STUDIES ON THE PROBLEM OF PRESERVATION OF MYELIN SHEATH ULTRASTRUCTURE: EVALUATION OF FIXATION, DEHYDRATION, AND EMBEDDING TECHNIQUES

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ABSTRACT

Currently accepted methods of tissue preparation for electron microscopy result in alterations of myelinated nerve fibers. In an attempt to minimize distortion of myelin, various fixation techniques, dehydration schedules, and embedding methods have been evaluated. It was found that the major damage to myelinated nerves occurs in the embedding procedure. A technique for embedding nerve tissue using the polyester Vestopal W is described which was found to result in improved preservation of myelin.

Although much of the early investigation in biological electron microscopy was devoted to studies of myelin sheath structure (1–5), methods and techniques for preparation of myelinated nervous tissue have not been significantly improved in recent years, and the same methods used initially are still being employed. These methods (6–8) are inadequate for the study of myelin sheaths. In an attempt to improve the preservation of myelin sheath structure, a systematic study was undertaken to determine the stage or stages in specimen preparation in which damage to myelin sheath structure occurs. It is the purpose of this paper to present an evaluation of the myelin sheath damage produced by variations of fixation, dehydration, and embedding techniques in the preparation of tissue for electron microscopy. We intend to attempt to establish that embedding is a critical step in this process, and that by employing Vestopal W myelin sheath damage can be minimized.

MATERIALS AND METHODS

Myelinated Nervous Tissue: Sciatic nerve and spinal cord tissue, obtained from rabbits, guinea pigs, and dogs anesthetized with nembutal, was the subject of this study. The exposed nervous tissue was excised, immersed in a fixative, and cut into 1 mm. cubes.

Fixatives and Fixation: $KMnO_4$ 1 per cent (9) and OsO_4 1 per cent, buffered with the Michaelis veronal acetate buffer (10, 11) at a pH between 7.3 and 7.5, were used as fixatives. When sucrose was added to the OsO_4 solution, it was prepared according to the method of Barrnett (12). All other fixative solutions were adjusted with NaCl so that the total osmolarity was 0.3. Tissues were fixed at temperatures ranging from 4°C. to 27°C. for periods of 2 to 36 hours.

Dehydration: Following fixation, tissue cubes were washed for approximately 5 minutes in veronal acetate buffer of 0.3 osmolarity. Methyl alcohol, ethyl alcohol, and acetone were used as dehydrating agents in conjunction with methacrylate and Araldite, while acetone was used exclusively with Vestopal W. Dehydrating schedules were varied.

Rapid, small step dehydration was attempted by running tissue through alcohol changes graded upward from 10 to 100 per cent in 10 per cent steps. In two experiments, tissues were soaked for 10 minutes in each change, and in two others they were rinsed twice in each change. In all cases the final dehydration was obtained by soaking for at least 1 hour in 100 per cent alcohol.

To insure the most gradual and constant changes in alcohol concentrations during dehydration, the

principle of gradient elution was also applied (13). An all-glass apparatus was constructed, consisting of an alcohol reservoir, a mixing flask or chamber, and a gooseneck side arm for the tissues. The reservoir was filled with absolute alcohol, the mixing chamber and side arm with veronal acetate buffer. The apparatus was placed on a magnetic stirring box and a magnetic stirring rod placed in the mixing chamber to insure nearly complete equilibration of the alcohol with the continuously diminishing concentration of the buffer. The gradient dehydration apparatus constituted a closed automatic siphon system, since the rate of addition of alcohol to the mixing chamber was dependent upon the rate of outflow at a stop-cock placed at the siphon end of the gooseneck. Complete dehydration of tissues was achieved in 20 minutes to 4 hours by varying the rate of outflow.

Embedding: Methacrylate, Araldite and Vestopal W were the embedding agents used in this study.

Methacrylate: Both unpolymerized and prepolymerized (14) solutions of *n*-butyl methacrylate and various combinations of methyl methacrylate and *n*-butyl methacrylate were used to embed the tissue blocks. Embedding techniques were based on the method of Swerdlow, as modified by Farquhar (6). Blocks were polymerized in an oven at temperatures ranging from 48°C. to 52°C., or placed over ultraviolet lamp (15, 16) and allowed to polymerize at room temperature overnight.

Araldite: Araldite embedding followed the schedule suggested by Glauert and the New York Society of Electron Microscopists (17, 18).

Vestopal Polyester: Vestopal W[1] embedding followed the technique suggested by Ryter and Kellenberger (19). A variation of this procedure was found to give better penetration of the tissue blocks and is outlined as the method of choice.

Buffered 1% OsO_4 (osmolarity 0.300)	2 to 4 hrs.
Veronal acetate buffer	2 to 5 min.
Dehydration:	
30% acetone	2 rinses
50% acetone	2 rinses
70% acetone	2 rinses
90% acetone	2 rinses
100% acetone	4 changes at 15 min./change
Vestopal:acetone	
1:3	½ hr.
1:1	½ hr.
3:1	½ hr.
Vestopal + Initiator 1% + Activator 0.5%	4 changes at ½ hr. intervals

[1] Martin Jaeger, Geneva, Switzerland.

Blocks were embedded in predried gelatin capsules by polymerizing the tissue-containing resin at 56–60°C. for 24 hours.

Effect of Fixation and Dehydration: In experiments to compare effects of fixation, the nervous tissue was taken from a single animal and cut into 1 mm. cubes. OsO_4 was employed for some of the blocks and $KMnO_4$ for others. Dehydration in alcohol and embedding in methacrylate were accomplished according to standard procedures. In experiments to study effects of varying the dehydration procedure, only the dehydration was varied; all other aspects of procurement of tissue, fixation, and embedding in methacrylate were held constant according to the standard methodology.

Effect of Embedding: In experiments to evaluate the embedding material on myelin sheath preservation, the tissue blocks were obtained each time from a single animal, fixed in OsO_4, dehydrated in alcohol when methacrylate was used and in acetone when Vestopal W was used, and embedded in the respective plastic. In an additional series of observations, acetone was compared to alcohol as dehydrating agent for tissues ultimately embedded in methacrylate.

Sectioning: Sections were cut using glass knives on a Servall Porter-Blum microtome or an LKB ultratome. Thick sections (0.5 to 2 microns) of each block were cut and stained by the Wright-Giemsa method of Condie *et al.* (20), for study under the light microscope.

Blocks were chosen at random from each experimental and control group, and the preservation evaluated without prior knowledge of the method which had been employed in their preparation. The myelin sheaths were judged to be well preserved, moderately well preserved, and poorly preserved, according to the following criteria:

Well Preserved

Gross appearance of myelin sheath
1. Compact
2. No breaks

Lamellar structures
1. Uniform spacing around entire sheath
2. No fragmentation
3. Unraveling of inner or outer sheath turns absent or minimal

Moderately Well Preserved

Gross appearance of myelin sheath
1. Compact by light microscopy
2. No breaks, or breaks extending only part-way through the sheath

Lamellar structure
1. Minimal amount of irregular spacing
2. No or minimal fragmentation
3. Slight or moderate amount of unraveling at inner or outer sheath turns

Poorly Preserved

Gross appearance of myelin sheath

1. Disruption of lamellar structure apparent by light microscopy
2. One or more breaks extending through the entire sheath

Lamellar structure

1. Wide and irregular spacing of many lamellae
2. Moderate to severe fragmentation, involving many lamellae
3. Marked lamellar unraveling at either inner or outer sheath turns

Those blocks showing the best myelin preservation for each variable under study were selected for examination in an RCA EMU 3-D electron microscope. Measurements of lamellar structures were calculated from electron microscope calibration figures obtained from photographs of a calibration grid with 28,800 lines/inch.

RESULTS

Sample observations of the preservation of large myelin sheaths, as visualized by light microscopy in 0.5 to 2 micron sections of sciatic nerve and spinal cord prepared in a variety of ways, are recorded in Table I. It will be seen from the table that none of the variations in fixation and dehydration procedures resulted in uniformly good preservation of myelin when methacrylate was used as the embedding medium. Some improvement was noted when small step dehydration in alcohol was used. No significant advantage could be attributed to prepolymerization of the methacrylate. Embedding in Araldite resulted in significant improvement in preservation of axons, but here, too, consistently good preservation was not obtained. When Vestopal W was used as the

TABLE I

Results of Experimentation with Techniques Used to Prepare Myelin Sheaths for Electron Microscopy

Experimental procedure	No. of axons counted	Well preserved	Moderately well preserved	Poorly preserved
		(Per cent)	*(Per cent)*	*(Per cent)*
1. Routine methacrylate embedding, using 1 per cent OsO_4* as fixative				
Large axons	1200	0	1	99
Small axons	1200	25	33	42
2. Routine methacrylate embedding, using 1 per cent $KMnO_4$ as fixative				
Large axons	1000	0	2	98
Small axons	1000	22	31	47
3. Rapid, small concentration step dehydration with methacrylate embedding				
Large axons	1200	4	14	82
Small axons	1200	43	31	26
4. Embedding, prepolymerized methacrylate				
Large axons	1300	2	4	94
Small axons	1300	16	32	52
5. Prolonged soaking in prepolymerized methacrylate with embedding in methacrylate				
Large axons	1000	0	2	98
Small axons	1000	9	5	86
6. Embedding in Araldite				
Large axons	1000	53	31	16
Small axons	1000	70	20	10
7. Embedding in Vestopal W				
Large axons	1900	78	11	11
Small axons	1900	95	4	1

* Except where noted, cell blocks were fixed in 1 per cent osmium tetroxide.

embedding medium, consistently good preservation of myelin sheath structure was found. Although the myelin sheaths of small fibers withstood embedding in methacrylate better than the myelin sheaths of large fibers, the preservation of the myelin sheaths of small fibers was improved by embedding in araldite or Vestopal W.

Methacrylate Embedding: Fig. 1 illustrates the distortion and fragmentation of the myelin sheaths regularly observed by light microscopy when routine methods for fixation, dehydration, and embedding of sciatic nerve in methacrylate are used. The damaged myelin sheaths, when viewed in the electron microscope, appear fragmented and swollen, and the myelin lamellae are often separated by what appear to be accumulations of embedding material (Fig. 7). Fig. 8 illustrates some of the better preservation observed among the methacrylate-embedded fibers. Even here, the swelling and distortion are apparent. Figs. 9 and 10 compare typical myelin sheaths of nerve fibers embedded in methacrylate and Vestopal W at comparable magnification. The greater uniformity of the lamellar structure in the fiber embedded in Vestopal W, and the fragmentation and dislocation characteristic of the methacrylate-embedded fiber are apparent.

With the exception of rapid stepwise or gradient alcohol dehydration, none of the variations in fixation or dehydration reduced myelin sheath damage in methacrylate embedding significantly. Experiments using either the gradient dehydration apparatus or rapid dehydration with multiple alcohol changes yielded some tissue in which myelin was moderately well preserved. Good results could not be obtained consistently. Figs. 2 and 9 illustrate some of the better preservation of myelin sheaths which we have obtained using ideal methods of dehydration together with mechacrylate embedding. Use of acetone or

FIGURE 1

Cross-section of normal rabbit sciatic nerve, fixed in 1 per cent OsO_4, embedded in methacrylate by routine methods. Sheath architecture of all heavily myelinated axons is almost totally destroyed. These axons would be classified in Table I as "poorly preserved." Thinly myelinated axons appear to be well preserved and would be classified as "well preserved." × 1200.

FIGURE 2

Longitudinal section of a normal rabbit sciatic nerve, fixed in 1 per cent OsO_4, dehydrated rapidly in small alcohol concentration steps, and embedded in methacrylate. Although the myelin appears fairly well preserved grossly, damage may be seen at various places along the sheaths. The sheath at (*a*) would be classified in Table I as well preserved. The sheath at (*b*) would be classified as moderately well preserved. × 1000.

FIGURE 3

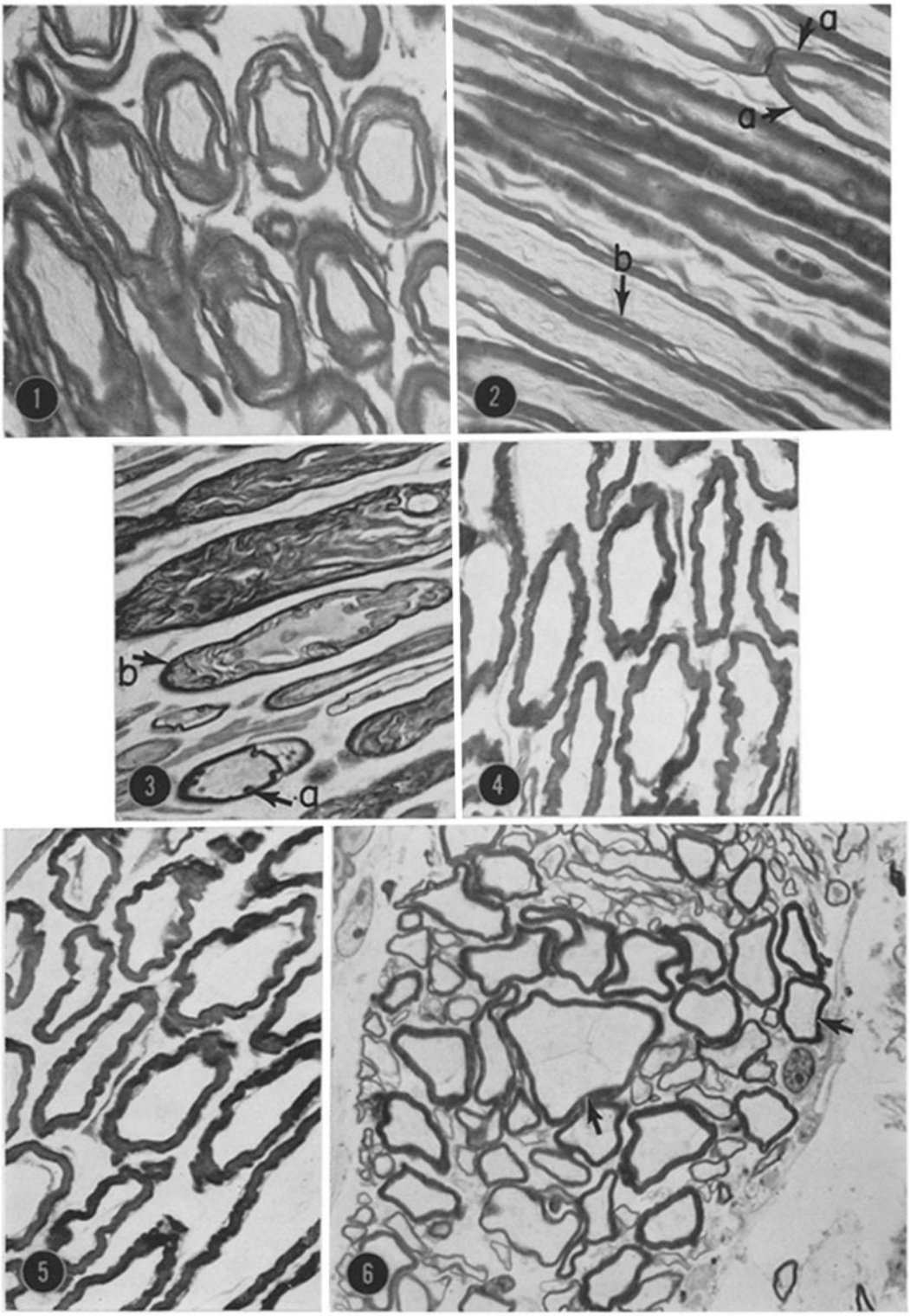
Diagonal section of normal rabbit sciatic nerve, fixed in $KMnO_4$, embedded in Araldite. These axons are better preserved than those embedded in methacrylate, but myelin sheath damage is present. (*a*) well preserved axon, (*b*) poorly preserved axon. × 1000.

FIGURES 4 and 5

Cross-sections of normal rabbit sciatic nerve, fixed in 1 per cent OsO_4, embedded in Vestopal W. Except for gross wrinkling, all myelin sheaths appear to be well preserved, showing only occasional disruption of lamellae. × 1000.

FIGURE 6

Cross-section of spinal cord of a dog, fixed in 1 per cent OsO_4, embedded in Vestopal W. Myelin sheaths, axon cytoplasm, and glial cell elements show good preservation. Arrows indicate examples of well preserved myelin sheaths. × 1000.


1
2
a
a
b
3
b
a
4
5
6

methanol for dehydration did not improve preservation of the sheaths.

Lamellar measurements were as follows:

Lamellar spacing	approximately 130 A
Width of dense bands	approximately 30 A
Width of intermediate lines	approximately 15 A

Araldite Embedding: The minimal shrinkage and more uniform polymerization characteristic of the epoxy resins (17, 18) would on theoretical grounds appear to offer material superior to methacrylate for the embedding and preservation of myelinated tissues, and the latest work using this plastic to embed myelinated nervous tissue supports this expectation (21). Although we often obtained good preservation using Araldite®[2] as an embedding agent, many of the myelin sheaths of large fibers showed distortion, and good preservation was seen among these large fibers in only slightly more than 50 per cent of the axons counted (Fig. 3).

Vestopal W Embedding: The polyester Vestopal W was used for embedding because, like Araldite, its properties of minimal shrinkage and uniform cross-linking polymerization (22–24) suggested that it might be suited for the preservation of myelin sheaths. Other potential advantages were the rigorous standardization of the Vestopal W embedding procedure by Ryter and Kellenberger (19), and the standardization of the embedding compounds by the manufacturer, insuring maximum uniformity. The results obtained to date in the study of myelin sheaths with this embedding material have been good (Table I).

[2] Obtained from the New York Society of Electron Microscopists.

When cut and examined under the light microscope, sections revealed uniform preservation of myelinated nerve fibers in their entirety (Figs. 4 to 6). Electron microscopic observation of Vestopal W–embedded nervous tissue also revealed most areas of the myelin sheaths to be intact (Figs. 10 to 13). A wavy configuration of the myelin sheaths (Figs. 4 to 6) and slight separation of the innermost lamellae of the sheaths in the region of the axolemma (Figs. 12 and 16) were often seen. These alterations in axon shape appeared in both large and small axons, but were most prominent in the large ones. Fine structure of central nervous system cortical cells and their processes (Figs. 14 and 15) appeared to be well preserved. The Schwann cells, as well as the axoplasm (Figs. 16 and 17), showed some evidence of extraction which may be related to acetone dehydration.

Lamellar spacing	approximately 115 A
Width of dense bands	approximately 30 A
Width of intermediate lines	approximately 15 A

DISCUSSION

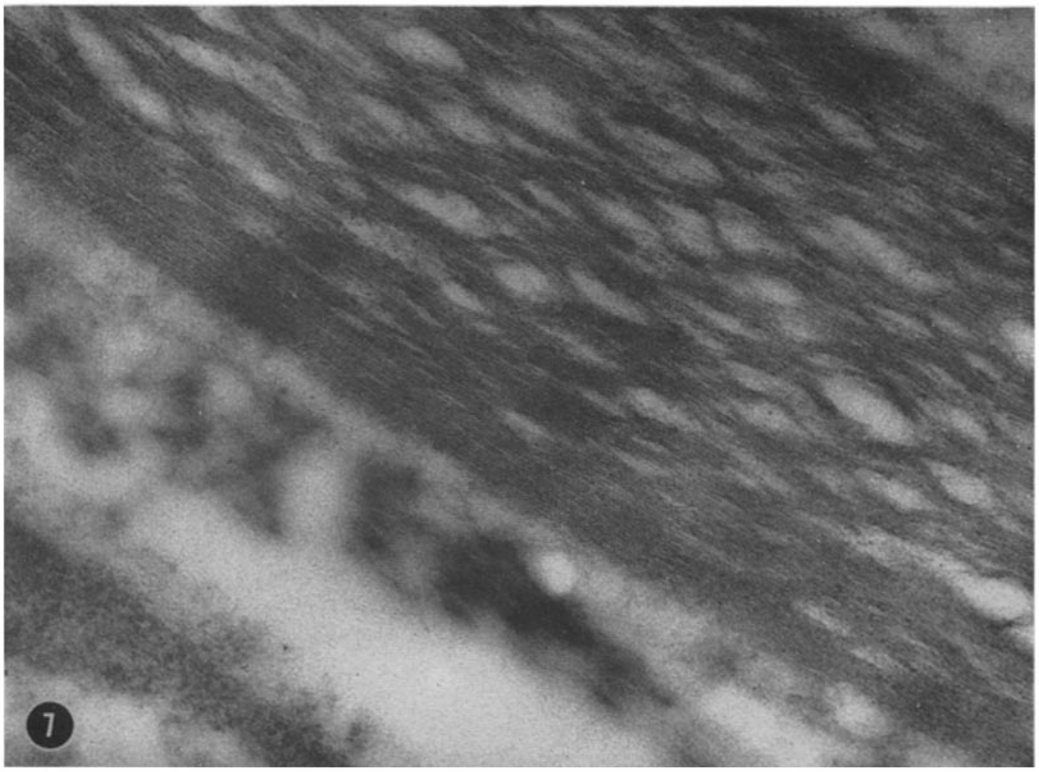
Preservation of the myelin sheath presents a most difficult problem to the investigator interested in the ultrastructure of myelin, mechanisms of myelination, and problems associated with demyelination. Methods of tissue preparation, which

FIGURE 7

Myelin sheath of a normal rabbit sciatic nerve axon, fixed in 1 per cent OsO_4, embedded in methacrylate. This sheath is typical of thickly myelinated axons embedded in methacrylate. Although individual lamellae are distinct and intact, the sheath is damaged grossly. Separation of the lamellae into bundles by methacrylate inclusions precludes effective study of the fine structure of the sheath. × 38,000.

FIGURE 8

Myelin sheath of a normal rabbit sciatic nerve axon, fixed in 1 per cent OsO_4, embedded in methacrylate. This thinly myelinated axon displays good preservation of its sheath as a whole. Upon close examination, however, individual lamellae show a tendency toward separation at various sites along the sheath (arrows). This figure demonstrates that good preservation of myelin sheath can at times be obtained with this embedding medium. × 38,000.



at present enjoy almost universal usage and acceptance among electron microscopists, are inadequate when employed for the preparation of myelinated tissues. The present study was undertaken to determine the stage in the preparation procedure during which damage to myelin was produced. Fixation and dehydration appear to play a minor role, although it is possible that damage to myelin could be introduced during either of these procedures. The results of our study indicate that it is during the embedding procedure that the most obvious artifacts are introduced. The damage can be traced indirectly to the nature of polymerization of the methacrylate. When tissues are embedded in methacrylate, electron microscopic examination of myelin sheaths reveals that certain areas may be grossly distorted, while others appear to be well preserved. Such damage might stem from forces of contraction resulting from three factors: (*a*) polymerization of this plastic begins in multiple centers, (*b*) polymerization proceeds at different rates in different areas, and (*c*) shrinkage occurs when polymerization takes place (25–27).

Although electron micrographs of other organs and tissues embedded in methacrylate are technically excellent, the artifact produced in myelin embedded in this plastic attests to considerable play of physical forces which probably results in some distortion in all tissues embedded in methacrylate. Most damage seen in methacrylate-embedded myelin sheaths is "explosion artifact" in which sheaths are torn apart to produce shredding and separation of lamellae. Destruction is particularly severe in heavily myelinated nerves. Very few of the electron micrographs presented in the literature to date display what can be considered to be satisfactory preservation of thickly myelinated nerve fibers, and many of these illustrations show widespread distortion and destruction of thinly myelinated fibers.

In order to be considered superior to methacrylate for embedding nervous tissue, a new embedding medium must fulfill two criteria. It must preserve myelin sheaths intact, and distort other cellular elements as little as possible. To different degrees, both Araldite and Vestopal W meet these requirements. Preservation in Araldite-embedded tissues was often good, but the results were disappointing since good preservation was not obtained consistently. Vestopal W proved to be superior to either Araldite or methacrylate. Myelin sheaths were compact and intact, and cytoplasmic details within cellular structures were well preserved, with a minimum of distortion. Indeed, with respect to membranous structures, Vestopal W embedding was associated with clearer detail than any other method used.

One important difference between myelin embedded in Vestopal W and myelin prepared in other ways merits further discussion. X-ray diffraction studies done on fresh nerve reveal diffraction bands at 170 A in amphibians to 185 A in mammals, as well as an 85.5 A band and several others (28, 29). The intralamellar spacing of mammalian myelin sheaths is thus thought to approximate 180 A, the other x-ray bands corresponding to structural subunits or multiple order reflections of the basic repeating unit (29, 30). Gelatin-embedded tissue reveals a 110 A spacing, and methacrylate-embedded tissue yields bands at about 130 A (30–32).

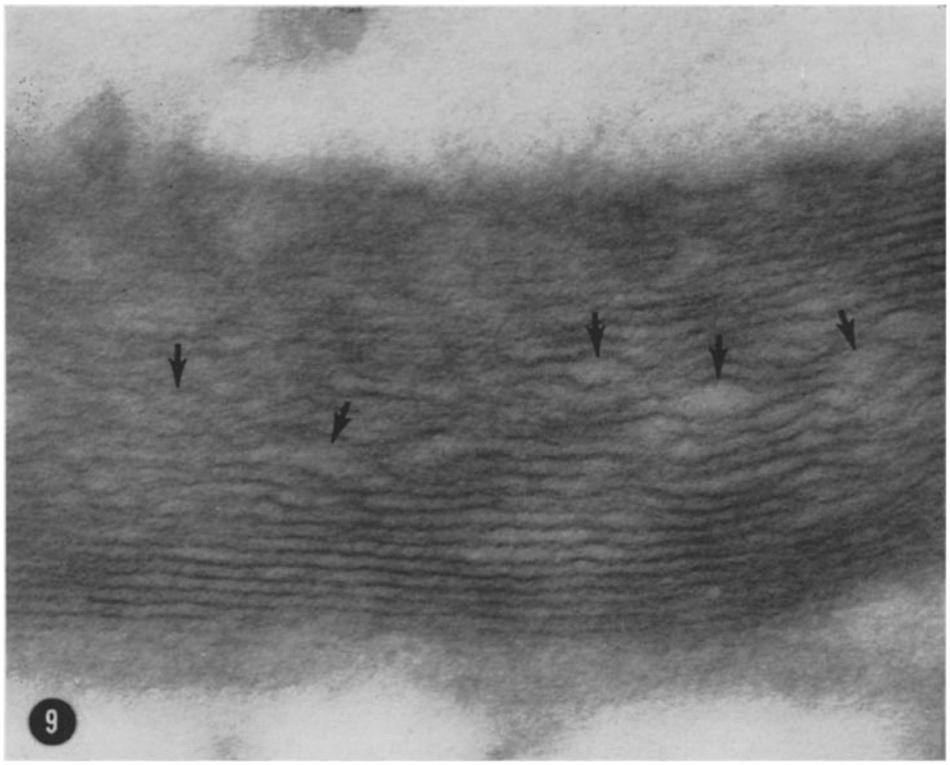
Intralamellar distances in methacrylate-embedded controls in our experiments measured 130 A, a value which agrees well with previously published results from work on methacrylate-

FIGURE 9

Myelin sheath of a normal rabbit sciatic nerve axon, fixed in 1 per cent OsO_4, embedded in methacrylate. Lamellar ultrastructure appears generally to be intact in this thinly myelinated axon. The arrows point, however, to several areas where lamellae are misshapen. × 138,000.

FIGURE 10

Myelin sheath of a normal rabbit sciatic nerve axon, fixed in 1 per cent OsO_4, embedded in Vestopal W. The well preserved, compact lamellae of this axon show greater uniformity of structure throughout the sheath than the lamellae of the methacrylate-embedded axon of similar size shown in Fig. 9. × 180,000.



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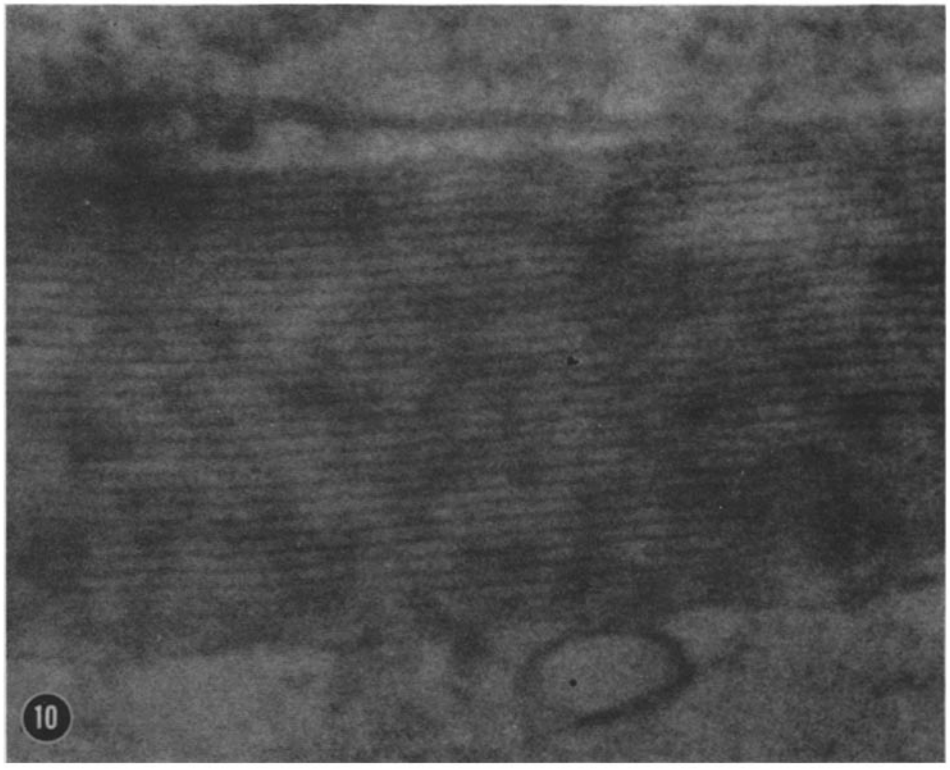

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embedded mammalian myelin sheaths. The smaller spacing of 115 A observed in Vestopal W–embedded tissue might result from either of two processes. First, dehydration of tissue with acetone for Vestopal W embedding might lead to a greater dehydration artifact than alcohol dehydration for methacrylate embedding. Control experiments using acetone dehydration with methacrylate embedding yielded myelin lamellar spacings of 130 A. Thus, dehydration with acetone does not appear to contribute to the smaller spacings observed in Vestopal W–embedded tissue.

In a series of experiments using various methods of drying nerve tissue for x-ray diffraction studies, Elkes and Finean (33) observed that myelin-lamellar spacings decreased during drying to the same final value regardless of the method of drying. According to x-ray diffraction and other studies undertaken by Fernández-Morán and Finean (30), myelin sheaths undergo a certain amount of shrinkage during alcohol dehydration and then re-expand during embedding in either methacrylate or gelatin. If similar changes occur when embedding myelin sheaths in Vestopal W, the 115 A spacing indicates that nerve axons swell less from the dehydrated state during Vestopal W polymerization than during methacrylate polymerization. The smaller re-expansion factor in Vestopal W embedding apparently prevents extensive sheath damage, just as it does in gelatin embedding. Thus, uniformity of preservation using Vestopal W is reflected on the ultrastructural level of myelin sheath architecture.

These findings, coupled with observations of uniform, undistorted preservation of non-myelinated nervous tissue embedded in Vestopal W, lead us to believe that Ryter and Kellenberger's development of this plastic as an embedding agent for biological electron microscopy contributes a method superior to any previously available for preservation of myelin sheath structure. Because of the simplicity and reproducibility of the method, it should find wide usage in embedding tissue for electron microscopy.

SUMMARY AND CONCLUSIONS

1. The roles of fixation, dehydration, and embedding in the preservation of myelin sheath structure have been evaluated. Results of this study indicate that damage to myelin sheath structure is produced during the embedding of tissue with either methacrylate or araldite.

2. Damage introduced during polymerization of methacrylate can be reduced by embedding following rapid stepwise and gradient dehydration. Prepolymerization of methacrylate offers no advantage over the routine methods of methacrylate embedding.

3. Embedding with the epoxy resin Araldite offers improvement in preservation of myelin sheath structure. Difficulty in reproducing results limits its usefulness in the embedding of myelinated nervous tissue.

4. Vestopal W, a polyester, consistently produces good results in preserving myelin sheath ultrastructure.

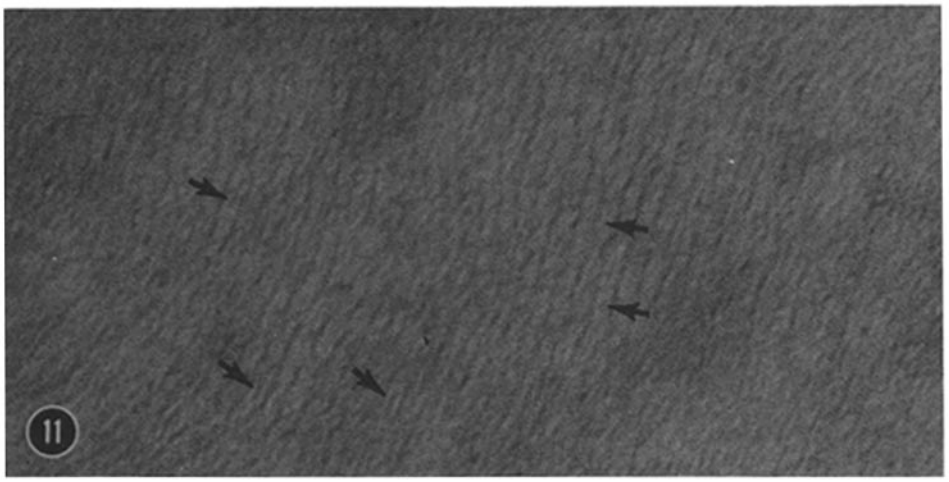
5. It is proposed that improved preservation of myelin observed when Vestopal W is used is due to more uniform cross-linking polymerization characteristic of polyester resins; and it is suggested that an additional factor may be the smaller degree of final expansion of the myelin sheaths from the dehydrated state.

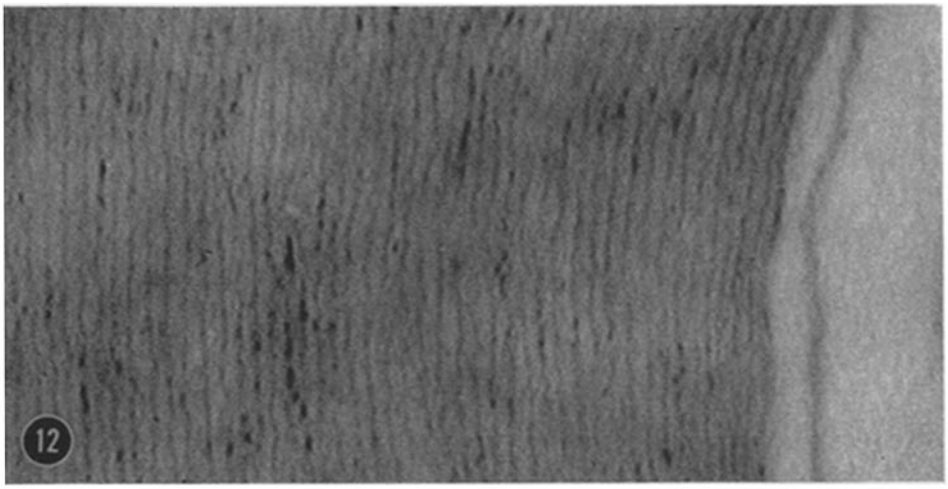
This work was supported by grants from the United States Public Health Service, the American Heart Association, and the Minnesota Heart Association.

Mr. Condie is Fellow of the Child Development Study Group; Mr. Howell is Medical Student Research Fellow in Pediatrics; and Dr. Good is American Legion Memorial Heart Research Professor of Pediatrics.
Received for publication, September 7, 1959.

FIGURES 11 to 13

Myelin sheaths of normal rabbit sciatic nerve axons, fixed in 1 per cent OsO_4, embedded in Vestopal W. Lamellae of the sheaths show almost no tendency to become separated within the sheath. Intralamellar distance, 117 A; dense band dimension, 34 A; intermediate lines (indicated by arrows), 15 A. Fig. 11, × 222,000; Fig. 12, × 196,000; Fig. 13, higher magnification of Fig. 12, × 282,000.





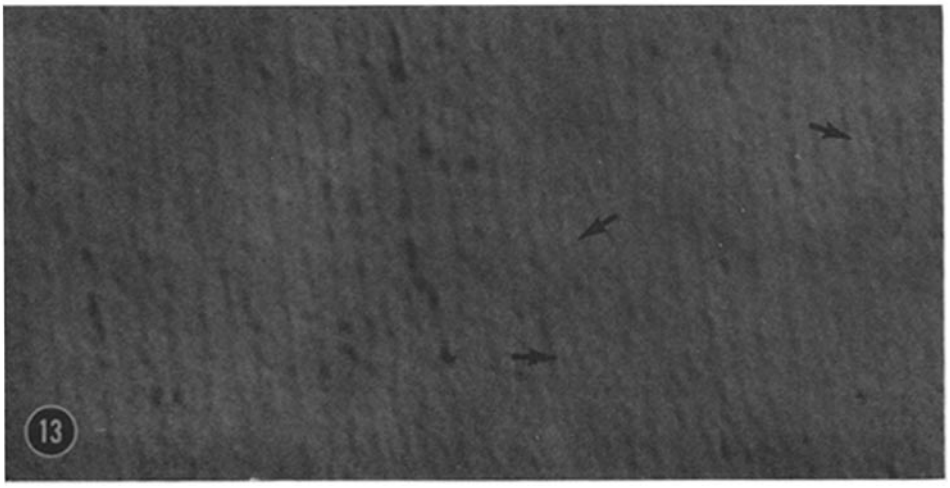


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FIGURE 14

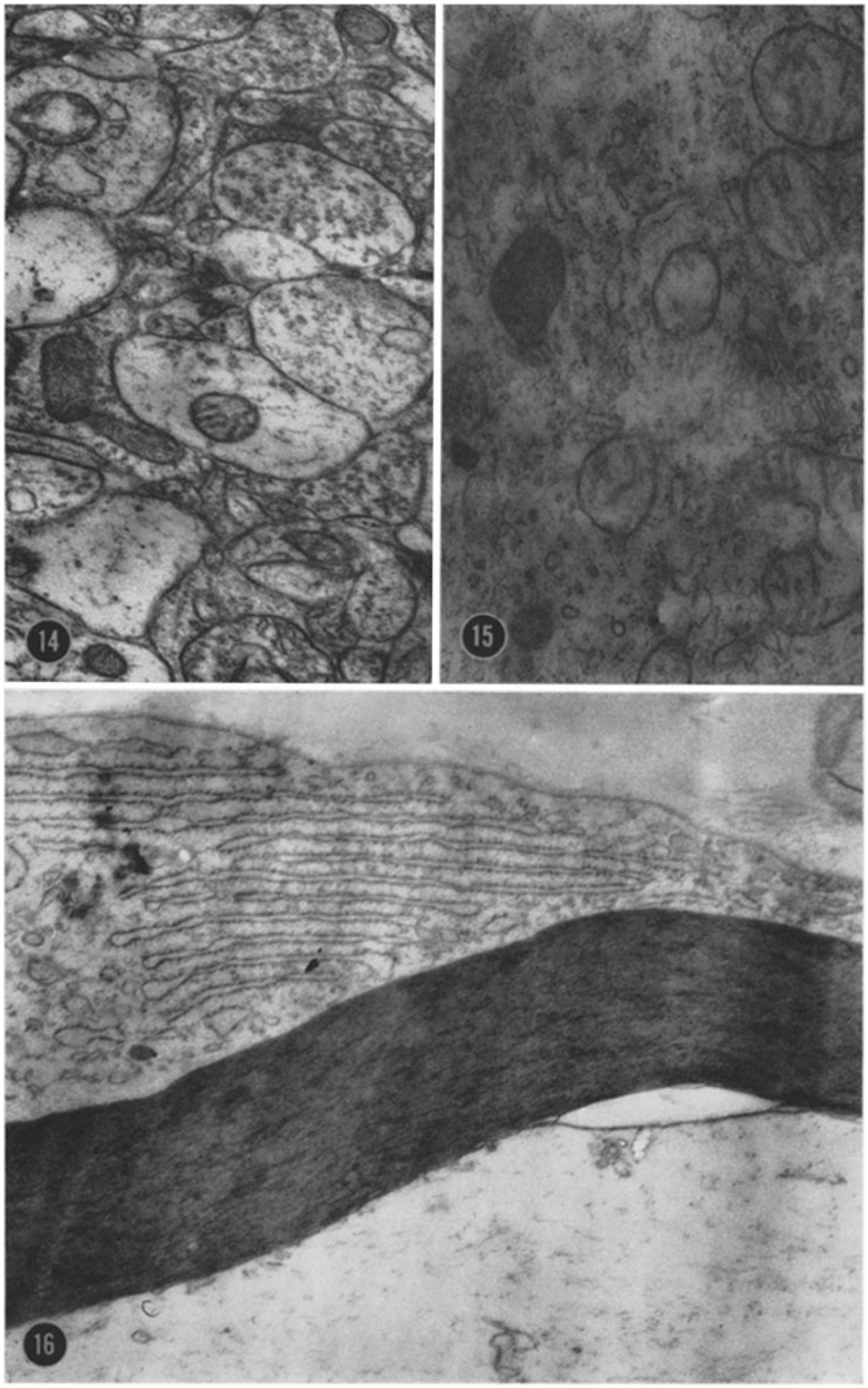
Area of cerebral cortex, fixed in 1 per cent OsO_4, embedded in Vestopal W. Processes show mitochondria, synaptic vesicles, and fibrillar structures. All of these elements, as well as cell membranes, show good preservation. × 32,000.

FIGURE 15

Cerebral glial cell cytoplasm, fixed in 1 per cent OsO_4, embedded in Vestopal W. Mitochondria, endoplasmic reticulum, and cytoplasmic granules compare favorably with methacrylate-embedded material. × 40,000.

FIGURE 16

Myelin sheath of a normal rabbit sciatic nerve axon, fixed in OsO_4, embedded in Vestopal W. This section shows good preservation of axon elements. The myelin sheath is intact and compact. Axon cytoplasm shows retraction away from the sheath at only one point. The Schwann cell cytoplasm is intact, containing vacuoles, an occasional mitochondrion, and endoplasmic reticulum, but shows some evidence of extraction. × 16,000.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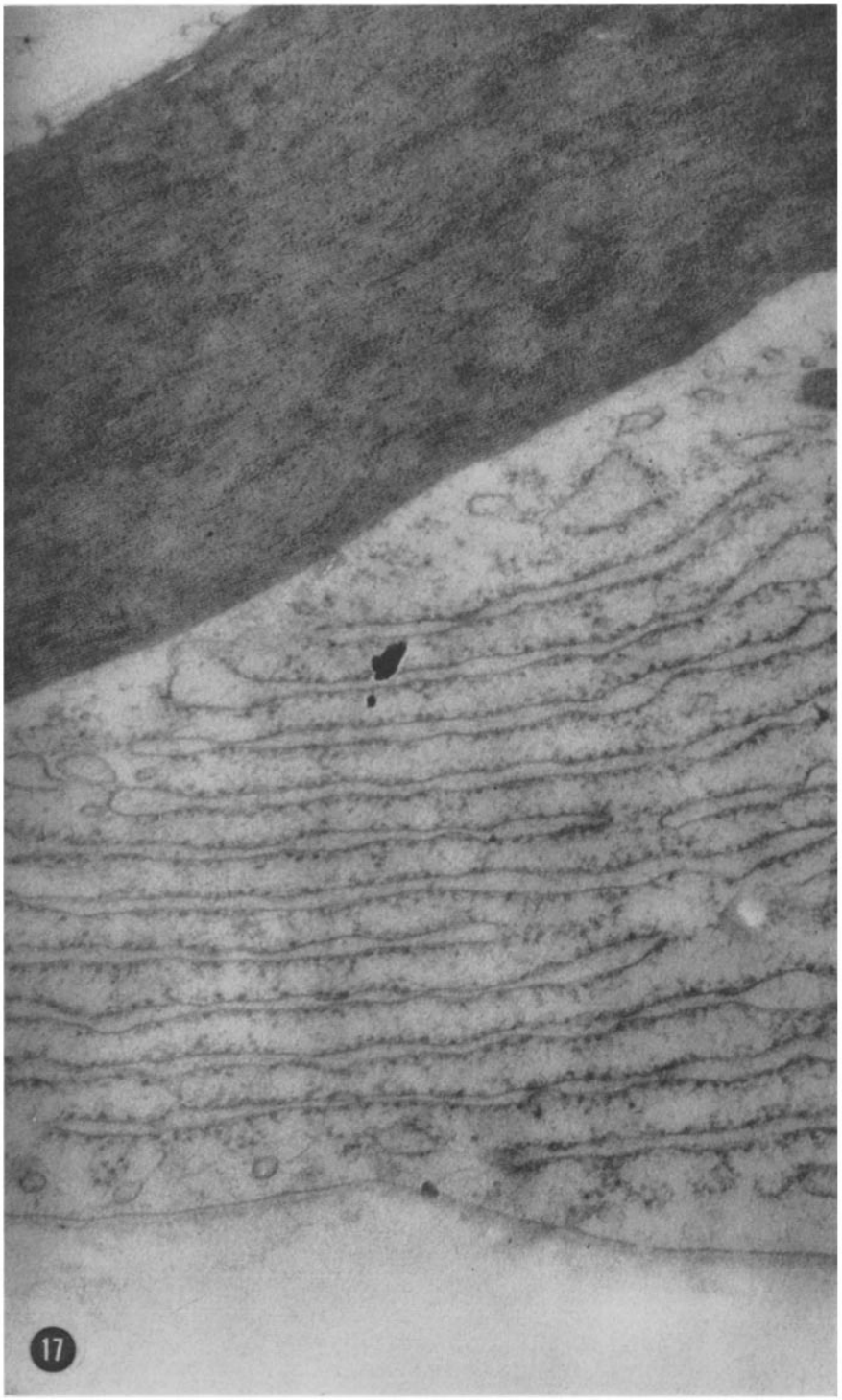
15
16

FIGURE 17

A higher magnification of Fig. 16, showing well preserved myelin sheath lamellae. If this figure is compared with Fig. 7, the differences between routine methacrylate and routine Vestopal W. embedding become clear. In the two figures the myelin sheaths are of approximately the same width, and the magnification is similar. × 43,000.


17