Probing tissue surfaces

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Abstract

This thesis on 'Probing tissue surfaces' describes work done on *in vivo* and *in vitro* models between the 1960s and thesis submission in 2018. Output is by book excerpts, chapters and journal articles. Chapters 1 to 4 are linked through cognate output: Chapters 2, 3 and 4 each contain a review article summarising much of the content. Collaborative links are highlighted, as is the author's changing contribution from entirely 'hands-on' laboratory work to a principal investigator or supervisory role.

Chapter 1 provides a background to the other Chapters, highlighting the use of transmission electron microscopy and a continuing interest in the interpretation of data from sectioned material. Applications include the use of microscopy for quality control and for reporting tissue responses to environmental challenge. Chapter 2 sets out contributions to the development of image interpretation in the then new field of scanning electron microscopy, particularly of soft tissues, with relevance to research and wider applications, including the effects of environmental challenge. Key findings include: early images and reports of intestinal villi, skin or isolated cells; correlative techniques to interpret surface information; optimisation of techniques and descriptions of the surface responses to developmental change or ulcerogenic and other agents.

Chapter 3 deals with the impact of external irradiation, through the exploration by microscopy of its effects on tissue surfaces and deeper structures, mainly alimentary, with relevance to clinical side effects of radiotherapy and to space flight. Key findings include radiation-induced villous collapse differing in extent from changes in proliferative compartments; and variations in responses of structures from all four basic tissue types to different radiation schedules. Chapter 4 addresses questions on microparticle uptake, with relevance mainly to the environmental impact of the Chernobyl incident. Key findings include: most early particle uptake *in vivo in situ* occurring not at Peyer's patches but through villous epithelium, possibly at tight junctions; ethanol and cooling *in vitro* producing different changes to uptake, tight junctions and junctional proteins; and uptake being higher in late pregnancy and early lactation, affected by age not species and increased by irradiation.

Chapter 5 brings together the content of Chapters 1 to 4, summarising their contributions and relevance, both at publication and thereafter. The thesis title reflects the use of probing at different levels, not only during image formation but also by an environmental challenge such as radiation, a pharmacological agent or atmospheric pollution.

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List of accompanying material

This list sets out the submissions in the order of their appearance in the thesis, not necessarily in exact chronological order, but instead chosen to develop the theme of the Chapter. Each submission is therefore identified by three numbers, according to Chapter, then Section, then order within its Section. An individual entry below begins with its number within the Section, then a note of how it appears in the text and then a record of its full reference in the Harvard format used in the thesis. The number in bold (in brackets) at the end of each reference signifies the position of the article in the Presubmission list, which is in chronological order.

The full references, in Harvard style, are also annotated to indicate the status of some of the authors, as follows, **only for an author in first or last position** –

* Student of thesis author, whether as sole or senior supervisor for either graduate or undergraduate student. In one case, the student continued in the group as a post-doctoral assistant: the asterisk sign is used here for all entries for her.

(*) Student co-supervised by the thesis author or supervised by another senior member of the collaborative group, but with the thesis author involved in guiding a microscopy aspect of the work. While Department Head or School Director, I formally appointed an Advisor for each research student: Advisors often became interested in the project and became co-authors, but this is not marked below.

† Technician in group.

Chapter 1 Ultrastructure

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1. Hodges et al, 1995b - Hodges, G.M., Carr, E.A., Hazzard, R.A. & Carr, K.E. 1995b, "Uptake and translocation of microparticles in small intestine - morphology and quantification of particle distribution", *Digestive diseases and sciences*, vol. 40, no. 5, pp. 967-975. **(72)**

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1. McCullough et al, 1995 - McCullough, J.S., Hodges, G.M., Dickson, G.R., Yarwood, A. & Carr, K.E. 1995, "A morphological and microanalytical investigation into the uptake of particulate iron across the gastrointestinal tract of rats", *Journal of submicroscopic cytology and pathology*, vol. 27, no. 1, pp. 119-124. **(74)**

2. Cartwright-Shamoon et al, 1995 - (*)Cartwright-Shamoon, J., Dickson, G.R., Dodge, J. & Carr, K.E. 1995, "Morphological aspects of particle translocation in vivo following ingestion of the yeast Saccharomyces boulardii", *Journal of drug targeting*, vol. 3, no. 1, pp. 61-63. **(71)**

3. Carr et al, 2001 - Carr K.E., Youngman M.J., Barr M., Colhoun L. & Harrison J.D. 2001, “Visualisation of fused aluminoscilicate microparticles taken up by intestinal tissue”, *Journal of anatomy*, vol. 199, pp. 225–226. **(92)**

Chapter 4 - Section 4 *Model 1, developments in methodology*

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2. McMinn et al, 1996 - *McMinn, L.H., Hodges, G.M. & Carr, K.E. 1996, "Gastrointestinal uptake and translocation of microparticles in the streptozotocin-diabetic rat", *Journal of anatomy,* vol. 189, no. 3, pp. 553-559. **(84)**

3. Carr et al, 1996c - Carr, K.E., Hazzard, R.A., Reid, S. & Hodges, G.M. 1996c, "The effect of size on uptake of orally administered latex microparticles in the small intestine and transport to mesenteric lymph nodes", *Pharmaceutical research,* vol. 13, no. 8, pp. 1205-1209. **(76)**

4. Cartwright-Shamoon et al, 1996 - (*)Cartwright-Shamoon, J., Dickson, G.R., Dodge, J. & Carr, K.E. 1996, "Uptake of yeast (Saccharomyces boulardii) in normal and rotavirus treated intestine", *Gut,* vol. 39, no. 2, pp. 204-209. **(78)**

5. Smyth et al, 2005 - *Smyth, S.H., Doyle-McCullough, M., Cox, O.T. & Carr, K.E. 2005, "Effect of reproductive status on uptake of latex microparticles in rat small intestine", *Life sciences,* vol. 77, no. 26, pp. 3287-3305. **(94)**

6. Doyle-McCullough et al, 2007 - *Doyle-McCullough, M., Smyth, S.H., Moyes, S.M. & Carr, K.E. 2007, "Factors influencing intestinal microparticle uptake in vivo", *International journal of pharmaceutics,* vol. 335, no. 1-2, pp. 79-89. **(95)**

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Chapter 4 - Section 5 *What model? Model 2, the standard in vitro model*

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Chapter 4 - Section 6 *Model 2, developments in methodology*

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3. Moyes et al, 2008 - *Moyes, S.M., Killick, E.M., Morris, J.F., Kadhim, M.A., Hill, M.A. & Carr, K.E. 2008, "Changes produced by external radiation in parameters influencing intestinal permeability and microparticle uptake in vitro", *International journal of radiation biology,* vol. 84, no. 6, pp. 467-486. **(97)**

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5. Carr, personal communication, 2018 **(103)**

Chapter 4 - Section 7 *What model? Invited Review on microparticle uptake*

1. Carr et al, 2012 - Carr, K.E., Smyth, S.H., McCullough, M.T., Morris, J.F. & *Moyes, S.M. 2012, "Morphological aspects of interactions between microparticles and mammalian cells: intestinal uptake and onward movement", *Progress in histochemistry and cytochemistry*, vol. 46, no. 4, pp. 185-252. **(102)**

Preface

The thesis is subdivided according to subject matter, all connected by the themes of probing surfaces and responses to environmental challenge, Chapter 1 on ultrastructure, Chapter 2 on emerging scanning electron microscopy, Chapter 3 on integrative responses to irradiation, Chapter 4 on microparticle uptake and Chapter 5 integrating the whole.

With respect to my contributions, in all submissions I was involved in some or all of the following: project initiation; protocol construction; data collection and analysis; and preparation and revision of the published manuscript. My role was, for Chapter 1, as electron microscopist and gut researcher, for Chapter 2 as correlative scanning electron microscopist, for Chapter 3 as morphologist, group leader and grant-holder in radiobiology topics and for Chapter 4 as group leader and grant-holder in a programme on microparticle uptake. All but 2 of the 103 submissions have been fully published, one an Abstract and one a personal communication on data currently being analysed.

With respect to author order, most of the 101 submissions were either sole (6), first (42) or last (33) author, signifying a major contribution to the work. Sole authorship was related to early work or review articles. First author signified: 'hands-on' involvement in data production, in this case image recording, analysis and interpretation; group leadership of a specific project; or prime responsibility for manuscript content and writing, due perhaps to a difficulty in allotting this position to one other author. Being in the last position often implied the first author place was a student or young colleague. The remaining submissions were middle position authorships (20), indicating: the order was chosen in the interests of another; a collaborator from another group was in the first or last position; or the paper arose from involvement in projects centred in another group. Further information on my role and on collaborative input is given in the Acknowledgements section and elsewhere.

In terms of organisation, the thesis is presented in three volumes, the first containing the front matter, linking text describing the work in context and a list of references. The other two volumes contain the accompanying papers and extracts from books, those for Chapters 1 and 2 in Volume 2 and those for Chapters 3 and 4 in Volume 3.

In terms of lay-out, in Volume 1, each Chapter has, at the beginning, an excerpt from the Presubmission Abstract. In Volumes 2 and 3, each Chapter has a 'Contents' page giving a tabular list of the papers, with Chapter number, Section number and the number of the paper in the order in which they are presented.

In terms of the content of the front matter, the list of accompanying material contains the Reference list in standard Harvard format as in the University recommendations, but also has information on the order of appearance of the submissions in the thesis, giving Chapter, Section and paper number. This does not necessarily present the publications chronologically as in the Presubmission papers, but matches the scientific development of the research theme. The chronological order is, however, also given.

With respect to content of Volume 1, the main text aims to place 'the whole work critically into perspective with the general state of knowledge in the field of investigation to which the candidate's researches are related' (Regulation 4). This has been done by putting each submission into context with the scientific knowledge at the time it was written and also with the ongoing literature. That was analysed through its citation history, using Scopus and Google Scholar, consulted in the months before thesis submission: only those citations available in full through Scopus or Google Scholar were used and mentioned in the text. Cross referencing within the text to other papers or parts of the thesis is done by providing information on which Chapter and Section are involved: this is explained in footnotes as the first examples appear. The Reference list at the end of the text is in Harvard format and contains in one alphabetically arranged document the list of accompanying material, the list of supporting references and the list of citations to the submissions.

With respect to Copyright, the thesis relies on the hard copy of approximately one hundred published submissions, on all of which I am an author. It has been assumed that these will not be made available on line, since this would be a large task. Obtaining Copyright permission would also be 'too onerous' (University guidelines) and in many cases impossible because of the passage of time and difficulty of identifying and contacting original authors and publishers or their representatives. In the text, citation of papers by others has relied on my interpretation of their content: direct quotation is obvious from the format and care has been taken to check accuracy. Permission has not been sought for citation of the papers or for quoting them: their use is little different from standard scientific practice in paper writing and some of the authors are long out of the scientific community, making it impossible to treat all in the same way. Apologies are sincerely offered to any authors omitted or misquoted and for any other errors in the referencing or citations.

In terms of binding, the text is bound in a separate volume from the accompanying material to allow for the reading of Volume 1, while the relevant paper is open in Volume 2 or 3.

Acknowledgements

The work would not have been possible without contributions from others, indicated here, in the Preface and 'Context and comment' subsections of the Chapters and, in the submissions, in the authorship order, academic addresses and Acknowledgements.

The research was carried out while on staff at the Universities of Glasgow[1] (1965 – 1967), Strathclyde (1968-1969), Glasgow[2] (1970 – 1985) and Queen's University Belfast (1985 to 1999). There were also attachments to other Institutions for laboratory visits, on sabbatical or after early retirement, as Guest Researcher/Academic Visitor at the Donner/Lawrence Berkeley Laboratory (LBL), California, USA (1982 to 1987), The MRC Radiation and Genome Stability Unit (RAGSU), Harwell (2001 – 2007) and The University of Oxford Department of Anatomy, now the Department of Physiology, Anatomy and Genetics (2001 to current as Academic Visitor in Anatomy). All dates are approximate.

The work was supported by Departmental or central microscope, laboratory, library and computing facilities at all the Institutions: senior staff involved included, among others: Professors George Wyburn, Robert Kenedi, Dame Kay Davies; Gary Love; Dudley Goodhead (Heads of Department, Faculty or Unit). For twelve years in Belfast, I was Head of the Department of Anatomy and then Director of the School of Biomedical Science. The University purchased a state-of-the-art scanning electron microscope, to add to the transmission microscope already there and complemented later by a confocal scanning laser microscope: many of the staff and students there contributed to the work. In research on imaging, excellent technical support is essential and this has been enthusiastically given in all Departments and Institutions, some technicians identified through authorship in the list of accompanying material, as are some research students, mostly doctoral.

Equipment, project grants, or travel support came, either Departmentally or personally, from, among others, the Scottish Home and Health Department, The Cancer Research Campaign, The Medical Research Council, The Imperial Cancer Research Fund, The Royal Society and particularly most recently the Department of Health Radiation Protection Research Programme. The initial research at LBL, when the international collaborations began, was additionally supported at first by the Lister Centenary Travelling Fellowship from the University of Glasgow and then by the Wellcome Trust.

There have been many collaborating scientists, both internal and external. These include senior colleagues who gave ideas and support in the beginning, academic and technical members of staff in the Department/School in Belfast and others, both staff and students,

who have been involved through the years in Glasgow, Strathclyde, Belfast, Harwell and Oxford. Many colleagues have contributed, but there is only space here to mention a few, such as those participating while the research was taking a new direction. These included, within the home institutions, Frances Boyle, Glenn Dickson, Roy Hamlet, Stephen McCullough, John Morris and Tony Nias: some of them moved on to other Institutions and continued to collaborate from there. Externally, they included John Ainsworth (LBL, then Armed Forces Radiobiology Research Institute, AFRRI), Tom Hayes (LBL), Gisele Hodges (Imperial Cancer Research Fund, ICRF, then Belfast), Sue Hume (Hammersmith Hospital, London), Tom Seed (Argonne National Laboratory, Illinois) and Udo Schumacher (University Medical Centre, Hamburg Eppendorf). There have been major contributions from students and research assistants. With them, as for colleagues, space limitations here required selection of some from this group. Concentrating on those whose authorships indicated continuing contributions to gut-centred papers, these included Babar Abbas, Patrick Brennan, Eúnan Carr (EC), Jalal Cartwright-Shamoon, Melissa Doyle-McCullough, Raj Ettarh, Rosemary Hazzard, Mallika Indran, Hassan Kamel, Sheila Nunn, Siobhan Moyes and Sharon Smyth. The collaborators have included the author's brother, Ian Carr, and husband, Peter Toner, but the former student EC is not a relative.

With respect to the writing of the thesis, the declaration makes clear that this was entirely my work: it is therefore considerably the poorer for being without the contributions and editing that come from others during the preparation of articles, book chapters or books. However, at the beginning of the project, the following three colleagues at Oxford read and commented on the first draft of the Presubmission Abstract: Maike Glitsch, Deborah Goberdhan and John Morris. At the end of the project, my husband Peter Toner assisted with the checking of the final reference list. Additionally, I gained substantially with respect to thesis construction from reference to DSc volumes in the Glasgow University Library.

To all these Institutions, funding bodies, individuals and colleagues or students, I am most grateful. In addition, since the work has extended over many years, being postponed for various academic reasons, I owe a debt of gratitude to family and to friends: some of these became scientific collaborators and in other cases collaborators became friends. Finally and in memoriam, two of the collaborating scientists mentioned above are now dead, Frances Boyle and Gisele Hodges: the writing of the thesis has been a reminder of their contributions and of the loss their passing has been to science and to friendship.

To them all, whether named collectively or individually, many thanks.

Author's declaration

The thesis 'Probing tissue surfaces' submitted to The University of Glasgow in fulfilment of the requirements of the degree of Doctor of Science in Medicine has been written entirely by myself.

None of the material included in the thesis has been submitted by me for any other Higher degree at any Institution.

Katharine E Carr

Abbreviations

Key techniques

Abbreviation	*Term in full*
BEI	Backscattered electron image/imaging
EM	Electron micrograph/microscope/microscopy
LM	Light micrograph/microscope/microscopy
SEM	Scanning electron micrograph/microscope/microscopy
TEM	Transmission electron micrograph/microscope/microscopy
XRMA	X-Ray microanalysis

Institutions, departments and grant bodies

Abbreviation	*Term in full*
AEA	Atomic Energy Authority, AEA Technology
AFRRI	Armed Forces Radiobiology Research Institute
CRC	Cancer Research Campaign
ICRF	Imperial Cancer Research Fund
IRSN	Institut de Radioprotection et de Sûreté Nucléaire
LBL	Lawrence Berkeley Laboratory
MIT	Massachusetts Institute of Technology
NRPB	National Radiological Protection Board
RAGSU	Radiation and Genome Stability Unit

Other abbreviations and definitions

Abbreviation	*Term in full/definition*
D-T	Deuterium-tritium
E cadherin	Epithelial calcium-dependent adhesion
EAP	Epithelial/epithelium associated particle

Abbreviation	*Term in full/definition*
FAP	Fused aluminosilicate particle
GALT	Gut-associated lymphoid tissue
Gy	Gray, derived SI unit of ionising radiation dose
IORT	Intra-operative radiotherapy
IS	Injury score, see RIS
LET	Linear energy transfer
M cells	Microfold cells
MALT	Mucosa-associated lymphoid tissue
MCJ	Multicellular junction
Microparticle	Particle with diameter measurable in micrometres
MLN	Mesenteric lymph node
MoI	Morphological Index
MODS	Multiple organ dysfunction syndrome
n	Sample number
Rad	Unit of absorbed radiation dose
RBE	Relative biological effectiveness, ratio of effectiveness of one type of ionising radiation relative to another
RIS	Radiation injury score
SCID	Severe compromised immunodeficiency
SD	Standard deviation
SMP	Submembranous particle
TNFα	Tumour necrosis factor alpha
UD	Ultrastructural Deviation, information on subcellular changes from controls
VIP	Vasoactive intestinal peptide/polypeptide
ZO-1	Zonula occludens 1

Chapter 1 Ultrastructure

Preface - extract from Presubmission Abstract (Chapter 1)

Since the work on scanning electron microscopy was facilitated by experience in the interpretation of light and transmission electron microscope images, some papers on these are presented in Chapter 1.

1.1 Fore-runner to surface studies and links to Chapters 2 and 3

The interpretation in the 1960s of the then 'new' images produced by scanning electron microscopy (SEM) and described in Chapter 2 was greatly assisted by a background in histology and in the images produced by transmission electron microscopy (TEM). Papers on tissue ultrastructure have therefore been included in this Chapter, to show the usefulness of images of sections in biological research, beginning with early papers but also including selected articles published through the years, not entirely in chronological order.

The early papers, centred in Anatomy in Glasgow University, applied transmission electron microscopy to several projects carried out in collaboration with other University Departments, beginning an ongoing interest in gastrointestinal questions, but also covering other topics. The papers contributed to the literature in various ways, including technical aspects, subcellular detail and information on environmental effects on human populations.

The first paper ([1]Carr et al, 1967), written in collaboration with the Department of Microbiology, showed that TEM was necessary for the identification of the damage produced by dispersion of isolated liver cells for biochemical tests, adding to the information obtained by phase contrast light microscopy (Jacob and Bhargava, 1962). This application of TEM for quality control has remained useful, providing a baseline of morphological integrity. Of the available citations in the Scopus listings, one commented that it had used electron microscopy to demonstrate cellular damage (Walter et al, 1973) and a paper on nuclear parameters quoted it in a Table (Suciu, 1985).

Two other early papers (Carr, 1967, Carr and Whur, 1968) applied previous experience in high resolution TEM imaging, gained during earlier studies of non-biological material

[1] The submitted papers are described in the order given in the List of Accompanying Material. When each appears for the first time in this way, cross reference information is not given.

(Carr, 1970a, b), to the study of the substructure of inclusions in 'granules' of rodent globule leucocytes, cells known to be associated with parasitic infestation. This project was already a topic of interest in The University of Glasgow's Anatomy Department (Toner, 1965, Whur and Johnston, 1967). In Carr (1967) the cells were found in mouse intestine, where the inclusions were crystalline. With respect to citations, [2]this paper and that by Carr and Whur (1968) was quoted by Vogel et al (2018) as one of the references relating to the presence of globule leucocytes in various organs and species. It was also cited as considering globule leucocytes to be derived from plasma cells, while other groups opted for mesenchymal or lymphocytic derivation (Heine und Schaeg, 1977). It was still being quoted more recently in the discussion of the development of ideas on the likely source of this cell type (Gorgani-Firouzjaee et al, 2015). The 'crystallite' appearance of the granules was also described as being 'of interest', possibly related to globulin (Hanker et al, 1977). Further ultrastructural evidence, in collaboration with the Veterinary School, (Carr and Whur, 1968) showed that the inclusions were different in rats, being fibrous rather than crystalline. In addition to the citation by Vogel et al (2018), mentioned above, Heine und Schaeg (1977) also quoted it with respect to the linking of increased numbers of globule leucocytes and parasitic infestation with anaphylactic responses. Both these papers on small intestinal mucosa would have benefited from scanning electron microscopy to record the presence of any surface parasites.

A fourth early paper illustrated the advantages of integrating ultrastructural studies with the TEM into established projects elsewhere in the University, in this case the Departments of Materia Medica and Medical Cardiology (Moore et al, 1975). The project related to the simultaneous collection of data on morphological and biochemical effects in rat cardiac tissues after exposure to the levels of lead then found in the soft drinking water in Glasgow: this was linked to earlier findings that there was a connection between cardiovascular problems and water hardness (Stitt et al, 1973). The parameters used were the ultrastructure of cardiac papillary muscle and the levels of the enzymes ferrochelatase and δ-aminolaevulinate dehydratase. The effects were greatest after treatment for 6 months. This paper was cited: as describing lead as damaging heart muscle (Harary and Masamitsu, 1978); for the time of maximum tissue lead levels (Quarterman and Morrison, 1978); and

[2] Cross reference information is not usually included when a paper is being discussed with respect to its citation history.

with reference to the risk that populations could be exposed to metals corroded from the water distribution system (Alam and Sadiq, 1989).

1.2 Ongoing studies of biological sections

Some more recent publications from the period 1989 to 1998 in Belfast have also been included in this Chapter, to show the continuation of the interest in the use of microscopy to study tissue sections, concentrating on intestine. The papers have been presented in two sets of pairs.

The first pair has been chosen to highlight the importance of plane of section, both stemming from initiatives within the group. The first paper, Ettarh and Carr (1996a), used standard preparations, producing longitudinal sections of the small intestinal villi, to complement the literature on the gastric effects of indomethacin (Robert, 1979). In this ulcerogenic two-dose study, full circumference resin histology sections of mouse duodenum, jejunum and ileum were used to investigate villi and crypts aligned in longitudinal section in non-ulcerated areas. Parameters measured included epithelial and crypt dimensions, cryptal mitotic activity and counts for a range of cryptal and villous cells, as used further in [3]Chapter 3 (Sections 3.8, 3.9). The treated intestine showed some cryptal changes and increased mitotic activity. The citations for this paper were technical as well as data-based, including adaptation of its indomethacin protocol (Venkatesh et al, 2014), mention of its reporting of the side effects (Unmack et al, 2001) and of its findings on Paneth cells (Legraverend and Jay, 2013)

This approach contrasted with one where control data were collected for small intestinal villi cut in transverse section and not longitudinally as above, thereby allowing good analysis of axial structures that varied in the apico-basal direction (Abbas et al, 1989), as seen for functional parameters (McElligott et al, 1975, Pothier and Hugon, 1980). This provided baseline images for comparison with resin casting (Chapter 2, Section 2.4) and control information for studying responses to environmental challenge such as radiation (Chapter 3, Section 3.6). The images were from serial sections, alternating study of semi-thin sections for ‘resin histology’ light microscopy (LM) with examination of ultrathin sections in a TEM. The data collected were from mouse jejunum for apical, middle and basal thirds for a range of parameters from villous cross-sectional area to microvillous

[3] Cross references to other Chapters include information on Chapter and Section.

length. There were several marked changes from base to apex, such as enterocyte widening and microvillous lengthening. Two citations from T. Mayhew's group for this paper included it as one of the references quoted for cellular changes as cells move from the crypt to villous tip (Zoubi et al, 1995a and b).

The next pair of papers illustrated continuing morphological contributions to other groups studying aspects of intestinal diet, including variations in fat or fibre components. In the dietary fat study (Sagher et al, 1991), measurements were made of rat jejunal and ileal villous height and crypt depth in standard wax histology sections. These were compared with the levels of regulatory peptides, to add to the literature on the effects of other interventions, such as starvation (Hopper et al, 1968), and feeding (McManus and Isselbacher, 1970). Maize or olive oil increased the ratios of villous height/crypt depth and also altered the tissue concentrations of somatostatin, neurotensin or substance P. The outcome varied with intestinal site, a topic returned to later (Chapter 3, Section 3.9). The citations for this paper included comments that it had linked relative saturation of fat with villous morphology (Tamura and Suzuki, 1997, Moussavi et al, 2008) and commented on the importance of the ratio of villous length/crypt depth (Peuranen et al, 2004). The second paper in this pair (McCullough, J.S. et al, 1998) took further the interest in the effect of dietary manipulation on intestinal function, this time with a different collaborative group, including R. Goodlad as one of the authors. Exposure to fibre supplementation was one of the investigative tools, another being variation in intestinal microflora in germ-free as well as conventional rats. The paper used resin histology for the study of rat small and large intestine: measurements of several parameters were made, as set out in reports of post-irradiation effects (Chapter 3, Section 3.8): data were also collected on crypt branching (Levi et al, 1992), with discussion of how this related to crypt fission. The presence of fibre increased crypt number and branching and also affected goblet cells. Citations for this paper included references to technical aspects, with the advantages pointed out of being able to carry out additional analyses on fixed tissues stored for later use (Goodlad, 2017). Citations based on data reporting included reference to various aspects of gut biology: examples were Montagne et al (2003), Olivera et al (2003), Fastinger et al (2008) and Brownleee (2011).

1.3 Context and comment

The theme of this introductory Chapter was centred on the interpretation of information from sectioned material, paving the way for the addition of detailed study of surfaces. The work submitted in this Chapter took place initially in the University of Glasgow and then in

Belfast. It covered thirty of the fifty years, the dates of the submissions running from 1967 to 1998.

Output destination was on the whole similar for the two subsets of articles, a mixture of anatomical journals, such as the Journal of Anatomy, and clinical journal or specialist journals, such as Gut.

Collaboration was also important. The addresses of the publications charted its continuing significance, demonstrating contributions to research in University clinical departments and also to output with gastrointestinal and imaging groups elsewhere. Departments or Laboratories involved included: in Glasgow University, Materia Medica, Medical Cardiology, Microbiology, Veterinary Histology and Embryology and also Pathology; in Queen's University Belfast, Child Health, Dental Surgery and Medicine; and elsewhere, Lawrence Berkeley Laboratory (LBL), California, USA, Imperial Cancer Research Fund (ICRF), London, Robert Gordon University, Aberdeen and University College, Dublin, the last-named being the next address of a student after graduation.

The thesis author's roles and contributions were seen from the order of the names on the two sets of four articles. The first four papers in this Chapter, published in the period 1967 to 1975, were the earliest output, taking projects on from the work of colleagues or joining collaborative projects already established by groups in other specialities. These papers were produced as a 'hands-on' electron microscopist, based in the Department of Anatomy of the University of Glasgow, contributing to the maintenance of standards in the TEM Unit there and to the use of TEM images to update collections of illustrations for the study of cells and tissues. The second group of papers showed that the research continued, in the period 1989 to 1998, to produce data from sectioned material. This illustrated the change of roles to supervisor and leader of a group involved in gastrointestinal research and therefore benefiting from collaborations with scientists working in cognate fields.

Links to the next Chapter were clear from the continuing interest in the use of morphology to explore soft tissue structures, especially those of the alimentary system. The title of the thesis, 'Probing tissue surfaces' was selected to reflect the content of the four main Chapters and has been discussed in Chapter 5. Chapter 1's concentration on the study of sectioned material has provided a baseline for the later Chapters.

Chapter 2 Scanning electron microscopy emerging as a biological probe

Preface - extract from Presubmission Abstract (Chapter 2)

Early papers on scanning electron microscopy covered small intestinal villous surfaces, among others, and early reviews described the technique and its applications. Methodological developments included: correlation of surface and sectioned images having investigative and clinical relevance; histochemical or immuno- 'staining'; low temperature X-ray microanalysis, surface cleaning, 360º detail and microvascular casting. Papers on scanning electron microscopy of the alimentary system often used it as one of several tools, as did some reviews and chapters that included such images, referred to as 'topographical histology'.

2.1 Early scanning electron microscopy and links to Chapter 1

The work presented here, not necessarily in chronological order, was carried out in the early days of the application of scanning electron microscopy (SEM) to biomedical sciences.

The instrument used initially, producing a publication in 1968, was a Cambridge Scientific Instrument's 'Stereoscan' in the BioEngineering Unit at the University of Strathclyde, sited at Canniesburn Hospital. Other groups were publishing in related fields, including Sikorski (1960), Jaques et al (1965), Hayes et al (1966), Clarke and Salisbury (1967), Boyde and Lester (1968) and Echlin (1968). However, the SEMs in Glasgow, in the Universities of Strathclyde and then Glasgow, were early in concentrating on soft tissues. On the establishment of the first instrument, advice was sought on best practice through visits to the Sikorski and Boyde laboratories by the author and a technician from the group respectively.

The early interest in the ultrastructure of cells of the small intestine seen in Chapter 1 (Carr, 1967, Carr and Whur, 1968) was extended to take advantage of the new SEM approach by applying it to the study of villous surfaces. Although there had been earlier illustrations of the intestinal surface (Jaques et al, 1965), the first paper in this Chapter (Carr and Toner, 1968) was one of the earliest publications concentrating on it, in a letter to Lancet. This demonstrated the use of SEM to give more detail of 3 dimensional structure and epithelial surface detail than had previously been possible with dissecting light microscopy, often in

the study of specimens of clinical interest (Rubin et al, 1960). Technical aspects of interest included dehydration by a form of air drying using a vacuum desiccator and coating the specimen surface at 45º with carbon-platinum, an adaptation of the 'shadowing' of surface replicas prior to study in a transmission electron microscopy (TEM). The letter commented on the clear imaging of villous shape and cell surfaces and on the possibility that greater detail could be made available during the study of human biopsies. This paper was followed six weeks later by a another letter to Lancet from a different group, using an alternative preparation technique, extending the detail seen to microvilli by vigorous syringing of the mucosal surface and the use of a higher microscope kilovoltage (Swift and Marsh, 1968): this second letter, however, confirmed the general morphology and possible relevance to clinical studies. Although short, the paper by Carr and Toner (1968) was cited by other early publications in key journals (Gastroenterology, Journal of Ultrastructure Research) as one of the sources for images of the surface structure of the small intestine (Balcerzak et al, 1970, Takeuchi and Zeller, 1972, respectively). It was also quoted over a substantial period, for example for the description of villous shape (Sherman et al, 1987).

The Lancet letter was followed quickly by collaborations with colleagues already working with other techniques on alimentary topics that could be explored further by detailed examination of surfaces using the 'new' technique of SEM. One example (Ferguson et al, 1969) was provision of a clear visual record of the three dimensional structure of the connective tissue cores of autolysed villous surfaces from human newborns, confirming the overall appearance seen earlier (Loehry and Creamer, 1966). This illustrated well the development of villi from finger-like to a mixture at birth of finger, tongue and leaf shapes. There were no available citations of interest to the SEM aspects of this paper. Another use of the technique was its application to experimental situations at natural surfaces, such as secretagogue-induced duodenal ulceration (Carr et al, 1979a). Here the SEM was used for the kind of investigation previously carried out by stereomicroscopy (Giampaolo et al, 1978), charting the details of healing from one to ten days after 24 hours exposure to pentagastrin and carbachol. Technical features of note included the use of: large samples, making full use of the SEM's capacious specimen stage, situated below all the de-magnifying lenses and therefore lacking the size constraints of the TEM; and montage techniques to record information about the surface of these samples, combining survey and detail in the final composite images. Eleven years on from the initial descriptions of villous surfaces in 1968, critical point drying and gold sputter coating were by then available as standard preparative techniques. SEM showed: variation in the extent of healing; sequential

mucosal damage and villous abnormalities at the ulcer margin; the honeycomb surface appearance of the immature re-epithelialised surface; and irregular villi. Some signs of gastric metaplasia were also seen. This paper also had no citations relevant to the use of SEM.

The next four papers illustrated the use of the SEM for a range of other applications. The first paper (Carr, 1970c), published two years after the first SEM paper mentioned above and using similar early preparative techniques, explored the three dimensional structure of human skin. This built on work already done on rodent wound healing by Forrester et al (1969), where the authors included T. Hayes, a future collaborator (Chapters 1, 2, 3). Rather than studying naturally occurring surfaces, as in small intestine, this study described the exposed surfaces of dewaxed sections 25 to 35µm thick. This revealed the three dimensional arrangement of dermal connective tissue fibres: faint cross-banding identified the larger collagen bundles, with elastin as the thinner coiled structures. The advantage of having previous knowledge of how such structures appeared when studied by light microscopy and TEM gave rise to an interest in correlative approaches, dealt with later ([4]Section 2.3). The citation record included reference to the paper in the description of skin (Wei et al, 1989), of dermis as a whole, with its collagen fibres (Tsuji et al, 1979) or of bundles of these fibres with intervening fibrils (Li, 2015): the continuing relevance of the paper was illustrated by the fact that the citations ran to 2015.

The paper by Carr and Carr (1970) also moved away from the study of natural, easily accessed surfaces, this time by examining isolated mouse peritoneal macrophages on a substrate such as glass, following up a suggestion that stimulation could alter their speed of movement (Dannenberg et al, 1963). This article was, like the skin paper, published only two years after the Lancet letter and was still using unsophisticated techniques for drying the samples, leading to loss of surface detail in the images. The stages of settling on glass, were, however, reported, as was the quicker full extension of cells from mice exposed to peritoneal stimulation. Citations included references to cells spreading or their cellular surface features (Witkowski and Brighton, 1971, Mantovani et al, 1972, Rajaraman et al, 1974). The most interesting citations were to the use of the observations as a pointer to the Kupffer cells of the liver being of the macrophage line, in reports from Niigata, Japan and Rome, Italy respectively, (Muto, 1975, Motta, 1975).

[4] Cross references within a Chapter only include the Section number.

The next two papers (Cook et al, 1979, Al-Jaff et al, 1982) moved on the time-frame of the work by ten years and illustrated further the way in which early scanning electron microscopists contributed to collaborative networks, in this case between the Departments of Agricultural Chemistry and Anatomy at the University of Glasgow, where an instrument had by now been installed. The research produced two publications on the effects of the herbicide aminotriazole on bracken: its ability to thrive on open terrain had been linked to a decrease in the number of stomata (Bright, 1928). Both papers have been included here, to illustrate the continuing use of SEM across such a collaborative grouping before there was a more widespread availability of such instruments. In Cook et al (1979), bracken fronds growing in three different shade conditions were studied by light microscopy of plastic sections as well as by SEM of upper and lower surfaces. Herbicide uptake was also assessed in the laboratory. Shaded fronds took up less herbicide and their lower surfaces had different patterns of distribution of hairs and stomata. Further work (Al-Jaff et al, 1982) extended the range of growing sites to four, at each comparing shaded and exposed bracken. In addition to the differences observed earlier, the shaded frond surfaces were also more corrugated, restricting herbicide spreading. Citations included references to environmental conditions and other factors affecting penetration (McPhail and Duncan, 1981, Alonso-Amelot and Rodulfo-Baechler, 1996, Chachalis et al, 2001).

Several of these early papers showed the importance of correlating SEM results with other data collected in parallel: this is expanded on below (Section 2.3).

2.2. Early reviews of biological scanning electron microscopy

Reviews published between 1971 and 1974 put the work in context with other early SEM reports (Section 2.1). The first (Carr, 1971), published in a mainstream biological review series, began with correlative images of biological surfaces, using light and transmission microscopy of sectioned material: surfaces were illustrated by dissecting microscopy, SEM and TEM of both replicas and freeze-dried samples. Thereafter, the review covered image formation and instruments; preservation and preparation of specimens; stereopairs and different scanning modes; and images from a range of biological specialties, some from other microscopists, showing the connections developing world-wide. The conclusion that this versatile instrument would have a useful future in biological research has been borne out by events. With respect to citations, there were several mentions of this review as one of a small number describing the emergence of SEM as a biological tool (Taylor and Anderson, 1972, Sanders and Singal, 1973). An important citation came in a Special Invited

Review (Hayes, 1974), published in the Journal of Microscopy by T. Hayes, himself one of the early leaders in the SEM field (Section 2.1, above), working at the University of California, Berkeley and a future collaborator. He introduced the subject by noting the developments at Cambridge (Oatley et al, 1965) and describing the SEM as useful in physical (Thornton, 1968) and biological sciences (Carr, 1971). Another group (Martin et al, 1975) advised that interpretation of SEM images would be helped by understanding how they were formed, citing two authors specialising in instrumentation (Thornton, 1965 and Crewe, 1971) and also the Carr review (1971). This indicated that the review was seen as a source for technique and instrumentation as well as biological applications.

In the same year, SEM images of intestinal surfaces also contributed, with text, to an ultrastructural atlas and review of the digestive system (Toner et al, 1971), including comments on the possible variations of human villous shape from finger-shaped through leaf to ridges and convoluted structures. Non-standard images were included, such as intervillous crypt openings, villous creases, sites of enterocyte junctional complexes, the surface pockmarks of goblet cells and the autolysed villous connective tissue framework. The mucosa of coeliac disease was also illustrated, showing the lack of villi and the raised mucosal rings around some crypt mouths. Scopus did not list this book, but it was Google Scholar's top listing, with 135 citations, ranging from 1974 to 2015 (see also Chapter 5, Section 5.2.2).

The last of the three early reviews, (Carr et al, 1974) concentrated entirely on SEM surface images, briefly describing not only small intestine, but also the flat mucosal surfaces of oesophagus, the gastric pits of the stomach and the opening of colonic crypts. There was a mention of intestinal surfaces in coeliac disease and the effects of irradiation, dealt with again in Chapter 3. The only citation of interest was in a paper on oesophageal surface morphology at the junction of squamous and Barrett's epithelium (Shields et al, 1993), where the cells were said to resemble some features reported in the literature, including those in this short review.

2.3 Correlation of SEM images with those from established techniques

It became important to identify surface features more accurately, to 'validate' SEM image interpretation (Brummer et al, 1975, Carter, 1980). Correlation began by simply using different microscopy techniques on other, similar specimens, but progressed to post-embedding screened SEM samples for light microscopy resin histology and for TEM. This

technique has been illustrated here through the study of one intestinal villus in these three ways (Carr et al, 1981a). A swollen 'bleb' on an irradiated villous surface was confirmed not to be an artefactual deposit of the glue used to affix the sample to the metal stub: it was instead a multinucleate giant epithelial cell (Chapter 3, Sections 3.1, 3.6). Post-SEM embedding was also used to illustrate the surface and internal features of large bowel biopsies (Carr et al, 1981b). Orientation identifiers were used and plastic sections depolymerised to permit the use of stains normally restricted to wax histology sections, including azure blue, haematoxylin and eosin, periodic acid-Schiff (PAS) or Mallory (Laschi and Govoni, 1978). The often-used secondary fixation with osmium tetroxide was avoided, since it interfered with these staining procedures. It was concluded that in a few cases, here Hirschsprung's disease and post-irradiation proctitis, the preliminary SEM screening provided unexpected information that might be useful. There were no available citations of interest to the correlative technique used in either of these papers.

Another twist to the correlative studies involved light microscopy as the first technique, with SEM thereafter. Two different approaches were developed. The first was used for cells from body cavity fluids (Saleh et al, 1982), identified in light microscopy with localising micro-coverslips. These then acted as finder grids to produce SEM images with greater surface detail, giving selected areas of a more useful size than previously (Domagala et al, 1979). The few citations were technical and from papers specialising in microscopy or optics (Wouters, 1987, Sandoz and Jacquot, 2011), an appropriate readership. The second approach (Carr et al, 1984a) also changed the order of the techniques used from that of post-scanning light microscopy (Fenger and Knoth, 1981). Features of interest were identified by light microscopy in wax histology sections from gastrointestinal Pathology files: the whole block was then dewaxed for SEM surface examination. While the quality of the surface image was good, no case was made for the addition of SEM to the routine diagnostic procedures. One further feature was the paper's publication in Scanning Electron Microscopy after oral presentation at the relevant annual international meeting in the USA. As in the standard journal reviewing procedure, changes to text or figures were requested before publication: an example in this paper was the addition of images from SEM backscattered (primary) or transmission detectors. An additional aspect, however, of this Journal's publication method was the inclusion at the end of each paper of the authors' responses to points raised by the reviewers, whose names were usually given, such as, in this case, Shirley Siew, an authority in the field of gastroenterology. Questions covered possible techniques for mucus removal, details of preparative techniques for SEM, non-

standard detectors in the SEM and the use of the terms ‘investigative’ or ‘diagnostic’ for the application to pathology of SEM or other tools. This paper was cited as part of an interesting discussion of techniques, where Roessler et al (1991) included it in a list of papers using what they called ‘intermicroscopic correlation’, defined as ‘the sequential examination of a specimen by different microscopic techniques’.

2.4 Development of scanning electron microscopy techniques

Following on from the paper above, where an image was shown from a backscattered (primary) electron detector ([5]Carr et al, 1984a, Section 2.3) and from a report of the use of silver nitrate to produce contrast in SEM images (Geissinger, 1972), a paper by Carr and McGadey (1974) adapted enzyme histochemistry techniques. This produced localised precipitates of substances that gave a high SEM signal, in this case of lead in sections of pancreas, epididymis and cerebellum. The results marked the development of SEM towards the asking of more precise morphological questions. There were two citations of interest, one from P. Nakane, well known for his work on electron microscope (EM) immunocytochemistry, quoting the paper as a source for the use of enzymes as smaller markers (Nakane and Hartman, 1980). A second citation, from a paper on atomic number contrast, quoted it as having used variable accelerating voltages and primary or secondary electron signals (Bowen et al, 1983). Another labelling protocol (Hodges et al, 1985) used surface conconavalin-A and gold on control and irradiated mouse bladder: this was done in collaboration with G. Hodges, then at Imperial Cancer Research Fund (ICRF) laboratories in London and involved in the development of biomedical SEM and cell surface markers (Hodges et al, 1982). The gold/con-A labelling in control samples was present at the regions of the aysymmetric unit cell membrane then known as interplaque and later as hinge (Liang et al, 1999), as opposed to the intervening plaque sites. Data on changes in the epithelium and binding patterns after irradiation have been included in Chapter 3 (Section 3.3). The few available citations to the immunolabelling paper made no particular reference to the technique of SEM labelling: references to the effects of irradiation have been given in Chapter 3 (Section 3.3).

The next group of five papers reported on the production of chemical information from X-ray microanalysis (XRMA) of gastrointestinal samples prepared by freeze-hydration: this

[5] Cross reference to specific papers in the accompanying material give the reference first, then Chapter and/or Section information.

protected them from chemical exposure and left tissue fluid *in situ* (Echlin, 1978). It also minimally disrupted the relationship between luminal contents and the apical surfaces of villous epithelium, a theme addressed again later in this Section through reports on specimen cleaning and surface exudate (this Section, below and Section 2.5 respectively). The five papers came from work done during a series of laboratory visits to T. Hayes at the Donner and Lawrence Berkeley Laboratories, this being one of only a small number of groups world-wide with a low temperature X-ray SEM microanalysis capability. This collaboration stemmed from earlier citation of each other's work, such as to the review above (Carr, 1971, Section 2.2) and from meeting at the annual SEM conferences. It led to the five publications between 1983 and 1987 on low temperature SEM, described here, and to another on related material (Abbas et al, 1989, Chapter 1, Section 1.2). It also produced other papers between 1987 and 1994, on the effects on small intestine of radiation, including Berkeley heavy ion beams, (Chapter 3, Sections 3.4, 3.6, 3.8).

The first of these five papers, Carr et al (1983a) described specimen preparation in high vacuum and at low temperatures in an AMray Biochamber (Pawley and Norton, 1978), followed by screening in an AMray SEM with a cold stage. Tissue compartments, cell types and some internal detail were identifiable. Specimens were subsequently freeze-dried in the microscope and then prepared for routine surface SEM, resin histology and TEM. The second and third papers (Carr et al, 1983b, Carr et al, 1984b) compared frozen-hydrated etched images with those from plasma etching of resin sections (Humphreys and Henk, 1979), using either secondary or backscatter (primary) mode and allowing a range of magnifications from survey work to imaging of organelles such as mitochondria. The third paper (Carr et al, 1984b) also contained pilot X-ray microanalysis data of chlorine levels in stomach contents and adjacent surface cells and had input from Glasgow University Departments of Medicine and Chemistry and from Hitachi Limited, for high resolution field emission SEM images. The fourth and fifth papers (Carr et al, 1986a, Carr et al, 1987a), had contributions from a scientist with an interest in the handling of large data sets, from the Glasgow University Department of Physics: these papers described the X-ray microanalysis procedures (Fuchs and Fuchs, 1980, Echlin et al, 1981) and also the resulting data. The levels of sodium, sulphur, chlorine and calcium increased from cell apex to central luminal contents, while the differences in sulphur in particular were greater between distal (upper) and proximal (lower) villous regions than between apical, middle and basal parts of a cell. The first paper (Carr et al, 1983a) was the only one of the five with available citations in the field: it was quoted as a source for low temperature SEM in biology (Inoué and Koike,

1989), during a comparison of techniques (Jongebloed et al, 1999) and in the study of mucus (Lumsden et al, 1994).

The next paper, Dickson et al (1989) also reported on the interface between the gastrointestinal mucosal interface and the luminal contents, concentrating on removal of exudate and debris (Goldstein et al, 1981). This allowed comparison of rat stomach mucosal surfaces before and after cleaning. The low citation rate was linked to the fact that most groups removed the exudate before SEM examination. The subsequent 'multiview' paper, also relating to mucosal surfaces, used the boundary with the stomach as a reference line and recorded the four main 'faces' of villi (Carr et al, 1989), taking further the concept of stereo-pair imaging (Wells, 1960, Boyde, 1974). Different types of surface creases were described, showing that, in control samples, they were mostly short and ran around the villous diameter. The lack of citations here was probably because stereopairs were less used than they should have been and most groups were therefore unlikely to use a 360º analysis.

The final paper in this Section, Abbas et al (1990a) changed the focus from the mucosal/luminal interface to the internal vascular trees, exposed by microvascular casting (Murakami, 1971): this involved perfusion of resin monomer and then digestion of non-vascular tissues from the polymerised cast. The control villous arteriolar supply divided apically into two to form an inverted U-shape from which came a capillary plexus and then venules, running to the submucosal veins: these observations were used as a baseline for the changes produced by irradiation (Chapter 3, Section 3.6). This paper was better cited (from 1998 to 2012) than the other papers on technical developments in SEM. Jahnson et al (1998) commented on the 'excellent perfusion quality' and Burrell et al, (2012) cited it as one of their sources for the microcasting technique.

2.5 Scanning around the alimentary system

An early article, (Carr et al, 1974, Section 2.2) had briefly reviewed the information that SEM could provide on alimentary tract mucosal surfaces. More detail, almost all on mucosal surfaces, has been included in this Section, often including correlative information from other techniques.

The first paper (Nunn et al, 1990) analysed the SEM appearance of luminal 'exudate', giving different information from that provided by low temperature analysis (Carr et al, 1983a, 1986a, 1987a, Section 2.4). There had already been some reports on exudate in the upper

tract (Siew and Goldstein, 1981), stomach ([6]Carr et al, 1974) and children's small intestine (Poley, 1983). The paper by Nunn et al (1990) scored mucus, micro-organisms/bacteria and cells/debris in rabbit exudate on uncleaned surfaces. It reported that there was more variation down the tract than across animals. All three components were found on the stomach surface, which also had the greatest amount. Features elsewhere included debris at the oesophageal/cardiac junction and caecum, but micro-organisms at upper oesophagus and transverse colon. Although the paper was not highly cited, no doubt, as for Dickson et al (1989), because exudate was usually removed, a review of mucus during Helicobacter pylori infection reproduced one of its stomach images (Bansil et al, 2013).

The next group of three papers described the microridges of the epithelium of the upper part of the tract (Laferla et al, 1988, Shasha'a et al, 1993, Johnston et al, 1996). This stratified squamous non-keratinising epithelium had already been described (Ackerman et al, 1976, Siew and Goldstein, 1981), but there was not a large literature on microridges. The first paper (Laferla et al, 1988) used the surface characteristics of human oesophagus to categorise it as either a form of squamous epithelium, with typical or atypical microridge patterns, or alternatively as non-squamous, more like gastric mucosa. The squamous and non-squamous groups were from patients respectively without or with oesophageal symptoms. The second paper on microridges (Shasha'a et al, 1993) compared the surface features of human lower oesophageal biopsies with those from rabbits of different ages. Surfaces were cleaned as above if necessary (Dickson et al, 1989, Section 2.4), stereopairs (Boyde, 1974) confirmed that microridges were indeed raised above the cell surface and the percentage taken up by microridges was calculated. During development, surfaces of rabbit oesophagus had an increasing percentage area of microridges. All oesophageal regions of older rabbits resembled the typical squamous group of human biopsies. Points raised by reviewers (G. M. Roomans, W.H. Wilborn), included the distinction between microvilli and microridges, rabbit oesophagus as a model and possible identification of gland openings. The third paper on microridges (Johnston et al, 1996) looked for a link between them and the features of gastro-oesophageal reflux disease (GORD, Branicki et al, 1984, Johnsson et al, 1987). No correlation was found and it was concluded that the role of the SEM was more qualitative. All three publications were poorly cited, reflecting the small literature, although

[6] Chapter and/or Section information is only given for the first mention of a paper in any Section.

the 1988 paper was quoted for the presence of microridges, usually in parallel rows (Raymond et al, 1991).

Some work had also been done on stomach (Carr et al, 1984b, Carr et al, 1989, Dickson et al, 1989, all Section 2.4, Nunn et al, 1990, this Section), but the only systematic study led to no publications. The next four papers described the mucosal surface of small intestine, three after experimental intervention and one on childhood diarrhoea.

The first experimental procedure (Ferguson et al, 1978) involved allograft rejection of mouse fetal small intestine from its transplant site under adult kidney capsule: this was regarded as a model of delayed hypersensitivity (Ferguson and Parrott, 1973). The image quality in this early paper was limited by the air drying technique. The SEM images showed that isografts contained well-formed villi, while allografts showed great surface variation, including stunted villi or a flattened mucosa. The allograft damage profile was more like coeliac disease or parasitic infestation than a post-irradiation surface. With respect to citations, none of those available mentioned SEM.

The second experimental paper on small intestine (Ettarh and Carr, 1993) was the group's first on indomethacin-induced changes (Kent et al, 1969, Satoh et al, 1981). It followed on from an earlier paper on ulcerogenesis, (Carr et al, 1979a, Section 2.1) and from work on radiation-induced ulcers (Chapter 3, Section 3.5). It predated the paper included in Chapter 1 to illustrate the importance of plane of section (Ettarh and Carr, 1996a, Section 1.2) and showed early 'butterfly' lesions at the gastroduodenal junction, with adjacent villous distortion. Oval or elliptical jejunal and ileal ulcers were also reported, with villous separation and irregular creasing. Re-epithelialisation occurred by 24hours after treatment. This was the highest cited paper in Scopus in Chapter 2. It was used as a protocol source by several groups, including Playford et al (1996, writing with R. Goodlad and N. Wright), Croci et al (2003) and Xue et al (2009). It was cited for indomethacin having produced gastric ulceration (Langenbach et al, 1995). Faucheron (1999) quoted it, in a 'review in depth', for greatest damage being at 20hrs and for being 'of special interest' in a list of annotated references, where it was noted as 'demonstrating a systemic effect of NSAIDs on gastrointestinal tract'.

The third experimental paper, on streptozotocin-induced diabetes in mice (Ettarh and Carr, 1997), was a fore-runner to the study of radiation responses in subjects suffering from an underlying pathology (Chapter 3, Section 3.9): changes had previously been described in several morphological parameters (Mayhew and Carson, 1989, Pinto et al, 1995). Jejunal

and ileal villi were distorted, with attached filamentous microorganisms. Several histology-based quantitative data sets were given, using an approach developed to report multi-parameter radiation-induced changes (Chapter 3, Section 3.8). This paper, with SEM as one of the techniques, was cited for weight increase (Osborne et al, 2000), small intestinal elongation (Domènech et al, 2011), structural and functional changes to the small intestine (Durmus-Altun et al, 2011) and responses in Paneth cells (Porter et al, 2002, Min et al, 2014).

The fourth paper on small intestine (McClean et al, 1996) described the clinical features of jejunal surfaces from otherwise healthy children with symptoms of diarrhoea (Davidson and Wasserman, 1966, Thomas et al, 1992). In addition to recording villous shape, an estimate was made of mucus and debris coverage. Overall, the SEM data were regarded as useful. Coeliac disease and giardiasis were identifiable and microorganisms were sometimes associated with glycocalyx loss and microvillous clumping. For citations, it was interesting that a paper on villi in childhood diarrhoea, with authors from Departments of Child Health and Pathology as well as Anatomy, attracted interest less in clinical than in modelling papers (Bhuiyan and Poston Sr, 2005, Willman et al, 2009), albeit quoting biological findings, on mucus or villous numbers respectively.

A paper on rat large intestine, (Galloway et al, 1987) highlighted environmental factors (Wynder and Shigematsu, 1967, Berg and Howell, 1974). It included SEM images of mucosa following carcinogenesis and variations in diet, a topic that continued to be of interest (Sagher et al, 1991, McCullough, J.S. et al, 1998, both Chapter 1, Section 1.2). Histology showed no crypt hyperplasia. The most marked changes in crypt unit integrity were for the high fat low fibre diet, using an SEM scoring method constructed for irradiated small intestine (Chapter 3, Section 3.5). With respect to citations, it formed part of a debate on the details of how cell proliferation was affected by the type of dietary fibre ingested (Zhang et al, 1998) and was quoted for the relationship between proliferation and colon cancer risk (Robblee et al, 1989). It was also cited for the similarity between human and animal findings on the effects of fat and fibre (Freudenhieim et al, 1990) and for its report on surface responses in the high risk diet containing high fat/low fibre (Ma et al, 1996).

The next paper, by Ettarh and Carr (1996b), although a study of small intestine, described serosal and not mucosal surfaces, concentrating on mesothelium, important during peritoneal dialysis (Raftery et al, 1989). It had previously been studied with electron microscopy (Odor, 1954, Baradi and Hope, 1964, Abu-Hijleh et al. 1994). The presence of

straggly serosal microvilli was confirmed. Two other features were reported, one a serosal exudate, the other mesenteric stomata joining the two mesothelial aspects, but without evidence of connection to the lymphatic system, as seen at the diaphragmatic peritoneum (Tsilibary and Wissig, 1977, Fukuo et al, 1990). This paper was cited for the report of mesenteric peritoneal stomata (Wassilev et al (1998). It was also quoted for the thickness of the layer covering the mesothelial surface, examinable through peritoneal 'washings', and also for its report that this was only observed if osmication were omitted (Flessner et al, 2001). Reference was also made to it for the biosynthetic nature of mesothelial cells (Bird, 2004, in a review), as shown by its description of micropinocytotic vesicles. Gilloteaux et al (2013) cited it for the similarity of the mesothelial surface in the nude mice they used and that found in other animals, with reference to the spread of cancer.

The last paper in this Section (Simpson et al, 1983) reported on cat pancreatic duct after bile salt perfusion, exploring further the mucosal barrier (Reber et al, 1979): an interesting feature of the surface was the presence of scattered single cilia. It was quoted for its reporting on the duct epithelium (Arendt, 1991). It was of interest that the citing author commented on the relevance of the epithelial tight junctions, given their possible plasticity during microparticle uptake (Chapter 4, especially Section 4.8.4).

2.6 Later reviews of biological scanning electron microscopy

The first review (Carr, 1980) was a description of macrophage surfaces, in a book on the reticuloendothelial system. It covered: preparative techniques; phagocytic cells *per se* and in organ systems; phagocytosis; and pathophysiology. The macrophage family members, found in a wide range of body sites, were described as rounder when resting, flatter when more mobile; and heavily ruffled when busy with phagocytosis, but larger and smoother after engulfing extraneous material. Stimulated cells spread faster than resting cells and surface receptors were found more often at peripheral veils and were less common during spreading. This chapter on macrophages was included to show involvement in the cell culture field and the range of scientists contributing illustrations.

The second paper in this Section (Carr et al, 1982a), was an invited review of scanning electron microscopy in the journal Histopathology. It provided an outline of different aspects of SEM, such as instrumentation, sample preparation and examples of the types of images produced with standard detectors: all the illustrations were in-house. It used the term 'topographical histology' for the study of cell and tissue surfaces, to push towards more

rigorous image descriptions. It concluded that SEM was part of the range of available investigative approaches, rather than a diagnostic tool.

Further integration of SEM imaging was found in the descriptive text and illustrations of a range of surfaces in the 3rd edition of a book on cell structure (Carr and Toner, 1982): such images had not been available for the first two editions (Toner and Carr, 1968, 1971). The material from it presented in the thesis has concentrated on SEM, including the preface, instrumentation, critical examination of electron micrographs and a number of figures including a few correlative images. Some of the pictures were drawn from the wider community of scanning electron microscopists, but many were in-house. It has been included to point to the work done in the days of expanding TEM (Chapter 1) and developing SEM (this Chapter). This has provided contributions to the body of knowledge underpinning much modern biomedical research and education, despite predictions in the early 1960s that electron microscopy would never have anything to contribute to the teaching of histology.

Finally, the integration of surface and internal structure of tissues was also seen in an account of the morphology of the intestinal mucosa (Carr and Toner, 1984), the first chapter in a volume of the Handbook of Experimental Pharmacology. SEM Figures illustrated in 'three dimensional' format the structures specialised for the main intestinal function of absorption or reabsorption at the luminal/mucosal interface. The chapter also dealt with other functions, including defence, mechanical propulsion and neural or endocrine control.

Citation ranking for these four submissions did not reflect the influence of such publications. However, both the Pharmacology chapter (Carr and Toner, 1984) and the book 'Cell Structure' (Carr and Toner, 1982), listed only in Google Scholar, were placed at similar levels of interest to some of the articles in Chapters 2 to 4. In particular, the book had citations up to 2015, covering a range of topics, most published in journals rather than chapters or books.

2.7 Context and comment

The theme of Chapter 2 built on the interest in learning about tissue and cell relationships through the study of sectioned material, described in Chapter 1, by exploring surface structure and its underlying tissues. This was done through the development of SEM to take its place as one of a battery of morphological approaches used in the scientific study of the interface between environmental challenge and biological cells and tissues. The

submissions were produced from work carried out originally in the Universities of Strathclyde and Glasgow. The move to Belfast brought access to a more sophisticated instrument which allowed, for example, the use of non-standard detectors and image analysis: the facilities of LBL were also used during laboratory visits. The work covered slightly under thirty years, similar to Chapter 1, with article publication dates spanning the period from 1968 to 1997.

With respect to destination of articles, there was continuing use of anatomical or more applied journals and the first papers using SEM told their story in Lancet, the Journal of Pathology and the Scandinavian Journal of Gastroenterology, rather than in the 'microscope' journals. Reviews, books and book chapters showed the broad interest in the developing applications of SEM.

Collaborations with other Institutions, Departments or individuals, were, as for the work reported in Chapter 1, important in maximising the quality and breadth of the output: some of these have already been named above. In general, collaborating groups while at Strathclyde and Glasgow Universities were found in other Departments and Centres, including: Agricultural Chemistry, Bacteriology and Immunology, Chemistry, Cytology, Gastrointestinal Centre (Southern General Hospital), Medicine, Pathology; Pharmacology, Radiobiology Research Group, Glasgow Institute of Radiotherapeutics and Oncology (Belvidere Hospital), Royal Hospital for Sick Children (Yorkhill) and Surgery. Groups in Queen's University Belfast included Child Health, Dental Surgery, Medicine and Pathology.

Collaborations external to the 'home' Institution included the Centre for Bioengineering, University of Washington, Seattle, the Centre for Applied Microbiology and Research, PHLS, Porton Down, the Department of Anatomy, University of Sheffield, the Imperial Cancer Research Fund (ICRF), London, the Lawrence Berkeley Laboratory (LBL), California, USA and the MRC Cyclotron Unit, Hammersmith Hospital, London. Some colleagues or graduate students moved elsewhere during the work and continued to contribute, particularly to the writing of the papers: centres involved included Calgary, Alberta, Canada, Anatomy Department, the University College, Dublin, the Gastrointestinal Unit (Western General Hospital) University of Edinburgh, King Saud University, Riyadh, Saudia Arabia and the University of Basrah, Iraq.

The changing roles and contributions were again obvious from the authorship order, beginning with 'hands-on' scanning electron microscopy, addressing early technical and

interpretative questions, in laboratories in the UK and the USA, but moving later into involvement as supervisor, group leader, Head of Department or Director of School, the latter two applying during the last ten years of the work in this Chapter. This still involved a central role in the work, from protocol design to publication, but with students or colleagues often taking the lead authorship position.

Links between chapters were highlighted by the last submission (Carr and Toner, 1984), which set the scene for the next Chapter, where radiation effects have been described more for intestine than for other organs. The increasing use of correlative techniques with the SEM in this Chapter led on to its application to explore radiation-induced changes, at surfaces and tissues deep to them, dealt with in Chapter 3.

Chapter 3 Correlative morphology of irradiated small intestinal surfaces

Preface - extract from Presubmission Abstract (Chapter 3)

Early papers on scanning electron microscopy of irradiated rodent small intestine revealed villous collapse. This was dependent on radiation modality and dose, but was not always to the same extent as the changes in counts per circumference of proliferative crypts, the method then used for estimating intestinal damage. Early reviews came from annual international workshops on radiation-induced 'topographical' and subcellular changes, relevant to radiotherapy side effects and space travel. Further papers recorded morphological effects in other organs, villous changes over large samples and links between cryptal abnormalities and radiation conditions. Modalities included γ- and X- rays, electrons, neutrons and heavy ions. The importance of non-proliferative responses was highlighted by the shorter time-frame of damage and recovery after hyperthermia and by comparison of the effects of irradiation and pharmacological agents. The progressive collapse of villous surface shape was charted using a scale linked to published histometric measurements of section area: this recorded 'direct' intestinal radiation effects and 'distant' abscopal 'ulceration' after thoracic irradiation.

Correlative work showed that surface responses were accompanied by subsurface changes, in stromal, epithelial and neuromuscular components. Numerical data for changes in histological parameters per intestinal circumference produced a single number for morphological effects: subcellular information could also be included. The outcomes were plotted after fixed doses or times and were dependent on radiation dose, time and modality. Individual changes were also tabulated, revealing: possible adaptive responses after protracted irradiation; the lack of additive effects for irradiation and experimentally induced diabetes; and variations in control parameter numbers at different sites proximo-

distally. Reviews highlighted the individual responses linked to radiation-induced physiological symptoms and topics needing further attention.

3.1 Villous radiation-induced damage and links to Chapter 2

Leading on directly from Chapter 2, the work in this Chapter has been arranged to describe the surface changes in small intestinal villous structure after external irradiation and how they related to the underlying tissue responses. The results of other radiation or mechanistic studies have also been included. As before, the accompanying publications have not necessarily been presented in exact chronological order, but rather setting out optimally the developing theme.

The introductory paper on scanning electron microscopy (SEM) of radiation-induced villous shape changes (Carr and Toner, 1972) appeared only four years after the first paper on the SEM of their basic shape (Carr and Toner, 1968). Following this, links were made with radiation physicists, radiobiologists or oncologists, to ensure good protocol construction of the treatments and clinical relevance to patient care. Thereafter, as space radiobiology became important, it was fortunate that one of the collaborating groups had access to heavy ion facilities, important for the work on the possible effects of galactic cosmic rays.

The addresses used for this work included Anatomy, University of Glasgow, Anatomy (latterly the School of Biomedical Science), Queens, Belfast and The MRC Radiation and Genome Stability Unit, Harwell.

As set out above, the first paper in this Chapter, Carr and Toner (1972) followed on from Chapter 2, listing the flurry of papers from 1968 onwards on the insight given by SEM into control rodent small intestinal villous surface structure and normal and coeliac human samples. This was done either by initial short reports (Carr and Toner, 1968, Chapter 2, Section 2.1, Swift and Marsh, 1968) or by more lengthy descriptions (Demling et al, 1969, Marsh and Swift, 1969, Balcerzak et al, 1970, also Toner et al, 1971, Chapter 2, Section 2.2, Carr and Toner, 1984, Chapter 2, Section 2.6). Although there had been earlier descriptions of intestinal radiation damage (Wierneik, 1966a, b), the paper by Carr and Toner (1972) was possibly the first account using SEM to provide three dimensional information on the collapse of villous shape after external irradiation. It described the effects on mouse small intestine at up to 100 hours after exposure to whole body cobalt gamma rays at supra-lethal doses, 1500 to 2500rads. Contrast enhancers such as ruthenium red were applied to improve

the coating (Luft, 1966), limiting surface charging and optimising image quality. There was little radiation-induced change within the first 48 hours, but thereafter the villi appeared sequentially clumped and less erect, then shorter and more conical with disorganised creasing: finally, after 90 to 100 hours, the villi were grossly abnormal, appearing as stunted projections. The irradiated surfaces were different from the flat mucosa and prominent crypt mouths seen in human coeliac disease (Toner et al, 1971). At the latter stages, the surfaces had flagellate parasitic infestations. This paper (Carr and Toner, 1972) was followed shortly afterwards by an account, from one of the USA radiobiology laboratories specialising in gut damage, of radiation effects on rat small intestine up to 12 days after 1000rads of gamma radiation (Anderson and Withers, 1973). With respect to available citations, it was quoted for abnormally sized and shaped villi, obvious structural radiation-induced changes and intestinal damage (Brady and Hayton, 1977a, b, c). Z. Somosy, himself a productive author in the field, cited it as one of a dozen of the group's papers on irradiation-induced changes in villous structure and related changes, in this case commenting on the paper's report of 3 to 7 days as the time of maximum damage post-irradiation (Somosy et al, 2002).

The next three papers, in collaboration with R. Hamlet and T. Nias, then at the Glasgow Institute of Radiotherapeutics and Oncology at Belvidere Hospital, compared the effects on villous structure: of two radiation qualities with fixed time/variable dose or variable time/fixed dose; and of one radiation quality with single or fractionated doses. The radiation qualities had different Linear Energy Transfer (LET) values, low for gamma or X-rays and higher for neutrons, where the clinical interest was to understand the side effects of this comparatively new therapeutic tool. Villous shape and the number of microcolonies (crypts)/circumference were compared (Withers and Elkind, 1970).

In the first of these papers, on dose variation (Hamlet et al, 1976), published in a UK radiology journal, mouse small intestine was collected 3.5 days after whole body irradiation with either ^{60}Co gamma rays or D-T neutrons, at doses producing equivalent crypt count damage (Withers et al, 1970), also related to cryptal changes (Hendry and Potten, 1974). Partly in response to the need for standardisation of technique (Anderson and Withers, 1973), it was confirmed that intestinal distention affected the outcome, but did not mimic radiation damage. The usually sequential stages in radiation-induced surface villous damage seen with SEM were defined qualitatively as lateral, vertical, conical and rudimentary collapse. In general, gamma irradiation produced bent, laterally collapsed villi, whereas neutron irradiated villi were more conical. Such changes were seen before the shoulder of the crypt counting curve and they increased with dose. SEM, therefore,

provided a more sensitive reflection of radiation damage, which differed with the type of radiation. This paper had a similar citation history to the original radiation paper (Carr and Toner, 1972).

The second paper in the series, Hamlet et al (1981), adapted the protocol to give variable times after fixed doses of 10Gy (1000rads) for X-rays and 5Gy (500rads) for neutrons. As above, gamma or neutron irradiation produced early lateral or vertical villous collapse respectively, both with further progressive changes. Other features included early changes at villous tips, possibly associated with the extrusion zone or epithelial/stromal disruption, later fused villi (Anderson and Withers, 1973), pericryptal cell mounds, later called collared crypts (Carr et al, 1990, Section 3.4) and giant cells (Carr et al, 1981a, Chapter 2, Section 2.3, Carr et al, 1981d, Section 3.6). The report of possible stromal involvement highlighted the need for correlative studies of villous surface changes, as recommended in Chapter 2 (Section 2. 3). An addition to the literature in 1980 had come from a doctoral thesis on radiation-induced changes to rat small intestine by L-G Friberg, one of the reviewers of the Hamlet et al (1981) paper, another being J. Anderson (Anderson and Withers, 1973). The questions touched on sample preparation, evidence for the cellular nature of giant cells, other radiation-induced changes and correlative studies. This paper was cited for radiation-induced intestinal epithelial damage, permitting bacterial translocation, an important aspect of sepsis (Elliott et al, 1995).

The third paper in the series (Carr et al, 1979b) related villous radiation damage after three fractionation schedules of low LET Cobalt^{60} irradiation (Withers et al, 1975). It noted that damage after high total doses sometimes included heaped circles of cells around crypt mouths (Hamlet et al, 1981), calling for comparison with coeliac disease (Toner et al, 1971, Wellwood and Jackson, 1973). A technical development was the use of a pilot assessment system, adding subjective scores for seven villous features. This showed that the imbalance continued between villous and crypt count changes (Hamlet et al, 1976), this time to a different extent across the three fractionation schedules. With respect to citations, the core point of the paper was identified by Zook et al in 1983, one of the authors being G. Casarett: they reported, in their study of the effects of neutrons or photons on dog alimentary organs, that it had ‘argued that crypt cell counts do not always correlate with villous cell death’.

3.2 Early reviews on 'topographical' and ultrastructural radiation effects

The early publications on radiation damage (Carr and Toner, 1972, Section 3.1, Anderson and Withers, 1973, Heinzmann et al, 1978, Porvaznik, 1979, Friberg, 1980) included several published through the annual Scanning Electron Microscopy conferences, one being an early review (Carr, 1981). Regular meetings were therefore established on 'ultrastructural radiobiology' at this forum, leading to the publication of a booklet (Carr and Seed, 1983) compiled by the session organisers: T. Seed was a radiobiologist at the Argonne National Laboratory, with electron microscope (EM) interests. These papers included the review mentioned above (Carr, 1981), in which intestinal radiation damage was compared to that produced by other challenges such as methotrexate (Altmann, 1974), ulcerogenic treatment (Carr et al, 1979a, Chapter 2, Section 2.1) and abnormalities such as human coeliac disease (Toner et al, 1971, Chapter 2, Section 2.2, Wellwood and Jackson, 1973). Intestinal radiation effects included ulceration (Friberg, 1980) vascular changes (Egawa and Ishioka, 1978) and responses in microorganism populations (Porvaznik, 1979, Friberg, 1980). The surface changes began with damage, through stages of villous change from lateral collapse to a flat mucosa. Damage was followed by repair. SEM scoring systems for radiation damage included one entirely villous (Carr et al, 1979b, Section 3.1) and another scoring on three levels (Friberg, 1980). The reaching of any of the villous stages could be used as an 'end-point' to produce radiobiological equivalent (RBE) doses to allow comparison across radiation qualities. The question was raised, however, of RBEs being non-standard for different component tissues or compartments, a disadvantage in their use for such a multi-parameter organ, a subject dealt with later (Section 3.8). Surface damage was again described as probably related to underlying stromal effects and the review also gave brief descriptions of radiation injury to other tissues, such as vascular changes or neural effects, as well as to other epithelial responses. With respect to citations, this review was quoted, like the group's other papers, by Somosy et al (2002), again for the time of maximum damage and also for the formation of radiation-induced giant cells. Two papers on radioprotection (Kanter and Akpolat, 2008, Akpolat et al, 2009), cited it as showing damage at subcellular level, such as lateral and basal epithelial projections at intercellular spaces.

Extracts from the booklet (Carr and Seed, 1983) covering the 1981 and 1982 'ultrastructural radiobiology' sessions have been selected for inclusion in the thesis, to show the range of topics and information on input from reviewers, who, with the authors, included many of those working in the field. The concluding remarks by Seed and Carr touched on the

variation in radiation responses and the range of ‘target’ cells, radiation conditions or analytical tools used. *In vivo* topics included heavy ion induced retinal lesions; the role of type II pneumocytes; the importance of LET in intestinal damage; and structural stages in the onset of myeloproliferatove disorders. *In vitro* topics included: membrane damage; modifications of lectin or hormone binding; chromatin anchoring sites and cell shape. The 1983 booklet had almost no citation record.

3.3 Radiation effects at different sites

Most of Chapter 3, as for Chapter 2, has so far involved the small intestine, but other organs were also studied, all lined or covered with complex epithelial layers

With respect to stratified squamous non-keratinising epithelium, the surface structure of oral mucosa had been studied previously by SEM (Matravers and Tyldesley, 1978, Dourov, 1984) and microridges have been described above (Chapter 2, Section 2.5). In Robertson et al (1987), non-invasive sample collection was made of buccal smears from radiotherapy patients after tumour excision, allowing SEM assessment of microridges before, during and after fractionated radiotherapy: groups included control and pre-irradiated smears, those irradiated in four dosage ranges from 3 to 60Gy and finally post-irradiation samples. Control microridges were usually in parallel, equally spaced arrangements, with variable mucus and low bacterial colonisation. The pre-irradiation cells were similar, but with slightly more ruffled microridges, abnormal microvilli and also mucus and bacteria. A possible relationship was described between patient radiotherapy dose and microridge patterns, either directly or through exposure of cells at different stages of differentiation, implying that the surface details of this cell type (Chapter 2, Section 2.5) could have reflected the state of the epithelium. The citations for this paper (Robertson et al, 1987) have been difficult to interpret. On the one hand, further work by one of the radiotherapists/oncologists involved (A. Robertson), working with a different group of morphologists, concluded strongly that SEM was not likely to be useful in this way (Badran et al, 1994, title). On the other hand, Asikainen et al (2014), in their experimental study of irradiated dog oral mucosa simply quoted the original Robertson et al (1987) paper along with others and themselves concluded that ‘radiation disrupts superficial cells of the oral mucosa’. The same group (Asikainen et al 2015) in a review article on microridges (or microplicae as they called them) again merely quoted Robertson et al (1987) for radiation changes in the epithelium or its surface features and concluded that ‘The three-dimensional findings with TEM and SEM offer the opportunity to re-evaluate the classic histopathology

of the oral mucosa and oral mucosal diseases using more refined microtopographical parameters'. They also recommended further study of the tips of 'microplicae' and the use of immuno-localisation at electron microscope level.

With respect to urothelium, the paper by Hodges et al (1985), already mentioned for SEM methodology (Section 2.4), described immunogold-labelled mouse bladder after 5Gy whole body neutron irradiation. Surface atypia included blebbing and pleomorphic microvilli. There was parallel redistribution of some conconavalin A receptor sites from inter-plaque (later termed hinge, Liang et al, 1999) to plaque regions, possibly because of alterations in the cytoskeleton (Oliver and Berlin, 1982) or stroma (Hodges, 1982), another sign that surface responses reflected deeper problems. Discussion with reviewers covered internal cellular changes, surface atypia and lectin binding. The citations used this paper for radiation-induced changes in bladder epithelium, specifically after neutron treatment and greatest at 1 to 5 days after treatment (Dörr, 1995) and for membrane lectin-binding (Somosy, 2000, review).

With respect to epidermis and wound repair, the last two papers in this Section described rat skin irradiated with an electron beam (Mould, 1986), of interest because of its depth-dose distribution. This involved either incisional healing, already recorded for control and X-irradiated situations (Messerschmidt, 1986) or intraoperative radiotherapy (IORT, Swanson et al, 1988) of a graft-bed. The project was initiated by a Chinese plastic surgeon, who arrived with ideas already formulated for his post-graduate project. Wang et al (1994) reported on wound healing, 1 to 14 days after an incision, itself 7 days after local, 9.6Gy electron irradiation. The SEM images charted the healing wound and measured granulation tissue via image analysis taken directly from the microscope. The delay in healing reflected reductions in granulation tissue formation, epithelial cell migration, fibroblast proliferation and the inflammatory response: collagen bundle formation was also slower. Wang et al (1996) studied a graft bed rotated 180º from its original site 1 to 21 days after irradiation with 10Gy 6MeV electrons delivered as IORT. Irradiation again delayed but did not stop wound healing, reducing deposition of fibrinogen, fibrin and fibronectin, affecting re-epithelialisation and granulation tissue formation and reducing collagen fibril diameter. This paper recorded that the procedures were approved in Beijing, reflecting the disruption of the Belfast group's work by anti-vivisection pressure: graft formation and irradiation were carried out in China, with specimens brought to Belfast for analysis. With respect to citations, Wang et al (1994) was used with respect to wound healing (Kurul et al, 1997, Bernatchez et al, 1998). Citations for the second skin paper (Wang et al, 1996) included

some to technique, such as grafting methods (Eckhaus et al, 2008). Others cited it as reporting that there was delay in wound healing and less fibrin and fibronectin (Gentilhomme et al, 1998), the latter also quoted by Currie et al (2001). Ferguson et al (1999) cited both papers, the first for reporting reductions in fibroblast proliferation and migration and also in collagen volume and the second for the collagen being structurally altered.

3.4 Qualitative radiation effects on small intestinal surfaces

Returning to irradiated small intestinal surfaces, the next three topics have been chosen to illustrate features of interest separate from the developing theme of scoring the changes: they involved irradiated duodenal papilla, collared crypts and giant cells.

With respect to the papilla, Indran et al (1988) used the capacious SEM stage to record large samples, with the gastroduodenal junction as a reference line, (Carr et al, 1989, Chapter 2, Section 2.4). SEM produced more information than was previously available on the papilla (Edemskiĭ, 1983, Edemskiĭ and Svischev, 1986). Its opening was expanded by radiation and became easily visible. This could have been produced by radiation-induced effects on neuromuscular components, taking forward the proposal (Hamlet et al, 1981, Section 3.1) that non-epithelial tissues were important, highlighted again below (Sections 3.6, 3.7, 3.8). The relaxation of the papillary opening could in turn have functional significance. The only available citation to this paper (Gorea et al, 2010) was as a source reporting the obvious alterations to the papilla.

With respect to collared crypts, Carr et al (1990) explored further these enlarged regions resembling ‘heaped up mounds of cells’ (Hamlet et al, 1981): they were found around crypt mouths in the intervillous basins or clefts (Cocco et al, 1966) and resembled somewhat those seen in human coeliac disease (Toner et al, 1971, Chapter 2, Section 2.2). The collared crypt paper explored the effect of increased linear energy transfer (LET) on their appearance and incidence in mice 3 to 7 days after irradiation at Berkeley. This extended the collaboration with T. Hayes to include J. Ainsworth. Control crypt mouths were unremarkable, with no marked collars and openings approximately 5μm in diameter, but the entire collared crypt measured 87μm across, with an opening of 10μm. They were seen in some circumstances after neon and iron but not silicon ion irradiation, adding to the earlier sightings after a single dose of neutron irradiation (Hamlet et al, 1981). The collared crypts after iron ion irradiation were so tall that they could have alternatively been described

as villi with a central orifice: this suggested that crypts and villi were definable not only by shape, but also by vascular arrangements, a crypt having a peripheral network and a villus a central core. After X irradiation, they were only found after a high, fractionated dose (Carr et al, 1979b, Section 3.1). They seemed, therefore, to be mainly associated with some high LET irradiation conditions. Resin histology and TEM revealed possibly equivalent cryptal regions, with dark cells and prominent shoulders. This paper (Carr et al, 1990) produced no available citations of interest.

With respect to giant cells, correlative techniques had shown these to be epithelial swellings (Carr et al, 1981a, Chapter 2, Section 2.3). Five days after irradiation with either X-rays or neutrons, they were seen with SEM to be outgrowths 25 - 60μm in diameter, similar in size to the collars described above. Resin histology and TEM showed that giant cells had several nuclear profiles, lipid inclusions, vacuoles, microvilli, boundaries with neighbouring enterocytes and attachment over lengths of 40 - 80 μm to the underlying basement membrane and stroma. They were possibly formed after radiation damage to cryptal progenitor cells, allowing continuing division but incomplete separation of daughter cells. They had not been reported *in vivo* by other groups but had been seen in *in vitro* Hela cells (Tolmach and Marcus, 1960) and fibroblasts (Nias and Paul, 1961). With respect to citations to this paper, Syljuåsen et al (1997, with R. Withers included as one of the authors) listing it as one of only two reports on these cells *in vivo*, stated that their formation 'has not received much attention compared to other pathways to cell death such as apoptosis' and that 'they are an important pathway to cell death for first generation single cells after irradiation'. Another citation (Bhattathiri et al, 1998) quoted Carr et al (1981) as linking giant cells to problems in cytokinesis.

3.5 Scoring of radiation-induced changes to intestinal surfaces

The two papers featured here took forward the concept of quantifying radiation-induced villous shape changes to a more objective scoring system. Carr et al (1983c), again in collaboration with R. Hamlet and T. Nias, set out the basic concepts, important for understanding patients' radiation injury (Berthrong and Fajardo, 1981). Previous small intestinal SEM scoring systems (Carr et al, 1979b, Section 3.1, Carr, 1981, Section 3.2) were dependent on the three dimensional appearance, whereas this development related these changes in mice directly to those recorded histometrically for rat villous area in sections (Altmann, 1974): the ratios of these 'two-dimensional' changes with time, 1/2.5/2.5, were used to quantify the villous shape changes. This scoring system was then

used in two ways. Firstly, mean values were plotted for different radiation schedules, using scores from 0 for control erect villi to 6 for rudimentary structures and to 8.5 by extrapolation for a flat mucosal surface. Alternatively, a ratio was calculated of the doses of two radiation schedules required to produce one stage of collapse, such as conical villi, using this as an 'end-point', to give RBE values. Application of either of these more quantifiable methods confirmed that neutron damaged villi were more abnormal than those produced by X-irradiation, in conditions calculated to be equivalent for crypt loss. The paper (Carr et al, 1983c) also related villous shape changes to alterations in two different stromal compartments, suggesting involvement of villous stromal pegs and the stromal pericryptal plate. Control mucosa had competent pegs and plate. The different radiation-induced shape abnormalities related to incompetence in one, the other or both of these. This paper (Carr et al, 1983c) produced citations to villous surface effects (Köst et al, 1998) epithelial responses (Höckerfelt et al, 2000) and 'well documented' structural changes (Freeman et al, 2001, with W.K MacNaughton, a notable expert in the field, as one of the authors).

The other paper, (Carr et al, 1986b), in collaboration with A. Michalowski in the Hammersmith Hospital, adapted the scoring system to analyse larger intestinal areas, from the gastroduodenal junction to the duodenal papilla. More detail was produced through 'villous mapping', a detailed but time-consuming technique used in only one other study (Chapter 3, Section 3.7). After irradiation of a non-abdominal region, such as the lower mediastinum, there was localised intestinal 'ulceration' at 'abscopal' i.e. distant sites (Michalowski et al, 1983). An acetate sheet with grid squares was placed over standard prints and each square allotted a villous score, from which was calculated the total for that sample. Three days after irradiation, there was little change, but by five days, a range of sizes of sharply defined lesions containing conical villi began at the stomach junction: at 28 days, variable lesions were still present. Proximal regions were more damaged than distal. Possible causes were damage to sphincteric, glandular, vascular or nervous structures. The lesions differed from those produced by secretagogue exposure (Carr et al, 1979a, Chapter 2, Section 2.1), in that the abscopal 'ulcerated areas' usually had an epithelial covering and were therefore not strictly speaking 'ulcers', despite appearances in light microscopy (Michalowski et al, 1983). The stages of lesion development and 'recovery' were also different in the two forms of 'ulcer'. The only citation of note to this paper used it as a source for variation in the damage seen (Höckerfelt et al, 2000).

3.6 Importance of underlying structures to surface responses

The next four papers explored the internal responses deep to the surface changes already described (Sections 3.1 and 3.5).

With respect to stroma and villous/cryptal relationships, the first paper (Carr et al, 1981c), used autolysis (Ferguson et al, 1969, Chapter 2, Section 2.1) to reveal sub-epithelial stromal detail. Control samples treated in this way had double rows of crypts, each opening from a tube which was sometimes divided by a partition. A test frame (Gunderson, 1977) over micrographs of mouse small intestine was used to calculate the ratio of crypts to villi (Hagemann et al, 1970): this was 5.01/1 SD 0.73, n 32, close to previous findings of 4.53±0.99 (Smith and Jarvis, 1980). After irradiation, it was not easy to identify either the crypt mouths or their double rows, as reported earlier for unautolysed rat intestinal samples (Anderson and Withers, 1973). Many were occluded by stromal material and the crypt/villus ratio could not be established. Other radiation-induced abnormalities included a villus formed of skeins of cells twisted helically around each other, suggesting a spiral epithelial path from crypt mouth on to and up an adjacent villus. Radiation was used here as a 'probe' to distort the normal situation and facilitate consideration of the control results. Citations for this paper included an editorial in the Journal of Paediatric Gastroenterology and Nutrition, where it was referenced for the ratio of crypts to villi being 5/1, for possible radiation-induced spiral migration of cells from crypts to villi and for the use of SEM to study healing coeliac sprue (Lev, 1983). It was also quoted as reporting on how post-mortem changes have been helpful in the study of the structure of small intestine (Pietzonka et al, 2002a). This 1981c paper was also mentioned by Ross and Mayhew, (1984) in a paper on the advantages of stereological methods, citing it to point to the problems of quantifying villous shape changes after irradiation, but possibly just pre-dating the publication of the scoring system (Carr et al, 1983c, Section 3.5) linking them to histometric data. The possibility of spiral cell migration pathways was again noted with respect to cell migration patterns in chimeras, where it was shown that the pathway of epithelial cells was straight from crypt to villus, in a series of papers all of which included B. Ponder and one of which had N. Wright as an author (Schmidt et al, 1985, Wilson et al, 1985, Ponder et al, 1986).

With respect to the epithelial-stromal boundary, Carr et al (1981d) gave more information on giant cells (Carr et al, 1981a, Chapter 2, Section 2.3, Hamlet et al, 1981, Section 3.3), one containing an intracytoplasmic desmosome, possibly indicating that cell fusion or incomplete cell division could have had a role in its formation. The stromal changes seen

by TEM also included contact between epithelial and stromal cells through basal lamina herniation, the basal lamina looping away from the base of the epithelial sheet and complete epithelial/stromal stripping, comparable with the swollen villous tips seen with SEM. These interface abnormalities were reported as possibly being associated with weakened enterocyte function. This paper (Carr et al, 1981d) was cited by Eigenbrodt et al (1995), as one of the sources for radiation-induced structural responses, including giant cell formation, epithelial/stromal abnormalities and villous shape changes.

With respect to stromal components, the next paper (Carr et al, 1985) used high doses (18-20Gy Cobalt^{60} gamma or 10Gy neutrons) to reveal where such damage might be seen 3.5 days after irradiation. Villous shape changes were recorded (Carr, 1981, Section 3.2) and, since the neutron irradiation completely destroyed villous structure in one of the animals, more attention was paid to the pericryptal plate (Carr et al, 1983c, Section 3.5). TEM showed that deep stromal fibroblasts in both irradiated groups were irregular with more cytoplasmic inclusions: these were pale after gamma irradiation and dark after neutron treatment, implying lipid and lysosomal responses respectively. Gamma and neutron irradiation also produced responses in muscle and nerve respectively and there were changes in endothelial flaps, particularly after gamma irradiation. Some of these responses were similar to previous reports, including radiation-induced damage in other gut models (Lieb et al, 1977), in nerve (Mastaglia et al, 1976), at the epithelial/stromal interface (Chomette et al, 1977, Lieb et al, 1977) or in vessels (Egawa and Ishioka, 1978, Eriksson et al, 1983). One conclusion was that more data were needed, from a range of doses, radiation qualities and time-points. With TEM, this would have been very time-consuming and, as a result, light microscopy was preferred (Section 3.8). This paper, like Hamlet et al (1981), was cited by Elliott et al (1995), who used it as a source for the different changes caused by neutrons and gamma photons to villous shape and the stromal pericryptal plate.

With respect to the basement membrane at the epithelial-stromal interface, Carr et al (1987b) used the heavy ion facility at Berkeley to explore further how internal and surface changes might relate to each other, in another comparison of low and high LET treatments (1600 or 1720 cGy X-rays and 1000 or 1240cGy neon ions). The interest in heavy ion irradiation was by 1987 related to space research as well as to cancer treatment (Steinberg et al, 1990), the previous trigger for research (Leith et al, 1982). The paper by Carr et al (1987b), built on the data available from crypt survival (Alpen et al, 1980) and late ultrastructural changes, where basement membranes responded differently after heavy ion and gamma irradiation (Fatemi et al, 1985). Villous scoring was carried out and parameters

were analysed per circumference in resin histology sections stained with periodic acid Schiff (PAS) or silver, as well as the routine toluidine blue: the structures assessed included crypt counts, villous profiles, goblet cells and basement membrane integrity. All changes were related to their own control values rather than producing ratios of effects for the two schedules. X-irradiation had a greater effect on crypt counting than predicted (Alpen et al, 1980), making it more notable when neon ion irradiation produced a larger response, such as at basement membranes: this reinforced the importance of the epithelial/stromal boundary. The reviewers included S. Siew (gastroenterologist), R Laschi (editor of the Journal of Submicroscopic Cytology and Pathology) and T. Seed (ultrastructural radiobiologist at the Argonne National Laboratory). Questions or comments related to doses, sample orientation, staining of resin sections, non-removal of mucus, collared crypts and comparison of crypt counts with those of Alpen et al (1980). Dörr et al (1999), in a report on pig lung response to accelerated ^{12}C ions, cited this paper as one of only a few with data from *in vivo* experiments.

Concentrating on stromal vessels, two papers addressed different aspects of the response. The first has already been discussed for the description of control microvascular casts of small intestinal villi (Abbas et al, 1990a, Chapter 2, Section 2.4). Three days after 10Gy X-rays whole body irradiation, the vessels were more tortuous and compressed apico-basally than in the controls. Destruction of the apical parts of the cast (Egawa and Ishioka, 1978) was not seen, possibly because of the glutaraldehyde fixation and critical point drying (Lametschwandtner et al, 1984). Resin histology and TEM revealed capillary dilatation and luminal endothelial extensions. The paper concluded that more data were needed on how separate the vascular response was from the epithelial changes. The technical citation record for this paper was set out in Chapter 2 (Section 2.4) on SEM methods. Data-based citations were also made to its reports of changes in capillary tortuosity and density (Jahnson et al, 1998) and to vascular constriction (Chikui et al, 2012).

Continuing the stromal vascular theme, Abbas et al (1990b) added the earlier time-points of 6hrs and 1 day after 10Gy partial body X-irradiation: the villous transverse sections for light microscopy and TEM allowed good morphometric comparison between irradiated capillaries and those in control villi (Abbas et al, 1989, Chapter 1, Section 1.2). There were consecutive capillary dilatation, constriction and then mixed results. Changes were also seen in endothelial thickness, fenestrae and processes and in the perivascular space. Similarities were noted between these intestinal vascular responses and those in skin (Hopewell, 1980) or cultured endothelial cells (Friedman et al, 1986). The changes

corresponded to functional alterations rather than cell death and could have contributed to the radiation-induced response of this multicellular organ. The citations to this paper included references to it for radiation-induced endothelial pseudopods (Lee and Schmid-Schonbein, 1995) and, as to the previous paper (Abbas et al, 1990a), for changes in vascular diameter (Chikui et al, 2012).

Two other papers dealt with changes to other parameters in villous and cryptal compartments. The first (Carr et al, 1984c) compared numbers for villous scores, intra-epithelial lymphocytes, mitotic figures, parasitised crypts and giant cell incidence 5 days after 10Gy X-ray and 5Gy neutron irradiation. Villous tips showed oedema and epithelial/stromal stripping after X and neutron irradiation respectively. The reviewers included WE Dewey, WR Hanson and T Seed, all with a radiobiology background, and they asked questions on sample preparation, the parameters used, the villous scoring method, giant cells, dose rates, RBE calculations and the use of 'end-points'. The authors commented that RBE values for neutrons vs X-rays probably could not be extrapolated from intestine to other organs, such as skin (Hornsey, 1970, Hornsey and Field, 1974): the paper proposed further that RBE calculations for 'any one manifestation' of damage to a functional unit will apply only to that 'manifestation' and not to other cell populations (Chamberlain et al, 1980). The difficulty of the end-point approach was more obvious as the range of responses for different parameters increased, making it a challenge to use them to comment on total damage. Reviewers also requested statistical information, previously avoided because of small samples and 'n' values: when this was provided, it showed that, in data from sections, only the mitotic figure values were significantly different across the two radiation qualities. This point was borne in mind when a system was developed to report changes in all four tissue types in the small intestinal wall (Section 3.8). Elliott et al (1995) cited this paper (Carr et al, 1984c), like Carr et al (1985) and Hamlet et al (1981) for neutrons causing more villous responses than X-rays, while Dublineau et al (2002) concentrated instead on its report of approximately parallel time-related responses.

Also with respect to parameters and the possibility of different response 'manifestations', the next paper asked whether constituent cell types in a compartment all responded similarly to each other and to the compartment as a whole. Information was already available on many of the cryptal cell types (Potten et al, 1983) and the Wyatt et al (1987) paper investigated one of these, the endocrine population. The influence of gut hormones was already of interest (Besterman et al, 1982): since the neural component responded (Carr et al, 1985), it was important to explore this other player in alimentary tract control. Groups

of mice studied included fed, fasted, sham-irradiated and ether sedated shams, but only at one time-point. Irradiated samples were collected 1 or 3 days after partial body X-irradiation with 6, 10 or 18Gy, these being on the plateau, shoulder and exponential parts respectively of the crypt count dose-response curve. Most endocrine cells were cryptal (Ferreira and LeBlond, 1971) and control villous shapes were as previously reported (Carr and Toner, 1968, Chapter 2, Section 2.1), although the fasted group had slender, 'cleaner' villi. After some irradiation schedules, epithelial/stromal stripping was inferred from SEM images (Carr et al, 1981d), confirming that this region was at risk. Endocrine numbers decreased. Both this and villous shape were more sensitive than crypt counting, showing that a response in this compartment did not reflect the changes in all its constituent cells. Reviewers included CP Sigdestad, F Bonvicini, MS Al-Tikriti and T Seed. There were questions or comments on: the functional significance of the endocrine changes; the sham/ether effect; crypt size; sampling of other parts of the small intestine and identifying specific types of endocrine cells; why the animal pool was female only; the choice of doses; and epithelial/stromal stripping. With respect to citations, Panozzo et al (1991) cited the paper for the endocrine cell number decreasing after irradiation.

3.7 Mechanisms of structural damage

The papers in this section compared radiation effects with those after other procedures, either those acting as useful comparators, such as hyperthermia, or those with known mechanisms, such as pharmacological agents.

With respect to hyperthermia, when the Glasgow neutron facility closed, a collaboration was established to continue that work with the radiobiologists at the Hammersmith Hospital, London and to contribute further morphological data to their hyperthermia programme: this aimed to optimise its possible role in cancer therapy (Hume et al, 1979a, b, Tsubouchi et al, 1984). The paper included here (Kamel et al, 1988) was only one of several from the group (Carr et al, 1982b, Kamel et al, 1985, Dickson et al, 1992). It used light microscopy and SEM villous scoring (Carr et al, 1983c, Section 3.5) to compare the effects on small intestine of hyperthermia at 43º and 10Gy whole body X-irradiation. These two treatments produced maximum damage by 2hrs and 3.5 days respectively: the corresponding times for substantial recovery were 24hrs and 7 to 9 days. This timescale for hyperthermia was too short for villous shape to be reconstructed entirely through epithelial proliferation. Some of the responses after both treatments could therefore have been related to changes in non-epithelial components such as smooth muscle. This paper was cited in technical terms by

Oliver et al (2012), who adapted the scoring system to assess *in vitro* segments. It was also quoted, mainly with respect to data relevant to irradiation, along with Carr et al (1983c) and other papers, for the documentation of the morphological changes produced in small intestine (Höckerfelt et al, 2000). Forsgren et al (2000, co-authored with Höckerfelt), cited it for villous damage, as did Somosy et al (2002), particularly with respect to responses after different types of treatment.

The next three papers tested the hypothesis that radiation-induced villous changes were related to neuromuscular effects. This was done, in collaboration with Frances Boyle at Glasgow University's Pharmacology Department, by comparing them with those produced by pharmacological agents that either mimic radiation or reduce its effects by acting on this part of the intestinal wall.

With respect to radiomimetic effects (Indran et al, 1985, 1991), the 'radiation' aspect related to radiation responses in epithelum (Quastler and Hampton, 1962, Friburg, 1980, Carr et al, 1984c, Section 3.6) or muscle (Wirtz et al, 1982). The 'mimetic' effects were represented by reserpine, which increased gut motility through loss of inhibitory control, producing changes to smooth muscle (Casteels and Login, 1983) or nervous tissue (Patel et al, 1979). The 1985 paper (Indran et al, 1985) confirmed that ascorbic acid, the reserpine vehicle, produced clean mucosal surfaces (Maugel et al, 1980). Both irradiated and reserpinised treatments were shown by SEM scoring and light microscopy to produce villous shape changes and smooth muscle abnormalities respectively. The reviewers included C.S Potten from Manchester, UK, with a reputation in analysing different intestinal epithelial cell types after irradiation and J.R. Poley from Ulm, Germany, with a background of paediatrics and SEM in clinical applications. Reviewers' questions or comments included interpretation of villous scores; brush border cytoskeleton; colchicine effects; crypt morphology; links between villous and muscle changes; fixation techniques; the use of duodenum; cell position in crypts; and epithelial and vascular damage. Indran et al (1985) was cited by one of the authorities in the field, Hauer-Jensen (1990), who, while stating in a review that the crypts were the main target, quoted it as reporting that other parts of the wall could respond, thus taking note of the key point of the thrust of the work. The 1991 paper reported villous scores as 'enhancement factors', since shams were available for all groups. Damage occurred later after irradiation than after reserpine. Resin histology and TEM showed smooth muscle changes, deep to sites of villous collapse: it was concluded that this tissue could have contributed to the changes up to villous conical collapse, but that the more advanced rudimentary villous shape after irradiation required additional factors such as epithelial

lethality. This underlined the need to record responses in as many components as possible of multicellular organs. For citations to this paper, Amer et al (2005), noted its reference to villous changes and neuromuscular effects.

With respect to 'radioprotective' effects, Carr et al (1991a) reported on responses to atropine, which produced opposite effects to those of reserpine, decreasing gut motility. It was also known to affect the radiation response (Larkin, 1949, Burnett et al, 1952). Mice were irradiated with and without atropine. The radiation-alone schedule produced more villous damage than the combined treatment. MacNaughton (2000) cited this paper in a review on the pathogenesis of intestinal responses to irradiation: he noted the 'detailed morphometric studies by Carr and colleagues'. Somosy et al (2002) included it in a list of publications describing a range of effects. The main point of the paper was quoted for atropine blocking radiation-induced villous shortening by MacNaughton (2000) and Basson (2003, also a review paper). Özyurt et al (2014) referred to it while reporting a non-significant trend in the radioprotectant effect of this cholinergic antagonist. Work was also under way on conventional radioprotectants misoprostol and WR-2721 in collaboration with a group in the USA specialising in this area, led by W. Hanson. This was only published in Abstract form (Carr et al, 1991b) and has not been included as a submitted paper in the thesis.

Overall, the mechanistic arm of the programme had delivered an outcome, in supporting the need to study all tissues of the organ: the work on mechanisms of radiation damage and radioprotection was not pursued further.

3.8 Summary of small intestinal responses

The material here followed on from the findings on radiation-induced changes to different components of the intestinal wall (Section 3.6) and on the time-frames of responses to hyperthermia and pharmacological agents (Section 3.7): this approach also reflected the links between morphological and biochemical changes (Nikjoo and Goodhead, 1989). It focussed on producing, with collaborators in the USA and the UK, detailed below, a standardised approach for collection of quantitative information from well-preserved tissue sections and on controlling the time taken for analyses, using respectively data/circumference and resin histology rather than TEM. The responses of the key cells and structures in the intestinal wall were collected and brought together in one figure (the Morphological Index, or M^oI). Six papers have been included here. The first, using neutron-

irradiated samples, set out the method for two data levels, carrying information on tissues and cells. The second and third covered X irradiation after different doses or times and a comparison of neutrons and X-rays respectively. The fourth added ultrastructural data, extending the Index to three levels, while the last two presented data relating to heavy ion irradiation.

The first paper (Carr et al, 1991c) collected information on different tissues (Fajardo, 1989, Rubin, 1989), using mouse duodenum samples at four time points after whole body neutron treatment. Control tissue was the comparator and the parameters were also assessed at one time after X-ray treatment. Irradiation was carried out by S. Hume and colleagues at the Hammersmith Hospital and the data handling method was devised by the thesis author and A. Nelson, sequentially at Lawrence Berkeley Laboratory (LBL), the Massuchusetts Institute of Technology (MIT) and the University of Washington, Seattle: he also set out the procedures mathematically in an equation. Data collection, performed in Belfast, included areas for the four main tissues (epithelium, connective tissue, muscle and nerve) and counts for ten parameters, each assigned to one of the tissues. All measurements were per circumference, as for the microcolony assay (Withers and Elkind, 1970). The data were set out in tables, one row per tissue, and showed the statistical significance, direction of any change and ratios of the data, treated as absolute numbers, for treated/control values, categorising this as a Ratio Score system (Section 3.10). Data for each tissue row, weighted for area, were multiplied to give one figure, which could then be added for the four tissues to give a single number, called the Morphological Index: this was calculated per group for mean values or per animal to provide error bars for graphs. Information on villous numbers, crypts and mitotic figures were often included in the table, but not in the Index equation.

Interpretation of the changes contributing to the final Index number were read from the tables, as follows for neutron irradiation. At 6hrs, there was little change, but at 1 day the decrease in the Index related to changes in endocrine cells and Auerbach's plexus. The further Index drop at 3 days was due to significant epithelial, stromal and nerve responses. By 7 days, the return towards a control level Index was reflected in the lack of individual changes, found only in the stromal cells. After both treatments, the epithelial Index was affected more than others, but the two sets of individual responses were different. The conclusion above (Sections 3.1, 3.5, 3.6, 3.7) that non-epithelial cells contributed to the overall changes was confirmed by these findings. This paper (Carr et al, 1991c), with its range of data, was quoted as one of a number describing changes in different individual

epithelial cell types (Somosy et al, 2002), including goblet cells (Kanter and Akpolat, 2008) and stromal cells (Ch'ang et al, 2012).

With respect to the Index and variations of dose and time after partial body X-irradiation, the multi-tissue approach continued to be used (Carr et al, 1992a), while others reported radiation-induced effects on small intestinal epithelium (Potten et al, 1983). The Index displays included additional multiple graphs, showing the complete lack of uniformity in the parameter responses. For the dose experiment 3d after irradiation, the total Index decreased with dose, although that for 10Gy Index overlapped with the others. Reading the tables showed that, after 6Gy, endocrine cells were affected, at 10Gy there were several epithelial responses, but after 20Gy only two such changes appeared. The 10Gy time experiment data showed that the 6hrs total Index was within the control range, with only submucosal arterioles responding. The 1d Index was somewhat lower, with fewer endocrine cells, but larger error bars: the 3day data were as for the dose experiment. Carr et al (1992a) was the earliest of three cited by Scanff et al (2004) for the 'well-known' diminished absorptive surface, associating this with impaired active transport. It was also quoted, like the paper above, for changes to goblet cells (Kanter and Akpolat, 2008).

With respect to the Index for two radiation qualities, whole body irradiation was used, 5Gy neutrons and 10Gy X-rays, sampled at four time-points (Carr et al, 1996a). The displays included Index graphs and a parameter Table. The literature had continued to expand, with respect to intestinal irradiation (Busch, 1990, Cobb and Galland, 1990, Flickinger et al, 1990) and multi-parameter or multi-level approaches following experimental exposure (Argenzio et al, 1990, Goodlad and Wright, 1990, Mayhew et al, 1990, Vigneulle et al, 1990). The 6hr Indices showed greater damage by neutrons than X-rays, possibly related to the number of parameters responding. After 1 day, the continuing greater neutron effect reflected changes in endocrine cells and Auerbach's plexus profiles, while the similar 3d Indices reflected the individual parameter situation. At 7d, the neutron Index was at control level, while that for X-irradiation was still lowered, possibly due to greater data variation. In conclusion, crypt depletion was not predictive for changes to other parameters or compartments: both schedules led to epithelial changes and while X-irradiation produced early vascular changes, neutron treatment gave neuromuscular effects. Citations to Carr et al (1996a) included protocol topics, such as parameter assessment (Patel et al, 2012) or choice of time-points (Lehy et al, 1998) and also general radiation-induced intestinal damage (Yan et al, 2009). It was also mentioned for the greater effect of neutrons than X-rays (Mizukami-Murata et al, 2010) and for data for one or more of the parameters, such as

endocrine cells (Lehy et al, 1998) or myenteric plexus profiles (François et al, 1999, Ropenga et al, 2004): authors of papers from the French radiobiology groupings included I. Dublineau and/or N. Griffiths. As further papers were published on the Morphological Index, more groups appeared to obtain from its data display results for different parameters of interest to them.

While work was being done on adding extra ultrastructural or topographical information (Carr et al, 1992b), there had continued to be interest elsewhere in responses in different compartments (Fajardo, 1989, Rubin, 1989, Wright et al 1989). Villous scoring was carried out as described in an earlier paper (Carr et al, 1983c, Section 3.5), which had also recorded some of the responses without constructing an Index display. The additional factor for ultrastructural deviation (UD) was calculated as for the resin histology data (Carr et al, 1991c), where the two-level Index was analytical, using statistically testable numbers. However, the three-level Index, although more informative, was empirical, since it also contained subjective micrographs assessment. Ultrastructural responses were seen for epithelium in nuclei, organelles and membranes, for connective tissue in cell separation and endothelial structure, for muscle in vacuolisation and myo-filamentous arrangements and for nerve in dense bodies. Study of the villous scores and other data showed again that villous shape could be related to responses in muscle tissue (Section 3.7). Reviewers included LG Friburg and M Albertson, both with an interest in ultrastructural radiobiology, and J R Poley, with experience in SEM of gastrointestinal material. Topics raised included: the choice of neutrons, dose and proximal small intestine; extrapolation to late effects in human tumour tissue; vascular changes; cell separation; reasons for the changes in Auerbach's plexus profiles; and the selection of the time-points. Carr et al (1992b) was quoted (like Carr et al, 1986b) for having reported radiation-induced morphological changes (Höckerfelt et al, 2000). Other groups already mentioned above as citing the group's work continued to do so (Kanter and Akpolat, 2008, Akpolat et al, 2009, 2011). Rubio and Jalnäs (1996) commented on the presence of SEM and TEM data, but reported that there was 'no conventional histology', with no apparent reference to the resin histology data displayed in the tables.

With respect to Indices for heavy ion effects, (Carr et al, 1993 and 1994), this related to interest in space research after the Berlin Wall came down in 1989. Open discussion was then possible between American and Soviet or Russian scientists on space flight data and risks (NRCP Report, 1989), on further topics for research and on what advice should be given to Governments on whether to plan missions to Mars or to the moon. The two papers

were presented at ‘space’ conferences, since the heavy ion results from the LBL collaboration were possibly important for space travel risks, although originally collected because of relevance to clinical topics, such as targeted treatment of intra-cranial lesions (Steinberg et al, 1990). The heavy ion results were also one of the few *in vivo* data sets then available. The two papers used the Index approach, building on an earlier, more qualitative description (Carr et al, 1987b, Section 3.6).

The first paper (Carr et al, 1993) compared data from samples exposed to different LET irradiation schedules, involving contributions for neutrons from the Hammersmith Hospital, London and for neon ions and various aspects of data interpretation from three scientists originally from LBL. T. Hayes was still on staff there, A. Nelson by then worked in his own scientific company and J. Ainsworth had moved to the Armed Forces Radiobiology Research Institute, Bethesda, where he was Scientific Director from 1989–1998. The data were compared directly and also entered into a three-level Morphological Index display, building on results published earlier before the MoI approach was developed (Carr et al, 1987b, Section 3.6). Responses included neon ion-induced collared crypts (Carr et al, 1990, Section 3.4). Ultrastructural analysis revealed more severe changes after neon ion irradiation in epithelium, fibroblasts, endothelium and at the epithelial/stromal interface. Nerve tissue responded only after neon ions, this time in Meissner’s plexus profiles. Bringing in the neutron and X irradiation, it seemed that higher LET treatment produced more changes. This paper also compared structural responses with radiation-induced functional pathophysiologies, linking malabsorption and ulcer risk with enterocyte and goblet cell problems and changes in muscle tone with damage to Auerbach’s plexus. This paper produced no available external citations of interest, although it was cited as part of an extensive literature in a chapter on radiation biology of space (Kiefer et al, 1996). The second paper (Carr et al, 1994) compared the effects of neon ions with those of heavier ion types, niobium of interest because of its LET and iron ions more relevant to space flight, like neon (NRCP Report, 1989). Review of the data showed that collared crypts were easily identified after neon and iron but not after niobium or, as reported earlier, silicon (Carr et al, 1990). Neon ions produced most damage to several cell types and niobium ions led to least damage to mitotic figures. These findings pointed to a break in the previously perceived link between rising LET and increased damage. This was confirmed by animal by animal Morphological Indices, where three out of the four niobium ion values were lower than all iron data. The results were relevant to variation across animal strains (Hanson et

al, 1987) and to work on anatomical models (Atwell, 1994). The citation profile was similar to that for the 1993 paper.

The Index data display had confirmed that non-epithelial changes were important in the villous response to irradiation and it would have been interesting to collect more data on the effects of varying LET, dose and time. However, the balance of the work was moving towards the importance, post-Chernobyl, of understanding the uptake route of large, potentially radioactive particles through the gut wall (Chapter 4). The approach was still applied to irradiated intestinal tissues, but with more emphasis on individual responses (Section 3.9).

3.9 Different cell populations

In the next three papers, the Morphological Index protocol was adapted for specific projects. Other groups were also providing a comprehensive picture of the multi-factorial intestinal responses (Rubio and Jalnäs, 1996).

Brennan et al (1998), in collaboration with T. Seed of the Argonne National Laboratory, as a result of links made through the annual SEM conferences (Section 3.2), compared small intestinal responses to acute and protracted schedules 6hrs, 1d and 3d after 5Gy whole body gamma irradiation. Qualitative observations on apoptosis (Ijiri and Potten, 1990) were added to the parameters and two tables were used, one for epithelial tissue and one for the others. 'Damage' responses (Kwan and Norman, 1977) 6hrs after both schedules included mitotic arrest and fewer lamina propria cells. By 1d, the reduction in enterocytes was regarded as 'damaging', but the return of mitotic activity during the protracted schedule reflected some recovery. The 3d data showed continued lamina propria depletion for the acute schedule, but a possible move towards normality for the protracted schedule. It was concluded that 'adaptive' processes (Specian and Oliver, 1991) were present during continuous irradiation with low doses. Citations to this paper referred to general responses, such as epithelial damage, after genotoxic stress including irradiation (Morgan et al, 2014), decreased numbers of enterocytes and lamina propria cells (MacNaughton, 2000) or changes in the Paneth cell population (Porter et al, 2002).

The next paper (Ettarh et al, 2000) applied in an experimental setting the relevance of underlying pathology when planning radiotherapy (Gerard et al, 1995, Mayr et al, 1997, Morris and Powell, 1997). Mice with established small intestinal changes due to streptozotocin-induced diabetes, already studied in rodents (Rakieten et al, 1963, Mayhew

and Carson, 1989, Ettarh and Carr, 1997, Chapter 2, Section 2.5) were exposed to X-irradiation and samples collected three days later. Resin histology was used for qualitative observations and collection of Morphological Index data (Carr et al, 1991c, Section 3.8). Statistical analysis to differentiate between the effects of the two treatments showed that diabetes did not enhance radiation-induced damage and perhaps counteracted it through improved repair. The fact that the experimental procedures were carried out in London, courtesy of Imperial Cancer Research Find (ICRF), probably related to the anti-vivisection pressure that caused problems for the group about that time (Wang et al, 1996, Section 3.3). For citations, Salin et al (2001) quoted this paper (Ettarh et al, 2000) for a definition of how to identify cryptal mitotic cells, while Epperly et al (2004) cited it with respect to general radiation effects in villi and cryptal cells.

The above report on diabetic irradiated intestine (Ettarh et al, 2000) included data on three parts of the small intestine, in line with interest on proximo-distal variation (Mayhew and Carson, 1989). The approach dovetailed with that of the next paper (Brennan et al, 1999), where the control small intestine was divided into seven equal parts. Parameters were counted as before (Carr et al, 1991c, Section 3.8), but additional structures were also assessed, including intraepithelial lymphocytes, Paneth cells, apoptotic bodies and intestinal circumference. Some parameters had no inter-site differences; some decreased caudally, some increased caudally and others peaked at the middle of the intestinal length. Correlations were noted between some of the parameter numbers and villous height or crypt number. The data, although more detailed especially for non-epithelial parameters, had a similar overall pattern to earlier results from studies of three sites (Cheng and Leblond, 1974). With respect to citations for this paper, its report that proximal villi were taller than those distally was linked to the greater proximal villous fragility in Ussing chamber experiments (Inagaki et al, 2005). Foureau et al (2010), in a paper on intestinal defensins, cited it for the greater numbers of ileal Paneth cells. Randall et al (2011), in a review of gastrointestinal explant culture, used it as a source for the caudal decrease in crypts and therefore in crypt/villous ratio.

3.10 Reviews of irradiated small intestine

The descriptions of collection and interpretation of data from several components of the small intestine, highlighting its multi-parameter make-up (Sections 3.8, 3.9), featured in two reviews produced seven years apart, both summarising much of the material in this chapter of the thesis.

3.10.1 Structure and function

The first review was published after a meeting on 'Radiation and the Gastrointestinal Tract' held in Washington DC (Carr et al, 1995). This paper stemmed from the opening talk in the session on 'Diarrhoea', giving it a clinical relevance (Busch, 1990, Flickinger et al, 1990, Levi and Hodgson, 1990). It compared radiation-induced epithelial and non-epithelial effects, included an Index table for two notional radiation schedules and used backscattered SEM images (Chapter 2, Sections 2.3, 2.4) to illustrate epithelial/stromal stripping, crypt disorganisation and villous tip enterocyte discontinuities. Two-level Morphological Index displays reviewed the changes with time, dose and radiation quality (Section 3.8) and took further the linking of experimental data with functional or clinical questions (Carr et al, 1993, Section 3.8), in that response rankings were given for each parameter. For those related to diarrhoea, three were important, high ranking endocrine cells, giving humoral input and moderate ranking goblet cells or myenteric nerve plexus profiles providing lubrication and input to peristalsis. This paper was listed only by Google Scholar. It was cited as the introductory reference for intestinal cellular injury in a paper on selenium compounds as radioprotectants (Kunwar et al, 2011) and also for its comparison of morphological intestinal changes produced by whole and partial body irradiation, by Freeman et al (2001), a paper which included W.K. MacNaughton in the author list. Lebrun et al (1998, including Nina Griffiths in the authorship), noticed its interest in linking radiation-induced diarrhoea with alterations in regulatory systems, while Griffiths (1999) commented on its reporting of changes in nerve or endocrine cell populations. Picard et al (2002, also including Griffiths), in their introductory comments on the diarrhoea or constipation occurring as side-effects of abdominal radiotherapy or treatment with 5-OH-tryptamine antagonists, noted the epithelial effects described.

3.10.2 Comprehensive review

The linking of structural changes and functional consequences continued in this Chapter's main review (Carr, 2001), which drew attention to topics of interest with illustrations, some from other groups. The addresses for this paper were Queen's University of Belfast *and* the MRC Radiation and Genome Stability Unit, Harwell, due to a two year sabbatical there. The account given here of the contents of this review has been selective, due to space constraints.

Part I reviewed the literature back to an early report of gut radiation injury (Walsh, 1897). Part II described the intestinal wall and epithelial responses to challenge, including multiple organ dysfunction syndrome, possibly associated with bacterial translocation (Niewenhuizen et al, 1996) and thus looking ahead to Chapter 4. Part II continued with a brief account of radiation, including mention of a 'new golden age' (Alpen, 1998) and the need for more 'translational research' (Coleman and Harris, 1998).

Part III on the effects of a single dose of partial body, low LET irradiation took most information from early time-points. Animal-based observations (Vigneulle et al, 1990) included a report of abnormalities associated with malfunctions of the alimentary system (Summers et al, 1991). Multiparameter results often led to Scoring Systems. One type used Injury Scores or Radiation Injury Scores (RIS), which increased with time, as damage became more obvious (Hauer-Jensen 1983a, Rubio and Jalnas, 1996): other factors were also considered by Hauer-Jensen's group (Richter et al, 1997, Wang et al, 1999). The second type calculated Ratio Scores from irradiated and control samples, as in the Morphological Index (Carr et al, 1991c, Section 3.8): these also increased initially with time, given the treatment of the data as absolute numbers. Another group compared SEM scores with data from contrast medium in serum and urine (Solheim et al 1991). Part III then considered changes in intestinal compartments (Becciolini et al, 1982) and epithelial cells (Wiernik and Plant, 1971 onwards). Stromal involvement (Rubio and Jalnäs, 1996, Wang et al, 1998, Section 3.6) included changes in fibres and fibrogenesis, the inflammatory and immune systems and vascular components (Chomette et al, 1977). The next set of topics involved changes in endocrine, neural and muscle components (Sprügel et al, 1977, Wyatt et al, 1987, Section 3.6, Summers et al, 1989, Geisinger et al, 1990, Carr et al, 1992a, Section 3.8, Höckerfelt et al, 2000).

Part IV on the effects of a single dose of unshielded low LET irradiation described functional effects (MacNaughton et al, 1997) and linked them to structural responses (Gunter-Smith, 1989). There were more data on gut associated lymphoid tissue (GALT, Chen et al, 1995) and endocrine cell responses (Linard et al, 1997, Dublineau et al, 1998, Lehy et al, 1998). Part V on the effects of a fractionated dose of low LET radiation noted that much of the information related to human radiotherapy schedules, often with little detailed treatment information provided. Side effects included almost immediate radiation enteritis (Classen et al, 1998), early diarrhoea, later bleeding and obstruction or fistula (Spitzer, 1995), confirmed in animal studies (Allgood et al, 1996, including M. Hauer-Jensen). Cellular changes included some early severe epithelial atypia (Wartiovaara and

Tarpila, 1977), continuing later, when there was also ulceration (Hauer-Jensen et al, 1983b). There were vascular (Kallfass et al, 1996) and neuroendocrine changes (Höckerfelt et al, 1999). Tumours also occurred (Ming and Goldman, 1998).

Part VI, on factors affecting the outcome, covered several topics which had not fitted comfortably in other sections. These included radiation volume (Trott et al, 1995) or type (Alpen et al, 1980) and ingested radioactive material (Harrison and Stather, 1996, Hunt 1998), dealt with in Chapter 4: also touched on were pathophysiology (Gerard et al, 1995) and the treatment of radiation-induced symptoms (Classen et al, 1998). The section finished with a summary of the radiation-induced changes.

The conclusions section of the review highlighted the need for more data: on different irradiation schedules; on interfaces, composite structures and inter-compartment relationships; on cross-talk, signalling molecules and 'bystander' input; and on underlying factors such as stress, diet, aging or pathophysiology.

Citations to Carr (2001) included one from Hauer-Jensen's group, linking it with radiation-induced changes in 'most intestinal wall compartments' (Wang and Hauer-Jensen, 2003). These and further aspects of the responses were cited by other groups (Parihar et al, 2006, Prabhakar et al, 2007, Williams et al, 2010, including Hauer-Jensen, Kanimozhi et al, 2012, Shanthakumar et al, 2012). It was also used as a reference for the time after treatment (Ropenga et al, 2004, with N. Griffiths) or for the involvement of reactive oxygen species (Senesse et al, 2009). There were also clinical papers, relating to colorectal cancer emergencies (Barnett et al (2013) or to intestinal effects after liver irradiation (Cameron et al, 2012). It was also used by Ishihara et al (2011) as a first general reference in the Introduction of their paper on the effect of anabolic steroids in post-irradiation regeneration, while Veerginadis et al (2017) used it as a citation to fibrosis. More recently, Hauer-Jensen et al (2014) referred the reader to it as a 'very comprehensive review' in their section on clinico-pathological aspects of radiation damage to the gut.

3.11 Context and comment

The theme of this Chapter was the application of SEM and associated correlative approaches from Chapter 2 to a specific topic, as tissues, mainly intestinal, responded to external challenge such as irradiation. The work was carried out in the Universities of Glasgow and Belfast, while visiting the Lawrence Berkeley Laboratory (LBL) and on sabbatical at the MRC Radiation and Genome Stability Unit, Harwell. The period involved covered slightly

under thirty years, about the same span as Chapters 1 and 2, with publication dates from 1972 to 2001.

With respect to article destination, there were fewer articles in anatomical journals, apart from Scanning Electron Microscopy and the Journal of Submicroscopic Cytology and Pathology. There were several publications in radiation and radiobiology journals and a few in general journals, review series or conference proceedings.

Collaborations continued to be important: some begun as internal to Glasgow University remained in place. Collaborating scientists and groups in other Departments and various branches of radiation science included, in Glasgow, Pharmacology, Pathology, The Glasgow Institute of Radiotherapeutics and Oncology/Radiology Research Group, Belvidere Hospital, The Glasgow Institute of Radiotherapy and Oncology, The Western Infirmary. Groups in Belfast included the School of Clinical Dentistry/Dental Surgery, the School of Clinical Medicine/Oncology. Collaborations external to the 'home' Institution at the time included in London, the MRC Cyclotron Unit, The Hammersmith Hospital and the Imperial Cancer Research Fund (ICRF); and in the USA, the Centre for Bioengineering, University of Washington, Seattle, the Radiation Haematology Group, the Argonne National Laboratory, Argonne, Illinois, USA, and LBL, California, USA.

Some colleagues or graduate students continued to contribute after moving to other centres. These included Anatomy, University College, Dublin (UCD); Anatomy, University of Sri Lanka Peradeniya Campus; The Armed Forces Radiobiology Research Institute (AFRRI), Bethesda, Maryland, US; the CRC Normal Tissue Radiobiology Research Group, the Research Institute, The Churchill Hospital, Oxford; Diagnostic Imaging, UCD; Life Sciences, Kingston; NeoPath, Bellevue, Washington, USA; the Richard DimblebyDepartment of Cancer Research, St Thomas's Hospital Medical School, London.

A special factor needing mentioned was anti-vivisection pressure in Belfast. This was distracting for both staff and students. However, radiation and associated experimental facilities were kindly provided by other laboratories, namely Imperial Cancer Research Fund (ICRF) in London and Beijing Medical University in China.

The roles and contributions involved were still obvious from the order of the authors in the submissions, again reflecting the change from 'hands-on' scanning and transmission electron microscopist, to one of encouraging others to use the SEM, especially students. As before, as the years went on, more of the data were collected by them, but there was

continuing involvement in thesis supervision, protocol design, the provision of technical training, data quality control and paper writing.

With respect to links to the next Chapter, the above output in the overlapping fields of radiation, morphology and the intestine led to invitations after the Chernobyl accident to attend workshops on its likely impact and to funding for research on large particle uptake, with a resulting gradual switch of effort, in the period from 1986 to 2001, away from radiation effects.

The work by then had come full circle, beginning with the study in Chapter 1 of some aspects of the small intestine, following on with the need to produce correlative images to relate to the additional detail of mucosal surfaces produced by SEM, then to the need to explain villous collapse of these surfaces after radiation and tie it in to internal changes. The full circle returned interest to the exploration of the small intestine after a new, but cognate, environmental challenge, that of exposure to large particles (Chapter 4), but with the knowledge that as a multi-parameter organ, all of its compartments and their constituent cells or structures were of possible relevance.

Chapter 4 Microparticles crossing intestinal surfaces

Preface - extract from Presubmission Abstract (Chapter 4)

Relating surface effects with morphological counts per circumference was useful in work on microparticle uptake, begun soon after the Chernobyl accident in 1986, the continuity with Chapter 3 being highlighted in a conference chapter. The pharmaceutical and medical relevance of uptake was also noted and the timing of the writing of a review of the morphological aspects underlined its environmental importance, given the 2011 Fukushima incident.

The post-Chernobyl work addressed two questions. Firstly, could particles the size of those released into the radioactive cloud enter the human body through an intestinal route? Secondly, were any population groups particularly at risk, specifically pregnant or lactating women? Two models were used. In vivo, human exposure was simulated by exposing rodent intestine in situ rather than exteriorised: particles were usually 2–20 µm fluorescent latex, identified after maceration or by confocal scanning laser and electron microscopy. The in vitro model comprised Caco-2 monolayers, allowing recording of transepithelial resistance and particle uptake, which was in the same range as that in the other model. M cell monolayers have also been used.

The first answer was that, after in vivo in situ exposures as short as 5 minutes, particles did cross the small intestinal epithelium, moving on to secondary organs such as lymph nodes: particle dose, size and volume were important. Entry was, unexpectedly, mostly through villous epithelium and not M cells of lymphocyte-rich Peyer's patches, with uptake not greatly reduced by severe compromised immunodeficiency (SCID). Particles were observed at intercellular junctions or spaces, highlighting enterocyte tight junctions as possibly involved. In vitro work addressed this, showing that ethanol loosened junctions and increased uptake, while cooling produced opposite effects and different changes in immunolabelled junctional proteins. Particle retention at intercellular junctions affected the protein disturbances.

The second answer was that in vivo in situ uptake: was not increased by diabetes; was more affected by age than species and sex; and could be higher in late pregnancy and early lactation. In vitro uptake could be increased by irradiation. The Chapter finishes with further reference to the 2012 review.

4.1 Questions on uptake and links to Chapter 3

As indicated above, involvement in this area of research stemmed from previous work on radiation effects on small intestine (Chapter 3). This was relevant to the topic at the centre of this Chapter, namely the exploration of the impact of the 1986 Chernobyl incident on humans and their food chain, since the radioactive microparticles produced could have used an intestinal route to enter body tissues of exposed populations, according to earlier publications (Volkheimer et al, 1965 and later, e.g. Volkheimer, 1977, 2001). Although no written record has been retained of this, memory has indicated that the grant body initiating and supporting the programme of work on microparticle uptake, the UK Department of Health, London, set two questions.

Question 1 'Do microparticles enter tissues by an intestinal route ?'

Question 2 'Are any groups 'at risk', such as pregnant and lactating females ?'

Initial consideration of these two questions led to the grant-holder and thesis author posing a **supplementary methodological question** – 'What model, what microparticles, how locate them, what animal or cells exposed ?' The answer to this question was the construction of two models, one with rodent small intestine *in vivo in situ* (Model 1, Sections 4.2 - 4.4, 4.7) and the other with an *in vitro* culture of a monolayer resembling a sheet of absorptive intestinal epithelial cells (Beaulieu, 1999, Model 2, Sections 4.5 - 4.7). The first was used mainly by what could be called a 'Belfast particle uptake group', while the second, later one was centred in Oxford. The models have been described first, while data from them have mainly been reported in relationship to Questions 1 and 2.

The subject of microparticle uptake has been introduced by relating it to three meetings after Chernobyl.

The first publication (Carr et al, 1997) stemmed from a meeting in Rome on recent advances in microscopy of cells, tissues and organ, organised by P. Motta. The paper related surface responses after an environmental challenge to changes in the underlying tissues, through morphological analysis, as used above (Chapter 3, Section 3.8). It dealt with radiation effects (Buell and Harding, 1989, Potten, 1990, Carr et al, 1991c, Chapter 3, Section 3.8), taking further the approach (Chapter 3, Section 3.10) of matching changes in function with total responses over the whole radiation programme. It also quantified microparticle uptake (Kreuter, 1991, Couvreur and Puisieux, 1993), using a Morphological Index method: numbers in different compartments and cell types were recorded per circumference, as for

radiation-induced changes in crypt/microcolony numbers (Withers and Elkind, 1970). Sampling for particle counting covered nine proximo-distal segments. The conference was in honour of Marcello Malpighi, a 17th century Italian biologist and physician, regarded as the father of microscopical anatomy and histology. Authors were asked to mention him in their presentations: the references quoted in the paper by Carr et al (1997), have been used in Chapter 5 (Section 5.3) for a different purpose (Malpighi, 1687). The paper had almost no citation record and was included to illustrate the link between Chapters 3 and 4.

The second meeting, concentrating on particle uptake and translocation across epithelial membranes, was the central Symposium at the 1995 summer meeting of the Anatomical Society, in Belfast, hosted by the thesis author. The abstracts were published in the Journal of Anatomy, as were many of the full papers in 1996, after peer review, producing a total of 14 articles, including a one-page Introduction written by the organising committee. This contained representatives of gut biology, pharmacy, surgery and toxicology, reflecting the relevance of uptake to a range of subjects (Carr et al, 1996b). This short 'paper' noted: variation in methodology and data; villous epithelium and also Peyer's patches, important parts of gut-associated lymphoid tissue (GALT); factors influencing uptake; and the sensitivity of some population subsets. It also had almost no citation record and was included to illustrate the relationship of the Belfast particle uptake group's work with that of others in the field. However, it was cited as one of an introductory group of three papers in a 'meta-review' by Hussain et al (2001): the authors included S. Florence, one of the main authorities on uptake in the field and one of the co-organisers of the Belfast Symposium.

The third meeting, held in London, was entirely on microparticle uptake. The papers were published in the Journal of Drug Targeting and included Hodges et al (1995a), which summarised the literature (O'Hagan, 1990, Kreuter, 1991, Couvreur and Puisieux, 1993) and described its own two approaches to particle quantification: one used 'maceration' to produce solubilised tissues (LeFevre et al, 1989, Ebel, 1990) and the other specific methods for microscopy. Figures and data Tables of small intestine 30mins after oral administration of 2μm latex microparticles included a confocal image of particles in the lumen and villous stroma, along with numbers per circumference in several villous and Peyer's patch compartments and also per gram of macerated tissue. More particles were associated with nearby villi than with the actual Peyer's patches and there was discussion of the interpretation of the data for the two techniques. The topics cited from this paper included the uptake of particulate material (Hillyer and Albrecht, 2001) and the lack of clear understanding of the uptake route of this process (Tabata et al, 1996), of such importance

to drug delivery. Several groups quoted it as having reported uptake through villous sites near Peyer's patches (Pietzonka et al, 2002b) or at enterocytes (Damge et al, 1996, Le Visage et al, 2001). Florence (1997) quoted it in a review as a source for percentage uptake.

Citation histories from now on have been mostly separated into the minority relating to techniques and the majority relevant to data: citations have not been given for all submissions and only some have them in both categories.

4.2 What model? Model 1, the standard *in vivo in situ* model

More information on this model was given in the next paper, published in a mainstream gut journal (Hodges et al, 1995b). As above, the baseline *in vivo* procedure involved oral exposure of rats to microparticles, with the intestine *in situ* and not as exteriorised loops. Particles were often 2 μm fluorescent latex. Mesenteric lymph nodes or other particle-poor regions were collected first, to avoid cross-contamination. Maceration counting on unfixed tissue (LeFevre et al, 1989, Ebel, 1990) was carried out on nodes and on proximal, middle and distal small intestinal 'Peyer's patch regions', which included not only domes but also adjacent villi. Epifluorescence and confocal microscopy were used for morphological analysis of full circumference fixed cryomicrotomy slices, from the proximal ninth only: these were termed 'Peyer's patch circumferences' and included domes and villi around the entire intestinal wall. The microscopy technique was more accurate than maceration, since it permitted identification and exclusion of luminal or surface particles and therefore allowed accurate quantification: this point has been touched on again below (Sections 4.8.1, 4.9.2). One of the citations to technique quoted this paper: it described the histology approach as only suitable as a semi-quantitative assay, although stating that 'precious information' was gathered by it (Delie, 1998). She pointed out the disadvantages of the technique and seemed to feel that quantitation through maceration was preferable (but see Sections 4.8.1, 4.9.2).

4.3 Model 1, variation in particle type

The principal particle type in the *in vivo in situ* model was, as stated above, fluorescent latex, often 2μm in diameter (Hodges et al, 1995a, 1995b, Sections 4.1, 4.2). Starch had also been used (Volkheimer and Schulz, 1968, Volkheimer et al, 1968a, b), but produced artefactual problems. Pilot experiments (McCullough et al, 1995) were also carried out, administering iron (Volkheimer et al, 1969, Parmley et al, 1984) as powder in milk and using light microscopy, backscattered detector electron imaging (BEI) with scanning

electron microscopy (SEM, Chapter 2, Sections 2.3, 2.4), transmission electron microscopy (TEM, Chapter 1) and X-ray microanalysis (XRMA, Chapter 2, Section 2.4*)*. The citations related to this paper included a detailed description by Nagata (2004) of the techniques used.

There was another and serendipitous observation of 'particle' uptake (Cartwright-Shamoon et al, 1995), during investigation of the yeast Saccharomyces boulardii (Maupus et al, 1983, Vidon et al, 1986) as an anti-diarrhoeal agent, in collaboration with the Belfast Department of Child Health, headed by J. Dodge. Yeast uptake was studied with light microscopy, secondary detector SEM imaging and BEI SEM images. With respect to technical citations, Samonis et al (2011) referred to it as showing the yeast taken up in animal experiments as opposed to therapeutically, giving details of its protocol.

The final particle type (Carr et al, 2001) was fused aluminosilicate (FAPs), 2.2µm in diameter, able to be labelled with ^{57}Co to make its effects more like those of nuclear incidents such as that at Chernobyl (Cuddihy et al, 1989, Wakeford 2011a). This was carried out in collaboration with C. Collier and also J. Harrison and M. Youngman of the then Atomic Energy Authority (AEA) Technology and National Radiological Protection Board respectively, both at Harwell. The FAPs were visualised in resin sections of rat intestine by BEI and by TEM of freeze-fracture replicas. The techniques, however, were time-consuming and Model 1 with fluorescent latex remained the standard approach. This submission had no citation history, being in Abstract format only.

4.4 Model 1, developments in methodology

The *in vivo in situ* model was established by the time the paper by Hodges et al (1995b, Section 4.2) was published. Microparticle exposure time was usually between 30mins and 24hrs. Various technical changes were introduced, described in brief here, in approximate chronological order.

More detailed study of onward particle movement to secondary organs included microscopical analysis of mesenteric lymph nodes (MLNs, Hazzard et al, 1996), liver and spleen (McMinn et al, 1996). The percentage of administered dose was calculated for particles of different size, giving consideration also to the volume and surface area of particles taken up as well as to the numbers involved (Carr et al, 1996c). Citations to this paper on particle size included some to techniques, such as to the maceration protocol (Delie, 1998).

TEM images were used to explore the site of small intestinal uptake of yeast in mice, some infected with rotavirus to induce diarrhoea, (Cartwright-Shamoon et al, 1996). It was also applied to locate latex microparticles, in Model 1 routine ultrathin sections as well as those pre-selected by vibrotome sectioning of regions known to be particle-rich (Smyth et al, 2005). This paper explored the question of whether pregnant and nursing mothers were more at risk, by comparing uptake into male, virgin female, pregnant and lactating rats 5 or 30mins after particle administration. Other developments in the same paper included the use of gavage rather than oral/pharyngeal administration, the collection of particle numbers in cardiac blood and the examination of nine proximo-distal segments and Peyer's and non-Peyer's circumferences. The range of intestinal diameters in animals of different species or ages led to the recording of intestinal lengths and areas, giving stacked profile displays of 'area under the curve' numbers for luminal, surface and tissue particles down the intestinal length (Doyle-McCullough et al, 2007). Proximo-distal particle distribution was also shown for sites with different lymphoid profiles in a study of immune status (Smyth et al, 2008). Several aspects of the technique were also validated (Smyth et al, 2005, Doyle-McCullough et al, 2007).

4.5 What model? Model 2, the standard *in vitro* model

A major question had been identified (Hodges et al, 1995a, 1995b, Sections 4.1, 4.2) as to whether uptake was principally through villous epithelium (McClean et al, 1998, Hillyer and Albrecht, 2001) or through Peyer's patch microfold (M) cells (Ermak et al, 1995, Thomas et al, 1996, Beier and Gebert, 1998). Villous uptake implicated passage at tight junctions (Björk et al, 1995, McCullough et al, 1995, Section 4.3, Wiesner et al, 2002), which have attracted attention for their many roles (Aijaz et al, 2006). An *in vitro* protocol (Moyes et al, 2007) was constructed to explore the role of tight junctions in uptake, becoming more 'blue sky' than applied research. The aim was to construct *in vitro* an uptake model that simulated the *in vivo in situ* particle uptake at villous epithelium.

The choice for Model 2 of Caco-2 cells, derived from a human **co**lonic adeno**ca**rcinoma (Delie and Rubas, 1997) was related to their resemblance to a small intestinal enterocyte sheet (Beaulieu, 1999). Particles were usually but not exclusively fluorescent latex. The Caco-2 monolayers were grown on Transwell® plates and the inserts had 3µm pores, allowing passage into the lower well of 2 µm microparticles. Recording was made of transepithelial resistance (TER), reflecting permeability and the extent of closure of the tight junctions (Madara, 1998). Fluorescence microscopy was used for particle counting of a

'crosswire' across the middle of the epithelial monolayer. Confocal scanning laser microscopy images gave measurements for cell dimensions and estimated the proportions of particles associated with or sitting on the epithelial monolayer. Particle location was noted, either epithelial-associated or submembranous (EAPs, SMPs respectively). Any monolayers with discontinuities were discarded.

Objectives of the first *in vitro* paper (Moyes et al, 2007) were: to explore whether uptake was accompanied by permeability changes or affected by the removal of apical particles, mimicking peristalsis-driven onward movement of an intestinal bolus; to study the effect of the vasoactive intestinal peptide/polypeptide (VIP); and to compare the uptake data with those for *in vivo in situ* experiments (Sections 4.2, 4.4). With respect to technical citations, reference was made to its use of TER (Uematsu et al, 2016) and to the usefulness of Caco-2 cells for the assessment of uptake as for drug delivery (Lyu and Park, 2008, Derakhshandeh et al, 2011, Iannuccelli et al, 2011).

4.6 Model 2, developments in methodology

As was the case for Model 1 (Sections 4.2 – 4.4), there were methodological developments over the years, including further studies of culture conditions (Moyes et al, 2010a), a topic of interest to several groups (Hidalgo, 1996, Delie, 1998, Ranaldi et al, 2003, Zucco et al, 2005, Volpe, 2008). The developments included changes in cell sourcing and exposure to 'pre-treatments', to connective tissue cells found deep to the epithelium *in vivo* or to irradiation.

With respect to the two pre-treatments (Moyes et al, 2010a, Moyes et al, 2011), these were ethanol and temperature variations associated with the incubator (37º), culture hood (24º) or chilling (0º). Both pre-treatments became useful as comparator procedures for particle addition in identifying changes in junctional proteins. The technique-related citation record for the first pre-treatment paper (Moyes et al, 2010a) included references to it for the study of Caco-2 cells with TER (Yuan et al, 2011) or their use as a model of the intestinal epithelium (Li and Zhu, 2016).

With respect to the combined effects of external irradiation (Chapter 3) and particle exposure (Moyes et al, 2008), this was carried out during an attachment to the MRC Radiation and Genome Stability Unit (RAGSU) at Harwell. The collaborating team there included M. Kadhim (radiobiologist) and M. Hill (physicist). The protocol used 2Gy X-rays (de Carvalho et al, 2006), at the level of one fraction in human radiotherapy

(International Commission on Radiological Protection, ICRP, 1990, published 1991), with or without subsequent particle exposure. The importance of sham treatment (Chandler et al, 1993, Oude Elferink et al, 1993, Armitage et al, 1994) was explored to control for the different temperature of the irradiation room (Moyes et al, 2008). Derived data assessed included cellular surface area and the volume of cells or intercellular spaces (Lieb et al, 1977, Porvaznik, 1979, Somosy et al, 1993, de Carvalho et al, 2006).

With respect to exposure to connective tissue cells, THP-1 derived macrophages (Manabe et al, 2003, Satsu et al, 2006) were used: these were known to open Caco-2 cell tight junctions (Kanzato et al, 2001) and to phagocytose particles (Hallab and Jacobs, 2009), moving them away from the epithelium (Wells et al, 1988). In Moyes et al (2010b), the two cell types were mutually exposed for 24hrs in indirect contact through the medium, more 'compartmentalised' culture (Parlesak et al, 2004) than 'co-culture'. Some technical citations for this paper were to the Caco-2 model itself: for instance, Stevanović et al (2012) quoted it for Caco-2 cells being useful for testing the efficiency of drug delivery.

Alternatively, Raji lymphocytes in the lower well produced monolayers of M cells (Kernéis et al, 1997, Gullberg et al, 2000, Tyrer et al, 2002, des Rieux et al, 2007, Miyazawa et al, 2010), as found in Peyer's patch epithelium (Carr, personal communication, 2018). These were also produced through a compartmentalised culture, since a true 'co-culture' contained gaps that distorted the data (Moyes et al, 2009). Uptake was compared with that in the Caco-2 model (above), which simulated a sheet of villous enterocytes, testing claims that it was greater across M cells (van der Lubben et al, 2002, Lai and D'Souza, 2008).

Developments in assays included adjustments to the protocols for both pre- and post-fixation stages. Pre-fixation developments included the addition of the measurement of cytokine levels in the medium (Moyes et al, 2010b). TER, which provided information on permeability (Delie and Rubas, 1997), but not epithelial integrity (Mukherjee et al, 2004) was originally reported as the recorded, 'raw data' (Delie and Rubas, 1997, Moyes et al, 2007). Other approaches involved the comparison of two values, either through subtraction, giving 'delta' values (Moyes et al, 2010a, b) or through ratios, producing percentages (Moyes et al, 2011).

Post-fixation developments relating to cell dimensions (Moyes et al, 2007, Moyes et al, 2008) included the production of diagrammatic representation of cell shapes. Junctional protein (JP) immunolocalisation at intercellular junctions, put in context by their relationships to actin (Shaw et al, 2005), was developed (Shi et al, 1993, Ferruzza et al,

1999, Musch et al, 2006) to produce uniform localisation of the proteins over a wide area of control monolayers, allowing comparison with treated samples (Moyes et al, 2010b, 2011). Tight junction (TJ) proteins occludin and zonula occludens (ZO-1) in belt format met these criteria, as did adhering junction (AJ) protein E cadherin, found as belts and spots.

Other post-fixation developments involved particle counting, initially mostly through epithelial-associated particle (EAPS, Moyes et al, 2007, 2008), then more through submembranous numbers (SMPs, Moyes et al, 2010a, 2010b, 2011) and recently through both, for Caco-2 and M cell monolayers (Carr, personal communication, 2018). There were also developments in comparisons of microparticle uptake *in vitro* and *in vivo in situ*, (Moyes et al, 2007).

4.7 What model? Invited Review on microparticle uptake

The review (Carr et al, 2012) was written around the time of the 2011 Fukushima incident (Wakeford, 2011b), underlining the field's long-running relevance (Wakeford, 2007). It contained more information on some aspects of uptake, such as Peyer's patches and their M cells (Cornes, 1965, Owen and Jones, 1974, Regoli et al, 1995) and the history of uptake and its terminology (Steffens, 1995), including 'persorption' (Volkheimer and Schulz 1968, Volkheimer et al, 1968a, b) or, given Herbst's early paper in 1844, 'the Herbst-Volkheimer effect', (Prokop, 1990, Beukeveld and Wolthers, 1995).

The review differed from those in Chapters 2 and 3 in containing proportionally more otherwise unpublished data and comment. Model 1 topics dealt with further included: particle characteristics and localisation; animal status; quality control and model validation; calculation of percentage uptake; and data interpretation. In addressing further the question of 'at risk' populations, in this case after radiation exposure following incidents like those at Chernobyl (Wakeford, 2011a), the protocol involved a dose of 10Gy whole body X-rays, given before a 30mins Model 1 exposure. For Model 2, there was further description of the formation of an M cell monolayer and of immunocytochemistry localisation of junctional proteins. The review also reported results of a pilot study, in collaboration with I. Dublineau of IRSN, Paris, of the effects on permeability of depleted uranium, iron and latex (Carr et al, 2012). The review also reported briefly on the hypotheses that intercellular junction proteins participated in uptake, a topic requiring further analysis (Carr, personal communication, 2018).

4.8 Q. 1 Do microparticles enter tissues by an intestinal route ?

4.8.1 *In vivo in situ* data (Model 1)

The answer to the first question was that, after *in vivo in situ* administration, particles did cross the small intestinal epithelium (Hodges et al, 1995a, b, Sections 4.1, 4.2). Morphological analysis showed particles in proximal small intestine, confirming their position by optical slicing to be in and not artefactually on the tissues. Validation checks (Smyth et al, 2005, Section 4.4) were satisfactory and particles were seen by TEM at the microvillous surface, between epithelial cells and in blood vessels. Particles were also identified in lymph nodes.

The review (Carr et al, 2012, Section 4.7) brought together values for several aspects of uptake. Mean figures for percentage administered dose were higher from maceration (1.80%) than from microscopy (0.13%), which were nearer to the even lower literature values (Ebel, 1990). Returning to the point discussed above (Section 4.2), maceration not only overestimated uptake by including luminal and tissue particles, but also underestimated it to a variable extent because of the fading of particle fluorescence after storage in fluid containing residual potassium hydroxide. There was inter-animal variation, possibly related to handling-related stress but fasting had little or no effect, unlike literature reports on this and diet (Worthington and Syrotuck, 1976, Ebel, 1990, Simon et al, 1997).

Further information below has been set out in five parts (Sections 4.8.2 to 4.8.6).

4.8.2 Uptake time-frame (Model 1)

Papers by Hodges et al (1995b, Section 4.2) and McMinn et al (1996, Section 4.4) showed that, in macerated tissue (LeFevre et al, 1989, Ebel, 1990), particles were found 30mins after administration, confirming reports of 30mins to 2hrs as significant (Kreuter et al, 1989, Jenkins et al, 1994). Microscopy showed that the proportion in the lumen fell with time from 2 to 8hrs, while that for epithelium increased from 0.5 to 4hrs: the proportion in non-epithelial tissues varied with time (Hodges et al, 1995b). With respect to available citation information, this paper was the top cited article in the thesis in Scopus. It was quoted by Bakhru et al (2013), as one of four papers from the group, being included in their Table reviewing the literature on uptake. They also quoted it with respect to uptake occurring within 30mins of administration.

Extension earlier of the time-frame showed uptake as early as 5mins after administration (Hazzard et al, 1996, Section 4.4), confirming previous reports of it taking place within a few minutes (Volkheimer and Schultz, 1968, Sass et al, 1990, Jenkins et al, 1994). With respect to citations, the Hazzard paper was quoted by Åkesson et al (2011) as describing uptake as 'rapid' and by Bakhru et al (2013) as reporting it as happening at 5mins. The 'speed of movement' of particles from lumen to serosa was up to 4 times per second the diameter of a particle, or 3-8μm/second, allowing passage through the wall within 2mins, the earliest exposure time studied (Carr et al, 2012, Section 4.7).

Extension of the last sampling time later to 5 weeks (ibid) showed a fall in total particle numbers and the proportions in villous sites, while those at Peyer's patches rose, particularly for lymphoid tissue, by then at 100%, albeit of a much smaller total number. In other words, the earlier the more villous, the later the more Peyer's, confirming that the predominant villous uptake was partly due to the comparatively early time-points originally sampled.

Also relevant, however, were data on transit (Carr et al, 2012), provided by J. Harrison (then National Radiological Protection Board, NRPB, later Health Protection Agency, Harwell) and C. Collier (then AEA, Harwell): these showed that at the often used 30mins time-point, 89% of 10μm particles were still in the oesophagus or stomach and only 6% had reached the small intestine. This showed that quoting uptake figures for only one time-point could not have revealed the whole amount taken up over time.

4.8.3 Uptake sites, proximo-distal and villous/Peyer's patches (Model 1)

For proximo-distal distribution (Hodges et al, 1995b, Section 4.2), 30 mins after exposure, substantial numbers were seen from segment 1 through to segment 5, with more at segment 3 and fewer distally (Carr et al, 2012, Section 4.7). Uptake of fused aluminosilicate particles (Carr et al, 2001, Section 4.3) occurred even more proximally and to a lesser extent than for latex. The paper by Hodges et al (1995b) was quoted as reporting uptake to be proximal (Hussain et al, 2001, Bakhru et al, 2013).

For the primary uptake site as in villous or Peyer's patches, microscopy quantification of numbers per proximal circumference (Hodges et al, 1995a, Section 4.1, 1995b) recorded that most was villous (Wells et al, 1988), although several groups had reported that Peyer's patches were the key sites (LeFevre et al, 1985, Owen et al, 1986, Pappo and Ermak, 1989, Eldridge et al, 1990, Pappo et al, 1991, Jepson et al, 1993). Although the observed predominance of villous uptake was pointed out clearly (Hodges et al, 1995b), attention was

distracted from this by the use of the terms ‘Peyer’s patch region’ and ‘Peyer’s patch circumference’ and several groups have misquoted the results: the details of this have not been included here. Citations relevant to the importance of villous uptake included one to the Hodges et al paper (1995b) by Norris et al (1998) which included a definition of the term ‘Peyer’s patch rings’ and a reproduction of one of the Tables. Other references to this paper describing uptake to be heavily villous or involving enterocytes included one by Lwin et al (2009) and a review by Snoeck et al (2005). Reineke et al (2013), in their Introduction, mentioned the paper on uptake and immune status (Smyth et al, 2008, Section 4.4) and the Carr et al (2012) review as identifying non-FAE tissue to be a site of uptake, commenting that, since M cells only made up a very small proportion of the surface cells, targeting them was not easy.

Confirmation of this important predominantly villous site in the *in vivo in situ* model within 30mins of microparticle administration came from three sets of experiments.

Firstly, there was no difference in uptake between Peyer’s and non-Peyer’s circumference rings (Smyth et al, 2005, Section 4.4). Secondly, Peyer’s patches were not essential for uptake, since mice with virtually none of these lymphoid aggregations did not have reduced levels of uptake, as shown through collaborative work with U. Schumacher’s group in Hamburg (Smyth et al, 2008). This studied microparticle exposure in combined immunodeficient (SCID) mice, known to take up micro-organisms (Ohsugi et al, 1996, Havell et al, 1999, Mutwiri et al, 2001) and parasites (Mead et al, 1991, Koudela et al, 1999, Umemiya et al, 2005). BALBc and SCID particle uptake were respectively ≥ 90% and 100% villous. The percentage of administered dose taken up at 30mins was 0.27% for BALBc mice, similar to those in the literature (Ebel, 1990, Jenkins et al, 1994): the corresponding value for SCID mice was not significantly different.

Thirdly, results from an inter-laboratory study (Carr et al, 2012) showed that uptake in entirely villous samples was observed by three groups. One was headed by T. Dayan, at St Bartholomew’s Hospital, another by J. Harrison, at the then National Radiological Protection Board (NRPB), Harwell and the third by the thesis author. All three laboratories detected substantial particle numbers in non-Peyer’s sites: data courtesy of Dayan, Simon, Harrison and Carr. The Belfast group also showed by microscopy that 93% of the tissue particles in proximal segments were villous.

4.8.4 Uptake pathway, paracellular/apical (Model 1)

Information on this has come from work on four particle types, all relating to villous locations.

Firstly, TEM showed latex particles within intercellular spaces (Smyth et al, 2005, Section 4.4), implying a paracellular pathway, but not excluding apical/lateral access. Secondly, iron deposits (McCullough et al, 1995, Section 4.3) were seen at microvilli and intercellular boundaries, confirming apical uptake of metallic substances (Lever and Duncumb, 1961, Volkheimer et al, 1969, Rehnberg et al, 1981, Nwokolo et al, 1992). The identification of iron near tight junctions was an early indicator of their possible involvement. The deposits were patchy, perhaps because of variation in either iron receptors or nutritional status (Parmley et al, 1984). With respect to citations, the data were quoted by Nagata (2004) as implicating both intracellular and paracellular pathways, a conclusion reached also by Löbenberg et al (1997), which included J. Kreuter as one of the authors. Other groups cited it more specifically as having pointed to the paracellular pathway, (Srinivasu et al, 2015) and/or to particle access through tight junctions (Hillyer and Albrecht, 2001, Zhang et al, 2003, Rohner et al, 2007).

Thirdly, freeze-fracture TEM data from preliminary FAP studies (Carr et al, 2001, Section 4.3) also pointed to villous tight junctions as possibly involved in uptake, supporting paracellular passage. As indicated above, this submission had no citations. Fourthly, Cartwright-Shamoon et al (1995, Section 4.3) showed yeast ‘particles’ of Saccharomyces boulardii (Maupus et al, 1983, Toothaker and Elmer, 1984, Vidon et al, 1986) within the villous mucosal epithelial layer, implying translocation as suggested by Stone et al (1974), but not specifying whether uptake was paracellular. TEM (Cartwright-Shamoon et al, 1996, Section 4.4) showed the yeast between and to a lesser extent within the epithelial cells. This suggested that the more important pathway was paracellular, implicating tight junctions again. Samonis et al (2011) cited the 1995 paper as evidence for the yeast travelling from the intestinal lumen through the mucosa. The 1996 paper was quoted as reporting yeast uptake in a review by Lewis and Freedman (1998), by Marteau and Shanahan (2003) and Llopis et al (2014) and also with respect to identifying it as a possible risk factor, especially for immune-suppressed individuals (McCullough, M.J. et al, 1998).

4.8.5 Onward movement (Model 1)

With respect to secondary organs (Section 4.4), small numbers were seen in mesenteric lymph nodes as early as 5mins after administration (Hazzard et al, 1996), supporting previous reports of onward movement (Jani et al, 1989, O'Hagan, 1990): various sites were involved. Particles were also found to a lesser extent at 30mins in liver and spleen (McMinn et al, 1996) and not in cardiac blood until that time-point (Smyth et al, 2005). With respect to citations, Snoeck et al (2005), in their review of the role of enterocytes in the intestinal barrier, quoted Hazzard et al (1996) for the onward movement of particles to mesenteric lymph nodes 5 to 30 minutes after exposure. Yang et al (1999) and Akande et al (2010) cited McMinn et al (1996) for movement to secondary organs.

4.8.6 Particle-associated factors (Model 1)

These included particle administration, size, aggregation or tissue loading. With respect to administration, as reported elsewhere (Ebel, 1990, Seifert et al, 1996), dilution in water of the particle dose decreased uptake (Carr et al, 2012, Section 4.7), but the use of milk (ibid) as the diluent decreased rather than increased uptake (Le Ray et al, 1994): the lack of agreement was possibly associated with protocol differences.

With respect to size, (LeFevre et al, 1980, Ebel, 1990, Eldridge et al, 1990, Jenkins et al, 1994, Florence al, 1995), the cut-off depended on assay technique (Carr et al, 1996c, Section 4.4) but more was taken up of the smaller latex particles, which also penetrated further than larger ones. However, consideration of their dimensions showed that a diameter of 6µm could be the most efficient for delivery to lymph nodes, a subject of interest to pharmaceutical specialists, among others. With respect to citations, it was quoted with respect to uptake at 'non-lymphoid intestinal tissue' (e.g. Behrens et al, 2002) and used as a source for uptake level being < 1% (Møller et al, 2008). Bakhru et al (2013) quoted it for greater numbers of smaller particles being taken up, with another inclusion in their Table of the group's work, through this paper, as for Hodges et al, 1995b (Section 4.2). Uddin et al (2009) also noted its report that, when volume was considered, the next larger size (5 to 6µm) was more efficient for onward movement to nodes. Hussain et al (2001) quoted it with respect to loading capacity. Size was also important for iron uptake (McCullough et al, 1995, Section 4.3), since the particles were described as 6 - 9µm in diameter, but some deposits were so small that they were 'nanoparticles' and less suitable than latex for microparticle uptake studies.

With respect to aggregation, standard latex particles were seen singly, in superficial linear arrays and in deeper clusters (Carr et al, 2012). For tissue loading, in TEM samples there was 1 particle/1000 cells and even in heavily laden sections harvested by vibrotome slicing, the ratio only rose to 1/50 cells. The imbalance between uptake and cell numbers was also important, goblet cells having higher uptake but fewer cells than enterocytes. Aggregation and loading could affect microdosimetry and tissue damage, with possible impact on the pathophysiology of the response to particle exposure, particularly for larger particles, which carried more dose per unit particle (Carr et al, 1996c). Tissue damage (Fabian, 1983, Freedman, 1991) was confirmed as occurring at fractures where particles were located, especially when aggregation was present.

4.8.7 Particle-free *in vitro* monolayers (Model 2)

Laboratory conditions were standardised to define control Caco-2 monolayer parameters (Moyes et al, 2007, 2010a, Sections 4.5, 4.6) for comparison with other reports (Delie and Rubas, 1997, Sambuy et al, 2005, Hughes et al, 2007, Volpe, 2008). The mean control TER value was 457 Ωcm^2, with a range of 328-728 Ωcm^2 (Moyes et al, 2010a), in line with the literature (Delie and Rubas, 1997, Ingels and Augustijns, 2003). Cell height was 27.2 µm, in keeping with other reported values for cells *in vitro* (Hidalgo et al, 1989, van't Hof and van Meer, 1990, Nicklin et al, 1992) or human *in vivo* cells (Delie and Rubas, 1997): the apical dimensions were smaller than those in the literature (des Rieux et al, 2005, de Carvalho et al, 2006), although the protocols were not identical. The citation record for the first pre-treatment paper (Moyes et al, 2010a) included reference to it for the height of a Caco-2 cell (Ozeki et al, 2015), used when calculating permeability coefficients. Citations for the macrophage paper (Moyes et al, 2010b, Section 4.6) included confirmation of its report for cell packing (Xiao et al, 2014), any one cell being surrounded by six others (Uskoković et al, 2012a).

With respect to immunolabelling (Moyes et al, 2010b), actin was arranged in apical, lateral and basal zones (Shaw et al, 2005) and apical cell shape was clearly outlined by junctional protein localisation (de Carvalho et al, 2006, Musch et al, 2006). This included occludin and ZO-1 at tight junctions (TJs), seen as single lines in apical belts, as was adhering junction (AJ) E cadherin (Ferruza et al, 1999, Obert et al, 2000, Musch et al, 2006), which was, however, also found as basal arrays of spots (Fujimoto, 1995). As observed with TEM (Farquar and Palade, 1963), the three belts were part of the junctional complex, along with the most apical E cadherin spot, the others being classified as desmosomes. Where more

than two cells met at junction points, some of these had brighter immunolabelling (Moyes et al, 2010b), termed multicellular junctions (MCJs, Moyes et al, 2011).

The two pre-treatments (Moyes et al, 2010a) caused different effects. Ethanol decreased the TER and increased permeability, confirming previous reports of TJ loosening and related changes (Bjarnason et al, 1984Ma et al, 1999, Banan et al, 2007, Joseph et al, 2008, Amin et al, 2009). As for canine kidney cells (MDCK, Armitage et al, 1994), cooling produced a temperature-dependent increase in TER. The paper was quoted with respect to data on ethanol-induced changes in junctional occludin, including its diffuse profiles, by Tria et al (2013a). A subsequent paper (Moyes et al, 2011, Section 4.6) showed that after exposure times of 60mins, ethanol loosened and disorganised TJs, while cooling did the opposite: the junctions were also made more diffuse or sharp, respectively.

For connective tissue cell compartmentalised culture, macrophages caused the Caco-2 TER to decrease (Moyes et al, 2010b), loosening tight junctions, possibly by cytokine effects (Satsu et al, 2006, Al-Sadi et al, 2009). Cells in the Caco-2/macrophage group were shorter and wider, with less regular outlines, some with double linear junctions. Occludin labelling was more diffuse and ZO-1 more irregular, as seen after exposure to TNFα (Ma et al, 2004), possibly related to actin redistribution (Fanning et al, 1998, 2002). Control M cell permeability was higher, the cells were shorter and more irregular and there were differences in junctional protein distribution between the two monolayers.

4.8.8 Monolayer uptake routes (Model 2)

Addition of particles to control Caco-2 monolayers produced an initial marked TER increase followed by a slight decrease: addition of medium produced only the initial increase (Moyes et al, 2007, Section 4.5). The increase was possibly related to cooling, since particles were stored at low temperature (Carr et al, 2012, Section 4.7): certainly, when cells were chilled to 0ºC for long enough, there was a TER decrease when particles, now warmer than cells, were added. The main decrease could have been associated with tight junction loosening and with uptake, as reported after exposure to other challenges (Stenson et al, 1993, Ferruzza et al, 1999, Araki et al, 2005) or to substances known to loosen tight junctions (González-Mariscal et al, 2005).

Increases in epithelial-associated particle numbers (EAPs, Moyes et al, 2007) took place over similar time-frames to the TER decrease. Submembranous particle numbers (SMPs) were higher after longer exposure (Moyes et al, 20010a, Section 4.6). Simulation of *in vivo*

peristalsis by removal of particles from the upper well caused a TER decrease and more EAPs in some groups (Moyes et al, 2007). Addition of vasoactive intestinal peptide (VIP) affected TER (Blais et al, 1997), but interpretation was complicated by its half-life (Gololobov et al, 1998) and the distance between the administration site and the receptors (Anderson et al, 2003). Citations to the data in this paper (Moyes et al, 2007) included references to particles reaching the epithelial surface through the unstirred water layer (Sugano, 2010), to its reports of paracellular uptake (Coppi et al, 2010, with Iannuccelli) by an enterocyte route (Iannuccelli et al, 2011, with Coppi) and to 'direct cellular uptake' with respect to micronutrients and nutroceuticals in a review by Velikov and Pelan (2008). Powell et al (2010), in their article on dietary nano and microparticles, made reference to it for the integrity of tight junctions being altered by particle 'endocytosis'.

Ethanol allowed more particles through the membrane, as well as loosening and disorganising TJs, while cooling had an opposite effect, but only after a sufficient exposure time (Moyes et al, 2011, Section 4.6). The first pre-treatment paper (Moyes et al, 2010a) was cited in a recent 'advanced review' on food-borne nanomaterials (Bouwmeester et al, 2018, with M. Jepson as an author): it was quoted for its report of *in vitro* effects of ethanol and for tight junction involvement, during comments on possible increased uptake associated with epithelial damage, such as at sites of cell turnover or in disease. Citations for data in the second pre-treatment paper (Moyes et al, 2011) included its use as a source for a TER increase/decrease after treatment (Tria et al, 2013a) and for the linkage of this to changes in ion permeable tight junction pores (Tria et al, 2013b).

After particle addition in the macrophage experiments (Moyes et al, 2010b, Section 4.6), Caco-2/macrophages had a lower TER. Submembranous particle numbers (SMPs) were high in the Caco-2/macrophage group, with many at or near the macrophages. These cells therefore loosened Caco-2 TJs and encouraged uptake, facilitated by their phagocytic abilities and the reduced epithelial cell height, possibly associated with actin remodelling. The macrophage paper (Moyes et al, 2010b) was cited by Uskoković et al (2012b), who referred to it during a description of the jagged nature of ZO-1 after particle exposure, categorising them and double line junctions as indicative of membrane disruption but not dysfunction. Li et al (2013) mentioned the relevance of the paper's report on increased uptake by Caco-2 monolayers in the presence of macrophages, especially for low permeability drugs.

After particle exposure, the M cell permeability increase was more obvious than that of Caco-2 cell monolayers. The initial report (Carr et al, 2012) that uptake was similar in both groups applied to the end of the experiment after exposure to particles for 120mins. However, in the early group, when the lack of an underlying connective tissue compartment as *in vivo* was less marked, M cell monolayers had more epithelium-associated particles and higher uptake rates (Carr, personal communication, 2018, Section 4.6). As in the macrophage experiments above (Moyes et al, 2010b), particles were often located at intercellular junctions of both control and treated groups. This was now described as producing two intercellular junction subsets, most without retained particles ('bare') and fewer with ('decorated'). Early particle-induced junctional protein plasticity occurred at adhering as well as at tight junctions and was often dissimilar at 'decorated' and 'bare' junctions. This suggested that uptake was paracellular, an echo of earlier TEM sightings there of latex particles (Smyth et al, 2005, Section 4.4), identifying the junctions as a possible uptake route. The higher early uptake by M cell monolayers could have been linked to cell shortening or the different junctional protein responses in apical edge levels or other parameters.

4.8.9 Particle-associated factors (Model 2)

Model 2 produced quantitative data, showing that most particles were often 'singles' and that the proportion of aggregates increased with exposure time (Moyes et al, 2008, Section 4.6, Carr et al, 2012, Section 4.7). *In vitro* and *in vivo in situ* particle numbers (Moyes et al, 2007, Section 4.5) were in the same range, especially for data calculated per 1000 cells (Moyes et al, 2008) or for ATCC cells (Carr et al, 2012). Particle numbers on and associated with the monolayer increased with time, more so than in the *in vivo* model, which had underlying connective tissue, facilitating onward particle movement to secondary organs, and avoiding build up at the epithelium. Data citations to the first pre-treatment paper (Moyes et al, 2010a, Section 4.6) included a reference to the good link between permeability *in vitro* and 'absorption' *in vivo* (Ferraro et al, 2014).

4.9 Q. 2 Are any groups 'at risk', such as pregnant or lactating females?

4.9.1 Pregnancy and lactation (Model 1)

The question of whether pregnant or nursing mothers and their offspring were more at risk after Chernobyl as in other exposures (Bhattacharya, 1983, Leazer et al, 2002) was an

important issue in drug delivery (Florence, 1997) and radionuclide dosimetry (Harrison, 1998). Proximo-distal profiles of uptake varied across groups with different reproductive profiles (Smyth et al, 2005, Section 4.4) and particle numbers for late pregnant and early lactating rats were higher than those for virgin female animals, significantly so 5 minutes after administration: the differences were related to numbers associated with villous enterocytes and goblet cells. Factors implicated included gastric emptying (Paterson et al, 2000), endocrine influence (Chang et al, 1995) and the role of mucus (Sharma et al, 1995): the higher particle numbers at goblet cells in early lactating groups could have reflected corresponding changes to mucus elsewhere (Gadegone and Bhiwagde, 1981). The main uptake route was confirmed to be villous for *in vivo in situ* uptake. This highlighted again the relevance of tight junctions, given their possible response to hormone modifiers and their leakiness in pregnant animals (Nguyen and Neville, 1998, Cereijido et al, 2000, Nusrat et al, 2000). Available data citations for the paper on reproductive status (Smyth et al, 2005) surprisingly did not include any on its main topic of 'at risk' groups. In terms of general citations, Wu et al (2011) quoted this and three others of the group's papers, along with two from S. Florence, with respect to several aspects of uptake, including the involvement of enterocytes as well as M cells. Kobori et al (2012) quoted it for uptake being probably paracellular through tight junctions, with respect to novel food particles being taken up.

As part of this work on groups with different reproductive history, pilot studies showed that particles were found in the stomachs of the three rat pups examined (Carr et al, 2012, Section 4.7).

4.9.2 Species, age and pathophysiology (Model 1)

The lack of difference in uptake across species (Doyle-McCullough et al, 2007, Section 4.4) was difficult to compare with the literature at the time, due to protocol variations (McClean et al, 1998). Age was, however, important: uptake in young adult rats was higher than that of young, mature and old groups, confirming some earlier data (Harrison and Fritsch, 1992, Seifert et al, 1996) but not others (LeFevre et al, 1989, Simon et al, 1994). Some young adult females, therefore, had a double risk of taking up harmful particles *in vivo in situ*, since this sensitive age coincided with their active reproductive period (Smyth et al, 2005, Section 4.4). Both factors were also important during dose calculations for vaccines or pharmacological agents. Citations were made to the Doyle-McCullough (2007) paper's data for particles being in the unstirred water layer on the intestinal surface in a review by Sugano and Terada (2015). There were references to it for evidence of uptake (Lai et al, 2008,

Jünemann and Dressman's 2012 review, Schleh et al, 2012) and for it taking place through 'direct cellular delivery (Velikov and Pelan's review, 2008) or at goblet cells (Johnson et al, 2011). There were also comments in a paper on dietary nano- and micro-particles (Powell et al, 2010), quoting the data, with those from two other papers from the group, as demonstrating a predominantly villous route. One paper highlighted correctly the core point of the paper, in its mention of the importance of the intestinal size and of the higher uptake in younger animals (Woitiski et al, 2008).

With respect to pathophysiology, the possible effect of particle exposure on individuals with another health problem (McMinn et al, 1996, Section 4.4), was explored using the model for streptozotocin-induced diabetes described by Ettarh and Carr (1997, Chapter 2, Section 2.5) prior to the study of post-irradiation responses (Ettarh et al, 2000, Section 3.9). Small intestines were longer and wider in diabetic groups, confirming earlier findings (Mantle et al, 1989). The maceration data wrongly implied that there were many more particles in the diabetic intestine, highlighting the need for microscopy, which showed that those supposed higher numbers were found only in luminal compartments and had not yet been taken into the tissues. The numbers in the different parts of the diabetic intestinal wall implied reduced particle movement, related perhaps to the thicker basal lamina (Mantle et al, 1989). For secondary organs, with no luminal compartments to complicate the issue, the maceration data were not inaccurate: lymph node and liver had fewer particles in diabetic groups and the microscopy data did not contradict this. The conclusion overall was therefore that diabetic individuals did not form another at-risk group. Data were cited for confirmation of 'non-lymphoid' uptake (Behrens et al, 2002) and secondary organ involvement (Yang et al, 1999, Akande et al, 2010). Lopes et al (2014) in a review article quoted it as demonstrating that diabetes could influence the extent of uptake and therefore the dosimetry associated with insulin delivery systems.

4.9.3 Irradiation and related exposures (Models 1 and 2)

Another 'at risk' situation related to the project having its roots in the accidental release of radioactive particles (Cuddihy et al, 1989, Wakeford, 2011a, b), when ambient radiation could be a factor. Studies were therefore carried out on radiation-induced changes in particle uptake.

For Model 1, microscopy of nodes showed more particles at the subcapsular sinuses 3 days after irradiation (Carr et al, 2012, Section 4.7). Other experiments indicated that reserpine

(ibid), known to affect intestinal motility (Plummer et al, 1955) and simulate irradiation effects (Indran et al, 1985, 1991, Section 3.7), increased total uptake, in keeping with the fact that atropine, with an opposite action (Carr et al, 1991a, Section 3.7), led to decreased uptake (Volkheimer et al, 1968c, Volkheimer, 1977).

For Model 2, the work related to radiation effects and tight junction responses (Moyes et al, 2008, Section 4.6). Radiation-induced changes in small intestine had been widely reported, including in reviews (Carr, 2001, Chapter 3, Section 3.10, Wang and Hauer-Jensen, 2003) and books, Galland and Spencer, 1990, Dubois et al, 1995, Fajardo et al, 2001). Tight junctions (TJs), implicated in particle uptake (McCullough et al, 1995, Section 4.3, Moyes et al, 2007, Section 4.5, Carr et al, 2012, Section 4.7), were disrupted by radiation (Porvaznik, 1979, Somosy et al, 1993). Immunocytochemistry had shown changes in the intercellular transmembrane proteins (Dublineau et al, 2004, de Carvalho et al, 2006). In Moyes et al (2008), irradiation produced lower and broader cells. The increase in calculated intercellular space was similar to that seen directly by TEM after radiation *in vivo* (Lieb et al, 1977) and *in vitro* (de Carvalho et al, 2006). This change was linked to the relocation of junctional proteins (Dublineau et al, 2004), possibly giving desmosomal loosening, which, if happening at hemi-desmosomes, could have produced disorganised basal laminae and epithelial/stromal stripping (Carr et al, 1981d, Chapter 3, Section 3.6) or loss of epithelial integrity (Dublineau et al, 2004). For submembranous (SMP) numbers, irradiated groups allowed more through than controls. In conclusion, external irradiation could have affected uptake, with possible links between this and changes in permeability. Citations to this paper included those to data for uptake being influenced by various factors, including external radiation (Wu et al, 2011) and radiation-induced alterations in permeability and microparticle uptake (Morini et al, 2017). References are also made to it for irradiation causing disruption or opening of tight junctions (Stern et al, 2013, Strup-Perrot et al, 2016). In the review already mentioned above for ethanol (Section 4.8.8), the paper was quoted for irradiation affecting paracellular uptake and tight junctions (Bouwmeester al 2018, with M. Jepson as an author).

In the pilot Model 2 study with I. Dublineau, depleted uranium, iron and latex all produced the increase/decrease TER response pattern, particularly the higher concentration of the uranium (Carr et al, 2012, data courtesy of Dublineau, Moyes, Steffani, Carr). This project was not continued when support ended as the grant body's radioprotection programme was reviewed.

4.10 Overview and conclusions

The review (Carr et al, 2012, Section 4.7) made several points, including: the advantages of the microscopy technique; the small percentage of administered particles taken up; the preponderant *in vivo in situ* villous uptake and the possible role of tight junctions, confirmed *in vitro*; the location of many particles near or at intercellular junctions; and the considerable plasticity of proteins at adhering as well as tight junctions.

This position in the account of Chapter 4 has been chosen to put the review (ibid) in context with the later literature. It has been quoted in articles on additional applications of uptake, including microsphere design (Kazazi-Hyseni et al, 2014), drug encapsulation (Ramazani et al, 2016), engineered nanomaterials in soils (Cornelis et al, 2014), regulation of nanoparticle drug delivery (Hussain, 2016), environmental plastics in food production (Bouwmeester et al, 2015) and satiety-simulating food additives (Naftalovich et al, 2016). Data topics cited included the villous, non-follicle-associated epithelial site of uptake (Braakhuis et al, 2015, Reineke et al, 2013), the significance of this with respect to the time after particle administration and whether or not to target drugs to M cells (Hussain, 2016), the possible involvement of tight junctions (Cornelis et al, 2014) and the percentage uptake (Bouwmeester et al, 2015). The article was also cited for the importance of particle size (Kazazi-Hyseni et al, 2014, Ramazani et al, 2016), while Naftalovich et al (2016), describing it as having made 'an extensive review of the topic', quoted it verbatim for the relationship between size, uptake and penetration. Types of citing articles included a review article (Braakhuis et al, 2015), a critical review (Bouwmeester et al, 2015) an article on 'unique insights' into the fate of microspheres (Reineke et al, 2013) and an article forming part of a special journal issue to honour the contributions of S. Florence to the field (Hussain, 2016).

In conclusion, the answers to the two questions posed post-Chernobyl were as follows.

To the first question, 'Do microparticles enter tissues by an intestinal route ?', the answer was that, after *in vivo in situ* exposures as short as 5 minutes, particles did cross the small intestinal epithelium, moving on to secondary organs such as lymph nodes: particle dose and size were important. Entry at times soon after uptake was, unexpectedly, mostly through villous epithelium and not the M cells of lymphocyte-rich Peyer's patches. Particles were observed at intercellular junctions or spaces, highlighting the possible involvement of enterocyte tight junctions. *In vitro* work addressed this, showing that ethanol loosened junctions and increased uptake, while cooling produced opposite effects and different

changes in immunolabelled junctional proteins. Particle retention at intercellular junctions was also seen in this Model, further implicating tight junctions and their associated proteins.

To the second question, 'Are any groups 'at risk', such as pregnant or lactating females ?', the answer was that uptake could be higher in late pregnancy and early lactation, was more affected by age than species, was not increased by diabetes, but could be increased by irradiation.

There has therefore been continuing evidence of the relevance of microparticle uptake, not only to diet and therefore environmental issues (Volkheimer and Schultz, 1968), but also to drug and vaccine delivery (O'Hagan, 1990), to clinical topics such as multiple organ dysfunction syndrome (Nieuwenhuijzen et al, 1996) and to the additional areas listed in the citations entries for the review (this section, above). The data have presented strong confirmation of the earlier firm statement that 'persorption of solid particles in the micrometer range is a fact' (Volkheimer, 1977) and the equally definite description of the process as 'neither exceptional nor unusual' (Florence, 1997).

4.11 Context and comment

The theme here was the application of the understanding of mammalian small intestine acquired in Chapters 1 and 2 and of the multi-parameter approach of Chapter 3 to the exploration of the sites and pathways involved in microparticle uptake across the small intestinal wall from lumen to serosa. The main addresses were Belfast, Harwell and Oxford, covering only twenty years, publications running from 1995 to 2012: this was a shorter span than Chapters 2 or 3 and included a smaller number of publications.

For output destination, fewer were in anatomical journals, although the main review was in Progress in Histochemistry and Cytochemistry. There was also a small number of publications in general biological journals and more in those specialising in radiobiology, gastroenterology, or pharmaceutical science.

With respect to collaborative research, the topic of microparticle uptake involved proportionally more in-house work, in Belfast and Oxford. There were, however, still important networks. These involved already established links, such as that with Imperial Cancer Research Fund (ICRF) in London, who moved one of their staff and accompanying equipment to Belfast during a reorganisation. There were collaborations with the National Radiological Board (NRPB), Atomic Energy Authority, St Bartolomew's Hospital, London and the University Medical Centre, Hamburg Eppendorf, Germany. These and other links

brought contacts with groups interested in toxicology, drug delivery, gastrointestinal biology and clinical applications, such as surgery. The establishment of the Caco-2 *in vitro* model relied on the advice and help of Professor Ian Sanderson and his colleagues, Queen Mary University, London.

Roles and contributions changed during this period, but in a slightly different way, given the end of the commitment as Director of School and a two-year sabbatical in Harwell and Oxford and then early retirement to allow these attachments to continue. Much of the work was done while supervisor, Head of Department/School or group leader, but there was a continuing involvement in protocol design, the provision of technical training, data quality control and writing of reports and papers. Most recently, the role has been as Principal Investigator on the Department of Health Grant: while Academic Visitor in Anatomy, Department of Physiology, Anatomy and Genetics, University of Oxford, involvement has included writing grant renewal proposals, presenting the outcome at annual funding body meetings, guiding the direction of the programme, supervising the younger scientists involved and contributing in a major way to the production of reports and papers: time was also once more available for contributions to data recording.

With respect to links to the next Chapter, the summary of the data on uptake has provided a good bridge to Chapter 5, which has been constructed to provide an overview of the contents of all four Chapters in context with the wider literature.

Chapter 5 Conclusions – key findings and significance

Preface - extract from Presubmission Abstract (Chapter 5)

The Chapters above concentrate on the scientific findings, from work done when on staff in Universities in Glasgow and Belfast and when a Guest researcher/Visitor in Berkeley, Harwell or Oxford. These Chapters also: comment on the work's significance; discuss article and journal choice; describe collaborative links with London, Chicago, Hamburg and others; and mention where 'hands-on' work gave way to supervisory input. Chapter 5 draws these all together and highlights the linking theme of the importance of probing surfaces as well as exploring underlying tissues.

5.1 Background to output

Now that all the data sets and their interpretation have been set out in Chapters 1 to 4, an overview has been constructed of their content and possible influence. As set out in the Preface, all but 2/103 submissions (Carr et al, 2001, Chapter 4, Section 4.3, Carr, personal communication, 2018, Chapter 4, Section 4.6) were published as articles in journals, the records of conference proceedings or as chapters or books. In the 101 full publications, 80% were in one or other 'lead' position in the authorship list. In only one (recollected to be Ettarh and Carr, 1996b, Chapter 2, Section 2.5), was input to writing minimal or non-existent, taking place after the graduate student had moved on to another Department: this paper was, however, included because it was the culmination of several mini-projects on the subject, initiated and supervised by the thesis author.

Output was published in journals associated with the disciplines of anatomy, histology or microscopy, including the Journal of Anatomy and the Journal of Microscopy, associated with the Anatomical and Royal Microscopical Societies respectively. The papers in Scanning Electron Microscopy were placed there because of the advantages of meeting other early scanning electron microscope (SEM) practitioners at the annual conferences where the pre-publication papers were delivered as oral presentations. Those in the Journal of Submicroscopic Cytology/Pathology were submitted there because of the excellence of their micrograph reproduction, a matter of importance when developments in imaging or interpretation were being reported.

During the time of publication of the work, there has been increasing emphasis on journal Impact Factors. Although these were not borne in mind during selection of output destination when the accompanying material was being worked on, an analysis has been made recently of the Factors for the years 1999, 2006 and 2016. None of the Journals used in the thesis was as highly cited as journals such as Nature or Cell. However, some of the Journals were well placed in their group, such as Gut, in the top first or second position in a group of between 87 and 134 Journals in the Gastroenterology subject area and the Journal of Investigative Dermatology in the top place in a group of more than 100 Journals. The Pharmaceutical Journals were also on the whole in good positions, Pharmaceutical Research in the top three in 1999, The International Journal of Pharmaceutics approximately in the top 10% and The Journal of Drug Targeting within the top 20%. The Journal of Anatomy and The Journal of Microscopy were less well placed, but usually in the top third of their lists.

5.2 Influence of publications in Chapters 1 to 4

Chapters 1 through 4 have given an account of the scientific flow and development of the work, including citations to the individual publications. This Subsection has looked at the publications more generally, in three ways, each aiming to comment on the most important five or so publications: this demanding criterion was used to minimise the space taken up by this aspect of the thesis.

5.2.1 Representative papers

These five papers have been selected to reflect retrospective interest in aspects of the main thrust of the work, taking it from the systematic observation of surfaces with correlative SEM, through analysis of irradiated surfaces and their underlying responses to uptake of microparticles by an ‘unexpected' route.

The first (Carr et al, 1983a, Chapter 2, Section 2.4) came from the account of the emergence of scanning electron microscopy. It was the introductory low temperature paper, representative because it was early in the use of its particular technique for the examination of small intestinal villi and had used correlative techniques to maximise the information produced.

The second and third papers were from the Chapter on correlative morphology of irradiated small intestinal surfaces. The second (Carr et al, 1983c, Chapter 3, Section 3.5) applied two

dimensional information from the literature to produce the objective scoring system used to take the description of radiation-induced villous collapse beyond the merely descriptive. It analysed data from the study of surfaces and linked them with possible underlying stromal changes, in this case defining villous pegs and pericryptal plates as important. The third paper (Carr et al, 1992a, Chapter 3, Section 3.8) took this theme further, in particular the importance of non-epithelial tissues. This was the use of the Morphological Index display to compare the effects of changing dose and time after X-irradiation. It extended data collection from crypt count assays to include several villous and cryptal parameters, illustrating in graphical form their wide range of responses as dose and time changed.

The fourth and fifth papers were from the Chapter on microparticles crossing intestinal surfaces. The fourth (Smyth et al, 2005, Chapter 4, Section 4.4) was representative of the *in vivo in situ* model for uptake in using maceration and microscopy. It used nine proximo-distal segments and gave proof of validation of the techniques and evidence for the predominant uptake pathway being villous and not at Peyer's patches. It illustrated with transmission electron microscopy the presence of latex microparticles in intercellular spaces, thus confirming a possible role for tight junctions. It also showed that particles had moved on to secondary compartments or organs. The fifth paper, in a study of the effect of macrophages on uptake in the *in vitro* model (Moyes et al, 2010b, Chapter 4, Section 4.6), illustrated a full range of typical *in vitro* techniques, including measurement of transepithelial resistance (TER), cytokine levels, cell dimensions, particle counts for the lower well and also immunolocalisation of junctional proteins. The data supported the involvement of tight junctions during exposure to particles and highlighted the importance to uptake of the underlying cells, in this case macrophages. It also showed particles associated with intercellular junctions, complementing their sighting *in vivo* in intercellular spaces (Smyth et al, 2005, above) and again implicating tight junctions and a paracellular uptake route.

All five, although selected to be representative scientifically, also summarised the research in other ways, through the scientific centres involved, collaborative input, student contributions or Journal choice. The addresses reflected the move from hands-on contribution as a Glasgow University staff member through involvement as School Director in Biomedical Science in Belfast to being part of a small research grouping in Oxford University. Collaborative links were also representative, including Radiotherapeutics in Glasgow, The MRC Cyclotron Unit at the Hammersmith Hospital, London, the Lawrence Berkeley Laboratory, California, USA and the University of Washington, Seattle, USA.

The composition of the author list was also typical of the whole, including academic colleagues in Belfast and Oxford, technicians in Glasgow and Belfast and graduate students in Belfast and Oxford. The Journal choice spanned the types of output destination used, with three anatomical, one clinical radiology and one radiation science journal.

In conclusion, these five papers represented the whole body of work, although they were selected by subjective criteria, relying entirely on the author's opinion of the papers. The use of this 'author's hindsight' approach to select subjects of interest has also drawn attention to the fact that some have received less attention than was optimal, partly due to research focus changing or unavailability of funding. Selection of one 'sidelined' topic in each main Chapter focussed attention on the following areas that required more research: the significance of microridges (Shasha'a et al, 1993, Chapter 2, Section 2.5, Robertson et al, 1987, Chapter 3, Section 3.3), the possible overlapping effects of existing abnormal conditions and external irradiation (Ettarh et al, 2000, Chapter 3, Section 3.9) and the effects of irradiation or radioactive and associated particles (Moyes et al, 2008, Carr et al, 2012, Chapter 4, Sections 4.6, 4.7)

A different subjective author's view of the whole output has been the identification of a common approach throughout. This was a tendency to look at additional topics that complemented what was regarded at the time as a main theme in the research field. For the SEM work, this involved an interest not only in the production of surface images, but also the use of non-standard images and correlative methods. For the subcellular radiobiology, it took the form of reporting not only what could have caused side-effects in developing therapeutic treatments, but also collecting data on non-proliferative structures not identified classically as 'targets'. For the particle uptake work, the finding that, as an early phenomenon *in vivo in situ*, it occurred predominantly at villous sites, led to interest in the plasticity of junctional proteins and to the observation by others that over-concentration on targeting M cells could be unproductive.

5.2.2 Highly cited papers

This analysis, carried out during thesis preparation in 2017, used the top five papers in the Scopus citation data base. Only the peer-reviewed publications put forward in the thesis were included. This involved disregarding three papers within the top eight, two being duplicate coverage of material already in submitted papers and one stemming from a colleague's research area. In addition, a note was made of papers not in the Scopus list, but

ranked by Google Scholar in the top fifteen, giving consideration to publications of a type not included in Scopus. None of the research fields was large and none of the papers attracted long lists of citations, the largest here being 75 in Scopus and 135 in Google Scholar: all those selected had 30 or more citations.

The first paper in the top five of the Scopus list, with 75 citations, described the mainly villous route of microparticle uptake *in vivo in situ*: it was published in Digestive Diseases and Sciences (Hodges et al, 1995b, Chapter 4, Section 4.2). The second in the list, with 66 citations, was on the effect of dietary fibre, also in a specialist gastrointestinal Journal, Gut (McCullough, J.S. et al, 1998, Chapter 1, Section 1.2). The third, with 65 citations, was a further paper on particles, this time on their size, in Pharmaceutical Research (Carr et al, 1996c, Chapter 4, Section 4.4). The fourth, with 35 citations, was another paper on diet, this time on fat and in the British Journal of Nutrition (Sagher et al, 1991, Chapter 1, Section 1.2). The fifth paper, with 33 citations, was again a microparticle uptake paper, on the effect of diabetes on microparticle uptake, in the Journal of Anatomy (McMinn et al, 1996, Chapter 4, Section 4.4).

Consultation of Google Scholar produced two other publications for consideration, again omitting papers from the top fifteen of a similar type to those excluded from the Scopus list. Its surprising top scorer with 135 citations was the book on the digestive system (Toner et al, 1971, Chapter 2, Section 2.2), with some early SEM images. Another publication in the same Chapter and not in the Scopus list, was the Letter to the Editor of Lancet, describing briefly with SEM images the three dimensional structure of small intestinal villi (Carr and Toner, 1968, Chapter 2, Section 2.1): it had 31 Google Scholar citations and in was in thirteenth place.

In conclusion, the Scopus top five were either in Chapter 1, reflecting contributions to strong collaborative groupings, or in Chapter 4, whose more ‘in-house’ subject of microparticle uptake has been of continuing interest recently, with relevance to environmental issues. The Google Scholar additions from Chapter 2 included here were from early SEM publications, often reaching a wide audience due to the images providing scientific and educational information in what was then a novel format. Another notable point was that although neither Chapter 2 nor Chapter 3 had any papers in the Scopus top five, when the filter was relaxed to include the top ten, one article in Chapter 2 and two in Chapter 3 would have been included. With respect to Journal choice, all but one of the Scopus top five were in specialist journals, mostly in the gastrointestinal field.

5.2.3 Citations of interest

The five publications here have also been chosen using Scopus and Google Scholar, usually from papers in the top 20% of the lists: the selection was made mainly because of the topic highlighted, although the standing of the citing group was also taken into account.

In the first two Chapters, one of the most interesting citations was to the work on cells settling on glass (Carr and Carr, 1970, Chapter 2, Section 2.1) for its role in the identification of the Kupffer cells of the liver as of the macrophage line (Muto, 1975, Motta, 1975). Another citation of note was to the SEM review (Carr, 1971, Chapter 2, Section 2), where it was used as one of three key papers setting the scene in an overview of 'trends and prospects' in SEM (Hayes, 1974): it was also of interest because of the reputation of the author quoting it in this way.

For the work on the effects of irradiation, the main review (Carr, 2001, Chapter 3, Section 3.10) was included, for its range of citation dates, up to 2017, but also for the range of topics quoted. These included citations to the different responses of constituent cells and tissues of the intestinal wall (Wang and Hauer-Jensen, 2003, Veerginadis et al, 2017), demonstrating that individuals with different interests were accessing information by reading the Index Tables as they had been designed. It was also cited with reference to mechanistic aspects (Senesse et al, 2009) and to clinical topics (Cameron et al, 2012, Barnett et al, 2013), including possible post-irradiation treatments (Ishihara et al, 2011). Another citation, by Hauer-Jensen (2014), one of the key authorities in the field, was also notable because of its description of the paper as a 'very comprehensive review'.

From the work on microparticle uptake, Doyle-McCullough et al (2007, Chapter 4, Section 4.4) was cited on its key finding that uptake was higher in young animals (Woitiski et al, 2008), relevant to pollution, drug delivery and multiple organ dysfunction syndrome (MODS): this paper also had a citation for percentage uptake and the predominance of villous involvement in a paper on dietary nano- and micro-particles (Powell et al, 2010), referring to it as part of the 'extensive studies from Kate (KE) Carr's group in Belfast, and now Oxford'. Citations to the review on microparticle uptake by Carr et al (2012, Chapter 4, Section 4.7) included one crucially covering the villous site of uptake and its relevance to whether or not drug targeting should be aimed at M cells (Hussain, 2016): it was also of interest because it came from a paper published as part of a special issue dedicated to S. Florence, marking his special contributions to particle uptake.

In conclusion, this selection was as subjective as the first one listing representative papers (Section 5.2.1), since it also depended entirely on the author's' choice, but it did have hidden semi-objective information on how other groups had viewed the work.

5.2.4 Summary of output analysis

All the publications were in academic outlets and of the seventeen selected, most were in standard Journals, a few in review series, one from a book and one a letter to an editor. Although original articles took all the Scopus top five citation places numerically, review articles appeared to have more influence overall than has recently been thought. Of the topics covered, most were entirely or mainly on gut, a few on macrophages or their influence on gut cells and one was methodological, dealing with instrumentation and image interpretation. Over 80% of the selected publications had the thesis author in one of the 'lead' positions, similar to the percentage for the whole published submission list. When comparison was made of the output selected by these three different ways of assessing their contributions, none featured in more than one list, leading to the unsurprising conclusion that what seemed to be of most importance or interest to an author did not necessarily attract attention from a wider readership.

5.3 Context and comment - probes and surfaces

The theme of probing has been found throughout the thesis, at more than one level, probing both to produce images and also for a response to environmental challenge, whether or not the research was more applied, as in post-Chernobyl *in vivo* microparticle work, or more 'blue sky' as in some of the corresponding *in vitro* experiments.

In both Chapters 1 and 2, the probing was mainly but not exclusively, at one level, relating therefore substantially to imaging. In Chapter 1, electrons or light produced images of sectioned material, working out the relationship of the constituent structures. In Chapter 2, one-level probing was by a de-magnified electron beam interacting with the superficial part of specimens and producing images of different types, often needing correlative data to interpret them. Malpighi (1687, in Carr et al, 1997, Chapter 4, Section 4.1) would perhaps have had a comment, 'Observation by means of the microscope will reveal more wonderful things than those viewed in regard to mere structure and connexion'.

In Chapter 3, the probing was usually at two levels, addressing further the point of 'structure and connexion'. Firstly, the sample was probed experimentally by external irradiation,

interacting with control multicellular mammalian tissues, to produce a range of outcomes and sometimes to facilitate interpretation of control structure. Secondly, these samples were then examined as in Chapters 1 or 2, probed by an electron beam to produce images of the radiation-induced responses, leading to correlation, not just structurally, but with parameters associated with the pathophysiology of intestinal function. The appropriate comment by Malpighi (ibid) could have been 'The solution of those problems may prepare the way for greater things and will place the operation of nature more clearly before the eyes'.

In Chapter 4, the 'greater things' could have been related to the fact that there were more examples of up to four types of probe, at two levels. Firstly, experimental probing involved the addition of microparticles, which explored the surfaces of mammalian tissues or cells and found pathways through to underlying tissues or compartments. A proportion of the submissions also had a second example of this type of experimental probing, such as subjects with immune or other pathophysiological issues or samples exposed to ethanol, ice or irradiation. Secondly, there was probing as in Chapters 1 and 2, by the production of confocal or electron microscope images, in some cases with a further level of probe, using immunocytochemistry to chart the presence and distribution of junctional proteins and explore their responses. And the final comment from Malpighi (ibid), perhaps with tongue in cheek, could have been 'And when we try to unravel the obscure things in the viscera of animals, at length by our efforts, and only with great weariness, we conclude that the truth of our observations is made out'.

Complete list of References in Harvard format

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Probing tissue surfaces

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Volume 2 of 3

Accompanying material

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Scn	Section
No	Number of publication in sequence in Section, as presented in the text
a/b	Designation of publication when more than one/year: in some cases there are additional publications in the same year, not included in the thesis, but cited in the text and given in the full list of References.
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Ch	Scn	No	Authors	Date	a/b	Title	Reference	PS
1	1	1	Carr KE, Arbuthnott JP, Toner PG, Gemmell CG	1967		The fine structure of rat liver cells in suspension	Z Zellforsch. 79: 265-71.	1
		2	Carr KE	1967		Fine structure of crystalline inclusions in the globule leucocyte of the mouse intestine	J Anat. 101: 793-803.	2
		3	Carr KE, Whur P	1968		Ultrastructure of globule leucocyte inclusions in the rat and mouse	Z Zellforsch. 86: 153-62.	3
		4	Moore MR, Meredith PA, Goldberg A, Carr KE, Toner PG, Lawrie TD	1975		Cardiac effects of lead in drinking water of rats	Clin Sci Mol Med. 49: 337-41.	13
1	2	1	Ettarh RR, Carr KE	1996	a	Morphometric analysis of the small intestinal epithelium in the indomethacin-treated mouse	J Anat. 189: 51-6.	75
		2	Abbas B, Hayes TL, Wilson DJ, Carr KE	1989		Internal structure of the intestinal villus: morphological and morphometric observations at different levels of the mouse villus	J Anat. 162: 263-73.	52
		3	Sagher FA, Dodge JA, Johnston CF, Shaw C, Buchanan KD, Carr KE	1991		Rat small intestinal morphology and tissue regulatory peptides: effects of high dietary fat	Br J Nutr. 65: 21-8.	59
		4	McCullough JS, Ratcliffe B, Mandir N, Carr KE, Goodlad RA	1998		Dietary fibre and intestinal microflora: effects on intestinal morphometry and crypt branching	Gut. 42: 799-806.	88

Ch	Scn	No	Authors	Date	a/b	Title	Reference	PS
2	1	1	Carr KE, Toner PG	1968		Scanning electron microscopy of rat intestinal villi	Lancet. 2: 570-571.	4
		2	Ferguson A, Maxwell JD, Carr KE	1969		Progressive changes in the small-intestinal villous pattern with increasing length of gestation	J Path. 99: 87-91.	5
		3	Carr KE, Joffe SN, Toner PG, Watt C	1979	a	A preliminary study of scanning electron microscopic changes of the duodenum during healing of duodenal ulcers	Scand J Gastroenterol Suppl. 54: 78-83.	16
		4	Carr KE	1970	c	Scanning electron microscope studies of human skin	Br J Plast Surg. 23: 66-72.	6
		5	Carr KE, Carr I	1970		How cells settle on glass: A study by light and scanning electron microscopy of some properties of normal and stimulated macrophages	Z. Zellforsch. 105: 234-241.	7
		6	Cook GT, Carr KE, Duncan HJ	1979		The influence of morphological differences in bracken pinnules on the foliar uptake of aminotriazole	Ann Appl Biol. 93: 311-317.	17
		7	Al-Jaff DMA, Cook, GT, Carr KE, Duncan HJ	1982		Further studies on bracken morphology in relation to herbicide uptake	In 'The Plant Cuticle' Eds Cutler, DE, Alvin KL, Price CE Academic Press, London. 293-301.	26
2	2	1	Carr KE	1971		Applications of scanning electron microscopy in biology	Int Rev Cytol. 30: 183-255.	8
		2	Toner PG, Carr KE, Wyburn GM	1971		The Digestive System - an Ultrastructural Atlas and Review Excerpt	Butterworths.	9
		3	Carr KE, Shaw Dunn J, Toner PG	1974		Scanning electron microscopy of the alimentary tract	Scot Med J. 19: 211-220.	11

Ch	Scn	No	Authors	Date	a/b	Title	Reference	PS
2	3	1	Carr KE, Hamlet R, Nias AH, Watt C	1981	a	Multinucleate giant enterocytes in small intestinal villi after irradiation.	J Microsc. 123: 169-76.	20
		2	Carr KE, Wong AL, Young DG, Toner PG, Watt C	1981	b	Scanning electron microscopy and resin histology of large bowel biopsies	Scot Med J. 26: 103-14.	21
		3	Saleh KM, Toner PG, Carr KE, Hughes HE	1982		An improved method for sequential light and scanning electron microscopy of the same cell using localising microcoverslips	J Clin Path. 35: 576-80.	27
		4	Carr KE, Kamel HM, Toner PG, McGadey J, Wong A	1984	a	Correlative scanning electron microscopy in gastrointestinal pathology	Scan Electron Microsc. 2: 761-72.	35

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2	4	1	Carr KE, McGadey J	1974		Staining of biological material for the scanning electron microscope	J Microsc.100: 323-30.	12
		2	Hodges GM, Carr KE, Hume SP, Marigold JC, Southgate J, Marshall JF	1985		Changes in surface structure and concanavalin A-binding capacity of urothelium in the mouse bladder after whole-body neutron irradiation	Scan Electron Microsc. 4: 1603-14.	39
		3	Carr KE, Hayes TL, McKoon M, Sprague M, Bastacky SJ	1983	a	Low temperature scanning electron microscope studies of mouse small intestine	J Microsc.132: 209-17.	30
		4	Carr KE, Hayes TL, Hume SP, Kamel HMH	1983	b	Qualitative and quantitative SEM of gastrointestinal tract	Proc 41st Ann Mtg EMSA. 496-499.	31
		5	Carr KE, Hayes TL, McKoon M, Bastacky SJ, Kamel HM	1984	b	Etched surfaces of plastic embedded and frozen hydrated gastrointestinal tissue	J Submicrosc Cytol. 16: 219-26.	36
		6	Carr KE, Hayes TL, Watt A, Bastacky SJ, Klein S, Fife MG	1986	a	Specimen handling and data interpretation in X-ray microanalysis of frozen hydrated etched gastrointestinal tract	J Electron Micr Techn. 4: 371-379.	42
		7	Carr KE, Hayes TL, Watt A, Klein S, McKoon M, Bastacky SJ, Ellis S	1987	a	Biological variation in cryo-microanalytical data from mouse small intestinal villi	J Electron Micr Techn. 5: 65-74.	44
		8	Dickson GR, McKenna S, McColl K, Carr KE	1989		Examination of mucosal surfaces by scanning electron microscopy before and after removal of debris	J Microsc. 153: 75-9.	53
		9	Carr KE, Ellis S, Hayes TL Ainsworth EJ	1989		Multiview technique for SEM study of intestinal villi	Scanning 11: 109-110.	54
		10	Abbas B, Boyle FC, Wilson DJ, Nelson AC, Carr KE.	1990	a	Radiation induced changes in the blood capillaries of rat duodenal villi: a corrosion cast, light and transmission electron microscopical study	J Submicrosc Cytol Pathol. 22: 63-70.	55

Ch	Scn	No	Authors	Date	a/b	Title	Reference	PS
2	5	1	Nunn S, Gilmore RS, Dodge JA, Carr KE	1990		Exudate variation in the rabbit gastrointestinal tract: a scanning electron microscope study	J Anat. 170: 87-98.	56
		2	Laferla GA, Shashaa S, Crean GC, Gilmore RS, Carr KE	1988		Topographical variation in the mucosal surface of oesophageal biopsies	J Submicrosc Cytol Pathol. 20: 731-9.	49
		3	Shasha'a S, Dickson GR, Gilmore RS, Crean GC, Butt MM, Carr KE	1993		Rabbit and human non-keratinising stratified squamous oesophageal epithelium displays similar microridge structure by scanning electron microscopy	Scanning Microsc. 7: 953-959.	66
		4	Johnston BT, Nunn S, Sloan JM, Collins JS, McFarland RJ, Parkin S, Carr KE, Collins BJ	1996		The application of microridge analysis in the diagnosis of gastro-oesophageal reflux disease	Scand J Gastroenterol. 31: 97-102.	78

Ch	Scn	No	Authors	Date	a/b	Title	Reference	PS
2	5	5	Ferguson A, Carr KE, MacDonald TT, Watt C	1978		Hypersensitivity reactions in the small intestine. IV. Influence of allograft rejection on small intestinal mucosal architecture: a scanning and transmission electron microscope study	Digestion.18: 56-63.	15
		6	Ettarh, RR, Carr KE	1993		Structural and morphometric analysis of murine small intestine after indomethacin administration	Scand J Gastroent 28: 795-802.	65
		7	Ettarh RR, Carr KE	1997		A morphological study of the enteric mucosal epithelium in the streptozotocin-diabetic mouse	Life Sci. 61: 1851-8.	86
		8	McClean P, Dodge JA, Nunn S, Carr KE, Sloan JM	1996		Surface features of small-intestinal mucosa in childhood diarrheal disorders	J Pediatr Gastroenterol Nutr. 23: 538-46.	77
		9	Galloway DJ, Jarrett, F, Boyle, P, Indran, M, Carr, K, Owen, RW, George, WD	1987		Morphological and cell kinetic effects of dietary manipulation during colorectal carcinogenesis	Gut. 28: 754-763.	45
		10	Ettarh RR, Carr KE.	1996	b	Ultrastructural observations on the peritoneum in the mouse	J Anat. 188: 211-5.	76
		11	Simpson CJ, Toner PG, Carr KE, Anderson JD, Carter DC	1983		Effect of bile salt perfusion and intraduct pressure on ionic flux and mucosal ultrastructure in the pancreatic duct of the cat	Virchows Arch (Cell Pathol) 42: 327-342.	32

Ch	Scn	No	Authors	Date	a/b	Title	Reference	PS
2	6	1	Carr KE	1980		Scanning electron microscopy of macrophages	In 'The reticuloendothelial System' Vol I. Eds I Carr, WT Daems. 259-295.	19
		2	Carr KE, Toner PG, Saleh KM	1982	a	Scanning electron microscopy	Histopathology. 6: 3-24.	28
		3	Carr KE, Toner PG	1982		Cell Structure An Introduction to Biomedical Electron Microscopy. 3rd edition. Excerpt	Longman Group.	29
		4	Carr KE, Toner PG	1984		Morphology of the intestinal mucosa	Handbook Exptl Pharmacol. 70/I: 1-50.	37

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3	1	1	Carr KE, Toner PG	1972		Surface studies of acute radiation injury in the mouse intestine	Virchows Arch B Cell Pathol. 11: 201-10.	10
		2	Hamlet R, Carr KE, Toner PG, Nias AH	1976		Scanning electron microscopy of mouse intestinal mucosa after cobalt 60 and D-T neutron irradiation	Br J Radiol. 49: 624-9.	14
		3	Hamlet R, Carr KE, Nias AH, Watt C	1981		Surface damage in the small intestine of the mouse after X - or neutron irradiation	Scan Electron Microsc. 4: 73-8.	22
		4	Carr KE, Hamlet R, Nias AH, Watt C	1979	b	Lack of correlation between villus and crypt damage in irradiated mouse intestine	Br J Radiol.52: 485-93.	18
3	2	1	Carr KE	1981		Scanning electron microscopy of tissue response to irradiation	Scan Electron Microsc. 4: 35-46.	23
		2	Carr KE, Seed TM	1983		Ultrastructural effects of radiation on tissues and cells Excerpt	Soft-cover book, 17 papers, reprinted from Scan Electron Microsc 1981, 1982.	33
3	3	1	Robertson AG, Wilson P, Wilson DJ, Carr KE, Hunter I	1987		Microplication patterns on human buccal epithelia following radiotherapy: a scanning electron microscopic analysis	J Submicrosc Cytol. 19: 515-21.	46
		2	Wang Q, Dickson GR, Abram WP, Carr KE	1994		Electron irradiation slows down wound repair in rat skin: a morphological investigation	Br J Dermatol. 130: 551-60.	68
		3	Wang Q, Dickson GR, Carr KE	1996		The effect of graft-bed irradiation on the healing of rat skin grafts	J Invest Dermatol. 106: 1053-7.	79
3	4	1	Indran M, Carr KE, Boyle FC	1988		Radiation-induced changes in mouse duodenal papilla	Br J Radiol. 61: 1039-42.	50
		2	Carr KE, Hayes TL, Abbas B, Ainsworth EJ	1990		Collared crypts in irradiated small intestine	J Submicrosc Cytol Pathol. 22:265-71.	57

Ch	Scn	No	Authors	Date	a/b	Title	Reference	PS
3	5	1	Carr KE, Hamlet R, Nias AH, Watt C	1983	c	Damage to the surface of the small intestinal villus: an objective scale of assessment of the effects of single and fractionated radiation doses	Br J Radiol.56: 467-75.	34
		2	Carr KE, Ellis S, Michalowski A	1986	b	Surface studies of duodenal lesions induced by thoracic irradiation	Scan Electron Microsc. 1: 209-19.	43
	6	1	Carr KE, Hamlet R, Watt C	1981	c	Scanning electron microscopy, autolysis, and irradiation as techniques for studying small intestinal morphology	J Microsc.123: 161-8.	24
		2	Carr KE, Toner PG, McLay AL, Hamlet R	1981	d	The ultrastructure of some gastrointestinal lesions in experimental animals and man	Scand J Gastroenterol Suppl. 70: 107-28.	25
		3	Carr KE, Hamlet R, Nias AH, Boyle FC, Fife MG	1985		Stromal damage in the mouse small intestine after Co60 gamma or D-T neutron irradiation	Scan Electron Microsc. 4: 1615-21.	41
		4	Carr KE, Hayes TL, Indran M, Bastacky SJ, McAlinden G, Ainsworth EJ, Ellis S	1987	b	Morphological criteria for comparing effects of X-rays and neon ions on mouse small intestine.	Scanning Microsc. 1: 799-809.	48
		5	Abbas, B, Hume SP, McCullough JS, Wilson DJ, Stewart PC, Carr KE	1990	b	Early morphological changes in blood capillaries of mouse duodenal villi induced by X-irradiation	J Submicrosc Cytol Pathol 22: 609-614.	58
		6	Carr KE, Hamlet R, Nias AH, Watt C	1984	c	Morphological differences in the response of mouse small intestine to radiobiologically equivalent doses of X and neutron irradiation	Scan Electron Microsc. 1: 445-54.	38
		7	Wyatt MG, Hume SP, Carr KE, Marigold JC	1987		A preliminary study of the role of gastrointestinal endocrine cells in the maintenance of villous structure following X-irradiation	Scanning Microsc. 1: 291-300.	47

Ch	Scn	No	Authors	Date	a/b	Title	Reference	PS
3	7	1	Kamel HM, Hume SP, Carr KE, Marigold JC, Michalowski A	1988		Development of villous damage in mouse small intestine after local hyperthermia or irradiation	J Submicrosc Cytol Pathol. 20: 185-93.	51
		2	Indran M, Boyle FC, Carr KE	1985		Simulation with pharmacological agents of radiation damage to small intestinal villi	Scan Electron Microsc. 3: 1165-75.	40
		3	Indran M, Carr KE, Gilmore RS, Boyle FC	1991		Mucosal changes in mouse duodenum after gamma-irradiation or reserpine treatment	J Submicrosc Cytol Pathol. 23: 267-78.	60
		4	Carr KE, Bullock C, Ryan SS, McAlinden MG, Boyle FC	1991	a	Radioprotectant effects of atropine on small intestinal villous shape	J Submicrosc Cytol Pathol. 23: 569-77.	61
3	8	1	Carr KE, McCullough JS, Nunn S, Hume SP, Nelson AC	1991	c	Neutron and X-ray effects on small intestine summarized by using a mathematical model or paradigm	Proc Roy Soc London B. 243: 187-94.	62
		2	Carr KE, Nelson AC, Hume SP, McCullough JS	1992	a	Characterization through a data display of the different cellular responses in X-irradiated small intestine	J Radiat Res. 33: 163-77.	63
		3	Carr KE, Hume SP, Nelson AC, O'Shea O, Hazzard RA, McCullough JS	1996	a	Morphological profiles of neutron and X-irradiated small intestine	J Radiat Res. 37: 38-48.	80
		4	Carr KE, McCullough JS, Nelson AC, Hume SP, Nunn S, Kamel HM	1992	b	Relationship between villous shape and mural structure in neutron irradiated small intestine	Scanning Microsc. 6: 561-72.	64
		5	Carr KE, McCullough JS, Gilmore R S, Abbas, B, Hume SP, Nelson AC, Hayes TL, Ainsworth EJ	1993		Effects of neon ions, neutrons and X rays on small intestine	Biological effects and physics of solar and galactic cosmic radiation Part A Eds Swenberg CE et al Plenum Press, New York. 203-216.	67
		6	Carr KE, McCullough JS, Brennan P, Hayes TL, Ainsworth EJ, Nelson AC	1994		Heavy ion induced changes in small intestinal parameters	Adv Space Res. 14: 521-30.	69

Ch	Scn	No	Authors	Date	a/b	Title	Reference	PS
3	9	1	Brennan PC, Carr KE, Seed T, McCullough JS	1998		Acute and protracted radiation effects on small intestinal morphological parameters	Int J Radiat Biol. 73: 691-8.	89
		2	Ettarh RR, Hodges GM, Carr KE	2000		Radiation effects in the small bowel of the diabetic mouse	Int J Radiat Biol. 76: 241-8.	91
		3	Brennan PC, McCullough JS, Carr KE	1999		Variations in cell and structure populations along the length of murine small intestine	Cells Tissues Organs. 164: 221-6.	90
	10	1	Carr KE, Hume SP, Ettarh R, Carr EA, McCullough JS	1995		Radiation-induced changes to epithelial and non-epithelial tissue	In Radiation and the Gastrointestinal Tract Eds A. Dubois. D.R. King; D.R. Livengood. Taylor and Francis: 113-128.	70
		2	Carr KE	2001		Effects of radiation damage on intestinal morphology	Int Rev Cytol. 208: 1-119.	92

Ch	Scn	No	Authors	Date	a/b	Title	Reference	PS
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