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Studies on the Mode of Binding of Histamine in the Tissues

The effect of tonicity on the rate and amount of histamine released from several *in vitro* preparations was studied. In hypertonic (1.2M) solutions of sucrose or mannitol, basic histamine liberators released significantly less histamine from dog liver particles, isolated mast cells, perfused guinea pig lungs, and perfused cat paws, than they did in isotonic solutions. When surface-active compounds were used as histamine liberators, no significant differences were found in the amount of histamine released in the two kinds of solution. The anaphylactic reaction and anaphylatoxin were examined for their ability to release histamine from guinea pig lungs perfused with isotonic and hypertonic Tyrode. The results were similar to those observed when basic liberators were used. The findings are discussed in the light of their application to the mode of histamine binding, and to the mechanism of its release.

The ability of soluble antigen-antibody complexes to release histamine, both *in vivo* and *in vitro* is reported and discussed.

Studies on the Mode of Binding of Histamine in Tissues

by

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To my wife, Betty, I

dedicate this thesis

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Literature Review

A. Sites of Histamine in the Body

1. Mast Cells

In 1953 Riley and West (1953a;b) published a series of papers in which they showed a convincing correlation between the relative number of mast cells and the histamine content of various tissues. Since that time workers in the field of histamine research have been consciously concerned with mast cells, their granules or tissues that are rich in mast cells. Because of the physiological implications of their findings, plus the fact that many of the observations made in this investigation were made using isolated mast cells and their granules, this thesis will begin with a fairly detailed summary of the literature about these cells. Mast cells have been much studied over the past sixty years and have been the object of several reviews, first by Michels (1937) and more recently by Riley (1954), Padawer (1957), and Fulton et al. (1957).

a. Morphology

The first problem to confront the writer is to define a mast cell. Ehrlich (1879) first described them as cells of the connective tissue, densely packed with granules which stain metachromatically with toluidine blue and certain other basic dyes. He believed them to be over-nourished cells and so conferred on them the name "Mastzellen". The cells were actually discovered by several other workers some years earlier, but it remained for Ehrlich to describe their meta-

chromatic staining properties. Some years later he described similar cells in the blood (loc. cit. Riley 1954) but he noted that they had different origins from the tissue mast cells. Asboe-Hansen (1954) has recently defined mast cells as "mesenchymal cells which contain cytoplasmic granules and a substance with mucopolysaccharide characteristics". Higginbotham and Dougherty (1956) have shown that cells staining metachromatically can be evoked by the injection of various polysaccharides and their complexes with basic substances. This process, which they refer to as "micellophagosis", leads to the formation of cells they have termed as quasi mast cells. It is inferred that this process operates physiologically in the generation of tissue mast cells. In later reports (Higginbotham & Dougherty 1955; Higginbotham, Dougherty & Jee 1956) these workers showed that fibroblasts ingest native granules from disrupted mast cells and ultimately become indistinguishable from the mast cells. Dougherty and Schneebeli (1958), using microcinematographic techniques, were able to show this process of granule ingestion by fibroblasts in living isolated loose connective tissues of mice.

Riley, on the other hand, observed that young mast cells do not stain metachromatically (Riley 1953b). However, some workers might argue that these cells are precursors and not true mast cells. Perhaps the best defination for mast cells would be; cells of the connective tissue heavily packed with cytoplasmic granules which contain physiologically active substances.

Mast cells are quite variable in size and shape. Bloom (1942) observed mast cells in dog mast cell tumors (mastocytoma). The cells varied from round to oval, polygonal, spindleshaped and rodshaped. According to Bloom, Friberg, and Larrson (1956) their size averages from 7 to 13 μ. These figures agree with those of earlier workers (Michels 1937). The cell membrane is a thin structure of about 50 to 60 Å. Bloom and his co-workers were not able, in this study, to detect a double structured membrane, although the same authors had earlier reported that mast cells of dog mastocytomas possess a typical double membrane (Bloom, Friberg & Larrson 1956). The electron microscope reveals a great number of finger-like protrusions from the cell membrane varying from 0.3 to 0.7 μ in length and 0.06 to 0.08 μ in thickness (Bloom, Friberg & Larrson 1956). The significance of these protrusions is as yet unknown, but they are certainly not unique to mast cells. Similar processes have been observed in the cells of thyroid (Braunsteiner, Fellinger & Pakesch 1953), gall bladder (Dalton, Kahler & Lloyd 1951), in the epithelium of the respiratory passages (Bloom & Engström 1953), in cells of the inner ear (Engström & Wersall 1953), cells of Henle's loop (Dalton, Kahler & Lloyd 1951) as well as other cells.

The nucleus is usually round to oval and 4 to 6 μ in diameter (Fulton et al. 1957). Quite often it can not be seen because of the large number of heavily staining granules. These granules have also been observed with the electron microscope. Asboe-Hansen (1954) reported them to be globular and consistently 0.3 μ, while Bloom (Bloom, Friberg & Larrson 1956) found them

to vary between o.6 and 0.7 μ in dog mast cells. Nakajima (1928) did not measure the size of the granules but he did observe that their size varied in different species. Zollinger (1950), using the light microscope, considered them to be giant mitochondria, but later work with the electron microscope (Rogers 1956) revealed mitochondria which were distinct from the granules.

b. Origin

The ontogeny of tissue mast cells has not been definitely established. They are first recognized in late embryonic life but their origin is uncertain (Hjelmann 1954). It is generally stated that mast cells occur in tissues in close apposition to small blood vessels. This is interpreted by some to be due to the fact that mast cells are derived from precursors located in the adventitia of blood vessels and that they gradually move away into avascular areas in the connective tissues (Riley 1953b; Fawcett 1955). Others believe that they develop from fibroblasts (Montagna & Malaragno 1953).

Mast cells apparently multiply by cellular division, since both mitotic and amitotic forms have been seen in dog mastocytomas (Bloom 1952) and tissue cultures of human skin (Zitcer et al. 1953; Zitcer & Kirk 1954). While Fawcett (1955) did not see any forms of cellular division in rat mesentary, he did note that following recovery from the action of 48/80 mast cells are found in closely approximated pairs. From these findings he suggests that cell division rather than cell differentiation is the usual mechanism for maintainence

of normal numbers of mast cells in adult rats. The same type of distribution has been reported to occur in hamster cheek pouch following X-irradiation (Fulton et al. 1957). Nakajima (1928) had earlier reported seeing mitosis in the mast cells of various laboratory animals. Arguments against cellular division were presented by Cramer and Simpson (1944) who were unable to find any mitotic forms in a series of skin carcinomas they induced, and by Padawer who found no decrease in mast cell numbers in rats treated with colchicine (Padawer & Gordon 1955) and other antimitotic agents (Padawer 1957).

c. Cytological Properties

Montagna and Nobeck (1948) made an extensive study of the cytology of mast cells in man, rat, hamster, dog and mouse. They found alkaline phosphate to be associated with the granules,while acid phosphate was present in both the granules and nucleus. Other enzymes found in the granules were cytochrome oxidase, lipase, and a pseudo peroxidase. Investigating the mast cells of man, rhesus monkey and rat, Wislocki and Dempsey (1946) could not detect the presence of glycogen in the cells; a finding which confirms the work of Nakajima (1928). In addition, they observed that ribonuclease did not affect the granules. Zollinger (1950) reaffirmed the fact that the granules were not disrupted by ribonuclease, and he also showed that basophilia is not abolished by desoxyribonuclease either. In fixed tissues hyaluronidase does not alter the metachromasia (Wislocki & Dempsey 1946), but the local injection of hyaluronidase in biopsy of human skin is said to cause dissolution

of the granules (Asboe-Hansen 1954). Compton (1952), however, could find no evidence for this in the hamster. Nakajima (1928) reported that mast cell granules are highly susceptible to the action of both pepsin and trypsin.

d. Distribution

Mast cells occur in all regions of the body. Their abundance is roughly related to the amount of connective tissue present (Nakajima 1928). They have been found in testis, ovary, salivary glands, lymph nodes, liver, spleen, pancreas, heart, prostate, bladder, lung, and omentum of various animals including man (Michels 1937); as well as skin, thymus, uterus, mama, and tongue (Staemmler 1921). There is a total absence of mast cells in scar tissue, cartilage, and bone, and few or none are present in uterine mucosa and placenta. Nakajima (1928), however, reported a fair abundance of mast cells in bone marrow. Recently mast cells were found in the "gastric section" of stomachs of albino rats (Campbell, Conroy & Sgouris 1952), presumably as a result of diapedesis.

In general, parenchymatous organs are poor in mast cells, exclusive of the connective tissue of the capsule and trabeculae (Riley & West 1953b). The exception to this rule is the wide spread distribution of mast cells throughout the parenchyma of dog liver (Nakajima 1928).

e. Mast Cells and Heparin

In a histological investigation of mast cells, Holmgren and Wilander (1937) found that toluidine blue developed the same color with mast cell granules as it does with heparin.

They further noted that the dye precipitated out heparin from dilute solutions and removed it from heparinized blood. The extreme affinity of toluidine blue for heparin, plus the specificity of the staining reaction, suggested to these workers that mast cells contained heparin. Jorpes and his associates (Jorpes, Holmgren & Wilander 1937) analyzed the heparin and mast cell content of large blood vessels. They found that the amount of heparin obtained was in proportion to the number of mast cells. Wilander (1938) continued the study to show that this correlation exists in other tissues. Oliver, Bloom and Mangiera (1947) reported the presence of an extremely high concentration of heparin in a dog mastocytoma. Cass and her associates (Cass et al. 1954) have also shown that mast cell tumors are very rich in heparin. A subcutaneous mastocytoma in a dog contained 30 times more heparin than corresponding normal tissue.

Heparin is released during anaphylaxis in dogs but its release depends on the presence of the liver (Jaques & Waters 1941), Extensive damage to mast cells of the liver was found after the shock.

While it is unanimously agreed that heparin is contained in tissue mast cells, its location within the cells is still a matter of controversy. Köksal (1953b)has isolated mast cell granules from mouse subcutaneous tissue and found them to contain a heparin like substance. Riley (1956) believes that mast cell granules are not mere polymerizations of heparin, but rather intracellular osmometers from which heparin can be released. Heparin is present in the large granule fraction of

dog liver homogenates and can be released by the same physical and chemical procedures that release histamine (Grossberg, Garcia-Arocha & McIlreath 1956). It is associated with the granules of rat peritoneal mast cells, but in this instance can only be released by the most severe chemical treatment (Garcia-Arocha 1958). Friberg (Friberg, Graf & Åberg 1951) has also expressed the opinion that heparin is contained within the mast cell granules.

Julén and her associates (Julén, Snellman & Sylvén 1950) have fractionated cells from ox liver capsule and state dogmatically that heparin is in the intergranular cytoplasm. The metachromasia of the granules, they say, is due to aggregates of metachromatic material attached to the granule wall, from which it can easily be washed off. Correspondingly, Hedbom and Snellman (1955) found much less heparin in purified mast cell granules than in the fresh unfractionated tissue. The present author is somewhat skeptical of their results because according to their scheme, they sedeminted the granules by centrifuging at 850 g., a force which will not separate even larger particles.

f. Mast Cells and Histamine

Riley reported that stilbamidine, a known histamine liberator, given in lethal doses to rats produced fluorescent spots along the blood vessels of loose connective tissue, an area where mast cells may be found (Riley 1953b). He and West (1953b) found that the histamine content of such tissues was reduced following disruption of mast cells by stilbamidine. A comparative study of mast cell abundance and histamine content of various tissues (Riley and West 1953 a,b) showed a very strong

correlation between these two. The correlation was strengthened further by investigations made on mastocytomas in dogs which showed histamine to be present in very high concentrations (Cass, et al. 1954; Bloom, Friberg & Larrson 1956; Larrson 1957). More recently high concentrations of histamine have been found in mast cell tumors in mice (Furth, Hagen & Hirsch 1957). The mouse tumor is transplantable and contains concentrations of histamine as high as 4.2 mg./gm. of tissue. Dunn and Potter (1957) have also reported on a transplantable mastocytoma in the mouse, but in this case, no intensely granulated cells were even found in the transplants of the tumor.

Meanwhile Graham and her co-workers, investigating the distribution of histamine in blood elements of humans, found fifty times more histamine in the basophils, weight for weight, than in any other type of leucocyte (Graham et al. 1952). This observation led them to investigate what correlation might exist between histamine and tissue basophils. Mast cells were counted in dog skin and the histamine content of the various layers was determined. It was found that both the histamine concentration and the mast cell count were highest in the outer layer. They calculated that if all the histamine were assigned to mast cells, the average content of a single cell would be 6 uug. (Graham et al. 1953). This value, together with an estimate of the mast cell size, indicates a content of about 1% histamine or a cell concentration of 100 mM/Kg. In a continuation of this study (Graham et al. 1955) they reported a confirmation of Riley and West's correlation of histamine

and mast cells in the dog liver capsule.

While only one report of mast cell tumors in man has been made (Hissard, Moncourier & Jacquet 1950), there is a more common pathological condition known as urticaria pigmentosa which resembles a mastocytoma. One such lesion removed from a child contained 950 µg. of histamine per gm. of tissue (Riley and West 1953a). The tissue was composed almost entirely of mast cells and it was completely devoid of eosinophils. Many cells were pleomorphic, and mitotic forms were occasionally seen.

Provided with such a rich source of histamine, many workers began to use mast cells to study the problems related to the mode of binding and release of this amine. Mota (1953) injected 48/80 and stilbamidine into the peritoneal cavity of rats. Histological examination of the mesentery following the treatment revealed that the mast cells were disrupted and their granules extruded. He also observed the action of 48/80 under the light microscope, in which he described a bubbling of the mast cell surface for a few seconds. Benditt et al. (1954) were able to show that ovomucoid injected intravenously into rats caused a disruption of mast cells in the skin of the dorsum of the foot, and the production of edema. Later, they showed that histamine is released from mast cells of rat peritoneal cavity after freezing and thawing the cells (Benditt et al. 1955).

Fawcett (1955) obtained mast cells from rat peritoneal cavity and found them to be rich in histamine. He also injected water and 48/80 into the cavity and found large quantities of

histamine in the fluid. Histological examination showed disruption of mast cells. When 48/80 was injected subsequent to distilled water treatment, it released no histamine. Norton (1954) also showed that 48/80 disrupts rat peritoneal mast cells, however, she did not make any determinations concerning histamine release.

Padawer (1955) has developed a method for the purification of mast cells obtained by peritoneal lavage in rats. The method has been modified by Glick and his associates (Glick, Bonting & DenBoer 1956) and by Benditt (Benditt et al. 1955). Other workers (Schayer 1958; Garcia-Arocha 1958) have preferred to use the unpurified washings of rat peritoneum. Incubation of such cell suspensions with various histamine liberators results in the discharge of large amounts of histamine (Garcia-Arocha 1958; McIlreath this thesis).

g. Mast Cells and Serotonin

Direct analysis of mast cells obtained from rat peritoneal fluid (Benditt et al. 1955) shows that they contain about 0.7 µg. of serotonin (5-hydroxytryptamine) per cubic millimeter of cells. Parratt and West (1957) have confirmed the association of serotonin with tissue mast cells in rats and mice. They also noted that this amine is not concentrated in mast cells of guinea pig, dog, man, rabbit, cow, hamster and cat. Indeed, even in rat skin where over 50% of the total serotonin of this animal is located, there seem to be places other than mast cells where serotonin is located. More recently Cass, Marshall and Riley (1958) have found serotonin to be present in a rat mastocytoma.

Lagunoff and his associates (1957) reported that a material which acted like serotonin on the rat colon appeared in rat skin which had been incubated with 5-hydroxytryptophan. Similar results were obtained with mast cell suspensions from rat peritoneum. It was concluded from these studies that serotonin is produced in mast cells of rats.

Bhattacharya and Lewis (1956b) found that an intraperitoneal injection of 48/80 in rats disrupted mast cells, causing a liberation of both histamine and serotonin. When 48/80 was injected into the perfused hindquarters of rats both amines appeared in the perfusate, but 48/80 released only histamine from perfused tissues of rabbits, cats, and dogs(Bhattacharya & Lewis 1956a). Serotonin was also released from perfused rat intestine by 48/80 or, if the animal had been sensitized, by the specific antigen (Garcia-Arocha 1958).

Mast cell tumors of mice contain large amounts of serotonin and histamine, while those of dogs are rich only in histamine (Sjoerdsma, Waalkes & Weissbach 1957). The same authors showed that skin from humans with urticaria pigmentosa contains much histamine but very little serotonin. Carcinoid tumors, on the other hand, are well known to contain large quantities of serotonin but only small amounts of histamine (Page 1958).

It is surprising that although serotonin is a known constituent of mast cells in the rat, its subcutaneous injection in this species causes mast cell disintegration (Rowley & Benditt 1956). Asboe-Hansen (1956) reported that its intraperitoneal injection in hamsters resulted in damage and degranulation to mast cells, and Feldberg and Smith (1953) found

serotonin released histamine from perfused skin flaps of cats and dogs.

Mota (1957) has shown that mast cells in the rat are disrupted during the anaphylactic reaction. Coupled with this is the finding of Garcia-Arocha (1958) that the addition of the specific antigen to perfused intestine of sensitized rats releases serotonin. This evidence may explain the paradox reported by Kellaway (1930), that while rat uterus relaxes upon the addition of histamine, it contracts in a Schultz-Dale reaction: for serotonin powerfully stimulates the smooth muscle of this tissue. Recently Fink (1956) has attributed the uterine contraction of a Schultz-Dale reaction in mouse to the action of released serotonin.

It appears from the foregoing review that serotonin is specifically associated with mast cells only in the rat and the mouse among the common laboratory animals. Serotonin is indeed released in rabbits during anaphylaxis (Waalkes et al. 1957) but quite likely it comes from platelets (Humphrey & Jaques 1955) and not from mast cells.

h. Theories Concerning the Function of Mast Cells

Theories concerning the function of the metachromatic granules in the mast cells are almost as numerous as the cells themselves. Michels (1937) in his monumental review of mast cells enumerated 25 theories concerning their function, and 5 more with regard to the cause of the metachromasia. However, it would be of little value to list them here. Since that time workers have been more cautious. Jorpes (1946) has referred to these cells as heparinocytes, and believes they function in

the production and secretion of heparin. Asboe-Hansen prefers to think that mast cells are concerned with the production of hyaluronic acid. He postulates that under hormonal influence mast cells secrete hyaluronic acid, perhaps by way of a heparin precursor (Asboe-Hansen 1954). In spite of the convincing evidence for the presence of histamine in the mast cells, Riley (1954) believes the riddle of the mast cells is still unsolved. There can be no doubt that mast cells can discharge their potent constituents in response to a variety of pathological or pharmacological stimuli; but evidence is sadly lacking that such a discharge occurs in response to any physiological stimulus.

2. Histamine in Cells Distinct From Mast Cells

a. Blood

In their original publication, Barsoum and Gaddum (1935) found six times as much histamine in the blood cells than in the plasma of the rabbit. Code (1937) made a detailed study of histamine in blood. He noted that when precautions were taken against trauma, clotting and deterioration of blood, much lower quantities of histamine were found in the plasma. This is especially true in rabbit blood, however, even when extreme care is taken some histamine is still present in the plasma of dogs, cats, rats, guinea pigs, and rabbits (Emmelin 1945). The histamine content of red cells is also very low. Code (1937) extracted histamine from pure red cells and found only very small amounts present. In addition, patients with erythremia, (a disease in which red cells are present in excessive numbers) have normal quantities of histamine in their blood (Valentine, Pearce & Dawrence 1950).

Platelets from the blood of most animals contain very little histamine (Code 1952). The exception is the rabbit. Code (1937) obtained deposits of rabbit platelets and found them to contain histamine, but he was reluctant to draw any conclusions from this observation. Later, Zon, Ceder and Crigler (1942) performed a series of experiments using anti-platelet sera. They found a definite correlation between the concentration of histamine and the number of platelets. Other workers, (Graña & Rocha e Silva 1945; Rocha e Silva, Graña & Porta 1945; Humphrey & Jaques 1955) have obtained evidence that most of the blood histamine of rabbits is located in platelets.

In other species, which all have low blood histamine levels compared to rabbits, seventy to one hundred percent of the extractable blood histamine is contained in the white cells (Code 1937). This observation has been confirmed many times (Minard 1941; Rose & Weil 1939). The non-granular series of leucocytes, however, contain insignificant amounts of histamine. Examination of pleural exudates containing 70-80% neutrophils, showed these cells to contain little histamine (Code 1937).

Graham and her associates (Graham et al. 1952) have made a study of the distribution of histamine in blood elements. They have found that basophils of normal humans contain 50 times more histamine than any other type cell. Moreover, in patients with myelogenous leukemia these cells contain 90% of the total blood histamine. It should be remembered that there is a definite histological resemblence between basophils

and tissue mast cells, indeed some workers have even referred to them as circulating mast cells.

The case for eosinophils has been valiantly defended by Code (1956), but the evidence stated above against their being the major site of histamine storage in the blood is, I feel, too strong.

b. Other Cells

There can be little doubt that histamine is not exclusively contained in mast cells, as has been shown in the above section. Even if one were to overlook histamine in blood, there are still tissues in the body - the stomach mucosa for example - which are rich in histamine but contain few mast cells. As yet no one has been able to associate histamine with any cellular component other than mast cells. Riley and West (1956) have suggested the goblet cells of the stomach mucosa as a possibility but only on the basis of their metachromasia. This by itself can not be considered as a serious argument, since cartilage which also stains metachromatically contains no histamine.

B. The Release of Histamine by Various Means

Histamine release both in vivo and in vitro has been studied extensively for many years. Analysis of the procedures used to bring about the discharge of histamine from its cellular sites can yield fruitful information concerning not only its mode of binding but also the mechanism by which it is released. The means available to liberate histamine are extremely variable, ranging from agents which cause general

cellular destruction to highly specific compounds which appear to act only on cells (or cell particles) which contain histamine and heparin. Since the studies outlined in this thesis consist in the use of several different means of releasing histamine it seems worthwhile to review the various methods of releasing histamine.

1. The Anaphylactic Reaction

The literature prior to 1940 regarding experimental anaphylaxis has been thoroughly reviewed by Dragstedt (1941) and Feldberg (1941). I shall therefore limit myself, for the most part, to a review of the more recent work done in this field. However, to refresh the reader it would first seem appropriate to cite some of the work which ultimately led to the final proof that histamine is released during anaphylaxis.

The first detailed analysis of anaphylaxis was made by Portier and Richet (1902). Later their observations were extended by Biedl and Kraus (1909) and Arthus (1909, 1910a,b). In their investigation of the symptomology of anaphylactic shock, the latter authors emphasized the fact that the sudden drop in blood pressure was not of cardiac origin. Dale and Laidlaw (1910) expressed "as a point of interest and possible significance" the resemblance between the pharmacological actions of histamine and those seen in anaphylaxis. However, at that time it had not been established that histamine was present in normal tissues, and it did not seem likely that sufficient amounts could be formed to account for the immediate and profound symptoms in anaphylaxis.

Manwaring and his associates (1925) reported that a smooth muscle stimulating substance was released during anaphylactic shock in dogs. Their conclusion was based on the fact that the blood from shocked animals could cause contractions of the urinary bladder and intestine when injected into a normal dog.

After Best and his associates (1927) had firmly established that histamine is a normal constituent of normal animal tissues, Watanabe (1931) analyzed the liver from shocked and non-shooked dogs for their histamine content. He found approximately four times as much histamine in three non-shocked dog livers than in four shocked dog livers. However, in the light of later findings (Feldberg & Kellaway 1937), that there are extreme variations in the amount of histamine normally present in any organ, these results seem to hold little significance.

Further evidence supporting the view that histamine is released during anaphylaxis was provided by Dragstedt and Gebauer-Feulnegg (1932). They observed in thoracic duct lymph of shocked dogs, the sudden appearance of a smooth muscle stimulant which possessed many of the properties of histamine. Four years later a histamine-like substance, whose activity could be abolished by histaminase, was detected in the blood of shocked dogs (Dragstedt & Mead 1936). Code (1939) found increased amounts of histamine in the blood from shocked guinea pigs. Still later Ojers, Holmes and Dragstedt (1941) demonstrated that histamine is discharged from dog liver during anaphylaxis. Before they injected the antigen, a piece of liver

was removed and its histamine extracted. A second piece was removed twenty minutes after the injection. A significant decrease was found in the liver histamine following the injection of the antigen; in one experiment the histamine content dropped from 60 to 6 µg/gm. of liver.

Many reports of histamine release from sensitized tissue in vitro can be found in the literature. Bartosch, Feldberg and Nagel (1932) detected increased amounts of histamine in the effluent of perfused guinea pig lungs following the addition of the specific antigen. Schild (1936a) confirmed these findings. He was also able to show that more histamine appeared in the perfusate than could be accounted for by the conversion of histidine present in the antigen he used (egg white). Other perfused organs from which antigen has been shown to release histamine are dog liver (Scroggie & Jaques 1949); dog skin (Feldberg & Schachter 1952); rabbit skin, liver and intestine (Schachter 1953) and rat intestine (Garcia-Arocha 1958).

Ungar and Parrot (1936) showed that it was sufficient merely to place a piece of sensitized guinea pig lung in contact with the antigen in vitro to bring about the discharge of histamine. Schild (1939) developed this simple diffusion technique to detect histamine liberation from isolated tissue. With it he was able to show quantitatively that histamine was set free from a variety of tissues by the action of the antigen. He observed too that no correlation existed between the histamine content of a tissue and the amount released by the

antigen. Emmelin, Kahlson and Lindström (1941) used similar techniques to demonstrate histamine liberation from sensitized dog tissue.

Histamine can also be discharged from blood cells of sensitized rabbits into the plasma when the specific antigen is incubated with whole rabbit blood. Katz (1940) was the first to show this type of in-vitro anaphylaxis. His results were confirmed and extended by Dragstedt (Dragstedt et al. 1940); Rose and Browne (1941); and Carryer and Code (1950).

More recently Humphrey and Jaques (1955) showed that histamine and serotonin are released from platelets of sensitized rabbits in the presence of plasma and the specific antigen. They emphasized the importance of plasma in the reaction, for even heating the plasma to 56°C. for 30 minutes or removing the calcium ions from it abolishes the reaction.

Rose (Rose & Weil 1939; Rose 1941) reported a reduction in the concentration of histamine in the whole blood of intact rabbits during anaphylaxis. These findings can be explained on the basis of diffusion into the tissues of histamine released from the platelets or by the simultaneous trapping of platelet thrombi in various organs (Fidler & Waters 1946).

A major contribution to the study of histamine release in anaphylaxis has been made by Mongar and Schild. In a recent series of publications they have made a detailed study of in vitro anaphylaxis in guinea pig tissues. They found that histamine can be readily released from finely minced lung tissue from sensitized guinea pigs when it is incubated with the specific antigen (Mongar & Schild 1954, 1955a). The

amount of release is roughly dependent on the tissue fragment size; the smaller the fragments the less the release. If the tissue is homogenized and the intracellular particles incubated with the antigen no histamine release occurs. Grossberg (1954) had earlier made the same observation using dog liver particles.

In a continuation of the study (Mongar & Schild 1955b; 1957) they investigated the effects of chemical inhibitors of anaphylaxis, as well as oxygen lack, in an attempt to determine the various steps involved in the anaphylactic reaction. The substances tested, which included metabolic inhibitors and antipyretics all inhibited histamine liberation even though desensitization occured with some of the agents. Histamine release induced by organic bases was potentiated by these compounds, indicating to Mongar and Schild that the mechanism of histamine release in anaphylaxis differs from that caused by the histamine liberators.

Anaphylactic shock has been experimentally investigated in a wide variety of animals and histamine release has been demonstrated in most of the animals. Of those used by early workers, only rats seemed to be resistant to anaphylaxis (Longcope 1922).. These conclusions were most probably the result of poor sensitization of animals, because later work (Parker & Parker 1924) revealed that anaphylactic shock could be produced in these animals. In recent years anaphylactic shock in rats has been observed by several workers (Halpern et al. 1955; Mota 1957; Sanyal & West 1958; Garcia-Arocha 1958). However, the reaction in rats is distinct from that in other species in that serotonin as well as histamine is released

(Sanyal & West 1958; Garcia-Arocha 1958).

2. Anaphylatoxin

The first experiments concerning anaphylatoxin were made by Friedberger (1909). He found that when the precipitate obtained by mixing antigen with antisera was incubated with normal guinea pig serum a toxic principle was formed. Injection of the activated serum into a normal guinea pig led to a shock condition which was almost identical with anaphylactic shock. He named the toxic principle responsible for the shock anaphylatoxin.

Bordet (1913) added to the facts concerning the genesis of anaphylatoxin. He found that it was not necessary to use antigen and antisera. Incubation of normal guinea pig serum with agar or starch was sufficient to activate anaphylatoxin, which he believed to exist in normal serum in the form of a precursor or matrix. Four years later Novy and deKruif (1917) discovered variations in the potency of anaphylatoxins prepared from different species. They observed that anaphylatoxin prepared from rat serum was four to ten times more potent than that produced from guinea pig serum, while those obtained from oxen, horse, dog and man were even less active.

In 1950 Hahn and Oberdorf (1950) showed that antihistaminics prevent the shock produced in guinea pigs by the intravenous injection of anaphylatoxin. This observation prompted Rocha e Silva (Rocha e Silva et al. 1951; Rocha e Silva 1952; Rocha e Silva & Aronson 1952) to look for histamine in the perfusate of organs injected with anaphylatoxin. Large quantities of histamine, as high as 100 µg., were found to be re-

leased from isolated guinea pig lungs perfused with Tyrode following the injection of 4 ml. of rat serum anaphylatoxin. Rocha e Silva and his colleagues noted that heating the serum to 55 - 60°C. for one hour completely destroyed its capacity to be activated. However, after the serum had been activated, it could be heated to 69°C. for one hour without destroying more than half of its activity. The anaphylatoxin is non-dialysable and appears to be a protein since precipitation of the proteins by ammonium sulphate or ethyl alcohol inactivates the serum. Another interesting observation made by Rocha e Silva and his co-workers was that citrated blood could not be activated to any great extent. The effect of the citrate is not due, they said, to the removal of calcium ions since blood decalcified by treatment with a cation exchange resin retains its capabilitity to be activated. Rothschild and Rocha e Silva (1954) made a systematic study of the effect of ionic strength on plasma activation. Their results showed that the inhibitory action of several anions paralleled their position in the lyotropic series. Mota (1957) reported that anaphylatoxin produces no mast cell alteration in rats either following its intravenous injection or its incubation with rat mesentery. However, when anaphylatoxin was incubated with guinea pig mesentery "very conspicuous alterations of mast cells occur".

The exact meaning of the findings obtained in experiments using anaphylatoxin is not clear. Friedberger believed anaphylatoxin was formed during anaphylactic shock, and on this assumption he developed the humoral theory of anaphylaxis. However, later work, particularly that of Schultz (1910),

Dale (1913), Bartosch et al. (1932) and Jaques and Waters (1941), which showed that blood is not necessary for the production of anaphylaxis cast doubt on the Friedberger theory. In defence of it, one might argue that a great deal of plasma protein, including presumably some anaphylatoxin precursor is left in the interstitial fluid of any tissue, however well its blood vessels have been rinsed free of blood by perfusion. One thing is clear, at any rate, and that is that anaphylatoxin, particularly that prepared from rat serum, is a potent histamine liberator.

3. The Soluble Antigen - Antibody Complex

Within the last year several publications have appeared reporting the production of anaphylaxis in non-sensitized animals through the use of soluble antigen-antibody complexes. The first and most extensive of these reports was made by Germuth and Mckinnon (1957). They investigated the effect of antigen-antibody complexes on intact unsensitized guinea pigs. Their results show that when the complex is dissolved in an excess of antigen, its intravenous injection caused symptoms of severe anaphylactic shock to develop. Only rarely did supernate fluid from mixtures of antigen and antibody prepared in excess antibody give rise to any symptoms. These observations demonstrate two very striking facts; 1. the shocking property of antigen-antibody mixtures is due to the presence of soluble antigen-antibody complexes, and 2. anaphylactic shock does not require interaction of the antigen with antibody "fixed" in the tissues.

Tokuda and Weisser (1958) produced anaphylaxis in white mice with soluble antigen-antibody complexes. They prepared the complex in two ways. In one method they incubated rabbit antisera with excess antigen. The amount of antigen used was eight times the amount determined to be present in a precipitate formed at the zone of equivalence. The second method used was to incubate the antisera with enough antigen to obtain the precipitate at the zone of equivalence. The precipitate was washed twice with cold physiological saline and then incubated with excess antigen in saline. The animals reacted in the same way uniformally to the injection of complexes prepared by these two methods. However, the complex prepared by the first method killed a higher percentage of the animals injected.

Although the second type of complex was not as lethal, it represents a more pure solution since it does not contain any of the other plasma proteins. This is particularly important in preparations such as perfused cat skin which sometimes shows histamine release when foreign proteins are injected for the first time (Feldberg & Schachter 1952).

The only other report concerning soluble complexes to which the author is aware was made by Ishizaka and Campbell (1958), who investigated the skin reactive properties of soluble complexes. They found that intradermal injections of mixtures of antisera and excess antigen into previously blued guinea pigs caused a typical local skin reaction to occur. Their results were not too impressive because injecting normal serum or antisera also produced some diffusion of

dye into the skin. To overcome this difficulty they separated out the various protein components of the antisera by starch electrophoresis. A slow γ globulin fraction was found to possess skin reactive properties when mixed with excess antigen. When a mixture of this fraction contained an excess of antibody, no local skin reaction occured.

It is not surprising that normal sera or antisera produced local skin reactions. Bliss and his associates (Bliss & Stewart 1957; Bliss et al. 1957; Bliss et al. 1959; Bliss and Walker 1959) have shown that homologous, non-autologous plasma from dogs and man contain a factor which produces an increase in permeability and the formation of a wheal when injected intradermally. They have conclusively shown that in dogs the local skin reactions produced by these plasmas are due to the release of histamine by the above mentioned factor. Once again these results point to the advantage of using soluble complexes prepared according to the second method of Tokuda and Weisser.

Thus far all the published reports concerning the use of soluble complexes deal only with symptoms which resemble those seen in anaphylaxis. In the experimental section of this thesis it will be shown that soluble complexes prepared in excess antigen are able to release histamine both in vivo and in vitro.

4. Chemical Agents

a. Basic substances

The first evidence that tissue histamine could be released by comparatively small doses of a simple well-defined

chemical compound was provided by Alam and his co-workers (Alam et al. 1939) when they found curare caused histamine to be released from dog skeletal muscle. They also noted that following repeated injection of curare the histamine content of the muscle was reduced. Several years later, Schild and Gregory (1947) reported that strychnine possessed the same powers. In the same year MacIntosh and Paton (1947) showed that many other organic bases were histamine liberators; and a little later (1949) published an extensive and thorough report of histamine release in intact dogs and cats using a series of diamines, diamidines, diguanidines, diisothioureas and monoamidines. Intravenous injection of these compounds resulted in the appearance of increased amounts of histamine in the plasma. In addition their injection closely mimicked the symptoms of anaphylaxis - a sudden but somewhat delayed drop in blood pressure, and in the dog, incoagulability of blood.

Many other compounds have since been added to the original list of basic histamine liberators. Paton (1951) reported that compound 48/80, which is a mixture of low polymers of p-methoxy-N-methylphenylethylamine, caused a typical delayed depressor response when injected intravenously into dogs or cats. Together with Feldberg (Feldberg & Paton 1951) he showed that 48/80 was very efficient in releasing histamine from perfused cat skin. One molecule of 48/80 under favorable circumstances released from 10 to 100 molecules of histamine.

In spite of the fact that its exact formula is still unknown, 48/80 has become one of the most widely used of all

histamine liberators. Its activity has been reported in: cat skin, muscle and lung (Feldberg & Mongar 1954), perfused cat paws (Högberg et al. 1956), rat lungs, hindquarters, intestine and isolated mast cells (Feldberg & Mongar 1954; Bhattacharya & Lewis 1956a; Garcia-Arocha 1958; McIlreath, this thesis) and guinea pig lung, hindquarters, and intracellular particles (Feldberg & Mongar 1954; Mongar & Schild 1956). In addition many authors have observed mast cell disintegration in rat mesentery, both in vivo and in vitro, following contact of the tissue with 48/80 (Mota et al. 1953; Norton 1954; Fawcett 1955; Högberg & Uvnäs 1957).

Not all tissues are susceptible to the action of 48/80. Feldberg and Talesnik (1953) found that repeated intraperitoneal injections of 48/80 in rats greatly reduced the histamine content of the skin, skeletal muscle and heart but had little effect on the histamine content of stomach, liver, and duodenum. Mota and Vugman (1956) reported that 48/80 in large intravenous or repeated intraperitoneal injections in guinea pigs caused no alteration in histamine content of various tissues. They noted that it produced pulmonary emphysema, and antihistaminics did not protect the animal from the lethal effects of the drug. Feldberg and Mongar (1954) had earlier reported that 48/80 released very little histamine from perfused guinea pig lungs, even at high doses.

The only feature common to all these compounds is that they contain one or more nitrogen atoms and they are all basic in nature. However, basicity per se does not confer activity on them, for MacIntosh and Paton (1949) reported that compounds such as ethylamine, hexamethonium and benzamidine are inactive.

As Paton (1957) has noted, repetition of the basic group invariably seems to engender activity. Any compound containing basic groups separated by 5 to 14 atoms has a good chance of being a histamine liberator. However, polybasicity is not essential; L 1935, one of the most powerful liberators known, is a monobasic compound (LeCompte 1955).

It is very difficult to assess the relative potency of these drugs because this varies considerably according to the test object used. Thus if one tests a:ω diamines in vivo, C-10 is the optimum separation of the nitrogen atoms, if minced guinea pig lung is used (Mongar & Schild 1953) the optimum is C-15, and if rabbit blood is used the most active is C-18 (McIntire, Roth & Sproull 1951).

b. Lytic and Surface Active Compounds

Any harsh treatment which would destroy cells will liberate histamine. In fact this is the basis for many extraction procedures (Barsoum & Gaddum 1935; Code 1937). While it is true that the substances which fit into this category also destroy cellular membranes, their action may be considered as being less severe. Snake venoms with hemolytic activity were first shown to release histamine by Feldberg and Kellaway (1937). The substance responsible for this action, they felt, was lysolecithin (Feldberg & Kellaway 1938a). Phospholipase, or lecithinase A as some workers prefer to call it (Fairbairn 1945), is an enzyme present in the venoms of poisonous snakes which converts lecithin into the lytic lysolecithin. The action of lysolecithin is probably due to damage to the lipoprotein mosaic of the cell membrane (Rocha e Silva 1955, pg.

96). Feldberg and Kellaway(1938b) next showed that lysolecithin prepared by the action of cobra venom on crude egg lecithin discharged histamine from suspensions of "liver debris". Recently Högberg and Uvnäs (1957) reported that lecithinase A purified from the venom of several snakes and from bee venom was extremely effective in liberating histamine from rat mesentery in vitro.

Feldberg and Kellaway (1938b) advanced the hypothesis that lysolecithin might be involved in the anaphylactic reaction. This possibility, however, has never been seriously considered by others.

Krantz (Krantz et al. 1948) showed that "Tween 20", a non-nitrogenous dispersing agent, produces a rise in plasma histamine and the anaphylactoid syndrome following its intravenous injection in dogs. It is interesting to note that while it is generally agreed that "Tween 20" acts through its surface action, it seems to be specific to dogs because its intradermal injection in guinea pigs, rats or man produces no wheal.

In 1952 Schachter (1952) established that bile salts, which are also surface-acting substances, release histamine from isolated perfused cat skin. Grossberg (1954) showed that saponin incubated with dog liver particles causes the discharge of histamine. Finally Paton (1957) suggested that octylamine may owe some of its releasing powers to its surface action in view of its tendency to produce edema and release potassium.

5. Physical Procedures

It is not necessary to employ chemicals to bring about histamine release. Any substance which would cause the rup-

ture of a surface membrane will liberate histamine. Trauma is known to bring about histamine release, as well as grinding up of tissues (Trethewie 1938). More subtle means such as perfusing a tissue with water (Schild 1936) or injecting distilled water intraperitoneally (Fawcett 1954) are quite effective in freeing histamine. Grossberg (loc. cit. MacIntosh 1956) noted that histamine release from dog liver particles suspended in hypotonic solutions increases as the molarity of the solution decreases. Other procedures such as freezing and thawing quantitatively release all the histamine from intracellular particles (Hagen 1954; Grossberg & Garcia-Arocha 1954). One may also add boiling to the list of physical procedures (Trethewie 1938).

C. Differences in Histamine Content and Release from Various Tissues and Species

Scarcely any organ in the body does not contain at least some histamine; however, the relative amounts of this amine found in any one tissue varies greatly from one species to another. Indeed even within the same species there is a wide range in the histamine content of a tissue. As Feldberg (1956) pointed out, the concentration of histamine in dog liver varies between 8 and 110 µg/gm. A further aspect of the problem which cannot be overlooked is the fact that there are distinct differences in the percent of histamine that can be released from the tissues.

Schild (1939) investigated histamine release from several tissues of sensitized guinea pigs by the action of the specific antigen. He could find no correlation between the amount of histamine in a tissue and the amount released. Recently he

and Mongar (Mongar & Schild 1952) repeated the experiments, comparing the effects of anaphylactic shock and histamine liberators. There was a similarity in the percent of histamine released by both the antigen and the organic bases but again no correlation was found between the total histamine content and the amount discharged. For example, an average of 43% of the total histamine was released from the diaphragm, which had an average content of 1.5 µg/gm., while only 10% was discharged from lungs with a total average of 40 µg./gm.

Feldberg and Paton (1951) showed that cat skin, which contained an average of 25 µg./gm. could be depleted of over 99% of its histamine with repeated injections of histamine liberators. With cat skeletal muscle they could only release two-thirds at the most.

Other organs of the cat such as the lung are very resistant to the action of histamine liberators (Feldberg & Mongar 1954), although they have a high histamine content - 23 to 57 µg./gm. (Tarras-Wahlberg 1937). The lungs of the monkey are also extremely rich in histamine (Feldberg & Kellaway 1937). In rats the histamine concentration of the lungs is much lower 3.5 to 10.5 µg./gm. (Martin & Valenta 1939; Götzl & Dragstedt 1940), yet they readily give up their histamine under the influence of organic bases.

For more complete data concerning the histamine content of tissues in various species the reader is referred to monographs of Feldberg (1956) and Rocha e Silva (1955). The prime purpose of this section of the review is to emphasize the importance of selecting the proper tissue or organ when perform-

ing experiments concerning the release or binding of histamine.

It is quite obvious that, from the title of this thesis, one important topic has not been covered in the introduction: Theories concerning the mode of histamine binding. This was not an oversite. It was found more convenient to consider this topic separately, in the introduction to the experimental work.

Experimental Work

A. Introduction

An understanding of the manner in which histamine is bound within the cell is of prime importance in our attempt to learn the mechanisms by which it is released. Prior to 1938, it was known that histamine could be extracted from tissues by action of strong acid (Barsoum and Gaddum 1935; Code 1937) or by perfusing tissue with distilled water (Schild 1936b). In that year, Trethewie (1938) showed that much histamine remained bound to cellular debris when dog liver was homogenized. This was the first evidence which suggested that histamine is contained in intracellular particles and not in the cellular sap. Copenhaver and his co-workers (1953) analyzed the distribution of histamine in the various cellular fractions. Their findings showed over half of the histamine was associated with the mitochondrial fraction. Hagen (1954) and Grossberg and Garcia-Arocha (1954) showed that histamine could be released from the large granule fraction of dog liver homogenates by basic histamine liberators or by certain purely physical procedures such as freezing and thawing. In addition, Hagen reported that suspensions of the large granules could be injected intravenously into cats without eliciting any of the pharmacological actions of histamine. These workers agreed that it is likely that histamine is bound within the particles. McIntire (1956) expressed the opinion that histamine is bound within the cell by a weak, probably ionic type, linkage which does not require the action of an enzyme for its release. MacIntosh (1956) suggested heparin in the granules as a possible anion to which histamine could be bound by polar or

Van der Waal forces.

Several other workers, notably Rocha e Silva (1956), are of the opinion that histamine is bound to a cellular protein by a primary chemical bond. This concept gained prominence with the finding that trypsin released histamine from perfused guinea pig lungs (Rocha e Silva 1938). After Hofmann and Bergmann (1941) reported that trypsin has a rather strict specificity toward synthetic substrates, requiring a basic amino acid (arginine or lysine) in the proximity of the peptide linkage ruptured by the enzyme, Rocha e Silva and Andrade (1943) subjected papain to many fractionation procedures and verified that its ability to release histamine from rabbit blood cells parallels its capacity to split benzoyl-L-arginine amide.

There are of course several other theoretical schemes one may draw concerning the mode of histamine binding but the two mentioned above have received widest acception. In an attempt to uncover more information regarding the mode of binding of histamine, I have studied the action of several histamine liberators in systems in which one of the physical properties had been altered, namely tonicity changes in the fluid media. The investigations cover histamine release from many types of preparations ranging from intracellular particles to more integrated physiological systems.

B. Materials and Methods

1. Materials

Appendix i. contains a list of the compounds used during the course of this research and the sources from which they were obtained. As a rule all solvents and ordinary chemicals were of reagent grade in purity. All aqueous solutions were made in glass distilled water.

The glassware used in these studies was cleaned first with detergent and then with chromic acid cleaning solution. Upon removal from the acid each piece was individually rinsed twelve times with tap water and two times with distilled water, and placed in a drying oven. Plastic centrifuge tubes, which were used in the experiments were cleaned with a detergent, rinsed as above and dried on racks in the open air. When any glassware was found to be grease coated, it was washed with a 20% solution of trisodium phosphate followed by the usual cleaning procedure.

2. Histamine Assays

The histamine content of the samples was determined using the bioassay method first described by Guggenheim and Loeffler (1916). This test is based on a comparison of the contractions produced on a guinea pig ileum by the samples and standard histamine. Although several chemical methods for histamine detection have been developed in recent years (Graham et al. 1951; McIntire et al. 1947), this method still proves to be the most sensitive one known.

a. Apparatus

This instrument has been described in detail by Ashwin

(1953) and Grossberg (1954), so it suffices to say that it was an isolated organ bath supplied by C.P. Palmer Ltd., London. The organ chamber itself had a volume of 7 ml. The ileum was suspended on a glass hook through which oxygen was bubbled. The temperature of the bath was maintained at 30 ± 2°C.

b. Buffer

The author has noted that while many workers refer to Tyrode solution, this seems to be only a convenient term since the formulae they use vary considerably. Several solutions were examined but the following one proved to be most satisfactory in providing good sensitivity and little spontaneous activity. Although the solution is called Tyrode, a more appropriate name would be modified Ringer's solution. The formula in gm./L is as follows: NaCl - 9, KCl - 0.42, $CaCl_2$ - 0.24, $MgCl_2$ - 0.005, $NaHCO_3$ - 0.5, and glucose - 1.0. The chlorides of calcium, potassium and magnesium were made up as stock solutions (20%, 20% and 0.5% respectively). Other chemicals were weighed out at the time of preparation. To prevent the precipitation of calcium ions in the presence of a concentrated bicarbonate solution (upon adding solid $NaHCO_3$, the concentration would be quite high in the immediate area where the substance was added), the bicarbonate was dissolved in a separate container and added to the other salts later. 0.5 ml. of a 10^{-4} solution of atropine sulphate was added to each liter of Tyrode. This was done to eliminate contractions of the ileum produced by acetylcholine or choline. Presumably these substances could be present in the samples or released spontaneously from nerve endings in the wall of the ileum.

c. Ileum

Guinea pigs of either sex weighing 300 to 500 gm. were killed by cranial fracture followed by decapitation. A section of the terminal ileum roughly 15 cm. long was removed and the ileum washed with Tyrode. From this, a strip about 2.5 cm. long was used for the assay. One end was attached to the glass hook by a ligature and a thread tied to the other end was passed around the writing lever. Although no study was made concerning the reactivity of the ileum, it seemed to the author that pieces having a light color and a dimpled appearance proved most suitable. Other workers (Minard 1941) have reported that sections of guinea pig ileum may be kept refrigerated for seven days and still be used for assay. The author, however, was only rarely able to use a piece of ileum that had been stored for 24 hours.

d. Assay

The ileum was sensitized by adding a large (2 - 3 µg.) dose of histamine to the bath. After a period of 15 to 20 minutes, doses ranging between 18 and 30 nanograms (ng.) were repeated until the sensitivity was steady. At this time the assay of the samples began. The author attempted to perform the four point assay discussed by Schild (1942), but because of the large number of samples to be assayed and the limited time available, this was not always possible. However, any dose of the unknown was repeated at least twice before it was bracketed between doses of the standard, to make sure the response was constant.

e. Neo-Antergan Test

Since the responses of the guinea pig ileum are not specific to histamine, it was necessary to use a method whereby it could be determined whether histamine was actually the drug responsible for the effects observed. One such technique is the use of specific antagonists; the method employed in these experiments.

pA_2 is defined as the negative logarithm to the base 10 of the molar concentration of an antagonist drug which will reduce the effects of a multiple dose (x) of an active drug to that of a single dose (Schild 1947). Atropine, for example, has a pA_2 of 8.8 against acetylcholine after 14 minutes contact with the ileum. Neo-Antergan, on the other hand, has a pA_2 of 4.9 against acetylcholine but a pA_2 of 9.3 against histamine. This means that neo-antergan at a concentration of $10^{-9.3}$ will reduce the response of 2H to that of H. Thus one can see that neo-antergan is quite specific in antagonizing histamine, and therefore was the drug used in these experiments.

The most reliable procedure to follow in identifying an unknown drug through the use of an antagonist is to see if the antagonist will reduce the response of the known and the unknown to the same extent. Further confidence can be added to the test if the known and unknown drugs recover from the effects of the antagonist at the same rate. Fig. 1 shows the results of a typical neo-antergan test. 0.2 ml. of neo-antergan at a concentration of 10^{-7} was added to the bath and allowed to remain in contact with the ileum for two minutes, then doses of both known and unknown samples were given. The process was repeated until the effect of the standard was completely abolished. The

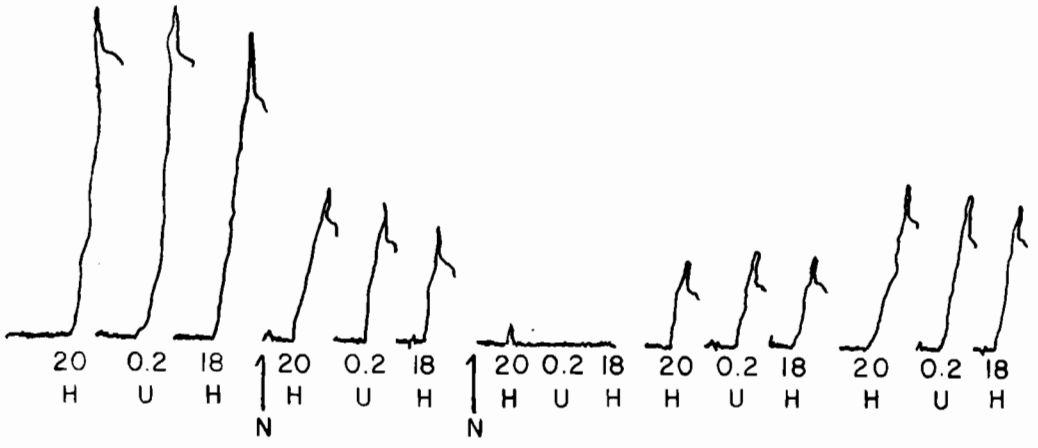


Fig. 1. This is a reproduction of a neo-antergan test done in one of the experiments described in this thesis. H is the histamine standard expressed as nanograms. U is the sample expressed as ml. N refers to the points where neo-antergan (20 mg.) was given.

muscle was washed thoroughly and after several minutes the same doses of the known and unknown were again given until the response of the standard had returned to near its original effect.

3. Statistical Analysis of the Experimental Findings

The experiments were designed in such a way that the results could be paired with regard to a single variable. Such a method of experimentation allows the results to be presented in a clear and concise manner, as well as simplifying statistical treatment of the findings.

The t test of Student (Fisher 1932) was the significance test used in these studies. This test as applied to paired observations is used to determine whether the mean difference is significantly different from 0. It is expressed by the formula:

$$t = \frac{\bar{d}}{\sqrt{\frac{(d - \bar{d})^2}{n(n - 1)}}}$$

The results were not considered to be significantly different unless t was greater than that given in the t Table at the confidence level P = 0.05.

C. The Effect of Tonicity Changes on Histamine Release from Dog Liver Particles

1. Preparation of the Dog Liver Particles

Trethewie (1938) made the original observation that "cell fragments", obtained by grinding liver with sand in Tyrode, contained bound histamine which can be freed by boiling. More recently Hagen (1954) and Grossberg & Garcia-Arocha (1954)

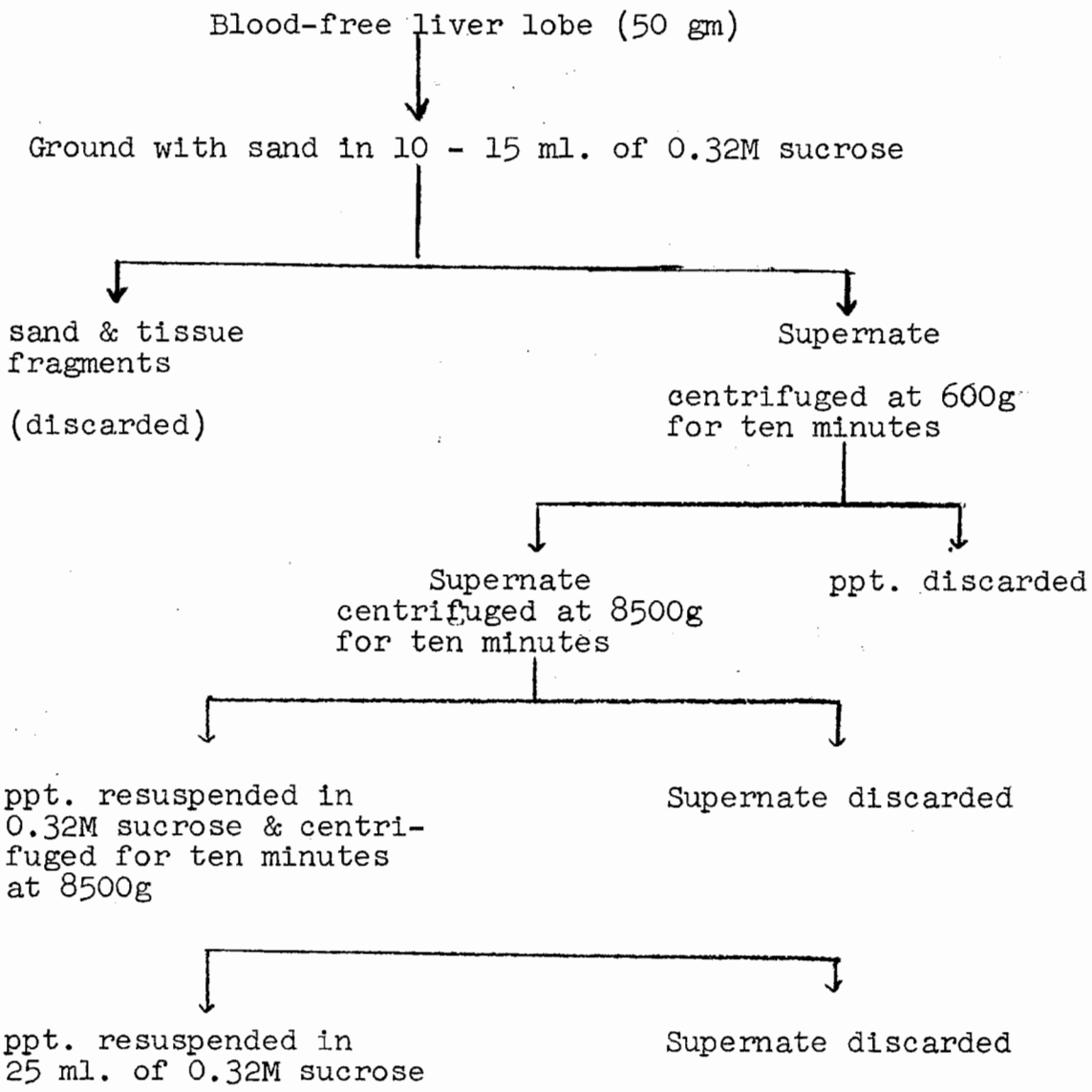
have confirmed this finding. Hagen showed that a suspension of these particles could be injected intravenously in the dog without producing the circulatory effects seen after the injection of histamine.

With such a system, the research worker has a preparation which is high in histamine content, stable and one from which he can produce uniform samples. In addition, he might easily devise experiments which could be well controlled with regard to parameters such as pH and temperature.

The procedure employed is outlined in Table 1. Mongrel dogs of either sex were used. Although no preference to physical size was made, usually only small dogs (i.e. under 11 Kg.) were available. The animals were anesthetized with sodium pentobarbitol at a dose of 0.54 mg./Kg. A mid-line incision was made from the sternum to the pubis, and the liver exposed. A loop of twine was passed over several lobes and tightened securely at the hilus. The bile duct was clamped with a hemostatic clamp and the lobes were removed and placed in ice-cold saline (0.9%). The animal was then killed by an intravenous injection of a saturated solution of $MgSO_4$. The gall bladder was dissected away from the lobes, which were then perfused with ice-cold saline until the perfusate appeared blood free. Three or four pieces of liver, approximately 50 gm. each, were minced and ground with 10 to 15 ml. of ice-cold isotonic sucrose (0.32M). Vigorous grinding for several periods of about 10 seconds each was usually sufficient to produce a fairly homogeneous mixture. The degree of grinding determined to some extent the amount of histamine present in the final suspension, and only through

TABLE 1

Preparation of Dog Liver Particle Suspension



experience was it possible to determine when the tissue had been gound sufficiently. Twenty to thirty ml. of cold sucrose was added and the mixture stirred gently. The sand was allowed to settle to the bottom and the fluid portion was decanted through several layers of gauze (three "Lisco" pads opened to 6" x 6"). This procedure was effective in removing all the intact cells and sand. Nuclei and cellular debris were removed by centrifuging at 600 g to ten minutes*. The large granule fraction was separated out by centrifuging at 8,500 g for ten minutes. The granules were washed twice with isotonic sucrose. A final suspension was made by adding a volume of isotonic sucrose to give a concentration of 2 gm./ml. with regard to the original tissue. The resuspension of the final material was facilitated by passing it through a gauze pad.

The suspension was either used at once or stored in a methanol-water cold bath at -2°C. The histamine content of the final suspension ranged between 3 and 10 µg./ml. The preparation was quite stable, the decrease in sedimentable histamine varied from 1 to 8% per day. The histamine lost from the particles could be accounted for by an increase in the free histamine and so could be considered as spontaneous release. It was noticed that when suspensions over one week old were incubated, the histamine was discharged more quickly than when fresh preparations were used. For this reason no suspensions over four days old were used in any of the experiments and in the majority of cases fresh suspension was used for each experiment.

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*All centrifugation in these experiments was performed in a Servall Refrigerated Centrifuge, Model No. RC 54651B, with an angle head SS-1. Fig. 2 is a calibrated curve for this centrifuge.

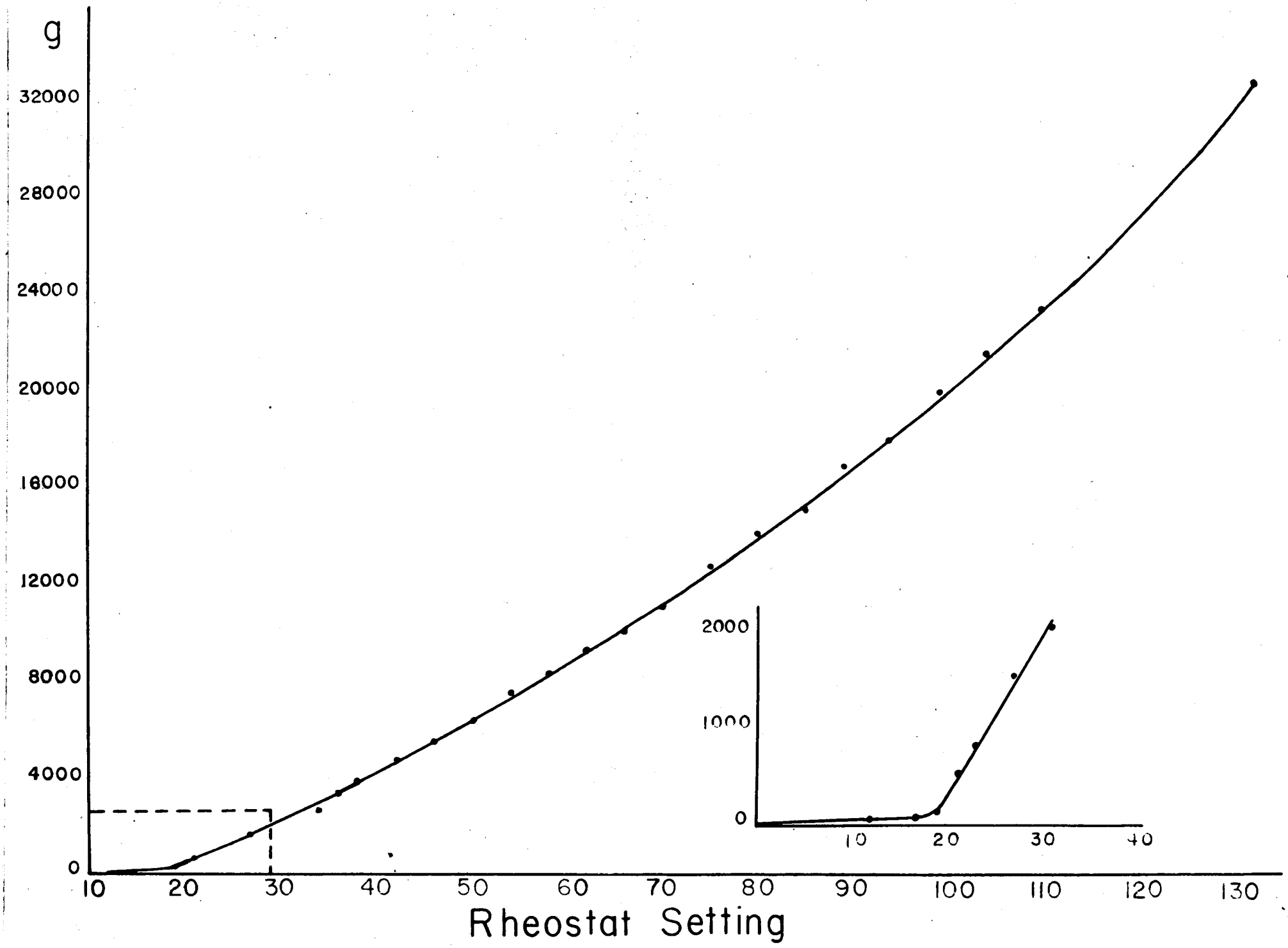

Fig. 2. This is a calibrated curve for the Servall Refrigerated Centrifuge used in these experiments. g, the gravitational force was calculated from the speed and size of the centrifuge head.

2. Methods

One ml. of suspension was added to four ml. of sucrose solution, buffered at pH 8 with "tris" buffer (tris hydroxymethylaminoethane) 0.07M and containing appropriate amounts of the liberator to be tested. The mixture was incubated for periods of 15 and 30 minutes. Control samples containing buffer but no liberator were treated in the same manner. In the first experiments incubations were carried out in a water bath, the particulate matter being kept well suspended by frequent shaking of the samples. In later experiments a Dubnoff Metabolic Shaking Incubator was used. Following incubation the samples were immediately centrifuged at 30,000 g for 5 minutes. The decanted samples were placed in a boiling water bath for two minutes. The purpose of routinely boiling the samples was to destroy to a reasonable extent any histaminase activity which would act in the samples between the incubation and the time of assay. The samples were then stored at -2°C.until they were assayed.

The total content of histamine in the suspension before incubation was determined by assaying a sample (1 ml. of suspension to four ml. of buffer) which had been heated to boiling in a water bath for two minutes. Of this total a small percentage was always found in the liquid phase. The quantity of this non-sedimentable or free histamine was determined by adding 1 ml. of suspension to 4 ml. of buffer and immediately centrifuging the mixture at 30,000 g for five minutes, the supernate was placed in the boiling water bath for two minutes and saved for assay. The amount of histamine in the sample supernates after incubation, minus the free histamine, represents the histamine released and is expressed as a percentage of the total bound histamine.

3. Studies Using Basic Histamine Liberators

a. DA_{10} (1,10 decamethylene diamine)

In the preliminary experiments concerning the effect of tonicity on the release of histamine from dog liver particles DA_{10} was used. This compound was first shown to be a histamine liberator by MacIntosh and Paton (1949), and later by Mongar and Schild (1953) and Grossberg (1954). A solution of this drug was made by adding the DA_{10} to water and then adding HCl dropwise until the compound was dissolved. This procedure results in the formation of the soluble dihydrochloride form of the drug. The pH of the solution can then be raised to 4.5 without precipitation of the material. With weaker solutions (10^{-4}) the pH can be raised even higher.

Samples of the liver particles were incubated with DA_{10} at a final concentration of 200 µg./ml., in solutions of 0.32M, 0.84M and 1.2M sucrose. No difference was found between the percent of histamine released in the isotonic (0.32M) and the 0.84M solutions, but a large difference was found between the histamine released in isotonic and 1.2M sucrose solutions. The experiment was then repeated several times to determine if this difference was real and significant. The results of ten experiments using DA_{10} as a histamine liberator (Fig. 3 and Table II) revealed an average discharge of 39 ±-9% in isotonic solution and 18 ± 5% in the 1.2M solution. These values are for 15 minutes of incubation; the difference is significant at the level P = 0.01. Spontaneous release of histamine from the heavy granule fraction did not significantly vary in the two solutions. This suggests the spontaneous release and DA_{10}

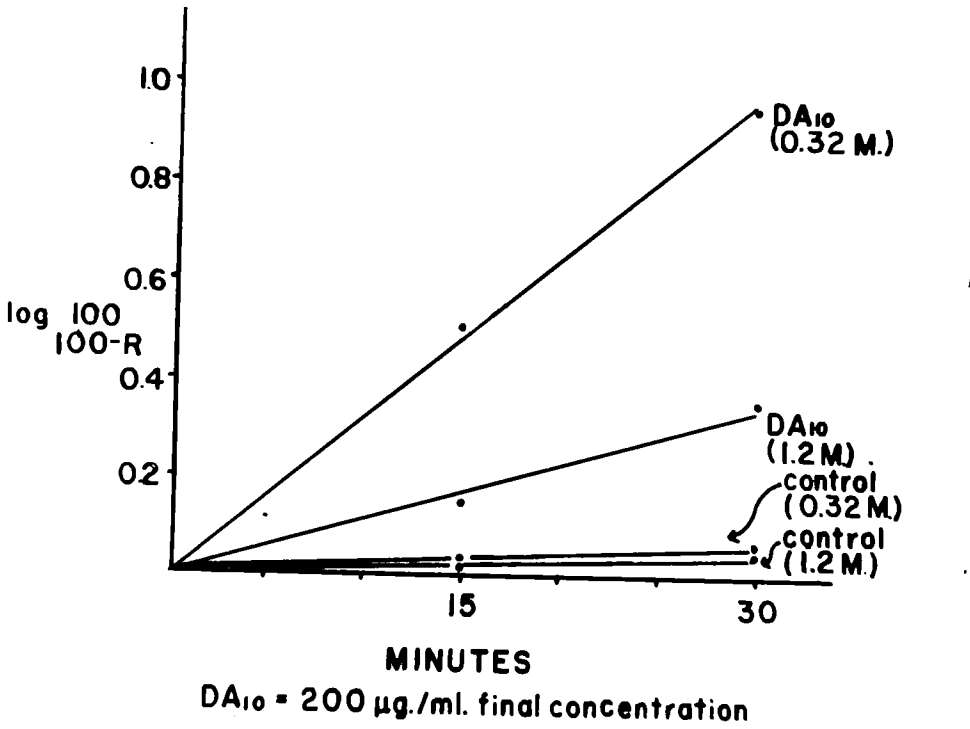

Fig. 3. Release of histamine from dog liver particles by DA_{10} in isotonic and hypertonic sucrose solutions showing the linear relationship with time. The particles were incubated at 37°C. Concentrations of the liberators are given below the graph. Control curves represent the spontaneous histamine release under these conditions.

TABLE II

Release of Histamine From Dog Liver Particle Suspensions by Various Histamine Liberators in 0.32M and 1.2M Sucrose Solutions

Liberator	No. Expts.	Conc. (µg/ml)	Incubation time (min.)	% Hist. Released (mean ± SM) 0.32M	1.2M	P
Control	10	-	15	7 ± 3	8 ± 2	>.05
			30	15 ± 5	13 ± 3	>.05
DA_{10}	10	200	15	39 ± 9	18 ± 5	<.01
			30	67 ± 9	29 ± 7	<.01
48/80	3	60	15	40 ± 13	22 ± 4	<.01
			30	66 ± 20	39 ± 9	<.05
stilbamidine	4	200	15	27 ± 3	16 ± 2	<.01
			30	60 ± 7	32 ± 2	<.01
Toluidine blue	5	10	15	23 ± 5	10 ± 2	<.01
			30	54 ± 4	23 ± 5	<.01
Saponin	5	400	15	49 ± 13	50 ± 13	>.05
			30	86 ± 11	82 ± 13	>.05
Sodium Taurocholate	4	1000	15	32 ± 5	36 ± 5	>.05
			30	65 ± 10	68 ± 8	>.05

induced discharge act through different mechanisms; that is, DA_{10} does not merely increase the rate of spontaneous release.

The rate of release in both solutions can be represented by a first order reaction constant, $K = \frac{C_o}{C_t} \cdot t^{-1}$ or $K = \frac{100}{100 - R} \cdot t^{-1}$. The discharge therefore can not be considered as explosive, but rather it takes place at an approximately constant rate over a considerable portion of the range of the percentage of the substrate available.

There is no doubt that the hypertonic sucrose solution in some way exerted an inhibitory action on histamine release from dog liver particles. The author therefore considered it necessary to continue the study using several other base type histamine liberators.

b. Toluidine blue (Dimethyltoluthionine)

Toluidine blue has been used since the time of Ehrlich to histologically stain tissue mast cells. Recently Grossberg (1954) showed that this compound was indeed a powerful histamine liberator in vitro. In the present studies toluidine blue at a final concentration of 10 µg./ml. released an average of 54% of the total bound histamine when incubated with dog liver particles in isotonic sucrose solutions (30 minute incubation). In 1.2M solutions it freed only 23% during the same period. The findings were consistent in five experiments and, as shown in Table II, the results are significant at the level P = 0.01. The dose used was not quite as effective as those of the other liberators, but it was purposely kept at this level to avoid any untoward effects on the guinea pig ileum produced by the toluidine blue.

c. Stilbamidine (4,4'-Diamidinostilbine Di-(β-hydroxyethane-sulfonate)

Originally reported to be a histamine liberator by MacIntosh and Paton (1949), the compound was used in these studies as a typical basic histamine liberator. The drug, added to the liver particles at a final concentration of 200 μg./ml., produced an average histamine release that very nearly approximated that of DA_{10} (Table II). In 1.2M solutions the rate of discharge was decreased by slightly over 50% (according to K_1 values, 0.32M K_1 = 11; 1.2M K_1 = 5.3, the decrease was on the average 52%). This difference was also significant at the level P = 0.01.

d. 48/80 (a mixture of low polymers of p-methoxyphenylethylmethylamine probably as dimer, trimer and tetramer)

Although the exact formula of this basic histamine liberator is still unknown its molecular weight is generally taken to be 511. 48/80 was first shown to be a histamine releaser by Paton (1951). On the basis of molecular weight it proved to be about as effective as toluidine blue in freeing histamine from the liver particles suspended in isotonic sucrose solutions. Used at a final concentration of 60 μg./ml., it had a K_1 of 15.1 in 0.32M sucrose and 7.3 in the hypertonic media. This represents a decrease of 51%, although, as can be seen in Table II, there was a wide variation in the percentage released from the different suspensions in isotonic media. For this reason the significance of the difference between histamine released in the two solutions was not as striking, but nevertheless still significant.

4. Experiments Using Surface Active Compounds

The premise established for the experiments reported below was: that by virtue of the lytic nature of these compounds, their ability to release histamine would not be affected by changes in permeability of any membrane involved in the reaction. At this stage of the work I held the opinion that the inhibition of histamine release in 1.2M sucrose solutions was the result of a decrease in the permeability of the granule membrane. This interpretation was later abandoned.

a. Saponin

MacIntosh and Paton (1949) first tested saponin as a possible histamine liberator in the intact cat, but they obtained only negative results. Grossberg (1954) confirmed their in vivo findings, but showed that saponin was quite effective in liberating histamine from dog liver particles.

In four experiments (Table II) saponin, at a final concentration of 400 µg./ml., was incubated with dog liver particles in 0.32M and 1.2M solutions of sucrose. A statistical treatment of the results showed no significant differences in the percent of histamine released between the two solutions.

There was a wide variation in the effectiveness of saponin as a histamine liberator in different liver particle suspensions used (Table II). In one experiment, for example, 81% of the total bound histamine was released in the first 15 minutes of incubation in isotonic sucrose, and 87% in the hypertonic solution. This experiment naturally distorted the linearity of the average results.

b. Sodium Taurocholate

The surface active properties of bile salts are well known. Recently Schachter (1952) showed that they would also liberate histamine. Rather than use the crude bile preparations, I decided to use the purified salts. In preliminary experiments I found, with the concentrations necessary to release histamine, sodium glycocholate had such adverse stimulating effects on the ileum that the samples could not be assayed. Therefore, only sodium taurocholate was used. In order to produce a rate of release comparable to that induced by DA_{10} in isotonic media (K_1 = 13) a concentration of 1 mg./ml. was required. Even at this high concentration, sodium taurocholate had no irritating effects on the ileum. The results of these experiments were essentially the same as those obtained with the other surface active compound (Table II). The action of this drug was much more consistent in its histamine releasing action than was saponin. In a total of four experiments no significant differences were found in the percent of histamine released in the isotonic and hypertonic solutions.

5. Studies Showing the Reversibility of the Inhibitory Action of the 1.2M Sucrose Solutions on Histamine Release

To determine whether the inhibition of histamine release in hypertonic solutions was permanent, the following experiment was devised.

Six samples of liver particles, prepared in the usual manner, were incubated with DA_{10} at a final concentration of 200 µg./ml. Three samples contained 0.32M sucrose and three in 1.2M sucrose. After 15 minutes incubation one sample of

each molarity was removed, centrifuged at 30,000 g for 5 minutes, resuspended in isotonic sucrose containing the same concentration of DA_{10}, and incubated for another 15 minutes. The other four samples served as controls; two (one of each molarity) were incubated for 15 minutes and two for 30 minutes. The results of this experiment are shown in Fig. 4. When the sample suspended in isotonic sucrose was resuspended in isotonic sucrose there was an increase in histamine release of 32% over its control. However, the increase in histamine release from the hypertonic sample resuspended in isotonic sucrose was 182% greater than its corresponding control. Undoubtedly, some of the increased histamine released was the result of mechanical damage incurred during the resuspension of the particles. Nevertheless, the results clearly show that the effects of the 1.2M sucrose solution on the particles is reversible.

6. Studies Using Mannitol Solutions

In 22 experiments the effect of isotonic and hypertonic (1.2M) mannitol solutions on the release of histamine from dog liver particles was studied. The procedure followed was the same as that outlined in part 2 of this section, and therefore it was not considered necessary to deal separately with the results obtained with each liberator. Table III shows the findings in this series of experiments, using mannitol as the solute in place of sucrose. It suffices to say that the results confirm those obtained using sucrose as the suspending media (Fig. 5). Significantly less histamine was released by DA_{10}, 48/80, stilbamidine, and toluidine blue in 1.2M solutions than

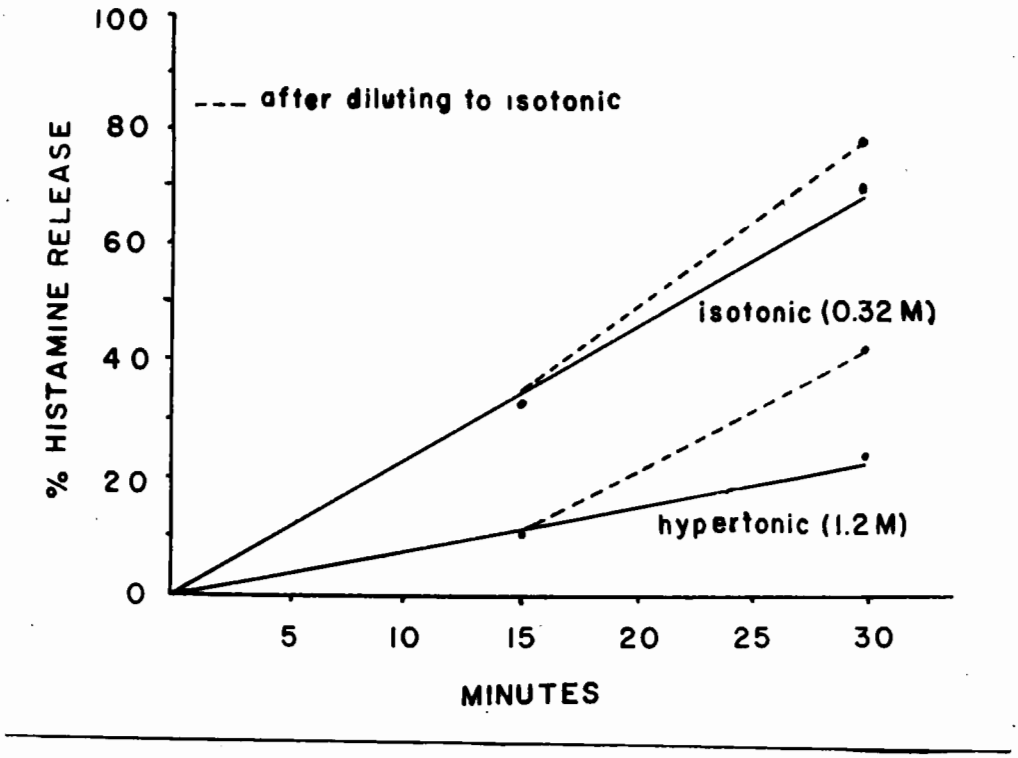

Fig. 4. Histamine release from dog liver particles incubated with DA_{10} (100 µg./ml.) in isotonic and hypertonic sucrose. The broken lines show the release from particles resuspended in isotonic sucrose.

TABLE III

Release of Histamine From Dog Liver Particles By Various Histamine Liberators in 0.32M and 1.2M Mannitol Solutions

Liberator	No. Expts.	Conc. µg/ml.	Incubation time (min.)	% Hist. Released mean ± sM 0.32M	% Hist. Released mean ± sM 1.2M	P
Control	4	-	15	10 ± 2	10 ± 2	>.05
			30	25 ± 3	24 ± 4	>.05
DA_{10}	4	200	15	39 ± 9	18 ± 5	<.01
			30	67 ± 9	29 ± 7	<.01
48/80	3	60	15	35 ± 10	13 ± 7	<.01
			30	64 ± 14	37 ± 9	<.05
stilbamidine	4	200	15	35 ± 3	19 ± 7	<.01
			30	66 ± 4	41 ± 13	<.01
Toluidine blue	4	10	15	25 ± 5	10 ± 7	<.01
			30	60 ± 12	29 ± 12	<.01
Saponin	4	400	15	54 ± 18	52 ± 19	>.05
			30	75 ± 16	73 ± 14	>.05
Sodium Taurocholate	4	1000	15	30 ± 8	30 ± 6	>.05
			30	64 ± 14	66 ± 10	>.05

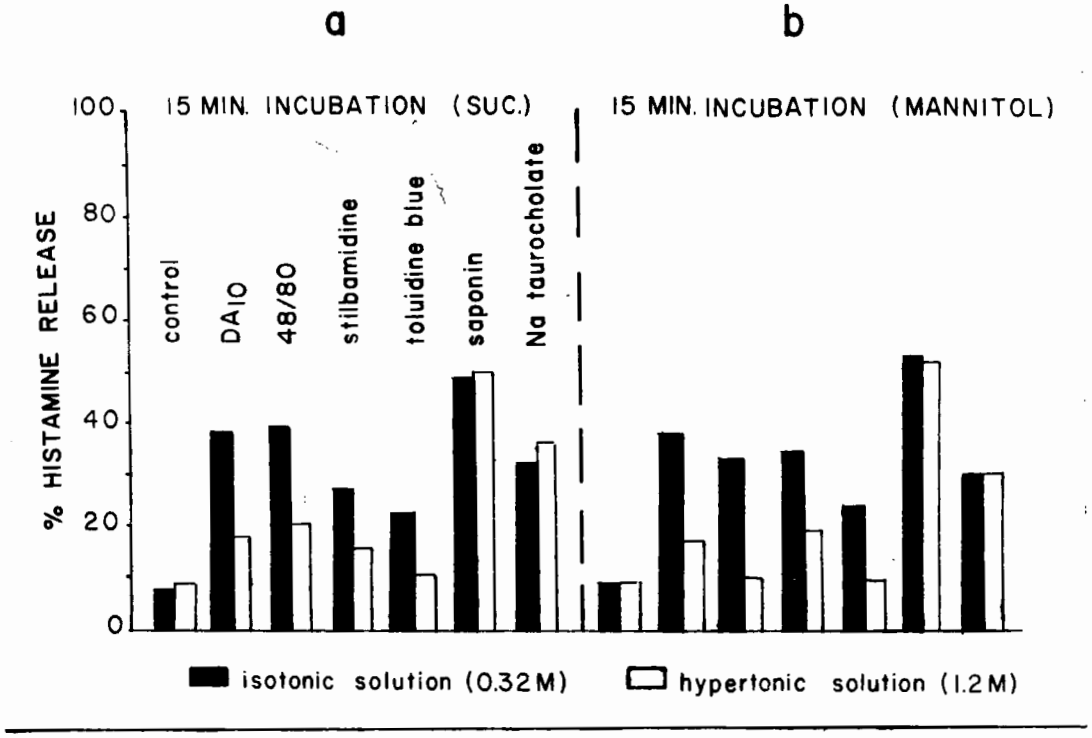

Fig. 5. Release of histamine from dog liver particles incubated at 37°C. with histamine liberators in isotonic (0.32M) and hypertonic (1.2M) solutions of (a) sucrose, and (b) mannitol.

in isotonic media. As in the previous studies no significant differences were found between the histamine released by the lytic agents saponin and sodium taurocholate. When the findings of these experiments were compared statistically with those obtained using sucrose solutions, they were found to be the same. This clearly indicates that sucrose per se was not responsible for the inhibition of histamine release seen in the hypertonic solutions.

7. Attempts Using Other Solutes

Thus far the solutes used were non-electrolytes of molecular size large enough to be considered non-penetrating with regard to permeability across a cell membrane. Attempts were therefore made to examine the effects of smaller molecules. Because of their physiological importance in the body, $CaCl_2$, KCl and NaCl were the solutes selected. In high concentrations, however, all of these cations have untoward effects on the guinea pig ileum. For this reason only samples containing isotonic NaCl could be assayed by this method. In this connection I was assisted by Mrs. Anne Wechsler, who performed several experiments in an attempt to extract the histamine from liver particles suspended in these salt solutions. The method of extraction she used was that reported by McIntire (McIntire, Roth & Sproull 1947). Her results were not reproducible and the experiments were abandoned.

Cation ion exchange resins could not be used since they would also alter the composition of the Tyrode in the muscle chamber when the samples were assayed. The only alternative the author could think of was to perform the experiment in

the usual manner, but instead of assaying for histamine in the supernate following the incubation, to discard it and assay for the histamine still bound in the particles. The results of these experiments were also inconclusive and therefore will not be reported here.

D. The Effect of Tonicity on the Release of Histamine From Isolated Perfused Cat Paws

The experiments described in this section were undertaken in the hope of studying histamine release from a more physiological source. The cat paw contains many of the various tissues found in the entire body. Its intact circulatory system permits the liberator to reach the cells concerned in the reaction in a more natural way. Another feature of this preparation is that it has an almost identical mate. This allows the study of histamine release from both paws; one serving as the control for the other. Högberg and his associates (Högberg *et al.* 1956) first used such a preparation to study histamine release. They found it to be not only a rich source of histamine but also a sturdy preparation which retained its histamine for considerable lengths of time.

1. Methods

The procedure was a modification of that of Högberg *et al.* (1956). Cats of either sex weighing 3 Kg. or more were used whenever possible. In a few experiments it was necessary from lack of choice to use smaller animals. The animals were anesthetized with ethyl chloride followed by ether. A femoral vein was cannulated and the animal injected with 100 mg. of chloralose

per Kg. of body weight. A circular incision was made just proximal to the radio-carpal joint and the radial artery cannulated with fine polyethylene tubing (PE 200). The cephalic vein was ligated and the paw removed. Saline was perfused through the paw until most of the blood was washed out. This usually required about 25 to 30 ml. of saline. A ligature was passed through one of the toe pads and the paw suspended, claws up, in an incubator at 37°C. It was perfused with fluid at the same temperature under sufficient pressure to produce a venous outflow of approximately 0.5 ml./minute. The perfusate dropped into a funnel and was collected in graduated tubes outside the incubator. Fig. 6 is a photograph of the incubator and the perfusion system.

The paws were perfused for one hour before the samples were collected. Following this period, three 5-minute control samples were collected. The liberator, dissolved in 0.1 or 0.15 ml. of saline, was injected into the cannula and the perfusion stopped. After 45 seconds the perfusion was resumed and 5-minute samples collected over the next 25 minutes. The samples were then placed in a boiling water bath for two minutes and then stored at 0°C. until assayed.

When the experiment was completed, the opposite paw was removed and the experiment repeated following the same procedure except that the perfusing fluid was changed. In each experiment one paw was perfused with an isotonic solution of either sucrose or mannitol and the opposite one with a hypertonic solution (1.2M) of the same solute. The tonicity of the fluid perfused into the paws was varied in each experiment; that

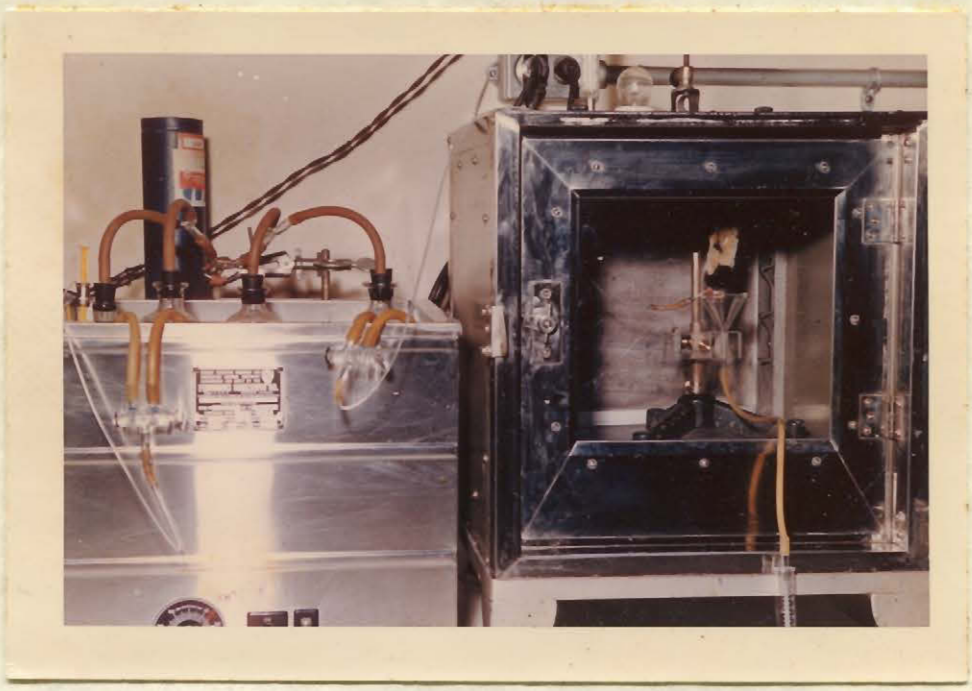

Fig. 6. A photograph showing the system used to perfuse cat paws. Fluid contained in bottles in the constant temperature bath was forced through the vascular bed of the paw under oxygen pressure. Venous effluent dropped into the funnel and was collected in the graduated tube outside the incubator.

is to say, the left paw was perfused with an isotonic solution only in every other experiment.

The injection of the liberator was made so that the point of the needle was very close to the tip of the cannula; the dead space was always less than 0.05 ml. Approximately 5 seconds were required after the injection before the perfusion could be stopped. By using various dyes (Azure A, Organe G, and Toluidine blue), it was found that circulation time for these preparations was about 15 seconds. From these figures it was calculated that, at the flow rate used in these experiments, the liberator was well into the vascular bed of the paw when the perfusion was stopped.

2. Studies Using Basic Histamine Liberators

a. DA_{10}

During the preliminary perfusion period the paws perfused with the 1.2M solution became dehydrated as was evidenced by a loss of weight of 3.5 to 5.0% between the 5th and 60th minutes of perfusion. No weight changes were noted in the paws perfused with the isotonic solutions. Fig. 7 shows the results of three experiments in which histamine was released with 100 µg. of DA_{10}. In the paws perfused with isotonic sucrose solutions, DA_{10} caused an immediate increase in the histamine content of the venous effluent. The amount of histamine found in successive samples decreased slowly over the 25 minute period following the injection, giving an average total release of 10 µg. of histamine. This figure does not represent the entire amount of histamine found in the samples but rather the amount in excess of the spontaneous release calculated to

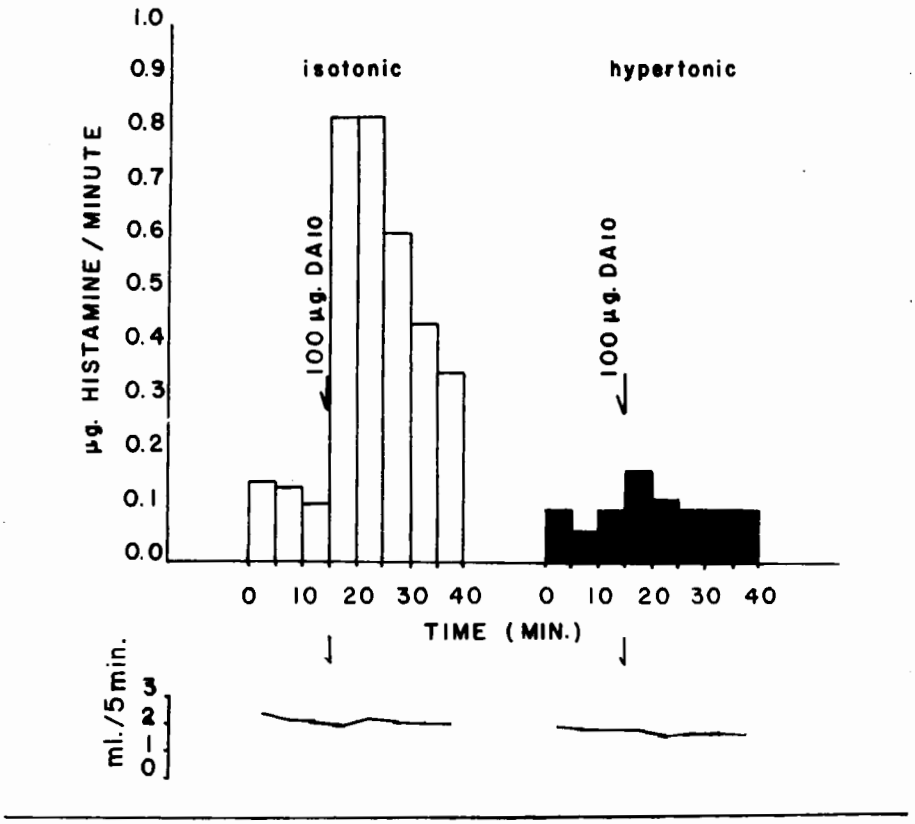

Fig. 7. The discharge of histamine from isolated cat paws perfused with isotonic and hypertonic sucrose solutions. Arrows indicate the time when 100 mg. of DA_{10} was injected into the perfusion stream. Below is the flow-rate curve.

histamine actually discharged by the liberator, There was only a small increase in the histamine content of the venous effluent from the paws perfused with the hypertonic media after the injection of DA_{10}. The average total release from these paws was only 0.15 µg.

The release of histamine from the perfused paws appeared to be exponential, indicating an explosive discharge of histamine into the extracellular spaces from where it is washed out by the perfusion fluid. This type of release was suggested by Feldberg and Paton (1951) using perfused cat gastrocnemius. Figure 8 shows the results of an experiment in which DA_{10} liberated histamine from a paw perfused with isotonic sucrose solution. The abscissa is the volume of perfusate expressed as a percent of the paw weight, assuming a fixed relationship between weight and volume. The slope of the curve very nearly approximates that of the English workers. The dropping off of the last two points on the graph can best be explained by the formation of edema which would effectively decrease the concentration of histamine in the interstitial space and therefore decrease the amount washed out by the perfusion fluid.

b. 48/80

Similar results were obtained with 48/80 but the dose used was less effective than that of DA_{10}. Figure 9 shows the results of three experiments in which 48/80 was used as the histamine liberator. An average of 6.0 µg. of histamine was cleared from paws perfused with the isotonic solution while only 1.3 µg was released from the paws perfused with 1.2M sucrose solutions. The results do not have the exponential

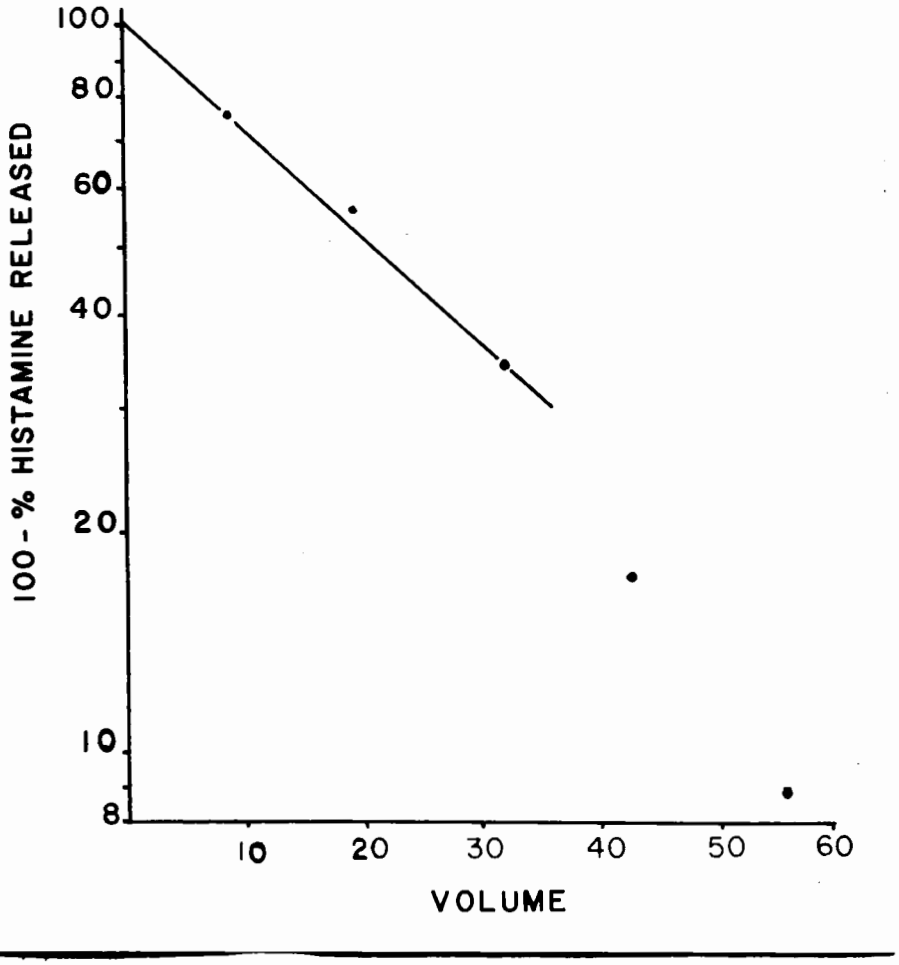

Fig. 8. Histamine output from perfused cat paws after the injection of 100 µg. of DA_{10}. Ordinate: the total output of histamine was taken as 100%, the points show the percentages of this histamine still remaining in the paw. Abscissa: the volume of the perfusate expressed as a percentage of the paw's weight.

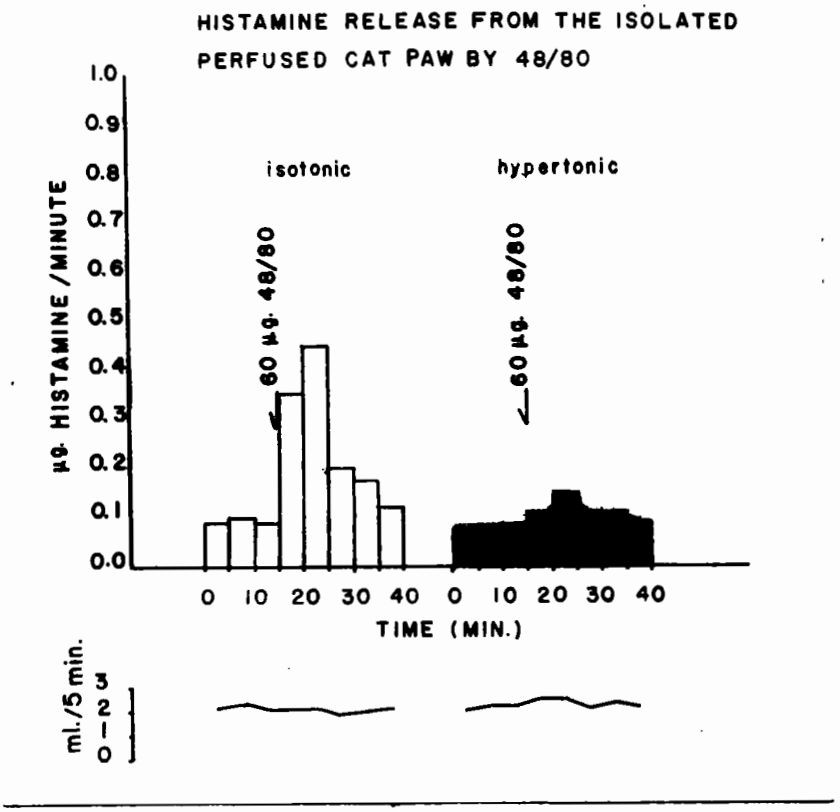

Fig. 9. The discharge of histamine from cat paws perfused with isotonic and hypertonic sucrose solutions. Arrows indicate the time when 60 µg. of 48/80 was injected into the perfusion stream. Below is the flow-rate curve.

form seen with DA_{10}. Most likely this was because with the smaller amounts released, experimental variations obscured the presence of such a curve.

3. Studies Using a Surface Active Compound: Saponin

The results of three experiments in which histamine was released from perfused cat paws by saponin are shown in Figure 10. Unlike DA_{10} and 48/80 which produced no change in the pressure-flow relationship, the injection of 1 mg. of saponin into the cannula increased the resistance to flow and resulted in the appearance of a bloody tint in the effluent. The reduction in flow might have been due to the clogging of small vessels by proteins or desquamated cellular material rather than to vasoconstriction. The decrease in flow occured in both solutions though to a much less degree in the hypertonic media. As might be expected from the slower flow rates, the concentration of histamine in the venous outflow rose more slowly and remained elevated longer. Again in these experiments there was no significant difference between the amounts of histamine released in the isotonic and hypertonic solutions. An average of 2.04 µg. was discharged in the isotonic media and an average of 1.98 µg. was released from the paws perfused with the hypertonic solution.

In the experiments with the liver particles the final concentrations of the liberators were known and it could easily be determined whether or not the ileum would be affected by these concentrations. However, in the experiments with cat paws the final concentrations of the liberators were not known. The highest possible concentration could occur if all of the liberator passed through the paw and appeared in the first

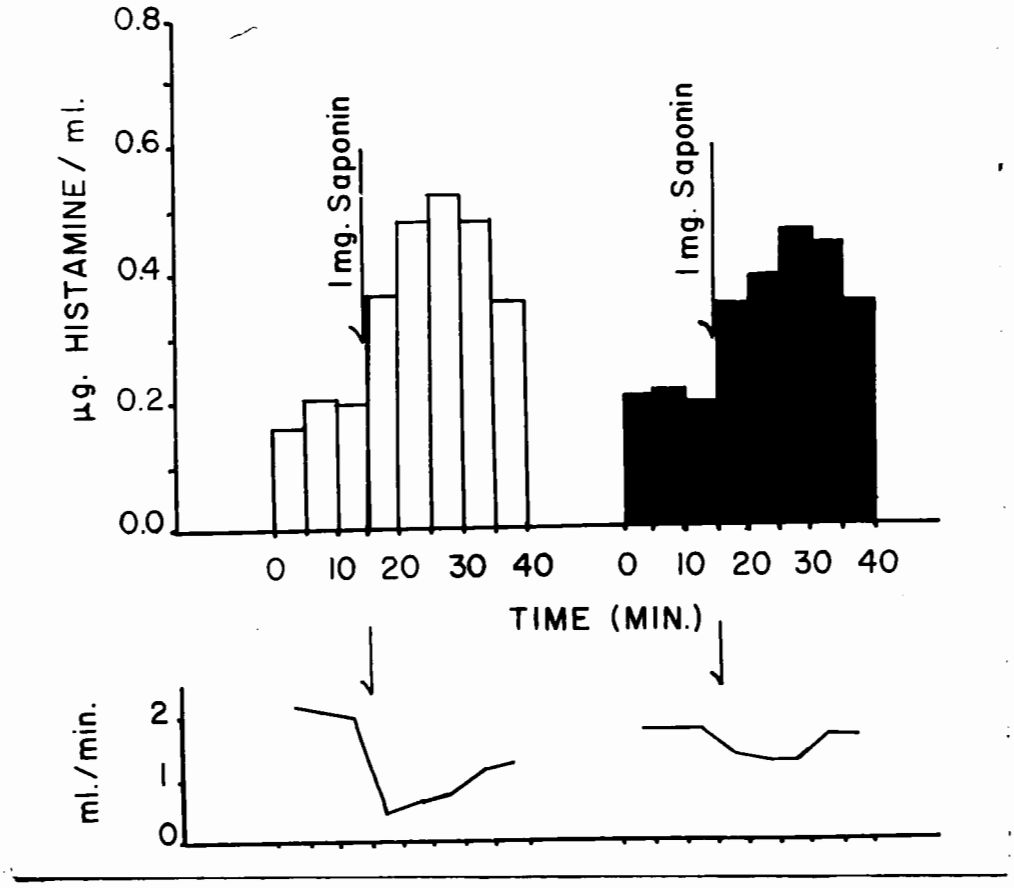

Fig. 10. Histamine liberation by saponin from isolated cat paws. The histogram on the left shows the release from paws perfused with 0.32M sucrose solutions; the one on the right that released from paws perfused with 1.2M solutions. Below are the flow-rate curves. The arrows indicate the time of injection.

sample. By calculating the maximum concentration that could have occured in the first sample of each experiment, it was found that only saponin had any effect on the ileum. This was a potentiating effect that was so small (22 nanograms (ng.) histamine standard = 20 ng. histamine standard plus saponin) that it was ignored.

4. The Effectiveness of 48/80 in Sucrose and Tyrode

The small amount of histamine released in these preparations by 48/80 is in extreme contrast to the effectiveness of 48/80 reported by Högberg and his associates (1956). One distinction between the experiments reported here and those of the Scandanavians was in the perfusion fluid used. Högberg and his co-workers perfused with Tyrode while I used sucrose solutions. Therefore, the following experiment was performed to ascertain if the discrepancies between our results could be accounted for in the difference in the perfusing media.

A cat was prepared in the usual manner. One paw was perfused with isotonic sucrose solution and the opposite one with Tyrode. The results of this experiment are seen in Figure 11. A total of 1.87 µg. of histamine were released from the paw perfused with the sucrose solution while 22.77 µg. were cleared from the opposite paw. In other words, roughly ten times more histamine was discharged from the paw when Tyrode was used. Mongar and Schild (1956) also found the same ratio when they studied the action of 48/80 and octylamine on histamine release from mitochondrial fraction of guinea pig lungs in sucrose and Tyrode.

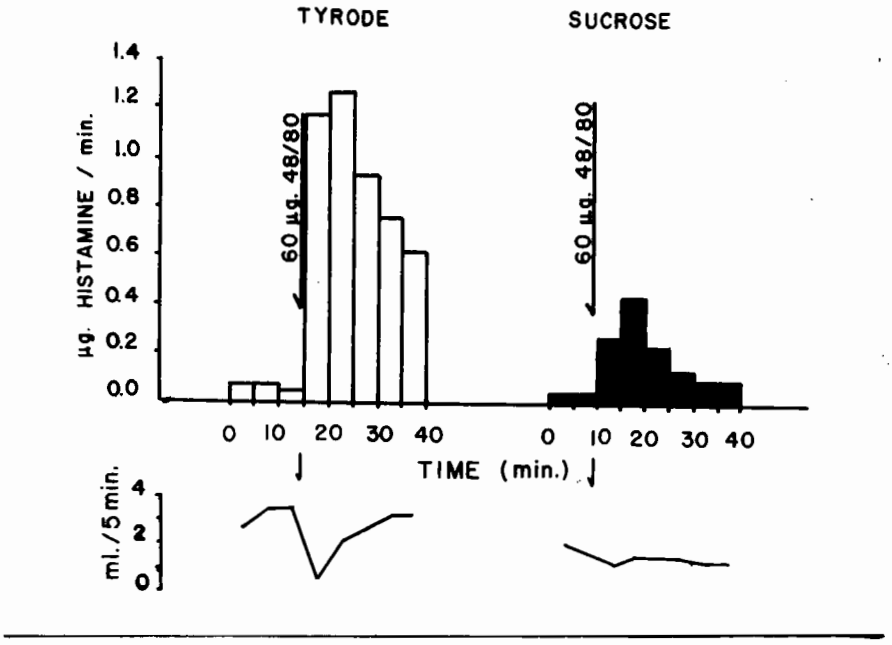

Fig. 11. Release of Histamine from a pair of cat paws after injection of 60 µg. of 48/80. The histogram on the left shows the rate of release from paw perfused with Tyrode; the one on the right shows the rate of release from paw perfused with isotonic sucrose solution. Below are the flow-rate curves.

5. Observations on the Vasoconstrictor Action of Histamine in Sucrose and Tyrode Solutions

As can be seen from the flow-rate curves in Figures 8 and 9, there was no evident vasoconstriction following the injection of either DA_{10} or 48/80. In several experiments in which histamine itself was injected into the cannula, doses as high as 1 µg. produced no decrease in the rate of venous outflow. In an experiment similar to the one described in the previous section it was found that DA_{10} or injected histamine would cause a decrease in the flow rate from the paws perfused with Tyrode. Further experiments revealed that histamine caused no vasoconstriction in paws perfused with sodium free Tyrode. Removal of the calcium or potassium had no effect on the vasoconstrictor action of histamine.

6. Studies Using Mannitol Solutions

Nine experiments, three each with DA_{10}, 48/80 and saponin, were performed to reconfirm the contention that the reductions in histamine release were not due to the presence of sucrose molecules per se, but rather they were the result of tonicity changes in the fluid caused by the increased concentrations of sucrose.

The results of these experiments, which were carried out in the same manner as outlined in part 1, of this section, closely resemble those obtained with sucrose solutions. There were no changes in the flow rate after the addition of 48/80 or DA_{10}, and the amounts of histamine discharged did not significantly differ from the amounts released in isotonic sucrose solutions. When saponin was used as a liberator, approximately equal

quantities of histamine were cleared with both the isotonic and hypertonic mannitol solutions (Fig. 12).

E. The Effect of Tonicity on the Release of Histamine From Isolated Cells of Rat Peritoneal Cavities

Association of histamine with tissue mast cells is now well established (Riley & West 1952; 1953a,b). Recently techniques have been developed which permit the removal of intact mast cells from the peritoneal cavity of rats (Padawer & Gordon 1955; Benditt et al. 1955; Glick, Bonting & DenBoer 1956). Therefore, a study was made concerning the effect of tonicity on histamine release from suspensions of mast cells. Use of such preparations eliminates, to a reasonable degree, any possible interference in the release mechanism produced by factors arising from other types of cells, yet it allows the study of histamine binding using intact living cells.

1. Preparation of the Cell Suspensions

Wistar rats, males weighing between 200 and 300 gm., were anesthetized with ether. Ten ml. of a 0.32M sucrose solution were injected intraperitoneally; the injection was made through the linea alba using a 24 gauge ½ inch needle. The drunken staggering of the animals as they recovered from the ether produced a gentle but highly effective massage of the abdominal cavity. About twenty minutes later the animals were reanesthetized with ether and killed either by decapitation or by injecting air intracardially.

A strip of skin about a half inch wide was removed from the midline, extending from the pubis to the sternum. This

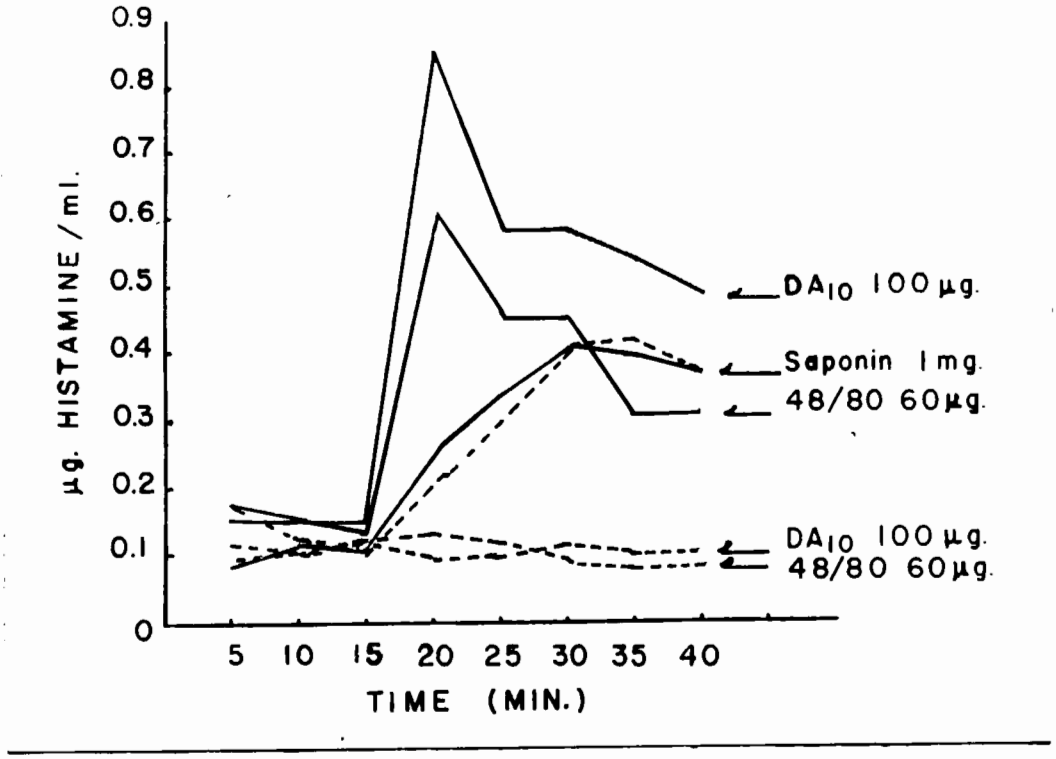

Fig. 12. Histamine release by DA10, Saponin, and 48/80 from cat paws perfused with mannitol solutions. The solid lines show the release in 0.32M media and the broken lines that in the 1.2M solutions. Injections were made at the 15 min. mark.

prevented any blood or hair from getting into the opened cavity. The abdomen was then opened through an incision in the linea alba, and the fluid removed with the aid of a Kahn suction pipette. Care was taken not to rupture any small blood vessels within the cavity, which would contaminate the fluid.

The sucrose solution was transferred to a 50 ml. plastic centrifuge tube kept in a small ice bath. Routinely six animals were used for each experiment and the peritoneal washings pooled. The cells were removed by centrifuging at 800g for 20 minutes, they were then washed twice with ice-cold sucrose soltuion (0.32M). Following the second wash, the cells were taken up in 6 to 8 ml. of sucrose buffered at pH 8 with "tris" buffer (0.07M), and filtered through several layers of gauze to remove any traces of fibrin. The volume was adjusted to 11 ml.

Microscopic examination of the suspensions revealed the presence of large numbers of typical mast cells with a fair percentage of leucocytes. Few erythrocytes and no epithelial cells were seen in any of the preparations. Dr. Garcia-Arocha made several attempt to separate the mast cells from the leucocytes in similar preparations. Using the method of Padawer (1955) and Glick (Glick, Bonting & DenBoer 1956), he was able to obtain suspensions containing only mast cells, but biological analysis of the cell suspension showed that they no longer contained any histamine. For this reason I made no attempts at further separation of the mast cells.

The histamine content of the final mast cell suspension averaged 15.3 µg./ml. or 28.05 µg./animal. Adding to this amount the histamine present in the combined washings (which

averaged 9.8% of the overall total), the average histamine content per animal was 30.9 µg. Using the figure for the histamine content of rat skin - 6 µµg./cell (Graham et al. 1953), it was calculated that approximately 5,150,000 mast cells were obtained from each animal.

2. Methods

Three histamine liberators were used in the experiments, two organic bases - DA_{10} (200 µg.) and 48/80 (60 µg.) and one surface active compound - saponin (1 mg.). Basically the experiments were very similar to those in which liver particles were used. One ml. of mast cell suspension was added to between 3 and 4 ml. of sucrose solution buffered to pH 8 with "tris buffer" (0.07M). The samples were allowed to stand in the cold for one hour, then appropriate amounts of the liberators were added and the samples, which then were uniformly 5 ml., were incubated at 37°C. for twenty minutes in a Dubnoff Metabolic Shaking Incubator. When the incubation period was completed the samples were centrifuged at 30,000g for 10 minutes. The supernates were decanted, placed in a boiling water bath for two minutes and stored at 0°C. until assayed. Control samples containing mast cells and buffer but no liberator were treated in the same manner. The total and free histamine in the samples before incubation were determined according to the procedures outlined in part 2 of section C.

3. Results

The findings of these experiments are shown in Table IV. It can be seen that the three liberators were equally effective in isotonic solutions, at the concentrations used. Wide variations

TABLE IV

Percent Histamine Release From Isolated Cells From Rat Peritoneal Cavity Incubated With Histamine Liberators in Isotonic and Hypertonic Solutions of Sucrose

Expt. No.	µg. Histamine		% Histamine Released							
	Total	Free	Spont.		48/80 12 µg./ml.		DA_{10} 40 µg./ml.		Saponin 200 µg./ml.	
			I†	H‡	I†	H‡	I†	H‡	I†	H‡
157	9.0	3.2	7.8	5.2	83.	31.	75.	31.	56	48.
160	14.0	1.65	9.1	16.7	59.	19.1	70.3	34.8	79	78.5
161	20.0	4.2	20.0	24.0	55.6	27.2	49.0	27.2	61	56.7
162	11.0	4.0	10.0	7.1	100	21.4	50.0	7.1	100	71.6
163	20.0	3.3	1.8	3.6	29.6	18.9	32.0	7.2	46	50.6
165	18.0	4.2	6.0	6.0	51.0	27.0	65.0	32.0	73	75.5
Ave.	15.6	3.43	9.1	10.6	63	24	57	23	71	63.5
t			1.08		5.2		9.2		1.46	

† isotonic

‡ hypertonic

were evident in the action of any one of the liberators in the different experiments, but these variations were fairly consistent for all three drugs. In experiment 157 for example, DA_{10} released 75% of the total bound histamine, 48/80 - 83% and saponin - 63%; and in experiment 163 the results were DA_{10} - 32%, 48/80 - 29.6% and saponin - 46%.

Comparing the action of the liberators in the isotonic and hypertonic solution, the same striking results were found again. The average percent histamine released by 48/80 in the isotonic media was 63% while the average was only 24% in the hypertonic fluid. With DA_{10} the averages were 57% in 0.32M sucrose and 23% in 1.2M sucrose. These differences are of course highly significant.

A comparison of the spontaneous release in the two solutions showed a slightly higher amount in the 1.2M solution - 9.1% to 10.6%. With saponin these figures were 71% in the 0.32M solution and 63% in the hypertonic media. These differences are not statistically significant however.

F. Experiments Using the Anaphylactic Reaction

1. Studies Using Guinea Pig Lungs

a. Methods

Guinea pigs weighing 100 - 200 gm. were sensitized to egg albumin according to the method suggested by Mongar and Schild (1957). Four to ten weeks were allowed for the sensitivity to develop. The animals were then anesthetized by an intraperitoneal injection of 0.5 ml. of nembutal. Artificial respiration was begun and the thorax opened. Both the inferior and superior vena cava were ligated and the pulmonary circuit was flushed by

injecting physiological saline into the right ventricle. The pulmonary veins were laid open from their entrance to the left atrium to the hilus of each lobe. The bifurcation of the pulmonary artery was located and the left and right branches cannulated using fine polyethylene tubing. The lungs were excised and mounted in an incubator at 37°C. in such a way that the venous effluent of the two lungs could be collected in separate containers (Fig. 13). They were perfused with oxygen-saturated Tyrode under sufficient pressure to produce a venous outflow of 0.5 to 1.0 ml./minute. One lung was perfused with normal Tyrode and the opposite one with Tyrode containing sucrose at a concentration of 300 mg./ml.

Before beginning each experiment the lungs were perfused for one hour to insure cellular dehydration in the lung perfused with the hypertonic Tyrode. Following this period, three 5 minute control samples were collected and 1 mg. of egg albumin (0.1 ml. of a 10^{-2} solution) was injected into the cannula. The perfusion was then stopped for 45 seconds to allow the antigen to diffuse into the tissue spaces. Perfusion was again started and five-minute samples collected over the next 25 minutes.

b. Results

Feldberg and Kellaway (1937) first noted that while there were extreme variations in the histamine content of the lungs of different guinea pigs, both lungs from any one animal contained essentially the same quantity of histamine. However, it was necessary in these experiments to determine whether the same amount of histamine would be released from both lungs by the action of the antigen. Figure 14 shows the results of such an experiment.

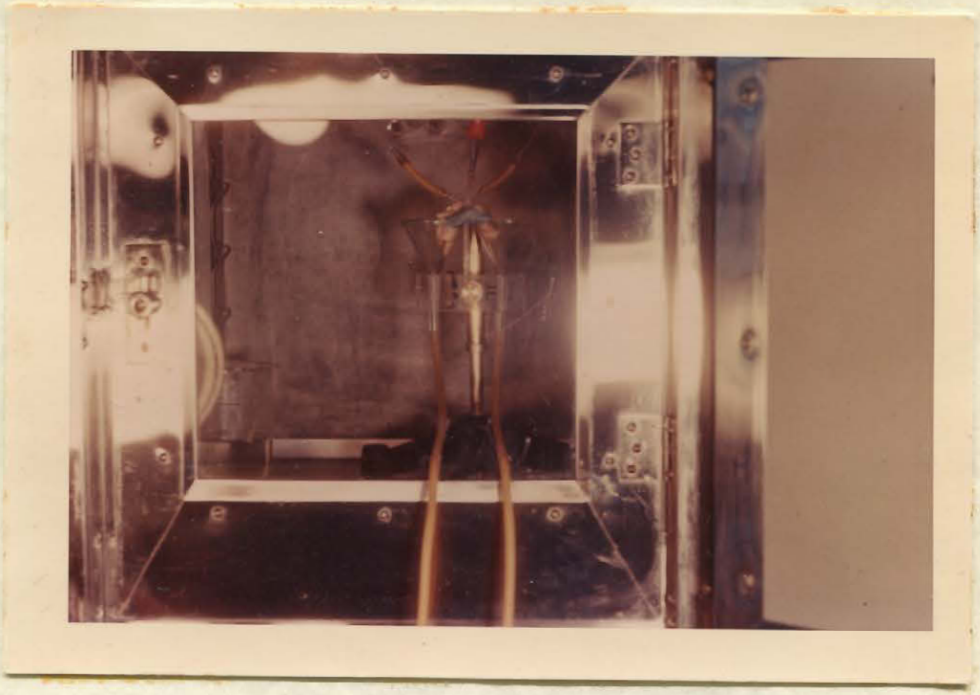

Fig. 13. A photograph showing apparatus for perfusing guinea pig lungs separately. Lungs were inflated rhythmatically by respiratory pump. Venous effluents were collected separately. Temperature of incubator was 37°C.

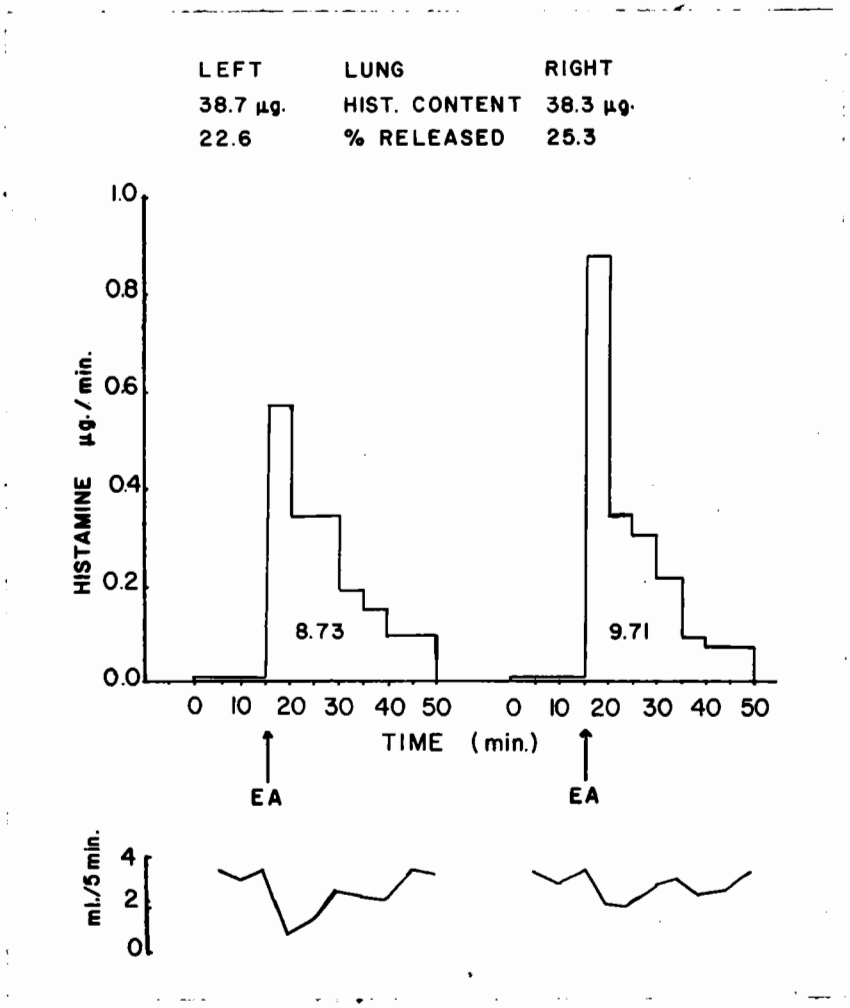

Fig. 14. Release of histamine from sensitized guinea pig lungs after injection of specific antigen (1 mg. egg albumen). Both lungs were perfused with oxygenated Tyrode at 37°C. Fig. in histogram represents the amount of histamine (µg.) released. Below are the flow-rate curves.

The total histamine content of the left lung was 38.7 µg. and that of the right lung was 38.3 µg. 22.6% of the total histamine was released from the left lung and 25.3% from the right. Vasoconstriction was more pronounced in the left lung and therefore the histamine was discharged more slowly than from the opposite lung.

A total of ten experiments were performed using the antigen as a histamine liberator in iso- and hypertonic solutions. Figure 15 shows the results of these experiments. Following the injection of the antigen there was a sudden increase in the histamine content of the venous outflow from the lungs perfused with normal Tyrode, while only very small amounts were cleared from their counterparts. One very striking feature was the relatively small amount of histamine discharged from the lungs perfused with the normal Tyrode. In the experiment shown in Figure 14, approximately 9 µg. were released from each lung but the average release from these ten experiments was only 1.69 µg. One possible explanation for this is that in the latter experiments there was a preliminary perfusion period of one hour. At the end of this time it was noted that both lungs were always quite enlarged with edema fluid. This no doubt would cut down the effective concentration of the antigen in the extracellular space.

To establish that basic histamine liberators would act in a similar manner in these preparations an experiment was conducted in which 48/80 was used as the liberator. The results of this experiment are shown in Figure 16. Feldberg and Mongar (1954) had reported that 48/80 was a very weak liberator in per-

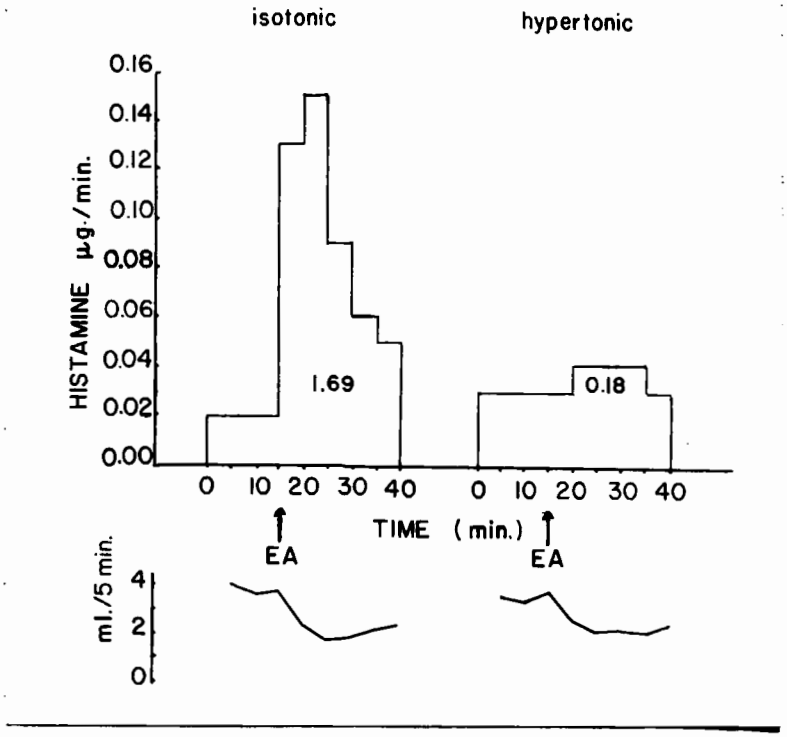

Fig. 17. Release of histamine from perfused lungs of sensitized guinea pigs. The graphs are the composite of ten experiments, the one on the left represents the rate of release from lungs perfused with oxygenated Tyrode after injection of 1 mg. egg albumen, while the one on the right shows the rate from lungs perfused with oxygenated Tyrode containing 300 mg./ml. sucrose. Figures shown in the histograms are the number of micrograms of histamine released. Below are the flow-rate curves.

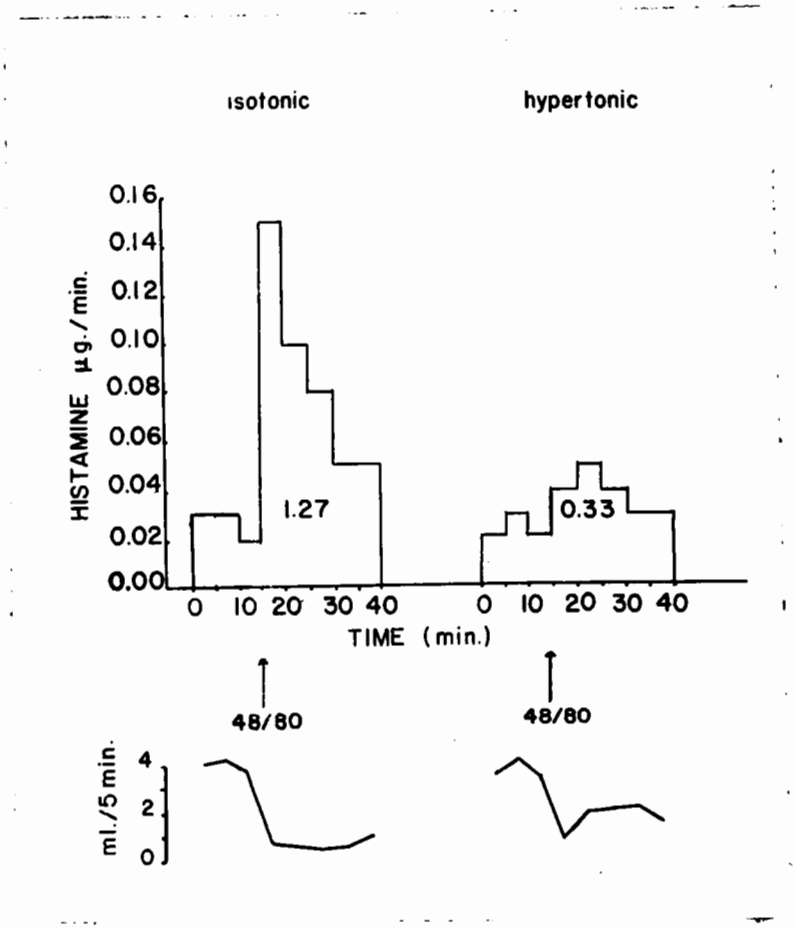

Fig. 16. Histamine released from perfused guinea pig lungs by the action of 48/80 (1 mg.). 1.27 µg. was released drom the lung perfused with normal Tyrode. The histogram on the right shows histamine released from the lung perfused with Tyrode containing 300 mg. sucrose/ml. Below are the flow-rate curves.

fused guinea pig lungs, therefore a much larger dose was used than in previous experiments with this compound. One mg. of 48/80 released 1.27 µg. of histamine from the lung perfused with normal Tyrode and 0.33 µg. from the opposite lung. These findings show that tonicity changes have the same effect on histamine release from such preparations by both organic bases and the anaphylactic reaction.

G. Experiments Using Anaphylatoxin as a Histamine Liberator

Anaphylatoxin has been shown by Rocha e Silva and his associates (1951; 1952) to bring about the discharge of large quantities of histamine from perfused guinea pig lungs. These findings, as interpreted by Rocha e Silva, suggested that anaphylatoxin or a similar substance may be formed during the anaphylactic reaction. The experiments to be reported in this section were undertaken to compare the actions of anaphylatoxin to the anaphylactic reaction. The source of histamine was again the guinea pig lung. Experiments were conducted in the same manner as described in the previous section.

In the first experiment sucrose solutions were used as the perfusing fluids. With this media, however, no histamine was released following the injection of anaphylatoxin. The perfusing fluid was therefore changed to Tyrode.

1. Preparation of Rat Serum Anaphylatoxin

Rat serum anaphylatoxin was prepared according to the method outlined by Rothschild and Rocha e Silva (1954). A 0.25% solution of agar was made and heated to boiling for a few minutes. One and a half ml. of this solution was added to 15 ml. of rat serum and the mixture incubated for 20 minutes at 37°C. It was then centrifuged at 850g for ten minutes and the

serum decanted. Two ml. of the activated serum was injected into each of three guinea pigs. Typical symptoms of anaphylaxis occurred; the animals all died within three minutes. Post mortem revealed the lungs to be fully distended.

2. Results

Fig. 17 shows the results of 4 experiments. Injection of 1 ml. of rat serum anaphylatoxin resulted in the sudden appearance of histamine in the venous effluent. An average of 3.8 µg. of histamine were released from the lungs perfused with normal Tyrode while an average of only 0.94 µg. was cleared from those perfused with the hypertonic media. The figure of 3.8 µg. is much smaller than that reported by Rocha e Silva but the dose used in the present experiments was only one fourth that used by him.

Two control experiments showed that fresh rat serum caused no release of histamine from guinea pig lungs. Two further experiments, however, showed some indication that aged rat serum was capable of releasing histamine from these preparations. In one experiment 6.9 µg. were discharged and in the other 0.22 µg. came out.

Calcium is known to play an important part in the anaphylactic reaction (Mongar & Schild 1958). In view of the failure of anaphylatoxin to release histamine from lungs perfused with sucrose solutions, it was decided to see what part, if any, calcium ions play in the release of histamine by anaphylatoxin. To do this the lungs of a guinea pig were set up in the usual fashion, one lung was perfused with normal Tyrode and the opposite lung with calcium free Tyrode. The results of this experiment are seen in Fig. 18. When the calcium ions were absent much less

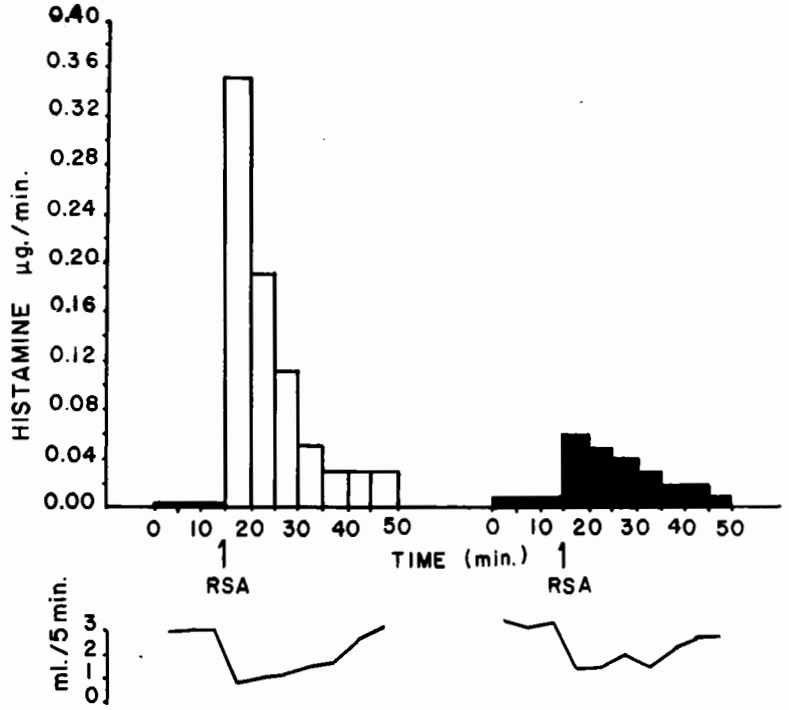

Fig. 17. Release of histamine from guinea pig lungs following the injection of 1 ml. of rat serum anaphylatoxin (RSA). The histogram on the left represents the average rate of histamine release from 5 lungs perfused with normal Tyrode; the one on the right; that from lungs perfused with Tyrode containing 300 mg. sucrose/ml. Below are the flow-rate curves.

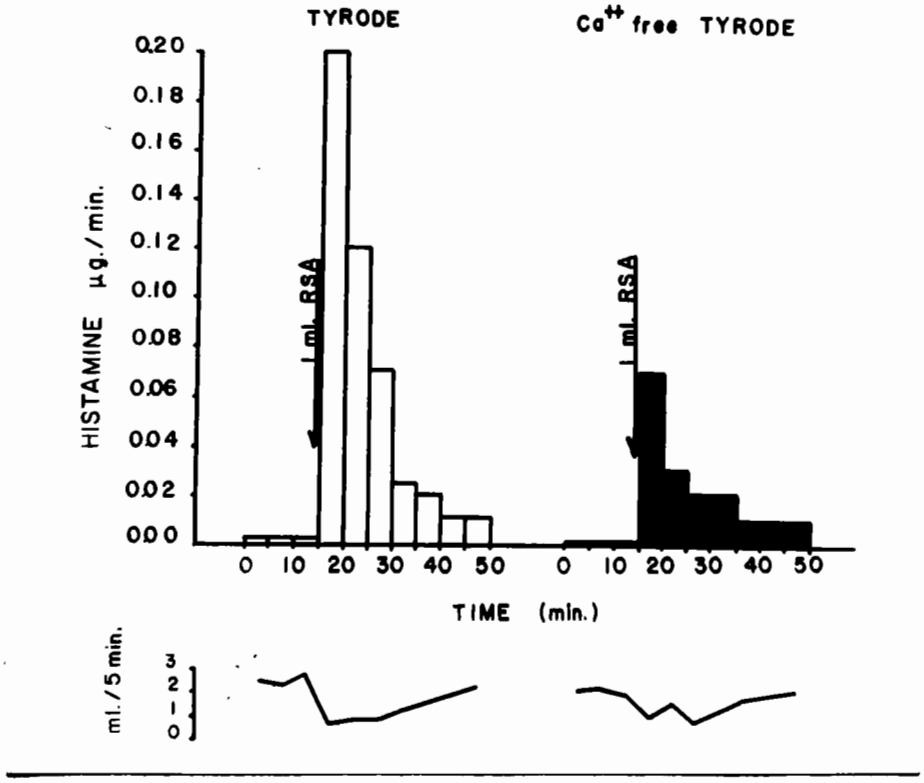

Fig. 13. Histamine released from perfused guinea pig lungs by rat serum anaphylatoxin in the presence and absence of calcium ions. Below are the flow-rate curves.

histamine was discharged by the action of rat serum anaphylatoxin. This further strengthens the similarity between these two entities.

H. Studies Concerning the Release of Histamine by Soluble Antigen-Antibody Complexes

The literature concerning the production of anaphylaxis in non-sensitized animals by the injection of antigen-antibody mixtures dissolved in excess antigen have been reviewed in this thesis. The importance of these mixtures in unraveling the mysteries of anaphylaxis should be self evident. It was decided to investigate whether the soluble antigen-antibody complex could be used as a histamine liberator *in vitro* with regard to the present studies.

1. Preparation of the Soluble Antigen-Antibody Mixtures

a. The Antisera

Nine rabbits were hyperimmunized by 12 intravenous injections (1 ml. each) of a 2.5% solution of crystallized bovine plasma albumin (Armour & Co.), given over a period of four weeks.

Beginning two days after the last injection, blood samples were taken and the antibody titer determined using the interfacial technique (ring test) described by Boyd (1947). When a titer greater than 1:64 was found, the animal was bled by cutting the marginal ear vein. In this way as much as 70 ml. could be obtained from each animal. This method of intravenous sensitization yielded antibody titers ranging from 1:320 to 1:1280 in seven of the nine animals used. Bloods having the same titer were mixed, and an approximation of the zone of equivalence was determined according to the method first described by Dean and Webb (*loc cit.* Boyd 1947).

b. The Complex

Two methods of preparing the soluble complexes were used, both of these methods were described by Tokuda and Weisser (1958). The first method was to add excess antigen to the antisera, the resulting complex was designated as Type 1. The quantity of antigen used in this method was eight times the amount present in the zone of equivalence. The mixture was incubated at 37°C. for 90 minutes, centrifuged for twenty minutes at 12,000g and the sera decanted and saved for tests.

The second complex (Type 11) was formed by incubating the antisera with a quantity of antigen, sufficient to give the precipitate at the zone of equivalence, for ninety minutes at 37°C. The precipitate was washed twice with large volumes of ice-cold physiological saline and then incubated at 45°C. for forty minutes in saline containing ten times as much antigen as present in the precipitate. The solution was centrifuged at 12,000g for twenty minutes and the fluid saved for tests.

2. Tests Indicating the Effectiveness of the Complex In Vivo

a. Rats

With Type 1 complex, injections ranging from 0.5 to 2.0 ml. killed 11 out of 12 animals or 91.7%. Type II, while it produced symptoms of anaphylaxis in all the animals, was not as lethal. Sixty-nine percent of the 19 rats injected with this complex died. There was a definite pattern in the onset of the symptoms. The animals immediately exhibited signs of extreme excitement. They appeared very nervous and apprehensive running about, exopthalmus was common. This phase lasted for approximately five minutes at which time they became suddenly quiet. The respiration became deep and slow. One of the animals exhibited signs of a typical

asthmatic attack at this time. From this point on the animals began to show symptoms of a slow progressive circulatory collapse. They became weak, cyanotic, breathing was deep and labored, micturation and defecation were common. Their color slowly turned ashen and they became prostrate. Death generally ensued within 3 to 5 hours, sometimes preceded by a convulsive seizure.

Autopsy revealed massive hemorrhages throughout the length of the small intestine, always most severe in the jejunum and proximal ileum. Quite often the hemorrhages occured in circular bands, a pattern which I have chosen to refer to as a "Zebra effect". The lumen was generally filled with blood. Occasionally the caecum was congested and contained numerous petechial hemorrhages. Figure 19 is a photograph showing the extent of the reaction compared to an untreated animal.

The only other tissues which were consistently hemorrhagic were the abdominal lymph nodes. The liver was congested and enlarged in most of the animals.

b. Guinea Pigs

Ten guinea pigs were injected intravenously (cephalic vein) with 2 ml. of the soluble complex, Type 1. All of the animals showed clinical signs of anaphylaxis: itching, sneezing, and dyspnea. Three of them died within minutes. Post mortem revealed the lungs to be over-distended. Four other animals died over a period of several hours. Autopsy showed that in addition to the inflated lungs, there were hemorrhages in the stomach and small intestine. The abdominal lymph nodes were enlarged but not severely congested.

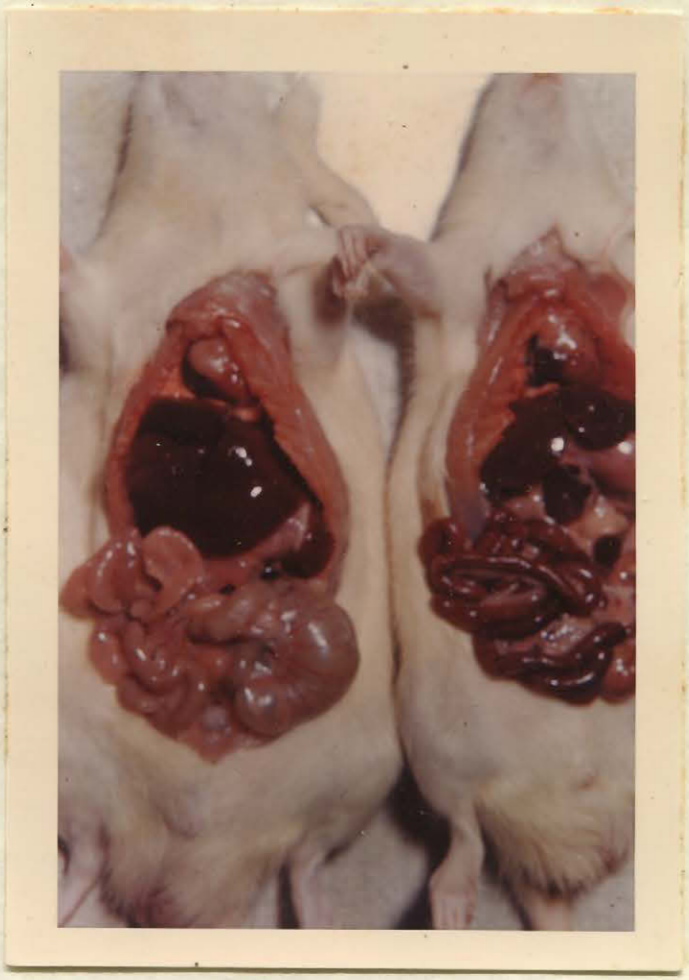

Fig. 19. A photograph showing the effects of soluble antigen-antibody complex in rats. The animal on the left is untreated; the animal on the right received 2 ml. of soluble complex intravenously.

c. Cats

A total of six cats were used, they were anesthetized by intravenous injection of chloralose (100 mg./Kg.), following ether induction. A carotid cannula was inserted for blood pressure recording and a femoral artery was cannulated to collect blood samples. A control blood sample was taken and 4 ml. of the soluble complex (Type II) injected into a femoral vein. In four of the six animals there was a sudden fall in arterial blood pressure (Figure 20), two cats died within four minutes. A second injection of the complex into one cat produced no pressure changes. Blood samples were taken at various times after the injection, usually at 30 seconds and 1, 2, 5, and 10 minutes. The blood was centrifuged and the plasma assayed immediately for its histamine content.

The results of these experiments are shown in Table V. In all cases where there was a drop in blood pressure there was a concomitant rise in plasma histamine. The highest concentration was found within 30 to 60 seconds. This corresponded to the maximal drop in blood pressure. It is interesting to note that no blood pressure changes or plasma histamine changes were seen after the second injection of the complex in cat No. 6. Neo-Antergen tests were performed on the samples from cats No. 5 and 6. They verified that the substance responsible for the contraction of the ileum was histamine.

3. Tests Using In Vitro Preparations

a. Isolated Cells from Rat Peritoneal Cavity

Suspensions of rat peritoneal mast cells were prepared according to the procedure outlined in part 1 of section E.

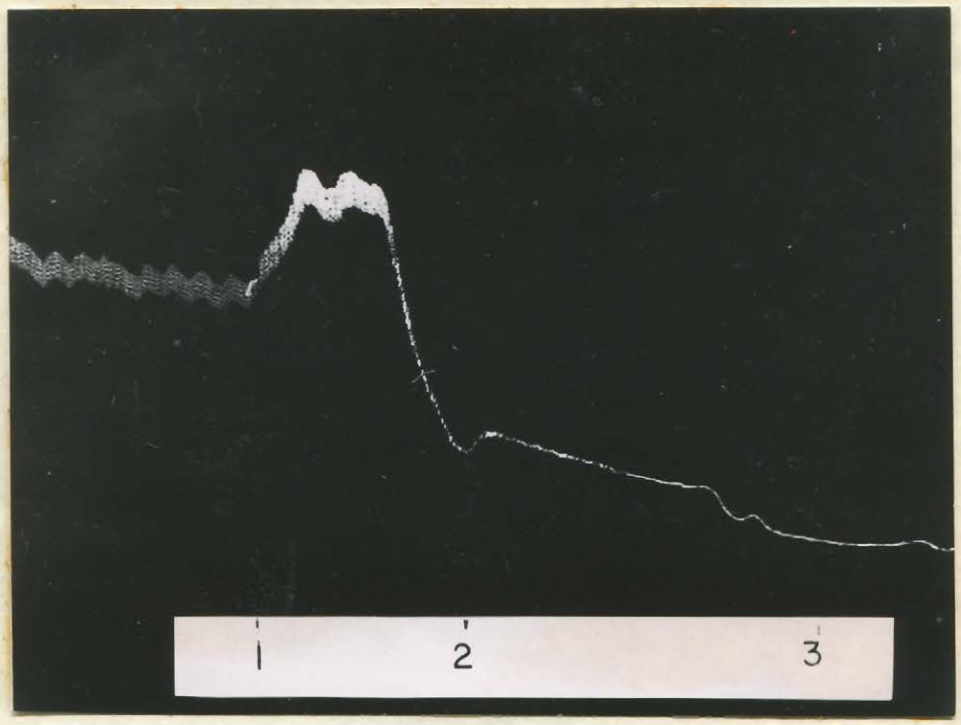

Fig. 20. Blood pressure of a cat injected with 4 ml. of soluble antigen-antibody (Type II). (1) indicates the time of injection of complex which was washed in with 4 ml. of saline. Pressure was 100 mm.Hg. (2) 30 sec. after injection, pressure 40 mm.Hg. (3) 2 minutes after injection, cat died.

TABLE V

The Release of Histamine in Cats Following the Intravenous Injection of Soluble Antigen-Antibody Complex

	Plasma Histamine ng./ml.					
Cat No.	1	2	3	4	5	6
Control	3 ng.	4 ng.	12 ng.	8 ng.	10 ng.	8 ng.
--	4 mg. Soluble Complex Injected					
30 sec.	12	21	12	8	22	10
1 min.	--	20	--	--	--	18
2	8*	16	--	8	14	--
4	--	10	--	--	22*	--
5	--	--	--	7	--	14
8	--	--	12	--	--	--
10	9	--	6	--	--	--
13	--	--	--	--	--	10
-	4 ml. Soluble Complex					
30 sec.	--	--	--	--	--	8
5 min.	--	--	--	--	--	8

* animal died

More care was taken in resuspending the cells after the final wash. This was probably the reason why the free histamine was much less (7.5%) than in the previous experiments with mast cells. One difference in these experiments and the earlier ones was that the cells were suspended in Tyrode rather than a sucrose solution.

In two preliminary experiments, incubation of the mast cells with soluble complex did not result in any increase in the histamine content of the sample supernates. It was felt that perhaps some serum factor was necessary in the reaction. Since rat serum has untoward actions on the guinea pig ileum, it was decided to add fresh cat serum to some of the samples. The samples were prepared according to the outline shown in Table VI. They were incubated in a Dubnoff metabolic shaking incubator at 37°C. for 30 minutes. The samples were then centrifuged at 11,000g for ten minutes and the supernates saved for assay. The assays were always conducted on the same day as the experiment.

The results of seven experiments with rat peritoneal cells are shown in Table VII. Type II complex was used in all the experiments. As can be seen in the Table, soluble complex alone released only slightly more histamine than was spontaneously released in the control samples. This slight increase was not significantly different from the control samples.

In the samples containing soluble complex plus fresh cat serum the average histamine release was 19.7% which is an increase of 14.7% over the control values. Statistical analysis showed these results to be highly significant; $P < 0.02$. Cat serum itself had no histamine liberating activity on the mast cells.

TABLE VI

Scheme for Incubation of Isolated Cells From Rat Peritoneal Cavity With Soluble Antigen-Antibody Complex (Type II)

Tube No.	Cell Suspension	Soluble Complex	Cat Serum	Tyrode	Other
1	1 ml.	---	---	4.0 ml.	---
2	1 ml.	0.5 ml.	---	3.5 ml.	---
3	1 ml.	0.5 ml.	0.5 ml.	3.0 ml.	---
4	1 ml.	---	0.5 ml.	3.5 ml.	---
5	1 ml.	0.5 ml.	0.5 ml.	---	3.0 ml. suc. + versene 0.3 ml.
6	1 ml.	---	---	3.7 ml.	48/80*
T†	1 ml.	---	---	4.0 ml.	---
F‡	1 ml.	---	---	4.0 ml.	---

* 300 µg.

† Total

‡ Free

TABLE VII

Histamine Release From Isolated Mast Cells Incubated with Soluble Antigen-Antibody Complex

Expt. No.	Hist. Cont. µg./ml. Total	Free	% Histamine released in 30 minutes Spont.	S. C.†	S. C. +ser.≠	S. C. +ser. +sucrose	48/80≠	Ser.
166	11.5	0.8	6.5	3.3	23.4	---	91	5.6
168	10.5	0.3	3.9	3.9	41.0	---	100	8.3
171	22.1	0.4	0.9	1.1	9.1	---	--	2.2
173	9.0	2.8	2.8	5.8	10.1	---	100	---
174	8.0	0.6	3.0	12.0	16.5	16.0	100	---
175	10.5	0.3	12.5	16.4	17.5	11.9	100	---
177	12.0	1.2	5.6	8.0	21.0	---	100	---
Ave.	11.9	0.9	5.0	7.2	19.7	13.95	98.5	5.5
t				0.7	3.5*			

† soluble complex

≠ fresh cat serum

≠ 60 µg.

* significant at P = 0.02

In an attempt to determine if calcium ions were necessary for this reaction as they are for the anaphylactic reaction, a sample containing mast cells, soluble complex and cat serum was incubated in 0.32M sucrose solution to which versene had been added. The results of two such experiments were inconclusive. In one there was a histamine release of 16%, a 13% increase over its control, but in the other experiment no increase was found. (Table VII).

Dr. Garcia-Arocha is presently continuing these studies in an attempt to ascertain the nature of the serum factor(s) involved. In one experiment thus far conducted he was able to show that when the serum was heated to 56°C. for 30 minutes prior to the incubation, no histamine was released over its control value.

b. Perfused Guinea Pig Lungs

The lungs of three guinea pigs were prepared for perfusion according to the method outlined in part 2 of section F. Into the perfusion stream of one lung was injected a mixture of 0.25 ml. of soluble complex (Type II) and 0.25 ml. of Tyrode, while a mixture of the complex and serum (taken from the same animal as the lungs) was injected into the opposite lung. A total of 0.867 ug. of histamine was discharged from the lung when the second mixture was injected and 0.337 ug. when the first mixture was used. With the dose used, the complex was at least as effective in releasing histamine as the specific antigen had been. The presence of residual blood in the lungs may explain why the soluble complex mixed with Tyrode released some histamine, though its action was less effective than when the complex was mixed with serum.

c. The Schultz-Dale Reaction

At the beginning of these studies several attempts were made to produce the Schultz-Dale reaction on the guinea pig ileum, but all attempts failed. In view of the later findings that apparently fresh serum is needed for the complex to release histamine, one more attempt was made. An ileum from a non-sensitized guinea pig was set up for histamine assay and the bath temperature adjusted to 37°C. Four tenths ml. of fresh dog serum was added to the chamber and the gut allowed to equilibrate for one minute. Now 0.4 ml. of soluble complex was added. After 30 seconds a maximal contraction occured which lasted for several minutes. After repeated washings the ileum relaxed. When the procedure was repeated no contractions occured. Figure 21 shows the results of this experiment.

It is regretted that sufficient quantities of the soluble complexes were not left to permit the logical continuation of the tonicity studies, however, the experiments which were completed established the fact that histamine is released by the action of these antigen-antibody mixtures and therefore is a significant contribution to the present studies.

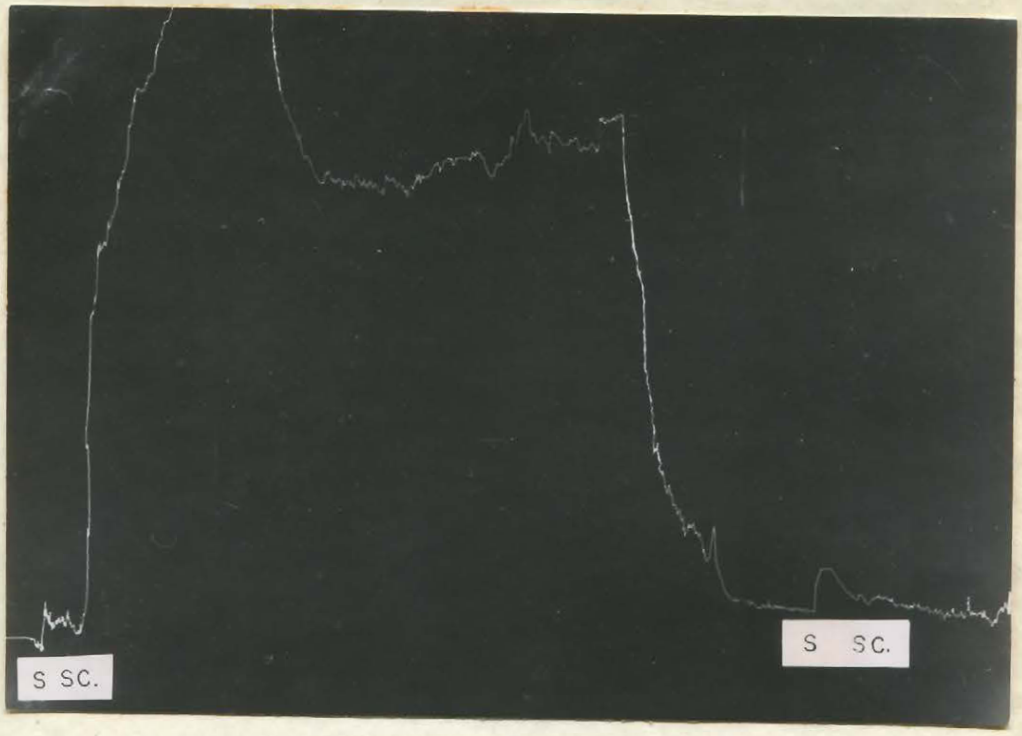

Fig. 21. Tracing of contractions of an isolated guinea pig ileum produced by soluble antigen-antibody complex.
S. 0.4 ml. of fresh dog serum
S.C. 0.4 ml. of soluble complex

Discussion

The results presented in the experimental section of this thesis are clear-cut and consistent. The task lies therefore in the correct interpretation of these findings. Throughout the program of studies the experiments were planned and conducted in the hope of providing evidence which could enlarge on our present knowledge of the manner in which histamine is bound in the tissues. The results of these investigations will be discussed in relation both to histamine binding in the mast cells and to the mechanism by which it is released from these cells. In a separate division of the disucssion the action of soluble antigen-antibody complexes in releasing histamine from *in vivo* and *in vitro* preparations will be considered.

1. Histamine Binding

With regard to the problem of histamine binding, one fact appears to be positively established. That is that much of the histamine in the body is stored in tissue mast cells. Beyond this much controversy exists. The tissues used in the present work were all rich in mast cells; and the evidence presented in the introduction justifies the conclusion, at least as a first approximation, that histamine release as it has been studied in this investigation was in fact a phenomenon concerned with mast cells.

The experimental findings of this thesis, as applied to the mode of binding of histamine, can be explained in several ways. The most plausible of these possibilities will be ex-

amined and an attempt made to establish whether or not they adequately explain the results.

The action of a series of organic bases in releasing histamine from dog liver particles was determined both in isotonic and hypertonic (1.2M) solutions of sucrose (Fig. 3). All the compounds reacted similarly; the initial rate of histamine release was reduced by approximately 50% in the hypertonic sucrose solutions. This finding, that less histamine is released in hypertonic solutions, was conspicuous in all of the other preparations used in the course of this study (Fig's. 7, 9, 15, 17; Table IV).

The decrease in the rate of transfer of histamine from the particulate to the liquid phase could possibly be the consequence of changes in the molecular configuration of proteins (to which some workers believe histamine is bound) brought about by the dehydrating action of the hypertonic solution. While it is true that dehydration can denature some proteins (Mirsky 1938), this does not seem to be the answer in view of results shown in Fig. 4. If the inhibitory action had been due to any denaturation, the effects would have been expected to be irreversible. And in general it would seem more likely that protein denaturants should diminish rather than increase the affinity of histamine for the material to which it is bound; thus protein precipitants like alcohol, trichloroacetic acid and $HgCl_2$ are all releasers of histamine, and have been used to extract histamine quantitatively from tissues. However, one could suppose that configuration of proteins may be reversibly altered by changes in ionic environment or in osmotic pressure, as in

salting-out effects; in this case ionic strength of cell interior might be increased, by concentration of intracellular K^+ ions. Such changes in the composition, as contrasted with the volume, of the intracellular fluid might influence the histamine-binding capacity of a hypothetical protein. But in general, it would be expected that the effect of increased ionic strength would be to dissociate a histamine-protein complex, rather than to increase its stability. Similarly, if histamine is held within cells by being bound with heparin (MacIntosh 1956) or some other polyanion like a nucleic acid or high-energy phosphate (Paton 1956) the effect of increased ionic strength on such a binding would presumably be to promote dissociation; in much the same way as the metachromatic complex of heparin and the base Toluidine blue is dissociated by salt (MacIntosh 1941).

From a cursory glance at Table II, one might suspect that the suppression in the rate of histamine release could be attributed to the direct action of the sucrose per se. For this reason many experiments were repeated using mannitol in place of sucrose. Comparable results in these experiments (Fig's. 5 and 12) precludes the possibility that sucrose by itself was responsible for the effects seen with the 1.2M solutions.

A third explanation for the depression in the rate of histamine release could be made on the basis of the osmotic pressure generated by the hypertonic solution. As far as is known the only effect of sucrose (or mannitol) which is a relatively non-penetrating solute, is to set up an osmotic gradient across a membrane causing a decrease in the volume of the structure enclosed by the membrane. This is my working hypothesis; it will be discussed more fully in the following paragraphs.

In striking contrast to the action of the basic histamine liberators, the surface-active compounds were equally effective in iso- and hypertonic solutions. The surface active properties of saponin and sodium taurocholate have been known for many years although the exact mechanism for their hemolytic action is still unknown. The evidence of Berheimer (1947) suggests that saponin and sodium taurocholate act similarly. According to Rideal and Taylor (1957), saponin and other anionic detergents cause hemolysis of red blood cells by attacking the lipoprotein-cholesterol complex. It is assumed that this occurs with other membranes. One must assume that histamine is either attached to the membrane itself or it is contained within the granule since the lytic agents act directly on the granule membrane. [Mast cell granules have been reported by several workers to possess a limiting boundary (Bloom, Larrson & Smith 1957; Smith & Lewis 1957). These observations along with those of Zollinger (1950) that the granules exhibit an osmotic behavior, strongly suggest that mast cell granules are clearly defined structures possessing a surrounding membrane.] That histamine is attached to the granule membrane seems unlikely in view of the decreased activity of the organic bases in hypertonic solutions, for it is inconceivable that mere crenation could inhibit the action of these compounds.

If, on the other hand, histamine is located within the granule it must be held by only a weak bond since it diffuses out so easily (McIntire 1956). A very weak linkage is also indicated by the wealth of data which shows that histamine can be released by purely physical procedures such as would not be expected to rupture a primary chemical bond. Distilled water is

an excellent histamine liberator both in vitro from perfused guinea pig lungs (Schild 1936b) or intraeellular particles (Hagen 1954; Grossberg & Garcia-Arocha 1954), and in vivo in rats (Riley & West 1953a; Fawcett 1954, 1955). Other procedures such as freezing and thawing (Hagen 1954), heating (Trethewie 1938), and treatment with acetone (Grossberg & Garcia-Arocha 1954) quantitatively release histamine from its tissue storage site.

The evidence which supports the idea that histamine is held by a peptide bond is based on the finding that histamine is released by trypsin (Rocha e Silva 1938). This concept leads to the assumption that histamine release requires the activation of a trypsin-like enzyme. However, it seems to be disproved by the observation that histamine can be released from dog liver particles in the presence of soy bean trypsin inhibitor at 0°C. (Grossberg & Garcia-Arocha 1954). The histamine-releasing action of trypsin could, of course, be explained on other bases. Thus trypsin could rupture peptide bonds in the protein component of a membrane and thus cause the membrane to rupture or change its permeability, allowing histamine held in storage behind the membrane to leak out. Or trypsin, since it acts on peptide bonds to which L-arginine or L-lysine has contributed a carboxyl group, might set free polypeptides containing these basic amino acids, which could well be active histamine liberators.

Histamine is a fairly strong base (it can be considered as an amine, a diamine or an amidine); it might not remain freely dissociated within the granule under physiological conditions. There are several large polyanions that might easily bind

histamine with a loose polar bond. Most notable of these is heparin. Jorpes (1946) has stated that heparin is a normal constituent of mast cell granules. While Julén and her co-workers (1950) disagree with this belief, most of the recent work shown in the introduction tends to agree with the opinion of Jorpes.

The idea that heparin and histamine will combine has been advanced several times. Germendás, Csefkó and Udvardy (1948) found that histamine opposed the anticoagulant activity of heparin. Later Parrot and Laborde (1951) noted not only that heparin antagonized the effects of histamine on the guinea pig ileum, but that when a histamine solution is dialyzed against a heparin solution the histamine concentrated in the heparin compartment. Sanyal and West (1956) have succeeded in combining histamine and heparin sufficiently strong enough that the complex will precipitate out of solution. In addition to combining with histamine heparin has a strong affinity for many basic histamine liberators. MacIntosh (1956) suggested that the attraction of the liberator for heparin could be responsible for the accumulation of the liberator molecules in the mast cell, which Riley (1953b) showed so well with stilbamidine.

Another possible candidate is ribonucleic acid. A good correlation exists between the histamine releasing activity of several organic bases and their ability to react with RNA (Paton 1956). Paton suggested that combination with this polyanion could explain the binding of histamine in cells other than mast cells. There are, however, several objections to this compound in a histamine trapper. First, RNA is found

mainly in the cytoplasm of cells, or in the extremely minute granules which Palade found adherent to the endoplasmic reticulum and which sediment with the microsome fraction in sucrose homogenates, whereas histamine has been found to sediment with the mitochondrial fraction in many preparations, and is therefore associated with a much larger kind of granule (Barnett, Hagen & Lee 1958). This makes it seem unlikely that RNA binds histamine in mast cells since it appears that histamine is associated with intracellular granules (Hagen 1954; Grossberg & Garcia-Arocha 1954; Mota et al. 1954; West 1955; Barnett, Hagen & Lee 1958). A second objection can be based on the specificity of histamine liberators. Riley (1953b) showed that stilbamidine concentrated only in mast cells. Pathological examination of tissues subjected to the action of histamine liberators reveals disintegration of mast cells while other cells appear normal (Fawcett 1955). Now all cells (other than mature erythrocytes) contain RNA, and there is no evidence that mast cells contain more than other cells. Were the liberators attracted by RNA, they would be expected to show no preference to mast cells, and, in general, much higher concentrations of the liberators would be required to bring about the release of histamine from tissues. Humphrey and Jaques (1955) showed that 48/80 does not release histamine from rabbit platelets, which of course contain RNA.

Adenosinetriphosphate (ATP) has also been considered as an anionic substrate to which histamine could attach itself. Darlow (1956) has stated that organic base histamine liberators will complex with ATP, but once again the same objections may be made against this compound. There is some evidence which

indicates that ATP could in some way be involved in the intracellular binding of histamine. Sanyal and West (1956) reported that when solutions of heparin and histamine are mixed under suitable conditions about 70% of the histamine is removed from solution. If ATP is added to the system, the uptake of histamine by the heparin is increased to over 90%. It would not be surprising that histamine, which contains two basic groups (one of them the amidine group which has good possibilities for forming resonance-stabilized complexes) might act as a kind of chemical "glue" causing increased association of polyanions with loose binding of the histamine itself.

Blascko and his associates (1956) found that ATP and catechol amines are concentrated in the large granule fraction of homogenates from bovine adrenal medulla. In normal platelets a proportionality was found between the amounts of ATP and 5HT before and after incubation with plasma containing 5HT (Born, Ingram & Stacey 1958). These observations are not concerned with the binding of histamine but they do demonstrate that some relationship may exist between ATP and the storage of physiologically active amines. This relationship may be to some extent one of direct binding. But the unique association between histamine and heparin in mast cells suggests, though it does not prove, that heparin is a better candidate for the role of histamine-binding polyanion. In other kinds of cellular element with the ability to bind histamine, other polyanions may be more important. Thus rabbit platelets do not, so far as I know, contain heparin, though they are rich in histamine; and it is perhaps relevant that 48/80 does not release histamine

from these platelets (Humphrey & Jaques, 1955). The same may be said of the gastric mucosa, on which, again, 48/80 has only a trivial histamine-releasing action (Smith 1953).

2. Mechanism of Release

The development of the use of histamine liberators in the past decade has stimulated research concerning the mechanism of histamine release. It is not surprising, therefore, that several theories have evolved from these studies. Basically these theories fall into two broad classes: enzymatic and non-enzymatic. In general, the enzymatic theories have arisen from the observation that proteolytic enzymes appear in blood during anaphylactic shock (Ungar 1947). I do not deny the participation of enzymatic reactions in the anaphylactic reaction. This seems to be clearly established by the recent work of Mongar and Schild (1958). Participation in this reaction, however, does not necessitate the assumption that an enzyme is the ultimate causal agent responsible for the release of histamine. Indeed, fibrinolysin and streptokinase both fail to release histamine from dog liver particles (MacIntosh 1956), and are at best only very weak in discharging histamine from rat mast cells (Högberg & Uvnäs 1957). Another objection to a protease theory for histamine release is the lack of evidence that histamine is bound to a protein in the mast cells. On the contrary, the evidence stated in the previous section is against the existence of such a firm binding. Ungar's latest modification of his theory (1956) is somewhat complicated, involving kinases in the blood and cells which convert proactivators into activators, which in turn transform protease precursors into active

proteases. The proteases are supposed to attack the polypetides which bind histamine, but unless it can be shown that histamine is attached to a protein, the whole scheme is pointless.

Feldberg and Kellaway (1938b) postulated that lysolecithin might be the active agent in anaphylaxis, suggesting that histamine might be linked to a lipoprotein from which it would be released by lysolecithin. The basis for this theory was that snake venoms which contain lecithinase A, an enzyme which converts lecithin to the lytic lysolecithin releases histamine. Högberg & Uvnäs (1957) recently showed that in a series of 25 enzymes they tested, only lecithinase A efficiently disrupts rat mast cells. It may well be that the action of lecithinase A is directly on the cell and granule membranes in a manner similar to that of the surface active compounds. This could explain why lecithinase A is such a poor releaser in perfused cat paws. It is well to point out here that saponin, which is a fair histamine liberator on rat mast cells (Table IV), was quite weak in perfused cat paws (Fig. 10).

A somewhat different enzyme theory than those discussed above, was recently proposed by Högberg and Uvnäs (1957; Uvnäs 1958) to explain the disruption of mast cells by 48/80. They envisage a lytic enzyme situated on the outer surface of the mast cell membrane. This enzyme is normally kept inactive by the presence of an inhibitor . 48/80 supposedly removes the inhibitor thus allowing the enzyme to attack the cell membrane. They presented evidence that the action of 48/80 is blocked by various enzyme inhibitors but Mongar and Schild

(1956) reported that oxygen lack or metabolic inhibitors potentiate the action of 48/80 on minced guinea pig lung.

One obvious weak point in this theory is that it does not explain how 48/80 releases histamine from isolated cell particles. Certainly the same explanation will not suffice. Therefore, if their theory is correct 48/80 must have two separate actions in releasing histamine or else the same enzyme-inhibitor complex must be postulated as being present on the outer surface of the granule as well as on the outer surface of the cell.

One of the theories canvassed by MacIntosh and Paton (1949) was that histamine liberation by bases involves a process of ionic exchange. Such a theory does not require the participation of any enzymes and fits well with the idea that histamine is held by a loose bond. It arose from the fact that all the histamine liberators tested by MacIntosh and Paton were basic substances. In the light of the experimental findings reported here, a possible displacement theory of histamine release (McIlreath 1959) will be discussed.

If histamine and heparin are assumed to be held inside the mast cell granule, the liberator must in some way induce a transfer of these compounds across the membrane. This could be accomplished in several ways. One possibility is that the liberator could inhibit an active transport system similar to the sodium pump of Hodgkin and Katz (1949) which might be supposed to normally keep histamine confined within the granule. Such a mechanism was considered by Darlow (1956) and McIntire (1956), and while such a system is theoretically possible, it seems highly improbable considering the very slow rate of

histamine release from dog liver particles stored for long periods at temperatures low enough to reduce the activity of any active transport system to a negligible low level.

Another possibility might be that the liberators could release histamine chemically bound within the granule, thus allowing its free diffusion across the granule membrane. Objections to this type of release were cited in the previous section. In addition, if the basic histamine liberators were mere base-exchangers, one would not expect to see the violent disintegration of the mast cells which occurs when histamine liberation results from the action of these compounds.

A third possibility is that the basic liberators could release the active amine by altering the physical structure of the cell membrane itself. Grossberg and Garcia-Arocha (1954) considered the action of the basic histamine liberators to be a lytic one, involving the rupture or damage of the mast cell granule membrane. If this is true, one would expect basic histamine liberators to show activity at other cell surfaces. Grossberg (1954) found, indeed, that most liberators were active in causing hemolysis of washed red blood cells, but the action was a very weak one; and in vivo. These substances do not cause hemolysis when injected intravenously even in lethal doses. Moreover, an action on the cell membrane alone is insufficient to explain histamine release, since mechanical disruption of the cells when a tissue is homogenized still leaves much histamine bound within granular cell components, from which it can be released by liberators.

Norton (1954) also suggested that 48/80 increased membrane permeability, but her reference was to the cell membrane. She based her reasoning on the finding that hypotonic solutions did not accelerate cellular disruption induced by 48/80, if an osmotic action were involved, she said, disruption should have been hastened. It is possible that her results are not valid, if one supposes that the hypotonicity itself increases the volume of the cell. Under these conditions the entry of a given amount of 48/80 would lead to a lower concentration of 48/80 than if the medium were isotonic.

Paton (1957) indicated that some of the histamine releasing activity of octylamine might be due to surface activity, because its time course of histamine release is much slower than that of 48/80, it releases potassium, and it produces more edema in perfused organs.

Another possibility is that the liberator molecules could aggregate inside the granule and through an osmotic action cause a mobilization of water molecules within the granule. This would result in a swelling of the granule and an increase in the permeability or rupture of the granule membrane. It is this mechanism that will be proposed here.

It is easy to imagine how the liberator molecules are accumulated within the granule; its affinity for heparin would be sufficient to cause this. But how would the liberator increase the osmotic pressure in the granule? If it is attracted into the granule by the presence of heparin it certainly would be expected to enter into a combination with it. Therefore, it might be argued that the liberator would contribute nothing

to the osmotic pressure. Suppose, however, that the histamine of the granules is normally combined with heparin by a loose polar bond and so contributes little to the osmotic pressure of the granule interior. And suppose, further, that the liberator has more affinity for heparin than histamine has, and will therefore displace it from its combinations. The displaced histamine molecules would therefore be, at least temporarily, freely dissolved in the water of the granule interior. Histamine (unlike the most active histamine liberators whose molecules all contain a large, predominantly hydrocarbon moiety) is probably poorly soluble in lipids; at any rate it is not extracted from aqueous solution by non-polar solvents, even at alkaline pH. It might therefore be expected to pass out of the granule interior only slowly; at any rate less rapidly than the liberator would be entering. Free histamine base would therefore tend to accumulate inside the granule, and this accumulation would have two effects. First, the pH of the granule water would be instantaneously raised by dissociation of water, just as introducing free ammonia into water produces an excess of OH^- ions. Secondly, the osmotic pressure of the granule fluid would increase, because of the osmotic effect of the histamine itself and also because of the anions that might move into the granules along the charge gradient set up by the histamine as it became freely ionized. The physical chemistry would be rather complex, but undoubtedly there would be a tendency for the granule to swell and for its contents to become more alkaline.

The number of osmotically active particles and the pH would increase in proportion to the number of histamine molecules freed from their heparin complex. Dougherty & Schneebeli (1958), indeed, have been able to show a swelling of the granules during the slow disintegration of subcutaneous mast cells from rats.

The concentration of histamine in mast cell granule is very high. Graham's (Graham et al. 1953) figure of 6 uug./cell can be used to roughly estimate the molar concentration of histamine in the mast cell granule*. Estimating the granule sap to comprise 50% of the cell volume, a figure which is consistent with the histological and electron micrographic evidence (Bloom Friberg and Larrson 1956), the histamine concentration would be 181 mM. This, if the histamine could be suddenly made osmotically active and balanced by anions, would, within limits, represent an isotonic solution. If all the histamine in 10% of the granules was suddenly released from its binding sites, and if osmotic readjustment were effected mainly by entry of water into the cell to replace the cell sap attracted into the granules, there would be at least a 10% increase in cell volume. If anions also entered the cell to balance the excess of (histamine) cation, the increase could reach 20%. Some increased influx of anions would probably have occurred along with the initial penetration of the histamine liberator, since the molecules of most liberators are partly ionized at the pH of the tissue fluids, this much of an increase in cell volume could rupture the membrane. The cell membrane of mast cells, it may be relevant

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*average diameter of mast 10 μ (Bloom, Friberg & Larrson) treating the mast cell as a sphere volume = $4/3\ \pi\ r^3 = 523\ \mu^3$.

to note, exhibits an irregular contour in electron micrographs, with small finger-like outward projections (Bloom, Friberg & Larrson 1956). If these are not artifacts of fixation, it is tempting to suppose that they might serve the purpose of allowing the cell to swell without much change in internal pressure and therefore without disruption, when for any reason a small amount of histamine-releasing substance entered. Under these circumstances, the amount of histamine set free would be, at a maximum, equal to the amount of liberator entering.

This then, can explain the rupture of the cell as a whole. What is the fate of granules in such a cell? They might, indeed, have already undergone osmotic ruption with release of their contents. If not, they would be bathed in a mixture of extracellular and intracellular fluid, in which a number of enzymes might well have become activated. It would not be surprising if their membranes suffered further damage. The change of Na^+ for some of the K^+ in the cytoplasmic reaction might have such an effect. Ionic changes can affect the stability of these granules; for instance, it has been shown that dog liver particles are much more stable when they are suspended in isotonic sucrose than when they are in 0.9% NaCl or Tyrode (Grossberg 1954).

This theory, although it involves base displacement, readily explains Feldberg and Paton's finding that one molecule of 48/80 releases 10 - 100 molecules of histamine. The liberator would only have to act on a fraction of the granules in a cell to release all the histamine in this cell. Since the granules are so numerous and so densely packed, the liberator would preferentially concentrate in the granules situated along the periphery of the cell.

It is implicit in this theory that mast-cell granules discharged by rupture of the cell will soon rupture themselves or at least release their charge of histamine. This idea is plausible, enough in itself, since isolated mast-cell granules rapidly lose their histamine in vitro unless special precautions are taken to preserve them. Numerous authors have described the histological appearance of disrupted mast cells with granules lying loose in the tissue fluid, and of metachromatic "haloes" which presumably represent heparin that has escaped from the granules and been trapped by temporary combination with tissue proteins. (Heparin indeed probably tends to remain attached to the structural protein of the granules by residual valences, and is not released nearly so rapidly as histamine, even from a preparation of isolated particles (Garcia-Arocha 1958).) The theory would imply further that the rate at which histamine was released into the circulation by the action of a histamine liberator would depend on the composition of the extracellular fluid for a medium that tended to preserve the integrity of the granules would delay or prevent the release of histamine from the granules that had not already burst. (Such granules, lying free in the tissue spaces, might not even at this time be too much swollen, since if the dose of liberator were not too great, it might have been mostly taken up by the heparin in the granules lying most peripherally in the cell.) One would expect, for instance, that if a sucrose solution were used for perfusion much less histamine would be released by a liberator than if Tyrode was used, since isolated granules are much more stable in sucrose than in Tyrode (Grossberg 1954). This is in fact the case, as Fig. 11 shows; the difference is a ten fold one. With sucrose perfusion, in fact,

it is probable that a molecule of 48/80 can immediately release, at best, one molecule of histamine; in other words that simple base-displacement operates. In the short perfusion time used in these experiments, one molecule of 48/80 released less than one molecule of histamine from paws perfused with isotonic sucrose. A further corallary of the theory would be that if a liberator were injected into the stream of sucrose solution perfusing limb, bringing about a release of some of the tissue histamine, then after the release were finished much more histamine could be released simply by changing over to Tyrode perfusion without further injection of liberator. Unfortunately this test did not occur to me until the experimental work for the thesis had been completed.

My interpretation for the suppression of histamine release in hypertonic solutions now becomes more clear; the action of the liberator tends to draw water into the cell while the hypertonic solution tends to remove it; then, swelling of the cell would occur less rapidly and histamine release would be retarded. The fact that the reduction in histamine release from dog liver particles was approximately the same with all the basic liberators tested (Fig. 3) makes this supposition more likely.

Undoubtedly, the surface-active compounds act through a different mechanism. They cause a break down of either the cellular or the granule membrane or very likely of both membranes by a direct action rather than by osmotic bursting. There is no reduction in histamine release in hypertonic solutions because after rupture of the membrane, no pressure gradient can be set up between the exterior and interior of the cell (or granule).

One consistent finding is that histamine is released abruptly from tissues of intact cells (Feldberg (Paton 1954; Garcia-Arocha 1958; McIlreath 1959) but slowly from cell particles (Grossberg loc. cit. MacIntosh 1956; Mongar & Schild 1956; McIlreath 1959). The cause for the explosive action in cells is that, as explained above, many molecules of histamine are released by a few molecules of the liberator. When cells are homogenized only a small portion of the granules remain intact. These may be the ones that are strongest physically and perhaps also those that are youngest and contain the least histamine and heparin. In this preparation the liberator can only release histamine from the granule in which it is concentrated. Also, there is probably a diffusion of displaced histamine from the particle rather than a rupture of the granule because the liberator will enter all the granules instead of only a few as in the case of intact cells making the effective concentration of liberator per granule lower and the swelling less severe. Such considerations as these may perhaps account for the somewhat awkward fact that the release of histamine from isolated particles when a liberator is added is slower by a couple of orders of magnitude, than the release that occurs when the same concentration of liberator reaches intact cells via the blood stream. If the course of release from the granules had been followed over the entire period, the curve might have tended to take a sigmoid shape. It is, in retrospect, unfortunate that this was not done.

It was found that when the anaphylactic reaction was used to release histamine from perfused guinea pig lungs, much less histamine was cleared from lungs perfused with hypertonic Tyrode than from those with normal Tyrode (Fig. 15). The similar-

ity between these results and those obtained with organic bases (Fig. 16) could be interpreted as meaning that as a consequence of the anaphylactic reaction an endogenous basic histamine liberator is formed. Mongar and Schild (1956) have also suggested this possibility. One may only speculate as to the nature of such a substance. Conceivably, it could be a polypeptide released as the result of proteolytic action on some protein substrate. Other explanations, however, are certainly possible.

I do not think at the present time it is possible to absolutely rule out anaphylatoxin as being the natural histamine liberator formed during the anaphylactic reaction. Results obtained in this study show that histamine release induced by rat serum anaphylatoxin in suppressed by hypertonic solutions (Fig. 17), compared to its action in isotonic solutions. In addition, the results suggest that calcium ions, which are so important in the anaphylactic reaction, may also be necessary for the release of histamine by anaphylatoxin (Fig. 18). This evidence adds to the many similarities already known about these reactions, although as Sir Henry Dale (1956) pointed out, they may be no more than similarities having no actual realtionship.

3. Soluble Complex

Research has now reached the point where experimental anaphylaxis can be produced in non-sensitized animals. This represents a major step in our attempt to learn the mechanism of the anaphylactic reaction. The most significant findings of this investigation are: (1) antibody from one species, when mixed with appropriate amounts of its specific antigen, will

release histamine in other species, and (2) a serum factor appears to be necessary in this reaction.

My own findings that histamine is released from both *in vivo* (Table V) and *in vitro* (Table VII) preparations in the presence of soluble complex indicates that anaphylactic reaction is basically the same in different species. From our present knowledge concerning this phenomena, however, it does not seem justifiable to assume, as Germuth and McKinnon (1957) did, that the soluble complex itself liberates the histmaine. This complex has to be a very large unit, with an equivalent molecular weight of the order of 200,000. It is a little difficult to imagine that a molecule of this size could traverse the capillary wall and cellular membrane with sufficient speed to account for the suddenness of the reaction. Experiments could (and should) easily be devised using metabolic inhibitors to determine at what stage of the anaphylactic reaction the soluble complex acts.

One major difference between this reaction and the *in vitro* anaphylactic reaction is that fresh serum is needed for the soluble complex to be active *in vitro*. It is possible that when the antigen and antibody combine in the body, sufficient co-factor(s) is provided to allow rapid completion of the reaction. However, when antigen and antibody combine *in vitro*, presumably the co-factor(s) is not present, and some sort of a catalyst is necessary to start the chain reaction when the soluble complex is introduced to the system. What this factor(s) is can not be determined on the basis of the experiments performed thus far. Because of the need for the serum to be fresh, and in view of the results of the previously mentioned experiment

of Dr. Garcia-Arocha, that heating the serum to 56°C. will destroy its ability to complete the reaction, it is tempting to guess that the factor might be complement. One puzzling feature clouds the issue: a second injection of the complex into a cat was without effect. It does not seem likely that all the complement could be exhausted by the first injection, especially since a second Dale-Schultz reaction could not be elicited in the guinea pig ileum even in the presence of fresh serum.

There is no doubt that these studies merit further investigation. More experiments must be performed to ascertain the nature of the serum factor(s) and to explain why only antigen-antibody mixtures dissolved in excess antigen are active as histamine liberators.

Summary

1. A review has been made of the literature pertinent to the present investigations. This includes a review of mast cells, which are now known to be a major site for the storage of histamine, and an examination of the present knowledge concerning the means available to study the ways in which histamine can be experimentally released.

2. The action of a series of organic bases in releasing histamine from dog liver particles was studied in isotonic and hypertonic (1.2M) solutions of sucrose. Statistical analysis of the results showed that significantly less histamine was released from liver particles suspended in hypertonic solutions.

3. Histamine release from dog liver particles incubated with basic histamine liberators could be represented by a straight line curve. This indicates a suppression in the release mechanism rather than a blocking of it. The effects of the hypertonic solution were found to be reversible.

4. The action of lytic agents is different from the organic bases. No significant differences were found in the amount of histamine released by saponin and sodium taurocholate in isotonic and hypertonic solutions of sucrose.

5. When mannitol was substituted for sucrose, essentially the same results were obtained. Efforts to analyze the actions of electrolytes (Na^+, K^+, Ca^+) in this respect were futile.

6. Histamine release was studied in perfused cat paws. The action of organic bases and surface active compounds in releasing histamine from paws perfused with isotonic and hypertonic solutions of sucrose and mannitol was the same as in the liver particles.

7. It was found that histamine (endogenous and exogenous) produced no constriction of the blood vessels of cat paws perfused with sucrose solutions. Further experiments performed to explain these results indicate that the presence of Na^+ ions is necessary for histamine to be able to cause vasoconstriction in cat paws.

8. Evidence presented in this thesis suggests that histamine is explosively released from perfused cat paws into the extracellular spaces and it is then slowly washed out by the perfusing fluid.

9. A method is described for the isolation and incubation of cells from rat peritoneal cavity. With this method the action of various histamine liberators was studied in isotonic and hypertonic sucrose solutions. The findings were the same as with the two previously mentioned procedures. It was calculated that approximately 5,150,000 mast cells were obtained from each animal.

10. A new method for the perfusion of guinea pig lungs, which permits the perfusates from each lung to be collected separately, was described. Using this technique, the action of the anaphylactic reaction was studied in isotonic and hypertonic Tyrode.

11. Control experiments showed that not only is the same quantity of histamine contained in both lungs, but that a given dose of the antigen will release equivalent amounts from each lung.

12. Rat serum anaphylatoxin was used to liberate histamine from perfused guinea pig lungs. Significantly less histamine was discharged from lungs perfused with hypertonic Tyrode.

13. Experimental findings indicate that calcium ions are necessary for anaphylatoxin to liberate histamine from perfused guinea pig lungs.

14. Antigen-antibody mixtures dissolved in excess antigen were prepared according to two different methods. Injection of these soluble complexes into rats and guinea pigs brought on symptoms of profound anaphylaxis, and death to most of the animals.

15. A sudden drop in arterial blood pressure and a concomitant rise in plasma histamine was observed in four of six cats injected with soluble complex. The shock proved fatal to two of the animals. In another, a second injection of the complex produced no changes in blood pressure or plasma histamine.

16. When isolated mast cells were incubated with soluble complexes, significant amounts of histamine were released if fresh serum was added. Attempts to ascertain whether calcium ions were necessary in the reaction were inconclusive.

17. Considerably more histamine was liberated from perfused guinea pig lungs when fresh serum was injected with the soluble complex.

18. A Schultz-Dale type reaction was elicted in a guinea pig ileum only when fresh serum and soluble complex were present in the organ bath at the same time. A second addition of the soluble complex did not cause a contraction of the ileum.

19. The results of the experiments were discussed with regard to the mode in which histamine is bound in mast cells and to the mechanism of its release.

20. Theories are proposed to explain the mode of histamine in the mast cells and the mechanism of its release by organic bases.

Claims to original research are included in the above summary.

Appendix

This is a list of the compounds, other than common chemicals, which were used in the studies reported here, and the source from which they were obtained.

1.	Agar (shredded)	British Drug Houses, Ltd., Montreal
2.	Albumen (egg) powder	British Drug Houses, Ltd., Montreal
3.	Crystallized Bovine Plasma Albumen	Armour & Co., Kankakee, Illinois
4.	Decamethyline diamine	H.M. Chemical Co., Ltd., Santa Monica, California
5.	48/80	Burroughs Wellcome & Co., Inc. Tuckahoe, New York
6.	Histamine (dihydrochloride)	Fisher Scientific Co., Montreal
7.	Neo-Antergan (maleate)	Poulenc Ltd., Paris, France
8.	Saponin (white)	British Drug Houses Ltd., Montreal
9.	Sodium taurocholate	Merck & Co., Inc., Montreal
10.	Stilbamidine (isethionate)	May & Baker, Ltd., Dagenham, England
11.	Toluidine Blue "O" certified for use in histology	Anaechemia Ltd., Montreal
12.	Tris(hydroxymethyl)aminoethane	Brickman & Co., Montreal

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