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Cholinesterase and the nerve supply to blood vessels in the rabbit's external ear

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In the course of observations on the heightened reactivity of denervated blood vessels, we recorded manometric estimations of the pseudo-cholinesterase activity of the central artery and main nerve trunk in the rabbit's ear (Armin, Grant, Thompson & Tickner, 1953). Enzyme activity in the artery seemed to be confined to the nerves since it was halved by sympathetic ganglionectomy and virtually abolished by the addition of sensory nerve section. The results also made it seem that the esterase is sited mainly, but not entirely, at the nerve endings. We therefore sought to display by histochemical means the site of enzyme activity and to determine how this is affected by interference with the nerves. This work led to the repetition and extension of the manometric estimations and to other observations on the vessels and nerves in the ear.

The results show that some of our previous conclusions require to be modified. Among other things it is found that cholinesterase activity is not concentrated towards the terminal parts of the nerves but is distributed throughout their course. It is present both in the axon and in the supporting structures and while it is much reduced it is not abolished by 'total' denervation. Some sympathetic fibres survive ganglionectomy and a few even total denervation. They are thought to arise from ganglia not removed at operation. Gross inhibition of the cholinesterase activity during life does not obviously interfere with the functions of the sympathetic or sensory nerves.

METHODS

As before, rabbits with half to three-quarter lop ears were used. In some the sympathetic nerve supply to one ear was interrupted by excising on that side both the superior cervical and stellate ganglia together with the intervening cord. In some, one ear was *partially* denervated by excising the superior cervical ganglion and portions of the posterior and great auricular nerves. In some, one ear was *totally* denervated by excising the same nerves as for partial denervation with portions of the auricular branch of the vagus, the auriculotemporal branch of the trigeminal nerve and the posterior facial branches. To prevent the rabbit reaching and eating the anaesthetic ear, this was shortened by folding the tip to the base and stitching it there. The ears were tested for completeness of sympathetic interruption by showing that the vessels no longer responded to changes of body temperature. To test for severance of sensory nerves, a faradic current was applied to the moistened skin (Grant, 1935). The increased reactivity provoked by ganglionectomy and denervation was displayed by noting the effect of the intravenous

infusion of adrenaline (0·1 μg./kg./min.) on the appearance and temperature of both the normal and denervated ears. The ears were depilated either by clipping or with a barium paste 24–48 h. beforehand. After killing the rabbits either by a blow on the head or by air embolism, the ears were cut off and freed from blood by brief perfusion with NaCl (0·9 g./100 ml. glass-distilled water), through a polythene catheter inserted into the central artery.

Manometric estimations of cholinesterase activity were made on fresh tissue as already described by Thompson & Tickner (1953) using the central artery, nerve trunk and perichondrium, though not all these were used in one experiment.

The central artery and the accompanying nerve trunks were dissected out as cleanly as possible after slitting the dorsal skin. To prepare perichondrium, a transverse strip $\frac{1}{2}$–$\frac{3}{4}$ in. wide was cut from about the middle of the ear. Under a dissecting microscope, the dorsal skin was removed and the exposed perichondrium separated from the underlying cartilage. In our previous experiments, the separated tissues were immersed in saline until submitted to the Warburg technique. In the present series, they were placed in a glass dish with a moist piece of filter-paper until dealt with, usually within an hour or two, but sometimes not until after refrigeration at 4° C. during the night. To avoid differences of technique which might affect cleanness of dissection and the moisture content of the tissues, the same person prepared the tissues from the normal as from the denervated ear. To obtain sufficient weight of artery and nerve for estimation, two or more rabbits were used for a single experiment. Acetyl-β-methylcholine chloride (0·03 M) and butyrylcholine perchlorate (0·03 M) were used as substrates for true and pseudocholinesterase respectively.

Cholinesterase activity was displayed histochemically by Gomori's (1952) modification of Koelle & Friedenwald's (1949) method, using perichondrium, skin and isolated artery and veins. Freed from blood, tissue was used either fresh or after perfusion of the vessels with 10 % formol saline injected into the central artery at a pressure of 100–200 mm. for 0·5–1 hr. while the venous outflow was obstructed by a rubber band around the base of the ear (Grant, 1930).

Transverse strips were cut from the ear and the dorsal skin dissected off. Short pieces of polythene tubes were inserted into the main artery and veins and these dissected out. The perichondrium was either left attached to, and so supported by, the underlying cartilage or was separated and extended by fixing with threads to a glass slide. Fresh tissue was then incubated; fixed tissue was first washed in running water for about $\frac{1}{2}$ hr.

Incubation at 37° C. was continued for 12–24 hr. with either acetylthiocholine or butyrylthiocholine iodide as substrate. To inhibit pseudocholinesterase, and so display the true, tissue was soaked for $\frac{1}{2}$ hr. at 37° C. in an aqueous solution (2×10^{-5} M) of mipafox (bis(mono*iso*propylamino) fluorophosphine oxide) before transferring to Gomori's solution plus the substrate. After incubation, tissue was washed in three changes of warm saturated sodium sulphate, immersed in dilute ammonium sulphide until the brown colour had fully developed and then thoroughly washed in distilled water. After dehydration, the perichondrium, if not already separated, was freed from the cartilage. The isolated vessels, still on the polythene tubes, were split longitudinally and spread flat. After clearing, preparations were mounted in Canada balsam or depex.

Other histological preparations. In some instances, the thiocholine-stained tissue was counterstained with methylene blue or Mayer's carmalum. To display coarsely myelinated fibres, Sudan black B (Stillwell, 1957) was applied to fixed tissue and used either alone or after thiocholine staining. Sudan black preparations were mounted in glycerine jelly. Tissue was also silver-impregnated by Richardson's (1960) method. For this, the vessels of the intact ear were perfused with glucose formalin under pressure for 0·5–1 hr., after which the tissues were separated as already described. Fixation was then continued for at least a week. To compare the histological pictures, neighbouring strips were in some instances stained by different methods. To ensure that normal and denervated tissues were treated alike, a piece of each ear was put through the staining process in the same dish simultaneously and mounted together.

In some rabbits, the superior cervical and the stellate ganglia were excised with their branches as complete as possible, pinned out under the dissecting microscope and fixed in formalin. The branches were teased out and then stained in methylene blue to display ganglion cells.

The effect of locally inhibiting cholinesterase activity during life was tested by perfusing in the anaesthetized animal a solution of DFP (di*iso*propyl fluorophosphonate) through the vessels of the ear while the blood circulation was arrested, and noting any change in the vascular reactions when the circulation was restored. A fine needle, connected by a polythene tube attached to it and to a motor-driven syringe, was inserted against the blood stream into the distal part of the central artery, and a wider needle with tube ending freely introduced into the posterior marginal vein. Ear circulation was then arrested either by a rubber band at the base or by a rubber-lined metal clamp applied across the blade of the ear. Perfusion of Tyrode's solution at a rate of 10–20 ml./hr. soon washed the blood from the ear, as shown by the clearing of the perfusate and the disappearance of blood colour from the transilluminated ear. Then perfusion of DFP 5×10^{-5} M in Tyrode's solution was substituted for periods of up to 1 hr. at the same rate and followed by Tyrode's solution alone for 5–10 min. The needles were removed and, whilst finger pressure was applied at their sites to minimize haematomata, the circulation was restored to the ear. After periods of up to 5 days, the rabbit was killed and the cholinesterase activity of the ear tissues displayed histochemically or estimated manometrically or both. As a control, the other ear was perfused in the same way and for the same times, but using Tyrode's solution only.

RESULTS

Histochemical preparations

Since our manometric estimations were made with fresh tissue, this was used initially for incubation with the thiocholine compounds. It was subsequently found that brief fixation with formalin under pressure, while not significantly reducing the staining, resulted in clearer histological pictures. This is due in part to reduction of diffusion haloes, and in part to wider separation of the tissue elements in the dilated vessels. Both formalin-fixed and fresh tissues mounted in balsam or depex deteriorate after a few months, the colour diffusing from the stained structures; those mounted in glycerine jelly last longer.

In the tissues used, the thiocholine method gives virtually a selective staining of the nerves. Other deeply stained elements are fat cells, but they are usually few, and sebaceous glands and hair roots, which are confined to the skin. Smooth muscle is faintly stained.

Perichondrial strips contain a cross-section of the vessels and nerves supplying the external ear spread out in almost a single layer. The main artery crosses the centre with the main nerve trunks beside it. A large vein runs along each margin. Between these main vessels are branching nerves, smaller arteries and veins and many arteriovenous anastomoses. Lymphatic vessels are also present. In the meshes of the network formed by the smaller arteries and veins lie numerous arterioles, capillaries and venules.

Because of the selective staining, whole mounts of perichondrium, when examined under the stereoscopic microscope with incident illumination, provide a readily followed map of the distribution of the nerves from the main bundles down to the fine network of the perivascular plexus. The blood vessels themselves are practically unstained, only the larger arteries are faintly brown, and counterstaining is required to display them and their relationship to the nerves. Nevertheless, as Pl. 1, fig. 1, shows, most of the arteries and veins can be located without counterstaining by the nerve strands accompanying them and the perivascular plexus arising from these strands. The network is denser over the arteries than over the veins and on the larger than on the smaller vessels (Pl. 1, fig. 1). It is arranged in two more or less distinct layers. The outer, of relatively coarse wide meshed strands, is superficial and gives rise to the inner one, deeper in the adventitia and consisting of finer and more closely meshed strands. In the larger arteries this inner layer is relatively thick so that only the most superficial strands come into focus in a photograph (Pl. 2, fig. 3). Details, however, are clearly seen in the thin-walled veins (Pl. 2, fig. 4) where the inner and outer layers are practically in one place. The plexus seems to be almost confined to the adventitia and enters the media little or not at all. In Pl. 2, figure 4, the sparse and faintly stained smooth muscle cells are visible beneath the nerve plexus. Moreover, in counterstained preparations of the arteries, while the isolated vessel is still on the polythene tube, the nerve net may be dissected off and yet leave the underlying media intact or almost so. Note that in Pl. 2, fig. 4, some of the fine nerve strands appear to end blindly.

Among the smaller vessels, the perivascular plexus is particularly dense over the arteriovenous anastomoses which, therefore, are conspicuous even under low-power magnification and more so in the fresh than in the fixed preparations (compare Pl. 3, figs. 5, 6). A characteristic feature of these anastomoses is the abrupt thinning out of the nerve plexus at the transition to vein. Only a few strands, hardly visible in the figures, continue on to the vein. As the arteries divide up to become arterioles, the perivascular plexus becomes sparser and disappears, but one or two strands continue alongside these small vessels. The capillaries do not appear to be supplied with thiocholine stained strands, but the histological picture in counterstained preparations is hardly clear enough for detailed analysis. A sparse plexus reappears on the small veins.

The nerves which leave the perichondrium to enter the skin (Pl. 3, fig. 6 shows the cut end of such a strand) here form another plexus which is shown in Pl. 4, fig. 7.

The large dark blobs indicate the sebaceous glands and the smaller ones the hair roots.

Thiocholine staining does not distinguish between axon, sheath and supporting connective tissue. In some places where the nerve is spread out, for example at cut ends or where it crosses a vessel, there is an indication of darker stained fibres lying in a lighter stained matrix. At the cut end of the main nerve strands staining in fresh tissue is deeper than along the course of the strand. In the fine perivascular network, counterstaining is required to display the nuclei, presumably of Schwann cells, that occupy the spindle shaped enlargements and points of branching of the strands. These strands form only one continous network and not two interlacing nets. To show this, an outline projection drawing was made in pencil of an area like that of Pl. 2, fig. 4. The area was then examined by transmitted light under higher magnification to decide whether meeting strands united or merely crossed. The network was followed from point to point and the outlines inked in where continuity seemed certain. In each instance, the whole network became inked in.

Cholinesterase activity in these preparations is mainly due to the pseudo- or non-specific type. Evidence for this is that the nerves are at least as well stained by butyrylthiocholine (hydrolysed by only the non-specific enzyme) as by acetylthiocholine (hydrolysed by both). Further, pretreatment with mipafox, which inhibits the pseudo-enzyme, prevents staining by butyryl and greatly reduces that by acetylthiocholine. From the staining reactions, there is no indication that one or other type of enzyme is concentrated in any part of the nerves.

Effects of denervation.

(*a*) *Sympathectomy.* Thiocholine preparations were made from a series of about forty rabbits killed at intervals ranging from 8 days to 20 months after ganglionectomy on one side. In all these animals, the vessels of the sympathectomized ear failed to constrict when body temperature was lowered. Most were given an intravenous infusion of adrenaline and in all its effect was greater in the sympathectomised than in the normal ear. It seems, therefore, that the ear vessels were deprived of their sympathetic nerve supply.

The stained preparations of the sympathectomized ear are a little lighter in colour to the naked eye than those of the normal. Microscopically, the main nerve bundles, the parallel nerve strands accompanying the vessels and the outer perivascular network are apparently unchanged, but the inner network is thinned out as is shown in Pl. 5, figs. 9 and 10, which illustrate comparable parts of the anterior marginal vein of the normal and sympathectomized ears of the same rabbit. As a result of this thinning, the arterio-venous anastomoses do not stand out as conspicuously as normally. Staining of fat cells, sebaceous glands and hair roots does not seem to be altered. The degree of thinning varies. Usually it is not great but sometimes almost all the inner network has disappeared; occasionally it is only a little less dense than normal.

(*b*) *Partial and total denervation.* In a series of twelve rabbits, one ear was partially denervated and the animals killed at intervals ranging from 21 to 110 days. This operation rendered the distal half of the ear insensitive to faradism. The ear

vessels failed to respond to changes of body temperature and were very sensitive to the constrictor effect of adrenaline.

Contrary to expectation from previous manometric estimations, this partial denervation did not abolish cholinesterase activity from the ear vessels, though it greatly reduced it. The stained preparations are lighter to the naked eye than those of a sympathectomized ear and much lighter than those of a normal ear. Microscopically, the main nerve trunks, the nerve strands accompanying the vessels and the outer perivascular network are still stained though more lightly than normally. Most of the inner plexus is wanting. As a result, in the perichondrial preparations it is difficult to follow the course of the arteries, and the arteriovenous anastomoses are hard to locate. Staining of fat cells, sebaceous glands and hair roots seems unaltered.

Since it seemed possible that the persisting cholinesterase activity might be due to the remaining intact nerves to the basal half of the ear, these were also cut in a series of fourteen rabbits, killed at intervals ranging from 21 to 84 days. This operation rendered both surfaces of the ear insensitive to faradism from tip to base; the ear vessels did not respond to changes in body temperature and were very sensitive to the constrictor effect of adrenaline. Cholinesterase staining in the perichondrium, central artery and skin remained as in the partially denervated ear. Pl. 1, figs. 1 and 2, show perichondrial preparations of the normal and totally denervated ears of the same rabbit. Pl. 6, fig. 11, shows the remnants of the perivascular plexus on a marginal vein of a totally denervated ear; the appearance of the normal ear was very like that of Pl. 5, fig. 9.

The assessment of the staining changes brought about by denervation is subjective. In an attempt to give them numerical values and thus allow comparison with the manometric estimations, Dr P. J. Warren, of the Department of Chemical Pathology, at our suggestion estimated the copper content of normal and denervated central arteries stained by the thiocholine method. He found (unpublished observations) that so much copper is invisibly adsorbed to the tissue as to outweigh by far that visible in the stained preparations.

Manometric estimations

The fact that considerable cholinesterase activity was detected histochemically in the arteries from even totally denervated ears, led us to repeat our earlier manometric estimations. As before arteries were used, and now also perichondrium and nerve trunk. Because of its large content of non-nervous active tissue, skin was not used. The results are summarized in Table 1. This shows that, as previously, activity in the artery is due mainly to the pseudo-enzyme and that the same holds good for the perichondrium and nerve. Non-specific activity is 4 to 5 times that of the true enzyme. Now, however, after sympathectomy about 70 % activity persists in the artery (Expts. 1 and 2) rather than the 50 % of earlier observations. About 70 % activity persists also in the perichondrium (Expts. 7 and 8). Further, partial and total denervation, instead of abolishing activity in the artery, reduce it to about 20–30 % (Expts. 4–6). Rather more, about 50 %, persists in perichondrium (Expts. 10 and 11).

It is to be noted that for the present estimations, the dissected tissues were kept only moist until minced. In two experiments (nos. 3 and 9), by error, the former

Table 1. *Cholinesterase activity of rabbit ear tissues*

(Results expressed as $\mu l.\ CO_2/g./hr.$)

Expt.	No. of rabbits	Tissue	Normal			Denervated			Type of denervation, duration in days	Remarks
			BuCh	MCh	BuCh/MCh	BuCh	MCh	BuCh/MCh		
1	6	Artery	1135	—	—	872 (77 %)*	—	—	Sympathectomy, 32–47	—
2	5	Artery	1772	—	—	1245 (70 %)	—	—	Sympathectomy, 180–293	—
3	5	Artery	908	196	4·6	1118 (?%)	301 (?%)	3·7	Sympathectomy, 194–362	Normal arteries immersed in saline until minced
4	4	Artery	790	—	—	192 (23 %)	—	—	Partial, 28	Tissues immersed in saline until minced
5	7	Artery	1158	385	3·0	308 (27 %)	17 (4·4 %)	18·1	Total, 28–33	—
6	5	Artery	945	170	5·5	323 (34 %)	88 (52 %)	3·7	Total, 68–79	—
7	6	Perichondrium	1173	161	7·2	795 (67 %)	83 (51 %)	9·5	Sympathectomy, 32–47	—
8	5	Perichondrium	954	191	5·0	758 (79 %)	103 (54 %)	7·3	Sympathectomy, 180–293	—
9	5	Perichondrium	583	107	4·5	588 (?%)	139 (?%)	4·2	Sympathectomy, 194–362	Normal perichondrium immersed in saline till minced
10	7	Perichondrium	356	82	4·3	161 (45 %)	26 (32 %)	6·2	Total, 28–33	—
11	5	Perichondrium	570	216	2·6	322 (56 %)	98 (45 %)	3·3	Total, 68–70	—
12	6	Nerve	3068	586	5·4	2895 (94 %)	446 (78 %)	6·5	Sympathectomy, 36–40	Fine mincing
13	4	Nerve	1750	—	—	1678 (96 %)	—	—	Sympathectomy, 60–97	Fine mincing
			2135	—	—	1565 (73 %)	—	—	—	Coarse mincing
										Coarse/Fine: Normal 1·2, Symp. 0·9
14	4	Nerve	2165	—	—	1140 (53 %)	—	—	Total, 28	Fine mincing
15	2	Nerve	2650	—	—	—	—	—	—	Fine mincing
			4120	—	—	—	—	—	—	Coarse mincing
										Coarse/Fine: Normal 1·6, Symp. —
16	3	Nerve	1745	—	—	—	—	—	—	Fine mincing
			4160	—	—	—	—	—	—	Coarse mincing
										Coarse/Fine: Normal 2·4, Symp. —

* Percentage normal.

technique was followed of immersing the dissected tissues in saline. Only the normal tissue was so immersed and this later was found to be 'juicier' than that from the sympathectomized ear. Activity in both normal artery and perichondrium lay below that of the opposite sympathectomized ear, a result not found in any other instance now or previously. Because immersion seemed to have considerably reduced the normal enzyme activity, a further experiment was made with normal and denervated arteries, both immersed in saline (Expt. 4) to see if this would yield the earlier result of disappearance of activity on the denervated side. On the contrary, activity proved to be not much less than that in the totally denervated and unimmersed arteries.

In our previous paper (Armin *et al.* 1953) we stated that the pseudo-cholinesterase activity of the main nerve trunk was in two experiments of the same order of that of the central artery. This statement requires modification. The values then determined were 1960 and 2320 μl. CO_2/g./hr., the mean being 2005. These are only a little less than those of the present series with fine mincing. In five experiments, as Table 1 shows, the values range from 1745 to 3068, with a mean of 2276. Both old and new values for the nerve trunks are nearly twice those for the central arteries (mean value old 1078, new 1252 (Expts. 1, 2, 5 and 6). The mean value for the perichondrium is 763, considerably less than that for the artery. This sequence of values is what might be expected from examination of the stained preparations. Manometrically, therefore, the results do not, as we earlier thought, suggest that the arteries contain some structure with an appreciably higher cholinesterase activity than that of the nerve.

One further point of technique requires mention. Since the histochemical preparations showed deeper staining about the cut ends of nerve bundles than along their course, it was thought that perhaps finely minced nerve would show greater activity than coarsely minced. In three experiments (nos. 13, 15 and 16) a portion of the nerves was finely minced, as was usual for all other experiments, and the other portion was cut into lengths of 1–2 mm. Activity is not greater in the finer but in the coarser mincing. In the sympathectomized nerve fine mincing gives little or no reduction from the normal activity (Expts. 12 and 13), a result in keeping with the unchanged appearance of the nerve histochemically. Total denervation reduces activity of the nerve to about 50 % (Expt. 14), the same level as in perichondrium.

Histological preparations

Since the thiocholine method does not differentiate the various elements in the nerve bundle, we cannot interpret the stained structures remaining after denervation without further evidence. To aid interpretation, we have made preparations stained with Sudan black, which picks out the myelinated fibres, also preparations impregnated with silver by Richardson's (1960) method. This yields dark staining of the axon with only a faint indication of the supporting structures and is particularly suitable for fine sympathetic fibres. We have found both methods reliable, which is important when normal and denervated structures are to be compared and the amount of material is limited.

Sudan black colours the myelin sheaths grey blue and the nodes of Ranvier are clearly visible. The blood vessels are faintly outlined in blue and the fat cells are

dark blue. As would be expected from their origin from the great and posterior auricular nerves, the nerve bundles crossing the perichondrial strips are largely composed of myelinated fibres. These seem to be distributed only to the skin. The nerve strands accompanying the blood vessels often contain one or more myelinated fibres but these continue to a cut end and do not seem to enter the perivascular plexus (Pl. 4, fig. 8). In preparations stained with thiocholine and then coloured with Sudan black, it can be seen that when a nerve branch passes from a bundle to supply a vessel, any myelinated fibres present do not enter the branch but continue in the bundle.

Sudan black reveals no difference between the normal and sympathectomized tissues. In the totally denervated ear, however, the myelin sheaths are replaced by the fatty globules associated with degeneration. In examining many preparations, we have failed to detect any intact myelinated fibres in the totally denervated ear.

Silver impregnation. In contrast to the thiocholine preparations, the nerves in the silver impregnations lie in a wealth of detail, chiefly nuclear. Cytoplasm is faintly stained and connective tissue fibres unstained. The various classes of blood vessels are readily recognized and it is easy in the perichondrial strips to pass from artery to vein either through arteriovenous anastomoses or through arterioles, capillaries and venules. The vessels being fixed in the dilated state, arterioles are about 20 μ diameter just before they break up into capillaries which are usually about 10 μ or less diameter. Varicose and valved lymphatic vessels are distinct.

The nerve branches, which appear as single strands in the thiocholine preparations, are seen to be bundles of parallel fibres of different diameters lying in a faintly stained sheath of connective tissue. In the fine bundles of the perivascular plexus a sheath is not obvious, though the Schwann cell nuclei are distinct. In the smallest nerve twigs of the inner perivascular plexus, consisting of apparently only one or two neurites, the Schwann cell nuclei are relatively far apart, for example 0·25 mm. Pl. 6, fig. 12, illustrates the fine neurites in a vein well.

The silver preparations show the same general pattern of nerve distribution as do the thiocholine ones. Strands accompany the blood vessels and give off branches forming the perivascular plexus with its inner and outer networks in the adventitia of the larger vessels. The visible neurites do not seem to penetrate between the muscle fibres but mainly to lie superficially to them. Some neurites are lost by fading to invisibility but most can be traced to join another strand. As in the thiocholine, so in the silver preparations, as the arteries branch to become arterioles, the perivascular plexus thins out and disappears. One or two fine bundles continue alongside the small vessels, branch occasionally and may leave the vessel for another, eventually rejoining another bundle. They do not seem to continue on to the capillaries, although a fine nerve bundle may lie alongside a capillary for a short distance. Fine bundles are found alongside most venules of 30 μ diameter and over. The arteriovenous anastomoses have a plexus obviously richer than that of their arteries of origin which thins out rather abruptly at the transition to the vein. The lymphatic vessels do not seem to be supplied with nerves.

In the *sympathectomized ear* the most obvious change is again the thinning out of

the inner perivascular network to a variable degree. The main nerve bundles and their branches are apparently unchanged.

In the *totally denervated ear* the darkly stained main nerve bundles are replaced by lightly stained strands with numerous nuclei. As these strands are traced peripherally and divide, they ultimately become lost in the general background staining. Only occasionally a quite sharply defined neurite can be seen in a small faintly stained branch. Some fine neurites with their Schwann cells can be found on the blood vessels.

These findings show that after sympathectomy a variable number of nerve fibres persist intact on the vessels and that even after total denervation a few survive. They suggest that the lightly stained structures persisting in the totally denervated thiocholine preparations consist mainly of supporting elements of the nerve bundle. Since no myelinated fibres are found in the Sudan black preparations, the persisting fibres probably belong to the sympathetic system.

Intermediate ganglia

The amount of inner perivascular plexus remaining after sympathectomy does not seem to be related to the time after operation at which the animals are killed. It does, however, seem to be related to the degree of increased reactivity of the vessels during life, as judged by the constrictor effect of adrenaline. In rabbits showing a greatly increased reactivity, little of the inner plexus is found after death. Those with a relatively slight increase usually show more of the plexus than is common. In one instance, slight reactivity following sympathectomy was considerably increased by sensory nerve section. It is known that the increased reactivity following preganglionic section is considerably less than that following postganglionic (Grant, 1935). This suggests the existence of ganglion cells distal to the main ganglia.

Examination of the ganglia and their branches, as described earlier, shows that in at least half the rabbits ganglion cells, isolated or in groups, are present at least as far as the cut ends of the excised branches, which may be 1 cm. long. In one instance, the superior cervical ganglion was replaced by two ganglia separated by a cord. In another, part of a ganglion was found at the end of a medial branch of the superior cervical ganglion where it had been torn through at operation. In still another, two small ganglia were found on the vertebral ramus of the stellate ganglion at its entrance into the vertebral canal. In sum, small ganglia or isolated cells were found in the branches of seven or fourteen superior cervical ganglia and in the vertebral ramus of five of ten stellate ganglia examined.

Local inhibition of cholinesterase activity

When the ear vessels have been perfused for 1 hr. and the rabbit is killed 1 hr. after circulation was restored to the ear, then incubation of the tissues with either acetyl or butyrylthiocholine fails to stain the nerves or other structures. By marking the margin of the clamp on the ear, it can be shown that the enzyme inhibition is closely limited to the perfused area. The line of transition in the stained tissue is remarkably sharp and is obvious to the naked eye. Immediately outside the perfused area the tissues are normally stained as are those of the opposite ear

perfused with Tyrode's solution alone. This inhibition gradually passes off. Staining is only faint in rabbits killed at 24 hr, much reduced at 48 hr. and still definitely but only slightly reduced at 5 days.

With a perfusion rate of about 20 ml./h. and a free outflow, only a little oedema develops in both ears during perfusion and increases for a time when circulation is restored. Removal of the clamp is followed by a great reactive hyperaemia which subsides over the next hour or two. The oedema passes off more gradually. When the reactive hyperaemia has sufficiently subsided, it can be seen that the vessels of the perfused ear react like those of the normal, though they do not constrict so readily and dilate more easily. Faradism applied to the moistened skin of the ears seems to provoke an equal response of the rabbit from both. By the following morning, about 20 hr. after perfusion, the rabbit has recovered from the anaesthetic and both ears seem normal except for the small haematomata at the needle punctures in artery and vein. Again faradism applied to the skin of the perfused area provokes a response from the animal apparently equal to that when applied to normal skin. When the rabbit is cool, the vessels of both ears are equally constricted. When the rabbit is warmed, the vessels of the DFP perfused ear relax first and for a time are distinctly more dilated than those of the normal ear. The latter, however, soon catch up and when the rabbit is warm, the vessels of both ears are equally dilated. When the warm rabbit is stimulated, the vessels of both ears contract, the normal a little before and to a greater degree than those affected by DFP. The difference between the ears gradually lessens and in the rabbit killed at 5 days was doubtfully detectable.

Two rabbits whose ears showed these changes at 20 hr., were killed at 22 hr. The central artery and two perichondrial strips were excised from each ear. The arteries and one pair of strips were stained by the thiocholine method, while cholinesterase activity was estimated manometrically in the other pair of strips. In both rabbits acetylthiocholine yielded only slight staining of the DFP-treated part of the artery and perichondrium but normal staining of the untreated parts and of the other ear perfused with Tyrode only. By manometric estimation activity in the perichondrium of the DFP-treated ear was reduced in one rabbit to 57 % (butyrylcholine) and in the other to 34 % (acetylcholine) of the normal.

It seems, therefore, that gross inhibition of cholinesterase during life, does not interfere obviously with sympathetic control of the blood vessels or with the function of the sensory nerves to the skin.

DISCUSSION

We can find but little previous reference to histochemical observations on cholinesterase activity in rabbit nerves. According to Koelle (1955) acetylcholinesterase is present in all types of rabbit nerves, but with lower activity in the adrenergic than in the cholinergic. Apparently, only Auerbach's plexus contains a high concentration of non-specific activity. Coupland & Holmes (1957) state that all nerve fibres examined so far in the rabbit and other animals have been found to contain both true and pseudo-cholinesterase; in most post-natal tissue, true and pseudo-activity are present in more or less equal amounts. Our histochemical agree

with our manometric findings in showing that the cholinesterase is mainly of the pseudo-type. Histochemistry shows that this activity is distributed throughout the whole course of the nerves, from entering trunk to the plexuses around the vessels and in the skin.

According to Coupland & Holmes (1957) the thiocholine method gives a complete picture of the nervous elements in a single preparation and is particularly suitable for demonstrating the nerve fibres around blood vessels. We find, however, that in our preparations, though the nerves are clearly shown, the blood vessels themselves are hardly visible and require counterstaining to define their walls. Again, although the picture of the nerves is apparently complete, it is so only in outline but not in detail. The stain resulting from cholinesterase activity is diffused throughout the nerve bundle and therefore axon, sheath and connecting tissue are not differentiated. To accomplish this other methods are required. It follows that the elements in the nerve responsible for the staining are not demonstrated. Other workers (Koelle, 1955; Sawyer, 1946; Cavanagh, Thompson & Webster, 1954) find that the two types of cholinesterase are variously distributed between axon, sheath and connecting tissue in other animals.

The thiocholine preparations aid in the interpretation of the manometric results. Since smooth muscle is apparently the only structure other than the nerves showing activity, and this but slight, manometric estimation of the excised central artery gives information about the enzyme activity of the sympathetic perivascular plexus. Manometric estimation of the nerve trunks represents the activity mainly of the sensory nerves and their supporting structures. The perichondrium contains both vascular nerves and portions of the sensory nerves on their way to the skin and in addition a variable, though usually small, amount of adipose tissue. Skin, however, contains so large a proportion of non-nervous tissue as to render manometry of little value in estimating nervous cholinesterase.

A prominent feature of the perichondrial strips is the large number of arteriovenous anastomoses with their rich nerve supply. Other workers have noted for the dog (Brown, 1937) and man (Masson, 1937) as well as for the rabbit (Grant, 1930) that this supply is richer than that of the artery origin and thins out abruptly at the transition to the vein. According to Hurley & Mescon (1956) a striking concentration of specific cholinesterase is found at the arteriovenous anastomoses of the human finger, while both specific and non-specific enzymes are absent from the other cutaneous vessels. But Beckett, Bourne & Montagna (1956) find that the cholinesterase activity at the anastomoses is non-specific rather than specific. This work prompted us to use perichondrium not only for staining but also for manometric estimation. If the cholinesterase activity of these numerous vessels were entirely due to the true enzyme, we thought we might find a greater proportion of this here than in the central artery. But neither manometrically nor histochemically do we find this to be so. For the same reason and also because Staubesand & Luckner (1950) report the presence of an acetylcholine-like substance in the coccygeal body (largely composed of arteriovenous anastomoses) we estimated manometrically the cholinesterase activity in a group of six coccygeal bodies excised from the cadaver by Dr G. A. K. Missen of the Department of Pathology of this School. Again, the activity was preponderatingly of the pseudo-type (unpublished observations).

The depth of staining of the histochemical preparations varies but little from animal to animal, provided times of incubation, etc., are kept constant. A feature of the manometric estimates, however, is the wide range of activity in different experiments. Thus, as Table 1 shows, in four experiments on the central artery and four on the nerve, all carried out without immersion and all with fine mincing, the highest value is not far short of twice the lowest (artery 945–1772, nerve 1745–3068 μl. CO_2/g./hr.), while in four experiments on the perichondrium, it is over three times as great (356–1175). Several factors are probably responsible for this range. One is the amount of inactive tissue, another is the variable fat content. These factors affect specially the perichondrium, but may also affect the central artery and nerve trunk, depending on how cleanly they are dissected out. On the other hand, too clean dissection may remove a part of the perivascular plexus around the artery. A third factor is the water content of the tissue. This may be added to by perfusing too long or under too high pressure when washing blood from the vessels, by moistening the tissue during dissection and by immersing isolated tissue until mincing. A fourth factor, to which Bonting & Rosenthal (1960) have drawn attention, is fineness of subdivision of the tissues during estimation. We have found that coarse mincing of the nerve yields an activity distinctly higher than that of fine mincing. The reason for this is unknown. Pavlin & Thompson (1961), however, have compared the estimated cholinesterase activity in sliced, minced and homogenized rat brain and spinal cord and find that the type of preparation may have a profound effect on the observed level of activity. They conclude that these variations are due partly to differences in the degree of dispersion of the tissue, particularly of the lipid-rich myelin, which may render enzyme centres inaccessible to the water-soluble substrates.

As has been seen, the thiocholine preparations led us to question our earlier manometric findings on the effects of denervation, which we have been unable entirely to repeat. But both histochemistry and manometry now agree in showing some reduction of cholinesterase activity after sympathectomy and a greater reduction but not disappearance after so called total denervation. The nervous structures in which cholinesterase staining persists after denervation are shown by other methods of staining to be in part the supporting elements of the nerve bundle and in part surviving fibres, probably sympathetic in origin. These fibres are thought to arise from ganglion cells lying distally to the main cervical ganglia. We exclude of course the ganglion cells shown by Douglas, Lywood & Straub (1960) to be constantly present in the cervical cord between the main ganglia. Little seems to be known about the so-called 'intermediate' ganglia in the rabbit. Wrete (1941), from the examination of embryos, does not find them ahead of the second thoracic nerve. Boyd (1957), however, apparently also from embryonic material, suggests that in the rabbit as in man, an intermediate ganglion is present on the ramus connecting the superior cervical ganglion to the second cervical nerve. We have found such ganglia on the branches of both the main ganglia supplying the ear vessels in half the rabbits examined and it is probable that more refined methods would reveal them in a much higher proportion. We do not know how far distally these ganglia extend. The effects of denervation suggest that most lie between the main sympathetic ganglia and the point of section of the sensory nerves at the root of the ear, but a few

must lie distal to that. It is to be remembered, however, that we have only indirect evidence that the persisting fibres arise from such ganglia. We know that the ganglia exist and may not be removed at operation. But, once the superior cervical and stellate ganglia are excised, we have subsequently no obvious means, short of serially sectioning a large bulk of tissue, for tracing the branches that arose from these ganglia to see if intermediate ganglia are present or to trace the origin of persisting fibres to these ganglia. The only other reference we have found to the effects of sympathectomy on the nerve supply to the ear vessels of the rabbit is that of Woollard, Weddell & Harpman (1940), and this deals with skin vessels. They find that after a unilateral sympathetic neurectomy, the only fibres and nerve endings not demonstrable by *intra vitam* methylene blue were those supplying limited portions of the capillaries and larger lymphatic vessels. They give no details of the neurectomy but remark that it was followed by a persistent Horner's syndrome. We have not studied the skin vessels but in the perichondrium we have been unable to demonstrate a nerve supply to the capillaries or lymphatic vessels.

We had hoped that histological examination after sympathetic ganglionectomy might reveal the extent of sensory nerve supply to the ear vessels. But it is clear that, as Boyd (1957) remarks, the histological identification of persisting fibres in a tissue after ganglionectomy does not permit the conclusion that the persisting elements cannot be sympathetic in nature. Moreover, although by the use of Sudan black, myelinated fibres are easily recognized and for a part of their course may accompany blood vessels, they seem to be distributed only in the skin. Unless some sensory fibres are too finely myelinated to be easily visible or unless they lose their sheath a considerable distance before they reach the vessels, we can only conclude that the vessels of the perichondrium lack a sensory supply. The whole of the perivascular plexus, therefore, is probably sympathetic in origin. According to our observations this plexus lies superficially to the smooth muscle of the vessel wall but the manner of the terminal distribution of this plexus, like that of the nerve supply to capillaries, is a matter of controversy requiring other methods for its resolution (see Mitchell, 1956; Stöhr, 1957; Richardson, 1960).

The observations on the inhibition of activity during life do not give any clear indication of what the function or functions of cholinesterase may be. If one of the functions is hydrolysis of acetylcholine, then the tendency of the DFP-treated vessels to dilate more readily than normally could be explained by the undue accumulation of this substance, for the normal presence of which in the vessel wall there is some evidence (Armin *et al.* 1953).

SUMMARY

1. Cholinesterase activity has been demonstrated histochemically (thiocholine method) and estimated manometrically in the rabbit's external ear, normal and after deprival of sympathetic and sensory nerves.

2. Histochemistry shows that, except in the skin, cholinesterase activity is sited mainly in the nerves.

3. The thiocholine method provides an outline picture of the distribution of the peripheral nerves. Demonstration of the elements of the nerve bundle requires other methods.

4. Both histochemistry and manometry show that cholinesterase activity is mainly pseudo in type, is moderately reduced by sympathetic ganglionectomy and greatly reduced, though not abolished, by total denervation.

5. The persisting activity is due to the survival of fibres, probably sympathetic in origin and arising from intermediate ganglia.

6. Gross inhibition of cholinesterase activity during life does not obviously interfere with the functions of the sympathetic or sensory nerves.

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. Perichondrium, normal ear; formalin fixed under pressure; incubated 24 hr. acetylthiocholine. A darkly stained stout nerve bundle runs diagonally; the perivascular plexus outlines a vein (lying horizontally) and an artery (running vertically) which branches. Several arteriovenous anastomoses arise from the branches and enter the vein.

Fig. 2. Perichondrium, totally denervated ear (21 days after operation); same rabbit as in Pl. 1, fig. 1. Shows several lightly stained stout nerve bundles and the remnants of the perivascular plexus around a vein (lying vertically at left) and a branching artery (parallel to the nerve bundles). Several anastomoses can hardly be distinguished.

PLATE 2

Fig. 3. Isolated normal central artery, viewed from adventitial aspect; fresh tissue; incubated 20 hr. acetylthiocholine. Shows the coarse strands of the outer perivascular plexus and numerous fine strands of the inner plexus of which only a few are in focus.

Fig. 4. Isolated normal marginal vein viewed from adventitial aspect; fresh tissue; incubated 24 hr. butyrylthiocholine. Shows the perivascular plexus and the faintly stained underlying smooth muscle cells.

PLATE 3

Fig. 5. Perichondrium normal ear; fresh tissue; incubated 18 hr. acetylthiocholine. A sausage-shaped, darkly stained arteriovenous anastomosis arises from an artery on left and appears cut short on the right where it becomes vein. Fat cells are seen indistinctly above at left and below at right.

Fig. 6. Perichondrium normal ear; formalin fixed under pressure; incubated 20 hr. acetylthiocholine. Shows nerve plexus around an arteriovenous anastomosis arising on left from an artery and passing into a vein on right. A few fat cells are seen on the right and also the cut end of a nerve bundle entering the skin.

PLATE 4

Fig. 7. Normal skin viewed from subcutaneous surface; fresh tissue; incubated 18 hours acetylthiocholine. Shows the dermal plexus, sebaceous glands and hair roots.

Fig. 8. Perichondrium normal ear; formalin fixed under pressure; stained Sudan black B. A small vessel, lying transversely about the middle of the figure, is crossed by a nerve bundle containing two myelinated fibres, one finer than the other. Neither gives off a branch to the artery. Several nodes of Ranvier are visible.

PLATE 5

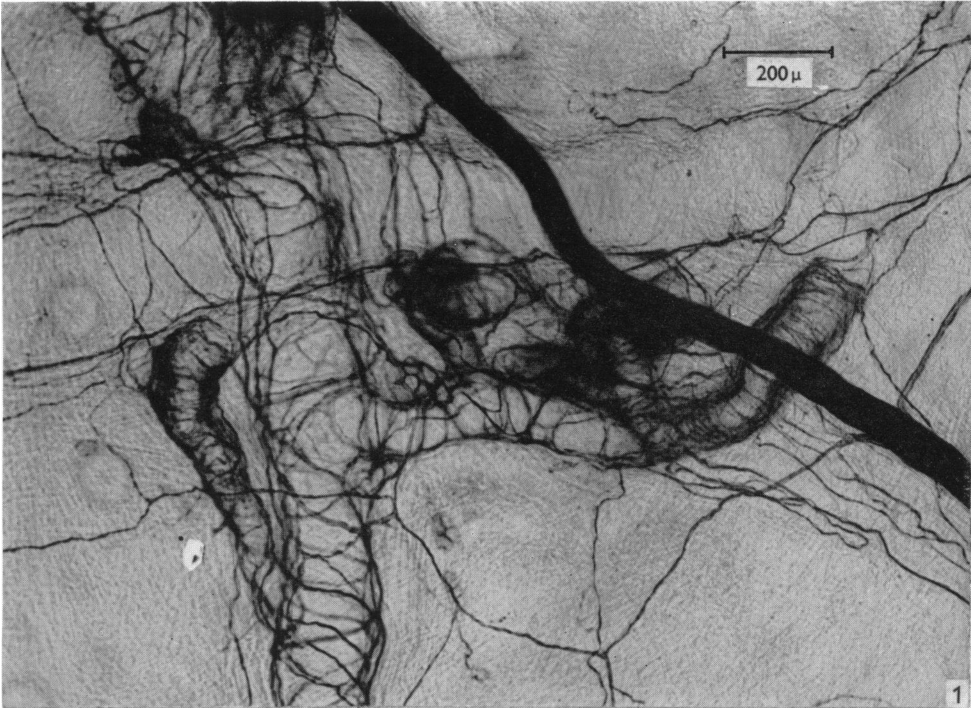
Fig. 9. Isolated marginal vein normal ear, viewed from adventitial aspect; formalin fixed under pressure; incubated 19 hr. acetylthiocholine. Shows the coarse and fine strands of the perivascular plexus.

Fig. 10. Corresponding portion of the vein from the sympathectomized ear (20 months after operation) of the same rabbit as in Pl. 5, fig. 9. Shows the considerable reduction of the fine strands of the perivascular plexus.

PLATE 6

Fig. 11. Corresponding portion of the vein from the totally denervated ear (20 days after operation) of another rabbit treated in the same way. Shows the lighter staining of the few persisting coarse strands and faint remnants of some of the fine strands. The plexus in the normal vein was like that shown in Pl. 5, fig. 9.

Fig. 12. Neighbouring portion of the same normal vein as that of Pl. 5, fig. 9, but silver-impregnated by Richardson's method and more highly magnified. The fine strands of the inner perivascular plexus are seen in places to be composed of bundles of fine neurites. A Schwann cell nucleus, with fine neurites surrounding it, occupies the centre of the figure.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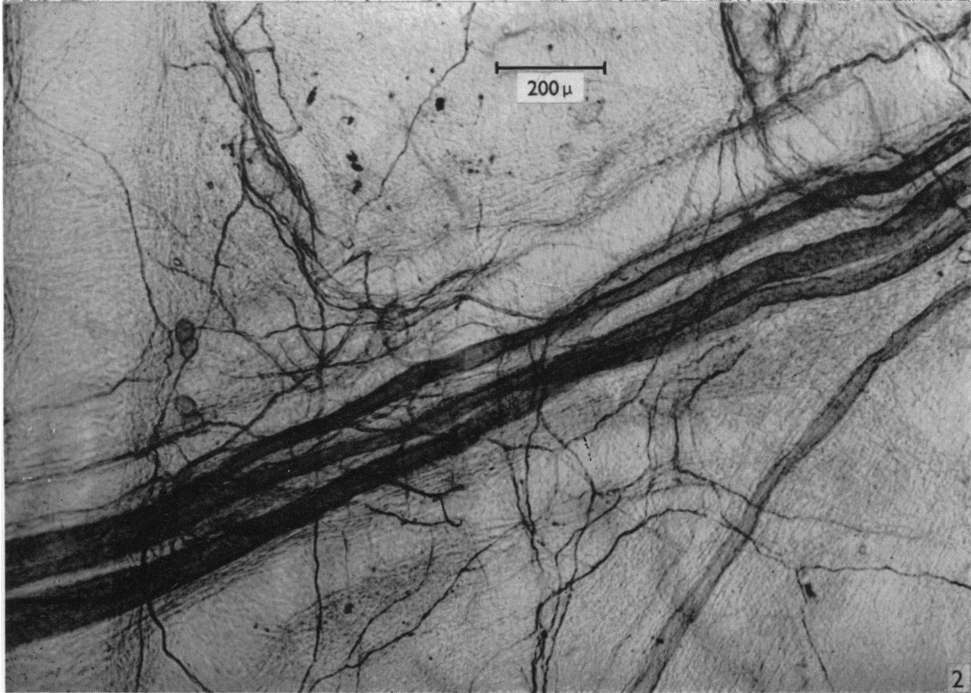
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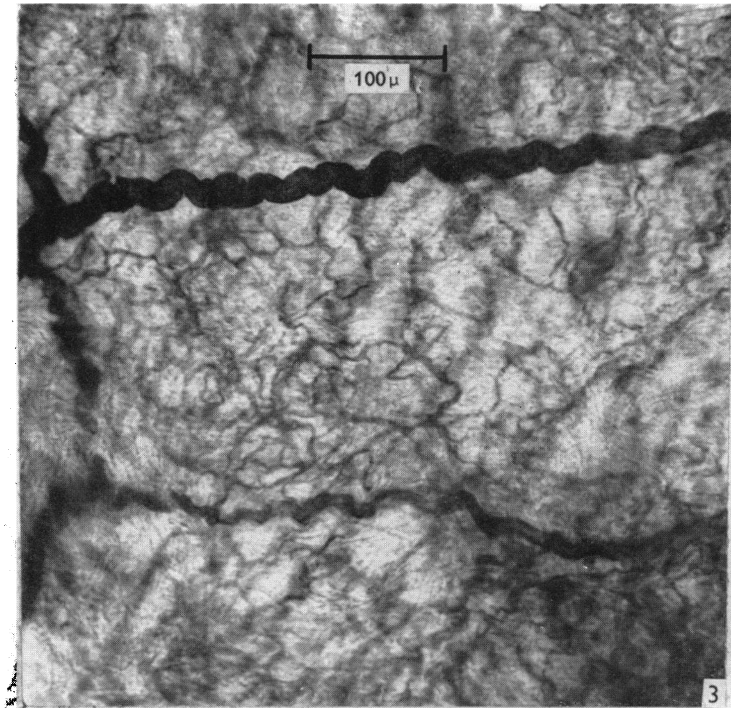

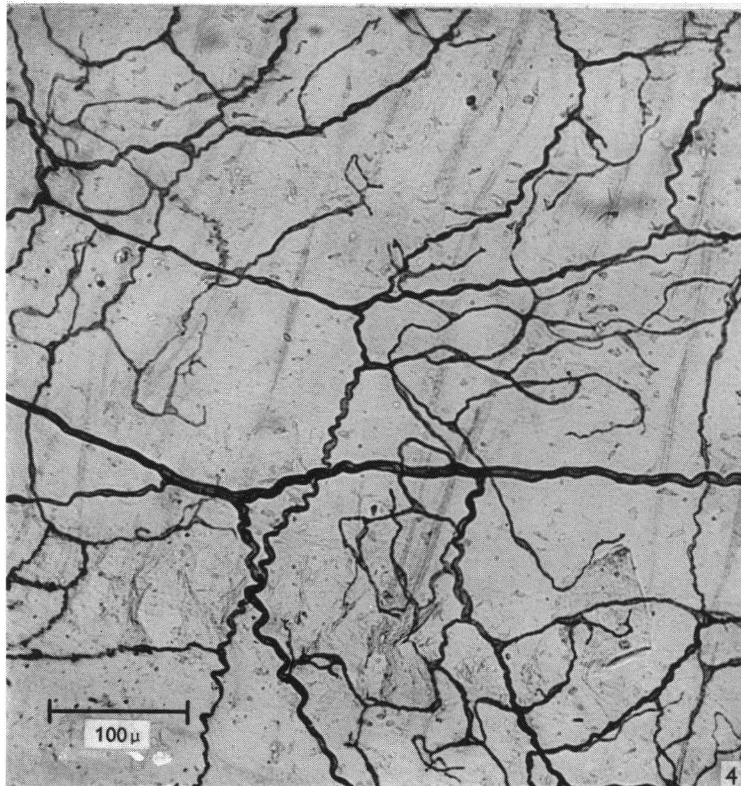

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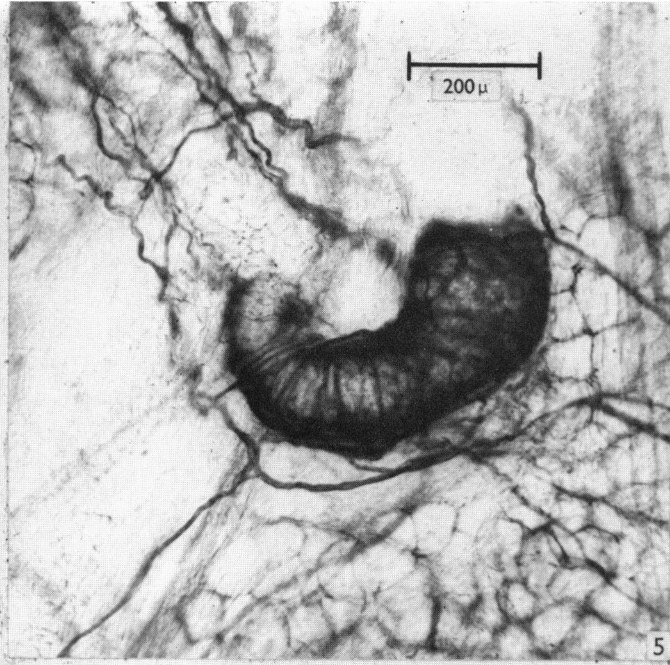

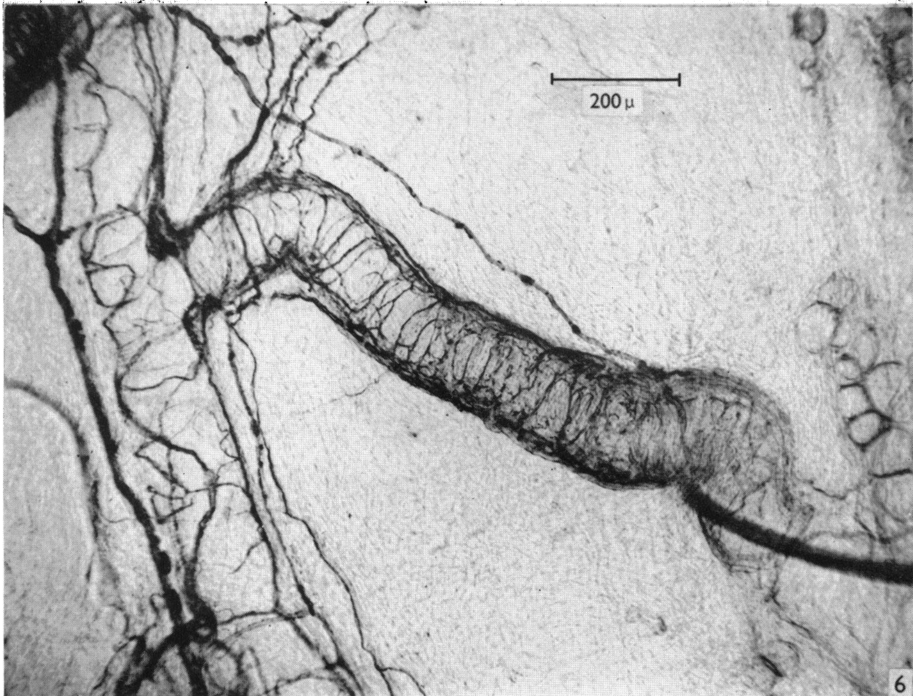

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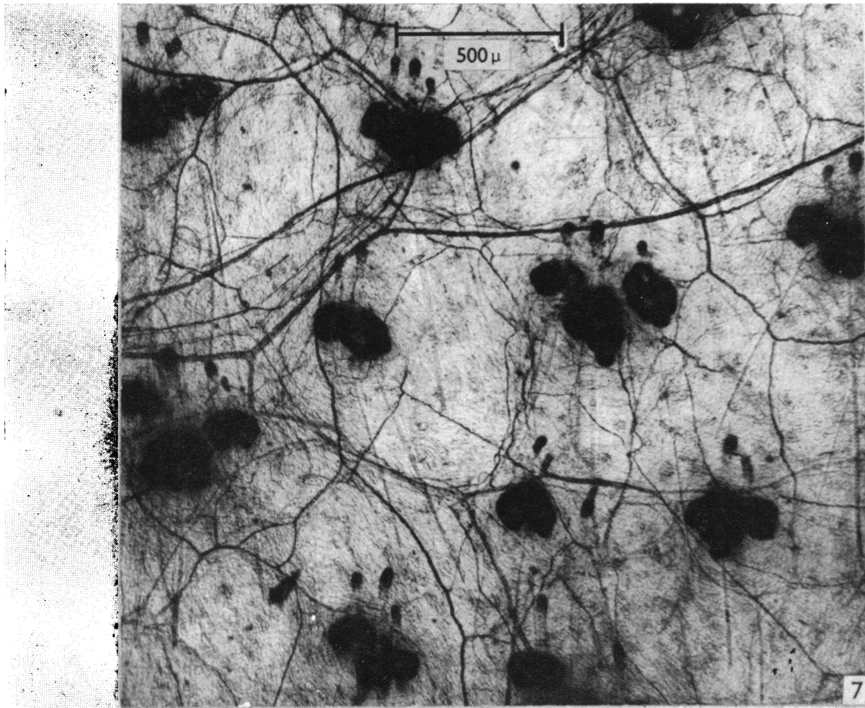

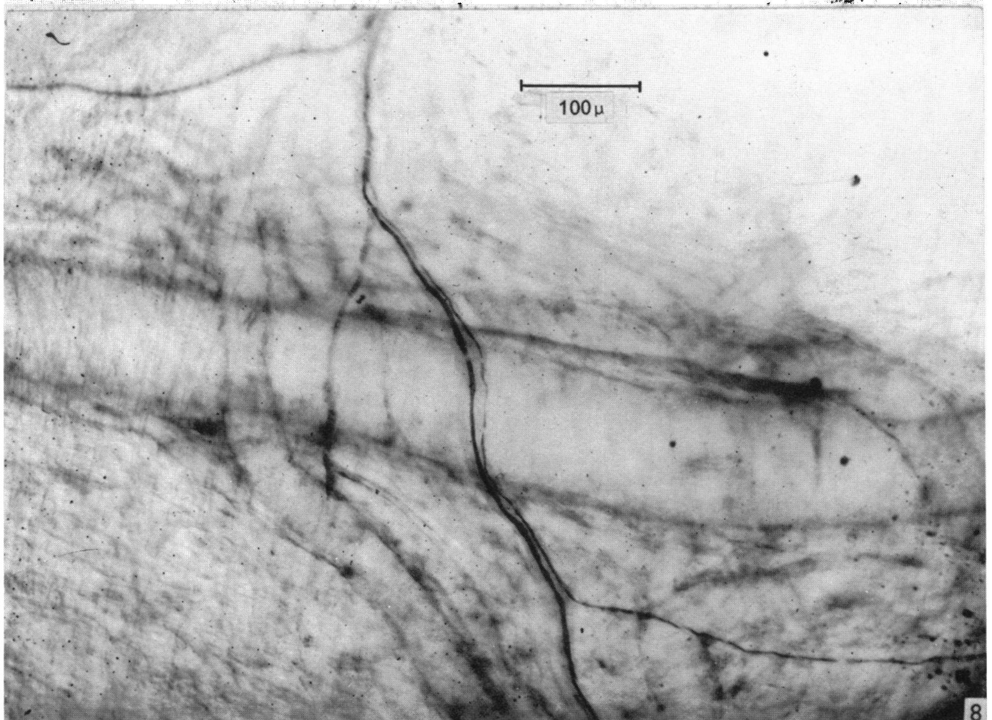

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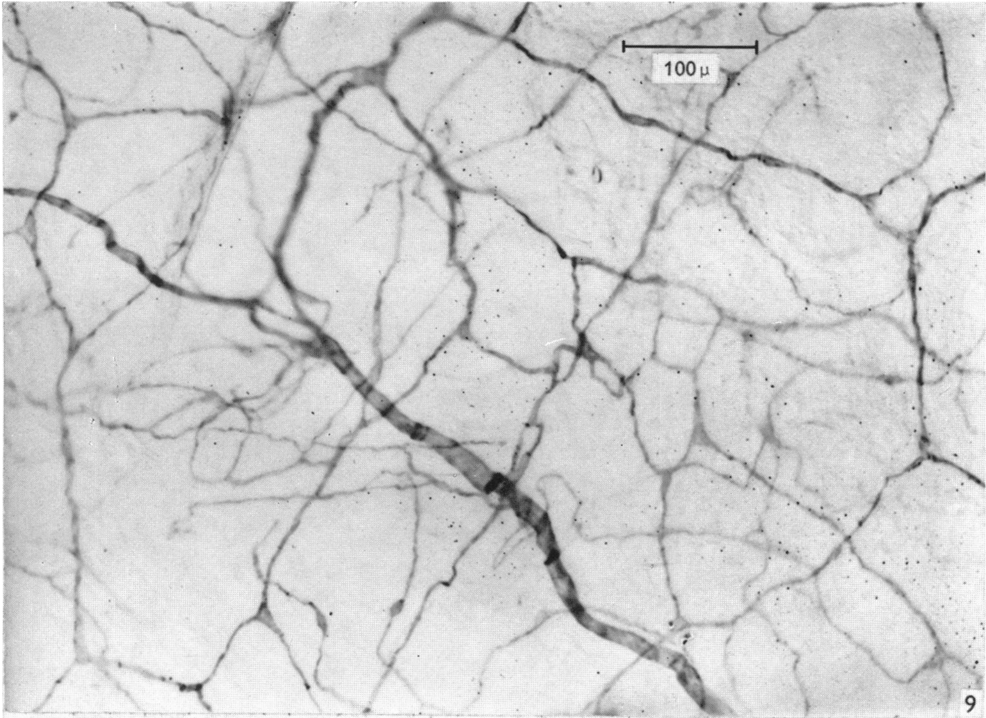

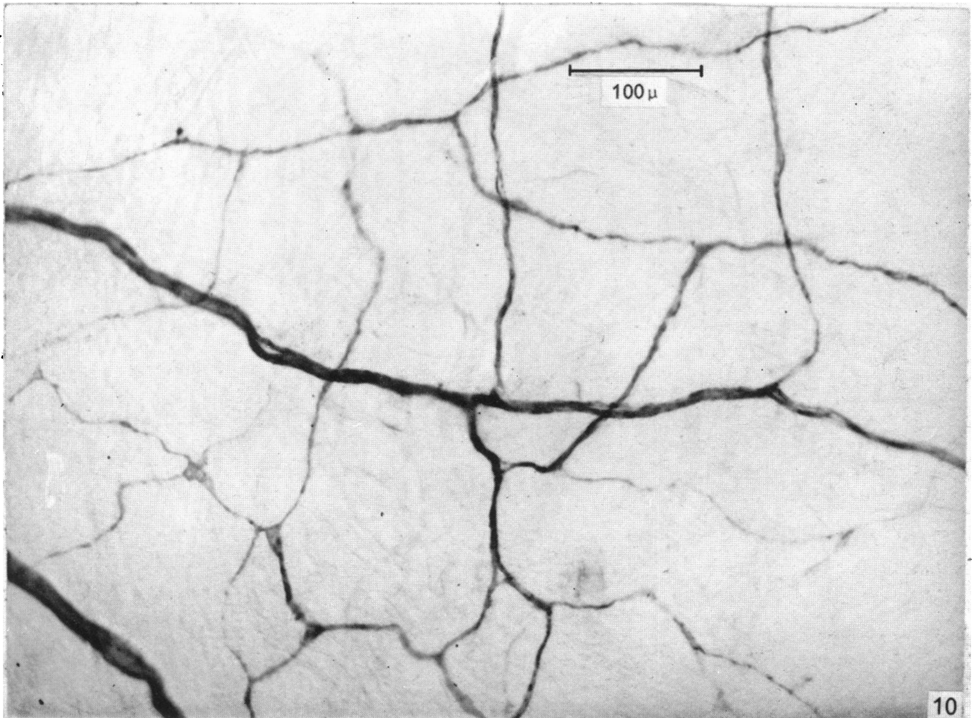

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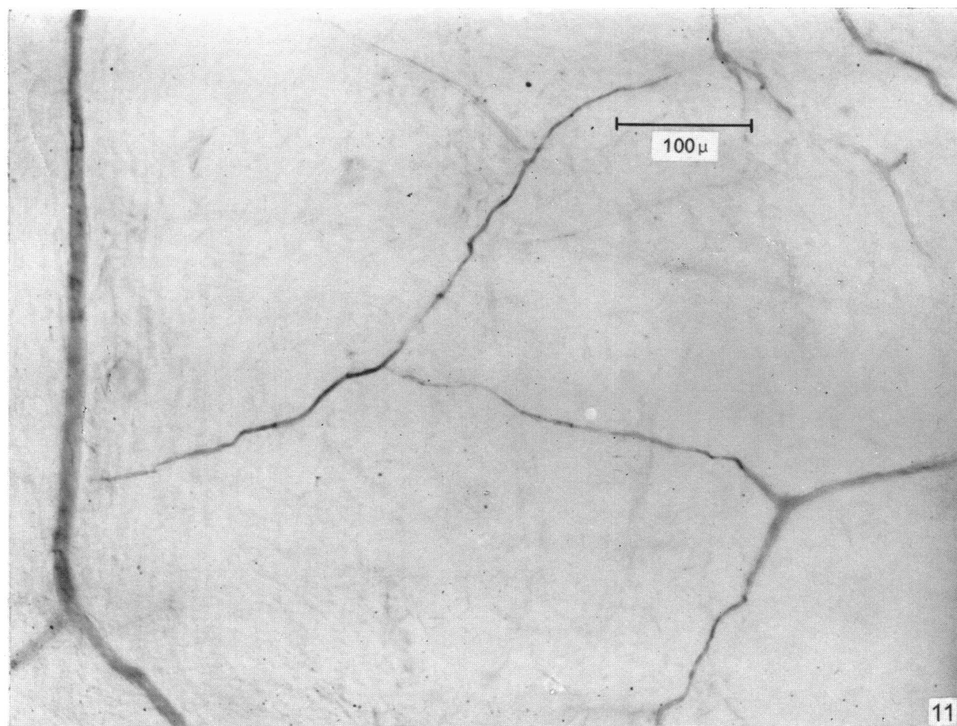
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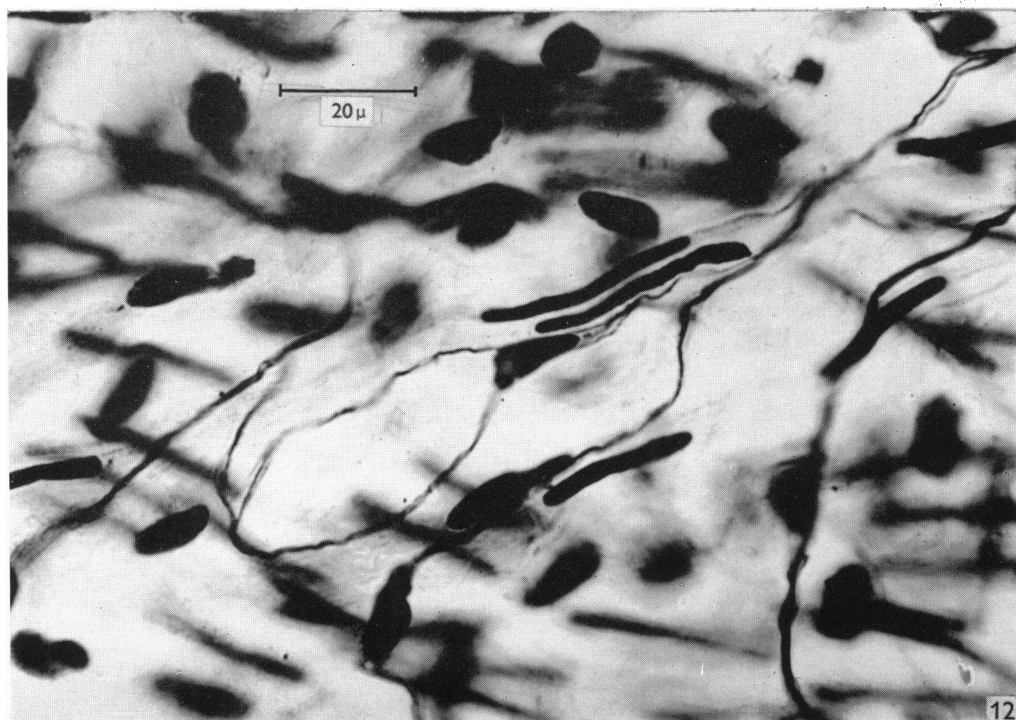
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