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Release of histamine from isolated mast cells by compound 48/80 and specific antigen

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RELEASE OF HISTAMINE FROM ISOLATED MAST CELLS BY COMPOUND 48/80 AND SPECIFIC ANTIGEN

by

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I. Introduction

The present study had its origin when Riley and co-workers made the correlation between histamine and mast cells. Since then studies have been conducted to elucidate the mechanism of histamine release from the mast cell. Early investigations were made on the tissues both *in vivo* and *in vitro*. Chemical and biological agents known to release histamine were employed to find the nature of the histamine complex in the mast cell.

Histamine liberation and mast cell degranulation have been associated with the anaphylactic process. Therefore, the action of specific antigen has been studied on various tissue preparations from sensitized animals. In a similar manner, naturally occurring histamine releasers such as certain snake venoms, extracts from *Cyanea capillata* and *Ascaris lumbricoides* have been tested. Chemical liberators, compound 48/80 in particular, have been objects of considerable study. In all the investigations, it was felt that in knowing the chemical structure of the histamine liberators and the conditions under which they release histamine, the nature of histamine binding in the mast cell could be determined.

Although the work on intact animals and in vitro preparations did much to clarify conditions under which histamine is released from mast cells, these preparations were not ideal. Agents causing histamine release and mast cell damage could have been working on some other factor in the tissue which was in turn elaborating a substance actually causing the effect. Therefore, a proper experimental design should be one in which the mast cells are isolated from other influences.

With this in mind, the present investigations were carried out. Mast cells were isolated from the peritoneal fluid of the rat and divided into aliquots. In this preparation of morphologically and physiologically intact mast cells, the effects of specific antigen and compound 48/80 were studied on isolated cells in an attempt to learn more about the mechanism of histamine release from the mast cell.

II. Historical background.

The first report of the mast cell as a specific entity was made by a young German medical student, Paul Ehrlich, in 1877. However, the earlier investigators of connective tissue, who mentioned perivascular cells with coarse granules, were most probably describing mast cells. Therefore credit must be given to v. Recklinghausen (1863), Kühne (1864), Koelliker (1867), Friedländer (1867), Cohnheim (1869), Rollet (1871), Schöbl (1871) and Flemming (1867) as the first ones to discover the tissue mast cell.(49)

In 1875, Waldeyer, in his studies of connective tissue reported certain cells which he called collectively plasma cells. By studying his colored plates one can see that many of the so-called plasma cells were in actuality mast cells (49).

One of the more prominent characteristics of the mast cell, its staining reaction, was the key to its specific discovery.

Paul Ehrlich tried a variety of dyes obtained from German manufacturers on connective tissue preparations and found that these cells contained granules which have an affinity for basic dyes and also exhibited metachromasia. Thus in his report before the Physiological Society of Berlin, Ehrlich made the differentiation between the plasma cells of Waldeyer and the Mastzellen. The name, which means well-fed, was chosen because of the enhanced nutritional status of the connective tissues in which the cells appeared (68). Opposition to the theory was presented by Ranvier who claimed that the Ehrlichian mast cells were a subdivision of his clasmocytes. However the work of Jolly and Maximow proved Ehrlich to be correct (49).

The mast cell has a polymorphous light staining cytoplasm which may vary in shape from round to oval, pyriform, spindle or star-shape. Its mean diameter has been described as 3.5 µ to 14.0 µ and 8.0 µ to 15.0 µ. The nucleus is round or oval and usually central, measuring from 4.0 to 6.0 µ in diameter regardless of the size of the cytoplasm. A nucleolus may be present in rare cases (49)(5).

The cytoplasmic granules are globular or occasionally oval shaped with a mean diameter of 0.2 to 0.4 µ. They vary in solubility from species to species. The least soluble granules are found in the mast cells of the guinea-pig, rat and mouse, while the most water soluble ones are found in the rabbit and certain fishes. The granules have both a basophilic and a metachromatic staining reaction. Metachromasia, a term introduced by Ehrlich, means that the substance to be stained stains with a tone different from that possessed by the dye. Thus a blue dye will impart a reddish tone and a red dye a yellowish tone to the granules. A distinct cellular membrane has been demonstrated with the electron microscope, while with the light microscope the outline of the cell is determined by the basophilic cytoplasmic granules (49) (5).

Mast cells are numerous in the reticular connective tissue which surrounds blood vessels and in the connective tissue which underlies epithelial, serous and synovial membranes. Their distribution and amounts vary with each species. In pathological connective tissue, mast cell tumours have been found in dogs, mice and horses. In man focal accumulations of mast cells are noted in urticaria pigmentosa (69).

For sixty years after Ehrlich's work with mast cells, their function remained unknown. Many theories were advanced as to their role in the organism. Some of the twenty-five hypotheses include: 1) the production of the mucin of connective tissue, 2) nutrition of the connective tissue (original theory proposed by Ehrlich), 3) fat absorption, 4) pigment production, 5) phagocytosis. Other theories include defensive reactions, oxidative capacities, metabolism of vitamins, defence reaction in cancer and so forth (5).

The theory which was nearest to the true function, proposed that the mast cells were actually monocellular glands. This idea was held by many of the early workers. Lehner noted that the granules were at times swollen and showed intracellular dissolution, the product leaving the cell.

In 1937, came the first real insight into the function of the tissue mast cell. Two years before, in 1935, Lison found that the metachromatic staining reaction was produced by sulfuric mucopolysaccharides (45). Jorpes in 1936 described the chemical structure of heparin as a poly-sulfuric acid ester (39). Thus the metachromatic granules of the mast cell were "rediscovered" and Holmgren and Wilander (1937) (34), Jorpes, Holmgren and Wilander (1937) (41) and Wilander (1939)(90) claimed that mast cells contained heparin. Positive correlations were found between the mast cell count and the heparin content of various tissues. Oliver _et al_. found a very large amount of heparin in a mast cell tumour (62).

The next advance in elucidating the function of the mast cell came in the early 1950's. Rocha e Silva noted that histamine was released at the same time as heparin from the liver of the dog in shock states.(73) The work of MacIntosh and Paton on (47) chemical histamine liberators gave James F. Riley an idea how to test his theory that mast cells contained histamine. With rapid intravenous injections of lethal doses of the fluorescent liberators, stilbamidine or 2-hydroxystilbamidine, fluorescent diamidine was shown in the cytoplasm of the peritoneal mast cells.(67) When given slowly, the same dosage caused disruption of the mast cells. Antihistamines were able to prevent disruption caused by diamidines.

The initial studies by Riley seemed to support this theory. Therefore, he and West undertook a series of pharmacological studies on the histamine content of various tissues.(71) There proved to be a strong correlation between the numbers of tissue mast cells and the histamine content as reported by Jorpes (40)(70). Further evidence to confirm this theory was the discovery that mast cell tumours were rich in histamine (71). These tumours were obtained from children and dogs. Intraperitoneal injections of ammonia produced changes in the amount of tissue histamine which corresponded to the morphological changes in the mast cells (72)(18).

III. Histamine liberation by compound 48/80.

With the evidence strongly supporting the localization of histamine in the mast cell, the next object of investigation was the mechanism of histamine release from the mast cell. This study has been facilitated by various chemical histamine liberators, especially by compound 48/80.

Riley used the fluorescent liberators stilbamidine and 2-hydroxystilbamidine to localize histamine in mast cells. In 1949 Baltzly, Buck, deBeer and Webb reported the preparation of a new histamine liberator, compound 48/80, a condensation product of phenylethylamine (6). Following the administration of 48/80 there was a temporary depletion of histamine which varied with the species. Unlike some of the other liberators, 48/80 is effective in most species. Paton found compound 48/80 to be a very potent liberator of histamine; it was able to release considerably more than its own molecular equivalent of histamine (65).

In the cat, a fall in blood pressure and increased plasma histamine was found (65). There was also a depletion of histamine from the skin (76). Similar effects were noted in the dog (12).

Mongar and Schild, in a study of histamine release by compound 48/80 and antigen, reported a parallelism of effects between the two agents. Both compound 48/80 and antigen released from 2 to 40% of the total histamine from isolated guinea pig tissue incubated in Tyrode's solution (50).

Compound 48/80, when injected subcutaneously in rats, produced a reduction of about 90% of the total skin histamine. Intraperitoneal injections resulted in signs of itching, cyanosis and prostration which often terminated in death (22). Edema in rats, which followed doses of 5 mg/kg of 48/80, was prevented by previous treatment with an antihistamine drug Neo-antergan (12).

Mast cells were disrupted by the chemical histamine liberator. The disruption was correlated with histamine release(57). Fawcett also observed the disruptive effect of 48/80. After treatment with 48/80 there was an increase in the amount of histamine that appeared in the peritoneal fluid of the rat which corresponded to the dose of 48/80. If the rat were depleted of mast cells by repeated doses of 48/80, then subsequent injections failed to release histamine from the tissues (18). Considering all the evidence of its effectiveness, compound 48/80 was selected by most workers as a tool for studying histamine release.

IV. Histamine liberation by specific antigen.

Sensitized animals and tissues provided another source for the study of histamine release from mast cells. In 1932 Dragstedt and Gebauer-Fuelnegg reported the release of histamine in severe or fatal anaphylactic shock in the dog (17). Histamine was known to be present in organs and tissues which were involved in the anaphylactic reaction. There was a good correlation among the amounts of histamine, time of release, effects of histamine etc. and the anaphylactic process (16). Mota found the amount of histamine in the plasma of shocked rats high (54)(55).

The discovery of histamine in mast cells by Riley implied that the mast cell was involved in the anaphylactic process. Indeed, as early as 1941, in studies on the liberations of heparin in the dog in anaphylactic shock, Jacques and Waters noted changes in the mast cells of the liver (38). Various investigators reported histamine release from isolated tissues incubated with specific antigen (50)(80).

In 1956 Mota and Vugman discovered that the injection of antigen into sensitized guinea pigs produced an anaphylactic reaction which caused a significant reduction in the number of mast cells (60). Many of the remaining cells showed degranulation and a decrease in the metachromasia of the granules. The amount of histamine in the guinea pig lung was correlated with the mast cell count; in anaphylactic shock both dropped. The investigations of Humphrey and Mota support these findings (37). The mast cells of sensitized mice showed degranulation when antigen was introduced (9). Mota conducted similar studies on sensitized rats. He found a definite alteration in the mast cells of the rats which manifested signs of anaphylaxis (54). In vitro work with mesentery from sensitized rats gave similar results.

Washed isolated mast cells from sensitized rats were also able to respond to the specific antigen by degranulation (59). Archer found that a labile substance was formed when antigen was added to fresh immune serum (in vitro) which was capable of disrupting the mast cells (4). The evidence was strong that histamine was released from mast cells in anaphylaxis.

V. Further studies of histamine liberation by compound 48/80 and specific antigen.

With the knowledge that mast cells were involved in anaphylactic histamine release and histamine release by chemical agents, more detailed studies were conducted in which such factors as concentration of liberator, pH, temperature, etc. were measured.

Mongar and Schild were able to demonstrate the release of three-quarters of the total histamine from isolated guinea-pig tissue by compound 48/80 in a concentration of 1 μg/ml (50).

Similar effects of 48/80 were reported by other workers; 90% of the skin histamine of the rat was depleted by an injection of 500 µg of 48/80 (23), 4 µg/ml 48/80 disrupted 78% of the mast cells in the perfused cat's paw (28), 0.5 µg/ml 48/80 degranulated 90-100% of the rat mesentery mast cells (31) and rat peritoneal mast cells released 50% of the total histamine when treated with 48/80 in a concentration 5.0 µg/ml (44). Uvnäs and Thon found that 2.5 µg/ml of 48/80 released 63% of the total histamine while a concentration as high as 62.5 µg/ml released 90% and as low as 0.1 µg/ml released 10% (85) from isolated cells. Further investigations by the same workers on isolated cells showed that approximately 8% of the total histamine could be liberated by 0.01 µg/ml 48/80 (86). The release of histamine and the disruption of mast cells caused by 48/80 was shown by many investigators to be dependent on the concentration of 48/80 (85)(44)(86)(87).

Histamine release in anaphylaxis was also effected by the concentration of antigen. Högberg and Uvnäs, in working with mesentery mast cells from sensitized rats, found that specific antigen in a concentration of 1:500 degranulated 90% of the mast cells; 1:1000, 85% and 1:2000, 70% (31). Chakravarty reported histamine release from lung pieces of sensitized guinea pigs rose almost linearly with the log dose of antigen. Thirty-six percent of the histamine was released by a 10^{-2} concentration of specific antigen. The lowest effective concentration was 10^{-7} which released approximately 1% (10).

Egg albumin, when added to minced guinea pig lung from sensitized guinea pigs, released 20 - 40% of the total histamine (53). Similar findings were demonstrated in isolated mast cells; 36% of the total histamine was released by antigen in a concentration of 1:100 (53). Garcia-Arocha found the concentration to be a most critical factor. Dilutions of 1:10, 1:50, 1:1000 and 1:10,000 were found ineffective; only a dilution of 1:20 proved successful in liberating histamine. Alterations in concentration by as little as 1:25 or 1:15 effected the amount of histamine released (25).

The incubation medium most frequently employed was Tyrode solution (10)(21)(50)(28). Variations of media included buffered saline (31)(89), isotonic sucrose (25) and Krebs-Ringer (91). The pH optimum for histamine release was in the majority of cases on the alkaline side. The range for optimal release with compound 48/80 was between 7.0 and 7.75 (rat mesentery, cat skin and isolated mast cells) (31)(89)(85). Junqueira and Beiguelman found that 48/80 was effective in rat mesentery only in neutral or alkaline media (42). Anaphylactic histamine release from the guinea pig was inhibited at a pH below 5.6 and above 9.5. The optimum was between 7.0 and 8.5 (10). Mongar and Schild, who were also working with sensitized guinea pigs, reported inhibition below pH 6.5 and diminished histamine release above pH 8.0 (50). They reported an optimal range from 7.5 - 8.0. Other workers found the optimum to be around 7.4 (33)(31). However, Uvnäs and Thon in later studies on isolated cells found that a slightly acid media was more favorable to histamine liberation by 48/80. The optimum reported by these workers was 6.5 - 7.0 (86).

An incubation temperature of 37°C was utilized in most procedures. Inhibition of histamine release by 48/80 was noted when preparations of rat mesentery were cooled below 25°C (30), cat skin below 20°C (89) and isolated mast cells below 4°C (8). This was not irreversible since rewarming restored the liberating power of 48/80. This was also true in anaphylactic histamine release; inhibition occurred at 17°C in guinea pig lung preparations (10). Uvnäs and Thon found that histamine release by 48/80 in isolated mast cells increased with rising temperature until it abruptly fell off at 42°C (86). This effect was also noted in rat mesentery incubated with 48/80 (45°-50°C - 0% released) (30), rat skin and mesentery (above 45°C - 0% released) (58) and cat skin (above 47.5°C - 0% released) (89). Again the effect was the same in anaphylactic histamine release. Release by antigen from rat mesentery and guinea pig lung was inhibited at 45°C and 47°C respectively. In all cases, heating above 42° - 45°C proved to have an irreversible effect. When temperatures were returned to 37°C there was no histamine release by 48/80 or antigen (30)(58)(31)(10)(44)(55)(56).

Histamine is released rapidly from the mast cells both in anaphylaxis and by 48/80. Uvnäs and Thon reported a peak of histamine release was reached in 1 - 2 minutes in isolated mast cells treated with 48/80 (86). Degranulation of mast cells occurred in 15 seconds followed by a significant difference in the total amount of mast cells in the tissue when guinea pig lung and mesentery were treated with specific antigen (56). The peak of release in guinea pig anaphylaxis was in 8 - 16 minutes according to Chakravarty (10). In the guinea pig 48/80 liberated three-fourths of the total histamine in the first 10 minutes (50) while Lagunoff and Benditt found a sharp loss of histamine from peritoneal mast cells in the first 30 minutes (44).

Calcium ions were reported to be necessary for both anaphylactic and chemical histamine release from rat skin and mesentery, guinea pig and rat lung, and guinea pig mesentery (33)(58)(10)(56). In preparations of peritoneal cells and isolated mast cells, this dependence on calcium ions was not found. VanArsdel and Bray reported that EDTA was ineffective in inhibiting histamine release from peritoneal mast cells (87). Lagunoff and Benditt used a low calcium Tyrode's solution for the incubation media in their studies (44).

Histamine release mediated by chemical and biological agents can be inhibited by a variety of substances which have specific blocking properties. Inhibitors such as ninhydrin and allicin, which are specific for amino groups and sulfhydryl groups respectively, have been reported as effective in blocking histamine release; ninhydrin and allicin blocked histamine release from cat skin by 48/80, from rat mesentery by 48/80 and antigen and from isolated mast cell by 48/80 (89)(86)(31). Glutathione restored the histamine liberating property of both antigen and 48/80 in the rat mesentery (31). 2-4 dinitrophenol, which blocks phosphorylation, inhibited anaphylactic histamine release (51). Sodium azide and sodium cyanide, which also block the phosphorylating process, were effective inhibitors of release by 48/80 and antigen (33). In the rat, Mongar and Schild found that anoxia inhibited histamine release in anaphylaxis and enhanced release by 48/80 (53). However, Chakravarty found that histamine liberation by both 48/80 and antigen was inhibited by anoxia, but that it had no effect on anaphylactic and chemical histamine release in the rat (10).

VI. <u>Theories of histamine release</u>.

Resultant from the many studies on histamine release are a variety of theories for the mechanism of release both by chemical agents and specific antigen. There is quite a variation among the various liberating agents and among the species as to their mode of action. One agent may act in one manner in the rat but apparently follow a different mechanism in the guinea pig.

One of the first theories was the protease theory propounded by Rocha e Silva. He had studied the work of Feldberg and Kellaway in which the release of histamine by snake venoms was reported (19). Noting that snake venoms contain proteolytic enzymes, Silva was able to show that trypsin and other proteolytic enzymes could release histamine from perfused isolated guinea pig lung (74). The proteolytic enzyme could theoretically displace the histamine by hydrolysing a peptide linkage; tertiary or quaternary bases might release histamine by displacing it from its cell connections.

Ungar's work in anaphylactic and peptone shock in vitro substantiated that of Rocha e Silva. Ungar felt there were kinases in the blood and tissues, which through a series of reactions, could transform protease precursors to active proteases which attack the polypeptides to which histamine is supposed to be attached (79).

However, this theory did not have good substantiating evidence. No quantitative relationship was found between protease activity and histamine release (20). When histamine was discovered in mast cells, the proteolytic enzymes were tested for their ability to disrupt the mast cells. They were not able to degranulate the cells which is further evidence against the protease theory (81).

Various agents such as ice-cold TCA, distilled water, etc. were able to release histamine which indicated that histamine was held in the mast cells by ionic linkages and not chemical bonds. Surface agents such as octylamine, decylamine etc. seemed to act in a non-enzymatic way in releasing histamine from the mast cells (33). The action of these liberators was destroyed by various enzyme inhibitors.

The "displacement theory" held by MacIntosh and Paton stated that 48/80 simply entered the cell and then the granules and replaced the histamine (47)(66). Histamine, a base, was believed to be held in the cell by association with a complex acid and any substance with a greater affinity for the acid could displace the histamine.

It was found, however, that 48/80, a typical histamine liberator, released several molecules of histamine, approximately in a 10:1 ratio. Compound 48/80 followed a pH curve which fit an enzyme theory, not a displacement theory. An agent such as decylamine, which is considered as non-enzymatic, had a straight line relationship of disruption vs. pH consistent with the displacement theory. This was true of rat and cat tissues. In the guinea pig, enzyme inhibitors did not block the action of 48/80 (14).

In 1955, Junqueira and Beiguelman held that 48/80 released histamine via an enzyme mechanism involving SH groups (42). There has been much substantiating evidence for an enzyme mechanism for 48/80. In the cat's paw non-specific enzyme inhibitors such as polysaccharides and polymeric salicylic acid as well as specific inhibitors such as sulfhydryl blocking agents (allicin) were able to block histamine release. Heating the paw to 45°C irreversibly abolished histamine release (11)(29)(28).

Studies with rat mesentery cells _in situ_ showed that pH, temperature, enzyme inhibitors, etc. had an influence on mast cell degranulation (30)(81). Work with isolated rat mast cells indicated that again 48/80 and antigen worked in an enzymatic process to release histamine (86). Ninhydrin (blocks NH_2 groups) and allicin (blocks SH groups) prevented histamine release. Glutathione abolished the inhibitory action of allicin. The action of dinitrophenol, a metabolic inhibitor, was not as strong on the isolated cell, but did manifest an inhibitory action. It would seem that SH groups, NH_2 groups and oxidative phosphorylation were implicated in histamine release. The action of metabolic inhibitors was counteracted in part by the presence of glucose (83).

It was found that phospholipase A was the only one of 35 enzymes tested that could degranulate mast cells (30)(32). Its action was blocked by the same inhibitors that blocked the action of 48/80 and antigen. Although phospholipase A did not require the presence of high energy compounds, DPN blocked it. Possibly the enzyme only sets off an energy-requiring histamine release mechanism. Where the energy step is needed remains unknown.

VanArsdel and Bray disagreed that histamine release by antigen was similar to chemical release by 48/80. They felt there wasn't enough evidence to support the enzyme theory of release. In their preparation of peritoneal mast cells, the inhibitors were not as effective as the reports by other workers. They saw little uniformity in the activity of the enzyme inhibitors and could not relate their action to a known enzyme system (87).

VIII. Isolation of mast cells.

It was important for the present study to select a method of isolating mast cells so that they could be studied without the influence of outside factors that would be encountered in tissue preparations.

The method developed by Padawer and Gordon (63) involved separation of the mast cells from the other cellular elements of the peritoneal fluid by a process of differential centrifugation. The cell suspension of low density was layered on a sucrose suspension of a high density. The mast cells, which have the highest density of the peritoneal cells, passed through the interface upon centrifugation leaving the other cells above the interface.

The cells were obtained from the peritoneal cavity by lavage with 0.9% saline solution. The saline increased the amount of cells obtained, raised the volume available to work with and lowered the density of the solution. The body wall was massaged to dislodge the mast cells and the fluid recovered was layered above a high density medium consisting of sucrose, gelatin, salt and distilled water. Centrifugation at a maximum speed of 740 r.p.m. was of 5 minutes duration. Following centrifugation, the high density solution below the interface was withdrawn.

The resulting suspension contained 75% mast cells as opposed to 3 - 5% in the peritoneal fluid. The authors stated there was some degranulation of the cells which occurred due to the high osmotic activity of the sucrose solution. However, the cells so isolated were reported to react to 48/80 in the same manner as the mast cell <u>in situ</u>.

Glick, Bonting and DenBoer (27) made modifications of the method of Padawer and Gordon. They sought a purer suspension of mast cells and a higher percentage of recovery. Major modifications consisted of 1) changing the peritoneal wash solution to Hank's solution, 2) elimination of gelatin from the gradients because it made them difficult to handle, 3) a different pattern of centrifugation. The isolated cells were washed in distilled water and resuspended in saline.

The authors reported that with this method the mast cells were separated in a distinct layer in the sucrose which was uncontaminated by other cells. They also claimed 87% recovery of the mast cells layered on. No mention was made as to the morphological or functional status of these isolated cells.

Lagunoff and Benditt in attempting to measure 5-hydroxytryptamine decarboxylase activity in isolated mast cells found it necessary to find a method of obtaining mast cells which did not result in damage to the cells (43). They adapted the method of Agranoff and Vallee which utilized albumin solution for cell separation (1). Cat serum, diluted to 50% with saline, was used as the peritoneal wash solution. This was layered on a 35% albumin solution, centrifuged and the isolated mast cells removed and washed.

Mast cells were obtained in a purity of 40 to 100%; the initial value was 5% in the peritoneal wash solution. Morphologically, the cells were in excellent condition; they presented a uniform size and round shape. Resuspension in the incubation medium did not cause disruption. However, cells isolated with sucrose gradients had irregular shapes and disintegrated when suspended in isotonic saline. Cells isolated with this method were found to have the same activity with regards to 5-hydroxytryptamine formation as unpurified mast cells, whereas cells isolated in sucrose did not show this activity.

The albumin method employed by Archer (3) was similar to that of Lagunoff and Benditt although his separations were done on a much smaller scale. The peritoneal wash solution was layered on a 4 cm column of 50% human albumin solution in a Wintrobe hematocrit tube and centrifuged. The mast cells were removed from the bottom of the tube. No report was given concerning the condition of the cells following the separation procedure.

In Sweden, Uvnäs and Thon developed a technique for the isolation of "biologically intact" mast cells which involved the use of a Ficoll gradient system (85). Ficoll is a water soluble substance with polysaccharide-like properties. It was prepared in concentrations of 30, 40 and 50% and placed in layers of 1 ml each in a test tube. The peritoneal wash solution was layered above this and centrifuged for 10 minutes at 1200 r.p.m. The mast cells were washed and resuspended in a solution of phosphate buffer which contained 0.2% glucose and 2% cat serum.

The authors reported that with the technique of Glick, Bonting and denBoer and with that of Padawer and Gordon, in which the mast cells were isolated with sucrose, much of the histamine was released into the sucrose and the remainder was lost during washing of the cells. When Ficoll gradients were employed, the mast cells did not lose histamine spontaneously.

MATERIALS and METHODS

A suspension of mast cells which were pure as to cell type and which were morphologically and physiologically intact was desired. It was necessary to separate these cells from the other cells of the rat's peritoneal fluid. A process of differential centrifugation was employed in which the mast cells, which have a greater density than the other peritoneal cells, were removed from the bottom of the centrifugation medium. They were then washed and resuspended in an incubation medium from which aliquots were made for various studies.

I. Isolation of mast cells

Two methods were used to isolate mast cells from the peritoneal cavity of the rat. The first was a slight modification of the method of Glick, Bonting and DenBoer (27) which was an adaptation of the method of Padawer and Gordon (63). This method did not prove to be satisfactory and a second method, that of Lagunoff and Benditt (43), was slightly modified for use. The overall procedures of both were similar, the main differences consisted of the medium used in the differential centrifugation. For the purposes of clarity and to avoid repetition, the method of Glick *et al.* has been referred to as Method A and that of Lagunoff and Benditt as Method B.

All rats used throughout the procedure were 200 to 350 g male albino rats of the Sprague-Dawley strain which were obtained from the Charles River Breeding Laboratory of North Wilmington, Massachusetts. The animals were maintained on

Purina Lab. Diet and water ad libitum prior to use.

The rats were decapitated by a rat decapitator and exsanguinated. They were then strapped to an animal board in supine position with the limbs extended. The fur covering the thorax and abdomen was wet and the skin cut in the midline from the neck to the pubis and reflected from the body by dissection.

A. Method A - Various solutions were used as the peritoneal wash solution during the course of the experimental work. In Method A heparinized physiological saline was first used. It consisted of 8.5 g sodium chloride and 50.0 µg/ml heparin (Hynson, Westcott and Dunning, Inc.) dissolved in 1.0 l distilled water.

A modified Hank's solution was next used. It consisted of the following ingredients:

Sodium chloride	8.0 g
Potassium chloride	0.4 g
Calcium chloride	0.2 g
Magnesium sulfate	0.2 g
Sodium phosphate, dibasic	0.1 g
Potassium phosphate, monobasic	0.1 g
Glucose	2.0 g

After sterilization by autoclaving, 1.0 ml of isotonic (1.4%) $NaHCO_3$ was added to each 1.0 ml of the salt solution. Carbon dioxide was bubbled through the solution to obtain the desired pH.

A leukocyte preservation media modified from that described by Tullis (78) was the third peritoneal wash solution used. It had the following formula:

Stock Solution 1

Sodium phosphate, dibasic	0.12 g
Potassium phosphate, monobasic	0.12 g
Sodium chloride	7.50 g
Potassium chloride	0.75 g
Potassium phosphate, tribasic	0.05 g

Made up to 1.0 l in distilled H_2O.

Stock Solution 2

Sodium Bicarbonate	4.00 g
Distilled water	100.00 ml

Solutions number 1 and 2 were autoclaved separately. Five ml of solution number 2 was added to 1.0 l of solution number 1. The pH was adjusted to 7.4 by bubbling in carbon dioxide. The solution was dispensed into flasks containing 1.0 l of solution 1 and 5 ml of stock solution 2. The following ingredients were added:

Sodium acetate	21.00 mg
Dextrose	42.00 mg
Ascorbic acid	0.35 mg

The contents of the flasks were then frozen for storage. For convenience, some of the solution was divided into tubes each containing 10.0 ml and upon use the following additions were made to each:

Heparin (100 µg/ml)	0.1 ml
Sterile gelatin solution (8.6% q. s. ad. 1 g %)	

In a few experiments, the pH of the Tullis' solution was adjusted to pH 6.0 by bubbling in carbon dioxide.

Four ml of the appropriate solution was injected into the abdominal cavity through the linea alba by inserting a #25 gauge needle attached to a 5.0 ml syringe.

A towel clip attached with a string to a clamp was clipped to the abdominal wall on each side of the midline so as to hold the abdomen in a slightly taut position. A midline incision was made from xiphoid to pubis with special care taken to avoid spilling the tissue juices into the peritoneal cavity. The towel clips were adjusted to hold the abdominal incision open and away from the viscera. Aspiration of the peritoneal wash solution was accomplished with a 2.0 ml transfer pipette which had been cut down and the ends fire polished. This was attached to a 1.0 ml screw-type pipette with rubber tubing. As the viscera were pushed gently aside to remove the fluid, care was taken to avoid tearing the mesenteries. The fluid was pooled in a 15.0 ml test tube.

Method A utilized a sucrose gradient system for cell separation and isolation. The three sucrose gradients were made according to the following formula:

Versene-phosphate buffer pH 7.4

Disodium EDTA	0.190 g
Disodium phosphate	2.860 g
Potassium phosphate, monobasic	0.272 g

The above ingredients were made up to 1.0 l in glass distilled water and stored in the cold (2 - 4°C).

Sucrose stock solution

Sucrose	268.50 g
Versene-phosphate buffer	231.50 ml

The sucrose stock solution was placed in a 500 ml bottle stored in the cold (2 - 4°C).

Using the two stock solutions described above, solutions were made with the following densities:

1.113	5.15 ml sucrose + 4.85 ml Versene-phosphate buffer
1.165	6.55 ml sucrose + 3.45 ml Versene-phosphate buffer
1.200	7.95 ml sucrose + 2.05 ml Versene-phosphate buffer

One ml of each solution was carefully layered in a test tube measuring 10 x 1 cm and stored at 2 - 4°C for 24 hours. The preparation of the gradients was varied in a few experiments by diluting each layer 1:5 with Versene-phosphate buffer. Two ml of the pooled washings which had been gently mixed with the pipette were layered on the gradients.

In the early experiments with sucrose gradients, the centrifugation was carried out in an International Horizontal Centrifuge with an International head number 235, cup number 303 and 16.2 cm radius of gyration. The speed was gradually brought up to 110 times gravity in 50 seconds and held at this speed (750 rpm) for 3.5 minutes and then gradually reduced to 0 in 50 seconds. Later a new centrifuge was obtained and calculations were made to obtain the same force of gravity. The new centrifuge was an International Horizontal Centrifuge, head #253, cup number 356 and 22.5 cm radius of gyration. The speed was gradually brought up to 650 rpm (110 x g) in 50 seconds and maintained at this speed for 3.3 minutes and then reduced to 0 in 50 seconds.

The mast cell layer was distinguished from the other cellular elements of the peritoneal fluid by its position in test tube. It was the bottom layer in a series of cellular layers consisting first of the top red blood cell layer, secondly of the dense white blood cell layer under which was the diffuse white blood cell layer and finally the mast cell layer.

Removal of the mast cell layer was accomplished by clamping the test tube to a lab jack and gently raising the tube so that the contents of the tube met a pipette whose tip was filled with wash solution. The tip of the pipette was placed in the correct layer and fluid containing the mast cells withdrawn by a syringe attached to the pipette.

The isolated cells were then washed in the solution which was to serve as the incubation medium. Washing consisted of adding the correct volume of wash solution and gently mixing the two solutions by careful slow inversion of the tube. This was followed by centrifugation for 5 minutes at 1500 rpm. The cells of the first experiments were washed in 0.85% saline in a volume equal to 3 times that of the mast cell layer. In succeeding experiments Tullis' solution, as previously described, was used in quantities equal to 2 to 3 times that of the mast cell layer. The pH of the Tullis' solution was 7.4 and 6.0. These solutions were also the incubation media. None of these solutions contained heparin.

The isolated mast cells were diluted up to 1.0 ml with the incubation solution and then were carefully mixed by drawing the suspension slowly in and out of a 1.0 ml pipette which was graduated in 0.1. Using the same pipette, 1.0 ml of the suspension was divided into 0.1 ml aliquots and placed in test tubes measuring 10 x 1 cm.

In some instances the cells were not washed. The aliquots were made from the sucrose mast cell suspension obtained upon differential centrifugation. In experiments where the amount of histamine in cells and supernatant was to be determined, the cells and supernatant were analyzed at 0 time. The 0.1 ml aliquots of these cells were diluted up to 1.0 ml with the incubation medium.

The cells were considered to be contained in the bottom 0.1 ml of the 1.0 ml mast cell suspension which had been centrifuged for 2 minutes at 2000 rpm. The top 0.9 ml, the supernatant, was withdrawn by a 1.0 ml pipette, graduated in 0.1, which was clamped to a ring stand and attached to a syringe. The test tube containing the mast cell suspension was clamped to a lab jack. The lab jack was gently raised so that the tip of the pipette was always on the surface of the liquid. The next samples of the supernatant were taken at 10, 20, 30, 40, 60 and 80 minute time intervals.

B. Method B - Method B was identical to Method A until the peritoneal wash solution was introduced into the peritoneal cavity. In this method, 0.85% saline buffered with phosphate buffer was used. It had the following composition:

Sodium chloride	8.5 g
Heparin (0.1 mg/ml)	
Phosphate buffer pH 7.4 (0.5 ml to 10 ml saline)	

The phosphate buffer was made in the following way:

Solution A

Potassium phosphate, monobasic (M/10)	13.605 g
Distilled water was added to 1.0 l	

Solution B

Sodium phosphate, dibasic 14.190 g
Distilled water was added to 1.0 l

One hundred ml of a solution with pH 7.4 was made by mixing 19.0 ml of Solution A with 81.0 ml of Solution B.

In the adaptation of Lagunoff and Benditt's method, Method B, an albumin solution was utilized for the separation of mast cells from the other cellular elements of the peritoneal fluid. The ingredients were:

> 30% bovine albumin (Bovine Albumin Solution 30%, Armour Pharmaceutical Company) and 0.5 g dried bovine albumin (Crystallized Bovine Plasma Albumin, Armour Pharmaceutical Company) made up to 10 ml total volume producing a solution containing 35% albumin with a density at room temperature (25^oC) of 1.090 - 1.094 and a viscosity of 16 centipoises.

Six ml of the pooled peritoneal washings which had been gently mixed were layered on 6.0 ml of the 35% bovine albumin solution in 15.0 ml lusteroid centrifuge tube.

Centrifugation in Method B took place in the second centrifuge described. The centrifuge tubes were spun for 20 minutes at 100 times gravity (645 rpm).

The mast cells were located in the bottom 4.0 ml of the 6.0 ml albumin solution used in the isolation. The layer was removed by a drawn-out 2.0 ml transfer pipette with a long fine tip. The pipette was attached to a mouth aspirator and the tip of the pipette was filled with the wash solution and gently lowered to the bottom of the mast cell layer.

The cells were washed in the same manner as described in

Method A. The washed cells were centrifuged at 645 rpm for 20 minutes, the supernatant solution removed, and the cells suspended in 1.0 ml of solution.

At first the mast cells were suspended in 0.1 M phosphate buffer for the incubation medium. The preparation of this buffer was described previously. A modification of Tyrode's solution was next used. It was made according to the following formula:

Modified Tyrode's Solution

Sodium chloride	8.0 g
Potassium chloride	0.2 g
Anhydrous calcium chloride	0.1 g
Sodium bicarbonate	1.0 g

In solutions containing sodium bicarbonate, the sodium bicarbonate had to be completely dissolved before the calcium chloride was added. The above was dissolved in 1.0 l of glass distilled water.

The final solution contained 9 parts of Tyrode's solution to 1 part phosphate buffer. Modifications of the solution consisted of the addition of normal horse serum in a final concentration of 2.0% (Normal Horse Serum, without preservative, Massachusetts Public Health Biologic Laboratories) and/or doubling the calcium chloride content.

The division of cells into aliquots and the analysis of cells and supernatant was as described in Method A.

II. <u>Effect of chemical and biological agents on isolated mast cells</u>.

After obtaining a satisfactory method for the isolation of intact cells, the effects of chemical and biological agents were tested on the isolated cells. The two substances tested were compound 48/80 and specific antigen, egg albumin.

Compound 48/80, a condensation product of p-methoxyphenylethylmethylamine with formaldehyde, obtained from the Research Laboratories, Park, Davis & Company, was weighed in 25.0 mg lots and dissolved in a total volume of 50.0 ml glass distilled water (0.5 mgm/ml). One ml of this solution was diluted to 100.0 ml with glass distilled water. This gave a concentration of 5.0 µg/ml or 1.0 g in 200,000.

This solution of compound 48/80 was added to the mast cell aliquots in volumes of 0.1 ml, 0.2 ml and 0.4 ml. The amount of the incubation medium was adjusted so that the final volume of cells, incubation medium and agent to be tested equaled 1.0 ml. The addition of test solution was done at 0 time (immediately before the first sample was to be analyzed for cellular and supernatant histamine). The incubation was carried out at room temperature (25°C).

Sensitivity in rats to hen's egg albumin was induced by subcutaneous injection of 1.0 ml of egg albumin and 1.0 to 1.5 ml *H. pertusis* vaccine given no less than 12 days before use. The egg albumin was prepared from fresh hen's eggs. The albumin was separated from the yolk and mixed with an equal volume of 0.85% saline and then run through a strainer. The *H. pertussis* vaccine was obtained from the Commonwealth of Massachusetts Department of Public Health, Division of Biologic Laboratories, Boston, Massachusetts and consisted of 35,000 million killed *H. pertussis* organisms per ml in lot WFD 125 A. In experiments using lot #21487-25, 1.0 ml of H. pertussis and 1.0 ml of a 1:1 egg albumin solution were mixed in a 2.0 ml syringe and injected subcutaneously in the neck of the rat. One and one half ml of H. pertussis and 1.0 ml of the 1:1 egg albumin-saline solution were used in experiments using lot # WDF 125 A. A period of 12 to 18 days after injection was considered as the optimum time for sensitivity.

Tests to determine the effectiveness of the sensitizations were run in the following way. Rats were placed under light ether anesthesia and 1.0 ml of egg albumin solution, as prepared for the sensitization, was introduced into the jugular vein. The wound was closed with sutures and the animal allowed to recover. If death had not occurred within 2 hours, the animal was sacrificed. Autopsy findings, consisting of the gross appearance of the small intestine, were recorded.

In testing the effect of specific antigen on mast cells, the same preparation of egg white as used in the sensitization was utilized. From this dilution, a 1:50 dilution was made from the stock solution.

The antigen was used in concentrations of 1:1000, 1:500, 1:100 and 1:25. The time of incubation was 2 hours from 0 time when the antigen was added. To insure proper mixing of the albumin and the mast cell suspension, the tube was gently tipped and rotated. A similar tube without antigen was handled in an identical manner so that the mechanical trauma to the cells could be measured. The cells and the supernatant were separated as previously described.

The supernatant fluid, obtained by either Method A or B, was placed in a test tube and to it was added 0.1 ml glass distilled water and 1.0 ml trichloroacetic acid. Nine tenths ml of water was added to the cells, which as previously stated, were contained in the remaining 0.1 ml in the bottom of the tube. One ml of trichloroacetic acid was also added to this preparation. All the samples were treated in this manner at the appropriate time. The samples were then ready for the histamine determination.

III. Histamine determinations

The extraction of histamine with the trichloroacetic acid was the first step in the chemical analysis of histamine described by Lowry *et al*. (46). This method was developed to measure histamine in blood plasma and cells. The histamine was concentrated and separated from many other amines by adsorption on a column of Decalso, a cation exchange resin. The histamine was eluted from the column with hot potassium bromide and colored with 2-4-dinitrofluorobenzene. Methyl-n-hexyl ketone was used to further concentrate and separate this product from dinitrofluorobenzene derivatives of other remaining amines. A final extraction was made in a small amount of hydrochloric acid. The yellow color was measured spectrophotometrically at 360 mμ.

A standard curve was plotted from the data presented by Lowry for complete determinations. Histamine standards consisting of 1.0, 3.0, 5.0, 10.0 and 15.0 μg histamine base were then run in complete determinations and eluates. (An eluate is the sample after it has been eluted from the Decalso with potassium bromide. In this instance, adsorption on the Decalso column was eliminated and the histamine standard added directly to the potassium bromide.) The % transmittance was plotted against the amount of histamine, expressed in µg. The tabulated data from which the curve was plotted is presented below.

Data of Lowry* (46)

Conc. histamine base µg/ml	Eluates %T	Complete %T
1.0	83.0	92.0
3.0	56.0	64.4
5.0	37.0	42.6
10.0	-	-
15.0	6.0	6.9

* Data converted to %T from O.D.

Data of Loew. (Eluates)

Conc. histamine base µg/ml	Number of determinations	%T	S. D.	Coef. Var.
1.0	8	88.3	1.8	2.0
3.0	16	63.7	4.7	7.4
5.0	8	45.7	2.9	6.3
10.0	16	19.1	2.9	14.9
15.0	8	10.1	3.6	35.2

Data of Loew. (Complete determinations)

Conc. histamine base µg/ml	Number of determinations	%T	S. D.	Coef. Var.
1.0	6	90.3	2.7	3.0
3.0	8	72.6	2.8	3.9
5.0	6	58.0	3.9	6.7
10.0	7	26.2	2.6	9.8
15.0	6	14.4	2.0	14.0

With each experiment a standard was run along with the unknowns. In a majority of the experiments the standard containing 3.0 μg histamine was selected. The values obtained with the use of this standard in each experiment presented in the results are given. The mean was 2.8 with a standard deviation of 0.64. A standard of 1.0 μg histamine was used for the remaining experiments. The mean obtained in this set of determinations was 0.9 with a standard deviation of 0.27.

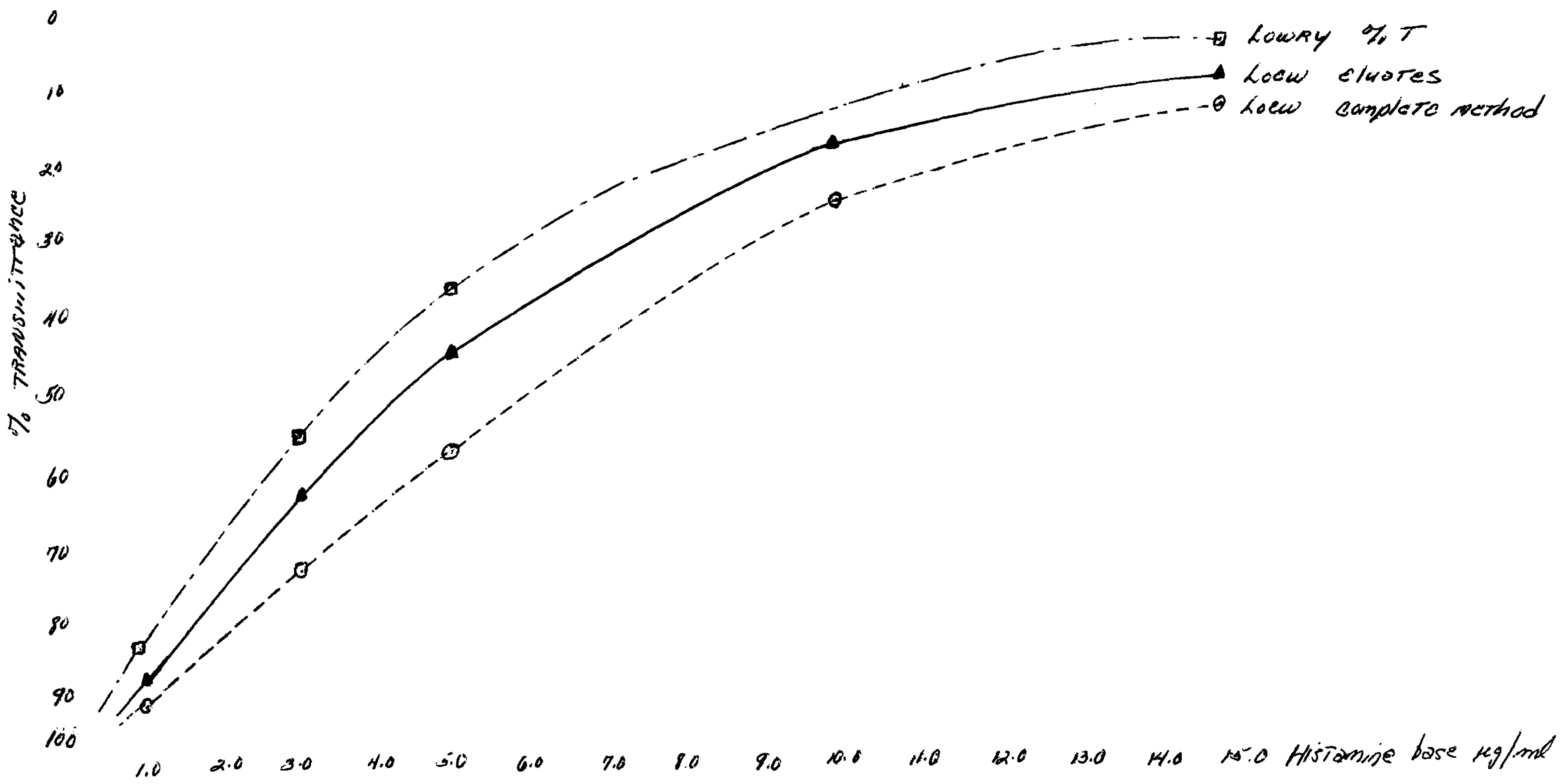
Standard (3 µg histamine base) from experimental data.

Exp. No.	µg histamine	Exp. No.	µg histamine
12	2.7	127	3.3
16	2.7	142	2.9
21	3.4	143	3.5
29	3.1	137	3.7
30	2.7	137	3.5
31	2.6	140	2.2
32	2.9	155	2.7
33	2.7	156	2.7
86	2.2	156	2.5
87	3.8	158	3.0
112	3.1	159	1.8
113	3.4	159	2.2
114	1.4	160	2.6
115	3.8	160	3.0
116	2.3	161	2.1
119	3.5	162	1.8
120	3.7	164	1.7
123	3.3	165	2.6
126	3.8	165	2.1
		Mean	2.8
		Stand. dev.	0.64

Standard (1 μg histamine base) from experimental data.

Exp. No.	μg histamine
7	1.0
76	1.1
77	1.1
78	1.2
79	0.9
81	0.9
84	0.4
Mean	0.9
Stand. dev.	0.27

	Conc. Hist. base µg/ml	Lowry (from control) Eluates %T	Lowry (from control) complete %T	Loew %T Eluates n	Loew %T Eluates %T ± S.D.	Loew %T Eluates Coef var.	Complete n	Complete %T ± S.D.	Complete coef var.
23	1.0	83 ± 15%	92.0	8	88.8 ± 1.8	2.0	6	90.3 ± 2.7	3.0
24	3.0	56 "	64.4	16	63.7 ± 4.7	7.4	8	72.6 ± 2.8	3.9
25	5.0	37 "	42.6	8	45.7 ± 2.9	6.3	6	58.0 ± 3.9	6.7
26	10.0	–	–	16	19.1 ± 2.9	14.9	7	26.2 ± 2.6	9.8
27	15.0	6 "	6.4	8	10.1 ± 3.6	35.2	6	14.4 ± 2.0	14.0



EXPERIMENTAL RESULTS

I. Isolation of mast cells with sucrose gradients. Method A

It was first necessary to determine if the mast cells, isolated with sucrose gradients, were able to retain histamine. The entire procedure of Method A was carried out. Heparinized saline was used as the peritoneal wash solution and the isolated mast cells were suspended in physiological saline. Determinations of the amounts of histamine in the cells and supernatant were made as soon as the separation was complete. This information is presented in Table 1 along with the % of the total histamine in the cells and supernatant.

It was evident that the washed mast cells did not retain histamine; only 23.5% of the total histamine was left in the cells. If the washing of the cells and not the centrifugation procedure were the cause of histamine loss from the mast cells, then a determination of histamine in the cells suspended in sucrose should show histamine retention by these cells. To obtain the data on the amounts of histamine in the cells and supernatant, as well as the percentage recovery shown in Table 2, the mast cell layer was removed, divided into aliquots, and the histamine determination done immediately, without washing the cells.

Although there was an improvement (23.5 to 51.8%) in the ability of the cells to retain histamine, this 50% loss represented some mechanical trauma to the cells which would interfere with the measurement of histamine loss by chemical or biological agents. The average % recovery of mast cells layered on the gradients was also poor and indicated that the cells were either disrupting and disappearing in the isolation process or they were not reaching the proper layer for separation.

The high molarity of the sucrose gradients was probably responsible for the poor morphological and physiological condition of the cells. It was felt that a less hypertonic solution would be a better medium for separation, therefore each gradient was diluted 1:5 with the Versene-phosphate buffer. However, to maintain an adequate separation, the temperature was held at 10°C throughout the separation to increase the viscosity of the diluted gradients.

A comparison was made between the recovery of cells isolated with diluted and undiluted gradients. Table 3 shows the % recovery of mast cells with both methods.

It can be seen that the average % recovery was only slightly improved when the diluted gradients were used for the separations. These cells were unwashed, therefore, it was desired to ascertain if washed cells, isolated with diluted gradients would retain histamine during an eighty minute time interval. The mast cells were washed and incubated in Tullis's (78) solution. This media was developed to prolong the viability of the leukocyte. Because the mast cells are found with leukocytes in the peritoneal fluid, it seemed that a successful preservation media for the white blood cells might prove beneficial to the mast cells. The pH was adjusted to 6.0 and 7.4 with phosphate buffer. The retention of histamine by the mast cells is shown on graphs 1 and 2.

At the end of the forty minute incubation perioa the mast cells incubated in Tullis' solution at pH 7.4 had a mean retention of total histamine of 22.7% while the mast cells incubated at pH 6.0 had a mean retention of total histamine of 23.3%. Although the cells did begin with a higher retention of histamine (66.7% at pH 7.4 and 67.7% at pH 6.0) they rapidly lost this histamine during the incubation period.

Judging from previous experiments in which the cells were unable to retain histamine when measured immediately following separation, it appeared that the spontaneous loss of histamine was due to the isolation procedure and not the incubation media. This conclusion is supported by the higher values for initial histamine retention.

At this time a new method for isolating mast cells appeared in the literature. Lagunoff and Benditt (43), as previously stated, were seeking a method of mast cell separation which would not damage the mast cells. Their use of a 35% albumin solution seemed wise as its tonicity was more compatable with that of the mast cell. They reported the absence of one of the functional activities of the mast cell, 5-hydroxytryptamine formation, when sucrose gradients were employed.

At approximately the same time Archer published a method for separating mast cells utilizing 50% albumin solution(3). Both groups of workers seemed satisfied with the recovery and morphology of the isolated cells. It was decided on the basis of the evidence presented in these reports that it would be worthwhile to adopt the albumin method of separation.

II. Isolation of mast cells with albumin solution. Method B

In the succeeding experiments, Method B was used to isolate the mast cells. The cells were suspended in phosphate buffer following the complete procedure of Method B to separate the cells. Table 4 shows the distribution of histamine in cells and supernatant for the two hour period.

These two experiments and a few initial experiments indicated that histamine retention by cells isolated with the albumin method was quite satisfactory. In later experiments where compound 48/80 was being tested on cells isolated by Method B, it can be seen that the control mast cells have retained histamine over a two hour period. (Table 4)

III. Effect of compound 48/80 on isolated mast cells.

The action of compound 48/80, a known histamine liberator, was studied on isolated mast cells. Compound 48/80 was added to the aliquots of isolated cells to give a final concentration of 0.5 µg/ml. A phosphate buffered Tyrode's solution (pH approx. 7.2) was considered a more physiological milieu for the mast cells. The amounts of histamine in the treated and untreated cells were measured at various time intervals and is reported in Table 5.

When a statistical analysis was made between the untreated mast cells at one and two hours and the treated cells at the same time, it was found that there was no significant difference between the two groups at either time. The P value at one hour was $< .50 > .10$ and $> .50$ at two hours.

This indicates that histamine was not being released from the mast cells at this concentration of 48/80. The ineffectiveness of 48/80 might be attributed to various factors, 1) Too low a concentration of liberator, 2) Poor functional condition of the cells, 3) Lack of some factor in the incubation media necessary for histamine liberation.

It seemed probable that the concentration of compound 48/80 was the cause, therefore it was increased to 2 µg/ml. Similar tests were run on these cells which were also suspended in Tyrode's solution. Recorded in Table 6 are the quantities of histamine in mast cells after one and two hour incubation in the presence and absence of compound 48/80.

The treated cells at both 1 and 2 hours had significantly less histamine than the control cells at the same period. ($P < .01$)

When the amounts of histamine in the mast cells from both one hour control groups (Tables 5 and 6) were compared, it was found there was no significant difference between the groups ($P > .50$). Therefore, it can be shown that compound 48/80 released significantly more histamine at a concentration of 2 µg/ml than 0.5 µg/ml ($P < .01$). At the two hour period a difference appeared in the controls so no comparison could be made of the effect of concentration at this time.

The higher concentration of 48/80 was not able to release all the histamine from the mast cells. At 1 hour, 30.8% of the total histamine remained in the cells and 29.2% was present at two hours. With the lower concentration of 48/80, 100% of the total histamine remained in the cells at 1 hour and 88.5% at 2 hours.

It was obvious that concentration of liberator was the factor involved. With the proper concentration of 48/80, 70% of the total histamine was liberated from the isolated cells. The untreated cells retained most of their histamine, whereas treatment with adequate amounts of 48/80 caused release of a large percentage of their total histamine.

IV. Effect of chemical and biological agents on isolated mast cells from sensitized and non-sensitized rats.

Since compound 48/80 was able to release histamine from isolated mast cells, it was decided to determine if specific antigen could release histamine under the same conditions. This would be helpful in determining the process of the anaphylactic reaction. Isolated cells would be free of the tissue factors which have been implicated in the process. The specific antigen selected for the study was albumin.

A. Sensitization of rats to albumin.

Rats were made sensitive to hen's egg albumin as previously described. *H. pertussis* vaccine was used because of its ability to enhance the anaphylactic reaction of rats and mice. Sanyal and West reported that 12 - 18 days was the time for optimal sensitivity (75).

Inspection of Table 7 shows that rats challenged with egg albumin 10 - 14 days after sensitization developed anaphylactic shock which terminated in death. Post mortem studies showed hyperemia of the small intestine. Normal rats when subjected to this same procedure recovered showing no signs of anaphylaxis and no hyperemia. It was concluded from these findings that the procedure of sensitization was effective.

B. Effect of albumin on isolated mast cells from non-sensitized rats.

The ability of albumin to elicit histamine release was first measured on mast cells isolated from non-sensitized rats. The cells were suspended in buffered Tyrode's solution as previously described. Table 8 shows the amounts of histamine in control mast cells and those treated with albumin in a concentration of 1:500 and 1:1000.

No significant difference was found between the treated and untreated cells at the two hour period. The P value for albumin treated cells (1:500 and 1:1000) and the controls was $>.50$. If the value of 84 µg is considered an aberrant value, then it is found that 100% of the histamine was retained by the cells when the albumin was at a concentration of 1:1000 and 91.5% when the albumin was at a concentration of 1:500. This indicates that albumin was unable to produce histamine release from the isolated cells. These results are in agreement with those found in the intact animal where albumin was unable to produce any signs of anaphylaxis.

C. Effect of albumin on isolated mast cells from sensitized rats.

The histamine releasing ability of specific antigen, albumin, was next measured on mast cells isolated from sensitized rats. (Table 9). No statistical difference was found between the control cells at two hours and the treated cells. The P value for a concentration of albumin of 1:1000 was $>.50$ and for albumin at 1:500 it was $>.10 < .50$. The cells treated with albumin at 1:1000 retained 85.4% of the controls amounts of histamine, while the cells treated with albumin in a concentration of 1:500 retained 76.8% of the total histamine. Again it was decided that the factor of concentration was involved.

D. Effect of increased concentrations of albumin on isolated mast cells from sensitized rats.

The concentration of albumin was increased to 1:100 and similar measurements of histamine were made. (Table 10). A significant difference was demonstrated between the amounts of histamine present in control mast cells at two hours and the histamine present in mast cells treated with albumin in a concentration of 1:100 ($P < .01$). Thirty-two per cent of the total histamine was released from the cells exposed to albumin.

Uvnäs and Thon reported that serum must be present in the wash and incubation medium for compound 48/80 and antigen to release histamine from the isolated cells (85). Although it was found in the preceeding experiments that serum was not necessary for histamine release by compound 48/80, it was felt that the addition of serum might enhance the action of the albumin and thereby release a higher percentage of the total histamine.

E. Effect of the addition of serum to wash and incubation medium on the histamine releasing ability of albumin.

Normal horse serum was added to the wash and incubation medium in a concentration of 2%. It was decided that the mechanical agitation involved in mixing the albumin might be responsible for some of the histamine release. Cells at 2 hours, which received the same mixing procedure as the cells treated with albumin, were used as controls. The effect of serum may be seen on Table 11.

The addition of serum did not enhance the action of albumin on the isolated cells. There was a significant difference in histamine release between the treated and untreated cells with regard to both antigen ($.02 < P < .05$) and compound 48/80 ($P < .01$), whereas mechanical agitation had no effect ($P > .50$). The percentage of total histamine released from the cells treated with antigen was 29.4, while without serum 31.0% of the total histamine was liberated. Apparently serum was not the necessary factor for histamine release.

It was next felt that if the cells were incubated at a temperature of 37°C, histamine release might be facilitated. This temperature was selected by several workers.

F. Effect of 37°C incubation and serum on the histamine releasing ability of albumin.

Albumin and compound 48/80 were added to the aliquots of mast cells and the temperature of incubation was maintained by a water bath. Serum was retained in the wash and incubation medium.

The incubation temperature of 37°C seemed to decrease the amount of histamine released from the cells treated with antigen (Table 12). There was no significant difference between the control cells and those treated with albumin ($P > .50$); however, 48/80 was able to release a significant amount of histamine ($P < .01$). The albumin treated cells retained 87.6% of the total histamine. Mechanical agitation had no effect on the cells ($P > .50$).

Various reports have been made on the effect of calcium (33)(58)(10)(56). It was found that for some undetermined reason calcium ions had to be present for histamine release in tissue preparations. Although Uvnäs and Thon did not find this dependence on calcium in their isolated preparations (85), it was felt that additional calcium might enhance the effect of albumin.

G. <u>Effect of calcium, 37°C incubation and serum on the histamine releasing ability of albumin.</u>

In the next set of experiments, all three factors were combined; additional calcium, 37°C incubation and serum. The amount of calcium was doubled. Measurements of the amounts of histamine in the treated and untreated cells are shown in Table 13.

Again, compound 48/80 released a significant amount of histamine, ($P < .05$). The cells treated with albumin were not significantly different from the controls ($P < .10 > .05$), but did liberate 35.1% of their total histamine. However, this relatively high release of histamine is heavily weighted by two low values.

Apparently histamine release was not enhanced by serum, increased temperature of incubation or additional calcium. In all the experiments, compound 48/80 released a significant amount of histamine; the percentages of total histamine released by the cells were 66.7%, 87.8% and 78.4%.

Table 1.

Histamine retention by isolated mast cells suspended in saline at zero time

Exp. No.	µg histamine cells	super.*	% of total cells	super.
7	0.85	6.94	10.9	89.1
12	1.84	9.32	16.5	83.5
16	1.26	4.14	23.3	76.7
21	1.62	3.24	33.3	66.7
Mean	1.39	5.91		
Mean % of total	19.0	81.0		

* supernatant

Table 2. Percentage recovery of mast cells in sucrose layer and histamine retention by these cells at zero time.

Exp. No.	ug histamine cells	ug histamine super.*	% total histamine cells	% total histamine super.	total mast cells x 10^3 layered on sucrose	total mast cells x 10^3 recovered	% recovery in mast cell layer
29	39.97	37.44	49.7	50.3	800	240	30.0
30	39.00	25.92	60.1	39.9	800	264	33.0
31	39.52	25.92	60.4	39.6	790	149	18.9
32	8.32	23.62	26.0	74.0	652	114	17.5
33	18.20	19.15	48.7	51.3	1040	435	41.8
Mean	28.40	26.05			816	240	
Mean % of total	52.2	47.8			Mean % recovery		29.4

* supernatant

Table 3

Percentage recovery of mast cells with undiluted and diluted sucrose gradients.

Undiluted gradients. 25°C

Exp. No.	Amount layered on x 10^3.	Mast cells recovered in mast cell layer x 10^3	% recovery of mast cells.	WBC in mast cell layer x 10^3.
60	400	114	28.5	10
61	824	532	64.6	675
62	1090	122	11.2	71
65	858	48	5.6	90
66	320	63	19.7	12
67	998	296	29.6	52
69	630	95	15.1	65

Diluted gradients. 10°C.

Exp. No.	Amount layered on x 10^3.	Mast cells recovered in mast cell layer x 10^3	% recovery of mast cells.	WBC in mast cell layer x 10^3.
63	802	365	45.5	162
64	776	232	29.9	80
68	314	137	43.6	259
70	902	426	47.2	0
72	410	181	44.1	102
73	400	129	32.2	87
74	456	133	29.2	100
75	1110	188	16.9	42

Undiluted gradients: Total layered on: 5120 x 10^3
mean: 731 x 10^3

Total mast cells recovered: 1270 x 10^3
mean: 181 x 10^3

Mean % recovery: 24.8

Mean WBC contamination: 50 x 10^3
(Exp. 61 not inc.)

Diluted gradients: Total layered on: 5170 x 10^3
mean : 646 x 10^3

Total mast cells recovered: 1791 x 10^3
mean: 224 x 10^3

Mean % recovery: 34.7

Mean WBC contamination: 104 x 10^3

Table 4

Histamine retention by mast cells isolated with the use of albumin solution.

μg histamine

	0 time		1 hour		2 hours	
Exp. No.	cells	super.*	cells	super.	cells	super.
112	17.10	0	15.66	2.75	16.38	0.90
113	15.84	5.40	18.90	6.12	16.60	4.00
114	2.07		3.42		4.68	
115	6.12		6.12		7.02	
116	10.08		11.34		8.82	
119	8.28		6.12			
120	9.72		9.00		5.76	
123	4.86		7.02		4.32	
126	11.70		11.16		9.54	
127	21.06		18.54		18.00	
Mean	10.68		10.73		10.12	
Mean % of total			100		94.8	

* supernatant

Table 5

Effect of compound 48/80 (0.5 µg/ml) on histamine release by isolated mast cells.

µg histamine per cell x 10^{-6}

Exp. No.	1 hr	1 hr + 48/80	2 hrs	2 hrs + 48/80
114	285	285	390	330
115	360	445	413	349
116	244	252	190	201
Mean	296	327	331	293
Mean % of total		100		88.5

Table 6

Effect of compound 48/80 (2 µg/ml) on histamine release by isolated mast cells.

µg histamine per cell x 10^{-6}

Exp. No.	1 hr	1 hr + 48/80	2 hrs	2 hrs + 48/80
119	141	41.4	248	-
120	295	177.0	189	70.8
123	234	45.0	144	54.0
126	318	61.5	272	51.3
127	279	64.0	271	86.6
Mean	253	77.9	225	65.7
Mean % of total		30.8		29.2

Table 7

Test of sensitivity of rats to hen's egg albumin.

Rat No.	Date of sensitization	Date of challenge	Interval	Signs of sensitization
1	March 14	March 23	10 days	Death and hyperemia of small intestine
2	"	"	"	"
3	"	"	"	"
4	April 7	April 20	14 days	"
5	"	"	"	"

Table 8

Effect of antigen on histamine release from mast cells isolated from non-sensitized rats.

μg histamine per cell x 10^{-6}

Exp. No.	2 hrs	2 hrs + albumin 1:500	2 hrs + albumin 1:1000
137	171	141	111
		137	
139	208	84	272
		272	240
Mean	200	159	208
Mean % of total		79.5	100

Table 9

Effect of antigen on histamine release from mast cells isolated from sensitized rats

μg histamine per cell x 10^{-6}

Exp. No.	2 hrs	2 hrs + albumin 1:500	2 hrs + albumin 1:1000
137	175	112 77.8	97.3
139	273	224 213	261 209
140	107	110 117	110 114
Mean	185	142	158
Mean % of total		76.8	85.4

Table 10

Effect of increased concentration of antigen on histamine release from mast cells isolated from sensitized rats

μg histamine per cell x 10^{-6}

Exp. No.	2 hrs	2 hrs + albumin 1:100
142	366	305
		238
		281
		256
143	283	180
		171
		137
		188
Mean	324	220
Mean % of total		67.9

Table 11

Effect of the addition of serum to wash and incubation medium on histamine release by antigen from mast cells isolated from sensitized rats by antigen.

ug histamine per cell x 10^{-6}

Exp. No.	2 hrs ≠ mechanical agitation	2 hrs	2 hrs ≠ albumin 1:25	2 hrs ≠ 48/80 0.2 mg/ml
155	171	208	205 115	52.7
156a	162	166	49.9 77.0	31.2
156b	201	232	205 108	55.4
158	110	103	123 123 76.7	
159a	252	279	184 164	76.5
159b	240	214	111 196	102
Mean	189	200	134	63
Mean % of total			70.9	33.3

Table 12

Effect of serum and 37°C incubation on histamine release by antigen from mast cells isolated from sensitized rats.

ug histamine per cell x 10^{-6}

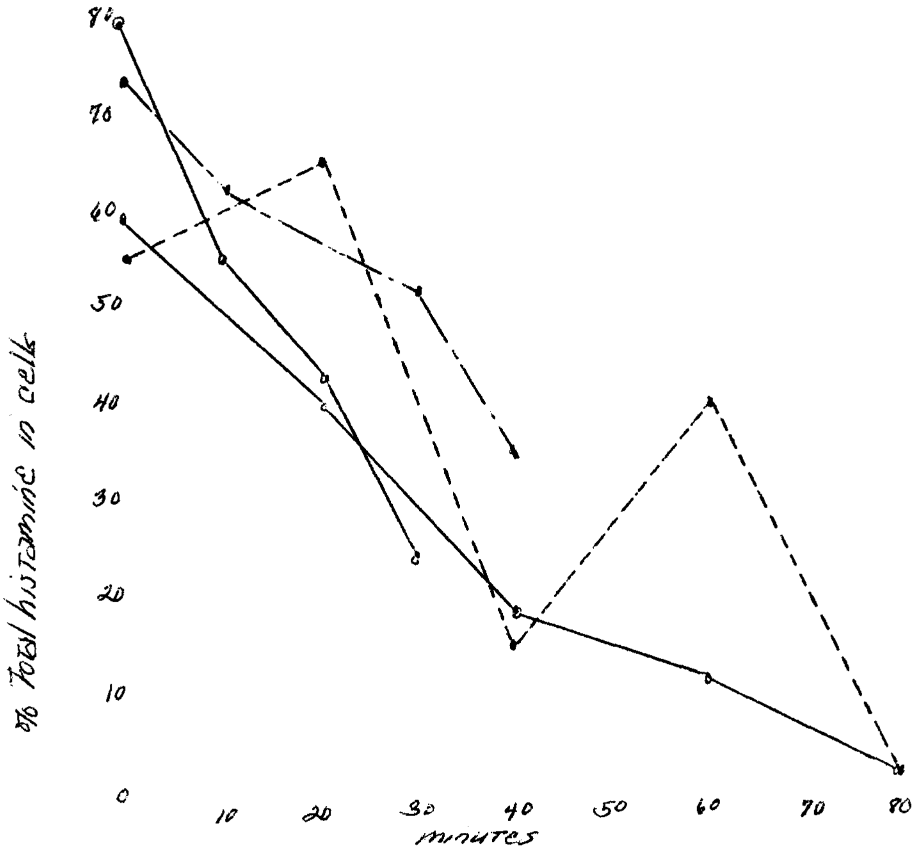
Exp. No.	2 hrs + mechanical agitation	2 hrs	2 hrs + albumin 1:25	2 hrs + 48/80 0.2 mg/ml
160a	215	225	132 125	16.1
160b	346	360	403 365	66.3
161	191	219	146	11.2
162	186	-	112 153	20.7
Mean	234	268	205	28.6
Mean % of total			87.6	12.2

Table 13

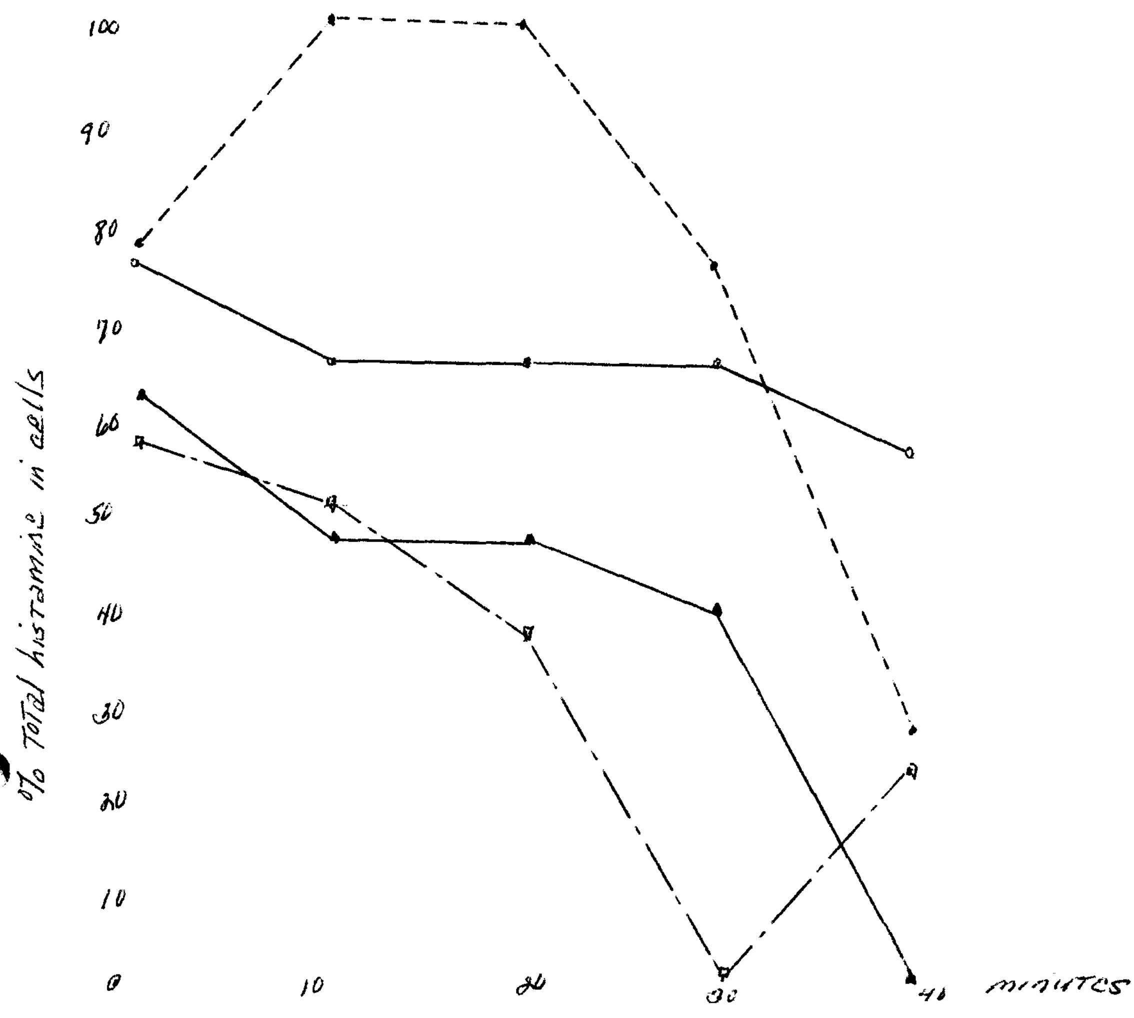
Effect of serum, 37°C incubation and additional calcium in wash and incubation medium on histamine release by antigen from mast cells isolated from sensitized rats.

Exp. No.	2 hrs ≠ mechanical agitation	2 hrs	2 hrs ≠ albumin 1:25	2 hrs ≠ 48/80 0.2 mg/ml
164	100	171	128 110	14.0
165a	229	326	134 212	57.2
165b	185	220	87.6 74.6	38.9
166	171	156	70.5 70.5	-
Mean	171	218	111	36.7
Mean % of total			64.9	21.6

Graph I. Histamine retention by mast cells suspended in Tullis's solution pH 7.4



Graph II. Histamine retention by mast cells suspended in Tullis's solution pH 6.0



Discussion

I. Isolation of mast cells.

Padawer and Gordon were the first investigators to adopt the process of gradient differential centrifugation for the isolation of mast cells (63). In theory, this process seems ideally suited for the separation of mast cells from other cellular elements of peritoneal fluid. It is dependent upon the sedimentation rate which is determined by the mass and surface area. Mast cells, having the most rapid sedimentation rate of peritoneal cells, can be separated in a distinct layer along the gradient. In practice the difficulty arose in selecting a proper gradient for the separation. Although a sucrose system was satisfactory for obtaining a pure suspension of mast cells, they were damaged in the process.

In this work it was found that washed isolated mast cells contained only 23.5% of the total histamine. It was first considered that the sudden change in osmolarity from the sucrose solution to the wash solution was responsible for the marked depletion of cellular histamine. The high molarity of the sucrose gradients was also considered to be a factor. When determinations were made of cellular histamine in mast cells taken directly from the mast cell layer without washing, it was found the cells contained 51.8% of the total histamine. This indicated that although washing was responsible for an additional 28% loss, the isolation procedure was sufficiently traumatic to the cells to effect a 50% loss.

In an attempt to increase recovery of cells by lowering the density, gradients were diluted 1:5. Mean % recovery was 24.8% with undiluted gradients and increased to only 34.7% with the diluted gradients. The cells, isolated with diluted gradients, retained only 22.7% of the total histamine at the end of the 40 minute incubation period. Lowering the pH of the incubation medium for these cells from 7.4 to 6.0 did not enhance histamine retention.

From these observations it was concluded that gradient differential centrifugation could be applied to the isolation of mast cells, but a less hypertonic medium was needed. Other investigators have reported difficulty in obtaining physiologically intact cells with the use of sucrose gradients (43)(85). Garcia-Arocha found that isolated cells resuspended in isotonic sucrose lost 80% of both histamine and 5-hydroxytryptamine but when examined microscopically, were found to have remained intact (24). Although some workers have continued the use of sucrose gradients for their studies, it is doubtful that any conclusions regarding the mechanism of histamine release would be valid considering the present findings and the reports of others. A more successful method was developed by Lagunoff and Benditt in which isolated mast cells were obtained through a process of differential centrifugation in an albumin solution (43). In the present study, all of the histamine was retained in the cells throughout a 2 hour incubation period.

II. Effect of compound 48/80 on isolated mast cells.

The data obtained from *in vivo* and *in vitro* tissue studies of the histamine releasing mechanism of compound 48/80 must be interpreted with the consideration that mast cells might require the presence of other tissue components for 48/80 to be effective. The investigation of Smith indicated that histamine may be released from mast cells without cellular disruption; the mast cell being a merocrine cell. If cellular destruction occurred in normal histamine release, histamine release would be impaired for a prolonged period of time (1 - 2 months). This is not true. When mast cells are degranulated and disrupted by 48/80, the effect may be interpreted as more than the normal response (77). Direct measurement of histamine release from intact mast cells is therefore a more appropriate method of studying the mechanism of histamine release by 48/80 than tissue preparations in which degranulation of mast cells and/or histamine release is determined.

In this study, compound 48/80 (2 µg/ml) released a significant amount of histamine from isolated mast cells. The amount liberated is in agreement with the report by Uvnäs and Thon at a similar concentration (2.5 µg/ml). Their isolated mast cells released 63% of the total histamine in one experiment and approximately 85% in another (85). In the investigations conducted by Archer, 55% of the total histamine was released by compound 48/80 (10 µg/ml) (3). In the present study, 70% of the histamine was liberated from the mast cells after one hour and 71% after 2 hours.

This indicated that isolated mast cells are able to release a high percentage of the total histamine. Uvnäs and Thon found that mast cells isolated with their ficoll gradient system required 2% serum in the wash and incubation medium for 48/80 to be effective (85). This was not true in this study. Compound 48/80 released a statistically significant amount of histamine when the concentration of liberator was increased from 0.5 µg/ml to 2 µg/ml. Tissue factors were not required for chemical histamine release. The necessity of having serum in the wash and incubation medium in the experiments of Uvnäs and Thon may be explained by their system of cell isolation. Ficoll, a substance with polysaccharide-like properties, was used as a separation medium and may have had a deleterious effect on the mast cells. In a later investigation, it was found that serum could be replaced by albumin (86). In the present study serum albumin was used as the isolation medium. It is possible that the presence of albumin in the isolation procedure may have had a sufficient protective effect to eliminate the necessity of adding either serum or albumin.

Mast cells isolated in concentrated albumin were reported by Archer to be refractory to releaser if more than 45 minutes elapsed from the time of collection until the time liberator was added (3). This effect was not found in these experiments. Approximately 1 hour passed before 48/80 was added to the mast cells, yet the cells responded by liberating 70% of their total histamine.

The pH of the incubation media for optimal release of histamine was within the range reported by other investigators. The temperature during the incubation period, 25°C, was lower than that selected for a majority of the other studies. Histamine release did not seem to be inhibited at this lower temperature. Seventy per cent release compares favorably with the release achieved in similar studies at 37°C. Perhaps histamine liberation would have been enhanced by raising the incubation temperature.

The maximum release was within the first hour, no further loss was found at 2 hours. Most workers report that 48/80 achieves its maximum effect within minutes (86)(56)(10)(50)(44). It was not surprising to find that all of the histamine was not liberated from the cells after two hours incubation considering the other reports. It would have been interesting to have tried several concentrations of 48/80 and thus obtain a dose response curve which would show if sufficiently large concentrations of 48/80 would release all of the histamine.

In the present study, then, isolated mast cells which retain histamine for an extended period of time, were able to release histamine when treated with a relatively low concentration of 48/80. There was no need for any additional factor in the wash and incubation medium nor was there any refractory period in which the cells were not responsive to 48/80. The amount of histamine released reached a maximum during the 1 hour incubation period. The conditions in which the cells release histamine by this chemical liberator indicate that no tissue factors are necessary to mediate histamine release. The conditions are in agreement with those found in the postulated enzymatic histamine release, although this study was not extensive enough to reach any conclusions regarding this theory.

III. Effect of antigen on isolated mast cells from sensitized rats.

The location of the antigen-antibody reaction which takes place in anaphylaxis, has long been of interest to investigators. Mast cell damage, occurring when specific antigen comes in contact with sensitized tissues, suggested that the site of the reaction may be the mast cells. Humphrey and Mota felt that antibody must be on or near the surface of the cells participating in the reaction (37). However, the presence of other tissue factors cannot be neglected in any theory resulting from these observations. Only mast cells, isolated from these factors, could provide a means of determining the location of the reaction. Considering that compound 48/80 proved to be an effective histamine liberator in isolated cells, it seemed that specific antigen might be equally effective.

Antigen dilutions of 1:500 and 1:1000 liberated 90% and 85% of the histamine present in rat mesentery from sensitized rats (31). These same dilutions were tested on isolated mast cells and proved ineffective. When the concentration was increased to 1:100, the concentration at which Uvnäs and Thon reported 36% release from isolated cells, a significant amount of histamine was released when compared with the controls. However, there was only a 32% release which is close to the limits of error in the method of histamine determination, and there are no control experiments in which cells from non-sensitized rats were exposed to 1:00 concentrations of antigen.

Further attempts to provide the proper environment for histamine release were not successful. Cells which were incubated with 2% horse serum added to the Tyrode solution released only 29% of the total histamine while these same cells responded with a significant release of histamine when exposed to 48/80. Incubation of cells at 37°C again resulted only in a 12% loss of histamine with the addition of antigen. In other reports, histamine release improved with increasing temperature (86). When additional calcium was added to the incubation media, in combination with the two other factors, a statistically significant amount of histamine was released but this is heavily weighted by two low values.

Various factors must be considered in an interpretation of the failure of antigen to release histamine from isolated mast cells. The first would be the physiological condition of the mast cells when the antigen was added. Compound 48/80 added at the same time as the antigen, in each instance released histamine in an amount averaging 77.6%. It would seem that the cells were capable of responding to a histamine liberator.

The second factor would be the incubation media. Tyrode solution was selected by a majority of investigators for tissue preparations. Judging from the response to antigen in tissues, Tyrode solution doesn't seem to have an inhibitory effect. The modified Tyrode solution did not contain glucose, which may have been necessary if release by antigen is an energy-requiring process (15). The pH, temperature and additional calcium all were similar to conditions in which histamine was liberated from tissue mast cells by antigen.

Concentration of antigen may also be a critical factor. Garcia-Arocha found that very slight alteration in concentration reduced the effectiveness of the antigen. Antigen dilutions of 1:10, 1:50, 1:100, 1:1000 and 1:10,000 did not cause histamine release, while a 1:20 dilution proved effective. Even such slight alterations in concentration as 1:25 or 1:15 reduced histamine release (25). If these observations are correct, then failure of antigen to release histamine from mast cells in the present study may be caused by the antigen dilution. Although a wide range of concentrations was tried (1:25 to 1:1000) it may be the effective dilution was not used.

It would seem, then, that the mast cells were physiologically active and were incubated in a favorable environment (pH, temperature, ionic concentration etc.). Under these same conditions tissue mast cells released histamine. It might be concluded that, 1) the mast cells were altered in the isolation process in such a way that only the process of anaphylactic release was damaged, 2) the environmental conditions for isolated cells are not the same as those for tissue cells, 3) mast cells are not the site for the entire antigen-antibody process.

There are very few reports in the literature of antigen induced histamine release from isolated mast cells. One of these is by Uvnäs and Thon in which mast cells isolated with Ficoll gradients released 36% of their total histamine (85). Assuming that with their method a 36% loss of histamine is significant, it must be noted that serum was a necessary factor in the Tyrode solution used as the wash and incubation media. Although the mast cell could be the site of the antigen-antibody reaction, some component of serum must be present for the initiation or completion of the process.

In the studies of Garcia-Arocha mast cells were not isolated from the other peritoneal cells (25). Again the mast cell could be the site of the antigen-antibody reaction and yet be dependent upon a factor released from the other cells present in the suspension.

In the investigations of Mota and daSilva, where isolated mast cells from sensitized rats were exposed to antigen, no histamine measurements were made. The cells, studied by phase contrast microscopy, lost their regular contour, the granules became more apparent and large empty spaces appeared among them, when exposed to antigen (59). No mention is made of the concentration of antigen and although it was reported that controls were run there is no report of the appearance of these cells.

There have been no demonstrations of antigen induced histamine release from isolated mast cells in which no additional factors were present. Until this has been demonstrated, it cannot be concluded that the mast cells are the site for the entire antigen-antibody reaction and subsequent histamine release. Whether the mast cell alone is the site of the antigen-antibody reaction and whether the complex thus formed releases histamine via an enzymatic process is still to be determined.

Summary

1. Mast cells were isolated from the cellular elements of rat peritoneal fluid through a process of differential centrifugation in albumin. The cells so isolated retained histamine through a 2 hour incubation period. Previous attempts to isolate the cells by gradient differential centrifugation in sucrose were unsuccessful. The mast cells spontaneously lost a high percentage of their total histamine, probably because of the hypertonicity of the sucrose solutions. Modifications of the procedure, designed to improve both recovery and histamine retention, were also unsuccessful. It was only with the albumin method that mast cells, suitable for studies of histamine releasing agents, were obtained.

2. Isolated mast cells, incubated in glucose-free Tyrode solution, released 70% of the total histamine after one hour following exposure to compound 48/80 (2 μg/ml). The cells were not dependent upon any tissue factor for histamine release. The conditions, under which histamine release was obtained, were similar to those reported by the proponents of an enzymatic mechanism in histamine release.

3. Mast cells, isolated from rats sensitized to hen's egg albumin, failed to release significant amounts of histamine when treated with specific antigen. Alterations in antigen dilution, as well as modifications in the incubation media (additional calcium ions, 2% horse serum, increased incubation temperature), did not change the response to antigen. Whether the mast cells need certain tissue factors to complete the antigen-antibody

induced histamine release, or whether the incubation conditions, which were the same as those for 48/80, need to be modified for release by antigen, remains to be determined.

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BOSTON UNIVERSITY

GRADUATE SCHOOL

Abstract of a Thesis

RELEASE OF HISTAMINE FROM ISOLATED MAST CELLS BY COMPOUND 48/80 AND SPECIFIC ANTIGEN

by

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Histamine liberation has long been associated with mast cells both in anaphylaxis and chemically induced histamine release. Although the work on intact animals and in vitro preparations did much to clarify conditions under which histamine is released, these preparations were not ideal. A proper experimental design would be one in which the mast cells were isolated from other tissue influences. With this in mind, the present investigations were carried out. Mast cells were isolated from the peritoneal fluid of the rat and divided into aliquots. In this preparation of morphologically and physiologically intact mast cells, the effects of specific antigen and compound 48/80 were studied on isolated cells in an attempt to learn more about the mechanism of histamine release from the mast cell.

The peritoneal cavity of the rat was washed with heparinized saline. The wash solution containing the cells was removed and layered over the sucrose density gradients. Following centrifugation, the appropriate layer of cells was drawn off, the cells washed and divided into aliquots. With this method the recovery of mast cells was poor and the cells retained only a small percentage of their total histamine. The hypertonicity of the sucrose solution was probably responsible for the loss of histamine. Modification of the method, which included alterations in the density and pH, did not significantly improve either recovery of cells or histamine retention.

At this time a new method was adopted in which an albumin solution was substituted for the sucrose gradients. The albumin solution and other slight modifications proved very beneficial; the mast cells retained histamine over a 2 hour incubation period.

After obtaining a satisfactory method for the isolation of intact cells, the effects of chemical and biological agents were tested on these cells. The 2 substances tested were compound 48/80 and specific antigen (hen's egg albumin). Compound 48/80, a condensation product of p-methoxyphenylethylmethylamine with formaldehyde, was used because of its known effectiveness as a histamine releasing agent in a variety of species. It was prepared in concentrations of 0.5 µg/ml and 2 µg/ml.

Compound 48/80, at a concentration of 0.5 µg/ml, did not release histamine from the isolated mast cells. The ineffectiveness of 48/80 might be attributed to a variety of factors, but concentration seemed the most likely. At 2 µg/ml, compound 48/80 released a significant amount of histamine from the treated cells as compared to the controls; 70% of the total histamine was liberated after 1 hour.

Isolated mast cells, which retain histamine for an extended period of time, were able to release histamine when treated with a relatively low concentration of 48/80. There was no need for any additional factor in the wash and incubation medium as reported by other workers. The amount of histamine released reached a maximum during a 1 hour incubation period. The conditions under which this chemical liberator releases histamine from cells indicate that no tissue factors are necessary to mediate histamine release.

The location of the antigen-antibody reaction which takes place in anaphylaxis has long been of interest to investigators. Mast cell damage, occurring when specific antigen comes in contact with sensitized tissues, suggested that one site of the reaction may be the mast cells. However, the presence of other tissue factors cannot be neglected. Only mast cells, isolated from these factors, could provide a means of determining the location of the reaction.

Sensitivity in the rat to hen's egg albumin was induced by subcutaneous injection of 1.0 ml egg albumin and 1.0 to 1.5 ml H. pertussis vaccine given no less than 12 days before use. Challenge with antigen proved this to be an effective sensitization procedure.

Mast cells were isolated from the sensitized rats in the usual manner. Antigen was prepared in concentrations of 1.000, 1:500, 1:100 and 1:25. Albumin in concentrations of 1:1000 and 1:500 was ineffective in producing a significant loss of histamine from mast cells isolated from non-sensitized rats and was also equally ineffective in mast cells isolated from sensitized rats.

When the concentration of antigen was increased to 1:100 there was a significant difference between the amounts of histamine in the control cells and the treated cells. However, there was only a 32% release which is close to the limits of error in the method of histamine determination.

Further attempts to provide a proper environment for histamine release was not successful. Cells which were incubated with 2% horse serum added to the Tyrode's solution released only 29% of the total histamine while these same cells responded with a significant release of histamine when exposed to compound 48/80. Incubation of cells at 37°C again resulted only in a 12% loss of histamine with the addition of antigen. When additional calcium was added to the incubation media, in combination with the two other factors, a statistically significant amount of histamine was released but this was heavily weighted by 2 low values.

Various factors must be considered in an interpretation of the failure of antigen to release histamine from isolated mast cells. It would seem that the physiological condition of the cells was good because 48/80 released histamine. The incubation media was that selected by most workers. The pH, temperature and concentrations of calcium all were similar to conditions in which histamine has been demonstrated to be liberated by antigen added to mast cells in the presence of other cells or tissues. Although a wide range of antigen concentrations was tried, the effective dilution might not have been used.

It appears that the mast cells were physiologically active and were incubated in a favorable environment (pH, temperature, ionic concentration, etc.). Under these same conditions tissue mast cells released histamine when treated with 48/80 and antigen.

It might be concluded that, 1) the mast cells were altered in the isolation process in such a way that only the process of anaphylactic release was damaged, 2) the environmental conditions for liberation of histamine from isolated mast cells are not the same as those for tissue mast cells, 3) mast cells are not the site for the entire antigen-antibody process.

There have been no demonstrations of antigen induced histamine release from isolated mast cells in which additional factors were not present. Until this has been demonstrated, it cannot be concluded that the mast cells are the site for the entire antigen-antibody reaction and subsequent histamine release. Whether the mast cell alone is the site of the antigen-antibody reaction and whether the complex thus formed releases histamine via an enzymatic process is still to be determined.