THE INTESTINAL MAST CELL
IN NORMAL AND PARASITISED RATS

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57 8

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CONTENTS

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GENERAL INTRODUCTION

Parasitic infections in man and animals continue to be the cause of severe social and economic problems on several continents; even in countries where highly efficient methods of agriculture have been developed, parasitism remains a source of great financial loss to the farming community. Measures to combat infection include treatment with anthelmintics, improvement of hygiene, elimination of vectors and intermediate hosts as well as efficient husbandry. But these methods are often costly and not always practicable and in many instances a different approach is required.

A major development in the prevention of parasitic infections was the introduction of vaccination techniques employing living irradiated larvae (Jarrett, Jennings, McIntyre, Mulligan and Urquhart, 1960; Urquhart, Jarrett and Mulligan, 1962). But, as yet, many of the fundamental aspects of the immunity conferred by such techniques remain poorly understood. This has led to a renewed interest in the immune mechanisms involved in the expulsion of parasites from the host.

It is essential to have an experimental system for exploratory studies which allows a detailed analysis of many of the factors involved. Large domestic animals are unsuitable because of the time and expense involved in quantitative experiments. Nippostrongylus brasiliensis in the rat offers an excellent model system as it is easily quantitated, produces a good immunity and the life cycle is on a reasonable time scale.

This thesis is a study of the role of the mast cell and the mediators released by it in the immune response of the rat to this parasite.

INTRODUCTION

The occurrence of immediate hypersensitivity reactions in a variety of hosts harbouring parasites has been noted for many years (see Review by Andrews, 1962). Relatively recently there have been suggestions that such phenomena could be beneficial to the host and evidence supporting this hypothesis continues to accumulate and is discussed in the appropriate part of this introduction.

Much research has been directed towards characterising the antibodies involved in mediating immediate hypersensitivity reactions and there has been a number of experiments to test the effects of these immunoglobulins on a target cell, the mast cell (see Review by Keller, 1966). Less attention has been directed towards the role of the mast cell in immediate hypersensitivity reactions in vivo and little is known about the role of this cell during parasitic infections.

The Mast Cell

The ubiquity of mast cells in vertebrates and the many publications on aspects of their morphology, cytochemistry and immunological and pharmacological properties are an indication that they serve an important function in the body. The present discussion is confined to certain relevant aspects of mast cell structure in relation to their possible function. Their historical background has already been the subject of numerous reviews of which the most noteworthy include those by Michels (1938), Riley (1959), Benditt and Lagunoff (1964), Selye (1965) and Keller (1966).

The mast cell is a connective tissue cell whose cytoplasm is packed with granules which stain metachromatically with toluidine blue (Michels, 1938). Jorpes, Holmgren and Wilander (1937) observed a relationship between mast cell numbers and the heparin content of various tissues. Since then it has been shown on several occasions that the mast cell granules contain heparin and possibly other, highly sulphated, acid mucopolysaccharides (Schiller and Dorfman, 1959; Schiller, 1963; Horofield and Summerly, 1966). Histological identification of mast cells has, until recently, relied upon the metachromasia of the granules after staining with basic dyes such as toluidine blue. With the development of new dyes and with a greater knowledge of the binding of polycations and polyanions in tissues, the basic dye techniques have been superseded by new methods which allow more specific recognition of polyanions; these are discussed in Section I.

A basic protein is present in mast cell granules and in some species may be identified by histochemical means (Spicer, 1963; Steward, 1968). The cationic protein and polyanionic heparin bound together by ionic linkages in the granules forms a relatively stable matrix; in suspensions of isolated mast cell granules moderately high concentrations of salt are required for the dissociation of this complex (Benditt, 1966). At least part of the basic protein is in the form of a proteolytic enzyme and in the rat this has chymotrypsin-like properties (Benditt and Arase, 1959; Lagunoff and Benditt, 1969).

Riley (1953) and Riley and West (1953, 1955) found a relationship between the histamine content of tissues and their mast cell numbers. They showed that agents which caused the degranulation of mast cells also depleted the tissue of histamine. Further evidence that histamine was contained within mast cells was

supplied by the correlation found between histamine content and mast cell numbers in pathological conditions such as mast cell tumours (Cass, Riley, West, Head and Strong, 1956) and urticaria pigmentosa (Riley, 1959). The more specific localisation of histamine was facilitated by isolating mast cells from the peritoneal cavity of rats (Uvnäs and Thon, 1959) and by the development of sensitive fluorescent techniques (Juhlin and Shelley, 1966).

Experiments using isolated mast cell granules suspended in solutions containing varying concentrations of salt, indicate that histamine is retained within the granule matrix by ionic bonding, although it is not clear whether it is linked to the acid mucopolysaccharide groupings or to the carboxyl groups of the basic protein (Åborg, Novotny and Uvnäs, 1967). The granules of the mast cells of the rat and mouse contain 5-hydroxytryptamine as well as histamine (Benditt and Lagunoff, 1956). 5-HT was identified in mast cells by Benditt, Wong, Arase and Roeper (1955) and there was found to be a close correlation between the 5-HT content of the tissues and the number of mast cells. With the development of the formaldehyde condensation method for the demonstration of monoamines (Falck, 1962; Falck, Hillarp, Thieme and Torp, 1962) the characteristic bright yellow fluorescence of carboline, the condensation product of paraformaldehyde and 5-HT, has been identified within mouse and rat mast cells on numerous occasions (Falck, 1962; Adams-Ray, Dahlström, Fuxe and Hillarp, 1964; Enerbäck, 1966d).

The amines in the mast cell granules are interchangeable and this was demonstrated by Uvnäs (1968) who found that histamine extracted from isolated granules by a process of ion exchange could be replaced by any one of a number of

basic amines when the granules were subsequently incubated in solutions containing suitable concentrations of the respective amines. It would seem, therefore, that mast cell granules having matrices consisting of mixtures of sulphated acid mucopolysaccharide and basic protein are ideally suited for the retention of basic amines such as histamine, 5-HT and dopamine. In the bovine and sheep, some of the mast cells contain a catecholamine which is probably dopamine (Falck, Nystedt, Rosengren and Stenflo, 1964).

The ultrastructure of mast cells has been studied on a number of occasions. The granules are the most prominent feature in the cytoplasm which otherwise contains few organelles. Characteristically, the granules are approximately 0.5 microns in diameter, are delimited by unit membranes and have homogeneous, electron-dense matrices (Benditt and Lagunoff, 1964; Singleton and Clark, 1965; Combs, 1966). The unit membrane provides a structural barrier to the escape of the granule matrices and is probably of considerable functional significance in the release mechanisms of the cell (Bloom and Haegermark, 1965).

Mast cells have poorly developed endoplasmic reticulum and small numbers of mitochondria which suggests that the cells secrete little protein and have a low metabolic activity (Benditt, 1968). Furthermore, the low turnover of labelled sulphate would indicate that the granules of adult connective tissue mast cells are relatively inactive (Combs, Lagunoff and Benditt, 1965). The adult population is remarkably stable since the cells in the normal mature animal rarely undergo mitosis (Allen, 1962; Combs et al., 1968; Blenkinsopp, 1967).

Functions of Mast Cells

Theories about mast cell functions are extraordinarily diverse, but the distribution of the cells close beside blood vessels (Riley, 1959) and their content of potent vasoactive substances have led many investigators to postulate a function for these cells in the controlling of the microcirculation (Selye, 1965; Keller, 1966). As Benditt and Lagunoff (1964) point out, a theory of mast cell function must embrace the activities of the known granule constituents. It must be consistent with the rate of production and rate of secretion of the constituents and additionally, should not only account for the distribution of mast cells in normal tissues, but should also adequately explain their presence or absence in a variety of pathological conditions.

Even now, little is known about the function, if any, of the mast cells in their normal inactive state and more attention has been directed towards discovering the effects of release of the granule components and of the underlying mechanisms involved. Thus, mast cell degranulation occurs locally in areas of trauma whether of mechanical, chemical or bacterial origin and the disappearance of mast cells has been recorded in various inflammatory conditions (Selye, 1965). Mast cells are degranulated in animals undergoing immediate type hypersensitivity reactions and their role in these reactions is discussed below.

The release of vasoactive amines from discharged mast cell granules brings about an increase in vascular permeability which is manifest as oedema when localised to a specific site or as an anaphylactoid or anaphylactic reaction when mast cell degranulating agents or antigen-antibody reaction respectively bring about generalised mast cell damage (Benditt and Lagunoff, 1964; Selye, 1965; Keller, 1966). The

amines, histamine and 5-HT cause increased vascular permeability but their effects are transient and the blood vessels become refractory to further stimulation (Spector and Willoughby, 1968). The functions of heparin when released from mast cell granules remain enigmatic, although its release has frequently been associated with reduced coagulability of the blood (Johansson, 1967). Benditt (1968) suggested that heparin might stabilise the chymotrypsin-like enzymes of the granules. It has been postulated that the mast cell proteases are active in the release mechanisms of the cell (see below) and that after release they might be involved in acting against components in the connective tissues, although evidence for this remains equivocal (Lagunoff, 1968).

Whilst mast cell function may, in part, be explained on the basis of the effects of degranulation, there remains the problem of why these cells should proliferate in the tissues. A variety of chemical agents causes an increase in the mast cell content of tissues; they increase in number in healing wounds and in many past inflammatory conditions (Benditt and Lagunoff, 1964). They have been found to proliferate in antigenically stimulated lymph nodes (Miller and Cole, 1968) as well as amongst isolated lymphoid cells (Ginsburg, 1968; Ginsburg and Lagunoff, 1967). Increased numbers of mast cells have frequently been reported during parasitic infections (see below) but little is known of the functions of these cells, or of the stimulus which brings about their proliferation.

The Role of Mast Cells in Immediate Type Hypersensitivity

Many hypersensitivity reactions are brought about by a specific sensitising or reaginic antibody which is now often termed "homocytotropic" antibody (HA) (Becker and Austen, 1966). HA attaches to the surface of a target cell, so that subsequent contact with antigen leads to release of pharmacologic agents from that target cell. The released agents act on primary shock tissues of the species such as smooth muscle and vascular structures, giving rise to the clinical manifestations of anaphylaxis or when confined to a limited area, causing a weal and flare reaction (Bloch, 1968). HA has been demonstrated in a number of species, including the guinea pig, man and the rat (Bloch, 1967).

Studies in the rat have shown that mast cells are the main target cells (Mota, 1964) although it is possible that other cell types, such as endothelial cells (Bloch, 1967) and enterochromaffin cells (Gershon and Ross, 1962) are also sensitised. Direct evidence for the involvement of mast cells in this type of reaction include the *in vitro* release of histamine from isolated, sensitised mast cells, the capacity of antihistamine and antiserotonin drugs to inhibit the hypersensitivity reaction, and the disappearance of mast cells from tissues after exposure to antigen (Mota, 1964). The attachment of HA to the target cell is indicated by its persistence at passively sensitised skin sites; by failure to remove it after repeated washing from isolated sensitised mast cells, and by the localisation of antigen on the surfaces of sensitised degranulating subcutaneous mast cells (Mota, 1964; Keller, 1966; Movat, Lovett and Talchman, 1966).

The mechanisms by which the interaction of antigen and of HA on the surface of the cell bring about the release of vasoactive amines are not entirely clear, but it has been shown that antigen-induced release of histamine from sensitised mast cells, is an energy requiring process which is sensitive to temperature and to enzyme inhibitors (Högberg and Uvnäs, 1960; Mota and Ishii, 1960). Becker and Austen (1966) used phosphonates to inhibit antigen induced release of histamine from mast cells and suggested that a mast cell esterase was activated to cause membrane lysis, although the relationship of this esterase to the mast cell chymase described by Benditt and Lagunoff (1969) was not established.

HA sensitisation of mast cells occurs in other species (Bloch, 1968) but not all immediate hypersensitivity reactions are brought about on the HA-target cell-effector axis (Keller, 1966). In some species, notably the pig and the rabbit, anaphylaxis may be the result of the precipitation of antigen-antibody aggregates in the lumina of blood vessels. The aggregates are phagocytosed by polymorphonuclear leukocytes and platelets with the concomitant release of hydrolytic enzymes and vasoactive amines. The combination of vascular obstruction and release of biogenic substances, probably brings about the severe systemic effects seen in this reaction (Movat, Uriuhara, Taichman, Rowsell and Mustard, 1968). Mota (1964) reported a similar reaction in the rat caused by precipitating antibodies without the participation of mast cells.

It is obvious that several mechanisms exist, and vary, not only between species, but also in the types of inducing antibody which may bring about immediate

hypersensitivity type reactions.

Mast Cells in Parasitized Hosts

Taliaferro and Sarles (1939) observed that the population of 'tissue basophils' in the intestinal mucosa of the rat increased in number during the course of infection with the nematode Nippostrongylus brasiliensis. Wells (1962) found that the mast cell population in the intestines of Nippostrongylus infected rats, decreased during infection and increased after the worms had been expelled and that the histamine levels in the bowel were likewise altered. In rats infected with Ampliacecum robertsi, the number of peritoneal mast cells decreased in the early stages of infection and later returned to normal levels (Archer and McGovern, 1968). Mast cells have been noted to increase in number in several species during the course of, and after, parasitic infections (Fernex and Fernex, 1962; Fernex, 1963; Dobson, 1967).

For a number of years, it has been known that extracts of Ascaris produce an effect in the body that closely resembles anaphylaxis and more recently it was shown that the extracts caused degranulation of mast cells (Uvnäs, Diamant, Högberg and Thon, 1960). Uvnäs and Wold (1967) isolated a polypeptide of approximately 2,000 - 3,000 molecular weight from ascaris extracts and showed that it caused degranulation of mast cells from germ-free rats, thereby excluding the possibility of prior sensitization.

The degranulation of mast cells brought about by the release of specific degranulators should be distinguished from antigen-induced damage to previously

sensitised mast cells. Briggs (1968) found that connective tissue mast cells of mice were actively sensitised by living infections of Trichinella spiralis and that the cells could also be passively sensitised with peritoneal fluid obtained from infected mice. Active sensitisation of connective tissue mast cells was reported in rats infected with Strongyloides ratti (Goldgraber and Lavart, 1966) and Wilson and Bloch (1968) demonstrated that peritoneal mast cells from Nippostrongylus infected rats were sensitised to release histamine on challenge with antigen. This second mechanism requires prior sensitisation of the cells before degranulation and histamine release takes place on exposure to parasitic antigens.

HA and Parasitism

Since Ogilvie (1964) reported relatively high levels of reagin-like antibodies in rats after infection with Nippostrongylus brasiliensis, HA has been found in a number of species harbouring a variety of parasites. These antibodies have been detected in rabbits infected with Schistosoma mansoni (Zwaifler, Sadun and Becker, 1966) and with Dirofilaria uniformis (Sadun, Duxbury, Gore and Stechschulte, 1967). HA has also been detected in humans, rhesus monkeys and chimpanzees infected with Schistosoma mansoni (Sadun, von Lichtenberg, Hickman, Bruce, Smith and Schoenbechler, 1966) as well as in mice and rabbits harbouring Trichinella spiralis (Sadun, Mota and Gore, 1968).

Homocytotropic antibodies produced in animals by living parasitic infections are similar in many of their properties to HA artificially induced in laboratory animals

in that they are inactivated by heating at 56°C or when treated with 2 - mercapto-ethanol and they persist at skin sites many days or weeks after injection in contrast to IgG antibodies which disappear within 1 or 2 days (Sadun, Mota and Gore, 1968). It is the persistence of these antibodies in the skin which provides the most useful indication of their presence. HA production is most readily stimulated by living worm infections and an anamnestic rise in the titre occurs on reinfection, but attempts to induce HA by vaccination with worm extracts have proved to be disappointing (Ogilvie, 1967).

The unusually high titres of HA induced by parasitic infections have excited much attention and it has been suggested that this could be the result of persistent antigenic stimulation by the parasites at sites where HA production is predominant (Ogilvie, 1967).

The presence of HA in Trichinella infected mice (Sadun et al., 1968) and in rats infected with Nippostrongylus (Ogilvie, 1964, 1967; Wilson and Bloch, 1968) corroborate the findings that the mouse connective tissue mast cells and rat peritoneal mast cells are sensitised (Briggs, 1968; Wilson and Bloch, 1968).

Immunity to Parasites

(a) Evidence of Immunity - There now exists substantial evidence that many parasitic infections are ended by an immunological reaction on the part of the host. The termination of Nippostrongylus infections in the rat is heralded by a drop in the egg production and is followed by expulsion of the parasites themselves (Africa, 1931;

Mulligan, Urquhart, Jennings and Nelleon, 1965; Jarrett, Jarrett and Urquhart, 1968). Oral infections with Trichinella spiralis are followed by loss of the adult parasites from the small intestine towards the end of the 2nd week of infection (Larsh, 1963). Chandler (1936) transferred adult Nippostrongylus worms which were about to be expelled from infected rats into the intestines of normal rats and found that the parasites recovered and lived for a further 8 - 10 days in the new host before expulsion.

Apart from the expulsion of primary infections, other manifestations of immunity included the establishment of reduced worm burdens on reinfection together with inhibition of development, stunting of growth and reduced fecundity and longevity of the worms in the reinfected animals (Chandler, 1937b). These criteria have been demonstrated in a number of host/parasite relationships including Dictyocaulus viviparus in cattle and in guinea pigs (Jarrett, Jennings, McIntyre, Mulligan, Thomas and Urquhart, 1959; Wilson, 1966) and Cooperia punctata in the calf (Stewart, 1968). Other evidence for the immune expulsion of parasites by the host, is provided by the ability of Cortisone (Coker, 1956; Weinstein, 1956; Campbell, 1968) and of whole body irradiation of the host (Larsh, 1967) to prevent the expulsion of the worms at the end of the primary infection.

(b) Parasitic Antigens - The variety and complexity of helminth antigens have long bedevilled attempts to identify those which might be responsible for stimulating the production of protective antibodies in the host. Excretory and secretory products of the parasites, larval exsheathing fluid and somatic extracts have all been used in

attempts to produce active immunity against a variety of parasitic infections, but in general, none has proved so effective as infections with viable larvae (see Review by Urquhart et al., 1962). Chandler (1937b) hypothesised that some of the antigens might be enzymes and this has been shown to be the case for several parasites (Soulsby, 1962). There remain the problems of how to purify such antigens and to maintain their immunogenicity and at the same time identify those which stimulate the production of protective antibodies.

Jones and Ogilvie (1967) and Wilson (1967) identified an allergen produced from somatic extracts or excretory-secretory products of adult Nippostrongylus worms which reacts with HA. However, there is no evidence that this allergen stimulates the production of protective antibodies.

(c) Evidence for Anti-parasitic Antibodies - Passive protection against Nippostrongylus infections using hyperimmune sera has been achieved on a number of occasions (Sarles and Taliaferro, 1936; Chandler, 1937a; Sarles, 1939; Mulligan et al., 1965) and passive protection has also been conferred to cattle against Dictyocaulus viviparus (Jarrett et al., 1955), and in a variety of other host/parasite relationships (Urquhart et al., 1962). By placing second stage larvae of Ascaris lumbricoides in millipore chambers, Crandall and Ascan (1964) were able to show that the larvae ceased to grow in the peritoneal cavities of immune mice, but on being placed into normal mice, the larvae resumed normal growth. Although the passive transfer of protection and the millipore chamber experiments demonstrate that antibody confers some protection against parasites, they do not show that humoral antibody per se is necessarily effective

in the elimination of parasitic infections.

It has been known for a number of years, that specific antibodies against a variety of antigens are secreted at mucous surfaces (see Review by Pierce, 1959). The development of monospecific antisera to the various classes of heavy chains has allowed the identification of the cells producing antibody in these sites and in the intestinal tract of man and the rabbit, plasma cells containing IgA are predominant (Crabbé, Corbonera and Heremans, 1966; Crandall, Cebra and Crandall, 1967). Not only are IgA producing cells present in large numbers, but there is evidence that IgA is preferentially secreted into the gastrointestinal and respiratory tract and is found in higher concentrations in the muco-antibodies than in the serum (Chodirker and Tomasi, 1963). Locally secreted IgA has been shown to be active against a number of agents including bacteria and viruses (Bellanti, 1968).

It is not clear whether similar mechanisms operate in parasitic infections; Crandall et al. (1967) noted that there was an increase in plasma cells containing IgM and IgG in the intestinal lamina propria of rabbits harbouring T. spiralis, although plasma cells showing fluorescence for IgA were predominant in the mucosa and remained so during the infection. Increases in the numbers of immunoglobulin-producing cells have been reported in the intestines of Nippostrongylus infected rats (Taliaferro and Sarles, 1939) and of sheep infected with Oesophagostomum columbianum (Dobson, 1967). Jarrett and Sharp (1963) observed an increase in the number of plasmablasts and plasma cells in the lungs of calves after infection with D. viviparus and Murray (1968) noted an increase in the population of these cells in the abomasal

mucosa of calves infected with Ostertagia ostertagi.

Dobson (1966a) found precipitating antibodies within the muscularis, the mucosa and sera of sheep infected with O. columbianum, and not in the mucus exudates, but he was able to detect a mucoantibody with haemagglutinating properties (Dobson, 1966b).

It remains to be shown whether local secretion of antibody in any way protects the host against parasites or whether it is merely a reaction to the variety of antigens released by the parasite.

Immediate Hypersensitivity and Acquired Immunity to Parasitic Infections

Stewart (1950; 1958; 1963) observed that ingestion of Haemonchus contortus larvae by sheep already infected with the adult parasite or with Trichostrongylus Spp. resulted in some cases in the expulsion of the adult parasites and this was accompanied by a transient rise in the blood histamine and a rapid development of oedema in the affected portion of the alimentary tract. Antihistaminics, in some instances, blocked the effect of freshly ingested larvae on the existing infection without affecting the subsequent rise in antibody titre. Injection of massive doses of larvae directly into the exposed abomasum of normal or of hypersensitive sheep, rapidly led to increased peristaltic segmentation and oedema, only in the hypersensitive group.

Urquhart,Mulligan, Eadie and Jennings (1965) noted speckles and patches of dark blue corresponding to areas of increased capillary permeability in the intestinal mucosa after giving Nippostrongylus infected rats intravenous injections of Evans blue.

When the dye was mixed with extracts of whole worms and given intravenously to rats at various intervals of time after infection, there was extensive bluing of the mucosa, macroscopically evident hyperaemia, and an increased amount of fluidity of deeply stained mucus.

Barth, Jarrett and Urquhart (1966) postulated that the increased capillary permeability associated with a local anaphylactic reaction might be important as a means of allowing a significant extravascular leak of plasma into the subepithelial spaces of the villi or into the intestinal lumen and that the antibody might then act directly on the worms. To test this hypothesis, they subjected rats harbouring a population of transplanted N. brasiliensis to a heterologous anaphylactic shock. Neither anaphylaxis per se nor the administration of hyperimmune serum alone had any effect on the expulsion rate of the population; the combination of the 2 factors accelerated the expulsion of the parasites. They concluded that 2 components might be responsible for worm expulsion. The first was the presence of a specific antibody and the second a local increase in mucosal permeability which might facilitate the transfer of such antibody into the intestinal lumen.

The role of immediate type hypersensitivity in the expulsive mechanisms was further suggested by the effects of antihistamines and antagonists and depletors of 5-HT which partially prevented the expulsion of Nippostrongylus and of Trichinella from the intestines of their hosts (Urquhart et al., 1965; Campbell, Hartman and Cuckler, 1969; Sharp and Jarrett, 1968). Cortisone and its derivatives also prevented the expulsion of parasites, even after the self cure process had started and,

at the same time, depleted the intestinal mucosa of mast cells (Jarrett, Jarrett, Miller and Urquhart, 1967a)and administration of these drugs suppressed artificially induced anaphylaxis (Urquhart et al., 1965; Briggs and Deglurst, 1966).

GENERAL MATERIALS AND METHODS

Experimental Animals

a) Strain and Source All rats used were the hooded Lister variety and were obtained from Animal Suppliers Ltd., London.

b) Age, Weight and Sex The animals were approximately ten weeks old, and weighed between 170 and 220 gms. . Female rats were used in all experiments.

c) Maintenance The rats were kept in wire cages with mesh floors suspended above trays containing sawdust so that they had no access to infected faeces. The animal house temperature was maintained at approximately 22°C. The rats were fed on a pelleted diet (Diet 41, supplied by Spearer Ltd., Glasgow); this and water were available ad libitum.

Histological Procedures

Rats were lightly anaesthetised with trichloroethylene and killed by a blow on the head or by cervical dislocation. Where tissues were at the same time being removed for electron microscopy, the rats were maintained under a deep anaesthesia. Tissues were rapidly excised and placed in fixative for 24 - 48 hours.

a) Fixation A range of fixatives was used and they were prepared as follows:-

(i) Carnoy's fluid (Clayden, 1953)

Chloroform 30 ml., Absolute alcohol 60 ml. and Glacial Acetic acid 10 ml.

(ii) Calcium acetate formalin (Lillie, 1965).

4% formaldehyde buffered with 2% calcium acetate.

(iii) Formol Sublimate (Carleton and Drury, 1957).

4% Formaldehyde in a saturated aqueous solution of mercuric chloride.

(iv) Mercuric chloride (Spicer, Staley, Wetzel and Wetzel, 1967).

6% $MgCl_2$ buffered with 1.3% sodium acetate.

(v) Isotonic formaldehyde - acetic acid (IFAA) (Ruenbleck, 1966a).

0.6% formaldehyde and 0.5% acetic acid.

The tissue blocks were dehydrated and cleared in an alcohol - amyl acetate - chloroform series and embedded in paraffin wax.

b) Staining Techniques Paraffin sections of approximately 6 microns were stained using the following techniques:-

(i) Thiazine dyes: Toluidine blue (E. Gurr Ltd., London) was used in 0.5% aqueous solution at pH 4 (McIlvaine's citric acid - disodium phosphate buffer (Lillie, 1965)). Staining time was 45 seconds (Ruenbleck, 1966b). Other sections were stained with a 0.1% aqueous solution at about pH 0.3 (dye diluted in 0.7 N HCl) for 10 minutes followed by rinsing in 0.7 N HCl for ten minutes (Ruenbleck, 1966b).

(ii) Copper Phthalocyanine dyes: Astra blue (G. T. Gurr, London) and Alcian blue 8 GX (E. Gurr Ltd.) were prepared as 0.5% solutions at about pH 0.3 (Ruenbleck, 1966b); staining time was 10 or 30 minutes and the sections

were subsequently immersed in 0.7 N HCl for ten minutes (Bloom and Kelly, 1960).

Some sections were counterstained for 30 seconds with 0.5% Safranin O (Hopkins and Williams Ltd.) in 0.125 N HCl (about pH 1) (Enerbäck, 1966b). For quantitative purposes, sections were stained with 0.5% Astra blue or Alcian blue at pH 0.3 for 30 minutes, rinsed in 0.7 N HCl for ten minutes and were counterstained with 0.5% Safranin O at pH 1 for 30 seconds.

(iii) Critical electrolyte concentration method (CEC) (Scott and Dorling, 1965). The following stock solutions were made up:-

1% Alcian blue 8 GX in distilled water was prepared on the day of use.

3.0 M magnesium chloride

0.1 M sodium acetate buffer

Staining solutions were prepared by mixing dye, buffer and distilled water to give 50 ml of solution containing the required concentration of $MgCl_2$.

Sections were immersed overnight at room temperature in upright Coplin jars containing 50 ml of 0.05% Alcian blue in 0.025 M sodium acetate buffer and $MgCl_2$. They were then individually rinsed in a stream of distilled water and transferred to a fresh distilled water bath.

(iv) Acridine orange: (G. T. Gurr Ltd.) was used as a 0.001% solution (Jagatic and Weinkopf, 1966) and as a 0.1% solution in distilled water (Saunders, 1964).

(v) Biebrich scarlet (G. T. Gurr Ltd.) (Spicer and Lillie, 1961). A 0.04% solution of this dye was made up in glycine buffer at pH 8.15, 8.9 and 9.9 (Lillie, 1965). Staining times of 30 - 90 minutes were used. Some sections were transferred directly to 95% alcohol, dehydrated and cleared. Others were rinsed in distilled water prior to dehydration.

(vi) Other stains: Paraffin sections were stained with haemalum and eosin, ~~pleromallory~~ picro-Mallory and periodic acid-Schiff (Pearse, 1960).

Sections were dehydrated through an ascending series of alcohols, cleared in xylene and mounted in D.P.X. (B.D.H., Poole).

c) Technique for Demonstration of Monoamines (Falck, Hillarp, Thieme and Torp, 1962; Falck, 1962).

Small blocks of tissue (maximum thickness 3 - 4 mm) were quenched in isopentane cooled by liquid nitrogen; they were then placed on the cooled module of an Edwards-Pearse Tissue Drier and were left overnight under a vacuum of 0.001 Torr at a temperature of -40°C. Two P_2O_5 vapour traps were placed in the chamber prior to processing the tissues.

The samples were then brought to room temperature and warmed to 37°C before being transferred to an oven where they were treated with paraformaldehyde vapour at 80°C for one hour. They were impregnated with degassed molten paraffin wax at 57 - 60°C in vacuo for fifteen minutes.

Some tissues were impregnated with an epon-araldite resin mixture using an Edwards-Pearse resin-embedding accessory. The resin mixture was hardened in the

usual manner (see below). Sections treated for monoamines and acridine orange stained sections were examined with a Leitz Orthoplux microscope; A BG 12 3 mm exciting filter and a K 530 barrier filter were used. The light source was a mercury vapour lamp (Watan HBO 200 w.)

Techniques for Electron Microscopy

Specimens were removed from rats anaesthetised with Trichloroethylene and were placed in drops of chilled fixative on blocks of dental wax. They were sliced into pieces 1 - 2 mm in thickness using grease-free razor blades and transferred to vials containing fixative at +4°C.

a) Fixation

(i) Glutaraldehyde. A stock solution of 25% glutaraldehyde purified by TAAB Laboratories and stabilised at pH 5 - 6 was used.

The fixative was a 4% solution in 0.067 M Sorensen's Phosphate buffer pH 7.2 - 7.4.

The buffer was made up as follows -

Na_2HPO_4	- 9.118 gm/litre	8 parts
KH_2PO_4	- 9.912 gm/litre	1 part

(ii) Osmium tetroxide (B. D. H., Poole). 1% OsO_4 was prepared in Millonig's Phosphate buffer

$NaH_2PO_4 2H_2O$	2.26%	83 ml
NaOH	2.52%	17 ml
H_2O		10 ml
Sucrose		0.54 gm

(iii) Uranyl acetate (May and Baker, Dagenham). 0.5% uranyl acetate was prepared as follows:-

Uranyl acetate	0.5 gm
Distilled water	100 ml
Sucrose	4.5 gm

Sodium hydroxide IN was added by drops to a pH of 4.9 - 5.0.

Tissues were fixed for 2 - 3 hours or 14 - 16 hours in 4% glutaraldehyde in 0.067 M phosphate buffer, pH 7.2 - 7.4 at +4°C. They were rinsed 3 times in Millonig's phosphate buffer for a total of 5 - 6 minutes. The samples were then kept in 1% osmium tetroxide in Millonig's phosphate buffer, pH 7.2 - 7.3, on ice for 60 - 90 minutes.

Some samples were dehydrated immediately but the majority were rinsed with Ringers solution three times for 3 minutes each. They were then kept in 0.5% uranyl acetate on ice for 90 minutes and were dehydrated.

b) Dehydration and Embedding

Samples were dehydrated through an ascending series of 70%, 95% and absolute ethanol. They were treated for 2 x 15 minutes with propylene oxide and placed in a 50/50 mixture of the final embedding media and propylene oxide. The mixture was left overnight in open vials and the tissues were transferred to gelatin capsules containing embedding media.

c) Embedding Resins

Two preparations were used:-

(i) Epon-Araldite

25 ml Epon 812 (G.T. Gurr Ltd.)
58 ml DDSA (Shell Chemicals)
15 ml Araldite resin (CIBA, Cambridge)
4 ml DButyl phthalate (BDH)

These were stirred thoroughly and stored at +4°C. For the final mixture 1.5% DMP 30 (accelerator, G.T. Gurr Ltd.) was added and thoroughly mixed.

Epon-Araldite was hardened by heating at 60°C for 36 hours.

(ii) Araldite

7 parts Araldite resin (CIBA)
3 parts Hardener HY/964 (CIBA)

These were mixed by stirring overnight and stored at +4°C. 2% DMP 30 (accelerator) was added and mixed to make the final preparation. Hardening was at 60°C for 48 hours.

d) Section Cutting and Staining

Thin sections were cut with an LKB Mark III ultratome and mounted on copper mesh grids. They were double stained with 20% uranyl acetate in methanol and with lead citrate (Reynolds, 1963). They were examined with an AEI Electron Microscope 6B.

Sections 1 - 1½µ thickness were taken from the same blocks and were mounted on glass slides; they were stained either with Azure II - methylene blue borax (Richardson, Jarett & Finke, 1960) or with basic fuschin in acetone (Ackerman and Hostetler, 1968).

Parasitological Techniques

Details of the strain of N. brasiliensis and the culture methods are described by Jarrett (1968).

Infection of Rats

Methods of counting larvae were those described by Jarrett (1968). Doses of 3,000 larvae in 1.0 ml of water were inoculated subcutaneously in the groin region.

SECTION 1

The Intestinal Mast Cell in the Normal Rat

Part 1

Fixation, Histochemistry and Morphology of the Intestinal Mast Cell

Since Maximow (1906) reported the presence of atypical mast cells in the intestinal mucosa of the rat, their origin and nature has been in dispute (Michels, 1938; Selye, 1965). Mota, Ferri and Yoneda (1956) thought that there were comparatively few mast cells in the intestinal mucosa and Wiwi and Gracie (1967) reached similar conclusions; Lindholm (1960) used similar histological procedures, but described a relative abundance of mast cells in this site. The controversy was partially resolved by Enerbäck (1966a) who studied the fixation of mast cells in different parts of the gastrointestinal tract. He found that they, unlike mast cells in the connective tissues, were not demonstrated by certain standard tests after fixation with the usual strength of formaldehyde; when a mixture of low concentrations of formaldehyde and acetic acid, various lead containing solutions or Carnoy's fluid were used, they could be visualised. He also found (Enerbäck, 1966a, 1966b, 1966d) that the mast cells in the gastrointestinal mucosa showed morphological as well as histochemical differences from the connective tissue mast cells.

The localisation of acid muco-substances in tissues by histochemical methods is dependent on the staining of free polyanionic groups; this in turn is governed by the availability of such groups after the tissues have been fixed, dehydrated and embedded. The possibilities that the acid muco-substance is extracted during these procedures or that the presence of polycations such as basic proteins may block the staining of the polyanions must both be considered when choosing methods suitable for fixation and staining of mast cells.

For many years metachromasia of the granules after staining with toluidine has been used for the identification of mast cells (Michels, 1938). This basic thiazine dye binds to acidic groups in the tissues and where the groups are close to one another there is a metachromatic shift from blue to violet and red. Metachromasia of this type signifies the presence of free electro-negative charges of minimum surface density but is not specific for any particular acidic groups (Pearse, 1968).

Muco-substances can be more readily characterised by the basic dye extraction method. Spicer (1960) used azure A over a range of pH to characterise rodent acid mucopolysaccharides. This method has the disadvantage that it may not distinguish between muco-substance carboxyl groups and protein carboxyl groups (Pearse, 1968). Both the toluidine blue and the basic dye extraction methods are unsatisfactory when acid muco-substances and basic proteins are found in close proximity because the polycations may in many instances block the basophilia of the anions (Scott, Dorling and Stockwell, 1968). These methods may therefore fail to demonstrate the polyanions even though they are abundant in the tissues.

For the classification and typing of muco-substances, Spicer, Leppi and Steward (1965) proposed that their affinity for the copper phthalocyanine dye Alcian blue 8 GX should be used together with their affinity for basic dyes and their susceptibility to testicular hyaluronidase and Vibrio cholerae sialidase. Alcian blue 8 GX carries at least two and possibly four positive charges per molecule and probably combines with polyanions by salt linkages (Scott, Quintarelli and Dellovo, 1964). When used at a low pH this dye has been found to be specific for sulphated acid mucopolysaccharides (AMPS) (Lev and Spicer, 1966) and a similar copper phthalocyanine dye, Astrablau, has

been shown to be specific for the sulphated AMPS in mast cells (Bloom and Kelly, 1960).

A method of differentiating between carboxyl, uronic and sulphate groups using Alcian blue has been described by Scott and Dorling (1965) who found that addition of electrolyte to solutions of Alcian blue enabled them to localise and identify polyanions. This same method was also found to be effective in dissociating polycation-polyanion complexes so that the Alcian blue was able to stain the unblocked polyanion (Scott et al., 1960).

The present experiment was designed in the first place to select a fixative which would give consistent fixation of intestinal mast cells (IM cells) for future experiments and secondly to characterise further the nature of the acid muco-substances in the granules of those cells. In view of the basic protein content of mast cell granules (Benditt and Lagunoff, 1964) and the histochemical demonstration of this protein in mast cells in certain sites (Spicer, 1963) similar methods were used to demonstrate basic proteins in the IM cell. The paraformaldehyde condensation method of Falck, Hillarp, Thieme and Torp (1962) was used to identify the monoamines present in the granules of the IM cells. In addition, the fixation, histochemical properties and morphology of the IM cell were compared with those of the connective tissue mast cell (CTM cell)of the tongue and skin.

Materials and Methods

Fifteen female hooded Lister rats weighing 180 - 210 gm. were used. 1½ - 2 cm. blocks of jejunum were excised from an area 10 - 20 cm. posterior to the pylorus; segments of tongue and dorsal skin were also taken. The samples from all rats were placed in Carnoy's fluid. Tissues from four of the rats were also fixed in calcium acetate formalin, formol sublimate, mercuric chloride and IFAA (see General Materials and Methods).

The staining techniques are described in General Materials and Methods. For the CEC method (Scott and Dorling, 1965) serial sections were cut and were immersed in a series of Coplin jars containing dye solution and increasing concentrations of electrolyte (0.1 M increments of $MgCl_2$). The range of molarities was from 0.0 - 1.4. Sections from jejunum fixed in calcium acetate formalin, mercuric chloride and formol sublimate were stained at the same time.

Five of the rats were given intraperitoneal injections of 3 x 40 mg/Kg d-L-DOPA (DL - 3 - 3, 4 Dihydroxyphenyl-alanine)(Koch-Light Laboratories Ltd., Colnbrook) dissolved in distilled water, at one hour intervals and were killed one hour later (Enexblick, 1966d). Small blocks of jejunum and tongue were rapidly excised from the treated rats and from 5 untreated rats and were examined for monoamines using the method of Falck et al, (1962). After freeze drying some of the blocks were placed in an oven at 80°C but without paraformaldehyde. These were used as controls to test the specificity of the monoamine reaction. The Falck method, and embedding procedures were carried out as described in General Materials and Methods.

Results

Fixation

The fixatives used and the staining properties of both types of mast cell are summarised in Table 1. Tissue sections were also stained with haemalum and eosin to evaluate the overall standards of fixation.

(a) Carnoy's Fluid Table 1 shows that this fixative was satisfactory for most of the histochemical tests employed. The overall fixation when compared with buffered formaldehyde or formol sublimate was not so good because the cytological detail was less clearly defined. There was no evidence of tissue shrinkage.

(b) Calcium Acetate Formalin This method provided good preservation of the detailed cytology without apparent swelling or shrinkage of the tissues. It was, however, quite inadequate for the demonstration of IM cells except by the critical electrolyte concentration (CEC) method (Table 1) whereas the CTM cells could be visualised using a variety of stains (Table 1).

(c) Formol Sublimate The preservation of cytological detail was excellent with this fixative. Although CTM cells could be readily demonstrated with a variety of stains for AMPS the staining of IM cells was at best extremely weak (Table 1).

(d) Mercuric Chloride Fixation by this method was poor and the sections were difficult to cut, tending to break up or tear away from the knife. IM cells were slightly more strongly stained for AMPS than they were in tissues fixed with formol sublimate. (Table 1.)

TABLE 1

	Alcian Blue / Astra Blue	Safranin O	Toluidine Blue pH 6.3		Toluidine Blue pH 4.0		Biebrich Scarlet pH 9.9		Acridine Orange 0.001%		CEC *	
	EMC	CTMC	EMC	CTMC	EMC	CTMC	EMC	CTMC	EMC	CTMC	EMC	CTMC
CAR	B+++	R+++ (B+)[a]	B++	P+++	RP+	P+++	OR++	-	?	O++	1.2	1.4
FCA	-	R+++ (B+)[a]	-	P+++	-	P+++	?	-	-	O++	0.9	ND
FS	(B+)	R+++ (B+)[a]	-	P+++	-	P+++	?	-	OY+	O++	-	ND
MC	B±	R+++ (B+)[a]	ND	ND	-	P+++	?	-	ND	ND	-	ND
IFAA	B+	R++ (B+)[a]	-	P++	RP++	P+++	?	-	ND	ND	ND	ND

* Molarity of $MgCl_2$ at which Alcian Blue staining is extinguished

a Some cells stained blue and others had a mixture of blue and red granules

CAR	Carnoy's fluid	B	Blue	ND	Not done	Intensity of Staining	- none
FCA	Formalin and calcium acetate	R	Red	?	Cell type not identified		+ weak
FS	Formol Sublimate	P	Purple				++ moderate
MC	Mercuric Chloride	O	Orange	()	Small numbers of cells		+++ strong
IFAA	Isotonic formalin and acetic acid mixture	Y	Yellow				

(c) FAA Even though the IM cells were strongly stained with toluidine blue, FAA provided poor overall fixation. The tissues after 24 - 48 hours fixation were soft, greasy to handle and difficult to trim. Histologically, there was swelling of the tissues and some of the cells in the mucosa appeared vacuolated.

Morphology

IM cells were mostly confined to the lamina propria of the small intestine although cells showing all the characteristics of IM cells were occasionally seen within the epithelium of the crypt region. Apart from the obvious differences in staining properties (Table 1) the IM cells showed a greater morphological variation than did the CTM cells. In the former the granules tended to be less densely packed so that the nucleus was often visible and the cell was frequently irregular in outline (Figure 1). The IM cells in the basal lamina propria and between the gland crypts tended to be larger and were more diverse in shape than the smaller more globular cells found in the villi. In contrast, the granules of the CTM cells were smaller and more densely packed, so that individual granules could not be readily visualised (Figure 3); in addition, they frequently obscured the nucleus (Figure 4).

Histochemistry

Acid mucopolysaccharide Unless otherwise stated, the staining techniques were carried out on Carnoy-fixed tissue. The results are summarised in Table 1. With the Alcian blue or Astra blue-safranin techniques, the IM cells stained blue (Figure 1) whereas the majority of CTM cells were red (Figure 4); a few small cells situated just below the epithelium had a mixture of red and blue granules. The staining of IM cells with toluidine blue was unsatisfactory with all fixatives apart

from FAA. When fixed in Carnoy's the IM cells showed very weak purple to red metachromasia at pH 4 (Figure 2) although moderately strong orthochromatic staining was evident at pH 0.3. The CTM cells showed deep purple metachromasia at both pHs.

With the CEC method of Alcian blue staining, the CTM cells stained at molarities as high as 1.4 whereas IM cells did not stain at more than 1.2 M. In jejunum fixed in calcium acetate-formalin mixture there was no staining of IM cells until the concentration of magnesium chloride was raised to 0.4 M and there was no staining at molarities higher than 0.9 M (Table 1).

Acridine orange was unsatisfactory when used with Carnoy-fixed tissue because it stained DNA, RNA and goblet cells strongly and IM cells could not be readily distinguished. When fixed in sublimate formol, the IM cells fluoresced orange yellow. CTM cells fixed by either method fluoresced orange (Table 1).

Basic protein IM cell granules when stained with biebrich scarlet and immediately dehydrated without rinsing in water were orange red. Many nuclei were stained less strongly with an orange metachromasia thus allowing the distinction between IM cells and eosinophils to be made. The nuclei of the latter were usually ring or band shaped and the granules did not stain with biebrich scarlet whereas IM cell nuclei were oval with some clumping of the chromatin and were characteristically surrounded by deep staining granules. If the stained sections were washed in distilled water prior to dehydration the nuclear staining was lost but the mast cell granules retained the dye strongly (Figure 3). CTM cells were not stained by biebrich scarlet with either technique.

When tissues were fixed in sublimate formol or in mercuric chloride there were many cells containing orange red granules scattered throughout the intestinal mucosa. More cells were coloured in this procedure than would be expected if only the IM cells had stained. There was, however, no nuclear staining and it was not possible to distinguish whether the granulated cells were eosinophils or mast cells. No staining of CTM cells was observed after fixation by these methods.

Monoamines Several fluorescent cells could be distinguished by their colour, by their morphology and by their distribution within sections from freeze-dried and paraformaldehyde-treated jejunum. At all levels within the epithelium, there were flask-shaped cells which fluoresced bright yellow and often had fine processes extending towards the lumen (Figure 6). Sometimes small granules which were at the limit of resolution could be visualised in the fine processes. These were identified as enterochromaffin cells.

Within the lamina propria there were granulated cells which were morphologically similar to and distributed in the same manner as IM cells identified by other methods (Figure 7). Those in the villi and upper crypt region fluoresced weak dull green; some in the lower crypt regions fluoresced similarly whereas others had a yellow fluorescence, a colour more typical of 5-hydroxytryptamine. The number of yellow fluorescent cells tended to vary; in some rats very few were found, whereas in others most of the cells in the crypt region had this characteristic fluorescence. Most cells in the submucosa, mesentery and in the tongue fluoresced a bright yellow (Figure 8).

There were several cells in the villus tips containing larger, irregular orange yellow fluorescent granules (Figure 6) but the intensity of their fluorescence did not diminish with prolonged exposure to ultra-violet light whereas the fluorescence of enterochromaffin as well as IM and CTM cells decreased markedly over the same period of time. The same type of cell continued to fluoresce in tissues which were freeze-dried and subsequently embedded in paraffin without paraformaldehyde treatment. There was no fluorescence in the enterochromaffin or IM cells in normal rats nor in rats which had been pre-treated with L-DOPA where paraformaldehyde treatment had been omitted.

The contrast between jejunum from rats given L-DOPA before sacrifice and normal rats was striking. In the former there was brilliant fluorescence of IM cells at all levels in the lamina propria (Figure 9). The majority fluoresced bright green but some in the lower crypt region remained yellow. CTM cells in both tongue and mesentery continued to fluoresce yellow as did the enterochromaffin cells in the jejunum.

When jejunum was embedded in Araldite-Epon and thin sections of approximately 1.5μ were cut, the intensity of IM cell fluorescence was much reduced. IM cells in the villus or between crypts were therefore difficult to distinguish but some in the basal mucosa could still be identified by the yellow fluorescence of their granules. Examination of these same individual cells using phase contrast or after re-staining the sections with azure II-methylene blue-borax showed them to be mast cells. Identification of IM cells was facilitated in epony embedded sections by pretreating the rats with L-DOPA. Individual granules in IM cells fluoresced bright green at all levels in the lamina propria except for a few with more yellow fluorescence in the

crypt region. Fluorescent cells were subsequently identified as mast cells by re-examination under phase contrast or by staining the same or immediately adjacent sections with basic dyes (Figures 10a, 10b).

Discussion

The eventual choice of fixatives for subsequent experiments was governed by several factors including the quality of fixation and the necessity of using simple but reliable staining methods to demonstrate IM cells. Whilst a mixture of low concentrations of formaldehyde and acetic acid (FAA) (Enerbäck, 1966a) proved to be sufficient for locating IM cells with aqueous toluidine blue, it was of less value when the copper phthalocyanine dyes were used since the staining was weak (Table 1). Additionally, the overall standard of fixation by this method was poor. Formaldehyde buffered with calcium acetate was tried in this experiment because the salt acts as a cationic precipitant for acid mucins (Pearse, 1968); this proved to be unsatisfactory for the demonstration of IM cells (Table 1). Formol sublimate was equally unsatisfactory while mercuric chloride permitted only weak staining of IM cells and provided extremely poor overall fixation.

Carnoy's fluid not only gave adequate fixation but facilitated the demonstration of IM cells by a variety of stains. Toluidine blue was of little value because only a weak metachromasia was obtained at pH 4; at pH 0.3 it provided a moderately strong orthochromatic staining. Because of the questionable reliability of different batches, the rather more time-consuming and elaborate buffering procedures required and

the difficulties sometimes encountered over dehydration (Pearse, 1968) toluidine blue was not used to any great extent for the demonstration of IM cells. By way of contrast, the strong staining of IM cells with the copper phthalocyanine dyes and the relatively uncomplicated staining method were considered to be adequate for routine demonstration of the cells. In addition, Carnoy's fluid proved to be satisfactory for the CEC method of Scott and Dorling (1965) and also allowed the demonstration of basic proteins within the granules of IM cells. For these reasons, Carnoy's fluid was the fixative of choice.

There were minor morphological differences between IM and CTM cells, for in the former the granules tended to be less densely packed so that the nucleus was readily distinguished. There also tended to be a greater variation in the size and the outline of IM cells than of CTM cells. This is in agreement with Enerbäck (1966a) who observed that IM cells tended to be smaller, less densely granulated and to have greatly varying shapes. The morphological differences are emphasised in the ultrastructural studies that follow (This Section Part 2).

The strong affinity of IM cells for copper phthalocyanine dyes at the low pH of 0.3 is evidence that their granules contain a sulphated acid mucopolysaccharide. The present findings and the earlier experiments by Enerbäck (1966a, b) show that there are histochemical differences between the CTM cell and the IM cell. Enerbäck (1966b) found that IM cells had a weaker affinity for thiazine dyes than CTM cells as was the case in the present experiment, but one point of difference from his data was that toluidine blue at pH 0.3 gave a moderately strong orthochromatic staining of the IM cells. Enerbäck (1966b) assumed that the differences between IM and CTM cells

reflected differences in the physical properties or chemical composition of the granules and on the basis of preliminary experiments with labelled sulphate, suggested that there might be a lower content of sulphate in the IM cells or that there was a different rate of turnover of sulphate in the 2 cell types.

The present findings suggest that the acid mucopolysaccharide of the IM cell is less strongly sulphated than its counterpart in the connective tissues because the staining of the former, using the CEC method of Scott and Dorling (1965) was cut off by lower molarities of electrolyte than the CTM cell. This is further supported by the strong affinity of IM cells for Alcian blue even when counterstained with safranin. The same technique was used by Combs et al. (1965) who showed that during the early stages of CTM cell maturation the granules were Alcian blue positive but as the cells matured the granules had an increasing affinity for safranin. The shift in staining affinity was associated with an increased turnover of radio-sulphate in the cells and was interpreted as initially reflecting the synthesis and accumulation of a heparin precursor in Alcian blue staining granules, which was then followed by the synthesis and accumulation of highly sulphated n-heparin. These findings have recently been substantiated by Meyer and Saunders (1968) who found a relationship between this change in staining from Alcian blue to safranin and the affinity of AMPS for acridine orange in different concentrations of electrolyte. Finally, the lower affinities of IM cells for acridine orange (Table 1) and for basic thiazine dyes (Table 1, Buxbiok, 1966b) would support the assumption that the acid mucopolysaccharide of the IM cell granules possesses fewer sulphate groupings than that in the CTM cell granule.

When the usual concentrations of formaldehyde were used to fix IM cells, there

was no staining of the AMPS in the granules. Enesbleik (1966a) suggested that the action of formalin might result from the blocking of basic groups in the protein moiety and so interfering with ionic linkages between the protein and AMPS, with the dissolution of the latter. The present results showed that this was not the case; IM cells could be stained with Alcian blue once the concentration of magnesium chloride was raised to 0.4 M, suggesting that the AMPS is bonded to the basic protein by salt linkages which can be disrupted by increased concentrations of electrolyte. Thus, the polyanions of the AMPS are probably masked by the basic protein when conventional staining techniques are used after formaldehyde fixation.

The strong staining of IM cells with the acid dye Biebrich scarlet, indicated the presence of a highly basic protein in the granule matrices. Biebrich scarlet has a strong affinity for highly basic proteins when used in glycine buffers at a high pH (Spicer and Lillie, 1961). Spicer (1963) noted that only certain mast cells in the rat stained with Biebrich scarlet, including those of the cervical lymph node, but he did not specify the other sites at which this histochemical property of the mast cell could be demonstrated. The CTM cells could not be stained by this method and it is not clear whether this absence of staining reflects a qualitative or quantitative difference in the protein content of the granules of the respective cell types. Their staining affinities may, however, reflect different ionic bonding between the protein and the AMPS in each cell. It is well established that the mast cell granules of the rat contain a basic protein which is probably linked to heparin by ionic bonds (Lagunoff, 1968). Biochemical assay of the granules (Benditt and Arase, 1959; Lagunoff and Benditt, 1968) and the identification of a substrate activity within intact isolated rat peritoneal

mast cells (Budd, Darzynciewicz and Barnard, 1967) have shown that part of the protein in the granules is composed of a chymotrypsin-like enzyme and in view of the basic protein content of the IM cell it is tempting to suggest that a similar enzyme exists within its granules.

The paraformaldehyde condensation method for the demonstration of monoamines (Falck et al., 1962) was used to study the monoamine content of the IM cells. Mast cells in the villus fluoresced a dull green whereas some of the cells in the crypt region fluoresced a moderately bright yellow. The high specificity and extreme sensitivity of the paraformaldehyde condensation method is well established (Corrodi and Jonsson, 1967), but the intensity of fluorescence depends not only on the nature of the amine, but also on its concentration in the cell, and in view of the rather close peaks of emission of the condensation products of 5-HT and catecholamines, their identification is more certain when microspectrofluorometric measurements are made (Corrodi and Jonsson, 1967). The dull green fluorescence of the IM cells has been attributed to the presence of a monoamine (Enerbäck, 1966d) but on the basis of available data, it is not possible to speculate further on the nature of this monoamine. The IM cells which showed yellow fluorescence although not as bright as the enterochromaffin cells or the CTM cells, presumably contained 5-hydroxytryptamine.

Pre-treatment of normal rats with L-DOPA caused the cells to fluoresce bright green, but those cells in the crypt region retained their yellow fluorescence as did the CTM cells in the tongue. Enerbäck (1966d) showed that IM cells fluoresced green after treatment with L-DOPA, but did not observe yellow fluorescent cells in the crypt

region of either treated or untreated rats and these differences may reflect the use of dissimilar strains of rat.

The specificity of the fluorescence was apparent from its abolition when para-formaldehyde treatment was omitted. Some cells had a non-specific fluorescence, but this continued after prolonged exposure to ultraviolet light whereas monoamine fluorescence was markedly reduced by such treatment; the latter being a well-recognised characteristic of monoamine fluorescent compounds (Conrodi and Jonsson, 1967). The use of 1.5 μ sections from epon-araldite embedded tissue enabled the fluorescent mast cells to be identified with certainty.

The present data emphasise and enlarge on the findings of Enerbäck (1966a, b, d) that the IM cell differs histochemically and to a lesser extent, morphologically, from the CTM cell. Even so, IM cells have all the features which assure the identification as mast cells since they contain cytoplasmic granules in which there is a sulphated acid mucopolysaccharide complexing with a highly basic protein and binding histamine (Hakanson, Owman and Sjöberg, 1967) as well as a monoamine. The unique relationship between the AMPS and the basic protein has frequently resulted in failure to detect IM cells by the usual histochemical methods (Mota, Yoneda and Ferri, 1966; Wuur, 1966a; Wuur and Grazia, 1967). However, the experiments of Enerbäck (1966a, b) and the present results show that IM cells can be readily detected provided that suitable fixation and histochemical techniques are used.

Part 2

Ultrastructure of the Intestinal Mast Cell

The fine structure of the connective tissue mast cell of the rat has been examined both *in situ* (Singleton and Clark, 1965; Fujita, 1966; Combs, 1966) and after isolation from the peritoneal cavity (Bloom and Haegermark, 1965). There is general agreement that rat mast cells characteristically are packed with granules whose matrices have no evident substructure, but are delimited by unit membranes (Benditt and Lagunoff, 1964; Combs, 1966). The granules vary in size from 0.5 to 2 μ diameter (Benditt and Lagunoff, 1964); other organelles are sparse although Golgi complexes are sometimes present (Smith, 1963).

It was necessary to examine the IM cells to establish whether morphological and histochemical differences from the CTM cell were reflected at the ultrastructural level. It was also important to ensure that the methods of fixation and embedding provided good preservation of IM cells so that results from further experiments could be satisfactorily evaluated.

Combs (1966) emphasized the importance of employing a suitable fixative for the interpretation of changes amongst mast cells, pointing out that osmium tetroxide and potassium permanganate caused extensive degranulation, whereas glutaraldehyde and formaldehyde provided adequate fixation of mature cells. Bloom and Haegermark (1965, 1967) achieved good preservation of isolated peritoneal mast cells using 4% glutaraldehyde. Other aldehydes, including acrolein and a combination of acrolein and glutaraldehyde (Fujita, 1965; Kent, 1966) have also provided adequate fixation of mast cells. Recently, metallic dyes, having an affinity for acid mucopolysaccharides, have been incorporated in the fixative; thus, Ruthenium red increased the contrast of

the granules (Gustafson and Pihl, 1967) and Alcian blue improved the preservation of immature mast cell granules (Combs, 1966).

Not only should the mast cell granules show adequate preservation, but the overall fixation of the tissues should also be satisfactory; attention should be paid to the osmolality of the solution as well as the concentration of the fixative (Maunsbach, 1966; Mazor, Powell and Philpott, 1967). Murray (1968) tested several fixatives using buffer systems to vary the osmolality and using different strengths of glutaraldehyde he found that 1.5 to 4% glutaraldehyde in 0.067 M phosphate buffer was satisfactory for bovine tissues and rat intestine.

In preliminary experiments, it was found that the lower concentrations of glutaraldehyde did not preserve IM cell granules and even though CTM cell fixation was adequate with2% glutaraldehyde, 4% glutaraldehyde provided the most satisfactory fixation of IM cells. Addition of Alcian blue 8 GX (Combs, 1966) or Ruthenium red (Gustafson and Pihl, 1967) proved to be of no particular advantage, presumably because of the relative impermeability of the intestinal epithelium since thick sections showed that these dyes did not penetrate beyond the surface epithelium (Miller, unpublished observations). Because no one fixative was entirely satisfactory, the morphology of the IM cells and the probable artefacts of fixation, are evaluated and discussed and the IM cells are compared with the connective tissue mast cells.

Materials and Methods

Tissues were taken from seven female hooded Lister rats weighing between 180 and 215 gm. Segments of jejunum (10 - 20 cm. posterior to the pylorus) and small blocks of tongue were removed from the rats under trichloroethylene anaesthesia. The procedure for fixation, dehydration, embedding and staining are described in General Materials and Methods.

Low power electronmicrographs (magnification x 1,500) of thin sections were compared with the adjacent thick sections. This allowed the same individual cells to be visualised ultrastructurally and by light microscopy. Thick sections (1 - 1.5μ) were stained by the methods of Richardson et al. (1960) and Ackerman and Hostetler (1968).

Results

Intestinal Mast Cells

Identification: By using stained epoxy-embedded sections approximately 1.5 μ in thickness for light microscopy and studying the adjacent thin sections in the electron microscope, it was possible to locate the same mast cell and compare its morphological and staining properties with its ultrastructural features. The granules of the majority of mast cells, either stained a deep blue with methylene blue-azure II-borax (Figures 11, 12, 13 and 14) or were coloured a deep orange-red with basic fuschin.

Distribution: Mast cells were found in the lamina propria of the villus, lying between crypts and below the crypts above the muscularis mucosa. They were more rarely

found in the submucosa and an occasional cell which had all the distinguishing features of a mast cell, was found intraepithelially (Figures 15, 16), usually in the gland crypts. Most of the IM cells were found in the vicinity of the basement membrane of the epithelium, or beside small blood vessels.

Morphology: There was considerable heterogeneity amongst IM cells with regard to size and shape as well as the content of granules. On the whole, the cells in the villus tended to be smaller and more globular than those in the crypt region. Oval, polygonal, elongated and spindle-shaped mast cells were found (Figures 11 - 14) and the nuclei of the oval and polygonal cells were usually encircled by granule-bearing cytoplasm, whereas those of the elongated and spindle-shaped mast cells were rarely completely surrounded by granules, having narrow rims of cytoplasm free of granules laterally. Some cells were large and were packed with many granules. Others were considerably smaller and contained few granules. Small finger-like processes were found at the borders of some of the IM cells (Figure 14).

Whereas the intergranular cytoplasm of the majority of mast cells contained sparse amounts of endoplasmic reticulum and few organelles (Figures 17, 18, 23, 24 and 28) there were a number of cells which had rather more dense cytoplasm containing small ribosome-like particles and dilated cisternae of rough surfaced endoplasmic reticulum (RSER) (Figures 19 - 21); often the perinuclear cisternae were likewise dilated. Many of these cells appeared to be closely related to, or forming syncytiae with fibroblast-like cells (Figures 20 and 21). IM cells, showing these various features formed approximately 10 - 15% of the total IM cell population.

Granules: The majority of IM cell granules had homogeneous matrices of moderate electron density although this varied with the type of embedding medium. Those embedded in Araldite were less electron-dense than granules of mast cells embedded in epoxy-araldite mixtures. The granules were delimited by trilaminar unit membranes and an occasional granule was bordered by a double unit membrane. Granule size varied from 0.4μ - 1.6μ, but in general measured about 0.6μ diameter. Although in the majority of cells the granule matrices had no substructure, there was an occasional cell containing one or two granules in which small vesicles or myelin configurations were visualized (Figures 22 and 23). In tissues that were well fixed as judged by other criteria, such as absence of myelin figures or mitochondrial swelling or of breakdown of cristae mitochondriales, the IM cell granules to a large extent showed corresponding satisfactory fixation and were of uniform density and homogeneity. In some of the cells, though, there was an occasional granule whose delimiting membrane was separated from the matrix by a clear space (Figure 24). These granules could often be detected in thick sections by light microscopy. Approximately 10 - 20% of IM cells showed this feature in one or at most, two, of their granules, the remainder having uniform fixation of the granules. More rarely, IM cells were vacuolated as if the matrices of several granules had been extracted (Figure 25). In the second type of mast cell with the more dense intergranular cytoplasm, the granules themselves had more granular or particulate matrices, many of which appeared to be partially extracted or eluted (Figures 21 and 26). Mast cells of this type were difficult to locate on thick sections because their granules stained weakly with basic dyes.

Golgi complex: Golgi complexes were found in many of the IM cells. They tended to vary in size and were usually situated in a paranuclear position. In some cells they were extensive, and were composed of several groups of 4 - 6 flattened parallel cisternae with small sack-like dilatations at the periphery (Figures 18, 21, 22, 23, 27 and 28). When a suitable plane of section through the centrioles was achieved, the cisternae were seen to be disposed in a semi-circular fashion around about the centrioles. Dense cored vacuoles (progranules) of various sizes, but smaller than the granules, were found in the Golgi regions. They were relatively numerous in the more elaborate complexes, but were less common in cells which only contained small Golgi regions. Each progranule had a dense central core separated from the surrounding unit membrane by a clear space (Figures 27 and 28). Progranules were occasionally found scattered amongst the intergranular cytoplasm. Swarms of uncoated vesicles were also located in the Golgi complex, and their size varied although the majority were approximately 600 Å diameter. Smaller numbers of coated vesicles were also found and were larger than the uncoated variety measuring approximately 700 - 1000 Å diameter and were distinguished by a bristle-like coat on their outer surface (Figure 28). Microtubules were occasionally noted in the Golgi region.

There was no clear-cut relationship between cell size, granule content and the extent of the Golgi complex, although, in general, cells which were densely packed with granules tended to have the least fully developed Golgi complexes.

Intergranular Cytoplasm The cytoplasm of the mast cells, apart from the content of granules, was unremarkable and usually only a few small strands of RSER could be detected in the majority of cells. Free ribosomes were also few in number. In the

second type of mast cell, the dilated cisternae of RSER and the perinuclear cisternae frequently contained small cytoplasmic peduncles (Figures 19, 21 and 26). These cells also contained a number of ribosome-like particles which gave them an appearance of increased density when compared with the majority of IM cells (Figures 19 and 20. cf. Figures 17 and 18).

The mitochondria were small, oval structures, and the cristae were arranged in parallel. Mitochondria were few in number, and were scattered throughout the cytoplasm.

Nucleus The nuclei tended to assume a shape similar to that of the cell, being elongated in the spindle-shaped cell and oval or polygonal in the more globoid IM cell. Reniform and irregularly shaped nuclei were commonly found. Nuclear chromatin was usually coarsely clumped and in some cases was fairly heavily marginated. Nucleoli were rarely seen.

Connective Tissue Mast Cells

The majority of CTM cells were oval and of fairly uniform size and were densely packed with granules. Usually the granule-bearing cytoplasm encircled the nuclei (Figure 29). The granules had homogeneous matrices and were delimited by unit membranes. Golgi complexes tended to be small and were observed with less frequency than in IM cells. The progranules and Golgi-associated vesicles were less prominent in the CTM cell than in the IM cell. The nuclei tended to be oval with evenly dispersed chromatin.

Other Granule-bearing Cells

CTM and IM cells were readily differentiated from other cells containing granules or inclusions. Eosinophils (Figure 30) were abundant in the intestinal lamina propria and contained smaller granules which characteristically had central dense crystalline bars. Other distinguishing features included the doughnut or bilobed shape of the nucleus and the refractile greenish colour of the granules in sections stained by the method of Richardson et al. (1960).

Neutrophils were rarely found in the intestinal lamina propria. They had small granules and a characteristic polymorphous nucleus (Figure 31).

There was a superficial resemblance between macrophages containing many inclusions and the mast cell which had few granules, but the macrophage inclusions tended to be very heterogeneous, including phagosomes, residual bodies, multi-vesicular bodies and lipid-like inclusions. Apart from the phagosomes, none of these inclusions stained with any intensity in the thick sections and allowed a distinction to be made between mast cells and macrophages. Macrophages tended to have more extensive strands of endoplasmic reticulum and more abundant vesiculated cytoplasm (see Section III, part 1 and part 6).

Discussion

Histochemically, the IM cells in the rat had shown all the features that ensured their identification as mast cells, namely that they contained numerous cytoplasmic granules whose matrices were composed of a sulphated acid mucopolysaccharide-basic protein complex (Section I, part 1). The present results showed that although there were morphological differences between the cells, the granules were indistinguishable

from those found in adult mast cells elsewhere in the body (Bloom and Haegermark, 1965; Combs, 1966). In this study both the CTM cell and the majority of IM cells contained granules with homogeneous electron-dense matrices and outlined by unit membranes.

The identification of mast cells was facilitated by examining thick sections stained with basic dyes and comparing them with adjacent thin sections, so that it was possible in some instances to identify the same individual cell by both light and electron microscopy. This method was used by Bloom and Haegermark (1965; 1967) who stained thick sections with toluidine blue and found that the majority of mast cell granules stained deep blue or purple. In the present experiment, the thick sections of the intestinal mucosa were stained with either methylene blue-azure II-borax (Richardson et al., 1960) or with basic fuschin in 50% acetone (Ackerman and Hostetler, 1968) and the IM cell and CTM cell granules stained deep blue or deep orange-red with the respective dyes. Thus, their characteristic morphology together with their staining properties in thick sections, enabled IM cells to be differentiated from other granule-bearing cells and from macrophages containing a variety of inclusions.

Stained thick sections proved to be of particular value when assessing the state of preservation of the mast cells after fixation because they allowed a much greater area of mucosa to be examined than would have been possible with thin sections alone. Bloom and Haegermark (1965, 1967) found that some of the isolated rat peritoneal mast cells contained several red or pink granules which they were able to correlate to swollen granules in thin sections having less electron-dense and more

particulate matrices than normal. They assumed that these merely represented normal granules that had been altered in some way during the process of isolation and fixation. They found, however, that 4% glutaraldehyde in a phosphate buffer gave optimal standards of fixation (Bloom and Haegermark, 1965;1967).

Four per cent. glutaraldehyde in phosphate buffer was used to fix the intestinal mucosa, but the results are not directly comparable to those obtained for isolated peritoneal mast cells. Fixation of the latter required minimal penetration by the fixative; in addition, the cells had undergone the potentially disrupting process of isolation. Adequate preservation of IM cells on the other hand required the fixative to penetrate some distance through the tissues. Even so, the standards of fixation appeared to be equivalent to those obtained by Bloom and Haegermark. Approximately 10 - 15% of the IM cells contained one or two granules whose delimiting membranes were separated from the matrices by an electrolucent area. These altered granules were visible in both thin and thick sections. The matrices of some of the granules ultrastructurally had a leached-out appearance and in other cells the matrices of some granules had disappeared completely. Since these changes were noted in only one or two granules in a small proportion of IM cells, they were judged to be an artefact of fixation rather than functional changes in the cells themselves. In view of the considerable difficulties that have been encountered in the fixation of mast cells (Combs, 1966) the present results were considered to provide satisfactory criteria for judging the preservation of IM cells in future experiments.

The IM cells showed some morphological heterogeneity and although a few IM cells had many densely packed granules in the cytoplasm, the majority contained fewer granules which were more sparsely distributed than was the case in CTM cells.

These morphological differences would explain the reported atypical appearance of the intestinal mast cells (Maximow, 1906; Enerbäck, 1966a). Other features which contrasted with the CTM cell were the rather more extensively developed Golgi complexes and the greater variations, not only in size, but in shape of the IM cells.

The reasons for such differences are not clear, but as far as the Golgi complex is concerned, it was well recognised that the latter, in granulocytes and maturing mast cells, expands markedly during the process of granule elaboration (Hainton, and Farquhar, 1966; Combs, 1966). The granules are manufactured in the Golgi complex and are redistributed in the cytoplasm and as the cells reach maturity, the Golgi decreases in size and ceases to elaborate granules.

It is not clear whether an analogy can be drawn for the IM cells with regard to the size of their Golgi complexes. For example, a large complex may mean that the cells are producing granules continually. On the other hand, it could reflect a young cell manufacturing granules and the variations in size and granule content of the IM cells may be indicative of different stages of maturation. In any event, it is tempting to speculate that these cells are subject to a variety of immunological stimuli following the exposure of the intestinal tract to a multiplicity of antigens. If such circumstances prevail, then it would be reasonable to postulate that the IM cells constitute a less stable population than the CTM cells and that the variations seen amongst them do indeed reflect the different stages of maturation.

The IM cells with dense cytoplasm were unusual in that they had a higher

content of ribosomes and RSER than did the majority of IM and CTM cells. In addition, their granule matrices tended to be more granular and less dense than was the case in the majority of mast cells. Many of the granules in this cell type showed evidence of extraction of the matrix. It was, perhaps, significant that this type of mast cell was difficult to locate on thick sections. Little is known about the affinity of epoxy-embedded acid mucosubstances for basic dyes, but it is tempting to suggest that the granules which had a reduced affinity for them, either have a smaller content of AMPS or that the granules differ in their chemical composition from those found in the majority of mast cells.

The present results delineate various features of mast cell structure and provide some criteria for the recognition of ultrastructural changes that might be the result of suboptimal fixation.

Part 3

The Action of Compound 48/80 on the Intestinal Mast Cell

In a series of experiments, Riley and West (1955) and Riley (1959) showed that compound 48/80 acted as a histamine liberator by causing the disruption of connective tissue mast cells. Since then, the specific effects of compound 48/80 on isolated rat peritoneal mast cells have been examined in some detail and a relationship between the dose given, the extent of mast cell damage and the amount of histamine released has been established. Thus, increasing doses of compound 48/80 resulted in progressively more severe mast cell disruption and concomitantly augmented release of histamine (Bloom and Haegermark, 1965). Riley and West (1955) gave rats treatment with 48/80 over a period of five days and found that there was a parallel relationship between the histamine value of a particular tissue and its mast cell content. Many workers have used this subacute treatment with 48/80 to assess the mast cell and non mast cell histamine content of various organs (Johnson, 1968). Histamine remains at normal levels in the intestine during 48/80 treatment (Riley, 1959; Johnson, 1968) and there have been repeated failures to demonstrate mast cells in the intestinal lamina propria (Mota, Ferri and Venedo, 1956) so it has often been concluded that the intestinal histamine content is stored in non-mast cell sites.

The validity of these conclusions must be re-examined in the light of Enerbäck's work (1966a, b, d) and the findings in the first part of this section where mast cells were demonstrated in more or less abundance in the intestinal lamina propria. Furthermore, Enerbäck (1966c) found that RM cells were resistant to the degranulating effects of 48/80 and that during subacute treatment with 48/80 RM cell numbers were not depleted but were, in fact, increased.

Because of these conflicting reports, it seemed relevant to re-examine the effects of compound 48/80 on the IM cells and accordingly, experiments were devised with two purposes in mind. In the first place, the short term action of 48/80 was investigated because it seemed likely that the time lapse between giving 48/80 and sacrificing the rat in previous experiments (Enerbäck, 1966a) might be too short for the detection of changes amongst IM cells. Furthermore, it has recently been shown that isolated rat peritoneal mast cells given two separate treatments with 48/80 undergo two separate phases of degranulation (Thon and Uvnäs, 1967). To investigate the short term action of 48/80, rats were killed one hour after two separate intra-peritoneal doses of 48/80.

In the light of previous results after subacute treatment with 48/80 (Enerbäck, 1966a) it was hoped that this might provide a way of artificially boosting the mast cell numbers using non-immunological methods, thus providing a system comparable to the effects of Nippostrongylus infections (see Section 3, part 2). An even longer term programme of intermittent 48/80 treatment was also devised and was directed towards the same end.

Materials and Methods

Experiment 1

Thirty-five adult female hooded Lister rats weighing 170 - 220 gm were used. Five were kept as controls and thirty were treated with compound 48/80. Compound 48/80 was dissolved in distilled water to give concentrations varying from 0.1 mg/ml to 0.5 mg/ml.

Rats were divided into four groups of five and one group (Group 1) of ten rats. All rats were given an intraperitoneal dose of 0.05 mg. of 48/80 followed 7 hours later by a second dose of 0.1 mg and those in Group 1 were killed 1 hour later. Rats in Groups 2, 3, 4 and 5 were treated with 0.15 and 0.2 mg. of 48/80 on the 2nd day and with 2 x 0.3 mg. on the 3rd day. The rats in Group 2 were sacrificed 1 hour after the last dose on the third day. 2 x 0.4 mg. and 2 x 0.5 mg. of 48/80 were given on days 4 and 5 respectively and the rats in Group 3 were sacrificed 1 hour after the last dose on the 5th day. Group 4 rats were given 2 x 0.5 mg. 48/80 each day until day 8 when only 1 injection was given, the rats being killed 4 hours later. Those in Group 5 were given no further treatment after the 5th day and were sacrificed at the same time as the rats in Group 4.

Histological Procedure: All rats were lightly anaesthetised with Trichloroethylene before being killed by cervical dislocation. Tissues were taken from jejunum, tongue and ear of each rat, placed in Carnoy's fluid and allowed to fix overnight. The subsequent dehydrating and embedding processes are described in General Materials and Methods.

The blocks of intestine were orientated to give longitudinal sections at right angles to the surface of the mucosa. The sections were stained with Astra blue safranin for counting purposes; the details of the counting procedures and of the measurements of mucosal expansion are described in Section 3, part 2.

Procedures for Electron Microscopy: Blocks of jejunum for electron microscopy were removed from rats in Group 1, anaesthetised with Trichloroethylene. The fixation and embedding procedures are described in detail in General Materials and Methods.

Experiment 2

Eight adult female hooded Lister rats, weighing 185 - 220 gms. were used. One group of four rats was given an intraperitoneal injection of 0.05 mg. of compound 48/80 once a week for a period of 6 weeks. The second group of four rats was left untreated as controls. Both groups were sacrificed one week after the last treatment with 48/80. Histological procedures and counting methods were as described for Experiment 1.

Results

Experiment 1

Clinical Signs: Within half an hour of the first treatment with 48/80, the majority of rats were more dull than usual, were unreactive to stimuli and tended to wash or scratch their faces. Five or six rats collapsed and were dyspnoeic, having swollen and reddened ears and snouts. Within two hours of treatment, the majority of rats

were fully recovered. The second dose of 48/80 seven hours later caused the collapse and prostration of many rats and two died within fifteen minutes of treatment. At post mortem, the latter had markedly congested serosae with some subserosal and mucosal haemorrhage, particularly in the colon and ileum. On the second and third days, many rats collapsed soon after treatment with 48/80 and a further two rats died. On the fourth day and thereafter, the treated rats showed little or no reaction to further doses of compound 48/80, although one rat died on the fifth day of treatment.

Rats necropsied one hour after treatment with 48/80 on the first and third days had congested or haemorrhagic serosae and the gastrointestinal tract was distended with gas. Subserosal and mucosal congestion and haemorrhage was most severe in the posterior ileum or colon, but was also noted occasionally in the jejunum and stomach and other abdominal blood vessels were often markedly congested.

Histological and Quantitative Findings

IM Cells: Group 1. There was a highly significant reduction in the overall numbers of IM cells (Table 2) although many of them as judged from Astra blue/Safranin stained sections, were unaltered. A proportion of the IM cells in three of the eight rats in Group 1 had migrated intraepithelially to become globule leukocytes (See Section 2). In all of the rats, there were IM cells which had shed their granules or were fragmented (Figure 33). Some of them showed reduced eletanophilia and others had pale-staining halos around the nuclei. The nuclei in a few cells were pyknotic and stained brightly with safranin. Blue-staining granule remnants and pink-staining debris were observed

TABLE 2

IM Cells in Rats Treated with Compound 48/80 (EXPERIMENT 1)

MAST CELLS / VILLUS-CRYPT UNIT

Control		GROUP I (Day 1)	GROUP II (Day 3)	GROUP III (Day 5)	GROUP IV (Day 8)	GROUP V (untreated) (Day 8)
12.7	7.1	5.3 + (2.4)[a]	6.3	10.9	10.8	8.2
11.1	5.2	5.5 + (3.8)	7.5	8.2	10.0	10.4
10.8	8.9	7.1 + (4.1)	6.3	9.6	11.2	11.1
12.1	5.6	D	6.0	10.1	11.2	10.0
11.8	5.1	D	D	D	10.0	9.6
Mean ± SE	11.7±0.3	7.5 ± 0.8[b]	6.5 ± 0.3	9.7 ± 0.6	10.6 ± 0.3	9.9 ± 0.5

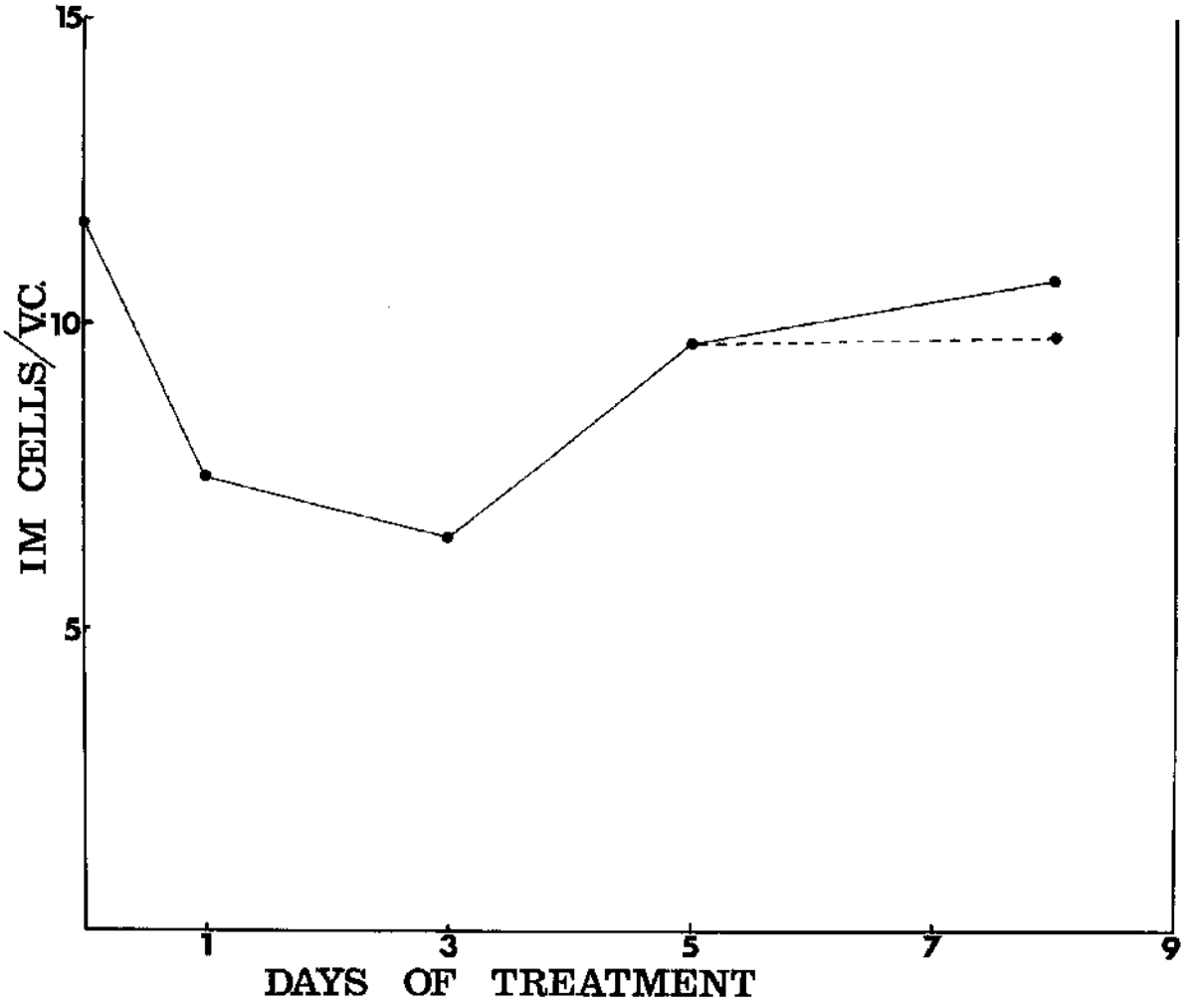
( )[a] = mean number of globule leukocytes

D = died

b = globule leukocytes included in total (see Section II)

Control V GPI	$P < 0.001$
Control V GPII	$P < 0.001$
Control V GPIII	$P < 0.02$
Control V GPIV	$P < 0.05$
Control V GPV	$P < 0.02$

Graph 1



Changes in the intestinal mast cell population during treatment with Compound 48/80.

within the cytoplasm of macrophage-like cells.

Group 2. The numbers of IM cells remained depressed on the third day of treatment (Table 2). There was, however, a proportion of apparently unaltered IM cells, although many stained weakly, especially in regions denuded of epithelium. Very few globule leukocytes were found within the epithelium.

Groups 3, 4 and 5. The population of mast cells was slightly depressed on the fifth day (Table 2) and the numbers remained at the same level in both groups on Day 8 (Table 2). No morphological difference could be detected between IM cells of the treated rats and the control rats, and there was no change in their distribution in the lamina propria. The results are recorded in Graph 1.

General Observations: Oedema was evident in a few villi in some rats in Group 1, but the majority showed little alteration in size or shape (Table 3). There was an increase in the number of safranin-staining inclusions within macrophages and the brightly staining nuclear and cytoplasmic debris in the lamina propria suggested that several cells might be undergoing cytolytic changes. Some of the villi in Group 2 on the third day of treatment were oedematous, the epithelium of many of the others was separated from the basement membrane and in many instances lost altogether. It was therefore not possible to measure villus expansion. The intestinal mucosa in Groups 3, 4 and 5 on the fifth day of treatment and thereafter was of normal appearance being indistinguishable from that in untreated rats and there was no evidence of villus oedema or mucosal expansion.

TABLE 3

Measurement of Villus Area in R. to Treated with

Compound 49/80 (EXPERIMENT 1)

Villus Area mm^2

Control	GROUP I
0.054	0.052
0.051	0.042
0.051	0.039
0.051	0.056
0.046	0.060
Mean ± SE 0.051 ± 0.001	0.050 ± 0.004

Control V GPI N.S.

CTM Cells: The response of the CTM cells varied amongst individual rats in Group 1. Some merely showed a decreased affinity for safranin, others were disrupted and the scattered granules were no longer coloured by safranin, but were stained with Astra blue (Figure 34). Often an amorphous material which stained pale blue was scattered around the discharged mast cells. In Group 2 on the third day of treatment, mast cell numbers were depleted and those that remained were small and contained a mixture of blue and red staining granules. The fifth day of treatment and thereafter showed a further reduction in the numbers of CTM cells with very few faintly blue-staining cells remaining, although one or two small safranin-staining cells were found beneath the epithelium. Larger numbers of small blue-staining cells were found in the tongues of the untreated rats of Group 5.

Ultrastructural Findings

The intestinal lamina propria was first examined using stained resin-embedded thick sections under the light microscope. The IM cells showed a variety of changes ranging from the alteration of 1 or 2 granules similar to that found in IM cells of normal rats to rather extensive vacuolation. It was clear, however, that cytolysis had occurred in the lamina propria since many macrophages contained ingested cell debris (Figures 35 and 36).

Ultrastructurally, the majority of mast cells were unaltered and only a few degenerating mast cells were found free in the lamina propria. These showed swelling of the cytoplasm with separation of the granules and the matrices of many

TABLE 4

IM Cells in Rats Treated with Compound 48/80 (EXPERIMENT 2)

MAST CELLS / VILLUS-CRYPT UNIT

Controls	Treated
13.9	16.5
13.4	16.6
13.8	24.1
13.4	13.2
Mean ± SE 13.6 ± 0.1	16.8 ± 1.6

Control V. Treated $P < 0.05$

of the granules themselves were partially eluted or were more granular than normal (Figures 37 and 38). Mast cell granules were identified within the phagosomes of some of the macrophages (Figure 39) but so also were remnants of other cell types including eosinophils and plasma cells (Figure 40).

Experiment 2

With each treatment, the four experimental rats showed mild clinical signs and within half an hour of receiving 48/80 were inclined to be dull and listless and to remain apart from each other; their fur was ruffled and they tended to scratch and to rub their faces. Occasionally one or two rats collapsed and remained prostrate for a short period of time, but they recovered rapidly. At post-mortem, there were no obvious abnormalities in the treated rats.

Histologically, the intestinal mucosa was of normal appearance in both the treated and control groups and there was no morphological or distributional difference amongst the IM cells. Quantitatively, there was a slight increase in the number of IM cells in the treated as opposed to the control rats (Table 4).

The connective tissue mast cells in both groups stained strongly with safranin.

Discussion

The results from these experiments emphasise the differences between the IM cells and their connective tissue counterparts. Acute treatment even with small doses of 48/80 intraperitoneally caused the disruption of the latter with scattering of their granules. Subacute treatment resulted in a marked depletion of the connective

tissue mast cells and these findings are in agreement with the results of earlier experiments (Riley and West, 1955; Riley, 1959). In contrast, the IM cells showed greater resistance to compound 48/80; the short term acute treatment reduced the overall numbers but without causing degranulation of a severity comparable to that seen in CTM cells. Of more significance was the ability of the IM cell to show a partial recovery and to persist in the mucosa during the course of subacute treatment with 48/80.

Enerbäck (1966c) found that IM cells were unaltered after treating rats with 48/80 but the interval of time between giving the drug intravenously and excising the tissue was short, and only a single treatment was given. In the present experiment, two treatments were given intraperitoneally over a period of several hours and the counts pointed to a significant reduction in the numbers of IM cells in the intestines of Group 1 rats after the first day of treatment. This observation was supported by both light microscopic and ultrastructural findings where IM cell damage was detected. The difference between the present results and those of Enerbäck (1966c) may, therefore, stem from the double treatment given over a period of several hours which might have allowed sufficient time for the destruction of a proportion of the IM cells. The strains of rats used in the respective experiments might also have had differing susceptibilities to 48/80.

Compound 48/80 has a direct effect on the CTM cell of the rat and is probably dependent on energy requiring enzymatic processes in the cells because its action is blocked by metabolic inhibitors (Högberg and Uvnäs, 1957; Mota and Ishii, 1960), and

by anoxia in the absence of glucose (Diamant and Uvnäs, 1961). It has been suggested therefore that the varying degranulation responses in IM and CTM cells may reflect the differing enzyme contents in the cells (Enerbäck, 1966c) and further work is required to resolve this question.

During the subacute treatment, the numbers of IM cells were reduced but not to the same extent as CTM cells. IM cells were therefore able to persist in the mucosa during the course of treatment and did not require a post treatment recovery period. In this respect, the reaction of the CTM cells to 48/80 was substantially different because the reappearance of mast cells in the tongue and ear was confined to the small, poorly granulated cells in Group 5, where treatment had ceased. The characteristic pale blue staining of their granules with Astra blue safranin would suggest that these cells were equivalent to the maturing cells found in embryonic and baby rats (Combs et al. 1963). Riley and West (1955) pointed out that the CTM cells recovered after the cessation of treatment with 48/80 and were derived either from large ghost cells or from very small cells apparently arising in the adventitia of the blood vessels.

Enerbäck (1966c) gave rats a subacute treatment with 48/80 and found that the IM cells increased to twice the normal numbers in the treated rats, but in the present experiment, the IM cell numbers were slightly depressed in the treated groups. The different results may reflect the strains of rats used or they may possibly relate to the methods of mast cell fixation, staining and quantitation adopted in the experiments.

Despite these discrepancies, the persistence of many IM cells during treatment would, as Enerbäck (1966c) pointed out, be an argument in favour of histamine in the

intestinal mucosa being present within the mast cells rather than in non mast cell sites and this location for histamine has been confirmed by histochemical means (Håkanson, Owman and Sjöberg, 1967).

The failure of subacute treatment with 48/80 to substantially increase mast cell content of the jejunum was disappointing and even though intermittent treatments with this drug caused a small rise in the numbers, it was not sufficient to merit further attention. These experiments had been designed to provide a method of artificially inducing an increase in the Mf cell population by non immunological means. The ability to do this might have been of use in the studies of local hypersensitivity reactions to Nippostrongylus brasiliensis.

SECTION II

The Globule Leukocyte and its Derivation from the Intestinal (Subepithelial) Mast Cell

The globule leukocyte (GL) is a cell of striking morphology whose origin has remained unknown and whose function is uncertain. It is found within epithelia and characteristically contains large acidophilic granules or globules. The GL occurs in many species and is particularly prominent in parasitic infections. Early studies of these cells resulted in conflicting opinions as to their nature; they were variously termed eosinophils, aberrant tissue mast cells, degranulating mast cells, plasma cells and Russell body cells (Michels, 1938).

Globule leukocytes are found in the intestinal epithelium of the rat during Nippostrongylus brasiliensis infections (Taliaferro and Sarles, 1939) and Whur (1966) reported that there was a relationship between the onset of self cure and the appearance of these cells in the epithelium.

It was recently suggested that the GL is derived from both the Russell body-containing cell (Whur and Johnston, 1967) and from the lymphocyte (Kent, 1952, 1966; Toner, 1965). Dobson (1966c) and Whur and Gracie (1967) considered mast cells and GL cells to be unrelated and that the GL was derived from immunoglobulin producing cells; they postulated that its function was to carry antibody into the intestinal lumen.

In the second part of this Section (Section 1, part 2), it was noted that a small proportion of IM cells were located within the epithelium; an observation also made by Enerbäck (1966a). A preliminary examination of Nippostrongylus infected rats had shown that there was an increase in the number of intra-epithelial cells with histochemical properties similar to IM cells. In view of these findings, a more detailed histochemical and ultrastructural examination of the relationship between IM cells and GL cells was undertaken.

The present findings show that GL cells are derived from IM cells and are in no way related to the family of immunoglobulin producing cells. The results have been published in conjoint papers with Jarrett and Murray (Jarrett, Miller and Murray, 1967b; Miller, Murray and Jarrett, 1967; Murray, Miller and Jarrett, 1966).

Materials and Methods

Eight female hooded Lister rats weighing 185 - 215 gms. were used. Five were infected subcutaneously in the groin with 3,000 N. brasiliensis larvae. They were killed nineteen days after infection. Three rats were hyperimmunised with successive doses of 3,000, 5,000, 8,000 and 10,000 larvae. They were sacrificed two weeks after the last infection.

Segments of jejunum approximately fifteen centimetres behind the pylorus were taken (this is a site where the parasites are found in large numbers during infection - see Section III). The histological and cytochemical procedures are described in General Materials and Methods. Jejunum was also taken for electron microscopy and the details are given in Materials and Methods.

Results

Histochemistry

Mast Cells - The histochemical properties of mast cells in normal rat intestine are described in Section I, part 1 (Table 5). The mast cells were more numerous in infected rats (Section III, part 2) (Figure 42).

Globule Leukocytes - GL cells were identified by their position within the epithelium (Figure 42). In sections stained with Haematoxylin and Eosin the granules of the GL, like those in the IM cell, were eosinophilic, but tended to be less densely packed within the cytoplasm and were in many instances larger than the granules of the IM cell.

Histochemically, GL cell granules had the same properties as those of the IM cell (Table 5). With toluidine blue at pH 0.3, the granules were not metachromatic

TABLE 5

Histochemistry of IM and GL Cells

(Tissues fixed in Carnoy's fluid)

		IM Cell	GL Cell
Toluidine Blue	pH 0.3	B ++	B ++
	pH 4.0	RP +	RP +
Astra or Alcian Blue/Safranin		B +++	B +++
Biebrich Scarlet	pH 9.9	OR ++	OR ++
Acridine Orange *	0.001%	OY +	OY +

* Fixed in formol sublimate

For Key see Table 1.

but they did stain orthochromatically, like those of the IM cell.

After the Falck technique for monoamines, the granules of some GL cells fluoresced with a weak green colour, not clearly attributable, either to 5-HT or catecholamine. Many of the GL cells failed to fluoresce at all (see Section III, part 3 for Figures).

Ultrastructure

Mast Cells - The fine structure of the normal IM cell has been described (Section I, part 2). In parasitised rats, the IM cells showed several ultrastructural differences. The granules tended to vary in size, some of them being larger than those in normal IM cells. In addition, the matrices were partially lost from some of the granules, leaving paracrystalline structures (Figure 44). Golgi complexes were larger, more highly developed and more frequently observed than in non parasitised animals. The granules tended to be less densely packed and the RSER was more prominent in some of the IM cells in parasitised rats than in normal rats (Figure 44).

Globule Leukocytes - GL cells were found at all levels within the epithelium. They were identified in thick sections stained with basic dyes (Figure 43 a and b) and were extremely common in the gland crypt epithelium (Section III, part 2). There were no junctional complexes between GL and epithelial cells and small cytoplasmic prolongations could be observed in the intercellular spaces (Figure 51). Granules were present in all GL cells examined, but their morphology varied. The following types were recognised:

Type 1. Granules which were indistinguishable from those of the subepithelial mast cell, having homogeneous matrices of moderate electron density and surrounded by unit membranes (Figures 46, 47, 49 and 51).

Type 2. Granules in which small areas or rims of less electron dense and more granular matrix separated the denser, homogeneous material from the delimiting membrane (Figures 48, 49 and 50). Small vesicles and myelin-like configurations were sometimes found in the periphery of these granules (Figure 49).

Type 3. The matrix was partially lost from these granules, leaving para-crystalline structures with electron density similar to the original matrix (Figures 46 and 48). Longitudinal striations with a periodicity of approximately 70 Å were present within the paracrystalline bodies. Often remnants of less dense but coarsely granular matrix were found within these granules (Figure 48). Small vesicles and myelin-like figures were occasionally seen within the perigranular membranes and these were frequently disrupted at various points (Figure 48).

Type 4. Granules from which the unit perigranular membranes were lost, leaving paracrystalline structures free within the cytoplasm (Figures 49, 50, 51 and 52).

In GL cells where granules of Type 3 and Type 4 predominated, the cytoplasm had a pseudo-vacuolated appearance (Figures 51 and 52). In some instances, there were degenerative changes affecting the nuclei and mitochondria of the GL cells. There was an even greater variation in granule size amongst GL cells than was found in the IM cells of parasitized rats. The largest granules, measuring approximately 3μ diameter,

Extensive Golgi complexes were found in many GL cells. They were similar to those of subepithelial mast cells in parasitised rats. In general, as in the fully granulated IM cell, RSER and ribosomes were sparse.

Many of the mast cells in the lamina propria lay very close to the basement membrane and projections of cytoplasm from these cells were occasionally found in direct contact through the epithelial basement membrane with similar projections from the basal part of the epithelial cells (Figure 58).

Discussion

Experimental infections with N. brasiliensis afford an excellent opportunity to study the GL in the rat, since it is well established that GL cells increase in number during infection (Taliaferro and Sarles, 1939; Wiwa, 1966; Jarrett et al., 1967a) It has also been shown that mast cells in the intestinal lamina propria increase in number in infected rats. (Taliaferro and Sarles, 1939; Wells, 1962), and that there is a quantitative relationship between the increase in mast cell numbers and the appearance of GL cells (Jarrett et al., 1967a; Section III, part 2).

The rat IM cell contains an acid mucopolysaccharide (AMPS) associated with a basic protein which differ histochemically from those in the CTMC (Section I, part 1). The AMPS of the IM cell has a weak affinity of thiazine dyes (Enerbäck, 1966b Section I, part 1) and yet stains strongly with copper phthalocyanine dyes. The present results show the intraepithelial GL cell has granules containing an AMPS which likewise stains weakly with thiazine dyes but retains Astra or Alcian blue when counterstained with safranin. Further similarities are shown by the staining of the granules with acridine

orange. The latter dye also, having an affinity for AMPS (Saunders, 1964). On the basis of these tests, the AMPS in the GL cell and IM cell are indistinguishable.

It has also been shown that the IM cells contain a highly basic protein within the granules (Section I, part 1). Using the acid dye, Biebrich scarlet, at high pH's the GL cells were found to contain a basic protein with similar affinities for this dye as the IM cells.

Some of the GL cells contained small amounts of monoamines, but in general, the fluorescence of the granules was of much lower intensity than in IM cells. The latter observation is probably the result of a functional change in the cells whereby monoamines are lost from the granules and this is discussed in Section III, part 5.

The morphological and histochemical similarities between IM and GL cells were striking and were further emphasised by the ultrastructural findings; granules delimited by unit membranes and indistinguishable from IM cell granules were observed in many of the GL cells. Other features that were common to both cell types, particularly in parasitised rats, included moderately large Golgi complexes, finger-like cytoplasmic projections and relatively small amounts of RSER.

The results show that the GL cell is closely related to the IM cell and that it is probably derived by migration of the IM cell into the epithelium. The migratory origin of the GL is indicated by the absence of any desmosomes or other junctions between it and the epithelial cells. The means by which GL cells reached their location in the epithelium was suggested by the points of contact between mast cells in the lamina propria and the basal parts of the epithelial cells. Presumably such a contact

would allow the migratory cell to break through the basement membrane and rest against the basal plasmolemma of the epithelium, a position in which many GL cells were found.

Bovine and sheep GL cells were also shown to be derived from subepithelial mast cells (Murray et al., 1968; Murray, 1968) and it was found that this transformation involved a loss of amine which was associated with a change in the relationship between AMPS and basic protein in the granules. In the bovine and sheep GL cells, the changes in the AMPS were recognised by the varying fluorescence of the granules when stained with acridine orange, and this was attributed to a change in the stacking of the dye on the altered AMPS. (Murray et al., 1968; Murray, 1968). In the rat, no such change was detected with acridine orange, but an alteration in the AMPS content of the granules was detected using the critical electrolyte method (Section III, part 5).

The histochemical evidence for granule discharge is further substantiated by the ultrastructural findings where type 2, 3 and 4 granules could all be stages in this process. The type 2 granule may reflect an early loss of components from the matrix which permits vesiculation of microvesicles and myelin-like configurations around the periphery of some of the granules; these are almost certainly related to the manufacture of the granules themselves (Section III, part 3). Further discharge of the GL is probably characterised by type 3 and 4 granules where the matrices are almost completely lost and the delimiting membranes are destroyed. The para-crystalline structures may be the remnants of the basic protein. In the bovine and sheep,

where histochemical data pointed to a loss of amine from the granules, their varied ultrastructure was thought to reflect this loss (Jarrett et al., 1967b; Miller et al., 1967; Murray et al., 1968).

Wiur and Johnston (1967) and Dobson (1966c) suggested that there were many similarities between the GL and immunoglobulin producing cells. They hypothesised that the globules contained immunoglobulin and that the function of the GL was to transport antibody across the epithelium to the target site. During this study, many plasma cells were seen, all of which were characterised by the presence of extensive well developed RSER (Figure 66). In some cells, the cisternae of RSER were markedly dilated to form Russell bodies (Figure 45). The latter were recognised by the presence of a rough surfaced membrane surrounding the dilated cisternae and were at all times easily distinguished from mast and GL cells.

It is clear from the quantitative (Section III, part 2), histochemical and ultrastructural data, that the GL is derived from the IM cell by the migration of the latter into the epithelium. The observed histochemical and ultrastructural differences between the two cell types, probably reflect a dynamic process of amine discharge and either a loss of AMPS or a change in the relationship between it and the basic protein (Murray et al., 1968; Section III, part 5). The possible mechanisms by which these changes are brought about and the functional significance that they might have are discussed in Section III and in the General Conclusions.

SECTION III

The Intestinal Mast Cell in the Parasitised Rat

Nippostrongylus brasiliensis: The life cycle of N. brasiliensis has been reviewed by Haley (1962). The parasites infect their natural host, the rat, as third stage larvae by penetrating the skin and they then migrate to the lungs. In this site, the larvae feed, grow and moult, they subsequently migrate via the respiratory tract, oesophagus and stomach to the small intestine. Four days after infection, the majority of parasites are found in the anterior part of the small intestine. They undergo a final moult and begin to lay eggs on about the fifth or sixth days of infection.

Once in the intestine the parasites localise in the jejunum but later in infection they are redistributed, and some of them migrate anteriorly into the duodenum (Bramhall, 1965). Egg laying reaches a peak between the eighth and tenth day of infection and declines just before the parasites are expelled. The worms are rejected in an exponential fashion between the eleventh and eighteenth days of infection. (Jarrett et al., 1968).

The kinetics of Nippostrongylus infections have been examined by Jarrett et al. (1968) and they described four phases:

1. Loss phase 1 (LP1) where a proportion of the infecting dose of larvae is immobilised or destroyed before, or during, the course of migration via the lungs to the intestine.
2. The plateau phase. The worms remain as a constant population in the predilection site.

3. Loss phase 2 (LP2) during which the parasites are expelled in an exponential fashion from the host by an immunological mechanism.

4. Threshold phase. A small residual population of the worm is not expelled and survives for a fairly prolonged period.

They also showed (Jarrett et al., 1968) that in animals undergoing second or third infections LP1 becomes greater, the plateau phase is shortened and the expulsion rate of LP2 increases.

The Intestinal Mast Cell in Nippostrongylus-infected rats: Taliaferro and Sarles (1939) described the histological changes in the intestines of Nippostrongylus-infected rats and noted that the numbers of "connective tissue basophils" and globule leukocytes increased as the parasites were expelled. They did not suggest any function for these cells. Wells (1962) quantitated the mast cells in the intestines of infected rats and found that they were depleted when the parasites were in the intestine, but that they increased in number after the latter had been expelled. She did not, however, distinguish between globule leukocytes and mast cells and it is not clear whether both cell types were included in her counts. Globule leukocytes were quantitated by Whur (1966) in rats during primary infections with N. brasiliensis and he found that they increased in number at a time when the worms were being expelled.

Recent experiments have established that the GL cells are derived from IM cells (Jarrett et al., 1967b; Miller et al., 1967; Murray et al., 1968). It was also shown that both cell types increased in number at the time of helminth expulsion (Jarrett et al., 1967a).

This Section is concerned with the changes amongst the mast cells in the intestinal mucosa of rats infected with Nippostrongylus brasiliensis. It is divided into six parts. The first part describes the IM cell and mucosal reaction associated with the invasion and colonisation of the intestine by the parasite between the fourth and eighth day of infection (i.e. plateau phase). The second, third, fourth and fifth parts are quantitative, ultrastructural and histochemical studies of the IM cell during the period of worm expulsion (i.e. loss phase 2) and threshold phase. The sixth part describes the mucosal changes associated with the expulsion of helminths.

In order to keep the experiments to a manageable size, and because the parasites move about within the intestinal lumen (Brambell, 1965) a constant site in the jejunum was examined, and a segment, approximately 1.5 cm. in length was used for quantitative, ultrastructural and histochemical studies.

Part 1

The Fate of the Intestinal Mast Cell During the Invasion of the Rat Intestine by N. brasiliensis

When the larvae of N. brasiliensis reach the small intestine on the third and fourth day after subcutaneous infection, they establish themselves in the jejunum about 10 to 20 cm. behind the pylorus (Bramhall, 1965; Jarrett, 1968). During this process of establishment, a number of characteristic changes take place in the intestinal mucosa; these include the alteration of villus shape and the development of oedema of the lamina propria which is manifest as a demonstrable vascular leak (Urquhart et al., 1965; Jarrett, E., 1968; Jarrett, W. - personal communication).

Earlier experiments had shown that the "mucosal leak lesion" was prominent at the sites where worms were established and minimal at non-worm sites (Jarrett, 1968) and it was suggested that these lesions were a direct consequence of the feeding processes of the larvae and of the adult worms. At the same time, quantitative results showed that there was a marked reduction in the number of MM cells in the affected mucosa and that subsequently during the period of immunological expulsion of the helminths, the mast cells reappeared in the lamina propria (Jarrett et al., 1967a).

Several questions arose from these previous results. For example, it was not clear whether the newly formed cells were derived from previously degranulated mast cells or whether degranulation was followed by complete lysis and destruction of the cells themselves. The present experiment was therefore designed to show the relationship between quantitative and morphological findings in order to establish the eventual fate of the MM cells. Some of the vascular changes and the ensuing inflammatory reaction that result from the intestinal invasion with Nippostrongylus brasiliensis were also examined.

The results show that IM cells are progressively destroyed and the remnants are subsequently engulfed by macrophages. Changes in the ultrastructure of the vasculature as well as the accompanying inflammatory reaction in the lamina propria are described.

To obtain more information on the mechanisms of mast cell degranulation, a group of normal rats was treated with metabolic products derived from adult worms and the results suggest that these metabolites may be responsible for the destruction of the mast cells.

Materials and Methods

Experiment 1

Thirty-one female hooded Lister rats weighing 170 - 210 gm. were used. Twenty-one rats were infected subcutaneously in the flank region with 3,000 larvae of N. brasiliensis. Ten rats were kept as controls.

Segments of jejunum 1 - 2 cm. in length were removed from an area 12 - 15 cm. posterior to the pylorus and were fixed in Carnoy's fluid (see General Materials and Methods). Tissues were also taken for electron microscopy; the details are given in General Materials and Methods.

The experimental plan is shown in Table 6.

TABLE 6

Day of Infection	Number of Rats	Histology for EM cell Quantitation	Electron Microscopy
4	2	2	2
5	7	7	5
6	2	-	2
7	4	4	4
8	6	6	-
Controls	10	10	7

Details of the procedures used for staining and counting mast cells are given in the second part of this Section. Paraffin embedded sections were also stained with haemalum and eosin and picro-Mallory (see General Materials and Methods).

Experiment 2

Worm metabolites were kindly supplied by Dr. Ellen Jarrett. Adult Nippostrongylus brasiliensis worms were incubated in physiological saline at 37°C for one hour. The supernatant containing metabolites was centrifuged to remove the eggs and other debris and was stored at -20°C.

One ml. of metabolite solution containing the equivalent of 3,000 worm units/ml was injected into the tail vein of four female hooded Lister rats weighing 185 - 200 gm. The rats were killed twenty-four hours later and jejunum was removed for histology. Procedures for staining and quantitation are described in this Section, part 2.

Results

General Observations

Macroscopically there was mild congestion of the serosal surface of the parasitised region of the intestine on the 4th day of infection. Nippostrongylus larvae were just visible to the naked eye and were confined to an area of the jejunum which began about 10 cm. behind the pylorus and extended some 15 - 20 cm. posteriorly. On the 5th and 6th days, the same approximate area of jejunum was more deeply congested and dilated and the contents were fluid and mucoid in nature

The immature and adult parasites were larger and readily visualised with the naked eye. Seven and eight days after infection, the worms were spread over a larger area of mucosa and the jejunum was dilated and congested 4 to 5 cm. both anterior and posterior to the original predilection site. By the eighth day, the majority of adult parasites were lying free in the lumen in which there was an excess of thin fluid and of yellowish mucus.

Morphology and Quantitation of IM Cells

The larvae were found to be intertwined amongst the villi, usually at a level midway between the villus tip and the crypt on the fourth day. The IM cells had disappeared in the immediate vicinity of the larvae and the adjacent IM cells were altered in a number of ways. Many of them showed a reduced affinity for copper phthalocyanine dyes and stained only faintly or else had a pale-staining alcianophilic halo around the nucleus. In some cells, the granules were clumped and were irregularly distributed around the nucleus, but in others, there were many vacuoles in the cytoplasm. Occasionally, granules were observed scattered in the lamina propria in the region of disrupted mast cells. Single granules, clumped granules or degenerate-appearing mast cells, were observed within the cytoplasm of macrophage-like cells. The changes were more marked on the 5th day and the mast cells in the crypt region were more severely damaged than those in the villus. Seven and eight days after infection, there was a progressive loss of IM cells from the intestinal lamina propria in the predilection site of the parasites. By the eighth day, no mast cells were found in the intestines of four of the six rats (Figure 54)

and in the remaining two, they showed extensive degenerative changes and were found in groups of two or three in the tips of several villi. The quantitative depletion of the mast cells is recorded in Table 7.

Ultrastructure of Mast Cell Degranulation

On the fourth day of infection, there were many mast cells in the lamina propria which were apparently unaltered although the granule matrices in a few of them showed a patchy decrease in electron density. More rarely, an occasional cell showed considerable degenerative changes; there was extensive vacuolation of the cytoplasm and the perigranular membranes were ruptured; at some points the plasmalemma itself was damaged and the granule contents leaked out into the extracellular space (Figures 55 and 56). By the fifth day of infection, normal MM cells were more difficult to locate. The alterations to damaged mast cells were extensive and often the disruption of the perigranular membrane had resulted in the fusion of the granule matrices to form a large homogeneous electron-dense deposit around which one or two unaltered granules remained, thus permitting identification of the cell which could also be detected in thick sections (Figures 58 and 59). Altered mast cells were rarely found lying free in the lamina propria, since most of them had been phagocytosed by macrophages (Figures 60, 61 and 62) or by less differentiated cells (Figure 57). Granule and nuclear remnants could frequently be identified within the phagosomes. Occasionally a macrophage-like cell containing mast cell remnants was found within the epithelium (Figure 63).

TABLE 7

Experiments 1 and 2

Quantitation of IM Cells in Nippostrongylus-Infected rats

and in Rats Treated with Worm Metabolites

Experiment 1

		CELLS/VILLUS-CRYPT UNIT		
Day of Infection	No. of Rats	IM Cells[a]	GL Cells[a]	Total[a]
Controls	10	11.9±0.2	-	11.9±0.2
4	2	7.1±0.4	-	7.1±0.4
5	7	5.2±1.3	0.3±0.3	5.5±1.4
7	4	1.3±1.2	-	1.3±1.2
8	6	1.2±0.8	-	1.2±0.8

Experiment 2

Treated with Metabolites	4	7.0±1.2	1.9±1.3	8.9±2.4

[a] Mean ± SE

Six and seven days after infection, very few normal IM cells were found in the mucosa. In addition, few granule remnants were found within macrophages at this time which was in contrast to the earlier days of infection.

Histological Changes in the Intestinal Mucosa

The larvae were intertwined amongst the villi on the fourth day of infection. The stromal elements of those villi adjacent to the parasites were separated by oedema fluid giving the affected villus a bulbous appearance. The epithelium at the tips of some villi was separated from the lamina propria by a fluid-filled space. As the numbers of parasites increased on the fifth and sixth days, villus oedema extended to the majority of villi within the predilection site, even where the parasites themselves were not readily visualised histologically. By this time, some of the villi were stunted and there was a higher mitotic rate amongst the crypt epithelial cells than normal (Figure 64) together with an increase in the ratio of crypt length to villus length. Where the parasites were adjacent to the villi, there was flattening of the villus epithelium which formed an indentation around the worm (Figure 65).

The changes were more severe on the seventh and eighth days of infection and the oedema of the lamina propria was such that the outlines of many villi were extensively altered. Characteristically, there was an increase in the crypt length and the epithelial cells showed a continued high mitotic rate. Many of the villus epithelial cells were cuboidal and were vacuolated (Figure 64).

In the normal rat, the lamina propria of the intestinal mucosa was populated

by macrophages and undifferentiated cells towards the tip of the villus; plasma cells were mostly confined to the lower villus and upper crypt regions. Eosinophils and lymphocytes were distributed at all levels in the lamina propria. Between the time when the parasites arrived in the intestine and the eighth day of infection, there was a marked change in the cell population of the lamina propria. Initially many cells underwent cytolysis, plasma cells were reduced in number and eosinophils frequently had small pyknotic nuclei and small amounts of intensely eosinophilic cytoplasm. Five and six days after infection, relatively few eosinophils or plasma cells were evident in the lamina propria. At the same time, macrophages with abundant cytoplasm containing a variety of inclusions, became prominent at all levels in the lamina propria and there was an increase of undifferentiated cells; a few polymorphonuclear leukocytes were also found.

By the eighth day, macrophages were still much in evidence, but increasing numbers of small lymphocytes and larger lymphoid cells with moderately abundant basophilic cytoplasm had begun to repopulate the lamina propria. Numerous eosinophils having band or ring shaped nuclei were migrating through the submucosa into the basal lamina propria and were found in small numbers in the basal crypt regions and scattered in the villi.

Ultrastructural Changes in the Intestinal Mucosa

Oedema of the lamina propria was observed as early as the fourth day of infection and was readily apparent thereafter. Whereas in the normal rat, the cells in the lamina propria lay close to one another (Figure 66), in the oedematous areas

blood vessels and lymphatics were markedly dilated and the cells in the lamina propria were separated by oedema fluid. Small intercellular gaps were found in the venular endothelium (Figure 69), and the endothelium of some capillaries was swollen and the cytoplasm was vacuolated and rarified (Figure 68) when compared with the capillaries in normal rats (Figure 67). Endothelial cells in infected rats showed increased numbers of small dense bodies and four and five days after infection there were many extravasated red blood cells in the lamina propria (Figure 70).

The epithelial cells covering the villi of infected rats were as described in the light microscopic studies, but the electron microscope showed that the microvilli were markedly reduced in size and in numbers (Figures 72 and 73; cf. Figure 71). In addition, there was marked dilatation of the lateral intercellular spaces (Figure 73; cf. Figure 71) which was particularly severe on the sixth day of infection.

Between the fourth and seventh days of infection, plasma cells, eosinophils and lymphocytes all suffered varying degrees of damage with their remnants being evident within phagosomes of macrophages and of activated undifferentiated cells (Figure 74). Eosinophils were often disrupted, but their granules generally remained intact, even some which were found within phagosomes (Figures 76 and 77). Macrophages were particularly abundant and had large amounts of vacuolated cytoplasm containing numerous dense lysosome-like inclusions and phagosomes. They also had multivesicular bodies and lipid droplets in the cytoplasm (Figures 60 and 61), but the rough surfaced endoplasmic reticulum was relatively sparse. Their nuclei

tended to be large and reniform with diffusely distributed chromatin. The undifferentiated cells had smaller amounts of cytoplasm having fewer organelles and dense bodies but containing more abundant rough surfaced endoplasmic reticulum (Figure 75).

Lymphocytes and lymphoid blast cells with more abundant cytoplasm containing many ribosomes were found within the lumen and in the close vicinity of both blood and lymphatic vessels, particularly on the sixth and seventh days of infection (Figures 78, 79 and 80). Polymorphonuclear leukocytes were also found in small numbers within blood vessels and distributed in the lamina propria.

By the seventh day, there was less evidence of cytolysis, and macrophages appeared to be slightly less active in that they were smaller and contained fewer phagosomes. At the same time a number of cells having cytoplasm with moderately well developed endoplasmic reticulum, but which could be readily distinguished from plasma cells, were found in the lamina propria. Eosinophils were more abundant than on the previous days of infection.

Experiment 2

The Effect of Worm Metabolites on Intestinal Mast Cells : Mast cells were quantitated in normal rats given an intravenous dose of Nippostrongylus metabolic products. Table 7 shows that there was not a significant reduction in the total numbers of cells in the mucosa of treated rats. When the GL cells were excluded from the total, the depletion of mast cells was significant $P < 0.01$. There was no evidence of villus oedema or mucosal expansion.

Discussion

In the first experiment, where rats were infected with N. brasiliensis larvae, the mast cells were damaged and began to disappear from the lamina propria as early as the fourth day after infection and thereafter were progressively destroyed up to the eighth day when very few were found. The merits of the villus/crypt method of IM cell quantitation are discussed in this Section, part 2, but in the present experiment, some caution is necessary in the interpretation of the results. Because of the gross villus distortion which became severe on the fifth day and persisted on the seventh and eighth days, it was not feasible to measure the villus areas and some allowance must therefore be made for the expansion of the mucosa. Even so, using the villus crypt as a unit of measurement, the volumetric expansion is accounted for in two planes and the resultant error is less than that obtained by the fixed objective field method. Despite the inherent difficulties of quantitation, it was evident that the majority of IM cells were destroyed by the eighth day of infection in the parasitised region of the jejunum. This is supported by the ultrastructural evidence where IM cells were numerous on the fourth day of infection, but on the fifth day there were a few normal cells and many were in the process of degeneration; by the seventh day, even granule remnants were difficult to find.

The cause of mast cell degranulation has not been clearly established. The results of the second experiment where GL cells were found and IM cell numbers were reduced in rats after treatment with Nippostrongylus metabolic products, point to the direct action of these metabolites on the IM cells. It would seem likely that the worm products contain a specific degranulator similar to the one isolated from

Ascaris extracts by Uvnäs and Wold (1967). This is supported by the histological observations where IM cells first disappeared from areas in the vicinity of the recently arrived larvae. The timing of mast cell degranulation and the results of the worm metabolite experiment, do not, therefore, support the hypothesis of Wilson and Bloch (1968) that these cells are sensitised prior to their degranulation during the early stages of Nippostrongylus infections.

Ultrastructural studies of mast cell degranulation during parasitic infections have not previously been reported. In the present experiment, a natural infection with N. brasiliensis was used to examine the ultrastructural changes in the mast cells. Unfortunately, IM cells showing early alterations were not found in sufficient numbers for the certain recognition of the earliest changes, but in some cells there was partial extraction of the granule matrices on the fourth day and it appeared as if the subsequent rupture of the perigranular membranes and the plasmalemma allowed the escape of the matrices, leaving a vacuolated cell. The damage was not confined to the granules, since other organelles, including the nucleus, underwent degenerative changes. In some cells, the granule matrices had fused to form large electron-dense conglomerations, which presumably were visible as the clumped granules in stained histological sections. The rapid uptake of damaged mast cells by macrophages and undifferentiated cells was striking. The sequence of events suggested that the IM cells, once ingested by the macrophages, were subsequently destroyed by intracellular digestion.

The phagocytosis of mast cell granules by other cells has been noted on several occasions (Higginbotham and Dougherty, 1955 and 1956; Burton, 1963) and it is hardly surprising in view of the profusion of macrophages in the intestinal lamina propria, that the IM cells suffer a similar fate. It is evident that the IM cells in the predilection sites of the parasite are destroyed and do not remain as viable cells which are subsequently able to regenerate and produce more granules.

The findings of villus oedema are in agreement with the description of Barth et al. (1966) and of Jarrett (1968). On the fourth day, oedema was usually confined to those villi which were immediately adjacent to parasites, whereas on subsequent days, a larger proportion of the villi was oedematous. The close association between the location of the parasite and the site of oedema, is probably significant, since it was from these areas that the IM cells first disappeared. Mast cell degranulation releases the vasoactive amine, histamine and 5-hydroxytryptamine which in the rat induce increased vascular permeability (Majno and Palade, 1961); the association between mast cell degranulation, the presence of a demonstrable vascular leak and oedema in the immediate vicinity of the parasite, raises the likelyhood that the leak lesion is at least initiated by degranulation of mast cells. The early localised oedema probably reflects the relatively small numbers of parasites adjacent to the mucosa and the subsequent more generalised oedema could result from the build-up in the worm numbers and their migration to different areas in the mucosa. The persistence of the leak lesion cannot be attributed to mast cell discharge alone, because the blood vessels become refractory to the action of the vasoactive

amines within a short period of time (Spector and Willoughby, 1968), although the action of mast cell proteases has not been clarified in this respect. In any event, the presence of haemorrhages as well as ultrastructural evidence of endothelial cell damage indicate that other factors are involved in the increased vascular permeability. It is possible that the parasites themselves produce a permeability inducing factor apart from the mast cell degranulator which, as postulated by Jarrett et al. (1967a) is necessary for the establishment and feeding of Nippostrongylus brasiliensis.

The degenerative changes in the lamina propria were not confined to the mast cells; eosinophils, plasma cells and lymphocytes all suffered varying degrees of damage and were phagocytosed by macrophages. There was a predominance of macrophages and of phagocytic undifferentiated cells between the fourth and seventh days of infection and this was followed by infiltration of the lamina propria with large lymphoid cells.

Larsh (1967) and Dineen, Ronai and Wagland (1968) have suggested that a delayed type hypersensitivity reaction might play a part in helminth expulsion. The present findings where lymphoid blast cells and macrophages were numerous in the lamina propria might represent such a response. On the other hand the mucosal changes could well be the preparatory stages of a local immune response, with a lymphoid cell - macrophage interaction preceding the eventual repopulation of the lamina propria with specific antibody producing cells. This is discussed in part 6 of this Section.

Part 2

The Intestinal Mast Cell During Immunological Expulsion of *Nippostrongylus brasiliensis* in the Rat

Quantitation and kinetics

Nippostrongylus infections are terminated towards the end of the second week by an immunological reaction known as self-cure (Africa, 1931; Mulligan et al., 1965) and recent experiments have shown that the parasites are expelled in an exponential fashion between the eleventh and eighteenth days (Jarrett et al., 1968).

At the commencement of worm expulsion mast cells reappear in the intestinal mucosa and increase in number (Jarrett et al., 1967a). The origin of these cells remains uncertain although Taliaferro and Sarles (1939) observed that they were derived from large stroma cells in the lamina propria. The same workers also noted an increase in the mucosal content of GL cells.

Wiwa (1966) found a relationship between the immune expulsion of N. brasiliensis and the appearance of GL cells in the intestinal epithelium. Jarrett et al. (1967a) showed that there was a numerical relationship between the IM and GL cells and it has been demonstrated that the GL cell is derived from the subepithelial mast cell (Miller et al., 1967; Murray et al., 1968 - Section II).

The purpose of the present experiment was to discover the origin of the IM cells during self cure. At the same time a more detailed study of the quantitative relationship between IM and GL cells was undertaken. The morphological findings and the population kinetics indicate that IM cells are derived by differentiation and division of precursor cells and by their subsequent maturation. The results also provide a quantitative basis for the subsequent ultrastructural and histochemical studies of the mast cell reaction at the time of worm expulsion (see this Section, parts 3, 4 and 5).

Materials and Methods

Fifty-five female hooded Lister rats weighing 170 - 220 gm. were used; fifty were infected subcutaneously in the groin region with 3,000 larvae of N. brasiliensis. They were anaesthetised on the appropriate days of infection and tissues were removed for histological and ultrastructural purposes before the animals were killed by cervical dislocation.

Two rats from each group and five uninfected controls were given intraperitoneal injections of 3 x 40 mg/Kg d-L-DOPA (DL - 3 - 3, 4, Dihydroxyphenyl-alanine) (Koch-Light Laboratories Ltd.) at hourly intervals and were sacrificed one hour later. The experimental plan is given in Table 8. The control group of ten rats was the same as that used in the first part of this section; they were obtained from the same source at the same time as the experimental groups.

A segment of jejunum approximately 1 cm in length was removed from a site 12 - 15 cm. behind the pylorus from each rat, and was opened longitudinally with fine scissors. The worm burden in the segment and in the remainder of the intestine was estimated, but not counted. Tissues were allowed to fix for 24 - 48 hours in Carnoy's fluid before dehydration and embedding. Blocks were trimmed and orientated to obtain sections along the long axis of the jejunum at right angles to the surface of the mucosa. Sections were cut at approximately 6 μ thickness and were stained with Astra blue/safranin (See General Materials and Methods).

TABLE 8

Experimental plan for the quantitative, histochemical and ultrastructural studies of the IM cell during the immunological expulsion of N. brasiliensis

Day of Infection	No. of Rats	No. of rats treated with L-DOPA	Tissues for IM cell quantitation & histochemistry	Tissues for Electron Microscopy and Falck Technique
Controls	15	5	15	10
Day 10	6	2	6	5
Day 11	5	-	5	5
Day 12	9	2	9	5
Day 14	8	2	8	5
Day 16	7	-	7	5
Day 19	7	2	7	5
Day 35	4	1	4	4

Method of Quantitation

The villi of the jejunum of the rat tend to be tongue-shaped with their broader axes at right angles to the long axis of the intestine (Reynolds, Brim and Sheehy, 1967; Nordstrom, Dahlqvist and Josefsson, 1968), so that in longitudinal sections they appear to be finger-shaped and regularly spaced, usually being separated basally by one gland crypt.

For quantitative purposes, the mucosa was divided into 'villus-crypt' units (VC). The number of mast cells lying between two gland crypts and in the lamina propria of the villus above were counted. Globule leukocytes were enumerated in the length of epithelium outlining that area of lamina propria. The latter was delimited basally by the muscularis mucosae. The whole area comprised a VC unit. Cells were quantitated only in those units which were sectioned longitudinally.

The granules of mast cells and GL cells were stained by Astra blue and the nuclei were outlined by safranin, but mitotic figures and nucleoli were strongly safranin positive. IM cells and GL cells were quantitated separately in each villus-crypt unit and the numbers for each rat were obtained by counting twenty units, and were expressed as the mean values. Total IM cell counts were taken as the sum of mean IM cell and mean GL cell numbers in individual rats. An analysis of regression (Snedecor, 1966) was carried out using the logarithms of the geometric mean of total IM cell numbers in each group.

To give some indication of the extent of mucosal expansion, villus length was measured from the tip to the muscularis mucosa and the width was measured midway

between the tip and the gland crypt region. The product of villus length and villus width, the villus area, was recorded for ten villi in each of five rats in a group except on day 35 where only four rats were examined. Mean villus area ± SE was tabulated for all groups.

Results

The expulsion of the worm burden followed the usual pattern described by Jarrett et al. (1968). Worms were present in large numbers on the tenth day and no obvious decrease could be observed until the twelfth day after infection. By the fourteenth day, the worm burden was reduced in all regions of the small intestine. Few parasites were found in rats on day 16 and on the nineteenth day, worms were difficult to locate. Thirty-five days after infection, only very small numbers could be found in the intestine of each rat.

Light Microscope Observations

Normal Rats: IM cells were found at all levels in the intestinal lamina propria and were evenly distributed amongst the VC units (Table 9); there tended to be a higher concentration in the crypt region than in the lamina propria of the villus (Figure 41). Intraepithelial GL cells were rarely observed. Treatment with L-DOPA did not affect the number or morphology of IM cells (Table 9).

Parasitised rats: On the eighth day the villi were severely distorted (this Section, part 1). Ten days after infection they had assumed a more normal shape although villus area was greater than it was in controls and the villi remained expanded throughout the course of the experiment (Table 10).

TABLE 9

The Quantitation of IM and GL cells in the Intestinal Mucosae of Rats during the Immune Expulsion of N. brasiliensis

Day of Infection	No. of Rats in Group	IM Cells* / VC	GL Cells* / VC	Total Cells* / VC	Log_{10} Total** cells / VC
Control	10	11.9 ± 0.2	-	11.9 ± 0.2	
Control L-DOPA Treated	5	11.7 ± 0.4	-	11.7 ± 0.4	
Day 10	6	2.1 ± 0.7	0.1 ± 0.1	2.2 ± 0.7	0.2029 ± 0.16
Day 11	5	6.8 ± 1.9	0.4 ± 0.3	7.3 ± 2.2	0.7847 ± 0.13
Day 12	9	11.7 ± 2.5	5.0 ± 2.2	16.7 ± 4.4	1.1262 ± 0.10
Day 14	8	51.3 ± 4.7	48.6 ± 5.1	99.9 ± 8.3	1.9874 ± 0.04
Day 16	7	50.8 ± 4.6	41.2 ± 7.0	92.0 ± 10.3	1.9475 ± 0.05
Day 19	7	53.2 ± 2.3	30.1 ± 5.0	83.3 ± 7.1	1.9116 ± 0.04
Day 35	4	26.8 ± 0.7	12.5 ± 0.4	39.4 ± 0.4	1.5952 ± 0.007

* = mean ± SE

** = log_{10} of the geometric mean ± SE

TABLE 10

Size of Villus-Crypt Unit mm^2

Day of Infection	Number of Rats	Villus Area mm^2 *
Control	5	0.049 ± 0.002
Day 10	5	0.074 ± 0.003
Day 11	5	0.071 ± 0.003
Day 12	5	0.070 ± 0.003
Day 14	5	0.070 ± 0.004
Day 16	5	0.074 ± 0.003
Day 19	5	0.074 ± 0.003
Day 35	4	0.069 ± 0.002

* = Mean ± SE

Control V Rats on Day 14 $P < .01$

Control V all other groups $P < .001$

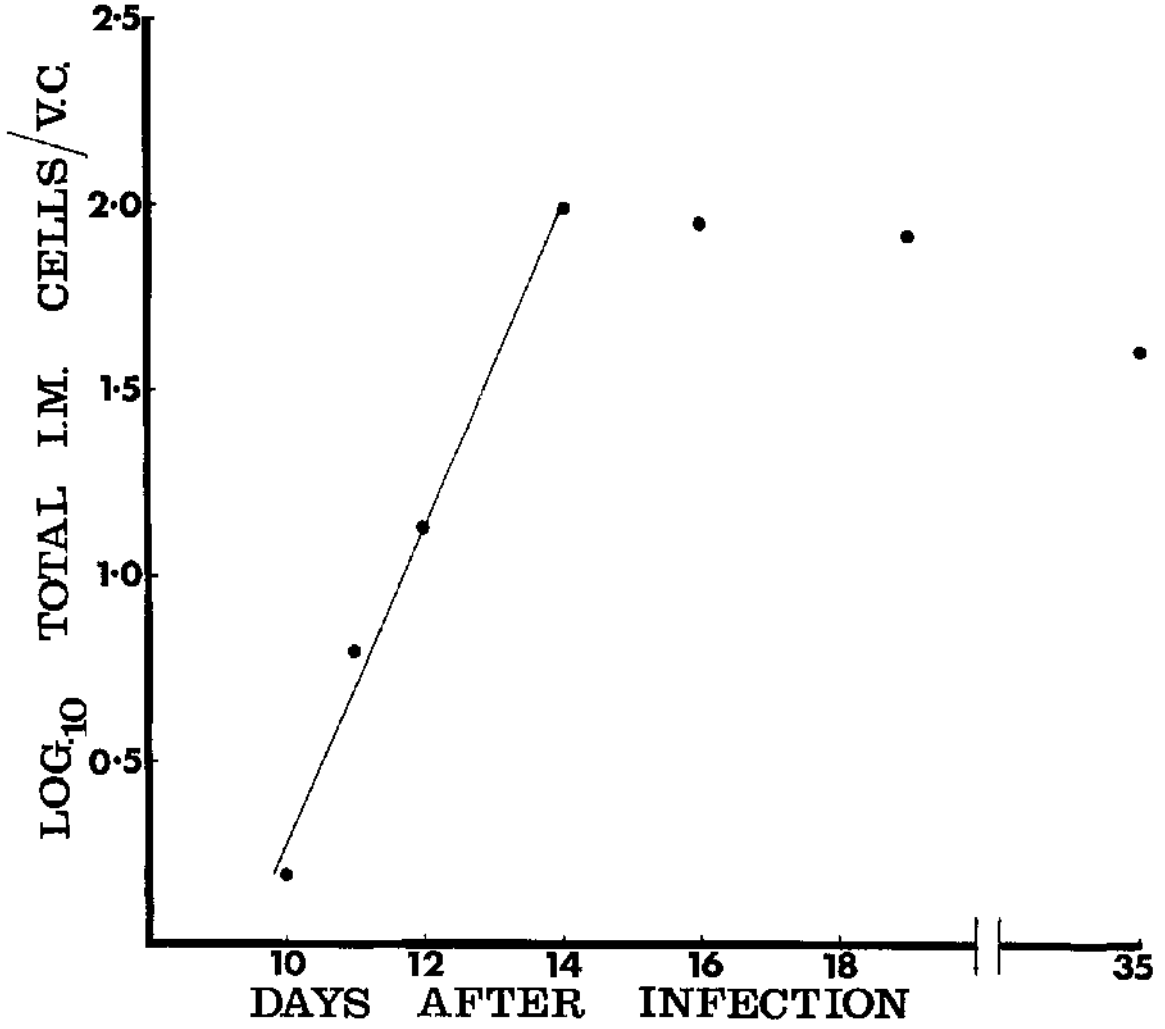
Small numbers of IM cells and GL cells were found in the lamina propria and epithelium respectively on the tenth day. Both cell types had large, often reniform, pale-staining nuclei with one or two prominent nucleoli. The cytoplasm characteristically contained only one or two Astra blue positive granules (Figure 81), although a few had larger complements of granules. The distribution of the cells was irregular, some VC units contained few, if any, whereas others had as many as five to ten. An occasional mitotic figure was observed in both IM and GL cells.

The population of granulated cells increased on the eleventh, twelfth and fourteenth days after infection. The geometric mean of total cell numbers and the $\log_{10}$ plot of these figures against time show that the rise was exponential in character with a regression coefficient $b = 0.44 \pm 0.08$ (Graph 2).

On the eleventh and twelfth days the IM cells and GL cells were morphologically similar to those on the tenth day, but some of them tended to have larger numbers of granules. Mitotic figures were increasingly evident (Figures 82 and 83) but still were not common. GL cells were found in greatest concentrations where IM cells were most abundant, although the distribution of both types tended to be extremely variable.

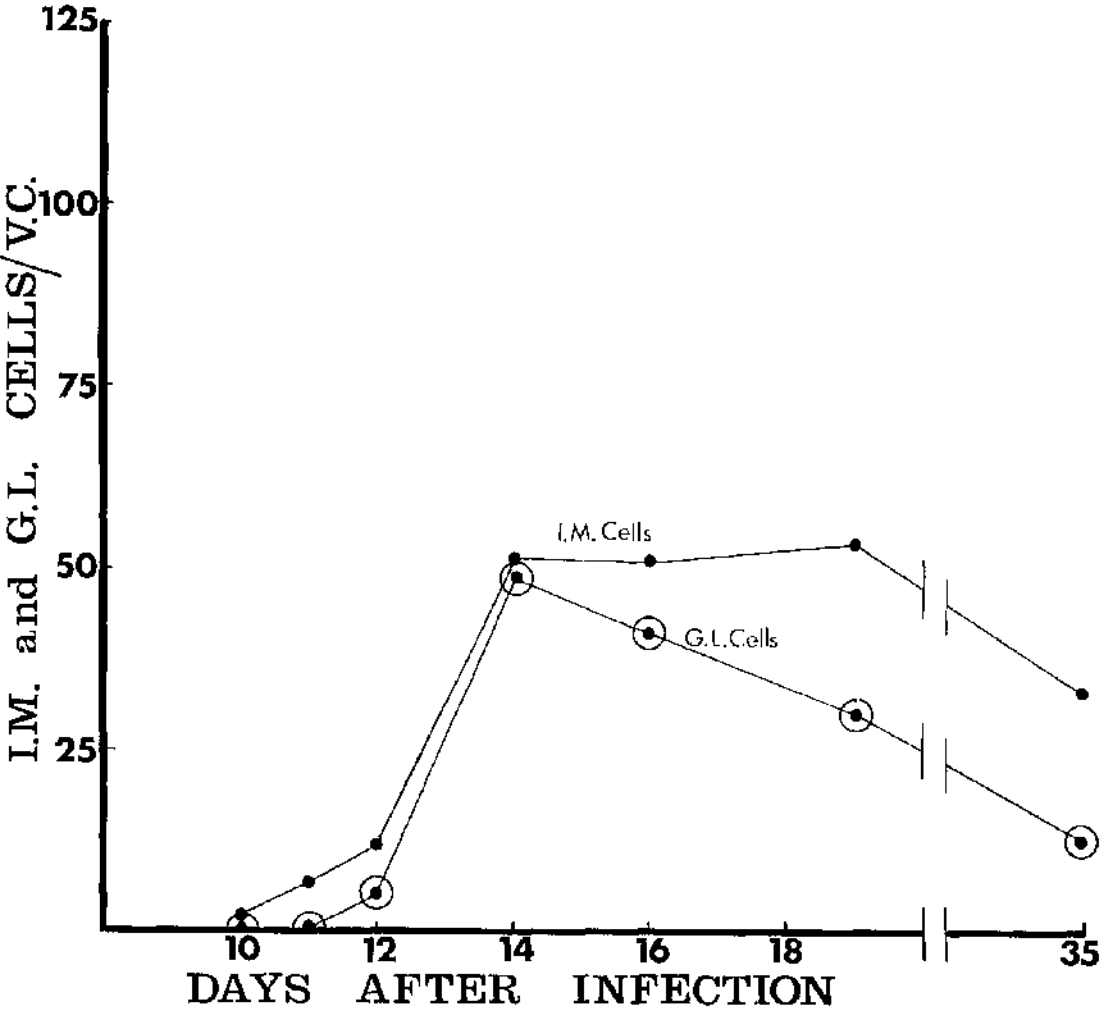
The maximum number of cells was observed on the fourteenth day after infection. Some were fully granulated and were indistinguishable from IM cells seen in normal rats; most of the GL cells contained fewer granules than their counterparts in the lamina propria and in some areas, many of the IM cells fragmented and were, for this reason, difficult to quantitate (Figure 84). Mitoses were readily observed in both GL cells and IM cells fourteen days after infection (Figure 85).

Graph 2



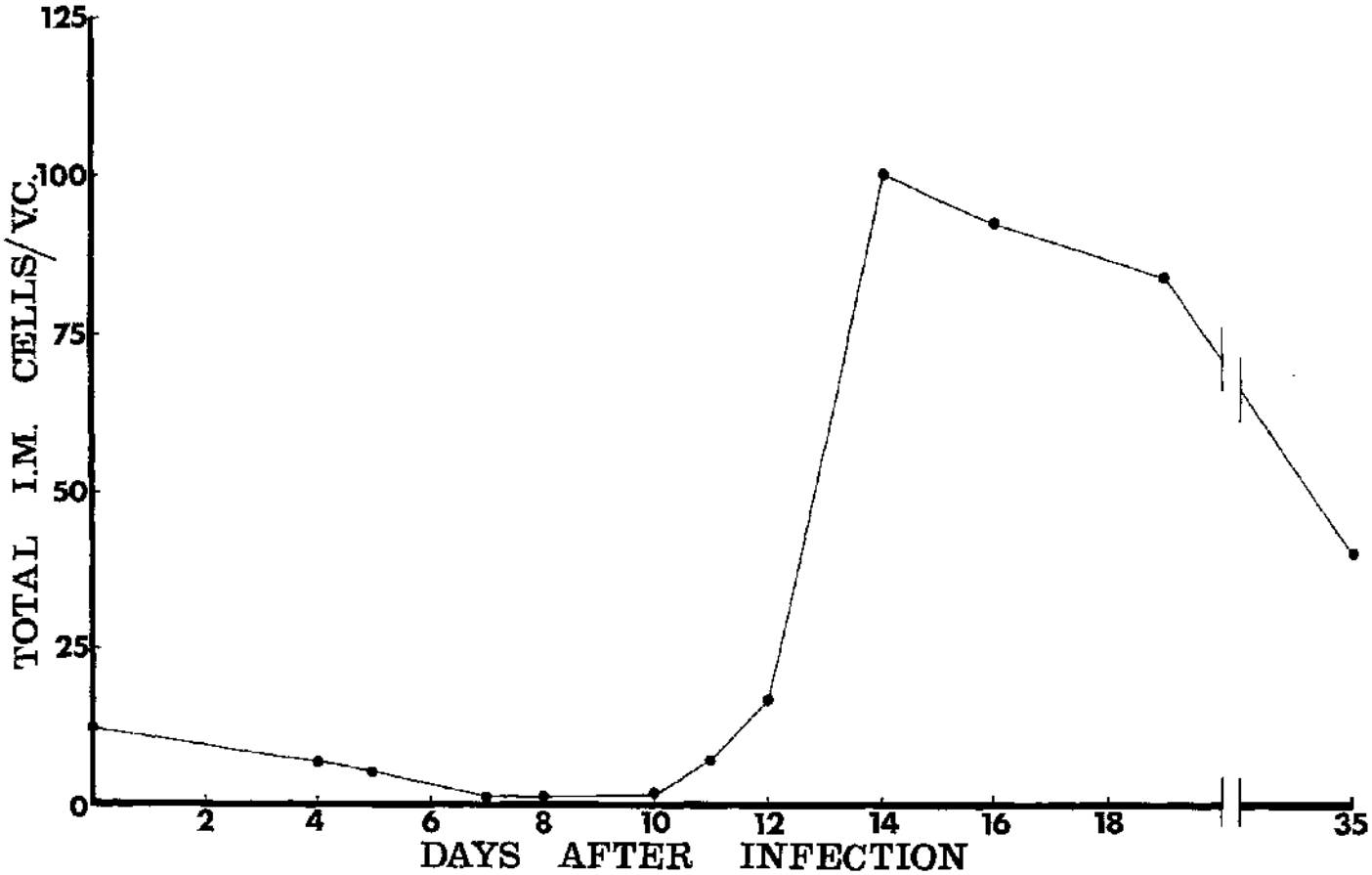
The kinetics of the total population of IM and GL cells during the immunological expulsion of N. brasiliensis.

Graph 3



The changes amongst the intestinal mast cell and globule leukocyte population during the immunological expulsion of N. brasiliensis.

Graph 4



Changes amongst the total population of IM and GL cells during infection with N. brasiliensis.

The greater part of the GL cell population was found in the upper parts of the gland crypt epithelium, very few were present in the epithelium overlying the villus and those that were, usually had very few granules. GL cells contributed an increasing proportion of the total from the tenth to the fourteenth day (Graph 3) by which time they formed approximately 49%. On the sixteenth day of infection, the distribution and morphology of the granulated cells were similar to those found in the mucosa on the fourteenth day. Mitoses were however, rare, although nucleoli could still be distinguished in many of the nuclei. The increase in the population of granulated cells ended after the fourteenth day. The number of IM cells remained at the same level until after the nineteenth day, but the proportion of GL cells decreased steadily so that by the thirty-fifth day they formed only 32% of the total (Table 9).

On the nineteenth and thirty-fifth day of infection, the IM cells were fully granulated and compact (Figure 86) and very few appeared to be fragmented. GL cells were morphologically similar to, and distributed in the same way as those on day 14.

The overall changes amongst the mast cell population in the intestinal mucosa during infection with Nippostrongylus brasiliensis are recorded in Graph 4.

Discussion

The purpose of this experiment was not only to delineate the kinetics of the IM cell changes but also to establish a quantitative relationship between the numbers of IM cells in the lamina propria and of GL cells in the epithelium during immunological expulsion of N. brasiliensis. Several factors had to be considered in choosing a method

for counting these cells including the distribution of the cells in the mucosa, alterations in tissue volume and the facility with which the two cell populations could be compared.

IMi cells were evenly dispersed in the mucosa in normal rats but during Nippostrongylus infections a large proportion of the cells was concentrated in the crypt region. Also during infection the villi were expanded so that the gaps between them were narrowed. The standard method of counting cells per objective field was discarded because of these changes and because it proved to be impracticable for the comparison of IM and GL cell numbers.

There were several advantages in the villus-crypt method. The subjective selection of fields which was difficult to avoid in the fixed field method was no longer a problem and it was also relatively easier to compare IM and GL cell numbers. The unit was flexible in the event of volume changes so that two of the three dimensional alterations were already included within the villus-crypt. It was imperative, however, that the villi should be sectioned longitudinally to avoid artefacts of cell distribution which could arise from oblique sections of the crypt region.

The IMcells in normal rats were evenly distributed amongst the villi and numerical differences between individual rats were small, but the villus-crypt method of quantitation demonstrated the uneven distribution of cells in the intestines of infected rats and it is reflected by the high standard errors of the means.

Very few mast cells remained in the intestine on the eighth day of infection, the majority having been phagocytosed by macrophages (this Section, part 1). The origin of the newly derived cells was therefore of considerable interest; they did in fact

differentiate from cells with large nuclei and prominent nucleoli. This is in agreement with the findings of Taliaferro and Sarles (1939) and in the next part of this Section, it will be shown that the precursor cells closely resemble lymphoid blast cells.

By the tenth day after infection, small numbers of granulated cells reappeared in the intestinal mucosa and thereafter increased in number exponentially in a period of rapid differentiation, division and maturation. The exponential character of the population expansion, the blast type of IM cell and the frequently observed mitotic figures are evidence that the numbers increased by division, differentiation and maturation rather than by an infiltration of granulated cells from elsewhere. The state of flux at this time is reflected in the range of IM cell counts between the tenth and twelfth days of infection and by the irregular distribution of cells within the lamina propria of individual rats. By the fourteenth day this wide range had narrowed and IM cells were more mature as was evident from the larger numbers of granules within individual cells, but even so, mitotic figures amongst granulated cells were readily found.

The stimuli required to bring about increased numbers of mast cells are not known. They have, however, been observed to develop from lymphoid cells of the thymus (Ginsburg, 1963) from thoracic duct cells, from cultures of lymph node cells (Ginsburg and Lagunoff, 1967) and to multiply within lymph nodes (Miller and Cole, 1968). The presence of mast cells within cultures of thoracic duct cells implies that they were derived by differentiation of precursor cells rather than by multiplication of an existing population of mast cells although Miller and Cole (1968)

postulated that both mechanisms might be functional. Proliferation of these cells was rather more extensive after antigenic stimulation (Ginsburg and Lagunoff, 1967; Miller and Cole, 1968). It is possible that N. brasiliensis also provides a continued antigenic stimulus which may be responsible for the redifferentiation and multiplication of the mast cells, although it is not known what influence the earlier degranulation of the mast cell population might have.

The number of mast cells in the mucosa did not increase after the fourteenth day even though mitotic activity was high at this time; in fact, a gradual decline in the population took place. Three factors may have contributed to this phase of decline.

1. The results show that increasing proportions of the cells migrate intra-epithelially to become GL cells during the period of expansion. Increased number of thelatter was noted by Talieferro and Sarles (1939) and Wiur (1966) observed that there was an association between the appearance of GL cells and the onset of self cure. It was recently shown, however, that the GL is derived from the sub-epithelial mast cell (Jarrett et al., 1967b; Miller et al., 1967; Murray et al., 1968, Section II) and in view of this origin GL cells were included in the total IM cell counts. Numerically, there is a striking correlation between the IM and GL cells because the latter were found in maximum numbers at a time when the total IM cell population was at a peak, thus on the fourteenth day of infection approximately half of the total was GL cells. The eventual fate of the latter has not yet been clarified, but the majority are partially discharged as they reach their location within the epithelium (Murray et al., 1968; Section II) and it is likely that as they are carried towards the villus tip between migrating epithelial cells they continue to discharge their granules. Eventually,

the complete loss of acid mucopolysaccharide from the granules may render the cells less easily detected by conventional histological methods. In any event, GL cells were readily visualised in the crypt region but were only found in small numbers higher up the villus. It is possible that GL cells are able to migrate back into the lamina propria but none was seen to migrate into the lumen. It is more than likely that the demise of these cells contributes to the overall decline in the number of recognisable mast cells.

2. Histologically, many IM cells were fragmented and ultrastructural as well as histochemical studies show that a large proportion of them were partially discharged or were even more severely damaged on the fourteenth and sixteenth days of infection (This section, parts 4 and 5). The severity of these changes particularly in rats with high counts was striking (this Section, part 4) and is presumably a major factor in the cessation of the expansion phase.

3. The rate of cell division and differentiation was probably declining. It was beyond the scope of the present experiment to estimate the mitotic activity of IM cells during Nippostrongylus infection, but many cells were seen in mitosis on the fourteenth day, whereas mitotic figures were rarely found on the sixteenth day and none was observed on later days of infection. Population growth by cell division had therefore probably almost ceased by the nineteenth day of infection.

There are three separate phases of IM cell activity which can be related to the kinetics of Nippostrongylus infections on a temporal basis; the mast cells are

degranulated when the parasites reach and establish themselves within the intestine; regeneration of IM cells begins just prior to the inception of and continues during the immunological expulsion of the helminths which occurs between the eleventh and sixteenth days of infection (Jarrett et al., 1968). The subsequent reduction of the population starts in the middle of the self cure reaction and continues well after the majority of the parasites have been expelled.

Part 3

The Intestinal Mast Cell During Immunological Expulsion of *Nippostrongylus brasiliensis* in the Rat

The ultrastructure of differentiating intestinal mast cells

In part 2 of this Section, quantitative and kinetic studies of the IM cell population in the intestinal mucosa showed that these cells increased in number in an exponential fashion between the tenth and fourteenth day of Nippostrongylus infections. They were derived from precursor cells with large pale-staining nuclei and prominent nucleoli and the immature mast cells were seen in mitosis throughout the course of population expansion.

The origin of the IM cell precursors was not identified with certainty although they had earlier been characterised as 'stroma' cells because they resembled reticular cells (Taliaferro and Sarles, 1939). The purpose of this ultrastructural study was to discover the origin of the newly differentiated IM cell and to establish whether an abrupt cessation of the differentiation and maturation processes was responsible for the sudden fall off in the rate of population growth between the fourteenth and sixteenth days of infection (this Section, part 2).

Whilst it is generally recognised that CTM cells represent a stable population amongst which cell division is rare (Benditt and Lagunoff, 1964), recent experiments have shown that differentiation and replication of mast cells occurs in a variety of lymphoid tissues after antigenic stimulation (Ginsburg and Lagunoff, 1967; Miller and Cole, 1968). It was of interest, therefore, to compare the process of IM cell differentiation and maturation with that seen in in vitro cultivation of mast cells from lymphoid tissue (Ginsburg and Lagunoff, 1967) and with the maturation of the CTMC in the rat (Combs, 1966). The ultrastructural features of IM cell maturation and differentiation are described and are compared with processes seen in the CTMC (Combs, 1966) and granulocytes of

various species. These findings are correlated with the histochemical features of the cells (this Section, part 5) and the probable mechanisms of granule elaboration are described.

Materials and Methods

The experimental regime is described in part 2 of this Section. Tissues for electron microscopy were obtained from the intestines of five rats in each group except on day 35 where only four rats were used. Small blocks of jejunum were removed with fine scissors from areas immediately adjacent to those examined quantitatively and histochemically and were immediately cut into small slices less than 1 mm in thickness in drops of chilled fixative. The fixation and embedding procedures are described in General Materials and Methods. Some of the tissues were embedded in Araldite and the remainder in Araldite/Epon (see General Materials and Methods). Thin sections were cut using an LKB Mark III ultratome, mounted on copper mesh grids and double stained with 20% uranyl acetate in methanol and with lead citrate (Reynolds, 1963). Thin sections were alternated with sections 1 - 1½ μ thick which were mounted on glass slides and stained by the methods of Richardson, Jarett and Finke(1960) or with basic Fuchsin in 50% acetone (Ackerman and Hostetler, 1968).

Results

Identification of Mast Cells

In the normal rat the IM cell was identified by the characteristic granules which were packed into the cytoplasm and in thick sections these granules stained a deep blue with the Methylene blue/azure 2 method and a bright orange red with basic fuchsin (Figures 13 and 14). The use of the same technique in infected rats enabled the immature IM cells with few granules to be distinguished from other inclusion-containing cells

such as macrophages and basophil-like cells which were particularly abundant in the lamina propria at this time.

The granules of maturing mast cells stained deeply with these dyes (Figures 93 and 94) whereas macrophage inclusions were faintly stained and were pale green or pink except occasional phagosomes containing ingested cell debris which could be identified ultrastructurally. Apart from their staining properties, the inclusions within macrophages tended to be more heterogeneous than those in IM cells, consisting of dense bodies of variable size, lipid droplets, multivesicular bodies and phagosomes. In general, the immature IM cells had abundant ribosomes and ribosomal aggregates whereas in macrophages the ribosomes were usually located on a few strands of RSER.

The basophil-like cells were more readily distinguished from the IM cell; they were most abundant from the tenth until the twelfth day after infection and contained a number of electron-dense inclusions ultrastructurally similar to IM cell granules but which did not stain with basic dyes, in addition these cells had multilobulated nuclei, very few ribosomes and deposits of glycogen in the cytoplasm (this Section, part 6).

The Precursor Cell

The precursor cells from which IM cells differentiated were recognised ultrastructurally because they were identical, apart from the absence of granules, to the immature IM cells containing one or two granules. They had relatively abundant cytoplasm in which there were numerous free ribosomes and ribosomal aggregates as well as a few strands of RSER (Figures 90 to 92). The Golgi complex was well

developed and centrioles were commonly found at its centre (Figures 91 and 92). Often small electron-opaque inclusions could be seen in the vicinity of the Golgi or else were irregularly dispersed in the cytoplasm (Figures 90 to 92). Mitochondria were usually fairly abundant and located in greatest numbers in the region of the Golgi complex. Microfibrils were found in the cytoplasm both around the nucleus (Figure 96) and in the marginal cytoplasm.

The nuclei were large and were oval, pear-shaped, or reniform. The chromatin was diffusely distributed but was marginated at the periphery of the nucleus and one or more nucleoli were commonly present (Figure 90). Nuclear pores were seen in tangential sections.

Typically, the precursor cells were abundant on the twelfth day after infection and could be found in moderate numbers on days ten and eleven. The derivation of the precursor cells was not clear-cut and in view of the large numbers of different cell types present in the lamina propria, the sequence of their development can only be tentatively suggested.

From as early as the sixth day after infection, lymphoid cells were found in the lamina propria in increasing numbers; some were small lymphocytes and had only small amounts of cytoplasm and rather coarsely clumped nuclear chromatin. However, a proportion of the infiltrating cells were larger, having abundant cytoplasm in which there were free ribosomes and several ribosomal aggregates (Figure 87). The nuclei of some cells were enlarged, the chromatin was marginated and nucleoli were prominent.

Further nuclear and cytoplasmic enlargement occurred together with an increase in the free and aggregated ribosomal content so that ten, eleven and twelve days after infection, an increasing proportion of the cells in the lamina propria were the blast type with large nuclei, prominent nucleoli and abundant cytoplasmic content of ribosomes (Figures 89 to 92). Some were seen in mitosis (Figure 88) and some were found in lymphatic and blood vessels (Figure 87). Further cytoplasmic changes included the appearance of one or two strands of RSER (Figures 89 to 92) and the enlargement of the Golgi complex which had been rudimentary or absent in the earlier stages. In the final stage prior to the appearance of granules in the cytoplasm these cells were the typical precursor cells described earlier.

The Maturation of IM Cells

Observations on the maturation of the IM cells were complicated by extensive IM cell degranulation and the description that follows is confined to those cells which showed little or no evidence of discharge. Because the process of maturation was not entirely synchronous, continuing at different speeds in different cells, the findings given here, although typical for the majority of cells, do not allow for the wide variation sometimes seen.

Between the tenth and twelfth days after infection, cells containing one or two granules were evident in the lamina propria and more rarely in the epithelium (Figure 93 and 94). They had abundant cytoplasm and, depending on the plane of section, extensive Golgi complexes as well as increased amounts of RSER. The granules were found in the vicinity of the Golgi complex and usually not more than four or five were

seen within a cell on the tenth day of infection.

The granule content of individual cells built up concomitantly with the density of the IM cell population so that by day twelve, there were some cells in which as many as ten to fifteen granules were visible in the plane of section. Their nuclei were similar to those of the precursor cells; mitoses were more readily seen in the granulated cells on day twelve than on the earlier days (Figure 95). No granulated cells were found in lymphatics or in blood vessels.

As the granule content within a cell increased there was an inverse relationship between it, the amount of RSER, and to a lesser extent the size of the Golgi complex so that fourteen days after infection, many of the cells contained moderately large numbers of granules which were sufficiently loosely packed to show the interlacing remnants of RSER and the few remaining ribosomes (Figure 102). Golgi complexes were still moderately large and mitotic figures amongst granulated cells were found with least difficulty at this time (Figures 103 to 104). Nucleoli were present but tended to be smaller, and the chromatin was more densely clumped and more heavily marginated than in the cells earlier in infection.

Sixteen and nineteen days post infection, individual cells were more densely packed with granules which were larger than those in mast cells of normal rats and fewer strands of RSER were lying in the intergranular cytoplasm (Figure 106). Golgi complexes were still in evidence but tended to be smaller than those on day fourteen and were reduced in complexity when compared with the Golgi regions in immature cells. Mitochondria were also reduced in number, were smaller and were more compact. No mitoses were seen on these days. Nuclear chromatin was more

densely precipitated towards the periphery of the nucleus. Nucleoli were seen in some IM cells on day sixteen (Figure 105) but were rare on the nineteenth day.

The majority of cells were packed with electron-dense granules by day 35. Golgi complexes when found, usually occupied only a small area in the paranuclear cytoplasm and the RSER was considerably reduced in amount. The distribution of nuclear chromatin was patchy and marginated and nucleoli were rarely seen.

The Golgi Complex and Granule Formation

The Golgi complex in the precursor and immature cells between the tenth and twelfth days of infection consisted of groups of four to six cisternae arranged in a circular or semi-circular fashion around the centrioles (Figures 92 and 96). The outer cisternae on the convex face of the complex were slightly dilated and empty whereas the inner cisternae on the concave face were flattened and had moderately electron-dense contents. In the more mature cells between the fourteenth and nineteenth days of infection, the complex was oriented similarly but tended to be smaller and less elaborate with fewer cisternae in each group (Figure 97), although large complexes were sometimes found in cells later in infection (Figure 107). Dense cored vacuoles (progranules) were present in moderate numbers in the complex. Some of the dense cores were separated from the delimiting membranes by electron-lucent areas (Figure 99). The progranules varied in size, those close to the cisternae were small with only moderately electron-dense central cores and were apparently derived by pinching off from the innermost flattened Golgi cisternum containing electron-dense material (Figure 98). Progranules in the peripheral parts of the complex and in the cytoplasm tended to be larger with cores of about the same

density as the granule matrices. These were often found in close association with the granules and in some instances appeared to be fusing with them (Figure 94). Small electron-dense foci approximately the same size as the progranule cores were observed within the delimiting membranes of some of the larger granules (Figures 95 and 100), but progranule aggregates of the type described by Bainton and Farquhar (1966) and Combs (1966) were not seen.

Vesicles were abundant in the golgi region and were of two varieties; small uncoated vesicles were apparently derived by budding from the cisternae and from transitional elements of the endoplasmic reticulum (Figure 98). They were found in close association with perigranular membranes and within the periphery of granule matrices (Figures 95 and 102). The second variety were coated vesicles which were larger and were less abundant than uncoated vesicles and budded from or fused with Golgi cisternae (Figure 107). Coated vesicles were also scattered in the cytoplasm and were occasionally seen to bud from the plasmalemma (Figure 101).

Within the vicinity of the Golgi complex, particularly of the immature cells, there were small accumulations of homogeneous material of approximately the same density as the granule matrices but which were often only partially delimited by a membrane (Figures 98 and 99). Progranules and small vesicles were clustered around them and the profiles of the latter could be distinguished within the delimiting membranes (Figure 99). A variety of appearances suggested that the addition of progranules and vesicles contributed to a further increase in size of the focal accumulations and that these eventually formed granules. Enlargement of the granules

was apparently by the fusion of progranules to the perigranular membranes with the deposition of the dense progranule core within the border of the granule matrix (Figures 94, 95 and 100).

Consistently present in the region of the Golgi complex were membrane delimited vacuoles of approximately the same size or larger than the granules but which had faintly electron dense contents and resembled the condensing vacuoles found in the pancreatic zymogen cells (Jamieson and Palade, 1967). Some of the vacuoles appeared to be in direct continuity with the Golgi cisternae (Figure 97) and vesicles of both the coated and uncoated variety were budding from or fusing with the vacuolar membranes (Figures 96 and 107). Intermediate stages between these vacuoles and fully formed granules were not identified although it was not clear whether the granules with less electron-dense and more particulate matrices (Figure 97) were in the process of condensation of whether they were artefacts of fixation.

One or two granules in the maturing IM cells had double delimiting membranes (Figure 100) and these were commonly found in the more fully granulated mast cell between days 14 and 19 after infection. Some granules, particularly in the more mature cells, had small vesicular and tubular elements distributed around the periphery of the matrix and myelin-like figures could be distinguished within the matrix (Figures 102 and 112).

In a few cells, particularly between the fourteenth and sixteenth days of infection there were several vacuoles in the cytoplasm which were surrounded by single or double unit membranes and contained a variety of cytoplasmic constituents (Figures 108 and 109). Vesicles and focal accumulations of dense material were present within

some of the vacuoles. Progranules were closely associated with some of the latter (Figure 110) and in others, electron dense material which was similar to the granule matrices had accumulated to such an extent that it partially obscured the constituents of the vacuole (Figure 111). Figures 108 to 112 suggest a possible sequence by which matrix could be added to the vacuoles eventually, perhaps, to form granules.

Discussion

The results reported here clarify the observations made in the quantitative studies of the IM cell population (this Section, part 2), namely that the reappearance of these cells in the lamina propria is the result of cell differentiation with granule production and of cell division. Ultrastructurally, differentiation and division were readily seen in the lamina propria and no granulated cells were found in blood vessels or lymphatics, suggesting that the majority of IM cells were derived from cells already in situ and not by infiltration of the mucosa by mast cells from other places. But, the presence of lymphoid and blast cells within blood vessels and lymphatics would suggest that the IM cell precursors could be derived from elsewhere in the body.

The use of thin sections examined ultrastructurally and compared with adjacent thick sections stained by basic dyes ensured that mast cells were identified. In a few instances the same individual cell could be located in both the thick and the thin section. Thus, the maturing IM cells had deeply basophilic granules whereas other cell types, such as macrophages and basophil-like cells contained inclusions which stained only faintly with basic dyes. Mast cell granules first appeared in the blast cells with relatively abundant cytoplasm containing many polyribosomes and with large

nuclei and prominent nucleoli. This is in agreement with the observations in part 2 of this Section and of Tallaferro and Sarles (1939) who noted that "connective tissue basophils" developed from large 'stroma' cells in the lamina propria.

There was, however, a striking resemblance between the IM cell precursors and blast cells found in lymph nodes, lymphatics and thoracic ducts of animals after antigenic stimulation which have been variously termed haemocytoblast (Feldman and Nordquist, 1967; Leduc, Avrameas and Boutelle, 1968) lymphoid blast cell (Hall, Morris, Moreno and Bessis, 1967) or immunoblast (Damashek, 1963).

The origins of the blast cell were difficult to trace although it was possible to suggest a series of transformations beginning with cells of the lymphoid series and ending with the typical blast cells, but in view of the large numbers of plasmablasts and plasma cells present in the mucosa, they may represent several unrelated lines of differentiation. Several workers have postulated that mast cells differentiate from lymphocytes (see review by Michels, 1939) and Ginsburg (1963) found that mast cells developed from large lymphoid blast cells in cultures of thymic lymphocytes. In a subsequent experiment, mast cells proliferated in cultures of lymphoid cells derived from antigenically stimulated mice, and were probably derived from small lymphocytes which had transformed into large lymphoid cells prior to the production of granules (Ginsburg and Lagunoff, 1967).

Blast cells are commonly found in animals sensitised to parasite antigens (Soulsby, 1967) and recently Jamuar, Kim and Hamilton (1968) reported a blast cell transformation of lymphocytes cultured *in vitro* and stimulated with antigen from

Trichinella spiralis. The cells described here are morphologically similar to those reported by Jamuar et al. (1968) and it is possible that the presence of Nippostrongylus in the intestine provides an antigenic stimulus which might be responsible for the transformation and differentiation processes.

Although differentiation and maturation of IM cells were not entirely synchronous, the build-up in the numbers of granules in individual cells was approximately parallel to the increase in the cell population. Mitotic figures were observed amongst the precursor cells, in the immature granulated cells and were still evident in even the more fully granulated mast cells. Ginsburg (1963) applied the term 'mastoblast' to the blast cells prior to the appearance of identifiable granules in their cytoplasm, but in view of the pluripotentiality of the blast cell it would seem preferable to reserve this term for the differentiated mast cell which is still capable of dividing.

The granules in the IM cell eventually filled the cytoplasm at the expense of the other cytoplasmic components, so the pattern of maturation was similar in many respects to that described for the CTMC (Combs, 1966), the polymorphonuclear leukocyte (PMN) (Bainton and Farquhar, 1966; Wetzel, Horn and Spicer, 1967), the eosinophil, and the basophil leukocyte (Wetzel et al., 1967). In the developing granulocyte the RSER becomes more prominent just prior to and during the production of granules and is subsequently reduced in size and amount at later stages of maturation (Ackerman, 1968) and typically in the maturing IM cell the RSER was progressively reduced in amount as the granules filled the cytoplasm.

The majority of the granules in the maturing IM cell appeared to be derived from

the Golgi complex which was considerably enlarged just before and during the early stage of granule production, but as the cells reached maturity, it decreased in size and in complexity. Even so, there were many cells in the mucosa on the sixteenth day of infection with sufficiently large and apparently active Golgi regions to suggest that the production of granules was still in progress. This was true for a smaller proportion of cells on the nineteenth day but by the thirty-fifth day, the complexes were mostly small and inactive.

In the CTM cell and in the rabbit polymorphonuclear leukocyte the progranules fused to form dense aggregates which become the precursors of the mast cell granule and of the azurophil granule in the PMN (Combs, 1966; Baluton and Farquhar, 1966), but no aggregates of this type were seen in the IM cell; instead the majority of the granules in the maturing cell were large and had homogeneous electron-dense matrices. Progranules were, however, seen to fuse with already large granules; they were found within the borders of the granule matrices, and were clustered around the small, partially membrane-delimited accumulations of matrix. It would appear reasonable to assume that the latter are granule precursors which enlarge to form full-sized granules by fusion with progranules, Golgi vesicles, and possibly other precursors. The moderately large vacuoles in the Golgi region which resembled condensing vacuoles found in the pancreatic zymogen cell (Jamieson and Palade, 1967) offer an alternative method of granule formation. Ackerman (1968) found similar vacuoles in the maturing cat neutrophil and observed that they enlarged and fused with other vacuoles to form azurophil granules. In the case of the IM cell the condensing vacuole appeared in some instances to be the dilated peripheral portions of the Golgi cisternae. This direct

continuity between the Golgi cisternae and the condensing vacuoles would presumably allow the accumulation of matrix which might then condense to form the typical mast cell granule. The variety of vesicles clustered around and fusing with the vacuole would allow further constituents to be added to the matrix.

The existence of a double unit membrane around some of the granules would be difficult to explain if these granules had been derived from condensing vacuoles, especially since progranules and vesicles were sometimes encompassed by these membranes. One possible explanation is that the matrix precursors become surrounded by membranes derived from the endoplasmic reticulum as has been observed in the formation of autophagic vacuoles (Elliot and Bak, 1964; Brandes, Buctow, Bertini and Malkoff, 1964; Novikoff and Shin, 1964; Swift and Hruban, 1964; Hugon and Borgers, 1966; Holtzman, Novikoff and Villaverde, 1967). The presence of vesicles within granules surrounded by a unit membrane is less readily understood. It is possible that they were originally surrounded by a double unit membrane and that the inner membrane was destroyed as has been postulated by Deduve and Wattiaux (1966). However, recent studies on the formation of multivesicular bodies and pinocytotic vesicles may shed some light on this mechanism. The vesicles in these organelles are internalised by migration into open-ended cup shaped membranes (Gordon, Miller and Bensch, 1965) by budding into the vacuole (Friend and Farquhar, 1967; Hirsch, Fedorko and Cohn, 1968) or by inclusion into autophagic vacuoles formed by membranes derived from GERL (Holtzmann et al., 1967). Further studies are required to find which, if any, of these mechanisms is functional in the maturing IM cell.

In the more mature cells there were a number of cytoplasmic inclusions which were indistinguishable from autophagic vacuoles described in other cell types (Ashford and Porter, 1962; Brandes et al., 1964; Swift and Hruban, 1964; Deduve and Wattiaux, 1966). Such vacuoles are commonly found in cells undergoing physiological and pathological alterations and their presence in the maturing mast cell which itself undergoes fairly extensive cytoplasmic remodelling was not therefore surprising. However, progranules were sometimes closely associated with these vacuoles and a homogeneous electron dense material appeared to accumulate in them. A variety of images suggested that the matrix continued to build up in this fashion so that the vacuoles ~~become~~ became virtually indistinguishable from granules manufactured by other methods. Indeed, if this represents a method of granule formation it would account for the presence of myelin-like figures in the altered granules of partially discharged cells (Murray et al., 1968; this Section, part 4).

It is clear that the maturation of the IM cell differs in many respects from the process in the CTM cell. The granule matrices in the latter, when it reaches maturity, cease to stain with Alcian blue and instead have a strong affinity for safranin (Combs et al., 1965). Combs (1966) showed that there was an ultrastructural reorganisation of the granule matrices at this time and suggested that as the mucopolysaccharide became fully sulphated, maximum ionic binding between the heparin polymer and basic proteins might lead to contraction of the granule matrix to form the dense relatively homogeneous granule seen in the mature cell. In the IM cell no major shift in the histochemical properties of the granules occurred (this Section, part 5) and there was no such dramatic reorganisation of the granule matrices. Combs (1966)

hypothesised that the basic proteins including chymase were added to the granule matrices by direct continuity between the rough surfaced endoplasmic reticulum and the perigranular membranes. In the present study direct continuity of this type was not established even though the RSER and perigranular membranes were often closely related to one another. Basic protein is, however, histochemically demonstrable within the maturing IM cell granule (this Section, part 5) and it is possible that it reaches the granule matrices via the Golgi vesicles as has been shown to occur in the pancreatic zymogen cell (Jamieson and Palade, 1967).

There were similarities between the maturation processes of the IM cell and the differentiation of mast cells from in vitro cultures of mouse lymphoid cells; focal accumulations of electron dense material and of vesicles within perigranular membranes were observed in both cell types and the in vitro mast cells, like the intestinal mast cell, stained blue in the Alcian blue/safranin sequence (Ginsburg and Lagunoff, 1967). Since both cell types appear to be derived from lymphoid tissue it is tempting to speculate that they represent a different population from the mast cells in connective tissues. It remains to be shown, however, whether there are functional differences between the mast cell derived from lymphoid tissue and the CTMC.

The present results show that differentiation from lymphoid blast cells and cell division are responsible for the exponential increase in the IM cell numbers between the tenth and fourteenth day of infection and confirm that not only does cell division occur on the fourteenth day but that granule production in, and maturation of the IM cells continue on the sixteenth and nineteenth days after infection. This

would tend to support the earlier assumption that a sudden decline in the rate of division, differentiation and maturation of the IM cells did not play a major part in the cessation of the growth phase after the fourteenth day of infection (this Section, part 2) and it will be shown that at this time there is total disruption and lysis of many of the granulated cells in both the lamina propria and the epithelium (this Section, part 4).

Part 4

The Intestinal Mast Cell during Immunological Expulsion of *Nippostrongylus brasiliensis* in the Rat

The ultrastructure of discharging cells

Just prior to the immunological expulsion of Nippostrongylus brasiliensis from the intestine of the rat, mast cells proliferated in the lamina propria and increased in number in an exponential fashion by processes of differentiation and cell division (this Section, part 2). On the fourteenth day of infection when many of the parasites were being expelled the increase in mast cell numbers stopped rather abruptly even though the granulated cells were dividing and maturing at the time (this Section, parts 2 and 3).

The termination of this phase of increased population was attributed to the migration of almost half of the total IM cell into the epithelium to become globule leukocytes and to the extensive destruction of both mast cells and globule leukocytes at this time (this Section, part 2).

The ultrastructural findings reported here confirm that there is extensive IM and GL cell disruption on the fourteenth and sixteenth days after infection and the features of the reaction are described and the possible underlying mechanisms are discussed.

Materials and Methods

The experimental regime was that described in parts 2 and 3 of this Section.

Results

Day 10 to Day 12 after Infection with N. brasiliensis

As was previously reported, developing mast cells were detected ten days after infection and in thick sections their granules stained with a deep basophilia (this Section, part 3). A small proportion of cells on the tenth and eleventh days had one or two granules which stained less strongly, or sometimes with a faint metachromasia. In some of the cells there were small vacuoles in which pale blue rod-like bodies could be visualised. On the twelfth day after infection an increasing number of cells had faint-staining granules or vacuoles in their cytoplasm (Figure 115 inset) although rarely more half of the granules were changed in a single cell. IM cells which had migrated intraepithelially to become GL cells were altered more extensively, but still retained several deep-staining granules. The findings were compatible in all the rats examined on the tenth to twelfth days.

Ultrastructural examination of the mucosa on the tenth and eleventh days after infection showed that the majority of maturing mast cells had homogeneous electron-dense granules. Twelve days after infection a proportion of the granules in many of the cells were altered and their delimiting membranes were disrupted (Figures 113 to 115). The matrices of some of the granules were partially lost and had become more particulate and less dense so that the outlines of the paracrystalline structures which were approximately the same electron density as the original unaltered matrices, became

visible (Figure 115). There was a complete loss of the matrix from some granules leaving an empty vacuole partially surrounded by membranes, in which paracrystalline structures were lying free (Figures 113 to 115). These were composed of parallel arrays of fibres which, on cross section, had a lattice arrangement (Figure 114); small dense particles were found within the borders of some of the altered granules (Figure 114). Apart from the alterations to the granules, there were no marked changes seen in the other cytoplasmic organelles or the nuclei of the maturing cells between the tenth and twelfth days of infection. Thus, in thick sections, the pale or faintly metachromatic granule was probably equivalent to the granule which ultrastructurally had the partially extracted matrix; the vacuoles in which small, pale blue bodies were seen in thick sections presumably represented the granules where the matrix has been lost to leave the characteristic paracrystalline structures visualised ultrastructurally.

Days 14 and 16 after Infection with N. brasiliensis

Large numbers of more fully granulated mast cells were found on the fourteenth and sixteenth days of infection and a far greater proportion of the total population was located intraepithelially than on previous days (this Section, part 2). In many of the cells there was a decrease in staining intensity of at least 50% of their granules (Figure 118); others were more extensively altered in that all their granules stained with a much reduced intensity and tended to be pale blue or greenish in colour (Figure 119). Some IM and GL cells were vacuolated (Figure 120), or were fragmented and in the regions where this occurred, there was disruption of other cells in the lamina propria and epithelium (Figure 124) (this Section, part 6).

The changes were not uniformly distributed in the mucosa and areas where IM and GL cells had approximately the same mild alterations described for the twelfth day of infection were sometimes located close to other areas where the cells were extensively damaged. Cytolysis was most severe in the rats with the highest total IM cell counts and was more extensive on the fourteenth than on the sixteenth day of infection. Only limited areas of mucosa could be examined by the thick section method, but cytolytic changes were found in all of the five rats on the fourteenth day and in two of the five on the sixteenth day. Apart from the overall severity of the cytolysis, there appeared to be little to distinguish between the fourteenth and sixteenth days of infection so the description that follows gives a composite picture of the findings on these days.

Ultrastructurally, some of the cells were only slightly altered and were similar to those seen earlier in infection; in most of them, however, there was a large proportion of disrupted granules (Figures 116 and 118). Small vesicles and occasional myelin figures were found around the borders of some of the altered granules (Figure 116) and in others, paracrystalline structures lay free in empty vacuoles. In many IM and GL cells, there was a breakdown of the plasmalemma which, although it retained its original outline, had a beaded appearance and was interrupted at numerous points (Figure 122). Where this had occurred, there was a general decrease in the density of the granules with the matrices being finely granular and the outlines of the paracrystalline structures becoming visible (Figures 117, 121 and 122). The delimiting membranes surrounding the granules were absent or were swollen and indistinct (Figure 122) and where the alterations to the cell and granules were of this severity, there were

degenerative changes amongst other organelles. Nuclear chromatin was coarsely clumped and heavily marginated (Figures 121, 122 and 123) and the perinuclear cisternae tended to be dilated and disrupted. Sometimes small oval structures were found within them (Figure 117) and similar structures could be visualised within the cisternae of RSER (Figure 117). The ribosomes on both the perinuclear cisternum and on the rough-surfaced endoplasmic reticulum, were swollen and indistinct (Figures 117, 122 and 123). The cisternae of RSER were no longer arranged in flattened sacs, but instead formed oval, circular or irregularly shaped structures with swollen and indistinct membranes (Figures 117, 122 and 123). Mitochondria were usually small and empty membrane-delimited blebs protruded from their borders.

Some of the IM and GL cells were almost totally destroyed and their plasmalemmata were either visible as small fragments outlining the remnants of the cells (Figure 125) or else were no longer evident around identifiable remains of the IM or GL cell (Figures 121 and 123). Where the disruption was of this severity, the original identity of the cell could only be established from the characteristic appearance of the few remaining paracrystalline structures or from the shadow outlines of altered granules (Figures 121, 123 and 125).

A number of individual cells, however, showed the complete range of changes with the different stages in granule disruption and alteration of other organelles all being found in the cytoplasm. Sometimes double delimiting membranes were present around some of the partially altered granules (Figures 117 and 123). In general, it appeared as if the nucleus, although extensively altered, was the last structure to undergo dissolution and often remained to the last, being surrounded by only a few small remnants of the cell cytoplasm (Figure 121).

Days 19 and 35 after infection with N. brasiliensis

Examination of thick sections on these days showed that most of the mast cells in the lamina propria were fully granulated and were indistinguishable from IM cells observed in normal rats. However, there were intraepithelial GL cells in which some of the granules had a reduced affinity for basic dyes and others were extensively vacuolated (Figure 43b).

Ultrastructurally, on the nineteenth day, there was a small proportion of cells in the lamina propria containing a few altered granules. The alterations were similar to those described for the twelfth day of infection; a typical example is shown in Figure 44, and although the cells contained more granules, only a relatively small proportion in an individual cell showed any alteration. the GL cells were similar to those described earlier (Section II).

Thirty-five days after infection, the IM cells in the lamina propria were indistingushable from those seen in normal rats. Some of the GL cells still had granules in which paracrystalline structures could be visualised, but others had granules where the matrices remained homogeneous, but tended to leak out through ruptures in the delimiting membranes (Figure 126).

There were no extensive cytolytic changes seen in any of the nine rats from which blocks of tissue were taken on these two days.

Discussion

The present findings show that there were three distinctive phases of IM cell activity which could be related to the extent of granule alteration. Total cell numbers had increased exponentially between the tenth and the fourteenth days of infection

(this Section, part 2) and the first phase in the present experiment extended from the tenth to the twelfth day where only minor changes amongst the granules of the cells occurred, but few other effects were noted. The second phase included the fourteenth and sixteenth days after infection when the majority of cells showed more extensive granule changes and many of the cells themselves were severely damaged. Not only did the exponential increase in the population cease over this period of time, but there was, in fact, a slight decline in the total numbers (this Section, part 2). In the final phase on the nineteenth and thirty-fifth days of infection the changes amongst the IM cells in the lamina propria were minimal, but the CL cells underwent the same processes of discharge as was described earlier (Section II) and at the same time the total IM cell population continued to decline.

The decrease in the staining intensity of mast cell granules in thick sections could be related to the decreased density of the granule matrices visualised ultrastructurally and similarly, the vacuoles containing paracrystalline structures were evident in both thin and thick sections. Bloom and Haegermark (1965 and 1967) and Bloom, Fredholm and Haegermark (1967) used similar techniques to study the effects of degranulating substances on isolated peritoneal mast cells of the rat and found that slightly altered granules stained metachromatically with toluidine blue, whereas those that were more severely damaged, stained pale pink. Even though different basic dyes were used in the present experiment, the results would appear to be analogous and the ultrastructural changes were reflected by the staining properties of the cells in thick sections. The technique proved to be more useful for wider surveys of the

mucosa than would have been practicable using thin sections only.

Thus, mast cell damage and destruction was most severe on the fourteenth day after infection and particularly in the rats with high mast cell counts. The increasing severity of the changes which progressed from a few altered granules in a mast cell on the tenth day to more or less total destruction of the granules and the cell itself on the fourteenth day, suggests that this was a true functional change, rather than an artefact of fixation and this is supported by the histochemical findings (this Section, part 5). The early granule changes seen on the tenth to the twelfth day were similar to the granule alterations in the more severely damaged cells and this would indicate that they, also, were early functional changes rather than fixation artefacts.

Probably a majority of the mast cells from the tenth to the twelfth day could be visualised with the copper phthalocyanine dyes since each cell contained several unaltered granules. Histochemical studies on the sixteenth day using the critical electrolyte method of Scott and Dorling (1965) (see part 5 of this Section) suggested that many of the cells retained their affinity for Alcian or Astra blue at pH 0.3 and the quantitative data therefore reflected the numbers of slightly altered and more diffusely altered cells, although it probably did not include those that were totally lysed. Bearing in mind the limited areas that can be examined with thick sections, the present findings tend to confirm, but do not necessarily prove, the hypothesis that the cessation of the mast cell multiplication phase is at least partially due to the destruction and subsequent disappearance of IM and GL cells between the fourteenth and

sixteenth days of infection (this Section, part 2). Since the changes amongst mast cells on the subsequent days were small, the gradual decline in the total numbers probably reflected the continuing migration and degranulation of GL cells.

In the various experiments where the ultrastructure of degranulating mast cells have been described, there were several differences from the present results. Singleton and Clark (1965) found that swelling and loss of density of mast cell granules occurred in rats treated with 48/80 and that the granules were subsequently extruded into the surrounding extracellular spaces. The extrusion of granules was a common sequel to the treatment of mast cells with 48/80 (Bloom and Haegermark, 1965; Bloom, Fredholm and Haegermark, 1967) and the shed granules were sometimes phagocytosed by leukocytes (Singleton and Clark, 1965). The phagocytosis of granules and even of partially-discharged whole cells has often been observed (see part 1 of this Section).

In the present study, IM cell discharge was not characterised by the extrusion of granules, nor in the more severely damaged areas were mast cell granules or partially discharged mast cells themselves phagocytosed by macrophages. The earliest change was the decrease in density of the granule matrix and the delimiting membrane around such a granule was never found to be intact; it should, however, be pointed out that delimiting membranes were not always readily visualised around apparently normal granules. The probable sequence of events began with the rupture of the perigranular membrane and the gradual loss of matrix which became less dense and rather more granular, eventually to disappear and leave the paracrystalline structures in an empty vacuole. More severe changes were noted particularly amongst the fully

granulated cells on the fourteenth day of infection, where virtually all the granules within an individual cell might be altered in situ, but not extruded although the cells themselves were often fragmented and there was disruption and dissolution not only of the perigranular membranes, but also of the plasmalemmata. Other organelles, including the nuclei, also showed extensive alterations.

In some respects, the changes resemble those caused by the n-decylamine on isolated rat peritoneal mast cells where rupture of the plasmalemma and of the perigranular membranes occurred and the total breakdown of the cell was associated with severe granule alteration in situ. In the latter instance it was postulated that the proteolytic enzymes released from the granules might play a part in the lytic changes in the cell (Bloom and Haegermark, 1967). Since the granules of the IM cell contain a basic protein (Section I, part 1) and presumably, like the CTM cell (Benditt and Arase, 1959; Lagunoff, 1968) also contain proteolytic enzymes, it is possible that the release of similar enzymes is partly responsible for the diffuse lytic changes.

It has been suggested that the basic protein is precipitated to form the para-crystalline structures (Murray et al, 1968) and Carr (1967) concluded that the crystalline inclusions found in GL cells of the mouse were protein. Paracrystalline structures were more clearly defined in the immature than in the fully granulated cells, as for example, on the 35th day of infection where the granule matrices of the GL cells tended to leak out through ruptured membranes, rather than be precipitated as para-crystalline bodies. It may be that these differences reflect the maturity of the cells where, in for example the mastoblast, the basic protein has not yet been sufficiently

admixed or stabilised and so may be more liable to precipitation than in mature cells.

The causes of the extensive mast cell damage at the time of immunological expulsion of Nippostrongylus brasiliensis from the intestinal tract, have not been clarified, but there are several possibilities.

1. The mast cells are sensitised with homocytotropic antibody and degranulate as a result of exposure to Nippostrongylus antigens. Homocytotropic antibody may be detected in the sera of rats shortly after infection with N. brasiliensis and the parasite itself produces an allergen which reacts with homocytotropic antibody (Jones and Ogilvie, 1967; Wilson, 1967). Wilson and Bloch (1968) showed that the peritoneal mast cells were sensitised to release histamine as early as ten days after infection and that increasing quantities of histamine were released from isolated peritoneal mast cells reaching a maximum on the twentieth day when homocytotropic antibody could be detected serologically. In view of these findings, it is reasonable to postulate that the mast cell reaction in the intestinal lamina propria and the mast cell to GL transformation are the consequence of sensitisation with homocytotropic antibody and exposure to worm antigen. Thus, the mild changes in the maturing cells on day twelve may be the result either of a relatively deficient supply of homocytotropic antibody since it is unlikely that allergen is in short supply with the bulk of the worm population still being present at this time or, of a functional immaturity of the cells. The increased severity of degranulation of IM cells on the fourteenth day may be the product of increased concentrations of homocytotropic antibody and the interaction of allergen causing the lysis of the perigranular membranes. The subsequent stability of the mast cells may

result from deficiency of allergen because by the nineteenth and thirty-fifth days, very few parasites remain in the intestine although homocytotropic antibody titres are high (Ogilvie, 1964; Ogilvie, 1967; Wilson and Bloch, 1968).

2. The parasites may continue to secrete the specific mast cell degranulator which presumably was responsible for the destruction of the IM cells during intestinal invasion by the parasite (this Section, part 1). The destruction of the new population of mast cells could clearly be caused by degranulator secreted by the parasites, but this is unlikely for several reasons. In the first instance, more than half of the worm burden had been expelled by the fourteenth day and very few parasites remain in the intestine on the sixteenth day. In addition, over this interval of time, they no longer lie close to the mucosa but tend to be redistributed in different sites in the intestine (Brambell, 1965). If the damage was caused by degranulator, then mast cell discharge could be expected to be more severe on the twelfth day when the parasites were concentrated in their predilection site in the jejunum.

3. The union of antigen with free antibody in the lamina propria may be a factor in the general cytolytic reaction that occurred in the intestinal mucosa during the expulsion of the parasites. Such a mechanism has been postulated for the severe superficial necrosis and sloughing of the mucosa that occurs in parasitic gastritis in cattle (Jarrett, 1966; Murray, 1968). And in view of the extensive structural damage that is found in the parasites at the time of self cure (Ogilvie and Hockley, 1968; Lee, 1969) there may well be a sufficient excess of antigens to bring about mucosal changes. A reaction of this type presumably would require host cells to release biogenic substances which might then act on the tissues.

It is possible that the amines and proteolytic enzymes escaping from damaged cells contribute by triggering the release of biogenic substances from previously undamaged mast cells. In addition, it is not known what influence the degranulation of eosinophils (this Section, part 6) might have on the overall reaction or whether they too are affected by the mast cell products. However, the comparative stability of IM cells on the nineteenth day after infection even though there are many present in the lamina propria would indicate that the presence of the parasites is necessary for degranulation. The underlying mechanism will not be clarified until changes caused by a mast cell degranulator produced by the worm can be differentiated from damage caused by mast cell sensitization and exposure to allergen.

As was briefly mentioned in the results the peak of mast cell destruction was associated with extensive mucosal damage. This reaction is described in detail in this Section, part 6.

Part 5

The Intestinal Mast Cell During Immunological Expulsion of *Nippostrongylus brasiliensis* from the Rat

Histochemistry

During the immunological expulsion of Nippostrongylus from the rat intestine, the mast cells in the mucosa increased in number in an exponential fashion, being derived from precursor cells by differentiation, division and maturation (this Section, Parts 2 and 3). The histochemistry and ultrastructure of the maturing connective tissue mast cell (CTMC) have been described (Combs et al., 1965; Combs, 1960) and since the intestinal mast cell (IM cell) differs both morphologically and histochemically from the CTMC, it seemed relevant to compare the histochemistry of the maturation of these two cell types.

Ultrastructurally, many of the IM cells even as they matured, were discharging, often without extruding their granules (this Section, part 4). However, it was not clear whether the quantitative methods included these partially discharged cells, or whether they only accounted for the intact mast cells. By using differential staining methods for the acid mucopolysaccharides and by examining the basic protein and monoamine content of these mast cells, the present experiment was designed to throw further light on the histochemical changes occurring in maturing and degranulating mast cells. The results show that the granules of the developing cells contain a highly sulphated acid mucopolysaccharide, a basic protein and a monoamine, which is probably 5-HT. The findings also point to depletion of acid mucopolysaccharide and loss of monoamines from the discharging cells between the fourteenth and sixteenth days of infection.

Materials and Methods

The experimental plan is given in Table 8. Tissues fixed in Carnoy's fluid were used for the detection of AMPS and basic protein. Staining with Toluidine blue, Alcian or Astra blue/safranin and Biebrich scarlet was carried out as described in General Materials and Methods. The CEC method (Scott and Dorling, 1965) was the same as that used in Section I, part 1, and is given in detail in General Materials and Methods. The Falck technique for detection of monamines was the same as that described in General Materials and Methods and in Section I, part 1.

Results

(a) Acid Mucopolysaccharides: The staining properties of the IM cells in infected rats are recorded in Table 11. IM cells in normal rats were used as controls (Table 11).

On the eleventh day of infection, the majority of mast cells had only small numbers of granules in their cytoplasm. But on the fourteenth, sixteenth and nineteenth days after infection, they were morphologically similar to the mature IM cell in the normal rat.

Table 12 records the affinity of Alcian blue 8GX for IM cells and CTM cells in normal and in infected rats when increasing molarities of $MgCl_2$ were added to the staining solution. Both IM and GL cells from rats on the eleventh day of infection, stained less intensely at high molarities than IM cells in normal rats (Table 12). In addition, the staining was cut off for some IM cells and a larger proportion of GL cells on the eleventh day of infection (Figures 129, cf. 130) at lower CEC than was the case with IM cells in normal rats (Figures 127, cf. 128 and 131, cf. 132) (Table 12). Many GL and some IM cells were cut off by lower molarities of electrolyte on day 16 (Figures 133, cf. 134) (Table 12).

TABLE 11

Histochemistry of IM and GL Cells during the Immunological Expulsion of *Nippostrongylus brasiliensis*

	Normal Rat	Infected Rats					
		Day 11		Days 14 and 16		Day 19	
	IM Cell	IM Cell	GL Cell	IM Cell	GL Cell	IM Cell	GL Cell
Toluidine Blue pH 0.3	B ++	B ++	B ++	B ++	B ++	B ++	B ++
pH 4.0	RP +	RP +	RP +	RP +	RP +	RP +	RP +
Astra or Alcian Blue / Safranin	B +++	B +++	B +++	B +++	B +++	B +++	B +++
Biebrich Scarlet pH 9.9	OR ++	OR +	OR +	OR ++	OR +(+)	OR ++	OR ++

For key, see Table 1

TABLE 12

The critical electrolyte concentrations for the staining of IM and GL Cells with Alcian blue 8GX during the immunological expulsion of N. brasiliensis.

Molarity of Electrolyte ($MgCl_2$)	Normal Rats		Infected Rats			
			Day 11		Day 16	
	IM Cell	CTM Cell	IM Cell	GL Cell	IM Cell	GL Cell
0.4	3	3	3	3	3	3
0.5	3	3	3	3	3	3
0.6	3	3	3	3	3	3
0.7	3	3	3	3	3-	3-
0.8	3	3	3-	2-	3-	2- -
0.9	2	3	2-	1- -	2 - -	1- -
1.0	2-	3	1- -	1- -	1- -	1- -
1.1	1- -	2	1- -	1- -	1- -	1- -
1.2	1- -	1	1- -	1- -	1- -	1- -
1.4	0	1- -	0	0	0	0

Staining intensity

3 = strong
2 = moderate
1 = weak
- = staining of a few cells cut out
- - = staining of many cells cut out
0 = no staining

(b) Basic Protein: The granules of both GL and IM cells were orange-red with Biebrich scarlet, but tended to stain less strongly in rats on the eleventh day of infection than in normal rats or in rats on the subsequent days of infection (Table 11). Some GL cells in infected rats stained less strongly than the mast cells in the lamina propria (Table 11).

(c) Monoamines: Normal rats. The IM cells in normal rats showed the same range of fluorescence as described earlier (Section I, part 1).

Infected rats - Day 10 to 12. IM and GL cells containing few granules were found in small numbers on the tenth day of infection, but were more numerous on the twelfth day (Figures 135 and 137). The granules of the IM cells had a moderately bright but dirty yellow fluorescence. Some of the GL cells had a similar bright fluorescence (Figure 136) but in others, the granules fluoresced dull green (Figure 138). Treatment with L-DOPA caused both the IM and GL cells to fluoresce a bright apple green (Figure 139).

Bright yellow fluorescent enterochromaffin cells (Figure 137) were readily distinguished from mast cells because of their characteristic flask shape and because the granules were not coarse globules, but instead were just resolvable by light microscopy. These cells were few in number on the tenth day after infection, but were more readily visualised on the twelfth day. In rats treated with L-DOPA there were cells morphologically similar to enterochromaffin cells, but having a green fluorescence more typical of catecholamines.

Day 14. The fluorescence of IM cells varied considerably amongst individuals, for in the mucosa of some rats, there were cells which fluoresced a moderately bright, but dirty yellow, whereas in others, the majority of cells had a dull green fluorescence (Figure 140). In the rats treated with L-DOPA, there were many cells in the lamina propria which had bright green fluorescence and often these cells appeared to be fragmented and isolated granules or clusters of granules appeared to have separated from the cells (Figure 141).

Although GL cells were as numerous as IM cells (this Section, part 2), very few showed any fluorescence and those that could be distinguished only emitted a faint dull green fluorescence (Figure 140). In the rats treated with L-DOPA, there were only one or two GL cells which showed any marked fluorescence, but these contained fewer granules than the mast cells in the lamina propria (Figure 141). Examination of 1.5 μ sections of tissues embedded in Araldite and of the same cells in adjacent sections, stained with basic dyes (Figures 145 a and b) confirmed that the cells in the lamina propria fluoresced brightly but the intraepithelial GL cells showed at best a weak fluorescence.

The enterochromaffin cells were more numerous than on the previous days of infection and the majority had a bright yellow fluorescence characteristic of 5-HT. In the L-DOPA treated rats, small numbers of enterochromaffin-like cells also showed green fluorescence.

Day 16. In all of the rats examined, the IM cells in the lamina propria of the upper crypt region and villus fluoresced a dull green, but there were a number of cells in the lower crypt region whose granules fluoresced yellow. The granules of both

fluorescent types were often scattered at a distance from whole cells (Figure 142). Only a few GL cells were visible and these emitted a very faint green fluorescence.

The numbers of yellow fluorescent enterochromaffin cells were approximately the same as found in uninfected rats.

Day 19. The most cells in the lamina propria of the villus and upper crypt region had a dull green fluorescence, but the majority in the basal crypt region fluoresced yellow. Fewer fluorescent granules were scattered freely in the lamina propria and IM cells were more compact than on the fourteenth and sixteenth days of infection (Figure 143). The IM cells in the upper crypt and villus regions of the L-DOPA treated rats fluoresced bright green, but many in the lower crypt region retained their yellow fluorescence.

Globule leukocytes had a dull green fluorescence of a much lower intensity than that observed in IM cells. In L-DOPA treated rats some of the GL cells fluoresced to a small extent (Figure 143).

The distribution of enterochromaffin cells was similar to that found in rats on the sixteenth day of infection. Very small numbers of enterochromaffin-like cells fluoresced green in L-DOPA treated rats.

Day 35. The fluorescence of IM and GL cells was similar to that seen on the nineteenth day after infection but approximately one third of the IM cell total fluoresced yellow and these cells were to a large extent located in the basal regions of the mucosa (Figure 144).

Discussion

In quantitative and ultrastructural studies (this Section, parts 2 and 3) it was shown that a new population of mast cells differentiated from blast cells and proliferated in the intestinal mucosa of rats during the immunological expulsion of Nippostrongylus brasiliensis from the intestinal lumen. In this histochemical study the granules in the newly differentiated cells, like the granules of mature intestinal mast cells were found to contain a highly sulphated acid mucopolysaccharide (AMPS), a basic protein and monoamines.

The AMPS was identified by its high affinity for copper phthalocyanine dyes at pH 0.3 and by its continued alcianophilia, even in high molarities of magnesium chloride.

The toluidine blue metachromasia at pH 4 and the strong orthochromasia at pH 0.3 also indicated that an acid mucopolysaccharide was present in the granules.

The maturing mast cell granules were less strongly stained with Alcian blue at the higher CE concentrations than were the granules of mast cells in normal rats and there may be several reasons for this weaker affinity. It has been shown that as the CTM cell matured, its granules changed from alcianophilic to having an affinity for safranin and this was associated with an increased uptake of labelled sulphate. It was suggested that this changeover in staining properties represented the final sulphation of a heparin precursor. (Combs et al., 1966). In a study of mast cell regeneration after treatment of rats with polymixin B, Meyer and Saunders (1969) found that as the cells matured, higher CE concentrations were required to change acridine orange

fluorescence of the granules from metachromatic to orthochromatic and they interpreted these findings as an increase, either in the number of repeating disaccharide units, each similarly sulphated and/or an increase in the sulphation per disaccharide. The present findings are probably analagous in that the mast cells are maturing cells (this Section, parts 2 and 3) and the weaker affinity for alcian blue of the maturing cell, probably reflects the maturation process. On the other hand, ultrastructurally some of these cells appeared to be discharging (this Section, part 4) and the staining of the granules may reflect this process, especially since those located intraepithelially (i.e. GL cells) were cut off at lower CEC than the IM cells.

By using Biebrich scarlet at pH 9.9, it was possible to demonstrate that the maturing mast cell granules contained a basic protein. The granules were less strongly stained than were the granules of IM cells in normal rats, but it is not clear whether there was a lower content of basic protein in them or whether the intragranular relationship between basic protein and acid mucopolysaccharide was different.

By comparison with IM cells in the upper crypt region and villus of normal rats which fluoresced a dull green, the maturing IM cells between the tenth and twelfth days of infection, fluoresced a moderately bright, but dirty yellow. As was pointed out in Section I, part 1, the interpretation of monoamine fluorescence requires microspectrofluorometric analysis together with biochemical assay of the monoamine content of the tissues. However, a similar fluorescence was reported in the islet cells of guinea pig pancreas and on the basis of the colour, and of monoamine assays, was attributed to a mixture of dopamine and 5-HT. It was postulated that primary catecholamines could be quantitatively transferred to their fluorophor whereas 5-HT, having

a lower activity in the paraformaldehyde condensation reaction, may be masked by green dopamine fluorescence (Cegrell, Falck and Rosengren, 1967). Similarly, the fluorescence in the maturing cells may represent a mixture of 5-HT and catecholamine within the granules. The green fluorescence of the cells after treatment with L-DOPA could, therefore, be the result of the quantitative transfer to catecholamine fluorophor rather than to the 5-HT condensation product. Recent assays on the intestinal mucosal content of 5-HT have shown increasing amounts of this monoamine at the time of Nippostrongylus expulsion (Murray, Miller, Sanford and Jarrett, to be published), but it is not known what proportion of 5-HT is in the mast cells and how much is found in other cell types such as enterochromaffin cells.

When IM cells migrate intraepithelially to become globule leukocytes, they lose some of their monoamines and there is a change in the relationship between the acid mucopolysaccharide and basic protein in the granules (Jarrett et al., 1967b; Miller et al., 1967; Murray et al., 1968; Section II). Table 12 shows that the staining of many GL and IM cells on the sixteenth day of infection was cut off at much lower electrolyte concentrations than was the case with IM cells in normal rats. These lower CE values would indicate either that there is a loss of acid mucopolysaccharide from the granules or that it is less fully sulphated. Ultrastructural findings pointed to a loss of matrix from the granules (this Section, part 4) and it would seem likely that there is, in fact, an overall reduction in their content of acid mucopolysaccharides.

Attempts to correlate ultrastructural and histochemical findings should take into account the different fixation and processing techniques employed in both methods. Ultrastructurally, many GL and IM cells showed extensive lysis of their perigranular membranes with apparent loss of granule matrix and in thick sections these cells no longer stained deeply with basic dyes (this Section, part 4). Although it provides satisfactory fixation of normal mast cells (Section I, part 2), glutaraldehyde may not be able to preserve the matrix of the structurally altered cells. On the other hand, Carnoy's fluid which has many reactive groups (Fuchtler, Waldrop, Connor and Terry, 1968) may be more readily able to precipitate the acid mucopolysaccharides even in damaged cells. This is evident from the fragmented appearance of the IM cells on the fourteenth day of infection and yet their granules still stained with Alcian blue at pH 0.3. Ultrastructurally, the granules of fragmented cells had shown extensive alteration. (this Section, part 4).

Half of the IM cell total was located intraepithelially on the fourteenth day after infection (this Section, part 2), yet very few GL cells showed monoamine fluorescence at this time and even in the rats treated with L-DOPA, there were few cells in the epithelium which fluoresced brightly; additionally, there was ultrastructural evidence of globule leukocyte and IM cell discharge (this Section, part 4). The staining of IM cells and GL cells with copper phthalocyanine dyes at pH 0.3 must, therefore, have been non-specific to the extent that partially discharged cells were included. This is supported by the staining affinities of these cells at different CE concentrations on the sixteenth day of infection.

Although most of the cells between the tenth and twelfth day of infection had a bright, but dirty yellow colour, on the fourteenth day the fluorescence of the majority had changed to dull green. This probably reflects an absolute loss of monoamines. It is possible, however, that it represents a qualitative change in fluorescence from the condensation products of 5-HT to the fluorophor of another monoamine. Sloroch and Uvnäs (1968) suggested that the reason why normal rat peritoneal and pleural mast cells contain small amounts of 5-HT and dopamine is the unavailability of the precursors. The same situation may exist in the intestinal mucosa because the mast cells were able to take up L-DOPA. The increasing numbers of mast cells in the basal part of the mucosa which fluoresced yellow during the later days of infection suggest that either 5-HT precursors were more available at this time and/or the cell population itself was more stable, the latter possibility being evident both ultrastructurally and quantitatively.

Enterochromaffin cells were few in number on the tenth day of infection, but by the sixteenth day were found in approximately the same concentration as in non-infected rats. Some enterochromaffin-like cells were able to take up L-DOPA and similar cells have been described in the gastric and duodenal mucosae of rats (Enerbäck, 1966d; Håkanson, Owman and Sjöberg, 1969). Enterochromaffin cells in the mouse have been implicated in anaphylaxis (Gershon and Rose, 1962) but the present findings indicate an increase in the mucosal content of these cells at a time when many mast cells are discharging.

These studies have underlined the necessity of using a variety of techniques to

examine the fate of mast cells in vivo. Cell counts showed an exponential increase in the cell population, but histological, histochemical and ultrastructural studies demonstrated that there was also massive discharge of these cells at a time when the parasites were being expelled from the intestine. The CEC method suggested that there was a loss of acid mucopolysaccharide from GL and IM cell granules and the Falck technique showed that most cells had lost their monoamines on the fourteenth day of infection. The histochemical findings give added support to the ultrastructural evidence that there is massive IM cell discharge between the fourteenth and sixteenth days of infection. This dramatic damage to the cells is, therefore, probably responsible for the rather abrupt cessation of the phase of population expansion just after the fourteenth day of infection (this Section, part 2).

It may, in future experiments, be possible to correlate the acid mucopolysaccharide changes with the depletion of monoamines and give a quantitative estimate of the proportion of discharging cells. In any event, these techniques, when used together, provide a sensitive index to the status of the mast cells in vivo.

Part 6

Mucosal Changes Associated with the Immunological Expulsion of *Nippostrongylus brasiliensis*

Previous studies of the immunological expulsion of N. brasiliensis had suggested that an anaphylactic component together with antiworm antibodies were important in the expression of the reaction (Urquhart et al., 1965; Barth et al., 1966; Jarrett, 1968). In the second to the fifth parts of this section it was found that the IM cells increased in number exponentially between the tenth and the fourteenth day of infection. From as early as the twelfth day these cells were discharging and on the fourteenth day there was massive disruption of IM cells and almost half the total had transformed to GL cells (this Section, parts 2, 4 and 5). There was a striking temporal relationship between worm expulsion (Jarrett et al., 1968) and the mast cell response (this Section, parts 2, 3, 4 and 5) and the latter presumably contributed the anaphylactic component to the self cure reaction. It remains uncertain, however, as to how a local anaphylactic reaction augments the antiworm antibody action, although Barth et al. (1966) postulated that it might increase the permeability of the mucosa.

In the fourth part of this Section, it was noted that disruption of mast cells was associated with focal cytolysis in the lamina propria and epithelium and this was apparently more severe on the fourteenth than on the sixteenth day of infection. The present experiment is a histological and ultrastructural study of the mucosal changes associated with the self cure reaction, and the mucosal cytolysis that occurs during the expulsion of the parasites are described in detail. Observations are also made on eosinophil and basophil-like cells; their possible functions together with the action of mast cells and other cell types in the mucosal changes are discussed.

Materials and Methods

The experimental plan was that described in the second part of this Section. Intestines from rats infected with doses of 2,000 and 5,000 Nippostrongylus larvae, fixed in sublimate formol were also examined. For histological purposes, paraffin sections were stained by haemalum and eosin and picro-Mallory (see General Materials and Methods). Carnoy fixed tissues stained with Astra blue /safranin, Toluidine blue and Biebrich scarlet were also examined. Details of these techniques are given in General Materials and Methods.

The ultrastructural methods and techniques for staining epoxy-embedded thick sections were those used in Section I, part 2 and in this Section, parts 3 and 4.

Results

General Observations

By the tenth day after infection the parasites had spread over a wider area of the small intestine than on previous days and were lying free in the lumen. An excess of mucus was found in the predilection site and in the posterior part of the intestine there was a thin, yellowish fluid. Both Peyers' patches and the mesenteric nodes were enlarged. Eleven days after infection little change was noted but on the twelfth day there was a reduction in the overall density of the worms. They were found in greatest concentration 3 to 4 cm posterior to the site from which tissues were taken, and some parasites had moved anteriorly into the duodenum. By the fourteenth day, the numbers were markedly reduced and the remaining worms were spread more

widely in the intestine, but again, the greatest worm density was just posterior to the segment of jejunum used in this study. Sixteen days after infection, there were few worms in the lumen and they were widely spread in the anterior half of the intestine. Very few parasites were found nineteen and thirty-five days after infection and they were randomly scattered in the anterior half of the small intestine.

Histological Findings

Ten days after infection, the villi were less distorted than on previous days but were still enlarged. The epithelial cells over the villus had reverted to a columnar shape but mitoses were numerous amongst the crypt epithelial cells and the gland crypts were elongated. The stromal elements in the lamina propria of some villi were still separated by oedema fluid although it was less pronounced than on the eighth day of infection.

The cell population had increased and plasma cells, plasmablasts and lymphoid cells were abundant, particularly lower in the villus. Macrophages and undifferentiated cells were numerous at all levels in the villus. At the junction of the villus and crypt region the lamina propria was populated by many eosinophils, by undifferentiated cells with large nuclei having in some instances prominent nucleoli and by a number of cells with multilobulated nuclei. The latter when stained with Biebrich scarlet had small granules which stained a faint orange. They were readily distinguished from maturing IM cells and did not stain with any of the dyes specific for AMPS. Ultrastructurally, these were identified as basophil-like cells and, with eosinophils, were infiltrating the basal mucosa and submucosa. These two cell types and a number of undifferentiated

cells were packed between the gland crypts.

From the tenth through to the twelfth day after infection, the mucosal changes were of degree rather than kind. Oedema became less evident and the plasma cell content of the mucosa continued to increase. Mitoses were frequently observed amongst the blast cells and undifferentiated cells in the lamina propria. Eosinophils were numerous and were found in all regions of the mucosa; the population of basophil-like cells remained at approximately the same level. Mast cells and globule leukocytes with faintly eosinophilic granules were also noted (see this Section, parts 2 to 5).

On the fourteenth day of infection, the epithelium of the gland crypts was heavily infiltrated with GL cells (this Section, parts 2 to 5) and goblet cells were prominent. The intestinal lamina propria was packed with IM cells, plasma cells, eosinophils and some macrophages. Lymphoid cells and undifferentiated cells although still present were not so numerous as they had been earlier in infection. Basophil-like cells were rarely observed.

On the sixteenth day there was no obvious change in the mucosa except that basophil-like cells were not found. Plasma cells, mast cells and eosinophils were abundant in the lamina propria and globule leukocytes were numerous (this Section, parts 2 to 5). Macrophages were found in greatest concentration at the tips of the villi. Nineteen and thirty-five days after infection there was a decrease in the numbers of globule leukocytes and mast cells (this Section, parts 2 to 5) and a decline in the mitotic rate amongst the crypt epithelial cells. Blast cells and undifferentiated

cells were less abundant and mitotic figures were rarely observed. Apart from the structural components (i.e. smooth muscle and fibroblasts), eosinophils, plasma cells, macrophages, lymphoid cells and mast cells formed the bulk of the population in the lamina propria.

Ultrastructural Findings

Days 10 to 12 after Infection : The wide lateral intercellular spaces observed between epithelial cells on the sixth and seventh days of infection had narrowed. The absorptive cells over the villus had assumed a columnar shape and the microvilli on their luminal surface were increased in length and were more densely packed. Many mitoses were found amongst the crypt epithelial cells.

The lamina propria was densely packed with cells although in some areas these were apparently separated by oedema fluid on the tenth day. Macrophages containing a variety of inclusions were numerous at all levels in the mucosa. There were many lymphoid and blast cells in the lamina propria and some of them were found within or in the vicinity of lymphatics. Plasmablasts, some of which were in mitosis, and plasmacytes were distributed in the lower part of the villus and at its junction with the gland crypts. Eosinophils were more numerous than on previous days and were concentrated in the basal mucosa and between the gland crypts; they were also found within blood vessels. The basophil-like cells were similarly distributed and like the eosinophils were often observed within blood vessels; one or two of these cells were noted within lymphatics and an occasional one was migrating amongst the epithelial cells of the gland crypts (Figure 150).

The basophil-like cells were distinguished by having multilobulated nuclei with coarsely clumped chromatin at their margins (Figures 148 to 151). Characteristically, they had moderately large granules measuring approximately 0.5μ diameter. The matrices of the majority were homogeneous and of approximately the same electron density as IM cell granules. Some had a crystalloid structure and in others a variety of vesicles or myelin figures were found. The intergranular cytoplasm contained few ribosomes or RSER and mitochondria were sparse (Figures 148 to 150). Basophil-like cells fixed for shorter intervals of time had dense glycogen particles in the cytoplasm (Figures 148 and 150). Golgi complexes, when seen, were well developed and located in a paranuclear position often being partially surrounded by the lobes of the nucleus.

In thick sections the granules of these cells stained pale green by the method of Richardson et al. (1960) (Figure 149) or a faint pink with basic fuschin (Ackerman and Hostetler, 1968) thus their tinctorial properties were quite different from both maturing IM cells and eosinophils. The granules of the polymorphonuclear leukocyte were not visible by light microscopy. Ultrastructurally, basophil-like cells were readily distinguished from maturing IM cells and from eosinophils. Polymorphonuclear leukocytes were rarely found in the mucosa at this time but the size of their granules differentiated them from basophil-like cells.

The changes amongst the cell population between the tenth and twelfth day of infection are described histologically. Ultrastructurally, there were several features of interest. On the eleventh and twelfth days after infection small numbers

of eosinophils were disrupted and their granules were released into the connective tissues (Figures 152 and 153). The majority of the granules were unaltered and the perigranular membranes remained intact. Eosinophils were rarely found within the epithelium.

By the twelfth day the cells were densely packed in the lamina propria. Plasma cells were abundant and many of them had dilated cisternae of RSER (Figures 146 and 147). The endothelial cells of many capillaries and venules were thickened and had relatively abundant RSER as well as increased numbers of lysosome-like bodies in their cytoplasm.

Days 14 and 16 after Infection: The fourteenth day of infection was marked by a variety of mucosal changes not only in the lamina propria, but also in the epithelium. The epithelial cells, particularly in the gland crypts, stained more strongly by the method of Richardson et al. (1960), the nuclei were more basophilic and were haphazardly arranged some of them lying much closer to each other than they did in normal rats (Figures 154 and 162). Gaps approximately the width of a single cell were often observed within the epithelial sheet (Figures 154 and 162). Some cells were sloughing off from the surface of the mucosa into the lumen of the crypt. Faint staining and vacuolated globule leukocytes could often be distinguished in these regions. Usually the cells in the adjacent lamina propria showed extensive alterations. Mast cells were fragmented, pale-staining, or vacuolated, and the outlines of the other cells were no longer clearly demarcated (Figures 124 and 154).

Ultrastructurally, the epithelial cells showed extensive alteration.

There was, however, a wide range of changes. In some regions the basal and lateral plasmalemmata of the epithelial cells were interrupted or were absent (Figures 155 to 158, 160 and 161). Their cytoplasm was diffusely granular and the cisternae of RSER were dilated,having indistinct membranes and swollen ribosomes,and their perinuclear cisternae were separated from the nuclei (Figures 157, 158, 160 and 161). Many mitochondria were enlarged and had membrane delimited but empty blebs protruding from their borders (Figures 158 and 161). The nuclei showed an increased electron density and the chromatin tended to be precipitated in dense patches around their margins (Figures 155, 158 and 161). Occasionally, the chromatin appeared to be spilling out from the nucleus itself (Figures 155 and 161). The epithelial basement membrane retained its integrity even in more severely damaged areas (Figures 155, 156 and 161). The changes were found most frequently in the gland crypts but they were also observed to a lesser extent in the epithelium overlying the villus.

In some parts of the gland crypts, epithelial cytolysis had occurred to such a degree that large gaps the width of one or more cells were found in the epithelial sheet (Figures 155 and 156). The cells bordering such gaps were severely damaged. In some instances, their cytoplasm was lost and the nuclei were lying in an empty space or close to the basement membrane.

Globule leukocytes were found in the damaged epithelium and were usually extensively altered, although this was not always the case because occasional GL cells, showing little evidence of discharge, were found within epithelium which was undergoing extensive lysis (Figure 160). Conversely, GL cells could be found within epithelium in which there was little morphological change (Figure 159).

Cytolytic alterations in the lamina propria were, like those in the epithelium, extensive and varied from several small interruptions in the plasmalemmata of cells to severe disruption, lysis, and eventual disappearance of their cytoplasm. Where damage was severe, few cells escaped lysis. Plasma cells no longer had well oriented cisternae of RSER, their plasmalemmata were absent, their cisternae were ruptured and small fragments of RSER had broken away from the cells (Figure 164). These tended to round up and form oval structures with swollen ribosomes and indistinct membranes. The changes in the other organelles and nuclei were similar to those described in epithelial cells.

Whereas on previous days of infection, one or two eosinophils had been disrupted, on the fourteenth day there was extensive lysis of these cells. Not only were the cell membranes dissolved and the granules of some cells scattered widely, but the peri-granular membranes were disrupted and the matrices had escaped leaving the dense crystalline bars. The granules were discharged in this fashion even when remaining in situ within the cell cytoplasm (Figures 163, 164 and 165).

Venular and capillary endothelium was also disrupted although again the basement membranes tended to remain intact (Figures 155 and 161). The endothelial cells, like the epithelial cells, showed a graded sequence of alteration from mild swelling of the endoplasmic reticulum and mitochondria to lysis and total disruption of the cells.

Macrophages, lymphoid cells, fibroblasts and undifferentiated cells all had the range of changes described for the epithelium. Often all that remained of the cell was

the nucleus surrounded by one or two remnants of organelles and it was impossible to identify whether it had originally been a mast cell, macrophage or some other mononucleated cell type (Figure 161).

The cytolysis in the epithelium was usually associated with changes of similar severity in the lamina propria, but this was not always the case. The changes were of such a focal character that even within the area of a single electron microscopic field, there were cells undergoing extensive lysis close beside areas where few changes could be found. Cytolysis was apparently more severe in rats which had high mast cell counts although it was found to a greater or lesser degree in all of the five rats examined on the fourteenth day; only two of the five rats on the sixteenth day had cytolytic changes of comparable severity. In the remaining three rats in this group, there were one or two small foci of cytolysis but in general the epithelium and lamina propria was not severely affected. Globule leukocytes were, however, numerous and the lamina propria was packed with plasma cells, mast cells, eosinophils, macrophages, lymphoid and undifferentiated cells (Figures 166, 167 and 168). No basophil-like cells were found at this time.

Days 19 and 33 after Infection : Globule leukocytes were present in the epithelium but no cytolytic changes were found in any of the rats examined on these days. The population of cells in the lamina propria is described in the histological findings.

Discussion

The mucosal changes described here include the interval of time between the eleventh and eighteenth day of infection when the parasites are expelled from the small intestine (Jarrett et al., 1968) and the findings throw some light on the possible mechanisms of immune expulsion.

Between the tenth and fourteenth days of infection there was an increase in the number of plasmablasts and plasma cells. This was also observed by Taliaferro and Sarles (1939) and an increase in the number of antibody-containing cells has been reported during several parasitic infections (Jarrett and Sharp, 1963; Dobson, 1967; Crandall et al., 1967; Murray, 1968). It was also evident that the Peyer's patches and mesenteric nodes were markedly enlarged at the time of worm expulsion and the ability of these lymphoid organs to respond to antigenic stimulation has recently been demonstrated by Cooper and Turner (1967). It has yet to be shown, however, whether such locally produced antibody is functionally effective in the rejection of parasites.

The presence of basophil-like cells has not previously been reported in Nippostrongylus infections. They were readily distinguished from other cell types both ultrastructurally and on the staining of their granules in thick sections. The weak affinity of their granules for Biebrich scarlet suggests that they might contain some basic protein. Their failure to stain dyes specific for AMPS distinguishes these cells from basophils in other species (Ackerman, 1963).

In the present study these cells were relatively abundant between the tenth and twelfth days of infection; few were found on the fourteenth day and none was seen on the sixteenth day and thereafter. Basophil-like cells were found within blood vessels and lymphatics and migrating through the epithelium. Presumably, therefore, they were derived from blood. There was no evidence that they degranulated in the mucosa.

Dobson (1968) observed an increase of the cells in the blood of rats infected with Amplicaecum robertsi and Chan (1965) reported that basophils were numerous in guinea-pigs treated with Ascaris body fluids. Basophils in the human are associated with delayed type hypersensitivity reactions (Wolf-Jürgenson, 1968). These cells in the guinea pig can be sensitised to release histamine (Greaves and Burdis, 1968) but in the rat, basophils supposedly are rare (Selye, 1965). Although the cells in the present study morphologically resembled basophils, there is no clue to their function in Nippostrongylus infected rats.

Eosinophilia is a constant feature in parasitised animals and the increase in eosinophils at the predilection site of the parasite has often been observed (Andrews, 1962). In the present experiment, numerous eosinophils were found in the intestinal lamina propria at the time of worm expulsion. Wells (1962) quantitated these cells in the intestines of Nippostrongylus infected rats and found that they increased in number and their location within capillaries and venules would suggest that they were derived from the blood; Murray (1968) in a study of bovine ostertagiasis reached a similar conclusion.

The functions of eosinophils have not been clarified although experimental data have suggested that they respond to the union of antigen and antibody as well as to antigen-antibody complexes. (Cohen, Sapp and Gallis, 1963; Litt, 1964). Eosinophils also phagocytose antigen-antibody aggregates and protein-coated particles (Sabesin, 1963; Kostage, Rizzo and Cohen, 1967).

There was no ultrastructural evidence that eosinophils were carrying out a phagocytic function in the intestinal lamina propria. During the early stages of infection, some eosinophils were disrupted and granules as well as whole cells were phagocytosed by macrophages (this Section, part 1). Between the tenth and twelfth days of infection, an occasional cell was disrupted and the granules were scattered in the lamina propria but these mostly remained intact. However, on the fourteenth day after infection, many eosinophils were disrupted, the perigranular membranes were ruptured and the granule matrices had escaped, leaving the dense central crystalline bars. Degranulation of this type was less marked on the sixteenth day and was not seen on subsequent days of infection.

The eosinophil reaction at the time of worm expulsion was similar to the changes found amongst the IM cells in that maximum degranulation occurred on the fourteenth day of infection. The perigranular membranes of the eosinophils like those of the mast cells were lysed, but the cause of such extensive degranulation is not clear. Perhaps eosinophils, like mast cells are able to be specifically sensitised. Such a hypothesis is made attractive by the recent findings of Zolov and Levine (1969) who demonstrated a specific association between blood eosinophilia and the presence of

skin sensitising antibodies in patients undergoing penicillin therapy. The presence of the parasites was necessary for eosinophil degranulation to occur because after the worms were expelled, the cells remained intact. Degranulating substances produced by the worm are unlikely to be effective since degranulation would then be more vigorous between the tenth and twelfth days of infection when parasites were numerous. Eosinophil disruption might, on the other hand, be brought about by the biogenic substances released from the numerous mast cells which were degranulating at the same time.

Whereas on the twelfth day the lamina propria was densely packed with many apparently viable cells and only one or two globule leukocytes were found in the epithelium, a dramatic change had taken place by the fourteenth day. Many cells both in the epithelium and in the lamina propria showed extensive lytic changes. The earliest alteration appeared to be the dissolution of the plasmalemma and this was followed by a loss of intracellular organisation. The cytoplasm of the more severely altered cells had a diffuse granularity, the cisternae of RSER and the perinuclear envelope were dilated and disrupted and the nuclei had an increased electron density. The final event appeared to be the complete dissolution of the cytoplasm to leave an isolated nucleus. In areas where cytolysis was severe, no cell type was free of damage. The lytic changes appeared to be most extensive in rats with high mast cell numbers although, even in these animals, the changes were focal and tended to be more severe in the region of the gland crypts.

The functional effects of this reaction are probably significant in the mechanisms

whereby the parasites are expelled from the intestine. The large gaps in the epithelial sheet and the lysis of epithelial cells could be responsible for a significant increase in the mucosal permeability. The damage to the vascular endothelium would allow free passage of plasma into the lamina propria and into the intestinal lumen. The extensive damage to the plasma cells presumably brings about a massive release of locally manufactured immunoglobulins. The increased vascular and epithelial permeability would permit free access for humoral antibody to the intestinal lumen and any locally produced antibody need only traverse the leaky epithelium. It is tempting to suggest that massive release of immunoglobuline by such mechanisms is to a large extent responsible for the rapid expulsion of the parasites.

The cause of the mucosal lysis is not certain, but there are several possibilities. The granules of the rat mast cell contain histamine, 5-HT and proteolytic enzymes (Benditt and Lagunoff, 1964). The intestinal mast cells also have histamine (Håkanson, Owman, and Sjöberg, 1967) and some contain 5-HT in their granules (this Section, part 5); they probably also contain proteolytic enzymes. Whilst it is recognised that 5-HT and histamine induce increased vascular permeability (Majno and Palade, 1961) little is known about the effects of release of proteases from mast cells. The changes observed in the mucosa are consistent with the activity of such enzymes since the most striking features were the extensive lysis of the cell membranes and the characteristic disruption of endoplasmic reticulum. The basement membrane seemed to suffer little damage and cell nuclei although altered, retained their gross structure even after the surrounding cytoplasm had been completely lysed. This damage occurred

at a time not only when mast cell numbers were at a maximum, but when a large proportion of these cells were discharging.

The damage was apparently more severe in rats with high mast cell counts and tended to be localised to a large extent in the crypt region; the site where mast cells are found in high concentration (this Section, part 2). These observations are, however, limited by the small areas of intestine that can be examined ultrastructurally, but they would indicate that mast cell products are at least partly responsible for the mucosal damage.

Eosinophils in the rat contain a variety of hydrolytic enzymes (Archer and Hirsch, 1963) which on disruption of the granules, could presumably contribute to the mucosal damage. Other cells containing lysosomes such as macrophages would, when disrupted, release their enzymes.

It is therefore reasonable to suggest that the mucosal damage is brought about by the release of histamine, 5-HT and various enzymes from mast cells, eosinophils and possibly other cell types. The relationship between mast cell and eosinophil disruption and mucosal damage was striking. The absence of mucosal damage on the nineteenth day when mast cells and eosinophils although abundant, showed little evidence of degranulation supports such a hypothesis.

GENERAL CONCLUSIONS

The presence of mast cells in the lamina propria of mucous surfaces has been reported on several occasions (Michels, 1938) but there was considerable doubt as to whether they existed in the intestinal mucosa of the rat (Mota, Ferri and Yoneda, 1956; Whur and Gracie, 1967). However, Enerbäck (1966a) found that the method of fixation used was critical for the demonstration of these cells. Furthermore, he demonstrated that a highly sulphated AMPS was present within their granules (Enerbäck, 1966b).

The histochemical studies in Section I, part 1 confirmed the presence of a highly sulphated AMPS in the IM cell granules, and at the same time, gave an indication as to how it was retained within the granule matrix. The demonstration of a strongly basic protein within the granules suggested that this might be linked ionically with the AMPS, as is known to occur in the connective tissue mast cell (Benditt and Lagunoff, 1964). Thus, the failure to demonstrate IM cells in formaldehyde-fixed tissues was almost certainly related to the masking of the polyanions by the polycationic protein; this was shown to be the case by applying the critical electrolyte technique of Scott and Dorling (1965). The majority of IM cells contain only small amounts of monoamines (Enerbäck, 1966d; Section I, part 1) although in the hooded Lister rat used in the present experiments some IM cells in the basal regions of the mucosa contained 5-HT.

The ultrastructural studies in part 2 of the first Section emphasised the variability of the IM cell morphology although the granules themselves were indistinguishable

from those found in the CTM cell. It was considered that the exposed locus of the IM cells rendered them liable to repeated immunological insults. Thus, the heterogeneity of the population might be attributed to a possibly higher rate of cell turnover than occurs amongst connective tissue mast cells.

In the third part of the first Section, it was shown that IM cells were more resistant to the degranulating effects of Compound 48/80 than their connective tissue counterparts. However, contrary to Enerbäck's (1966c) findings, the population of IM cells did not increase during the course of subacute treatment with 48/80.

The ability to recognise IM cells and to have an understanding of their biochemistry was of paramount importance in subsequent studies of their functions during infections with the intestinal nematode N. brasiliensis. Thus, a relationship was established between the IM cell and a cell of hitherto doubtful origin, the globule leukocyte. The latter is found within epithelia of mucous membranes, increases in number during parasitic infections and has acidophilic granules. In Section II, using histochemical and ultrastructural techniques, it was shown that the globule leukocyte was derived by the migration of IM cells into the epithelium. During the course of this migration, the granules discharged their monoamines and probably also some of their acid mucopolysaccharide (Section III, part 5). The end result of this process was a cell whose granules had a reduced basophilia, but which remained strongly acidophilic.

The main purpose behind this work was to define a role for the IM cell during the course of a Nippostrongylus infection. Suggestive evidence that it might play a part in the mucosal reaction was provided by earlier reports that IM cells increased in number during, or after infection with this parasite (Tallaferro and Sarles, 1939; Wells, 1962) and that immediate-type hypersensitivity reactions were important in the self-cure phenomenon (Urquhart et al., 1965; Barth et al., 1966). Accordingly, the fate of the IM cell was followed during the course of a Nippostrongylus infection.

The parasite, once established in the intestine, caused the disruption of the IM cells and they were subsequently phagocytosed by macrophages and undifferentiated cells (Section III, part 1). Their demise was attributed to a mast cell - degranulating substance secreted by the worms. Associated with their disruption was the development of oedema of the intestinal lamina propria; this was initially localised to the immediate vicinity of the worms but later became generalised in the predilection site of the parasite. It seemed likely that the initiation of the oedema was the consequence of IM cell degranulation; the ensuing reaction was probably potentiated by permeability-inducing factors secreted by the worms (Jarrett et al. 1967a) since ultrastructurally there was evidence of vascular damage (Section III, part 1).

Just prior to the immunological expulsion of the worms, IM cells reappeared in the intestinal mucosa. At first they contained few granules, they were derived from large blast cells in the lamina propria, and some were in mitosis. When the

granulated cells were counted they were found to increase in number exponentially and between the tenth and fourteenth day of infection the population rose from less than five cells within a villus to nearly one hundred (Section III, part 2). Differential counts demonstrated that on the fourteenth day of infection, nearly half the total was located intraepithelially (i.e. was GL cells). Thus, at least 50% of the cells were discharging their amines during the period of worm expulsion (Jarrett et al., 1968).

Ultrastructural studies (Section III, part 3) of the differentiation process showed that the IM cells developed from blast cells and that these were indistinguishable from lymphoid blast cells. There was ultrastructural evidence to suggest that the IM cell precursor cells were, in fact, derived by transformation of lymphoid cells (Section III, part 3). The production of granules in the maturing IM cell was similar in many respects to that reported to occur in granulocytes of various species and in the maturing connective tissue mast cell (Combs, 1966). The quantitative and ultrastructural data, therefore, indicated that the new IM cell population was derived from lymphoid blast cells by cell division, differentiation, and maturation.

Jarrett, Miller and Murray (1969) have suggested that the parasites produce a factor which, like Bordetella pertussis, causes the stimulation and transformation of lymphocytes; these might then home on the lamina propria of the gut and differentiate into IM cells. In view of their origin, IM cells, like plasma cells, may be immunologically committed and might possibly even be a source of reaginic antibodies.

The increase in the IM cell population ended on, or soon after, the fourteenth day of infection, although mitosis amongst granulated cells was still evident at this time (Section III, part 2). There was, apart from the massive transformation

of IM cells into globule leukocytes, extensive lysis of the mast cells in the lamina propria on the fourteenth day (Section III, part 4). Thus, three factors probably contributed to the cessation of the phase of population expansion: (1) the mast cell - globule leukocyte transformation; (2) the lysis and disruption of the IM cells in the lamina propria and (3) a decline in the rate of cell division and differentiation. The ultrastructural and histochemical findings (Section III, parts 4 and 5) suggest that (1) and (2) were the major factors.

It was not clear whether the degranulation of the IM cells was brought about by their sensitisation and exposure to allergen or whether the degranulator produced by the parasite earlier in infection was still functional. There is, of course, a third possibility that an antibody-antigen interaction in an excess of antigen might be responsible for the IM cell lysis (Jarrett et al., 1969).

The relationship between the mast cell reaction and the expulsion of the parasite was striking, but it must be emphasised that in the present study only one relatively small area of the mucosa was examined. There is evidence that the IM cell response occurs at different times in different parts of the mucosa and that it occurs later in male rats than females (Murray, Miller, Jarrett and Sanford, 1969 - to be published). Thus, a more definitive relationship between the timing of worm expulsion and the changes amongst the IM cells must be established over a wider area of the mucosa before definite conclusions can be drawn as to the role of these cells in the rejection of the parasite.

The present findings do, however, suggest a function for IM cells in the self cure reaction: when their numbers were maximal on the fourteenth day of infection and many of them were disrupted, there was lysis, not only of the epithelial cells, but also of the vascular endothelium and of cells in the lamina propria (Section III, part 6). Since this work was completed, Murray, Jarrett and Jennings, (1969 - to be published) have demonstrated that in male rats, labelled polyvinylpyrrolidone, when injected intravenously, leaks into the intestinal lumen during self cure. The maximum leak occurred on the fourteenth day of infection. The present ultrastructural findings are entirely compatible with a state of increased mucosal permeability. It might also be envisaged that the disruption of the plasma cells during the cytolytic reaction would allow rapid release of locally produced antibody and that this, together with any humoral antibody, might act against the parasites.

It is not clear whether the biogenic amines released from the mast cells contribute significantly to the mucosal changes, but they do have a role in the expulsion of the parasites because antihistaminics (Urquhart et al., 1965) and depletors of 5-HT (Sharp and Jarrett, 1968) inhibit the self-cure reaction. The mucosal lysis was more consistent with alterations which might be produced by proteolytic enzymes. Indeed, it is tempting to suggest that these are present in the IM cell granules and that their release might be responsible for the mucosal cytolysis. Proteases have been found in the serum and urine of several species undergoing anaphylactic or anaphylactoid reactions (Ungar, Tamura, Isola and Kohrin, 1961). Possibly, experiments carried out along similar lines in the rat, during self cure, might throw further light on the action of mast cell proteases.

Although the findings suggest that IM cells play a major part in causing increased mucosal permeability at the time of worm expulsion, the functions of other cell types, especially the eosinophil, have yet to be clarified. Furthermore, it remains to be shown whether IM cell degranulation is the consequence of sensitisation and exposure to antigen or whether it is brought about directly by substances produced by the parasite.

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APPENDICES

Appendix to Table 7

Experiment 1.

Quantitation of intestinal mast cells in rats infected with N. brasiliensis

Cells/Villus-Crypt Unit

	IM Cells	GL Cells	Total
Day 4	7.5	-	7.5
	6.7	-	6.7
Mean ± SE	7.1 ± 0.4	-	7.1 ± 0.4

	IM Cells	GL Cells	Total
Day 5	9.2	-	9.2
	4.4	-	4.4
	3.5	-	3.5
	9.4	2.0	11.4
	6.2	-	6.2
	3.6	-	3.6
	-	-	-
Mean ± SE	5.2 ± 1.3	0.3 ± 0.3	5.5 ± 1.4

	IM Cells	GL Cells	Total
Day 7	4.8	-	4.8
	-	-	-
	0.1	-	0.1
	0.3	-	0.3
Mean ± SE	1.3 ± 1.2	-	1.3 ± 1.2

	IM Cells	GL Cells	Total
Day 8	4.8	-	4.8
	2.7	-	2.7
	-	-	-
	-	-	-
	-	-	-
	-	-	-
Mean ± SE	1.2 ± 0.8	-	1.2 ± 0.8

Appendix to Table 7

Experiment 2.

Quantitation of intestinal mast cells in normal rats treated with metabolites from N. brasiliensis

Cells/Villus-Crypt Unit

	IM Cells	GL Cells	Total
	6.3	-	6.3
	8.4	1.8	10.2
	9.3	5.7	15.0
	4.0	-	4.0
Mean ± SE	7.0 ± 1.2	1.9 ± 1.3	8.9 ± 2.4

Appendix to Tables 7 and 9

Quantitation of Intestinal Mast Cells in Normal Rats

and in Rats Treated with L-DOPA

IM Cells/Villus-Crypt Unit

	Untreated Rats		Treated Rats
	11.5	11.8	11.6
	12.2	11.8	11.7
	11.8	10.4	12.5
	11.6	12.9	10.1
	12.6	12.6	12.5
Mean ± SE	11.9 ± 0.2		11.7 ± 0.4

Appendix to Table 9

The Quantitation of IM and GL Cells during the Immune Expulsion of *N. brasiliensis*. Mean Cell Numbers per Villus-Crypt

	IM Cells	GL Cells	Total	Log_{10} Total
Day 10	2.0	-	2.0	0.3010
	0.7	0.1	0.8	$\bar{1}$.9031
	4.2	0.1	4.3	0.6335
	4.3	0.2	4.5	0.6532
	0.9	0.1	1.0	0.0000
	0.5	-	0.5	$\bar{1}$.6990
Mean ± SE	2.1±0.7	0.1±0.1	2.2±0.7	0.2029±0.16

	IM Cells	GL Cells	Total	Log_{10} Total
Day 11	7.7	0.3	8.0	0.9031
	3.0	0.1	3.1	0.4914
	13.7	1.6	15.3	1.1847
	6.3	0.2	6.5	0.8129
	3.4	-	3.4	0.5315
Mean ± SE	6.8±1.9	0.4±0.3	7.3±2.2	0.7847±0.13

	IM Cells	GL Cells	Total	Log_{10} Total
Day 12	4.8	0.7	5.5	0.7404
	7.3	1.3	8.6	0.9345
	13.5	8.3	21.8	1.3385
	20.8	2.7	23.5	1.3711
	8.3	1.9	10.2	1.0086
	7.5	1.9	9.4	0.9731
	26.8	21.3	48.1	1.6821
	10.1	5.4	15.5	1.1903
	5.9	2.0	7.9	0.8976
Mean ± SE	11.7±2.5	5.0±2.2	16.7±4.4	1.1262± 0.1

Appendix to Table 9 (continued)

	IM Cells	GL Cells	Total	Log_{10} Total
	36.2	31.9	68.1	1.8331
	66.8	41.8	108.6	2.0359
	29.9	31.9	61.8	1.7910
Day 14	62.5	52.8	115.3	2.0618
	46.3	75.0	121.3	2.0838
	56.4	49.6	106.0	2.0253
	49.2	45.8	95.0	1.9777
	63.2	60.0	123.2	2.0906
Mean ± SE	51.3 ± 4.7	48.6 ± 5.1	99.9 ± 8.3	1.9874 ± 0.04

	IM Cells	GL Cells	Total	Log_{10} Total
	59.1	56.3	115.4	2.0622
	67.2	38.6	105.8	2.0245
	33.8	31.3	65.1	1.8136
Day 16	36.0	23.6	59.6	1.7752
	57.7	75.5	133.2	2.1245
	49.8	37.7	87.5	1.9420
	52.0	25.7	77.7	1.8904
Mean ± SE	50.8 ± 4.6	41.2 ± 7.0	92 ± 10.3	1.9475 ± 0.05

	IM Cells	GL Cells	Total	Log_{10} Total
	66.0	43.2	109.2	2.0381
	49.2	21.4	70.6	1.8488
Day 19	55.5	51.6	107.1	2.0298
	49.0	20.7	69.7	1.8432
	54.9	35.2	90.1	1.9547
	52.1	20.0	72.1	1.8579
	45.7	18.7	64.4	1.8089
Mean ± SE	53.2 ± 2.3	30.1 ± 5.0	83.3 ± 7.1	1.9116 ± .04

Appendix to Table 9 (Continued)

	IM Cells	GL Cells	Total	Log_{10} Total
	27.8	11.7	39.5	1.5966
Day 35	26.8	13.1	39.9	1.6010
	24.9	13.2	38.1	1.5809
	27.9	12.1	40.0	1.6021
Mean ± SE	26.8 ± 0.7	12.5 ± 0.4	39.4 ± 0.4	1.5952 ± 0.007

Appendix to Table 10

Size of Villus-Crypt Unit mm^2

Controls	Day 10	Day 11	Day 12	Day 14	Day 16	Day 19	Day 35
0.057	0.067	0.068	0.077	0.062	0.079	0.080	0.065
0.050	0.077	0.062	0.061	0.072	0.081	0.074	0.066
0.048	0.077	0.075	0.068	0.078	0.067	0.074	0.072
0.045	0.083	0.077	0.069	0.078	0.072	0.071	0.075
0.046	0.065	0.075	0.075	0.058	0.069	0.072	
Mean $\pm$ SE	0.049 $\pm$ 0.002	0.074 $\pm$ 0.003	0.071 $\pm$ 0.003	0.070 $\pm$ 0.003	0.070 $\pm$ 0.004	0.074 $\pm$ 0.003	0.074 $\pm$ 0.003	0.069 $\pm$ 0.002

The Intestinal Mast Cell in Normal and Parasitised Rats

by

Hugh R. P. Miller, B.V.M.S., M.R.C.V.S.

A Summary of a thesis submitted for the Degree of Doctor of Philosophy in the Faculty of Veterinary Medicine, University of Glasgow. July, 1969.

This work is divided into three sections. In the first section, the morphology histochemistry and ultrastructure of the mast cell (IM cell) in the intestinal lamina propria of the rat are described; the action of compound 48/80 on these cells is also examined. Section II shows the relationship between IM cells and globule leukocytes. Section III is a study of the IM cell during infection with Nippostrongylus brasiliensis.

Section I: Fixation with Carnoy's fluid is optimal for the demonstration of IM cells. Their granules contain a highly sulphated acid mucopolysaccharide, a strongly basic protein and monoamines. Ultrastructurally the IM cell granules are indistinguishable from those of the connective tissue mast cell, but there is greater morphological heterogeneity amongst the cells in the intestinal lamina propria. On the basis of their histochemistry and morphology, IM cells are considered to have all the features that ensure their identification as mast cells.

Compound 48/80 causes some disruption of IM cells, but they are more

massive disruption of IM and GL cells, but the changes become progressively less severe thereafter. The mechanisms of this reaction are discussed.

In part 5 of Section III, the maturing cells are shown to contain a highly sulphated acid mucopolysaccharide, a basic protein, and monoamines. The loss of monoamines and depletion of acid mucopolysaccharide from the disrupted cells are demonstrated histochemically.

There is, during self cure and associated with the peak of mast cell damage, extensive lysis of epithelial cells and of the cells in the lamina propria of the intestine. These changes are described in the sixth part of Section III. It is concluded that the IM cell reaction is largely responsible for an increase in mucosal permeability during self-cure, thus permitting the release into the intestinal lumen, of antibodies directed against the parasite.

The IM cells were quantitated during the immunological expulsion of the parasite (self-cure). The second part of Section III describes the kinetics of the IM cell population during self-cure. The number of cells increases exponentially between the tenth and fourteenth day of infection. The IM cells are derived from blast cells in the intestinal lamina propria and increase in number by differentiation and cell division. When numbers are at a maximum on the fourteenth day of infection almost half of the total population is located intraepithelially and many IM cells in the lamina propria are fragmented. Although mitoses are commonly noted amongst the granulated cells on the fourteenth day, there is gradual decrease in their numbers later in infection. It is concluded that the IM cell-GL transformation, the disruption of the IM cells in the lamina propria and a slower rate of cell division and differentiation, are responsible for the subsequent decline of the population.

Ultrastructural studies in the third part of Section III show that the newly differentiated cells are derived from blast cells which are indistinguishable from lymphoid blast cells. A sequence of cell transformation from lymphoid to blast cells is suggested. The processes of granule elaboration are described and are similar in many respects to those seen in granulocytes of various species.

In the fourth part of Section III, the ultrastructure of disrupted IM and GL cells is described. There is evidence of some granule discharge in maturing cells between the tenth and twelfth days of infection; on the fourteenth day there is

resistant to treatment with this drug than connective tissue mast cells. During subacute treatment with 48/80 IM cells, unlike their connective tissue counterparts, persist in the mucosa; the population is only slightly reduced when compared with that of untreated rats.

Section II: The globule leukocyte (GL) is found within epithelia and contains large acidophilic granules. Ultrastructural and cytochemical data are presented to show that it is derived from the IM cell. Experimental infections with Nippostrongylus brasiliensis are used to induce the formation of GL cells; they are shown to be the end-products of the migration of IM cells into the epithelium. During the course of migration there is a change in the relationship between the acid mucopolysaccharide and basic protein in the granules and a depletion of monoamines; the final product of these changes is a cell with a strong affinity for acid dyes - the globule leukocyte.

Section III: In the first part of this section, the fate of the IM cell is followed during intestinal invasion and colonisation by N. brasiliensis. IM cells are disrupted and many of the damaged cells are phagocytosed; the majority of cells are destroyed in the predilection site of the parasite. The IM cell population is also reduced in normal rats given intravenous injections of metabolic products obtained from adult worms. It is suggested that degranulating substances produced by the parasites are responsible for the disruption of these cells.

THE INTESTINAL MAST CELL

IN NORMAL AND PARASITISED RATS

VOLUME 2

PLATES

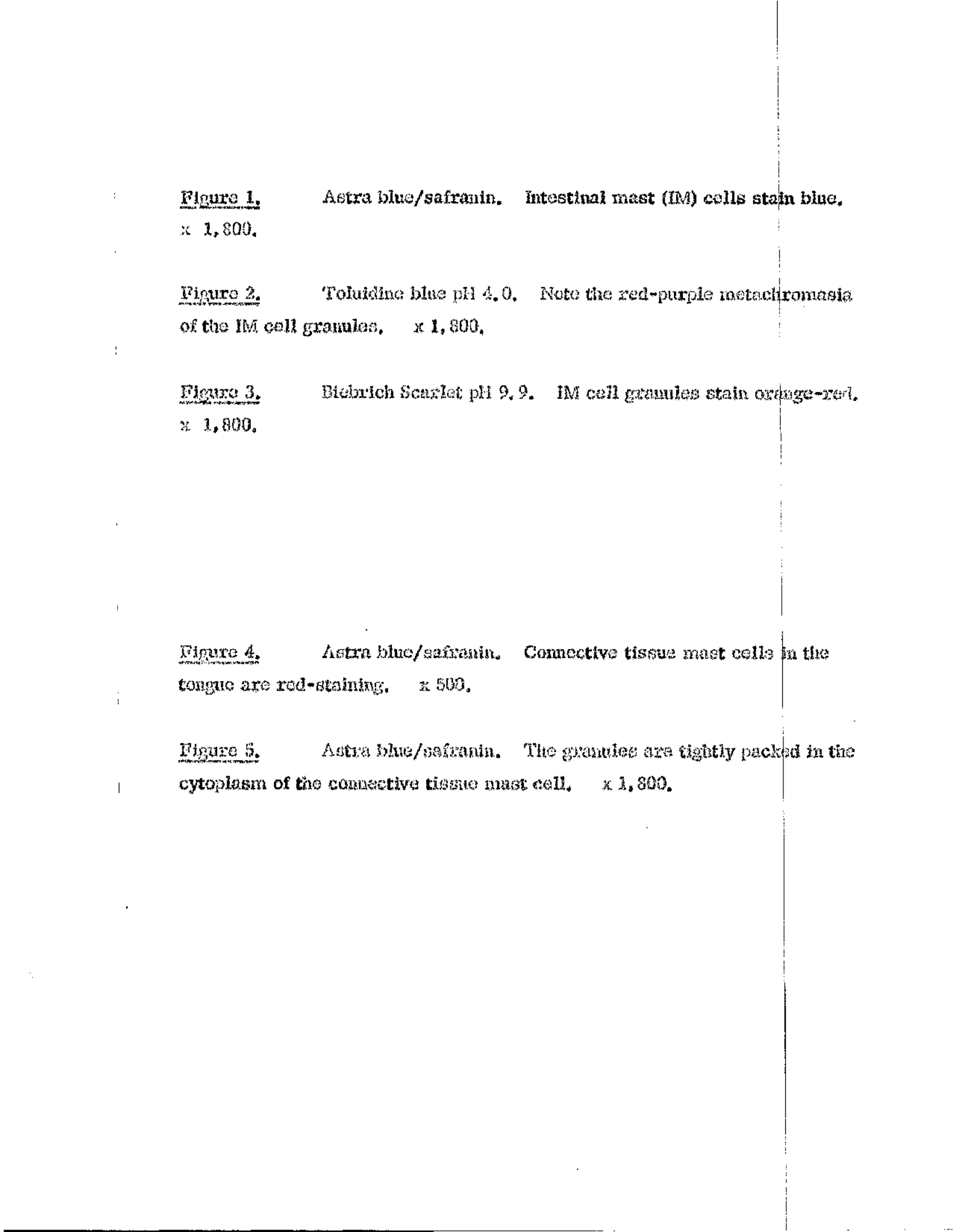


Figure 1. Astra blue/safranin. Intestinal mast (IM) cells stain blue. x 1,800.

Figure 2. Toluidine blue pH 4.0. Note the red-purple metachromasia of the IM cell granules. x 1,800.

Figure 3. Biebrich Scarlet pH 9.9. IM cell granules stain orange-red. x 1,800.

Figure 4. Astra blue/safranin. Connective tissue mast cells in the tongue are red-staining. x 500.

Figure 5. Astra blue/safranin. The granules are tightly packed in the cytoplasm of the connective tissue mast cell. x 1,800.

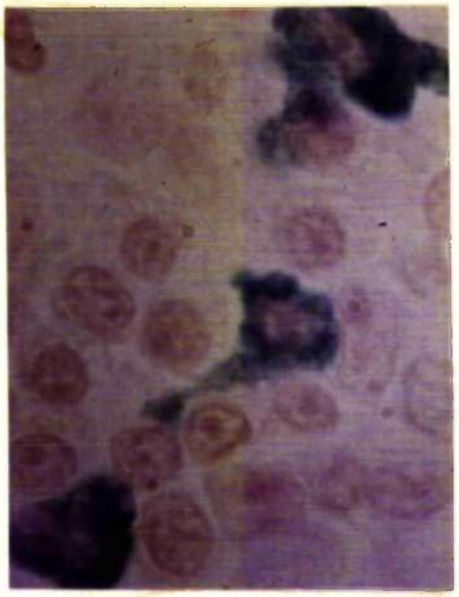


Figure 1.

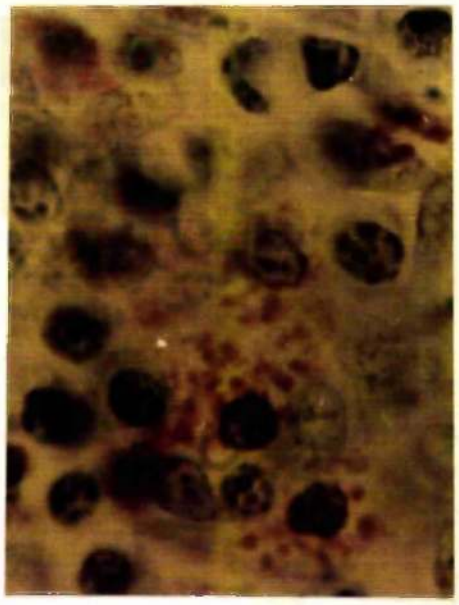


Figure 2.

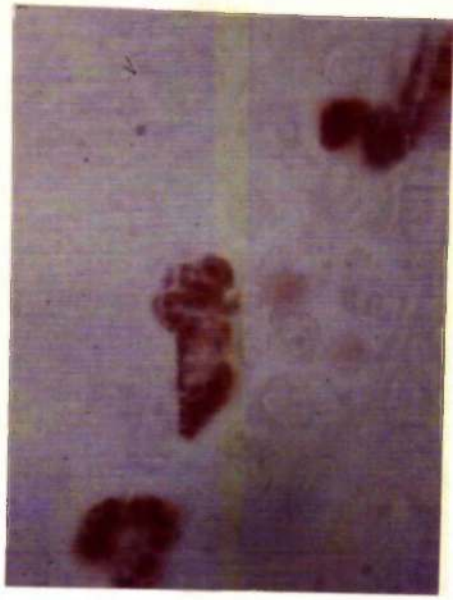


Figure 3.

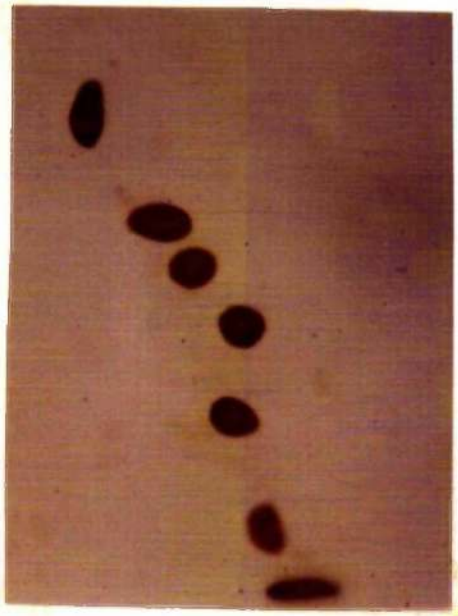


Figure 4.

Figure 5.

Figure 6. Falck reaction for monoamines. An enterochromaffin cell (E) towards the tip of the villus has a bright yellow fluorescence. There are many large non-specific fluorescent granules in the lamina propria. x 300.

Figure 7. Falck reaction for monoamines. Mast cells and enterochromaffin cells in the region of the gland crypts. A few of the basally situated mast cells (B) have a bright yellow fluorescence. Others fluoresce a weak green. x 300.

Figure 8. Falck reaction for monoamines. Connective tissue mast cells in the tongue have a bright yellow fluorescence. Green fluorescent adrenergic nerve fibres can also be distinguished. x 130.

Figure 9. Falck reaction for monoamines. Bright green fluorescence of mast cells in the villus of a rat treated with L-DOPA. The epithelial brush border has a non-specific orange fluorescence. x 300.

Figure 10a. Falck reaction for monoamines. The tissues were embedded in Araldite-Epon and the individual fluorescent granules of the intestinal mast cells are easily distinguished. x 1,800.

Figure 10b. The section immediately adjacent to 10a, but stained with Azure II - methylene blue - borax; the mast cell granules are deeply basophilic. x 1,800.

Figure 6.

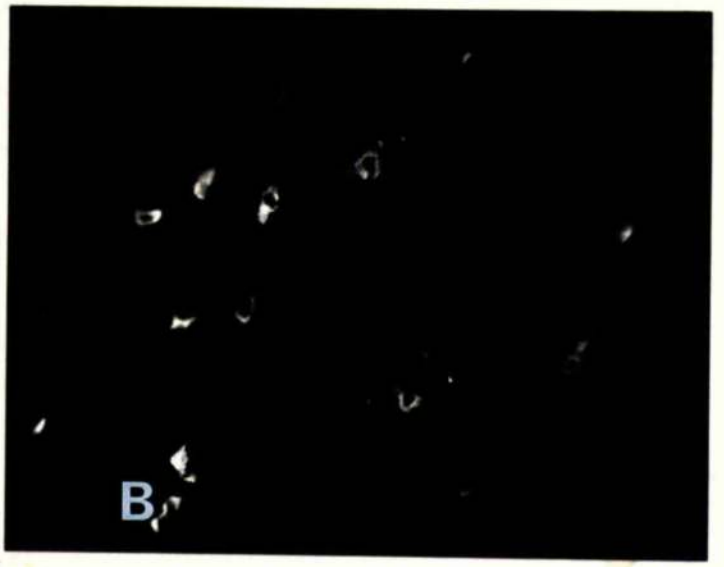


Figure 7

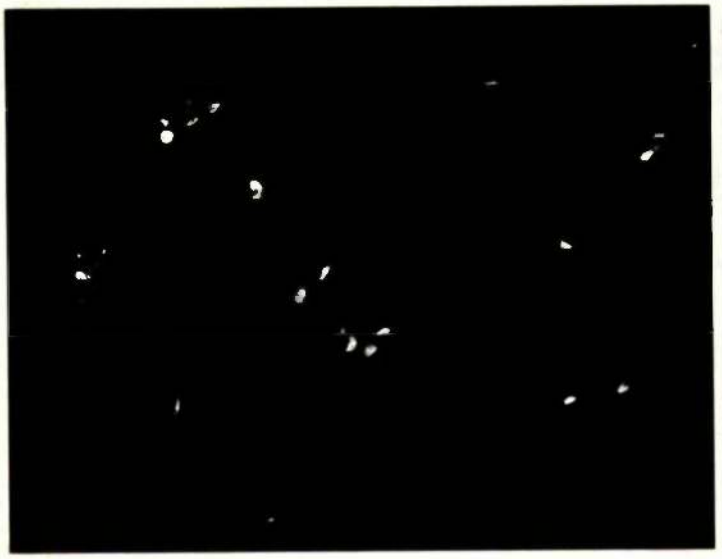


Figure 8.

Figure 9.

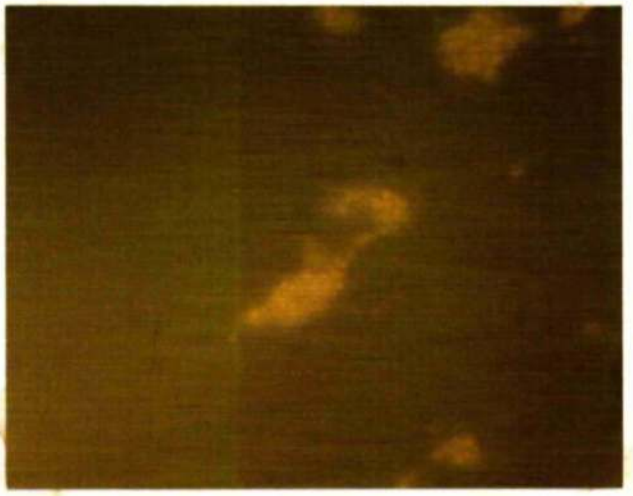


Figure 10a.

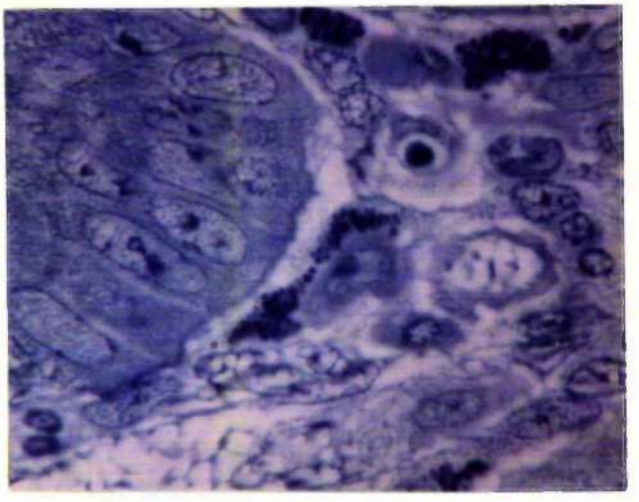


Figure 10b.

Figure 11. (Top Left). Light micrograph of a spindle-shaped intestinal mast cell. 1.5μ Araldite-Epon section stained with Azure II - methylene blue - borax. x 1,800.

Figure 12. (Top Right). A group of intestinal mast cells visualised by the techniques used in Figure 11. x 1,800.

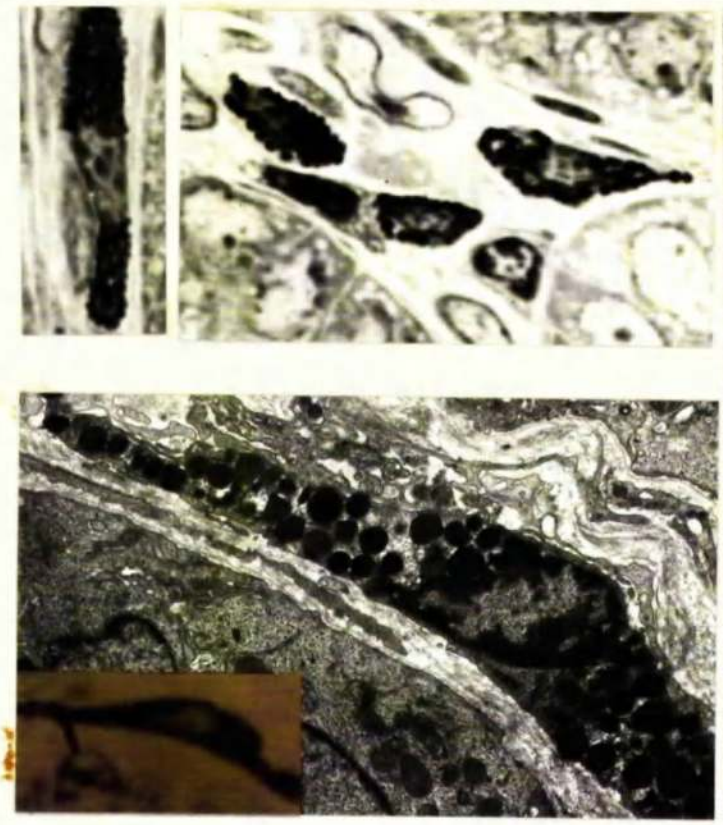
Figure 13. An electron micrograph of an intestinal mast cell lying close to the basement membrane of the gland crypt epithelium. x 5,400.
INSET shows the same cell located in a 1.5μ section stained with Azure II - methylene blue - borax. x 1,800.

Figure 14. Electron-micrograph of an intestinal mast cell; the granules are uniformly electron-dense. Small finger-like processes (arrows) protrude from the plasmalemma. x 11,250.
INSET. The same cell visualised by light microscopy (for methods, see Figure 11). x 1,800.

Figure 15. Light-micrograph of a mast cell within gland crypt epithelium. 1.5μ Araldite-Epon section stained with Azure II - methylene blue - borax. x 1,800.

Figure 16. Electron-micrograph of an intestinal mast cell within gland crypt epithelium. The matrices of some of the granules have escaped into the cytoplasm (arrows) but others appear to be intact (arrowheads). x 13,120.

NOTE All resin-embedded tissues, unless stated to the contrary, were fixed in 4% glutaraldehyde and post fixed in osmium tetroxide and uranyl acetate. They were embedded in Araldite-Epon.



Figures 11 - 13

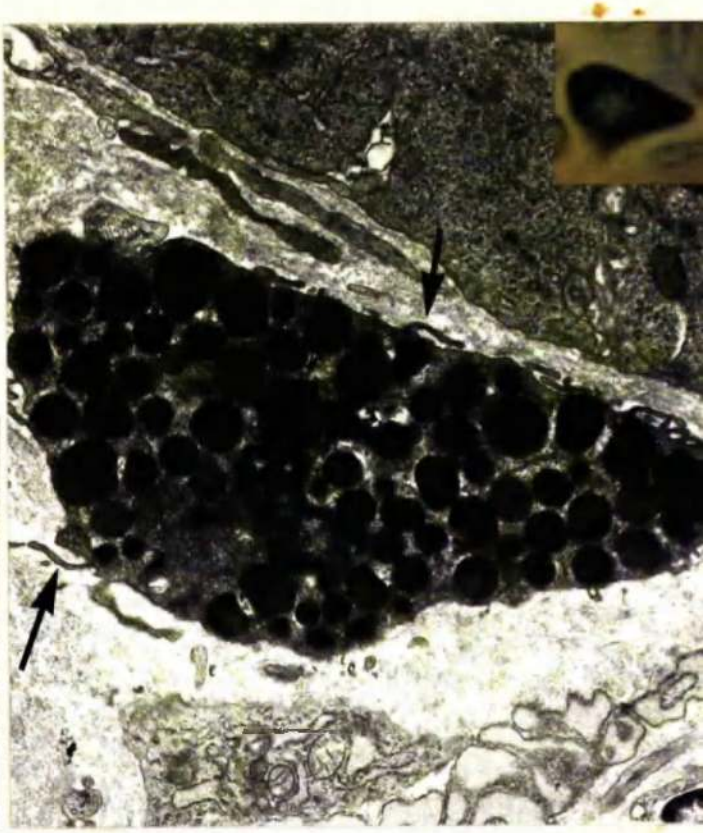


Figure 14.

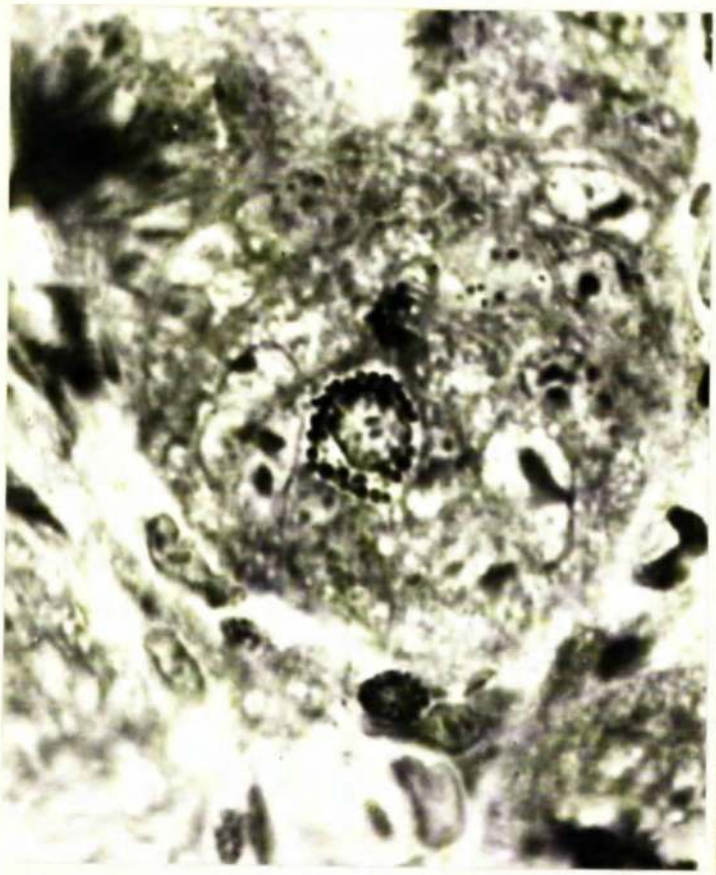


Figure 15

Figure 16

Figure 17. Intestinal mast cells containing many granules of uniform electron-density. The intergranular cytoplasm has only a few strands of RSER (arrow) and an occasional ribosomal aggregate (arrowhead). Several mitochondria are also present. x 11,250.

Figure 18. The cytoplasm of this IM cell is sparsely populated with granules. The Golgi complex (G) is well developed. x 15,000.

Figure 19. IM cell with dense intergranular cytoplasm. The cisternae of RSER are dilated (arrowhead) and small cytoplasmic peduncles (arrows) protrude into the dilated perinuclear cisternum. x 17,500.

Figure 20. IM cells (IM) lying closely apposed to fibroblasts (F). Both IM cells have relatively dense intergranular cytoplasm and dilated perinuclear cisternae. x 11,250.

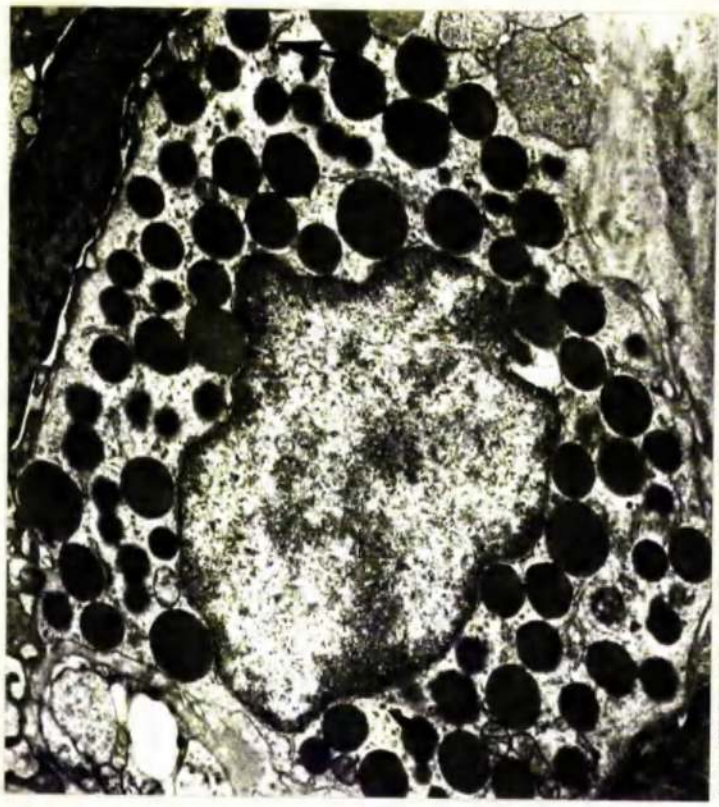


Figure 17

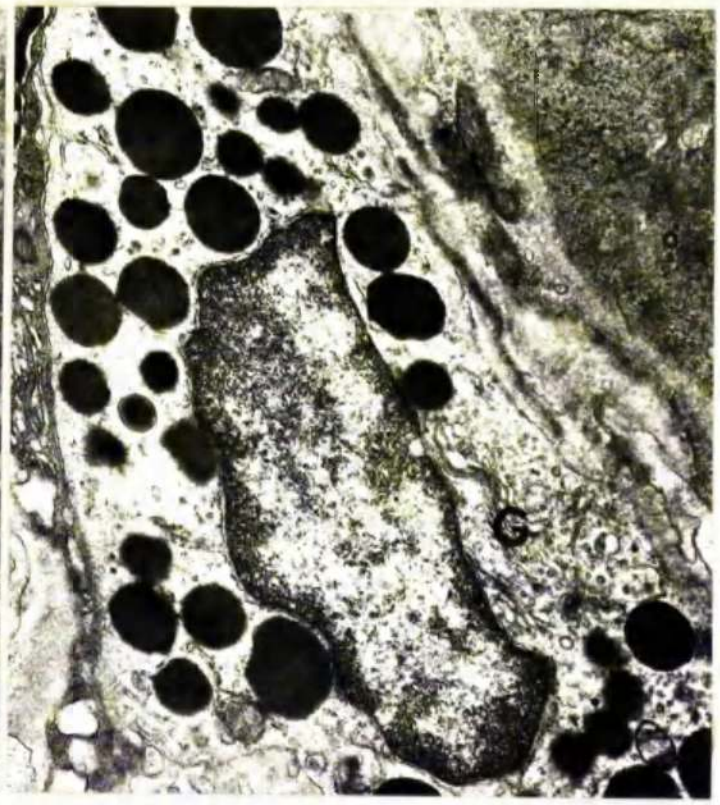


Figure 18.

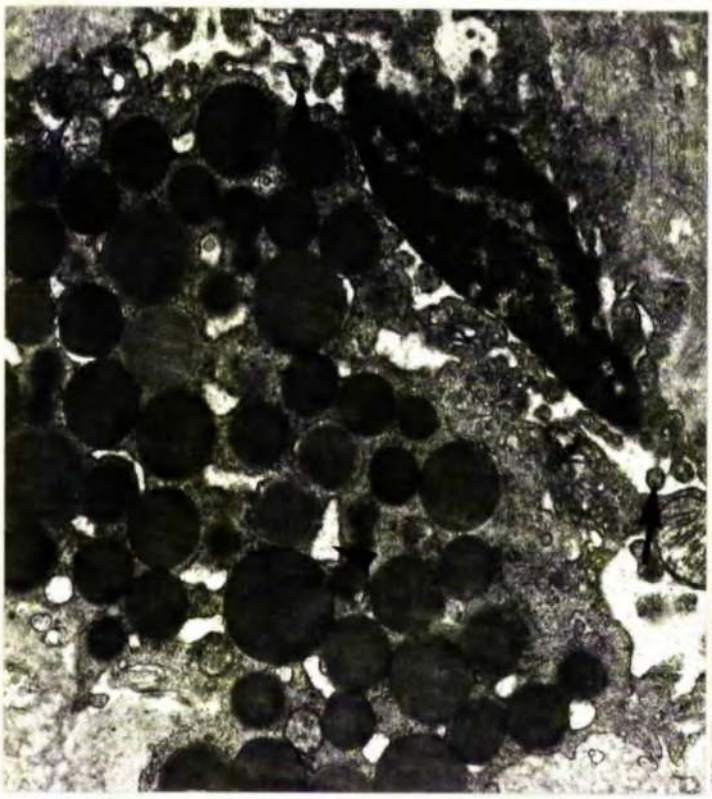


Figure 19.

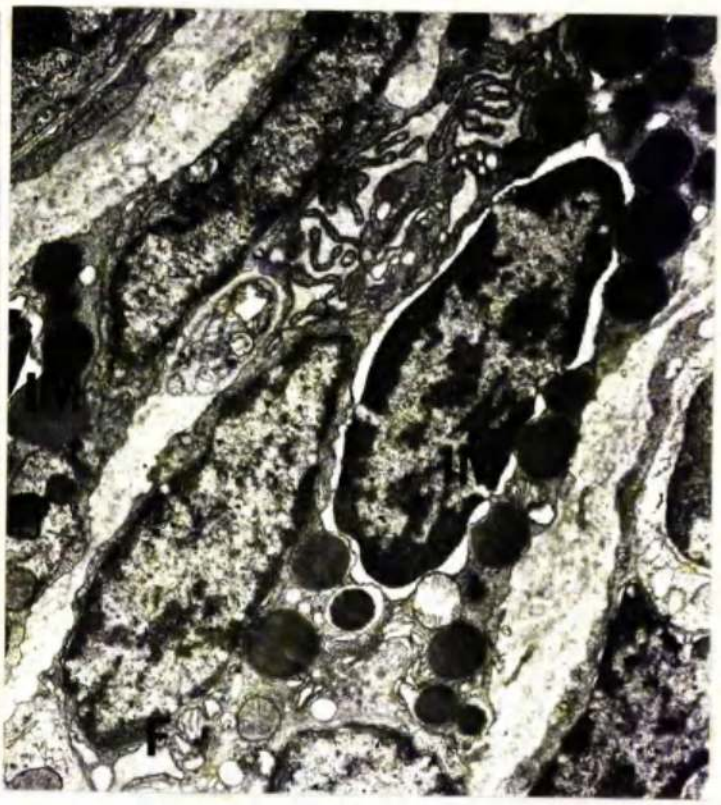


Figure 20.

Figure 21. IM cell closely related to a fibroblast (F) and to the basement membrane (BM) of a capillary. The matrices of some of the granules (arrows) are granular and are less electron-dense than usual. (G), Golgi complex. x 13,750.

Figure 22. The Golgi complex of an IM cell. Golgi cisternae and vesicles surround the centrioles (C). Trilaminar structures (arrow) are seen in the matrix of a granule. There was no post-fixation with uranyl acetate. Embedded in Araldite. x 40,000.

Figure 23. IM cell lying close beside a capillary. There are myelin-like configurations (arrow) within one of the granules. The Golgi complex is moderately well developed. x 22,500.

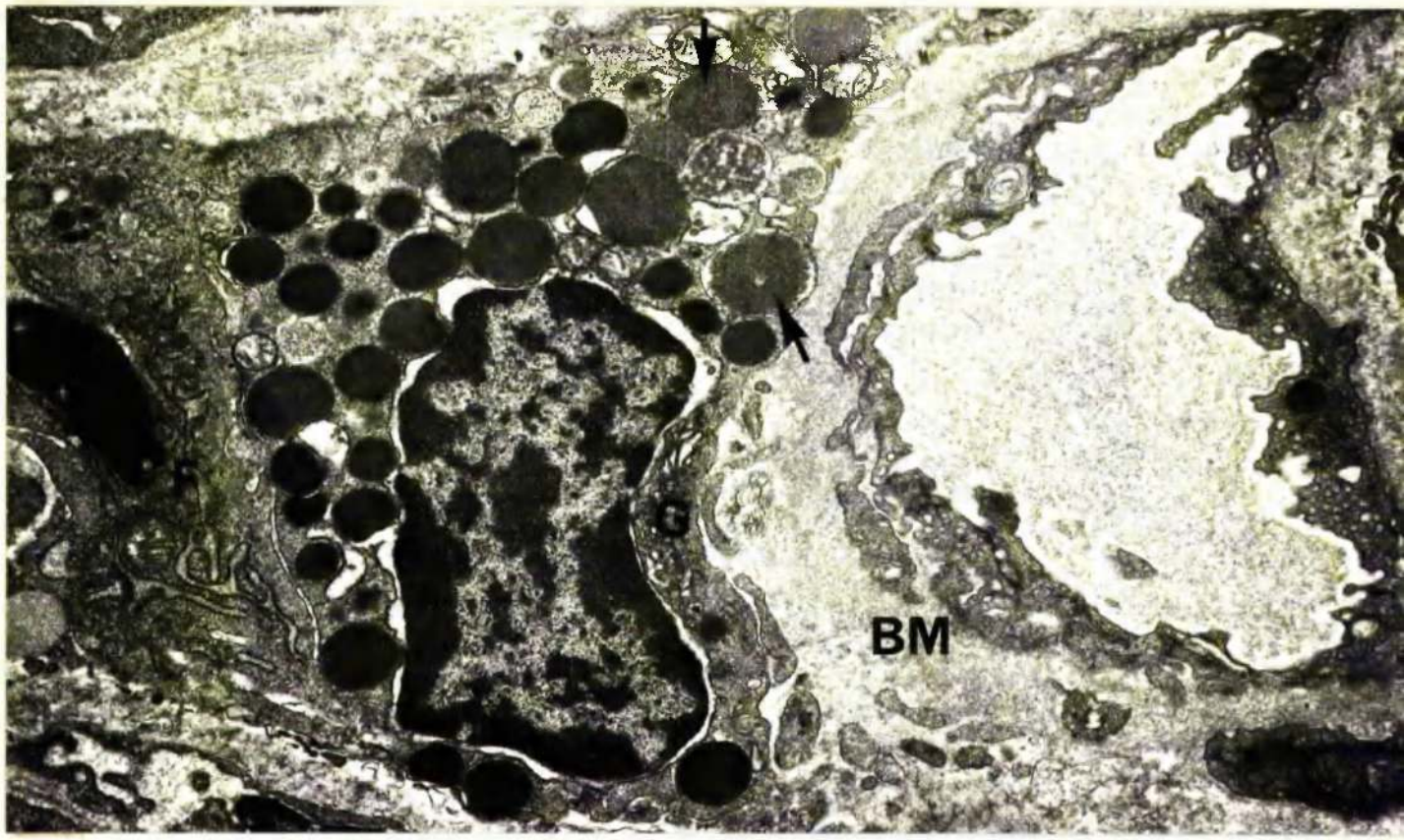


Figure 21.

Figure 22.

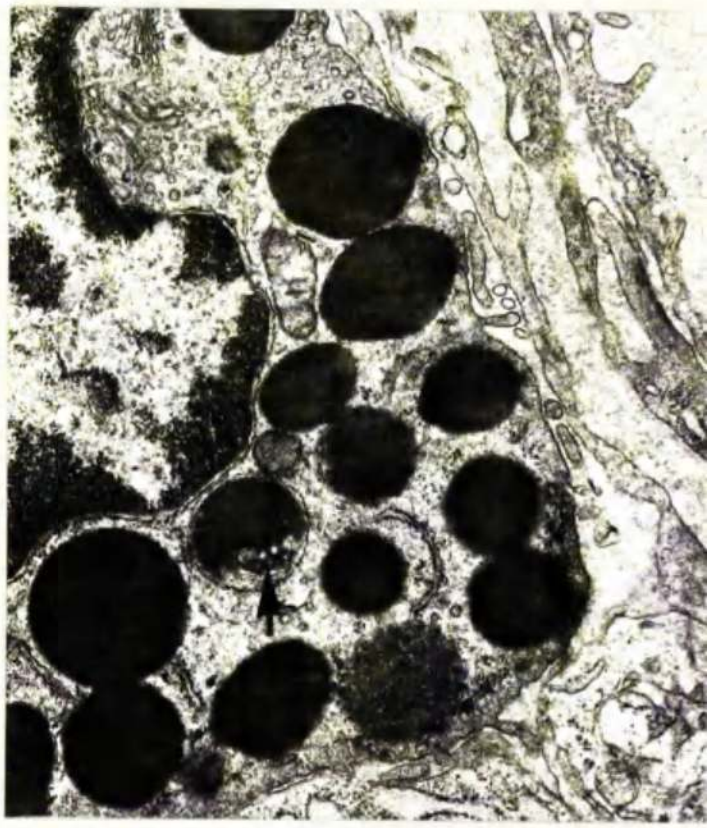


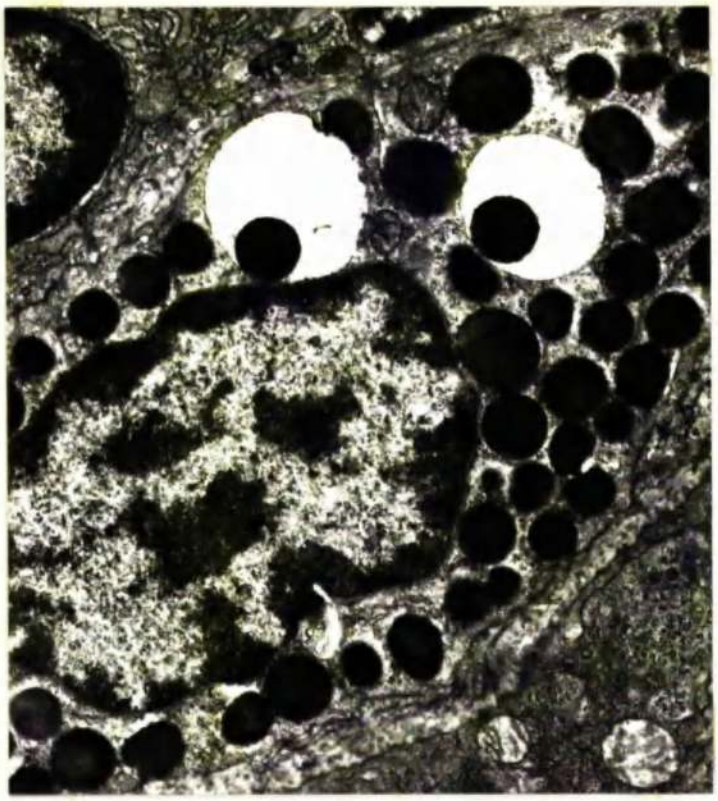
Figure 23.

Figure 24. The delimiting membranes of two of the IM cell granules are widely separated from the matrices. The remainder show uniform fixation. x 13,100.

Figure 25. IM cell adjacent to a plasma cell (P). The cytoplasm of the mast cell is vacuolated as if the matrices of several granules had been eluted. In one or two granules (arrow) the matrices have a granular structure. x 13,100.

Figure 26. IM cell with dilated and branching cisternae of RSER (R). Peduncles of cytoplasm (arrows) extend into these and into the perinuclear cisternum. This cell is either binucleate (N) or has formed a syncytium with another cell containing a large Golgi complex (G). x 17,500.

Figure 27. The cytoplasm of this IM cell is sparsely populated with granules. A progranule (p) lies within an elaborate Golgi complex. Strands of RSER and free and aggregated ribosomes are scattered in the cytoplasm. x 17,000.



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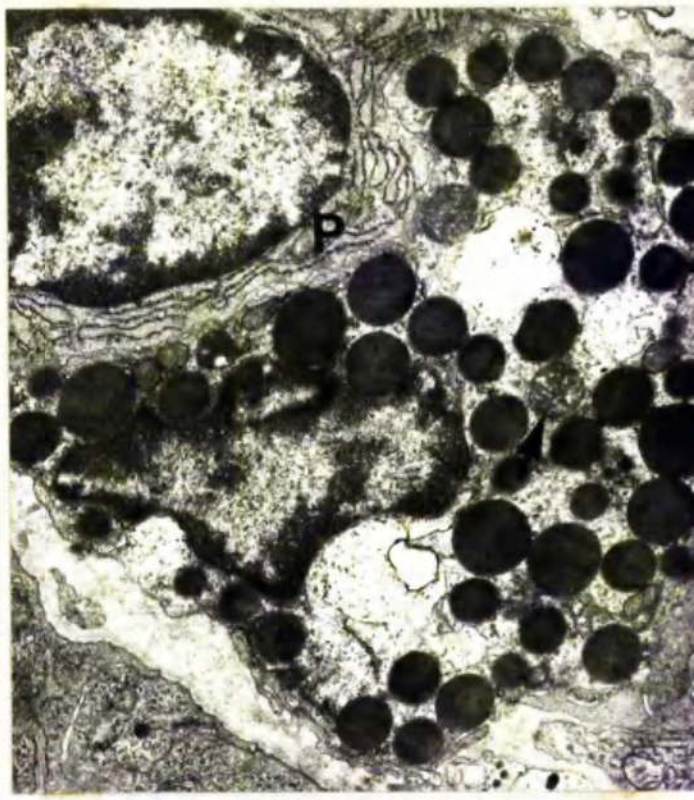

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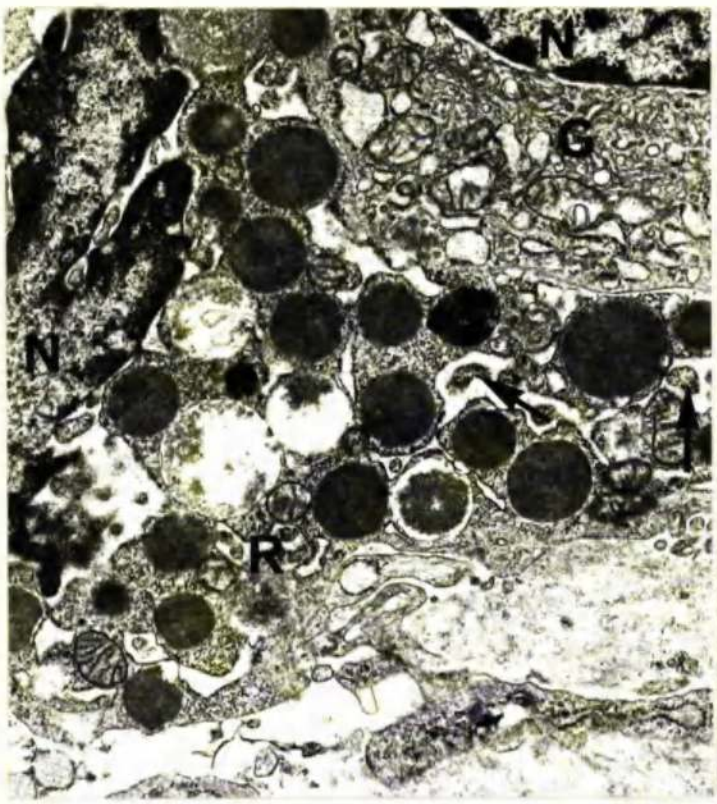


Figure 26.

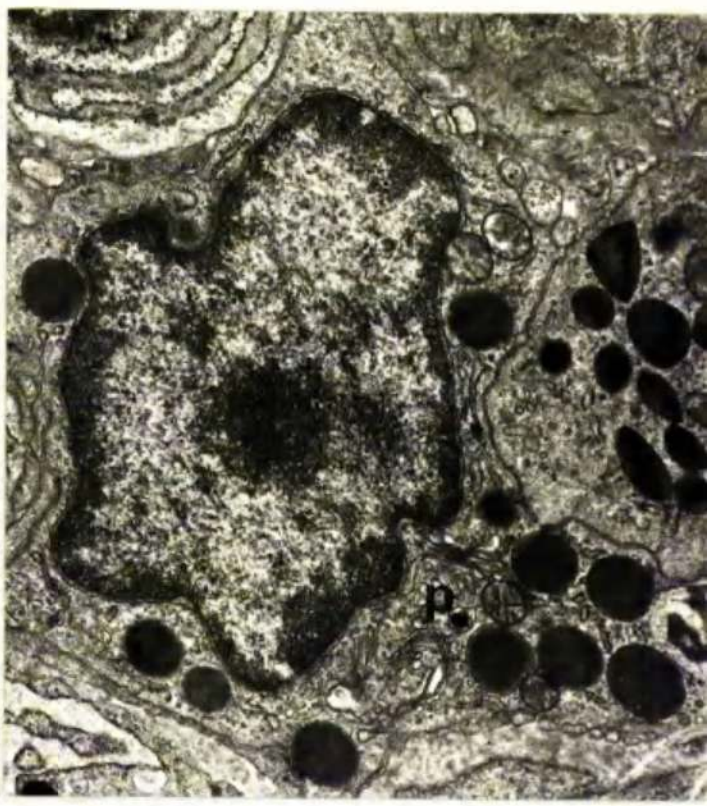


Figure 27.

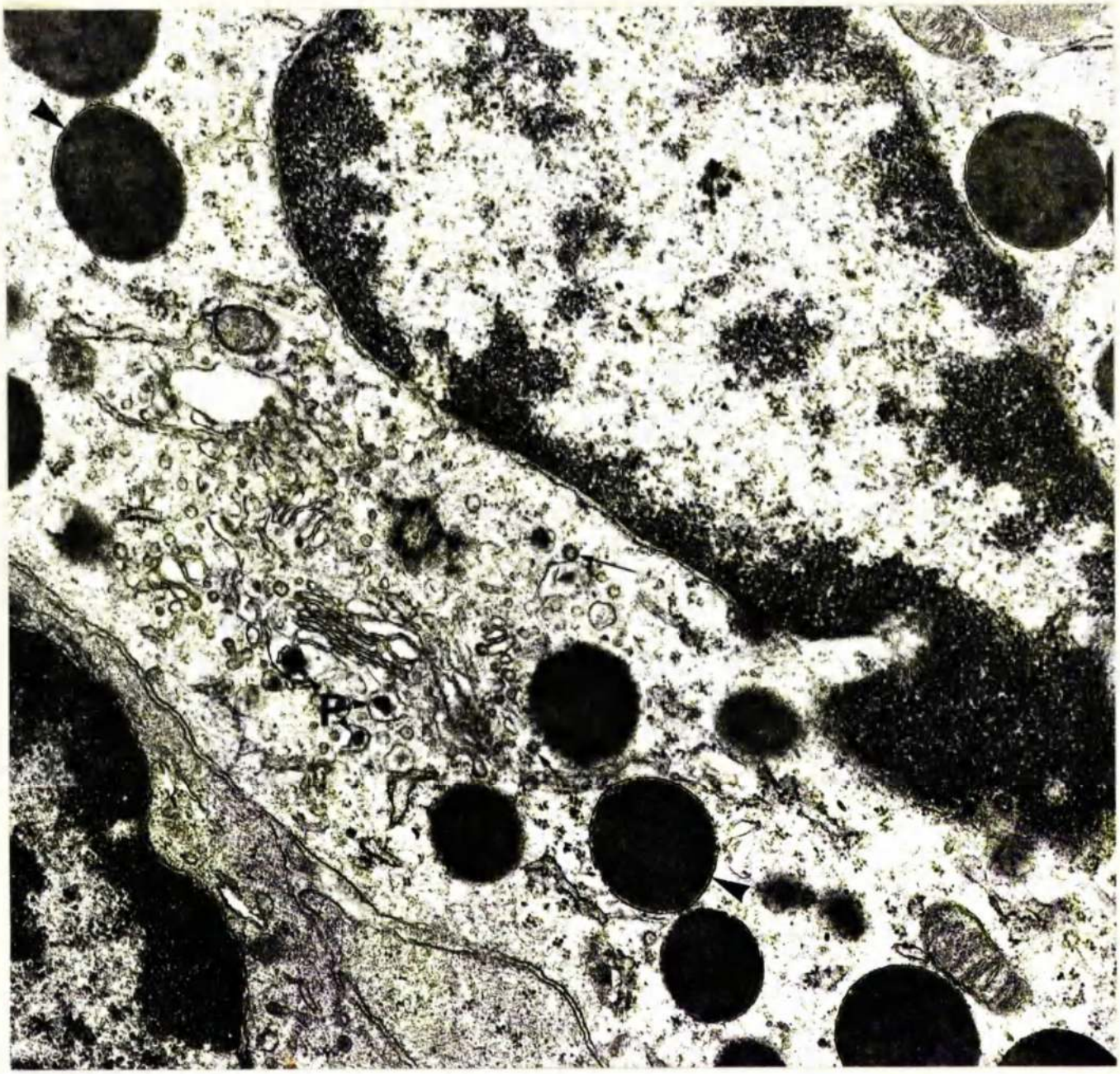


Figure 28. Intestinal mast cell. The Golgi cisternae are distributed around the centriole and numerous small uncoated vesicles are scattered amongst them. Several coated vesicles (arrows) and progranules (P) are present within the Golgi region. Unit membranes (arrowheads) are visualised around several granules. x 30,000.

Figure 29. Connective tissue mast cells (MC) in rat tongue. Embedded in Araldite. Post-fixation with uranyl acetate was omitted. x 8,000.

Figure 30. Eosinophil; rat intestinal lamina propria. The granules are oval and many have dense central crystalline bars. x 12,700.

Figure 31. Neutrophil within the lumen of a venule of a Nippostrongylus-infected rat. Two lobes of the nucleus are visible in the plane of section. Note the heterogeneity and small size of the granules. x 13,750.

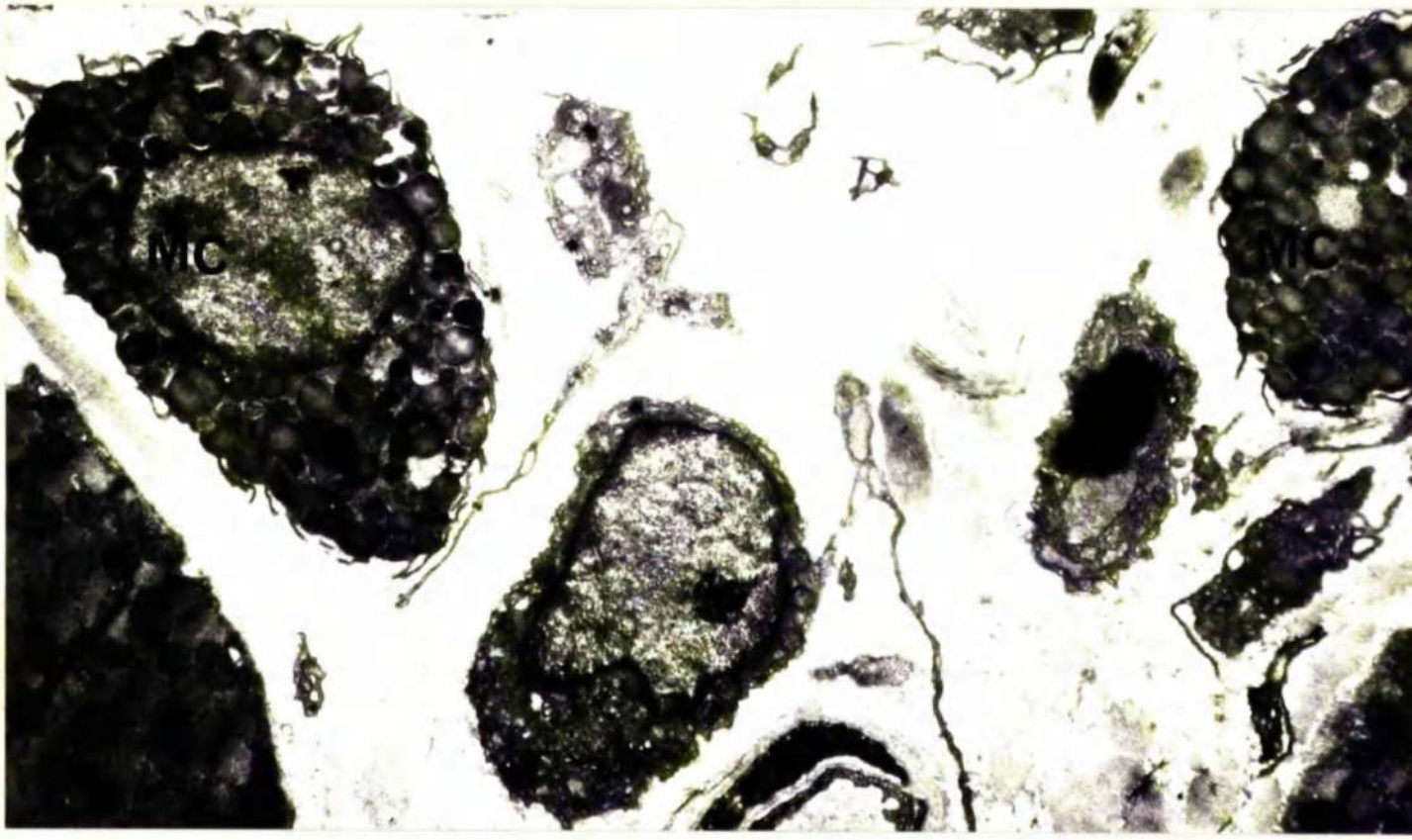


Figure 29.

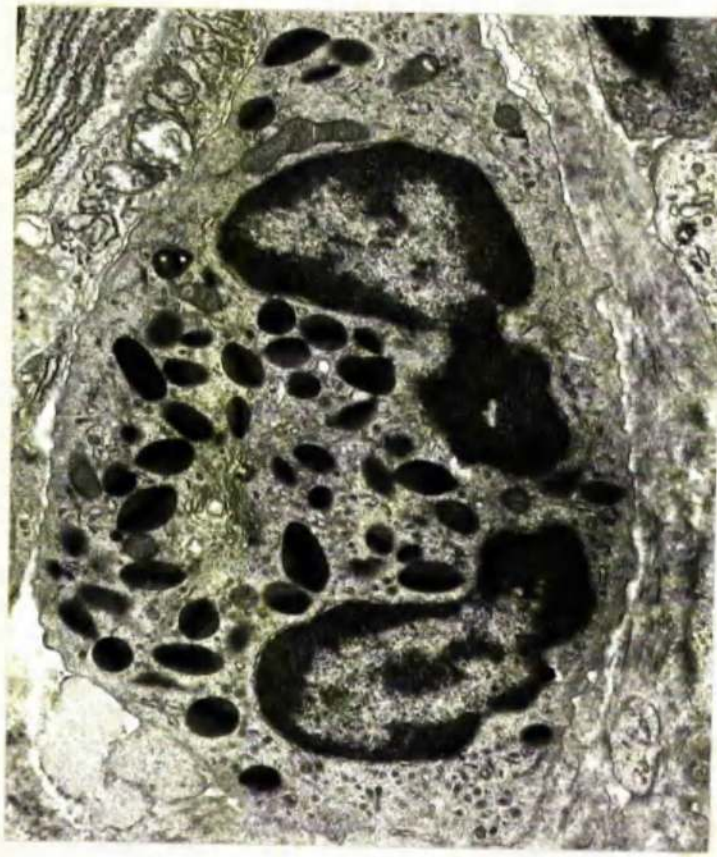


Figure 30.

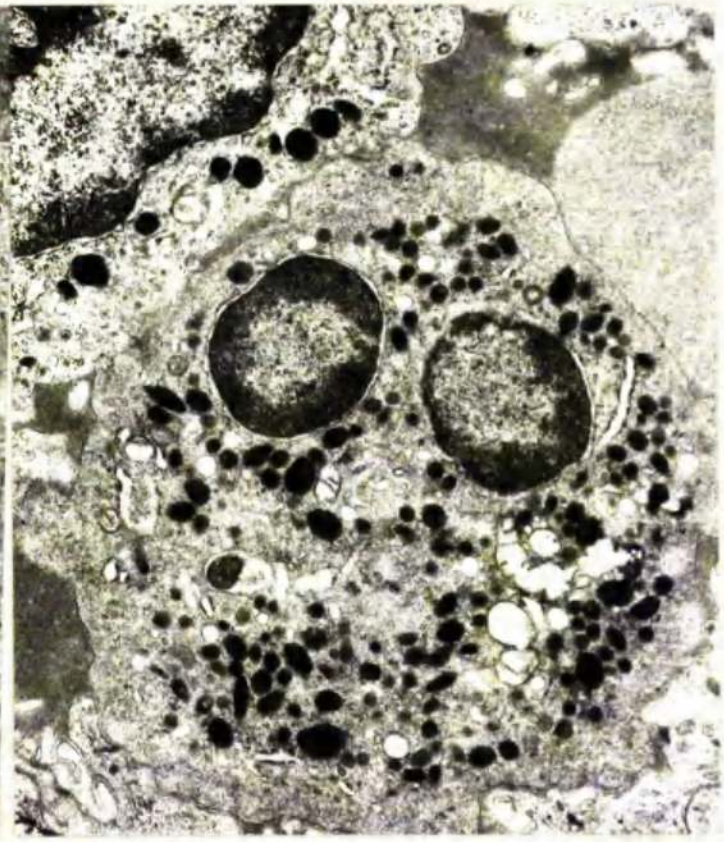


Figure 31.

Figure 32. Light micrograph of IM cells in a normal untreated rat. Astra blue/safranin. x 700.

Figure 33. Mast cells within the villus tip of a 48/80 treated rat (Group I, day 1). Blue-staining granule fragments (arrows) are scattered in the lamina propria and several inclusions which stain brightly with safranin (arrow-head) are also evident. Astra blue/safranin. x 700.

Figure 34. Disrupted connective tissue mast cells in the tongue of a treated rat (Group I, day 1). Astra blue/safranin. x 700.

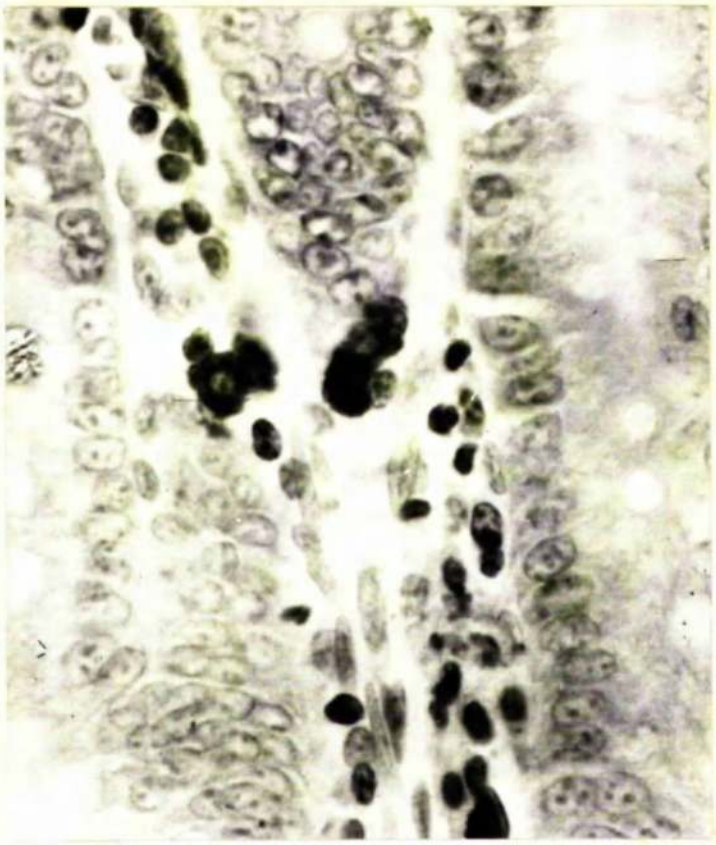


Figure 32.

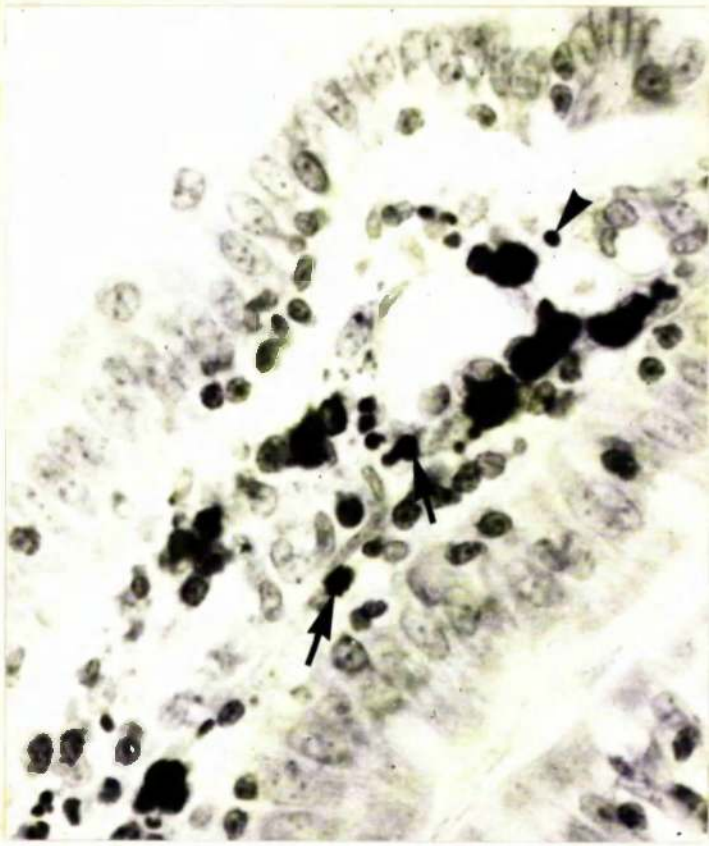


Figure 33.

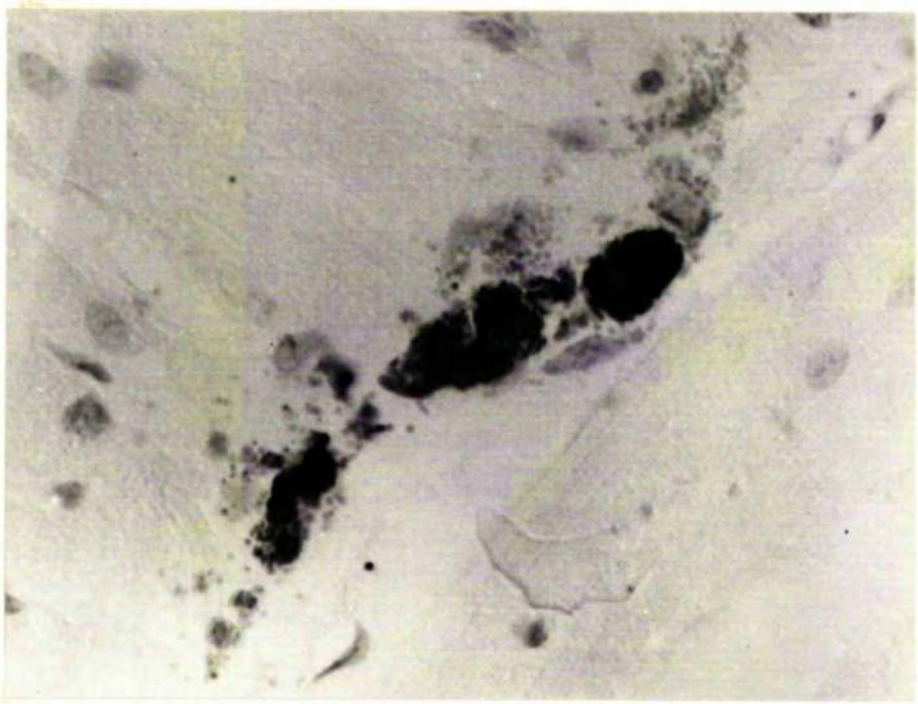


Figure 34.

Figure 35. Intestine of a 48/80 treated rat (Group I, day 1). A mast cell (M) is lying in the basal part of the gland crypt epithelium. Deep staining inclusions and a vacuolated mast cell (arrow) are present in the lamina propria. 1.5μ Araldite-Epon section. Azure II - methylene blue - borax. x 1,000.

Figure 36. Intestine of a 48/80 treated rat (Group I, day 1). Numerous deep staining inclusions are scattered in the lamina propria. Preparation as above. x 700.

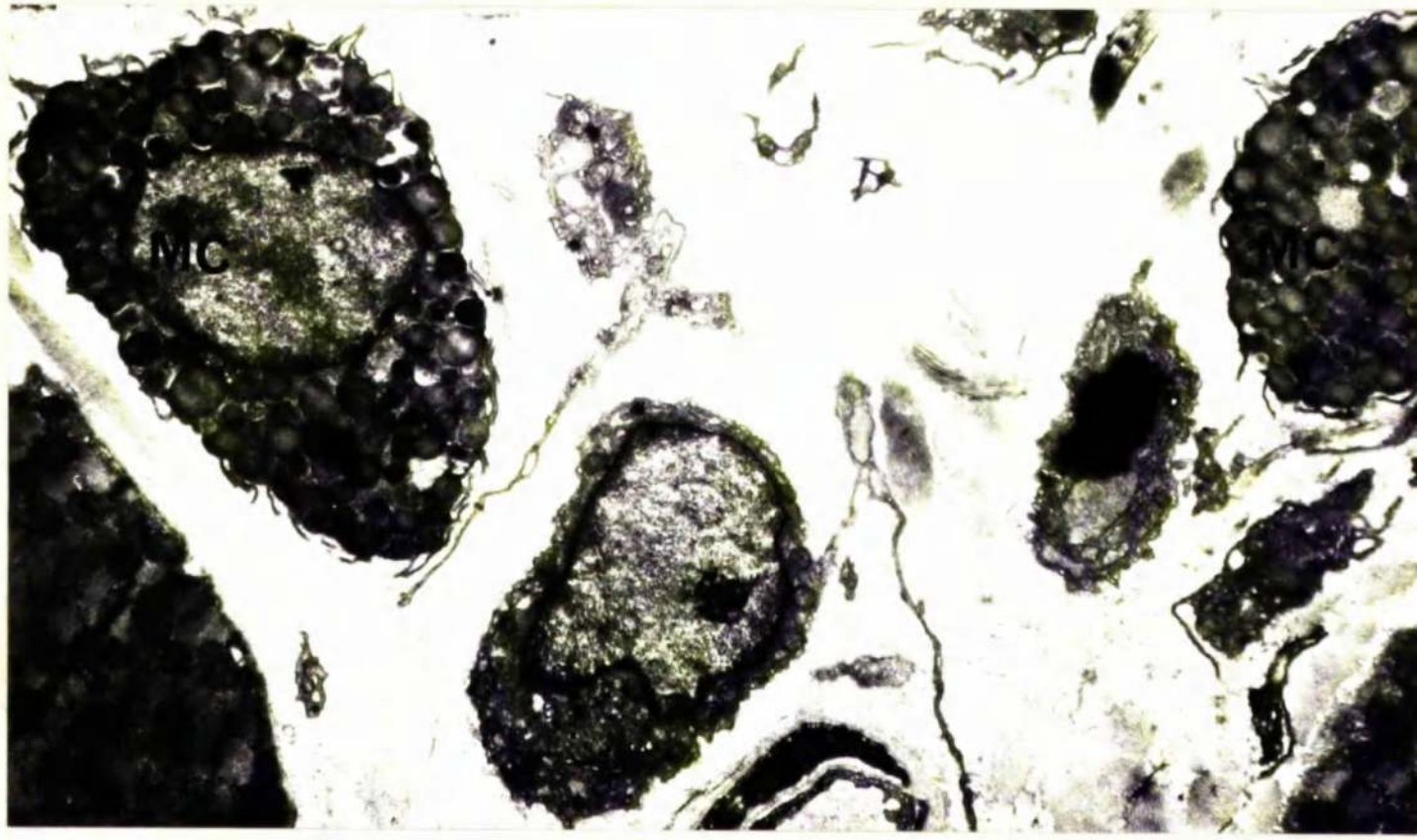
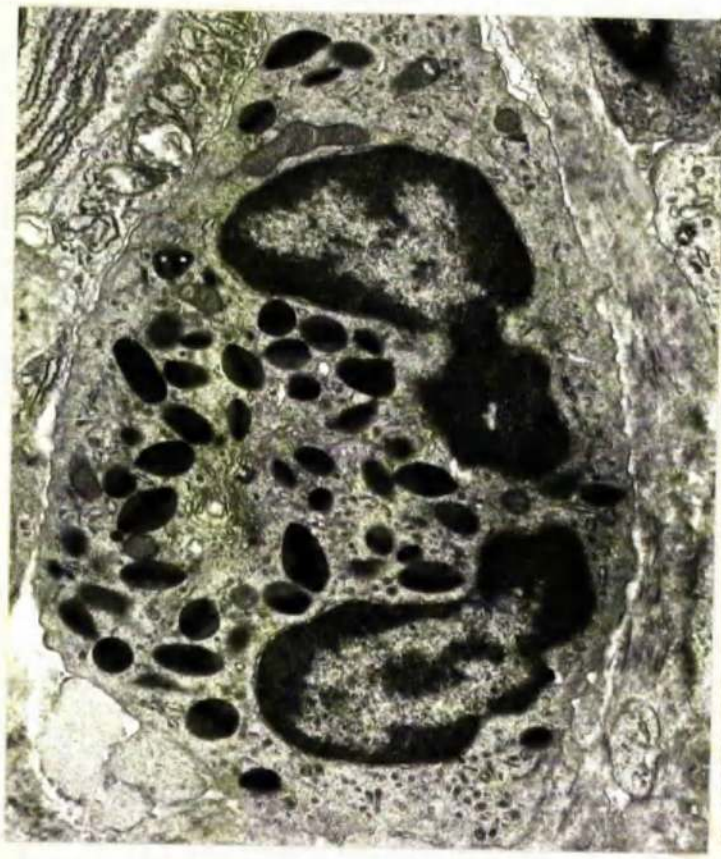


Figure 29.



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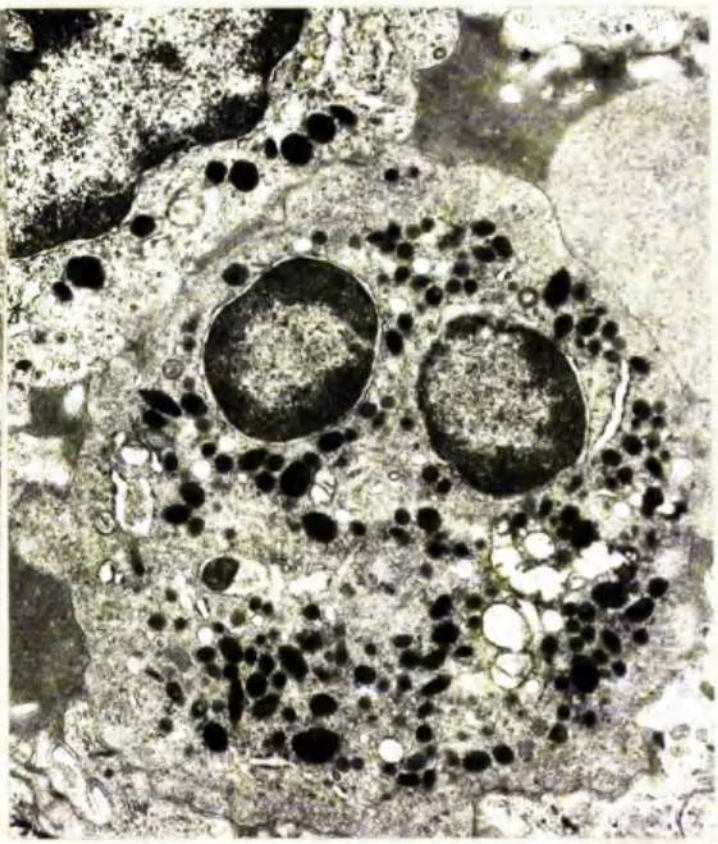

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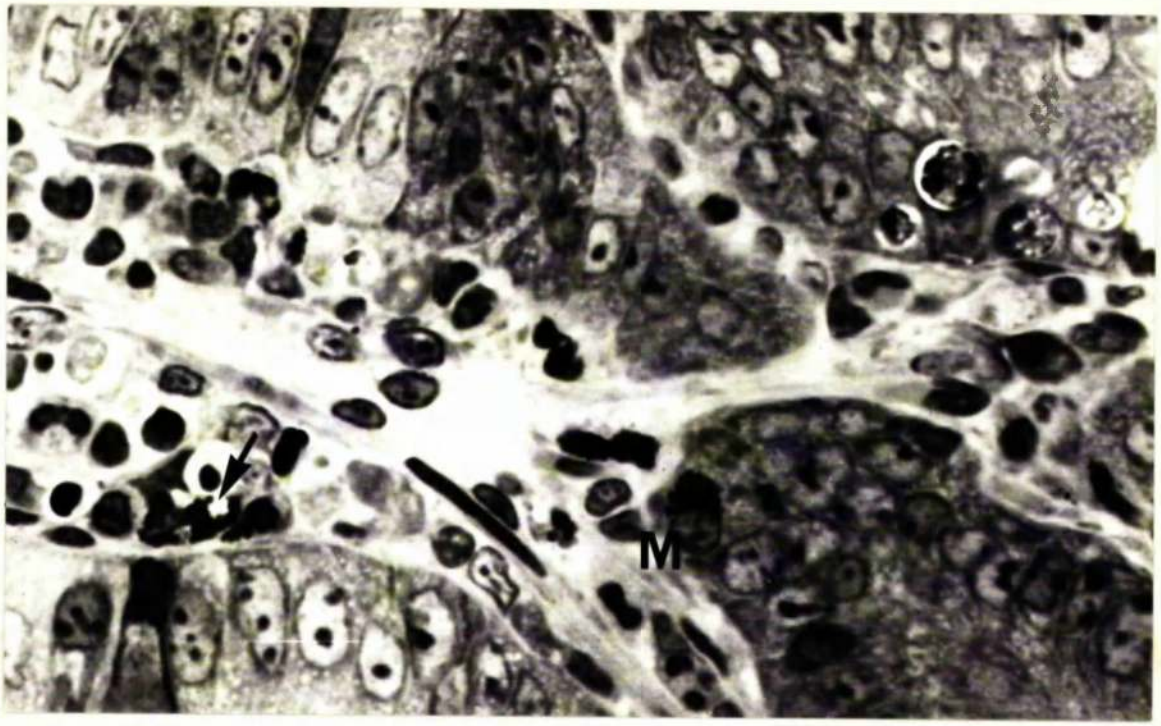

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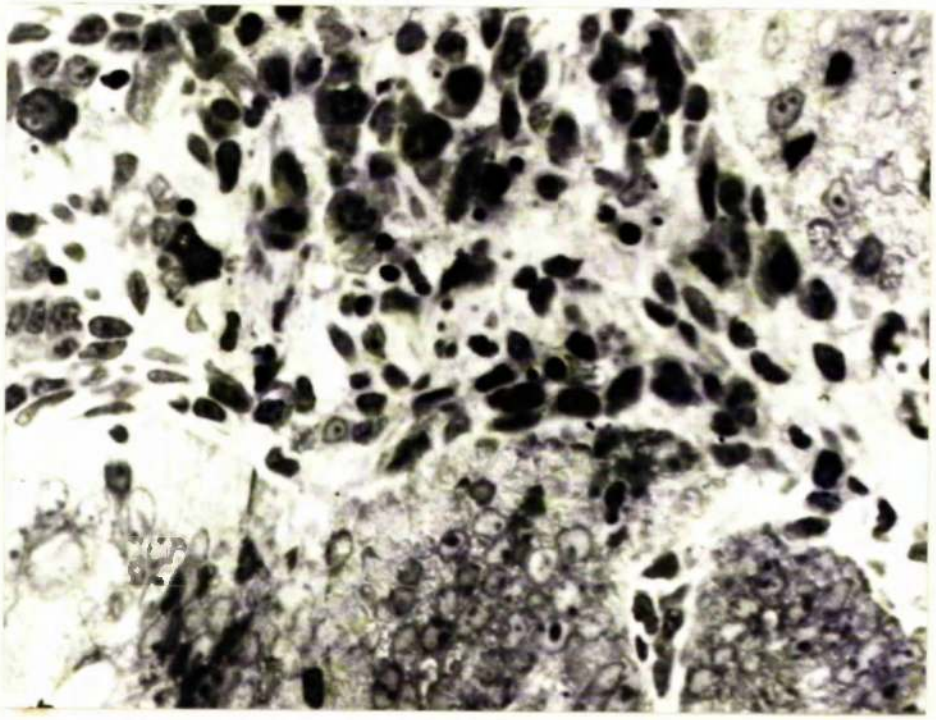


Figure 36.

Figure 37. Electron-micrograph of an IM cell in a 48/80 treated rat (Group I, day 1). The matrices of some granules have been lost, but electron-dense central cores remain in others and are surrounded by rims of less dense and granular matrix (arrows). x 17,500.

Figure 38. IM cell in a rat treated with 48/80 (Group I, day 1). The cytoplasm is extensively vacuolated and only a few unaltered granules remain. x 13,100.

Figure 39. Mast cell remnants within the phagosome of a macrophage. Intestinal lamina propria of a rat treated with 48/80 (Group I, day 1). x 8,750.

Figure 40. A disrupted eosinophil is identified by the dense crystalline bars of the granules. Intestinal lamina propria of a rat treated with 48/80 (Group I, day 1). x 17,500.

Figure 37.

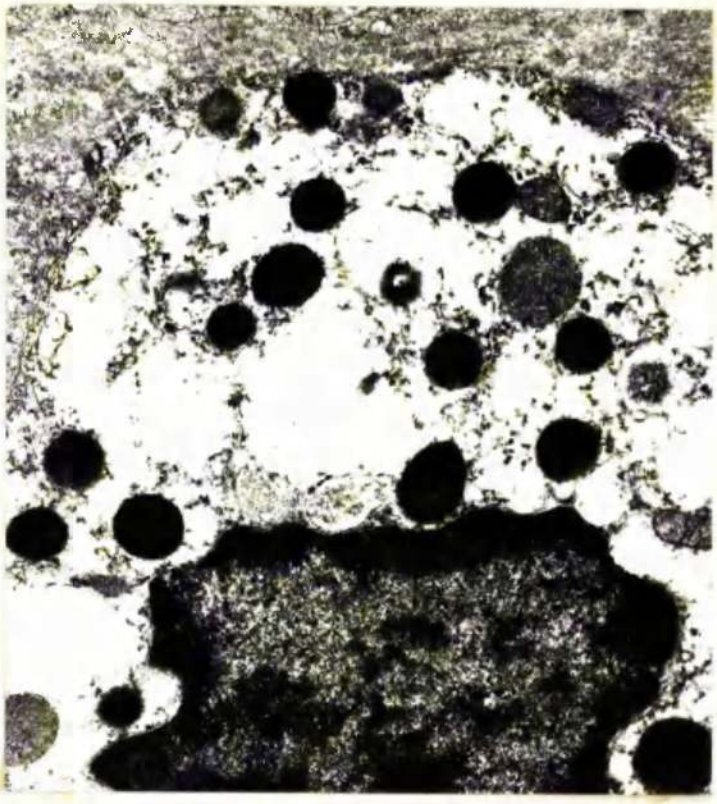


Figure 38.

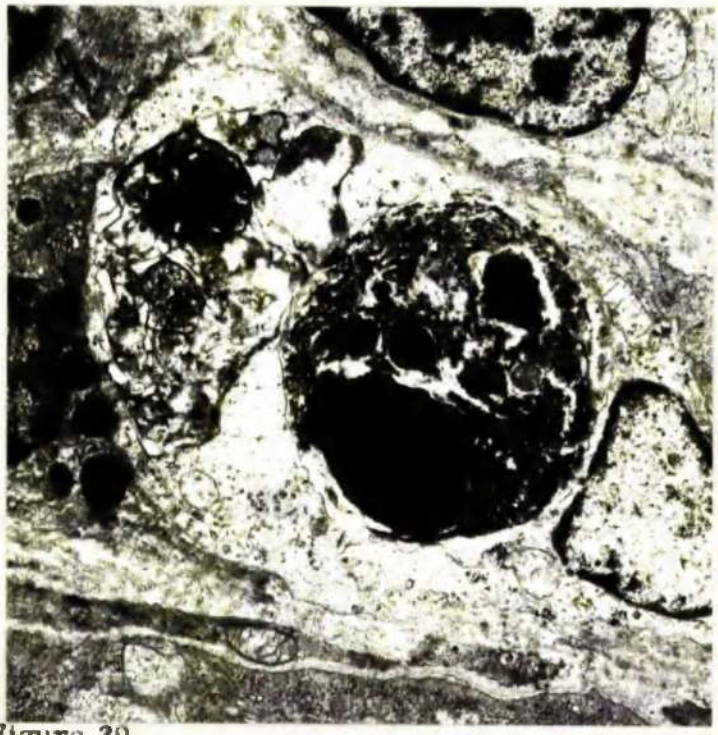


Figure.39.

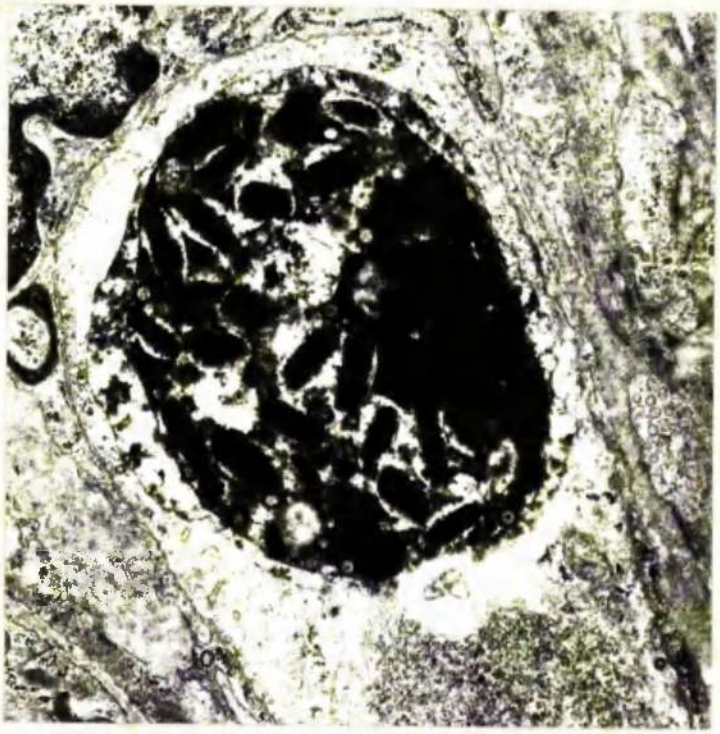


Figure 40.

Figure 41. Intestinal mucosa of a normal rat. Mast cells are found at all levels in the lamina propria. Astra blue/safranin x 150.

Figure 42. Intestinal mucosa of a rat nineteen days after infection with N. brasiliensis. The number of IM cells in the lamina propria is markedly increased and GL cells are abundant in the epithelium of the gland crypts. Astra blue/safranin. x 150.

Figure 43 (a) GL within the epithelium of a gland crypt. Day 19 of infection. 1.5μ Araldite section. Azure II - methylene blue - borax. x 1,500.

(b) A vacuolated GL with few deep-staining granules located in gland crypt epithelium. Nineteenth day after infection. 1.5μ Araldite section. Azure II - methylene blue - borax. x 1,500.

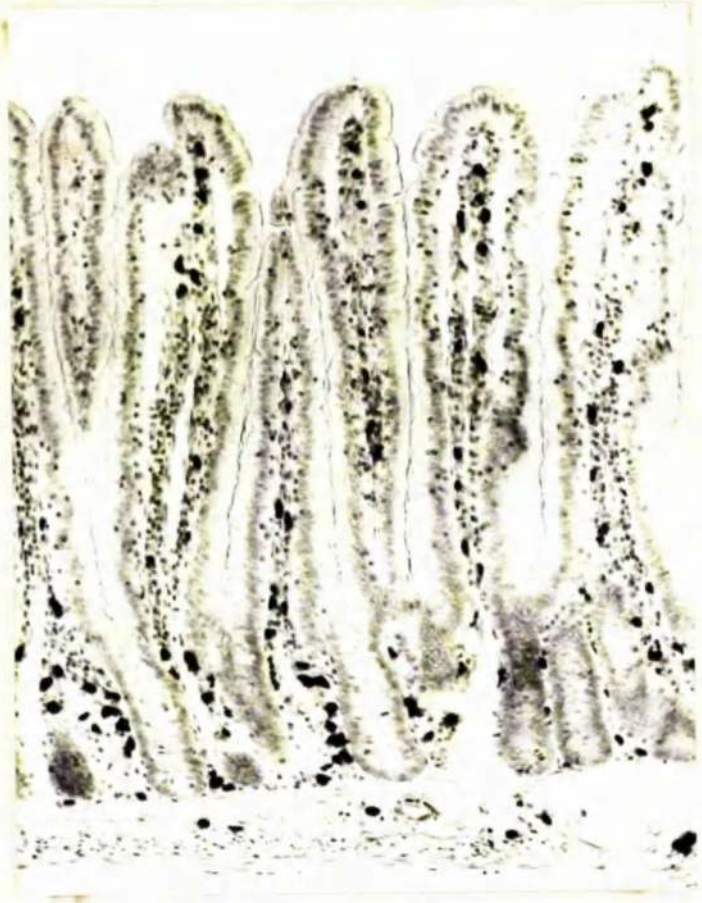


Figure 41.

Figure 42.

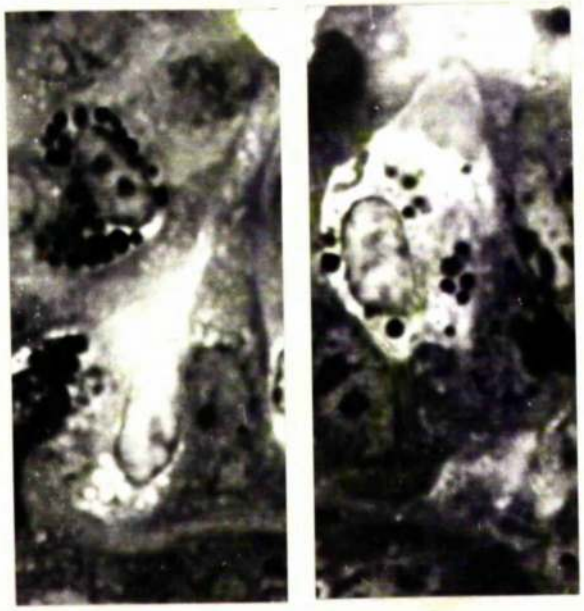


Figure 43 (a) (b)

Figure 44. IM cell in parasitised rat (nineteenth day of infection). There are paracrystalline structures (arrows) within several of the granules. The Golgi complex is well developed. x 13,100.

Figure 45. Plasma cell with markedly dilated cisternae of RSER (Russell bodies). Note the rough-surfaced membranes surrounding the cisternae. x 13,100.

Figure 46. GL situated basally in the gland crypt epithelium of a parasitised rat. Most of the granules have homogeneous electron-dense matrices and are indistinguishable from IM cell granules. x 8,750.

Figure 47. GL within gland crypt epithelium and located near to the lumen. The Golgi complex is well developed and there are several strands of RSER in the cytoplasm. Parasitised rat; nineteenth day of infection. x 8,750.

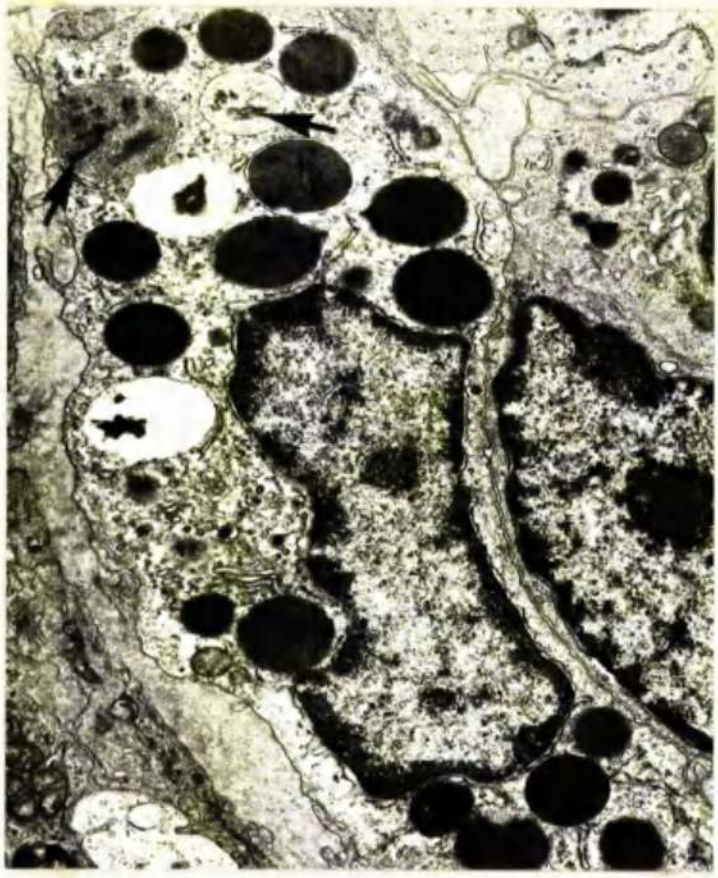


Figure 44.

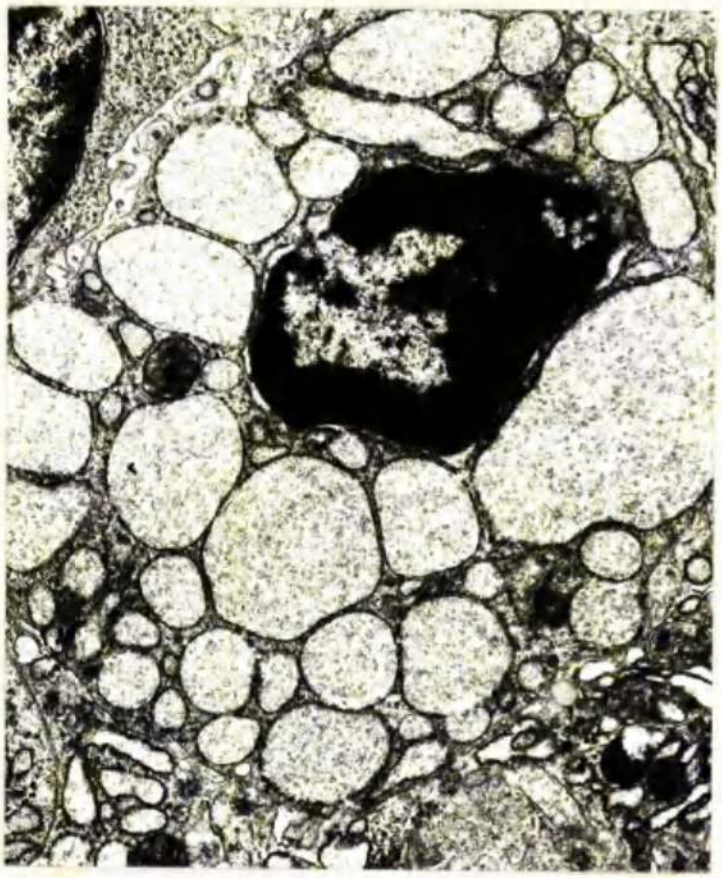


Figure 45.

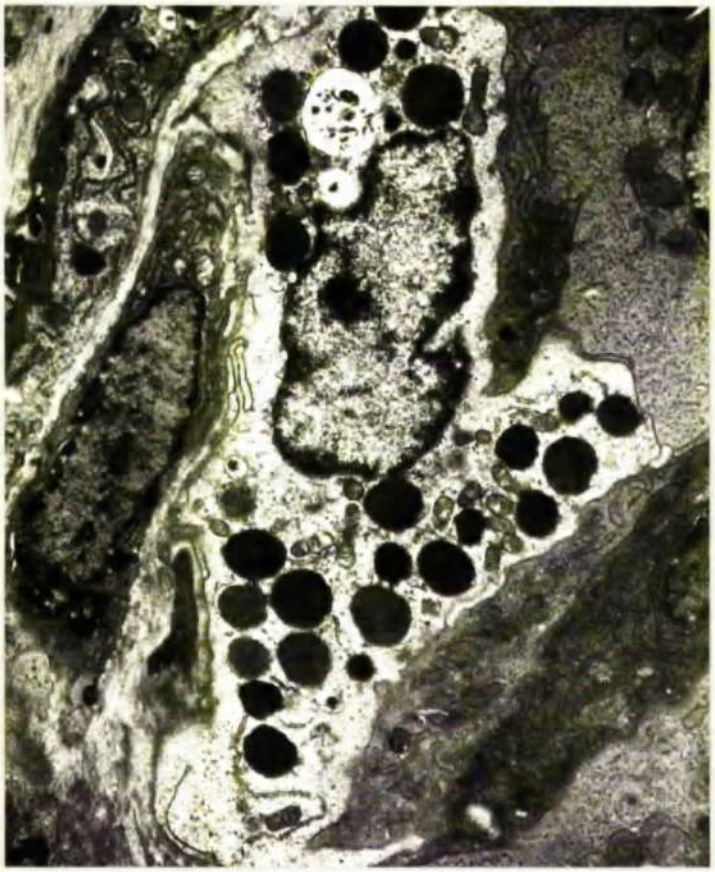


Figure 46.

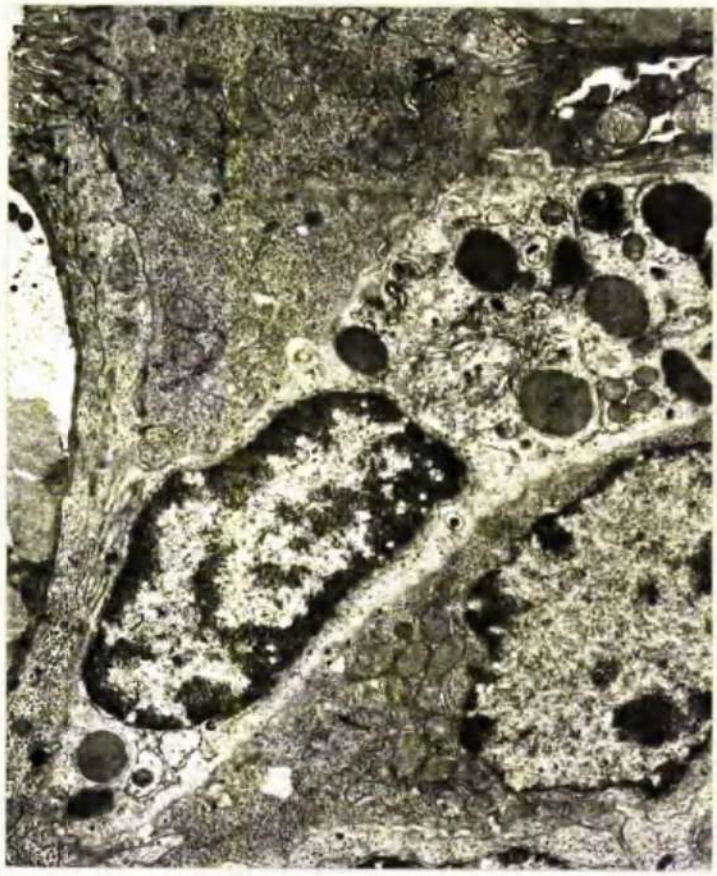


Figure 47.

Figure 48. Globule leukocyte within small intestinal epithelium of a parasitised rat showing details of granule type. Unit membranes (arrow) invest homogeneous electron-dense granules (1). Type 2 granules (2) have rims or areas of less electron-dense matrix. Type 3 granules (3) which contain paracrystalline structures (PC) are surrounded by unit membranes which are disrupted at various points (arrowheads) or else fused with other granule membranes. A type 4 granule contains only paracrystalline structures. V, vesicle; E, epithelial cell; p, progranule. x 41,250.

Figure 49. There are several type 1 granules in the cytoplasm of this GL and there are vesicles around the periphery of a type 2 granule (2). 4, type 4 granule. Parasitised rat, nineteenth day of infection. x 27,500.

Figure 50. Type 2 (2), 3 (3) and 4 (4) granules within a GL of a parasitised rat on the nineteenth day of infection. x 27,500.

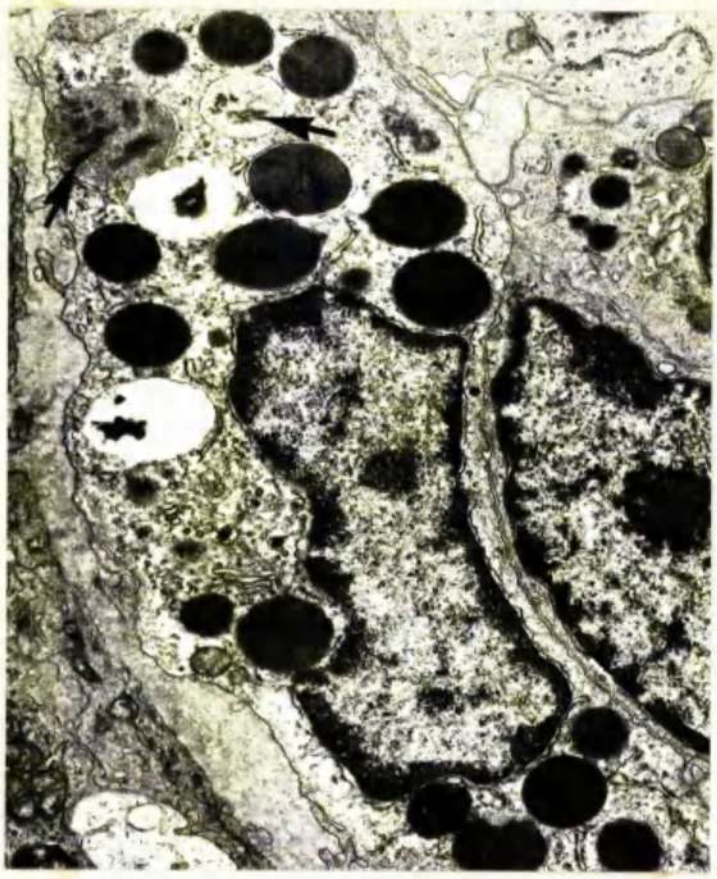
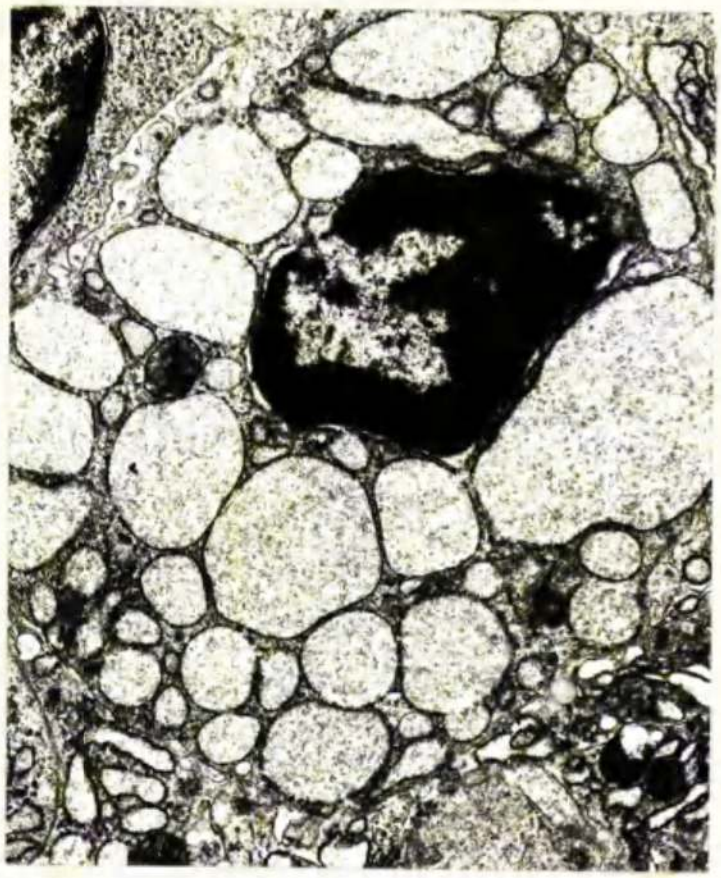


Figure 44.



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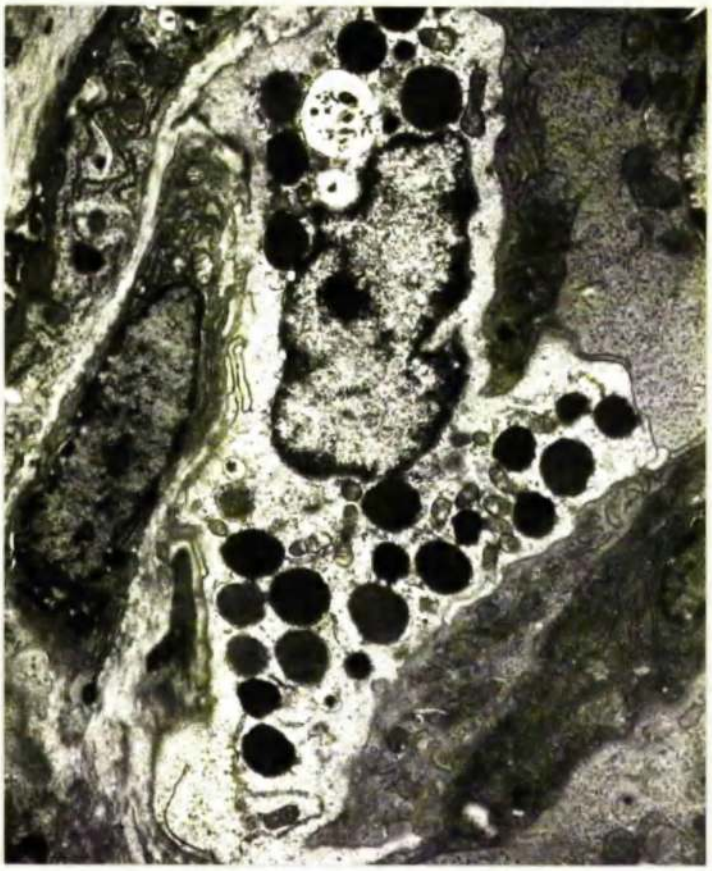


Figure 46.

Figure 47.

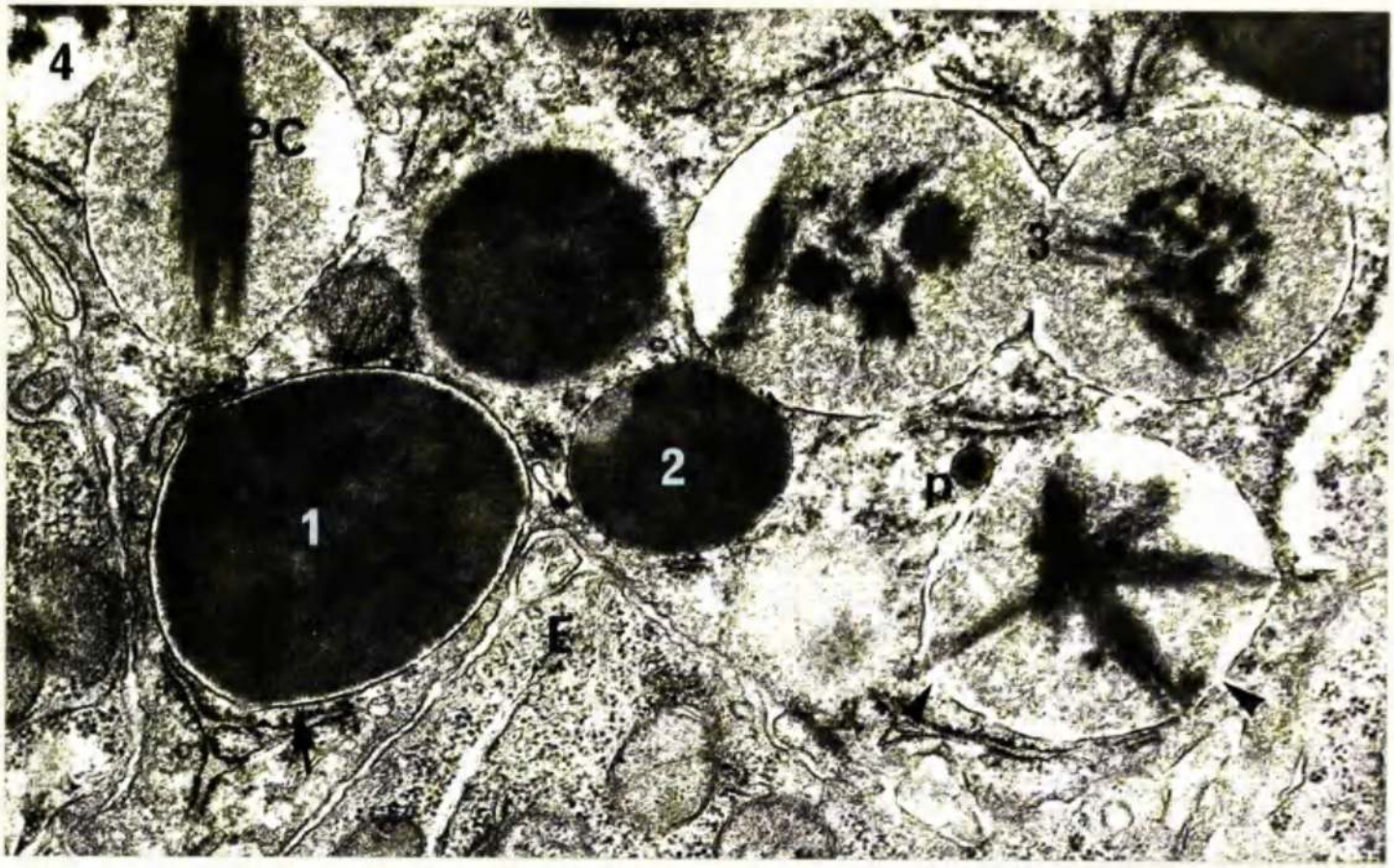


Figure 48.

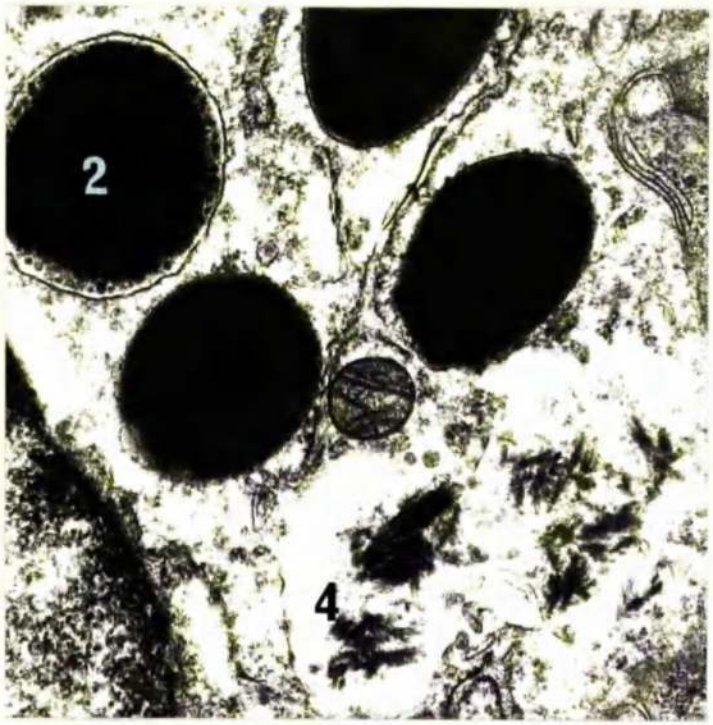


Figure 49.

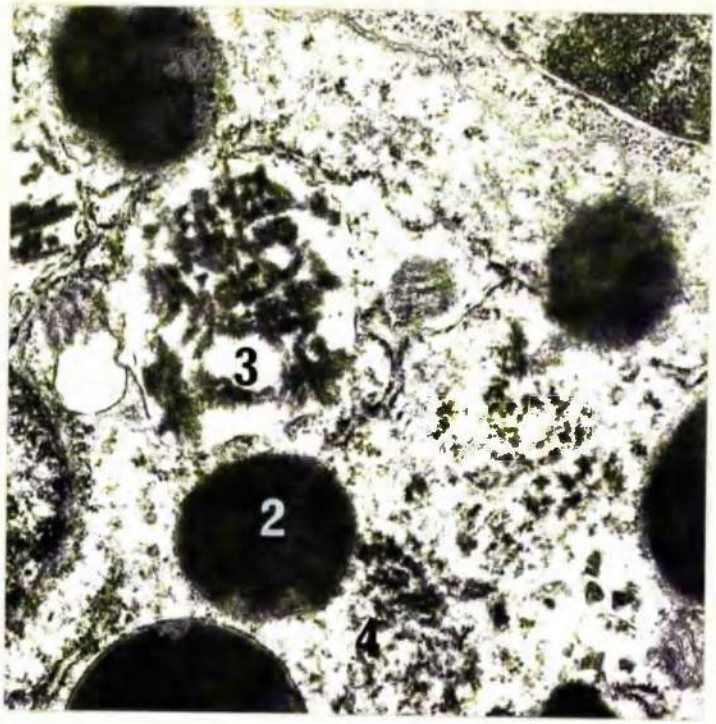
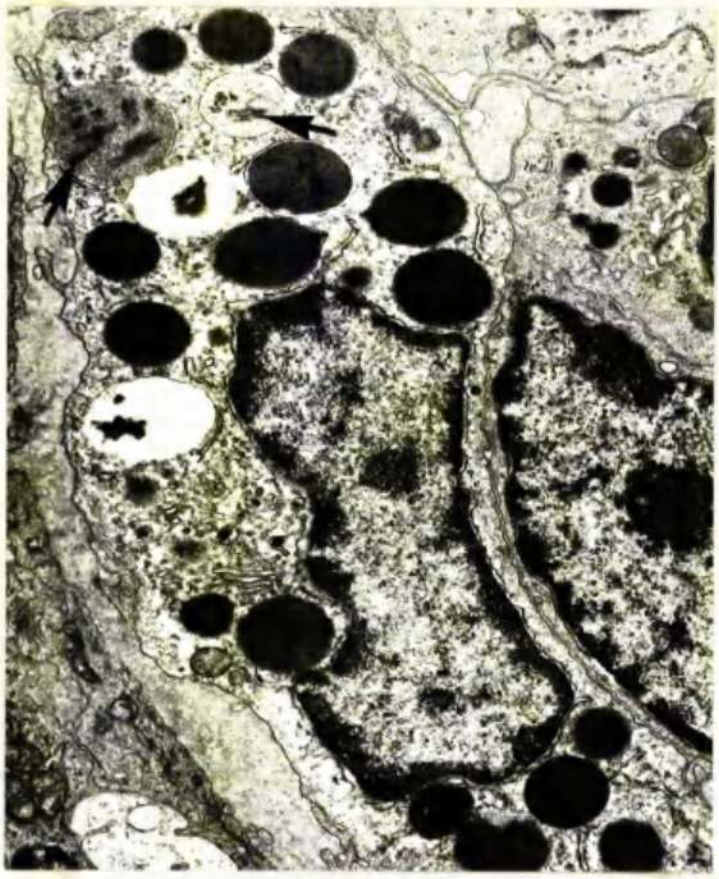


Figure 50.



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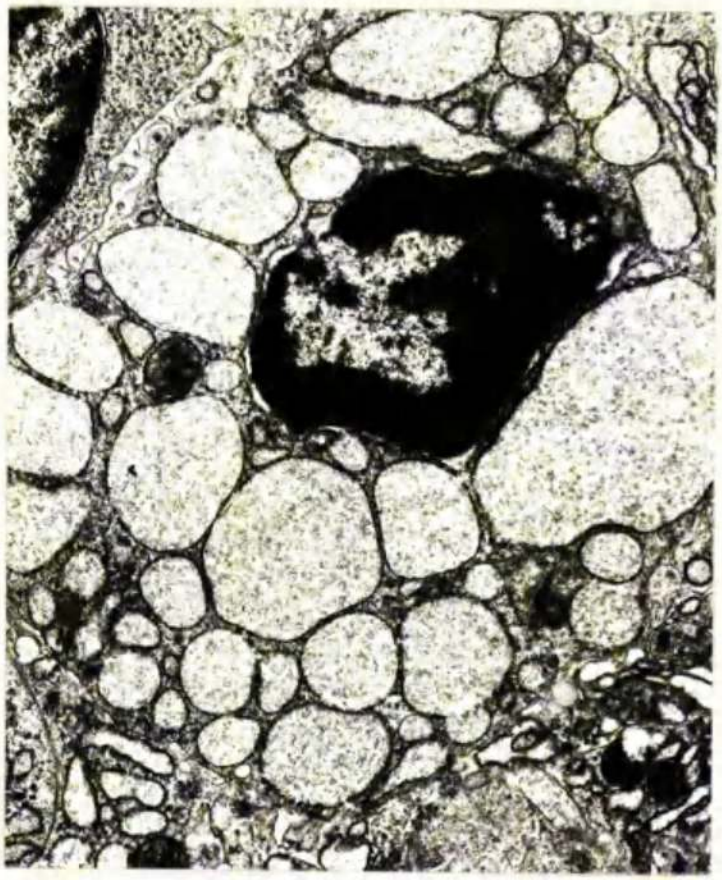


Figure 45.

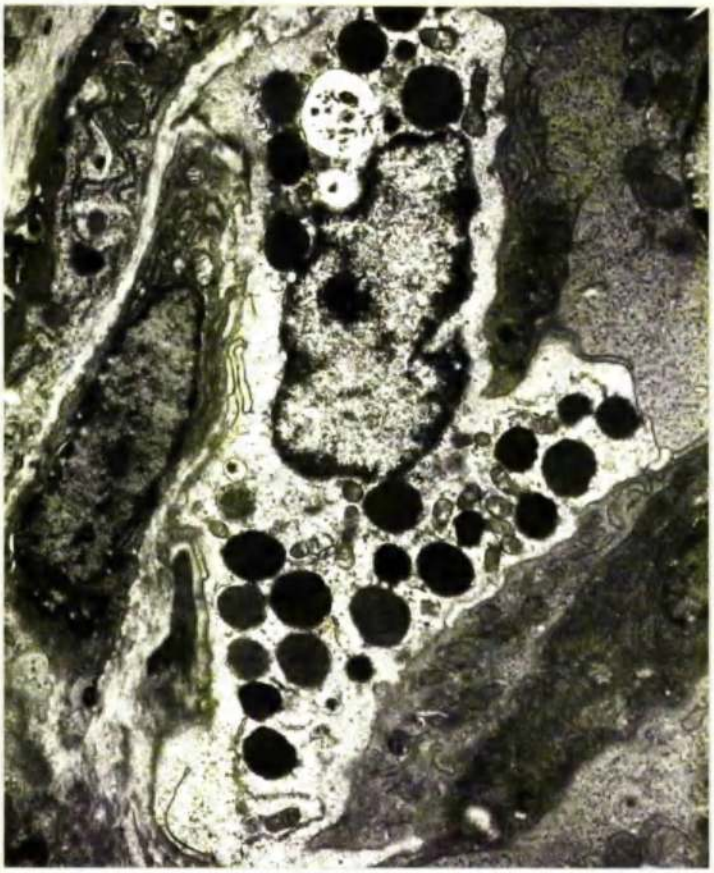


Figure 46.

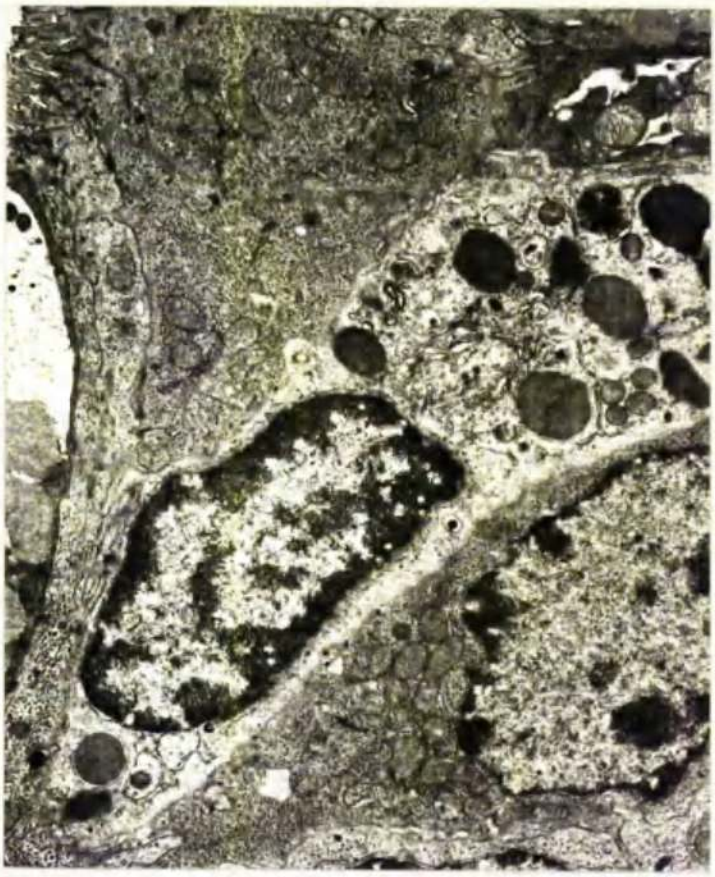


Figure 47.

Figure 51. GL within the intestinal epithelium of a parasitised rat (day 19) showing the complete range of granule types. The Golgi complex is moderately well developed. x 13, 100.

Figure 52. GL with pseudovacuolated cytoplasm and containing a variety of granule types. Parasitised rat nineteenth day of infection. x 13, 100.

Figure 53. A point of contact (arrow) has been established between a pseudopod extending from the basal part of an epithelial cell and a mast cell in the lamina propria. BM, epithelial basement membrane. Day nineteen of infection with N. brasiliensis. x 13, 750.

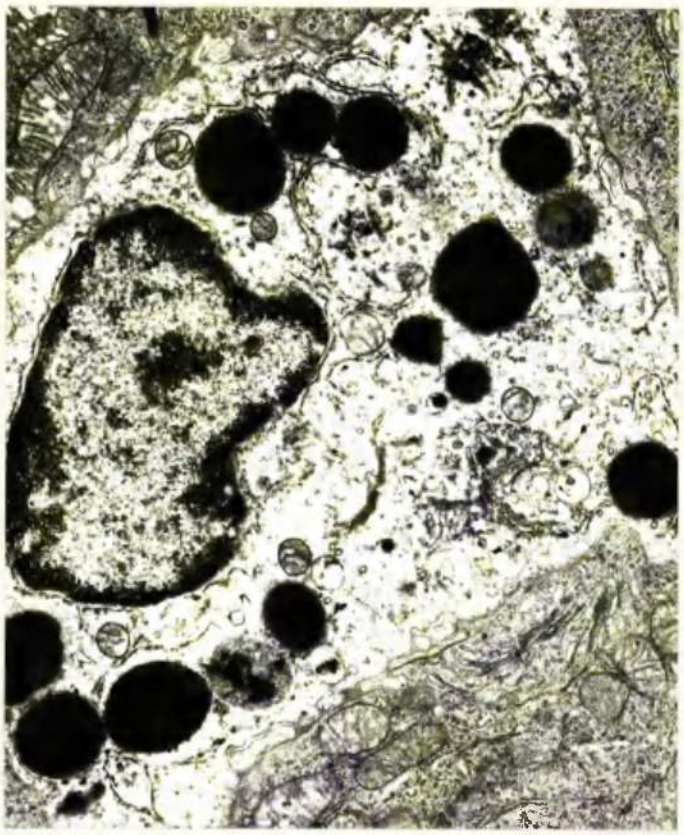


Figure 51.

Figure 52.

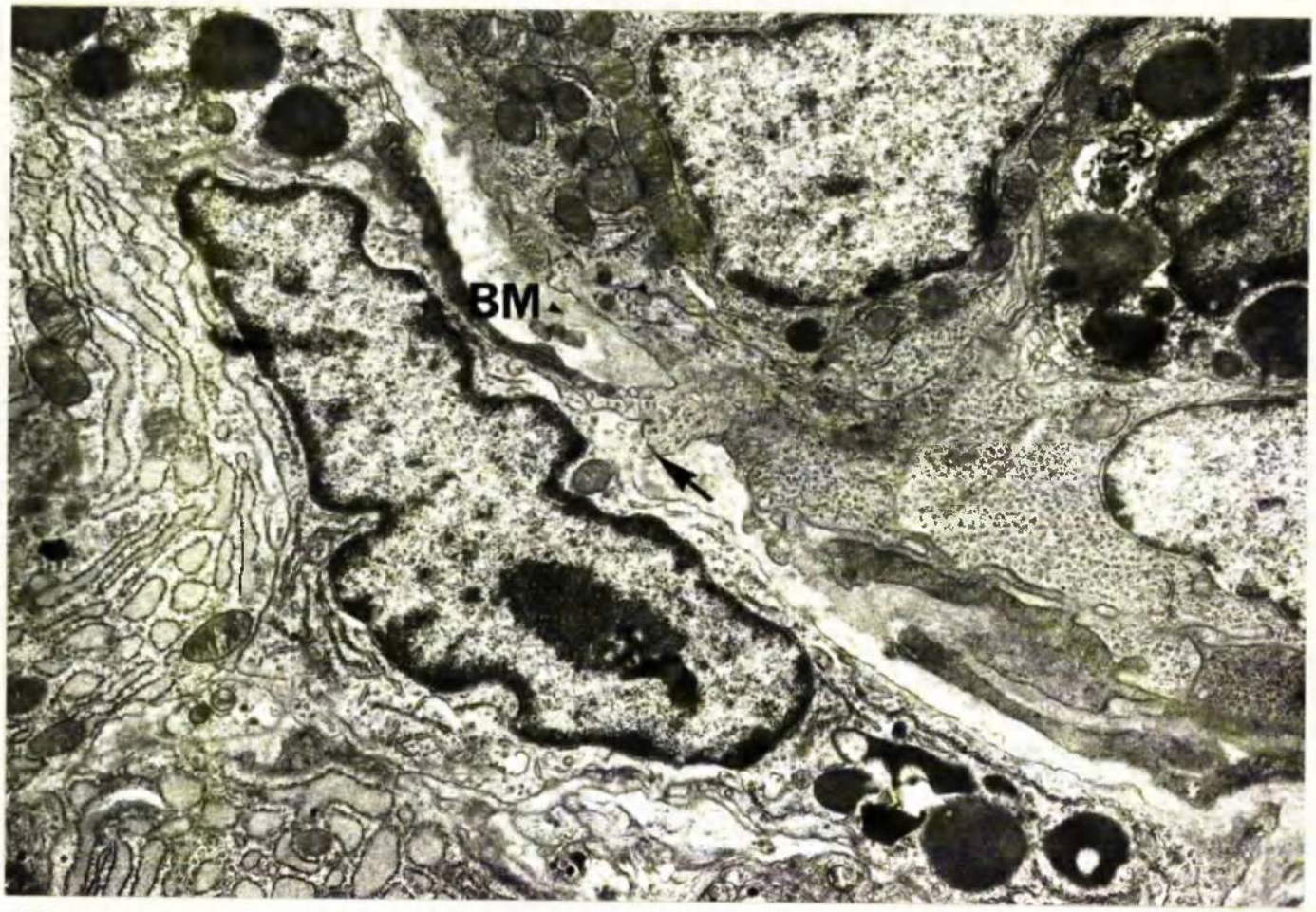


Figure 53.

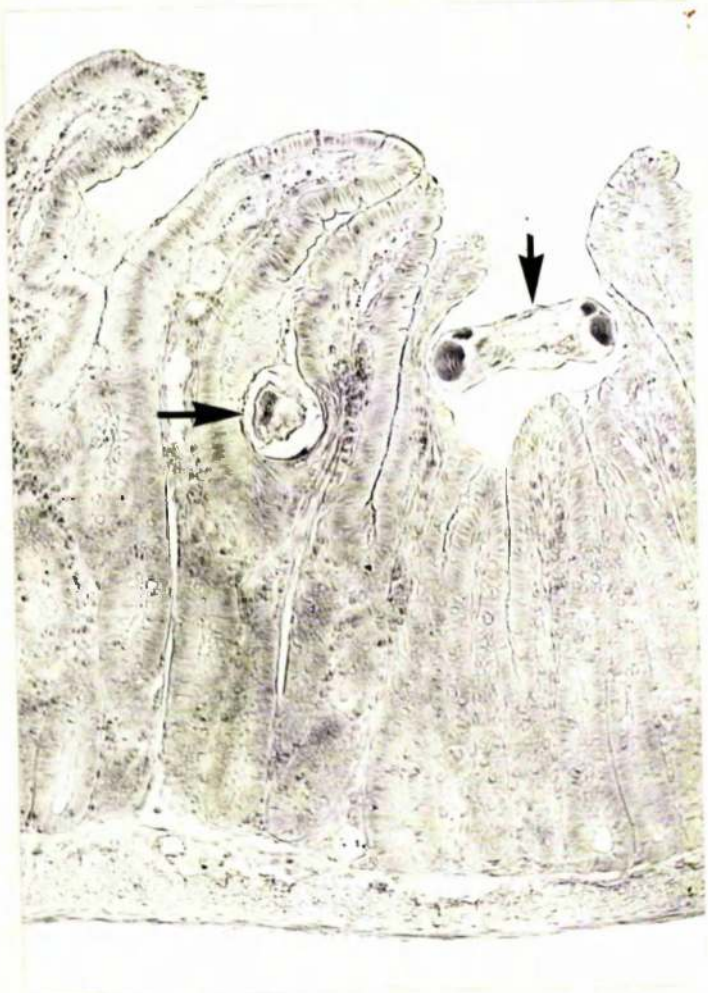


Figure 54. Intestinal mucosa of a Nippostrongylus infected rat (Day 8). Mast cells are absent from the lamina propria and the villus outline has been altered in the region of the parasites. (arrows). Astra blue/safranin. x 150.

Figure 55. Electron-micrograph of discharging IM cells. Fourth day of a Nippostrongylus infection. The plasmalemma of the cell at the top is interrupted at several points and the matrices of granules (arrows) in both cells are escaping into the lamina propria. There is vacuolation of the cytoplasm and the perinuclear cisternum (C) is dilated. x 27,500.

Figure 56. A serial section of the same cell shown in Figure 55. Note the dense clumping of the nuclear chromatin. x 8,750.

Figure 57. IM cell granules are enclosed within the phagosome of an undifferentiated cell (U). Fifth day of infection. x 13,100.

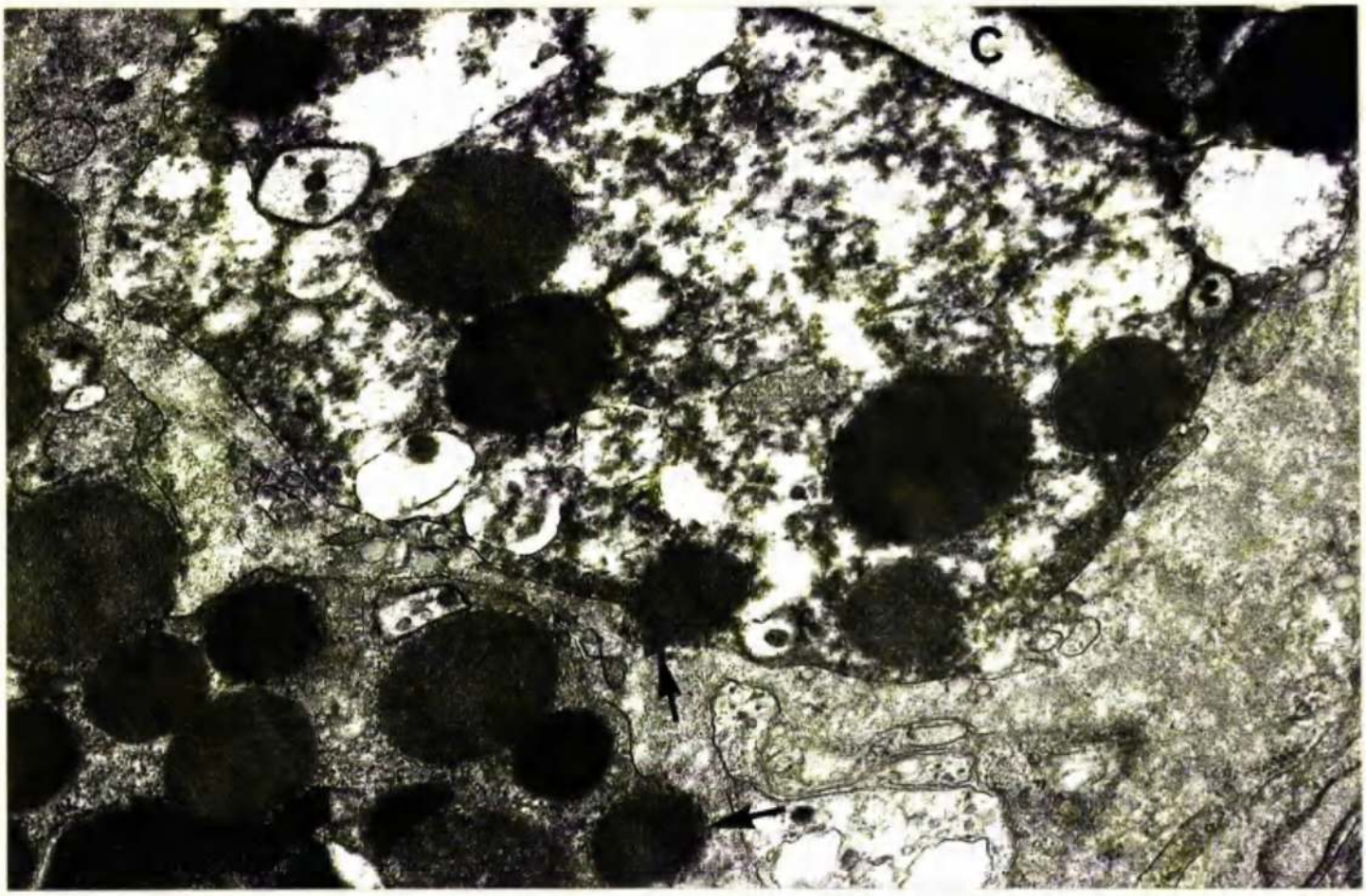


Figure 55.

Figure 56.

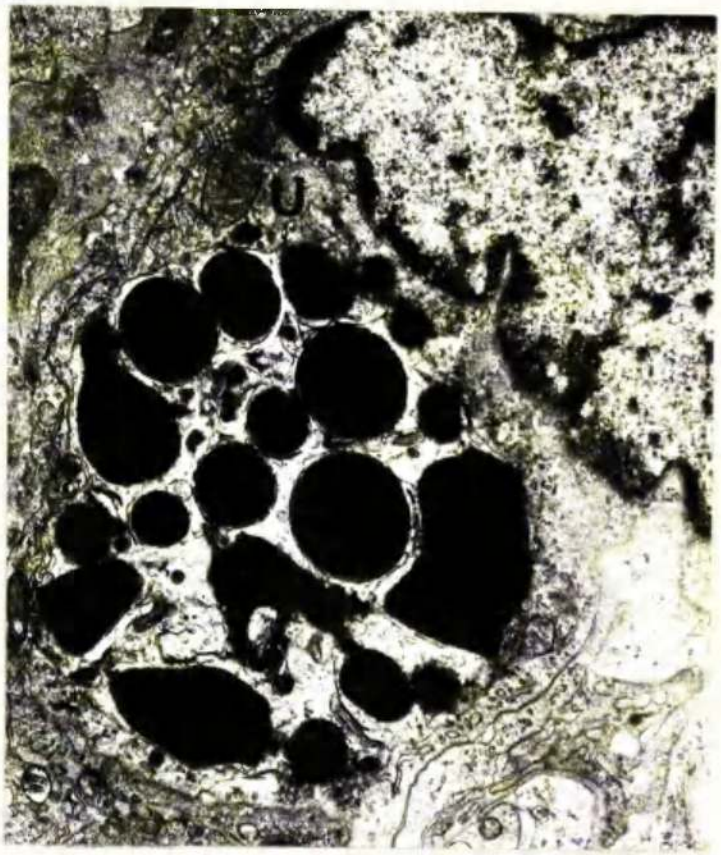


Figure 57.

Figure 58. Light micrograph of a disrupted IM cell; basophilic granules and a large deposit of basophilic material are seen. Fourth day of infection with N. brasiliensis. 1.5μ Araldite-Epon section. Azure II - Methylene blue - borax. x 1,800.

Figure 59. An electron-micrograph of the cell visualised in Figure 58. Several granules surround an amorphous deposit of approximately the same electron-density as the granule matrices. x 13,100.

Figure 60. Two IM cells have been phagocytosed by a macrophage. Fourth day of infection. x 8,750.

Figure 61. IM cell granules (arrows) lie within the phagosomes of a macrophage. Note the variety of inclusions within the cytoplasm of the macrophage. x 13,100.

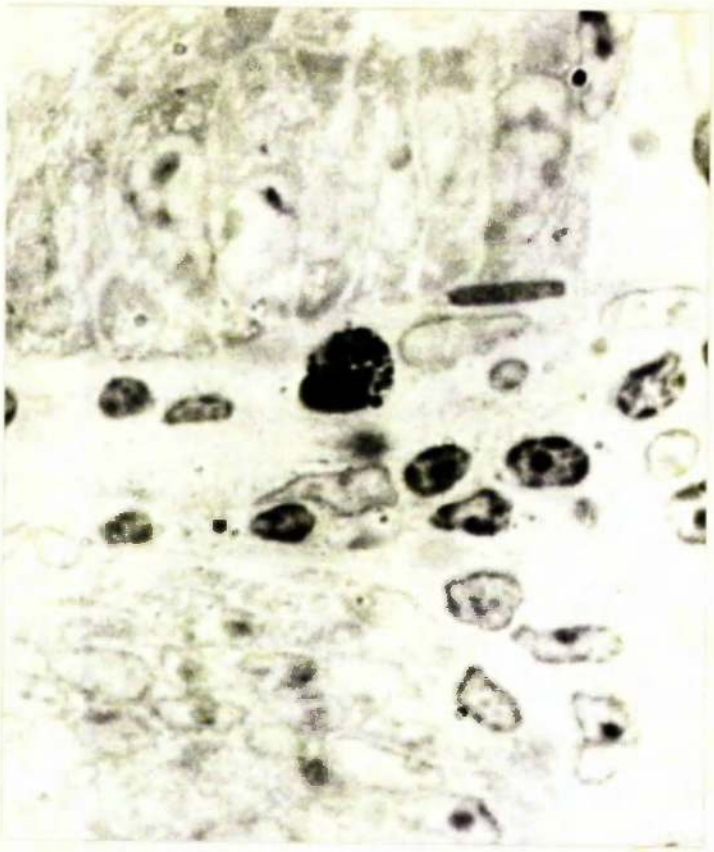


Figure 58.

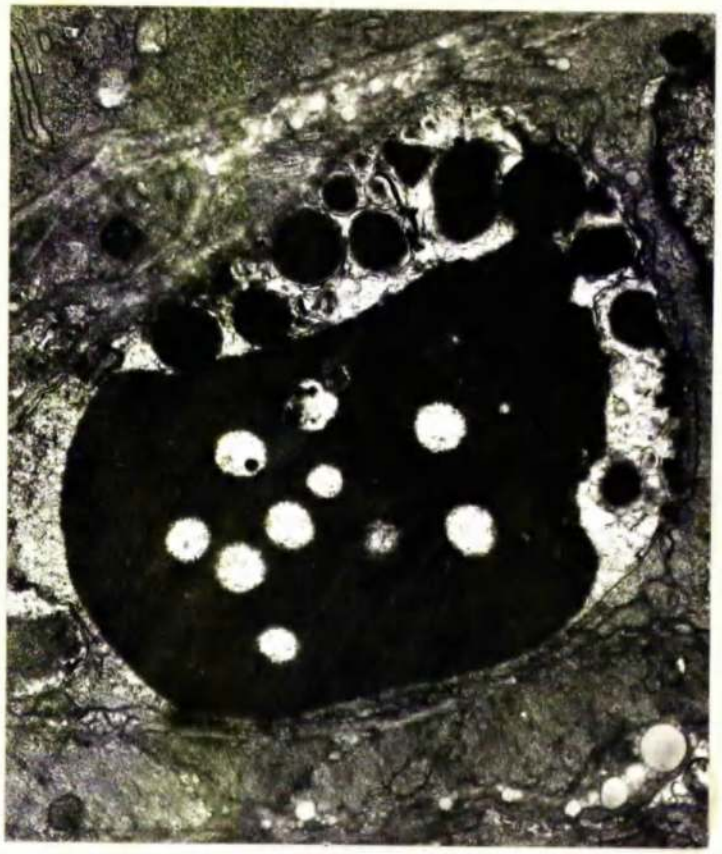


Figure 59.

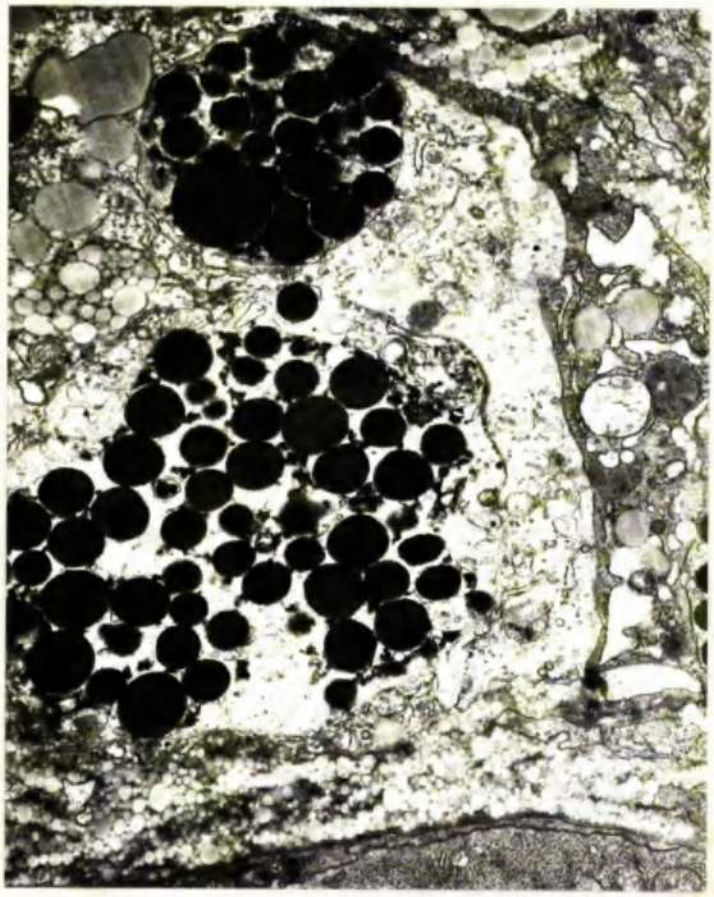


Figure 60.

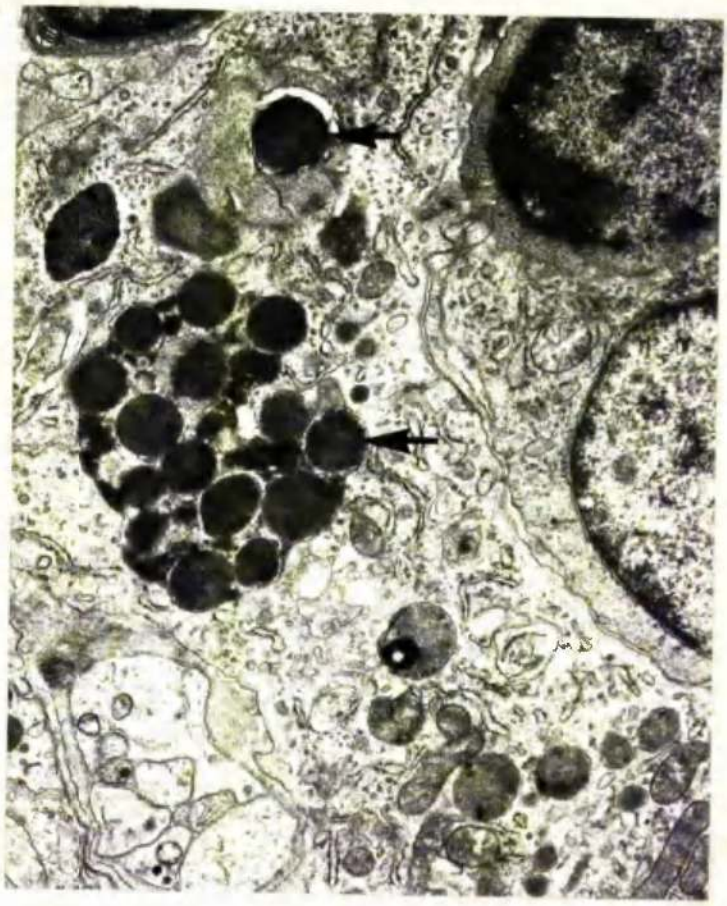


Figure 61.

Figure 62. There are several IM cell granules (arrows) within a phagosome. Note the unit membrane (arrowhead) delineating the phagosome. Fifth day of a Nippostrongylus infection. x 11,250.

Figure 63. Characteristic IM cell granule remnants are evident within the phagosome of a macrophage-like cell which has migrated intraepithelially. Fifth day of infection. x 8,750.

Figure 64. Light micrograph of the intestinal epithelium on the fifth day after infection with N. brasiliensis. The epithelial cells are vacuolated and are cuboidal. Several mitotic figures (arrows) are evident. The cells in the lamina propria are separated by oedema fluid. H.E. x 700.

Figure 65. The epithelial cells of the villus are markedly flattened where the parasite lies close to them. Sixth day after infection. H.E. x 450.

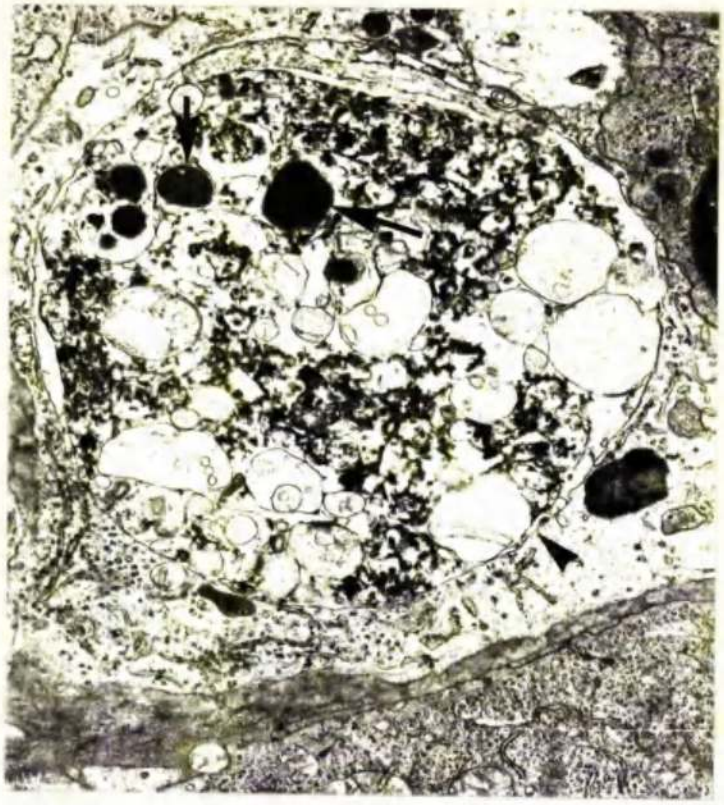


Figure 62.

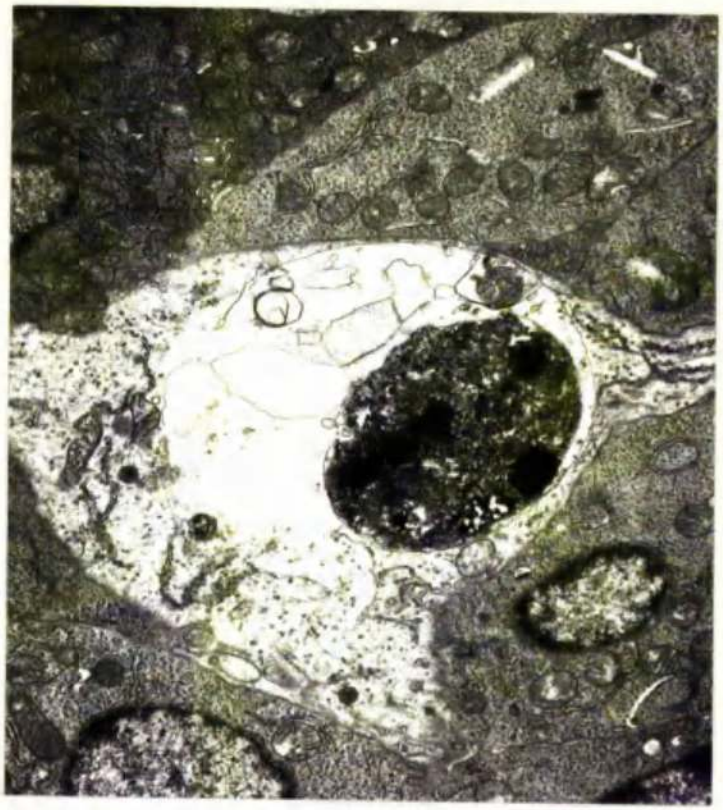


Figure 63.

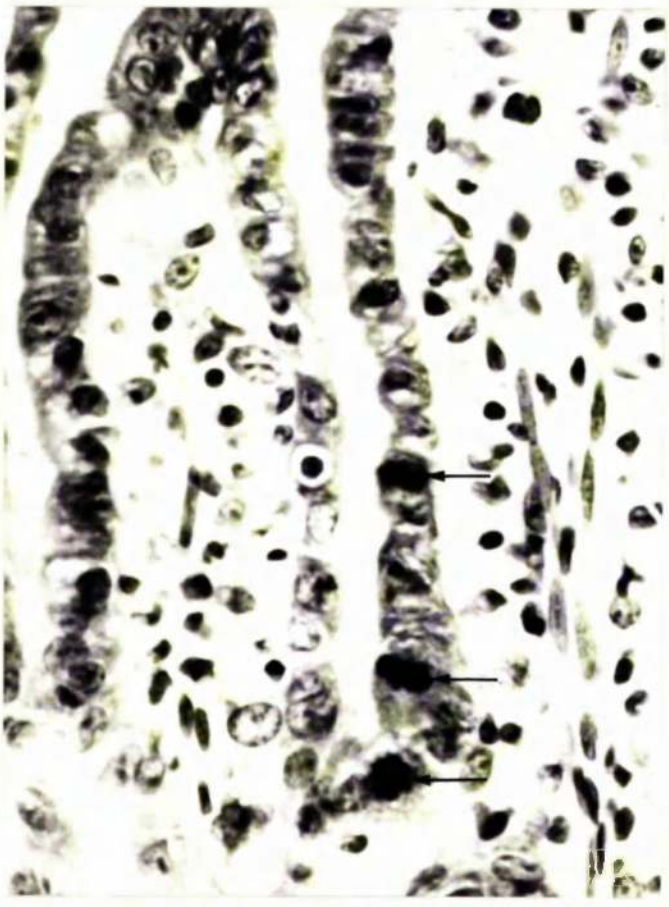


Figure 64.

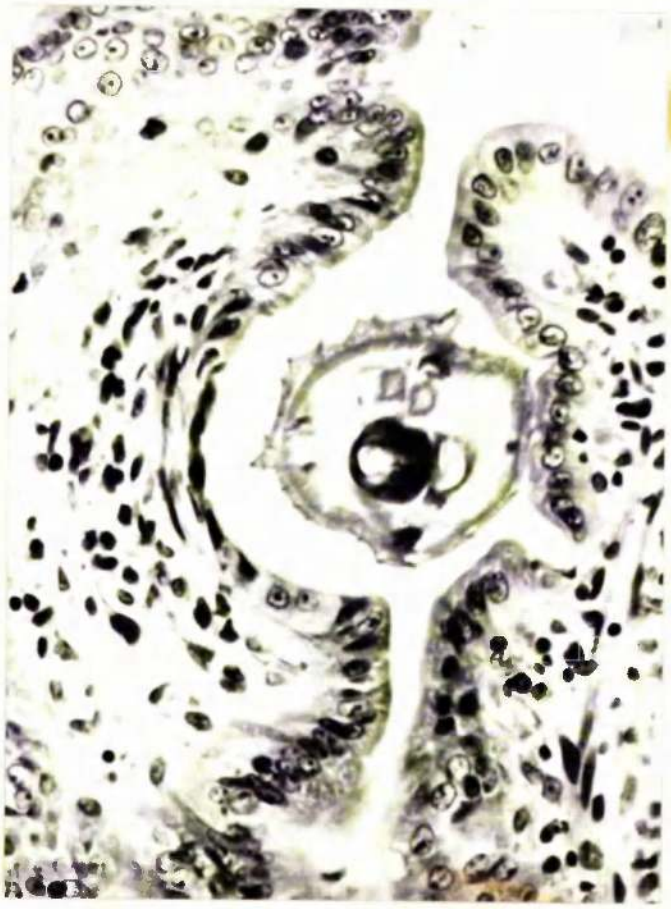


Figure 65.

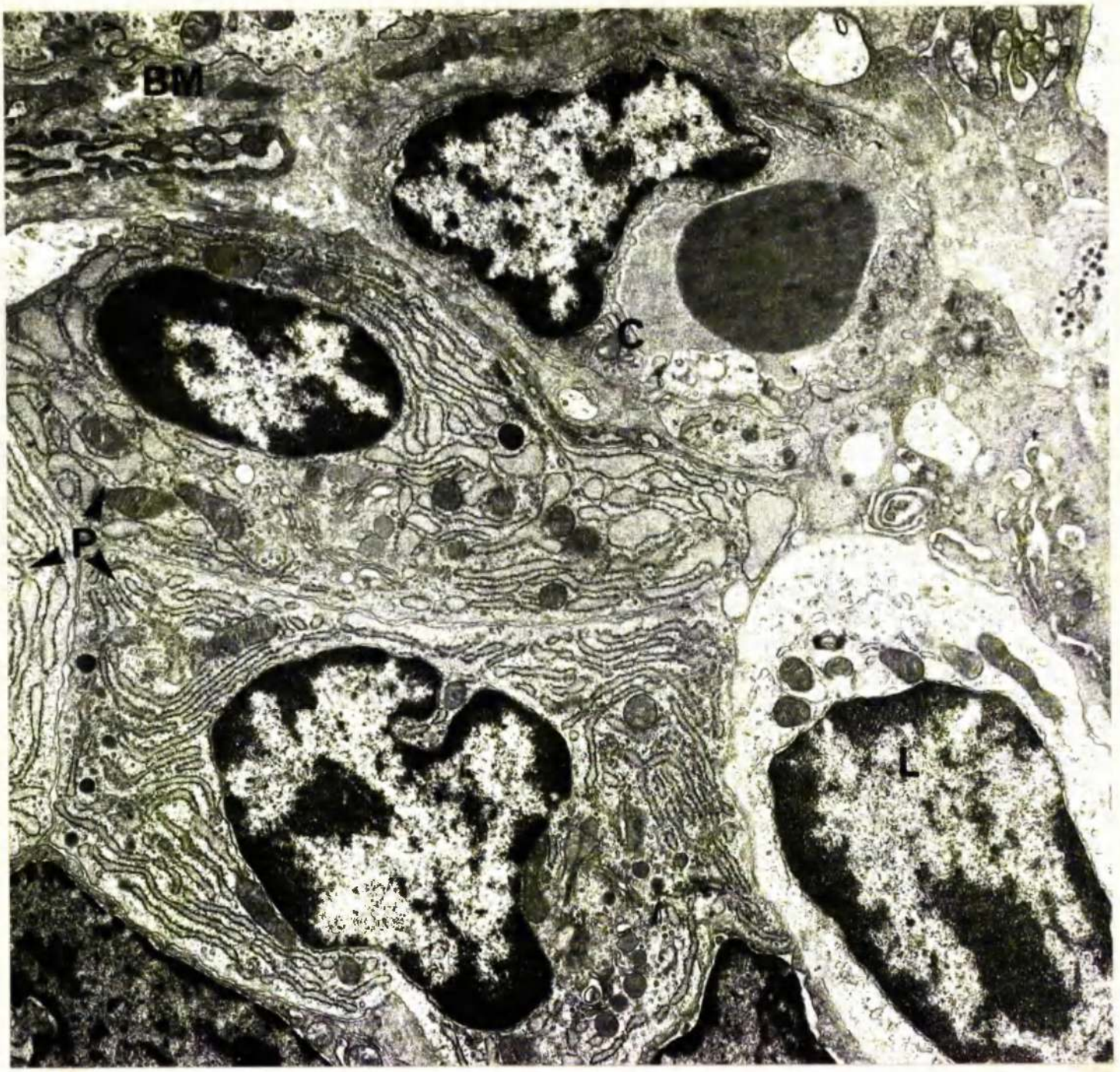


Figure 66.

The intestinal lamina propria of a normal rat. Plasma cells (P) and a lymphoid cell (L) are closely related to one another. C, Capillary; BM, Epithelial basement membrane. x 13,750.

Figure 67. Capillary in the intestinal lamina propria of the normal rat. R, red blood cell. x 20,000.

Figure 68. Altered capillary in the intestinal lamina propria of a Nippostrongylus infected rat (Day 5). The endothelium is swollen and a bleb of rarified cytoplasm (arrow) extends into the lumen. x 18,750.

Figure 69. There are several gaps (arrows) in the venular endothelium. Fifth day of infection. R, red blood cell. x 15,000.

Figure 70. A red blood cell (R) has escaped into the lamina propria. Fifth day of infection. x 8,400.

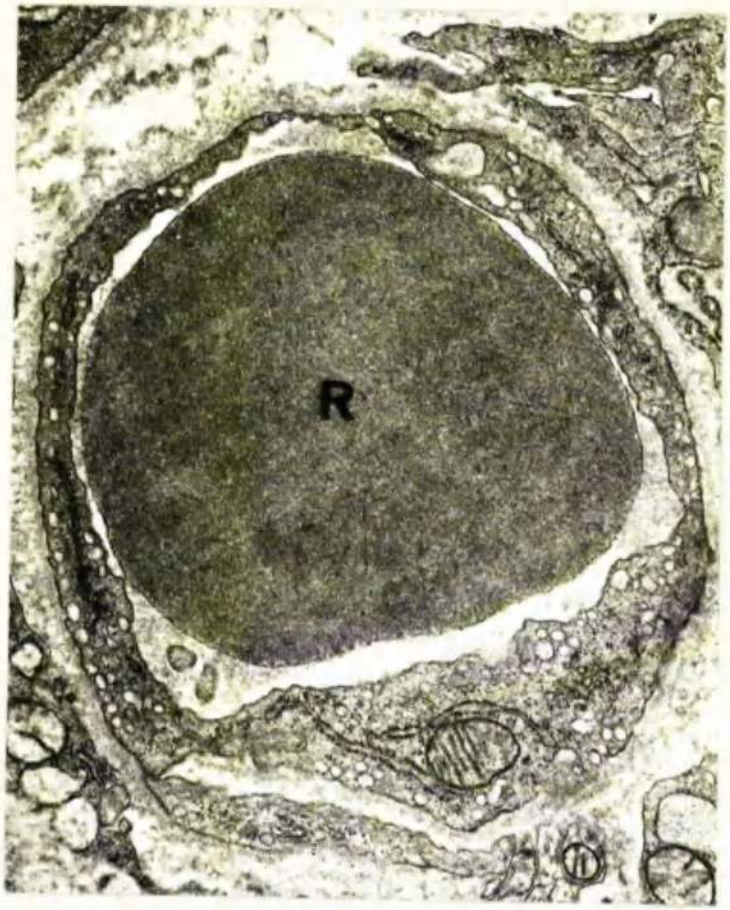


Figure 67.

Figure 68.

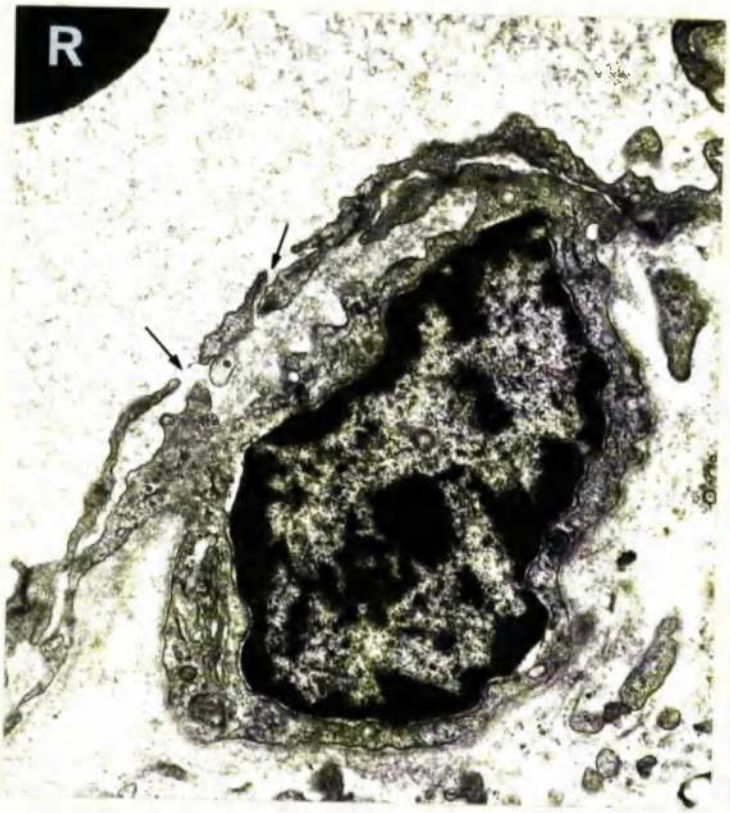


Figure 69.

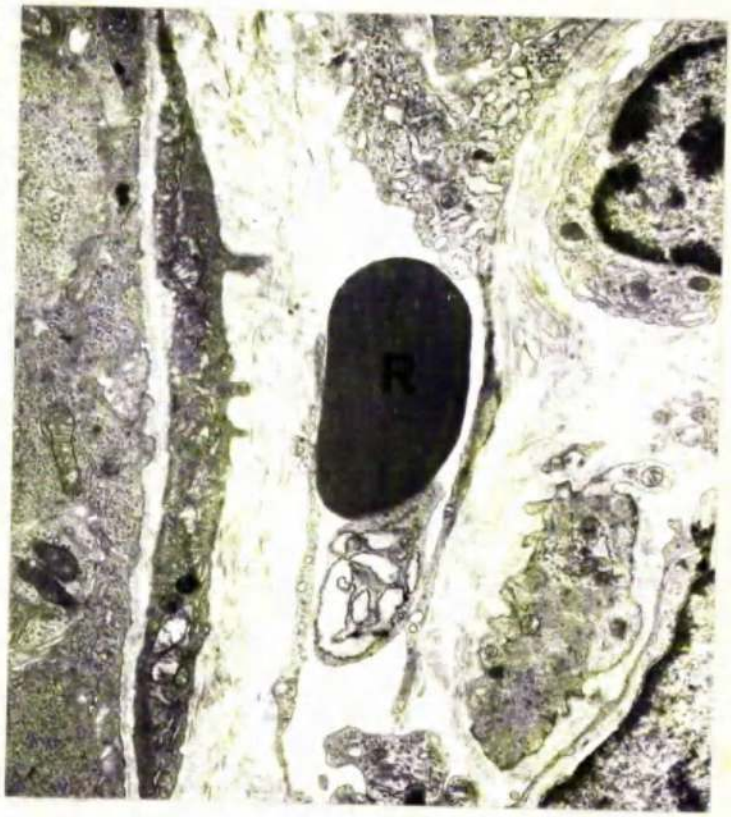


Figure 70.

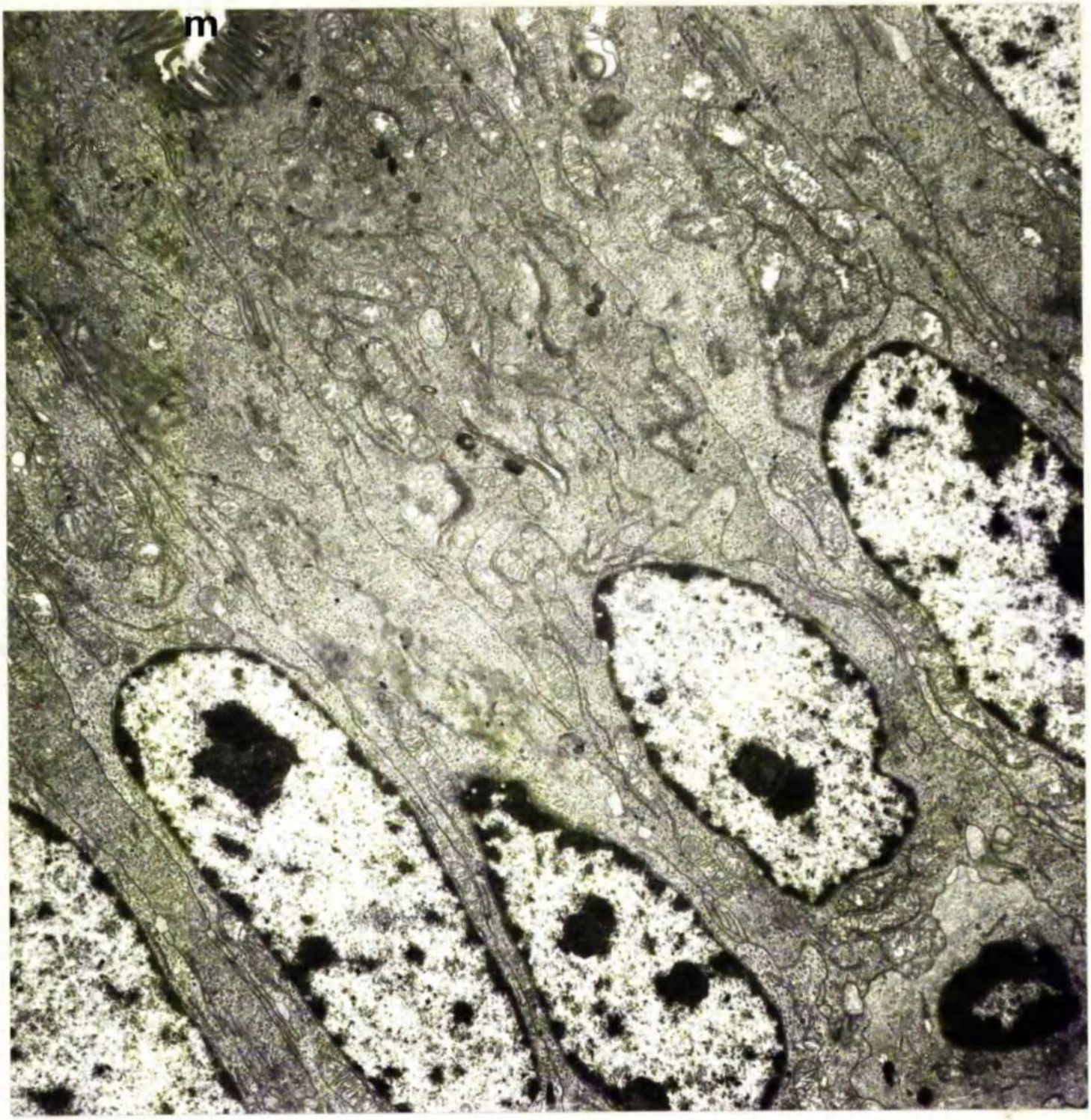


Figure 71.

Intestinal epithelium of a normal rat. Mid villus region. The lateral plasmalemmata are in close apposition. Finger-like microvilli (m) extend from the luminal surface. x 8,250.

Figure 72. A parasite (P) is in contact with the villus epithelium (E). The cuticle of the parasite abuts upon the shortened microvilli. Fifth day of infection. x 16,800.

Figure 73. Villus epithelium of an infected rat (Day 6). The lateral intercellular spaces (IS) are dilated, the microvilli (m) are blunt and several vacuoles containing lipid (L) are present in the cytoplasm. x 4,500.

Figure 74. Degenerating plasma cells (p) lie within the cytoplasm of macrophage-like cells. Intestinal lamina propria, seventh day of infection. x 8,400.

Figure 75. An undifferentiated cell in the intestinal lamina propria. Fifth day of infection. x 11,250.

Figure 72.

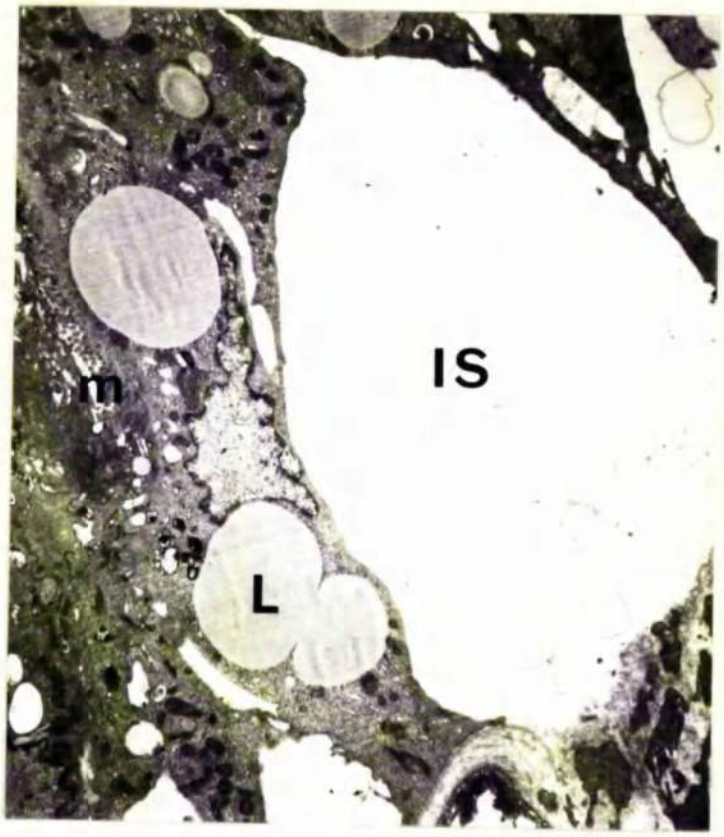


Figure 73.

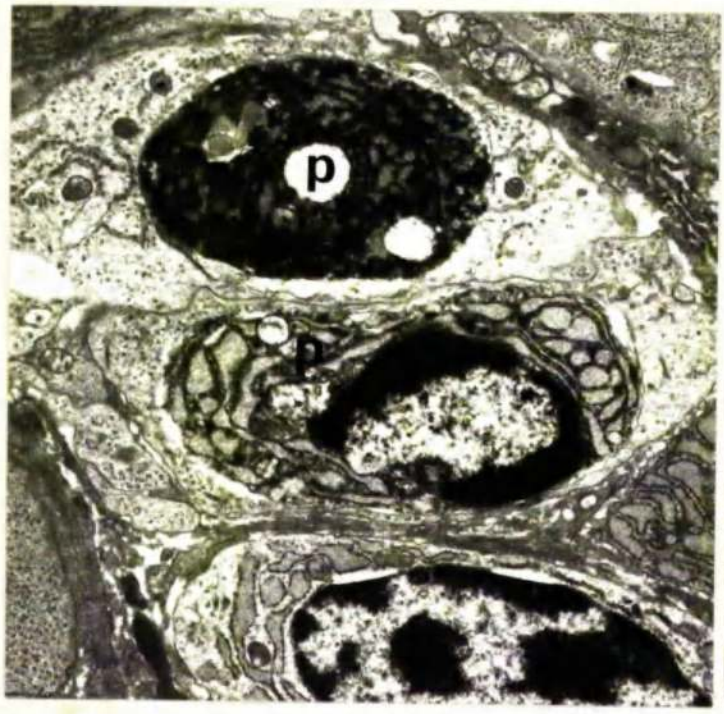


Figure 74.

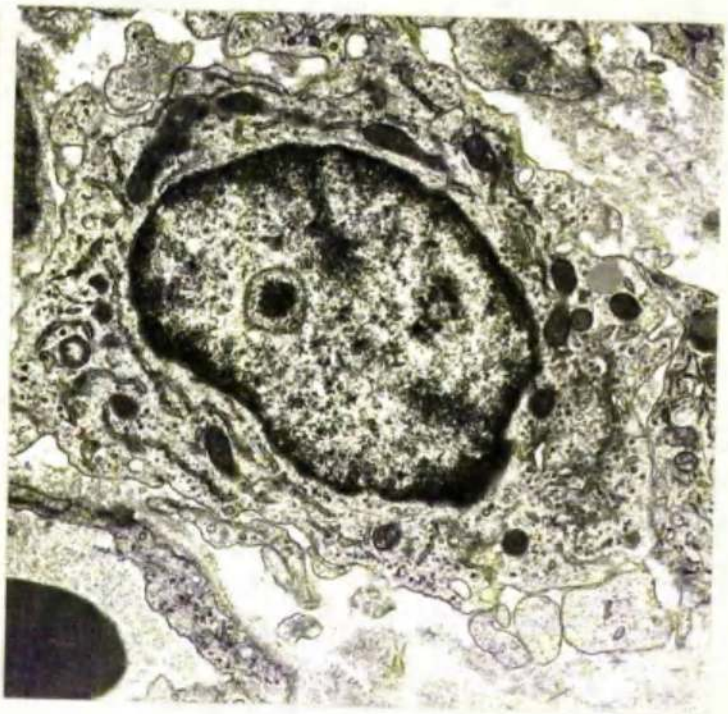


Figure 75.

Figure 76. The cytoplasm of an eosinophil has been lysed and the granules are scattered in the lamina propria. The majority of granules are unaltered and their delimiting membranes remain intact. Fifth day of infection. x 13,100.

Figure 77. Intact eosinophil granules (arrows) within the phagosome of an undifferentiated cell in the intestinal lamina propria. Five days after infection with N. brasiliensis. x 26,000.

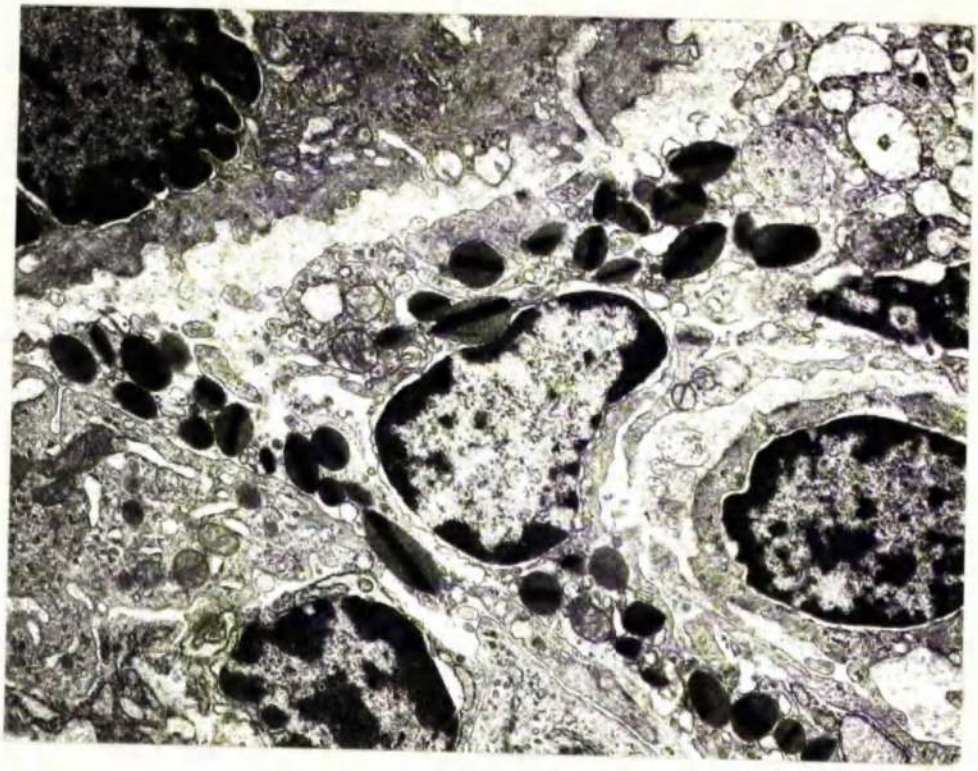


Figure 76.

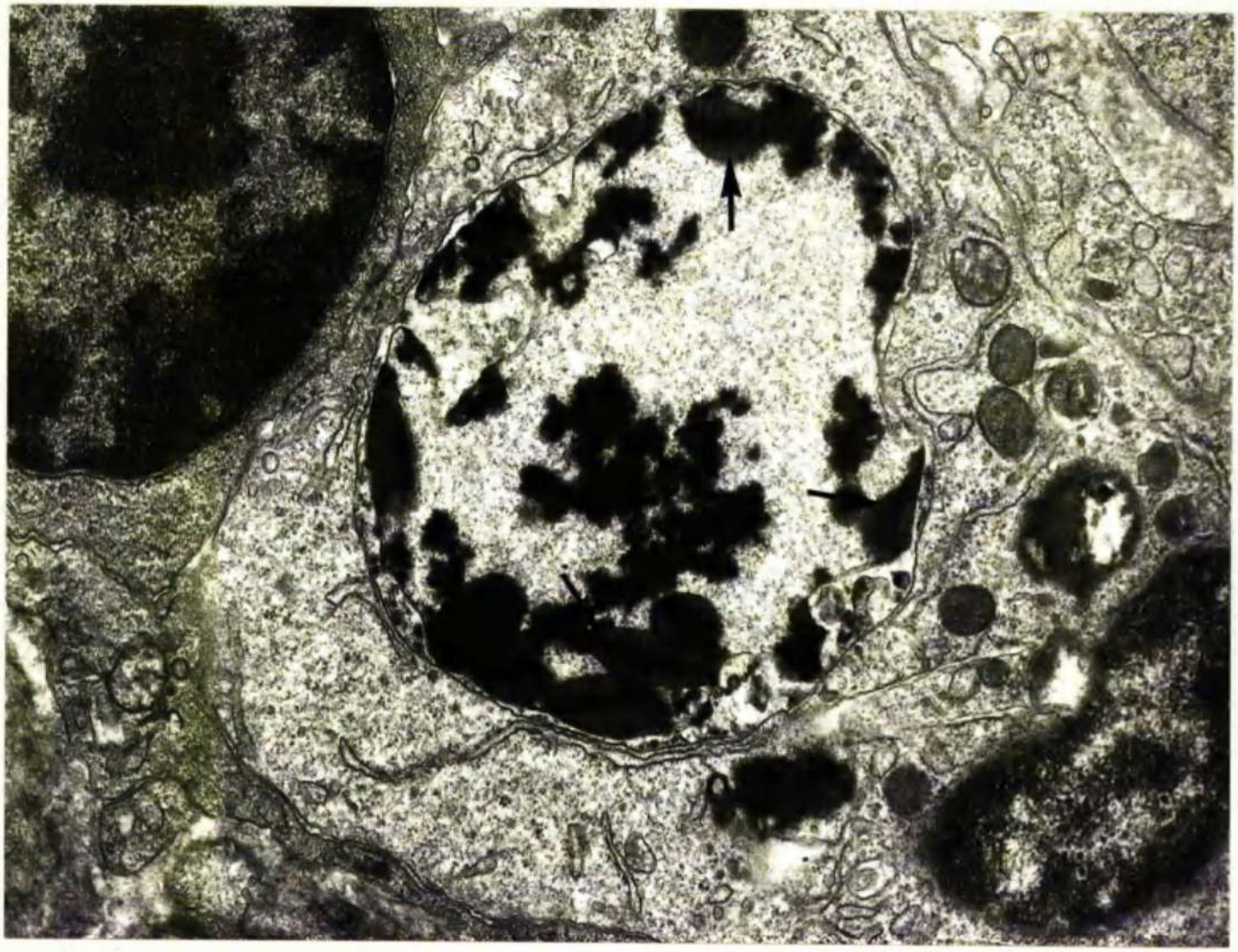


Figure 77.

Figure 78. Lymphocyte in a capillary. Fifth day of infection. x 10,500.

Figure 79. Blast cell in intestinal lamina propria six days after infection. Note the ribosomal aggregates in the cytoplasm. x 7,000.

Figure 80. Blast cell at an opened junction of a lacteal. Arrows, endothelium; L, lumen. Seventh day of infection. x 10,500.

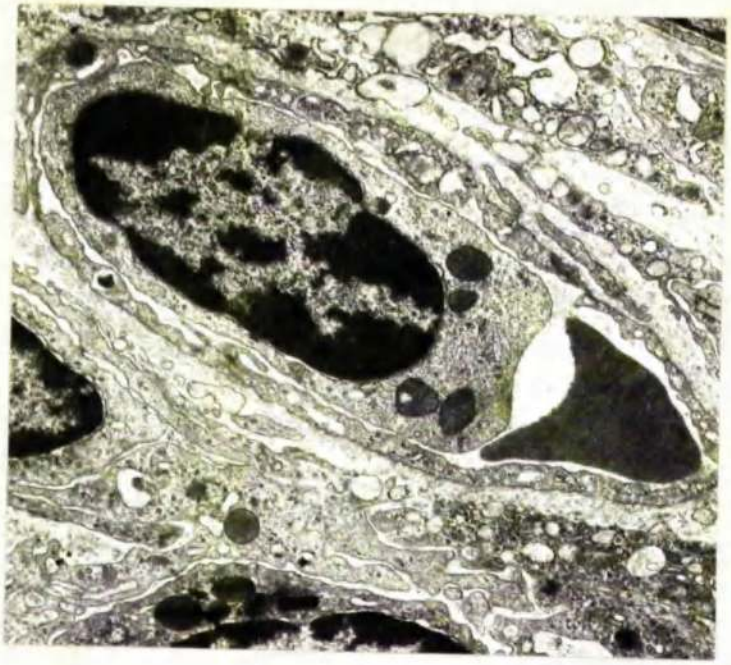


Figure 78.

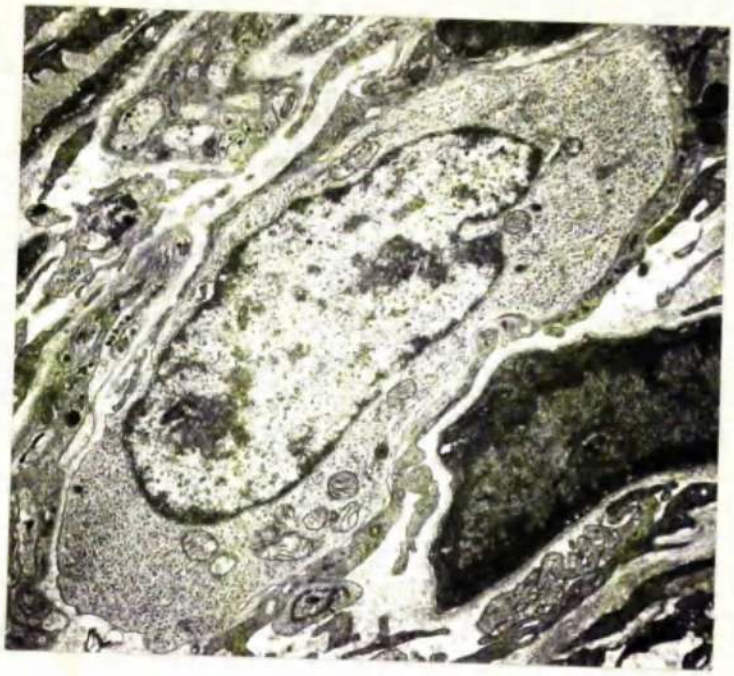


Figure 79.

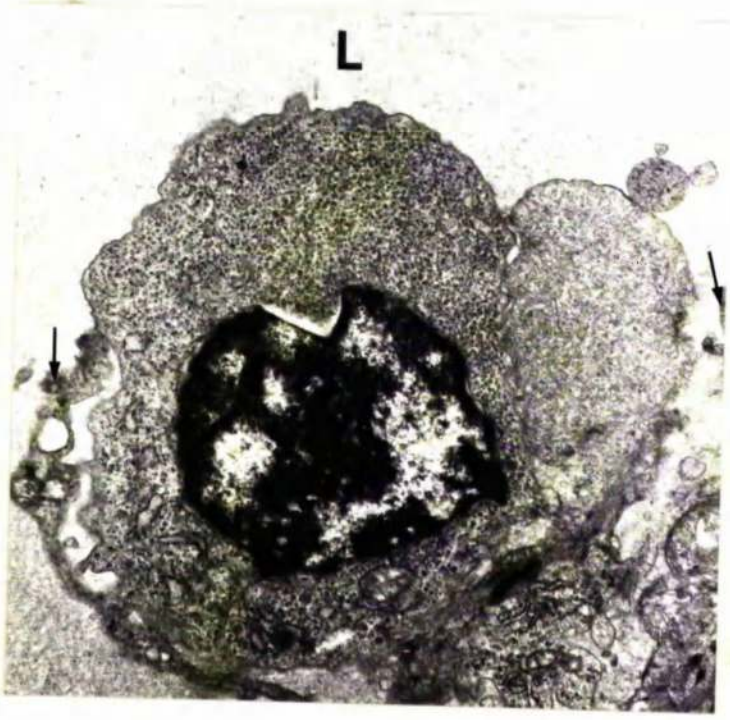


Figure 80.

Figure 81. Light micrograph of maturing IM cells in the intestinal lamina propria on the eleventh day after infection with N. brasiliensis. In one cell (arrow) there are very few granules. The more fully granulated IM cell has a prominent nucleolus (arrowhead). Astra blue/safranin. x 840.

Figure 82. A dividing globule leukocyte has mitotic figures which are stained strongly with safranin. The granules stain blue. Twelve days after infection with N. brasiliensis. Astra blue/safranin. x 600.

Figure 83. A high power micrograph of the dividing cell in Figure 82. Note the prominent nucleolus (arrow) of a nearby granulated cell. Astra blue/safranin. x 2,000.

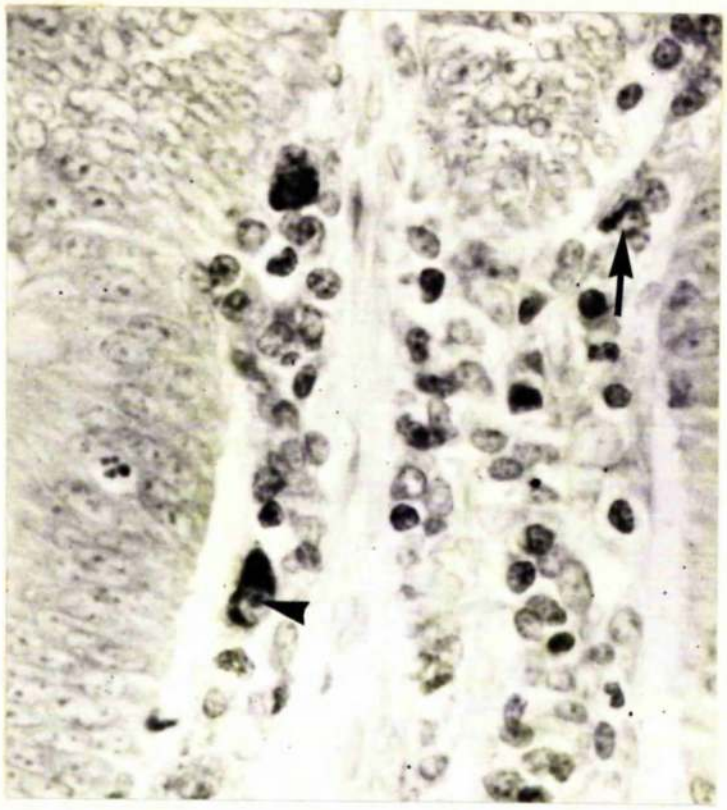


Figure 81.

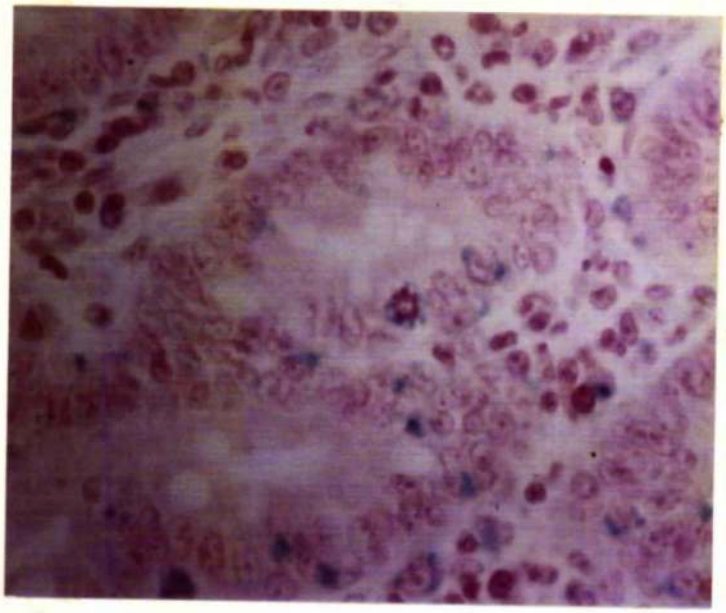


Figure 82.

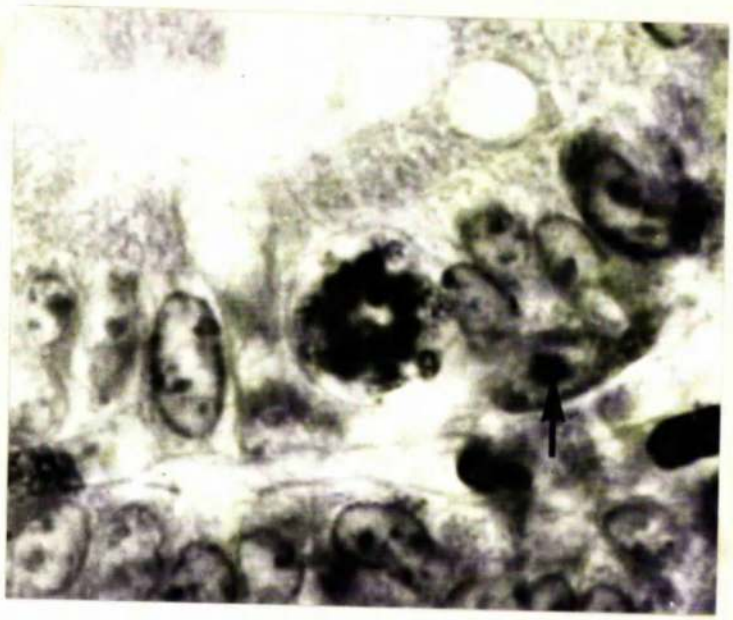


Figure 83.

Figure 84. Fourteen days after infection the mucosa is densely populated with IM cells (M) and globule leukocytes (G). Some of the IM cells are fragmented (arrow) and the GL cells contain fewer granules than their counterparts in the lamina propria. Astra blue/safranin. x 500.

Figure 85. Two IM cells are in mitosis. The mitotic figures (arrows) stain strongly with safranin. Fourteenth day of infection. Astra blue/safranin. x 840.

Figure 86. IM and GL cells are abundant in the mucosa nineteen days after infection. Astra blue/safranin. x 150.

Figure 84.

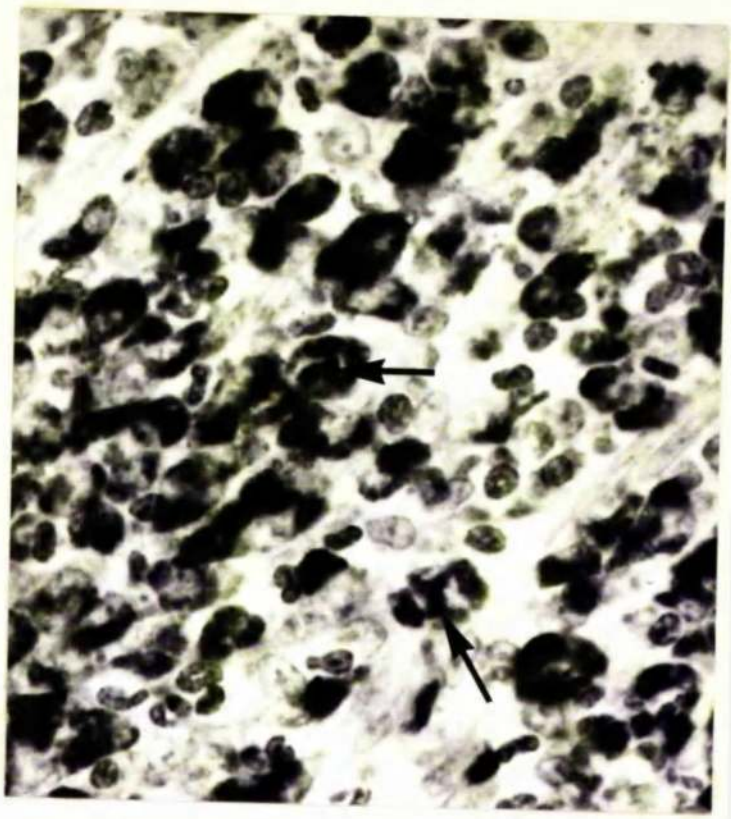


Figure 85.

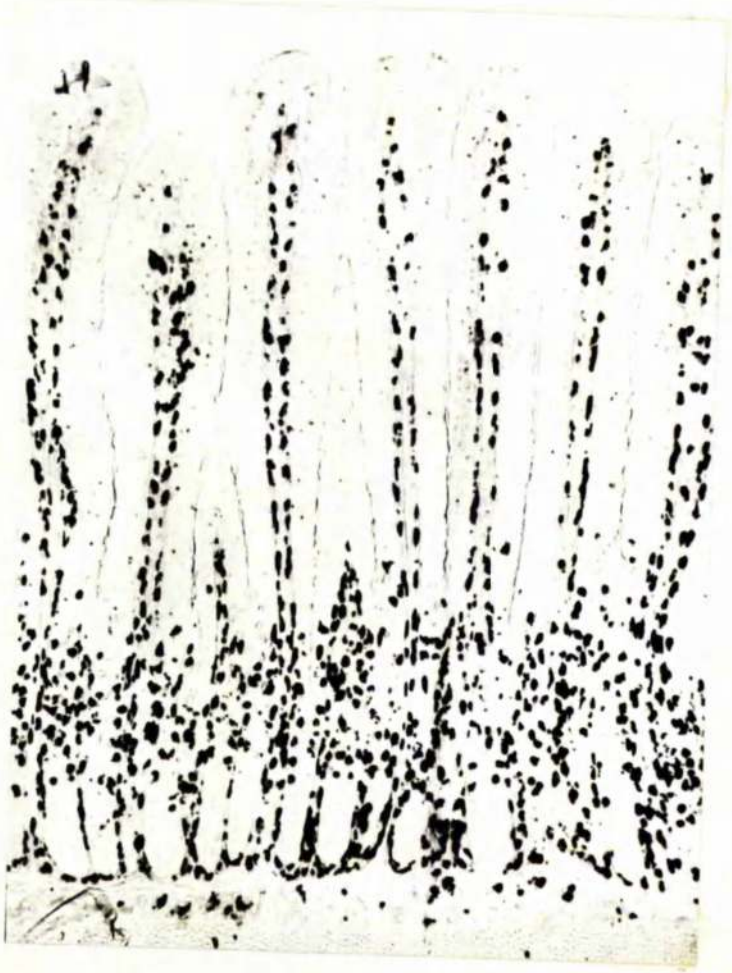


Figure 86.

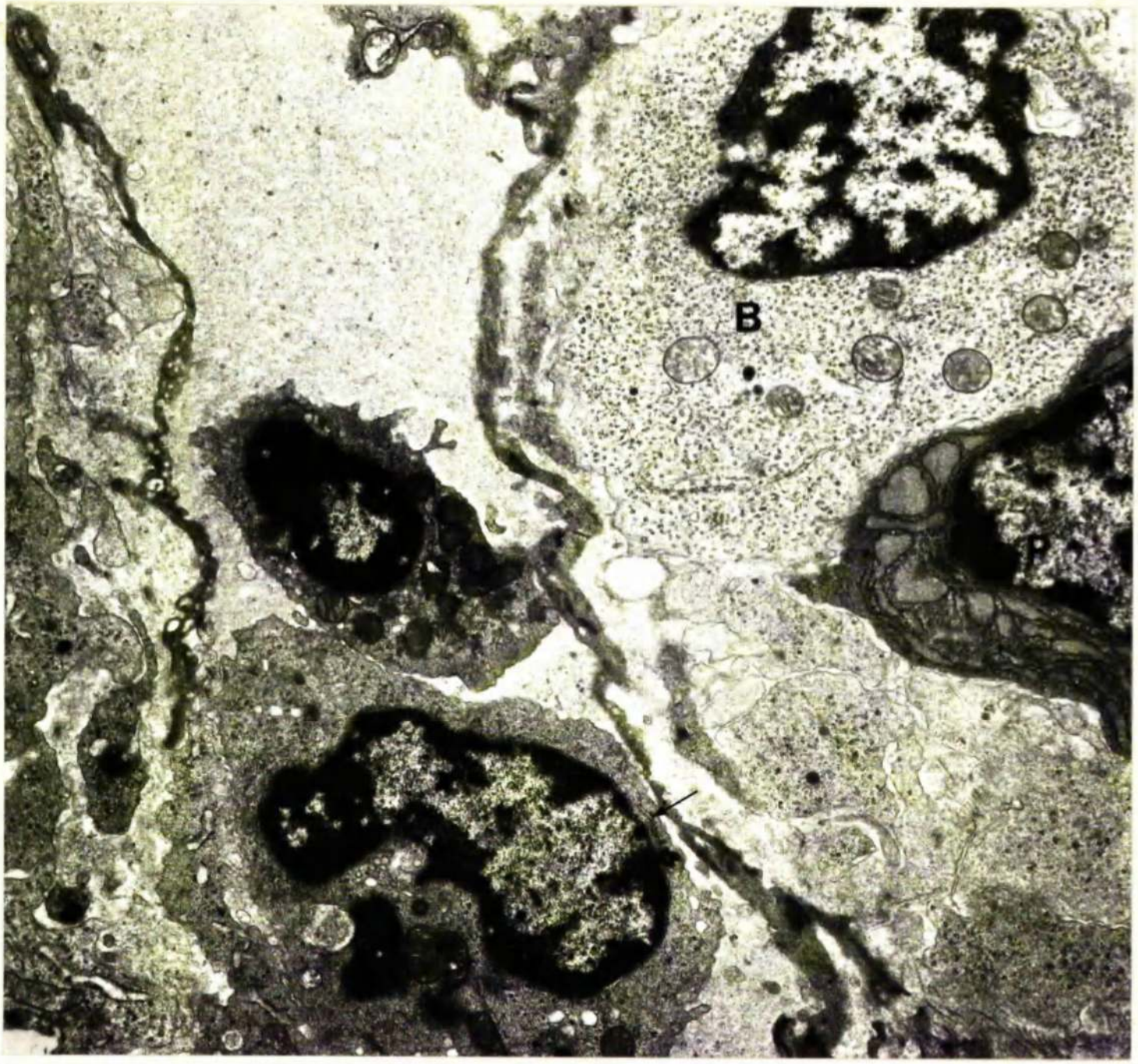


Figure 87. Two lymphoid cells are located in a lacteal and in one of them there are several ribosomal aggregates and a strand of RSER (arrow). A blast cell (B) is adjacent to the lacteal endothelium. Twelfth day of infection. P, plasma cell. x 12,500.

Figure 88. Dividing cell in intestinal lamina propria; twelfth day of infection. Single ribosomes and polyribosomes are present in the cytoplasm. d, dense body. x 14,100.

Figure 89. Blast cell in the lamina propria twelve days after infection with N. brasiliensis. Polyribosomes are abundant and there are several strands of RSER in the cytoplasm. The Golgi complex (G) is small. Note the diffuse distribution of chromatin and its margination at the periphery of the nucleus. x 10,000.

Figure 90. A large nucleolus (N) is present in this blast cell. Twelfth day of infection. x 10,000.

Figure 91. Blast cell with a more elaborate Golgi complex and moderately abundant RSER. x 10,000.

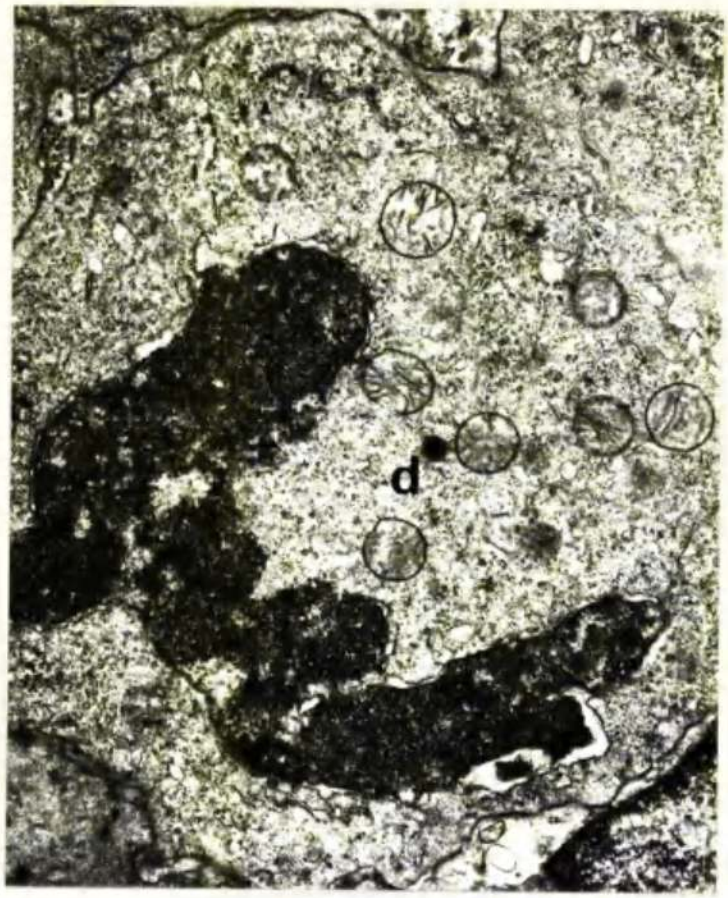


Figure 88.

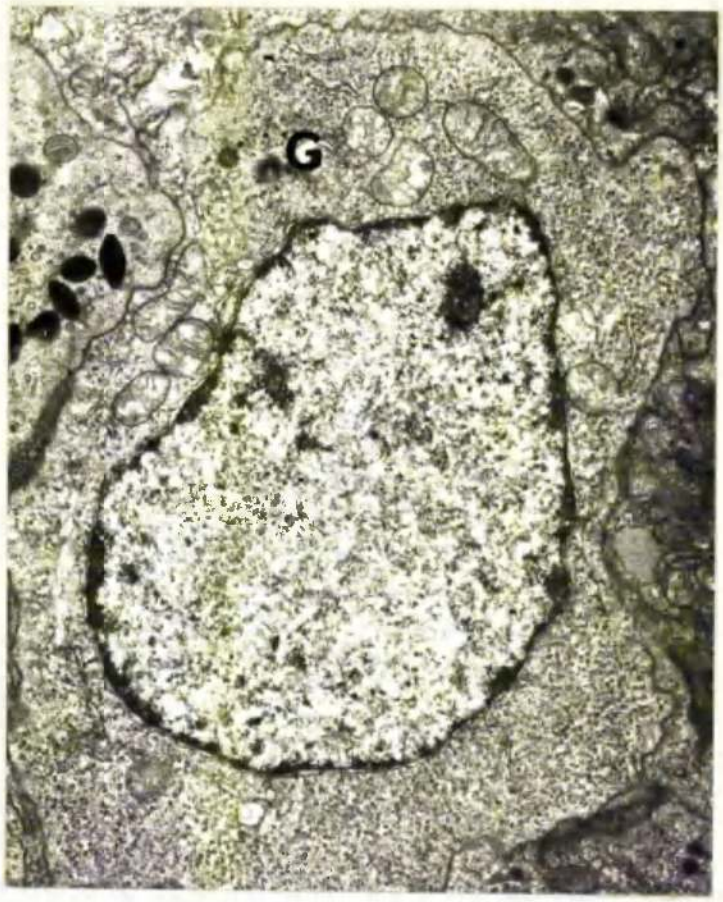


Figure 89.

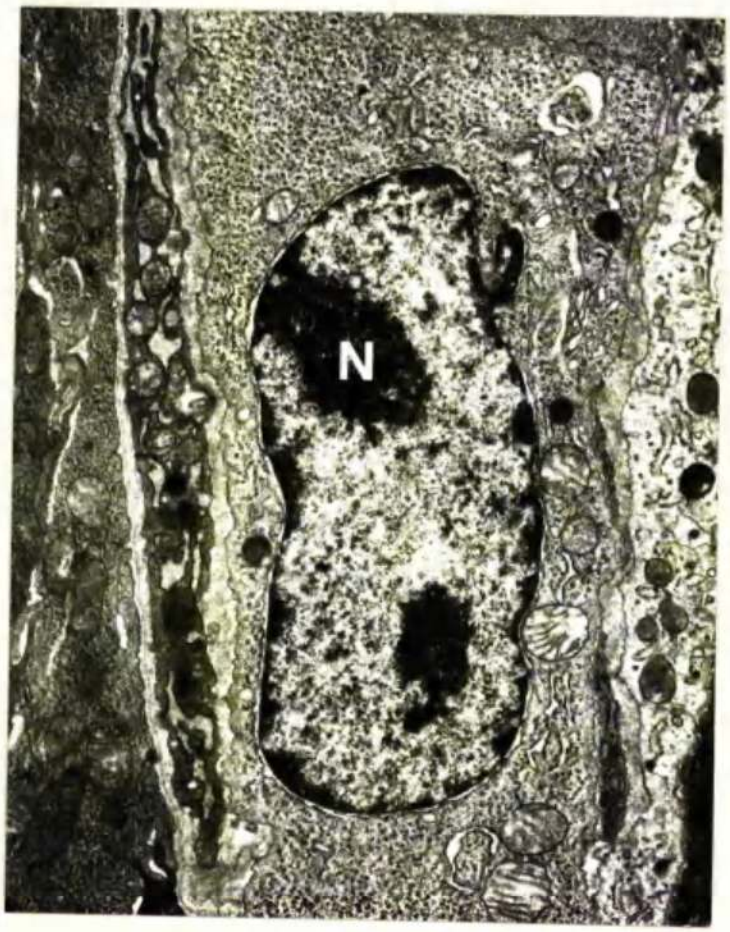


Figure 90.

Figure 91.

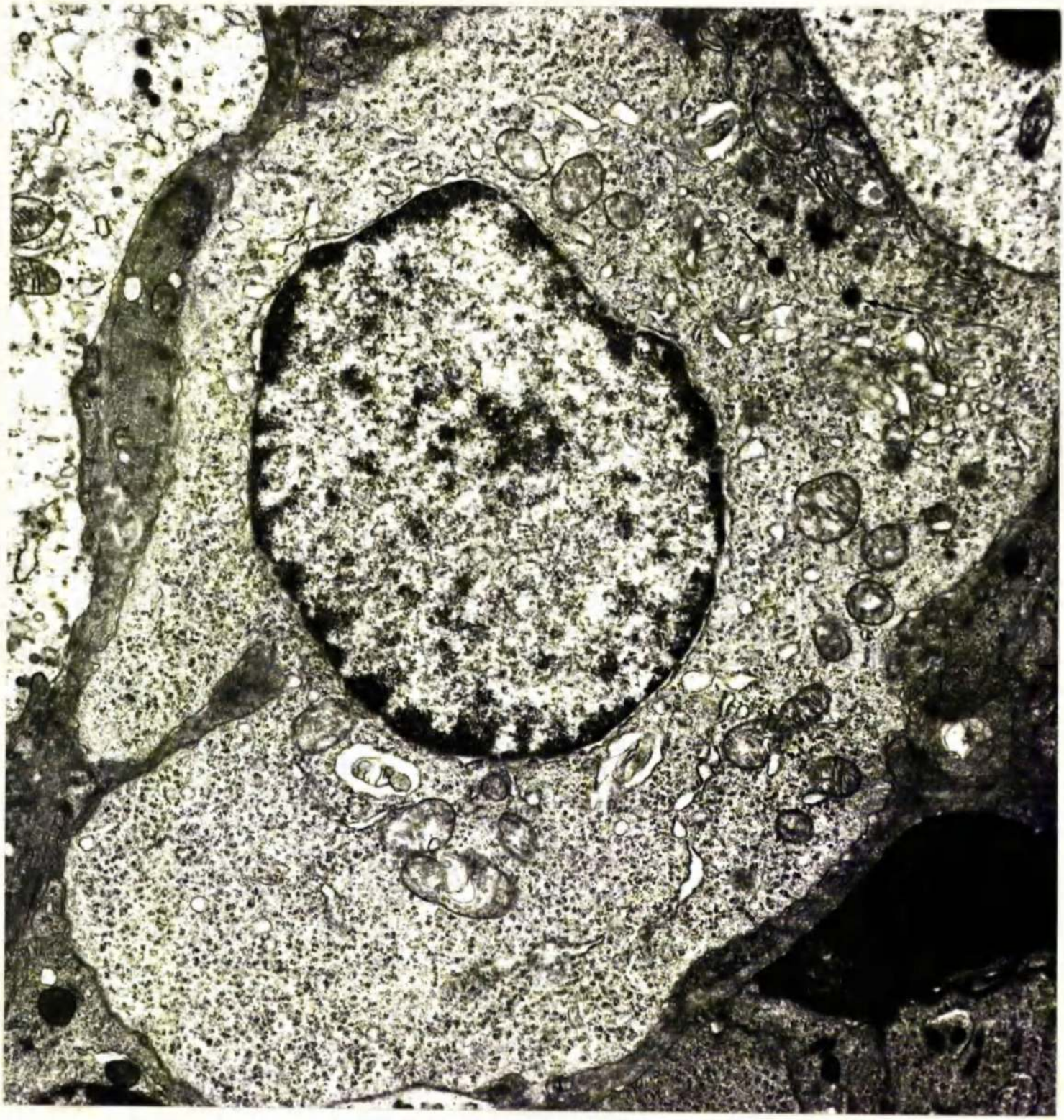


Figure 92. Twelve days after infection. Blast cell with abundant cytoplasm. Progranules (arrows) are located within a moderately well developed Golgi complex. x 14,000.

Figure 93. A maturing IM cell in the intestinal lamina propria. Twelve days after infection. Homogeneous electron-dense granules are distributed in the cytoplasm. Other features are similar to the blast cells described earlier. x 8,700.

INSET shows the same cell located in a thick section; note the deep basophilia of the granules. Azure II - methylene blue - borax. x 1,900.

Figure 94. GL cell in the basal epithelium (twelfth day of infection). A progranule (arrow) is fusing with a large granule. Progranules (P) are numerous in the cytoplasm. There are many strands of RSER as well as polyribosomes. The cisternae of the Golgi complex are partially encircled by the granules. x 8,800.

INSET. The same cell viewed by light microscopy. Azure II - methylene blue - borax. x 1,900.

Figure 95. Part of an IM cell in mitosis (Day 12). Small electron-dense foci are present within the borders of several granules. The profiles of vesicles (arrow) can also be distinguished. x 25,000.

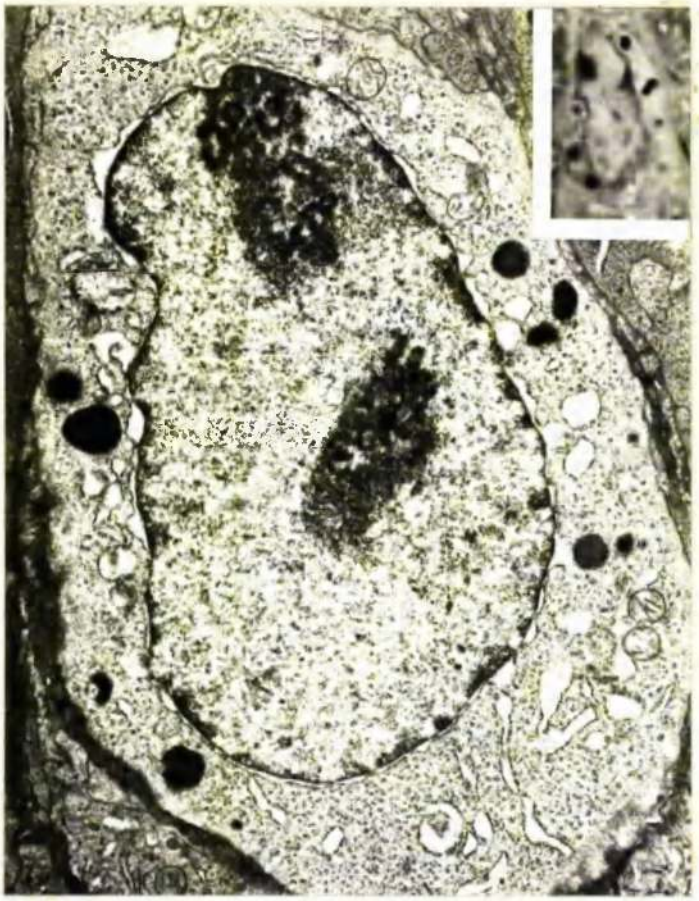


Figure 93.

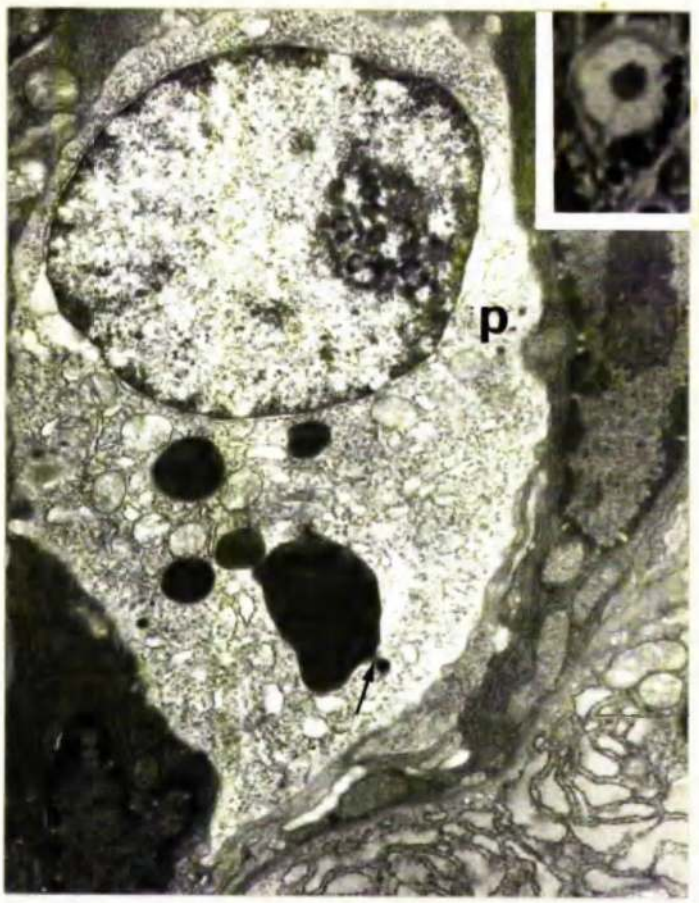


Figure 94.

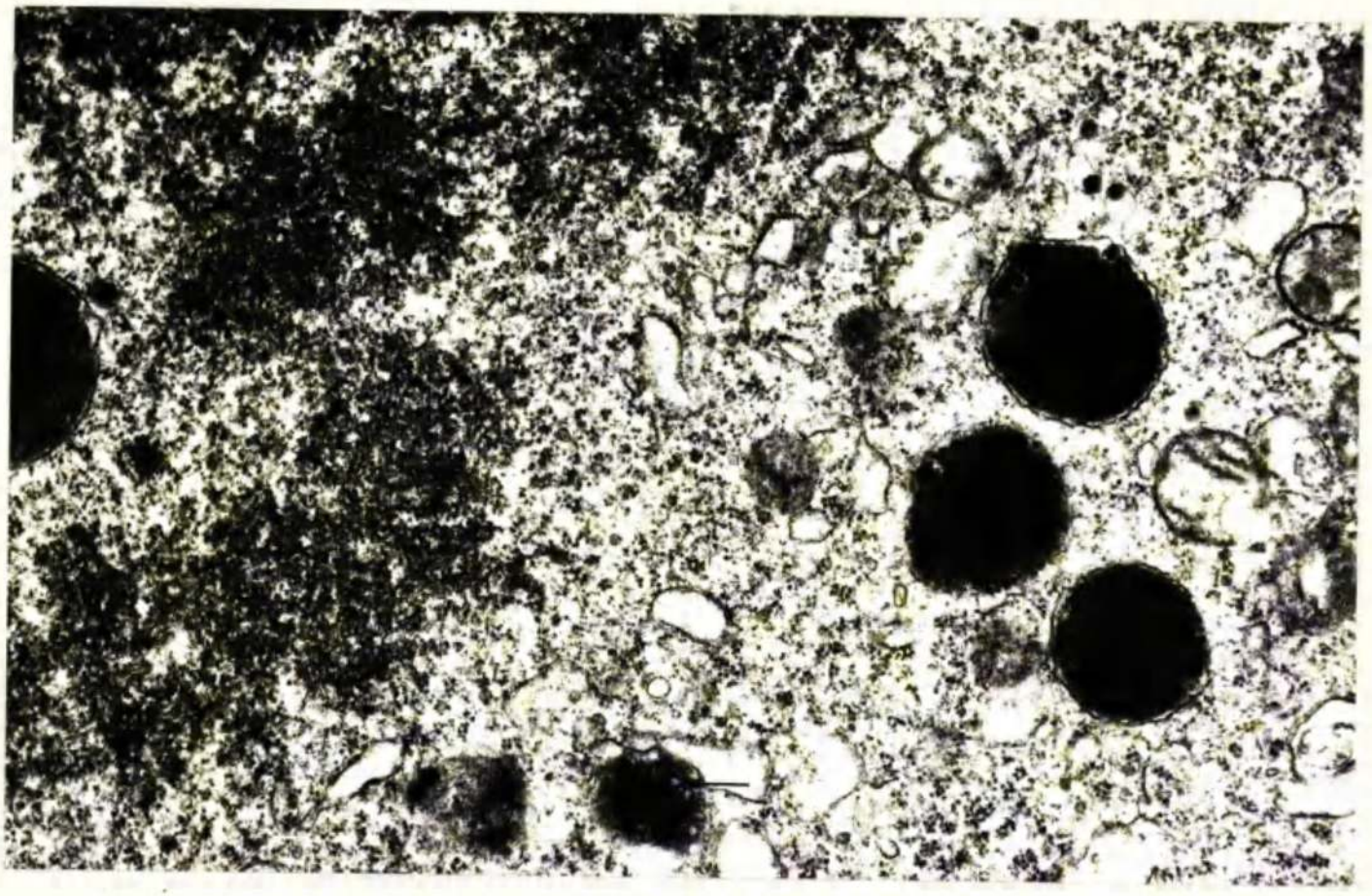


Figure 95.

Figure 96. The Golgi complex of a maturing IM cell, twelve days after infection with N. brasiliensis. Vesicles (arrows) are fusing with a condensing vacuole. The granules are moderately large and the majority have homogeneous matrices. Progranules and vesicles are abundant within the complex and the surrounding cytoplasm is densely packed with polyribosomes. x 16,750.

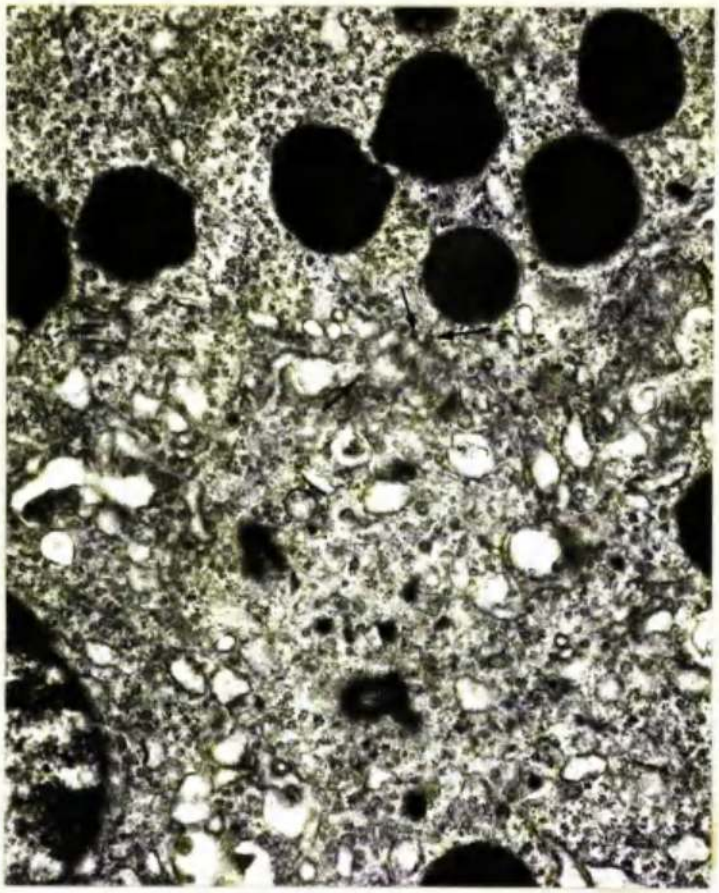
Figure 97. The Golgi complex in this IM cell fourteen days after infection is less elaborate and polyribosomes and RSER are reduced in amount. C, condensing vacuole. x 15,000.

Figure 98. Several progranules surround a small accumulation of matrix which is only partially delimited by a unit membrane (arrow). Material of similar density (arrowhead) is pinching off from the periphery of a Golgi cisternum. T, transitional ER. C, coated vesicle. (Day 12). x 37,500.

Figure 99. The profiles of vesicles (arrows) can be distinguished within the borders of a granule precursor and in the adjacent cytoplasm. (Day 12). x 26,250.

Figure 100. A granule is delimited by a double unit membrane (arrow). Two electron dense foci are located within the membrane of a nearby granule (Day 12). x 30,000.

Figure 101. A coated vesicle (C) is budding from the plasmalemma of an IM cell. Nineteen days after infection. x 30,000.



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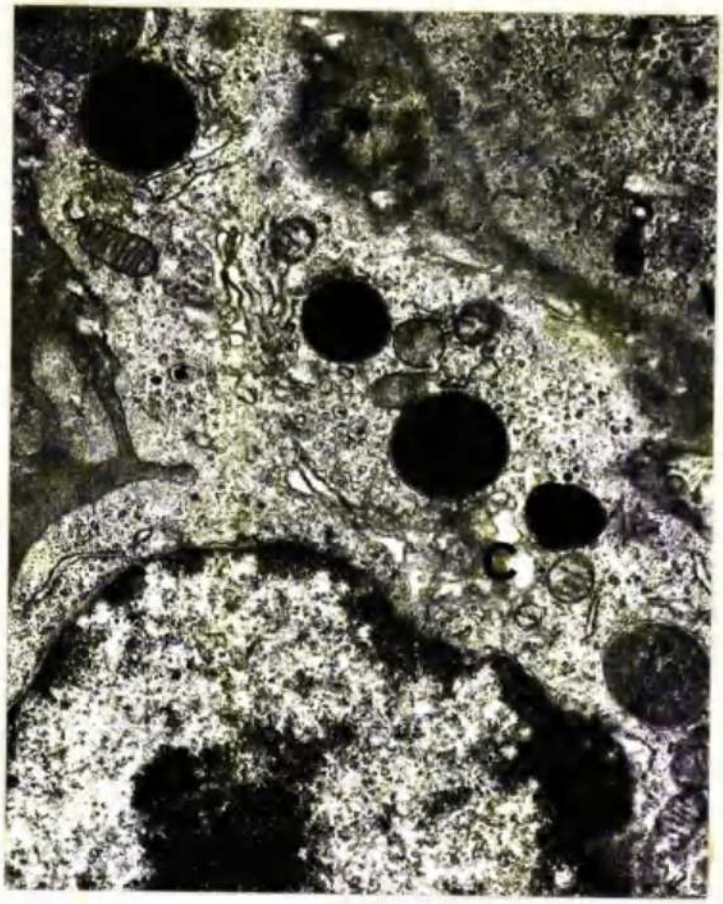


Figure 97.

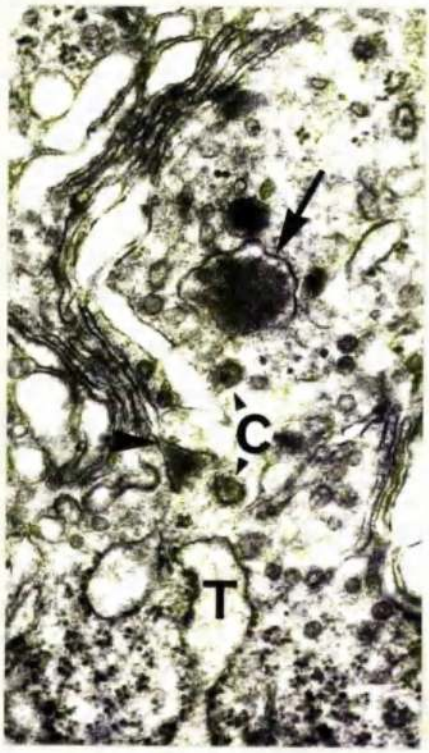
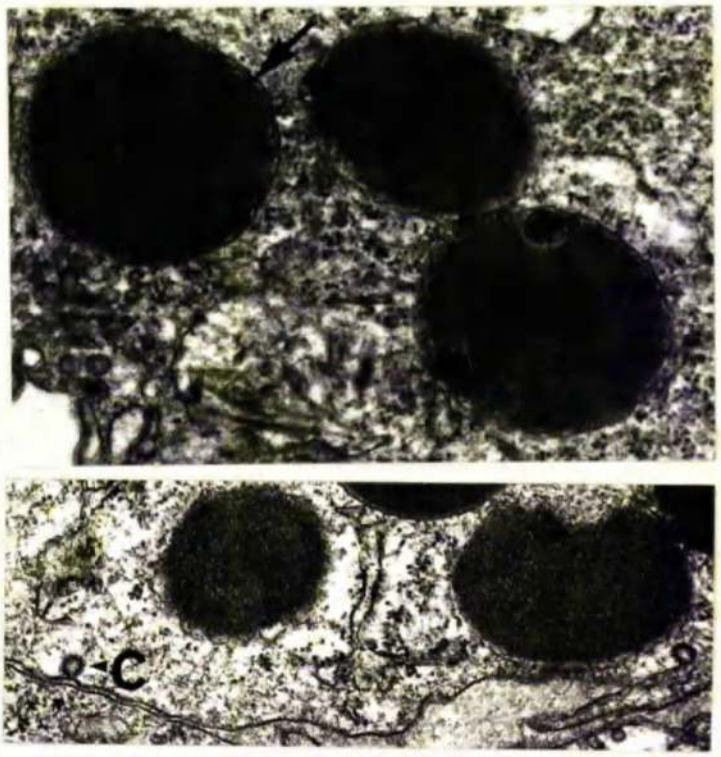


Figure 98.

Figure 99.



Figures 100 and 101.

Figure 102. IM cell fourteen days after infection; the granules are more numerous but the cytoplasmic content of polyribosomes has decreased when compared with cells on previous days of infection. Cisternae of RSER are relatively abundant. Vesicles (arrow) are clustered around the border of a granule. x 12,500.

Figure 103. Light-micrograph of a GL in mitosis on the fourteenth day of infection. 1.5μ Araldite-Epon section. Azure II - methylene blue - borax. x 1,800.

Figure 104. IM cell in mitosis. Day 14. Preparation the same as Figure 103. x 1,600.

Figure 105. Sixteen days after infection. Nucleoli (arrows) are present in several IM cells. Note the variable size of the granules. Preparation the same as Figure 103. x 1,800.

Figure 106. Electron-micrograph of an IM cell sixteen days after infection. The cytoplasm is densely populated by granules. x 10,000.

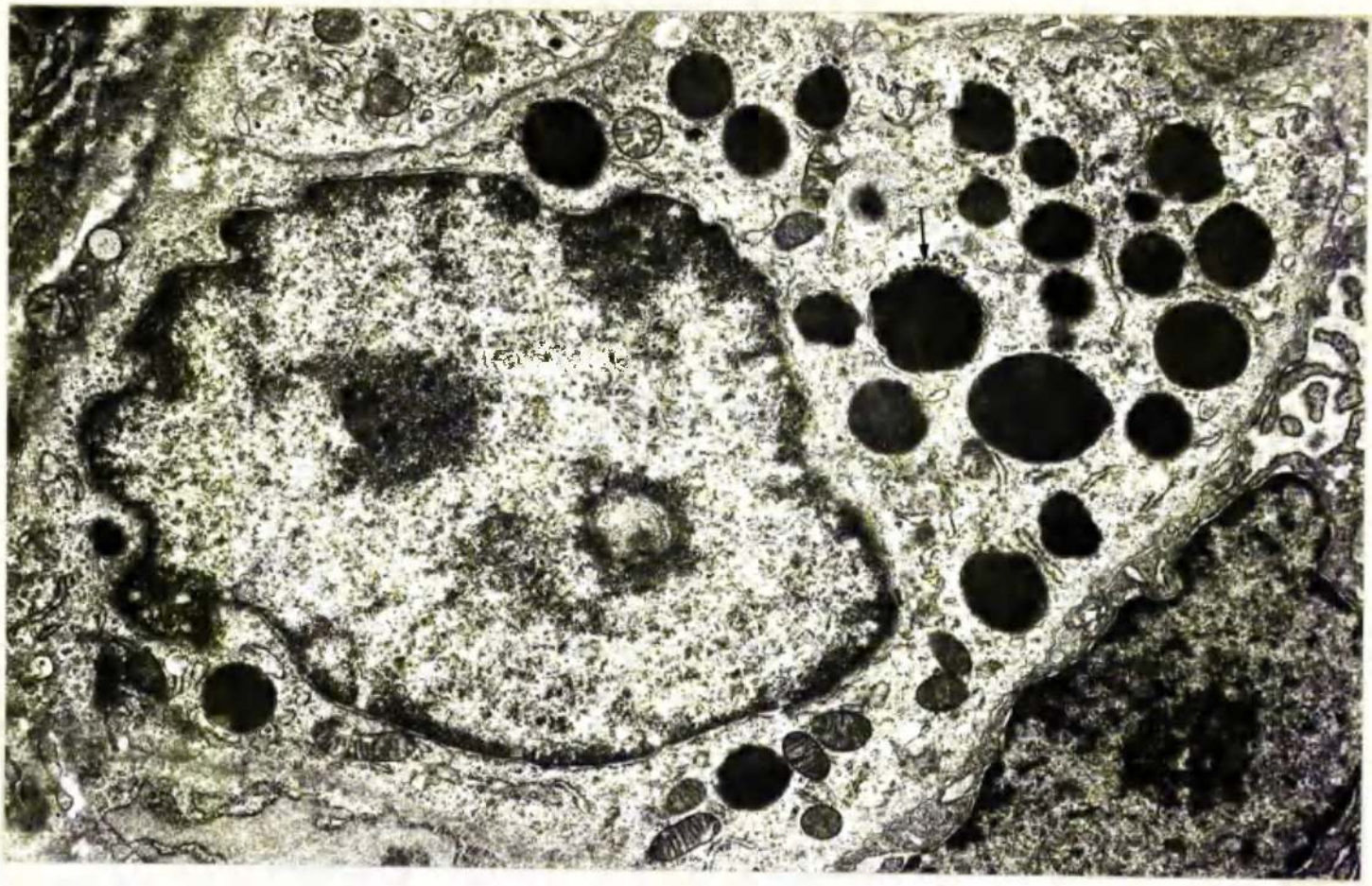
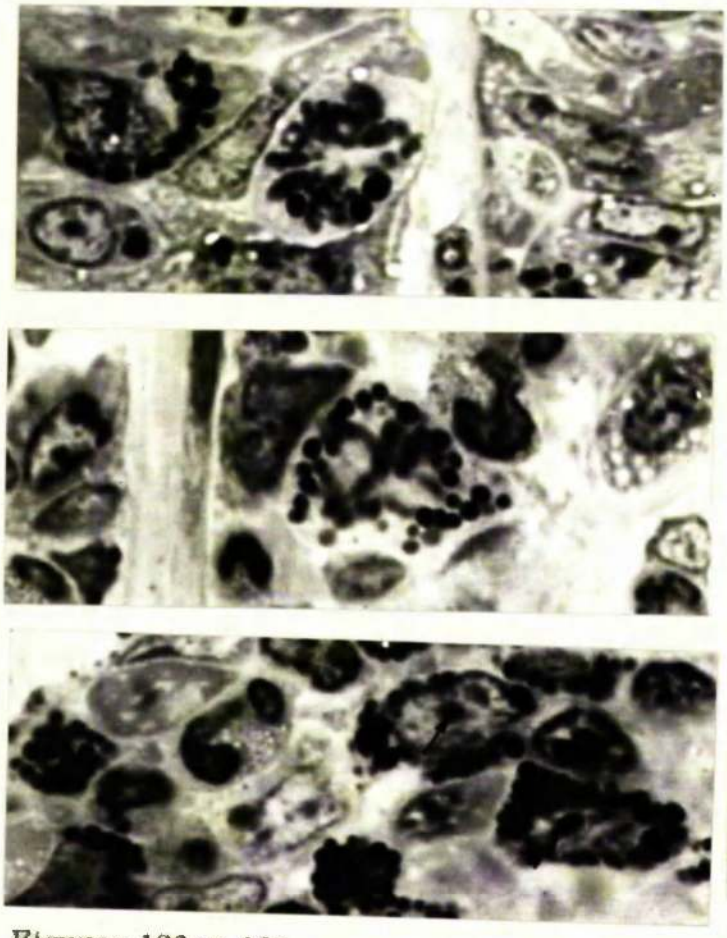


Figure 102.



Figures 103 to 105.

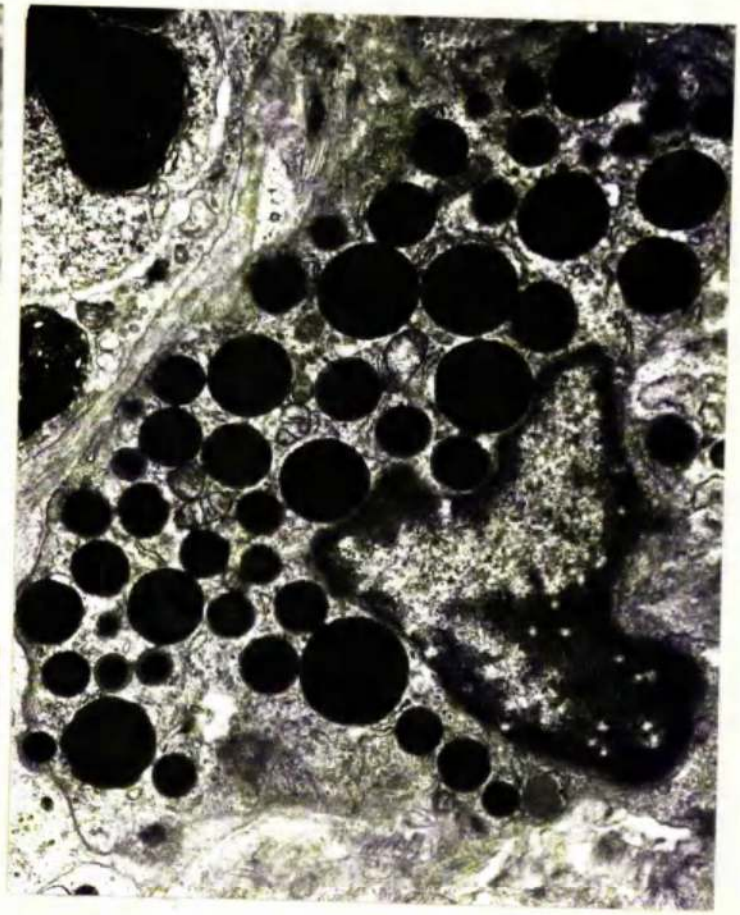


Figure 106.

Figure 107. The Golgi complex of an IM cell sixteen days after infection. Coated vesicles (arrowheads) are distributed in the cytoplasm and are fusing with or budding from (arrows) a cisternum (C) containing faintly electron-dense material. x 30,000.

Figure 108. Vacuole (V) delimited by several unit membranes and containing strands of RSER. Fourteenth day of infection. x 25,700.

Figure 109. Vesicles, dense bodies and cytoplasm enclosed by a vacuolar membrane (arrow). Fourteenth day of infection. x 18,750.

Figure 110. Point of fusion (arrow) of a progranule with vacuole containing a membrane-delimited dense body. Day nineteen of infection. x 30,000.

Day 111. Accumulation of matrix within a vacuole in which cytoplasmic and membranous components are also included. x 25,000.

Figure 112. Myelin-like configurations, vesicles and tubular structures within a GL cell granule. x 24,000.

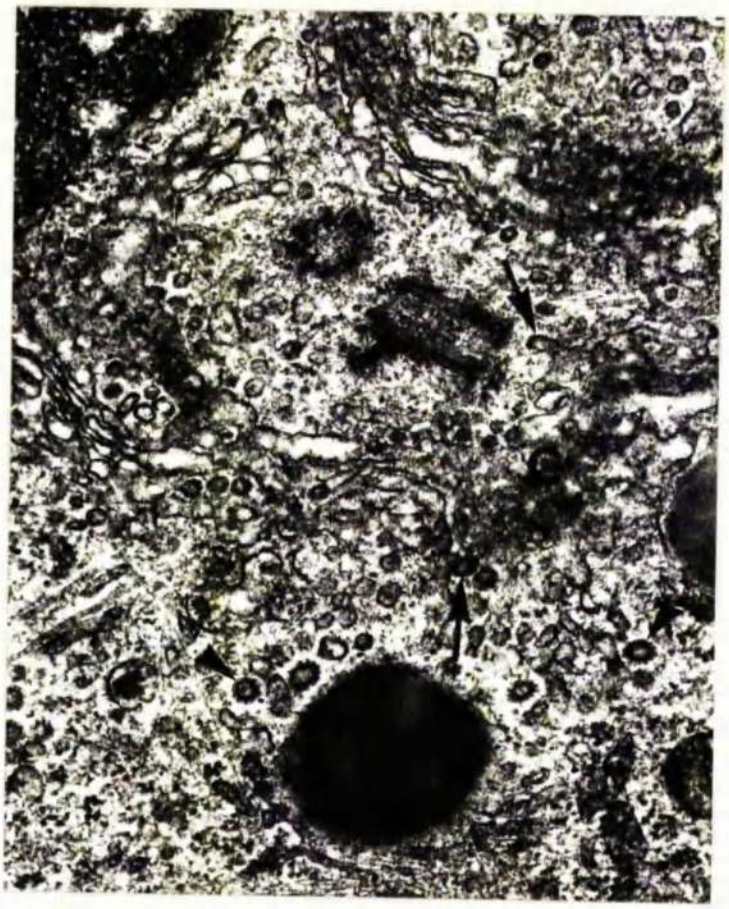


Figure 107.

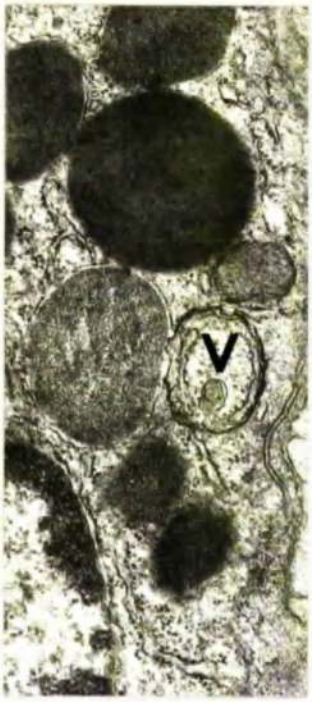
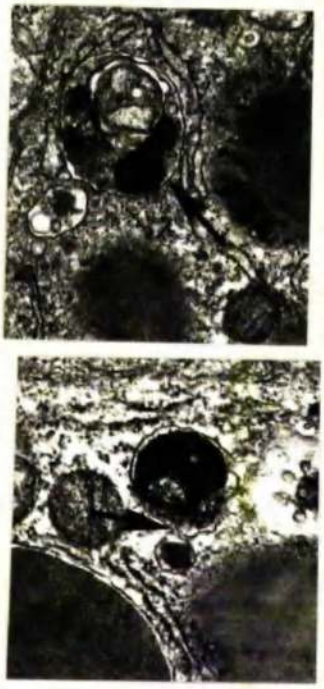


Figure 108.



Figures 109 and 110

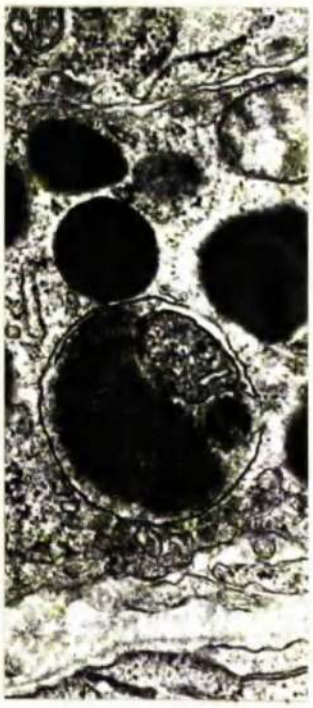


Figure 111.

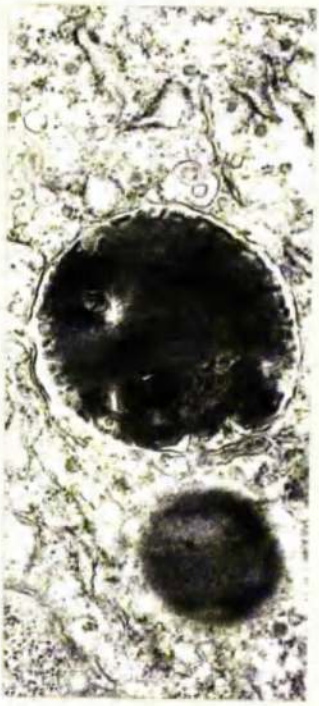


Figure 112.

Figure 113. Maturing IM cell on the twelfth day of infection. The matrices have been lost from several of the granules (arrows) and paracrystalline structures remain in the vacuoles. x 8,750.
INSET. Light-micrograph of the same cell. Note the deep basophilia of one of the granules; the other stains less strongly. Azure II - methylene blue - borax. x 1,700.

Figure 114. Paracrystalline structures in an altered granule of a maturing mast cell (twelfth day of infection). Note the parallel array of fibres which, on cross section, have a lattice arrangement (arrow). A trilaminar unit membrane (arrowhead) only partially surrounds the vacuole. x 70,000.

Figure 115. The sequential granule changes are suggested in this micrograph. The electron density of the matrix is slightly decreased and the paracrystalline structures are just visible (1). A further decrease in the density of the matrix (2) is followed by its complete loss from the granule (3). The membranes surrounding the altered granules are disrupted at several points (arrowheads). Twelfth day of infection. x 24,200.
INSET. Light-micrograph of a similar cell in which weak-staining granules (arrows) and vacuoles (arrowheads) can be distinguished. Twelfth day of infection. Azure II - methylene blue - borax. x 1,800.

Figure 116. GL in mitosis, fourteenth day of infection. The granules show the range of change described in Figure 115. A myelin figure (arrow) and vesicles can be distinguished at the borders of several granules. C, chromatin. x 22,500.

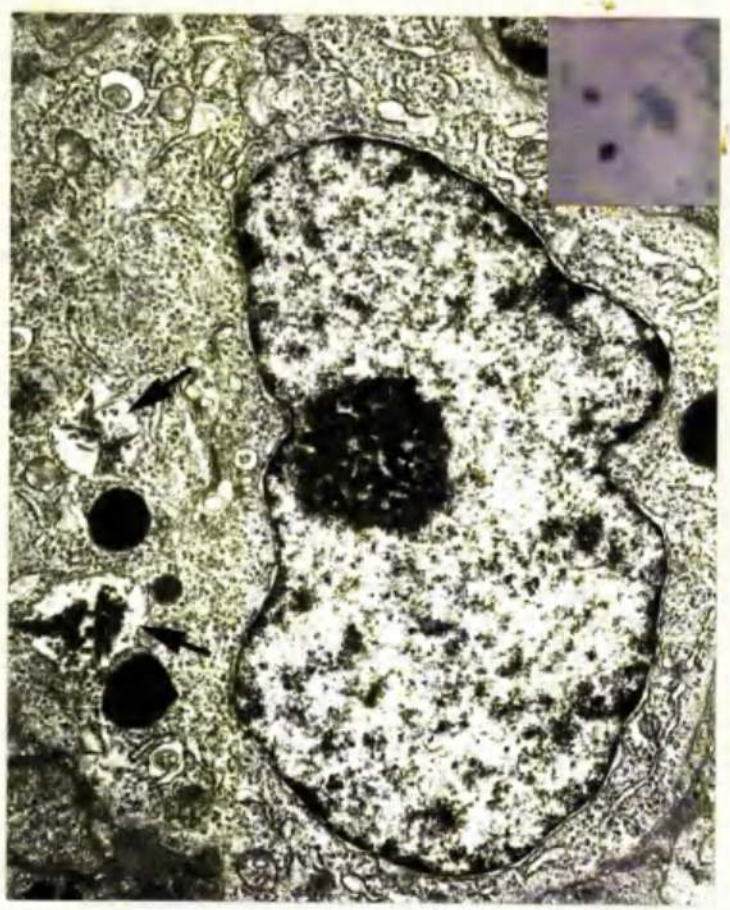


Figure 113.

Figure 114.

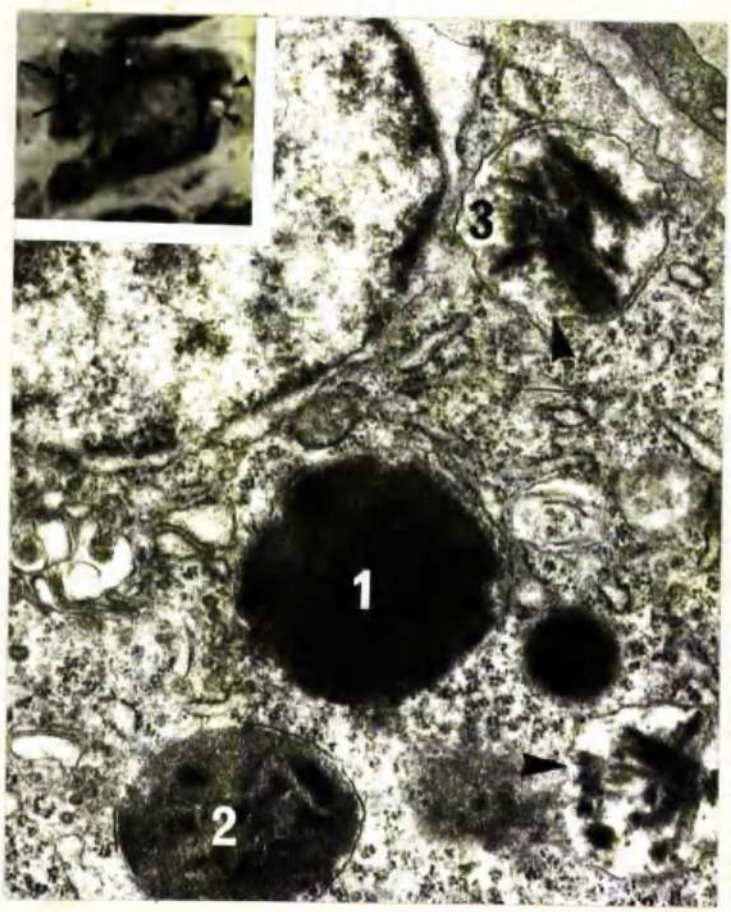


Figure 115.

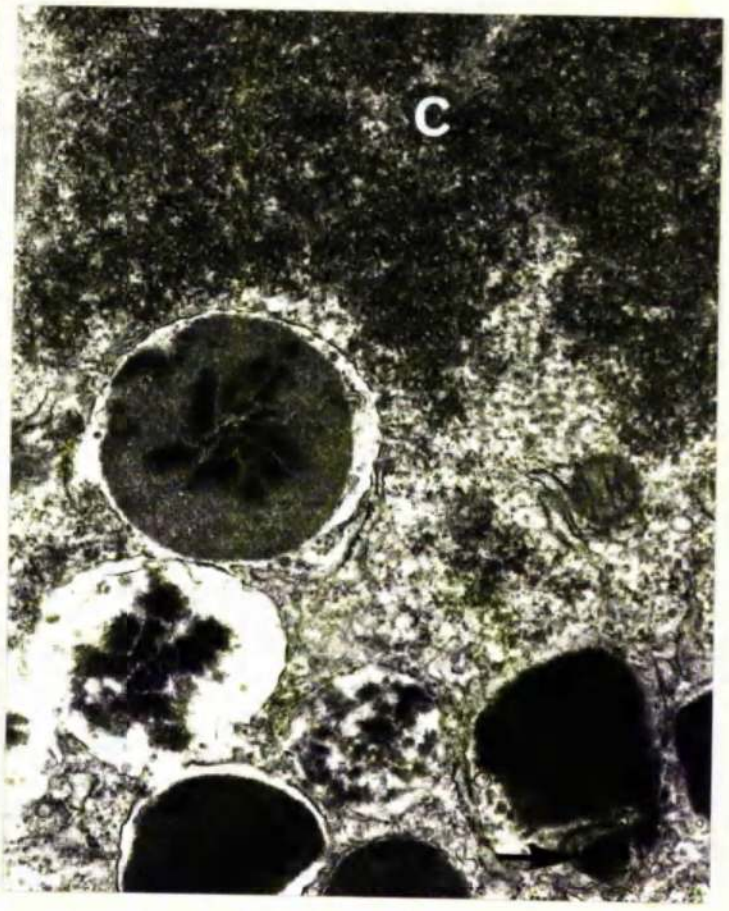


Figure 116.

Figure 117. IM cell fourteen days after infection. The granule matrices have low electron-density and paracrystalline structures can be distinguished within them. A double delimiting membrane (arrowhead) partially surrounds an altered granule. The perinuclear cisternum and the cisternae of RSER are dilated and oval structures (arrows) lie within them. x 15,000.

Figure 118. Light-micrograph of a vacuolated IM cell (arrow). The granules of a cell nearby (arrowhead) stain with a reduced intensity. Fourteenth day of infection. Azure II - Methylene blue - borax. x 1,800.

Figure 119. A mast cell (M) has faint staining granules. The globule leukocyte (G) also has a weak affinity for basic dyes. Day 14. Azure II - methylene blue - borax. x 1,800.

Figure 120. Extensively vacuolated IM cells (arrows) in the lamina propria on the fourteenth day of infection. The GL (G) is relatively unaltered. Azure II - methylene blue - borax. x 1,700.

Figure 121 The granules in an IM cell have low electron-density and para-crystalline structures are evident in the matrices. The plasmalemma is interrupted at several points (arrowhead). The vestiges of a more severely altered cell, identifiable by the paracrystalline structures (arrow) lie nearby. The nuclear chromatin of both cells is coarsely precipitated. Day fourteen. x 8,500.

Figure 122. High power micrograph of the cell in Figure 121. Note the beaded appearance of the plasmalemma, the swollen perigranular membranes, the altered RSER and the disrupted perinuclear cisternum. x 16,700.

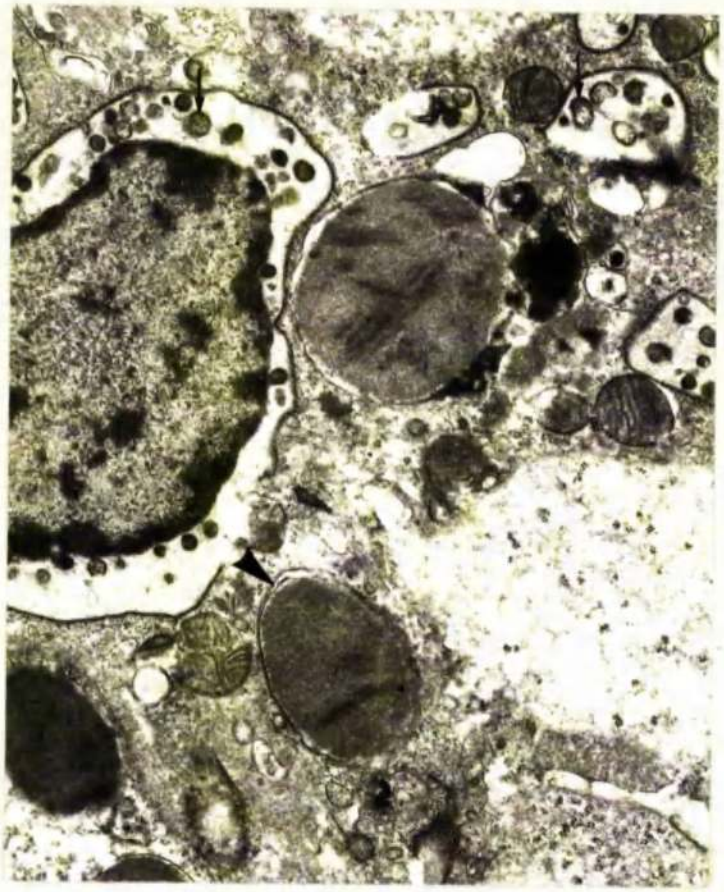
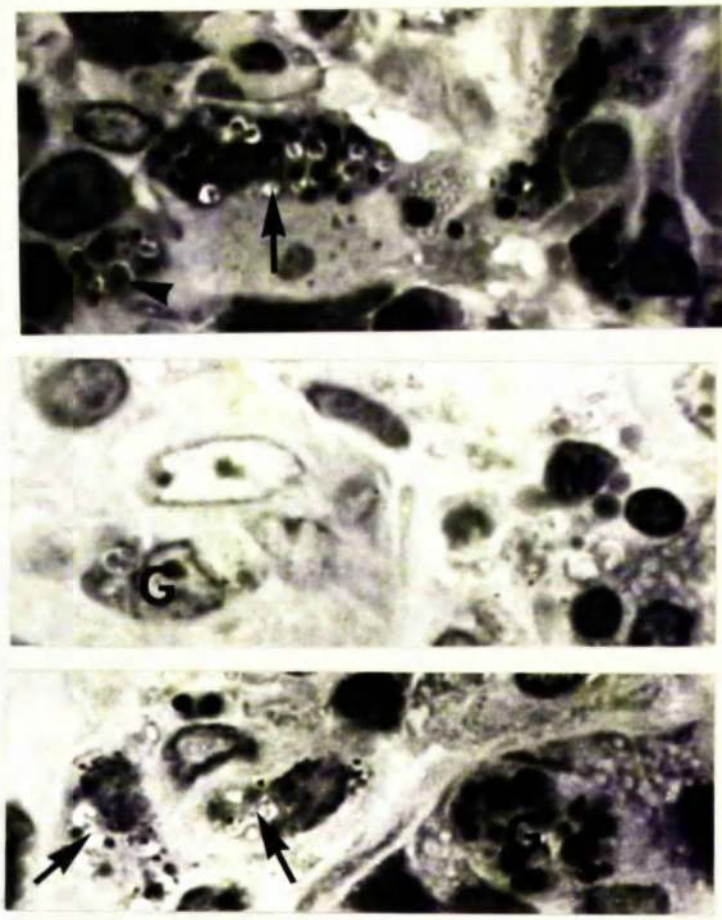


Figure 117.



Figures 118 to 120

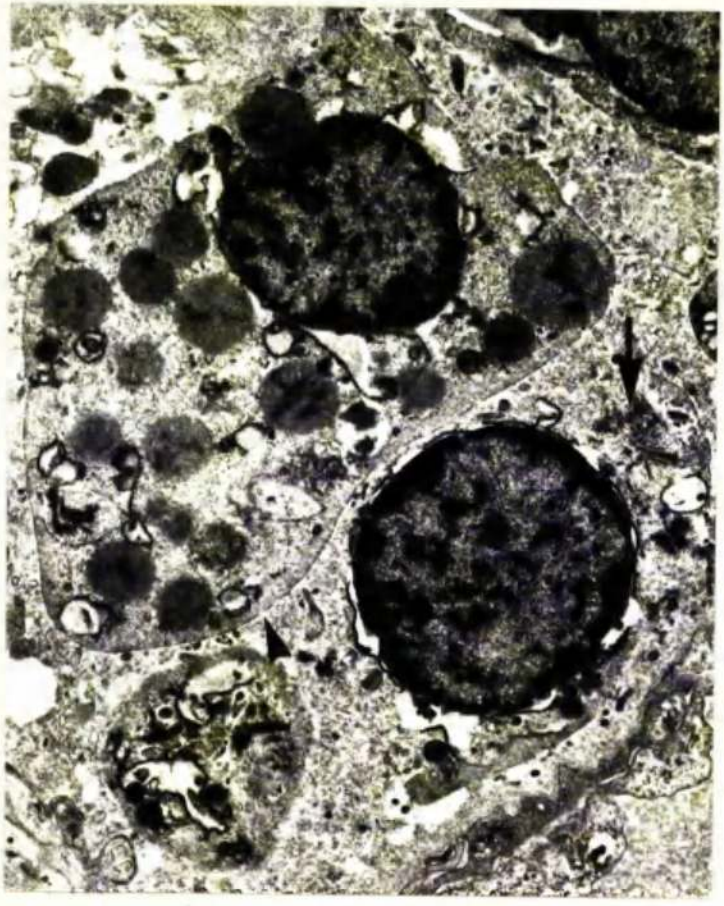


Figure 121.

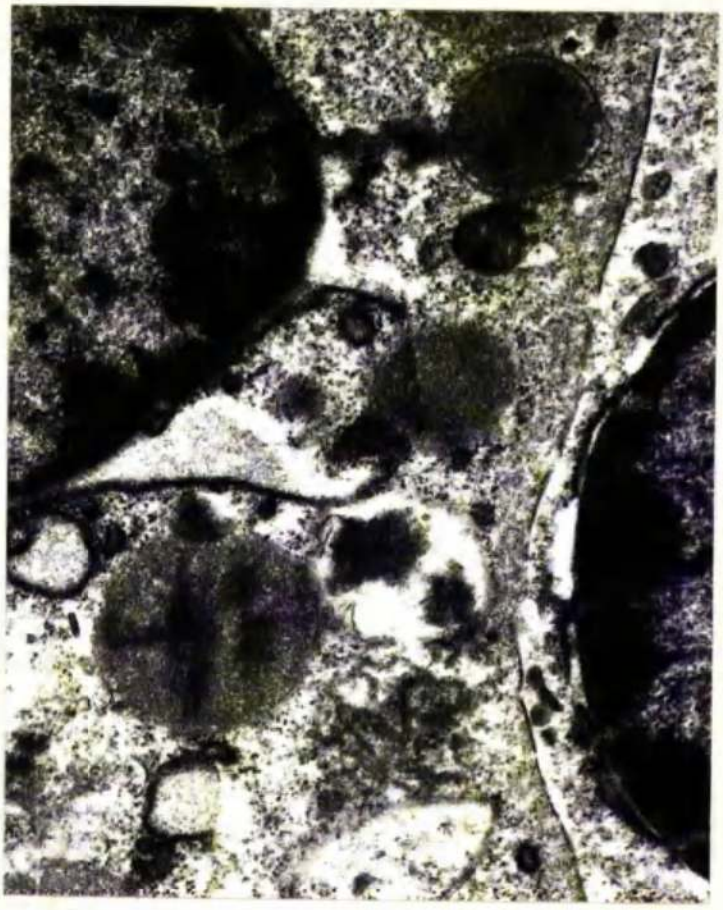


Figure 122.

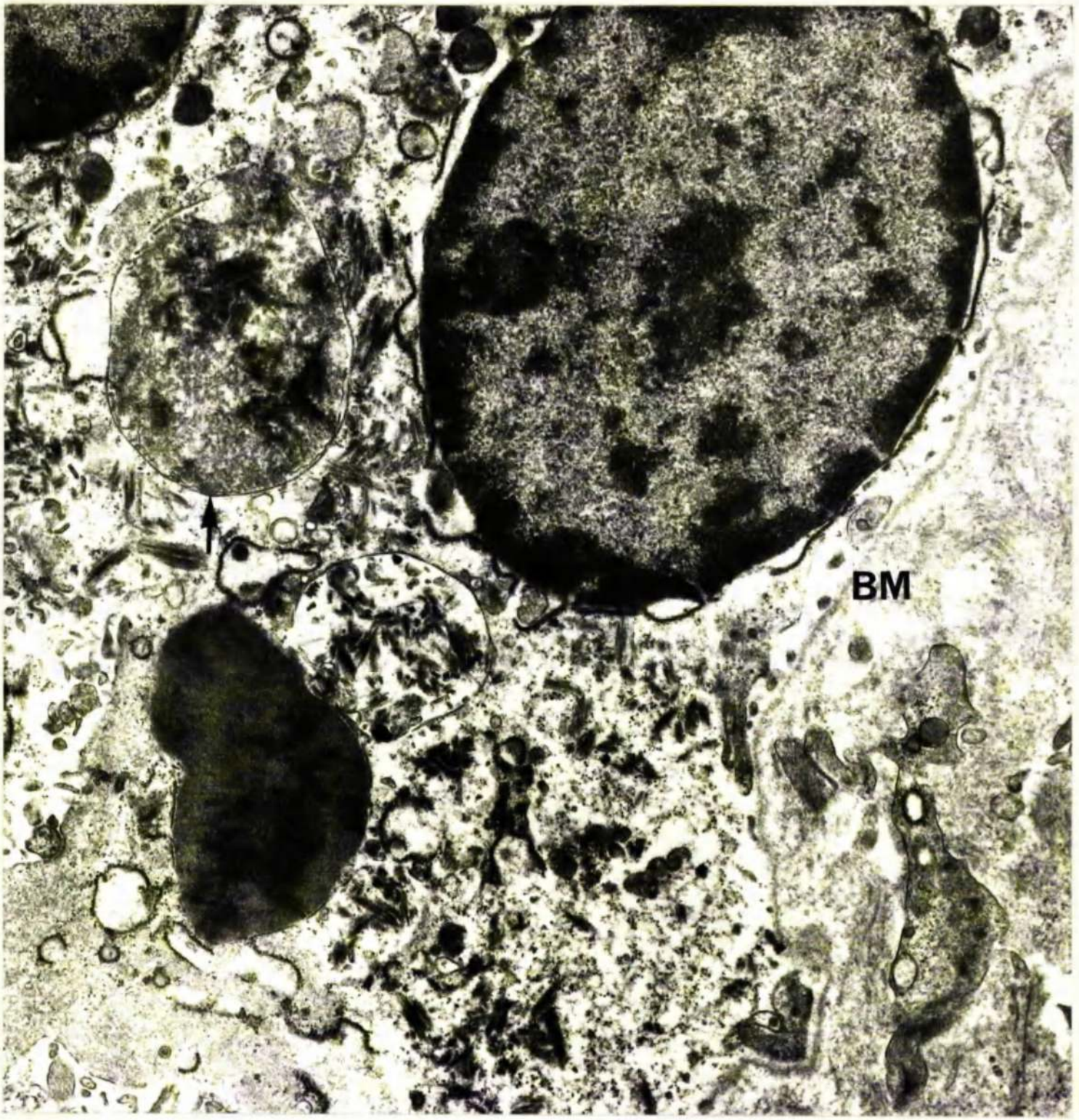


Figure 123. Discharging GL. Fourteenth day of infection. A double unit membrane (arrow) partially surrounds an altered granule. BM, epithelial basement membrane. x 21,000.

Figure 124. Light micrograph of mucosal cytolysis. There are several fragmented IM cells with pale-staining granules (arrows). A disrupted eosinophil (E) and an area of cytolysis (C) are also apparent. There is a gap (G) in the epithelium which extends to the level of the epithelial basement membrane. Fourteenth day of infection. Azure II - methylene blue - borax. × 1,800.

Figure 125. A few membrane fragments (arrows) outline an IM cell which has undergone diffuse lysis. The dense crystalline bars of disrupted eosinophil granules (E) are scattered in the lamina propria. Sixteenth day of infection. × 10,000.

Figure 126. Intestinal mucosa thirty-five days after infection. The majority of IM cells have uniformly electron-dense and homogeneous granules. G, globule leukocyte. × 3,750.

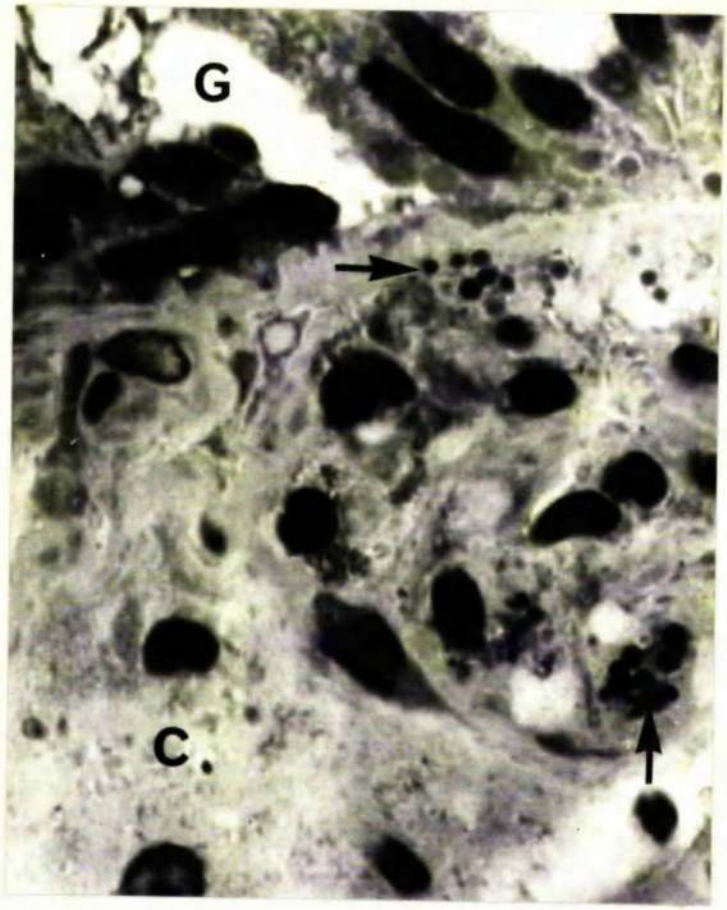


Figure 124.

Figure 125.

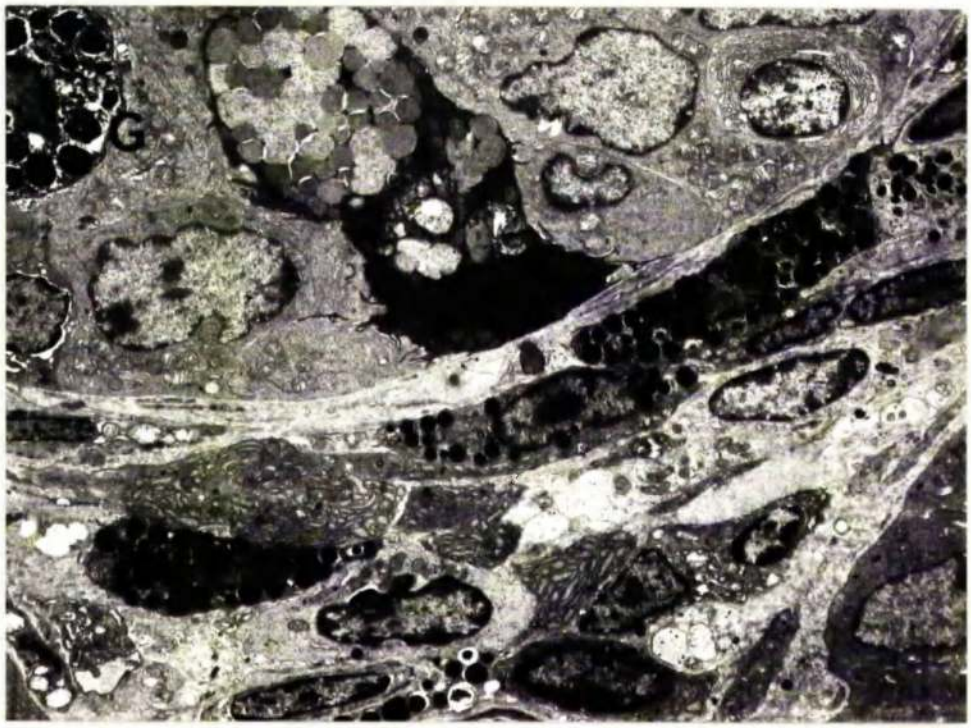


Figure 126.

Figure 127. IM cells in the intestinal lamina propria of a normal rat. Alcian blue 8 GX. 0.4 M $MgCl_2$. x 430.

Figure 128. As in Figure 127, but with 0.8 M $MgCl_2$. x 430.

Figure 129. IM and GL cells eleven days after infection with N. brasiliensis. Alcian blue 8 GX. 0.4 M $MgCl_2$. x 430.

Figure 130. As in Figure 129, but with 0.8 M $MgCl_2$. x 430.

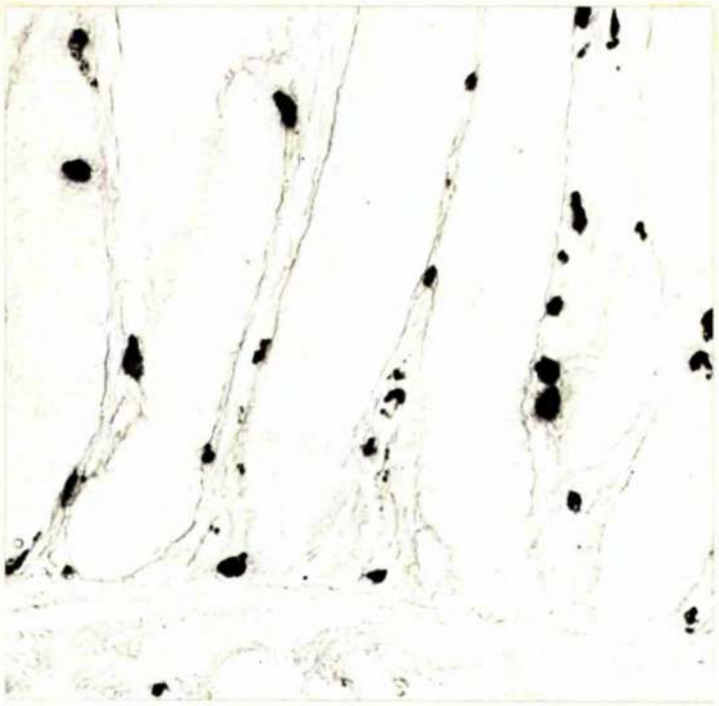


Figure 127.

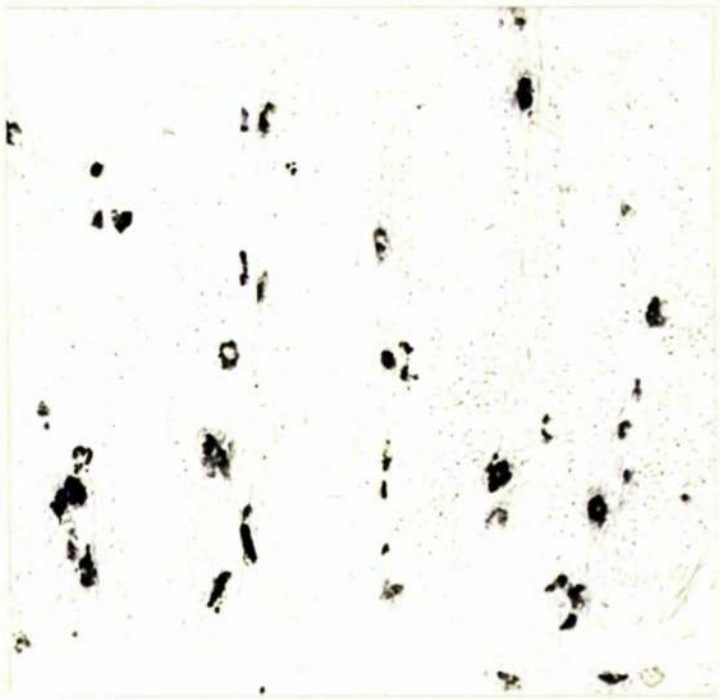


Figure 128.

Figure 129.

Figure 130.

Figure 131. Intestinal mucosa of a normal rat. Alcian blue 8 GX. 0.4 M $MgCl_2$. x 150.

Figure 132. As above, but with 0.8 M $MgCl_2$. x 150.

Figure 133. IM and GL cells in the mucosa on the sixteenth day of infection. Alcian blue 8 GX. 0.4 M $MgCl_2$. x 150.

Figure 134. The staining of the majority of GL cells and of a proportion of IM cells has been cut out by the higher molarity. Alcian blue 8 GX. 0.8 M $MgCl_2$. x 150.

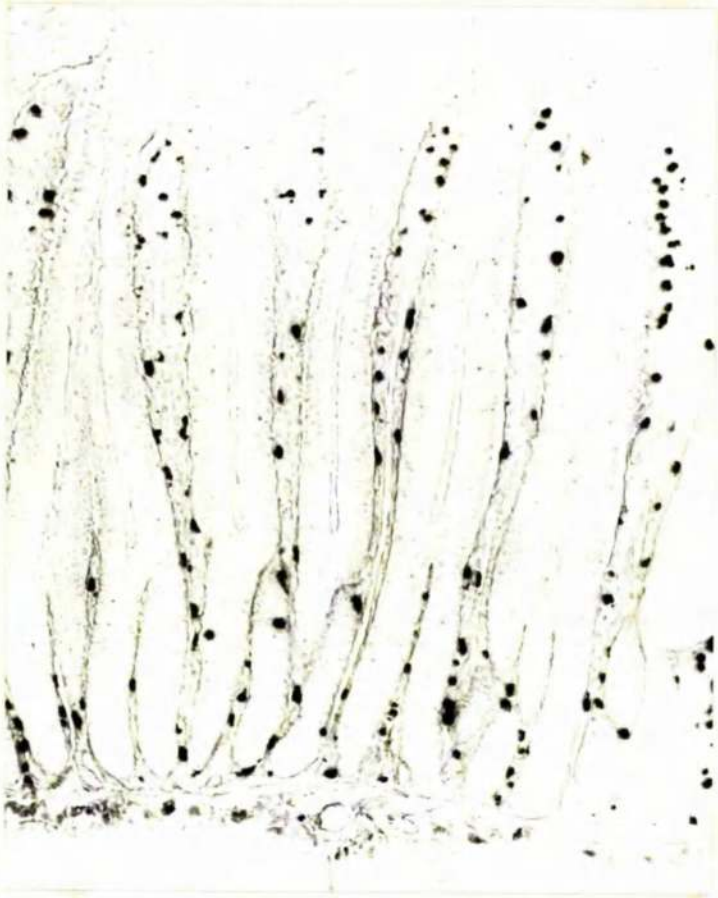


Figure 131.

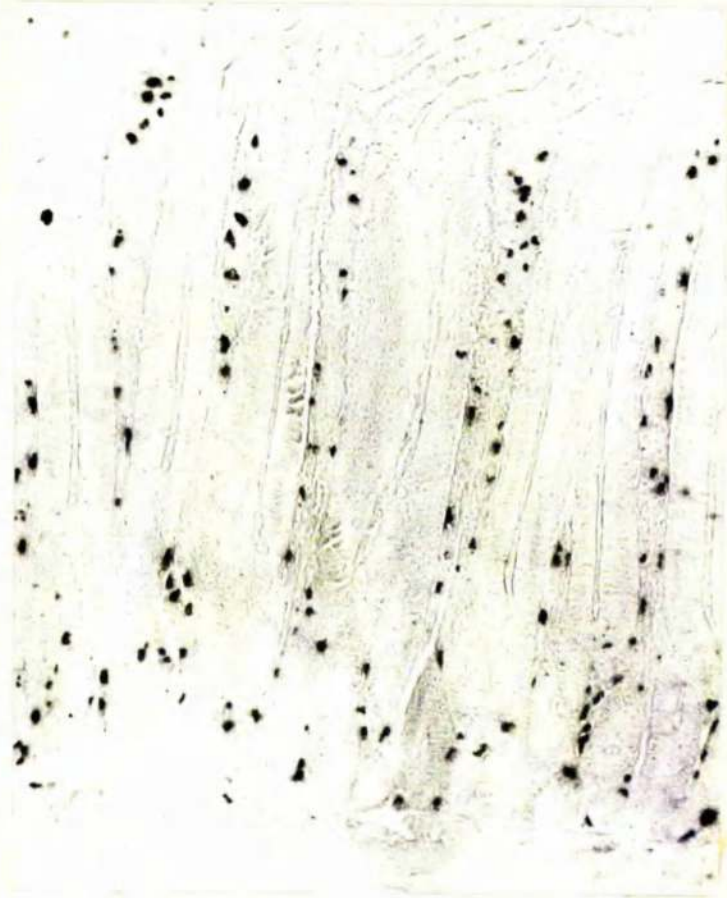


Figure 132.

Figure 133.

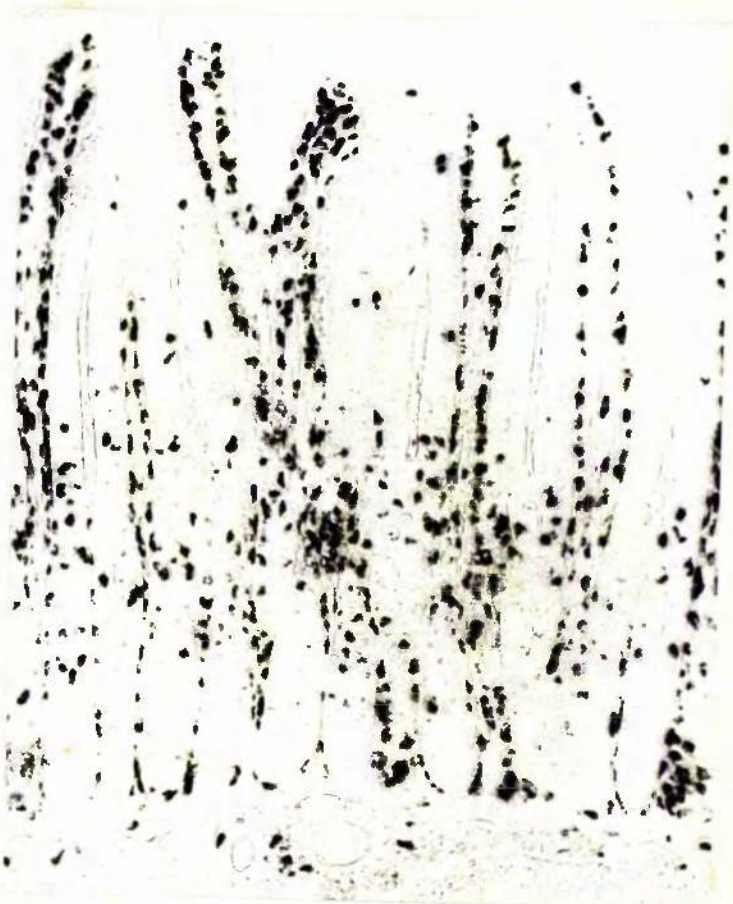


Figure 134.

Figure 135. Falck technique for monoamines. Twelfth day of infection. The cells contain relatively few granules, but have a moderately bright dirty yellow fluorescence. A GL (g) also fluoresces brightly. x 350.

Figure 136. Falck technique for monoamines. Twelfth day of infection. A brightly fluorescent GL x 480.

Figure 137. Falck technique for monoamines. IM and GL cells have a range of fluorescence. Twelve days after infection. e, enterochromaffin cell. x 350.

Figure 138. Falck technique for monoamines. IM cells (m) contain few granules, but fluoresce moderately brightly; the GL cells (g) fluoresce weakly. Twelfth day of infection. x 480.

Figure 139. Falck technique for monoamines. Rat pretreated with L-DOPA; twelfth day of infection. Individual granules of a GL cell which fluoresce a bright green can be distinguished. x 1,400.

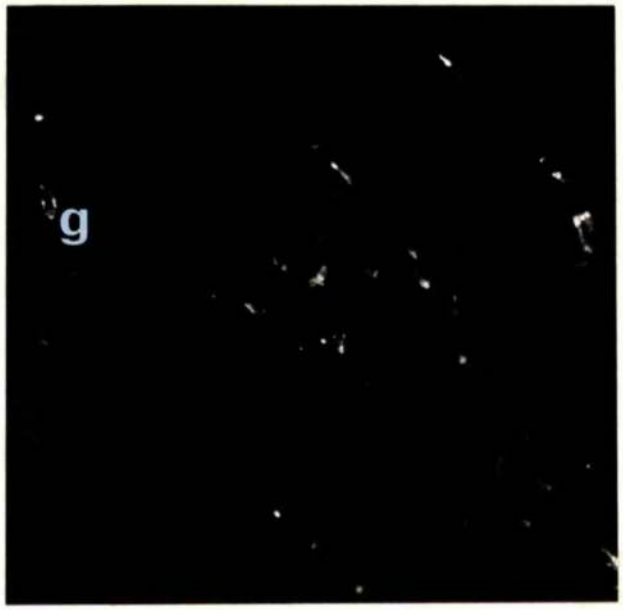


Figure 135.

Figure 136.

Figure 137.

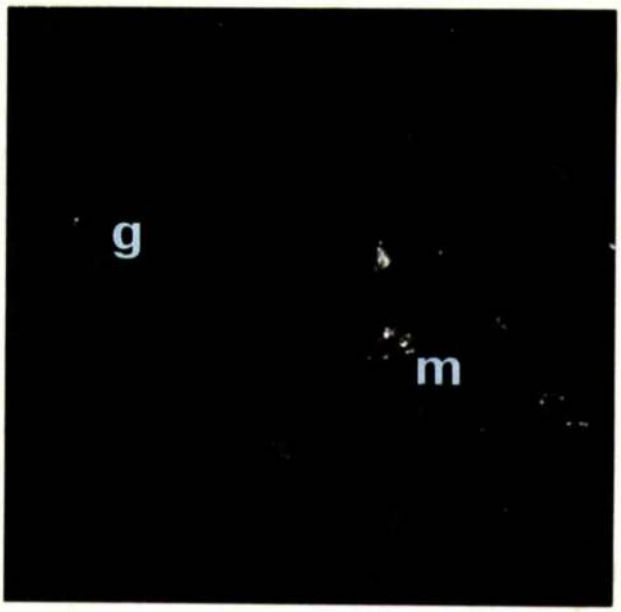


Figure 138.

Figure 139.

Figure 140. Falck technique for monoamines. Fourteenth day of infection. A few cells have retained a moderately bright fluorescence, the others are only just visible. Extremely feeble fluorescence is emitted from several GL cells (g). x 480.

Figure 141. Falck technique for monoamines. Fourteen day of infection after treatment with L-DOPA. There are many IM cells in the lamina propria which have a brilliant green fluorescence. Some of the fragmented cells and isolated granules are also brightly fluorescent. GL cells (g) contain fewer and less bright granules. x 350.

Figure 142. Falck technique for monoamines. Sixteen days after infection; IM cells show variable fluorescence and the granules are scattered in the lamina propria. Few GL cells can be visualised. e, enterochromaffin cell. x 350.

Figure 143. Falck technique for monoamines. Nineteenth day of infection and after treatment with L-DOPA. The IM cells are compact and fluoresce brightly. Globule leukocytes(g) contain few granules and have a weak fluorescence. x 350.

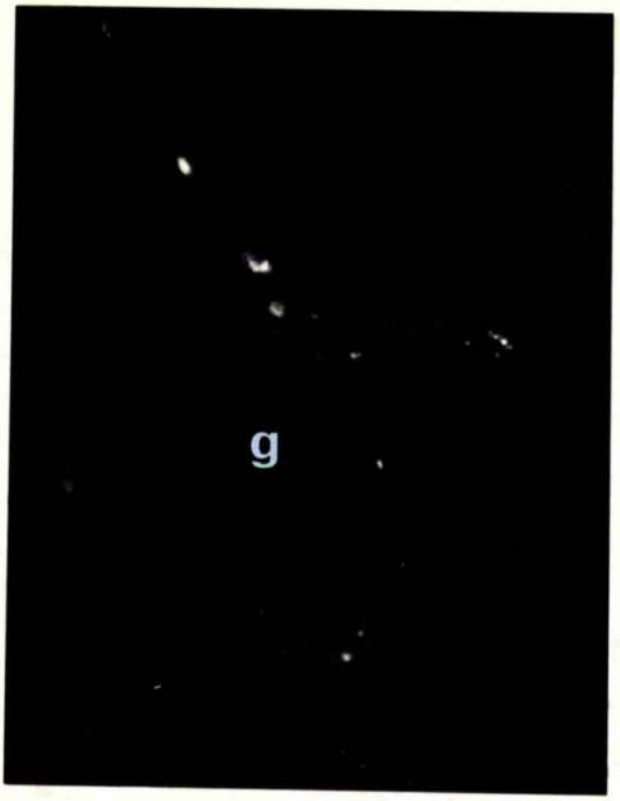


Figure 140.

Figure 141.

Figure 142.

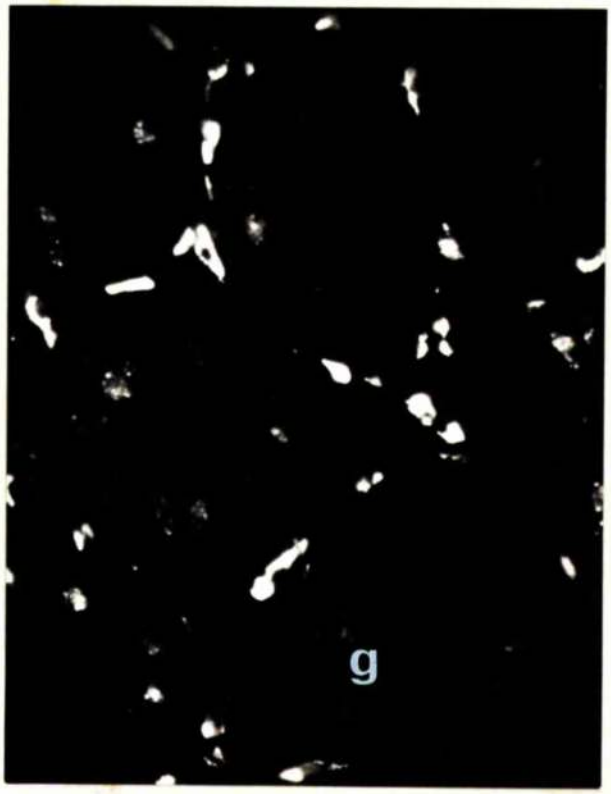


Figure 143.

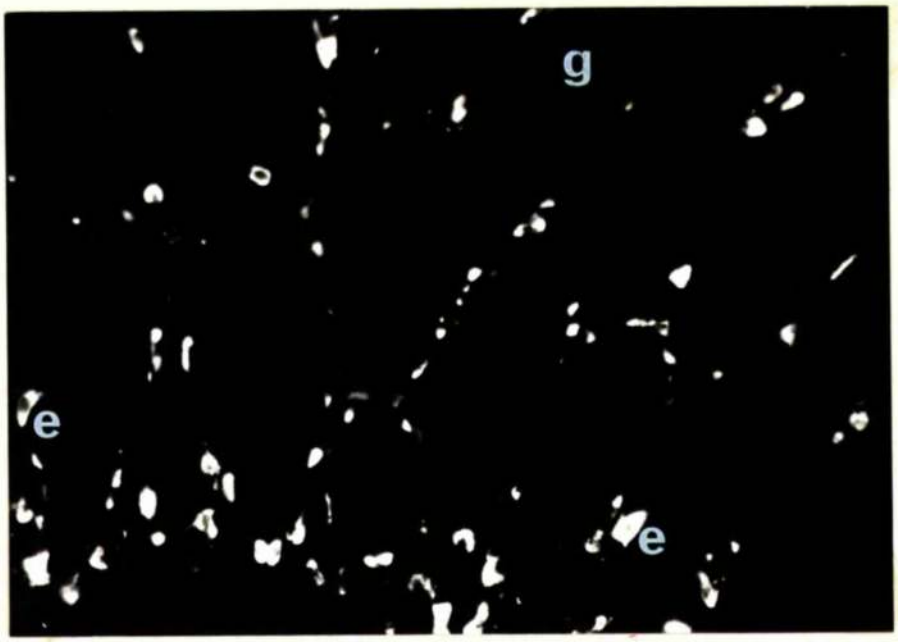
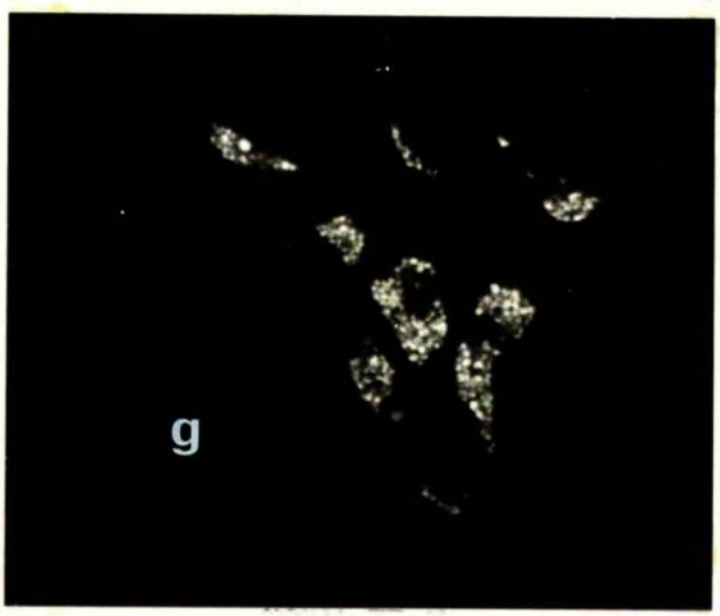


Figure 144. Falck reaction for monoamines. Yellow fluorescent IM cells and enterochromaffin cells (e). Some IM cells have only a weak fluorescence and GL cells (g) can only just be distinguished. Thirty-five days after infection. x 200.



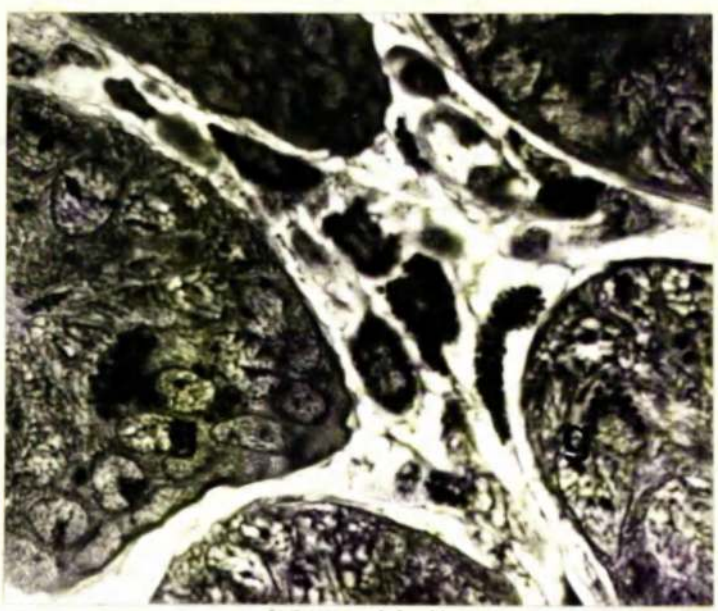


Figure 145 (a)

Figure 145 (b)

Figure 145 (a) Falck reaction for monoamines. Individual granules of IM cells fluoresce strongly. A GL (g) emits a faint fluorescence. Thirty-fifth day of infection. The rat was pretreated with L-DOPA. 1.5μ Araldite-Epon section. x 1,500.

Figure 145 (b) Shows the same cells as Figure 144 (a) in an immediately adjacent section. g, Globule leukocyte. Azure II - methylene blue - borax. x 1,500.

Figure 146. Light micrograph of the intestinal lamina propria twelve days after infection with N. brasiliensis. Maturing IM cells, plasma cells, macrophages and eosinophils (e) can be distinguished. A basophil-like cell (b) with a multilobulate nucleus is also present. x 1,500.

Figure 147. Electron-micrograph of the intestinal lamina propria twelve days after infection. Plasma cells, blast cells, macrophages (m) and eosinophils are tightly packed together. x 3,000.

Figure 148. Basophil-like cell. Note the multilobulate nucleus, the relatively large electron dense granules and the small dense particles (arrow) in the cytoplasm. x 15,000.

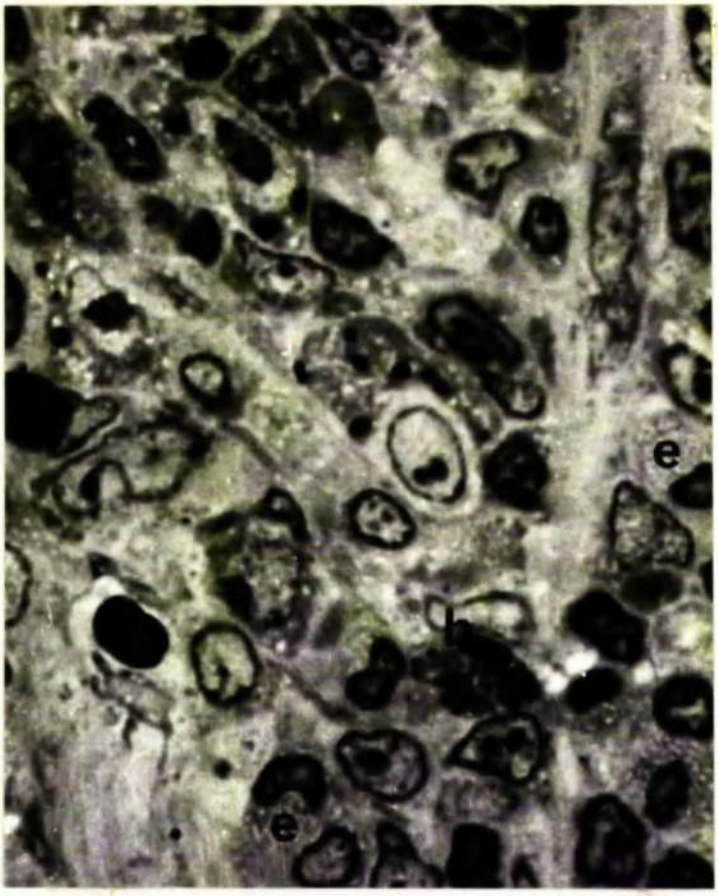


Figure 146.

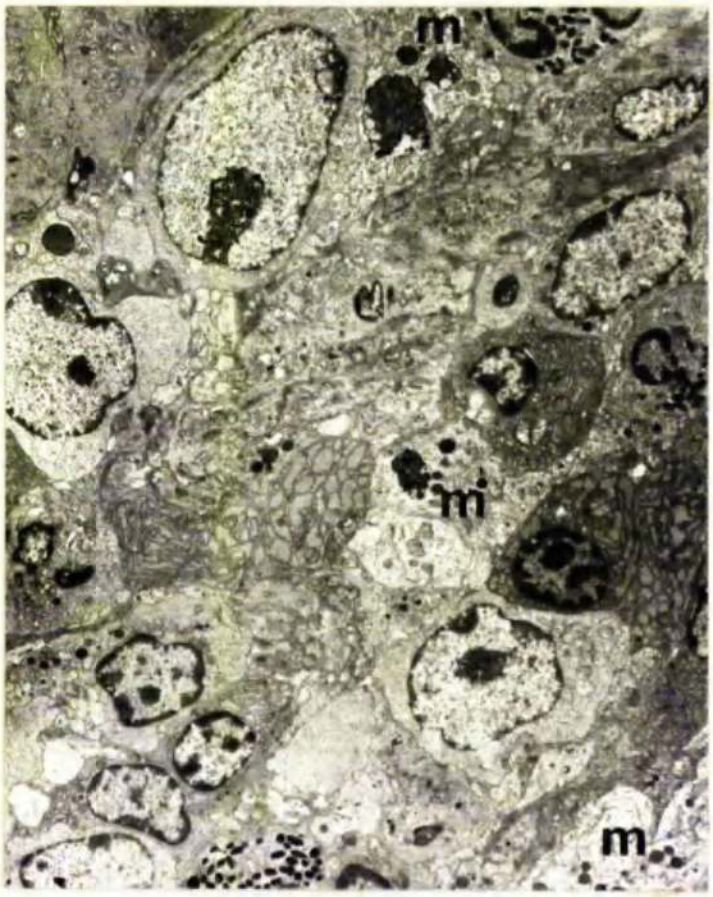


Figure 147.

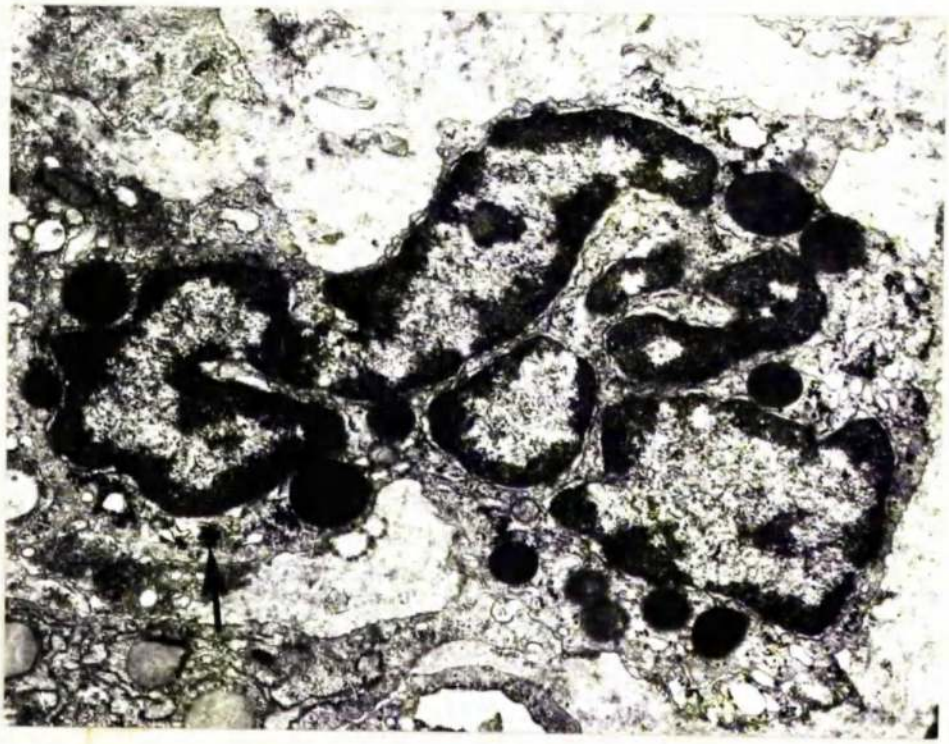


Figure 148.

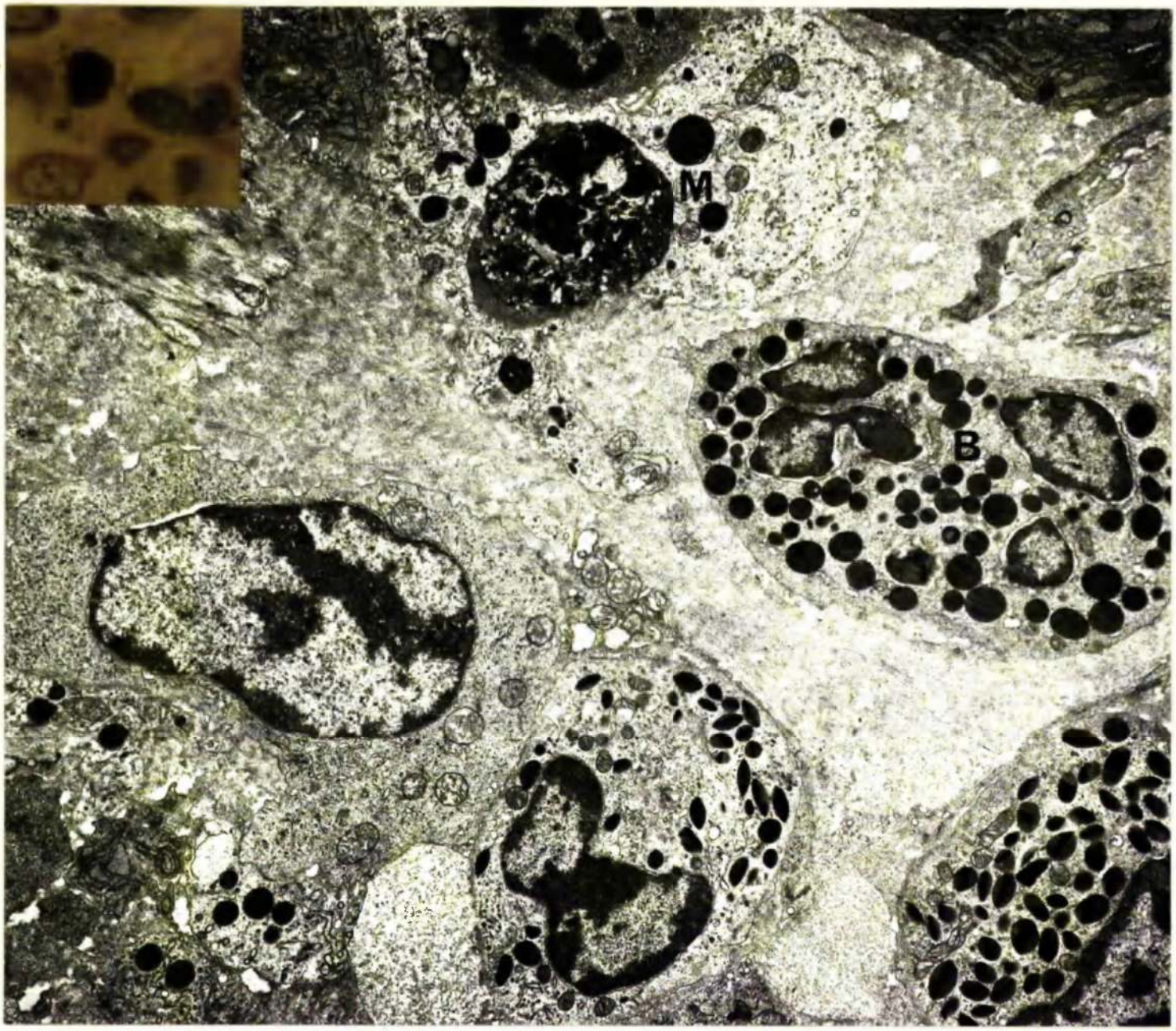


Figure 149. Basophil-like cell (B) in the lamina propria on the twelfth day of infection. A macrophage (M) contains ingested cell debris. x 7,500.
INSET. The granules of the same basophil-like cell stain a faint greenish colour. The adjacent phagosome is deeply basophilic. Azure II - methylene blue - borax. x 1,800.

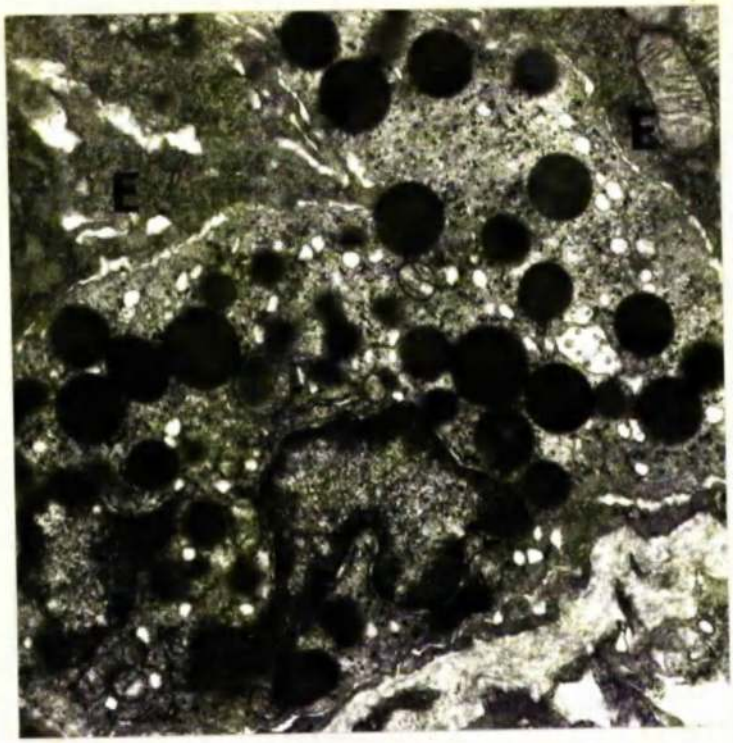


Figure 150.

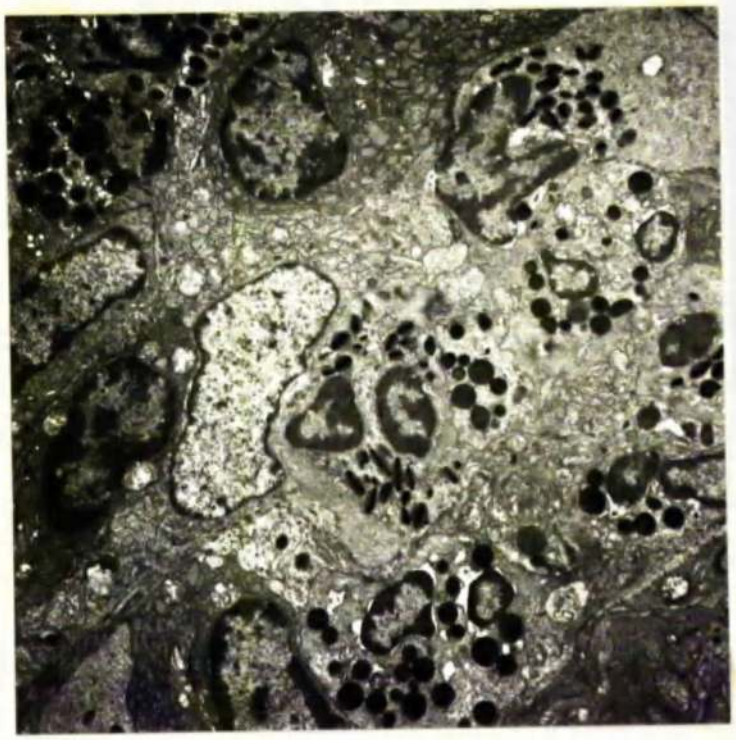


Figure 151.

Figure 150. Basophil-like cell in the basal epithelium (E). Twelfth day of infection. x 15,000.

Figure 151. Lamina propria on the twelfth day of infection. There are several basophil-like cells and eosinophils amongst the plasma cells. x 4,500.

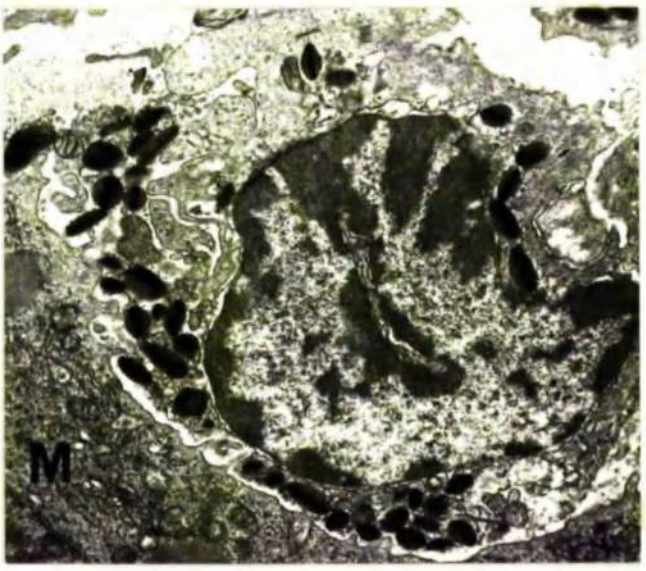


Figure 152.

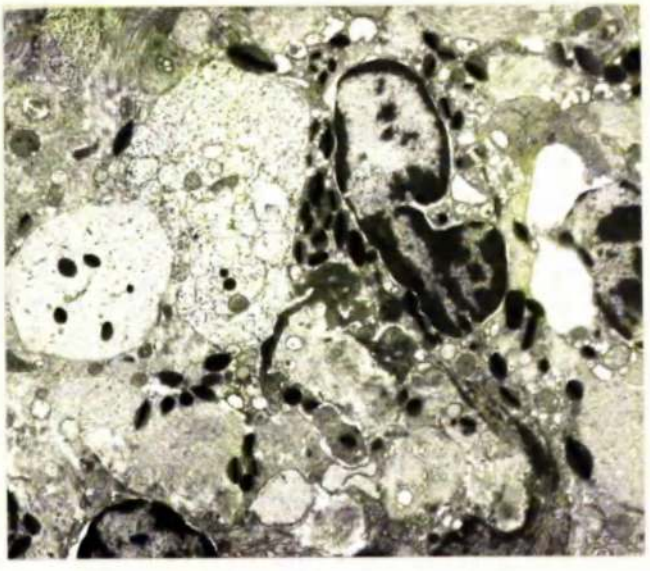


Figure 153.

Figure 152. Eleventh day of infection. A disrupted eosinophil lies adjacent to a maturing IM cell (M). Note that the granules remain intact. x 8,000.

Figure 153. A disrupted eosinophil on the twelfth day. The majority of granules are unaltered. x 5,000.

Figure 154. Light-micrograph of the intestinal mucosa fourteen days after infection. There is a gap (G) in the epithelium. Several GL and IM cells (M) have weakly basophilic or vacuolated granules. Note the increased basophilia of many nuclei. x 1,700.

Figure 155. Electron-micrograph of the same area shown in Figure 154. The endothelium of the venule has been disrupted at several points. Note the discharging eosinophil (e) and mast cells (m). G, gap in epithelium. An epithelial cell nucleus (arrow) is bulging into the lamina propria. x 3,000.

Figure 156. There are many cells both in the lamina propria and epithelium which are extensively damaged. A break in the epithelium extends almost to the lumen (L) of the gland crypt. The epithelial basement membrane (arrow) appears to be unaltered. Fourteenth day of infection. x 3,000.

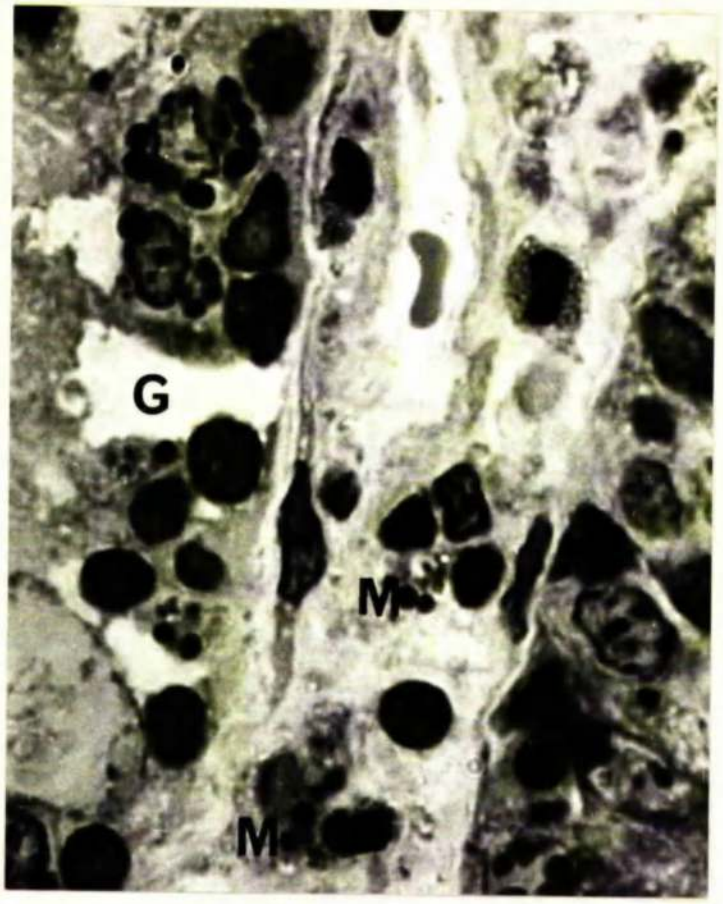


Figure 154.

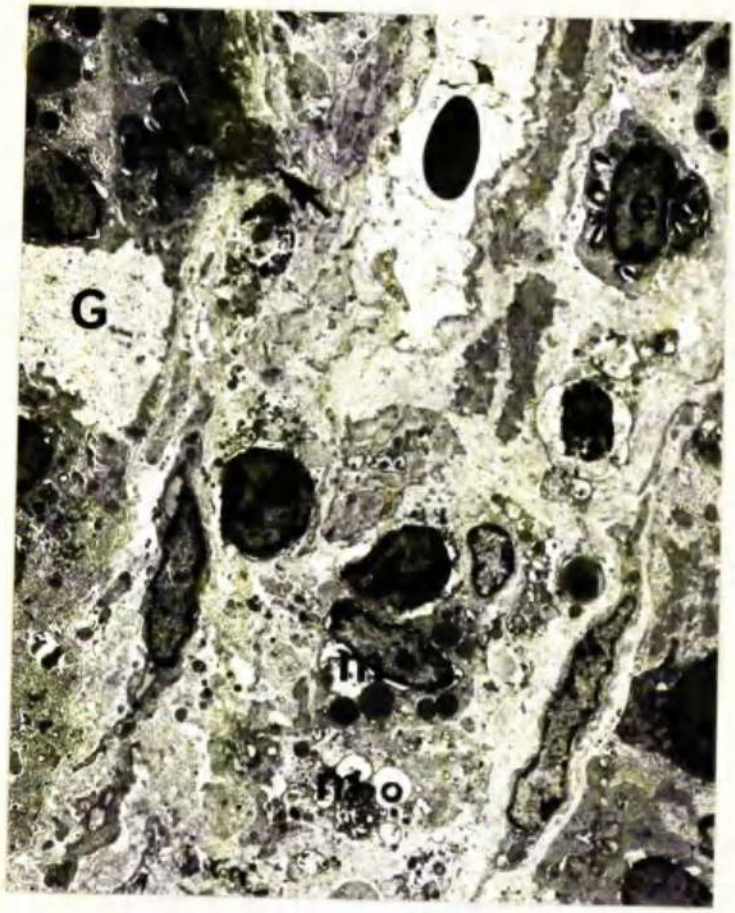


Figure 155.

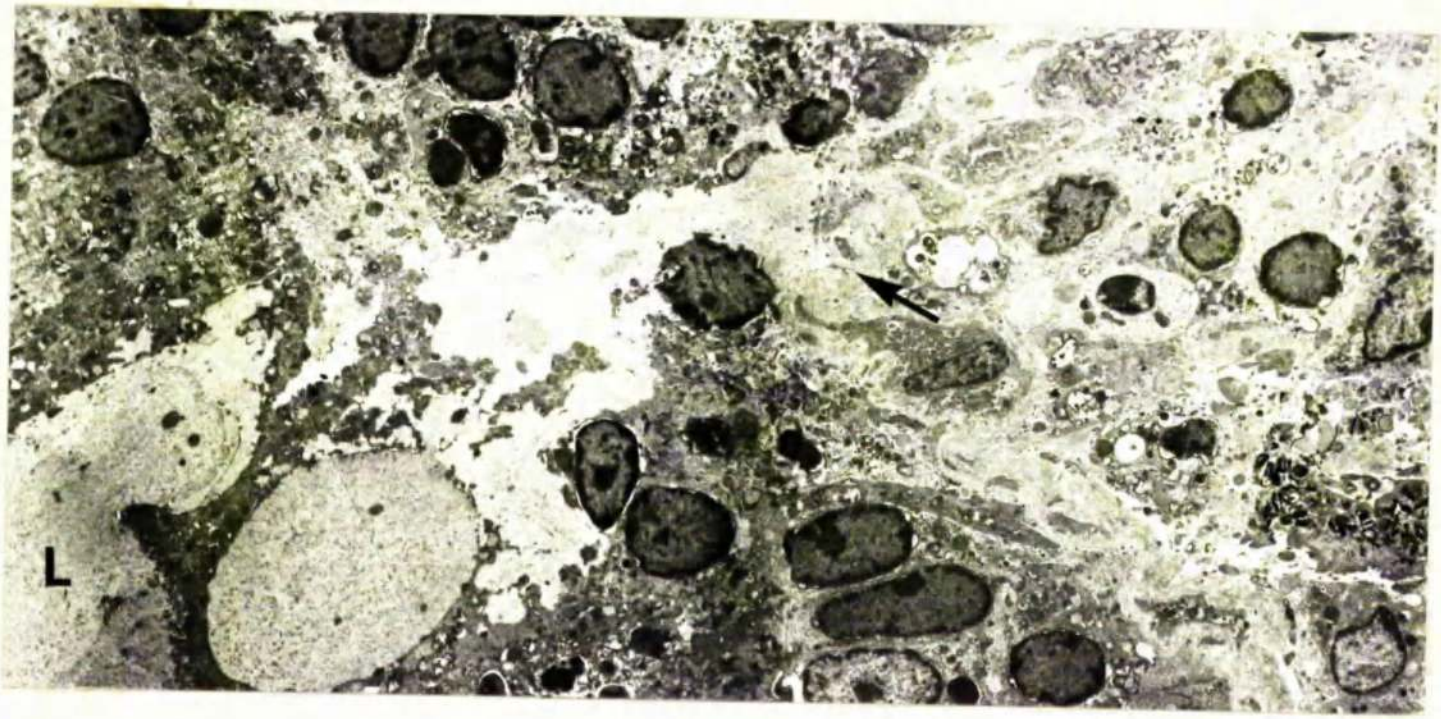


Figure 156.

Figure 157. Epithelial cells in the mid-villus region. The plasmalemmata extend only a short distance basally and thereafter are fragmented (arrows). Fourteenth day of infection. x 10,000.

Figure 158. Altered epithelial cell in a gland crypt. Note the diffuse granularity of the cytoplasm, the blebbing of the mitochondria and the dilatation and disruption of the RSER and perinuclear cisternum. Nuclear chromatin is coarsely clumped and the plasmalemmata are lysed. Day sixteen of infection. x 13,100.

Figure 159. The gland crypt epithelial cells show little alteration although GL cells are numerous. Fourteenth day of infection. x 4,500.

Figure 160. The lateral plasmalemmata are lysed and the epithelial changes are similar to those in Figure 158. The cell membrane of a GL appears to be intact. Sixteenth day of infection. x 7,500.

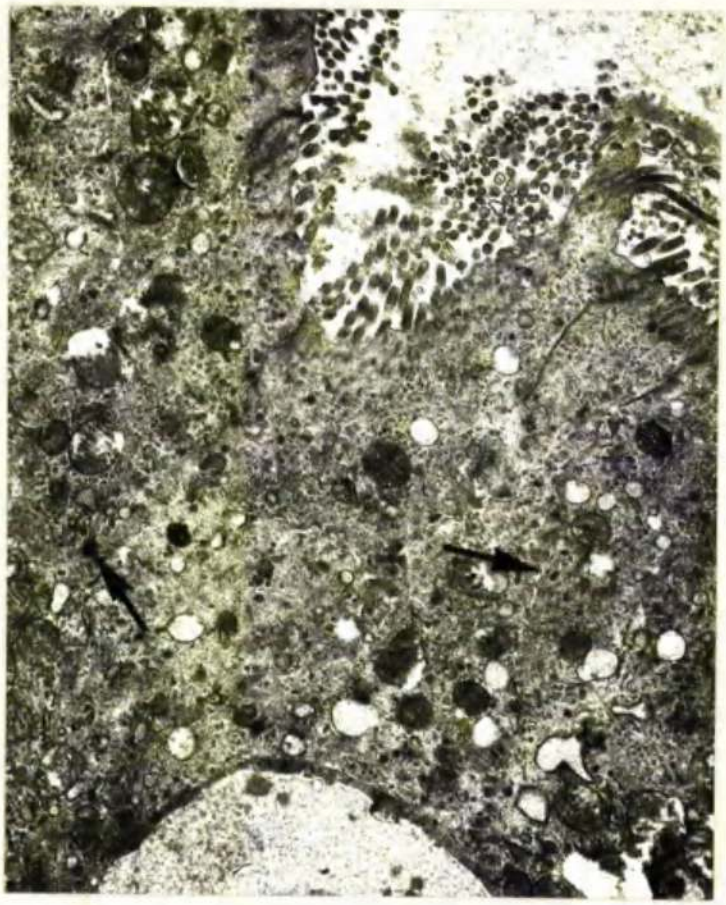


Figure 157.

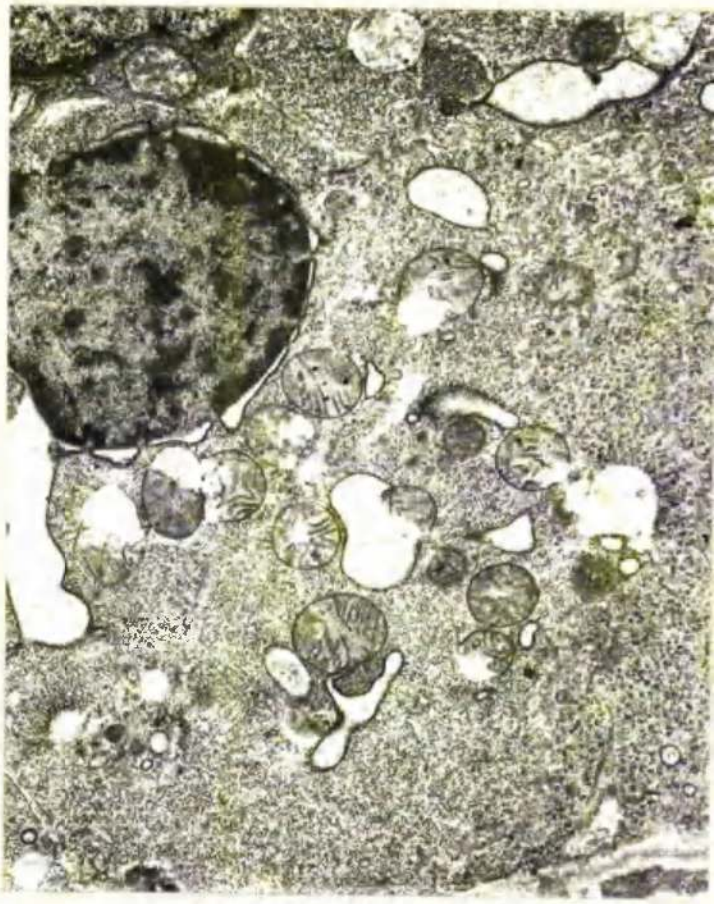


Figure 158.

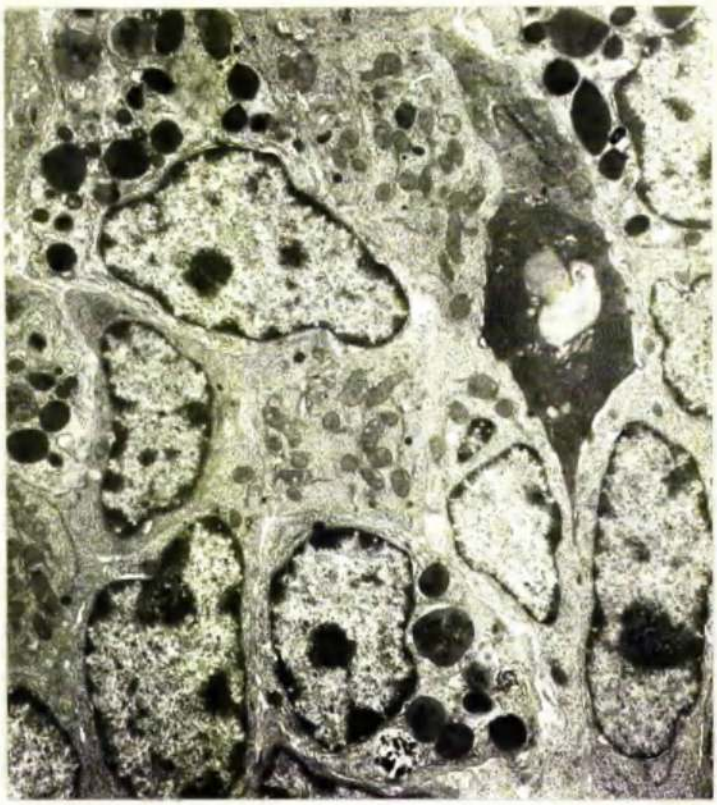


Figure 159.

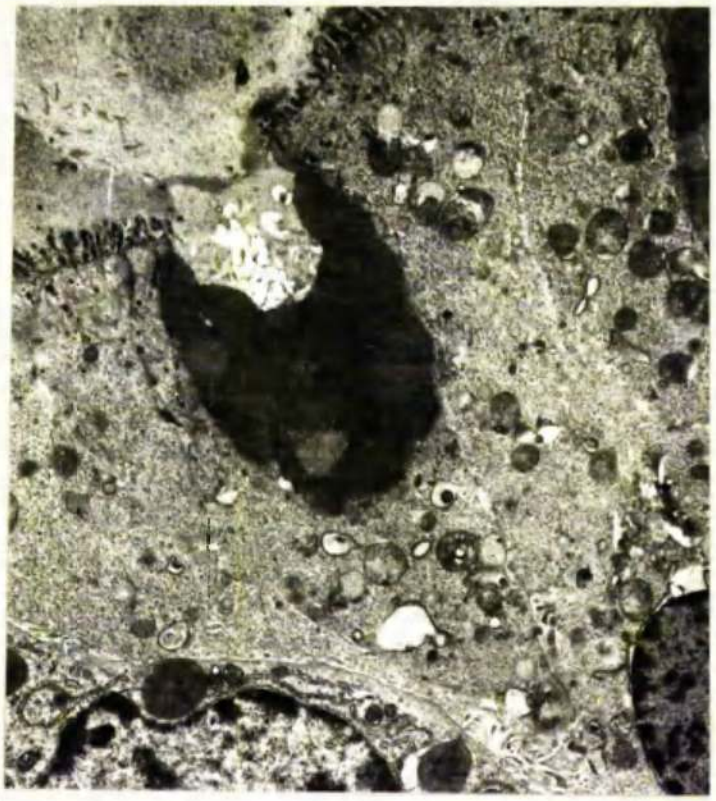


Figure 160.

Figure 161. Mucosal changes on the fourteenth day of infection. There are isolated nuclei in the lamina propria which are surrounded by a few remnants of organelles. The perinuclear cisternae have been disrupted and the chromatin is leaking out of one nucleus (arrow). Vascular endothelium (E) is shattered but the basement membrane (arrowhead) remains. The gland crypt epithelium shows the range of changes described in Figures 157, 158 and 160, and the epithelial basement membrane is apparently unaltered. x 12,500.

Figure 162. Light micrograph of an area of mucosal cytolysis. Arrows indicate regions where the integrity of the epithelium has been breached. Fourteenth day of infection. 1.5μ Araldite-Epon section. Azure II - methylene blue - borax. x 400.

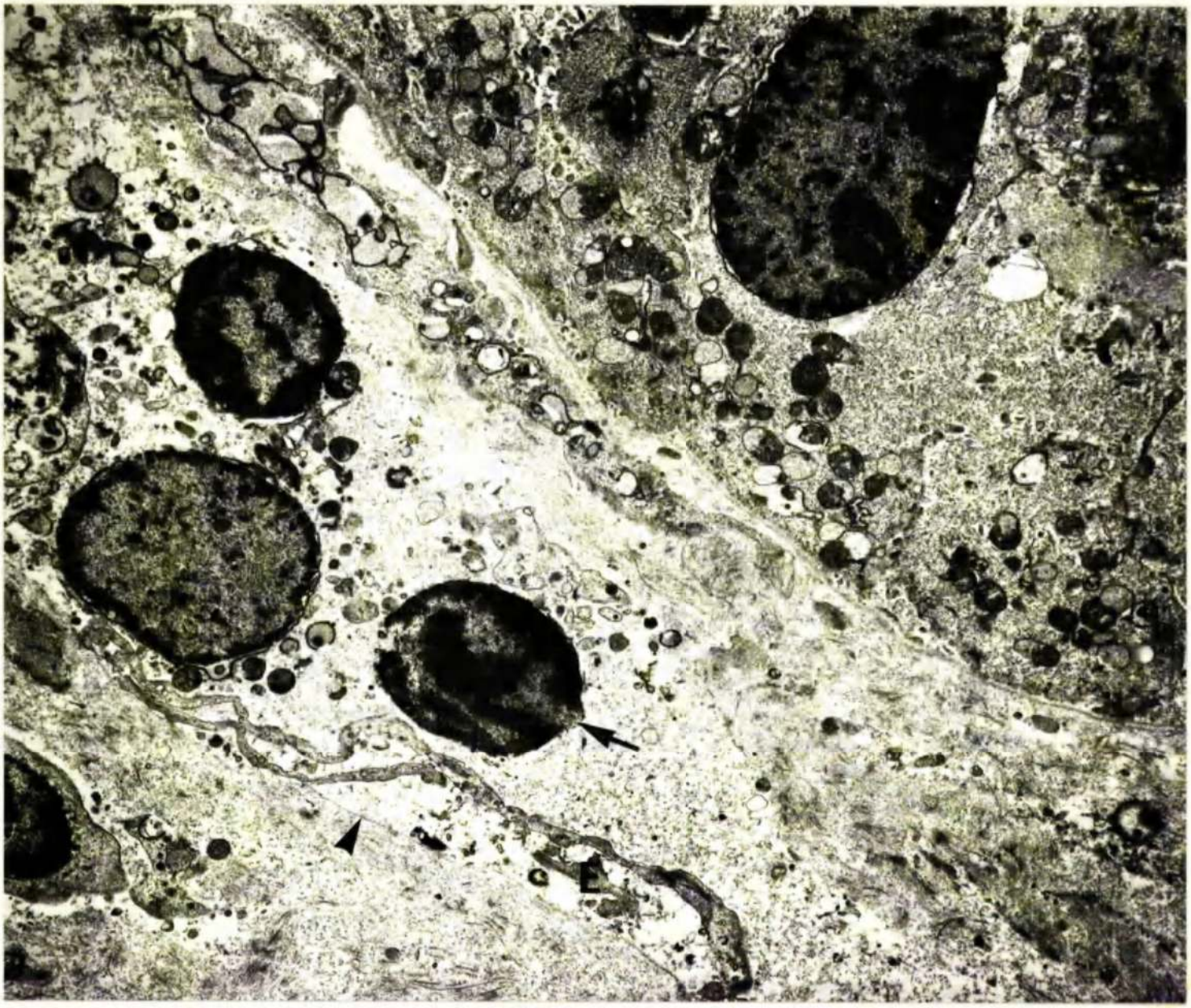


Figure 161.

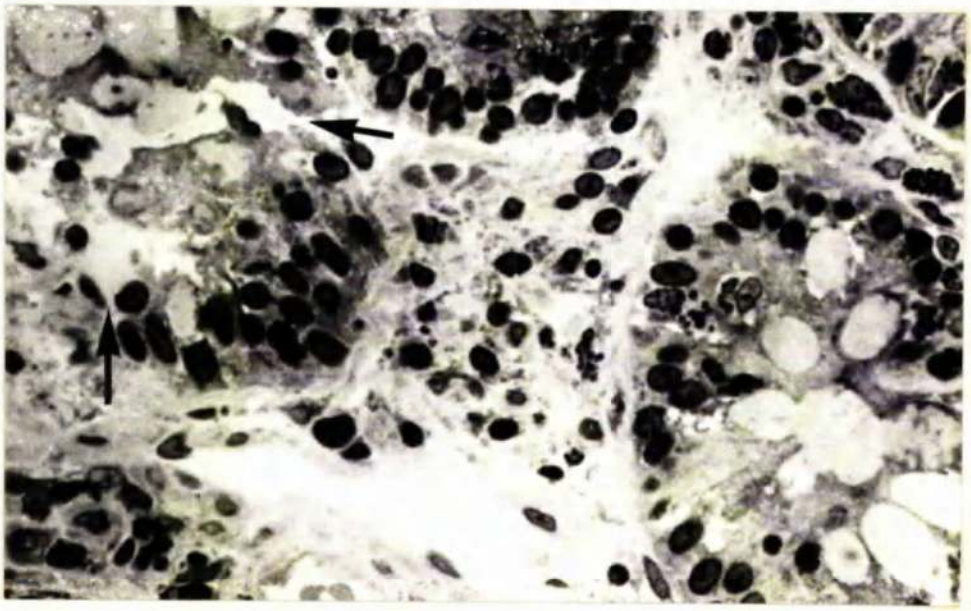


Figure 162.

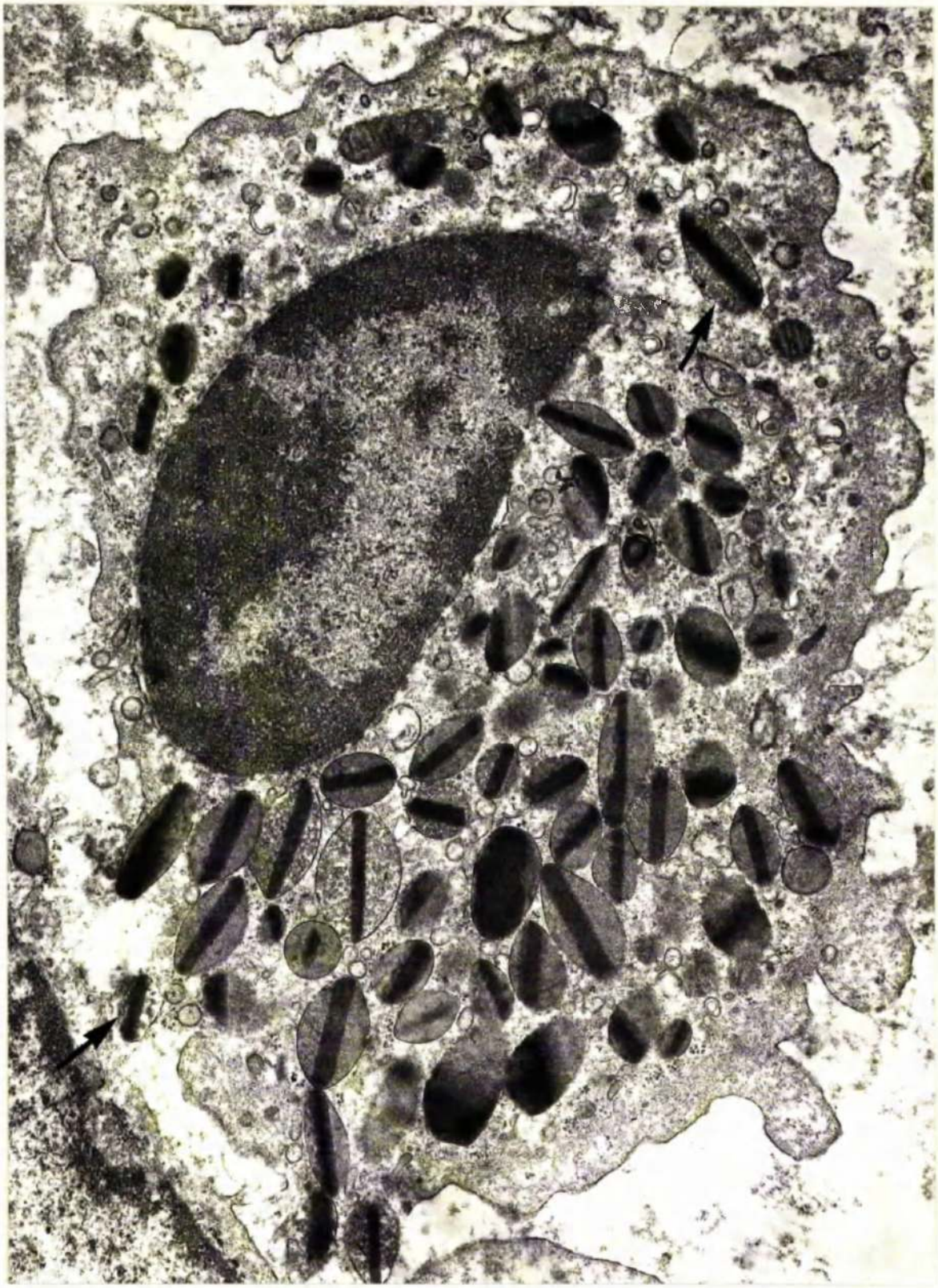


Figure 163. Early degranulation of an eosinophil. Only one or two granules are altered, but the perigranular membranes have been lysed (arrows). Day sixteen. x 24,000.

Figure 164. Plasma cells (P) are severely disrupted and the cisternae of RSER are breaking away from the cell borders. The granules of an eosinophil are extensively altered in situ. Fourteenth day of infection. x 6,000.

Figure 165. Degranulating eosinophil. Many of the granules have lost their matrices without being released from the cell. Fourteenth day of infection. x 14,000.

Figure 166. Intestinal mucosa on the sixteenth day of infection. Fully granulated IM cells and several G L cells are present and there is no evidence of cytolysis. Sixteen days after infection. 1.5μ Araldite-Epon section. Azure II - methylene blue - borax. x 570.

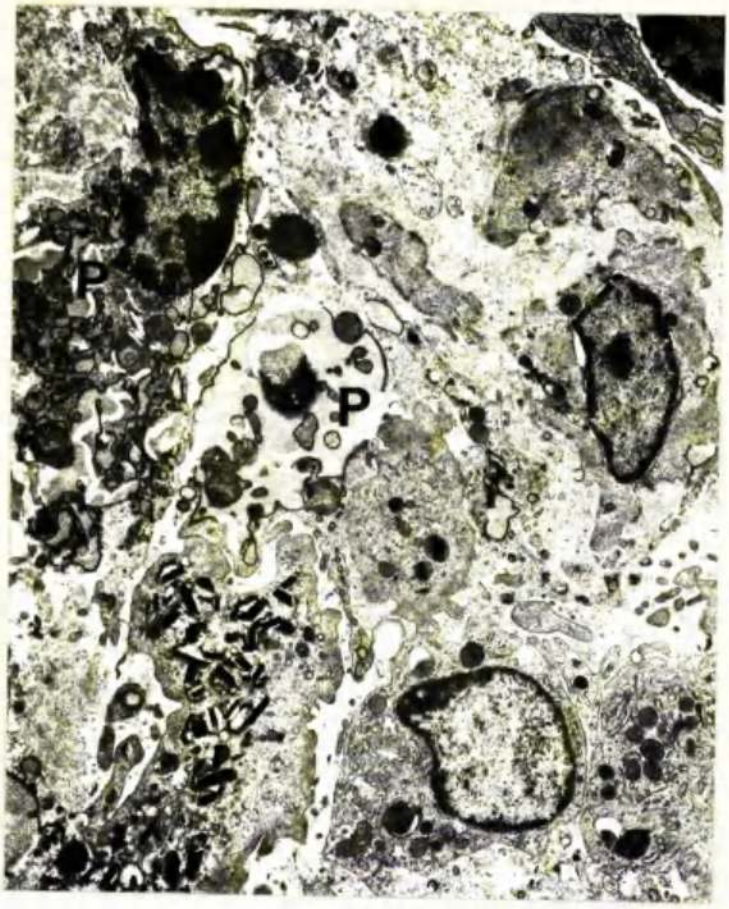


Figure 164.

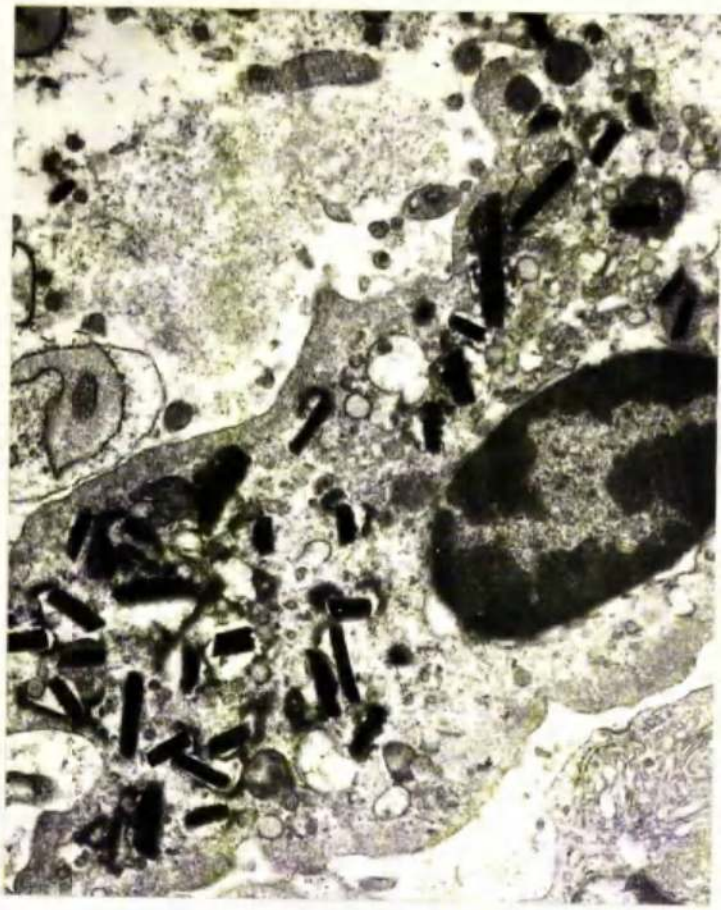


Figure 165.

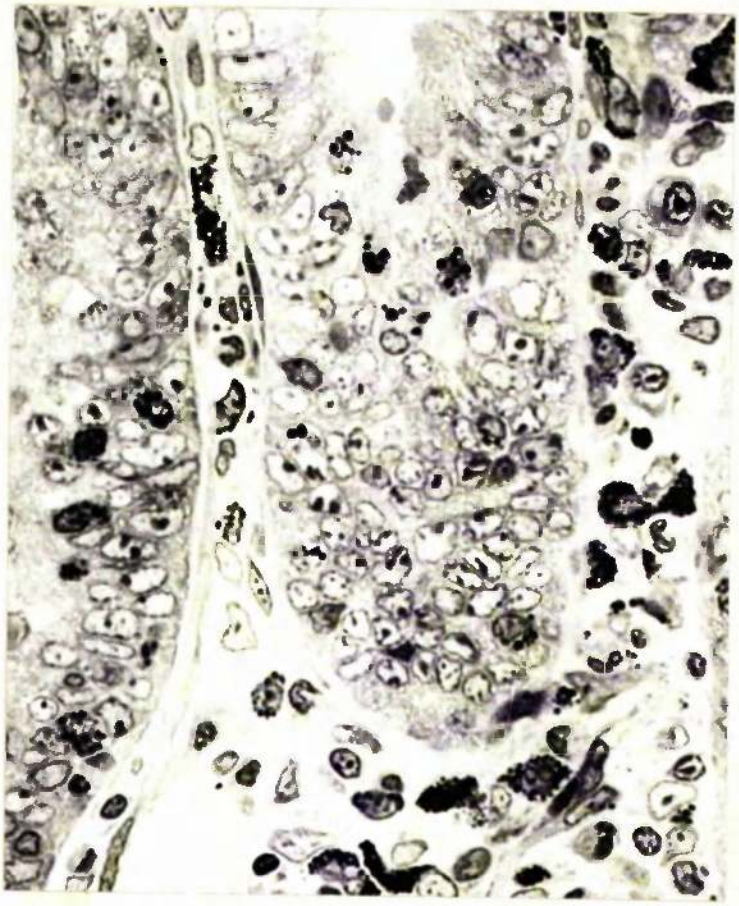


Figure 166.

Figure 167. Eosinophil and a mast cell (IM) in the lamina propria on the sixteenth day of infection. Ma, macrophage. x 14,000.

Figure 168. Plasma cells in the lamina propria sixteen days after infection. x 7,500.

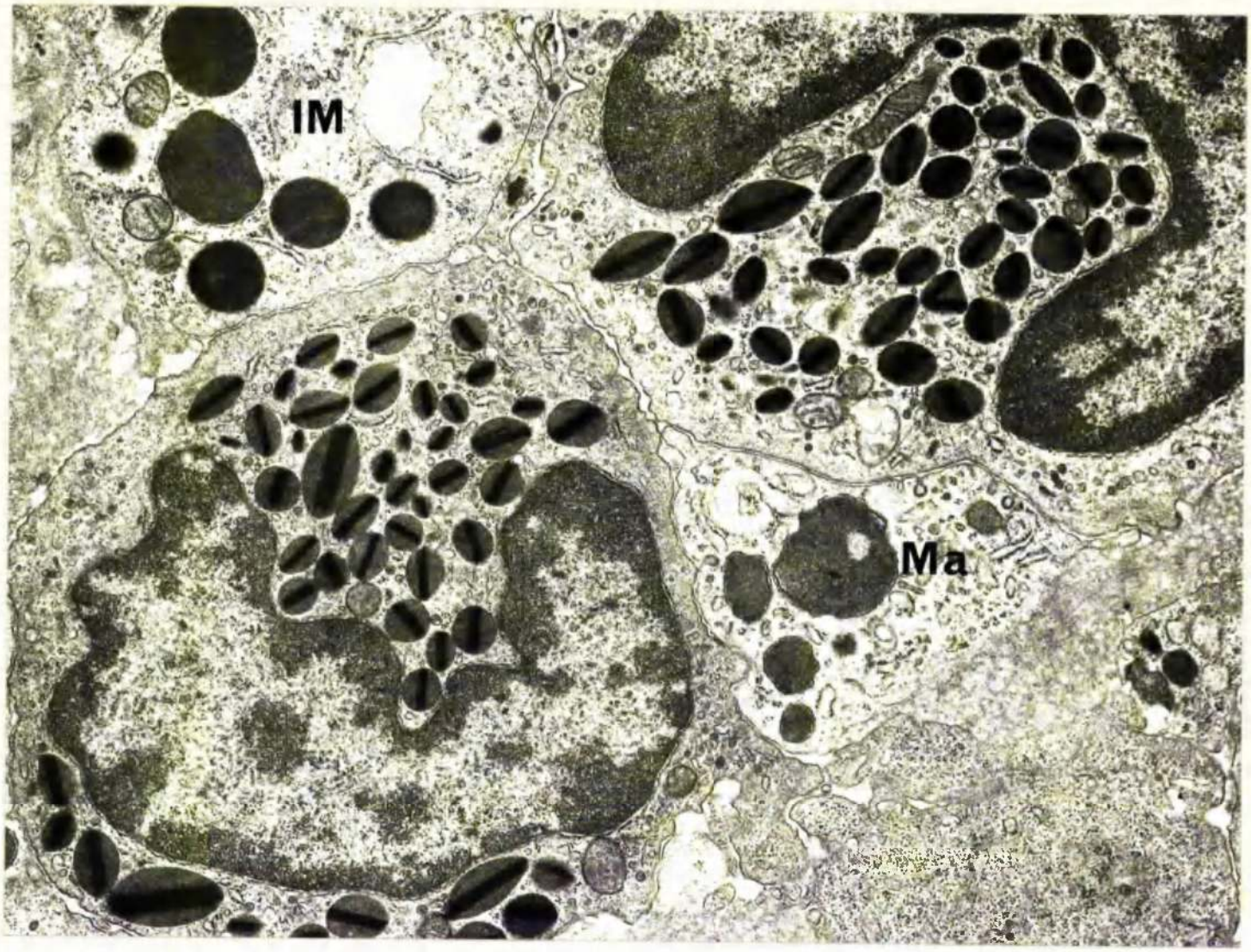


Figure 167.

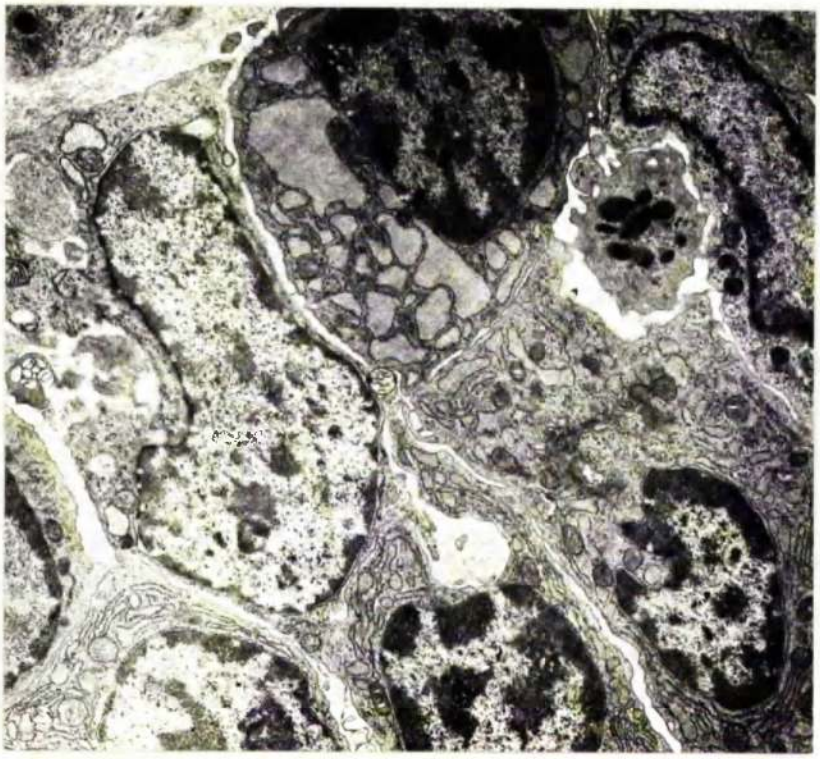


Figure 168.