ELECTRON MICROSCOPE AND X-RAY DIFFRACTION STUDIES OF THE EFFECTS OF DEHYDRATION ON THE STRUCTURE OF NERVE MYELIN

I · Peripheral Nerve

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ABSTRACT

The dehydration of frog sciatic nerve has been studied by allowing specimens to become partially or fully dried before fixation and preparation for electron microscopy. Low magnification electron micrographs of OsO_4-fixed preparations showed marked tissue shrinkage which could be correlated quantitatively with the loss of water during the preliminary drying. $KMnO_4$-fixation appeared to cause a rehydration of the dried tissue. Higher magnification electron micrographs of the OsO_4-fixed preparations showed a sequence of modifications of the myelin layers which could be correlated with changes in the small-angle x-ray diffraction data which were recorded during drying. An intermediate stage of drying was characterised by a partial collapse of layers and a disappearance of the intraperiod dense line in some regions of the myelin sheath. Continuity between collapsed and non-collapsed layers was maintained throughout the sheath. The fully dried preparation showed two main modifications of the myelin layers. In many regions the layers (principal layers) resembled those of normal preparations, but showed an intensification and frequently a doubling of the intraperiod dense line. In addition, there was a very extensive system of fine (40 A periodicity) dense layers, some of which could be demonstrated to be continuous with the principal layers. In such cases it was observed that two of the fine layers were related to each principal layer. The correlation between diffraction data and electron microscope data is discussed, and some speculations are made concerning the molecular significance of the observations.

The effects of dehydration on the molecular structure of peripheral nerve myelin have previously been studied by small-angle x-ray diffraction methods (2, 7) and the kinetics of the drying process have also been examined (7). Complementary electron microscope studies have now been made, and correlation with the earlier work has been facilitated by further small-angle x-ray diffraction studies. A preliminary mention of these experiments was made in an earlier publication (9).

MATERIALS AND METHODS

Frog sciatic nerve was used in all experiments.

Freshly isolated segments of nerve were suspended under slight tension and allowed to dry freely at

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room temperature for various periods of time before immersion in chilled fixative (1 per cent OsO_4 in veronal-acetate buffer (pH 7.2) for 4 hours, or 0.6 per cent $KMnO_4$ (14) in veronal-acetate buffer for 3 hours). In every case a segment of the fixed tissue was set aside for the immediate recording of the small-angle diffraction pattern, and the remainder was dehydrated in a graded alcohol series and embedded in araldite casting resin (13). The embedded nerve preparation was also examined by x-ray diffraction. The x-ray cameras have been described in previous publications (4).

Thin sections for electron microscopy were cut using glass knives and either a Porter-Blum or a Philips ultramicrotome. The sections were deposited on carbon-covered specimen grids and examined in a Siemens electron microscope, type Elmiskop I.

A study was also made of the effects of re-immersion of the partially dried nerve specimens in Ringer's solution for 60 minutes, and of subsequent re-drying. Desiccator-dried specimens and preparations subsequently treated with dry acetone were also investigated.

In separate experiments, serial x-ray diffraction patterns were recorded during the drying of the nerve specimen. This was a repetition of earlier experiments but the camera resolution was higher than could be obtained in the original work. The duration of each exposure was 15 or 20 minutes.

A further correlation with previous work was established by simultaneously repeating experiments in which the rate of loss of weight of such preparations was measured.

This publication also provides an opportunity to report a previously unpublished study of the drying of nerve preparations in which the changing diffraction pattern was scanned every 3 minutes using a Geiger counter technique. This study was carried out at the Venezuelan Institute for Neurology and Brain Research in collaboration with Dr. H. Fernández-Morán.

RESULTS

X-Ray Diffraction Data

Diffraction Studies during Drying: The x-ray diffraction studies of unfixed material and the kinetics of the drying process were in complete agreement with earlier experiments (2, 7). The higher resolution achieved in these recent experiments has established that there is little change in the intensity of the first order diffraction of the principal structural unit during the intermediate stages of drying and that no new reflections occur in the 100 to 150 A region.

The experiments in which Geiger counter recording was used to follow the drying changes, showed conclusively that the apparent intensification of the third order reflection in the early stages of drying was due to the appearance of a new reflection at a spacing only slightly greater than that of the normally weak third order diffraction of the principal structural unit. This new reflection corresponded to a spacing very close to 60 A.

OsO_4-Fixed, and Fixed and Embedded Preparations: The diffraction data from the corresponding fixed, and fixed and embedded material, and also the electron microscope observations, permitted

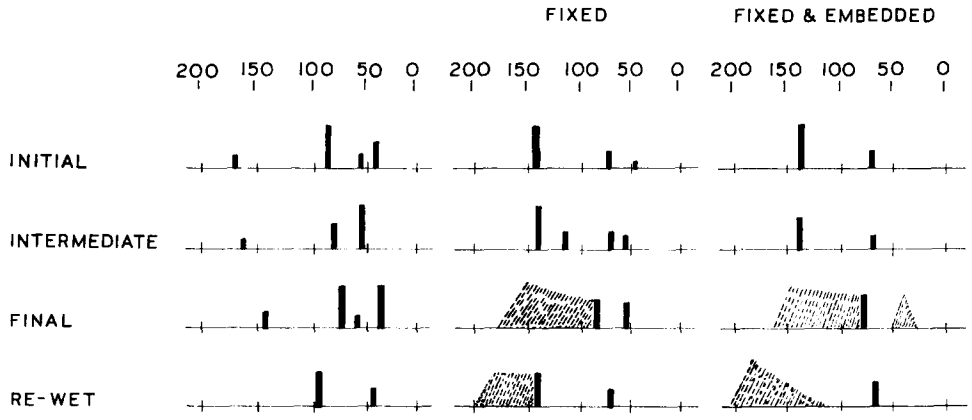


FIGURE 1

Diagrammatic summary of small-angle diffraction data. The diffraction spacings in A units, intensities, and definitions are indicated for each type of diffraction pattern recorded. Diffuse scatter is represented by intermittent line shading.

the distinguishing of only one well defined intermediate stage of drying, and the results presented in Fig. 1 are, therefore, referred to a simplified subdivision of the drying series.

The diffraction pattern of nerve which had been fixed with osmium tetroxide immediately following isolation showed first, second, and third order diffractions of a fundamental repeat of about 148 A (Fig. 2 *d*) (6). The corresponding araldite-embedded preparation gave a more diffuse pattern showing only first and second order diffractions of a slightly lower fundamental period.

Nerve specimens fixed at intermediate stages of drying showed two additional small-angle x-ray reflections which could be considered as first and second order diffractions arising from a new fundamental repeat of about 115 A (Fig. 3 *g*). These additional diffractions first became apparent after the nerve specimens had undergone a preliminary drying period of 15 to 30 minutes before fixation. Their intensities gradually increased as the preliminary drying period was extended, but the diffractions relating to the principal structural unit remained equally intense. In the diffraction patterns of the fixed and embedded specimens diffractions corresponding to the additional structural unit were never clearly distinguished.

Many preparations which had been dried for 40 to 80 minutes before fixing gave poorly defined diffraction data, but ultimately a further well defined pattern could be distinguished which appeared to be typical of specimens in the final stages of drying. The pattern showed considerable small-angle scatter, but diffractions at about 85 and 57 A could be clearly distinguished. The diffraction patterns of the corresponding embedded preparations showed a sharp reflection at 75 to 80 A and a rather more diffuse one at 40 to 45 A (Fig. 5 *c*). This latter reflection provided the most readily identifiable characteristic of the final stages of drying.

Treatment of desiccator-dried nerve specimens with acetone under various conditions produced no further changes in diffraction data which might be attributed to a completion of the drying of the tissue. The effects of lipid extraction by the acetone under some of the applied conditions will be considered separately.

Rehydration: Present studies have supported the earlier conclusion (2) that the structural changes associated with the drying process become irreversible approximately at the stage characterised by the reappearance of a 40 to 45 A band in the diffraction pattern of the drying preparation. Specimens dried to this limit and then re-immersed in Ringer's solution gave diffraction patterns which were indistinguishable from that of the fresh nerve, and the patterns of the corresponding OsO_4-fixed and fixed and embedded preparations also indicated complete reversibility. Thus the changes associated with the intermediate stage as represented in Fig. 1 are considered to be reversible.

Diffraction data derived from preparations which had been taken to a more advanced state of drying before re-immersion in Ringer's solution differed significantly from the initial data. Data from such re-wet preparations are included in Fig. 1.

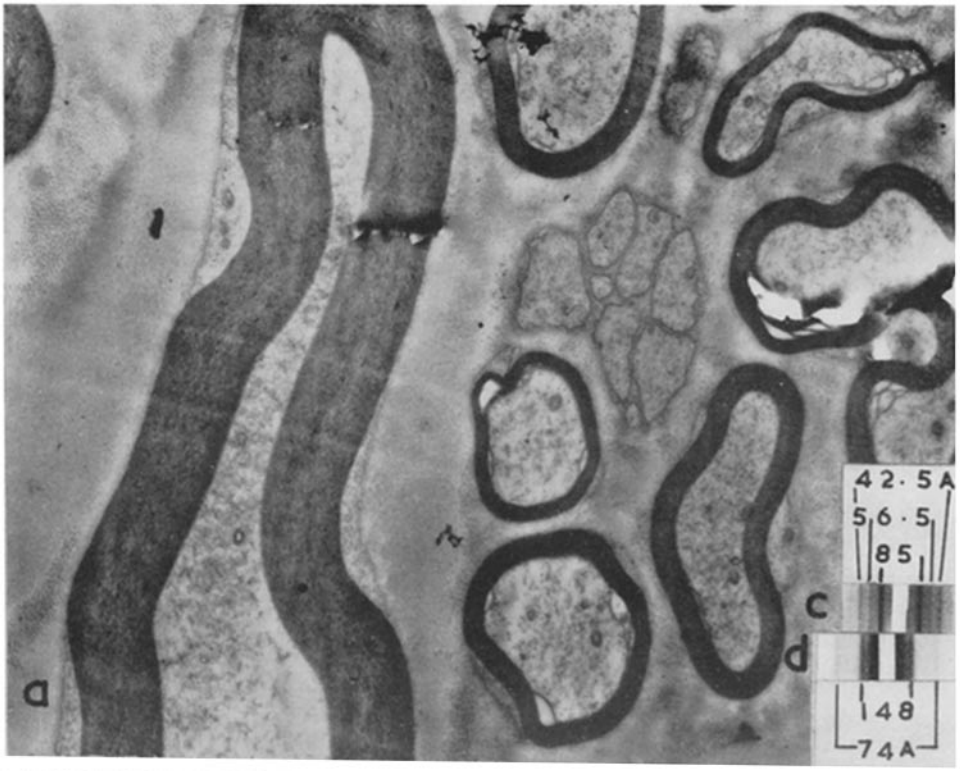
$KMnO_4$-Fixed Preparations: Preparations fixed with buffered $KMnO_4$ solution immediately after isolation gave a diffraction pattern showing first and second order reflections corresponding to a 150 A repeating unit. The first order was partially obscured by small-angle x-ray scatter, but the second order was intense and well defined. An essentially identical pattern was obtained from specimens which had been dried for an hour or even more before fixation, but after more prolonged preliminary drying a new fixed nerve diffraction pattern could be distinguished. This featured two fairly sharp diffractions at 90 to 100 A and 55 to 75 A. The diffraction patterns obtained from the corresponding araldite-embedded preparations differed from those described for the fixed specimens mainly in that the diffraction bands were not so well defined.

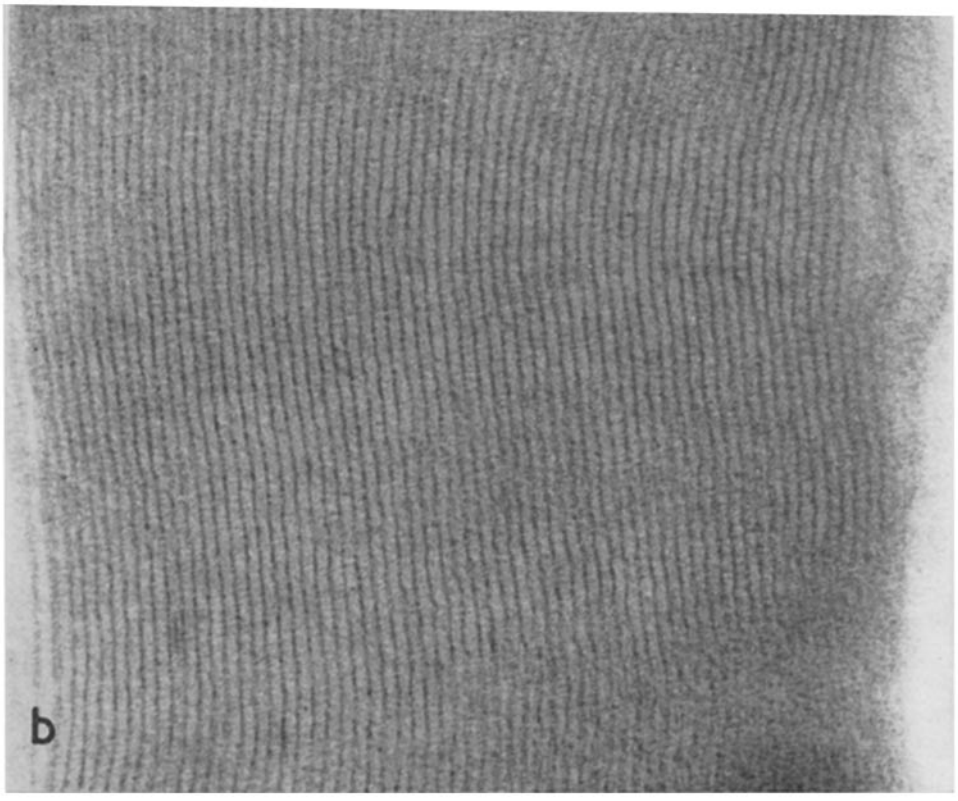
ELECTRON MICROSCOPY

OsO_4-Fixed Preparations

Changes in Relative Volumes of Nerve Components: Striking changes in the relative areas occupied by the different nerve components were apparent in electron micrographs of cross-sections of nerve preparations representing various stages of drying. In order to facilitate correlation with the over-all shrinkage of the tissue as deduced from weight loss measured in parallel experiments, these relative areas were considered as an acceptable approximation to the relative volumes, and they are used as such in subsequent calculations.

Measurements made on five normal preparations (*i.e.* those fixed immediately following


42·5A
56·5
85
c
d
48
74A
a


b

isolation) used as controls in these dehydration experiments, showed that myelinated fibres accounted for about 50 per cent of the tissue volume, and the average myelin to axon area ratio deduced from a consideration of about 100 myelinated fibres in these preparations was approximately 3:2. The electron micrograph reproduced in Fig. 2 *a* is typical of these preparations.

After a preliminary drying period of 20 to 30 minutes (corresponding approximately to the beginning of the intermediate stage as indicated in Fig. 1) the contribution of the myelinated fibres to the total tissue volume had increased to about 80 per cent and the myelin to axon ratio to 7:1 (Fig. 3 *a*). In the fully dried tissue the corresponding figures were 90 per cent and 10:1. Figs. 4 *a* and 5 *a* both relate to this final stage of dehydration.

Preparations which had been completely dried and then rehydrated before fixation (Fig. 8 *a*) showed only a partial re-expansion of the tissue and the myelin to axon ratio remained considerably higher than in the normal preparation.

Changes in the Myelin Layers: High magnification electron micrographs of the normal preparation showed a very precise and uniform layering of the myelin sheaths of the nerve fibres (Fig. 2 *b*). The thickness of the repeating layer was invariably in the region of 115 A and there was no significant variation between small and large fibres. The repeating layer consisted of a principal dense line about 30 A thick and a broad, light zone. An intraperiod dense line was frequently observed, but this did not seem to be a constant feature of the normal preparation.

Preparations which had undergone a preliminary drying for periods up to 20 minutes generally showed layering which was indistinguishable from the normal. However, at about this stage of drying the electron micrographs began to reveal regions in which the myelin layering was collapsed to about two-thirds of the normal thickness (Figs. 3 *c*, *d*, and *e*). In such preparations the principal layering retained the normal dimension of about 115 A but now showed an intense and well defined intraperiod dense line (Fig. 3 *b*). The definition of the main dense line was also improved. The dense line of the collapsed layering appeared to be slightly broader than that of the principal layering but was undoubtedly continuous with it. The collapsed layers showed no intraperiod dense line.

The extent of this collapsing of the myelin layers increased as the preliminary drying period was extended but never affected more than about one-half of the layering. Collapsed regions were not readily detected in the smallest fibres but among the larger fibres the collapsed regions appeared never to account for more than about 50 per cent of the total in any one fibre and to show no systematic distribution.

At a later stage, when the low magnification electron micrographs (Fig. 4 *a*) seemed to indicate a fragmentation of the myelin, the higher magnification micrographs (Figs. 4 *b* and *c*; Fig. 7) showed significantly different modifications of the myelin layering. Again a principal layering could be distinguished but the layers were somewhat thicker than those of the normal preparation. In preparations which had been dried for about 2 hours before fixation, the electron micrographs showed principal layers averaging 145 A in thickness, but, in other cases, particularly where drying had been completed in a desiccator or by means of cold acetone, the layer thickness was as high as 160 A. The intraperiod dense line was prominent but somewhat variable both in intensity and in

FIGURE 2

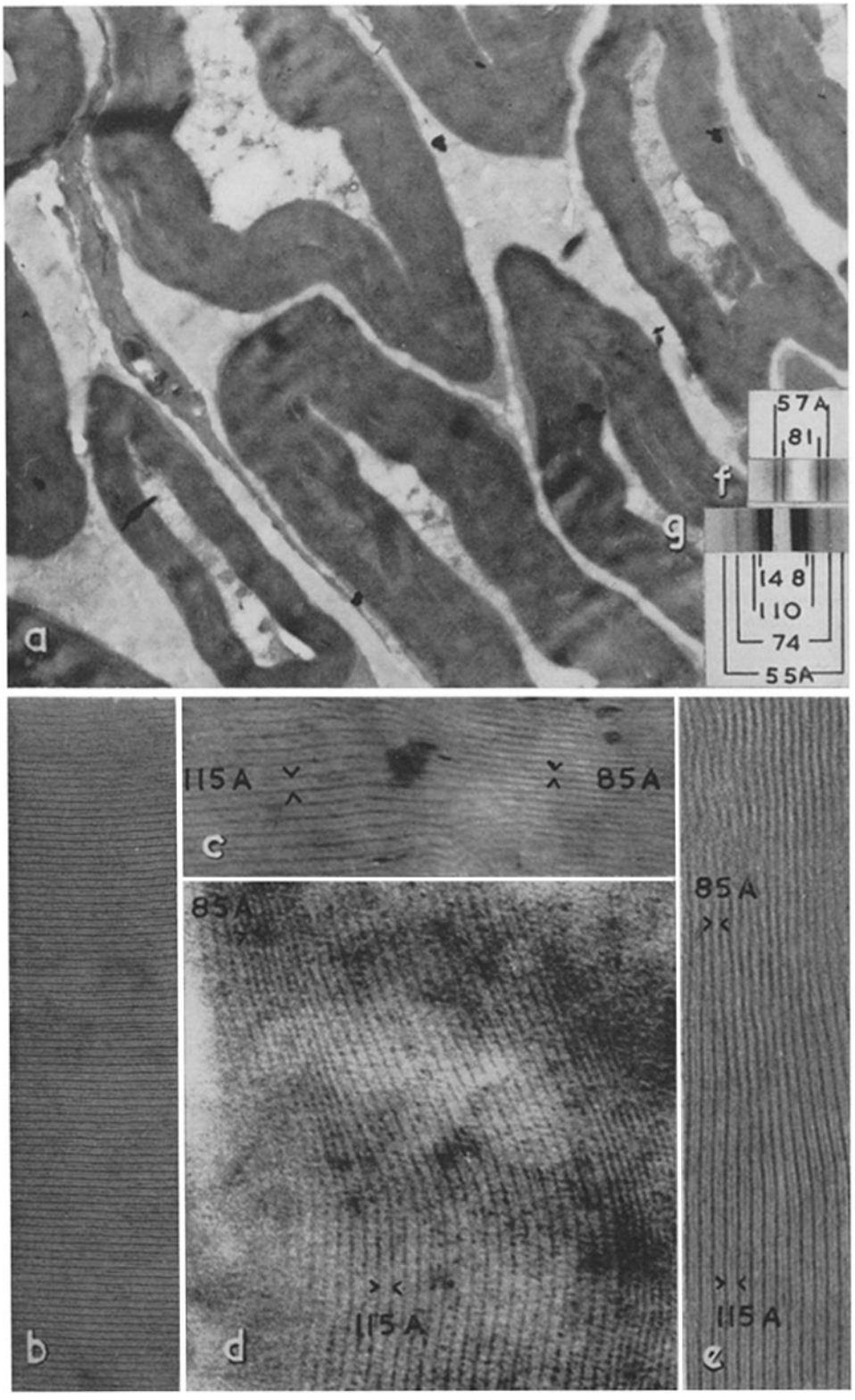
Electron micrographs of "normal" osmium tetroxide–fixed frog sciatic nerve preparation and related small-angle x-ray diffraction patterns.

a. Low magnification electron micrograph showing a large myelinated nerve fibre, several small ones, and also unmyelinated nerves. × 10,000.

b. High magnification electron micrograph of a section of myelin sheath. Layer spacing ~ 115 A. × 160,000.

c. Small-angle x-ray diffraction pattern recorded from frog sciatic nerve during the first fifteen minutes of drying.

d. Small-angle x-ray diffraction pattern of a nerve preparation fixed with OsO_4 after a preliminary drying period of up to 15 minutes.


a
57A
81
f
g
148
110
74
55A
115A
85A
c
85A
d
115A
b
85A
e
115A

thickness. In some regions main dense lines and intraperiod dense lines were hardly distinguishable (Fig. 4 *b*, upper right), and when the layer thickness was of the order of 160 A the intraperiod dense line was often seen to be a doublet (Fig. 7 *b*). These regions of principal layering were the ones that appeared dense in the low magnification electron micrographs (Fig. 4 *a*) and would be estimated to account for about 50 per cent of the myelin volume.

The remaining less dense zones, when studied at high magnifications, were found to feature a very fine layering which gave a periodicity of about 40 A. This period was divided about equally between dense and light bands. A frequently encountered additional feature of such regions consisted of irregular strands of high density superimposed on the patches of fine layering.

A close study was made of the boundaries between the principal and fine layering, and whilst in most cases the layering in the boundary region was ill defined, there were many cases such as the one illustrated in Fig. 7 *b* where a continuity between principal and fine layers was clearly indicated. Here the transition from principal to fine layering appeared to involve a collapsing of the principal layer to about 80 A together with a narrowing and intensification of the intraperiod line so as to halve the periodicity.

Rehydration: Electron micrographs of preparations which had been partially dried and then re-immersed in Ringer's solution prior to fixation again distinguished two stages of drying. When the preliminary drying period was such as would be expected to produce those modifications of the myelin layering described for the "intermediate stage," the re-immersed specimens showed no trace of such modifications. The myelin layers were of normal dimension and appearance, and it was also noted that the fibres were relatively widely separated and the axons not appreciably collapsed. Preparations which had been taken to an advanced stage of drying before re-immersion in Ringer's solution eventually yielded electron micrographs such as the ones reproduced in Fig. 8. The nerve fibres were again relatively widely separated and the axons were of normal appearance, but the myelin sheaths showed areas of disorganised structure. Higher magnification electron micrographs showed that precisely ordered layering persisted in large areas but that this was of modified appearance. The layer thickness averaged about 130 A, and the intraperiod dense line was only slightly less intense than the principal dense line (Fig. 8 *b*). In the disorganised regions the layering was grossly distorted but appeared to be of the same general type as that of the well ordered regions.

Preparations which were fully dried, re-immersed, and again dried before fixation still showed regions featuring two different types of layering. The fine layering was essentially as described earlier, but the principal layering showed a still greater expansion to 180 to 200 A, and both dense and intraperiod lines appeared to be doublets.

$KMnO_4$-Fixed Preparations

The general appearance of the normal preparation was very similar to that of the corresponding OsO_4-fixed material but the axons of the larger nerve fibres appeared more collapsed in the OsO_4-fixed preparation than they did after

FIGURE 3

Electron micrographs of frog sciatic nerve preparations fixed with OsO_4 after a preliminary drying period of 20 to 30 minutes, together with the corresponding small-angle x-ray diffraction patterns.

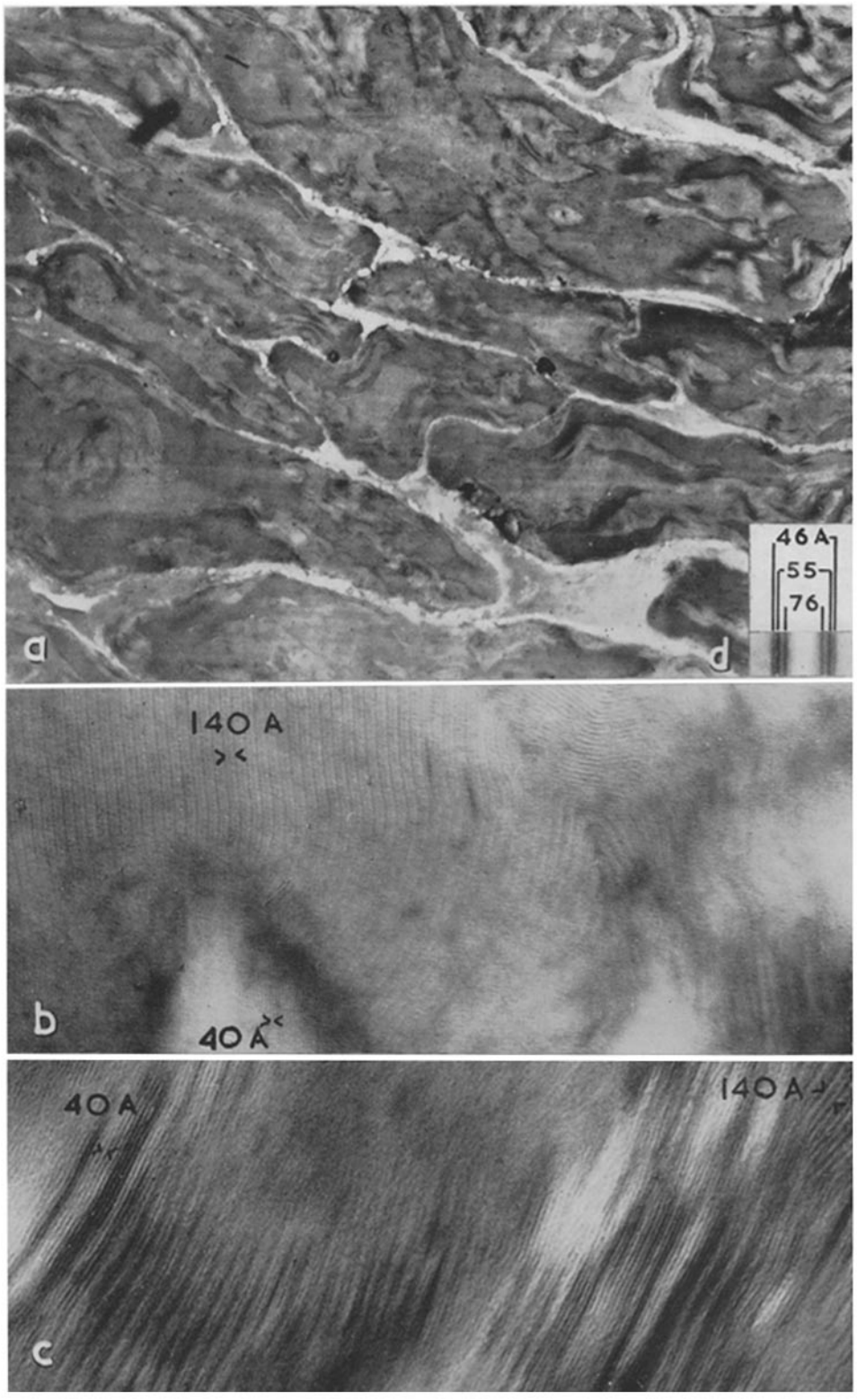
a. Low magnification electron micrograph showing partially collapsed nerve fibres. × 10,000.

b. High magnification of section of myelin sheath showing the principal layering—spacing ~115 A. × 100,000.

c, *d*, and *e*. Examples of condensation of myelin layers. Principal layer spacing ~115 A: condensed layer spacing ~85 A. × 160,000.

f. Small-angle x-ray diffraction pattern recorded from frog sciatic nerve between the tenth and thirtieth minutes of drying.

g. Small-angle x-ray diffraction pattern of nerve preparation fixed with OsO_4 after a preliminary drying period of 20 to 30 minutes.


a
46 A
55
76
d
140 A
b
40 A
40 A
140 A
c

$KMnO_4$-fixation. In the $KMnO_4$-fixed preparation the intraperiod dense line was very prominent.

Specimens which had been partially dried before fixation with $KMnO_4$ showed appreciably less over-all shrinkage than the corresponding OsO_4-fixed specimens, but the electron micrographs revealed a much greater distortion of the myelin sheaths (Fig. 6). In these early stages, however, the appearance of the individual layers was essentially normal (Fig. 6, inset). Preparations dried to the point where most of the water had been removed were observed to re-expand when placed in the $KMnO_4$-fixative, and individual nerve components were scarcely recognisable in the electron micrographs eventually obtained. It was clear that a re-hydration was being effected by the $KMnO_4$-fixative and hence that this method of fixation was not suitable for studies of dehydration phenomena by electron microscopy.

Weighing Experiments

A detailed report of such experiments has been given in a previous publication (7). For the purposes of the present study it was necessary only to obtain values for weight loss corresponding to intermediate and final stages of drying as defined with reference to the diffraction data. The range of values obtained in five experiments was 40 to 50 per cent for the intermediate stage of drying and 65 to 75 per cent for the final stage. These figures are in agreement with the more extensive studies made earlier (7).

DISCUSSION

The additional x-ray diffraction data and the electron microscope studies reported in this paper have necessitated a reconsideration of the effects of drying on the nerve tissue in general, and in particular on the structural changes in the nerve myelin.

Implications of the Relative Shrinkages of Nerve Components during Drying

The changes in the relative volumes of nerve components as observed in the electron micrographs of OsO_4-fixed material can be readily correlated with the drying kinetics reported previously (7) and also repeated in the course of the present experiments.

As the myelin period remains unchanged during the first phase of drying (say the first 20 minutes), it may be inferred that the total myelin volume will not have changed appreciably. However, its contribution to the total volume of the tissue has increased from 30 to 70 per cent during this period, which means that the total volume must have decreased by about 57 per cent. Considering the possible variations in drying conditions and also the preparative effects, this is in reasonable agreement with the estimate of 40 to 50 per cent derived from measurements of weight loss in parallel experiments. A similar calculation from figures obtained from electron micrographs of fully dried preparations leads to an estimate of about 67 per cent for the over-all shrinkage of the tissue, compared with 65 to 75 per cent estimated from weight loss. It would, therefore, seem that these electron micrographs probably give a fairly accurate picture of the changes in relative volumes of nerve components during dehydration, and there is some justification for manipulating the figures further in order to obtain an estimate of the water content of the myelin sheath.

During drying, the myelin to axon volume

FIGURE 4

Electron micrographs of frog sciatic nerve preparations fixed with OsO_4 after a preliminary drying period of 60 minutes, together with the corresponding small-angle x-ray diffraction patterns.

a. Low magnification electron micrograph showing numerous collapsed and distorted myelinated nerve fibres. × 10,000.

b. High magnification detail of myelin layering showing several different types of layers. Principal layer spacing ~140 A; fine layer spacing ~40 A. × 120,000.

c. High magnification electron micrograph showing examples of principal (~140 A) and fine (~40 A) layerings. × 160,000.

d. Small-angle x-ray diffraction pattern recorded from frog sciatic nerve between the 40th and 60th minutes of drying.

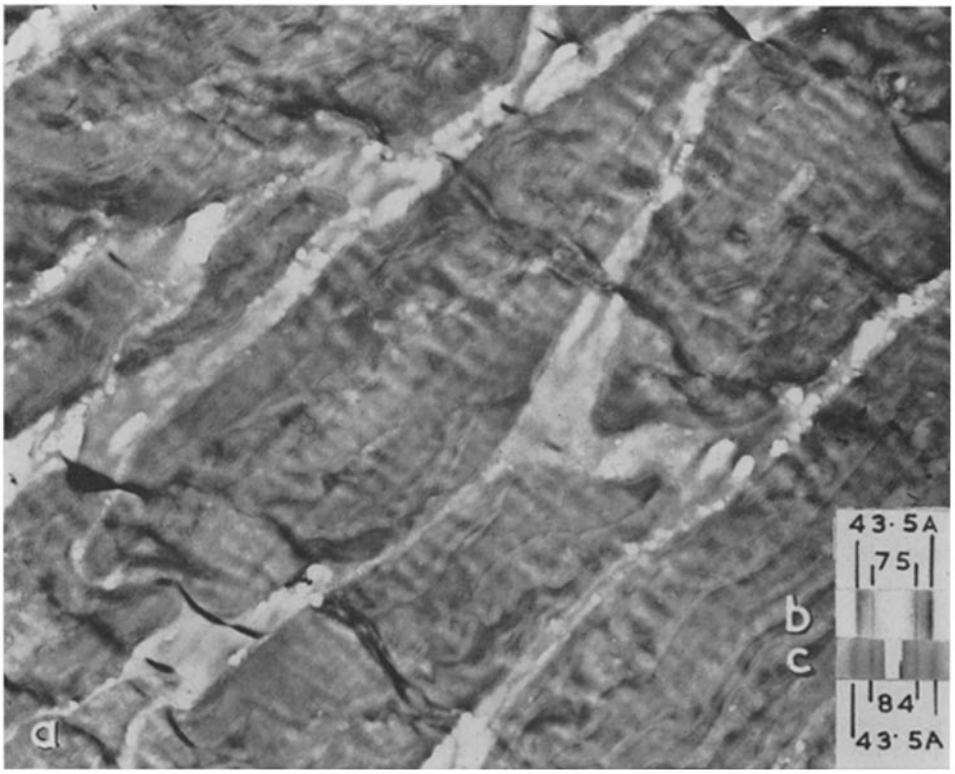


FIGURE 5

Electron micrograph of frog sciatic nerve preparation fixed with OsO_4 after a preliminary drying period of 100 minutes, together with corresponding small-angle x-ray diffraction patterns.

a. Low magnification electron micrograph showing collapsed nerve fibres. × 10,000.

b. Small-angle x-ray diffraction pattern recorded from frog sciatic nerve between the 80th and 100th minutes of drying.

c. Small-angle x-ray diffraction pattern of nerve preparation fixed with OsO_4 and embedded in araldite after a preliminary drying period of 100 minutes.

ratio in myelinated fibres increases from 3:2 to 9:1. Axon material from isolated giant fibres has been found to contain approximately 90 per cent water, and if this figure can be applied to the present case, then the decrease in axon volume during drying would be expected to be 90 per cent. In order to account for the observed change in volume ratio, the shrinkage of the myelin would then have to be 40 per cent.

The chief source of possible error in these deductions is probably the assumption that the effects of the preparative techniques are constant. A comparison of diffraction measurements derived from the unfixed material and measurements made on the corrsponding electron micrographs shows that whereas the fresh myelin period is about 40 per cent higher than that eventually measured in electron micrographs (170 A as compared with 115 A), the periodicities relating to the dried nerve are almost identical (148 A as compared with 140 to 160 A). The magnitude of the error that might arise from this discrepancy can be estimated by increasing the initial volume of the myelin by one-third. This would increase the initial myelin:axon ratio from 3:2 to 2:1 which would in turn raise the estimated water content of the myelin to 50 per cent. However, a comparison of $KMnO_4$-fixed and OsO_4-fixed normal preparations has indicated that the axon volume is considerably reduced by OsO_4-fixation. The initial myelin to axon ratio is therefore almost certainly less than 2 to 1 and the estimate of 50 per cent water in myelin can probably be regarded as a maximum. This conclusion agrees

with estimates previously made from diffraction data and drying kinetics.

Correlation of X-Ray Diffraction Data and Electron Micrographs

Correlations between data derived from normal preparations have been discussed in detail in previous publications (3, 10). Briefly, the 170 A radial repeat of fresh myelin (frog nerve) is reduced to about 148 A by fixation with osmium tetroxide, and the density distribution within this unit is modified. In electron micrographs this unit is represented as a repeating layer measuring only 115 A in the radial direction. As compared with the fresh myelin, it is assumed that the myelin layers have contracted and that the density distribution has been modified by the preparative procedures employed in electron microscopy.

A similar correlation is readily established between diffraction patterns recorded at intermediate stages of drying and the diffraction patterns and electron micrographs of preparations which had been partially dried before fixation with osmium tetroxide. The additional diffraction band which appears at about 60 A in the partially dried nerve (Figs. 3 *f* and 4 *d*) can be confidently related to the additional 110 and 55 A reflections in the diffraction pattern of the corresponding fixed preparation (Fig. 3 *g*) and to the regions of collapsed (85 A) layering observed in the electron micrographs. If the 60 A band is assumed to be a second order diffraction relating to a 120 A unit in the unfixed material then the shrinkage and change in density distribution of this unit during preparation for electron microscopy are very similar to those shown by the normal diffracting unit.

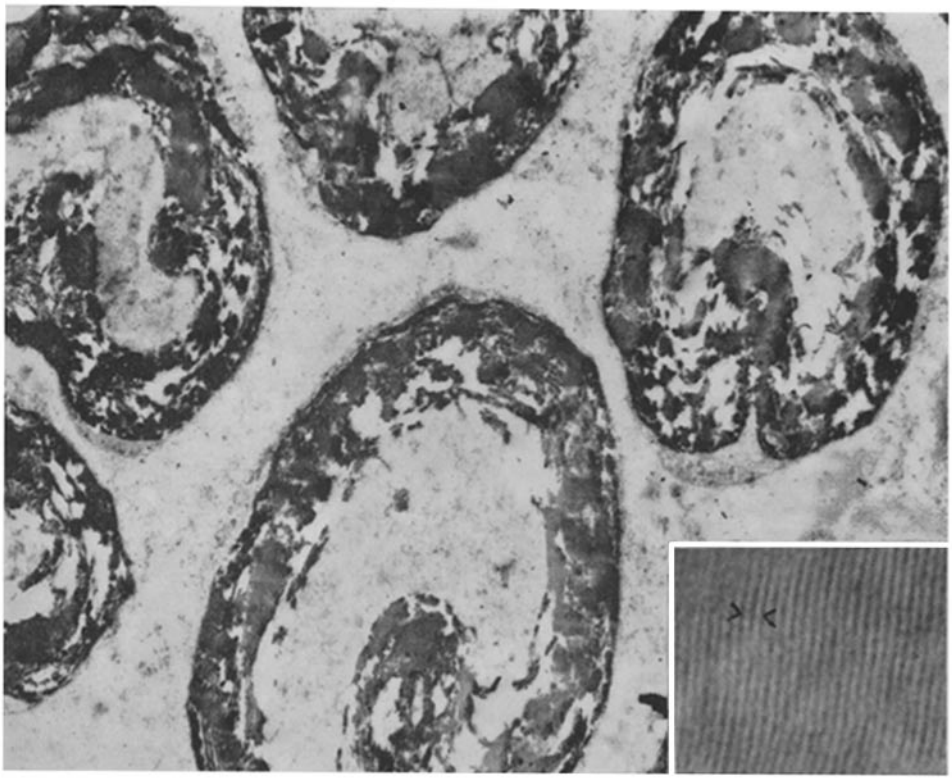


FIGURE 6

Low magnification (× 10,000) electron micrograph of frog sciatic nerve preparation fixed with $KMnO_4$ after a preliminary drying period of 100 minutes. There is appreciable separation of the nerve fibres and fragmentation of the myelin sheaths. The axons are not obviously collapsed though in most fibres there is an infolding of the myelin sheath which reduces the axon volume. The myelin to axon volume ratio is higher than is found in normal preparations. Inset: High magnification (× 240,000) of myelin layering. Periodicity is ~140 A and features a very dense intraperiod line.

In the case of the fully dried nerve preparations three components are clearly distinguished in the diffraction pattern of the unfixed preparation but only two in the fixed material and in the electron micrographs. The bands at about 74 and 148 A in the diffraction pattern of the unfixed material (Fig. 5 *b*) have previously been considered as first and second order diffractions of a 148 A residual lipoprotein layer (2, 5) and it seems most likely that this unit corresponds to the principal layering (140 to 160 A) seen in the electron micrographs. The 34.5 A band of the dried nerve diffraction pattern has previously been attributed to cholesterol, and as this component does not readily interact with osmium tetroxide it may not be distinguished in the electron micrographs. Furthermore, some extraction of cholesterol by the dehydrating solvents and by the embedding medium can be readily demonstrated. Consequently, it would seem that the 40 A layering seen in the electron micrographs of dried nerve is more likely to be related to the remaining component detected in dried nerve diffraction patterns, namely the one responsible for 40 and 60 A reflections. It has previously been demonstrated that the two reflections are interdependant and probably related to alternative polymorphic forms of a lipid phase which could include cerebroside and phospholipid (10). Such a polymorphism has been demonstrated in isolated phospholipid and in mixed lipid systems which included both phospholipid and cerebroside. Furthermore, electron microscope studies of various OsO_4-fixed phospholipid preparations have demonstrated that such compounds do form layered systems of very similar appearance and dimensions (8, 11, 17).

The diffraction pattern of rehydrated nerve shows that the myelin layers are somewhat swollen as compared with the fresh myelin layers, and the odd order diffractions are markedly weakened (Fig. 8 *c*). Corresponding effects are not readily discernible in the diffraction patterns of the fixed and fixed and embedded preparations, but the electron micrographs do show layering which is expanded as compared with the corresponding normal preparation, and the observed intensification of the intraperiod line would seem to be related to the weakening of the odd order diffractions in the pattern of the unfixed preparation.

In general, there is excellent correlation between diffraction and electron microscope data derived from OsO_4-fixed material but not from that fixed with $KMnO_4$.

Molecular Interpretation of the Changes in Myelin Structure during Drying

Normal Myelin: Previous considerations of the diffraction characteristics of myelin in relation to information available on its chemical composition led to the suggestion that its structural repeating unit included two lipoprotein layers, each featuring a bimolecular leaflet of mixed lipids sandwiched between monolayers of protein (5). In order to account for the inclusion of both lipoprotein layers in the fundamental repeat some difference in detail had to be assumed, and this was referred to as the "difference factor" (see reference 10 for more detailed discussion).

When electron microscope studies later demonstrated that the myelin unit was derived from two oppositely oriented layers of Schwann cell membrane (12) it immediately became apparent that the "difference factor" was related to an asymmetry of the Schwann cell membrane. This "difference factor" is markedly exaggerated by the action of osmium tetroxide, and in the electron micrographs of OsO_4-fixed preparations it is reflected in the density difference between the so called "main" and "intraperiod" dense lines.

Recent histochemical evidence (18, 19) has emphasised the presence of polysaccharide in the myelin sheath and two suggestions have already been made as to its localisation in relation to the general structural features already discussed. The presence of polysaccharide at the surface of the Schwann cell has been demonstrated histochemi-

FIGURE 7

High magnification electron micrographs from the myelin sheaths of nerve preparations fixed with OsO_4 after drying for 24 hours over P_2O_5 in an evacuated desiccator. The principal layers (140 to 160 A) frequently display a double intraperiod line and appear to be continuous with the fine (~40 A) layered system. There is also a concentration of dense material, frequently in the form of strands, in the fine layered phase immediately adjacent to the principal layers. × 160,000.

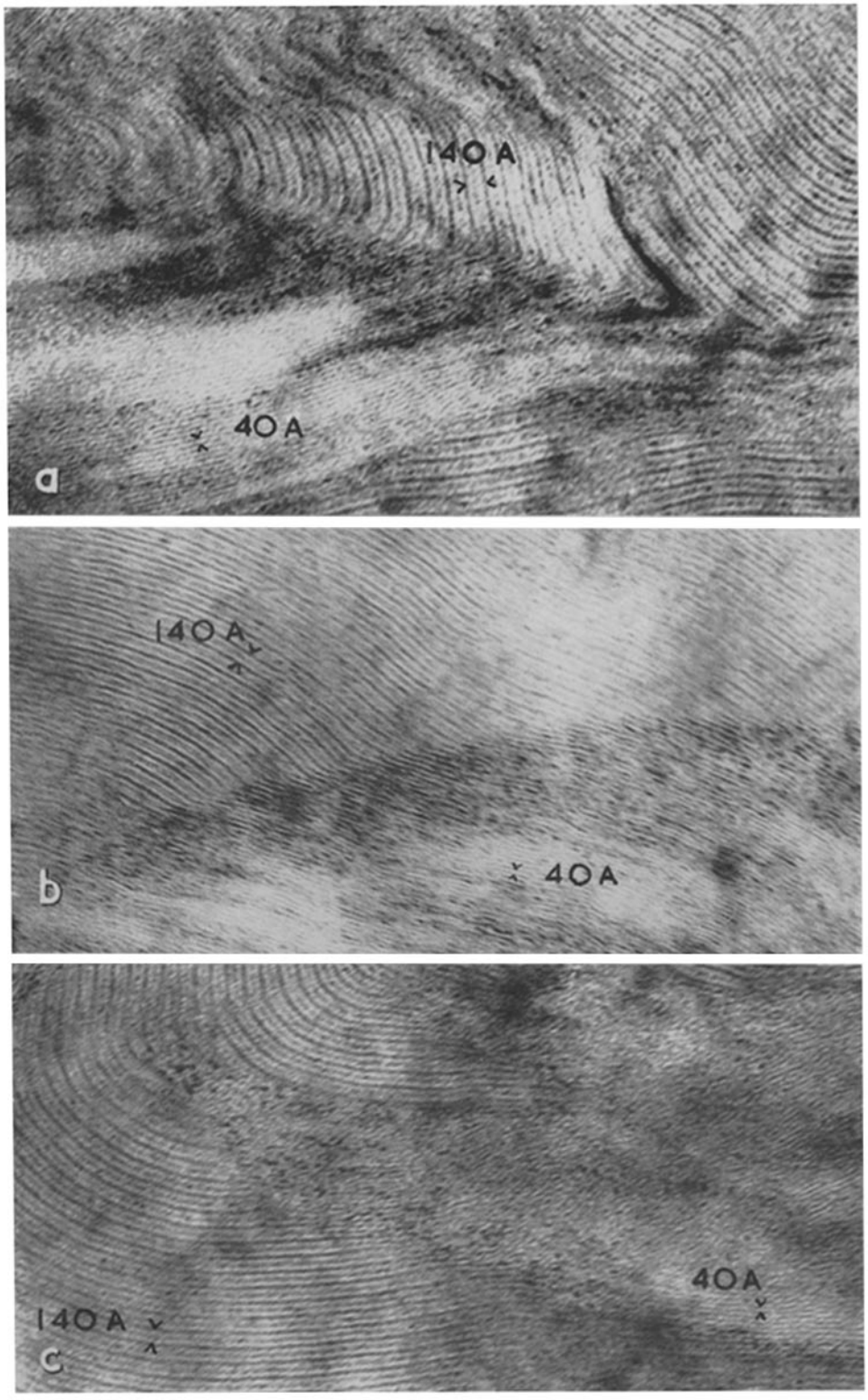
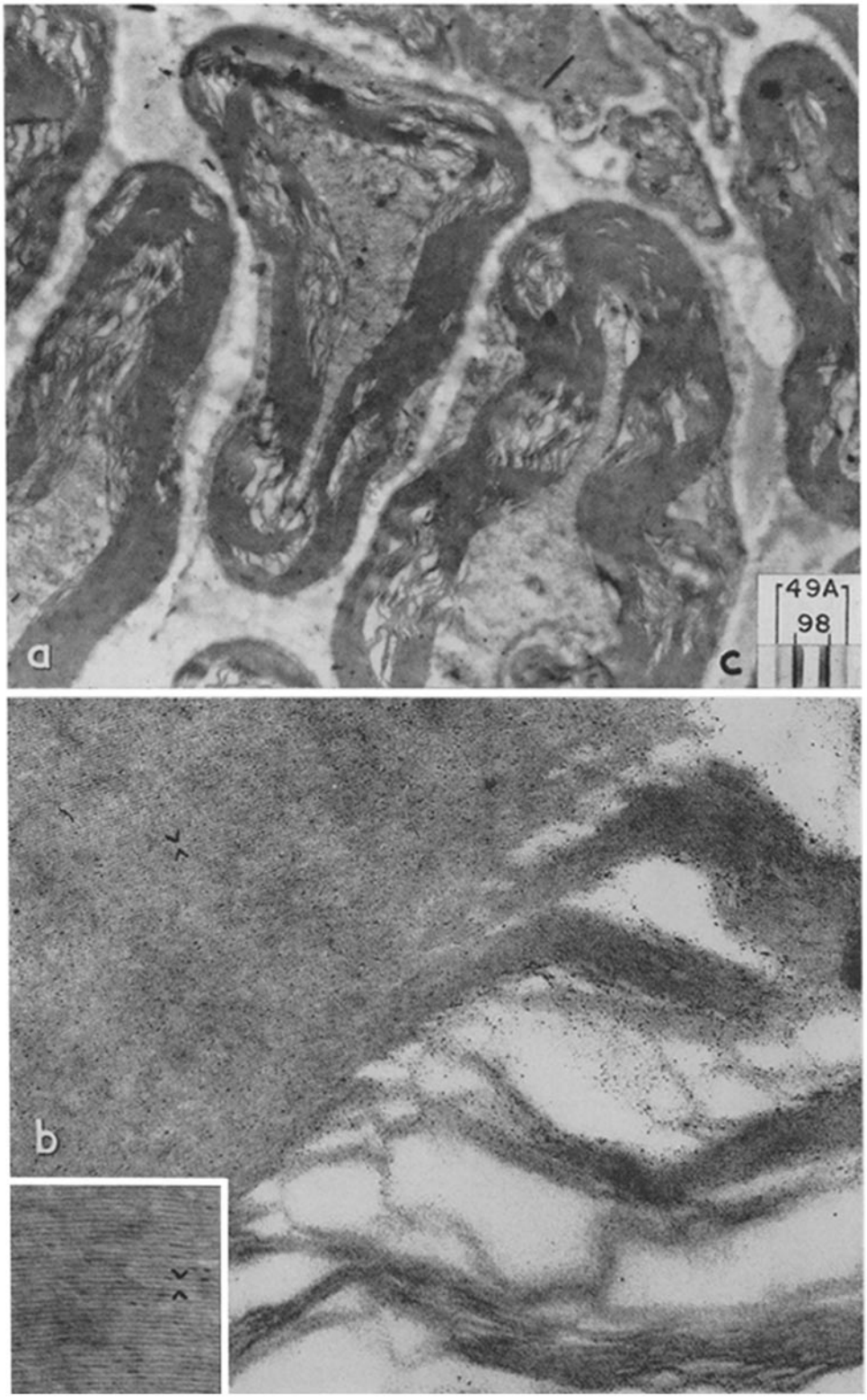


FIGURE 7


a
c
49A
98
b

cally (1), and Robertson (15, 16) has suggested that a monolayer of polysaccharide may be incorporated in the outer surface of the Schwann cell membrane and hence appear in the intraperiod dense line of the compact myelin. Wolman and Hestrin-Lerner (20), on the other hand, have suggested that the polysaccharide component (myelosaccharide) is located at the site of the main dense line in the compact myelin. However, this conclusion is reached from correlations between histochemical experiments and not wholly relevant electron microscope data, and some of the assumptions made are scarcely justifiable.[1]

Intermediate Stage of Drying: From x-ray diffraction studies it has already been suggested that during drying the myelin layers become contracted (from 170 to 148 A) through the loss of water layers and/or tilting of lipid molecules, and that at the same time some "labile" lipid components become separated from it to form a second phase (5, 10).

The general conclusion to be drawn from electron micrographs representing the intermediate stage of drying would seem to be that a molecular rearrangement has taken place within the myelin layers which has led to a collapsing of layers and a disappearance of the intraperiod dense line in some regions, whilst causing an intensification of the intraperiod dense line in others. A simple explanation of these changes and their reversibility could be achieved by assuming that a hydrated layer of protein or polysaccharide is located at the site of the intraperiod line and that during dehydration this layer contracts within the plane of the layers. This would lead to an increased concentration of this component in some regions and account for the intensification of the intraperiod line there, but it would be withdrawn from other regions which would consequently become collapsed and show no intraperiod line.

The corresponding parts of the diffraction data could be re-interpreted satisfactorily in these terms.

Final Stage of Drying: In the case of the completely dried tissue x-ray diffraction studies have provided substantial evidence of marked chemical differences between components represented in the diffraction pattern (5, 10). It was suggested that they included a "residual lipoprotein" component and a mixed phospholipid-cerebroside component, and these are now suggested to correlate with the principal layers and the fine layers seen in the electron micrographs of preparations of fully dried tissue. Considered separately, molecular explanations of these layers as they appear in electron micrographs are readily produced. The

[1] This particular suggestion is derived mainly from the observation that P.A.S.-positive material remained prominent in the myelin sheaths of nerve preparations which had been extracted with 2:1 chloroform: methanol. The significance of this observation in relation to electron microscopy has been considered by attempting to correlate it with electron micrographs of alcohol-extracted tissue published by Fernández-Morán and Finean (3). It was assumed that the closely spaced dense lines observed in some areas of the tissue were to be identified with the main dense lines of the normal myelin from which components represented by the intraperiod dense line and adjacent less dense areas had been extracted. Such a conclusion from the data presented is scarcely justifiable. Furthermore, it would seem to the present author that the only indication of a relationship to be found in such electron micrographs is the continuity between 70 A spaced dense layers and 40 A spaced dense layers. From experience with acetone-extracted tissue and the dehydrated tissue this would tend to suggest that the intraperiod dense line continues to be represented in the condensed layering of the extracted tissue. These grounds for allocating the polysaccharide component to the main dense line in the electron micrographs of myelin are, therefore, highly suspect.

FIGURE 8

Electron micrographs of frog sciatic nerve preparation fixed with OsO_4 after a preliminary drying period of 100 minutes followed by re-immersion in Ringer's solution for 60 minutes, together with the corresponding small-angle x-ray diffraction pattern.

a. Low magnification electron micrograph showing several partially re-expanded nerve fibres. × 10,000.

b. Detail of myelin showing layer spacing of 130 to 140 A. × 80,000. Inset × 120,000.

c. Small-angle x-ray diffraction pattern of a nerve preparation that had been dried for 100 minutes and then re-immersed for 60 minutes in Ringer's solution.

principal layers are very similar to the layers seen in normal preparations of myelin and to the principal layers of the intermediate stage of drying. The main difference is that the intraperiod dense line now appears as a doublet but this is not unexpected in view of the identification of the intraperiod dense line as the site of coalescence or apposition of outer surfaces of Schwann cell membrane during myelin formation. Similarly, there is no difficulty in considering the fine (40 A) layering as representing a simple lipid phase in view of the identical appearance of comparable preparations of isolated phospholipids.

However, the problem becomes very complex when consideration is given to the demonstrated continuities between these two types of layering and also to their relationships with the layers observed during the intermediate stages of drying.

It should be noted that a very similar continuity of principal (~140 A) and fine (~40 A) layers has previously been observed in electron micrographs obtained from moist nerve preparations which had been treated with acetone (3), but in this case some lipid extraction was also effected and became a factor in the attempts to interpret the changes in the appearance of the myelin layering. A more detailed diffraction and electron microscope re-investigation of solvent effects on fresh tissue (unpublished data) has established that the first action of many solvents, including acetone, is one of dehydration, and that condensation of layering such as that observed previously is essentially a dehydration phenomenon.

The continuity of principal layers and fine layers is actually observed only occasionally, and there is still the possibility that much of the material appearing as 40 A layering is independent of the principal layering. Consequently it is not necessarily finally established that the fine layering which retains continuity is to be identified with the purely lipid phase whose presence is indicated by the diffraction data. Nevertheless, these continuities still require explanation, and they constitute a most interesting development in the general attempt to establish the molecular constitution of the myelin layers through a study of the modifications that they can be made to undergo.

In connection with the solvent extraction experiments it was considered that the components present at the main dense line and the intraperiod dense line of the principal layering were the ones that continued into the fine layered system and that these components were probably the non-lipid components, protein or polysaccharide. Considering this interpretation in relation to the present dehydration series one is left wondering how this might be related to the intermediate stage of dehydration in which the extent of collapsing of the myelin layers is very similar but the intraperiod dense line is eliminated. Moreover, at this stage there would seem to be no possibility of a third phase which might represent ejected lipid material. It would, therefore, seem that at least part of the lipid must remain in the collapsed layering.

If the collapsed layering of the intermediate stage of drying is to be related to that of the final stage, it is necessary to account for the return of the intraperiod dense line without increasing the dimension. The possibility of restoring intraperiod dense line material that was removed by the early stages of drying seems rather remote. A more plausible explanation might be a further removal from, or change in activity of, the collapsed layers which enables osmium tetroxide to deposit more freely at the central line.

A point which might be further considered is that similar densities in electron micrographs do not necessarily mean identical chemical constitutions. One cannot readily distinguish between dense lines in a lipid system and dense lines in a lipoprotein system, and a continuity of dense lines such as that between the principal layers and fine layers in fully dried myelin might, for instance, simply represent a continuity of lipid-lipid ionic regions and lipid-protein ionic regions. In this case, the maintenance of continuity might be more significantly assigned to the less dense layers representing the lipid hydrocarbon chains common to both systems, and the non-lipid material might be identified with the irregular dense strands often seen to be superimposed on the fine layering.

An alternative interpretation which considers the dense lines to be associated with deposition of osmium compounds in the hydrocarbon layers is not favoured because of the difficulty of reconciling the extreme variability of the intraperiod dense line with a lipid hydrocarbon region (3).

Rehydration: The results of rehydration provide critical information concerning the drying changes. The fact that the layers can apparently be restored to normal configuration after being

taken to intermediate stages of drying must mean that the initial changes are relatively simple and particularly that they do not involve breaking the continuity of the layered system. It would also seem that a large measure of continuity must remain even at the final stage of drying, for again the available evidence suggests that a single type of layering is restored by rehydration and this layering is, in many respects, similar to the normal myelin layer. The observation is thus in keeping with the importance attached to the continuities observed at all stages of dehydration.

CONCLUSION

The present conclusion is that the simple molecular picture of myelin layers so far developed, although still generally satisfactory, does not offer a ready explanation of many detailed aspects of the modifications associated with dehydration. The uncertainties that still exist regarding the chemical composition of the myelin layer and the action of osmium tetroxide prevent a systematic elimination of possibilities to the point of deducing a unique detailed solution. Nevertheless, the introduction of the electron microscope data on dehydration has helped to clarify many aspects of the general picture of the molecular changes involved, and further relevant data is being sought from studies of solvent extraction of dried tissue as well as from other methods of dehydration.

REFERENCES

1. ABOOD, L. G., and ABUL-HOJ, S. K., *J. Neurochem.*, 1956, **1**, 119.
2. ELKES, J., and FINEAN, J. B., *in* Lipoproteins, Discussions of the Faraday Society, No. 6, 1949, 134.
3. FERNÁNDEZ-MORÁN, H., and FINEAN, J. B., *J. Biophysic. and Biochem. Cytol.*, 1957, **5**, 725.
4. FINEAN, J. B., *J. Scient. Instr.*, 1953, **30**, 60.
5. FINEAN, J. B., *Exp. Cell. Research*, 1953, **5**, 202.
6. FINEAN, J. B., *Exp. Cell Research*, 1954, **6**, 283.
7. FINEAN, J. B., *J. Biophysic. and Biochem. Cytol.*, 1957, **3**, 95.
8. FINEAN, J. B., *J. Biophysic. and Biochem. Cytol.*, 1959, **6**, 123.
9. FINEAN, J. B., The ultrastructure of cells, Anatomical Society Symposium, London, Arnold, 1960, in press.
10. FINEAN, J. B., *in* Modern Scientific Aspects of Neurology, (J. H. Cummings, editor), London, Arnold, 1960, 232.
11. GEREN, B. B., and SCHMITT, F. O., *J. Appl. Physics*, 1953, **24**, 1421.
12. GEREN, B. B., *Exp. Cell Research*, 1954, **7**, 588.
13. GLAUERT, A. M., ROGERS, G. E., and GLAUERT, R. H., *Nature*, 1956, **178**, 803.
14. LUFT, J. H., *J. Biophysic. and Biochem. Cytol.*, 1956, **2**, 799.
15. ROBERTSON, J. D., *J. Biophysic. and Biochem. Cytol.*, 1958, **24**, 349.
16. ROBERTSON, J. D., *Biochem. Soc. Symp.*, No. 16, 1959, 3.
17. STOECKENIUS, W., *J. Biophysic. and Biochem. Cytol.*, 1959, **3**, 491.
18. WOLMAN, M., *J. Histochem.*, 1956, **4**, 195.
19. WOLMAN, M., *J. Neurochem.*, 1957, **1**, 370.
20. WOLMAN, M., and HESTRIN-LERNER, S., *J. Neurochem.*, 1960, **5**, 114.