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Soft-tissue specimen shrinkage during the preparation for scanning electron microscopy

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Keywords: Electron microscopy; scanning electron microscopy; techniques in electron microscopy

SUMMARY

The amount of shrinkage and the scanning electron microscope appearance of specimens of rat tongue, soft palate and small intestine, dehydrated by air-drying, the critical point technique, the camphene technique and freeze drying were compared. In the specimens of tongue the area shrinkage ranged between 11,8 and 30,4 per cent, in the soft palate from 19,8 to 37,4 per cent and in the small intestine from 26,0 to 51,4 per cent. The least amount of shrinkage was in tissues dehydrated using freeze-drying. Shrinkage due to the critical point technique itself was tongue 17,4 per cent, soft palate 15 per cent and small intestine 14,4 per cent. This was half the total shrinkage seen in the tongue and soft palate and a quarter of that in the small intestine. It was not possible to distinguish between the scanning electron microscope appearances of comparable samples dehydrated with the various techniques.

OPSOMMING

Die hoeveelheid inkrimping sowel as die aftaselektronmikroskopiese beeld van tong, sagteverhemelte en kleinderm monsters van rotte wat deur middel van lugdroging, die kritiesepunttegniek, die kamfeentegniek en vriesdroging ontwater is, word vergelyk. Die inkrimping het in tongweefsel tussen 11,8 - 30,4 persent, in sagteverhemelteweefsel tussen 19, 19,8 - 37,4 8,37,4 persent, en in kleindermweefsel tussen 26,0 - 51,4 persent gewissel. Die minste inkrimping het in weefsel wat deur vriesdroging ontwater is plaasgevind. Inkrimping as gevolg van die kritiesepunttegniek self was soos volg: tong 17,4 persent, sagteverhemelte 15 persent en kleinderm 14,4 persent. Dit staan gelyk aan helfte van die totale inkrimping van tong- en sagteverhemelteweefsel en 'n kwart van dié wat in die kleindermweefsel plaasgevind het. Dit was onmoontlik om die aftaselektronmikroskopiese beelde van die vergelykbare monsters wat volgens die verskillende tegnieke ontwater is, van mekaar te onderskei.

When a soft specimen dries it shrinks producing distortions of two types, namely volume changes and surface changes (Cohen 1974).

In the early days of scanning electron microscopy soft specimens were dehydrated by air drying but later other techniques namely freeze drying, sublimation and the critical point technique were felt to produce less distortion (Watters and Buck 1971, Waterman 1972, Hollenberg and Erickson 1973). These conclusions seem, for the most part, to be based on subjective observations.

Only two studies appear to have included quantitation of tissue shrinkage. Waterman (1972) reported that linear measurements showed that embryological specimens dried by the critical point technique retained approximately 80 per cent of their original measurements. In contrast, air dried samples were reduced to approximately 45 per cent of their original size. He noted that most of the shrinkage occurred during drying since fixation and dehydration through either 100 per cent acetone or isoamyl acetate produced only 6-8 per cent shrinkage.

More recently, Wheeler, Gavin and Seelye (1975) investigated the scanning electron microscope appearance and dimensional changes of endocardium prepared by freeze drying from water or tertiary butanol and the critical point technique. The per cent reduction in surface area with the three techniques was: freeze-drying from water 6,8 per cent, freeze-drying from t-butanol 15,4 per cent and critical point drying 22,0 per cent.

The present investigation was carried out

a. to determine the degree of shrinkage in three varieties of soft tissues using air-drying, the critical point technique, a modified form of the camphene technique of Watters and Buck (1971) and three methods of freeze drying, and
b. to compare the scanning electron microscope appearances of the tissues prepared with the various techniques.

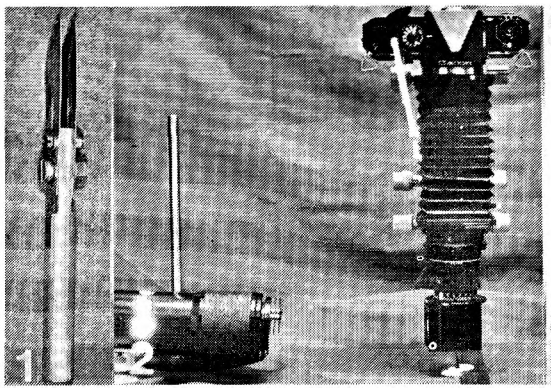


Fig. 1. **Double bladed scalpel used to cut standard specimens.**

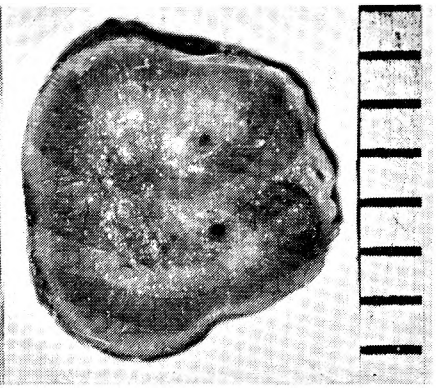


Fig. 2. **Macrophotography system used to photograph specimens and a photograph of a specimen of tongue together with the 1 mm scale rule.**

MATERIALS AND METHODS

Specimens of Wistar strain albino rat soft palates, tongue and small intestine were excised and fixed for one week in 10 per cent neutral buffered formol saline. Standard sized specimens of each tissue were then cut using scalpel blades held 5 mm apart (Fig. 1). A total of 5 specimens of each tissue was used for each of the methods being investigated.

The specimens were removed from the fixative, lightly blotted and, together with an engineer's scale, photographed using standardized lighting and a Canon F1 35 mm camera with a bellows attachment set at constant magnification to which was attached a 65 mm macrolens. Illumination by direct lighting from above, to obviate shadows, was obtained through the use of a mirror attachment attached to the lens (Fig. 2). Photographs of all dimensions were taken of the tongue and small intestine specimens but, because of its thickness of 1 mm, only the flat surface of the soft palate. Once dehydration had been completed the specimens were rephotographed. Using the image of the engineer's scale as an aid, photographs were printed at x 10 enlargement. A cut surface of the specimen was carefully outlined with ink and the area within this outline was measured using a planimeter. The length of the specimens was measured on the photographs using an engineer's scale.

Area shrinkage was calculated directly from the planimeter area recordings. Volume shrinkage was determined after calculating the specimen volume as follows: Volume of specimen = area of cut surface x specimen height.

The sequence of events in the dehydrations, which made use of a short series of alcohols (Brain 1966, Culling 1974, Drury and Wallington 1976, Nunn 1970, Pease 1964), was as follows:

(i) **Air Drying** The specimens were passed through two changes of each of 70 per cent ethanol (90 min), 96 per cent ethanol (90 min), then three changes of 100 per cent ethanol (60 min) and finally two changes of ether (60 min). The ether was allowed to evaporate in air at about 22°C.

(ii) **Critical Point Technique** Two changes of 70 per cent ethanol (90 min) and 96 per cent ethanol (90 min) were followed by three of 100 per cent ethanol (60 min) and two of amyl acetate (60 min). In this section of the study 4 of the 5 specimens of each tissue were also photographed at the end of the amyl acetate stage. The fifth specimen served as a control. The specimens were finally dried in a Polaron E2000 Critical Point Apparatus using liquid CO_2 at 1200 psi and 41.0°C.

(iii) **A Camphene Technique based on that of Watters and Buck** (1971) Following two changes of 70 per cent (90 min) and 96 per cent (90 min) ethanol the specimens were passed through two changes of acetone (30 min) and benzene (30 min), one of each of benzene + 1,2-epoxypropane (20 min), 1,2-epoxypropane (20 min at 45°C) and camphene (20 min at 45°C). After cooling to room temperature (about 22°C) they were kept under vacuum overnight at 20 psi.

(iv) **Freeze-Drying** Three types of freeze drying apparatus were used but the initial preparative technique was identical for each. After removal from the fixative the specimens were washed overnight. They were then placed into one change of chloroform in water (30 min) to nucleate ice crystal formation (Boyde 1972), before being quenched in isopentane pre-cooled in liquid nitrogen.

The three freeze-drying apparatuses were an Edwards-Pearse tissue dryer, an Edwards E12E4 vacuum coating unit (Edwards Ltd., Crawley, Kent, U.K.) and a Gallenkamp freeze drying apparatus (A. Gallenkamp, London, U.K.). The specimens were dehydrated in each apparatus for 8 hours.

Phosphorous pentoxide was used in the Edwards-Pearse tissue dryer while in the Edwards E12E4 vacuum coating unit the frozen specimens were placed into numbered compartments in a brass block pre-cooled in liquid nitrogen.

All the dehydrated specimens, irrespective of the technique used, were individually wrapped in metal foil and stored in a desiccator over silicon dioxide.

Table I. **Percentage reduction in surface area of blocks (mean ± SD)**

Method of Dehydration	Tissue		
	Tongue	Small Intestine	Soft Palate
Critical point	30,4 ± 1,5	51,4 ± 3,7	32,8 ± 1,6
Ether-air drying	29,6 ± 1,5	50,0 ± 6,2	35,2 ± 4,4
Camphene	29,4 ± 2,3	50,8 ± 3,7	37,4 ± 4,1
Freeze Drying			
(i) Edwards-Pearse	17,4 ± 5,1**	27,8 ± 89*	19,8 + 1,5*
(ii) Edwards E12E4	11,8 ± 2,2**	26,9 ± 3,8*	21,4 ± 5,6
(iii) Gallenkamp	20,0 ± 4,1*	43,0 ± 5,4	29,6 ± 6,6

*P< 0,01 **P< 0,001

Table II. **Percentage area shrinkage after amyl acetate and the critical point technique (mean ± SD)**

Tissue	After amyl acetate	After critical point technique	Difference i.e. shrinkage during critical point technique
Tongue	13,0 ± 1,8	30,4 ± 1,5	17,4
Small Intestine	37,0 ± 10,1	51,4 ± 3,7	14,4
Soft palate	17,8 ± 2,4	32,8 ± 1,6	15,0

Table III. **Percentage reduction in volume of blocks (mean ± SD)**

Method of Dehydration	Tissue	
	Tongue	Small Intestine
Critical point	43,0 ± 0,7	65,4 ± 2,3
Ether-air drying	42,4 ± 2,6	64,4 ± 5,7
Camphene	42,8 ± 2,2	65,8 ± 4,6
Freeze-drying		
(i) Edwards-Pearse	29,4 ± 6,5*	29,4 ± 8,1*
(ii) Edwards E12E4	23,4 ± 2,2*	34,2 ± 3,3*
(iii) Gallenkamp	35,2 ± 6,8	52,4 ± 4,8

*P< 0,01

For the scanning electron microscopy part of the study the specimens were mounted on aluminium stubs with colloidal graphite, coated with gold palladium and finally examined in a Cambridge S4 Stereoscan operated at 20 kV.

Statistical analysis was carried out using the analysis of variance, Scheffe's test and Student's t test.

RESULTS

The amount of shrinkage seen depended both on the technique used and the tissue involved.

(i) **Area Shrinkage** The percentage area shrinkage in each tissue obtained with each dehydration method is shown in Table I. The amount of shrinkage in the tongue and soft palate specimens were similar except for those dehydrated in the Edwards E12E4 apparatus where the tongue shrank less than the soft palate (P<0,001). The small intestine specimens all shrank considerably more than the tongue and soft palate (P<0,001). Comparing the effect of the different techniques on the same type of tissue the amount of shrinkage was similar in all the non-freeze drying techniques i.e. the critical point, camphene and air-drying. Likewise, with one exception, the freeze drying shrinkages were similar. The only exception was in the small intestine where the Gallenkamp shrinkage was significantly more than the Edwards-Pearse or the Edwards E12E4 (P< 0,001). In the tongue specimens the freeze drying techniques produced siginificantly less shrinkage than the other techniques (P<0,001). In the soft palate this was the case only with the air-drying and camphene techniques and the Edwards-Pearse and Edwards E12E4 apparatus (P<0,001). Small intestine shrinkage using the Gallenkamp apparatus was similar to that obtained with the non-freeze drying techniques. However, the two Edwards apparatuses produced significantly less shrinkage (P< 0.01).

During the dehydration using the critical point technique the amount of shrinkage after the amyl acetate stage, i.e. from the critical point apparatus itself was 14,4 per cent for the small intestine, 15 per cent for the soft palate and 17,4 per cent for the tongue (Table II). Thus in the tongue and soft palate specimens about half the shrinkage occurred in the critical point technique and about a quarter of the shrinkage in the small intestine specimens.

(ii) **Volume Shrinkage** The soft palate was not included in this portion of the study. As a result of its thinness it was not possible to photograph the lateral aspect.

In the tongue specimens the shrinkage with the critical point, air drying and camphene techniques were very similar (Table III). Of the freeze drying techniques the Gallenkamp apparatus resembled this. The Edwards-Pearse and Edwards E12E4 freeze drying apparatus showed significantly less shrinkage (P<0,01). The freeze drying techniques did not differ significantly from each other.

In the small intestine the volume reduction was greater than that of the tongue (except for the Edwards-Pearse technique) but the pattern of shrinkage was similar.

(iii) **Scanning electron microscopy** Examination of the various tissues in this study revealed that it was not possible to say from the SEM image which technique had been used except that occasional cracks in the specimen surface identified those that had been freeze-dried. Examples of scanning electron micrographs are shown in Figures 3-10.

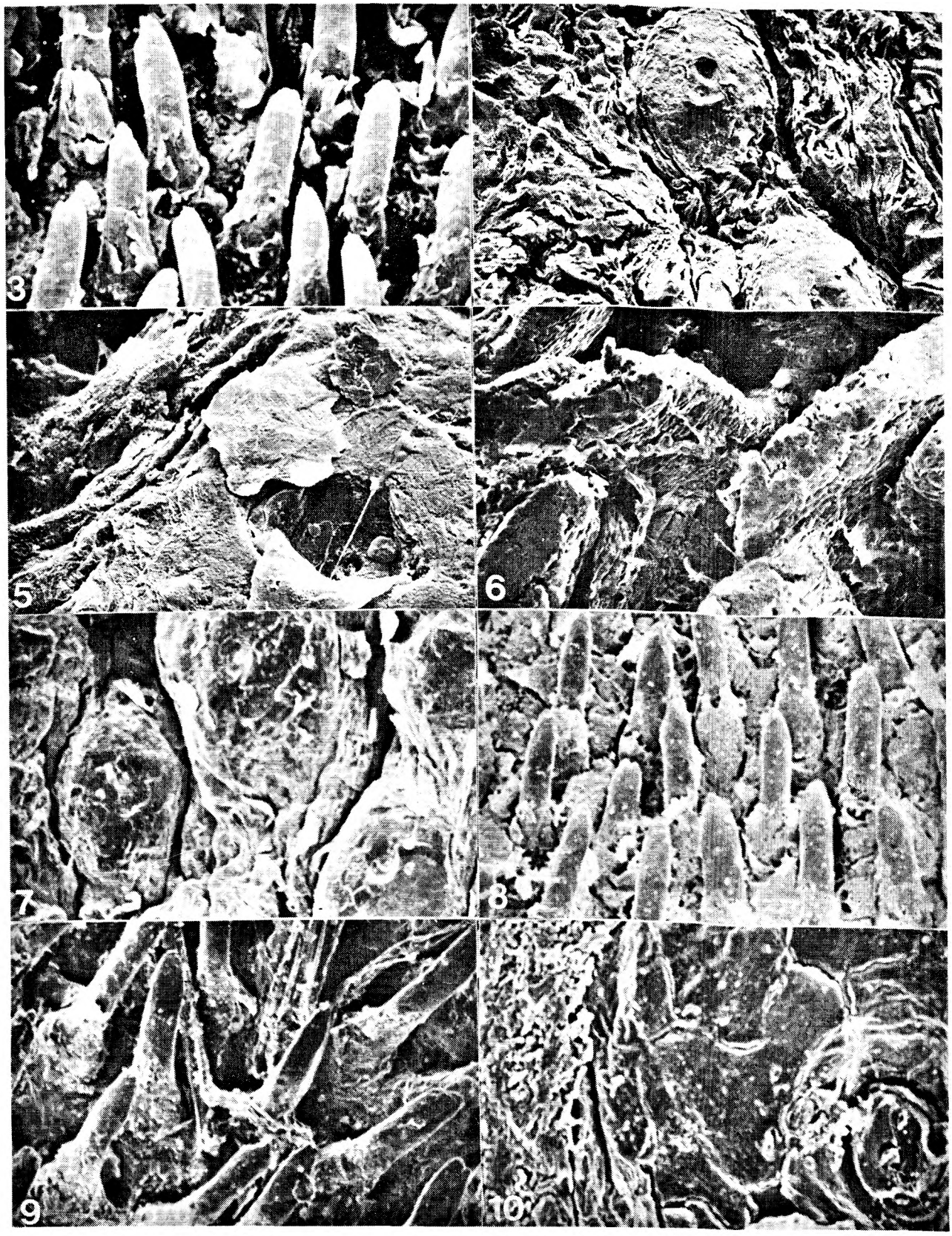


Fig. 3. **Filiform papillae of rat tongue. Critical point method. SEM X 240.**

Fig. 4. **Fungiform-like papillae on surface of rat soft palate. Air dried. SEM X 184.**

Fig. 5. **High magnification of the surface of a soft palate papilla. Air dried. SEM X 880.**

Fig. 6. **Villi in rat small intestine. Camphene. SEM X 115.**

Fig. 7. **Fungiform-like papillae in rat soft palate. Freeze dried in Edwards-Pearse apparatus. SEM X 192.**

Fig. 8. **Filiform papillae of rat tongue. Freeze dried in Edwards E12E4 apparatus. SEM X 216.**

Fig. 9. **Filiform papillae of rat tongue. Freeze dried in Gallenkamp apparatus. SEM X 232.**

Fig. 10. **High magnification of the surface of a soft palate papilla. Freeze dried in Edwards E12E4 apparatus. SEM X 960.**

DISCUSSION

This study confirms quantitatively the observation of Boyde and Wood (1969) that there is less tissue shrinkage when tissues are freeze-dried than with any of the other fundamental SEM preparative techniques used today.

The amount of shrinkage varied from tissue to tissue which could account for the differences between the area shrinkage results obtained in our study and those of Wheeler *et al* (1975) who used canine endocardium dehydrated with freeze drying and critical point techniques. A similar explanation may apply to the marked differences in shrinkage results obtained in our study and by Waterman (1972) in his embryological material.

Boyde (1972) mentioned that a small amount of shrinkage occurs during the critical point during process proper while Waterman (1972) felt that a good deal of shrinkage occurred at this stage. Our study has confirmed Waterman's viewpoint.

Wheeler *et al* (1975) were unable to distinguish between the SEM appearances of specimens dehydrated by freeze drying and the critical point techniques. A similar finding in our study with the freeze-drying, critical point and camphene techniques was not unexpected. What was unusual however was that the air-dried specimens could not be identified among those prepared with the other techniques.

From this study it is concluded that the freeze-drying technique produces less tissue shrinkage than the air-drying, critical point and camphene techniques. The amount of shrinkage could not be correlated with the appearance of the tissues in the scanning electron microscope.

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