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Shrinkage in Preparatory Steps for SEM

A Study on Rabbit Corneal Endothelium *

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Abstract. Since specular microscopy of the cornea offers the opportunity to observe and measure cells in vivo without any outside interference this method forms an unrivalled basis for estimation of tissue shrinkage during various preparatory methods. Therefore a study was performed with the purpose of evaluating the degree of artifacts in each preparatory step from the living tissue "in vivo" to the final SEM specimen.

The study was performed on rabbit corneas, the endothelium serving as measuring target. The in vivo state was recorded by specular microscopy. Unfixed corneas were studied by light microscopy unstained and stained by alizarin red S or silver nitrate. Fixation was performed intracamerally with 1.5% glutaraldehyde (Gla) by a pH, osmolarity, viscosity and intraocular pressure identical with the physiological values of rabbit eyes. Fixation was completed by immersion in 2.5% Gla for $^1/_2$ h. Gla-fixed corneas were evaluated as above before osmification.

Dehydration was performed either by graded acetone, by acetone in a gradient-free system, both followed by critical point drying (CPD).

At all steps cells were counted using the same reference frame. The number of cells/mm^2 was estimated and statistical analysis showed a shrinkage of 22 per cent (area) in unfixed tissue, 26 per cent (area) in normally dehydrated tissue and 37 per cent (area) in gradient free dehydrated tissue processed for SEM.

Introduction

It is known that any preparation of biological tissue for morphological study leads to changes in size and shape. To evaluate the tissue in question, it is important to be familiar with these changes qualitatively as well as quantitatively.

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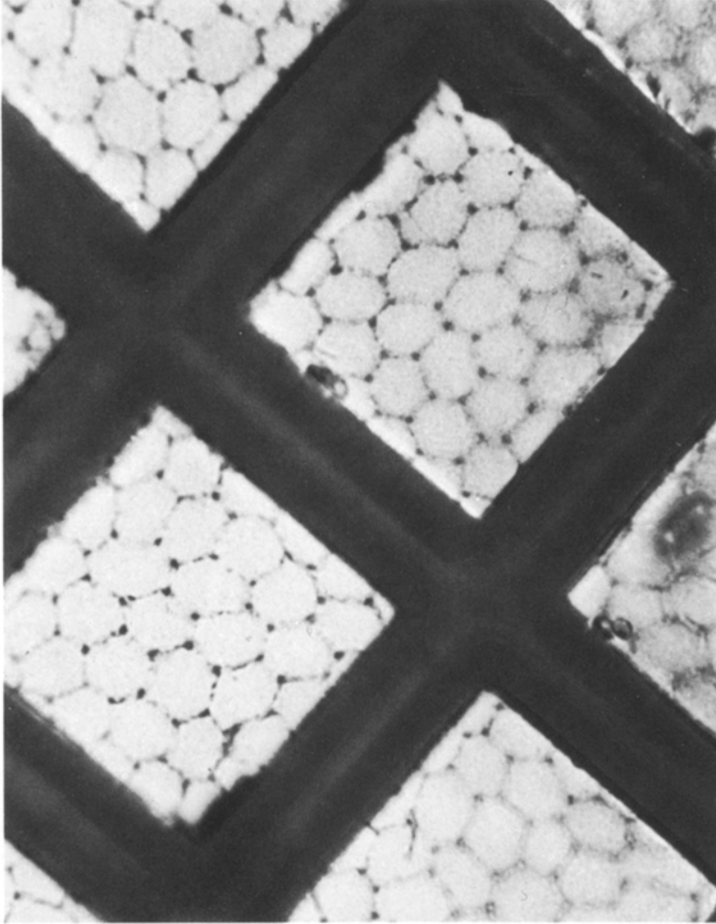

Fig. 1. Unfixed cornea stained with 1% alizarin red S for 1 min. A 200-mesh copper grid is placed on the endothelium as a reference frame for counting. (Rabbit No. 7, Lab.No. 824/79, × 1280)

Quite a substantial amount of work has been done in this field, particularly by Boyde et al. (1977) and Boyde (1978a), who provides a valuable survey of previous publications.

The work on dimensional changes during SEM preparation has been performed mainly with embryonic tissue, using mouse embryo heads, limbs and conceptuses, rat brain and liver blocks, human blood corpuscles and cultured muscle cells, as reviewed by Boyde (1978b). Shrinkage of rabbit and human corneal endothelium has been studied recently by Binder et al. (in press).

In all the above-mentioned works, the original cell tissue used for establishing initial reference measurements was removed from its natural environment.

Following the invention of the specular microscope, it has been possible to study and measure undisturbed cells, i.e. corneal endothelial cells in vivo.

The aim of the present work was therefore to measure the shrinkage of corneal endothelial cells in the process of various preparation- and staining

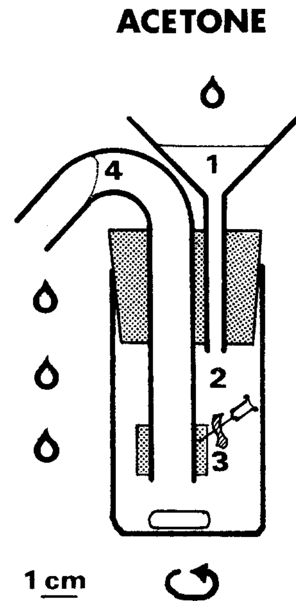

Fig. 2. Apparatus for gradient-free dehydration. 1. Funnel-intake for acetone. 2. Beaker. 3. Specimen pinned to rubber ring. 4. Outlet of mixture. (Cp. text)

methods – and mainly during preparation for SEM using the critical-point drying (CPD) method.

Material and Methods

Fourteen albino rabbits of both sexes each weighing about 2,500 g and raised on a standard chow were used.

The experimental animals were anaesthesized by intramuscular gluteal injection of ketamine chloride (50 mg/kg) and pentobarbitone sodium (30 mg/kg).

The anaesthesized animal was placed on its belly in front of a Heyer Schulte contact specular microscope. Several areas of the central corneal endothelium were photographed.

The animals were sacrificed by intravenous pentobarbitone sodium and the cornea of one eye was immediately excised and divided into quarters between razor blades.

Unfixed and unstained specimens were studied by interference contrast microscopy after the method of Nomarski in a Leitz Orthomat equipped with a Leitz interference contrast device T for transmitted light. In all light microscopy studies, a reference frame was established by placing a 200-mesh copper grid directly on the endothelium and taking photographs of at least five fields (Fig. 1).

Unfixed specimens were stained with 1% alizarin red S after the method of Sperling (1977) or with 1% $AgNO_3$, both for 1 and 5 min.

Simultaneously, the other cornea was fixed intracamerally by perfusion for 30 min with 1.25% glutaraldehyde (Gla) in sodium cacodylate buffer, the perfusion solution having an osmolarity of 275 mOsm, a pH of 7.2 and a viscosity (measured in a Ubbelohde capillary viscometer) similar to that of rabbit aqueous humour.

The perfusion-fixed cornea was excised and divided into quarters, one quarter being studied by interference contrast as above, unstained and stained, whereas the remaining parts were further fixed by immersion in 2.5% cacodyltate buffered Gla of pH 7.2 at room temperature for 1 h and postfixed in 2% OsO_4 for 1 h before dehydrating one quarter conventionally by graded acetone (30, 60, 90, 100% for 10, 20, 10, 15 min), the remainder being dehydrated in a simple gradient-free system (Fig. 2). This system consists of a small beaker filled with distilled water, which is replaced gradually by acetone over 24 h. The specimens are pinned to a rubber ring by a 26 gauge needle. Magnetic stirring ensures complete mixing of water and acetone.

Table 1. Survey of Material and Methods

Rabbit No.	Specul. Micr.	Unfix. Unstain.	Unfix. Stain.	Fix. Unstain.	Fix. Stain.	Grad. Dehydr.	Grad.-free Dehydr.	CPD	SEM	TEM
1	×	×	×	×	×					
2	×		×		×					
3	×	×								
4	×		×		×					
5	×	×				×	×	×	×	
6	×	×	×	×	×	×	×	×	×	
7	×	×	×		×	×		×	×	
8	×		×		×	×	×	×	×	×
9	×		×		×	×	×	×	×	×
10	×									
11	×			×		×	×	×	×	×
12	×									
13	×				×		×	×	×	
14	×				×		×	×	×	
Total	14	5	7	3	9	6	7	8	8	3

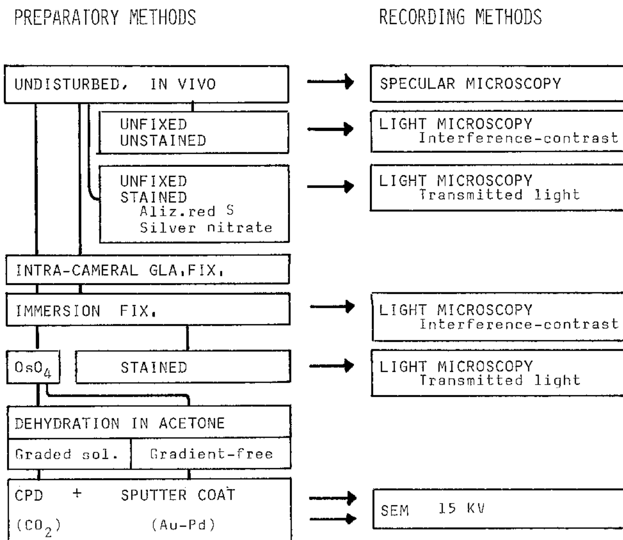

Fig. 3

Critical-point drying (CPD) was performed in a Polaron critical-point drying apparatus E 3000, the acetone being replaced by liquid CO_2 and brought through the critical point to about 1600 psi and 40° C.

The specimens were glued to metal stubs with conducting colloidal silver and coated with 50 nm gold-palladium in a Polaron E 5100 Series II Cool Sputter Coater.

The number of corneas studied after the various procedures is listed in Table 1, and a flow chart illustrating the sequence of preparatory steps is shown in Fig. 3.

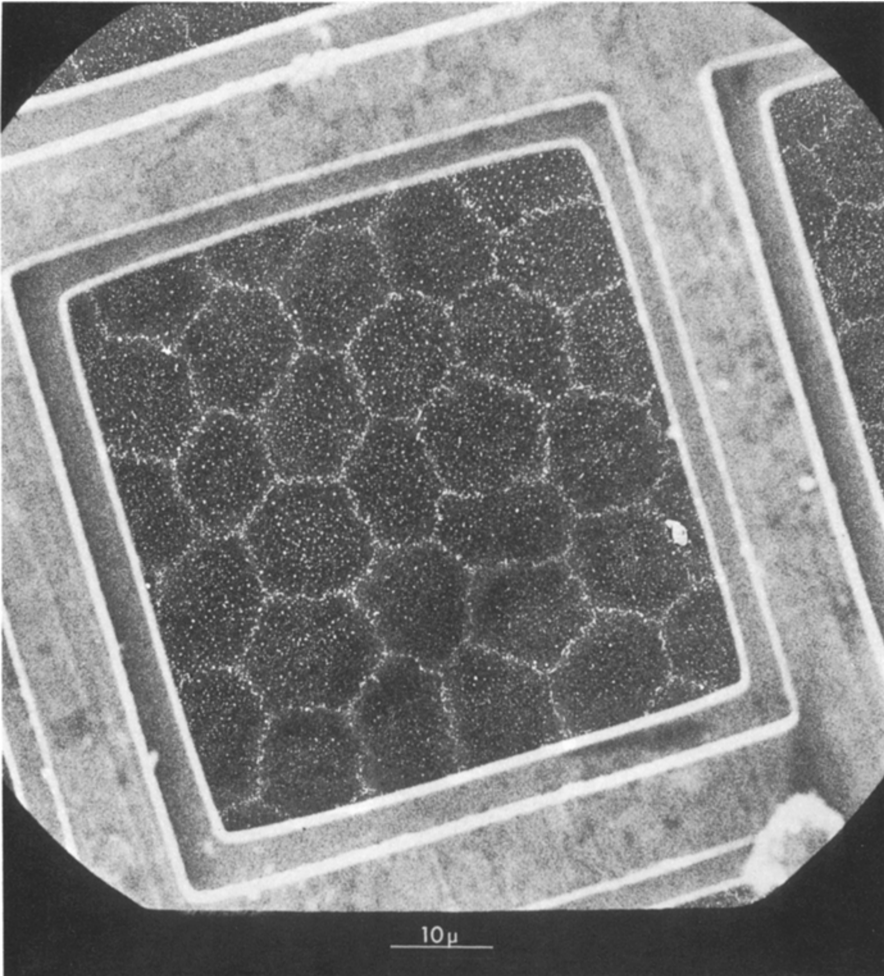

Fig. 4. SEM picture of conventionally dehydrated cornea. A 200-mesh copper grid is placed on the endothelium as a reference frame for counting. (Rabbit No. 9, Lab.No. 900/79, SEM 454, ×1360

A 200-mesh copper grid was also placed on all SEM specimens and served as a counting frame after photography (Fig. 4). The grid was fastened to the coated specimen by double tape or silver paste. The size of the frame was 9.266×10^{-3} mm^2.

By rotating and tilting the specimen the quadrangular mesh hole could be made to appear on the screen, ensuring that the endothelium was observed at a 90° angle exactly as in the light microscope. However, exact adjustment to achieve equal sides is not necessary as long as the same reference frame is used, and a correcting factor is easily calculated. The specimens were examined in a JEOL JSM-35 scanning electron microscope at 15 kV. Recording was on Agfapan 100 professional (ASA 100/DIN 21) film at a magnification of 540 times, and positive copies were enlarged three-and-a-half times.

Several corneas cut transversally were studied by transmission electron microscopy (TEM). The tissue was removed from the SEM stubs with a razor blade, placed in a propylene-epon mixture (1:1) for 4 h in vacuum and in pure epon for 2 h before polymerization at 60° C for

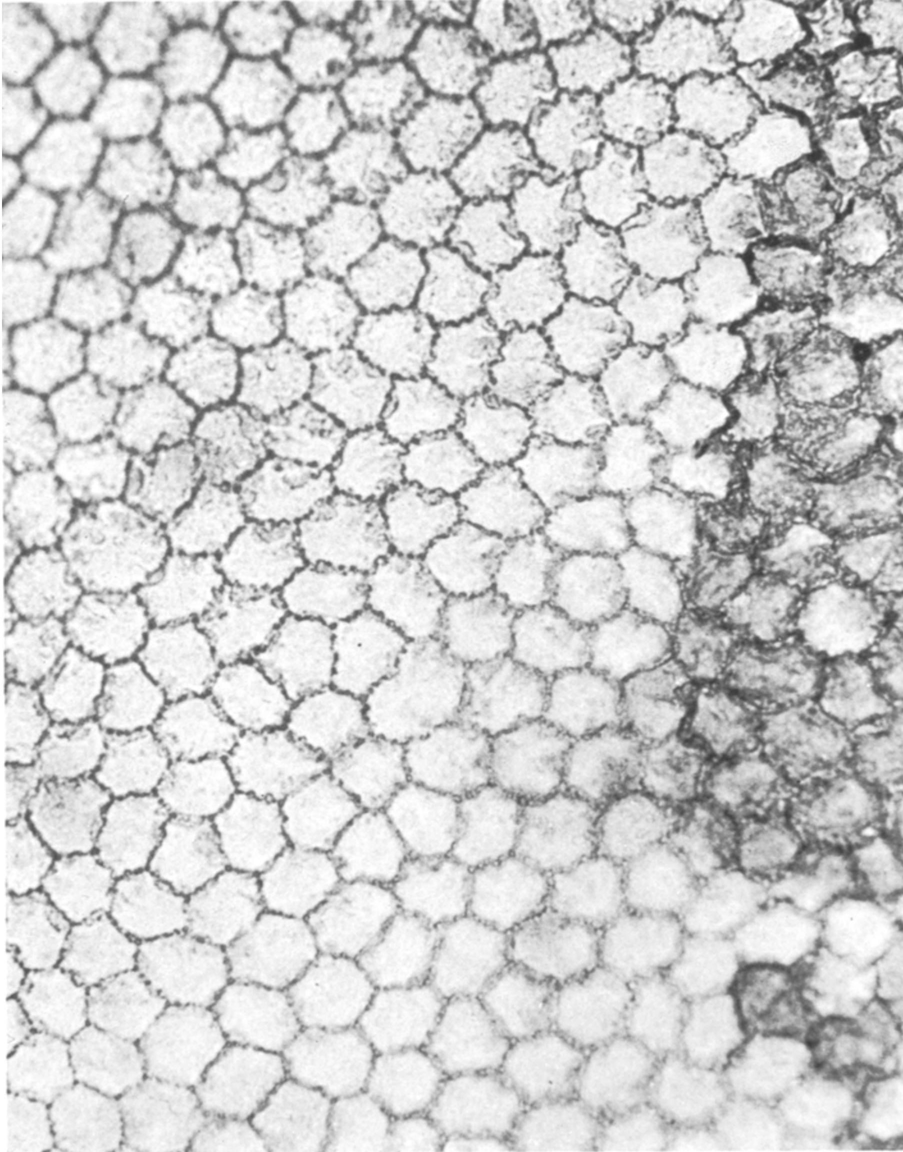

Fig. 5. Unfixed cornea stained by 1% $AgNO_3$ for 1 min. Note the distinct cell borders. (Rabbit No. 12, Lab.No. 197/80, ×1600

48 h. Survey sections were stained with toluidine blue, and ultrafine sections with uranyl acetate for 45 min and lead citrate for 5 min, the latter sections being examined in a Zeiss EM 9 S-2 or a JEOL JEM 100 C electron microscope at 60 and 80 kV.

In the specular microscope, the fixed frame, the size of which was 20.161×10^{-3} mm^2, was used for counting in the specular microscopy photographs.

Five or more frames were selected from each cornea on the basis of highest photographic clarity and counted according to the criteria given by Sperling and Gundersen (1978).

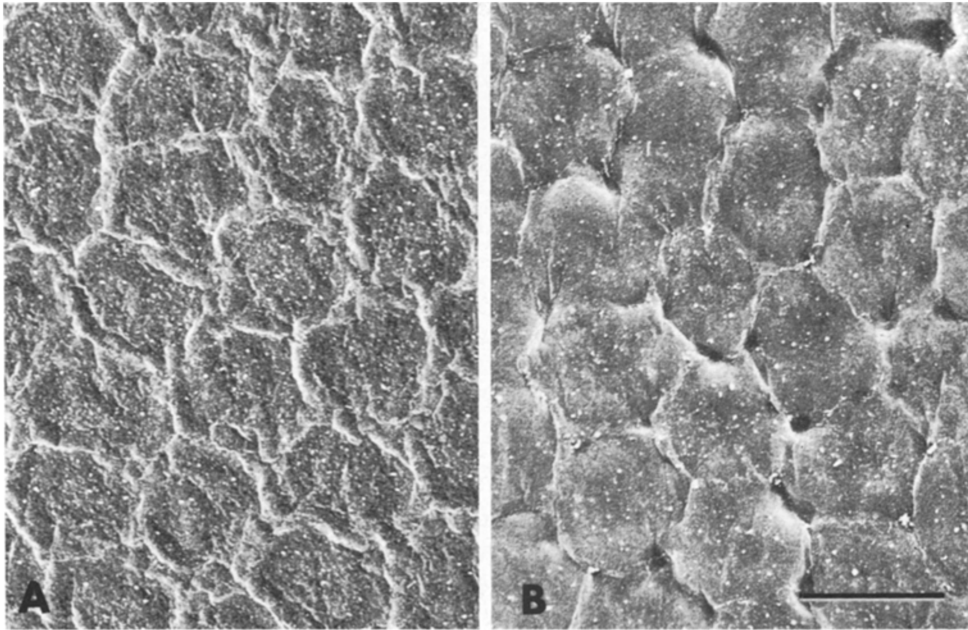

Fig. 6. Comparison between conventionally and gradient-free dehydrated cornea. **A** Conventional dehydration by 30, 60, 90 and 100% acetone for 10, 20, 10 and 15 min. (Rabbit No. 5, Lab. No. 667/79, SEM 419, ×1800). **B** Gradient-free dehydrated endothelium, distilled water being progressively replaced by acetone over 24 h. Note less-shrunken cell borders. (Rabbit No. 11, Lab. No. 88/80, SEM 472, ×1800). Scale bar 1 μm

This method eliminates the edge effect, and the counting is unbiased. With the sizes of the frames selected, the cell counts ranged from 25 to 80 cells per frame. Estimation of the central cell density is then carried out with less than five per cent error. Each cornea served as its own control, since the in vivo cell density was known from the specular microscopy. Variance analysis demonstrated significant deviations in the distributions, and non-parametric statistics were preferred. The paired Wilcoxon signed test was used. The results were given as median with 25- and 75-percentiles. The Mann-Whitney rank sum test was used for testing the difference between groups.

Results

The endothelium of the unfixed, unstained corneas was very difficult to study and to photograph, although we found the interference contrast method the most applicable. The material was insufficient for counting, and was therefore omitted in Table 2 (Counting results). This holds true also for the fixed, unstained cornea, the endothelium of which was even more difficult to photograph.

Staining of unfixed endothelium was optimal when 1% solutions of either alizarin red S (Fig. 1) or silver nitrate were used for 1 min.

As concerns the unfixed cornea, 1 min staining time was optimal. Silver nitrate staining appeared to give the most distinct picture (Fig. 5).

As concerns the fixed cornea, alizarin red S and silver nitrate staining for 1 min showed the alizarin red S staining to be far better than the silver nitrate

Table 2. Density of corneal endothelial cells and percentage of shrinkage median with 25- & 75-percentiles)

	Number of cells/mm^2	Shrinkage	(per cent)	
		Area	Volume	n
1. Specular microscopy	3114 (2770–3234)	0	0	27
2. Fixed (+stained)	3388 (2772–4004)	–	–	8
3. Unfixed (+stained)	4014 (3226–4737)	22	32	9
4. SEM normal	4230 (3605–5024)	26	37	5
5. SEM gradient-free	4961 (4239–5583)	37	50	5

n = number of corneas
3, 4, 5 ≠ 1, $2p < 0.025$, Wilcoxon signed rank test.
Each cornea served as its own control, since the in vivo cell density was known from the specular microscopy.

staining, whereas a staining time of 5 min gave the reverse result, the silver nitrate staining producing a very delicate network. We found this to be the preferable staining for fixed corneal endothelium.

In estimating the morphology of conventionally dehydrated endothelium and that dehydrated by our gradient-free method we found very little difference, but the cell borders appeared to be better preserved and the cytoplasm less wrinkled in the gradient-free dehydrated specimens (Fig. 6). In TEM examination the endothelium was quite well preserved, in no instance showing a cobblestone appearance, a shrinkage artifact particularly notable in endo- and epithelia. Neither was a prominent nucleus observed.

The counting results are shown in Table 2. The unfixed tissue and the tissue processed for SEM showed a significant increase in the number of cells per unit of corneal area. There was no significant difference between the two methods of dehydration used for SEM. The shrinkage was 22% (area) in unfixed tissue, 26% (area) in normally dehydrated tissue and 37% (area) in gradient-free dehydrated tissue processed for SEM. For all the 28 corneas the coefficient of variation (CV%) was 10.3%, and for all the central test areas the CV% was 11.9%.

The mutually-compared groups 3, 4, 5 showed no significant differences.

Discussion

The endothelium of the unfixed, and particularly of the fixed but unstained cornea is difficult to study and to photograph. In pilot studies, we tested different methods including phase contrast as advocated by Binder et al. (in press). In our hands the interference contrast method gave the best result, although optimal results were never achieved.

Staining of unfixed corneas has been extensively studied by Sperling (1977). We found his recommended solution of alizarin red S extremely useful. However, a pH of 7.4 in the staining solution is mandatory, as the authors found in co-operation with Sperling.

Fixed corneas could also be stained with the alizarin red S solution, but more distinct cell borders were observed by using 1% silver nitrate.

Comparison between counts of endothelial cells in a specular microscope and in histological preparations has been performed in only a few studies. Laule et al. (1978) used a 0.01% aqueous solution of toluidine blue O for 2 min on unfixed bank corneas, and counted the cells by light microscopy (LM) at 200× and by phase contrast. They found a difference between LM-counts and counts in a non-contact specular microscope of $+0.78 \pm 2.28\%$ (percentage error ± SEM).

This is not in agreement with our findings, since we found a shrinkage of approximately 22%. This could result from our use of fresh rabbit corneas instead of human bank corneas, and also from different staining procedures. The difference in counts obtained by specular microscopy and by the other procedures cannot be caused by non-identical counting areas, since several investigations have shown that there is no difference between peripheral and central cell densities when four or more areas are counted, each containing between 20 and 80 cells (Laing et al. 1976; Blackwell et al. 1977; Laule et al. 1978; Sperling 1978; Sperling and Gundersen 1978; Sturrock et al. 1978; Binder et al. (in press).

Shrinkage induced by the SEM procedure evaluated with the aid of corneal tissue has been studied by Binder et al. (in press). However, they did not use specular microscopy of the corneas evaluated for shrinkage. They used instead freshly enucleated human and rabbit corneas stored in a McCarey-Kaufman medium and photographed in phase contrast as a basis for their measurements. Dehydration was performed in graded alcohol and CPD from CO_2. They found a shrinkage of between 5–50%, and advocated use of the mean value of 31% for area shrinkage. This figure is in agreement with our findings based on in vivo measurements.

Shrinkage artifacts during preparation for SEM have been studied with increasing intensity over the last few years. Most investigators have used tissue blocks (Madge 1974; Boyde et al. 1977; Clark et al. 1980), embryonal tissue (Boyde et al. 1977; Boyde 1978a), blood cells (Gushnard and Kirschner 1977; Schneider et al. 1978) or cultured cells (Lee et al. 1979).

Measurements of living cells in situ have not previously been used as a basis of calculation, primarily for technical reasons. To avoid this step can induce errors in estimation of the shrinkage as shown in the present paper. The shrinkage involved in the process of removing corneal tissue from the eye and preparing it for light microscopy is approximately 22%, in spite of precautions to prevent swelling or dehydration. Few authors (Schneider et al. 1978) have used glutaraldehyde-fixed cells as a basis, and according to our findings there is no shrinkage between in vivo tissue and Gla-fixed tissue, provided temperature, perfusion pressure, pH and viscosity are kept at physiological levels. The use of fixed cells can therefore be accepted if it is not possible to perform the optimal procedure: the measurement of living cells in situ.

Boyde et al. (1977) and Boyde (1978a, 1978b) have found that use of various dehydration solutions results in a variable amount of shrinkage, and that maximal shrinkage is found in the steps between 70% and 100% of the solvent.

The above authors have also shown that little shrinkage during dehydration is followed by increased shrinkage during CPD, and vice versa, the end-result in all cases being approximately 50% of initial area.

To prevent the above-mentioned shrinkage occurring during dehydration in stages from 70–100% solvents, a gradient-free system was designed. This method unfortunately did not result in reduced shrinkage, the final shrinkage being the same as with conventional methods, i.e. 30%. However, the SEM examination showed a better-preserved cell surface with less artifacts (Fig. 6). Other advantages of the gradient-free system are its ease of use, the low cost of construction and the saving in man-power, the procedure taking place automatically during the night.

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