Volume 260, number 2, 187–194 FEB 08036 January 1990

Video enhanced imaging of the fluorescent Na^+ probe SBFI indicates that colonic crypts absorb fluid by generating a hypertonic interstitial fluid

R.J. Naftalin and K.C. Pedley

Department of Physiology, King's College London; Strand and Kensington Campuses, London WC2 2LS, England

Received 23 October 1989; revised version received 22 November 1989

Extracellular accumulation of Na^+ detected by video-enhanced microscopic imaging of the impermeant fluorescent probe SBFI[1,2] confirms the view that colonic crypts produce a hypertonic absorbate ca 1000 $mOsm \cdot kg^{-1}$, thereby generating a large osmotic pressure across the crypt wall. This creates a high fluid tension within the crypt lumen, sufficient to dehydrate faeces [3,4]. When bathed in Tyrode the SBFI·Na^+ fluorescence indicates a [Na^+] ca 750 mM within the interstitial space of metabolizing rat descending colon. There is no evidence of interstitial Na^+ accumulation in octanol (2 mM) or in rabbit colon incubated with 1.0 mM ouabain and no evidence of Na^+ secretion via the crypt lumen during absorption [5].

Fluorescent imaging; Sodium-ion; Colon; Absorption

1. INTRODUCTION

Extracellular accumulations of Na^+ have been monitored previously in epithelial tissues using ion-sensitive microelectrodes [6] and X-ray microprobe microanalysis of frozen hydrated sections [7]. Use of the impermeant acid form of SBFI, a fluorescent probe for Na^+, along with high resolution video-enhanced microscopy to detect the fluorescent emission of SBFI, is a more sensitive and convenient tool for detecting extracellular Na^+ than these other methods [1,2] and its use provides an opportunity to test a new hypothesis relating to colonic absorptive function. Rabbit descending colon has been shown to generate a very hypertonic absorbate (~1000 $mOsm \cdot kg^{-1}$) [3] confirming previous findings with rat descending colon in vivo and in vitro [8,9]; the hypertonic interstitial fluid is predicted to produce the large fluid tension within the crypt lumen which is required to suck fluid from faeces; thereby transforming faecal slurry into hard faeces, containing approximately 60% water [4]. The narrow crypt lumen provides both the low hydraulic conductance and isolation from the more permeable surface mucosa which is required to generate and maintain the hypertonic absorbate and high fluid tension within. The conventional view of colonic function is that colonic crypts are routes of fluid and electrolyte secretion [5]. Hence, accurate determination of the concentration of Na^+ within the crypt lumen and interstitial fluid using the fluorescent marker SBFI should discriminate between these two views. If crypts have an absorptive function, then the crypt lumen should be depleted of Na^+ and the interstitial fluid contain a higher concentration of Na salts than in the bulk solution; whereas if the crypts have a secretory function, then the crypt lumen should contain fluid hypertonic to the interstitial fluid, i.e. a high concentration of Na salts.

Correspondence address: R.J. Naftalin, Department of Physiology, King's College London, Strand, London WC2 2LS, England

2. MATERIALS AND METHODS

Rat (Wistar) or rabbit (New Zealand White) descending colonic mucosa was stripped of its muscle layer and mounted in a perfusion chamber, then perifused with isotonic Tyrode solutions supplemented with SBFI free acid (10 μM). Low Na^+ (4.8 mM) Tyrode solution contained 3.9 mM $NaHCO_3$; 1.8 mM $CaCl_2$; 0.9 mM $MgCl_2$; 0.9 mM NaH_2PO_4; 3.9 mM $KHCO_3$; 135 mM *N*-methyl-glucamine acetate; 5.6 mM glucose; pH 7.4 when gassed with 95% O_2/5% CO_2; normal Tyrode contained 140 mM NaCl and zero *N*-methyl-glucamine. SBFI (Molecular Probes Inc., Eugene, OR, USA) is an agent which binds Na^+ with a K_d of 18 mM in K^+ containing solutions [1,2]. When Na^+ is bound the reagent has a fluorescent excitation in the range 340–360 nm. The microscope viewing chamber was mounted on the stage of a Leitz Dialux 20 microscope equipped with a Zeiss 16 × multi-immersion Planar Achromat objective. The microscope also had switchable interference excitation filters and a silicon-intensified target (SIT) camera (Javelin TC1030/HX, Javelin Instr., CA, USA). Images were digitised in real time, averaged and stored by a Hamamatsu C1966 image processor, as well as being stored on magnetic tape (Sony VO-5800PS U-Matic VTR). Stored images were subsequently processed using a 32-bit RISC computer (Acorn Computers, Cambridge) and displayed in pseudocolour or monochrome at varying grey levels. The local concentrations of Na^+ were quantified by determining regional fluorescence intensity using the computer-averaged fluorescence excitation of SBFI · Na^+ complex

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/90/$3.50

(340–360 nm) after background fluorescence from the cells and interstitial fluid was subtracted. Comparison of the local emission is obtained with respect to background fluorescence of either the whole field or a neighbouring field of low intensity. This was done by monitoring the fluorescence intensity under a line chosen to traverse the area of interest (see fig.2), or of a circumscribed area within the field. This can be displayed graphically as a histogram of fluorescence intensity versus position along the line.

2.1. *Estimation of the [Na^+] from the ratio of local fluorescence to background fluorescence*

If there are local regions ('l') of extracellular or interstitial fluid in which the steady state [Na^+] is maintained at a higher (or lower) level than the bulk solution [Na_b^+] by the local activity of the tissue Na-pump and the concentration of free SBFI (unbound to Na^+) at steady-state is uniformly distributed in all extracellular compartments, i.e. at steady-state [$SBFI_b$] = [$SBFI_l$] then the following rela-

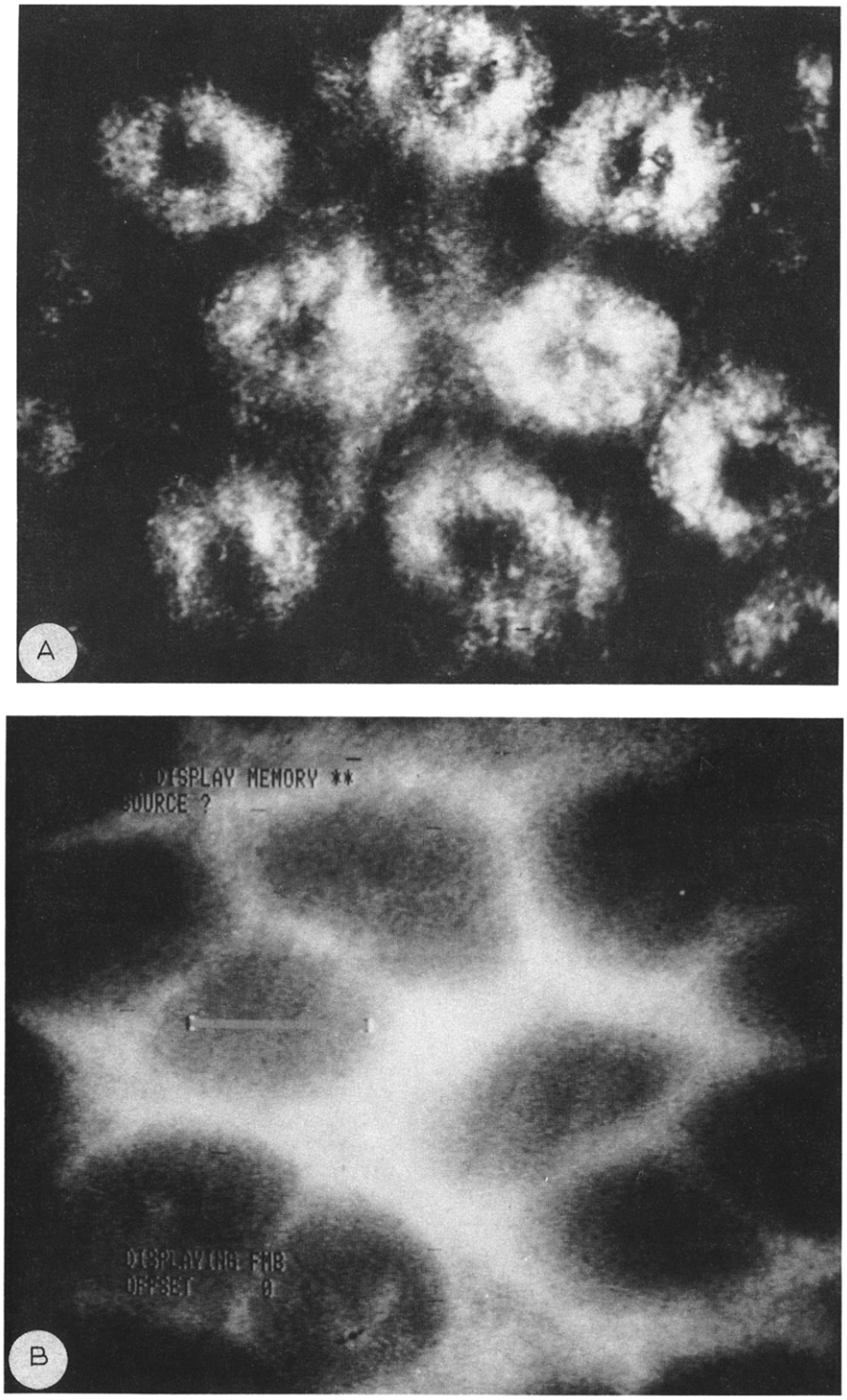


Fig.1. Illustrations are photographs of the video-screen image. The width of the microscopic image in this and all the other plates and figures is 300 μm. (A) A bright-field illuminated portion of rat descending colonic mucosa. (B) The fluorescence intensity is shown in monochrome grey scale.

tionships may be deduced, where T = total bound and free SBFI in bulk solution:

$$T = [SBFI_b] + [SBFI \cdot Na_b^+] \quad (1)$$

and

$$[SBFI] \cdot [Na^+]/[SBFI \cdot Na^+] = K_d \quad (2)$$

K_d is the dissociation constant of SBFI for Na^+ = 18 mM [1,2].

It follows that

$$[SBFI \cdot Na_i^+]/[SBFI_i] = [Na_i^+]/K_d \quad (3)$$

and

$$[SBFI_b] = K_d \cdot T/\{[Na_b^+] + K_d\} \quad (4)$$

also

$$[SBFI \cdot Na_b^+] = T \cdot [Na_b^+]/\{[Na_b^+] + K_d\} \quad (5)$$

hence

$$[SBFI \cdot Na_i^+] = T \cdot [Na_i^+]/\{[Na_b^+] + K_d\} \quad (6)$$

Eqn 6 indicates that the local fluorescence intensity $[SBFI \cdot Na_i^+]$ is linearly related to $[Na_i^+]$ and because free $[SBFI_b]$ falls as the bulk $[Na_b^+]$ is raised, fluorescence is inversely related to $[Na_b^+]$; hence optimal discrimination is obtained when $[Na_b^+] < K_d$. It follows from eqns 5 and 6 that

$$[SBFI \cdot Na_i^+]/[SBFL \cdot Na_b^+] = [Na_i^+]/[Na_b^+] \quad (7)$$

These equations may be applied to determine the local $[Na^+]$ as follows:

$$[Na^+_{local}] = [Na^+_{(bulk)}] \cdot \{I_{(local)} - I_{(background)}\}/\{I_{(bulk)} - I_{(background)}\}$$

I is the fluorescence intensity of the image as detected by the SIT.

It should be noted that discrimination of extracellular $[Na_i^+]$ by SBFI is over a much wider concentration range than is possible with intracellular SBFI loaded using the permeant esterified AM form of the dye. With intracellular SBFI the total amount of SBFI, T is fixed and the relationship of $[SBFI \cdot Na_{in}]$ is a saturable function:

$$[SBFI \cdot Na_{in}^+] = T \cdot [Na_{in}^+]/\{[Na_{in}^+] + K_d\} \quad (8)$$

3. RESULTS

A group of 8 adjacent crypts from rat descending colonic mucosa perifused with low Na^+ Tyrode (4.8 mM Na^+) is shown in fig.1A with bright-field illumination. The crypts appear as rosettes of illuminated cells surrounding a central dark lumen; the crypts themselves are surrounded by dark interstitial fluid. The fluorescence emission of the same field with background fluorescence from the cells and interstitial fluid subtracted is shown in fig.1B. The maximal fluorescent intensity is situated in the interstitial fluid surrounding the crypts. There is virtually no emission from the crypt cells or from the crypt lumen. In plate 1 the fluorescent intensity is displayed in pseudocolour. This indicates $[Na^+_{(I)}]$ = 30–50 mM within the interstitial fluid. This degree of hypertonicity can be sustained for periods of 3–4 h. During this time there is no evidence of any Na^+ accumulation above background levels within the crypt lumens.

When the tissue is perifused with solution containing 150 mM Na^+ there is a decreased luminal cross-sectional area as has been previously reported [3] and a loss of contrast between areas showing maximal and minimal fluorescence, due to the raised level of background fluorescence; however, the interstitial fluid still has regions of fluorescence intensity which are 4–6-fold above background indicating a local interstitial $[Na^+_{(I)}]$ (600–1000 mM) (fig.2). A histogram showing the relative intensity of fluorescence emission

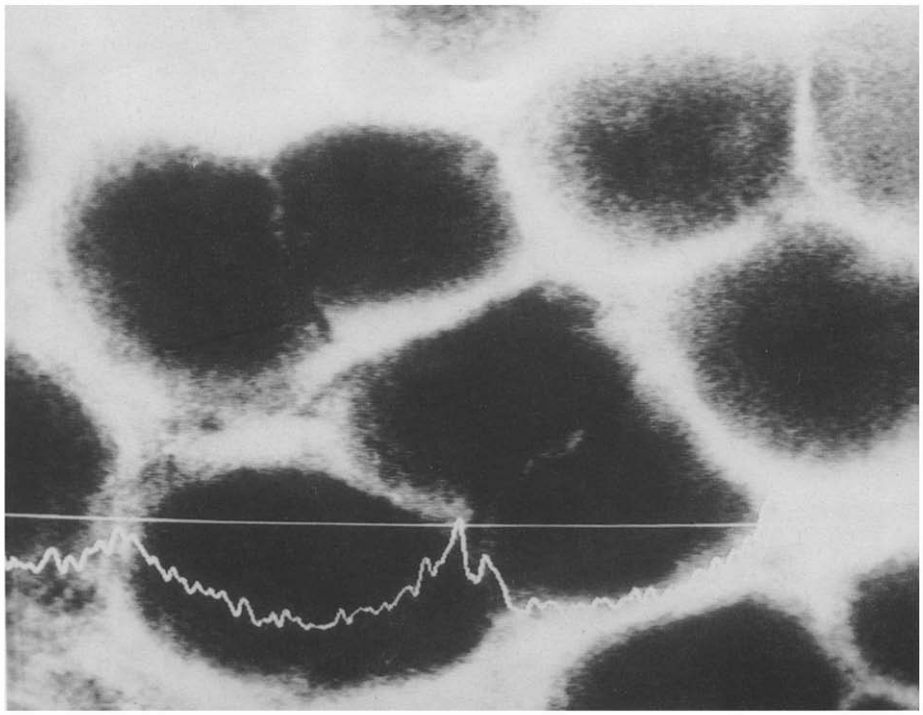


Fig.2. The fluorescence intensity of colonic crypts after preincubation with 140 mM $[Na^+]$ for 60 min. The histogram shows the relative intensity of SBFI · Na fluorescence with distance along the line. The scale illustrates the intensity of fluorescence relative to background = 140 mM Na^+. The horizontal scale of the histogram relates to the corresponding point on the line through the tissue.

at the corresponding point on the line is superimposed. This requires the colonic absorbate tonicity to be similar to the tonicity previously estimated, i.e. ~1000 mOsm·kg^{-1}.

3.1. *Effect of octanol (2 mM) on rat colonic interstitial [Na^+]*

Following exposure to 4.8 mM [Na^+] containing octanol (2 mM) for 90 min, an agent which causes closure of intercellular gap junctions [10], and which reduces the rates of fluid and solute absorption and the tonicity of the rabbit colonic absorbate within 30–60 min [3], there is almost total loss of the high intensity fluorescence from the interstitial fluid. Only punctate areas of high intensity emission remain at the basal surface of the crypts. The $t_{1/2}$ for loss of interstitial Na^+ following exposure to octanol is approximately 30 min (fig.3 and plate 2).

3.2. *Effect of ouabain (1 mM) on rabbit colonic interstitial [Na^+]*

Fig.4A shows the bright-field image of rabbit colonic crypts following incubation with 4.8 mM Na^+ Tyrode and SBFI (10 μM) for 1 h and fig.4B shows the fluorescent intensity within the interstitial fluid of the same field. In figs 4C and D the effects of incubation with ouabain (1.0 mM) for 90 min are shown. In control tissue Na^+ accumulates within the interstitial fluid to a higher concentration than in the bulk solution – ~30 mM; this level is sustained for at least 3 h. With ouabain present the interstitial fluorescence decreases almost to background levels following 1–2 h incubation, indicating that the enhanced fluorescence located within the interstitial fluid of control tissue is ouabain-sensitive. In contrast to the effect of octanol no residual localized Na^+ pump activity remains at the basal surface of the crypts after ouabain treatment. With ouabain present it can also be observed that the crypt colonocytes are swollen in comparison to control crypts, as previously observed [3].

4. DISCUSSION

Rat descending colon gives a clearer spatial resolution of extracellular [Na^+] than rabbit because the rat crypts are straighter and more tightly packed than those of rabbit. The decrease in Na^+-dependent fluorescence seen in fig.3 and plate 2, following exposure to octanol (2 mM) illustrates that the high levels of fluorescence within the interstitial fluid, seen in fig.1B and plate 1, are due to crypt wall colonocyte Na^+-pump activity, rather than to non-specific binding of SBFI to extracellular matrix. The residual punctate areas of high intensity fluorescence at the basal surface of the crypts indicate that the source of the high interstitial [Na^+] is the Na^+-pump activity at the basal surface of the crypts. This view is further supported by ouabain-sensitive decrease in interstitial fluorescence observed with rabbit colon (figs 4C and D).

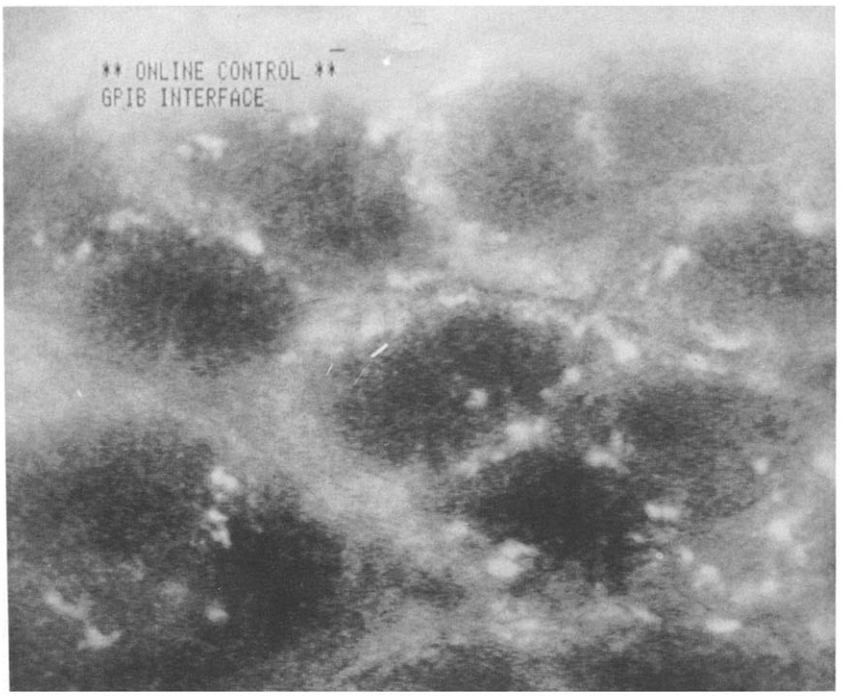


Fig.3. The fluorescence intensity is shown in monochrome fig.3 and in pseudocolour (plate 2) following exposure of the tissue to octanol (2 mM) for 90 min. No change is observed in control tissue over the same period.

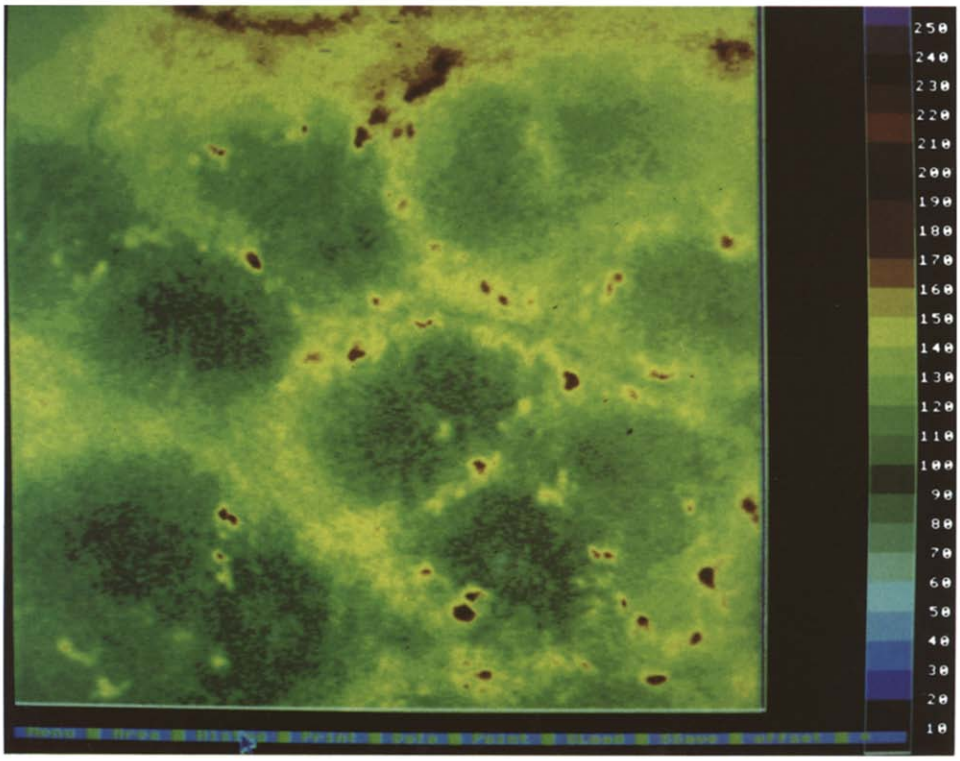


Plate 1. The same data as in fig.1B in pseudocolour; the purple end of the spectrum has a [Na^+] 50 mM and blue/black < 4.8 mM.

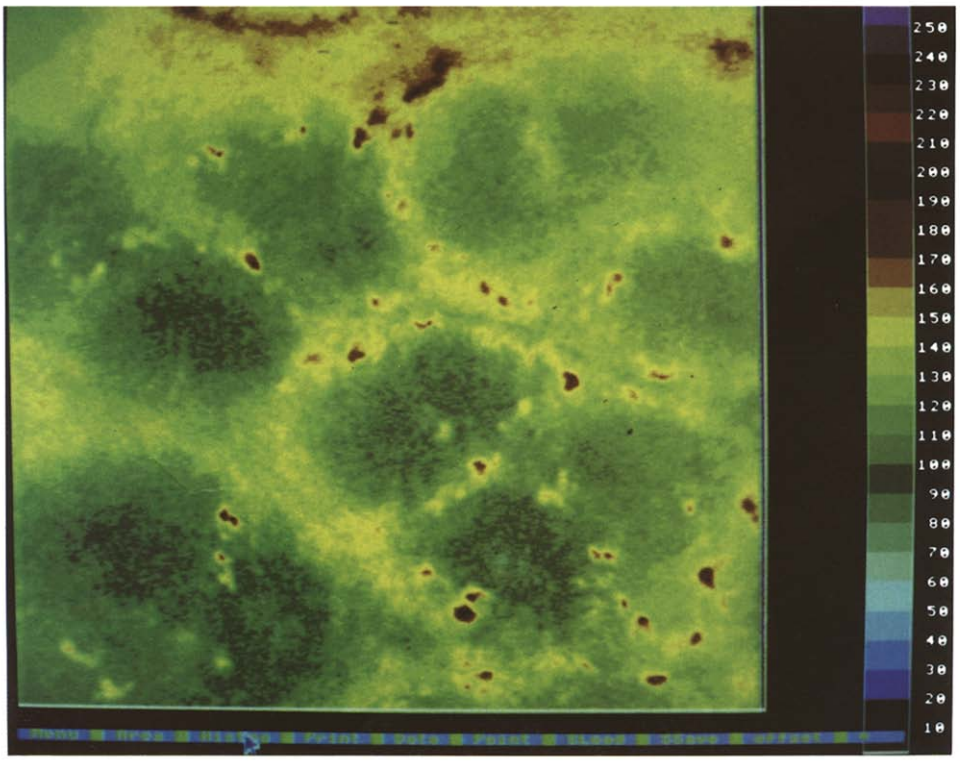


Plate 2. The fluorescence intensity in pseudocolour following exposure of the tissue to octanol (2 mM) for 90 min. No change is observed in control tissue over the same period.

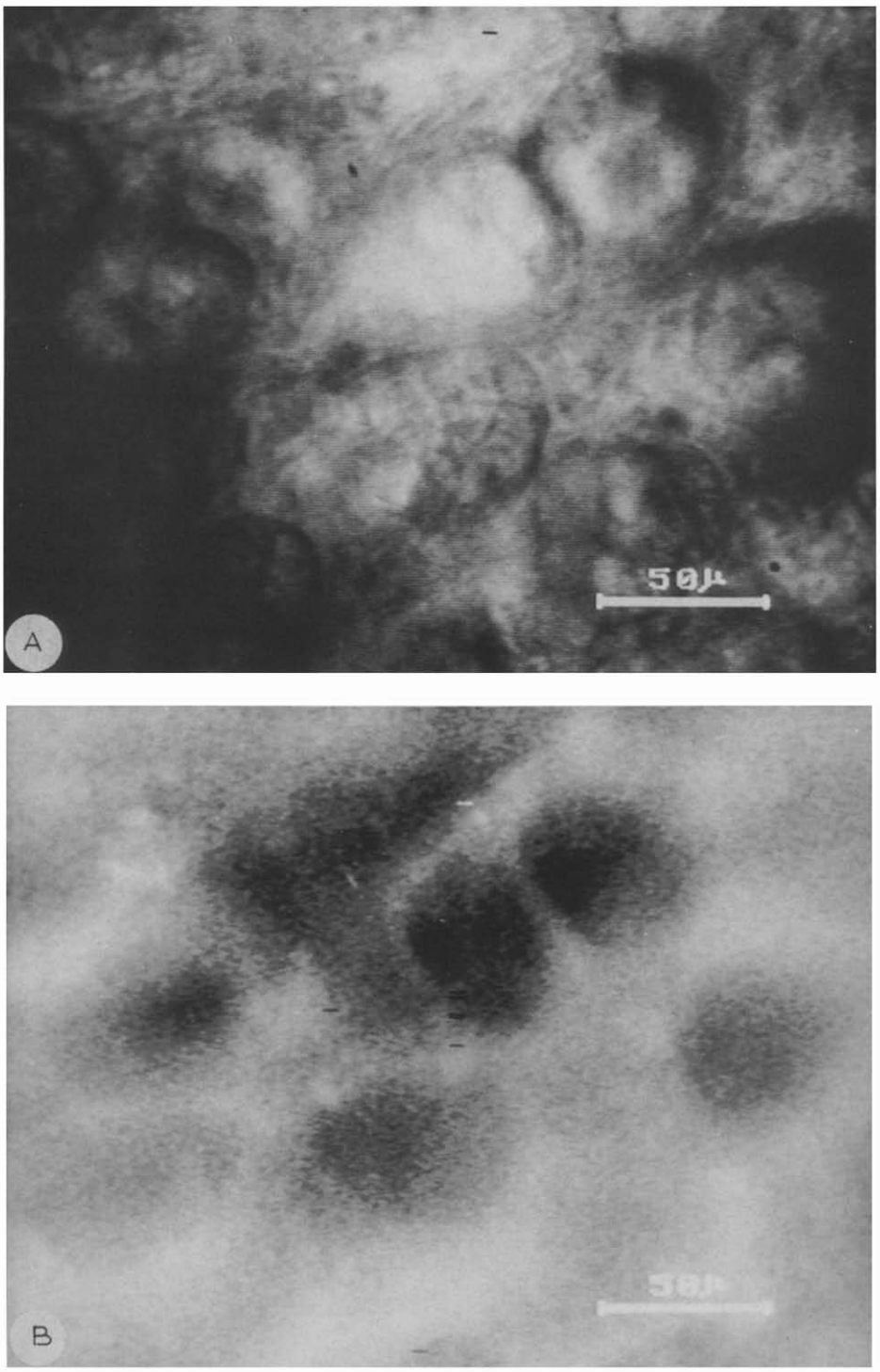
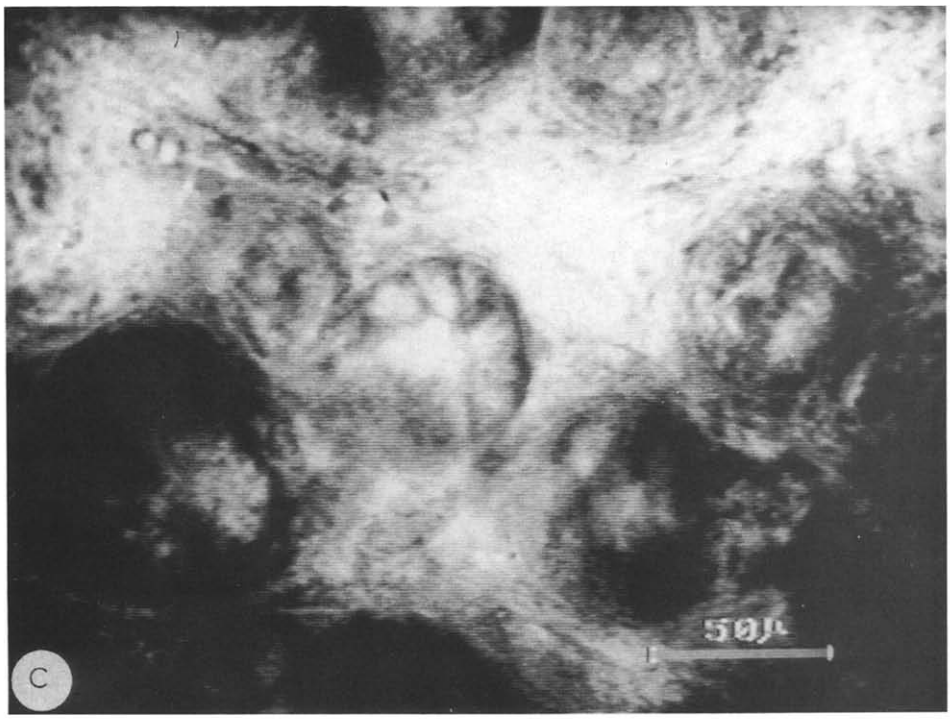
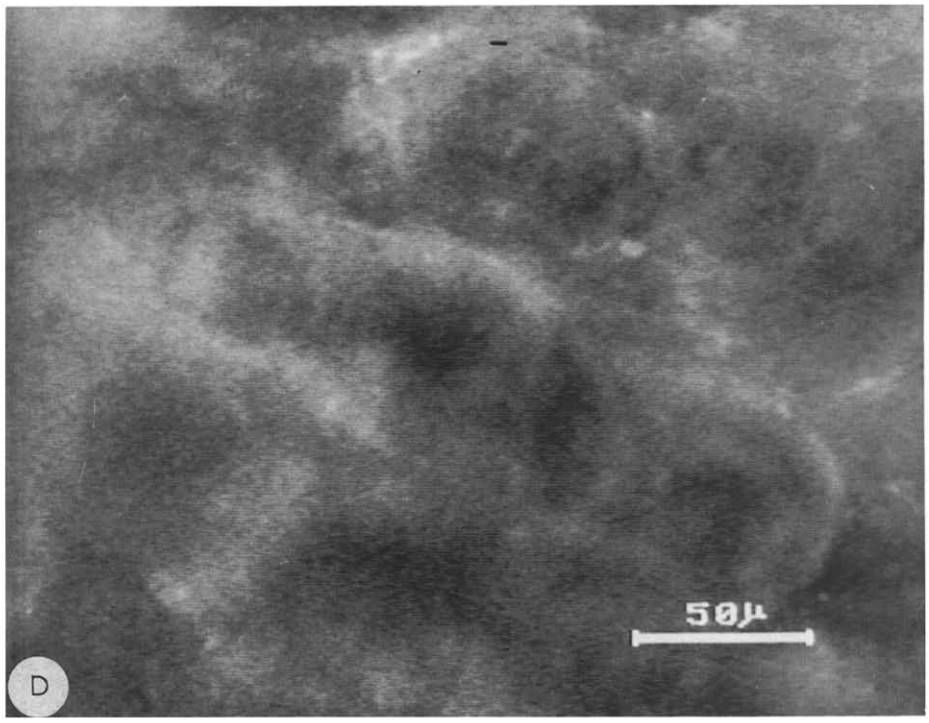


Fig.4. (A) The bright-field image of a group of rabbit colonic crypts bathed in Tyrode containing 4.8 mM Na^+ and SBFI (10 μM) for 1 h. (B) The SBFI (10 μM) fluorescence within the interstitial fluid the crypts. The interstitial Na^+ is ~30 mM. (C) The bright-field image of rabbit colonic crypts bathed for 90 min in Tyrode (4.8 mM) Na^+ + ouabain (1.0 mM) together with SBFI (10 μM). (D) SBFI (10 μM) fluorescence within the interstitial fluid surrounding a group of crypts from rabbit descending colon bathed in Tyrode containing 4.8 mM Na^+ + ouabain (1.0 mM) for 90 min.

In active tissue, the highest intensity fluorescence is in the central area of the interstitial space at a distance of 5–10 μm from the basal surface (plate 1, fig.2), whereas in the octanol-inhibited tissue the highest intensity regions of fluorescence are on the basal surface of the crypt colonocytes (plate 2). Displacement of the highest concentration of Na^+ from the membrane surface to the centre of the interstitial space implies that





fluid outflow from the crypts of actively transporting tissue dilutes the [Na^+] at the membrane surface. The accumulation of [Na^+] in the core region of the interstitial fluid is due to accumulation of solute within the convective stream as it flows past the crypt surface towards the serosal bathing solution. The interstitial accumulation of high concentrations of [Na^+] implies that the crypts have a very low hydraulic conductance [3]. In the octanol-inhibited tissue there is insufficient interstitial hypertonicity to generate a convective stream, hence the maximal concentration of Na^+ resides at the basal surface of the crypts.

The absence of any raised fluorescence within the crypt lumen when the tissue has been incubated bilaterally with either 4.8 mM, or 150 mM Na^+ Tyrode indicates that the steady-state crypt luminal [Na^+] does

not exceed and may be lower than found in the mucosal bathing solution and much lower than in the interstitial fluid. These results are consistent with the view that the crypts normally absorb via the lumen and lend no support to the view that the colonic crypts are secretory during the absorptive mode of colonic function [5].

Acknowledgement: The authors are grateful to the Wellcome Trust for financial support.

REFERENCES

[1] Minta, A., Harootunian, A.T., Koo, J.P.Y. and Tsien, R.Y. (1987) J. Cell Biol. 105, 89a.
[2] Tsien, R.Y. (1989) Annu. Rev. Neurosci. 12, 227–253.
[3] Bleakman, D. and Naftalin, R.J. (1990) J. Physiol. (Lond.) 410, 41P.
[4] McKie, A., Powrie, W. and Naftalin, R.J. (1990) Am. J. Physiol., in press.
[4a] McKie, A., Powrie, W. and Naftalin, R.J. (1989) J. Physiol. 410, 41P.
[5] Welsh, M.J., Smith, P.L., Fromm, M. and Frizzell, R.A. (1982) Science 218, 1219–1221.
[6] Zeuthen, T. (1983) J. Membrane Biol. 76, 113–122.
[7] Gupta, B.J., Hall, T.A. and Naftalin, R.J. (1978) Nature 272, 70–73.
[8] Parsons, D.S. (1956) Q. J. Exp. Physiol. 41, 410–420.
[9] Powell, D.W. and Malawer, S.J. (1968) Am. J. Physiol. 215, 49–55.
[10] Spray, D.C. and Bennett, M.V.L. (1985) Annu. Rev. Physiol. 47, 281–303.