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ADRENERGIC RE-INNERVATION OF SMOOTH MUSCLE OF NICTITATING MEMBRANE BY PREGANGLIONIC SYMPATHETIC FIBRES

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SUMMARY

1. The preganglionic and post-ganglionic trunks of the cervical sympathetic nerve were joined in an end-to-end anastomosis after excision of the superior cervical ganglion in the cat.

2. Seventy-five days after the anastomosis the diameter of the pupil was nearly normal and there was almost complete recovery of the prolapsed palpebra and of the nictitating membrane. The contraction of the nictitating membrane, induced by electrical stimulation, caudally to the point of anastomosis, showed that the smooth muscle of the nictitating membrane had been re-innervated.

3. Neither hexamethonium nor nicotine had any marked effect on the contraction of the nictitating membrane. Severing the regenerated nerve trunk produced a degeneration contraction. These results are strong evidence that the denervated membranes were re-innervated by true cholinergic preganglionic fibres.

4. Our pharmacological studies indicated that in the re-innervated preparations neuromuscular transmission was adrenergic in the sense that it was blocked by phentolamine and not by atropine. These results were confirmed by the histochemical-fluorescence studies which showed that the endings of the regenerated axons contained high concentrations of catecholamines.

5. Electron microscopy showed that the regenerated terminals contained none of the small dense-core vesicles, considered to be typical of adrenergic nerve endings, but contained clear synaptic vesicles and an unusually great number of large granular vesicles.

6. Our results suggest that the denervated nictitating membranes were re-innervated by cholinergic presynaptic sympathetic fibres that had been

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modified so that they could release catecholamines in addition to, or instead of, acetylcholine.

INTRODUCTION

Experimental morphologists and neurophysiologists have been interested for years whether preganglionic sympathetic fibres are able to regenerate and to re-innervate peripheral organs without ganglionic relay. The nictitating membrane is a convenient organ in which to study this question and many investigators have excised the superior cervical ganglion of rabbits or cats and tried to re-innervate the denervated nictitating membrane with preganglionic fibres of the sympathetic cervical nerve. The results of these experiments are conflicting. Some early workers obtained no functional recovery of the denervated nictitating membrane (Pye-Smith, 1887; Langley, 1900; Meltzer, 1907; Butson, 1950); others obtained partial recovery in cats (Langendorff, 1901; Langley & Anderson, 1904), but attributed it to incomplete ganglionectomy (Langley & Anderson, 1904). More recently Berselli & Rossi (1953) have reported more convincing evidence in cats for re-innervation of the nictitating membrane by preganglionic fibres; about half of their animals showed almost complete functional recovery. These authors claimed that the regenerated sympathetic nerve contained mainly preganglionic fibres although the presence of some post-ganglionic fibres was not completely ruled out.

The earlier investigators were mainly concerned with obtaining a successful re-innervation of the peripheral structures by sympathetic preganglionic fibres and none of them tried to determine the nature of the neuromuscular transmitter that supported the recovery of the peripheral functions. It is especially important to know this where the normal adrenergic innervation has been replaced by normally cholinergic fibres. The present investigation is concerned not only with whether or not sympathetic preganglionic fibres can directly innervate peripheral structures, but also with the nature of the transmitter in the re-innervated structures. To these ends we examined the physio-pharmacological properties, the histochemical characteristics and the ultrastructure of the smooth muscle of the re-innervated nictitating membrane of cats.

The smooth muscle of the nictitating membrane of cats is a particularly suitable tissue for this experiment. Although the nerve supply to the membrane contains a small component of cholinergic fibres (Bacq & Fredericq, 1935; Burn & Trendelenburg, 1954; Burn & Rand, 1960), it is generally claimed that practically all the nerve terminals in the smooth muscle are adrenergic. A few acetylcholinesterase-positive nerve fibres occur within the nictitating membrane (Gardiner, Hellmann & Thompson,

1962; Jacobovitz & Koelle, 1965), but none of these esterase-positive axons has a close relationship with the smooth muscle cells. A few of these fibres may end on the small blood vessels (Gardiner *et al.* 1962; Esterhuizen, Graham, Lever & Spriggs, 1968) while most pass through the smooth muscle to the Harderian gland (Gardiner *et al.* 1962). At present there is no strong evidence for a cholinergic component to neuromuscular transmission in the smooth muscle of the nictitating membrane (Cervoni, West & Fink, 1956; Gardiner *et al.* 1962; Esterhuizen, Graham, Lever & Spriggs, 1967; Esterhuizen *et al.* 1968).

METHODS

Surgical procedure: Gangliectomy

The right superior cervical ganglion was excised under aseptic conditions from sixteen adult cats of either sex (average weight, 2500 g) under Nembutal anaesthesia (35 mg/kg I.P.). Approximately 2 cm of the vagus-sympathetic trunk, including the nodose ganglion and the superior cervical ganglion, was exposed and the two nerve trunks carefully separated from each other. The completeness of the separation was checked by observing the response of the pupil to electrical stimulation of each of the separated nerves. The superior cervical ganglion with about 2 mm of the pre- and post-ganglionic nerve trunk were then excised, and the stumps of the two nerve trunks joined in an end-to-end anastomosis by passing a single stitch (Ethicon 6–0) through the perineurium as illustrated in Text-fig. 1. As a control in four of these sixteen animals, the left superior cervical ganglion and 2 mm of the post-ganglionic trunk were also removed along with 2 cm of the preganglionic nerve. To ensure that the regenerating axons did not reach the nictitating membrane the central stump, previously tied, was lifted up and fixed on top of the muscle plane.

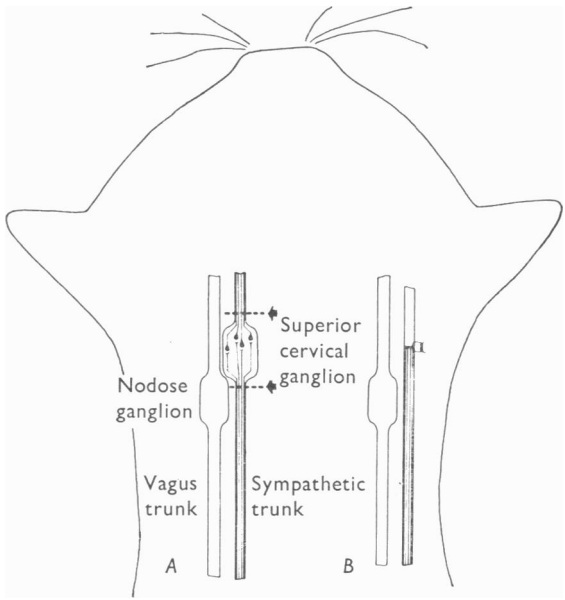
After the operation the cats were kept in single cages, and during the post-operative period (about 4 days) they received liquid and antibiotics parenterally. To check the pupillary diameter, the degree of the palpebral ptosis and the relaxation of the nictitating membrane, the cats were periodically photographed under constant and uniform illumination.

The contraction of the nictitating membrane in response to drugs and to electrical stimulation of the cervical sympathetic trunk was checked in twelve of these animals from 75 to 120 days after surgery. Following these tests the animals were sacrificed as described below and the nictitating membrane prepared for the histochemical analysis of catecholamines or for electron microscopy. In two other cats the cervical sympathetic nerve was stimulated electrically and the pupillary response was checked visually. Then the sympathetic nerve was severed 6–7 cm caudally to the point of anastomosis, and the animals were allowed to recover.

Stimulation and recording procedures: cervical-sympathetic nerve nictitating membrane preparation

In cats anaesthetized with chloralose (70 mg/kg I.V.) the trachea was cannulated, about 2 cm of the right vagus-sympathetic trunk was exposed at the base of the neck and the two nerves were separated. The cervical sympathetic nerve was carefully isolated from the surrounding tissues and, except for the nicotine experiments, tied as far caudally as possible in order to block the propagation of impulses into the spinal cord. In some animals both the right and the left cervical-sympathetic trunks were exposed and prepared for stimulation. The nerve trunks were covered with

warm (about 37° C) liquid paraffin and were stimulated through platinum electrodes by supermaximal shocks 0·3 msec in duration applied at frequencies from 1 to 25/sec for 20–30 sec every 5–6 min. The contractions of the nictitating membrane were recorded with a kymograph; the free edges of the membranes were connected to frontal isotonic levers by thin silk threads that passed through pulleys. The weights of the nictitating membranes were approximately 5 g and the contractions were amplified about 10 times. When the contractions of both nictitating membranes were recorded simultaneously, the weights and the amplifications of the two levers were made equal.



Text-fig. 1. Scheme of the pre-post-ganglionic anastomosis (*B*). The superior cervical ganglion is excised and the stumps of the two sympathetic trunks are joined in an end to end anastomosis. Normal anatomical relationships are shown schematically in *A*.

The blood pressure was recorded from the femoral artery, and drugs were injected into the cannulated femoral vein. Adrenalectomy was carried out through a mid line abdominal incision.

Catecholamine histochemistry

At the end of recording experiments both nictitating membranes were quickly dissected from six animals (Acheson, 1938; Thompson, 1958, 1961) and prepared for visualization of their content of catecholamines by the histochemical fluorescence technique of Falck & Owman (1965). A portion of the nictitating membrane that included the inferior smooth muscle was frozen in freon cooled with liquid nitrogen, cooled further in pure liquid nitrogen, and finally transferred to an Edwards/Pearse tissue freeze dryer at $-35°$ C and dried over P_2O_5 at a pressure of 1×10^{-3} mm Hg for 4–5 days. The specimens were then exposed to paraformaldehyde fumes at 80° C for 2 hr and embedded under vacuum in paraffin. Sections about 10 μ were cut and

mounted in Entellan (Merk) on microscope slides and examined in a Leitz Ortholux fluorescence microscope with a mercury vapour lamp as light source and a dark field condenser. Excitation was at 430 mμ and the emitted light below 490 mμ was cut off with Leitz K-530 barrier filter. Photomicrographs were taken on Kodak Ektachrome (day light) film.

Tissue preparation for electron microscopy

The nictitating membranes of six cats were prepared for electron microscopy after their contractions had been recorded. The animals were rapidly perfused via the abdominal aorta with approximately 1 l. Ringer-lactate solution at 18 ± 2° C containing heparin (1000 u./l.) and procaine hydrochloride (0·2 %), and then they were perfused (Ceccarelli & Pensa, 1968) with a solution of 2·5 % glutaraldehyde and 2 % formaldehyde in 0·12 M phosphate buffer at pH 7·4 (Karnovsky, 1965). The osmolarity of the fixative was about 1330 ± 10 m-osmole. The amount of the fixative solution perfused was about double the animals' weight. Both nictitating membranes were dissected from each cat, and the regions that included the inferior smooth muscle (Acheson, 1938; Thompson, 1958, 1961) were cut into small pieces and post-fixed for approximately 2 hr at 4° C in 1 % osmium tetroxide in 0·12 M phosphate buffer at pH 7·4. Specimens were embedded in Epon 812 and sections were cut with a diamond knife, stained with uranyl acetate and lead citrate (Venable & Coggeshall, 1965) and examined with a Philips EM 300 Electron Microscope.

RESULTS

Catecholamine histochemistry and ultrastructure of normal nictitating membrane

In agreement with previous investigators (Spriggs, Lever, Rees & Graham, 1966; Esterhuizen *et al.* 1967, 1968; Van Orden, Bensch, Langer & Trendelenburg, 1967; Weiner, Langer & Trendelenburg, 1967) we found that the normal nictitating membrane showed a dense plexus of pale green strongly fluorescent fibres (Figs. 1–3 of Plate 1) when treated according to the paraformaldehyde-fluorescence technique for the demonstration of biogenic amines. The fluorescence was restricted to the layer of the smooth muscle and to the walls of the blood vessels. At a high magnification one could see many intensely fluorescent, irregularly shaped spots that represent nerve terminals or axon varicosities with their large stores of catecholamines.

The fine structure of the smooth muscle layer of the cat nictitating membrane has been described by many authors (Lever & Graham, 1964; Evans & Evans, 1964; Taxi, 1965; Spriggs, Lever & Graham, 1967; Van Orden *et al.* 1967; Esterhuizen *et al.* 1968) and does not require a further detailed description. We shall briefly summarize the subcellular organization of the nerve fibres and nerve endings and their relationship with the smooth muscle cells.

Profiles of nerve terminals and unmyelinated preterminal axons are found, often free of Schwann cell processes, in the spaces between adjacent

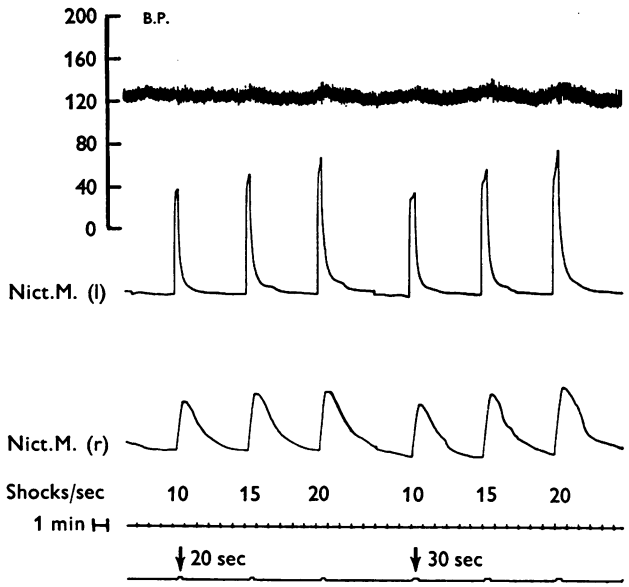
smooth muscle cells. The terminals never show modified regions of contact characteristic of synapses, and they rarely approach the muscle cells more closely than 300–400 Å. Indeed, the density of innervation is quite low in the smooth muscle of the nictitating membrane and it is likely that many muscle cells are not closely associated with any nerve terminals (Burnstock, 1970). Three kinds of vesicles are seen in the profiles of the preterminal axons. The most frequent kind is the small dense-core vesicle with a diameter of 300–450 Å that is characteristic of adrenergic nerve endings (Clementi, 1965; Van Orden, Bloom, Barrnett & Giarman, 1966; Burnstock, 1970; Jaim-Etcheverry & Zieher, 1971), the second kind is the usual agranular vesicles with a diameter of 300–400 Å, and the third kind is a large granular vesicle, 750–1000 Å in diameter, with a round, uniformly dense core that occupies almost all of the area within the vesicular membrane. These latter vesicles occur infrequently and account for only about 3 % of the total number of vesicles in the nerve terminals of the smooth muscle (Van Orden *et al.* 1967). Two types of preterminal axon can be distinguished according to their vesicular contents. The more frequent type contains a large proportion of the small dense core vesicles and lesser proportion of the agranular vesicles, whereas the other type of preterminal axon, which is rare, contains mostly agranular vesicles and almost no small dense core vesicle. Both types of preterminal axon profile occasionally contain a few of the large granular vesicles.

Physiopharmacological, histochemical and ultrastructural observation 75–120 days after gangliectomy

Fourteen of sixteen animals showed clear signs of recovery 75 days after the anastomosis; the diameter of the right pupil was nearly normal and there was almost complete recovery of the prolapsed right palpebra and nictitating membrane. By contrast, no recovery occurred in those four animals in which re-innervation was prevented on the left side.

These visual signs of recovery were confirmed by recording the contraction of the nictitating membrane induced by electrical stimulation of the right sympathetic trunk 6–7 cm caudally to the point of anastomosis. In the twelve animals tested, stimulation at frequencies of 1/sec or more always produced marked contraction of the membrane (Text-figs. 2, 5). The amplitude of the contraction was often slightly smaller, and the rates of contraction and relaxation slower, on the operated side than on the normal side (Text-figs. 2–4). The contraction of the nictitating membrane to stimulation of the sympathetic trunk showed that the smooth muscle of the membrane had been re-innervated. However, we could not be sure that the re-innervation had been accomplished by presynaptic fibres, for

the re-innervating fibres may have been post-synaptic fibres derived from extraganglionic neurones. If this had been the case, then transmission along the nerve trunk should have been blocked by hexamethonium. However, we found that hexamethonium (hexamethonium bitartrate 2·5–7·5 mg/kg) did not affect the contractions of the re-innervated membrane (Text-figs. 3, 5) while it greatly reduced the contractions of the normally innervated membrane (Text-fig. 3). This suggests that the new fibres were not derived from extraganglionic neurones located distally to the point of stimulation.

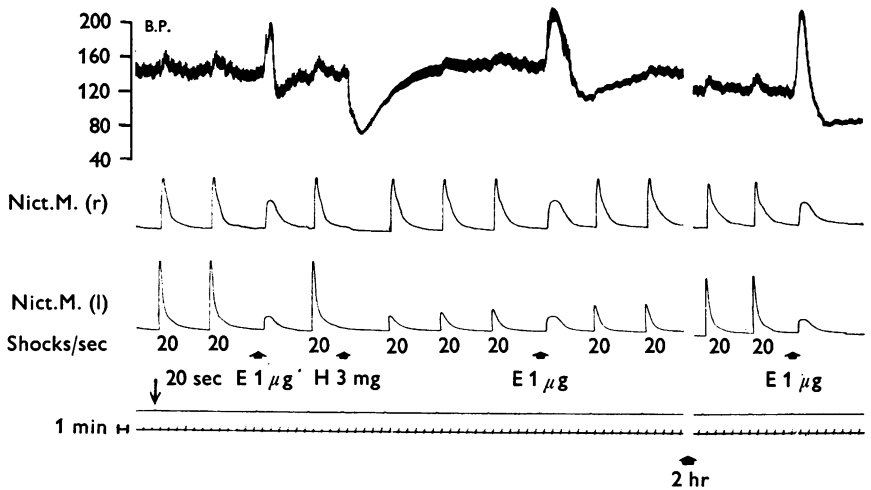


Text-fig. 2. Cat 75 days after pre-post-ganglionic anastomosis. B.P. = blood pressure. Nict.M. (l): record of contractions of the left, normal, nictitating membrane. Nict.M. (r): record of contractions of the right, re-innervated, nictitating membrane. Contractions of the nictitating membranes were elicited by electrical stimulation, at various frequencies, of the pre-ganglionic sympathetic trunk.

Additional evidence against the existence of synapses distal to the point of stimulation is given by experiments in which the regenerated nerve were severed again 90 days after the initial gangliectomy. In these animals the pre-anastomotic sympathectomy induced the reappearance of all the ocular signs of cervical sympathetic paralysis, and in addition, produced a transient degeneration contraction of the nictitating membrane that was evident from 20 to 26 hr after the new denervation. The occurrence of this transient degeneration contraction is important because it shows that the second sympathectomy produces a true denervation, and not a simple

decentralization, of the nictitating membrane (Langer, 1966; Langer & Trendelenburg, 1966) and this implies that there were no synapses between the cut and the periphery.

We injected nicotine (100–250 μg/kg) to rule out the presence of synapses central to the point of stimulation. The response of the nictitating membrane to nicotine includes at least three components. One component is due to the stimulation of neurones in the sympathetic trunk, a second is due to the release of catecholamines from the adrenal medulla and a third

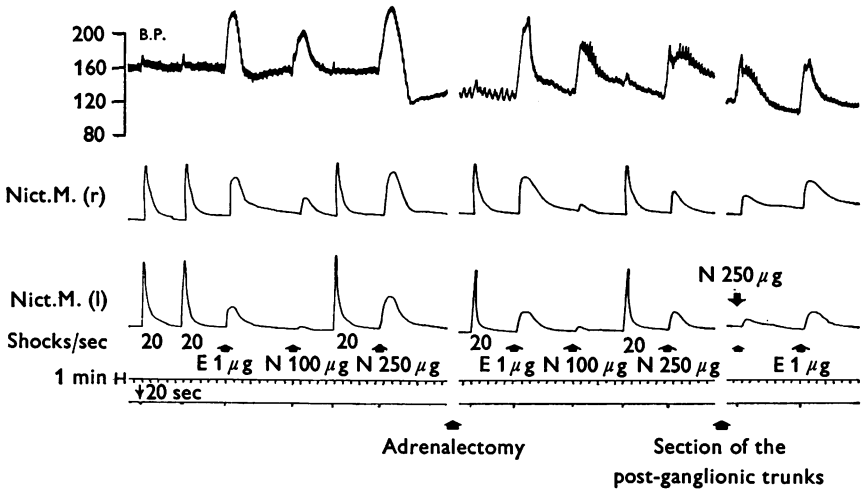


Text-fig. 3. Cat 90 days after pre-post-ganglionic anastomosis. From top to bottom: blood pressure (B.P.), and contractions of right, re-innervated (r), and left, normal (l), nictitating membranes (Nict.M.). Contractions of nictitating membranes are elicited by either electrical stimulation of the sympathetic pre-ganglionic trunks or by I.V. injections of adrenaline (E). The I.V. injection of hexamethonium (H) does not affect the contractions of the right nictitating membrane, but nearly abolishes the contractions of the normal left nictitating membrane. Doses of drugs given in μg/kg or mg/kg.

is due to the release of catecholamines from adrenergic nerve endings (Thompson, 1958; Khan, Mantegazza & Piccinini, 1965). An estimate of the neuronal contribution is given by the difference between the responses to nicotine obtained before and after section of the post-ganglionic nerve in adrenalectomized animals. The results of such an experiment are shown in Text-fig. 4. It is clear that sectioning the post-ganglionic trunk almost completely abolished the response of the normally innervated nictitating membrane to nicotine and had no effect on the response of the re-innervated membrane. This result indicates that most of the fibres re-innervating the nictitating membrane were not derived from extraganglionic neurones distributed along the sympathetic trunk. The relatively large contraction

of the re-innervated membrane elicited by nicotine was probably an expression of the slight supersensitivity to catecholamines that we commonly found in the re-innervated nictitating membrane (Text-figs. 3–4).

These results strongly suggest that the nictitating membrane was re-innervated by nerve fibres that were preganglionic in the functional sense. Since preganglionic sympathetic fibres are normally cholinergic whereas the post-ganglionic sympathetic fibres are adrenergic, it was important

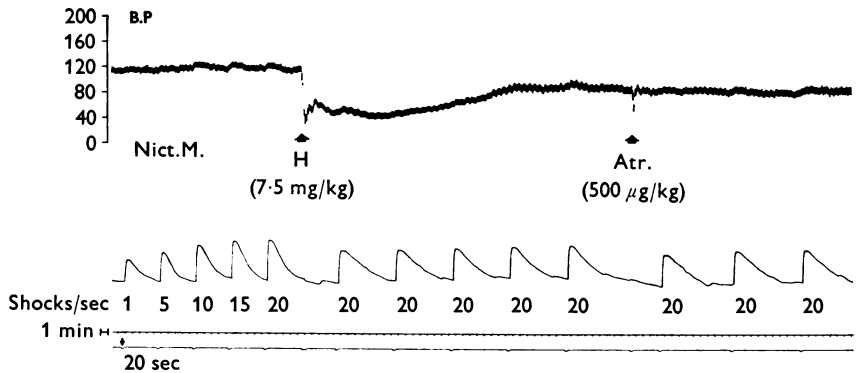


Text-fig. 4. Cat 120 days after the pre-post-ganglionic anastomosis. Contractions of the nictitating membranes are elicited either by electrical stimulations of pre-ganglionic sympathetic trunks, or by I.V. injections of adrenaline (E) and nicotine (N). Nicotine elicits contractions of the nictitating membranes on both sides. Adrenalectomy remarkably reduces bilaterally the nicotine-evoked responses. The section of both post-ganglionic trunks does not affect the nicotinic responses on the right (r) re-innervated side, but almost abolishes the nicotinic response on the left (l) normal side. Doses of drugs given in μg/kg.

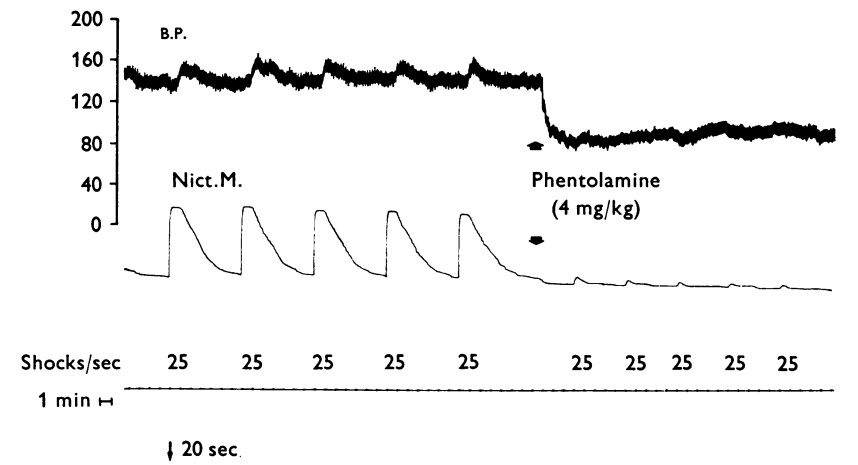
to establish the nature of the neural transmitter in the re-innervated membrane and our experiments were first directed at determining the pharmacological properties of the re-innervated membrane. We found that anticholinergic drugs as atropine, even at large dose (atropine sulphate 500 μg/kg), had no effect on the electrically evoked response of the re-innervated side (Text-fig. 5) whereas the α-adrenergic blocking agent, phentolamine (phentolamine mesylate), completely abolished this response at doses larger than 2·5 mg/kg (Text-fig. 6). Thus it appears that in the re-innervated membrane neuromuscular transmission is adrenergic.

The results of these pharmacological studies were substantiated by our histochemical studies of the distribution of catecholamines in the smooth

muscle of the re-innervated membranes. Between 75 and 120 days after the anastomosis the re-innervated muscle showed again the intense pale green fluorescence characteristic of catecholamines. The distribution of the fluorescence in the re-innervated muscle (Figs. 4–6 of Pl. 2) was similar to, but less dense than, that of the control muscle with their normal post-ganglionic nerve supply. When the re-innervated muscles were examined at high magnification intensely fluorescent individual nerve endings or axon varicosities stood out clearly in the dark intercellular spaces (Fig. 6,



Text-fig. 5. Cat 96 days after pre-post-ganglionic anastomosis. Upper record: blood pressure (B.P.). Lower record: re-innervated nictitating membrane (Nict.M.). The contractions evoked by electrical stimulation at various frequencies of pre-anastomotic trunk are affected neither by I.V. injections of hexamethonium (H) nor by atropine (Atr.).



Text-fig. 6. Cat 87 days after pre-post-ganglionic anastomosis. Upper record: blood pressure (B.P.). Lower record: re-innervated nictitating membrane (Nict.M.). The contractions evoked by electrical stimulation of pre-anastomotic trunk are abolished by phentolamine.

of Pl. 2), but the number of these varicosities was usually less than in the normally innervated muscle. In agreement with the results of other workers (Spriggs *et al.* 1966; Van Orden *et al.* 1967; Weiner *et al.* 1967; Esterhuizen *et al.* 1968), we found no specific fluorescence in the smooth muscles from the left sides of the four cats in which regeneration was prevented.

Thus the electrophysiological, pharmacological and histochemical studies indicate that the denervated nictitating membrane had been re-innervated by preganglionic fibres modified so as to release catecholamines. Since adrenergic and cholinergic nerve terminals usually display characteristic differences in their ultrastructure, it was of interest to examine the ultrastructures of the regenerated axons.

Figs. 7, 8, 9 of Pl. 3 are typical electron micrographs of re-innervated inferior smooth muscle of nictitating membranes. The re-innervated muscle shows preterminal axon profiles and axon varicosities scattered among the muscle cells but the number of these profiles seems less than in normal tissue. The regenerated nerve endings or axon varicosities resemble the normal structures in that they never display specialized regions of contact with the smooth muscle cells. However, other features of the subcellular organization of the regenerated axons, especially their complement of vesicles, are quite different from those of normal adrenergic axons. In the profiles of the regenerated preterminal we could never find the small dense core vesicles generally considered to be a typical subcellular component of adrenergic nerve endings. Besides containing a relatively large but variable number of agranular vesicles (300–500 Å), the regenerated preterminals also contain an unusually great number of large granular vesicles (Figs. 7–9 of Pl. 3). The large granular vesicles in regenerated preterminals have large diameters (1000–1600 Å) and denser cores than the large granular vesicles in normal preterminals.

We found no structures that we could definitely identify as nerve fibres or nerve endings in the smooth muscle of those nictitating membranes in which re-innervation was prevented.

DISCUSSION

The results obtained in the present investigation show that functional neuronal connexions are re-established in the nictitating membrane after removal of the superior cervical ganglion and anastomosis of the pre- and post-ganglionic cervical sympathetic trunks. Our results basically agree with the results of previous workers (Langendorff, 1901; Langley & Anderson, 1904; Berselli & Rossi, 1953), the major differences being the greater number of successful re-innervations we obtained and the greater

speed with which recovery occurred in our animals. These differences are probably due to differences in surgical techniques, especially to the fact that we joined the nerve trunks together after gangliectomy, whereas others left a gap of 1 cm or more between the nerve stumps (Langendorff, 1901; Langley & Anderson, 1904).

Although the stimulation experiments clearly indicate that the re-innervating fibres originated in the preganglionic cervical sympathetic trunk, it does not follow that these fibres are presynaptic in the functional sense for there is ample evidence, both anatomical (Foley, 1945; Jacobowitz & Woodward, 1968) and electrophysiological (Douglas & Ritchie, 1956; Douglas, Lywood & Straub, 1960), that extraganglionic neurones are distributed along the sympathetic trunk for several millimetres or more on each side of the superior cervical ganglion. Hence it is possible that the re-innervating fibres, though located in the preganglionic trunk, were post-synaptic fibres growing out from some of these extraganglionic cell bodies. However, we feel that the experiments with hexamethonium, the preanastomotic sympathectomy and the experiments with nicotine make this possibility unlikely. If the nictitating membrane were re-innervated by post-synaptic fibres, then: (*a*) hexamethonium should block the synapses and inhibit that part of the contraction of the nictitating membrane that was mediated by those synapses located between the membrane and the point of stimulation, (*b*) cutting the regenerated sympathetic trunk should produce effects characteristic of decentralization, but not of denervation, and (*c*) nicotine should induce a contraction of the membrane by exciting all neurones located between the membrane and the spinal cord. In fact neither hexamethonium nor nicotine had any marked effect on the contraction of the nictitating membrane and severing the regenerated nerve trunk produced the degeneration contraction that accompanies post-ganglionic sympathectomy in normally innervated preparations. The results of these three kinds of experiments are strong evidence that the denervated nictitating membranes were re-innervated by true pre-synaptic fibres and not by post-synaptic fibres originating from extraganglionic neurones. The hypothesis that post-synaptic fibres are not involved in the re-innervation is supported also by the experiments of Berselli & Rossi (1953). After a successful gangliectomy and anastomosis of the pre- and post-ganglionic trunks they severed the ansa subclaviae and found that the regenerated fibres degenerated again and that the nictitating membrane no longer contracted when the sympathetic trunk was stimulated.

Thus we have good evidence that in our experiments the smooth muscle of the nictitating membrane was re-innervated by the normally cholinergic preganglionic fibres. However, our pharmacological studies indicate that

in the re-innervated preparations neuromuscular transmission is adrenergic in the sense that it is blocked by phentolamine and not by atropine. This interpretation of the results of the pharmacological experiments is supported by the histochemical studies which show that the endings of the regenerated axons contain high concentrations of catecholamines. Taken altogether our results suggest that the denervated nictitating membrane was re-innervated by normally cholinergic presynaptic sympathetic fibres that had been modified so that they could release catecholamines in addition to, or instead of, acetylcholine.

Although the regenerated axons appear to be adrenergic in a functional sense, the electron micrographs show that the regenerated terminals contain none of the small dense-core vesicles generally considered to be the primary and typical component of an adrenergic nerve ending. The regenerated terminals contain clear synaptic vesicles and an unusually great number of large granular vesicles. The diameter of these large vesicles and the densities of their cores appear to be greater in regenerated terminals that in normal terminals. An increase in the number of large granular vesicles also occurs in the proximal stump of regenerating axons after compression of the afferent preganglionic fibres of the superior cervical ganglion on the rat (Pellegrino de Iraldi & de Robertis, 1968).

There is now an increasing body of evidence suggesting that in adrenergic terminals the large granular vesicles, as well as the small dense-core vesicles, can store amines (Tranzer & Thoenen, 1968; Geffen & Ostberg, 1969; Bisby & Fillenz, 1971; Jaim-Etcheverry & Zieher, 1971). Whether biogenic amines are stored in the analogous vesicles present in some cholinergic nerve terminals is not certain (Tranzer & Thoenen, 1968; Jaim-Etcheverry & Zieher, 1971), even though some indirect data seem to favour this hypothesis (Clementi, Mantegazza & Botturi, 1966; Pellegrino de Iraldi & de Robertis, 1968). Our results also favour this hypothesis in that the increase in the number size, and density of the large granular vesicles occurs together with the reappearance of fluorescence in the re-innervated smooth muscle. Perhaps these relatively numerous granular vesicles in the regenerated terminals contain the stores of catecholamines that we demonstrated in our histochemical studies.

This is not the first time that the nictitating membrane has been successfully re-innervated by cholinergic nerve fibres, for Vera, Vial & Luco (1957) and Vera & Luco (1967) re-innervated the membrane by somatomotor fibres or by sensory fibres of the vagus. However, in these earlier studies neuromuscular transmission appeared to be cholinergic. It is difficult to account for these two sets of results that appear to be so different. One possible explanation may be that the degree of specialization or differentiation is not the same in all adult neurones and it may be

possible for some neurones to dedifferentiate under some conditions and return to a more primitive state of development. It may be that the relative abundance of the various populations of vesicles in cholinergic endings is one indication of the degree of specialization of the neurones. For instance, the terminals of mammalian motor nerve fibres, one of the types of fibre used by Vera *et al.* (1957), contain an almost pure population of clear synaptic vesicles and large granular vesicles are rarely found. This may indicate that motoneurones are highly specialized and able to synthesize only acetylcholine. On the other hand, large granular vesicles are relatively numerous in the cholinergic nerve terminals of the axodendritic synapses within the superior cervical ganglion (Clementi *et al.* 1966; Ceccarelli, Clementi & Mantegazza, 1971). The presence of these vesicles in some cholinergic terminals may be a morphological sign that the neurone contains latent mechanisms for the synthesis, uptake, and storage of biogenic amines. These mechanisms might be activated during regeneration and modulated by the local environment around the nerve ending and by trophic interaction between muscle and nerve. This interpretation is an extension of the concepts of synaptic plasticity and neuronal adaptation and is strengthened by evidence that the synapse is a dynamic structure which can undergo relevant biochemical, structural and functional changes even in adult neurones (Giacobini, 1971).

The original assumption of Langley & Anderson (1904) that functional recovery is to be attributed to post-synaptic fibres originating from extraganglionic cells is the simplest way of accounting for the adrenergic nature of neuromuscular transmission in the re-innervated nictitating membrane. Nevertheless our results seem to exclude this explanation and suggest as an alternative an intriguing possibility, that sympathetic preganglionic cholinergic fibres can directly re-innervate the smooth muscle of the nictitating membrane with an adrenergic mechanism.

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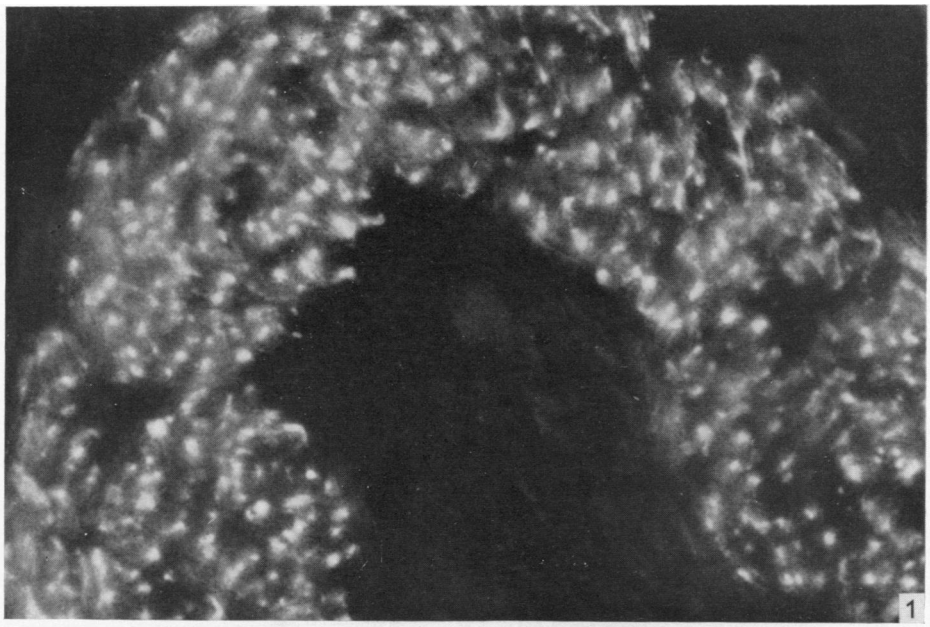
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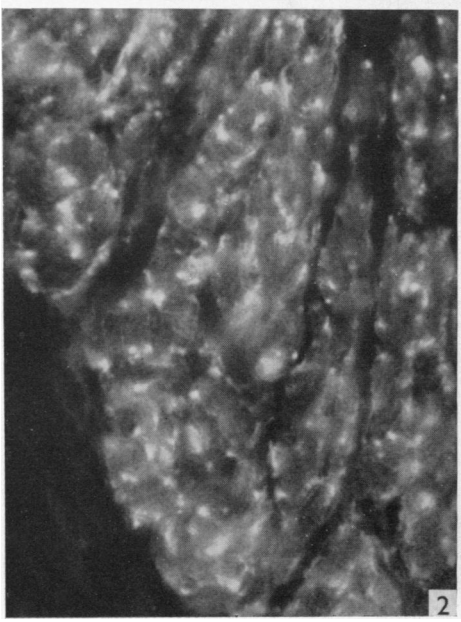
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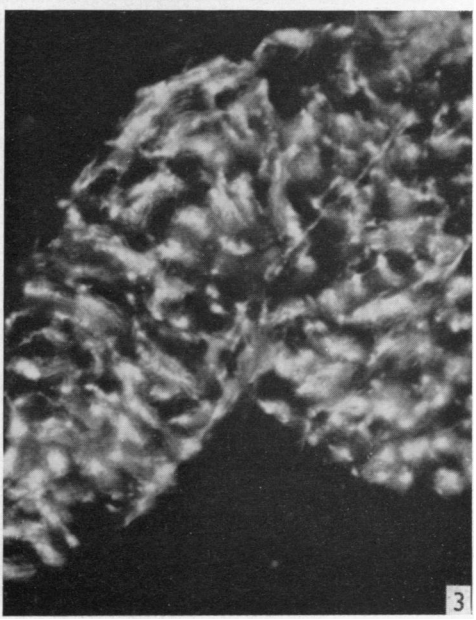
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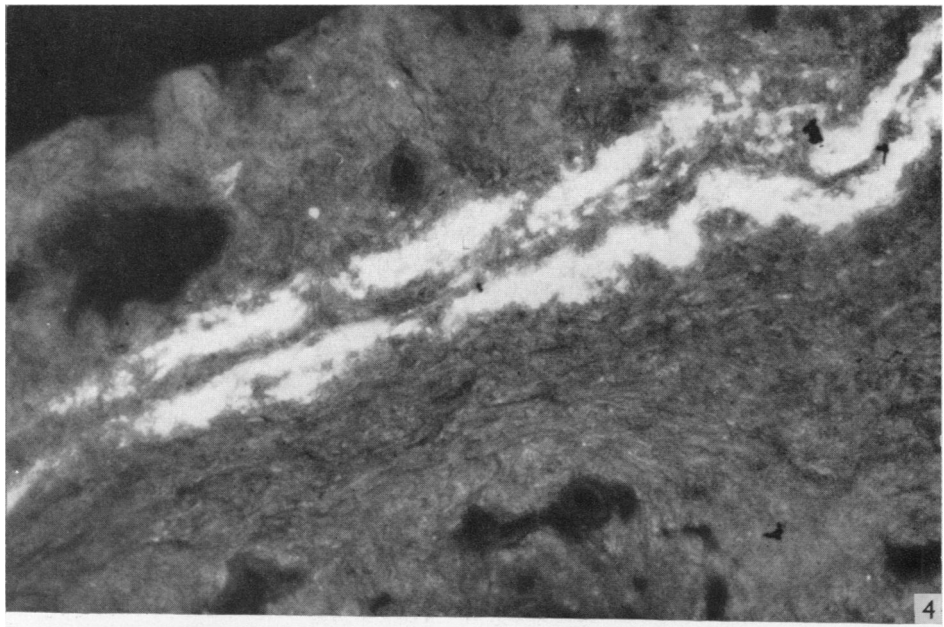
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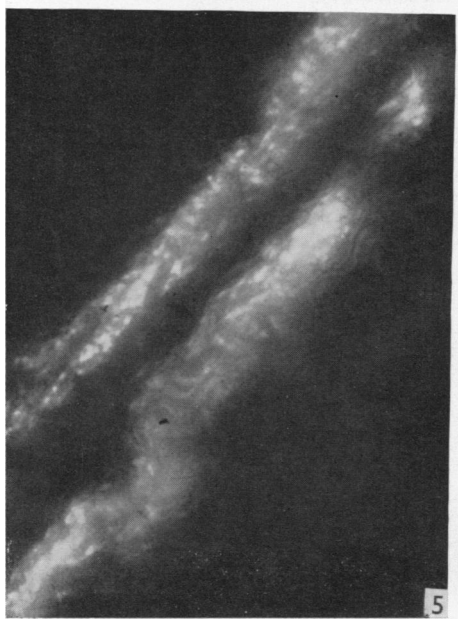
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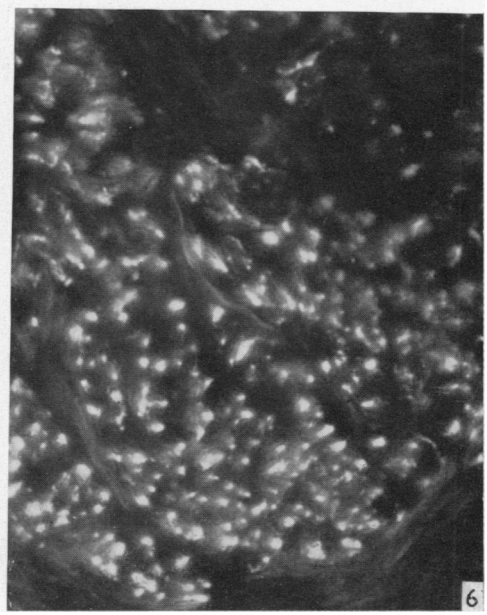
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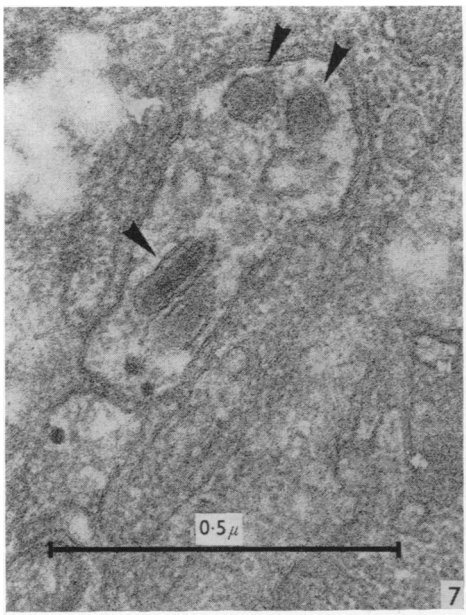
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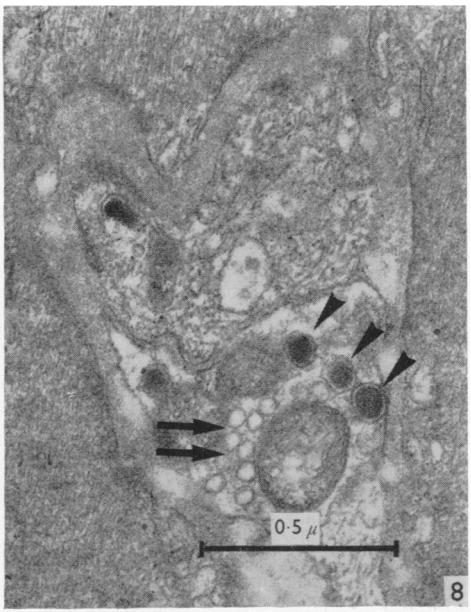
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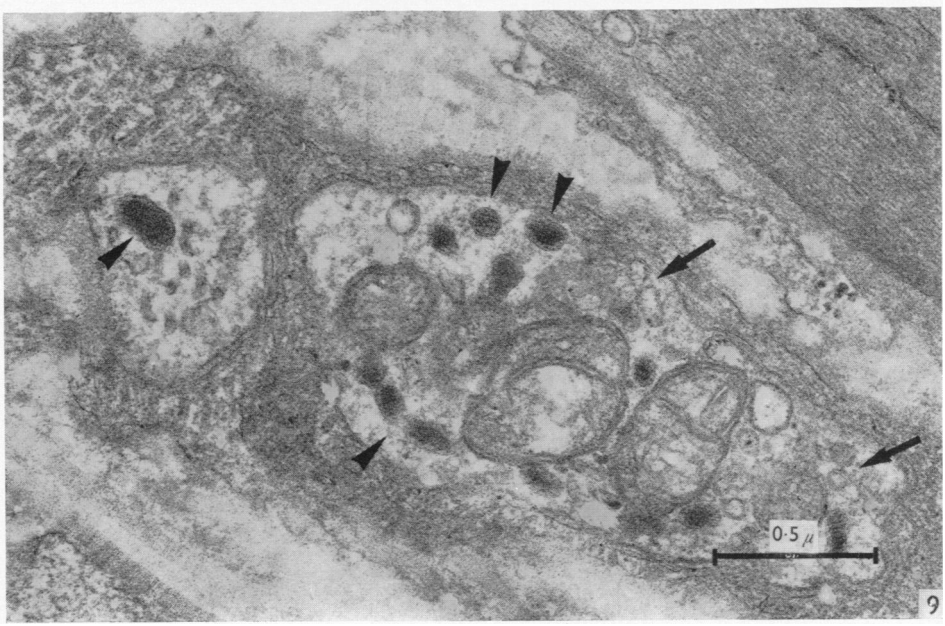
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EXPLANATION OF PLATES

PLATE 1

Figs. 1–3. Fluorescence micrographs of the normal nictitating membrane. The fluorescence is restricted to the layer of the smooth muscle. Fig. 1 × 250, Figs. 2 and 3 × 500.

PLATE 2

Figs. 4–6. Fluorescence micrographs of the re-innervated nictitating membrane. The distribution of fluorescence is similar to that of the control muscle. At higher magnification nerve endings or axon varicosities can be observed as individual units in the dark intercellular space. Fig. 4 × 80, Fig. 5 × 180, Fig. 6 × 600.

PLATE 3

Figs. 7–9. The figures show typical axon varicosities or nerve endings in the inferior smooth muscle of the re-innervated nictitating membrane. Beside containing a relatively large but variable number of agranular vesicles (➡) the regenerated terminals also contain a great number of large dense-core vesicles (➤).

Fig. 7 × 90,000, Fig. 8 × 50,000, Fig. 9 × 42,000.