RECENT DEVELOPMENTS IN METHACRYLATE EMBEDDING

I. A Study of the Polymerization Damage Phenomenon by Phase Contrast Microscopy*

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Plates 1 and 2

The increasing use of thin sections of methacrylate-embedded specimens (1) as a means for the study of the fine structure of cells and tissues with the electron microscope has accentuated the problem of the elimination of preparation artifacts. The high resolution of this relatively new instrument has made it necessary to think in terms of the preservation of structures with dimensions well below the limit of resolution of the optical microscope. One approach to this problem has been provided by a technique, developed by Borysko and Sapranauskas, that permits the continuous optical examination of cells, grown in tissue culture, through all phases of the methacrylate embedding process, from the living state to the final embedded condition (2). Although this technique is limited to the optical level of resolution, it can be reasonably assumed that submicroscopic artifacts would be minimal when the appearance of the cells in the embedded condition is optically identical with their living aspect as seen just prior to fixation.

Originally, Borysko and Sapranauskas did not detect any alterations in the appearance of the cells throughout the entire methacrylate embedding process. However, subsequent experience revealed that cells in many embeddings were obviously badly distorted, and a re-evaluation of the method was undertaken. It was found that, though considerable shrinkage of spherical cells occurred during dehydration with ethanol, the most serious distortions frequently occurred during the polymerization of the embedding matrix. In this report, a study of the characteristics of polymerization damage is presented and a method for the production of relatively undamaged embedded specimens is described. A hypothesis for the mechanism of polymerization damage is proposed, based on the data obtained and the physical changes that are known to take place in methacrylate during polymerization.

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Materials and Methods

In general, the methods used in this work were the same as those already published (2). Normal rat fibroblasts, cell strain 14pf, were grown in drilled slides for 2 to 4 days. The upper coverslips of the drilled slides were then removed and the cells were fixed, dehydrated with ethanol, impregnated with methacrylate monomer and embedded by polymerization while they remained attached to the coverslips on which they were grown. Visual observations and comparative photomicrographs were made of the living cells just prior to fixation and at the end of each phase of the process. To facilitate comparisons, the same cells that were photographed in the living state were also photographed during processing whenever possible.

Preliminary observations were made on four preparations processed according to the following "basic" schedule:

1. Fixation.—10 minutes in a 1 per cent isotonic solution of osmium tetroxide buffered to pH 7.3 with a veronal buffer at room temperature (3).

2. Washing.—10 minutes in tap water.

3. Dehydration.—10 minutes in 70, 85, and 95 per cent ethanol followed by 10 minutes in each of 3 changes of absolute alcohol.

4. Impregnation.—10 minutes in each of 2 changes of methacrylate monomer followed by storage in a third change for 0.5 to 2 hours prior to embedding. A 4:1 mixture of *n*-butyl and methyl methacrylate monomers was used. The inhibitor (hydroquinone) was removed from the monomer mixture by washing approximately 5 parts of monomer with about 2 parts of a concentrated (20 to 30 per cent) aqueous solution of sodium hydroxide in a separatory funnel. The monomer was then washed with an equal volume of tap water to remove remaining traces of the sodium hydroxide solution. The inhibitor-free monomer was dried with anhydrous calcium chloride and filtered. One per cent (weight/volume) of luperco CDB paste was dissolved in the inhibitor-free monomer. After addition of the catalyst, the monomer was filtered and stored in a refrigerator.

5. Embedding.—A quantity of the catalyzed monomer was partially polymerized to a thick, syrupy consistency in the manner previously described (2). The cavity of the drilled slides was drained of impregnating monomer and filled with the embedding syrup. The upper coverslip was replaced and polymerization was carried to completion in a convection type oven at 45°C. The embeddings were kept in the oven for a minimum length of time of about 5 hours.

After the time of occurrence and some of the characteristics of distortions of the cells had been established by the foregoing method, experiments were performed to determine whether the embedding process could be altered in a manner that would better preserve the appearance of the cells. The excellent preservation of structure originally obtained by Borysko and Sapranauskas, using an almost identical processing schedule, suggested that relatively minor factors were responsible for the observed distortions. Hence, changes in processing were limited to varying the time, temperature, method of preparation or composition of the processing fluids rather than major changes in the types of fluids used. The following alterations in the "basic" schedule were tried:

1. Fixation.—Fixation times of 30 seconds; 3, 6, 10, 13, and 21 minutes; 5, 9, and 10 hours were used.

2. Washing.—The washing time was varied from a quick rinse of a few seconds duration to 10 minutes. Both distilled and tap water were used.

3. Dehydration.—The time in 70 per cent alcohol was varied from 10 minutes to 1 month. In the 85, 95, and 100 per cent changes of alcohol, the time was held at about 10 minutes.

4. Impregnation.—

A. The monomer mixture was prepared for use in the following manner: The inhibitor was removed by washing alternately with 5 per cent NaOH, distilled water, 5 per cent $NaHCO_3$, and distilled water. This cycle was repeated 6 times. Two per cent (weight/volume) of luperco CDB paste was added to the washed monomer and it was dried with anhydrous Na_2SO_4, filtered and stored in a refrigerator.

B. During removal of inhibitor by the method used in the "basic" schedule, distilled water was used instead of tap water.

C. The catalyst was added to the inhibitor-free monomer just prior to use instead of the usual practice of storing activated monomer in a refrigerator for indefinite periods of time prior to use.

D. *N*-Butyl methacrylate was used alone in place of the usual mixture of 4 parts of *n*-butyl and 1 part of methyl methacrylate.

E. The dibutyl phthalate was removed from the luperco CDB paste and relatively pure 2,4-dichlorobenzoyl peroxide was used as a catalyst. This was done by suspending about 50 gm. of paste in about 250 ml. of absolute alcohol and filtering the suspension in a Büchner funnel. The dibutyl phthalate, being soluble in absolute alcohol, was thus separated from the relatively insoluble peroxide. The catalyst was washed with about 500 ml. of absolute alcohol and air-dried before adding it to the monomer.

F. The concentration of catalyst in the monomer was varied. The following percentages (weight/volume) were used: 0.5, 1, 2, 2.5, and 4.

5. Embedding.—

A. The viscosity of the embedding syrup was varied from that of the monomer (partial polymerization omitted entirely) to a viscosity so great that the mass barely flowed. When partial polymerization was omitted, the upper coverslip was sealed in place with paraffin to prevent evaporation of the monomer in the polymerization oven.

B. Mechanical vibration was used to accelerate polymerization. In a series of experiments designed to study factors influencing the rate of the polymerization reaction, it was discovered that mechanical vibration of moderate frequency and amplitude could almost double the rate of the polymerization reaction. Polymerization was carried out by placing the preparations on a plywood platform that was set into vibration by a permanent magnet radio loudspeaker mechanism vibrating at 60 cycles per second. The platform was placed in a polymerization oven heated at 45°C.

C. Polymerization was carried out at 60° and 80°C.

Strict control of any step in the process was assumed to be unimportant when it was found that drastic changes in the step did not have a visible effect on the quality of preservation of the cells. Thus, after determining that storage in 70 per cent alcohol for prolonged periods of time had no visible effect on the appearance of the cells as compared to 10 minute storage, preparations were allowed to remain in 70 per cent alcohol for any convenient time, usually overnight.

Electron Microscopy.—Electron micrographs were made of cells that had suffered various degrees of polymerization damage. Sections were cut in a plane normal to the plane of the base of the cells, using a motor-driven Servall

ultra-microtome (5). Microtome settings of 500 and 250 A were used and the sections were mounted serially on slot-type grids.[1]

OBSERVATIONS

The data obtained using the "basic" processing schedule will be considered together with the data obtained after experimental changes in the process were made. A summary of the work is shown in Table I. Minor changes in the process, such as whether tap or distilled water was used to wash the monomer, were not tabulated.

1. Fixation.—After fixation, no gross changes in the cells were observed other than the appearance of a slight brownish color and the cessation of the motion of small particles in the cytoplasm (Figs. 1 and 2). Nuclear membranes appeared to be more distinct, but it is not known whether this was due to a physical change produced by the action of the fixative, or whether it was an optical effect caused by a change in the refractive index of the surrounding medium. Mitochondria, lipide droplets, and vacuoles in the cytoplasm retained a life-like appearance, unaltered in size, shape, or relative position by the action of the fixative. The lipide droplets were considerably darker than in life, probably because of the staining action of osmium tetroxide. The number, position, shape, and size of nucleoli and the distribution of "chromatin" threads in interphase nuclei remained essentially unchanged. The quality of preservation appeared to be independent of fixation time since it was as good after fixing for 30 seconds as it was after fixing for 10 hours.

2. Dehydration.—The first readily apparent distortion of the specimens appeared during dehydration. A symmetrical decrease in the diameters of spherical cells in the colonies was observed when the cells were immersed in 70 per cent ethanol (Figs. 1 and 2). The shrinkage apparently occurred during the first few minutes of immersion since it was never seen to be in progress at the end of 10 minutes nor did prolonged storage in 70 per cent ethanol cause any greater shrinkage. Approximate comparative measurements of the diameters of 5 completely dehydrated spherical cells indicated that the decrease in diameter during dehydration was of the order of 5 to 10 per cent, as shown in Table II. From these measurements, it is evident that spherical cells may suffer a decrease in volume of as much as 30 per cent during dehydration. Dehydration shrinkage was not detectable in the flat cells that made up the bulk of the population of the colonies. In these cells, the shape and relative positions of all cytological structures remained much the same, after dehydration, as in the living cells.

The length of time that specimens were stored in 70 per cent ethanol did

[1] Slot-type grids were made to order by Smethurst High-light Ltd., Bolton, Lancs., England. They are now carried in stock.

TABLE I

Results of Experimental Changes in the Methacrylate-Embedding Technique

Prep. No.	Fix.	70 per cent alcohol	Monomer	Per cent conc. cat.	DBPh	Pre-poly.	Poly. temp.	Vibr.	Dam-age
							°C.		
1, 12, 13, 14	10 min.	10 min.	4:1	0.5	+	+	45	−	+
10, 11	13 min.	36 hrs.	4:1	0.5	+	+	45	−	+
15	3 min.	10 min.	4:1	0.5	+	−	45	−	++
16	3 min.	10 min.	4:1	0.5	+	+	45	−	+
17–18	3 min.	10 min.	4:1	0.5	+	++	45	−	+
19–20	3 min.	22 hrs.	4:1	0.5	+	++	45	−	+
21	5 hrs.	10 min.	4:1	0.5	+	++	45	−	+
22	9 hrs.	10 min.	4:1	0.5	+	++	45	−	−
25	9.5 hrs.	10 min.	4:1	0.5	+	++	45	−	+
26	10 hrs.	10 min.	4:1	0.5	+	++	45	−	+
27	21 min.	—	4:1	1.0	+	+	45	−	+
28	6 min.	—	4:1	1.0	+	+	45	−	+
31	30 sec.	1 mo.	4:1	2.0	−	+	45	−	+
33	3.5 min.	18 hrs.	4:1	2.0	−	−	45	−	++
34	3.5 min.	18 hrs.	4:1*	1.0	+	−	45	−	++
35	3.5 min.	18 hrs.	4:1	2.0	−	+	45	−	+
36	3 min.	5 hrs.	4:1	2.0	−	+	45	+	+
37	3 min.	4.5 hrs.	4:1	2.0	−	+	45	+	+
38	3 min.	20 min.	4:1	2.0	−	+	45	+	+
40–41	3 min.	—	4:1	2.0	−	+	45	+	−
42	3 min.	—	4:1	1.0	+	+	45	+	+
43	3 min.	46 hrs.	*n*-Butyl	4.0	−	+	60	−	−
46	3 min.	12 hrs.	*n*-Butyl	4.0	−	+	45	+	−
47	3 min.	11 hrs.	*n*-Butyl	2.0	−	+	45	+	+
48	3 min.	11 hrs.	*n*-Butyl	2.0	−	+	60	−	−
53	11 hrs.	11 hrs.	*n*-Butyl	2.5	−	+	60	−	−
54–55	3 min.	4 hrs.	4:1	1.0	−	+	60	−	−
56–57	3 min.	4 hrs.	*n*-Butyl	2.5	−	+	60	−	−
58–60	3 min.	12 hrs.	4:1	2.0	−	+	60	−	−
63–64	3 min.	11 hrs.	4:1	2.0	−	+	60	−	−
66–70	3 min.	24 hrs.	4:1	2.0	−	+	60	−	−
71–72	3 min.	15 hrs.	4:1	2.0	−	+	60	−	−
75–77	3 min.	17 hrs.	4:1	2.0	−	+	80	−	−
78–79	3 min.	17 hrs.	4:1	2.0	−	+	60	−	−

* Monomer prepared by long method.

DBPh = dibutyl phthalate; Prepoly. = prepolymerized methacrylate; Poly. temp. = polymerization temperature; Vibr. = vibration during polymerization. In DBPh and Vibr. columns, + indicates use. In Prepoly. column, + indicates normal viscosity, ++ indicates greater than normal viscosity and − indicates that prepolymerized methacrylate was not used. In Damage column, + indicates slight to moderate damage, ++ indicates complete destruction, − indicates no apparent damage.

not seem to be of great importance. Flat cells were just as well preserved and the shrinkage of spherical cells was about the same after 1 month of storage as after 10 minutes. It also did not seem to matter whether the fixative was washed off with a quick rinse or by soaking for 10 minutes in tap water before placing the preparations in alcohol.

3. Impregnation.—Impregnation with monomer had no apparent effect on the appearance of the dehydrated cells. This was true for all of the 7 different monomer mixtures used in this work. Neither the appearance of flattened cells nor the diameters of spherical cells were altered by this phase of the process.

4. Embedding.—The greatest distortion of the cells occurred, in some embeddings, during the hardening of the embedding matrix when a polymerization temperature of 45°C. was used. Periodic observations were made in this

TABLE II

Approximate Percentage Decrease in the Size of Five Spherical Cells during Dehydration with Ethanol. Units in Millimeters after Photographic Enlargement

Living		Dehydrated		Per cent decrease	
Diameter	Volume	Diameter	Volume	Diameter	Volume
10.0	523.6	9.0	381.7	10.0	27.1
6.5	143.9	6.0	113.2	7.7	21.3
6.7	157.6	6.4	137.3	4.5	12.9
7.2	195.9	6.8	165.0	5.5	15.8
11.2	736.2	10.0	523.6	10.7	28.8

phase of the process and it was determined that the cells were damaged when the embedding matrix had achieved a hardness approaching that of the finished product. The degree of damage was extremely variable from one embedding to the next, ranging from a barely perceptible distortion to a rather complete destruction of the cells. In a few embeddings, no damage could be detected. In general, the damage appeared in the form of a swelling of the cells with a loss of surface projections and structural detail (Figs. 1, 2, 3).

Of 30 embeddings that were polymerized at 45°C., only 4 (13.3 per cent) appeared to be undamaged. One of the 4 undamaged specimens was processed by using a long fixation time (9 hours) as the only change from the "basic" schedule. However, 3 other specimens that had been fixed for long periods of time (5, 9.5, and 10 hours) showed typical polymerization damage. Two other undamaged specimens were processed by using a fixation time of 3 minutes, 2 per cent catalyst without dibutyl phthalate in the monomer and mechanical vibration to accelerate polymerization. Three specimens that were similarly processed were damaged during polymerization. The fourth undamaged speci-

men was prepared by using 3 minute fixation, *n*-butyl methacrylate monomer containing 4 per cent catalyst without dibutyl phthalate and mechanical vibration to accelerate polymerization.

It is interesting to note that 3 of the 4 undamaged specimens in this 45°C. series were obtained by using mechanical vibration during polymerization. Of a total of 8 preparations embedded with vibration at 45°C., 3 were undamaged, a yield of 37.5 per cent. Only 1 of 22 embeddings polymerized without vibration at 45°C. was undamaged, a yield of 4.5 per cent.

The characteristic swelling of the cells that occurred during polymerization at 45°C. could not be expressed in quantitative terms. It not only varied from one preparation to the next but also from cell to cell within a single

TABLE III

Approximate Increase in Size of Nuclei in a Single Cell Colony After Polymerization of the Embedding Matrix. Units in Millimeters after Photographic Enlargement

Living		Embedded		Per cent increase	
Diameter	Volume	Diameter	Volume	Diameter	Volume
8.8	355.8	8.8	355.8	0.0	0.0
7.6	299.8	8.0	268.2	5.3	16.7
5.2	73.8	5.7	97.6	9.6	32.3
4.5	47.8	5.8	102.5	28.8	114.2
5.0	65.8	5.8	102.5	15.9	55.8
5.1	70.5	5.6	92.6	9.8	31.3
5.7	97.5	7.0	180.0	22.7	84.6
6.0	113.5	7.5	221.0	25.0	95.0
4.8	58.0	5.0	65.8	4.2	13.4
7.2	195.5	8.0	268.2	8.3	37.3

preparation. In one preparation showing moderate polymerization damage, the average diameter of 10 nuclei was increased 12.9 per cent when compared to the diameters of the same nuclei in the living cells (Table III). However, the increases in diameter ranged from 0.0 to 28.8 per cent. With such a wide variation from the mean, the average increase in diameter would be of little use as a correction factor in dimensional studies involving single cells.

Variations in the degree of damage were observed to occur not only between the cells in a single embedding but also between structures within a single cell. This was most clearly demonstrated by the almost complete destruction of nucleoli in cells which were otherwise only moderately damaged as shown in Figs. 4 and 5. Though the dimensions of the nucleus of the giant cell in these photomicrographs increased slightly during embedding, the normally dense nucleoli became barely discernible, appearing to have been swollen and fragmented almost beyond recognition. Nucleoli in neighboring cells, however,

appear to be fairly well preserved, emphasizing the local variability in the degree of damage that can occur within a single preparation.

The greatest degree of damage to the cells occurred in 3 specimens that were prepared using monomeric methacrylate instead of a partially polymerized embedding syrup at the beginning of the polymerization of the matrix. The damaged cells in these preparations appeared to have been completely destroyed (Fig. 6) and made utterly worthless for cytological studies. The typical polygonal shape of the living cells was altered so that the embedded cells appeared as roughly spherical masses in which polymorphous remains of the originally ovoid nuclei could be distinguished. Mitochondria, lipide droplets, and nucleoli were completely destroyed and surface projections were obliterated.

The only change in processing that resulted in the consistent production of preparations that were not visibly damaged during polymerization was the increase in embedding temperature from 45°C. to 60° and 80°C. (Figs. 7 and 8). Polymerization damage could not be detected in any of 20 embeddings polymerized at 60°C. or 3 embeddings polymerized at 80°C. The characteristic swelling of spherical cells was entirely absent from these preparations. In fact, the use of higher polymerization temperatures succeeded in preserving the shrinkage of spherical cells that had occurred during dehydration. The diameters of nuclei and nucleoli remained essentially the same as in the living cells. Though some decrease in contrast was observed, the shape and general positions of mitochondria and lipide droplets were unaltered. Delicate projections from the borders of the cells, measuring less than a micron in width, appeared to be perfectly preserved. The outlines of the embedded cells were almost identical with those of the living cells. Complete identity could not be achieved because of the natural movements occurring in the period between the taking of the picture of the living cell and its immobilization by fixation.

Variations in the time of addition of catalyst to the monomer prior to polymerization had no apparent effect on the damage phenomenon. When a polymerization temperature of 45°C. was used, damage was observed in specimens prepared from freshly catalyzed monomer as well as from catalyzed monomer that had been stored for several weeks in a refrigerator. The undamaged specimens obtained by high temperature polymerization were made from monomer that had been catalyzed anywhere from 1 hour to 1 month prior to use. Similarly, neither the concentrations of catalyst nor of dibutyl phthalate could be related to the presence or absence of damage in the embedded specimens.

5. *Electron Microscopy.*—In the phase contrast microscope, the cells in an embedded block selected to represent a high degree of polymerization damage appeared to be grossly swollen and almost completely devoid of surface projections. Though nuclear outlines were apparent, many of the normally dense

nucleoli could barely be seen. The only structures visible in the cytoplasm were a few dense lipide droplets. Thin sections of this preparation, examined in the electron microscope, showed that the damage consisted of a general fragmentation and scattering of the cell substance (Fig. 9). The structure of the cells was so thoroughly broken up that it was impossible to recognize whole mitochondria or the elements of the endoplasmic reticulum. However, lipide droplets were resistant to the general fragmentation, appearing as large dense bodies in the granular ruins of the cells. Projections from the surfaces of the cells (invisible in the optical microscope) consisted of masses of small granules and short fragments of membranes. Most of the granules making up the damaged cells were less than 0.2 micron in size, which would account for the optical disappearance of normally visible structures. Stretches of unbroken cell or nuclear membranes, parts of mitochondria, and bits of the endoplasmic reticulum were occasionally seen, indicating that the degree of damage was quite variable from point to point within the cells.

A similar fragmentation was seen in thin sections of a less severely damaged specimen (optically: slightly swollen with no loss of surface projections or internal structure), as shown in Fig. 10. However, the fragments were of such a size and distribution that most of the structures within the cells could be easily recognized. The damage here was characterized by numerous discontinuities in the various membranous components without loss of identity.

Thin sections of an undamaged specimen which had been embedded at 60°C. showed a remarkable compactness of structure that was quite different from the general appearance of the sections of damaged cells (Fig. 11). Discontinuities in the various membranes were almost entirely absent and the ground substance of the cytoplasm seemed to form a relatively homogeneous matrix in which a wealth of fine detail could be distinguished. These fine details were not apparent in any of the damaged specimens that were examined.

In some of the sections of the undamaged preparations, many irregularly shaped empty spaces were seen throughout the cells. By using different portions of the glass knife and by varying the cutting speed, it was determined that many of these spaces were produced during sectioning and were not related to the polymerization damage phenomenon.

DISCUSSION

Until recently, electron microscopists working with thin sections of biological materials have almost ignored specimen changes that might be induced after fixation. Emphasis was placed mainly on postmortem changes, the composition of the fixative, and the thermal and temporal conditions of fixation with very little regard for dehydration shrinkage or the variable degree of specimen damage that might occur during the polymerization of the embedding matrix (3, 4, 6, 7, 8).

The fact that cells and tissues shrink during dehydration with ethanol has been known for some time (9). This phenomenon presents a major obstacle to accurate dimensional studies of cell structure with the electron microscope primarily because it cannot be assumed that the various components of a cell suffer a change in dimension proportional to that of the cell as a whole. The failure of Borysko and Sapranauskas (2) to observe dehydration shrinkage in their time-lapse movie studies was probably due to the exclusive use, at that time, of thin, flat cells in order to obtain good photographic records of cytological details. In this work, the shrinkage was noticed only in the rounded cells. It is quite possible that shrinkage also occurs in the flat cells in a direction parallel to the optical axis of the microscope, resulting in a slight shift in focal plane that would be difficult to detect.

The polymerization damage phenomenon was first reported by Newman, Borysko, and Swerdlow in their original paper describing methacrylate embedding (1). However, these authors did not offer either a description of the damage at the cellular level or a possible explanation for its occurrence. The observations reported here show that the damage usually consists of a variable degree of swelling of the cells, involving a fragmentation of the fixed cell substance, occurring during the latter stages of the hardening of the embedding matrix. The history of treatment of the specimens prior to the polymerization of the embedding matrix does not seem to be connected in any way with the appearance of this type of distortion. It was only when the conditions of polymerization were altered that any effect on the phenomenon was noted. Thus, it can be assumed that the damage was caused by the physical or chemical changes that take place in the methacrylate during polymerization.

All of the methacrylates used for embedding decrease in volume by about 15 to 20 per cent when they are converted from the monomeric to the polymeric state (10). Swelling of the cells in such a contracting matrix can conceivably occur if the rate of contraction outside of the cells is greater than the rate of contraction of the methacrylate within the cells. During the early phases of polymerization, when the viscosity is low, the stresses set up by these local differences in rates of contraction can be relieved by flow but, as the viscosity increases, a point is reached where flow can no longer act to relieve the stresses. The resultant forces literally pull the cells apart, breaking them up into the minute fragments shown in Fig. 9.

If this interpretation of the mechanism of polymerization damage is correct, then it is obvious that the damage can be minimized by maintaining the methacrylate in a fluid condition throughout the entire polymerization reaction. Internal stresses would then be relieved by flow and the forces required to disrupt the cells could not be set up. This condition was achieved by the use of polymerization temperatures that were sufficiently high to keep the forming polymer in a fluid condition by virtue of its thermoplastic nature. Polymeriza-

tion damage was not seen in any of the high temperature embeddings, attesting to the validity of the interpretation.

When it had been established that polymerization damage in tissue culture cells could be minimized by the use of high polymerization temperatures, several other workers in our laboratory applied this modification in technique to their own specimens (lung, bone, nasal epithelium, and other tissues) without altering any other step in their processing schedules. They all reported a definite improvement in the appearance of their specimens as seen in sections in the electron microscope, and have adopted high polymerization temperatures as a standard part of their procedures. These workers used processing schedules involving such variations in technique as cold fixation and dehydration, an "isotonic" wash after fixation, acetone dehydration, and constant gentle agitation in all of the processing fluids. The general improvement in the appearance of their sections when high polymerization temperatures were used provides supporting evidence for the observation that the history of treatment of the specimen prior to embedding is not connected with the polymerization damage phenomenon.

In our laboratory, the routine use of high embedding temperatures over the past year has revealed that a number of factors other than the temperature of the polymerization oven must be taken into consideration for the consistent production of undamaged specimens. We have found, for example, that it is extremely important to use prepolymerized methacrylate at the start of embedding. The prepolymerized methacrylate must be prepared with frequent agitation to ensure a relatively uniform distribution of active polymerization nuclei in the matrix surrounding the cells or tissues. The exact temperature of the polymerization oven does not seem to be of importance so long as it is sufficiently high to maintain the methacrylate in a soft condition and yet not so high that the embedding mass becomes hot enough, due to the heat of reaction, to volatilize the monomeric component. The minimum oven temperature that can be used is, of course, dependent on the second order transition temperature of the methacrylate used and the rate of loss of the heat of reaction from the embedding mold. The latter factor determines, to a large extent, the temperature achieved by the embedding mass during the polymerization reaction.

The main value of this work, in the opinion of the author, lies in the emphasis it has placed on the idea that major artifacts may be produced at any stage in the processing of tissues for study in the electron microscope. The work of Williams and Kallman (11) and Morgan (12) carries this thesis even further, focusing attention on distortions that may be produced during sectioning and the examination of the sections in the electron beam. An understanding of the various distortions that can be produced is a prerequisite to any analysis of the fine structure of cells or tissues.

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EXPLANATION OF PLATES

PLATE 1

FIG. 1. Typical appearance of living fibroblasts as seen in phase contrast. A spherical cell lies to the right of center.

FIG. 2. The same group of cells photographed after osmic fixation and partial dehydration in 70 per cent ethanol. Note the excellent preservation of details in the flattened cells and the ethanol-induced shrinkage of the spherical cell.

FIG. 3. The same cells photographed after embedding in methacrylate, showing severe swelling produced during polymerization of the matrix at 45°C. The spherical cell is now considerably larger than in the living state. The flattened cells exhibit a general loss of fine detail and an enlargement of nuclei and nucleoli.

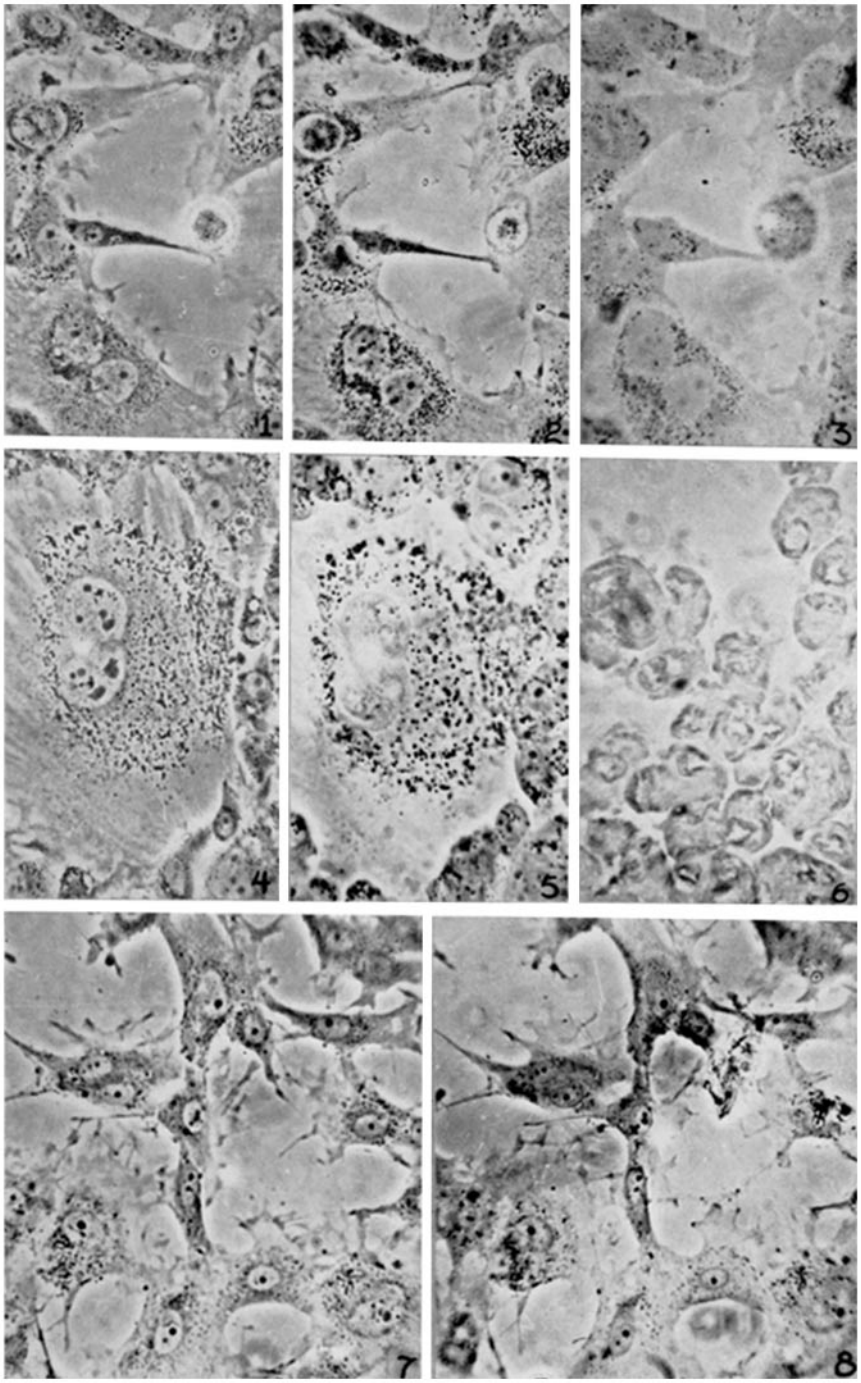
FIG. 4. Appearance of a living giant cell in a colony of fibroblasts.

FIG. 5. The same cell photographed after osmic fixation, ethanol dehydration, and 45°C. embedding in methacrylate. Note the almost complete destruction of the nucleoli in the giant cell though the nucleoli in surrounding cells appear to be well preserved.

FIG. 6. Complete destruction of cells embedded in methacrylate at 45°C. without using the prepolymerization technique. In life, these cells were comparable in appearance to those shown in Figs. 1, 4, and 7.

FIG. 7. Appearance of a group of living fibroblasts.

FIG. 8. The same group of cells after osmium fixation, ethanol dehydration, and embedding in methacrylate at 60°C., showing excellent preservation of structure that was obtained by embedding at high temperature.



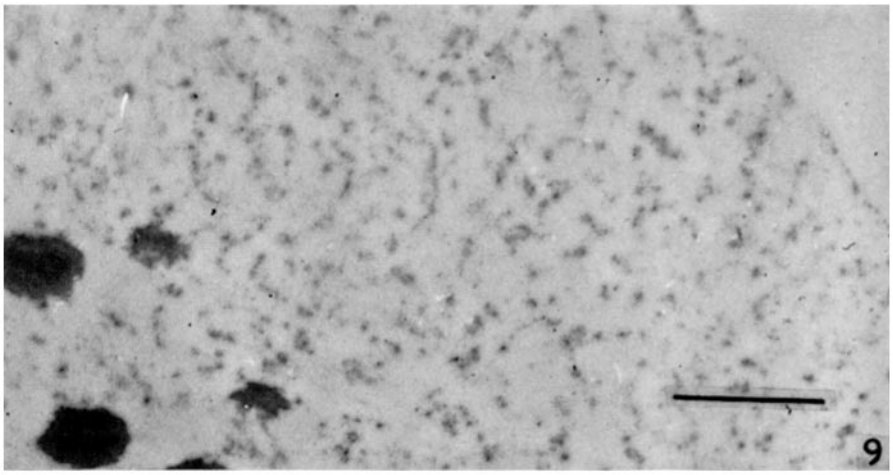
(Borysko: Methacrylate embedding. I)

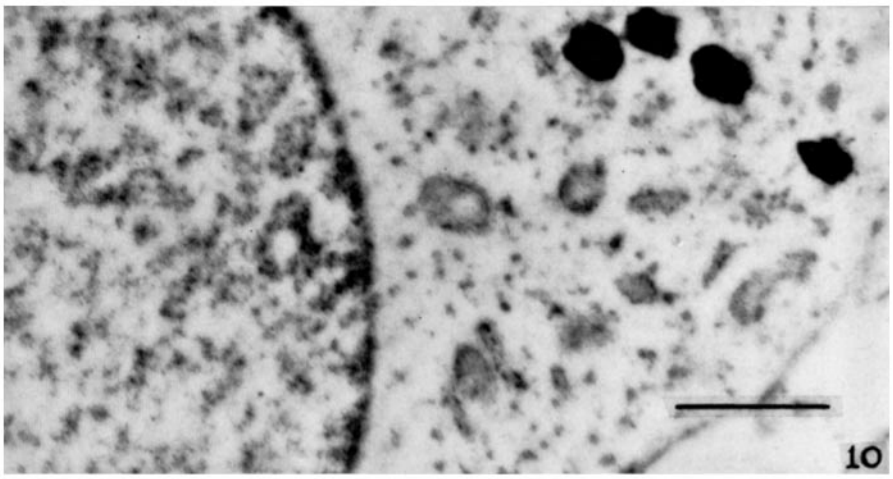
PLATE 2

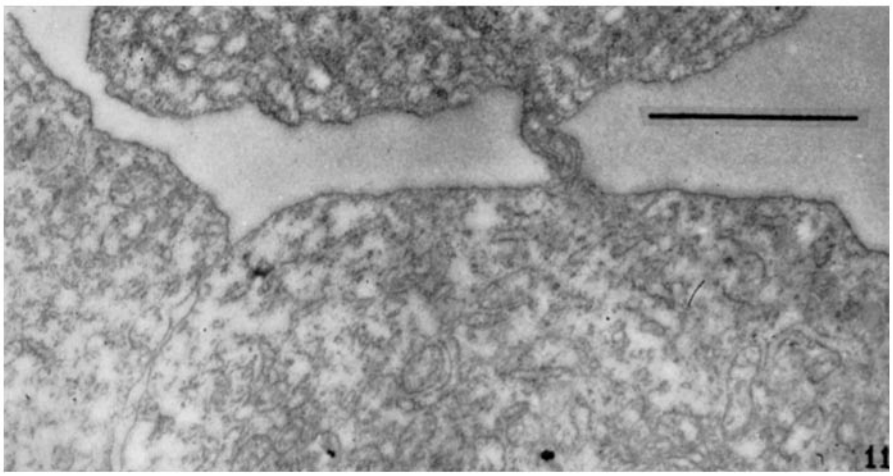
FIG. 9. Electron micrograph of a thin section of a fibroblast that had been severely damaged during 45°C. embedding, showing general fragmentation of the fixed cell substance. A portion of the cell membrane appears in the upper right corner. Lipide droplets (lower left) do not appear to be affected.

FIG. 10. Thin section of a fibroblast that had been slightly damaged during 45°C. embedding. A part of the nucleus appears on the left and a segment of the cell membrane can be seen at the lower right of the photograph. The fragmentation characteristic of polymerization damage appears here as numerous discontinuities in the membranes of the mitochondria, the endoplasmic reticulum, the nucleus, and the cell surface.

FIG. 11. Thin section of well preserved fibroblasts embedded at 60°C. All membranes appear to be continuous and there is a general compactness of structure that is lacking in the damaged preparations.







(Borysko: Methacrylate embedding. I)