
# Plastic Embedding Techniques for Light Microscopy Histological Studies

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## ABBREVIATIONS

| | |
|---|---|
| ABC | avidin biotin complex |
| ACTH | adrenocorticotrophin |
| APES | 3-amino propyltriethoxysilane |
| BMA | N-butyl methacrylate |
| BrdU | bromodeoxyuridine |
| DAB | 3,3-diaiminobenzidine tetra-hydrochloride |
| DMA | N-N-dimethylaniline |
| DMBA | 4-tert-butyl-N,N-dimethylaniline |
| DMPT | N-N-dimethyl *para*-toluidine |
| DMSX | N-N 3,5-tetramethylaniline |
| DNA | deoxyribonucleic acid |
| EDTA | ethylenediamine tetra-acetic acid |
| EM | electron microscopy |
| FSH | follicle-stimulating hormone |
| GMA | glycol methacrylate |
| H&E | Haematoxylin and Eosin |
| HEMA | 2-hydroxyethyl methacrylate |
| HGH | human growth hormone |
| HP | high power (magnification greater than x325) |
| ICC | immunocytochemistry |
| IHC | immunohistochemistry |
| IGSS | immunogold silver staining |
| ISH | in-situ hybridisation |

| | |
|---|---|
| LH | luteinizing hormone |
| LM | light microscopy |
| LP | low power (magnification x50-125) |
| LS | longitudinal section |
| MAR | microwave antigen retrieval |
| MMA | methyl methacrylate |
| MP | medium power (magnification x126-325) |
| mRNA | messenger ribonucleic acid |
| NFP | neurofilament protein |
| PC | pressure cooking |
| PCR | polymerase chain reaction |
| PMMA | polymethyl methacrylate |
| SDS | sodium dodecyl sulphate |
| SSC | saline sodium citrate buffer |
| TAT | turnaround time |
| TBS | Tris buffered saline |
| TMA | tissue microarray |
| TS | transverse section |
| TSH | thyroid-stimulating hormone |
| UV | ultraviolet |

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**Abstract**

This thesis collates, records and reviews pioneering studies performed by the author on the formulation, use, advantages, and applications of acrylic plastic embedding media for the histological examination of tissue using light microscopy, with particular emphasis on histochemical and molecular techniques. Nine key papers by the author are reviewed. The first relates to a number of modifications of an established method for the histological examination and investigation of metabolic bone diseases. As a result it was reported that the procedure had resulted in significant time saving without detrimentally affecting quality, and has been used for investigating approximately 10,000 cases of metabolic bone diseases for diagnosis.

In a further separate development, the enzymes lactase and sucrase were first reported in plastic-embedded jejunum. Subsequently it was shown that these two important disaccharidases for assessing malabsorption were sensitive to specific processing agents and could easily be lost, resulting in false negative staining. However, by using a specific processing schedule and times, the author was able to demonstrate how best tissue could be processed to retain these and other enzymes in various plastic embedding media.

A number of procedures were initiated for the application of IHC on plastic-embedded tissue that included the development of a modified plastic with new preparatory techniques, and changes in immunocytochemical staining protocols to enable IHC to be routinely performed. Previously, this had been regarded as technically not possible. Later in a series of papers published, procedures were described where the use and evaluation of enzyme, and/or antigen retrieval techniques using microwave heating and/or pressure cooking were assessed. Numerous antigens were successfully demonstrated, and subsequently specific diagnostic applications on various tissues were reported, including extensive routine use on approximately 35,000 cases to date of bone marrow trephines.

In a further development, the application of ISH techniques for the detection of mRNA in chick tissue for the demonstration of transcription *Sox* genes 11 and 21 was reported, which subsequently was combined with IHC for bromodeoxyuridine or neurofilament protein for simultaneous double staining on the same section.

**Acknowledgements**

I would like to thank the many people over many years where I have worked in both Cardiff and Nottingham for allowing me to carry out activities that have stimulated and later enabled me to pursue research interests, which forms the basis for this PhD thesis by published works. More recently to Mr T. Scriven and Mrs L. Finnerty at Nottingham University Hospitals who permitted me the use of computer facilities for the writing of this thesis. Also, in particular I thank Mr K. Morrell and Dr R. Church in Nottingham and Mr D. Blythe and Mr P. Jackson in Leeds for their technical contributions, and Dr R. Allibone and Professor J. Lowe (Nottingham) as a source of advice and encouragement. In addition, I appreciate the interest and support of Dr A. Jack from the Haematological Malignancy Diagnostic Unit at Leeds in the immunohistochemical concepts and techniques developed. To my colleagues Mr J. Ratliff and Mr P. Seifert in America and Ms Lynn Doverty in Aberdeen, thanks for your help in supplying useful technical and/or workload information.

Many of the photomicrographs were transposed from my personal collection of transparencies or computer images, which are supported by plates 47-49 from Mr D. Blythe. I am grateful to computer and photographic assistance relating to these by Mr T. Gray, Mrs C. Boag and Ms C. Townsend in the Histopathology Department at Queen's Medical Centre campus in Nottingham and Mr K. Premji and Mr C. Pendlebury in Pathology IT at City campus in Nottingham. In addition Mr C. Pendlebury has been extremely helpful with suggestions and assistance on the drawing of the figures within the thesis.

I wish to express my appreciation to my supervisors Dr S. Raleigh and Dr S. Allen at the University of Northampton who have prompted me and provided helpful suggestions during the writing of this thesis. In addition, Dr P. Antunes (internal examiner) and Dr P. Gerrits (external examiner) have offered advice especially with the chemical equations relating to the polymerisation of acrylic monomers.

Finally, I wish to record my thanks to the late Professor I. Dawson of the Histopathology Department at Queen's Medical Centre, Nottingham for suggesting many years ago after completing my M.Phil that I should think of submitting a PhD, and to Dr S. Jahav for allowing me to belatedly continue to pursue this long held vision, when he and his medical staff at City Hospital, Nottingham nursed me to back to health after the writing of this thesis was interrupted following a heart attack.

**Declaration**

The work carried out in this thesis was carried out exclusively by the author unless otherwise stated

All information sources are acknowledged by means of reference

None of the data contained herein have been submitted in full or in part for any other degree unless otherwise stated

## Dedication

To the many thousands of patients who have had their bone marrow trephine biopsies embedded in plastic using pioneering procedures developed by this author in the belief by pathologists that this contributed to best practice for accurate histological and immunohistochemical diagnosis to enable subsequent assessment of appropriate clinical treatment to those patients.

# CHAPTER 1

# PURPOSE OF THESIS

## 1.1 INTRODUCTION

The purpose of this thesis was to collate, record and review how the studies performed by Hand on the formulation, use, advantages, limitations and applications of plastic embedding media have had, and continue to have a relevance for the histological examination of tissue using light microscopy (LM). Though these studies included modifications to an existing method for the diagnosis of metabolic bone diseases, particular emphasis has been placed on several other new techniques for the application of enzyme histochemistry, immunohistochemistry (IHC) and *in situ* hybridisation (ISH). The author has been instrumental in publishing numerous papers and articles to describe these techniques with a series of pioneering and innovative developments. In addition, the studies performed are reviewed and examined with various other concepts and techniques found in similar contemporary publications, noting how these developments may be related. In another development, the author has seen the incorporation and application of some of these techniques in a diagnostic service for the management of diagnosis and subsequent therapy of patients admitted to hospital for medical examination. For metabolic bone studies (see chapter 6) the use of 6 µm or 13 µm thick sections were used, but for high resolution LM, 2 - 4 µm sections (commonly referred to as semithin sections) were used. All sections were produced via microtomy.

### 1.1.1 Evolution of Author's Research

The employment record by the author of this thesis as a Biomedical Scientist within Histopathology in the National Health Service enabled him to gain valuable experience and knowledge about histological techniques and practices, including the embedding of undecalcified bone samples, especially for the diagnosis of metabolic bone diseases. In the period 1971 – 1978, this involved using a polyester plastic embedding procedure that was not formally published until 1983 by Mawhinney and Ellis. Following a career move from the University Hospital of Wales (Cardiff) to Queens Medical Centre (Nottingham) in 1978, the author again came across a similar service in the Histopathology department, but this time the acrylic polymethyl methacrylate (PMMA) was used as the embedding medium.

Later when the development and widespread interest in plastic embedding using semithin plastic sections became evident, the author was involved in setting up a diagnostic service for producing high resolution LM. Many of the samples were bone marrow trephine biopsies for the diagnosis of various myelo and lymphoproliferative diseases. The development and interest of this work stimulated further studies culminating in the award of a Master of Philosophy (Hand, 1998b), which laid the foundations for future areas of research and development, many of which will be discussed in this thesis.

### 1.1.2 Outline of Studies Examined in this Thesis

Histology of tissue is routinely examined and assessed with the aid of a microscope (Stevens and Lowe, 2004). For most preparations the sample is required to be stained to allow structures to become visible, but first in order to

reach this stage, a sequence of preparatory procedures culminating in the embedding and subsequent production of paraffin wax sections are necessary. Typical procedures have been collectively described by Bancroft and Stevens (1982) and later updated by Bancroft and Gamble (2008) and Suvarna *et al.,* (2013). The fundamental procedures have changed little since their introduction during the latter part of the nineteenth century, when a series of scientific and technological advances (including the introduction of paraffin wax embedding by Bourne in 1882), each contributed and impacted on developing techniques for producing samples satisfactory for microscopic examination. However, whilst embedding tissue in paraffin wax was and still is perfectly satisfactory for many examinations (Suvarna *et al.,* 2013), other specific investigations have necessitated embedding instead in a plastic or resin as it is sometimes called. Numerous publications have been cited (some are provided in this thesis), but those by Burns and Bretschneider (1981), Sanderson (1997), Troiano and Kacena (2006) and Singhrao *et al.,* (2012) illustrate the range and diversity of techniques during the last 30 years.

This thesis has focussed on the studies and publications by the author that were associated with a range of plastic embedding procedures, with particular emphasis on those for enzyme, immunohistochemical and molecular techniques. To begin with, the histological examination and investigation of metabolic bone diseases such as osteoporosis, osteomalacia and Paget's disease, has for many years relied on the preferred option to examine samples by preparing undecalcified sections of iliac crest bone biopsies (Callis, 2008; Recker, 1983; Sanderson, 1997). Based on a method previously published by Difford (1974)

where PMMA was used to embed undecalcified bone biopsies, a number of modifications were described by this author (Hand, 1996). These included modifying the PMMA plastic mix, non-removal of the inhibitor, a reduced processing time schedule, quick polymerisation of the plastic and a modified microtome clamp that permitted blocks to fit existing equipment without prior shaping for subsequent sectioning. When investigating whether the method by Difford could be simplified and improved, Hand also considered whether a quicker turnaround time (TAT) to process biopsies and the quality could both be improved. Both criteria are important national requirements for accreditation of diagnostic laboratories in the UK (Clinical Pathology Accreditation UK Ltd, 2010). The modifications described by Hand (1996) which included a reduced time for processing biopsies from 14 to 3 days, concluded that significant savings in time could be achieved without detrimentally affecting quality, and reported that at the time, the improvements had been used for investigating approximately 10,000 cases of metabolic bone diseases for diagnostic purposes.

In a further separate development, plastic embedding using semithin sections has long been suggested for high resolution LM studies (Ashley and Feder, 1966; Burns, 1973). Since this time, Hand and colleagues have published a series of articles where several pioneering techniques were developed, described and advocated for the application of enzyme histochemistry, IHC and ISH on tissue embedded in a diverse range of acrylic plastics (Hand, 1987; Hand, 1988a; Hand, *et al.,* 1996; Blythe, et al., 1997; Church *et al.,* 1997; Hand and Church, 1997; Church *et al.,* 1998; Hand and Church, 1998).

In 1986 and 1987, the histochemical demonstration of the enzymes lactase and sucrase was first reported in plastic-embedded jejunum (Hand, 1986 and 1987). In these reports, these two important disaccharidases for assessing malabsorption (Lojda, 1983) were initially demonstrated in the proprietary glycol methacrylate (GMA) plastic JB-4 (Polysciences Inc., USA). In a subsequent paper it was shown that these two enzymes were sensitive to specific processing and embedding agents and could easily be lost, resulting in false negative staining (Hand, 1988a). By using a specific processing schedule and times, Hand (1988a) was able to demonstrate how best tissue could be processed to retain these and other enzymes in the plastic embedding media JB-4 (Polysciences Inc., USA), Historesin / Technovit 7100 (Heraeus Kulzer, Germany) and Histocryl (London Resin Company, UK).

The application of IHC in plastic-embedded tissue has long been acknowledged as troublesome and idiosyncratic (Vogt, *et al.,* 1976; Gerrits, 1988; Hand, 1988b). In an effort to overcome these difficulties, a number of procedures were initiated by Hand that included the development of a modified plastic embedding procedure, together with new associated preparatory techniques, and changes in immunocytochemical staining protocols to enable IHC to be routinely performed (Hand *et al.,* 1989; Hand and Morrell, 1990). Previously this been regarded as technically not possible (van Goor *et al.,* 1988).

Later in a series of papers published, pretreatment procedures were firstly described where the use and evaluation of enzyme, and/or antigen retrieval techniques using microwave heating (Hand *et al.*, 1996) and pressure cooking

(Hand and Church, 1998) were assessed. Subsequently Blythe *et al.,* (1997) and Hand and Church, (1997) described the successful demonstration of numerous antigens on various tissues for specific diagnostic applications. In a separate article, Jack *et al.,* (1993) stated that this plastic procedure was used routinely for embedding all bone marrow trephine biopsies received in their department, and acknowledged that the method had originated and was developed by Hand. Jack *et al.,* (1993) also commented on the good tissue morphology and high quality IHC that was achieved using this procedure, which is central to modern histopathological reporting of bone marrow trephine biopsies (Naresh *et al.,* 2006) in the management of patients with lymphoma and leukaemia. Currently this plastic embedding procedure is still used at some centres including the regional haematological malignancy service based in Leeds, UK, where to date it has been used as the method choice for approximately bone marrow 30,000 cases, making this probably the most prolific plastic embedding procedure for IHC — a testimony to its diagnostic value (D. Blythe, personal communications).

In a further development this author was involved with Church *et al.,* (1997) who reported the application of non-isotopic ISH techniques for the detection of mRNA in chick tissue for the demonstration of the transcription Sox genes 11 and 21 on tissue embedded in methyl methacrylate (MMA). Subsequently, (Church *et al.,* 1998), described sequencially the simultaneous double staining of both ISH for Sox 11 and 21 and IHC for bromodeoxyuridine or neurofilament protein on the same section. Previously only isotopic ISH had been successfully performed using the same plastic medium with no reports of the application of

simultaneous ISH and IHC.

Throughout the investigations by the author where techniques and applications for plastic embedding were developed, it was suspected that the choice and concentration of the amine accelerator used to induce polymerisation of the plastic could affect staining for IHC and ISH. This was reported firstly as an observation in some papers, but later scientific evidence was produced to prove this hypothesis (Church *et al*. 1997; Hand and Church, 1997; Church *et al*. 1998; Hand and Church, 1998).

### 1.1.3 Impact of Studies

The above studies by Hand outline the publications that form the basis of this thesis. In addition, the thesis examines and records collectively in chapter 11 the impact, significance, applications and value of these scientific publications. Also included are the past, present and possible future role, uses and function of plastic embedding in histopathological practice. The thesis also collectively collates and records numerous verbal presentations the author has been fortunate to be invited to give, whether they were for continuing personal development courses, educational teaching programmes, workshops and/or lectures at symposia.

# CHAPTER 2

# AIMS OF THIS THESIS

## 2.1 INTRODUCTION

In chapter 1 it was stated that the purpose of this thesis is to collate, record and review studies performed by the author on formulation, use, advantages, limitations and applications of plastic embedding media for LM. In addition, the studies performed are reviewed and examined with other contemporary publications, noting how these developments may be related. Also included in chapter 11 are the past, present and possible future role, uses and function of plastic embedding in histopathological practice.

All of the studies involved “thin” 6 µm (except for autofluorescence of tetracycline) or “semithin” (2 - 4 µm) sections for high resolution microscopy and were produced by microtomy using equipment from a routine laboratory that was readily available. This was more applicable than the use of very specialised and expensive equipment that is required for producing ground sections such as that developed and used by Donath (1990) which is only found in a few specialised laboratories. However, ground sections will be briefly discussed because of their contribution to understanding more about plastic embedding media. Similarly, some transmission electron microscopy (hereafter in this thesis referred to as EM) investigations have been published which have impacted on LM studies especially where dual purpose plastics have been used, although the main focus of the author’s research has been for LM. The overall aims of this study are

summarised as follows:-

### 2.1.1 Aims of this Study

1. To collate and describe the pioneering research relating to plastic embedding techniques performed by the author for high resolution light microscopy. In particular, the development and application of a range of immunohistochemical techniques that has led to their extensive routine diagnostic use on bone marrow trephine biopsies for investigation of haematological malignancies. Previously this type of service on plastic embedded tissue was thought to be technically not possible.

2. To critically review the impact, significance, applications and value of the research for tinctorial, enzyme, immunohistochemical and molecular staining performed by the author on tissue embedded in plastic.

3. To assess current practice on the use and value of plastic embedding media including that performed by the author.

## 2.2 PUBLICATIONS SUBMITTED (Chronological order)

**1.** Hand, NM. (1987) Enzyme histochemistry on jejunal tissue embedded in resin. *Journal of Clinical Pathology.* **40:**346-47.

**2.** Hand, NM. (1988a) Enzyme histochemical demonstration of lactase and sucrase activity in resin sections: the influence of fixation and processing. *Medical Laboratory Sciences.* **45:**125-30.

**3.** Hand, NM. (1996) Embedding undecalcified bone in polymethyl methacrylate: an improved method. *British Journal of Biomedical Science.* **53:**238-240.

**4.** Hand, NM. Blythe, D. and Jackson, P. (1996) Antigen unmasking using microwave heating on formalin fixed tissue embedded in methyl methacrylate. *Journal of Cellular Pathology*. **1:**31-37.

**5.** Blythe, D. Hand, NM. Jackson, P. Barrans, SL. Bradbury, RD. and Jack, AS. (1997) The use of methyl methacrylate resin for embedding bone marrow trephine biopsies. *Journal of Clinical Pathology.* **50:**45-49.

**6.** Hand, NM. and Church, RJ. (1997) Immunocytochemical demonstration of hormones in pancreatic and pituitary tissue embedded in methyl methacrylate. *Journal of Histotechnology.* **20:**35-38.

**7.** Church, RJ. Hand, NM. Rex, M. and Scotting, PJ. (1997) Non-isotopic *in situ* hybridization to detect chick Sox gene mRNA in plastic embedded tissue. *Histochemical Journal.* **29:**625-629.

**8.** Hand, NM. and Church, RJ. (1998) Superheating using pressure cooking: its use and application in unmasking antigens embedded in methyl methacrylate. *Journal of Histotechnology.* **21:**231-236.

**9.** Church, RJ. Hand, NM. Rex, M. and Scotting, PJ. (1998) Double labelling using non-isotopic *in situ* hybridisation and immunohistochemistry on plastic embedded tissue. *Journal of Cellular Pathology.* **3:**11-16. (Eratum **3:**84).

# CHAPTER 3

# EXISTING METHODS AND THE NEED FOR OTHER TECHNOLOGIES

## 3.1 INTRODUCTION

The study of minute anatomical structures within tissue, known as histology, necessitates viewing the sample with a microscope (Stevens and Lowe, 2004). Usually when the sample is solid tissue (as opposed to a collection of cells or organisms), a thin slice is prepared which is then subsequently examined. For most preparations the sample is required to be stained to allow structures to become visible, but first in order to reach this stage a sequence of preparatory procedures are necessary. These procedures are collectively described in standard textbooks (Kiernan, 1999; Suvarna *et al.,* 2013) and have changed little since their introduction during the latter part of the nineteenth century when a series of scientific and technological advances each had an individual impact on developing techniques for producing samples satisfactory for microscopic examination. None of these advances alone resolved problems in isolation, nor did they happen simultaneously or sequentially, but they all contributed to preparing tissue by the fundamental procedures of fixation, dehydration and embedding in paraffin wax, which nowadays are accepted routine practices. In addition, equipment for producing thin slices of tissue was invented, stains and associated staining techniques devised, and improved optics on microscopes developed. All culminated in superior histological examination of tissue, leading to the study of abnormalities and changes in diseases referred to as histopathology.

## 3.2 EXISTING METHODS

It is not the purpose of this thesis to reiterate in chronological detail the developments within histological practice including those of the nineteenth century, but it is worth outlining the fundamental procedures required in order to appreciate and understand in context the background to the current study.

### 3.2.1 Fixation

When tissue is examined it should ideally be as close to the living state as possible (Hopwood, 1977; Rhodes, 2013). Unless tissue is prepared for frozen sections, or cells are cultured, or a smear is produced, it is usual that it is required to be preserved by the process of fixation. Fixation is a series of complex chemical processes that varies depending on the fixative employed and the chemical substances and structures present in the tissue, but in essence it should preserve tissue by preventing autolysis (breakdown of dead tissue by enzyme activity), changes in size and shape, loss of components, and should also allow subsequent histological staining (Baker, 1960; Hopwood, 1977; Rhodes, 2013).

Though some knowledge of fixation and the effects of certain fixatives on tissue through embalming have been known for over 2,000 years, it was only when histological procedures were being developed and evolved in the latter part of the nineteenth century, along with knowledge gained from the leather tanning industry, that systematic investigations were carried out (Hopwood, 1977). It was then realised that no one fixative was ideal so numerous mixtures were introduced, some of which have been recommended for specific tissues. One of

the most important fixatives was formaldehyde introduced by Blum in 1893 (see Baker, 1960). This is a colourless gas that is very soluble in water (40%) which is sold in an aqueous solution commonly referred to by the tradename formalin (Baker, 1960), and can be used either alone e.g. 10% formalin (4% formaldehyde) or in various mixtures e.g. formol saline, formol calcium, and neutral buffered formalin (Hopwood, 1977). In this thesis, the papers submitted by this author for review have used a variety of formalin variants, partly because the studies often employed the routine fixative that was at the time routinely used by the department, and partly because in collaborative studies there was different usage by other departments. In reality, for routine LM histological examination, there is little difference although as will be seen later in this thesis, formol calcium has been favoured for enzyme histochemical studies. In addition, for non-routine histological practice, where the purity of formaldehyde is considered important, e.g. molecular studies and EM, paraformaldehyde (methanol-free formaldehyde has been recommended (Sterchi and Astbury, 2013; Woods and Stirling, 2013).

In recent years there have been attempts to streamline and standardise the choice of fixative employed for routine investigations in laboratories, and nowadays it is unusual to find a laboratory that does not use “formalin” for their routine specimens (Rhodes, 2013). Though formalin is not perfect for all structures, satisfactory morphology can be relatively easily and quickly be achieved on many tissues which will subsequently permit numerous staining techniques to be performed. The details of the precise chemical reactions by all components for each fixative are not always known, but the general principles where tissue is stabilised by forming crosslinks, especially between protein, are

understood (French and Edsall, 1945; Baker, 1960). This includes reactions between formaldehyde and amino acids of proteins, which in addition to the general properties of the fixative, may be particularly useful to know when performing histochemical and molecular studies. An overview of fixatives and fixation was provided by Hopwood (1977) and subsequently updated by Grizzle *et al.,* (2008) and Rhodes (2013).

It is important to realise that much of histological technique depends on correct and good fixation as subsequent procedures are influenced by it, and ultimately if sub-optimal, diagnosis may be affected. As such, fixation should therefore be regarded as a cornerstone in histological practice.

### 3.2.2 Tissue Processing

It is necessary to impregnate and embed tissue in a solid support medium to facilitate the production of sections for microscopy (Gordon and Bradbury, 1977), and this process of preparing tissue for impregnating and subsequent embedding is referred to as tissue processing. Often tissue requires water to be removed as the embedding medium is immiscible with aqueous fixatives such as formalin, and therefore it is usual that a series of industrial alcohols are employed to dehydrate the tissue (Spencer and Bancroft, 2013a). Paraffin wax is by far the most popular embedding medium, but because it too is immiscible with alcohol, an intermediate solvent is required such as xylene. Other dehydrating and intermediate solvents can be employed, but alcohol and xylene are the most common pairing (Gordon and Bradbury, 1977).

When tissue was first processed through to molten wax, the steps required were manually performed, but for many years equipment has been available that is able to automate these activities. In addition to the obvious time saving and convenience, this approach may also involve agitation, heat, vacuum of the tissue, all of which can improve the processing quality of the tissue (Spencer and Bancroft, 2013a).

The quality of the processing also influences subsequent histological procedures, e.g. staining of tissue later will not be optimal if this is inadequate and unsatisfactory. An overview of tissue processing, reagents used and developments has been described by Gordon and Bradbury (1977) and Spencer and Bancroft (2008 and 2013a).

### 3.2.3 Embedding Media

Control of the physical state of paraffin wax by temperature alone is simple as the melting point, usually about 56$^{o}$C, enables easy and rapid conversion from solid to liquid and vice versa (Kiernan, 1999; Spencer and Bancroft, 2013a). Klebs (1869) introduced the process of impregnating and embedding specimens in paraffin wax, but the procedure did not become established until published by Bourne (1882). Paraffin wax is a mixture of solid hydrocarbons, having a general formula $C_nH_{2n+2}$ and it is the proportions of these mixtures resulting in different melting points, which forms the basis for their classification (Gordon and Bradbury, 1977; Spencer and Bancroft, 2013a). Although other types of waxes such as water soluble waxes and ester waxes have been introduced for embedding biological specimens, they have never replaced paraffin wax and are

seldom used nowadays. The advantages of paraffin wax for routine histological use over other embedding media such as agar, celloidin and gelatin include:-

(i) easy and rapid conversion from liquid to solid state and vice versa through changes in temperature
(ii) sections easily at 3 - 8 μm
(iii) embedded blocks require no attention during storage
(iv) permits the application of numerous staining procedures
(v) relatively inexpensive

In more recent times, embedding centres have been introduced to facilitate easy and rapid preparation of wax blocks by having both molten wax on tap and cooling areas readily available (Gordon and Bradbury, 1977; Spencer and Bancroft, 2008 and 2013a). This has enabled blocks of varying sizes to be produced depending on the size of mould selected and is widely used in most laboratories. As a result of the properties and advantages of paraffin wax for light microscopy studies and its widespread use, it would be appropriate to describe it as “The Universal Embedding Medium”.

However, with the advent of EM it was soon realised that paraffin wax (despite the numerous types and modifications tried) was unstable in the electron beam and was not suitable as an embedding medium. The poor penetrating power of an electron beam and the large depth of field involved in EM, make specimen structures difficult to interpret with sections that are over a fraction of a micron thick (Woods and Stirling, 2013). In the early days of thin sectioning for EM, the

conventional paraffin waxes tested evaporated and were too soft to enable sections sufficiently thin (30-90 nm) to be cut, but some success was achieved using a double embedding technique of celloidin followed by paraffin wax (Pease and Baker, 1948) or harder ester wax (Flewett and Challice, 1951). The most significant technological advance however occurred when Newman, Borysko and Swerdlow (1949) introduced the resin or plastic medium n-butyl methacrylate (BMA). During the early 1950s several modifications of the final mix were used, notably those which increased the hardness of the block by the addition of ethyl and/or methyl methacrylate. Although the use of these acrylic media presented certain difficulties, superior EM was achieved and the demise of wax as an embedding medium for this type of preparation became irreversible.

There are many types of plastic embedding media depending on their chemical composition, and in 1956 Glauert, Rogers and Glauert developed the epoxy resin/plastic Araldite as an embedding medium for EM. Subsequently Epon (Finck, 1960) and Spurr (Spurr, 1969) were also introduced, with various recipes and ingredients, and both Araldite and Epon such as Epon 812 still remain popular plastics for EM because of the quality that can be achieved. In addition, several polyester resins/plastics were also introduced (Kellenberger *et al.,* 1956; Ryter *et al.,* 1958) but these are nowadays seldom used.

### 3.2.4 Section Preparation

For histological examination of tissue using a microscope, a thin slice or section is prepared and stained. To aid production of sections of suitable thickness, tissue is produced as previously outlined and the resulting embedded blocks cut

on a microtome. Various types of microtomes have been developed along with a variety of different types of knives, and though the combination employed depends on the application required, for paraffin blocks it is now common practice to use a rotary type microtome (Spencer and Bancroft, 2013b). During the last 30 years the metal knives previously used have given way to disposal feather blades in most laboratories which allow sections to be cut at 3-8 μm by a skilled technician. Paraffin sections are floated on to a heated waterbath (approximately 56 $^{o}$C) to allow them to expand and flatten, and then picked up on a glass microscope slide. When adequately dry and usually after heating the sections, the wax is removed prior to staining the tissue. For cutting plastic sections whether semithin (LM) or especially ultrathin (approximately 50-100 nm) for EM, glass knives are superior (Latta and Hartmann, 1950; Bennett, *et al.,* 1976; Linder and Richards, 1978; Hand, 2013; Woods and Stirling, 2013).

In some instances ground sections may be prepared for a few specialised applications when sectioning is either unsuitable or impossible by conventional microtomy. Following fixation and processing, this requires embedding the sample in a plastic and cutting a relatively thick slice e.g. 100 μm with the aid of a saw, followed by “grinding” and “polishing” the section to an appropriate thickness. The procedures are nowadays achieved using expensive, hi-technology precision equipment which is found only in very few specialised laboratories. Ground sections have been used for bone, tooth and implants (Rijke *et al.,* 1971; Emmanual *et al.,* 1987; van der Lubbbe, 1988; Donath, 1990; Sanderson, 2010). Futher details are discussed on page 21.

### 3.2.5 Staining

Tissue sections may be stained with dyes to permit their structure to be visible. There are a few natural dyes e.g. haematoxylin, and saffron, but most dyes used are synthetic with many produced through the textile industry. Hundreds of histological staining techniques using numerous staining recipes and solutions have been devised, enabling tinctorial staining of tissue via chemical reactions (Bancroft and Cook, 1994). In some cases, specific histochemical reactions can be exploited to localise specific substances such as carbohydrates and enzymes, although it should be noted that because enzymes are labile, most are destroyed or lost during paraffin wax preparations and consequently frozen sections are used (Nestor and Bancroft, 2008).

A further type of staining involves the application of antibodies to tissue sections known IHC or immunocytochemistry (ICC) to detect specific antigens by selective attachment of a specific antibody. The antibodies may be tagged with a marker that can subsequently enable their visible localisation either naturally for example via a fluorescent dye or more commonly through induced staining. In the latter, the attached enzyme peroxidase can be easily coloured through a chemical reaction with the chromogen 3,3-diaiminobenzidine tetra-hydrochloride (DAB), giving rise to the more specific term immunoperoxidase. As a result of numerous improved reagents, technologies and techniques, along with the application of many antibodies on routinely prepared paraffin sections, and the simultaneous recognition of the importance in diagnostic histopathology of IHC (Jackson and Blythe, 2013), its growth in recent years has been exponential.

More recently it has been shown that ISH may also be applicable on paraffin sections where specific probes are able to localise and identify specific DNA/RNA targets. Though a specialised procedure, this too is becoming an increasingly more important diagnostic tool (Sterchi and Astbury, 2013).

The above procedures describe briefly the preparatory steps required in producing paraffin wax sections and illustrates the vast range of techniques that are available to demonstrate tissue structures. Since the introduction of paraffin wax as an embedding medium in the nineteenth century along with associated developments, the production of paraffin wax sections for histological practice has become a well established and routine procedure for most applications. Using formalin-fixed tissue embedded in paraffin wax, high quality sections can be achieved with satisfactory morphology for many tissue structures, although it should be emphasised that artefacts are still produced, so it is more a case of minimising these to an acceptable level. After 150 years this approach has become the standardised way of producing histological sections, and is still a major function of every histology laboratory.

## 3.3 THE NEED FOR OTHER TECHNOLOGIES

Paraffin wax is an unsatisfactory embedding medium for bone in its normal undecalcified state (unless minute) as it does not provide adequate support for quality sections to be produced. Following embedding of undecalcified bone in the acrylic medium MMA for autoradiography investigations by Kidman *et al.,* (1952), further developments were described by Arnold and Jee (1954), Woodruff and Norris (1955) and Yaeger (1958) where sectioning and staining

were performed. Subsequently numerous scientific papers relating to embedding undecalcified bone in plastic have been published. These have included its use for the histological examination and investigation of metabolic bone diseases such as osteoporosis, osteomalacia and Paget's disease where it is important to qualitatively and quantitatively assess mineralised bone, osteoid, and cellular activity of osteoblasts and osteoclasts (Stevens, Lowe and Scott, 2009). If paraffin wax sections are used, it is necessary to first decalcify the bone to enable good sections to be cut, leaving bone after the removal of calcium unmineralised and consequently unable to be quantified by staining techniques such as von Kossa that depend on the presence of calcium.

In a further development, bone or tooth can be embedded in plastic for ground sections to be produced, and is particularly useful if the sample contains an implant which may be too hard or too fragile to cut by conventional microtomy (Hand, 2013). Several types of implant materials have been used e.g. metal, ceramic, polymers and though simple equipment was used in early studies (Emmanuel *et al.,* 1987; van der Lubbe *et al.,* 1988; Allison and Sugar, 1990), nowadays superior results are achieved with more sophisticated equipment (Donath, 1990; Knabe *et al.,* 2006). Detailed descriptions of the procedures required have been described by Plenk (1986), and Troiano and Kacena (2006) who reviewed the processing, embedding and cutting techniques used for bone implants. Selection of the best monomer for implant pathology was discussed by Jenkins and Marcum (2006), and Alves and Therin (1997) described a plastic embedding procedure for assessing tissue response to biomaterials. Another type of metallic implant is a vascular stent and using a modification of the plastic

embedding medium used by Wolf *et al.,* (1992), sections of stent have been prepared by both ground sections and microtomy (Seifert *et al.,* 2001). A comparison of methodologies for arteries containing metallic stents was later discussed by Rippstein *et al.,* (2006).

In 1960 Rosenberg *et al*., and Wichterle *et al.,* introduced the water-miscible acrylic monomer 2-hydroxyethyl methacrylate (HEMA) or GMA as it is more commonly called for EM, in the hope that its properties would produce less artefacts and enable tissue to resemble more closely its life-like state, so that histochemical studies could be performed. In reality, tissue morphology was inferior to the epoxy plastics Araldite and Epon and as a result these are still the preferred choice for ultrastructural studies (Woods and Stirling, 2013). The introduction of the higher homologue 2-hydroxypropyl methacrylate by Leduc *et al.,* (1963) and Leduc and Holt, (1965) produced better results than GMA as reported by Leduc and Bernhard (1967), but its use for EM was only temporary. However, unlike epoxy plastics, GMA does permit tissue to be easily stained with many tinctorial stains, and consequently it was soon recommended for LM studies (Ashley and Feder, 1966). In addition, it was realised that GMA could be polymerised at low temperature and this useful property enabled some enzymes to be histochemically localised and demonstrated. Initial studies focused on tinctorial staining and/or enzyme histochemical staining (Feder, 1963: Ashley and Feder; 1966; Hoshino, 1971; Ashford *et al.,* 1972), but later reports for IHC (e.g. Hoshino and Kobayashi, 1972; Spaur *et al.,* 1975), lectins (Beckstead *et al.,* 1986) and ISH (Cau and Beckstead, 1989) also began to appear. Senoo (1978) reported on a method for several histological investigations on a single modified

GMA block. New and improved modifications of the plastic mix were beginning to be described (Ruddell, 1967a, 1967b), and subsequently other mixes were investigated (Ruddell, 1971; Gerrits and Smid, 1983; Gerrits *et al.,* 1991) with some of these becoming proprietary products under the names of JB-4 (Polyciences Inc.), Technovit 7100 and Technovit 8100 (Heraeus Kulzer).

It was also observed during the 1960s that if thinner sections than the normal 5 µm paraffin sections were cut, then semithin sections (2/3 µm) could provide greater information (Ashley and Feder, 1966; Zambernald *et al.,* 1969). This practice of preparing semithin sections for high resolution LM had long been recognised for localising specific areas and structures before subsequent EM, e.g. when sections are stained with Toluidine blue (Eastham and Essex, 1969; Zamboni, 1972; Burns, 1973; Hoffmann and Flores, 1981). Since the properties and quality of paraffin wax at that time made semithin sectioning difficult, proprietary GMA kits soon became available, along with the introduction of heavy motorised microtomes suitable for sectioning plastic blocks. Not since the introduction of paraffin wax in the nineteenth century had there been such a significant new embedding medium for LM. Now not only was tinctorial staining possible, but so too was enzyme histochemistry — something which was not possible with paraffin wax. An indication of the rapid growth of plastic embedding with GMA can be ascertained by 53 staining techniques listed by Cole in 1982. A revolution it seemed was beginning to happen with workshops being arranged and prompting Murray (1988) to suggest that plastic embedding could be the way forward in the future. Some even dared to suggest that paraffin as an embedding medium was over (Murray and Ewen, 1989a).

# CHAPTER 4

# DEVELOPMENT AND CHARACTERISTICS OF PLASTIC EMBEDDING MEDIA

## 4.1 INTRODUCTION

In the previous chapter, it was stated that plastic embedding media were required as an alternative to paraffin wax for certain specialised techniques. The basic chemical composition of epoxy, polyester and acrylic plastics vary significantly resulting in different characteristics, but further alterations by the incorporation of additional specific ingredients into the plastic mix can also have an effect, which as will be shown in this thesis, is probably the most obvious with acrylics. For example, hardness, stability and colouration of blocks, along with the mode and temperature used for polymerisation of the plastic and the use of different ingredients can all vary. As a consequence staining can also be affected, so it is important for the scientist to understand this when various histological techniques are required. In this chapter and later in the thesis, it will be shown how in particular, the development of acrylic plastics and techniques have evolved to enable a diverse range of investigations to be performed.

### 4.1.1 Resin or Plastic: Their Description

In histological practice the terms “resin” or “plastic” have both been liberally used to describe appropriate embedding media. Neither is more correct, but the use of the word plastic has in recent years gained more popularity, possibly because many publications have been from USA where the description plastic is more

common, and also perhaps because the term resin is often associated with adhesive-like properties of a substance or products obtained from the sap of certain plants and trees. For the sake of clarity and consistency, the term plastic will from now on be used throughout this thesis.

### 4.1.2 Types and Terms of Plastics

Plastics are classified according to their chemical composition into epoxy, polyester, or acrylic (Hand, 2013). To produce suitable solid blocks, several chemical components are required, which can affect the properties of the embedding medium and may determine whether the plastic is hydrophobic (repels water) or hydrophilic (attracts water); sometimes referred to as non-polar and polar respectively (Causton, 1984; Newman, 1987). Some of the components can present potential health and safety problems, and it is imperative that all chemicals used in the formulation of plastics are handled in accordance with local and legal safety requirements. The chemical reactions between various components in plastic embedding media and the process of polymerisation is complex, but it is useful to understand the fundamental role and limitations of particular components used in acrylic plastics to appreciate their properties.

Simple molecules called monomers (derived from the Greek word *mono* meaning ‘single’ and *mer* meaning ‘part’), can be joined together to produce a complex macromolecule made up of repeating units termed polymer (‘many parts’). The change in the physical state of an embedding medium from liquid to solid is called polymerisation or curing, and in order to stimulate this, a

catalyst (initiator) in conjunction with a polymerisation agent to speed up the reaction, called an accelerator (sometimes activator or hardener) or is often suggested or necessary (Janes, 1979).

### 4.1.3 Epoxy Plastics

Epoxy plastics were introduced as embedding media by Glauert, Rogers and Glauert (Aradite), Finck (Epon), and Spurr (Spurr) in 1956, 1960 and 1969 respectively. The constituents, molecular structure and properties of epoxy plastics including their hazards and toxicity were discussed by several authors (Kay, 1967; Causton, 1980; Causton, 1981; Glauert, 1984; Germaine and Stevens, 1996). They have been the embedding media of choice for EM, especially Aradite and Epon (Woods and Stirling, 2012), because the polymerised plastic is sufficiently hard to permit sections as thin as 30-40 nm to be cut and is stable in the electron beam. Epoxy plastics derive their name from the active ring structure through which they polymerise where epoxide groups (R-CHO=$CH_2$) can be attached to an almost infinite number of chemical structures to form a three-dimensional polymer of great mechanical strength (Germaine and Stevens, 1996). The three types of epoxy plastic used in microscopy are based on either bisphenol A (Araldite), glycerol (Epon), or cyclohexene dioxide (Spurr). The tradenames in parentheses are those in common usage by microscopists and do not convey any structural property. Aquon (Gibbons, 1959) and Durcupan (Stäubli, 1960) are two examples of a water soluble epoxy plastic that were introduced, although the former is no longer available.

Epoxy embedding media are a carefully balanced mixture of epoxy plastic, catalyst, and accelerator, each component having a direct impact on the physical and mechanical properties of the polymerised plastic. The type of catalyst used can influence the properties either by acting as a plasticiser to make blocks that are tough but flexible, or by producing hard but inflexible blocks by using a rigid molecule to stiffen the plastic.

The rate at which each epoxy plastic infiltrates the tissue depends on the size (and hence the viscosity) of the diffusing molecules, and the density of the tissue. Infiltration by Araldite is slow, partly because the epoxy plastic molecule is large, and partly because of the formation with the accelerator of three-dimensional structures. Epon and Spurr have lower viscosities and shrinkage of epoxy plastics can be as low as 2% after polymerisation (Glauert, 1984). The physical properties of epoxy plastics depend on the number of cross-links formed between polymers, which can be influenced by the polymerisation temperature which in turn affects the rate of polymerisation. Care is needed to provide a section with the right level of cross-linking to permit subsequent staining of the tissue.

To an experienced observer in tissue interpretation, Toluidine blue is the most useful and informative stain on epoxy-embedded tissue for LM (Germaine and Stevens, 1996). If the stain is heated and used at high alkaline pH, it easily penetrates the plastic and stains various tissue components a blue colour of differing shades and intensities, with no appreciable staining of the embedding medium. Polychromatic stains, e.g. Paragon, can be used to resemble

Haematoxylin and Eosin (H&E) staining. Various reagents known as “etching agents” including a mixture of sodium hydroxide, benzene and methanol (Mayor *et al.,* 1961) and alcoholic sodium hydroxide (Lane and Europa, 1965) have been suggested to reduce the cross-link density which then allows expansion of the plastic to improve access of stains and antibodies to the tissue. Janes (1979) reported that several staining techniques could be applied after the surface plastic has been ‘etched’ using alcoholic sodium hydroxide, but stated that the results were inconsistent. Johnson and Tam (1973) were able to achieve successful staining of undecalcified bone embedded in Spurr using an unmodified H&E, von Kossa and Masson trichrome without any pretreatment. Another type of pretreatment consisted of oxidising osmium-fixed tissue sequentially with potassium permanganate and oxalic acid (Shires *et al.,* 1969; Bourne and St John, 1978) so that aqueous solutions could be stained more consistently. The application of several staining methods to tissue embedded in Araldite and Epon was described by Aparico and Marsden (1969) who pretreated osmium-fixed tissue sections with 15% hydrogen peroxide, but did not remove the plastic. It was reported that no modifications of the staining methods were required other than adjusting the times to achieve maximum colour contrast.

Several reports publications also described the application of IHC to epoxy sections for LM studies following treatment with sodium ethoxide/methoxide (Rodning *et al.,* 1980; Dell’orto, *et al.,* 1982; Giddings *et al.,* 1982; Pedraza *et al.,* 1984; Smart and Millard, 1985; McCluggage *et al.,* 1995; Krenacs *et al.,* 1996; Krenacs *et al.,* 2005), although this practice is now seldom used. In

reality the difficulties of tinctorial and immunohistochemical staining described in these papers resulted in only limited success, and in general when high-resolution LM was required, acrylic plastic sections were preferred because of the potential easier handling and improved tinctorial staining. However, as will be seen later in this thesis (especially IHC), some techniques have still presented formidable challenges. A comparison of IHC on araldite and glycol methacrylate sections was reported by Britten *et al.,* (1993).

### 4.1.4 Polyester Plastics

These plastics were originally introduced in the mid-1950s for EM, as they polymerise uniformly with little shrinkage. Originally a polyester mixture known as Vinox K3 was developed by Kellenberger *et al.,* (1956) but this was superceded by another mixture containing Vestopal W (Ryter and Kellenberger, 1958). Polyester plastics are now rarely used for microscopy, although Mawhinney and Ellis (1983) reported using the proprietary plastic Polymaster 1209 (Bondaglass-Voss Ltd, UK) for embedding undecalcified bone for LM investigations of metabolic bone diseases.

### 4.1.5 Acrylic Plastics

The acrylic plastics used for microscopy are esters of acrylic acid ($CH_2$=CH-COOH) or more commonly methacrylic acid ($CH_2$=C($CH_3$)-COOH), and are often referred to as acrylates and methacrylates respectively (Janes, 1979). Numerous mixes have been devised to produce plastics that have provided a wide range of properties, with a consequence that there is a diverse range of potential uses. Acrylics have been used extensively for LM, but some have

been formulated so that EM can be performed, either in addition or exclusively. BMA, MMA and GMA were all introduced for EM, but are now not used for this purpose (unless as a component of a mix) because the plastic is unstable in the electron beam, although Kushida (1961) showed that by adding a cross-linking agent e.g. divinyl benzene to a BMA/MMA embedding mixture, a one-dimentional structure could be changed to a more stable three-dimensional structure when polymerised. For staining of semithin sections for high-resolution LM, greater success has been achieved with acrylics, and in particular GMA, which is extremely hydrophilic and therefore allows many aqueous tinctorial staining methods to be applied without its removal, but is tough enough when dehydrated to section well.

### 4.1.6 Components and Formulation of Acrylic Plastics

To produce an acrylic plastic suitable as an embedding medium for biological material and its subsequent histological examination, several components are required. In addition to the monomer, a catalyst for inducing polymerisation is required. The most common catalyst is dibenzoyl peroxide which when dry is explosive and is therefore supplied damped with water, or as a paste, or as plasticised particles. In some mixes, the water is required to be removed and care must be taken to dry aliquots away from direct sunlight or heat. Azobisisobutyronitrile and bis (4-tert-butylcyclohexyl) peroxydicarbonate (tradename Perkadox 16) are other catalysts that have been used. In addition, light-sensitive photocatalysts such as benzil and benzoin (various types) can be used for polymerisation of some acrylics e.g. LR Gold and Lowicryl plastics (derscribed in more detail later in this chapter) at sub-zero temperature using

short wavelength ultraviolet (UV) light. Sanderson (1995) described polymerisation of MMA containing Perkadox 16 using UV irradiation.

Several other components are also often necessary in addition to the monomer and catalyst. To improve sectioning qualities of acrylic blocks, softeners or plasticisers such as 2-butoxyethanol, 2-isopropoxyethanol, polyethylene glycol 200/400, nonlyphenol-polyglycoletheracetate (tradename Plastoid N) and dibutyl phthalate are often added to the mix. Some proprietary acrylic mixes contain a small amount of a cross-linker such as a dimethacrylate compound. For example, the cross-linking agent found in some Lowicryl plastics to stabilise the matrix of the plastic against physical damage caused by the electron beam is triethylene glycol dimethacrylate (Carlemalm *et al.,* 1982), whereas in Technovit 8100 ethylene glycol dimethacrylate is incorporated to provide stability against staining solutions (Gerrits *et al.,* 1991). Unlike epoxides, the viscosity of acrylics is low and hence short infiltration times are possible, although the size and nature of tissue, together with the processing and embedding temperature will affect the times required. In Figs 1.1 – 1.8 (pp 32 – 35), the basic chemical formulae of the reagents methyl methacrylate, the higher homologue n-butyl methacrylate, 2-hydroxyl ethylmethacrylate, dibenzoyl peroxide and various amine accelerators are illustrated.

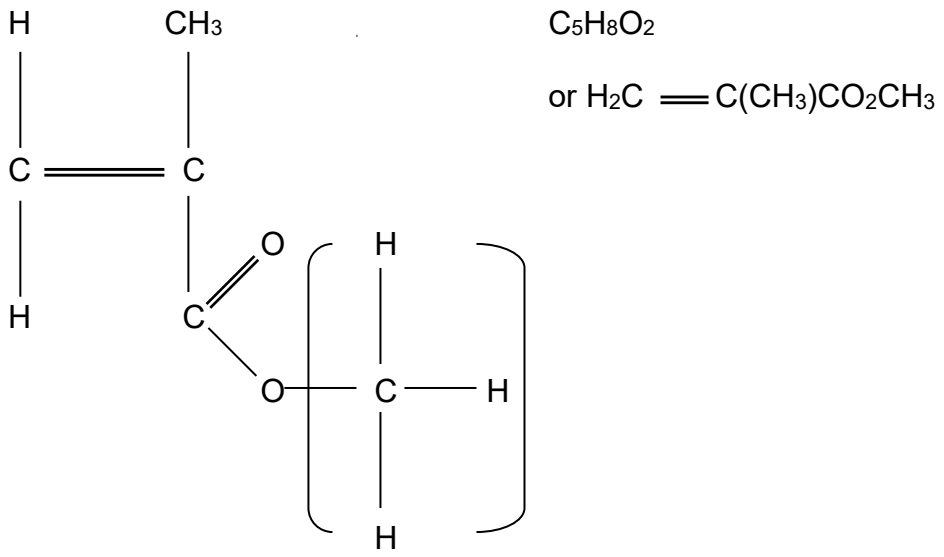



**Fig. 1.1** Methyl methacrylate

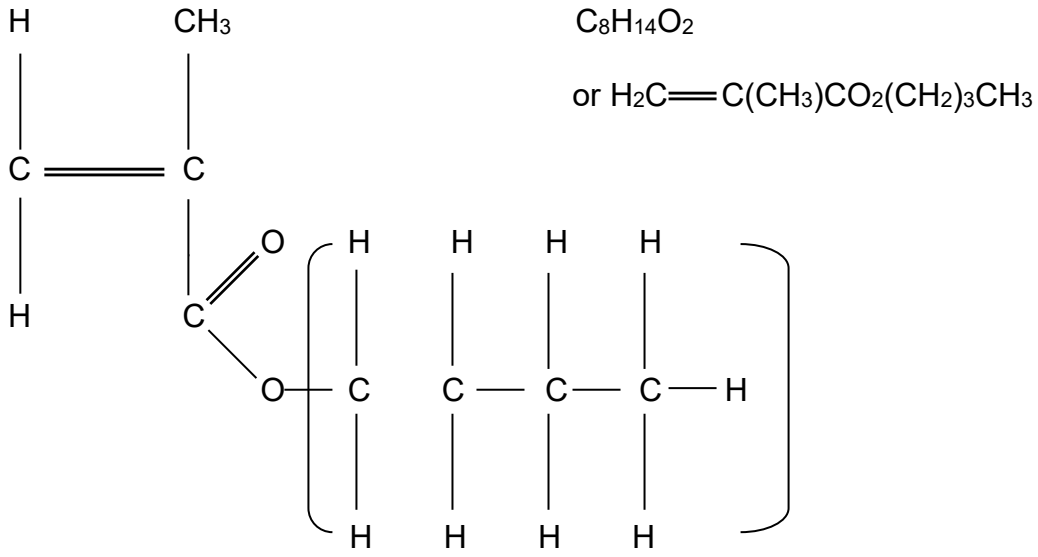



**Fig. 1.2** n-Butyl methacrylate

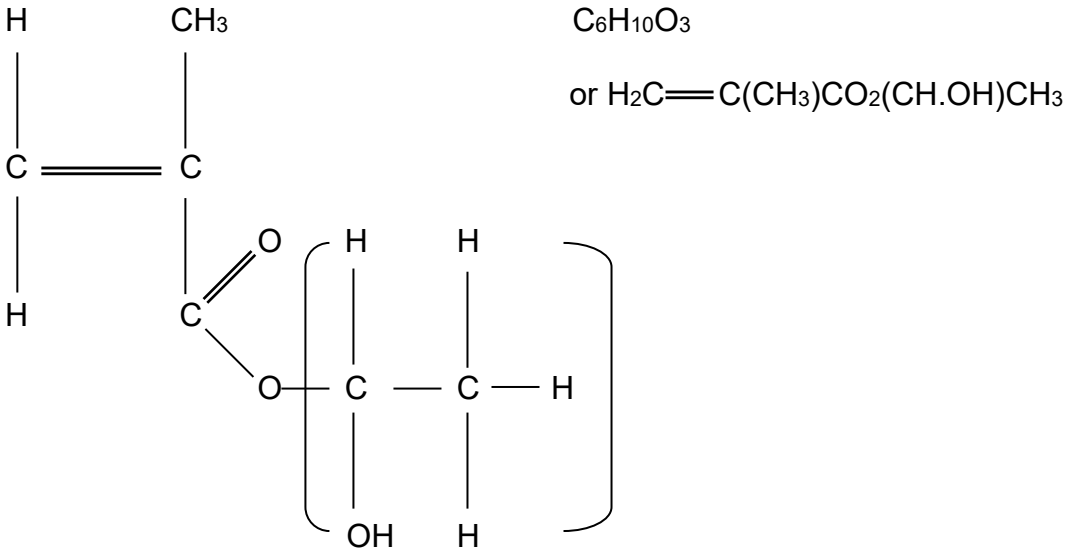



**Fig. 1.3** 2-hydroxyethyl methacrylate or glycol methacrylate

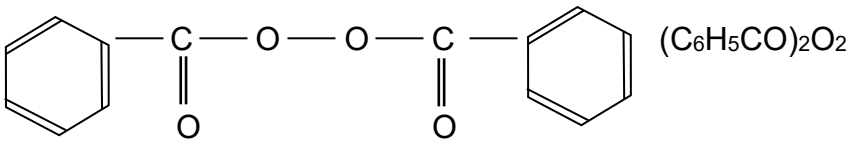
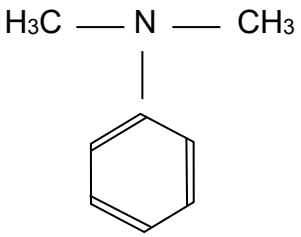


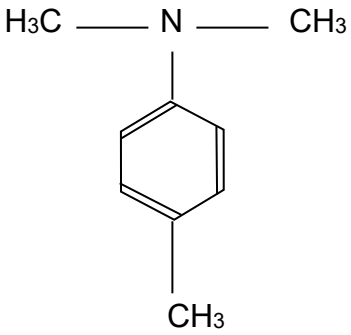

**Fig. 1.4** Dibenzoyl peroxide



$C_8H_{11}N$

or $C_6H_5N(CH_3)_2$

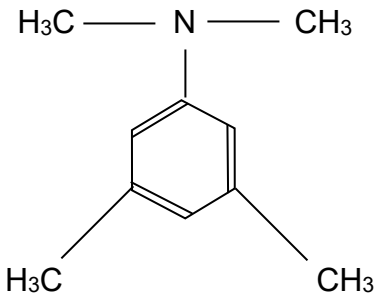
**Fig. 1.5** N,N-dimethylaniline



$C_9H_{13}N$

or $CH_3C_6H_4N(CH_3)_2$

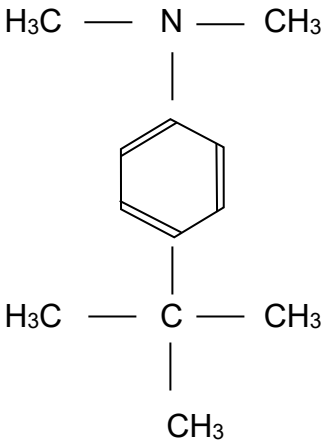
**Fig. 1.6** N,N-dimethyl *para*-toluidine




$C_{10}H_{15}N$

or $(CH_3)_2C_6H_3N(CH_3)_2$

**Fig. 1.7** N,N-3, 5-tetramethylaniline




$C_{12}H_{19}N$

or $(CH_3)_3CC_6H_4N(CH_3)_2$

**Fig. 1.8** 4-tert-butyl-N,N-dimethylaniline

### 4.1.7 Polymerisation of Acrylic Plastics

Polymerisation of acrylics occurs by the production of free radicals. Radicals can also be produced by light or heat, and consequently acrylic plastics and their monomers should be stored in dark bottles in a cool place. Acrylics contain variable quantities of the inhibitor hydroquinone or more recently hydroquinone monomethyl ether in the same concentration (0.01% – 0.03% / 100 – 300 parts per million) to prevent spontaneous polymerisation, and for many applications this remains when preparing the embedding mixes. The presence of hydroquinone prevents spontaneous polymerisation by combining with free radicals to produce stabilisation by resonance (Gerrits and van Leeuwen, 1985).

The change in the physical state of an acrylic liquid to solid is synthesised by joining together molecules of the monomer to produce a polymer. Acrylics are polymerised by a free-radical chain addition mechanism where free radicals are produced, in a process where polymerisation consists of the three steps: initiation, propagation and termination. Dibenzoyl peroxide breaks down at 50-60°C, but the addition of a tertiary aromatic amine, e.g. N,N-dimethylaniline (DMA) or dimethyl *para*-toluidine (DMPT), can cause the peroxide to break down into radicals at 0°C, so that the plastic can be polymerised at room temperature or lower. As the amine stimulates polymerisation to proceed at a faster rate, these chemicals are often termed either activators or more commonly accelerators, and can be responsible for inducing discolouration of polymerised blocks. Gerrits *et al.,* (1991) investigated two other amine accelerators N,N-3,5-

tetramethylaniline (DMSX) and 4-tert-butyl-N,N-dimethylaniline (DMBA) for polymerisation of GMA, and sulfinic acid and some barbiturates have also been used (Gerrits and Smid, 1983). A review of the properties of various amine accelerators for methacrylate sysytems was discussed by Bowen and Argentar (1971). Polymerisation can be carried out by means of heat, UV light in the presence of suitable free radical producing catalysts, or by free radical producing agents, which can be promoted by an accelerator. The production of free radicals by the decomposition of dibenzoyl peroxide in the presence of the accelerator DMA is shown in Fig 1.9 on page 38 (Gerrits, 1987).

In the studies by Gerrits and van Leeuwen (1985) it was shown that the concentration of inhibitor hydroquinone in GMA and dibenzoyl peroxide used can both affect the rate of polymerisation and the temperature produced during polymerisation. Generally an increased concentration of dibenzoly peroxide and/or reduced concentrations of inhibitor resulted in an increase in the temperature with polymerisation occurring faster in monomer that contained a lower concentration of inhibitor.

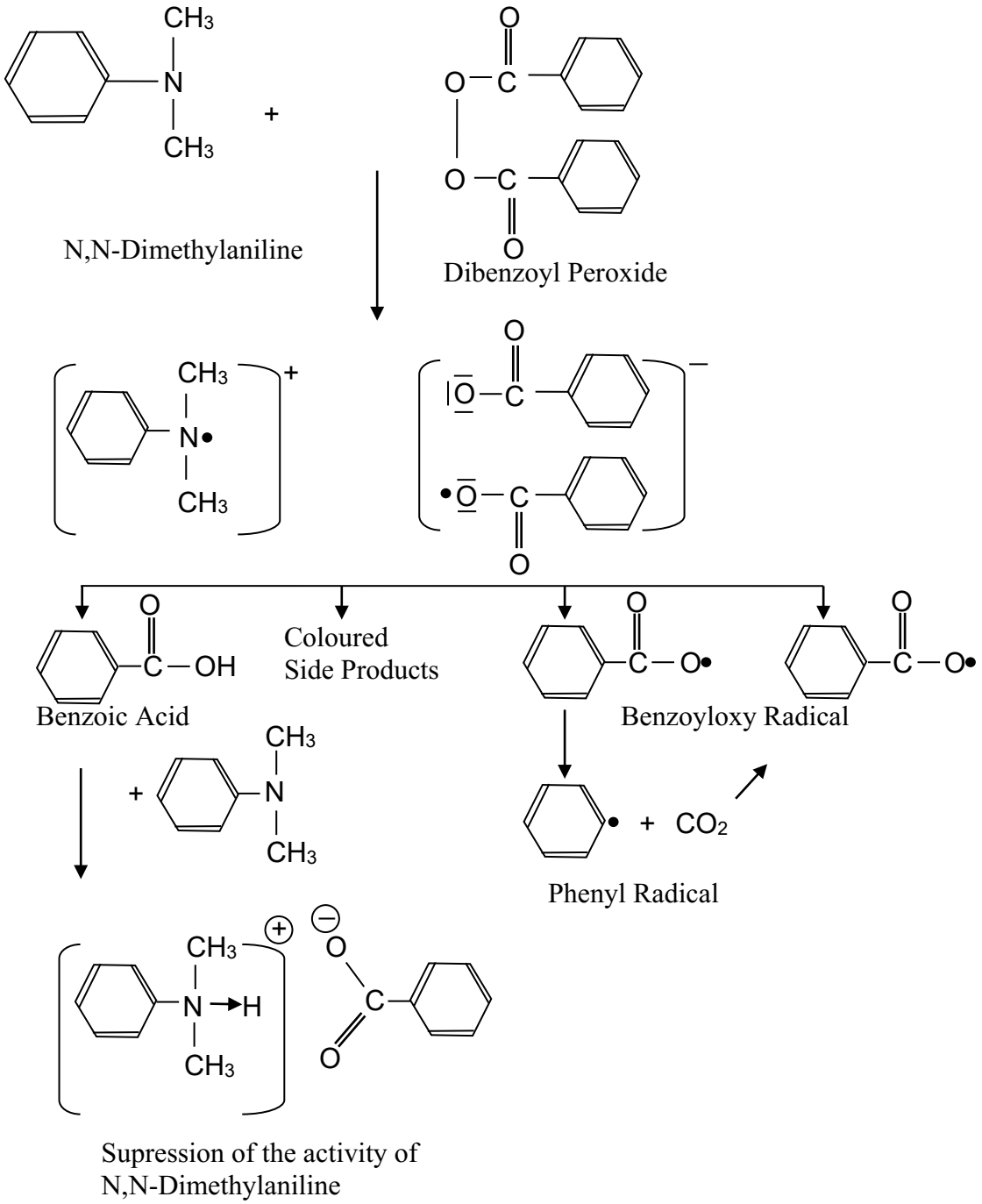



**Fig 1.9** Decompostion of dibenzoyl peroxide to produce benzoyloxy radicals in the presence of N,N-dimethylaniline (Gerrits, 1987).

During polymerisation via free-radical chain reactions, the intermediate chemicals formed have an incomplete number of electrons. The monomer is exposed to a source of phenyl (benzoyloxy) radicals which react with the double bond of the acrylic monomer (initiation), with a result that the electrons relocate to a different position on the monomer which then becomes a radical in turns. This now acts as an active site, attracting and joining another monomer by repeating the process of opening the carbon-carbon double bond and forming a dimeric radical via a covalent bond (propagation). Successively more monomer molecules are added to produce a long aliphatic chain and form a polymer. These chains can entangle with each other. Simplified schemes to demonstrate the reaction mechanisms of initiation and propagation of GMA are shown in Figs 1.10 and 1.11 on page 40 (Gerrits, 1987). Propagation is terminated on reaction of pairs of polymer radicals leading to dead polymer chains, and occurs by one of two mechanisms known as termination coupling or combination (Fig 1.12 A) and termination by disproportionation (Fig 1.12 B) as shown in on page 41 (Gerrits, 1987). Some monomers terminate by one of these mechanisms or by both (http//chem.chemrochester.edu/-chem421/frterm.htm).

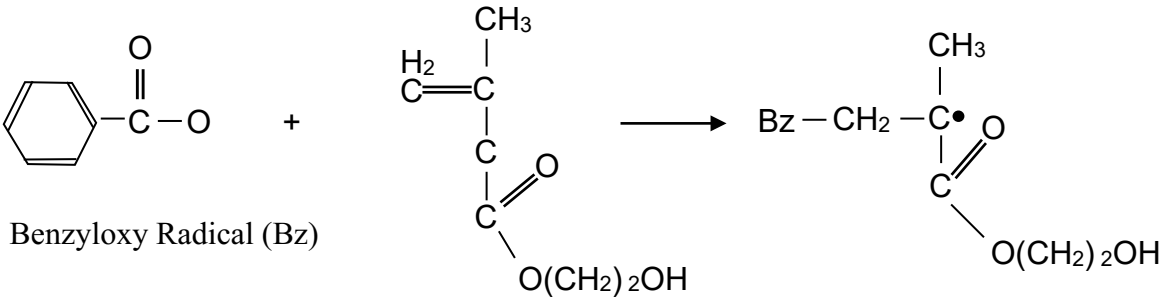



**Fig 1.10** Initiation of glycol methacrylate (Gerrits, 1987).

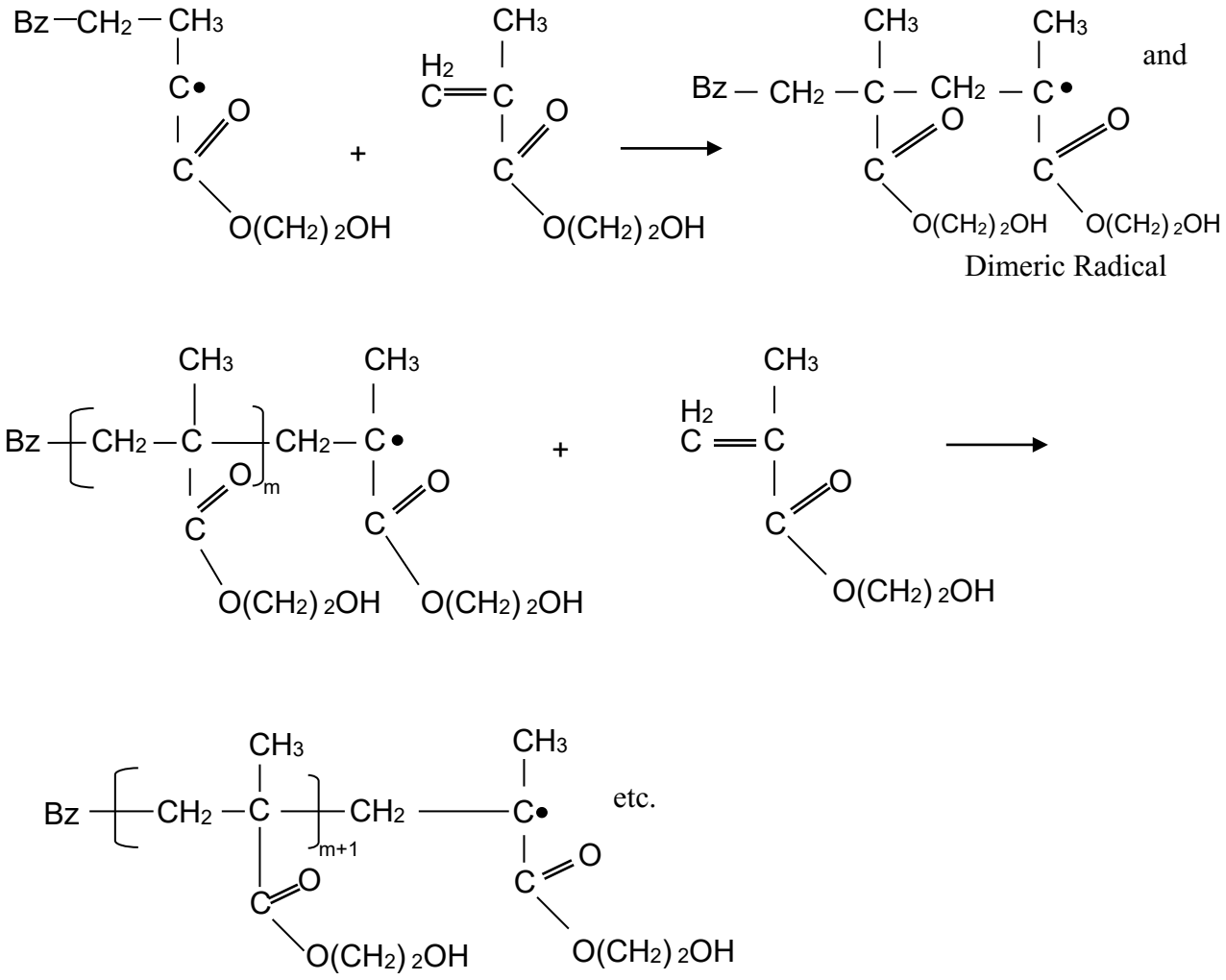
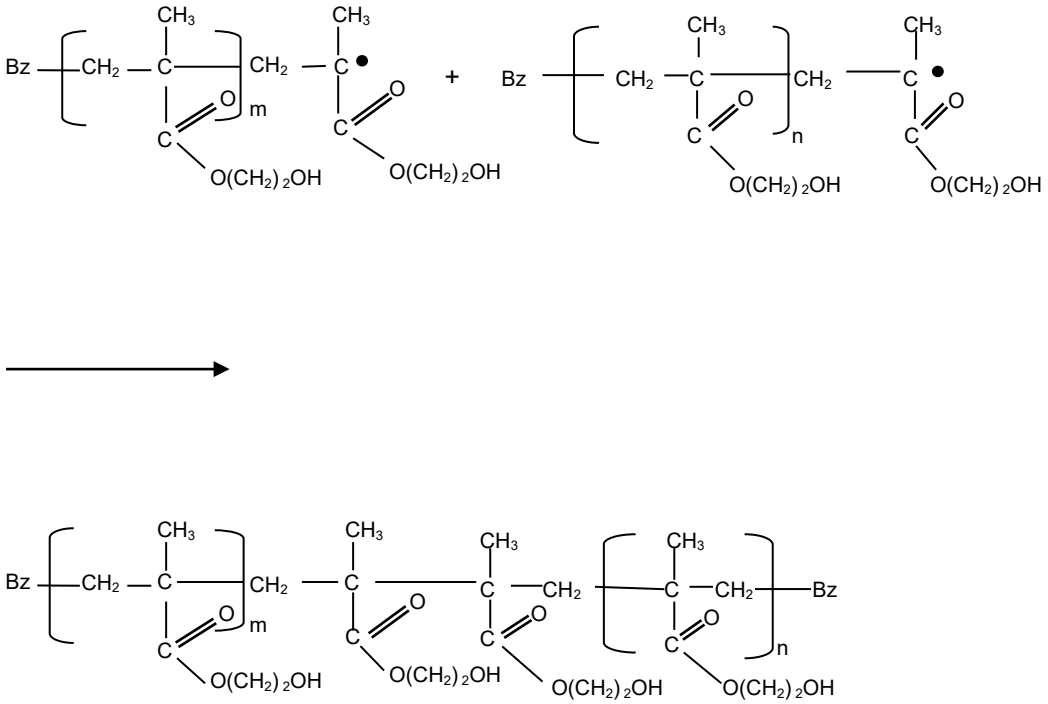



**Fig 1.11** Propagation of glycol methacrylate (Gerrits, 1987).

A. Termination by combination



B. Termination by disproportionation

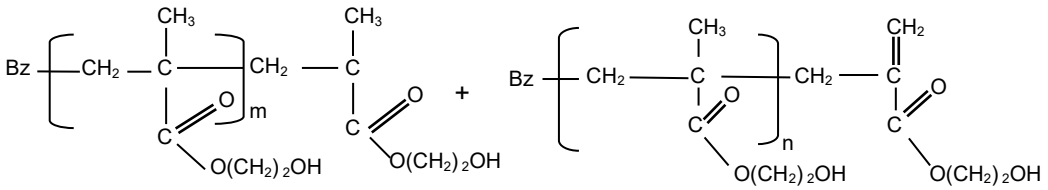


**Fig 1.12** Termination of the propagation of glycol methacrylate (Gerrits, 1987).

Brauer *et al.,* (1956) described a mechanism for the decomposition by benzoyl peroxide by the accelerator DMA via a series of complex unstable intermediates to produce free radicals. However, Imoto and Choe (1955) suggested the formation at the start of a benzoyl peroxide-dimethylaniline complex before proceeding via further reactions. In spite of the different mechanisms of benzoyl peroxide proposed, both studies agreed that for polymerisation to occur, one or both of the free electrons on the nitrogen of the amine is transferred to dibenzoyl peroxide. A more recent discussion on the polymerisation of different methacrylates was provided by Sideridou *et al.,* (2006). Both oxygen and acetone prevent attachment of radicals and should therefore be avoided during polymerisation.

The conversion of a double bond to a single bond is accompanied by an exothermic “heat of polymerisation”. The resulting acrylic polymers also become more dense producing shrinkage in acrylics of up to about 20% (Glauert, 1984), but the amount of contraction depends on the mode of polymerisation and the plastic. For example, cold cure acrylic plastics produce less exothermic heat and consequently less shrinkage than heat cured systems (Osborne and Wilson, 1970). This means polymers with shorter chains and a lower molecular weight are produced. According to Osborne and Wilson (1970), the hardness of cold cured plastics is less than if heat cured when tested immediately after processing, but will harden to an equal degree after a further two weeks, indicating that cold cured acrylics are not initially completely polymerized. Polymers may branch and interconnect with other branches to give structural rigidity, and separate polymers or part of the same polymer are attracted to each

other by van der Waals forces (Rodriguezs, 1970).

### 4.1.8 Proprietary Single and Dual Purpose Acrylic Kits

During the last three decades or so, several proprietary plastic embedding kits have become available. Some are suitable for either LM or EM only, but others are dual purpose acrylics proposed for both applications. BMA is now rarely used for any histological purpose unless as an ingredient in an acrylic mix, since it has proved unreliable and produces tissue artifact during polymerisation (Kay, 1967; Janes, 1979) but it is present in Bioacryl, now re-named Unicryl (British BioCell International, UK), introduced by Scala *et al.,* (1992).

Since Arnold and Jee (1954), MMA has been widely used as the ideal embedding medium for either hard tissue such as undecalcified bone, or tissue containing stents or implants for LM studies because of its hardness. For these purposes several proprietary kits containing MMA such as Technovit 9100 now superceded by Technovit 9100 New (Heraeus Kulzer), Osteo-Bed/Osteo-Bed Plus (Polysciences Inc.), K Plast (Medim, Germany) and Acrylosin (Dorn & Hart Microedge Inc., USA) have been introduced. Technovit 9100 was supplied with Perkadox 16, but this catalyst has now been replaced with dibenzoyl peroxide in Technovit 9100 New, although both kits consist of PMMA powder to stiffen the mix to produce a harder block. It is polymerised via the amine accelerator DMSX which can be used at sub-zero temperatures. Osteo-Bed and Osteo-Bed Plus consists of two components, MMA monomer containing a plasticiser and dibenzoyl peroxide. Osteo-Bed Plus produces harder blocks and is therefore more suitable for larger pieces of undecalcified bone and/or tissue

containing metal implants, grafts and stents (Polysciences Inc.). Polymerisation of Osteo-Bed and Osteo-Bed Plus is achieved either with gentle heat or by mixing excess catalyst to induce polymerisation naturally. K Plast (Meeuwsen, 1986) and (soft and hard) Acrylosin which use dibenzoyl peroxide and Perkadox 16 as the catalyst repectively are similar plastics that are polymerised via gentle heat, and as a result of not using an amine accelerator the blocks produced are colourless (Medim, Germany; Dorn & Hart Microedge Inc., USA).

Various GMA mixes have been reported, with a result that some may be either prepared from the components or purchased as a proprietary kit with several based on the classic recipe published by Ruddell (1967b). All kits contain the monomer HEMA, but the proportion and variety of ingredients vary, leading to different characteristics. The monomer can be contaminated with methacrylic acid, which has been suggested should be removed (Frater, 1979; Franklin *et al.,* 1981), but the background staining that occurs can be reduced by purchasing “low-acid” HEMA or a high-quality proprietary kit. In 1967 Ruddell published two recipes for producing a GMA block. In the first paper (Ruddell, 1967a) HEMA with polyethylene glycol 400 as a plasticiser and dibenzoyl peroxide as the catalyst was polymerised using the accelerator DMA, whereas in the second paper (Ruddell, 1967b) an improved recipe using 2-butoxyethanol as the plasticiser was used. The latter formulation has resulted in well known proprietary GMA kits becoming available such as JB-4 (Polysciences Inc.) which have been widely used in many laboratories. Subsequently Ruddell (1971) also reported the use of pyridine as an alternative accelerator for polymerisation of GMA, although no proprietary kit is available that uses this

accelerator. Several publications have described using individual ingredients to produce various GMA blocks to a specific recipe (Green, 1970; Cole and Sykes, 1974; Sims, 1974; Murgatroyd, 1976; Lee, 1977), whilst other preferred to use MMA (Cathey, 1963; Phillpotts, 1972; Potter, 1974) or BMA combined with paraffin wax (Engen and Wheeler, 1978; Wechbanjong *et al.,* 1979; McMillan *et al.,* 1983). Janes (1979) reviewed three plastic embedding techniques using either a BMA/MMA mix, GMA or the epoxy Spurr plastic. The relative ease of using GMA, together with its availability later as a kit (Sorvall Resin, Sorvall; JB-4, Polysciences Inc.), prompted many laboratories to begin using GMA as the plastic embedding medium of choice for LM purposes. Gerrits and Smid (1983) described a less toxic system where polymerisation of GMA was initiated with a barbituric acid derivative in combination with chloride ions and dibenzoyl peroxide. The non-toxic catalyst system produced clear blocks which could be serial sectioned. The GMA mix was marketed as a kit under the proprietary brand of Technovit 7100 (Heraeus Kulzer) or Historesin (Leica, UK). Further studies later by Gerrits *et al.,* (1991) suggested a safer alternative amine accelerator to DMA by the introduction of RES G20 which later became known as Technovit 8100 (Heraeus Kulzer). JB-4 Plus and ImmunoBed, (Polysciences Inc.) were two other kits based on GMA and similar to JB-4, with the manufacturers stating that the former produced clearer blocks at a lower polymerisation temperature, whereas the latter was more suitable for IHC studies.

An alternative interesting hydrophilic plastic was suggested by Frater (1985) who formulated a mixture containing acrylonitrile, dimethyl acrylamide and

methyl methacrylate. Dithiothreitol was added to limit the degree of polymerisation, which was achieved using benzoin and irradiation with UV light (wavelength 360 nm). The resulting polymerised plastic was soluble in dimethyl formamide, although if dithiothreitol was absent from the mix, the resulting polymer did not completely dissolve. The paper reported on sections stained with tintorial dyes.

Causton *et al.,* (1980) reported on the design of a low toxicity plastic for EM that consisted of a high aromatic content provided by styrene to achieve beam stability. Styrene also had the advantage of low viscosity and hence rapid tissue penetration. However, polystyrene was too brittle for EM sectioning and so a long chain chain methacrylate was added as a plasticiser. Finally to reduce shrinkage during polymerisation and "craze" formation during sectioning, an aromatic dimethacrylate was incorporated. Aromatic dimethacrylates have a very low toxicity and are used in dental filings, but also have very good beam stability. It would seem from this and subsequent publications including information from material hazard data sheets that these reagents formed the basis of LR White and LR Gold manufactured by London Resin Company. LR White contains catalyst dibenzoyl peroxide and may be polymerised by the addition of the accelerator DMPT, whereas LR Gold developed in 1983 (Germaine and Stevens, 1996) is cured by the addition of benzil and exposure to UV light using a quartz halogen lamp specifically for sub-zero temperature embedding at -25$^{o}$C. In addition, these "aromatic polyhydroxy dimethacrylate resins" were supplemented by Histocryl for LM purposes (London Resin Co., data sheet), whereas LR White and LR Gold can

be used both for LM and EM since they combine hydrophilicity with electron beam stability (London Resin Co., data sheets).

Carlemalm *et al.,* (1982) described the development of the aliphatic Lowicryl plastics HM20 (hydrophobic) and K4M (hydrophilic) for low temperature embedding. Subsequently HM23 (hydrophobic) and K11M (hydrophilic) were also developed for similar purposes (Carlmalm *et al.,* 1985; Acetarin *et al.,* 1986), and though all Lowicryl plastics were intended for EM, Al-Nawab and Davies (1989) reported the use of K4M for some specific LM studies. The Lowicryls can be cured at low temperature by the addition of the photocatalyst benzoin and exposure to UV light. Various types of benzoin were recommended depending on their effect at sub-zero temperatures ranging from -30$^{o}$C to -80$^{o}$C (Carlmalm *et al.,* 1982 & 1985; Acetarin *et al.,* 1986). The detailed description of these Lowicryl plastics provided greater insight into the formulation and characteristics of acrylics and stimulated further developments. Recently K4M Plus has been introduced for EM which is a light curable epoxy-acrylate product combining rapid polymerisation of an acrylic with the high strength of an epoxy (Polysciences Inc.). Both benzil and benzoin decompose when subjected to UV irradiation to produce free-radicals that stimulates the polymerisation of the acrylic (Polysciences Inc., data sheet 370).

Another dual purpose plastic introduced where specific components were incorporated to induce specific properties was Bioacryl, later renamed as Unicryl (Scala *et al.,* 1992). This plastic consisted of 2-hydroxyethyl methacrylate, 2-hydroxypropyl methacrylate, n-butyl methacrylate, styrene and

dibenzoyl peroxide. It was suggested it should be polymerised using UV light at 4ºC over a period of 72 hours and stated that staining for both LM and EM was superior to that achieved with Lowicryl K4M.

Various plastic embedding kits have been marketed under different names (especially the Technovit range), leading to confusion which this author attempted to clarify (Hand, 1995b). In addition, there has been a constant introduction of new proprietary kits each claiming their suitability for a specific study, which often makes it difficult for the scientist to know which to choose. Currently many of these kits are available from TABB, UK and/or Polysciences Inc., USA.

### 4.1.9 Designer Plastics

Understanding the basic components of acrylic plastics has enabled the histologist to potentially design and create a plastic embedding medium suitable for a specific need. During the last 50 years, the literature confirms that a variety of acrylics have been formulated and a range of techniques applied (Hand, 2013). For example, the flexibility of acrylics in particular has been exploited to produce plastics of different hardness depending on the tissue or implant to be embedded, and/or whether sections are to be prepared by production of sections cut on a microtome or by ground sections. Other criteria that should be considered include the quality of sectioning possible, whether sections will “float out,” the ability of sections to adhere to a microscope slide and the solubility of the plastic, in addition to the obvious fundamental requirement of allowing good morphology and subsequent staining of the tissue. Additional properties of

acrylics have also included the ability to polymerise at low temperature for enzyme histochemical studies, control of the rate of polymerisation and/or the stabilisation of the plastic in an electron beam by the addition of specific ingredients such as a cross-linking agent present in Lowicryl plastics and Bioacryl/Unicryl.

### 4.1.10 Health and Safety

In recent years, the health and safety issues relating to the use of the components used in plastic embedding media has become increasingly important considerations (Janes, 1979; Causton, 1981; *et al.,* 1981; Tobler and Freiburghaus, 1990), which has been highlighted by the introduction of low toxicity Technovit 7100 (Gerrits and Smid, 1983) and Technovit 8100 (Gerrits *et al.,* 1991). In addition, some studies e.g. Gerrits *et al.,* (1990) and Gerrits *et al.,* (1991) have focused on discussing specific safety data of individual components. The plasticiser dibutyl phthalate is subject to legislative control by both the European Union and United States with the latter adding the chemical to the California Proposition 65 (1986) list of suspected teratogens in November 2006 (http://oehha.ca.gov/prop65.html). It is also well known that amine accelerators are toxic, and several components such as MMA may be flammable. Many of the reagents used in plastic embedding media are unpleasant and/or harmful, so attention to safety must at all times be observed in their handling which should always be in a fume cupboard avoiding inhalation of the vapours and skin contact by the wearing disposable gloves. Disposal of reagents and polymers must comply with local and national regulations.

### 4.1.11 Other Studies

In addition to the numerous publications describing the formulation of various acrylics and the application of staining protocols on plastic-embedded tissue, there has been a variety of other papers that have reported on the specific characteristics and properties of these plastics. Many of these reports focused on GMA which included assessment of the stability of sections (Gerrits and van Leeuwen, 1987) and variations in section thickness (Helander, 1983), as well as the effects on the final dimensions of the sections either as a result of using the monomer as a dehydration agent (Gerrits *et al.,* 1992), or the effects of various plasticisers and catalyst systems (Gerrits and van Leeuwen, 1984), or through embedding (Hanstede and Gerrits, 1982), or from floating on a water bath (Gerrits *et al.,* 1987). Whilst it is acknowledged that these were interesting studies, they are not discussed further in this thesis as similar studies were not investigated by this author.

# CHAPTER 5

# REVIEW OF PUBLICATIONS

## 5.1 INTRODUCTION

From the preceding two chapters, it can be seen how the development of plastic embedding evolved and how their properties influenced what type of plastic was most suitable for a particular function or study. For LM, acrylics provided the best potential to achieve staining and resolution, which in the studies by the author included techniques beyond traditional tinctorial staining such as enzyme histochemistry, IHC and ISH. During the last 20 years as the latter two in particular have undergone rapid development and expansion on routine frozen and especially paraffin sections, so too has there been a parallel evolution of similar techniques on plastic embedded tissue. The author has been instrumental in publishing numerous papers and articles to describe and publicise these techniques, some of which have been utilised and integrated in to a diagnostic service. In this chapter the papers central to these procedures are listed as shown below and will be reviewed in chapters 6 – 10. For a copy of the complete manuscript of each reviewed paper, the reader is advised to refer to pages 164-217 in the Appendix of this thesis.

## 5.2 PUBLICATIONS SUBMITTED

**1.** Hand, NM. (1996) Embedding undecalcified bone in polymethyl methacrylate: an improved method. *British Journal of Biomedical Science.* **53:**238-240. (ISSN 0967-4845).

**2.** Hand, NM. (1987) Enzyme histochemistry on jejunal tissue embedded in resin. *Journal of Clinical Pathology.* **40:**346-47. (ISSN 1472-4146).

**3.** Hand, NM. (1988a) Enzyme histochemical demonstration of lactase and sucrase activity in resin sections: the influence of fixation and processing. *Medical Laboratory Sciences.* **45:**125-30. (ISSN 0308-3616).

**4.** Hand, NM. Blythe, D. Jackson, P. (1996) Antigen unmasking using microwave heating on formalin fixed tissue embedded in methyl methacrylate. *Journal of Cellular Pathology*. **1:**31-37. (ISSN 1359-7388).

**5.** Hand, NM. Church, RJ. (1998) Superheating using pressure cooking: its use and application in unmasking antigens embedded in methyl methacrylate. *The Journal of Histotechnology.* **21:**231-236. (ISSN 0147-8885).

**6.** Blythe, D. Hand, NM. Jackson, P. Barrans, SL. Bradbury, RD. Jack, AS. (1997) The use of methyl methacrylate resin for embedding bone marrow trephine biopsies. *Journal of Clinical Pathology.* **50:**45-49. (ISSN 1472-4146).

**7.** Hand, NM. Church, RJ. (1997) Immunocytochemical demonstration of hormones in pancreatic and pituitary tissue embedded in methyl methacrylate. *The Journal of Histotechnology.* **20:**35-38. (ISSN 0147-8885)

**8.** Church, RJ. Hand, NM. Rex, M. Scotting, PJ. (1997) Non-isotopic *in situ* hybridization to detect chick Sox gene mRNA in plastic embedded tissue. *Histochemical Journal.* **29:**625-629. (ISSN 0018-2214).

**9.** Church, RJ. Hand, NM. Rex, M. Scotting, PJ. (1998) Double labelling using non-isotopic *in situ* hybridisation and immunohistochemistry on plastic embedded tissue. *Journal of Cellular Pathology.* **3:**11-16. (ISSN 1359-7388).

## 5.3 CLASSIFICATION OF REVIEW

From the titles of the nine publications listed (not in chronological order) in section 5.2, it can be seen that whilst all were related to plastic embedding techniques, there were well defined and distinct specific details that enabled their segregation. In this section the papers are divided as shown below according to:-

(i) the plastic employed
(ii) the type of section prepared
(iii) a description of the investigation and use.

For clarity, the publications listed in section 5.2 will be referred to as paper 1 – 9.

### 5.3.1 Paper 1

- Polymethyl methacrylate
- Thin sections of bone (6 &13 μm)
- Methodology and technique for metabolic bone disease

### 5.3.2 Papers 2 and 3

- Glycol methacrylate and Histocryl
- Semithin sections (3 μm)
- Methodology and application of enzyme photochemistry

### 5.3.3 Papers 4 and 5

- Methyl methacrylate
- Semithin sections (2 μm)
- Methodology and techniques for immunohistochemistry

### 5.3.4 Papers 6 and 7

- Methyl methacrylate
- Semithin sections (2 - 4 µm)
- Methodology and applications of immunohistochemistry on hard and soft tissues

### 5.3.5 Papers 8 and 9

- Methyl methacrylate
- Semithin sections (2 & 4 µm)
- Methodology and application of *in-situ* hybridisation including in combination with immunohistochemistry

# CHAPTER 6

# REVIEW OF PAPER 1

## 6.1 REVIEW OF PAPER 1

### 6.1.1 Embedding undecalcified bone in polymethyl methacrylate: an improved method. (Hand, 1996; *British Journal of Biomedical Science*, 53:238 - 240).

### 6.1.2 Introduction

For many years the histological examination and investigation of metabolic bone diseases such as osteoporosis, osteomalacia and Paget's disease has relied on the preferred option to examine samples by preparing undecalcified sections of iliac crest bone biopsies that have been embedded in plastic (Callis, 2008; Recker, 1983; Sanderson, 1997). In the above paper by Hand (1996), a number of improvements were described for the preparation of plastic sections of undecalcified bone specifically for the diagnosis of metabolic bone diseases. Though several previous publications had reported using acrylic and polyester plastics e.g. Recker, (1983) and Mawhinney and Ellis, (1983) respectively, this paper developed further ideas using PMMA based on a recipe described by Difford (1974) that had previously been routinely and extensively used in the author's laboratory. To enable this author to implement all the modifications and improvements in a diagnostic environment, it was also necessary to consider in particular whether a quicker TAT to process the tissue was possible, and whether the quality of the morphology and staining produced could be improved.

### 6.1.3 Method

In the preparation of undecalcified bone sections, iliac crest biopsy cores of up to 20 mm long and 8 mm in diameter were sampled, but tissue of this size necessitated producing a plastic block that was sufficiently hard enough to enable quality sections to be produced. In preliminary studies, various mixtures of the conponents were investigated to produce an optimal block, and to assist with hardness and ensure that the shrinkage associated with MMA was reduced, a partially polymerised syrup-like mix based on that described by Difford (1974) was preferred. In the original mix by Difford this was created solely by the addition of “low molecular weight” PMMA beads, but unfortunately for 20 $mm^2$ blocks, this mix proved too hard cut on a Reichert-Jung 1140 Autocut, without producing the sectioning “chattering” artefact, and consequently the plasticiser dibutyl phthalate was added. It was decided not to remove the hydroquinone inhibitor present (0.01% / 100 parts per million) in the MMA monomer which in similar bone studies both Kenner *et al.,* (1982) and Buijs and Arend (1983) also did not remove. During the time this plastic mix was being routinely used, the PMMA beads became no longer available and PMMA powder was instead used. Although the viscosity differed slightly, even following reformulation from a concentration of 30% PMMA beads to 40% powder, the latter dissolved more easily and produced similar results. Sufficient dibenzoyl peroxide catalyst was added to permit rapid and controlled overnight polymerisation using a 60$^oC$ waterbath. No bubble artefact was produced, which was probably assisted by the inclusion of the inhibitor. However, when increased volumes of plastic were required for larger blocks of up to 50 $mm^2$, polymerisation was performed in a waterbath at the lower temperature of 37$^oC$ over two to five days to avoid the

potential of a violent exothermic reaction. These blocks were then further hardened at 60°C for 24 hours. The procedures used were as follows:-

Specimens were either fixed in formol calcium for at least 24 hr or 70% ethanol if the patient had previously been administered in vivo tetracycline drugs. A processing schedule for fixed biopsies consisted of 70% ethanol 1 hr x2, 90% ethanol 1 hr x2, 100% ethanol 2 hr x2, 100% ethanol overnight, infiltrated firstly with solution 1 (previously-prepared) containing MMA monomer (70%), dibutyl phthalate (30%), with dried dibenzoyl peroxide (2%) 4 hr x2, and secondly infiltrated in solution 1 containing 40% low molecular weight PMMA powder (solution 2 previously-prepared) 23 hr. (Solutions 1 and 2 were kept in the refidgerator at 4°C). The specimens were processed at room temperature in 20 $mm^2$ diameter glass vials (vol 10 cc) which were agitated at all stages on a roller mixer in a fume cupboard. Subsequently the specimens were allowed to stand at room temperature for 1 hr to remove air bubbles in the plastic mix, before the vial was capped and placed in a 60°C waterbath for polymerisation to proceed overnight. Plate 1 (p 60) shows a glass vial and an iliac crest core embedded in a polymerised PMMA block. In preliminary studies, attempts were made to produce rectangular blocks but as these were unsuccessful, circular shaped blocks using the glass vial as a mould were instead. To release the colourless polymerised block, the vial was cooled to -20°C in the deep freeze compartment of a refrigerator for 15 mins, and the glass then carefully broken by tapping with a hammer. For larger pieces of bone of up to 50 $mm^2$, the above schedule was unsuitable and optimal results were achieved by extending each processing stage to 24 hr with 48 hr for the infiltration steps in solutions 1 and 2.

Polymerisation was as previously described but at 37°C. Sections for tinctorial staining or those labelled with tetracycline were cut at 6 µm or 13 µm respectively either on a Reichert-Jung Autocut 1140 microtome with a specially designed modified block clamp (plate 2, p 61) or for larger blocks on a Jung K using a D profile tungsten carbide knife with 30% ethanol as a lubricant. This solution was also used to store the sections until they were required for staining. The sections were stained free-floating without removal of the embedding medium, and mounted in Picomount (R. A. Lamb, UK) on a glass microscope slide after being blot dried and cleared in methylcyclohexane. To flatten the sections, the slide was clamped overnight in a polythene wrap. The staining techniques used included von Kossa/van Gieson, Goldner's Trichrome, Solochrome Azurine and tetracycline flourescence on unstained sections.

### 6.1.4 Results

For many years, the laboratory had used PMMA embedding which had proved a satisfactory embedding medium for undecalcified bone in the diagnosis of metabolic bone diseases, and consequently it was decided to continue its use. In preliminary studies it had been established that the plastic used needed to be suitable for large blocks and easy sectioning, whilst maintaining sufficient hardness to produce good morphology of undecalcified bone. The modified plastic mix that incorporated the plasticiser dibutyl phthalate described in this paper satisfied both these criteria. In addition, other improvements were investigated, which included non-removal of the inhibitor, a reduced processing time schedule, quick polymerisation of the plastic (concentration of dibenzoyl peroxide was increased from 1% - 2%) and a modified microtome clamp that

permitted blocks to be sectioned on a Reichert-Jung Autocut without prior shaping to see whether a quicker TAT to produce the sections could be achieved. When using blocks of this type, the identification label (arched over the bone core) remained embedded in the block. Previously, the blocks were shaped on a mechanical sander where in addition to exposure of harmful PMMA dust and the risk of injury, the removal of the label with the potential of an error occurring were serious disadvantages. It was found in initial studies that as MMA reacted with substances such as silicone rubber that could be used for making a mould, the simplest and most effective mould for biopsies was to continue to use a 20 $mm^2$ glass vial. During polymerisation it was important that the vials were capped for the PMMA to cure properly.

Though PMMA can be dissolved, superior results with various stains using free-floating sections without removal of the embedding medium were achieved. Successful staining techniques used included von Kossa/van Gieson, Goldner's Trichrome, Solochrome Azurine and tetracycline flourescence on unstained sections (see plates 3 - 8 on pp 61 - 64).

The overall processing time for biopsies was reduced from 14 days to three, although for larger pieces of bone measuring up to 50 $mm^2$ a longer processing and polymerisation schedule was necessary. The modifications described resulted in considerable saving in time without any compromise in quality, and it was reported that modifications had been used for investigating approximately 10,000 cases of metabolic bone diseases for diagnostic purposes.

### 6.1.5 Conclusions

This paper focussed on a number of modifications to an existing method that was used to create a more streamline procedure and improve the results for the investigation of metabolic bone diseases. The preparation of the plastic mix was easier and quicker to prepare with the removal of hydroquinone unnecessary, and as a result exposure to harmful MMA fumes was reduced. The overall processing schedule was reduced from 14 to three days and the design and use of the modified block holder averted the need to shape the block. This reduced (i) personnel to exposure of harmful PMMA dust, (ii) the risk of error due to non-removal of identification label and (iii) the risk of injury. The series of improvements that had been used for investigating approximately 10,000 cases of metabolic bone diseases resulted in a quicker TAT of work whilst maintaining high quality tissue morphology and staining.

## 6.2 PHOTOMICROGRAPHS

**Plate 1:** Glass vial with PMMA block. Glass vial used to process and embed an iliac crest bone biopsy in PMMA to produce 20 $mm^2$ plastic block

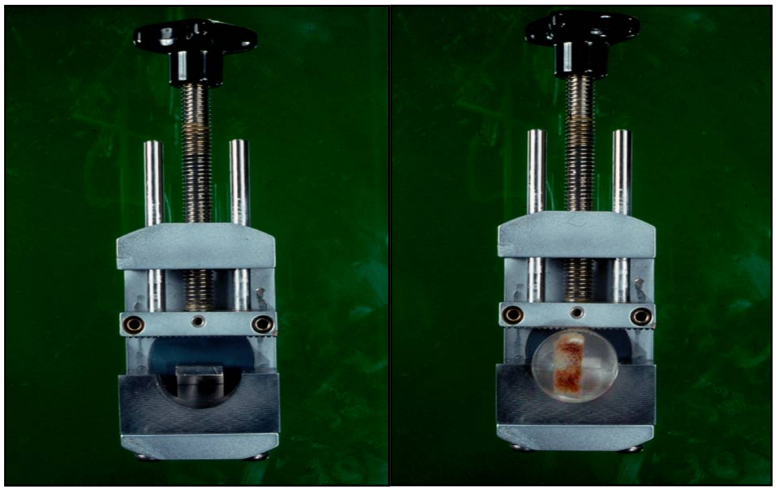


**Plate 2:** Modified clamp without and with block. Modified clamp for Reichert-Jung Autocut without and with PMMA block containing an iliac crest bone biopsy.

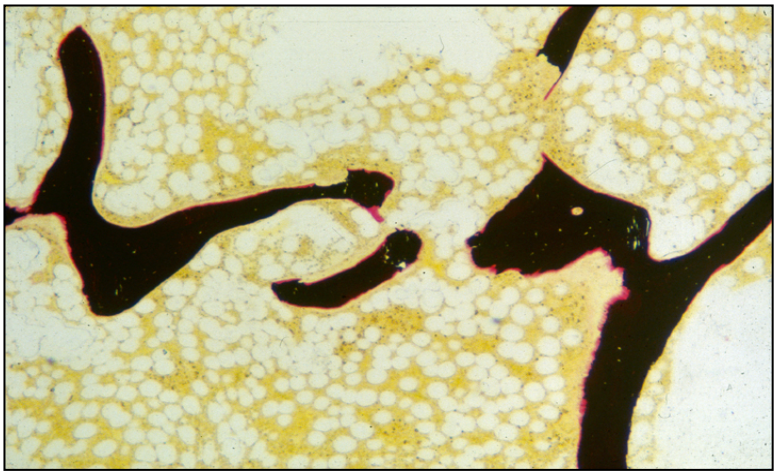


**Plate 3:** von Kossa/van Gieson – normal bone (LP). Iliac crest bone fixed in formol calcium and embedded in PMMA without decalcification showing mineralised bone (black) and osteoid (red). Bone marrow is yellow.

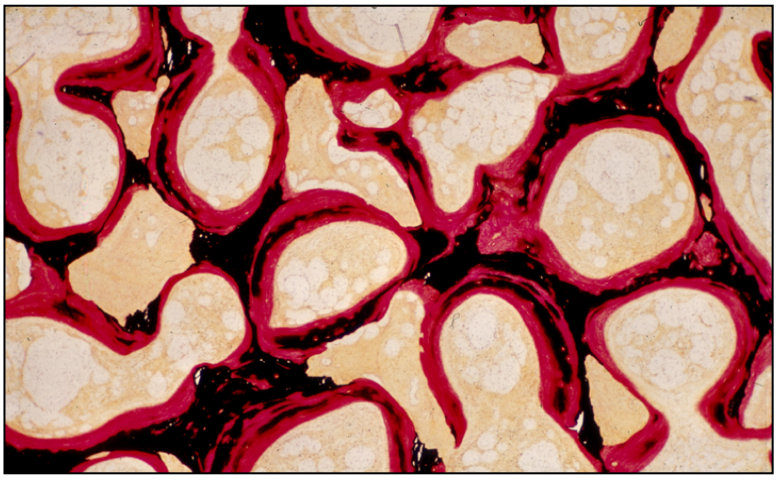


**Plate 4:** von Kossa/van Gieson – osteomalacia (MP). Iliac crest bone fixed in formol calcium and embedded in PMMA without decalcification showing mineralised bone (black) and excess osteoid (red). Bone marrow is yellow.

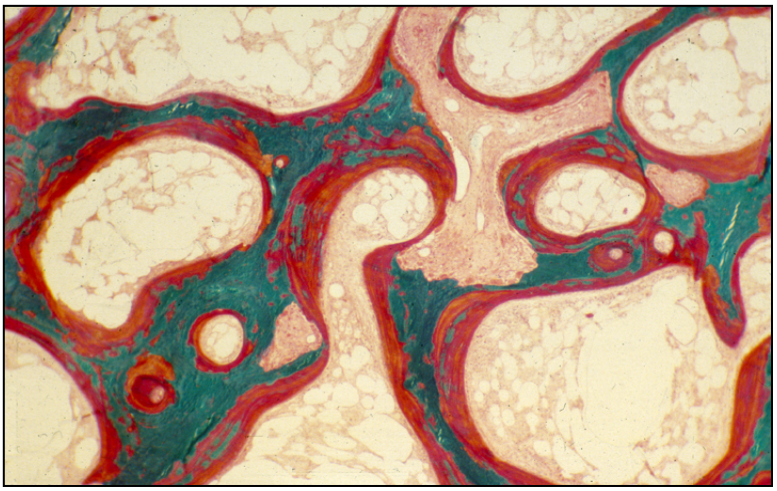


**Plate 5:** Goldner Trichrome – osteomalacia (MP). Iliac crest bone fixed in formol calcium and embedded in PMMA without decalcification showing cellular components in the marrow and mineralised bone (green) with an excess of osteoid (red).

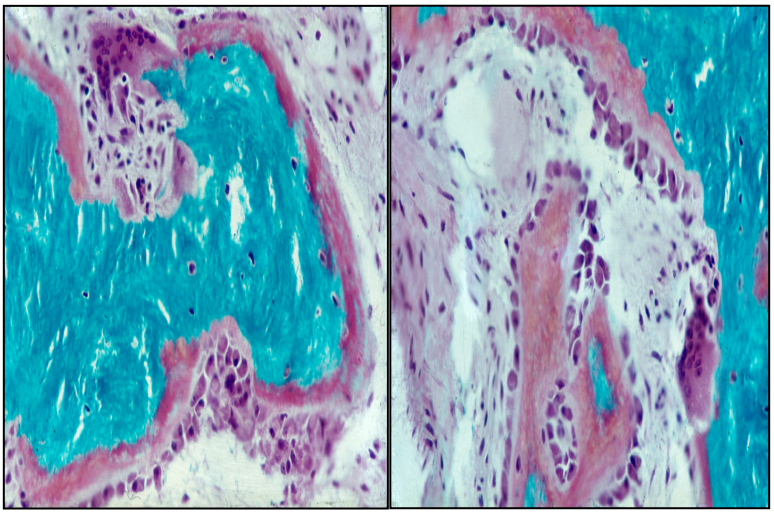


**Plate 6:** Goldner Trichrome – Paget's Disease (HP). Iliac crest bone fixed in formol calcium and embedded in PMMA without decalcification showing mineralised bone (green) and excess osteoid (red) with resoprtion of bone by multi-nucleated osteoclasts and deposition of new bone by osteoblasts.

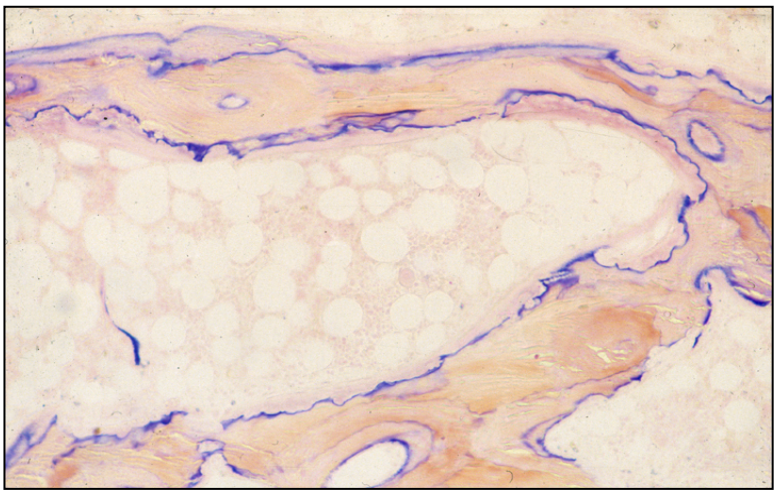


**Plate 7:** Aluminium – Solochrome Azurine (MP). Iliac crest bone fixed in formol calcium and embedded in PMMA without decalcification showing deposition of aluminium (purple) in the trabecular bone.

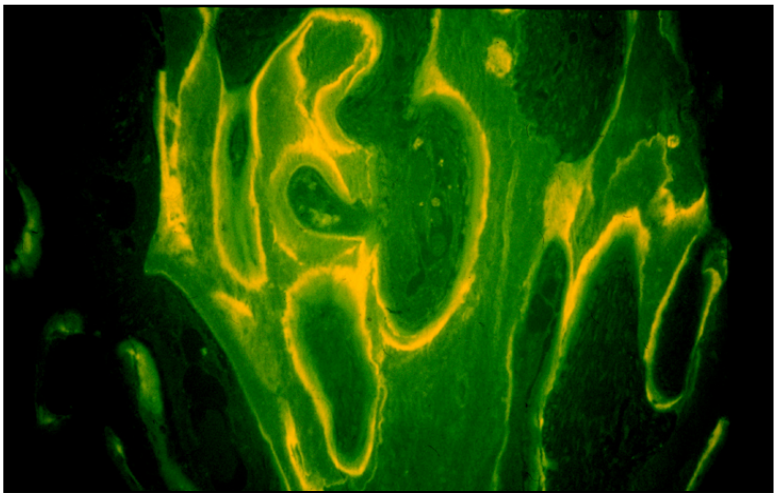


**Plate 8:** Tetracycline fluorescence (LP). Iliac crest bone fixed in 70% ethanol and embedded in PMMA without decalcification viewed with fluorescent light and showing tetracycline fluorescence (yellow) in mineralised bone (green) of an unstained section.

# CHAPTER 7

# REVIEW OF PAPERS 2 AND 3

## 7.1 REVIEW OF PAPER 2

### 7.1.1 Enzyme histochemistry on jejunal tissue embedded in resin. (Hand, 1987; *Journal of Clinical Pathology,* 40:346 - 47).

### 7.1.2 Introduction

It has been acknowledged earlier in chapter 3 of this thesis that the potential to process and polymerise acrylic plastics at low temperature had enabled the publication of several papers describing the demonstration of a number of enzymes (Litwin, 1985). In the above paper in 1987, Hand reported on the histochemical demonstration of the disaccharidases lactase and sucrase, neither of which had previously been shown in plastic embedded tissue. Both of these enzymes are important for assessing suspected malabsorption, and have become the most popular disaccharidases as they represent a range of sensitivity to injury of enterocytes (Lojda, 1983). Based on an awareness that lactase and sucrase could withstand formalin post-fixation on frozen sections, (routine laboratory practice) Hand, (1986 and 1987) described the demonstration of these enzymes in plastic sections of formol calcium-fixed tissue that had been processed and embedded in JB-4 (Polysciences Inc.). In addition to staining for lactase and sucrase, acid phosphatase, alkaline phosphatase, and leucine aminopeptidase were also demonstrated on semithin sections. The above paper (Hand, 1987) expanded in more detail on what had previously been briefly reported in a poster presentation (Hand, 1986).

### 7.1.3 Method

Fresh jejunal tissue was received and a longitudinal edge produced if absent by bisecting the biopsy, so that the maximum width was 2 mm. The tissue was fixed in cold (4°C) formol calcium as recommended by Dawson (1972) for 4 hr, followed by washing in cold (4°C) 3% sucrose in cacodylate buffer pH 7.4 for 16 hr. Subsequently the tissue was processed via three changes of JB-4 monomer at -20°C each for 30 mins, infiltrated with JB-4 monomer containing 0.4% dibenzoyl peroxide at -20°C for 30 mins, and then embedded in catalysed monomer (0.4%) and accelerator (40:1) at 4°C. The tissue was orientated with pre-chilled forceps in an open plastic moulding tray (Polysciences Inc.) and plastic block stubs (CellPath plc, UK) used (plates 9 – 10; p 73) so that the villi were sectioned longitudinally. Polymerisation of the block was achieved at 4°C in a dessicator containing cold water in which an open moulding tray was partially immersed to minimise and dissipate any heat produced, and the dessicator then filled with nitrogen as oxygen inhibits polymerisation (plate 11; p 74). Following polymerisation, the 12 x 6 x 5 mm block (plate 12; p 74) was allowed to reach room temperature and 3 µm sections cut on a Reichert-Jung Autocut 1140 using a 10 mm width Latta-Hartmann glass knife. The sections were floated out on an ambient temperature waterbath and picked up on a cover glass coated with 0.1% poly-L-lysine (Mol wt. 90,000) and allowed to air-dry. Excess water on the cover glass was removed by careful blotting around the section to avoid delaying staining longer than 1 hr.

Sections were stained with haematoxylin and eosin (H&E) for microanatomical structure, and enzyme histochemistry performed at 37°C for the demonstration of

lactase, sucrase, acid phosphatase, alkaline phosphatase and leucine aiminopeptidase using well documented techniques (see Hand, 1987). The stained sections were rinsed in water, blotted, air dried and then rinsed in xylene before mounting in Permount.

### 7.1.4 Results

The results achieved showed that excellent histochemical staining of all enzymes including lactase and sucrase was possible on jejunum embedded in JB-4. Neither lactase nor sucrase had previously been demonstrated in plastic sections. Unpublished observations by Hand had previously indicated that disccharidases were more sensitive to processing than most other hydrolytic enzymes, but in the current study it was shown that the opportunity to process tissue via the monomer produced satisfactory preservation and staining of these enzymes. As expected, tissue morphology was excellent. The paper also suggested that the procedure may be a practical and useful alternative to preparing frozen blocks, especially as the latter can sometimes be troublesome when handling small biopsies. Examples of sections stained for the enzymes including lactase and sucrase using the indigogenic and 6-Bromo-2 naphthyl-$\alpha$-D-glucoside procedures respectively are shown in plates 13 – 21 (pp 75 – 79), although plates 13, 15 and 16 are not JB-4.

### 7.1.5 Conclusions

The poster of 1986 and subsequent paper in 1987 by the author demonstrated that high quality enzyme histochemical staining of lactase and sucrase in jejunum embedded in the proprietary plastic JB-4 was possible. Previously neither of

these disaccharidases had been reported in plastic sections. In addition, high quality tissue morphology and excellent enzyme histochemical staining of acid phosphatase, alkaline phosphatase and leucine aminopeptidase were also demonstrated. The paper suggested that the results offered potentially an alternative procedure to using frozen sections for the above enzymes.

## 7.2 REVIEW OF PAPER 3

### 7.2.1 Enzyme histochemical demonstration of lactase and sucrase activity in resin sections: the influence of fixation and processing. (Hand, 1988a; *Medical Laboratory Sciences,* 45:125 - 30).

### 7.2.2 Introduction.

In this paper, a closer examination of the effects that fixation, processing reagents and components present in different acrylic plastics had on the enzymes lactase and sucrase were investigated. This was significant because in preliminary and hitherto unpublished studies it had been observed that the demonstration of lactase and sucrase appeared to be more sensitive than other enzymes with reduced or no staining (plate 22; p 79), which had been previously noted (Hand, 1987). The above paper also commented on the effects of formol calcium fixation and processing had on the presence of acid and alkaline phosphatase — two other enzymes that might be useful when assessing malabsorption. The plastics investigated were JB-4 (Polysciences Inc.), Technovit 7100 / Historesin (Heraeus Kulzer) and Histocryl (London Resin Co.). Although further details were later extensively reported for several enzymes (Hand, 1988b), this paper (Hand, 1988a) together with the previous paper (Hand,

1987) examined whether a general approach for the optimal histochemical demonstration of lactase and sucrase (together with other enzymes) in various plastic sections could be provided. Using the knowledge gained in the first part of this study, staining was subsequently performed on a jejunal biopsy to test the theory. This paper (Hand, 1988a) expanded in more detail on what had been previously briefly reported in a poster presentation (Hand, 1986).

### 7.2.3 Method

A series of (i) frozen tissue blocks were prepared from transverse slices of rat jejunum that were fixed in cold (4°C) formol calcium for various times (30, 60, 120, and 240 mins) with and without post-fixation washing in gum sucrose (Holt *et al.* 1960) for 16 hr. In the first part of the study, cryostat sections were then prepared and stained for lactase and sucrase using the indigogenic and 6-Bromo-2 naphthyl-α-D-glucoside procedures respectively, and then subjectively assessed. Unfixed frozen sections were used as controls.

In the second part of the study, fixed frozen sections tissue that had been fixed in cold (4°C) formol calcium (60 mins) followed by gum sucrose overnight (16 hr) were then incubated in different processing reagents and constituents of plastic embedding media. Firstly, sections were incubated in either the cold (4°C) processing solutions ethanol or acetone for 15, 30, 60, 120, 180, 240 mins and then stained for lactase and sucrase. The results were then subjectively assessed. All the results were compared against staining performed on unfixed frozen control sections.

Secondly, similar frozen sections (formol calcium 1 hr / gum sucrose 16 hr) were also incubated in various catalysed plastic solutions and their associated accelerators at 4°C for 4 hr and 16 hr, and then stained for lactase and sucrase which was then subjectively assessed. The plastics investigated (JB-4, Historesin / Technovit 7100, and Histocryl) had 0.4% dried dibenzoyl peroxide (catalyst) added.

To test the optimal results achieved above, fresh human jejunum samples each approximately 5 x 2 x 2 mm were fixed in cold (4°C) formol calcium followed by washing in cold (4°C) 3% sucrose in 0.2M cacodylate buffer at pH 7.4. (Preliminary studies had shown that gum sucrose used for frozen sections was too viscous to allow good processing of tissue for plastic embedding). Tissue was processed at -20°C via acetone 15 min, 100% acetone/plastic 1:1, 15 min, monomer x2 30 min each, and monomer +0.4% dibenzoyl peroxide 30 min. To produce polymerisation for JB-4 and Historesin, a ratio of 40:1 with the relevant accelerator was used. For Histocryl, one drop of the accelerator was delivered from the specialised dropper bottle supplied with kit. Embedding and polymerisation procedures were at 4°C using a plastic moulding tray as described previously (7.1.3), and are illustrated in plates 9 – 12 (pp 73 - 74). Subsequently sections were prepared and stained for lactase and sucrase as described in section 7.1.3.

### 7.2.4 Results

From the results achieved with frozen sections, it was shown that lactase and sucrase staining decreased after 1 hr fixation, with lactase being the more

sensitive enzyme. Demonstration of both enzymes was considerably improved following washing in gum sucrose, with tissue fixed for 1, 2, 3 and 4 hr all giving similar staining intensity. The best results were achieved in tissue fixed for 1 hr followed by gum sucrose for 16 hr sequence and were comparable to the staining achieved in the unfixed control sections.

In the second part of the investigation, it was shown that ethanol had a critical effect on enzyme activity with complete loss of lactase within 15 mins of exposure, but could be well demonstrated after a similar time in acetone, although longer exposure did progressively reduce lactase staining. (plates 23 – 24 on p 80 shows similar staining patterns but for clarity, staining has been performed on plastic sections). The effects of ethanol and acetone on sucrase were less dramatic, although acetone was also superior to ethanol for the retention of this enzyme. With the exception of the Historesin accelerator, all reagents allowed strong staining of both lactase and sucrase even after 16 hr, with best results achieved from Histocryl constituents. The results clearly demonstrated that the schedule and reagents used for fixing and processing of the tissue was critical if accurate localisation of these enzymes were to be achieved, as staining of lactase could be easily lost and sucrase severely reduced. The paper also commented on the effects of formalin fixation and processing had on acid and alkaline phosphatase staining — two other enzymes that might be useful when assessing malabsorption, but stated that these enzymes were less sensitive than disccharidases.

When staining was tested on tissue embedded in JB-4, Historesin and Histocryl and processed under controlled conditions via acetone, good demonstration of both lactase and sucrase was shown in all plastics, (see plates 13, 15 and 16; pp 73 - 74) although Histocryl sections tended to lift off the coverglass during staining.

### 7.2.5 Conclusions

A number of parameters were investigated in this paper relating to the effects of fixation, processing and polymerisation on the staining of lactase and sucrase in jejunum embedded in JB-4, Historesin and Histocryl. It was shown that formol calcium retains the presence of lactase and sucrase, but processing could critically affect enzyme histochemical staining of lactase and sucrose; ethanol removed lactase activity even after only 15 mins exposure, but sucrase was less affected. Acetone retained lactase activity although eventually this became diminished, whereas sucrase was well preserved. As a result, it was stated that ethanol must not be used to process tissue because of potential false negative staining that could occur, but under controlled conditions acetone was satisfactory. Both lactase and sucrase could be demonstrated in JB-4, Historesin and Histocryl. The paper provided a template on how tissue should be processed for the demonstration of these enzymes whilst maintaining high quality tissue morphology.

## 7.3 PHOTOMICROGRAPHS

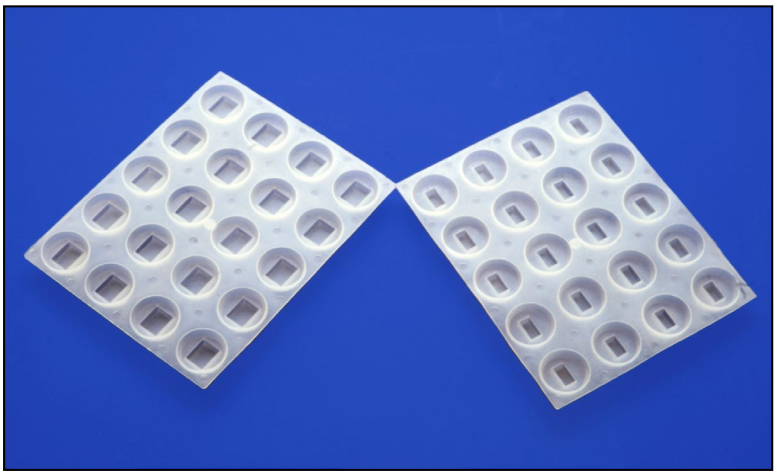


**Plate 9:** Moulding Trays. Proprietary polyethylene moulding trays of sizes 16 x 12 x 5 mm and 12 x 6 x 5 mm used for embedding.

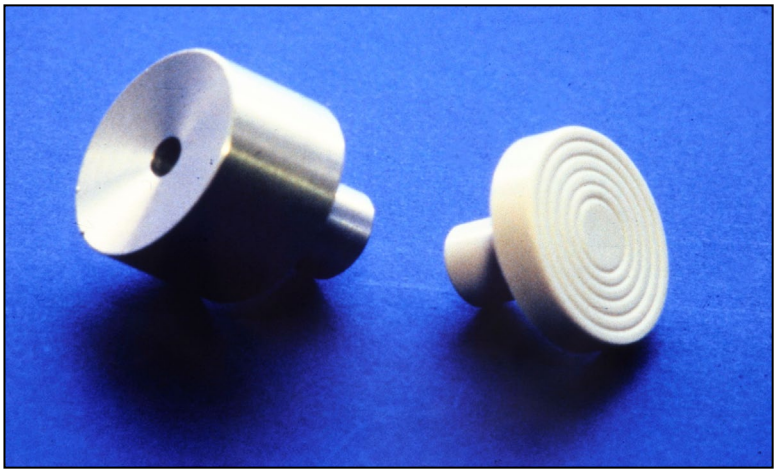


**Plate 10:** Block stubs. Proprietary aluminium and plastic block stubs

**Plate 11:** Dessicator being filled with oxygen-free nitrogen. Glass dessicator partially filled with water and the chamber filled with oxygen-free nitrogen.

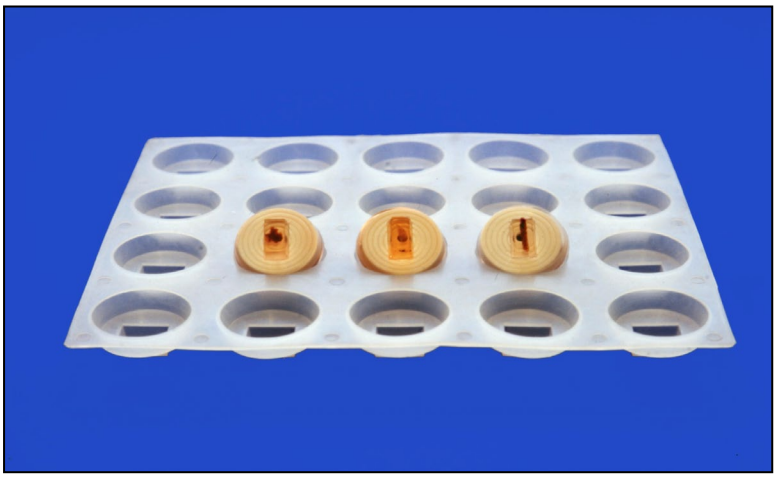


**Plate 12:** Plastic blocks. Polymerised plastic blocks of size 12 x 6 x 5 mm attached to plastic block stubs.

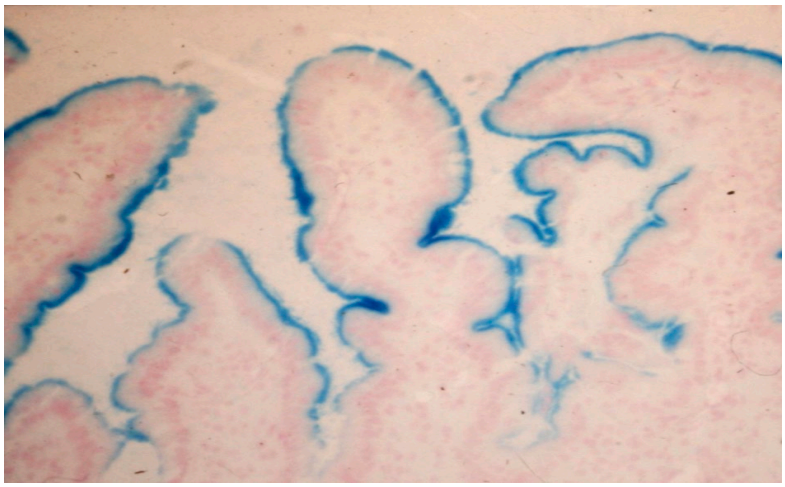


**Plate 13:** LS of villi – lactase (LP). LS section of jejunum processed via acetone and embedded in Histocryl showing histochemical staining of lactase by the indigogenic procedure on brush border of villi. Counterstain Nuclear Fast red.

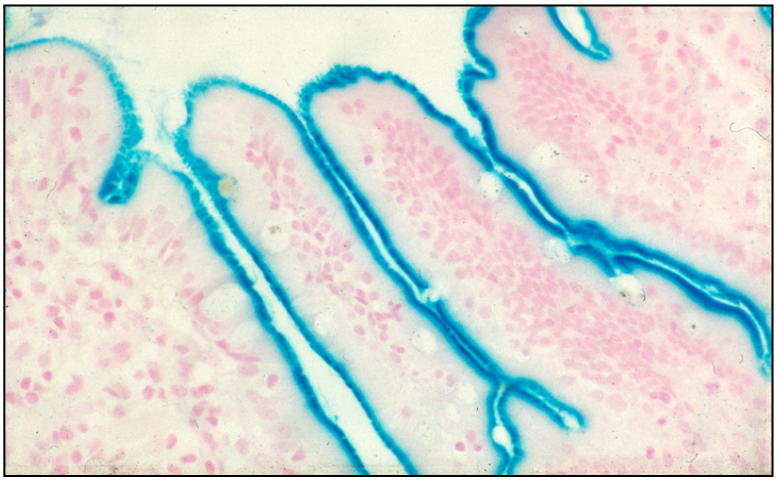


**Plate 14:** LS of villi – lactase (HP). LS section of jejunum processed via JB-4 monomer and embedded in JB-4 showing histochemical staining of lactase by the indigogenic procedure on brush border of villi. Counterstain Nuclear Fast red.

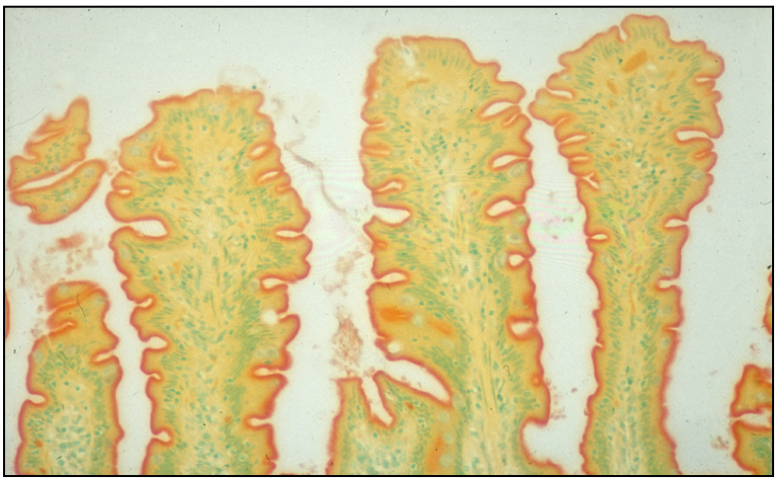


**Plate 15:** LS of villi – sucrase (MP). LS section of jejunum processed via acetone and embedded in Historesin / Technovit 7100 showing histochemical staining of sucrase by the 6-Bromo-2 naphthyl-α-D-glucoside procedure on brush border of villi. Counterstain methyl green.

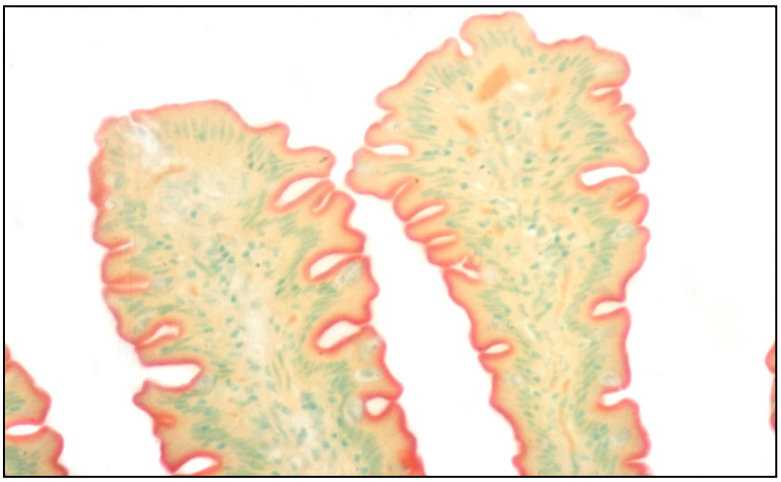


**Plate 16:** LS of villi – sucrase (HP). LS section of jejunum processed via acetone and embedded in Historesin / Technovit 7100 showing histochemical staining of sucrase by the 6-Bromo-2 naphthyl-α-D-glucoside procedure on brush border of villi. Counterstain methyl green.

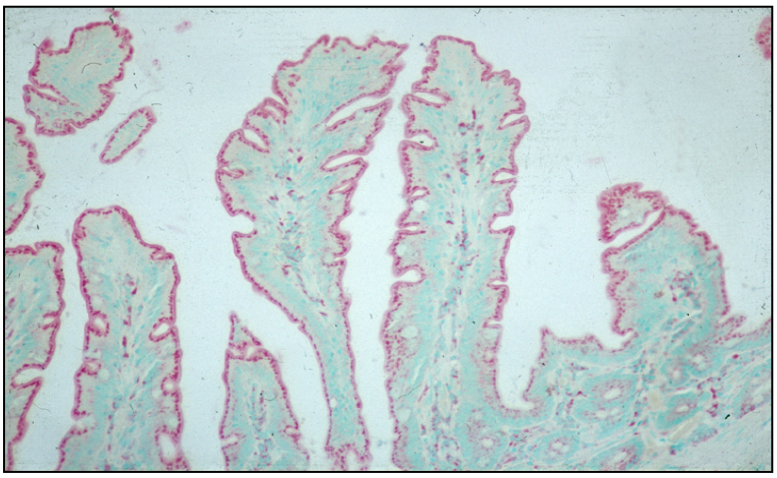


**Plate 17:** LS of villi – acid phosphatase (LP). LS section of jejunum embedded in JB-4 showing histochemical staining of acid phosphatase by the AS-BI naphthol phosphate procedure on brush border of villi. Counterstain methyl green.

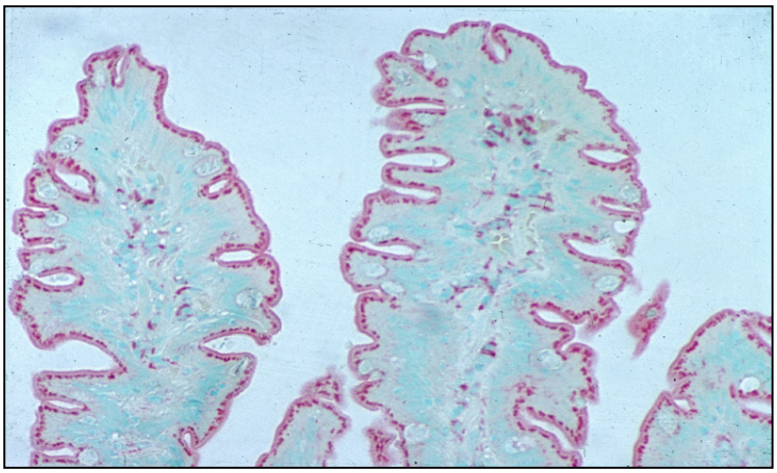


**Plate 18:** LS of villi – acid phosphatase (HP). LS section of jejunum embedded in JB-4 showing histochemical staining of acid phosphatase by the AS-BI naphthol phosphate procedure on brush border of villi. Counterstain methyl green. Reproduced by permission of American Society of Clinical Pathologists, Chicargo.

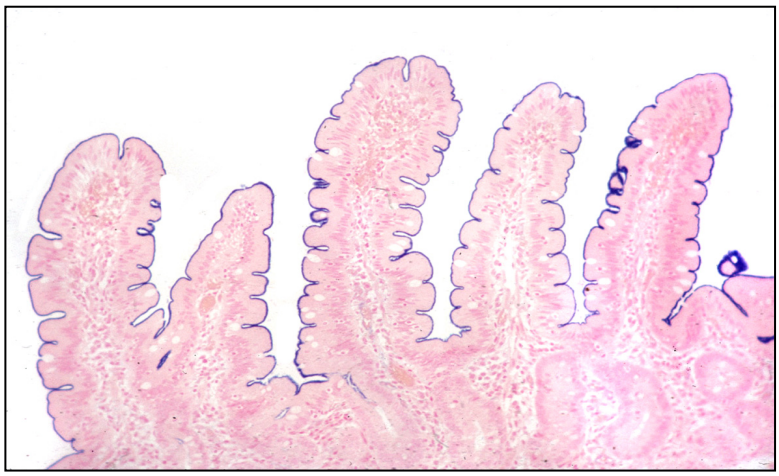


**Plate 19:** LS of villi – alkaline phosphatase (LP). LS section of jejunum embedded in JB-4 showing histochemical staining of alkaline phosphatase by the naphthol AS phosphate and Fast blue BB procedure on brush border of villi. Counterstain Neutral red.

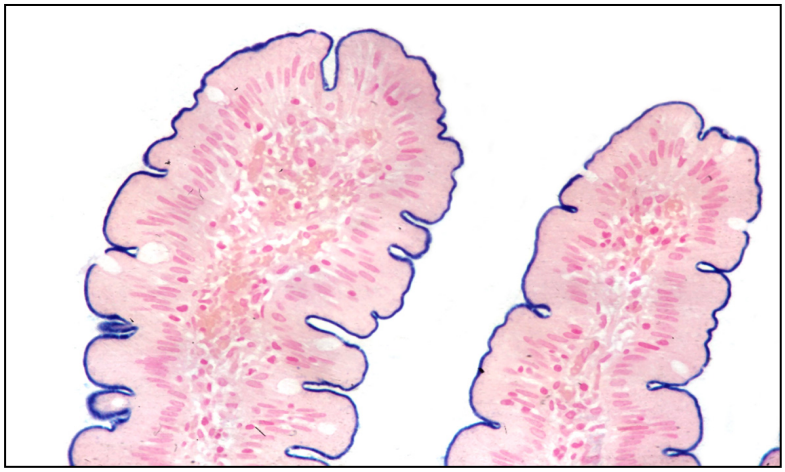


**Plate 20:** LS of villi – alkaline phosphatase (HP). LS section of jejunum embedded in JB-4 showing histochemical staining of alkaline phosphatase by the naphthol AS phosphate and Fast blue BB procedure on brush border of villi. Counterstain Neutral red.

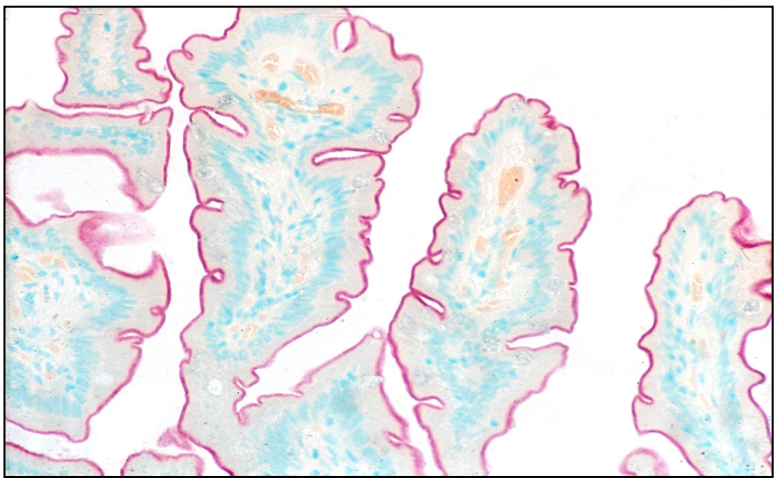


**Plate 21:** LS of villi – leucine aminopeptidase (MP). LS section of jejunum embedded in JB-4 showing histochemical staining of leucine aminopeptidase by the β naphthylamine / Fast blue B procedure on brush border of villi. Counterstain methyl green.

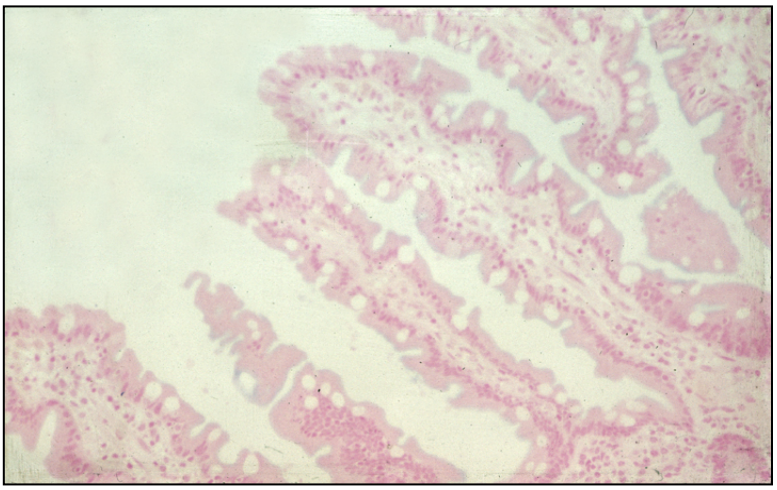


**Plate 22:** LS of villi – lactase negative after ethanol (LP). LS section of jejunum processed via ethanol and embedded in JB-4 stained for lactase by the indigogenic procedure. Note the absence of staining on brush border of villi. Counterstain Nuclear Fast red.

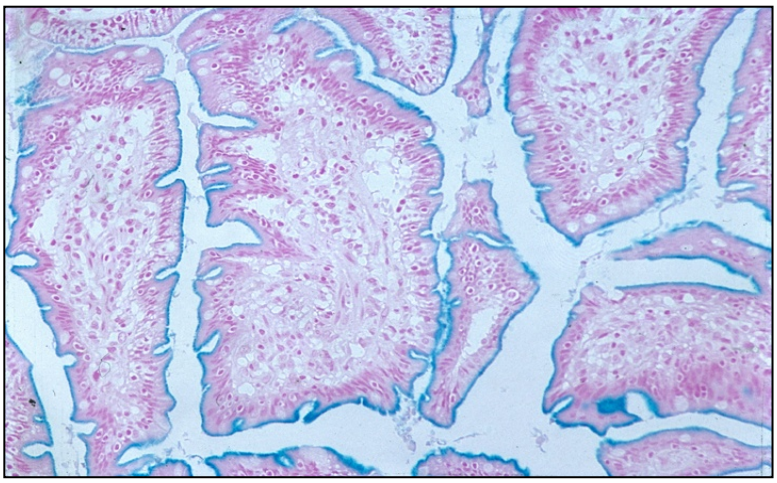


**Plate 23:** TS of villi – lactase (MP). TS section of jejunum processed via acetone and embedded in JB-4 stained for lactase by the indigogenic procedure. Note the presence of staining on brush border of villi. Counterstain Nuclear Fast red.

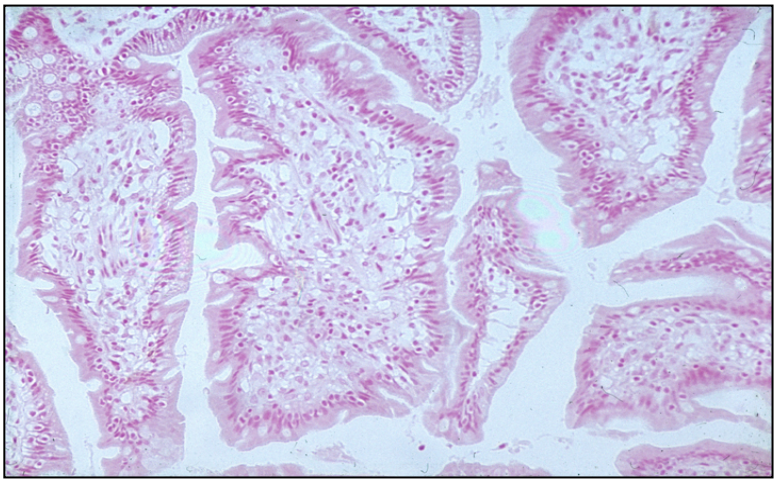


**Plate 24:** TS of villi – lactase negative after ethanol (MP). TS section of jejunum processed via acetone, embedded in JB-4 and incubated in ethanol which was then stained for lactase by the indigogenic procedure. Note the absence of staining on brush border of villi. Counterstain Nuclear Fast red.

# CHAPTER 8

# REVIEW OF PAPERS 4 AND 5

## 8.1 REVIEW OF PAPER 4

### 8.1.1 Antigen unmasking using microwave heating on formalin fixed tissue embedded in methyl methacrylate. (Hand, Blythe and Jackson, 1996; *Journal of Cellular Pathology,* 1:31 - 37).

### 8.1.2 Introduction

Since the introduction of acrylic plastics for high resolution studies, there have been numerous reports relating to the application of immunohistochemisry, which as stated in chapter 3 began about 1972 (Hoshino and Kobayashi). Further papers are reviewed and discussed more fully in chapter 11. However, it was evident from several sources including previous publications such as Vogt *et al.,* (1976), Takimiya *et al.,* (1980), Gerrits, (1988), (Hand, 1988b) and from several conversations by this author with scientific colleagues that immunohistochemistry on acrylic plastic embedded tissue was problematical and difficult to achieve. As a result, the practicality of using MMA was explored as an alternative to the more popular insoluble GMA and LR White plastics as MMA can be dissolved after polymerisation. A specially devised formulation of MMA had previously been described (Hand *et al.,* 1989; Hand and Morrell, 1990), which had enabled blocks to be simply prepared and quickly polymerised using a chemical accelerator that could then be easily sectioned. Following removal of the plastic from the section (which in this type of preparation was adhered to a glass slide),

immunohistochemical staining was applied. In the above paper Hand *et al.,* (1996) described the application of a pretreatment prior to immunohistocemical staining either using a microwave oven for the heating of sodium citrate and/or enzyme digestion for the unmasking of antigens in formalin-fixed tissue embedded in MMA. Though these forms of pretreatment had become established procedures for paraffin wax sections of tissue fixed in formalin (Curran and Gregory, 1977; Mepham *et al.,* 1979; Shi *et al.,* 1991; Gerdes *et al.,* 1992; Cattoretti *et al.,* 1993; Cuevas *et al.,* 1994), and had also been reported on epoxy plastic sections (Pedraza, *et al.,* 1984; Smart and Millard, 1985; McCluggage *et al.,* 1995, Krenacs *et al.,* 1996), neither had previously been reported on acrylic plastic sections. The aims of the paper by Hand *et al.,* (1996) were:-

(i) to assess whether microwave antigen retrieval (MAR) was applicable to tissue embedded in MMA
(ii) to evaluate whether MAR was useful and beneficial for immuno-histochemical staining
(iii) to compare immunohistochemical staining using either MAR and/or enzyme (trypsin) digestion
(iv) to develop suitable pretreatment protocols for MMA-embedded tissue

### 8.1.3 Method

The paper reported how during a two year period, a variety of tissues including bone marrow, breast, kidney, lymph node, skin and uterus had been collected and fixed in formalin for approximately 18-24 hr. Various formalin solutions had

been used including 10% formalin, 10% formol calcium and 10% formol saline as the samples had come from different locations. Except for the bone marrow trephines, all tissues were sliced to a maximum thickness of 2 mm and manually processed via graded ethanol which consisted of 50%, 70%, 90% and three changes of 100% ethanol each for 1 hr, followed by two changes of catalysed MMA first for 1 hr and then overnight before embedding in a thick translucent polyethylene moulding cup tray (Polysciences Inc.) partially immersed in water inside a glass desiccator. All samples were processed at room temperature and agitated on a roller mixer. The catalysed MMA consisted of MMA monomer and dibutyl phthalate mixed 3:1 v/w containing 5% dried dibenzoyl peroxide. For embedding, fresh catalysed MMA was used to which 125 µl of DMA was added to each 10 g aliquot to induce polymerisation. Optimal polymerisation was achieved in an anaerobic environment by filling the dessicator with oxygen-free nitrogen. The wells in the tray were filled using a Pasteur pipette so that when the plastic block stub (CellPath plc, UK) was placed in position, none or little of the embedding mix flowed on top of the stub. All processing and polymerisation was carried out inside a fume cupboard to comply with safety regulations. To discard waste MMA, it was first polymerised. The procedures and equipment were similar to that previously used for enzyme histochemical studies shown in plates 9 – 12 on pp 73 – 74.

Following polymerisation of the MMA (approximately 2 hr) the blocks (12 x 6 x 5 mm or 16 x 12 x 5 mm) were cut either on a Reichert-Jung Autocut using a 10 or 12 mm width Latta-Hartmann knife or Ralph knife respectively, and 2 µm sections floated out on a 54ºC distilled waterbath. The sections were picked up on slides

that had previously been coated with 2% 3-amino propyltriethoxysilane (APES), drained and dried either at 37°C overnight or on a 60°C hotplate for 20 mins.

Prior to immunohistochemical staining, the MMA plastic was removed with two changes of xylene at 37°C each for 10 mins and the slides then rinsed in ethanol. Subsequently the sections were incubated in 0.5% hydrogen peroxide in methanol to block endogenous peroxidase before antigen retrieval techniques were commenced. A variety of antigens were investigated and in general those antibodies which the supplier recommended pretreatment of the antigens in wax sections, also required pretreatment on MMA sections. The following pretreatments were investigated:-

(i) 0.025% and 0.1% trypsin digestion
(ii) MAR using 10 mM (0.01M) sodium citrate buffer pH 6
(iii) a combination of both procedures

Both trypsin solutions were prepared in 0.1% calcium chloride made up in 0.005M Tris buffered saline (TBS) adjusted to pH 7.4 and used at 37°C for various times up to 30 mins. Different microwave ovens were used either with progressive heating of 10 mM (0.01M) sodium citrate buffer without stopping, or a heat / cool sequence. When both trypsin and MAR were used in sequence on the same slide, the former was employed first. The concentration of the trypsin solutions and the times used for MAR were based on routinely practices used for paraffin sections. Subsequently the sections were thoroughly washed in cold running water before incubating with 20% normal serum (20 mins) and then

without washing the slide drained and primary antibody added for 1 hr. A variety of immunoperoxidase methods were used including Avidin Biotin Complex (ABC) or Streptavidin biotin (Dako, UK) using DAB as the chromogen. Both primary and secondary antibodies were diluted in 5% normal serum with incubation in secondary antibody (Dako, UK) and ABC each for 30 mins. Between incubations, sections were washed in 0.005M TBS for 5 mins. Staining was intensified with 0.5% copper sulphate and the sections counterstained with haematoxylin. Immunostaining of numerous antigens using a variety of antibodies were investigated.

### 8.1.4 Results

From the results achieved, pretreatment clearly improved staining of many antigens, but the best protocol depended on the specific antibody employed and the time that had elapsed since the tissue was embedded. The stronger 0.1% trypsin produced superior staining than using 0.025% trypsin. The use of 0.1% trypsin or MAR produced satisfactory staining with AE1/AE3, CAM5.2 and vimentin, but was more reproducible with MAR particularly if the tissue had been embedded a while, when it was noticed antigenicity either decreased or became negative, but could be restored using MAR. For the demonstration of CD45RO optimal staining was achieved with short (5 mins) MAR, as longer incubation resulted in increased nuclear and non-specific staining.

Though several antigens could be demonstrated without pretreatment, staining was more intense and reproducible following MAR. Similar results were not seen after trypsin digestion alone. To localise Ki67 antigen using the antibody clone

MIB1, it was reported that positive staining using MAR alone was only possible within a few days after the tissue had been embedded in MMA, but strong staining could be retrieved if trypsin and MAR were employed in sequence. This observation was not apparent with any other antibodies investigated. Although the protocols suggested for bcl-2 and CD79a (MAR and 0.1% trypsin/MAR respectively) produced the best staining, the results were often weak or negative especially in archival tissue.

The paper reported that MAR which had been used on over 1,000 cases, and had found that MAR either alone or in combination with trypsin digestion was an extremely useful technique for optimising the localisation of numerous antigens that had been fixed in formalin and embedded in MMA. The staining produced had been excellent and mainly consistent, and had enabled an expansion of the range of antibodies that could be successfully applied to MMA-embedded tissue for the demonstration of many antigens. Various antigens are illustrated in plates 25 – 44 on pp 92 – 101 including immunogold silver staining (IGSS) in plates 43 and 44 (unpublished), although this work did not form part of the above study (Hand *et al.,* 1996).

### 8.1.5 Conclusions

The results in this paper established that MAR with and without enzyme digestion was applicable to MMA-embedded tissue to enable the demonstration of a variety of antigens. Previously the use of MAR and/or enzyme digestion had not been reported on MMA sections. Staining was more intense and reproducible following MAR as similar results were not seen after trypsin digestion alone.

However, when trypsin was used in conjunction with MAR, the stronger concentration of 0.1% trypsin produced superior staining than 0.025% trypsin. Optimal staining of CD45RO was achieved with shorter MAR, but for Ki67 using MIB1 staining was only possible using MAR alone within a few days after embedding, although strong staining could be retrieved if trypsin and MAR were employed in sequence. This observation was not seen with any other antibodies investigated. For bcl-2 and CD79a the best staining produced was often weak and sometimes negative in archival tissue. The results in this paper showed that the use of a pretreatment improved staining or enabled more antigens to be demonstrated as similarly experienced with paraffin sections. Furthermore, it provided a framework to be able to achieve reliable immunohistochemical staining on MMA-embedded tissue.

## 8.2 REVIEW OF PAPER 5

### 8.2.1 Superheating using pressure cooking: its use and application in unmasking antigens embedded in methyl methacrylate. (Hand and Church, 1998; *Journal of Histotechnology,* 21:231 - 236).

### 8.2.2 Introduction.

In addition to the use of a microwave oven for the heating of various solutions to antigen unmask antigens in paraffin sections (Shi *et al.,* 1991; Cattoretti, *et al.,* 1993; Cuevas, *et al.,* 1994), it has also been suggested that the use of superheating using pressure cooking (PC) can either act as an alternative procedure, and / or produce improved staining (Norton *et al.,* 1994; Miller *et al.,* 1995). More recently Krenacs *et al.,* (2004) described PC applied to epoxy

plastic sections. In the above paper, Hand and Church (1998) investigated whether pressure cooking was applicable to formalin-fixed tissue embedded in MMA to achieve IHC, and in particular for the antigens bcl-2, CD3, CD79a and Ki67, which in the previous paper (Hand *et al*. 1996) had shown disappointing or unsatisfactory staining. Furthermore, as it had been previously observed that immunocytochemical staining may be affected by the choice and concentration of the accelerator employed (unpublished), staining was applied to tissue from blocks that had been polymerised with different accelerators at various concentrations to ascertain whether differential staining could be achieved. In summary therefore, the purpose of the study was to:-

(i) assess whether superheating was applicable to MMA-embedded tissue.

(ii) evaluate whether the technique was beneficial in the staining of bcl-2, CD3, CD79a and Ki67

(iii) ascertain whether differential staining occurred if different accelerators were employed to polymerise the plastic

### 8.2.3 Method

A series of fresh tonsil slices were prepared and fixed in 10% formol calcium (routine fixative used in the department) for 24 hr. The tissue was processed via graded concentrations of ethanol and infiltrated with MMA mix as described previously (Hand and Church, 1997). Blocks were polymerised using either the amine accelerator DMA (D14,575-0, Aldrich, UK) or DMSX (T3642, Sigma, UK) by adding 100µl (1%), 125µl (1.25%), 150µl (1.5%), and 200µl (2%) to 10g aliquots of catalysed MMA. When using these two accelerators the plastic

required up to 2 hr for polymerisation which was achieved in an anaerobic atmosphere inside a glass dessicator as previously described (Hand *et al.,* 1996). Only blocks measuring 5 x 2 x 5 mm were prepared using a plastic moulding tray (Polysciences Inc.), and subsequently 2 µm sections cut with a Latta-Hartmann knife were picked up on slides coated with APES from a heated waterbath and dried as previously described (Hand and Church, 1997).

Sections were deplasticised with two changes of xylene at 37°C each for 10 mins and the slides then rinsed in ethanol, endogenous peroxidase blocked using 0.6% hydrogen peroxide in methanol for 15 mins and then subjected to either 0.1% trypsin (37°C), or MAR or PC using a microwave oven or pressure cooker respectively to heat 10 mM (0.01M) sodium citrate buffer at pH 6. Following preliminary studies, trypsin was employed only in conjunction with PC for the demonstration of CD3 antigen. The protocols employed for pretreatment were identical to what was routinely used for paraffin sections and included either 0.1% trypsin at 37°C for 20 mins, or progressive heating to boiling of 650 ml of sodium citrate solution for 20mins in a microwave oven (700W) or 3mins superheating in a pressure cooker.

Following pretreatment and a thorough washing in cold running tap water, the sections were initially incubated in 20% normal serum made up in TBS pH 7.6 and a Streptavidin Biotin Complex (Dako) procedure applied as previously described (Hand and Church, 1997), where the primary antibody, secondary antibody and Streptavidin were incubated for 60 mins, 30 mins and 30 mins respectively. The optimal dilution for the antibodies bcl-2, CD3, CD79a and Ki67

(clone MIB1) had been pre-determined as referred to in a previous paper (Hand *et al.,* 1996). Peroxidase activity was detected using DAB with imidazole added, and then further enhanced using copper sulphate solution both for 10 mins.

### 8.2.4 Results

From the results subjectively assessed, it was shown that in general the antigens bcl-2, CD3, CD79a and Ki67 exhibited stronger staining with PC than MAR. In addition, DMSX produced superior staining than DMA which also increased as the concentration of the accelerator increased. However, improved staining of CD3 was achieved if enzyme pretreatment with trypsin prior to pressure cooking was adopted. This combination produced superior staining to trypsin followed by MAR, which had previously provided the best results (Hand *et al.* 1996). In the same study it was shown that staining with MIB1 in archival tissue also required trypsin digestion when MAR was used, but in this investigation no similar enzyme pretreatment was necessary when PC was employed. Various antigens are illustrated in plates 31, 34, 35 and 37 – 40 on pp 95 – 99 following pretreatment with PC.

The paper also and confirmed the author's views that the choice and concentration of accelerator used to induce polymerisation of the plastic could critically affect immunohistochemical staining. (see plates 38 – 40 on pp 98 – 99) with DMSX producing superior staining to DMA. It was suggested that a concentration of 1.5% DMSX produced satisfactory processing and polymerisation in 1-2 hr, and also subsequently enabled good IHC to be performed.

### 8.2.5 Conclusions

From the results in this paper it was established that superheating using a pressure cooker with and without enzyme digestion was applicable to MMA-embedded tissue to enable the demonstration of antigens studied. Antibodies investigated focused on particularly those that had previously produced disappointing or problematic staining using MAR. For bcl-2, CD3, CD79a and Ki67 the use of PC resulted in excellent stronger staining although for CD3 improved staining was achieved if enzyme pretreatment with trypsin prior to PC was also adopted. For this antibody, trypsin / pressure cooking produced superior staining to trypsin / MAR sequence. No trypsin pretreatment was necessary when PC was employed with MIB1 in archival tissue and non-specific background staining was none or insignificant with the other antibodies studied. Antigen retrieval using superheating with a pressure cooker had previously not been reported on any plastic sections, but its use enabled a greater number of antigens to be reliably demonstrated. The choice and concentration of accelerator used to induce polymerisation of the plastic was found to influence immunohistochemical staining, and in general higher concentrations of accelerator produced superior staining. The use of the amine accelerator DMSX produced superior staining than DMA.

## 8.3 PHOTOMICROGRAPHS

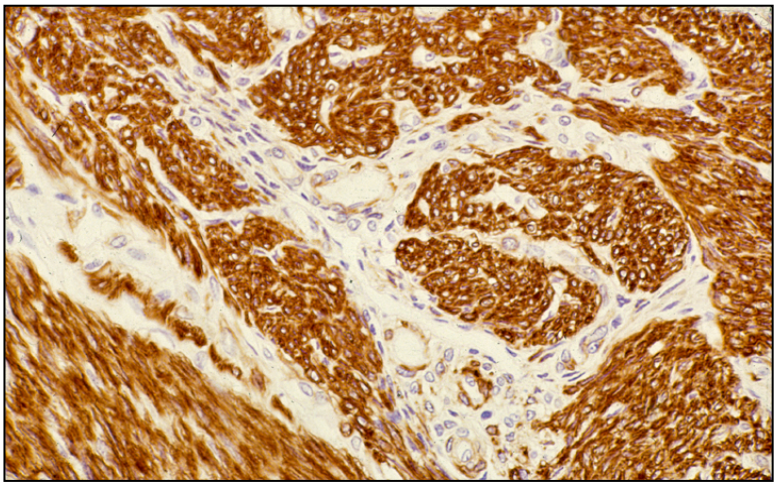


**Plate 25:** Smooth Muscle Actin, microwave antigen retrieval (MAR) pretreatment: (MP). Formalin fixed tissue embedded in MMA polymerised with DMA and stained with an antibody for smooth muscle actin (Dako, clone 1A4) with a Streptavidin biotin procedure with DAB chromogen following pretreatment with citrate buffer pH 6.0 heated in a microwave oven. Counterstain Gill's haematoxylin.

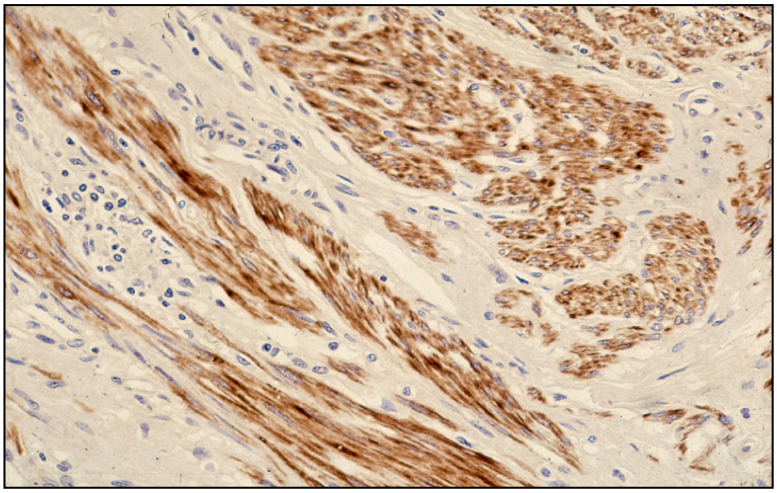


**Plate 26:** Desmin, (MP). Formalin fixed tissue embedded in MMA polymerised with DMA and stained with an antibody for desmin (Dako, clone D33) with a Streptavidin biotin procedure with DAB chromogen without pretreatment. Counterstain Gill's haematoxylin

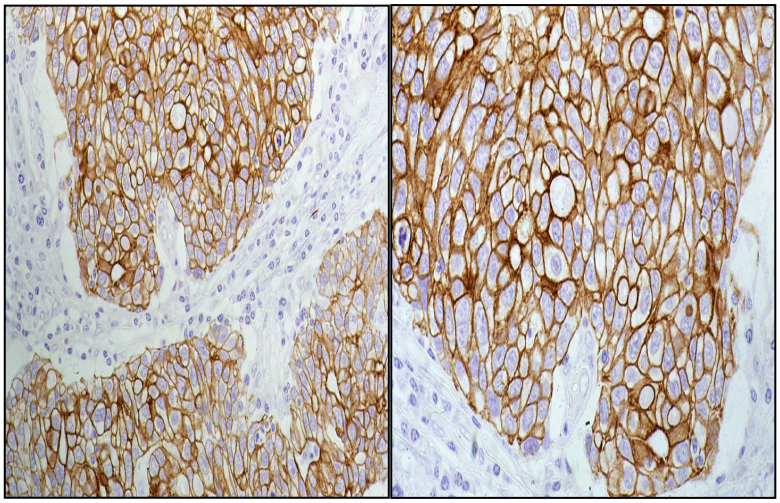


**Plate 27:** Cytokeratin 7, MAR pretreatment: (LP & HP). Formalin fixed ovarian tumour embedded in MMA polymerised with DMA and stained with an antibody for cytokeratin 7 (Dako, clone OV-TI 12/30) with a Streptavidin biotin procedure with DAB chromogen following pretreatment with citrate buffer pH 6.0 heated in a microwave oven. Counterstain Gill's haematoxylin.

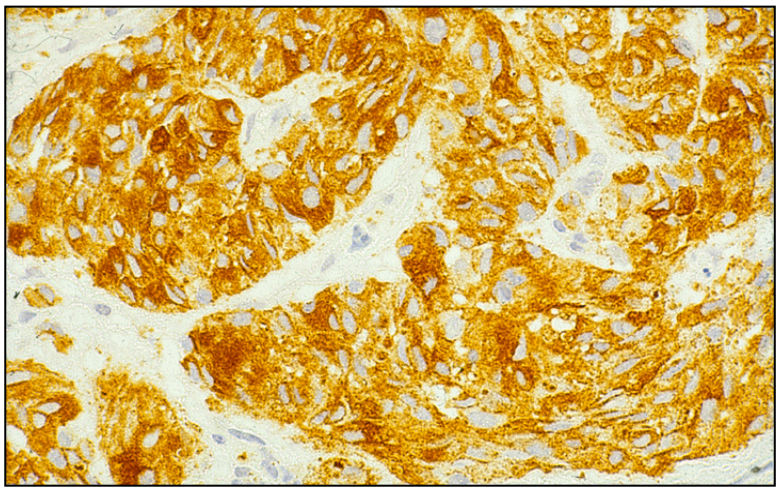


**Plate 28:** HMB-45, MAR pretreatment: (MP). Formalin fixed melanoma embedded in MMA polymerised with DMA and stained with an antibody for melanocytes (Dako, clone HMB 45) with a Streptavidin biotin procedure with DAB chromogen following pretreatment with citrate buffer pH 6.0 heated in a microwave oven. Counterstain Gill's haematoxylin.

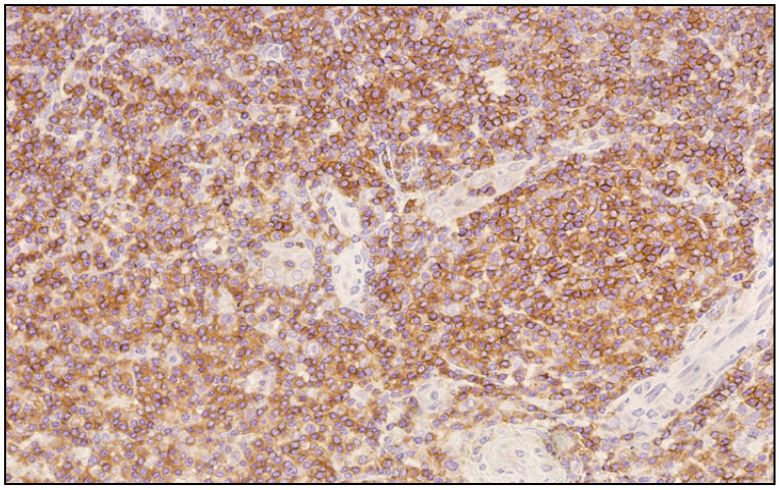


**Plate 29:** Leucocyte Common Antigen, MAR pretreatment: (LP). Formalin fixed tonsil embedded in MMA polymerised with DMA and stained with an antibody for leucocyte common antigen (Dako, clone 2B11 + PD7/26) with a Streptavidin biotin procedure with DAB chromogen following pretreatment with citrate buffer pH 6.0 heated in a microwave oven. Counterstain Gill's haematoxylin.

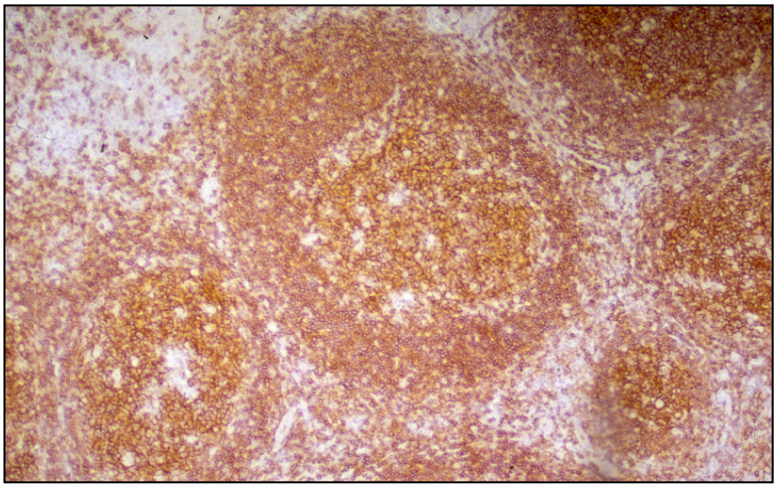


**Plate 30:** CD20, MAR pretreatment: (LP). Formalin fixed tonsil embedded in MMA polymerised with DMA and stained with CD20 antibody for B lymphocytes (Dako, clone L26) with a Streptavidin biotin procedure with DAB chromogen following pretreatment with citrate buffer pH 6.0 heated in a microwave oven. Counterstain Gill's haematoxylin.

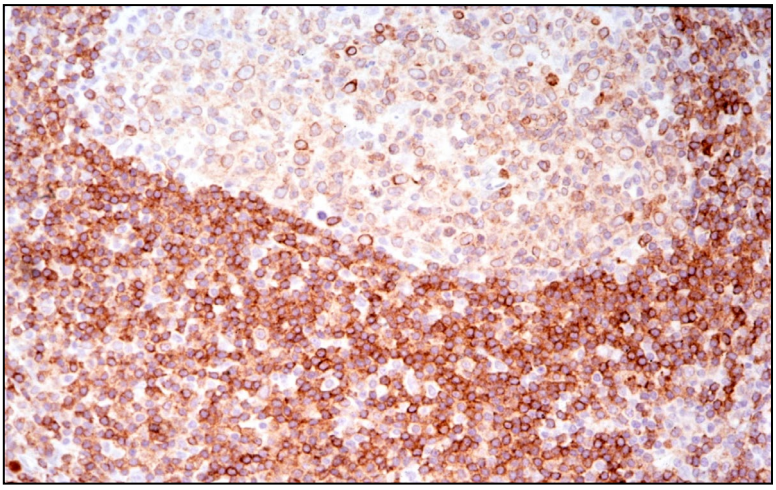


**Plate 31:** CD79a, pressure cooking (PC) pretreatment: (MP). Formalin fixed tonsil embedded in MMA polymerised with DMSX and stained with CD79a antibody for B lymphocytes (Dako, clone JCB-117) with a Streptavidin biotin procedure with DAB chromogen following pretreatment with citrate buffer pH 6.0 heated in a pressure cooker. Counterstain Gill's haematoxylin.

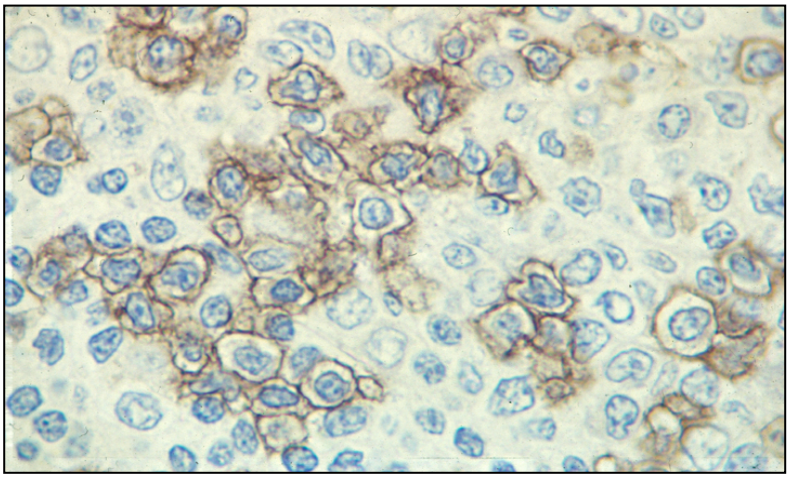


**Plate 32:** CD45RO, MAR pretreatment: (HP). Formalin fixed tonsil embedded in MMA polymerised with DMA and stained with CD45RO antibody for T lymphocytes (Dako, clone UCHL1) with a Streptavidin biotin procedure with DAB chromogen following pretreatment with citrate buffer pH 6.0 heated in a microwave oven. Counterstain Gill's haematoxylin.

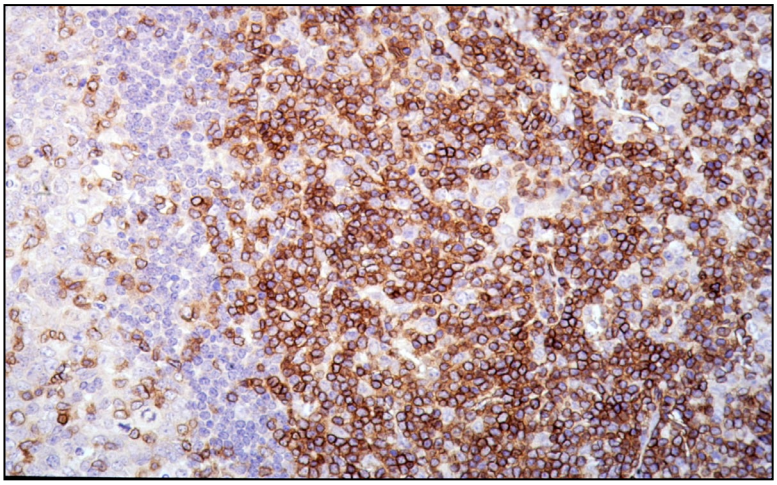


**Plate 33:** CD3, Trypsin + MAR pretreatment: (LP). Formalin fixed tonsil embedded in MMA polymerised with DMA and stained with CD3 antibody for T lymphocytes (Dako, clone LN10) with a Streptavidin biotin procedure with DAB chromogen following sequencial pretreatment with trypsin (37$^{o}$C) and citrate buffer pH 6.0 heated in a microwave oven. Counterstain Gill's haematoxylin.

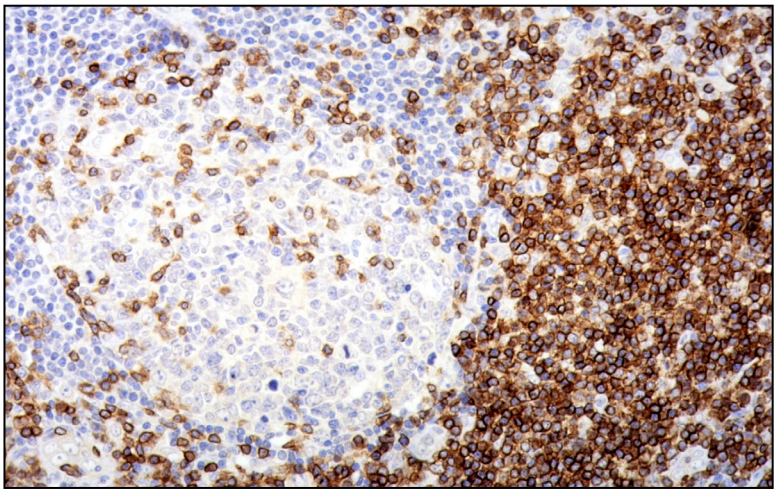


**Plate 34:** CD3, Trypsin + PC: (LP). Formalin fixed tonsil embedded in MMA polymerised with DMA and stained with CD3 antibody for T lymphocytes (Dako, clone LN10) with a Streptavidin biotin procedure with DAB chromogen following sequencial pretreatment with trypsin (37$^{o}$C) and citrate buffer pH 6.0 heated in a pressure cooker. Counterstain Gill's haematoxylin. Note superior staining to plate 33.

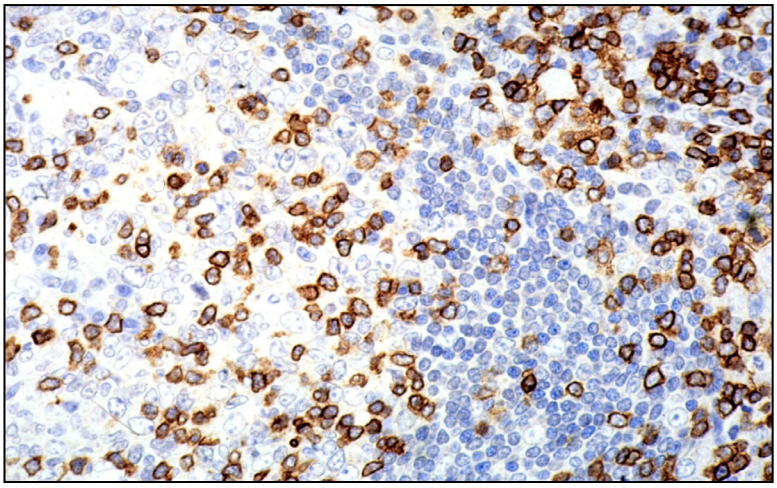


**Plate 35:** CD3, Trypsin + PC pretreatment: (MP). Formalin fixed tonsil embedded in MMA polymerised with DMA and stained with CD3 antibody for T lymphocytes (Dako, clone LN10) with a Streptavidin biotin procedure with DAB chromogen following sequencial pretreatment with trypsin (37°C) and citrate buffer pH 6.0 heated in a pressure cooker. Counterstain Gill's haematoxylin. Note superior staining to plate 33.

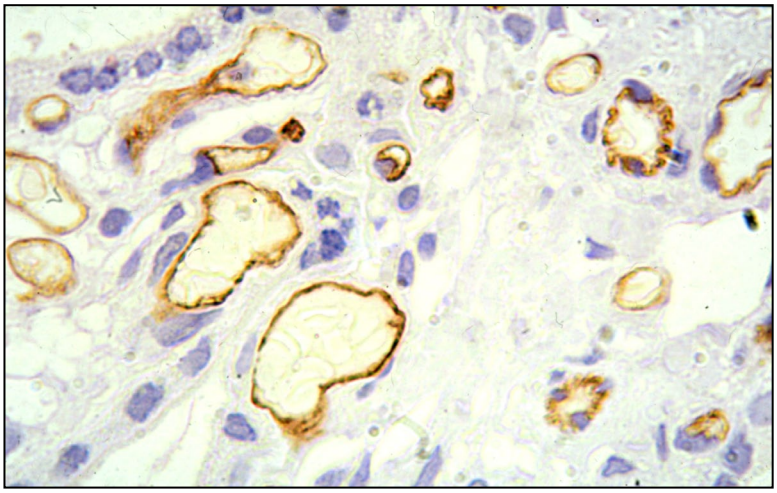


**Plate 36:** CD34, MAR pretreatment: (HP). Formalin fixed placenta embedded in MMA polymerised with DMA and stained with CD34 antibody for endothelial cells (Leica/Novocastra, clone QB-End/10) with a Streptavidin biotin procedure with DAB chromogen following pretreatment with citrate buffer pH 6.0 heated in a microwave oven. Counterstain Gill's haematoxylin.

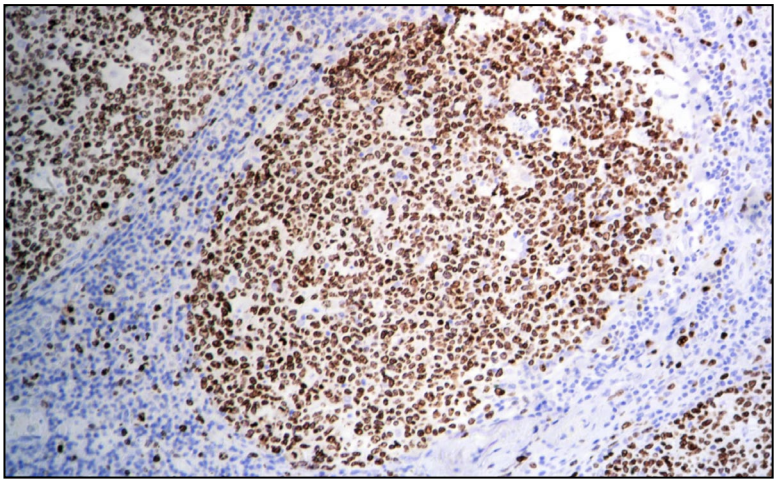


**Plate 37:** Ki67, PC pretreatment: (MP). Formalin fixed tonsil embedded in MMA polymerised with DMA and stained with Ki67 antibody for proliferating cells (Dako, clone MIB1) with a Streptavidin biotin procedure with DAB chromogen following pretreatment with citrate buffer pH 6.0 heated in a pressure cooker. Counterstain Gill's haematoxylin.

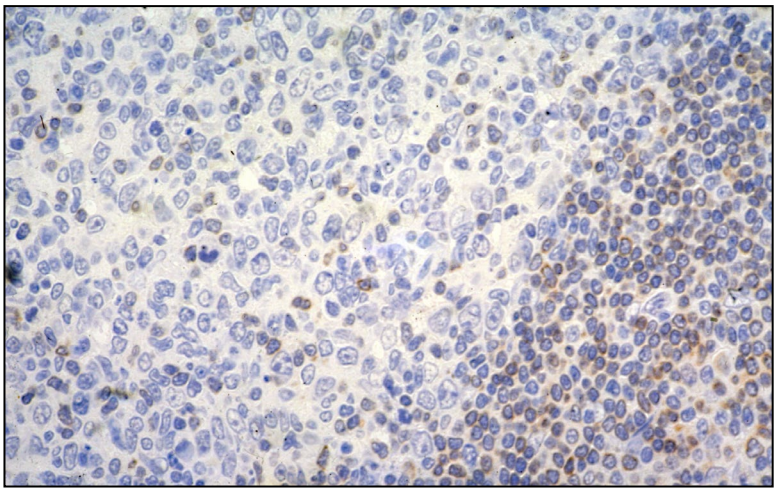


**Plate 38:** Bcl-2, PC pretreatment: (MP). Formalin fixed tonsil embedded in MMA polymerised with DMA and stained with bcl-2 (Dako, clone 124) with a Streptavidin biotin procedure with DAB chromogen following pretreatment with citrate buffer pH 6.0 heated in a pressure cooker. Counterstain Gill's haematoxylin.

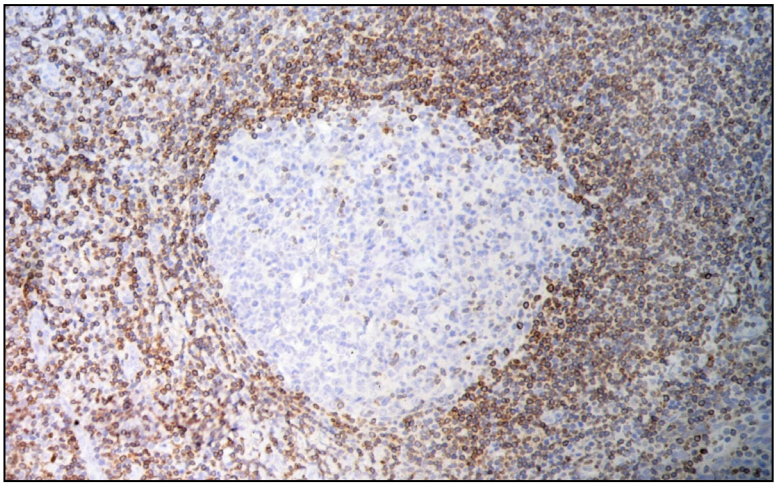


**Plate 39:** Bcl-2, PC pretreatment: (LP). Formalin fixed tonsil embedded in MMA polymerised with DMSX and stained with bcl-2 (Dako, clone 124) with a Streptavidin biotin procedure with DAB chromogen following pretreatment with citrate buffer pH 6.0 heated in a pressure cooker. Note superior staining to plate 38. Counterstain Gill's haematoxylin.

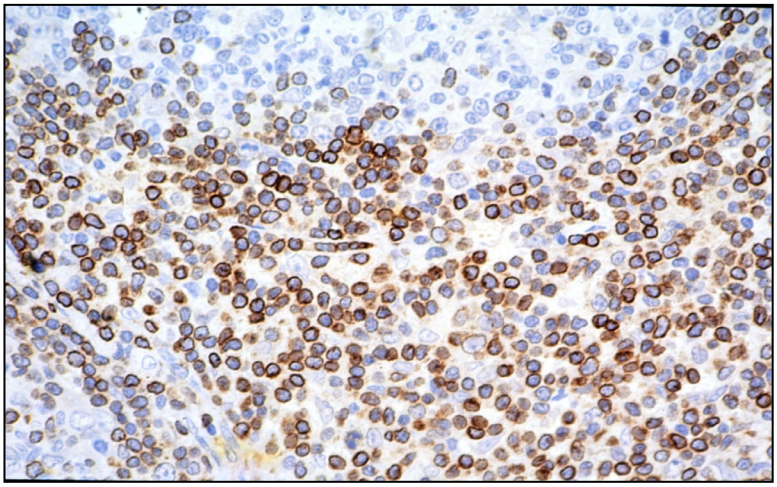


**Plate 40:** Bcl-2, PC pretreatment: (MP). Formalin fixed tonsil embedded in MMA polymerised with DMSX and stained with bcl-2 (Dako, clone 124) with a Streptavidin biotin procedure with DAB chromogen following pretreatment with citrate buffer pH 6.0 heated in a pressure cooker. Note superior staining to plate 38. Counterstain Gill's haematoxylin.

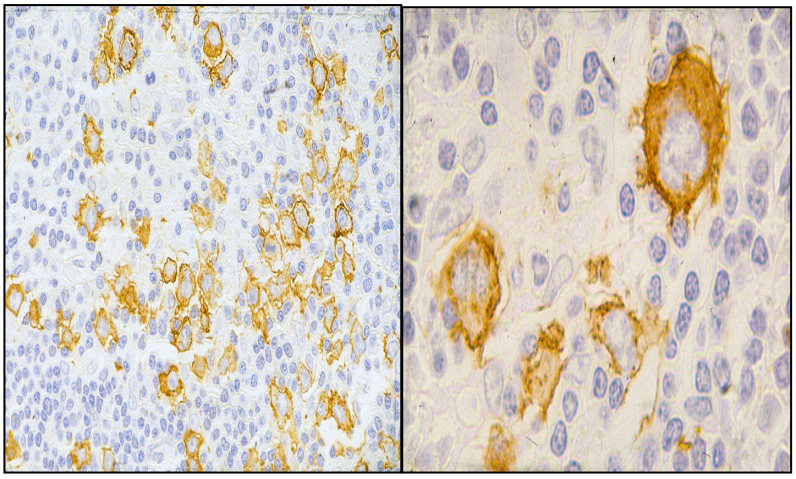


**Plate 41:** CD15, MAR pretreatment: (LP & HP). Formalin fixed Hodgkin's lymphoma embedded in MMA polymerised with DMA and stained with antibody CD15 for Reed Sternberg cells (Becton Dickinson, clone Leu M1) with a Streptavidin biotin procedure with DAB chromogen following pretreatment with citrate buffer pH 6.0 heated in a microwave oven. Counterstain Gill's haematoxylin.

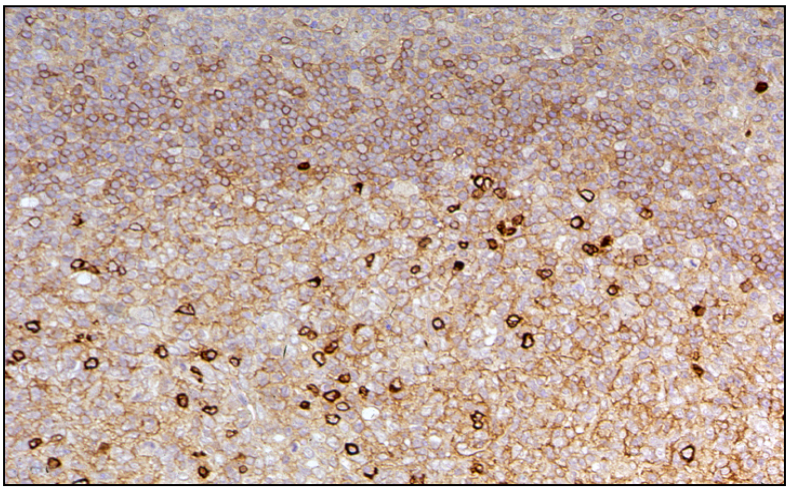


**Plate 42:** Lambda Light chains, Trypsin + MAR: (MP). Formalin fixed tonsil embedded in MMA polymerised with DMA and stained with a polyclonal antibody for lambda light chains (Dako) with a Streptavidin biotin procedure with DAB chromogen following sequencial pretreatment with trypsin (37$^{o}$C) and citrate buffer pH 6.0 heated in a microwave oven. Counterstain Gill's haematoxylin.

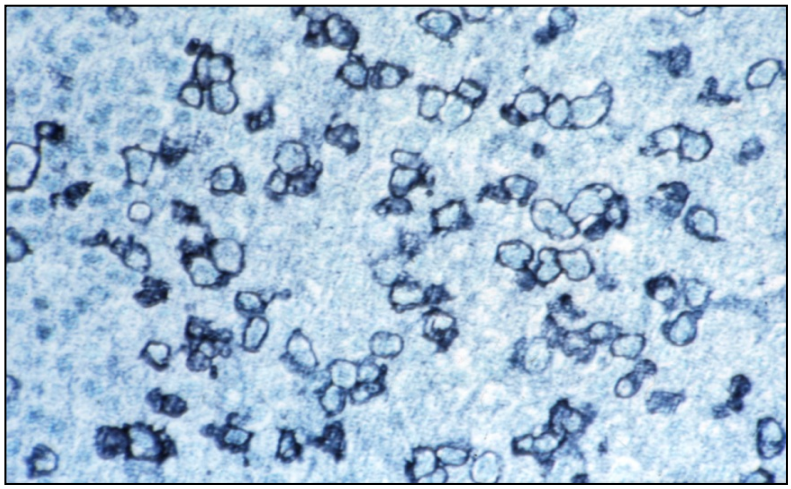


**Plate 43:** CD45RO / IGSS, (HP). Acetic formalin fixed tonsil embedded in MMA polymerised with DMA showing surface staining of T lymphocytes with CD45RO demonstated by immunogold silver staining. Counterstain with methyl green. No antigen retrieval pretreatment was necessary.

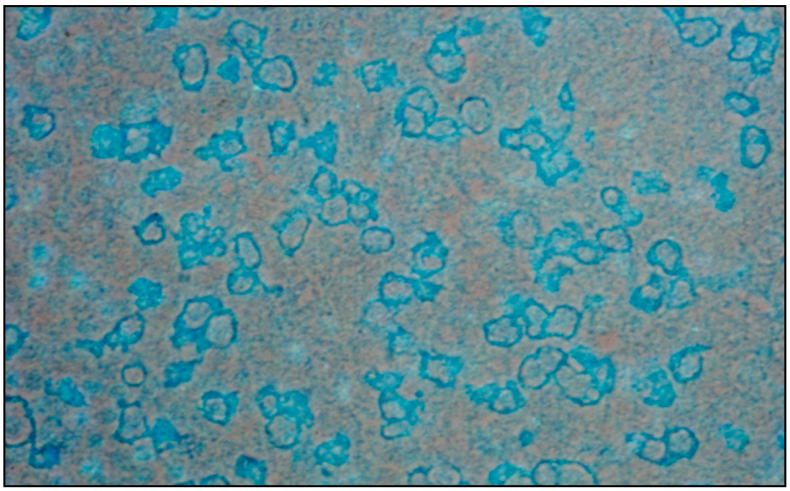


**Plate 44:** CD45RO / IGSS, (HP). Same stained section as plate 43 viewed by epi-polarised light.

# CHAPTER 9

# REVIEW OF PAPERS 6 AND 7

## 9.1 REVIEW OF PAPER 6

### 9.1.1 The use of methyl methacrylate resin for embedding bone marrow trephine biopsies. (Blythe, Hand, Jackson, Barrans, Bradbury and Jack, 1997; *Journal of Clinical Pathology,* 50:45 - 49).

### 9.1.2 Introduction

Based on the procedure previously developed and the immunocytochemical staining achieved (Hand *et al.,* 1989; Hand and Morrell, 1990; Johns *et al.,* 1992; Hand *et al.,* 1996), the above paper described a diagnostic application for undecalcified bone marrow trephine biopsies. In this paper it was reported that the use of MMA as an embedding medium had been used for approximately 2,500 undecalcified bone marrow trephine biopsies in the Haematological Malignancy Diagnostic Unit at Leeds, UK. Though it stated that decalcified bone biopsy specimens embedded in paraffin wax could be used, it claimed this technique had the disadvantage that morphology and antigenicity may be poorly preserved. It also stated that whilst both acrylic and epoxy plastic embedding media had previously been advocated as suitable for high resolution LM (Burkhardt, 1971; Moosavi *et al.,* 1981; Beckstead, 1986; Clarke, 1991), it agreed with others that when using the popular GMA medium, the immunohistochemical techniques reported were complicated, troublesome and impractical in a routine laboratory (Vincic *et al.,* 1989; Vincic, 1990). As a consequence, this paper

described the procedures and experience of using MMA embedding as an alternative procedure based on that originally developed by Hand *et al.,* (1989) and Hand and Morrell, (1990) for the diagnosis of various myelo and lymphoproliferative diseases using tinctorial stains and a wide range of 46 lymphoid markers for immunocytochemical staining on bone marrow trephine biopsies.

### 9.1.3 Method

Bone marrow trephine biopsies had been fixed in several variants of formalin for a minimum of 18 hr with similar results and then manually processed in small glass vials on a roller mixer. The tissue was dehydrated through 50%, 70%, 95% ethanol (1 hr each) and 100% ethanol for a minimum of 2 hr, and then infiltrated overnight in the plastic mix consisting of a 3:1 solution of MMA and dibutyl phthalate on the roller mixer. The biopsies were embedded in thick polyethylene moulding cup tray with a block stub (Polysciences Inc.) and an identification label attached using an embedding mix that consisted of fresh infiltration solution to which 5% pre-dried dibenzoyl peroxide and 1% DMA had been added. (Note: as this paper was written before the use of DMSX previously discussed in paper 5 was investigated, only the accerator DMA was used). Polymerisation occurred inside a dessicator with the block stub proving sufficiently satisfactory to prevent contact with oxygen in the air. (See plates 9 – 12, pp 71 – 72). Following polymerisation (approx 2 hr), sections were cut on a Ralph knife using a Reichert-Jung Autocut either at 3 µm for tinctorial and immunohistochemical staining or 4 µm for reticulum staining. The sections were floated out on a 56$^{o}$C waterbath and picked up on APES coated slides, drained and placed on a hot

plate for a minimum of 10 mins. Other tinctorial / histochemical staining techniques mentioned included H&E, May-Grunward Giemsa, Ziehl-Neelsen and Perls Prussian blue. For staining to occur, the plastic was first removed from the sections with xylene at 37°C for 20 mins, and sections then rinsed in alcohol and washed in water before proceeding.

For immunocytochemical staining endogenous peroxidase activity was blocked using 0.5% hydrogen peroxide in methanol for 30 mins. The optimal antigen retrieval pretreatment procedures depended on the antibodies investigated, but consisted of MAR using 0.01M sodium citrate (pH 6.0), or 0.025% trypsin at 37°C for 6 mins, or a combination of trypsin followed by MAR, or none. Following antigen retrieval, sections were rinsed in 0.005M TBS (pH 7.6), incubated in 20% normal serum for 10 mins and then without rinsing, excess serum was drained prior to incubation in the primary antibody. Over 46 antibodies were investigated, each incubated for 1 hr and a Streptavidin biotin horseradish peroxidase procedure used (Dako, Duet kit) where the secondary antibody and complex were both incubated for 30 mins. Peroxidase activity was visualised with DAB / copper suphate sequence and sections counterstained with haematoxylin. Throughout the staining protocol 0.1% Tween 20 in TBS was used in the buffer to either wash the sections or as the antibody diluent.

### 9.1.4 Results

This paper described the diagnostic application of tinctorial and immuno-histochemical staining that was used routinely for bone marrow trephines in the Haematological Malignancy Diagnostic Service at Leeds General Infirmary.

Using this procedure for the reporting of various myelo and lymphoproliferative diseases, it stated that approximately 2,500 undecalcifed bone biopsies had been embedded in MMA which produced an annual workload of about 3,000 slides. A wide range of lymphoid markers for immunocytochemical staining were employed with excellent staining achieved (plates 45 – 49, pp 110 – 112). The results showed with the exception of myeloperoxidase and CD57 that trypsin, MAR or both were either essential, or greatly improved staining intensity and reproducibility. Almost all the antibodies that were used on paraffin sections, also worked well on MMA sections except CD40. In this thesis plates 47 – 49, (pp 111 – 112) are photomicrographs of ICC performed at a later date which used “high pH” antigen retrieval solution (Vector) heated with a pressure cooker.

The paper also reported that no biopsy specimens embedded in MMA had been difficult to interpret because of technical failure. The standard morphological preservation was consistently better than that on decalcified tissue embedded in paraffin wax, and TAT of MMA sections compared favourably with that achieved using decalcification followed by paraffin wax processing and embedding.

### 9.1.5 Conclusions

The paper reported on the experience of the using MMA for the embedding of 2,500 undecalcifed bone marrow trephine biopsies, which was the routine method of choice at the Haematological Malignancy Diagnostic Service in Leeds General Infirmary at the time of publication. A variety of tinctorial stains and a wide range of 46 lymphoid markers for immunocytochemical staining were employed for investigating various myelo and lymphoproliferative diseases that

produced an annual workload of about 3,000 slides. The paper stated that excellent staining was achieved with all the antibodies that were used on paraffin sections except for CD40. For myeloperoxidase and CD57 no pretreatment was necessary, but the use of trypsin, MAR or both were either essential or greatly improved the staining and reproducibility of the other antigens investigated. In addition, it reported that morphological preservation was consistently better than decalcified paraffin wax sections and TATs of MMA sections compared favourably with the use of paraffin sections. In conclusion, the paper stated that the use of MMA as an embedding medium for processing and performing immunocytochemical staining of bone marrow trephine biopsy specimens had many advantages over other embedding media and recommended its use.

## 9.2 REVIEW OF PAPER 7

### 9.2.1 Immunocytochemical demonstration of hormones in pancreatic and pituitary tissue embedded in methyl methacrylate. (Hand and Church, 1997; *Journal of Histotechnology,* 20:35 - 38).

### 9.2.2 Introduction

In the above paper Hand and Church described the diagnostic application of a broad spectrum of antibodies for the demonstration of several hormones on pancreas and pituitary tissue embedded in MMA. Although several other investigations had previously reported the immunocytochemical demonstration of various hormones on plastic-embedded material, e.g. Spaur *et al.,* (1975); Newman *et al.,* (1983); and Pedraza *et al.,* (1984), these studies did not include using MMA. In this study, Hand and Church (1997) used identical antibody titres

and techniques as those that were routinely employed for paraffin sections, which also included the absence of any pretreatment required to induce antigen retrieval. The paper also commented on the observation that some immunocytochemical staining might be affected by the choice of accelerator used to polymerise the plastic, and as a result both DMA and DMSX were used to polymerise a duplicate set of blocks to ascertain whether there was differential staining.

### 9.2.3 Method

Fresh post mortem pancreas and pituitary tissue of maximum thickness 2 mm was fixed in 10% formol saline for 48 hr, and then processed via graded ethanol and embedded as previously described Hand *et al.,* 1996). However, in this study, one set of blocks were polymerised by adding 125 µl of DMA and for a second set 125 µl of DMSX both to 10 g aliquots of catalysed MMA/dibutyl phthalate mix. Only blocks measuring 12 x 6 x 5 mm were cast which polymerised in approximately 2hr at room temperature using an open moulding tray (Polysciences Inc.). Subsequently 2 µm plastic sections were cut, deplasticised and immunocytochemical staining performed using a Streptavidin Biotin (Dako) immunoperoxidase procedure. All processing, embedding sectioning and staining details were as previously described (Hand *et al.,* 1996). The antibodies assessed were glucagon, insulin, pro-amylin N, somatostatin and substance P on pancreas, and adrenocorticotrophin (ACTH), beta endorphin, follicle-stimulating hormone (FSH), human growth hormone (HGH, luteinizing hormone (LH), prolactin and thyroid-stimulating hormone (TSH) on pituitary. The antibody titres in conjunction with a Streptavidin biotin method used was identical

as that used routinely for paraffin sections, which included no pretreatment to unmask antigenicity. Peroxidase activity was detected using DAB with imidazole followed by enhancement with copper sulphate, and sections counterstained with haematoxylin. It was stated that during staining it was important not to allow the sections to dry out, which occurred more readily than paraffin sections.

### 9.2.4 Results

Excellent staining with each hormone was easily achieved on all plastic sections of pancreas, although on pituitary tissue moderate non-specific background staining was encountered, that was significantly decreased by reducing the incubation time in primary antibody from 60 mins to 30 mins. The paper also reported that excellent staining could also be achieved in archival material that had been embedded in MMA and polymerised with DMA 10 years earlier.

Staining was equally strong in sections where the MMA had been polymerised with DMA or DMSX, and neither accelerator caused any difference in the morphology of the tissue. It was suggested that the lack of differential staining by either accelerator may be because hormones appeared much less sensitive to these chemicals than other antigens.

The results showed that immunocytochemical staining of hormones in the pancreas and pituitary could be easily and quickly achieved, (see plates 50 – 52, pp 112 - 113) combining excellent tissue morphology that was expected using plastic sections with strong staining associated with paraffin sections.

### 9.2.5 Conclusions

This paper reported on the excellent immunocyotochemical staining achieved on pancreas and pituitary tissue using similar antibody titres and a methodology as that used in the laboratory routinely for paraffin sections. This included the non-requirement to use any pretreatment prior to immunostaining. For pituitary tissue the higher background non-specific staining was decreased if the incubation time of the primary antibody was reduced. Both the accelerators DMA and DMSX produced equally strong immunocytochemical staining, and as a result it appeared that hormones were much less sensitive to the of accelerator selected than other antigens. No difference in the tissue morphology with either accelerator was observed

## 9.3 PHOTOMICROGRAPHS

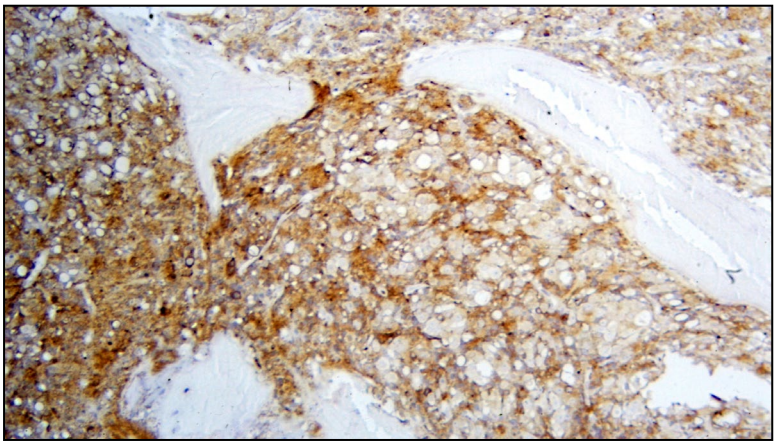


**Plate 45:** Prostatic Acid Phosphatase, MAR pretreatment: (LP). Formalin fixed bone marrow trephine embedded in MMA polymerised with DMA and stained with a polyclonal antibody for prostatic acid phosphatase (Dako) with a Streptavidin biotin procedure using DAB chromogen following pretreatment with citrate buffer pH 6.0 heated in a microwave oven. Counterstain Harris's haematoxylin.

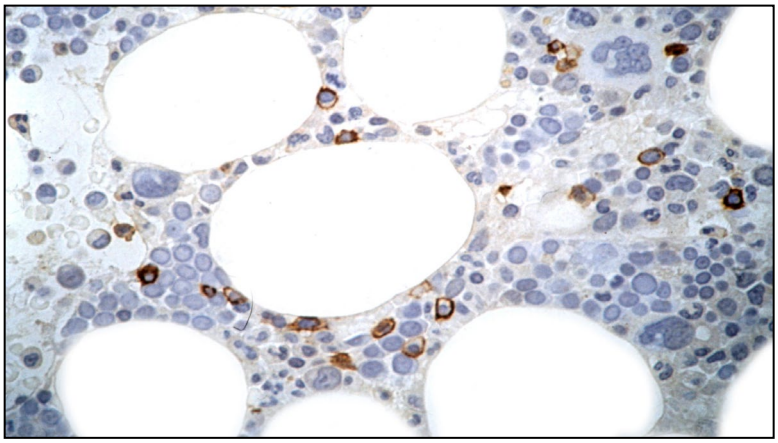


**Plate 46:** CD45RO, MAR pretreatment: (MP). Formalin fixed bone marrow trephine embedded in MMA polymerised with DMA and stained with an antibody for prostatic acid phosphatase (Dako, clone UCHL1) with a Streptavidin biotin procedure using DAB chromogen following pretreatment with citrate buffer pH 6.0 heated in a microwave oven. Counterstain Harris's haematoxylin.

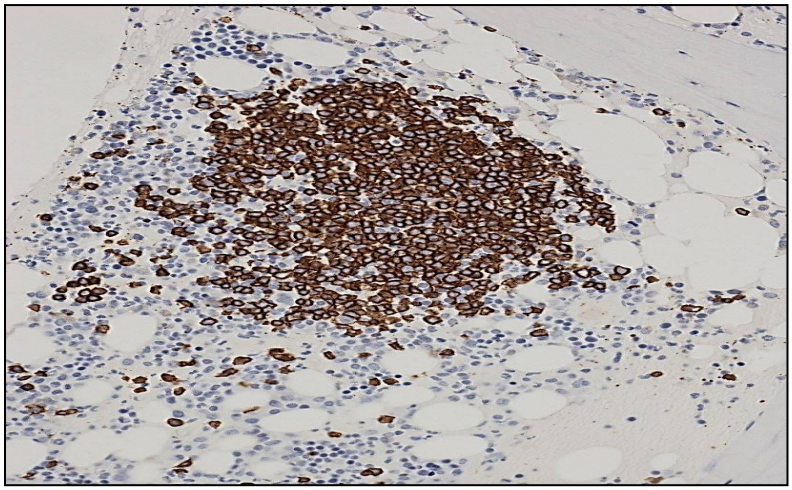


**Plate 47:** CD20, PC pretreatment: (LP). Formalin fixed undecalcified bone marrow trephine embedded in MMA polymerised with DMA and stained with an antibody for CD20 (Dako, clone L26) with a Streptavidin biotin procedure using DAB chromogen following pretreatment with high pH antigen retrieval solution (Vector) heated in a pressure cooker. Counterstain Harris's haematoxylin.

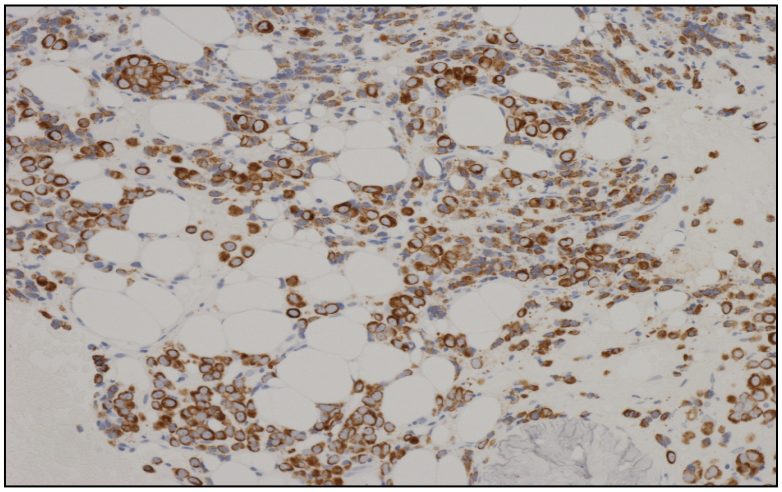


**Plate 48:** Cytokeratin, PC pretreatment: (LP). Formalin fixed undecalcified bone marrow trephine embedded in MMA polymerised with DMA and stained with an antibody for cytokeratin (Dako, clone MNF116) with a Streptavidin biotin procedure using DAB chromogen following pretreatment with high pH antigen retrieval solution (Vector) heated in a pressure cooker. Counterstain Harris's haematoxylin.

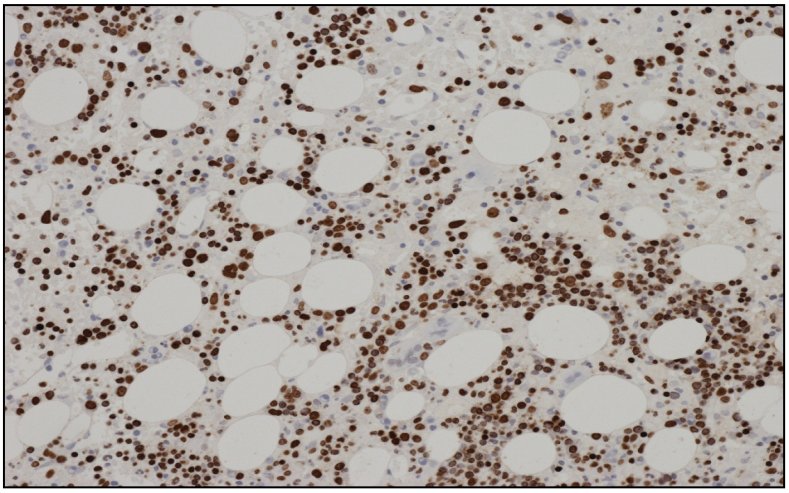


**Plate 49:** Ki67, PC pretreatment: (LP). Formalin fixed undecalcified bone marrow trephine embedded in MMA polymerised with DMA stained with an antibody for Ki67 (Dako, clone MIB1) with a Streptavidin biotin procedure using DAB chromogen following pretreatment with high pH antigen retrieval solution (Vector) heated in a pressure cooker. Counterstain Harris's haematoxylin.

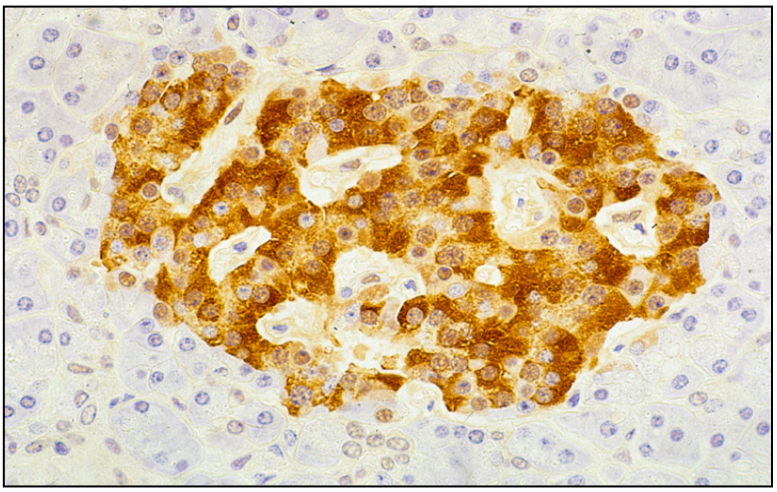


**Plate 50:** Insulin, (MP). Formol saline fixed pancreas embedded in MMA polymerised with DMA and stained with a polyclonal antibody for insulin (Prague University) with a Streptavidin biotin procedure using DAB chromogen following no pretreatment. Counterstain Gill's haematoxylin.

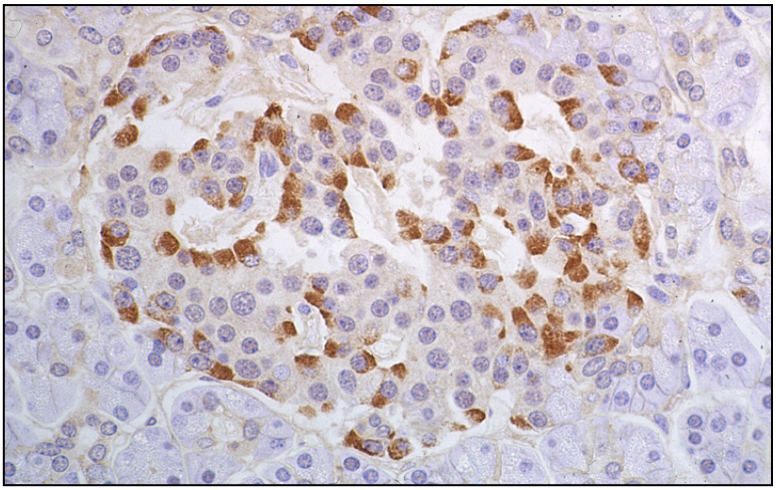


**Plate 51:** Glucagon, (MP). Formol saline fixed pancreas embedded in MMA polymerised with DMA and stained with a polyclonal antibody for glucagon (Dako) with a Streptavidin biotin procedure using DAB chromogen without pretreatment. Counterstain Gill's haematoxylin.

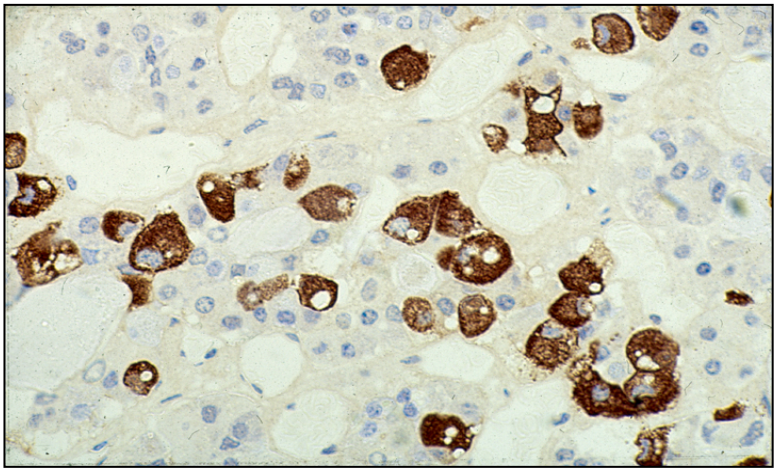


**Plate 52:** ACTH, (HP). Formol saline fixed pituitary embedded in MMA polymerised with DMA and stained with a polyclonal antibody for ACTH (Dako) with a Streptavidin biotin procedure using DAB chromogen without pretreatment. Counterstain Gill's haematoxylin.

# CHAPTER 10

# REVIEW OF PAPERS 8 AND 9

## 10.1 REVIEW OF PAPER 8

### 10.1.1 Non-isotopic *in situ* hybridization to detect chick Sox gene mRNA in plastic embedded tissue. (Church, Hand, Rex and Scotting, 1997; *Histochemical Journal,* 29:625 - 629).

### 10.1.2 Introduction

The above paper stated that the literature had previously contained only a few reports of ISH on plastic embedded tissue using either non-isotopic techniques (Cau and Beckstead, 1989; Kibbelaar *et al.,* 1992; Ding *et al.,* 1996) or isotopic techniques (Jamrich *et al.,* 1984; Wide *et al.,* 1989). Though one of these studies (Jamrich *et al.,* 1984) had used MMA, the authors of above paper claimed that the method was not as simple or applicable to tissue as their procedure. In this publication, methods were described for the demonstration of two *Sox* gene mRNAs (*Sox* 11 and *Sox* 21) in MMA-embedded chick tissue using non-isotopic ISH. Previously Uwanogho *et al.,* (1995) had studied both *Sox* 11 and *Sox* 21, although the results for the latter were unpublished. Both genes are members of the *Sox* gene family that encode transcription factors, which are related to the transition of cells of the nervous system from proliferation to differentiation and though readily detectable in frozen and paraffin sections, their demonstration in plastic sections was investigated to improve cellular resolution.

Church *et al.,* (1997) also examined in this paper whether the choice of accelerator to polymerise the plastic, and whether pretreatment of the sections with proteinase K, or heat mediated techniques either with a microwave oven or a pressure cooker could affect ISH staining.

### 10.1.3 Method

Six day old chick embryo samples were fixed in 4% paraformadehyde made up in phosphate-buffered saline (PBS), pH 7.3 at 4$^{o}$C for 24 hr, and then processed and embedded in MMA as described previously (Hand *et al.* 1996). However, to induce polymerisation, either the amine accelerator DMA or DMSX was used at a concentration of both 150 µl per 10 g aliquot of catalysed MMA mix. Following polymerisation (approximately 2 hr), the blocks were sectioned at 2 µm and 4 µm on a Reichert-Jung Autocut using a 10 mm wide Latta-Hartmann knife, and sections drained and dried as previously described (Hand *et al.* 1996).

Riboprobes were synthesized according to the method recommended by the suppliers of digoxigenin-labelled nucleotides (Boehringer Mannheim). Plasmid DNA was digested with single restriction enzyme to linearise, and was then incubated with either T3 or T7 RNA polymerase to generate sense or antisense riboprobe. Probes were between 0.5 and 1.5 kb in length. The dioxigenin-labelled riboprobes were diluted in hybridisation solution that consisted of 50% formamide, 5 x saline sodium citrate (SSC), 1% sodium dodecyl sulphate (SDS), 50 mg ml$^{-1}$ yeast tRNA and 50 mg ml$^{-1}$ heparin. The optimum dilution factor was established empirically.

It was stated that during staining the sections should not be allowed to dry out. The sections were first deplasticised in xylene at 37°C for 30 mins, hydrated through decreasing concentrations of ethanol and water before undergoing pretreatment either using proteinase K, or heating sodium citrate solution in a microwave oven (Hand *et al.* 1996) or in a pressure cooker (Hand and Church, 1997). For enzyme pretreatment the slides were overlaid with approximately 100 µl of proteinase K solution (10 mg $ml^{-1,}$ pre-warmed to 37°C) and left at room temperature for 15 mins. Proteinase K was removed by washing for 1 min (x3) in PBS containing 0.1% Tween 20. The slides were post-fixed with 4% paraformadehyde in PBS for 20 mins.

The method for ISH was generally as described by Uwanogho *et al.,* (1995). A 100 µl sample of diluted probe was added to each slide for incubation at 70°C overnight under a coverglass in a humidified chamber. The slides were then washed twice with 50% formamide, 5x SSC, 1% SDS at 65°C for 30 mins each; three times with 50% formamide, 2x SSC at 65°C for 30 mins each, and finally three times in 1 x TBST (140 nM Tris-HCl buffer, pH 7.5, 300 nM levamisole. 0.1% Tween 20) at room temperature for 5 mins each.

For detection the slides were submerged in 1% blocking reagent for nucleic acid hybribridisation in TBST for 30 mins at room temperature, and then in a 1:2000 dilution of pre-absorbed sheep anti-digoxygenin-alkaline phosphatase Fab fragments for incubation at 4°C overnight. They were then washed in three changes of freshly prepared NTMT buffer (0.1M Tris-HCl, pH 9.5, 50 mg $MgCl_{2,}$ 0.1M NaCl, 0.1% Tween 20, 2mM levamisole). The sections were stained with

X-phosphate-NitroBT (45 µl of 75 mg $ml^{-1}$ NitroBT in 70% dimethylformamide, 35 µl of 50 mg $ml^{-1}$ X-phosphate in dimethylformamide, 100 ml of NTMT), and the colour allowed to fully develop (7 days) in the dark at room temperature. The reaction was stopped by incubating in 10 mM Tris-HCl, pH 7.8, containing 10 mM ethylenediamine tetra-acetic acid (EDTA). The sections were mounted in Mowiol mounting medium (Calbiochem-Novabiochem, USA).

### 10.1.4 Results

The results showed that both *Sox* gene mRNAs were demonstrated in MMA-embedded chick tissue using non-isotopic ISH techniques in each of the MMA mixes. However, whereas *Sox* 21 was readily detected using any of the three pretreatments investigated, *Sox* 11 was only detected strongly after superheating with a pressure cooker (plate 53, p 122). The pretreatments investigated produced variable staining intensity of each *Sox* gene with PC superior to proteinase K digestion and microwave heating (see plates 54 – 56, pp 122 – 123). Although proteinase K produced moderate staining for both probes, microwave heating produced strong staining only for *Sox* 21. Similar staining intensity using either accelerator was observed for the detection of both mRNAs, although for *Sox* 11, slightly superior staining was produced with DMSX. Unsurprisingly stronger staining was more evident in 4 µm sections than 2 µm sections, and though decreased staining in archival blocks prepared six months earlier was seen in sections pretreated with either proteinase K or using microwave heating, the use of pressure cooking restored staining to an acceptable level.

As a result of differential staining for the demonstration of *Sox* 11 and *Sox* 21, where the choice of pretreatment (and to a lesser extent the accelerator) had a critical influence on the former, it was suggested that both criteria should be considered when ISH studies are required in the future.

### 10.1.5 Conclusions

This paper reported on the application of ISH for the demonstration of *Sox* gene mRNA using non-isoptopic ISH in MMA-embedded tissue. Previously such techniques had not been reported on MMA-embedded tissue. Both *Sox* genes 11 and 21 were demonstrated in tissue embedded in each MMA mix that had been polymerised using different accelerators. *Sox* 21 was detected using all three pretreatments investigated but *Sox* 11 was only detected strongly after superheating sodium citrate with a pressure cooker. Antigen retrevial using a microwave oven produced poor staining for *Sox* 11 and overall the use of pressure cooking produced best staining and was recommended. Superior staining was achieved in blocks polymerised with DMSX and not unexpectedly stronger staining was more evident in 4 µm sections than 2 µm sections. It was noticed that in archival blocks decreased staining occurred, but the use of PC restored ISH staining.

## 10.2 REVIEW OF PAPER 9

### 10.2.1 Double labelling using non-isotopic *in situ* hybridisation and Immunohistochemistry on plastic embedded tissue. (Church, Hand, Rex and Scotting, 1998; *Journal of Cellular Pathology*, 3:11- 16).

### 10.2.2 Introduction

Following reporting of the demonstration of Sox gene mRNAs in MMA-embedded tissue using ISH techniques (Church *et al.,* 1997), the same authors described in the subsequent above paper (Church *et al.,* 1998), double staining consisting of combining ISH and IHC. As *Sox* 11 and *Sox* 21 in the previous study were related to the transition of cells of the nervous system from proliferation to differentiation, their staining was combined with the immunocytochemical localisation of bromodeoxyuridine (BrdU) to demonstrate proliferation and neurofilament protein (NFP) to demonstrate differentiation. The script of the above paper was supported by colour photomicrographs to enable differential staining to be easily observed.

### 10.2.3 Method

Briefly, 6-8 day old chick embryos were labelled with BrdU by injection with approximately 5-30 µg/g body weight. The tissues were then fixed, processed and embedded as described previously (Church *et al.,* 1997) using 150 µl DMSX to polymerise the MMA mix. Similarly 4 µm sections from blocks measuring 12 x 6 x 5 mm were cut, dried and the plastic removed prior to staining as reported (Church *et al.,* 1997).

In preliminary investigations, it had been determined that no pretreatment was necessary using the NFP antibody specified (Milab), whereas the BrdU (Dako) antibody benefitted from heat induced antigen retrieval. As a result, a single pretreatment was chosen using either proteinase K for ISH and NFP, or PC to heat sodium citrate solution for ISH and BrdU. The methodology used for both

pretreatments and ISH were as previously described (Church *et al.,* 1997). The slides were left unmounted in distilled water and then washed in 0.005M TBS to allow subsequent immunohistochemical staining to be applied. For immunoperoxidase staining of BrdU, sections were treated with 20% normal serum diluted in TBS for 20 mins, drained and then incubated in 1:25 diution of BrdU (made up in 5% normal serum in TBS) for 1 hr. A standard Streptavidin biotin (Dako) procedure was applied using reagents, protocols and detection of peroxidase activity using the procedures previously described (Hand *et al.* 1996). The sections were not counterstained, but washed in water, rinsed very quickly in ethanol, air dried and mounted in Loctite 358 fast-curing UV adhesive that was polymerised under UV light for 2 mins.

For immunostaining of BrdU and NFP using alkaline phosphatase, a similar incubation and procedure as that described above for immunoperoxidase was employed, although the NFP antibody did not require dilution. Following a wash in TBS, the sections were incubated in sheep anti-mouse biotinylated secondary antibody diluted 1:100 in TBS for 30 mins, washed again in TBS, and then the sections incubated in alkaline phosphatase streptavidin (Vector) diluted 1:500 in TBS for 30 mins. After a further wash in TBS, alkaline phosphatase activity was detected using freshly prepared Fast red TR until the colour was satisfactory (8 mins). The sections were then washed in water and ethanol (quickly), air dried and mounted without counterstaining as described above for immunoperoxidase staining of BrdU.

### 10.2.4 Results

From the results produced, excellent demonstration of *Sox* gene mRNA and tissue antigens were simultaneously achieved in MMA-embedded chick tissue (see plates 57 – 59, pp 124 – 125), although superior staining when combined with ISH was observed using alkaline phosphatase rather than peroxidase because it was cleaner and more intense. Though in preliminary investigations BrdU had been successfully demonstrated with either pepsin digestion or MAR, neither produced staining as good as that achieved using PC. The choice of DMSX as the accelerator to polymerise the MMA confirmed previous results for IHC (Hand and Church, 1997) and ISH (Church *et al.,* 1997), where it was reported that DMSX produced superior staining.

### 10.2.5 Conclusions

This paper established that ISH and IHC could be simultaneously achieved in MMA-embedded tissue on the same section, which previously had been unreported on plastic sections. Superior immunocytochemical staining was observed using alkaline phosphatase rather than peroxidase, and antigen retrieval using PC produced the best staining. The best staining was achieved in blocks polymerised with the accelerator DMSX

## 10.3 PHOTOMICROGRAPHGS

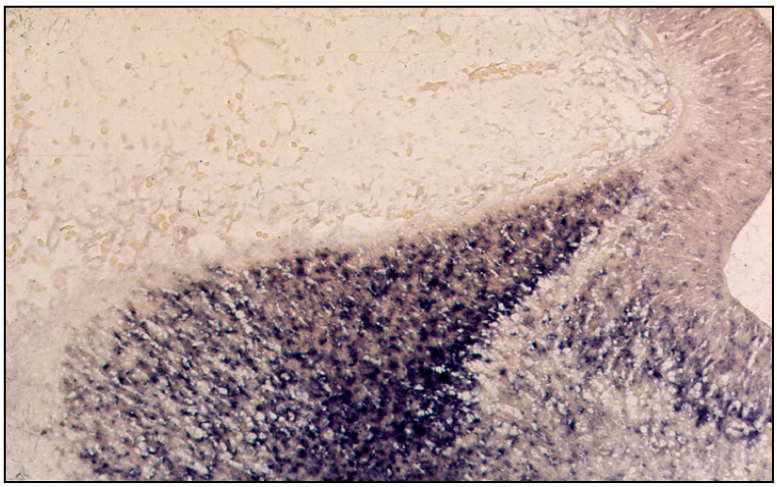


**Plate 53:** *Sox* 11, PC pretreatment: (LP). *Sox* 11 staining (blue) in chick tissue embedded in MMA which was polymerised using DMSX. Staining was achieved with TNBT following pretreatment with heated 0.001M sodium citrate pH 6 in a pressure cooker, which was the only pretreatment to produce strong staining for *Sox* 11.

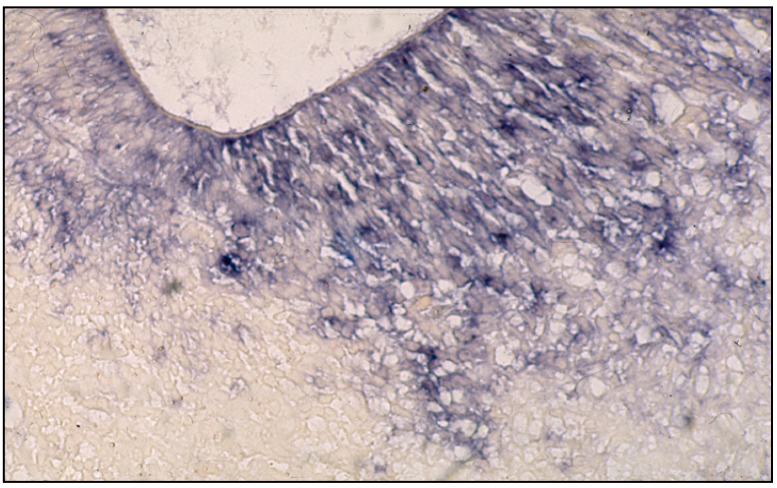


**Plate 54:** *Sox* 21, PC pretreatment: (MP). *Sox* 21 staining (blue) in chick tissue embedded in MMA which was polymerised using DMSX. Staining was achieved with TNBT following pretreatment with heated 0.01M sodium citrate pH 6 in a pressure cooker. Note strong and superior staining to plates 55 & 56.

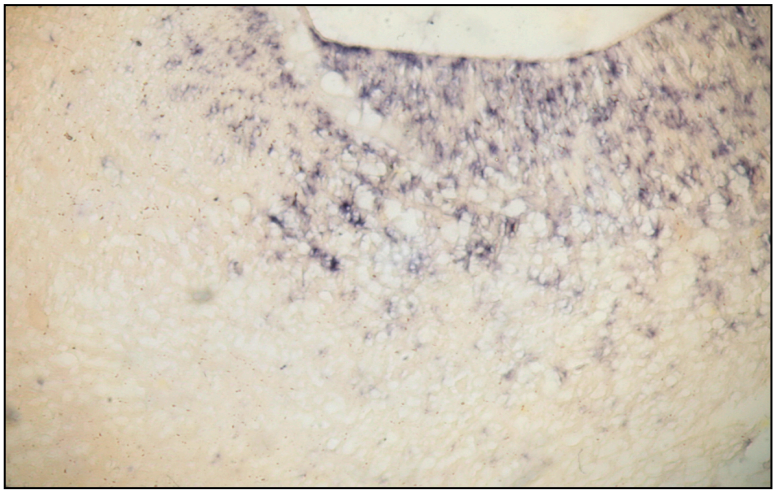


**Plate 55:** *Sox* 21, Proteinase K pretreatment: (MP). *Sox* 21 staining (blue) in chick tissue embedded in MMA which was polymerised using DMSX. Staining was achieved with TNBT following pretreatment with Proteinase K. Note inferior staining to plate 54.

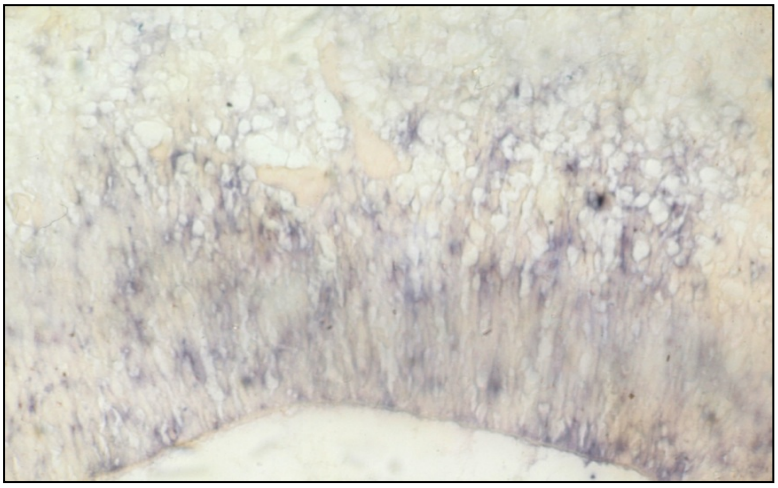


**Plate 56:** *Sox* 21, MAR pretreatment: (MP). *Sox* 21 staining (blue) in chick tissue embedded in MMA which was polymerised using DMSX. Staining was achieved with TNBT following pretreatment with heated 0.01M sodium citrate pH 6 in a microwave oven. Note inferior staining to plate 54.

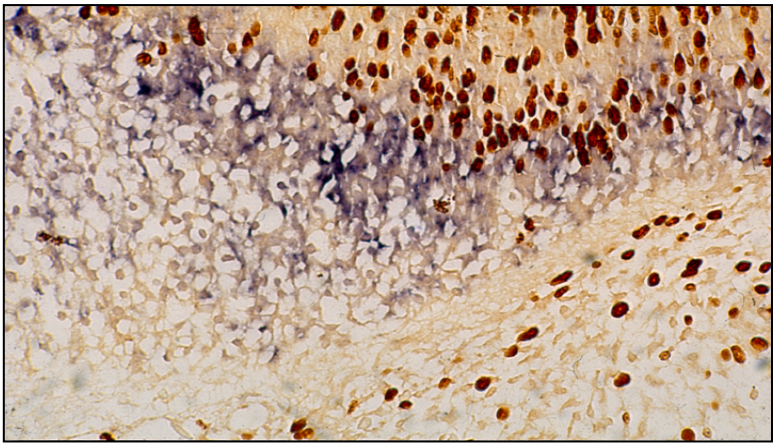


**Plate 57:** *Sox* 11 / BrdU, PC pretreatment: (MP). Transverse section of chick neural tube embedded in MMA polymerised with DMSX showing *Sox* 11 (blue) stained with TNBT and immunoperoxidase staining of BrdU with DAB (brown) following pretreatment with heated 0.01M sodium citrate pH 6 in a pressure cooker. Reproduced by permission of Greenwich Medical Media Ltd, London.

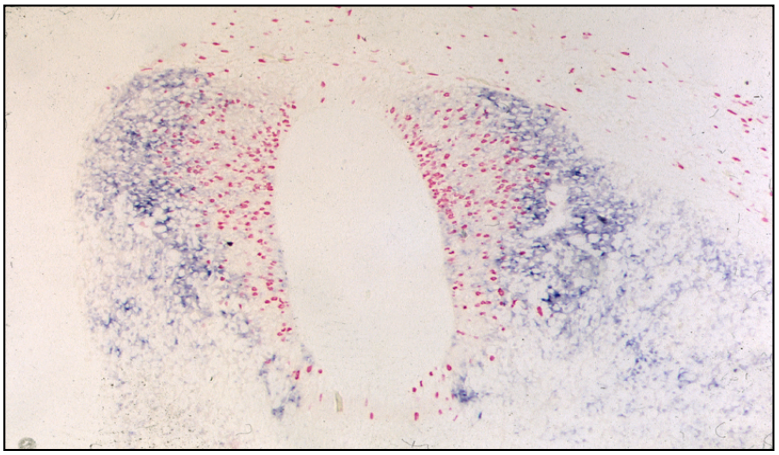


**Plate 58:** *Sox* 11 / BrdU, PC pretreatment: (LP). Transverse section of chick neural tube embedded in MMA polymerised with DMSX showing *Sox* 11 (blue) stained with TNBT and immunocytochemical staining of BrdU (red) using alkaline phosphatase visualised with Fast red TR following pretreatment with heated 0.01M sodium citrate pH 6 in a pressure cooker. Reproduced by permission of Greenwich Medical Media Ltd, London.

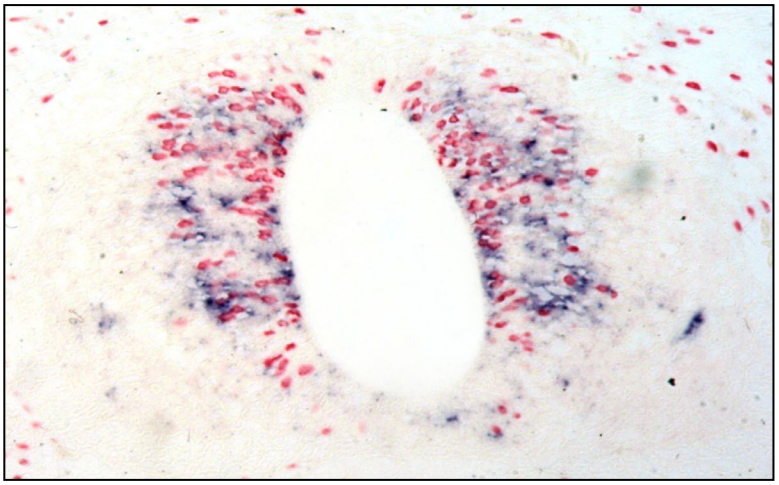


**Plate 59:** *Sox* 21 / BrdU, PC pretreatment: (MP). Transverse section of chick neural tube embedded in MMA polymerised with DMSX showing *Sox* 21 (blue) stained with TNBT and immunocytochemical staining of BrdU (red) using alkaline phosphatase visualised with Fast red TR following pretreatment with heated 0.01M sodium citrate pH 6 in a pressure cooker. Reproduced by permission of Greenwich Medical Media Ltd, London.

# CHAPTER 11

# GENERAL DISCUSSION

## 11.1 INTRODUCTION

In this thesis, it has been stated that the development of plastic embedding originated from the need to use an alternative embedding medium to paraffin wax for EM. Originally an acrylic plastic was used (Newman *et al.,* 1949), but this was superceded by epoxy (Glauert *et al.,* 1956) and polyester plastics (Kellenberger *et al.,* 1956), and for ultrastructural purposes the epoxy plastics still remain the embedding media of choice. As plastics and techniques for EM were developed, useful applications also evolved for LM. Many of the plastics for these purposes were acrylics as these were found to be the most suitable. During the 1950s, embedding undecalcified bone in plastic was introduced (Kidman *et al.,* 1952; Woodruff and Norris, 1955; Yaeger, 1958) and later in the 1960s high resolution LM studies using semithin sections started to become a major area of interest and development (Feder, 1963; Ashley and Feder, 1966; Ruddell, 1967a, 1967b). The author of this thesis has published several papers relating to the development of these two applications that forms the subject of this thesis and which will now be discussed.

In chapters 6 - 10 an outline description of specific papers that involved the author was presented which included the purpose and value of the particular study. Having worked in various diagnostic laboratories, there was an awareness of the need to develop uncomplicated procedures that would deliver

timely results, and therefore many of the techniques and protocols described were developed with this in mind. The approach was systematic and several procedures evolved from similar concepts that already existed, although some of these did not relate specifically to plastic embedding. Further details are discussed in this chapter about the techniques and methodology employed, and how they relate to other plastic and non-plastic publications. The plastic embedding studies and publications that have been collated and collectively described in this thesis, include procedures for undecalcified bone, enzyme histochemistry, immunohistochemistry and *in situ* hybridisation.

The development of plastic embedding procedures for LM studies led to the introduction of specialised equipment. Fundamentally this consisted of microtomes and knives that would enable thin and semithin sections to be produced by microtomy. In addition, sophisticated equipment has also been developed such as that manufactured by EXAKT for the production of ground sections (Donath, 1990), which is especially useful when the tissue is undecalcified bone, tooth and/or contains implants. However, such very expensive equipment is only found in isolated specialised laboratories, whereas the studies in this thesis have focussed on using equipment suitable for microtomy, as this had been readily available and was more appropriate for the specific diagnostic applications investigated.

### 11.1.1 Embedding Undecalcified Bone

The histological examination and investigation of metabolic bone diseases such as osteoporosis, osteomalacia and Paget's disease has utilised plastic

embedding to enable undecalcified bone sections to be cut and stained (Recker, 1983; Malluche and Faugere, 1987). Numerous procedures have been published describing various techniques, some of which were reviewed by Sanderson (1997). Also different plastics have been suggested (Fallon and Teitelbaum, 1981; Mawhinney and Ellis, 1983; Recker, 1983). Though the authors of many publications have stated their reasons why their technique was preferred, often they were developed in specialised research institutions that resulted in producing a time-consuming and labour-intensive methodology which was not compatible with a diagnostic environment. As a consequence Stevens and Palmer (1985) advocated the use of frozen sections instead for bone biopsies in metabolic and other diseases. However, cutting frozen sections of undecalcifed bone is not easy and can result in poor morphology (Pearse and Gardner, 1972), and as a result there have been several attempts instead to streamline the preferred option of using a plastic embedding procedure (Frost, 1958; Chapman, Kirkham and Schiller, 1984; Emmanual, 1988). In the experience of this author, the procedure based on using PMMA as described by Difford (1974) had proved satisfactory in the laboratory for routine diagnostic use, but it was also recognised that a number of improvements to the technique were necessary that could benefit the service and ultimately the patient. Specific technical advantages of the modification introduced have previously been listed in chapter 6, but other significant merits included the presentation of a simpler method, reduced preparation time, reduced cost, higher throughput of cases and quicker diagnosis. For example, in the paper that collectively described these improvements (Hand, 1996) it was reported that the inhibitor hydroquinone was left in the monomer, whereas Sissons (1968) and Difford (1974) recommended

its removal. In another example, several publications that have used MMA for embedding bone have reported that the sections should be adhered to a gelatinised microscope slide and the plastic removed prior to staining (Delling, 1972; Recker, 1983; Malluche and Faugere, 1986), whereas excellent tinctorial staining has been achieved "free-floating" sections by Sissons (1968) and this author with the plastic *in situ.* When sectioning large and heavily calcified bone samples, this author has found through experience that to achieve good morphology of the tissue, it has been necessary for the plastic mix to be "stiffened" by using PMMA, but often the harder mix did not adhere well to glass slides. An advantage of using "free-floating" sections was that the time-consuming clamping procedures required for adherence were avoided. In the paper reviewed, (Hand, 1996) it was reported that the traditional staining techniques of Goldners trichrome, Solochrome azurine, Toluidine blue and von Kossa/van Gieson, and the demonstration of tetracycline by fluorescent microscopy were all applicable.

Though current practice for assessing and diagnosing metabolic bone diseases is now performed by bone densitometry, it was concluded that the modifications described, which evolved through service needs and were tested on a large number of cases (10,000), had at the time impacted significantly on the diagnostic service by providing greater efficiency and quicker reporting without compromising quality. In addition, some of the knowledge and skills gained from handling MMA was to prove influencial and beneficial later in the development of techniques by the author for IHC and ISH studies.

### 11.1.2 High resolution studies

The literature contains several reports on the advantages of using semithin plastic sections for high resolution studies (Eastham and Essex, 1969; Zamboni, 1972; Agodoa *et al.,* 1975; Hoffmann and Flores, 1981; Hoffmann *et al.,* 1982; Woodruff and Greenfield, 1982; Lumb, 1983; Mason and Mackie, 1985; Ferrell and Beckstead, 1988; Casey *et al.,* 1990a; Gerrits and Suurmeijer, 1991) with several of these publications focussed on the use of GMA. Other reports included combining tinctorial staining procedures with more exacting enzyme histochemical techniques using carefully controlled fixed tissue and low temperature processing (Beckstead and Bainton, 1980; Beckstead *et al.,* 1981; Beckstead, 1983; Islam and Henderson, 1987). In other enzyme studies freeze drying (Murray, *et al.,* 1988a; Murray and Ewen, 1989b) and freeze substitution (Murray and Ewen, 1991) had been used, prompting one of the authors to ask whether paraffin wax might be on the wane because of its limited application for enzyme histochemistry studies (Murray, 1988).

The use of a harder embedding medium than paraffin wax attracted the obvious use for the application of embedding undecalcified bone marrow trephine biopsies for the diagnosis of non-metabolic bone diseases. However, there have been several reports where decalcified bone samples embedded in paraffin wax have remained the preferred method for examination (Gatter *et al.,* 1987; Vincic, Weston and Riddell, 1989; Schmid and Issacson, 1992), although others have stated their preference for plastic embedding using undecalcified cores (Hott and Marie, 1987; Islam and Frisch, 1985; Jack, Roberts and Scott, 1993). Later in

this discussion, possible explanations will be presented to suggest the reasons for the differing opinions.

### 11.1.3 Enzyme Histochemistry

The traditional histological procedure to demonstrate enzyme activity within tissue is to use frozen sections (Nestor and Bancroft, 2008), and for several enzymes improved morphology and localisation can be achieved if the sections are first subjected to light fixation prior to enzyme histochemical staining. Based on numerous previous studies where the effects of fixation have been systematically collated and assessed (Chayen *et al.,* 1973), it was shown that mild fixation with a cold aldehyde fixative permitted subsequent demonstration of many enzymes via their histochemical activity. Formalin has frequently been used and Dawson (1974) preferred formol calcium which this author had also found effective and satisfactory. Subsequent washing of the tissue in gum sucrose for frozen sections or 3% sucrose in 0.2M cacodylate buffer for plastic embedded tissue produced enhanced enzyme histochemistry. Except for a few hardy enzymes such as chloroacetate esterase and peroxidase, the standard schedules that involve fixation, processing and embedding in hot wax to produce paraffin blocks are not conducive for the preservation of enzyme activity (Nestor and Bancroft, 2008). However, the ability to process and polymerise acrylic plastics at low temperature, usually following mild fixation, can be effective for their retention. As a result, numerous publications, (often ultilising acrylic GMA sections) have described procedures for enzyme histochemistry, (Hoshino, 1971; Ashford *et al.,* 1972; Troyer and Nusbickel, 1975; Brinn and Picket, 1979; Huguchi *et al.,* 1979; Nusbickel and Swartz, 1979; Horton *et al.,* 1980; Chappard,

1985; Murray *et al.,* 1987; Pretlow *et al.,* 1987a; Gruber *et al.,* 1988). In some cases useful diagnostic applications have also been suggested (Beckstead *et al.,* 1981; Beckstead, 1983; Soufleris, *et al.,* 1983; Islam and Henderson, 1987; Murray and Ewen, 1991). Scrutiny of the methodology reveals a wide range of preparatory procedures, with many (including some of those referenced above) based on the general approach of low temperature aldehyde fixation followed by processing and embedding also at low temperature. The plastic was then polymerised with a chemical accelerator or irradiated with light, although in the studies by Höpfel-Kreiner and von Mayerbach (1978) and von Mayerbach and Höpfel-Kreiner (1978) it was stated that UV light caused inactivation of the enzymes.

A further development saw the introduction of LR Gold (Thompson and Germaine, 1984) where unfixed tissue could be processed at sub-zero temperature and the plastic polymerised by irradiation with blue light. This procedure enabled a range of hydrolytic enzymes in addition to the fixation-sensitive enzyme succinic dehydrogenase to be demonstrated, although no other oxidative enzyme has been demonstrated using this tedious technique, which this author has found time-consuming and not applicable for diagnostic use. However, LR Gold has also been used on fixed tissue for the demonstration of other oxidative enzymes (Murray *et al.,* 1988a and 1988b). A similar procedure to that used by Thompson and Germaine (1984) was also described by Dilsanete *et al.,* (1992) for demonstration of acid and alkaline phosphatase in fresh, unfixed undecalcified samples of rat tooth embedded in LR Gold. Succinic

dehydrogenase has been demonstrated in fixed GMA-embedded tissue, but incubation in the staining solutions occurred prior to fixation (Weber, 1974).

Oxidative enzymes have rarely been localised in aldehyde-fixed tissue, although Pretlow *et al.,* (1987b) reported the demonstration of aldehyde dehydrogenase, and NADPH tetrazolium reductase, but not glucose-6-phosphate dehydrogenase. In a series of articles by Murray and colleagues, freeze drying (where tissue was immersed in liquid nitrogen and water removed at -40$^{o}$C) and freeze substitution (where tissue was fixed at -40$^{o}$C with acetone or alcohol) has produced greater success. Investigations included the demonstration of several oxidative enzymes using both freeze drying (Murray *et al.,* 1988a; Murray and Ewen*,* 1989b) and freeze substitution (Murray and Ewen, 1991). A comparison of aldehyde-fixed tissue and freeze dried tissue was reported by Murray *et al.,* (1988a), with the latter producing superior results. One notable enzyme that failed to be demonstrated by freeze drying was succinic dehydrogenase (Murray *et al.,* 1988a). LR Gold and LR White have been used for embedding fixed tissue and stained for NADH dehydrogenases, but the results were inferior to those achieved with GMA (Murray *et al.,* 1988b). The authors also stated in this paper that GMA was the plastic of choice when enzyme histochemistry was required, and advocated the use of JB-4 as it has the advantage of being able to process tissue via the monomer/plasticiser solution. The use of freeze drying and embedding in GMA had previously been investigated (Mitrenga *et al.,* 1974; von Höpfel-Kreiner and Mayerbach, 1978) with variable results.

Other developments have seen the use of methyl methacrylate (MMA) for

enzyme studies (Westen *et al.,* 1981; Chappard *et al.,* 1983, 1987; Bernhards *et al.,* Gruber *et al.,* 1988; 1992; Liu, 1987; Wolf *et al.,* 1992; Erben, 1997), although some of these plastic mixes have also incorporated BMA or GMA. For those mixtures incorporating GMA, staining occurred with the plastic in situ, whereas with non-GMA mixes the plastic was removed. Ruhl-Fehlert and Ludl (1987) reported on the use of the MMA based plastic K-Plast (Medim) for the demonstration of acid and alkaline phosphatase in bone tissue.

As indicated, a wide range of preparatory protocols and plastics have been ultilised for enzyme histochemical investigations on plastic-embedded tissue, but it has been shown that for some enzymes the results could be critically affected by specific reagents. In this thesis, studies relating to the demonstration of lactase and sucrase by the author were reported on plastic-embedded tissue, and though these disaccharidases were known to survive mild formalin fixation during their routine demonstration on frozen sections, it has been shown that both enzymes were sensitive to certain processing reagents, and particularly ethanol (Hand 1988a and 1988b) when processed for plastic embedding. Other studies have also demonstrated differential staining with different reagents (Beckstead and Bainton, 1980; Gerrits and Zuideveld 1983; Murray *et al.,* 1988b), although when the investigations by this author were carried out, these studies were unknown. No investigations on lactase and sucrase in plastic-embedded tissue previously existed, but as it was shown that enzyme staining can be influenced by the processing schedule adopted, it is imperative for diagnostic use that the technique and protocol employed must ensure maximum retention of an enzyme for the results to be valid. This was best achieved by

using acetone or processing via JB-4 monomer. Murray *et al.,* (1988a) also stated that processing tissue at 4°C was less effective than embedding at -20°C in preserving enzyme activity. In addition, the use of GMA sections provided better adherence to a glass slide or coverglass. For confidence in the results, this researcher would continue to recommend the use of frozen sections on diagnostic cases for the demonstration of the majority of enzymes to avoid any inconsistency and/or potential false negative staining.

### 11.1.4 Immunohistochemistry

During the last 25 years, stimulated by the tens of thousands of publications produced, there has been a worldwide exponential growth in the use and applications of IHC in histopathology, with the realisation that on an increasing number of occasions this type of investigation has at the very least proved useful, and in many cases essential for diagnosis (Dabbs, 2006). A multitude of techniques and reagents have been introduced that have enabled IHC to be routinely performed in many histopathology laboratories (Jackson and Blythe, 2013). Originally frozen sections were ultilsed, but as technical developments advanced, so too have procedures been devised that have resulted in most IHC now being performed routinely on formalin-fixed paraffin sections. Such is the importance of IHC, that it hard to imagine a histopathology laboratory now where this service is not required. In addition, there have always been research developments that depend on using IHC, which has assisted in the rapid and dynamic expansion of this speciality.

As techniques and applications for IHC, lectins and ISH have evolved and

developed on routine frozen and paraffin sections, so too has there been a parallel evolution of similar techniques beyond the traditional tinctorial staining procedures on plastic embedded tissue. In the development of acrylic plastic embedding procedures, several papers have been published describing the application of IHC including using a BMA/paraffin wax medium (Wechbanjong *et al.,* 1979), although it is probably those by Beckstead (1985) and Casey *et al.,* (1988) using GMA that are the best known. However, despite several other reports claiming successful immunohistochemical staining on GMA-embedded tissue, e.g. Hoshino, and Kobayashi, (1972); van de Velde, (1980); Zerpa *et al.,* (1981); Mozdzen and Keren, (1982); Casanova *et al.,* (1983), Archimbald *et al.,* (1987); Colbatzky and Hermanns, 1987; Lazzaro *et al.,* (1988); Islam *et al.,* (1988); van Goor *et al.,* (1988); Jackson, (1989); Burgio *et al.,* (1991); van Goor, (1993); (van Pelt-Verkuil, (1995), there have also been others stating that the methods were idiosyncratic, problematic and unreliable (Vincic *et al.,* 1989; Vincic, 1990; Hand, 1993; Hand, 1995a; Blythe *et al.,* 1997). Curiously, in one of the above papers that described a method for immunostaining, van Goor *et al.,* (1988) also quoted that it was “impossible to predict whether positive staining might be achieved.” In early studies some of the difficulties encountered were discussed (Avrameas *et al.,* 1976; Vogt, 1976; Takimiya *et al.,* 1978; Takimya *et al.,* 1979; Takimya *et al.,* 1980) that lead to Franklin and Martin (1980 and 1981) to suggest the use of pre-embedding immunohistochemical staining procedures. In an attempt to produce post-embedding immunostaining, numerous specialised techniques and protocols have since been proposed, which have included pretreatment of the sections with proteolytic enzymes (Vogt *et al.,* 1976; Takimiya *et al.,* 1980; Casanova *et al.,* 1983), low temperature embedding

(Beckstead, 1985), the addition of methyl benzoate to the infiltration solutions (Casey *et al.,* 1988), replacement of the commonly used plasticiser butoxyethanol with butandiol monoacrylate (van Goor *et al.,* 1988) and the use of immunogold silver staining (van de Kant *et al.,* 1988; Jackson, 1989; van de Kant *et al.,* 1990; Tacha *et al.,* 1993). Polysciences introduced the GMA based plastic ImmunoBed specifically for immunohistochemistry, but for this author, the experience has been that the results were extremely disappointing and ranged from poor staining to none. Some of the possibilities and limitations were summarised by Gerrits (1988), but it is reasonable to say that the debate on whether IHC can be performed on acrylic sections has been one of the most controversial in recent years within histological practice.

Several publications have reported the use of immunocytochemical staining on tissue embedded in LR White and especially those that originated from the group of scientists based in Cardiff, UK (Newman *et al.,* 1982; Newman *et al.,* 1983; Newman and Jasani, 1984a and 1984b; Bowdler *et al.,* 1989; Newman and Hobot, 1993). To increase optical density and ensure visualisation of immunostaining when using LR White, Newman, *et al.,* (1882 and 1983); and Germaine (1991) suggested enhancement of DAB. Thompson and Germaine (1984) also reported IHC on LR Gold sections. Other reports have appeared using the Lowicryl plastic K4M (Al Nawab and Davies, 1989) and Bioacryl (Scarla *et al.,* 1992). Though many of these non-GMA plastics can also be used for EM, these specific studies referred to techniques for LM. However, the complex procedures involved, together with their limited applications in a routine laboratory and a general lack of success by many scientists in achieving IHC,

has resulted in the plastics listed not being widely used in histological practice for LM studies. Detailed advice and additional information for those wishing to use Lowicryl plastics or LR Gold/White was provided in the excellent publications by Newman and Jasani (1984b), Newman (1987), Newman and Hobot (1987) and Newman and Hobot (1993), although the paper in 1987 by Newman and Hobot related to electron immunohistochemistry only.

A major disadvantage of many acrylics including GMA, LR Gold, LR White, Histocryl, Lowicryl K4M, K11M, HM20, and HM23, and Bioacryl (Unicryl) is that when these plastics are polymerised, they become insoluble and remain present during staining. This has presented significant and formidable difficulties especially when large molecules are required to penetrate through the plastic matrix to achieve immunocytochemical staining. Casey *et al.,* (1988) also reported that the plastic matrix becomes super-polymerised over time to form a closer and tighter network. In addition, as the chemical reactions between the ingredients are complex and not fully understood (and can vary depending on conditions), it is virtually impossible to consistently reproduce similarly structured polymerised plastic blocks leading to variations in staining. For these reasons, this author has long considered it is plausible that for many of the techniques and protocols previously published, embedding at low temperature was beneficial because gentle polymerisation produced a plastic with a more loosely bound matrix that was more conducive for large molecules to penetrate. The need to produce a less dense structure has also been noted by others (Causton, 1984; Newman and Jasani, 1984b; Newman, 1987; Gerrits, 1988; Newman and Hobot, 1993). Whilst it would be too simplistic an explanation to claim that cross-linking

leading to an insoluble polymer was solely responsible for poor or no immunostaining, it was undoubtedly an obvious and significant reason. Gerrits (1988) and Gerrits *et al.,* (1990) stated that dyes with a molecular weight less than 500 daltons penetrate the GMA plastic matrix quickly, but those of 1300 daltons cannot. Immunhistochemical procedures use macromolecules with molecular weights of at least 150,000 daltons (Gerrits, 1988). In addition, some publications have added to the confusion with statements such as “etching is not required” (Scala *et al.,* 1992), or “acrylics resins such as glycol methacrylate do not require etching prior to staining” (Wilkins, 1995) implying that it is optional, when factually due to cross-linking of the plastic, they are insoluble. Such comments to those less experienced could be misleading.

In an effort to overcome these difficulties, investigations into the possibilities of using MMA were made, because in some circumstances it can be dissolved when polymerised. Several initial reports were published (Hand *et al.,* 1989; Hand and Morrell, 1990; Johns *et al.,* 1992; Hand, 1995a) where the MMA embedding medium was removed (rather like paraffin wax) prior to immunohistochemical staining. Using a specially devised formulation where the MMA was polymerised quickly, new preparatory techniques for producing the sections were introduced and immunohistochemical staining applied. By comparison with previous staining attempts, the results were spectacular. Originally some of the investigations utilised tissue which was not fixed in formalin and so did not require pretreatment techniques to unmask antigenicity caused by the effects of fixation. Previously, Rodriguez *et al.,* (1984) had reported the application of IHC using immunoperoxidase enhanced with silver

methamine staining on tissue embedded in a BMA/MMA mix although the plastic was polymerised using either with dibenzoyl peroxide or benzoin and UV light over a period of 24-36 hr.

In early (unpublished) studies by Hand when immunogold silver techniques were topical, some success was also achieved using this technique on MMA-embedded tissue (see plates 43 and 44, p101). Though several other publications reinforced that IHC was possible on MMA-embedded tissue, including immunogold silver staining by van de Kant *et al.,* (1988 and 1990), it was interesting to note that Islam (1987) and Frisch and Bartl (1990) have stated that IHC could not be performed due to the shortcomings of MMA. Following yet another paper by Schmid and Issacson (1992) which continued to perpetuate this myth, Hand (1993) and Jack *et al.,* (1993) replied in an attempt to correct this misunderstanding.

It has long been known that enzyme pretreatment of formalin-fixed tissue could greatly enhance immunohistochemical staining on paraffin sections (Curran and Gregory, 1977; Mepham *et al.,* 1979). For many years this was an accepted practice, but the introduction of heat induced epitope retrieval techniques by Shi *et al.,* (1991) revolutionised staining protocols, especially when rapid developments soon led to the use 10 mM sodium citrate and 10 mM EDTA (not assessed or used in the studies presented in this thesis) at pH 6 and pH 8-9 respectively (Gerdes *et al.,* 1992; Cattoretti *et al.,* 1993). These solutions which were safe, easy to prepare and inexpensive to use became widely adopted and could be conveniently heated in a microwave oven. Though the rationale behind

heat pretreatment is unclear with different theories suggested (Jackson and Blythe, 2013), the results for paraffin sections led to significant advantages with enhanced staining, and microwave antigen retrieval as it became known is now common practice. However, there are still occasions for certain antibodies when enzyme pretreatment or where other devices for producing heat induced pretreatments are useful and/or preferred, either as an alternative to using a microwave oven or to produce better staining. One procedure described was the use of superheating solutions with a pressure cooker (Norton *et al.,* 1984).

Following the initial immunohistochemical studies on MMA-embedded tissue referred to previously in this thesis, this author and colleagues then investigated the application of various pretreatments to assess whether these would be beneficial for immunostaining. Both microwave antigen retrieval (Hand *et al.,* 1996) and pressure cooking (Hand and Church, 1998) were found to be extremely useful and resulted in significant enhanced staining with neither pretreatment previously described for MMA-embedded tissue. As a result, the number of antibodies that could be used to reliably demonstrate a variety of antigens was dramatically increased, and provided an opportunity to investigate whether embedding in MMA could be realistically used for diagnostic applications (Blythe *et al.,* 1997; Hand and Church, 1997).

One very useful and obvious application was for embedding undecalcified bone marrow trephine biopsies for the diagnosis of haematological malignancies. Traditionally this type of investigation has been performed on decalcified tissue which is then embedded in paraffin wax, and though reported by many as

satisfactory (Gatter *et al.,* 1987; Schmid and Issacson 1992; Naresh *et al.,* 2006), others have stated that the use of plastic embedding offers superior morphology (Burkhardt, 1971; Green, 1970; Islam and Frisch, 1985; Frisch and Bartl, 1990; Blythe *et al.,* 1997; Jack *et al.,* 1993). However, a major disadvantage of many plastic embedding procedures as has been discussed is the restriction of not being able to routinely perform IHC, but this author has shown that by using MMA in a specified procedure, high quality IHC can be achieved. As a result of combining plastic embedding with IHC, Jack *et al.,* (1993) stated that in their opinion the use of MMA was the method of choice for embedding bone marrow trephine biopsies.

However, it is acknowledged that the use of MMA has also been used by others for IHC studies (Rodrigues *et al.,* 1984; Schröder and Delling, 1986; Hahn *et al.,* 1991; Bernhards *et al.,* 1992; Wolf *et al.,* 1992; Erben, 1997). All have stated the advantages and merits of using MMA for IHC, but the procedures described were far more complicated and time-consuming than those advocated by this author and the range of antibodies investigated less comprehensive. When discussing the use of methacrylate embedding media for early EM studies Nunn (1970) reported the “polymerisation damage” that occurred to various tissue structures. Using the mild preparatory procedures described in this thesis with MMA, no obvious morphological abnormalities or defects have been noticed.

Producing MMA sections that adhere easily and readily to glass slides can be challenging, and in some procedures lengthy clamping of the sections has been suggested (Delling, 1972; Islam and Frisch, 1985; Schröder and Delling, 1986;

Hahn *et al.,* 1991; Erben, 1997) often using gelatinised slides. In addition, Seifert (personal communication) reported that when Haupt's gelatin/fixative was used, excessive background staining resulted. However, when using the MMA formulation described in the papers by Hand and colleagues, the sections which were easily flattened on a heated water-bath and subsequently picked up on slides remained attached, although the use of slides coated with APES was recommended (Hand *et al.,* 1996; Blythe *et al.,* 1997; Church *et al.,* 1997). Naturally some tissues e.g. undecalcified bone, presented additional challenges, but the routine use of the procedure for bone marrow trephine biopsies in the Haematological Malignancy Diagnostic Unit in Leeds is evidence that it can be reliably performed. The use of the readily available solvent xylene to deplasticise (sometimes called deacrylation) MMA sections has been found satisfactory, although other workers have suggested in conjunction with xylene the use of 1-acetoxy-2-methoxy-ethane (Yang *et al.,* 2003) or 2-methoxyacetate (Wittenburg *et al.,* 2009) when using the proprietary MMA kit Technovit 9100 New (Heraeus Kulzer). According to Seifert (personal communication) Technovit 9100 New requires the deplasticising solutions to be heated which results in producing wrinkled sections. In a study by Bernhards *et al.,* (1992) where a modified MMA was used, both acetone and methoxyethyl acetate were investigated as a solvent and though neither affected the reactivity after incubation for 6 and 12 hours of the enzyme chloroacetate esterase and various antigens investigated, patchy residential polymer was present after acetone alone.

In the studies by Hand, the ability also to mimic similar well-established immunohistochemical staining protocols has been advantageous and fortuitous

in being able to develop a technique applicable in a routine laboratory. Originally ABC procedures were used but later in line with modern practice, immunological polymer detections systems for the localisation of antigens have been successfully employed (D. Blythe, personal communication). The introduction of heat mediated procedures for formalin-fixed plastic sections, which other authors did not consider, has certainly assisted in extending the repertoire of antibodies (except CD64; D. Blythe, personal communication) and improving the quality of staining achieved on MMA-embedded tissue. However, more recent studies by Howat *et al.,* (2005) and Howat and Wilson (2010) have used heat mediated antigen retrieval procedures on formalin-fixed tissue microarrays (TMAs) embedded in GMA. Good immunostaining was achieved on both formalin fixed and acetone-fixed material (no antigen retrieval required) and represented the first application of plastic embedding for TMA. For MMA-embedded tissue, Blythe (personal communication) now routinely uses 1% Vector high pH unmasking solution (Vector Laboratories, UK) heated for 6 mins in a pressure cooker at 15 psi.

### 11.1.5 In Situ Hybridisation

The results that Hand and colleagues achieved with IHC on MMA-embedded tissue prompted investigations into whether ISH was applicable. Based on previous studies by co-workers using paraffin sections (Uwanogho *et al.,* 1998), it was shown that *Sox* genes 11 and 21 could be demonstrated in chick tissue embedded in MMA. Previously MMA had only been reported for isotopic ISH (Jamrich *et al.,* 1984). In the studies by Church *et al.,* (1997) it was shown that the choice of accelerator used to polymerise the plastic mix and pretreatment

used prior to ISH could affect staining. These were important findings which could impact significantly if diagnostic investigations are required. In a subsequent paper, simultaneous ISH and IHC were demonstrated on the same MMA section (Church *et al.,* 1998), when the best results were achieved using the same pretreatment and accelerator as previously used. Consequently, it would appear that generally both pressure cooking and the accelerator DMSX offer the potential to produce the best staining for ISH and IHC on MMA-embedded tissue.

### 11.1.6 Development of Designer Plastics

In chapter 3, it was described how acrylic plastics evolved from being initially used for EM to extensive use for LM studies. As the properties and characteristics that were outlined in chapter 4 became better understood, leading to a plethora of publications describing various applications for tinctorial, enzyme and immunohistochemical techniques, so too was there a realisation that acrylics could be formulated to numerous permutations that could reflect specific characteristics. From this, it is plausible that a plastic could be made which might be more appropriate for a particular function or requirement. In this thesis, this knowledge has been capitalised to formulate either an improved plastic e.g. for metabolic bone studies (Hand, 1996) or new modifications for specific IHC and ISH techniques (Hand *et al.,* 1988; Hand and Morrell, 1989; Blythe *et al.,* 1997; Church *et al.,* 1997; Hand and Church, 1997; Church *et al.,* 1998). Previously for EM studies, Causton, *et al.,* (1980), Carlemalm *et al.,* (1982) and Scala *et al.,* (1992) have presented descriptions and reasoning relating to the formulation of LR White, Lowicryl plastics and Bioacryl (Unicryl) respectively. These

publications which provided a valuable insight into polymer chemistry and their formation have assisted this author in initiating concepts for IHC studies.

The fundamental process in plastic embedding is to convert a monomer into a polymer by the process of polymerisation. This can be induced by incorporating a catalyst which reacts with an agent such as heat, a chemical accelerator or light. However, for a plastic to be a suitable embedding medium for histological investigations, many other features are required that include the ability to allow tissue to become properly processed, or the ability to produce a plastic of suitable hardness for the tissue, or the ability to produce suitable sections, or the preservation of tissue structures and substances, or the stability of the plastic against either reagents and/or an electron beam, or the ability to be able to perform histological staining techniques, or the ability to produce stable blocks within an appropriate time scale. In more recent times other issues such as the colour of the plastic and the toxicity of the reagents have also become important and relevant (Gerrits and Smid, 1983; Gerrits *et al.,* 1991).

The plastic mixes suggested by Hand for embedding undecalcified bone with PMMA (Hand, 1996) and those for IHC/ISH studies (Hand *et al.,* 1989, Church *et al.,* 1997) incorporated the plasticiser dibutyl phthalate. Another way to produce a softer block would have been to include in the mix a softer monomer such as butyl methacrylate, but this author considered that the co-polymer could adversely affect tissue structures more and/or create additional problems during polymerisation. For semithin sections of bone marrow trephine biopsies where IHC studies may be required, Blythe (personal communication) has since

increased the MMA content from 15ml per 20 ml aliquot to 17 ml per 22 ml aliquot. The MMA monomer contains 0.01% (100 parts per million) of the inhibitor hydroquinone monomethyl ether.

A range of accelerators have been used for GMA systems which have included the use of pyridine (Ruddell, 1971), although Green (1977) found this unsatisfactory. Gerrits *et al.,* (1991) assessed various plasticisers and accelerators with the emphasis on producing a low toxicity GMA embedding medium for various histological and histochemical staining procedures. The accelerators examined by Gerrits *et al.,* (1991) were DMA, DMPT, DMSX and DMBA, which the author of this thesis along with colleagues have also utilised and assessed in the development of specialised MMA mixes. In preliminary (unpublished) studies by Hand, DMBA was found to be unsatisfactory with MMA, which also agreed with similar conclusions that Gerrits *et al.,* (1991) found with GMA. However, both DMA and later DMSX have been shown to be excellent accelerators in a MMA system, where the plastic was polymerised similarly to GMA, but also had the significant advantage of being dissolved. This proved to be extremely useful and beneficial especially for IHC and ISH studies, and the use of these accelerators has been a major factor in designing a suitable plastic practical for these purposes. Also, the studies by Church *et al.,* (1997), Hand and Church, (1997) and Church *et al.,* (1998) have shown that improved staining could be achieved using DMSX.

According to Casey and Beckstead (1990) Technovit 7100 (Historesin) did not perform consistently for IHC. However, in the development of various GMA

media, different formulations have been suggested in the literature to improve immunocytochemical staining such as the replacement of the plasticiser 2-butoxyethanol with butandiol monoacrylate (van Goor *et al.,* 1988), and the introduction of ImmunoBed and Technovit 8100 by Polysciences Inc. and Heraeus Kulzer respectively. For Technovit 8100, known as RES G20 during development, (Gerrits *et al.,* 1991) this was evident from several photomicrographs in a later publication (van Goor, 1993; van Pelt-Verkuil, 1995).

The use of 5% dibenzoyl peroxide has in the author's experience also produced consistent and reliable immunostaining on MMA-embedded tissue, prompting initial thoughts about whether this high concentration of catalyst provided a similar protection to tissue antigens as the inclusion of methyl benzoate during processing with GMA as suggested by Casey *et al.,* (1988). As Blythe (personal communication) currently only adds the catalyst to the embedding MMA mix, this would appear not to be a correct hypothesis. The high concentration of dibenzoyl peroxide has been retained as lower concentrations had in preliminary unpublished studies by the author indicated variable immunostaining.

A major disadvantage of many of the ingredients in plastic embedding media relates to the safety and toxicity of the reagents. In some cases, especially kits of unknown composition, this can present problems, and certainly the chemicals employed in the production of MMA blocks used in the studies by this author are no exception. MMA monomer has a strong pungent odour and is flammable, and dibenzoyl peroxide is an irritant that is potentially explosive, although the non-explosive Perkadox 16 has been used in MMA systems (Buijs and Arend, 1983).

Gerrits *et al.,* (1990) investigated several plasticisers used in GMA embedding and described a numerical procedure for selecting a low toxicity reagent based on a range of physical and chemical parameters. The plasticiser dibutyl phthalate is subject to legislative control by both the European Union and United States with the latter adding the chemical to the California Proposition 65 (1986) list of suspected teratogens in November 2006 (http://oehha.ca.gov/prop65.html). Hard and soft grades of Acrylosin contain 3% and 10% w/v of dibutyl phthalate (J. Ratliff, personal communication). It is also well known that amines are toxic and based on the hydrophobicity of a compound (Dearfield *et al.,* 1989), it has been suggested that DMA would be the most toxic and DMSX would be the least (Gerrits *et al.,* 1991). It is thought therefore that DMSX should be used in the future with MMA, especially as the results with Technovit 8100 and those presented in this thesis, also indicate superior immunostaining than DMA.

The use of an accelerator provides control over the rate of polymerisation and in conjunction with the concentration of catalyst dibenzoyl peroxide, an effective and practical time to achieve curing can be established. In the author's experience, approximately two hours is suitable as a decreased time has resulted in inadequately processed tissue. Also if polymerisation is too rapid, there is a likelihood that the temperature produced during the exothermic reaction will increase to an undesirable level. Several studies have been published to illustrate the conditions and factors which influence this (Gerrits and van Leeuwen, 1985; Hand, 1988b; Bernhards *et al.,* 1992; Gerrits *et al.,* 1991), and Spijker (1978) described a specially designed mould when using MMA, but unlike many GMA studies when either enzyme histochemistry was required

and/or producing a loose plastic matrix for IHC was important, neither were significant in the studies by Hand when the plastic was removed prior to staining. This author has over many years been challenged about whether deplasticised sections are indeed really plastic sections, but he has argued that this is a matter of perception, as paraffin wax sections which also require the removal of the embedding medium are universally called “paraffin sections” or “wax sections.”

In the paper by Gerrits *et al.,* (1991), the hardness of GMA blocks was also considered where it was suggested that the ability of Technovit 7100 (Historesin) to produce ribbons of sections was due to the softer surface layer of this plastic. A further consideration is the discolouration of polymerised blocks, which was influenced in GMA blocks by the choice of accelerator used (Gerrits *et al.,* 1991) with DMA producing a yellowish colour which developed in to a deep brown after a short period exposed to daylight. The other accelerators investigated (DMBA, DMPT and DMSX) also produced a light yellow which darkened to deeper shade, with DMSX the palest. This author has found similar colouration produced with MMA but the high concentration of dibenzoyl peroxide did produce further intensification. Both Technovit 7100 which ultilises a non-amine accelerator and JB-4 Plus produce clear or pale yellow blocks respectively.

### 11.1.7 Diagnostic Uses of Plastic Embedding

This thesis has provided details on how plastics have evolved as embedding media for the use of histological techniques. The properties of acrylics allow many LM techniques to be performed, and these are therefore the plastics of choice for this purpose. Though dual purpose plastics such as the Lowicryls,

Bioacryl and LR Gold/White can in some instances be used also for EM, their formulation is more restrictive than some other acrylics for many LM techniques. Incorporation of a crosslinking agent for electron beam stability, not unexpectedly reduces usage for certain procedures, although over the years (as it has been reported in this thesis) numerous attempts have been published where a methodology has been contrived that could produce results. Unfortunately many of these methods are far too complex, time-consuming and unreliable for widespread application in a routine diagnostic laboratory.

For over 50 years, undecalcified bone has been embedded in a plastic to enable histological assessment of metabolic bone diseases. This has proved a valuable procedure, but as discussed earlier in this thesis, many of the techniques published were cumbersome, prompting this author to consider refinement. As a result numerous modifications were introduced to streamline an existing method so that it was more applicable for a diagnostic service. In this environment, TAT and reduced costs through greater efficiency, in addition to high quality, were constant drivers and consequently publication of an improved technique was justified. The paper by Hand (1996) reported its use on a large number of cases (10,000) and obviously this figure has increased in the past 17 years, although nowadays assessment and diagnosis of bone for metabolic diseases is performed by bone densitometry.

The introduction of GMA in the early 1960s for originally EM (Rosenberg *et al.,* 1960; Wicherle *et al.,* 1960*)* and later for LM (Ashford and Feder, 1966) provided an embedding procedure where semithin tissue sections could be easily

produced and stained with many existing tinctorial techniques. At the time, the concept of being able to deliver semithin sections with improved morphology seemed an attractive option, especially as high resolution was considered increasingly useful for diagnosis (Burns, 1973; Chi and Smuckler, 1976; Ferrell and Beckstead, 1990). In addition, the recognition that GMA could be polymerised at low temperature and potentially provide a procedure that could allow enzyme histochemistry to be performed was another significant attractive advantage (Litwin, 1985).

In 1967 the papers by Ruddell (1967a, 1967b) on improved formulations of GMA mixes were a major stimulus for high resolution plastic embedding, and soon proprietary GMA kits and specialised equipment started to become available. Later other acrylic plastics were developed, either for LM or EM alone or in combination. Until this time, equipment was limited to what was then available for handling paraffin and epoxy (EM) blocks, which was illustrated by articles published describing how existing equipment could be used or improvised to produce plastic blocks and/or cut semithin sections (Cole and Sykes, 1974; Murgatroyd, 1976; Green, 1977; Semba, 1979) or offer a cheaper alternative (Janes, 1980). The introduction of a motorised microtome such as the Reichert-Jung Autocut, glass knife makers for triangular (Latta-Hartmann) and extended edge (Ralph) knives, moulds and block holders, together with packaged embedding kits containing all the reagents necessary to produce plastic blocks stimulated interest and provided a ready and easy means of implementing a plastic embedding service. Soon a new age of plastic embedding was widespread as many laboratories adopted such practices to supplement their

core paraffin work (Cole and Sykes, 1974). Bennett *et al.,* (1976) encapsulated the mood of the time with their detailed paper describing the preparation of sections of tissue embedded in GMA. Subsequently many details relating to the principles and trouble-shooting of using GMA were reviewed by Gerrits and Horobin (1996) with further advice about its use in diagnostic pathology (Gerrits and Suurmeijer, 1991).

Many tinctorial staining techniques could be applied to GMA-embedded tissue with some only requiring slight modifications (Burns and Bretschneider, 1981), although other procedures such as silver impregnation for reticulin and trichromes proved more problematic (Geritts, 1981). Frozen sections were still predominantly used for routine enzyme histochemical staining, but plastic sections were suggested for diagnostic applications (Beckstead and Bainton, 1980; Beckstead *et al.,* 1981; Beckstead, 1983; Soufleris, *et al.,* 1983; Islam and Henderson, 1987; Murray and Ewen, 1991), and also employed by some for specialised research activities e.g. Burnett, (1982). As an alternative to GMA, the polyhydroxy aromatic dimethacrylate plastics Histocryl, LR White and LR Gold were introduced by the London Resin company, with the latter two also suitable for EM. However, as demand for molecular techniques that could be applied to paraffin sections began increasing, due to their diagnostic importance, it was a natural progression that similar techniques would also be wanted on plastic sections (Beckstead *et al.,* 1986; Casey *et al.,* 1990). As has been shown in this thesis, this presented formidable difficulties to many workers, and prompted some to suggest that plastic embedding should be abandoned in favour of paraffin wax either because plastic embedding was an unnecessary

departure from normal routine paraffin processing since special equipment, different preparatory protocols and technical expertise were required, or because the difficulties encountered in performing IHC proved too unattractive to consider using (Gatter *et al.,* 1987; Vincic *et al.,* 1989; Vincic, 1990). Even though from time-to-time the merits of superior morphology were publicised, especially for bone, (Gatter *et al.,* 1987; Murray and Ewen, 1989a; Kingsley and King, 1989; Schmid and Issacson, 1992) it seemed that the current decline of plastic embedding was inevitable. It is however, interesting to note that in an effort to overcome these difficulties, Islam (1987) suggested that bone marrow cores should be divided so that one half could be processed into MMA "for optimal morphology" and the other half into GMA "for enzyme or immunohistology." Other important considerations for the decline in plastic embedding are likely to have been that the quality of paraffin waxes and microtomes have improved considerably during the last 40 years to the point that now 2 and 3 μm semithin sections are able to be reliably cut, which previously was much more challenging.

In a unique study by Hillmann *et al.,* (1991), staining for enzyme, lectin and immunohistochemistry was performed on undecalcified bone and tooth embedded in the (one component) plastic Technovit 7200 (Heraeus Kulzer) based on isobornyl methacrylate that was polymerised with blue light. Ground sections of 10 μm were produced and successful staining achieved. (Further technical information about the component is unavailable).

This author has shown that by using a new approach where the plastic was removed prior to staining, both excellent tissue morphology and the ability to

perform molecular staining techniques were possible. A plastic based on MMA was produced using reagents, equipment and rationale that in many ways resembled GMA procedures, but whereas the latter formed cross-links during polymerisation that prevented it from being dissolved, the use of MMA in a simple formulation as suggested by Hand provided an alternative and practical technique for high resolution where the plastic could be removed from the section. Recognition of the advantages of MMA and the feasibility of integrating the technique in to a diagnostic laboratory led to its adoption by some laboratories including the Haematological Malignancy Diagnostic Service in Leeds, UK. Since the start of this service in 1991, approximately 30,000 bone marrow trephine samples up to 2013 have been processed (currently approximately 7,000 blocks per annum; D. Blythe, personal communication), which their consultant medical staff have advocated is the method of choice for the histopathological reporting of these biopsies in the management and therapy of patients with lymphoma and leukaemia. The same procedure has also been extensively used since 1992 at Aberdeen Royal Infirmary and currently (2013) processes approximately 370 bone marrow trephine biopsies per annum (L. Doverty, personal communication). For a plastic to be useful medium for embedding tissue for diagnosis, it is imperative nowadays that IHC can be reliably performed, which the studies by this author referred to in this thesis and the continued routine use of the procedure are testimony to its feasibility and practicality. Although other diagnostic applications for plastic embedding have been suggested by Hand and Church (1997), Randall and Foster (2007) and Doverty (corneal biopsies and epiretinal membranes, personal communication), it is however probably for undecalcified bone marrow trephine biopsies, where

MMA is most beneficial and useful.

### 11.1.8 Impact of Studies

The existence of paraffin wax as a routine embedding medium for tissue has remained a widely used and accepted universal practice. However, it was realised 40 years ago, that severe deficiencies of wax existed at that time in being both unsuitable for providing semithin sections and inadequate for supporting hard tissue such as undecalcified bone (Green, 1970; Burkhardt, *et al.;* 1971). The introduction in the 1950s and 1960s of plastic embedding for light microscopy studies, which combined thin sectioning of initially epoxy plastics (Burns, 1973) and later with acrylics, was an attempt to resolve these issues, and for many years the associated procedures, especially for acrylics were topical and frequently used for a variety of studies (Te Velde *et al.,* 1977; Ferrell and Beckstead, 1990; Randall and Foster, 2007; Callis, 2008; Singhrao *et al.,* 2012). Many studies focussed on using plastic embedding for undecalcified bone marrow cores e.g. Islam and Henderson, (1987); Islam *et al.,* (1988); Burgio *et al.* (1991). So attractive was the versatility and results that could be achieved with acrylic plastics, that some suggested that plastic embedding would be the way forward and could perhaps even replace paraffin wax (Murray, 1988; Murray and Ewen, 1989). The frequent publications that soon became available was an indication of the euphoria of an alternative embedding medium to paraffin wax after 100 years.

In reality it has transpired that the use of plastic sections in histological practice has declined. For the assessment and diagnosis of metabolic bone diseases,

non-histological procedures using bone densitometry are instead nowadays performed, although the studies by Hand (1996) have still proved a useful reference in several later studies such as that on murine bone specimens (Kacena *et al.,* 2004 and 2006). The usefulness of providing thinner sections has led to the introduction of proprietary waxes that have been reinforced by the inclusion of polymers to enable quality semithin sections to be produced. As a result, for the examination of leukaemia and other non-metabolic bone diseases in bone marrow trephine biopsies, it has become a routine practice to now use decalcified samples embedded in wax (Naresh *et al.,* 2006). Also as outlined previously, there were significant problems in being able to perform the essential requirement of IHC on plastic sections, and this undoubtedly had a major impact on many scientists to abandon plastic embedding (Vincic *et al.,* 1989; Vincic, 1990). Other factors that have contributed to a decrease in the use of plastic embedding have included the health and safety concerns of specific reagents, the need for specialised equipment and skilled staff, along with higher costs (Vincic *et al.,* 1989). However, the fact that some of the techniques developed and pioneered by this author are still being extensively used, indicate the advantages and successes of procedures that some still believe plastic embedding have for certain histological examinations (Jack *et al.,* 1993). In addition, several recent publications have either referred to or ultilised procedures previously reported in publications by Hand. For example, the paper by Blythe *et al.,* (1997) has been cited numerous times including in recent studies by Naresh *et al.,* (2006); Torgersen *et al.,* (2009); Yamashita, (2007). Other authors including Gerrits *et al.,* (1991); Gerrits and Horobin, (1996); Buesa,

(2005); Zhang *et al.,* (2010); Leong *et al.,* (2011) have also referred to and acknowledged the impact of studies by Hand.

For certain research projects or for when there are stents / implants, plastic embedding is still a useful technique. For example, Seifert *et al., (2001)* and Baker *et al.,* (2009) have utilised plastic embedding studies that have been assisted from publications by this author. In further studies by Howat *et al.,* (2005); Randall and Foster, (2007) and Howat and Wilson, (2010), antigen retrieval procedures (Hand *et al.,* 1996; Hand and Church, 1997) were used to assist diagnosis and the application of specific techniques. From an original idea conceived from previous experience and knowledge, a technique was developed that has been reliably used for the routine diagnosis of thousands of bone biopsies, which has recently been adapted for use on automated immunostainers, although the removal of plastic and antigen retrieval are required to be manually performed (D. Blythe, personal communication). Since the paper by Blythe *et al.,* (1997) several further developments and modifications have been introduced for bone marrow trephine biopsies (some of which have been mentioned in this thesis) including changes to the plastic mix, antigen retrieval protocols, immunological detection technique and an expansion of the repertoire of antibodies applied. There are bound to be further advancements and developments in the future, but it is hoped that those introduced by this author and collectively described in this thesis will continue to impact on future histological studies.

### 11.1.9 Plastic Embedding in the Future

Diagnostic histopathology has undergone significance change within the last 40 years with the range of diagnostic tests performed on paraffin wax sections greatly extended, which has been supplemented by considerable immunohistochemical investigations. In addition, there is a far greater emphasis on throughput with cost and TAT now increasingly important considerations. The use of plastic embedding is seldom used, although the associated reagent costs for MMA are low and TAT for plastic embedding and staining of bone marrow trephine biopsies comparable to paraffin sections as the extra time for processing and polymerisation is offset by the absence of decalcification. Proprietary MMA embedding kits however, such as Technovit 9100 New (Heraeus Kulzer) are more expensive than buying the components and the preparatory procedures advocated are more complicated than those described by Hand. The inclusion of PMMA is also not ideal for small trephine samples or soft tissue (P. Seifert, personal communication). For optimal interpretation of bone marrow trephine specimens, Naresh *et al.,* (2006) stated that the ability to extract DNA of a quality sufficient to carry out polymerase chain reaction (PCR)-based analyses was crucial. Though Krenacs *et al.,* (2004) was successful with the epoxy plastic Durcupan, limited investigations by Blythe on MMA-embedded material have so far been unsuccessful (personal communication).

For routine histological diagnosis, the use of paraffin wax sections rightly remains widely used, and as such is still the universal embedding medium even after 150 years. In recent years improvements in the quality of histological waxes have enabled semithin sections to be reliably cut, e.g. in spring of 2013, IMEB

(International Medical Equipment) Inc. will launch their new wax “paraffin 53” which it is claimed has increased hardness that will allow sectioning down to 1.5 μm. The improved waxes available, together with the difficulties in performing molecular techniques on acrylic sections experienced by many workers, and the health and safety concerns of some reagents required has probably been mainly responsible for the decline in the use of plastic embedding. Many of the reagents in plastic embedding media to produce blocks are unpleasant and/or harmful, so it is imperative that safety advice is followed at all times, and they are handled in a fume cupboard. The purchasing of suitable new equipment including microtomes and glass knife makers, especially for Ralph knives, has become problematic because the decreased demand has led to some manufacturers stopping production of certain items. This illustrates clearly and strongly how market forces can influence what equipment workers use and perhaps ultimately what protocols and procedures are employed. The decline in interest and use in recent years is also reflected in the decreased number of papers published relating to plastic embedding, compared to 1970-80 when it seemed to dominate the literature in many histopathology journals.

No embedding medium exists that enables the application of all histological techniques, but this thesis has shown that a wide variety of techniques are possible on acrylic plastic sections, although the choice of plastic can critically influence which techniques are applicable. For the majority of histological investigations paraffin wax is a suitable embedding medium, but there are several recent specialised areas where plastic embedding either for diagnostic or research purposes may be necessary and/or beneficial (Yang *et al.,* 2003;

Singhrao *et al.,* 2009; Singhrao *et al.,* 2010; Singhrao *et al.,* 2012; Wittenberg *et al.,* 2009). Several of these papers reported the application of immunohistochemistry and ultilised Technovit 9100 New (Heraeus Kulzer). For many years the use of plastic embedding with GMA has been advocated for the examination of bone marrow trephine biopsies, but the virtual inability to be able to perform immunohistochemical techniques limited its use. This thesis has focussed on the development of IHC and ISH techniques by this author using MMA which have enabled the extensive use of molecular procedures on bone marrow trephine biopsies. Although it would seem that paraffin wax is still rightfully the universal embedding medium in histopathology for most routine investigations, it is hoped that the increased knowledge and greater understanding of these plastic embedding procedures will continue to be of use and benefit to other studies where plastic embedding is required.

# APPENDIX

## Complete List of Publications by Hand Relating to Plastic Embedding

## (Chronological Order)


**1.** HAND, N.M. (1986) An alternative approach to the diagnosis of malabsorption syndrome. *Medical Laboratory Sciences.* **43:**Suppl 1, S7.

**2.** HAND, N.M. (1987) Enzyme histochemistry on jejunal tissue embedded in resin. *Journal of Clinical Pathology.* **40:**346-347. (ISSN 1472-4146)

**3.** HAND, N.M. (1988a) *A Study of the Use of Hydrophilic Resins in Diagnostic Histopathology.* M.Phil Thesis: Nottingham University.

**4**. HAND, N.M. (1988b). Enzyme histochemical demonstration of lactase and sucrase activity in resin sections: the influence of fixation and processing. *Medical Laboratory Sciences.* **45:**125-30. (ISSN 0308-3616)

**5**. HAND, N,M., MORRELL K.J. and MacLENNAN KA. (1989). Immunohistochemistry on resin embedded tissue for light microscopy: a novel post-embedding procedure. *Proceedings of the Royal Microscopical Society.* January **24:**A54-A55.

**6.** HAND, N,M. and MORRELL K.J. (1990) Immunocytochemistry on plastic sections for light microscopy: a new technique. *Proceedings of the Royal Microscopical Society.* March 1990;**25:**111.

**7**. JOHNS, L., HAND, N.M., FISH, D.C.W. and MILLER, K.D. (1992) Immunocytochemistry on methyl methacrylate embedded tissue. *Journal of Pathology.* **167:**suppl 154.

**8.** HAND, N.M. (1993a) Misunderstandings about methyl methacrylate. *Journal of Clinical Pathology.* (letter), **46:**285.

**9.** HAND, N.M. (1993b) Immunocytochemistry on resin sections for light microscopy. British Journal of Biomedical Science. **50:**162-163.

**10.** HAND, N.M. Diagnostic immunocytochemistry on resin-embedded tissue. UK NEQAS Newsletter. 1995a;**6:**13-16. (No ISSN)

**11.** HAND, NM. (1995b) The naming and types of acrylic resins. UK NEQAS Newsletter. **6:**15 (letter).

**12.** BLYTHE, D., JACKSON, P. and HAND N.M. (1996). Heat-mediated pre-treatment procedures for immunocytochemical staining on resin sections. *British Journal of Biomedical Science.* **53**:6.

**13.** HAND, N.M., JACKSON, P. and BLYTHE, D. (1996) Methyl methacrylate: is it a suitable embedding medium for immunocytochemical studies? *British Journal of Biomedical Science.* **53**:12.

**14.** HAND, N.M., BLYTHE, D. and JACKSON, P. (1996) Antigen unmasking using microwave heating on formalin fixed tissue embedded in methyl methacrylate. *Journal of Cellular Pathology.* **1:**31-37. (ISSN 1359-7388)

**15.** HAND, N.M. (1996) Embedding undecalcified bone in polymethyl methacrylate: an improved method. *British Journal of Biomedical Science.* **53:**238-240. (ISSN 0967-4845)

**16**. BLYTHE, D. HAND, N.M., JACKSON, P., BARRANS, S.L., BRADBURY, R.D. and JACK, A.S. (1997) The use of methyl methacrylate resin for embedding bone marrow trephine biopsies. *Journal of Clinical Pathology.* **50:**45-49. (ISSN 1472-4146)

**17.** HAND, N.M. and CHURCH R.J. (1997) Immunocytochemical demonstration of hormones in pancreatic and pituitary tissue embedded in methyl methacrylate. Journal of Histotechnology**20:**35-38. (ISSN 0147-8885)

**18.** CHURCH, R.J., HAND, N.M., REX ,M. and SCOTTING, P.J. (1997) Non-isotopic in situ hybridization to detect chick Sox gene mRNA in plastic embedded tissue. The Histochemical Journal. **29:**625-629. (ISSN 0018-2214)

**19.** CHURCH, R.J., HAND, N.M., REX, M. and SCOTTING P.J. (1998) Double labelling using non-isotopic in situ hybridisation and immunohistochemistry on plastic embedded tissue. *Journal of Cellular Pathology.* **3:**11-16. (ISSN 1359-7388)

**20.** HAND, N.M. and CHURCH, R.J. (1998) Superheating using pressure cooking: its use and application in unmasking antigens embedded in methyl methacrylate. *Journal of Histotechnology.* **21:**231-236. (ISSN 0147-8885)

**21.** HAND, N.M. (1999) *Plastic embedding for light microscopy: a guide for the histotechnologist.* ASCP Sample Tech teaching programme. Histotechnology No. HT-6, 29-35. (ISSN 1056-6678)

**22.** HAND, N.M. Plastic Embedding Media and Techniques. (2001) In: Bancroft, J.D. and Gamble, M. (eds.) *Theory and Practice of Histological Techniques*. 5th edn, London: Harcourt Health Sciences. (ISBN 0-443-06435-0)

**23.** HAND, N.M. Plastic Embedding Techniques for Light Microscopy. (2008) In: Bancroft, J.D. and Gamble, M. (eds.) *Theory and Practice of Histological Techniques*. 6th edn Oxford: Churchill Livingstone, Elsevier. (ISBN 978-0-443-10279-0)

**24.** HAND, N.M. Plastic Embedding Techniques for Light Microscopy. (2013) In: *Bancroft's Theory and Practice of Histological Techniques.* (eds.) Suvarna, Layton, and Bancroft, J.D. 7th edn Oxford:Churchill Livingstone, Elsevier.

## List of Publications Submitted (Chronological Order)


**1.** Hand, NM. (1987) Enzyme histochemistry on jejunal tissue embedded in resin. *Journal of Clinical Pathology.* **40:**346-47.

**2.** Hand, NM. (1988a) Enzyme histochemical demonstration of lactase and sucrase activity in resin sections: the influence of fixation and processing. *Medical Laboratory Sciences.* **45:**125-30.

**3.** Hand, NM. (1996) Embedding undecalcified bone in polymethyl methacrylate: an improved method. *British Journal of Biomedical Science.* **53:**238-240.

**4.** Hand, NM. Blythe, D. and Jackson, P. (1996) Antigen unmasking using microwave heating on formalin fixed tissue embedded in methyl methacrylate. *Journal of Cellular Pathology*. **1:**31-37.

**5.** Blythe, D. Hand, NM. Jackson, P. Barrans, SL. Bradbury, RD. and Jack, AS. (1997) The use of methyl methacrylate resin for embedding bone marrow trephine biopsies. *Journal of Clinical Pathology.* **50:**45-49.

**6.** Hand, NM. and Church, RJ. (1997) Immunocytochemical demonstration of hormones in pancreatic and pituitary tissue embedded in methyl methacrylate. *The Journal of Histotechnology.* **20:**35-38.

**7.** Church, RJ. Hand, NM. Rex, M. and Scotting, PJ. (1997) Non-isotopic *in situ* hybridization to detect chick Sox gene mRNA in plastic embedded tissue. *Histochemical Journal.* **29:**625-629.

**8.** Hand, NM. and Church, RJ. (1998) Superheating using pressure cooking: its use and application in unmasking antigens embedded in methyl methacrylate. *The Journal of Histotechnology.* **21:**231-236.

**9.** Church, RJ. Hand, NM. Rex, M. and Scotting, PJ. (1998) Double labelling using non-isotopic *in situ* hybridisation and immunohistochemistry on plastic embedded tissue. *Journal of Cellular Pathology.* **3:**11-16. Eratum **3:**84.

**Manuscript of Publications Submitted**

Note: Article contents are not included in this electronic version.

Hand, NM. (1987) Enzyme histochemistry on jejunal tissue embedded in resin. *Journal of Clinical Pathology.* **40:**346-47.

Hand, NM. (1988a) Enzyme histochemical demonstration of lactase and sucrase activity in resin sections: the influence of fixation and processing. *Medical Laboratory Sciences.* **45:**125-30.

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Hand, NM. and Church, RJ. (1997) Immunocytochemical demonstration of hormones in pancreatic and pituitary tissue embedded in methyl methacrylate. *The Journal of Histotechnology.* **20:**35-38.

Church, RJ. Hand, NM. Rex, M. and Scotting, PJ. (1997) Non-isotopic *in situ* hybridization to detect chick Sox gene mRNA in plastic embedded tissue. *Histochemical Journal.* **29:**625-629.

Hand, NM. and Church, RJ. (1998) Superheating using pressure cooking: its use and application in unmasking antigens embedded in methyl methacrylate. *The Journal of Histotechnology.* **21:**231-236.

Church, RJ. Hand, NM. Rex, M. and Scotting, PJ. (1998) Double labelling using non-isotopic *in situ* hybridisation and immunohistochemistry on plastic embedded tissue. *Journal of Cellular Pathology.* **3:**11-16. Eratum **3:**84.

## Dissemination of the Research: Lectures, Seminars, Courses, Conferences and Posters Presented that Included Plastic Embedding

### Local

- Departmental lecture on enzyme histochemistry (Queen's Medical Centre, Nottingham)
- Departmental lecture on immunohistochemistry (Queen's Medical Centre, Nottingham)
- Lecturer to EC students seconded to the Medical School (QMC, Nottingham)

### National

- Lecturer and technical adviser at LKB resin workshop (Leicester, 1984)
- Lecturer at East Anglia Histopathology Symposium (Chelmsford, 1988)
- Lecture presented at the Yorkshire Area Microscope User Group at Leeds University entitled "Immunohistochemistry on semi-thin resin sections for light microscopy". Prize winner, Royal Microscopical Society (Leeds, 1988)
- Presented paper at Royal Microscopical Society Histochemistry meeting (London, 1989)
- Speaker at Cellular Pathology Discussion Groups at Leeds, Cambridge, Nottingham, Preston, London and Leicester (1989/92)
- Lecturer at IGSS meetings at Leeds, Edinburgh and Cardiff (1989/90)
- Lecturer at Royal Microscopical Society Gold Label Users meeting (London, 1989)
- Lecturer at Resin Workshop (Edinburgh, 1989)
- Lecturer at Cellular Pathology symposium (York, 1990)
- Lecturer at South East Thames Cellular Pathology meeting (Maidstone, 1990)
- Lecturer at IMLS. Triennial conference (Liverpool, 1992).
- Lecturer at Industrial Histology conference (Llandudno, (1992)
- Lecturer at UK NEQAS Immunocytochemistry seminar (Leeds, 1994)
- Lecturer at IBMS Congress (Birmingham, 1995).
- Lecturer at 1st Biomen Cellular Pathology symposium (Oxford, 1995)
- Lecturer at Industrial Histology conference (Blackpool, 1996)

**International**

- Lecturer at National Society of Histotechnology (Albuquerque, New Mexico, 1996)
- Lecturer at National Society of Histotechnology (Columbus, Ohio, 1997)
- Lecturer at National Society of Histotechnology (Salt Lake City, Utah, 1998)
- Lecturer at National Society of Histotechnology (Providence, Rhode Island, 1999)
- Lecturer at National Society of Histotechnology (Charlotte, North Carolina, 2001)
- Lecturer at National Society of Histotechnology (Long Beach, California, 2002)
- Lecturer at National Society of Histotechnology (Louisville, Kentucky, 2003)
- Lecturer at National Histology Conference (Melbourne, Australia, 2006)
- Lecturer at National Society of Histotechnology (Vancouver, Canada, 2012)*

*(Unable to attend Vancouver due to illness)

**Poster**

- Poster presentation at IMLS Triennial conference (Southampton, 1986)
- Poster presentation at Pathological Society (London, 1992)
- Poster presentation at IBMS Congress (Birmingham, 1995)

**Teaching and CPD (Many required detailed handouts and/or abstracts).**

- Lecturer for MSc course on Advanced Immunocytochemistry (Trent University, Nottingham).
- Lecturer for CPD courses on Advanced Immunocytochemistry (Leeds, 1991-2005).
- Lecturer for CPD courses on Resin Embedding (Leeds, 1996).
- Lecturer for CPD courses on Microwave antigen retrieval in immunocyto-chemistry (Leeds, 1994-2001).

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