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J. Stephen
University of Birmingham

M. P. Osborne
University of Birmingham

A. J. Spencer
University of Birmingham

A. Warley
University of Birmingham

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FROM HELA CELL DIVISION TO INFECTIOUS DIARRHOEA

J STEPHEN[1]*, M P OSBORNE[2], A J SPENCER[2], and A WARLEY[1,3]

Departments of Microbiology[1] and Physiology[2], University of Birmingham, PO Box 363, Birmingham B15 2TT, U.K.

[3]Present address: Division of Anatomy, UMDS (ST. Thomas's Campus) Lambeth Palace Rd, London, SE1 7EH

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Abstract

Hela S3 cells were grown in suspension both randomly and, synchronously using hydroxyurea which blocks cells at the G1/S interface. Cryosections were prepared, freeze-dried and analyzed by X-ray microanalysis. As cells moved into S and through M phases [Na] and [Cl] increased; both returned to normal levels upon re-entering G1 phase. The Na/K ratio was 1:1 in G1 phase. Infection of HeLa S3 cells in G1 phase with vaccinia virus resulted in no change in intracellular [Na].

Infection of neonatal mice with murine rotavirus was localized to villus tip enterocytes and gave rise to diarrhoea which was maximal at 72h post-infection (p.i.). Diarrhoea was preceded by ischemia of villi (18-42h p.i.) and villus shortening (maximal at 42h p.i.), and was also coincident with a dramatic regrowth of villi. At 48h p.i. a proliferative zone of electron lucent cells was observed in villus base regions. Cryosections of infected gut, taken before, during, and after infection, together with corresponding age-matched controls, were freeze-dried and analysed by X-ray microanalysis. At 48h p.i. electron lucent villus base cells were shown to be more hydrated, and, to contain higher levels of both Na and Cl and lower levels of P, S, K and Mg than corresponding control cells.

These studies (we argue) increase confidence in the use of X-ray microanalysis in studying biological systems, provide some insight into the process of cell division, and constitute the basis of a new concept of diarrhoeal secretion

Key Words: X-ray microanalysis,Hela cells,rotavirus, cell division,gut secretion,hypersecretion,infectious diarrhoea.

*Address for correspondence: J Stephen, Department of Microbiology, School of Biological Sciences,The University of Birmingham,PO BOX 363, Birmingham B15, 2TT,UK. Phone: 021-414-6550

Introduction

In this article we outline two contributions which superficially bear little relationship with each other, yet, in a serendipitous way one proved seminal to the other and both demonstrate the power of quantitative X-ray micro-analysis of freeze-dried sections in the solution of biological problems.

HeLa Cells

During the late 1970's and early 80's, considerable efforts were made to elucidate the basis of selective inhibition of protein synthesis by cytopathic viruses. How could host-cell protein synthesis be inhibited without affecting virus-directed protein synthesis which depends absolutely upon the functional integrity of the host biosynthetic machine? As summarized by Shatkin (1983) several ideas were put forward to explain this selectivity. One of these ideas centred around virus-induced changes in membrane permeability with a concomitant influx of Na^+ into infected cells. This would (if it happened) give rise to conditions which would favour translation of virus-specific as opposed to host-specific messenger RNAs; indirect evidence suggested that this was, at least for some viruses, a possibility (see Norrie *et al.*, 1982). However, the direct evidence that significant ion changes actually occurred was missing. Norrie *et al.* (1982) set out to obtain such evidence for vaccinia-infected HeLa cells by flame photometric bulk cell analysis. Their data showed that although changes did occur, they occurred too late for these to be the cause of the changes observed in patterns of protein synthesis. Either, the theory was wrong, or the technique of bulk cell analysis was incapable of detecting relevant' changes in intracompartmental ion concentrations. To address this possibility, Warley *et al.* (1983*a,b;* 1985) applied X-ray microanalysis to the problem. To obtain interpretable data it was necessary to work with synchronized cells. This was achieved using hydroxyurea which arrests cells at the G1/S interface: several important findings

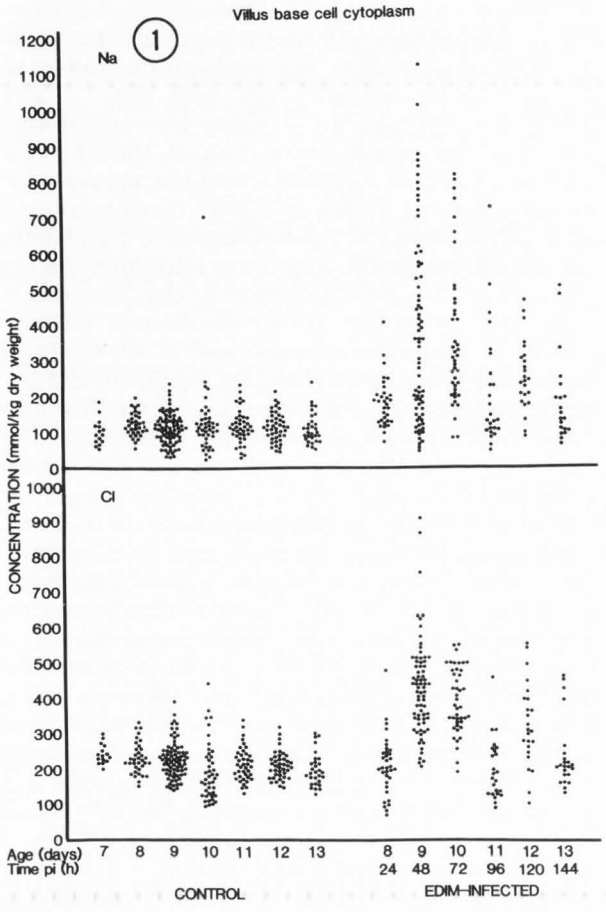
emerged.

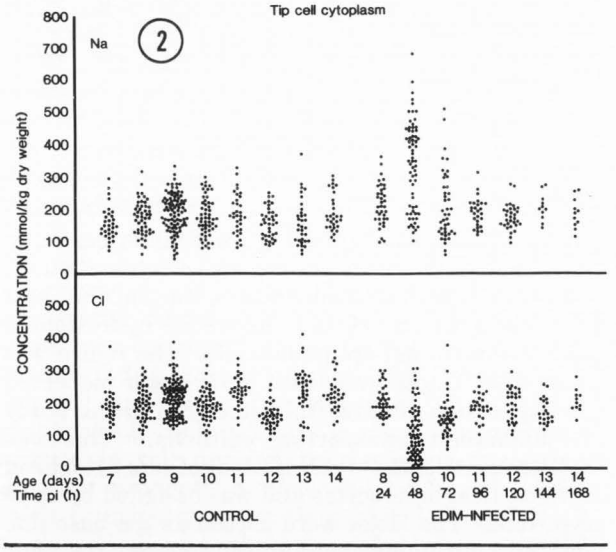
First, analysis of approximately 10 cells per datum point yielded data which were in broad agreement with the flame photometric measurements based on 10^6 cells per datum point. Second, one significant difference was observed; the late increase in [Na^+] which was thought to occur in vaccinia-infected HeLa cells was probably an artifact of the method used to prepare HeLa cell pellets for bulk analysis. Third, when cells were infected in G1 phase (during which phase [Na] was least variable) no evidence was obtained from the X-ray microanalytical study to sustain the theory of virus-induced changes in elemental concentrations in cell compartments: at this point commitment to the theory that virus-induced changes in ion concentrations were responsible for selective inhibition of protein synthesis was abandoned. Fourth, and potentially the most important result of this study was the observation that significant changes in [Na] and [Cl] occurred as uninfected cells went into S and through M phases of the cell cycle. Commitment to DNA synthesis (i.e. removal of hydroxyurea from cells blocked at G1/S interface) was either triggered or accompanied by, transient increases in both [Na] (384±35 to 566±44 mmol/kg dry wt. ±SEM) and [Cl] (224±31 to 518±30 mmol/kg dry wt. ±SEM); this was maintained through G2 and M phases. Progress from M to G1 phase was accompanied by a return to G1 levels of [Na] and [Cl]. Other workers using quite different techniques have observed increases in [Na] associated with S phase (Frantz *et al.*,1981; Jung and Rothstein, 1967; Cuff and Lichtman, 1975; Moolenaar *et al., 1981;* Rozengurt *et al.,* 1981; Mummery *et al.*,1982). While the pattern of changes in [Na] and [Cl] in HeLa cells was reproducible and, as alluded to for Na, in good agreement with the data obtained by other workers, the K/Na ratio of HeLa S3 cells was low in G1 phase - approximately 1:1. It was concluded that this was a feature of HeLa S3 cells grown in suspension, since good agreement was obtained between X-ray analysis and flame photometric measurements of standards. Also, K/Na ratios in erythrocytes and oubain-treated HeLa cells (as measured by X-ray analysis) were as expected: 7.5:1 and 1:8 respectively.

Infectious Diarrhoea

The initiation of the second project discussed in this paper overlapped with the conclusion of the first. The objective was to elucidate both the pathogenesis and pathophysiology of rotavirus-induced diarrhoea in neonatal mice — a model for infant human rotavirus-induced diarrhoea which is responsible for several million fatalities annually in young children. A multidisciplinary approach was mounted involving: quantitative measurements of virus infection in different regions of the gut throughout the course of infection and convalescence (Starkey *et al.,* 1986); a transmission and scanning electron microscopic study of changes in gut villi (Osborne *et al.,* 1988); changes in profiles of enzymes of known or potential importance in gut transport (Collins *et al.,* 1988; 1990); changes in the transport properties of infected gut analysed *in vitro* (Starkey *et al.,* 1990*a,b*); X-ray microanalysis of freeze-dried cryosections of rotavirus-infected mouse gut (Spencer *et al.,* 1989; 1990); changes in the microcirculation of rotavirus-infected mouse intestine (Stephen and Osborne, 1988). Arising from all these integrated studies a new concept of the pathophysiology of rotavirus-induced diarrhoea has emerged (Spencer *et al.,* 1990) which by extension is also applicable to other infectious diarrhoeas (Stephen and Osborne, 1988). To appreciate fully the crucial contribution made by X-ray microanalytical studies to the development of the new concept, and, to demonstrate the power of this technology when applied appropriately to biological systems, it is necessary to set such analyses in context: a brief summary of the main findings of these multidisciplinary studies ensues.

Oral infection of rotavirus-antibody free 7 day-old mice with mouse rotavirus (Cambridge strain of epizootic diarrhoea of infant mice rotavirus) resulted in diarrhoeal disease; the attack rate was 100%. Virus infection was biphasic. The first peak occurred at 48h post infection (p.i.) mainly but not exclusively in the mid-small intestine; a second, lesser peak occurred at around 120h p.i.. Preceding and during major virus replication, a progressive ischaemia of villi occurred between 18-42h p.i. (Stephen and Osborne 1988; Osborne *et al.*,1990*a,b*). Scanning electron microscopy revealed at 48h p.i., a dramatic shortening of villi followed by their explosive regeneration such that they regained full height by around 72h p.i.. Transmission electron microscopy consistently revealed a band of electron lucent cells, rich in dividing cells, associated with villus bases. Enzymological studies showed that levels of thymidine kinase (a marker of DNA synthesis) increased over control values and peaked at 72h p.i., the time at which diarrhoea was most severe. Diarrhoeal disease was prolonged over another 3 to 4 days and then stopped. Prevailing conventional wisdom would have predicted maximal diarrhoea at 48h p.i., corresponding with maximum shortening of villi, and hence with maximum loss of "absorptive" enterocytes; the concomitant loss of lactase enzyme would lead to lactose intolerance. Measurements of lactase and lactose levels, and, *in vitro* study of the transport properties of mid small intestine disproved both these predictions (Collins *et al.,* 1988; Starkey *et al.,* 1990 *a,b*). Maximum diarrhoeal secretion was associated with the period





Figures 1 and 2. For X-ray microanalysis, gut tissues were mounted on aluminium specimen stubs (Reichert) and plunge-frozen in liquid propane in a Reichert KF80 cryofixation apparatus. Cryosections, produced with a Reichert FC4/Ultracut E cryoultramicrotome, were mounted on titanium grids and freeze-dried before examination in a JEOL 120CXII fitted with an LaB_6 filament and TEMSCAN. EDAX analyses were performed in STEM mode with a Link Systems 860 series 30mm^2 Si[Li] detector and multichannel analyser. Quantitative data were calculated using the Link Systems "QUANTEM/FLS" programme based on the continuum method of TA Hall.

Figure 1. Villus base cells. Concentration profiles of Na and Cl in the cytoplasm of villus base cells in the middle small intestine of control and EDIM rotavirus-infected mice. Results are from three animals (8-, 9-,and 10-day data) or two animals (7-, 11-, 12-, 13-,and 14-day data). Each point represents one analysis from an individual cell. Figures 1 and 2 are taken from Spencer AJ *et al.*, (1990), Journal of Pediatric Gastroenterology and Nutrition, 10: 516-29.

Figure 2. Villus tip cells. As for figure 1 .

of rapid regrowth of villi i.e. after 48h and upto 72h p.i.. This explosive regeneration of villi induced by virus is similar to that reported by Robinson *et al.* (1981) following experimentally-induced ischaemia of small intestine.

Based on the data from dividing Hela cells described above, it was predicted that cells in the electron lucent zone would have elevated Na and Cl profiles: this was looked for and indeed found (Spencer *et al.*, 1990). Freeze-dried cryosections of basal regions of villi revealed that electron lucent cells contained up to 10 times the normal levels of both Na and Cl (Fig.1). The considerable vertical scatter in the data points for individual cells was interpreted as evidence that these cells had been arrested on freezing in different stages of the cell cycle. In contrast to tissue culture cells, epithelial cells are polarized with respect to ion traffic and associated movement of water. Thus it is possible that excess Na^+ and Cl^- are secreted into the gut lumen, and with them water, giving rise to hypersecretory diarrhoea. In sharp contrast to the situation in villus base cells, few changes in elemental profiles were observed in crypt cells, but changes of a different nature were seen in villus tip cells : increases in [Na] occurred simultaneously with decreases in [Cl] (Fig.2). This occurred when villi were ischaemic and presumably hypoxic. It is thus possible that a switch to anaerobic/glycolytic type metabolism occurred with a predictable rise in intracellular [H^+]. This would imbalance the Na^+/H^+, Cl^-/HCO_3^- antiports — coupled through metabolically generated CO_2 and hence H_2CO_3. The concentrations of H^+ and HCO_3^- will normally be determined mainly by the dissociation of the weak acid H_2CO_3. However, increasing [H^+] by other means would upset this tightly controlled

equilibrium, which in turn would imbalance the uptake of Na^+ and Cl^-: intracellular [Na^+] would rise and [Cl^-] would fall (Charney and Feldman, 1984).

Conclusions

From these quantitative studies confidence has been strengthened in X-ray microanalysis as a tool for biological research, new insights into aspects of cell biology have been gained, and important new — indeed quite revolutionary concepts of the pathophysiology of a very important disease have been developed. These are summarized below.

There is still a residuum of suspicion in accepting the usefulness of elemental concentrations measured by X-ray microanalytical techniques applied to cryosections; this is particularly so with respect to highly mobile ions such as Na^+, K^+ and Cl^-. Since the controlled transport of these ions lies at the heart of some organ functions (e.g. gut and kidney) it is essential that data obtained by X-ray microanalysis is reliable, otherwise physiologically meaningful extrapolations cannot be made. Clearly, the biological significance must be dependent upon the quality of structural preservation achieved during the preparative procedures. We believe that the work on mouse gut shows that this goal can be accomplished with complex tissues and not just homogeneous cell populations. In support of this we make three points. First, with EDAX the concentrations of all detectable elements are measured simultaneously from the area irradiated by the electron probe. Therefore, relative concentration values (if not absolute values) must be valid and constrained only by the quality of preservation of the tissues and the detection limits for each element. The Na/K ratios in gut enterocytes (see Spencer *et al.*, 1990) for control tissues lay in the range of 1:5-8 which is similar to values derived by other techniques for other cell types; such values are accepted as the 'norm'. Second, there is clearly tight clustering of individual elemental concentrations in control cells when these are compared with cells from infected gut (Figs.1,2). It is possible, however, that some variation exists within the data for villus tips (Fig 2). We do not think that this is wholly due to technical scatter although there must (as in all such experiments) be an element of this involved. It could reflect the fact that, as von Zglinicki and Roomans (1989) have shown, there are differences in elemental concentrations in different locations within enterocytes. Also, as cells move up villi they differentiate from "secretory" to "absortive" cells, senesce and are shed. Therefore, scatter could reflect changes in physiological state. Third, differences in [Na] and [Cl] were maximal between infected villus base cells and corresponding controls; differences were also observed within the same tissue between villus bases and tips. These differences are capable of being interpreted in a biologically meaningful way. Fourth, the HeLa cell work showed that confidence could be expressed in the data derived from a relatively small number of cells. The profile of relative change in elemental concentrations obtained by X-ray microanalysis was (in general) remarkably similar to that obtained by bulk cell analysis. Clearly one cannot measure directly, absolute concentrations of elements in freeze-dried sections. Hydrated sections would be preferable, but to date insufficient studies have been made on such materials for evaluation. This leads us to consider the significance of our data in the context of cell biology.

At present, one can only speculate as to the biochemical mechanism underlying the transient rise in Na and Cl in cells as they enter into S and pass through M phases. However Spencer *et al.*, (1990) put forward a plausible interpretation of the biological significance of this rise in [Na] and [Cl] which is summarized as follows. Somlyo *et al.*, (1979) calculated relative degrees of hydration of intracellular organelles based on the continuum counts derived from such organelles in single cells in the same freeze-dried section. We felt it was necessary to attempt to make some estimates of degrees of hydration and hence element wet weight concentrations. In this rotavirus work comparisons were made between cytoplasm in different cells within the same section using the same mathematical transformation (Somlyo *et al.*,1979); this is illustrated as follows for electron lucent (villus base) cells :

$$\%H_2O_{vb} = 100 \times [1 - 0.28 \times (Co_{vb}/Co_c)]$$

where Co_{vb} and Co_c are, respectively, the continuum counts originating from villus base and crypt cell cytoplasm in a freeze-dried cryosection. A value of 72% hydration was used as the best available estimate of the degree of hydration of "normal" intestinal epithelial cells. The figure was taken from Gupta and Hall, (1979) who pioneered the application of X-ray microanalysis to the study of mammalian transporting epithelia. This was their estimate of the water content of core cytoplasm of rabbit ileal enterocytes and was assigned by us to crypt cells. The latter were chosen as the base line since, in both uninfected and infected gut the ionic (and hence one presumes water) contents of such cells were practically the same (Spencer *et al.*,1990). The value 72% was used in order to place the data into some meaningful range. However, even if the absolute value for neonatal mouse cells was different, it would not affect the validity of the

comparison between cells from control and infected animals. The concentration (C) of elements in villus base cells, in mmol/kg H_2O, was then calculated assuming that all elements are dissolved in cell H_2O. Thus:

$$C\ (\text{mmol/kg } H_2O) = C\ (\text{mmol/kg dry weight}) \times \frac{1-(\%\ \text{hydration}/100)}{\%\text{hydration}/100}$$

It transpired that electron lucent villus base cells were more hydrated than other (more electron opaque) cells in the mucosa (mean ± SEM: 77.9 ± 0.7% vs 69.5 ± 1.6 %). Moreover, conversion to wet weight values (mmol/kg H_2O) showed that both [Na] and [Cl] in villus base cells of infected animals were significantly greater than in villus base cells from uninfected animals (means ± SEM: Na, 137 ± 7 vs 38 ± 4; Cl, 121 ± 4 vs 89 ± 6; $p < 0.001$). The wet weight concentrations of other elements in electron lucent cells were lower than in corresponding cells from uninfected animals (means ± SEM: P, 196 ± 9 vs 305 ± 27; S, 46 ± 3 vs 73 ± 6; K,191 ± 6 vs 304 ± 9; Mg,19 ± 3 vs 24 ± 4; for all except Mg, $p < 0.001$). This is entirely consistent with the view that during this phase of the cell cycle, there is an osmotically assisted mechanism — driven by a transient increase in intracellular [Na] and [Cl] — for expansion of cell volume at a time when one cell gives rise to two daughter cells. For unavoidable logistical reasons, these strict comparisons of cells within a single section were only possible on a small sample. However, similar treatment of the data reported by Spencer *et al.*, (1989) (obtained from different sections) gives essentially similar results.

Finally, these studies have suggested a totally new concept of the pathophysiology of rotavirus-induced diarrhoea. It is not caused by the passive efflux from mucosa to lumen of water due to high levels of unabsorbed lactose arising from lack of lactase (resulting from destruction and shedding of enterocytes). It is more likely to result from an exaggerated homeostatic response necessary to reconstruct damaged, shortened villi. It must be remembered that the gut epithelium is normally a high cell turnover tissue; migration of cells from crypt to villus tip is 3-5 days in mice. The dramatic increase in cell division in the villus base/crypt assembly produces a zone of proliferating cells which transiently accumulate high levels of NaCl which we believe is the origin of the ions secreted into the lumen and with them water. The prolongation of diarrhoea beyond this acute peak phase is due, we suggest (Osborne *et al.*, 1990 *a,b*) to an entirely different mechanism which involves the restoration of a perturbed villus microcirculation and hence is beyond the scope of this paper. We have confined this contribution to successful applications of X-ray microanalysis to biological problems.

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Discussion with Reviewers

G.M.Roomans: The spread in the data presented in Figs 1 and 2 (even in the controls) is considerable. To what factors do you attribute the spread?

B.L.Gupta: Your base-line control values for Na in the villus-tip cells are in the range of 50-300 mM/kg dry wt. Using an average value of 20% dry wt these approximate to 10-60 mM/kg wet wt. Does this large spread represent technical scatter in the data, differences in the sites probed within cells, differences in the functional state of the cells, or ionic gradients within cells?

Authors: These are the data obtained and clearly there must be (as in all such measurements) an element of technical scatter. However, we do not think this is the whole explanation. As cells progress from mid-villus to villus tip they become senescent and eventually shed; it therefore follows that they are likley to be in different states. What is to us most significant is that the data are much less scattered than data from villus bases and tips in infected animals. It is the large differences between and the directional trends of data (as seen in infected animals) to which we ascribe significance.

T.Von Zglinicki: It is suggested that villus base cells secrete salt and water during virus-induced diarrhoea. However, normally the primary event in secretion is the opening of a Cl channel in the luminal membrane. This would exclude accumulation of Cl in secreting cells above electrochemical equilibrium. Do you suggest a different secretory mechanism in virus-induced diarrhoea?

Authors: Your statement is in line with contemporary dogma which we have sought to challenge and replace with a new concept which is fully expounded in Spencer *et al.*,1990. Opening a Cl channel would merely dissipate, over a very short period of time, existing levels of Cl. Our mechanism is based on observations (summarised in this paper) which demonstrate a huge transient increase in [Cl] in a population of cells within villi undergoing a response to infection.