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Blood-nervous Tissue Barriers In The Peripheral Nervous System

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ABSTRACT

After years of investigation into blood-nervous tissue barriers controversies remain regarding the permeability of blood vessels to macromolecules in the enteric nervous system, the endoneurium of peripheral nerve, and the spaces between satellite cells and neurons in sensory and sympathetic ganglia.

The permeability of the above areas of the peripheral nervous system was investigated in rats using the following intravenously administered tracers: rhodamine-labelled bovine albumin; horseradish peroxidase; acriflavine and ethidium. The last two, which are fluorescent cationic dyes were shown to bind to serum proteins. In addition immunohistochemical staining for endogenous albumin was performed. One long-term study was done in which rhodamine-labelled bovine albumin was injected subcutaneously, once daily, for one week. With all these methods, it was possible to show that blood vessels in the brain were impermeable, whereas those in circumventricular organs were permeable, thus validating their application to regions in which the existence of permeable vessels was questionable.

Rhodamine-labelled bovine albumin was seen in all the extracellular spaces in sympathetic and sensory ganglia,

even after short times in the circulation. The endoneurium of peripheral nerve contained this tracer only in rats in which it had been injected daily for one week. At no time did this fluorescent albumin enter the enteric ganglia. Positive immunohistochemical staining for endogenous albumin was present in the enteric nervous system, in the endoneurium of peripheral nerve, in the spaces between satellite cells and neurons in sensory ganglia, and around neurons in sympathetic ganglia. Horseradish peroxidase, which is present in the blood for about 5 minutes following intravenous injection, penetrated the enteric nervous system, and the extracellular spaces between satellite cells and neurons in sensory ganglia, but it did not enter the endoneurium of peripheral nerve. The fluorochromes acriflavine and ethidium entered enteric, sympathetic and sensory ganglia, but not the endoneurium of peripheral nerve.

In conclusion, the enteric nervous system, sympathetic and sensory ganglia are permeable to most circulating macromolecules. The endoneurium of peripheral nerve is permeable only to macromolecules that are present in the circulation for at least one week, so the transudation or diffusion in this tissue must occur more slowly than elsewhere. These permeabilities may explain how some pathogens enter the nervous system.

To My Parents and My Brother Norman

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LIST OF ABBREVIATIONS

CFAP = glial fibrillary acidic protein

CNS = central nervous system

CSF = cerebrospinal fluid

DAB = diaminobenzidine

EAGMD = experimental autoimmune grey matter disease

EAMND = experimental autoimmune motor neuron disease

EM = electron microscopy

ENS = enteric nervous system

HRP = horseradish peroxidase

ICC = Interstitial Cell of Cajal

IgG = Immunogammaglobulin

LM = light microscopy

NGS = normal goal serum

PAGE = polyacrylamide gel electrophoresis

PAP = peroxidase antiperoxidase

PBS = phosphate buffered saline

RBA = rhodamine labelled bovine albumin

CHAPTER 1 INTRODUCTION

BLOOD-TISSUE BARRIERS IN THE NERVOUS SYSTEM

Functional barriers prevent the entrance of blood-borne macromolecules into the nervous system. There are two such barriers: (1) the blood-brain barrier, which also exists in the spinal cord, and (2) the blood-nerve barrier in the peripheral nervous system.

1.1 Central Nervous System

The blood-brain barrier is the mechanism by which macromolecules in the blood are excluded from the central nervous system. These same substances readily enter the extravascular spaces of non-nervous tissue. The structural basis of this barrier is the existence of zonulae occludentes between adjacent endothelial cells. There are regions in the brain where the blood-brain barrier is absent, as is the case in the circumventricular organs. During the prenatal and perinatal development of the brain in some animals the blood-brain barrier is absent.

1.1.1 Blood-Brain Barrier

The first evidence for the blood-brain barrier came from Ehrlich's experiment (1885) in which he injected animals with a dye, coerulein-S. This stained all the

tissues examined except the brain. Further studies revealed that intravenously injected dyes that stained the brain were also taken up by fat (Ehrlich, 1902) and that basic dye entered the brain, whereas most acid dyes were excluded (Ehrlich, 1902; Spatz, 1933; Friedemann, 1937; 1942). In 1955, Rodriguez-Peralta proved wrong the generalization that basic dyes cross the blood-brain barrier, by showing that some fluorescent basic acridine dyes, notably proflavine and acriflavine, did not cross the blood-brain barrier in rodents. Other basic dyes that do not enter the CNS are certain cyanines including true blue (Van der Krans and Hoogland, 1983; Leong and Ling, 1990) and phenanthridines such as ethidium bromide (Cesarini et al., 1985).

Trypan blue, an acid dye. failed to stain the ordinary brain tissue following intravenous injection in rodents. When the same dye was injected into the ventricles or subarachnoid space, it readily stained the ordinary brain tissue, which was normally protected by a blood-brain barrier (Goldmann, 1909; 1913; Spatz, 1933). Goldmann (1909; 1913) considered it likely that the blood-brain barrier to trypan blue was the glial sheath around the capillaries. Spatz (1933), on the other hand, noted that trypan blue injected into the subarachnoid space was not confined to the Virchow-Robin spaces, the spaces between the leptomeningeal cells and the glia limitans externa, but readily penetrated around all blood vessels except the capillaries. Thus Spatz (1933) concluded that the capillary

endothelium was the site of the barrier to trypan blue. At about the same time, Walter (1933) made similar observations and came to the same conclusion. Further support for a barrier to dyes at the luminal side of the capillary endothelium was provided by Rodriguez-Peralta (1955; 1957). He injected rodents either intravenously or intraventricularly with fluorescent dyes that bound to the nuclei of cells of tissues with permeable blood vessels and found that the nuclei of the endothelial cells of the capillaries in the brain were not stained after intravenous injection. The absence of staining of the endothelial cell nuclei may have been due to the failure of the selective transport systems in the brain endothelial cells to take up these dyes from the blood. However, when the dyes were given intraventricularly, they stained the nuclei of neurons, glial cells, and capillary endothelial cells. Brightman (1965) showed by EM that the blood-brain barrier to ferritin, a protein (500 kd) that is electron-opaque, because it contains iron, was on the luminal side of the capillary endothelium. After an intraventricular injection in rodents, ferritin permeated extensively among the cells of the brain, even between the astroglial end feet and the capillary basal lamina. Reese and Karnovsky (1967) confirmed that the blood-brain barrier to proteins was at the luminal surface of the capillary endothelium. They injected horseradish peroxidase (HRP), a 40 kd protein, intravenously, as an electron microscopic tracer. Zonulae

occludentes between capillary endothelial cells were seen to block the passage of the HRP.

1.1.2 Blood-Cerebrospinal Fluid Barrier

The blood-cerebrospinal fluid (CSF) barrier is similar to the blood-brain barrier, but there is no CSF-brain barrier. Goldmann (1909) discovered these facts when he injected several small mammals intravenously with trypan blue and did not find any of the dye in the brain or the CSF. In a second experiment, he administered the dye intraventricularly and found it in the CSF and the brain. Although Stern and Gautier (1921; 1922) obtained similar results, these authors failed to recognize that macromolecules cannot pass from the blood into the CSF. They interpreted the data, as the CSF being the medium by which essential substances entered the brain. In two separate reviews, Walter (1933) and Spatz (1933) pointed out that this interpretation was wrong as macromolecules did not cross from the blood into the CSF and hence it was not the route by which essential substances entered the brain. Rodriguez-Peralta (1955) located the blood-CSF barrier by intravenously administering a fluorescent dye which stained the nuclei of cells of capillary endothelium and connective tissue of the choroid plexus, but not the choroid ependyma. He concluded that the blood-CSF barrier was at the basal poles of the choroid epithelial cells. These observations were confirmed and extended by Brightman and Reese (1967; 1968; 1969). They injected HRP intravenously into rodents

and found that the enzyme went between the choroidal ependymal cells as far as the zonulae occludentes at their apical poles. Also HRP did not enter the CSF and brain. On the other hand, when they injected HRP into the lateral ventricle, they found extensive penetration of the extracellular space of the brain, but not between the choroidal ependymal cells.

In addition, CSF in the subarachnoid space is prevented from mixing with the extracellular fluid of the dura mater by a barrier composed of arachnoid mesothelial cells (Nabeshima et al., 1975).

1.1.3 Comparative Anatomy

In the brains of vertebrates, the brain capillary endothelium is surrounded by a layer of glial end feet. Cserr and Bundgaard (1984) found that brain endothelial cells in cyclostomes are joined by tight junctions which are impermeable to HRP and microperoxidase (a 2 kd protein) at the electron microscope level. When HRP was injected intravenously into teleosts (bony fishes), amphibians, reptiles, birds and mammals, it did not leave the lumina of the blood vessels and pass into the brain, except in the circumventricular organs (see section 1.1.2). The HRP was stopped by tight junctions between brain capillary endothelial cells (see Cserr and Bundgaard, 1984; Abbott, Bundgaard and Cserr, 1986). In contrast, in elasmobranchs (cartilaginous fishes, such as sharks and skates) HRP passed between the capillary endothelial cells in the brain and was

only stopped by the tight junctions between the glial end feet. These prevented the HRP from penetrating deeper into the brain (see Cserr and Bundgaard, 1984; Abbott, Bundgaard and Cserr, 1986).

1.1.4 Areas of the Brain with no Blood-Brain Barrier

These areas are the circumventricular organs (the area postrema, median eminence, subfornical organ, organum vasculosum laminae terminalis, neurohypophysis and the pineal gland). Their lack of a blood-brain barrier is attributed to fenestrated capillaries. This was demonstrated by a number of investigators by administering trypan blue or silver nitrate, by various routes to a variety of mammals (Wislocki and Putnam, 1920; Cappel, 1929; Wislocki and King, 1936; Wislocki and Leduc, 1952; 1954). They found the tracers in the capillary lumina and in the spaces between the cells in the circumventricular organs, but no staining in the rest of the brain, indicating that the circumventricular organs had permeable blood vessels. This was confirmed at the electron microscope level which revealed fenestrated capillaries in the circumventricular organs (Dempsey and Wislocki, 1955; Palay, 1955; Lederis, 1965; Wolfe, 1965; Rohr, 1966; Dempsey, 1973) and penetration of proteins such as ferritin and HRP (see Koella and Sutin, 1967).

1.1.5 Development of the Blood-Brain Barrier

Stewart and Wiley (1981) found that structural and functional characteristics were determined by the

surrounding astrocytes. They transplanted a piece of embryonic quail's brain (prior to vascularization) into the coelomic cavity of 3-day old chick embryo. This enabled them to determine the origin of the blood vessels growing into the grafts because the nuclei of quail cells are easily recognized. The grafts were not rejected because the immune system of the chick embryo was not yet developed. Stewart and Wiley examined the recipients after eighteen days by electron microscopy. The capillaries in the transplanted quail tissue were derived from the chick's abdominal vessels, but they had tight junctions between their endothelial cells. The capillaries in the transplanted piece of brain excluded trypan blue which had been injected intramuscularly into the host, and hence demonstrated the existence of a functional blood-brain barrier in the piece of ectopically transplanted quail's brain. They concluded that it was the brain tissue (neuroglia cells and/or neurons) that was responsible for inducing the formation of zonulae occludentes in blood vessels and not the blood vessels themselves.

The inductive action of astrocytes upon the formation of tight junctions between endothelial cells has been confirmed in experiments in which glial and mesodermal cells were grown together in tissue culture (Janzer and Raff, 1984; Arthur et al., 1987; Dehouck et al., 1990; see also review by Risau and Wolburg, 1990).

The time of maturation of the mammalian blood-brain

barrier is controversial. Olsson and coworkers (1968) found an effective barrier to fluorescein labelled bovine albumin (injected into an umbilical artery) as early as the fifteenth day after fertilization. No experiment was done prior to the fifteenth day after fertilization. Although these authors saw the fluorescent tracer inside cerebral blood vessels, they did not record observations on the permeability of any circumventricular organs.

In contrast to the observations cited above, other investigators have found that in fetal rats and mice up to two weeks after birth the blood-brain barrier is permeable to ferrocyanide ions (Stern and Peyrot, 1927), and HRP (Broadwell and Brightman, 1976; Stewart and Hayakawa, 1987). Mollgard and Saunders (1975) found that the capillary endothelia in the brains of fetal sheep were permeable to HRP.

Stewart and Hayakawa (1987) injected one group of mice with HRP intraperitoneally and 4 hours later killed them and measured the HRP activity in the extract of the homogenate of each brain. In a second group of mice age-matched to the first, the HRP-injected mice were killed and the ultrastructure of the tight junctions between endothelial cells was examined. They measured the "vessel density" (number of vessels / mm^2 tissue section) and calculated the "permeability index" (HRP activity in brain/vessel density). They found a gradual decrease to normal levels in the permeability of the brain capillary endothelium to HRP in

mice from the 15th day in utero up to 2 weeks after birth. They also found that the zonulae occludentes between brain capillary endothelial cells, occupied more of the interendothelial junctions, and the junctional clefts decreased in size up to 2 weeks after birth.

Risau and coworkers (1986; 1990), who also used mice, agreed with Stewart and Hayakawa (1987) in observing that the development of the blood-brain barrier was a gradual process occurring during embryonic, fetal,and early postnatal development.

Mollgard and Saunders (1986) and Saunders, Dziegielewska and Mollgard (1991) criticized those investigators who found no blood-brain barrier in fetal and early post-natal animals. They suggested that too high of a concentration of HRP was used as a tracer and that the HRP damaged the blood vessels and made them leak. However, Wakai and Hirokawa (1978) and Risau and Wolburg (1991) did not observe any damage to the blood vessels in the brains of fetal mice and chicks in ovo respectively. It has also been pointed out that immunohistochemical staining for the endogenous plasma proteins may be used to determine whether or not the blood-brain barrier is functional (Mollgard and Saunders, 1986; Saurders et al., 1991). The problem with this is that many endogenous plasma proteins are also synthesized in the brain itself as pointed out by Ali et al., 1983; Aldred et al., 1987; Risau and Wolburg, 1991.

A possible explanation for finding a blood-brain

barrier to fluorescein in fetal and neonatal rats (Olsson and coworkers, 1968) and to trypan blue in neonatal mice, rats, rabbits and cats (Stern and Rapoport, 1927; Cappell, 1929) is that both these tracers are known to bind to albumin (Tschirgi, 1950). The resulting complex molecule may be too big to penetrate between the capillary endothelial cells of the brain, or the complexes may not be transported across the endothelium.

1.1.6 Structural Basis of the Blood-Brain Barrier

The structural basis is the zonulae occludentes between endothelial cells in the capillaries and also selective transport across the endothelium (Reese and Karnovsky, 1967; Brightman and Reese, 1969; Van Deurs, 1980).

Scanning electron microscopy of freeze fracture replicas of cerebral endothelial cells reveals the three-dimensional structure of zonulae occludentes. The inner protoplasmic (P) face of an endothelial cell in the brain has rows of anastomosing strands, whereas the exte al (E) face has furrows which are complementary to the strands on the (P) face (Shivers et al., 1984; Arthur et al., 1987). The neural tissue environment has an influence on the development of the blood brain barrier (Stewart and Wiley, 1981). Glia, in particular astrocytes and/or their products have an inductive effect on the formation of zonulae occludentes between endothelial cells (Arthur et al., 1987; Janzer and Raff, 1987).

Transendothelial transport is the process of transporting macromolecules from the luminal side of the endothelium by forming membrane-bound vesicles containing macromolecules, moving these vesicle in the endothelial cytoplasm to the abluminal side of the endothelium, and releasing the contents of the vesicles on the abluminal side. Blood-borne tracers, such as HRP have been observed in vesicles in cerebral endothelial cells in mice and rats (Westergaard and Brightman, 1973; Hansson et al., 1975; Shivers et al., 1984). Coomber and Stewart (1986), using ultrathin sections of mouse brain, have observed fused vesicles connected to the luminal surface of brain endothelial cells but no transendothelial channels. Frokjaer-Jensen et al. (1988) showed that the vesicles observed in either aldehyde fixed or rapidly frozen endothelium were not artifacts of aldehyde fixation as suggested by other investigators (Bungaard et al., 1979; Balin et al., 1987; Broadwell, 1989). These later investigators did not use ultrathin sections of the brain endothelium which resulted in underestimating the free and fused vesicles in the endothelial cells. However, no one to date has observed transendothelial channels in brain endothelium.

In the past, the blood brain barrier was thought to prevent plasma proteins from entering the brain. However, more recent work has revealed that cationized albumin, either with or without a covalently linked peptide, and

cationized IgG do enter the brain, *in vivo* (Kumagai et al., 1987; Triguero et al., 1989; Pardridge et al., 1990). These investigators found that cationized plasma proteins were taken up by cerebral endothelial cells from the luminal side of the blood vessels, transported across the endothelial cells, and released on the abluminal side. They suggested that the uptake of these cationzied proteins may be receptor mediated.

1.2 Peripheral Nervous System

The peripheral nervous system consists of: the cranial nerves and their branches and sensory ganglia; spinal nerves and their branches and sensory ganglia; the afferents connecting the sensory end organs to the CNS; the efferents connecting the CNS to the effector; the sympathetic trunks and their ganglia; all other autonomic ganglia and their nerves (Johnston et al., 1958).

1.2.1 Ganglia

The blood vessels in sensory and sympathetic ganglia are permeable. However, a controversy still remains as to whether or not large molecules can penetrate from the blood into the spaces between the neurons and their ensheathing satellite cells. Intravenously injected Evan's blue albumin or fluorescently labelled dextrans readily entered the extracellular spaces around the neurons and between satellite cells and neurons in the hamster and rat (Arvidson et al., 1973; Olsson et al., 1975; Hultstrom et al., 1983). These fluorescent substances were also seen in the spaces

between the myelinated fibres in the adjacent nerves.

At the electron microscope level, it has been shown that both HRP and ferritin enter the spaces around the neurons and between the satellite cells and the neurons in rats and mice (Jacobs et al., 1976; Arvidson, 1979; Ten Tusscher et al., 1989). In contrast to these results, Depace (1982) failed to find HRP in the extracellular spaces of autonomic ganglia taken from rats injected intravenously with HRP. Depace noted that HRP was confined to the blood vessels in these ganglia and claimed that the endothelium of these blood vessels was continuous and that adjacent endothelial cells were joined by tight junctions. Depace is the only investigator to have made this observation.

Azzi et al. (1990) immunized rats to HRP by giving them intramuscular injections of HRP and determined that the serum antibodies to HRP were IgG. The presence of these IgG molecules to HRP in the tissues was detected by HRP molecules binding to the antiserum and developed by the DAB reaction. Azzi et al. (1990) did not find any antibodies to HRP between the satellite cells and neurons despite finding these antibodies around the neurons.

1.2.2 Blood-Nerve Barrier

Many investigators have injected mice, rats, and rabbits with fluorescent proteins or HRP. They have all found the tracers in the epineurium and the outer layer of the perineurium but not in the endoneurium of peripheral nerves (Doinikov, 1913; Olsson, 1966; 1971; Reese and

Olsson, 1970; Kristensson and Olsson, 1971). Fluorescent proteins, ferritin or HRP injected into the tissue surrounding a peripheral nerve entered the epineurium and outer layers of the perineurium but not the innermost perine al layer and endoneurium (Klemm, 1970; Reese and Olsson, 1970; Kristensson and Olsson, 1971; Olsson and Reese, 1971). These observations indicated that the innermost layer of perineurial cells constituted a barrier to diffusion of large molecules into the endoneurial compartment.

It is therefore widely agreed that the blood vessels in the endoneurium and the innermost layer of perineurial cells are impermeable to macromolecules and together, constitute a blood-nerve barrier (see Olsson, 1990).

Electron microscopic studies using HRP revealed that the blood-nerve barrier consisted of zonulae occludentes between adjacent endothelial cells of the blood vessels in the endoneurium and zonulae occludentes between cells in the innermost layer of the perineurium (Reese and Olsson, 1970; Kristensson and Olsson, 1971; Olsson and Reese, 1971; Reale et al., 1975; Akerte et al., 1976; Shinowara et al., 1982).

However, Olsson (1971) observed some leakage of injected bovine albumin and also a gamma globulin into the endoneurium in guinea pigs, hamsters, hens, rabbits, cats, and monkeys. The distribution of these plasma proteins in the endoneurium varied within and among groups of animals. These results conflicted with those of the preceding

investigators and with results of Waksman and Adams (1956); Waksman and Mansmann (1957); Waksman (1961) in rabbits. More recently, other researchers detected endogenous albumin in the endoneurium around the nerve fibres and in the perineurium and epineurium of sciatic nerve (Mata et al., 1987) and spinal nerve roots (Pettersson et al., 1990) in the rat. In addition, microinjection of lanthanum nitrate into epineurium of peripheral nerves in rats, resulted in penetration of the electron-dense metal ions into the perineurium and endoneurium (Mackenzie et al., 1987).

1.3 Enteric Nervous System

The enteric nervous system (ENS) consists of the myenteric plexus which lies between the two muscle layers and the submucous plexus in the submucosa. There are no blood vessels in these plexuses and nutrients must diffuse from the blood vessels in the muscle layers and submucosa to the enteric nervous system (Gabella, 1972).

1.3.1 Structure of the Enteric Nervous System

The enteric nervous system consists of the myenteric and submucous plexuses. The ganglia contain glial cells, neurons, and the processes of both cell types which form a densely-packed neuropil. The surface of ganglia and tracts are covered by a 20-60 nm thick basal lamina, which does not envelop the parts of the cells or processes not exposed to the surface (Gabella, 1972; 1981; Komuro et al., 1982). No blood vessels are present within the ganglia and tracts of

the ENS, in contrast to other peripheral ganglia. The nearest blood vessels are those in the adjacent muscle layers and submucosa (Clementi and Palade, 1969; Simionescu et al., 1972; 1974).

The neurons vary in size (9-35 μm) and shape (unipolar, bipolar and multipolar) and are less numerous than the glial cells (Schofield, 1968; Gabella, 1972; 1982; Cook and Burnstock, 1976; Gershon, 1981; Komuro, 1982; Jessen and Mirsky, 1983; Gabella and Trigg, 1984). The neurons have a spherical to ovoid nucleus with dispersed chromatin, and in the cytoplasm lots of rough endoplasmic reticulum, neurofilaments, and on the cell membranes, synapses. Glial cells are distinguished from neurons by their smaller cell size, elongated to ovoid nuclei with clump of condensed chromatin near the nuclear membrane, and their processes which envelop the cell processes of neurons (Gabella, 1972; 1981; 1982; Cook and Burnstock, 1976; Komuro et al., 1982). Glial cells are also more numerous than neurons.

Interstitial cells of Cajal (ICCs) make contact with nerve varicosities in the myenteric plexus, each other, and smooth muscle cells. They occur at all levels of the gastrointestinal tract where they act as pacemaker cells determining the rate of contraction of the smooth muscle (Thuneberg et al., 1982; Daniel and Daniel, 1984; Faussone-Pellegrini, 1987; Berezin et al., 1988; Barajas-Lopez et al., 1989; Faussone-Pellegrini et al., 1990; Berezin et al., 1990). There are gap junctions between adjacent ICCs, and

ICCs and smooth muscle cells. (Daniel and Daniel, 1984; Berezin et al., 1988; Komuro, 1989). An ICC has a stellate or fusiform cell body with thin lateral processes covered by a discontinuous or continuous basal lamina and an ovoid nucleus with dispersed chromatin. The ICC cytoplasm contains numerous mitochondria and lots of smooth endoplasmic reticulum, filaments, and caveolae (Komuro et al., 1982; Faussone-Pellegrini, 1987; Komuro, 1989; Berezin et al., 1990).

1.3.2 Similarities of the Enteric and Central Nervous Systems

The enteric nervous system resembles the CNS in four ways: (1) the glia of the ENS and the astrocytes of the CNS both contain glial fibrillary acidic proteins (GFAP); (2) both the ENS and CNS have dense neuropil; (3) both ENS and CNS have a multiplicity of neurotransmitters (Gabella, 1972; 1979; Cook and Burnstock, 1976; Furness and Costa, 1980; Jessen and Mirsky, 1980; 1983; 1984; Burnstock, 1981; Bjorklund et al., 1984); Jessen et al., 1984; (4) both enteric glia and astrocytes do not have individual basal laminae. In the ENS, the basal lamina covers the surfaces of the ganglia and tracts, and in the CNS the basal lamina covers the astrocytic endfeet near blood vessels (Gabella, 1972; 1981; 1982; Cook and Burnstock, 1976; Peters, Palay and Webster, 1991).

Enteric glia, Remak cells (Schwann cells that do not form myelin) in peripheral nerves, satellite cells in

sympathetic and sensory ganglia, and astrocytes in the CNS contain filaments of glial fibrillary acidic protein in their cytoplasm (Jessen and Mirsky, 1980; 1983; 1984). By the use of a monoclonal antibody to astrocytic GFAP, Jessen, Thorpe and Mirsky (1984) showed that only some glial cells in the myenteric plexus have the same GFAP as astrocytes. The other enteric glia have the same GFAP as Remak cells, although this has yet to be tested with a monoclonal antibody to GFAP in Remak cells.

1.3.3 Permeability of Enteric Nervous System

The ganglia and tracts of the enteric nervous system do not contain any blood vessels (Gershon et al., 1979; Gabella, 1985). The nearest blood vessels to the myenteric plexus are in the circular and longitudinal muscle layers. The penetration of this part of the enteric nervous system has been investigated. Jacobs (1977) injected HRP intravenously into rats and guinea pigs and found the HRP reaction product in the extracellular spaces of the myenteric ganglia, tracts, and the muscle layers of the intestine. In marked contrast, Gershon and Bursztajn (1978) failed to detect the same HRP reaction product in the myenteric plexus in the mouse, although they did detect the enzyme's reaction product in the submucous ganglia and tracts in the submucosa and mucosa. Hence these investigators concluded that impermeable capillaries in the adjacent smooth muscle prevented diffusion of protein from the blood into the myenteric plexus.

CHAPTER 2

RATIONALE AND PLAN FOR THE INVESTIGATION

Although blood-nervous tissue barriers have been studied for about ninety years, some controversies remain regarding the enteric nervous system, peripheral nerve, and sensory ganglia. These three areas were, therefore investigated.

2.1 Enteric Nervous System

In order to resolve the controversy concerning the penetration of intravenously injected horseradish peroxidase (HRP) into the extracellular spaces of the myenteric plexus, the original experiment of Gershon and Bursztajn (1978) was repeated. Jacob's (1977) original experiment was not repeated because it seemed that the HRP she saw in the myenteric plexus was the result of perfusion damage to the zonulae occludentes between the capillary endothelial cells in the circular muscle layer, which allowed these capillaries to leak HRP. The results obtained from repeating Gershon's and Bursztajn's experiment showed a patchy distribution of HRP. For this reason their experiment was modified as follows: phosphate buffer was used instead of cacodylate buffer; a mixture of 1% glutaraldehyde and 1% formaldehyde (paraformaldehyde)

replaced a mixture of 5% of glutaraldehyde and 4% formaldehyde (paraformaldehyde) and vibratome sections were used in place of tissue chopper sections.

Since HRP only has a molecular weight of 40,000 daltons it remained to be determined whether or not larger molecular weight exogenous proteins would enter the extracellular spaces of enteric ganglia. For this reason, and also to determine the effect of circulation on tracer distribution rhodamine labelled bovine albumin was administered to rats (see table 1) and allowed to circulate for various times. In order to determine whether other foreign substances could enter enteric ganglia, ethidium bromide and acriflavine, which bind to plasma proteins were also administered intravenously.

Since HRP and rhodamine labelled bovine albumin were exogenous proteins their distribution in the animal may not be identical to that of circulating endogenous proteins. For this reason and also to determine whether endogenous plasma proteins could enter the enteric nervous system, the following experiments were done: (1) The rabbit anti rat albumin and the PAP method were used to detect the presence of endogenous albumin in ileal tissue; and (2) bovine albumin was injected into rats and detected immunohistochemically but due to the antibody to bovine albumin cross-reacting with rat albumin, this experiment was abandoned.

The following control experiments were performed: (1) saline injected animals were used as controls for comparison in each tracer experiment and the control tissues were processed using the same procedure as that used for tissues from tracer injected animals; and (2) for each tracer used the permeability of the enteric nervous system was compared to that of areas of known permeability in the brain and peripheral nervous system.

2.2 Peripheral Nerve and Sensory and Autonomic Ganglia

Controversy regarding blood-tissue barriers in the peripheral nervous system is not confined to the enteric nervous system. The extracellular spaces around axons and their ensheathing supporting cells in peripheral nerves are not penetrated by HRP or fluorescently labelled proteins, but they do contain some plasma proteins (section 1). Therefore the effect of the circulation time of the tracer was varied in order to determine whether the tracer would be present in the endoneurium after long circulation times, but absent after shorter times.

Another controversy in the peripheral nervous system, in particular in sensory and automatic ganglia, is whether a tracer can enter the extracellular spaces between satellite cells and the neurons they ensheath in these ganglia. These ganglia contain permeable blood vessels. Here too the length of time of the tracer in the bloodstream was thought to have an effect on whether or not the tracer would be present in these extracellular spaces in the ganglia.

Therefore in order to investigate the permeability of the endoneurium of peripheral nerve and the accessibility of extracellular spaces between satellite cells and the neurons they ensheath in sensory and autonomic ganglia, the following experiments were done:

1. The tracers rhodamine labelled bovine albumin, bovine albumin (abandoned due to cross-reactivity of the rabbit antibovine albumin antibody with rat albumin), enthidium bromide and acriflavine were allowed to circulate for short times (5-30 min); 2. for peripheral nerve, HRP was allowed to circulate for 5, 10 and 30 min;

3. rhodamine labelled bovine albumin was injected daily for one week into rats (long circulation time);

4. endogenous rat albumin was detected immunohistochemically (long circulation time); 5. saline-injected animals served as controls and were compared with those injected with tracers. For each tracer used the permeability of the endoneurium and sensory and sympathetic ganglia were compared with known permeable areas of the nervous system (circumventri-cular organs) and to known impermeable areas of the central nervous system (dorsal motor nucleus of the vagus and the arcuate nucleus).

2.3 Do Ethidium Bromide and Acriflavine Behave as Protein Tracers in the Circulation?

In order to determine whether ethidium bromide and acriflavine would behave as fluorescent protein tracers in the circulation it was necessary to determine the percent

binding of these substances to bovine albumin and rat's serum. The binding of ethidium bromide and acriflavine to bovine albumin was determined by dialyzing a solution of each dye and albumin for eight days because this resembled long circulation times. The binding of rhodamine B isothiocyanate and Evans blue to bovine albumin served as comparisons. Albumin alone inside the dialysis tubing was used as a control.

In order to ascertain the percent binding of ethidium bromide and acriflavine to rat's serum for short circulation times a solution of each dye and rat's serum were mixed together and allowed to equilibrate for five days and then filtered (centricon ultrafiltration unit) under 2600 g force by centrifugation. The binding of each of these basic dyes in the free state to the filter was not investigated, because neither basic nor acidic drugs in the free state bind to these filters at detectable levels in free drug assays (personal communication, Ward, 1991).

2.4 Does Rhodamine Labelled Bovine Albumin Circulate as a Single Molecule or a Complex of Molecules in the Bloodstream?

In order to address this question RBA was subjected to polyacrylamide gel electrophoresis to determine the number of components and the molecular weight of each component. These results were compared to those HRP, bovine albumin and known molecular weight standards.

CHAPTER 3

MATERIALS AND METHODS

3.1 Animals

Six male Long Evans rats (250-300 g) were used for each experiment unless stated otherwise. The animals were housed in plastic cages (1-3 rats/cage) and fed Purina Rat Chow and water ad libitum with 12 hours of light per day.

The rats were anaesthetized with intramuscular ketamine (75 mg/kg) and xylazine (8 mg/kg) (Green, 1982) for all intravenous injections prior to killings. The animals were killed by exsanguinating from the large vessels of the neck (External and Internal carotid Arteries and External and Internal Jugular Veins).

3.2 Administration of Intravascular Tracers

Rats were injected intravenously (penile vein) with 1 ml of one of the tracers (Table I) or 1 ml of saline.

In the case of RBA, another experiment was performed, in which four rats were injected subcutaneously with 1 ml of RBA per day for 7 days.

Table I - Intravascular Tracers

Number of rat	Compound	Solution and Dose per rat	Circulation Time	Fixative (sectioning)
4	HRP[1]	1 ml of 320mg/kg in saline	5m	1% Glu + 1% Form[3] (Vibratome)
4	HRP	1 ml of 320mg/kg in saline	10m	1% Glu + 1% Form (Vibratome)
4	HRP	1 ml of 320mg/kg in saline	30m	1% Glu + 1% Form (Vibratome)
6	RBA[2]	1 ml of 10% solution	5m	4% Form[4] (Paraffin)
6	RBA	1 ml of 10% solution	30m	4% Form (Paraffin)
4	RBA	1 ml of 10% solution	7 days	4% Form (Paraffin)
4	bovine albumin[1]	1 ml of 1% solution in saline	45m	2% Form +Picric acid[5] (Paraffin)
6	acriflavine hydrochloride[1]	1 ml of 0.15% solution in saline	5m	4% Form[4] (Cryostat)
6	acriflavine hydrochloride	1 ml of 0.15% solution in saline	30m	4% Form (Cryostat)
6	ethidium bromide[1]	1 ml in 0.1% solution in saline	5m	4% Form[4] (Cryostat)
6	ethidium bromide	1 ml in 0.1% solution in saline	30m	4% Form (Cryostat)

[1]Sigma Chemical Company, St. Louis, Mo. USA.

[2]Rhodamine labelled bovine albumin (RBA) was synthesized according to the method of Heinicke and Kiernan (1978).

[3]1% Glu + 1% Form = 1% glutaraldehyde and 1 % formaldehyde (from paraformaldehyde) in 0.1M phosphate buffer, pH 7.4.

[4]4% Form = 4% formaldehyde in 0.1M phosphate buffer, pH 7.2.

[5]2% Form + Picric acid = 2% formaldehyde and picric acid in 0.1M phosphate buffer, pH 7.3.

3.2.1 Determination of Optimal Acriflavine Dose and Ethidium Bromide Dose

One ml of the following acriflavine hydrochloride concentrations: 0.0015%, 0.015%, 0.15% and 0.75% was injected intravenously into rats. The 0.15% dose yielded results of only staining areas known to be permeable to macromolecules was used in all subsequent experiments.

Although the doses of 0.1% and 0.5% ethidium bromide were tried, only the 0.1% dose yielded the expected results in areas of the nervous system known to be permeable or impermeable.

3.3 Dissection, Fixation and Processing

3.3.1 Parts Removed

The following tissues were removed and processed for light microscopy: the brain, pituitary gland, trigeminal ganglia, liver, celiac and superior mesenteric ganglia (preaortic ganglia), ileum (10-20 cm from the ileocecal junction), and sciatic nerves. The hypothalamus and caudal end of the medulla were processed for light microscopy because these pieces contain the median eminence and area postrema respectively. Other pieces of area postrema taken from the medulla, in addition to pieces of trigeminal ganglion, ileum, and sciatic nerve were processed for electron microscopy.

3.3.2 Fixation

Animals that had been injected with fluorescent dyes (and the corresponding controls injected with saline) were dissected and the removed parts were immersed in phosphate-buffered 4% formaldehyde, pH 7.2 (Kiernan, 1990, p.25) for 24 hours at 4°C.

For subsequent immunohistochemistry, the parts removed from rats injected with bovine albumin and also non-injected rats were fixed by immersion in phosphate-buffered 2% formaldehyde with picric acid (Stefanini, deMartino and Zamboni, 1967) for 24 hours at 4°C. Non-injected rats that were processed for immunohistochemistry at the electron microscopic level were fixed in a solution of 1% glutaraldehyde and 1% formaldehyde (from paraformaldehyde) in phosphate buffer, pH 7.4 (Palay and Palay, 1974, p.327).

Specimens from animals injected with HRP and from the saline-injected rats that served as their controls were immersed for 18 hours at 4°C in a solution of 1% glutaraldehyde and 1% formaldehyde (from paraformaldehyde) in phosphate buffer, pH 7.4 (Palay and Palay, 1974, p.327).

3.3.3 Processing

Fixed tissues from rats injected with rhodamine-labelled bovine albumin (RBA), or unlabelled bovine albumin from saline-injected controls and from non-injected rats were dehydrated in graded alcohols, cleared in toluene, and embedded in paraffin (Kiernan, 1990, p.37), and sections 4-5 μm thick were cut.

Fixed specimens from animals injected with ethidium bromide or acriflavine hydrochloride were immersed in 10% sucrose in 0.1M phosphate buffer (pH 7.4) for 6-8 hours at 4°C and then, in 30% sucrose solution in the same buffer for 24 hours at 4°C. The sucrose was used as a cryoprotectant. These specimens were then frozen in tissue tek O.C.T. compound (Canlab), an embedding medium for frozen tissue, on dry ice and stored at -70°C until cut on the cryostat (Leitz Kryostat 1720 Digital) at -25°C (cabinet, knife and block temperature). These sections were collected on slides that had been subbed with chrome-gelatin (Kiernan, 1990, p.44).

Sections of tissues to be processed for electron microscopy were cut at 40 μm (non-injected rats) and 100 μm (HRP or saline-injected rats) in 0.1M phosphate buffer, pH 7.4 on a vibratome (Oxford Vibratome) with a cutting speed setting of six. The sections were collected, immersed in 0.1M phosphate buffer, pH 7.4, and used immediately for peroxidase histochemistry or immunohistochemistry. The sections were subsequently trimmed to make blocks about 1x2 mm for electron microscopy. These small pieces were washed for 10 minutes in 5.4% sucrose in 0.1M phosphate buffer, pH 7.4 with 0.2mM calcium chloride (x2) and osmicated in 1% osmium tetroxide, 5.4% sucrose, 0.02mM calcium chloride, 0.1M phosphate buffer, pH 7.4 (Palay and Palay, 1974, p.327) for 1 hour at 4°C. They were then rinsed in distilled water (3x10 minutes), stained (section 3.4.2), dehydrated, rinsed in propylene oxide (2x5 minutes), and embedded in a mixture

of Epon812 and araldite (Mollenhauser, 1964). The blocks were left to polymerize at 60°C for 2 days.

3.4 Examination of Sections

3.4.1 Mounting

Sections of specimens taken from rats injected with RBA were dewaxed, hydrated through graded alcohols, mounted in glycerol, and examined for fluorescence (Table II) on a Leitz epifluorescence microscope. Then the sections on the slides were stained (section 3.4.2), mounted in DPX and reexamined.

Sections of tissues from rats injected with bovine albumin and from non-injected rats were stained immunohistochemically (section 3.4.5), mounted in glycerol and examined on a Leitz light microscope.

Sections of specimens from rats injected with ethidium bromide or acriflavine hydrochloride were put in distilled water for 5 minutes at room temperature to remove the tissue tek O.C.T. compound, mounted in glycerol and examined for fluorescence.

Table II - Excitation Wavelength and Emission Wavelength for Fluorochromes

Dye	Exciting λ (nm)	Exciting Filter λ (nm) Range	Emission λ (nm)	Emission Filter λ (nm) Range
RBA[1]	555	BG 36 + S 546 520-546	579	TK 580 / K 580 580 nm and above
ethidium bromide[2]	482	BG 36 + S 546 520-546	616	TK 580 / K 580 580 nm and above
acriflavine[3]	445	3 mm BG 12 325-500	539	TK 510 / K 515 515 nm and above
neutral red[2]	540	3 mm BG 12 325-500	orange (probably 497)	TK 510 / K 515 515 nm and above

[1]Haugland (1989).

[2]Pearse (1980).

[3]Roch (1972).

3.4.2 Staining

The sections of trigeminal and preaortic ganglia and of the ileum from rats injected with RBA were stained with 0.005% neutral red (a freshly made 1/100 dilution in distilled water of a stock solution containing 0.5% neutral red (C.I. 50040) in 0.1M acetate buffer, pH 5) for 2 minutes. These sections of tissue were rinsed in tap water, blotted dry with filter paper, dehydrated in 2 changes of 100% ethanol, cleared in xylene, mounted in DPX, and examined for fluorescence. The dilute neutral red provided a fluorescent stain for basophilic components of the tissue, notably nuclei and Nissl substances.

Sections of sciatic nerves from rats injected with fluorescent dyes were stained with Weigert's haematoxylin (for black nuclei) followed by 0.1% eosin Y (C.I. 45380) for pink background, (Kiernan, 1990, pp.96-98).

Alkaline toluidine blue (C.I. 52040) was used as a solution of the dye in 1% aqueous borax and applied for 1 minute at approximately 80°C to the 0.5-1 μm plastic sections for light microscopy. The plastic sections on the slide were then rinsed in hot (45-50°C) tap water, dried at 80°C for 1 minute without further dehydration and cleaning, and mounted in DPX (Hunter, 1984).

After peroxidase histochemistry and immuno-histochemistry, the tissues to be prepared for electron microscopy were stained en bloc with 2% uranyl

acetate for 1 hour at room temperature (Mollenhauser, 1964). The grids bearing ultrathin sections (see section 3.3.3) were floated for 1 minute on a drop of 0.4% lead citrate on a slab of dental wax. The staining was done in a closed petri dish containing pellets of potassium hydroxide to prevent the formation of lead carbonate (Millonig, 1976, p.54, method 2). The grids were then rinsed in distilled water, blotted with filter paper and dried. The treatment with uranyl acetate and lead citrate increased the opacity of the sectioned tissues to electrons.

3.4.3 Peroxidase Histochemistry

The 100μm vibratome sections from rats that had been injected with HRP and from their saline-injected controls were incubated in 3,3'-diaminobenzidine tetrahydrochloride (DAB, Polysciences Inc., 50 mg) in 0.1M phosphate buffer, pH 7.4 (100 ml) for 10 minutes at room temperature on a shaker table. Hydrogen peroxide was added to the DAB solution to make a 0.003% solution and the sections were incubated for a further 15 minutes. The sections were then rinsed (3 × 10 minutes) in 0.1M phosphate buffer, pH 7.4 (Graham and Karnovsky, 1966).

Sections from normal non-injected rats were also cut on the vibratome and processed for the immunohistochemical demonstration at the EM level of endogenous albumin by the peroxidase anti-peroxidase (PAP) method (see section 3.4.5). These sections were then processed for the localization of peroxidase activity as described in the preceding paragraph

with the following exceptions: the pre-incubation step with DAB was omitted; and the reaction proceeded for 5 minutes.

For subsequent light microscopic examination, the PAP method of peroxidase histochemistry was performed on sections from normal non-injected rats and rats injected with bovine albumin after immunohistochemistry. The incubation time was 10 minutes in 0.05% DAB, and an additional 5 minutes after adding enough hydrogen peroxide to make a 0.01% solution. Finally the sections were rinsed in distilled water (3x10 minutes).

3.4.4. Electron Microscopy

Sections (0.5-1 μm) were cut from the epon blocks for light microscopy and examined both unstained and stained. Then ultrathin (silver) sections were cut, collected onto clean, uncoated copper grids (mesh 200 i.e. 200 open squares/grid), dried, and stained. These grids were then examined for the presence of DAB reaction product using a Jeol JEM-100 CXII electron microscope at 60 kV.

3.4.5 Immunohistochemistry

The peroxidase anti-peroxidase (PAP) immunohistochemical staining method (Sternberger, Hardy, Cuculis and Meyer, 1970) was used to detect endogenous albumin in the sections of tissues from non-injected rats and to detect bovine albumin in the sections of tissues for rats injected with that protein. This procedure involves five steps: (1) the blocking of endogenous peroxidase; (2) the blocking of non-specific binding; (3) application of the primary

antiserum; (4) application of the secondary antiserum; and (5) application of the PAP complex. At the end of the incubation with each antiserum the sections were washed (3x10 minutes) with 0.1M phosphate-buffered saline, pH 7.4 (PBS). The reagents were made up in PBS and the solutions of antisera also contained 1% normal goat serum and 0.001% triton-X 100.

In step 1, the slides of tissues were dewaxed in xylene for 15 minutes, rinsed in xylene and two changes of 100% ethanol and then immersed in 0.3% hydrogen peroxide in 100% methanol for 30 minutes at room temperature to block the endogenous peroxidase activity (Polak and Van Noorden, 1984). The slides were then rinsed in distilled water (3x5 minutes) followed by PBS (10 minutes).

In the second step, the sections were immersed in 10% normal goat serum (NGS) (Sigma Immumologicals, Sigma Chemical Company) in PBS for 30 minutes at room temperature to block sites that would bind proteins non-specifically.

In step 3, rabbit antiserum to rat's albumin (Nordic Immunology, Cedarlane Laboratories Ltd.) or rabbit antiserum to bovine albumin (Nordic Immunology, Cedarlane Laboratories) was applied to the sections. Initially dilutions of 1/20, 1/50, 1/100. 1/500, 1/1000, 1/5000, 1/10000 were used before determining that the 1/1000 dilution of the rabbit anti-rat's albumin antiserum and the 1/500 dilution of the rabbit anti bovine albumin antiserum yielded optimal reactivity. The lyophilized antisera were

reconstituted with distilled water and diluted with PBS with the final solution containing 1% NGS and 0.001% triton-X. The primary antisera were applied for 48 hours at 4°C after which the sections of tissues were rinsed in PBS (3x10 minutes).

In the fourth step, the secondary antiserum, a goat antiserum to the Fc fragment of the rabbit IgG (Cappel, Organonteknika Corporation) was reconstituted with distilled water. The sections of tissues were incubated in a dilution of 1/75 of this antiserum in PBS with final 1% NGS and 0.001% triton-X content for 30 minutes at room temperature and then rinsed in PBS (3x10 minutes).

In the fifth step, PAP (ICN Immunobiologicals) each unit of which is composed of three molecules of HRP + two molecules of rabbit anti-HRP (Sternberger, 1979) was used in a dilution of 1/50 in PBS with final concentrations of 1% NGS and 0.001% triton-X. The sections of tissues were incubated in this dilution for 30 minutes at room temperature followed by rinsing in PBS (3x10 minutes). Finally, the peroxidase activity of the localized PAP was demonstrated histochemically (see section 3.3.3).

The same immunohistochemical procedure was used for the vibratome sections processed for electron microscopy with the following exceptions: the procedure was performed in 1 ml Eppendorf tubes; the first step involving blocking of endogenous peroxidase was omitted; the primary antiserum was used in the dilutions of 1/500 and 1/1000; the secondary

antiserum, goat anti rabbit IgG (Fc specific) was used in the dilution of 1/20; and the PAP complex was used in a dilution of 1/50. The peroxidase histochemical procedure was employed as in section 3.4.3.

3.4.5.1 Controls

The following control procedures were performed to establish the specificity of immunohistochemical staining:

1) Detection of endogenous peroxidase activity by incubating the sections in a mixture of DAB and hydrogen peroxide.

2) The primary antiserum was omitted, in order to detect any non-specific binding of the secondary antiserum and/or PAP.

3) The secondary antiserum was omitted to detect any non-specific binding of PAP.

4) Normal non-immune whole rabbit serum (ICN Immunobiologicals) was substituted for the rabbit primary antisera in order to detect any binding of components of the primary antiserum other than the desired antibodies.

5) Lyophilized rat's serum (Polysciences Inc.) (final concentration in diluted antiserum: 2 mg/ml, 10 mg/ml) was added to the 1/1000 dilution of rabbit anti-rat albumin antiserum, and bovine albumin (Cohn's fraction V; final concentration in diluted antiserum: 10 mg/ml, 20 mg/ml) was added to the 1/500 dilution of rabbit anti bovine albumin antiserum. Any precipitate was

removed by centrifugation and the resulting mixture was used as the primary antiserum in the PAP procedure. In this control, the antibodies to rat's albumin and bovine albumin had been absorbed by the excess of added antigen. Any subsequent immunohistochemical stain would, therefore, be due to other, unwanted antibodies in the primary antisera.

6) Lyophilized rat's albumin (Sigma) (final concentration in diluted antiserum: 1 mg/50 ml) was added to the 1/1000 dilution of rabbit anti rat's albumin antiserum.

7) In order to detect cross-reactivity of the rabbit anti bovine albumin antiserum with rat's albumin this primary antiserum was used on sections from rats that had not received injections of BSA.

3.5 Binding Studies

The binding of ethidium bromide and acriflavine hydrochloride to bovine albumin and rat's serum proteins was investigated by dialysis and ultrafiltration. The binding of rhodamine B isothiocyanate (Sigma) and Evans blue (Sigma) to bovine albumin served as controls.

Solutions of rhodamine B isothiocyanate (0.04%), ethidium bromide (0.10%), Evans blue (0.43%), and acriflavine hydrochloride (0.15%) were made up in 10% bovine albumin in PBS. One ml of each solution was dialysed (6mm Spectrapor dialysis tubing, molecular weight cut-off at

12 000) against 15 ml of PBS for eight days at 4°C and the resulting solutions were examined by ultraviolet/visible spectroscopy (200-800 nm).

Solutions of ethidium bromide (0.10%) and acriflavine hydrochloride (0.15%) were made up in 6.53% lyophilized rat's serum in PBS (6.53% is the average concentration of serum proteins in Long Evans rats (Mitruka and Rawnsley, 1981)). After the solutions of dyes and serum had equilibrated for five days, a 2 ml aliquot of each solution was transferred to a centricon ultrafiltration unit and centrifuged for 2 hours at 2600 g and 4200 rpm (GSA rotor, 5.75 cm radius, 28° fixed angle; Sorvall RC-5B centrifuge). The 6.53% solution of lyophilized rat's serum (by weight) in PBS was used as a control and PBS was used as the background. The filtrates were examined by UV-visible spectroscopy.

3.6 Analysis of Bovine Albumin (Cohn fraction V), HRP (type II) and Rhodamine labelled Bovine Albumin by Electrophoresis

The solutions were made up in 0.01M trisHCl buffer (Sigma), pH 8.0 containing 0.001M EDTA, 2.5% sodium dodecylsulfate (SDS), 5% mercaptoethanol, and 0.01% bromophenol blue. The following samples and concentrations were used: a mixture of low molecular weight standards (3.5 μg/μl), a mixture of high molecular weight standards (3.5 μg/μl), bovine albumin (1 mg/ml and μg/ml), RBA

(8 mg/ml and 4 mg/ml), and HRP (2 mg/ml and 1 mg/ml). Then the samples were heated in Eppendorf tubes in boiling water for 10 minutes. The samples were loaded onto a 0.45 mm preformed gradient acrylamide gel. The gel had a 32 mm long linear gradient of 8-25% acrylamide (2% cross-linking) in the separating gel and 6% acrylamide in the 13 mm stacking gel. The proteins were separated by electrophoresis (SDS-PAGE) on an automated horizontal apparatus (Phast system, Pharmacia) using preformed gels and buffer strips (Phast Gels, Phast Gel SDS buffer strips, Pharmacia) with a constant current for 95 volt hours. The gels were stained with Coomassie blue R350 (Phast Gel Blue R350, Pharmacia) in the developing unit of the Phast system (Heukeshoven and Dernick, 1988). The molecular weight of a given protein band was calculated from a standard curve of $\log_{10}$ (molecular weight) plotted versus migration distance (mm) of standard proteins which were run on the same gel.

CHAPTER 4 RESULTS

The distribution of plasma albumin and of injected tracers are described for various parts of the nervous system: median eminence and hypothalamus; area postrema; preaortic ganglia; trigeminal ganglia; sciatic nerve; and ileum.

4.1 Median Eminence and Hypothalamus

4.1.1 Endogenous Albumin

Sections of median eminence and hypothalamus stained immunohistochemically by the PAP method for endogenous rat's albumin were examined under phase contrast and bright-field conditions. Staining in the median eminence was observed inside and outside of the blood vessels (Figure 1 A and B). However, the nearby arcuate nucleus exhibited immunoreactivity to albumin only within the blood vessels (Figure 1 B).

Evidence that the staining was due only to the presence of rat albumin came from the control observations. No staining was observed with the following:

(a) The primary antiserum, rabbit anti rat albumin was omitted.

(b) Non-immune rabbit serum was substituted for primary antiserum.

(c) The primary antiserum was absorbed with rat's albumin and then used instead of the primary antiserum.

Positive controls of the liver showed staining in the cytoplasm of the hepatocytes and around the cells and in intravascular plasma. No staining was observed in any of the other tissues of the negative controls previously mentioned.

4.1.2 Fluorescent Rhodamine Labelled Bovine Albumin (RBA)

Five or thirty minutes after the intravenous injection of RBA, the orange-red fluorescence of this tracer was conspicuous in blood vessels of all sizes and was confined to these sites in all parts of the hypothalamus (Figure 1 C and D). In the median eminence however, fluorescence was present inside and outside the blood vessels. The extravascular fluorescence was due to the rhodamine label because it was absent from sections of the same area taken from saline-injected control rats.

Exactly the same appearances were seen when RBA had circulated for 5 or 30 minutes, or after daily injections for one week.

4.1.3 Basic Fluorochromes

After an injection of ethidium bromide, rats were sacrificed at 5 and 30 minutes. Identical results were obtained with both circulation times. The same sections

Figure 1

A. (x80) and B. (x650) Hypothalamus and median eminence showing immunoreactivity to endogenous albumin. In the median eminence there was staining inside and outside blood vessels, but in the adjacent arcuate nucleus staining was confined to the blood vessels.

C. (x150) and D. (x360) fluorescence in the median eminence was inside and outside the blood vessels, RBA fluorescence in the adjacent hypothalalmus was only present within blood vessels. These sections were from rats killed 30 minutes after injection rhodamine labelled albumin.

AN = arcuate nucleus; bv = blood vessel;

ME = median eminence; V = 3rd ventricle.

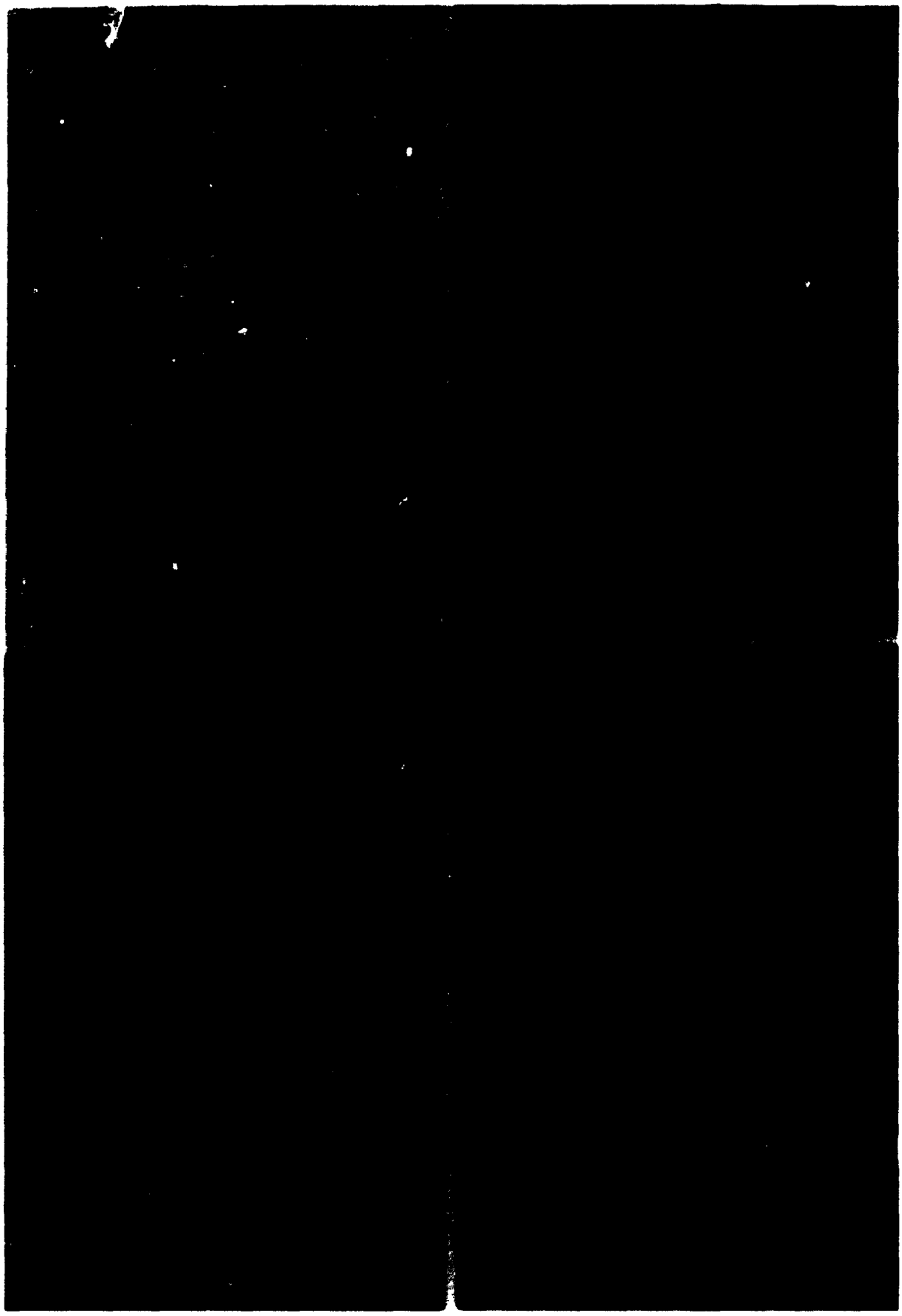
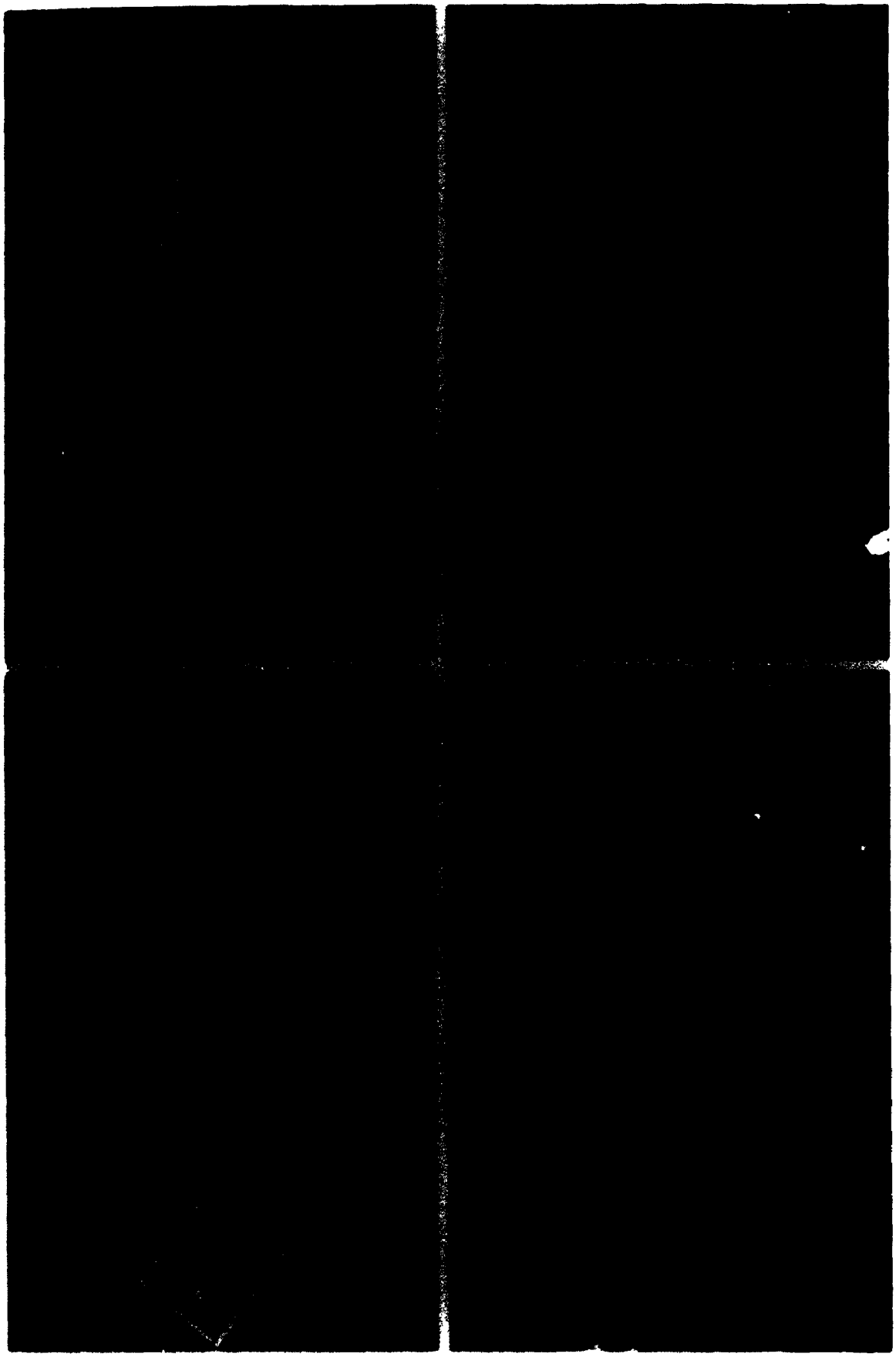


Figure 2

A. (x150) and B. (x360) Red fluorescence was present in the nuclei of cells in the median eminence of this rat killed 5 minutes after an intravenous injection of ethidium bromide. There was no fluorescence inside blood vessels or in the adjacent hypothalamus.

C. (x150) and D. (x360) From a rat killed five minutes after intravenous injection of acriflavine. Fluorescence was present in the nuclei of cells in the median eminence and in the nuclei of many cells in the adjacent hypothalamus. In D. the background fluorescence was due to autofluorescence: no fluorescence was observed inside the blood vessels. HT = hypothalamus, ME = medium eminence, V = 3rd ventricle, bv = blood vessel.



were compared under blue light for autofluorescence and green light for red fluorescence. Red fluorescence was present in the nuclei and nucleoli of tanycytes and other cells and in the processes of tanycytes (Figure 2 A and B). The tanycytes were recognized by their location in the floor of the third ventricle. The processes of tanycytes were long thin fluorescent structures close to blood vessels. It was not possible to identify the other cell types unequivocally. No fluorescence was observed inside the blood vessels. The adjacent hypothalamus did not exhibit any fluorescence.

When acriflavine (Figure 2 C and D) was used instead of ethidium bromide, yellow-green fluorescence was observed in the same locations in the median eminence as the red fluorescence observed with ethidium bromide. However, in contrast to the results obtained with ethidium bromide, the adjacent hypothalamus exhibited fluorescence in the nuclei of many cells. The results for both circulation times were the same.

4.2 Area Postrema and Adjacent Medulla

4.2.1 Endogenous Albumin

The immunohistochemically stained sections were examined by bright-field and by phase contrast microscopy. In the ordinary tissue of the medulla, staining was confined to the blood vessels (Figure 3 A). In the area postrema however, staining was present inside and outside the blood vessels (Figure 3 B). Negative and positive control

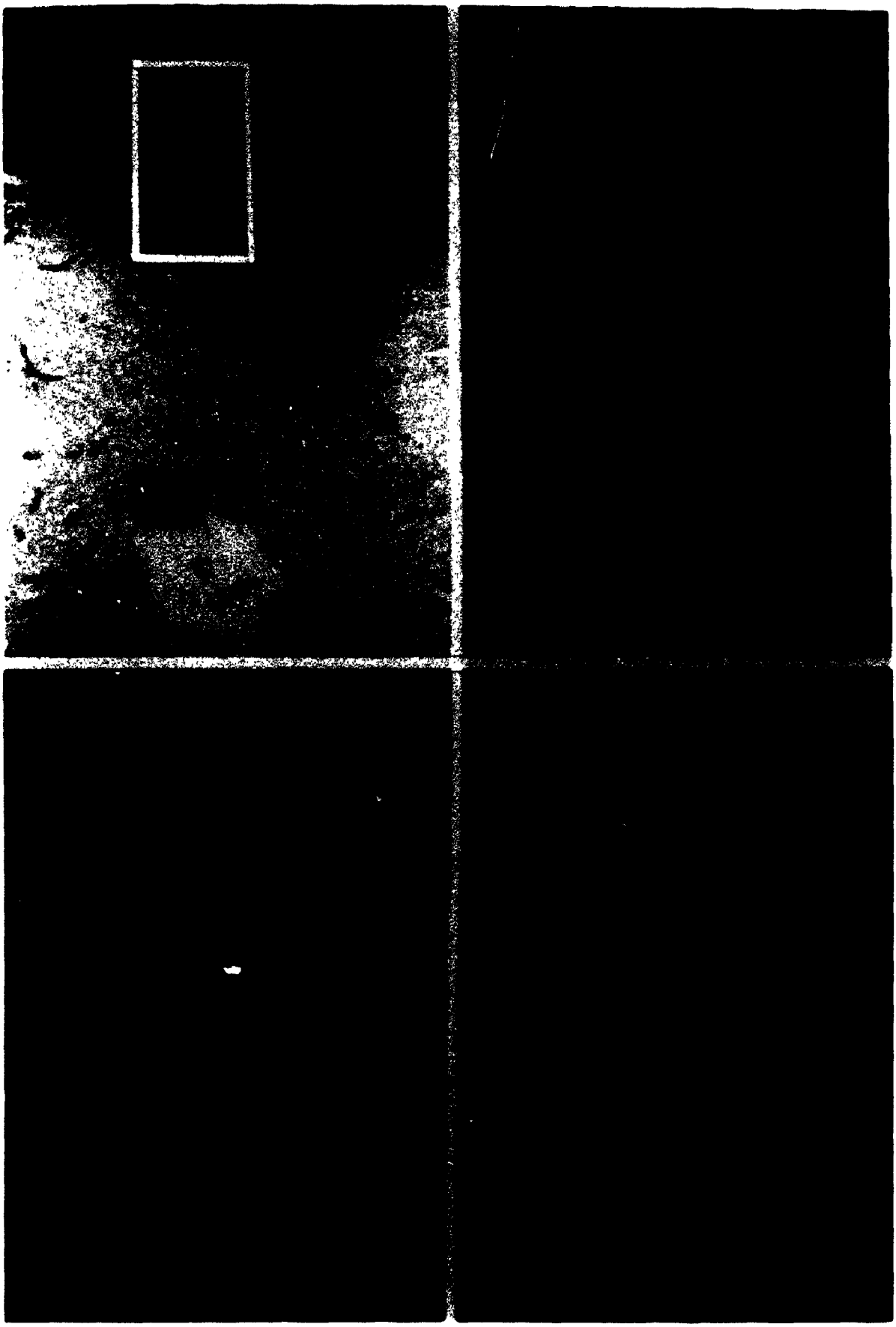
Figure 3

A. (x140) and B. (x540) Immunopositive staining for endogenous albumin was present inside and outside the blood vessels in the area postrema, but was only present inside the blood vessels of adjacent medulla.

C. (x360) and D. (x360) Area postrema and adjacent medulla from a rat killed 5 minutes after intravenous injection of the fluorescent dye, rhodamine labelled albumin. In the area postrema fluorescence was present inside and outside the blood vessels, but in the adjacent medulla, fluorescence was confined to the lumina of the blood vessels.

AP = area postrema; bv or arrowhead indicates blood vessel; C = central canal; DMV = dorsal motor nucleus of vagus; HGN = hypoglossal nucleus;

M = medulla.



procedure confirmed the specificity for rat's albumin (see section 3.4.5.1 and 4.1.1).

4.2.2 Fluorescent Rhodamine Labelled Bovine Albumin (RBA)

Sections from animals injected with RBA or saline were examined for red fluorescence with excitation by green light. The red blood cells and cytoplasm of the neurons in the dorsal motor nuclei of the vagus and hypoglossal nuclei in saline controls exhibited a weak red fluorescence. Five minutes after an intravenous injection of RBA, red fluorescence was present both inside and outside the blood vessels of the area postrema (Figure 3 C). In the adjacent medulla the fluorescence was confined to the blood vessels (Figure 3 D). The appearance after the circulation of RBA for 30 minutes was identical to that after 5 minutes. Somewhat different results were obtained when RBA was administered daily for one week (Figure 4 A and B). Red fluorescence attributable to RBA was visible inside and outside the blood vessels and also in the cytoplasm of a few cells of the area postrema. The adjacent medulla only contained fluorescence in its blood vessels.

4.2.3 Horseradish Peroxidase

The area postrema had fenestrated blood vessels which were surrounded by large perivascular spaces (Figure 5 A and B). The endothelial cells were covered by a basal lamina and surrounded by pericytes which contained free ribosomes, some dense bodies, mitochondria, and endoplasmic reticulum. Astrocytic end feet which were covered by basal lamina

Figure 4

A. (x220) and B. (x520) Area postrema from a rat which was killed after one week of daily subcutaneous injection of rhodamine labelled albumin. Red fluorescence was present inside and outside the blood vessels in the area postrema, but was confined to the lumina of the blood vessels in the adjacent medulla.

AP = area postrema; bv = blood vessel; C = central canal; M = medulla; arrow indicates fluorescent cell.

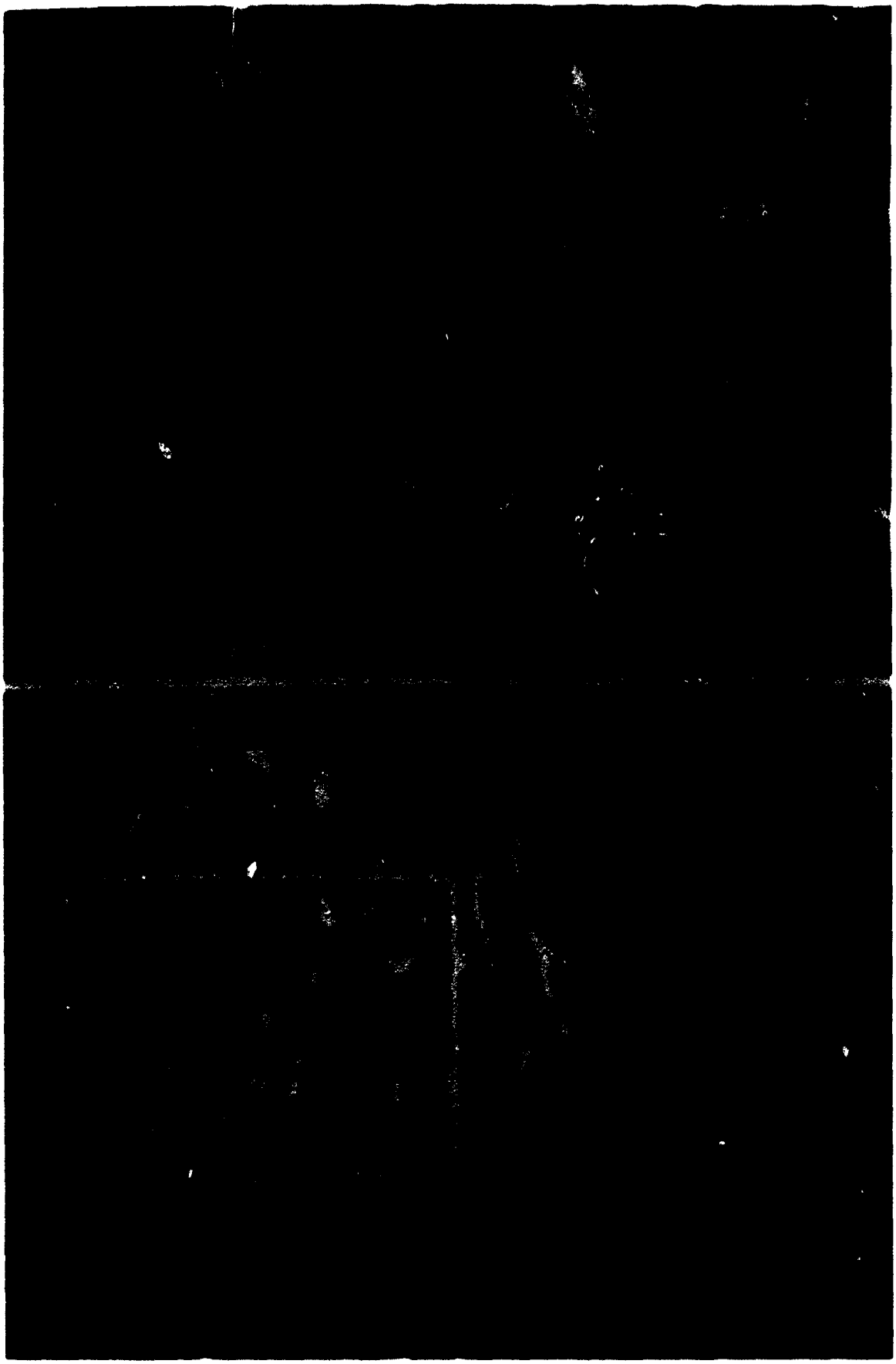


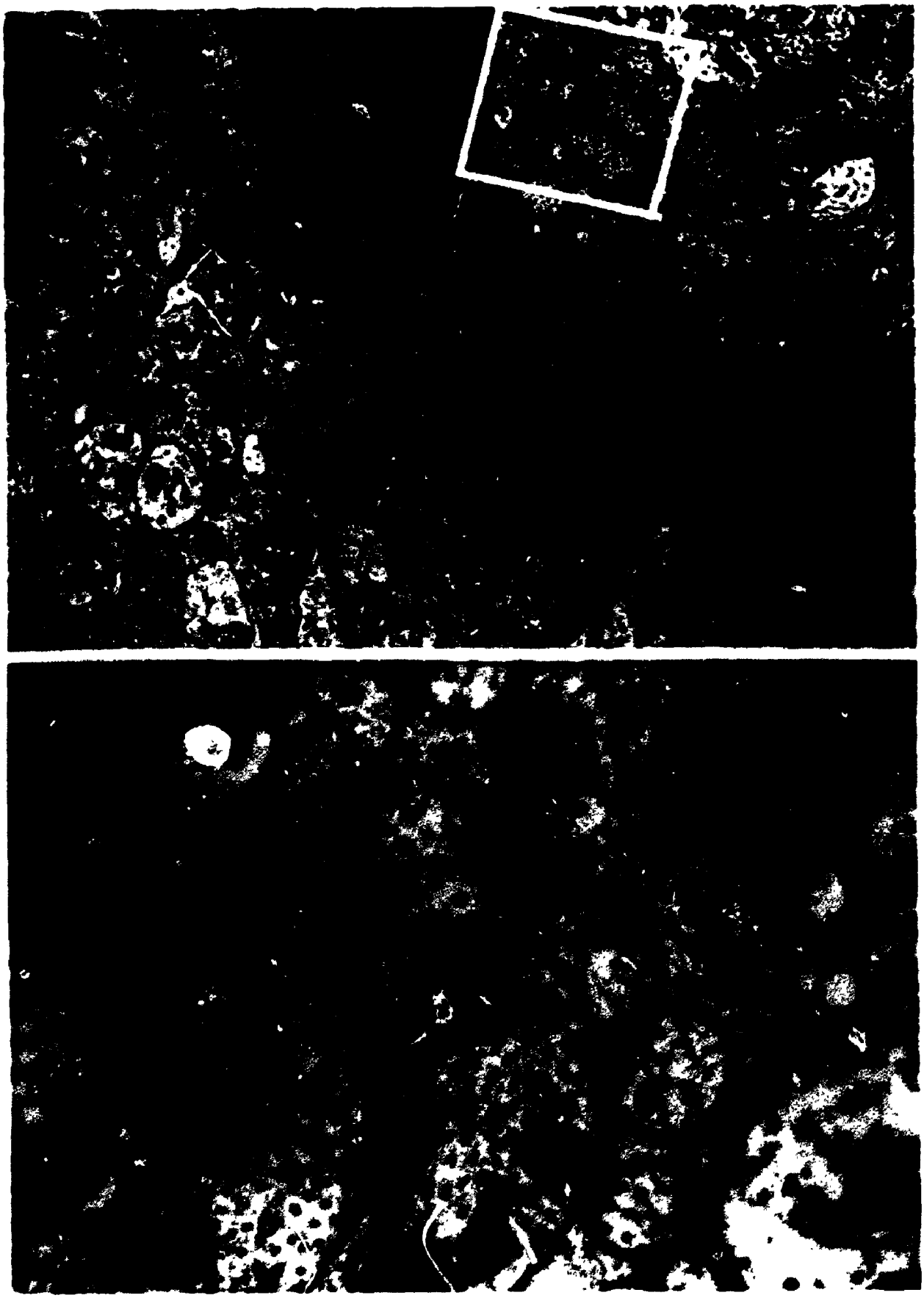
Figure 5

A. (x11,000) The DAB reaction product in perivascular spaces (open arrowheads) around the blood vessel and between cell membranes in neuropil of the area postrema. This open section was from a rat which was killed 10 min. after the HRP injection.

B. (x47,000) Area in white square.

A = axon; As = astrocytic process; bv = blood vessel; D = dendrite; E = endothelial cell;

m = microtubules; N = neuron; Pa = punctum adherens; P = pericyte; open arrowhead indicates glycogen granule; solid arrowhead indicates basal lamina; open arrowheads indicate perivascular spaces containing the DAB reaction product.



surrounded the endothelial cells of the blood vessels. The processes of astrocytes were recognized by their enveloping many other processes (Figure 5 B), their pale cytoplasm which contained 8 nm filaments (Peters, Palay and Webster, 1991), dense 33 nm glycogen granules (Peters, Palay and Webster, 1991), and mitochondria. There were puncta adherentes between adjacent astrocytic processes. Sometimes nerve endings were present in the perivascular spaces between the astrocytes and pericytes. The neuropil of the area postrema contained only unmyelinated fibres. The membranes in the neuropil were 20 nm apart. Dendrites were recognized by their regular arrays of 22 nm microtubules (Peter, Palay and Webster, 1976), mitochondria, fragments of agranular endoplasmic reticulum, neurofilaments in the cytoplasm, and synaptic thickenings. Axons were identified by the presence of synaptic vesicles, free ribosomes, 10 nm neurofilaments in the cytoplasm, and synaptic thickenings. Some neurons were present in the area postrema. The cytoplasm of the neuronal soma was identified by the presence of an abundance of Nissl bodies and ribosomes, some mitochondria, microtubules, and neurofilaments.

Ten minutes after intravenous injection of HRP, the rats were killed and the brains (caudel end of the medulla) were processed as stated in sections 3.3.2 and 3.4.3. The diaminobenzidine (DAB) reaction product was present in the blood vessels, perivascular spaces, and neuropil of the area postrema (Figure 5 A and B). Dark deposits were visible in

some vesicles inside the endothelial cells , in their basal lamina, as well as between the endothelial cells and adjacent pericytes. The basal lamina of the pericytes also contained the DAB reaction product, as did the spaces between the pericytes and the astrocytic feet and the basal lamina around the end feet. In the neuropil the reaction product of HRP was present between astrocytic processes, dendrites, and axons. The spaces between neuronal somata and astrocytic processes, axons, and dendrites also exhibited the dense deposits.

The same results were obtained after 5 minutes. By 30 minutes very little reaction product of HRP was visible in the area postrema.

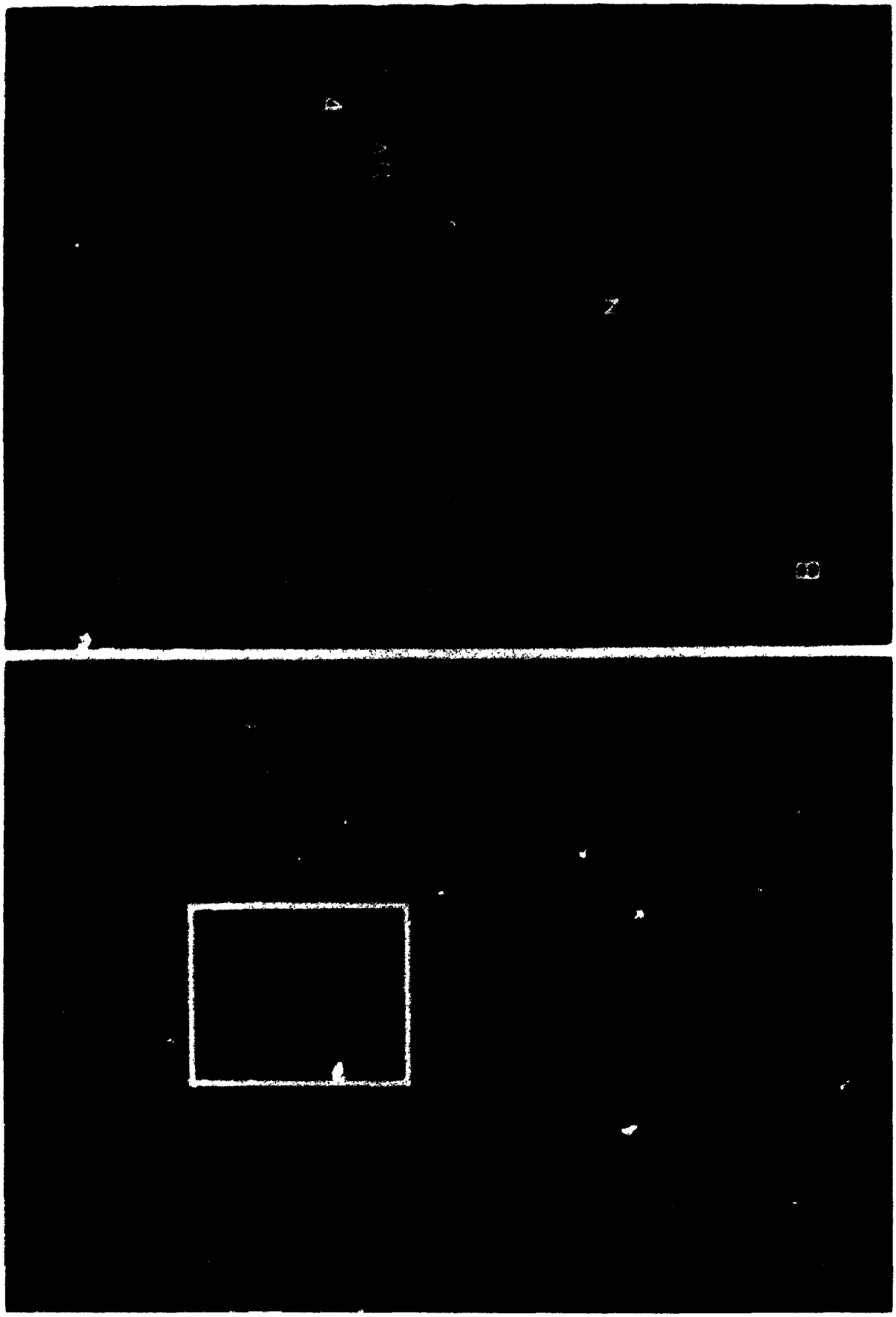
4.2.4 Basic Fluorochromes

Intravenously injected acriflavine, after circulating for 5 minutes, imparted bright yellow-green fluorescence to the nuclei and cytoplasm of endothelial cells and to the cells of the nervous tissue in the area postrema. Probably the nuclei of all the neurons and glial cells were stained, but cell-types could not be identified with any certainty (Figure 6 A and B). Endothelial cells were easily identified on the basis of their alignment in the walls of the blood vessels and elongated nuclei. Some neurons had typical granular cytoplasm similar to that seen in Nissl preparation and large fluorescent nucleoli in otherwise non-fluorescent nuclei. The rest of the medulla did not

Figure 6

A. (x230) and B. (x860) The area postrema from a rat killed 5 minutes after an intravenous injection of acriflavine. Fluorescence was observed in the nuclei and cytoplasm of the cells in the area postrema. No fluorescence was present inside the blood vessels, but the nuclei and cytoplasm of the endothelial cells (arrowhead) fluoresced.

AP = area postrema; M = medulla; C = central canal; bv = blood vessel; N = neuron; arrowhead indicates endothelial cell's nucleus.



fluoresce. The appearances observed after 30 minutes circulation of acriflavine were identical to those for 5 minutes.

When ethidium bromide was used instead of acriflavine, identical results were obtained.

4.3 Pituitary Gland

4.3.1 Endogenous Albumin

The sections which were stained immunohistochemically for rat albumin were examined using bright-field and phase contrast conditions. The walls and lumina of the sinusoids of the pars distalis were stained (Figure 7 A). Staining was also observed around cells of the parenchyma in this area. The connective tissue septa of the pars intermedia were stained and the brown colour extended into a pattern of lines around the cells. Immunopositive material was also present in the lumina and walls of the few small blood vessels near the edges of the pars intermedia. The cells in the pars distalis and the pars intermedia were unstained. In contrast many cells in the pars nervosa were stained. Strong staining was present in the walls and some lumina of blood vessels.

4.3.2 Fluorescent Bovine Albumin (RBA)

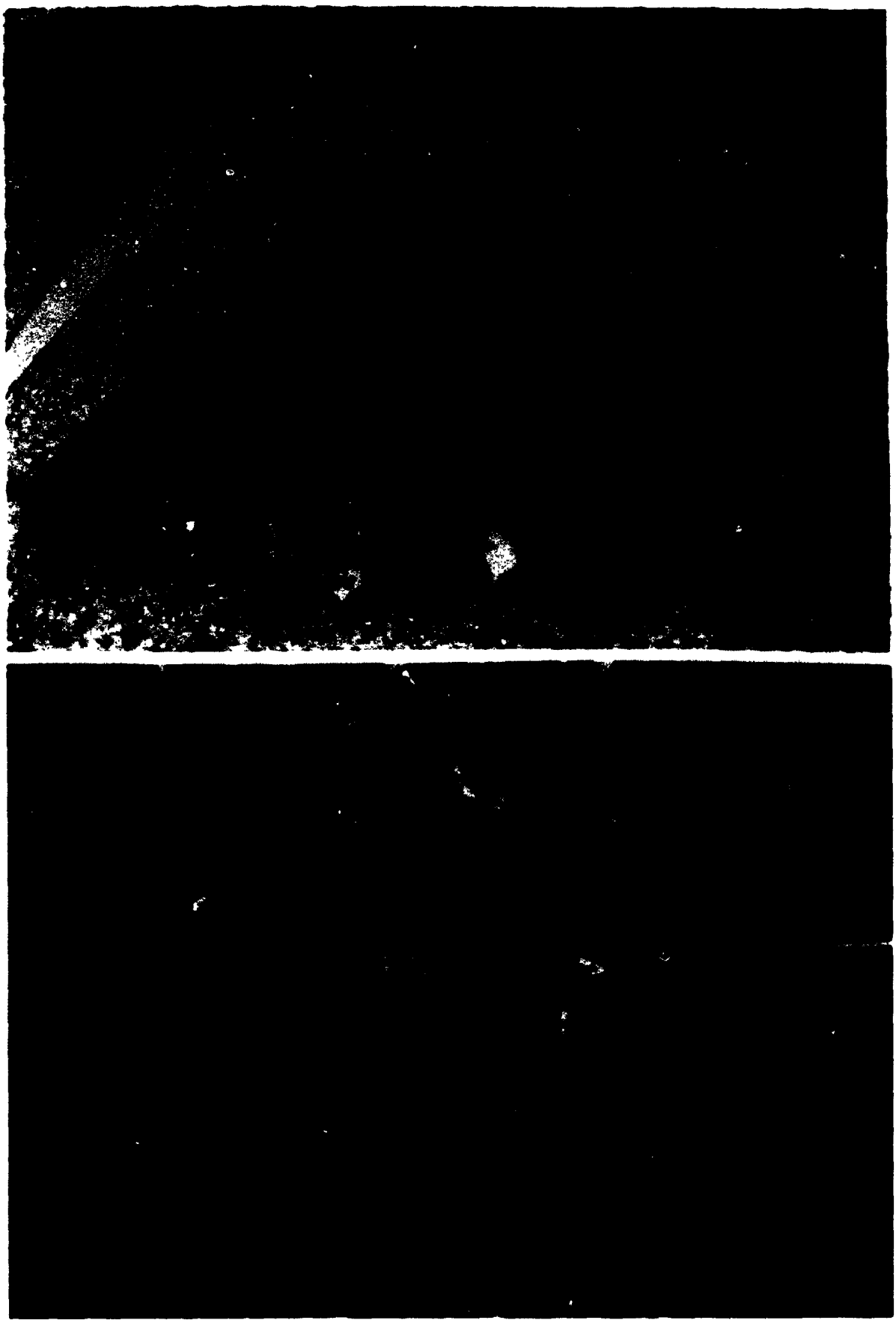
The red fluorescent sections were compared with the green autofluorescent sections to determine the location of the cells. Five minutes after an intravenous injection of RBA, fluorescence was visible both inside and outside the

Figure 7

A. (x200) Immunoreactivity for endogenous albumin in the pituitary. In the pars distalis staining was present in the walls and lumina of the sinusoids and around the cells in the parenchyma. In the pars intermedia, the connective tissue septa were stained, as were the areas around the cells. In the pars nervosa staining was present in many cells and in the walls and lumina of blood vessels.

B. (x530) The pituitary from a rat which was killed 5 minutes after an intravenous injection of rhodamine labelled albumin. In the pars nervosa fluorescence was present inside and outside the blood vessels and around the cells. Fluorescence was observed in the connective tissue septa in the pars intermedia and around cells adjacent to these septa.

PD = pars distalis; PI = pars intermedia; PN = pars nervosa; C = cleft; bv = blood vessel.



blood vessels and around the cells of the pars nervosa (Figure 7 B). The pars distalis exhibited the same distribution of fluorescence as the pars nervosa. Fluorescence was seen in the connective tissue septa and around cells near these septa in the pars intermedia. The fluorescence in the pars nervosa and pars distalis was much brighter than that observed around cells in the pars intermedia.

Identical results were obtained after RBA had circulated for 30 minutes. After one week the fluorescence was considerably brighter but in the same location.

4.4 Preaortic Ganglia

The preaortic ganglia, which lie near major visceral arteries arising from the aorta include the celiac, superior, and inferior mesenteric ganglia. These ganglia and their nerves form the major part of the abdominal plexus. In the rat, the celiac and superior mesenteric ganglia do not have discrete boundaries between them. However, the inferior mesenteric ganglion can be distinguished from the other preaortic ganglia (Gabella, 1985).

4.4.1 Endogenous Albumin

The stained sections were examined under bright-field and phase contrast illumination (Figure 8 A and B). These sections were also compared with the controls which confirmed the positive staining for rat albumin.

Figure 8

A. (x570) Preaortic ganglion showing immunoreactivity for endogenous albumin. Staining was present in the connective tissue capsule and around the neurons.

B. (x570) Phase contrast of A.

N_1 = neuron with positive staining in cytoplasm;

N_2 = neuron with no intracellular staining.

C = connective tissue capsule

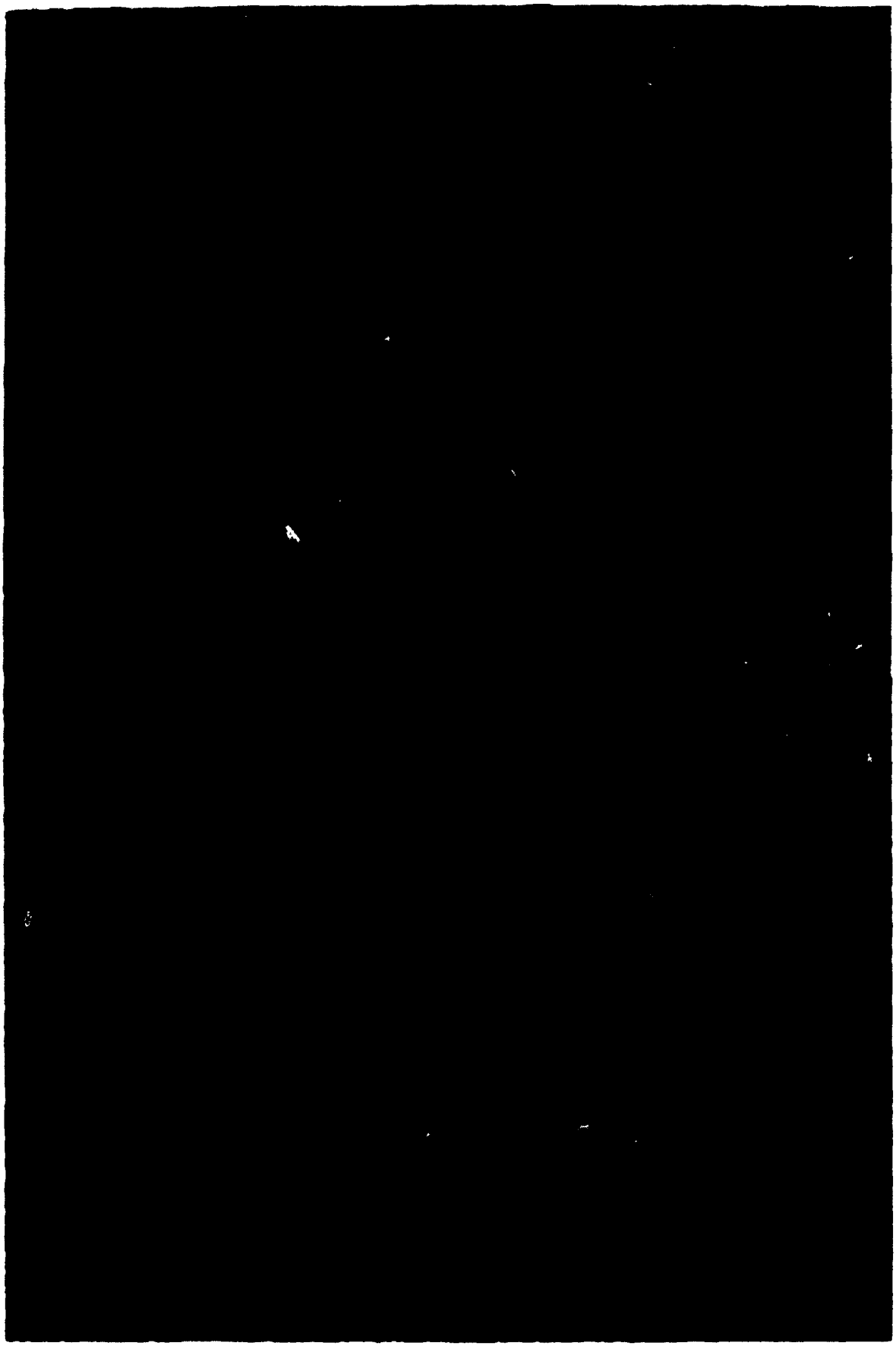


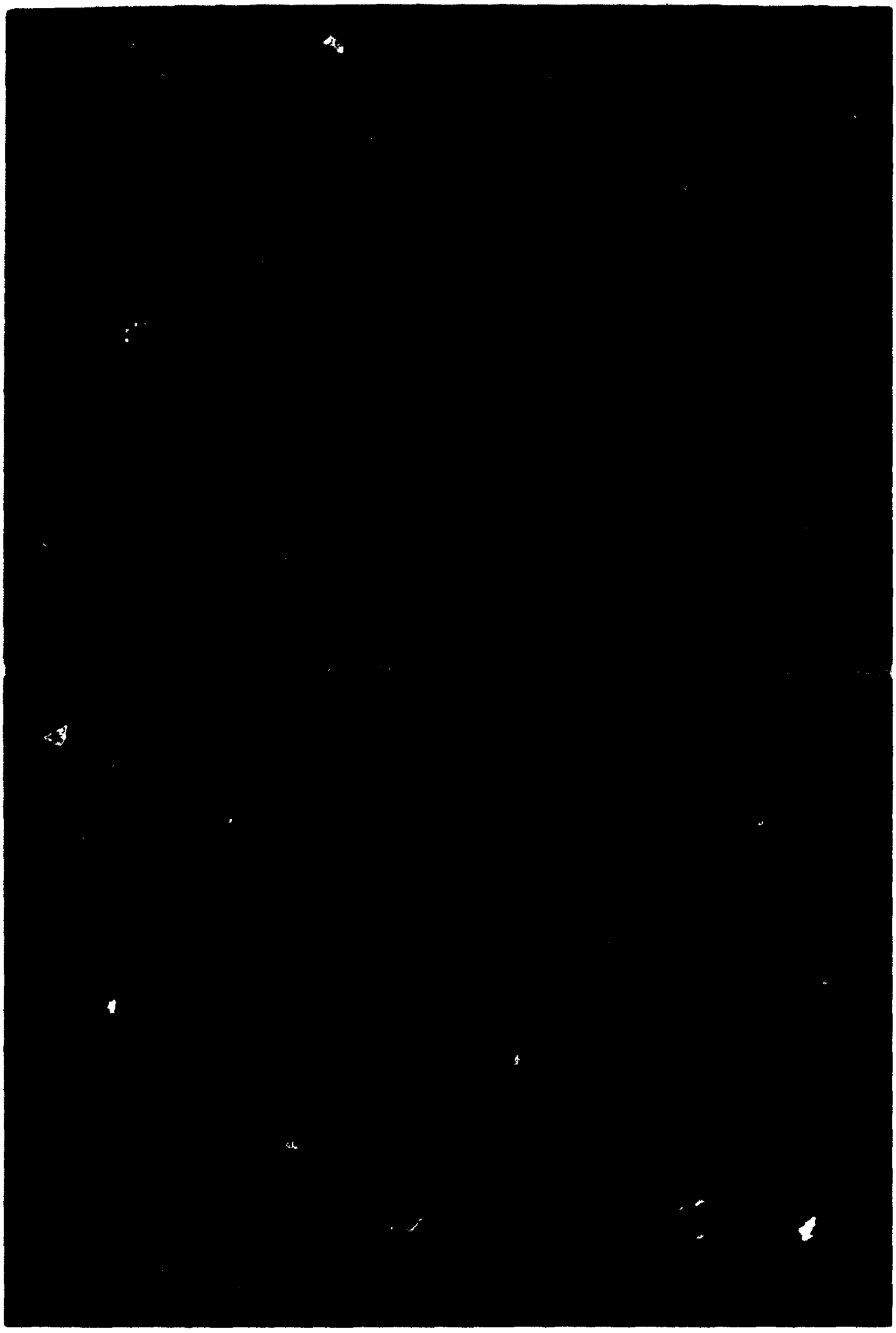
Figure 9

A. (x520) Preaortic ganglion from a rat killed 30 minutes after an intravenous injection of rhodamine-labelled albumin. Fluorescence was present in the connective tissue capsule, inside blood vessels, around neurons, and between neurons and their satellite cells.

B. (x520) Dull green autofluorescence of A. observed with blue exciting light.

C = connective tissue sheath; N = neuron;

bv = blood vessel; solid arrowheads indicate satellite cells; clear arrowhead indicates neuronal nucleus.



Immunohistochemical staining for endogenous albumin was present in the connective tissue capsule and walls and lumina of blood vessels in the preaortic ganglia. Staining was present around neurons and in the cytoplasm of some neurons next to the connective tissue capsule (Figure 8 A). In contrast the cytoplasm of other neurons was negative.

4.4.2 Fluorescent Bovine Albumin

The red fluorescence of RBA was observed, after circulating for 30 minutes, in the connective tissue capsule and blood vessels of the preaortic ganglia (Figure 9 A). Comparison with the autofluorescence showed that RBA was present around the neurons, and between neurons, and their satellite cells (Figure 9 B). The nerves that connected with the ganglia did not fluoresce. The same results were obtained after 5 minutes.

One week of daily subcutaneous injections of RBA (Figure 10 A) yielded a brighter fluorescence than that observed after 5 or 30 minutes. Comparison of the RBA fluorescence with that of sections counterstained with neutral red (Figure 10 A and B) revealed that the satellite cells were separated by bright lines of RBA fluorescence delineating them from the neurons. This was more easily seen than after the shorter circulation times. In addition red fluorescent lines were clearly visible around the cell processes in the neuropil (Figure 10 A). The nerves attached to the ganglion exhibited fluorescence in the epineurium, perineurium, and endoneurium. This agreed with

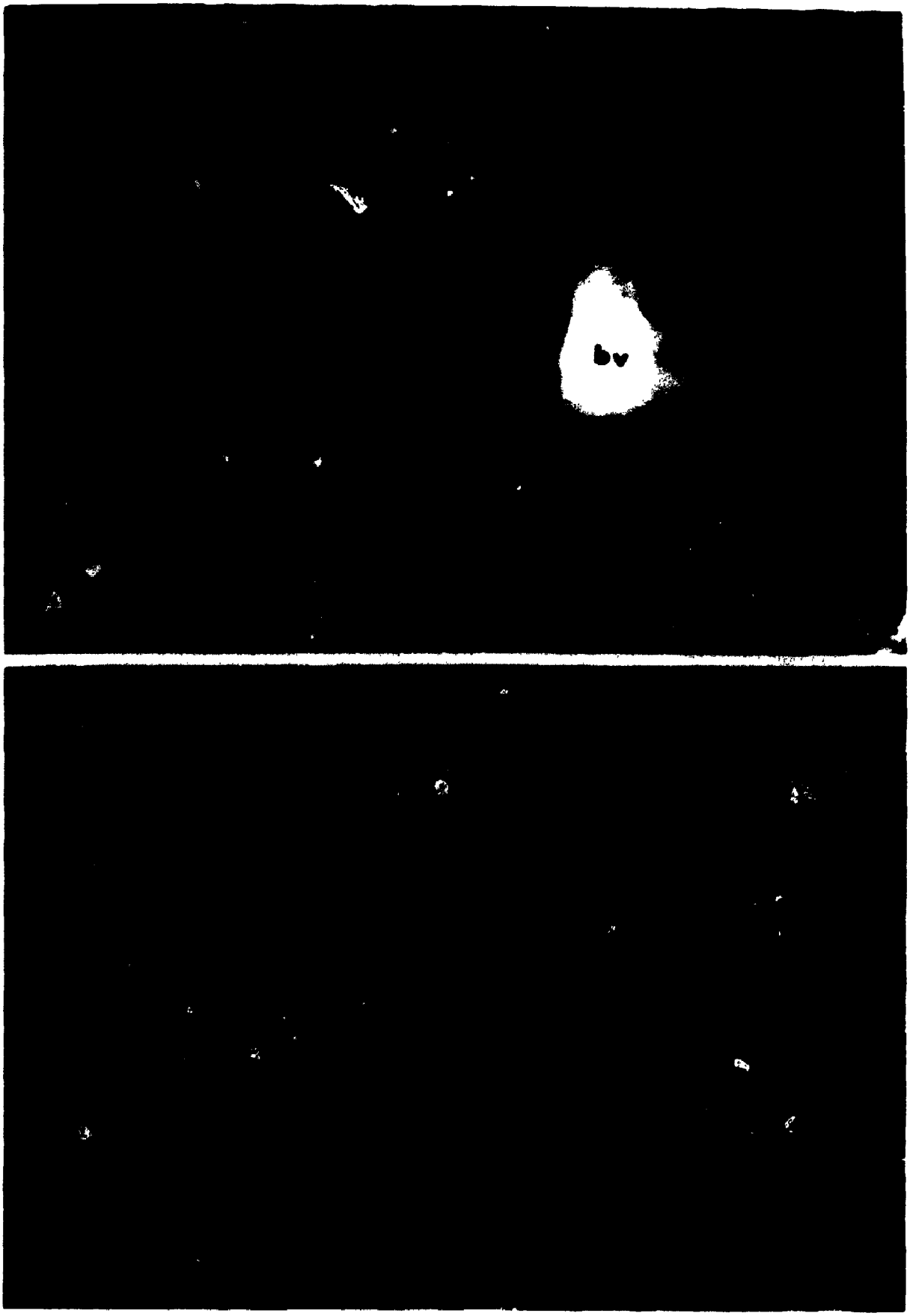
Figure 10

A. (x880) Fluorescence of preaortic ganglion from a rat that was killed after one week of daily injections of rhodamine labelled albumin. Fluorescence was present in the connective tissue sheath, around the neurons, and between neurons and their satellite cells and in the neuropil.

B. (x880) Fluorescent neutral red stained section of A showed the morphology of the ganglion. The cytoplasm and nucleoli of neurons and the nuclei of satellite cells were stained. The connective tissue sheath was also stained.

bv = blood vessel; c = connective tissue sheath;

N = neuron; arrowheads indicate satellite cells.


bv

the results obtained for the trigeminal (section 4.5.2) and sciatic nerves (section 4.6.2). The presence of RBA in the endoneurium was the most conspicuous qualitative difference associated with prolonged circulation of this fluorescent protein tracer.

4.4.3 Basic Fluorochromes

Rats were sacrificed after acriflavine circulated for 5 or 30 minutes. The yellow-green fluorescent nuclei of capillary endothelial cells were recognized by their shapes and orientation. In small arteries, the nuclei of smooth muscle cells were perpendicular to those of endothelial cells. Fluorescence was also observed in the nuclei of satellite cells, and also in the neuronal nucleoli, but not in the nucleoplasm of the neurons (Figure 11 A and B). The neurons also had fluorescent cytoplasm. The nerves attached to the ganglion exhibited fluorescent Schwann cell nuclei. Fluorescence was also seen in the nuclei of fibroblasts in the connective tissue sheath. The fluorescence of acriflavine was never seen in collagen fibres or in any other extracellular structures. The distribution of the fluorescence was the same for 5 and 30 minutes, but the fluorescence was less intense after 30 minutes circulation.

The results obtained with ethidium bromide differed somewhat from those for acriflavine despite using the same circulation times. In the connective tissue capsule, the nuclei and cytoplasm of fibroblasts fluoresced under green exciting light (Figure 11 C and D). Fluorescence was also

Figure 11

A. (x240) Preaortic ganglion from a rat that was killed 5 minutes after intravenous injection of acriflavine. Fluorescence was present in the nuclei of satellite cells and in the cytoplasm and nucleoli of neurons, but not in neuronal nucleoplasm.

B. (x410) 30 minutes after intravenous injection of acriflavine. The distribution of fluorescence was similar to that in A.

C. (x400) 5 minutes after intravenous injection of ethidium bromide. Fluorescence was observed in the nuclei and cytoplasm of fibroblasts in the connective tissue sheath, in the nuclei and cytoplasm of satellite cells, and in the nucleoli and cytoplasm of neurons, but not in neuronal nucleoplasm.

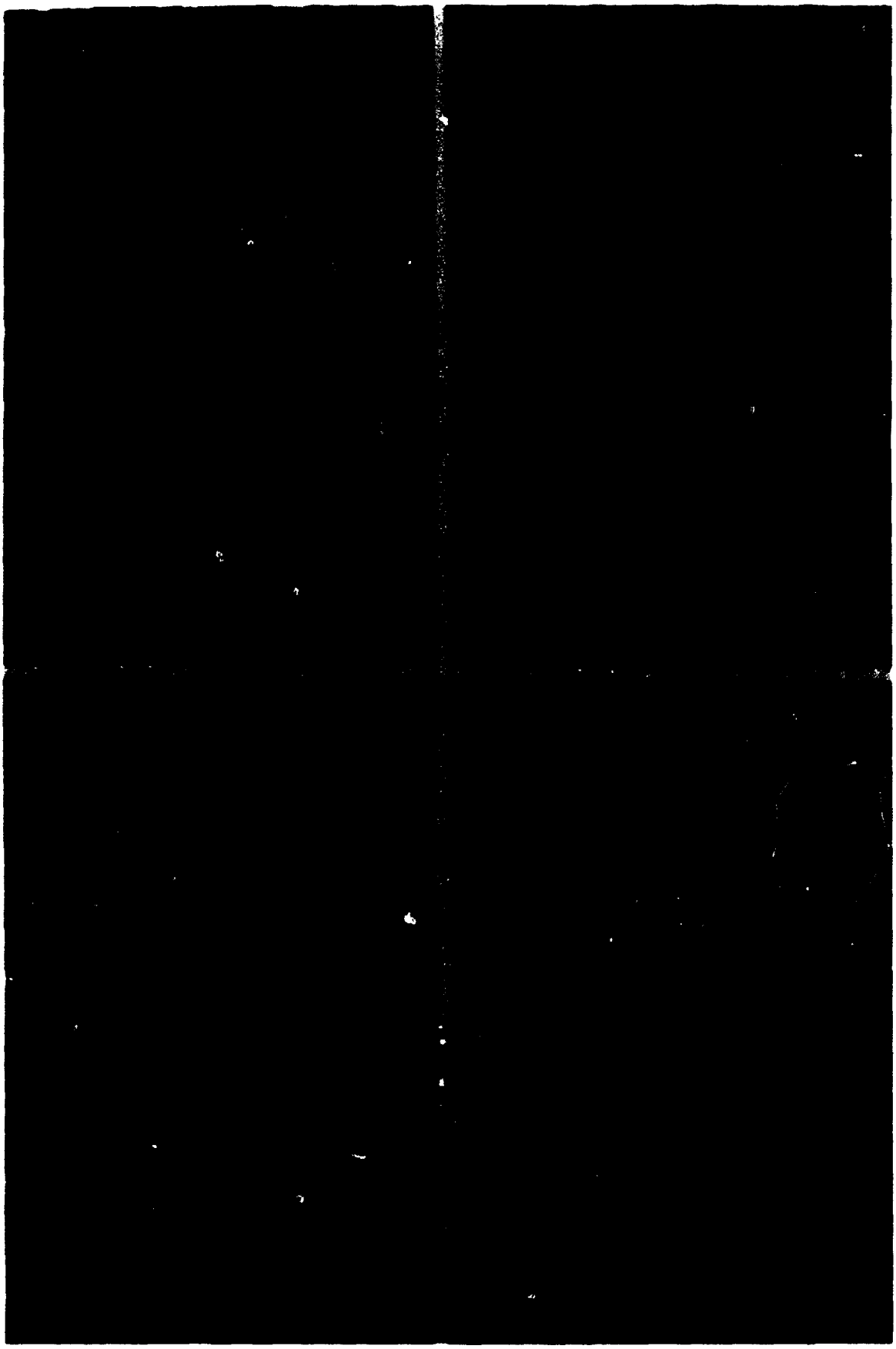
D. (x160) 30 minutes after an intravenous injection of ethidium bromide. The same distribution of fluorescence was observed as that in C.

N = neuron; arrowheads indicate satellite cells.

n = nerve

F = fibroblast

C = connective tissue capsule



observed in the nucleoli, nucleoplasm, and cytoplasm of satellite cells. The distribution of the rest of the fluorescence with ethidium bromide, for both circulation times, was identical to that of acriflavine.

4.5 Trigeminal Ganglion

4.5.1 Endogenous Albumin

Positive immunostaining was present in the thick collagen fibres of the connective tissue capsule of the ganglion and dark brown lines surrounded and separated the neurons. The cytoplasm of some neurons was stained but not the nuclei (Figure 12 A). However, most neurons did not stain immunohistochemically for endogenous albumin. No particular features of either structure or location were noted to be different for the unstained neurons. In addition, strong staining was observed around the satellite cells and between them and the neurons. The cytoplasm and nuclei of satellite cells were negative. Staining was also seen in the walls and lumina of blood vessels. The areas of the section containing bundles of nerve fibres also exhibited positive staining between the fibres.

4.5.2 Fluorescent Bovine Albumin (RBA)

Five or thirty minutes after an intravenous injection of RBA, red fluorescence was visible in the connective tissue sheath and in the walls and lumina of blood vessels of the ganglion (Figure 12 B). Neurons and satellite cells were surrounded by fluorescent lines. The location of these

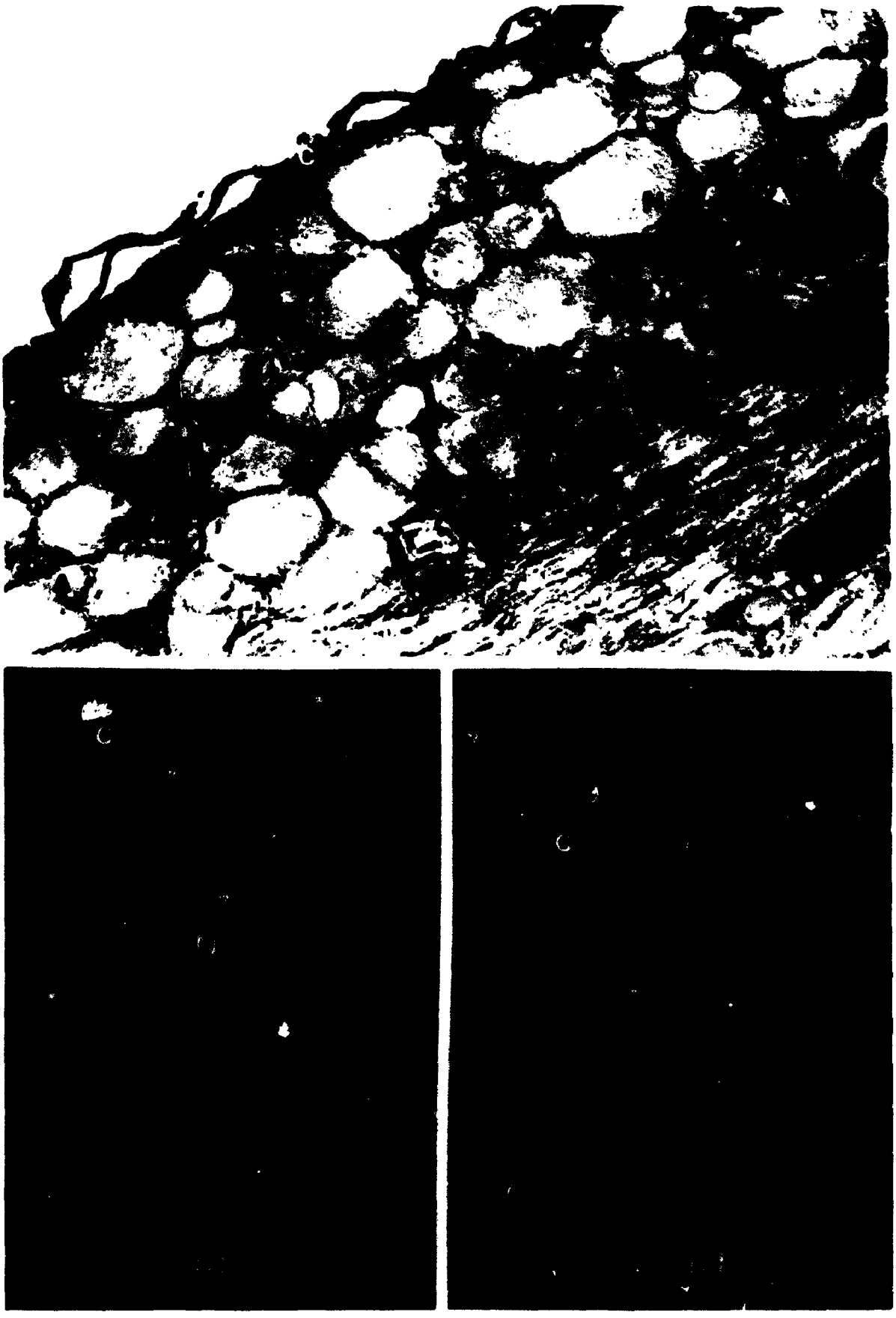
Figure 12

A. (x500) Trigeminal ganglion showing immunoreactivity for endogenous albumin, using phase contrast microscopy. Staining was present in the connective tissue sheath, in the walls and lumina of blood vessels, and around the neurons. The cytoplasm of some neurons was stained, as were the spaces between nerve fibers in the adjacent nerve.

B. (x360) Trigeminal ganglion from a rat killed 30 minutes after an intravenous injection of rhodamine labelled albumin. Fluorescence was seen in the connective tissue capsule, in the walls and lumina of blood vessels, around neurons, and between neurons and their satellite cells.

C. (x360) Fluorescent neutral red staining of B showing the morphology and location of neurons and satellite cells.

bv = blood vessel; N_1 = neuron with no positive staining in cytoplasm; N_2 = neuron with positive staining in cytoplasm; N = neuron; nf = nerve fibres; arrowheads indicate satellite cells; C = connective tissue sheath.



cells were determined by comparison with the same sections subsequently stained with neutral red (Figure 12 C). No red fluorescence due to RBA was observed in the bundles of trigeminal nerve fibres adjacent to the ganglion. In marked contrast to these results, after one week of daily subcutaneous injections of RBA, fluorescence was observed in the trigeminal nerve (Figure 13 A), as revealed by comparison with the neutral red stained section (Figure 13 B). The red fluorescence was present between the nerve fibres. Although the fluorescence observed after one week was brighter than for the shorter times, the fluorescence elsewhere in the ganglion was in the same locations as that observed for 5 or 30 minutes.

4.5.3 Electron Microscopy of Trigeminal Ganglion From Saline Injected Rats

The trigeminal ganglion was composed of neurons and their encapsulating satellite cells and their processes (Figure 14 A and B). Blood vessels were also present in the ganglia. The extracellular spaces between adjacent ensheathed neurons contained some collagen fibres. The neurons were easily recognized by their large size, cytoplasm, and nuclei. The cytoplasm contained many Nissl bodies, ribosomes, mitochondria, and microtubules. The nucleus had uniformly distributed chromatin except for the odd small clumps of chromatin occasionally observed. The neurons did not have a basal lamina between them and their satellite cells. Satellite cells were easily distinguished

Figure 13

A. (x880) Trigeminal ganglion from a rat killed after one week of daily injections of rhodamine-labelled albumin. Fluorescence was observed in the connective tissue sheath, around the neurons and between the neurons and their satellite cells. In the adjacent nerve fiber bundle the space between the nerve fibers fluoresced.

B. (x880) Fluorescent neutral red stained section of A showing the morphology and location of neurons and satellite cells.

bv = blood vessel; c = connective tissue sheath;

s = satellite cell; N = neuron; nf = nerve fibres.

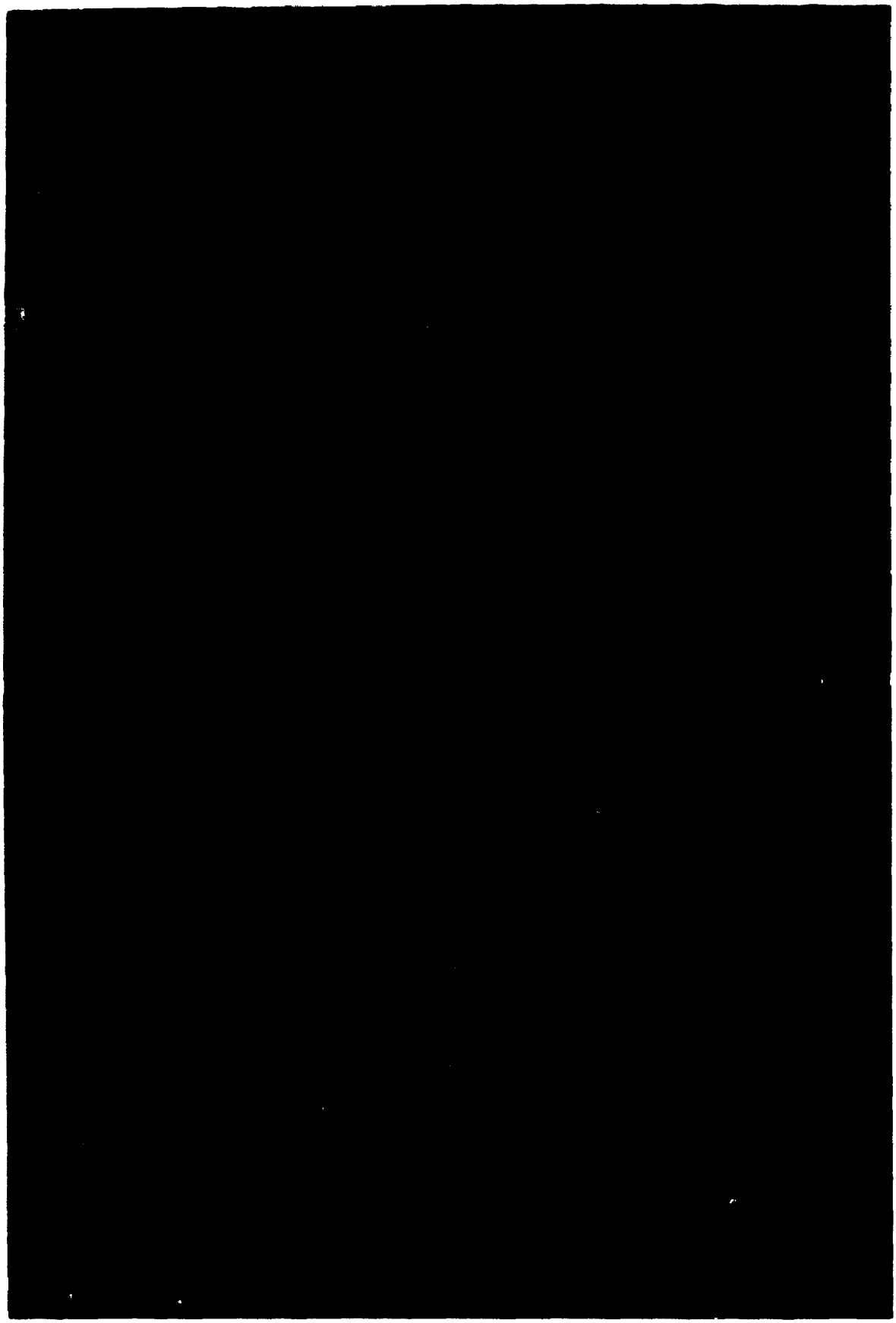
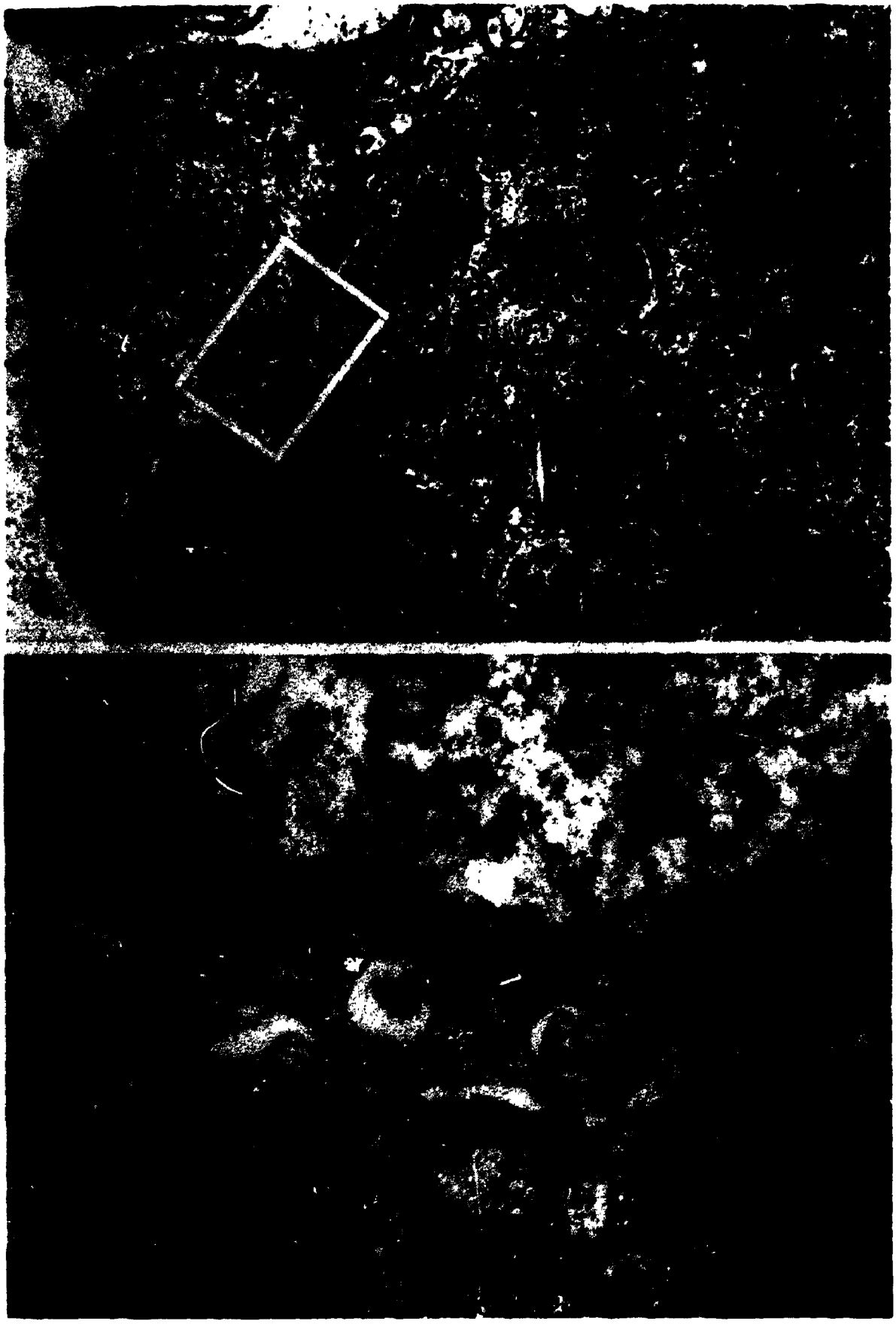


Figure 14

A. (x8,700) A Neuron and a Satellite cell in the Trigeminal ganglion after an intravenous injection of saline. The extracellular spaces around ensheathed neurons were very pale and empty-looking.

B. (x47,000) Enlargement of rectangular area in A showed the empty extracellular spaces between a neuron and its satellite cells.

N = neuron; M = mitochondrion; Nb = Nissl bodies; Ly = lysosome; s = satellite cell; arrows indicate basal lamina; r = ribosomes.



from neurons by their smaller size (6μm x 16μm), their location, basal lamina, cytoplasm, and nuclei. Their cytoplasm contained glycogen granules, lysosomes, ribosomes, and endoplasmic reticulum but no Nissl bodies. The nuclei of satellite cells had more concentrated chromatin than the nuclei of neurons. In addition, clumps of chromatin were present at the inner nuclear membrane.

The electron micrographs of the trigeminal ganglia from control animals (Figure 14) were compared with those from animals injected with horseradish peroxidase. The satellite cells and their processes however, had a basal lamina covering them on the sides which were farthest from the neurons that they encapsulated. No basal lamina was present between adjacent satellite cells.

4.5.4 Horseradish Peroxidase

Plastic sections of trigeminal ganglia, obtained from rats in which HRP had circulated for 10 minutes, exhibited DAB reaction product in the walls and lumina of the blood vessels (Figure 15 A). On comparing these unstained plastic sections with the corresponding toluidine blue-stained sections (Figure 15 B) the location of the HRP reaction product between the neurons and satellite cells was confirmed. Dark lines surrounded the neurons and encircled the satellite cells (Figure 15 A). No reaction product was present in the adjacent bundles of nerve fibres. After a shorter time of 5 minutes, very little dark reaction product was visible between ensheathing satellite cells and their

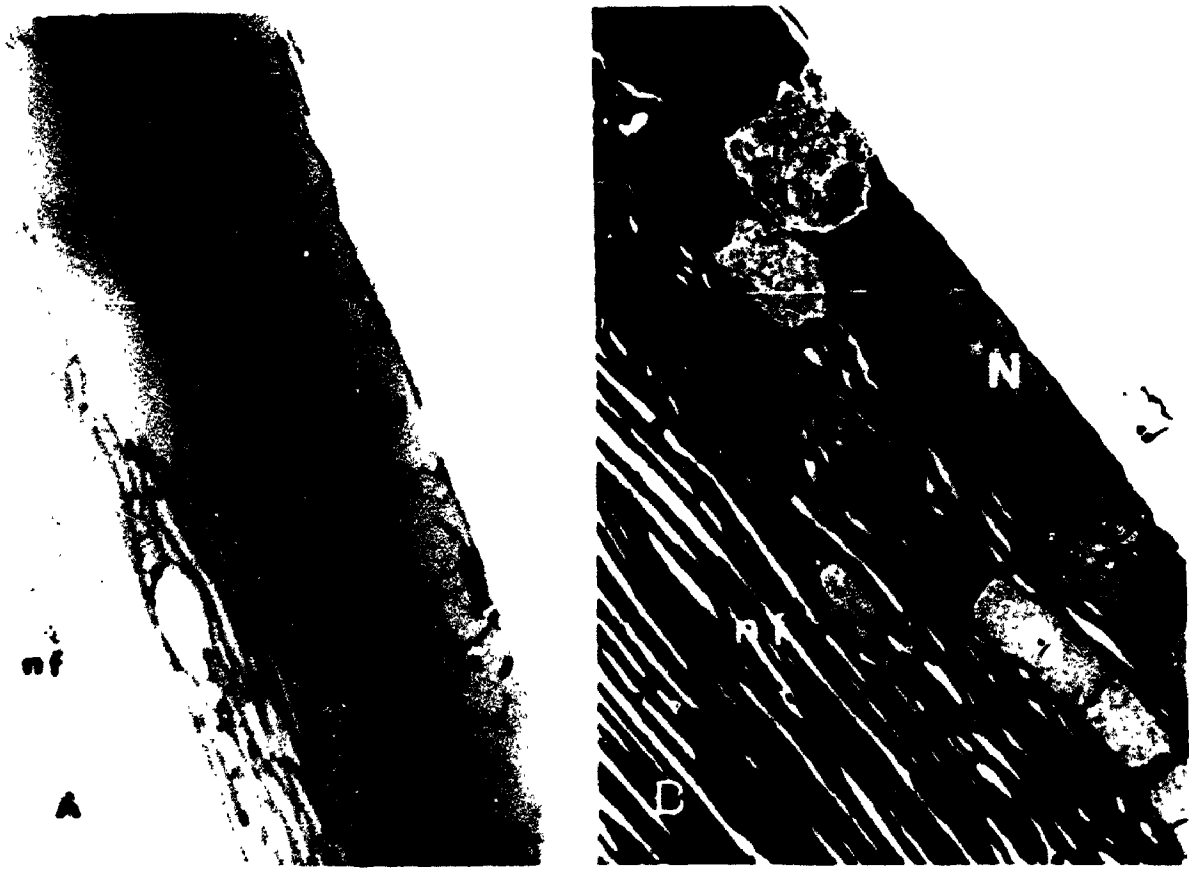
Figure 15

A. (x340) Trigeminal ganglion from a rat killed 10 minutes after an intravenous injection of HRP (1 μm plastic section). DAB reaction product was present in the walls and lumina of blood vessels, around neurons and between neurons and their satellite cells. No reaction product was present between the nerve fibers in the adjacent nerve.

B. (x340) Toluidine blue staining of A showed the morphology and location of the neurons.

C. (x500) 30 minutes after an intravenous injection of HRP (1 μm plastic section). No reaction product was visible in the tissue and blood vessels.

bv = blood vessel; N = neuron; s = satellite cell; nf = nerve fibres.


nf
A
N
B


N
nf
C

neurons. However, some deposits of reaction product were present between neurons and inside and just outside the blood vessels. Longer circulation of HRP (30 minutes) resulted in no dense reaction product in the tissue or inside the blood vessels (Figure 15 C).

Electron microscopy of tissues, obtained from animals in which HRP circulated for 10 minutes, revealed DAB reaction product between adjacent encapsulated neurons and satellite cells and their encapsulated neurons (Figure 16 A and B, 17 A and B). The extracellular spaces of the adjacent nerve fibres exhibited dark deposits around them. HRP reaction product was also present in the lumina of blood vessels, in the clefts between adjacent endothelial cells, and in the spaces around blood vessels and pericytes (Figure 16 A).

After a shorter circulation time of 5 minutes, less DAB reaction product was observed and none was present between satellite cells and the neurons ensheathed by them. In contrast to the results obtained with the shorter circulation times, no HRP reaction product was visible in the trigeminal ganglia taken from animals in which HRP had circulated for 30 minutes (Figure 18 A and B).

4.5.5 Basic Fluorochromes

The cytoplasm and nuclei of fibroblasts in the connective tissue sheath of the trigeminal ganglion fluoresced red after ethidium bromide had circulated for

Figure 16

A. (x6,000) Trigeminal ganglion from rat killed 10 minutes after an injection of HRP. Dark DAB reaction product was present in the extracellular spaces around neurons and satellite cells, around adjacent nerve fibres, in the lumina of the blood vessel, in clefts between adjacent endothelial cells, and in the spaces around the blood vessel and pericytes.

B. (x47,000) Enlargement of rectangular area in A showed the dark reaction product in the extracellular spaces between a satellite cell and the neurons that it ensheathed.

bv = blood vessel; N = neuron; s = satellite cell; Nb = Nissl bodies; M = mitochondrion.

open arrowheads indicate location of dark DAB reaction product.

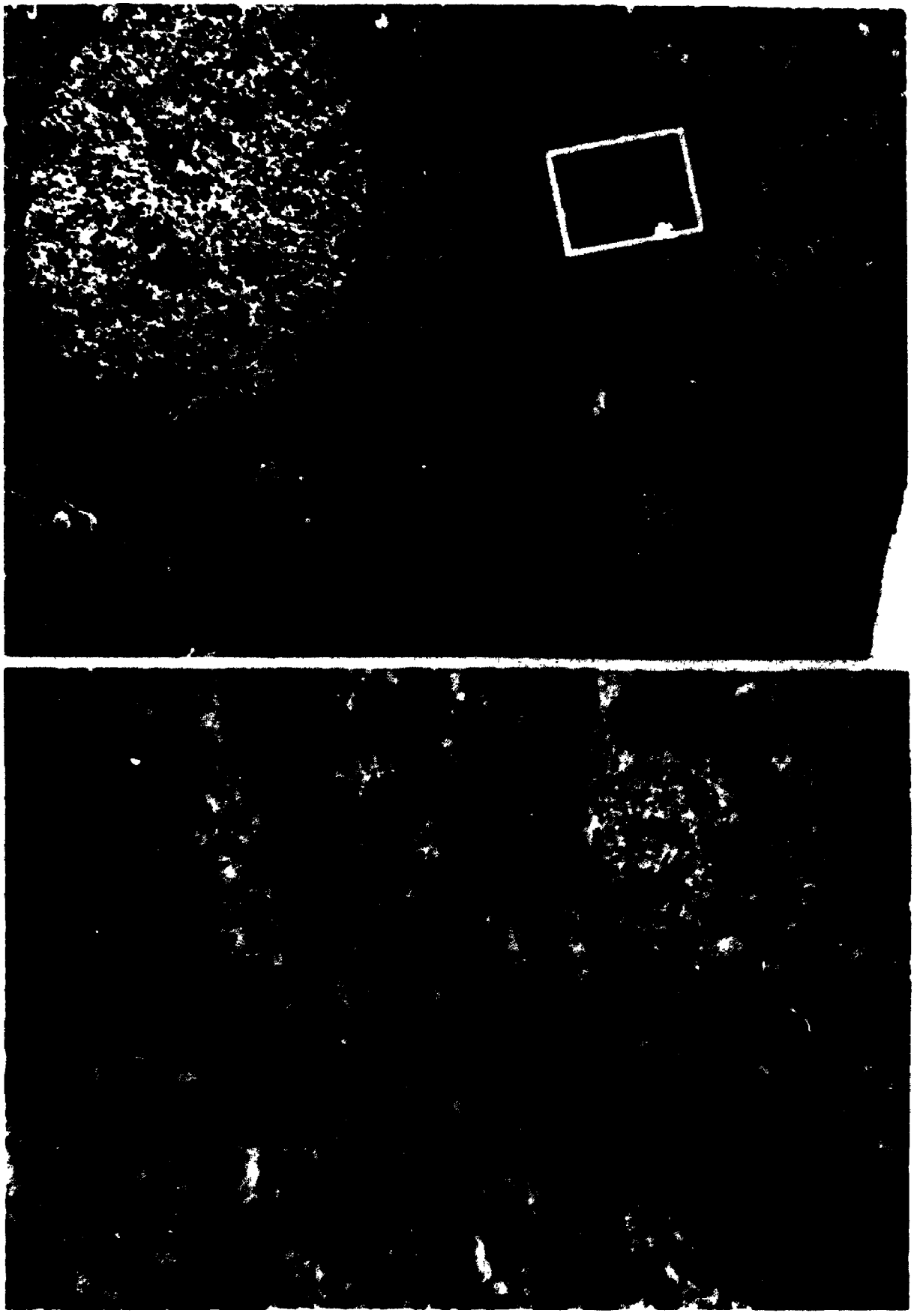


Figure 17

A. (x8,800) Trigeminal ganglion from a rat killed 10 minutes after an injection of HRP. Dark DAB reaction product was present in the extracellular spaces around neurons and satellite cells.

B. (x47,000) Enlargement of rectangle in A showed the reaction product between the satellite cells and the neurons that they ensheathed.

N = neuron; s = satellite cell; Nb = Nissl bodies; M = mitochondrion; r = ribosomes.

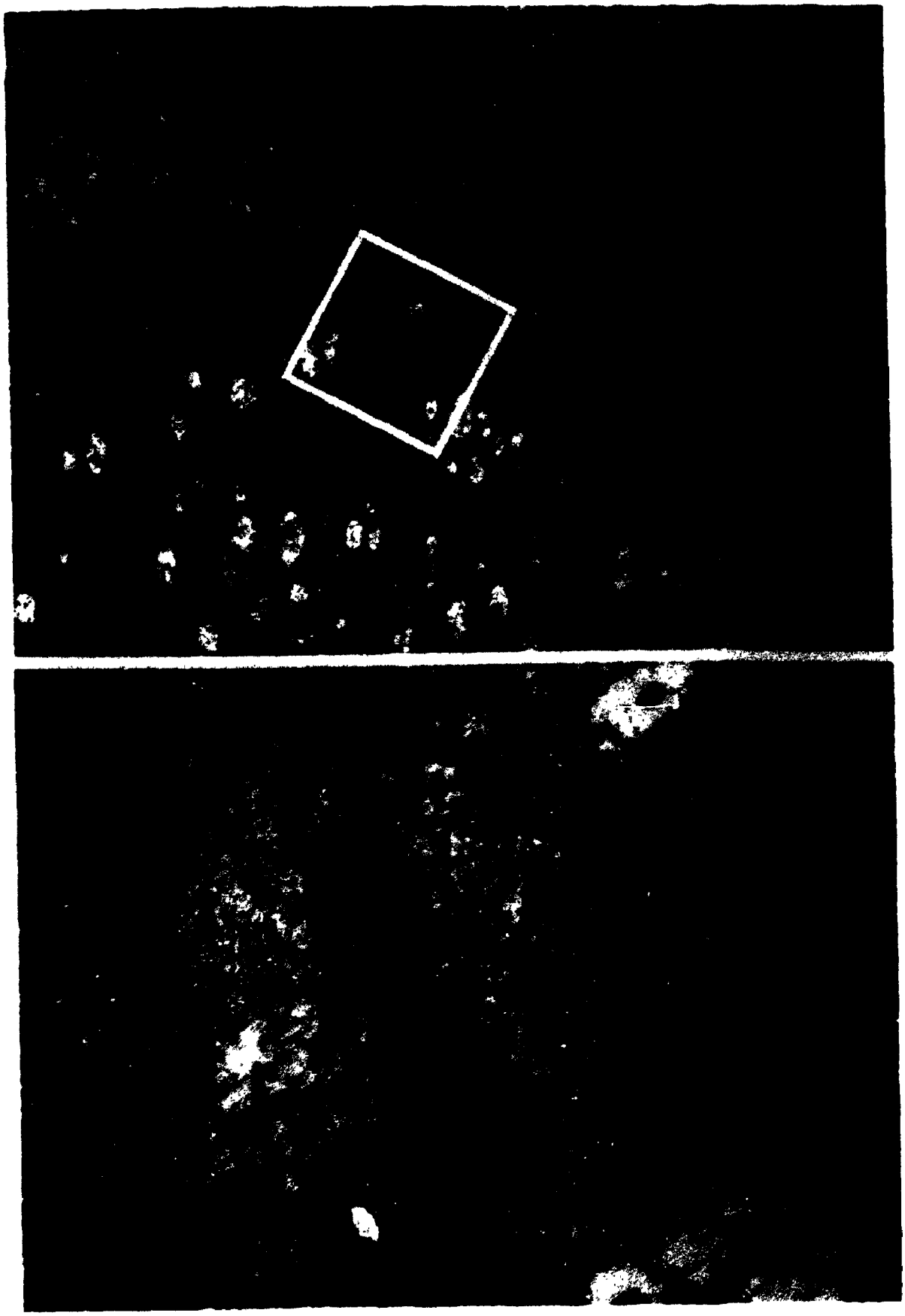


Figure 18

A. (x9,900) Trigeminal ganglion from a rat killed 30 minutes after an intravenous injection of HRP. Note the absence of dark reaction product in the extracellular spaces around the neuron and the satellite cell, and between the neuron and adjacent nerve fibres.

B. (x37,000) Enlargement of rectangle in A showed absence of the reaction product between the satellite cell and the neuron, and also between the Schwann cell and the ensheathed neuron.

N = neuron; Nb = Nissl bodies; M = mitochondrion; BL = basal lamina; s = satellite cell; Sc = Schwann cell.

Figure 19

A. (x540) Trigeminal ganglion from a rat that was killed 5 minutes after an intravenous injection of ethidium bromide. Fluorescence was present in the cytoplasm and nucleoli of neurons and in the nuclei and cytoplasm of satellite cells.

B. (x540) 30 minutes after an injection of ethidium bromide. Fluorescence had the same distribution as in A. Fluorescence was also visible in the connective tissue sheath and in the walls of the blood vessel.

C. (x430) 5 minutes after an intravenous injection of acriflavine. Fluorescence was present in the cytoplasm and nucleoli of neurons and in the nuclei and cytoplasm of satellite cells, and in the connective tissue sheath.

D. (x430) 30 minutes after an injection of acriflavine. The distribution of fluorescence was similar to that in C.

bv = blood vessel; c = connective tissue sheath;

N = neuron; s = satellite cell; asterisk indicates intranuclear rod.

5 or 30 minutes (Figure 19 A and B). Fluorescence was observed in the walls of the blood vessels in the endothelial cells' cytoplasm and nuclei. The neurons exhibited fluorescent nuclei and some had a fluorescent baton in the nucleus. Diffuse and punctate red fluorescence was present in the neuronal cytoplasm. The punctate fluorescence was associated with the Nissl substance as determined by comparison with adjacent toluidine blue stained sections. Fluorescence was also observed in the cytoplasm and nuclei of satellite cells and between the nerve fibres adjacent to the ganglion. However, in the adjacent trigeminal nerve, the bundles of nerve fibres and their blood vessels did not fluoresce. The fluorescence after the circulation of ethidium bromide for 30 minutes was not as bright as that after 5 minutes.

When acriflavine was used instead of ethidium bromide, the distribution of fluorescence was the same (Figure 19 C and D). However, in contrast to the results with ethidium bromide, the fluorescence observed after 30 minutes was brighter than that after 5 minutes.

4.6 Sciatic Nerve

4.6.1 Endogenous Albumin

The stained sections of sciatic nerve were compared with the controls in which (1) the primary antiserum (rabbit anti rat albumin) was omitted, or (2) normal rabbit serum was substituted for the primary antiserum, or (3) the

primary antiserum preabsorbed with rat albumin was used instead of the primary antiserum (Figure 20 A). All the controls were negative.

Positive staining for albumin was present in the collagen fibres, in the cytoplasm of fibroblasts and perineurial cells, in the epineurium and perineurium respectively (Figure 20 B). The walls and lumina of the small arteries and veins in the epineurium and capillaries of the endoneurium were stained. In the endoneurium, positive staining was observed around the nerve fibres and in some axons but not in the myelin sheath.

4.6.2 Fluorescent Albumin

The sections from RBA-injected animals were compared with those from saline-injected controls. Only a dull red background fluorescence was observed in the sciatic nerves of control rats. Red blood cells displayed somewhat stronger autofluorescence than other cells or connective tissue fibres (Figure 21 A). The identification of components of the nerve in fluorescence microscopy was confirmed by comparison with H and E stained sections (Figure 21 A and B).

Fluorescence was visible in the epineurium, in the lumina and walls of the blood vessels and in the connective tissue, 5 minutes after the administration of RBA (Figure 21 C; cf. Figure 21 D). No fluorescence was observed in the perineurium but blood vessels with fluorescent lumina were seen in the endoneurium. The same results were obtained

Figure 20

A. (x520) Sciatic nerve stained with preabsorbed rabbit antirat albumin (Transmitted light). Note the absence of stain in the epineurium, perineurium, and in the endoneurium around the nerve fibres in the nerve fasicle.

B. (x830) Immunoreactivitv for endogenous albumin (phase contrast). Dark reaction product was present in the epineurium , perineurium, and in the endoneurium around the nerve fibres in the nerve fasicle, and also in the walls and lumen of the blood vessel.

bv = blood vessel; E = epineurium; F = fibroblast; P = perineurium; nf = nerve fasicle containing nerve fibres and endoneurium.

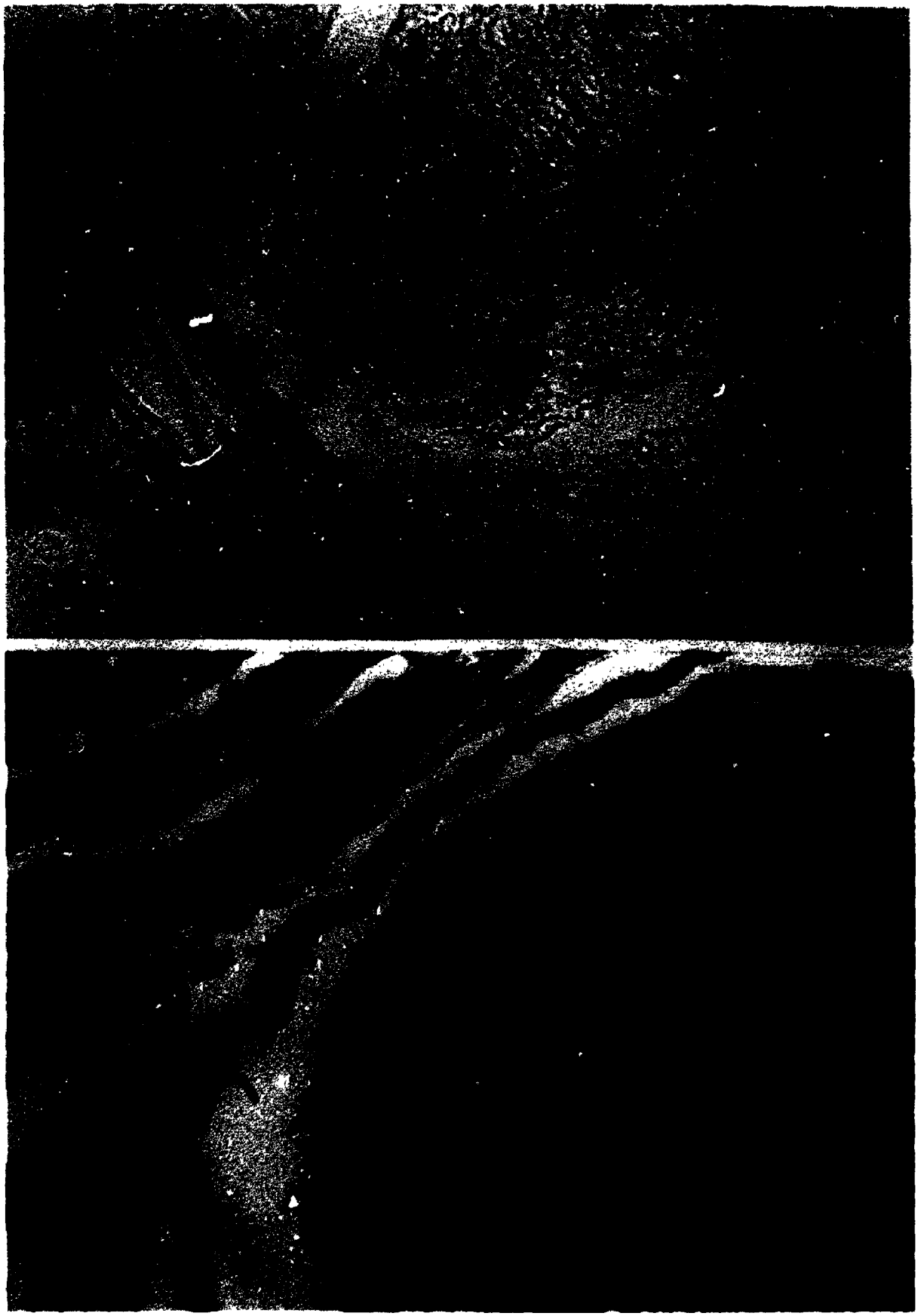


Figure 21

A. (x150) Shows autofluorescence in the epineurium, perineurium and a nerve fasicle of the sciatic nerve after an intravenous injection of saline. Note the fluorescent red blood cells.

B. (x150) Haematoxylin and eosin stained section of A showing morphology.

C. (x360) Sciatic nerve from rat killed 5 minutes after an intravenous injection of rhodamine labelled albumin. Fluorescence was observed in the epineurium and in the walls and lumina of blood vessels. No fluorescence was observed in the perineurium, but blood vessels with fluorescent lumina were observed in the endoneurium.

D. (x340) Haematoxylin and eosin stained section of C showing morphology.

E = epineurium; P = perineurium; bv = blood vessel; Asterisk indicates red blood cells in blood vessel; Nf = nerve fasicle containing nerve fibres and endoneurium.

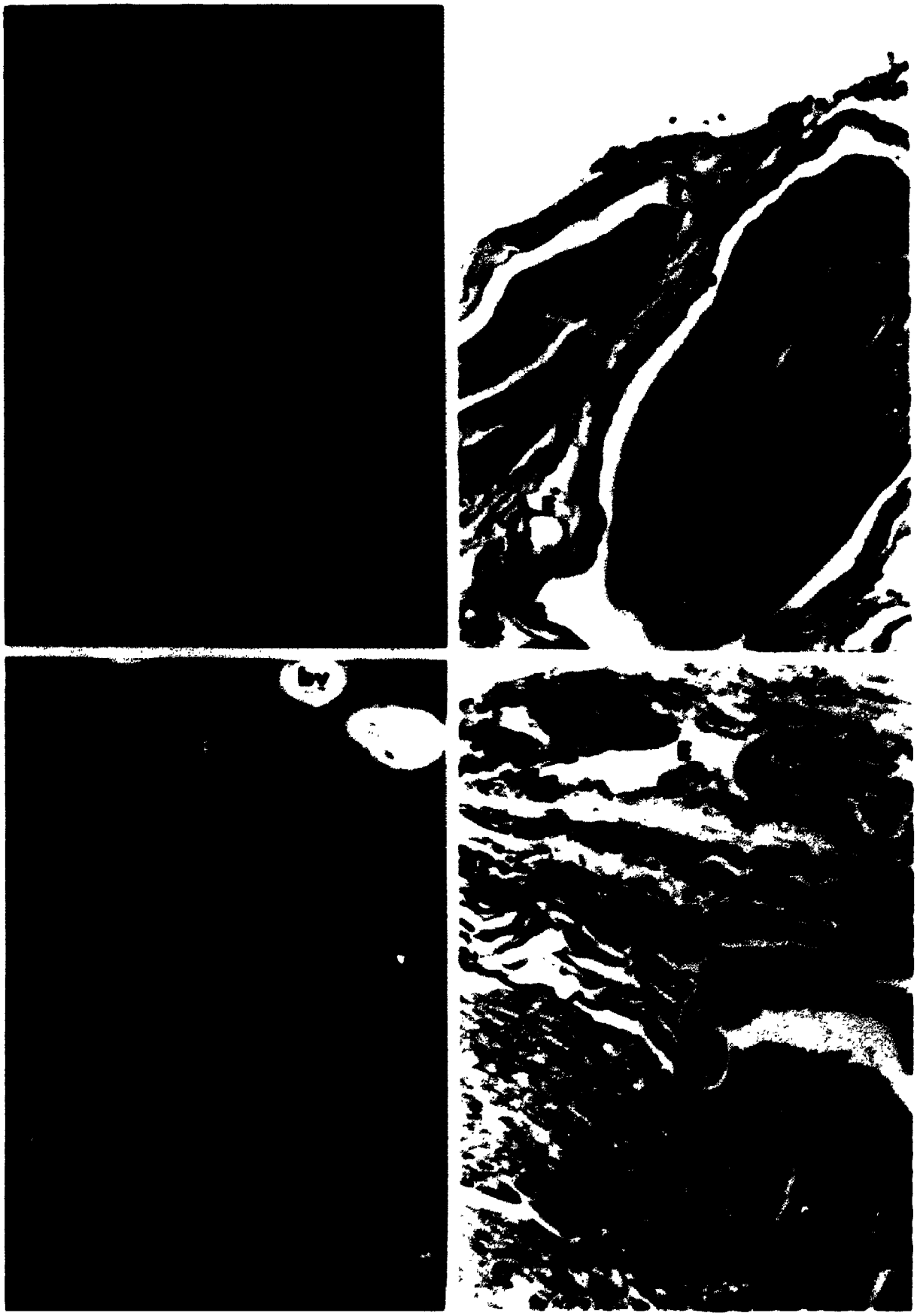


Figure 22

A. (x360) Sciatic nerve from a rat killed 30 minutes after an intravenous injection of rhodamine labelled albumin. Fluorescence was present in the epineurium and in the walls and lumina of blood vessels, but not in the perineurium. In the endoneurium fluorescence was confined to the lumina of the blood vessels.

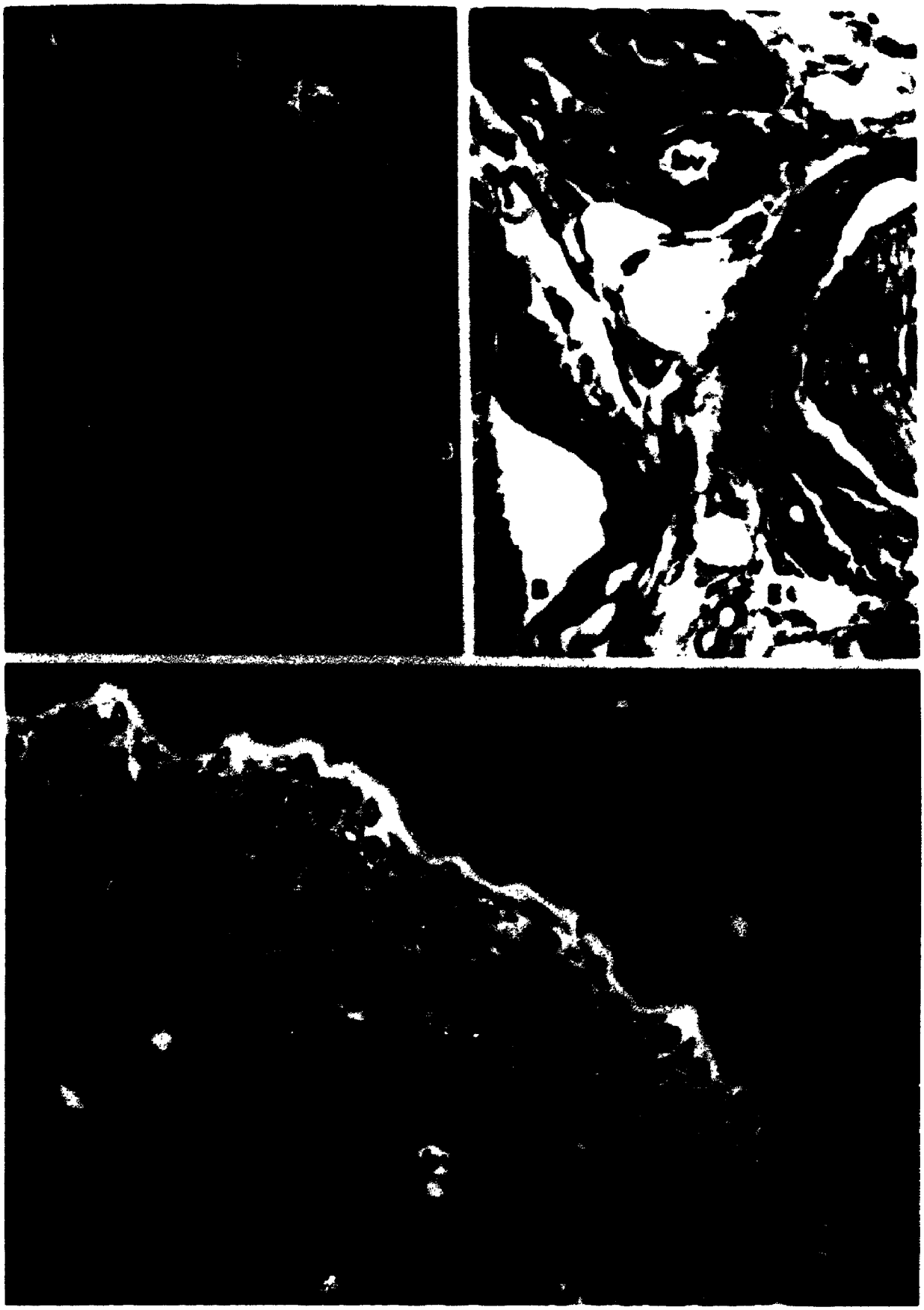
B. (x360) Haematoxylin and eosin stained section of A showing morphology.

C. (x520) Sciatic nerve from a rat killed after one week of daily injections of rhodamine labelled albumin. Fluorescence was observed in the epineurium, perineurium and in the endoneurium around the nerve fibres, and in many of the axons, but not in the myelin sheaths.

bv = blood vessel; En = endoneurium;

E = epineurium; P = perineurium; F = fibroblast;

Nf = nerve fasicle.



after RBA had circulated for 30 minutes (Figure 22 A; cf. Figure 22 B).

One week of daily injections resulted in brighter fluorescence in the connective tissue of the epineurium as well as the cytoplasm of fibroblasts. In marked contrast to the sciatic nerves from rats that had received only one injection of RBA, the perineurium and endoneurium fluoresced brightly (Figure 22 C). The lumina and walls of blood vessels and many axons in the endoneurium fluoresced, but the myelin sheaths stood out as dark zones encircling the axons.

4.6.3. Horseradish Peroxidase

The electron micrcgraphs of sciatic nerve obtained from HRP injected rats were compared with those from control animals. The fibroblasts in the connective tissue sheaths were easily recognized by their spindle-shaped cell bodies, nuclei, and their long thin cytoplasmic processes. The epineurium was distinguished from the perineurium by the interspersing of collagen fibres with fibroblasts which lacked basal laminae. The perineurium consisted of compact layers of fibroblasts (Figure 23). Some collagen fibres were visible between the layers of perineurial fibroblasts. The endoneurial fibroblasts were distinguished from those in the perineurium by their being adjacent to Schwann cells. The endoneurium also contained collagen fibres, myelinated and unmyelinated axons, and their Schwann cells. The Schwann cells were recognized by being in contact with and

Figure 23

Sciatic nerve from a rat killed 10 minutes after an injection of HRP. Note the dark reaction product amongst the co_lagen fibres in the epineurium and in the basal lamina along the outermost layer of cells of the perineurium. No reaction product is present in the perineurium or endoneurium (x21,000).

BL = basal lamina; C = collagen; En = endoneurium; E = epineurium; F = fibroblast; M = myelin sheath; P = perineurium; Sc = Schwann cell.

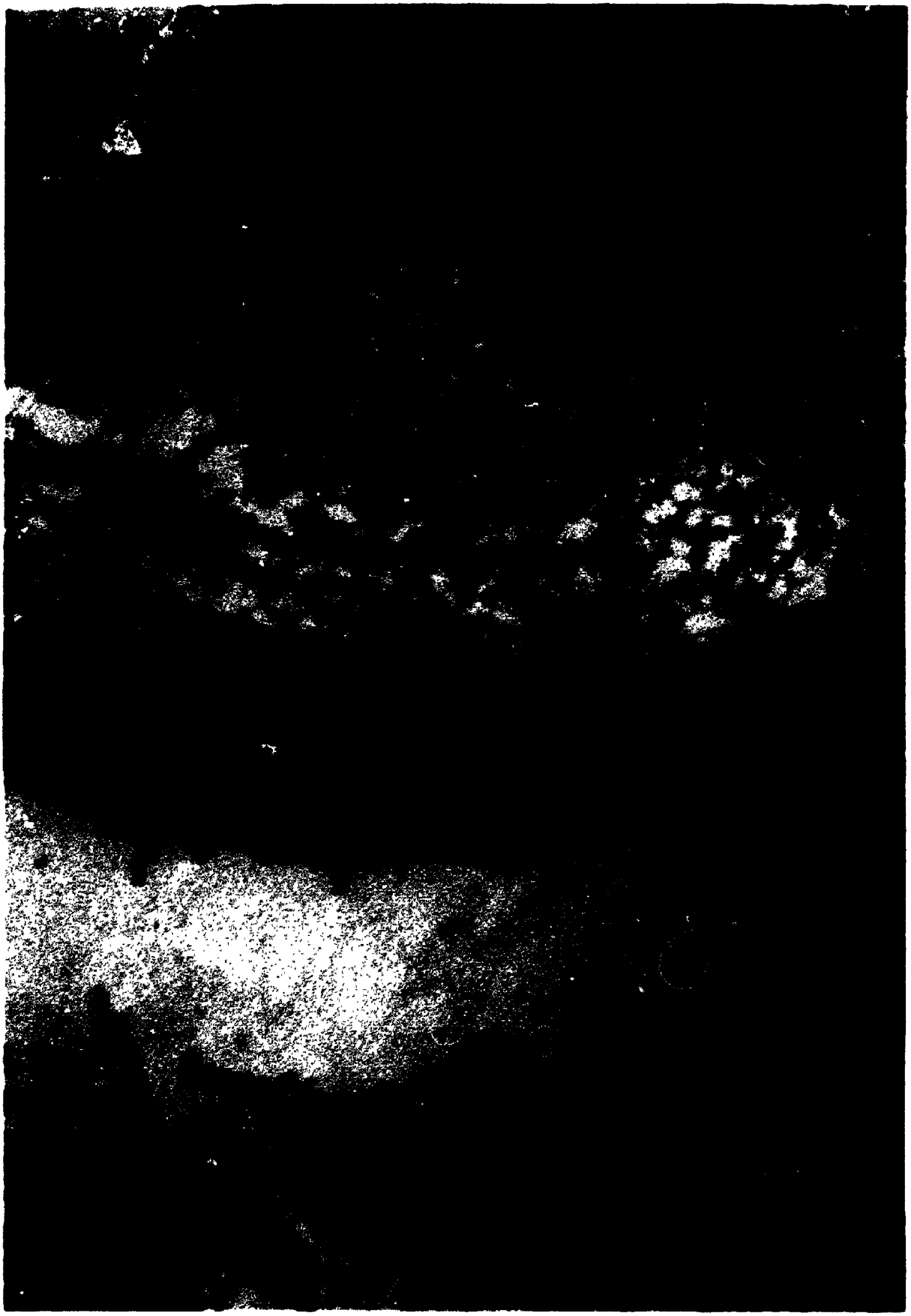
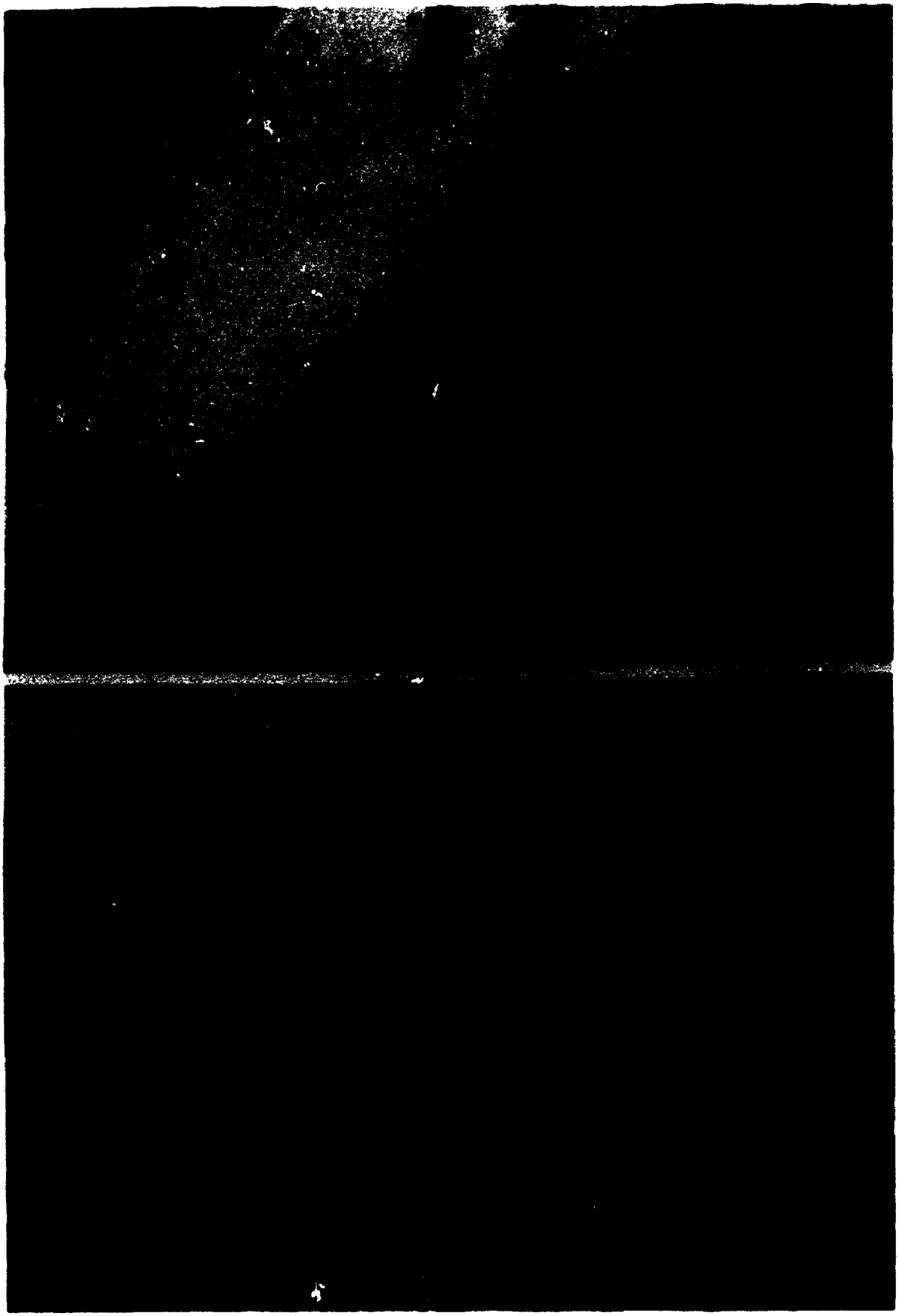


Figure 24

A. (x12,000) and B. Enlargement of A. (x17,000) Sciatic nerve from a rat killed 30 minutes after an injection of HRP. No dense reaction product was present in the epineurium, perineurium and endoneurium.

C = collagen; En = endoneurium; E = epineurium; M = myelin sheath; F = fibroblast; BL = basal lamina; P = perineurium.



surrounding single myelinated axons or multiple unmyelinated axons.

Sciatic nerves from rats in which HRP had circulated for 10 minutes, exhibited DAB reaction product around the collagen fibres in the epineurium and in the adjacent basal lamina of the fibroblasts constituting the outermost layer of the perineurium (Figure 23). No reaction product was present between the layers of the perineurium or within the endoneurium. Five or thirty minutes circulation of HRP (Figure 24) made a marked difference in the results. At five minutes some reaction product was present in the epineurium but at thirty minutes very little reaction product was observed in the epineurium and none was observed in the perineurium or endoneurium at either time.

4.6.4 Basic Fluorochromes

Rats injected with ethidium bromide were sacrificed 5 and 30 minutes afterwards. Identical results were obtained for both times. Identification of cell types was facilitated by examining the sections of sciatic nerves under blue exciting light and comparing the same sections under green exciting light. Red fluorescence was observed in the nuclei of fibroblasts in the epineurium (Figure 25 A). Mast cells in the epineurium exhibited fluorescent nuclei and cytoplasm with fluorescent dots. The more oval cell shapes and the fluorescent cytoplasmic dots distinguished the mast cells from the fibroblasts. The blood vessels' walls fluoresced but not their lumina. No

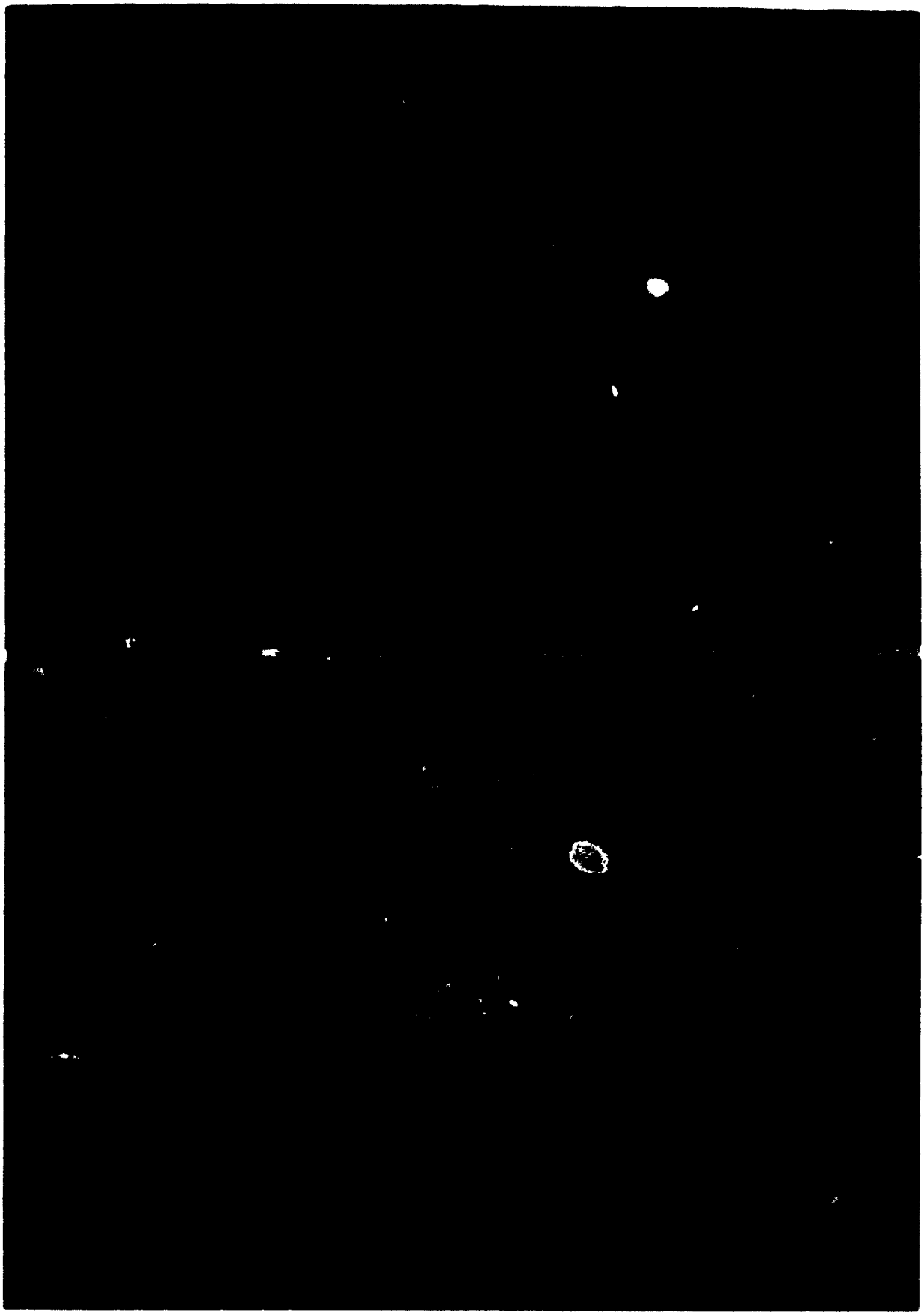
Figure 25

A. (x510) Sciatic nerve from a rat killed 30 minutes after an intravenous injection of ethidium bromide and fluorescence was observed in the nuclei of fibroblasts in the epineurium and in the walls of blood vessels, but not in their lumina. No fluorescence was observed in the perineurium or endoneurium.

B. (x500) Five minutes after injection of acriflavine. The distribution of fluorescence was the same as that observed in A.

bv = blood vessel; E = epineurium;

En = endoneurium; F = fibroblast; Mc = mast cell; sm = smooth muscle cell.



fluorescence was observed in the extracellular spaces, collagen, perineurium, or endoneurium.

When acriflavine was substituted for ethidium bromide, the yellow-green fluorescence was confined to cells and was mainly in nuclei. In the epineurium, fluorescence was observed in the nuclei and cytoplasm of endothelial cells and smooth muscle cells of blood vessels (Figure 25 B). The endothelial cells' nuclei were elongated with respect to the lumina of blood vessels, whereas, nuclei of muscle cells were oval as they were cut in cross-section. The fibroblasts' nuclei and cytoplasm also exhibited fluorescence in the epineurium. No fluorescence was present in extracellular spaces, collagen, perineurium, and endoneurium (Figure 25 B).

4.7 Enteric Nervous System

4.7.1 Endogenous Albumin

Immunostained sections of ileum were examined with bright-field illumination and phase contrast microscopy, which was useful for the identification of unstained components of the tissue. The controls included sections in which (i) the primary antiserum (rabbit anti rat albumin) was omitted, (ii) the primary antiserum was preabsorbed with rat's albumin and used instead of the primary alone, and (iii) rabbit's serum was substituted for the primary antiserum. All these were negative.

Figure 26

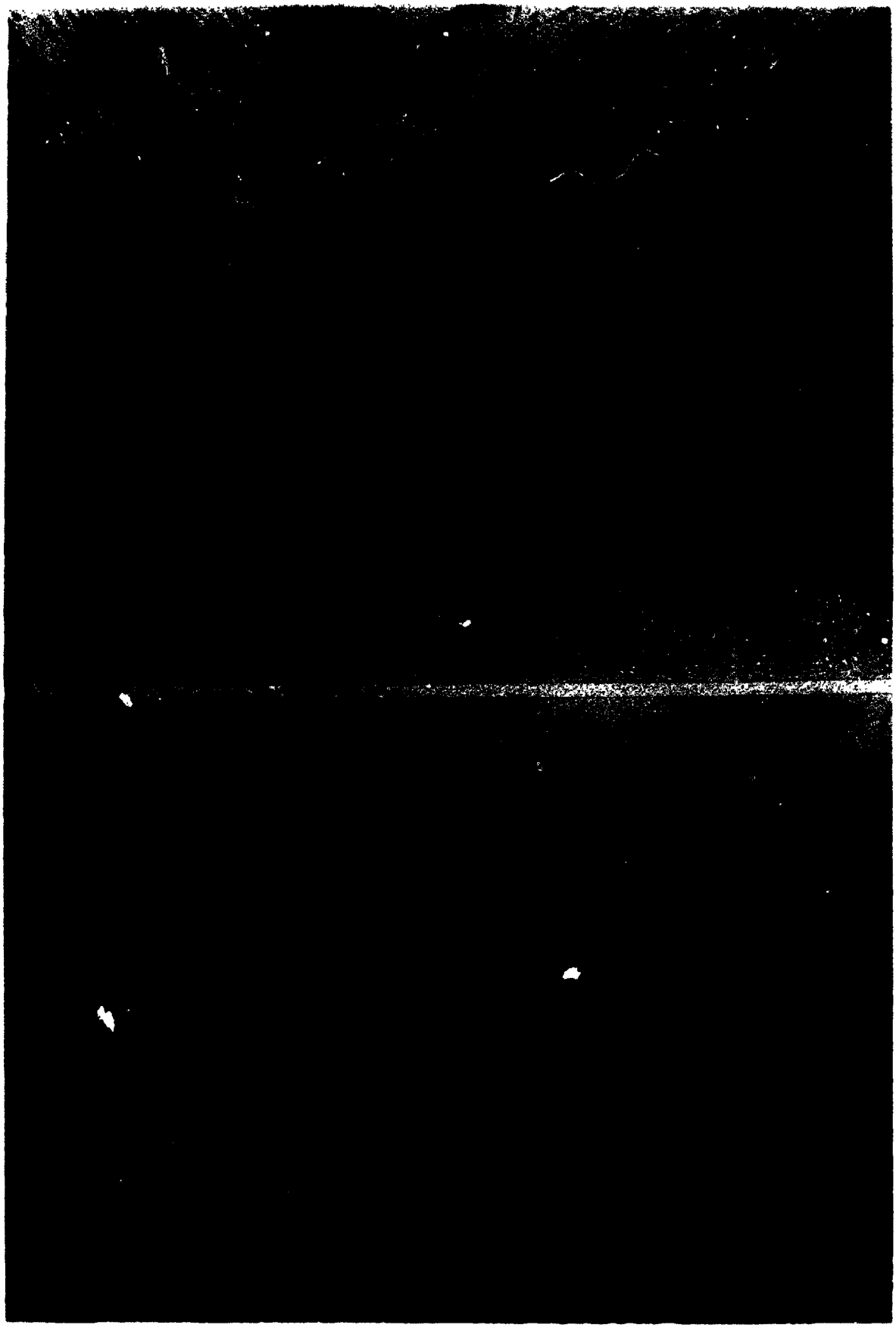
A. (x820) Ileal myenteric ganglion

B. (x820) Submucous ganglia.

Positive staining for endogenous albumin was present around the muscle cells in the muscle layers, in the adventitiae and lumina of the blood vessels and in the submucosa. Intense staining was observed in both ganglia and in the cytoplasm of neurons.

bv = blood vessel; LM = longitudinal muscle;

MG = myenteric ganglion; N = neuron; s = submucosa; SG = submucous ganglion.



In the muscularis externa, fine lines of positive staining outlined the muscle cells (Figure 26 A and B). The serosa also showed some staining in its connective tissue. The lumina of blood vessels and their adventitiae were also stained. In the myenteric and submucous ganglia, the cytoplasm of a number of neurons was strongly stained. However, other enteric ganglionic neurons were negative. The submucosa also exhibited positive staining in the connective tissue. In the mucosa, staining was present as fine lines in the muscularis mucosa and between adjacent epithelial cells.

Toluidine blue stained plastic sections were used to locate the enteric ganglia which were identified by the neurons containing vesicular nuclei and stained Nissl substance. At the EM level, neurons (Figure 31 B, 32 A, 33 A and B) were distinguished from glia by their nuclei having dispersed chromatin, their generally larger size, and cytoplasmic Nissl bodies and synapses . The glia had nuclei with denser, clumped chromatin, and cell processes that enveloped many processes of neurons (Figure 34 A). The ganglia and their tracts were covered by a basal lamina. The myenteric ganglia lay between two layers of smooth muscle cells which had a high density of cytoplasmic filaments.

After staining immunohistochemically the 40 μm thick vibratome sections of ileum for endogenous albumin, the specimens were processed for EM. Electron microscopy

revealed electron dense deposits around the muscle cells of the muscularis externa, in the basal lamina of myenteric ganglia, and of tracts of unmyelinated fibres lying between the longitudinal and circular muscle layers. The histochemical reaction product was observed between glial cell processes and neuronal processes in the tracts (Figure 27) and ganglia.

The submucous ganglia also exhibited electron dense reaction product, indicative of the presence of albumin immunoreactivity, in the same locations as in the myenteric ganglia.

4.7.2 Fluorescent Bovine Albumin

Five or thirty minutes after intravenous injection of RBA, strong fluorescence was observed in the plasma inside blood vessels of all sizes (Figure 28 A). Extravascular fluorescence of almost equal intensity was seen in the mesentery and in the muscularis externa where bright lines outline the dark profiles of muscle cells (Figure 28 and 29). Fluorescence was also present in the connective tissue and around the smooth muscle cells in the mucosa. The myenteric ganglia did not fluoresce, but each one was encapsulated by a conspicuous line of fluorescence (Figure 28 A and 29 A). This line was continuous with the thinner fluorescence line that separated the longitudinal and circular muscle layers of the muscularis externa. The submucous ganglia stood out as non-fluorescent areas within the strongly fluorescent zone that separated the circular

Figure 27

Immunoreactivity for endogenous albumin in myenteric plexus of ileum. Dark reaction product was present in the basal lamina and in the extracellular spaces around neuronal processes and glial cell processes. Reaction product was also observed around the muscles in the muscle layer (x24,000).

LM = longitudinal muscle; A = axon; G = glial cell; Gp = glial cell process; D = dendrite.

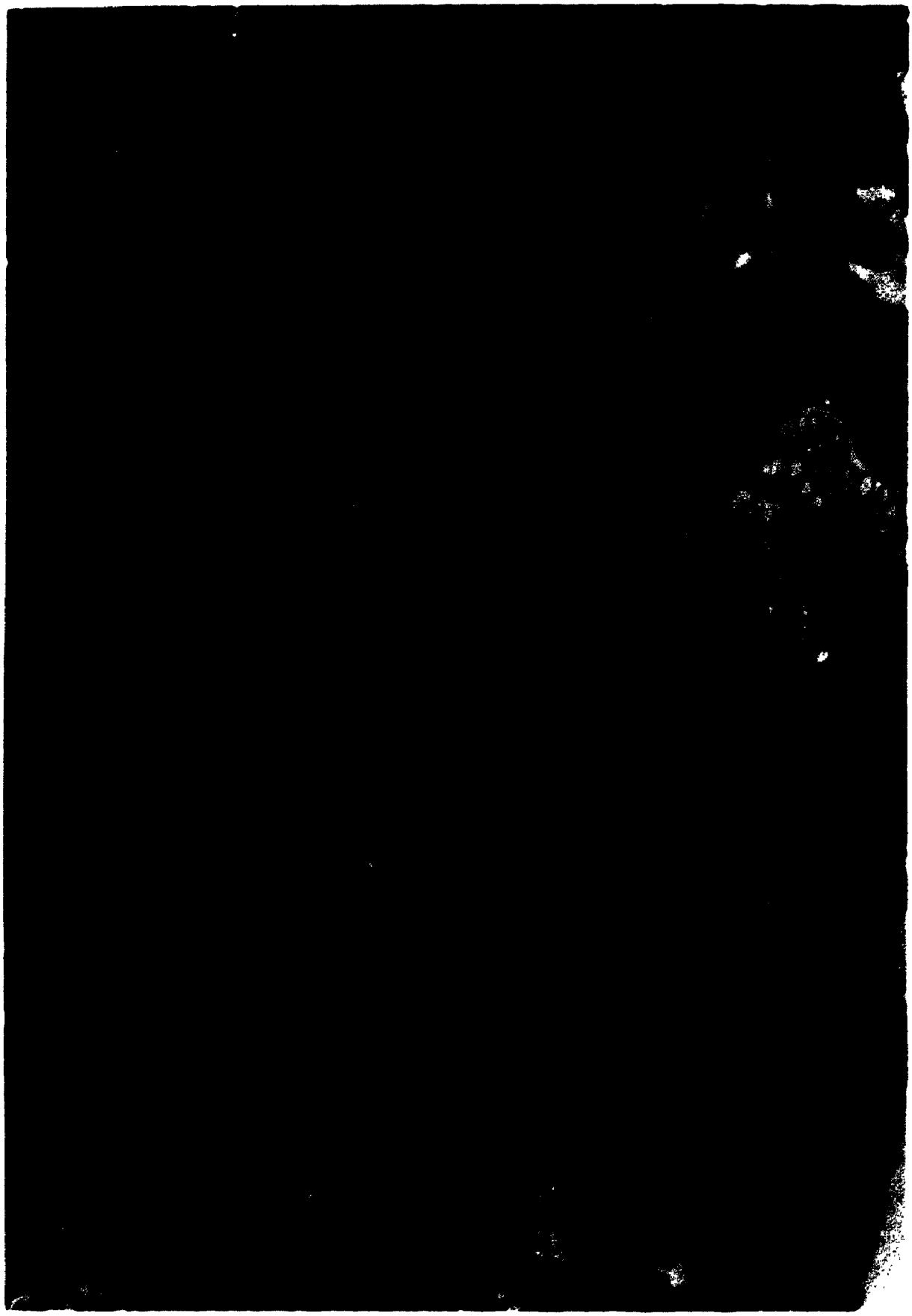


Figure 28

A. (x820) Ileum from a rat killed 5 minutes after an intravenous injection of rhodamine labelled albumin. Fluorescence was present around the muscle cells in the muscle layers, in the lumina of the blood vessels, and in the submucosa. No fluorescence was observed in the myenteric or submucous ganglia, but a fluorescent ring surrounded these ganglia.

B. (x820) Fluorescent neutral red stained section of A showing morphology. Note the myenteric and submucous ganglia.

bv = blood vessel; MG = myenteric ganglion;

SG = submucous ganglion; s = submucosa; N = neuron.

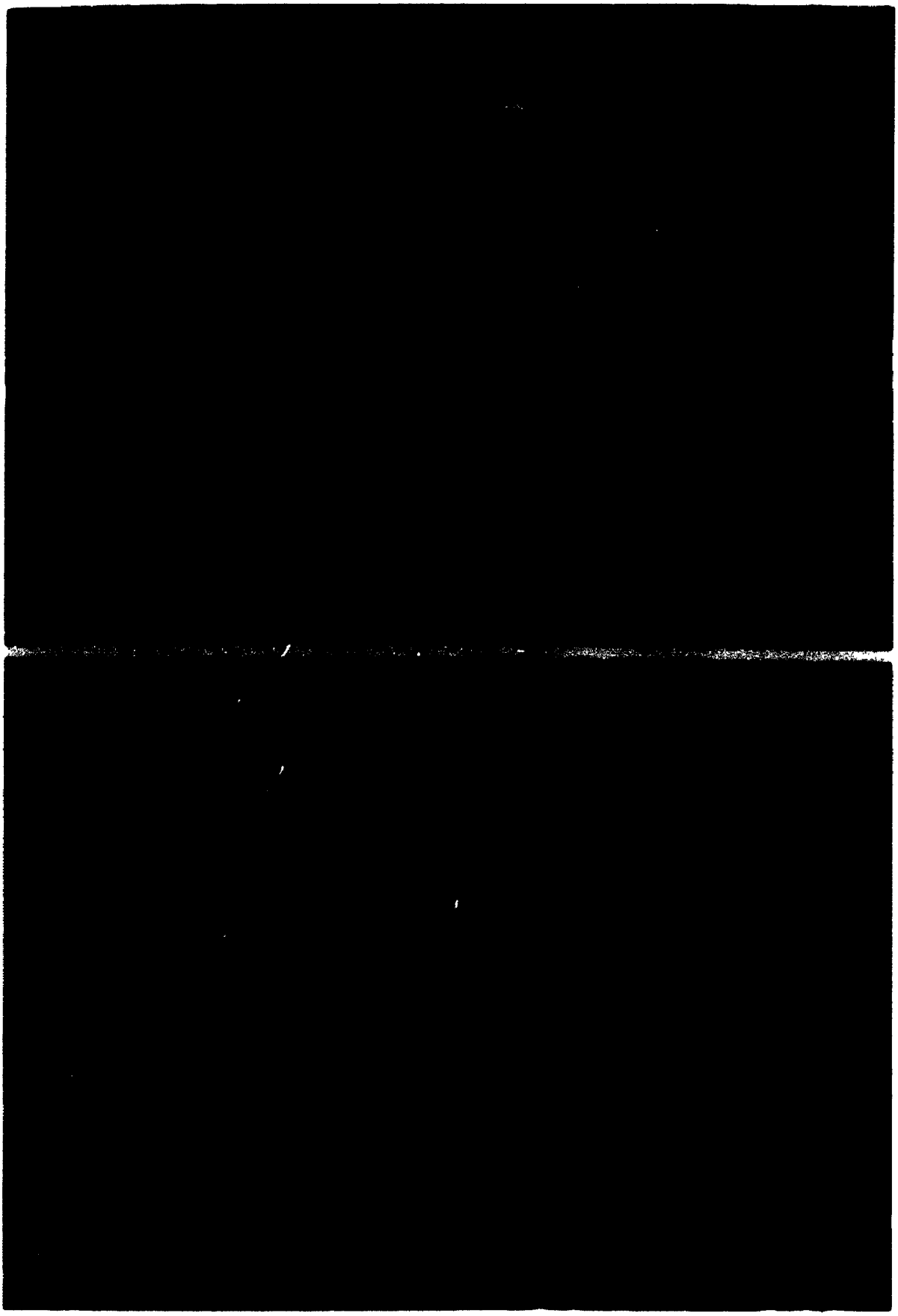
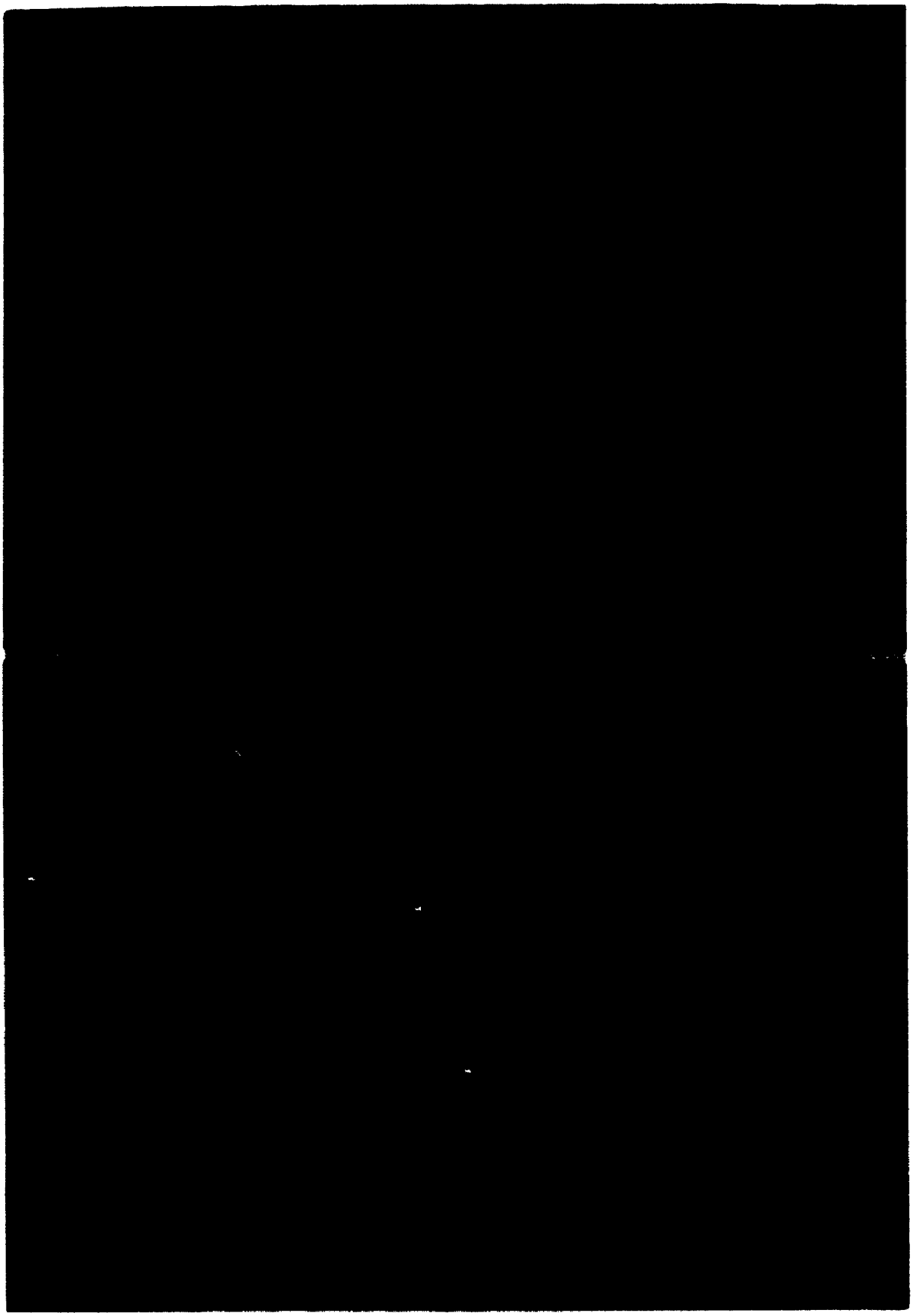


Figure 29

A. (x840) Ileum from a rat killed 30 minutes after an injection of rhodamine labelled albumin. Note the fluorescence around the muscle cells in the muscle layers, in the lumen of a blood vessel and in the submucosa. No fluorescence was observed in the myenteric ganglion, but it was surrounded by a ring of fluorescence.

B. (x840) Neutral red stained section of A showing the neurons and the myenteric ganglion.

bv = blood vessel; s = submucosa; MG = myenteric ganglion; N = neuron.



muscle from the muscularis mucosa. It was not possible to see fluorescent capsules around submucosa ganglia because the surrounding collagenous connective tissue, which contained many large blood vessels, was brightly fluorescent.

The locations of the enteric ganglia were confirmed by staining the same sections, which had been examined under green exciting light, with neutral red.

Although the red fluorescence was much brighter after one week than that after the shorter times, the distribution of fluorescence was the same (Figure 30).

4.7.3 Horseradish Peroxidase

In 0.5-1.0 μm sections of ileum, the peroxidase reaction product was present inside blood vessels of all sizes and in all layers of larger vessels. Extravascular deposits were conspicuous around the muscle cells in the muscle layers and in the connective tissue of the submucosa and mucosa. Myenteric ganglia, which lie between the two muscle layers, were encapsulated by thick dark lines with finer and paler lines extending into the ganglia among the cells (Figure 31 A). The submucosa ganglia had the same appearance as the myenteric ganglia. Peroxidase activity was not detected within any cells. The appearances were the same when HRP had circulated for 5 or 10 minutes. In rats sacrificed 30 minutes after injecting the enzyme, very little peroxidase activity was present.

Figure 30

A. (x880) Ileum from a rat killed after one week of daily injections of rhodamine labelled albumin. Note the absence of fluorescence in the myenteric and submucous ganglia, which had a ring of fluorescence around them. Fluorescence was present in the muscle layers in the submucosa and in the mucosa.

B. (x880) Neutral red stained section of A showing the myenteric and submucous ganglia.

bv = blood vessel; MG = myenteric ganglion;

SG = submucous ganglion; N = neuron.

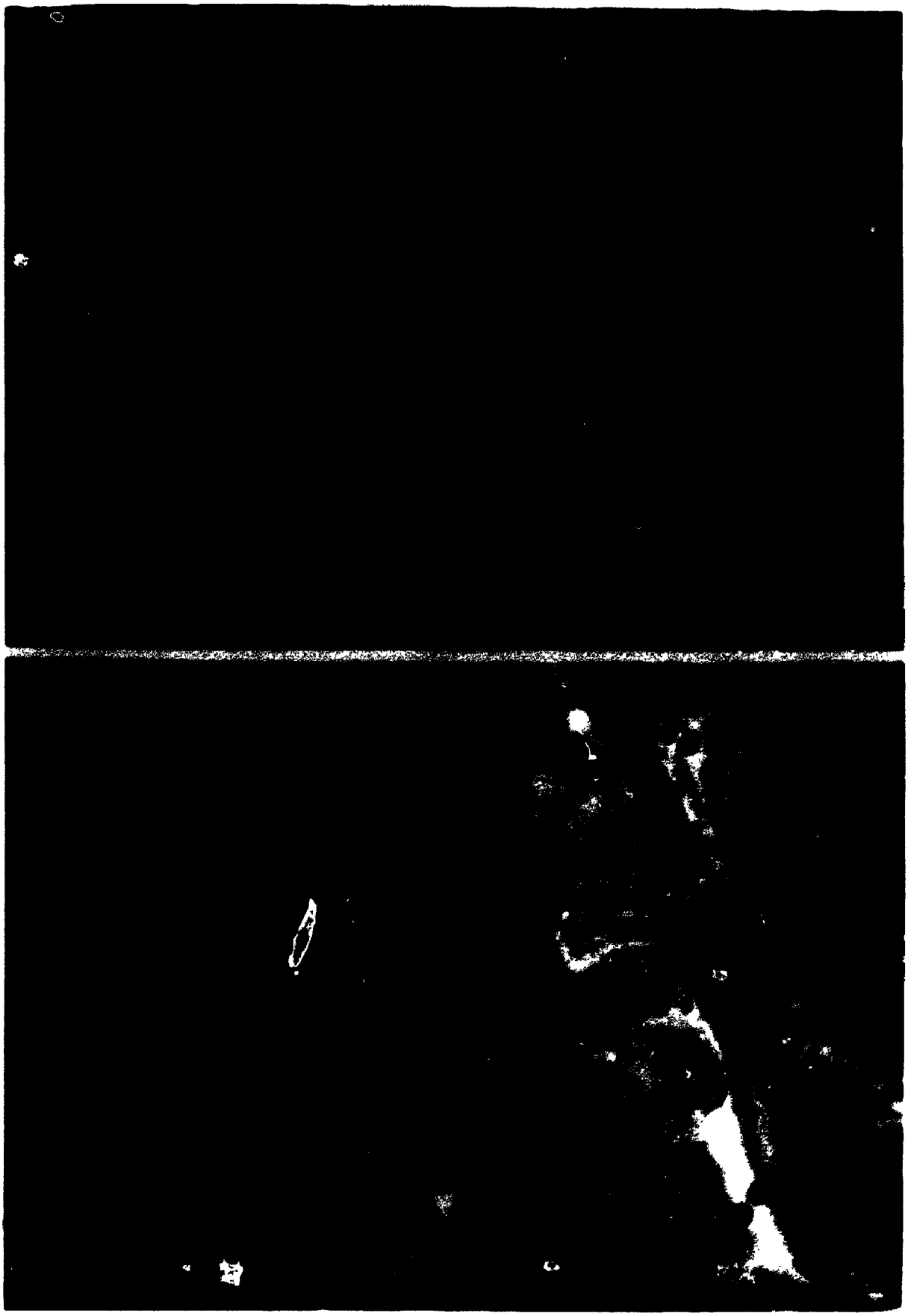


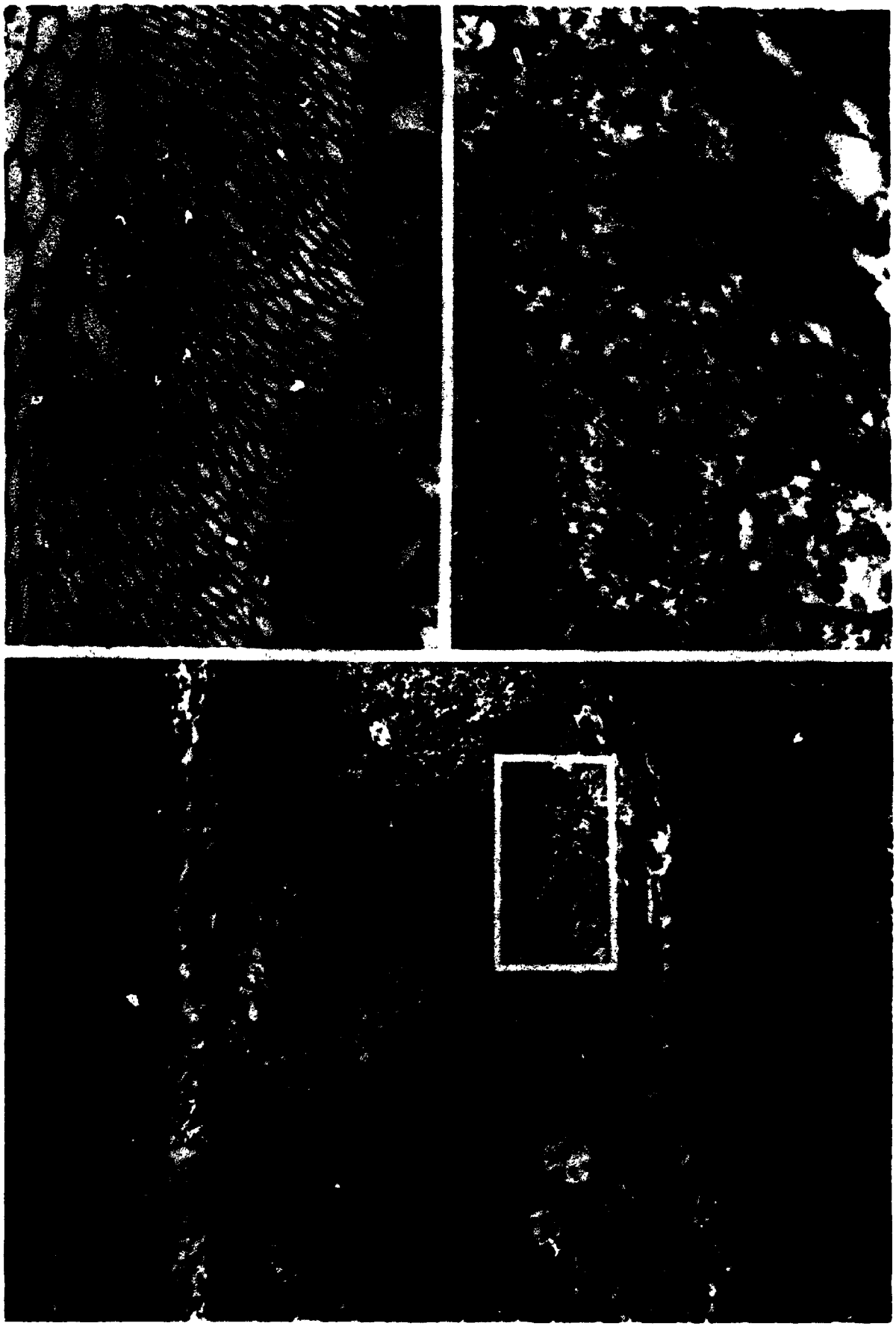
Figure 31

A. (x700) Unstained plastic section of ileum from a rat killed 10 minutes after an intravenous injection of HRP. Note the dark reaction product around the muscle cells, in the muscle layers, in the walls and lumina of blood vessels and in the submucosa. The reaction product was present around the cells in the ganglia and forms a dark ring around the myenteric ganglion.

B. (x10,000) Electron micrograph of A. The dense reaction product was visible around the muscle cells and in the extracellular spaces of the myenteric ganglion (dark line enlarged in C.).

C. (x35,000) Enlargement of rectangular area in B. Note the dark reaction product between the neuron and glial cell process, and in the extracellular spaces of the neuropil.

MG = myenteric ganglion; bv = blood vessel; s = submucosa; SG = submucous ganglion; N = neuron; G = glial cell; F = fibroblast; Gp = glial cell process; D = dendrite; A = axon; BL = basal lamina; arrowhead indicates a synapse.



Electron microscopy of ileum, taken from animals in which HRP had circulated for 5 or 10 minutes, revealed HRP reaction product in the lumina of blood vessels, in the extravascular spaces around them, and in all layers of the ileum. The reaction product was also present in the clefts between adjacent endothelial cells and in the cytoplasm of endothelial cells (Figure 32 B). No blood vessels were in the enteric ganglia or any of the fibre tracts.

The extracellular spaces around the muscle cells in the muscularis externa exhibited dark reaction product, as did the spaces around the collagen fibres in the submucosa and mucosa. The myenteric ganglia, which lie between the longitudinal and circular muscle layers, had basal lamina covering them that showed DAB reaction product (Figure 31 B and C, 32 A). Peroxidase activity was detected in these ganglia in the spaces between neurons and glia, and neurons and glial processes (Figure 31 B and C, 32 A). Neurons were recognized by their nuclei containing dispersed chromatin, their darker cytoplasm which contained Nissl bodies, and lots of free ribosomes and synapses (Figure 31 B and C, 32 A). Glia were identified by their denser nuclei with clumps of chromatin, paler cytoplasm, and processes that enveloped many other cell processes. DAB reaction product was observed between glial processes, dendrites, and axons (Figure 31 B and C, 32 A). The dendrites were recognized by their regular array of microtubules, whereas axons were identified by their synaptic vesicles and synapses.

Figure 32

A. (x47,000) HRP reaction product was present in the extracellular spaces between the neuron and glial cell and in the neuropil in myenteric ganglion of ileum from a rat killed 5 minutes after injection.

B. (x47,000) Reaction product was observed in the capillary lumen, between adjacent endothelial cells, and around the capillary in circular muscle of the ileum from a rat killed 5 minutes after an injection of HRP.

A = axon; G = glial cell; N = neuron;

Er = endoplasmic reticulum; r = ribosomes;

Nb = Nissl bodies; Ly = lysosomes; CL = capillary lumen;

cm = circular muscle; E = endothelial cell; V = vesicle.

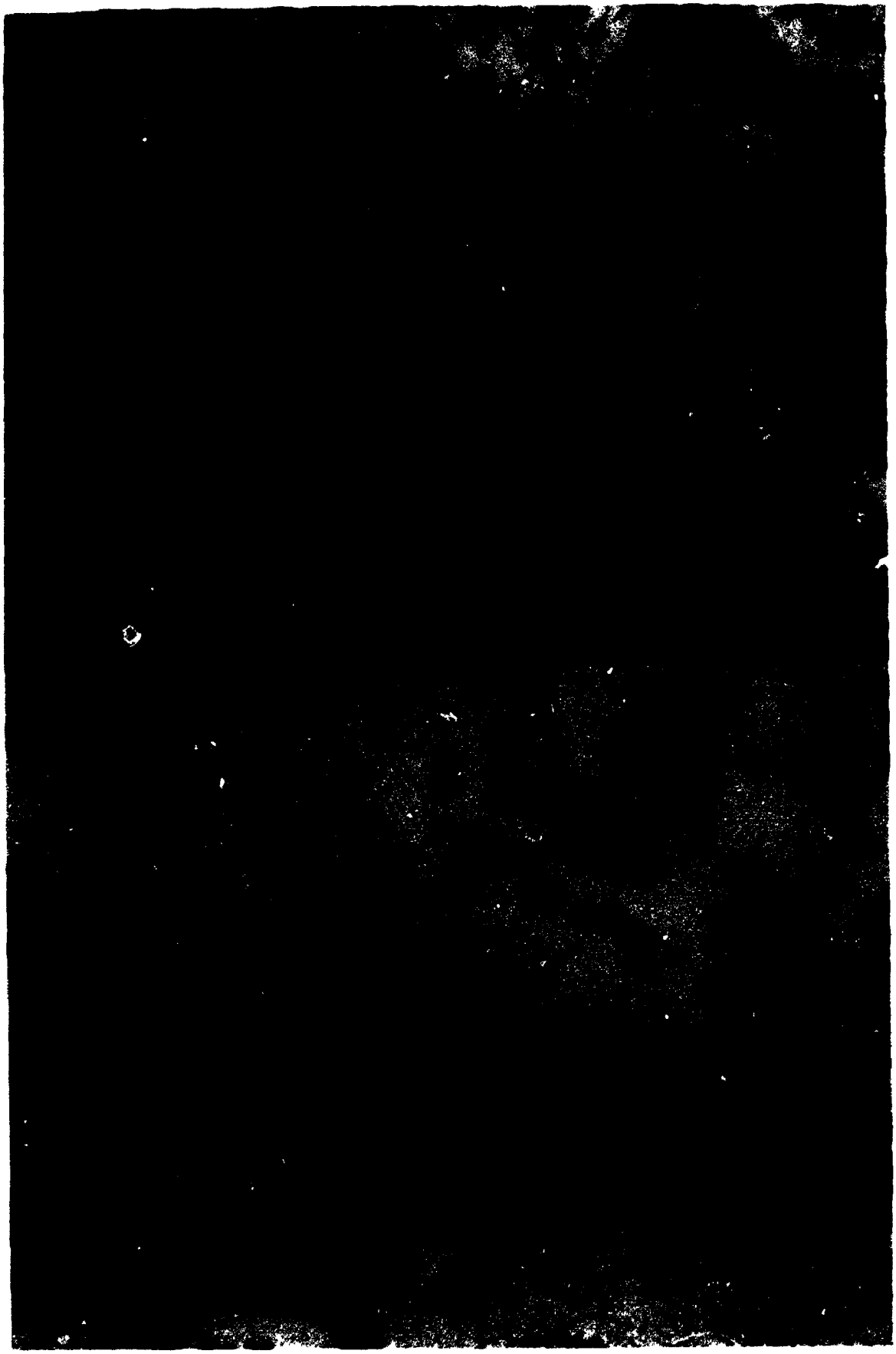


Figure 33

A. (x87,000) Submucous ganglion in ileum from a rat killed 5 minutes after an injection of HRP. Dark reaction product was present around the muscle cells in the muscle layer, around the ganglion and in the extracellular spaces between neurons and glial cells.
B. (x35,000) Enlargement of rectangular area in A showing reaction product between neurons and a glial cell process and in the neuropil.
cm = circular muscle; C = collagen; BL = basal lamina; N = neuron; G = glial cell; GP = glial cell process; m = mitochondrion; Nb = Nissl bodies.

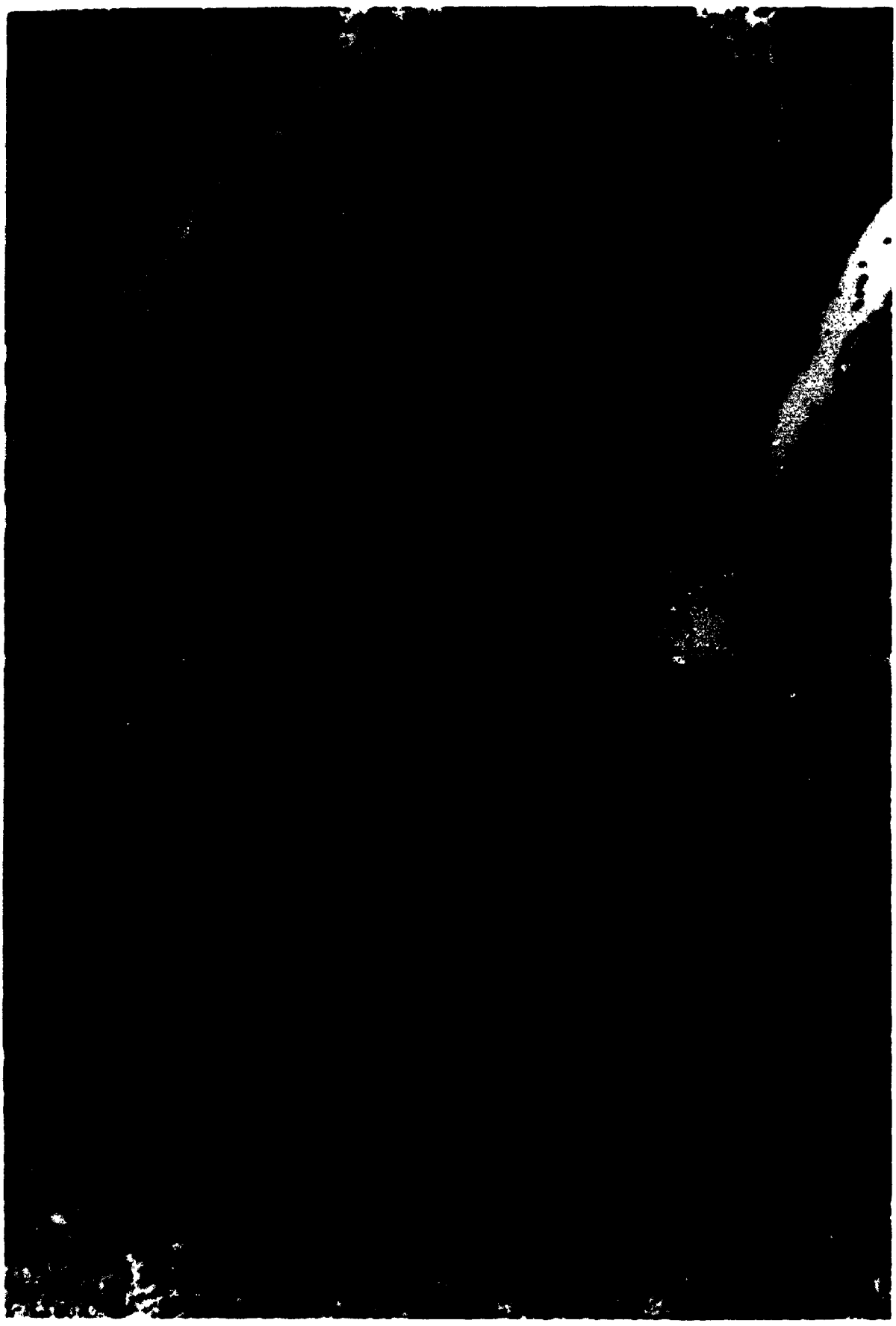
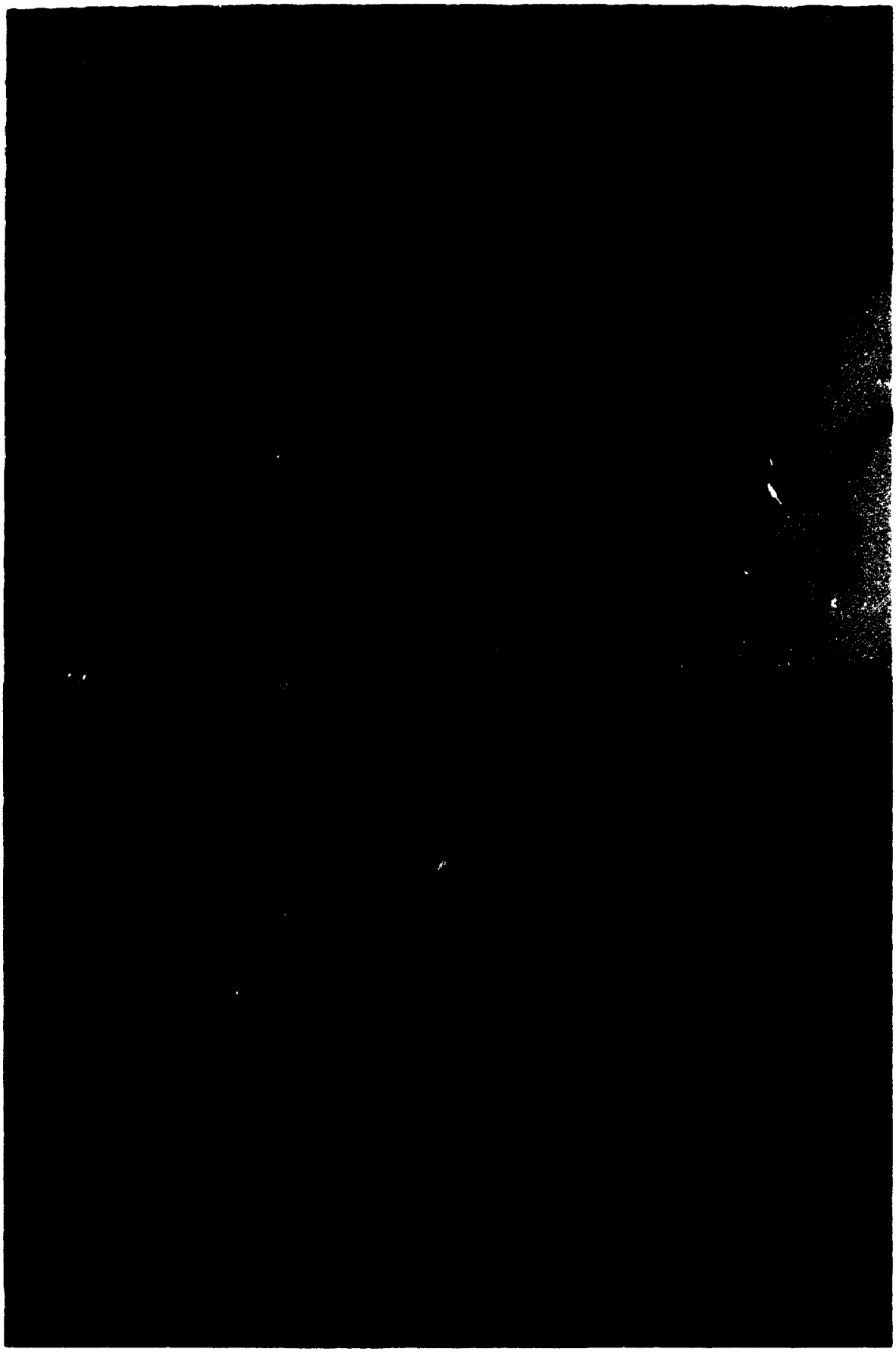


Figure 34

A. (x47,000) Submucous ganglion in ileum from a rat killed 10 minutes after an injection of HRP. Note the dark reaction product between the neuron and glial cell process and in the extracellular spaces of the neuropil.
B. (x87,000) Submucous ganglia 30 minutes after an injection of HRP. No reaction product was present in the extracellular spaces between the neuron and glial cell, or in the neuropil.
N = neuron; Gp = glial cell process; BL = basal lamina; A = axon.



Peroxidase activity was also detected in the fibre tracts of the enteric nervous system. The submucosa ganglia exhibited the same distribution of HRP reaction product (Figure 33 A and B, 34 A), as that in myenteric ganglia with 5 or 10 minutes circulation times. Identical results were obtained with these shorter times. In ileum from rats sacrificed 30 minutes after injecting HRP, very little reaction product was visible in the muscle layers, submucosa, and mucosa. No peroxidase activity was detected in the myenteric and submucous ganglia (Figure 34 B) and their fibre tracts.

4.7.4 Basic Fluorochromes

The distribution of red fluorescence, due to ethidium bromide, was compared with the autofluorescence emitted when the sections were excited by blue light to facilitate anatomical orientation. Red fluorescence was observed in the nuclei of cells in the connective tissue of the submucosa and mucosa, in the nuclei of epithelial cells, and in the cytoplasm of these cells. The nuclei and cytoplasm of the cells composing the walls of blood vessels fluoresced but the plasma in the lumina did not.

Ileum from rats, sacrificed 5 or 30 minutes after injection of ethidium bromide, exhibited red fluorescence in the nuclei of muscle cells. In the myenteric and submucosa ganglia, red fluorescence was observed in the nuclei and cytoplasm of neurons and the nuclei of glia (Figure 35 A and

Figure 35

A. (x340) Myenteric ganglion in ileum from a rat killed 5 minutes after an injection of ethidium bromide. Fluorescence was present in the nuclei of muscle cells in the muscle layer, and in the cytoplasm and nuclei of neurons in the ganglion.

B. (x380) Submucous ganglion in ileum from a rat killed 5 minutes after an injection of ethidium bromide. Note the fluorescence in the cytoplasm and nuclei of epithelial cells, in nuclei of cells in mucosa and submucosa in nuclei of muscle cells, and in the nuclei and cytoplasm of neurons in the ganglion.

C. (x410) Myenteric ganglion in ileum from a rat killed 5 minutes after an injection of acriflavine. Note the fluorescence in he cytoplasm and nuclei of neurons, in the nuclei of muscle cells, the nuclei of connective tissue cells in the mucosa and submucosa, and in the cytoplasm and nuclei of epithelial cells.

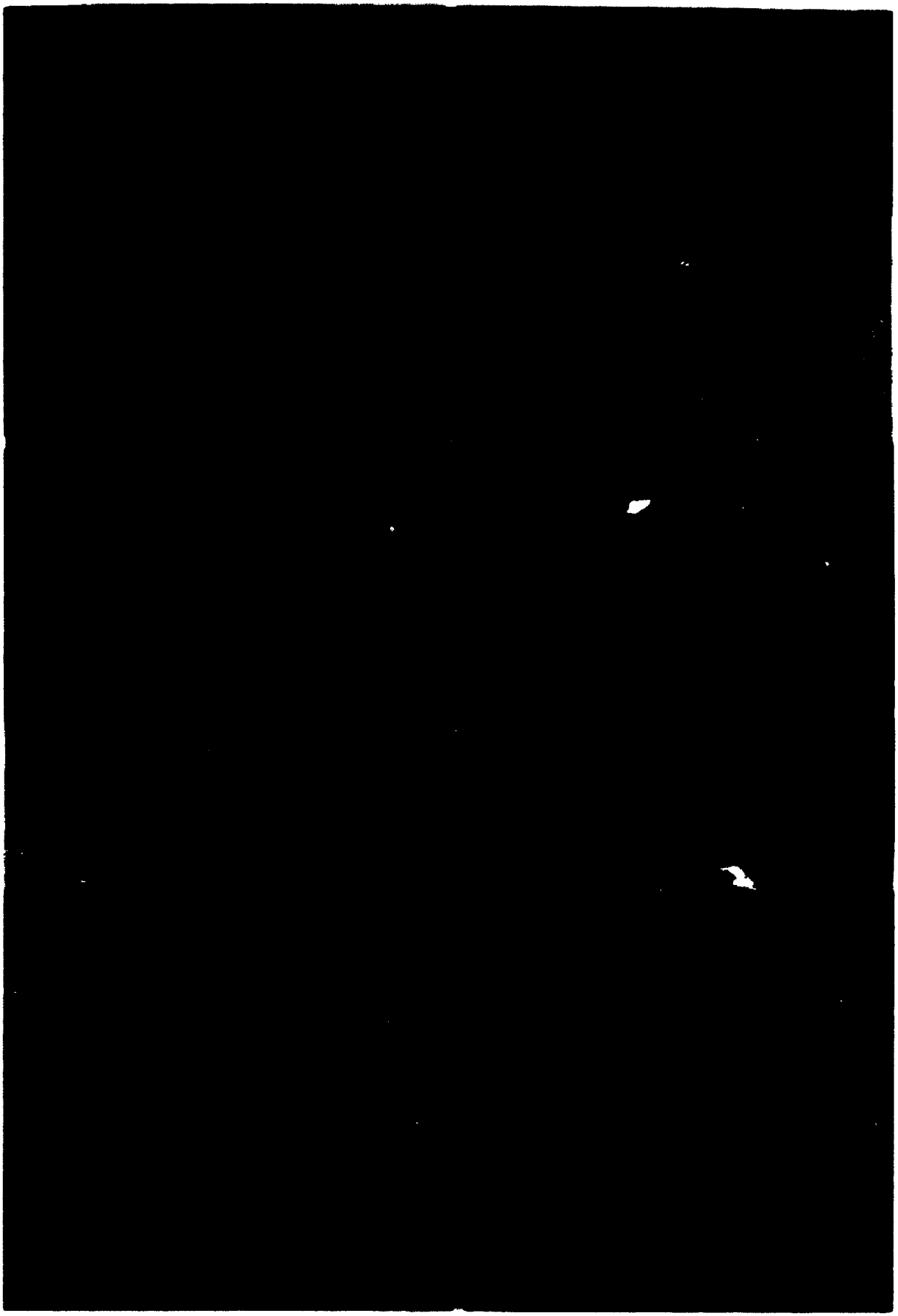
D. (x410) Submucous ganglion in ileum from a rat killed 5 minutes after injection of acriflavine. A similar distribution of fluorescence was observed as in C.

E. (x580) Myenteric ganglion in ileum from a rat killed 30 minutes after an injection of acriflavine. The distribution of fluorescence was the same as that in C.

F. (x380) Myenteric and submucous ganglia in ileum from a rat killed 30 minutes after an injection of acriflavine. The distribution of fluorescence was the same as that in C and D.

MG = myenteric ganglion; N = neuron; SG = submucous ganglion.

Acriflavine has bright apple green fluorescence whereas autofluorescence is dull green fluorescence.



B). The larger neurons were recognized by their size and vesicular nuclei.

The same distribution of fluorescence was observed after injection of acriflavine except that the fluorescence was excited by blue light and the emitted colour was yellow-green (Figure 35 C-F).

4.8 Immunohistochemical Staining for Bovine Albumin

Intravenous BSA was used as a protein tracer in rats and paraffin sections were stained immunohistochemically using a rabbit anti bovine albumin antiserum and the PAP method. Sections of the median eminence, area postrema, preaortic ganglia, trigeminal ganglion, sciatic nerve, and intestine all exhibited staining identical to that observed for rat albumin (see Sections 4.1.1, 4.2.1, 4.3.1, 4.4.1, 4.5.1, 4.6.1, 4.7.1).

These results were compared with the appearances of identically stained tissues from animals that had not been injected with BSA. Exactly the same distribution of staining was observed. This showed that the rabbit anti bovine albumin antiserum recognized both BSA and rat albumin. It was not possible, therefore, to determine the distribution of injected unlabelled bovine albumin using the antiserum that was available.

4.9 Polyacrylamide Gel Electrophoresis (PAGE) of some Protein Tracers

The objective of the PAGE analyses was to determine the molecular weights of the protein tracers used, and to find out whether conjugation of bovine albumin with rhodamine B changed the size of the major component of bovine albumin. The results of electrophoreses are shown in Figure 36 and Table III. The distance travelled by each major band was recorded and the $\log_{10}$ (molecular weight of the standard) (Y-axis) was plotted versus the distance travelled (cm) (X-axis). A least squares regression line was fitted to the points. The molecular weights of the major bands from BSA, RBA, and HRP were determined from this line (Figure 37).

Figure 36

PAGE of molecular weight standards and bovine albumin, rhodamine labelled albumin, and HRP. The molecular weights are shown on the left side (low molecular weight standards) and on the right side (high molecular weights) (x3). Low molecular weight standards: α-lactalbumin (14,400 daltons); trypsin inhibitor (20,100); carbonic anhydrase (30,000); ovalbumin (43,000); BSA (67,000); phosphorrylase (94,000). High molecular weight standards: glutamic dehydrogenase (53,000 daltons); transferrin (76,000); β-galactosidase (116,000); α_2-macroglobulin (170,000); myosin (212,000). The substances subjected to PAGE are shown below the PAGE. BSA = bovine albumin; RBA = rhodamine labelled bovine albumin; HRP = horseradish peroxidase.

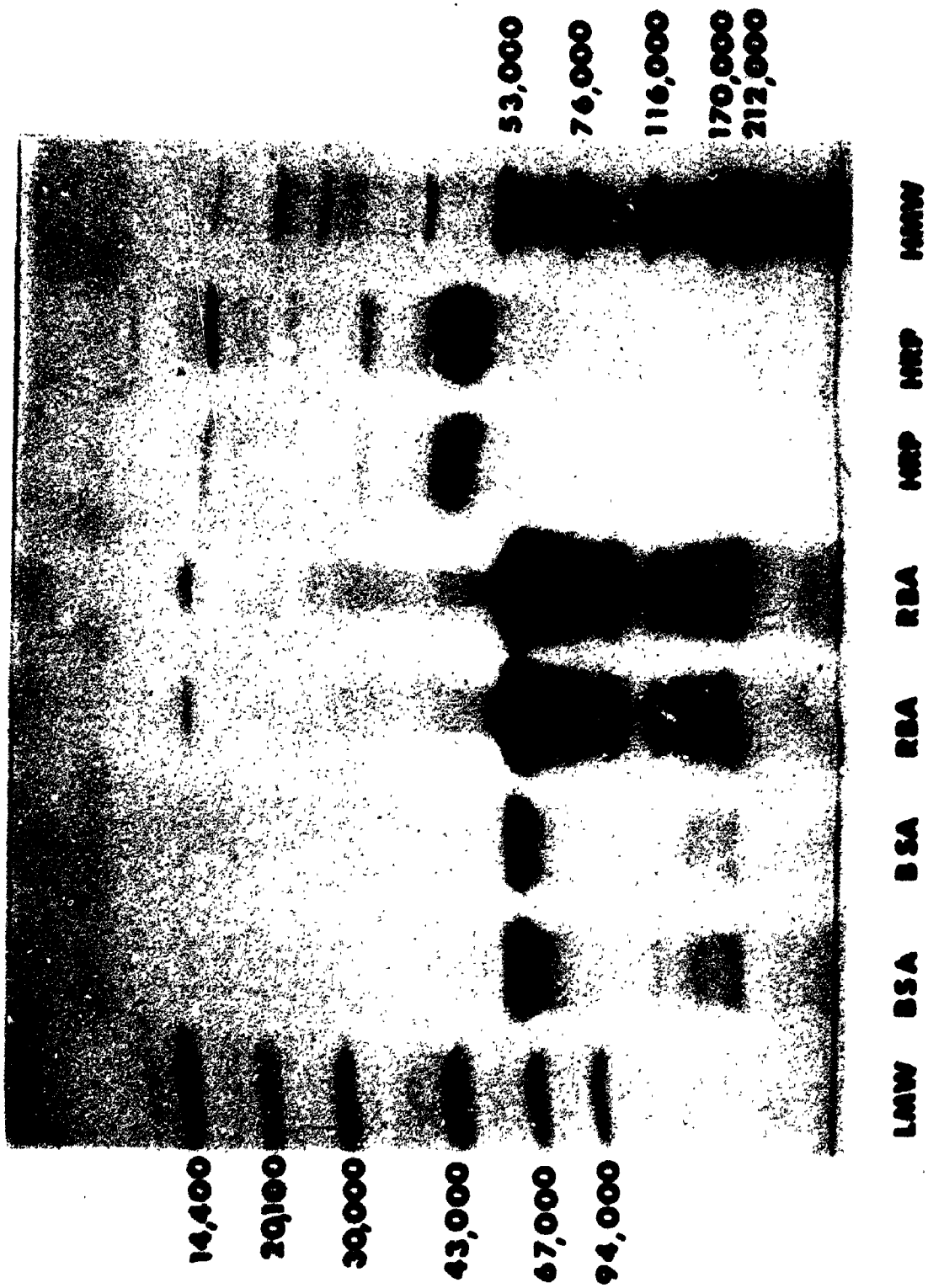
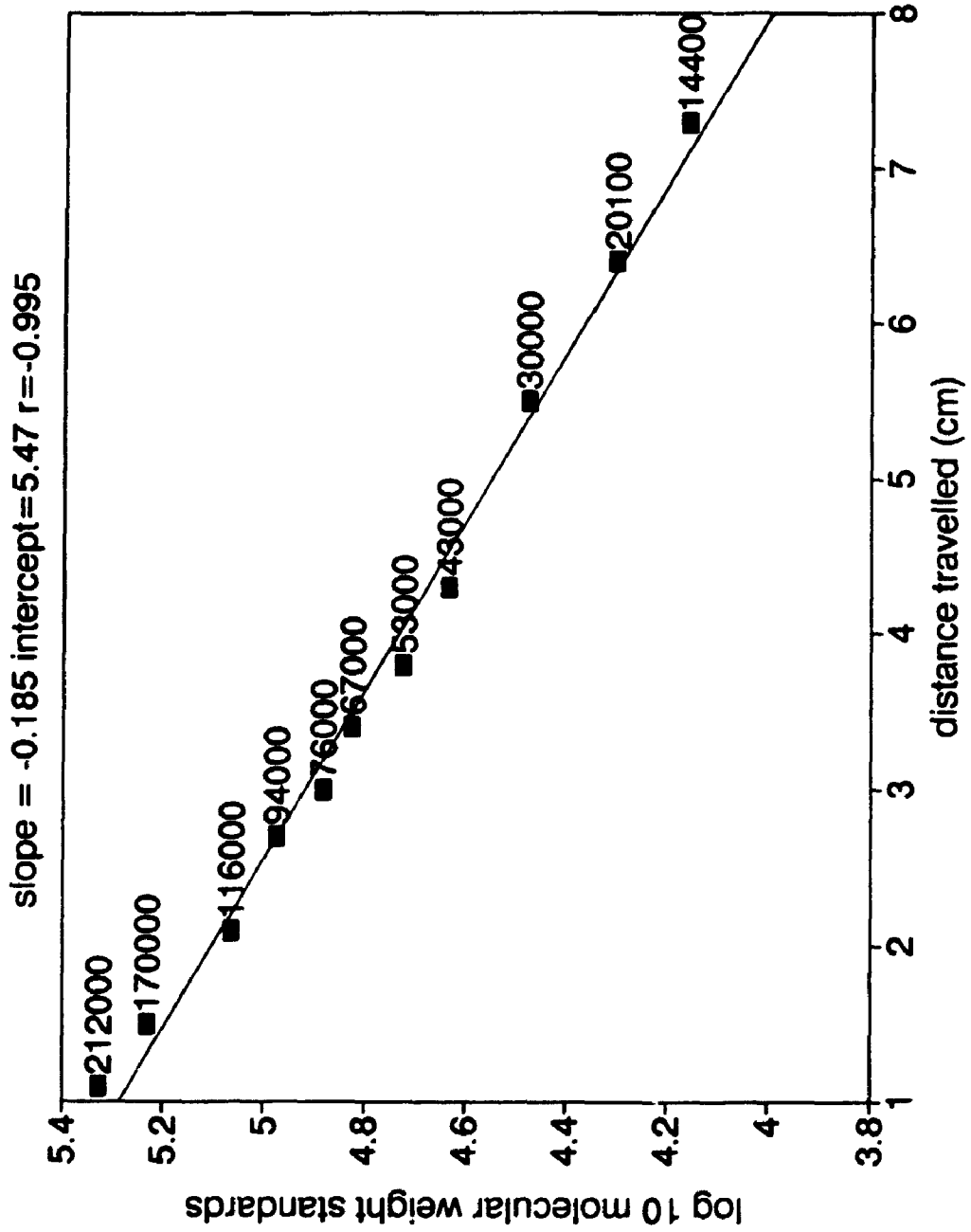

LMW
BSA
BSA
BSA
BSA
HRP
HRP
MMW
94,000
67,000
43,000
30,000
20100
14,400
53,000
79,000
110,000
170,000
212,000

Table III Proteins Analyzed by PAGE Showing the Components Listed in Order of Relative Abundance and Molecular Weights of Components

STD	Molecular Weight	Distance (cm)
α-lactalbumin	14 400	7.3
trypsin inhibitor	20 100	6.4
carbonic anhydrase	30 000	5.5
ovalbumin	43 000	4.3
glutamic dehydrogenase	53 000	3.8
BSA	67 000	3.4
transferrin	76 000	3.0
phosphorylase	94 000	2.7
β-galactosidase	116 000	2.1
α_2-macroglobulin	170 000	1.5
myosin	212 000	1.1
<u>BSA LANE</u>		
band 1 - most abundant	64 000	3.5
band 2	212 000	1.1
band 3	156 000	1.5
band 4 - least abundant	126 000	2.0
<u>RBA LANE</u>		
band 1 - most abundant	64 000	3.6
band 2	102 000	2.5
band 3	212 000	1.1
band 4	121 000	2.1
band 5	86 000	2.9
band 6	144 000	1.7
band 7 - least abundant	14 000	7.4
<u>HRP LANE</u>		
band 1 - most abundant	47 000	4.3
band 2	14 000	7.1
band 3	31 000	5.3
band 4	22 000	6.1
band 5 - least abundant	10 000	8.0

Figure 37

Polyacrylamide gel electrophoresis of molecular weight standards plotted versus distance travelled. Linear regression analysis yielded a slope = -0.185, an intercept = 5.47, and a r = -0.995. This line was used to calculate the molecular weights of bovine albumin, RBA and HRP.


slope = -0.185 intercept=5.47 r=-0.995
log 10 molecular weight standards
distance travelled (cm)
212000
170000
116000
94000
76000
67000
53000
43000
30000
20100
14400
5.4
5.2
5
4.8
4.6
4.4
4.2
4
3.8
1
2
3
4
5
6
7
8

4.10 Dye Binding Studies

Ethidium bromide, acriflavine, rhodamine B isothiocyanate, and Evans blue were dissolved in 10% BSA in PBS, pH 7.4 to yield dye solutions with the concentrations shown in Table I for the first three dyes and a 0.43% solution of Evans blue by weight. One ml of each of these dye solutions was dialysed against 15 ml of PBS, pH 7.4. The dialysis tubing had a molecular weight cutoff of 12000 daltons. As a control, one ml of 10 % BSA in PBS was also dialysed against 15 ml of PBS. These solutions were dialysed for one week at 4°C (see section 3.5).

Since ethidium bromide, acriflavine and Evans blue obey Beer's law, the concentrations of these dyes in the PBS solutions outside the dialysis tubing were calculated from their absorbances at the wavelengths of maximum absorption (λ_{max}), and known molar extinction coefficients for each of these dyes. The absorption maxima and their wavelengths (λ_{max}) were determined for each dye by performing UV-visible spectrophotometry on a solution of known concentration of the dye. The absorption maxima of a dye in the solution outside the dialysis tubing was obtained by UV-visible spectrophotometry. The percentages of free and bound dye were calculated from the concentration of free dye, the total volume (inside and outside the dialysis tubing), and the known number of moles of dye put into the dialysis tubing. The results are shown in Table IV. Ethidium bromide (90%) and acriflavine (93%) remained mainly in the

free state, whereas Evan's blue (80%) and rhodamine B isothiocyanate (72%) were mainly bound.

Ethidium bromide and acriflavine were dissolved in rat's serum to yield solutions with the concentrations shown in Table I and 1 ml of the resulting solution was dialysed (molecular weight cutoff 12 000 daltons) against 15 ml of PBS, pH 7.4 for one week at 4°C. In order to control for leakage of protein from the tubing, 1 ml of rat's serum was also dialysed against 15 ml of PBS using the same conditions (see section 3.5).

The concentration of ethidium bromide or acriflavine in the solution outside the dialysis tubing was determined by UV-visible spectrophotometry. The percentages of free and bound dye were calculated the same way as were those for the previous experiment in which 10% BSA was used. The results using rat's serum are shown in Table IV. As with bovine serum, ethidium bromide (96%) and acriflavine (71%) remained mainly in the free state.

Table IV Binding of Dyes to Proteins as Determined by Dialysis

Dye	Protein Solution	% Bound*	% Free*
acriflavine	10 % BSA+	7 %	93 %
ethidium bromide	10 % BSA	10 %	90 %
Evan's blue	10 % BSA	80 %	20 %
rhodamine B isothiocyanate	10 % BSA	72 %	28 %
acriflavine	rat serum+	29 %	71 %
ethidium bromide	rat serum	4 %	96 %

* (% by moles).

+ No leakage of BSA or rat serum out of the tubing was observed by spectrophotometry.

Table V Binding of Dyes to Rat Serum as Determined by Filtration+ and Centrifugation

Dye	% Bound*	% Free*
acriflavine	79 %	21 %
ethidium bromide	67 %	33 %

* (% by moles). Binding of free dye to the filter was assumed to be negligible because in free drug assays neither basic nor acidic drugs have bound to the filter at a detectable level (personal communication, Ward, 1991; Amicon, 1991).

+ No rat serum was present in the filtrate.

The percentages of bound and free ethidium bromide and acriflavine were also determined using the Amicon ultracentron-10. This apparatus consisted of a reservoir above a filter with a molecular weight cutoff of 10 000 daltons and a tube under the filter to catch the filtrate. The dye, dissolved in rat's serum, was placed in the reservoir above the filter and the entire apparatus was centrifuged. As a control rat's serum was placed in a reservoir and centrifuged. This procedure provided a much faster separation of bound dye (remaining above the filter) from free dye (in the filtrate) than was possible using traditional dialysis. No fluid remained above the filter.* Filtration and centrifugation also allowed less time for equilibration of bound and free dye. Each filtrate was analyzed by UV-visible spectrophotometry and the percentages of bound and free dye were calculated from the concentration of free dye, the total volume, and the number of moles of dye put into the reservoir.

The percentages of bound and free dyes determined by filtration and centrifugation are shown in Table V. In contrast to the results obtained by dialysis, most of the acriflavine (79%) and ethidium bromide (67%) remained bound to large molecules in serum. Due to the short circulation times used the results shown in Table V are a more realistic

* These filters were used in free drug analyses in sera at the University Hospital, London and no problems were encountered with the filters clogging (personal communication, Pat Ward, Head of Dept. of Clinical Biochemistry).

representation of what happens in vivo than those obtained by dialysis.

CHAPTER 5 DISCUSSION

5.1 Blood-Brain Barrier

The distribution of injected proteins and of certain fluorescent cationic dyes and also of endogenous albumin in the brain has been described by numerous investigators (Wislocki and Putnam, 1920; Wislocki and King, 1936; Rodriguez-Peralta, 1955; Reese and Karnovsky, 1967; Clasen et al., 1970; Cesarini et al., 1985). Observations made in the present study agreed with these findings: the blood vessels in most parts of the brain were impermeable, but in the circumventricular organs, the vessels were permeable. The observed penetration of dyes and proteins was the same, irrespective of the time of circulation in the blood. Impermeability of ordinary capillaries in the central nervous system is attributed to tight junctions between the endothelial cells, which block the passage of large molecules such as proteins (Brightman, 1965; Reese and Karnovsky, 1967). A somewhat different explanation is needed for the exclusion of cationic fluorochromes from the brain.

Ethidium and acriflavine did not stain endothelial nuclei at the doses that effectively demonstrated the blood-

brain barrier in the brain. However, with higher doses (~5x; see section 3.2) generalized staining of all cell nuclei in the brain was observed. This may have been due to saturation of the binding capacity of plasma proteins (most likely albumin) for these basic fluorochromes, because they circulated for short times in approximately 70-80% bound form (bound dye/total dye; see sections 3.5 and 4.1). There is more than enough protein present to bind the dye. Therefore this explanation is rejected.

Assuming a binding ratio of 1:1 at the optimal doses, ethidium bromide (1 ml of 0.1% solution) and acriflavine (1 ml of 0.15% solution) would bind to only 0.15% and 0.23% respectively of the total serum proteins (0.653g in 10 ml of blood which is the total blood volume). The 0.15% saturation of serum proteins by ethidium bromide at a dose of 1 ml of 0.1% solution of the dye was calculated as follows: the number of mg of dye injected was divided by the number of mg of serum proteins in 10 ml of rat blood. The resulting ratio was multiplied by 100% to give the percentage of serum proteins by weight of the total that would bind all the dye.

The most likely explanation of the generalized staining of all cell nuclei in the brain observed with five times the optimal dose of dye was due to the increased amount of free dye in the blood to a detectable level when the dye left the blood vessels and bound to the nucleic acids in the cells. However, if bound dye was taken up by the cells, the dye

would readily bind to the nuclei acids in the cells for which it has a greater affinity than for albumin.

Acridine dyes bind to albumin and globulins (Kaldor et al., 1961; Stastny and Horejsi, 1961). In rat's serum 79% of the total amount of acriflavine was bound to the serum proteins (see sections 3.5 and 4.10). Similar results were obtained with ethidium bromide (see sections 3.5 and 4.10).

Actinomycin D which binds to plasma proteins is taken up by living cells and intercholates in the DNA (Goodman and Gilman, 1990). Acriflavine and ethidium probably behave in the same way as actinomycin D. These substances are probably taken up by cells in the bound form, as in known to occur with albumin (Kumagai et al., 1987); Pardridge et al., 1990). Diaminoacridine dyes are taken up by living cells and stain the nucleic acids (Robbins, 1960).

Ethidium bromide and acriflavine yielded clearer pictures of permeable and non-permeable areas than did a fluorescent protein tracer or the histochemical demonstration of exogenous HRP or endogenous albumin. The greater clarity was attributable to concentration of the cationic fluorochromes in the nuclei of cells (Lerman, 1963; Gersch and Jordan, 1965; Le Pecq and Paoletti, 1967; Waring, 1968; 1981; Cozzarelli, 1977). Another advantage is that the results can be obtained quickly. Ethidium bromide was preferable to acriflavine, because the fluorescence could

not be confused with autofluorescence of the tissue, and there was less fading.

The fact that five different methods for demonstrating vascular permeability to macromolecules gave the expected results in the central nervous system justified the application of the same techniques to peripheral tissues in which the existence of vascular barriers was uncertain.

5.2 Blood-Nerve Barrier

5.2.1 Effect of Circulation Time

Rhodamine-labelled bovine albumin, HRP, ethidium bromide and acriflavine did not enter the endoneurium or perineurium, but were confined to the epineurium and lumina of capillaries, after circulating for 5 or 30 minutes. These short circulation times may not have been valid tests of the presence of a barrier to blood-borne macromolecules in the peripheral nervous system. Longer times, such as a week for fluorescent albumin or an indefinitely long time for endogenous albumin, were more representative of plasma proteins circulating naturally in the blood. The biological half life of rat albumin is 2.5 days (Dixon et al., 1953). The fluorescent rhodamine labelled albumin would be metabolized in the liver to other fluorescent metabolites which would also behave as intravascular tracers and contribute to the observed fluorescence. After one week of daily injections rhodamine-labelled bovine albumin was observed in the epineurium, perineurium, endoneurium and in

many axons. Kristensson (1970) and his coworkers (Kristensson et al., 1971; Kristensson and Sjostrand, 1972; Kristensson and Olsson, 1973) provided evidence that Evan's blue-labelled bovine albumin can be taken up by axons in the periphery, when they locally injected this fluorescently-labelled albumin into muscle and found the tracer in the perikarya of neurons. Endogenous albumin was also detected immunohistochemically in the endoneurium and in some axons of the rat's sciatic nerve, an observation in agreement with Mata et al. (1987).

Therefore the blood-nerve barrier is not as restrictive to the passage of macromolecules across it as is the blood-brain barrier. It is preferable to use the term blood-nerve interface as proposed by Weerasuriya et al. (1989). These investigators showed that approximately 30% of the endoneurial albumin is turned over per day. Since the radio-labelled albumin was administered intravenously in the brachial vein, the sciatic nerves were not damaged by direct injection, thus eliminating capillary leakage due to damage to the endoneurium. This is in marked contrast to the central nervous system in which no albumin was detected in the tissue except in the circumventricular organs because the rate of entry of albumin to the brain is very slow (Kumagai et al, 1987). The fact that albumin enters the endoneurium within 30, 60, or 120 minutes raises the question of how it enters. Since these are short circulation times, two possible routes of entry exist:

(1) transport across the endoneurial capillaries; and
(2) passage between adjacent capillary endothelial cells.

5.2.2 Possible Routes of Entry of Albumin into the Endoneurium

There are three possible routes of entry of albumin into the endoneurium after long circulation times: (1) by entering the open end of the peripheral nerve sheath and diffusing retrogradely; (2) by transperineurial transport of macromolecules; and (3) by leaking across endoneurial blood vessels.

Albumin may have entered the endoneurium at the end of the peripheral nerve where the perineurium eventually becomes discontinuous just before the neuromuscular junction (Peters, Palay and Webster, 1991). This route of entry would account for failure to detect fluorescent albumin that had circulated for only 5 or 30 minutes, because a longer time would obviously be needed for retrograde diffusion in the endoneurium along the length of the nerve. The rate of movement of the fluorescent albumin within the endoneurium could be determined by examining transverse sections of the sciatic nerve taken at various distances from the ends of some of its branches (including intramuscular fascia), at different times after systemic injection.

Transperineurial transport of HRP, ferritin and ^{125}I-albumin (by EM autoradiography) has been described in peripheral nerves of the rat by Oldfors and Sourander, 1978; Oldfors and Johansson, 1979; Oldfors, 1981. However,

transperineurial transport of HRP was not observed in either this study or by some other researchers (Klemm, 1970; Reese and Olsson, 1970; Kristensson and Olsson, 1971). Perhaps the transperineurial transport of proteins observed by the first group of investigators was a result of ischemic damage. This could be tested by fixing the peripheral nerve at different times after killing the animal and examining the nerve with the electron microscope.

The disputed occurrence of transperineurial transport cannot be attributed to differences in time (10-120 minutes) of killing the animal after giving the local HRP injection, or to different methods of fixation. It may be that the tight junctions of the inner perineurial layer are not all of the occluding type, so that proteins do cross slowly from the epineurium into the endoneurium. This would provide a long term circulation of plasma-derived proteins within the extracellular fluid around the nerve fibres.

The third possible route of entry for proteins into the endoneurium is across endoneurial vessels. Endothelial vesicles containing HRP were seen by Arvidson (1977; 1984) in mice 2 minutes-4 hours after injection of the enzyme at all doses. However, this leakage was markedly increased with a dose of 4 mg HRP/10 g body weight or more (8 mg/10 g). Endoneurial macrophages in Arvidson's animals also contained HRP. It should be noted that even at the higher doses of HRP (4 mg/10 g or more) the brain capillaries did not leak. It may be that at 4 mg/10 g or more HRP has a

toxic effect on the endothelium of the endoneurium. Although Arvidson claimed that the interendothelial cell tight junctions were still intact, it is possible that some of them may have been macula adherentes or if they were tight junctions they may have been opened up. This could be tested by repeating the experiment and measuring the distances between the plasma membranes at the tight junctions of adjacent endoneurial endothelial cells.

Another protein that leaves endoneurial blood vessels in the rat, over the course of one day, is albumin. Weerasuriya et al. (1989) found that the turnover of endoneurial [^{125}I] albumin is about 30% per day. Biochemical analysis of mammalian endoneurial fluid also revealed the presence of albumin in the endoneurium of nerves (Poctuslo et al., 1985). Further support for albumin in the endoneurium of nerves came from its detection immunohisto-chemically in my investigation and also in that of Mata et al. (1987).

It is known that cationized albumin and IgG are readily taken up by cerebral capillary endothelial cells and transported intracellularly to the albuminal side where they are extruded. This may be also occurring in the endoneurial capillaries in peripheral nerve. It may be that the covalently linked rhodamine albumin yields a cationized albumin which is readily taken up by the endothelial cells of the endoneurial capillaries and transported inside the endothelial cells to the albuminal side and then exocytosed

into the endoneurium. This same process may also occur with fluorescent metabolites of RBA.

In addition lanthanum ions which bind to fibrinogen (Pedersen et al., 1980) do not cross the perineurium, but do leave the endoneurial capillaries by passing between adjacent cells and/or by vesicular transport (Shinowara et al., 1982; Mackenzie et al., 1987). Hence proteins of greatly different molecular size (fibrinogen 341 kd; albumin 67 kd) can leave the endoneurial capillaries. These data point to the endoneurial blood vessels as the most likely site of egress of plasma proteins. If this is so, the tight junctions between the endothelial cells may not form complete zonulae occludentes, or their extent may change with age. Vesicular transport may also be involved.

5.2.3 Intraneuronal Plasma Proteins

Endogenous albumin was present in about two thirds of the axons. There are three possible explanations for this: (1) some of the axons have taken up the albumin at their terminals or laterally from the endoneurial space; (2) some of the neurons whose axons are present in the nerve synthesize albumin; and (3) some substance in these axons cross-reacts with the rabbit anti-rat antiserum to albumin. The rhodamine-labelled albumin which circulated for a week was also present in a number of the axons. The fluorescent albumin and its fluorescent metabolites were probably taken up by the axons.

Axonal endings in the periphery and synaptic boutons in the central nervous system can take up proteins and transport them retrogradely (Kristensson et al., 1971; La Vail et al., 1973). Proteins such as albumin and IgG have been detected in the perikarya of neurons in the central and peripheral nervous systems (Sparrow, 1981; Vidal, 1983; Wenthold et al., 1986; Fabian and Petroff, 1987; Fabian, 1988). Their presence in the cell bodies is a result of uptake by the nerve endings and retrograde axonal transport. Fluorescent albumin is similarly taken up by the nerve endings and transported back to the neuronal perikarya (Kristensson, 1970; Kristensson et al., 1971; Kristensson and Sjostrand, 1972; Kristensson and Olsson, 1973; Moos et al., 1991).

The second possible explanation for the presence of albumin in axons is intraneuronal synthesis. Immunohistochemistry, isolation of messenger RNA for albumin and other plasma proteins, and in vitro synthesis of the plasma proteins by the specific messenger RNAs showed that some plasma proteins were synthesized in fetal and adult neurons in the brain (diencephalon; basal forebrain) and in motor neurons in the spinal cord in humans (Mollgard and Jacobsen, 1984; Dziegielewska et al., 1985; 1986; Mollgard et al., 1988). Plasma proteins were also synthesized in neurons in the brains of neonatal and adult rats (Ali et al., 1983; Aldred et al., 1987). It is therefore possible that neurons with axons in the sciatic nerve,

synthesized albumin and transported the protein in positive immunostaining for albumin in these axons. Intraneuronal synthesis cannot, of course, account for the detection of fluorescently-labelled bovine albumin and its fluorescent metabolites in the axons of nerves of animals that received daily injections of this protein for a week. It is unlikely that the rhodamine was dissociated from the albumin and taken up by the axon, because the rhodamine was covalently bound to the albumin.

The third suggested explanation of positive immunostaining for albumin in axons is that the antiserum may have cross-reacted with some other substance present in the axons.

5.2.4 Disorders Resulting from Retrograde Axonal Transport and Autoimmunity

The fact that plasma proteins can enter the endoneurium and axons may explain how antibodies to different nervous tissue components enter the nerve and result in neuropathies. The ability of axons to take up proteins and transport them retrogradely to the neuronal perikarya may account for the neurological problems produced by tetanus (Carroll et al., 1978) and motor neuron disease (Yamamoto, 1987).

Tetanus toxin was shown by immunohistochemical techniques to be transported retrogradely in axons of sciatic nerve (Carroll et al., 1978). Autoradiography showed that [^{125}I] tetanus toxin passed from the somata and

dendrites of spinal motor neurons to the presynaptic terminals (Schwab and Thoenen, 1976).

Guinea pigs injected with isolated bovine motor neurons developed antibodies to their own motor neurons. The resulting paralytic disease was called experimental autoimmune motor neuron disease (EAMND) (Engelhardt et al., 1990). Another experimental autoimmune disease named experimental autoimmune grey matter disease (EAGMD) is produced by inoculating animals with homogenate of the spinal ventral horn (Engelhardt et al., 1990). Immunoglobulins from animals with EAGMD injected into mice produced marked limb weakness and muscular atrophy within one month. Immunohistochemistry revealed the presence of the EAGMD immunoglobulins in motor neurons of the spinal cord and brain stem in the inoculated mice (Appel et al., 1991). A likely explanation of these events is that the antibodies from the EAGMD animal cross-reacted with epitopes of motor neurons in the inoculated mice which elicited an immune response from the inoculated mice. In this way inoculated mice were made to produce antibodies of their own against their own motor neurons. It is probable that the antibodies from the EAGMD animal were taken up by axons in the periphery and transported retrogradely to the motor neurons' perikarya of the inoculated mice. In addition, the antibodies may have crossed the endoneurial capillaries and may have been taken up by the axolemma. Both explanations

are in accord with the observed presence of albumin in the endoneurium of peripheral nerves in this thesis.

Antibodies to gangliosides and to myelin proteins have been found in patients with the Guillain-Barre syndrome, which is a late complication of some viral infections. In this disease, there is demyelination of segments of peripheral nerves. The auto antibodies are abnormal plasma proteins that produce a neuropathy. The accessibility of peripheral nerves to plasma proteins found in this thesis may provide some explanation of the cause of this disorder.

5.3 Permeability of Ganglia to Blood-Borne Macromolecules

5.3.1 Ganglia

Even after short circulation times rhodamine-labelled albumin penetrated between neurons and their satellite cells in both sensory and sympathetic ganglia. Endogenous albumin was detected immunohistochemically in the same sites. HRP also entered the extracellular clefts between satellite cells and neurons even after circulating in the blood for only 5 minutes. Therefore the intercellular spaces of these ganglia are much more freely accessible to plasma proteins than is the endoneurium of a peripheral nerve. These results agree with those of most other investigators (Arvidson et al., 1973; Arvidson, 1979; Olsson et al., 1975; Jacobs et al., 1976; Hultstrom et al., 1983), although others have made conflicting observations. Ten Tusscher

et al. (1989) found HRP between satellite cells and neurons in sensory ganglia after either direct injection into the ganglion or intravenous injection, but they failed to find HRP in the equivalent location in sympathetic ganglia.

Azzi et al. (1990) immunized rats to HRP and killed them. They used HRP and the diaminobenzidine chromogen to detect the presence of the antibodies to HRP in the tissue sections of sensory ganglia from the immunized rats. They did not detect any antibodies to HRP between satellite cells and neurons in sensory ganglia. This may have beer due to the antibody molecules (IgG ~170 kd) being too large to enter the 20 nm extracellular space between neurons and satellite cells. The ability of ferritin (500 kd, 100 nm diameter) to enter this perineuronal space is questionable (Arvidson, 1979).

The observations of Depace (1982) differ from those of all other investigators. He did not detect HRP anywhere inside the sympathetic ganglia 5-15 minutes after intravenous injection in rats despite finding some in the connective tissue sheath of the ganglia and in some blood vessels in the ganglia. This anomalous finding may have been due to too low a dose of HRP (0.08-0.15 mg/g rather than 0.3-0.8 mg/g used by most other investigators). Depace's histochemical technique may have failed to detect small amounts of peroxidase activity around neurons and satellite cells in pieces of specimen (cut up with a razor blade) too large to be adequately penetrated by the

chromogen (diaminobenzidine) used to detect the enzymatic activity. Another possible explanation is that the glutealdehyde concentration used produced some inhibition of the peroxidase enzyme which would decrease its detection levels.

5.3.2 Plasma Proteins in Ganglionic Neurons

Some neuronal perikarya stained immunohistochemically for albumin in both sympathetic and sensory ganglia. This observation has also been made by Vidal (1983). Plasma albumin and globulins are also immunohistochemically demonstrable in the perikarya of motor neurons in the spinal cord and in the motor and parasympathetic nuclei of the medulla in the rat (Sparrow, 1981). As discussed in section 5.2.3 albumin could be taken up by the nerve endings and transported retrogradely to the perikarya, or it might be synthesized by the neurons themselves.

5.3.3 Enteric Nervous System

Acriflavine, ethidium, HRP and endogenous albumin could all be demonstrated within the myenteric and submucous ganglia. In contrast, rhodamine-labelled albumin, even after a week of daily injections, could never be detected in the enteric ganglia, which appeared as conspicuous dark areas surrounded by fluorescent rings. The basal laminae around these ganglia appeared to block the diffusion of the fluorescent albumin from the adjacent connective tissue layers and muscle layers of the intestine into the ganglia. All blood vessels have basal laminae and these do not

generally impede the diffusion of macromolecules that have passed through the endothelium. However, some basal laminae may be more obstructive. For example, the basal lamina of the choroidal epithelium impedes the diffusion of silver ions (given as silver nitrate ($AgNO_3$) in drinking water to rats), which are known to bind to albumin (Dempsey and Wislocki, 1955 a and b). Perhaps multiple silver-albumin complexes are associated with each other forming large particles which would be trapped in the net-like lamina of collagen IV and laminin molecules. The rhodamine-bovine albumin complex may also be associated with other rhodamine-albumin complexes forming a large particle which would be unable to penetrate the basal laminae around enteric ganglia. This hypothesis could be tested by injecting animals with ferritin (500 kd) or lanthanum which binds to fibrinogen (341 kd) and using electron microscopy to determine whether or not these tracers entered the enteric ganglia. If these latter two tracers do enter the enteric ganglia then the results would support the hypothesis that multiple complexes of rhodamine albumin or silver-albumin form large particles. However, if lanthanum and ferritin do not enter enteric ganglia, it may be that they are too large or they also form multiple complexes to form a large particle. Then fluorescent dextrans with molecular weights ranging from 40 kd to 500 kd could be used as intravenous tracers to determine the molecular weight at which penetration of enteric ganglia is prevented.

HRP penetrated myenteric ganglia and the fibre tracts of the plexus. These results agree with Jacobs (1977), but contradict Gershon and Bursztajn (1978), who failed to find any HRP in either the myenteric ganglia or the fibre tracts of the plexus. Gershan and Bursztajn (1978) also failed to find HRP in the muscularis externa and interpreted these data as being due to impermeable capillaries in the adjacent parts of the muscle layers. While other investigators have noted that the endothelium of the capillaries in the muscularis externa lacks fenestrae (continuous endothelium) (Landis and Pappenheimer, 1963; Clementi and Palade, 1969). Simionescu et al. (1972) pointed out that absence of fenestrae in capillary endothelial cells does not preclude capillary permeability, as muscle capillaries also lack fenestrae and yet are permeable (Bruns and Palade, 1968).

Cotrans and Karnovsky (1967) showed that HRP injected locally or intravenously produced vascular leakage of trypan-blue (bound to albumin) and colloidal carbon. They observed that the leaking of the blood vessels was augmented by histamine and inhibited by antagonists of histamine and serotonin. However, HRP produced no gross signs of vascular leakage (i.e. erythema and edema of the paws, snout and ears), and no vascular damage resembling that produced by histamine was visible on electron microscopic examination. These changes would have been: (1) partial removal of diaphragms in fenestrated blood vessels; (2) detachment of endothelium from the basement membrane; and (3) separation

of endothelial intercellular junctions (Clementi and Palade, 1969). Mast cells are the principal source of the histamine and serotonin in rodents that mediate inflammatory responses to allergens and "histamine releasing" compounds (see Selye, 1963). No mast cells have been observed in the muscularis externa, but they are plentiful in the mucosa and submucosa of the intestine in the rat (Maximow, 1906; Weill, 1920).

Gershon and Bursztajn (1978) found no histochemically detectable HRP in the myenteric ganglia 5 minutes after intravenous injection of an adequate dose of the enzyme. I have obtained the opposite result, as did Jacobs (1977). Their failure to detect HRP in enteric ganglia may have been due to inadequate penetration of the chromogen (diaminobenzidine) into the pieces of intestine (chopped up with a tissue chopper). When I chopped up the intestine with a tissue chopper and processed the tissue histochemically to detect HRP, I found a patchy distribution of the HRP reaction product in the submucosa and mucosa and very little in the muscle layers and enteric ganglia. Thereafter I always used a vibrating microtome for sectioning the intestine.

Another factor working against Gershon and Bursztajn (1978) was the short half-life of HRP (5 minutes) in the plasma of the rat (Rodmann et al., 1978). This would be another reason why they did not see any HRP reaction product after 45 minutes in the muscle layers and myenteric ganglia, and only a little in the submucous ganglia. I also found

that after 30 minutes very little HRP was detected in the ganglia and muscle layers of the intestine.

5.4 Significance of Blood-Nervous Tissue Barriers

The functional significance of protecting the nervous system from blood-borne macromolecules are: (1) to protect some cells from possible toxic effects of serum proteins; (2) to protect against autoimmunity; (3) to maintain a constant osmotic environment; and (4) to maintain selective transendothelial transport.

Oligodendrocytes grown in culture are destroyed by complement from serum added to the nutrient medium in the absence of any antibody-antigen complex (Wren and Noble, 1989). The susceptibility of oligodendrocytes is thought to be due to the lack of some cell surface molecules which are known to protect other cells from complement attack (Wren and Noble, 1989). If this is true in vivo, then the blood-brain barrier would prevent components of the complement system from reaching oligodendrocytes. Wren and Noble (1989) and Compston et al. (1991) think that the complement system may attack oligodendrocytes in multiple sclerosis. One argument against complement attack on oligodendrocytes occurring in vivo is in the case of transient hypertension or hyperosmotic shock in rats when serum proteins do enter the brain (Hansson et al., 1975) and oligodendrocytes are not killed.

Blood-nervous tissue barriers may be required to prevent the body from making antibodies to potentially antigenic proteins. Autoimmune diseases occur in both the peripheral and central nervous systems. Experimental allergic encephalomyelitis (EAE) is known to be caused by antibodies to myelin basic protein (MBP) and results in demyelination in the CNS (Kibler and Barnes, 1962; Laatsch et al., 1962). Multiple sclerosis resembles EAE in that there also are autoantibodies to MBP that can cause demyelination (Fredrikson et al., 1991; Compston et al., 1991). Autoantibodies to basic proteins of peripheral nerve myelin cause experimental allergic neuritis (EAN) (Abramsky et al., 1975). Guillian-Barre syndrome is a human demyelinating disease caused by autoantibodies to peripheral nerve myelin proteins (Koski, 1990; Quarles et al., 1990). An argument against the avoidance of possible autoimmunity as a major reason for having impermeable blood vessels in nervous tissue is that in my study, I demonstrated that albumin enters the endoneurium of normal peripheral nerve without any deleterious effects. Also the normal enteric nervous system has plasma proteins present in it and so do sensory and autonomic ganglia, and the circumventricular organs. Autoimmune diseases of ganglia are known, but are initiated by antigens of invading microorganisms, not by nervous tissue (Teixeira et al., 1980; Wood et al., 1982).

A likely reason for having the blood-brain barrier is to maintain a constant osmotic environment because large

changes in osmotic pressure result in cell death. Davson and coworkers (1961; 1963; see 1988) found that the concentrations of radioactive glucose and radioactive ions were not equal in the extracellular spaces of the brain to the concentrations of these solutes in the plasma. He concluded that the solutes encountered a permeability barrier which was the blood-brain barrier. Controlled permeability is necessary to maintain a relatively constant osmotic pressure in the brain. Homeostasis of osmotic pressure is also important in nerves, because either edema or loss of extracellular fluid could damage the axons and interfere with impulse transmission.

The brain has selective transport mechanisms located on the luminal side of capillary endothelium. These transport systems are: (1) carrier-mediated, which occurs with glucose; (2) receptor-mediated, which is the case with insulin; and (3) plasma protein-mediated, which occurs with steroid hormones (see Bradbury, 1985; see Pardridge, 1988). If proteins could diffuse out of brain capillaries so would smaller molecules and ions and their egress could not be controlled by carriers located in the endothelial cells.

5.4.1 Circumventricular Organs

Permeable capillaries are necessary for neurosecretory function which occurs in the median eminence and posterior lobe of the pituitary, and for chemoreceptors to detect different substances in the blood, as occurs in the area postrema (Broadwell et al., 1987; see Miselis et al., 1987).

CHAPTER 6 SUMMARY

Despite many years of investigation into blood nervous tissue barriers a number of questions remain unanswered.

(1) Do intravenously injected macromolecules penetrate the enteric nervous system? To answer this question rats were injected with rhodamine-labelled bovine albumin. This fluorescent tracer did not enter enteric ganglia, which was probably due to the formation of large complexes. Therefore immunohistochemical staining for endogenous albumin was performed on ileum. Positive staining was present around neurons in the enteric ganglia, as well as in the cytoplasm of some of the neurons. It is possible that neurons took up albumin at their terminals and transported it retrogradely. Electron microscopy revealed immunoreactivity for endogenous albumin in the enteric ganglia and fibre tracts, but not in the neuronal cytoplasm. The absence of reaction product inside neurons may have been due to poor penetration of reagents in the thick sections used for the immunohistochemical procedure prior to electron microscopy.

Rats were injected with HRP and electron microscopy was performed on ileum. HRP penetrated the enteric ganglia and fibre tracts. The basic fluorochromes acriflavine and ethidium stained the cells in the enteric ganglia after intravenous injection.

(2) Is the endoneurium of peripheral nerve permeable to macromolecules present for a long time in the circulation? To answer this rats were injected daily with rhodamine-labelled albumin for a week. The fluorescent albumin penetrated the endoneurium of peripheral nerve and was present around the myelinated nerve fibres and inside many axons, but not in myelin sheaths. The fluorescent tracer may have entered the axons by uptake from their peripheral endings.

Immunohistochemical staining for endogenous albumin was positive around the nerve fibres in the endoneurium of sciatic nerve and in many axons. The immunoreactivity in the axons may be explained by uptake of endogenous albumin from the periphery.

(3) Are the extracellular spaces between satellite cells and neurons in sympathetic and sensory ganglia penetrated by macromolecules from the blood? Rats were injected with rhodamine-labelled albumin and their sympathetic and sensory ganglia were examined by fluorescence microscopy. The fluorescent tracer was present throughout the ganglia.

Immunohistochemical staining for endogenous albumin in sensory and sympathetic ganglia was present throughout the ganglia.

Electron microscopy revealed that intravenously infused HRP entered and diffused throughout the sensory ganglia.

(4) Do the basic fluorochromes acriflavine and ethidium, which are sometimes used in histological studies of vascular permeability, behave as proteins would with regards to known blood-nervous tissue barriers? Solutions of ethidium (0.10%) and acriflavine (0.15%) were made up in 6.53% lyophilized rat serum and the binding of ethidium (67%) and acriflavine (79%) to serum proteins was determined. When these fluorochromes were injected into rats they behaved as proteins would with regards to known blood-nervous tissue barriers.

In conclusion: (a) the enteric nervous system is permeable to blood-borne macromolecules; (b) macromolecules circulating for a long time penetrate the endoneurium of peripheral nerves; (c) the extracellular spaces between satellite cells and neurons in sensory and sympathetic ganglia are permeable to macromolecules derived from the bloodstream; (d) protein bound acriflavine and ethidium behave as proteins with regards to known blood-nervous tissue barriers.

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