A Method for Avoiding Centrifugation in the Imbedding of Suspensions in Polymethacrylates.

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The imbedding of suspensions of particles like blood cells, bacteria, mitochondria, or virus particles in polymethacrylates necessitates a number of centrifugations varying between 10 and 12 before the monomer containing the cells in suspension is set to polymerise (1, 2) unless one strong centrifugation is done after fixation and fragments of the pellet are treated like tissue fragments (3). The number of centrifugations depends on how gradually the dehydration is to be done, whether one or more changes are desired for the absolute ethanol, and how gradually the pure ethanol is to be replaced by the pure monomer containing the initiator.

The procedure involving centrifugation, removal of the supernatant, and resuspension of the pellet in the next liquid presents considerable difficulties when the particles to be imbedded are very small, of low density, or especially fragile.

Centrifugation is also a handicap when it is important to know the distance between neighbouring cells at the moment of fixation. This distance is of great importance in the study of agglutination of erythrocytes, leucocytes, thrombocytes, bacteria, etc. In these cases centrifugation can cause packing of the cells and give rise to closer contacts than would have existed if the centrifugal force had not been applied. On the other hand, when agglutinating forces are relatively small, repeated centrifugation and resuspension can bring about dispersion of the agglutinated cells and their repacking in different configurations (unpublished observations). In the following a method of imbedding of suspensions, avoiding centrifugation is described. In this method the suspending medium is gradually replaced as desired, using dialysis through a cellophane membrane as in a procedure reported previously (4) for preparing erythrocytes ghosts by gradual hemolysis.

Method

Dialyzing tubing, ¾ inch size and 25 cm. long,[1] is formed into a bag by tying it at one end, and is then treated in the following manner in order to increase the rate of diffusion through it. The open end of the tubing is attached to an ordinary water line tap and the tap is opened very carefully till the bag is filled. The rate of filling is then controlled even more carefully as the bag is swelling. When the bag reaches about two times its initial volume the tap is closed. After the bag has remained on the tap for 2 minutes it is taken off, emptied, and wiped. The membrane is now considered "stretched" and ready for use. Its diffusion time is approximately half that of the original dialyzing tube. The tube is mounted on a short section of glass cylinder so that it can be stoppered. It is then suspended by strings attached to two hooks (Text-fig. 1).

To a volume of 2 to 2.5 ml. of the fixed cell suspension an equal amount of 96 per cent ethanol is added drop by drop. The suspension is now introduced into the dialyzing bag and the bag is bent back on itself to obtain a U form (Text-fig. 1). This avoids settling of the cells in the folds near the knot and increases the area of diffusion. The U-form bag, with the suspension now in a medium of about 45 per cent to 50 per cent ethanol, is suspended in an Erlenmeyer flask containing 250 ml. of 96 per cent ethanol, as shown in Fig. 1. The Erlenmeyer is then stoppered so that the threads suspending the dialysis bag are held between the glass and the stopper. The height is adjusted so that the glass cylinder is above the level of the 96 per cent ethanol surrounding the bag. The ethanol is stirred by a magnetic stirrer, so as to cause a slight shaking of the bag. After dialysis for 10 minutes at room temperature the volume inside the bag will be markedly reduced and the ethanol inside will be about 95 per cent. The dialysis bag is now dipped into another Erlenmeyer, previously desiccated in an oven at 100°C., containing 250 ml. of absolute ethanol. The stirring is repeated as before, and after 30 minutes the dialysis bag should contain a suspension in more than 99 ethanol.[2] Two and one-half ml. of a mixture of butyl methacrylate and methyl methacrylate in the proportion 4:1 (free of inhibitor and dehy-

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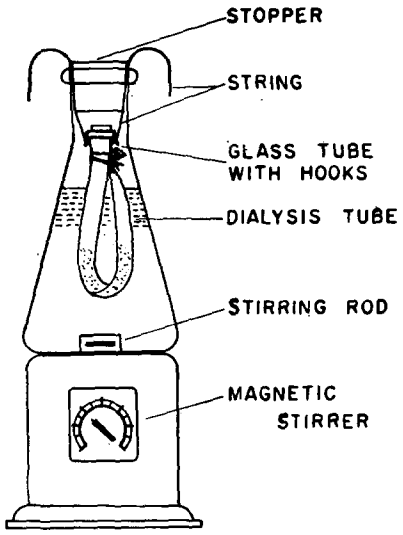
[1] Purchased from Fisher Scientific Company, New York, No. 8-667.

[2] As determined by Karl Fischer method. The authors are indebted to Mr. Z. Luz for the titrations.



TEXT-FIG. 1

drated by the usual methods) are then introduced into the dialysis bag containing the suspension in absolute ethanol. The bag is now suspended in front of a fan, so that it will be continually shaken by the flow of air. The fan is left on till the volume of suspension is reduced to 2.5 ml. or less (an operation which takes approximately 30 minutes). By then, practically all the ethanol, and traces of water which might have remained, are evaporated. The bag now contains a suspension in practically pure monomer. The next step is to add to the suspension in the dialysis bag another 0.5 ml. of the above mixture of monomers to which 25 mg. of initiator (benzoyl peroxide) has been added. The final concentration of initiator in the monomer within the bag is approximately 1 per cent. The bag is now gently shaken for a few minutes and aliquots of the suspension are taken directly from it into gelatin capsules previously dehydrated in a desiccator for 24 hours. The capsules are put in an oven at 50 to 55°C. for polymerisation.

If the particles in suspension are sufficiently dense to sediment before the polymerising monomers have become too viscous, as in the case of red blood cells, then the cell concentration in the bottom of the capsule is sufficient for sectioning and observation. If, however, the particles are not dense enough, one centrifugation is necessary to concentrate them. In this case the procedure used is as follows. When the liquid in the dialysis bag in front of the fan has reached the desired volume at which practically all the ethanol has evaporated, 0.5 ml. of the mixture of monomers containing 25 mg. of initiator is added, the suspension is centrifuged at the minimal speed and time necessary to throw down the particles. The supernatant is discarded and samples taken from the bottom of the centrifuge tube are introduced into the capsules (1 drop per capsule No. 3). The capsules are set to prepolymerise, and when the drop has become viscous the capsules are filled with a mixture of monomers containing 1 per cent initiator. We find that concentrating the suspension and replacing the supernatant by new monomer containing initiator is expedient since it ensures adequate concentration of material in the bottom of the capsule.

The polymerisation is practically complete after 24 hours. The blocks are transparent; no bubbles are observed. The degree of hardness can be controlled by the proportion of butyl and methyl methacrylate as in the usual method.

Fig. 1 represents electron micrographs of ultrathin sections of normal human thrombocytes. Blood was taken and fixed according to the method of Feissly *et al.* (3), using the Palade fixation (6). For Fig. 1 *a* the preparation was dehydrated and imbedded as described by Feissly *et al.* (3). The dehydration and imbedding was done as for tissue fragments. For Fig. 1 *b* the thrombocytes were fixed by the same method. They were dehydrated and imbedded by the above described method. Sections were cut by a new mechanically advanced microtome of our own design (5). Observations were made with an RCA-EMU 2A electron microscope using Canalco projector and objective apertures of 250 μ and 50 μ respectively.

SUMMARY

A method of imbedding cell suspensions in polymethacrylates in which the suspending medium is gradually changed by dialysis is described.

This procedure avoids centrifugation and resuspension for changing liquids. Representative electron micographs of sections of suspensions imbedded by this and one other method are presented.

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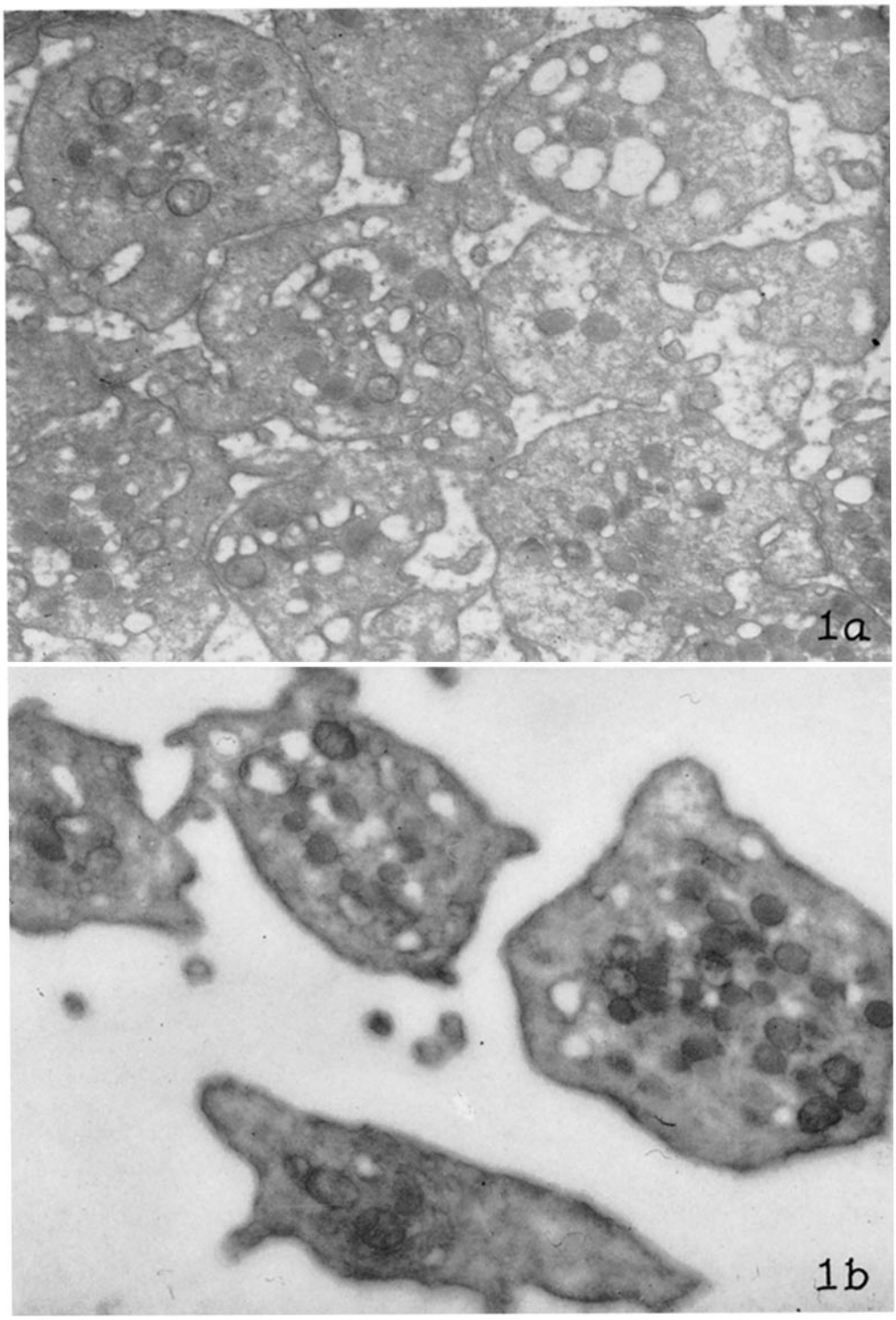
3. Feissly, R., Gautier, A., and Marcovici, I., *Rev. hematol.*, 1957, **12,** 397.
4. Danon, D., Nevo, A., and Marikovsky, Y., *Bull. Res. Counc. Israel*, 1956, **6E,** 36.
5. Danon, D., to be published.
6. Palade, G. E., *J. Exp. Med.*, 1952, **95,** 285.

Explanation of Plate 155

Fig. 1 *a*. Normal human thrombocytes, fixed, dehydrated, and imbedded according to the method described by Feissly *et al.* (3) in which the thrombocytes are centrifuged after "prefixation" and then the pellet is cut into small fragments that are subsequently treated like tissue fragments for the dehydration and imbedding. × 22,000.

Fig. 1 *b*. Normal human thrombocytes fixed as in 1 *a* (same blood) dehydrated and imbedded without centrifugation, using the method described in the text. × 22,000.



(Danon and Marikovsky: Method for avoiding centrifugation)