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Stretch-Induced Changes in Geometry and Ultrastructure of Transporting Surfaces of Toad Bladder

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Summary. Methods were developed for estimation of the area of the microscopic and ultramicroscopic surface of the toad bladder epithelium at various degrees of stretch. Bladder sacs fixed while containing 2.5, 5, 25 or 50 ml of mucosal fluid were studied. For a perfect, hollow elastic sphere, this range of volume corresponds to a sevenfold range of surface area. In the bladder, this increase could be achieved by unfolding of surface irregularities, with no change in surface area, or by stretching the epithelial membrane. The measured microscopic surface area increased three- instead of sevenfold, but the ultramicroscopic surface remained constant. Thus the bladder stretches (1) by unfolding of the mucosal epithelium, and (2) by flattening of the microvilli. From measurements of the apparent thickness of the mucosal epithelium alone and of the entire bladder, we conclude that the former behaves like a flexible inelastic sheet, whereas the submucosa and serosa stretch elastically. Lateral intercellular spaces do not widen with stretch, but they do become more convoluted because of thinning of the epithelium. Thinning is unlikely to explain the increased sodium transport which follows stretching, because cytoplasmic resistance cannot approach total transepithelial resistance of this preparation.

Epithelia capable of active transport of fluid and electrolytes often exhibit a highly irregular surface, both in terms of microscopic foldings encompassing several cells, and specialized processes of ultramicroscopic dimensions which increase the surface area of individual cells. Epithelial area is usually employed as a referent base in quantitative analysis of transport. The area so measured is neither the microscopic nor ultramicroscopic surface, but rather a macroscopic measurement based on the overall dimensions of the sample of tissue under study and the apparatus employed. This referent suffers from the fact that a given sample of tissue can usually be stretched to a variable degree. The toad bladder, in particular, can maintain an appreciable wall tension over an approximately 50-fold range of intraluminal volume.

Expression of rates of transport in terms of tissue weight circumvents this difficulty to some extent. However, an estimate of transporting surface area is required for any analysis of solute transport based on the laws of diffusion, with or without the presence of convection. An additional difficulty with weight as a referent is that transport per unit weight may vary with the degree of stretch. This possibility was first proposed in the hypothesis of Gertz, Mangos, Braun and Pagel (1965) on the mechanism of glomerulotubular balance in the proximal nephron. The role of stretch in determining renal tubular transport is still controversial. In toad bladder sacs, however, reversible stimulation of sodium transport per unit weight by stretch has been clearly demonstrated (Walser, 1969). For example, a threefold increase in mucosal volume leads to a rise in short circuit current of 10 μamp/cm^2, within 30 min. Surface area in this study was calculated as gross macroscopic area, based on the assumption of simple spherical geometry. Clearly the area so estimated is only a crude approximation of the actual epithelial surface.

The purpose of the present work was threefold: (1) to develop methods for measuring microscopic and ultramicroscopic epithelial surface and relating these quantities to macroscopic surface; (2) to determine how the toad bladder epithelium stretches in terms of its microscopic and ultramicroscopic geometry; and (3) to seek a morphological explanation for the stimulation of sodium transport by stretch.

Materials and Methods

Twenty-four hemibladders were studied. They were removed from the toads and mounted on glass cannulas as described by Walser (1969) and filled with 2.5 or 5.0 ml amphibian Ringer's solution. After recording a stable short-circuit current (SCC) and potential difference (PD) (30 to 45 min after mounting), the mucosal volume was increased (except in controls) to 25 ml, 50 ml, or the maximal mucosal volume attainable without applying more than 4 cm H_2O hydrostatic pressure (about 65 ml). The values of the electrical parameters and their response to stretching were similar to those observed earlier (Walser, 1969). The bladders were fixed after stretching by adding osmium tetroxide to the mucosal fluid to a final concentration of 0.8 %. They were removed from the cannulas after 2 to 4 min, and then remained in ice-cold osmium solution for 1.5 to 2 hr. The tissue was then cut into 3×3-mm pieces, dehydrated in ethanol and propylene oxide, and embedded in Araldite. Sections for light and electron microscopy were cut on a Servall Porter-Blum ultramicrotome MT-1. Sections (1 to 2 μ) were stained with toluidine blue; ultrathin sections were stained in 1:25 lead acetate solution and viewed in a RCA EMU 3F electron microscope.

Measurements and Principles

We were unable to find previously published methods by which the epithelial surface of a hollow organ such as the toad bladder can be esti-

mated from microscopic sections. Stereological methods, as developed b Elias, Hennig and Elias (1961), Loud (1962), and Weibel, Kistler an Scherle (1966), were considered but found to be inapplicable because th first premise of these methods is that the sections be randomly oriente with respect to the surfaces under study, and that the planes of the surface be randomly oriented in space.

We have defined mucosal surface of the toad bladder in three ways *macroscopic surface*, *microscopic surface*, and *ultramicroscopic surface*.

Macroscopic surface is the surface calculated for a given mucosal volum based on the assumption that the toad bladder is an ideal hollow sphere Since radius $r = \sqrt[3]{\frac{3 \times \text{volume}}{4\pi}}$, the surface S can be calculated fron $S = 4\pi r^2$. Current density (μamp/cm^2) and resistance (Ω cm^2) were cal culated from this surface area (Walser, 1969). In this earlier study, th *macroscopic thickness* D of the wall was calculated as the ratio of weight W to surface, assuming unit density.

Since the mucosal surface of the toad bladder is folded in unstretchec bladders, the *macroscopic surface* is an underestimate. A closer approxi mation to the true surface is given by the *microscopic surface* S' which wa defined as the surface visible in the light microscope. We measured th degree of folding in light micrographs as follows: A straight line of arbitrary length a (encompassing several mucosal folds) was measured parallel tc the mucosal surface; by means of a map measure, the length b of the apical cell surface across the same distance was estimated, and a factor (f_1) wa calculated as b/a. S' is the product of f_1 and S. The reason for not squaring f_1 in this calculation is explained below. A *microscopic thickness* (D') could, in principle, be calculated as W/S'. However, owing to the fact that W could not be measured directly in these fixed bladders, D' was instead measured directly from light microscopic sections in the following way.

Twenty-seven measurements were randomly taken over at least three samples from different parts of the bladders. The shortest distances (M) between mucosal and serosal surfaces were measured and averaged. D' is not equal to M, but rather to $M/\sqrt{f_1}$. The principle underlying this calculation is illustrated in Fig. 1. In estimating from oblique sections the true thickness D' of a flat, unfolded sheet, the measured thickness M differs from D' by $1/\cos \alpha$, where α is the angle between the plane of section and the plane normal to the surface of the sheet (Fig. 1). In a section optimally oriented, M will equal D'. In such a sheet, the surface as estimated by length measurements in oblique sections is nevertheless identical with the true surface ($f_1 = b/a = 1$). In a folded or irregular surface of a sheet of

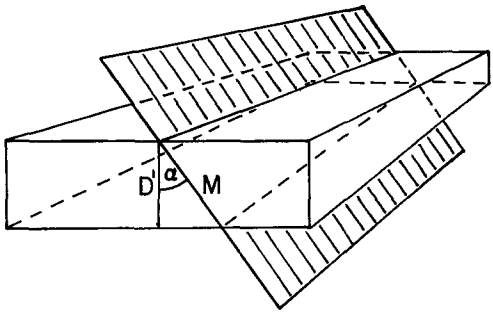

Fig. 1. Sectioning an unfolded sheet of uniform thickness by an oblique section (shaded plane). D' true thickness. α angle between plane of section and perpendicular to the surface of the sheet. M measurement (apparent thickness)

uniform thickness, however, an additional problem arises. No matter how the plane of section is oriented, M is necessarily larger than D' because the plane cannot be optimal for all portions of the surface. The discrepancy increases with the degree of folding, since the plane of section is normal to a progressively smaller fraction of the surface. In algebraic terms, M/D' varies with b/a. Consider first a sheet of uniform thickness folded only in two dimensions and sectioned normal to this plane (Fig. 2). Length b measured along the surface is greater than length a of a corresponding straight line. Average thickness $\overline{M}$ measured in a direction normal to the axis a, is greater than true thickness D', which is assumed to be uniform. The triangles PQR and QSR are congruent; hence $\Delta b/\Delta a = M_i/D'$, where M_i is a single measurement of oblique thickness. As Δa approaches 0, the length PQ approaches RT; therefore, both lengths may be represented as M_i.

$$b = \int_0^a \frac{db}{da}\,da = \frac{a}{n}\sum_{i=0}^{n}\left(\frac{db}{da}\right)_i = \frac{a}{n}\sum_{i=0}^{n}\left(\frac{\Delta b}{\Delta a}\right)_i = \frac{a}{n}\sum_{i=0}^{n}\frac{M_i}{D'} = \frac{a}{D'}\,\overline{M}\,.$$

Hence $\overline{M}/D = b/a$.

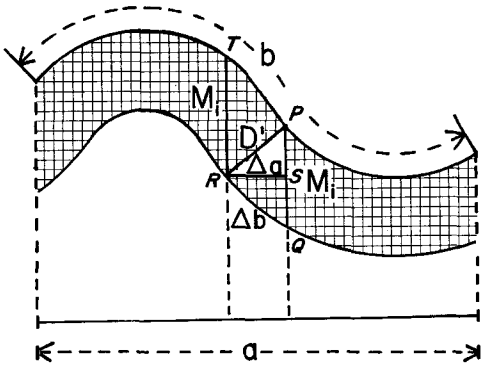

Fig. 2. Apparent thickness (M) and true thickness (D') of a sheet folded in one dimension

Consider now a surface folded in three dimensions such as the bladder. Here the empirical measurement b_v^m/a of the irregularity of the surface overestimates the average deviation from a horizontal plane, because the section can no longer be normal to a plane of folding. Thus b_v^m/a overestimates surface irregularity by a factor b/a, or $b_v^m/a = \sqrt{b/a} \cdot \sqrt{b/a}$. Hence b_v^m/a is the quantity by which $\bar{M}$ must be multiplied to obtain D'. It is not, however, the correction factor for surface, because the surface extends in three dimensions instead of two. The square of the quantity $\sqrt{b_v^m/a}$ (or b_v^m/a or f_1) is the factor by which S must be multiplied to obtain S'. Inspection of Fig. 3 shows that the length of a corrugated surface measured in this plane exceeds the true length. $1/\cos \alpha$ is a measure of the degree of overestimation.

Ultramicroscopic surface S'' is defined as that surface visible in section by electron microscopy. The contribution of microvilli to surface area was estimated from randomly placed electron micrographs ($\times 38{,}000$). The length c of a straight line parallel to the mucosal surface was compared with the length d of the apical cell membrane over the same region. Over the short distances used (5 μ or less), *microscopic foldings* were negligible. The results were expressed as a factor f_2 calculated as c/d. Unlike f_1, f_2 is not an overestimate of surface irregularity because the thickness of the section exceeds the dimensions of the microvilli. Thus the irregularity is visible in its entirety, and there is no problem of overestimating surface owing to oblique sections. Hence $S'' = S' f_2^2$.

The *microscopic thickness* D_e' of the mucosal epithelial cell layer was obtained in the same manner as D'. A *macroscopic thickness* of this layer cannot the calculated. The volume V_e of the mucosal epithelial cell layer was calculated as $D_e' S'$.

The degree of folding of the lateral spaces was measured by comparing the length e of a straight line perpendicular to the apical cell surface on randomly placed electron micrographs ($\times 35{,}000$) with the length g of the corresponding section of lateral plasma-membrane as obtained with a map

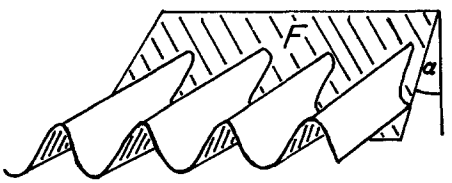

Fig. 3. Illustration showing how an oblique section can lead to an overestimate of the length of a folded surface

measure. A factor f_3 was calculated as g/e. Then the length L of the spaces is equal to $D'_e f_3$. *Ultramicroscopic thickness* does not differ appreciably from *microscopic thickness* D'.

Results

Qualitative impressions of the changes with stretching in morphology of the toad bladder can be gained from examination of Figs. 4–11. The light micrographs in Figs. 4–7 show the extent of microscopic folding of the mucosal epithelium in unstretched sacs, as compared with those which

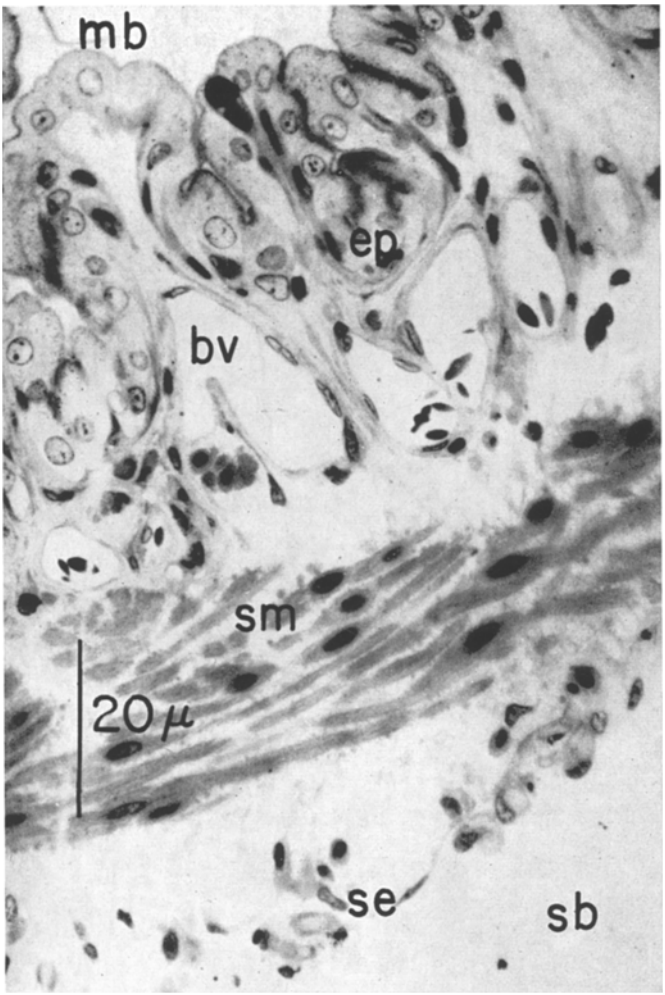

Fig. 4. Light micrograph of a minimally stretched bladder (mucosal volume = 2.5 ml). *mb* location of mucosal bath; *ep* mucosal epithelium; *bv* blood vessel; *sm* smooth muscle bundles cut longitudinally; *se* serosa; *sb* location of serosal bath

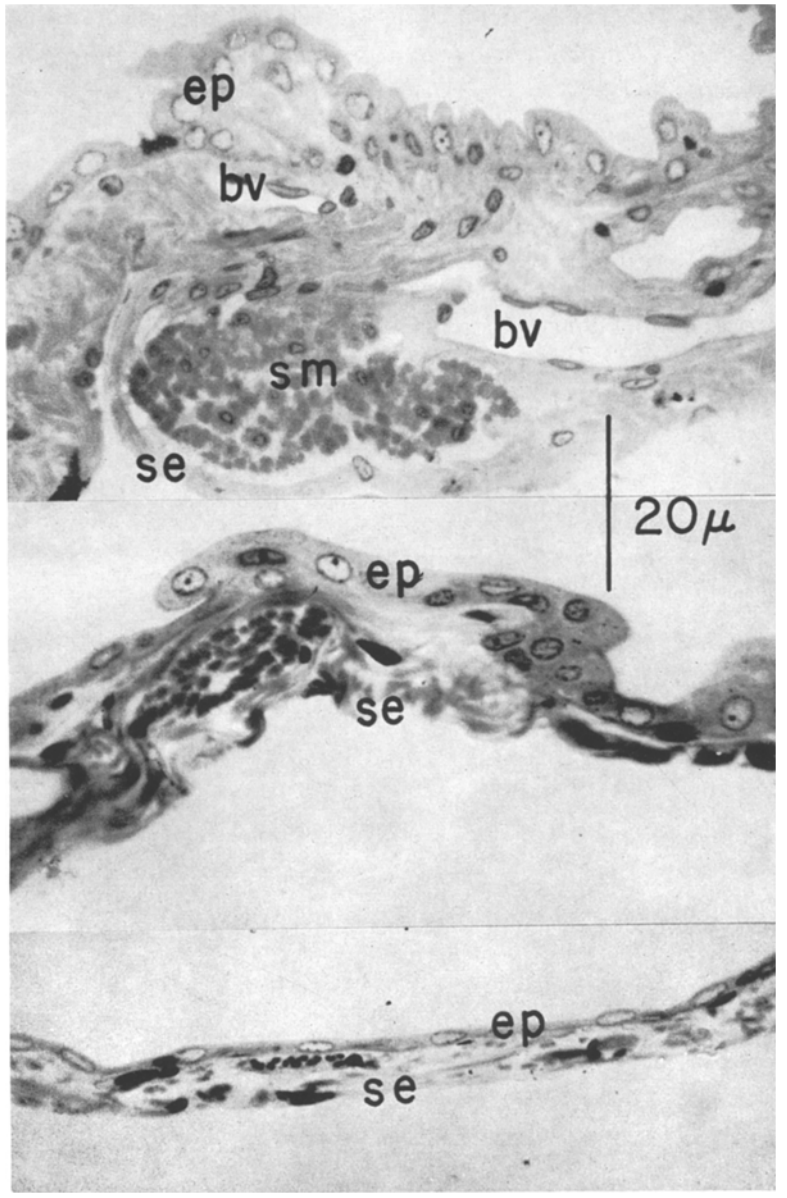

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Fig. 5. Section of a bladder containing 5 ml mucosal volume. Symbols and magnification as in Fig. 4

Fig. 6. Section of a bladder containing 25 ml mucosal volume. Symbols and magnification as in Figs. 4 and 5

Fig. 7. Section of a bladder containing 50 ml mucosal volume. Symbols and magnification as in Figs. 4, 5 and 6

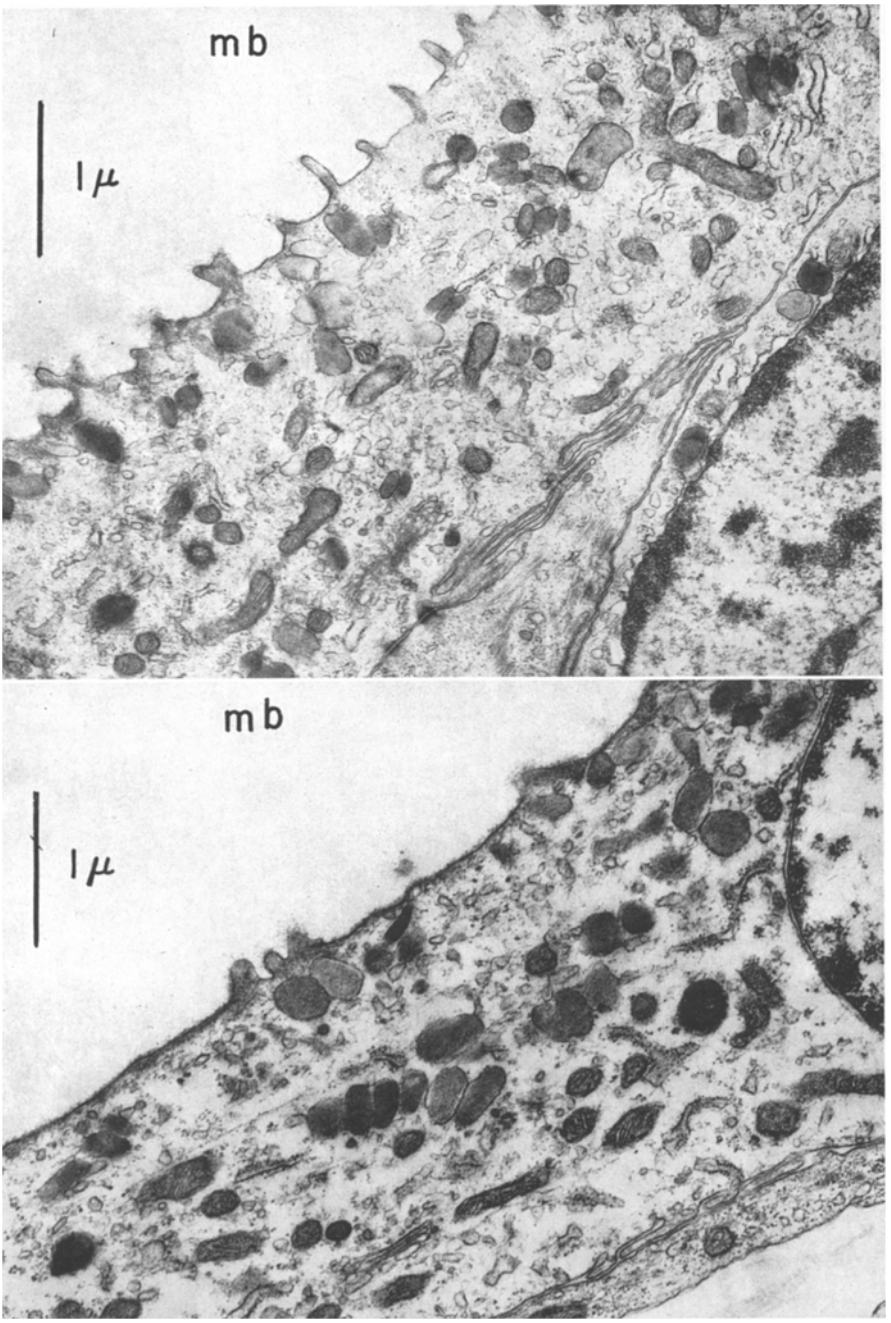
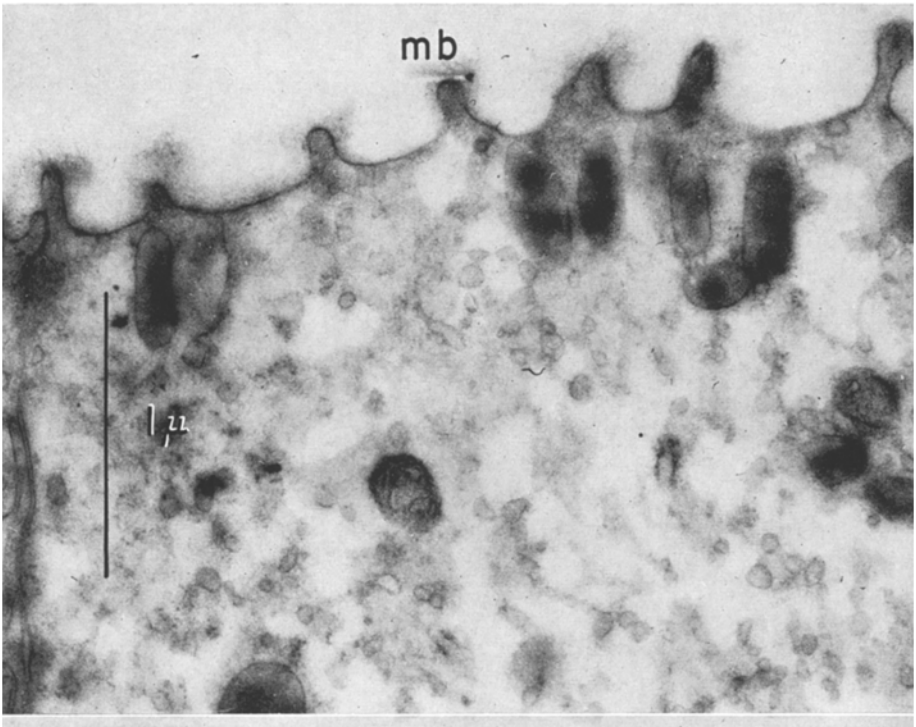


Fig. 8. Electron micrograph of a mucosal granular cell in a bladder containing 5 ml mucosal volume (× 25,000, by reproduction reduced to 4/5). Note the numerous microvilli protruding into the mucosal bath (*mb*)

Fig. 9. Mucosal epithelial cell of a bladder stretched to 25 ml mucosal volume (× 25,000, by reproduction reduced to 4/5). The microvilli are less numerous and shorter than in Fig. 7



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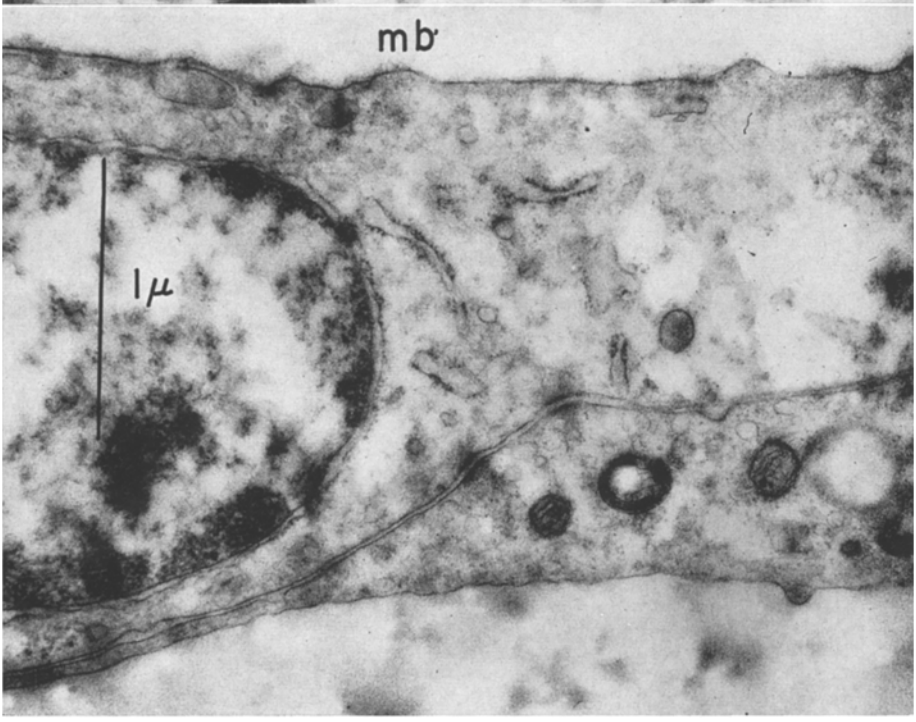

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Fig. 10. Apical cell membrane of a bladder containing 2.5 ml mucosal volume (× 47,000, by reproduction reduced to 4/5). Note the similarity with Fig. 8

Fig. 11. Mucosal epithelium of a bladder stretched to 50 ml mucosal volume (× 47,000, by reproduction reduced to 4/5). Note the complete absence of microvilli

were stretched. Occluded pockets of mucosal fluid, which might impede transport, were seen only in bladders containing less than 5 ml. The electron micrographs show the reduction in the extent of microvilli in stretched bladders (Figs. 8–11).

Table 1 summarizes the principal measurements. The *macroscopic surface S*, calculated from mucosal volume, increases from 8.9 to 65.6 cm^2. The *microscopic surface S'*, calculated from S and the measured folding factor f_1, increases from 24.3 to 68.7 cm^2. This means that, despite the considerable degree of unfolding which occurs, there is nevertheless a substantial increase in *microscopic surface* from 5 ml on. Between 2.5 and 5 ml, only unfolding occurs, as shown by the unchanging value for S'.

Ultramicroscopic surface S'' did not increase with volume, because microvilli flattened and virtually disappeared with increasing stretch. The extent of microvillous surface is indicated by f_2, which decreases from 1.69 to 1.035.

Apparent thickness of the mucosal epithelium M_e decreases by a factor of four, but when correction is made for the obliquity of sectioning (D'_e), the decrease is only threefold. One way to evaluate the relative validity of these two measures of thickness is to estimate the volume of mucosal epithelium. If epithelial thickness were indeed constant at all parts of the given bladder, as is assumed in calculating D'_e, the product of microscopic surface

Table 1. *Surface and thickness measurements on bladders containing differing volumes*

No. of hemi-bladders	Volume (ml)	S [a] (cm^2)	f_1	S' (cm^2)	f_2	S'' (cm^2)	M_e (μ)	D'_e (μ)	M (μ)	D' (μ)
3	2.5	8.9	2.73 ±0.24	24.3 ±2.2	1.69 [b]	79.7 [b]	14.8 ±0.5	9.1 ±0.6	109.8 ±14.9	67.6 ±12.0
5 [c]	5.0	14.1	1.82 ±0.06	25.6 ±0.6	1.58 ±0.13	65.6 ±12.1	8.8 ±1.2	6.5 ±2.1	51.5 ±4.7	38.8 ±3.8
11 [d]	25.0	41.4	1.14 ±0.03	46.9 ±1.3	1.17 ±0.022	64.4 ±3.8	5.4 ±0.4	5.1 ±0.3	41.4 ±3.9	38.5 ±3.5
3	50.0	65.6	1.04 ±0.01	68.7 ±0.8	1.035 [b]	74.2 [b]	3.4 ±0.3	3.1 ±0.5	26.7 ±2.3	26.0 ±2.3

[a] Abbreviations used: S, macroscopic surface; f_1, folding factor for microscopic surface; S', microscopic surface; f_2, folding factor for ultramicroscopic surface; S'', ultramicroscopic surface; M_e, apparent epithelial thickness; D'_e, "true" epithelial thickness; M, apparent bladder thickness; D', "true" bladder thickness.

[b] Two hemibladders.

[c] One bladder excluded since it was 30 × SEM off of the mean of the others.

[d] One bladder excluded since it was 17 × SEM off of the mean of the others.

Table 2. *Epithelial thickness and folding of the lateral interspaces*

Bladder volume (ml)	Epithelial thickness D'_e	f_3	$D'_e \times f_3$
5	9.8	3.4	34.3
	6.1	3.8	23.2
25	5.8	5.0	29.0
	5.7	3.4	19.4
	4.4	6.1	26.8
	4.4	4.5	19.8
	3.7	6.4	23.7
	Mean calculated length of the lateral interspaces ±SEM		25.2±1.9

and D'_e would yield the volume of the mucosal cells. Whether cell volume does change with stretching is conjectural, but it is unlikely to change radically. Mucosal cell volumes calculated from the data in Table 1 are 0.022, 0.017, 0.024, and 0.021 cm^3 at mucosal bath volumes of 2.5, 5, 25, and 50 ml, respectively. Thus cell volume estimated in this way is fairly constant. If M_e instead of D'_e is used, the estimated volume decreases with stretch. Thus the model of a folded sheet of uniform thickness is a fair approximation.

The last two columns give apparent (M) and "true" (D') thicknesses of the bladder wall. Since the model of a folded sheet of uniform thickness is clearly an inaccurate representation of the bladder as a whole, neither of these estimates has any exact physical meaning, and tissue volume cannot be calculated from them.

Table 2 shows the results of measurements of the degree of folding of the lateral intercellular spaces, expressed as the factor f_3. This measure increases markedly with stretch, as expected. The length of the lateral spaces, estimated as $D'_e f_3$, does not change with stretch, and averages 25 μ. A quantitative analysis of the width of the lateral spaces is currently under study.

Discussion

These results show that the toad bladder accommodates itself to increases in volume by unfolding of the mucosal epithelium and by flattening of the microvillous irregularities: ultramicroscopic surface appears to remain constant.

Estimates of mean bladder thickness from tissue weights and mucosal volumes, as used by Walser (1969, 1970) would be more reliable than microscopic measurements. We were unable to obtain adequate weights of these bladders because they were fixed while still mounted. Total bladder thickness is clearly less important physiologically than the thickness of the mucosal epithelium. It has been demonstrated that active transport processes as well as the barrier to passive movement of ions are located within the mucosal epithelium (Leaf, 1965).

We have assumed, in calculating the macroscopic surface, that the bladder sac is a perfect sphere. This is only approximately true. However, the bladder sacs used in this study appeared to be nearly spherical and did not change their shape with stretch.

The measurements establish that elastic deformation plays little if any role in the adaption of the mucosal epithelium to changes in volume. The force which produces folding must be the elasticity of the submucosa. The characteristic pressure-volume relationship of the toad bladder (Walser, 1969) must also be attributable to this layer.

Several possible explanations of the stimulation of sodium transport by stretch can be entertained. (1) Occluded pockets of mucosal fluid might limit transport by virtue of changes in their composition. As noted above, such pockets were seen only in the least stretched bladders. The stretch response, however, is seen in bladders with much greater initial volumes of mucosal solution (Walser, 1969). There is, undoubtedly, an unstirred layer of fluid at the mucosal surface. It is conceivable that the changing shape of microvilli may modify the thickness of this layer, with stretch, and thereby alter rates of transport. Current knowledge is not adequate to evaluate the significance of this possibility.

(2) The amount of surface area exposed to the mucosal bath might influence the availability of sodium to the transport mechanism. No increase in ultramicroscopic surface occured. However, the membrane near the base of microvilli may stretch when they flatten, so that there may be local regions of stretched membrane even though the total ultramicroscopic surface is unaltered.

(3) Lateral intercellular spaces might become widened by stretching. No such widening was observed. Our measurements of lateral intercellular spaces were made in the absence of vasopressin. It has not been clearly established if any water movement accompanies salt transport in the toad bladder under these conditions. Consequently our data have little bearing on the question of solute-solvent coupling. The results do demonstrate, however, that increased sodium transport (in response to stretch) can

occur without widening of intercellular spaces. DiBona and Civan (1969 have also reported that dilatation of the lateral interspaces occurs in respons to vasopressin when both media are isotonic.

(4) The thinner epithelium might provide a shorter path for trans epithelial movement of sodium ions. Diamond and Bossert (1967) hav suggested (in the case of the rabbit gallbladder) that sodium is first pumpe into lateral intercellular spaces. The path from this point on is unaltere by stretch, since the length of these spaces remains constant. The possibilit has previously been examined that increased access of sodium to th transport mechanism in response to stretch might account for the increas in sodium movement (Walser, 1969). Subsequently, the electrical resistanc of the bladder has been found to be considerably higher when edge damag is eliminated (Walser, 1970). Resistance measured in this way average $12{,}000\,\Omega\,\mathrm{cm}^2$. Thus, the cytoplasmic resistance is unlikely to approach th total transepithelial resistance. Nevertheless, the possibility that partia conductance for sodium through a critical and rate-limiting path might b altered by stretch cannot be excluded.

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