figures, the abscissa gives, in minutes, the differences in survival time between the injected animals and their controls. The difference is given as positive, if the treated animal survived longer than the control animal; as negative, if the control animal survived longer than the treated animal; and as 0, if the difference was 30 seconds or less, or, if both animals survived the 20 minutes exposure time. Each square represents 1 guinea pig and the numbers in the squares indicate the survival time (to the nearest minute).

vival time of the controls was 6.9 minutes while that of the treated was 11.7 minutes. Statistical analysis showed that the mean survival time of the treated animals was significantly longer than that of the corresponding controls ($p < 0.05$).

b. The Ethyl Acetate Soluble Fraction of the Chloroform:Methanol Extract

According to Broome et al. (1962), the antihistamine-like principle(s) could be re-extracted from the chloroform:methanol (2:1, v/v) dry residue with chloroform. The results of 25 experiments obtained in this laboratory did not indicate that chloroform was efficacious in extracting the active principle(s) from the chloroform:methanol residue. A number of other solvents were tried for the re-extraction of the active principle(s) from the chloroform:methanol (2:1, v/v) residue and eventually, ethyl acetate was found to be the most efficacious solvent. Accordingly, a method had been routinely adapted by which the dry chloroform:methanol residue was extracted three times with ethyl acetate (50 ml/100 gm oak gall powder) and the filtrate was taken to dryness. The dry weight of the different batches of oak gall extract averaged 600 mg/100 gm of oak gall powder.

An aliquot of the ethyl acetate soluble residue was taken up in isopropyl myristate and injected intraperitoneally into guinea pigs (each animal received an equivalent of 10 gm oak gall powder or an average of 60 mg extract per guinea pig). The control animals were similarly injected with the same amount of the vehicle. Each treated animal together with its control was exposed to 0.15% histamine aerosol 5 hours after injection.

The results of 16 experiments obtained with 8 batches of the ethyl acetate soluble fraction are shown in Fig. 4. In 15 of the 16 experiments, the treated animals survived longer than the controls and 5 of the treated guinea pigs survived the 20 minutes period of exposure. The mean survival time of the control guinea pigs was 7.7 minutes while that of the treated animals was 13.4 minutes. Statistical analysis showed that the mean survival time of the treated animals was significantly greater than that of the control animals, when both groups of animals were exposed to a lethal dose of histamine aerosol ($p < 0.02$).

c. Chlorophyll Removal from the Ethyl Acetate Extract

Chlorophyll, which constitutes a substantial portion of the total dry weight of the ethyl acetate soluble fraction, was removed by washing the residue (600 mg/100 gm oak gall powder) twice with ether absolute (20 ml/100 gm oak gall powder) in room temperature. The dry weight of the different batches averaged at 480 mg/100 gm oak gall powder. An aliquot of the dried ether insoluble residue was taken up in isopropyl myristate and injected intraperitoneally into guinea pigs (each animal received an equivalent of 10 gm oak gall powder or an average of 48 mg per animal). The controls were similarly injected with the same amount of the vehicle. Each treated animal together with its control was exposed to 0.15% histamine aerosol 5 hours following the injection.

The results of 20 experiments obtained with 10 batches of the chlorophyll-free ethyl acetate extract are shown in Fig. 5. In 17 of the 20 experiments, the treated animals survived longer than the controls and 7 of the treated guinea pigs survived the 20 minutes period of exposure. The mean survival time of the

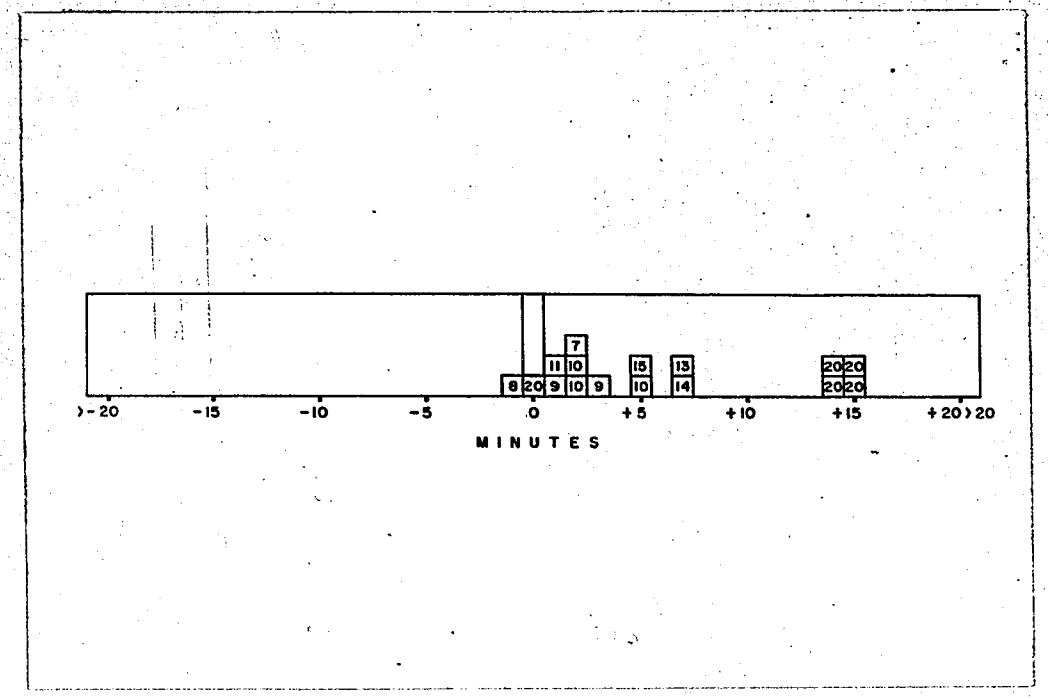


Figure 4

Protection produced by intraperitoneal injection of the extract prepared from the Hungarian oak galls by re-extracting the chloroform:methanol (2:1, v/v) residue with anhydrous ethyl acetate in guinea pigs exposed to 0.15% histamine aerosol. Details of this figure are the same as for figure 2.

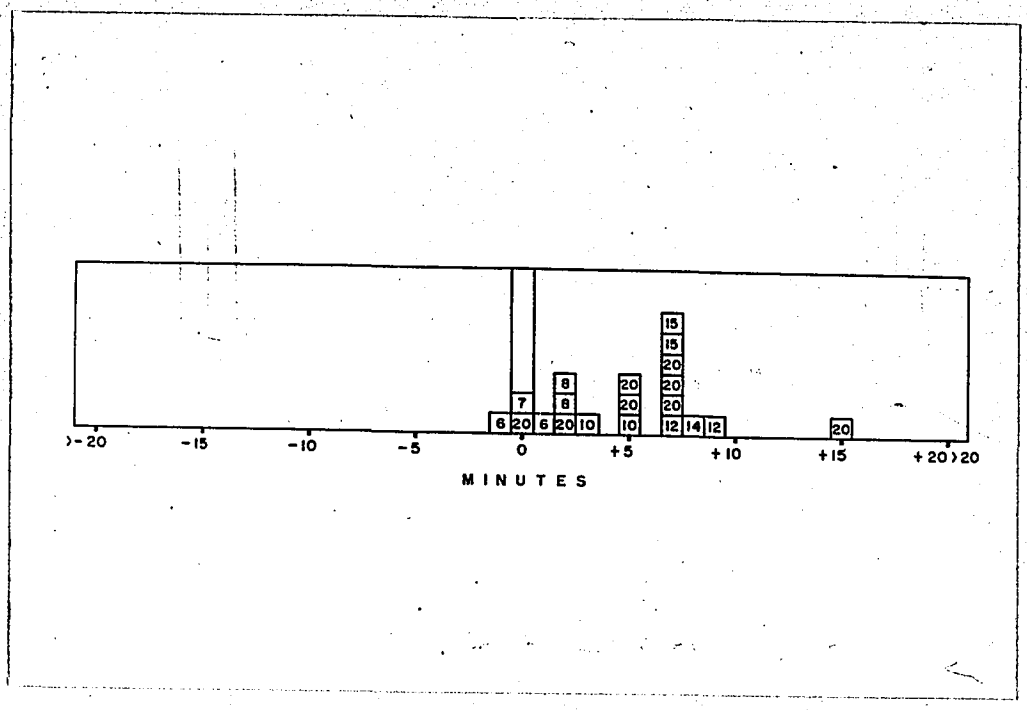


Figure 5

Protection produced in guinea pigs exposed to 0.15% histamine aerosol by intraperitoneal injection of extract prepared from the Hungarian oak galls after most of the chlorophyll in the anhydrous ethyl acetate residue was removed by ether absolute. Details of this figure are the same as for figure 2.

controls was 9.2 minutes while that of the treated animals was 14.1 minutes. Statistical analysis showed that the mean survival time of the treated animals was significantly greater than that of the controls ($p < 0.05$).

d. Tannin-free Crude Extract

The chlorophyll-free ethyl acetate extract (averaged at 480 mg/100 gm oak gall powder) was dissolved in 60 ml ethyl acetate and applied onto a silica gel G column (4 x 30 cm). The column was eluted with ethyl acetate with a flow rate of 10 ml/min. A single fraction was collected until the appearance of traces of tannin (determined by $FeCl_3$ reaction) and the tannin-free effluent (about 800 ml.) was thoroughly dried. The yield of 8 different batches of the tannin-free crude extract is listed in Table II.

An aliquot of the dried tannin-free extract was dissolved in isopropyl myristate in a concentration of 40 mg/ml and injected intraperitoneally into guinea pigs at a dose of 80 mg/kg of body weight. The control animals were similarly injected with the same amount of the vehicle. Each treated guinea pig together with its control was exposed to 0.15% histamine aerosol 5 hours after the injection.

The results of 21 experiments obtained with 10 batches of the tannin-free extracts are shown in Fig. 6. In 16 of the 21 experiments, the treated animals survived longer than the controls and 7 of the treated guinea pigs survived the 20 minutes period of exposure. The mean survival time of the treated animals was 12.4 minutes while that of the controls was 7.6 minutes. Statistical

TABLE II.

Yield of the Tannin-free Crude Extract

Amount of Oak Gall Powder (in grams)	Yields of the Tannin-free Crude Extract (Crude X-4) in % (w/w)		
300	0.042	=	126 mg
300	0.065	=	195 mg
300	0.053	=	159 mg
200	0.052	=	104 mg
200	0.042	=	84 mg
150	0.036	=	54 mg
150	0.086	=	129 mg
150	0.054	=	81 mg
	Mean $\pm$ 0.053	$\pm$ 0.005	53 mg/100 gm oak gall powder

Table II compares the yields of the tannin-free crude extracts prepared from 8 batches of Hungarian oak galls. These yields were representative of all the tannin-free crude extracts prepared in these studies. The range was 0.036 - 0.086 % (w/w).

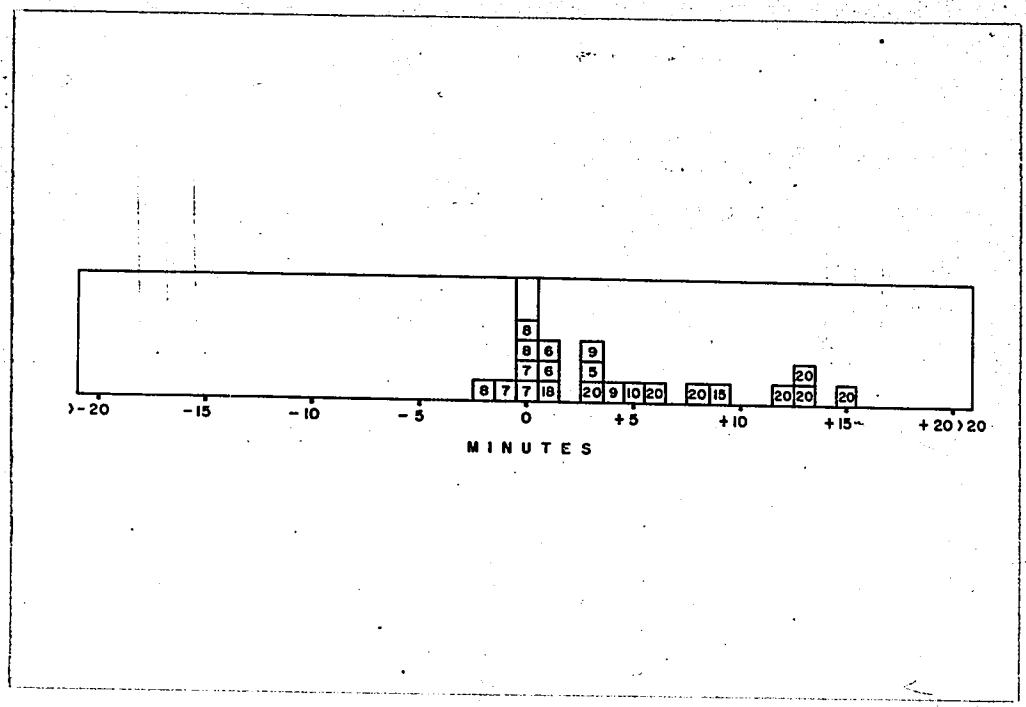


Figure 6.

Protection produced in guinea pigs exposed to 0.15% histamine aerosol by intraperitoneal injection of the tannin-free extract of the Hungarian oak galls. Details of this figure are the same as for figure 2.

analysis showed that the mean survival time of the treated animals was significantly longer than that of the control animals ($p < 0.01$).

e. Methanol Soluble Extract of the Tannin-free Crude Residue

The tannin-free residue (about 160 mg) was extracted 3 times with 40 ml of methanol. The clear filtrate of the methanol soluble fraction was taken to dryness and the methanol insoluble residue was discarded. An aliqout of the dried methanol soluble fraction (about 110 mg) was taken up in isopropyl myristate in a concentration of 30 mg/ml and injected intraperitoneally into guinea pigs in a dose of 50 mg/kg of body weight. The control animals were similarly injected with an equal amount of the vehicle. Each treated guinea pig together with its control was exposed to 0.15% histamine aerosol 5 hours following the injection.

The results of 20 experiments obtained with 10 different batches of the methanol soluble fraction are shown in Fig. 7. In 17 of the 20 experiments, the treated animals survived longer than the controls and 12 of the treated guinea pigs survived the 20 minutes period of exposure. The mean survival time of the treated animals was 16.4 minutes while that of the controls was 10.5 minutes. Statistical analysis showed that the mean survival time of the treated animals was significantly longer than that of the control animals ($p < 0.001$).

f. Sephadex LH-20 Column Chromatography of the Methanolic Extract

The methanol soluble extract (110 mg) was dissolved in 5 ml methanol and applied

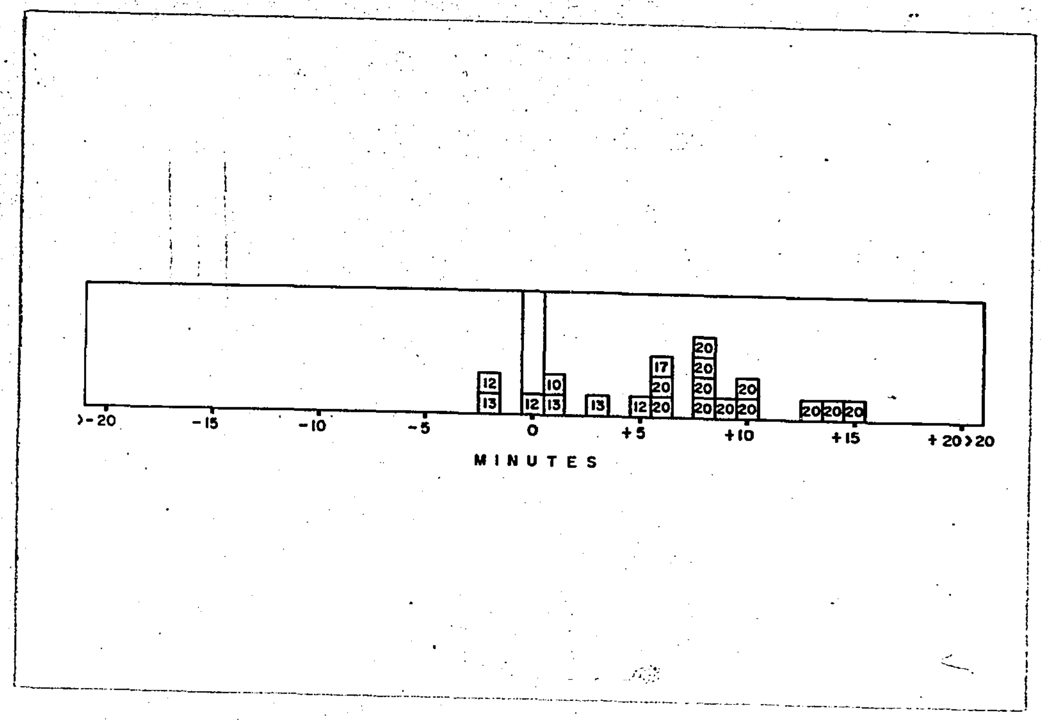


Figure 7

Protection produced in guinea pigs exposed to 0.15% histamine aerosol by intraperitoneal injection of the extract prepared from the Hungarian oak galls by re-extracting the tannin-free residue with anhydrous methanol. Details of this figure are the same as for figure 2.

onto a Sephadex LH-20 water jacketted column (2.5 x 80.0 cm). The column was eluted with methanol at a flow rate of 2 ml/min. The effluent fractions were collected in 10 ml aliquots and analysed by the Coleman Jr. Spectrophotometer (470 mμ) and the DU-Spectrophotometer (280 mμ). Representative spectrophotometric results obtained are seen in Fig. 8, which shows that two peaks were obtained with the Coleman Jr. Spectrophotometric method (470 mμ) and five peaks were obtained with the DU-Spectrophotometric method (280 mμ). The effluent fractions were also investigated by the ninhydrin, anthrone, concentrated sulphuric acid and the tryptophan methods. The results obtained were, however, not easily reproduceable. Thus the effluent fractions of the Sephadex LH-20 column were pooled into 6 fractions according to the DU-spectrophotometric peaks and each fraction was thoroughly dried.

An aliquot of the dried fractions was dissolved in isopropyl myristate in a concentration of 10 mg/ml and the solutions were injected intraperitoneally into guinea pigs in a dose of 10 - 20 mg/kg of body weight. The control animals were similarly injected with the same amount of the vehicle. Each treated animal together with its control was exposed to 0.15% histamine aerosol 5 hours after the injection. Of all the fractions tested, only Fraction I and Fraction VI showed antihistamine-like activity. The yields of Fraction I and Fraction VI are shown in Table III.

The results of 13 experiments in which the guinea pigs were treated with Fraction I are shown in Fig. 9A. In 10 of the 13 experiments, the treated animals survived longer than the controls; and 7 of the treated guinea pigs survived the 20 minutes period of exposure. The mean survival time of the control animals

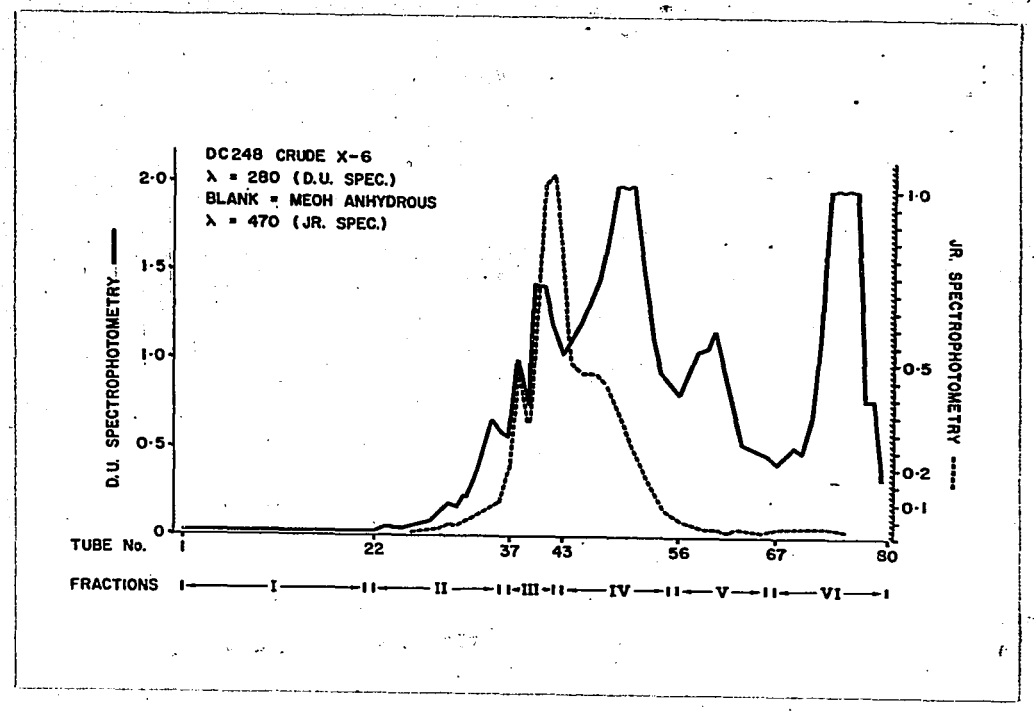


Figure 8

Gel Filtration of the methanol soluble extract of the Hungarian oak gall on sephadex LH-20 column (2.5 x 80.0 cm). The column was eluted with methanol at a flow rate of 120 ml/hr. The effluent was collected in 10 ml aliquots. Effluent tubes were pooled into 6 fractions (I-VI) according to the optical densities obtained with the visible and UV spectrophotometric analysis. For details, see text.

TABLE III

Yields of Fraction I and Fraction VI

Amount of Oak Gall Powder (in grams)	Fraction I (%)	Fraction VI (%)
300	0.0030	0.0060
300	0.0041	0.0081
300	0.0024	0.0100
600	0.0030	0.0060
600	0.0026	0.0059
900	0.0025	0.0049
	Mean $\pm$ 0.0029 $\pm$ 0.0003	0.0068 $\pm$ 0.0007

Table III compares the yields of Fraction I and Fraction VI prepared from 6 batches of Hungarian Oak Galls. These yields were representative of all the Fraction I and Fraction VI prepared in this studies.

was 11.9 minutes, while that of the treated animals was 17.0 minutes. Statistical analysis showed that the mean survival time of the treated animals was significantly longer than that of the controls ($p < 0.05$).

The results of 15 experiments in which guinea pigs were treated with Fraction VI are shown in Fig. 98. In all of the 15 experiments, the treated animals survived longer than the controls and 7 of the treated guinea pigs survived the 20 minutes period of exposure. The mean survival time of the control animals was 8.8 minutes, while that of the treated animals was 16.0 minutes. Statistical analysis showed that the mean survival time of the treated animals was significantly longer than that of the controls ($p < 0.001$).

g) In Vitro Assay - The Isolated Guinea Pig Ileum Preparation

The antihistamine-like activity of the Hungarian oak gall extracts (the tannin-free extract and Fraction I and Fraction VI of the Sephadex LH-20 column) were tested for *in vitro* activity using the isolated guinea pig ileum preparation. The purified oak gall extracts were taken up in ethanol (60%) at a concentration of 1.6 mg/ml and the bath concentrations ranged from 2 ug/ml to 100 ug/ml. Controls were obtained by administering the corresponding volume of the vehicle into the organ bath.

The results obtained from both the tannin-free extract and Fraction I and Fraction VI of the sephadex LH-20 column showed that the purified oak gall extracts could not inhibit the histamine-induced contractions of the isolated guinea pig ileum preparation. The results of a typical *in vitro* assay of the

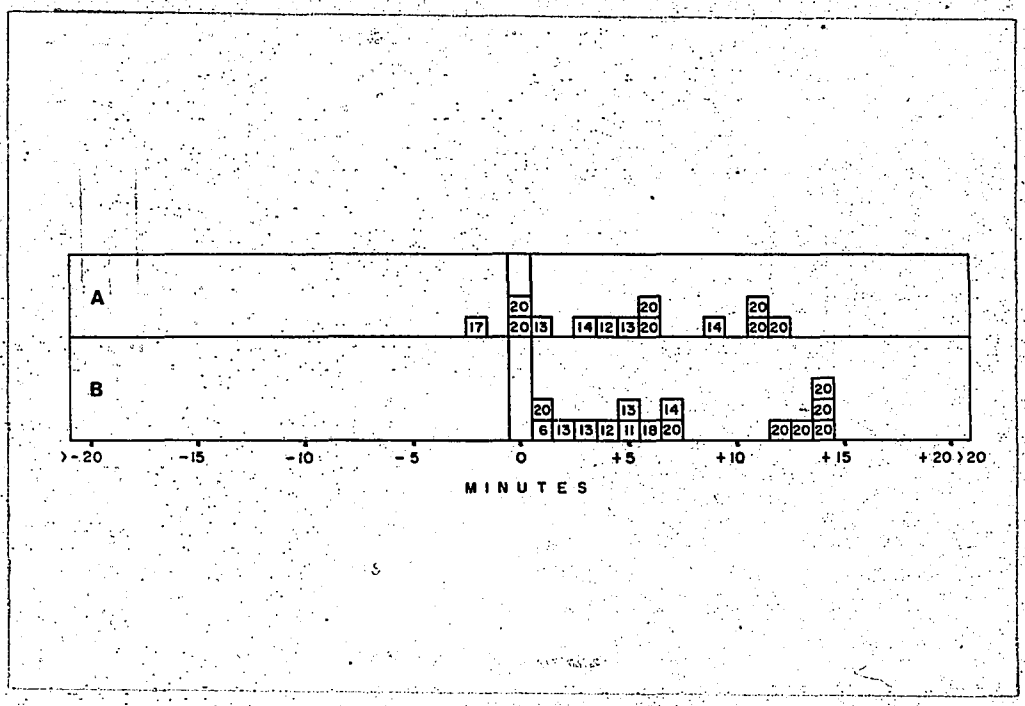


Figure 9

Protection produced in guinea pigs exposed to 0.15% histamine aerosol by intraperitoneal injection of the two active fractions (I and VI) of the sephadex LH-20 column chromatography of the crude extracts prepared from the Hungarian oak galls. Details of this figure are the same as for figure 2., and for further details of the figure, see text.

purified oak gall extracts are seen in Fig. 10. It can be seen that the first dose of histamine following the administration of the sample was inhibited. However, since the controls gave similar results, the observed inhibition can be attributed to the solvent.

h) Thin Layer Chromatography (TLC) of the Tannin-free Extract and the Active Fractions of the Sephadex LH-20 Column Effluent.

Aliquots of 100 µg of the tannin-free extract, Fraction I and Fraction VI were spotted on sheets precoated with silica gel and the chromatograms were developed in the water:methanol (2 3/4:1, v/v) solvent mixture.

Fig. 11A shows the TLC results of the tannin-free extract. The chromatogram was stained by o-phosphoric acid (47%) and heated in 65°C for 5 minutes. 10 - 14 components were regularly obtained with this extract.

Fig. 11B shows the TLC results of Fraction I of the sephadex LH-20 column. The chromatogram was stained in o-phosphoric acid (47%) and heated in 65°C for 5 minutes. Only one compound which remains at the base was seen.

Fig. 11C shows the TLC results of Fraction VI of the sephadex LH-20 column. The chromatogram, which was stained in o-phosphoric acid (47%) and heated in 65°C for 5 minutes, showed at least four components.

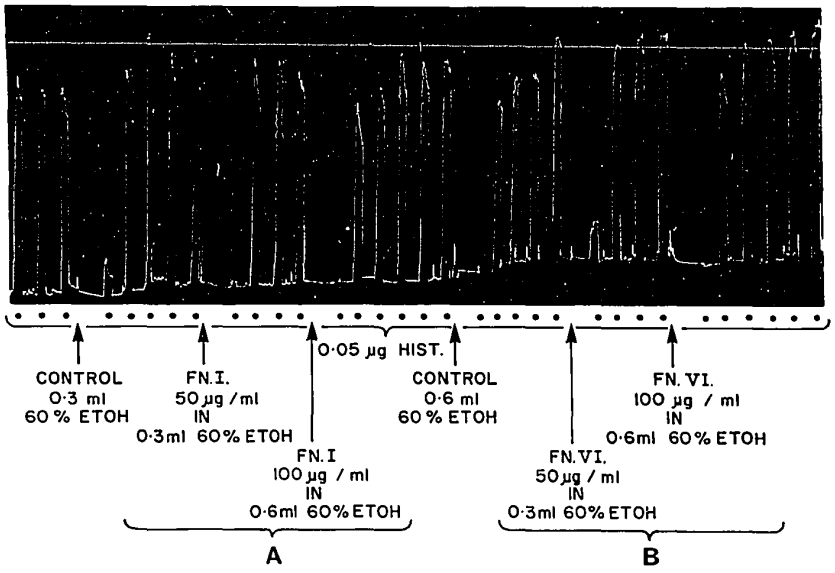


Figure 10

Responses of the guinea pig ileum preparation to histamine (black dots) before and after the addition of (A) Fraction I (5×10^{-5} gm/ml, and 1×10^{-4} gm/ml) and (B) Fraction VI (5×10^{-5} gm/ml, and 1×10^{-4} gm/ml) of the sephadex LH-20 column. Intervals of 3 minutes elapsed between each administration of histamine. In each instance, the drum was temporarily stopped after washing out the organ bath and restarted 30 seconds before the next dose of histamine. The contact time was 20 seconds for histamine and 2 minutes for the purified oak gall extracts.

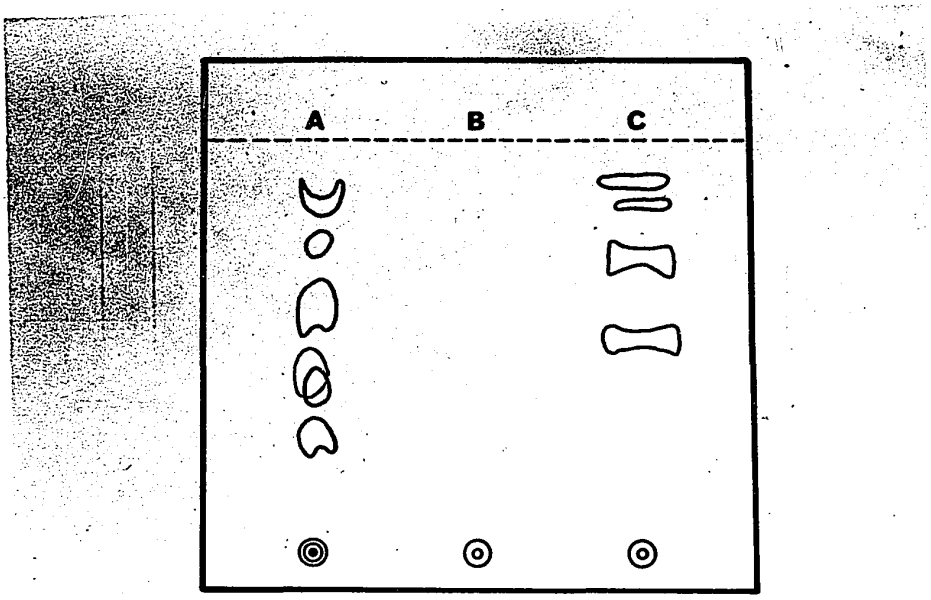


Figure 11

A diagrammatic thin layer chromatography representation of (A) tannin-free extract, (B) Fraction I and (C) Fraction VI. A sheet (20 x 20 cm) precoated with silica gel was used as the thin layer chromatogram and developed with water:methanol (2 3/4:1, v/v). The chromatogram was stained with o-phosphoric acid (47%).

Section C. A Comparative Study of the Antihistamine-like Activity of the Purified Hungarian Oak Gall Extract, Tomatine and Gomatine

The Hungarian oak gall, considered to be a form of plant tumour, was found to possess *in vivo* antihistamine-like activity. Since similar activity was observed with substances recently isolated from a different type of plant tumour, it was of interest to compare their antihistamine-like activity in terms of potency and toxicity.

There had been very little investigation into the biological activity of tomatine (Fontaine et al., 1948) and gomatine (Wakkary, 1968). It had been reported that both tomatine and gomatine possess antihistamine-like activity (Calam and Callow, 1964; Wakkary, 1968). These reports, however, were either conflicting, e.g., regarding the status of tomatine as having antihistamine-like activity (Calam and Callow, 1964; Kovacs et al.,1964; Wakkary, 1968) or the nature of the investigation so preliminary that no definite conclusion about their antihistamine-like activity could be drawn. The tomatine and gomatine used in the present study were isolated by the methods described by Wakkary (1968) and the purified oak gall extracts were prepared according to the method previously described (Part II, Section E).

The following methods were employed to evaluate the antihistamine-like activity of these three substances: 1. bronchoconstriction induced by histamine; 2. systemic anaphylactic shock; 3. capillary permeability induced by histamine and bradykinin; and 4. PCA reaction in guinea pigs.

a. Antihistamine-like Activity of the Purified Hungarian Oak Gall Extract

1. Histamine Aerosol

The tannin-free crude extract of the Hungarian oak gall was taken up in isopropyl myristate and injected intraperitoneally in doses of 40 mg/kg and 80 mg/kg of body weight into guinea pigs. The control animals were similarly treated with the same volume of the vehicle. Each treated animal together with its control was exposed to 0.15% histamine aerosol 5 hours after the injection.

The results of 15 experiments in which guinea pigs were treated with 40 mg/kg of purified oak gall extract are shown in Fig. 12B. In 13 of the 15 animals , the treated ones survived longer than the controls and 5 of the treated animals survived the 20 minutes period of exposure.

The results of 15 experiments in which guinea pigs were treated with 80 mg/kg of the purified oak gall extract are shown in Fig. 12A. In 14 of the 15 animals the treated ones survived longer than the controls and 7 of the treated guinea pigs survived the 20 minutes period of exposure.

2. Anaphylactic Shock

Guinea pigs sensitized to egg albumen were injected intraperitoneally with 40 mg/kg and 80 mg/kg of purified oak gall extract in isopropyl myristate. The sensitized controls were similarly injected with the same amount of the vehicle. The treated and the control animals were injected intracardiacally with the

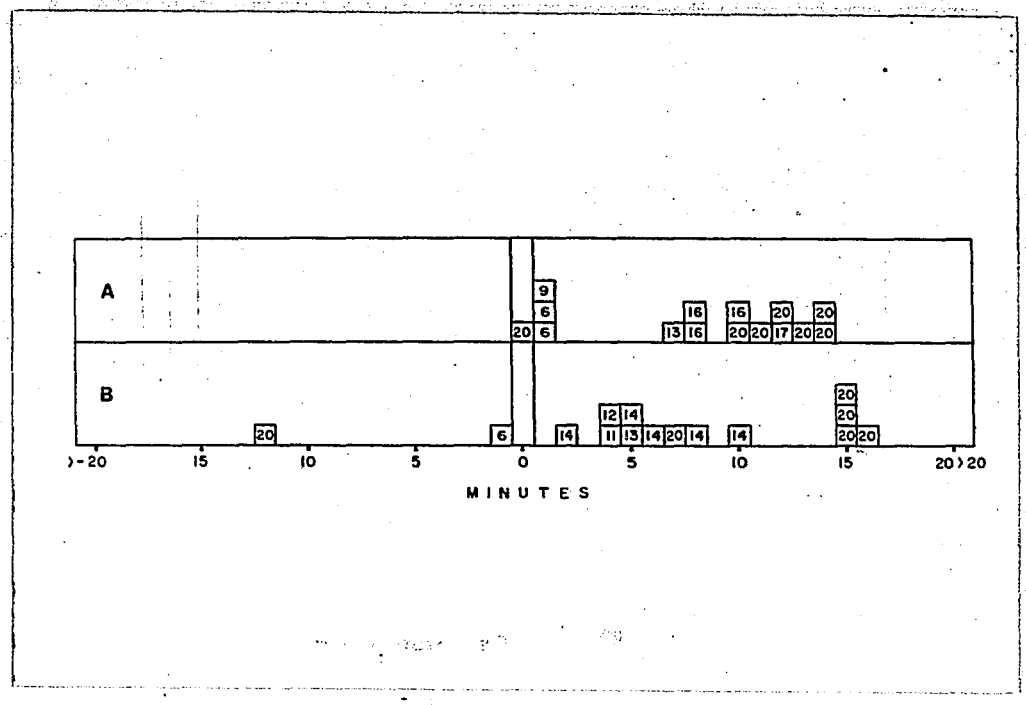


Figure 12

Protection produced in guinea pigs exposed to 0.15% histamine aerosol by intraperitoneal injection of the purified oak gall extract (A) 80 mg/kg and (B) 40 mg/kg of body weight.

challenging dose of the egg albumen (2 mg/kg of body weight) 5 hours after the treatment with the purified oak gall extract or the vehicle.

The results of 12 experiments in which 12 guinea pigs were treated with 80 mg/kg purified oak gall extract and 10 guinea pigs were treated with the same amount of the vehicle showed that 10 of the treated animals were definitely protected (they survived the 30 minutes of observation time with no signs of dyspnea following the antigenic challenge). The animals treated with 40 mg/kg purified oak gall extract were not protected against the anaphylactic shock, since their mean survival time was identical to that of the control animals, which had a mean death time of 4 minutes following the administration of the antigen.

3. Capillary Permeability

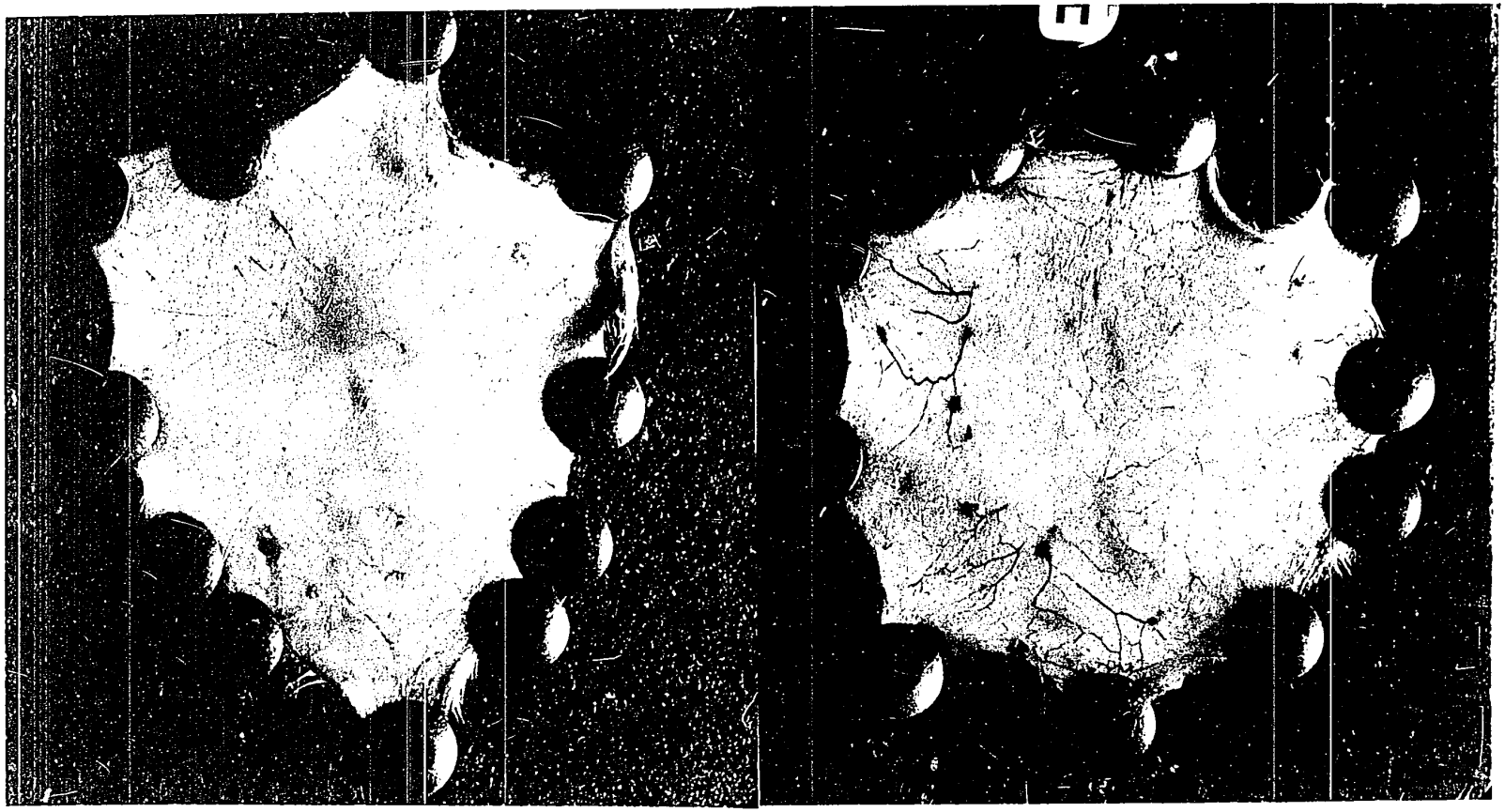
The effect of purified oak gall extracts on the increased capillary permeability, induced by the intradermal injection of histamine and bradykinin in 13 guinea pigs, was investigated. The purified oak gall extract was taken up in isopropyl myristate in a concentration of 40 mg/ml and injected intraperitoneally into albino guinea pigs in doses of 40 mg/kg and 80 mg/kg of body weight. The control animals were similarly injected with the same amount of the vehicle. Five hours after the injection, each animal received 30 mg/kg pontamine sky blue intracardiacally followed immediately by the intradermal administration of 0.1 µg and 0.5 µg of histamine or bradykinin.

The results of 5 experiments in which guinea pigs were treated with 40 mg/kg purified oak gall extract are shown in Table IV. The mean area of blueing

produced by 0.1 µg histamine in the treated animals was 177 mm^2 while that of the controls was 189 mm^2. Similarly, the mean area of blueing produced by 0.5 ug histamine in the treated guinea pigs was 437 mm^2 while that of the controls was 435 mm^2. No inhibition of the increase in capillary permeability by 40 mg/kg purified oak gall extract was found.

The results of 8 experiments, in which guinea pigs were treated with 80 mg/kg purified oak gall extract, are shown in Table V and Fig. 13. The mean area of blueing produced by 0.1 µg histamine in the treated animals was 34 mm^2 while that of the controls was 235 mm^2. Similarly, the mean area of blueing produced by 0.5 µg histamine in the treated animals was 102 mm^2 while that of the controls was 515 mm^2. The inhibition of the increase in capillary permeability by the 80 mg/kg purified oak gall extract was highly significant ($p < 0.001$).

The results of 8 experiments in which guinea pigs were treated with 80 mg/kg purified oak gall extract are shown in Table VI. The mean area of blueing produced by 0.1 µg bradykinin in the treated animals was 226 mm^2 while that of the controls was 258 mm^2. Similarly, the mean area of blueing produced by 0.5 µg bradykinin in the treated animals was 545 mm^2 while that of the controls was 543 mm^2. Statistical analysis showed that the inhibition of the increase in capillary permeability by 80 mg/kg purified oak gall extract was completely lacking.



A

Figure 13.

B

Blue patches of the internal surface of guinea pig skins, which received pontamine sky blue (30 mg/kg) intracardiacally followed by separate intradermal injection of 0.1 µg (upper half of photograph) and 0.5 µg (lower half of photograph) histamine. (A) responses of a normal guinea pig and (B) responses of a guinea pig treated with the purified oak gall extract (80 mg/kg) intraperitoneally 5 hours before the injection of histamine.

TABLE IV

Area of blueing as a measure of the increase in capillary permeability induced by histamine injected intradermally into guinea pigs.

Treatment	Purified Oak Gall Extract (40 mg/kg)		Vehicle	
Animal Number	EXTRAVASATION OF DYE (mm^2) BY HISTAMINE (μg)			
	0.1 μg	0.5 μg	0.1 μg	0.5 μg
1	135	342	180	365
2	140	310	153	418
3	228	494	208	449
4	246	560	198	535
5	138	482	206	410
	Mean $\pm$ 177 $\pm$ 22	437 $\pm$ 44	189 $\pm$ 11	435 $\pm$ 27
	P NS	NS		

TABLE V

Area of blueing as a measure of the increase in capillary permeability induced by histamine injected intradermally into guinea pigs.

Treatment	Purified Oak Gall Extract (80 mg/kg)		Vehicle	
Animal Number	EXTRAVASATION OF DYE (mm^2) BY HISTAMINE (μg)			
	0.1 μg	0.5 μg	0.1 μg	0.5 μg
1	21	45	144	478
2	40	108	308	860
3	48	100	209	527
4	42	144	340	522
5	19	114	283	458
6	34	96	202	410
7	27	90	160	325
8	42	126	234	538
	Mean $\pm$ 34 $\pm$ 4	103 $\pm$ 10	235 $\pm$ 23	515 $\pm$ 51
	$P < 0.001$	< 0.001		

TABLE VI

Area of blueing as a measure of the increase in capillary permeability induced by bradykinin injected intradermally into guinea pigs.

Treatment	Purified Oak Gall Extract (80 mg/kg)		Vehicle	
Animal Number	EXTRAVASATION OF DYE (mm^2) BY BRADYKININ (μg)			
	0.1 μg	0.5 μg	0.1 μg	0.5 μg
1	198	720	238	336
2	150	408	273	625
3	168	560	372	608
4	204	589	280	561
5	304	660	210	548
6	288	551	180	642
7	204	480	248	481
8	292	392	262	542
	Mean $\pm$ 226 $\pm$ 20	545 $\pm$ 38	258 $\pm$ 26	543 $\pm$ 33
	P NS	NS		

4. Passive Cutaneous Anaphylactic Reaction (PCA)

The effect of the purified oak gall extract on the increase in capillary permeability induced by antigen-antibody reaction was investigated. The antibody (anti-HSA) was injected intradermally in the following dilutions: 1:100; 1:1000; 1:10000; and 1:100000 and was allowed 4 hours to be fixed to the guinea pig skin (Fig. 14). The purified oak gall extract was taken up in isopropyl myristate in a concentration of 40 mg/ml and injected intraperitoneally into albino guinea pigs in a dose of 80 mg/kg of body weight one hour before the intradermal injection of the anti-HSA. The controls were similarly injected with the same amount of the vehicle. Five hours following the administration of the purified oak gall extract, the antigen (HSA), in a dose of 2 mg/kg was given intracardiacally as a mixture with 30 mg/kg pontamine sky blue into the treated and control animals.

The results of 7 experiments in which the passively sensitized guinea pigs were treated with 80 mg/kg of purified oak gall extract are shown in Table VII. The purified oak gall extract did not inhibit the increase in capillary permeability induced by the antigen-antibody reaction.

b. Antihistamine-like Activity of Tomatine

1. Histamine aerosol

Tomatine was dissolved in water acidified to pH2 by 12N HCl in a concentration of 30 mg/ml and injected into guinea pigs, intraperitoneally, in a dose of

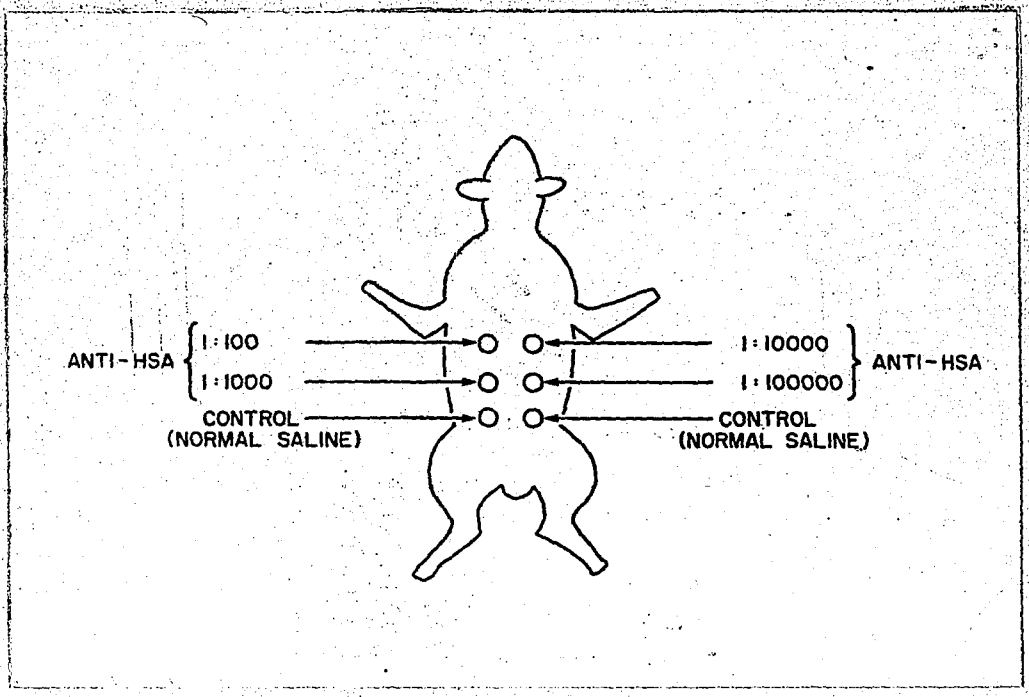


Figure 14

Sites of intradermal injections of the antibody (anti-HSA) in the guinea pig for PCA reaction.

TABLE VII

Area of blueing as a measure of the PCA reactions induced by the HSA and Anti-HSA systems in the guinea pigs.

Treatment	Purified Oak Gall Extract (80mg/kg)			Vehicle		
Animal Number	AREA OF DYE EXTRAVASATION (mm^2)					
	ANTIBODY DILUTIONS					
	1:100	1:1000	1:10000	1:100	1:1000	1:10000
1	320	221	144	234	180	49
2	180	168	42	284	180	30
3	224	154	35	365	210	88
4	360	265	110	420	230	120
5	484	280	88	540	254	110
6	410	200	96	390	160	94
7	348	168	80	386	145	70
	Mean $\pm$ 332 $\pm$ 37	186 $\pm$ 39	85 $\pm$ 13	374 $\pm$ 25	194 $\pm$ 14	80 $\pm$ 11
	P NS	NS	NS			

30 mg/kg of body weight. The control animals were similarly injected with the same volume of the vehicle. Each treated animal together with its control was exposed to 0.15% histamine aerosol 3 hours following the injection.

Fig. 15 summarizes the results obtained from 4 batches of tomatine preparations. in 10 of the 13 animals, the treated animals survived longer than the controls. The mean survival time of the control animals was 8.2 minutes, while that of the treated animals was 12.0 minutes. Statistical analysis showed that the mean survival time of the treated animals was significantly greater than that of the controls. ($p < 0.05$).

2. Anaphylactic Shock

Guinea pigs sensitized to egg (chicken) albumen were injected intraperitoneally with 30 mg/kg tomatine, which was dissolved in water acidified to pH2 with 12N HCl in a concentration of 30 mg/ml. The sensitized control animals were similarly injected with the same amount of the vehicle. The treated and the control guinea pigs were injected intracardiacally with the challenging dose of egg albumen (2 mg/kg of body weight) 3 hours following the treatment with tomatine or the vehicle.

The results of 13 experiments showed that none of the treated animals survived the 30 minutes of observation time. However, the death time of the treated animals was slightly prolonged (mean time of death being 9.0 minutes) compared to that of the control guinea pigs (mean time of death being 4.0 minutes).

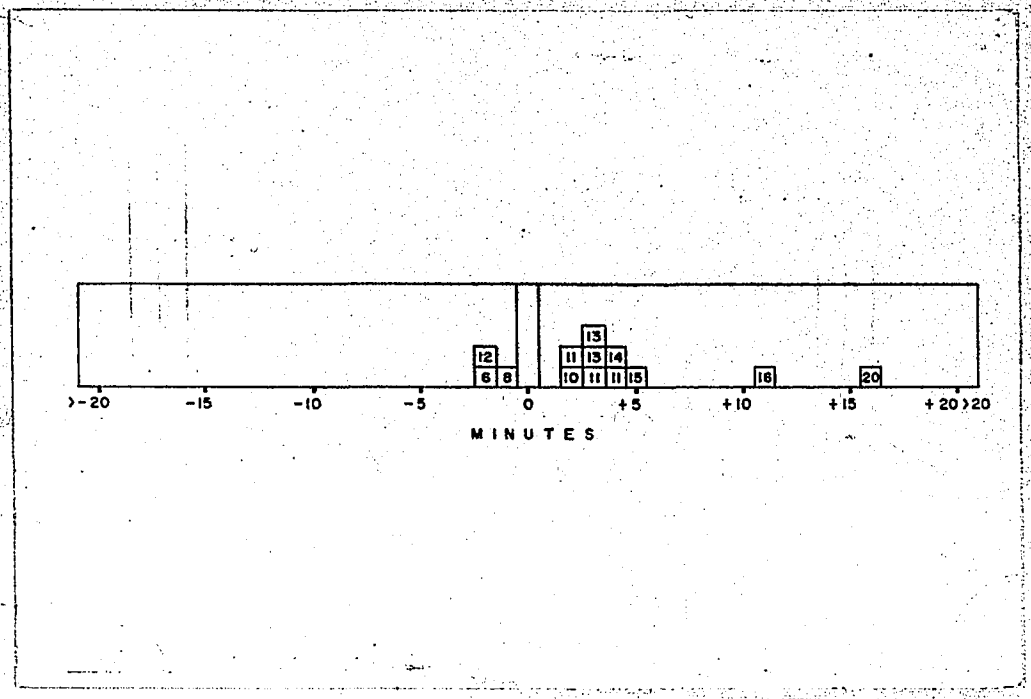


Figure 15

Protection produced in guinea pigs exposed to 0.15% histamine aerosol by intraperitoneal injection of 30 mg/kg tomatine.

3. Capillary Permeability

The effect of tomatine on the increase in capillary permeability, brought about by the intradermal injection of histamine and bradykinin, was investigated. Tomatine was dissolved in water acidified to pH2 with 12N HCl in a concentration of 30 mg/ml and administered intraperitoneally in doses of 15 mg/kg and 30 mg/kg of body weight. The control animals were similarly treated with the same amount of the vehicle. Three hours later, each animal received 30 mg/kg pontamine sky blue intracardiacally and followed immediately by injecting 0.1 µg and 0.5 µg histamine or bradykinin intradermally.

The results of 8 experiments in which the guinea pigs were treated with 15 mg/kg tomatine are shown in Table VIII. Statistical analysis shows that the inhibition of the increase in capillary permeability by 15 mg/kg tomatine was not significant at the 5% level.

The results of 13 experiments in which guinea pigs were treated with 30 mg/kg tomatine are shown in Table IX and Fig. 16. The mean area of blueing produced by 0.1 µg histamine in the treated animals was 101 mm^2 while that of the controls was 326 mm^2. Similarly, the mean area of blueing produced by 0.5 µg histamine in the treated animals was 234 mm^2 while that of the controls was 548 mm^2. Statistical analysis showed that the inhibition of the increase in capillary permeability, induced by 0.1 µg and 0.5 µg histamine was significant ($p < 0.001$).

The results of 8 experiments in which guinea pigs were treated with 30 mg/kg tomatine are shown in Table X. The mean area of blueing produced by 0.1 µg

TABLE VIII

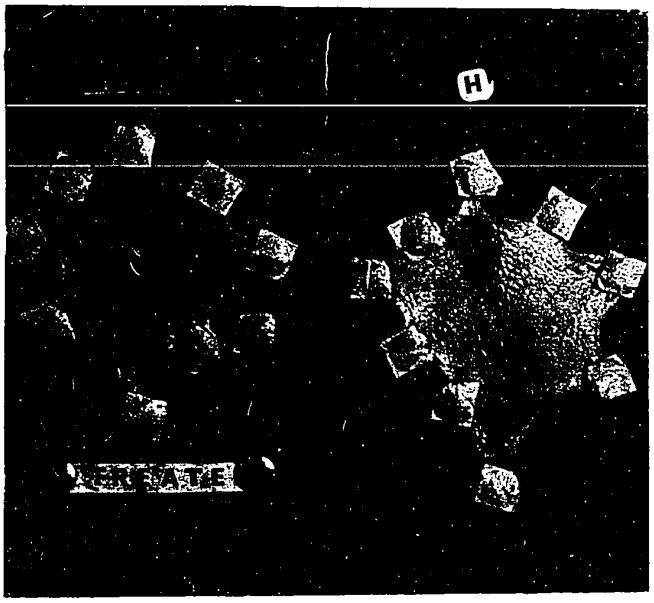
Area of blueing as a measure of the increase in capillary permeability induced by histamine injected intradermally into guinea pigs.

Treatment	Tomatine (15 mg/kg)		Vehicle	
Animal Number	EXTRAVASATION OF DYE (mm^2) BY HISTAMINE (μg)			
	0.1 μg	0.5 μg	0.1 μg	0.5 μg
1	231	512	299	544
2	220	392	360	560
3	532	700	231	304
4	48	108	198	575
5	9	60	200	490
6	324	420	--	--
7	49	160	--	--
8	180	465	--	--
	Mean $\pm$ 199 $\pm$ 58	352 $\pm$ 74	258 $\pm$ 27	495 $\pm$ 42
	P NS	NS		

TABLE IX

Area of blueing as a measure of the increase in capillary permeability induced by histamine injected intradermally into guinea pigs.

Treatment	Tomatine (30 mg/kg)		Vehicle	
Animal Number	EXTRAVASATION OF DYE (mm^2) BY HISTAMINE (µg)			
	0.1 µg	0.5 µg	0.1 µg	0.5 µg
1	15	80	432	814
2	25	108	320	680
3	130	190	225	565
4	149	460	280	612
5	78	150	189	594
6	80	117	204	430
7	94	198	196	480
8	117	400	288	544
	Mean ± 86 ± 16	213 ± 61	267 ± 27	596 ± 25
	P < 0.001	< 0.001		



A B

Figure 16

Blue patches of the internal surface of the guinea pig skin which had received pontamine sky blue (30 mg/kg) intracardiacally followed by separate intradermal injections of 0.1 µg (upper half of photograph) and 0.5 µg (lower half of the photograph) histamine.

(A) responses of a guinea pig treated with tomatine (30 mg/kg) and

(B) responses of the control animal.

TABLE X

Area of blueing as a measure of the increase in capillary permeability induced by bradykinin injected intradermally into guinea pigs.

Treatment	Tomatine (30 mg/kg)		Vehicle	
Animal Number	EXTRAVASATION OF DYE (mm^2) BY BRADYKININ (µg)			
	0.1 µg	0.5 µg	0.1 µg	0.5 µg
1	15	80	432	814
2	25	108	320	680
3	130	190	225	565
4	149	460	280	612
5	78	150	189	594
6	80	117	204	430
7	94	198	196	480
8	117	400	288	544
	Mean ± 86 ± 16	213 ± 45	267 ± 27	596 ± 25
	P < 0.001	< 0.001		

bradykinin in the treated animals was 86 mm^2 while that of the controls was 267 mm^2. Similarly, the area of blueing produced by 0.5 µg bradykinin in the treated animals was 213 mm^2 while that of the controls was 596 mm^2. Statistical analysis showed that the inhibition of the increase in capillary permeability induced by 0.1 µg and 0.5 µg bradykinin was significant, ($P < 0.001$).

4. Passive Cutaneous Anaphylactic Reaction (PCA)

Antibody (anti-HSA) was injected intradermally into 7 guinea pigs in dilutions of: 1:100; 1:1000; 1:10000; and 100000 and allowed 4 hours to be fixed to the skin. Tomatine was dissolved in water acidified to pH2 with 12N HCl in a concentration of 30 mg/ml and injected intraperitoneally into the guinea pigs in a dose of 30 mg/kg of body weight 1 hour following the intradermal injection of the anti-HSA. The sensitized control animals were similarly injected with the same amount of the vehicle. Three hours following the tomatine administration, 2 mg/kg of the antigen (HSA) mixed with 30 mg/kg of pontamine sky blue, was injected intracardiacally into the treated and the control animals.

The results of 7 experiments are shown in Table XI. There was no difference in the mean area of blueing in the two groups of animals at any antibody dilutions.

c. Antihistamine-like Activity of Gomatine

1. Histamine Aerosol

Gomatine was dissolved in 95% ethanol to give a concentration of 30 mg/ml amd

TABLE XI

Area of blueing as a measure of the PCA reactions induced by the HSA and anti-HSA system in the guinea pig.

Treatment	Tomatine (30 mg/kg)			Vehicle		
Animal Number	AREA OF DYE EXTRAVASATION (mm^2)					
	ANTIBODY DILUTIONS					
	1:100	1:1000	1:10000	1:100	1:1000	1:10000
1	320	245	136	368	280	135
2	391	265	150	340	270	130
3	360	235	120	376	210	130
4	160	80	25	400	264	126
5	700	240	120	382	244	154
6	342	143	36	330	200	84
7	380	210	100	500	104	108
	Mean ± 379 ±57	202 ±24	98 ± 17	385 ±20	224 ± 23	123 ± 10
	P NS	NS	NS			

injected intraperitoneally into guinea pigs in a dose of 20 mg/kg of body weight. The control animals were similarly injected with the same amount of the vehicle. Each treated animal together with its control was exposed to 0.15% histamine aerosol 3 hours following the injection.

The results of 24 experiments obtained with 18 batches of gomatine preparations are shown in Fig. 17. In 19 of the 24 experiments the treated animals survived longer than the controls. The mean survival time of the treated animals was 16.4 minutes while that of the controls was 10.1 minutes. Statistical analysis showed that the mean survival time of the treated animals was significantly greater than that of the controls ($p < 0.001$).

2. Anaphylactic Shock

Guinea pigs sensitized to egg albumen were injected intraperitoneally with 20 mg/kg of the ethanolic solution of gomatine (30 mg/ml). The sensitized control animals were injected with the same amount of the vehicle. The treated and the control animals were injected intracardiacally with the challenging dose of egg albumen (2 mg/kg of body weight) 3 hours after the treatment with tomatine or the vehicle.

The results of 18 experiments, in which the guinea pigs were treated with 20 mg/kg gomatine, showed that 8 of the treated animals were definitely protected against the anaphylactic shock, since they survived the 30 minutes period of observation time without the development of severe dyspnea following the antigenic challenge. One of the treated animals developed severe dyspnea

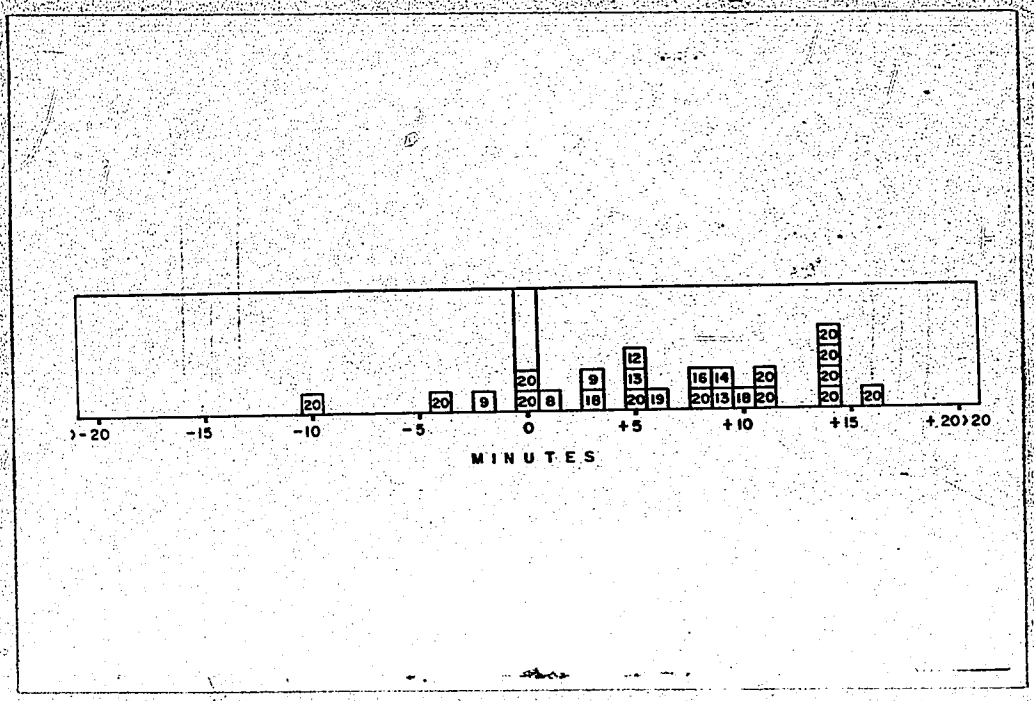


Figure 17

Protection produced in guinea pigs exposed to 0.15% histamine aerosol by intraperitoneal injection of 20 mg/kg gomatine.

within 5 minutes after the administration of the antigen but it gradually recovered. The response of the other 9 treated animals was not significantly different from the 14 controls, in which the anaphylactic shock symptoms were fully developed in the first minute with a mean death time of 5 minutes.

3. Capillary Permeability

The effect of g omatine on the increase in capillary permeability brought about by the intradermal injection of histamine and bradykinin was investigated. Gomatine was dissolved in ethanol (95%) in a concentration of 30 mg/ml and administered into albino guinea pigs in doses of 10 mg/kg and 20 mg/kg of body weight. The controls were similarly treated with the same amount of the vehicle. Three hours later, each animal received 30 mg/kg pontamine sky blue intracardiacally followed immediately by injecting 0.1 µg and 0.5 µg histamine or bradykinin intradermally.

The results of 8 experiments in which guinea pigs were treated with 10 mg/kg gomatine and tested against histamine are shown in Table XII. Statistical analysis showed that the inhibition of the increase in capillary permeability by 10 mg/kg gomatine was not significant at the 5% level.

The results of 8 experiments in which the guinea pigs were treated with 20 mg/kg gomatine and tested against histamine are shown in Table XIII and Figure 18. The mean area of blueing produced by 0.1 µg histamine in the treated animals was 80 mm^2 while that of the controls was 244 mm^2. Similarly, the mean area of blueing produced by 0.5 µg histamine in the treated animals was 162 mm^2

TABLE XII

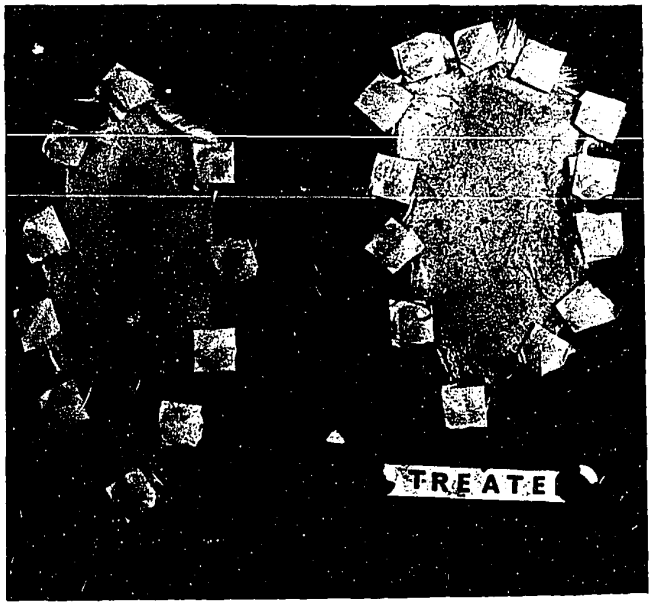
Area of blueing as a measure of the increase in capillary permeability induced by histamine injected intradermally into guinea pigs.

Treatment	Gomatine (10 mg/kg)		Vehicle	
Animal Number	EXTRAVASATION OF DYE (mm^2) BY HISTAMINE (µg)			
	0.1 µg	0.5 µg	0.1 µg	0.5 µg
1	148	440	166	404
2	52	189	204	420
3	144	243	485	888
4	144	192	345	646
5	536	777	389	627
6	378	805	297	733
7	231	497	210	460
8	24	90	150	410
	Mean ± 232 ± 45	404 ± 84	231 ± 40	574 ±
	P NS	NS		

TABLE XIII

Area of blueing as a measure of the increase in capillary permeability induced by histamine injected intradermally into guinea pigs.

Treatment	Gomatine (20 mg/kg)		Vehicle	
Animal Number	EXTRAVASATION OF DYE (mm^2) BY HISTAMINE (µg)			
	0.1 µg	0.5 µg	0.1 µg	0.5 µg
1	84	144	154	414
2	96	105	254	663
3	60	140	218	304
4	48	144	204	402
5	100	277	264	520
6	78	172	310	485
7	82	184	300	388
8	90	135	248	425
	Mean ± 80 ± 4	163 ± 17	244 ± 17	450 ± 36
	P < 0.001	< 0.001		



A B

Figure 18

Blue patches of the internal surface of guinea pig skins, which received pontamine sky blue (30 mg/kg) intracardiacally followed by separate intradermal injections of 0.1 µg (upper half of photograph) and 0.5 µg (lower half of photograph) histamine. (A) responses of a normal guinea pig and (B) responses of a guinea pig treated with gomatine (20 mg/kg) intraperitoneally 3 hours before the injection of histamine.

while that of the controls was 450 mm^2. Statistical analysis showed that the inhibition of the increase in capillary permeability induced by 0.1 µg and 0.5 µg was significant ($p < 0.001$).

The results of 13 experiments in which guinea pigs were treated with 20 mg/kg gomatine and tested against bradykinin are shown in Table XIV. The mean area of blueing produced by 0.1 µg bradykinin in 13 treated animals was 105 mm^2 while that of the controls was 241 mm^2. Similarly, the mean area of blueing produced by 0.5 µg bradykinin in the treated animals was 365 mm^2 while that of the controls was 642 mm^2. Statistical analysis showed that the inhibition of the increase in capillary permeability induced by 0.1 µg and 0.5 µg bradykinin was significant at the 1% and 5% levels respectively.

4. Passive Cutaneous Anaphylactic Reaction (PCA).

The effect of gomatine on the increase in capillary permeability induced by antigen-antibody reaction was investigated in 6 guinea pigs. The antibody (anti-HSA) was injected intradermally in the following dilutions: 1:100; 1:1000; 1:10000 and 1:100000 and allowed 4 hours to be fixed to the guinea pig skin. Gomatine, in a concentration of 20 mg/ml in ethanol (95%), was injected intraperitoneally into albino guinea pigs in a dose of 20 mg/kg of body weight one hour following the intradermal injection of the anti-HSA. The control animals were similarly injected with the same amount of the vehicle. Three hours following the administration of gomatine, the antigen (HSA) in a dose of 2 mg/kg, was given intracardiacally as a mixture with 30 mg/kg pontamine sky blue into the treated and the control animals.

TABLE XIV

Area of blueing as a measure of the increase in capillary permeability induced by bradykinin injected intradermally into guinea pigs.

Treatment	Gomatine (20 mg/kg)		Vehicle	
Animal Number	EXTRAVASATION OF DYE (mm^2) BY BRADYKININ (µg)			
	0.1 µg	0.5 µg	0.1 µg	0.5 µg
1	176	651	247	874
2	60	234	236	400
3	168	540	171	322
4	198	741	162	680
5	238	882	260	700
6	27	156	189	594
7	48	165	425	924
8	119	464	--	--
9	72	204	--	--
10	21	180	--	--
11	63	165	--	--
12	50	168	--	--
13	126	190	--	--
	Mean $\pm$ 105 $\pm$ 23	365 $\pm$ 81	241 $\pm$ 29	642 $\pm$ 56
	P $<$ 0.01	$<$ 0.05		

The results of 6 experiments are shown in Table XV. Gomatine did not inhibit the increase in capillary permeability induced by the PCA reaction.

TABLE XV

Area of blueing as a measure of the PCA reactions induced by the HSA and Anti-HSA systems in the guinea pigs.

Treatment	Gomatine (20 mg/kg)			Vehicle		
Animal Number	AREA OF DYE EXTRAVASATION (mm^2)					
	ANTIBODY DILUTIONS					
	1:100	1:1000	1:10000	1:100	1:1000	1:10000
1	500	130	49	240	120	30
2	260	170	55	420	180	90
3	340	160	70	540	160	64
4	680	216	144	312	208	96
5	308	182	100	364	188	160
6	320	288	100	410	175	120
Mean ±	401 ± 61	191 ± 20	86 ± 14	381 ± 38	172 ± 11	93 ± 17
P	NS	NS	NS			

PART IV. DISCUSSION

a. Purification of an Antihistamine-like Substance(s) from the Hungarian Oak Galls.

The results of the present studies confirmed previous reports that the extract of the Hungarian oak galls exerted an antihistamine-like activity (Kovacs and Sgabadi, 1950; Kovacs et al., 1952; Feldberg and Kovacs 1960; and Broome et al., 1962). Although several attempts were made to isolate the active principle(s) from the oak gall extracts, as indicated by the results, the study fell short of this objective and resulted, instead, in obtaining a highly purified, tannin-free oak gall extract with an antihistamine-like activity.

In the past 18 years, several methods had been published by which crude extract could be obtained from several species of galls. Kovacs and Szabadi (1950) reported that an extract with antihistamine-like activity was obtained when the 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). Feldberg and Kovacs (1960) confirmed the activity of the oak gall extract, but also noticed 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 O_2 inactivates the antihistamine-like principle(s). 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 of serotonin. In the same year, Broome et al. (1962) obtained a stable extract of the Hungarian oak galls, using the chloroform:methanol (2:1, v/v) mixture for extraction. Calam (1966), who confirmed the antihistamine-like activity of extracts prepared either by ethanol or by the chloroform:methanol method, also reported that the guinea pigs receiving the oak gall extract showed toxic reaction and the toxicity of the extracts appeared to vary according to the extracting solvents. He thus concluded that the observed antihistamine-like activity of the oak gall extract was probably a non-specific effect, produced by the interaction between a toxic substance(s) (probably tannin and 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 serotonin, as reported by Berry et al. (1962), further supported this assumption of non-specific action. However, he did not make an attempt to explain the finding of the same authors, who showed that the extract was ineffective against acetylcholine (Berry et al., 1962). In our view, this observation, in fact, suggested a certain degree of specificity. Furthermore, Calam's explanation did not take into account the previous experimental findings that the tannin-free extract retained its antihistamine-like activity with an increase in specific activity and a concomitant decrease in toxicity (Kovacs et al., 1952; Feldberg and Kovacs, 1960; Broome et al., 1962).

In view of Calam's (1966) conclusion, it appeared important to make an attempt to resolve the question whether the observed antihistamine-like activity of the oak gall extract was in fact, exerted by some specific antihistamine-like substance(s) or that the effect was brought about by some non-specific toxic

reaction. The resolution of this question involves the further purification and possible isolation of the antihistamine-like principle(s). In this respect, it seemed important first to find a method by which a stable and active crude extract could regularly be obtained. The results obtained showed that the method of Broome et al. (1962), subjected to slight modification, was reproduceable. The modification instituted was the increase in extraction time from 1.5 hours to 16.0 hours by the chloroform:methanol (2:1, v/v) mixture. While Broome et al. did not allude to the importance of time as a determining factor in the complete extraction of the antihistamine-like principle(s), we found that the time of extraction was important and that 16.0 hours appeared to be the optimum time.

The toxic effect of tannin present in the crude extract was recognized by early workers (Kovacs and Szabadi, 1950). Two methods had been developed to obtain tannin-free extracts of the oak galls. In 1952, Kovacs et al. (1952) removed the tannic acid by precipitation with lead hydroxide and the antihistamine-like activity of the tannin-free extract was also confirmed by Feldberg and Kovacs (1960). Broome et al. (1962) worked out a method to obtain a stable tannin-free extract by re-extracting the dry chloroform:methanol residue with chloroform. This method had been applied in our laboratory but was abandoned, when the antihistamine-like activity could not be consistently transferred into the chloroform phase. We are not able to offer any explanation at present for this lack of reproduceability. However, it is possible that the great variability in the dry weight of the extract may involve a change in the ratio of the components present in the extract which could influence the solubility of the active substance(s) in chloroform. Ethyl acetate, on the other hand,

was found to be efficacious since all the antihistamine-like activity from the chloroform:methanol residue could be transferred to the ethyl acetate soluble fraction with an increase in specific activity. The ethyl acetate soluble fraction was composed of substances ranging from strongly hydrophilic (e.g., tannin) to highly hydrophobic (e.g., chlorophyll) compounds. As indicated by the results, after the extraction of the ethyl acetate soluble fraction with diethylether, the active principle(s) was found to be present in the ether insoluble residue, which also contains the tannin. This finding could be interpreted that either the a ctive principle(s) has solubility properties similar to that of tannic acid or that the active principle(s) is in some ways bound to the tannin molecules.

Although two methods had been reported by which active tannin-free extracts could be obtained, such preparations were also known to be very labile, making any further purification steps practically impossible (Feldberg and Kovacs, 1960; Broome et al., 1962). Therefore, several methods were tried by which tannin present in the either insoluble fraction could be removed and at the same time, a stable and active tannin-free extract could be obtained. The application of the ether insoluble fraction onto a silica gel G column and the use of ethyl acetate for elution resulted in the separation of the active principle(s), which moves with the solvent front, from the tannin which was strongly adsorbed to the silica gel. The bioassay results of the tannin-free fraction conclusively show that tannin does not contribute to the antihistamine-like activity of the extract and confirmed similar reports by other investigators (Kovacs et al., 1952; Feldberg and Kovacs, 1960; and Broome et al., 1962). The observed antihistamine-like activity of the preparation was accompanied by a marked increase in the specific activity of the extract.

As indicated by the results obtained with the effluent of the sephadex LH-20 column, Fraction I and Fraction VI showed consistent biological activity with a marked increase in the specific activity. In spite of these favourable results, however, there were several disturbing factors, which strongly influenced the outcome of the problem of isolation. The presence of two active fractions could either mean that the oak gall extracts contain at least two active principles or more likely, that the system used resulted in the splitting of the single active principle into two chromatographically distinct areas. Further attempts to isolate the active principle(s) were also hindered by the lack of a reliable a) in vitro or b) chemical assay method by which the active principle(s) could be located in small quantities.

The results of the in vitro experiments showed that the purified oak gall extract did not inhibit the histamine-induced contraction of the isolated guinea pig ileum. In view of the strong in vivo activity of the extract, the reason for the lack of in vitro activity is puzzling. However, it may indicate that the active substance(s) per se has no antihistaminic activity and that the active substance(s) may, in fact, be a metabolite which exists as an inactive precusor in the extract. The possibility that the active principle(s) may be a metabolite is further suggested by the fact that guinea pigs tested 3 hours following the intraperitoneal injection of the extract are less protected from the lethal effect of the histamine aerosol than guinea pigs tested 5 hours later. In contrast to the rather extended period that the oak gall extract requires for optimal activity, most synthetic antihistamines, which are active per se, are optimally effective within 30 minutes after administration.

The tannin-free effluent developed with water:methanol (2 3/4:1, v/v) on the

precoated TLC sheet gave a well defined pattern when treated with o-phosphoric acid (47%). Although 10 - 14 spots were consistently obtained with this fraction, we have not been able to determine which spot(s) was associated with the antihistamine-like activity. Furthermore, a large number of staining methods were used in our attempts to identify the reactive groups associated with Fraction I and Fraction VI, but we were not able to arrive at a satisfactory conclusion.

Probably the most important reason for failing to isolate so far the active principle(s) from the Hungarian oak gall was the unfortunate fact that when the sephadex LH-20 purification method was established, the stock of the Hungarian oak galls was exhausted. Due to the many factors involved in obtaining fresh galls from Hungary, the arrival of the ordered galls was unexpectedly delayed.

Nevertheless, the results of the present studies confirmed the original assumption that the Hungarian oak gall extract contains one or more active substance(s) which exert an antihistamine-like activity. The probability that this antihistamine-like activity is brought about by some as yet unknown substance rather than by some non-specific general toxic effects of the injected extracts as suggested by Calam (1966) is indicated by the continual increase in specific activity with a concomitant decrease in toxicity as the purification of the active principle(s) present in the extract progressed. In this respect our conclusion contradicts Calam's.

b. The Antihistamine-like Activity of Tomatine, Gomatine and the Purified Oak Gall Extract

As the results obtained indicate, the sensitivity of the normal untreated guinea pigs to 0.15% histamine aerosol was essentially identical to that observed by other investigators (Broome et al., 1962; Calam and Callow, 1964; Wakkary, 1968). In the course of our investigations we found, as others did, that the symptoms leading to bronchoconstriction and asphyxial death developed in a fairly uniform sequence in the guinea pigs: cough, dyspnea, swaying, falling, lying and death. A number of studies have been reported in which any one of these symptoms was regarded as the end point of the experiment (Halpern, 1942; Loew et al., 1945; Herxheimer, 1951; Broome et al., 1962; Herxheimer and Stresemen, 1963; Calam and Callow, 1964; Wakkary, 1968). In most instances, the appearance of dyspnea has been taken as the end point. According to Halpern (1942) and Lish et al. (1966), if guinea pigs exposed to histamine aerosol were removed from the aerosol chamber when signs of coughing or dyspnea appeared, the animals could be re-exposed 2 - 4 hours later without significant change in the predyspneic interval. On the other hand, Wakkary (1968) reported that the dyspnea time of guinea pigs on second exposure, within 2 - 4 hours, to the histamine aerosol changed in a large percentage of animals. In view of the foregoing contradiction, the method of Broome et al. (1962) that is, the cessation of respiration as the end point of the experiment, was selected as a routine method of testing.

The capillary permeability test is a common method used to evaluate the specificity and potency of potential antihistamines. Bain et al. (1948) reported

that there is an inverse linear relationship between the logarithm of the dose of the antihistamines and the area of the flare induced by histamine. As indicated by the results, all three substances - the purified oak gall extract, tomatine and gomatine - were able to antagonize the histamine-induced increase of the capillary permeability. This finding is interesting, since it is well established that only the most specific and potent antihistamines can antagonize this phenomenon (Celice and Durel, 1942; Parrot and Lefebvre, 1943; Rocha e Silva, 1955).

The use of the systemic anaphylactic shock to evaluate antihistaminic drugs was initiated as early as 1937 by Staub and Bovet (1937) and has since been used by many others (Halpern, 1942, 1947; Mayer et al., 1945; Feinberg et al., 1950). It is well established that potent antihistamines can prevent the death of guinea pigs from anaphylactic shock and this fact serves to indicate that histamine during anaphylactic shock plays a major role in the death of the guinea pigs (Nodine and Seigler, 1964; Kabats and Mayer, 1961). In our experience, the mean death time of the sensitized guinea pigs receiving 2 mg/kg egg albumen intracardiacally was 4.2 minutes, with 86.5% expiring between 2 - 4 minutes; 10.8% expiring between 4 - 30 minutes; and 2.7% surviving the 30 minutes period of observation time. Thus, the anaphylactic shock method provided a sensitive indicator for testing the activity of the antihistamine-like substances.

The passive cutaneous anaphylactic (PCA) reaction, described first by Ovary ( ), utilizes one of the fundamental characteristics of the immediate allergic reaction, i.e., the liberation of vasoactive substances and their action

on the minute vessels of the skin. If the amount of the antibody used for the intradermal sensitization is not very great, potent antihistamines may inhibit the reaction (Ovary, 1958; Rocha e Silva, 1966; Movat et al., 1967). The animal of choice for the PCA reaction is the guinea pig, because of the ease with which good PCA reactions are elicited and of the major role played by histamine in this species (Ovary, 1964). In connection with our study, we found that the PCA reactions were easily produced and the size of the wheals ranged from 30 mm^2 to 160 mm^2. This wide range of reaction in the guinea pig is, unfortunately, inherent and is ammended only by using an adequate number of animals for a greater statistical certainty.

The results of the present study, using the histamine aerosol, anaphylactic shock, capillary permeability and PCA methods, confirmed the previous observations that all three substances, i.e., the highly purified oak gall extract, tomatine and gomatine exerted antihistamine-like activity (Kovacs and Szabadi, 1950; Kovacs et al., 1951; Broome et al., 1962; Wakkary, 1968). In contrast to the findings of Wakkary (1968) in our <u>in vivo</u> experimental results it showed that tomatine exerted antihistamine-like activity when tested by the histamine aerosol or the capillary permeability method, but it did not protect the sensitized guinea pigs from a lethal anaphylactic shock. Dr. T.H. Chen (Dept. Chemistry, McGill University), who is presently engaged in the elucidation of the chemical structure of gomatine, indicated that the so-called chemically pure tomatine obtained from the crown-gall of tomato stalks, is contaminated by gomatine, a substance which bears close chemical resemblance to tomatine and possesses antihistamine-like activity (Wakkary, 1968). Our observation that the antihistamine-like activity of freshly prepared tomatine decreased

with time and the finding that antihistamine-like activity of gomatine quickly deteriorated (Wakkary, 1968), might indicate that the ability of tomatine to inhibit both the histamine-induced bronchoconstriction and increased capillary permeability could simply have been brought about by the presence of a minute quantity of gomatine in the tomatine preparation. While it cannot be stated that gomatine, at the dose level investigated, is a very potent antihistamine, our finding is in accord with those of Wakkary (1968) who also reported that gomatine exerted a strong protection against the lethal effects of a histamine aerosol.

In the present study, care was taken to use only freshly prepared gomatine for all the tests, because of the ease with which the substance was inactivated. As was the case with tomatine, the intraperitoneal injection of gomatine in a dose of 20 mg/kg of body weight into guinea pigs produced abdominal tension and irritation. These symptoms cannot be accounted for by the 80% ethanol used as vehicle to dissolve gomatine, because the symptoms were not observed immediately after the intraperitoneal injection but were detected one hour after the injection. Furthermore, control guinea pigs receiving the same amount of the vehicle showed no signs of abdominal irritation and tension. In view of the observed toxic symptoms, the dose of gomatine was not increased beyond the 20 mg/kg level. Gomatine also inhibited the increased capillary permeability effect of intradermally injected bradykinin. These results confirmed the <u>in vitro</u> findings of Wakkary (1968) that gomatine was equally effective in antagonizing the contraction of the isolated guinea pig ileum induced by histamine and bradykinin. Under these circumstances, the specificity of gomatine is not at all clear. On the one hand, Wakkary (1968) was able to demonstrate clearly

in his in vitro tests that the effect of gomatine is dose dependent. On the other hand, its almost equal inhibiting capacity against a number of chemical mediators is disturbing, since if this is really the case, then gomatine should, theoretically, be a potent anti-anaphylactic agent, which it is not.

In contrast to the toxic effects imparted by tomatine and gomatine, the purified oak gall extract showed very little toxicity. Central effect, which was frequently observed in guinea pigs treated with tomatine and gomatine, was absent in animals treated with the purified oak gall extracts.

Since the active principle(s) has not been isolated, it is not possible to state whether the antihistamine-like activity of the purified extract is the attribute of a single or a group of closely related substances. However, within the parameter of this preliminary investigation, there is some evidence which indicates that the observed antihistamine-like activity of the purified oak gall extract is probably specific. It was clearly demonstrated in the anaphylactic shock and capillary permeability tests that the effect of the extract was dose dependent. The complete lack of activity against the bradykinin-induced increase in capillary permeability, further suggests that the purified oak gall extract probably acts more or less specifically against histamine.

Results obtained from the PCA reaction showed that none of the three substances tested antagonized the increase in capillary permeability induced by the antigen-antibody reaction. The present study, however, only investigated the heterologus PCA reaction (HSA and anti-HSA system), which is known to be quite insensitive to antihistamines (Craig and Wilhelm, 1962; Harada et al., 1966).

Due to the great variability in the reactivity of the guinea pigs, even if a slight inhibition did occur, it would have not been detectable. Admittedly, the effect of the purified oak gall extract should have been investigated in homologous PCA reaction, especially since Movat et al. (1967) showed that the cutaneous anaphylactic reaction in the guinea pig is mediated through at least two antibody types: the anaphylactic γ_1 antibody and the anaphylactic γ_2 antibody. They were able to demonstrate that PCA mediated through the γ_1 antibody was suppressed by antihistamines because γ_1 sensitizes and disrupts the mast cells to release histamine, which in turn provoked the observed cutaneous anaphylaxis, while the PCA mediated through the γ_2 antibody did not respond to antihistamine pretreatment because the γ_2 PCA is mediated through the release of lysosomes from the polymorphonuclear leucocytes.

SUMMARY

1. The method described by Broome et al. (1962), using the chloroform:methanol mixture (2:1, v/v) to obtain a stable crude extract of the Hungarian oak galls, exerting antihistamine-like activity, was adopted as the starting point of the purification procedures.

2. The active stable crude extract obtained with the method described by Broome et al. (1962) was further purified by extracting the chloroform: methanol residue with anhydrous ethyl acetate.

3. In order to remove the biologically inactive chlorophyll from the ethyl acetate soluble residue, it was extracted with ether absolute.

4. The active principle(s) was separated from the biologically inactive and highly toxic tannin by submitting the chlorophyll-free ethyl acetate soluble fraction to silica gel G column chromatography.

5. Further purification of the active principle(s) was achieved by re-extracting the tannin-free residue with anhydrous methanol, in which the active principle(s) was soluble.

6. The methanol soluble fraction was applied onto a sephadex LH-20 column and the effluent was divided into 6 fractions on the basis of their optical densities. The results of the bioassays showed that only Fraction I and Fraction VI possessed antihistamine-like activity. According to the TLC

studies, these active fractions contained at least 4 - 8 components.

7. The active fractions from the sephadex LH-20 column chromatography showed a marked increase in specific activity when compared to the crude extract obtained with the method of Broome et al. (1962). 20 mg/kg of Fraction I or Fraction VI provided the same protection as 550 mg/kg of the crude extract.

8. Neither the purified oak gall extract nor the two active fractions from the sephadex LH-20 column chromatography, added to the organ bath in a bath concentration of 10^{-4} gm/ml, inhibited or modified the histamine-induced contractions of the isolated guinea pig ileum preparation.

9. The antihistamine-like activity of the purified oak gall extract was compared to those of tomatine and gomatine (isolated by the method described by Wakkary, 1968) and it was found that a single intraperitoneal injection of all three substances into guinea pigs, in doses of 80 mg/kg, 30 mg/kg and 20 mg/kg respectively, significantly increased the survival time of the treated animals when exposed to the lethal effect of a 0.15% histamine aerosol.

10. The increase in capillary permeability induced by histamine was significantly inhibited in guinea pigs, when pretreated with 80 mg/kg purified oak gall extract, 30 mg/kg tomatine and 20 mg/kg gomatine respectively.

11. The increase in capillary permeability induced by bradykinin was inhibited

in guinea pigs pretreated with 30 mg/kg tomatine or 20 mg/kg gomatine (Wakkary, 1968) but not by 80 mg/kg purified oak gall extract.

12. Actively sensitized guinea pigs pretreated with 80 mg/kg purified oak gall extract or 20 mg/kg gomatine were protected from anaphylactic shock following the antigenic challenge.

13. Pretreatment of guinea pigs with either 80 mg/kg purified oak gall extract, 30 mg/kg tomatine or 20 mg/kg gomatine did not inhibit or modify the heterogeneous PCA reaction.

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