Br. J. exp. Path (1974) **55**, 595

DEGENERATION AND REGENERATION OF NERVES FOLLOWING CRYOSURGERY

D. K. WHITTAKER

From the Department of Oral Biology, Dental School, Heath, Cardiff

Received for publication July 23, 1974

Summaries.—Ventral epithelium of the tongue was frozen by means of a surface applied cryosurgical probe. Nerves in the sub-epithelial connective tissue were examined in the electron microscope at various time intervals after thawing. Degeneration commenced immediately on thawing of the tissues and regeneration was well advanced within one week of the injury. Degeneration occurred before ischaemia could be expected to have produced nerve damage and it is suggested that the initial insult is a cryolytic one. Although nerve function was not studied in the present experiments, the structural changes were in accord with clinical findings of reduced sensitivity during and immediately following cyrosurgery and also with recovery of function within 7–14 days of the injury.

IT HAS generally been assumed that cryosurgery is a painless procedure because of the immediate blockage of nerve transmission in the area (Feder and Stratigos, 1971). Other workers, however, have considered that local anaesthesia should be used (Emmings, Koepf and Gage, 1967; Goldstein, 1970).

Low temperatures have been shown to be capable of blocking neural transmission but the reversibility of this procedure seems to be under debate. Cold is better than heat in the production of reversible or irreversible interruption of nerve transmission (Bernstein, 1963) and has been used for blocking neural transmission by local freezing of the brain (Le Beau, 1962). Although Cooper (1965) initially took the view that there was no permanent damage to nerves following freezing, he later commented (Wisniewska *et al.*, 1970) that 9 days after cryosurgery there was disintegration of myelin sheaths and axons. In contrast, studies on the monkey labyrinth indicated that nerve fibres were normal postoperatively (Cutt *et al.*, 1968).

Severe changes on thawing of frozen nerves have been demonstrated at the ultrastructural level (Menz, 1971) using *in vitro* techniques. The regeneration of nerves following freezing has received little attention although a study carried out on exposed nerves of rats (Carter *et al.*, 1972) demonstrated return of function due to healing by the 14th day.

There appears to be little information available on the histological or ultrastructural changes occurring in peripheral nerves subjected to cryosurgery and the present studies were carried out under conditions of *in vivo* surface freezing, which are comparable to those used in cryosurgical operations.

MATERIALS AND METHODS

Ventral epithelium of the tongue of 27 golden hamsters (*Mesochricetus auratus*) was frozen by means of an Amoils cryo-pencil (Spembly Ltd, Andover) operating at −70° and

placed in light contact with the wet mucosa. Freezing was for 1 min and has previously been shown to result in a typical cryosurgical lesion (Whittaker, 1972). Three animals were sacrificed at each of the following times after cryosurgery: immediately, 2, 10 and 30 min. 1, 2 and 5 h, 7 and 14 days. A further 12 animals were used as controls and to check fixation and embedding procedures.

Specimens from the area of cryosurgery were excised by means of parallel welded scalpel blades 1 mm apart and were cut into mm^3 blocks, under 2·5% glutaraldehyde in phosphate buffer at 4°. Fixation was continued for 2·5 h and specimens were washed in phosphate buffer, post-fixed in 1% osmium tetroxide, dehydrated in ethanol and embedded in Araldite. One μm sections were cut for optical microscopy and stained with toluidine blue. Nerves were orientated transversely and thin sections cut for electron microscopy using glass knives on an LKB ultratome. Staining of sections on the grids was by uranyl acetate and lead citrate.

RESULTS

Immediately on thawing

In comparison with control specimens (Fig. 1), myelinated nerves in the experimental tissues consistently showed changes which could be attributed to the freeze thaw injury. Contraction of the axon from the myelin sheath had occurred, as indicated by a spacing of up to 0·2 μm between the inner leaflet of the sheath and the axolemma (Fig. 2).

The axolemma appeared to be intact although in some areas there was poor resolution of the membranes. Mitochondria of the axon were swollen and there was disruption or loss of cristae. Microfilaments appeared to be intact although the outline of cross sections of microtubules was indistinct. There was early evidence of myelin disruption in the sheath, as indicated by an increased width between adjacent leaflets resulting in a corrugated appearance.

2–10 *min after thawing* (Fig. 3)

Damage to myelinated nerves was considerable. Mitochondria were enlarged and the majority had lost their cristae. In the majority of cases distortion of the myelin sheath was severe and spacing between the leaflets reached 0·09 μm. The cytoplasm of the Schwann cells was less distinct than in earlier specimens and mitochondria were greatly enlarged. In some cases swollen mitochondria were

---

EXPLANATION OF PLATES

FIG. 1.—Electron micrograph of myelinated nerve from control unoperated animal. Even spacing of myelin leaflets, intact mitochondria in axon and Schwann cell are evident. EM. × 26,500.

FIG. 2.—Electron micrograph of myelinated nerve immediately on thawing. Note contraction of axon from myelin sheath and irregular spacing of myelin leaflets. EM. × 20,000.

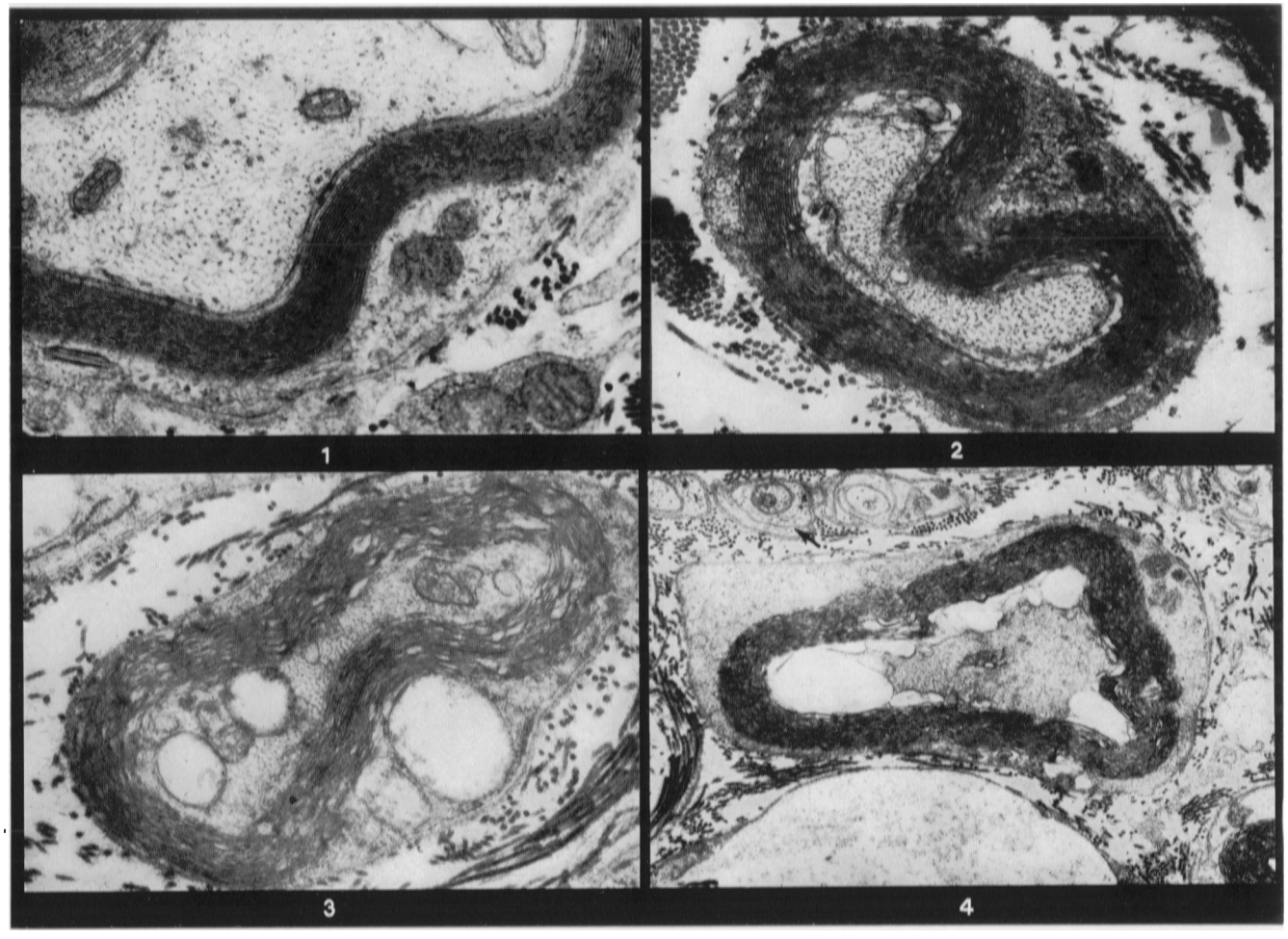
FIG. 3.—Myelinated nerve 10 min after thawing. Irregular spacing of myelin leaflets and disrupted mitochondria in Schwann cell and axon are evident. EM. × 13,500.

FIG. 4.—Nerve 2 h after thawing. Severe damage is evident in axon. Schwann cell cytoplasm is necrotic and ballooned. Non-myelinated fibres (arrow) appear intact. EM. × 8250

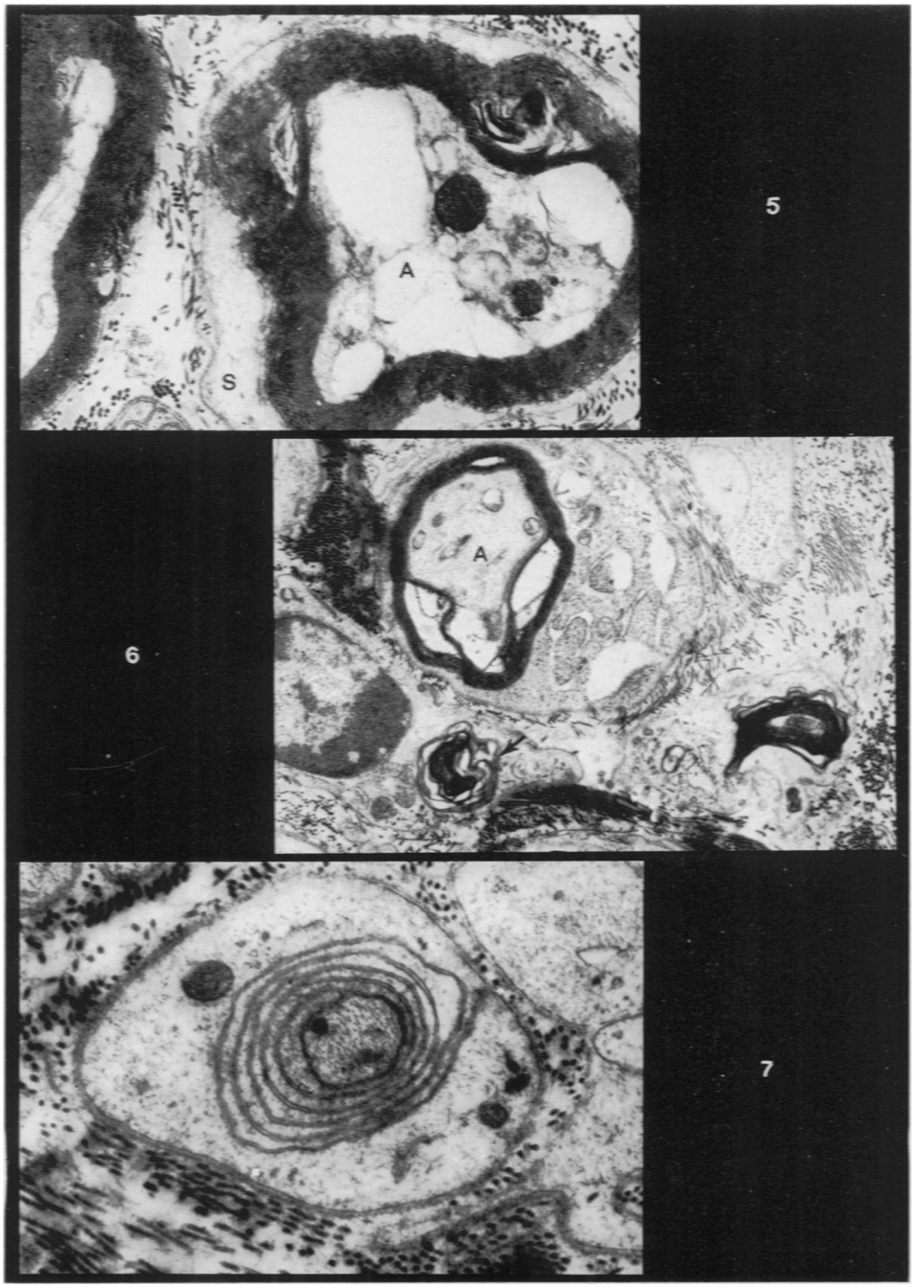
FIG. 5.—Section of nerve 5 h after thawing. Schwann cell (S) and axon (A) are necrotic. EM. × 13,250.

FIG. 6.—Nerve bundle 7 days after cryosurgery. Damaged axon (A) is present along with membrane bounded mass of degenerating myelin in adjacent Schwann cell (arrowed). EM. × 6625.

FIG. 7.—Commencement of myelination in a peripheral nerve. Condensation of myelin leaflets has not yet occurred. EM. × 20,000.



Whittakor


A
S
5
A
6
7

1 μm in diameter. The cytoplasmic membrane of the Schwann cells appeared to be intact as was the lamina densa.

30 *min–2 h after thawing* (Fig. 4)

Damage to the peripheral nerve bundles was severe following this time lapse. The cytoplasm of the Schwann cells was ballooned out into large vacuoles, which in some cases had compressed the myelin surrounded axon. There was considerable loss of structure in the cytoplasm of the axons, with loss of microtubules and microfilaments. Few mitochondria could be distinguished and there was severe distortion of the axoplasm, which was surrounded by an apparently intact axolemma but had contracted away from the myelin sheath to form vacuoles up to 1·5 μm in diameter.

The nuclei of the Schwann cells at this stage exhibited heavy clumping of the chromatin and there was widening of the perinuclear cisternae. A striking feature of this material was the normal appearance of the unmyelinated fibres which were within the same bundles as the severely damaged myelinated fibres.

5 *h post-thawing* (Fig. 5)

Disruption of axons in myelinated nerves was complete and only a finely dispersed flocculent precipitate remained, with no evidence of the axolemma. The degree of corrugation and distortion of the myelin sheath was slightly more severe than in the earlier material and some spaces between the myelin leaflets were 0·13 μm in size. Disruption of the Schwann cell cytoplasm had occurred.

7–14 *days after cryosurgery*

A feature of the specimens examined at this time interval was the presence both of degenerating and regenerating nerves. In the early post thaw material it was clear that axonal destruction preceded myelin degeneration. One week after freezing myelin destruction was severe although present only in a proportion of the axons studied. Fragments of myelin were present within the cytoplasm of the Schwann cells (Fig. 6) in the form of rounded bodies which maintained a laminated structure. The endoplasmic reticulum of Schwann cells associated with degenerating nerves was richly developed compared with that in the control specimens and numerous pale and darkly staining globules were present along with clear vacuoles and those containing the fragments of disorganized myelin. The lamina densa surrounding the Schwann cells appeared to be intact.

In the specimens obtained 7 days after cryosurgery regeneration was proceeding in association with Schwann cells, in which degenerating myelin was being absorbed. At the 14-day interval regeneration was more obvious than degeneration. Some Schwann cells surrounded numerous regenerating nerves. Many regenerating axons had commenced to myelinate and where this was occurring the Schwann cell responsible for the myelin formation appeared to be associated with only one axon (Fig. 7). The microtubules and microfilaments of the regenerating axons were distributed in a normal pattern. Early myelination with between 2 and 7 myelin leaflets surrounding an axon was seen in axons up to 1·5 μm diameter in the 7-day material. In the 14-day specimens larger axons up to 3 μm diameter were present and possessed a myelin sheath which was thinner than that seen in axons of comparable diameter in the control specimens.

DISCUSSION

The present studies have demonstrated that under the conditions of freezing used in these experiments there were some changes in nerves immediately after thawing. These consisted mainly of contraction of the axon away from the myelin sheath along with distortion of the myelin leaflets and damage to Schwann cell elements. It is not possible to say whether this change is sufficient to account for lack of transmission of nerve impulses and hence a pain-free post-operative situation but taken in conjunction with clinical evidence (Ostergard, Townsend and Hirose, 1967; Gage *et al.*, 1966; Mayers, Tussing and Wentz, 1971) this appears to be the case.

The increased periodicity of the myelin leaflets on thawing compares with the results of Joy and Finean (1963), who demonstrated that similar changes in nerves frozen and thawed *in vitro* were comparable with those produced by transferring nerves from isotonic to hypertonic solutions. The immediate damage to nerves seen in the present experiments could therefore be explained on the grounds of osmotic effects during freezing and thawing rather than physical disruption by ice crystals.

The increasingly severe nerve damage evident between 10 min and 3 h after freezing is comparable with that described in brain tissue following freezing (Walder, 1970).

Final dissolution of axons seen at 5 h post-freezing is in agreement with previous light microscope studies on exposed nerves (Carter *et al.*, 1972) or using less energetic methods of freezing (Denny-Brown *et al.*, 1945). The former workers speculated that the damage might be due primarily to ischaemia but admitted that freezing might have a direct cryolytic effect on myelin. Harkin and Skinner (1970) took the same view but commented that the problem has been inadequately examined at the electron microscope level. The present studies have demonstrated that nerve damage is apparent within 1 min of freezing and thawing whilst studies on tissues subjected to the same degree of freezing have indicated that ischaemic phenomena are not apparent at this early stage (Whittaker, 1973). It seems likely that the primary cause of nerve damage is the freezing episode itself although ischaemic changes may later assume importance. The appearance of frozen–thawed nerves 1 week after cryosurgery is similar to that described following cutting or crushing of peripheral nerves (Barton, 1962; Harkin and Skinner, 1970). The digestion of myelin by the Schwann cells is in accord with their comment that axon degeneration precedes loss of myelin. There has in the past been some debate as to whether digestion of myelin is carried out exclusively by Schwann cells or by macrophages invading the area. Most authorities take the former view (Nathaniel and Pease, 1963; Hudson, Morris and Weddell, 1970) whilst Harkin and Skinner believe that macrophages are involved. In the present study no evidence of involvement of cells other than the Schwann cells was seen.

Previous studies of the functional recovery of nerves after cryosurgery (Gaster *et al.*, 1971) have shown that the rate is similar to that observed after crushing injuries. Recovery ranges between 7–18 days, depending on the temperature, but demyelination is less severe following freezing. In a study of the effect of cryosurgery on the spinal cord nerve fibres were seen penetrating the lesion 2 weeks after the injury (Pinner-Poole *et al.*, 1966). The present experiments do

not enable the chronology of regeneration to be described accurately but remyelination of axons was at an early stage in the 7-day post-operative specimens and was still continuing at 14 days, as judged by the thickness of the myelin sheath in relation to axon diameter (Morris, Hudson and Weddell, 1972).

The structural resistence of unmyelinated nerves in the present study is of interest. Although it is not possible to correlate this with continuing functional activity, there is some clinical evidence that autonomic nerves do survive freezing better than myelinated nerves (Denny-Brown *et al.*, 1945). The present experiments confirm the findings of the earlier workers in this respect.

I am grateful to Professor B. E. D. Cooke for facilities and encouragement to carry out this work.

REFERENCES

BARTON, A. A. (1962) An Electron Microscopic Study of Degeneration and Regeneration of Nerve. *Brain*, **85**, 799.

BERNSTEIN, E. F. (1963) Cryogenic Surgery. *Med. J. Aust.*, **5352**, 267.

CARTER, D. C., LEE, P. W. R., GILL, W. & JOHNSON, R. J. (1972) The Effect of Cryosurgery on Peripheral Nerve Function. *Jl R. Coll. Surg. Edinb.*, **17**, 25.

COOPER, I. S. (1965) Cryogenic Surgery for Cancer. *Fedn Proc.*, **24**, 5237.

CUTT, R. A., ISHIYAMA, E., MYERS, E. N. & WOLFSON, R. J. (1968) Histology of the Monkey Labyrinth following Experimental Cryosurgery. *Ann. Otol. Rhinol. Lar.*, **77**, 275.

DENNY-BROWN, D., ADAMS, R. D., BRENNE, C. & DOHERTY, M. M. (1945) Pathology of Injury of Nerve induced by Cold. *J. Neuropath. exp. Neurol.*, **4**, 305.

EMMINGS, F. G., KOEPF, S. W. & GAGE, A. A. (1967) Cryotherapy for Benign Lesions of the Oral Cavity. *J. oral Surg.*, **25**, 320.

FEDER, M. & STRATIGOS, G. T. (1971) Current Status of Cryosurgery for Benign Oral Neoplasms. *J. Can. dent. Ass.*, **37**, 46.

GAGE, A. A., GREENE, G. W., NEIDERS, M. & EMMINGS, F. G. (1966) Freezing Bone without Excision. An Experimental Study of Bone Cell Destruction and Manner of Regrowth in Dogs. *J. Am. med. Ass.*, **196**, 770.

GASTER, R. N., DAVIDSON, T. M., RAND, R. W. & FONKALSRUD, E. W. (1971) Comparison of Nerve Regeneration Rates following Controlled Freezing or Crushing. *Archs Surg.*, **103**, 378.

GOLDSTEIN, J. C. (1970) Cryotherapy in Head and Neck Cancer. *Laryngoscope*, **80**, 1046.

HARKIN, J. S. & SKINNER, M. S. (1970) Experimental and Electron Microscopic Studies of Nerve Regeneration. *Ann. Otol. Rhinol. Lar.*, **79**, 218.

HUDSON, A., MORRIS, J. & WEDDELL, G. (1970) An Electron Microscopic Study of Regeneration in Sutured Rat Sciatic Nerves. *Surg. Forum*, **21**, 451.

JOY, R. T. & FINEAN, J. B. (1963) A Comparison of the Effects of Freezing and of Treatment with Hypertonic Solutions on the Structure of Nerve Myelin. *J. ultrastruct. Res.*, **8**, 264.

LE BEAU, J. (1962) Selective and Reversible Block of Cerebral Structures by Local Cooling. *Confinia neurol.*, **22**, 341.

MAYERS, P. D., TUSSING, G. & WENTZ, F. M. (1971) The Histological Reaction of Clinically Normal Gingiva to Freezing. *J. Periodont.*, **42**, 346.

MENZ, L. J. (1971) Structural Changes and Impairment of Function Associated with Freezing and Thawing in Muscle, Nerve and Leucocytes. *Cryobiology*, **8**, 1.

MORRIS, J. H., HUDSON, A. F. & WEDDELL, G. (1972) A Study of Degeneration and Regeneration in the Divided Rat Sciatic Nerve based on Electron Microscopy II. The Development of the " Regenerating Unit ". *Z. Zellforsch.*, **124**, 76.

NATHANIEL, E. J. H. & PEASE, D. C. (1963) Degenerative Changes in Rat Dorsal Roots during Wallerian Degeneration. *J. ultrastruct. Res.*, **9**, 511.

OSTERGARD, D. R., TOWNSEND, D. E. & HIROSE, F. M. (1967) The Treatment of Chronic Cervicitis by Cryotherapy. *Cryobiology*, **4**, 97.

PINNER-POOLE, B., TOMASULA, J. J., DECRESCITO, V. & CAMPBELL, J. B. (1966) Preliminary Observations of the Regenerative Potentials of the Spinal Cord after Cryogenic Surgery. *Proc. Ann. clin. Spinal Cord Inj. Conf.*, **15**, 1.

WALDER, H. A. D. (1970) The Effect of Freezing and Rewarming on Feline Brain Tissue: an Electron Microscope Study. In *The Frozen Cell*. Eds. G. E. W. Wolstenholme and M. O'Connor. London: Churchill. p. 251.

WHITTAKER, D. K. (1972) Cryosurgery of the Oral Mucosa: a Study of the Mechanisms of Tissue Damage. *D. Pract.*, **22**, 445.

WHITTAKER, D. K. (1973) An Experimental Study of the Effects of Cryosurgery on the Oral Mucous Membrane. *Ph.D. Thesis*, University of Wales.

WISNIEWSKA, K., SAMRA, K., WALTZ, J. M., TERRY, J. & COOPER, I. S. (1970) Microscopic Pattern of Healing of the Cryogenic Thalamic Lesion. *Cryobiology*, **6**, 354.