J. Physiol. (1966), **183**, *pp.* 501–512
With 3 plates
Printed in Great Britain

THE DISTRIBUTION OF CHOLINESTERASES IN THE CAT CAROTID BODY

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(*Received* 27 *September* 1965)

SUMMARY

1. The distribution of acetyl- and butyrylcholinesterase in the carotid body of the cat has been examined histochemically. Studies were made on normal carotid bodies and on carotid bodies from cats in which certain nerves had been cut some time previously. The nerves sectioned were the sinus nerve, the post-ganglionic sympathetic branch of the superior cervical ganglion or the preganglionic cervical sympathetic trunk.

2. It was confirmed that more butyrylcholinesterase than acetylcholinesterase is present. Both enzymes are found in three sites: (i) as strands, (ii) as plexuses, (iii) inside a few cells.

3. The distribution is unaffected by cutting the sinus nerve or preganglionic cervical sympathetic nerves. Disorganization and depletion of the cholinesterases in the strands and plexuses occurs when the post-ganglionic branch of the superior cervical ganglion is cut. The cholinesterase in cells is unaffected.

4. In carotid bodies in which vessels were filled with red blood cells or in which the vascular bed was injected with carmine-gelatine, it was seen that strands and plexuses are associated with blood vessels, and with blood vessels and cells respectively.

5. It is suggested that a cholinergic pathway controlling carotid body blood vessels runs in the post-ganglionic cervical sympathetic.

INTRODUCTION

There has for some years been a controversy about the way in which impulses are initiated in the carotid body chemoreceptors (see Biscoe & Taylor, 1963, for discussion of this problem). It was suggested by Schweitzer & Wright (1938) that acetylcholine (ACh) may be acting as a chemical mediator and this suggestion has been supported by some authors (Euler,

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Liljestrand & Zotterman, 1939; Liljestrand, 1954) and attacked by others (Douglas, 1952, 1954; Daly, 1954). The position has been reviewed by Heymans & Neil (1958) and Anichkov & Belen'kii (1963). Very recently Eyzaguirre, Koyano & Taylor (1965) have found an acetylcholine-like substance in the cat carotid body. The distribution of cholinesterases in the carotid body may be relevant to the interpretation of the function of any acetylcholine present, and has been studied by several workers. Hollinshead & Sawyer (1945) showed that the carotid body contains more pseudocholinesterase than true cholinesterase, the enzymes being defined biochemically, by their hydrolysis of benzoylcholine chloride and acetyl-β-methylcholine chloride, respectively. Koelle (1950) confirmed this observation histochemically and later examined the distribution of non-specific, or pseudocholinesterase, more precisely (Koelle, 1951). He found it in the peripheral cytoplasm of small groups of cells and in nerve fibres. Ross (1957) used the myristoylcholine technique (Gomori, 1948) and confirmed that cholinesterase was present in chemoreceptor cell groups.

We have combined histochemical studies with experiments on nerve section in the carotid region, in an attempt to localize accurately the sites of cholinesterase activity. Some of these findings have been demonstrated to the Physiological Society (Biscoe & Silver, 1965).

METHODS

Histochemistry

Carotid bodies were removed from fourteen cats anaesthetized with sodium pentobarbitone, 30–40 mg/kg intraperitoneally. The trachea was cannulated and the larynx and pharynx reflected in the mid line so that the carotid bodies could be approached from the medial side. In the first two experiments the carotid bodies were examined without attempting to wash out the blood vessels. In the next five, the carotid body on one side was perfused with isotonic sodium sulphate solution (16·76 g/l.) in order to remove the blood; the carotid body on the other side was not perfused. The distribution of cholinesterases on the two sides was comparable but red blood cells present in the non-perfused organ sometimes confused the interpretation. In all other experiments therefore, carotid bodies were perfused to eliminate blood corpuscles. The following vessels were tied off before the start of perfusion: the external carotid artery, the ascending pharyngeal artery, the internal carotid artery when present, and any branches from the sinus region to muscle. The occipital artery was tied beyond the branch to the carotid body. The carotid artery was cannulated and the carotid body slowly perfused until the fluid flowing from the carotid body vein was relatively blood-free. In one cat, one carotid body was perfused with carmine-gelatine (Moore, 1929) to fill the blood vessels; both carotid bodies were stained for cholinesterases as described below.

As soon as the perfusate was clear, the carotid bodies with the sinus nerves attached were excised, mounted in cat or guinea-pig liver and frozen on to a cryostat chuck with carbon dioxide gas. The chucks were stored in the cryostat at $-20°$ C until the tissues were sectioned. No difference in the distribution of cholinesterases was found between sections cut within 1–2 hr of removal from the animal, and those left for five days before sectioning. Usually blocks were sectioned within three days of removal. Adjacent serial sections,

routinely 6 μ thick, were cut in a Slee cryostat at between -20 and $-15°$ C; thicker sections (8 or 12 μ) were occasionally examined. The volume of carotid body tissue sectioned from each block was calculated to be about 90 % of the total volume; this calculation was based on the assumption that the body was a sphere with a total diameter of approximately 1 mm.

The sections were lifted off the knife with clean coverslips and when dry were exposed to formaldehyde vapour for 5 min. Groups of adjacent sections were first treated for 45 min in 0·2 M sodium acetate: acetic acid buffer of pH 6·5 with or without a cholinesterase inhibitor, and then incubated for 3 hr with the substrate according to the scheme shown in Table 1. When a cholinesterase inhibitor was used it was present in the same concentration during both pre-treatment and incubation. The incubation medium (substrate concentration 6·3 mM) was based on Lewis's (1961) modification of Koelle's (1950) solution (for preparation, see Krnjević & Silver, 1965). The substrates, acetylthiocholine (AcThCh) and butyrylthiocholine (BuThCh), were obtained, as the iodides, from L. Light and Co. Ltd; the cholinesterase inhibitor di-isopropylphosphorofluoridate (DFP) was kindly given by Dr B. C. Saunders. In an attempt to minimize diffusion artifacts, the optimal pH for each substrate solution, 5·3 for AcThCh and 5·5 for BuThCh, was determined in preliminary experiments, as was the duration of incubation.

TABLE 1. Schedule of treatment of sections

No. of sections	Substrate	Concentration of DFP (M)	Cholinesterase demonstrated
8	BuThCh	—	Butyrylcholinesterase (BuChE)
8	AcThCh	—	BuChE and acetylcholinesterase (AChE)
8	BuThCh	5×10^{-7}	Nil
16	AcThCh	5×10^{-7}	AChE
8	AcThCh	5×10^{-6}	Nil

At the end of the incubation, sections were well washed in water, developed for 2 min in a solution of sodium sulphide (see Krnjević & Silver, 1965), rinsed again in water and allowed to dry. They were cleared in xylol, taken through descending grades of alcohol to water, counterstained with aqueous haematoxylin and eosin and finally mounted in Gurr's damar-xylol.

Nerve section experiments

Aseptic operations were performed on six of the cats, anaesthetized with sodium pentobarbitone (30 mg/kg) given intraperitoneally. In all experiments in which a nerve was cut, at least 3 mm of the nerve was removed at operation. In two cats, the left sinus nerve was cut; in one of these the right sinus region was also exposed but the nerve left intact to determine any effect of scar tissue formation. In a third cat the right sinus nerve was cut; all of these cats were killed 14 days after operation. The post-ganglionic branch of the left superior cervical ganglion to the carotid body was cut in two cats one of which was killed 11 days, and the other 17 days later. In another cat the left preganglionic cervical sympathetic nerve was severed at the level of the superior thyroid artery; the cat was killed 14 days later.

Before the carotid bodies were removed for histochemical examination, the sinus region was exposed so that any activity in the intact sinus nerve or in its stump could be recorded. Recording was done with bipolar platinum wire electrodes leading to a high input impedance a.c. pre-amplifier (Tektronix type 122) and an oscilloscope with audio-monitoring of the signal. Chemoreceptor activity was tested by clamping the carotid artery or by closing the trachea or by allowing the animal to breathe 100 % oxygen. The carotid body from each side was then perfused and examined histochemically for cholinesterases.

The glossopharyngeal ganglia of Andersch & Ehrenritter (Cajal, 1909) from one control cat, and from one cat in which the sinus nerve had been cut, were stained for cholinesterases in the same way as the carotid bodies.

RESULTS

Comments on the technique

Diffusion of the cholinesterases or of their reaction products can seriously interfere with the precise localization of the enzyme in any tissue. We found that diffusion could occur in the carotid body under conditions in which the enzyme in the supporting liver tissue was very precisely localized, its distribution being identical with that shown in liver sections by Koelle (1951). The method we finally adopted was a compromise between methods which gave strong but diffuse staining and those which gave very weak but localized activity. Some diffusion, and a rather crystalline type of staining (cf. Pl. 1 *f*) still occurred in a few preparations despite the precautions.

Post-fixation of sections with formalin vapour limited the diffusion artifacts and only slightly reduced the enzyme activity. There was little difference between formalin-fixed sections incubated at once and those incubated 40 min after formalin treatment. The best results in terms of definition and minimal diffusion were obtained with formalin-treated sections incubated for 3 hr at pH 5·5 (BuThCh) or pH 5·3 (AcThCh). The use of a lower pH did not improve definition appreciably, and the staining was weak unless the incubation was considerably prolonged.

Distribution of cholinesterases in normal carotid bodies

Plate 1 *a* illustrates a carotid body stained for both BuChE and AChE; the separate distribution of the two enzymes is shown in Pl. 1 *b* and *c*. Comparison of *b* with *c* shows that the localization of the two enzymes is similar but BuChE activity is greater than AChE activity. This difference is not due to the higher pH used with BuThCh; it was still marked when the same pH was used for both substrates. Similar observations were reported by Hollinshead & Sawyer (1945), and by Koelle (1950, 1951).

Examination of sections under higher-power magnification confirms that AChE and BuChE are present in similar sites. The distribution of AChE is illustrated in Pl. 1 *d–f*. First, cholinesterase is found in complex plexuses which have an interwoven appearance (Pl. 1 *d*). These plexuses are outside the glomus cells and the apparent spaces within them are due to the counterstained nuclei showing through. Secondly, cholinesterase is localized in fine strands as shown in Pl. 1 *e*; these strands are also clearly visible in the low-power views of Pl. 1 *a* and *b*. They are distributed throughout the carotid body and are sometimes associated with the plexuses but often they are quite separate from them. Frequently strands are found running parallel to each other (Pl. 1 *a*, *b* and *e*). All sections examined were found to contain both AChE and BuChE in these two sites.

Thirdly, and more rarely, AChE and BuChE are found inside the cytoplasm of some cells (Pl. 1*f*). These cells are usually about 20–40 μ in diameter and are often found near the periphery of the tissue. In some carotid bodies there were no such cells in the portion examined, but in one carotid body we found twenty of them.

AChE and BuChE were also found in a few strands within the sinus nerve (Pl. 2*f*) and stained cells which resembled those just described were occasionally found in the sinus nerve (Pl. 2*d*).

Because we thought that the cholinesterase-containing strands which lay parallel to each other might be related to blood vessels, we looked for inadequately perfused vessels containing red blood cells. Several of these were found (Pl. 1*e*; Pl. 2*a*) and in all cases parallel strands of cholinesterase were present either adjacent to or within the blood vessel walls. In the field shown in Pl. 1*e*, there seems to be a vessel cut transversely which continues to one side in tangential section. Plate 2*b* gives another example of staining apparently associated with a blood vessel. In addition, the low-power views of Pl. 1*a* and *b* show numerous structures outlined with cholinesterase which appear to be blood vessels all converging towards the main artery.

To obtain further evidence on this point the blood vessels were filled with carmine-gelatine in one experiment. The extreme vascularity of the carotid body (see de Castro, 1951) was clearly shown. It was found that strands containing both AChE and BuChE were usually associated with blood vessels (Pl. 2*c*) and often completely encircled a vessel cut in transverse or tangential section. In addition, it was seen that most of the cholinesterase-containing plexuses were closely related to complexes of small blood vessels and cells, and not to cells alone as might appear from Pl. 1*d*. The contrast between carmine-gelatine and cholinesterase staining was very poor in black-and-white photomicrographs; hence this observation is not illustrated here.

The effects of cutting nerves associated with the carotid body

Carotid body chemoreceptor activity. No chemoreceptor activity was recorded from the distal cut end of the sinus nerves which had been severed fourteen days previously. The chemoreceptors did, however, respond to changes in arterial gas concentrations in the cat in which the nerve was exposed at operation but left intact. After section of the preganglionic cervical sympathetic or of the post-ganglionic branch to the carotid body, the chemoreceptors also responded to changes in arterial gas concentration.

Cholinesterases. There was no change in the distribution of cholinesterases within the carotid body fourteen days after section of the sinus

nerve. Plexuses and strands were present in their organized form and were as frequent and as well stained as on the control side. Stained, normal-looking cells were observed and no degenerating cells were seen in any of the sections. Cutting the preganglionic cervical sympathetic nerve also had no detectable effect on the cholinesterase staining. In contrast, when the post-ganglionic branch from the superior cervical ganglion to the carotid body was cut, there were marked changes in the cholinesterase staining; these are shown in Pl. 3.

When Pl. 3*a* is compared with *b*, it is obvious that there has been a considerable depletion of total cholinesterases in the carotid body. The effect produced on the BuChE can be assessed from the comparison of Pl. 3*c* with *d*. It is clear that much of the staining has been lost and the strands which gave an impression of running towards a focal point have disappeared. The same changes are seen under low-power magnification in sections stained for AChE; Pl. 3*e* and *f* illustrate high-power views of AChE staining in control and post-ganglionically denervated carotid bodies. The typical picture of strands apparently running next to a blood vessel is seen on the control side *e*, while *f* shows the disorganized appearance of the cut side. In the first cat, none of the sections from the denervated side contained any normal, unfragmented stained plexuses or strands although a few cholinesterase-containing cells were present. In the second cat, as many as thirteen cholinesterase-containing cells were found in one section from the operated side. This was the largest number counted in any one carotid body section in the whole series; one such cell is shown in Pl. 1*f*. In this second cat some of the cholinesterase staining persisted in an organized form especially in the vicinity of these cells but most of it was fragmented as in the first cat.

The glossopharyngeal nerve and ganglia of Andersch & Ehrenritter

AChE was found in strands within the IXth nerve and in some of the cells of these ganglia, as shown in Pl. 2*e* and *g*. In addition, many cells contained BuChE.

DISCUSSION

Our results confirm previous reports that the carotid body contains more BuChE than AChE. While it is unwise to interpret histochemical findings quantitatively it should be emphasized that the carotid body is not at all rich in AChE. In view of the relative paucity of this enzyme it is surprising that the carotid body contains as much as 20–30 μg/g acetylcholine (Eyzaguirre *et al.* 1965).

In contrast to the observations of Koelle (1951) in the cat, and those of Rogers (1965) in embryonic rats, we did not find that AChE was mainly

localized in chemoreceptor cells. In our preparations we saw a few stained cells in some cats but most of the enzyme was present in strands, and in plexuses round groups of cells and blood vessels. Because the counter-stained nuclei show through the plexuses, low-power views may give the impression that the cholinesterase is present in the perinuclear cytoplasm of glomus and other cells. Careful focusing shows that the cytoplasm of the glomus cells is unstained and that most of the cholinesterase is outside the cells. We do not think that this extracellular enzyme can be attributed to intracellular enzyme which has diffused out of the cells. Koelle (1950, cf. 1951) showed that uncontrolled diffusion produced nuclear rather than peripheral cytoplasmic staining in the carotid body. Evidence that the stained plexuses are not situated within the glomus cells is provided by the finding that they fragment after chronic section of the post-ganglionic branch of the superior cervical ganglion to the carotid body. If the cholinesterases were located inside cells they should be unaffected by the operation except in the unlikely event that enzyme-containing cells send processes along the nerve.

The few stained cells which we did find occurred too infrequently to be glomus cells. They may well be aberrant sympathetic ganglion cells which are known to occur outside the main sympathetic ganglia (Streckfuss, 1931; Kuntz, 1956; Kuntz, Hoffman & Napolitano, 1957; Pokrovskaya, 1958). They certainly resemble the ganglion cells illustrated by Koelle (1955), Koelle & Koelle (1959) and Cauna, Naik, Leaming & Alberti (1961). Moreover, Kohn (1900) described what he called ganglion cells in the carotid body and de Castro (1926, 1927) has identified groups of ganglion cells in and around the carotid body; in addition Rogers (1965) described cells in the rat embryo which migrate from the superior cervical ganglion into the carotid body and Pryse-Davies, Dawson & Westbury (1964) have described AChE in ganglion cells within the carotid body.

Since staining in these cells was unaffected by any of the operations it seems probable that their axons terminate within the carotid body. Section of preganglionic fibres to such cells is unlikely to alter their enzyme content (Koelle, 1955; Brown, 1958; Koelle & Koelle, 1959; Holmstedt, Lungren & Sjöqvist, 1963; Härkönen, 1964; Eränkö & Härkönen, 1965) but if their axons were travelling in any of the nerves that were cut it is probable that the staining would have been reduced (Brown, 1958; Fredricsson & Sjöqvist, 1962; Sjöqvist, 1963; Härkönen, 1964; Eränkö & Härkönen, 1965). Additional support for the view that axons from these cells end in the carotid body was provided by the experiment in which some well-organized cholinesterase-containing strands survived in the carotid body after section of the post-ganglionic branch. An exceptional number of stained cells were present in this carotid body and it seems

probable that the intact strands were the axons of these cells (see de Castro, 1926, 1951).

Cutting the sinus nerve had no effect on the distribution of cholinesterase within the carotid body, which suggests that the enzyme is not localized in chemoreceptor afferent pathways. Similarly, the cholinesterase is probably not related to the cervical sympathetic preganglionic trunk to any major extent since the enzyme remains normal after this nerve is cut.

The few cholinesterase-filled cells in the sinus nerve, and the strands of cholinesterase staining also found there, suggest that some branches of the sympathetic continue along this nerve, as already shown by Eyzaguirre & Lewin (1961).

Although these experiments help to define the sites of cholinesterase activity they cannot provide unequivocal evidence about the role of ACh in the carotid body. Some of the cholinesterase found in close association with blood vessels may be present in the muscle of the vessels themselves (cf. Pl. 2*b* and see Koelle, 1950; Crook, 1963) but we suggest that the stained strands and plexuses may represent a direct or indirect cholinergic supply to the vessels and may be concerned in the modification of blood flow within the carotid body. It appears that this supply is composed of post-ganglionic fibres from the superior cervical ganglion but we have not eliminated the possibility that preganglionic fibres from the medulla reach the carotid body through the vagus, superior cervical ganglion and its post-ganglionic trunk (see Shashirina, 1963). This does not seem likely, however, since the post-ganglionic branch of the superior cervical ganglion to the carotid body contains very few myelinated fibres (Gerard & Billingsley, 1923; Eyzaguirre & Uchizono, 1961). The plexuses which are described here may be the same as those shown to persist after section of the glossopharyngeal nerve (Meijling, 1938). If the presumed cholinergic pathway is concerned in the modification of blood flow and if it is the only such pathway, an increase in its activity should cause vasoconstriction (Daly, Lambertsen & Schweitzer, 1954), leading to an increase in chemoreceptor activity. Increased chemoreceptor activity follows stimulation of the cervical sympathetic (Floyd & Neil, 1952; Eyzaguirre & Lewin, 1961). The existence of a single cholinergic mechanism controlling blood flow might explain the observations that tetramethylammonium (Moe, Capo & Peralta, 1948) and hexamethonium (Douglas, 1952) block the response of the carotid body chemoreceptors to acetylcholine, nicotine and lobeline, but do not prevent the hyperpnoea produced by hypoxia, hypercapnia and asphyxia. On the other hand there may be an adrenergic vasoconstrictor innervation of vessels, running in the post-ganglionic branch, and vasomotor tone would then depend on the balance between adrenergic vasoconstriction and cholinergic vasodilatation.

This study does not elucidate the function of the phenolic amines found in the carotid body (Lever, Lewis & Boyd, 1959; Priimak, 1959; Muscholl, Rahn & Watzka, 1960; Rahn, 1961; Rodríguez-Pérez, 1961; Pryse-Davies, Dawson & Westbury, 1964). They may be concerned in blood vessel control through the acetylcholinesterase-containing pathway which we have described or they may be directly involved in nerve impulse initiation. The role of BuChE in the carotid body, as elsewhere, remains unresolved.

We are grateful to Mr George Marshall for his skilled assistance during the histochemical processing and to Mr A. L. Gallup and Mr A. Shaw for taking the photomicrographs.

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EXPLANATION OF PLATES

All sections counterstained with haematoxylin and eosin.

PLATE 1

a. Section of carotid body stained for AChE and BuChE. Scale bar, 250 μ.

b. Section of carotid body stained for BuChE. Scale bar, 250 μ.

c. Section of carotid body stained for AChE. Scale bar, 250 μ.

d. AChE-containing plexuses surrounding glomus cells in the carotid body. Scale bar, 25 μ.

e. AChE-containing strands associated with a blood vessel (b.v.) in the carotid body. Scale bar, 50 μ.

f. Ganglionic type of cell in carotid body stained for AChE. The post-ganglionic branch from the superior cervical ganglion to the carotid body had been cut seventeen days previously. Scale bar, 20 μ.

PLATE 2

a. AChE-containing strands associated with inadequately perfused blood vessels (b.v.) in the carotid body. Scale bar, 50 μ.

b. Cholinesterase in walls of blood vessels (b.v.) in a carotid body stained for AChE and BuChE. Scale bar, 50 μ.

c. AChE-containing strands running parallel to a carotid body blood vessel (b.v.) injected with carmine-gelatine. Scale bar, 50 μ.

d. AChE staining in cells in the sinus nerve. Scale bar, 20 μ.

e. AChE-containing fibres in the IXth nerve. Scale bar, 100 μ.

f. Cholinesterase-containing fibres in sinus nerve stained for AChE and BuChE. Scale bar, 100 μ.

g. AChE-containing cells in the ganglion of the IXth nerve. Scale bar, 100 μ.

PLATE 3

a. Section of a normal carotid body stained for AChE and BuChE. Scale bar, 250 μ.

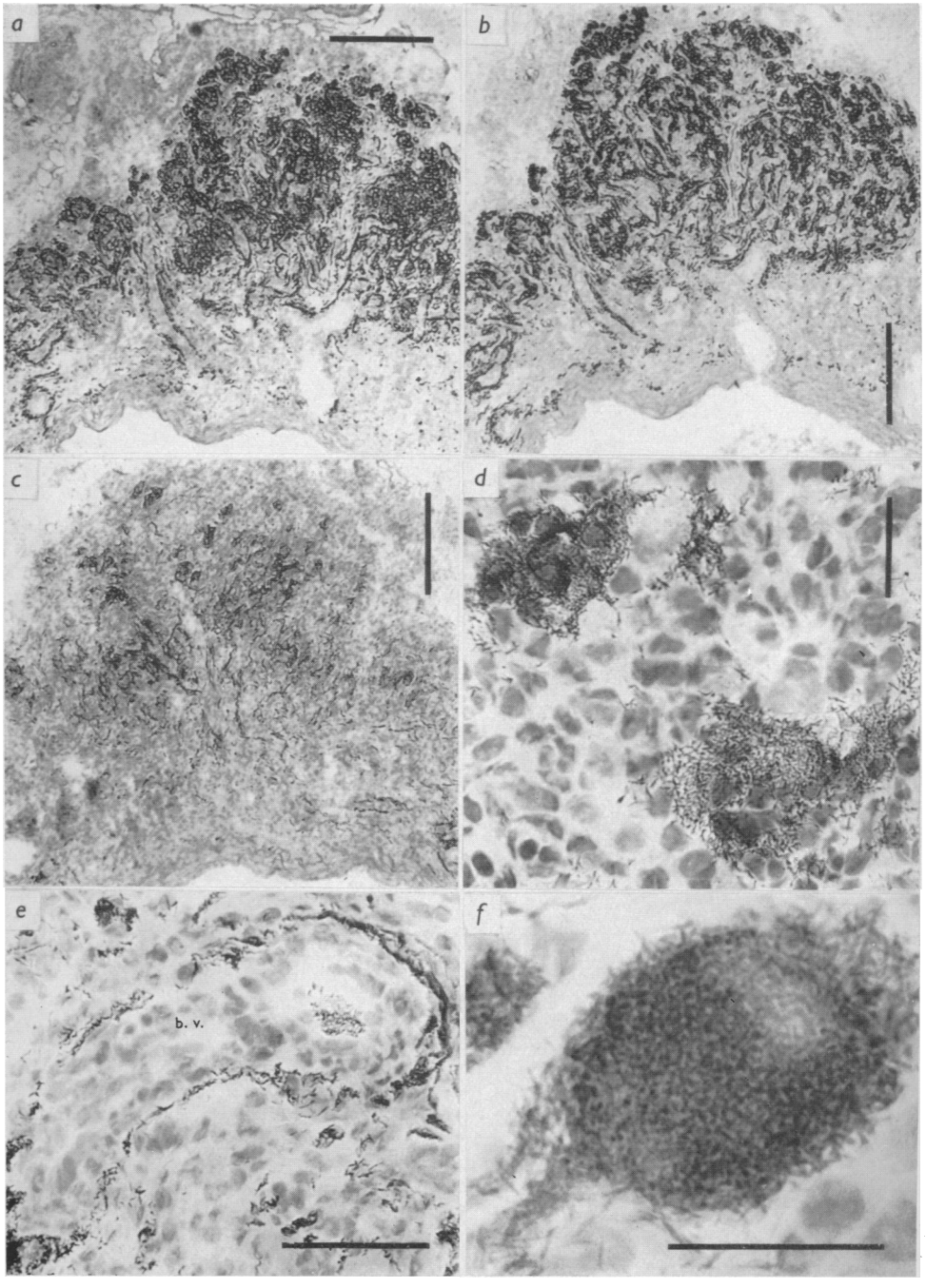
b. Section of the contralateral carotid body stained for AChE and BuChE. The post-ganglionic branch from the superior cervical ganglion to the carotid body had been cut eleven days previously. Scale bar, 250 μ.

c. Section of a normal carotid body stained for BuChE. Scale bar, 250 μ.

d. Section of the contralateral carotid body stained for BuChE, eleven days after the operation described under *b*. Scale bar, 250 μ.

e. AChE-containing strands associated with a blood vessel (b.v.) in a normal carotid body. Scale bar, 50 μ.

f. Fragments of AChE-containing elements in the contralateral carotid body, eleven days after the operation described under *b*. Scale bar, 50 μ.



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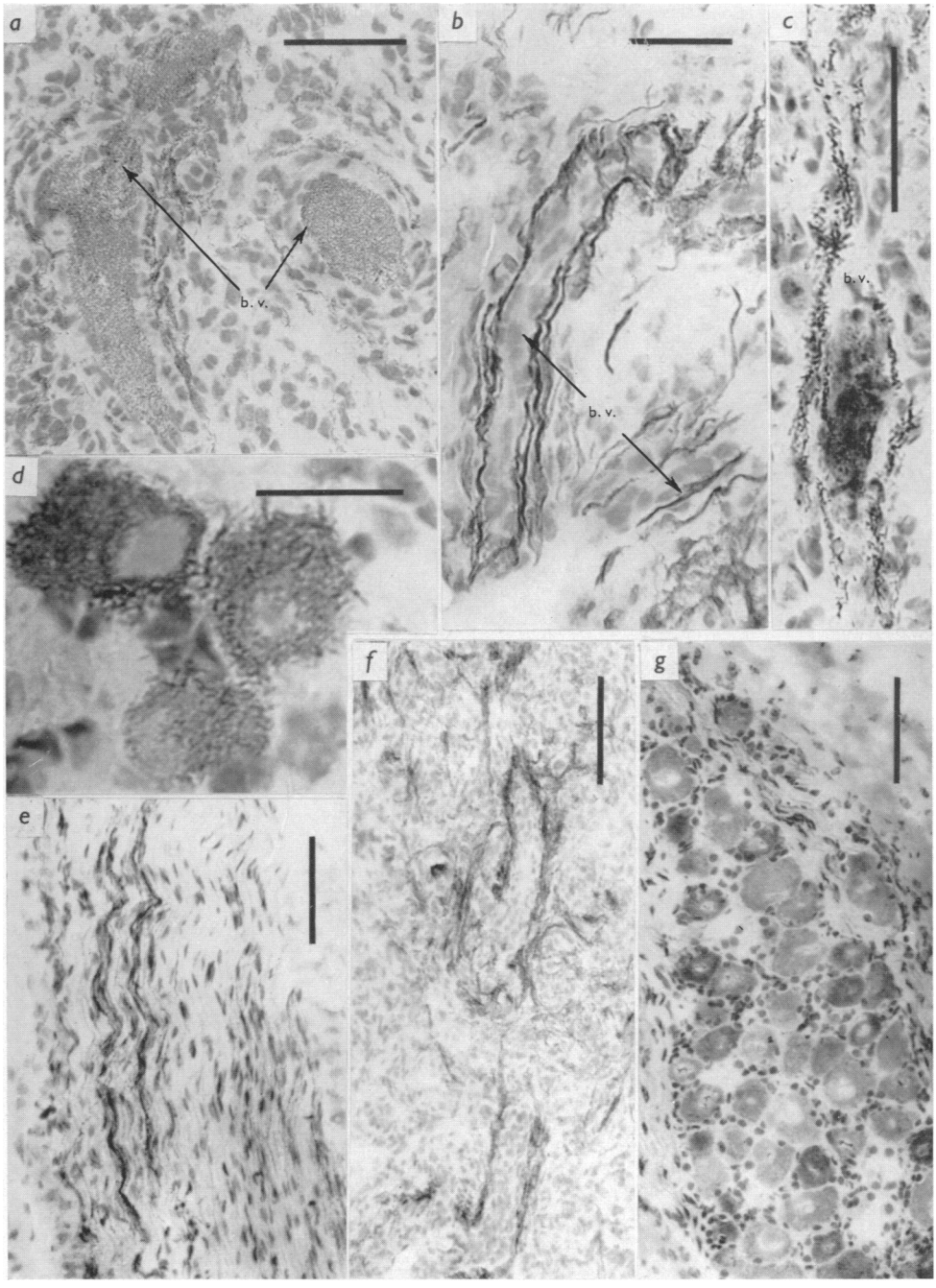

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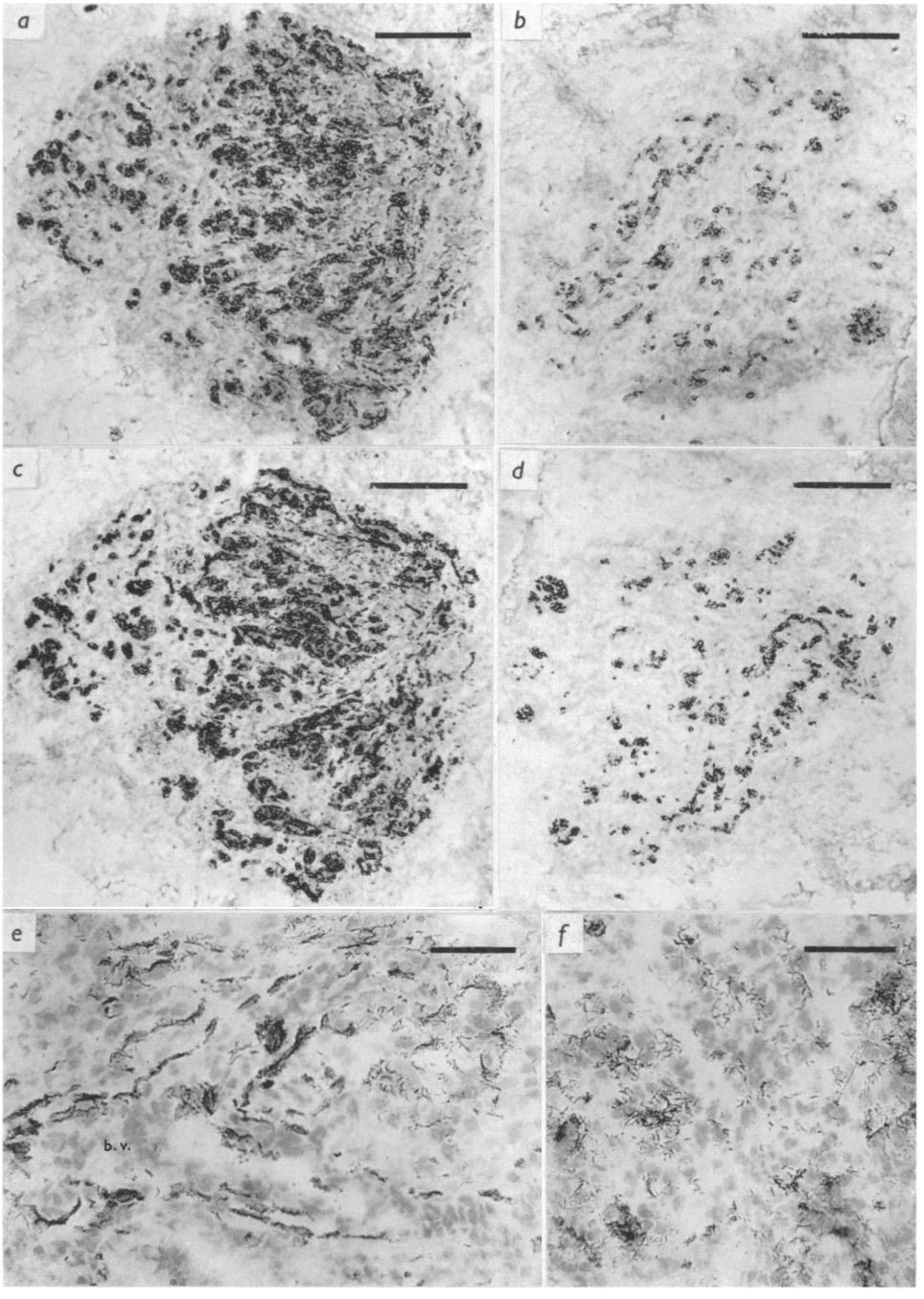

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