Scanning Microscopy

Volume 9 | Number 4 Article 9

8-31-1995

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Scanning Microscopy, Vol. 9, No. 4, 1995 (Pages 1027-1040) 0891-7035/95$5.00+.25
Scanning Microscopy International, Chicago (AMF O'Hare), IL 60666 USA

CELLS ARE NOT JUST BAGS OF WATER: A PERSONAL APPRECIATION OF THE WORK OF DR. BRIJ L. GUPTA AND HIS CONTRIBUTION TO BIOLOGICAL X-RAY MICROANALYSIS

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(Received for publication May 7, 1995 and in revised form August 31, 1995)

Abstract

This paper surveys the contribution made by the work of Dr. B.L. Gupta to the science of biological X-ray microanalysis. A brief biographical sketch of Brij's early years is given, this is followed by considerations about the models for water transport across epithelia. The ultrastructural and histochemical studies carried out by Brij Gupta and colleagues are reviewed to introduce the historical need for the use of EPXMA for the study of ion and water transport in epithelia. The outstanding contribution made by Brij Gupta's work in this field is outlined, and his understanding of the subcellular distribution ions in other cell types and in the pericellular environment is acknowledged.

Key Words: Electron probe X-ray microanalysis, biology, epithelial transport, ions.

Introduction

I was privileged to be asked by the organisers of Scanning Microscopy to present a tribute to the work of Dr. Brij L. Gupta (Fig. 1) who died early last year. I came to know Brij during the last decade of his life through a joint interest in the use of electron probe X-ray microanalysis (EPXMA) for the study of biological specimens and, for this reason, the contribution made by Brij in this field will be the main topic of this appreciation. However, although to many of us Brij is inextricably associated with biological applications of EPXMA, this was not his sole contribution in the scientific field. Brij was accomplished in the use of both light and electron microscopy, and adept in histochemical procedures and their application to invertebrate tissues when the use of EPXMA to study biological specimens seemed little more than a pipe dream. In addition, Brij was an excellent teacher, and a warm hearted friend. I hope that this paper can do justice to him.

Brief resume of the early years

Brij Gupta was born and educated in India. He obtained the Degrees of B.Sc. and M.Sc. from Panjab University at Chandigarh where he established facilities for all types of light microscopy. He already had a considerable publication list, consisting of some 34 papers (see Appendix), when, in 1961, he moved to the Department of Zoology in Cambridge as a research student on a Commonwealth Scholarship. Here, under the supervision of Dr. Lawrence Picken, he produced his Ph.D. thesis on spermatogenesis, which was considered by the examiners to be outstanding, and the most complete account of sperm morphogenesis up to that time.

Brij then moved to the University of Charlottesville in Virginia to carry out post-doctoral research. It was here that his interest in epithelial transport began. Working with Dr. Colin Little, Brij undertook a study on the uptake of nutrients by the marine worms of the Phylum *Pogonophora*. Since they possess no gut, the exact mechanisms by which these animals obtained their

Figure 1. Brij L Gupta taken at Churchill, his Cambridge College.

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nutrients was not clear, but Brij's work showed that they were able to take up organic nutrients directly through their skin (Little and Gupta, 1968).

After Charlottesville, Brij Gupta returned to Cambridge to set up an electron microscope facility in the Department of Veterinary Anatomy, but was soon poached by the Head of the Zoology Department, Professor Weis-Fogh, and he remained in the Zoology Department to the end of his career. It was here that he carried out his important work using EPXMA to study the mechanisms of transport across epithelia.

Transport Across Epithelia

Background: questions, models and structure

The transport of solutes and water across epithelia in the processes of absorption and excretion is fundamental to enable multicellular (animal) organisms to maintain a stable internal environment. Epithelial transport is also important in the production of a number of glandular secretions. The work of Brij Gupta made a considerable contribution to our understanding of the mechanisms involved in transport across epithelia, particularly in the transport of water.

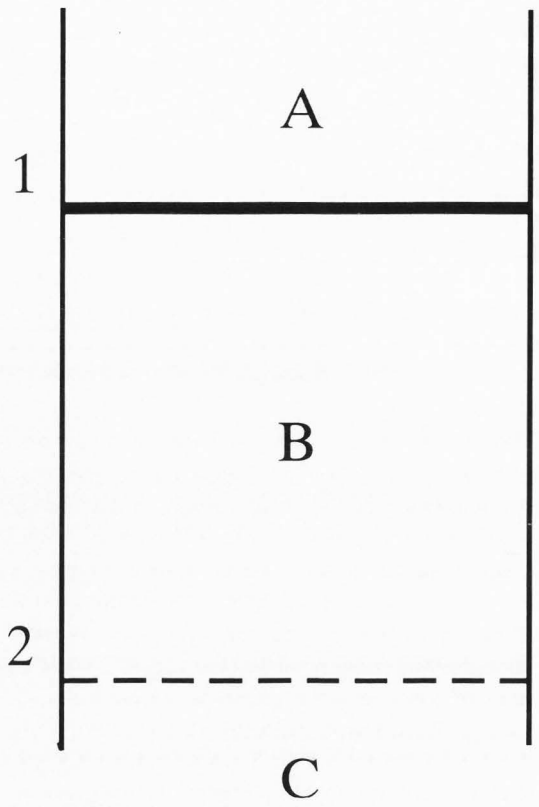

Figure 2. Curran's model to account for the co-transport of solute and fluid across epithelia. Transport occurs from compartment A to compartment C through compartment B. Compartments A and B are separated by barrier 1 which is selectively permeable and is the site of active transport of solute from A to B. Barrier 2 separates B and C and is permeable to both solute and solvent (although it is sufficient of a barrier to retard movement). Active transport of solute across barrier 1 leads to an increase in the concentration of osmotically active particles in B resulting in water also being drawn in through this barrier. The resulting increase in hydrostatic pressure in B drives both solute and water into compartment C. (Redrawn from Curran, 1960).

The history of the development of theories to explain the mechanisms of transport of water across epithelia has been reviewed extensively by Brij Gupta himself (Gupta, 1976, 1984; Gupta *et al.*, 1977). In his review for the 50th anniversary meeting of the Society for Experimental Biology, Gupta (1976) described the changes in attitudes to the fundamental question: How does water traverse the lipid membrane to enter cells and thus cross the epithelial barrier? Studies published in the 8th symposium of the Society held in 1953 had concluded that the active transport of water was unavoidable, but by the time of the 19th symposium in

1965, this general agreement had changed. In many tissues, experimental work *in vivo* had shown that transport of water was almost invariably accompanied by the transport of ions, suggesting a link between the two. So there was a need to explain how the transport of water occurred if it was not actively transported, and how the movement of water was coupled to the movement of solute.

In 1960 Curran proposed the "double membrane theory" (Fig. 2) to explain fluid absorption in rat ileum, and this became a very popular model applied by many investigators to explain transport across numerous types of epithelia. The double membrane model was very successful probably because it was just that, a model, and the original paper did not ascribe any particular known cellular structures to any of the compartments; it was left to the electron microscopists to provide the structural basis for epithelial transport.

At that time, investigations of many vertebrate systems were showing that transporting epithelia had many structural components in common (Keynes, 1969; Berridge and Oschmann, 1972; Gupta, 1976). They often, but not always, consisted of a single cell layer with recognisable basal and apical surfaces giving the cells polarity. Tight junctions were present near the apical surface binding the cells together, and both basal and apical surface areas were greatly increased by either the presence of microvilli or infoldings of the basal surface. Many showed the presence of blind channels in the form of the lateral intercellular spaces (LIS) or intracellular canaliculi. The cells also contained numerous mitochondria. In 1967, Berridge and Gupta published a detailed study on the rectal papillae of *Calliphora* which are responsible for the absorption of water and potassium secreted by the Malpighian tubule. They showed that the structure of this tissue conformed to the pattern for transporting epithelia outlined above. The papillae enclosed an extensive system of intercellular spaces which communicated with the haemocoel and ramified between the lateral boundaries of the cells. They noted that the lateral boundaries were infolded in a complex manner to form stacks which were closely associated with mitochondria in the cytoplasm. Berridge and Gupta (1967) proposed that these stacks were the site of a mechanism which actively pumped ions into the intercellular spaces and suggested that the presence of ions in the narrow intercellular spaces would create a high ionic concentration to draw water from the lumen of the rectum. Their subsequent localisation of Mg^{2+} activated ATPase on the cytoplasmic face of the lateral plasma membranes (Berridge and Gupta, 1968) added further evidence to support this interpretation. Berridge and Gupta (1967) verified experimentally that change in composition of the rectal contents were reflected by

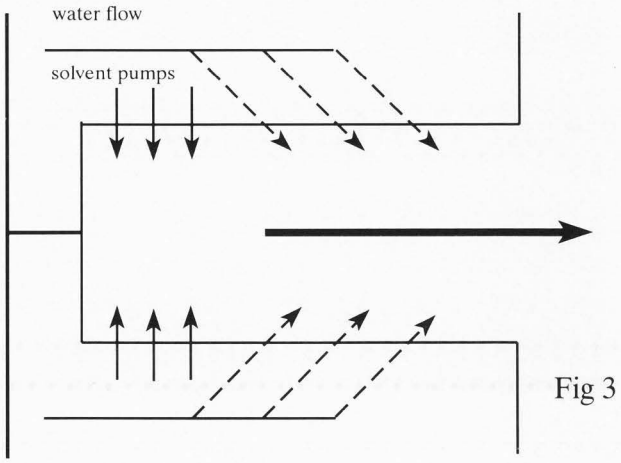

Fig 3

Figure 3. The standing gradient model for the transport of water. This model accounts for the presence of blind-ended channels (canaliculi/ lateral intercellular spaces) in the epithelium. Solute pumps are situated towards the blind end of the channel and pump solute from the cell into the channel causing hypertonicity which draws water through the cell osmotically. The contents are gradually diluted by the inflow of water causing the gradient of concentration along the length of the channel. (Redrawn from Diamond and Bossert, 1968).

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structural changes in the rectal papillae, particularly in the spatial arrangement of the lateral plasma membranes and the results were interpreted in terms of the Curran double membrane model.

The structural studies of Berridge and Gupta (1967, 1968) on invertebrate tissues were in agreement with those from vertebrates which suggested that transport of water was a consequence of active transport of ions into narrow intercellular spaces, with the resulting high concentrations causing water to be drawn into the spaces by osmosis. The recognition that, in the gall bladder, the lateral intracellular spaces act as channels for fluid transport had led Diamond and Bossert (1967, 1968) to develop the standing gradient model to explain coupled transport of solute and water (Fig. 3). This model accounted for the presence of blind channels in the structure of the epithelium. Diamond and Bossert (1967, 1968) suggested that solute was actively pumped into these channels creating a localized high concentration of solute to draw water through the cells by osmotic flow. In this way, the fluid in the spaces would be gradually diluted until it became isosmolar. Thus, by 1969, when Keynes produced a major review on epithelial transport, physiologists were able to measure the concentrations of osmotically active particles on either

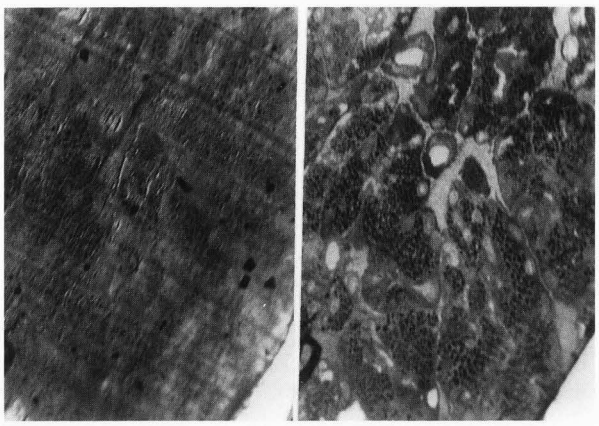

Figure 4. STEM images from a 1 μm thick cryosection of cockroach salivary gland: fully hydrated (**left**) and after freeze drying (**right**).

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side of the epithelial cells and had produced good models which could adequately account for the coupled movement of solute and water across epithelia, and there were numerous electron microscopical studies showing a common structural arrangement in the epithelial tissues. But, as cautioned by Gupta (Berridge and Gupta, 1967) "functional interpretations of structure (and vice versa) were still conjectural." There was a requirement for experimental data on the concentration of ions both inside epithelial cells and in the LIS to provide support for the models of transport and allow a direct relationship to be established between measured element concentrations and known structural details of epithelial cell. The work of Brij Gupta contributed greatly to this.

EPXMA technical developments

In their paper, Diamond and Bossert (1968) outlined several predictions, one of which was that fluid in the basal infolding or lateral intercellular spaces will be hypertonic to the intracellular fluid in forwards transporting epithelia, and hypotonic in backwards transporting epithelia, and they noted that "the testing of this hypothesis requires quick frozen tissue to prevent the disruption of gradients." So it is perhaps not surprising that, as a practicing microscopist aware of the developments of EPXMA that were happening in Cambridge, Brij Gupta was looking towards the possibility of using EPXMA to analyze fluid transporting tissues from invertebrate species. This would be no mean task. The technical problems that were faced are probably best summed up by the words of Brij's long time collaborator Ted Hall (Hall, 1994). "In 1970, Weis-Fogh and Gupta were intrigued by the possibility of direct measurement of the concentrations of diffusible electrolytes *in situ* within cells and within extracellular spaces in frozen-hydrated tissue sections by means of EPXMA. Clearly such measurements would be very valuable, but at the time no one knew if the electrolytes could be frozen in place; EPXMA was not well developed for biological specimens; there was no established method for quantitative EPXMA of tissue sections; and quantitative EPXMA had not yet succeeded with any specimens at the low concentrations anticipated. Furthermore, there was no evidence that the frozen-hydrated sections would be stable during analysis and no evidence that the scanning microscope could provide useable images of such sections." The interest in epithelial transport, and the considerable expertise in electron microscopy and EPXMA present in Cambridge at that time, led to the setting up of the Biological Microprobe Laboratory (BML) as an interdepartmental project with funding obtained from the Science Research Council. T. Weis-Fogh, B.L.Gupta, T.A. Hall, R.B. Moreton and P. Echlin were listed as the Principal Investigators.

The few sentences above summarise what must have been formidable technical problems, and a historical perspective about how these problems were tackled and solved was published by Brij Gupta himself in his own tribute to the work of Ted Hall (Gupta, 1991). In this latter paper, Brij Gupta pointed to the **necessity** of using frozen-**hydrated** sections when measurements are required in matrix free extracellular spaces, and he documented the instruments and the methods used for the preparation of and analysis of the frozen sections, and the early experiments which showed that this approach was indeed feasible. Because these aspects have been so well covered, I will not pursue them further here. Suffice to say that, as one who has had experience of the type of microtome used, I am still amazed by the quality of the sections which were obtained (see, for example, Fig. 4) which are equal to many of those produced today. I also think that it is a tribute to the true nature of the collaboration between Hall and Gupta that it is impossible for anyone not directly involved in the work to know the exact contribution of either partner in the development of these techniques, but I am sure that Brij played no mean part. The approach used and advocated by Brij Gupta was not to wait for the perfect technique before beginning the experiment, but to use the methods available to answer the questions posed by biological problems. This meant that he was well aware of the necessity of properly understanding all aspects of the techniques in use, their limitations, as well as their advantages. This led to a number of excellent publications, usually in co-authorship with Ted Hall, outlining the theory and practice of X-ray microanalysis (see, for example, Gupta and Hall, 1979, 1981, 1982; Hall and Gupta, 1983, 1986) which are regarded as cornerstone contributions by many of us practicing electron probe analysts.

Table 1. Element concentrations (mmol/kg wet weight) in control and 5-HT stimulated epithelial cells of *Rhodnius* Malphigian tubules. Data from Gupta *et al.* (1976).

Position	Control			Stimulated		
	Na	K	Cl	Na	K	Cl
Serosa	132	20	160	132	20	160
Basal Lamina	82	120	97	94	110	74
Basal Region	10	92	77	40	109	65
Cytoplasm	13	103	31	42	102	61
Apical Cytoplasm	41	158	85	24	125	81
Microvillus (base)	38	119	83	60	103	99
Microvillus (tip)	53	133	100	96	139	160
Lumen	48		140	90	90	180

In 1974, the technical developments bore fruit with the publication in Nature of the methods and analysis of frozen hydrated sections of salivary gland from larvae of the blowfly *Calliphora* (Moreton *et al.*, 1974). This was the first report of analysis of frozen hydrated sections showing the stability of the specimen under the electron beam and the first scanning transmission electron microscope (STEM) picture of frozen hydrated material. The results in this paper showed that any dehydration suffered by the specimen during preparation, transfer or examination was tolerable (< 10%) and demonstrated the feasibility of the chosen technique.

EPXMA of transporting epithelia: cells, intercellular spaces and channels

Brij Gupta had, for a long time, chided physiologists for the black box approach of their models and pleaded the need for these to be reconciled with electron microscopical studies revealing the detailed fine structure of the cell (this theme, which was to recur in many of his publications, is the origin of the title of this paper). In 1976, he pointed out that despite its wide acceptance the standing gradient hypothesis, as recognised by its originator, was only a model, and contained several assumptions. He outlined these as:

(1) That the junctions at the blind end were tight.

(2) That there was limited localisation of the solute pumps.

(3) That there was a standing gradient.

(4) That solute and water enter the epithelial cell through the basement membrane.

By that time, experimental evidence that the first two of these assumptions might not hold was already beginning to accumulate. Structural studies had suggested that the so called tight junctions were not necessarily tight (see e.g., Lane and Treherne, 1972; Machen *et al.*, 1972; Claude and Goodenough, 1973). As pointed out in Gupta (1976), this mitigates the blind nature of the channel, and the effects of leakiness on fluid transport would need to be assessed. In addition, histochemical studies on the localization of ion pumps such as those carried out by Berridge and Gupta (1968) had shown that these were not confined to the closed end of the channel. The ability to analyze frozen hydrated sections would allow the assumptions on the presence of standing gradients and pathways of water and ion movements through cells to be tested. Over the next few years Brij Gupta and colleagues carried out experiments to determine local element concentrations both inside the cells and in the extracellular spaces in transporting epithelia largely from insects, beginning in 1976 with work on *Rhodnius* Malphigian tubules (Gupta *et al.*, 1976), followed by Malphigian tubules (Gupta, 1976; Gupta *et al.*, 1976), salivary glands (Gupta *et al.*, 1977, 1978a) and rectal papillae (Gupta *et al.*, 1977, 1980; Gupta and Hall, 1979) from *Calliphora*; in addition, rabbit ileum was also studied (Gupta *et al.*, 1978b). Much of this work is summarised in Gupta and Hall (1979). It is not possible to go into the detail from all of the individual studies here but the results from a few of them will be summarised briefly, and their contribution to understanding epithelial transport outlined.

Rhodnius Malphigian tubules

The Malphighian tubules of Rhodnius can be stimulated to secrete *in vitro* by the application of 5-hydroxytryptamine (5-HT). When bathed in a Ringer containing 20 mM K and 132 mM Na the stimulated cells secrete a fluid which contains equimolar (90 mM) Na and K. Gupta *et al.* (1976) used EPXMA of frozen hydrated sections to try and determine the mechanisms by which this secretion occurred (Table 1). They were able to show an uneven distribution of elements in the cytosol of both stimulated and unstimulated cells with the lowest concentrations of Na and K occurring in the perinuclear region. The effect of stimulation was to increase cytosolic Na by approximately 30 mM. There was no gradient of concentrations in the microvillus region in unstimulated cells, but in the stimulated cells the concentrations of Na, K and Cl increased towards the lumen. This distribution was inverse to that expected for explanation by the standing gradient hypothesis. It was also noted that the basal lamina contained higher concentrations of K than the dextran Ringer and lower concentrations of Na and Cl.

When the Malphigian tubules were stimulated with 5-HT in the presence of K free medium, the level of

intracellular Na increased and there was a high rate of secretion of a Na rich fluid, suggesting that the tubular secretion was driven by an apical pump which transported Na and K in proportion to their availability.

Calliphora salivary gland

The salivary gland of *Calliphora* is a simple epithelium which when immersed in Ringer containing 140 mM Na and 20 mM K secretes a fluid into the lumen which is almost isosmotic with the bathing medium but in which the concentrations of Na and K are reversed. This has the technical advantage that, since the concentrations in the extracellular fluids are known, measurements made in these spaces allow the assessment of adequacy of EPXMA. This approach was advocated and used by Gupta (Gupta, 1976).

Secretion in the salivary gland of *Calliphora* occurs from one cell type which is characterised by the presence of deeply infolded canaliculi. Gupta *et al.* (1978a) used EPXMA to analyze the concentrations of Na, K, Cl, P and Ca (see Table 2) and microelectrodes to analyze for K and Cl in the major tissue compartments. For K, there was good agreement in the values obtained from both techniques for both stimulated and unstimulated cells. The finding that the concentration of K in the canaliculi exceeded that in the cytoplasm indicated that these were the sites where the osmotic gradient was generated. However, the results from a number of analyses along the length of the canaliculus were unable to support the idea of a standing gradient, and it was suggested that final dilution of the salivary fluid could be due to flux of water through a paracellular route (discussed in Gupta and Hall, 1979). An interesting observation was that the water content of the stimulated cells was less than the unstimulated cells suggesting that the secretory cells were slightly hypertonic to the bathing medium; it was suggested that this could also provide a gradient for the osmotic movement of water. Again, higher than expected levels of elements were found associated with the basement membrane.

Rabbit ileum

Gupta's work was not confined solely to the study of insect tissues, and he carried out studies on the rabbit ileum in collaboration with Dr. Richard Naftalin (Gupta *et al.*, 1978b). In contrast to the secretory epithelia discussed above, this tissue is an absorptive epithelium which is capable of transporting water from mucosa to serosa across a substantial osmotic gradient. Microprobe analysis studies showed that average microprobe values for Na K and Cl under different conditions of stimulation agreed well with other published values but these average values concealed the fact that the distribution of these elements and of dry mass within the cells was not homogeneous. A diagrammatic representation

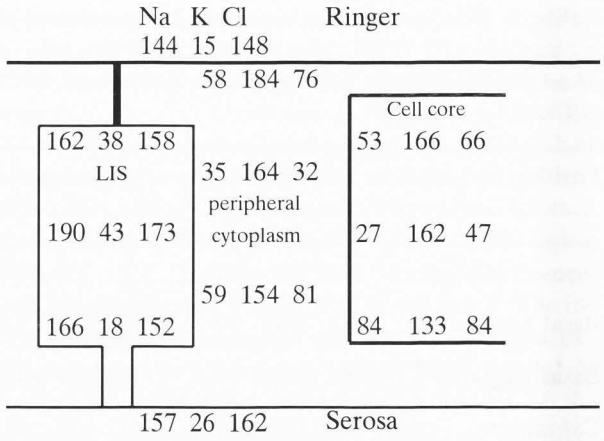

Figure 5. Summary of concentrations of Na, K and Cl (mmol/l water, standard errors omitted) determined by EPXMA of galactose stimulated rabbit ileum, showing the gradients in the LIS and peripheral cytoplasm. (Redrawn from Gupta and Hall, 1979).

Table 2. Element concentrations (mmol/l water) in control and 5-HT stimulated epithelial cells from *Calliphora* salivary gland. Data from Gupta *et al.* (1978).

Position	Control			Stimulated		
	Na	K	Cl	Na	K	Cl
Serosa	132	20	158	132	20	158
Basal lamina	76	76	134	80	129	134
Intracellular	24	135	39	19	161	30
Canaliculi	19	178	127	18	187	117

of the results obtained for element contributions in enterocytes after stimulation by 10 mM galactose is given in Figure 5. The cell could be considered to consist of a narrow (approximately 0.5-1 μm wide) band of cytoplasm and an organelle rich cell core with greater dry mass. The concentration of K was much higher in the peripheral zone and intermediate between that of the bathing medium and the LIS, and it was suggested that this peripheral zone might act as a channel for the conductance of ions between the apical surface and the LIS. Concentrations of the osmotically active ions Na and K were significantly higher in the LIS than in the external bathing solutions, but analysis of the profile along the LIS showed that highest osmotic concentrations were found in the mid region, and thus, these results were not consistent with the standing gradient hypothesis. The profile was interpreted as being due to dilution of the contents of the lateral intracellular spaces by the flow of water across the cell junctions.

Table 3. Concentrations of Na and K {mmol/kg wet weight, mean ± standard error of mean (SE)} in nucleus (N) and cytoplasm (C) of various different tissues.

Tissue	Compartment	Concentration		Reference
		Na	K	
Malphigian tubules (*Rhodnius*)	N	36 ± 2	196 ± 12	Gupta *et al.* (1976)
	C	47 ± 5	149 ± 10	
Salivary glands (*Calliphora*)	N	7 ± 1	192 ± 6	Gupta *et al.* (1978a)
	C	15 ± 1	125 ± 7	
Salivary glands (*Periplaneta*)	N	10 ± 2	158 ± 5	Gupta and Hall (1983)
	C	16 ± 2	88 ± 3	
Ileum (Rabbit)	N	20 ± 2	138 ± 9	Gupta *et al.* (1978b)
	C	36 ± 3	115 ± 6	
Erythrocytes (Chick)	N	40 ± 5	112 ± 9	Jones *et al.* (1979)
	C	78 ± 8	136 ± 13	

Epithelial transport: conclusions

The above are just three examples of studies on transporting epithelia carried out by Gupta and colleagues. Although the epithelia are quite different, there are common points which emerge. Chiefly, these studies show that the invaginated spaces (canaliculi/LIS) are hypertonic confirming that these are the sites where the gradients for osmotic flow of water are established. Gupta and colleagues were unable to demonstrate the presence of a standing gradient as suggested by Diamond and Bossert (1967, 1968). Their results did, however, suggest a pathway for the flow of water through the lateral junctions which would modify, to a variable extent, any gradient which had been set up. Their results demonstrated unequivocally that elements were not distributed uniformly within the epithelial cells. The presence of gradients of concentration both of elements and water content within the stimulated cells confirmed a transcellular route for the absorbance of both water and ions, but, as outlined above, evidence was also accumulated for a paracellular route for the movement of water. The studies of Brij Gupta and colleagues have made a major contribution to understanding epithelial transport because of the ability to measure element content at known localizations within the tissue, and thus, directly relate element content, structure and function. Their findings are perhaps best summarised realistically by Brij Gupta (Gupta and Hall, 1983; Gupta, 1984) who felt that the movement of water across epithelia was best accounted for by mechanisms which incorporated local osmosis, but that it was probably futile to try and fit everything into one general model and the routes of water transport might well differ in different epithelia possessing different structural geometries.

EPXMA of transporting epithelia: the pericellular environment

The conclusion that mechanisms of transport may differ in different tissues may seem somewhat disappointing to those who would like grand theories unifying everything, but the observations made on the various tissues led to one of the most important contributions that Brij Gupta has made, that is the idea that the connective tissue elements surrounding the cell (glycocalyx, basal lamina) may play an important role in modifying epithelial transport. The microprobe studies carried out at the BML had invariably shown the basal lamina contained high concentrations of K and Ca which were often intermediate between those of the extracellular and intracellular environments. Additionally, it was found that after freeze drying, the fluid spaces (canaliculi/LIS) contained a residual amount of dry mass ($< 5\%$ to $> 30\%$). Initially, it was thought that this might be due to overlap of the probe with hidden cellular compartments, but later Gupta realised that it more likely represented connective tissue elements (Gupta, 1989a, 1989b). Experimental evidence from microelectrode measurements showed concentrations for K which were lower than those measured in the same preparations by EPXMA. Since microelectrodes only measure free ions, and EPXMA total concentrations, it was reasonable to suggest that the difference was made up by ions bound by the highly ionic constituents of the connective tissue components. Thus, in 1982, Gupta (Gupta and Hall, 1982) stated with reference to earlier results: "This table also shows the limitations of EPXMA in such work be-

cause it measures the total elemental concentrations and not osmotic or ionic activities. The narrow interspaces also contain a matrix of fixed negative charges. It is therefore not simple to decide how much of the excess Na, K and Cl in such spaces contributes to the osmotic activities." Studies on toad bladder had suggested that recycling of K in the pericellular environment was important in the transport of Na across this tissue, and would explain some of the anomalies found on exposure to K free medium (Civan *et al.*, 1980). Later, Gupta (1989b) proposed that the K-sequestering abilities of the pericellular mucoids might be a general mechanism by which K leaking out of the cells was conserved and this would reduce the energy load for active transport. His interest in the pericellular environment, was in advance of the interest currently being shown in intercellular adhesion molecules.

Subcellular localisation of Na and Ca

One of the significant general products of the numerous microprobe studies which were carried out by Gupta and colleagues was to challenge some of the views in cell physiology that were held at that time. For instance, despite it being known that the normal ratio of K/Na in the cytoplasm of most viable cells was about 10/1, there was a common belief that the cell nucleus contained high concentrations of Na. In discussing their results from studies on transporting epithelia, Gupta often referred to the finding of nuclear concentrations of Na more in line with those of the cytoplasm (Table 3). In a study on chick erythrocytes, Jones *et al.* (1979) compared element concentrations in the nuclei of frozen hydrated sections of chick erythrocytes with those in nuclei which had been subject to an isolation procedure which minimised electrolyte loss. The results showed that the nuclei in the sectioned cells had low concentrations of Na whereas the isolated nuclei were characterised by high concentrations of Na; it was concluded that the high levels of Na were a result of the isolation procedure.

In a similar vein, many of the early results from transporting epithelia predicted some of the views on cytoplasmic stores of Ca which are held today. Biochemical studies (Lehninger, 1970) suggested that mitochondria were the subcellular site for the storage of Ca released in response to stimulatory signals. In their studies on *Calliphora* salivary gland Gupta and colleagues (Gupta *et al.*, 1978a; Gupta and Hall, 1978) were interested in identifying the presumptive Ca stores. Histochemical precipitating techniques involving oxalate ions supported the biochemical observations suggesting that mitochondria were the intracellular store. These observations, however, were not confirmed in cryoprepared specimens analysed directly by electron microprobe. In these preparations, the Ca concentration in the mitochondria from healthy cells was no different to that for their surrounding cytoplasm, but hot-spots of Ca were found near the nuclear envelope and in the perinuclear cytoplasm. High levels of mitochondrial Ca were only found to be associated with damaged cells typically containing an inverse K/Na ratio in their cytoplasm. The authors concluded that "mitochondria can soak up Ca^{2+} if it becomes freely available as in damaged cells. However, in our view, the identity of intracellular Ca stores remains an open question and sites like the nuclear envelope (continuous with the rough endoplasmic reticulum) should also be considered." These observations predict later work using fluorescence dyes and digital imaging which showed Ca release from this site in chromaffin cells (O'Sullivan *et al.*, 1989). The problem of localisation of Ca stores was investigated systematically by Somlyo's group (Somlyo, 1984; Somlyo *et al.*, 1985; Bond *et al.*, 1987) who were able to show unequivocally that intracellular calcium stores were associated with the endoplasmic reticulum and not with mitochondria.

The Contribution of Brij Gupta to Biological X-Ray Microanalysis

What then was Brij Gupta's contribution to biology and X-ray microanalysis? In the above discussions, I have talked about the enormous contribution that Brij Gupta made to the development of techniques for X-ray microanalysis of biological specimens, to the application of these methods to produce relevant results on transporting epithelia, and his development of new ideas about the interactions of cells with their environment. His work certainly shows that cells are not just bags of water, and that even the study of fixed tissues can give insight into their dynamic nature. The review above gives the highlights of Brij's work but does not claim to be comprehensive.

Brij Gupta's contribution to biology and biological X-ray microanalysis was, however, not limited to his published work, although his papers are an education in themselves. (Experiments were not usually confined solely to one technique but different methods were combined to give maximum information. The discussions in these papers consider, with reasoning, not only his own point of view, but all sides of an argument.)

On several occasions when stumbling on problems, I had recourse to seek his help, and he was always helpful and encouraging. He read widely and his broad knowledge and kindly attitude made him an ideal referee for many journals. After we held our first meeting of the U.K. Biological X-ray Microanalysis Group (in London, March 1988, before its formal setting up), Brij

encouraged us to continue and we were pleased that he attended the meetings and shared his knowledge so readily with newcomers to the field. Brij Gupta was truly a scholar and a gentleman.

Acknowledgements

I would like to thank Scanning Microscopy International for the opportunity of making this tribute to Brij Gupta, and to thank them and Oxford Instruments Microanalysis Group for sponsoring my attendance at the Scanning Microscopy 1995 meeting at Houston, Texas. I would also like to thank Dr. T.A. Hall for reading the manuscript and making helpful suggestions, and the Senior Tutor of Churchill College, Cambridge, Dr. Andrew Putnis, Cambridge for providing the picture of Brij for Figure 1 and his complete bibliography.

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