RECOVERY AND ANALYSIS OF DNA FROM FIXED TISSUE

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by

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Dedication

This thesis is dedicated to the memory of my father, Dr. Ernest Warford, who kindled my interest in science and demonstrated by example what is possible through diligent application.

Praise God, from whom all blessings flow;
Praise Him, all creatures here below;
Praise Him above, ye heavenly host;
Praise Father, Son, and Holy Ghost!

Thomas Ken, 1637-1710

Abstract

This research was undertaken to: 1) Develop techniques for the recovery of DNA from fixed and paraffin wax embedded tissues. 2) Assess the potential for use of this DNA for the diagnosis of lymphoma. 3) Contribute to the understanding of the effects of fixation on DNA.

Initial investigations used pure DNA and lymphocytes to determine the effect of five fixatives on the integrity, recovery and restriction endonuclease digestion of the nucleic acid. Two fixatives, Bouin and formol sublimate, proved unsuitable for further analysis. DNA recovery and Southern analysis was then attempted using Carnoy, formol saline and neutral buffered formalin fixed and paraffin wax embedded tissue. From this an optimised method featuring prolonged incubation of tissue with Proteinase K and SDS at 37°C followed by purification was developed. Within limits imposed by fixation this DNA was suitable for Southern analysis and amplification by PCR. A simplified DNA recovery method involving overnight incubation of paraffin wax sections in Proteinase K at 55°C without purification was also evaluated. This gave satisfactory results by PCR but the maximum product size obtained was 500 bp compared with 1250 bp using the optimised method.

DNA was better preserved after Carnoy than following formalin fixation and this was reflected in consistently superior Southern and PCR results. Formalin fixation induced degradation of DNA, which increased as fixation time was extended. This made Southern analysis for the identification of B and T cell rearrangements in lymphomas unreliable. However, the t(14;18) translocation was demonstrated successfully by PCR in formalin fixed follicular lymphomas.

The results of these investigations show the importance of the pH of fixatives on the preservation of DNA. They also suggest that chemical interactions with DNA and associated proteins occur using fixatives containing formalin and mercury.

Forward

This thesis describes research that was undertaken part-time whilst I was employed in the Department of Pathology at the University of Leicester. I was initially registered as a student with De Montfort University. My studies commenced in late 1986 at a time when Southern Blotting was the main technique used for the molecular analysis of DNA. Accordingly my initial research focused on developing methods to recover DNA from fixed and paraffin wax embedded tissue for subsequent Southern analysis. Part of this work was published in 1988 (Appendix). From that time until 1992 I continued to investigate refinements to the DNA recovery procedure, analysis of lymphoma samples and the effect of extended fixation on the quality of nucleic acid recovered. Again all of these studies culminated in Southern analysis. From 1992 until early 1997 I was unable to continue my studies due to new work commitments. During that period my supervisor left De Montfort University and my period of registration lapsed. In early 1997 I registered as a PhD student with Leicester University and undertook new studies to conclude the research by investigating the amplification of DNA recovered from fixed tissue using the polymerase chain reaction. I am now able to submit this thesis "Recovery and Analysis of DNA from Fixed Tissue".

Anthony Warford
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My thanks are also due to my wife Anne and children Dan and Becci who have shown great patience over the past years.

Abbreviations

3'	3 prime orientation of polynucleotide
5'	5 prime orientation of polynucleotide
A	Adenine base
B cell	B lymphocyte
B-5	Fixative containing mercuric chloride, formalin and sodium acetate
bp	Nucleotide base pair
C	Cytosine base
dAMP	Deoxyadenosine monophosphate
dATP	Deoxyadenosine triphosphate
dCTP	Cytidine triphosphate
dGMP	Deoxyguanosine monophosphate
dGTP	Deoxyguanosine triphosphate
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Mix of deoxynucleotides
dTTP	Deoxythymidine triphosphate
EDTA	Ethylenediaminetetraacetic acid
FP1	Forward primer 1
FP2	Forward primer 2
FP3	Forward primer 3
FP4	Forward primer 4
FP5	Forward primer 5
G	Guanine base
IMS	Industrial methylated spirits
IPA	Isopropyl alcohol
JH	Joining region of immunoglobulin heavy chain
kb	Kilobase
MAR	Matrix associated regions of chromatin loops
MBR	Major breakpoint region

MCS	Minor cluster sequence
N	Random nucleotide
NHL	Non-Hodgkin's lymphoma
NP	Nucleoprotein
OD	Optical density
PE	Buffer containing Tris, sodium pyrophosphate, SDS, bovine serum albumin, polyvinyl pyrolidone, ficoll and EDTA
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
RNase	Ribonuclease
RP	Reverse primer 1
RPMI	Rosewell Park Memorial Institute
RT	Room temperature
SAR	Scaffold attached regions of chromatin loops
SDS	Sodium dodecyl sulphate
SE	Buffer containing EDTA and sodium hydroxide
SSC	Standard saline citrate
T	Thymine base
Tβ	Beta chain of T lymphocyte antigen receptor
TAE	Buffer containing Tris, acetic acid and EDTA
T cell	T lymphocyte
TE	Buffer containing Tris and EDTA
Taq	DNA polymerase from *Thermus aquaticus*
Tm	Melt temperature of DNA
Topo II	Topoisomerase II
Tris	Tris (hydroxymethyl) aminomethane

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Chapter 1 Introduction

1.1 Objectives of the research

The demonstration of specific DNA sequences using extracted nucleic acid by Southern blotting and the polymerase chain reaction (PCR) is making an increasingly important contribution to diagnostic and research pathology, (Young,1992, Arends and Bird, 1992, Kawasaki, 1992 and Pan *et al,* 1995). These methods were originally described for use with DNA prepared from unfixed fresh or frozen cells and tissues (Southern, 1975 and Saiki *et al,* 1985). Effectively this limited applications to prospective diagnostic investigations involving blood, body fluids or especially collected tissue. In histopathology laboratories worldwide files of fixed and paraffin embedded tissues have been carefully maintained for many years. Potentially these storehouses could provide a valuable source of DNA for the retrospective analysis of disease. Furthermore, as the majority of surgical tissues are prepared in this way, the development of methods for DNA analysis using these preparations would be highly convenient.

In 1985 Goelz *et al* described a method for the extraction and Southern analysis of DNA from formalin fixed and paraffin wax embedded tissue. This was followed by other preliminary descriptions of methods to recover and analyse similar material (Dubeau *et al,* 1986). Two years after the description of the PCR technique its application to paraffin wax embedded tissues was described by Impraim *et al* (1987). Accordingly the potential of using DNA recovered from archived paraffin wax embedded tissue had been established.

The objectives of this research were to:

Optimise extraction techniques for the recovery of DNA from routinely fixed and paraffin wax embedded tissues.

Define conditions by which extracted DNA could be used by Southern blotting and PCR as an aid to the diagnosis of lymphoma.

Through the application of these molecular methods obtain information that would contribute to the understanding of the effects of chemical fixation on DNA.

1.2 The structure and organisation of DNA in the nucleus

As described by Watson and Crick (1953) DNA is a structure comprising nucleotide bases linked together by a sugar phosphate backbone and assembled via base pairing into a double helix. Base pairing is ordered with the purines adenine (A) and guanine (G) forming hydrogen bonds with, respectively, the pyrimidines thymine (T) and cytosine (C). The only known chemical modification, which occurs to the DNA bases, is methylation. This usually occurs on position 5 of cytosine and in animal cells about 5% of these bases are so modified, Van Holde (1989).

In vivo DNA is present in two main conformations. In the predominant B-form there is a 36° right hand helical twist between adjacent base pairs with an average of 10 nucleotides per full turn. In contrast the Z-form is a left handed helix that is irregular in outline and in which the backbone follows a zigzag path. This form is favoured when alternating C and G bases comprise the backbone and in situations where the former are methylated. A further right handed conformation, the A-form, which provides for a shorter and wider helix (36Å versus 19Å) than the B-form is found *in vivo* when DNA is paired with RNA. To form this conformation *in vitro* between complementary strands of DNA requires strong denaturing conditions that are unlikely to pertain *in vivo*. The formation of the double helix results in external major and minor groves that under physiological conditions are hydrated. The association of proteins with DNA and sequence dependent bending, such as that induced by AAAAANNNNN, can alter conformations significantly. Accordingly the forms of DNA as described should be considered as summary rather than definitive.

Hydrogen bonding allows the strands of the helix to be separated exposing the genetic code of the bases for replication of the DNA or transcription of RNA. This is achieved *in vivo* by enzyme complexes. In contrast to this sequence specific opening, transient 'breathing' of individual base pairs has been proposed as a continuous process by Frank-Kamenetskii

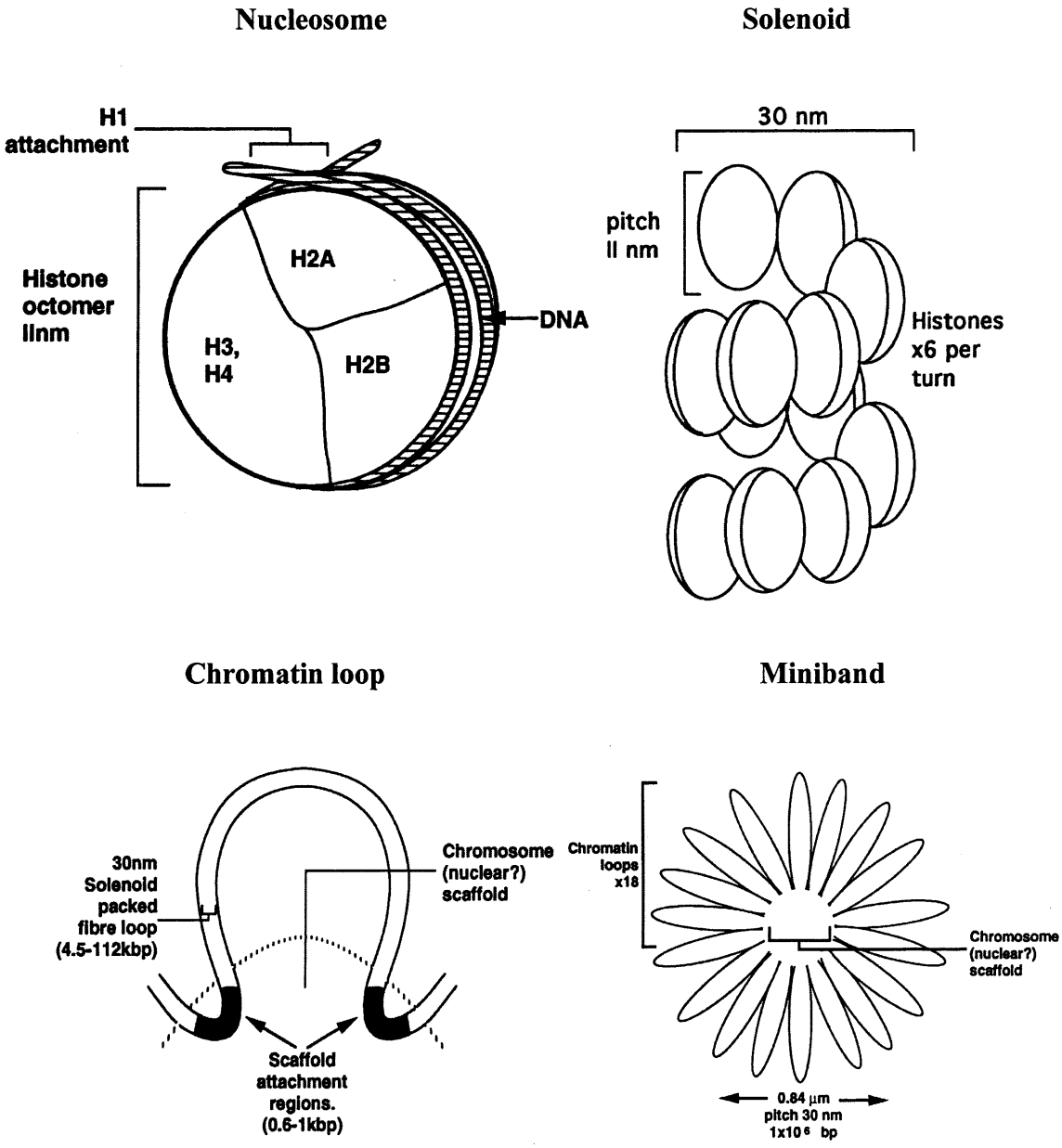
(1985). Evidence in support of this has been derived from theoretical and practical investigations using formaldehyde as a probe for opened DNA (Vologodskii and Frank-Kamenetskii, 1975, Lukashin *et al,* 1976) and from hydrogen exchange experiments (Guéron *et al,* 1987). In a commentary on the latter Frank-Kamenetskii (1987) gave an estimate of 10^{-7} sec for the lifetime of an individual opened base pair. Double helix opening due is also energetically favoured by the presence of inverted repeat sequences resulting in formation of cruciform structures (Van Holde, 1989).

In vitro increased heat and salt concentration, respectively, promote and antagonise base pair opening. Under conditions of low pH DNA is denatured then depolymerised. At pH 2.5 almost all hydrogen bonds are irreversibly disrupted due to the protonation of base amino groups (Geiduschek, 1958). This is followed by the hydrolysis of purines and slower hydrolysis of the phosphoester bonds of the nucleic acid backbone (Thomas and Doty, 1956). Low pH conditions will also remove histones from DNA. At pH 1.8 histone types 1 and 5 are removed whilst at pH 1 histone types 2B, 3 and 4 are dissociated from the nucleic acid (Holtzman, 1965, Darzynkiewicz *et al,* 1975). These conditions may become important in the preservation of DNA during fixation.

The DNA of the human genome has been estimated to consist of 3.6×10^9 haploid genome base pairs distributed over the 46 chromosomes present in the diploid cell. If these were joined together the total length would be about 3 metres. Clearly packaging is required to fit DNA into an average nucleus with a 5 μm diameter. This is achieved through the specific association of proteins with DNA, for which the term chromatin is used. A hierarchical order of packaging has emerged. This is illustrated in Fig 1.1 and described below. It has also been shown that the association of DNA with proteins is vital to the function of the nucleic acid.

The primary packaging unit of chromatin is the nucleosome, which was first described by Noll (1974). This results in a 30 to 40 fold compression of DNA length. The nucleosome consists of a flattened octomeric complex of core histones around which 147 bp of DNA is wrapped in 1.65 turns of a left-hand helix. The minor and major groves line up and form

Fig 1.1 Chromatin packaging



channels through which the N terminals of the histones protrude. Contiguous A or T residues are present at the ends of the nucleosomal DNA whilst dinucleotides of AA and TT present in the minor grove are directed towards the histones and GC dinucleotides are preferentially directed outwards (Dilworth and Dingwall, 1988). Four pairs of histones, H2A in combination with H2B and H3 in combination with H4 form the octamer. These histones are between 102 and 135 amino acids in length, have highly conserved sequences and are rich in lysine and arginine. Enzyme catalysed acetylation and deacetylation of the N terminal lysines is important for the regulation of gene activity (Grunstein, 1997). During transcription histones must disassociate from the surrounding DNA but evidence from formaldehyde histone H4/DNA binding investigations indicates that repositioning is local (Solomon *et al,* 1988). By electron microscopy nucleosomes are visualised as 'beads on a string' with the, micrococcal nuclease sensitive, intervening nucleic acid referred to as linker DNA.

Secondary packaging of chromatin involves the arrangement of nucleosomes in ordered helical turns. One complete turn containing six nucleosomes is termed a solenoid (Finch and Klug, 1976). The stacking of these helices results in the formation of a 30 nm diameter chromatin fibre which is stabilised by histone HI interactions (Thoma *et al*, 1979, Allan *et al*, 1982). Like the other histones HI carries many basic charges and has an average length of 220 amino acids. A central globular portion interacts with the outside of the nucleosome histones whilst the extended terminals bind to linker DNA. During transcription the removal of histone HI allows opening of the solenoid structure. At intervals the solenoid structure is interrupted by areas free of nucleosomes that are particularly vulnerable to DNase I digestion. These 'nuclease hypersensitive regions' of DNA often contain motifs that are recognised by sequence specific proteins. Solenoid packaging would reduce the average chromosome to a length of 1 mm and still grossly oversized to fit in a nucleus.

The tertiary packaging of chromatin into loops was first proposed by Cook in 1976. In each loop the 30 nm solenoid fibre is anchored at its base to the nuclear scaffold, or as it is alternatively known, the nuclear matrix. Loops vary considerably in length and contain gene sequences. Several DNA sequences are associated with the base of the loops. These

include the A-T rich 250 to 1000 bp scaffold attached -or matrix association – regions and the 156 bp Topo II boxes. The S(M)ARs bind topoisomerase II whilst the Topo II boxes provide a cleavage site for the enzyme. Topoisomerase II is vitally important in DNA replication, recombination and gene expression (Poljak and Käs, 1995). Many enhancer sequences and over 70% of DNA replication sites have also been located in the DNA of the base regions.

In binding to the nuclear matrix Topoisomerase II also has a structural role acting as the fastener for the chromatin loops (Garrard, 1990, Poljak and Käs, 1995). The nuclear matrix is revealed after removal of chromatin and phospholipid and comprises residual nuclear pore complexes and an internal fibrogranular protein and RNA containing network. The proteins associated with the matrix represent approximately 10% of the total nuclear protein. The nuclear matrix has been defined by Nelson *et al* (1986) as a "dynamic structural sub-component of the nucleus that directs the functional organisation of DNA into domains and provides sites for the specific control of nucleic acids". Its biological importance is underlined by it being a major site for steroid receptor binding and the target for transforming proteins and oncogene products. Over 95% of heterogeneous RNAs are also associated with the nuclear matrix. Combining these features with those described for the DNA in the chromatin loops it would appear that this region is of pivotal importance in the control of genetic function.

The miniband has been proposed by Pienta and Coffey (1984) for the packaging of chromatin into an arrangement consistent with the known width of condensed chromosomes. In this chromatin loops are arranged once more in ordered helical turns. One complete turn containing eighteen chromatin loops or 6×10^4 DNA base pairs. The authors also suggest that during interphase the telomeres of chromosomes may be attached to the nuclear membrane leaving the remaining chromosomal material to traverse the nucleus. In this model a central channel is defined by the base of the loops could provide for communication with the nuclear pores (Gasser and Laemmli, 1987). However, at this level several alternatives for the structural organisation of chromosomes and transcription of genes have been proposed (Park and De Boni, 1999).

Other structures, which can be readily identified in the interphase nucleus, are the nucleolus and the nuclear membrane. The nucleolus represents the functional congregation and transcription of tandem repeat ribosomal RNA encoding genes, which in the human, are distributed on five chromosomes. The nuclear membrane is a double layered phospholipid membrane which, via the nuclear pores, is continuous externally with the endoplasmic reticulum and, as has been noted, may be internally connected through the same structures with the nuclear matrix. The inner aspect of the nuclear membrane is also lined by an orthogonal mesh of lamin filaments that may have a role in the anchorage of chromatin (Laskey and Leno, 1990).

1.3 The preparation of tissue for histological examination

During this century the vast majority of tissues have been prepared for histological examination using paraffin wax embedding. The success of the embedding media may be attributed its ease of use, support provided during sectioning and suitability for long term storage at room temperature. This preparation method consists of three essential steps fixation, processing and embedding. Following these microtomy, staining of the tissue sections and microscopy complete the process of histological examination.

When tissues are removed from a living organism deterioration of structure and loss of chemical constituents follow. The rate at which these occur in an individual tissue will depend on its susceptibility to putrefaction and autolysis. Putrefaction describes tissue destruction caused by microrganisms whilst autolysis denotes the break up of cells due to endogenous factors such as the uncontrolled action or release of enzymes. The primary purpose of fixation is to halt these processes as soon as possible. The chemicals used as fixatives, however, need to act more than just preservatives. These reagents must also allow for the subsequent manipulation of tissue during processing, embedding and microtomy to be achieved without further structural alteration. They must also render chemical groups within the tissue reactive to dye staining so that microscopy can be undertaken. Fixation is usually undertaken by immersing tissue in the fixative for a length of time sufficient to ensure that it will survive subsequent steps. This time will vary

according to the size and consistency of the tissue sample and the rate of penetration and fixation of the fixative itself. Typically a tissue biopsy can be adequately fixed within 24 h whilst a resected specimen make require several days fixation. Exceptionally tissues, for example whole brains, may be kept in fixative for several months before processing. The choice of fixative used and duration of fixation can have a profound influence not only on the microscopic preservation and dye staining of tissue but also on its constituent chemicals as will be discussed in the next section.

Processing for paraffin wax embedding involves the removal of water from tissue immersed in aqueous based fixatives and the infiltration of the specimen with paraffin wax. To ensure this is completed satisfactorily tissue dimensions must be carefully controlled necessitating the selection of representative samples from resected specimens. Typically processing involves dehydration in ascending grades of industrial methylated spirits or isopropyl alcohol followed by the use of an anti-medium to replace the dehydration fluid with a solvent miscible with paraffin wax. Examples of anti-media are chloroform, toluene and xylene. However, due to safety considerations proprietary non-toxic and non-inflammable solutions are replacing these reagents. Dehydration and exposure of tissue to anti-medium may be undertaken at room temperature. However, the infiltration of tissue with paraffin wax necessitates the use of elevated temperatures, typically in the region of 55 to 65°C. Until recently routine histology processing was usually undertaken automatically using an overnight schedule. However, rapid automated schedules employing elevated temperatures at all stages, are being adopted to reduce the 'turn around time' for diagnosis.

Whilst tissue shrinkage and the loss of free lipid during exposure to the anti-medium is inevitable, processing should not produce further structural or chemical changes to adequately fixed tissues. However, DNA melting may begin within the temperature range used for paraffin wax infiltration (Hopwood, 1977) and this could allow any residual fixative to react with the nucleic acid. Only chemically bound fixative would remain at this stage for any reaction to occur. However, when rapid processing schedules are used the

fixative, often used as an initial reagent or available in decreasing concentrations in the dehydration solutions, could potentially interact directly with DNA.

Following processing embedding is undertaken by placing the tissue in a suitable mould and surrounding it with molten paraffin wax. After the wax has set microtomy may be undertaken and the block then stored at room temperature. With respect to the subsequent production of sections and their dye staining deterioration due to storage, even after many years, is seldom observed.

1.4 Chemical interactions of fixatives

In the preceding section the main requirements for fixation were outlined. At the time of the introduction of most fixatives, in the second half of the nineteenth century, a further factor, tissue 'hardening', was regarded as important. This was due to the incomplete development of embedding procedures and microtomes and meant that sections could be cut with greater ease from a suitably hardened specimen. Although no longer important this factor contributed to the initial selection of fixatives, many of which continue in use to this present day. It will be noted that none of the criteria that have been mentioned make any specific reference to chemical interactions, though these must underlay the fixation process. This reflects the timing of the introduction of fixatives when practical considerations took precedence and chemical understanding was limited.

Without the stabilisation of proteins the structure of cells and tissues cannot be maintained. Accordingly, all chemicals that have been used successfully as fixatives are able to induce this stabilisation. Fixatives have been classified according to their macroscopic and chemical action on proteins as follows:

> Coagulant or non-coagulant, as shown by macroscopic interaction with solutions of purified proteins.

Additive, when fixatives form chemical bonds, or non-additive when fixation is induced by dehydration.

The retention of carbohydrates, lipids and nucleic acids, is influenced by choice of fixative and may not parallel that of protein fixation.

Fixatives have been frequently combined for use as mixtures. These have usually been determined empirically according to the resultant improvement in tissue preservation. In situations where mixtures are used the precise definition of chemical interactions may become difficult not only due to reagent combinations but also to the pH of the solution employed. As an alternative to the use of mixtures, the sequential immersion of a tissue in two fixatives has been employed. This process is usually referred to as 'post fixation'. A factor, which should be considered in this situation, is the time of exposure to each reagent. Some fixatives and fixative mixtures include an indifferent chemical, such as salt, which does not itself contribute chemically to the fixation process but has been shown to improve subsequent histology.

In this research the influence of five fixative preparations on the recovery and analysis of DNA was investigated. Four of these, formol saline, neutral buffered formalin, formol sublimate and Bouin are aqueous solutions that have been extensively employed in histology. The fifth fixative used was Carnoy, a non-aqueous fixative that has been used mainly in cytological preparation. The physical and chemical interactions of the fixatives contained within these solutions, acetic acid, ethanol, formalin, mercuric chloride and picric acid are discussed below and possible mechanisms for their interaction when employed as mixtures is also presented. With respect to chemical interactions discussion has been limited to proteins and nucleic acids as these constitute the vast majority of nuclear material.

1.4.1 Acetic acid

Clarke introduced acetic acid as a fixative in 1851 by mixing it in the proportion of one part to three parts wine. The latter component was later replaced with ethanol. Acetic acid features as a component of several fixative mixtures of which Baker (1958) lists thirteen aqueous solutions and two non-aqueous, Clarke's and Carnoy. In contrast to Clarke's fixative the concentration of acetic acid in these mixtures, at 0.12 to 10%, is much lower.

The chemistry and effects of acetic acid fixation were reviewed by Baker (1958). Acetic acid is an additive non-coagulant fixative. The pH of a 5% solution is between 2.3 and 2.4. In such solutions proteins are swollen through the breakage of salt groups and the formation of hydrogen bonds between adjacent amide groups on separate protein chains. Physically this results in marked tissue swelling whilst leaving the tissue softer than any other fixative.

Using a variety of cytochemical methods Fraschini *et al* (1981) showed that DNA was retained and nuclear proteins largely removed after tissue fixation in 45% acetic acid or in solutions comprising 25% acetic acid and 75% methanol. Dick and Johns (1968), Retief and Rüchel (1977) and Krejcí *et al* (1980) have also reported the removal of histones using similar solutions.

1.4.2 Ethanol

The Egyptians used ethanol as an embalming fluid but not as the principle preservative. As noted above it was introduced as a histological fixative in combination with acetic acid by Clarke in 1851.

Ethanol causes protein denaturation through the removal of water from free carboxyl, hydroxyl, amino, amido and imino groups of proteins (Baker, 1958). This results macroscopically in protein coagulation and gross tissue shrinkage. However, whilst nucleic acid is precipitated macroscopic coagulation of nucleoprotein is not observed.

In this investigation ethanol was used as a constituent of Carnoy fixative. This mixture comprises 10% acetic acid, 30% chloroform and 60% ethanol. The pH of this solution as used was between 3.0 and 3.2. The mixture, described by Carnoy in 1887, has since been employed chiefly in situations where the preservation of cytological rather than morphological detail is required.

The actions of acetic acid and ethanol in this fixative are complementary. Both constituents retain nucleic acids whilst tissue shrinkage produced by ethanol is countered by the action of acetic acid. Merriam (1958) recorded no loss of nucleic acid when acetic acid was used in combination with ethanol and Dick and Johns (1968) observed that when 25% acetic acid was combined with 75% ethanol most histone proteins were retained but that this did not confer subsequent protection from acid extraction. The potential stabilising effect of ethanol has also been reported by Holtzman (1965) who demonstrated that histone extraction by 0.25N HCl could be considerably reduced when ethanol was added to the acid. However, Krejcí *et al* (1980) recorded equivalent loss of proteins from deoxyribonuclearprotein when 25% acetic acid was combined with either methanol or ethanol. With this exception the results of these investigations suggest that pH mediated removal of histones by acetic acid is slowed by the addition of ethanol but this does not confer protection against their subsequent removal by acid treatment. Chemical interactions during fixation with chloroform have not been described but loss of free lipid may be expected.

1.4.3 Formaldehyde

Formaldehyde is a colourless gas, which is soluble in water to 37%. In commercial preparations such solutions also contain 0.05% formic acid and between 6 to 15% methanol (Baker, 1958). It has become convention to refer to this solution as concentrated formalin and for use in histology a one in ten aqueous dilution is usually employed which is termed 4% formalin. In this diluted solution a hydrated monomeric form predominates with the formula $HOCH_2OH$, (Baker, 1958).

Formalin was introduced as a fixative by Blum in 1893 who noted its hardening properties and undertook microscopy on several celloidin embedded tissues. Formalin has a fast penetration rate and renders the cytoplasm acidic thereby promoting staining with basic dyes. During fixation tissue volume is maintained but formalin does not protect against subsequent shrinkage during processing. Used alone histological preservation is not optimal but this is significantly improved if salt is added. Accordingly formol saline, comprising 4% formalin and 0.9% sodium chloride, has been extensively used in histology. At the concentration used the action of sodium chloride may be to maintain tissue in an isotonic state until formalin penetrates in sufficient concentration to fix cells. The pH of formol saline is acid and in this investigation was between 3.5 and 3.8. Formalin is also widely employed as a 4% solution buffered to neutrality with sodium phosphate. The pH of this solution favours additive reactions of formaldehyde with proteins. The fixative has also been used frequently as a constituent of mixtures, two of which were used in this investigation. These were formol sublimate in which formalin is combined with mercuric chloride and Bouin in which the fixative is combined with picric acid and acetic acid.

Baker (1958) reviewed the reaction of formaldehyde with proteins. The most important reaction is with the side chain amino group of lysine. This is additive resulting in the initial formation of an unstable hydroxymethyl adduct. Subsequent methylene bridge formation may also occur according to the following formulations:

$$\text{R-NH}_2 + \text{HCHO} \rightarrow \text{R-NH-CH}_2\text{(OH)}$$

$$\downarrow$$

$$\text{R-NH-CH}_2\text{(OH)} + \text{R'-NH}_2 \rightarrow \text{R-NH-CH}_2\text{-NH-R'} + \text{H}_2\text{O}$$

The formation of the hydroxymethyl adduct is maximal between pH 7.5 and 8 but very slow below pH 3. Even in the optimal pH range the interaction of formaldehyde with amino groups is slow and the formation of methylene bridges slower still. The importance of the latter reaction is that it produces relatively stable cross-links between proteins reinforcing fixation induced by the primary reaction. Reactions with arginine and

sulphydryl groups of cysteine (Pearse, 1980) have also been demonstrated but only when the pH is higher than 8.

In reviews of histological fixation covering the period 1960 to 1982 no additional information on the interaction of formaldehyde with proteins was highlighted (Hopwood, 1969, 1985). However, during this period formaldehyde was extensively employed as a reagent to investigate histone-histone and histone-DNA interactions. Research was also undertaken into the reaction of formaldehyde with mono- and poly-nucleotides and DNA. It should be noted that in most of these formaldehyde was used at lower concentrations than employed in histology and under conditions, which would not be reproduced during the normal fixation of tissues.

The reaction of formaldehyde with nucleotides was investigated in detail by McGhee and von Hippel (1975a and b). With nucleotides possessing exocyclic amino groups the formation of mono- and di-hydroxymethyl adducts with dAMP and dCMP was observed. In the case of the adenine adducts, a lifetime of 100 min was recorded. With these nucleotides two isomeric products could form. One, preferred by a ratio of 20:1, would block hydrogen bond base pairing of DNA whilst the other would protrude into the major grove of the double helix. The reaction with dGMP was more complex due to the presence of three potential reaction sites. Reaction with the proton of N-1 in the heterocyclic ring occurred first followed by additions to the two protons of the C-2 amino group. The former reaction was pH dependent increasing rapidly above 7. The amino reaction was pH independent, slower, and the only reaction to occur below pH 7. Using 1M (3%) formaldehyde reactions, for the three nucleotides, were 50% complete in 10 to 60 min. Reaction with the endocylic imino group of thymidine was demonstrated with the N-3 proton being replaced by a hydroxymethyl group. This reaction was pH independent and between 10^3 and 10^4 times faster than the amino group reactions of the other nucleotides. However, product formation was five fold lower than amino containing compounds and, at 40°C, the life of the adduct was one tenth of a second. The authors also observed that for the reaction to occur in DNA base pair opening would be a necessary prerequisite. These

results confirmed and expanded earlier work reported by Haselkorn and Doty (l96l) and Eyring and Ofengand (1967).

The reaction of synthetic polynucleotides with formaldehyde has also been investigated. Using poly A Haselkorn and Doty (l96l) demonstrated rapid denaturation followed by the addition of formaldehyde to the polynucleotide. With 1% formaldehyde the melt temperature of poly A was reduced by 18°C whilst this parameter was reduced by 50°C in reactions incorporating 2.76% formaldehyde. Identical reactions with poly T to those of equivalent mononucleoside was reported by Eyring and Ofengand (1967). McGhee and von Hippel (1977a) also recorded similar reactions with polynucleotides to nucleotides and noted that base stacking was not prevented by the reaction with formaldehyde.

Interaction of formaldehyde with DNA is effectively controlled by base pairing. When DNA is denatured reaction with formaldehyde is rapid and renders renaturation impossible (Stollar and Grossman, l962, Feldman, l973). The presence of formaldehyde also lowers the melt temperature of DNA in a concentration dependent manner. Accordingly at 45°C a 1% solution of formaldehyde was unreactive with T4 DNA but when used at 15% reaction occurred (Berns and Thomas, l96l). No evidence has been recorded for formaldehyde being able to sufficiently lower the melt temperature of DNA to enable a room temperature reaction to occur. McGhee and von Hippel (1977b) proposed the initial reactions occur with opened A-T rich regions of DNA and suggested three favoured mechanisms:

Reaction with the thymine imino group.
Hydroxymethylation of the N-6 adenine amino group *anti* to N-7
Hydroxymethylation of the N-6 adenine amino group *syn* to N-7

Only the latter would allow subsequent reformation of a, less stable, base pair. McGhee and von Hippel (1977b) also allow that a reaction not requiring base pair opening could take place. This would involve the replacement by formaldehyde of the outside proton of the N-6 amino group of adenine resulting in a hydroxymethyl group syn to N-7 and protruding into the major grove. This they argue is unlikely as the chemistry of the

formaldehyde interaction with aromatic amines is not supportive. However, this stance is contested by Frank-Kamenetskii (1985).

In summary formaldehyde forms similar hydroxymethyl adducts with amino and imino groups of mono- and poly-nucleotides. Formaldehyde also reacts rapidly with denatured DNA. However, whilst the melt temperature of DNA is lowered by the presence of formaldehyde this is not sufficient to produce conditions of denaturation at room temperature. Accordingly unless the disputed N-6 adenine amino reaction is allowed or other conditions pertain, such as DNA 'breathing' or pH induced denaturation, reaction of formaldehyde at room temperature would appear to be unfavourable.

Formaldehyde has been used to elucidate the structural relationships between histones and histone with DNA. Romakov and Bozhko (1967) observed that after 30 day exposure to 4% formaldehyde at pH 7 nucleoprotein (NP) became insoluble whilst solubility was retained at pH 4. In the same study it was shown that as formaldehyde concentration increased, to a maximum of 8%, less NP could be precipitated by sodium chloride. The authors concluded that these results indicated that formaldehyde was reacting with histones and thereby inhibiting electrostatic interactions with DNA. The dimerisation and formation of polymers of histones after exposure to formaldehyde was subsequently demonstrated by electrophoretic analysis by Van Lente *et al* (1975) and, Jackson (1978), and shown by SDS centrifugation by Ohba *et al* (1979).

Evidence for the interaction of formaldehyde induced histone-DNA cross-links was first presented by Brutlag *et al* (1969). In reactions employing pea bud nucleohistone with 0.05% formaldehyde at pH 7.2 and 4°C for 24 h coupled with analysis by buoyant density centrifugation they demonstrated that 30% of the protein remained attached to DNA. This result was reinforced by the observation in the same study that after exposure to formaldehyde some histones were no longer acid extractable. In subsequent studies similar results were reported using the former (Doenecke, 1978), and latter methods of analysis (Chalkley and Hunter, 1975, Varricchio and Jamieson, 1977, Jackson, 1978). Other evidence for the formaldehyde induced histone DNA cross-linking has been provided

through changes reported in circular dichroism spectra (Senior and Olins, 1975, Ohba *et al,* 1979) and DNA melting point changes (Li, 1972). Wilkins and MacLeod (1976) reported the binding of non-histone proteins to E coli DNA by formaldehyde, suggesting that the cross-linked proteins were most likely to be DNA and RNA polymerases.

The diverse range of experimental conditions used in the above studies makes analysis of the histone type and extent of reaction with formaldehyde difficult. Generally it can be stated that all histone types can be 'fixed' by formaldehyde but the interaction, as judged by percentage formation of histone dimers and polymers, is incomplete. Histone-DNA cross-links can be reversed by dialysis against SDS (Jackson, 1978) or by proteolytic digestion (Chalkley and Hunter, 1975, Doenecke, 1978). The ε amino group of lysine has been suggested by several authors as the primary reactive group for histone-histone formaldehyde interactions with hydroxymethylation proceeding to the formation of methylene bridges. In support of this Ohba *et al* (1979) demonstrated a rapid reduction of histone free amines on exposure to formaldehyde whilst Siomin *et al* (1973) demonstrated the binding by formaldehyde of lysine to denatured DNA. For histone-DNA cross-links evidence for the importance of the N terminal region of histones in binding to the nucleic acid has been provided by the resistance of this trypsin sensitive region to digestion after formaldehyde exposure (Doenecke, 1978, Jackson, 1978).

1.4.4 Mercuric chloride

Mercuric chloride was introduced as a fixative by Blanchard in 1847 who added the chemical to sea water to fix marine Turbellaria. In subsequent use mercuric chloride has always been incorporated a component of a fixative mixture, 19 of which are listed by Hopwood (1972). Amongst these Helly, Susa and Zenker mixtures have been extensively used in histology over the past one hundred years on account of their pronounced enhancement of acid dye staining and excellent micro-anatomical preservation. Formol sublimate, a solution comprising 10% formalin in saturated aqueous mercuric chloride, has also been employed in the United Kingdom and this fixative was recommended by Lendrum *et al* (1962) for the dye staining of fibrin. Lillie (1965) describes a modification

of this mixture incorporating sodium acetate. This fixative, which has become known as B-5, has been used for the immunocytochemical demonstration of cytoplasmic antigens and in-particular immunoglobulins. Due to the safety hazards associated with the use of mercury the use of fixatives containing this metal has diminished substantially in recent years.

Baker (1958) and Hopwood (1972) have reviewed the reaction of mercuric chloride with protein. Mercuric chloride is an additive coagulant fixative. Two types of additive reaction occur, binding to sulphydryl groups and interaction with amino groups. With the former reaction hydrogen ions are released and cross-linking via mercury may occur between adjacent proteins. By limiting the concentration of mercuric chloride the reaction with sulphydryl groups can be shown to take precedence over that with amino groups. The interaction of mercury with amino groups is pH dependent. The pH of formol sublimate recorded during this investigation was between 2.4 and 3.0. Under such conditions whilst reaction with sulphydryl groups occurs, interaction with amino groups would involve only proteins with iso electric points in this pH range and as such could be expected to be extremely limited. Such reactions would involve the uptake of mercury as $[HgCl_4]^=$ resulting in a very loose bond being formed. The pH of formol sublimate does not preclude the interaction of formalin with proteins but, as previously described, conditions are not optimal. Furthermore the general denaturing effect of low pH should also be considered as contributing to the fixation of proteins under these conditions.

Mercuric chloride has been shown to react with nucleic acids. Yamane and Davidson (1961) observed spectral and viscosity changes with pure DNA together with the release of hydrogen ions for which they proposed the following reaction:

$$R_2NH + Hg^{2+} \rightarrow R_2N{:}Hg^{+} + H^{+}$$

Importantly they also suggested that one mercury ion could react with the amino groups of two bases which may indicate that the metal can intercalate with the bases of DNA. In the same study the authors reported spectral changes induced in A-T polymers which could be

reversed on exposure to excess chloride. Similar observations for mercury with a guanine oligonucleotide were reported by Lipsett (1964) whilst Millar (1968) in experiments using poly uridine provided evidence that Hg^{2+} could replace native hydrogen bonds. The interaction of mercury with nucleosides was investigated by Eichorn and Clark (1963) who demonstrated binding to the N_1O^6 groups of uridine and guanosine. In the same study it was observed that when this reaction was blocked by formaldehyde, mercury bound to the amino groups of adenosine and cytosine. Evidence for a physiological effect for mercuric chloride has been demonstrated through the inactivation of Tobacco Mosaic Virus RNA by mercuric chloride as recorded by Katz and Santilli (1962). This inactivation could be reversed by exposure to excess chloride ions.

In summary fixation with mercuric chloride is primarily dependent on the interaction of the metal with sulphydryl groups of proteins. Mercury can also react with the amino groups of proteins and has been shown to interact reversibly with nucleic acid bases. When used as formol sublimate the low pH of the fixative may preclude an amino acid reaction but this may be compensated by formalin interactions.

1.4.5 Picric acid

Picric acid was introduced as a hardening agent by Ranvier in 1875 although Baker (1958) observes that it leaves tissue considerably softer than most other fixatives. As a fixative picric acid has been mainly used in an aqueous mixture with formalin and acetic acid named after its inventor Bouin (1897). This fixative mixture comprises 9% formalin, 1% picric acid and 5% acetic acid. It provides excellent tinctorial staining with acid dyes but can result in inferior results with basic dyes. Due to the very slow penetration of tissue by picric acid its use is effectively restricted to biopsy sized samples.

Picric acid coagulates protein but no chemical interaction for picric acid with proteins has been described and Baker (1958) remarks that DNA is left in solution after exposure to the reagent. When incorporated in Bouin the pH of the fixative mixture is between 1.3 to 1.6, Baker (1958) – 1.5 to 1.6 in this investigation. According to the same author this pH is too

acid for formaldehyde to fix effectively whilst below pH 1.4 acetic acid macerates nucleoproteins. A further consideration is that below pH 2.5 DNA will begin to disintegrate due to depurination. Accordingly, it is suggested that pH of the fixative rather than the action of its constituents will dominate its effects on DNA.

1.5 Molecular methods for the identification of DNA

1.5.1 Southern analysis

As originally described by Southern (1975) this technique involved the transfer of restriction endonuclease digested DNA from an electrophoretic gel using high salt concentrations to a nitro-cellulose filter. Following immobilisation of the DNA to the filter by baking specific restriction fragments were identified by the hybridisation of RNA radiolabelled probes. Apart from the substitution of nylon membranes for the fragile and background prone nitro-cellulose filters the transfer procedure has remained unaltered. Most applications however, now employ recombinant DNA or oligonucleotide probes for hybridisation.

The availability of DNA of high molecular weight DNA and purity is essential for the successful analysis of DNA by Southern blotting. The presence of residual proteins attached to the DNA, can hinder sequence recognition by restriction enzymes and accordingly prevent or diminish the efficiency of digestion. The degree of DNA degradation will determine the maximum restriction fragment length of the nucleic acid that can be resolved by hybridisation. This can result in a smear rather than a discrete hybridisation band being visualised or when degradation is more pronounced the absence of hybridisation.

1.5.2 DNA extraction

The most efficient methods for the preparation of high purity DNA involve the use of proteolytic enzymes. Several enzymes have been used for this purpose but that most

commonly employed is Proteinase K, isolated from the fungus *Tritirachium album* Limber (Ebeling *et al,* 1974). This enzyme cleaves peptide bonds adjacent to aliphatic, aromatic and hydrophobic amino acids of native and denatured proteins and has several features that promote its practical use. These include; stability, when dissolved in 50 mM Tris/HCl pH 8, for 12 months, activity over a broad pH range of 7.5 to 12 and to a temperature of 65°C. Furthermore Proteinase K activity is enhanced in the presence of denaturing agents such as sodium dodecyl sulphate and urea.

For the extraction of DNA from fresh or frozen tissues incubation times as short as 1 h in Proteinase K at 37°C, without the presence of a denaturing agent, is sufficient to yield large quantities of high molecular weight DNA (Jackson *et al,* 1990). Removal of digested proteins and other contaminants is usually undertaken by phenol and chloroform washing followed by precipitation of the DNA with cold ethanol and salt. Providing extracts are redissolved in nuclease free water or buffer the DNA should remain intact for several years stored at 4°C.

1.5.3 Polymerase chain reaction

The polymerase chain reaction (PCR) relies on repeated primer directed amplification of short sequences of DNA. As a consequence only a few intact double stranded DNA sequences need to be present in the sample to provide an adequate initial template for amplification.

As initially described by Saiki *et al* (1985) the polymerase chain reaction involves repetition of the following cycle of steps:

Heat denaturation of target DNA
Annealing of oligonucleotide primers to both strands of the DNA
Enzyme catalysed assembly (extension) of complementary strands of DNA.

As the oligonucleotides are designed to hybridise in a 5' to 3' direction the newly synthesised DNA reproduces the original template. Accordingly at the end of the first cycle the number of 'target' DNA sequences which are available for subsequent hybridisation to the oligonucleotide primers should have doubled. In theory each time the PCR cycle is repeated similar amplification should be accomplished. As a result of this exponential amplification it is possible to calculate that a maximum of 2×10^7 copies of a single original target can be synthesised after 20 PCR cycles. Although this efficiency cannot be achieved in practice the PCR method is extremely sensitive and indeed often allows amplification products to be detected by gel electrophoresis. However, with sensitivity come issues of specificity and accordingly PCR amplification must be carefully controlled if non-specific products are to be avoided.

Klenow polymerase, a DNA polymerase with no exonuclease activity, was initially employed in the extension step. Whilst biochemically suited for use the enzyme is not thermostable and accordingly this meant that it had to be added fresh during each cycle. Furthermore extension had to be undertaken at 37°C and under these conditions of low stringency the production of non-specific primer extensions was a frequent occurrence. This problem was overcome in large measure by the substitution of a thermostable DNA polymerase obtained from *Thermus aquaticus (Taq)* (Saiki *et al,* 1988). The enzyme allowed PCR amplification to be undertaken with a single addition of the reagent and using extension temperatures that favoured specific priming. In a further development it was demonstrated that when Taq polymerase was added to the PCR reaction mix at the annealing temperature after the initial denaturation of the DNA this contributed significantly to a reduction in non-specific extension products (Erlich *et al,* 1991). This so called 'hot start' now forms a standard component of the PCR technique. With these modifications it became possible to reliably generate specific PCR products which could be resolved using gel electrophoresis alone. This contrasted with the previous requirement to identify specific PCR products by Southern transfer and hybridisation using a probe internal to the amplified region.

Optimisation of the components of the PCR reaction mix is important to produce efficient amplification. Furthermore control of the temperatures and times employed in the PCR cycle together with the number of cycles used will also contribute to the overall efficiency of the method. The effects of these factors have been practically reviewed by Harris and Jones (1997).

The intrinsic sensitivity of PCR leads to the possibility of minute contamination of samples generating false positive products indistinguishable from those of true positive samples. Accordingly the inclusion of a control to demonstrate that there has been no contamination of the PCR reaction mix is an essential component of any investigation. This may be simply achieved by undertaking PCR with the reaction mix in the absence of DNA. Care should also be taken to ensure that during the preparation of DNA sample cross contamination is avoided. The problems of false positives and steps that may be employed to reduce their occurrence have been reviewed by Kwok and Higuchi (1989).

1.5.4 *In situ* hybridisation

The analysis of DNA *without* prior extraction may also be undertaken in tissues and cells using *in situ* hybridisation. For this method cytogenetic, smear, as well as frozen or paraffin wax embedded sections may be used. Essential steps in the method are:

Fixation and pre-treatment to optimise the retention and exposure of the target DNA.
Hybridisation to the nucleic acid of a labelled probe.
Detection of the label to reveal the cytological localisation of the target DNA.

Mitchell *et al* (1992) and Warford (1996) have reviewed the principals and technical procedures involved in *in situ* hybridisation. In the context of the present investigation it is important to note that the target nucleic acid does not need to be intact but it must be of sufficient length and free of protein to allow specific hybridisation to occur.

1.6 Contribution of molecular biological techniques to the diagnosis of lymphoma

Lymphomas represent malignant proliferations of the cells of the lymphocyte series. Histologically they are classified as Hodgkin's disease or non-Hodgkin's lymphoma (NHL). These are then divided according to microscopic appearance into several sub categories. This sub classification is relatively simple for Hodgkin's disease but for NHL more than 20 types have been proposed in a recent classification system (Harris *et al,* 1994). Accurate diagnosis is important as the prognosis and treatment regimes vary considerably for the lymphoma sub types (The non-Hodgkin's Lymphoma Classification Project, 1997).

Hodgkin's disease is characterised by the presence of the Reed-Sternberg cell and identification of this is essential for histological diagnosis. In most instances Reed–Sternberg cells can be identified in haematoxylin and eosin preparations but immunocytochemical identification of the CD30 antigen may assist in the visualisation of this cell.

The accurate classification of NHL is more demanding. This is due to each lymphoma reflecting the cytology of its normal lymphoid cell counterpart. Accordingly, on purely morphological grounds it is not possible to distinguish between a B and T cell lymphoma composed of small lymphocytes. As a consequence immunocytochemistry and molecular biology methods are extensively used as an aid to accurate classification.

B cell NHLs will normally express immunoglobulin on their cell membrane or within the cytoplasm. In line with the clonal expansion theory for the origin of malignancy the immunoglobulin should be of a single light chain type. This light chain restriction can be demonstrated using immunocytochemistry or by *in situ* hybridisation (Jordan *et al,* 1995). Immunocytochemistry may also be employed to differentiate T from B cells and aid the identification of particular NHLs. Examples of the latter include the demonstration of cyclin D1 in mantle cell lymphoma and the oncogene product Bcl2 in follicular lymphoma. However, using immunocytochemistry it is not possible to demonstrate the clonal origin of

T cell lymphomas and antigen demonstration in NHLs may not be unequivocal. In these instances the use of molecular biological methods can make an important contribution to accurate diagnosis (Ashton-Key *et al,* 1995).

During T and B lymphocyte development genes coding for immunoglobulin and T cell receptors are rearranged to provide for the cell membrane expression of an antigen receptor of single conformation on each cell. The rearrangement process involves the systematic juxtaposing of variable (V), joining (J), diversity (D) - in some instances - and constant (C) region genes from different chromosomes to produce a functional receptor. The diversity engendered by this process ensures that antigen recognition is possible and in a normal lymphoid cell population each unstimulated lymphocyte carries a distinctive antigen receptor. When stimulated by antigen lymphocyte proliferation occurs of cells to which the antigen has bound, the result being a polyclonal immune response. However, in a lymphoma originating from the monoclonal expansion of a single lymphocyte all the tumour cells will possess the same gene rearrangement and in this situation Southern and PCR analysis methods can be used to demonstrate these.

Identification of gene rearrangement using Southern analysis is based on the demonstration of alterations of the position and number of restriction endonuclease sites due to the rearrangement process. In an unstimulated or reactive lymphoid cell population the number of cells with a similar gene rearrangement will be insufficient to show these alterations. Instead a germline restriction fragment pattern will be demonstrated due to the predominance of cells in which gene rearrangement has not occurred. Using selected restriction endonucleases in combination with specific cDNA probes these germline fragments can be reproducibly demonstrated (Table 1.1). In lymphomas, as the tumour cell population increases, Southern analysis will reveal a new restriction fragment pattern reflecting the single gene rearrangement. The sensitivity of Southern analysis to identify such rearrangements has been estimated as requiring the presence of between 1 to 10%, lymphoma cells in a population (Arnold *et al,* 1983, Lowe, 1986). Interpretation of restriction fragments patterns as demonstrated by Southern analysis must be undertaken with care (Cossman *et al,* 1988). In particular the presence of a restriction site

Table 1.1 Germline restriction fragment patterns

Probe	Restriction enzyme	Germline fragment/s
Joining Heavy (JH)*	*Bgl* II	4 kb
	Hind III	9.2 kb
T cell beta receptor (Tβ)**	*Eco*R I	4.2, 9, and 12 kb
	Hind III	3.5, 7.6 and 11.5 kb
	*Bam*H I	24 kb

* Used to identify B cell rearrangements, Ravetch *et al* (1981)

** Used to identify T cell rearrangements, Yanagi *et al* (1985)

polymorphism in germline DNA can result in the apparent appearance of rearranged restriction fragment. To guard against this possible misinterpretation the authors warn that rearrangement should not be concluded unless evidence of restriction fragment alteration is present after two separate restriction enzymes digests have been hybridised with the same cDNA probe.

Due to the large number of genes available for rearrangement PCR methods for the demonstration of B and T cell clonal expansion usually employ several consensus primers in semi-nested PCR or multiple PCR reactions. For B cell rearrangement the use of consensus primers for the framework 3 region of the immunoglobulin variable heavy chain together with a universal JH primer are favoured (Inghirami *et al,* 1993, Pan *et al*, 1994). PCR methods have also been described for the demonstration of the clonal expression of the β and γ T cell receptors (McCarthy *et al,* 1991, McCarthy *et al,* 1992, Dippel *et al,* 1999).

In contrast to the complexities of T and B cell gene rearrangement identification by PCR the demonstration of the t(14;18) translocation as an aid to the diagnosis of follicular lymphoma is straightforward. The translocation involves the movement of the *Bcl2* oncogene sequence from a major or minor breakpoint cluster on chromosome 18 into or 5' to the JH region on chromosome 14. Ensuing transcription of Bcl2 confers protection of cells from apoptosis. PCR demonstration involves the use of a single pair of primers, one adjacent to the major or minor breakpoint region on chromosome 18 and the other a consensus primer to the JH sequences on chromosome 14. A PCR product is only formed when translocation has occurred and this can usually be visualised directly by gel electrophoresis.

The incidence of t(14;18) translocation in follicular lymphomas by cytogenetic analysis has been reported as between 44 and 84% (Pezzella *et al,* 1990). The translocation is also present in 20 to 30% of diffuse large cell lymphomas and in 50% of adult undifferentiated lymphomas (Weiss *et al,* 1987, Crescenzi *et al,* 1988). PCR for the translocation has been used for the detection of minimal residual disease (Crescenzi *et al,* 1988). However, as

reported by Berinstein *et al,* 1993 t(14;18) translocation may be detected by PCR in a proportion of normal lymphocytes and accordingly monitoring for minimal residual disease requires comparison of sequence analysis of the PCR product with that of the original tumour (Miettinen and Lasota, 1997).

1.7 Research strategy

Initial investigations (Chapter 3) were undertaken with pure DNA and lymphocytes to determine the effect of the five fixative mixtures on the integrity, restriction endonuclease digestion and recovery of the nucleic acid. Extraction of DNA and Southern analysis was then attempted from Carnoy, formol saline and neutral buffered formalin fixed and paraffin wax embedded tonsil tissue (Chapters 3 and 4). Restriction enzyme and nucleic acid probe combinations were selected to provide information on the integrity of recovered DNA and to allow for assessment their potential diagnostic application in the identification of T and B cell lymphomas using Southern blotting. For PCR based investigations (Chapter 5) *Bcl2* primer combinations were used to study the influence of fixation on the size of recovered DNA and hence amplified product. The same primers were also employed to demonstrate the practical application of this technology to the identification of follicular lymphomas.

Chapter 2 Materials and Methods

2.1 Materials

2.1.1 cDNA Probes

cDNA sequences as listed in Table 2.1.1 were restricted from their vectors, separated by low melting point gel electrophoresis and recovered into sterile water.

2.1.2 Primers used in polymerase chain reaction amplification

Primers were selected using GCG Primer software according to sequence data provided for the Bcl2 gene as identified by Seto *et al* (1988). The hsbcl2c.em-hum1 RNA sequence of 6030 bases was used to define the primers shown in Table 2.1.2 Each combination of primer set spanned the major break point region for the Bcl2 gene.

The primers were synthesised by Genosys Biotechnologies (Europe) Ltd and received lyophilised. Each primer was resuspended in 1 x TE at 200 pmol/µl (stock) and 10 pmol/µl (working) then aliquoted and stored at -20°C.

For the demonstration of the t(14;18) translocation in follicular lymphomas the JMSW primer detailed in Table 2.1.2 was used. This primer was synthesised at the University of Leicester and stored at 10 pmol/µl at -20°C.

Table 2.1.1 cDNA probes

Probe	Reference	Chromosomal distribution
JH	Ravetch *et al* (1981)	Single copy on long arm C14
Tβ	Yanagi *et al* (1985)	Single copy on long arm C7
pHY 2.1	Cooke *et al* (1982)	2000 repeats on long arm of Y and 100-200 copies on X and autosomes

Table 2.1.2 Bcl2 primers: Sequence, characteristics and products

Primer	Sequence 5' - 3'	Tm	PCR product
Reverse Primer (3271-3288)*	ATA GCA GCA CAG GAT TGG	58.7	
Forward Primer 1 (3017-3034)	TTT CAA CAC AGA CCC ACC	59.5	272 bp
Forward Primer 2 (2776-2793)	GTG CAT TTC CAC GTC AAC	60.1	513 bp
Forward Primer 3 (2446-2464)	GAT GGA ATA ACT CTG TGG C	57.0	843 bp
Forward Primer 4 (2018-2035)	AGA AGG ACA TGG TGA AGG	57.4	1271 bp
Forward Primer 5 (1375-1392)	GTT GGG AAC TTC AGA TGG	57.9	1914 bp
JMSW	AAC TGC AGA GGA GAC GGT GAC C	68.9	

* Numbers in brackets represent position within hsbcl2c sequence

2.1.3 Lymphoma cases

a) Initial cases

Reference	Fixation (duration between embedding and extraction)	Tissue and histological Diagnosis	Immunocyto-chemistry	Frozen tissue Rearrangements
A	Formalin (unknown)*	Lymph node, possible immunoblastic lymphoma	Not known	Not undertaken
B	Not known (unknown)*	Lymph node, Large cell infiltrate, diagnosis not established	T cell	Not undertaken
C	Formol saline (21 months)	Spleen, hairy cell leukaemia	Not known	B cell; *Bgl* II and *Hind* III with JH probe
D	Formol saline (16 months)	Lymph node, high grade T cell lymphoma	T cell	T cell; *Eco*R I and *Bam*H I with Tβ probe
E	Formol saline (17 months)	Lymph node, possible T zone lymphoma (Lennert)	Not known	Results inconclusive
F	Not known (unknown)*	Not known, Possible T cell lymphoma	Not known	Not undertaken
G	Formol saline (21 months)	Lymph node, T cell lymphoma	Not known	T cell; *Eco*R I and *Bam*H I with Tβ probe
H	Not known (unknown)*	Lymph node, Hodgkin's disease or peripheral T cell lymphoma	Not known	Not undertaken
I	Formol saline (17 months)	Spleen, B cell intermediate or lymphocytic lymphoma	IgM/D kappa monoclonal expression	B cell; *Bgl* II, *Bam*H I and *Hind* III with JH probe
J	Formol saline (15 months)	Lymph node, centroblastic B cell	IgM/D lambda monoclonal expression	B cell; *Eco*R I and *Bam*H I with JH probe

* Referred cases

b) Lymphomas with known fixation conditions

Reference	Fixation (duration)	Tissue and histological Diagnosis	Immunocyto-chemistry	Frozen tissue Rearrangements
K	Formol saline (24 h)	Lymph node, lymphoblastic lymphoma	IgM/ kappa	B cell
L	Formol saline (24 h)	Lymph node, high grade non-Hodgkin's lymphoma, probable T cell	Not known	T cell
M and O*	Formol saline (72 h)	Lymph node, lymphoma	Not known	T cell
N and P*	Formol saline (48 h)	Lymph node, diffuse large B cell lymphoma	Not known	B cell

* Tissue from same case but fixed for 24 h in formol saline from frozen tissue stored at time of biopsy

c) Follicular Lymphomas

Reference	Tissue	Immuno-cytochemistry	Light chain *in situ* hybridisation
Q	Lymph node	B cell, Bcl-2 positive	Not undertaken
R	Lymph node	B cell, Bcl-2 positive	Kappa restricted
S	Lymph node	Not undertaken	Not undertaken
T	Lymph node	Bcl-2 positive	Not undertaken
U	Lymph node	B cell	Lambda restricted
V	Lymph node,	B cell	Not undertaken
W	Salivary gland	B cell, Bcl-2 positive	Not undertaken
X	Lymph node	B cell, Bcl-2 positive	Not undertaken
Y	Lymph node	B cell, Bcl-2 positive	Not undertaken

2.1.4 Common reagents

Reagent	Supplier
Acetic acid	Merck 10001
Agarose	Seakem HGT Boehringer Mannheim 1388991
Bovine serum albumin - standard grade	Sigma A7906
Bovine serum albumin - molecular biology grade	Boehringer Mannheim 711454
Chloroform	Merck 10077
Dimethyl sulphoxide	Merck 10323
Di-sodium hydrogen phosphate	Merck 10249
Ethidium bromide	Fisons E/P800/03
Ficoll lymphocyte separation medium	Flow Laboratories 16-920-54
Ficoll MW 400,000	Sigma F4375
Foetal calf serum	Tissue Culture services 402004
Formaldehyde solution	Merck 10113
Isopropyl alcohol	Merck 29694
Methylated spirits	Genta Medical
Paraffin wax	Sherwood medical paraplast
Penicillin streptomycin	Flow Laboratories 16-700-49
Polyethylene glycol 6000	Merck 44271
Polyvinyl pyrolidone MW 140,000	Agar Aids R1286
RPMI culture medium	Flow Laboratories 12-604-49
Sodium chloride	Merck 10241
Sodium dihydrogen phosphate di-hydrate	Hopkin & Williams 1072 226239
Tris equilibrated phenol	Fisons P2318
Xylene	Western Solvents 1307

2.1.5 Molecular biology reagents

Reagent	Supplier
φX174 RF DNA *Hae* III size marker	Advanced Biotechnologies AB-0389
100 bp ladder size marker	Gibco/BRL 15628-050
α^{32}p dCTP	Amersham PB 10205
dATP	Pharmacia 27-2050
dATP, dCTP, dGTP and dTTP for PCR	Advanced Biotechnologies AB-0315
dGTP	Pharmacia 27-2070
dTTP	Pharmacia 27-2080
Hexadeoxyribonucleotides	Pharmacia 27-2166
Klenow DNA polymerase	Life Technologies 510-8012
Lambda DNA	Boehringer 745 782
Protease VII	Sigma P5255
Protease XXIV	Sigma P5147
Proteinase K	Boehringer Mannheim 745723 and 1000 144 for DNA extraction DAKO S3004 for PCR DNA extraction only
RNase type 1A	Sigma R4875
Salmon sperm DNA	Sigma D1626
Taq polymerase	Advanced Biotechnologies AB-0192 Promega M166B/747460

2.1.6 Restriction enzymes

*Bam*H I	BRL 510-5201
Bgl II	Pharmacia 27-0946
*Bst*N I	New England Biolabs 168
*Eco*R I	Pharmacia 27-0854
Hind III	BRL 510-5207
Msp I	BRL 510-5419

2.1.7 Fixatives

These were prepared according to methods described By Hopwood (1977).

The pH of each solution was recorded on preparation and immediately before use.

Bouin

Formaldehyde solution	25 ml
Acetic acid	5 ml
Saturated aqueous picric acid	75 ml

Carnoy

Absolute ethanol	60 ml
Chloroform	30 ml
Acetic acid	10 ml

Formol saline

Formaldehyde solution	10 ml
Sodium chloride	0.9 g
Reverse osmosis treated water	90 ml

Formol sublimate

Formaldehyde solution	10 ml
Saturated aqueous mercuric chloride	90 ml

Neutral buffered formalin

Formaldehyde solution	10 ml
Di-sodium hydrogen phosphate	0.65 g
Sodium dihydrogen phosphate di-hydrate	0.452 g
Reverse osmosis treated water	90 ml

2.1.8 Solutions

Note: All solutions marked * were sterile

Acid - alcohol

99% IMS	700 ml
Concentrated hydrochloric acid	10 ml
Pure water	290 ml

Choroform, isoamyl alcohol

Chloroform	96 ml
Isoamyl alcohol	4 ml

Eosin

Aqueous solution containing in 1 litre:

Eosin Y	10 g
40% formaldehyde	1 ml

Gel Loading Buffer (Standard 5x)

Bromophenol blue	20 mg
Xylene cyanol FF	20 mg
50 x TAE	2 ml
Pure water	8 ml
Glycerol	10 ml

Gel Loading Buffer for PCR

Standard loading buffer	
without Xylene cynanol FF	2 ml
50 x TAE	2 ml
Pure water	4 ml
Glycerol	12 ml

Mayer's Haematoxylin

Aqueous solution containing in 1 litre:	
Haematoxylin	1 g
Aluminium potassium sulphate	50 g
Sodium iodate	0.2 g
Citric acid	0.2 g
Chloral hydrate	50 g

Oligonucleotide Labelling Buffer *

This solution comprises:

Solution A	200 µl
Solution B	500 µl
Solution C	300 µl

The solution was stored in 25 µl aliquots at -20°C and used within 6 months.

Solution O

Tris/HCl, pH 8.0	1.25 M
Magnesium chloride	125 mM

This solution was stored at 4°C.

Solution A

Add to 1 ml of Solution O

2-mercaptoethanol	18 µl
0.1 M dATP in 1 x TE	5 µl
0.1 M dGTP in 1 x TE	5 µl
0.1 M dTTP in 1 x TE	5 µl

This solution was stored at -20°C.

Solution B

2 M Hepes, titrated to pH 6.6 with 4 M sodium hydroxide.
This solution was stored at 4°C.

Solution C

Hexadeoxyribonucleotides, dissolved in 1 x TE at 90 OD units/ml.
This solution was stored at -20°C.

PCR Extraction Buffer *

Potassium chloride	50 mM
Tris, pH 8.3	10 mM
Magnesium chloride	2.5 mM
Tween 20	0.45%
NP40	0.45%

PCR Reaction buffer *

This was prepared as a 10 x concentrate according to the following formulation:

Tris, pH 8.8	450 mM
Ammonium sulphate	110 mM
Magnesium chloride	45 mM
dNTP mix*	2 mM
Bovine serum albumin – molecular biology grade	1.1 mg/ml
Mercaptoethanol	0.67 M
EDTA	0.44 mM

* dATP, dCTP, dGTP and dTTP each at 500 µM

The reaction buffer was aliquoted in 100 µl volumes and stored at -20°C.

PE buffer *

Tris/HCl, pH 7.5	50 mM
Sodium pyrophosphate	0.1%
Sodium dodecyl sulphate	1%
Bovine serum albumin	0.2%
Polyvinyl pyrolidone MW 140,000	0.2%
Ficoll MW 400,000	1%
EDTA	5 mM

Phenol, chloroform, isoamyl alcohol

Tris equilibrated phenol	50 ml
Chloroform	48 ml
Isoamyl alcohol	2 ml

This solution was stored at 4°C

Protease digestion buffer *

1 x SE, pH 8.0	9 ml
10% sodium dodecyl sulphate	1 ml

Proteinase K digestion buffer *

50 mM Tris/HCl, pH 8.0	9 ml
10% sodium dodecyl sulphate	1 ml

RNase digestion solution *

RNase type 1A	40 mg/ml
Dissolved in:	
Tris/HCl, pH 7.5	10 mM
Sodium Chloride	15 mM

Note: To destroy any contaminating DNase activity the preparation was boiled for 15 min then allowed to cool to room temperature.

SE *

EDTA	100 mM
sodium hydroxide	150 mM

SSC

Sodium chloride	150 mM
Trisodium citrate	15 mM

Adjusted to pH 7.0

Stop buffer *

Sodium chloride	20 mM
Tris/HCl, pH 7.5	20 mM
EDTA	2 mM
Sodium dodecyl sulphate	0.25%

TAE buffer

Tris	20 mM
Acetic acid	10 mM
EDTA	5 mM

TE buffer *

Tris/HCl, pH 8.0	10 mM
EDTA	1 mM

Urea based digestion solution (Applied Biosystems 400456) *

Urea	4M
Tris/HCl, pH 8.0	100 mM
Sodium dodecyl sulphate	0.5%
Diaminocyclohexane tetra-acetic acid	10 mM

2.2 Methods

2.2.1 Preparation of Lymphocyte Enriched Mononuclear Cells

These were prepared after the method of Böyum (1968).

Briefly, 40 ml of heparinised (10 U/ml) peripheral blood was divided equally into six universals and an equal volume of RPMI medium added. A half volume of ficoll lymphocyte separation medium was then carefully added to each sample and the tubes were centrifuged at 400 g for 20 min. Mononuclear cells were carefully pipetted from just above the ficoll/plasma interface and transferred to 10 ml tubes and centrifuged again for 5 min at 350 g. Following discard of the supernatant, cells were resuspended in 10 ml of RPMI medium containing 2% penicillin streptomycin and 2% foetal calf serum and tubes centrifuged for 5 min at 200 g. After discarding the supernatant, the samples were pooled, made up to 5 ml with the RPMI medium plus additives solution and a sample withdrawn for haemocytometer counting. The suspension was made up to 10 ml and centrifuged for 5 min at 90 g. Following discard of supernatant, samples were resuspended in freezing medium comprising 64% RPMI medium, 18% foetal calf serum, 8% penicillin streptomycin and 10% dimethyl sulphoxide to give a concentration of 1 or 2 x 10^7 cells/ml. The aliquots were then placed at -70°C for 2 h prior to storage in vapour phase liquid nitrogen.

2.2.2 Collection, Initial Preparation and Paraffin wax embedding of Tissue

Fresh tissue specimens of palatine tonsil were transported dry from the theatre with the minimum of delay in sealed containers. Details of age and sex of tissue was recorded and each tissue was given a separate reference number from the laboratory daybook. A cryotube or plastic bag was also labelled with the reference number. For each sample representative pieces of tissue were dissected, each not exceeding 0.5 x 0.5 x 0.2 cm.

These were either frozen for a minimum of 30 seconds in isopentane pre-cooled in liquid nitrogen then transferred to the appropriate cryotube or bag and held in liquid nitrogen or immersed in appropriate fixative. Frozen tissue was transferred from liquid nitrogen to a -70°C freezer for storage.

Tissue was removed from fixative at specified intervals and paraffin wax processed as described below or excess fixative washed from the tissue prior to DNA extraction (Method 2.2.7b).

For paraffin wax embedding tissue was transferred to labelled processing cassettes and held in 70% isopropyl alcohol (IPA)in water before initiating processing the same day. Care was taken to ensure that tissues that had been immersed in different fixatives were held in separate containers of IPA.

Paraffin wax processing was undertaken using a Shandon 2L carousel tissue processor using following schedule: 70% IPA in tap water for 2 h, 95% IPA in tap water 1 h, absolute IPA 6 changes of 1 h, xylene 2 changes of 1.5 h and paraffin wax (56°C melting point) at 65°C for 2 changes of 2 h. The tissue was then embedded in paraffin wax using a suitable mould and stored at room temperature.

2.2.3 Paraffin Wax Sectioning

A disposable microtome blade (Feather S35) was moistened with xylene to remove its protective coating then secured in the knife holder on the microtome (Reichart Jung 2030). The wax block was then secured in the microtome chuck. Using the coarse advance excess wax was the trimmed away from the surface of the tissue to expose the embedded tissue. The surface of the block was then polished by cutting a few sections at 4-10 μm and removed from the microtome and placed on ice to cool.

To cut sections the block was removed from the ice and resecured in the microtome chuck. A new disposable blade was also secured in the microtome holder. Section thickness was

the adjusted to 4 μm for cutting sections for histological staining or thicker if sections were to be used for DNA extraction. To avoid possible cross contamination of samples the microtome blade was cleaned with xylene between the cutting of each tissue block and wax trimmings were removed from the microtome using a tissue soaked in the solvent. Similarly, forceps used to transfer cut sections were also cleaned with xylene before use with a new tissue block.

For histological staining sections were transferred to a floating out bath containing reverse osmosis treated water held at just below the melting temperature of the paraffin wax. The sections were picked up on labelled standard 76 x 22 mm microscope slides, dried at 37°C or 60°C then stored at room temperature prior to staining. For DNA preparation the sections were transferred into labelled screw capped sterile polypropylene tubes and stored at room temperature.

2.2.4 Haematoxylin and Eosin Staining

Paraffin wax sections were dewaxed and rehydrated by sequential immersion in xylene, 2 changes of 3 min, 99% Industrial methylated spirits (IMS), 2 changes of 1 min, 95% IMS in water for 1 min and running tap water for 1 min

Slides (or smears) were immersed in Mayer's Haematoxylin for 5 min then washed in running tap water for 1 to 2 min. Microscopic examination was then undertaken to check for the intensity and distribution of Haematoxylin staining. If staining was confined to the nucleus the slides were taken forward for eosin staining. However, when cytoplasmic and interstitial Haematoxylin staining was present the slides were immersed in acid - alcohol for 1-2 seconds to remove excess stain. Following a running tap water wash for 1 to 2 min the slides were re-examined microscopically. The steps of acid - alcohol, washing and examination were repeated until only nuclear staining was observed.

The slides were immersed in eosin for 1 min then briefly washed in running tap water. Dehydration was undertaken by sequential immersion in 95% IMS in tap water for 15 secs, 99% IMS, 2 changes of 1 min. The slides were then cleared by immersion in xylene, 2 changes of 3 min, and the sections were coverslipped using a resinous mountant.

2.2.5 Assessment of the Reaction of Fixatives with Lambda DNA

Lambda DNA was dissolved in TE at a concentration of 100 µg/ml. To 10 µl aliquots of this solution 90 µl of fixatives or control solutions were added. The solutions were then incubated for 5, 15 and 60 min at 4°C, room temperature or 37°C. Reaction was terminated by precipitation of the nucleic acid by the addition of sodium chloride to 100 mM, 200 µl absolute ethanol (pre-cooled to -20°C), and storage for at least 30 min at -70°C. Samples were then centrifuged at 12000 g for 10 min at 4°C, the precipitate washed in 80% ethanol/20% pure water (pre-cooled to -20°C), re-centrifuged as before and resuspended after vacuum drying for 15 min in 10 µl TE at 4°C.

Resuspended samples were restricted in a solution containing the nucleic acid in 17 µl TE, 1 µl of 25 U/µl *Hind* III and 2 µl of 10 times reaction buffer for 4 h at 37°C. Following incubation samples were analysed by electrophoresis according to method 2.2.10.

Note: Phosphate containing solutions inhibited restriction endonuclease digestion. Accordingly this component was removed by centrifuging appropriate samples at 1600 g through a 1 ml spun column according to the method described by Sambrook *et al* (1989).

2.2.6 Optimised Method for the Extraction of DNA From Cells

100 µl (2 x 10^6 cells) aliquots of lymphocyte enriched mononuclear cells were transferred to Eppendorf tubes and fixed with 900 µl of fixative at room temperature for the required time. Fixative was removed by washing three times in 1 ml of 50 mM Tris/HCL, pH 7.6, each wash being preceded by pelleting of samples by centrifugation for 1 min at 2800 g. After discard of the last wash solution the cells were resuspended in 1 ml of appropriate digestion buffer and 200 µg of protease VII or Proteinase K added. The preparations were incubated at 37°C for 2 h for Carnoy fixed preparations and 48 h for formol saline fixed

samples. Additional enzyme (200 µg) was added to the 48 h digestion preparations after 24 and 44 h incubation.

Digests were purified by three 800 µl phenol/chloroform/isoamyl alcohol extractions followed by removal of phenol by two 800 µl chloroform/isoamyl alcohol separations. For the first two 'phenol' extractions the layers were separated by standing. For the third 'phenol' and both 'chloroform' separations, the interface was formed by centrifugation at 12000 g for 5 min. To improve the final yields of nucleic acid, the samples were back extracted with 600 µl of relevant digestion buffer.

Primary and back extractions were pooled and nucleic acids precipitated by addition of sodium chloride to 0.2 M, 2 volumes of absolute ethanol (pre-cooled to –20°C) and storage at –70°C for 30 min. Nucleic acid was pelleted by centrifugation at 12000 g for 5 min at 4°C. The supernatant was discarded and the pellet washed with 1 ml of 80% ethanol/20% pure water (pre-cooled to –20°C) to remove salt. After mixing, the samples were centrifuged as above, supernatant discarded and pellets vacuum dried for 15 min before resuspension and storage in 1.5 ml 1 x TE at 4°C.

2.2.7 Preparation of Tissue for DNA Extraction

a) Fresh frozen tissue

A piece of tissue was removed from storage at -70°C, weighed then diced and placed in a 15 ml polypropylene tube.

b) Fixed, unprocessed tissue

A piece of tissue was removed from fixative and weighed. The pieces were diced and transferred into a sterile 15 ml polypropylene tube. To remove extraneous fixative, the fragments were then washed in three 1 h changes of sterile 50 mM Tris/HCl, pH 8.0. For each step, 10 ml of buffer was added and centrifugation at 2000 g for 5 min was employed between each solution change to pellet the fragments.

c) Paraffin wax embedded tissue

The dry weight of the tissue for DNA extraction was recorded, the tissue was diced, placed in a sterile 15 ml polypropylene tube and rehydrated according to the following schedule: xylene, 1 change 30 min then 2 changes 15 min, 99% industrial methylated spirit (IMS), 1 change 30 min then 2 changes 15 min, 95% IMS, 1 change 30 min then 1 change 15 min and sterile 50 mM Tris/HCl, pH 8.0, 1 change 30 min then 2 changes 15 min. For each step, 10 ml of solvent was added and centrifugation at 2000 g for 5 min was employed between each solution change to pellet the fragments. Rehydrated samples were:

Homogenised in digestion buffer for 1 min using a Ystral X10/20 homogeniser fitted with a 20T shaft and fine generator set at low speed.

or

Finely diced into pieces no larger than 2 mm^3 then placed into the digestion buffer.

d) Paraffin wax sections

Excess paraffin wax was trimmed from the embedded block of tissue then sections were cut at 15 μm and transferred to a sterile 30 ml Corex tube (DuPont 03163). The sections were rehydrated by sequential immersion for 10 min each in xylene (3 changes), 99% industrial methylated spirits (3 changes) and 95% IMS in water (2 changes) and sterile water (2 changes). Centrifugation at 2000 g for 5 min was employed between each solution change to pellet the fragments. Following the removal of the water the sections were resuspended in the digestion buffer.

e) Dialysis against amino acids and buffer

100 mg quantities of tissue sections were rehydrated separately according to the method d) and dialysed in three changes of 200 mM glycine, cysteine or 50 mM Tris/HCl pH 8.0 over

a period of 24 h. Following dialysis the preparations were then washed in three 40 ml changes of 50 mM Tris/HCl pH 8.0 to remove amino acids with centrifugation between each wash.

2.2.8 Extraction of DNA from Fixed Tissues

Tissue preparations were digested in 50 mM Tris/HCl, pH 8.0 containing 1% sodium dodecyl sulphate and 100 μg/ml of Proteinase K or protease XXIV. Digestion was undertaken at 37°C with gentle agitation. For unfixed samples overnight incubation was employed whilst for fixed tissue digestion times varied from 24 to 168 h. In the case of fixed tissue digestions the proteolytic enzyme was replenished by the additional 100 μg/ml of Proteinase K or protease XXIV at 24 h intervals.

Nucleic acid was purified from the digested proteins using three 3 ml changes of phenol/chloroform/isoamyl alcohol and three 3 ml changes of chloroform/iso amyl alcohol. For each step, the organic solutions were added to the aqueous digest solution and the polypropylene tubes gently inverted to mix the phases. To separate the organic and aqueous phases the tubes were centrifuged for 30 min in the case of the phenol solutions and 15 min in the case of the chloroform solutions at 2000 g. Care was taken to transfer the aqueous phase without disturbing the interphase with the organic layer and all samples were back extracted with 2 ml of sterile 50 mM Tris/HCl, pH 8.0. After the final chloroform wash the aqueous phase was transferred to 30 ml sterile Corex tubes.

To the solutions in the Corex tubes sodium chloride was added to give a final concentration of 0.1 M and two volumes of absolute ethanol (pre-cooled to -20°C) to precipitate the DNA. Following overnight storage at -20°C to allow maximum precipitation of DNA the preparations were centrifuged at 17000 g for 10 min at 10°C to pellet extracted DNA. After discarding the supernatant the pellets were washed in 2 ml of 80% absolute ethanol/20% pure water (pre-cooled to -20°C) and centrifuged as before. Following the discard of the supernatant the sample tubes were inverted for 15 min to drain away excess ethanol before adding 1 x TE to resuspend the nucleic acid at 4°C. Once resuspension was

complete the solutions were transferred to sterile Eppendorf tubes and stored at 4°C

2.2.9 Quantitative Analysis of DNA Preparations by Optical Density Measurement

Dilutions of DNA preparations were prepared in 1 x TE. All readings were made in quartz cuvettes against a blank of 1 x TE using a dual beam Pye Unicam SP1700 ultraviolet spectrophotometer. The optical density of each preparation was recorded at 230, 260 and 280 nm and from these the following were calculated:

Double stranded nucleic acid in µg/ml

OD 260 nm x dilution of preparation x 50

Carbohydrate contamination

$$\frac{\text{OD 230 nm}}{\text{OD 260 nm}}$$

For minimally contaminated preparations a ratio of 0.5 was expected.

Protein contamination

$$\frac{\text{OD 260 nm}}{\text{OD 280 nm}}$$

For minimally contaminated preparations a ratio of 1.8 was expected. Lower ratio values indicated protein contamination, ratios around 2.0 indicated the presence of RNA in the sample.

2.2.10 Qualitative Analysis of Extracted DNA by Gel Electrophoresis

Extracts were prepared for electrophoresis by:

Concentration (if required) by precipitation with 0.2 M sodium chloride and a minimum of 2 volumes of absolute ethanol (pre-cooled at -20°C) followed by storage at -70°C for a minimum of 30 min. The extracts were then centrifuged at 12000 g for 10 min at 4°C and the supernatant discarded. 0.5 ml of 80% absolute ethanol/20% pure water (pre-cooled at -20°C) was added and centrifugation repeated as above. The supernatant was discarded and the preparations vacuum dried for 15 min prior to resuspension in sterile pure water.

Dilution (typically 5µg) in pure water and 5 x gel loading buffer in ratio of 4 parts to 1 part respectively.

Pre-treatment of 5µg of extract with 4µg of RNase digestion solution for 30 min at 37°C followed by concentration and dilution as described above.

Gels were prepared using 0.8% agarose in 1 x TAE and when set submerged in an electrophoresis tank containing the same buffer. DNA extracts, prepared as previously described, were heated at 65°C for 10 min along with *Hin*d III digested lambda phage size marker then cooled on ice prior to loading onto the gel. Electrophoresis was then undertaken at constant voltage for the required time.

For staining the gel was immersed in 1 x TAE and ethidium bromide added to a concentration of 0.1 µg/ml. Staining was undertaken with gentle agitation for 45 min. The gel was then rinsed with 3 changes of 1 x TAE then placed on a ultra violet transilluminator (UV Products inc. TM-40) for examination. A photographic record of results was made using a Polaroid model MP-4 Land camera fitted with a UV filter and Polaroid type 667 film.

2.2.11 Restriction Enzyme Digestion of DNA Extracts

Aliquots of the DNA extracts were incubated for 4 or 24 h at 37°C (*Bst*N I 60°C) with 2.5 units of restriction enzyme per 1 μg of DNA in appropriate 1x reaction buffer. In 4 h incubations 4 mM spermidine was added after 30 min incubation.

To establish that restriction had been successfully achieved a 2.5 μg aliquot of each preparation was analysed electrophoretically as described in 2.2.10. A photographic record was made then the gels were discarded.

DNA in the remaining preparations was precipitated by adding sodium chloride to a final concentration of 0.1 M and two volumes of absolute ethanol (pre-cooled to -20°C) followed by storage at -70°C for 30 min. The precipitates were pelleted by centrifugation at 12000 g for 10 min at 4°C, washed in 100 μl of 80% ethanol/20% pure water (pre-cooled to -20°C) recentrifuged as before then dried under vacuum for 15 min before resuspension in sterile 1 x TE, at 4°C.

To check that resuspension had been successfully achieved a further 2.5 μg aliquot of each preparation was analysed electrophoretically. A photographic record was made then the gels were discarded.

Each of the remaining preparations was electrophoresed. A photographic record was made and gels retained for Southern blotting according to the method described in 2.2.12.

2.2.12 Southern Blotting of Restricted DNA Preparations

The following methods modified from that described by Southern (1975) were used.

a) Short transfer method

Gels carrying restricted DNA were partially depurinated in 0.25 M HCl for 10 min, rinsed in pure water then denatured in two changes of 30 min of 1.5 M sodium chloride, 0.5 M sodium hydroxide. Following a rinse in pure water the gels were neutralised in 1.5 M sodium chloride, 1 M Tris/HCl, pH 8.0 for 60 min then rinsed again in pure water.

DNA was transferred onto nylon membranes (Amersham RPN 203N) using 20 x SSC for 2 h. The membrane was then removed, dried between sheets of 3MM chromatography paper (Whatman 3030917), baked at 65°C or 80°C for 10 min, wrapped in Saran wrap and DNA immobilised by exposure to UV light at 302 nm for 30 sec. To check efficiency of transfer gels were re-stained and a photographic record made according to method 2.2.10.

b) Long transfer method

The method was identical to method 1 with the following differences; gels were depurinated in 0.25 M HCl for 7 min and DNA was transferred overnight.

2.2.13 Hybridisation of Transferred DNA

a) Church and Gilbert (1984)

Membranes were pre-hybridised for 15 min at 65°C in closed perspex chambers in 0.5 M sodium phosphate buffer, pH 7.2, 1 mM EDTA, 7% sodium dodecyl sulphate and 36% polyethylene glycol 6000.

α^{32}p dCTP labelled cDNA probes (method 2.2.14) were heat denatured then added at a concentration of 0.5 ng/ml to the pre-hybridisation solution and hybridisation undertaken at 65°C overnight.

Post hybridisation washes at 65°C consisted of two changes of 5 min each in 40 mM sodium phosphate buffer, pH 7.2, 1 mM EDTA, 5% sodium dodecyl sulphate and 0.5% bovine serum albumin followed by eight changes of 5 min in 40 mM sodium phosphate buffer, pH 7.2, 1 mM EDTA and 1% sodium dodecyl sulphate.

After drying on 3 mm chromatography paper (Whatman 3030917) and wrapping in Saran, the membranes were autoradiographed using Kodak X-Omat film (1651454) with intensification at -70°C and developed using a Kodak RP X-Omat processor.

b)

Membranes were pre-hybridised for 1 h at 65°C in closed perspex chambers or in a hybridisation oven (Hybaid maxi oven HB-MSOV1-220) in 5 x SSC, 1 x PE, 200 µg/ml sonicated and denatured salmon sperm DNA and 10% polyethylene glycol 6000.

α^{32}p dCTP labelled cDNA probes (method 2.2.14) were heat denatured in 200 µg/ml salmon sperm DNA then added at a concentration of 0.5 ng/ml to the pre-hybridisation solution and hybridisation undertaken at 65°C overnight.

Post hybridisation washes at 65°C consisted of four changes of 10 min each in 2 x SSC, 0.1% sodium dodecyl sulphate then 0.2 x SSC, 0.1% sodium dodecyl sulphate.

After drying on 3 mm chromatography paper and wrapping in Saran, the membranes were autoradiographed using Fuji RX film with intensification at -70°C and developed using a Kodak RP X-Omat processor.

2.2.14 Labelling of cDNA Probes

Probes were labelled using the random primer reaction after the method described by Feinberg and Vogelstein (1984) as follows:

10 ng of cDNA probe was boiled in 10 µl of pure water for 5 min. Immediately following removal from the heat source the following additions were made in the order given:

Oligonucleotide buffer	3 µl
Bovine serum albumin - molecular biology grade	0.6 µl
α^{32}p dCTP, specific activity 3000 ci/mMol	1 µl
Klenow DNA polymerase	0.6 µl

The solution was then incubated overnight at room temperature and the reaction stopped by the addition of 85 µl of stop buffer.

A total DPM count was recorded before separation of the labelled sequences from unincorporated nucleotides using an Oncor Probe Counter. Separation of labelled

sequences was undertaken by exclusion filtration using G50 Sephadex with 1 x TE. The Sephadex was held either in a plugged Pasteur pipette column or contained in a 1 ml spun column prepared according to the method described by Sambrook *et al* (1989). When the column was used twenty 100 µl fractions were collected. Labelled sequences were usually recovered in fractions 8-11 followed by unincorporated nucleotides in fractions 13 onwards. When the spun column was used the labelled sequences were recovered in one 100 µl eluent. The percentage radioactive incorporation was calculated by dividing the sum of the labelled nucleic acid eluent/s by the total DPM reading taken before filtration. Labelled probes were used on the same day as their preparation.

2.2.15 Rapid DNA Preparation Method for use in Polymerase Chain Reaction Amplification

5µm paraffin wax sections were placed into a sterile 500 µl Eppendorf tubes and rehydrated by sequential addition of 400 µl of xylene (2 changes), 99% industrial methylated spirits (3 changes) and 95% industrial methylated spirits (2 changes). The samples were incubated for 5 min in each solvent then centrifuged at 12000 g for 3 min. Solvents were removed using a new pipette tip for each sample and care was taken not to dislodge the tissue pellet. New solvent was then added and the tissue pellet resuspended. Following removal of the last solvent the preparations were vacuum dried for 10 min.

For digestion 400 µl of extraction buffer containing 200 µg Proteinase K was added and the preparations incubated overnight at 58°C. The activity of Proteinase K was then terminated by incubating the preparations at 95°C for 15 min and the samples were stored at 4°C.

2.2.16 Polymerase Chain Reaction Amplification

Preparation of reaction solutions.

Each PCR reaction was undertaken in a total volume of 50 µl in sterile 500 µl Eppendorf tubes. To assist in standardisation of reaction conditions between tubes a master mix was prepared containing reagents common to all PCR reactions. This contained:

10 x reaction buffer	5 µl x number of reactions required
Primer (10 pmol)	1 µl x number of reactions required
Sterile water	x µl to give final reaction volume per tube of 49 µl

Following the aliquoting of the master mix into each reaction tube DNA, diluted (if required) in 1 x TE, was added. This was followed by the addition of 1 µl (10 pmol) of the appropriate second primer. For each primer combination a negative control was also set up by substituting the DNA with same volume of sterile water. The solutions were overlaid with 50 µl of sterile paraffin oil.

Amplification conditions

All amplifications were performed using a Hybaid 'Omnigene' Thermal Cyler. Standard conditions were as follows:

For initiation DNA was denatured at 98°C for 3 min. The programme was then held at an annealing temperature of 58°C to allow 'Hot Start' addition of Taq polymerase. This was followed by extension at 72°C for 2 min.

Each amplification cycle featured; denaturation of DNA at 94°C for 1 min followed by annealing at 58°C for 30 secs and extension at 72°C for 2 min. The total number of cycles inclusive of initial hot start was 30.

At the end of amplification the samples were 'held' at room temperature until removed for storage at 4°C. The products of amplification were then analysed by gel electrophoresis according to the method described in 2.2.17

Note: In some investigations alternative annealing temperatures, extension times and number of cycles were employed. These variations are detailed under the specific investigations recorded in Chapter 5.

2.2.17 Analysis of Products of Polymerase Chain Reaction Amplification by Gel Electrophoresis

Gels were prepared using 3% agarose in 1x TAE and when set submerged in an electrophoresis tank containing the same buffer. To 18µl of each PCR product 2µl of PCR loading buffer was added. The samples were then heated for 10 min at 65°C along with aliquots of 1µg/20µl φX174 RF DNA *Hae* III size marker prior to loading onto the gel. Alternatively the samples were loaded without heating with aliquots of 1µg/20µl 100 bp ladder size marker.

Electrophoresis was undertaken at constant voltage for the required time. For staining the gel was immersed in 1 x TAE and ethidium bromide added to a concentration of 0.1 µg/ml. Staining was undertaken with gentle agitation for 45 min. The gel was then rinsed with 3 changes of 1 x TAE and placed on a ultra violet transilluminator for examination. Images were recorded using a UV Products Image Store 5000 Gel Documentation System coupled with a Sony digital camera. Print outs of the images were made at the time of gel examination and saved to disk as Tiff files.

Chapter 3 Development of methods to recover DNA from fixed cells and tissues and analysis using Southern blotting

3.1 Introduction

In initial investigations the effect of Bouin, Carnoy, formol saline, formol sublimate and neutral buffered formalin fixatives on Lambda DNA and extraction of DNA from fixed cells were studied. The investigations with Lambda DNA provided information on the influence of the fixatives on the molecular weight and restriction endonuclease digestion of the nucleic acid. Additional information on the ease of recovery of DNA from fixed cells by protease digestion was provided using lymphocyte enriched cell populations (Sections 3.2 and 3.3).

In the remaining sections of this chapter investigations using Carnoy, formol saline and neutral buffered formalin fixed and paraffin embedded tissue are recorded. In section 3.4 the compatibility of these preparations with recovery of DNA by protease digestion and Southern analysis for single- and multi-copy genes was assessed. Section 3.5 examines variations to the DNA recovery method which were evaluated with the objectives of shortening the technique and improving the quality of nucleic acid recovered.

3.2 Reaction of fixatives with pure DNA

3.2.1. Experimental design

Fixative mixtures of Bouin, Carnoy, formol saline, formol sublimate, and neutral buffered formalin were prepared according to the formulae provided in 2.1.7. Control solutions of 0.9% sodium chloride, 40 mM sodium phosphate buffer, pH 7.0 and deionised and reversed osmosis treated (pure) water were also prepared and autoclaved to destroy any DNase contamination.

Fixative and control solutions were incubated with Lambda DNA for 5, 15 and 60 min at 4°C, room temperature or 37°C. Following concentration of the nucleic acid samples were digested with *Hin*d III restriction endonuclease then electrophoresed and photographed to assess for any changes. The method is fully described in 2.2.5.

3.2.2 Results

Lambda DNA treated with pure water, 0.9% sodium chloride or 40 mM phosphate buffer pH 7.0 at 4°C, room temperature and 37°C for 5, 15 or 60 min gave identical *Hin*d III restriction patterns to endonuclease digestion without pre-treatment. This restriction pattern consisted of six discrete bands representing size fragments of 23130, 9416, 6557, 4361, 2322 and 2027 base pairs (Fig 3.2.1)

The restriction endonuclease digestion patterns observed after pre-treatment with fixatives are summarised in Table 3.2.1 and illustrated in Fig 3.2.2

Results obtained with Bouin's fixative were temperature dependent. Used at 4°C this fixative had no effect on the restriction of Lambda DNA. However, at room temperature and 37°C, the respective observations of a nucleic acid smear and undigested DNA indicated shearing and inhibition of restriction of DNA.

The effects of pre-treatment of Lambda DNA with formol sublimate were also temperature dependent. Full restriction was only obtained after 4°C pre-treatment and partial restriction was observed in only one sample exposed to the fixative at room temperature. After 37°C pre-treatment whilst the DNA appeared intact, no evidence of restriction was obtained.

The restriction of Lambda DNA by *Hin*d III was essentially unaffected by exposure of the nucleic acid to Carnoy, formol saline and neutral buffered formalin fixatives. A full complement of restriction digest fragments were demonstrated throughout the various time and temperatures of reaction.

Table 3.2.1 *Hind* III restriction endonuclease digestion of Lambda DNA after pre-treatment with fixatives

Fixative	Temperature of pre-treatment (°C)	Time of pre-treatment (min) 5	15	60
Bouin	4	R	R	R
	RT	ND	S	S
	37	NR	NR	ND
Carnoy	4	R	R	R
	RT	R	R	R
	37	PR	R	R
Formol Saline	4	NR	R	R
	RT	R	R	R
	37	R	ND	NR
Formol Sublimate	4	ND	R	R
	RT	ND	NR	PR
	37	NR	NR	ND
Neutral Buffered Formalin	4	R	R	ND
	RT	ND	R	R
	37	R	ND	R

Key:

RT = Room temperature

R = Restricted with size fragments of 23130, 9416, 6557, 4361, 2322 and 2027 base pairs visible

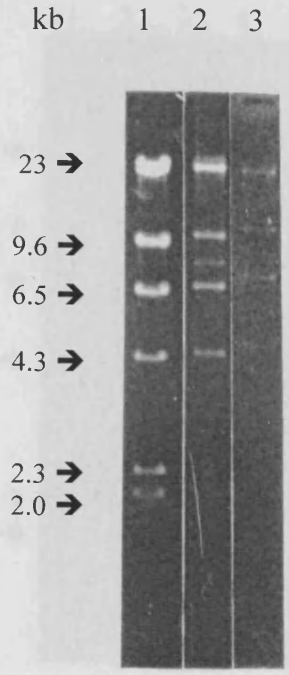
PR = Restricted without full complement of size fragments

NR = DNA not digested by endonuclease

S = Smear of DNA, no restriction bands

ND = No DNA detected in gel

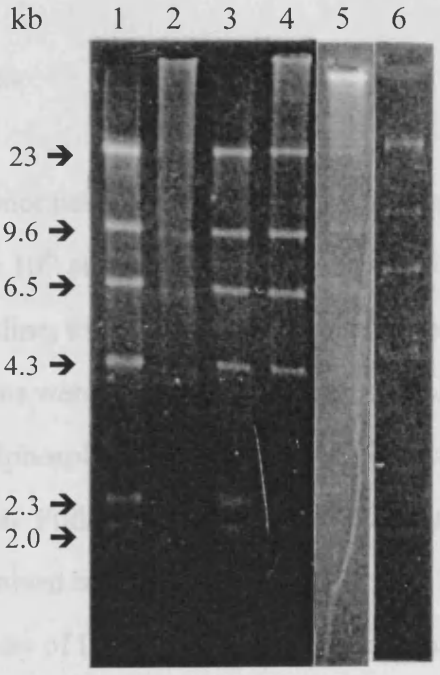
Fig 3.2.1 Electrophoretic analysis of Lambda DNA digested with *Hin*d III restriction endonuclease - Control preparations



Key
Lane 1 Pure water
Lane 2 0.9% Sodium chloride
Lane 3 40 mM Phosphate buffer

All preparations were treated with the reagents for 15 min at room temperature

Fig 3.2.2 Electrophoretic analysis of Lambda DNA digested with *Hind* III restriction endonuclease - after pre-treatment with fixatives



Key

Lane 1 Pure water	Lane 4 Formol Saline
Lane 2 Bouin	Lane 5 Formol Sublimate
Lane 3 Carnoy	Lane 6 Neutral buffered formalin

All preparations were treated in the reagents for 15 min at room temperature

3.3 Recovery of DNA from fixed cells

3.3.1 Experimental design

Lymphocyte enriched mononuclear cell samples (2.2.1), were used in each experiment as a source of intact cells. 2 x 10^6 aliquots of these cells were fixed at room temperature in Bouin, Carnoy, formol saline, formol sublimate or neutral buffered formalin according to investigation. Cell aliquots were also exposed to control solutions of 40 mM sodium phosphate buffer pH 7.0 (phosphate buffer), 0.9% sodium chloride (saline) or sterile water under the same conditions. Following washing and centrifugation to remove the fixative smears were made and stained by haematoxylin and eosin (2.2.4) to assess cell numbers and preservation. Recovery of DNA was then attempted using either protease VII or Proteinase K digestion in the presence of 1% sodium dodecyl sulphate. The appearance of each solution was noted at the commencement of digestion, during incubation and at the end of the digestion period. Extracted DNA was then purified from digested cellular components using phenol and chloroform washes.

A quantitative assessment of the DNA recovered and its purity was made according to the method described in 2.2.9. This was followed by concentration of the preparations and submerged gel electrophoresis (2.2.10) to provide a qualitative assessment of the recovered DNA. In some investigations *Eco*R 1 restriction enzyme digestion was attempted and in one investigation the products of digestion were further analysed by Southern blotting (2.2.12). A summary of methods used in the investigations is provided in Table 3.3.1 and a description of the optimised method for recovery of DNA from fixed cells is described in 2.2.6.

3.3.2 Results

Several steps were found to influence the recovery of DNA from cell preparations. Choice of buffer for washing following fixation influenced the number and preservation of cells (Table 3.3.2). Of the buffers evaluated 50 mM Tris/HCl, pH 7.6 was found to give best conservation of cell numbers and cytology. Choice of enzyme for digestion did not affect the recovery of DNA. However, duration of incubation time was important. Retention of nucleic acid during phenol/chloroform purification was enhanced by avoidance of centrifugation to separate the aqueous and organic layers and the use of back extraction procedures.

In investigation 1 and 2 the Lymphocyte enriched cell preparations were fixed in Bouin, Carnoy, formol saline, formol sublimate and neutral buffered formalin for 30 min at room temperature. Control preparations were exposed to phosphate buffer, saline and sterile water. Digestion was undertaken for 2 h using protease VII. During digestion Carnoy and control preparations became viscous and at the end of the digestion period no cell fragments remained in any solution. Whilst optical density readings indicated the recovery of nucleic acid in all preparations DNA was consistently observed by gel electrophoresis only in control and Carnoy preparations. The DNA in these preparations was of high molecular weight and present at the exclusion zone (Fig 3.3.1). DNA was observed only in one Bouin preparation (Fig 3.3.2). In contrast to the other preparations a smear of DNA originating at about 6 kb was present. These gels also provided evidence of successful but not consistent *Eco*R I restriction.

Table 3.3.1
Summary of methods used in investigations to recover DNA from fixed cells

Investigation	Fixatives and controls	Fixation	Wash buffer	Digestion	1° Purification		2° Purification
					Reagents	Centrifugation	
1	B,C,Fsal, Fsub, NBF, Sal, PO4, H_2O	30 min	SE	200 _g protease XXIV in SE, 2hr	2 ml P/C/AA x 2 1.5 ml C/AA x 2	17000g, 10°C, 15 min 1500g, RT, 10 min	Sodium acetate/ethanol precipitation, dialysis with TE 64 hr
2	As in 1	As in 1	PBS	As in 1	0.5 ml P/C/AA x 2 0.5 ml C/AA x 2 with back extraction	12000g, RT, 5 min 12000g, RT, 5 min	Dialysis overnight with TE
3	Carnoy	0.5, 2, 4 and 24 hr	Tris, pH 7.6	As in 1	1 ml P/C/AA x 3 1 ml C/AA x 3 with back extraction	12000g, RT, 10 min 12000g, RT, 10 min	Sodium chloride and ethanol precipitation
4	Fsal, Fsub, NBF, H_2O	As in 1	As in 3	200 _g/ml proteinase K in Tris, pH 7.6, 2 and 24 hr	As in 3	As in 3	As in 3
5	Fsal, Sal	As in 1	As in 3	200 _g/ml proteinase K in Tris 2, 24 and 48 hr	P/C/AA x 4 C/AA x 3	As in 2	As in 3
6	Fsal, Sal	As in 1	As in 3	100 _g/ml proteinase K in Tris or 100 _g/ml protease XXIV in SE 2, 24 and 48 hr	0.5 ml P/C/AA standing separation x 2 0.5 ml P/C/AA x 1 0.5 ml C/AA standing separation x 2 0.5 ml C/AA x 1 with back extraction	12000g, RT, 10 min 12000g, RT, 10 min	As in 3

Notes: All digestion solutions contained 1% sodium dodecyl sulphate and were incubated at 37°C
Back extraction was undertaken using the same buffer as used in digestion

Abbreviations: Fixatives B = Bouin, C = Carnoy, Fsal = Formol saline, Fsub = Formol sublimate, NBF = Neutral buffered formalin.
Controls Sal = Saline, PO4 = 40 mM Phosphate buffer pH7.2, H_2O = Sterile water
Tris = 50 mM Tris/HCl, P/C/AA = Phenol/Chloroform/Isoamyl alcohol, C/AA = Chloroform/Isoamyl/alcohol
RT = Room temperature

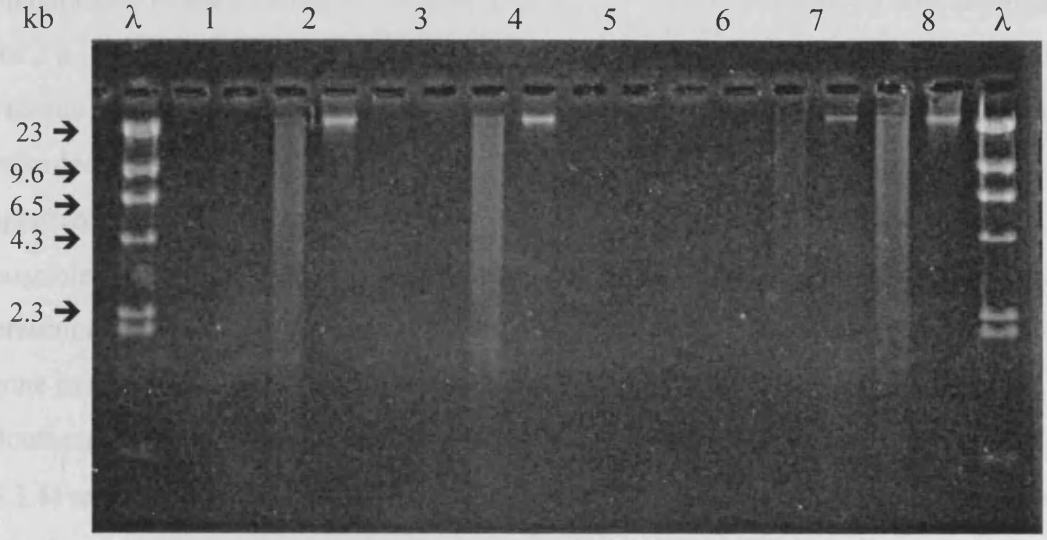
Table 3.3.2 Appearance of cell smears after exposure to "wash" solutions

Solution	Estimate of cell numbers	Cytology of cells
Phosphate buffer	Several	Some well preserved others "ghosts" *
Phosphate, saline	Several	Some well preserved others "ghosts"
Saline	Few	Some well preserved others "ghosts"
SE	Few	Nuclei often smeared, no cytoplasm
100 mM EDTA	Several	Nuclei often smeared, no cytoplasm
50 mM Tris/HCl, pH7.6	Many	Some well preserved others "ghosts"
Freezing medium **	Many	Some well preserved others "ghosts"

* "Ghosts" described cells in which only the remnants of cell membranes remained.

** This comprised 64% RPMI medium, 18% foetal calf serum, 8% penicillin streptomycin and 10% dimethyl sulphoxide.

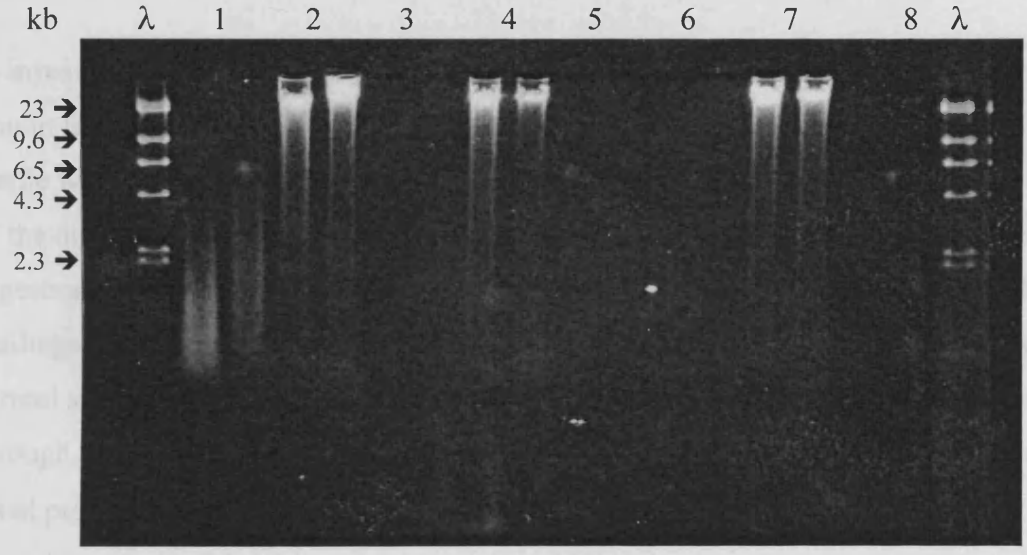
Fig 3.3.1 Investigation 1: Electrophoretic analysis of preparations digested with protease VII for 2 h



Key: Note: All samples restricted then unrestricted

λ Lambda *Hind* III size marker	1 Bouin	2 Carnoy
3 Formol saline	4 Saline	5 F Sublimate
6 Buffered formalin	7 Phosphate buffer	8 Water

Fig 3.3.2 Investigation 2: Electrophoretic analysis of preparations digested with protease VII for 2 h



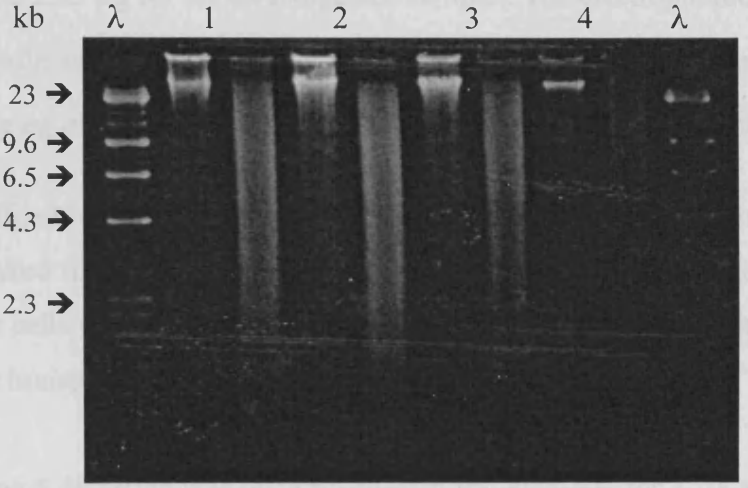
Key: See Fig 3.3.1

In investigation 3 the effect of prolonged fixation in Carnoy was assessed by exposing cell preparations to the fixative for 30 min, 2, 4, and 24 h prior to digestion with protease VII for 2 h. At the end of the digestion period all solutions were clear of cell fragments and viscous. Optical density measurements were abnormal with very high readings being recorded at 230 nm decreasing through 260 and 280 nm. This may explain why the apparent DNA concentration in all preparations exceeded that of the 12 μg theoretically possible from the 2×10^6 cells submitted to fixation. Electrophoretic analysis revealed the presence of high molecular weight DNA divided between the loading well and exclusion zone in all unrestricted samples together with a *Eco*R I restriction smear (Fig 3.3.3). Southern blotting of this gel with a Tβ probe was undertaken. The results obtained (Fig 3.3.4) revealed expected germline bands at 12 and 4.2 kb in the restricted preparations. An additional band at approximately 6 kb was present in unrestricted and restricted preparations. This may indicate the presence of contaminating plasmid in one of the solutions used in the extraction procedures.

In contrast to the ease with which DNA was recovered from Carnoy fixed cells the nucleic acid proved difficult to extract from preparations exposed to cross-linking fixatives.

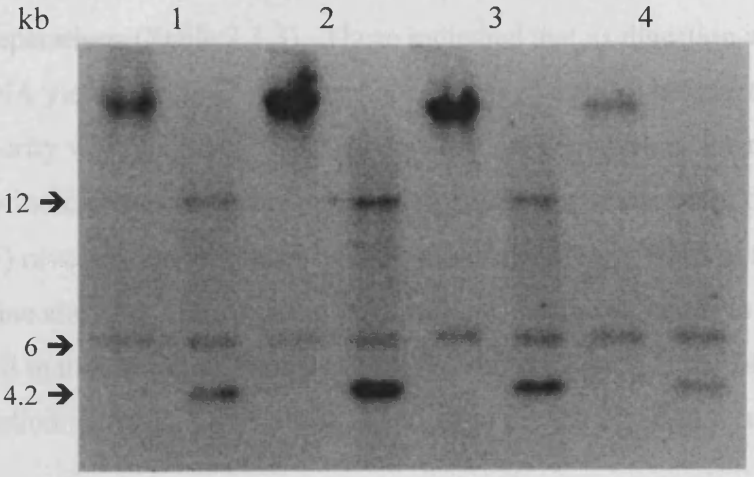
In investigation 4 recovery of DNA was attempted using Proteinase K after fixation for 30 min in formol saline, formol sublimate and neutral buffered formalin. Cells exposed to sterile water were used as controls and digestion times of 2 and 24 h were used. At the end of the digestion periods all solutions were clear of cell fragments. In contrast to the 2 h digestion preparations the 24 h digested fixed samples were viscous. Low optical density readings were recorded in all fixed preparations and the measurements were abnormal for formol sublimate samples with highest readings being recorded at 230 nm decreasing through 260 and 280 nm. A yield of DNA of 1.85 μg, was recorded for the formol saline fixed preparation subject to 24 h digestion.

Fig 3.3.3 Investigation 3: Electrophoretic analysis of Carnoy fixed preparations digested with protease VII for 2 h



Key: Note: All samples unrestricted then restricted
λ Lambda *Hin*d III size marker 1 24 h fixation 2 4 h fixation
3 2 h fixation 4 30 min fixation

Fig 3.3.4 Investigation 3: Southern analysis of Carnoy preparations



Key: See Fig 3.3.3

DNA yields for the water control samples were higher at 4.0 μg for the 2 h digested preparation and 2.05 μg for the 24 h digested sample. The electrophoretic gel of the preparations reflected the optical density measurements with faint high molecular weight DNA bands being observed in the 24 h formol saline and both water control samples only.

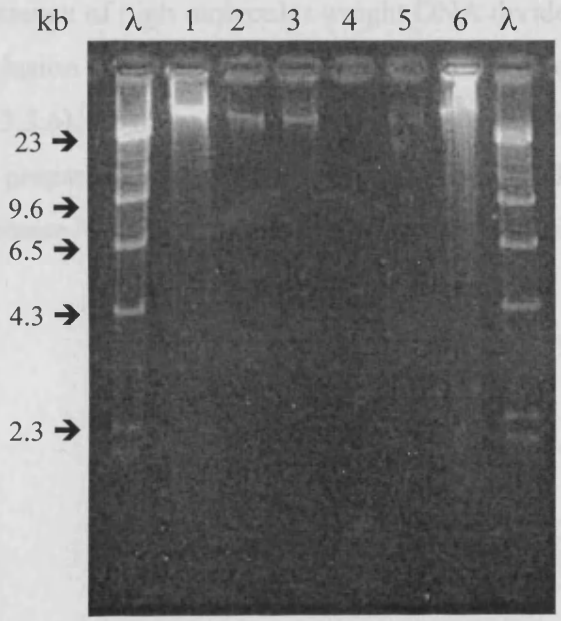
Due to the difficulties outlined above the final investigations (5 and 6) to recover DNA from cross-linked fixed cells were limited to the use of formol saline fixative. In these investigations cells were exposed to saline as a control. Exposure to fixative and control solutions was limited to 30 min.

In investigation 5 digestion was undertaken with Proteinase K for 2, 24 and 48 h with additional enzyme being added to the 48 h sample after 24 h incubation. At end of the digestion periods all preparations were viscous and clear of cell fragments. Optical density readings for the 2 h digested preparations were atypical, very high 230 nm readings being followed by high 260 nm and low 280 nm readings. Consequently these readings were disregarded. In contrast expected optical density readings were obtained for the 24 and 48 h digested preparations (Table 3.3.3). These indicated that as digestion was extended from 24 to 48 h DNA yield increased. For formol saline preparations satisfactory carbohydrate and protein purity was obtained. For the saline control preparations the ratio obtained for protein purity indicated the possible presence of RNA. Examination of the electrophoretic gel (Fig 3.3 5) revealed intense bands of high molecular weight DNA at the exclusion zones of the 2 h saline and 48 h formol saline preparations. Moderate bands in the same region were observed in the 24 and 48 h saline controls and weak band in the 24 h digested formol saline preparation. No nucleic acid was observed in the 2 h digested formol saline sample.

Table 3.3.3 Investigation 5: DNA concentration and protein purity of preparations

Preparation	Digestion	DNA μg	Protein purity
Formol saline	24 h	2.35	1.9
Formol saline	48 h	5.95	1.7
Saline	24 h	2.30	2.19
Saline	48 h	3.25	1.97

Fig 3.3.5 Investigation 5: Electrophoretic analysis of preparations



Key

λ Lambda *Hind* III size marker

Lanes 1-3 Samples pre-treated with saline for 30 min, digested with Proteinase K for 2, 24 and 48 h

Lanes 4-6 Samples fixed in formol saline for 30 min, digested with Proteinase K for 2, 24 and 48 h

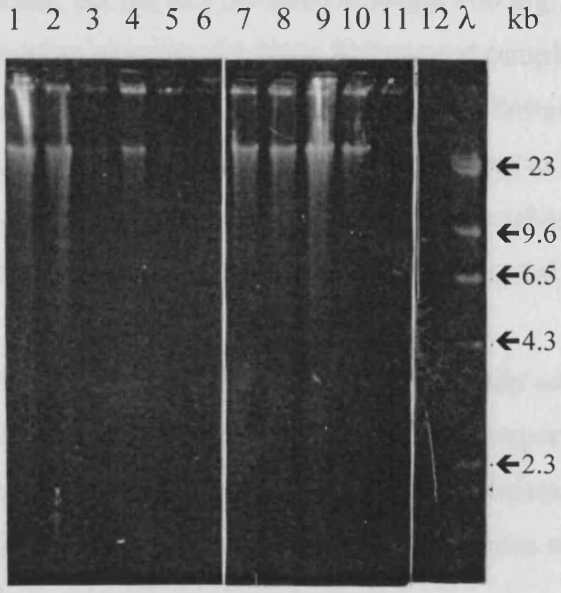
In investigation 6 Proteinase K and protease VII digests were set up with incubation times of 4, 24 and 48 h being employed. Additional enzyme was added to the 24 h incubations after 16 h and 48 h incubations after 24 and 44 h incubation. At end of the digestion periods all preparations were viscous and clear of cell fragments.

DNA yields and protein purity of the preparations are recorded in Table 3.3.4. With the exception of the 4 h protease VII digested preparation yields of DNA from saline control samples were similar. In contrast yields of DNA from formol saline fixed preparations increased with prolonged digestion time. The protein purity of all preparations was satisfactory. The presence of high molecular weight DNA divided equally between the loading well and exclusion zone was observed in all saline preparations submitted to gel electrophoresis (Fig 3.3.6). In these samples the most intense bands was associated with the 4 h Proteinase K preparation. In contrast DNA was observed only in the 48 h Proteinase K and protease VII digested formol saline fixed samples.

Table 3.3.4 Investigation 6: DNA concentration and protein purity of preparations

Preparation	Enzyme	Digestion	DNA μg	Protein purity
Formol saline	Proteinase K	4 h	2.10	1.83
Formol saline	"	24 h	2.45	1.89
Formol saline	"	48 h	4.65	1.69
Saline	"	4 h	6.45	1.65
Saline	"	24 h	6.20	1.82
Saline	"	48 h	5.55	1.79
Formol saline	Protease VII	4 h	1.85	1.76
Formol saline	"	24 h	3.25	1.67
Formol saline	"	48 h	3.45	1.97
Saline	"	4 h	1.85	1.76
Saline	"	24 h	6.70	1.72
Saline	"	48 h	8.75	1.86

Fig 3.3.6 Investigation 6: Electrophoretic analysis of preparations



Key

Lanes 1-3 Samples pre-treated with saline for 30 min, digested with protease VII for 48, 24 and 4 h

Lanes 4-6 Samples fixed in formol saline for 30 min, digested with protease VII for 48, 24 and 4 h

Lanes 7-9 Samples pre-treated with saline for 30 min, digested with Proteinase K for 48, 24 and 4 h

Lanes10-12 Samples fixed in formol saline for 30 min, digested with Proteinase K for 48, 24 and 4 h

λ Lambda *Hin*d III size marker

3.4 Recovery and Southern Blot Analysis of DNA from Fixed and Paraffin Wax Embedded Tissue

3.4.1 Experimental design

Fresh palatine tonsil was obtained and processed according to the method described in 2.2.2. Unfixed tissue blocks were stored at -70°C. The remaining tissue was fixed for 6 or 24 h in Carnoy, formol saline or neutral buffered formalin then processed for paraffin wax embedding.

With reference to a haematoxylin and eosin stained section (2.2.4) an area of tonsil rich in lymphoid tissue was selected, cut out and trimmed to weigh 100 mg. The sample was then diced and rehydrated according to method 2.2.7c. Rehydrated samples, together with 100 mg of case matched unfixed tissue, were homogenised prior to Proteinase K or protease XXIV digestion. The duration of digestion time varied according to sample and investigation. Digests were purified using phenol chloroform washes and nucleic acid recovered by salt and ethanol precipitation. This method is recorded in 2.2.8.

A quantitative assessment of the recovered nucleic acid was made according to the method described in 2.2.9. This was followed by concentration of the preparations and gel electrophoresis (2.2.10) to provide a qualitative assessment of the nucleic acid recovered. To distinguish low molecular weight DNA from RNA preparations of the extracts were pre-treated with RNase Type 1A before electrophoresis.

Aliquots of the extracts were incubated with the following restriction enzymes in the presence of 4 mM spermidine: *Msp* I and *Bst*N I for subsequent pHY 2.1 probing, *Eco*R I and *Bam*H I for Tβ probing and *Hin*d III and *Bgl* II for JH probing. Southern blotting was undertaken according to the method described in 2.2.12. cDNA probes were labelled with 32_p dCTP using the random primer method (2.2.14) and hybridisation was undertaken using the methods described in 2.2.13.

3.4.2 Results

Carnoy fixed tissue

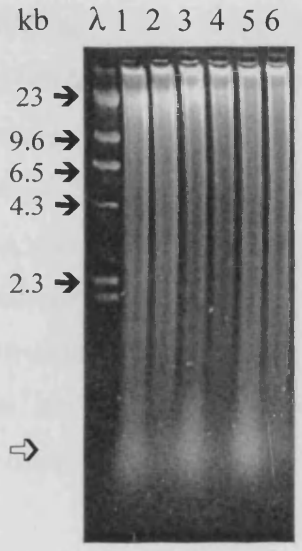
The results obtained with the extraction of DNA from Carnoy fixed cells indicated that short proteolytic digestion times would be sufficient to extract the nucleic acid from paraffin wax embedded tissue. Accordingly preparations from tissue fixed for 6 and 24 h were digested with Proteinase K and protease XXIV for 24, 48 and 96 h. Unfixed control tissue sample was digested for 16 h. At the start of digestion of fixed tissue fine pieces of tissue approximately 1 mm^3 were dispersed in clear fluid. After 24 h the fluid was viscous and contained fewer fragments. The digestion solution remained viscous thereafter and by 96 h no tissue fragments remained.

Nucleic acid was recovered from all samples. Yields after Proteinase K digestion were consistent over the digestion times. The highest yield at 12.1 μg/mg tissue was recorded after 2 day digestion of 24 h fixed tissue. Similar yields, maximum 13.1 μg/mg tissue after 2 day digestion of 6 h fixed tissue, were recorded after protease XXIV digestion for 48 or 96 h. The recovery of nucleic acid using this enzyme was reduced by about 50% when 24 h incubations were employed.

Qualitative analysis by electrophoresis revealed high molecular weight DNA in all extracts which was associated with some lower molecular weight components (Fig 3.4.1a). RNA was also demonstrated in all extracts.

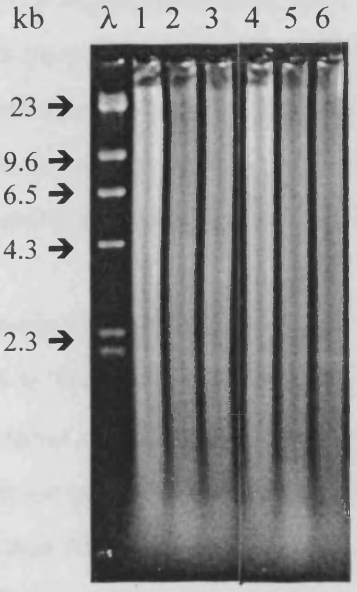
Proteinase K and protease XXIV extractions gave identical restriction and hybridisation results. In comparison with unfixed restriction digests those obtained with Carnoy fixed extracts were more weakly stained. The electrophoretic mobility of the fixed digests was, however, identical to the unfixed preparations (fig 3.4.1b).

Fig 3.4.1a Electrophoretic analysis of 24 h Carnoy fixed preparations digested with Proteinase K



Key: λ Lambda *Hin*d III size marker
Lanes 1-2 96 h digestion Lanes 3-4 48 h digestion Lanes 5-6 24 h digestion
Note: RNA marked by open arrow, present in lanes 1, 3, 5 and removed by pre-treatment with RNase in lanes 2, 4, 6.

Fig 3.4.1b Electrophoretic analysis of 24 h Carnoy fixed preparations digested with restriction endonucleases



Key: λ Lambda *Hin*d III size marker
Lanes 1 & 4 unfixed control Lanes 2 & 5 24 h extract Lanes 3 & 6 96 h extract
Lanes 1-3 *Bgl* II restriction Lanes 4-6 *Hin*d III restriction

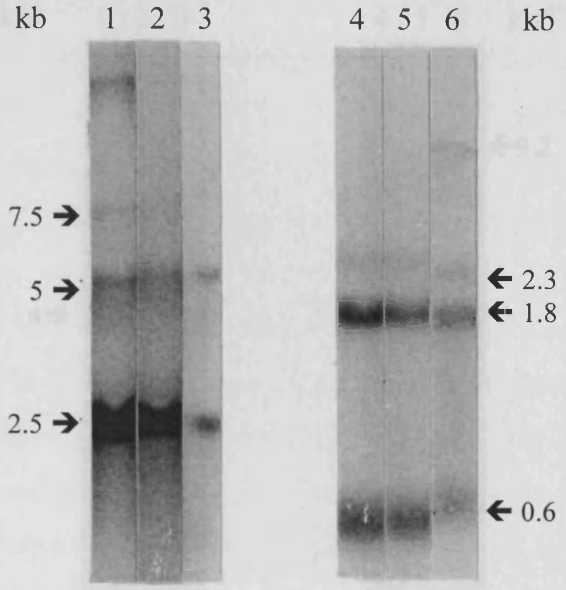
No differences were observed between the transfer of Carnoy fixed DNA and unfixed nucleic acid. Hybridisation, using method (a) as described in 2.2.13, with the pHY 2.1 probe was successfully achieved (Fig 3.4.2a). Using the Tβ probe identical *Eco*R I restriction fragments to unfixed DNA were observed when using the 6 and 24 h extracts, albeit at reduced intensity. However, the 24 kb restriction fragment associated with the *Bam*H I digest was not observed in the Carnoy fixed preparations (Fig 3.4 2b). Hybridisation with the JH probe using method (a) resulted in very high background staining of the membrane which rendered interpretation difficult. However, using hybridisation method (b) restriction fragments were observed with 6 and 24 h fixed extracts digested with *Bgl* II and *Hin*d III preparations. In comparison with the unfixed preparation these were identical, but of substantially weaker intensity (Fig 3.4 2c).

Formol saline fixed tissue

The lymphocyte cell results indicated that the recovery of DNA from fixed tissue would require extended proteolytic digestion. Accordingly preparations fixed in formol saline for 6 and 24 h were digested for 24, 96 and 168 h. The unfixed control tissue sample was digested for 16 h. At the start of digestion of the fixed samples fine pieces of tissue approximately 1 mm^3 were dispersed in clear liquid. After 24 h the tissue fragments had coalesced into a loose gelatinous lump. As digestion continued this dispersed into fine fragments which were present in an increasingly viscous solution. After 168 h digestion some fragments remained, especially in the protease XXIV samples.

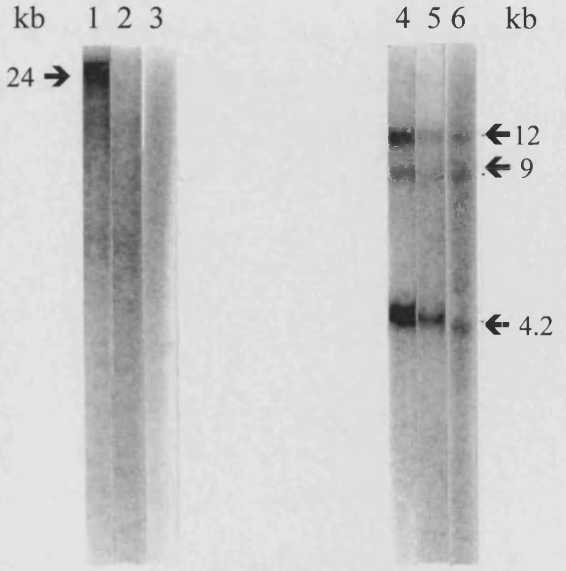
Nucleic acid was recovered from all samples. Highest yields were recorded after Proteinase K digestion for 96 h. The maximum value was 17 μg/mg tissue obtained from the 24 h fixed sample. In contrast the amount of nucleic acid recovered using protease XXIV showed a gradual increase to give a maximum yield after 168 h digestion. With this enzyme the maximum value was 13 μg/mg tissue obtained from the 24 h fixed sample. The 260/280 ratio of Proteinase K extracts suggested the presence of RNA. This was confirmed when the samples were analysed electrophoretically (Fig 3.4.3a). The gels also

Fig 3.4.2a Southern analysis of Carnoy fixed DNA hybridised with pHY 2.1 probe



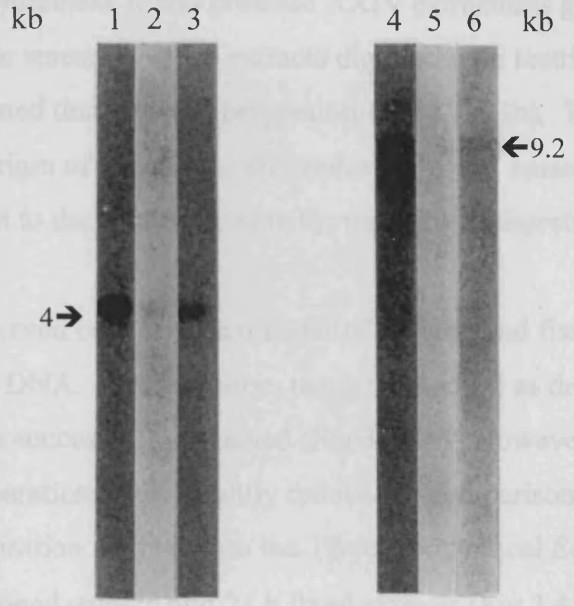
Key
Lanes 1-3 *Msp* I restriction Lanes 4-6 *Bst*N I restriction
Lanes 1 & 4 unfixed controls Lanes 2 & 5 6 h fixed extract
Lanes 3 & 6 24 h fixed extract

Fig 3.4.2b Southern analysis of Carnoy fixed DNA hybridised with Tβ probe



Key
Lanes 1-3 *Bam*H *I* restriction Lanes 4-6 *Eco*R I restriction
Lanes 1 & 4 unfixed controls Lanes 2 & 5 6 h fixed extract
Lanes 3 & 6 24 h fixed extract

Fig 3.4.2c Southern analysis of Carnoy fixed DNA hybridised with JH probe



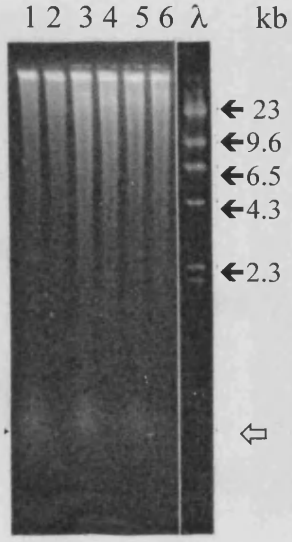
Key

Lanes 1-3 *Bgl* II restriction Lanes 4-6 *Hin*d III restriction
Lanes 1 & 4 unfixed controls Lanes 2 & 5 6 h fixed extract
Lanes 3 & 6 24 h fixed extract

demonstrated the presence of high molecular weight DNA with some lower molecular weight components. Proteinase K and protease XXIV extractions gave identical restriction results. Electrophoretic smears of fixed extracts digested with restriction endonucleases were more weakly stained than unfixed preparations (Fig 3.4.3b). With the exception of the *Bst*N I digest the origin of the smears differed with 'fixed' smears beginning at the loading well in contrast to the exclusion zone for the unfixed digests.

No difference was observed between the transfer of unfixed and fixed restriction endonuclease digested DNA. Hybridisation, using method (a) as described in 2.2.13, with the pHY 2.1 probe was successfully achieved (Fig 3.4.4a). However, the intensity of the bands in the fixed preparations were slightly reduced in comparison with the unfixed DNA. Using the same hybridisation method with the Tβ probe identical *Eco*R I banding to unfixed DNA was obtained using 6 and 24 h fixed extracts (Fig 3.4.4b). In contrast the 24 kb *Bam*H I band was shown clearly only in the unfixed and 6 h fixed extracts. As with the Carnoy extracts it proved necessary to use the alternative hybridisation method when employing the JH probe. No difference was observed in *Bgl* II and *Hin*d III banding between the 6 h fixed and unfixed extracts (Fig 3.4.4c). However, whilst the 4 kb *Bgl* II restriction fragment was demonstrated in the 24 h fixed extract the 9.2 kb *Hin*d III restriction fragment was not observed with this preparation.

Fig 3.4.3a Electrophoretic analysis of 24 h formol saline fixed preparations digested with Proteinase K



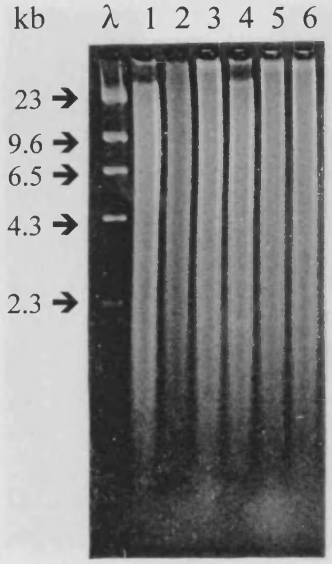
Key

λ Lambda *Hind* III size marker

Lanes 1-2 48 h digestion Lanes 3-4 96 h digestion Lanes 5-6 168 h digestion

Note: RNA marked by open arrow, present in lanes 1, 3, 5 and removed by pre-treatment with RNase in lanes 2, 4, 6.

Fig 3.4.3b Electrophoretic analysis of 24 h formol saline fixed preparations digested with restriction endonucleases



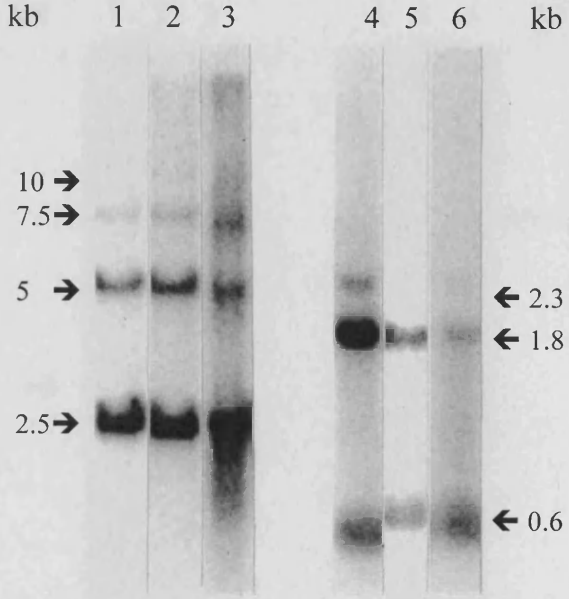
Key

λ Lambda *Hind* III size marker

Lanes 1 & 4 unfixed control Lanes 2 & 5 96 h extract Lanes 3 & 6 168 h extract

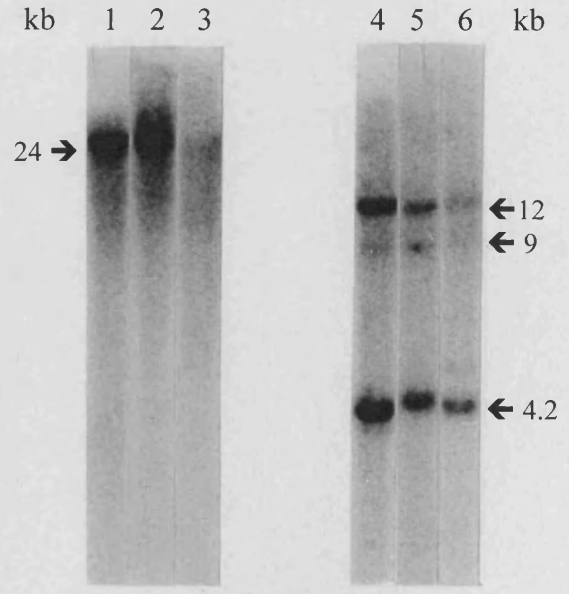
Lanes 1-3 *Bgl* II restriction Lanes 4-6 *Hind* III restriction

Fig 3.4.4a Southern analysis of formol saline fixed DNA hybridised with pHY 2.1 probe



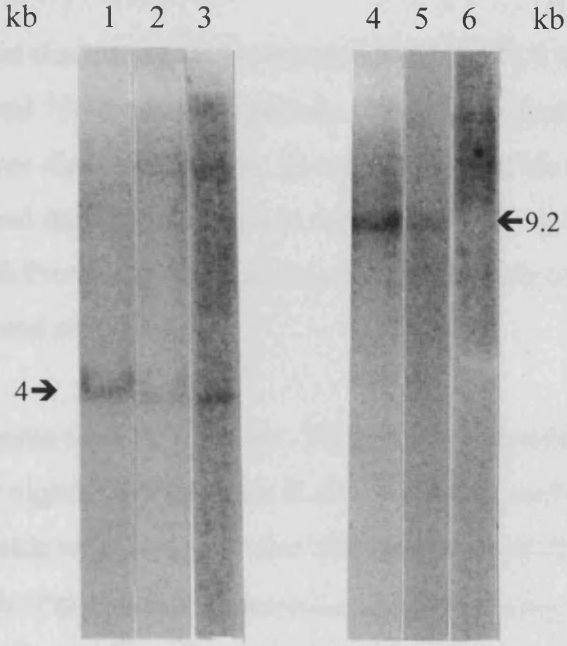
Key
Lanes 1-3 *Msp* I restriction Lanes 4-6 *Bst*N I restriction
Lanes 1 & 4 unfixed controls Lanes 2 & 5 6 h fixed extract
Lanes 3 & 6 24 h fixed extract

Fig 3.4.4b Southern analysis of formol saline fixed DNA hybridised with Tβ probe



Key
Lanes 1-3 *Bam*H I restriction Lanes 4-6 *Eco*R I restriction
Lanes 1 & 4 unfixed controls Lanes 2 & 5 6 h fixed extract
Lanes 3 & 6 24 h fixed extract

Fig 3.4.4c Southern analysis of formol saline fixed DNA hybridised with JH probe



Key
Lanes 1-3 *Bgl* II restriction Lanes 4-6 *Hin*d III restriction
Lanes 1 & 4 unfixed controls Lanes 2 & 5 6 h fixed extract
Lanes 3 & 6 24 h fixed extract

Neutral buffered formalin fixed tissue

Recovery of nucleic acid was attempted from tissue fixed for 24 h in neutral buffered formalin using 48, 96 and 168 h digestion periods. As in other preparations the unfixed control tissue sample was digested for 16 h. The appearance of the fixed samples at the initiation of digestion and during incubation in the enzymes was similar to formol saline preparations. The 168 h Proteinase K digest was the only sample to be completely clear of tissue fragments at the end of digestion.

Nucleic acid was recovered from all samples. Yields increased with digestion time and these were consistently higher for Proteinase K preparations at each digestion end point. Accordingly highest yields were obtained after 168 h digestion with 32.7 μg/mg tissue being recovered from the Proteinase K preparation and 22.6 μg/mg tissue recovered with protease XXIV. The OD 260/280 ratio for all Proteinase K extracts at 1.7 indicated a high degree of protein purity. However, the same ratio varied between 1.4 and 1.8 in protease XXIV preparations.

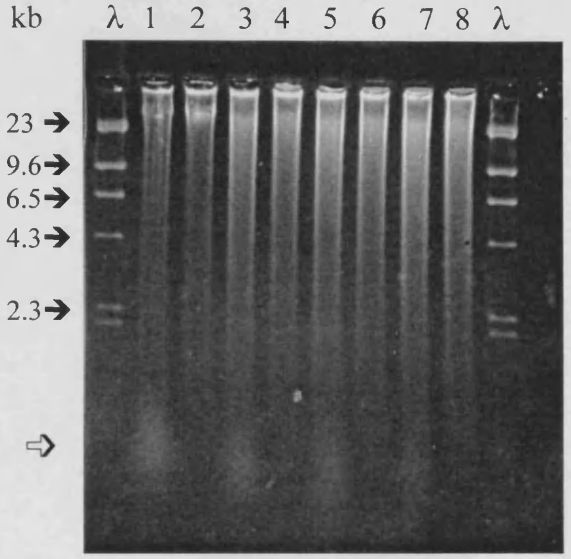
Qualitative analysis by electrophoresis revealed high molecular weight DNA in all extracts. High molecular weight DNA was observed at the loading well and exclusion zone in unfixed and fixed samples. This was accompanied by a smear of lower molecular weight nucleic acid and RNA (Fig 3.4.5a).

Proteinase K and protease XXIV extracts of unfixed and fixed preparations were successfully restricted (Fig 3.4.5b). In all fixed samples the restriction smear originated at the loading well. A similar smear origin was observed with unfixed samples with the exception of preparations digested with *Eco*R I and *Bam*H I. In these the restriction smear originated at the exclusion zone.

No difference was observed between the transfer of unfixed and fixed restriction endonuclease digested DNA. Hybridisation with the pHY 2.1 probe was undertaken using method (b) as described in 2.2.13. Successful hybridisation was achieved though the

intensity of bands in fixed preparations was slightly less than those observed in unfixed samples. Furthermore in *Msp* I preparations the 10 kb restriction fragment clearly visible in unfixed samples were absent in fixed preparations (Fig 3.4.6a). Tβ and JH hybridisation was undertaken using method (a) as described in 2.2.13. A strong 24 kb *Bam*H *I*/Tβ restriction fragment was present in the unfixed preparations and weak band in fixed samples prepared from tissue digested for 168 h (Fig 3.4.6b). This band was absent in preparations in which shorter digestion times had been used to recover the nucleic acid. Moderate to strong 4.2 and 12 kb *Eco*R 1/Tβ restriction fragments were observed in unfixed samples. However, in similar fixed preparations only weak 4.2 kb restriction fragments were observed. Hybridisation bands of expected size and of moderate to strong intensity were observed in unfixed preparations hybridised with the JH probe (Fig 3.4.6c). However, in fixed samples the 9.2 kb *Hin*d III restriction fragment was observed only in the 168 h digested protease XXIV preparation and the 4 kb *Bgl* II restriction fragment was present only in the 168 h digested Proteinase K preparation. In both instances the hybridisation bands were of weak intensity.

Fig 3.4.5a Electrophoretic analysis of 24 h neutral buffered formalin fixed preparations digested with Proteinase K



Key

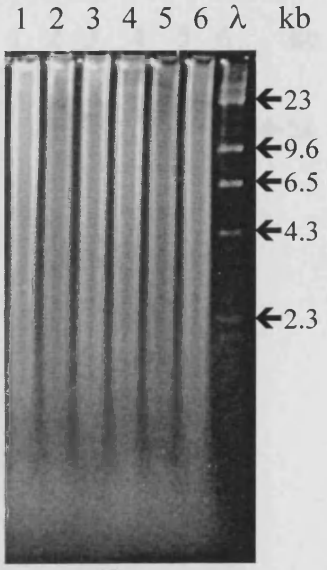
λ Lambda *Hind* III size marker

Lanes 1-2 unfixed DNA Lanes 2-8 Fixed DNA

Lanes 3-4 48 h digestion Lanes 5-6 96 h digestion Lanes 7-8 168 h digestion

Note: RNA marked by open arrow, present in lanes 1, 3, 5, 7 and removed by pre-treatment with RNase in lanes 2, 4 ,6, 8.

Fig 3.4.5b Electrophoretic analysis of 24 h neutral buffered formalin fixed preparations digested with restriction endonucleases



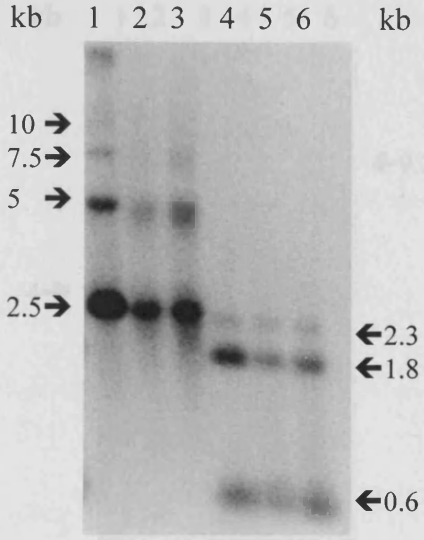
Key

λ Lambda *Hind* III size marker

Lanes 1 & 4 unfixed control Lanes 2 & 5 48 h extract Lanes 3 & 6 168 h extract

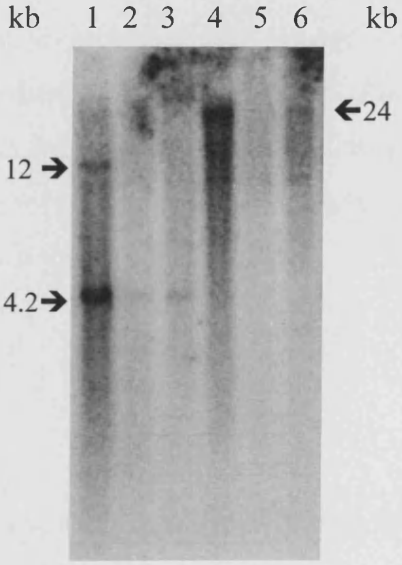
Lanes 1-3 *Bgl* II restriction Lanes 4-6 *Hind* III restriction

Fig 3.4.6a Southern analysis of neutral buffered formalin fixed DNA hybridised with pHY 2.1 probe



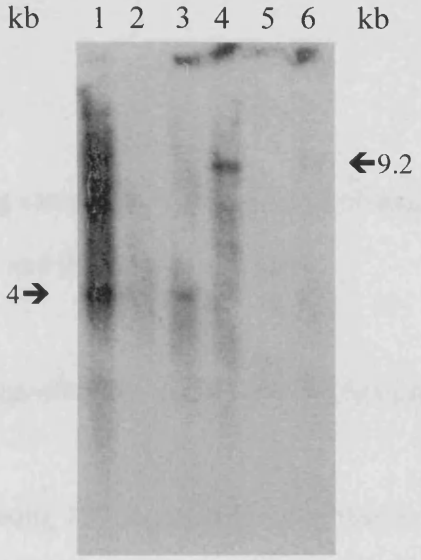
Key
Lanes 1-3 *Msp* I restriction Lanes 4-6 *Bst*N I restriction
Lanes 1 & 4 unfixed controls Lanes 2 & 5 48 h digest, fixed tissue
Lanes 3 & 6 168 h digest, fixed tissue

Fig 3.4.6b Southern analysis of neutral buffered formalin fixed DNA hybridised with Tβ probe



Key
Lanes 1-3 *Eco*R I restriction Lanes 4-6 *Bam*H I restriction
Lanes 1 & 4 unfixed controls Lanes 2 & 5 96 h digest, fixed tissue
Lanes 3 & 6 168 h digest, fixed tissue

Fig 3.4.6c Southern analysis of neutral buffered formalin fixed DNA hybridised with JH probe



Key
Lanes 1-3 *Bgl* II restriction Lanes 4-6 *Hin*d III restriction
Lanes 1 & 4 unfixed controls Lanes 2 & 5 48 h digest, fixed tissue
Lanes 3 & 6 168 h digest, fixed tissue

3.5 Refinement of DNA Recovery Techniques

3.5.1 Experimental design

The influence of the following variations in preparation of tissues and DNA recovery methods as described in 2.2.7 and 2.2.8 were assessed.

Preparation of tissue by homogenisation, dicing and sectioning.

This was undertaken using 150 mg quantities of tissue, which had been fixed for 24 h in Carnoy or formol saline prior to paraffin wax embedding.

Comparison of nucleic acid obtained by salt and ethanol precipitation

This was undertaken as part of the investigation comparing the preparation of tissue by homogenisation, dicing and sectioning. Before salt and ethanol precipitation of purified nucleic acid the preparations were divided equally. One preparation (total nucleic acid) was processed as described in 2.2.8. From the other any floating nucleic acid produced at the time of precipitation was removed immediately and redissolved in TE. The remaining solution was processed by the standard method and resultant nucleic acid termed 'stored' DNA.

Proteinase K digestion at 65°C

This was undertaken using 100 mg samples of homogenised tissue, which had been fixed for 6 and 24 h in Carnoy or formol saline prior to paraffin wax embedding. As a control unfixed tissue was also digested overnight at this temperature. With

the exception of the variation of digestion temperature the DNA recovery method was as described in 2.2.8.

Dialysis of tissue with amino acids prior to proteolytic digestion.

Tissue fixed in formol saline for 24 and 48 h prior to paraffin wax embedding was used. The purpose of the investigation was to assess if the pre-treatments could reverse any fixation induced cross-links between DNA and proteins. Dialysis was undertaken against 200 mM glycine, 200 mM cysteine and 50 mM Tris/HCl, pH 8.0 for 24 h prior to digestion with Proteinase K for 96 h. As controls unfixed tissue was digested overnight and fixed tissue, without pre-treatment, was digested for 48, 96 and 168 h.

A quantitative and qualitative assessment of the recovered nucleic acid was made according to the methods described in 2.2.9 and 2.2.10 respectively. Restriction enzyme digestion, Southern blotting and hybridisation was also undertaken according to the methods 2.2.11, 2.2.12 and 2.2.13.

3.5.2 Results

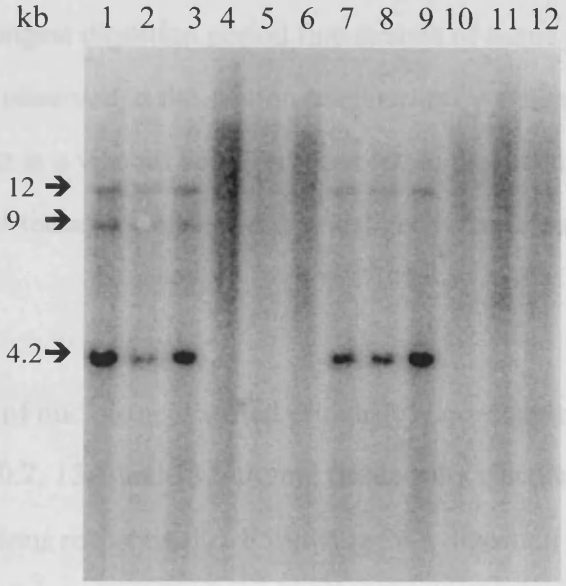
Preparation of tissue by homogenisation, sectioning and dicing.

For Carnoy fixed preparations Proteinase K digestion was undertaken for 24 and 48 h. At the end of the longest digestion period homogenised and section preparations were clear of all initial tissue fragments and non-viscous. In contrast some tissue fragments remained in the diced preparations which were slightly viscous.

Quantitative recovery of nucleic acid varied according to preparation. Section preparations gave highest values at 20.4 and 25 μg/mg tissue after 24 and 48 h digestion respectively. Comparative yields for homogenised preparations were 15.2 and 14 μg/mg tissue. In contrast recovery of nucleic acid from diced preparations at 20 μg/mg tissue was higher after 24 h digestion than after 48 h digestion for which a value of 15.2 μg/mg was obtained. The protein purity of 24 h digested preparations varied from 1.7 to 1.8 whilst after 48 h extraction ratios of 1.95 to 2.01 were obtained. Carbohydrate purity ratios were 0.57 to 0.62 after 24 h digestion and 0.41 to 0.51 after 48 h extraction.

Electrophoresis of the preparations revealed no difference between the preparations. Common features were extensive shearing of the DNA and the presence of degraded RNA. Successful restriction endonuclease digestion with *Eco*R I and *Bam*H I was achieved with all preparations and identical results were obtained with Southern blot hybridisation employing the Tβ probe (Fig 3.5.1). For *Eco*R I 12, 9 and 4.2 kb restriction fragments were observed in all 48 h digests and 12 and 4.2 kb bands only in the 24 h digest preparations. No *Bam*H I restriction fragments were observed in any preparation.

Fig 3.5.1 Southern analysis of Carnoy fixed DNA hybridised with Tβ probe



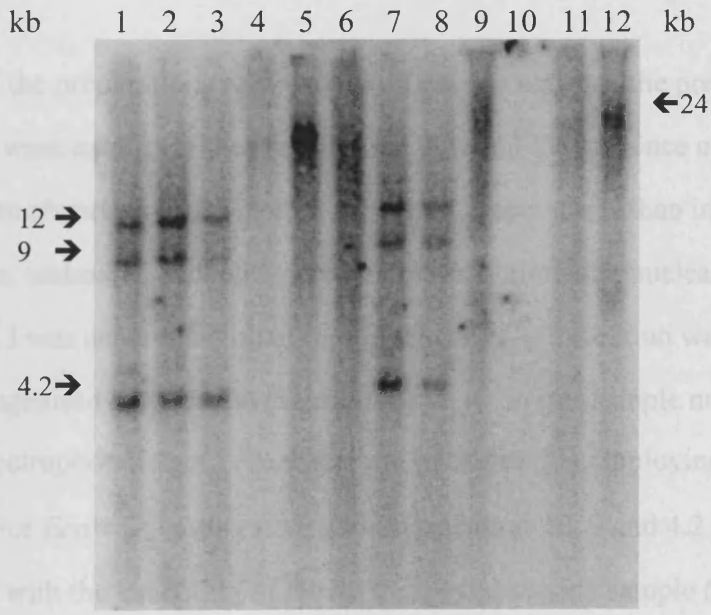
Key

Lanes 1-3, 7-9 *Eco*R I restriction Lanes 4-6, 10-12 *Bam*H I restriction

Lanes 1-6 48 h digest Lanes 7-12 24 h digest

Lanes 1,4,7,10 homogenised Lanes 2,5,8,11 diced Lanes 3,6,9,12 section

Fig 3.5.2 Southern analysis of formol saline fixed DNA hybridised with Tβ probe



Key

Lanes 1-3, 7-9 *Eco*R I restriction Lanes 4-6, 10-12 *Bam*H I restriction

Lanes 1-6 96 h digest Lanes 7-12 48 h digest

Lanes 1,4,7,10 homogenised Lanes 2,5,8,11 diced Lanes 3,6,9,12 section

For formol saline fixed preparations Proteinase K digestion was undertaken for 48 and 96 h. At the end of the longest digestion period fine strands of tissue present in a markedly viscous solution were observed in the section preparation. At the same time point pale pieces of tissue present in a viscous solution remained in the diced preparation whilst several larger pieces of tissue were observed in a slightly viscous solution in the homogenised sample.

Quantitative recovery of nucleic acid varied primarily according to digestion time. After 48 h digestion yields at 20.2, 15.4 and 18.6 μg/mg tissue were calculated for section, diced and homogenised preparations respectively. Following 96 h digestion yields were 26.2, 25.6 and 25.4 μg/mg tissue for the same order of preparations. Protein purity ratios at 1.76 to 1.84 showed little variation across all preparations. Carbohydrate purity ratios at 0.40 to 0.43 after 48 h digestion contrasted slightly with values of 0.45 to 0.47 obtained in the 96 h digestion preparations.

Electrophoresis of the preparations revealed no difference between the preparations. Common features were extensive shearing of the DNA and the presence of degraded RNA. The latter was more abundant in the section and diced preparations than in homogenised samples. With one, technical, exception successful restriction endonuclease digestion with *Eco*R I and *Bam*H I was achieved with all preparations. The exception was the *Bam*H I digest of the homogenised preparation digested for 96 h. In this sample no DNA was observed in the electrophoretic gel. Southern blot hybridisation employing the Tβ probe was undertaken. For *Eco*R I digests restriction fragments at 12, 9 and 4.2 kb were observed in all preparations with the exception of the 48 h digested section sample (Fig 3.5.2). In this a failure of restriction endonuclease digestion was indicated. The 24 kb *Bam*H I/Tβ restriction fragment was observed in the 96 h digested diced and 48h digested section preparations only.

Comparison of nucleic acid obtained by salt and ethanol precipitation

At the time of ethanol and salt precipitation floating DNA was formed in all Carnoy and formol saline fixed preparations. Following storage at -70°C additional flocculent material was observed in all preparations. Optical density measurements revealed differences between the amount of DNA in each fraction primarily according to fixation used (Table 3.5.1). In all Carnoy fixed preparations higher percentages of DNA were recovered in the 'stored' samples whereas the percentage recovery in formol saline fixed samples was always higher in the 'floating' nucleic acid removed immediately after precipitation.

The protein and carbohydrate ratios for Carnoy preparations digested for 24 h indicated higher purity in the 'floating' DNA samples. However, in samples digested for 48 h the ratios were similar indicating good protein and carbohydrate purity coupled with expected presence of RNA. For formol saline preparations no consistent difference in the ratios was observed between 'floating' or 'stored' DNA. These indicated moderate to good protein and carbohydrate purity.

Electrophoresis revealed no significant differences between the quality of DNA present in the 'floating' and 'stored' samples with respect to Carnoy fixed preparations (Fig 3.5.3). In these extensive shearing of the DNA and the presence of degraded RNA was observed. In formol saline fixed preparations electrophoresis revealed slight differences between the preparations (Fig 3.5.4). In samples prepared by homogenisation or dicing the 'stored' DNA was less sheared and more (degraded) RNA was present than in the equivalent floating preparations. However, whilst more RNA was present in equivalent section preparations the degree of shearing of DNA was similar. As the overall quality of the nucleic acid closely matched that of preparations in which the DNA had not been separated at the time of precipitation further analysis of the samples was not undertaken.

Table 3.5.1 Yield of floating and flocculent DNA

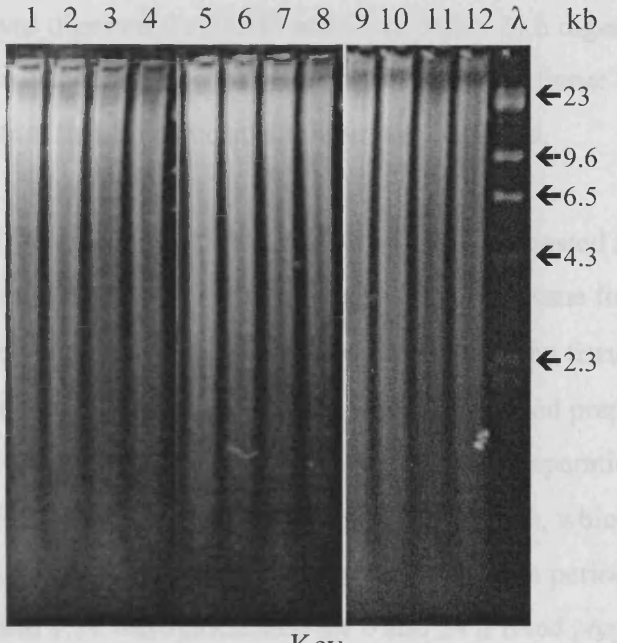
a) 24 h Carnoy fixation

Preparation	Floating DNA µg/mg tissue	% of total	Stored DNA µg/mg tissue	% of total
Homogenised 24 h extraction	3.6	25	10.8	75
Homogenised 48 h extraction	2.4	24	7.6	76
Diced 24 h extraction	9.2	43	12.0	57
Diced 48 h extraction	4.4	39	6.8	61
Section 24 h extraction	8.4	39	13.2	61
Section 48 h extraction	9	39	14.2	61

b) 24 h formol saline fixation

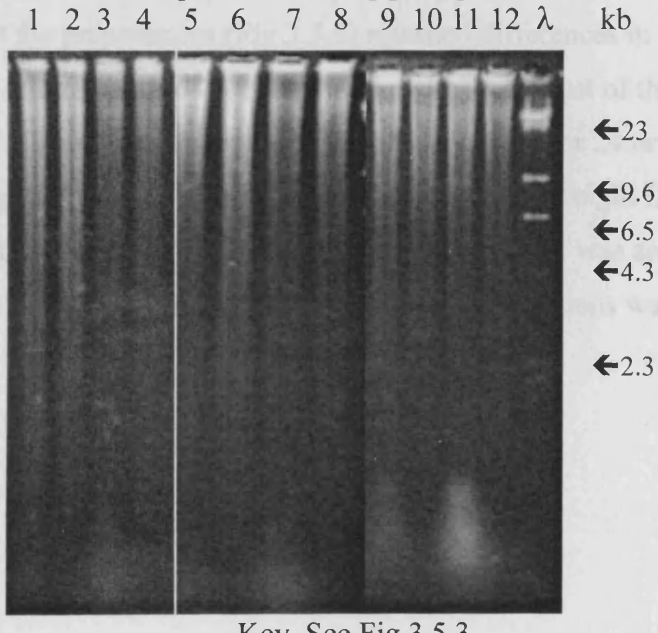
Preparation	Floating DNA µg/mg tissue	% of total	Stored DNA µg/mg tissue	% of total
Homogenised 48 h extraction	14.0	78	4.0	22
Homogenised 96 h extraction	17.1	77	5.0	23
Diced 48 h extraction	12.8	66	6.6	34
Diced 96 h extraction	19.4	79	5.2	21
Section 48 h extraction	20.6	85	3.6	15
Section 96 h extraction	20.4	77	6.1	23

Fig 3.5.3 Electrophoretic analysis of Carnoy fixed DNA digested for 24 h and separated during precipitation



Key
λ Lambda *Hind* III size marker
Lanes 1-2, 5-6, 9-10 Floating DNA Lanes 3-4, 7-8, 11-12 Stored DNA
Lanes 1-4 homogenised preparation Lanes 5-8 diced preparation
Lanes 9-12 section preparation Note: Even lanes RNase pre-treated

Fig 3.5.4 Electrophoretic analysis of formol saline fixed DNA digested for 48 h and separated during precipitation



Key See Fig 3.5.3

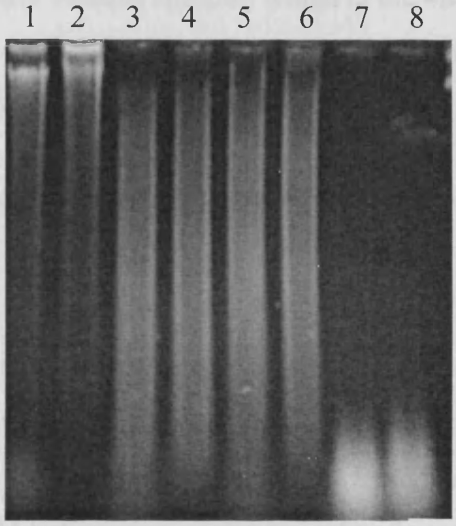
Proteinase K digestion at 65°C

Carnoy fixed tissue was digested for 24, 48 and 96 h. After 24 h digestion pieces of tissue were present in a translucent solution, at the 48 h time point no tissue fragments remained and at 96 h a clear rather than translucent solution was observed.

Maximum yields of DNA were associated with preparations digested for 48 h. These were 24.2 μg/mg tissue for the 6 h fixed preparation and 23 μg/mg tissue for the 24 h fixed sample. Lowest yield for the 6 h fixed preparation at 14.9 μg/mg tissue was recorded for the 24 h digest sample. In contrast lowest yield for the 24 h fixed preparation at 11.8 μg/mg tissue was recorded for the 96 h digest sample. In all preparations protein purity ratios were below 1.8 and carbohydrate ratios above 0.5. Those, which most closely approached these ratios, were associated with the 96 h digestion period. In these protein purity ratios of 1.76 and 1.71 were calculated for 6 and 24 h fixed preparations respectively whilst for the same samples the carbohydrate purity ratios were 0.52 and 0.57. Those, which differed most, were linked to the 48 h digest samples. In these protein purity ratios of 1.58 and 1.56 were calculated for 6 and 24 h fixed preparations respectively whilst for the same samples the carbohydrate purity ratios were 0.7 and 0.64.

Electrophoresis of the preparations (Fig 3.5.5) revealed differences in the quality of DNA recovered. In the unfixed control extract, digested for 24 h, most of the DNA was of high molecular weight. In contrast all fixed preparations digested for 24 or 48 h showed extensive shearing of the DNA. Furthermore high molecular weight DNA was absent in samples subjected to 96 h digestion. This degradation of DNA was accentuated in the 24 h fixed sample. No further analysis of the Carnoy fixed preparations was undertaken.

Fig 3.5.5 Electrophoretic analysis of Carnoy fixed DNA digested at 65°C



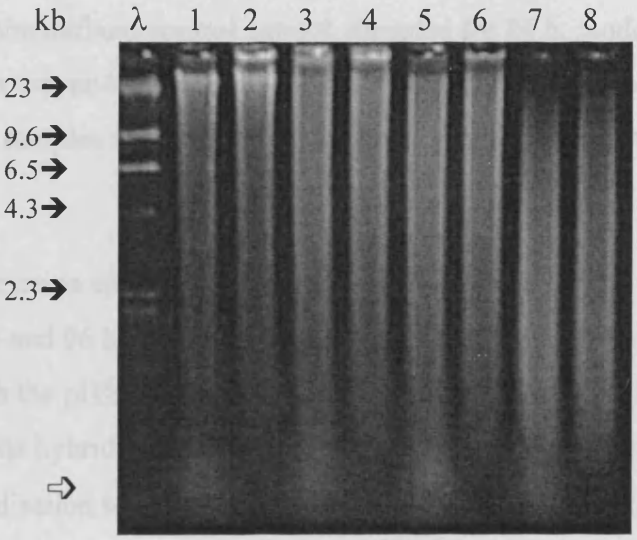
Key

Lanes 1-2 unfixed DNA Lanes 3-8 24 h fixed DNA

Lanes 1-4 24 h digestion Lanes 5-6 48 h digestion Lanes 7-8 96 h digestion

Note: RNA marked by open arrow, present in lanes 1, 3, 5 and removed by pre-treatment with RNase in lanes 2, 4 ,6.

Fig 3.5.6 Electrophoretic analysis of formol saline fixed DNA digested at 65°C



Key

See Fig 3.5.5

λ Lambda *Hin*d III size marker

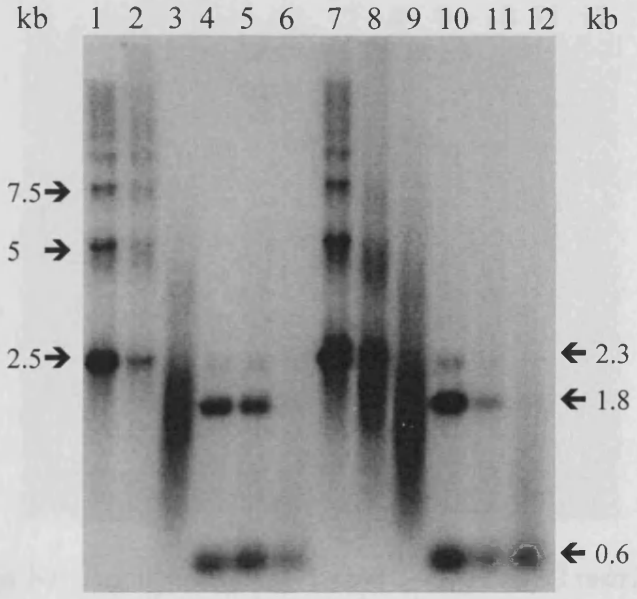
Formol saline fixed tissue was digested for 24, 48 and 96 h. After 24 h digestion pieces of tissue were present in a slightly viscous solution whilst at the 48 h time point no tissue fragments remained and the preparations were translucent and viscous. At 96 h the preparations were translucent but no longer viscous.

Yields of DNA from 6 h fixed preparations increased with digestion time. After 24 h digestion 5.3 μg/mg tissue was calculated whilst after 96 h digestion a value of 8.3 μg/mg tissue was obtained. Highest yield for the 24 h fixed preparation at 8.8 μg/mg tissue was recorded for the 48 h digest sample. Other yields were 7.2 μg/mg tissue after 24 h digestion and 6.6 μg/mg tissue following 96 h digestion. Protein purity ratios were closely banded, the lowest ratio was 1.75 and the highest 1.82. Carbohydrate purity ratios ranged from 0.45, 6 h fixation and 24 h digestion, to 0.6, 24 h fixation and 24 h digestion. Preparations fixed for 6 and 24 h then digested for 48 h had ratios of 0.53 and 0.51 respectively. Whilst ratios for similar preparations digested for 96 h were 0.5 and 0.56 respectively.

Electrophoresis of the preparations (Fig 3.5.6) revealed differences in the quality of DNA recovered. In the unfixed control extract, digested for 24 h, moderate shearing of DNA was observed. This was accentuated in fixed preparations digested for 24 or 48 h and most pronounced in samples subjected to 96 h digestion. Degraded RNA was present in all preparations.

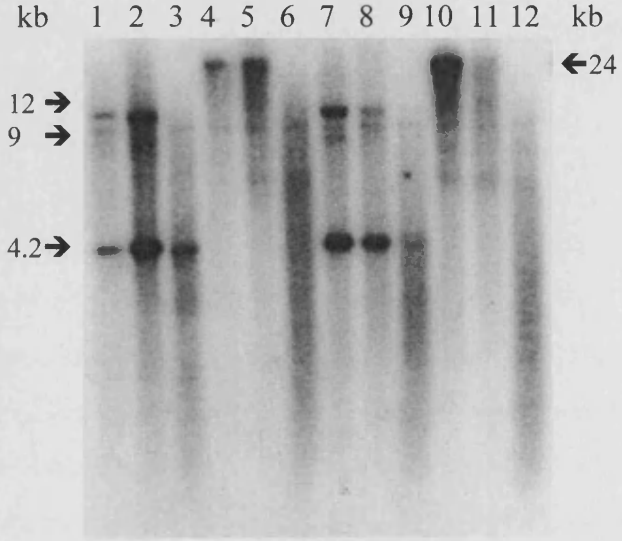
Successful restriction endonuclease digestion was undertaken on unfixed and fixed samples digested for 24 and 96 h. Southern blots prepared with *Msp* I/*Bst*N I transfers were hybridised with the pHY 2.1 probe using method a) as described in 2.2.13 and *Eco*R I/*Bam*H I digests hybridised using the Tβ probe by the same method. For *Hind* III/*Bgl* II transfers hybridisation with the JH probe was undertaken using method b) as described in 2.2.13. Optimal results were obtained with unfixed preparations. In terms of the presence and intensity of hybridisation bands fixed preparations were inferior. Least affected were samples that had been fixed for 6 h and digested for 24 h whilst most affected were preparations which had been fixed for 24 h and digested for 96 h. The results are illustrated in Fig 3.5.7.

Fig 3.5.7a Southern analysis of unfixed and formol saline fixed preparations digested with Proteinase K at 65°C hybridised with pHY 2.1 probe



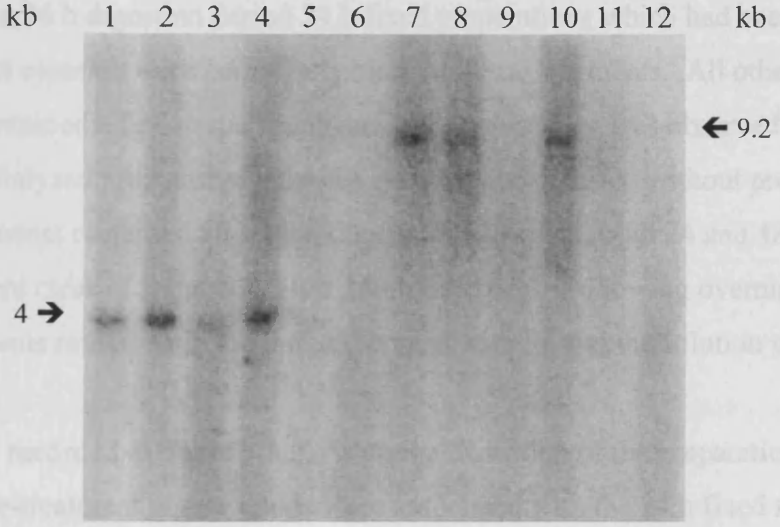
Key
Lanes 1-6, 7-9 *Msp* I restriction Lanes 4-6,10-12 *Bst*N I restriction
Lanes 1,4,7,10 unfixed Lanes 2, 3, 5, 6 6 h fixation Lanes 8, 9, 11, 12 24 h fixation
Lanes 2, 5, 8, 11 24 h digest Lanes 3, 6, 9, 12 96 h digest

Fig 3.5.7b Southern analysis of unfixed and formol saline fixed preparations digested with Proteinase K at 65°C hybridised with Tβ probe



Key
As 3.5.7a except
Lanes 1-3, 7-9 *Eco*R I restriction Lanes 4-6,10-12 *Bam*H I restriction

Fig 3.5.7c Southern analysis of unfixed and formol saline fixed preparations digested with Proteinase K at 65°C hybridised with JH probe



Key

Lanes 1-6 *Bgl* II restriction Lanes 7-12 *Hind* III restriction

Lanes 1,4,7,10 unfixed Lanes 1-3, 7-9 6 h fixation Lanes 4-6, 10-12 24 h fixation

Lanes 2, 5, 8, 11 24 h digest Lanes 3, 6, 9, 12 96 h digest

Dialysis of tissue with amino acids prior to proteolytic digestion.

At the end of the 96 h digestion period 24 h fixed preparations which had been dialysed with glycine and cysteine were completely clear of tissue fragments. All other dialysis preparations contained a few tissue fragments. Slight viscosity was observed in the 24 h fixed and Tris dialysed preparation only. In preparations digested without pre-treatment a few tissue fragments remained after 96 h digestion. However, both 24 and 48 h fixed preparations were clear of fragments after 168 h digestion. Following overnight digestion no tissue fragments remained in the unfixed control sample and the solution was viscous.

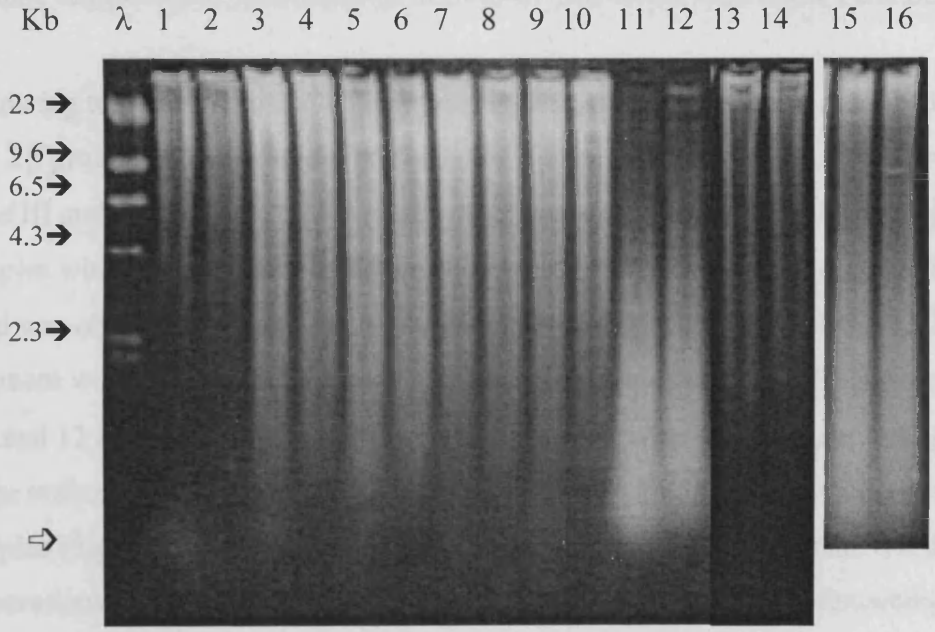
DNA yields are recorded in Table 3.5.2. With the exception of the preparation digested for 96 h without pre-treatment higher yields were associated with the 24 h fixed samples. For 24 h fixed preparations digested for 96 h similar yields were recorded for Tris and cysteine dialysed and non-pre-treated samples. In comparison the yield for the glycine dialysed preparation was considerably lower. For 48 h fixed preparations digested for 96 h the highest DNA yield was associated with the non-pretreated sample, an intermediate yield with the Tris dialysed sample and lowest yields with those preparations dialysed against the amino acids. The protein purity ratios of the 24 h fixed preparations ranged from 1.80 to 1.87. In comparison the ratios obtained after 48 h fixation ranged from 1.67 to 1.87. In each set the highest ratio was associated with the non-pre-treated preparations digested for 96 h and the lowest ratio with the sample dialysed with cysteine. The carbohydrate purity ratios of the 24 and 48 h fixed samples ranged from 0.44 to 0.53.

Electrophoresis of the preparations revealed differences in the quality of DNA recovered (Fig 3.5.8). The majority of DNA obtained from unfixed tissue was of high molecular weight. Some shearing of the nucleic acid was visible in the 24 h fixed preparation digested for 48 h without pre-treatment. In the equivalent 48 h fixed preparation further degradation was noted. In the remainder of the non pre-treated samples and those dialysed with Tris and glycine extensive shearing of the DNA was observed irrespective of fixation time. Only low molecular weight DNA was present in the 24 h fixed cysteine dialysed

Table 3.5.2 DNA yields for formol saline fixed tissue digested with or without dialysis pre-treatment

Preparation	Digest time h	24 h fixation µg/mg tissue	48 h fixation µg/mg tissue
Tris dialysis	96	16.7	11.34
Glycine dialysis	96	9.0	7.7
Cysteine dialysis	96	14.8	7.6
Standard	48	8.0	8.2
Standard	96	14.3	15.3
Standard	168	14.4	10.4
Unfixed	24	16.5	

Fig 3.5.8 Electrophoretic analysis of DNA digests of formol saline fixed tissue with and without dialysis pre-treatment



Key

λ Lambda *Hind* III size marker

Lanes 1-12 24 h fixation Lanes 13, 14 unfixed Lanes 15, 16 48 h fixation

Lanes 1, 2 48 h digestion no pre-treatment Lanes 3, 4 96 h digestion no pre-treatment

Lanes 5, 6 168 h digestion no pre-treatment

Lanes 7, 8 96 h digestion Tris dialysed Lanes 9, 10 96 h digestion glycine dialysed

Lanes 11,12, 15, 16 96 h digestion cysteine dialysed

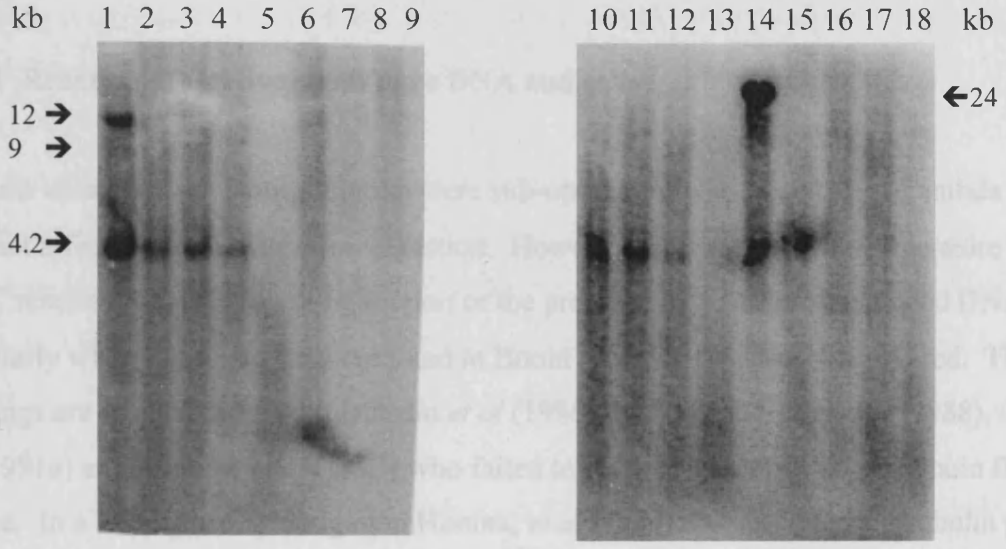
Note: RNA marked by open arrow, present in lanes 1, 3, 5, 7, 9 and removed by pre-treatment with RNase in lanes 2, 4 ,6, 8, 10.

preparation but in the equivalent 48 h fixed sample some high molecular weight nucleic acid was observed. Degraded RNA was visible in the 24 h fixed preparations that had been digested without pre-treatment or dialysed against Tris or glycine only. No RNA was observed in the unfixed sample or in any 48 h fixed preparation.

Restriction endonuclease digestions were undertaken with *Eco*R I, *Bam*H I, *Hind* III and *Bgl* II for 24 h using DNA from the unfixed sample and all fixed preparations that had been digested for 96 h. With cysteine dialysed preparations the initial degradation of the samples made it impossible to judge if restriction had taken place. In unfixed and all 24 h fixed samples good restriction smears were obtained. In 48 h fixed preparations good smears were obtained with *Hind* III and *Bgl* II but tracking of DNA at the margins of each gel lane suggested the possibility of incomplete restriction with *Eco*R I and *Bam*H I.

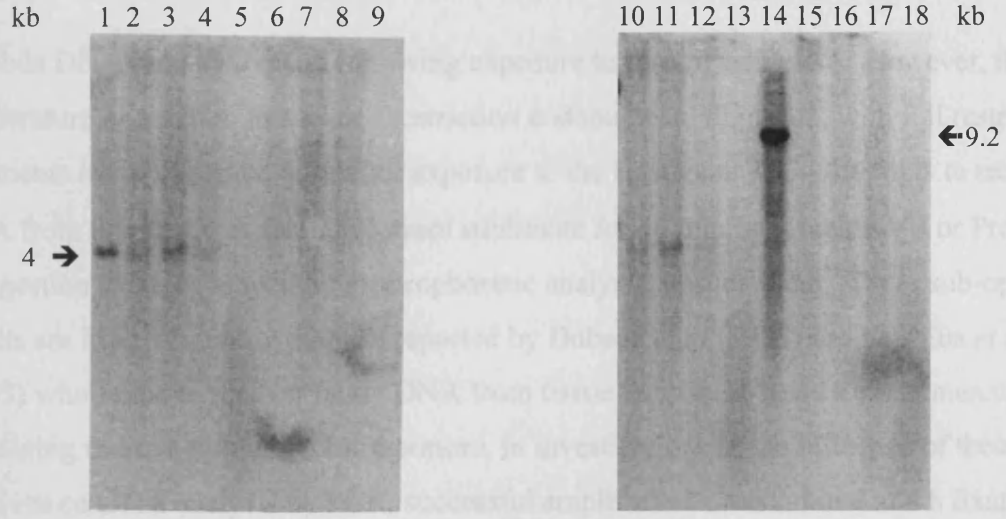
Following transfer hybridisation was undertaken using method b) as described in 2.2.13. The Tβ probe was hybridised with the *Eco*R I, *Bam*H I transfers and the JH probe with the *Hind* III and *Bgl* II blots. No restriction fragments were observed in cysteine pre-treated samples with any restriction enzyme/probe combination. The 24 kb *Bam*H I/Tβ restriction band was observed in the unfixed preparation only (Fig 3.5.9a). A strong 4.2 kb restriction fragment was observed in all *Eco*R I preparations hybridised with Tβ. However, the bands at 9 and 12 kb expected with this restriction enzyme/probe combination were present only in the unfixed preparation. The 4 kb *Bgl* II/JH restriction fragment was present in all samples (Fig 3.5.9b). This was of moderate intensity in the unfixed and Tris dialysed preparations. The same hybridisation band was of weak intensity in remaining samples. In the unfixed *Hind* III restricted preparation hybridised with the JH probe a 9.2 kb restriction fragment of strong intensity was observed. This band was observed in two 24 h fixed preparations only; Tris dialysed (weak band) and glycine dialysed (very weak band). No restriction fragments were observed in 48 h fixed preparations using the *Hind* III restriction enzyme and JH probe combination.

Fig 3.5.9a Southern analysis of formol saline fixed preparations with and without pre-treatment hybridised with Tβ probe



Key
Lanes 1-5 , 10-13 *Eco*R I restriction Lanes 6-9, 14-18 *Bam*H I restriction
Lanes 1, 14 unfixed controls Lanes 2-9 24 h fixation, 96 h digestion
Lanes 10-13, 15-18 48 h fixation, 96 h digestion
Lanes 2, 6, 10, 15 no pre-treatment Lanes 3, 7, 11, 16 Tris dialysed
Lanes 4, 8, 12, 17 glycine dialysed Lanes 5, 9, 13, 18 cysteine dialysed

Fig 3.5.9b Southern analysis of formol saline fixed preparations with and without pre-treatment hybridised with JH probe



Key
As 3.5.9a except
Lanes 1-5 , 10-13 *Bgl* II restriction Lanes 6-9, 14-18 *Hin*d III restriction

3.6 Discussion

3.6.1 Reaction of fixatives with pure DNA and cells

Results obtained with Bouin fixation were sub-optimal. Fixation at 4°C of Lambda DNA did not affect *Hind* III restriction digestion. However, fixation at room temperature or 37°C resulted in inhibition of restriction or the presence of a smear of degraded DNA. Similarly when lymphocytes were fixed in Bouin intact DNA was not recovered. These findings are in agreement with Dubeau *et al* (1986), Nuovo and Silverstein (1988), Greer *et al* (1991a) and Koshiba *et al* (1993) who failed to recover intact DNA from Bouin fixed tissue. In a PCR based investigation Honma, *et al* (1993) demonstrated a α tubulin product of 268 bp after 24 h Bouin fixation at 4°C and room temperature. Hostein *et al* (1998) also demonstrated t(14;18) translocation by PCR using DNA prepared from tissue fixed in Bouin. However, using the same fixative O'Leary *et al* (1994) obtained a 110 bp β globin PCR product in only one reaction whilst Ben-Ezra *et al* (1991) failed to demonstrate a 250 bp β actin amplification product in DNA recovered from breast tissue fixed in Bouin. Together these findings indicate that DNA is degraded by Bouin fixation.

Lambda DNA remained intact following exposure to formol sublimate. However, the temperature of reaction influenced restriction endonuclease digestion, with full restriction fragments being observed only after exposure to the fixative at 4°C. Attempts to recover DNA from lymphocytes fixed in formol sublimate for 30 min by protease VII or Proteinase K digestion were, as judged by electrophoretic analysis, unsuccessful. These sub-optimal results are in agreement with those reported by Dubeau *et al* (1986) and Koshiba *et al* (1993) who failed to recover intact DNA from tissue fixed in B-5 and Zenker mercury containing fixative mixtures. Furthermore, in investigations of the influence of these fixatives on DNA analysis by PCR, successful amplification was limited to 4 h fixation (Greer *et al* 1991a) and to products of less than 300 bp (Crisan *et al* 1990, Crisan and Mattson 1992). However, Limpens *et al* (1993) successfully demonstrated t(14;18) translocation by PCR using DNA prepared from tissue fixed in B-5.

The absence of high molecular weight DNA after Bouin fixation and failure to recover DNA from formol sublimate from fixed lymphocytes effectively precluded the use of these fixatives for Southern analysis and further investigations using these solutions were not undertaken.

Fixation in Carnoy had no effect on the integrity of Lambda DNA or subsequent restriction endonuclease digestion with *Hin*d III. High molecular weight DNA was also recovered with ease from lymphocytes fixed in Carnoy for 30 min to 24 h after digestion for 2 h in protease VII. This DNA restricted successfully with *Eco*R I and after Southern analysis germline Tβ restriction fragments were demonstrated.

Under the conditions used in the investigation, 5 to 60 min at 4°C, room temperature and 37°C, restriction endonuclease digestion of Lambda DNA with *Hin*d III was unaffected by exposure to formol saline and neutral buffered formalin. However, Tokuda *et al* (1990) reported partial restriction endonuclease digestion with *Hin*d III and *Hinf* I following 1 day room temperature fixation with buffered formalin, pH 7.0, whilst observing no degradation of the DNA. This finding was confirmed by Koshiba *et al* (1993) and Hamazaki *et al* (1993) who demonstrated consistent alteration of restriction endonuclease digestion patterns. These authors also explored the effect of unbuffered formalin, pH 4.3, fixation on Lambda DNA. This fixative resulted in the degradation of the nucleic acid within 1 day when used at temperatures above 10°C. However, when neutralised or after inclusion of sodium or potassium chloride the integrity of the DNA, after exposure to the modified fixative at room temperature for 1 day, was maintained.

In contrast to Carnoy fixed preparations recovery of DNA from lymphocytes fixed in formol saline for 30 min required prolonged proteolytic digestion. A minimum of 24 h digestion was required before any DNA could be visualised electrophoretically. After 48 h digestion yields were improved but in contrast to Carnoy extracts these were insufficient to allow for Southern analysis. However, the DNA recovered from the formol saline fixed cells was of high molecular weight with no evidence of degradation. The influence of fixation in 10% formalin at pH 3.5 on the recovery of DNA from HTLV-1 infected

cultured cells fixed has been reported by Ohara *et al* (1992). Using overnight Proteinase K digestion in the presence of 1% SDS at 37 °C followed by phenol washing and ethanol precipitation they demonstrated that high molecular weight DNA, with the increasing presence of degraded DNA, could be recovered from cells fixed from 30 min to 4 days at room temperature. In extracts fixed for 10 and 30 days the DNA recovered was below 2 kb in sequence length.

Taken together the findings with Carnoy, formol saline and neutral buffered formalin indicated that these fixatives were compatible with the recovery of high molecular weight DNA. Accordingly, these fixatives were taken forward for further evaluation.

3.6.2 Recovery and Southern analysis of DNA from fixed and paraffin wax embedded tissue

For this investigation tonsil tissue was fixed in Carnoy, formol saline and neutral buffered formalin for 6 and 24 h then paraffin wax embedded. Recovery of DNA was attempted using Proteinase K and protease XXIV digestion and a quantitative and qualitative assessment made of the extracted nucleic acid. Southern analysis for multiple, pHY 2.1 probe, and single copy, Tβ and JH, gene sequences was then undertaken.

The protocol used for the recovery of DNA combined features of the first methods described for the recovery of nucleic acid from paraffin wax embedded tissue. The method described by Goelz *et al* (1985) involved Proteinase K incubation of diced paraffin tissue in the presence of 1% SDS at 50°C. Following digestion the extract was syringed, phenol extracted and precipitated with ethanol before resuspension and storage. By contrast, Dubeau *et al* (1986) described a relatively complicated method designed to remove low molecular weight nucleic acid from the digestion solution. In their protocol rehydrated paraffin wax sections were subjected to two digestions in Proteinase K in the presence of 1% SDS at 37°C and one with pronase at the same temperature. Between the Proteinase K digestions low molecular weight contaminants were removed and before incubation in pronase, RNA was digested. After the final enzyme digestion and phenol washing DNA

was recovered immediately after precipitation by 'spooling'. In its relative simplicity the method used in this investigation matches more closely that of Goelz *et al* (1985). However, like Dubeau *et al* (1986) rehydrated rather than diced paraffin tissue was used and no syringing step was included. Rehydration was employed to aid the action of the proteolytic enzymes and syringing avoided due the possibility of this shearing extracted DNA. However, it should be noted that Goelz *et al* (1985) and Jackson *et al* (1990) reported that tissue rehydration had no influence on the recovery of DNA.

Both Proteinase K and protease XXIV proved suitable for the recovery of DNA. However, the nucleic acid was extracted more quickly using the former enzyme, DNA yields were higher and in the case of neutral buffered formalin preparations OD 260/280 ratios indicated better protein purity. As with fixed lymphocytes DNA was recovered more easily from Carnoy fixed tissue. At the end of the digestion periods, 24 to 96 h, no tissue fragments were present in these preparations whilst some tissue fragments remained in the majority of formalin fixed digests even after 168 h digestion. DNA yields were consistent after Carnoy fixation but with formalin fixed preparations the quantity of nucleic acid increased as the time of proteolytic digestion was extended. The latter results concur with those of Jackson *et al* (1990) who reported that the yield and molecular weight of DNA obtained from formalin fixed and paraffin wax embedded tissue improved with extended proteolytic digestion. In contrast to the electrophoretic appearance of DNA recovered from fixed lymphocytes the DNA prepared from paraffin wax tissue, irrespective of fixative used, was moderately degraded and accompanied by degraded RNA, a feature also observed by Forsthoefel *et al* (1992).

Restriction endonuclease digestion and Southern transfer were achieved without difficulty. In contrast to unfixed and Carnoy preparations the origin of the restriction endonuclease digest in the majority of the formol saline and in all neutral buffered formalin preparations was at the loading well rather than at the exclusion zone. Goelz *et al* (1985), Dubeau *et al* (1986) and Moerkerk *et al* (1990) also reported similar reduced electrophoretic mobility. Successful hybridisation results were obtained for multi and single copy genes. However, comparison with matched unfixed DNA preparations revealed an overall reduction in

intensity of hybridisation signal and in some instances the absence of restriction fragments. Examples include the reduction in intensity of the 24 kb *Bam*H I/Tβ restriction fragment in all fixed preparations and the absence of the 12 kb *Eco*R I/Tβ hybridisation band in the neutral buffered formalin fixed preparation. The hybridisation results also indicate that as fixation time was increased from 6 to 24 h these features were accentuated. The absence of the 9.2 kb *Hind* III/JH restriction fragment in the 24 h fixed formol saline fixed preparation and loss of the 24 kb *Bam*H I/Tβ hybridisation band in the 24 h fixed Carnoy and formol saline fixed extracts serve as examples. These results are consistent with the observed partial degradation of the DNA.

The partial degradation of DNA recovered from formalin fixed and paraffin wax embedded tissue was also observed by Goelz *et al* (1985), Dubeau *et al* (1986), Jackson *et al* (1990) and Moerkerk *et al* (1990). In the influence of fixation time on the quality of DNA recovered, the results of this investigation agree with Dubeau *et al* (1986) and Jackson *et al* (1990) who observed increased DNA degradation with extended fixation. In contrast Goelz *et al* (1985) reported no effect, even when fixation was of several weeks' duration. It is possible that this observation was due to the Southern demonstration of restriction fragments below 1 kb and the absence of high molecular weight DNA in their preparations.

Variations to the DNA recovery protocol were explored with the objective of improving the quality of DNA recovered and reducing digestion time with consequent shortening of the recovery method.

Comparison of tissue preparation by homogenisation with use of sections and diced tissue was undertaken to assess if the former procedure had introduced or promoted DNA shearing. For this investigation 24 h fixed and paraffin embedded tissue was used coupled with digestion times of 24 and 48 h with Carnoy and 48 and 96 h with formol saline fixed preparations. With Carnoy preparations higher yields of DNA were obtained with section preparations and lowest with homogenised samples. Similar results were obtained with formol saline fixed preparations digested for 48 h but after 96 h digestion there was no

appreciable difference in yields. However, no improvement in the quality of DNA as assessed by electrophoresis and Southern analysis was observed in any preparation.

Dubeau *et al* (1986) emphasised that recovery of high molecular weight DNA was dependent on the immediate 'spooling' of visible nucleic acid after ethanol precipitation. Accordingly, an investigation was undertaken to compare 'floating', aka, 'spooled' DNA removed immediately after addition of salt and ethanol with that which was collected after storage of the same solution. Yields of DNA differed according to fixative and mode of DNA recovery. With Carnoy preparations yields were consistently higher in the 'floating' DNA fractions whilst the opposite was true in all formol saline samples. However, electrophoresis did not reveal any marked differences in the quality of DNA recovered. This finding is in agreement with that of Moerkerk *et al* (1990), who observed no difference in the molecular weight of DNA recovered from 'spooled' and 'non-spooled' preparations.

In model experiments Siomin *et al* (1973) and Semin *et al* (1974) demonstrated that incorporation of glycine altered the reaction of DNA and formaldehyde. Accordingly, dialysis was attempted using this amino acid, with cysteine and Tris buffer alone as controls, to investigate if the potential transfer of formaldehyde to the amino acid would alter the quality of recovered DNA. For this investigation sections of 24 and 48 h formol saline fixed tissue were dialysed for 24 h prior to digestion for 96 h. Electrophoretic analysis did not reveal any difference between the Tris and glycine dialysed preparations which showed DNA degradation. After cysteine dialysis more extensive DNA degradation was observed. A possible explanation for this could be nuclease contamination of the amino acid. The absence of high molecular weight restriction fragments and reduced intensity of lower molecular weight fragments in subsequent Southern hybridisations confirmed the degradation of the DNA.

In an attempt to shorten the DNA recovery protocol the effect of Proteinase K digestion at 65°C was evaluated. The investigation was undertaken using 6 and 24 h fixed Carnoy and formol saline tissue and matched unfixed tissue. Digestion times of 24, 48 and 96 h were

employed for the fixed tissue and 24 h for the unfixed samples. Results with Carnoy demonstrated no qualitative improvement over digestion at 37°C and after 96 h digestion only very low molecular weight DNA remained. Similar results were observed with the formol saline preparations although the extent of DNA degradation was less marked after 96 h digestion. Subsequent Southern analysis of the formol saline extracts confirmed that the DNA was degraded.

In summary, the results obtained with variation of the DNA extraction protocol did not improve the quality of nucleic acid recovered or indicated that there would be any advantage in using Proteinase K at 65°C. The only consistent improvement noted was in DNA yield when sections were used in the place of homogenised tissue as the starting material for digestion. Importantly, the results of these investigations indicate that fixation rather than subsequent manipulation of tissue is the primary factor determining the quality of DNA recovered.

Chapter 4 Southern Blot analysis of lymphoma specimens and investigation of prolonged fixation and tissue processing on quality of nucleic acid

4.1 Introduction

In the previous chapter a protocol for the recovery of DNA from fixed and paraffin embedded tissue was developed. Although the recovered DNA was partially degraded successful Southern analysis for single and multi-copy sequences was demonstrated. Restriction endonuclease and probe combinations used for single copy sequences identified germline T and B cell sequences. Accordingly, this suggested that the demonstration of gene rearrangements in DNA from fixed and paraffin wax embedded lymphoma samples might be possible. An assessment of this application using 14 known or suspected lymphoma cases is described in section 4.2 of this chapter.

Fixation times of 6 and 24 h prior to paraffin wax embedding had been used in the investigations to develop the protocol for DNA recovery. In section 4.3 of this chapter the effect of fixation in Carnoy, formol saline and neutral buffered formalin for 24 to 168 h and the influence of paraffin wax processing on the quality of recovered DNA is explored.

4.2 Analysis of lymphoma specimens

4.2.1 Experimental design

Analysis of initial lymphoma cases.

DNA extraction was attempted from 10 suspected or known lymphoma cases. Details of these cases, coded as A-J, are given in 2.1.3a. Sections were cut from each case amounting

to 100 mg dry weight and prepared for digestion with Proteinase K according to the methods (2.2.7d and 2.2.8). After 96 h digestion 5 ml of the solution was removed for purification whilst the remaining solution was digested for a further 72 h before phenol/chloroform treatment.

Analysis of lymphoma cases with known fixation conditions.

DNA extraction was attempted from a further 4 known lymphoma cases which had been fixed for 24 to 72 h in formol saline prior to paraffin wax embedding. Details of these cases, coded as K-N, are recorded in 2.1.3b. These tissues had been stored for several months as paraffin wax blocks prior to extraction. Tissue was also removed from frozen samples of cases M and N stored at the time of biopsy. This tissue, coded as O and P respectively, was fixed for 24 h in formol saline prior to paraffin embedding. Extraction of DNA from this tissue, together with the other cases, was undertaken immediately.

From each case two 50 mg dry weight quantities of sections were cut and prepared for digestion (method 2.2.7d). Digestion of one section set was undertaken using the method described in 2.2.8. Digestion of the remaining sections was undertaken using the same method except for the replacement of the 50 mM Tris/HCl, pH 8.0 digestion buffer with a commercial urea based solution. In both instances a 96 h digestion period was employed.

For both investigations a quantitative and qualitative assessment of the recovered nucleic acid was made (methods 2.2.9 and 2.2.10). Restriction enzyme digestion, Southern blotting and hybridisation, using method b, was also undertaken (methods 2.2.11, 2.2.12 and 2.2.13).

4.2.2 Results

Analysis of initial lymphoma cases.

The yields of DNA (Table 4.2.1) showed considerable variation. In 5 samples maximum yields were noted after 96 h digestion whilst in the remaining cases higher yields were obtained after 168 h digestion. Yields of over 10 μg/mg tissue were obtained in 4 samples incubated for 168 h but in only in case J digested for 96 h. In case E the yield of DNA at 168 h was over 10 fold less than that calculated at 96 h possibly indicating that the majority of recoverable nucleic acid had been obtained by the end of the initial digestion period. Protein purity ratios (Table 4.2.1) for the 96 h digests varied from 1.73 to 1.97 with 6 cases being in the range of 1.8 to 1.9. The ratios calculated for the 168 h digestion samples ranged from 0.28 to 4.3 with 6 cases with ratios between 1.5 and 2.2. Carbohydrate purity ratios (Table 4.2.1) for the 96 h digestion samples varied from 0.48 to 0.58 in 6 cases whilst in 3 preparations ratios above 1 were calculated. Ratios for carbohydrate purity in 168 h preparations ranged from 0.2 to 9.44 with poor correlation with those of the 96 h samples. Overall the results suggest that recovery of DNA was highly case dependant and in terms of protein and carbohydrate purity that 96 h digestion was optimal.

Electrophoretic analysis of the preparations digested for 96 h revealed material in the gel loading well in all samples with the exception of case I. Extensive DNA degradation was present in cases C, and H-J whilst in the remaining preparations moderate degradation of the nucleic acid was observed. Degraded RNA was present in cases A-E whilst the possible presence of the nucleic acid was observed in cases G and H. After 168 h digestion more extensive shearing of the DNA was observed in the electrophoretic gels. Furthermore no material was observed in the gel loading well for cases I and J whilst little material was observed in the gel loading well in cases C, G and H. Degraded RNA was present in cases A and B whilst the possible presence of the nucleic acid was observed in cases C, F and G. Representative examples of the electrophoretic gels are shown in Fig 4.2.1.

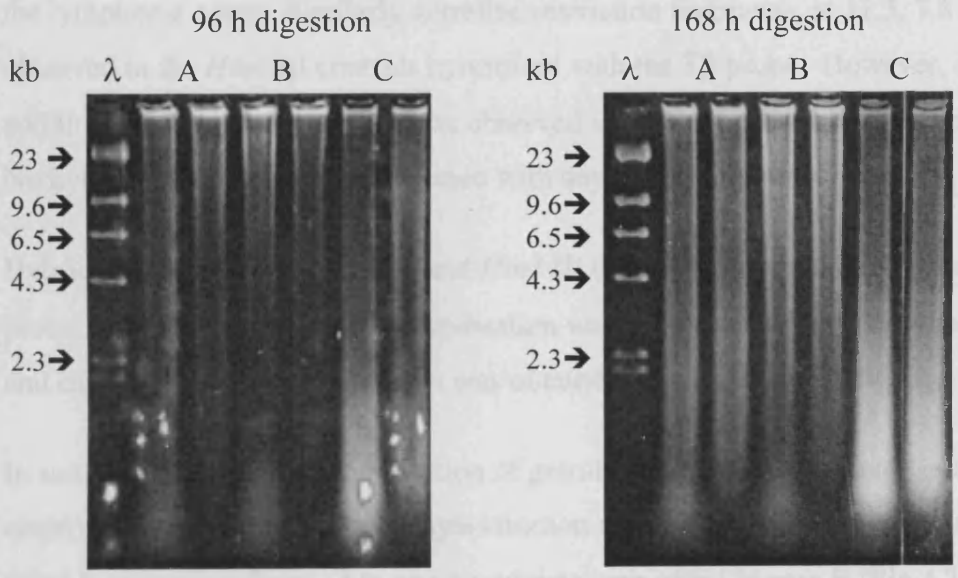
Restriction endonuclease digestion was attempted using the 96 h digest samples with the exception of case I. This case was not included as electrophoresis of the recovered nucleic acid indicated that only low molecular weight DNA was present. Initial restrictions were undertaken using 15 μg of DNA with *Bgl* II, *Hin*d III and *Eco*R I for 4 h. Unfixed DNA was used as a control to validate each restriction enzyme. The *Hin*d III digests were set up in duplicate to allow for JH and Tβ hybridisation. With the latter germline restriction fragments of 11.5, 7.6 and 3.5 kb were expected and it was considered that these would be more likely to be present in degraded DNA than the 24 kb germline *Bam*H I band which had been the subject of previous Tβ hybridisations. Restriction smears were observed in the control preparations. However, in the lymphoma cases a restriction smear was obtained with *Hin*d III in case B only.

A further attempt was made to obtain successful digestion by incubating 17 μg of DNA from cases B, C, F-H and J with the same enzymes overnight. These cases were selected due to their satisfactory protein and carbohydrate purity ratios. DNA recovered from fresh tissue and 24 h formol saline fixed paraffin embedded tissue was used to validate each restriction digest. Electrophoretic analysis of 2 μg of each digest revealed smears in the controls, case B with *Bgl* II and cases G and H with *Bgl* II and *Hin*d III. Equivocal restriction of case B with *Eco*R I and *Hin*d III and case F with all enzymes was also observed. However, in the remaining restriction enzyme/sample combinations no evidence of successful digestion was obtained. Overnight re-restriction of the same samples was attempted and electrophoretic analysis of these preparations was undertaken in total. In the controls and case B results were as observed in the initial overnight restrictions. Possible restriction with *Bgl* II with case G and *Hin*d III with case H was also observed whilst only low molecular weight DNA was observed in the latter case restricted with *Eco*R I (Fig 4.2.2). In the remaining restriction enzyme/sample combinations no evidence of successful digestion was obtained. Southern transfer of these gels was undertaken.

Table 4.2.1 DNA yields and purity of initial lymphoma cases

Cases	96 h digestion			168 h digestion		
	DNA yield (μg/mg tissue)	Protein and carbo-hydrate ratios		DNA yield (μg/mg tissue)	Protein and carbo-hydrate ratios	
A	2.8	1.83	1.27	2.4	2.80	0.44
B	4.9	1.86	0.50	11.6	2.10	0.24
C	6.1	1.83	0.54	3.8	3.00	0.49
D	1.8	1.97	3.50	5.3	1.50	4.48
E	2.4	1.92	1.19	0.2	0.28	9.44
F	3.6	1.73	0.58	2.0	4.30	0.78
G	5.6	1.83	0.52	9.4	2.20	0.32
H	8.0	1.80	0.48	11.1	2.11	0.34
I	9.7	1.78	0.50	16.1	1.96	0.31
J	12.3	1.82	0.73	10.3	2.14	0.20

Fig 4.2.1 Electrophoretic analysis of initial lymphoma preparations, cases A-C



Key
λ Lambda *Hind* III size marker
Note: Second lane for each case RNase pre-treated

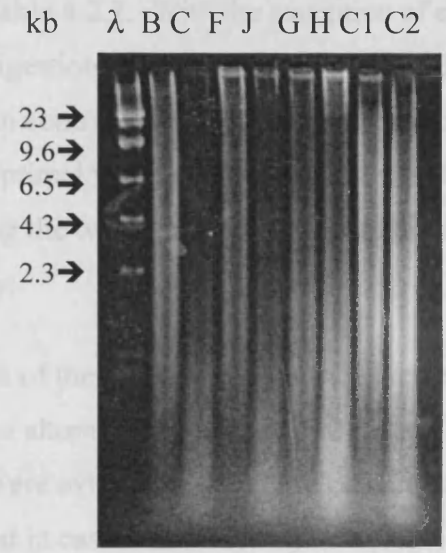
Initial hybridisation results were unsatisfactory. No hybridisation was observed with *Bgl* II and *Hin*d III digests hybridised with the JH probe and strong non-specific smears were obtained with the *Eco*R I and (separate) *Hin*d III digests hybridised with the Tβ probe. Due to the latter result new overnight restrictions were set up using 15 μg of each DNA sample. Electrophoretic analysis of these preparations was undertaken in total. In the controls and case B smears were observed with the *Eco*R I and *Hin*d III enzymes. In the remaining lymphoma cases possible restriction with *Eco*R I with cases C, F and J only was observed. No evidence of successful *Hin*d III restriction in these cases was observed. Southern transfer of these gels was undertaken.

Re-hybridisation of the initial transfers was undertaken. *Eco*R I and *Hin*d III digests were hybridised with the Tβ probe whilst *Bgl* II and (separate) *Hind* III digests were hybridised with the JH probe. Germline restriction fragments were observed with the JH probe in the controls and case B. No hybridisation bands were obtained with any other lymphoma case. In autoradiographs hybridised with the Tβ probe germline restriction fragments were observed in the fresh and fixed *Eco*R I digested controls but no bands were obtained with the lymphoma cases. Similarly germline restriction fragments, at 11.5, 7.6 and 3.5 kb, were observed in the *Hin*d III controls hybridised with the Tβ probe. However, in case B additional hybridisation at 3 kb was observed indicating a possible T cell rearrangement. No hybridisation bands were obtained with any other lymphoma case.

Hybridisation of the new *Eco*R I and *Hin*d III digests was also undertaken with the Tβ probe. With *Eco*R I germline hybridisation was observed with the fresh and fixed controls and case B only. No hybridisation was obtained with the *Hin*d III digests.

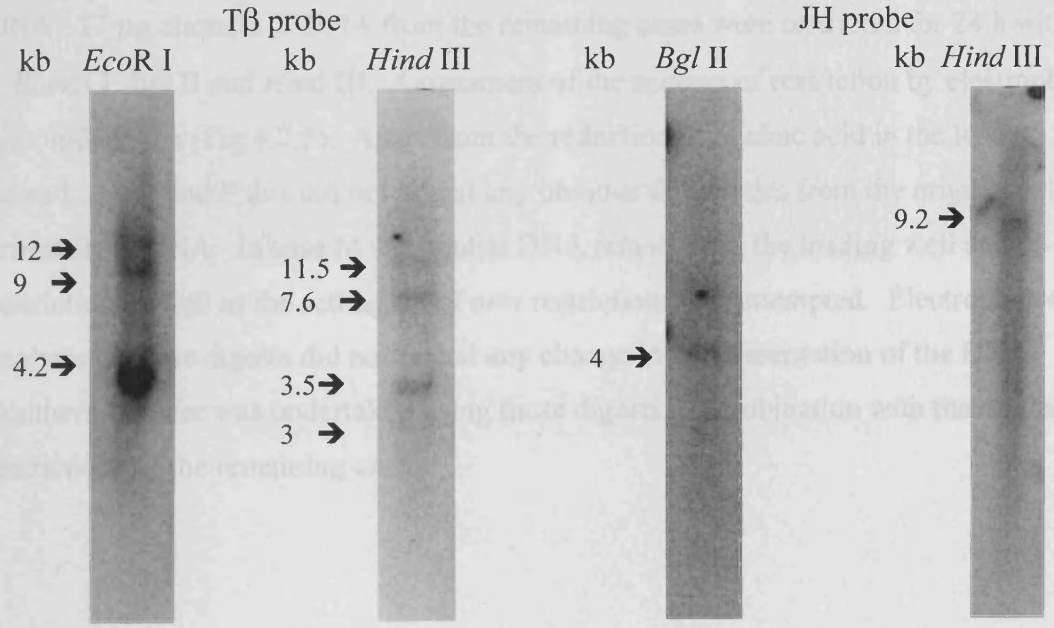
In summary successful demonstration of germline restriction fragments in fresh and fixed control DNA was obtained after hybridisation with Tβ and JH probes. However, hybridisation was observed in one lymphoma case only. In case B (Fig 4.2.3) germline hybridisation bands were observed for *Bgl* II and *Hin*d III digests probed with JH and *EcoR* I probed with Tβ. However, in one Tβ hybridisation with *Hin*d *III* digestion the expected germline restriction fragments were supplemented by the presence of an additional band suggesting a T cell rearrangement or the demonstration of a restriction site polymorphism.

Fig 4.2.2 Electrophoretic analysis of *Eco*R I re-restricted initial lymphoma preparations



Key
λ Lambda *Hind* III size marker
Lymphoma cases indicated by code
C1 = fresh tissue control, C2 = formol saline control

Fig 4.2.3 Southern analysis of lymphoma case B



Analysis of lymphoma cases with known fixation conditions.

DNA yields together with the protein and carbohydrate purity ratios for the lymphoma cases are provided in Table 4.2.2. With the exception of case K the yield of DNA was similar using the two digestion buffers. In this case the DNA yield was almost doubled using the Tris buffer. In contrast the protein and carbohydrate purity ratios calculated for case K were closer to optimal values after use of the urea buffer. In all other samples these ratios were similar using the two digestion buffers and in cases M-P indicated high protein and carbohydrate purity.

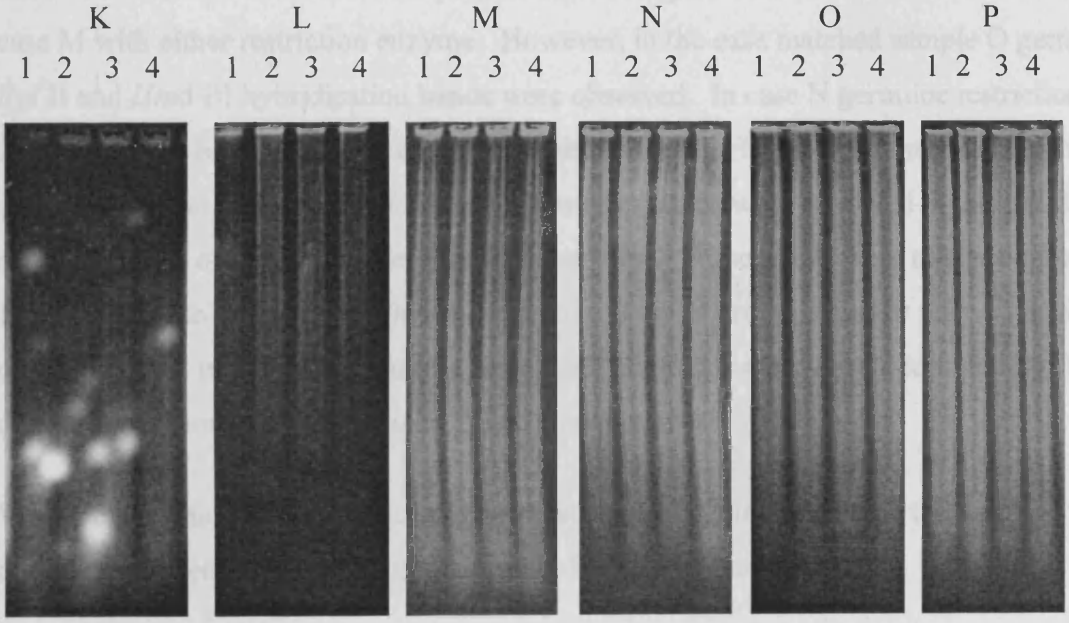
Electrophoretic analysis of the samples (Fig 4.2.4) revealed no differences in the quality of DNA obtained using the alternative digestion buffers and RNA was not demonstrated. However, differences were evident from case to case. In case K only low molecular weight DNA was present whilst in case L the majority of nucleic acid was present in the gel loading well. In the remaining cases the DNA was extensively degraded. No difference was observed in the quality of DNA recovered from case N (stored paraffin block) and case P (extracted without storage). However, with case M (stored paraffin block) less high molecular weight DNA was observed than case O (extracted without storage).

No further analysis of case K was undertaken due to the absence of high molecular weight DNA. 17 μg aliquots of DNA from the remaining cases were restricted for 24 h with *Eco*R I, *Bam*H I, *Bgl* II and *Hin*d III. Assessment of the success of restriction by electrophoresis was undertaken (Fig 4.2.5). Apart from the reduction of nucleic acid in the loading well in cases L, N, O and P this did not reveal any obvious differences from the original gels of unrestricted DNA. In case M substantial DNA remained in the loading well and re-restriction as well as the setting up of new restrictions was attempted. Electrophoretic analysis of these digests did not reveal any change in the presentation of the DNA. Southern transfer was undertaken using these digests in combination with the original restrictions of the remaining cases.

Table 4.2.2 DNA yields and purity of lymphoma cases with known fixation conditions

Cases	96 h digestion, Tris buffer			96 h digestion, Urea buffer		
	DNA yield (μg/mg tissue)	Protein and carbo-hydrate ratios		DNA yield (μg/mg tissue)	Protein and carbo-hydrate ratios	
K	9.1	1.56	0.76	5.1	1.77	0.61
L	13.9	1.39	0.67	12.9	1.33	0.70
M	10.0	1.77	0.51	11.2	1.78	0.48
N	16.2	1.84	0.47	12.1	1.87	0.46
O	16.4	1.80	0.50	14.7	1.82	0.47
P	10.1	1.75	0.51	12.0	1.82	0.47

Fig 4.2.4 Electrophoretic analysis of lymphoma cases with known fixation conditions



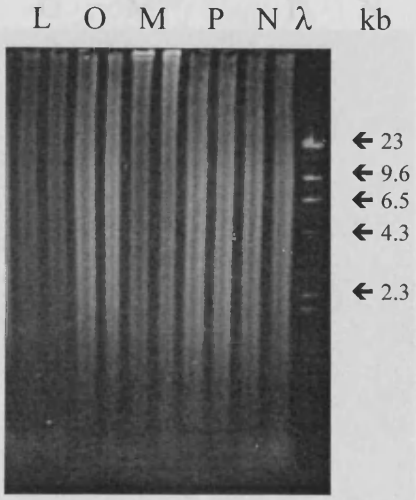
Key
Lanes 1 and 2 urea digestion buffer
Lanes 3 and 4 Tris digestion buffer
Note: Even lanes for each case RNase pre-treated

Hybridisation with the Tβ probe of the *EcoR* I digests revealed germline restriction fragments only (Fig 4.2.6). In cases L and M only 4 and 6 kb bands were demonstrated whilst in the other cases restriction fragments at 4, 6, 9 and 12 kb were observed. No hybridisation bands were demonstrated in the *BamH* I transfers hybridised with the Tβ probe. This was probably due to the degradation of DNA. New *Hin*d III restriction digests for case O were prepared, transferred and hybridised with the Tβ probe in an attempt to demonstrate gene rearrangement in this known T cell lymphoma. In the resultant autoradiograph germline restriction fragments were demonstrated.

The *Bgl* II and *Hin*d III digests were hybridised with the JH probe. The resultant autoradiographs are presented in Fig 4.2.7 and 4.2.8. In case L a weak 4 kb germline restriction fragment was observed in the *Bgl* II autoradiograph but no hybridisation bands were demonstrated in the *Hin*d III preparation. No hybridisation bands were obtained for case M with either restriction enzyme. However, in the case matched sample O germline *Bgl* II and *Hin*d III hybridisation bands were observed. In case N germline restriction fragments with both restriction enzymes were observed. However, in the case matched sample P several additional high molecular weight bands were observed in the *Bgl* II autoradiograph of the Tris buffer digest preparation. These bands were at approximately 10, 12 and 20 kb and suggest identification of a B cell rearrangement or restriction site polymorphism. In this case germline restriction fragments were observed in the *Bgl* II urea digest preparation and in the *Hin*d III restricted samples.

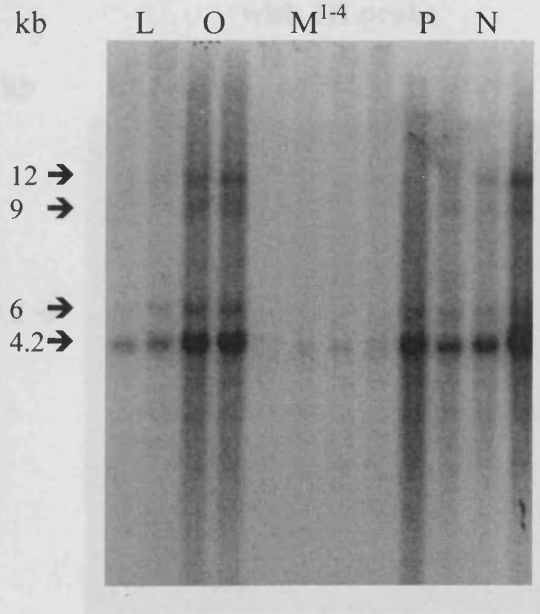
With the exception recorded above hybridisation results obtained using the Tris and urea digests were identical or differed marginally in band intensity.

Fig 4.2.5 Electrophoretic analysis of *Bgl* II lymphoma cases with known fixation conditions



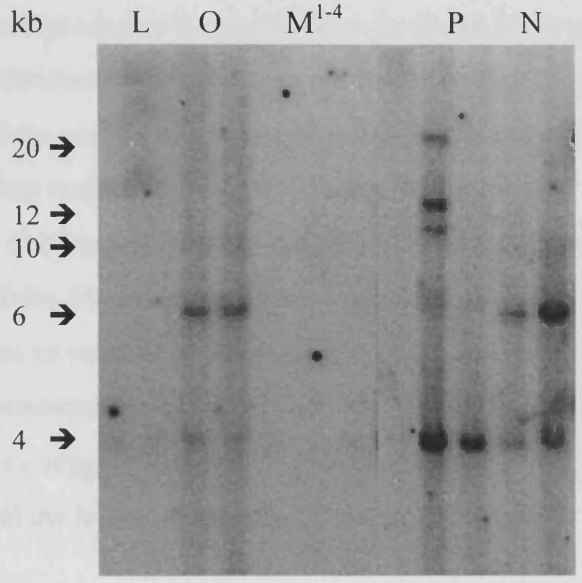
Key
First lane for each case Tris digestion buffer preparation
Second lane for each case urea digestion buffer preparation

Fig 4.2.6 Southern analysis of *Eco*R I restricted lymphoma cases L-P hybridised with Tβprobe



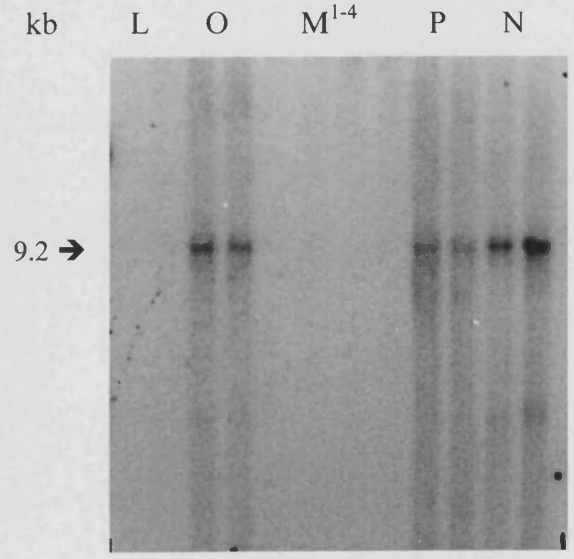
Key
First lane for each case Tris digestion buffer preparation
Second lane for each case urea digestion buffer preparation
M^{1-2} = second restriction, M^{3-4} = new restriction

Fig 4.2.7 Southern analysis of *Bgl* II restricted lymphoma cases L-P hybridised with JH probe



Key
See Fig 4.2.6
Note: Multiple hybridisation bands present in Tris digest preparation of case P

Fig 4.2.8 Southern analysis of *Hin*d III restricted lymphoma cases L-P hybridised with JH probe



Key
See Fig 4.2.6

In summary DNA was successfully recovered from cases K-N in which short fixation had been followed by the storage of paraffin wax blocks before extraction. However, in case K the DNA was too degraded to be analysed by Southern blotting and in cases L and M the restriction and hybridisation results indicate that very little of the nucleic acid was in a form suitable for Southern analysis. In contrast DNA obtained from case O, case matched with sample M, germline restriction fragments were demonstrated. Germline hybridisation results were also obtained in case N. However, in the case matched sample P, in which tissue was fixed from frozen, embedded in wax then extracted immediately evidence of a B cell rearrangement or restriction site polymorphism was obtained in the *Bgl II* digest probed with JH. The demonstration of bands between 10 and 20 kb in distinction from the 4 kb germline band may suggest that the DNA in the routinely fixed paraffin block was too degraded to reveal the higher molecular restriction fragments.

4.3 Investigation of the effect of extended fixation and tissue processing on the quality of DNA recovered from fixed tissue

4.3.1 Experimental design

Palatine tonsil from two independent samples was processed according to the method described in 2.2.2. Unfixed tissue was stored at -70°C. The remaining tissue was fixed in Carnoy, formol saline and neutral buffered formalin. Tissue was removed from the fixatives after 24, 96 and 168 h and processed for paraffin embedding according to the method detailed in 2.2.2. For DNA extraction the dry weight of the tissue was recorded then the tissue was diced and rehydrated according to method 2.2.7c. In addition after 24 and 168 h fixation, a piece of tissue was removed from each of the fixative solutions and weighed. These pieces were diced and washed in 50mM Tris/HCl, pH 8.0 to remove fixative prior to digestion according to the method described in 2.2.7b. As a control fresh frozen tissue was removed from storage, weighed then diced prior to digestion. Each preparation was digested using Proteinase K according to method 2.2.8. Fixed tissues were digested for 168 h whilst unfixed tissue was incubated for 24 h.

A quantitative and qualitative assessment of the recovered nucleic acid was made according to methods 2.2.9 and 2.2.10. Restriction enzyme digestion, Southern blotting and hybridisation, using method b, was also undertaken (methods 2.2.11, 2.2.12 and 2.2.13).

4.3.2 Results

Extraction of DNA

During digestion initially white tissue fragments became translucent then transparent. In Carnoy fixed preparations complete digestion of the tissue was obtained. All samples were slightly viscous at the end of the 168 h digestion with the exception of the 24 h fixed and

paraffin preparations in which no viscosity was observed. Moderately sized floating masses of DNA were observed immediately after the addition of the precipitating reagents in Carnoy fixed extracts with the exception of the 168 h fixed preparations made from the first tonsil. In these the quantity of floating DNA was smaller. After overnight storage at -20°C additional flocculent material was observed in the 24 h fixed and paraffin processed extracts but no change was noted in the other samples.

Tissue fragments remained at the end of the digestion period in all formaldehyde fixed samples. The 24 h fixed and unprocessed neutral buffered formalin preparations were viscous from 72 h of incubation in proteinase K in contrast to the slight viscosity observed in all other samples at the end of the digestion. Moderately sized floating masses of DNA were observed immediately after the addition of the precipitating reagents to the 24 h fixed and unprocessed formol saline fixed digests. In equivalent paraffin processed samples small masses of DNA were observed but in all other preparations no precipitate was noted. In contrast an initial precipitate of DNA was observed in all neutral buffered formalin fixed preparations. This was of small size except for the 24 h fixed and unprocessed samples in which a moderately sized floating mass of DNA was observed.

Translucent fragments of tissue suspended in a slightly viscous solution remained in the unfixed preparation at the end of the 24 h digestion period. Small masses of floating DNA were observed upon the addition of the precipitation reagents.

Quantitative and qualitative assessment of extracted DNA

The yields of DNA (Table 4.3.1) were calculated by dividing the total yield of DNA with the weight of sample before extraction. It should be noted that the weight of the paraffin wax preparations was a dry weight in contrast to a wet weight for the unprocessed samples. Consistently higher DNA yields were obtained from the paraffin processed tissues. This was particularly emphasised in the 168 h fixed preparations from tonsil 1 in which the yield of DNA was more than double that of the unprocessed tissue. No consistent difference in DNA yield was observed in relation to fixative used.

Protein purity ratios (Table 4.3.1) were usually higher for the paraffin preparations than the unprocessed samples. As with DNA yield no consistent difference in protein purity was observed in relation to fixative used. Carbohydrate purity ratios could not be calculated for some preparations due to negative optical density readings being obtained at 230 nm. In the preparations in which these ratios were obtained (Table 4.3.1) there was no apparent correlation with fixative used or subsequent tissue processing.

Electrophoretic analysis of the extracts (Fig 4.3.1) revealed some marked differences between individual preparations, which were reproduced identically in both tonsil samples. The origin of the electrophoretic smear was, with the exception of the formalin fixed and paraffin processed preparations, at the exclusion zone. In the excepted preparations the origin of the smear was at the gel loading well. Fixative, fixation time and paraffin processing influenced the quality of smear. After 24h fixation the integrity of the DNA was better preserved than in preparations fixed for longer periods. The integrity of the DNA was marginally affected by paraffin processing in the Carnoy fixed preparations. However, in the formalin fixed extracts the 24 h fixed unprocessed extracts gave smears indicating better preservation of DNA in comparison with paraffin processed counterparts. After 168 h formol saline fixation only low molecular weight DNA was present in the unprocessed tissue in comparison with the presence of an even smear in the equivalent paraffin processed samples. By contrast in neutral buffered formalin preparations after 168 h fixation even smears of DNA were present in both the unprocessed and paraffin processed samples.

Table 4.3.1 DNA yields and purity of extracts

a) Unprocessed preparations

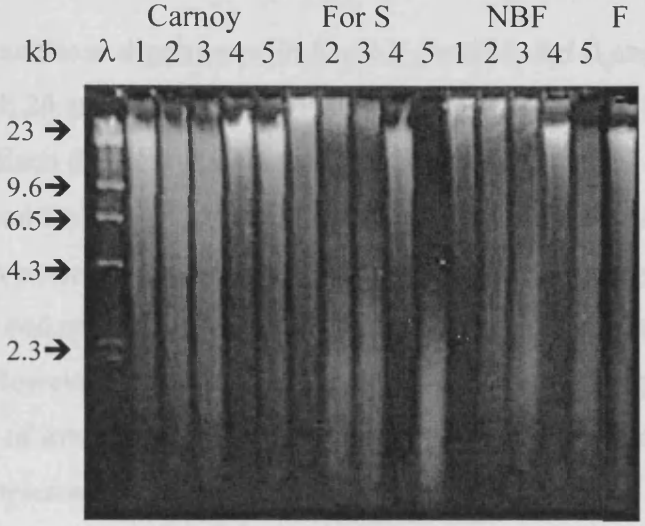
Fixative and time	Tonsil 1			Tonsil 2		
	DNA yield (μg/mg tissue)	Protein and carbo-hydrate ratios		DNA yield (μg/mg tissue)	Protein and carbo-hydrate ratios	
Carnoy 24 h	18.5	1.78	0.60	7.9	1.81	0.19
Carnoy 168 h	15.3	1.79	0.41	18.2	1.76	0.54
Formol saline 24 h	13.1	1.99	0.70	13.3	1.88	0.52
Formol saline 168 h	16.5	1.64	-	21.5	1.92	0.62
NBF 24 h	9.1	1.66	-	16.7	1.76	0.53
NBF 168 h	9.5	1.70	-	11.8	2.22	0.93
Fresh frozen	6.3	1.60	-	7.9	1.78	0.67

b) Paraffin processed preparations

Fixative and time	Tonsil 1			Tonsil 2		
	DNA yield (μg/mg tissue)	Protein and carbo-hydrate ratios		DNA yield (μg/mg tissue)	Protein and carbo-hydrate ratios	
Carnoy 24 h	22.1	2.07	0.80	14.0	2.05	1.06
Carnoy 96 h	20.5	2.00	0.66	17.8	2.21	0.87
Carnoy 168 h	30.9	1.74	0.48	24.2	1.94	0.67
Formol saline 24 h	20.7	2.19	0.85	18.1	2.59	1.20
Formol saline 96 h	17.9	2.04	0.93	11.7	1.36	-
Formol saline 168 h	43.7	1.84	0.61	31.6	1.56	0.14
NBF 24 h	23.0	1.80	0.78	24.2	1.89	0.47
NBF 96 h	16.5	1.81	0.50	21.9	2.20	1.48
NBF 168 h	29.6	1.60	0.13	33.4	1.84	0.54

Key
NBF Neutral buffered formalin
- Calculation not possible due to negative optical density reading at 230 nm

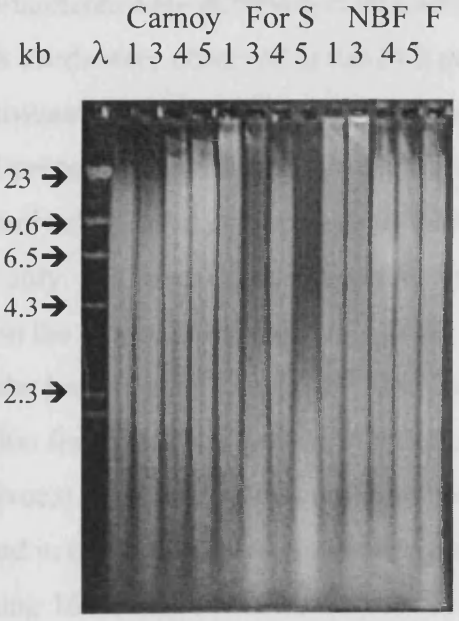
Fig 4.3.1 Electrophoretic analysis of DNA extracts from tonsil 1



Key

For S = Formol saline, NBF = Neutral buffered formalin, F = Frozen
λ = Lambda *Hin*d III size marker, 1 = 24 h fixation, paraffin processed
2 = 96 h fixation, paraffin processed, 3 = 168 h fixation, paraffin processed
4 = 24 h fixation, unprocessed, 5 = 168 h fixation, unprocessed

Fig 4.3.2 Electrophoretic analysis of *Hin*d III restricted DNA from tonsil 1



Key

See Fig 4.3.1

Restriction digestion and Southern Blotting

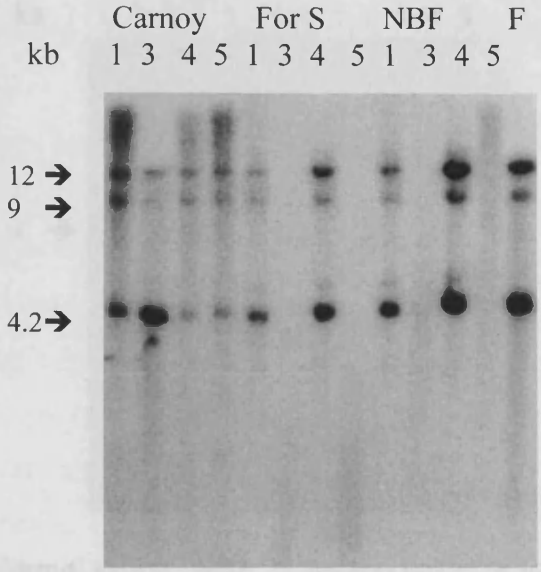
Restriction endonuclease digestion with *Eco*R I, *Bam*H I, *Bgl* II and *Hin*d III was undertaken for all 24 and 168 h fixed preparations and the unfixed DNA extracts from both tonsil samples. Each digest was set up with 20 μg of DNA and 50 units of enzyme and allowed to proceed for 24 h. Evidence of successful restriction was provided by the presence of an even smear of DNA in all 24 h fixed and unfixed preparations (Fig 4.3.2). In 168 h Carnoy and neutral buffered formalin fixed samples similar evidence of digestion was observed. However, in the 168 h formol saline fixed paraffin processed samples most of the DNA was of low molecular weight and the appearance of the DNA in the unprocessed samples was unchanged from the original extract.

*Eco*R I restricted samples were probed with Tβ. Broadly similar results were observed in both tonsils. However, a partial restriction band just above the 4.2 kb restriction fragment was present in tonsil 1 preparations and the 9 kb restriction fragment was not consistently present in samples from tonsil 2. In the unfixed preparations the bands were of strong intensity. Restriction fragments were observed in all Carnoy fixed preparations (Fig 4.3.3). In tonsil 1 the strongest bands were observed in the 24 h paraffin processed preparation and weakest in the equivalent 168 h fixed digest. However, these differences in intensity were slight. In tonsil 2 preparations uniformly strong restriction fragments at 4 and 12 kb were observed. In formol saline fixed preparations from both tonsils bands were noted in the 24 h fixed samples only. In tonsil 1 preparations the intensity of the restriction fragments was greater in the unprocessed sample. Results with neutral buffered formalin fixed digests were similar but not identical. In the 24 h fixed preparations of tonsil 1 the intensity of the restriction fragments was greater in the unprocessed samples. In the 168 h fixed preparations equivocal restriction fragments were observed in the paraffin processed sample from tonsil 1 and in the unprocessed sample from tonsil 2. No hybridisation was observed in the remaining 168 h neutral buffered formalin digests.

The *Bam*H I digests were hybridised with the Tβ probe and more sensitive results were obtained with tonsil 1 preparations. In these preparations a 24 kb restriction fragment was observed in the 24 h Carnoy fixed unprocessed sample only (Fig 4.3.4). However, in some other digests the presence of a smear at the expected site of the restriction fragment suggested that hybridisation to degraded DNA fragments had occurred. In the unfixed, 24 h Carnoy fixed paraffin processed preparation and 168 h Carnoy unprocessed sample this smear was of strong intensity. In contrast in the 168 h Carnoy fixed paraffin processed digest a smear of weak intensity was observed. Hybridisation smears were present only in formalin preparations fixed for 24 h. These were of strong intensity. In tonsil 2 preparations the 24 kb *Bam*H I restriction fragment was observed in the unfixed sample only. In the fixed sample smears in the region of the expected band were present in some samples. A smear of moderate intensity was recorded in the 24 h Carnoy fixed unprocessed preparation whilst in the other Carnoy samples the smears were of weak intensity. In formalin fixed preparations very weak smears were observed in the 24 h formalin fixed preparations only

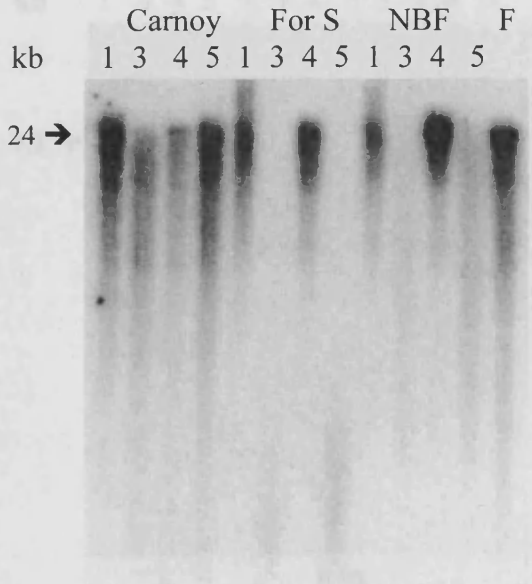
Slightly more sensitive hybridisation results were obtained for *Bgl* II digests probed with JH with preparations from tonsil 1. Strong hybridisation to the 4 kb restriction fragments were observed in the unfixed and all Carnoy fixed preparations (Fig 4.3.5). In the formalin fixed samples restriction fragments of similar intensity were observed in the 24 h fixed unprocessed digests. However, in the equivalent paraffin processed preparations restriction fragments of moderate intensity were noted. No hybridisation was observed in the 168 h formalin fixed samples. In tonsil 2 preparations the 4 kb restriction fragment was of strong intensity in the unfixed and 24 h Carnoy fixed unprocessed sample. In the remaining Carnoy preparations restriction fragments of moderate intensity were observed. In the formalin fixed preparations of this tonsil a strong restriction fragment was noted in the unprocessed 24 h neutral buffered formalin fixed sample whilst in the remaining 24 h fixed digests the 4 kb restriction fragment was of weak intensity. No hybridisation was observed in the 168 h formalin fixed preparations.

Fig 4.3.3 Southern Analysis of *Eco*R I digested DNA from tonsil 1 hybridised with Tβ probe



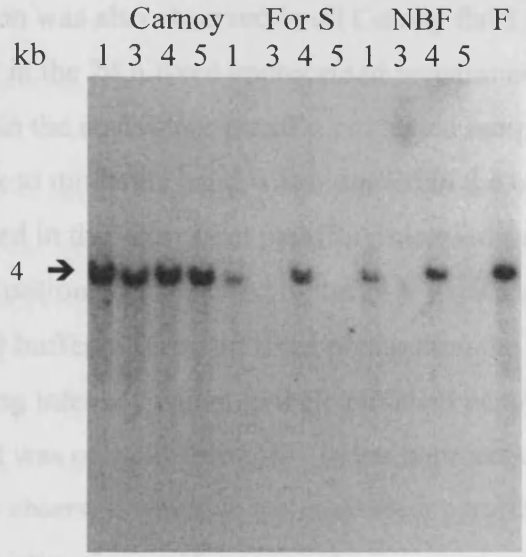
Key
For S = Formol saline, NBF = Neutral buffered formalin, F = Frozen
1 = 24 h fixation, paraffin processed, 3 = 168 h fixation, paraffin processed
4 = 24 h fixation, unprocessed, 5 = 168 h fixation, unprocessed

Fig 4.3.4 Southern Analysis of *Bam*H I digested DNA from tonsil 1 hybridised with Tβ probe



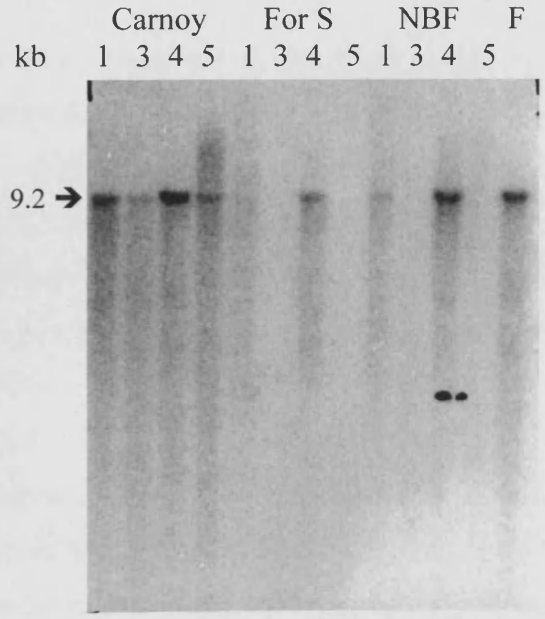
Key
See Fig 4.3.3

Fig 4.3.5 Southern analysis of *Bgl* II restricted DNA from tonsil 1 hybridised with JH probe



Key
For S = Formol saline, NBF = Neutral buffered formalin, F = Frozen
1 = 24 h fixation, paraffin processed, 3 = 168 h fixation, paraffin processed
4 = 24 h fixation, unprocessed, 5 = 168 h fixation, unprocessed

Fig 4.3.6 Southern analysis of *Hind* III restricted DNA from tonsil 1 hybridised with JH probe



Key
See Fig 4.3.5

For *Hind* III digests probed with JH the expected restriction fragments were observed in tonsil 1 only. In the unfixed preparation a strong 9 kb restriction fragment was noted (Fig 4.3.6). Hybridisation was also observed in all Carnoy fixed samples. A band of strong intensity was noted in the 24 h fixed unprocessed preparation and a band of weak to moderate intensity in the equivalent paraffin processed sample. In the 168 h fixed Carnoy preparations a weak to moderate band was observed in the unprocessed sample whilst a weak band was noted in the equivalent paraffin processed sample. In the formalin fixed preparations hybridisation was observed in the 24 h fixed samples only. In the unprocessed neutral buffered formalin fixed preparation the 9 kb restriction fragment was of moderate to strong intensity whilst in the equivalent paraffin processed sample the restriction fragment was of weak intensity. In the unprocessed formol saline preparation a moderate band was observed whilst in the equivalent paraffin processed sample an equivocal 9 kb restriction fragment was noted.

4.4 Discussion

4.4.1 Analysis of lymphoma specimens

Initial assessment was undertaken using 10 known or suspected lymphomas (2.1.3a). Six of these cases had been received as routine surgical specimens, fixed in formol saline for an undetermined period prior to paraffin embedding and then stored for 15 to 21 months before DNA extraction. The remaining cases were referred for analysis, one of these being identified as formalin fixed. The results obtained with these cases were disappointing and may be summarised as follows:

1. The DNA of all cases was degraded and in some cases markedly so.
2. Restriction endonuclease digestion was problematic.
3. Restriction fragments, including a possible T cell rearrangement or demonstration of a restriction site polymorphism, were demonstrated in one case only.

Due to the difficulties encountered with the initial lymphoma cases a further four cases were selected for analysis for which fixation conditions were known. These represented two T and two B cell lymphomas in which gene rearrangement using unfixed tissue had been demonstrated (2.1.3b). Fixation had been for 24 to 72 h in formol saline prior to paraffin wax embedding. Frozen unfixed tissue was available for two cases and portions of these were fixed for 24 h in formol saline, paraffin embedded and DNA was recovered immediately.

When compared with the initial cases the results were improved but not optimal. DNA degradation was again observed in all cases and in one was so extensive as to preclude further analysis. Due to this degradation it was difficult to assess if restriction endonuclease digestion had been successful. However, germline restriction fragments were

demonstrated in two cases and in one of these, fixed from frozen, evidence of a possible B cell rearrangement or demonstration of a restriction site polymorphism was obtained.

In an analysis of an unspecified number of buffered formalin fixed paraffin wax embedded lymphoma cases Dubeau *et al* (1988) stated an overall success rate of 75% for the demonstration of B cell gene rearrangements. In a case report of Warthin's tumour with malignant lymphoma Bunker and Locker (1989) also demonstrated B cell rearrangement by deliberately employing restriction endonuclease and probe combinations compatible with the analysis of degraded DNA. However, the authors reported problems with *Eco*R I and *Hin*d III restriction of the extracted DNA. Problems, due to the degradation of DNA, in the identification of restriction fragments in formalin fixed paraffin wax embedded tissue have also reported by Handt *et al* (1990) and Wu *et al* (1990).

Combining these observations with those of the present assessment indicate that Southern analysis of routine formalin fixed paraffin wax embedded lymphoma specimens is, as concluded by Handt *et al* (1990) and Wu *et al* (1990), not reliable. The results also suggest that 'extended' fixation or the storage of embedded tissue had a marked influence on the quality of DNA recovered. The influence of the former is discussed below.

4.4.2 Effect of extended fixation and tissue processing on DNA quality

For this investigation blocks of tissue from two tonsil samples were fixed in Carnoy, formol saline and neutral buffered formalin for 24, 96 and 168 h and paraffin wax embedded. Additional blocks of the same tissues were also fixed for 24 and 168 h in the same fixatives then washed prior to the recovery of DNA by Proteinase K digestion for 168 h.

As previously observed complete tissue digestion was obtained with Carnoy fixed preparations whereas tissue fragments remained in the formalin samples at the end of the digestion period. DNA yield was consistently higher in paraffin wax embedded tissue regardless of fixation (Table 4.3.1). However, as these values were calculated with reference to dry weight of tissue compared with wet weight for the unprocessed samples

this may be an apparent rather than real difference. There was no consistent difference in DNA yield according to fixative employed. This contrasts with values reported by Jackson *et al* (1990) who recovered from 24 h fixed Carnoy tissue 20 times as much DNA as from equivalent formalin fixed preparations. These authors also noted that the yield of DNA from unbuffered formalin fixed tissue decreased markedly within 24 h. However, in this report the duration of proteolytic digestion is not recorded and, as observed in chapter 3, this can influence the rate of release of DNA.

In summary electrophoretic analysis revealed consistent differences in the quality of recovered DNA. These were:

1. Least DNA degradation in 24 h fixed preparations.
2. Progressive degradation of formalin fixed DNA with extended fixation in comparison with slight alteration after prolonged Carnoy fixation.
3. Apparent reduction in the molecular weight of DNA in 168 h formol saline fixed unprocessed sample compared with the equivalent paraffin embedded preparation.
4. Reduced electrophoretic mobility of formalin fixed paraffin embedded DNA samples in comparison with all Carnoy and unprocessed preparations.

Restriction endonuclease digestion, undertaken with all 24 and 168 h fixed preparations, was successful. However, Southern analysis demonstrated that the preservation of DNA was influenced, in order of importance, by fixative, duration of fixation and paraffin processing. Overall the best results were obtained with Carnoy fixed preparations. Hybridisation was consistently observed in 24 h and 168 h fixed samples. The intensity of signal indicated that after extended fixation some degradation of DNA had occurred and that this was emphasised in the paraffin processed samples. The influence of extended fixation time was particularly shown in the formalin fixed preparations. In these, hybridisation was usually absent in the 168 h fixed samples. In contrast adequate hybridisation results were recorded in the majority of the 24 h fixed preparations whilst comparison of the band intensity indicated that paraffin processed resulted in further degradation of the DNA. Comparison of the intensity of the hybridisation signals also

suggested that fixation in neutral buffered formalin was slightly less detrimental to the preservation of DNA than the use of formol saline.

The deleterious effect of prolonged fixation on the quality of recovered DNA has also been reported in several investigations. In model experiments employing overnight and 48 h Carnoy, unbuffered (pH 4.3) and buffered formalin (pH 7.2) fixation at 4°C and room temperature Koshiba *et al* (1993) reported degradation of the DNA unless low temperature fixation was employed. However, extended exposure to buffered formalin at 4°C resulted in a gradual degradation and only low molecular weight DNA was recovered from samples fixed for 144 h. These authors also observed more extensive degradation of DNA recovered from paraffin wax compared with unprocessed formalin fixed tissue. In a model experiment in which several tissue types were fixed in 2, 4 or 8% unbuffered formalin from 1 to 70 days Wiegand *et al* (1996) also recorded a progressive decrease in amount and molecular weight of DNA recovered as fixation time was extended. Yagi *et al* (1996) investigated the effect of buffered formalin fixation at 4°C, room temperature and 37°C on the recovery and Southern analysis of DNA from spleenic tissue. A v-H-ras restriction fragment of over 10 kb was consistently demonstrated in DNA recovered from tissue fixed for 168 h at 4°C. In contrast this restriction fragment was unequivocally demonstrated only after 24 h fixation at room temperature and was not observed in DNA analysed from tissue fixed for the same length of time at 37°C.

Together these observations emphasise that formalin fixation results in time and temperature dependant degradation of DNA. At room temperature this process is relatively rapid and is exacerbated by subsequent paraffin wax embedding. Accordingly the results offer an explanation for the failure to demonstrate T and B cell rearrangements in the formalin fixed paraffin wax tissues evaluated in the previous section of this Chapter. Practically they suggest that unless conditions of formalin fixation are precisely controlled and restriction endonuclease and probe combinations chosen to identify low molecular weight restriction fragments then Southern analysis will be sub-optimal.

Chapter 5 The effect of tissue fixation on polymerase chain reaction efficiency: A study of *bcl2* sequences

5.1 Introduction

In the previous chapter fixation induced degradation of DNA was shown to place practical limitations on the application of Southern analysis for T and B cell gene rearrangements. The polymerase chain reaction (PCR) provides an alternative analysis method based on the primer directed amplification of relatively short sequences of DNA. Accordingly, in this chapter investigations were undertaken to determine the compatibility of PCR with DNA recovered from fixed and paraffin wax embedded tissue. The influence of Carnoy, formol saline and neutral buffered formalin fixation, paraffin wax processing and different methods for the preparation of DNA on PCR for the amplification of *Bcl2* sequences recovered from normal lymphoid tissue are presented. The chapter concludes with an assessment of the application of PCR for the identification of t(14;18) translocation in follicular lymphomas using DNA recovered from 9 fixed and paraffin embedded cases.

5.2 Experimental design

DNA, which had been prepared from tonsil 1, as described in 4.3, was used as 'standard' DNA in the investigations. In addition 'rapid' DNA extracts were prepared from paraffin wax sections of the same tonsil according to the method described in 2.2.15.

'Rapid' DNA extracts were also prepared from nine formol saline fixed and paraffin processed cases of follicular lymphoma as detailed in 2.1.3c. The dimensions of tissue presented in the paraffin blocks varied according to case and to compensate for this a variable number of 5 μm sections were cut from each block for DNA extraction (Table 5.2.1).

Table 5.2.1 Dimensions and sections cut from follicular lymphoma cases for DNA preparation

Case code	Maximum dimensions	Sections cut
Q	20 x 20 mm	5
R	15 x 15 mm	5
S	12 x 7 mm	10
T	17 x 11 mm	5
U	17 x 8 mm	5
V	22 x 12 mm	8
W	15 x 12 mm	5
X	17 x 14 mm	5
Y	10 x 10 mm	10

Primers for amplification of the *Bcl2* gene were selected using sequence data as identified by Seto *et al* (1988). The hsbcl2c.em-hum1 RNA sequence of 6030 bases was used to define a single reverse primer and 5 forward primers that spanned the major break point region for the *Bcl2* gene situated on chromosome 18. The characteristics of these primers are detailed in Table 2.1.2. Combination of the single reverse and 5 forward primers, respectively, allowed the potential amplification of 272, 513, 843, 1271 and 1914 bp sequences. This range was chosen to investigate the influence of fixation and DNA preparation methods on the size of amplifiable DNA product.

For the demonstration of the t(14;18) translocation in follicular lymphomas forward primer 1 was combined with a (JMSW) primer as detailed in Table 2.1.2. This primer hybridised to a JH consensus sequence present on chromosome 14 and accordingly allowed for the potential formation of a PCR product in situations of t(14;18) translocation.

Each PCR reaction was undertaken in accordance with the 'hot start' method described in 2.2.16. Standard reaction conditions for reverse/forward primer combinations were as follows:

Template DNA	100 ng
Taq Polymerase	1 unit
Primers	10 pmol each
Denaturation temperature and time	94°C, 1 min
Annealing temperature and time	58°C, 30 secs
Extension temperature	72°C, 2 min
Amplification cycles	30

PCR conditions for the demonstration of t(14;18) translocation differed only in the use of an annealing temperature of 62°C. The products of amplification were analysed by gel electrophoresis as described in 2.2.17.

100 ng of 'standard' unfixed DNA was used as a positive control for all reverse/forward primer PCR. For all primer combinations a water blank was set up by substituting the DNA with the same volume of sterile water. In no instance was a PCR product observed in this control preparation.

5.3 Results

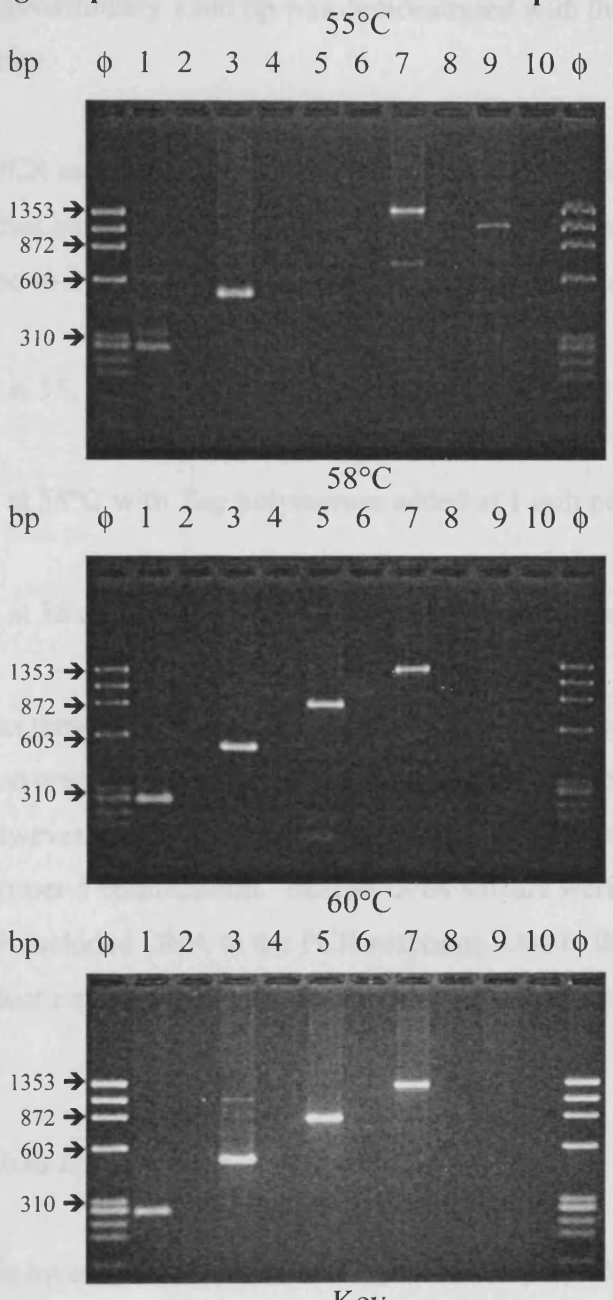
Optimisation of PCR parameters for different reverse/forward primer sets using unfixed DNA

The purpose of this investigation was to determine the optimal annealing temperature for each primer set by demonstration of a single amplification product. The 'standard' unfixed DNA preparation was used at 100 ng per PCR reaction which were undertaken using annealing temperatures of 55, 58 and 60°C.

Single amplification bands were obtained with reverse and forward primer 1-4 combinations in reactions employing an annealing temperature of 58°C. The bands were of the expected size (fig 5.3.1) and strongly stained. Similar results were obtained for reverse and forward primer 1, 3 and 4 combinations in reactions employing an annealing temperature of 60°C. However, with reverse and forward primer 2 combination the strong specific PCR product was supplemented by additional weak bands of approximately 900 and 1100 bp. No amplification bands were demonstrated with the reverse/forward primer 5 combination annealed at 58 and 60°C.

Electrophoretic analysis of PCR products employing an annealing temperature of 55°C demonstrated a more complex pattern. With reverse primer/forward primer 1 a band of appropriate size and moderate intensity was supplemented a weak bands of approximately 320 and 620 bp. A specific product was present in the reverse/forward primer 2 PCR but no PCR product was observed with the reverse primer/forward primer 3 combination. With reverse primer/forward primer 4 a specific PCR product, of strong intensity, was

Fig 5.3.1 Electrophoretic analysis of PCR products to determine the optimal annealing temperature of the forward/reverse primer combinations



Key

ϕ = phi x 174 *Hae* III size marker

Lanes 1-2, FP1/RP1 with unfixed DNA and water blank

Lanes 3-4, FP2/RP1 with unfixed DNA and water blank

Lanes 5-6, FP3/RP1 with unfixed DNA and water blank

Lanes 7-8, FP4/RP1 with unfixed DNA and water blank

Lanes 9-10, FP5/RP1 with unfixed DNA and water blank

accompanied by a weak band of approximately 700 bp. A weak, inappropriately sized, PCR product of approximately 1200 bp was demonstrated with the reverse primer/forward primer 5 combination.

Variations to the PCR technique were undertaken in an attempt to produce a specific amplification product using the reverse/forward primer 5 combination. In these template DNA was increased to 250 and 500 ng. Variations explored included:

Annealing at 55, 58 and 60°C with *Taq* polymerase added at 0.5 U per reaction.

Annealing at 58°C with *Taq* polymerase added at 1 unit per reaction.

Annealing at 58 and 60°C with the extension time increased from 2 to 4 min.

At the same time as these variations were undertaken PCR reactions employing other reverse and forward primer combinations were successfully completed thus verifying the reagents used. However, gel analysis failed to reveal any specific PCR products using the reverse/forward primer 5 combination. Instead DNA smears were visualised in all preparations which included DNA in the PCR reactions. Due to the failure to obtain a specific PCR product no further investigations were undertaken using this primer combination.

Amplification of fixed DNA extracts

The purpose of this investigation was to determine if PCR products could be obtained with primer 1-4 combinations using DNA extracted from fixed unprocessed and fixed paraffin processed samples. For this investigation the following 'standard' DNA preparations were used in each PCR reaction:

Unprocessed tissue fixed in Carnoy, formol saline and neutral buffered formalin for 24 and 168 h.

Paraffin processed tissue fixed in Carnoy, formol saline and neutral buffered formalin for 24, 96 and 168 h.

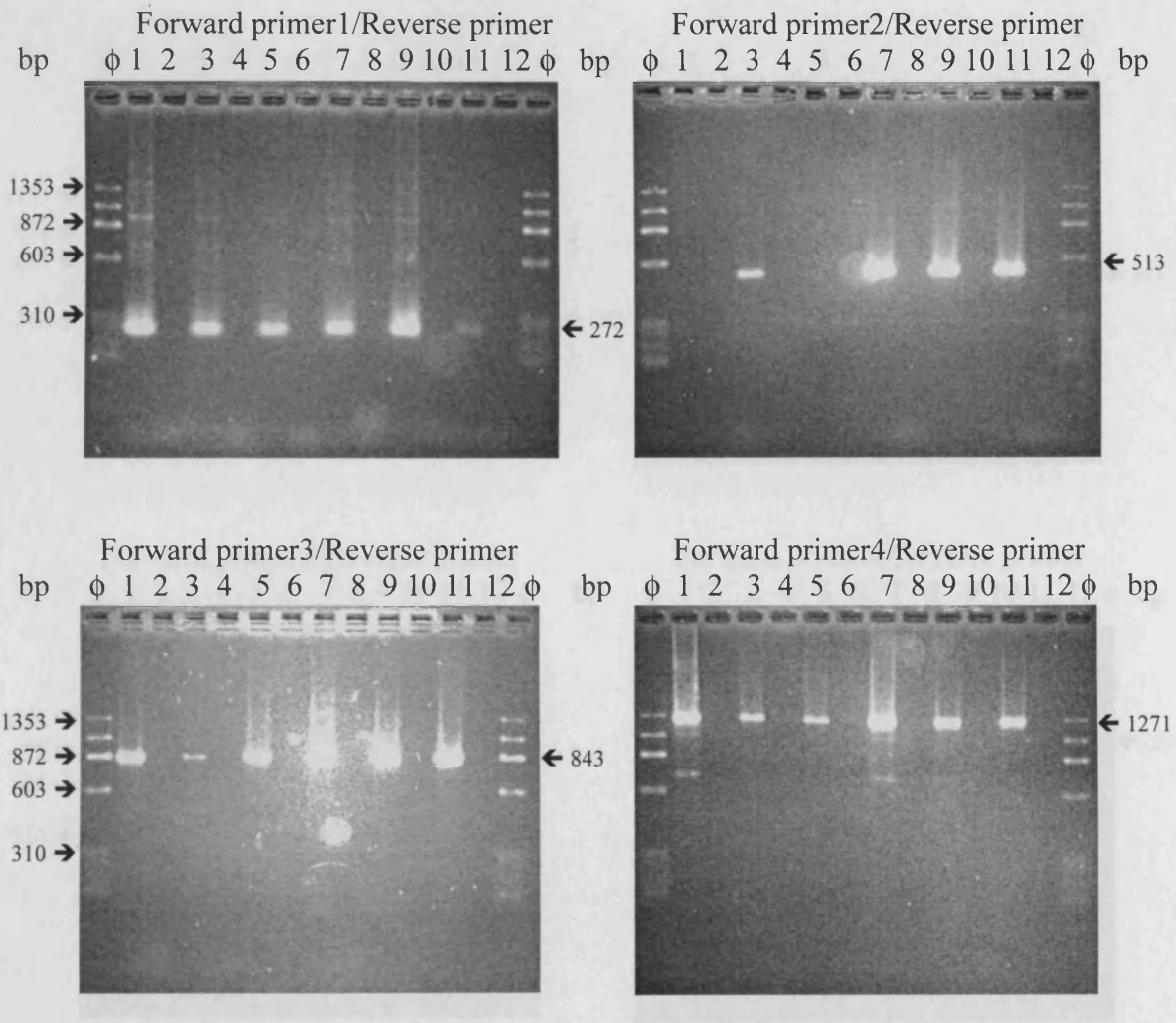
Specific PCR products were obtained with unfixed DNA with all reverse and forward primer combinations.

In preparations from Carnoy fixed tissue specific PCR products, of strong intensity, were obtained with the majority of extracts (figure 5.3.2). Only 24 and 168 h paraffin processed extracts in combination with reverse primer/forward primer 2 failed to provide any visible amplification product. The 96 h paraffin processed extract with reverse primer/forward primer 3 gave a weak but specific product. In addition to the specific PCR product observed with reverse primer/forward primer 1 multiple weak bands of larger size were present in each preparation. The presence of these additional bands may indicate that the PCR conditions for this primer combination were not entirely optimal. Only specific PCR products were visualised with reverse primer/forward primer 2 and 3 combinations. With the reverse primer/forward primer 4 combination inappropriately sized amplification bands of moderate intensity and smaller size than the specific PCR product were present in the 24 h fixed unprocessed and paraffin processed preparations.

In formol saline fixed preparations specific PCR products were demonstrated in the 24 h fixed unprocessed and paraffin processed preparations only (figure 5.3.3). The amplification product was of strong intensity with the reverse/forward primer 3 combination and of moderate intensity when forward primer 2 was used. With the other primer combinations the specific PCR products were of weak intensity.

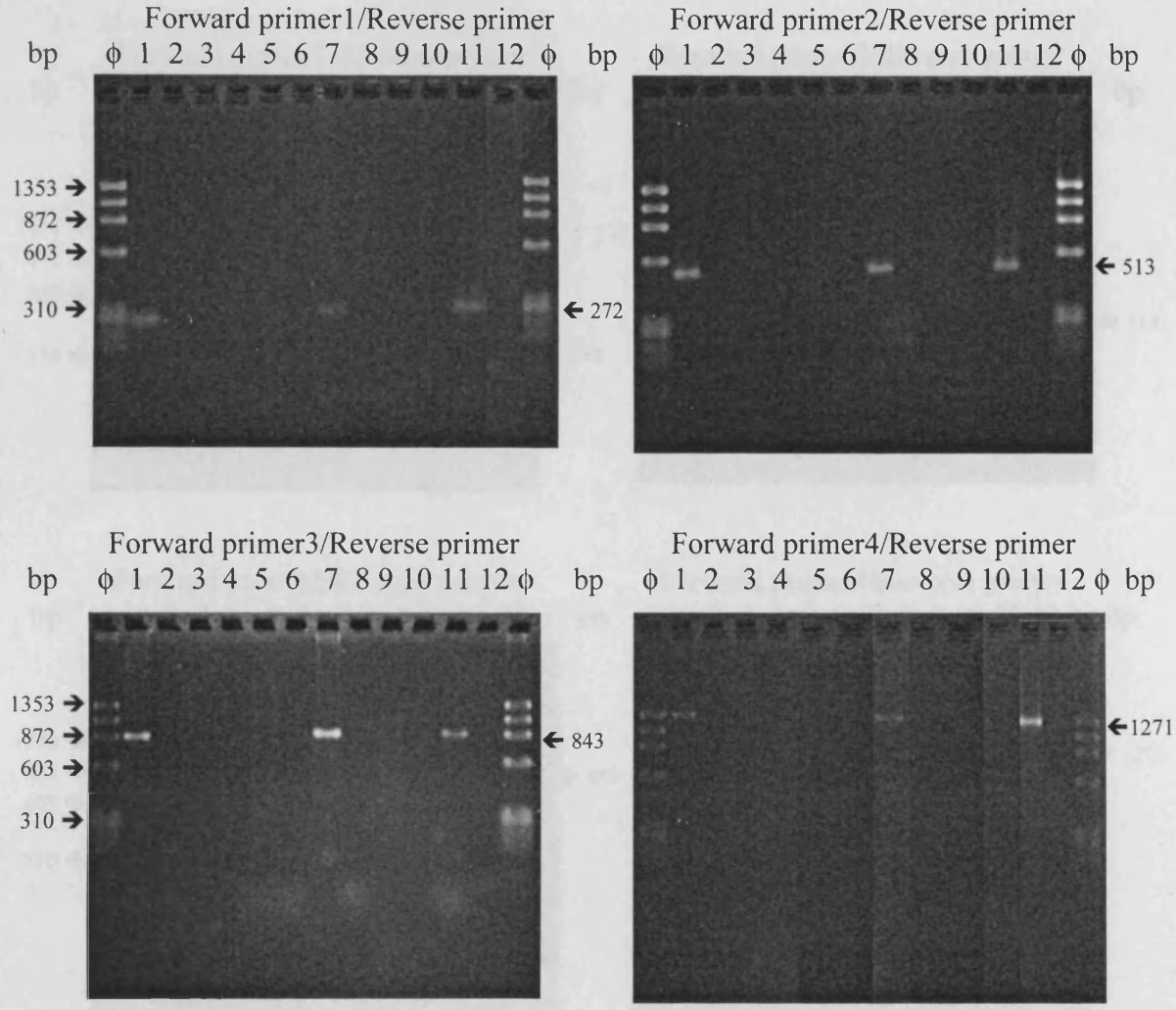
Specific PCR products were demonstrated with all neutral buffered formalin extracts used in combination with reverse and forward primer 1 (fig 5.3.4). An amplification product of moderate intensity was observed with the 24 h fixed unprocessed sample but in remaining preparations the PCR products were of weak intensity. With reverse/forward primer 2 specific PCR products, of predominantly weak intensity, were observed with all extracts with the exception of the 24 h paraffin processed sample in which no amplification band

Fig 5.3.2 Electrophoretic analysis of PCR products from Carnoy fixed DNA



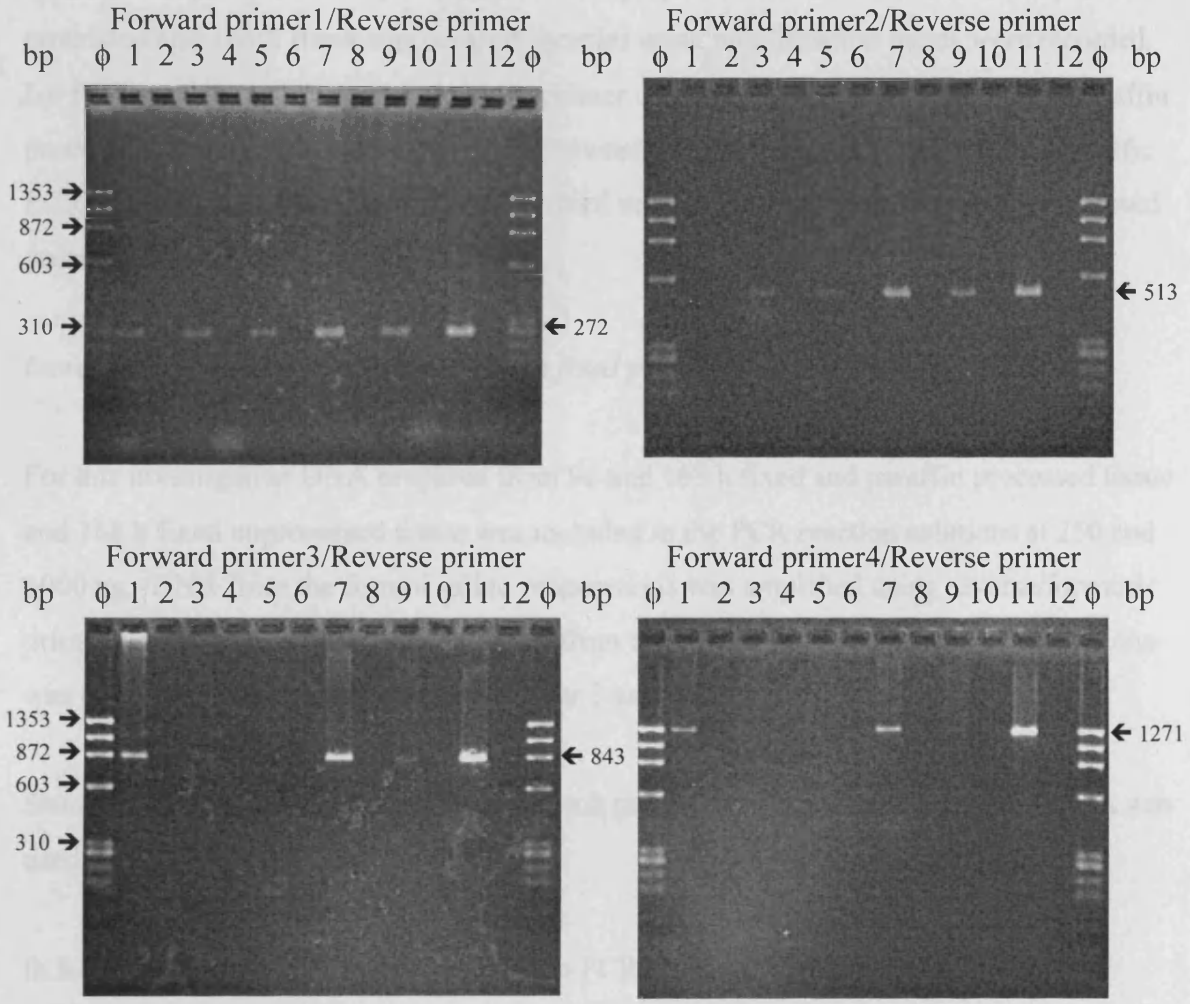
Key
ϕ = phi x 174 *Hae* III size marker
Odd lanes DNA included in reaction
Even lanes water blank controls
Lane 1, 24 h paraffin processed DNA
Lane 3, 96 h paraffin processed DNA
Lane 5, 168 h paraffin processed DNA
Lane 7, 24 h unprocessed DNA
Lane 9, 168 h unprocessed DNA
Lane 11, unfixed DNA

Fig 5.3.3 Electrophoretic analysis of PCR products from formol saline fixed DNA



Key
ϕ = phi x 174 *Hae* III size marker
Odd lanes DNA included in reaction
Even lanes water blank controls
Lane 1, 24 h paraffin processed DNA
Lane 3, 96 h paraffin processed DNA
Lane 5, 168 h paraffin processed DNA
Lane 7, 24 h unprocessed DNA
Lane 9, 168 h unprocessed DNA
Lane 11, unfixed DNA

Fig 5.3.4 Electrophoretic analysis of PCR products from neutral buffered formalin fixed DNA



Key

ϕ = phi x 174 *Hae* III size marker

Odd lanes DNA included in reaction

Even lanes water blank controls

Lane 1, 24 h paraffin processed DNA

Lane 3, 96 h paraffin processed DNA

Lane 5, 168 h paraffin processed DNA

Lane 7, 24 h unprocessed DNA

Lane 9, 168 h unprocessed DNA

Lane 11, unfixed DNA

was visible. With reverse/forward primer 3 combination specific amplification bands were visualised at varying intensities. PCR products of strong intensity were observed with the 24 h fixed unprocessed and paraffin processed preparations. With the 96 h fixed paraffin processed and 168 h fixed unprocessed samples weak amplification bands were recorded. No PCR product was observed with this primer combination using the 168 h fixed paraffin processed preparation. With the reverse /forward primer 4 combination moderate specific PCR products were observed in the 24 h fixed unprocessed sample and paraffin processed preparations only.

Increase in template DNA using formalin fixed preparations

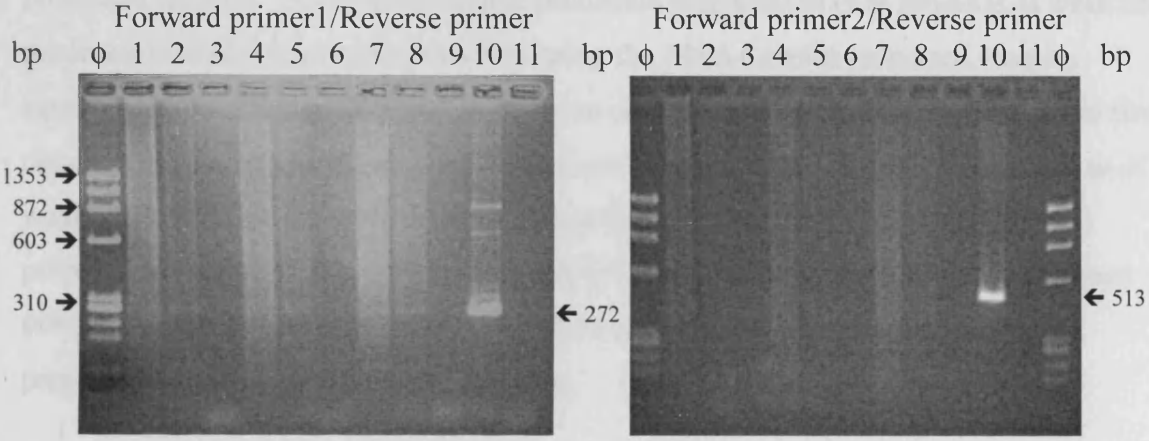
For this investigation DNA prepared from 96 and 168 h fixed and paraffin processed tissue and 168 h fixed unprocessed tissue was included in the PCR reaction solutions at 250 and 1000 ng. DNA from the formol saline preparations was amplified using reverse/forward primer 1 and 2 combinations whilst DNA from the neutral buffered formalin preparations was amplified using reverse/forward primer 3 and 4 sets.

Strong products were demonstrated with each primer combination when unfixed DNA was used.

In formol saline fixed preparations specific PCR products were observed with the reverse/forward primer 1 combination only (Fig 5.3.5). In 168 h fixed unprocessed preparations weak amplification bands were obtained using 250 and 1000 ng of template DNA. With the 96 h paraffin processed sample a weak PCR product was observed with 250 ng of template DNA only. In the 168 h fixed paraffin processed preparation a weak PCR product was recorded after amplification using 250 ng of template DNA only.

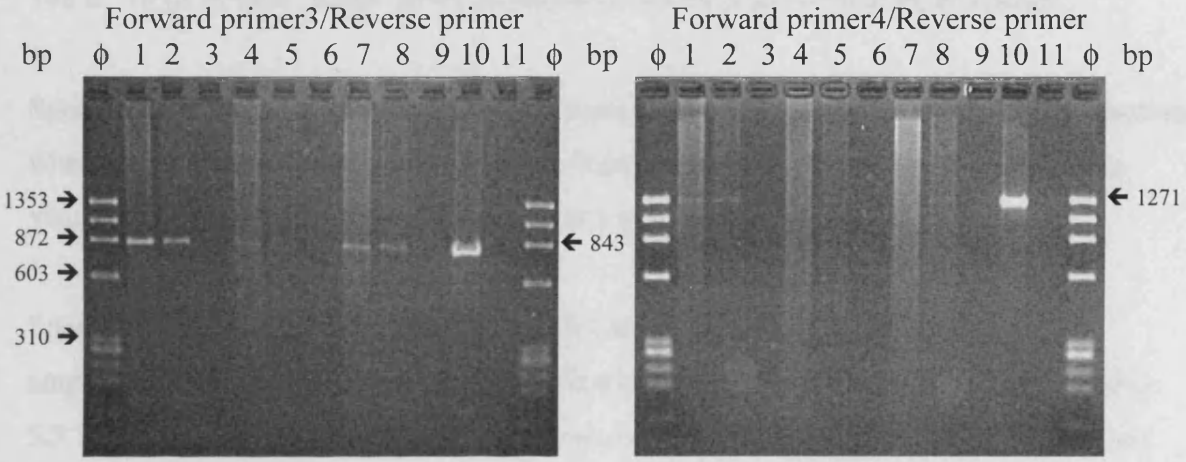
Specific PCR products were obtained in all neutral buffered formalin fixed preparations amplified using the reverse/forward primer 3 combination (Fig 5.3.6). In all instances the PCR products were more intensely stained in the 1000 ng template DNA preparations.

Figure 5.3.5 Electrophoretic analysis of PCR products from formol saline fixed DNA



Key
ϕ = phi x 174 *Hae* III size marker
Lanes 1-3, 96 h paraffin processed 1000, 250 ng DNA and water blank
Lanes 4-6, 168 h paraffin processed 1000, 250 ng DNA and water blank
Lanes 7-9, 168 h unprocessed 1000, 250 ng DNA and water blank
Lanes 10-11, unfixed 100 ng DNA and water blank

Figure 5.3.6 Electrophoretic analysis of PCR products from neutral buffered formalin fixed DNA



Key
See Fig 5.3.5

PCR products of moderate and strong intensity were observed with the 96 h fixed paraffin processed samples. With 168 h fixed unprocessed preparations PCR products of weak and moderate intensity were recorded whilst using the 168 h paraffin processed samples equivocal and weak amplification bands were observed. Specific PCR products were also obtained in some preparations using the reverse/forward primer 4 combination. Bands of weak intensity were observed after amplification of the 96 h fixed paraffin processed preparation using 250 and 1000 ng template DNA and with the 168 h fixed unprocessed sample using 250 ng template DNA. No PCR products were observed in remaining preparations using this primer combination.

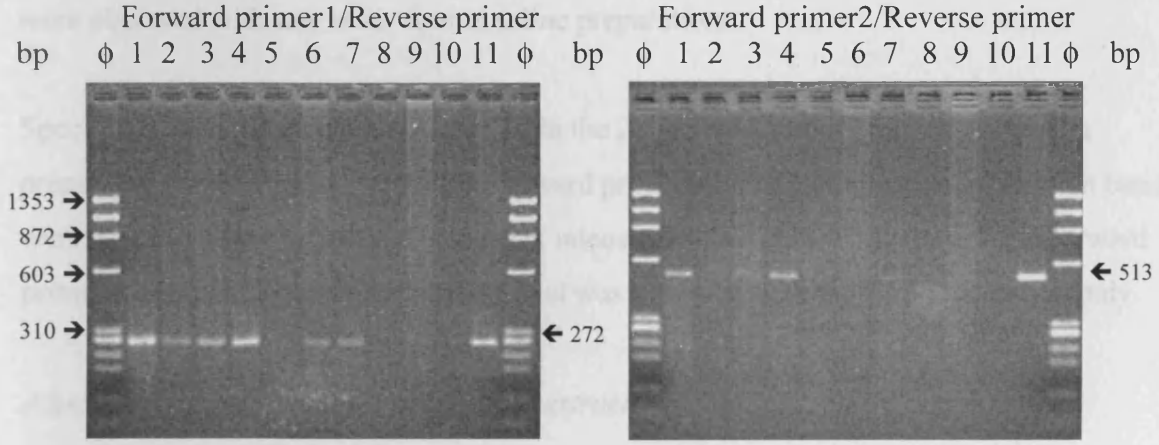
Amplification of 'rapid' DNA extracts of paraffin wax sections fixed in Carnoy, formol saline and neutral buffered formalin.

The purpose of this investigation was to determine if specific PCR products could be obtained with reverse and forward primer 1-4 combinations using DNA prepared by a 'rapid' method from fixed and paraffin processed samples. DNA was prepared from tissue that had been fixed in Carnoy, formol saline and neutral buffered formalin for 24, 96 and 168 h. 10 µl of each 'rapid' DNA preparation was included in each PCR reaction.

Specific PCR products of strong intensity were demonstrated with each primer combination when unfixed DNA was employed. In the fixed preparations amplification bands were visualised using reverse and forward primer 1 and 2 combinations only.

Specific PCR products were observed in all Carnoy preparations, the intensity of amplification bands varying according to fixation time and primer combination used (Fig 5.3.7). PCR products of strong intensity were observed in the 24 h and 96 h preparations with the reverse/forward primer 1 combination whilst using the 168 h sample a weak specific PCR product was obtained. With the reverse/forward primer 2 combination specific PCR products of moderate intensity were obtained with 24 and 96 h preparations whilst an equivocal band was observed using the 168 h sample.

Fig 5.3.7 Electrophoretic analysis of PCR products from 'rapid' DNA extracts



Key

ϕ = phi x 174 *Hae* III size marker

Lanes 1-3, 24 h fixation in Carnoy, formol saline and neutral buffered formalin

Lanes 4-6, 96 h fixation in Carnoy, formol saline and neutral buffered formalin

Lanes 7-9, 168 h fixation in Carnoy, formol saline and neutral buffered formalin

Lane 10, water blank

Lane 11, unfixed 100ng DNA

In formol saline preparations a specific PCR product, of moderate intensity, was observed with the 24 h sample using the reverse/forward primer 1 combination. No PCR products were observed with any other formol saline preparations.

Specific PCR products were observed with the 24 and 96 h neutral buffered formalin preparations only. Using the reverse/forward primer 1 combination the amplification bands were, respectively, of moderate and weak intensity. After PCR using the reverse/forward primer 2 combination an equivocal product was observed with the 24 h preparation only.

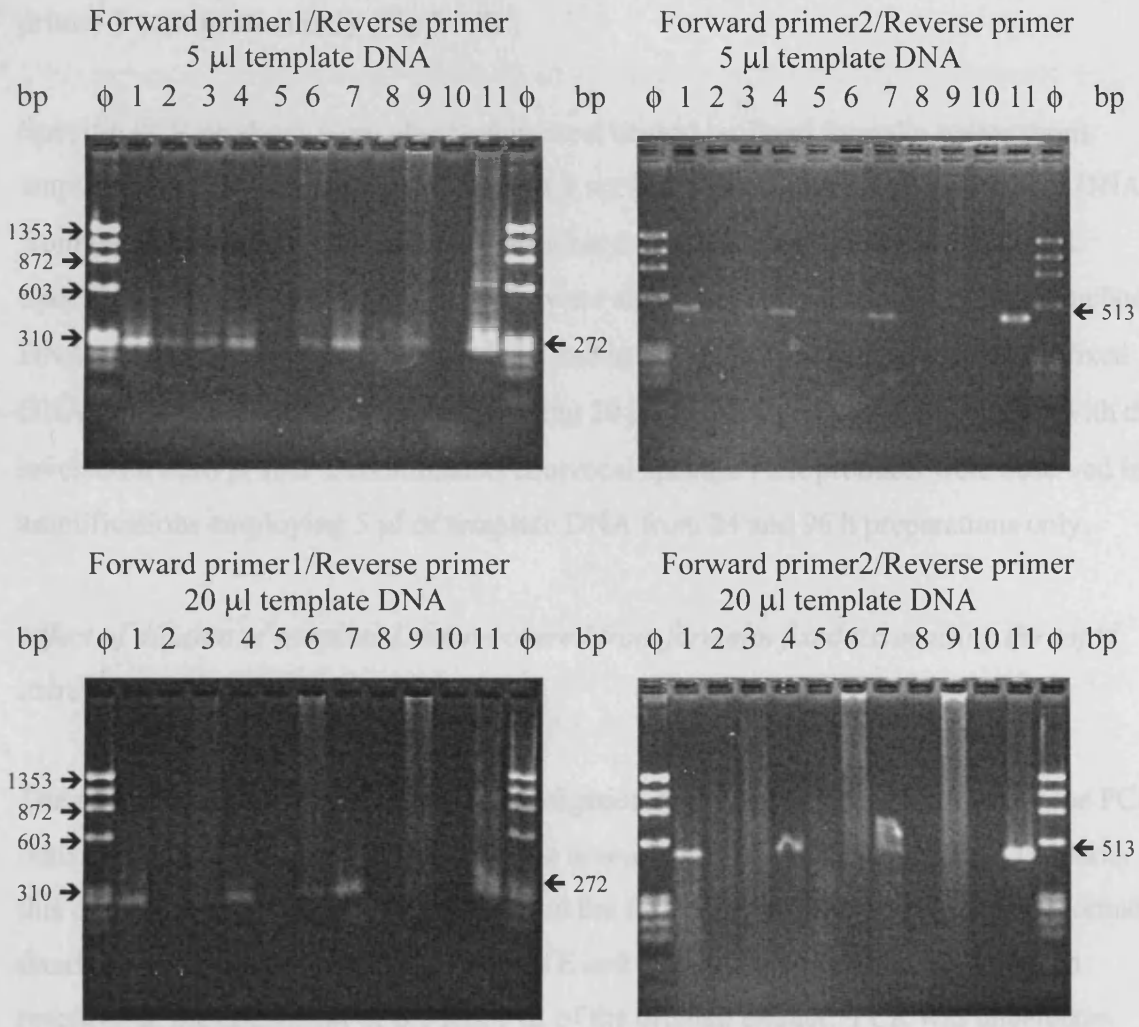
Adjustment of template DNA using rapid extracts

The purpose of this investigation was to determine if variation of template DNA would influence the efficiency of PCR amplification. DNA from the 'rapid' extracts was included at 5 and 20 µl in the amplification reaction using reverse/forward primer 1 and 2 combinations only.

Specific PCR products, of strong intensity, were demonstrated with each primer combination when unfixed DNA was employed. In one reverse/forward primer 1 PCR inappropriate, weaker, non-specific products of 500, 700 and 900 bp (Fig 5.3.8) were also observed.

Specific PCR products were observed in all Carnoy fixed preparations (Fig 5.3.8). With the reverse/forward primer 1 combination amplification products were, respectively, of strong and weak intensity with 5 and 20 µl of template DNA from the 24 h preparation. With the 96 and 168 h preparations identical PCR products were obtained. These were, respectively, of moderate and weak intensity using 5 and 20 µl of template DNA. At each fixation time point reciprocal PCR product intensities were observed using the reverse/forward primer 2 set.

Fig 5.3.8 Electrophoretic analysis of PCR products from 'rapid' DNA extracts: Use of 5 and 20 μl of template DNA



Key

ϕ = phi x 174 *Hae* III size marker

Lanes 1-3, 24 h fixation in Carnoy, formol saline and neutral buffered formalin

Lanes 4-6, 96 h fixation in Carnoy, formol saline and neutral buffered formalin

Lanes 7-9, 168 h fixation in Carnoy, formol saline and neutral buffered formalin

Lane 10, water blank

Lane 11, unfixed 100ng DNA

In formol saline fixed preparations a specific PCR product, of weak intensity, was observed using 5 µl of template DNA from the 24 h sample amplified with the reverse/forward primer 1 combination only (Fig 5.3.8).

Specific PCR products were observed in most neutral buffered formalin preparations amplified with the reverse/forward primer 1 set (Fig 5.3.8). Using 5 µl of template DNA from the 24 h preparation an amplification band of moderate intensity was obtained. Specific PCR products of weak intensity were also observed when 20 µl of 24 h template DNA was used, in both 96 h preparations and in the PCR employing 5 µl of 168 h fixed DNA. No PCR product was observed using 20 µl of 168 h fixed template DNA. With the reverse/forward primer 2 combination equivocal specific PCR products were observed in amplifications employing 5 µl of template DNA from 24 and 96 h preparations only.

Effect of dilution of template DNA recovered from formalin fixed tissue using the rapid extraction method

The results obtained in the previous investigation suggested that the efficiency of the PCR reaction could be inversely affected by the amount of DNA template present. To clarify this observation 'rapid' DNA extracts from the formol saline and neutral buffered formalin fixed preparations were prediluted in 1x TE and then included in PCR amplification reactions at the equivalent of 0.1 and 1 µl of the original extract. PCR was undertaken using reverse/forward primer 1 and 2 combinations.

Strong specific PCR products were demonstrated with each primer set when unfixed DNA was employed. No PCR products were observed in any preparation using the diluted fixed DNA extracts.

Influence of presence of DNA extracted from formalin fixed preparations on the amplification of DNA recovered from unfixed tissue.

The purpose of this investigation was to establish if the addition of DNA extracted from formalin fixed preparations would influence PCR amplification of unfixed DNA. Three sets of amplification reactions using the reverse and forward primer 1 combination and including 0.001, 0.01, 0.1, 1, 10 and 100 ng amounts of 'standard' unfixed DNA were prepared. To two sets 10 µl of the 96 or 168 h formol saline 'rapid' DNA extracts were also added to each reaction solution. These fixed extracts were chosen as they had not previously yielded PCR products and were therefore unlikely to supplement the unfixed reaction products. However, due their preparation conditions, there was a high probability that they would include any inhibitory component that might be present.

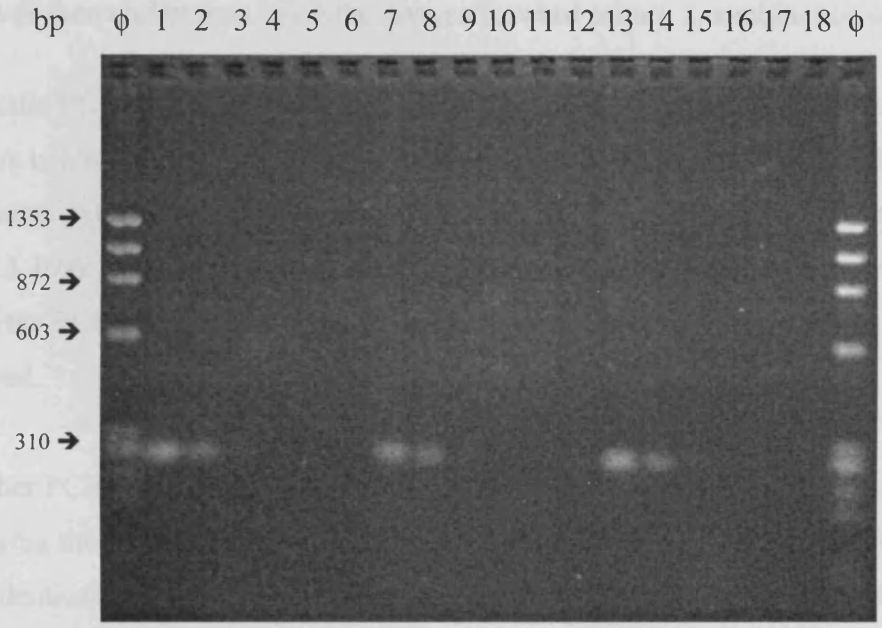
In PCR preparations of 'standard' DNA alone specific PCR products were demonstrated in reactions incorporating DNA at 1, 10 and 100 ng only. The bands were, respectively, of equivocal, weak and moderate intensity (Fig 5.3.9). Amplification results were unaffected by the presence of fixed DNA.

Amplification of follicular lymphoma extracts using reverse/forward primer combinations

The purpose of this investigation was to establish the suitability of the DNA prepared from the follicular lymphoma cases for amplification by PCR. In an initial investigation 10 µl of each lymphoma extract was added to amplification reactions which included reverse and forward primer combinations 1-4.

Specific PCR products were obtained with the unfixed DNA positive control with all reverse and forward primer combinations. No amplification products were observed in the follicular lymphoma preparations. Instead a nucleic acid smear, which was similar for each case, irrespective of primer combination, was visualised.

Fig 5.3.9 Electrophoretic analysis of PCR products from forward primer1/reverse primer with unfixed DNA amplified in the presence of fixed DNA



Key

ϕ = phi x 174 *Hae* III size marker

Lanes 1-6, unfixed DNA at 100, 10, 1, 0.1, 0.01 and 0.001 ng

Lanes 7-12, unfixed DNA, at same concentrations, amplified in the presence of 10 µl 96 h formol saline fixed rapid extract

Lanes 13-18, unfixed DNA, at same concentrations, amplified in the presence of 10 µl 168 h formol saline fixed rapid extract

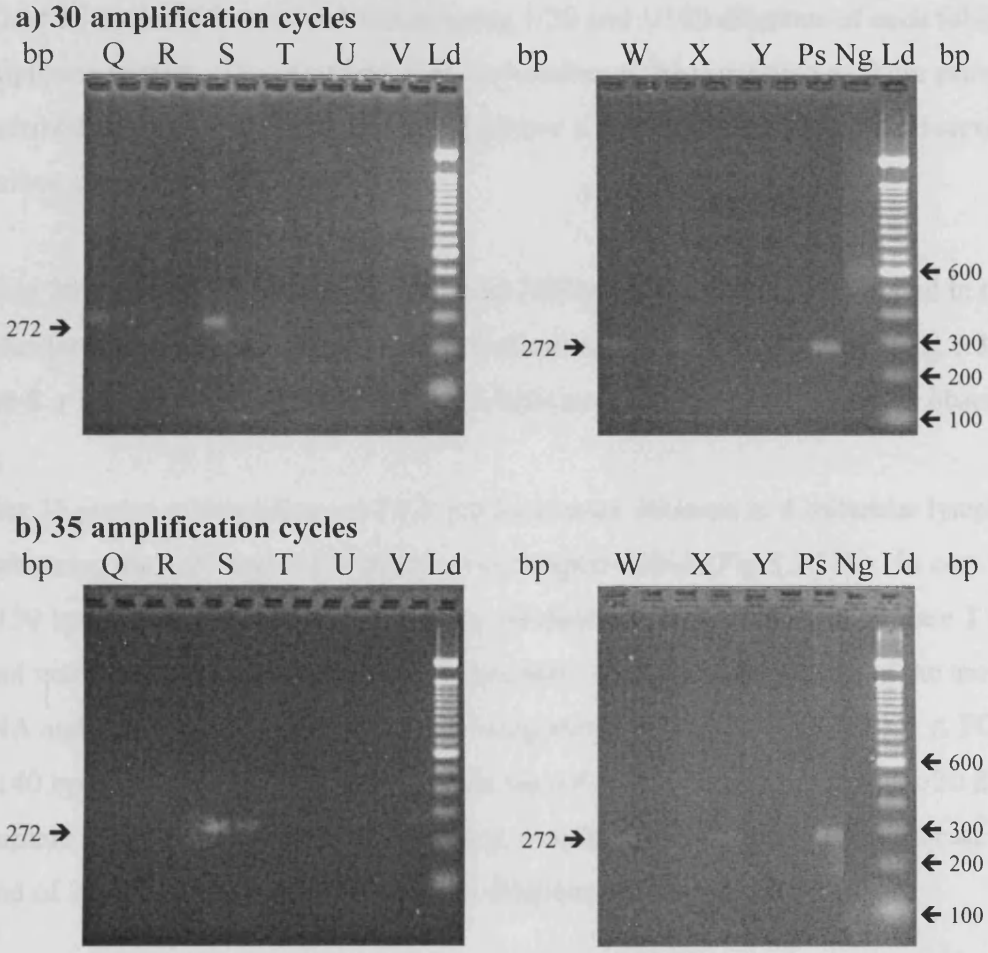
To clarify if the amount of DNA included in the initial PCR was inappropriate 1/20 and 1/100 dilutions for each of the follicular lymphoma extract were prepared using 1 x TE. PCR was then undertaken using the reverse/forward primer 1 combination only.

A specific PCR product, of moderate intensity, was obtained with the unfixed DNA positive control. Specific PCR products were also demonstrated in five of the follicular lymphoma extracts but only in reactions employing the 1/20 dilution of the template DNA (Fig 5.3.10a). In case S the amplification band was of moderate intensity, in cases Q, W and X the PCR product was of weak intensity whilst in case R an equivocal band was observed.

A further PCR reaction was undertaken to determine if results could be improved by increasing the number of amplification cycles. Sample preparation and PCR conditions were identical to those employed previously with the exception of the use of 35 amplification cycles.

A specific PCR product, of moderate intensity, was obtained with the unfixed DNA positive control. Specific PCR products were also demonstrated in three of the follicular lymphoma extracts (Fig 5.3.10b). In case S an amplification band, of moderate intensity, was obtained with the 1/20 and 1/100 dilutions of template DNA. In case W the amplification band was of weak intensity and present only in the reaction with the 1/20 dilution of template DNA. In contrast in case X a weak specific PCR product was observed with the 1/100 dilution of template DNA only.

Figure 5.3.10 Electrophoretic analysis of PCR products using forward primer1/reverse primer with, follicular lymphoma cases Q-Y



Key

Follicular lymphoma order for each case is 1/20 then 1/100 dilution of DNA

Ps = 100 ng unfixed DNA

Ng = water blank control

Ld = 100 bp ladder

Demonstration of t(14;18) translocation in follicular lymphoma extracts using forward primer 1/JMSW primer combination

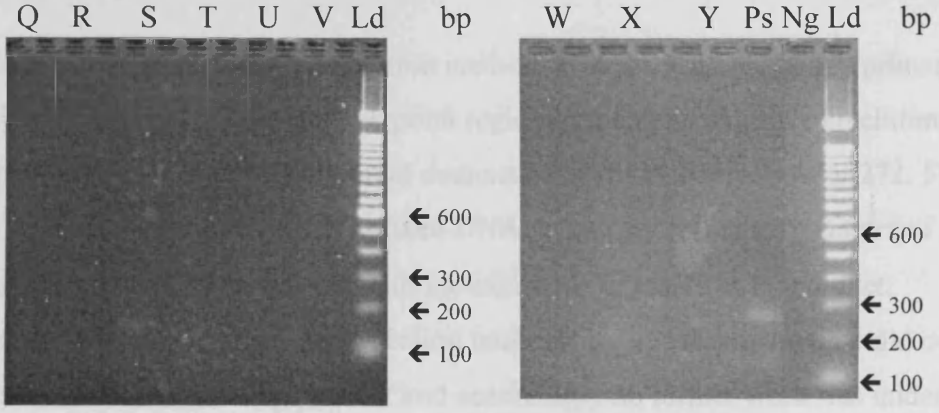
30 and 35 cycle PCR was undertaken using 1/20 and 1/100 dilutions of each follicular lymphoma extract. The reactions were undertaken at the same time as those previously described using the reverse and forward primer 1 primer combination which served as a positive control for amplifiable DNA.

Using 30 cycles of amplification equivocal PCR products were demonstrated in two follicular lymphoma cases using the 1/20 dilution of template DNA only (Fig 5.3.11a). In case S a 150 bp band was demonstrated whilst in case X a 200 bp band was observed.

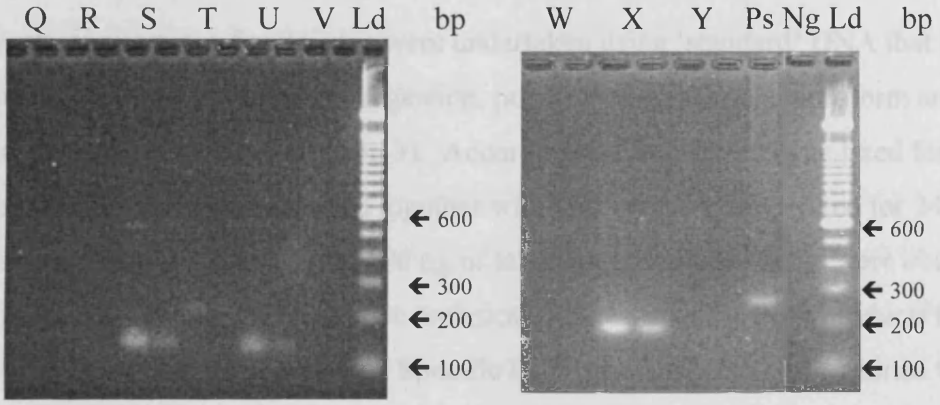
After 35 cycles of amplification PCR products were obtained in 4 follicular lymphoma cases using the 1/20 and 1/100 dilutions of template DNA (Fig 5.3.11b). In case S a band at 150 bp of moderate and weak intensity, respectively, was observed. In case T a 210 bp band was visualised. This was of weak intensity with the 1/20 dilution of the template DNA and equivocal after amplification using template DNA diluted 1/100. A PCR product at 140 bp was obtained with case U. This was of strong intensity with the 1/20 dilution of template DNA and of weak intensity using template DNA diluted 1/100. In case X a strong band of 200 bp was observed using both dilutions of template DNA.

Figure 5.3.11 Electrophoretic analysis of PCR products using forward primer1/JMSW primer with follicular lymphoma cases Q-Y

a) 30 amplification cycles



b) 35 amplification cycles



Key

Follicular lymphoma order for each case is 1/20 then 1/100 dilution of DNA
Ps = 100 ng unfixed DNA amplified with forward primer 1/reverse primer 1
Ng = water blank control forward primer 1/JMSW primer
Ld = 100 bp ladder

5.4 Discussion

5.4.1 Effect of fixation and DNA preparation methods on size of PCR product

The effect of fixation and DNA preparation methods was assessed using five primer pairs combinations spanning the major break point region of the *Bcl2* sequence on chromosome 18. The primers allowed for the potential demonstration of PCR products of 272, 513, 843, 1271 and 1914 bp. Using 100 ng of unfixed DNA and an annealing temperature of 58°C these were specifically demonstrated with the exception of the 1914 bp product. Adjustment of DNA concentration, annealing temperature and extension time did not result in the generation of the 1914 bp product and accordingly no further work was undertaken using this primer combination.

Initial investigations using fixed tissue were undertaken using 'standard' DNA that had been prepared by 168 h Proteinase K digestion, purified using phenol chloroform and ethanol precipitated (Chapter 4, section 3). Accordingly, DNA from tissue fixed for 24, 96 and 168 h and paraffin wax embedded together with unprocessed tissue fixed for 24 and 168 h was available for study. Using 100 ng of template DNA best results were obtained with Carnoy extracts with, excepting two technical failures, specific PCR products being observed with all primer combinations. Specific PCR products were also obtained with the majority of neutral buffered formalin extracts. With these preparations a trend was observed with better results being observed after shorter fixation and in unprocessed preparations. In contrast, after formol saline fixation PCR products, covering the full sequence range, were observed with the 24 h fixed preparations only. Increasing template DNA to 250 and 1000 ng with the formalin extracts resulted in the demonstration of additional specific PCR products. For formol saline the 272 bp product was observed in 96 h paraffin and 168 h paraffin and unprocessed samples, but no 513 bp product was demonstrated using increased template DNA with these preparations. With neutral buffered formalin fixed extracts the 843 bp product was observed in the 168 h fixed paraffin wax preparation and the 1271 bp product with the 96 h fixed paraffin wax and 168 h fixed unprocessed samples for the first time.

DNA was also prepared from the same paraffin wax embedded tissue using a 'rapid' recovery method involving overnight Proteinase K digestion without purification. Accordingly, DNA preparations from tissue fixed for 24, 96 and 168 h was available for study. Using 10 μl aliquots of these preparations, only the 272 and 513 bp PCR products were demonstrated. Specific PCR products were obtained with all Carnoy fixed preparations but the intensity of these diminished with increased fixation time and in relation to amplified sequence length. With neutral buffered formalin fixed preparations 272 and 513 bp products were obtained using the 24 h fixed preparations and the 272 bp product with the 96 h fixed sample only. As with the 'standard' DNA preparations worst results were obtained with formol saline preparations with the 272 bp product being demonstrated with the 24 h fixed preparation only. Adjustment of template DNA did not alter the results obtained with Carnoy and formol saline fixed preparations. However, using 5 μl of the neutral buffered formalin DNA preparations did allow the demonstration of a weak 272 bp PCR product in the 168 h fixed sample whilst an equivocal 513 bp product was observed in the 96 h fixed sample.

These results parallel those observed with Southern analysis of the same DNA extracts (Chapter 4, section 3) with fixation type, time and paraffin processing, in descending order of importance, influencing the intensity and maximum size of amplified PCR product. They also indicate that DNA preparation method can also significantly influence the sensitivity and success of PCR.

The effect of fixation type and time on the efficiency of PCR using DNA prepared using Proteinase K digestion over several days with phenol purification and precipitation of paraffin embedded tissue has been the subject of several investigations. In model experiments employing normal spleen Honma *et al* (1993) attempted the amplification of a 286 bp α tubulin PCR product using serial dilutions of template DNA. Similar results were observed using DNA prepared from tissue fixed in Carnoy for 96 and 240 h whilst a marked decline in PCR sensitivity was observed using extended buffered or unbuffered formalin fixation. In contrast after 4°C fixation for the same periods Carnoy and buffered formalin results were similar whilst a marked decrease in PCR sensitivity was recorded in

equivalent unbuffered formalin fixed preparations. O'Leary, *et al* (1994) also compared Carnoy with formalin fixation of tonsil for up to 168 h at 0-4, 21 and 37°C. After room temperature fixation demonstration of a 110 bp β globin PCR product was observed in all Carnoy fixed preparations whilst this amplification product was limited to 48 h fixation in buffered formalin, unbuffered formalin and formol saline preparations. Following low temperature and 37°C fixation inconsistent results were recorded with all fixatives. The effect of buffered formalin fixation of kidney and cervical cancer samples was studied by Karlsen *et al* (1994). PCR with the renal tissue extracts was attempted using two primer pairs for chromosome 3 codon D3S32. The longer 415 bp sequence was demonstrated in tissue fixed for up to 70 h, whilst the shorter PCR product of 214-235 bp was observed in tissue fixed for up to 170 h. Amplification of a range of HPV 16 sequences from 118 to 1190 bp was attempted with the cervical cancer DNA preparations. The maximum length of amplified product observed in fixed tissue was 449 bp and this was no longer demonstrated in tissue fixed for 8 h. However, 220 and 118 bp products were observed in 48 and 96 h fixed preparations. Karlsen *et al* (1994) also extracted DNA from archival prostatic carcinoma tissues for which fixation times had been recorded. Amplification of two p53 sequences of 171 and 191 bp was successfully achieved in samples fixed for up to 96 h whilst the 415 bp chromosome 3 sequence was absent in many samples fixed for 24 h.

In several other investigations of the effects of fixation DNA was recovered by overnight Proteinase K digestion, often at temperatures above 37°C, followed by heat inactivation of the enzyme or phenol purification. These reports provide a suitable comparison with the results obtained using the 'rapid' DNA preparation method described in the present investigation. Using a DNA extraction protocol almost identical to the 'rapid' technique Inghirami *et al* (1993) successfully demonstrated immunoglobulin gene rearrangements, with PCR products of up to 450 bp, in 24 to 48 h buffered formalin fixed paraffin embedded tissue. In a study of the effect of buffered formalin described by Greer *et al* (1991b) tonsil tissue was fixed for 2 h to 30 d then paraffin embedded and PCR attempted for β globin products ranging from 268 to 1327 bp. All PCR products were demonstrated in 2 and 24 h formalin fixed preparations, after 72 h fixation the 1327 bp product was not amplified whilst after 8 day fixation only equivocal demonstration of the 989 bp product

was obtained. After 30 day formalin fixation only the 268 bp PCR product was demonstrated. In model experiments described by Alaibac *et al* (1997) and Giannella *et al* (1997) DNA was recovered from skin and breast tissue fixed for 24 or 72 h then paraffin embedded. The maximum sequence length of PCR product was reduced by increased formalin fixation time. Accordingly, in breast and skin extracts a 530 bp phosphoglycerokinase gene product amplified in the majority of 24 h fixed samples was demonstrated in only half the preparations fixed for 72 h. In contrast in breast preparations a 280 bp androgen receptor PCR product was demonstrated in the majority of samples after 72 h fixation. The effect of extended formalin fixation on PCR has been investigated by Hamazaki *et al* (1993) and Kösel and Graeber (1994). In model experiments Hamazaki *et al* (1993) DNA was recovered from liver fixed in unbuffered formalin for 7 days to 2 years without subsequent paraffin embedding. Using these preparations they were able to demonstrate a 128 bp c-ki-ras PCR product in tissue fixed for up to six months. Kösel and Graeber (1994) extracted DNA from brain tissue fixed for up to 26 years in formalin or embedded in paraffin and stored for equivalent times and obtained PCR products for several nuclear and mitochondrial sequences. Products of less than 300 bp could be routinely demonstrated but those in excess of 500 bp were also frequently obtained.

Together the results of these studies correlate with those of the present investigation and emphasise the detrimental effects of prolonged formalin fixation on PCR efficiency. However, they also suggest that the maximum size of PCR product may be related to the target DNA, tissue type or technical aspects of the amplification reaction. The possible influence of target DNA or tissue is emphasised in the differential effect of formalin fixation time on the demonstration of equivalent sequence length PCR products for HPV in cervical tissue and a chromosome 3 codon in renal tissue as reported by Karlsen *et al* (1994). Demonstration of nuclear and mitochondrial sequences in brain tissue fixed for several years by Kösel and Graeber (1994) also suggests that target DNA may influence PCR results. These results also suggest a correlation with the successful amplification of mitochondrial DNA in ancient tissue samples (Pääbo, 1989 and Fisher *et al,* 1993). The demonstration of relatively long β globin PCR products by Greer *et al* (1991b) in comparison with the results obtained in the present investigation using the equivalent

'rapid' DNA recovery method may represent another example of the differential susceptibility of target DNA to formalin fixation. However, Greer *et al* (1991b) employed 40 amplification cycles and 2.5 units of *Taq* polymerase in their PCR reactions compared with the 30 cycles and 1 unit of the enzyme used in the present investigation. Accordingly, these technical factors may be suggested as an alternative explanation for the increased sensitivity of the results.

5.4.2 Inhibition of PCR

A further factor that should be considered is the presence of a PCR 'inhibitor' in extracted DNA. The possibility of the formalin fixed 'rapid' DNA preparations containing a PCR 'inhibitor' was investigated in the present study by amplifying unfixed 'standard' DNA at 0.001 to 100 ng in the presence or absence of 10 µl of 96 and 168 h formol saline fixed extracts. No difference in results was observed with the 272 bp PCR product being demonstrated in reactions which included DNA at 100, 10 and 1 ng. This result concurs with the findings of Greer *et al* (1991a) who undertook a very similar investigation amplifying a HPV sequence in the presence of DNA prepared from tissue fixed in a variety of solutions. However, in other studies evidence for the presence of an 'inhibitor' has been presented (An and Fleming, 1991, Forsthoefel *et al*, 1992) . Furthermore in the present investigation PCR reactions with the follicular lymphomas were only successful after dilution of the template DNA indicating the possible presence of an inhibitor in these samples.

An and Fleming (1991) demonstrated the influence of a template DNA concentration dependant 'inhibitor' using extracts prepared by boiling from a variety of unfixed and formalin fixed and paraffin embedded tissues. This inhibitor' was removed when DNA extracts were purified by phenol washing or centrifuged through a Centricon 30 filter. The beneficial influence of phenol purification and ethanol precipitation on the removal of fixation induced 'inhibitors' has also been noted by Wright and Manos (1990).

Frank *et al* (1996) suggest that detergents, such as SDS, used in extraction procedure may represent an additional type of PCR 'inhibitor' and that their removal using purification procedures is important. The presence of such an 'inhibitor' may explain the results reported by Forsthoefel *et al* (1992) who, using unpurified Proteinase K and SDS digested extracts, obtained PCR amplification only when less than 1 ng of template DNA was used.

5.4.3 Demonstration of t(14;18) translocation in follicular lymphomas

For this investigation DNA was prepared using the 'rapid' extraction procedure from 9 formol saline fixed and paraffin wax embedded cases of follicular lymphoma. Two primer combinations were used for PCR. The first was identical to that used in the fixation investigations to demonstrate a 272 bp sequence spanning the major breakpoint region of the *Bcl2* sequence on chromosome 18. The second combination used the same forward primer combined with a consensus primer hybridising to the JH region on chromosome 14 and accordingly allowing a PCR product to be formed only in situations of t(14;18) translocation. The relative sensitivity of 30 and 35 cycle PCR was investigated using both primer combinations.

Successful demonstration of the 272 bp PCR product was obtained only after careful titration of the DNA extracts with optimal results being obtained using 0.5 μl of template DNA. A specific PCR product was demonstrated in five of the follicular lymphoma cases when 30 amplification cycles were employed. After 35 cycle PCR the overall sensitivity was reduced with a PCR product being demonstrated in only three cases.

Using 30 amplification cycle PCR t(14:18) products were demonstrated in two cases. However, using 35 cycle PCR demonstration of this translocation was obtained in two additional lymphoma cases. The successful identification of t(14;18) translocation in 4 of the 9 (44%) of the follicular lymphoma cases used in the present investigation is comparable with larger case studies involving similar DNA and PCR methods of 32% (Mitani *et al,* 1993) 40% (Miettinen and Lasota, 1997), 41% (Pezzella *et al,* 1990), 50% (Liu *et al,* 1993) and 53% (Shibata *et al,* 1990).

It is of interest that two of the lymphoma cases in which a t(14;18) PCR product was identified had not produced a 272 bp product. This result suggests that the extracted DNA was only suitable for the demonstration of the shorter sequence length translocation products. Conversely it is possible that in cases in which the 272 bp product was demonstrated but a t(14;18) PCR product was absent that this was due to the translocated sequence being of a greater length and accordingly not detectable in degraded DNA. Evidence for this has been provided by Liu *et al* (1993) who demonstrated t(14;18) translocation PCR products of over 1 kb in several unfixed DNA preparations which were not detectable using DNA recovered from matched formalin fixed paraffin embedded tissue. However, factors not related to the influence of fixation could also account for failure to demonstrate the t(14;18) translocation and these are considered in context of the diagnostic value of the method in section 2 of the general discussion (Chapter 6).

Chapter 6 General Discussion

6.1 Recovery of DNA from fixed tissue

The investigations recorded in this thesis were undertaken with the practical objective of developing a method for the recovery of DNA from fixed tissue in a form suitable for analysis using Southern blotting and PCR. Within each investigation identical DNA, cell or tissue samples were used and unfixed preparations included. This allowed an internal comparison to be made of the effect of experimental conditions. Replicate experiments were, with one exception (Chapter 4, section 3), not set up and accordingly statistical validation of results is not possible. However, the systematic nature of the investigations allowed consistent trends to be identified which support the interpretations offered and the method for the recovery of DNA suggested.

Initial investigations (Chapter 3, sections 2 and 3) used pure (Lambda) DNA and lymphocyte enriched cell populations to determine the effect of five fixatives on the integrity, recovery and restriction endonuclease digestion of the nucleic acid. Two fixatives Bouin and formol sublimate proved unsuitable for further analysis. In subsequent investigations the effect of Carnoy, formol saline and neutral buffered formalin fixatives on palatine tonsil was assessed. The development of a method for the recovery of high molecular weight DNA from tissue so fixed and paraffin wax embedded is described in Chapter 3, section 4. This method is based on the prolonged incubation of tissue with a protease in the presence of SDS at 37°C followed by standard purification using phenol/chloroform and ethanol precipitation. Two proteolytic enzymes, Proteinase K and protease XXIV were evaluated using paraffin wax tissue that had been initially fixed for 6 and 24 h. Both gave satisfactory results but, in matched preparations, Proteinase K allowed DNA to be recovered more quickly, gave higher nucleic acid yields and, after neutral buffered formalin fixation, provided DNA of higher protein purity.

The quality of recovered DNA was unaffected by variations in methods for the initial preparation of paraffin wax embedded tissue (Chapter 3, section 5). Whilst homogenised,

diced and section preparations gave identical results, DNA yields were slightly improved using the latter preparation method which also conferred the benefit of not interfering with the structure of the embedded tissue block. Consequently the section method of initial tissue preparation was adopted. Comparison of DNA recovered immediately after ethanol precipitation (spoolable DNA) with that obtained after storage of the solution did not reveal any differences in the quality of the nucleic acid. This result argued that there was no advantage to introducing a spooling step, as recommended by Dubeau *et al* (1986), as a means of promoting the recovery of high molecular weight DNA.

Within the limits imposed by fixation induced degradation of the DNA the nucleic acid recovered by prolonged incubation was suitable for restriction endonuclease digestion and Southern blotting. An obvious disadvantage of using the method is the time taken to recover the DNA. The use of Proteinase K at 65°C coupled with standard purification was evaluated as a potential way of reducing the digestion time. However, the quality of DNA recovered was compromised and gave inferior Southern analysis results. Accordingly for Southern analysis prolonged incubation at 37°C is recommended. DNA obtained by prolonged incubation of tissue samples at 37°C also gave optimal PCR results. However, coupling a preparation method extending to a week or more with this rapid analysis technique is obviously not conducive to potential diagnostic applications. In recognition of this a 'rapid' DNA preparation method involving the incubation of paraffin wax sections in Proteinase K at 55°C without subsequent purification was evaluated. This gave satisfactory results but significantly reduced the maximum size of PCR product from over 1250 bp to approximately 500 bp.

Ideally it would be an advantageous in diagnostic situations if the time taken for DNA recovery exactly matched that of the PCR and subsequent gel analysis steps. Kiene, *et al* (1992) and Sepp, *et al* (1994) have critically compared several such short preparation methods. In the investigation reported by Kiene *et al* (1992) DNA recovery was attempted from sections of formalin fixed and paraffin wax embedded tissue using rehydration followed by exposure to a detergent, rehydration followed by Proteinase K digestion at 55°C for 3 h and subsequent boiling or phenol/chloroform purification and boiling with or

without the inclusion of a chelating resin. In subsequent PCR analysis amplification was obtained for a variety of viral targets between 88 and 450 bp using DNA prepared by Proteinase K digestion with purification or after boiling with the chelating resin. Very similar DNA recovery methods were assessed by Sepp *et al* (1994) using sections prepared from buffered formalin and paraffin embedded tissue that had been initially fixed for 24 to 48 h. The methods used were, boiling of rehydrated or non-rehydrated sections in the presence or absence of a chelating resin and Proteinase K digestion at 55°C for 3 h and subsequent boiling with or without the inclusion of Chelex-100. Electrophoretic assessment of the recovered DNA revealed the average length to be between 100-200 bp after boiling, 100-600 bp after boiling with the inclusion of the chelating resin and 100 to 4000 bp after use of the Proteinase K methods. The maximum size of PCR product paralleled these results with a 408 bp sequence being demonstrated after boiling, an amplification product of 647 bp after boiling with chelation and a 983 bp product with use of the Proteinase K extracted DNA.

Another short DNA preparation method employing Proteinase K and sonication was first described by Heller *et al* (1991). Using this method Heller *et al* (1991) were able to amplify β globin sequences of up to 500 bp with DNA prepared from single sections of formalin fixed and paraffin embedded surgical and post mortem tissue. Comparing DNA recovery methods Frank *et al* (1996) found the technique superior to boiling but inferior to overnight Proteinase K extraction at 37°C followed by boiling.

Together these results indicate that recovery methods employing short Proteinase K digestion at elevated temperature, Proteinase K and sonication or boiling with the inclusion of a chelating resin can provide DNA within a few hours which is suitable for PCR amplification of sequences of up to 500 bp. Boiling of sections with or without initial rehydration may also be considered if shorter sequences are to be amplified (Coates *et al,* 1991) but variable results using this preparation method have been reported by several investigators (Jackson *et al,* 1990, O'Leary *et al,* 1994 and Frank *et al,* 1996).

6.2 Use of DNA for the diagnosis of Lymphoma

The identification of B and T cell rearrangements by Southern analysis was assessed using DNA recovered from 12 paraffin embedded tissues of known or suspected lymphomas. All tissues had been obtained surgically, 8 of the cases had been fixed in formol saline, one in formalin whilst fixation conditions for the remaining referred specimens were unknown. Excessive DNA degradation or unsuccessful restriction endonuclease digestion were encountered in the majority of preparations and (equivocal) evidence of gene rearrangement was demonstrated in 2 cases only. The sub-optimal results highlighted problems of using degraded DNA induced by formalin fixation with an analysis method requiring high molecular weight nucleic acid. Practically they indicate that unless formalin fixation conditions can be controlled and restriction endonuclease and probe combinations are chosen to demonstrate low molecular weight restriction fragments Southern analysis for T and B cell gene rearrangements using this DNA cannot be recommended as a reliable diagnostic method.

A possible alternative strategy, suggested by the results obtained with Carnoy fixation, would be to change fixative. In this context ethanol has been evaluated and shown to be capable of providing acceptable results. Bramwell and Burns (1988) demonstrated that following ethanol fixation to 168 h it was possible to obtain high yields of DNA and using this to demonstrate T and B cell rearrangements. However, after paraffin wax embedding DNA yields were dramatically reduced. Wu *et al* (1990) demonstrated gene rearrangements in DNA extracted from tissues that had been stored at room temperature in ethanol for 2 years. In contrast to the findings of Bramwell and Burns (1988), Wu *et al* (1990) also demonstrated identical gene rearrangements using DNA extracted from 48 h ethanol fixed and paraffin wax embedded tissue to that obtained with DNA from matched frozen tissue. Accordingly, these investigations suggest that ethanol could be used to circumvent the problems of formalin induced DNA degradation. However, in comparison with formalin histology after ethanol fixation is sub-optimal and would pose great difficulties in interpretation. Perhaps for this reason both investigators suggest that ethanol

is used alongside routine formalin fixation as an effective storage medium allowing Southern analysis to be undertaken when required.

The introduction of PCR and the development of methods to demonstrate T and B cell gene rearrangements using DNA recovered from formalin fixed and paraffin wax embedded tissue offers a further alternative. The advantage of using DNA recovered from the same tissue as used in morphological diagnosis is obvious and microdissection methods allow rearrangements to be identified using as few as 25 cells (Pan *et al*, 1994, Magalini *et al,* 1998). However, due to the large number of possible rearrangements the PCR techniques are complex and therefore probably best suited for use in reference centres.

In contrast to the complexities of T and B cell gene rearrangement identification by PCR the demonstration of the t(14;18) translocation as an aid to the diagnosis of follicular lymphoma requires only one set of primers. In the present investigation DNA recovered from 9 formol saline and paraffin embedded follicular lymphomas was used to assess the sensitivity of this method. Amplifiable DNA was obtained in 7 of the cases and the translocation was demonstrated in 4 of these.

As discussed in Chapter 5, section 5.4.3, this frequency of translocation identification at 44% is comparable with larger case studies involving similar DNA and PCR methods in which frequencies of 32 to 53% have been reported (Shibata *et al,* 1990, Pezzella *et al,* 1990, Limpens *et al*, 1993, Liu *et al,* 1993, Mitani *et al,* 1993, Aston-Key *et al,* 1995, Miettinen and Lasota, 1997). These percentages fall short of the 44 to 84% incidence for t(14;18) translocation as determined by cytogenetic analysis (Pezzella et al, 1990) and accordingly explanations for the failure to achieve better results using this PCR method should be sought.

Failure to detect rearranged DNA sequences due to the degradation of the nucleic acid after formalin fixation, as discussed in Chapter 5, can account for some PCR failures. However, the frequency of t(14;18) translocation identification using DNA from unfixed DNA is still lower than that revealed by cytogenetic analysis (Liu *et al*, 1993). As in the present

research some investigators have used primers for the major breakpoint region (MBR) only (Shibata *et al*, 1990, Limpens, *et al,* 1993, Aston-Key *et al*, 1995) and accordingly translocations involving the minor cluster sequence (MCS) region would not have been demonstrated. However, studies using unfixed and fixed cases in which PCR has been undertaken using primers to detect MCS translocations have recorded a very low incidence (Pezzella *et al*, 1990, Liu *et al*, 1993, Mitani *et al*, 1993, Miettinen and Lasota, 1997).

A further possibility is the occurrence of breakpoints outside the MBR and MCS regions. Evidence in support of this is provided by Limpens *et al* (1993) who demonstrated the translocation in 66% of follicular lymphomas using Southern blotting and in 53% of these cases using a MBR PCR method. A much higher overall incidence of 89% was reported by Weiss *et al* (1987) in a study employing Southern blotting only and using probes for the MBR (58% positive cases) and MCS (30% positive cases) regions. The high incidence MCS translocations is at variance to the low frequency of this t(14;18) rearrangement reported by others (see above) and has led several investigators to propose that this may be attributable to geographical variations in the tumour (Pezzella *et al*, 1990, Liu, *et al*, 1993, Mitani *et al*, 1993).

Practically the relatively low incidence of detection of the t(14;18) translocation by PCR means that method should not be used alone to aid the morphological diagnosis of follicular lymphoma. This conclusion is also reached by Ashton-Key *et al* (1995) who undertook a practical review of value of immunocytochemical and PCR methods to differentiate follicular lymphomas from follicular hyperplasia. They suggest that immunostaining for Bcl2 protein expression, centroblast identification using the antibody MIB-1 and demonstration of light chain immunoglobulin restriction by immunocytochemistry should be used as a first line of attack with PCR methods for B cell gene rearrangement and t(14;18) translocation held in reserve for difficult cases.

6.3 Fixation mechanisms

6.3.1 Bouin

Lambda DNA was severely degraded by exposure to Bouin fixative at room temperature and 37°C. This observation was also noted in DNA recovered from lymphocytes fixed in this solution for 30 min. As previously discussed the extent of DNA degradation (Chapter 3, section 3.6.1) precluded the use of DNA recovered from Bouin fixed tissue for Southern analysis. However, using techniques which do not require intact nucleic acid, DNA from Bouin fixed tissue has, on occasions, been successfully amplified by PCR (Honma *et al,* 1993, Hostein *et al,* 1998) or demonstrated by *in situ* hybridisation (Pringle *et al,* 1987, Weiss and Chen, 1991).

The Bouin fixative mixture contains 9% formalin, 1% picric acid and 5% acetic acid in an aqueous solution. Lambda and lymphocyte DNA was not degraded by fixatives containing formalin alone and it is therefore suggested that this constituent is unlikely to be the cause of DNA degradation. Acetic acid was also used, at higher concentration, as a component of Carnoy fixative which preserved DNA better than any other solution used in the present investigation. Accordingly, it is reasonable to propose that the remaining constituent, picric acid, is responsible for DNA degradation. Furthermore in the absence of any known chemical interaction of picric acid with DNA (Baker, 1958) it is suggested that the action of this constituent is indirect and mediated by the low pH, 1.3-1.6, it confers on the fixative mixture. At this pH denaturation, depurination and hydrolysis of DNA will occur (Thomas and Doty, 1956, Geiduschek, 1958) whilst histones are removed (Holtzman, 1965, Darzynkiewicz *et al,* 1975). Such events would readily explain the rapid degradation of the DNA and the relative ease with which the nucleic acid was recovered from fixed lymphocytes.

6.3.2 Carnoy fixation

This fixative mixture gave the better results than any other solution used in the present investigation. Lambda DNA remained intact after exposure to Carnoy and restriction endonuclease digestion was unaffected. High molecular weight DNA, in a form suitable for Southern analysis, was recovered from lymphocytes fixed for 30 min to 24 h without difficulty using a short proteolytic digestion method. DNA was also recovered from tissue fixed in Carnoy for 6 to 168 h much more readily than equivalent formalin fixed preparations. As fixation time was extended partial degradation was observed but the demonstration of restriction fragments of up to 12 kb was possible using DNA recovered from paraffin wax embedded tissue fixed in Carnoy for 168 h. Using the same DNA *Bcl2* sequences of 272 to 1271 bp were successfully amplified using PCR. DNA from paraffin embedded tissue initially fixed for 24 to 168 h in Carnoy and recovered using a 'rapid' extraction protocol was also successfully amplified though the maximum size of PCR product was limited to 513 bp. The ease of recovery of DNA from Carnoy fixed tissue and its utility in PCR analysis has been reported by several investigators (Jackson *et al*, 1990, Honma *et al*, 1993, O'Leary *et al,* 1994).

Carnoy is a mixture comprising 10% acetic acid, 30% chloroform and 60% ethanol. No fixation reaction for chloroform has been described but acetic acid and ethanol fix tissue by mechanisms which favour the retention of DNA and extraction of nuclear proteins (Holtzman, 1965, Dick and Johns, 1968, Retief and Rüchel, 1977, Krejcí *et al,* 1980, Fraschini *et al,* 1981). Consequently, the ease with which DNA was recovered from Carnoy fixed preparations accords with these fixation reactions.

DNA recovered from lymphocytes fixed for up to 24 h in Carnoy was of high molecular weight but partial degradation of the nucleic acid extracted from tissue fixed for the same length of time was observed. When tissue fixation time was extended from 24 to 168 h this degradation became more pronounced. For lymphocyte fixation cells were thawed and the fixed immediately. Accordingly, there was no opportunity for autolysis or putrefaction to occur. Tissue samples however, were obtained dry and immersed in fixative within an hour

after removal. This delay may have allowed nuclease degradation of DNA to begin but it does not explain why this was progressive. Continued nuclease activity could have occurred if the maximum thickness of the tissue impeded access of the fixative. However at 2 mm maximum thickness, and according to the rate of penetration of ethanol and acetic acid (Hopwood, 1977), all of the tissue should have been bathed in the fixative within 1 h. Alternatively it may be suggested that the progressive degradation of the DNA was promoted by the low pH of the fixative mixture conferred by the presence of acetic acid. Thus in tissue the inevitable delay in initial fixation may have allowed nuclease degradation to begin which then could have been accentuated by the low pH of the solution. This explanation would also concur with the observations, as discussed above, that high molecular weight DNA can be recovered and used for Southern analysis after extended storage in ethanol alone (Bramwell and Burns, 1988, Wu *et al*, 1990).

6.3.3 Formalin fixation

Two fixatives were used in the investigation that contained formalin only, these were formol saline and neutral buffered formalin. Both fixatives were aqueous solutions and included 4% formalin. Formol saline also contained sodium chloride at 0.9% and the resultant solution had a pH between 3.5 to 3.8. Buffered formalin was adjusted to neutrality using sodium phosphate.

Lambda DNA remained intact after exposure to formol saline and neutral buffered formalin for 5 to 60 min at 4°C, room temperature and 37°C and subsequent restriction endonuclease digestion was unaffected. High molecular weight DNA was also recovered from lymphocytes fixed for 30 min in formol saline. However, in contrast to Carnoy fixed preparations, this required prolonged protease digestion. Extended protease digestion was also necessary to release DNA from tissue fixed in formol saline and neutral buffered formalin. This DNA was progressively degraded as fixation time was extended from 6 to 168 h, a feature more noticeable in extracts prepared from tissue initially fixed in formol saline. Successful Southern analysis was effectively limited to tissue fixed for up to 24 h only. Using the DNA recovered from tissue fixed from 24 to 168 h by prolonged

Proteinase K extraction and purification *Bcl2* sequences of 272 to 1271 bp were amplified by PCR in the majority of neutral buffered formalin fixed preparations but only in the minority of formol saline fixed extracts. DNA from paraffin embedded tissue initially fixed for 24 to 168 h was also recovered using a 'rapid' extraction protocol and assessed using PCR. The maximum size of PCR product demonstrated with neutral buffered formalin preparations was 513 bp after 24 h fixation and 272 bp after 96 h fixation whilst no PCR product was obtained using the 168 h fixed sample. Successful amplification using formol saline preparations was limited to 24 h fixation and a PCR product size of 272 bp.

As discussed elsewhere (Chapter 3, section 3.6, Chapter 4, section 4.2 and Chapter 5, section 5.4.1) these results agree with other investigators. Together they demonstrate that DNA degradation in formalin is progressive and accentuated by increase in fixation temperature or with the use of the fixative in acid conditions.

Endogenous nuclease activity can be rejected as a prime cause of DNA degradation in the formalin fixed tissues used in the present investigation for the same reasons as discussed for Carnoy fixation and this has been confirmed by model experiments (Koshiba *et al,* 1993, Yagi *et al,* 1996). Rather the findings indicate a prime role for the fixative with a contribution related to the pH at which it is used.

Evidence for the direct interaction of formalin with DNA is provided through the Lambda DNA studies reported by Tokuda *et al* (1990), Hamazaki *et al* (1993) and Koshiba *et al* (1993). In these investigations DNA exposed to buffered or unbuffered formalin for 1 day subsequently gave rise to partial restriction endonuclease patterns. In the absence of associated protein these findings argue strongly that interaction of formalin with the DNA had resulted in restriction sites becoming unavailable for the enzymes. Accordingly, the results may support a N-6 adenine amino reaction (McGhee and von Hippel, 1977b) or a more general reaction with the bases promoted by DNA breathing (Frank-Kamenetskii, 1985). Such interaction may also explain why restriction endonuclease digestion was not obtained in several of the formalin fixed lymphoma cases.

As reviewed in Chapter 1, section 1.4.3 substantial evidence for the formation of DNA-protein cross-links has been presented by investigators who have used formaldehyde as a tool to probe nucleosome structure. The origin of electrophoretic smears of unrestricted and restriction endonuclease digested DNA recovered from formalin fixed and paraffin embedded tissue at the loading well instead of the exclusion zone has been suggested as providing evidence for the residual attachment of protein (Moerkerk *et al,* 1990). However in this investigation the electrophoretic smears of DNA recovered from formalin fixed but unprocessed tissue originated at the exclusion zone implying that the change in electrophoretic pattern was due to the effects of paraffin wax processing. Dubeau *et al* (1986), Moerkerk *et al* (1990) and Karlsen *et al* (1994) suggest that these differences may rather reflect the partial polymerisation of DNA, rendered single stranded by fixation, by exposure to elevated temperatures during the wax infiltration steps. However, no apparent fixation mechanism can be suggested in support of this proposal.

Alternatively the progressive degradation of DNA by formalin fixation may be related to the effects of DNA-protein cross-link formation if, as suggested by Noguchi *et al* (1997), these render the nucleic acid more susceptible to mechanical damage. Yagi *et al* (1996) reviewing the beneficial effect of the addition of EDTA on the preservation of high molecular weight DNA in formalin fixed tissue proposed that the chelating agent may reduce histone-DNA interactions and thus render the nucleic acid less susceptible to this type of damage. However, in this investigation comparison of homogenised, with the potentially less disruptive, dicing and section methods of tissue preparation failed to influence the quality of DNA subsequently recovered.

Degradation of DNA was more marked in DNA prepared from formol saline fixed preparations than in equivalent neutral buffered formalin fixed samples. This suggests that the acid pH of the former fixative enhanced this process. However, this factor cannot on its own account for the marked degradation of DNA as the nucleic acid was less degraded after Carnoy fixation which was undertaken at slightly lower pH. Accordingly, it is suggested that the acid pH conferred by the use of unbuffered formalin may have promoted

the opening of the DNA helix thereby allowing formaldehyde to react more efficiently with exposed bases and potentially with histone proteins.

In summary evidence for the direct interaction of formalin with DNA has been provided. However, with respect to the main feature of formalin fixation, that of progressive DNA degradation, no obvious explanation for this phenomenon can be presently offered.

6.3.4 Formol Sublimate fixation

Lambda DNA remained intact after exposure to formol sublimate. However, after exposure to this fixative at room temperature and 37°C restriction endonuclease digestion was impaired. Furthermore DNA was not recovered from lymphocytes fixed in formol sublimate. As previously discussed (Chapter 3 section 3.6.1) the failure to recover high molecular weight DNA is in agreement with other investigators. Reports of successful PCR amplification following brief fixation (Greer *et al*, 1991a) or of short DNA sequences (Crisan *et al*, 1990, Crisan and Mattson, 1992, Limpens *et al*, 1993) however, indicate that some DNA can be recovered. *In situ* hybridisation demonstration of DNA in paraffin wax tissue fixed in formol sublimate or its sodium acetate containing derivative B-5 has also been reported by Pringle *et al* (1987) and Weiss and Chen (1991). However, in comparison with formalin alone the results obtained were sub-optimal in terms of signal strength and numbers of positive cells. Furthermore Pringle *et al* (1987) noted that the Proteinase K concentration used in pre-treatment of the tissue sections had to be increased in comparison with formalin fixed preparations to reveal the target DNA.

Together these findings suggest that DNA is either directly or indirectly fixed by mercury containing fixatives and that this fixation is particularly efficient making subsequent DNA recovery or demonstration *in situ* difficult. For direct fixation the intercalation of mercury between the amino groups of bases as suggested by Yamane and Davidson (1961) or binding of the same to nucleotides (Eichorn and Clark, 1963, Lipsett, 1964, Millar, 1968) would explain the resistance of DNA to subsequent endonuclease digestion. These

reactions may also have been enhanced by the availability of partially denatured DNA due to the low pH (2.4-3.0) of the fixative as used.

The difficulty experienced in recovery of the nucleic acid from cells and tissue fixed in mercury containing solutions also suggests that an indirect fixation mechanism involving DNA-protein links is operative. At the pH used in the present investigation, 2.4 to 3.0, the predominant reaction of mercury, as mercuric chloride, would be with sulphydryl groups (Baker, 1958). The interaction of these groups in histone H3 with mercury has been used to fractionate transcriptionally active histones (Chen *et al*, 1991, Bazett Jones *et al*, 1996). However, in inactive DNA these groups are embedded within the histone octomer and accordingly the formation of protein-DNA cross-links in cells fixed in formol sublimate would be unlikely. A further possibility is that the formalin component of the fixative could contribute to the formation of DNA-protein links. As discussed above this is possible but on its own it would not explain the difficulty experienced in recovery of DNA from formol sublimate fixed tissue or its demonstration *in situ*.

6.4 Conclusions and future investigations

The protocols developed and assessed in this thesis together with alternatives described by many investigators offer a full range of methods for the recovery of DNA from fixed tissue. Though limitations were shown for the diagnostic application of this DNA using Southern blotting approaches the investigation has confirmed the utility of PCR as a valuable analysis method.

PCR analysis of fixed and paraffin wax embedded tissue can assist with lymphoma diagnosis. However, due to the underlying biology of gene rearrangement, the technique will probably remain as a relatively complicated procedure employing several primer pairs to ensure identification of clonality. The recent introduction of automated PCR assays, exploiting the 5' exonuclease activity of *Taq* polymerase and providing for real time detection of products, may offer the potential of enhanced efficiency, sensitivity and specificity (Evans *et al*, 1998, Luthra *et al,* 1998, Dippel *et al,* 1999, Zhou, *et al*, 1999). Accordingly, it is suggested that future investigations could be undertaken to develop an

integrated system for the preparation and automated analysis of DNA from fixed and paraffin embedded lymphoma samples. Potentially this system could provide a rapid, comprehensive and definitive method for the diagnosis and sub-typing of lymphomas.

The results obtained with Bouin, Carnoy and formol sublimate have confirmed previously reported fixation reactions with DNA and associated proteins. However, with respect to formalin, the fixative most commonly used and on which there is the most abundant reaction information, no satisfactory explanation can be given for the progressive DNA degradation that occurs in this fixative. If this could be prevented or retarded in routine specimens molecular assays would be more reliable and consequently yield better diagnostic information. Accordingly, it is suggested that future investigations should focus on understanding and seeking to minimise this problem.

With respect to understanding the interaction of formalin with DNA and nuclear proteins molecular modelling could provide precise spatial and energetic information on potential reactive groups. This is now feasible for the nucleosome (Luger *et al,* 1997) and could clarify, for example, if methylene bridges could form between the ε amino groups of the terminal lysines of histones and surrounding DNA. Enzyme digestion could also be used as a means of probing the reaction of formalin. In this context S1 nuclease digestion could be employed to determine if DNA is rendered single stranded by fixation whilst resistance of formalin treated chromatin to micrococcal nuclease digestion would imply a reaction with spacer DNA. At a microscopic level chromatin preparations could also be exposed to formalin under controlled conditions and then examined using electron microscopy to determine if structural changes are random or discrete.

Formalin induced DNA degradation could be analysed using gel electrophoresis, restriction endonuclease digestion and PCR analysis, as employed in this thesis. This work could be undertaken using pure DNA, chromatin, and nucleic acid recovered from fixed cells and tissues. Using this systematic approach the effects of pH, temperature and time of formalin fixation could be assessed and a base line set by which the benefit of any modification to the fixation procedure can be properly assessed.

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Appendix

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RAPID COMMUNICATION

SOUTHERN BLOT ANALYSIS OF DNA EXTRACTED FROM FORMOL–SALINE FIXED AND PARAFFIN WAX EMBEDDED TISSUE

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SUMMARY

Model experiments were designed to assess whether DNA could be recovered from formol–saline fixed peripheral blood lymphocytes and tonsil tissue for use in Southern blot gene analysis. Lymphocytes were fixed for 30 min and tonsil for 6 and 24 h, then paraffin embedded. High molecular weight DNA was extracted by prolonged digestion (2–7 days) with proteinase K or protease XXIV in the presence of 1 per cent sodium dodecyl sulphate. Restriction, transfer and hydridization were possible without modification of standard procedures. Multiple copy sequences were demonstrated using Mspl and Bst Nl restriction and hybridization for the Y chromosome (pHY 2·1 probe), single copy genes using EcoRI and BamHl restriction for the T-cell receptor β chain (Tβ probe), and Bgl II and Hind III for the immunoglobulin heavy chain (JH probe). Identical banding to unfixed tissue was achieved except when 24 h fixed extracts were used. With these, demonstration of the 24 KB Bam Hl/Tβ and 9·2 KB Hind III/JH bands was not obtained. These findings suggest that as the fixation time is extended, alterations to DNA will limit the available range of restriction enzyme/probe combinations. However, with careful choice of these the extraction of DNA from formalin fixed and paraffin embedded pathological tissue for Southern blotting should be profitable.

KEY WORDS—Fixation, DNA extraction, Southern blotting.

INTRODUCTION

The investigation of cells and tissues by molecular biology methods such as Southern and Northern blotting, dot blots, and *in-situ* hybridization is making an increasingly important contribution to diagnostic and research pathology.[1] Southern blotting, in which restriction endonuclease digested DNA gene sequences immobilized on membranes are visualized by radioactively labelled complementary probes, has found application in several areas. These include identification of viral infections,[2] prenatal diagnosis of inherited diseases,[3] analysis of genetic disorders,[4] identification of monoclonal B and T-cell proliferations in leukaemias and lymphomas,[5] DNA fingerprinting,[6,7] and oncogene activation.[8,9]

A limitation of Southern blotting has been its reliance on fresh tissue as a source of DNA. This has made retrospective analysis difficult and has especially hindered the investigation of rare disorders. However, recent investigations[10,11] have shown that DNA can be recovered from neutral buffered formalin fixed and paraffin embedded tissues and that it is suitable for Southern blot analysis.

In these studies, markedly different approaches were used to recover DNA. Goelz *et al.*[10] used simple prolonged proteolytic digestion. Dubeau *et al.*[11] however, described a complicated method yielding DNA free from the contaminants associated with the former procedure. Observations on the effect of fixation time on the recovery of DNA also differed. Goelz *et al.*[10] claimed that the quality of recovered DNA was not affected by the fixation time whereas Dubeau *et al.*[11] demonstrated a reduction in the amount of DNA extracted when the fixation time exceeded 5 days. There is, therefore, a need to clarify the technical parameters surrounding this methodology.

In this paper we describe model experiments using formol–saline fixed lymphocytes and tonsil

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tissue. We confirm that DNA can be recovered by simple prolonged protease digestion, but that its suitability for use with some restriction enzymes is reduced as the fixation time is extended. Information is also provided on the purity of extracted DNA, and its suitability for Southern blot detection of multi- and single copy genes is demonstrated.

MATERIALS AND METHOD

Samples

Lymphocyte-enriched mononuclear cell samples were prepared using the Ficoll–Paque method of Böyum[12] and stored at a concentration of 2×10^7 cells/ml at −70°C in a freezing medium comprising 64 per cent RPMI (Gibco 041-1875H), 18 per cent fetal calf serum (Tissue Culture Services 402004), 8 per cent penicillin and streptomycin (Gibco 043-5140H), and 10 per cent dimethyl sulphoxide.

Palatine tonsil obtained at routine tonsillectomy was divided into 0·5 × 0·5 × 0·3 cm blocks. 'Fresh' samples were frozen in isopentane precooled in liquid nitrogen and stored at −70°C. The remaining tissue was fixed for 6 or 24 h in 10 per cent formol–saline (4 per cent v/v formaldehyde in 0·9 per cent w/v saline) and processed using the following schedule: 70 per cent isopropyl alcohol (IPA), 2 h; 95 per cent IPA, 1 h; absolute IPA, 6 × 1 h; xylene, 2 × 1·5 h, and paraffin wax, 2 × 2 h at 65°C. Tissues were embedded and stored at room temperature.

Extraction of nucleic acid

(a) Lymphocyte cell suspension—Individual 100 μl (2×10^6 cells) aliquots of thawed samples were transferred to Eppendorf tubes and fixed with 900 μl of 10 per cent formol–saline for 30 min at room temperature. Controls were treated with 0·9 per cent saline only. Fixative was removed by washing three times in 1 ml of 0·05 M Tris/HCl, pH 7·6, each wash being preceded by pelleting of samples by centrifugation for 1 min at 2800 x*g*. The cells were then pelleted as before and resuspended in either 900 μl of 0·05 M Tris/HCl, pH 8·0, for proteinase K (BCL 745723) digestion or 1 × SE (0·1 M w/v EDTA and 0·15 M w/v sodium hydroxide), pH 8·0, for protease VII (Sigma P5255) digestion. 100 μl of 10 per cent sodium dodecyl sulphate (SDS) and 0·2 mg of enzymes were added and samples incubated at 37°C for 2, 4, 24, and 48 h. Additional enzyme (0·2 mg) was added to the 24 h samples after 16 h and to the 48 h preparations after 24 and 44 h incubation.

Products of enzymatic digestion were removed from the nucleic acids by three 800 μl phenol/chloroform/isoamyl alcohol extractions[13] followed by removal of phenol by two 800 μl chloroform/isoamyl alcohol (24:1, v/v) separations. For the first two 'phenol' extractions the layers were separated by standing. For the third 'phenol' and both 'chloroform' separations, the interface was formed by centrifugation at 12 000 x*g* for 5 min. To improve the final yields of nucleic acid, the samples were back-extracted with 600 μl of relevant digestion buffer. Primary and back extractions were pooled and nucleic acids precipitated by addition of sodium chloride to 0·2 M, 2 volumes of cold absolute ethanol, and storage at −70°C for 30 min. Nucleic acid was pelleted by centrifugation at 12 000 *g* for 5 min at 4°C. The supernatant was discarded and the pellet washed with 1 ml of 80 per cent (v/v) ethanol to remove salt. After mixing, the samples were centrifuged as above, the supernatant was discarded, and the pellets were vacuum dried for 15 min and resuspended in 1·5 ml 1 × TE, pH 8·0 (10 mM w/v Tris/HCl, 1 mM w/v EDTA) at 4°C overnight.

(b) Paraffin wax tissue—An area of tonsil rich in lymphoid tissue was selected, with reference to the corresponding haematoxylin and eosin stained section, cut out and trimmed to weigh 100 mg. The sample was diced, placed in a processing cassette, and rehydrated by immersion for 30 min in each of the following: xyelene, three changes; 99 per cent alcohol, two changes; and one change each in 95 per cent alcohol and running tap water.

Rehydrated sections were transferred directly to the digestion solutions. Tissue from the block rehydration method and 100 mg of tissue cut from unfixed 'fresh' samples were first homogenized in digestion buffer for 1 min using an Ystral X10/20 homogenizer with 20 T shaft and fine generator set at the lowest speed. The digestion solution contained 9 ml of buffer (see cell methods), 1 ml of 10 per cent SDS, and 1 mg of proteinase K or protease XXIV (Sigma P5147). The latter enzyme replaced protease VII as the nearest equivalent when this product was withdrawn from sale. Digests were incubated in 50 ml polypropylene tubes and 1 mg amounts of enzyme were added at 24 h intervals. For temperature and time of incubations, see the Results section.

Digests were purified by three extractions with 7·5 ml phenol/chloroform/isoamyl alcohol followed by three times with 5 ml chloroform/isoamyl alcohol. The 'phenol' extracts were centrifuged at

Table I—cDNA probes and restriction enzymes used for Southern blotting of fixed tissue extracts

Probe	Reference	Chromosomal distribution	Appropriate restriction	Source
pHY 2·1	17	2000 repeats on long arm of Y and 100–200 copies on X and autosomes	Msp I Bst Nl	BRL, 510-5419 New England Biolabs, 168
JH	18	Single copy on long arm C14	Bgl II Hind III	Pharmacia, 27-0946 BRL, 510-5207
Tβ	19	Single copy on long arm C7	EcoRI BamH1	Pharmacia, 27-0854 BRL, 510-5201

2000 *g* for 30 min to separate the phases and the 'chloroform' extracts 2000 *g* for 15 min. Preparations were also back-extracted with 5 ml of relevant digestion buffer. Primary and back-extract solutions were pooled in corex tubes and nucleic acid was precipitated as described for lymphocyte cell suspensions. Maximum yields were obtained when the tubes were subsequently stored at −20°C overnight.

Precipitates were pelleted by centrifugation at 17 000 *g* for 10 min at 10°C. The supernatant was discarded and replaced by 1 ml of 80 per cent cold ethanol. After mixing and centrifugation using the same parameters, the supernatant was discarded and the tubes were inverted to drain for 10 min. Nucleic acid was resuspended in 1·5 ml of 1 × TE and stored at 4°C.

Quantitation of DNA yield and assessment of sample purity

Spectrophotometer measurements at 260 and 280 nm were used to calculate the DNA yields and the degree of protein contamination of samples.

Quantitative analysis of the molecular weight of DNA was assessed electrophoretically by running 5 μg aliquots of extracts in submerged 0·8 per cent agarose gels, and photography after ethidium bromide staining. To distinguish low molecular weight DNA from RNA, 5 μg of tissue extract samples was pretreated with 10 μg of RNAase Type 1A (Sigma R4875) for 30 min at 37°C before electrophoresis as above.

Southern blotting

Ten μg aliquots of tissue extracts were reacted for 4 h at 37°C (Bst Nl at 60°C) with 25 units of each of the restriction enzymes listed in Table I in the presence of 4 mM spermidine. Samples were then electrophoresed as described above.

Gels were depurinated by immersion in 0·25 M hydrochloric acid for 10 min, denatured in 0·5 M sodium hydroxide (two changes of 30 mins), and neutralized in 1·5 M sodium chloride dissolved in 1 M Tris/HCl, pH 8·0, for 60 min. Nucleic acid was transferred onto nylon membrane (Hybond, Amersham) using 20 × SSC (3 M w/v sodium chloride, 0·3 M w/v trisodium citrate) after the method of Southern[14] over a period of 2 h, then immobilized by exposure to UV light at 302 nm for 30 s.

Probes (Table I) were labelled with α^{32}p dCTP of specific activity 3000 Ci/mmol (Amersham PB10205) by the random primer method[15] and used at a concentration of 0·5 ng/ml in the hybridization method of Church and Gilbert.[16]

Hybridized membranes were autoradiographed using Kodak X-Omat film with intensification at −70°C and developed using a Kodak RP X-Omat processor.

RESULTS

Lymphocyte cell suspensions

Enzyme digest times did not affect the amount of nucleic acid obtained from the samples treated with saline alone. An average yield of 3 μg/10^6 cells was recovered using either enzyme. This compares well with a theoretical maximum yield of approximately

6 μg from the same sample size. The extract was of good purity and shown to be high molecular weight DNA by electrophoresis (Fig. 1).

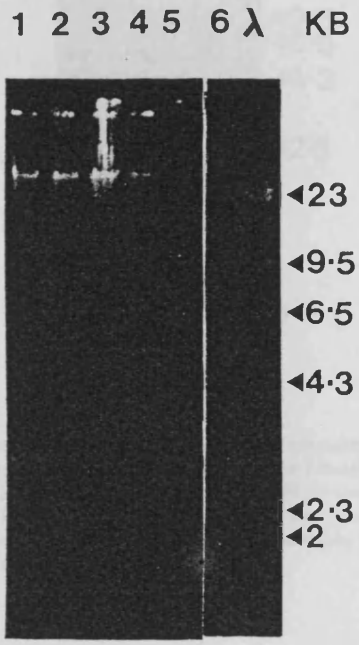

Fig. 1—Electrophoretic gel of DNA extracted from lymphocytes by proteinase K digestion. Lanes 1–3: samples pretreated in saline for 30 min and digested for 48, 24, and 4 h, respectively. Lanes 4–6: samples pretreated with formol–saline for 30 min and digested for 48, 24, and 4 h, respectively. Hind III digested λ DNA size marker is included to the right of lane 6

In comparison, very little nucleic acid was recovered from cells treated with formol–saline, unless digestion was prolonged. Indeed, only after overnight incubation did these samples become viscous, an indication of the presence of free high molecular weight nucleic acid. The highest yields (2 μg/10^6 cells) were obtained after 48 h digestion with proteinase K. Using protease VII under the same conditions, less nucleic acid (1·5 μg/10^6 cells) was extracted. The 260/280 nm ratios indicated moderate protein contamination, and electrophoresis (Fig. 1) showed the presence of high molecular weight DNA.

Paraffin wax tissue

(a) Extraction—The lymphocyte suspension results indicated that if high molecular weight DNA were to be recovered from fixed and paraffin embedded tissue long proteolytic enzyme incubations would be required. Consequently, rehydrated and homogenized preparations from tissue fixed for 6 and 24 h were digested with proteinase K and protease XXIV for 2, 4, and 7 days at 37°C. An unfixed tissue sample, digested for 16 h, was included as a control. At the start of digestion of fixed samples, fine pieces of tissue (1 mm^3) were dispersed in clear liquid. After 24 h, the tissue fragments had coalesced into a loose gelatinous lump. As digestion continued, this dispersed into fine fragments which were present in an increasingly viscous solution. After 7 days, some small tissue fragments remained, especially in the protease XXIV samples.

Nucleic acid was recovered from all samples. The highest yields were recorded using proteinase K at 4 days, i.e., 17 μg/mg tissue from the 24 h sample. In contrast, the amount of nucleic acid recovered using protease XXIV showed a gradual increase to give a maximum yield at 7 days, i.e., 13 μg/mg tissue from the 24 h sample.

The 260/280 nm ratio of the proteinase K extracts indicated that a significant amount of RNA was to be expected. This was confirmed when samples were examined electrophoretically (Fig. 2). The gels also demonstrated that in addition to the presence of high molecular weight DNA some lower molecular weight components were present.

A feature of proteinase K is its stability at high temperatures.[20] This characteristic was used to explore the possibility of reducing the digestion times by incubating 6 and 24 h fixed and homogenized samples for 1, 2, and 4 days at 65°C in the presence of the enzyme. Using this method, nucleic acid was liberated more quickly but it was associated with progressive DNA shearing which in the 4-day sample resulted in the complete loss of high molecular weight DNA.

(b) Restriction and Southern blotting—Proteinase K and protease XXIV extractions gave identical restriction and hybridization results. Electrophoretic smears (Fig. 3) of fixed extracts digested with restriction endonucleases were more weakly stained with ethidium bromide than unfixed preparations. With the exception of BstNI, the electrophoretic origin of the smears differed (Fig. 3), with 'fixed' smears beginning at the loading well in contrast to the exclusion zone for 'unfixed' digests.

No difference was observed between transfer of unfixed and fixed restriction endonuclease digested DNA. Hybridization with the pHY 2·1 probe on male extracts was investigated first to determine

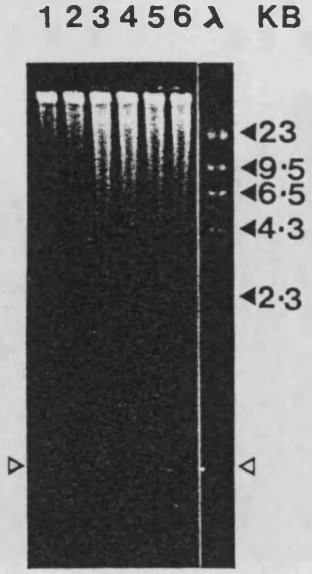

Fig. 2—Electrophoretic gel of nucleic acid extracted by proteinase K digestion from tonsil fixed for 24 h in formol–saline. The digestion times were for lanes 1 and 2, 2 days; lanes 3 and 4, 4 days; and lanes 5 and 6, 7 days. Note RNA, marked by open arrows, present in lanes 1, 3, and 5 but removed by pretreatment with RNAase in lanes 2, 4, and 6

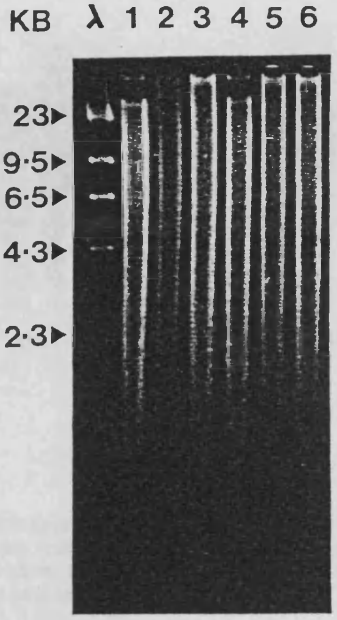

Fig. 3—Electrophoretic gel of extracted nucleic acids digested with Bgl II (lanes 1–3) and Hind III (lanes 4–6). Note the origin of smears in 24 h fixed samples prepared after 2 and 7 days' digestion in proteinase K (lanes 2, 3, 5, and 6) in comparison with unfixed controls in lanes 1 and 4

whether high copy numbers could be detected in fixed extracts. This proved successful (Fig. 4) although the strength of the signal was slightly reduced in comparison with unfixed controls. Successful banding was also achieved, with a similar decrease in signal, when detecting single copy gene sequences in 6 h fixed extracts (Figs 5 and 6). Using 24 h fixed extracts and JH probing, the 4 KB Bgl II restriction band was demonstrated but not the 9·2 KB Hind III fragment (Fig. 5). Tβ probing of 24 h fixed extracts demonstrated all three EcoRI restriction bands (4·2, 9, and 12 KB); however, the 24 KB Bam HI band was not detected (Fig. 6).

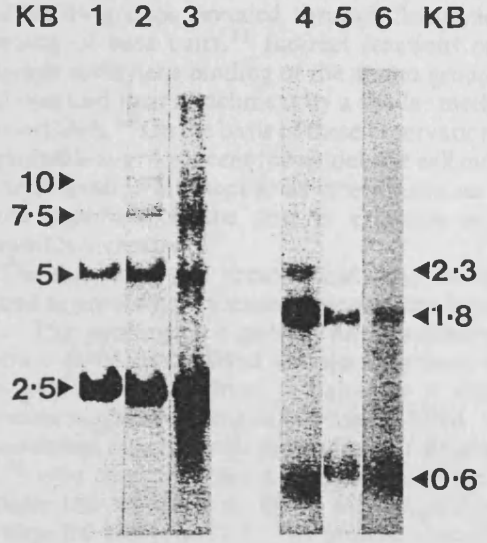

Fig. 4—Autoradiograph of MspI (lanes 1–3) and Bst NI (lanes 4–6) restriction endonuclease digests probed with pHY 2·1. Lanes 1 and 4: unfixed controls; lanes 2 and 5, 6 h fixed extracts; lanes 3 and 6, 24 h fixed extracts

DISCUSSION

The reactivity of formaldehyde with mono- and polynucleotides has been extensively studied.[21] The reaction proceeds in two stages: rapid but reversible hydroxymethylation of imino and amino groups on bases, then slow formation over a period of days, of methylene bridges between bases. These reactions are modulated by the concentration of reactants which effectively determines which product predominates. They are also enhanced by an increase in the reaction temperature.

The effect of temperature becomes particularly important when considering possible reactions

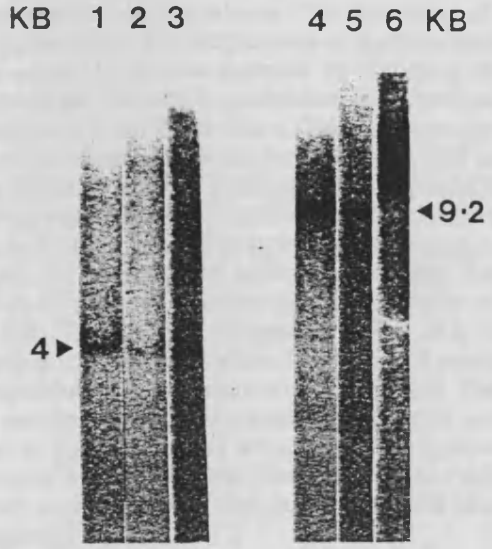

Fig. 5—Autoradiograph of Bgl II (lanes 1–3) and Hind III (lanes 4–6) restriction endonuclease digests probed with JH. Lane identification as for Fig. 4. Note the absence of a Hind III band (lane 6) in the 24 h fixed extract

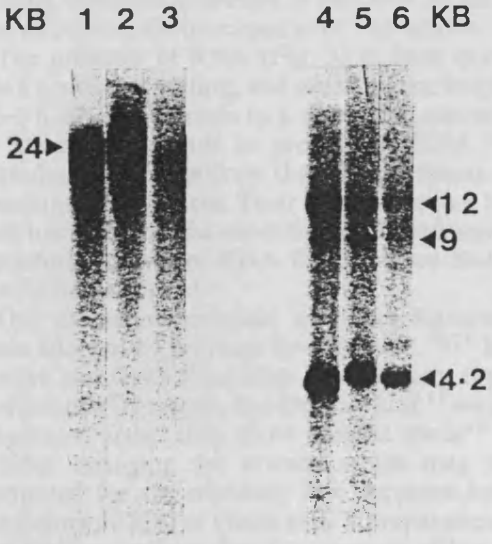

Fig. 6—Autoradiograph of BamHl (lanes 1–3) and EcoRI (lanes 4–6) restriction endonuclease digests probed with Tβ. Lane identification as for Fig. 4. Note the absence of a Bam Hl band (lane 3) in the 24 h fixed extract

between formaldehyde and native DNA. At room temperature, the double helix of DNA is present as two complementary polynucleotide chains linked together by base pair hydrogen bonding. On heating to 65°C, however, these bonds begin to break and eventually, at about 90°C, two single-stranded molecules are produced. In this denatured state, formaldehyde reacts rapidly, through hydroxymethylation, and this inhibits reannealing on cooling.

These observations have previously been used to suggest that little or no reaction occurs between formaldehyde and DNA at room temperature.[22] However, there is now evidence that both direct and indirect reactions occur. The former involves exocyclic amino groups which are exposed in the double helix and the presence of some endocyclic amino and imino groups revealed through fluctuational opening of base pairs.[23] Indirect reactions occur through methylene binding of the amino groups of histones and their attachment by a similar mechanism to DNA.[24] On the basis of these observations, it is probable that 4 per cent formaldehyde will induce indirect fixation and that some direct reactions will occur especially as the time of exposure to the reagent is increased.

The results of this present study can be interpreted as providing evidence to support this hypothesis. The prolonged digestion time necessary to liberate DNA in the fixed samples combined with the gradual change from a liquid to a viscous solution suggests binding of histones to DNA. This observation concurs with the findings of Brutlag *et al.*,[25] who demonstrated a residual 4 per cent of histone still attached to DNA after digestion in pronase for 4 days at 37°C. The presence of a DNA smear originating from the loading well in restriction enzyme digested fixed samples (Fig. 3) may also be evidence of residual attachment of histone to DNA.

Alternatively, as Dubeau *et al.*[11] suggest, the elevated temperature of wax impregnation during processing may account for reduced electrophoretic mobility. Using ethanol 'fixed' samples, they demonstrated restriction smears similar to formalin fixed extracts. We have also observed similar phenomena using extracts prepared from Carnoy fixed tissue (unpublished observations). There is therefore a possibility that during wax impregnation some single-stranded DNA is produced which is then unavailable for restriction endonuclease digestion.

That fixation and paraffin embedding results in some physical alteration of DNA is apparent from the presence of medium molecular weight DNA in non-digested samples (Fig. 2). It has been shown[26] that chromatin may be sheared by mechanical

homogenization procedures. The possibility of this step contributing to the presence of medium molecular weight DNA was assessed by digesting 15 μm rehydrated sections (unpublished observations) in proteinase K for 2 and 4 days. The resultant electrophoretic patterns of non-restricted DNA and banding obtained after Tβ probing of Bam HI and Hind III restriction endonuclease digests were identical to those of homogenized samples (results not shown). Thus the presence of medium molecular weight DNA in all fixed extracts and the absence of the 24 KB Tβ/Hind III fragment from 24 h fixed samples cannot be explained in terms of mechanically induced fragmentation of nucleic acid. The use of sections as a starting material for DNA extraction is, however, useful when the cell population of interest is intimately associated with other cells or when conservation of the architecture of a block is required.

The failure to detect the 9·2 KB JH/Hind III restriction endonuclease band (Fig. 5) and the 24 KB Tβ/Bam HI fragment (Fig. 6) in 24 h fixed extracts suggests that this DNA is of limited suitability for Southern blotting. A probable explanation of these results is that direct or indirect (histone binding) chemical alteration of the DNA makes certain restriction endonuclease sites unavailable.

The presence of RNA (Fig. 3) in fixed extracts was a consistent finding, and the sequence length of 0·3–0·6 KB corresponds to a ribosomal source. As no attempt was made to protect the RNA from degradation, it is unlikely that these extracts represent intact sequences. Their demonstration, however, indicates that the use of formalin fixed tissue as a potential source of RNA for Northern blotting should be considered.

Our extraction protocol combines features of those adopted by previous investigators.[10,11] In its relative simplicity it matches more closely that of Goelz *et al.*[10] However, like Dubeau *et al.*[11] we used rehydrated rather than diced paraffin tissue[10] and avoided syringing the extract, which may have accounted for the relatively low sequence length (maximum 10 KB) of Goelz *et al.*'s preparations.[10] Our findings indicate that the presence of low and medium molecular weight nucleic acid in extracts has no effect on restriction endonuclease digestion and hybridization. The complicated protocol of Dubeau *et al.*[11] in which these were removed during digestion would, therefore, appear unnecessary.

Points of similarity with the former investigations[10,11] in the restriction endonuclease digestion and hybridization results include the ease with which extracted DNA could be used in Southern blot procedures and the decreased electrophoretic mobility of restricted extracts. In the effect of fixation time on the quality of extracted DNA, our results agree only with those of Dubeau *et al.*,[11] who found a progressive diminution in usable DNA as fixation was extended to 5 days. In contrast, Goelz *et al.*[10] reported no effect, even when fixation was of several weeks' duration. It is possible that this observation was due to fortuitous restriction enzyme/probe combinations together with the absence of long sequence DNA in which alterations in the nucleic acid would have been more readily observed.

Further research is clearly required to provide a more complete understanding of the effects of fixation and processing on the recovery of DNA from tissue so treated. In particular, there is a need to examine several fixatives using model experiments and to determine to what extent DNA can be recovered from routinely fixed material. In the latter context, we have recently extracted DNA from formol–saline fixed and paraffin embedded CIN cervical tissue and successfully demonstrated the presence of human papilloma virus type 16.[27]

In conclusion, we have shown that DNA can be extracted from formalin fixed, paraffin embedded tissue by extension of standard digest protocols; and that this may be digested under normal conditions using restriction endonucleases and transferred and hybridized without modification of established methods. Extension of the fixation time may prove a limiting factor both in terms of the maximum sequence length of DNA available for probing and chemical modification of nucleic acid making it inaccessible for restriction enzymes. With careful selection of restriction endonuclease and probe combinations, however, it should be possible to circumvent these limitations and explore usefully the vast storehouse of material available in paraffin wax block files.

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