Thin Sections

I. A Study of Section Thickness and Physical Distortion Produced during Microtomy

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ABSTRACT

Knowledge of the thickness of sections is important for proper interpretation of electron micrographs. Therefore, the thicknesses of sections of *n*-butyl methacrylate polymer were determined by ellipsometry, and correlated with the color shown in reflected light. The results are: gray, thinner than 60 mμ; silver, 60 to 90 mμ; gold, 90 to 150 mμ; purple, 150 to 190 mμ; blue, 190 to 240 mμ; green, 240 to 280 mμ; and yellow, 280 to 320 mμ. These results agree well with optical theory and with previous published data for thin films. Sections, after cutting, are 30 to 40 per cent shorter than the face of the block from which they were cut. Only a small improvement results from allowing the sections to remain in the collecting trough at room temperature. Heating above room temperature, however, reduces this shortening, with a corresponding improvement in dimensions and spatial relationships in the sections. When the thickness of the section is considered in interpreting electron micrographs instead of considering the section to be two-dimensional, a more accurate interpretation is possible. The consideration of electron micrographs as arising from projections of many profiles from throughout the whole thickness of the section explains the apparent lack of continuity often observed in serial sections. It is believed that serial sections are actually continuous, but that the change in size of structure through the thickness of one section and the consideration of only the largest profile shown in the micrograph can account for the lack of continuity previously observed.

GENERAL INTRODUCTION

In recent years, the cutting of thin sections of embedded specimens for electron microscopy has become routine in many laboratories. Several good microtomes have been designed (1–5), and some are available commercially.[1] Improvements in knives (6, 7) and methods of mounting sections (8) have made excellent results in producing good specimens readily attainable. In spite of advances in the technique of microtomy, knowledge of the mechanism and effects of the cutting process is quite scant. The accurate interpretation of electron micrographs of thin sections and the attainment of the maximum amount of information on the material being examined require knowledge of many factors of the preparative process. Among these factors are such physical properties of the section as general thickness and local variations in thickness (knife marks, "chatter," etc.).

Some of the microtomes mentioned above have calibrated advances, but, because of thermal effects in the microtome and in the specimen, and effects inherent in the cutting process, the calibration does not give a true measure of section thickness. Consequently, the microtomist does not have accurate knowledge of the thickness of the sections he cuts. It has become standard practice to evaluate the thickness of sections by observing the interference

[1] The Moran ultra microtome is made by E. Leitz and is available in the United States from E. Leitz, Inc., New York. The Porter-Blum microtome is made and distributed by Ivan Sorvall, Inc., Norwalk, Connecticut. The Sjöstrand ultra microtome is made by LKB-Produkter, Stockholm, Sweden, and distributed in the United States by Ivan Sorvall, Inc., Norwalk, Connecticut.

colors in light reflected from the sections as they float in the collecting trough. This procedure, however, gives only a *relative* measure of section thickness. The present study was carried out to provide an accurate correlation of color and thickness, providing the microtomist with a convenient measure of *absolute* section thickness.

MEASUREMENT OF SECTION THICKNESS

Background Information:

The literature contains several estimates of section thickness which are based on either a microtome advance calibration or shadowing of the edge of the section (3–5, 9). Since the use of the advance calibration is not precise, for reasons already stated, and shadowing gives only a measure of the thickness of the edge of the section, it is to be expected that these estimates of section thickness would tend to be inaccurate. For example, Eaves and Flewett have concluded that sections in order to be colorless must be thinner than 15 mμ (9). This conclusion is incorrect and appears to be based on data which do not apply to the case in question in which there is a phase change on reflection. Indeed, there seems to be a general tendency in the literature to *underestimate* the thickness of sections, as has been pointed out by Cosslett (10), and is borne out by the results of the present study.

Occasionally, the material being examined provides an accurate "internal standard" by which section thickness can be estimated. This is seen to advantage in the work of Huxley on myofilaments, in which section thicknesses of the order of 20 mμ are demonstrated, using the myofilaments as the internal standards (11). However, it is obvious from examination of many published micrographs that sections this thin are not in general use, and it is probable that most estimates of section thickness in this range are inaccurate.

Interference colors provide a convenient way to judge the relative thicknesses of sections, and are therefore of tremendous practical importance. Interference phenomena in thin films are covered in many elementary optical texts, but discussions of the origin of interference colors are not so readily available. Therefore, a brief treatment of the origin of these colors will be given here.

The colors seen in the light reflected from the section are produced by interference between the rays of light that are reflected from the top surface of the section and the rays that are reflected from the section-water interface. The former rays, since they approach their reflecting surface from the side of lower index of refraction, undergo a phase change of π radians upon reflection, while the latter rays do not. For a section with a thickness of t and an index of refraction of n, and at normal incidence, there will be destructive interference at wave lengths, λ, given by

$$m\lambda = 2nt \tag{1}$$

in which m is any integer, and is called the order of the interference. For any particular value of t, those portions of the spectrum with wave lengths around λ's given by Equation 1 are suppressed, giving a relative enhancement to other portions of the spectrum.

In addition, at wave lengths defined by

$$(m - \tfrac{1}{2})\lambda = 2nt \tag{2}$$

there is constructive interference, and the wave lengths around these λ's are reinforced.

As t increases, the constructive and destructive interference peaks move to longer wave lengths, giving a series of colors, each of which corresponds to a particular thickness. As the order of interference increases, the color series repeats, with some changes. The thicknesses of interest in electron microscopy are covered by the first order and the beginning of the second order.

The thinnest sections that show definite color are those that are about 100 mμ thick (optical thickness, nt, about 150 mμ, since methacrylate has an index of refraction of 1.5). According to Equation 1, this gives destructive interference in the region of wave lengths around 300 mμ, that is, near the blue end of the visual spectrum. There is also a constructive interference peak, according to Equation 2, in the region of 600 mμ, near the red end of the spectrum. The result is a shift of the spectral range observed toward the long wave length end, and a predominance of yellow and red is observed, giving the sections a reddish yellow or gold color. For sections thinner than this (less than 100 mμ), only the first order constructive interference peak is effective. When it is near the center of the spectrum, the sections appear bright silver (80 to 90 mμ), and as the peak moves toward the blue and eventually out of the visual range, the sections become bright lavender gray (70 to 80 mμ) and then dull or dark lavender gray (60 mμ or less). As zero thickness is approached ($t=m=o$), all wave lengths destructively interfere, because of the half-wave length phase change on reflection, and the sections become darker gray and eventually black.

For sections thicker than gold sections, the destructive interference peak moves toward the longer wave length region of the spectrum, until at a thickness of about 180 mμ (optical thickness about 270 mμ) it is centered in the green region, giving a relative enhancement to the red and blue, and producing the purple

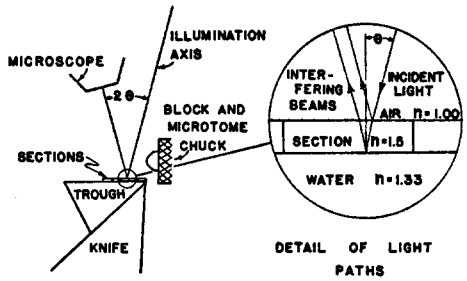
color observed at this thickness. At greater thicknesses, the destructive interference peak moves farther toward the long wave lengths, until at a thickness of 230 mμ, it is suppressing the red end of the spectrum and the second order constructive interference peak is beginning to intensify the blue end of the spectrum, giving a bright blue color to sections of this thickness. As the second order constructive interference peak moves through the spectrum with increasing thickness, the sections show colors of increasing wave length: green, yellow, and red, in that order. These sections are out of the range of thickness normally used in electron microscopy, except at high accelerating voltages (100 kv.), because lower energy electrons do not penetrate such thick sections sufficiently well.

Some optical data on interference in thin films of air and other substances have been published, and these data can be compared to the present data. Since most recent articles on the subject either directly or indirectly quote data from papers by Newton (12), Quincke (13), or Rollett (14), the data from these three papers, together with the more recent data of Mönch (15), were used in compiling the color scale (straight line in Text-fig. 2) to which the data on sections from the present work will be compared.

Experimentation:

Microtomy.—Sections were cut on a Porter-Blum microtome from blocks of *n*-butyl methacrylate polymerized in a 60°C. oven from monomer containing 2 per cent catalyst (50-50 2,4-dichlorobenzoyl peroxide and dibutyl phthalate[2]). There were no specimens embedded in the blocks used to produce sections for thickness measurements. The glass knives used were prepared by the method of Cameron (6), and superior edges were selected by inspection at 250× in a light microscope, using light reflected from the edge of the knife in a manner similar to that recently described by Sheldon (16). A metal trough, affixed to the knife and filled with water, was used to collect the sections (see Text-fig. 1). The section area was fairly large (2 to 3 mm. square) to aid accurate thickness measurements, and only sections that appeared uniform in thickness and free from defects such as knife marks were selected for measurement. The use of such large sections prohibited the cutting of extremely thin sections, but it was possible to cover the thickness range down to the dark gray sections.

Observation of Sections.—A 20× binocular microscope was used to observe the cutting process and the sections floating on the water surface. The microscope was adjusted so that the angle between the microscope axis and the illumination was kept small



TEXT-FIG. 1. Diagram of microtome setup, showing light paths used viewing in sections with reflected light. Insert shows light paths and reflections from section surfaces which give rise to the interfering light rays.

(30°). Thus the apparent change in thickness due to the oblique incidence of the light was kept constant and known. The arrangement is shown in Text-fig. 1. The color of each section was recorded, for comparison with its measured thickness.

Thickness Measurement.—The sections were picked up from the water surface on a Langmuir-Blodgett step-wedge prepared with Ba-stearate double layers (17). This wedge served as both a support for the sections and as a calibration standard. Thickness measurements were made on an ellipsometer of the Trurnit type (18).[3] In the ellipsometer, light is obliquely incident on the section and plane polarized at 45° to the plane of incidence. This light becomes elliptically polarized after reflection from the section. The phase and amplitude relationships of the components of the reflected light perpendicular and parallel to the plane of incidence are very sensitive to changes in section thickness, and thus the ellipsometer, which measures these parameters, gives a very accurate measure of section thickness.

Results:

Table I shows the observed colors and measured thicknesses of the sections. Since the interference colors were viewed at an angle from the normal to the section, the light producing the colors passed obliquely through the section. Therefore, before accurate correlation between color and thickness can be made, the actual (measured) thickness must be corrected to give the effective thickness or half-path length difference for the interfering light beams which determined the color observed. The two thicknesses are related by

$$t_e = t\left(1 - \frac{\sin^2\theta}{n^2}\right)^{1/2} \qquad (3)$$

[2] Luperco CDB, made by the Lucidol Division of Wallace and Tiernan, Inc., Buffalo, New York.

[3] Recording ellipsometer, model 441, O. C. Rudolph and Sons, Caldwell, New Jersey.

TABLE I

Results of Thickness Measurements

Measured thickness	Color observed at 15° to normal	Effective thickness for $\theta = 15°$
mμ		mμ
49	Gray	48
49	Gray	48
49	Gray	48
49	Gray	48
64	Grayish silver	63
64	Silver	63
83	Silver	82
85	Silver	84
88	Silver	87
92	Silver	91
95	Gold	94
110	Silver	108
125	Gold-silver	123
125	Gold	123
127	Gold	125
128	Gold	126
135	Reddish gold	133
166	Reddish gold	164
200	Purple	197
239	Sky-blue	235
264	Yellow	260
278	Yellow-green	274

in which t_e = the effective thickness or half-path length difference for the interfering light beams, t = the actual thickness of the section, n = the index of refraction of the section, and θ = the angle between the illumination axis and the normal to the section (see Text-fig. 1). It should be noted that, although the distance traversed by the light through the section increases as the illumination becomes more oblique, the effective thickness decreases.

Column 3 of Table I gives the calculated effective thicknesses of the sections for $\theta = 15°$, the angle used throughout this study. This is the thickness that should be correlated with the colors in Column 2.

Comparison with Published Optical Data.—These results can be compared with the published optical data referred to above. The optical phenomena are comparable in each case, since there is a phase change on reflection at only one surface. In Text-fig. 2, the abscissa represents thickness and the ordinate is a color scale based on the published optical data referred to above, corrected to an index of refraction of 1.5, and plotted on the same scale as the abscissa. The solid line is drawn with a slope of 1, and therefore represents the published optical data. For comparison with the published data, the data in Table I of the present study are plotted on the same graph (circles). It can be seen that the correlation between the two sets of data is good.

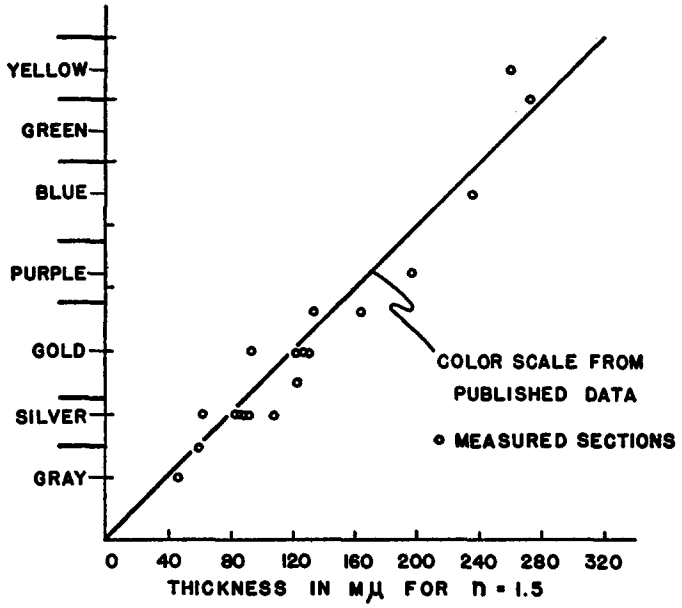
The color scale is summarized in Table II. The thickness range from 0 to 320 mμ has been divided into seven color bands, using the terminology presently used by electron microscopists. It must be emphasized that the color scale is actually continuous, and that all gradations and combinations of adjacent colors can be observed. For example, a light gold section can be said to be 90 to 110 mμ thick and a reddish gold one, 130 to 150 mμ; while a pure gold colored section showing no trace of red will have a thickness near the center of the gold band, or 110 to 130 mμ. Thus, the thickness of a section can very easily be estimated to within 10 to 20 mμ, using this color scale.

MICROTOMY ARTIFACTS

Background Information:

Williams and Kallman have described a "compression" in sections in a direction parallel to the direction of cutting, and attributed this compression to a fine scale wrinkling of the sections (19).[4] (In the present paper this dimension will be referred to as the length of the section, the dimension parallel to the knife edge as the width, and the dimension orthogonal to these as the thickness). Morgan *et al.* (20) have shown a periodic banding across the width of sections with an average periodicity of 0.2 μ. The banding largely disappears when the section is exposed to the beam of an electron microscope, but the compression artifact remains, since the section is already attached to the support film at many places and therefore cannot expand lengthwise. The section merely flattens down on the substrate, giving the distortion of structures commonly observed in electron micrographs. If the points of attachment were widely spaced, there could be expansion of small areas compensated by further compression in other areas. This would lead to variability in compression within one section, as has been reported by Morgan *et al.* (21). The fol-

[4] The word *compression*, as used by Williams and Kallman and as used in the present paper, refers to a one-dimensional shortening with a concomitant increase in thickness, and does not imply an increase in density, as in the thermodynamic meaning of the word.



TEXT-FIG. 2. Correlation between section thickness measurements and published color data. The ordinate is a color scale compiled from previously reported data on interference in thin films (12–15). The data of the present study are plotted (circles) corresponding to the observed color and measured thickness. Each point is plotted at the center of the range of the color, although the color covers a whole band of thicknesses. In the case of mixed colors, the point is plotted midway between the two colors.

If the correlation between the color scale, which is based on optical data, and the measurements on sections is good, the points should lie along the 45° line drawn on the graph. It can be seen that the correlation is good.

TABLE II

Color-Thickness Correlation

Color	Thickness range n = 1.5
	mμ
Gray	<60
Silver	60 to 90
Gold	90 to 150
Purple	150 to 190
Blue	190 to 240
Green	240 to 280
Yellow	280 to 320

lowing experiments give evidence that the mechanism of compression is really a folding of the section, and provide a method of reducing the compression artifact.

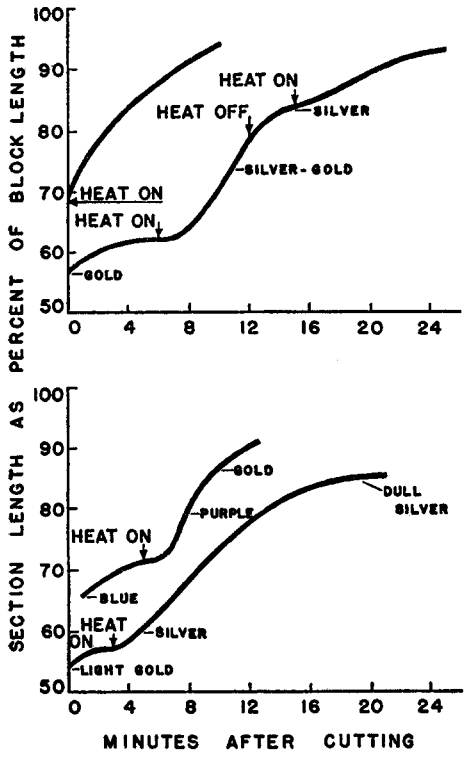
Experimentation:

The compression of sections was quantitated in the present study by measuring the length of the cut face of the blocks and the length of the sections as they floated in the trough. Measurements were made with a reticule in the eyepiece of the viewing microscope.

Sections floating in the collecting trough were found to expand when exposed to an RFL-2 reflector photoflood lamp at a distance of 20 cm. for 1 to 10 minutes. This was evidenced by a change in color and an expansion, in the direction parallel to the cut, to a length more nearly equal to the corresponding dimension of the block. The expansion of sections of different thicknesses was studied as a function of time, both with and without heat treatment.

Since, presumably, the sections after this treatment are flatter and their thickness more nearly equals the thickness removed from the block by the knife, the treatment was used throughout this study.

The effect of this treatment on the electron microscopic appearance of sections of embedded tissue was determined in the following way. Serial sections of various tissues were cut. Individual sections were measured in length and picked up on carbon-coated specimen grids at various times after being cut, and after various times of exposure to the heat from the photoflood lamp. During subsequent examination of these sections in an electron microscope, information on effects of the treatment on both the methacrylate and the embedded tissue was obtained. Corresponding structures in the different sections of the series were measured, and the results were correlated with the lengths of the sections as determined prior to picking up the sections on the specimen grids.



TEXT-FIG. 3. Plot of section length as a function of time during section flattening with heat. Arrows indicate times at which the heat source was turned on or off. The interference colors of the sections at various degrees of flattening are indicated. The top curve represents the sections which appear in Figs. 1 to 4.

Results:

Text-fig. 3 shows the changes in section length with time for four representative sections with different original thicknesses. It can be seen that a section, when cut, is considerably shorter in length than the face of the block. For example, a section showing gold interference color, when first cut, is usually reduced in length to about 60 per cent of the corresponding dimension of the block. Generally, the thinner the section, the more serious is the compression.

Leaving the sections floating in the trough at room temperature for several minutes removes less than one-quarter of the sectioning distortion. Exposure to heat from the photoflood lamp for 5 to 10 minutes, however, removes most of the compression, restoring the section to about 90 per cent of the length expected from the dimensions of the block face.

TABLE III

Heat Expansion of Sections

Figure number	Time of treatment	Sarcomere length	Section length as per cent of block length	Corrected sarcomere length
	min.	*μ*	*per cent*	*μ*
1	0	1.07	68	1.57
2	1	1.26	75	1.68
3	5	1.35	86	1.57
4	10	1.38	94	1.47

No change is found in the width of the sections either during the cutting or during the subsequent heat treatment. Since the length of the section is reduced to less than that of the block face while the width remains the same, the section must in some way be thicker than the piece removed from the block. During a 10 minute exposure to the heat, the temperature of the water in the boat rises about 10°C. (from room temperature). This would be expected to soften the methacrylate, but not to melt it. If the sections were truly thicker, a softening of the material would be expected to be followed by a two-dimensional expansion under the influence of surface tension forces, or no change in dimensions at all. Since the observed effect is a one-dimensional expansion, the compression must be anisotropic with respect to the length and width of the section. This fact, plus the fact that this artifact can readily be reduced by treatment of the sections with heat, would indicate that the sections are not actually thicker, but folded, with only an apparent increase in thickness due to the folding. This folding probably arises from a sticking and piling up of the methacrylate on the top surface of the knife as the section is cut, or more accurately, *scraped* from the block.

The effect of the reduction of this artifact by heat treatment on the fine structure of sections is illustrated in Figs. 1 to 4, which is a series of electron micrographs of serial sections (not necessarily adjacent sections) of muscle sectioned longitudinally (knife travelling parallel to myofibrils), and heat treated for varying lengths of time. The result of the heat treatment is an increase in dimensions along the myofibrils, as shown by an increase in sarcomere length. No apparent destruction of tissue elements or loss of methacrylate results from the heat treatment.

In Table III, the sarcomere lengths for corresponding areas in the sections in Figs. 1 to 4 are

tabulated, along with the total length of the section. The ratio of the two lengths should remain constant if the expansion is uniform, that is, if the expansion is the same inside the tissue structures as in areas that are mostly or all methacrylate. As seen in Table III, this ratio does remain constant, indicating that the expansion is uniform in this case. It is possible, however, that the expansion would be less uniform in a more heterogeneous tissue.

Sarcomere lengths in Figs. 1 to 4 have been corrected on the basis of the section length data to give values which correspond to the length in the embedded tissue before sectioning. The results are tabulated in Table III. The error which results from making such measurements in unexpanded sections without considering compression is obvious.

It was thought that possibly the compression shown here was not universal, but associated with the particular combination of microtome, knives, etc., used. However, a reexamination of electron micrographs of thin sections published by many authors using other equipment, and a measurement of the axial ratios of structures which are presumably spherical (nuclei in some tissues, isolated mitochondria, etc.) has revealed that a section compression of about 30 to 40 per cent has been common if not universal.

DISCUSSION

These data are somewhat at variance with previous estimates of section thickness. The present data must be taken as correct, however, since they were determined by accurate physical means, and since they closely correlate with optical data and theory.

Part of the previous underestimation of thickness may be due to the use of the microtome advance calibration in making the estimates, and the fact that the microtome would be most likely to produce good sections when extraneous thermal effects were causing additional advance. When these extraneous effects were opposing the advance, probably no section at all or only poor sections would be cut, and the microtomist would temporarily discontinue the sectioning.

Several points must be kept in mind in using the color-thickness data presented here to estimate section thickness. First, it must be remembered that the thickness values are for colors viewed normal to the section. Whenever the microscope axis and illumination are inclined away from the normal to the section, the colors observed will not exactly correspond to the thickness of the section. The error will, however, be less than 2 per cent for θ less than 15°, and can therefore be neglected in view of the subjective nature of the color estimation. If, however, the angle should be very large, the microtomist can measure the angle between the illumination axis and the microscope axis, divide by two to obtain the half-angle (θ), and calculate the true thicknesses from Equation 3. These thicknesses can then be tabulated for use with the particular viewing system in estimating true thickness.

It also must be remembered that the color observed depends on the color-content of the illumination used. The colors reported here were observed using incandescent light. Fluorescent light gives similar results, although there is some shift of the colors observed toward the blue end of the spectrum, due to the higher short wave length content of the fluorescent light.

The removal of the compression artifact has several beneficial effects. One is the restoration of structure dimensions to values which are closer to those in the embedded blocks. While, due to volume changes during fixation and dehydration, these values are not necessarily the same as those in the original tissue, at least no additional artifact is introduced by the sectioning. This not only gives more meaning to the measurement of dimensions in thin sections, but improves the shape and spatial relationships of the structures as observed in the micrographs.

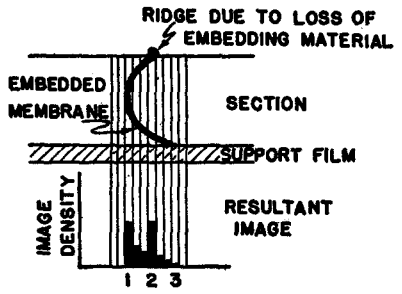
Another advantage of section flattening is associated with the relationships between specimen thickness and the resolution attainable in the electron microscope. Ornstein (22) and Cosslett (10) have pointed out that the thinner the specimen, the better is the attainable resolution. There are advantages, however, to the use of fairly thick sections from the standpoint of contrast and the inclusion of sufficient material in the section for structural analysis. We must concern ourselves here with two thicknesses. One is of interest in considering the resolution of the optical system, and is the thickness of the microscope specimen. The other is of interest in interpreting the micrographs, and is the amount of material which is removed from the blocks. Thus, it is desirable to remove a fairly thick section from the blocks, but to have a fairly thin specimen for the microscope. Compression during sectioning clearly is a step

in the wrong direction. Section flattening improves the situation by allowing a thicker piece to be cut from the block without impairing resolution with an excessively thick specimen.

Another method of reducing compression in sections has been described by Satir and Peachey (23), and involves a softening of the floating sections with solvent vapor, allowing surface tension forces to expand the section. This method is considerably easier than the heat method described above, and has the added advantage that it does not cause thermal effects in the microtome. The fact that this solvent-softening also causes a one-dimensional expansion of the section, adds further support to the hypothesis that the section length is reduced by a folding, and not a true thickening of the section.

We can now consider the use of information on section thickness in interpreting electron micrographs. The ratio of lateral dimension to thickness of a 1 mm. square section, 100 mμ thick, is 10^4. Even when this section is enlarged in the light microscope and a lateral dimension of 10 μ is considered, its ratio to the section thickness is 100. Therefore, it is reasonable to consider the section in these instances as a two-dimensional section with no thickness. However, when a section of this thickness is put into an electron microscope and structures of 1 μ and 0.1 μ are considered, the above ratio becomes 10 and 1 respectively. It is not valid to think of the section as being infinitesimally thin.

The problem of overlap of structures is very important in interpreting electron micrographs and should not be neglected. In many cases, overlap would not be expected to lead to confusion in interpretation. For example, in Fig. 5 there is an area of the longitudinal array of myofilaments which is overlaid with some profiles of the sarcoplasmic reticulum (see *A*, Fig. 5). On the basis of the knowledge that the contractile filaments and the sarcoplasmic reticulum are two separate systems with distinct locations in the muscle cell, it does not seem likely that this picture would be interpreted as showing elements of the reticulum included inside the myofibril. It is obvious that there are two separate structures superimposed. At *B* in the same figure, however, there are sections of elements of the sarcoplasmic reticulum which could easily be misinterpreted as tubules whose interior is slightly denser than the surrounding medium, if the thickness of the section were ignored. A consideration of the section thickness



TEXT-FIG. 4. Origin of image contrast in thin section of curved membrane. The section including the membrane is shown in side view, along with the support film. Estimated image densities are shown for different parts of the image. The numbers refer to three types of image boundaries which can occur, and correspond to the lines marked in Fig. 5.

and the possible orientations of the structure within the thickness of the section leads to a far more likely interpretation of the structure as being composed of cisternae which have dimensions somewhat larger than the section thickness. A pair of dense lines with an area between which is slightly denser than the surrounding area of the micrograph represents not a tubule wholly included in the section, but a single membrane obliquely sectioned. A portion of the membrane at *B* has been reconstructed in the sketch inserted in the plate legend, which shows how the membrane twists through the section. This interpretation is supported by a consideration of the origin of the dense lines seen bounding the structure in the micrograph. The origin of each of these lines is shown diagrammatically in Text-fig. 4. The section is shown in side view, with the membrane curving through it. The expected density of the image is shown below the section. The numbers correspond to the numbers of the lines in Fig. 5. Line *1* is the portion of the membrane, as it curves through the section, where the electron beam traverses the greatest length of membrane material. This is at the outside of the image, and forms one of its boundaries. It appears as a dense line, because of the orientation of the membrane parallel to the electron beam. Line *2* represents one of the cut margins of the membrane. It derives its density from the loss of embedding material in the electron beam, which leaves the membrane standing up as a ridge on the section. Very often in pictures of this kind, one of the cut edges stands out with more contrast than the other. The other cut margin of the membrane (line *3*) in Fig. 5 does not appear

with such great contrast, since it is at the section surface which is in contact with the carbon support film, where there is little or no loss of embedding material (24).

An apparent "lack of continuity" has been observed between adjacent serial sections in the electron microscope. The effect observed is that a structure is seen with considerable dimensions in one section, and is almost or entirely absent in the immediately adjacent section. Williams and Kallmann (19) offer as explanations for the phenomenon, sublimation of embedding material in the electron beam and gouging from the block of material which does not appear in the section. While the latter is possibly a contributing factor, it does not appear to be sufficient to explain the tremendous apparent discrepancy shown in the serial sections. The present author believes that this lack of continuity is not due to a loss of material, but rather to a lack of consideration of the thickness of the sections. It is probable that the sections are in reality continuous, but that the electron microscope image consists of a projection of profiles of the structure throughout the whole thickness of the section. Therefore, a section in the region of the end of a rounded structure shows a projected profile which is larger than the smallest profile of the structure present in the section. The next section, then, has as its largest profile one close in size to the smallest profile of the previous section. In the extreme case in which the end of the structure is just included in one section, a large profile is shown in one section and nothing in the next. This is compatible with the observation that the "lack of continuity" is most often observed in small structures (dimensions of the order of the section thickness or smaller) and in the region of the end of longer structures, where the size of the sectioned profile changes rapidly from level to level, and the boundaries of the structure are sharply inclined in the section.

All sections, in the present study, were cut from polymerized *n*-butyl methacrylate. Epoxy resins are coming into increased use as embedding materials for electron microscopy, and show considerable promise (25, 26). The color-thickness data presented here will also apply to thin sections of epoxy resins, except possibly for a correction for index of refraction. The compression properties of epoxy resins have not been studied, but it is our intention to examine them in the near future.

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Explanation of Plates

Plate 125

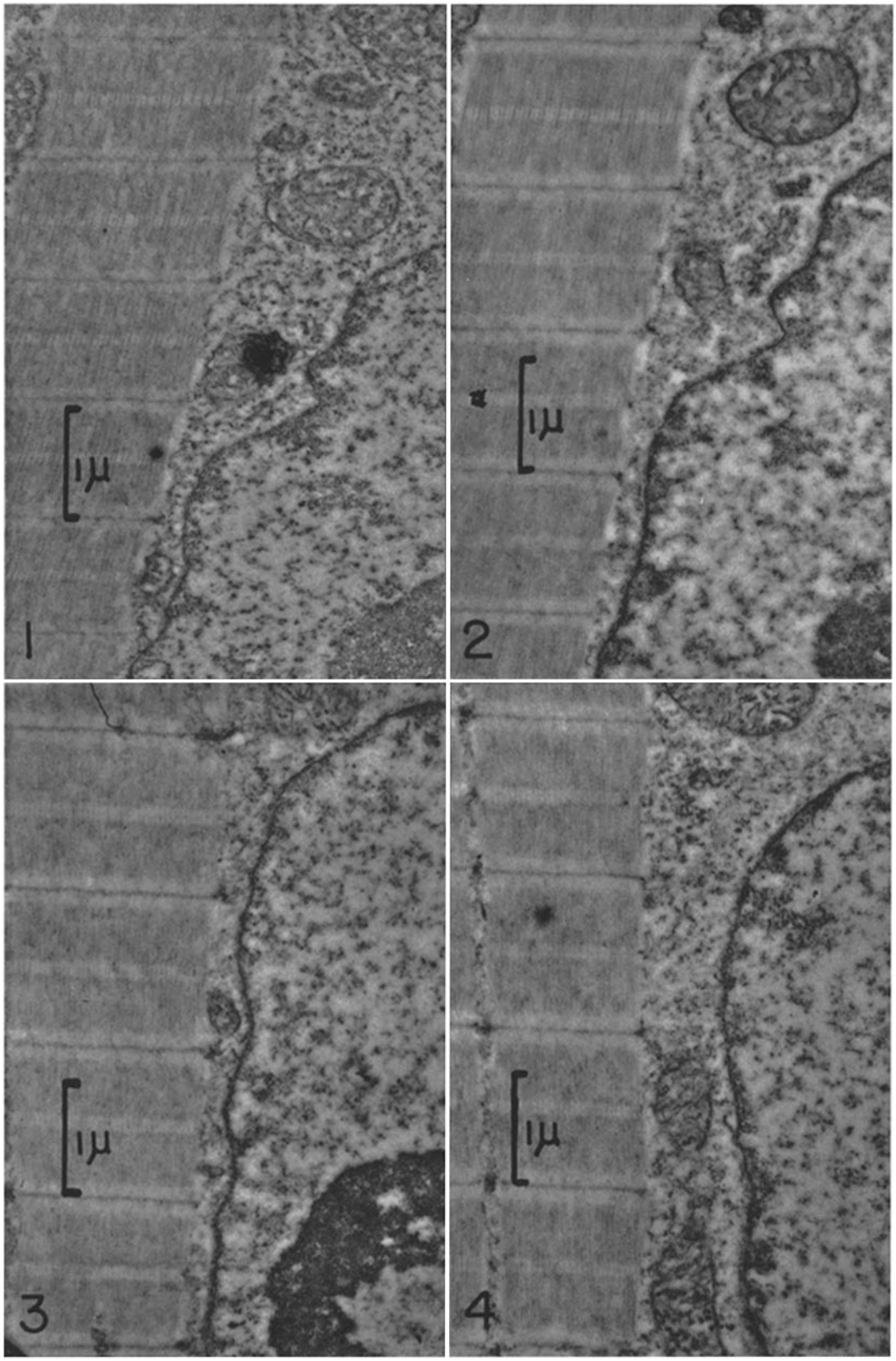
Figs. 1 to 4. A set of four serial sections (not necessarily consecutive) of muscle from the tail of a *Rana sylvatica* larva, sectioned longitudinally (parallel to the myofibrils), and heat-treated for different lengths of time. These are the sections represented by the top curve of Text-fig. 3. A portion of the same nucleus and the myofibrils adjacent to it are shown in each section. The lack of consecutiveness of the sections accounts for the rather large changes in the shape of the nucleus and the positions of the mitochondria. The important point here is that all four figures show a portion of the same nucleus, so measurements of sarcomere lengths in each figure are made on myofibrils from the same region of the same cell. The fibrils shown are uncontracted. This muscle has I bands which are short relative to the A bands. The vertical length of the face of the block from which the sections were cut was 1.5 mm. × 18,000.

Fig. 1. Section shortly after cutting—no heat treatment. The length of the section was 1.0 mm., or 68 per cent of the block face length. The sarcomere length is 1.07 μ.

Fig. 2. Section after 1 minute of heat treatment. Section length 1.1 mm., or 75 per cent of the block face length. The sarcomere length has increased to 1.26 μ.

Fig. 3. Section after 5 minutes of heat treatment. Section length 1.3 mm., or 86 per cent of the block face length. The sarcomere length has further increased to 1.35 μ.

Fig. 4. Section after 10 minutes of heat treatment. Section length 1.5 mm., or 94 per cent of the block face length. The sarcomere length has increased to 1.38 μ.

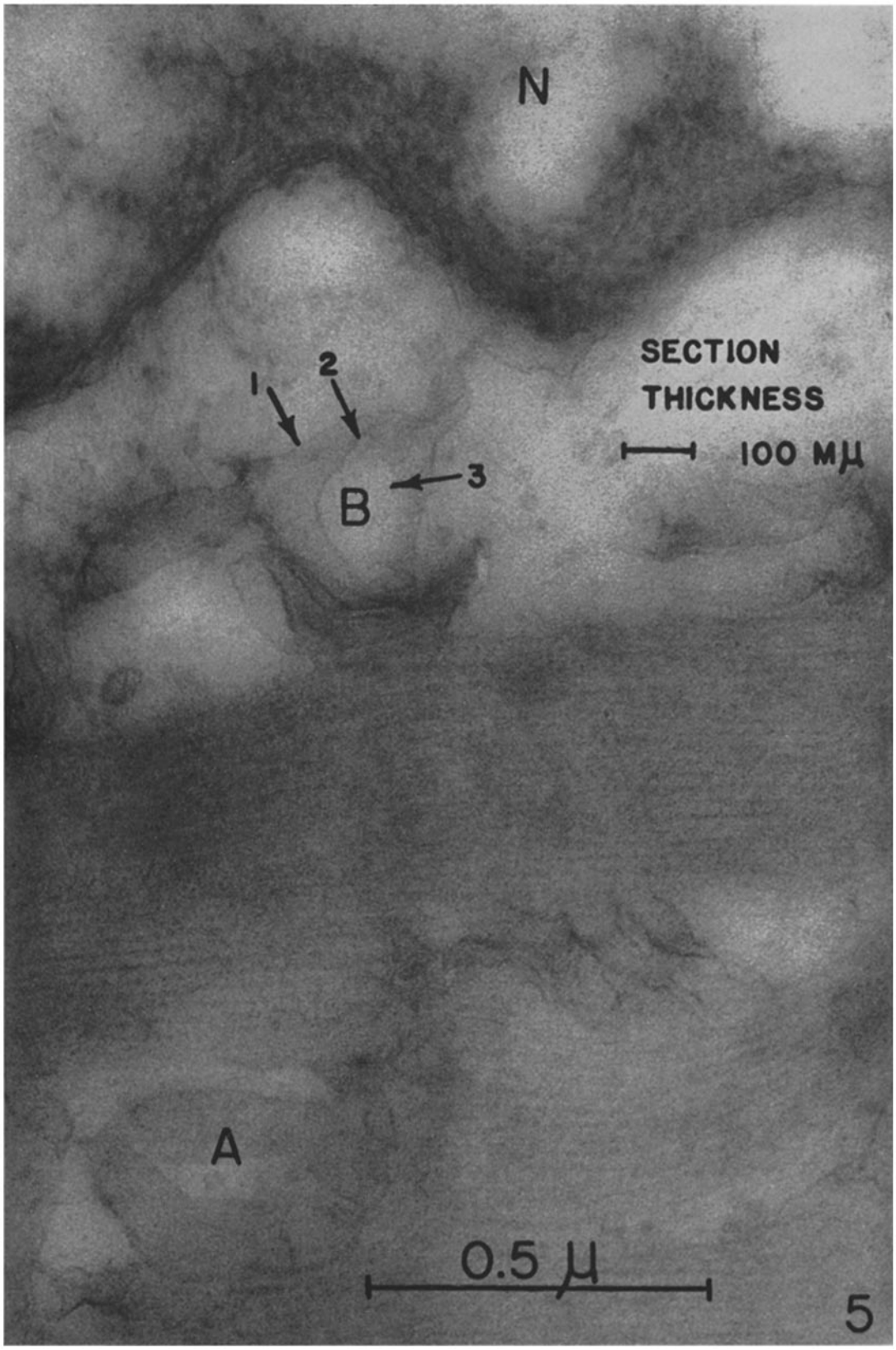


(Peachey: Thin sections. I)

PLATE 126

FIG. 5. A section of the muscle flexor ossis metatarsi digiti III of adult *Rana pipiens*. At *A*, elements of the sarcoplasmic reticulum are superimposed on myofilaments. At *B*, an element of the sarcoplasmic reticulum is shown which is interpreted to be a large cisterna extending through the section so that both cut edges of the inclined, bounding membrane can be seen. The section thickness was 100 mμ. This dimension is indicated in the figure.

The insert shows a three-dimensional reconstruction of a portion of the membrane at *B*, indicating how the membrane twists within the section. The contrast of the upper dark line in the micrograph (line *1*) arises from the portion of the curved sheet which lies roughly parallel to the electron beam. The contrast of line *2* (also dark) is due to loss of embedding material at the free surface of the section, which leaves a portion of the membrane projecting from the section surface as a ridge. Line *3* represents the cut edge of the membrane at the face of the section which is in contact with the carbon support film. The lack of a dark line at this boundary is due to the fact that there is little or no loss of embedding material at this surface. × 110,000.



(Peachey: Thin sections. I)