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An electron microscope study of the unmyelinated nerve fibres in normal baboon median nerves: negative effect of vitamin-B_{12} depletion

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INTRODUCTION

Captive primates fed vegetarian diets may become depleted in vitamin B_{12} and develop neurological defects in both peripheral and central nervous systems (Oxnard, 1967; Oxnard & Smith, 1966). The main lesion observed in the peripheral nervous system was segmental demyelination; Wallerian degeneration was much less common. There was no predominance of distal lesions and thus no indication of a 'dying-back' neuropathy (Torres, Smith & Oxnard, 1971).

The role that vitamin B_{12} plays in the maintenance of neural tissue has not been established. It has been suggested that because the vitamin is essential for the metabolism of choline and methionine (Williams, 1955) it may help maintain the myelin sheath and the function of related axons. Vitamin B_{12}, cyanide and thiocyanate are all metabolically interlinked and it has been suggested that at least some of the neurological changes observed in vitamin B_{12} depletion may be caused by cyanide neurotoxicity (Wilson & Langman, 1966; Matthews & Wilson, 1971).

In view of these findings the peripheral nerves of five groups of baboons have been investigated. All the groups were fed a diet deficient in vitamin B_{12}, but different groups were given injections of vitamin B_{12} (hydroxocobalamin), cyanide or thiocyanate.

This paper describes observations made on the unmyelinated nerve fibres, which have not been studied previously in vitamin B_{12}-depleted animals.

Torres *et al.* (1971) showed that the main peripheral nerve lesion in vitamin B_{12}-depleted primates was segmental demyelination. Thus, Schwann cells rather than axons were primarily affected, although myelin changes may well have a secondary effect on the associated axon. Unmyelinated fibres are closely associated with Schwann cells, but are unlike myelinated fibres in that variable numbers of axons are associated with the same Schwann cell. In the developing peripheral nerve both the immature myelinated fibres (as yet without a myelin sheath) and the immature unmyelinated fibres show a similar association with Schwann cells and it is not possible to distinguish which axons will become myelinated and which will remain unmyelinated (Peters & Muir, 1958; Ochoa, 1971; Webster, Martin & O'Connell, 1973).

MATERIALS AND METHODS

The work described in the present paper is part of a larger experiment (Matthews & Linnell, 1971; Matthews & Wilson, 1971) involving a total of 36 baboons. Many details of the maintenance of these animals have been given by Crees & Payne (1973) and full details of the diet will be described elsewhere by the Wellcome Trust Research Group (Crampton *et al.*, to be published). It should, however, be noted that the basic vitamin B_{12}-depleted diet never contained more than 3 μg vitamin B_{12} per kg. Observations were made on the median nerves of 19 adult male animals, all of which were of a similar age. They had been captured as juveniles in East Africa and sent to England where they were kept in the British Industrial Biological Research Association (BIBRA) Laboratories at Carshalton, Surrey, from 1968 until they were killed in either late 1972 or early 1973. The animals were kept in separate cages and were in five experimental groups as follows.

Group 1. Four animals (from a total of seven animals) were fed on a basic vitamin B_{12}-depleted diet, supplemented with vitamin B_{12} (10 μg of hydroxccobalamin intramuscularly every week).

Group 2. Five animals (from a total of eight animals) were fed on the basic depleted diet, supplemented with vitamin B_{12} (as Group 1) but were also receiving potassium cyanide injections (1 mg per kg body weight subcutaneously) five days per week.

Group 3. Three animals (from a total of seven animals) were fed the basic depleted diet and were not supplemented in any way.

Group 4. Two animals (from a total of four animals) were fed the basic depleted diet, unsupplemented with vitamin B_{12}, but received potassium thiocyanate injections (40 μmol per kg body weight subcutaneously) 5 days per week.

Group 5. Five animals (from a total of ten animals) were fed the basic deficient diet, unsupplemented with vitamin B_{12}, but received potassium cyanide injections (as in Group 2).

Throughout the experiments all animals remained in good health and weight gains in each group were very similar. In animals from the groups not supplemented with hydroxocobalamin injections, the plasma total vitamin B_{12} declined rapidly at first and within 9 months had fallen to levels below the normal limit for man. Plasma methyl cobalamin became disproportionately reduced, as in human vitamin B_{12} deficiency (Linnell, Mackenzie, Wilson & Matthews, 1969; Linnell, Hoffbrand, Peters & Matthews, 1971); methylmalonate excretion after valine was slightly increased. Haematological evidence of vitamin B_{12} depletion was absent. At necropsy organs from the vitamin B_{12}-depleted animals contained only 25–50 % of the total vitamin B_{12} of organs from control animals. Small amounts of cyanocobalamin were found in most tissues from all groups. Details of the cobalamin and other studies are to be reported elsewhere.

The animals were killed at the BIBRA Laboratories in six batches over a period of 8 months and those killed at any one time were chosen at random. All the animals were first tranquillized by an intramuscular injection of 1·5 mg per kg body weight of phencyclidine and then given an intravenous injection of 10–20 mg/kg body weight of Nembutal. It was decided not to perfuse–fix the peripheral nerves, as this

would have interfered with biochemical observations being made elsewhere on a wide variety of the baboons' tissues.

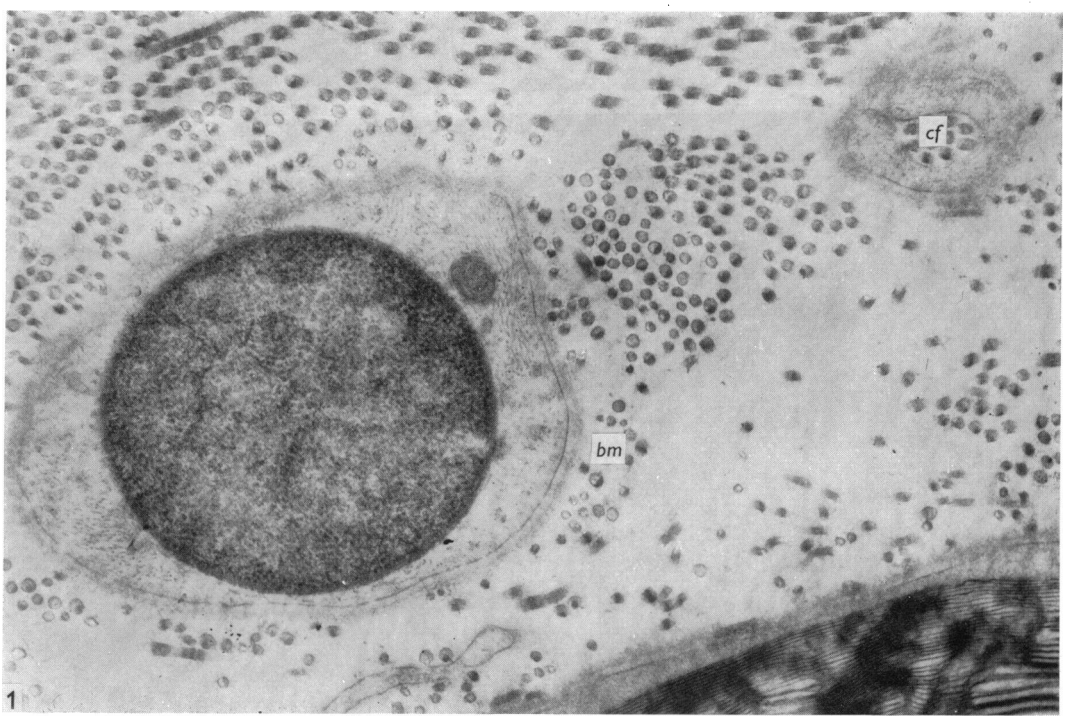
The distal part of the median nerve was used because it was found by trial and error that the conditions under which the necropsies were carried out enabled this nerve to be fixed and removed most conveniently. The best fixation possible under the circumstances was achieved by exposing the distal part of the median nerve near to the wrist (proximal to the flexor retinaculum) and flooding the nerve *in situ* with fixative for about 5 minutes. A 2–3 cm segment of nerve was then carefully removed, left in fixative for at least 2 hours, and then cut into 1–2 mm pieces that were left in fixative overnight. The fixative was 1·5% glutaraldehyde in 0·11 M s-collidine buffer at pH 7·4 (Weibel, 1969). After fixation the nerve segments were allowed to wash in 0·2 M s-collidine buffer for 1–2 days before post-fixation in osmium tetroxide fixative (Caulfield, 1957), dehydration in alcohol and embedding in Araldite. Thin sections were cut on a Reichert OmU2 ultramicrotome, mounted on uncoated copper grids, double-stained with lead citrate and uranyl acetate and examined using an AEI EM 6B electron microscope.

For quantitative measurements, photographs of transverse sections were taken at a magnification of ×2000 and printed at a final magnification of ×8000. The magnifications were checked by taking photographs of a calibrated grid. In transverse sections myelinated and unmyelinated fibres had a patchy distribution as did those in human sural nerve (Ochoa & Mair, 1969*a*). In order to eliminate any effect of this, the photographs were taken adjacent to one another in a linear manner so that the area of section photographed was a strip one plate in width at ×2000. The position on the section of the first photograph to be taken was decided at random. The area of nerve section photographed varied for each nerve from 13000 μm^2 to 54000 μm^2 but in most cases an area of 16000–18000 μm^2 was taken. The diameters of unmyelinated fibres on the photographic prints were measured using a Zeiss TGZ 23 without making any correction for shrinkage during processing. The number of unmyelinated axons associated with each Schwann cell and the number of myelinated fibres present were also counted. Search for qualitative changes of the nerve structures were carried out on the same low power photographs as those used in the quantitative study, and on photographs taken at higher powers after scanning the sections directly under the electron microscope. The method of fixation used did not give as satisfactory results as some other workers have obtained using perfusion fixation, and the larger myelinated fibres showed some artefactual changes; however, for the most part the unmyelinated fibres and their associated Schwann cells did not appear to be affected.

RESULTS

Qualitative examination

The unmyelinated fibres did not show unequivocal evidence of degeneration in either the axons or the associated Schwann cells. In the nerves from all of the animals a few swollen unmyelinated axons with watery cytoplasm were seen; such swelling was most likely a fixation artefact and probably would not have been present if perfusion fixation had been possible.



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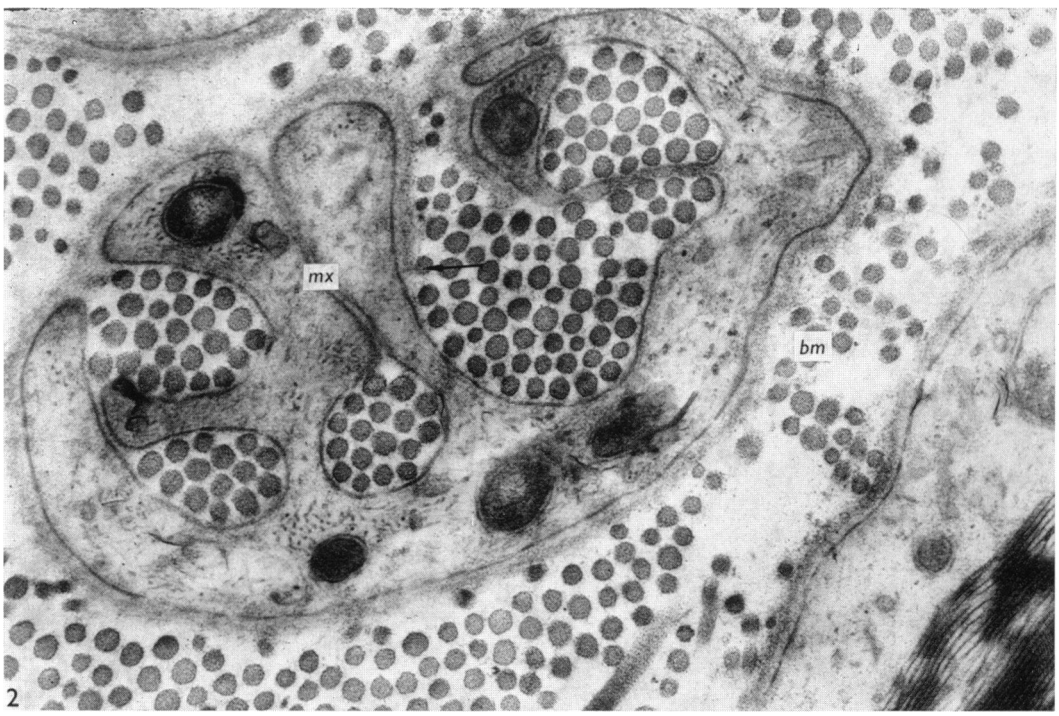

2

Bundles containing several Schwann cell processes, surrounded by a single basement membrane, were present in all nerves examined. These Schwann cell processes were almost always associated with unmyelinated axons, although occasionally a process unassociated with an axon was seen. Bundles of Schwann cell processes in which no process was associated with an axon were not seen. There were occasional small, isolated Schwann cell processes, and their profiles were sometimes irregular in shape (Fig. 2), but were often circular and contained a nucleus (Fig. 1). The presence of a nucleus and surrounding basement membrane, and the fact that similar cell profiles which were associated with unmyelinated fibres were also seen, indicated that these were almost certainly Schwann cell processes. In two nerves from Group 5, isolated Schwann cell processes occurred in greater numbers than in other animals. In the other three animals in Group 5 isolated Schwann cell processes were seen only occasionally, as in all other baboons.

Unmyelinated axons with more than one mesaxon were a feature of the nerves of almost every animal examined (Fig. 3). Bifurcated mesaxons were seen with a lesser frequency, but they were observed in animals from all groups except the vitamin B_{12}-depleted and thiocyanate-supplemented Group 4; this is probably not a significant observation in view of the infrequency of bifurcated mesaxons. Reversed mesaxons (Fig. 4) were rare. Pockets of collagen in invaginations of Schwann cell cytoplasm were occasionally noted in control and experimental animals, both in Schwann cells associated with unmyelinated nerve fibres (Fig. 5) and in Schwann cell processes unassociated with any fibres (Fig. 2).

Quantitative examination

The mean number of axons per mm^2 (Fig. 6) for each of the treated groups did not differ significantly from that of the normal group (Group 1), which had a mean of 25987 axons per mm^2 (Student's t test). The vitamin B_{12}-depleted Group 3 which differed from the control group by the greatest number of axons per mm^2 did so with a non-significant probability of $0{\cdot}1 < P < 0{\cdot}2$.

The mean unmyelinated nerve fibre diameter for each of the treated groups (Fig. 7) was not significantly different from that of the normal group mean of 0·82 μm (Student's t test).

The percentage distribution of unmyelinated nerve fibre diameters in the different size categories is shown in Figure 8. The distribution of diameters of each of the treated groups was tested against that of the normal group, using χ^2, but no significant differences were found. A unimodal unmyelinated fibre spectrum was present

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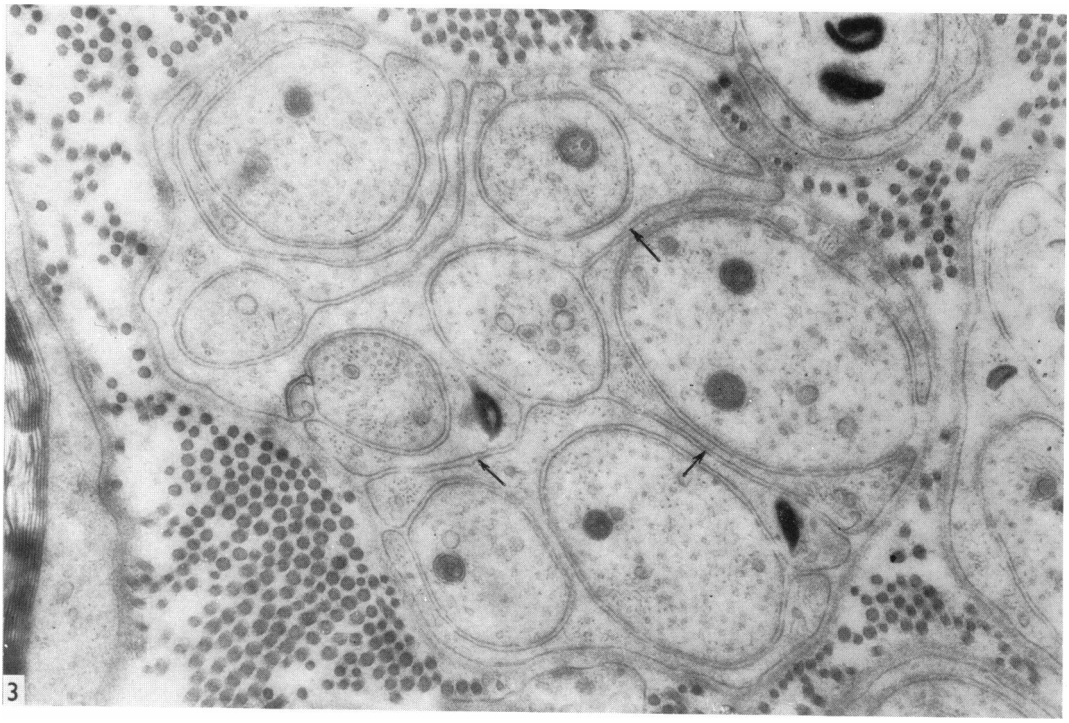
Fig. 1. Two isolated Schwann cell processes. The larger contains a nucleus and a mitochondrion and the smaller envelops a group of collagen fibres (*cf*). Both processes are surrounded by a basement membrane (*bm*) but the enclosed collagen fibres are not. Uranyl acetate and lead citrate ×42900.

Fig. 2. A small isolated Schwann cell process surrounds, or partially surrounds, several groups of collagen fibres, some of which are suspended by a short 'mesaxon' (*mx*). The Schwann cell process is surrounded by basement membrane (*bm*) but the collagen-containing pockets are not, although the basement membrane extends a short distance into the largest pocket (arrow). ×54300.



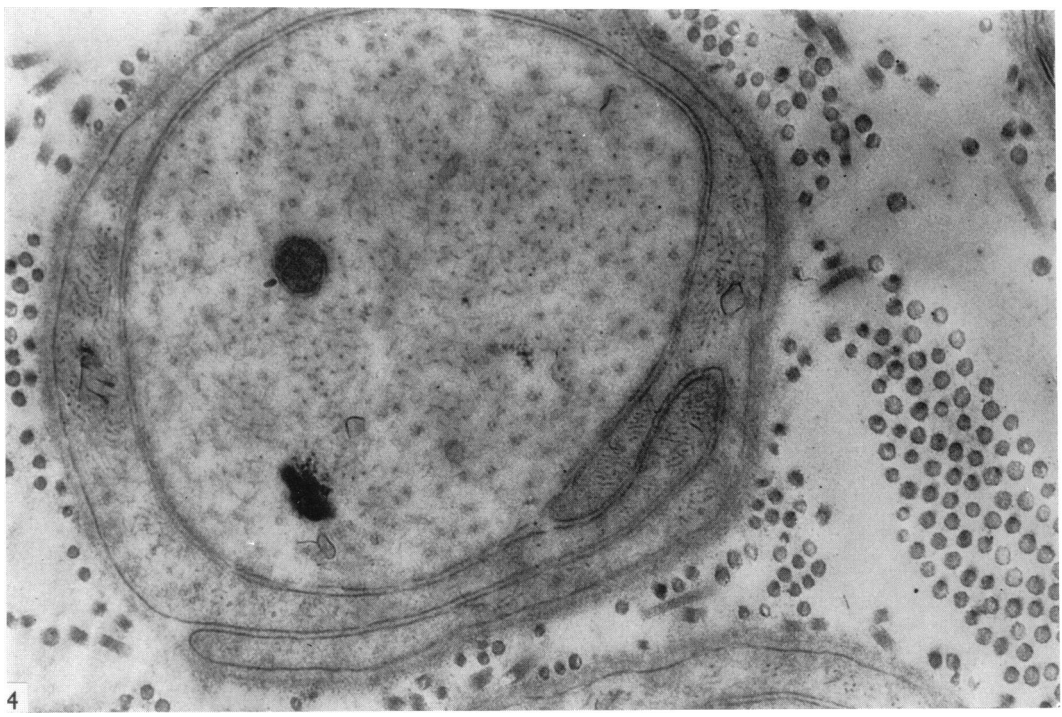


Fig. 3. A Schwann cell process is associated with several unmyelinated axons, one of which is associated with three separate 'mesaxons' (arrows). × 37 900.

Fig. 4. A single unmyelinated axon is surrounded by a Schwann cell process. The 'mesaxon' is turned back on itself or 'reversed'. × 56 000.

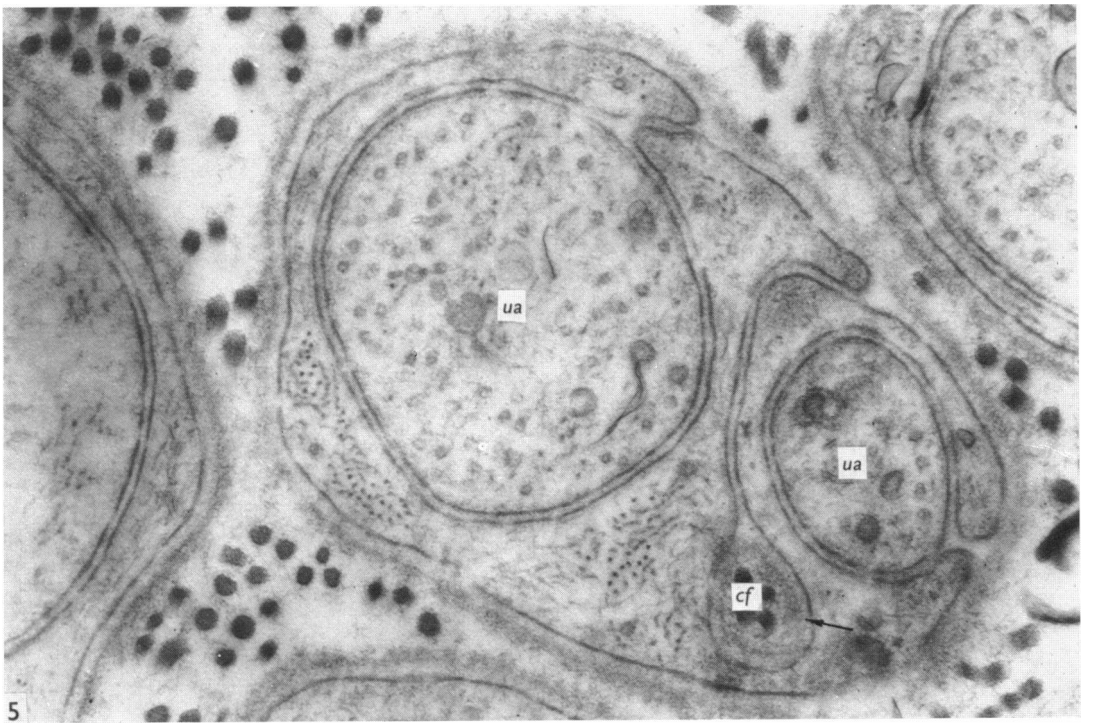


Fig. 5. A single Schwann process is associated with two unmyelinated axons (*ua*) and a group of collagen fibres (*cf*). The collagen fibres are suspended by a long 'mesaxon' and appear to be surrounded by a basement membrane (arrow). × 59700.

in all the nerves. The peak diameters varied only slightly between the treated groups and all were between 0·5 and 0·8 μm. The fibres ranged in diameter from 0·2 μm to about 3·8 μm, but very few were greater than 2 μm.

The average ratio of unmyelinated nerve fibres to myelinated nerve fibres (Fig. 9) in each of the treated groups did not differ significantly from that of the normal group (Student's *t* test).

The percentage distribution of Schwann cells associated with different numbers of unmyelinated nerve fibres is illustrated for each of the experimental groups in Figure 10. The distribution observed for each treated group was compared with that observed for the normal group, but no significant difference was indicated (χ^2). The greatest number of Schwann cells (excluding those associated with myelinated fibres) was associated with a single fibre and most were associated with five or less unmyelinated fibres.

In considering the results of these quantitative investigations it should be noted that the numbers of animals in each group were not sufficiently great to permit detection of any *slight* changes which might have been brought about by the various treatments.

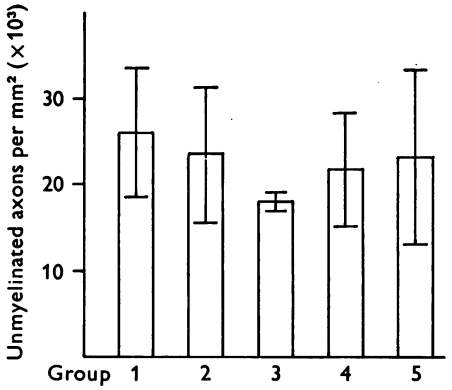


Fig. 6

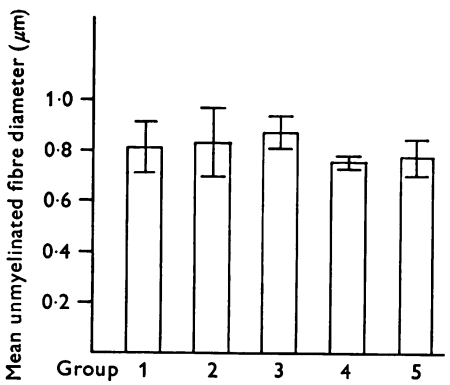


Fig. 7

Fig. 6. The mean number of axons per mm² of transverse sectional area of the median nerve, in each of the experimental groups. Long clamps indicate the standard deviation.

Fig. 7. The mean diameter of unmyelinated axons in nerves from each experimental group.

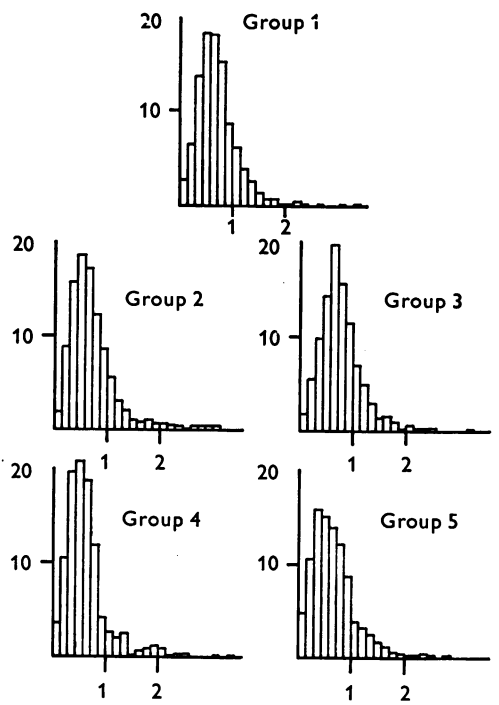


Fig. 8. The percentage distribution of different unmyelinated axon diameters in each experimental group (abscissa: unmyelinated fibre diameter in μm; ordinate: percentage of unmyelinated fibres).

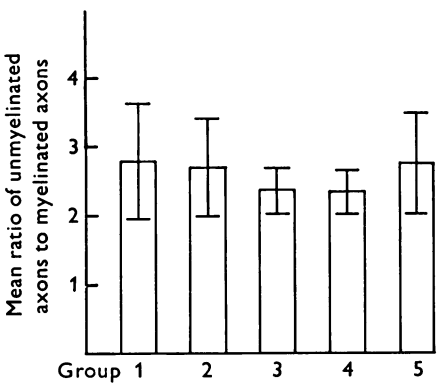


Fig. 9

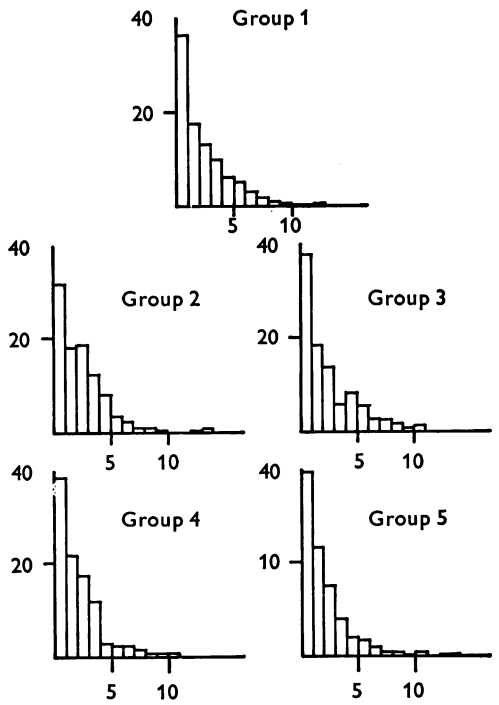


Fig. 10

Fig. 9. The mean ratio of unmyelinated axons to myelinated axons in each experimental group.

Fig. 10. The percentage distribution of Schwann cells associated with different numbers of unmyelinated axons in each experimental group (abscissa: number of unmyelinated fibres per Schwann cell; ordinate: percentage of Schwann cells).

DISCUSSION

This work has not shown any significant differences between normal and test groups. It has, however, established base-line information on normal baboon peripheral nerves which should be of value to subsequent workers. A recent investigation carried out in Kenya on the effect of vitamin B_{12} depletion on baboons failed to produce detectable neurological signs in animals kept on a vitamin B_{12}-depleted diet for 2 years (Siddons, 1974), although earlier work had suggested that the neuropathies developing in captive primates on a vegetarian diet might have been caused by vitamin B_{12} deficiency (Oxnard, 1967; Oxnard & Smith, 1966).

Qualitative examination

The finding that bundles consisting of several Schwann cell processes were enclosed within the same basement membrane is not regarded as evidence of a pathological change. Such bundles were seen in all nerves examined and have been noted by others in the human medial cutaneous nerve: 'careful inspection of bundles of unmyelinated nerve fibres shows that larger bundles are almost always of several

Schwann cell processes enclosed by a basement membrane' (Gamble & Eames, 1964). Ochoa & Mair (1969*b*) described bands of Büngner devoid of any axons in elderly human nerves and Ochoa & Vial (1967) described similar structures in several chronic neuropathies. No such structures were seen in the present investigation, and in fact Schwann cell processes devoid of axons were uncommon.

The only difference between the nerves of the various experimental baboons was the increase in small isolated Schwann cell processes in two baboons from Group 5. The other three baboons in this group did not show any such increase. It is not certain what the significance of isolated Schwann processes is: they may be associated with Schwann cell proliferation, because during mitosis Schwann cells become long and spindle-shaped and at anaphase a slender process is produced by each daughter cell (Martin & Webster, 1973). However, there was no direct evidence of mitosis and the several examples seen of the presence of a nucleus almost filling a small process (Fig. 1) does not support the idea that these isolated profiles are sections of dividing cells.

Bifurcated mesaxons, multiple mesaxons and reversed mesaxons are not regarded as pathological features as they have been described in normal human nerves as well as in pathological human nerves by Ochoa & Vial (1967). Collagen pockets similar to those that were associated with some of the Schwann cells have also been described in normal human nerves by Gamble (1964) and by Ochoa & Mair (1969*a*) and in normal and pathological human nerves by Ochoa & Vial (1967). Thomas (1973) claimed that there is reason to believe that collagen pockets are the site of degenerated unmyelinated axons, and supports this claim with evidence that collagen pockets are a similar size to unmyelinated axons, that they increase in frequency with age (Sharma, 1971, cited by Thomas, 1973) and that they become more numerous in neuropathies. Electron micrographs of normal nerves showing partially invaginated groups of collagen fibrils (Fig. 2, and Eames & Gamble, 1970) and the long complex suspending mesaxons illustrated by Ochoa & Vial (1967) are against the idea that collagen pockets are the sites of unmyelinated fibre degeneration.

Quantitative examination

None of the five groups of baboons showed any appreciable change in the number of unmyelinated axons per mm^2 as seen in the transversely sectioned nerve. The density of unmyelinated fibres in baboon nerves has not been published previously but Ochoa & Mair (1969*a*, *b*) found that in *human* sural nerve the average density was 29000 per mm^2 for a group of five individuals between 15 and 32 years old, and approximately 25000 per mm^2 for two males 35 and 59 years of age. When corrected to exclude regenerating nerve sprouts, the values for these older men were between 19000 and 21000 per mm^2. The only reports of a pathologically induced reduction in the numbers of unmyelinated fibres in a nerve appear to be those by Dyck & Lambert (1968) and Thomas & King (1974), who examined nerve biopsies of patients with amyloidosis and found that unmyelinated fibres were almost completely lost; by Ochoa (1970), who examined sural nerve biopsies of patients with isoniazid neuropathy and found that the numbers of unmyelinated fibres were considerably reduced when corrected for regenerating sprouts; and by Aguayo,

Nair & Bray (1971), who examined the sural nerve in the Riley–Day syndrome and found greatly decreased numbers.

The mean unmyelinated nerve-fibre diameter and the percentage distribution of unmyelinated fibres in different size groups did not differ significantly between control and treatment groups. This indicates that there has not been an increase in small fibres, such as might be expected if axonal sprouting had taken place, or indeed if there had been a selective increase or decrease of any size group of unmyelinated axons (e.g. Bray, Aguayo & Peyronnard, 1971; Ochoa, 1970). Fowler & Ochoa (personal communication) found that the unmyelinated fibres of the medial popliteal nerve of the baboon have a unimodal fibre-diameter spectrum with a peak at 1 μm, and that the fibre-diameters lay within a range of 0·2–2 μm. The findings of the present investigation were similar to these, though not identical; all animals in the present work show unimodal fibre-diameter spectra; the range of diameters was somewhat greater than that observed by Fowler & Ochoa, being from less than 0·2 up to 3·8 μm, although only a small number of fibres with diameters greater than 2 μm were observed. The peaks of the unmyelinated fibre-diameter spectra were between 0·5 μm and 0·8 μm, which are less than those observed by Fowler & Ochoa. This would seem to indicate that in baboons the unmyelinated fibre populations of the medial popliteal nerves, and the distal part of the median nerves, have slightly different fibre-diameter structures. Sex and age differences between the animals used in the different investigations could also have been a factor. In the present work only males were used and Fowler & Ochoa investigated only females. Ochoa & Mair (1969*a*, *b*) found that in human sural nerves the range of unmyelinated fibre-diameters was 0·2–3·0 μm and that the fibre-diameter spectra were unimodal with a peak at 1·4 μm. In older individuals the spectrum was bimodal, with a second peak at about 0·5 μm, this second peak being due to regenerating fibres formed subsequent to unmyelinated fibre degeneration. Although such changes may also be expected in normal ageing baboons they were not seen in those used in the present experiment, in which the baboons were all similar in age, thus excluding the possibility that normal age changes were being mistaken for changes induced by the experiment. An increase in the number of small unmyelinated axons due to axonal regeneration, resulting in a bimodal unmyelinated fibre-diameter spectrum, has also been described both in isoniazid neuropathy in human sural nerves (Ochoa, 1970) and following experimental transection of the rabbit anterior mesenteric nerve (Bray *et al.* 1971; Bray, Peyronnard & Aguayo, 1972).

The average ratio of unmyelinated to myelinated nerve fibres was not significantly different from that of the control group in any of the experimental groups. The ratio would be expected to change only if there was a selective loss or gain of any one type of fibre. The average ratio of unmyelinated to myelinated fibres varied from 2·3:1 to 2·8:1. This ratio has not previously been calculated for baboon peripheral nerve. Ranson, Droegenmueller, Davenport & Fisher (1935) examined several different human nerves and found great variation in the ratio of unmyelinated to myelinated nerves. In the median nerve at the wrist, a site very similar to that in which baboon median nerve was taken in the present investigation, they found the ratio to be 2·1:1, e.g. slightly less than the ratio in baboons. Ochoa & Mair (1969*a*) found that in human sural nerves from individuals between 15 and 32 years of age

the average ratio was 3·67:1 (2·59–4·03:1) and in a man of 59 years it was as high as 4·72:1. This latter high value was due to regenerating axonal sprouts, but when these were discounted the ratio fell to 3·83:1, which is similar to that in younger individuals. In sural nerves in the Riley–Day syndrome the ratio of unmyelinated to myelinated fibres changed from 4·4:1 to 0·38:1. This indicates a severe loss of unmyelinated fibres (Aguayo *et al.* 1971). In amyloidosis there is an almost complete loss of unmyelinated fibres (Dyck & Lambert, 1968); the numbers of myelinated fibres are also decreased, but not in proportion.

The percentage distribution of Schwann cells associated with different numbers of unmyelinated axons was similar in the control and experimental groups. Bray *et al.* (1971) showed that in regenerating anterior mesenteric nerves of rabbits there was an increase in the number of unmyelinated axons associated with each Schwann cell. Similarly, in regenerating nerves in rabbit skin autografts the mean number of unmyelinated axons associated with each Schwann cell was increased compared with donor and recipient controls (Orgel, Aguayo & Williams, 1972). The absence of such changes in the median nerve of the baboons examined in the present investigation reinforces the impression gained from the other observations that the experimental conditions have not induced any significant degenerative or regenerative lesions in the unmyelinated fibres of peripheral nerves*.

SUMMARY

The unmyelinated nerve fibres in the distal part of the median nerve of normal baboons (Group 1) and of baboons kept on diets deficient in vitamin B_{12}, and supplemented with potassium cyanide and potassium thiocyanate injections (Groups 2–5), have been examined under the electron microscope. Qualitative and quantitative observations on the distribution, size and Schwann cell relationships of the unmyelinated fibres were made. There were no significant differences between the nerves of the normal and experimental groups. New data about normal baboon peripheral nerve are presented and discussed.

This work was financed by the Wellcome Trust (to which the author is deeply grateful) and is part of the work of a Research Group under the Chairmanship of Professor R. H. S. Thompson, F.R.S. The animals were housed at the laboratories of the British Industrial Biological Research Association, and the author is indebted to the Director, Dr R. F. Crampton. Members of the Research Group whose contributions in different branches of laboratory medicine have made the above findings possible are Dr I. Gaunt, Dr Ruth Harris, Professor M. J. S. Langman, Dr J. Linnell, Professor D. M. Matthews, Professor D. Mollin, Professor W. T. Smith, Dr A. Waters and Dr J. Wilson; Dr Wilson has coordinated the multidisciplinary activities of the Group. Thanks are also due to Dr T. Fowler and Dr J. Ochoa for allowing me to mention their unpublished findings, to Mrs Ruth Glad for technical assistance and to Miss G. L. Parkinson for typing the manuscript.

* Since acceptance of the present paper the work of Fowler and Ochoa has been published (Fowler, T. J. & Ochoa, J. 1975. Unmyelinated fibres in normal and compressed peripheral nerves of the baboon. A quantitative electron microscopic study. Neuropathology and Applied Neurobiology, **1**, 247–265).

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