Ph.D. Experimental Medicine

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ISOLATION OF BIOLOGICALLY ACTIVE SUBSTANCES FROM CROWN-GALL TUMOR OF TOMATO.

ABSTRACT

The work presented in this thesis describes methods by which biologically active substances could be isolated from extracts of crown-gall infected tomato stalks. Two active principles: tomatine and gomatine were isolated.

Tomatine isolated from crown-gall infected tomato stalk extract was found to inhibit contractions of the isolated guinea-pig ileum preparation induced by: histamine, acetylcholine, bradykinin and barium chloride.

Gomatine when assayed in vivo in guinea-pigs exerted significant protection against the effect of a lethal histamine aerosol. Gomatine also protected guinea-pigs actively sensitized to egg albumin against anaphylactic shock. When assayed in vitro, gomatine inhibited the contractions induced by histamine, bradykinin, acetylcholine, 5-HT, and SRS-A. Some chemical properties (including melting point, infrared spectrum and elementary analysis) of gomatine were determined.

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-by-

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THESIS

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INTRODUCTION

The observation that extracts of plant tumors such as oak-gall and crown-gall when injected into guinea-pigs protected the animals against a subsequent lethal dose of histamine aerosol has been confirmed by several workers in the past 16 years. It was also reported recently that the protection elicited by a single intraperitoneal injection of partially purified extracts prepared from crown-gall infected tomato plants lasted for several days and sometimes for a few weeks. These findings demonstrated that the extracts contained active principle(s) which exerted some unusual and novel biological activity.

In the present study an attempt was made to isolate the active principle(s) from extracts of crown-gall infected tomato stalks. Methods by which crystalline substance(s) were isolated and some chemical and biological characterizations of the isolated substance(s) are the subject of this thesis.

PART I. HISTORICAL REVIEW

Section A. Anaphylaxis and Allergy.

a. Introduction.

A shock-like condition produced in animals by a single injection of proteose-peptone mixture was observed by Schmidt-Mulheim as early as 1880 and by Fano in 1881. However, the striking similarity of symptoms produced by intravenous injection of the peptone mixture and that of anaphylactic shock was recognized only in 1909 (Biedl and Kraus), after the general acceptance of Richet's basic discovery which he named anaphylaxis.

Richet's first observations in the field of anaphylaxis was made in 1898 using the serum of eels. This serum when given intravenously to a dog was somewhat toxic on first administration but caused sudden death whenever it was injected into the same animal for the second time. Other observations which were made by Richet and Portier (1902) concerned the toxicity of extracts of actinaria. From these extracts Richet obtained, by alcoholic precipitation, a substance which, when injected into dogs, produced intense congestion of the organs with hemorrhages into the peritoneum, and which he therefore called "congestine". Like the eel serum, congestine was toxic on the first dose, but much more toxic when treatment with it was repeated. Richet called this reaction to the second dose "anaphylaxis".

In 1903, Arthus observed that when small doses of horse serum were injected subcutaneously into rabbits at weekly intervals, the latter

doses in such a series were followed by local areas of inflammation which was extensive and severe, frequently progressing to necrosis. This peculiar reaction of sensitized rabbits to subcutaneous treatment with the specific protein is frequently referred to as the "Arthus Phenomenon". In 1904, Theobald Smith (Lewis, 1908) observed that guinea-pigs treated repeatedly with small amounts of horse serum, frequently died suddenly after a later injection. For this sudden death he had no explanation but two years later, in 1906, Otto published his work on "Das Theobald Schmithsche Phänomen der Serumüberempfindlichkeit", in which he described what we now recognize as clear cut anaphylaxis.

In 1906, Rosenau and Anderson described the first of their beautifully planned experiments, published between 1906-1910, in which they worked out the details of many of the fundamental features of anaphylaxis. At the same time, Von Pirquet (1906) recognized, on the basis of clinical observations and experiments, 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".

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

positive skin reactions with the extracts of the offending substances.

From 1910 on, research on anaphylaxis rapidly extended into many different phases of the problem. Numerous theories have been put forward to explain this phenomenon. The two most important theories,, formulated nearly at the same time, will be discussed here. They are the so called "humoral" or "anaphylatoxin theory", put forward in 1909 by Friedberger and the "cellular theory" brought to life following the observations of Schultz in 1910 and of Dale in 1912.

b. Humoral or Anaphylatoxin Theory of Anaphylaxis.

Though the anaphylatoxin theory had been neglected for many years it will be discussed first. The reason for this is a renewed interest in the subject in the past 15 years and also the fact that this theory was put forward earlier than the cellular theory. The first comprehensive theory of anaphylaxis was in fact the humoral or anaphylatoxin theory. However, it soon fell into general discredit not only because it could not explain the experimental fact that anaphylaxis in the guinea-pig was a phenomenon of smooth muscle stimulation, but mainly because it had been early established by Dale and others that in well washed sensitized guinea-pig tissues, free of apparent serum requirement, anaphylaxis could still take place. Anaphylatoxin was first described by Friedberger (1909). He incubated normal guinea-pig serum with washed, preformed immune precipitate, spun the preparation, and injected the supernatant into guinea-pigs. The animals developed an acute bronchoconstriction, characteristic

of anaphylaxis in this species.

The toxic principle formed in the serum during incubation with the immune precipitate was named "anaphylatoxin" by Friedberger. He also demonstrated that heating of the serum at 56°C would destroy its capacity of generating anaphylatoxin, when incubated with the immune precipitate. This, according to Friedberger, was an indication that complement might play a part in the formation of the toxic principle. However, Keysser and Wasserman (1911) demonstrated that a similar toxic principle was generated when the serum was treated with inert dust-like materials, such as kaolin and barium sulphate. Furthermore, Bordet (1913) showed that the incubation of the serum with agar suspension also generated anaphylatoxin. Likewise, this substance could be generated by incubating the serum with starch or inulin (Jobling and Petersen, 1915).

The explanations of the mechanism by which normal guinea-pig serum could be rendered toxic by contact with such diverse substances as agar, immune precipitates, kaolin, barium sulphate, starch and inulin, varied considerably, among the numerous workers on that subject. Practically all of them agreed that a chemical or enzymatic mechanism was involved and that the enzymatic process was mainly one of proteolysis. However, in the original form of the theory, there was the assumption that the proteolysis of the antigen-antibody complex generating toxic principles would be the consequence of the incubation of the serum with that complex. The fact that agar as well as starch and inulin were capable of generating

anaphylatoxin led to a new development of the theory by assuming that the normally occuring proteases of the plasma would release the toxic product. In this form, the theory was strongly supported by Jobling and Petersen (1915) and Bronfenbrenner (1915). According to this theory, the plasma proteins themselves and the split products would actually constitute the anaphylatoxin. However, the anaphylatoxin theory fell into general discredit after the important publication by Dale and Kellaway, in 1922. In this paper they demonstrated that upon the addition of anaphylatoxin to the isolated guinea-pig uterus preparation, the uterus showed only an irregular contraction. On the other hand the addition of the specific antigen to the isolated uterus of a sensitized guinea-pig, which had been thoroughly washed and was definitely free of any serum factor, brought about a strong contraction.

During the following 30 years, the humoral theory was almost completely forgotten. Then, in 1950, Hahn and Oberdorf (1950) demonstrated that antihistamines were able to protect guinea-pigs against the systemic effect of anaphylatoxin. This observation provided an entirely new basis for reinvestigation of the mechanism of action of anaphylatoxin. One year later, Rocha e Silva et al. (1951) made the observation that anaphylatoxin released large quantities of histamine from the isolated, perfused guinea-pig lung. Giertz et al. (1961) reported that the intravenous injection of anaphylatoxin into guinea-pigs resulted in an increased plasma histamine level and the animals frequently died. They also observed that as they increased the dose of anaphylatoxin injected

to these animals, the plasma histamine level and incidence of mortality correspondingly increased. On the other hand they did not find any increase in plasma histamine level of the same species during a lethal anaphylactic shock. From these findings, they concluded that during anaphylatoxin shock in guinea-pig, extrapulmonary histamine was predominantly involved, whereas in anaphylactic shock, intrapulmonary histamine was most probably responsible for the reaction. This observation was later confirmed and extended by Schmutzler et al. (1963). They showed that blood histaminase activity was also increased during anaphylatoxin shock and to a lesser degree during anaphylactic shock in guinea-pigs. These workers suggested that the plasma histamine level could be determined accurately by the use of histaminase inhibitor such as, aminoguanidine, added to the blood.

Osler et al. (1959) demonstrated that parenteral administration of EDTA into rats and guinea pigs prevented the production of anaphylatoxin. Vogt (1963) confirmed this observation and suggested that the formation of anaphylatoxin in the plasma was brought about by a metal requiring enzyme. Vogt and Schmidt (1966) presented evidence that anaphylatoxin was formed in rat plasma by the action of a specific enzyme on its substrate anaphylatoxinogen. They found the enzyme to be a metallo-protein with essential carboxyl groups.

Mota (1959) demonstrated that heating the sensitized guinea-pig ileum at 46°C abolished its response to the specific antigen but did not suppress the response to anaphylatoxin. Stegemann et al. (1964a) demonstrated

that anaphylatoxin resisted heating at 80°C and pH 2 for one hour, and at 60°C and pH 11 for 30 min. On the other hand, they showed that anaphylatoxin could be destroyed by proteinases and carboxypeptidases. From its behavior on ion exchangers, they suggested that anaphylatoxin had basic properties. Friedberg et al. (1963) were able to purify anaphylatoxin obtained from rat plasma by gel filtration on Sephadex G-100. An active fraction was obtained which produced fatal shock in guinea-pig at a dose of 30 µg/100 grams body weight. Stegemann et al. (1964b) showed that the N-terminal amino acid of anaphylatoxin from rat plasma was arginine and that the sequence was probably Arg-Leu-Ala. Hog anaphylatoxin, however, probably possessed the sequence: Arg-Glu-Ala or Arg-Asp-Ala. Stegemann et al. (1965) reported that mannose and an unidentified sugar were probably essential components of anaphylatoxin.

According to Austen and Humphrey (1963) serum anaphylatoxin, at best, offers an alternative pathway to histamine release.

In summary, all the available evidence points to the fact that anaphylatoxin most probably belongs to a class of compounds capable of liberating histamine.

c. Cellular Theory and Histamine in Anaphylaxis and Allergy.

In contrast to the "humoral theory" which states that the anaphylactic reaction starts with the union of antigen and antibody in the serum and that the antigen-antibody complex thus formed results in the formation of anaphylatoxin, the "cellular theory" postulates that the union of antigen and

antibody takes place on the cell surface and that the combination of the antigen with its antibody leads to the release of "histamine-like substances" which cause the anaphylactic or allergic syndromes.

The first experimental evidence in favour of the "cellular theory" was presented by Schultz, in 1910. He showed that an isolated preparation of small intestine of a sensitized guinea-pig responded by contraction to the application of the specific antigen. However, the most convincing piece of evidence, and actual formulation of the theory, was put forward by Dale in 1912. He demonstrated that the addition of the specific antigen to a thoroughly washed (i.e., free of any serum or blood), sensitized guinea-pig uterus, elicited a strong contraction of the tissue. Dale also demonstrated that the contraction on exposure to the antigen was a specific response and could not be obtained even by higher concentrations of indifferent protein.

The release of histamine-like substances resulting from the combination of the specific antigen with its antibody on the cells of the so called "shock organ" of a sensitized animal, as will be discussed later on in this chapter, constitutes the main argument in favour of the cellular theory of anaphylaxis.

Although histamine had been synthesized by Windaus and Vogt in 1907, its role in anaphylaxis was not known until it was extensively investigated by Dale and Laidlaw, in 1910. They observed that guinea-pigs, when injected with histamine, developed a condition which strongly resembled peptone and anaphylactic shock. They also showed that the uterus of the virgin guinea-pig would react to histamine in a concentration as

low as 4×10^{-9} g/ml. These observations were subsequently confirmed and extended by many workers.

The important role of histamine in anaphylactic and allergic symptoms was further supported by the classical observations made by Sir Thomas Lewis between 1924-1927. He found that the intradermal injection of histamine induced the sequence of phenomena named "triple response". He also demonstrated that the so called urticarial reaction which developed in the skin of allergic individuals after injection of the specific antigen was similar to the triple response elicited by histamine. On the basis of such an impressive analogy Lewis postulated that a substance similar to histamine (H-substance) would be liberated in the skin of allergic individuals during allergic manifestations.

In 1927, Harris, and Best et al. established beyond doubt that histamine is a natural constituent of the body tissues. In the same year, Dale (1927a, 1927b, 1927c) argued that Lewis' "H-substance" was histamine itself.

The first direct and conclusive evidence which proved that the liberation of histamine was a consequence of the combination of the antigen with its antibody in the tissue of the sensitized guinea-pig was presented by Bartosch et al. (1932) and by Dragstedt and Gebauer-Fuelnegg (1932). They perfused the lungs of a sensitized guinea-pig with Tyrode solution. Artificial respiration was used and the respiratory movements were recorded. When the antigen was added to the perfusing Tyrode solution, the respiratory movements of the animal

stopped. The perfusate of the sensitized guinea-pig was collected. When this perfusate was added to the perfusing Tyrode solution of a similarly prepared non-sensitized guinea-pig, its respiratory movements also stopped. The symptoms observed in the non-sensitized guinea-pig were similar to those of the sensitized guinea-pigs 1. cessation of respiratory movements; 2. strong bronchoconstriction. When the same antigen was added to the perfusing Tyrode solution of the similarly prepared non-sensitized guinea-pig, its respiratory movements did not stop and no bronchoconstriction was observed. The substance appearing in the collected perfusate of the sensitized guinea-pig was found to be pharmacologically identical with histamine, in its action upon the isolated guinea-pig ileum preparation, blood pressure of the anesthetized cat and also in its capacity of releasing epinephrine from the adrenal gland of the cat.

In subsequent years, the release of histamine in various species during the anaphylactic reaction was demonstrated successfully by many workers. However, the role of histamine in the symptoms exhibited in anaphylaxis varies greatly from species to species.

d. The Role of Histamine in Anaphylaxis.

1. Guinea-pig.

That the lung is the "shock organ" and the circulatory collapse secondary, is shown by the fact that bronchiolar constriction and acute emphysema can be produced in vitro in the perfused guinea-pig lung (Dale, 1912).

As mentioned above, Bartosch et al. (1932) found histamine in the perfusates of sensitized guinea-pig lungs. This evidence was confirmed and extended by Daly et al. (1934) who also showed that the yield of histamine in the perfusates of 38 guinea-pigs varied from 0.17-12.8 ug when egg albumin was used as the antigen. This basic experiment has been confirmed by many workers (Daly and Schild, 1934; Bartosch, 1935; Brocklehurst, 1960). Histamine release has also been demonstrated in passive anaphylaxis (Schild, 1936a) as well as in chopped sensitized guinea-pig tissues, such as lungs, large arteries, veins and small intestines. From these sensitized chopped tissues suspended in physiological solutions, large amounts of histamine were liberated following the application of the antigen (Ungar and Parrot, 1936; Schild, 1939; Mongar and Schild, 1952; Brocklehurst, 1960; Austen and Brocklehurst, 1960). The liberation of histamine has also been demonstrated on isolated preparation of uterus, small intestine and colon of sensitized guinea-pig, when challenged with the antigen (Alberty and Schiede, 1953; Paton, 1958).

2. Dog.

In 1925, Manwaring et al. in an ingenious set of cross-circulation experiments demonstrated the antigen-induced release of a vasodepressor, smooth muscle stimulating principle from dog liver. Dragstedt and his associates (1932, 1936) subsequently identified histamine in the thoracic duct lymph and peripheral blood, and extraction of histamine from peripheral blood of dogs in anaphylactic shock was accomplished by Code (1939).

Support for the contention that liver is the "shock organ" and histamine the mediator comes from several further observations. It was demonstrated by Ojers et al. (1941) and by Scroggie and Jaques (1949) that histamine was released from the sensitized dog liver in vitro after the appropriate antigen was added to the perfusate.

Feldberg (1956) showed that dog liver contained a relatively large amount of histamine in comparison to that found in most other species.

Akcasu and West (1960) demonstrated that the depletion of liver histamine by 48/80 pretreatment in sensitized dogs abolished the usual anaphylactic reaction to a challenge with antigen for at least 17 days.

3. Rabbit.

In rabbits the anaphylactic shock is characterized by circulatory collapse. Auer (1911) and Scott (1911) suggested that the anaphylactic circulatory collapse of rabbits was related to acute dilatation of the right heart. Airila (1914) attributed the right heart dilatation to an increase in pulmonary artery pressure. The experimental works of Dragstedt et al. (1940) and of Rose (1941a), however, indicated that the circulatory collapse was most probably due to the mechanical plugging of pulmonary capillaries brought about by microthrombi.

Katz (1940) demonstrated that addition of antigen to sensitized rabbit blood caused a shift of histamine from cells to plasma. This observation was confirmed almost immediately by Dragstedt et al. (1940)

and by Rose and Browne (1941).

Humphrey and Jaques (1955) demonstrated that antigen-antibody interaction in vitro released histamine from rabbit platelets.

Waalkes et al. (1957), Udenfriend and Waalkes (1959), Waalkes and Coburn (1959, 1960b) observed that anaphylactic shock in the rabbit was associated with an increase of plasma histamine, a reduction in total blood histamine because of a diminution in circulating platelets and leucocytes, and an increase in lung histamine in association with platelet-leucocyte clumps.

4. Rat.

It has been known for a long time that rats are difficult to sensitize to foreign proteins (Longcope, 1922). When sensitization is induced with a mixture of the antigen and an accessory agent such as Bacillus pertussis vaccine, anaphylactic shock occurs when the challenge is made with the specific antigen.

The anaphylactic shock in rat is characterized by circulatory collapse and increased peristaltic activity. West (1959) has suggested that in this species the gut is the "shock organ".

Mota (1957) demonstrated a rise in plasma histamine concentration, during active anaphylaxis of the rat sensitized with horse serum. Furthermore, Mota and Ishii (1960) in their in vitro experiments, using mesenteries from actively sensitized rats (B. pertussis and horse serum) demonstrated that the addition of the antigen resulted

in mast cell degranulation and histamine release. However, in passively sensitized rats, Mota (1962) observed neither rise in plasma histamine nor histamine release from mesenteries upon the application of the specific antigen. Austen and Humphrey (1961) reported that addition of antigen to isolated peritoneal mast cells or to preparation of mesentery from passively sensitized rats failed to release histamine, when bovine serum albumin or human gamma globulin were employed as antigens.

5. Mouse.

Anaphylaxis in the mouse is characterized by respiratory distress (Waalkes and Coburn, 1960a) and prostration (Gershon and Ross, 1962).

It was demonstrated by Mayer and Brousseau (1946) and by Pittman (1951) that normal mouse tissue was fairly insensitive to histamine.

Waalkes and Coburn (1960a) observed an increase in blood and lung histamine during systemic anaphylaxis in the mouse. However, in their in vitro experiment using the isolated lung of a sensitized mouse no histamine could be detected following the addition of specific antigen.

6. Man.

Rose (1941b) studied patients with various forms of allergy including asthma and observed that the blood histamine level was unstable relative to normals, but it did not increase during an asthmatic

attack when measured in blood samples taken from the femoral artery or right heart (Rose et al., 1950).

Evidence was presented by Schild et al. (1951) that isolated human bronchial muscle preparations, obtained from an asthmatic patient, contracted and released histamine when specific allergen was added to the organ bath,in which the bronchial chain was suspended. Though bronchial rings obtained from normal persons were fairly sensitive to histamine, they did not respond to the specific allergen. An antihistaminic agent (mepyramine) in a very low concentration (10^{-9} g/ml) halved the contractions elicited by histamine. However, the contractions of the bronchial chains elicited by the allergen were only slightly affected even by much higher concentration of mepyramine (10^{-5} g/ml). These experiments clearly showed that in asthmatic patients, not only histamine, but some other smooth muscle stimulating substance(s) was also released.

Some further evidence about the possible role of histamine in acute allergic reactions in man came from the study of Lecomte (1956). The injection of histamine releasers produced a reaction which was characterized by pruritus, skin erythema, a reduction in blood pressure, supra and infra orbital edema, and finally full blown generalized angioneurotic edema with or without nausea or vomiting. The reaction was associated with a three-fold rise in plasma histamine and could be abolished by pretreatment with antihistamines. Thus, at least, the pruritus, urticaria and some portion of the hypotension seen

during anaphylaxis in man, can probably be attributed to histamine release.

In summary, the fact that histamine is present in substantial amounts in the "shock organ" of guinea-pig and dog and the fact that in anaphylaxis it is liberated from these organs, suggest that histamine is an important mediator in the anaphylactic reactions in these species. On the other hand, histamine does not seem to play an important role in systemic anaphylactic reactions in rat and mouse, mainly because of the insensitivity of the tissues of these species to histamine. In the rabbit, in anaphylactic shock, although histamine is probably released directly into the pulmonary circulation, it is possible that the pulmonary emboli composed of immune precipitates may be more important than the appearance of this chemical mediator. Austen and Humphrey (1963) expressed the view that in man histamine probably accounts for only a limited number of the manifestations of anaphylaxis, e.g., pruritus, urticaria, angioneurotic edema and some hypotension.

e. Mechanism of Histamine Release.

In spite of the vast number of papers published in the past 40 years concerning this subject, in fact we know very little about the intimate mechanism of histamine release following the reaction of antigen with cell bound antibody. However, today we know that most of the released histamine originates from one special type of cells: the mast cells.

The classical paper of MacIntosh and Paton (1949) suggested to Riley (1953) a means of discovering whether a release of histamine was in fact accompanied by morphological changes in the mast cells. Riley and West, in 1953, demonstrated that tissues from rats treated with compound 48/80 showed a decrease in mast cell content which was accompanied by a fall in tissue histamine. On the other hand, mouse skin treated with carcinogen showed an increase in mast cell content accompanied by an increase in tissue histamine. They found a few mast cells in embryonic tissues and little histamine; whereas in certain adult tissues, e.g., in ox liver capsule and pleura, they found many mast cells and much histamine (200-300 µg/g). In pathological tissues, i.e., mast cell "tumor" from a child, they found large amounts of histamine (950 µg/g) and for a series of mast cell tumors from dogs they found values up to 1290 µg of histamine/g tissue.

Though Riley and West's (1953) discovery, which proved that the mast cells are the main source of the released histamine in the body, initiated many interesting new studies, the cytological basis of histamine release is still imperfectly understood. While it is not within the scope of this thesis to review in detail the theories of the mechanism of histamine release, for the sake of completeness, we shall summarize the most important data known concerning the liberation of histamine from the mast cells.

At present it seems a well established fact that the combination of a specific antigen with cell bound antibody, as well as a large

number of different substances, liberate histamine from the granules of mast cells. These substances may be grouped as follows:

1. Sensitizing compounds, which typically need more than one application to an animal to cause histamine release, which produce specific antibodies and which are usually of protein nature or are able to react with protein in the body to form an effective antigen.
2. Those compounds which produce damage to tissues, such as venoms and bacterial toxins.
3. Proteolytic enzymes, e.g., trypsin.
4. A group of surface active substances. Typical members are the detergent Tween 20 which Krantz et al. (1948) showed could cause generalized urticaria in the dog as well as other anaphylactoid signs and bile salts which Schachter (1952) found to release histamine from isolated, perfused cat skin.
5. Large molecular compounds, such as dextran in the rat, reported by Edlund et al. (1952), horse serum in the cat, shown by Feldberg and Schachter (1952), polyvinylpyrrolidone in the dog, as demonstrated by Halpern and Briot (1953) and also anaphylatoxin.
6. The group of bases known as histamine liberators.

The last group of compounds stemmed from the work of Alam et al. (1939), who showed in the course of studies on muscle, that curarine was able to release histamine from this tissue. It was later found by MacIntosh and Paton (1949) that the ability to release histamine extended to a very wide range of organic bases. They found that any compound pos-

sessing two or more basic groups carried on and separated by a sufficient aliphatic or aromatic scaffold is liable to have histamine releasing property. As demonstrated by MacIntosh and Paton, these organic bases had a rather characteristic type of action, exemplified in a simple way in the so called "delayed depressor response". When injected into a cat under chloralose anesthesia, histamine caused a fall in blood pressure in 5-8 sec. However, during this time, the histamine liberators showed no action at all; it was not until 20 or 30 seconds had elapsed that a depressor response occurred. When it came, however, it was often as rapid as with histamine alone. The absence of action during the initial latent period was a nice demonstration that the drug itself had no direct effects on the blood vessels.

On the basis of these observations, Paton (1956) expressed "the possibility, that there is in fact no single mechanism, even at the most fundamental level". He suggested three basic mechanisms to account for the release of histamine:

i) antigen-antibody interaction produces a membrane disturbance which in turn leads to histamine leakage through a damaged membrane.

ii) antigen-antibody interaction causes protease activation, which results in splitting off of histamine from a protein.

iii) antigen-antibody interaction causes protease activation, which leads to the release of peptides and bases. The latter compete with histamine for the "complex tissue acid" on the basis of ion exchange. Thus any base with a greater affinity for the acid would free histamine.

f. Discrepancies in the Histamine Theory of Anaphylaxis and the Possible Role of Other Pharmacologically Active Substances in Anaphylactic and Allergic Manifestations.

The principal feature of the anaphylactic reaction of an isolated smooth muscle preparation is the contraction of the muscle, supposedly due to histamine release. However, there are a number of situations where histamine either does not induce contraction or actually causes relaxation of smooth muscle, while the specific antigen causes contraction. For example, Kellaway showed as early as 1930 that histamine administration induced a relaxation of the isolated sensitized rat uterus preparation, however, the latter responded with a strong contraction to the application of the specific antigen.

Schild (1936b) demonstrated that the histamine desensitized isolated uterus of a sensitized guinea-pig gave a maximal contraction in response to the addition of specific antigen to the organ bath in which the uterus was suspended.

The discovery of the specific antihistaminic agents (Bovet and Staub, 1937), which will be discussed later on in this section, has permitted a more direct approach to the study of the role of histamine in anaphylactic and allergic manifestations.

Wells et al. (1946), in their in vitro studies using antihistamines, reported that these drugs were less effective in antagonizing the anaphylactic reaction than they were in antagonizing the effects of histamine. Schild et al. (1951), using human asthmatic bronchial

chains, demonstrated that low concentrations of mepyramine (10^{-9} g/ml) halved the contractions elicited by histamine, while the reaction to the specific antigen was not abolished completely even by much higher concentrations (10^{-5} g/ml) of the same drug.

In an effort to explain these discrepancies, Dale (1950) suggested the possibility that histamine would be maximally effective when released in, or in a most intimate relation to, the smooth muscle cell. Under these conditions the histamine would have access to receptor sites on the cell which could not be reached by antihistamines or by histamine added from the outside. However, a more probable explanation was put forward by Schild (1956), who attributed these discrepancies to the release of smooth muscle stimulants or histamine-like substances, the actions of which were not antagonized by antihistamine drugs.

A number of mediators have been isolated in the past 15 years. Most of these substances have pharmacological activity very similar to histamine and all of them seem to be released during anaphylaxis. Their possible role in anaphylaxis and allergy will be discussed next.

1. <u>5-Hydroxytryptamine (5-HT), Serotonin</u>.

5-HT was first described by Erspamer in 1940 and was then called "enteramine". Enteramine was regarded as an active principle found in tissue extracts, chiefly in extracts obtained from the gastrointestinal tract (argentaffin cells). In 1948, Rapport et al. isolated a

substance which appeared in clotted blood under certain conditions which they called "serotonin". Serotonin was identified as 5-HT and later enteramine was found to be identical with 5-HT (Erspamer and Asero, 1952).

Humphrey and Jaques (1955) made the observation that the addition of antigen to rabbit platelets suspended in heparinized plasma which contained antibody was followed by release of histamine and 5-HT from the platelets. This observation naturally stimulated further investigations about the possible role of 5-HT in the anaphylactic and allergic manifestations.

The role of 5-HT in anaphylaxis is more important in rats and mice than in other species. These animals are known to be very insensitive to histamine. It was logical, therefore, to assume that in these species 5-HT has the same role that histamine has in other species. This assumption seemed even more probable when Benditt et al. (1955) reported that the mast cells of rats and mice contained not only histamine but also 5-HT in fairly large amounts.

All experimental data, however, showed that 5-HT plays a very limited role, if any, in antigen-antibody induced anaphylaxis in rat. Previous injection of a 5-HT antagonist, lysergic-acid-diethylamide (LSD), did not influence the increased capillary permeability brought about by the antigen-antibody reaction. Similarly, depletion of 5-HT and histamine by pretreating the animals with reserpine and 48/80 did not prevent anaphylactic shock. Experiments performed on isolated or-

gans gave similar results. When a sensitized rat uterus was suspended in an organ bath, the addition of the specific antigen produced a full contraction in spite of the following treatments: i) the animal was pretreated with 5-HT and histamine releasers, before its uterus was suspended in the organ bath. ii) prior to antigen administration, LSD and antihistamine were added to the bath.

On the other hand in mice the release of 5-HT seems to be definitely responsible for most of the symptoms seen in anaphylactic shock. Using the isolated uterus preparation of a sensitized mouse (Fink, 1956), a previous administration of LSD to the bath completely inhibited the antigen-induced contraction. Similarly, using sensitized animals, LSD pretreatment prevented anaphylactic shock (Fox et al., 1958). Furthermore, Gershon and Ross (1962) demonstrated that 5-HT depletion by reserpine and methyl DOPA, suppressed the anaphylactic reaction.

The role of 5-HT in anaphylaxis in the guinea-pig seems to be uncertain. Herxheimer (1955) demonstrated that LSD does not influence the anaphylactic bronchoconstriction in this species. Furthermore Fink and Gardner (1956) and Brocklehurst (1958b), in their perfusion experiments with sensitized guinea-pig lung preparation, could not detect any 5-HT in the perfusate. Brocklehurst (1958a) also reported that guinea-pig bronchiole was only moderately sensitive to 5-HT and Udenfriend and Waalkes (1959) showed that the concentration of 5-HT in normal guinea-pig lung was negligible.

On the other hand, Sanyal and West (1958), using the method of Brocklehurst (1958b), did find a substance in the perfusate which they thought to be 5-HT. The perfusate elicited a contraction when added to the isolated rat uterus preparation and this contraction could be antagonized by LSD. Geiger and Alpers (1959) also supported Sanyal and West's (1958) finding, using the sensitized guinea-pig ileum preparation. They showed that the addition of specific antigen to the organ bath induced a contraction of the preparation. This contraction was not completely abolished if an antihistamine was added to the bath prior to the specific antigen, but the administration of LSD and antihistamine prior to the antigen prevented this contraction.

All available data in the literature indicate that 5-HT release does not play an important role in the anaphylactic reaction in the dog. Although dog liver contains an appreciable amount of 5-HT, systemic anaphylaxis does not diminish the 5-HT content of the liver and sensitized dog liver also fails to release 5-HT in vitro when exposed to specific antigen (Akcasu and West, 1960).

The observation of Humphrey and Jaques (1955) that antigen-antibody interaction in vitro can release 5-HT as well as histamine from rabbit platelets prompted Waalkes and his associates (1957) to study this amine during rabbit anaphylaxis in vivo. A significant pulmonary 5-HT concentration was found in normal rabbit lung (Udenfriend and Waalkes, 1959) which increased during anaphylaxis (Waalkes et al., 1957).

This increase was associated with a rise in plasma 5-HT and a fall in total blood 5-HT due to the reduction in platelet count (Waalkes et al., 1957; Waalkes and Coburn, 1959). On the other hand Fisher and Lecomte (1956) demonstrated that the depletion of 5-HT by reserpine administration did not ameliorate anaphylaxis in the rabbit.

There is at present no information as to whether or not 5-HT release occurs during anaphylaxis in man (Udenfriend and Waalkes, 1959). Human lung like guinea-pig lung, contains virtually no 5-HT and the mast cells of man like those of most species are devoid of 5-HT (Sjoerdsma et al., 1957). Though the disappearance of human platelets during antigen-antibody reaction has been demonstrated, there is no evidence that this is associated with the release of 5-HT (Storck et al., 1955).

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

Slow reacting substance was first described by Feldberg and Kellaway in 1938. Using the in situ guinea-pig lung preparation, they added snake venom to the perfusing fluid, because the symptoms of snake venom poisoning resembled strongly those of anaphylactic shock. The perfusate was then tested on the isolated guinea-pig ileum preparation. It was found that the addition of snake venom to the perfusing fluid caused the appearance in the perfusate of histamine and of a substance(s) which produced slow contraction and transient changes in the excitability of the guinea-pig ileum.

Kellaway and Trethewie (1940) demonstrated that fresh saline

extract of sensitized guinea-pig lung, after incubation with the antigen, contained a substance or substances, which caused slow contraction when tested on the isolated guinea-pig ileum preparation. They called the substance(s) "Slow Reacting Substance of Anaphylaxis" or "SRS-A". They suggested that anaphylactic contraction of smooth muscle was in part due to liberation of histamine and probably in part also to SRS-A which was liberated in the course of antigen-antibody reaction. They also showed that the sensitized guinea-pig lung did not contain SRS-A prior to the addition of specific antigen.

A detailed study of the occurence of SRS-A during anaphylactic shock was published by Brocklehurst (1960). His findings can be summarized as follows: i) the time course of the output of SRS-A from the perfused lung of a sensitized guinea-pig, after the addition of specific antigen, is slower in onset and more prolonged than that of histamine; ii) whereas the antigen-antibody reaction releases preformed tissue histamine, SRS-A is both formed and released in the lung as a result of antigen-antibody reaction; iii) SRS-A originates from the lung tissue, while platelets and other constituents of blood are not the source of SRS-A and are not necessary for its occurence; iv) when formation of SRS-A is studied in chopped tissue from various organs of sensitized guinea-pig it is found to occur chiefly in lung and vascular tissues: some formation occurs in salivary glands, pancreas, uterus, but little or none is detectable in ileum, trachea and skin; v) SRS-A is also present in the effluent after antigen has been added to the perfusing fluid of the lung of sensitized rabbit and monkey but is not present in

the effluent from the perfused lung of sensitized rat.

Brocklehurst (1956, 1960) also performed experiments with human asthmatic lung similar to those used by Schild et al. (1951), as described previously in the histamine section. He found that, after addition of the specific antigen, the perfusate not only contained histamine but also SRS-A. Since SRS-A induced contraction of the isolated human bronchial chain preparation was not antagonized by antihistamine drugs, Brocklehurst suggested that SRS-A plays an important role in the development of bronchoconstriction in asthmatic patients.

Herxheimer and Streseman (1963a) showed that an aerosol of purified SRS-A inhaled by human subjects produced a slowly developing but long lasting bronchoconstriction.

Middleton and Phillips (1964) demonstrated a striking similarity between the capacity of specific antigen and of cobra venom to give rise to SRS-A activity in tissues of sensitized guinea-pig. By either method of treatment, lung was found to be the major source of SRS-A. Aorta, ileum, spleen and uterus showed considerably less capacity to form SRS-A.

Recent experiments of Movat et al. (1967) and Orange et al. (1967) demonstrated that polymorphonuclear leucocytes played an important role in the release of SRS-A and the presence of these cells were required for optimal SRS-A generation at least in rats and guinea-pigs.

The chemical structure of SRS-A has not yet been elucidated.

Brocklehurst (1964) suggested that it is acidic and lipid. It is obtained rather firmly associated with some protein.

3. Kinins.

Kinins refer to a group of biologically active polypeptides. They originate from animal tissues, mostly from plasma protein and possess characteristic pharmacological actions: i) they produce hypotension; ii) they increase capillary permeability; iii) they produce pain when applied to blister base on human skin; iv) they are spasmogenic for most isolated smooth muscle preparations.

Frey and associates (1928-1933) were the first to demonstrate the presence of a blood pressure depressive substance in the extracts of urine and pancreas. They observed that these extracts, when injected intravenously into dogs, elicited a fall in blood pressure. The active principle was termed "kallikrein". At that time, they did not realize that the active principle extracted from urine and pancreas was not a blood pressure depressive substance itself, but an enzyme which acted on the plasma globulin of dogs and produced the release of an active polypeptide from the plasma, which caused a fall in blood pressure. The realization of this fact came only in 1949, when Rocha e Silva et al. (1949) reported that the incubation of plasma with trypsin resulted in the formation of a polypeptide, the action of which was similar to that of kallikrein. They found that incubation of trypsin or snake venom with plasma globulin brought about the formation of a polypeptide which caused a fall in blood pressure and contraction of smooth muscle preparations. They called this polypeptide "bradykinin" so as to cha-

racterize its slow contracting effect on the isolated guinea-pig ileum preparation, in contrast to that of the fast contracting effect of histamine. Following these publications, Werle (1961) incubated kallikrein with globulin and found that an active substance was formed which showed the same characteristic behavior as bradykinin. He called this pharmacologically active polypeptide "kallidin".

The chemical and pharmacological characteristics of bradykinin and kallidin are practically identical (Holdstock, et al., 1957; Pierce and Webster, 1961). Snake venom and trypsin act on the same globulin site as kallikrein does. Thus, if serum was first incubated with trypsin or snake venom so as to cause formation of bradykinin, the second incubation of the same serum with kallikrein would not result in kallidin formation.

Bradykinin was isolated by Elliot et al. (1960) and synthesized by Boisonnas et al. (1960). Bradykinin is a polypeptide which has the following amino acid sequence: Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg.

Werle (1961) isolated kallidin and found that kallidin had practically the same structure as bradykinin.

Erspamer and Anastasi (1962) reported the isolation of another polypeptide which they called eledoisin. Eledoisin consists of eleven amino acids and has the same biological activity as bradykinin but is 50-100 times more potent than the latter.

The probability that plasma kinins may play an important role in anaphylactic and allergic manifestations can be based on the follow-

ing experimental data: i) their pharmacological actions are practically identical to those of histamine; ii) the smooth muscle contracting effects of kinins are not antagonized by antihistamine or anti-5-HT; iii) kinins are very easily formed in the body by kinin forming enzymes and these enzymes can be activated by simple physical alteration; e.g., bradykinin formation occurs when plasma comes in contact with glass or is simply diluted.

As early as 1950, Beraldo demonstrated that during anaphylactic shock in the dog a substance was released into the blood which possessed bradykinin-like activity on the isolated guinea-pig ileum preparation, however, he could not prove its identity with bradykinin because there was no pure bradykinin available at that time.

Brocklehurst and Lahiri (1962) have shown that significant amounts of plasma kinin appeared in the blood of sensitized guinea-pigs, rats and rabbits within 2-5 minutes of injecting the specific antigen intravenously. These workers also found that the effluent from perfused, shocked guinea-pig lung contained no detectable bradykinin, but produced bradykinin when incubated with plasma pseudoglobulin which had been heated to destroy kinin-inactivating enzymes.

These experiments did not prove directly whether bradykinin is involved in anaphylactic reaction but only that bradykinin is released during anaphylaxis. Nevertheless, as pointed out by Feldberg (1961), the properties of the polypeptide bradykinin, i.e., its potent biological activity and ready activation from the plasma globulins and rapid inactivation, suggest that bradykinin is a very suitable candidate for

an anaphylactic mediator.

4. Heparin, Acetylcholine and Fatty-acid-like Substances.

i) Heparin.

According to our present knowledge most of the histamine released during anaphylaxis originates from the mast cells. It is also well established (Jorpes et al., 1937) that mast cells contain not only histamine but heparin as well. Thus heparin release may account for the blood incoagulability observed in anaphylaxis in several species, particularly in dogs (Biedl and Kraus, 1909). Indeed, Waters et al. (1938) demonstrated that heparin release from the liver of the dog is the factor responsible for the blood incoagulability in anaphylactic shock.

Monkhouse et al. (1952) demonstrated that heparin was increased in the blood in anaphylactic shock in guinea-pigs, rabbits and dogs. The increase was sufficient to change the clotting time of blood in dogs and rabbits but not in guinea-pigs.

ii) Acetylcholine.

The possible participation of acetylcholine in anaphylactic reactions was suggested by Nakamura (1941) and Danielopolu et al. (1948). This proposal was based on the evidence that acetylcholine antagonists, e.g., atropine, suppressed and eserine enhanced the in vitro anaphylactic reaction. However, the role of acetylcholine in

anaphylaxis is probably minimal and very little is known about it.

iii) Fatty-acid-like Substances.

A number of biologically active substances exerting somewhat similar pharmacological actions to that of histamine have been found in extracts of different organs and body fluids. Fatty-acid-like substances such as "Darmstoff", "Prostaglandin" and "Irin" may also play some role in the development of anaphylactic and allergic manifestations, but at present there is no experimental evidence available to support this hypothesis.

g. Substances with Antianaphylactic and Antiallergic Activity.

All the accumulated evidence described in the previous chapters, support the generally accepted assumption that the release of histamine and other biologically active substances play an essential role in the development of anaphylactic and allergic manifestations. The recognition of this fact naturally stimulated a great deal of work to find drugs which either prevent the release of these biologically active substances or antagonize their actions on smooth muscles and blood vessels.

The three most important and useful groups of agents employed to combat the symptoms of allergic diseases at present are:

1. Physiological antagonists (epinephrine and related substances).
2. Pharmacological antagonists (synthetic antihistamines).
3. ACTH and Corticosteroids.

1. Physiological Antagonists (Epinephrine and Related Substances).

As pointed out by Clark (1933), in the case of physiological antagonism, the agonist and the antagonist exert opposite effect on the same organ system. The main characteristic of this type of antagonism lies in the fact that the agonist and the antagonist act upon different receptors. A classical example of physiological antagonism is that of epinephrine and histamine. Thus, histamine stimulates the smooth muscles of the alimentary-tract, bronchiole, small and large veins, dilates capillaries and produces a fall in blood pressure. In contrast to these actions, epinephrine relaxes the smooth muscle of the gut and bronchiole, contracts the small arteries and raises blood pressure. Due to these facts, epinephrine and its derivatives, particularly those with powerful actions on beta-receptors (e.g., isoproterenol), are the mainstay in the symptomatic treatment of allergic diseases and first of all in the treatment of asthma. Though epinephrine and its derivatives are extremely useful to relieve the symptoms of acute hypersensitivity reactions, their usefulness is limited because: i) their duration of action is a very short lasting one; ii) after repeated administration tolerance may develop (epinephrine fastness).

2. Pharmacological Antagonists (Synthetic Antihistamines).

The recognition that histamine plays an important role in anaphylactic and allergic manifestations stimulated the search for sub-

stances which can specifically antagonize the effects of histamine.

Systematic work on synthetic compounds with antihistaminic activity was begun in 1937 by a team of French chemists and pharmacologists, notably by Bovet and his co-workers. Their work originated from the observation of Ungar et al. (1937) that the 2 compounds prosympal and piperoxan, which antagonized the pressor effect of epinephrine, exerted antihistaminic action on the isolated guinea-pig ileum preparation.

The basic study relating antihistaminic activity to chemical structure was done by Staub (1939) following the observation of Bovet and Staub (1937) that thymoxy-ethyl-diethylamine, or "F 929", was able to protect guinea-pigs against fatal anaphylactic shock. The replacement of the ether oxygen atom in the compound F 929 by an imino group led to the discovery of other antihistamines, among which the most potent was "F 1571" or N-ethylaniline-N-diethyl-ethylene-diamine (Staub, 1939). However, these two products could not be used in clinical practice because of their low therapeutic index. Nevertheless, this work soon led to the discovery of two types of potent antagonists of histamine, i.e., phenolic ethers, related to F 929 (Loew et al., 1945), and substituted ethylene diamines related to F 1571 (Halpern, 1942). In the next 20 years a large number of antihistaminic preparations have been put on the market.

The most frequently used synthetic antihistamines are derivatives of ethylene-diamine, dialkylaminoalkyl ether and propylamine (Raynaud, 1960).

Most of the antihistamines belong to that group of pharmacological antagonists that appear to act by occupying the "receptor sites" on the effector cells by the exclusion of the agonist. Presumably the antihistamines have such structures that allow them to bind with the "histamine receptor" without initiating any response. Thus it is generally accepted today that the bulk of antihistamines most probably act as "competitive antagonists" to histamine (Marshall, 1955).

The antihistamines antagonize, in varying degree, most but not all pharmacological actions of histamine. They can also reduce the intensity of allergic and anaphylactic manifestations and it is this property that provides the basis for their major therapeutic applications.

The methods employed to assay potential histamine antagonists are based on experiments in which their action can be tested against the following basic pharmacological actions of histamine:

i) Action on smooth muscle.

Histamine exerts a highly characteristic effect on smooth muscle. According to Dale and Laidlaw (1910): "The fundamental and characteristic feature of its action is its direct stimulant effect on plain muscle, in which it produces exaggeration of rhythm with increased tonus or steady maximal tonus unbroken by rhythm, according to the concentration of which it is applied. The sensitiveness of plain muscle from different organs and in different species varies within wide limits. The most sensitive of all appears to be the

plain muscle of the uterus, the non-pregnant uterus of some species responds to the drug in extreme dilution. The muscular coats of the bronchioles are also highly sensitive to the action, especially in rodents. The plain muscle of the intestinal wall, of the arterioles and of the spleen appears to occupy an intermediary position as regards responsiveness, that of the bladder and the iris was not affected by the direct action of such doses as we employed". Antihistamines can fairly specifically antagonize the smooth muscle stimulating effects of histamine both in vitro and in vivo. The actions of these drugs may be tested in vitro on a number of smooth muscle preparations such as the ileum, uterus and bronchial chain of the guinea-pig. The potent antihistamines can abolish the effect of histamine in a concentration as low as 10^{-9} g/ml when tested on the isolated guinea-pig ileum preparation. The high sensitivity of guinea-pig bronchial muscle to histamine lends itself to the in vivo method of assaying antihistamines. Histamine can be administered in the form of an aerosol. Administered this way, histamine produces cough, dyspnea, swaying, falling, lying and death by bronchoconstriction in the guinea-pig. The aerosol technique was originally described by Alexander et al. (1926) and Kallos and Pagel (1937) to study the development of anaphylactic shock in sensitized guinea-pig, modified later by Halpern (1942) and adapted for testing of antihistaminic compounds. The most potent antihistamines are able to antagonize the lethal effect of histamine aerosol in guinea-pigs in doses as low as 1-5 mg/kg body weight. The intravenous or intraperitoneal administration

of histamine in sufficient doses exerts similar effects on the bronchial muscle of the guinea-pig. Thus the potency of different antihistamines is frequently measured by injecting multiple lethal doses of histamine intravenously into antihistamine pretreated guinea-pigs with intervals of 5-10 minutes until death occurs (Staub, 1939). Using this method it was found that potent antihistamines, like phenergan protected the guinea-pigs against up to 1500 times lethal doses of histamine.

ii) Action on blood pressure and capillaries.

Histamine exerts a profound action on blood vessels in most species. Its effect is most characteristic on the capillaries. Histamine given intradermally dilates the capillaries and at the same time increases their permeability. The effect of histamine upon the capillaries is less influenced by antihistamines than its effect upon smooth muscles. It was only by the introduction of more potent antihistamines that a definite diminishing effect upon the whealing produced by histamine (triple response) could be evidenced by Celice and Durel (1942). The most specific and very potent antihistamines (neoantergan and phenergan) are very powerful in inhibiting the histamine effects not only upon the muscle but also upon the capillaries. Phenergan when tested in rabbits was found to be effective against the Schwartzman phenomenon (Bovet et al., 1944) and against the Arthus reaction (Benacerraf and Fischel, 1949).

iii) Action on circulation.

When injected intravenously in small doses (1-10 µg/kg) into

anesthetized cat or dog histamine brings about a transient sharp fall in blood pressure. The administration of large doses (3-10 mg/kg) usually produces a triphasic effect: i.e., an immediate steep fall in blood pressure, an incomplete recovery and, a renewed fall in blood pressure leading to shock and death. Most antihistamines have a relatively weak effect in antagonizing the action of histamine on circulation. Very small doses (4 µg) of histamine should be injected in the dog in order to have any appreciable reduction of the hypotensive effect after treatment of the animal with large doses of the antihistamines (Rosenthal and Minard, 1939). Even with potent antihistaminic drugs, such as neoantergan, only partial inhibition of the hypotensive effects of small doses of histamine (10 to 50 µg) can be observed (Bovet and Walthert, 1944).

iv) Action on gastric secretion.

Popielski (1920) was the first to demonstrate that histamine is a powerful stimulant of gastric secretion in doses as low as 3-4 µg/kg of body weight. In the next 40 years it has been clearly established by many workers that histamine is one of the most potent stimulators of gastric secretion, though there are still many doubts about the manner in which it acts as a physiological mediator of gastric secretion (Code, 1965). Antihistamines were found to be completely inactive upon the secretory effects of histamine. This might 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).

The synthetic antihistamines have been employed in the symptomatic treatment of various allergic disorders in which their usefulness is clearly attributable to their most characteristic action, i.e., antagonism of histamine. However, none of these synthetic antihistamines have been proved fully satisfactory from the clinical point of view due to a number of disadvantages. They are highly useful in urticaria, but less so in hay fever, most dermatoses and asthma. Their relative effectiveness is as follows (Cutting, 1964):

urticaria, angioneurotic edema, anaphylaxis	80%
hay fever, vasomotor rhinitis	50%
allergic dermatitis	20%
asthma	1%

In addition, the synthetic antihistamines may have unpleasant or dangerous side effects in therapeutic doses. Sedation is a common factor varying from a light state of dizziness to the stuporous state. Finally, the action of the synthetic antihistamines is short lasting, usually for a few hours only.

3. ACTH and Corticosteroids.

It is well known that symptoms of hypersensitivity reactions of the immediate type, such as hay fever, atopic asthma and serum sickness may be dramatically relieved in man by the administration of corticosteroids (Rose, 1954).

It is not surprising, therefore, that many studies were made on the possible effects of corticosteroids on the pharmacological actions of histamine as well as in anaphylaxis. A sound basis for the possible involvement of steroid in histamine dependent allergic responses was suggested by the observations that: i) adrenalectomy leads to considerable increase in tissue histamine (Rose and Browne, 1941); ii) adrenalectomy increases manyfold the toxicity of histamine in rats and mice (Halpern et al., 1952). However, in spite of some controversial data, it is a generally accepted fact that corticosteroids do not influence the pharmacological actions of histamine. Neither flare nor wheal reactions produced by the intradermal injection of histamine nor those produced by antigen, can be significantly inhibited by the administration of corticosteroids. Similarly, neither ACTH nor corticosteroids affect the evolution of acute anaphylactic shock or histamine induced bronchoconstriction in guinea-pigs (Leger et al., 1948; Halpern, 1964).

Though corticosteroids do not exert any significant effect on the actions of chemical mediators in acute experiments, they can influence both antibody synthesis and histamine biosynthesis in chronic experiments. It has been found that corticosteroids can i) depress antibody formation (Germuth et al., 1951; Bjorneboe et al., 1951); ii) depress the transformation of histidine to histamine (Halpern et al., 1953; Schayer et al., 1955); iii) deplete the tissue histamine content, at least in guinea-pigs (Kovacs, 1965; Hicks, 1965). However, these effects can be obtained on-

ly after prolonged treatment (7-15 days) and with doses which are usually much higher than those applied in clinical therapy. Thus the acute and dramatic therapeutic relief seen in man within 24 hours after the administration of corticosteroids remains an unresolved problem.

Although the immediate therapeutic effects are remarkable and dramatic, these hormones are not curative, since they do not eliminate the cause of the symptoms. Thus, while they are of great value in hypersensitivity reactions of the immediate type, continuous therapy is required for those related to intrinsic allergens such as infection. Furthermore, these hormones possess the following disadvantages: i) on withdrawal of the hormone, the symptoms frequently return, often in a more severe form; ii) side effects, ranging from minor and tolerable manifestations of hypercorticoism, to dangerous states even more hazardous to the patient than the disease itself for which treatment was instituted; iii) the tendency of the disease to become resistant to a suppressive dose of corticoid medication.

Section B. Naturally Occurring Antihistamine-like Substance(s).

a. Antihistamine-like Activity of Mammalian Tissue and Urine Extracts.

The presence of substance(s) in extracts of eosinophil rich leucocyte suspensions with antihistamine-like action both *in vivo* and *in vitro* was first reported by Kovacs (1950) and Kovacs and Kovacs-Juhasz (1951, 1952). This observation was confirmed by Vercauteren and Peeters (1952) and Vercauteren (1953). Experiments carried out with highly purified extracts of bovine eosinophils indicated that the active principle was most probably a steroid-like substance (Kovacs and Kovacs-Juhasz, 1955). Archer (1963) in his book summarized the results of his experiments performed between 1959 and 1963 concerning the antihistamine-like action of horse eosinophil extracts. He not only confirmed the previous findings but also found that these extracts were capable of antagonizing the local edema formation elicited by histamine, bradykinin and serotonin.

The fact that eosinophils contain an antihistamine-like principle suggested the possibility that the same or similar substances may occur in other tissues. Indeed, it was reported in 1962 (Kovacs and Melville, 1962) that partially purified extracts prepared from human urine were capable of antagonizing the histamine, 5-HT and bradykinin induced contractions when tested on the isolated guinea-pig ileum preparation. Kovacs and Melville (1963) also reported that not only human, but mammalian urine extracts (dog, horse, guinea-pig etc) in general

contain the same or similar active principle(s). Furthermore, they showed that partially purified extracts of horse urine given orally or intraperitoneally to guinea-pigs protected the animals against a lethal histamine aerosol and prevented the increased capillary permeability brought about by intradermal injection of histamine or bradykinin. Kovacs et al. (1963) and Francis et al. (1963) reported that extracts prepared from diverse human organs also contain the same or similar antihistamine-like principle(s). Recently, Kovacs and Voith (1966) showed that partially purified extracts of horse urine which exert antihistamine-like activity can also antagonize the histamine induced acid hypersecretion at least in guinea-pigs. Furthermore, they found that these extracts administered orally or intraperitoneally into rats prevented or strongly reduced the development of ulcer formation induced either by the Shay or restraint (stress) method.

b. Previous Work on Antihistamine-like Activity of Plant Tumor Extracts.

The observation that severe burns lead to shock with symptoms strikingly similar to those seen following the intravenous injection of histamine into experimental animals, led Barsoum and Gaddum (1936) to investigate the changes of blood histamine in patients with extensive cutaneous burn. They found an extensive rise of blood histamine at the time when secondary shock was liable to develop. Rose and Browne (1940) confirmed this finding and Dekanski (1945) observed in mice a strong increase in blood histamine 10 minutes after extensive experimental cutaneous burns.

Tannic acid was introduced by Davidson (1925) in the local treatment of burns. It seemed possible, therefore, that the therapeutic effect of tannic acid was brought about by the neutralization of the released histamine or that this mechanism was at least partly responsible for its therapeutic effect. To test this hypothesis the effect of tannic acid against a lethal histamine aerosol was investigated in guinea-pigs (Gyure and Kovacs, 1949). An intraperitoneal injection of a dose of 20-40 mg/kg body weight of commercial tannic acid gave a definite protection against the lethal histamine aerosol, when the injected guinea-pigs were tested 1-2 hours later. However, when the tannic acid was purified, this antihistamine effect decreased parallel with the purification and with the most purified preparations the antihistamine effect was no longer obtainable. It seemed possible, therefore, that the antihistaminic effect of the commercial tannic acid, which in Hungary is prepared from oak-gall, was most likely due to some impurities derived from the oak-galls.

Kovacs and Szabadi (1950) made the observation that a simple ethanolic extract of oak galls contained approximately ten times more of the active principle than commercial tannic acid. In addition they observed that the protection following a single intraperitoneal injection of the extract into guinea-pigs which were subsequently exposed to lethal histamine aerosol lasted for 18-20 hours.

In 1951, Kovacs et al. reported that galls of rose, poplar and willow also contained the antihistaminic substance, whereas extracts

prepared from non-tumorous parts of the same plants did not reveal antihistaminic activity.

In 1952, Kovacs et al. described a method by which tannin-free extracts of oak-gall could be obtained.

Feldberg and Kovacs (1960) demonstrated that the ethanolic extract of oak-gall, when injected intraperitoneally into guinea-pigs, protected the animals against a lethal dose of histamine aerosol and that the protection lasted for at least 24 hours, sometimes as long as four days. It was also reported that the protection did not result from the tannic acid present in the extract, because tannic acid injected intraperitoneally into guinea-pigs did not protect the animals, whereas the ethanolic extract of oak-galls, from which the tannic acid has been removed by lead hydroxide precipitation, produced protection.

Galls are hypertrophies of plant tissues caused by insects, mites, bacteria, fungi and possibly by other organisms. These organisms may initiate either mechanical or chemical stimulus. The latter is probably the more important in the production of galls. The irritant fluid or chemical may be in the eggs as in the case of sow flies, because the galls grow to full size before the eggs hatch. Oak galls are examples of galls caused by wasps, whereas crown-galls are induced by bacteria. The induction of crown-gall tumors by agrobacteria tumefaciens was discovered by Smith and Townsend, in 1907. They produced the crown-gall by puncture inoculation of agrobacteria tumefaciens on the Paris daisy, tobacco, tomato, root of sugar beet and root of peach tree. The tumor grew ra-

pidly only in young fleshy organs. The organism attacked both root and shoot. It frequently induced abnormal growth on the wounded parts of young cuttings. They also observed that the mature daisy galls were similar to the peach galls.

Naturally occuring crown-galls are found most frequently on the stems of tomato, tobacco and sun flower. It is considered as a malignant tumor which produces metastasis and frequently kills the host.

In 1962, Broome et al. made the observation that the ethanolic extract of the crown-gall tumor of tomato plants, when injected intraperitoneally into guinea-pigs, protected the animals against a lethal histamine aerosol. It was also shown that a single intraperitoneal injection of the extract exerted a protection which lasted sometimes as long as 47 days. However, when the chloroform extract was taken up in saline and added to the bath in which the isolated guinea-pig ileum was suspended, no antihistamine-like activity could be detected. Amounts equivalent to 2 mg of the chloroform soluble material did not antagonize but rather potentiated the effect of histamine on the isolated guinea-pig ileum. When a higher amount equivalent to 5 mg of chloroform soluble material was added to the bath it produced a slow contraction of the ileum but no reduction of the contractions elicited by histamine.

In early studies (Kovacs et al., 1964) a crystalline substance was isolated from crown-gall infected tomato stalks whose chemical properties resembled those of tomatine. The substance showed

in vitro antihistamine-like activity. Calam and Callow (1964) independently also demonstrated that tomatine isolated from both crown-gall infected and normal tomato stalks when injected intraperitoneally into guinea-pigs protected the animals against a lethal dose of histamine aerosol. Since tomatine forms part of the subject of this thesis it will be dealt with separately (Section C).

Section C. Tomatine and Related Substances.

Gottlieb (1943) observed that the expressed juice of tomato plant retarded the growth of Fusarium oxysporum f. lycopersici, an organism which caused the wilting of tomato plant. Since tomato species vary in their susceptibility to this organism, Irving et al. (1945) postulated that this might be due to differences in the ability of these plants to produce a substance or substances which either neutralized the toxin directly or inhibited the growth of the fungus. Thus, in 1945, Irving et al. reported that an antibiotic agent which they called "lycopersicin" could be obtained from the expressed juice of Pan America tomato plant. However, they later changed the name to "tomatine" to avoid confusion, since "lycopersicin" was once used as a synonym for "lycopene", the red pigment of tomato.

One year later, Irving et al. (1946) demonstrated that extracts containing tomatine effectively inhibited the growth of staphylococcus aureus, bacilus subtilis, phytomonas solanacearum, aspergillus clavatus, candida albicans, trichaphyton mentographytes, epidermophyton placcosum and microsporum andanini cultures. They suggested that tomatine might be used in the treatment of certain fungus infections both in man and animals.

It was reported by Ma and Fontaine (1948) that tomatine was a very effective inhibitor against the growth of candida albicans. They also showed that rutin and quercetin, which normally occurred in crude to-

matine concentrate were capable of neutralizing the inhibitory effect of crystalline tomatine toward these organisms. They found that quercetin, the aglycone of rutin, was more effective than rutin in counteracting the inhibitory activity of crystalline tomatine toward the organism candida albicans.

In searching for the fungistatic active principle(s), Fontaine et al. (1948) succeeded in the isolation of tomatine, in 1948. They also demonstrated that tomatine possessed a high antifungi but low antibacterial activity. The chemical characteristic of tomatine were described as follows: 1. Melting point 263-7°C; 2. Insoluble in aqueous medium at pH 10 but soluble at pH 4; 3. Minimal molecular weight was 1050.

In the same year, Kuhn and Löw (1948) isolated an alkaloid glycoside from the leaves of a wild tomato plant with a melting point of 270°C having a molecular weight of 1018.

Kuhn et al. (1950) and Ma and Fontaine (1950) reported that the hydrolysis of tomatine yielded two molecules of glucose, one molecule of galactose, one mole of xylose and the aglycone, tomatidine.

Prokoshev et al. (1950) reported the comparative study of three closely related alkaloid glycosides: solanine, demissine and tomatine. These workers found that solanine contained a trisaccharide whereas tomatine and demissine contained a tetrasaccharide unit.

Tomatidine was isolated from the roots of the Rutger tomato plant by Brink and Folkers (1951).

Sato et al. (1951) established the structure of the steroidal moiety of tomatidine and the point of attachment of the portion containing a secondary nitrogen. In the same year, Fontaine et al. (1951) presented evidence that tomatidine was a steroid secondary amine, because with digitonin it formed an ethanol insoluble complex which is characteristic of 3-beta-ol sterol configuration and by acetylation, tomatidine yielded an N, O-diacetyl derivative which indicated the presence of a secondary amino group. The absolute configuration of the nitrogen-containing ring F has only recently been confirmed (Boll and Sjöberg, 1963) and a formal total synthesis of tomatidine published (Schreiber and Adam, 1963).

Galinovsky and Wagner (1951) isolated tomatidine from the leaves of cultivated tomatoes. Acetylation of tomatidine gave an N, O-diacetyl derivative identical to that obtained by Fontaine et al. (1951).

The variability of tomatine content in the leaves of various tomato plants was investigated by Prokoshev et al. (1952). They found that lycopersicon pimpinellifolium contained tomatine in the highest quantity.

Kuhn et al. (1952) reported the synthesis of acetylpregnenolone from tomatidine. Dusek et al. (1966) patented the synthesis of dehydropregnenolone acetate from tomatidine.

The possibility of using tomatine for quantitative determination of cholesterol was thoroughly explored by Schulz and Sander (1957).

They found that: 1. Tomatine-cholesterol complex is only sparingly soluble in 96% ethanol; 2. Tomatine-cholesterol complex is much less soluble in 96% ethanol than the digitonin-cholesterol complex; 3. In comparison to digitonin, tomatine is simpler to obtain and easier to purify. On the basis of these findings they proposed the use of tomatine instead of digitonin for cholesterol precipitation. They also observed that cholesterol did not precipitate tomatidine; therefore, cholesterol can be used to separate tomatidine from tomatine. Kabara et al. (1961) compared the use of digitonin and tomatine for the quantitative microdetermination of cholesterol. They found that tomatine was more specific than digitonin in precipitating cholesterol. Among the 40 steroids studied, four were found to be precipitated by tomatine whereas digitonin precipitated nine. Tomatine, in contrast to seven other glycosides studied, gave a minimum color with Liebermann-Burchard reagent. In addition the solution of tomatine could be prepared easily and kept for a few months without any sign of precipitation or cloudiness. These results were confirmed by Edwards et al. (1964).

The observation that cholesterol quantitatively precipitates out tomatine from an ethanolic solution has been successfully used to isolate a new glycoside from the whole plant of Solanum Polyadenium (Schreiber, 1964). This new glycoside "polyanine" does not form a complex with cholesterol though its chemical structure is closely related to that of tomatine. Both polyanine and tomatine have similar aglycones

and they differ only in that tomatine has a tetrasaccharide while polyanine has a trisaccharide unit.

The possible pharmacological effects of tomatine are almost unknown. The only data found in the literature dealing with the pharmacological actions of tomatine are those of Wilson et al. (1961). They summarized their findings on the toxic and pharmacologic properties of tomatine, tomatidine and a derivative dihydrotomatidine as follows: "These compounds are not toxic orally, except in very high acute dosage. A 200 day continued feeding test with rats caused no reactions. A tomatine ointment did not irritate the skin, nor did tomatine sensitize the skin of guinea-pigs. Irritant properties were apparent when tomatine was applied to the rabbit eye, and abscesses were produced when the material was injected subcutaneously. The damage to the eye was reversed on cessation of treatment. Intravenously, in rats and rabbits, these compounds caused a sudden, short-lived hypotension without great effect on heart rate, and an increased respiratory rate and depth. In the rat, this blood pressure effect was largely eliminated or reversed by vagotomy. Hemolysis was observed <u>in vivo</u> and <u>in vitro</u>".

Calam and Callow (1964) demonstrated that tomatine isolated from both crown-gall infected and normal tomato plants, when injected into guinea-pigs in doses between 2-3 mg/guinea-pig protected the animals against a lethal dose of histamine aerosol. In contrast, tomatidine, the aglycone of tomatine, isolated from root extracts of both normal and infected tomatoes, was found to be ineffective.

Pure tomatine tested *in vitro* on the isolated guinea-pig ileum preparation in amounts as high as 50 µg was found to be without activity.

On the other hand, (Kovacs et al., 1964; Wakkary et al., 1966; Wakkary et al., 1967) a crystalline substance was isolated from crown-gall infected tomato plants, which when tested *in vitro* in a concentration of 3 µg/ml inhibited contractions induced by histamine. The crystalline crown-gall substance and tomatine showed the same efficacy in inhibiting contractions due to bradykinin, 5-HT and acetylcholine as those due to histamine. The chemical characteristics of the crystalline substance resembled closely those of tomatine. However, commercial crystalline tomatine tested in guinea-pigs by intraperitoneal injections in a dose range of 10-20 mg/kg did not show significant protection of the animals against the lethal effects of 0.15% histamine aerosol. The relationship of tomatine and other constituents of crown-gall extract to *in vitro* and *in vivo* antihistamine-like activity formed the principle aim of the present study.

PART II. GENERAL METHODS.

Section A. Production of Crown-gall Tumor of Tomato Plants.

a. Preparation of the Medium.

Dehydrated potato dextrose agar (Fisher Scientific) was suspended in distilled water in a concentration of 13 g/liter, thoroughly mixed and boiled until the agar dissolved completely. The solution and the Petri dishes were sterilized at 115°C under 40 psi steam pressure for 15 minutes, in a Barnstead sterilizer, Single Wall, Steam Heated (Fisher Scientific). The hot liquid culture medium was poured into sterile Petri dishes and allowed to harden at room temperature before use. In all experiments freshly made culture media were used.

b. Preparation of the Culture.

Agrobacteria tumefaciens strain M25 Un. Edinburgh originally received from Scotland was generously supplied to us by Dr. S. Vas, Department of Bacteriology, McGill University, who kept the bacteria in the freeze-dried form in sterile vials. Immediately before use one vial was opened and the bacteria was spread onto the freshly prepared sterile potato dextrose agar medium in one Petri dish. The Petri dish containing the culture medium was incubated at 28 ± 0.5°C in an incubator "Anhydro" National (Fisher Scientific). After 7 days incubation, the surface of the medium was completely covered with

bacteria. The bacteria grown in the first Petri dish were then respread into 40 Petri dishes containing freshly made sterile medium. After seven days incubation at 28°C, the bacteria were harvested by adding a few ml of saline into each Petri dish and gently scraping off the surface of the medium with a spatula, thereby detaching the bacterial colonies. The saline containing the bacteria was transferred into an Erlenmeyer flask. The bacterial suspension was diluted to a concentration of 10 Petri dishes per one liter saline. Immediately before use the bacterial suspension was thoroughly mixed by vigorous shaking of the container. Freshly prepared bacteria suspensions were used throughout the experiments.

c. Cultivation of Tomato Plants.

During the period of 1963-1967, approximately 6000 tomato plants of the "Best of All" variety were cultivated in the green house of the Horticulture Department of Macdonald College, McGill University. The plant seeds were purchased from Best of All Sutton Ltd., Reading, England.

Approximately 20 days after seedling, the young plants were potted and arranged in a 100 plants capacity bench. The pH of the soil was 7.0. The plants were fertilized daily with Nitrogen 10 parts, Phosphorus 52 parts, Potassium 17 parts, at the age of between 20-60 days; with N 19 parts, P 28 parts, K 14 parts, at the age of between 60-70 days; with N 20 parts, P 20 parts, K 20 parts, at the age of

70 days and over, and with Fe_2O_3 at the age of 100 days and over. Watering and pruning of the plants were carried out daily.

d. Production and Harvesting of Crown-gall Tumor.

About 5 mm deep longitudinal cuts were made with a sharp blade on the stalks of about 70 day-old tomato plants. Lots of 100 cut plants were sprayed with the suspension of agrobacteria tumefaciens by means of a compressed air sprayer. Crown-gall tumor began to appear on the stalks within 2 weeks after inoculation.

Fig. 1 shows the stalks of a normal control and an infected plant. The stalks of control plants was each measured about 1 cm in diameter, while those of the crown-gall infected tomatoes about 3 cm in diameter.

In order to determine the optimum time for harvesting the plants, 2 weeks after the tomato plants were inoculated with agrobacteria tumefaciens, 2-4 stalks (200 g) both from infected and normal plants were collected. Extracts from both the infected and normal stalks were prepared according to the method described in Section D,c. The extracts were assayed for antihistamine-like activity in guinea-pigs, using the histamine aerosol technique (Section C,a). This procedure was repeated once a week for the following 6 weeks and at least twice a week thereafter, until the crown-gall extracts exerted significant protection of guinea-pigs against the effects of a lethal histamine aerosol. At this time the crown-gall tumors were harvested. All the non infected parts of the

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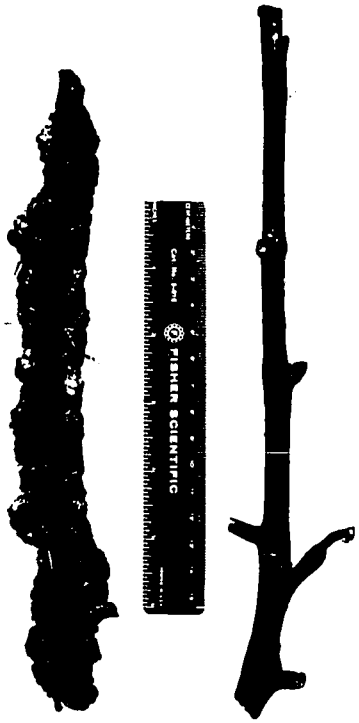


Figure 1

Photograph of crown-gall tumors on tomato stalk inoculated with Agrobacterium tumefaciens and of normal tomato stalk.

plants were trimmed and discarded. The infected parts of the plants were transferred to green plastic bags which were sealed immediately. The bags containing the crown-gall infected tomato stalks were stored in a chest filled with dry ice (-70°C).

Section B. In Vitro Methods for Testing the Biological Activity of the Extracts.

a. Pharmacological Agents.

The following pharmacological agents, except SRS-A, were of high purity: histamine dihydrochloride (Fisher Scientific), synthetic bradykinin (Sandoz), 5-hydroxytryptamine creatinine sulfate (Biochemical Research Corporation), acetylcholine chloride (Merck), tomatine (Nutritional Biochemicals Ltd.; Chemical Concentrates), promethazine (Poulenc Ltd.), atropine sulphate (Glaxo-Allenburys Ltd.), crude SRS-A (Fisons Pharmaceuticals Ltd.), Tomatidine (K and K Laboratories, Inc.).

Unless otherwise stated, the concentrations of the pharmacological agents were calculated as base.

b. Apparatus.

A constant temperature water bath (Palmer Co.) made of plexiglass with metal reinforcement with dimensions of 20x23x30 cm was employed for the *in vitro* testing. The water bath was equipped with a thermostat, a heating element, an electric stirrer, a thermometer, an organ bath and coiled polyethylene tubing.

The 20 ml organ bath was supported by the glass outflow tube, the latter led to a rubber draining tube. The inlet tube of the organ bath was connected to the Tyrode reservoir, through coiled polyethylene

tubing and a ground glass stop-cock. The flow of the Tyrode solution was regulated by the ground glass stop-cock. 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 ileum strip within the organ bath. The other end of the ileum was fixed by means of a small hook attached to a thread, which in turn was fixed to the writing lever located directly above the organ bath. The magnification of the writing lever was 3:1. A cable releaser, at the fulcrum of the lever, permitted the fulcrum to be held when the organ bath fluid was renewed. A kymograph (Palmer Co.), adjustable to various speeds, was used for recording. In some experiments the contractions were recorded isometrically by a Grass FT 03 C force-displacement transducer and a Grass Mod 7 polygraph. The applied tension on the muscle, usually 500 mg, was adjusted to give maximum contractions.

c. Preparation of Tyrode Solution.

The Tyrode solution was prepared by dissolving:

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 containing 0.4 mg atropine sulphate per ampule,	10 ml of 5% NaH_2PO_4,

in 10 liters distilled water. $NaHCO_3$ was dissolved first, since precipitation might occur otherwise. Atropine sulphate was incorporated

in the Tyrode, except for testing antiacetylcholine activity.

d. Preparation of Standard Solutions.

1. Histamine.

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 if stored at 4°C. The standard solution of histamine was prepared by diluting 1 ml of the stock solution to 100 ml with Tyrode. Freshly prepared standard solution of histamine was employed for each assay.

2. Bradykinin.

Ampules containing 80 µg/ml synthetic bradykinin were routinely used. The standard solution was prepared by diluting this stock solution 8 times with Tyrode. The standard solution of bradykinin was freshly made for each test.

3. 5-Hydroxytryptamine.

A 100 µg/ml stock solution of 5-HT was prepared by dissolving 23 mg of 5-HT creatinine sulfate in 100 ml distilled water. The standard solution of 5-HT in the concentration of 1 µg/ml was then prepared by diluting 1 ml stock solution to 100 ml with Tyrode.

4. Acetylcholine.

The stock solution of acetylcholine was prepared by dissolving

10 mg of acetylcholine chloride in 100 ml distilled water. The standard solution of acetylcholine was prepared by diluting 1 ml of the stock solution to 100 ml with Tyrode. Freshly prepared standard solution of acetylcholine was routinely employed.

5. Barium Chloride.

The standard solution of barium chloride was prepared by dissolving 1.0 g of the salt in 100 ml distilled water.

6. SRS-A.

The crude SRS-A was obtained as the freeze-dried product of 2 ml of sensitized guinea-pig lung perfusate per ampule. The powder, therefore, contained SRS-A, histamine, Tyrode salts and some proteinaceous matter, e.g., the sensitizing antigen egg albumin. The preparation was standardized by the supplier according to the method described by Smith (1962) who defined the unit of activity as follows: "At a concentration of 1 unit /ml, SRS-A produced contractions of guinea-pig ileum equivalent to histamine at a concentration of 10 ng/ml". According to the supplier the potency of the powder was 3.32 units/mg (the potency of the original perfusate was 23 units/ml). Since some SRS-A might have been lost in the freeze-drying process to prepare the standard solution approximately 7 mg of the crude powder were dissolved in 1 ml distilled water as recommended by the supplier.

e. The Isolated Guinea-pig Ileum Preparation.

A guinea-pig of either sex, fed with normal diet and weighing between 250-300 g was killed by a blow on the head. Immediately afterwards, the abdomen was opened 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 as well as the outside of the ileum strip was thoroughly washed with Tyrode. The end of the ileum nearest to the caecum was marked by a gauge no. 21 needle. Since this section is most sensitive to histamine, a 5-6 cm ileum strip was cut out from this end. All portions of the ileum which were handled during the procedure were rejected. The other section, which was not used immediately, was kept immersed in Tyrode at room temperature and discarded after 6 hours. Each end of the 5-6 cm ileum strip was fixed through the hooks, in such a way that the lumen remained open at both ends. All these operations were carried out while the ileum was immersed in Tyrode. The ileum preparation was transferred to the organ bath previously adjusted to 34 ± 1°C. A mixture of 95% O_2 and 5% CO_2 was bubbled through the oxygen inlet tube. The pressure of the mixture was adjusted by the pressure regulator to approximately 2 bubbles/second and maintained at this rate throughout the experiment. The writing lever was balanced with plasticine. The load of the tissue was approximately 350 mg.

f. Testing the Activity of the Extracts.

1. Antihistamine Activity.

Immediately after the preparation was set up, the sensitivity of the ileum was first tested as follows. The kymograph was switched on and the standard histamine solution (0.05 µg) was added to the organ bath and left in contact with the ileum for 20 seconds. During this time the contraction was recorded. The kymograph was then switched off and the organ bath was drained of histamine and Tyrode, and refilled with fresh Tyrode. Twenty seconds before the next administration of 0.05 µg standard histamine solution, the kymograph was switched on and kept on until the 20 seconds contact time of the standard histamine solution with the ileum 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 of which it was capable at this dose. If the tenth administration caused no contraction or only a very small one, larger doses of histamine were given. This procedure was repeated until the full contraction of the ileum recorded on the smoked drum was about 5 cm in height.

Having established the sensitivity of the ileum, first the solvent employed to prepare the extract solutions and then the extract solutions in different doses were added to the organ bath immediately after the replacement of the histamine containing Tyrode with the fresh

Tyrode. The extract was left in contact with the ileum for 2 minutes, after which additions of the standard histamine solution were repeated at intervals of 3 minutes until the contractions were at least 50% of the original standards.

2. Antibradykinin Activity.

The sensitivity of the isolated guinea-pig ileum to bradykinin was established as described previously for histamine. The amount of bradykinin added to the organ bath varied between 0.5-1.0 µg depending on the sensitivity of the ileum. The standard dose of bradykinin was kept in contact with the ileum for 40 seconds. Intervals of 4 minutes elapsed between each administration of the standard dose of bradykinin.

The assay of antibradykinin activity of the extracts was carried out by the similar method described earlier for testing the antihistamine activity. The contact time of the extract was 2 minutes.

3. Anti5-Hydroxytryptamine Activity.

The sensitivity of the ileum was established as described previously. The amount of 5-HT added to the bath varied between 0.2-0.5 µg, depending on the sensitivity of the ileum. The standard 5-HT solution was kept in contact with the ileum for 90 seconds. Intervals of 5 minutes elapsed between each administration of the standard dose of 5-HT.

The testing of the anti5-HT activity was carried out in the same manner as described for testing the antihistamine activity. Contact time between the ileum and the extract was 2 minutes.

4. Antiacetylcholine Activity.

The sensitivity of the ileum was established as described previously. The amount of acetylcholine added to the organ bath varied between 0.05-0.20 µg depending on the sensitivity of the ileum preparation. The standard acetylcholine solution was kept in contact with the ileum for 20 seconds. Intervals of 3 minutes elapsed between each addition of standard acetylcholine solution to the organ bath.

The assay of antiacetylcholine activity of the extracts was carried out by the similar method as that used for antihistamine activity.

5. Antibarium-chloride Activity.

After the preparation was set up, the sensitivity of the ileum was established as described previously. The amount of barium chloride added to the organ bath varied between 0.5-2.0 mg, depending on the sensitivity of the ileum. The standard barium chloride solution was kept in contact with the ileum for 40 seconds. Intervals of 4 minutes elapsed between each administration of the standard dose of barium chloride.

The testing of antibarium-chloride activity was carried out in

the same way as described for testing the antihistamine activity. Contact time between the ileum and the extract was 2 minutes.

6. Anti SRS-A Activity.

The same method described above was utilized, with the following modifications:

i) Magnification of the writing lever was 9 times.

ii) The temperature of the organ bath was maintained at 30 ± 1°C.

iii) A 5-6 cm strip of guinea-pig ileum, from about 20 cm proximal to the ileocaecal junction was employed.

iv) Two minutes prior to each addition of the standard SRS-A solution 4 ug promethazine solution was added to the organ bath.

v) The contact time between SRS-A and the ileum was 2-3 minutes and the relaxation time about the same. As the ileum only partly relaxed after each wash, 3 washes per minute for 3-4 minutes were required to return the ileum to steady state.

vi) The interval of addition of the standard SRS-A solution was 7 minutes. A strict time interval gave the most satisfactory results.

g. Determination of the Activity of the Extracts by the pA and R_{50} Methods.

The activity of crystalline tomatine in antagonizing histamine, bradykinin, acetylcholine and barium chloride were determined in terms of pA by the method of Schild (1947). pA_2 and pA_4 were determined on

different segments of the same gut using at least 4 segments for each determination. The extract to be tested was kept in contact with the ileum for exactly 2 minutes. The bath fluid was then replaced 6 times with fresh Tyrode and a double or quadruple of the stimulating drug was added to the preparation at intervals of exactly 3 minutes in the case of histamine and acetylcholine, 4 minutes in the case of bradykinin and barium chloride.

Since the molecular weight of substances tested apart from tomatine was unknown, a modification of the pA method described by Rocha e Silva and Beraldo (1948) was chosen to measure the activity of extracts against the effect of different agonists. The sensitivity of the ileum was established as described previously. The height of the uniform submaximal contractions was arbritrarily taken as 100% response. The amount of the antagonist to be tested was added to the organ bath and kept in contact with the ileum for exactly 2 minutes. Then the bath fluid was replaced 6 times with fresh Tyrode and the first dose of the stimulating agent (histamine, bradykinin, acetylcholine or barium chloride) was added exactly 2 minutes following the removal of the antagonist. The contraction of the muscle and changing of Tyrode took 30 seconds; after that time, a resting period of $2\frac{1}{2}$ minutes was allowed between the addition of the same dose of the agonist; 30 seconds and $2\frac{1}{2}$ minutes elapsed before the third addition and so on. The time of the first addition was taken as origin (0) and the time in seconds required for 50% recovery of sensitivity of

the ileum preparation (based on the height of the standard contractions) was taken as R_{50}. The R_{50}'s obtained for various doses of extract were plotted against dose of extract on a semilog scale.

Section C. In Vivo Methods for Testing the Biological Activity of the Extracts.

Guinea-pigs.

Normal guinea-pigs (Quebec Breeding Farm), of either sex, weighing between 250-300 g were used throughout the experiments. The animals, usually received a few days prior to the experiment, were kept on normal pellet diets until used.

a. Histamine Aerosol.

1. Apparatus.

The wooden chamber employed was of dimensions 30x30x60 cm and was provided with a sliding door on top and with 2 glass walls on opposite sides to allow observation of the animals while inside the chamber. The chamber had 2 holes, each 1 cm in diameter: one of the holes was used for ventilation and the other as inlet of the aerosol to the chamber. The histamine solution was nebulized using the Jouan histamine aerosol apparatus (capacity 20 ml/hour and particle size 1-3 μ).

2. Testing Procedure.

A freshly prepared 0.15% histamine solution was poured into the nebulizer. In order to secure uniform conditions, the chamber was saturated with histamine aerosol prior to placing the animals into the chamber.

A treated animal together with its control were put into the chamber and the time was immediately noted by means of a stop watch. The histamine aerosol exerted the following effects on the animals:

i) coughing, dyspnea and gasping

ii) swaying, falling and convulsive struggling

iii) lying on the floor of the chamber

iv) cessation of respiration.

The time of each of these four stages was recorded. Stage 4, cessation of respiration was chosen as the end point of the histamine effect. The maximum exposure time of the animals to histamine aerosol in the chamber described above was arbitrarily set at 20 minutes. The animals which survived longer than 20 minutes were re-exposed to histamine aerosol a few days later.

b. Anaphylactic Shock.

Guinea-pigs were sensitized with 2 mg crystalline egg albumin (Pentex Inc.). The sensitizing antigen was given intraperitoneally from a freshly prepared 0.2% solution twice on 2 subsequent days.

Twenty-one days after the second injection, the guinea-pigs were injected intraperitoneally with the extracts. The control animals received the same amount of the vehicle. Three hours after the administration of the extract or the vehicle, the challenging dose (0.2 ml of 0.2% crystalline egg albumin solution) was injected intracardially. The time of injection of the challenging dose was recorded. The guinea-

pigs were observed for the appearance of the following symptoms of anaphylaxis:

i) chewing

ii) scratching of the nose

iii) dyspnea and gasping

iv) swaying, falling and convulsive struggling

v) lying

vi) cessation of respiration.

c. Capillary Permeability.

The abdominal skin area of guinea-pigs was carefully shaved 24 hours prior to intraperitoneal injection of either crown-gall extract (treated group) or vehicle (control group). Three hours later the animals were injected intracardially using a 26 gauge needle with 0.15-0.20 ml of a saline solution of pontamine sky blue (60 mg/kg). Immediately following the injection of the dye, duplicate sites, one on each side of the shaved abdominal area were separately injected intradermally with 0.05 ml and 0.10 ml saline solutions of histamine and bradykinin (both solutions: 10 µg/ml). Thirty minutes later the animals were killed by a blow on the head, the corresponding area of the skin was carefully cut, the inner side cleaved of the soft tissues and the area of blueing measured for both groups of animals.

d. Statistical Analysis of Data.

Means and standard errors were calculated by standard procedures.

The significance of differences between means were estimated by Student's t test (Hoel, 1960).

Since in the histamine aerosol experiments the maximum time of exposure was arbritrarily limited to 20 minutes, in all those instances in which the animal survived longer than 20 minutes, for the statistical analysis of the data their survival time was taken as 20 minutes.

Section D. Preparation of Extracts from Normal and Crown-gall Infected Tomato Stalks.

a. Reagents and Solvents.

All reagents and solvents were of analytical reagent grade: absolute ethanol (Fisher Scientific), absolute diethylether (Malincrodt), chloroform (Malinckrodt), anhydrous methanol (Malinckrodt), sodium hydroxide (Fisher Scientific), hydrochloric acid (Malinckrodt), cholesterol (British Drug Houses), argon gas (Canadian Liquid Air Limited).

A solvent mixture of chloroform-ethanol (3:1,v/v) was prepared by mixing the required volume of each solvent in a separatory funnel.

A stock solution of cholesterol was prepared by dissolving 2.0 g of cholesterol in 1 liter of absolute diethylether.

b. Apparatus.

A two speed Waring blendor was employed to grind the stalks. Thermostatically controlled water baths adjusted to 70 ± 1°C and to 37 ± 1°C were employed for heating the extracts. A rotary evaporator (Buchler Instruments) connected to a Duo-Seal vacuum pump (Fisher Scientific) through dry ice traps was employed for all drying procedures. A pH meter (Corning) was employed for pH adjustments. A refrigerated centrifuged (International PR-2) was employed for all centrifugations.

c. Standard Crude Extract.

The tomato stalks (normal or crown-gall infected) were allowed to thaw at room temperature, cut into small pieces and weighed. The fragments were suspended in a known quantity of chloroform-ethanol (3:1,v/v) sufficient to permit 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 made up to 300 ml/100 g tissue. The pulp was thoroughly mixed, heated in a 70°C water bath for 5 minutes and filtered through a Buchner funnel using Whatman number 3 filter paper. The clear filtrate was taken to dryness by rotary evaporation. The dried extract was taken up in distilled water (15 ml/100 g tissue) and the resultant mixture was well blended, using a spatula to scrape off the residue which tended to stick to the wall of the flask, transferred to a beaker and adjusted to pH 2 with 12N HCl. The mixture was filtered through a Buchner funnel with Whatman number 2 filter paper, the residue discarded and the clear filtrate adjusted to pH 8. A precipitate formed on adjusting the solution to pH 8. The resulting suspension was extracted 3 times using for each extraction 15 ml/100 g tissue of chloroform-ethanol (6:1,v/v). The aqueous layer was discarded and the organic layers were combined and dried in a tared round bottom flask by rotary evaporation. Immediately after weighing the flask was filled with argon, stoppered and stored at -15°C.

d. Cholesterol-treated Standard Crude Extract.

The standard crude extract or Sephadex LH-20 fraction I derived

from crown-gall infected stalks was dissolved in anhydrous methanol (2 mg/ml) and to the solution (100 ml) was added 100 ml of the stock cholesterol solution. After thorough mixing and incubation of the clear mixture at room temperature for 15 minutes, 20 ml distilled water were added with thorough mixing to ensure complete precipitation of the tomatinide (tomatine-cholesterol complex). After incubation at least for 1 hour at -15°C the suspension was centrifuged at 2000 RPM for 15 minutes at 20°C. Upon second cholesterol treatment, the supernatant failed to yield additional precipitate. The clear supernatant obtained was taken to dryness by rotary evaporation. To remove excess cholesterol, the dried residue was washed with 20 ml diethylether repeatedly until the washings gave negative Liebermann-Burchard reaction (Section F,c). The residue was dried by rotary evaporation, weighed and stored under argon at -15°C.

Section E. Column Chromatography.

a. Gel Filtration on Sephadex G-25.

1. Material and Apparatus.

The resin consisted of Sephadex G-25, fine, of size 20-80 μ (Pharmacia, Montreal).

A chromatographic column (Scientific Glass Blowing Reg'd., Montreal) of dimensions 0.9x30 cm was provided with coarse glass filter disc. The column was connected to a fraction collector (Spinco) by polyethylene tubing.

2. Procedure.

Sephadex G-25 was suspended in dilute HCl pH 3.2. The latter was previously deaerated using a Duo-Seal vacuum pump for 10 minutes. The "fines" were removed by suction and the gel resuspended in the dilute HCl solution; this procedure was repeated until the slurry became relatively free of fine particles. The slurry was poured into the chromatographic column and allowed to pack by gravity. A reservoir of dilute HCl pH 3.2 (eluent) was connected to the column by polyethylene tubing. The eluent was allowed to pass through the column overnight at a fixed flow rate.

After overnight equilibration, the flow of the eluent was stopped and the reservoir disconnected from the column. The eluent was allowed to run out of the column until its surface coincided with that of the

packed Sephadex. A saturated solution of the sample to be chromatographed prepared in the eluent, was applied to the Sephadex dropwise, without disturbing its surface and allowed to penetrate the gel. The same volume of eluent was applied to the column and allowed to penetrate the Sephadex: this washing-in procedure was repeated twice. The column was filled with eluent, reconnected to the reservoir and the chromatogram developed at the same flow rate as that employed for equilibration. Effluent fractions (in 2 ml volumes) were collected in a Spinco fraction collector and monitored by the ninhydrin colorimetric method (Section F,a). Void volumes were determined by measuring the peak effluent volumes obtained with blue dextran (Pharmacia, Montreal). The effluents were examined at 625 mμ with a Coleman Jr. spectrophotometer.

b. Silica-gel Column Chromatography.

1. Material and Apparatus.

The resin consisted of silicic-acid of size 200-320 mesh (Bio-Rad, California).

Anhydrous methanol and ethylacetate (Malinckrodt) of analytical reagent grade were employed without further purification. A solvent mixture of ethylacetate-methanol (65:35,v/v) was prepared by mixing the required volume of each solvent in a separatory funnel.

A chromatographic column of dimensions 2.5x80 cm was provided with a glass wool plug and teflon stopcock. The column was connected

at the top to 1 liter capacity round bottom flask which served as reservoir by means of a ground glass ball joint. An LKB fraction collector was placed directly under the column for collection of effluent fractions.

2. Procedure.

Silicic-acid was slurried in the ethylacetate-methanol (65:35,v/v) solvent, poured into the column, and allowed to pack by gravity. The reservoir was connected and the ethylacetate-methanol solvent (eluent) was passed through the column for at least 20 hours at a fixed flow rate adjusted by the teflon stopcock. Following equilibration, 4 ml sample solution (prepared in the ethylacetate-methanol solvent) was applied dropwise so as not to disturb the surface and allowed to diffuse into the gel. The same volume of the eluent was applied to the column and allowed to diffuse into the gel, this washing-in procedure was repeated twice. The column was filled with eluent, the reservoir reconnected and the flow rate of the column was adjusted to that used for equilibration. Effluent fractions (in 4 ml volumes) were collected directly in an LKB fraction collector and monitored by anthrone, Liebermann-Burchard and visible spectrophotometric methods (Section F, b,c,e).

c. Sephadex LH-20 Column Chromatography.

1. Material and Apparatus.

The resin consisted of Sephadex LH-20, of size 25-100 μ (Pharmacia, Montreal).

Anhydrous methanol (Malinckrodt) of analytical reagent grade was employed without further purification.

Chromatographic columns of dimensions 2.5x80 cm and 4x100 cm were each provided with a coarse glass filter disc and teflon stopcock. Each column was connected to a 1 liter capacity round bottom flask by means of ground glass ball joint. An LKB fraction collector, placed directly under the column was employed to collect the effluent fractions.

2. Procedure.

Sephadex LH-20 was suspended in anhydrous methanol and allowed to swell for 2 hours. The gel was "de-fined" as described for Sephadex G-25 (Section E,a) 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 flow rate (adjusted by the teflon stopcock) until 2 liters of effluent were collected.

After equilibration of the resin, a saturated solution of the sample(prepared in methanol) was applied dropwise without disturbing the surface. The sample solution volumes were 5 ml and 10 ml for the 2.5x80 cm and 4x100 cm columns respectively. The sample solution was allowed to diffuse into the gel by gravity and wash-in 3 times using equal volumes of methanol. The chromatogram was developed with anhydrous methanol and effluent fractions in 5 or 10 ml volumes were collected directly in an LKB fraction collector, monitored by the ninhydrin, anthrone, sulphuric acid and visible absorption methods (Section F,a,b,d,e).

Section F. 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 employed without further purification: ninhydrin (Fisher Scientific), anhydrous hydrindantin (Brickman and Company), sodium acetate (Fisher Scientific) and methylcellosolve (Fisher Scientific).

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

The ninhydrin reagent was prepared as follows: 20 g of ninhydrin and 3 g of hydrindantin were dissolved in 750 ml of methylcellosolve (monomethylether of ethyleneglycol). Sodium acetate buffer 250 ml of pH 5.5 was added and the resulting solution was immediately transferred to a 1 liter dark glass reservoir. The reservoir was arranged to permit the reagent to be stored under nitrogen and was connected to an automatic pipet (Brewer).

2. Procedure.

Aliquots of 2% (by volume) of the effluents were transferred to test tubes of the same size and optical transparency. Into each tube was added 1 ml of the ninhydrin solution by means of the automatic pipet. The tubes were capped, briefly shaken by hands 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 (50:50,v/v) using the automatic pipet. The tubes were thoroughly shaken on a reciprocal shaker for 5 minutes and read on a Coleman Jr. spectrophotometer at 570 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 effluents.

1. Materials.

Anthrone (Fisher Scientific) and sulfuric acid (Malinckrodt) were of analytical reagent grade and were used without further purification.

A 0.1% anthrone solution was prepared by dissolving 1 g anthrone in 1 liter concentrated sulfuric acid. This solution was used within 24 hours after preparation.

2. Procedure.

Aliquots of 2% (by volume) of the effluents were transferred to test tubes of same size and optical transparency. Into each tube was added 4 ml of the 0.1% anthrone solution. The tubes were shaken by hands, allowed to stand for 30 minutes, and read in a Coleman Jr. spectrophotometer at 625 mμ. A blank was similarly prepared using the eluting solvent in place of the effluent fractions.

c. Liebermann-Burchard Colorimetric Method.

The method of Liebermann (1885) as described by Fieser and Fieser (1959) for cholesterol determination was adapted for monitoring column chromatographic effluents.

1. Materials.

Sulfuric acid (Malinckrodt) and acetic anhydride (Fisher Scientific) were of analytical reagent grade and were employed without further purification.

The Liebermann-Burchard reagent consisted of a mixture of acetic anhydride and sulfuric acid in a ratio of 10:1, v/v. The sulfuric acid was added slowly to the ice cooled acetic anhydride and the reagent, after cooling to room temperature, was used within 1 hour of preparation.

2. Procedure.

Aliquots of 2% (by volume) of the effluents were transferred to test tubes of the same size and optical transparency. Into each tube

was added 4 ml of the Liebermann-Burchard reagent. The tubes were shaken by hands, allowed to stand for 20 minutes and read in a Coleman Jr. spectrophotometer at 625 mμ. A blank was similarly prepared using the eluting solvent in place of the effluent fractions.

d. Concentrated Sulfuric Acid Colorimetric Method.

The method of Metz (1961) for spot detection of thin layer chromatograms was adapted for monitoring column chromatographic effluents.

1. Materials.

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

2. Procedure.

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

e. Visible Spectrophotometric Method.

For analysis by visible spectrophotometric method, the tube of effluent containing the highest optical density was determined by visual

estimation in the pigmented region of the effluents. The optical density of this tube of effluent at various wavelengths in the visible region was read in the Coleman Jr. spectrophotometer. The eluting solvent served as a blank. The wavelength (660 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 Coleman Jr. spectrophotometer at this wavelength.

Section G. 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 (Chromagram) were purchased from Eastman Kodak Co., N.Y. The apparatus for developing the chromatograms was obtained from Fisher Scientific, Montreal and consisted of metalic racks, solvent troughs and sandwich type developing chambers.

Ethylacetate and anhydrous methanol (Malinckrodt) were of analytical reagent grade, and were employed without further purification. Various mixtures of ethylacetate-methanol were used as developing solvents.

2. Procedure.

A light pencil line was drawn parallel and about 2 cm from the edge of a precoated sheet (20x20 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 cross mark by means of a micropipet (Lang-Levy) using a hair dryer to evaporate the methanol. The size of the area occupied by each sample was less than 5 mm in diameter. The sheet was placed in the developing chamber and the latter 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 trough, immediately opened and the solvent front marked. The chromatogram was dried at room temperature.

Components were detected on the dried chromatogram by the iodine vapor method (Brante, 1949) as follows: Iodine crystalls (5 g) were placed in a glass cylinder of dimensions 23x25 cm. The cylinder was saturated with iodine vapor and the dried chromatogram was placed in the cylinder until yellow spots became visible. The chromatogram was removed and the spots outlined in pencil. Rf values were determined using the formula:

$$Rf = \frac{\text{distance of migration of spot}}{\text{distance of migration of solvent front}}$$

b. Preparative TLC Using Sheets Precoated with Silica-gel.

1. Materials and Apparatus.

The precoated sheets solvents and apparatus were as described in Section G,a. A mixture of ethylacetate-methanol (65:35,v/v) was employed as developing solvent.

2. Procedure.

A light pencil line was drawn parallel to and about 2 cm from the edge of a 20x20 cm precoated sheet. A methanolic solution of sample was streaked along the pencil line by means of a micropipet. The sheet was freed of methanol by evaporating at room temperature and deposited in a sandwich type developing chamber. The chamber was placed in the solvent trough and the chromatogram developed until the solvent front travel approximately 17 cm. The developing chamber was then removed

from the trough, opened immediately and the solvent front marked. The chromatogram was dried with a draft of air at room temperature. A guide strip of 1x20 cm was cut out from the middle (perpendicular to the starting line) of the dried chromatogram, stained with iodine vapor as described previously (Section G,a) and the Rf's determined. The remainder of the chromatogram was cut according to the number and location of the spots on the guide strip. Equivalent cut from several chromatograms were pooled, extracted with methanol and the extract was dried by rotary evaporation. The dried extracts were stored in a tightly stoppered flask under argon at -15°C.

c. TLC Using Glass Plates Coated with Silica-gel G According to Stahl (Schreiber et al., 1963).

1. Material and Apparatus.

Methanol and chloroform (Malinckrodt) were of analytical reagent grade and were employed without further purification. Various mixtures of methanol-chloroform were employed as developing solvents.

Thin layer chromatographic apparatus (Desaga, Heidelberg) consisted of : glass plates (20x20 cm and 5x20 cm), an applicator, a platic template, developing chambers (of dimensions 21x21x10 cm) and a metallic rack.

Silica-gel G according to Stahl (Canlab) was slurried in distilled water. Coating of a single glass plate (20x20 cm) with 0.25 mm adsorbent thickness required 13 g of the gel slurried in 34 ml distilled water.

The glass plates (20x20 cm) were placed on the plastic template and a 5x20 cm glass plate was placed in front. The slurry was poured into the applicator which was previously adjusted to give 0.25 mm adsorbent thickness. The applicator was immediately opened and run over the glass plates with a single constant movement. The adsorbent was allowed to settle on the plates for 30 minutes. The plates were placed in the metallic rack and dried at 110°C for 1 hour.

2. Procedure.

Methanolic solution of samples were applied 2 cm from a starting edge of a coated glass plate, 4 cm from the side edges and 4 cm apart, by means of a micropipet. The methanol was evaporated from the sample spots at room temperature. The plates were immediately placed in the developing chamber (previously equilibrated with 250 ml developing solvent) in such a way that the starting edge was immersed in the developing solvent. The cover was replaced and the chromatogram allowed to develop until the solvent front had travelled about 17 cm. The plate was then removed from the developing chamber, the solvent front marked and the chromatogram dried at room temperature.

Components were detected by phosphomolybdate reagent method of Kaufmann and Makus (1960). Phosphomolybdic acid solution (10%) in methanol was employed. The dried chromatogram was sprayed with the 10% solution and heated in the chromatography oven (National Appliances Co.) at 120°C. The spots were marked and the Rf's determined.

Components were also detected by the concentrated sulfuric acid method of Metz (1961). The chromatograms were sprayed with concentrated sulfuric acid and heated at 110°C for 15 minutes. The spots were marked and the Rf's determined.

PART III. EXPERIMENTS AND RESULTS

Section A. Antihistamine-like Activity of Normal and Crown-gall Infected Tomato Stalks.

Broome et al. (1962) reported that extracts prepared from normal and crown-gall infected tomato stalks exerted protection in guinea-pigs against the effect of a lethal histamine aerosol. These workers, however, did not carry out a systematic study to compare this biological activity of extracts of normal with that of crown-gall infected tomato stalks. As the main aim of the study described in this thesis was to isolate and characterize the active principle(s) in such extracts, it was of interest at the outset to employ starting material providing the highest specific activity in the crude extract. Therefore, studies were done to compare the antihistamine-like activity of extracts of normal and crown-gall infected tomato stalks using the histamine aerosol method (Part II, Section C,a) for bioassay (for further experimental data on the histamine aerosol method see Appendix p.197).

a. Preparation of the Extracts.

The normal and crown-gall infected tomato plants were cultivated at the same time and under the same conditions. At day 70, some of the plants were inoculated with agrobacteria tumefaciens. The normal and infected plants were simultaneously collected at the various time intervals following inoculation as shown in Table I. The corresponding stalks were extracted according to the method of preparation of standard crude extract described in Part II (Section D,c). The mean yields of the extracts obtained are listed in Table I. The differences in mean yields for normal compared to infected stalks were found to be statistically significant ($p<0.05$).

TABLE I

Mean Yields of Extracts of Normal and Crown-gall Infected Tomato Stalks.

No. of expt.	Time after inoculation	Normal stalks	Infected stalks
	weeks	% (w/w) ± S.E.*	% (w/w) ± S.E.*
3	2-4	0.015 ± 0.003	0.050 ± 0.012
6	4-8	0.031 ± 0.004	0.084 ± 0.017
6	8-12	0.039 ± 0.004	0.056 ± 0.006

*S.E., Standard Error.

b. Antihistamine-like Activity of the Extracts.

Each extract was suspended in 1% acetic acid or distilled water acidified to pH 3.2 with HCl to a concentration of 30 mg/ml and the solution was injected intraperitoneally into guinea-pigs in a dose of 4 mg/100 g of body weight; the pH of the suspension was 5.9. The control animals were injected intraperitoneally with the same amount of the vehicle. Each treated animal together with its control were exposed to 0.15% histamine aerosol 3 hours following injection.

1. Activity of Extracts from Normal Tomato Stalks.

Fig. 2 summarizes the results obtained in studies of the biological activity of the extracts from normal tomato stalks.

In the experiments of Fig. 2A the animals were injected with extracts obtained from 3 different batches of normal tomato stalks collected 2-4 weeks after inoculation. In 4 of the 9 experiments in this series, the treated animals survived longer than their controls. In 3 experiments the control animals survived longer than their corresponding treated animals and in 2 experiments the difference in survival time between the treated animal and its control was less than 30 seconds. The protection was not significant ($p<0.60$).

In the experiments of Fig. 2B the animals were injected with extracts obtained from 6 different batches of normal stalks collected 4-8 weeks after inoculation. In 6 of the 8 experiments in this series the treated animals survived longer than their controls and 2 animals

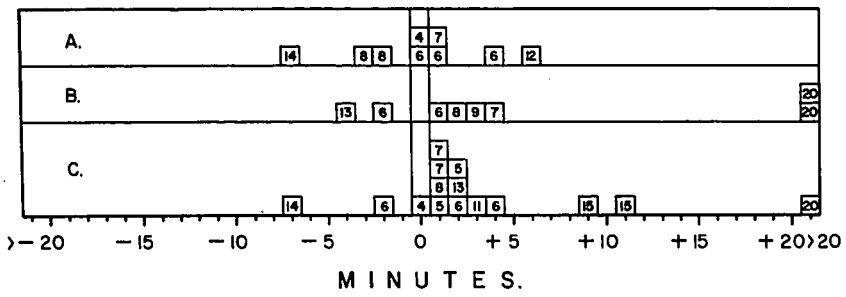


Figure 2

Protection of guinea-pigs against 0.15% histamine aerosol by intraperitoneal injection of the extracts prepared from non-infected tomato stalks collected together with the infected plants: (A) 2-4 weeks; (B) 4-8 weeks; (C) 8-12 weeks after inoculation. For details see text. In this and the following figures the abscissa gives, in minutes, the difference in survival time between treated animals and their controls. The difference is given as positive, if the treated survived longer than the control animal, as negative if the control survived longer than the treated animal, and as 0, if the difference was 30 seconds or less, or if both animals survived the 20 minute exposure time. Each square represents a pair of guinea-pigs (control and treated guinea-pig) and the number in the square indicates the survival time (to the nearest minute) of the longest surviving animal. The numbers in the squares above zero represent the mean survival time between the treated and control animals.

survived the 20 minute exposure time; the mean survival time of their controls was 7 minutes. In 2 experiments the control animals survived longer than the treated animals. The protection was not significant ($p<0.20$).

In the experiments of Fig. 2C the animals were injected with extracts obtained from 6 different batches of normal stalks collected 8-12 weeks after inoculation. In 12 of the 15 experiments in this series the treated animals survived longer than their controls but only one treated animal survived the 20 minute exposure time; the survival time of its control was 14 minutes. In 2 experiments the control animals survived longer than the treated animals and in 1 experiment the difference in survival time between the treated animal and its control was less than 30 seconds. The protection was not significant ($p<0.40$).

These results demonstrated that extracts of normal tomato stalks (up to 12 weeks after inoculation) showed no significant protection of the guinea-pigs against the effect of a lethal dose of histamine aerosol.

2. Activity of Extracts from Crown-gall Infected Tomato Stalks.

Fig. 3 summarizes the results obtained in studies on the biological activity of the extracts from crown-gall infected tomato stalks.

In the experiments of Fig. 3A the animals were injected with the extracts obtained from 3 different batches of crown-gall infected tomato stalks collected 2-4 weeks following inoculation. In 4 of the 9 experiments the treated animals survived longer than their corresponding controls

survived the 20 minute exposure time; the mean survival time of their controls was 7 minutes. In 2 experiments the control animals survived longer than the treated animals. The protection was not significant ($p<0.20$).

In the experiments of Fig. 2C the animals were injected with extracts obtained from 6 different batches of normal stalks collected 8-12 weeks after inoculation. In 12 of the 15 experiments in this series the treated animals survived longer than their controls but only one treated animal survived the 20 minute exposure time; the survival time of its control was 14 minutes. In 2 experiments the control animals survived longer than the treated animals and in 1 experiment the difference in survival time between the treated animal and its control was less than 30 seconds. The protection was not significant ($p<0.40$).

These results demonstrated that extracts of normal tomato stalks (up to 12 weeks after inoculation) showed no significant protection of the guinea-pigs against the effect of a lethal dose of histamine aerosol.

2. Activity of Extracts from Crown-gall Infected Tomato Stalks.

Fig. 3 summarizes the results obtained in studies on the biological activity of the extracts from crown-gall infected tomato stalks.

In the experiments of Fig. 3A the animals were injected with the extracts obtained from 3 different batches of crown-gall infected tomato stalks collected 2-4 weeks following inoculation. In 4 of the 9 experiments the treated animals survived longer than their corresponding controls

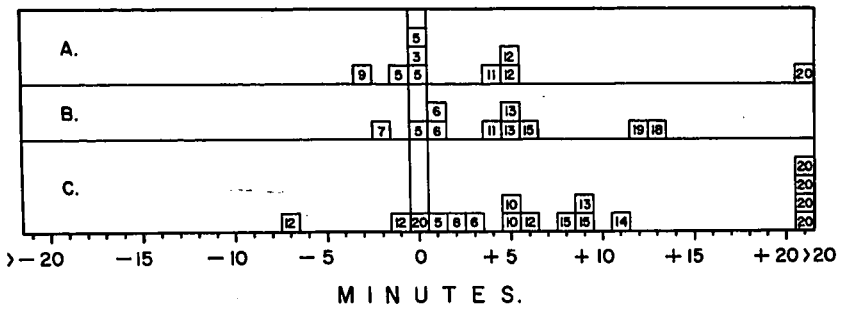


Figure 3

Protection of guinea-pigs injected with the extract prepared from the crown-gall infected tomato stalks harvested: (A) 2-4 weeks; (B) 4-8 weeks; (C) 8-12 weeks following inoculation. For details see text. Details of this figure are the same as in Fig. 2.

and 1 of the treated animals in this group survived the 20 minute exposure; the survival time of its control was 4 minutes. In 2 experiments in this series, the control animals survived longer than the treated animals and in 3 experiments the difference in survival time between the treated animal and its control was less than 30 seconds. The protection was not significant ($p<0.20$).

In the experiments of Fig. 3B the animals were injected with the extracts obtained from 6 different batches of crown-gall infected stalks harvested 4-8 weeks following inoculation. In 8 of the 10 experiments the treated guinea-pigs survived longer than their controls, but none of the treated animals survived the 20 minute period of exposure. Only in 1 experiment did the control survive longer than the treated animal and in 1 experiment the difference in survival time between the treated animal and its control was less than 30 seconds. The protection was significant ($p<0.05$).

In the experiments of Fig. 3C the animals were injected with extracts obtained from 6 different batches of crown-gall infected stalks harvested 8-12 weeks following inoculation. In 14 of the 17 experiments the treated animals survived longer than their corresponding controls and 4 of the treated animals survived the 20 minute exposure period; the mean survival time of their controls was 8 minutes. Only in 2 experiments did the control animals survive longer than their corresponding treated animals and in 1 experiment the difference in survival time between the treated animal and its control was less that 30 seconds. The protection was significant ($p<0.01$).

From the results of these experiments it was concluded that maximum

development of the biological activity occurred 8-12 weeks following inoculation of the plants.

c. Discussion.

The mean yields of extracts obtained from normal tomato stalks were generally lower than the mean yields of extracts of crown-gall infected stalks. Furthermore, the difference in mean yields of the 2 types of extracts was found to be statistically significant. The results of this study indicated that the older the normal plants the higher the yield and specific activity of the extractable material. Statistical analysis of the data, however, showed that the protective effect of the normal stalk extracts against the effect of a lethal histamine aerosol was insignificant. These findings are in agreement with that of Broome et al. (1962) who reported that extracts prepared from very young normal stems produced no protection in guinea-pigs against the effect of a lethal histamine aerosol, while extracts prepared from stems of older tomato plants exerted "some protection but to a lesser degree than that produced by the extracts prepared from infected plants".

In the present study the specific activity of the extracts prepared from the infected stalks appeared to increase in time following inoculation. The extracts prepared from 4-8 week old crown-gall infected stalks exerted protection against a lethal histamine aerosol at the 95% confidence level, while extracts prepared from the 8-12 week old infected stalks produced protection at the 99% confidence level. However, the

mean yields of the crown-gall extracts obtained from infected plants at different time intervals showed no definite pattern and the dry weight of the extracts obtained per unit weight of plants (mg/100 g) showed no correlation with specific activity.

The results presented in this section clearly demonstrated that the extractable specific activity of crown-gall infected tomato stalks and particularly of the older (8-12 weeks) plants was much higher than that of non-infected or freshly infected tomato stalks. The comparative study could not be continued beyond the 8-12 week interval owing to the death of the infected plants. On the basis of this study it seemed reasonable to conclude that the 8-12 week old crown-gall infected plants would provide the most efficacious starting material for the isolation of the antihistamine-like active principle(s) and this type and age of plant was employed in all subsequent experiments.

Section B. The Standard Crude Extract: Preparation and Antihistamine-like Activity.

The primary objective of the experiments described in this section was to establish an efficacious method for extracting the antihistamine-like activity from crown-gall infected tomato stalks. The method of Broome et al. (1962) was employed for the initial step in the extraction procedure, with the modification that the stalks were extracted with chloroform-ethanol (3:1,v/v). Studies were subsequently done on the optimum pH for aqueous dissolution of the active chloroform-ethanol extract and for partition of the antihistamine-like principle(s) from the aqueous to organic phases. The results of *in vitro* and *in vivo* assays of the final "standard crude extract" are also presented.

a. Chloroform-ethanol Extraction of Crown-gall Infected Tomato Stalks: Antihistamine-like Activity of the Extracts.

For the initial step in the extraction procedure, the stalks were extracted with chloroform-ethanol (3:1,v/v). The dried chloroform-ethanol extract was taken up in 1% acetic acid and 2 ml aliquots (each equivalent to 50 g wet weight of crown-gall infected stalk tissues) were injected intraperitoneally into guinea-pigs. Control animals were similarly injected with the same amount of the vehicle. Each treated animal together with its control were exposed to 0.15% histamine aerosol 3 hours following injection.

The results of 20 experiments obtained with 5 different batches of the chloroform-ethanol (3:1,v/v) extracts are shown in Fig. 4. In 19 of the 20 experiments the treated animals survived longer than their corresponding controls while 2 survived the 20 minute exposure period; the mean survival time of their controls was 10 minutes. In 1 experiment the control survived the 20 minute exposure period while the survival time of its treated animal was 13 minutes. The protection of the animals against the lethal effect of histamine aerosol was significant ($p<0.01$).

b. Aqueous Dissolution of the Chloroform-ethanol Extract: pH Requirement.

The chloroform-ethanol (3:1,v/v) extract obtained from approximately 400 g of crown-gall infected tomato stalks was devided into 4 equal portions. Each aliquot was separately taken up in 15 ml distilled water and well blended. The mixtures were adjusted with 12 N HCl to pH 1, pH 2, pH 4, and pH 5 respectively. Each mixture was filtered, dried and taken up in 2 ml of 1% acetic acid and injected intraperitoneally into guinea-pigs in a dose equivalent to 50 g original tissue. The control animals were similarly injected with the same amount of the vehicle. Each treated animal together with its control were exposed to 0.15% histamine aerosol 3 hours following injection. The results of 13 experiments are summarized in Fig. 5.

In 4 experiments (Fig. 5A) the animals were injected with 2 different batches of the pH 5 aqueous extract. In 2 of the 4 experiments the treated guinea-pigs survived longer than their controls and 1 treated animal survived the 20 minute period of exposure; the survival time of its control was 7

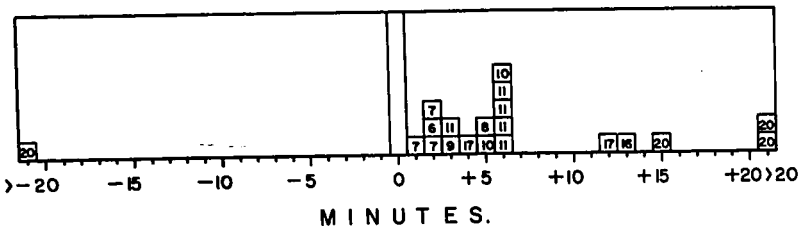


Figure 4

Protection produced by intraperitoneal injection of chloroform-ethanol (3:1,v/v) extract prepared from crown-gall infected tomato stalks in guinea-pigs exposed to 0.15% histamine aerosol. For details see text. Details of this figure are the same as Fig. 2.

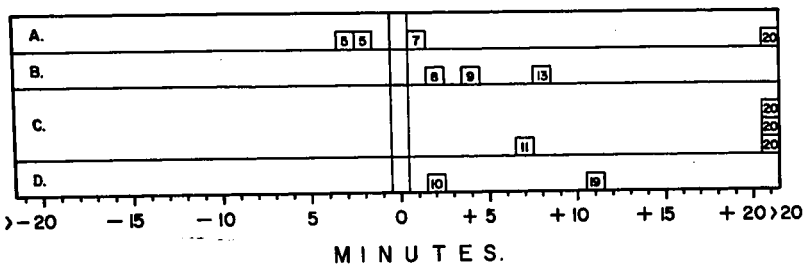


Figure 5

Protection produced by intraperitoneal injection of extract prepared from crown-gall infected stalks by reextracting the chloroform-ethanol (3:1,v/v) residue with water at pH 5 (Figure A), pH 4 (Figure B), pH 2 (Figure C) and pH 1 (Figure D) in guinea-pigs exposed to 0.15% histamine aerosol. For details see text. Details of this figure are the same as Fig. 2.

minutes. In 2 experiments the controls survived longer than their corresponding treated animals. The protection was not significant ($p<0.70$).

In 3 experiments (Fig. 5B) the animals were injected with 2 different batches of the pH 4 aqueous extract, all treated guinea-pigs survived longer than their controls, the protection was significant ($p<0.05$).

In 4 experiments (Fig. 5C) the animals were treated with 2 different batches of the pH 2 aqueous extract. All treated animals survived longer than their corresponding controls and, of these, 3 survived the 20 minute exposure period; the mean survival time of their controls was 5 minutes. The protection was significant ($p<0.01$).

In experiments of Fig. 5D the animals were injected with 2 different batches of the pH 1 aqueous extract, 2 of the 4 treated animals died about 2 hours following injection, the other 2 treated animals survived longer than their corresponding controls but the protection was not significant ($p<0.20$). Furthermore, the surviving treated animals showed sign of toxic side effects (abdominal irritation and tension).

The results of these experiments demonstrated that in the pH range studied, pH 2 was most suitable for dissolving the active principle(s) in aqueous medium.

c. Partition of the Antihistamine-like Principle(s) from the Aqueous to Organic Phases: Efficacy of Various Chloroform-ethanol Mixtures.

The pH 2 aqueous extract (60 ml) equivalent to 400 g of crown-gall infected tomato stalks was devided into 4 equal portions. Each portion was adjusted to pH 8. The 4 aliquots were separately extracted

3 times with 15 ml chloroform-ethanol mixtures of the following v:v proportions: 3:1 (aliquot no. 1); 5:1 (aliquot no. 2); 6:1 (aliquot no. 3); 9:1 (aliquot no. 4). The pool of organic layers obtained from each aliquot was dried individually. The dried extracts were dissolved in 1% acetic acid (20 mg/ml) and injected intraperitoneally into guinea-pigs in a dose of 4 mg/100 g of body weight. The control animals were injected intraperitoneally with the same amount of the vehicle. The treated animals together with their respective control animals were exposed to 0.15% histamine aerosol 3 hours following injection. The results obtained with 3 batches of extracts injected into 24 guinea-pigs are summarized in Fig. 6.

In 7 experiments (Fig. 6A) the animals were injected with 3 different batches of the 3:1 (v/v) chloroform-ethanol extract. Of the 7 treated guinea-pigs, 5 survived longer than their controls and of these 1 survived the 20 minute exposure period; the survival time of its control was 5 minutes. In 1 experiment in this series, the difference in survival time between the treated animal and its control was less than 30 seconds and in another the control survived longer than the treated animal. The protection was not significant ($p<0.10$).

In 5 experiments (Fig. 6B) the animals were injected with 3 different batches of the 5:1 (v/v) chloroform-ethanol extract. Of the 5 treated guinea-pigs, 4 survived longer than their corresponding controls but none of the treated animals survived the 20 minute exposure period. In 1 experiment in this series the difference in survival time between

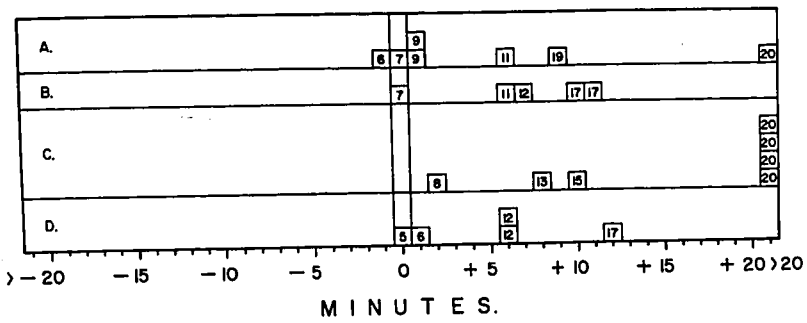


Figure 6

Protection produced in guinea-pigs exposed to 0.15% histamine aerosol by intraperitoneal injection of extract prepared from crown-gall infected stalks by reextracting the pH 2 aqueous extract with chloroform-ethanol, 3:1 (v/v) Figure A; 5:1 (v/v) Figure B; 6:1 (v/v) Figure C; and 9:1 (v/v) Figure D. For details see text. Details of this figure are the same as Fig. 2.

the treated animal and its control was less than 30 seconds. The protection was significant ($p<0.02$).

Fig. 6C shows the results obtained from guinea-pigs treated with 3 different batches of the 6:1 (v/v) chloroform-ethanol extract. All of the 7 treated guinea-pigs survived longer than their corresponding controls and 4 of these survived the 20 minute exposure period; the mean survival time of their controls was 6 minutes. The protection of the animals from the effect of a lethal histamine aerosol exerted by this extract was significant ($p<0.01$).

In 5 experiments (Fig. 6D) in which the animals were injected with 3 different batches of the 9:1 (v/v) chloroform-ethanol extract, 4 of the 5 treated animals survived longer than their corresponding controls. In 1 experiment in this series the difference in survival time between the treated animal and its control was less than 30 seconds. The protection was significant ($p<0.05$).

The results of these experiments demonstrated that the 6:1 (v/v) chloroform-ethanol solvent mixture was most efficacious for extraction of the active principle(s) from the pH 2 aqueous solution.

d. Yields of Standard Crude Extract.

The yields of standard crude extracts obtained from crown-gall infected tomato stalks by the method of preparation described above varied considerably. Table II compares the yields of standard crude extracts obtained from 10 batches of crown-gall infected tomato stalks. These yields were typical for all the standard crude extracts prepared in these studies, the lowest yield was 0.022% (w/w) while the highest was 0.085% and the mean yield was 0.061%.

TABLE II

Yields of the Standard Crude Extract.

Amount of crown-gall infected tomato stalks (wet weight in grams)	Yields of standard crude extract %(w/w)
1700	0.048
1200	0.022
1200	0.085
560	0.071
560	0.066
600	0.075
450	0.078
565	0.054
1175	0.053
1140	0.053
Mean ± S.E.	0.061 ± 0.012

e. Antihistamine-like Activity of the Standard Crude Extract.

1. In Vitro Activity.

A typical example of the effect of the standard crude extract on the isolated guinea-pig ileum preparation (Part II, Section B,e,f) against histamine-induced contractions is shown in Fig. 7. The standard crude extract was dissolved in 1% acetic acid to a concentration in the range of 1-2.5 mg/ml. The amount added to the organ bath varied between 0.1-0.3 ml, since it was found that addition of the same amount of 1% acetic acid did not influence the histamine-induced contractions. The addition of the extract in a bath concentration of 10^{-5} g/ml did not influence the contractions induced by the standard dose of histamine. On the other hand, the addition of the standard crude extract in a bath concentration of 2.5×10^{-5} g/ml itself elicited a contraction of the ileum. However, the responses to the subsequent repeated additions of the standard dose of 0.2 µg histamine became smaller. It was necessary to double the dose of histamine to obtain responses similar to those of the standard controls.

2. In Vivo Activity.

The standard crude extract was tested for antihistamine-like activity in guinea-pigs by the histamine aerosol method (Part II, Section C,a). The dried extract was suspended in 1% acetic acid to a concentration of 30 mg/ml and injected intraperitoneally into guinea-pigs in doses of 2, 3 and 4 mg/100 g of body weight; the pH of the suspension was 5.9. The

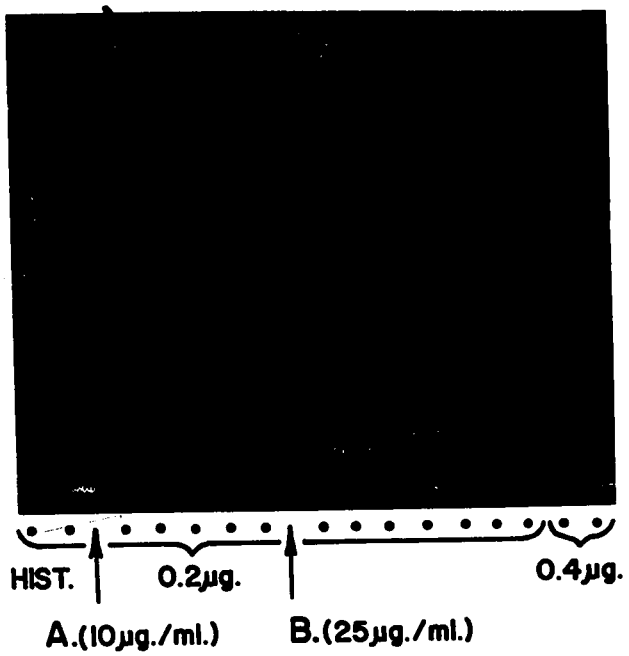


Figure 7

Responses of guinea-pig ileum preparation to histamine before and after the addition of the standard crude extract (as marked at arrows) in bath concentrations of 10^{-5} g/ml and 2.5×10^{-5} g/ml. Intervals of 3 minutes elapsed between each addition of histamine, indicated by the black dots. The histamine was left in contact with the preparation for 20 seconds and the standard crude extract 2 minutes. The organ bath was washed out between each addition of histamine, the drum being temporarily stopped and restarted 20 seconds before the next addition.

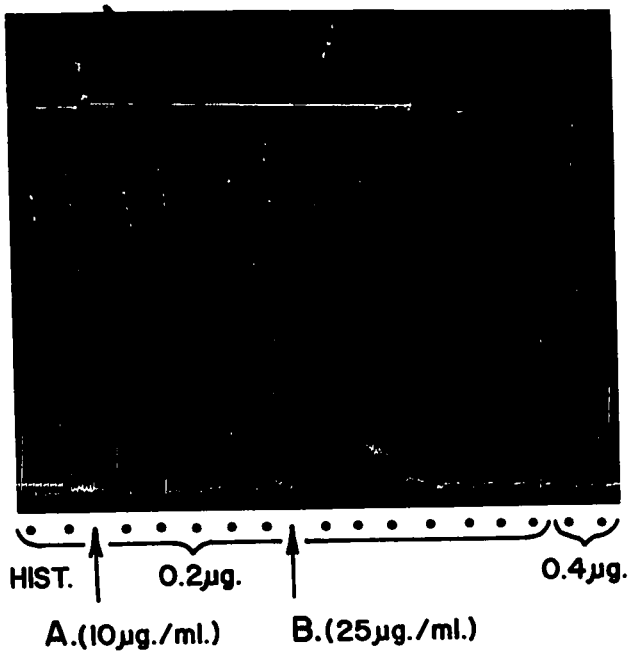


Figure 7

Responses of guinea-pig ileum preparation to histamine before and after the addition of the standard crude extract (as marked at arrows) in bath concentrations of 10^{-5} g/ml and 2.5×10^{-5} g/ml. Intervals of 3 minutes elapsed between each addition of histamine, indicated by the black dots. The histamine was left in contact with the preparation for 20 seconds and the standard crude extract 2 minutes. The organ bath was washed out between each addition of histamine, the drum being temporarily stopped and restarted 20 seconds before the next addition.

control animals were similarly injected with the same amount of the vehicle. Each treated animal together with its control were exposed to a 0.15% histamine aerosol 3 hours following injection. The results obtained from 40 different batches of standard crude extracts in 81 experiments are summarized in Fig. 8.

In 28 experiments (Fig. 8A) the animals were injected with a dose of 4 mg/100 g of body weight. In 20 experiments in this series, the treated animals survived longer than their corresponding controls and 8 treated animals survived the 20 minute exposure time; the mean survival time of their controls was 5 minutes. In 8 experiments the control animals survived longer than their corresponding treated animals and 2 control animals survived the 20 minute exposure period; the mean survival time of their treated was 6 minutes. The protection of the animals against the effect of a lethal histamine aerosol was significant ($p<0.01$).

In 25 experiments (Fig. 8B) the animals were injected with a dose of 3 mg/100 g of body weight. In 19 experiments in this series, the treated animals survived longer than their corresponding controls and 8 treated guinea-pigs survived the 20 minute exposure time; the mean survival time of their controls was 5 minutes. In 4 experiments the control animals survived longer than their corresponding treated animals and 2 control animals survived the 20 minute exposure period; the mean survival time of their treated animals was 10 minutes. In 2 experiments, the difference in survival time between the treated animal and its control was less than 30 seconds. The protection of the animals against the effect

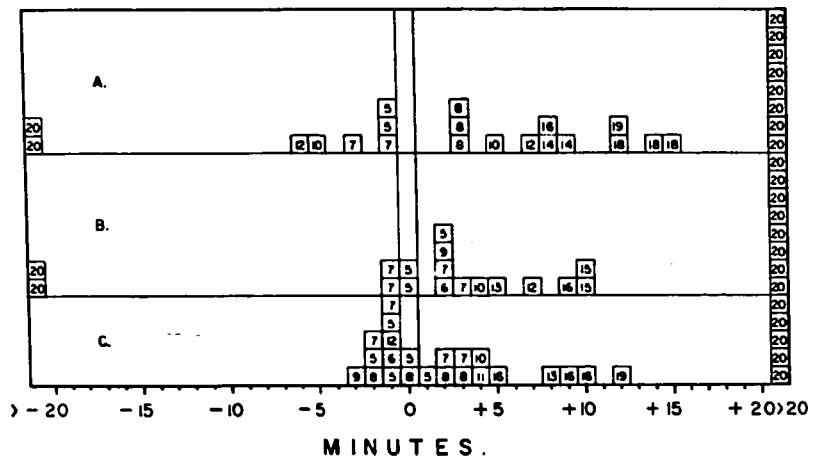


Figure 8

Protection of guinea-pigs against 0.15% histamine aerosol by intraperitoneal injection of the standard crude extract: (A) 4 mg/100 g ; (B) 3 mg/100 g ; (C) 2 mg/100 g of body weight. For details see text. Details of this figure are the same as in Fig. 2.

of a lethal histamine aerosol was significant ($p<0.01$).

In 28 experiments (Fig. 8C) the animals received a dose of 2 mg/100 g of body weight. In 17 experiments in this series, the treated animals survived longer than their corresponding controls but only 5 treated animals survived the 20 minute exposure period; the mean survival time of their controls was 7 minutes. In 9 experiments the control animals survived longer than their corresponding treated animals. In 2 experiments the difference in survival time between the treated animal and its control was less than 30 seconds. The protection of the animals against the effect of a lethal dose of histamine aerosol was significant ($p<0.01$).

f. Long-term Protection Against Lethal Histamine Aerosol.

Broome et al. (1962) reported that a single intraperitoneal injection of partially purified crown-gall extracts protected guinea-pigs against the effect of a lethal histamine aerosol "if injected in sufficient amounts sometimes for a few weeks". Calam and Callow (1964) attempted to confirm this finding by retesting 74 guinea-pigs which had been injected with either plant extracts or tomatine. From the results obtained they concluded that "There might be a slight protection in the injected animals; 53 gave positive results and 21 negative".

In view of the potential practical as well as theoretical significance, if substantiated, of long-term protection resulting from a single injection of plant tumor extract, it was of interest to repeat

these experiments using the extracts prepared in the present study.

For this purpose, 44 guinea-pigs (group I) which in previous tests had been pretreated with extracts of crown-gall infected tomato stalks and which subsequently had survived exposure to 0.15% histamine aerosol for 20 minutes, were re-exposed for a second time to the aerosol 5-53 days later. Eight guinea-pigs (group II) which had in previous tests served as controls and which had survived the 20 minute exposure period were also re-exposed to the histamine aerosol 6-13 days later. Each guinea-pig from the 2 groups was re-exposed only once to the aerosol along with a (fresh) control guinea-pig. The results of these experiments are summarized in Fig. 9.

As shown in Fig. 9A, only 4 of the 8 guinea-pigs in group II survived longer than their corresponding controls during the second exposure. Only 1 of the 8 animals survived the 20 minute exposure period; the survival time of its control was 5 minutes. The protection was not significant ($p<0.10$).

As shown in Fig. 9B, 36 of the 44 guinea-pigs in group I survived longer than their corresponding controls on re-exposure to the aerosol and 12 again survived the 20 minute exposure period; the mean survival time of their control was 5 minutes. The protection was significant ($p<0.01$).

g. Discussion.

The studies presented in this section were aimed at establishing

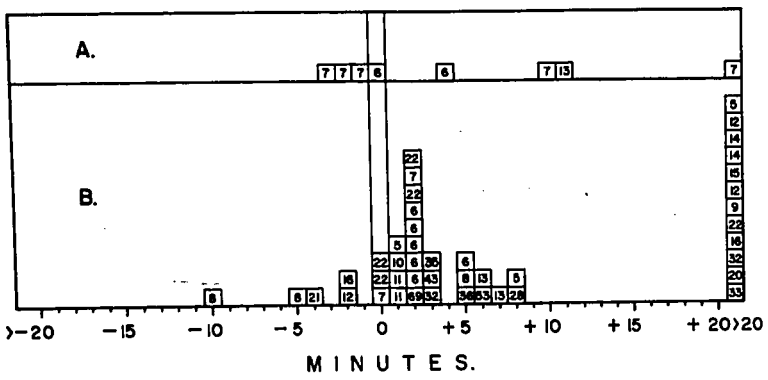


Figure 9

Protection of guinea-pigs against 0.15% histamine aerosol on retest. All animals had survived 1 exposure to the aerosol for 20 minutes. (A) control animals; (B) animals injected intraperitoneally with extracts of crown-gall infected tomato stalks. The abscissa gives the difference in survival time in minutes between the re-exposed animal and its control. The difference is given as positive if the re-exposed animal survived longer, as negative if the control survived longer, and as zero if the difference was less than 30 seconds or if both animals survived the 20 minutes exposure time. The numbers in the squares represent the time elapsed in days between the first and the second exposure.

a reliable and reproducible method by which the active principle(s) could be regularly extracted from the crown-gall infected tomato stalks. At the beginning of these studies i.e., sections a and b (and only in these) the activity of the extracts was calculated on the basis of the original weight of the plant tissues (50 g wet weight). Such a method of expressing activity had been frequently used in the past by other investigators (e.g., Euler and Gaddum, 1931). This method of expressing activity seemed to be the most reliable at this stage of the study since the extractable dry weight showed a wide variation depending upon the solvents or solvent mixtures employed.

To extract the active principle(s) from the plant tissues, the use of a number of organic solvents and solvent mixtures not described in the experimental part was explored but these solvents were not as efficacious as chloroform-ethanol (3:1,v/v) in extracting the maximum biological activity from the crown-gall infected tomato stalks. The latter solvent mixture was therefore routinely employed in these studies.

Due to the fact that the chloroform-ethanol (3:1,v/v) extract contained large amounts of chlorophyls means of separating chlorophyls from the active fractions were attempted. Distilled water acidified to pH 2.0 was found to be suitable for this purpose because most of the chlorophyls were insoluble, while the active principle(s) were soluble in this solvent. In order to make the acidified aqueous solution suitable for bioassay, its pH was adjusted to 8.0 with concomittant precipitate formation. The precipitate was separated from the aqueous solution by

repeated extractions with a chloroform-ethanol (6:1,v/v) mixture. Bioassay of the resulting organic layers showed that the antihistamine-like activity was confined to the organic layer. The totality of the procedures described in this section resulted in the method of preparation of the "standard crude extract" (Part II, Section D,c). Some 40 different preparations of standard crude extract obtained by this method, at this stage of the study, were almost invariably active in vitro (Fig. 7) at a dose of approximately 2.5×10^{-5} g/ml and in vivo (Fig. 8) at 2-4 mg/100 g of body weight.

In addition to protecting guinea-pigs against the effect of the first exposure to the lethal histamine aerosol, the extracts of crown-gall infected tomato stalks showed long-term significant protective activity (Fig. 9) against a second exposure to the aerosol, in agreement with the finding of Broome et al. (1962). (The possible significance of this observation is discussed in detail in the general discussion pages 186-191). One of the ultimate objectives of the present studies was to determine if the biologically active principle(s) in the standard crude extract would display both immediate and long-term antihistamine-like activity in the isolated purified state. The isolation and chemical and biological properties of such principle(s) (tomatine and gomatine) are described in the succeeding sections.

Section C. Isolation and Chemical and Biological Characterization of Tomatine.

In the previous section, a method was described for the preparation of an extract of crown-gall infected tomato stalks which displayed both *in vitro* and *in vivo* antihistamine-like activity. This standard crude extract formed the starting material for the subsequent isolation of the biologically active principles. One of these principles was identified as the steroid alkaloid glycoside, tomatine.

Early in these studies, evidence was obtained for the presence in extracts of crown-gall infected tomato stalks of a component which resembled tomatine and displayed antihistamine-like activity when tested on the isolated guinea-pig ileum preparation. The results obtained in these initial studies are presented in the first part of this section. Following this we shall give results obtained in studies on the isolation of tomatine from the standard crude extract along with chemical and biological characterization of this alkaloid.

I. Evidence Suggesting Tomatine as One of the Active Principles in Extracts of Crown-gall Infected Tomato Stalks.

a. Isolation of a Crystalline Product with Antihistamine-like Activity.

In initial exploratory studies, a crystalline product was isolated from extracts of crown-gall infected tomato stalks by gel filtration on

Sephadex G-25.

The extract employed in this study was prepared as follows: Crown-gall infected tomato stalks (350 g) were ground in a Waring blendor and extracted 3 times with 350 ml portions of diethylether-ethanol (3:1,v/v). The tissue homogenate was heated at 70°C for 10 minutes and filtered. The clear diethylether-ethanol filtrate was dried and the residue was extracted with distilled water (15 ml/100 g stalks). The aqueous extract was filtered and the filtrate was adjusted to pH 8.0 with 1 N NaOH to give a precipitate (approximate yield: 175 mg).

Ten mg of the precipitate was dissolved in dilute HCl (pH 3.2) and the solution applied to a column (0.9x30 cm) of Sephadex G-25. The chromatogram was eluted with dilute HCl (pH 3.2) at a flow rate of 30 ml/ hour. Some pigmented material remained strongly bound to the gel and could not be eluted using acidified water. The effluent was contained in 2 ml aliquots and assayed by the ninhydrin colorimetric method: a single ninhydrin positive peak was obtained (Fig. 10). The peak effluent volume was equal to the void volume (14 ml) of the column obtained by gel filtration of blue dextran.

To locate the biological activity by <u>in vitro</u> testing, an aliquot from each effluent tube was assayed on the isolated guinea-pig ileum preparation. The volume of the sample assayed was 5% (0.1 ml) of the total tube content. The clear effluent, usually freshly collected from the column, was added to the organ bath without further treatment, since it was found that addition of the same amount (0.1 ml) of similarly pre-

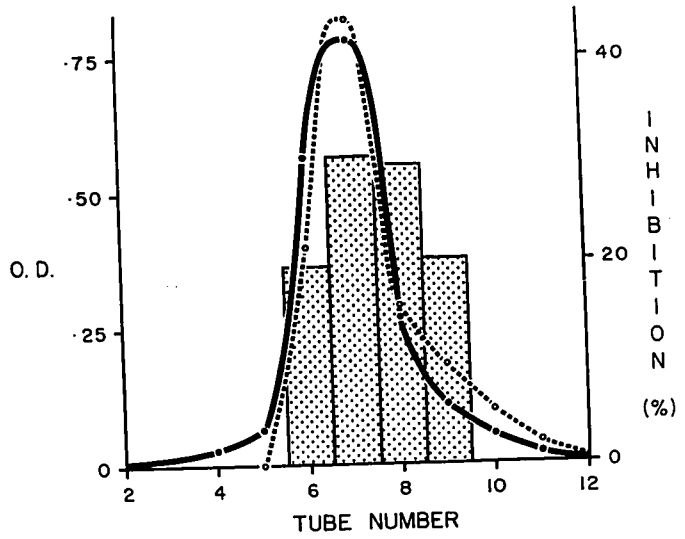


Figure 10

Gel filtration of crude crown-gall infected tomato stalk extract (precipitate) on Sephadex G-25. The chromatogram, with column dimensions of 0.9x30 cm, was developed with dilute HCl pH 3.2 at a flow rate of 30 ml/hour. The effluent was collected in 2 ml portions. The solid line indicates the distribution of the ninhydrin positive material at 570 mμ and the dotted line gives the distribution of blue dextran at 625 mμ. The histogram gives the % inhibitory activity of each effluent tube estimated from Fig. 11.

pared effluent (dilute HCl pH 3.2) did not influence the histamine-induced contractions. Fig. 11 shows the responses of a guinea-pig ileum preparation to histamine-induced contractions before and after the addition of the aliquots from tubes 6-9, as indicated by the arrows. Addition of these aliquots itself elicited contractions of the ileum. The responses to the subsequent standard dose of 0.05-0.40 µg of histamine with repeated washings, became smaller. To reduce the time required for each assay it was necessary to double the original doses of histamine to obtain responses similar to those of standard controls. The in vitro activity was largely confined to the descending side of the ninhydrin positive peak, suggesting slight retardation of the active principle(s) on Sephadex G-25.

The effluent tubes containing the ninhydrin positive substance(s) were pooled. The solution was adjusted to pH 8.0 with 2 N NaOH and extracted 3 times with 10 ml aliquots of a chloroform-ethanol (4:1,v/v) mixture. The pool of the chloroform-ethanol extracts was dried and the yellowish crystalline product obtained was assayed on the isolated guinea-pig ileum preparation. For in vitro assay the crystalline substance was dissolved in water acidified to pH 3.2. The volume of the solution added to the 20 ml organ bath varied from 0.1-0.3 ml, since it was found that addition of 0.1-0.3 ml water at the same pH did not influence the histamine-induced contractions. A typical example of the effect obtained on contractions induced by histamine is shown in Fig. 12. The addition of the substance to a bath concentration of 3×10^{-6} g/ml itself elicited

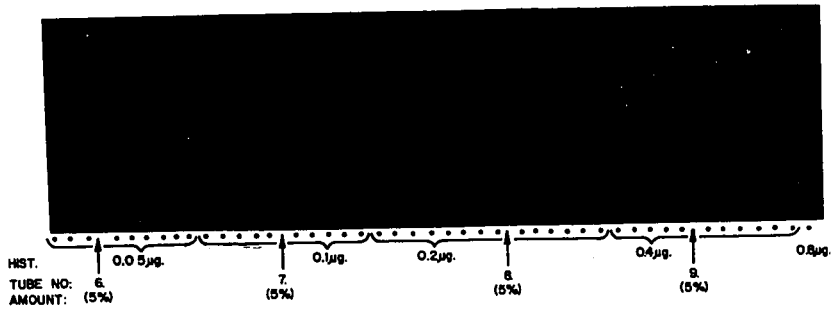


Figure 11

Responses of guinea-pig ileum preparation to histamine (Hist. at black dots) before and after the addition of the effluents as marked at arrows. The arrows are numbered according to the effluent tube number. From each effluent tube 5% (0.1 ml) of the total content was added to the organ bath. Intervals of 3 minutes elapsed between each addition of histamine. In each instance the drum was temporarily stopped after washing out the organ bath and restarted 20 seconds before the next addition. The histamine was left in contact with the ileum for 20 seconds and the effluent for 2 minutes.

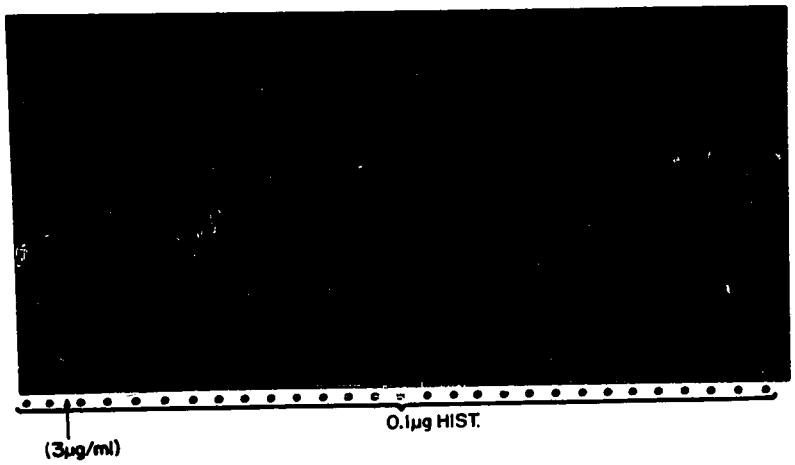


Figure 12

Responses of a guinea-pig ileum preparation to histamine before and after the addition of crystalline crown-gall substance (as marked at arrow) in a concentration of 3×10^{-6} g/ml. Intervals of 3 minutes elapsed between each addition of histamine (indicated by the black dots). The histamine was left in contact with the preparation for 20 seconds, and the crown-gall substance for 2 minutes. The organ bath was washed out between each addition of histamine, the drum being stopped temporarily and restarted 20 seconds before the next addition.

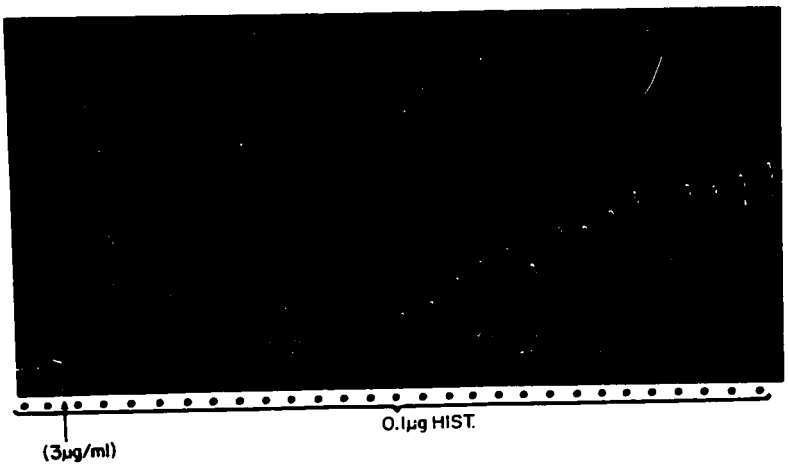


Figure 12

Responses of a guinea-pig ileum preparation to histamine before and after the addition of crystalline crown-gall substance (as marked at arrow) in a concentration of 3×10^{-6} g/ml. Intervals of 3 minutes elapsed between each addition of histamine (indicated by the black dots). The histamine was left in contact with the preparation for 20 seconds, and the crown-gall substance for 2 minutes. The organ bath was washed out between each addition of histamine, the drum being stopped temporarily and restarted 20 seconds before the next addition.

an initial contraction similar to that of histamine. However, the responses to the subsequent standard dose of 0.1 µg of histamine, with repeated washings, were almost completely inhibited. The sensitivity of the ileum remained much reduced for periods in excess of 1 hour.

b. In Vitro Antihistamine-like Activity of a Commercial Tomatine Preparation.

Gel filtration on Sephadex G-25 of an extract of crown-gall infected tomato stalks resulted in the isolation of a single ninhydrin-positive fraction with antihistamine-like activity when assayed on the isolated guinea-pig ileum preparation. As the in vitro activity was largely confined to the descending side of the eluted ninhydrin positive peak, the constituent active component(s) appeared to have a molecular weight of less than 5000, the latter being the exclusion limit of Sephadex G-25. The active fraction was readily soluble in acidified water (pH 1-3.2) but precipitated out from neutral or slightly alkaline media (pH 7-8).

From these results it was inferred that the active fraction might contain tomatine (a known constituent of normal tomato plant) of molecular weight 1035 and having similar solubility properties. This inference was made more plausible by the results obtained by in vitro assays of a commercial tomatine preparation.

A crystalline tomatine preparation (Nutritional Biochemicals Corp.) was tested for antihistamine-like activity on the isolated guinea-pig ileum preparation. For the assay the sample was dissolved in water acidified to pH 3.2 and added to the organ bath in volumes between 0.1-0.3 ml.

Fig. 13 shows the effect of the commercial tomatine preparation on histamine-induced contractions, when administered to bath concentrations of $3x10^{-6}$ g/ml and $4x10^{-6}$ g/ml. It can be seen that the inhibitory activity of the commercial tomatine was very similar to that of the crystalline crown-gall product. To reduce the time required for each assay, it was necessary to double the original dose of histamine to obtain responses similar to those of the standard controls.

c. Discussion.

In the present study we regularly observed antihistamine-like activity when the crystalline crown-gall product and commercial tomatine were separately tested on the isolated guinea-pig ileum preparation. In contrast, Broome et al. (1962) reported that chloroform soluble material derived from crown-gall infected tomato stalks, when taken up in saline, showed no such inhibitory activity. The discrepancy in the findings with respect to in vitro activity might be due to variation in the degree of purity of the active principle(s) in the respective extracts tested. The discrepancy might also be due to differences in solubility of the active principle(s) at different pH's. Broome et al. (1962) employed saline to dissolve the chloroform soluble extract, whereas in the present study the crystalline product was dissolved in water at pH 3.2.

The crystalline product and commercial tomatine preparation were equally potent in inhibiting the histamine-induced contractions of the isolated guinea-pig ileum preparations and both substances displayed a

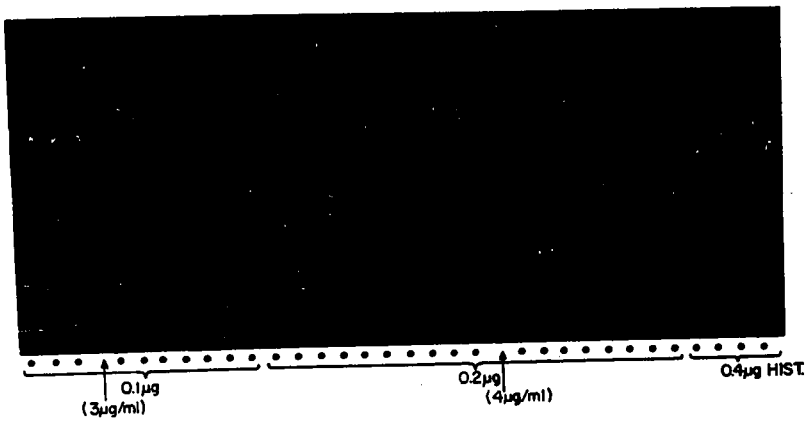


Figure 13

Responses of guinea-pig ileum preparation to histamine (Hist. at black dots) before and after the administration of crystalline tomatine as marked at arrows, in two different bath concentrations ($3x10^{-6}$ and $4x10^{-6}$ gm/ml). Intervals of 3 minutes elapsed between each administration of histamine. In each instance the drum was stopped temporarily after washing out the organ bath and restarted 20 seconds before the next dose. The contact times were 20 seconds for histamine and 2 minutes for tomatine.

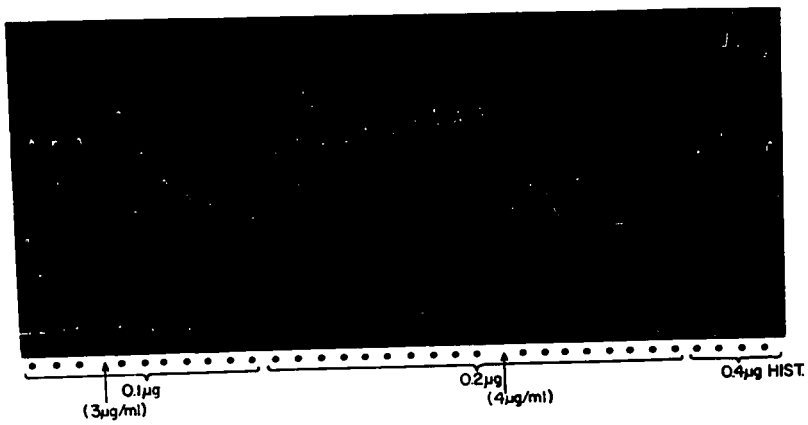


Figure 13

Responses of guinea-pig ileum preparation to histamine (Hist. at black dots) before and after the administration of crystalline tomatine as marked at arrows, in two different bath concentrations ($3x10^{-6}$ and $4x10^{-6}$ gm/ml). Intervals of 3 minutes elapsed between each administration of histamine. In each instance the drum was stopped temporarily after washing out the organ bath and restarted 20 seconds before the next dose. The contact times were 20 seconds for histamine and 2 minutes for tomatine.

characteristic behavior of eliciting a histamine-like contraction of the gut immediately following their separate administration to the organ bath. Since the crystalline product and commercial tomatine preparation each revealed multiple spots by paper chromatography, it was possible that the histamine-like contractions induced by these preparations were due to components other than the inhibitory principle(s). Some attempts were made to further purify these preparations by partition between aqueous and organic phases and by recrystallization from a variety of organic solvents. However, these attempts proved unsuccessful inasmuch as they failed to separate the histamine-like from the inhibitory effects of the preparations and to separate pigmented from non-pigmented components present in both preparations.

II. Isolation of Tomatine from Standard Crude Extract.

While no definitive conclusion could be drawn from the initial studies, described in the previous section, the results were strongly suggestive that tomatine was present in crown-gall infected tomato stalk extract and was active in inhibiting histamine-induced contractions of the isolated guinea-pig ileum preparation.

In subsequent studies the standard crude extract was employed as starting material for the isolation of tomatine. In view of the difficulties encountered in the purification of the crystalline product obtained by gel filtration of extract of crown-gall infected tomato stalks, a different

methodological procedure (cholesterol precipitation and fission of the tomatine-cholesterol complex) was applied to the standard crude extract. The latter method proved efficacious for the isolation of pure tomatine in an amount required for the chemical and biological characterization of this steroid alkaloid.

a. Isolation of Tomatine.

The standard crude extract (472 mg) was treated with cholesterol (472 mg) to obtain the tomatine-cholesterol complex (Part II, Section D, d). The tomatine-cholesterol complex (217 mg) was dissolved in 16 ml pyridine. The solution was refluxed for 1 hour in a water bath maintained at 100°C (Schulz and Sander, 1957). On cooling to room temperature, diethylether was added until precipitation ceased. The amount of diethylether added was about 10 times the volume of pyridine employed to dissolved the tomatine-cholesterol complex. A precipitate which formed on addition of diethylether was centrifuged at 2000 RPM for 15 minutes at room temperature and supernatant decanted. To remove all contaminating cholesterol, the precipitate was washed with diethylether until the washings showed a negative Liebermann-Burchard reaction. The mean yield of diethylether washed precipitate was 86% of the theoretical yield of tomatine (Table III). The precipitate (133 mg) was dissolved in a minimum volume of hot methanol (60°C) and filtered through a fluted filter paper (Whatman no. 2) in a large funnel with a short, wide stem. The solution was kept at -15°C overnight, centrifuged at 2000 RPM for 15 minutes at -15°C and the mother

liquor decanted. The crystals were dried in a vacuum desiccator over anhydrous $CaCl_2$.

The procedure of recrystallization was repeated and the melting point (corrected) obtained for each batch of crystals was determined on a Fisher-Johns melting point apparatus. Table IV lists the typical yields obtained on recrystallization and the corresponding melting point of the crystals. The melting point reached a constant value by the 4th or 5th recrystallization. For further chemical characterization and biological studies a 5 times recrystallized sample was employed.

TABLE III

Yield of Diethylether Washed Precipitate Obtained from Fission of Tomatine-cholesterol Complex.

Tomatine-cholesterol complex	Diethylether washed precipitate
mg	mg/100 g complex
294	61
1008	68
600	62
1300	62
Mean ± S.E.	63 ± 2

TABLE IV

Yields Obtained on Recrystallization of the Isolated Substance and the Melting Points of the Crystals.

Number of recrystallization	Yield		Melting point
	(mg)	(%)	°C
0	75.5	--	248-50
1st	55.5	73	260-2
2nd	43.9	58	266-9
3rd	37.1	49	272-4
4th	32.1	43	273-4
5th	22.1	29	273-4

The results of elementary analysis of the crystals, performed by Mikroanalytisches Laboratorium, Bonn, are given in Table V. There was close agreement between the percentage of carbon, hydrogen, oxygen and nitrogen obtained for the crystalline substance and the corresponding theoretical values calculated for tomatine. The crystalline substance and 30 times recrystallized tomatine (kindly supplied to us by Dr. J.J. Kabara, University of Detroit, hereafter referred to as authentic tomatine) were examined in the infrared in the KBr pellet form, using a Perkin Elmer Model 221 infrared spectrophotometer. Identical spectra were obtained for both substances (Fig. 14).

Data obtained from elementary and infrared analysis demonstrated that the 5 times recrystallized product was pure tomatine.

b. In Vitro Antihistamine-like Activity of Tomatine.

For in vitro assays (Part II, Section B) the 5 times recrystallized product (tomatine) was dissolved in dilute HCl, pH 3.2, or in 80% ethanol to a concentration of 1-2 mg/ml. The amount of solution added to the organ bath varied between 0.1-0.3 ml, since it was found that the addition of the same amount of the solvent to the bath did not influence the contractions induced by the agonists.

TABLE V

The Percentage of Carbon, Hydrogen, Oxygen and Nitrogen of Crystalline Crown-gall Substance and Tomatine.

Elements	% Composition	
	Crystalline crown-gall substance[1]	Tomatine[2]
Carbon	57.38	58.07
Hydrogen	7.91	8.09
Oxygen	33.08	32.49
Nitrogen	1.27	1.35

[1] Microanalytisches Laboratorium.

[2] Calculated from the formula $C_{50}H_{83}O_{24}N$ (Fontaine et al., 1951).

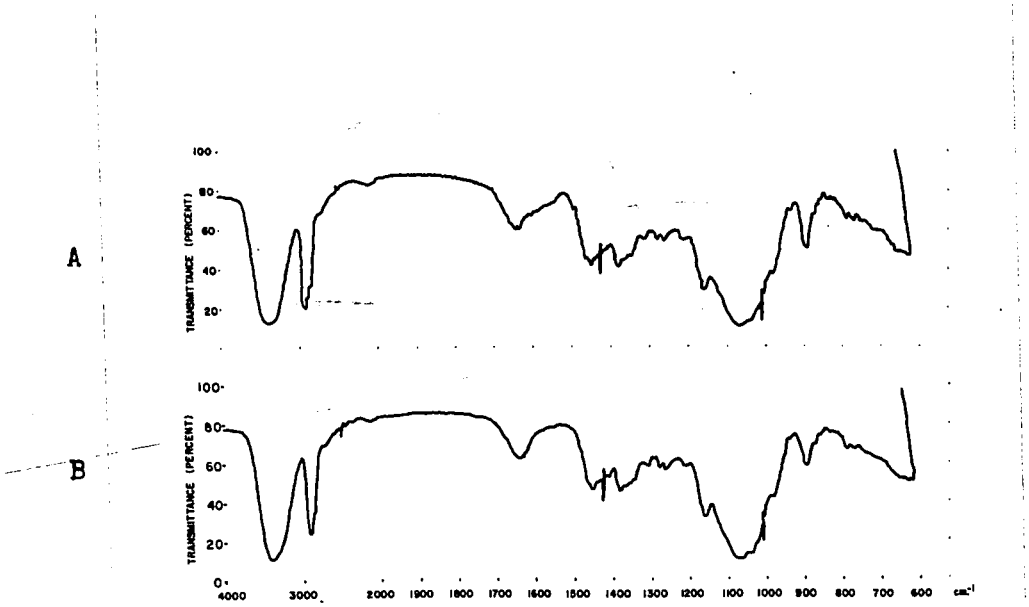


Figure 14

The infrared spectra of the crystalline crown-gall substance (A) and pure tomatine (B). The abscissa gives the wave numbers in cm^{-1} and the ordinate gives the transmittance in percentage.

Fig. 15 shows a typical example of the effect of tomatine in a bath concentration of 4×10^{-6} g/ml against the contractions induced by histamine. The addition of tomatine to the bath itself elicited an initial contraction of the ileum. However, with repeated washings the contraction to the subsequent standard dose of 0.25 µg of histamine became progressively smaller. The sensitivity of the ileum remained reduced for period in excess of 1 hour.

In order to determine whether tomatine-histamine antagonism was a competitive or a non-competitive type, the log dose/response curves for histamine with and without tomatine pretreatment of the ileum was determined. Fig. 16 summarizes the results obtained. The ordinate gives the percentage response of the ileum and the abscissa gives the logarithm of the concentration of histamine (bath concentration). The experiments were so conducted that each ileum preparation served as its own control. Prior to administration of tomatine, the log dose/response curve for histamine was first determined. The maximum response of the segment to the addition of histamine was arbritrarily designated as 100% response to facilitate computation of results. The preparation was then washed several times with Tyrode and the concentration of histamine which elicited approximately 50% response of the maximal contraction was established. After the sensitivity of the preparation to the standard dose of histamine had been established, the tomatine solution was added to the bath. Tomatine was left in contact with the preparation for 2 minutes and washed several times to remove the unbound tomatine. Histamine was added

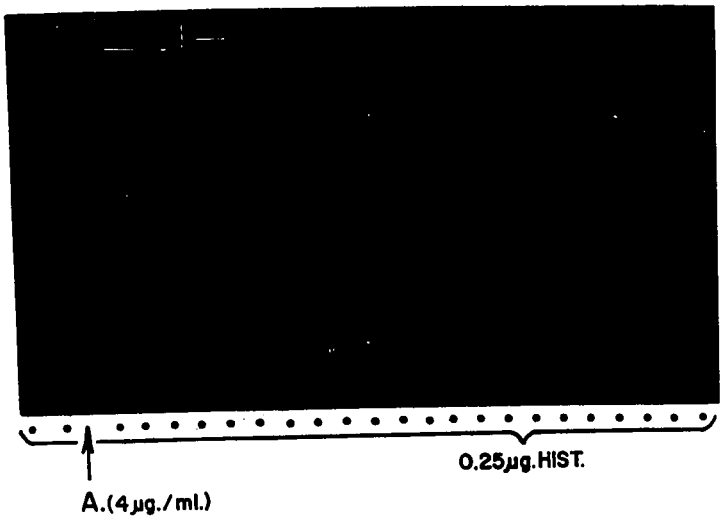


Figure 15

Responses of guinea-pig ileum preparation to histamine (Hist. at black dots) before and after the addition of tomatine (marked at arrow) in a concentration of 4×10^{-6} g/ml. 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 20 seconds before the next dose. The contact times were 20 seconds for histamine and 2 minutes for tomatine.

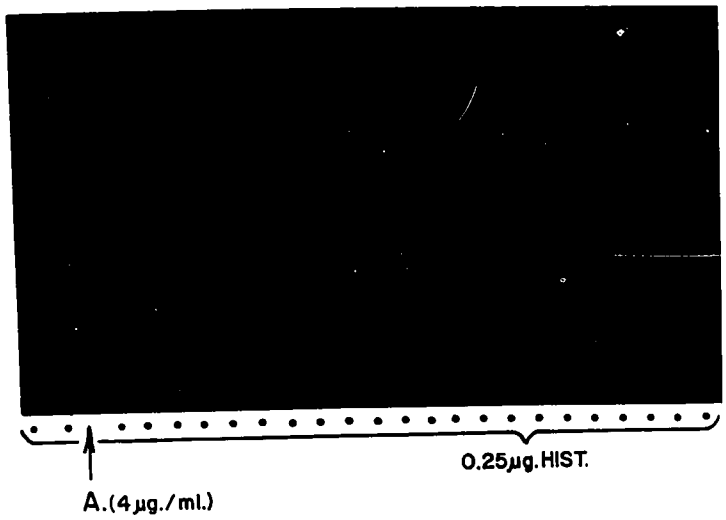


Figure 15

Responses of guinea-pig ileum preparation to histamine (Hist. at black dots) before and after the addition of tomatine (marked at arrow) in a concentration of $4x10^{-6}$ g/ml. 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 20 seconds before the next dose. The contact times were 20 seconds for histamine and 2 minutes for tomatine.

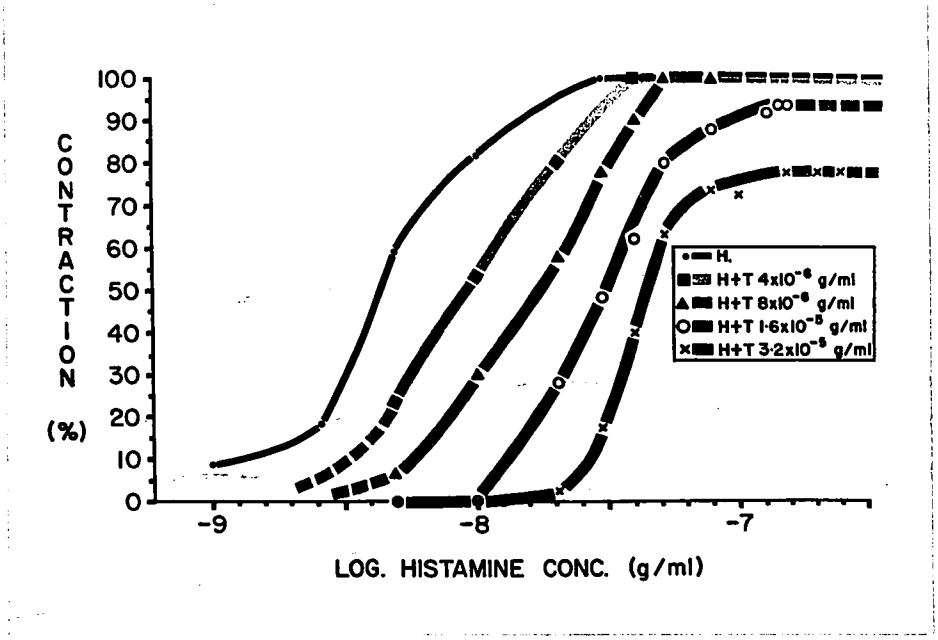


Figure 16

The log dose/response curves of histamine (H) with tomatine (T). The ordinate gives the percentage response of the ileum. The abscissa gives the logarithm concentration of histamine. The left hand curve was in the absence of tomatine. For detail see text.

in increasing doses at 3 minute intervals until a maximum response of the ileum was obtained. To identify the maximum response, doses of histamine were successively doubled and the maximum was considered to have been reached when a doubling of the dose produced no increase in response. Each point given in the figure represent an average of 2 contractions elicited by the same dose of histamine. Fresh segment was employed for each concentration of tomatine. As can be seen the log dose/response curves for histamine could be shifted along the agonist dose axis by low concentrations of tomatine (4,8 and 16×10^{-6} g/ml) without changing either its slope or asymptote significantly. However, with a higher concentration of tomatine (3.2×10^{-5} g/ml) the maximum of the curve became somewhat lower, and was attained using a histamine concentration nearly 10 times that required for the original (uninhibited) contractions.

The specificity of inhibitory action of tomatine was determined by comparison of pA_x values for tomatine against contractions induced by histamine, bradykinin, acetylcholine and barium chloride of the isolated guinea-pig ileum preparation. The term pA_x (Schild, 1947) is defined as the negative logarithm to the base 10 of the molar concentration of an antagonistic drug which will reduce the effect of a multiple dose (x) of an agonist to that of a single dose. The method (Part II, Section B,g) consisted of finding 2 mean concentrations of the antagonistic drug, one of which reduced the effect of a multiple dose (x) of the agonist to slightly less and the other to slightly more than the effect of a single dose. The negative logarithm of the molar concentration of the antagonist corresponding

to pA_x was then obtained by interpolation.

Table VI lists the mean negative log molar concentrations of tomatine and their corresponding mean percentage inhibitory effects. The positive % effects represent inhibitory effect of tomatine against a multiple dose (x) of the agonist to slightly more and the negative % effects to slightly less than the standard contractions induced by a single dose of the agonist. The pA_x values listed were obtained by interpolation as indicated by the arrows shown in Figs. 17, 18, 19 and 20, for histamine, bradykinin, acetylcholine and barium chloride, respectively. It is apparent from table VI that the pA_2 of tomatine for histamine, bradykinin and acetylcholine were fairly close to each other whereas that for barium chloride was lower.

c. In Vivo Antihistamine-like Activity of Tomatine.

Tomatine (recrystallized 5 times) was assayed in vivo using the histamine aerosol method. For this purpose, tomatine was dissolved in dilute HCl, pH 3.2, or 80% ethanol to a concentration ranging from 10-100 mg/ml and the solution (pH = 5.4) was injected intraperitoneally into guinea-pigs in doses in the range of 0.3-1.0 mg tomatine/100 g of body weight. The control animals were similarly injected with the same amount of the vehicle. Each treated animal together with its control were exposed to 0.15% histamine aerosol 3 hours following injection.

In 6 of the 12 experiments shown in Fig. 21 the treated animals survived longer than their corresponding controls but none of the treated animals survived the 20 minute exposure period. In 3 experiments the

TABLE VI.

The pA_x values for Tomatine in Antagonizing Histamine, Bradykinin, Acetylcholine and Barium Chloride Induced Contractions of the Isolated Guinea-pig Ileum Preparation.

Agonist	No. of determinations	Neg. log molar concentrations of tomatine ± S.E.	% Effect ± S.E.	pA_2	pA_4
Histamine	12	5.737 ± 0.039	+ 19.0 ± 12.5		
	10	5.642 ± 0.028	- 24.0 ± 13.6	5.688	
	3	5.440 ± 0.006	+ 19.0 ± 22.4		
	5	5.423 ± 0.004	- 9.0 ± 5.6		5.429
Bradykinin	3	5.738 ± 0.003	+ 14.0 ± 5.8		
	6	5.671 ± 0.023	- 11.0 ± 5.3	5.722	
	4	5.528 ± 0.018	+ 12.0 ± 9.8		
	3	5.472 ± 0.007	- 21.0 ± 8.7		5.508
Acetylcholine	4	5.771 ± 0.014	+ 16.0 ± 9.4		
	3	5.705 ± 0.006	- 11.0 ± 7.4	5.726	
Barium chloride	1	5.491	+ 16.0		
	3	5.443 ± 0.007	- 7.0 ± 3.0	5.461	

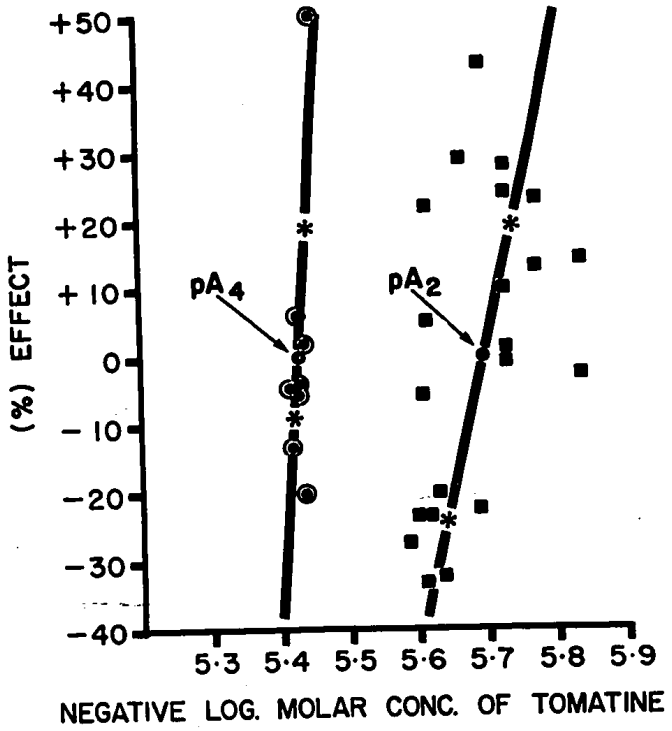


Figure 17

The pA_2 and pA_4 for tomatine against histamine on isolated guinea-pig ileum preparation. The abscissa gives the negative logarithm to base 10 of molar concentration of tomatine. The ordinate gives the percentage inhibitory effect elicited by tomatine against multiple (2 and 4) doses of histamine to slightly less (-% effect) and to slightly more (+% effect) than the standard contractions induced by a single dose of histamine. The aterisks represent mean percentage effects, circles and squares represent the experimental percentage effects. The arrows indicate the pA_2 and pA_4 values obtained by interpolation of the mean percentage effects.

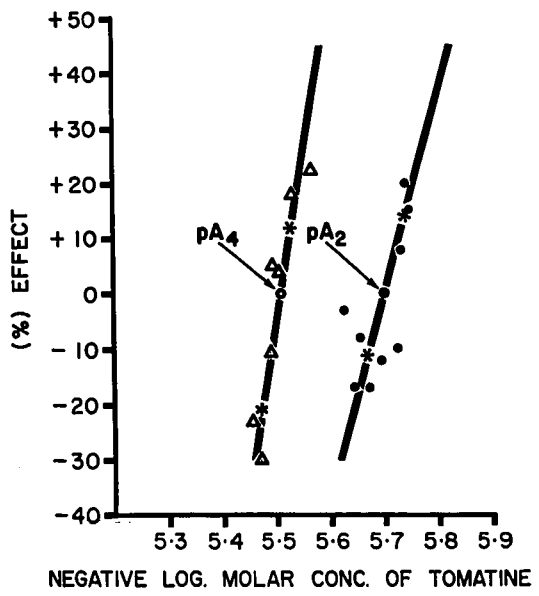


Figure 18

The pA_2 and pA_4 for tomatine against bradykinin on isolated guinea-pig ileum preparation. The abscissa gives the negative logarithm to the base 10 of molar concentration of tomatine. The ordinate gives the percentage inhibitory effects elicited by tomatine against a multiple (2 and 4) dose of bradykinin to slightly less (-% effect) and to slightly more (+% effect) than the standard contractions induced by a single dose of histamine. The aterisks represent mean percentage effects, triangles and dots represent the experimental percentage effects. The arrows indicate the pA_2 and pA_4 obtained by interpolation of the mean percentage effects.

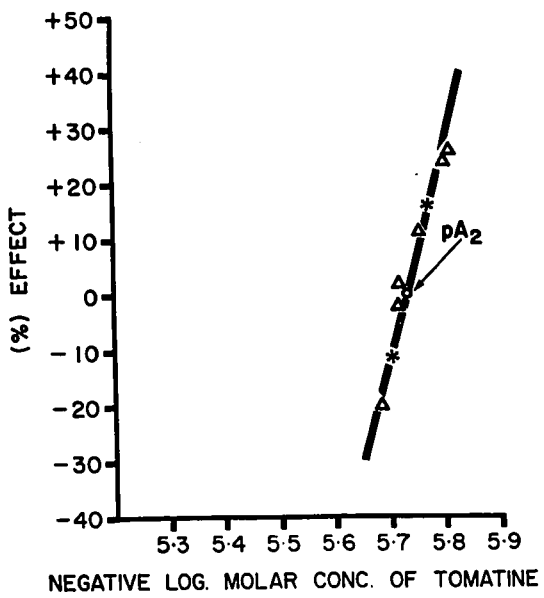


Figure 19

The pA_2 for tomatine against acetylcholine-induced contractions of the isolated guinea-pig ileum preparation. The abscissa gives the negative logarithm to the base 10 of molar concentration of tomatine. The ordinate gives the percentage inhibitory effects elicited by tomatine against a multiple (2) dose of acetylcholine to slightly less (-% effect) and to slightly more (+% effect) than the standard contractions induced by a single dose of acetylcholine. The aterisks represent mean percentage effects, the triangles represent the experimental percentage effects. The arrow indicates the pA_2 obtained by interpolation of the mean percentage effects.

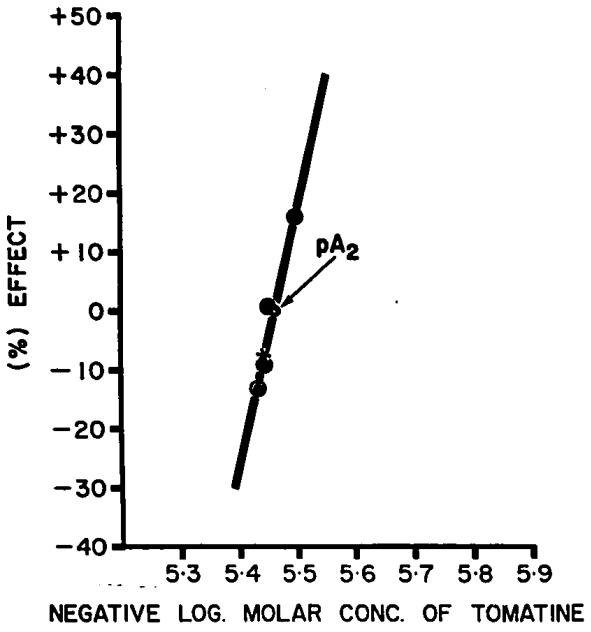


Figure 20

The pA_2 for tomatine against barium chloride-induced contractions of the isolated guinea-pig ileum preparation. The abscissa gives the negative logarithm to the base 10 of molar concentration of tomatine. The ordinate gives the percentage inhibitory effects elicited by tomatine against a multiple (2) dose of barium chloride to slightly less (-% effect) and to slightly more (+% effect) than the standard contractions induced by a single dose of barium chloride. The aterisk represents mean percentage effect the circles represent experimental percentage effects. The arrow indicates the pA_2 obtained by interpolation.

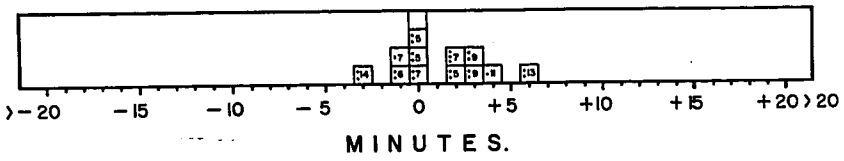


Figure 21

Protection produced by intraperitoneal injection of tomatine in doses between 0.3-1.0 mg/100 g of body weight, in guinea-pigs exposed to 0.15% histamine aerosol. The dots in the squares indicate the dose level injected, 0.3 mg/100 g (1 dot), 0.5 mg/100 g (2 dots) and 1.0 mg/100 g of body weight (3 dots). Details of this figure are the same as in Fig. 2.

control animals survived longer than their corresponding treated animals. In 3 experiments the difference in survival time between the treated animal and its control was less than 30 seconds. Two animals injected with a dose of 1 mg/100 g of body weight died within 3 hours following injection due to toxic effects of tomatine. The protection was insignificant ($p<0.20$).

d. Discussion.

The results of the studies presented in this section demonstrated that the method of cholesterol precipitation followed by fission of the insoluble complex was efficacious for the isolation of tomatine from the standard crude extract. Under the conditions employed, a single treatment of the extract with cholesterol quantitatively removed the cholesterol precipitable material from the extract, since a second treatment with cholesterol (Part II, Section D,d) invariably failed to yield any additional precipitate. Based on the theoretical yield for tomatine, 86% fission of the precipitated complex was obtained by refluxing a pyridine solution of the complex for the 1 hour period employed by Schulz and Sander (1957). The isolated product, following recrystallization, showed a sharp melting point of 273-4°C; however, no comparison with the literature values could be made since divergent melting points for tomatine have been reported (Fontaine et al., 1948; Kuhn and Löw, 1948). The isolated product reacted positively with the anthrone reagent indicating the presence of a carbohydrate moiety. Finally, the isolated product had an elementary composition close to theoretical for tomatine and an infrared spectrum indistinguishable from authentic tomatine.

Our observation that the tomatine obtained from the standard crude extract, when tested on the isolated guinea-pig ileum preparation, was able to antagonize the contractions induced by histamine, bradykinin, acetylcholine and bariumchloride, demonstrated a new biological activity for this steroid glycoside. Some preliminary studies were done to determine if the mechanism of action of tomatine against the agonist was of a specific or non-specific, competitive or non-competitive nature.

It is well known that competitive antagonist produce parallel log dose/response curves (Schild and Arunlaksana, 1959), though such curve do not constitute proof of a competitive antagonism (indeed they may occur in neutralization antagonism; Gaddum, 1943). On the other hand, in the case of non-competitive antagonism the log dose/response curves are not parallel but become progressively flatter and their maxima decline (Schild, 1954). In the present studies the log dose/response curves were parallel for low concentrations of tomatine (4,8 and 16×10^{-6} g/ml) but the maximum was reduced in the present of a higher concentration of tomatine (3.2×10^{-5} g/ml). Furthermore, increasing the dose of histamine (approximately 10 fold) failed to restore the ileum to its original maximum contractions.

According to Schild (1947), if different agonists act on the same receptors they could be expected to be antagonized by the same antagonist and to produce with the antagonist the same pA_x. The pA_x values can consequently be used to determine the specificity of an antagonist. Should the antagonist give the same pA_x with agonists acting on different receptors the antagonist can be considered to have a non-specific mechanism of action.

In the present studies the pA_2 values for tomatine in antagonizing histamine, bradykinin, acetylcholine-induced contractions of the isolated guinea-pig ileum preparation were very close to each other. This would suggest that tomatine exerted its effect against the different smooth muscle stimulants most probably in a non-specific manner. However, the log dose/response curves for histamine remained parallel over a considerable range of tomatine concentration. Therefore, the question of whether tomatine acts as a non-equilibrium (Nickerson, 1956, 1957) or as an irreversible competitive antagonist (Furchgott, 1954, 1966) can be decided by further studies only.

Regarding the in vitro antihistamine-like activity of tomatine, our findings are not in accord with those reported by Calam and Callow (1964). These workers reported that tomatine exerted no antihistamine-like activity when assayed on the isolated guinea-pig ileum preparation. Unfortunately, they did not give any details in their publication concerning the experimental procedure employed in their in vitro experiments but simply stated in the "Discussion" of their paper that "pure tomatine (50 µg) was also found to be without activity". Their finding of a lack of in vitro activity might have been due to the use of an insufficiently high dose of tomatine administered to the organ bath.

In contrast to the findings of the present studies that tomatine did not exert any significant protection in vivo against the effect of a lethal histamine aerosol, Calam and Callow (1964) reported that chemically pure tomatine was strongly protective. The in vivo protection observed

by the latter workers might have been due to the presence, in their tomatine preparation, of a component chemically similar to tomatine. Evidence for the presence of such an active component in extracts of crown-gall infected tomato stalks and studies done to isolate it are presented in the following section.

Section D. Purification and Chemical and Biological Characterization of Gomatine.

The data presented in the previous section showed that the standard crude extract exerted a stronger protection against the effect of a lethal histamine aerosol than cholesterol precipitable tomatine when injected intraperitoneally into guinea-pigs. On the basis of these observations, studies were done which demonstrated that the standard crude extract contained biologically active substance(s) other than cholesterol precipitable tomatine. The results of these studies are presented in the first part of this section. The second part is devoted to the purification of one of the active principle(s) (tentatively called "gomatine") and to its chemical and biological characterization.

I. Evidence for the Presence of Antihistamine-like Principle(s) in Cholesterol-treated Standard Crude Extract.

a. Biological Activity of Cholesterol-treated Standard Crude Extract.

Standard crude extract was treated with cholesterol as described in Part II (Section D,d) to quantitatively remove cholesterol precipitable tomatine. The yields of cholesterol-treated standard crude extracts obtained are shown in Table VII. The yields from 6 batches of crown-gall infected stalks varied appreciably (mean yield ± S.E.:20 ± 3 mg/100 g stalks).

The cholesterol-treated standard crude extracts were tested for

TABLE VII

Yields of Cholesterol-treated Standard Crude Extracts.

Crown-gall infected stalks	Standard crude extract	Cholesterol-treated standard crude extract.
(wet weight)	% (w/w)	% (w/w)
2000	0.068	0.025
3000	0.071	0.027
1600	0.050	0.009
1000	0.042	0.025
1000	0.062	0.021
1000	0.036	0.012
Mean ± S.E.	0.055 ± 0.006	0.020 ± 0.003

antihistamine-like activity in vivo in guinea-pigs. For this purpose, the extracts were separately dissolved in 80% ethanol (20 mg/ml) since the extracts could not be rendered soluble in aqueous medium in the pH range 1-3.2. The alcoholic solutions were injected intraperitoneally at 2 dosage levels of 2.5 ± 0.5 mg and 4.0 ± 0.5 mg/100 g of body weight. The control animals were similarly injected with the same volume of the vehicle. Each treated guinea-pig together with its control were exposed to 0.15% histamine aerosol 3 hours following injection. The results of 81 experiments obtained from 40 different batches of cholesterol-treated standard crude extracts are shown in Fig. 22.

In 42 experiments (Fig. 22A) the animals were injected with a dose of 2.5 ± 0.5 mg/100 g of body weight. In 26 experiments in this series the treated animals survived longer than their corresponding controls and 7 treated guinea-pigs survived the 20 minute exposure time; the mean survival time of their controls was 7 minutes. In 10 experiments the control animals survived longer than the treated animals and in 6 experiments the difference in survival time between the treated and its control was less than 30 seconds. The protection was significant ($p<0.01$).

In 39 experiments (Fig. 22B) the animals were injected with a dose of 4.0 ± 0.5 mg/100 g of body weight. In 26 experiments in this series the treated animals survived longer than their corresponding controls and 11 treated guinea-pigs survived the 20 minute exposure period; the mean survival time of their controls was 7 minutes. In 8 experiments the control animals survived longer than the treated animals and in 5 experiments the difference

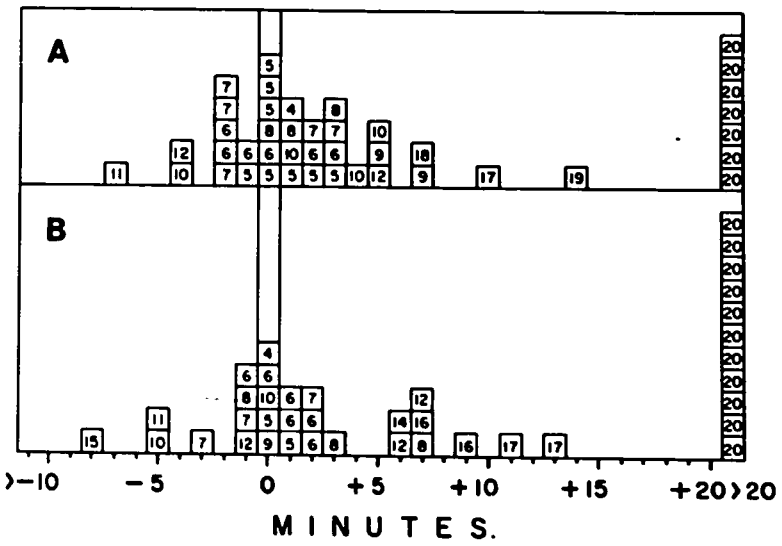


Figure 22

Protection produced by intraperitoneal injection of cholesterol-treated standard crude extract (A. 2.5 ± 0.5 mg/100 g of body weight; B. 4.0 ± 0.5 mg/100 g of body weight) in guinea-pigs exposed to 0.15% histamine aerosol. For details see text. Details of this figure are the same as in Fig. 2.

in survival time between the treated animal and its control was less than 30 seconds. The protection was significant ($p<0.01$).

b. Thin Layer Chromatography (TLC) of Cholesterol-treated Standard Crude Extract.

1. Qualitative TLC.

In preliminary studies TLC (Part II, section G,a) using sheet precoated with silica-gel was employed to establish the solvent system best able to resolve the component present in the cholesterol-treated standard crude extracts. Aliquots of 50 µg of the extract were spotted and the chromatograms developed using various mixtures of ethylacetate-methanol. The chromatograms were stained with iodine vapor and the Rf of the iodine-positive component were determined. Of the 7 solvent systems listed in Table VIII (A-G) ethylacetate-methanol (65:35,v/v) (solvent system C) resolved the extract into 3 widely separated major components. This solvent system was employed in silica-gel preparative TLC and column chromatography of the cholesterol-treated standard crude extract.

2. Preparative TLC of Cholesterol-treated Standard Crude Extract.

As shown above the cholesterol-treated standard crude extract was resolved by qualitative silica-gel TLC into 3 widely separated components. Experiments using preparative TLC were done to determine which of the components was associated with the in vivo antihistamine-like activity of the extract. For this purpose the cholesterol-treated standard crude extract

TABLE VIII

TLC of Cholesterol-treated Standard Crude Extract on Sheets Precoated with Silica-gel.

Solvent System	Component	Rf	
		Cholesterol-treated standard crude extract	Tomatine
A. EtOAc	1	0.000	0.000
	2	0.079	
	3	0.333	
	4	0.698	
B. EtOAc-MeOH (80:20,v/v)	1	0.000	0.023
	2	0.031	
	3	0.324	
	4	0.465	
	5	0.662	
C. EtOAc-MeOH (65:35,v/v)	1	0.000	0.206
	2	0.230	
	3	0.662	
D. EtOAc-MeOH (60:40,v/v)	1	0.000	0.265
	2	0.045	
	3	0.269	
	4	0.642	
E. EtOAc-MeOH (50:50,v/v)	1	0.000	0.436
	2	0.076	
	3	0.413	
	4	0.666	
F. EtOAc-MeOH (40:60,v/v)	1	0.000	0.492
	2	0.492	
	3	0.715	
G. MeOH	1	0.640	0.633

was dissolved in anhydrous methanol to a concentration of 20 mg/ml and the solution applied on 20x20 cm precoated silica-gel sheets (10 mg/sheet). The chromatograms were developed with ethylacetate-methanol (65:35,v/v) which resolved the extract into 3 components with Rf values given in Table VIII. Each of these components was eluted to give (from 10 mg extract chromatographed) 1.5-1.9 mg of component 1 (Rf, 0.000), 3.9-5.8 mg of component 2 (Rf, 0.230) and 2.7-3.9 mg of component 3 (Rf, 0.662). The range in yields for each of the components was obtained from at least 6 separate preparative TLC experiments.

For bioassay, each of the components derived from 10 mg of cholesterol-treated standard crude extract (eluted from a single chromatographic sheet) was dissolved in 0.5 ml of 80% ethanol and injected intraperitoneally into a guinea-pig. The control animal was similarly injected with the same amount of the vehicle. Each treated guinea-pig together with its control were exposed to 0.15% histamine aerosol 3 hours after injection. Fig. 23 summarizes the results obtained.

In 14 experiments (Fig. 23A) the animals were injected with component 3 (Rf, 0.662). In 10 of the 14 experiments the treated animals survived longer than the controls and 3 treated animals survived the 20 minute exposure; the mean survival time of their controls was 7 minutes. In 3 experiments the control animals survived longer than the treated animals and in 1 experiment the difference in survival time between the treated animal and its control was less than 30 seconds. The protection exerted by component 3 was significant ($p<0.05$).

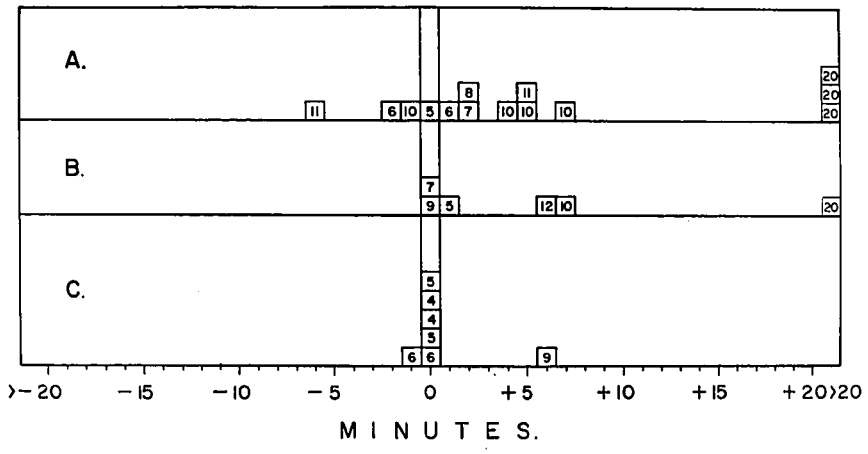


Figure 23

Protection of guinea-pigs against 0.15% histamine aerosol by intraperitoneal injection of : (A) TLC component 3; (B) TLC component 2; (C) TLC component 1. For details see text. Details of this figure are the same as Fig. 2.

In 6 experiments (Fig. 23B) the animals were injected with component 2 (Rf 0.230). In 4 of the 6 experiments the treated animals survived longer than their controls and one animal survived the 20 minute exposure; the survival time of its control was 7 minutes. In 2 experiments the difference in survival time between the treated animal and its control was less than 30 seconds. The protection, however, was non-significant ($p<0.10$).

In 7 experiments (Fig. 23C) the animals were injected with component 1 (Rf, 0.000). Only in 1 of the 7 experiments did the treated animal survive longer than its control. In 1 experiment the control animal survived longer than the treated animal and in 5 experiments the difference in survival time between the treated animal and its control was less than 30 seconds. The protection was not significant ($p<0.40$).

Thus, only 1 of the 3 major components of cholesterol-treated standard crude extract separated by TLC, significantly protected guinea-pigs from the effect of a lethal histamine aerosol.

c. Silica-gel Column Chromatography of Cholesterol-treated Standard Crude Extract.

Since the preparative TLC studies showed that silica-gel brought about a fairly good separation of the major components of the cholesterol-treated standard crude extract it was of interest to determine if silica-gel column chromatography could provide a more efficacious method than TLC for isolation of the active principle(s). For this purpose, the extract

(145 mg) was applied to silica-gel column (2.5x100 cm) and the chromatogram was developed with ethylacetate-methanol (65:35, v/v) at a flow rate of 60 ml/hour. The effluent was collected in 5 ml aliquots and analyzed by the Liebermann-Burchard reagent, visible absorption and the anthrone reagent. The results shown in Fig. 24 are typical of 6 silica-gel column chromatography runs. The effluents were pooled into 4 fractions (I-IV) and the pooled fractions were individually taken to dryness. The dry weight of each fraction is shown in Table IX and typical for all the chromatography runs.

TABLE IX

Dry Weights of Silica-gel Fractions of Cholesterol-treated Standard Crude Extract.

Fraction number	Recovery[1]	
	(mg)	(%)
I	39	27
II	28	19
III	35	24
IV	29	20
Total	111	90

[1] Based on dry weight of 145 mg of cholesterol-treated standard crude extract applied to the silica-gel column.

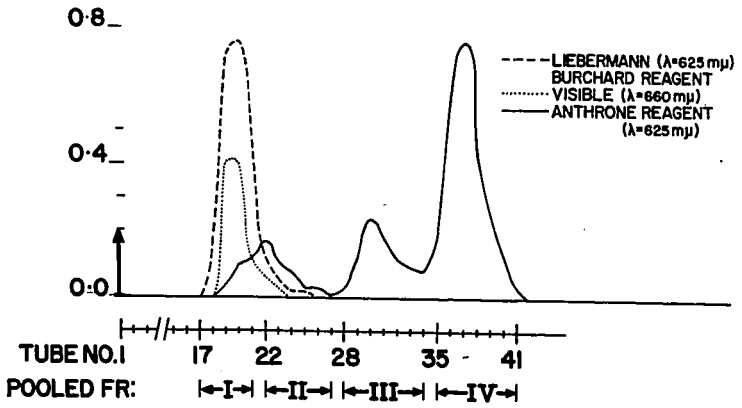


Figure 24

Fractionation of cholesterol-treated standard crude extract on a silica-gel column (2.5x100 cm). The column was eluted with ethylacetate-methanol (65:35,v/v) at a flow rate of 60 ml/hour. The effluent was collected in 45 tubes of 5 ml portions. Effluent tubes were pooled into 4 fractions (I-IV). The distribution of Liebermann-Burchard positive materials at 625 mμ is given by the broken line, of visible at 660 mμ by the dotted line and of anthrone positive material at 625 mμ by the solid line.

For bioassay, the dried silica-gel fractions were dissolved in 80% ethanol to a concentration of 20 mg/ml and the corresponding solutions were injected intraperitoneally into guinea-pigs in a dose of 1.5 mg/100 g of body weight. The control animals were injected intraperitoneally with the same amount of the vehicle. Each treated animal together with its control were exposed to 0.15% histamine aerosol 3 hours following injection. Of the 4 fractions tested only fraction I exerted significant protection against the effect of a lethal histamine aerosol. Fig. 25 shows the results of 12 experiments in which guinea-pigs were injected with fraction I. Of the 12 treated animals 11 survived longer than their corresponding controls and 2 of these survived the 20 minute period of exposure; the mean survival time of their controls was 10 minutes. Only in 1 experiment, the difference in survival time between the treated and the control animal was less than 30 seconds. The protection of the animals exerted by fraction I was significant ($p<0.01$).

The active silica-gel fraction I along with inactive fraction II, III and IV and tomatine (100 µg) each were analyzed using qualitative TLC on glass plate coated with silica-gel G according to Stahl (Part II, Section G,c). Ethylacetate-methanol (65:35, v/v) was employed as developing solvent. The chromatograms were sprayed with concentrated sulfuric acid and heated at 110°C for 15 minutes to develop the spots. Typical results obtained are shown in Fig. 26. It can be seen that the <u>in vivo</u> active fraction I showed no spot corresponding to cholesterol precipitable tomatine.

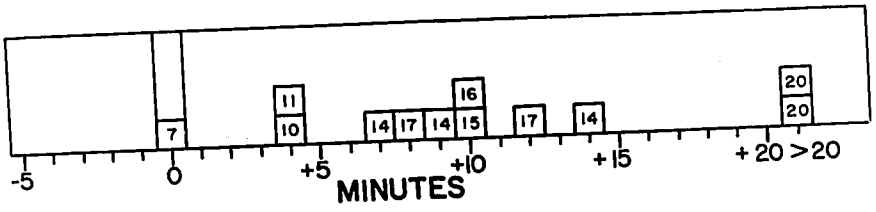


Figure 25

Protection produced by intraperitoneal injection of silica-gel fraction I into guinea-pigs exposed to 0.15% histamine aerosol. For details see text. Details of this figure are the same as in Fig. 2.

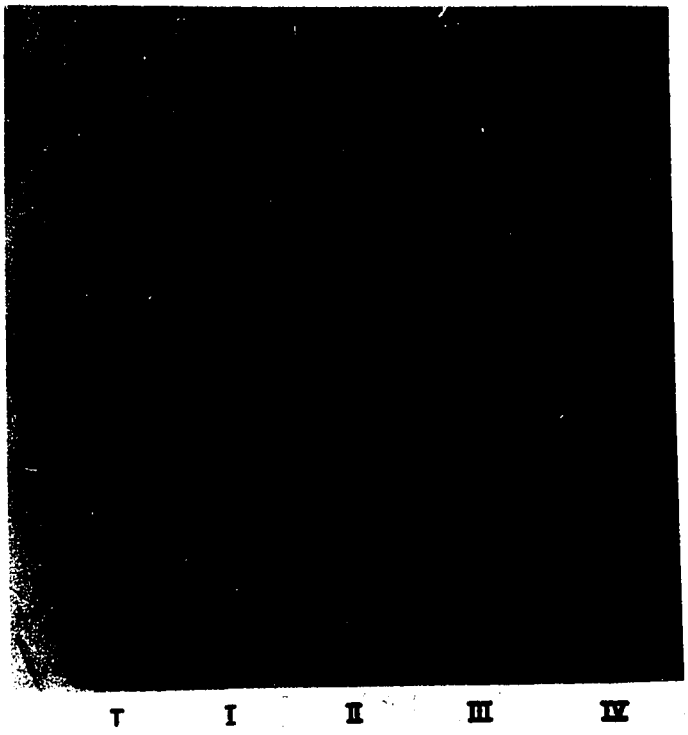


Figure 26

Thin layer chromatography of tomatine and silica-gel fractions (I-IV). The glass plate (20x20 cm) was coated with silica-gel G (according to Stahl) with layer thickness of 0.25 mm. The plate was developed with ethylacetate-methanol (65:35,v/v) and stained with concentrated sulfuric acid. (T) refers to tomatine and (I), (II), (III) and (IV) to silica-gel fractions I, II, III and IV, respectively.

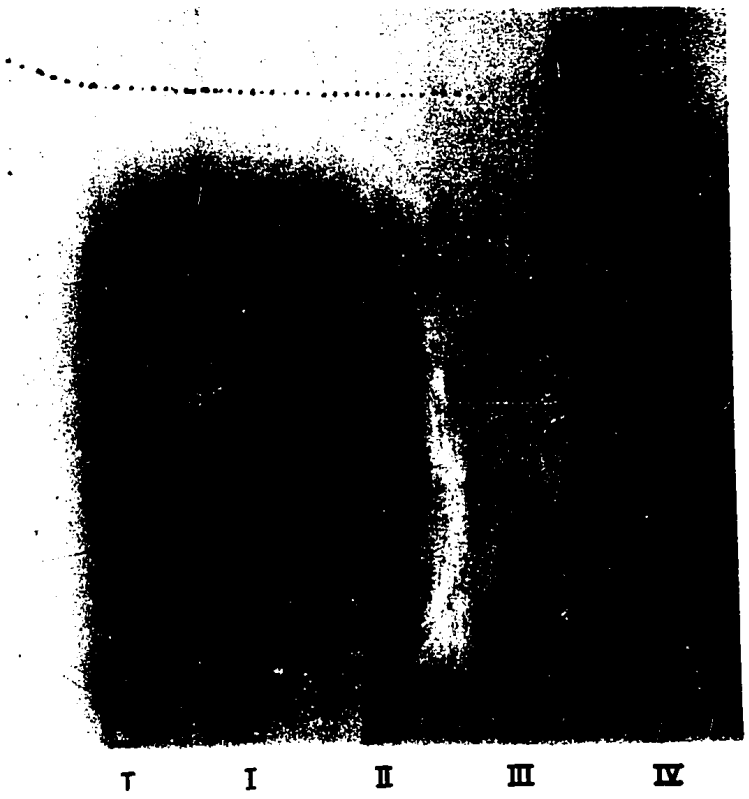


Figure 26

Thin layer chromatography of tomatine and silica-gel fractions (I-IV). The glass plate (20x20 cm) was coated with silica-gel G (according to Stahl) with layer thickness of 0.25 mm. The plate was developed with ethylacetate-methanol (65:35,v/v) and stained with concentrated sulfuric acid. (T) refers to tomatine and (I), (II), (III) and (IV) to silica-gel fractions I, II, III and IV, respectively.

Attempts to recrystallize fraction I from hot methanol by dropwise addition of water failed and resulted only in the formation of an amorphous pigmented material.

d. Discussion.

The results presented in this section demonstrated that standard crude extracts void of cholesterol precipitable tomatine exerted a definite protection against the effect of a lethal histamine aerosol in guinea-pigs. Two major methods, silica-gel preparative chromatography and column chromatography were employed in attempts to isolate the active principle(s) from the cholesterol-treated extract.

Although the preparative TLC method resulted in the isolation of an active and relatively pure fraction, it was abandoned because most of the active fraction which could be obtained by this technique was just sufficient for bioassay purposes. Thus, it was difficult to collect enough of the fraction for further purification studies. Repeated attempts to crystallize the active fraction resulted only in an amorphous product contaminated with pigments.

Though silica-gel column chromatography resulted in the isolation from the cholesterol-treated extract of a purified fraction (Fraction I) the method was considered unsuitable for isolation purposes because of the high losses of activity. Furthermore, the active fraction I contained at least 2 substances, one of which consisted of a pigment. Repeated attempts to crystallize the fraction resulted, as was found for the TLC

active fraction, in an amorphous pigmented product.

At this juncture in the studies, a new resin, Sephadex LH-20, which was developed for use with organic solvents, became available commercially. Gel filtration on Sephadex LH-20 proved to be the most efficacious of the methods employed for isolation of an *in vivo* active principle from the standard crude extract.

II. Purification and Chemical and Biological Characterization of Gomatine.

In the course of the purification studies, Sephadex LH-20, a dextran gel suitable for column chromatography with organic solvents became available. Since experiments using silica-gel preparative thin layer and column chromatography failed to yield the active principle(s) in a form which was readily crystallizable, it was of interest to investigate the efficacy of gel filtration on Sephadex LH-20 for purification purposes. In initial exploratory studies, the cholesterol-treated standard crude extract was applied to the Sephadex LH-20 column, however, this method yielded an active product containing a strongly bound and not readily dissociable pigment. The application of the standard crude extract directly to the Sephadex LH-20 column proved to be the most efficacious of the methods employed for the purification of the active principle(s).

a. Gel Filtration of Standard Crude Extract on Sephadex LH-20.

The following procedure was routinely employed: the standard crude extract (175 mg) dissolved in 5 ml methanol was applied to a Sephadex LH-20 column (4x100 cm). The column was eluted with methanol with a flow rate of 90 ml/hour. The effluent was collected in 10 ml aliquots and analyzed by the anthrone, ninhydrin, concentrated sulfuric acid method and visible absorption (Part II, Section F). Typical results obtained are shown in Fig. 27. The effluent tubes were pooled into 4 fractions (I-IV) according to the aforementioned analysis and taken to dryness. The dry weight of each fraction is listed in Table X.

b. In Vivo Antihistamine-like Activity of Sephadex LH-20 Fractions.

The dried fractions were dissolved in 80% ethanol in a concentration of 20 mg/ml and the solutions were separately injected into guinea-pigs intraperitoneally in a dose of 1.7 mg/100 g of body weight. The control animals were similarly injected with the same amount of the vehicle. Each treated animal together with its control were exposed to 0.15% histamine aerosol 3 hours following injection. The results obtained from 10 Sephadex LH-20 fraction I are summarized in Fig. 28. In 14 of 19 experiments the treated animals survived longer than their corresponding control animals and 9 guinea-pigs survived the 20 minute period of exposure; the mean survival time of their controls was 10 minutes. In 4 experiments the control survived longer than their corresponding treated animals and 2 of these controls survived the 20 minute exposure period;

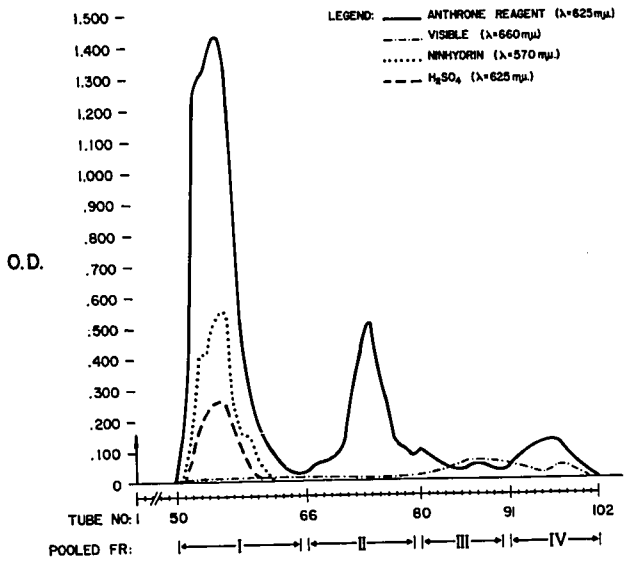


Figure 27

Gel filtration of standard crude extract on Sephadex LH-20 column (4x100 cm). The column was eluted with methanol at a flow rate of 90 ml/hour. The effluent was collected in 10 ml aliquots. Effluent tubes were pooled into 4 fractions (I-IV) according to the optical density curves obtained with the anthrone, visible, ninhydrin and sulfuric acid spectrophotometric methods. For details see text.

TABLE X

Yield of Sephadex LH-20 Fractions of Standard Crude Extract.

Fraction number	Dry weight	
	(mg)	% Recovery[1]
I	108.1	61.8
II	15.6	8.9
III	21.0	12.0
IV	11.6	6.6
Total	156.3	89.3

[1]Based on dry weight of 175 mg of standard crude extract applied to the Sephadex LH-20 column.

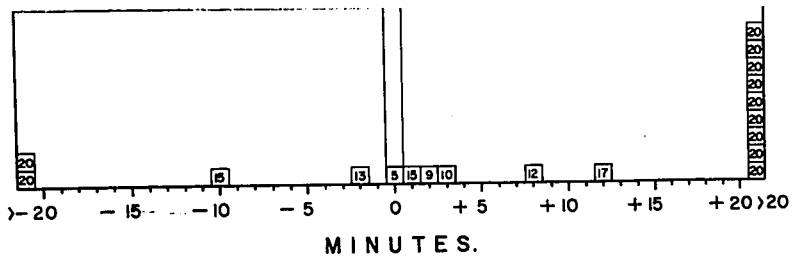


Figure 28

Protection of guinea-pigs against 0.15% histamine aerosol by intraperitoneal injection of Sephadex LH-20 fraction I. For details see text. Details of this figure are the same as in Fig. 2.

the mean survival time of their treated animals was 14 minutes. In 1 experiment the difference in survival time between the treated and its control was less than 30 seconds. The protection was significant ($p<0.01$). Fractions II, III and IV did not exert significant activity in vivo.

c. Further Purification of Sephadex LH-20 Fraction I.

Fraction I obtained by gel filtration of standard crude extract on Sephadex LH-20 was recrystallized 3-5 times from methanol until pigment free. The white crystalline substance was treated with cholesterol (Part II, Section D,d) to eliminate cholesterol precipitable tomatine. After removal of insoluble tomatine-cholesterol complex the supernatant was dried by rotary evaporation and washed with ether to remove excess cholesterol. The residue (hereafter referred to as "gomatine") was dried by rotary evaporation and weighed. Table XI lists the yields of gomatine obtained from various preparation of 3-5 times recrystallized fraction I.

d. Chemical Properties of Gomatine.

For chemical characterization, gomatine was recrystallized 3 times from hot methanol by dropwise addition of diethylether. The 3 times recrystallized product and tomatine (100 µg each) were compared by TLC using glass plates coated with silica-gel G according to Stahl (Part II, Section G,c). Ethylacetate-methanol (65:35,v/v) was employed as developing solvent. The chromatograms were sprayed with concentrated sulfuric

TABLE XI

The Yields of Gomatine Preparation.

Recrystallized Sephadex LH-20 fraction I	Gomatine	
(mg)	(mg)	(% of Fr. I)
117.0	33.9	29.0
135.0	35.0	25.9
161.5	31.1	19.3
154.7	25.5	16.5
208.2	70.5	33.9
242.0	84.0	34.7
205.4	51.0	24.8
Mean ± S.E.	--	26.3 ± 2.4

acid and heated at 110°C for 15 minutes to develop the spots. Typical results obtained are shown in Fig. 29. While gomatine gave a single spot under the chromatographic conditions employed, it was not possible to determine its accurate Rf value because of marked trailing. By visible inspection, however, it appeared that gomatine migrated slightly faster than tomatine.

The 3 times re-crystallized product was sent to the United States Testing Company (New Jersey) for determination of melting point, infrared spectrum and elementary analysis. The results obtained (U.S. Testing Company, file no. 79307) were as follows:

1. Melting point. First determination: 187°C; second determination: 186°C

2. Infra-red spectrum. The sample was incorporated into a potassium bromide pellet and the spectrum determined in a Perkin-Elmer Model 21 Infrared spectrophotometer. Fig. 30 compares the infrared spectrum of gomatine to that of tomatine. The spectrum differed from that of tomatine in the "fingerprint region".

3. Elementary analysis. Carbon and hydrogen were determined using the "Combustion Method" and found to be 58.1% and 8.4%, respectively. Nitrogen was determined by the Dumas procedure and was found in "trace" amounts only, however, according to this company the nitrogen determination was not accurate because the sample was insufficient.

Two gomatine samples were then sent to Mikroanalytisches Laboratorium with the following results. Sample I: C 58.17%; H 8.62%; O 30.60%;

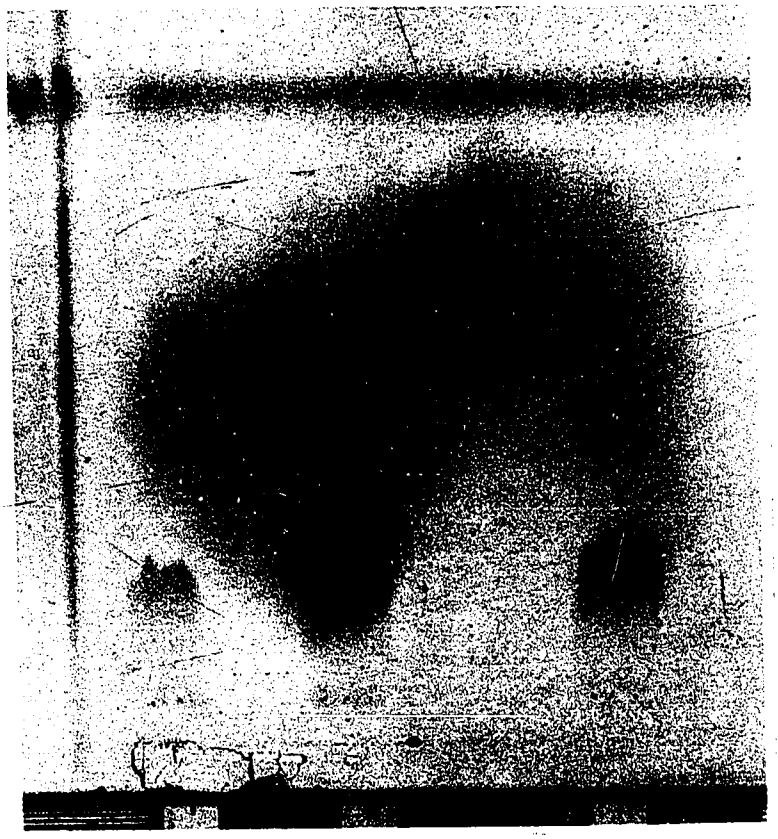


Figure 29

Thin layer chromatography of tomatine and gomatine. The glass plate (20x20 cm) was coated with silica-gel G (according to Stahl) with layer thickness of 0.25 mm. The plate was developed with ethyl-acetate-methanol (65:35,v/v) and stained with concentrated sulfuric acid. T refers to tomatine and G to gomatine.

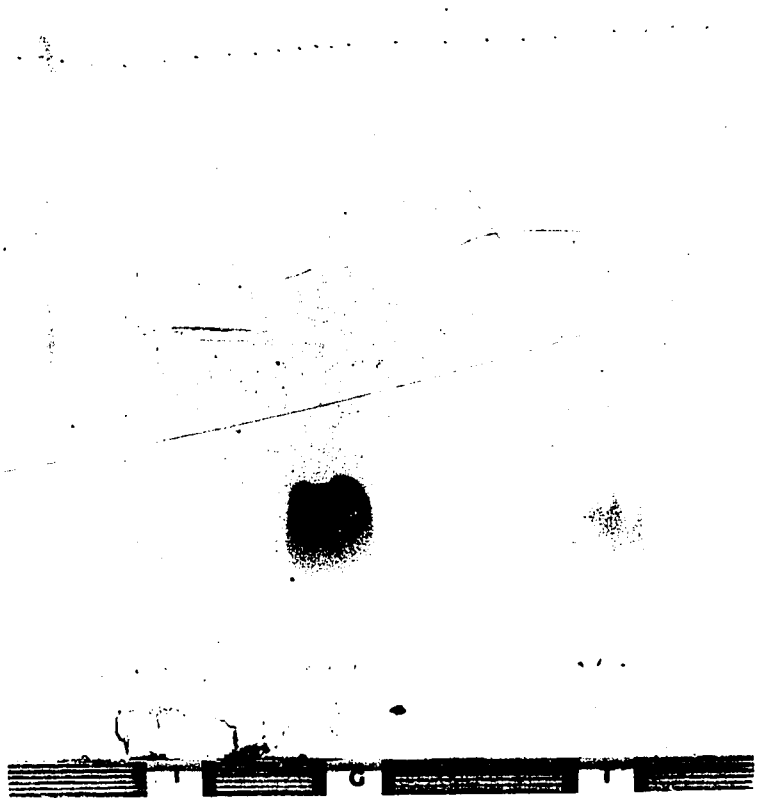


Figure 2°

Thin layer chromatography of tomatine and gomatine. The glass plate (20x20 cm) was coated with silica-gel G (according to Stahl) with layer thickness of 0.25 mm. The plate was developed with ethyl-acetate-methanol (65:35,v/v) and stained with concentrated sulfuric acid. T refers to tomatine and G to gomatine.

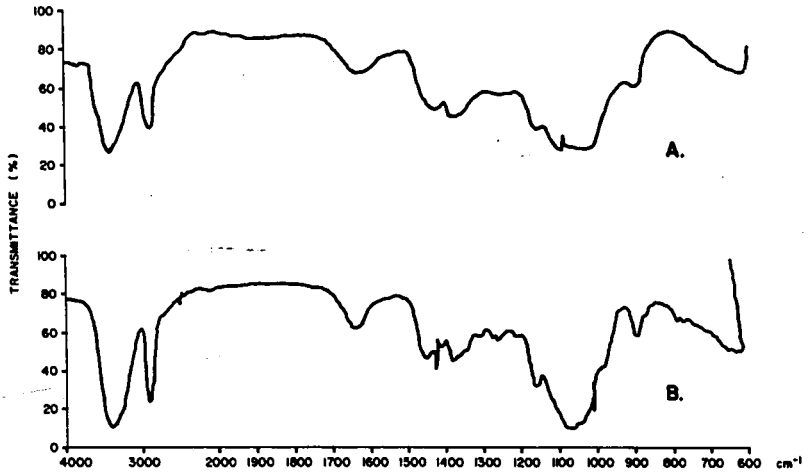


Figure 30

Infrared spectra of gomatine (A) and tomatine (B). The abscissa gives the wave numbers in cm^{-1}.

N 2.18%. Sample II: C 59.52%; H 8.19%; O 30.21%; N 2.08%.

e. Antihistamine-like Activity of Gomatine.

The antihistamine-like activity of gomatine was tested in vitro using the isolated guinea-pig ileum preparation and in vivo in guinea-pigs using histamine aerosol, anaphylactic shock and histamine and bradykinin-increased capillary permeability. For bioassay gomatine was dissolved in 80% ethanol owing to its insolubility in aqueous medium at pH 3.2. For the in vitro assay volumes of 0.1-0.3 ml and for the in vivo assays an average of 0.3 ml of the ethanolic solution of gomatine was employed. Control animals similarly received the same volume of the solvent.

1. In Vitro.

Gomatine was found to be equally effective when tested in a bath concentration of 1.4×10^{-5} g/ml on the isolated guinea-pig ileum preparation against contractions induced by bradykinin, histamine and 5-HT (Fig. 31).

The effects of gomatine against histamine, bradykinin and barium chloride-induced contraction were also studied on the isolated guinea-pig ileum preparation, by the R_{50} method of Rocha e Silva and Beraldo (1948) (Part II, Section B,g). This method was chosen since the molecular weight of gomatine was unknown. The results obtained for gomatine and tomatine are shown in Fig. 32. It can be seen that the efficacy of gomatine against contractions induced by histamine and bradykinin was less than that

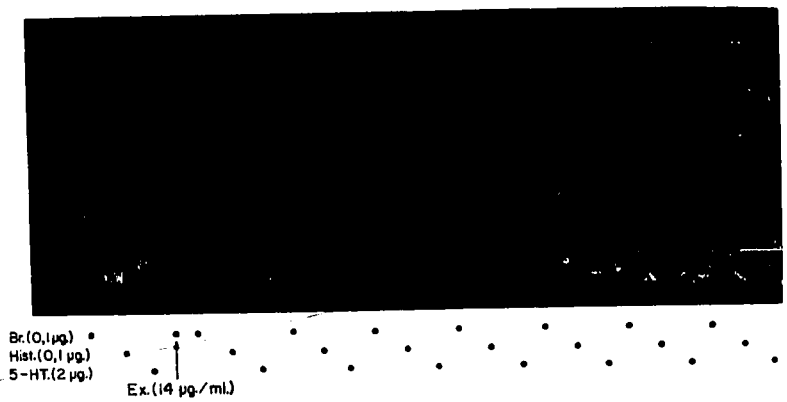
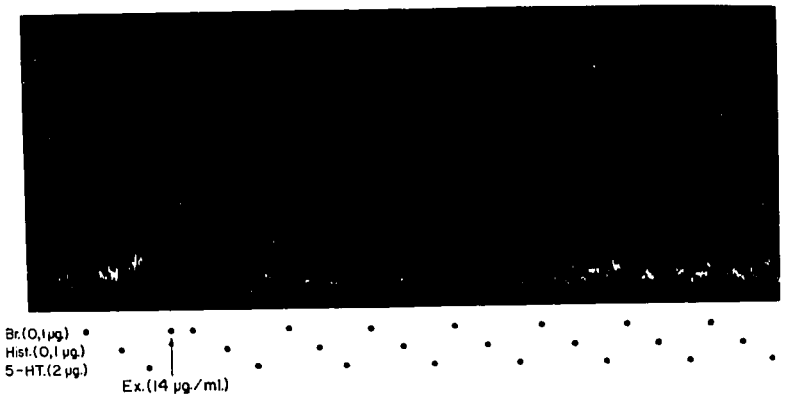


Figure 31

Responses of guinea-pig ileum preparation to histamine (Hist.), bradykinin (Br.) and 5-HT (5-HT) before and after the addition of gomatine (at arrow) in a concentration of 1.4×10^{-5} g/ml. Intervals of 3 minutes elapsed between administration of each drug (marked at black dots). In each instance the drum was temporarily stopped after washing out the organ bath and restarted 20 seconds before the next dose. The contact times were 20 seconds for histamine, 40 seconds for both bradykinin and 5-HT, and 2 minutes for gomatine.



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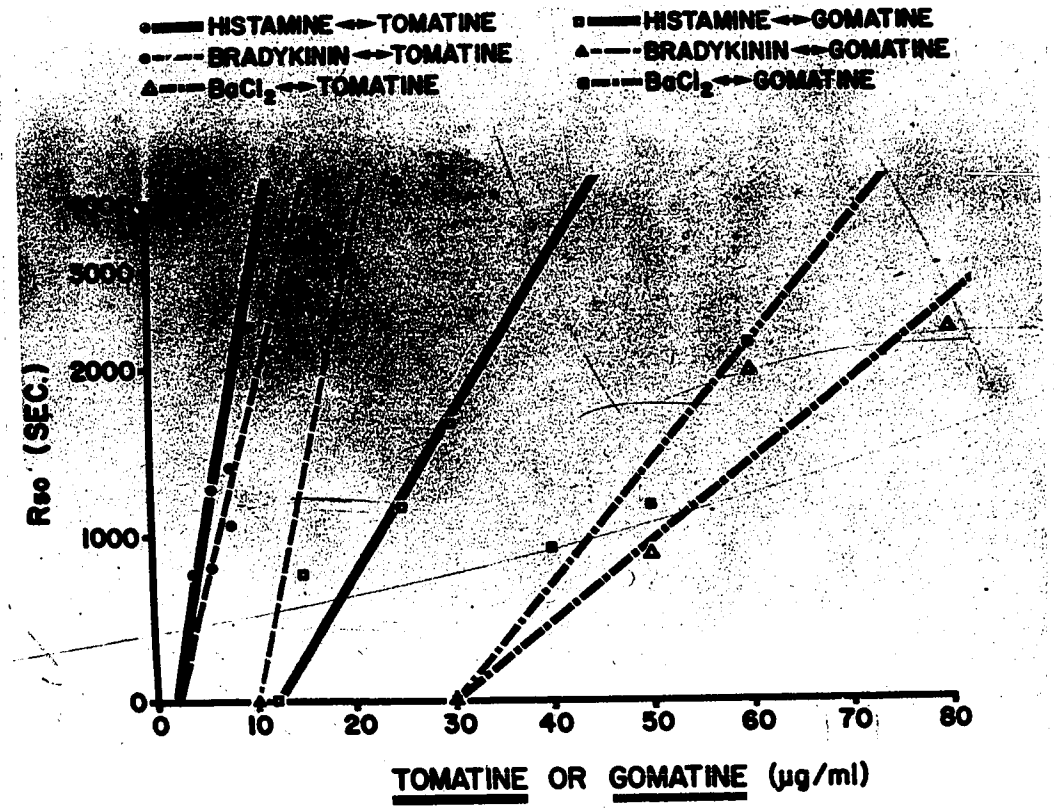

Figure 32

The R_{50} values of gomatine and tomatine obtained using isolated guinea-pig ileum preparations. The R_{50}'s are plotted against concentrations of gomatine or tomatine (µg/ml) on logarithmic scale. The abscissa gives the concentrations of gomatine or tomatine (µg/ml). The ordinate gives the R_{50} in seconds, which is the time required for 50% recovery of the standard contractions of the ileum. For details see text.

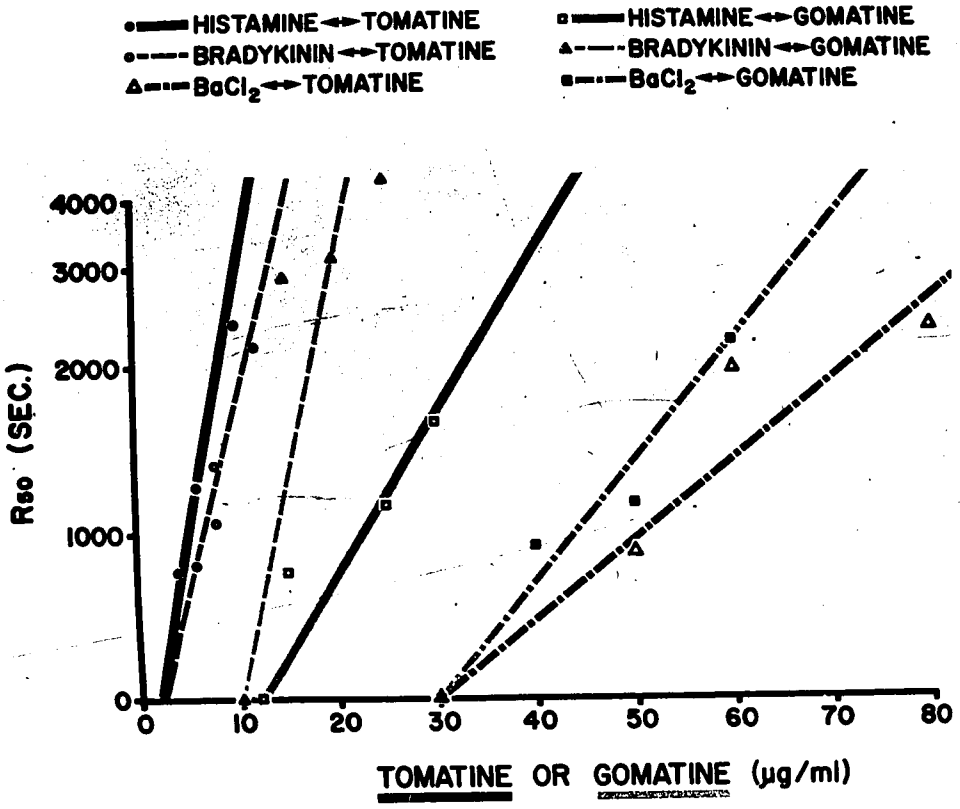

Figure 32

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of 5 times recrystallized tomatine, the latter being approximately 3-5 times more potent (on the weight basis). Both tomatine and gomatine were almost equally potent against barium chloride-induced contractions.

The results of studies done to determine the effect of gomatine against contractions of the isolated guinea-pig ileum preparation induced by SRS-A are shown in Fig. 33. In these studies a synthetic antihistamine (promethazine) in bath concentration of 2×10^{-7} g/ml was added to the bath immediately preceding the addition of gomatine and 2 minutes prior to the additions of SRS-A. In the presence of promethazine the addition of gomatine in a bath concentration of 1.4×10^{-5} g/ml itself did not induce contraction of the ileum similar to those observed in the experiments of Fig. 31. It can be seen from Fig. 33 that the contractions of the ileum upon the subsequent additions of crude SRS-A were almost completely inhibited; after repeated washings the sensitivity of the preparation returned to that of the initial controls.

2. In Vivo.

i) Histamine Aerosol.

Gomatine was dissolved in 80% ethanol to a concentration of 20 mg/ml and injected intraperitoneally into guinea-pigs in a dose of 1.7 mg/100 g of body weight. The control animals were similarly injected with the same amount of the vehicle. Each treated guinea-pig together with its control were exposed to 0.15% histamine aerosol 3 hours following injection. Fig. 34 summarizes the results obtained from 10 different batches of gomatine

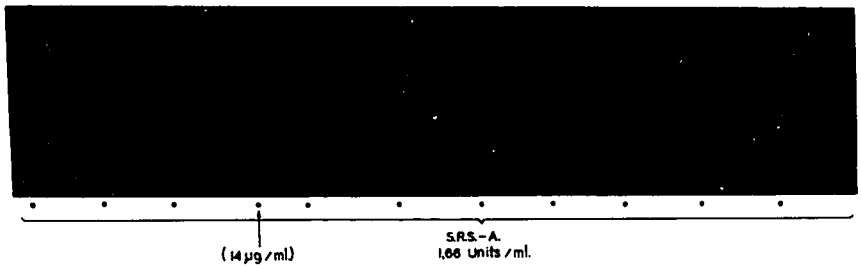


Figure 33

The responses of the guinea-pig ileum preparation to crude SRS-A (SRS-A at black dots) before and after the addition of gomatine (as marked at arrow) in a bath concentration of 1.4×10^{-5} g/ml. Intervals of 7 minutes elapsed between each addition of crude SRS-A. In each instance the drum was temporarily stopped after repeated washings and restarted 30 seconds before the next dose. The contact times were 3 minutes for crude SRS-A and 6 minutes for gomatine.

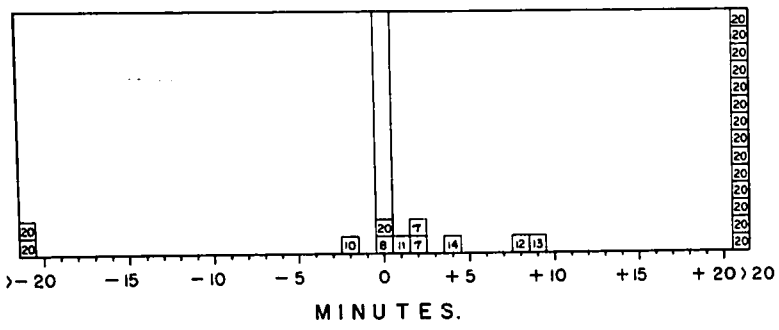


Figure 34

Protection produced by intraperitoneal injection of gomatine (1.7 mg/100 g of body weight) in guinea-pigs against 0.15% histamine aerosol. For details see text. Details of this figure are the same as in Fig. 2.

preparations. In 20 of the 25 experiments the treated animals survived longer than their corresponding control animals and 14 treated guinea-pigs survived the 20 minute exposure period; the mean survival time of their controls was 8 minutes. In 3 experiments the control animals survived longer than the treated animals and 2 of these control animals survived the 20 minute period of exposure; the mean survival time of their treated animals was 11 minutes. In 1 experiment both the treated and its control survived the 20 minute exposure period and in 1 experiment the difference in survival time between the treated and its control was less than 30 seconds. The protection was significant ($p<0.01$).

ii) Anaphylactic Shock.

Gomatine was dissolved in 80% ethanol to a concentration of 20 mg/ml and injected in a dose of 2 mg/100 g of body weight into 8 guinea-pigs sensitized to egg albumin. The control sensitized guinea-pigs were similarly injected with the same amount of the vehicle (about 0.3 ml). Three hours later the treated and control animals were injected intracardially with the challenging dose of egg albumin. Eight of the 10 control animals died within 5 minutes and 1 within 30 minutes following the intracardial injection of the egg albumin. One surviving control animal showed severe symptoms (bronchoconstriction, lying on its side etc.) for more than 3 hours after injection of the antigen.

Four of the 8 animals treated with gomatine showed only very mild symptoms of anaphylaxis (slight dyspnea). Signs of severe dyspnea was seen

in 1 animal within 5 minutes following challenge with the antigen. The symptoms, however, gradually disappeared and the animal recovered. Three of the treated animals died within 5 minutes after the intracardial injection of the antigen.

iii) Capillary permeability.

The effect of gomatine on the increased capillary permeability due to intradermal injection of histamine and bradykinin in guinea-pigs was investigated by the method described in Part II (Section C,c). For this purpose, gomatine was dissolved in 80% ethanol to a concentration of 20 mg/ml and injected intraperitoneally into guinea-pigs in a dose of 1.7 mg/100 g of body weight. The control animals were similarly injected with the same amount of the vehicle (about 0.3 ml). Fig. 35 shows a typical example of the results obtained in 10 experiments. The upper half of the figure shows the effect of 0.5 µg and the lower half that of 1 µg of histamine and bradykinin respectively. It is apparent from the figure that gomatine seemed to reduce the histamine and to a lesser degree the bradykinin-increased capillary permeability.

f. Discussion.

Experiments using cholesterol precipitation method as well as silica-gel preparative thin layer and column chromatography clearly suggested the presence in the standard crude extract of antihistamine-like principle(s) apart from cholesterol precipitable tomatine. The results obtained using

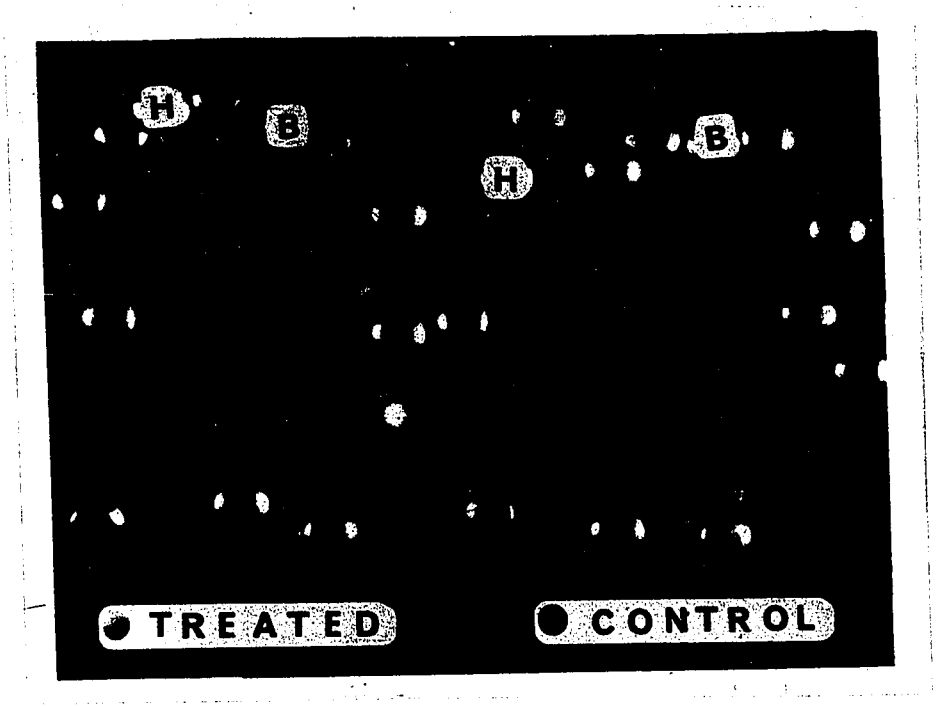


Figure 35

Blue patches of the internal surface of the skin of guinea-pigs which had received pontamine sky blue (60 mg/kg) intracardially followed by separate intradermal injections of 0.05 ml solutions (upper half of the figure) and 0.1 ml solutions (lower half of the figure) containing histamine (H) and bradykinin (B) in concentration of 10 µg/ml. On the right responses in a normal guinea-pig and on the left responses in a guinea-pig treated with gomatine (17 mg/kg) intraperitoneally 3 hours prior to intradermal injections of histamine and bradykinin.

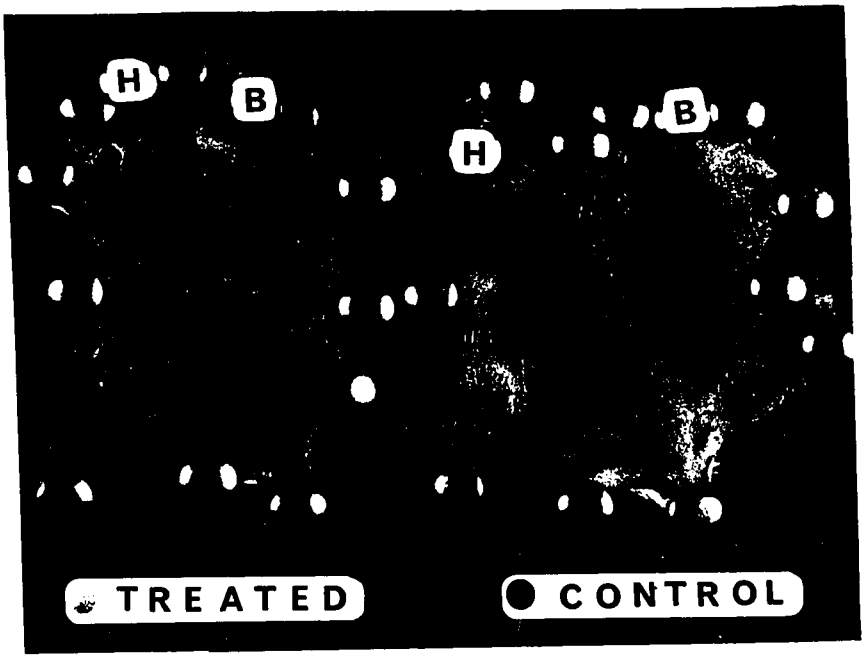


Figure 35

Blue patches of the internal surface of the skin of guinea-pigs which had received pontamine sky blue (60 mg/kg) intracardially followed by separate intradermal injections of 0.05 ml solutions (upper half of the figure) and 0.1 ml solutions (lower half of the figure) containing histamine (H) and bradykinin (B) in concentration of 10 µg/ml. On the right responses in a normal guinea-pig and on the left responses in a guinea-pig treated with gomatine (17 mg/kg) intraperitoneally 3 hours prior to intradermal injections of histamine and bradykinin.

gel filtration of the standard crude extract on Sephadex LH-20 were even more suggestive. Although Sephadex LH-20 fraction I could be crystallized, it contained at least 2 components. One of the components was most probably tomatine. Thus, fraction I gave a positive ninhydrin reaction, as would be expected with tomatine due to its imino group. In addition, fraction I also showed a positive reaction with anthrone demonstrating the presence of carbohydrate (mono or polysaccharide) perhaps due to the glycosidic moiety of tomatine and it showed a positive reaction with H_2SO_4, indicating most probably the presence of the steroidal moiety of tomatine. Furthermore, on treatment of the Sephadex LH-20 fraction I with cholesterol an average of 74% of the fraction was precipitated out, most probably due to the formation of tomatine-cholesterol complex.

The chemical properties of the isolated substance(s) which we have called gomatine were close to that of tomatine. The finding that gomatine was eluted from Sephadex LH-20 in the same region as tomatine, suggested that gomatine had a molecular weight close to tomatine, which might perhaps explain the observation of Calam and Callow (1964) who located the <u>in vivo</u> antihistamine-like activity of crown-gall extract following gel filtration on Sephadex G-25 on a single peak together with tomatine. In addition, the infrared spectra of gomatine (Fig. 30) was found similar to, though not identical with that of tomatine. Furthermore, Dr. T.H. Chan of the Chemistry Department, McGill University, analyzed quantitatively the carbohydrate content of gomatine and the isolated tomatine using gas chromatography. According to the data of Dr. Chan, the carbohydrate content of the 2

substances were the same and in the same ratio, i.e., 2 glucose, 1 galactose and 1 xylose.

On the other hand, gomatine was found to be less active than tomatine in vitro. Tomatine in a bath concentration of 4×10^{-6} g/ml nearly completely inhibited the smooth muscle contracting action of histamine, while gomatine was equally effective only in approximately 5 times the concentration of tomatine. Unfortunately these 2 substances could not be compared in term of pA_x (Schild, 1947) since the molecular weight of gomatine was unknown. As was found for tomatine, however, when assayed in vitro gomatine equally inhibited contractions of the isolated guinea-pig ileum preparation induced by histamine, bradykinin and 5-HT. When assayed in vitro against contractions induced by crude SRS-A gomatine nearly completely inhibited the contractions induced by subsequent standard dose of SRS-A. The most striking feature was that the addition of gomatine to the organ bath itself did not elicit an initial contraction as in the case of in vitro assay of gomatine against contractions induced by histamine, bradykinin and 5-HT (Fig. 31). In the course of in vitro assay against SRS-A, however, promethazine was added to the organ bath immediately following the addition of gomatine and 2 minutes prior to each addition of SRS-A. Therefore, the absence of an initial contraction in this case was most probably due to histamine liberating property of gomatine. When injected intraperitoneally into guinea-pigs (1.7 mg/100 g of body weight) gomatine exerted a significant protection against the effect of a lethal dose of histamine aerosol. Gomatine injected intraperitoneally into guinea-pigs in doses between 1.7-2.5 mg/100 g of body

weight did not produce abdominal tension and irritation as was found in the case of tomatine. Although 80% ethanol was employed as vehicle to dissolve gomatine, this would most probably not account for the difference, since tomatine dissolved in the same vehicle did produce abdominal irritation and tension. An immediate toxic effect (abdominal tension and irritation) was not observed for gomatine. However, a delayed toxic effect was observed in some of the animals which had received gomatine and which had survived the 20 minute period of exposure to the aerosol: the animals died within 7 days following intraperitoneal injection of gomatine. The long-term protection of the animals against the effect of a lethal dose of histamine aerosol brought about by a single intraperitoneal injection of gomatine was also studied. However, no conclusion could be drawn from these results since an insufficient number of animals survived beyond 7 days following administration of gomatine. The possible mechanism of action of gomatine in protecting guinea-pigs against the effect of a lethal histamine aerosol was not investigated in detail. Preliminary experiments, however, showed that animals which were pretreated with a beta blocker (pronethalol) plus gomatine when exposed to the histamine aerosol did not have significant difference in survival time as compared to animals injected with gomatine without pronethalol. These results suggest that the protective action of gomatine was most probably not mediated through adrenaline release.

From the results of the present study it may be tentatively concluded that tomatine and gomatine are closely related steroid alkaloids having distinct biological properties.

PART IV. GENERAL DISCUSSION

The results of the present study confirm previous observations (Kovacs et al., 1951; Broome et al., 1962) that extracts prepared from crown-gall infected tomato plants when injected intraperitoneally into guinea-pigs protected the animals against the effect of a lethal histamine aerosol.

The comparative study described in Part III (Section A) did not clarify whether or not the active principle(s) is intrinsically related to crown-gall tumor. This aspect of the problem is extremely important for the study of the pathophysiology of crown-gall per se. The aim of the present study, however, was simply to determine which type of tomato plant tissues would provide the best source for the isolation of the active principle(s). To this question the experiments described seemed to provide satisfactory answer. As the activity of the extracts prepared from crown-gall infected tomato stalks was found to be significantly higher than the corresponding extracts prepared from normal, non-infected tomato stalks, the crown-gall infected tomato stalks (8-12 weeks following inoculation) served as starting material for the isolation of the active principle(s). A potent stable crude extract ("standard crude extract") was obtained from the crown-gall infected tomato stalks.

The long-term protection of the guinea-pigs against the effect of a lethal histamine aerosol which resulted from a single intraperitoneal injection of the extracts of crown-gall infected tomato stalks suggested

that the active principle (s) present in this extract had a different mode of action from that of the synthetic antihistamines. However, it is futile to speculate on the mechanism of the long-term protection, unless a large number of carefully designed experiments could be performed. This action could be rather non-specific, brought about by the intraperiteneal injection of the extract containing a number of constituents. Further experiments might show that neither of the chemically pure active principle(s) alone would produce a long-term protection in the experimental animals. Thus, this observation would be significant only if similar results could be obtained with chemically pure active principle(s) under carefully controlled experimental conditions. Though at present no explanation can be offered for the long-term protection exerted by the extract we would like to discuss briefly one important aspect of this study. It is well known that repeated exposure of experimental animals to a number of pharmacologically active agents including histamine may result in desensitization of the animals. The phenomena of histamine desensitization have been studied by many investigators in a number of animal species (Ambrus et al., 1951; Beznak et al., 1948; Jancso, 1947; Karady and Rapcsak, 1953), however, their findings did not give a clear answer to the problem of desensitization. Some investigators deny the existence of histamine desensitization (Csaky and Kovach, 1947; Feinberg, 1946; Wells et al, 1942). Recently Maslinski and Wisniewska (1965)described the conditions under which guinea-pigs can be desensitized to histamine aerosol. According to these workers guinea-pigs have to be exposed to

histamine aerosol daily for at least 14 to 20 days to obtain desensitization. Even then animals could be desensitized only if they were exposed to an optimum histamine concentrations suitable for the individual animal. Since in the present study the animals were exposed only twice to the aerosol and a minimum of 5 days elapsed between the first and the second exposure, the histamine desensitization as a possible explanation for the long-term protection can certainly be excluded.

The first substance isolated (by cholesterol precipitation method) from the standard crude extract was identified as tomatine, a known constituent of normal tomato plant. In contrast to the finding of Calam and Callow (1964) that tomatine had no antihistamine-like activity in vitro, in the present study it was found that tomatine when tested on the isolated guinea-pig ileum preparation antagonized the contractions induced not only by histamine, but also by bradykinin, acetylcholine and barium chloride. An initial exciting and subsequent long lasting depressive effect exerted by tomatine against histamine and other smooth muscle stimulating agents was regularly observed and has revealed a new biological activity of this steroid alkaloid. While the mechanism of action of tomatine on the isolated organ preparations was not studied in detail, some preliminary investigations employing the pA_x and the log dose/response curves method of analysis suggested that the mechanism of action was most probably a non-specific and non-competitive.

According to Calam and Callow (1964) of the 3 known constituents of normal tomato plant (tomatine, tomatidine and rutin), only tomatine

exerted protection in guinea-pigs against the effect of a lethal histamine aerosol. On the other hand, Wilson et al. (1951) reported that rutin exerted slight protection in guinea-pigs against shock produced by injection of a lethal dose of histamine. In contrast to the findings of Calam and Callow (1964), that tomatine had antihistamine-like activity in vivo, in the present study it was found that tomatine when injected intraperitoneally into guinea-pigs exerted a rather weak and uncertain protection of the animals against the effect of a lethal histamine aerosol. When injected intraperitoneally into guinea-pigs in doses between 0.3-2.0 mg/100 g of body weight, tomatine produced abdominal irritation and tension, diarrhea, central effects loss of righting reflexes and ruffling of coats, symptoms similar to that observed by Broome et al. (1962) and by Calam and Callow (1964). These side effects observed in guinea-pigs treated with tomatine resembled the symptoms reported by Forsyth (1954) of solanine poisoning in animals which have eaten green potatoes. Higher doses of tomatine (5-20 mg/100 g of body weight) were observed to kill the animals within 2½ hours following intraperitoneal injection. Thus, the observations of Broome et al. (1962) that a relatively large amount of extracts of crown-gall infected tomato stalks injected intraperitoneally into guinea-pigs resulted in death of the animals was most probably due to tomatine present in these extracts. Our findings that tomatine exerted a much weaker protection than partially purified extract (standard crude extract) of crown-gall infected tomato stalks in guinea-pigs against the effect of a lethal histamine aerosol indicated that

tomatine is one but most probably not the most important of the principle(s) active *in vivo* and present in these extracts.

Several methods, e.g., silica-gel column and preparative thin layer chromatography as well as cholesterol precipitation method were employed to obtain an active preparation void of cholesterol precipitable tomatine from the standard crude extract. The application of Sephadex LH-20 gel filtration for the purification of the standard crude extract resulted in the isolation of crystalline product tentatively called gomatine. The chemical properties of gomatine were close to that of tomatine, suggesting that tomatine and gomatine are closely related steroid alkaloids. Owing to the fact that the isolation of gomatine has been accomplished recently, only few preliminary investigations could be performed concerning its pharmacological actions. The results showed that gomatine was effective in protecting the animals against the effect of a lethal histamine aerosol and anaphylactic shock. Unlike tomatine, gomatine (1.7 mg/100 g of body weight) did not display abdominal tension and irritation. However, some of the animals which had received gomatine and which had survived the 20 minute exposure period died within 7 days following intraperitoneal injection of gomatine. Thus, no conclusion could be drawn concerning the long-term protection exerted by gomatine due to insufficient number of animals survived beyond 7 days. Gomatine when tested on the isolated guinea-pig ileum preparation was found to be equally effective in inhibiting the smooth muscle stimulating activity of histamine, bradykinin, 5-HT and SRS-A. The latter substances are considered to play an essential role

in the development of anaphylactic and allergic manifestations. If, in further investigations using other species of animals, gomatine proved to be efficacious it might be of interest to investigate the effect of gomatine in alleviating the manifestations of allergic diseases in man.

SUMMARY

1. Methods for producing crown-gall infected tomato stalks by inoculation of normal tomato stalks with agrobacteria tumefaciens are described.

2. Methods are described for the preparation of a potent stable extract (standard crude extract) with antihistamine-like activity from crown-gall infected tomato stalks.

3. A single intraperitoneal injection into guinea-pigs of the extracts of crown-gall infected tomato stalks seemed to exert a long-term protection against the effect of a lethal histamine aerosol.

4. The level of antihistamine-like activity of normal and crown-gall infected tomato stalks at various time intervals following inoculation of the plants was determined.

5. Methods were developed for the isolation of tomatine from crown-gall infected tomato stalks.

6. Tomatine added in a concentration of 4×10^{-6} g/ml to the isolated guinea-pig ileum preparation elicited an initial stimulation and a subsequent long-lasting depressive effect against the contractions induced by histamine.

7. Tomatine was found to be equally effective in inhibiting contractions induced by histamine, bradykinin, acetylcholine and barium chloride on the isolated guinea-pig ileum preparation.

8. When investigated on the isolated guinea-pig ileum preparation

using the log dose/response curves method of analysis it was found that the mechanism of action of tomatine was most probably non-competitive.

9. Tomatine injected intraperitoneally into guinea-pigs in doses between 0.3-2.0 mg/100 g of body weight exerted at best only a mild protection of the animals against the effect of a lethal histamine aerosol.

10. Extract of crown-gall infected tomato stalks rendered free of cholesterol-precipitable-tomatine was assayed in vivo in guinea-pigs using the histamine aerosol method and shown to exert significant protection of the animals against the effect of a lethal histamine aerosol.

11. A cholesterol-precipitable-tomatine-free fraction obtained by silica-gel column chromatography of cholesterol-treated standard crude extract, when assayed in vivo in guinea-pigs protected the animals against the effect of a lethal histamine aerosol.

12. Methods were developed for the isolation of gomatine from standard crude extract.

13. Gomatine when tested on the isolated guinea-pig ileum preparation was found to be effective in inhibiting contractions induced by histamine, bradykinin, 5-HT, SRS-A and barium chloride.

14. Gomatine when injected intraperitoneally into guinea-pigs protected the animals against the effect of a lethal histamine aerosol.

15. Guinea-pigs actively sensitized to egg albumin were protected

by the intraperitoneal injection of gomatine (2 mg/100 g of body weight) from anaphylactic shock on challenge with the antigen.

16. Elementary analysis of gomatine for carbon, hydrogen, oxygen and nitrogen gave: I. C 58.17%; H 8.62%; O 30.60%; N 2.18%.

II. C 59.52%; H 8.19%; O 30.21%; N 2.08%.

17. The melting point of gomatine was found to be 186-7°C.

18. The infrared spectrum of gomatine was similar to but not identical with that of tomatine.

CLAIMS TO ORIGINALITY

1. Two substances with antihistamine-like activity were isolated from crown-gall infected tomato stalks. One was identified as the steroid glycoside alkaloid, tomatine and the other is tentatively called gomatine. The latter showed chemical properties close to that of tomatine.

2. Tomatine, a known constituent of tomato plant was isolated in 1948, but its antihistamine-like activity on isolated guinea-pig ileum preparation has not been reported previously.

3. Tomatine was found to inhibit contractions of the isolated guinea-pig ileum preparation induced by: histamine, bradykinin, acetylcholine and barium chloride.

4. The mechanism of inhibitory action of tomatine on isolated guinea-pig ileum preparation is most probably non-specific and non-competitive.

5. Methods were developed for the isolation of gomatine from crown-gall infected tomato stalks.

6. It was demonstrated that gomatine when injected intraperitoneally into guinea-pigs, significantly protected the animals against the effect of a lethal histamine aerosol.

7. Guinea-pigs actively sensitized to egg albumin were protected by the intraperitoneal injection of gomatine from anaphylactic shock on challenge with the antigen.

8. Gomatine was equally effective in inhibiting contractions induced by histamine, bradykinin, 5-HT as well as SRS-A.

9. Gomatine was shown to antagonize the activity of SRS-A and it represents the first substance described with such an effect.

10. The elementary composition, melting point, infrared spectrum and carbohydrate content of gomatine were determined.

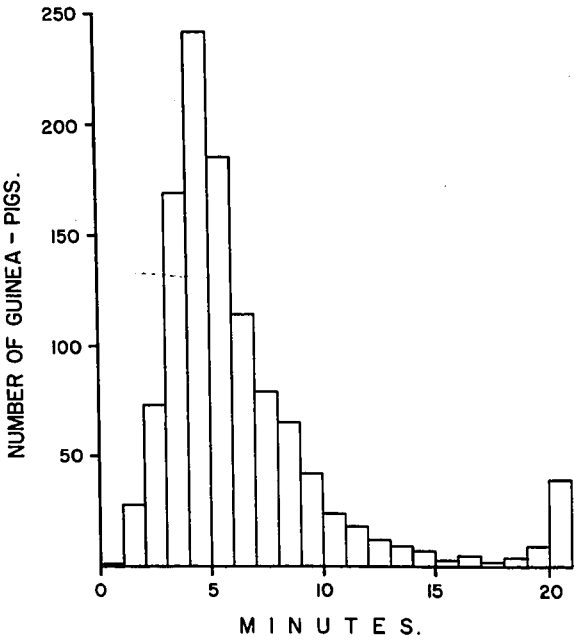
APPENDIX

Histamine Aerosol.

It was evident early in the present study that the expiration time of normal guinea-pigs exposed to 0.15% histamine aerosol fell within a fairly narrow time interval. This was confirmed by the data obtained in the course of testing control guinea-pigs over a 4 year period. The individual variation in survival time of 1029 control guinea-pigs is shown in APPENDIX Fig.1. It is apparent from the figure that an appreciable number of the control animals expired at the 2-10 minute interval.

The percentage of the total number of control guinea-pigs which survived for various time was calculated from APPENDIX Fig.1 and the results are shown in APPENDIX Table I. Respiration ceased during the first 5 minutes for nearly half the animals (46%) for 89% during the first 10 minutes and 95% during the first 15 minutes. Only 3% survived the 20 minute period of exposure.

It is difficult to evaluate and compare data obtained by workers from different laboratories in studies employing the histamine aerosol technique. This is due to the fact that the development and the severity of bronchoconstriction induced by inhaled histamine aerosol depends on the concentration of the histamine solution, the pressure employed to spray the solution and the size of the particles (Jemski and Phillips, 1965). The results obtained in the present studies, however, are in close agreement with



APPENDIX Figure 1.

Variation in sensitivity of 1029 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 during which the animal respiration ceased. The ordinate gives the number of animals.

APPENDIX TABLE I

Number (%) of Guinea-pigs Expiring After Exposure to 0.15% Histamine Aerosol for Various Times.

Time of cessation of respiration (minutes)	Number (%) of guinea-pigs	
	(number)	(%)
0-5	470	46
5-10	439	43
10-15	62	6
15-20	24	2
> 20	34	3

that of Broome et al. (1962): i.e., in approximately 90% of the control animals respiration ceased furing the first 10 minutes of exposure to the aerosol. We regularly observed that the symptoms leading to bronchoconstriction and 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 employed as the end point of the experiment (Halpern, 1942; Loew et al., 1945, 1946; Herxheimer, 1951; Broome et al., 1962; Herxheimer and Stresseman, 1963b; Calam and Callow, 1964). In most instances the appearence of dyspnea has been taken as the end point. According to Halpern (1942) and Lish et al. (1966) if guinea-pigs subjected to histamine aerosol were immediately 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 of the predyspneic interval. In contrast, in the present study we have observed that the time of appearence of dyspnea on the second exposure in the same guinea-pig changed in large percentage of the animals. Therefore, the dyspnea time as the end point of the experiment and the use of the animal as its own control did not seem to be sufficiently reliable. Thus, the method of Broome et al. (1962) that is the cessation of respiration taken as the end point of the experiment was selected as a routine method of testing.

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