AN APPRAISAL OF THE STABILITY OF SEX CHROMATIN AND THE H-Y MOLECULE IN FORENSIC CONTEXTS

by

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B.A., Simon Fraser University, 1986

THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF ARTS (ARCHAEOLOGY)

in the Department

of

Archaeology

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SIMON FRASER UNIVERSITY

November 1989

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ABSTRACT

Forensic investigations may involve recovery of remains so severely damaged that gender can no longer be determined from macroscopic examination of hard or soft tissue. In this event gender differentiation can sometimes be accomplished at the microscopic level by cytological probing of remnant tissue. This investigation is aimed at the application of such cytological sexing procedures to some simulated forensic environments.

Two procedures were developed using human tooth pulp tissue, one detecting X and Y chromatin in the cell nucleus and the other detecting a male molecule, histocompatibility -Y (H-Y) located on the cell membrane. Simulated forensic environments included burial of teeth, exposure on the surface, storing teeth at room temperature,and heat treatment. The jaws and heads of pigs (*Sus scrofa*)substituted for human tissue for the comparison of environmental effects on pulps from extracted teeth versus pulps from unextracted teeth.

Stained X and Y-chromatin discriminated gender for no longer than two weeks in teeth buried or exposed out of doors. No substantial difference in decomposition rates was recorded between human and pig extracted dental pulps, and pig unextracted dental pulps in outdoor environments. In teeth held at room temperature, sex chromatin remained stable for more than one year. The pulps of extracted human teeth, subjected to temperatures above 100C over a period of one hour,lost endonuclear granulation and with it sex

chromatin staining characteristics, whereas pulps in unextracted pigs teeth retained nuclear granularity after exposure to temperatures of 300C over the same period. A maximum temperature, reached but not sustained within the human or pig pulp chamber, at which sex could still be diagnosed, was assessed by a thermocouple probe to be 50C. Unextracted pig's pulp chambers registered this temperature in an open fire, when the temperature of the fire reached 600-700C. H-Y, detected by immunocytochemical staining, proved to be highly unstable, rapidly losing antibody binding capacity in any extracorporeal environment.

The results of the study indicate that whereas a cytological mode of sex discrimination has potential application to any forensic situation, its most common application is likely to be to situations where recovery of remains is fairly rapid, as in mass disaster or fire. Forensic tissue samples submitted for analysis should be stored as found without any fixative or fluid additive, and transported packed in ice.

ACKNOWLEDGEMENTS

I have been very fortunate in the help I have received for this research. I am especially grateful to my thesis advisers Dr. Mark Skinner, and Dr. Erle Nelson, Archaeology, SFU, and Dr. J. Douglas Waterfield, Oral Biology, UBC, for their outstanding support and guidance.

I would also like to thank those people who helped me with the photography. Ron Long, (Instructional Media Centre, SFU) in particular, gave his time and skills in making excellent enlargements from often poor quality negatives. I also wish to acknowledge the help of Leslie Western, Harold Taegar and Bruce McCaughey (UBC Dentistry) for their help with photography and graphics, Vic Bourne (Microbiology, SFU) for the use of a camera mounted microscope.

For technical advice I wish to thank Helena Schroeder of the Cancer Control Agency of British Columbia for familiarising me with chromosome staining methods. Further help at UBC Dentistry came from Noëlla Deslauriers,and Maureen Fairhurst who offered their advice and technical help in immunology, as did Barbara Tait in histology, and Trish Kapitan in animal experimentation. I also appreciate the advice and technical assistance in the use of thermocouples which came from Jean-Paul Sweeney and Dr. Babak Chehroudik.

I wish to acknowledge the donation of teeth from maxillo-facial surgeons in the Lower Mainland, namely Dr. Michal Kaburda (New Westminster), Dr. B.H. Goldstein (Vancouver) and Drs. E.

Marks and P.H. Tresher (North Vancouver). Dental auxillary personnel from North Vancouver who offered exceptional help in the collection of teeth were Merry Ann Carlysle and Donna Robinson.

Translation of articles from Japanese, and Russian to English are to be credited to Jim Spafford, Eung K.Pae and Tanya Abramovitch. Bonfires for testing the effects of temperature on pig dental pulp were set up courtesy of Louis Duffy and Kitty Koski. Finally I wish to thank my husband, Patrick Duffy, for his facility with a shovel and trowel in the field work for this research, for his computer expertise, and for his unfailing patience and encouragement.

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CHAPTER ONE

INTRODUCTION

Determination of sex is an essential part of any forensic investigation. Usually it is assessed macroscopically from soft tissue or skeletal morphology. In the case of adult skeletal material, when it is well preserved, there is about a 95-98% probability of correct sex determination from the combined metric and non-metric measures of the skull and pelvis (Rathbun and Buikstra 1984:213; Krogman and Isçan 1986:189). Unfortunately, the osteological areas of the skull traditionally used for sex determination by discriminant function analysis, such as the zygomatic arches, are often damaged. Similarly the pelvic bones preserve poorly, especially the diagnostically important pubic areas. Morphometric studies have contributed measures facilitating gender discrimination from a broad range of skeletal elements,but with less reliability, for example about 80-90% for long bones,89% for the sternum, 89-96% for the clavicle, 79-96% for the sacrum, and 79-89% for the talus and calcaneus (Rathbun and Buikstra 1984: 214; Krogman and Isçan 1986: 259).

In reviewing the osteological measures used to determine gender,Buikstra and Mielke (1985:374) caution that the available standards should not be applied uncritically because patterns of sexual dimorphism are variable. Skeletal dimensions of adult males and females exhibit a proportional difference of about 8% (females being the smaller), but overlap often occurs between individual traits (Krogman and Isçan 1986:190),and considerable variation is described between populations, some are more typically gracile and

others more typically robust. Further problems arise if the remains are from victims of fire, because fire can damage bones and produce bone shrinkage at temperatures of 700C and beyond. Shrinkage has led to an artificially high percentage of females reported from archaeological cremation sites (Stewart 1979: 65). The amount of shrinkage increases with temperature rise: according to Van Vark (cited in Stewart 1979: 65). Femoral shrinkage at temperatures between 700-900C may vary between 2-12%, however, Buikstra and Swegle (n.d.) report lower figures (0.7-5.6% femoral shrinkage at temperatures between 600-900C).

Immature skeletal material is even more difficult, if not impossible, to assess, because definitive sexual characteristics are not well developed in the skeleton until puberty. Some dimorphism is described for the pelvis as early as foetal life, but reports tend to be contradictory, and in most studies the data generated have not been subject to multivariate statistical analysis. Boucher (1955: 51; 1957: 581) found significantly larger subpubic angles and sciatic notch sizes in the American Black and British White female foetuses as compared to males (these same inequalities serve to separate the sexes in adult skeletons), but no similar dimorphism was found in American whites.

Talheimer (cited in Krogman and Iscan 1986 : 201) using an Italian sample reported the ilia to be longer in female foetuses and the male ilia broader. In contrast, however, Weaver (1980: 191) analysing remains from individuals of different racial affinity (predominantly Black and White), found no

significant differences in sciatic notch and ilia measures between foetuses of different sex, but estimated that the elevated auricular surface in females, permits an accuracy of 92%. This level of accuracy, however, was not maintained in postnatal samples. Choi and Trotter (1970: 307) developed a discriminant function statistic based on foetal longbone ratios of a combined sample of American Blacks and Whites. The 72% accuracy reported for the method is not very high. Reynolds (1945:321;1947: 165), determined that among American whites, male foetuses are larger in the inner structure of the pelvis,including having a large pelvic inlet. In prepuberal children he found that boys' overall pelvic structures are larger, whereas "girls tend to be either absolutely or relatively (i.e. in consideration of their general overall smaller size) larger in measurements relating to the inner structure of the pelvis, including the inlet" (1947: 199).

Hunt and Gleiser (1955:389) have compared differential dental development and radiographs of differential maturation of hand and wrist bones in preadolescent males and females (males exhibit slighty more rapid development). When specimens of unknown gender are compared to these norms, the sex for which the standards agree best,is accepted.In practice this method is rarely applied because the small bones of the hand are commonly lost in the disarranged skeleton. Multivariate measures for estimating sex from the morphology of tooth crowns in permanent and deciduous teeth have been attempted with some success (Black 1978: 227) but norms have mostly been derived from prehistoric populations rather than a broad range of modern populations. Ditch and Rose (1972: 61) using

a prehistoric population from the Dixon Mound Site (North American Indian), found a range of concordance of 95% with a combination of maxillary and mandibular canines. They did not recommend, however, that tooth measures be used in isolation, rather they should be used in conjunction with as many other criteria as possible because of the limitation imposed on accuracy by the sample of origin.

Gonda's (1959: 47) statistical analysis from measurements of 6,000 teeth from a modern population sample, also found the most sexually diagnostic teeth to be the upper and lower canines. He found about a 6% difference in total canine length between the sexes. However, use of these measures beyond the population of origin (in this case Japanese) again reduces accuracy because of significant ethnic differences in tooth size. Garn et al.(1979: 115) using radiographs and an optical scanner on a population of 83 subadults (16-17 year old students from the University of Michigan, ethnic origin not given), achieved a maximum of 87% accuracy from a combination of crown dimension and root lengths from the jaw.

Scattering of immature and adult remains occurs in homicide cases when buried or exposed bodies are scavenged by feral and domestic animals (Morse 1983: 124,148; Haglund et al. 1988:985). Certain catastrophic events such as explosions and high impact collisions, often accompanied by fire, can produce the same effects. Such situations may impede macroscopic soft as well as hard tissue evaluation of gender. Given, then, that the present state of the art of forensic gender analysis reveals important

limitations when applied to fragmentary remains, alternative methods of gender determination at the microscopic level can provide a useful adjunct to osteological analysis.

Contingent upon the degree of decomposition and/or incineration of soft tissue, gender may be discriminated at the microscopic level by investigation of certain characteristics of somatic cells. The fluorescent staining properties of the chromatin of the distal portion of the long arm of the Y (male) chromosome (termed the F-body), and the basic staining properties of the chromatin of the inactivated X (female) chromosome (termed the Barr body) have already been the subject of study. Various tissues have been tested to determine some of the circumstances under which sex chromatin diagnosis may have forensic application. The literature list is much too extensive to be given in full; a few examples are epithelia and cartilage (Dixon and Torr 1956a: 161) brain, thyroid, heart muscle, skeletal muscle, smooth muscle,testis (Tishler and Javier 1973:587), bloodstains (Phillips and Gitsham 1974:47), teeth (Seno 1977: 172) and buccal mucosa and hair root (Nagamori et al 1986:119).

A second characteristic of somatic cells, considered here for its potential application to forensic gender studies,is the minor histocompatibility - Y molecule, (H-Y), anchored on the cell membrane and considered specific for males. Its molecular components are being characterized as to structure and function (Burgoyne et al. 1986: 170; Brunner et al. 1987b: 181; Farber et al. 1988: 204; Heslop et al. 1989: 99). However, its stability in necrotic tissue has not been investigated. In this study, a

control for the stability of cell membrane molecules under circumstances of tissue degradation, will consist of the detection of β_2-microglobulin, a molecular component of the major histocompatibility complex, expressed on all cell surfaces, and considered to be the anchorage site of the H-Y molecule (Ohno 1977: 59; Fellous et al. 1978: 58).

Both of the somatic cell traits described above are common to the tissues of children and adults; in this aspect they hold a particular advantage over skeletal measures.The accuracy of these traits for discriminating gender, at least in fresh tissue, is very high. The mis-diagnosed population consists of two groups: 1. those individuals who carry chromosomal abnormalities such as mosaicism (sex chromatin and H-Y ambiguity): 6 per 1,000 (Kelly 1986: 143), and 2. some normal males who exhibit no discernible F-bodies with fluorochrome stains,(less than 1%,Phillips and Gitsham 1974: 47; Brøgger and Urdal 1978: 421) as a result of normal variation in the size of the Y chromosome. Moreover, occasionally brightly fluorescent autosomes can be mistaken for F-bodies when the Y chromosome is very small, as can contaminating bacteria which also fluoresce. According to de la Chappelle (1983: 193) 10-25% of samples screened for F-body fluorescence in clinical practice are difficult to interpret and must be repeated or assessed by karyotyping.

Research Design

The research to be presented here, which uses tooth pulp tissue from extracted human teeth as the substratum for sexual

characterization, and pig pulps for comparison of tissue deterioration in unextracted pulps, was designed to expand current knowledge on the constraints pertaining to sex discrimination in pulp tissue deposited in simulated forensic environments. Both sex chromatin and H-Y cell components were tested for their stability in necrotic or putrefactive pulp tissue. Methods were adjusted expediently throughout the course of the project.

The first reason for selecting tooth pulp as the preferred tissue for this study is its sequestered location. Embedded in a chamber surrounded by intact highly mineralized dentine and enamel,the pulp is vulnerable to the external environment only at a small apical aperture. Tooth enamel is known to be virtually indestructible in the ground from the evidence of archaeological sites (Mann and Wood 1970: 123), and dentine, which is less mineralised, is also very tough, but does eventually become brittle over many millenia (Hillson 1986: 156). Sequestration of the pulp in these protective materials occasionally results in unusual pulpal preservation. For example, remnants of odontoblastic pulp cell processes in dentinal tubules have been reported in a histological study of Bronze age teeth (Falin 1961: 5), and naturally mummified pulp tissue adhering to dentine in corners of the pulp cavity has been reported in teeth from Belgium, *circa* seventh to ninth century A.D.(Werelds 1961: 559).

The second reason for the selection of tooth pulp is its availability. Human teeth, mostly third molars, were obtainable courtesy of maxillofacial surgeons within the city of Vancouver, and pig jaws and heads could be garnered from local meat packers.

Supplementing these two reasons for selecting dental pulp is the suitability of the majority of the cells of the pulp for gender characterization.

Deposition of isolated human teeth is not typical of the reality of the forensic situation. Teeth are usually embedded in the gums, and the tooth roots encompassing the pulps, are supported by alveolar bone. These structures are then further encased in external facial tissue. To best approximate this reality, comparisons were made between decomposing, dry, or heat exposed pulp from extracted human teeth, and those from pigs' teeth,both extracted and unextracted in heads or jaws.

A limitation on human-pig comparison is that pig pulps cannot be so readily used for sex determination. Although H-Y is so highly conserved in the genome that its molecular structure has been found to be all but identical in all mammals studied (Wachtel 1983: 57), the fluorescent properties of Y-chromatin are limited to humans and gorillas (Pearson and Bobrow 1971: 326). X-chromatin bodies, however, are discernible in pig neuronal cells and in amniotic membranes but other tissues have proved refractory because most suid nuclei, whether male or female, exhibit multiple chromatin masses similar in appearance to Barr bodies (Hay and Moore 1961: 289; Harvey 1971: 273).

Preliminary testing was carried out by making smears from fresh or "fresh frozen" pulp in order to establish appropriate techniques. Once established, these methods were than applied to pulp tissues deposited in simulated forensic environments. Samples of human teeth, pig teeth, and pig jaws were buried at 30 cm

depth in British Columbian coastal humic soils and in interior sandy soil (the coastal region has a high rainfall compared to the relatively arid interior). Similar samples were exposed on the ground surface. Teeth were deposited at varying seasons, and retrieved sequentially for periods varying from a few days to two months. Experiments were discontinued when pulps exhibited either an advanced state of decomposition or pulp chambers showed total tissue loss. Further samples of human teeth, pig teeth, and pig jaws were stored at room temperature (about 25C), and retrieved at intervals of three months over a period of one year. In this situation pulps dehydrated rapidly in extracted teeth: within about 24 hours. Human and pig teeth, and pig jaws were also subjected to temperatures in the range of 100-350C sustained for a period of one hour. In addition chromel-alumel thermocouple probes inserted into human and pig pulp chambers were used to assess the maximum temperature that could be reached but not sustained, under which sex chromatin remains stable in a fire.

Once pulps began to decompose or dehydrate, pulp cells could no longer be evaluated by simply preparing smears. Gender characterization and analysis of the process of decomposition in wet pulp tissue retrieved from buried or exposed teeth, were found to be facilitated by paraffin embedding and sectioning prior to staining (Tishler and Javier 1973: 587; Culling 1976: 924). In dehydrated pulp tissue,from room dried or heat treated samples, it was found necessary to separate cells from the supporting pulp matrix in order to produce a monolayer of cells for staining. This was accomplished by rehydrating specimens in Ruffer's mummy fluid

(Sandison 1955: 277), then digesting the matrix with enzymes. It was also found to be expedient to exchange a mouse monclonal antibody to H-Y with a rat polyclonal, in order to discriminate the sex of human pulp fibroblast membranes.

CHAPTER TWO GENERAL THEORETICAL BACKGROUND

The Dental Pulp

The main function of the dental pulp is to house and nurture the cells which build and maintain the dentine (Veis 1985: 552). Dental pulp is comprised of a loose connective tissue with a structure consisting of an arrangement of cells, fibres and ground substance. Pulp cells are predominantly fibroblasts, such as odontoblasts and fibroblastic interstitial cells; other cells include nerve cells, some histiocytes and mast cells, red and white blood cells, and the endothelial lining cells of the blood vessels. The fibrous matrix is composed of reticulin and collagen fibres, whereas the ground substance is predominantly made up of proteoglycans and glycoproteins together with large amounts of water (Mjör and Pindborg 1973: 55).

The sequestered location of the pulp, enclosed in dentine except at the narrow apical foramen, makes it somewhat unusual. It is regarded as a special type of connective tissue. Moreover, it appears to be a relatively immature type of connective tissue based on the large proportion of reticulin fibers and small proportion of collagen fibers, and its high frequency of undifferentiated mesenchymal cells (Moss-Salentijn and Hendricks-Klyvert 1985: 201). In most of its components, pulp tissue is arranged in a manner similar to any other loose connective tissue, except that proximal to the coronal predentine is a layer of specialised odontoblasts with cytoplasmic processes which extend into the dentinal tubules, the function of which is to maintain

the dentine. Inferior to this layer is an almost cell free zone. Deeper within the pulp is a cell rich zone of fibroblasts which surrounds the regular pulp tissue (Stanley and Weaver 1968:4). The pulp tissue is highly vascularized; blood vessels and nerves enter or exit the pulp at the apical foramen.

To reiterate, most of the cells of the pulp, with the exception of the cells of the haemopoietic system, can be used to detect sex chromatin (Mittwoch 1974: 74). All the cell types found in the pulp are reported to carry H-Y molecules, even the red blood cells (Shalev et al. 1978: 303). Furthermore, fibroblasts, the predominant cell in the pulp, are known to carry a remarkable abundance of H-Y molecules on their cell membranes (Nagamine et al. 1984: 13; Shelton and Goldberg 1984: 7). Teeth from any position in the mouth can be used for this type of investigation. Some do offer advantages over others. Molars have larger pulp cavities and thus more pulp tissue than do anterior teeth. The size of the cavity is influenced by the age of the teeth; throughout life the dental pulp is depositing layers of secondary dentin which gradually reduce cavity size (Wheeler 1965: 292).

The Process of Soft Tissue Decay

At the histological level two types of cell death, apoptosis and necrosis, have been described, and the underlying mechanisms of their mode of cellular disintegration analysed (Wyllie 1981: 9). Apoptosis is the type of cell death associated with normal cell turnover. In this process cell junctions dissociate, the

cytoplasm becomes condensed and the nucleus pyknotic: the nuclear chromatin marginates forming into large masses. Endogenous endonucleases are known to be the catalysts for the nuclear changes observed (Bär et al. 1988: 60). The dying cell eventually breaks down into several membrane-bound apoptotic bodies; is extruded from the tissue surface, and phagocytosed by macrophages.

Necrosis, the type of cell death of interest in this thesis, results from environmental perturbation (Wyllie 1981: 12). In contrast to apoptosis, necrotic cells remain adherent to each other, and the cytoplasm swells as a result of a loss of selective membrane permeability. The nucleus remains relatively unaffected except for the margination of multiple small chromatin aggregates. Cell rupture eventually results from release of lysosomal hydrolases. With time, in the course of decomposition, this form of autolytic cell death initiates the process of putrefactive decay.

Putrefaction signifies bacterially induced reduction and liquefaction of organs. In the early stages of decomposition, necrosis and putrefaction can sometimes be difficult to distinguish histologically, as they appear to overlap (Janssen 1984: 17). Burn (1934: 395), in a classic study of the organisms involved in putrefaction, found that certain bacteria are more commonly isolated at autopsy, these include spore forming anaerobes (particularly *Clostridium welchii*), coliforms, micrococci, diptheroids and proteus organisms. Since such organisms are known to be normally present as commensals in the respiratory and intestinal tract, Burn proposed that the

physicochemical changes produced in autolysis allow these organisms to penetrate the mucosa and rapidly disseminate throughout the body principally *via* the blood stream. Cellular decomposition then proceeds in a mosaic fashion: for example epithelial cells are usually destroyed by putrefaction before connective tissue components, and glia cells and leukocytes are considered to be more resistant to decay than any other cells in the body (Janssen 1984: 18).

As putrefaction progresses, a diachronic series of changes at the gross level are observed (Adelson 1974: 171; Gorden et al. 1988: 43). Within a few hours of death, the body exhibits a blue-green discolouration as a result of haemolysis in the blood vessels releasing haemoglobin into the surrounding tissue. This phenomenon is followed by evolution of gases which cause the body to bloat. Tissue softening and collapse later ensue leading to tissue liquefaction. Finally a dry stage is described in which only dry skin cartilage and bone remain (Payne 1965: 592; Roderiguez and Bass 1983: 423). Simpson (1974: 7) gives the usual diachronic order of decomposition at the gross level as:

1. Intestines, stomach, liver blood, heart blood and circulation, heart muscle.
2. Air passages, lungs, liver.
3. Brain and chord.
4. Kidneys and bladder.
5. Testis.
6. Voluntary muscles.
7. Uterus.

8. Prostate.

A second set of factors of external origin also contribute to decompositional changes in the body's morphology. These include external micro-organisms present on the body's surface or in the surrounding environment, such as bacteria and fungi, together with invasion by insects which appear on the remains in predictable waves of succession on a body left in the open (Payne 1965: 592; Nuorteva 1977:1072; Rodriguez and Bass 1983:423). This phenomenon is sometimes used in forensic investigations to estimate the time elapsed since death. Dipterous flies may start laying eggs on a body within minutes of death, and some even reach buried remains, provided that the burials are shallow (Fisher 1973: 21; Rodriguez and Bass 1985: 848). Rodriguez and Bass stated that carrion insect activity was observed on human cadavers in East Tennessee buried at a depth of 30 cm, although not in deeper burials. The insects were identified as the larvae, pupae and adults of *Calliphoridae* and *Sarcophidae*. Further destruction in the open or in shallow burials may ensue in the form of disarticulation, and gnawing and chewing of remains by scavenging animals (Morse 1983:124,148; Haglund et al. 1988: 985).

The rate of tissue decay is affected by a host of variables. Age and nutritional status affect decay rates: putrefaction is relatively slow in unfed newborns and is relatively rapid in obese individuals. Other prexisting health conditions may favour decay such as acute infections or septicemia. The cause of death can also affect the decomposition rate in other ways, for example a bullet wound in the abdomen,

rupturing the intestines, is more likely to initiate putrefaction than a bullet wound to the head. (Whiteford, cited in Krogman and Işçan 1986: 28; Gorden et al. 1988: 45).

The depositional environment of the cadaver is a pivotal factor in decomposition rates, variables include land or water, shelter, exposure, scavenger access, exposure time prior to burial, burial depth, and type of burial container if any, all of which may function to retard or accelerate the decay process. Water is believed to retard decay, but is subject to variables such as presence or absence of sewage organisms, water temperature, and the prevalence of destructive aquatic fauna. Deep burial and the presence of coffins also delay decomposition, as does soil type: moist clay, clay-loess, porous sand, dry wood humus, acidic marshes, and the presence of algae or saprophytes hasten decomposition, whereas lime-soil retards the process (Krogman and Işçan 1986: 29). Ambient temperature, seasonal temperature changes, altitudinal temperature variability, and humidity are also major contributors to a mosaic of decomposition rates. High temperatures, and moist or humid conditions are conducive to putrefaction, in that they favour bacterial and fungal proliferation and insect activity (Gordon et al. 1988: 45).

Exposure of tissue to ultraviolet (U.V.) radiation in the 290-400 nm wavelength range for any length of time has been found to have an adverse effect on DNA (Lai et al. 1987: 143; Rosenstein 1988: 313; Peak and Peak 1989: 1). Experimentally, over periods of 20-24 hours, damage to DNA in human skin fibroblasts exposed to these solar ultraviolet wavelengths has

been shown to take the form of single strand breaks and DNA-protein crosslinking, although in dried bloodstains exposed to UV light, DNA restriction fragment length polymorphisms have proved stable over at least five days (McNally et al. 1989: 1059).

Some exceptional environmental conditions markedly inhibit decay. For example under certain damp environmental conditions such as wet soil, swamp, or water, the decomposition process includes the formation of adipocere in locations where fatty tissue normally occurs anatomically. The constituents of adipocere are described by Mant and Furbank as "a mixture of fatty acids formed by the *post-mortem* hydrolysis of body fats together with mummified remains of muscles, fibrous and nervous tissues, and a little soap" (1957: 18); histological section reveals that no defined cell structures remain (Evans 1962: 155). Putrefactive organisms are known to be involved in this process of saponification. Enzymes which split fat to fatty acids are produced by some putrefactive bacteria. Experimental research has implicated the lecithinase produced by *Clostridium welchii*, but other organisms also produce appropriate enzymes (Gotouda et al. 1988: 249). Saponification, once it has occurred (onset may be as early as three and one half weeks after death (Adelson 1974: 175)), creates an acidic environment which inhibits further bacterial degradation, sometimes for many months or years (Fisher 1973: 23).

In exceptional subsurface environments,such as those found in anaerobic bogs, tannic acid may preserve soft tissue for millenia, as exemplified in the iron age bog bodies recovered in

Europe (Glob 1965). Moreover, cold temperatures are bacteriostatic, sometimes allowing spectacular preservation such as those seen in the recovery of bodies from the Franklin expedition (Beattie and Geiger 1988). Another form of modification of the putrefactive process is that of mummification. Mummification is characterized by dehydration which produces dessication of tissues and viscera after death. This situation has been noted when corpses are exposed in dry windy places,in desert burials,in graveyards where soil is porous, or when bodies are exposed to warm dry atmosphere shortly after death, for example when corpses are deposited in lofts and chimneys,or concealed in boxes or trunks (Zivanovic 1982: 251; Gordon et al. 1988: 54).

Because of the multifactorial influences which dictate decay rates it is not possible to deduce a general rule for the rate of putrefaction. However, Fisher states that a "rule of thumb" for decay rates in temperate climates is that "one week in the air equals two weeks in the water or eight weeks in the soil" (1973: 21).

Anticipated Effects of Outdoor Decay Rate Variables on the Outcome of this Study

The early part of the decay process, namely necrosis and the preliminary stages of putrefaction prior to substantial nuclear damage (affecting sex chromatin) or plasma membrane destruction (affecting H-Y analysis), are germane to the present study. Hypothetically, the decay process is anticipated to accelerate in buried teeth in coastal British Columbia because

the climate is characterized by mild temperatures, and high rainfall which result in acid soils. The interior of the province, by contrast, is characterized by hot, arid summers and relatively cold winters, conditions conducive to better preservation of human remains. The decay process is expected to be prolonged by dehydration in extracted teeth held at room temperature, and in teeth heated to temperatures below the threshold of carbonization. No damaging effect from sunlight UV radiation is expected in sequestered pulpal DNA.

Effects of Elevated Temperature on Soft Tissue Destruction

In forensic circumstances involving conflagration such as building fires, explosions, motor vehicle accidents or plane disasters, many variables, often specific to the individual event, affect rate of exposure to heat and the incineration temperature reached (Wilson and Massey 1987: 32). Fires on open ground, such as camp fires are reported to exhibit temperature ranges between 400-800C (Tylecote 1962: 25; Brain and Sillen 1988: 464;). Juniper and oak fires gave temperatures of 680-820C (Buikstra and Swegle (n.d.). In fires confined inside buildings, at approximately 600C flashover often occurs, which is defined as a rapid transition takes place from local burning to conflagration of all combustible elements in the enclosure (Babrauskas 1986: 21).

Experimental vehicle fires have been shown to reach temperatures of 800-900C, but often not until more than one hour had elapsed from the initial time of ignition (Hyrnchuk 1978: 15).

Aircraft accidents are considered to cause the greatest damage to the human body of any commonly occurring disasters as a result of high impact forces, frequently very high incineration temperatures (some greater than 1000C), and fires of long duration (Sopher 1976: 47). Explosions, whether intentional or accidental, may also cause severe burns when confined in buildings (Rajs et al. 1987: 1), or traumatic damage to the body when they occur out of doors. In Sweden, the bodies of six out of twenty five individuals who committed suicide using solid explosives, were "totally disintegrated and parts could be found up to 100 m from the site of the explosion" (Rajs et al. 1987: 1).

The maximum temperature reached by a fire is not necessarily the relevant variable in studies dealing with the affects of heat on tissue. The most important variable is generally the temperature recorded for the tissue of interest (Shipman et al. 1984: 307), for example, in the studies reported in this thesis, the dental pulp. Meat insulates bones, and the surrounding soft and hard tissues insulate the dental pulp. In Buikstra and Swegle's study (unpublished) the authors showed that it took two hours for fleshed or skeletonized remains to reach the maximum temperature of the heating device inserted in the fire. For example within a fire of oak logs in which the temperature of the fire registered 900C, the flesh of a dog carcass burned at 580-650C, while the bones charred at 400-500C.

In certain forensic contexts of incineration, pathologists have great difficulty in determining the sex of the individual as a result of shrinkage, and charring or fracture damage to the

bones (Stewart 1979: 65; Pert 1980: 187;Barsley et al. 1985: 128; Clark 1986: 317). Resort is then made to less common approaches to sexing, such as anthropometric measures or soft tissue analysis (Wolcott and Hanson 1980: 1034; Torre and Varetto 1984: 339) including sex chromatin evaluation (Thomsen 1977: 235). Criteria for soft tissue selection are those areas afforded the best protection from carbonization. In Thomsen's study of Y-chromatin staining on severely burned corpses, of the five tissues tested (thyroid, liver, striated muscle, cerebral cortex and kidney), the author obtained the optimal results from the kidney tissue because this area of the body was well protected from scorching heat. He also established by experiment that Y-chromatin staining capacity was lost when kidney tissue was heated beyond 150C.

Dental tissue is reported to be fairly well protected in victims of death by fire. According to Botha "If death has occurred before exposure to fire teeth may remain almost intact due to the protective action of lips and tongue even though limbs are destroyed" (1985: 39). However, when death ensues as a result of fire, initially the lips swell, closing the mouth, subsequently the lips retract but the tongue often protrudes and partly protects the anterior teeth, posteriorly molars are protected by cheek tissue (Gustafson 1966: 178; Sopher 1976: 61).

Data on the effects of heat on sex chromatin in dental pulp are largely lacking. In extracted teeth, pulp is reported to carbonize at 215C (Basauri 1961: 45). Seno (1977: 172) and Ionesiy (1980: 27) both report that sex chromatin counts can be

made from extracted teeth heated for one hour at 100C but not at higher temperatures. Experiments indicate that loss of tooth weight is greatest between 100 and 300C, under fractionation cremation by which temperature is raised gradually to 1000C. This phenomenon is probably the result of moisture loss (Mannerberg cited in Gustafson 1966: 185). Most experimental work to date has been directed towards determining the effects of heat on hard dental tissue, and almost without exception has been conducted on extracted teeth.

Pig Tissue As A Measure for Comparison of Variables Affecting Human Tissue Destruction

It is unfortunate that most research to date, on the deterioration of dental tissue in simulated forensic situations, has been conducted on extracted teeth. The important factors of putrefaction and carbonization rates which are known to be affected by the tissue's location in the body have not been fully studied for dental tissue. In addition, decomposition of tissues varies not only with cell type but also in relation to organs, as noted in the aforementioned text. It would appear probable that in regard to forensic depositional environments, the hard and soft tissue around the teeth may aid in retaining moisture in the pulp cavity. In the case of simulated fire-studies involving extracted teeth, Gustafson (1966) correctly notes that "results cannot be compared directly with what happens to a body in a real fire where the teeth are protected by soft tissues". Surrounding tissues may contribute to temperature

variation in relation to heat penetration of the pulp tissue as compared to a situation involving extracted teeth. For this reason it was decided to compare 1. pulp decomposition in human and pig extracted teeth, and 2. unextracted pigs teeth in either in jaws or whole heads. Elevated temperature studies were conducted involving either heated conditions at varying temperatures in an oven, or the insertion of chromel-alumel thermocouple probes into extracted human and pigs' teeth, and unextracted pigs' teeth under conditions of combustion.

Although not closely related phylogenetically, with the exception of primates, the pig, (*Sus scrofa*), is more similar to humans in anatomy, morphology and physiology than any other mammals (Weaver et al.1962: 17; Pond and Houpt 1978: 31). As a result the pig has been used as a model for human infectious and metabolic diseases, for physiological and nutritional studies, and in dental research.

The pig, in common with humans, is an omnivore. The growth pattern of its teeth closely parallels that of humans as is its dental physiology, including mastication, jaw movements, adaptability to environmental change, and resistance to infection. *Sus scrofa* possesses both deciduous (I3/3, C1/1, M3/3) and permanent (I3/3, C1/1, PM4/4, M3/3) dentition. The primary dentition is present at birth or erupts within the first few weeks. The first permanent tooth (a molar) erupts at 5 months, and the permanent dentition is complete between 18-20 months. The transition period of mixed dentition spans about one year.

The pig model for studies involving human dentition does

have its limitations. In particular, the adult jaw is larger than in humans, and the surrounding soft tissue is thicker, the teeth are larger but much more brittle, perhaps because the enamel is thinner, or because the enamel structure confers less strength,(the arrangement of enamel rods is irregular in pigs, and parallel in humans (Furuhata and Yamamoto 1967: 55)). More important to the study discussed in this thesis, it should be noted that pig tissue could not be included for sex determination experiments for a number of reasons. First, the sex of the pigs was unknown in almost all the specimens studied. This was primarily due to the fact that pig remains were acquired from meat processing plants,and by the time of purchase, slaughter and butchering of the carcass had already taken place. Although adult pigs can be sexually differentiated on the basis of canine size (the permanent canine of the male is open rooted and its growth is permanent; females have small canines with closed roots (Schmid 1972: 80)), most pigs at slaughter represent juveniles from five to six months of age, and the canine morphology is not sexually diagnostic. Juvenile pigs, however, do better approximate humans in jaw size, tongue and jaw tissue thickness, and root size.

A second factor precluding the use of pig dental tissue for sex determination studies is due to the fact that cytological sex diagnosis is more difficult. With reference to sex chromatin,(as previously noted, p.8), the pig lacks a fluorescent male F-body. It does, however, have detectable X-chromatin,but this Barr body in pigs has been found to be clearly recognizable only in nerve

and amnion tissue (Harvey 1971: 273). In other tissues female chromatin is obscured by large dark staining granules that cannot readily be distinguished from Barr bodies (Hay and Moore 1961: 289; McFeely 1966: 18). Some polymorphonuclear leukocytes in the peripheral blood of the sow do, however, exhibit a nuclear "drumstick", previously described as a female sex characteristic in humans (Sergovich 1976: 1378; Pond and Houpt 1978: 256). Constraints also apply to H-Y testing in the pig, for although H-Y molecules are virtually identical in all male mammals, including pigs (Fellous et al. 1978: 58), interspecies variability is greater in β_2-microglobulin, about 61-68% homology has been shown by amino acid sequencing between mammals tested: mouse, guinea pig, rabbit, human (Orr 1982: 7). Moreover anti-pig β_2-microglobulin is not commercially available. Interestingly in spite of the large amount of molecular evolution that has occurred between phylogenetically distant species, murine B_2-microglobulin can effect the expression of HLA Class I molecules on the surface of human/mouse hybrid cells. In other words the interspecies evolutionary divergence has affected molecular structure without affecting function (Jones et al. 1976: 483; Chamberlain et al. 1988: 1285).

CHAPTER THREE: SECTION A.

SEX CHROMATIN IN HUMANS

Sexual differentiation in humans is genetically determined by he number of chromosomes, and by the presence or absence of the Y-chromosome. The karyotype of the normal female is 46,XX and the normal male is 46,XY. Chromatin is the complex composed of DNA and protein that was originally recognised in the nucleus of an interphase cell by its reaction with stains specific for DNA. The term sex chromatin refers to certain differential morphologic features of the chromatin found only on the sex chromosomes. Female chromatin was first described by Barr and Bertram (1949: 676) in the nerve cells of cats. In reference to this seminal work, X-chromatin is often called by the synonym "Barr body". When viewed under a light microscope, it is usually seen against the periphery of the nuclear membrane (occasionally in other situations) as a darkly staining structure, planoconvex in shape and measuring about 1.2 by 0.7 um (Yunis and Chandler 1979: 829).

In 1962 Mary Lyon (1962: 135) developed a hypothesis based on her research on the genetics of mice. She reasoned that given two X chromosomes in the female, any X structural gene products of the female should be double the products of the male. Lyon found that this was not the case in female mice, the products in both sexes were the same. She concluded that early in embryogenesis (now known to be at the time of embryonic implantation in the uterus (Yunis and Chandler 1979: 830) in the XX female), one of the two

X's in each cell is inactivated. This phenomenon is considered to be randomly distributed, that is the same X is inactivated in all the progeny of that cell. Evidence supporting this proposition comes from studying inherited conditions known to be X-linked. For example female carriers of the gene for glucose-6-phosphate dehydrogenase (G-6-PD) have been shown to support two populations of red blood cells: one line showing normal activity and the other being deficient for the enzyme (Beutler et al. 1962 :9; Davidson et al. 1963: 481). The Barr body is the inactivated X chromosome in a condensed state,and this condensation is only observed during prophase or interphase when the cells are not dividing (Ohno et al.1959: 415). All X chromosomes in excess of one are inactivated in both the abnormal male and the normal and aneuploidic female, therefore 46,XX females have one Barr body in their cell nuclei, 47,XXY males also have one Barr body, but 47, XXX females have two (Kelly 1986: 128).

Barr bodies are amenable to any nuclear stain. These include haematoxylin-eosin, Papincolaou, Feulgen, cresyl echt violet, aceto-orcein,and carbol-fuchsin (Mittwoch 1974: 73). Barr bodies also fluoresce,for example with acridine orange, but when stained with quinacrine dihydrochloride and quinacrine mustard at a low pH (Korf et al.1975:145) fluorescence of X chromatin is much less intense than for Y-chromatin. In females with normal karyotypes Barr bodies have been noted in about 30% of cell nuclei (range 15-40%) (Yunis and Chandler 1979: 830). In fact Barr bodies are probably more frequent than the findings suggest, but because they adhere to the nuclear membrane, in slide preparations, they tend

to fall behind or in front of the nucleoplasm and cannot be identified with certainty. In fresh tissue preparations of normal males no equivalent chromatin masses are reported, except in cases of genetic mosaicism.

The fluorescent staining property of Y-chromatin was first reported in 1970 in the nuclei of buccal mucosal cells, lymphocytes, and fibroblasts by Pearson et al.(1970: 78). It was found that the distal portion of the long arm of the Y-chromosome fluoresces so intensely that it is easily recognized in both interphase and mitotic nuclei as a distinctive fluorescent structure or F-body. The F-body is typically described as a single fluorescent spot, approximately 0.25 um in diameter and of variable length, located halfway between the periphery and the centre of the nucleus. The length of the long arm is closely correlated with the size of the fluorescent segment at interphase and metaphase. Range of variation in the F-body size is between double the normal size and completely obscured (Yunis and Chandler 1979: 829). A famous familial pattern characterized as a of lack of F-body staining, known as the "Beiler Y", was traced among many males of an Old Order Amish family named Beiler (Kelly 1986: 125). Lack of F-body in humans has no currently known functional consequence in that the genetic material of Y-chromatin constitutes a non-coding region of DNA.

The Y chromosome is considered to encompass four broad regions. On the distal short arm a pairing region has been detected, containing a subregion of sequence identity where recombination with the X is possible, followed by a pericentric

region containing the sex determining genes. The long arm has a euchromatic region believed to contain factors affecting spermatogenesis and a "functionless" distal portion, the fluorescent Y-body (Tiepolo and Zuffardi 1976: 119; Goodfellow et al. 1985: 329). When eukaryotic DNA is centrifuged on a density gradient of cesium chloride, most of the genome appears in one broad peak in the form of a continuum of fragments corresponding to the average guanine-cytosine content of the genome. Sometimes an additional smaller peak (or peaks) is seen at a different value. This material is called satellite DNA. Radioactive labelling of satellites reveal that they are most commonly located in the heterochromatic regions of the chromosome that are permanently tightly coiled, inert, and in the form of very short sequences, repeated many times in tandem (Lewin 1985: 382; Willard et al. 1986: 5611). Y-chromatin, termed human satellite I, is made up of many repeating sequences (Jones 1977: 295). Recent experiments using reassociation techniques and restriction enzymes analysis have revealed that a major component is a 3.56 kb tandem repeat sequence (DYZ1), as characterized by the HaeIII restriction enzyme. Variation in copy number of the DYZ1 sequence has been correlated with variation in size of the quinacrine fluorescent portion of the Y chromosome long arm (McKay et al. 1978: 19; Cooke et al. 1982: 491; Goodfellow et al. 1985: 329; Gill 1987: 35).

The cytochemistry of fluorochrome staining of chromosomes is not well understood. Caspersson et al. (1968: 219) postulated that quinacrine acting as a fluorescent alkylating agent might attack

N-7 atoms of guanine and therefore preferentially accumulate, at least temporarily, in the guanine rich segments of DNA. Barr and Ellison (1971: 190) report that quinacrine staining of chromosomes correlates with higher proportions of adenine-thymine base pairs. The latter interaction has also been claimed for the fluorochrome distamycin/DAPI (Schweizer 1981: 1). However, whereas quinacrine staining of Y-chromatin is limited to humans and gorillas, with distamycin/DAPI staining, all species of apes show brilliant Y-chromatin fluorescence (Weinberg and Stanyon 1987: 445). This finding suggests that the two fluorochromes may have different affinities. Staining with quinacrine mustard or quinacrine dihydrochloride is accomplished at an acidic pH of about 5.5 (Korf et al. 1975: 145). The range of fluorescent cells reported in males is between 25 and 92% (Pearson et al. 1970: 78; Seno 1977: 172). In females anomolous fluorescence can be as high as 10% (Tishler and Javier 1973: 587).

Forensic Studies on Dental Pulp Sex Chromatin

Some investigation of the stability of sex chromatin in tooth pulp has already been accomplished. Starting in 1956, Dixon and Torr used routine histological techniques to identify X-chromatin. They examined the cells of the dental pulp in extracted teeth, anticipating that "being virtually sealed from their surroundings, nuclear detail might be preserved for an extended period" (1956b: 797). They then went on to report that sex chromatin persists for no longer than 24 hours in isolated teeth (no environmental context given), and for no longer

than one week in teeth immersed in physiological saline,(considered by these authors to represent the environmental conditions of teeth *in situ*)).

Seno and Ishizu (1973: 8) discuss Y-chromatin fluorescence in isolated permanent teeth "left standing" (presumably on the laboratory bench), up to five months. After removal of the tissue from the pulp chamber, cells were separated from the surrounding tissue matrix by soaking the tissue in dilute acetic acid and abrading it with a pestle and mortar. The cells were then stained with quinacrine dihyrochloride. These researchers reported successful gender determination in teeth extracted from any position in the tooth row. Males exhibited chromatin fluorescence in 30-72 % of cells, and female overlap was between 0 and 4%. Post extraction time-lapse did not seem to have any appreciable effect on percent cell fluorescence.

In 1975 Whittaker et al. (1975: 403) followed up Seno and Ishizu's work, using the same methodology but adding magnesium ions to their preparations to enhance the fluorescence. Their study was double blind, and included pulps from deciduous and permanent teeth. Pulps were allowed to putrefy in a "humid atmosphere" (no further environmental information provided) for up to 10 weeks. Pulps were retrieved at intervals and Y-chromatin data compared to data from pulps removed from freshly extracted teeth. In some material these investigators report a maximum female overlap of 16%, but not more than 6% in tissues allowed to putrefy for less than seven weeks. Fluorescent pulp cells in males ranged between 17 and 51%, and appeared to be independent of

of the time allowed for of putrefaction. The authors consider that the discrepancy between their results and those of Seno and Ishizu - higher cell counts and greater female overlap - may be accounted for by a larger sample size, inclusion of infected carious teeth (i.e.contaminated with non-specific fluorescing bacteria),and lack of bias in a double blind study. However, not only did they experience some difficulty with fluorescent debris in using the cell separation method developed by the previous authors, but their teeth were exposed in a "humid atmosphere" to putrefy as opposed to "left standing", possibly to mummify in a dry environment.

Seno (1977: 172) later extended the original study on Y-chromatin fluorescence to a larger sample of teeth, including deciduous teeth, which were then exposed to more varied environmental conditions. Teeth were left standing at room temperature, stored in mud, immersed in running water, and exposed to heat. At room temperature, after one month male fluorescence ranged between 52 and 92%, with female overlap 0-3%, after three months Y-chromatin was observed in 47-64% of male teeth(female overlap 1-7%),a further decrease in frequency was reported at six months (males 15-65%,females 0-7%),and so on; however, gender determination was possible up to three years. Teeth were also buried at a depth of 25 cm. in a can filled with mud and sand in the outdoor environment. No specific data was provided with regards to environmental factors. In four out of seven male teeth, and four out of seven female teeth, gender could still be determined after one month (male fluorescent cell range 11-39%).

After three months sex diagnosis was greatly reduced to one of seven male teeth and two of ten female teeth presumably due to cellular decomposition. Teeth immersed in running water demonstrated complete cell structure deteriorated within three days. Male teeth heated to 100C for one hour,exhibited a range of sex chromatin fluorescence between 43 and 68%, and female overlap was 1-7%. In contrast at 200C for one hour no fluorescence was observed in any pulp tissue.

Dange et al. (1978: 115) "stored" (no further explanation given) a sample of permanent teeth including representatives from various positions in the tooth row. They then stained unfixed paraffin embedded pulp sections for male and female chromatin using quinacrine dihydrochloride and aceto-orcein respectively. Discrimination of gender was reported up to four years post extraction but no differential cell counts were provided.

Most recently, Ionesiy (1980: 27) repeated the methods of Seno and Ishizu, with the addition of X-chromatin staining for comparison. In fresh teeth, this author reports fluorescence in a range of 75-89% in males with an overlap of 0.5%, and X-chromatin staining in 30-52% of female cells with an overlap of 6%. Teeth were stored at room temperature for one year without any marked decrease in sex chromatin staining capacity. In teeth "buried in soil" (no further details given) up to 20 days, the percentage of cells retaining staining capacity decreased to 18-23% in males and 10-21% in females. Ionesiy's results on teeth immersed in running water accord with Seno's: cell nuclei are destroyed in a few days. Teeth heated to 100 and 200C also confirmed Seno's findings

(gender identifiable at 100C but not at 200C). A further tooth sample was refrigerated at -4 to -8C and pulps retrieved at 15, 30 and 40 days. Gender determination was found possible up to 30 days.

CHAPTER THREE: SECTION B.

THE H-Y MOLECULE

H-Y Antigen and β_2-Microglobulin Discrimination in Dental Pulp Tissue

The existence of the histocompatibility-Y (H-Y) molecule was first indicated by skin transplantation studies in inbred strains of mice. Eichwald and Silmser (1955: 148) discovered that in some strains of mice, skin grafts transplanted from males to females were invariably rejected. This male specific factor was named H-Y antigen (Billingham and Silvers 1960: 14). Skin grafting also induces female mice to produce cytotoxic T-lymphocytes which kill male target cells *in vitro* (Gorden et al. 1975: 1108). The T-lymphocytes, however, must share certain major histocompatibility complex (MHC) antigens with the target cells for killing to be effected; that is the reaction is described as MHC restricted. The serology of H-Y antigen began in 1971 when Goldberg et al. (1971: 478), found that female mice which rejected multiple male skin grafts developed cytotoxic antibodies to mouse sperm. It is still a matter of debate as to whether the H-Y antigen that induces graft rejection and elicits specific killer T-cell proliferation, is the same antigen as the H-Y which provokes the B-cells to produce the H-Y specific antibodies detected by serology (Goodfellow et al. 1985: 329; Ropers 1987: 133; Heslop et al. 1989: 99).

The H-Y molecule, as defined by antisera rather than

transplantation, is present at the early eight-cell stage in mouse embryonic development (Shelton and Goldberg 1984: 7) and is highly conserved in evolution. Wachtel et al. (1975: 270). concluded the cell surface component conferring H-Y antigenicity in the mouse "has been conserved through some 300 million years of evolution spanning the Carboniferous radiation of amphibians and the Pleistocene emergence of man" Their evidence is the ubiquitous cross reactivity of the mouse anti-H-Y antibody to cell surfaces of widely divergent species. The sperm cytotoxicity test proved cross-reactive in all mammals tested, for example mouse, rat, hamster, marmoset, baboon, dog, cat, horse, cow,and donkey. Moreover cross-reactivity was also observed in many non-mammalian vertebrates: those tested include the chicken, quail, turtles, frogs, toads, and fish. Even invertebrates such as lobsters, cockroaches, and beetles show the same cross reactivity (Wachtel 1983: 55).

Among the non-mammalian vertebrates,in some birds, reptiles, fish and amphibians, the female is the heterogametic sex. Male sex chromosomes are designated ZZ and females ZW. H-Y serological cross reactivity is then directed to cell surface molecules in the female. In these animals, when males are treated with an oestrogen compound (estradiol benzoate) during early ontogenetic development they become positive for H-Y antigen (Müller et al. 1979a: 142; Müller et al. 1980: 129; Wachtel et al. 1980: 859). Administration of testosterone to the female results in masculinization (lack of H-Y) (Reinboth et al. 1987: 13). This points to the fact that animals bearing a heterogametic ovary

share gene(s) for H-Y in both sexes, suggesting an autosomal location of the gene. The same is probably true of the mammals who bear a heterogametic testis. In fact chromosome mapping by Lau et al. (1986: A142), has detected a structural gene for H-Y serological specificity on the short arm of chromosome 6 in humans, in close proximity to the region on the same chromosome where the MHC gene complex is known to be located.

A second gene (or genes), believed to be testis determining, has recently been localised to a 140-kb region on the short arm of the Y chromosome (Page et al. 1987: 1091). It does not seem to code for a cell-surface protein, but shows homology with genes known to to code for zinc-binding "finger" proteins (i.e.paired cysteines and paired histidines are thought to be pulled into a tetrahedral coordination complex with a zinc cation). These proteins act as transcription regulators (similar tandemly repeating multiple "finger" proteins were first described in frog transcription factor 111A). Genes controlling spermatogenesis have been assigned to the proximal long arm of the Y through analysis of patients with structurally aberrant Y chromosomes (Tiepolo and Zuffardi 1976: 119). Although further elucidation of the genetics of sex differentiation is still required, it has been suggested given that the structural gene for H-Y is autosomal, that this locus is up-regulated by a Y-linked gene and down regulated by an X-linked gene (Wolf 1985: 81).

The serological H-Y molecule in mice has been analysed by treatments which alter protein and carbohydrate structure. From these studies it has been determined that it is a glycoprotein

(Shapiro and Erickson 1981: 503; Shapiro and Goldberg 1984: 209). Isolation of antibody-binding molecules, however, has produced confusing results. Western blot assay was used by Farber et al. (1988: 204) to analyse the pattern of H-Y antigens recognised by antisera. Lysates from human mononuclear cells were subjected to electrophoretic separation, then reacted with mouse anti-H-Y antibodies. The mouse sera revealed three bands (relative molecular mass (Mr): 15-20 kD) paradoxically detected in both males and females and a single male specific band (Mr:32-34 kD). The explanations proposed for their findings is that the cross reactive bands might correspond to structures present in both mice and humans or they may represent an incomplete form of the 32-34 kD molecule.

It is known that they are not Fc (antibody) receptor fragments because Fc sites are blocked on nitrocellulose strips incubated with normal mouse serum. The authors go on to point out the discrepancy in the Mr of the male specific antigen specified, and the 19 kD antigen produced by Daudi cells (a male Burkitt lymphoma cell line), also claimed by many authors to be the male specific H-Y. For example Heslop et al. (1989: 99) find a good correspondence between immunoaffinity-purified male-specific proteins from sheep testis (Mr: 19 kD), male specific molecules isolated from mouse liver by isoelectric focusing, and male specific antigens isolated from Daudi cells.

Extraordinary evolutionary conservation infers a critical function in sex determination for the H-Y antigen. Although this function is not completely understood, the serologically detected

antigen is currently conceived as a testis organiser involved in the formation of seminiferous tubules by mediating cell to cell recognition in the indifferent gonad (Ohno 1977: 59; Ohno 1978: 217). In an elegant experiment Ohno demonstrated his postulated role for the serological antigen. His *in vitro* experiment was based on the phenomenon of "capping". Ohno observed that in the gonadal cells of new-born mice, plasma membrane antigens gather over one pole of the cell (the cap), in the presence of an excess of anti-H-Y antibodies. Subsequently capped antigens are autophagocytosed by the cell. Uncapped gonadal cells organised themselves into seminiferous tubule-like structures, but the same gonadal cells stripped of the H-Y by capping and endocytosis, organised into follicular-like structures. In addition Müller and Urban (1981: 104) found that when dispersed cells of the neonatal rat ovary are exposed to soluble H-Y, they form testicular tubules in rotatory cultures, a phenomenon that is blocked by monoclonal antibodies to H-Y.

Ohno (1977: 59) envisages cell to cell association, in which MHC molecules, composed of two heavy chains and two β_2-microglobulin light chains, act as anchorage sites for regulatory plasma membrane proteins, (including H-Y), thereby directing organogenesis and cell differentiation. Further evidence in favour of this hypothesis is that Sertoli cells, which by interconnecting in embryonic development establish the walls of seminiferous tubules, secrete H-Y serological antigens, and along with Leydig cells (testosterone secretors), have receptors for binding the H-Y serological antigen. Furthermore, in contrast to the

situation in non-gonadal organs, gonadal cell binding of exogenous H-Y is independent of β_2-microglobulin (Müller et al. 1979b: 331).These special gonadal receptors lend support to Ohno's claim for serological H-Y as a testis organizer. Burgoyne et al. (1986:170) consider that the transplantation and cytotoxic T-cell antigen has a different function from the serological antigen, having a probable role in spermatogenesis. Mice have been described which develop testes but lack the cell surface molecule defined by the T-cell mediated transplantation tests. Burgoyne's group showed that mice lacking this transplantation antigen fail to produce sperm.

It is unlikely, however, that the functions of H-Y are limited only to testis development or spermatogenesis. As Heslop et al.(1989: 99) point out, H-Y is ubiquitously distributed on all somatic cells, not just those of the gonad, and it can be detected in the embryo prior to any gonadal differentiation. These researchers postulate that sex determination in mammals is a function of embryonic growth rate in general, and gonadal growth rate in particular. H-Y may serve as a major regulator of embryonic growth: the male embryo triggering maternal cells to produce a factor that stimulates a more rapid growth of male than female embryonic cells. The disparity between male and female growth rates is not in question, only its function, which Mittwoch (1986: 103) suggests may be that of a trigger for the development of Sertoli cells. Indirect evidence cited supports this contention, as does Heslop et al.'s *ad hoc* experimental evidence: *in vitro* substantial growth enhancement was noted in

male fibroblasts cultured with immune female anti-male serum. This result indicates that females, when immunized with H-Y, produce a growth-promoting factor along with an immune response.

Interestingly,it has been suggested that differences in foetal metabolic rate affect sex differentiation in species without morphologically identifiable sex chromosomes. In these animals (crocodiles, alligators, and some turtles), differentiation of the indifferent gonad is influenced by environmental factors implying that a similar physiological mechanism may underlie both (Mittwoch 1986: 103).

Research Rationale for H-Y Serology

In regard to the applicability of the serological H-Y to forensic investigations, not only has no study been applied to the detection of the molecule on pulp cell membranes, but no study has been applied to determine the preservation properties of this molecule in unfixed necrotic tissue. In theory, however, this approach to sex discrimination has the potential for success: fibroblastic cells are reported to be H-Y antigen enriched, and all the cells of the pulp carry H-Y molecules (Nagamine et al. 1984: 16). Moreover, whereas sex chromatin detection requires a structurally intact nucleus, H-Y detection does not. Membrane remnants should be the only necessary requirement. In addition to the exceptional sequestration of the odontoblastic processes which extend into the dentinal tubules, it is known that Class I MHC, and lymphocyte marker molecules on plasma membranes are remarkably stable in some environmental contexts.

HLA molecules have been detected on intact cells up to 36 hours after death in cool-kept cadavers (Henke et al. 1982: 267),and on extracts from dried and freshly thawed lymphocytes (Bishara and Brautbar 1989:99); lymphocyte marker antigens have been located on intact cells up to 72 hours *post-mortem* (Palleson and Knudsen 1985: 791). Moreover,in mummified tissue HLA antigens have been claimed to be detected in pre-Columbian specimens between 500 and 2,000 years old (Stastny 1974: 864), and in Greenland Inuit *circa* 500 years old (Hansen and Gürtler 1983: 447).

One obvious disadvantage of an approach to sex determination involving the H-Y molecule is that, unlike sex chromatin which has male and female diagnostic equivalents that can serve as controls for each other in diagnostic tests, H-Y serology offers no such heterogenous control. The fact that the H-Y molecule is not polymorphic, at least at any readily detectable level either within or between species, is inextricably tied to its function in sex determination. It is this property, however, not its sex specificity alone, which makes it a potentially useful tool for sex investigation. A sex-specific female equivalent of H-Y, histocompatibility molecule H-X, carried on the X chromosome, but is known to be highly polymorphic (Bailey 1963: 70; Bailey 1964: 203; Berryman et al. 1979: 363), thereby eliminating its value as a tool for forensic investigation. In order to help mitigate this situation, it was decided to test for the β_2-microglobulin protein along with the H-Y. The rationale for this decision was that when necrotic tissue proved to be H-Y

negative and β_2-microglobulin positive, it might be possible to infer a female on the grounds that the plasma membrane molecules had not yet succumbed to putrefactive decay.

A second disadvantage to the H-Y approach to forensic studies is that the literature contains many accounts of the technical difficulties encountered in H-Y serological testing (see Zenzes et al. 1984: 103 for a review). Anti-H-Y antisera commonly exhibit low reactivity (titer),perhaps as a result of the H-Y antigen carbohydrate moiety: it is well known that it can be very difficult to raise antibodies to some sugar structures (Klein 1982: 349). Or perhaps because the quantity of antigen on the cell surface is small. In rat liver cells, for example,the ratio of H-Y molecules to MHC Class I molecules is in the order of 1: 100 (Heslop et al. 1989: 99).The quantity of H-Y, however,is variable, and is probably enhanced in direct relation to the cell's proliferative activity. To help overcome this problem, researchers have resorted to highly sensitive serological techniques such as radioimmunoassay, peroxidase-antiperoxidase (PAP) and enzyme-linked immunoadsorbent assay (ELISA) (Müller and Bross 1979: 143; Meck and Goldberg 1984: 293; Moreira-Filho and Wachtel 1985: 525). However, in 1985 Bradley and Heslop (1985: 634) claimed this situation to be ameliorated. They obtained a transient anti-H-Y response (1-2 weeks) of very high titer in inbred rats by intrasplenic implantation of female rats with syngeneic male skin.

Anti-H-Y antisera also suffer from low specificity: they are commonly contaminated with heteroantibodies (natural antibodies to cell surface antigens from individuals of other species),and with

autoantibodies produced by hyperimmunization. Attempts to overcome the low specificity by adsorption of sera on female cells, lowers titers even further and sometimes results in loss of all reactivity (Crichton and Steel 1980:597; Brunner et al 1987b: 181-191). The development of monoclonal antibodies to H-Y, initially hailed as an answer to the problem of specificity (Koo 1981: 18), has not proved as predicted. Findings have been ambiguous, with some authors achieving much better results than others (Simpson 1982: 404; Brunner and Wachtel 1988: 49).

CHAPTER FOUR: SECTION A

MATERIALS AND METHODS, SEX CHROMATIN

Preliminary Experiments on Human Pulp

Human teeth were obtained from maxillofacial surgeons in the vicinity of Vancouver, British Columbia. Teeth were collected weekly, when available. Storage prior to collection was at -20C or at room temperature, depending on the facilities available. Experience in the laboratory showed deterioration of cell structure occurred in whole teeth stored at -20C for longer than six weeks, and this observation is consonant with Ionesiy's report noted previously (1980: 27). An attempt to prolong pulpal preservation by storing whole teeth at -70C proved unsuccessful. Deterioration of cell structure occurred in less than two weeks, perhaps because of the rapid formation of ice crystals in the absence of any low temperature storage medium such as glycerol or DMSO (dimethylsulfoxide). Pulps could have been extracted and stored at low temperature in liquid nitrogen, but this option was rejected because it would have introduced inconsistency in sample treatment.

Frozen teeth were stored in plastic bags labelled, and colour coded for male and female, (blue and pink); unfrozen teeth were placed in similarly coded brown bags, stored in a plastic container, dated, and set aside for later retrieval. These labelled bags were provided to dental surgeons to facilitate separation of teeth by sex, and to promote hygienic handling of specimens. As a

further precaution against infection, plastic gloves were worn at all times when it was necessary to handle any teeth.

Frozen teeth were thawed at room temperature for about half an hour prior to pulp removal. Pulp extraction was accomplished by placing individual teeth in plastic bags to prevent fragment scattering, then placing the bag on a wooden block in which shallow depressions had been whittled to accommodate tooth shape; the tooth was fractured by a sharp blow with a steel chisel and wooden mallet to expose the pulp chamber. Since pulps, whether wet or dry, adhere to the walls of the pulp chamber, the tissue was gently prized loose with a needle and removed with a pair of forceps. Each sample of pulp was first tested to establish its suitability for further study by examining a wet smear,or a fragment of dry tissue made by the aceto-orcein nuclear fixing and staining procedure. All slides used in any of the ensuing experiments were always precleaned by standing overnight in acid alcohol (concentrated hydrochloric acid and 70% ethanol, 1: 100)(Sergovich 1976: 1436).

A aceto-orcein stain fixative (~2%) was prepared using the procedure described by Culling (1976: 1437). Specifically, fresh fixative was prepared by adding 2 g of natural orcein (Fisher Scientific, Fairlawn, N.J.) to 45 mls of boiling glacial acetic acid, and the mixture was allowed to reflux for two hours. After refluxing was completed, the mixture was cooled, made up to 100 mls with distilled water, left to stand at room temperature for at least 24 hours, and filtered before use. A drop of aceto-orcein was pipetted onto the cells, a coverslip was added, and the

preparation left to stand for about 10 minutes. Aceto-orcein stains the cell nuclei a deep wine red, and Barr bodies and other smaller nuclear granules are seen as dark masses. Experiments were continued on a particular pulp if cells appeared to be plentiful, mostly undamaged,and not heavily contaminated with bacteria. Teeth which were strongly suspected of harbouring infected pulps, such as those with amalgam restorations in the proximity of the pulp cavity, were discarded without testing.

To stain thawed pulps for Y-chromatin, wet smears were made on a glass slide. The slide was left to air dry, fixed with methanol for 10 minutes, air dried again, and flooded with 0.5% aqueous quinacrine dihydrochloride (Sigma Chemicals, St.Louis,Mo.) for 20-30 minutes. The working solution was found to have a shelf life of about one week when stored at 4C, and staining was enhanced by warming to about 36C just prior to use. Slides were subsequently washed three times by dipping up and down in tap water,then differential staining was accomplished by immersing them in McIlvaine's 0.1 molar citric acid-phosphate buffer pH 5.5 for 3 minutes. F-bodies appeared on the nuclei as brilliant yellow fluorescent spots against a pale fluorescent background (Ishizu and Hayakawa 1977: 248). The preparation was mounted with a coverslip in the same buffer and viewed under an epi-fluorescence microscope (Zeiss standard lab 16 microscope with BP 436, FT 460 and LP 470 exciter filters) under oil immersion (100 X objective, 10 X eyepiece).

Controls consisted of female pulp smears prepared concomitantly. The frequency of Y-chromatin positive cells in

fresh specimens was established by counting approximately 100 cells per pulp from five male and five female teeth. These temporary preparations could be stored in a humid chamber at 4C overnight without significant loss of chromatin fluorescence.

Although it is noted that X chromatin can be stained by using the same procedures detailed previously with a simple change of buffer pH from 5.5 to 3, nevertheless, an alternative stain, carbol-fuchsin, was selected in order to eliminate any possibility of confusion which might result from artifactual staining in putrefactive tissue. Whittaker (1975: 403), as previously stated (p.31), reported artifactual fluorescence in up to 16% of female cells in pulps putrefying longer than seven weeks.

Briefly, air dried pulp smears were stained with carbol-fuchsin after the methods described by Eskelund(1956) and Carr and Walker (1961). A mixture was prepared containing 0.3 g basic fuchsin (Fisher Scientific, Fairlawn, N.J.) 10 ml of 70% ethanol, 10 ml, glacial acetic acid, 10 ml 37% formaldehyde and 70 ml of 5% phenol. (The working solution of carbol-fuchsin was allowed to stand at least 24 hours before use, and then had a shelf life of about one month stored at room temperature). Slides were stained for 10 minutes, immersed in 95% ethanol for two to three minutes, dehydrated in 100% ethanol for one minute, rinsed twice in xylene and mounted in permount. This procedure permitted differentiation of the Barr bodies and nuclear granules as dark granules against a purple background under an incident light microscope using oil immersion (100 X objective, 10X eyepiece). The specimens were then dehydrated in absolute ethanol for one

minute, cleared in two changes of xylene, mounted with Permount (Fisher Scientific, Fairlawn, N.J.), and the Barr bodies scored. Sample size was the same as for Y-chromatin noted in the aforementioned section above.

Preliminary Experiments on Pig Pulp

Pigs' jaws were purchased at slaughter from local meat packers. The dental pulp was examined in the laboratory the same day. Using common dental tools, peridontal membrane elevator and third molar extractor, anterior tooth extraction was readily accomplished in fresh remains. However, cheek teeth proved refractory, and extra force was required to lift them from the socket which usually resulted in tooth fracture. They could, however, be removed without damage after putrefactive soft tissue loss. Fresh pig pulp smears were stained with carbol-fuchsin (see p.47, for methodology) or with Giemsa stain (Sigma Chemicals, St.Louis, Mo..) made up according to the manufacturers instructions. Some permanent preparations served to establish the fresh state of the pulp, and as controls for evaluation of necrotic tissue.

Elements Interred or Exposed in Outdoor Environments

Samples of human teeth, pigs' teeth, and pigs' jaws or heads were buried at 30 cm depth or surface exposed, and retrieved at various time intervals (for experimental details see Appendix). The purpose of the pig depositions was to determine, by way of comparison, any variability in decay rates between extracted and

unextracted teeth. The pig elements were deposited on the day of slaughter whenever possible, otherwise they were held on ice overnight. It was considered that interred remains recovered by forensic investigators are usually those buried in haste; they are therefore likely to be located in shallow graves.

Some samples were deposited on the ground surface, on the author's property in Vancouver,in both summer and winter periods (June-July 1988, May 1989, October-December 1987, October to January 1988, November-December 1988). Other samples were deposited in the summer (August-September 1988), in the interior of British Columbia, near Lytton. Environmental variables (see Appendix) recorded for all specimen samples included: soil type, soil pH (Green Valley colour-coded soil test kit (Sudbury Laboratory, Sudbury,Mass.)), soil temperature at maximum burial depth, altitude and climate: ambient temperature, and precipitation, in the form of monthly summaries supplied by Environment Canada's Atmospheric Environment Service, Vancouver, (site distance to Burnaby Metrotown approximately 3 km, Vancouver weather station approximately 10 km, to Lytton weather station approximately 12 km).

Two locations were selected for experimental depositions to reveal any enhancement of soft tissue preservation resulting from the more arid conditions prevailing in the British Columbia interior in the summer. The coastal area of British Columbia experiences mild temperatures year round with a high rainfall: mean daily temperature in January 0 to 5C, and in July 16C to 18C, mean annual precipitation 150 to 200 cm (Farley

1979: 43,45). The interior by contrast, has hot arid summers and cold winters: mean daily temperature in January 0C to -5C,and in July 20C to 22C, mean annual precipitation 40 to 50 cm (Farley 1979: 43,45).

Human samples in the coastal environment (Vancouver), consisted of five male teeth and five female teeth per retrieval (mostly third molars). Winter retrievals (Appendix, Exp.3, November-December 1987) were at one week and two week intervals. Summer retrievals (June-July 1988,Appendix,Exp.1) were also at one, and two week intervals. Sex chromatin counts to end point were made on a representative sample of specimens of surface and subsurface deposited remains (May 24-31st, 1989, Appendix, Exp.10). The sample consisted of two male and two female teeth per daily retrieval. Pulps from these teeth were fixed in 3: 1 methanol to acetic acid, paraffin embedded and sectioned (p.53 below). Staining was conducted with carbol-fuchsin or quinacrine dihydrochloride, and the sex chromatin bodies counted from approximately 100 cells per slide.

Pigs' teeth and jaws exposed to Vancouver environmental test sites were apportioned with winter and summer samples of both extracted and unextracted material deposited surface and subsurface, and retrieved at various intervals (daily, weekly and monthly). Sampling details are noted in the appendix (Experiments 4,6,7,and 9). All buried teeth, pig or human, were relocated and exhumed using an archaeological sieve. Plastic gloves were worn at all times during exhumations. Teeth from both pigs and humans, with the exception of the paraffin sectioned samples (see p.53

below), were excised and examined as smears or tissue fragments, by staining with aceto-orcein, and either carbol fuchsin or Giemsa (Sigma Chemicals, St. Louis, Mo.).

An attempt was made in August 1988 to set up an "interior" deposition of dental samples in Manning Park, British Columbia, (approximately 220 km east of Vancouver). Samples of human teeth, and pigs jaws and teeth were set out at the site, either buried or exposed. Upon returning to the experimental site one week later, it was found that the sample had been disturbed by animals. Exposed pigs' jaws were defleshed and scattered, and gnaw marks were noted on the bones. At the burial site there was evidence of an animal scrabbling in the ground to gain access to the buried pigs' jaws, and none of the buried jaws could be located. The experiment was abandoned.

Subsequently a similar experiment was set up near Lytton (about 240 km northeast of Vancouver) at the end of August, as stated, but precautions were taken to protect the sample from wild animals. A wire mesh was set up over the buried material, and secured with pegs before refilling the burial cavity with earth. Large stones were then placed on the surface. Exposed remains were also covered with a heavy wire mesh, and secured with pegs and boulders at the periphery. A return visit to the site one week later again revealed animal disturbance. The burial site was intact, but many animal tracks were observed nearby. The mesh covering the exposed remains was torn out of the ground, some of the pigs' jaws were missing, teeth were scattered, and the remaining jaws revealed animal chewing on the mandibular condyles.

Obviously animals can detect the smell of meat in a shallow burial. When human remains are deposited in outdoor environments it would appear likely that their fate is scattering and destruction by wildlife.

For detailed information concerning sample deposition and retrieval of surface and subsurface pig remains at the Lytton site refer to the Appendix (Experiments 5 and 8). Environmental variables were monitored in the same way as that noted for the coastal samples, and post retrieval processing of dental pulps was as described above.

Laboratory Methods for Preparing Necrotic Tissue

In buried and exposed pig or human teeth, if pulpal tissue remained at all, it was usually in a wet putrefactive condition. Aceto-orcein smears showed very few cells. However, aceto-orcein staining of squash preparations of tissue fragments revealed many cells. This phenomenon may result from more rapid autolysis of the cells on the tissue surface. A similar observation is reported by Janssen (1984: 28) in *post-mortem* haematological smears where autolytic changes were observed in cells after two to three hours and all cells had disappeared by 24 hours. However, in histological sections of the same specimen, autolysis was seen to be substantially retarded. It was therefore decided to prepare paraffin sections of any putrefying pulps.

Paraffin embedding for sex chromatin counts was accomplished by fixing pulp tissue for at least 24 hours in 3: 1 methanol acetic acid (Tishler and Javier 1973: 587),or for histological

examination in Bouin's fluid (Harleco Chemicals,Toronto): staining method p.54 below. Dehydration,clearing and wax impregnation was done over three hours in an automatic tissue processor (Autotechnicon,Technicon Co., Chauncey, N.Y.). Pulps were then embedded by immersion in liquified Paraplast (MP 56C) (Sherwood Medical, St. Louis Mo.). Blocks were stored at -20C, and sections 7 um thick were prepared from frozen blocks (cold blocks facilitate cutting). Sections were transferred to acid-alcohol cleaned slides by floating sections in a warm (45C) water bath. Slides were dried for at least 24 hours in a 37C incubator, dewaxed using two changes of xylene (five minutes each), rehydrated through varying concentrations of alcohol to water, then stained for Barr body counts with carbol-fuchsin (as previously described p.48), or for F-body counting by immersion in McIlvaine's buffer (pH 5.5) for five minutes, followed by staining with quinacrine dihydrochloride (see p.46).

Laboratory Methods for Analysis of the Putrefactive Process

Post-mortem changes in human dental pulp from teeth deposited surface or subsurface out of doors, were analysed from histological sections of Bouin's-fixed tissue stained with either haemotoxylin and eosin, or carbol-fuchsin (Culling 1976: 924):

1. Sections were dewaxed in two changes of xylene, then brought through varying grades of alcohol to water.

2. Sections were stained in filtered Harris's haematoxylin (Fisher,Fairlawn, N.J.) for two minutes,then rinsed in water.

3. Sections were dipped in 1% aqueous lithium carbonate until the sections turned blue (about 30 seconds), and rinsed again in water.

4. Sections were then stained in acidified 1% aqueous eosin Y (Fisher, Fairlawn,N.J.) for about one minute, taken through three changes of absolute ethanol, two of xylene, and then mounted with Permount (Fisher Scientific, Fairlawn, N.J.).

Experiments On "Shelf Stored" Dental Tissue

Teeth which had been heated or stored at room temperature posed special problems for chromatin counts. In any dehydrated tissue, pulp cells become firmly embedded in the fibrous pulp matrix. An attempt was made to separate the cells by the method outlined by Seno and Ishizu (1973: 8), in which pulps were soaked in 20% acetic acid and mechanically abraded with a pestle and mortar. This technique proved to have some disadvantages in terms of nuclear damage to cells rendered fragile by *post-mortem* decomposition, as well as from the inclusion of a confusing assortment of fluorescent matrix debris impeding the examination of intact nuclei. The same problem was encountered by Whittaker et al. (1975: 403), as stated previously (p.31,32).

Consequently an alternative method was therefore devised, based on modification of other procedures. Individual pulp samples were first softened and rehydrated by allowing them to incubate at 25C in 2mls of Modified Ruffer's fluid (30:50:20; v/v/v ratio of 90% ethanol, 1% formaldehyde, and 5% aqueous sodium carbonate

(w/v) respectively from four to 24 hours (Sandison 1955: 227). This buffer was originally developed for the preparation of mummy tissue for histological study: the dilute alkali (sodium carbonate) swells the tissue slightly, and the alcohol and formaldehyde act as fixatives.

Once softened, pulps were washed twice with 0.2M phosphate buffered saline,(PBS),pH 7.2 made up to a final volume of 4 mls,to which was added 1 ml of freshly prepared enzyme mix consisting of 5 mg of collagenase Type 1A, and 4 mg of hyaluronidase,Type 1VS (both purchased from Sigma Chemicals,St. Louis, Mo.). The enzyme mix is a modified version of an enzyme combination found appropriate for the isolation of rabbit dental pulp fibroblasts for *in vitro* cultivation (Shuttleworth et al. 1980: 201). Collagenase breaks down the fibrous pulp matrix and hyaluronidase breaks down the proteoglycans and glycoproteins comprising the ground substance.

Capped tubes containing pulps and enzymes were incubated at 37C in a shaking water bath for 2.5 hours. Pulps were subsequently washed twice in PBS and then passed through a plastic mesh sieve (pore size: 1 mm square) by gently rubbing tissue through the mesh with the round tip of a glass rod, in the presence of PBS. The tissue suspension was then centrifuged at 1,500 rpm for 10 minutes. PBS was removed by aspiration, and a cell preparation was prepared by resuspending cells in in a few drops of PBS and spread onto gelatin-coated glass slide. The slide preparation was dried at room temperature overnight, fixed in methanol for 20 minutes, air dried, mounted and stained with

quinacrine dihydrochloride or carbol-fuchsin. Cell counts were performed on samples as previously described (pp.48). In this case, it should be noted that only intact cells, revealing no evidence of damage from the cell separation procedure, were included in the counts. Samples of dry teeth, stored at room temperature, consisting of five male and five female pulps per time interval, were processed and counted after storage intervals of three months, six months, nine months and one year.

In human teeth held at room temperature it was observed that pulp tissue dried rapidly, within 24 hours. A sample of eight human molars, eight extracted pigs' teeth, and three pigs' jaws store at room temperature (25C approximately), were evaluated at two day intervals over eight days to compare the variability in the rate of tissue dehydration between the three sample sets. Two anterior teeth were extracted at each interval.

Oven Heated Dental Materials

Pigs' jaws, extracted pigs' teeth, and extracted human teeth were heated at temperature intervals of 50C between 100 to 350C, for time periods ranging between one and three hours. Excised pulps were then processed as previously described for dehydrated teeth (pp.55-56). Sample size consisted of one jaw from which anterior teeth were examined, five male and five female human teeth, or five pigs' teeth per temperature interval. When possible pulp cell counts on human teeth were made from five pulp samples of each sex. Extracted pigs' teeth and pigs' jaws were included to evaluate the retardation of heat penetration afforded the pulp

by the surrounding soft and hard tissue of a fleshed jaw.

Outdoor Incineration

Further experiments were designed to elucidate the critical temperature attained within the pulp chamber at which sex chromatin was still detectable in extracted human and pigs' teeth and unextracted pigs' teeth. Human third molars and pig incisors, one per experiment, were wrapped around the roots with paper, held by the roots in a vice, and penetrated with a dental burr through the occlusal surface to the human tooth pulp chamber, or through the lateral surface of the crown in the case of pigs' incisors, leaving an aperture large enough to receive thermocouple wires. A type K (0-1370C range) 36 inch chromel versus alumel wire, with fishspine ceramic beads on one leg (Valax Scientific and Engineering, Vancouver) was then inserted to the level of the pulp chamber. The wires were held in place at the occlusal surface by a glass ionomer bonding cement (G.C. Dental and Industrial Corporation, Tokyo). A small piece of cotton batting was inserted just below the cement surface to ensure that the glass ionomer did not seep into the pulp chamber before hardening.

Glass ionomers have been highly developed for dental restorations. These ionomers composed of both silicates and zinc polyacrylates, combine the properties of high mechanical strength, and low solubility, with excellent tooth surface binding potential (Craig 1985: 58). They have the additional advantages in that they are good thermal insulators as compared to other cements (Tay and Braden 1987: 1040), have a comparatively

low coefficient of thermal expansion preventing microleakage at the tooth cement interface (Bullard et al 1988: 871), and they are the most electrically conductive of the cements having values similar to dentine (resistivity of human dentin 0.7-6.0 x 10^4 ohm.cm, glass ionomer is 0.8-2.5 x 10^4 ohm.cm)(Craig 1985: 52).

The glass ionomer was tested prior to the experiments for its ability to withstand high temperatures by exposing some bonded teeth to an open flame. Considerable damage to the dental pulp occurred from heat friction and penetration of the dental burr when it was used without coolant. Therefore teeth, incorporating thermocouple wires served only to determine the temperature in the pulp cavity, and were not used for subsequent analysis.

Five teeth per experiment, either five male or five female human teeth, which included incisors and premolars as well as molars, or five pig incisors, were placed on an asbestos pad over a bunsen burner in close proximity to a thermocoupled tooth of the same species. The thermocouple was connected to a digital type K thermometer (Wahl Instruments, Culver City, Ca.). Great care was taken to ensure that the thermocouple wires were free of surface contact of either apparatus or other teeth, and therefore an accurate pulp chamber temperature was assessed. Four sample temperatures (25C, 50C, 75C,and 100C) were tested such that as each temperature was attained in the thermocouple embedded tooth, one or more teeth were removed from the heat and allowed to cool at room temperature. For a brief period after retrieval the temperature within each chamber continued to climb by an increment of approximately 25C.

The heated pulps, in the temperature range tested, remained moist, and suitable for sectioning. They were fixed in 3: 1 methanol to acetic acid, then paraffin embedded, sectioned and stained, either with haematoxylin and eosin (pp.54,55), to evaluate cell damage, or with carbol fuchsin or quinacrine dihydrochloride for sex chromatin counts (pp. 48). From these experiments it was determined that 25C-75C, as registered in the chamber at the moment of retrieval, was the range which encompassed the critical temperature during which carbonization of the pulp produced loss of sex chromatin staining capacity.

Using this temperature range as the indicator for additional experiments, the heads of five young pigs (five to six months old as determined from assessment of tooth development; Bone 1982: 133), were purchased from a local slaughter house, and taken immediately to an open field where a log fire of Douglas fir and pine had been prepared. The thickness of tissue from the lingual gingival-crown junction of the lower right I_2, to the same point on the external surface of the jaw, and the maximum thickness of the tip of the tongue at the midline were recorded on all five pigs, using a Lange skinfold caliper (Cambridge Scientific Instruments, Cambridge, Maryland). This specific data helped establish the approximate thickness of hard and soft tissue surrounding the dental pulp. These measurements were then compared to a sample of five male and five female human volunteers.

Three pigs were then prepared by inserting thermocouple wires bonded with glass ionomer (as described above) into the pulp

chamber through the buccal surface of the crown of the lower right I_2. The pigs' tongues were extended to cover the right lower incisors (in human victims of fire the tongue is often protruded, helping to retard heat penetration to the teeth (see p.21). Heads were placed within freshly prepared log piles, with the supposition that this placement strategy best approximates the circumstance of a victims' body in a burning building (Buikstra and Swegle n.d.). A second thermocouple probe (type K long reaction surface probe, Wahl Instruments, Culver City, Ca.) was used to record the temperature of the fire. This probe was placed as close as possible to the lower right anterior teeth. Care was taken to ensure that the wires were free of all surface contact. The fires were then lit, and the temperatures within the pulp chambers monitored until the appropriate temperature was reached in each pig: 25C, 50C, or 75C,at which time the heads were rapidly removed from the fires. The temperatures of the fires were recorded at the exact time the samples were removed.

The temperature inside the pulp chamber rose by approximately 25C upon retrieval of the heads. It should be noted that the design of this experiment precludes the accurate assessment of pulp chamber temperature rise, since the samples are composed of pigs killed previously (one hour), and therefore represent colder initial body temperatures. One therefore expects that pulp chamber temperature rise would be more rapid in victims facing actual death by fire since the initial temperatures would be normal 37C body temperatures. After retrieval of the heads the lower I_1's were extracted with a peridontal membrane elevator.The

dental pulp was removed, fixed in 3: 1 methanol to acetic acid, paraffin embedded and stained either with haematoxylin and eosin (pp. 54,55), or carbol fuchsin (p.48), to evaluate cell and nuclear damage.

CHAPTER FOUR: SECTION B

MATERIALS AND METHODS, H-Y MOLECULE

Two principal methods were employed here, one, a peroxidase-antiperoxidase (PAP) test using a terminal enzyme-labelled antibody, and the other, an indirect fluorescent antibody test (IFA) using a terminal fluorescent-labelled antibody. The PAP antibodies consisted of adsorbed monoclonal antisera to H-Y (kindly donated by Stephen Wachtel, Centre for Reproductive Biology, Collierville, Tennessee) and a monoclonal anti-B2 microglobulin. This test is considered to be extremely sensitive (Moriarty et al. 1973: 825; Vandesande 1983: 107). The antibodies for the IFA test consisted of polyclonal adsorbed antisera to H-Y, raised by intrasplenic implantation, and a monoclonal anti-B_2 microglobulin. This test is less sensitive than the PAP but is appropriate when a very potent primary antibody, such as the polyclonal anti-H-Y described above, is available.

Preliminary Peroxidase-Antiperoxidase Testing

Antigen sources for testing of PAP included smears of human tonsil tissue (liquid nitrogen stored), and human male and female fresh tooth pulp smears, applied to wells on 0.5% gelatin-coated multitest slides (Flow Labs, McLean,Va.), and subsequently fixed for 20 minutes in acetone. The primary anti-H-Y antibody, provided by Stephen Wachtel,consisted of a mouse IgG_2a monoclonal secreted by hybridoma gw-16 (Brunner et al. 1984: 615). This hybridoma was

produced by the method of Köhler and Milstein (1975: 495)[1].

The donated gw-16 preparation was adsorbed on mouse thymocytes, prior to testing, by the method reported by Bradley and Heslop (1985: 634). A female chocolate brown agouti mouse was sacrificed and thymectomized. Thymic cells were then separated by teasing the tissue apart with needles in 2-3 mls of PBS containing 5% GG-free foetal calf serum (GIBCO Labs,Grand Island,N.Y.). The preparation was washed twice in the same medium and 1 x 10^8 cells added to the 0.5 ml of antiserum. The mixture was then incubated for two hours at 37C and the antiserum removed by centrifugation (1,500 rpm for 10 minutes).

The primary anti-human B_2-microblobulin antibody was purchased from ICN ImmunoBiologicals (Lisle, Ill.). The PAP test was a slightly modified version of that outlined by Müller and Bross (1979: 143)and is noted in the following:

1. Acetone fixed cells were washed 3 times in NKH buffer:(8.5g NaCl, 0.4g KCl/l,buffered with Hepes (Grand Island,N.Y.) pH 7.4) containing 0.1% gelatin. All subsequent steps were followed by the same washing procedure.

2.Endogenous peroxidase activity was blocked by immersing slides in methanol containing 0.3% hydrogen peroxide (v/v) for 20 minutes (Mason and Taylor 1975: 594).

1. gw-16 is described as the product of fused spleen cells from male-sensitized C57BL/6 mice with P3/NS1/1-Ag4-1 myeloma cells. The hybridoma cells were subsequently transferred as ascites tumours into (BALB X B6)F1 female mice. Ascites fluid was then purified on a column of protein-A sepharose (protein-A has a high affinity for Fc receptors).

3. All subsequent steps except the final one were carried out on ice in a wet chamber. All sera were applied in a volume of 10 ul per multitest well. Slides were blocked for non-specific staining with normal rabbit serum (1: 50) for one hour(Matthews 1987: 189).

4. All antisera were diluted in NKH buffer containing 10% GG-free foetal calf serum (PBS/calf).The primary monoclonal gw-16, was tested in four-fold dilutions in a range 1:32 to 1:2,000. The primary anti-B_2 microglobulin was used at a dilution of 1: 500. Each was incubated for 20 minutes.

5. Cells were then incubated for 20 minutes with the bridging antibody, rabbit anti-mouse IgG (Sigma Chemicals,St.Louis,Mo.), diluted 1:500.

6. All cell samples were next incubated for 20 minutes with a second bridging antibody, goat anti-rabbit IgG (Sigma Chemicals, St. Louis,Mo.), diluted 1:8.

7. The final 20 minute incubation was with a 1: 128 dilution of PAP immune complex (Sigma Chemicals, St. Louis, Mo.).

8. Staining by 3,3'-diaminobenzidine tetra hydrochloride (DAB)(Sigma Chemicals, St. Louis, Mo.) was accomplished by adding a fresh preparation of 0.05%,and hydrogen peroxide 0.003% in 0.05M Tris-HCl-0.13m NaCl, pH 7.6. Incubation was for one hour at room temperature.

9. The slides were then mounted with 50% glycerin-0.1% glutaraldehyde in NKH buffer, and viewed with oil immersion(100X

objective, 10X eyepiece) under a light microscope. If the PAP complex was bound, the reaction of the enzyme with the DAB substrate produced a brown stain on the surface of the cells. Controls consisted of male and female cell preparations, those with normal rabbit serum replacing the primary antiserum, and those in which the primary antiserum was excluded and replaced by buffer. All tests were repeated at least three times.

Preliminary Indirect Immunofluorescence Testing

Antigen sources for indirect immunofluorescence (IFA) testing consisted of positive control smears of human male leukocytes isolated from peripheral blood on a Ficoll-Hypaque gradient (Bøyum 1968: 77), and smears of fresh and thawed male and female dental pulp, applied to 0.5% gelatin-coated multitest slides. All slides were subsequently fixed with acetone. The polyclonal anti-H-Y antibody for this test was prepared by the intrasplenic immunization method of Bradley and Heslop (1985: 634). Briefly, two to three month old Lewis rats (Charles River Labs, Quebec), three males and three virgin females were used for intrasplenic immunization. Animals were anaesthetized with Fluothane (1.5% through a nose cone). Depilated dorsal skin snippets, about 1 mm diameter were removed from the males by tenting the skin and slicing off the raised tissue. Female spleens were delivered by subcostal incision. The skin grafts were implanted into the female spleens by impaling them on an 18 g needle, inserting the needle into the spleen, then displacing the skin tissue from the needle with forceps, and pushing it into the splenic incision until it

appeared to be firmly embedded. The abdominal incision was then closed in two layers. Both males and females were bled once from the tail prior to immunization (to provide control serum) and females at one, two, three, and four weeks post implant.

According to Bradley and Heslop, who used an enzyme linked immunosorbent assay (ELISA) test system, intrasplenic transplantation elicits a "rapid antibody response with titer reaching maximal values (1: 2,000 - 1: 4,000) in the first one to two weeks, and thereafter rapidly declining by the fifth week" (1985: 635). Moreover, the activity of this antiserum resides in the IgG fraction (Bradley et al. 1987: 352). One and two week post-transplant sera were therefore selected for these experiments. Some aliquots were adsorbed on mouse thymocytes before use, as described for the PAP test. The IFA test is not nearly as sensitive as the PAP test; estimates of sensitivity suggest a difference of 100 to 1,000 times (Sternberger et al. 1970: 315; Taylor 1978: 113). It was decided to use this test for two reasons 1. high titers are produced as reported by Bradley and Heslop, and 2. the method is simple and permits sources of error to be more readily monitored. The anti-β_2 microglobulin monoclonal was used in the same manner as for the PAP test.

The test was performed with minor modification by the method described by Galbraith et al.(1978: 25) and is noted in the following:

1. Acetone fixed cell smears were washed three times in 0.02M PBS pH7.2.The washing procedure was repeated after each step of the experiment. All sera were applied in volumes of 10 ul per

multitest well.

2. The rest of the experiment was conducted in a wet chamber at room temperature. The cells were blocked for non-specific staining with normal rabbit serum 1: 25 for one hour (Matthews 1987: 189).

3. Polyclonal rat anti-H-Y, diluted four-fold in a range 1: 8 - 1: 2,000 and/or monoclonal mouse anti-human B_2-microglobulin, 1:500 in PBS/calf were applied to the cells. The slides were incubated for 40 minutes.

4. Fluoroscein isothiocyanate (FITC) labelled rabbit anti-rat IgG (Zymed Labs.,San Francisco) 1: 25 was then applied to the cells being tested for H-Y, and FITC labelled goat anti-mouse IgG (Kirkegaard and Perry Labs., Gaithersburg,Md.) 1: 100 to the cells being tested for β_2-microglobulin. Incubation time was 30 minutes.

5.Finally slides were mounted in PBS and viewed with oil immersion (100X objective, 10X eyepiece) using an epi-fluorescence microscope (Zeiss standard lab 16 microscope with 450-490 nm, FT 510, LP 520 filters). If antibodies are bound to the cells, a yellow-green fluorescence is observed on the cell surfaces.

Negative controls consisted of female pulp smears, normal Lewis serum substituting for the primary Lewis antiserum, normal rabbit serum substituting for the primary Lewis antiserum, and buffer substituting for primary Lewis antiserum. All tests were repeated at least three times.

CHAPTER FIVE: SECTION A

RESULTS OF SEX CHROMATIN EXPERIMENTS

Results of Preliminary Sex Chromatin Counts

Fresh human pulp smears were made to establish the sex chromatin staining technique, and used as a primary control for further experimentation (see Materials and Methods pp. 46-47, Table 1, Graph 1, and Fig. 1 below).

TABLE 1 Sex Chromatin Counts on Fresh Human Pulps

Sex Chromatin Counts on Fresh Pulps

FEMALE BARR BODY				MALE F-BODY			
Pulp No.	Pos.	Neg.	% Pos.	Pulp No.	Pos.	Neg.	% Pos
1	14	86	14	1	96	167	37
2	24	76	24	2	81	122	40
3	28	72	29	3	128	62	67
4	14	114	11	4	94	31	75
5	18	118	13	5	100	96	51
MALE OVERLAP				FEMALE OVERLAP			
1	3	101	3	1	2	173	1
2	1	99	1	2	0	180	0
3	1	103	1	3	0	78	0
4	1	99	1	4	2	88	2
5	3	100	3	5	1	89	1

Barr bodies average (%): 18.6	F-bodies average (%): 54
Range (%): 11-29	Range (%): 37-75
Average male overlap (%):1.8	Average female overlap (%): 0.8
Standard Deviation: 6.2	Standard Deviation: 14.8
S.D. Male Overlap: 0.9	S.D. Female Overlap: 0.7
Male Overlap difference:	Female Overlap difference:
(t-test) $P < .0005$	(t-test) $P < 0.0005$

Graph 1

Sex Chromatin Counts on Fresh Pulp Smears

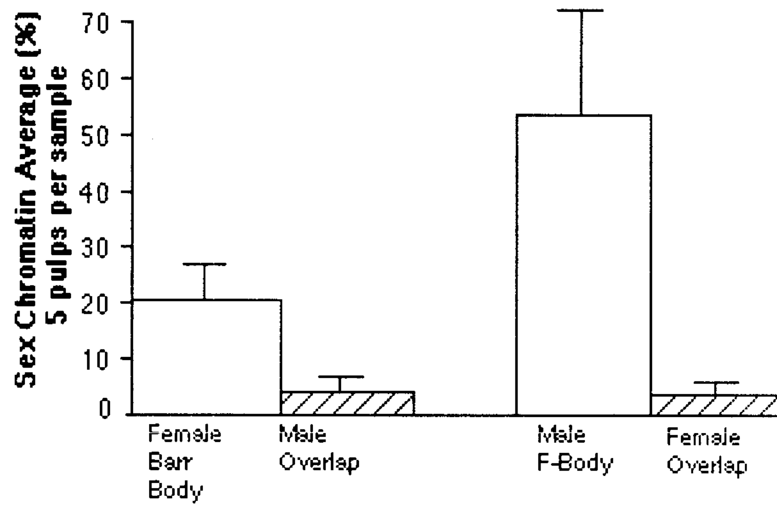


Figure 1a

Human Pulp Fibroblast Nucleus from a Male Showing Brightly Stained F-Body (Quinacrine Stain, Original Magnification X 1000).

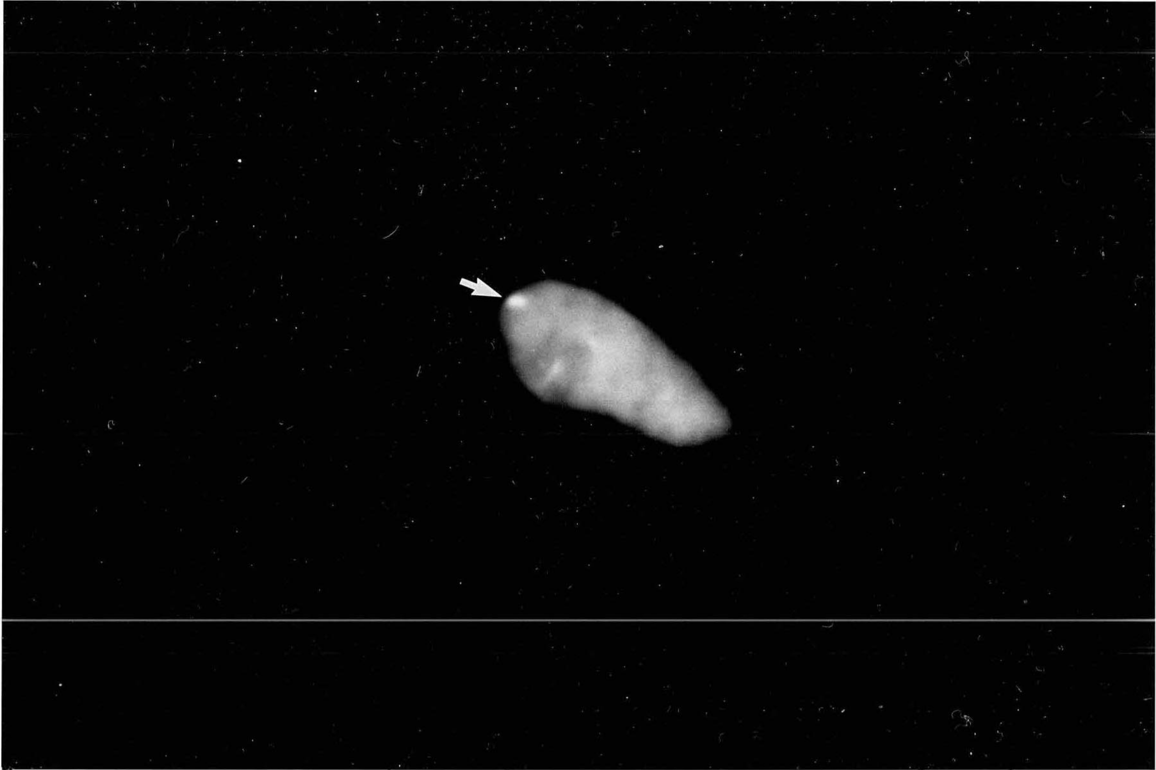


Figure 1b

Human Pulp Fibroblast Nucleus from a Male, the Nucleus Shows Background Fluorescence but No F-Body, (Quinacrine Stain, Original Magnification X 1000).

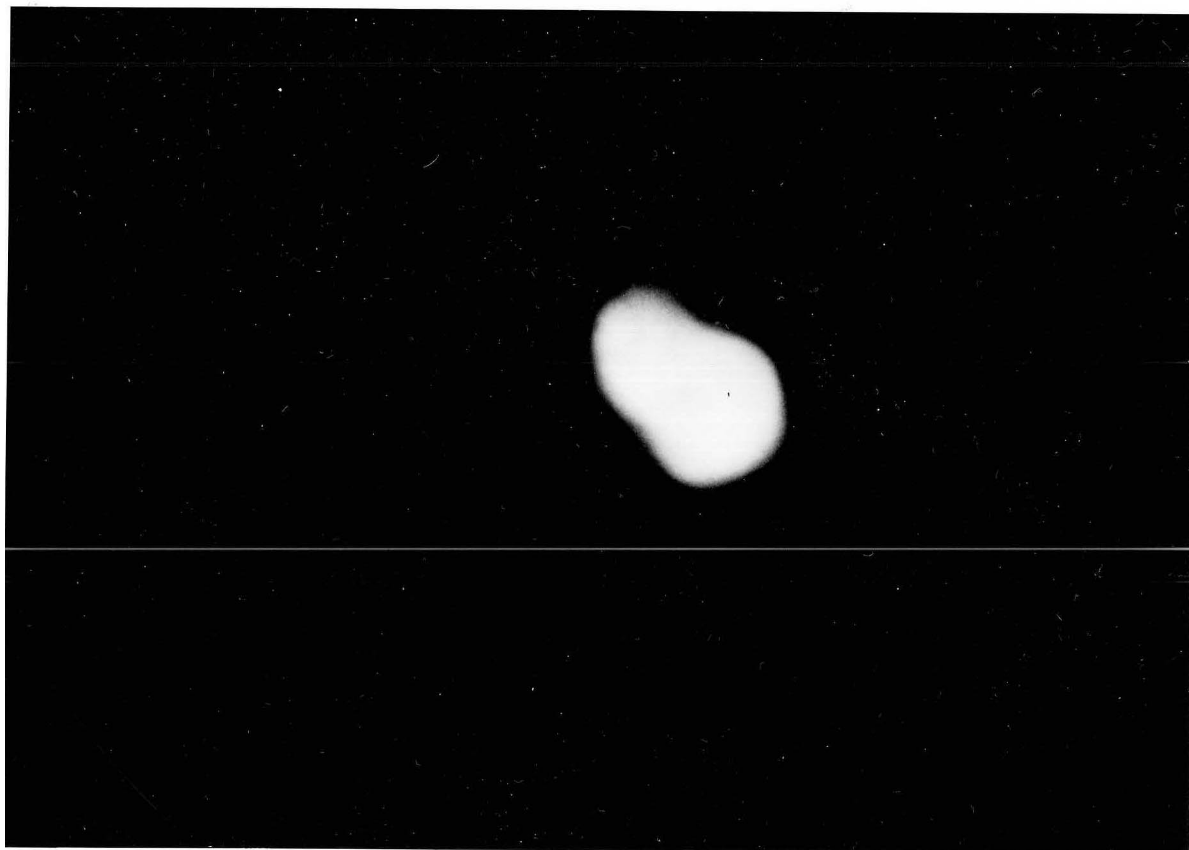


Figure 1c

Human Pulp Fibroblast Nucleus from a Female Showing a Barr Body (darkly stained mass), (Carbol-Fuchsin Stain, Original Magnification X 1000).

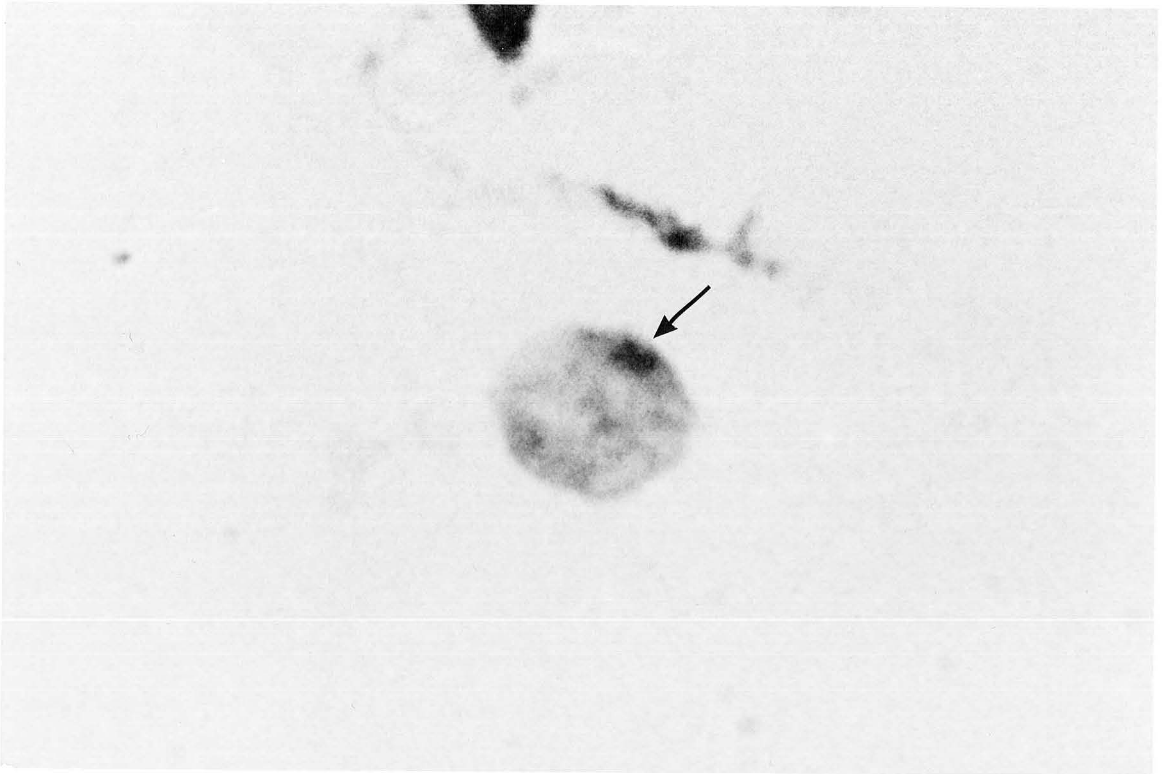
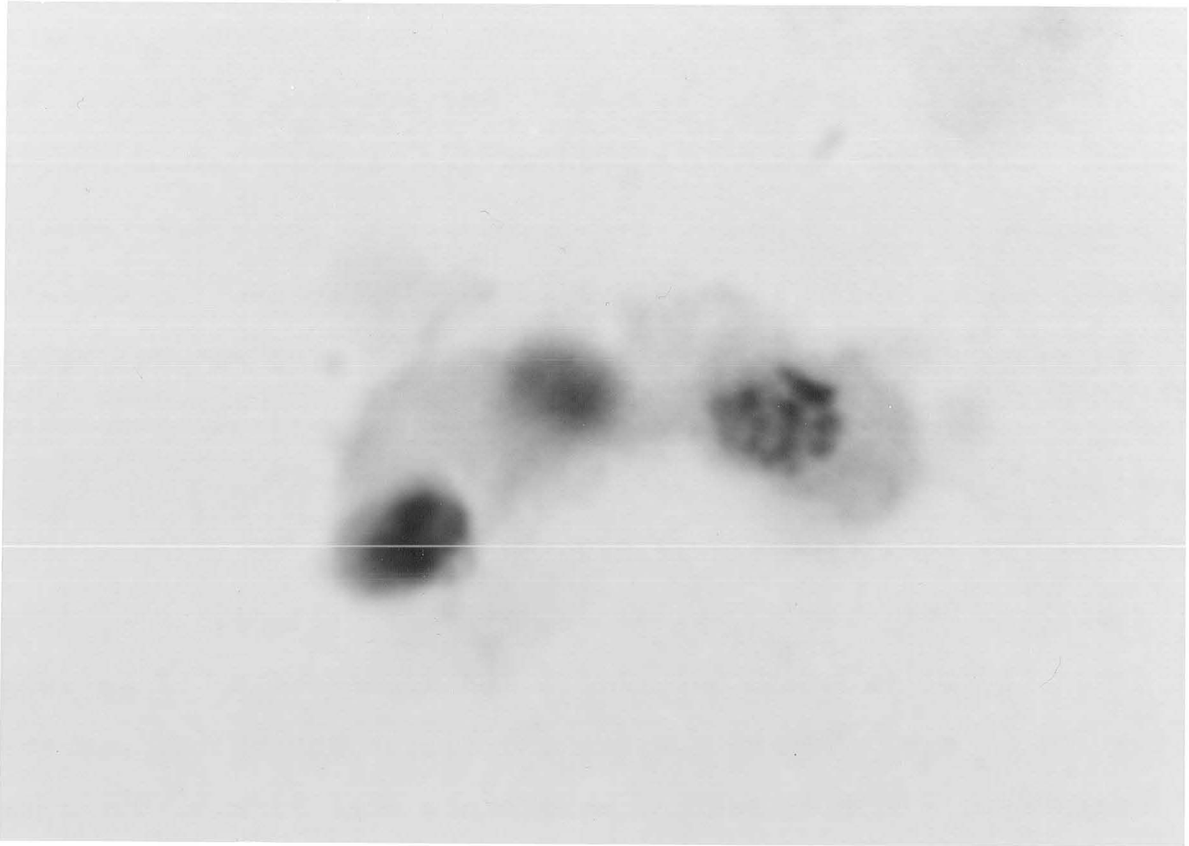


Figure 1d

Human Pulp Fibroblast Nucleus from a Female Without a Barr Body (Carbol-Fuchsin Stain, Original Magnification X 1000).



It should be noted that the counting method used is subjective. The evaluation of any cell as positive or negative for Barr bodies or F-Bodies is a judgement by the individual doing the counting. The estimate here - 12-28% Barr,37-75% F-body - is conservative in comparison to some reports in the literature: 15-40% Barr bodies (Yunis and Chandler 1979: 83) and 50-92% F-bodies, (Pearson et al. 1970: 78; Seno 1977: 172). Variation in sex chromatin enumeration between studies probably reflects variation in tissue type (Mittwoch 1974: 73), the stage of mitotic division (Barr bodies are found only in prophase and interphase, whereas F-bodies are seen at mitotic and interphase stages (Sharma 1972: 346),) or methods of tissue preparation, and inter-observer error.

From Graph 1 it is clear that Y chromatin is the better discriminator of sex in that F-bodies are more numerous than Barr bodies. In paraffin sections, however, Barr body estimates are reported to approximate 50% (Yunis and Chandler 1979: 830); this finding is borne out in the present study (see p.90 below). Testing for both male and female chromatin of the same sample is advantageous because it can corroborate sex diagnosis in the forensic field situation where tissue decomposition can produce a false negative result.

Pulp Cell Decomposition Over Elapsed Time In Outdoor Environments

Observations on the deterioration rate of tooth pulp cells, made from material exposed to surface or subsurface (30 cm at base) "simulated forensic" depositional environments, reveals that

in British Columbia putrefaction of pulp cells, beyond the possibility of generating valid sex chromatin counts almost always occurs within two weeks or less. This observation was consistent regardless of the location of the burial, season, soil type or pH, and site elevation. Moreover, no notable variation in deterioration rate was observed between extracted human, extracted pig, and unextracted pig pulps (in heads or jaws).

DATA SUMMARY (for details refer to Appendix pp. 182-204)

Decomposition of Tooth Pulp in Outdoor Environments

TABLE 2

	SUMMER	
	SUBSURFACE (30 cm)	SURFACE
Human Teeth		
Experiment 1, Appendix (Catalogue O.P.)		
Vancouver :	No cells remaining at one week (July '88)	1-2% of cells remaining at one week (July'88)
Average ambient temperature: 15.0C , Rainfall for period: 16.7 mm		
Average temperature subsurface: 13.3C, Soil pH over period: 6		
Experiment 2, Appendix (Catalogue T.U.)		
Lytton :	1-5% of cells remaining at one week (Aug./Sept.'88) acellular at one month	10-15% of cells remaining at one week(Aug./Sept.'88) acellular at one month
Average ambient temperature: 24C, Rainfall for period: 0		
Average temperature subsurface: 26C, Soil pH over period: 7		
Extracted Pig Teeth		
Experiment 4, Appendix (Catalogue Q.R.)		
Vancouver :	2-3% of cells remaining at one week (July '88) autolysis apparent at four days	2-5% of cells remaining at one week (July '88) autolysis apparent at four days
Average ambient temperature: 14C, Rainfall for period: 11.3 mm		
Average temperature subsurface: 12C, Soil pH over period: 6		

	SUBSURFACE (30 cm)	SURFACE
Experiment 5, Appendix (Catalogue T.U.)		
<u>Lytton</u> :	10-20% of cells remaining one week (Aug./Sept.'88) acellular at one month	>50% of cells still intact at one week(Aug./Sept.'88) acellular at one month
Average ambient temperature (one week): 24C, Rainfall for period: 0		
Average temperature subsurface: 26C, Soil pH over period: 7		
	Unextracted Pig Teeth	
Experiment 7, Appendix (Catalogue Q.R.I.)		
<u>Vancouver</u> :	>50% of cells at one week (July '88), still a few cells at two weeks	1-5% of cells remaining by five days (July '88)
Average ambient temperature: 15C (1st week), 20C (2nd week) Soil pH over period: 6, Rainfall for period:11.3 mm (1st week),0 (2nd week), Average temperature subsurface: 12C		
Experiment 8, Appendix (Catalogue T.U.)		
<u>Lytton</u>:	Acellular at one week (Aug./Sept.'88)	Acellular at one week (Aug./Sept.'88)
Average ambient temperature: 24C, Rainfall for period: 0		
Average temperature subsurface: 26C, Soil pH over period: 7		

WINTER

	SUBSURFACE (30 cm)	SURFACE

Human Teeth

Experiment 3, Appendix (Catalogue Y.Z.)

Vancouver :	10-20% cells at one week, a few at two weeks (Nov./Dec.'88)	15-20% cells at one week, a few at two weeks (Nov./Dec.'88)

Average ambient temperature: 5.9C, Rainfall for period: 6.3 mm

Average temperature subsurface: 7.3C, Soil pH over period: 6.5-7

Extracted Pig Teeth

Experiment 6, Appendix (Catalogue D.W.)

Vancouver :	Acellular at one week (Oct.'87)	2-3% cells at four days cell remnants at one week(Oct.'88)

Subsurface (seven days):

Average ambient temperature: 9.9C, Rainfall for period: 0.2 mm

Average temperature subsurface: 17C, Soil pH over period: 5.6

Surface (four days):

Average ambient temperature: 13C, Rainfall for period: 0

Unextracted Pig Teeth

Experiment 9, Appendix (Catalogue D.X.)

Vancouver :	Acellular at one week (Oct.'87)	1-5% cells at one week (Nov.'88)

Subsurface:

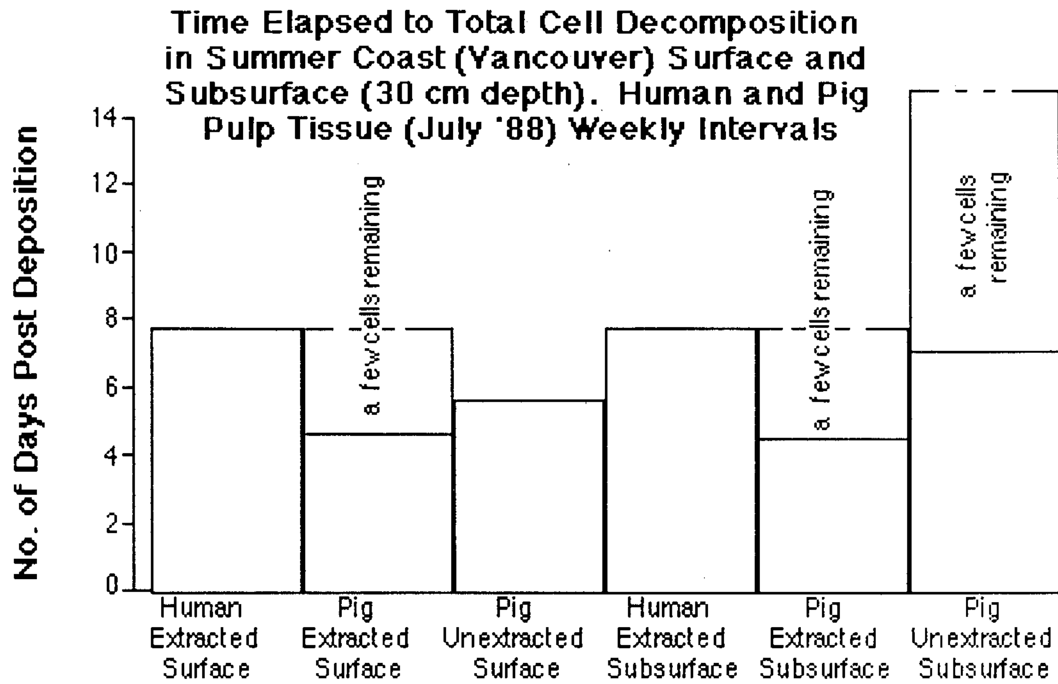
Average ambient temperature: 9.9C, Rainfall for period: 0.2 mm

Average temperature subsurface: 17C, Soil pH over period: 5.6

Surface:

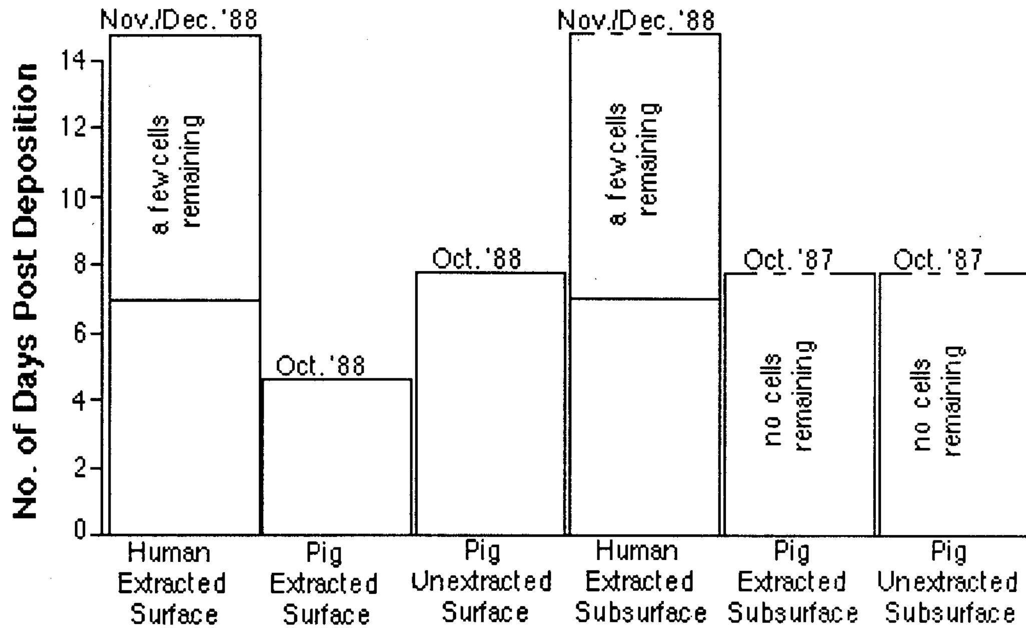
Average ambient temperature: 6.5C, Rainfall for period: 34.3 mm

Graph 2



Graph 3

Time Elapsed to Total Cell Decomposition in Winter Coast (Vancouver) Surface and Subsurface (30 cm depth). Human and Pig Pulp Tissue. Weekly Intervals



The longest observed pulp cell preservation of two weeks, occurred in the winter during the coldest period recorded in these experiments: average temperature 5.9C (Appendix Expt.3;Table 2, and Graph 3 above). Bacterial and mycotic activity was probably inhibited by the low temperatures. In the summer period the best preservation was noted for extracted teeth deposited on the ground surface at the Lytton site (Appendix Expt.5, and Table 2 above). This cellular preservation can most likely be attributed to the high temperatures (average 24C Appendix Expt.2, and Table 2 above), and lack of rainfall in the first week that contributed to a dessicated environment thus inhibiting microbial putrefaction. Cells in the extracted human teeth were not as well preserved at one week as those from the pigs, perhaps because pre-deposition storage at -20C had rendered them more fragile. After the first week at Lytton the temperature dropped (average temperature 15.5C between September 5th, and September 25th), and rainfall ensued (19 mm on the 16th of September and 13 mm on the 25th), creating the conditions for rapid putrefaction. Since no teeth were retrieved at two weeks from the Lytton site no end point for cell stability was recorded, however, retrieval at one month showed total pulp cell degeneration.

Cell stability in the subsurface sample at Lytton was no better than in the Vancouver coastal environment,(Table 2, and Graphs 2,3,above) in spite of the difference in the chemistry of the surrounding soil matrix. Acidic soils, such as those of the found in coastal locations, are considered to contribute to rapid decomposition of organic materials (Krogman and Işçan 1986: 28).

It should be noted that soil pH readings using a colour coded test kit, as is the case in these experiments, gives only an approximate estimate (± 0.5). A pH range between 5 and 7 was recorded in the coast experiments. This difference may reflect the varied location of the burials on the author's property as much as any affect of climate variation on soil pH. Summer readings at one site (Appendix, Expt.4,) in July 1988 were all 6. Winter readings at another site, recorded between October 1987 and January 1988 (Expt.6,), varied between 5 and 5.6, whereas in November/December 1988 at yet another location, the pH varied between 6.5 and 7 (Appendix Expt.3,). In these two winter experiments, with average rainfalls of 0.2 mm and 6.3 mm respectively, the lesser rainfall was recorded with the more acid pH.

The soil pH at the Lytton site varied between 7 and 8.5 (Appendix, Expt.2), reflecting not only the more basic soil chemistry of the interior environment, but also the possibility of much greater fluctuation in soil pH. The large increase in soil pH occurred after a hot dry summer followed by heavy rain. Deposition of remains at the site was at the bottom of an incline. Slope wash induced by heavy rain may therefore have contributed to the pH fluctuation.

Phenomena such as these have been described in the literature. Dystric Brunisol (loamy sand) is reported to become more acidic on Burnaby Mountain in times of low rainfall (Crampton 1980: 385). The author also states that drying of Ortho Humo-Ferric Podzol (sandy loam) has been found to increase soil acidity, and in addition that sandy soils show more marked

variation in pH than do loamy soils.

Daily Sex Chromatin Counts in Vancouver

On the coast daily sex chromatin counts to end point were made on a representative data set (two male and two female pulps per retrieval) over a period of seven days (see Materials and Methods p.51, and Appendix, Experiment 10, and Table 3 below for details).

TABLE 3

Daily Sex Chromatin Counts on Surface and Subsurface Remains

FEMALE BARR BODY

SURFACE DEPOSITION

Day	Positive	Negative	% Pos.
0	38	67	36
1	19	138	12
2	39	61	39
3	25	82	23
4	38	69	35
5	0	0	0
MALE OVERLAP			
0	7	99	7
1	4	105	4
2	5	95	5
3	5	106	5
4	3	100	3
5	0	0	0

MALE F-BODY

SURFACE DEPOSITION

Day	Positive	Negative	% Pos.
0	49	69	46
1	60	86	41
2	50	104	32
3	31	102	30
4	43	95	31
5	0	0	0
FEMALE OVERLAP			
0	3	81	4
1	2	110	2
2	3	105	3
3	4	100	4
4	6	96	6
5	0	0	0

SUBSURFACE DEPOSITION

BARR BODY

Day	Positive	Negative	% Pos.
0	38	67	36
1	23	95	20
2	14	100	14
3	0	0	0

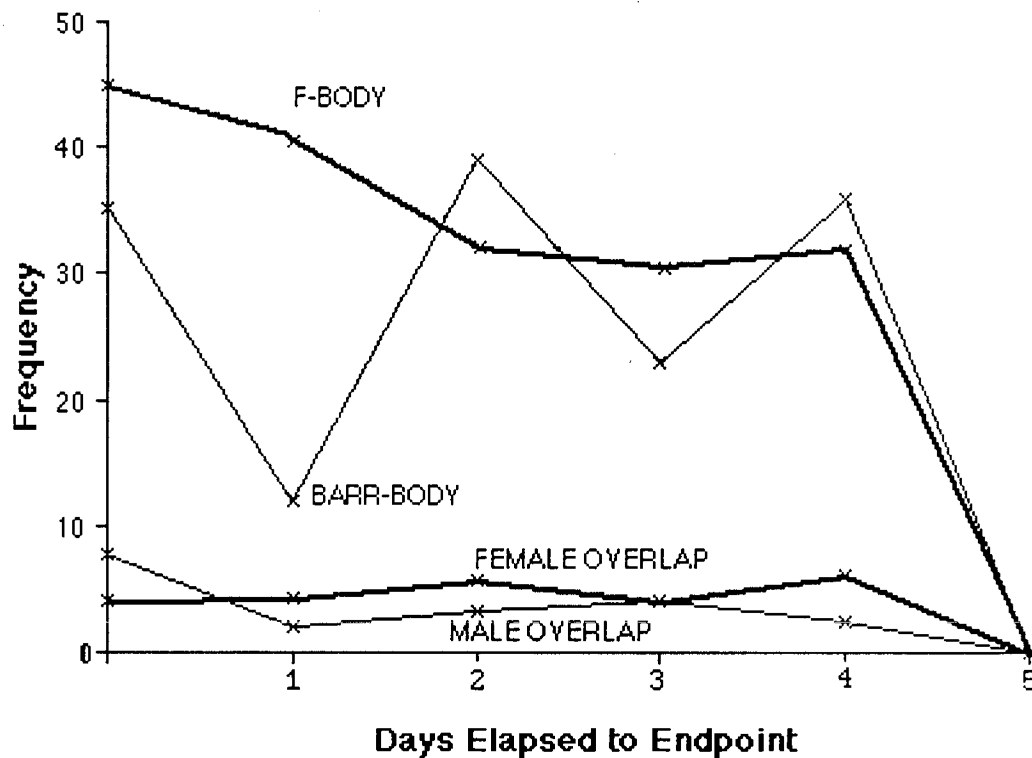
SUBSURFACE DEPOSITION

F-BODY

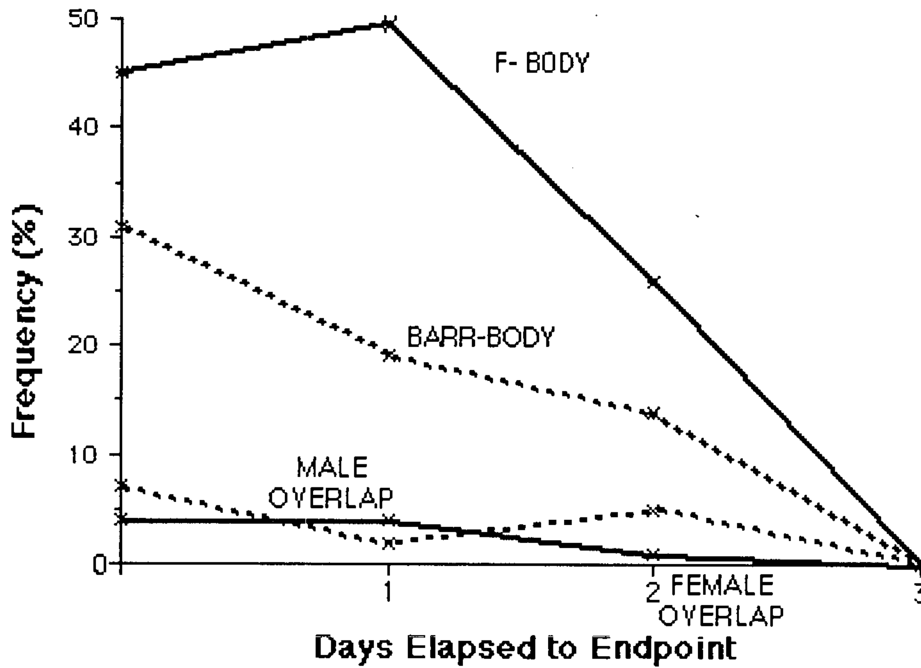
Day	Positive	Negative	% Pos
0	49	69	42
1	60	63	48
2	30	90	25
3	0	0	0

MALE OVERLAP				FEMALE OVERLAP			
0	7	94	7	0	3	81	4
1	2	99	2	1	3	102	3
2	6	106	5	2	0	102	0
3	0	0	0	3	0	0	0

Graph 4



Graph 5



Evaluation of methanol acetic acid-fixed paraffin sections revealed that decomposition beyond the possibility of generating valid sex chromatin counts occurred over a 24 hour period. In this case it took place in the surface sample between the fourth and fifth day (28th-29th May, 1989), and in the subsurface sample between the second and third day (26-27th May, 1989).

Although most of the chromatin counts appear to show a decrease before end point, the Barr body count on the surface sample fluctuates from 14 to 39% between days one and two, then drops to 23% and returns to 35% on days three and four. This erratic result may reflect some deterioration in individual pulps at the time of burial: teeth were stored frozen by dental surgeons prior to use. Alternatively it may represent an artifact of the small sample size (two pulps per sample). Pulp from the sample in question was tested prior to deposition (Day 0, positive 38, negative 67)giving a 36% positive count. This figure is quite high (reported range 15-40% see p.27/28), suggesting good preservation for the tooth batch, and is commensurate with counts made on paraffin sectioned tissue, i.e. smear preparations tend to give lower counts (as is the case in this thesis, fresh smears average Barr body range 11-29 % positive p.70) as a result of Barr bodies falling in front or behind the nucleoplasm where they go undetected (see p.27/28).

Histological Description Of Cellular Necrosis in Human Pulps

The state of decomposition is described here as necrotic, although the process also includes putrefaction, but in the early

stages of decomposition, necrosis is eventually overlapped by putrefaction and the two are then impossible to distinguish. Human pulps were exposed on the ground surface in the spring of 1988 (see Appendix; Experiment 10, May 24th-May 31st) and Fig.2a,2b,2c and 2d below), two pulps were retrieved daily, paraffin embedded, sectioned and stained with haematoxylin and eosin:

Day 1. Odontoblasts: Many nuclei were still intact, but some exhibited atrophy and pyknosis (shrinking and hyperchromatinism), or karyolysis (nuclear disruption).

Pulp Cells: Some cell sheets showed nuclei substantially intact, but chromatin margination (multiple aggregates of condensed chromatin at the nuclear periphery) was noted in certain cells, as well as vacuolation. Other cell sheets revealed nuclear ghosts (only nuclear outlines evident).

Day 2. Odontoblasts: Nuclei showed various stages of necrosis, from virtually undamaged chromatin arrangement, to chromatin margination,hypertrophy,and karyolysis.

Pulp Cells: A few areas with relatively intact nuclei,many sheets of autolytic ghost cells, some areas showing only nuclear debris. The fibrous pulp matrix appeared vacuolated.

Day 3. Both odontoblasts and pulp cell nuclei appeared either as ghosts or converted by karyolysis to debris. The fibrous matrix was vacuolated.

Days 4,5,6 as for, **day 3** except that one pulp on day six revealed a few intact nuclei. Chromatin in these cells was marginated and the nuclei showed vacuolation.

Figure 2a

Human Pulp Fibroblasts Held in an Outdoor Environment Day One: Nuclei Granular and Intact (Carbol-Fuchsin Stain, Original Magnification X 400).

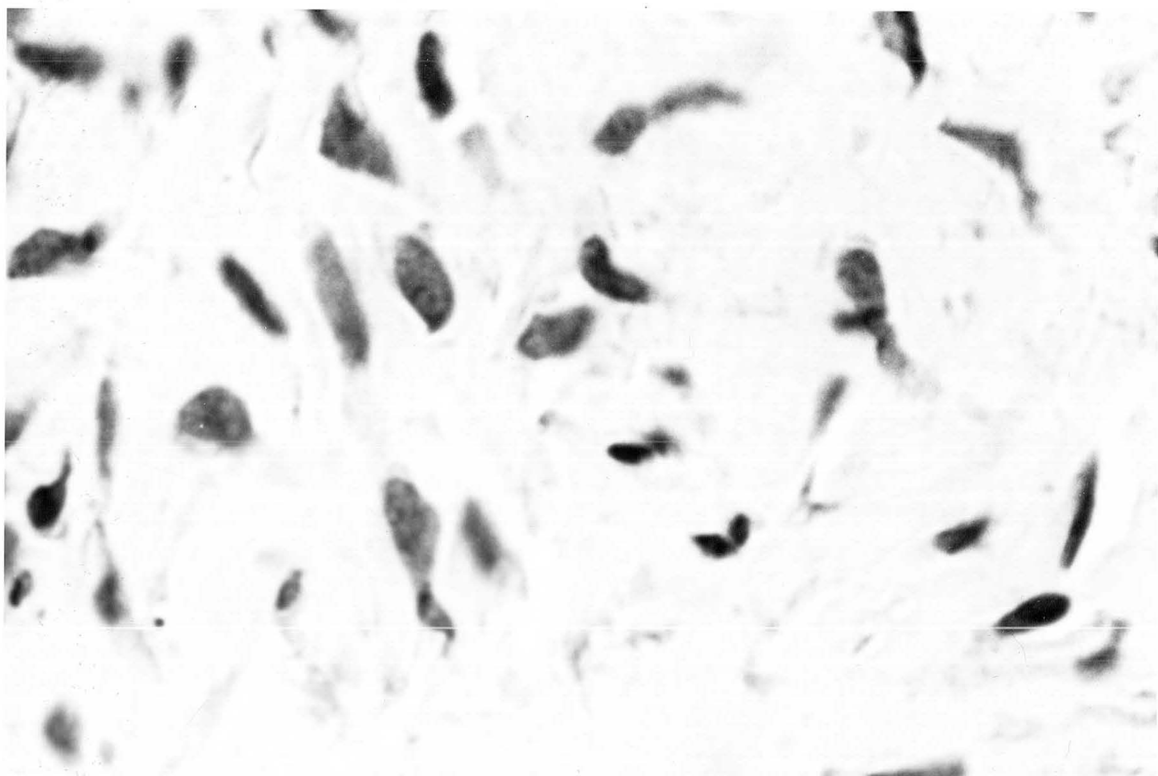


Figure 2b

Human Pulp Fibroblasts Held in an Outdoor Environment Showing Nuclear Pyknosis in the Early Stages of Putrefaction (Carbol-Fuchsin Stain, Original Magnification X 400).

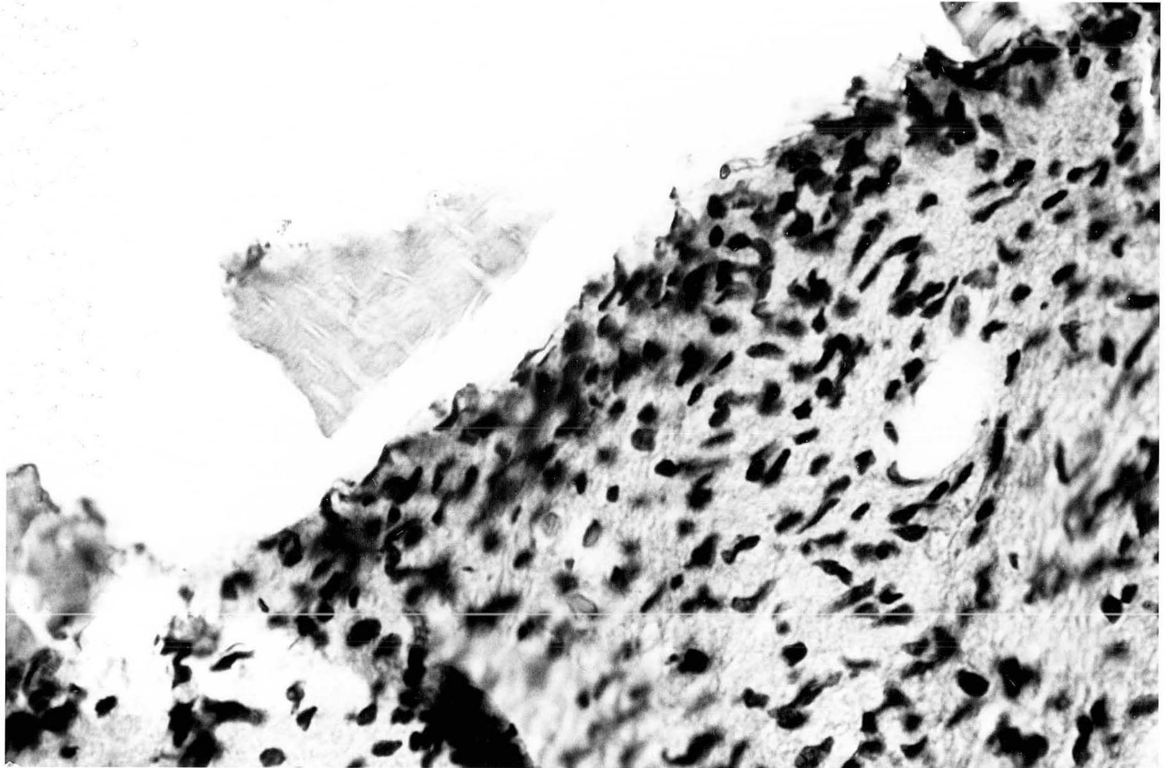


Figure 2c

Human Pulp Fibroblasts Held in an Outdoor Environment Showing Nuclear Debris in the Early Stages of Putrefaction (Carbol-Fuchsin Stain, Original Magnification X 400).

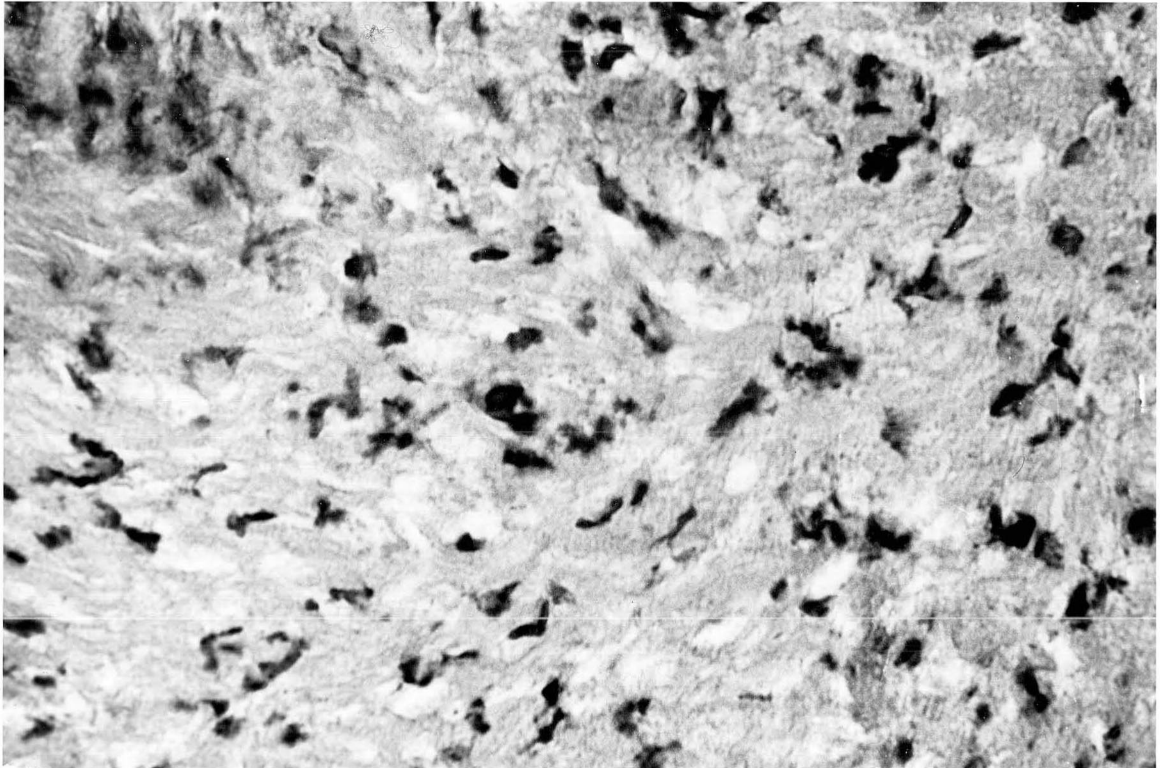
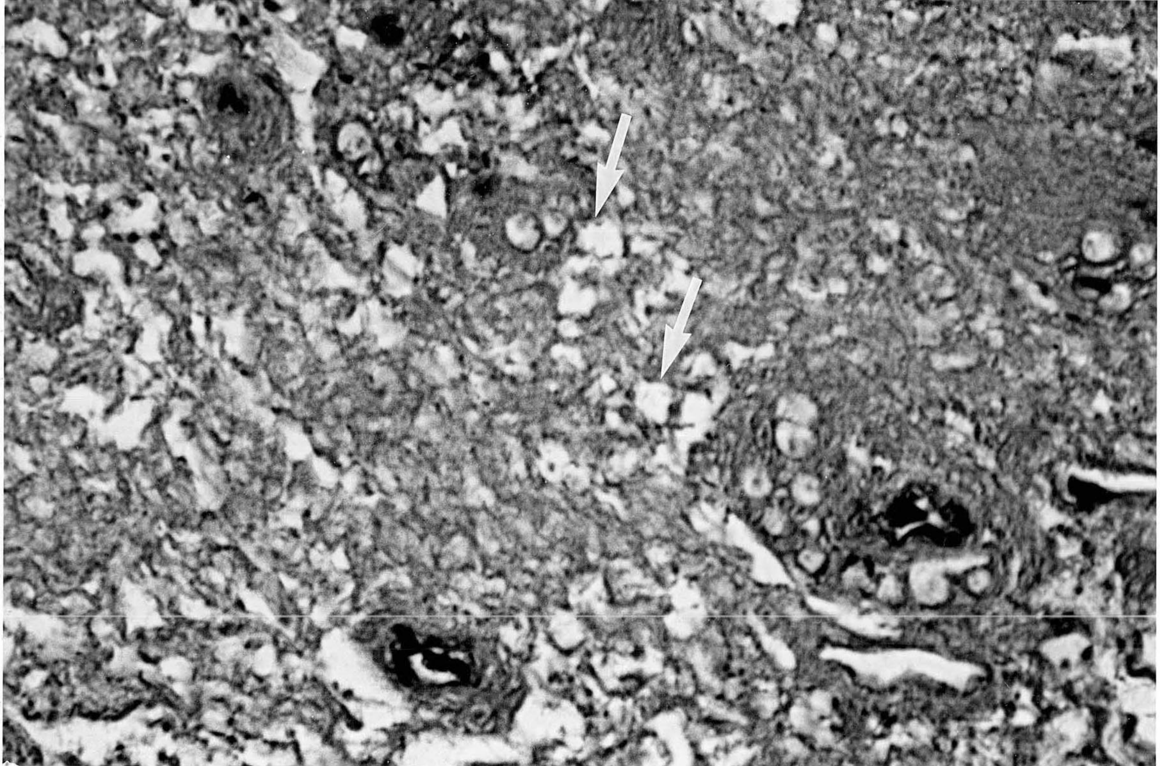


Figure 2d

Human Pulp Fibroblasts Held in an Outdoor Environment. Putrefaction of the Nuclei Leaves Tissue Spaces, "Cell Ghosts" (Carbol-Fuchsin Stain, Original Magnification X 400).



The experiment showed that even at day one some cellular destruction had occurred and that by the sixth day autolysis had claimed most of the cells of the pulp. No appreciable difference was observed in the rate of nuclear disintegration between odontoblasts and pulp cells. Yamamoto (1959:1) reported that in human tooth pulps exposed to the air in outdoor environments, nuclear atrophy in odontoblasts occurred on the first day, but not in the pulp cells until the second day. He states that odontoblast nuclei vacuolated and disappeared on the fourth day, but pulp cell nuclei remained to the fifteenth day. In teeth buried at 50 cm in sand, in a moisture controlled environment, he also found differential preservation in the two cell types: odontoblasts atrophied on the first day; pulp cells not until the fourth. This discrepancy may reflect the difference in climate between British Columbia (where decomposition is particularly rapid) and the locale in Japan. No climate data are recorded in the Japanese experiment. The surface tooth sample, however, was sheltered in a slatted box on a raised stand, and the buried sample was interred at a greater depth, 50 cm, as compared to the British Columbian material at 30 cm. Preservation of tissue over time is known to increase with increase in burial depth (Rodriguez and Bass 1985: 836). It should be noted that the human tooth sample used in the present experiment had been stored frozen for one week prior to the deposition, and it is possible that this treatment hastened still further the subsequent necrotic changes.

Sex Chromatin Preservation in Human Tooth Pulps Stored at Room Temperature

Pulps from human teeth stored at room temperature (~25C) on the laboratory bench could be used to determine sex for at least one year post storage, by performing chromatin counts on enzymatically digested tissue (Chap.4, Section A, Materials and Methods pp.55-56, Fig. 3a, 3b below). After the first day any decline in frequency of sex chromatin bodies did not follow a linear trend in relation to elapsed time (Table 4, and Graph 6 below).

Figure 3a

Enzyme Digestion of Fibres Frees the Pulp Cells from the Dry Tissue Matrix (Quinacrine Stain, Original Magnification X 400).

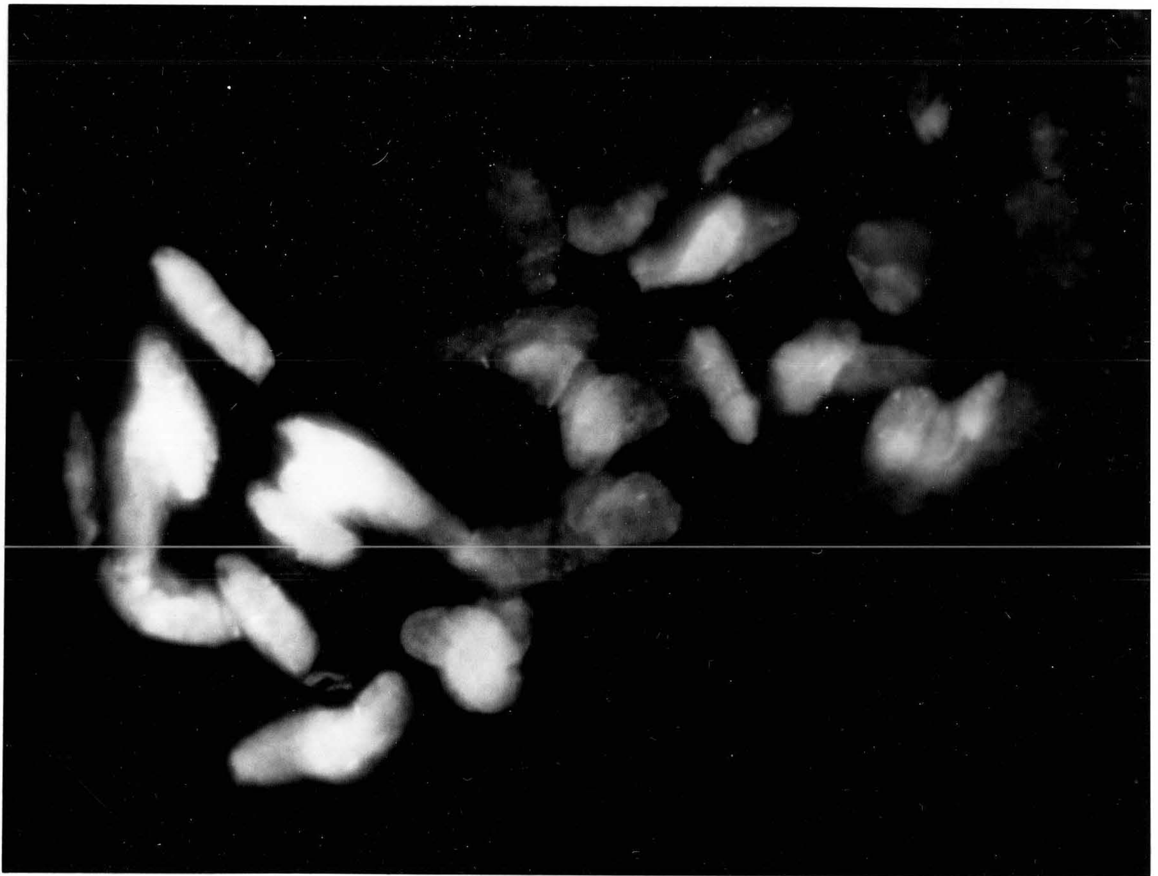
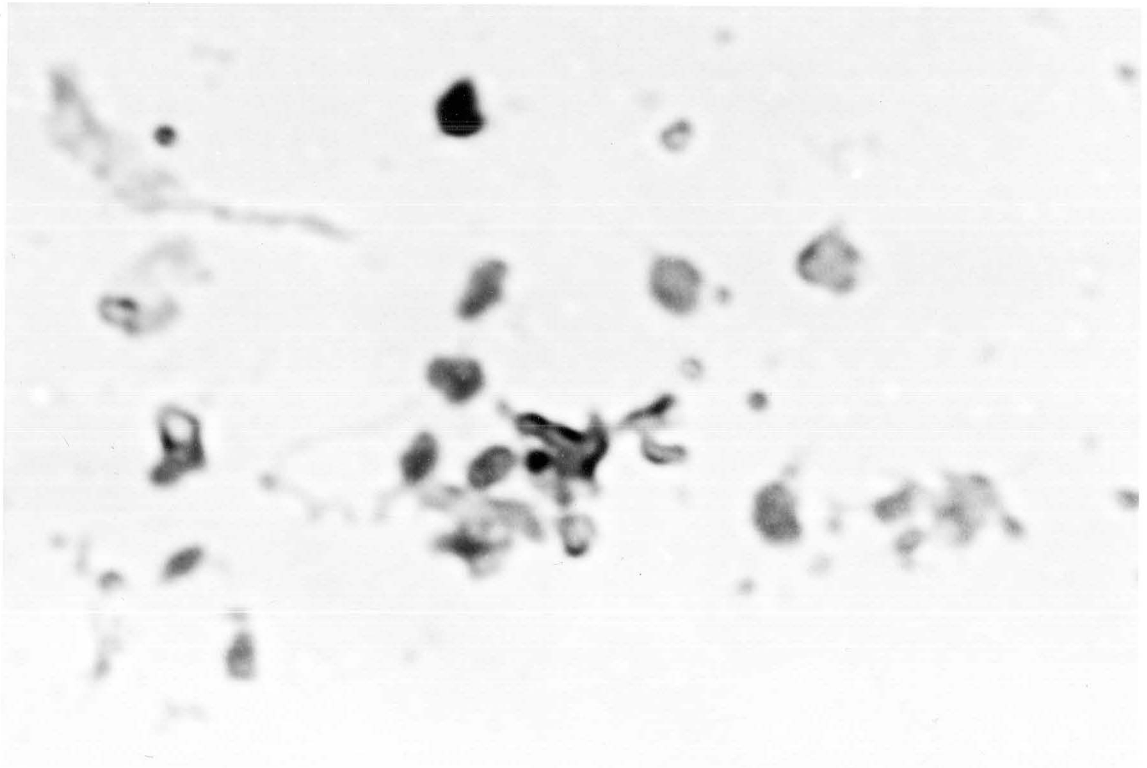


Figure 3b

Enzyme Digestion of Fibres Frees the Pulp Cells from the Dry Tissue Matrix (Carbol-Fuchsin Stain, Original Magnification X 400).



Sex Chromatin Counts on Human Dental Pulp Held at Room Temperature(25C)

TABLE 4

Pulp No.	Positive	Negative	% Positive	Average % Positive	SD.
FEMALE BARR BODY (Day 0)					
1	14	86	14	18.4	6.2
2	24	76	24		
3	28	72	28		
4	14	100	12		
5	18	100	15		
MALE OVERLAP (Day 0)					
1	3	101	3	1.7	0.9
2	1	99	1		
3	1	103	1		
4	1	99	1		
5	3	103	3		
Male Overlap difference: (t-test) P < 0.0005					
FEMALE BARR BODY (3 Months)					
1	12	81	13	11.0	2.1
2	10	100	9		
3	10	100	9		
4	11	100	10		
5	16	100	14		

Pulp No.	Positive	Negative	% Positive	Average % Positive	SD.
MALE OVERLAP (3 Months)					
1	2	101	2	3.1	0.7
2	3	107	3		
3	4	100	4		
4	4	100	4		
5	3	100	3		
Male Overlap difference: P < 0.0005					
FEMALE BARR BODY (6 Months)					
1	12	94	11	11.5	1.5
2	14	92	13		
3	15	100	13		
4	12	100	11		
5	10	100	9		
MALE OVERLAP (6 Months)					
1	0	100	0	1.7	1.6
2	4	110	4		
3	2	97	2		
4	0	99	0		
5	3	100	3		
Male Overlap difference (t-test) P < 0.0005					

FEMALE BARR BODY (9 Months)

Pulp No.	Positive	Negative	% Positive	Average % Positive	SD.
1	21	90	19	14.1	3.4
2	17	101	14		
3	19	103	16		
4	14	100	12		
5	10	98	9		

MALE OVERLAP (9 Months)

Pulp No.	Positive	Negative	% Positive	Average % Positive	SD.
1	4	99	4	3.3	0.4
2	4	114	3		
3	4	112	3		
4	3	102	3		
5	3	101	3		

Male Overlap difference: (t-test) $P < 0.0005$

FEMALE BARR BODY (12 Months)

Pulp No.	Positive	Negative	% Positive	Average % Positive	SD.
1	13	96	12	11.6	1.2
2	14	100	12		
3	10	100	9		
4	14	100	12		
5	14	100	12		

MALE OVERLAP (12 Months)

Pulp No.	Positive	Negative	% Positive	Average % Positive	SD.
1	4	102	4	2.7	1.2
2	4	100	4		
3	1	100	1		
4	3	102	3		
5	2	103	2		

Male Overlap difference(t-test) $P < .0005$

Sex Chromatin Counts on Dental Pulp Cells at Room Temperature(25C)

TABLE 5

MALE F-BODY (Day 0)

Pulp No.	Positive	Negative	% Positive	Average % Positive	SD.
1	96	167	37	54	14.8
2	81	122	40		
3	128	62	67		
4	94	31	75		
5	100	96	51		

FEMALE OVERLAP (Day 0)

Pulp No.	Positive	Negative	% Positive	Average % Positive	SD.
1	2	173	1	0.9	0.7
2	0	180	0		
3	0	78	0		
4	2	88	2		
5	1	89	1		

Female Overlap difference: (t-test) $P < 0.0005$

MALE F-BODY (3 Months)					
1	56	48	54	47.9	4.5
2	50	74	40		
3	49	50	49		
4	62	70	47		
5	57	60	49		

FEMALE OVERLAP (3 Months)					
1	6	101	6	4.6	1.2
2	4	97	4		
3	3	97	3		
4	6	100	6		
5	5	99	5		

Female Overlap difference: (t-test) $P < .0005$

MALE F-BODY (6 Months)					
1	54	43	56	47.3	5.3
2	51	52	50		
3	45	65	41		
4	45	60	43		
5	62	68	48		

FEMALE OVERLAP (6 Months)					
1	1	111	1	3.2	1.3
2	4	98	4		
3	4	139	3		
4	6	115	5		
5	4	116	3		

Female Overlap difference: $P < 0.0005$

MALE F-BODY (9 Months)

Pulp No.	Positive	Negative	% Positive	Average % Positive	SD.
1	70	43	62	58.3	3.9
2	66	55	55		
3	65	39	63		
4	71	49	59		
5	51	45	53		

FEMALE OVERLAP (9 Months)

Pulp No.	Positive	Negative	% Positive	Average % Positive	SD.
1	4	111	3	3.2	1.1
2	6	108	5		
3	3	123	2		
4	3	100	3		
5	2	103	2		

Female Overlap difference: $P < 0.0005$

MALE F-BODY (12 Months)

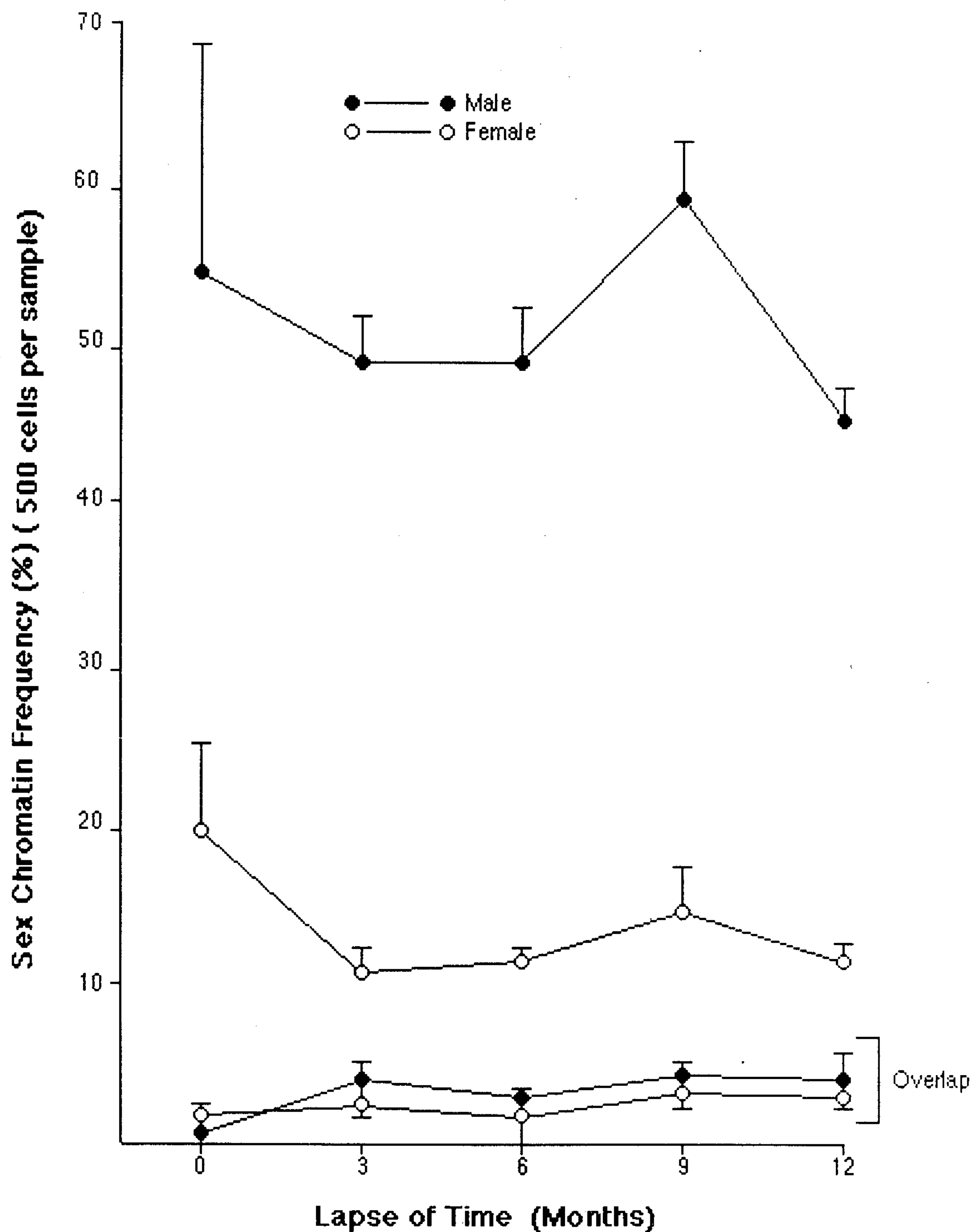
Pulp No.	Positive	Negative	% Positive	Average % Positive	SD.
1	64	76	46	44.5	2.1
2	57	70	45		
3	43	59	42		
4	44	60	42		
5	53	59	47		

FEMALE OVERLAP (12 Months)

Pulp No.	Positive	Negative	% Positive	Average % Positive	SD.
1	4	96	4	3.2	1.5
2	4	101	4		
3	2	101	2		
4	6	104	5		
5	1	101	1		

Female Overlap difference: (t-test) $P < 0.0005$

Graph 6



The variability in the frequency of Barr bodies (9-28%)over one year was in a lower range than reported by Ionesiy: (1980: 27) 29-52% for dental pulp stored in the same dry environmental context, although male overlap range (0-6%) was similar to that reported here (1-4%). The frequency of F-bodies, however, (37-75%, female overlap 0.9-4.6%) was lower than, but overlapped Ionesiy's finding of 60-89%, and was easily encompassed in the very broad range 15-92% reported by Seno (1977: 172) over the same period of elapsed time. Variation in frequency of intrasex chromatin bodies is usually accounted for by variation in tissue type (Mittwoch 1974: 73), but in this case all the tissue is dental pulp. The disparity between the data reported here and in the literature most likely reflects inter-observer error since sex chromatin evaluation involves subjectivity, as previously stated (p.76). Intersex variation, favouring higher counts for F-bodies, probably results from the stage of cell division: Barr bodies are detected only during prophase and interphase (Sharma 1972: 346), and F-bodies in both mitotic and interphase stages (Seno and Ishizu 1973:9).

The maximum variation in percent frequency of Barr bodies recorded at one time event was 14-28% (SD.6.2) at day 0 (Table 4 above). Ionesiy also recorded maximum variation at day 0 (31-52%). For F-bodies the maximum variation also occurs at day 0 (37-51% SD.14.8) in this study, in Ionesiy's work (75-89%), and in Seno's data 52-90% at the outset of the experiment: days 4-19. This may be explained by the highest sex chromatin counts being recorded in fresh tissue, followed by a decrease consequent on tissue

deterioration involving autolysis and putrefaction which continues until the tissue dried. Then the proportion of sex chromatin bodies becomes more uniform because of stasis of autolytic enzymes and diminished microbial activity from inaccessibility of the food supply; bacteria and fungi require moderate controlled moisture for their proliferation (Hedges et al. 1978: 377; Zivanovic 1982: 18).

Pulp tissue stored on the laboratory bench in extracted teeth dehydrates within 24 hours. This result was obtained from examining pulps from eight human and eight extracted pig teeth (see Chap.4,Section A,p.57). Unextracted pig teeth, removed from three pig jaws (two teeth per sample) in a time sequence of two days, four days, six days, and eight days, revealed wet pulps with decomposition of pulpal tissue and external tissue of the jaw, well under way within six days, and advancing rapidly. The process of decomposition was not examined in detail; it served only to reinforce the proposition that the explanation for the remarkable preservation observed in the bench stored human pulps is best explained by their rapid dessication.

Undoubtedly this experiment could have been continued beyond one year's storage; no endpoint was reached when the experiment concluded. Furthermore, Seno's experiments detecting F-bodies,were successfully extended over a three year period still without encountering any constraint in relation to elapsed time.

Results of the Effects of Heat on Dental Pulp Subjected to Sustained Heat

Extracted human and pig teeth and unextracted pig teeth were heated at controlled temperatures (100-350C) for sustained periods (one to three hours) (Materials and Methods, Chap.4, Section A,p.57). Extracted human teeth were heated for one hour at temperature intervals of 50C, in a range from 100-350C. At 100C the pulps were still firmly attached to the walls of the pulp cavity; their gross appearance was of a brown gelatinous mass of fibrous tissue within which the cells were firmly embedded. At 150C shrinkage had detached the pulps from the walls of the pulp cavity. They appeared brown, gelatinous and more dried. The pulps were also detached, and brown coloured at 200 and 300C, but at this temperature they appeared dry and papery.

Cells were separated from the pulps by enzymatic digestion (Materials and Methods,Chap.4, Section A, pp.56-57) and examined microscopically with aceto-orcein stain. At 100C both pig and human pulp cells appeared normal, at 150C almost acellular, and beyond that temperature, enzymatic digestion produced negative results.

Tissue subjected to 100C was selected for sex chromatin staining (Materials and Methods, Chap 4, Section A,pp.55-56; Table 5 and Graph 7 below).

TABLE 5

Proportion of Human Dental Pulps with Demonstrable Sex Chromatin in Teeth Held at 100C for One Hour

Pulp No.	Positive	Negative	% Positive	Average % Positive	S.D.
FEMALE BARR BODY					
1	20	80	20.0	13.6	3.4
2	13	100	11.5		
3	13	109	10.6		
4	13	100	11.5		
5	15	90	14.3		
MALE OVERLAP					
1	4	98	3.9	2.7	1.1
2	2	102	1.9		
3	3	100	2.9		
4	4	103	3.7		
5	1	106	0.9		
MALE F-BODY					
1	49	59	45.3	40.2	5.2
2	45	60	42.8		
3	33	76	30.3		
4	40	55	42.0		
5	42	62	40.4		

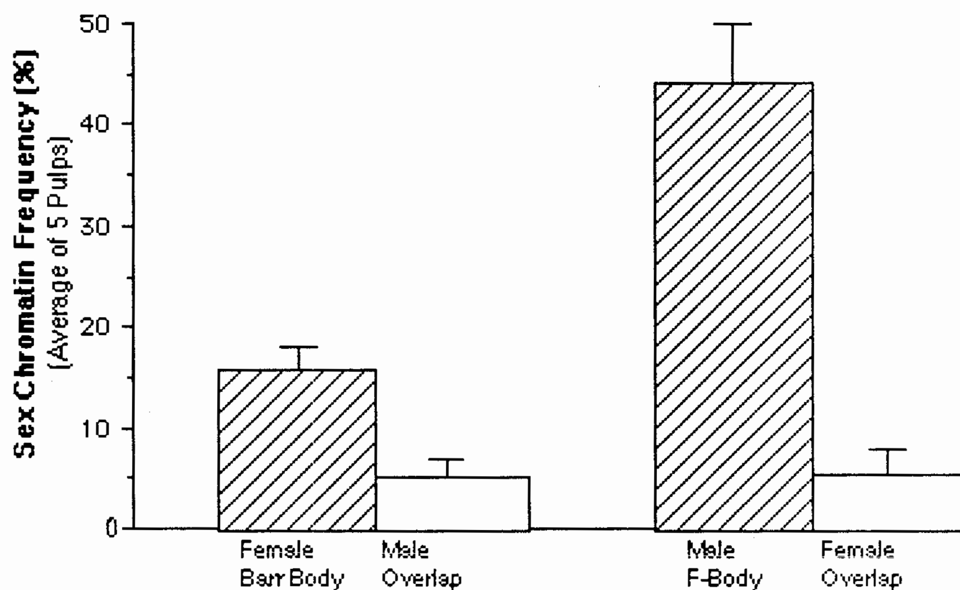
Male Overlap difference:(t-test) $P < 0.0005$

FEMALE OVERLAP

Pulp No.	Positive	Negative	% Positive	Average % Positive	SD.
1	1	101	1.0	2.9	1.3
2	2	100	2.0		
3	3	99	2.9		
4	4	102	3.8		
5	5	104	4.6		

Female Overlap difference:(t-test) $P < 0.0005$

Graph 7



The range of Barr bodies reported here, 10.6-20% (Table 5 above) is considerably lower than that reported by Ionesiy (1980: 27):33-49%, who also heated extracted teeth at 100C for one hour. When Barr body counts are made from tissue sections the percent frequency sometimes reaches close to 50% (see p.74) but Ionesiy's counts were made on cell smears separated from the fibrous matrix by softening the tissue in acetic acid and then breaking it apart with a pestle and mortar. His male overlap(0-4%) is similar to this report (0.9-3.9), making this large variation in Barr counts difficult to dismiss in terms of inter-observer error. His Barr-body enumeration for pulp tissue dried at room temperature was also high and outside the range reported here.

Ionesiy's range for F-bodies is also higher than reported here: 71-83% against 55-76% in this study. In contrast, Seno (1977: 172), reports F-bodies in teeth similarly heated in a range 43-65%. The concensus,however, in this research is that 100C for one hour is the maximum temperature that allows sex chromatin bodies to be evaluated in extracted human teeth.

Extracted pig teeth and fleshed pig jaws were also subjected to heat at varying temperature and time periods. Since pig teeth could not be assessed for sex chromatin (see p.8), the comparison was confined to the gross appearance of the tissue and microscopic tissue morphology. Pulp from extracted pig teeth heated for one hour at 50C intervals between 100-300C presented a more dry and papery gross appearance than did human pulps, but appeared similar histologically to human pulps at the same temperature. However, in teeth heated in fleshed pig jaws at 300C for one hour, the

gross appearance and microscopic morphology of the pulpal tissue was similar to that of extracted pig and human teeth at 100C (Table 6,and Figs.4a,4b below).At 100C the jaw tissue looked red, wet and almost fresh. Cell smears were made without any tissue processing. At 150C and 200C it was still possible to make smears although the tissue appeared more gelatinous. From 250 to 350C the cells were sufficiently encased in the fibrous matrix to make enzymatic processing necessary to free the cells for further examination. The tissue was then brown in appearance,but still adhering to the walls of the pulp chamber.

At the microscopic level,by aceto-orcein staining,many intact cells were observed to 300C,but not beyond. Fleshed pigs jaws were further tested at 300C. At one hour and one and a half hours nuclear morphology appeared normal: most nuclear membranes were intact and the nuclear granules looked normally distributed. By two, and two and a half hours most nuclear membranes were still intact but the granules looked distorted when compared to a fresh control. At three hours few intact nuclei remained. This set of experiments shows that heat penetration is very much retarded by the surrounding soft and hard tissue of the jaw. It is not possible from these experiments to deduce the temperature of the pulp itself under these conditions of heat. Moreover,heating material in an oven does not closely approximate a forensic situation involving fire. These deficiencies were to some extent rectified by conducting further experiments applying direct heat and determining the temperature in the pulp chambers by inserting thermocouple probes.

TABLE 6

HEATED HUMAN AND PIG ELEMENTS

Tooth Type	Heating Time	Critical T° for Nuclear Integrity					
		100C	150c	200C	250C	300C	350C
Human Extracted	1 hour	X					
Pig Extracted	1 hour	X					
Pig Unextracted	> 1 hour					X	

Figure 4a

Fresh Pig Pulp Fibroblast (Carbol Fuchsin-Stain, Original Magnification X 1000).

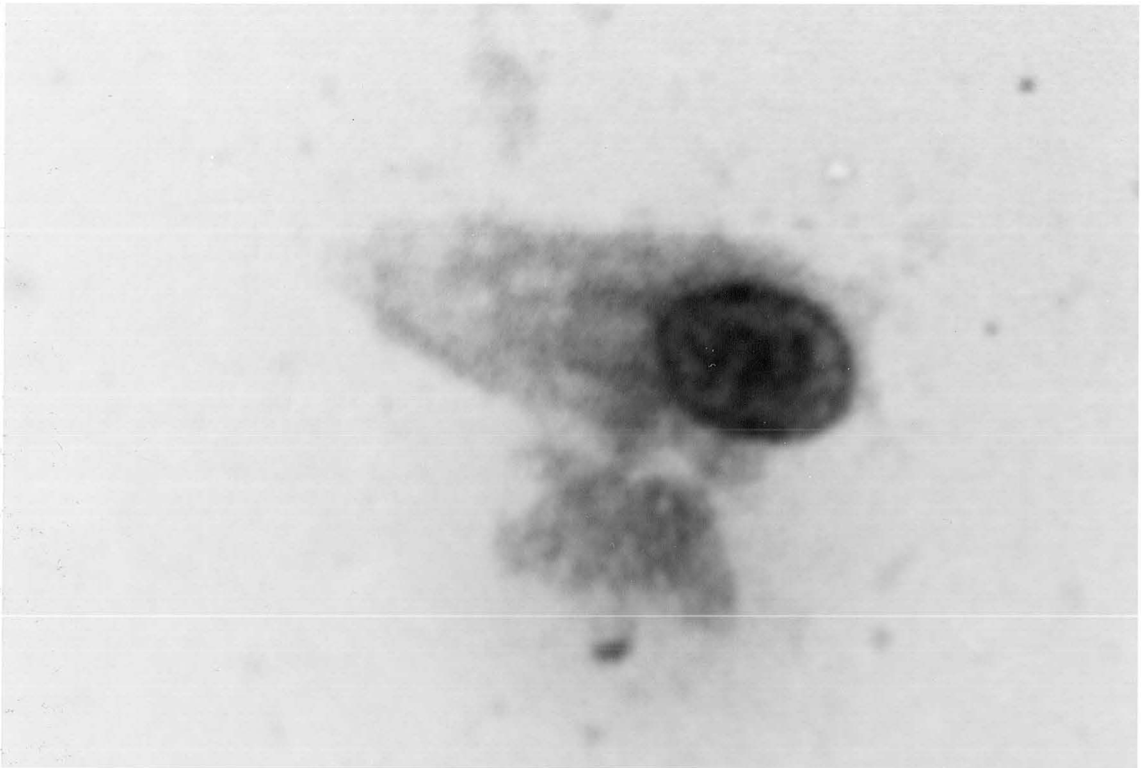
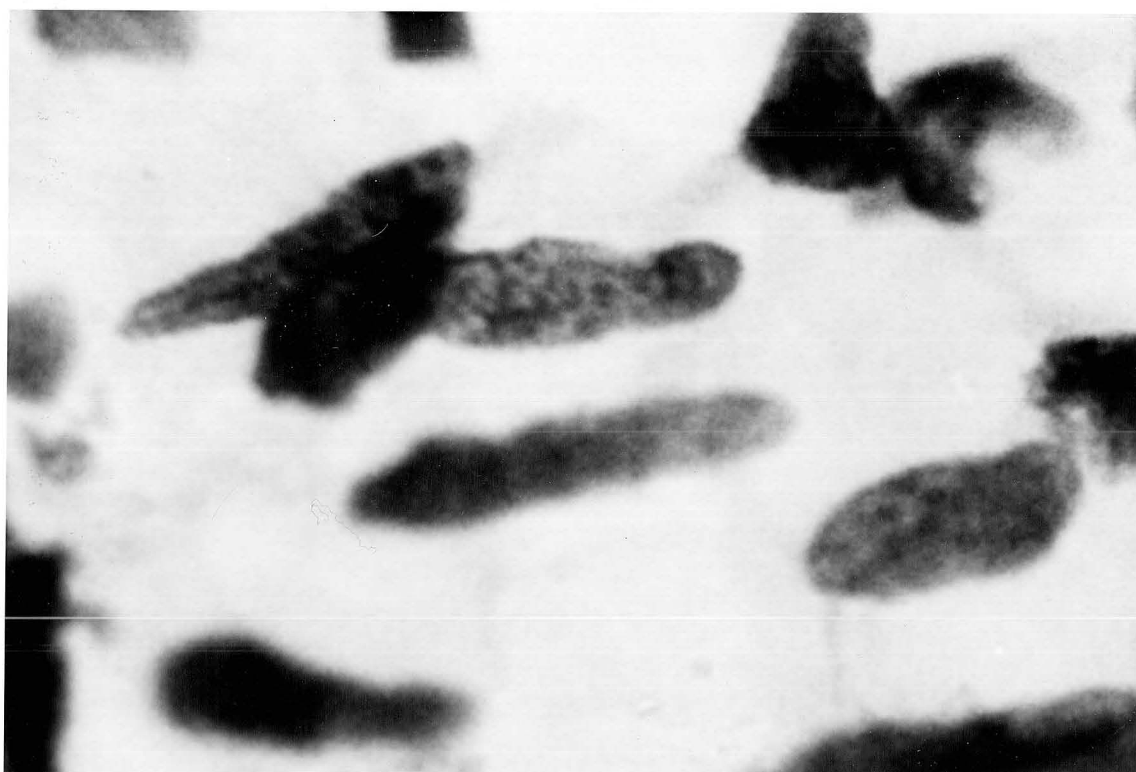


Figure 4b

Pig Pulp Fibroblasts from Unextracted Teeth Held at 300C for One Hour (Carbol-Fuchsin Stain, Original Magnification X 1000).



Results of Preliminary Laboratory Experiments on Human Teeth Prior to Outdoor Incineration

Preliminary experiments, designed to determine the critical temperature beyond which sex chromatin diagnosis is no longer possible, were conducted on samples of human male and female teeth, of which one per sample of five had a thermocouple wire incorporated in the pulp chamber. The teeth were subjected to direct heat over a bunsen burner flame (Materials and Methods, Chap.4, Section A., p.59). Temperatures of 50C increments were tested in a range 25-100C. Macroscopic and microscopic examination of the pulp tissue showed that:

1. At 25C pulps looked red, wet and fresh and the cells were undamaged.

2. At 50C, pulps were brown,but most of the nuclei appeared normal. Some premolars (four) and incisors (one) included in this experiment showed more evidence of cell damage than the molars; probably anterior pulps are more rapidly penetrated by heat because of their smaller size. However, pulps from molars, premolar and incisors all proved equally suitable for this purpose (Table 7 and Graph 8 below). 50C was therefore selected as representing the maximum temperature at which sex chromatin could be evaluated. It should be noted that when teeth were removed from the heat source the temperature within the cavity continued to rise to 75C (as previously stated p.58).

3. At 75C pulps were brown, but still moist. However, many cells

were disrupted, fibroblast and odontoblast nuclei on the periphery of the pulp i.e. proximal to the coronal dentine, were less damaged than the fibroblasts at a deeper level within the pulp, perhaps because the apical aperture allowed more rapid heat penetration to this area. A few cells were still intact and their nuclei presented a normal appearance.

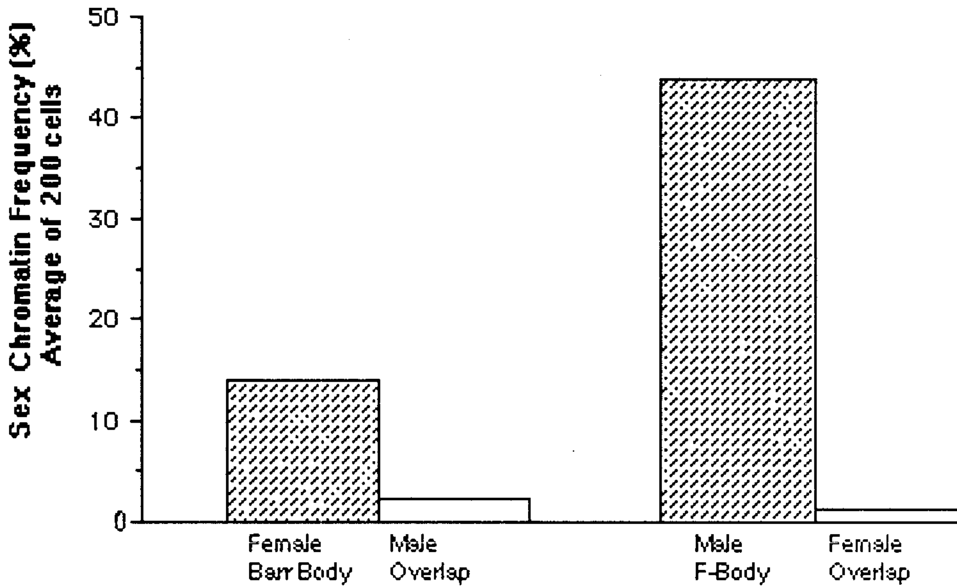
4.At 100C pulps were brown and moist, but most nuclei were destroyed, or disrupted, and of the remaining intact nuclei most were degranulated.

TABLE 7

Proportion of Sex Chromatin in Thermocoupled Human Pulps at Temperatures Between 50 to 75C

Tooth Type	Positive	Negative	% Positive	Average % Positive
BARR BODIES				
Molars (N=1)	14	87	14	13.0
Premolars (N=4)	12	89	12	
MALE OVERLAP				
Molars (N=1)	3	102	3	1.5
Premolars (N=4)	0	102	0	
F-BODIES				
Molars (N=3)	44	67	40	41.5
Incisors (N=1)	43	57	43	
FEMALE OVERLAP				
MOLARS (N=3)	2	101	2	1.0
INCISORS (N=1)	1	99	1	

Graph 8



Results of Preliminary Laboratory Experiments on Incinerated Pig Pulp

Comparison of pulps both macroscopically and microscopically showed little difference in pulpal damage between human and pig extracted teeth at the same temperatures (25,50 and 75C). At 25C the pulps were wet and fresh looking and the cells appeared normal against a fresh control. At 50C the tissues were still red and moist and most of the cell nuclei were intact and granular. By 75C the pulps were moist and brown; although some nuclei were still intact they had lost their granulation. The temperature in the pulp chamber rose by about 25C upon removal from the heat source.

Results of Field Experiments: Pig Heads in an Open Fire

Based on the results of the laboratory experiments above, three pigs' heads (mean initial temperature 15.5C) were placed in a log fire of Douglas fir and pine with thermocouple wires inserted into their lower right I_2's. Each head was retrieved rapidly from the heat source as soon as the critical temperature was achieved in the pulp chamber (25C, 50C and 75C). The temperature within each pulp chamber still continued to rise after retrieval by an increment of approximately 25C.

The thickness of soft and hard tissue around the pulp creates a barrier that affords some protection against rapid heat penetration. The surrounding thickness of pig and human jaw and tongue tissue was compared to establish any variation which might account for differential damage to pulps. The overall thickness measures proved to be very similar at the gross level(Tables 8 and

9 below), although variation may exist in relation to tissue type i.e. soft tissue, enamel or dentine.

TABLE 8

Thickness of tissue from the lingual gingival-crown junction of the lower right I_2, to the same point on the external surface of the jaw:

HUMANS (cm)		PIGS(cm)
Females	Males	Sex Unknown
1.6	1.5	1.6
1.3	1.2	1.5
0.2	1.2	1.5
1.8	1.3	1.6
0.3	1.4	1.5
Average (males plus females): 1.38		Average: 1.54

TABLE 9

Maximum thickness of the tip of the tongue at midline.

HUMANS (cm)		PIGS (cm)
Females	Males	Sex Unknown
1.0	0.5	0.8
1.0	0.7	0.9
0.9	1.0	0.9
1.1	1.0	0.8
0.7	0.7	0.9
Average (male plus female): 0.86		Average: 0.86

The time elapsed between lighting the fire and achieving the temperature of interest within the pulp chamber was about 8.5 minutes. The temperature of the fire when the pulp chamber registered 25C was 529C, but fluctuated between 450 and 600C because of air currents. When the pulp chamber registered 50C, the temperature of the fire had risen to 600C, fluctuating between 500 and 700C, and at a pulp chamber reading of 75C, the fire temperature had risen to 675C, fluctuating between 650-750C (Table 10 below).

Incisors (I_1's) were extracted from the incinerated pig heads for further study. The pulps heated to 25C appeared red and wet. Paraffin sections showed them to be undamaged. At 50C, pulps were brown, and moist; in section most nuclei appeared intact and granular (Fig. 5a below). At 75C the pulps were brown and moist, in section many nuclei were disrupted and of those still intact the granules appeared disorganized, with many of them clumped (Fig. 5b below).

TABLE 10

Outdoor Incineration of Pig Heads with Thermocoupled I_2

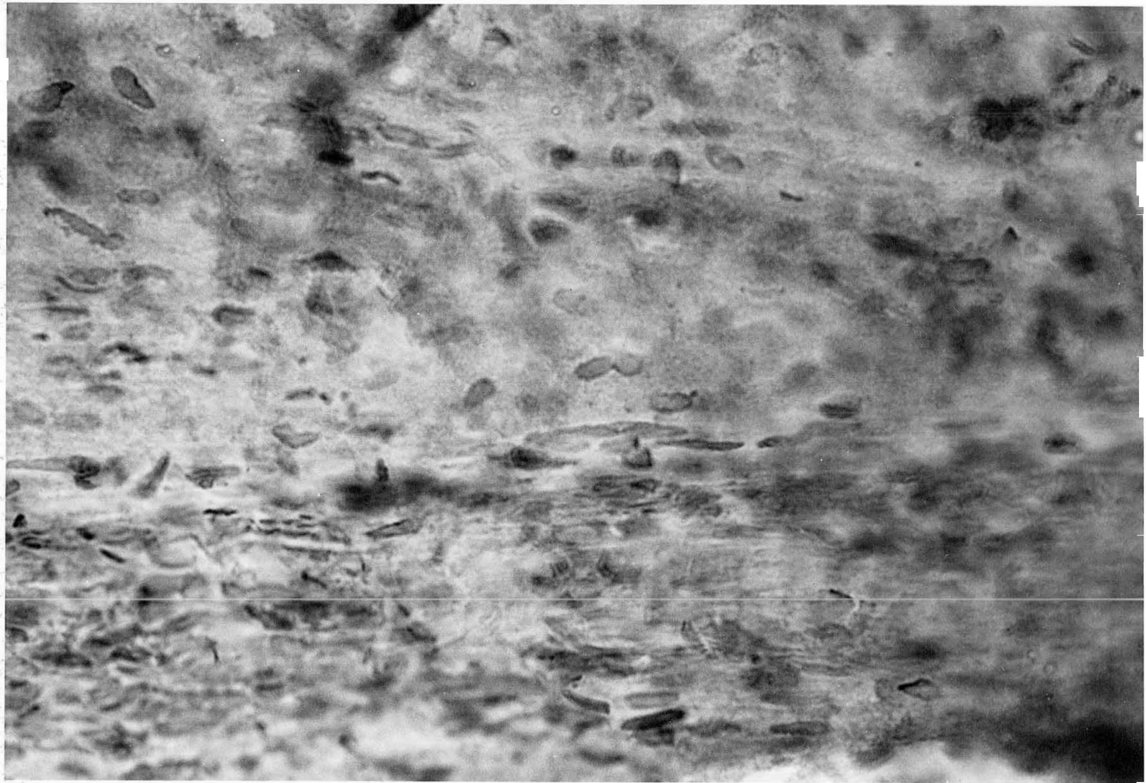
T° of I_2 Chamber	T° Rise Post-Retrieval	T° of Fire	Pulp Status
25C	25C	529C (450-600C)	Undamaged
50C	25C	600C (500-700C)	Mostly Undamaged
75C	25C	675C (650-750C)	Severely Damaged

Figure 5a

Pig Pulp Tissue from Unextracted Teeth Taken from an Open Fire When the Pulp Chamber Registered 50-75C (Carbol-Fuchsin Stain, Original Magnification X 400). The Cell Nuclei Are Still Intact and Granular

Figure 5b

Pig Pulp Tissue from Unextracted Teeth Taken from an Open Fire When the Pulp Chamber Registered 75-100C (Carbol-Fuchsin Stain, Original Magnification X 400). The Cell Nuclei Are Degranulated



The results of these experiments suggest that a temperature between 50C and 75C within the pulp chamber is the critical range beyond which severe nuclear damage occurs in both pig and human pulps under high temperature conditions. The findings of the final experiment, however, at best represent preliminary results since the data is based on a small sample size (N=3).

CHAPTER FIVE: SECTION B

TEST RESULTS FOR THE H-Y MOLECULE AND β_2-MICROGLOBULIN

Results of Preliminary Peroxidase-Antiperoxidase (PAP) Testing for H-Y and B_2-Microglobulin

The results of the Peroxidase-Antiperoxidase (PAP) testing the binding of the gw-16 mouse anti-H-Y monoclonal from ascites fluid to fresh pulp cells (Materials and Methods, Chap.4, Section B,pp.63-65) were less than satisfactory, failing to discriminate sexually; positive pulp cells stained weakly and both males and female pulp cells were positive to an endpoint titer of 1: 128. Controls were all negative. The mouse antihuman B_2-microglobulin fared better, staining male and female pulp cells darkly at the manufacturer's recommended dilution (1: 500). All controls were negative (see Graph 9,and Figs.6a,6b below).

Graph 9

Human Dental Pulp Fibroblasts Binding gw-16 (Anti-H-Y) and anti-B Microglobulin Reactions in a Peroxidase-Antiperoxidase Test (gw-16 shows no Sex Specific Reactivity)

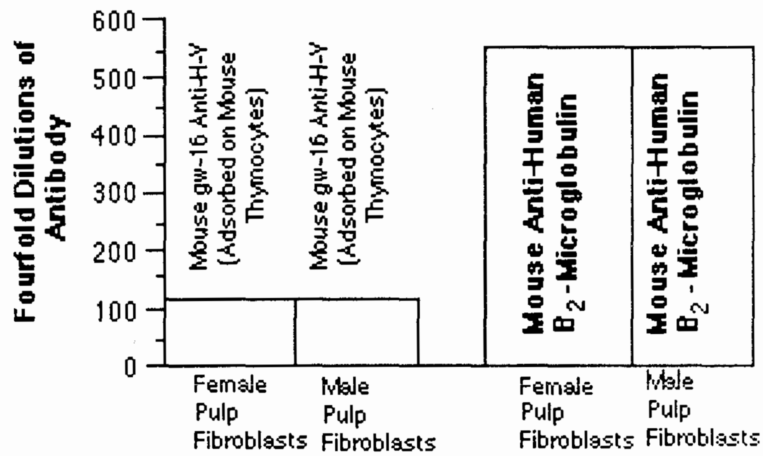


Figure 6a

Human Pulp Cells Bind Antibodies to H-Y Molecules in a PAP Test. Both Males and Females Show the Same Positive Reaction (Original Magnification X 1000).

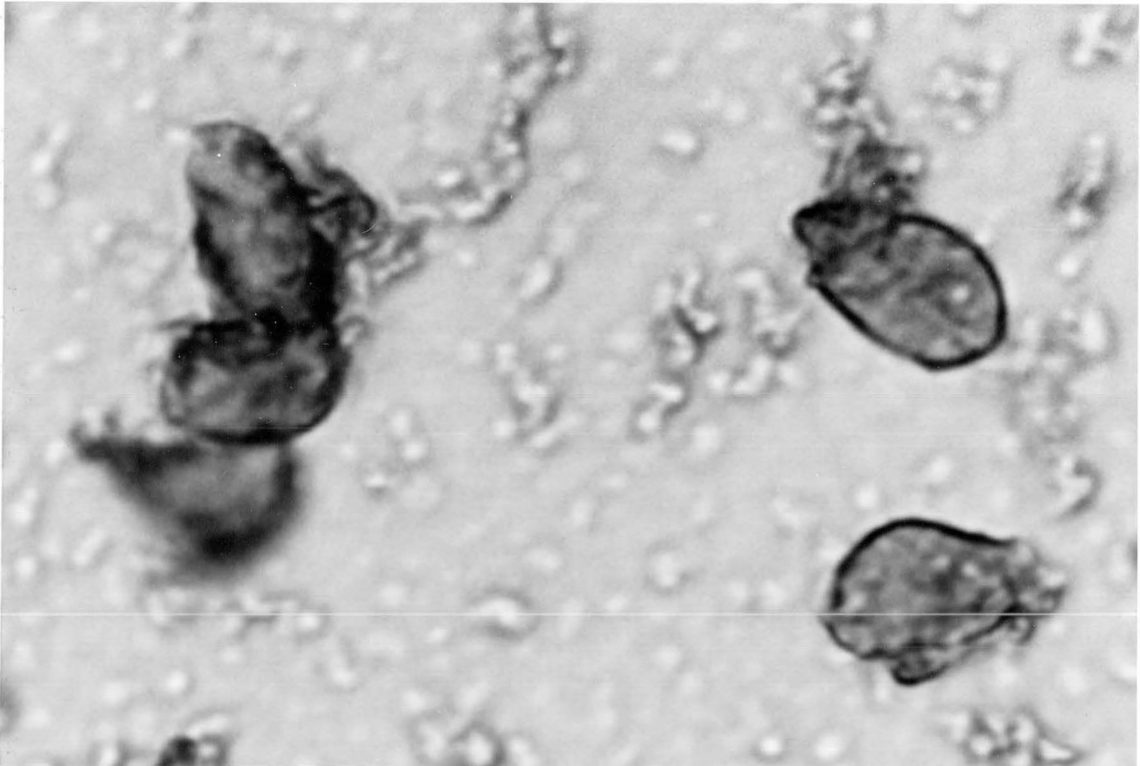
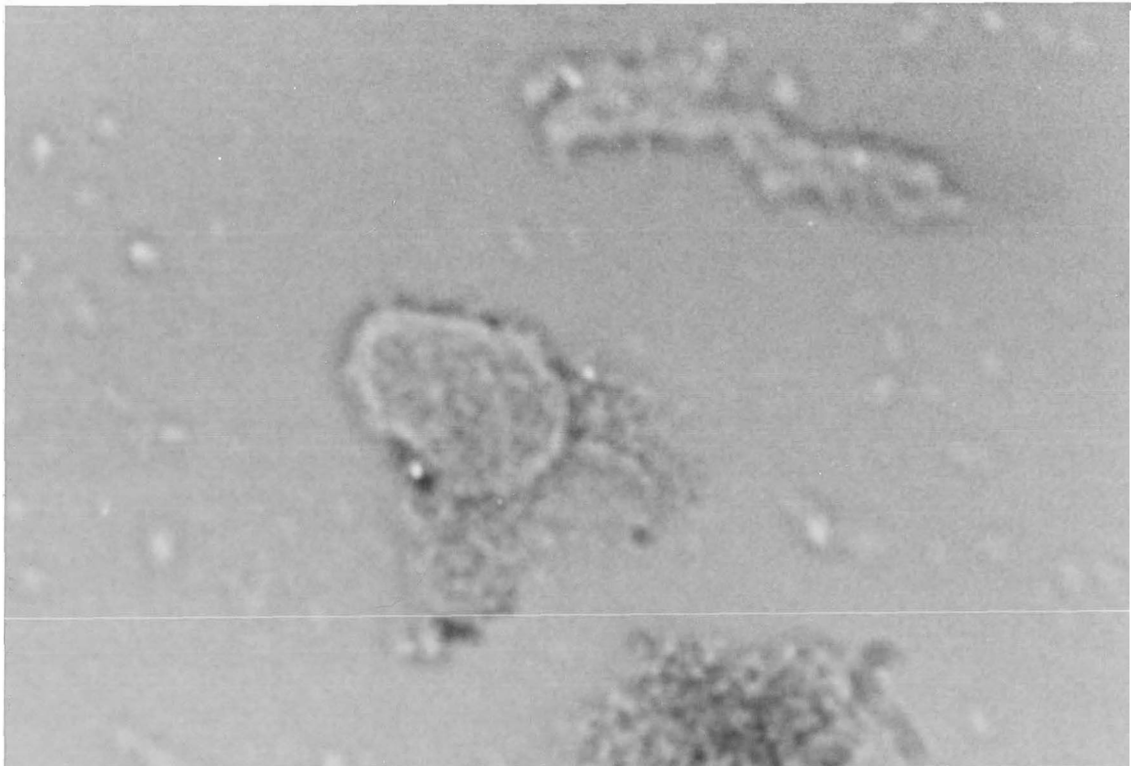


Figure 6b

Human Pulp Cells, Unstained Controls (No Primary Antibody) in a PAP Test (Original Magnification X 1000).



The gw-16 monoclonal has given low titers in other test systems (Brunner et al. 1987 a: 1[illegible]; Brunner et al. 1987b: 122; Jaswaney and Wachtel 1987: 115). Moreover, it is necessary to adsorb it to remove as many potentially cross- reactive contaminants as possible. Such contaminants can arise from the process of monoclonal antibody production, for example non-specific IgG in ascites fluid (Brunner et al.1987b: 122), or from interactions intrinsic to the test system: heterophile antibodies cross reacting between mouse and human, or autoantibodies induced by sensitization of male mice with female spleen cells (Jaswaney and Wachtel 1987: 115).In addition, the adsorption procedure itself (in this case gw-16 on female mouse thymocytes) may release cross reactive or blocking contaminants from the adsorbing cells (Brunner et al. 1987a: 181); it also further dilutes the antibody and in so doing may produce a reduction in the titer.

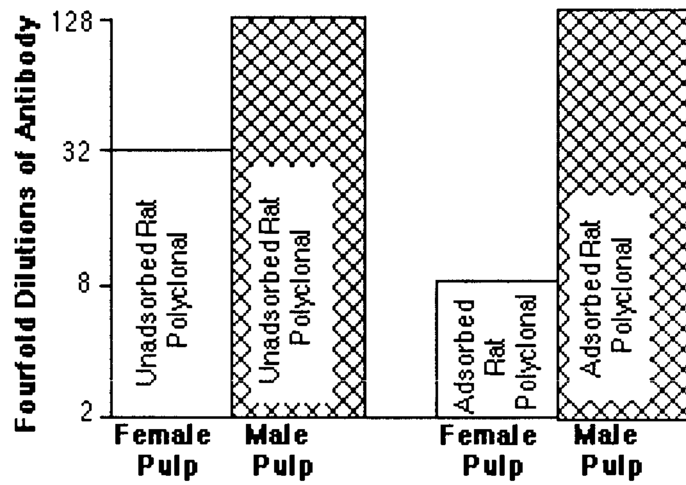
The failure of gw-16 to discriminate sex in the PAP test presented here, may be contingent on a weak antibody with a differential male-female binding capacity that is not detected in a coarse-grained fourfold dilution in a PAP test, scored simply as positive or negative. This system is perhaps less sensitive than the methods used by other researchers working with gw-16,such as the enzyme linked immunosorbent assay (ELISA),in which scoring is based on an optical density reading, or flow cytometry which measures intensity of fluorescence.

Results of Preliminary Indirect Immunofluorescence Testing (IFA) for H-Y and B_2-Microglobulin

After the failure of the gw-16 monoclonal to discriminate sexually in the PAP test, the procedure was abandoned in favour of a polyclonal antibody raised in our laboratory in Lewis rats and an indirect immunofluorescence (IFA) test (Materials and Methods, Chap.4, Section B, pp.64-66). Unadsorbed rat antisera discriminated between males and females (male titer 1: 128, female titer 1: 32), after adsorption the male titer remained the same but the female titer dropped to 1: 8. The positive control consisting of male peripheral leukocytes also gave a titer of 1: 128, as did mouse antihuman B_2-microglobulin in the same test system. All negative controls were satisfactory (Graphs 10,11 and Figs. 7a,7b below).

Graph 10

Indirect Immunofluorescence Antibody Test: Unadsorbed and Adsorbed Rat Polyclonal Anti - H-Y Binding to Human Pulp Cells



Graph 11

Indirect Immunofluorescence Antibody Test: Anti - B_2-Microglobulin Binding to Human Pulp Cells

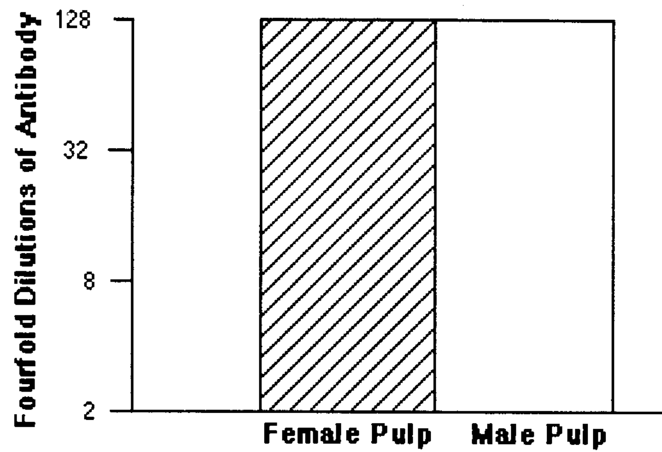


Figure 7a

Human Male Pulp Cells Bind Antibodies to H-Y Molecules (FITC Tagged Antibody, Original Magnification X 1000). Cells from Females Were Unstained.

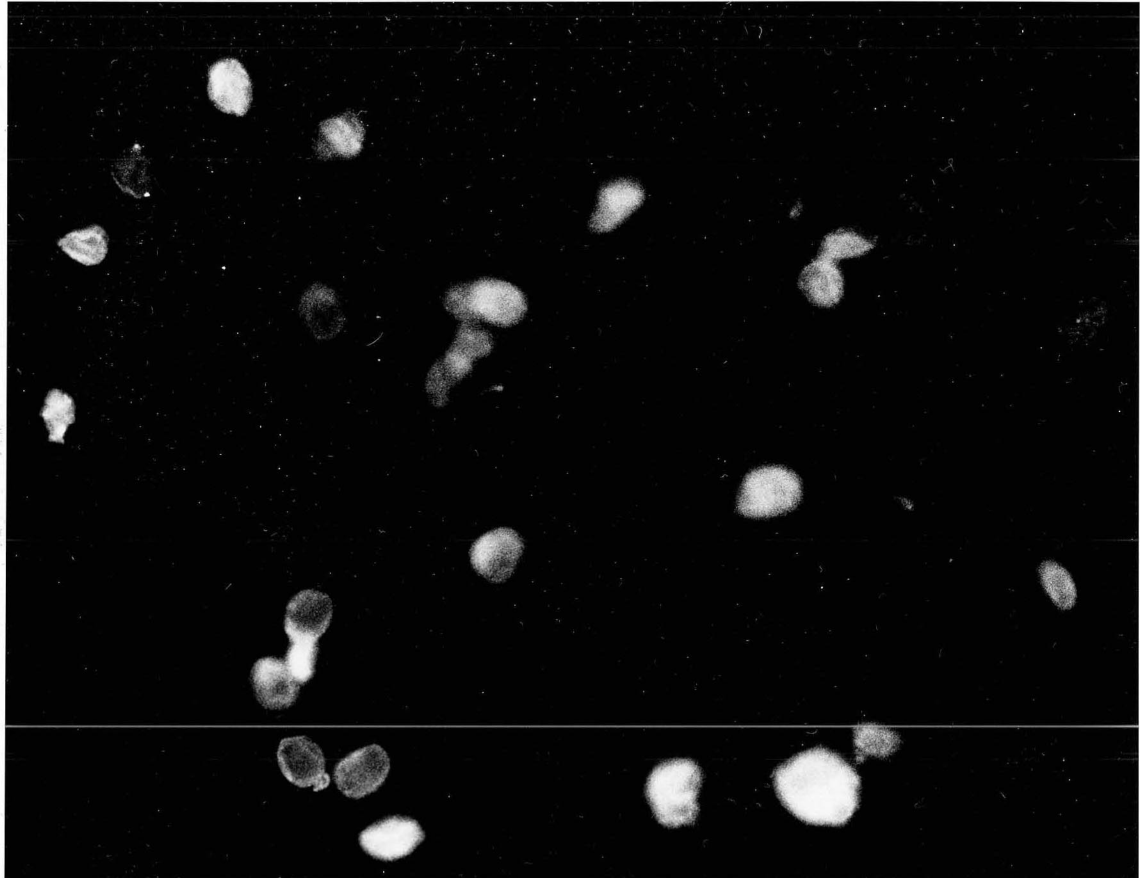
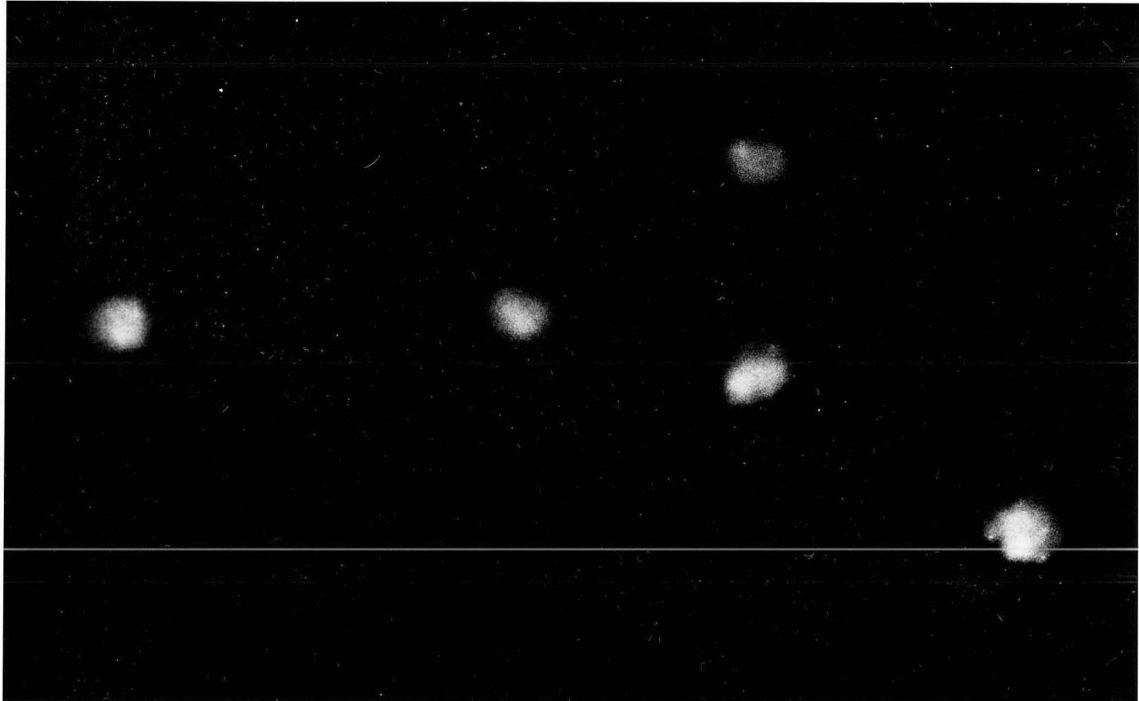


Figure 7b

Human Male Pulp Cells Bind Antibodies to β_2-Microglobulin (FITC Tagged Antibody, Original Magnification X 1000).



The fairly low H-Y titers recorded for this test, despite the avidity ascribed to the polyclonal antibody raised by intrasplenic immunization, are best explained by the fact that the IFA is a much less sensitive test than the PAP or the ELISA (the test system used in the original development of the immunization protocol). The insensitivity of the IFA also explains the reduction in titer of the B_2-microblobulin antiserum from 1: 500 (manufacturers recommended dilution) in the PAP to 1: 128 by IFA. Regardless of whether the rat antiserum was adsorbed or unadsorbed, the titer for male pulp cells by IFA was the same, i.e. 1: 128. However, a reduction in titer may be masked by a fourfold dilution increment, or the IFA may be too insensitive to detect a relatively small reduction.

Although this test proved to be successful in detecting the male specific component of H-Y, and B_2-microglobulin in fresh tissue, extending the test to thawed tissue resulted in retention of the B_2-microglobulin titer, but total loss of H-Y antibody binding. The fresh dental pulp consisted of teeth from the University of British Columbia dental clinic, processed within a few minutes post-extraction. Smears of pulp cells were rapidly fixed, and slides, wrapped in parafilm stored at -20C. The peripheral blood leukocytes comprised cells which, immediately after the blood sample was taken, were separated on ficoll-hypaque, and stored in liquid nitrogen while the cells were still viable. This material upon retrieval gave successful results. Teeth which were refrigerated overnight, by a Lower Mainland dental surgeon, then transported to the laboratory on ice gave

pulp smears negative for H-Y antigen, as did pulps stored frozen at -20C.

CHAPTER SIX

SUMMARY AND DISCUSSION

Summary

Two procedures were set up for sex determination from soft tissue, in this case dental pulp. The diagnostic characteristics were then tested for their stability in forensic contexts. The first procedure detected X and Y sex chromatin in the nuclei of pulp cell fibroblasts by carbol fuchsin or quinacrine staining respectively. In outdoor environments, sex chromatin markers proved stable for two weeks or less. Very similar decomposition rates pertained whether teeth were deposited surface or subsurface (30 cm) in British Columbia. The same rate of decomposition was also observed in the pulps of unextracted pig teeth deposited concomitantly.

Detection of sex chromatin could be accomplished for at least one year in pulpal tissue stored on the laboratory bench at an ambient temperature of about 25C. Presumably tissue stability is attributed to rapid dessication of pulpal tissue: within 24 hours. The pulp tissue in unextracted pig teeth rapidly decomposed in the same environment; presumably putrefaction occurred because the surrounding tissue of the jaw maintained a moist environment in the pulp chamber.

Sex chromatin remained stable in the dental fibroblasts of human teeth heated to 100C for one hour, but not longer. In unextracted pig teeth this temperature could be raised to 300C for

more than one hour before severe pulpal damage was detected histologically. A critical temperature range between 50 and 75C could be reached in the pulp chamber, but not sustained (determined by insertion of a thermocouple probe), when human teeth were incinerated in the laboratory. This same temperature range was reached in unextracted pig pulp chambers in an open fire after eight and one half minutes of incineration. The temperature of the fire reached 600C in the vicinity of the teeth; aircurrents caused temperature fluctuations between 500-700C. Histological examination of the pig pulps revealed a comparable level of heat damage to that noted in human teeth incinerated in the laboratory when similar temperatures were registered in the pulp chamber.

The second sexing procedure adopted for this study involved the application of immunocytochemistry in the detection of the male-specific H-Y and β_2-microglobulin located on the surface of human pulp fibroblasts. The H-Y molecule, however, proved highly unstable, being shed from the plasma membrane within a few hours of tooth extraction.

Discussion

The Rate of Decomposition in Surface and Subsurface Tissue

The rate of decomposition of organic materials in the early stages is multifactorial. Among these factors are the chemistry of the environment, the action of endogeous hydrolytic enzymes and putrefactive organisms, and the depth of burial, if any. These

several factors are in turn affected by climatic factors such as heat, cold, moisture and aridity. Comparison of the effects of climate in the experiments described here would have been served best if all "summer" and "winter" depositions had been made over the same period of time. This, however, was not possible since deposition dates were dependent on the availability of teeth. Moreover, the delayed "summer" (August/September) Lytton deposition was a consequence of the experiment in Manning Park which was abandoned because of animal disturbance (Materials and Methods p.52).

Longer pulp cell stability in outdoor environments beyond the two week limit recorded in this study have been documented. Yamamoto (1959: 1, see p.96) in Japan, reported preservation of pulp cell fibroblasts, but not odontoblasts, buried for 15 days beneath 50 cm of sand. More recently Seno,(1977: 172, see p.32) also in Japan, made F-body counts on pulp cells from teeth buried one month in mud at a depth of 25 cm. Finally Ionesiy (1980: 27, see p.33) made sex chromatin counts on pulps buried in soil for 20 days.

Clearly studies conducted in this report indicate that the rate of decomposition of pulp cells is partially due to environmental factors. Experiments conducted in the past often represent widely disparate regions of the world, with no environmental information, other than soil type and interrment depth recorded. In the absence of climatic data, altitude, or soil pH, it is not possible to meaningfully interpret the particular influences that may impact on the decay rate of any one

of these environmental variables. It would be extremely useful if future studies of decay rates included all relevant micro- and macro-environmental data.

Further testing under controlled laboratory or field conditions could yield much more specific information on the relative influence on decay rates of soil type, soil chemistry, burial depth, and climatic factors. The accumulation of this kind of information may have broader application than that of sex chromatin evaluation. Synthesis of comparable data might allow elucidation of the role of individual variables, or variable combinations in promoting or retarding decay, such that predictions could be made not only of the decomposition status of the tissue of interest,but also of the elapsed time since death.

The shallow depth of burial (30 cm) undoubtedly contributed to rapid decomposition of interred elements. Burial depth is known to be a principal factor in decay rate variability (Rodriguez and Bass 1985: 836). Selection of 30 cm for burial depth for this study, was made on the presumption that most burials of concern to forensic scientists are made in haste and would representative of a shallow grave.

The relatively rapid loss of cellular integrity, within two weeks or less, limits the use of the chromatin sexing method to situations where recovery of remains is rapid but sex determination is still difficult to establish. Circumstances such as these may arise when animal disturbance of surface or shallowly buried remains leads to disarticulation and fragmentation, involving incomplete or scanty recovery of elements (see p.4);

fragmentation and rapid recovery may also pertain in cases of suicide involving solid explosives where incineration is not extensive (see p.20), or in some instances of mass disaster such as aircraft crashes or air craft explosions.

Shelf Stored Dental Tissue

The excellent sex chromatin preservation in dental pulp stored for one year at room temperature (25C) is not an original finding, but is in accord with results reported previously in the literature (see Results, Chap.5, Section A,). What is new is the technique developed for isolating the cells from the fibrous tissue matrix by rehydration in Ruffer's fluid, followed by enzymatic digestion (see Materials and Methods, Chap.4, Section A, pp. 55, 56). This method gives a much cleaner preparation with less cell damage than conventional cell separation methods employing acetic acid to soften dessicated tissue, followed by release of cells by mechanical abrasion with a pestle and mortar (Seno and Ishizu 1973:8).

In British Columbia, isolated teeth are rarely located in situations conducive to rapid soft tissue dessication, such as within a building which has a temperature controlled environment. If the teeth are still in the alveolar sockets, pulps will probably rapidly putrefy, because the microenvironment will keep them moist for an extended period, as was shown to be the case in pig jaws stored on the laboratory bench (see Results, Chap. 5, Section A,).

The new enzymatic digestion methodology, however, may prove

useful in other areas of anthropological research. Mummified tissue has been subjected to ABO (Allison et al. 1978: 139) and HLA typing (Stastny 1974: 864; Hansen and Gürtler 1983: 447) because it aids in characterizing populations on the basis of their genes. Research along these lines can lead to inferences about human genetic evolution, prehistoric migrations, or population movements.

HLA antigens are gene products of the MHC whose Class I determinants are present on the plasma membranes of all somatic cells. Stastny's study on skin tissue of pre-Columbian mummies, 500-2,000 years old suggested remarkable similarity in the HLA profile of prehistoric and modern native Americans populations, including continuity of regional variation. Hansen and Gürtler established family identity and tested genetic continuity in eight naturally mummified Inuit remains from Greenland, dating to A.D.1460. Two families could be distinguished. The finding of some alleles rarely detected among modern Inuit led the authors to suggest the possibility of Caucasian admixture, maybe even from Viking settlements in West Greenland. In the Old World, isolation of DNA fragments from an Egyptian mummy (pMUM 2:9) by Pääbo (1985: 664) led to the identification of a 382 bp region almost identical to the 5'-end of the first intron of HLA-DQA1 (DQa),a highly polymorphic MHC Class II gene. Yet this 2,440-year-old mummy sequence shows 97% identity with HLA-DQA1-cDNA from a modern cell line (DR4.4) (Del Pozzo and Guardiolo (1989: 431) suggesting greater evolutionary conservation than previously suspected.

In fresh tissue, HLA typing is accomplished by tests (most often serological) designed to detect directly the specific antigenic determinants present on cell surfaces (usually lymphocyte cultures) (Kissmeyer-Neilsen 1982: 14). Intact cells are not readily isolated from mummified tissue. To overcome this difficulty the pre-Columbian and Inuit tissues were shredded, or powdered to a crude antigenic mixture, allowing the possible introduction of cross-reactive contaminants of internal or external origin. This technical problem has been encountered in forensic research contexts where high non-specific backgrounds accompany attempts to detect HLA antigens extracted from dry bloodstains (Bishara and Brautbar 1989:99). It was therefore necessary in the mummy testing to resort to an indirect, rather than a direct, test to detect the presence of HLA molecules. Essentially HLA antibodies were adsorbed on mummy tissue and tested for their remaining cytotoxic properties in relation to fresh lymphocytes *in vitro*.

Direct serological testing using mono- or polyclonal antibodies to cell surfaces might detect HLA antigens in rapidly dessicated tissue containing intact cells, provided that cells can be adequately isolated from the tissue matrix, and provided that the method in no way damages any preserved antigenic determinants. Ruffer's solution would need to be modified with immunologically suitable fixatives (Collings et al. 1984: 227). The concentrations of collagenase and hyaluronidase enzymes employed in the present study would be unlikely to affect the integrity of cell membrane antigens since they have proved harmless to living cells: the mix

was originally used in the isolation of rabbit dental fibroblasts for *in vitro* cultivation (Shuttleworth et al. 1980: 201). However, only future testing can ascertain the success of such procedures on dessicated plasma membranes.

The Effects of Heat on Dental Pulp

Previous studies have already shown that when extracted teeth are heated for a sustained period of one hour in an oven, the pulps are still sufficiently undamaged for a sex diagnosis to be made (Seno 1977: 172; Ionesiy 1980: 27). In the current study this research has been extended to examine the effects of heat in unextracted teeth, the more typical forensic situation. It was determined that in unextracted pig teeth, heat penetration is retarded by the greater surrounding tissue depth such that the temperature can be raised to 300C for the same length of time (see Table 6,). In addition pulp chamber temperatures can be raised to between 50-75C without loss of sexually diagnostic features. Furthermore, this critical temperature range was reached in unextracted pig pulp chambers in an open fire of Douglas fir and pine logs, at a fire temperature between 500-700C (see Table 10,).

The question still remains unanswered as to what forensic conditions of incineration produce a temperature in the pulp chamber of 75C or less? The ability to determine sex in an actual forensic situation of this kind is dependent on the particular circumstances of the individual event. As Binford so aptly stated when discussing the variables involved in structural alteration of bones recovered from archaeological cremation sites - a statement

equally applicable to any tissue protected within the body - the alteration:

" is a function of the length of time in the fire, the intensity of the heat, the thickness of the protecting muscle tissue, and the position of the (element) in relation to the point of oxidation of the consuming flame" (1963: 100).

The potential application of sex diagnosis in the context of conflagrations can be only very generally stated. In light of this problem it might be best to assess the potential for pulp sexing whenever trauma or incineration has precluded diagnosis. It would be of interest in terms of future research to test the potential for dental pulp sex diagnosis in relation to fire by examining forensic specimens from actual incineration events. Furthermore, it would not be advantageous to confine such a study to dental pulp tissue, rather than any tissue showing the minimum alteration after a conflagration. Thomsen's (1977: 235) study (see p.21) of severely burned bodies showed the kidneys to be well protected. He experimented by heating some kidney tissue (method not given), and reported that at 150C for 10 minutes, it was still possible to make F-body counts, although there was a marked decline in counts over fresh tissue. This work, however, suffers from the same drawback as the studies of the effects of heat on oven heated extracted teeth, in that the kidney tissue was isolated from the cadaver, and it was not determined what temperature or length of time is required for the renal tissue to reach 150C, when the kidney is *in situ*, in a real situation involving incineration.

The H-Y Molecule and β_2-Microglobulin

The inability to detect the H-Y molecule in all but fresh tissue was not the anticipated finding (see Results, Chap.5, Section B,). The results of the immunological tests with the rat polyclonal antibody prevented any possible application of this molecule to forensic study. Despite the constraints on H-Y reported here, the results hold interest in the context of basic research, since the H-Y molecule has not previously been tested in non-viable tissue. It has been reported, however, that in human and animal cadavers kept in cold storage, HLA typing is possible up to 36 hours post-mortem (Henke et al. 1982: 267-274). Furthermore, in cryostat sections of tissue from cold-storage cadavers, the essential markers of human leukocytes were detected by monoclonal antibodies in an immunoperoxidase assay up to at least 72 hours after death (see p.42). Testing was not continued to endpoint (Pallesen and Knudsen 1985: 791-804).

MHC molecules are plasma membrane integrated glycoproteins, i.e. they have a transmembrane domain, locked in the lipid bilayer by charged amino acid residues. B_2-microglobulin is non-covalently associated with the Class I molecule, but has no plasma membrane anchorage (Hood et al. 1984: 190). The exact conformational association of H-Y to the MHC, or to B_2-microglobulin, and to the plasma membrane has not yet been determined.

In vitro studies have shown that in humans only Sertoli and Daudi cells secrete H-Y molecules which can be detected in supernatant culture media (Müller et al. 1978: 203; Zenzes et al.

1978: 297). Sertoli cells' secretion of H-Y is functional: the H-Y molecules secreted bind to receptors on both Sertoli and Leydig germ cells as part of a process initiating male differentiation. Daudi is a pseudodiploid cell line from a human Burkitt lymphoma. It lacks HLA and B_2-microblobulin on its plasma membrane (Wachtel 1983: 121; Ohno et al. 1984: 26). Presumably H-Y molecules on Daudi cells have no plasma membrane anchorage sites. Ohno 1978: 217) and Wachtel (1983: 123) have suggested that H-Y does not possess a membrane integrated hydrophobic core on the basis of its putative role as a "short-range" hormone, triggering male differentiation. If H-Y is not membrane integrated, it may be more loosely associated with the MHC molecules (or the plasma membrane) than is B_2-microglobulin. If this is the case, then once the somatic cells die, H-Y molecules may be rapidly shed from the cell surface, which would explain the results of the immunological tests in this study.

The next research step would be to test the hypothesis of rapid *post-mortem* shedding of H-Y molecules. A suitable assay for detecting H-Y molecules shed from cell membranes is a sandwich or "capture" ELISA (Brunner and Wachtel 1988: 49; Bishara and Brautbar 1989: 99). Using this technique, any remaining H-Y molecules could be tested for in extracts of pulpal tissue at sequential time intervals post-tooth extraction, and in supernatant fluids from *in vitro* incubation of male dental pulp fibroblasts both in the viable and non-viable state. Controls for these experiments would consist of fresh male and female pulp extracts, and for cultured cells, Sertoli or Daudi cell lines, as

well as female dental fibroblasts. Briefly, in the "capture" ELISA, antibody (rat anti H-Y in this case) is coated to a microtiter plate, varying amounts of putative antigen source (H-Y) are introduced, and the amount of bound antigen is determined by the introduction of biotin-conjugated antibody (rat anti H-Y) and an avidin-biotinylated peroxidase indicator.

Some level of male-female cross reactivity in H-Y antibodies has been invariably reported throughout the literature for both mono- and polyclonal antibodies to H-Y (Farber et al.1988: 204), the present study being no exception. Even though a significant level of male-female overlap has been documented by authors using gw-16 in their test systems, male-female discrimination was always possible. Several explanations for high female overlap have been proposed with respect to gw-16. For example a hypothesis promulgated by Adinolfini (1982: 1) is invoked in which autosomal structural genes for H-Y produce a precursor substance H-Yp, that is glycosylated by genes of the Y chromosome. This form, designated H-Ya, is considered the antigenic testis inducer. H-Yp under this hypothesis can also be glycosylated by the X chromosome to produce H-Yr (H-Y related), an antigen which is not functional in specifying maleness. H-Yp and H-Ya are sufficiently similar as to be cross reactive and H-Yp molecules are postulated to be present, if not in as great abundance, on normal female cells as well as those of males.

Another scenario stems from Ohno's (1977: 59) hypothesis (see p.,39) that the MHC in conjunction with B_2-microglobulin serves as an anchorage site for H-Y molecules. H-Y recognition by cytotoxic

T-cells in transplant rejection is known to be MHC dependent suggesting that both antigen systems may be juxtaposed at the cell surface. It may not be the H-Y molecule that is recognised, but an H-Y plus MHC association, such that MHC-restricted recognition may involve a conformational modification of both molecules. A similar situation may exist for serological systems: if monoclonal H-Y is MHC-restricted and recognizes a particular MHC plus H-Y complex, it allows the possiblity of "errors" in recognition to be made, given the exceptional polymorphism exhibited by the MHC.

Theories are not as satisfying as hard evidence. The recent finding of Farber et al. (1988: 204) previously noted (see p.38) in which a Western blot analysis of human mononuclear cells by H-Y antisera revealed three bands (Mr: 15-20 kD), common to both sexes, and one band, (Mr: 32-34 kD), with exclusively male specificity, provides empirical evidence on which to base an explanation. Perhaps the monoclonal gw-16 is directed to one of the shared molecules, or is cross-reactive and is directed to any or all three of the 15-19 kD antigenic components. The most telling observation made on gw-16 is that "the reactivity of our antibody gw-16 could be abolished by repeated absorption (*sic*) with spleen cells from C57BL/6 female mice" (Brunner et al. 1987a: 181). This suggests that gw-16 is binding to antigenic determinants common to both male and female plasma membranes, but exhibiting preferential binding to the male cells, perhaps because more binding sites are present on the male membrane.

It is not in itself surprising to find that males and females share a common H-Y component. The evolutionary history of sex

differentiation suggests such a finding, in that among some ancestral non-mammalian vertebrates the female is the heterogametic sex (see p. 36). Moreover the gene identified by Lau (1986: A 142) as specifying H-Y is located, not on the male Y chromosome, but on an autosome, chromosome 6 (see p.37). Interestingly this gene codes for a protein with an approximate Mr. of 19 kD (Heslop et al. 1989: 99): the same Mr. as the cross reactive components detected by Farber et al. (1988: 204) above. Moreover, Ohno (1984: 17) has detected a Daudi-excreted H-Y antigen molecule of 18 kD on specific H-Y receptor sites of mammalian ovarian cells of foetal origin.

Whether or not this is a precursor molecule as Ohno suggested, the fact remains that the mechanism proposed for sex differentiation by binding of H-Y to a gonadal receptor is apparently contradicted by the finding that both male and female gonadal receptors bind an 18-20 kD H-Y molecule. A possible alternative or concomitant mechanism for initiation of sexual differentiation may be a variant physiological response such as differential tissue growth rate triggered by an unknown factor (Mittwoch 1986: 103) (see p.41). Heslop and Lyttle (1976: 209) infer such a factor from their observations that male skin grafts, undergoing rejection by syngeneic female hosts of the HS rat strain, show a striking hyperplasia of dermal fibroblasts and surface epithelial cells of graft provenance. This response is not seen in female to male,male to male, or female to female intrastrain grafts.

What is surprising is the seeming paradox that among inbred

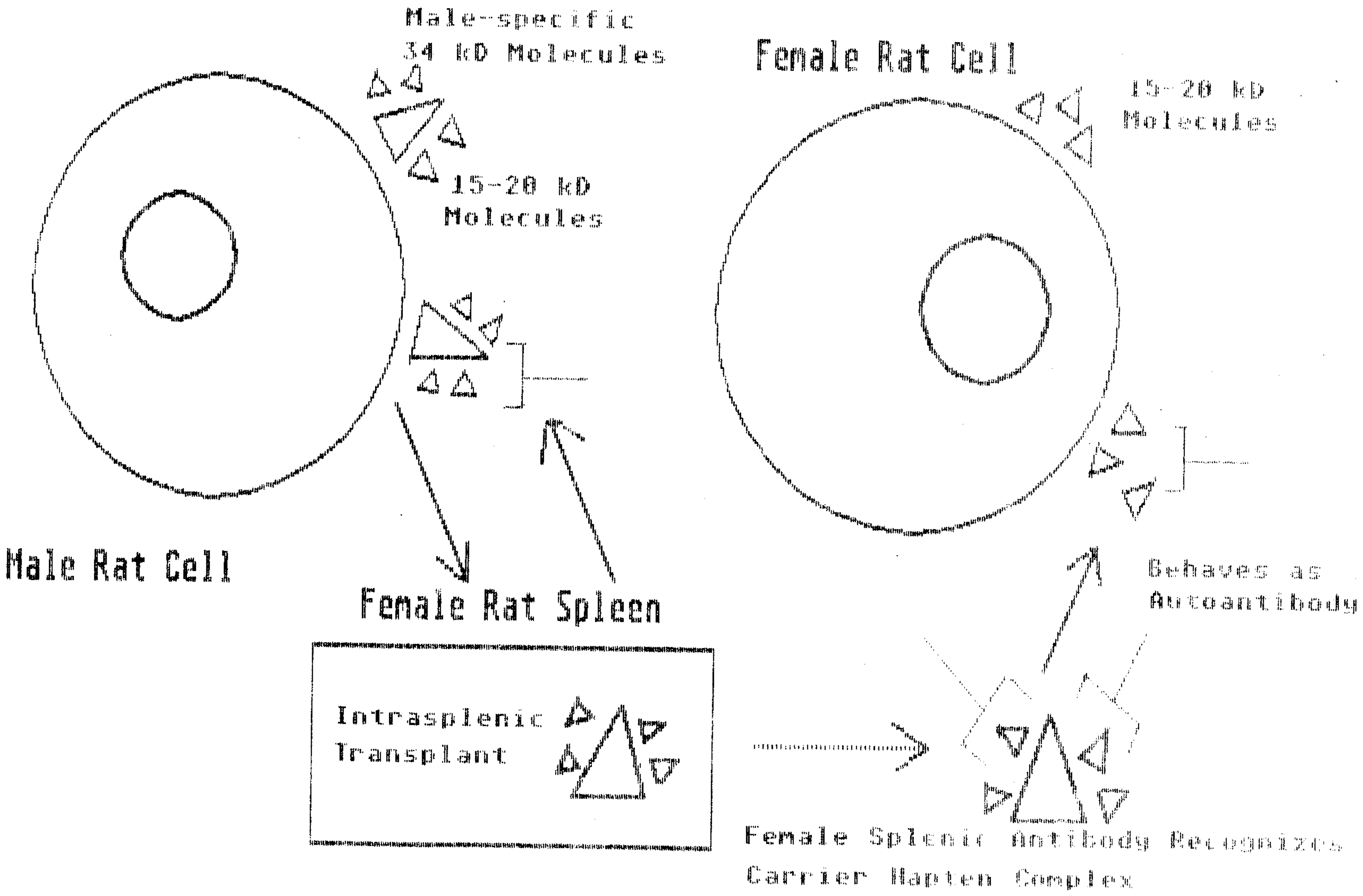
rats or mice, females mounting an antibody response to male H-Y transplants, or splenocytic inocula, generate an antibody which binds to putative H-Y molecules in both sexes: in other words the females make a self-directed autoantibody. An attempt by this author to account for this phenomenon follows; the reader is first requested to bear in mind that the induction of H-Y antibody by male grafts represents an artificial environment never encountered in the animal under normal cicumstances, except perhaps *in utero*. In mounting an immune response to any antigenic material many molecular species of antibody are elicited, and each will react with a different spectrum of antigens. Every antibody will react with the inducing antigen, but each kind of antibody molecule will differ in the spectrum of antigens that it will bind. When an autoantibody is produced it represents a failure of immunological tolerance to self. Sometimes the introduction of a foreign antigen, with a self antigen attached, can break self tolerance by inducing a helper T-cell that can stimulate self-reactive B-cells to produce antibodies. This phenomenon is usually encountered in response to haptens (antigens of small size < 5 kD), but according to Hood et al.: "In general, whenever self and foreign antigenic determinants are linked together, they have the potential for inducing self-reactive antibodies" (1984:455).

The largest molecule detected by Farber et al. (1988: 204) of 32-34 kD was found to a be male-specific H-Y. If this molecule is linked to any of the smaller 15-20 kD molecules, it may be speculated that it can elicit an autoantibody able to detect the

selfsame 15-20 kD molecules on females cells. The lower molecular weight molecule, or molecules, may always be present on the surface of female cells, but are only detectable when autoantibodies are produced in response to the 34 kD haptenic linked complex (see Diagram 1 below). Moreover, the male specific 32-34 kD H-Y component may hold the answer to the controversy as to whether the H-Y antigen that induces specific killer T-cell proliferation is the same antigen which provokes B-cells to produce H-Y antibodies, since this molecule appears to be a good candidate for the component involved in graft rejection.

Diagram 1

Hypothetical Induction of Autoantibody to the H-Y Molecular Complex



Y-Specific DNA Probes

Sex determination has been attempted in soft tissue for forensic purposes using methods other than sex chromatin staining. Analysis of polymorphonuclear "drumsticks" (Davidson 1960: 14), and sex-hormone ratios (Szendrenyi and Foldes 1980: 263; Brown 1981: 766; Ishizu and Yamamoto 1983: 127) have been tried. At the present time, with the advent of the application of recombinant DNA technology to forensic science, many research publications have appeared in which testing has been carried out to evaluate the application of repetitive DNA to forensic biology (see Craig et al. 1988 for a review). Research has included testing various Y chromosome-specific probes to determine the stability of DNA for sex determination in forensic samples. Y-specific probes have been applied to the determination of sex in dried bloodstains (Tyler et al. 1986: 267; Gill 1987: 35; Horiuchi et al. 1988: 351; He 1989: 346). The most commonly used probe (pY 3.4) is directed to the 3.56 kb locus (DYZ1). Cooke (1983: 48) has estimated that there are about 5,000 copies of the DYZ1 repeat, characterized by HaeIII sites, on the Y chromosome (see Theoretical Background p.29). Variation in the copy number of the 3.56 kb sequence has been correlated with variation in the size of the heterochromatic region of the chromosome, the same region which stains brightly with quinacrine (Cooke 1982: 492; Gill 1987: 35). For this reason the potential for successful sex diagnosis of male tissue in forensic specimens is likely to be very similar using either Y-chromatin fluorescence or a Y-specific probe.

As for the application of DNA probes to the determination of

female sex, although an X-specific region has been described for the X chromosome (Yang et al. 1982: 6593; Willard et al. 1983: 2017), no X-probes are used as an adjunct in these tests, since males also carry an X chromosome. The diagnosis of female tissue is therefore inferred by a negative result. This represents a source of potential error in that it cannot be determined if a negative result with a Y-specific probe represents a female diagnosis, or biodegradation of the forensic specimen.

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APPENDIX

VANCOUVER

Summer Subsurface and Surface Deposition of Human Teeth

Experiment 1, (Catalogue O.P.).

Period of Experiment : 2 weeks (June 26th to July 10th 1988), retrieval at 1 and 2 weeks (July 3rd, July 10th).

Location : Vancouver.

Elevation : less than 150 metres above sea level.

Burial Depth : 30 cm.

Sample Size : 20 molars (10 male and 10 female) surface, and subsurface.

Soil Temperature at Burial Depth : 15.5C (at burial), 14C (at 1 week), 11C (at 2 weeks).

Soil pH : 6 (at burial), 6 (at 1 week), 6 (at 2 weeks).

Soil Type: Humic.

Climate Recorded for the Experimental Period (about 3 km from the site) at Burnaby Metrotown, by AES Climate Services for the Experimental Period.

	Temperature (centigrade)		Rainfall (mm)
	Maximum	Minimum	
June 26th	19.5	12.0	
27th	19.0	13.5	
28th	18.5	8.0	
29th	18.0	9.0	
30th	20.5	10.0	

July 1st	15.5	13.0	4.6
2nd	21.5	12.5	0.4
3rd	19.5	10.0	
4th	18.0	7.5	1.2
5th	15.0	10.5	8.5
6th	18.5	10.0	0.4
7th	22.5	8.0	
8th	27.5	11.0	
9th	24.5	11.0	
10th	21.0	12.0	1.6

Results : Control cell preparations made prior to tooth burial showed the cells of the pulp to be intact. Ten subsurface teeth retrieved at 1 week (July 3rd) revealed wet pulp tissue devoid of cells. Ten subsurface teeth retrieved after 2 weeks (July 10th) contained pulp tissue with the consistency of jelly.This material was acellular. Ten teeth retrieved after 1 week on the ground surface revealed dry fibrous pulp with a few cells still apparent. Many bacteria were observed. After 2 weeks pulp chambers were either devoid of all tissue or the tissue was acellular. The anomaly of the drop in temperature at burial depth from 14C at 1 week to 11C at 2 weeks, in spite of the warming ambient temperature recorded at the weather station for the same period, may be explained by rainfall cooling the soil (8.5 mm fell on July 5th).

Lytton

Summer Subsurface and Surface Deposition of Human Teeth

Experiment 2, (Catalogue T.U.).

Period of Experiment : 4 weeks (August 29th to September 25th), retrieval at 1 and 4 weeks (September 4th, September 25th).

Location : Lytton.

Elevation : 1087 metres above sea level.

Burial Depth : 30 cm.

Sample Size : 32 molars (15 female, 17 male) subsurface, 15 molars (8 male, 7 female) surface.

Soil Temperature at Burial Depth : 26C (at burial), 26C (at 1 week), 13C (at 4 weeks).

Soil pH : 7 (at burial), 7 (at 1 week), 8.5 (at 4 weeks).

Soil Type : Sandy.

Climate Recorded for the Experimental Period (about 12 km from the site) at Lytton, by AES Climate Services.

	Temperature (centigrade)		Rainfall (mm)
	Maximum	Minimum	
August 29th	27.0	18.6	
30th	24.8	13.7	
31st	30.5	10.6	
September 1st	34.3	11.0	
2nd	38.0	25.9	
3rd	38.7	14.4	
4th	37.1	14.0	

September 5th	33.6	18.4	
6th	23.8	15.2	0.2
7th	21.7	11.1	1.4
8th	23.7	12.1	
9th	22.6	8.6	2.0
10th	23.0	5.9	
11th	27.0	4.8	
12th	31.3	8.3	
13th	31.1	10.1	
14th	32.8	13.1	
15th	24.1	15.2	
16th	15.6	7.2	19.0
17th	19.7	6.6	Trace
18th	12.9	5.4	1.0
19th	16.7	6.7	2.4
20th	20.2	4.6	
21st	20.2	5.5	
22nd	18.6	6.8	Trace
23rd	14.4	8.5	4.4
24th	13.3	6.7	0.4
25th	15.2	6.7	13.0

Results : Control cell preparations made prior to tooth deposition showed the cells of the pulp to be intact. Six subsurface teeth (4 female and 2 male), retrieved at 1 week (September 4th), showed either total cell loss or cells in a late stage of putrefaction. The remaining pulp tissue varied in appearance between teeth, either remaining wet and exhibiting a whitish colour or becoming

dry, red,and papery in consistency. Eight subsurface teeth (4 male and 4 female molars) retrieved at 4 weeks (September 25th), revealed acellular pulps which appeared damp and papery. Five surface teeth at 1 week showed some cells still intact but decomposition was rapidly advancing. At 1 month, 4 teeth recovered from the ground surface revealed damp acellular pulps adhering to the chamber walls.

Interestingly, although the 1 month period of the experiment,in fact represents a seasonal transition from late summer to early autumn, showing temperature decrease and rainfall September 6th (day 9), the first 7 days in which cellular decomposition occurred (August 9th to September 4th) represent the typical interior summer climate with high temperatures and no rainfall. Yet heat and aridity did not preserve the pulps.The teeth left at the ground surface fared best, many of their pulp cells were still intact at the end of 1 week,although putrefaction rather than mummification was the ongoing process. The sandy soil used for interrment was dry to burial depth. The temperature recorded at the time of interrment and at 1 week was 26C. At 1 month the site was damp from surface to base and the temperature at burial depth had fallen to 13C. The increase in soil pH from 7 at 1 week, to 8.5 at 4 weeks, may have been caused by the rain, which fell heavily on September 16th and 25th, (19 and 13mm respectively) creating slopewash. The site position is at the base of a sandy bank. The incline supports a sparse population of small poplars and Ponderosa pine, as well as *Equisetum arvaise* (horsetail),and *Tecucrium canadense* (sagebrush).

VANCOUVER

Winter Subsurface and Surface Deposition of Human Teeth

Experiment 3, (Catalogue Y.Z.).

Period of Experiment : 2 weeks (November 26th to December 11th 1988, retrieval at 1 and 2 weeks (December 4th, December 11th).

Location : Vancouver.

Elevation : less then 150 metres above sea level.

Burial Depth : 30 cm.

Sample Size : 20 molars (10 male and 10 female) subsurface, 17 molars (10 male and 7 female) surface.

Soil Temperature at Burial Depth : 7C (at burial), 7.5C (at 1 week), 7.5C (at 2 weeks).

Soil pH : 6.5 (at burial), 7 (at one week), 7 (at 2 weeks).

Soil Type : Humic.

Climate Recorded for the Experimental Period (about 3 km from the site) at Burnaby Metrotown, by AES Climate Services.

	Temperature (centigrade)		Rainfall (mm)
	Maximum	Minimum	
November 26th	5.0	-5.0	13.8
27th	7.0	2.0	1.5
28th	7.5	2.0	0.8
29th	6.5	4.0	
30th	9.5	3.0	
December 1st	13.0	4.0	12.0
2nd	6.5	1.5	0.4
3rd	12.0	1.5	

December 4th	12.5	2.0	7.8
5th	9.0	6.5	13.2
6th	9.0	7.0	6.6
7th	9.0	6.5	4.3
8th	8.0	6.5	12.0
9th	9.0	6.5	3.7
10th	8.0	4.5	1.2
11th	8.0	4.5	23.2

Results : Control cell preparations made prior to tooth deposition showed the cells of the pulp to be intact. Ten subsurface teeth (5 male and 5 female) retrieved at 1 week (December 4th) revealed putrefactive pulp tissue in which remaining cells appeared swollen,and also exhibited loss of nuclear granulation. Many bacteria were observed in the cell preparations. Ten subsurface teeth (5 male and 5 female) retrieved at 2 weeks (December 11th) showed some cells still present. Teeth retrieved from the ground surface at 1 week and 2 weeks (5 male and 3 female for each time interval) also showed some intact cells remaining.

The slightly prolonged cellular preservation (a few cells at 2 weeks), may have resulted from the cold temperatures inhibiting bacterial and fungal proliferation. The pulp tissue from this experiment was paraffin sectioned in an attempt to detect sex chromatin. The degenerating cells did not take up quinacrine dihydrochloride stain. Carbol fuchsin stained the preparations but female chromatin counts were negligible.

VANCOUVER

Summer Subsurface and Surface Deposition of Extracted Pig Teeth

Experiment 4, (Catalogue Q.R.).

Period of Experiment : 1 week (July 11th to July 17th 1988), retrieval daily to 1 week.

Location : Vancouver.

Elevation : less than 150 metres above sea level.

Burial Depth : 30 cm.

Sample Size : 15 anterior teeth (subsurface), 15 anterior teeth (surface).

Soil Temperature at Burial Depth: 11C (at burial), 13C (at 1 week).

Soil pH : 6 (at burial), 6 (at 1 week).

Soil Type : Humic.

Climate Recorded for the Experimental Period (about 3 km from the site) at Burnaby Metrotown, by AES Climate Services.

	Temperature (centigrade)		Rainfall
	Maximum	Minimum	
July 11th	15.0	11.5	4.7
12th	14.5	11.0	6.6
13th	19.5	11.5	
14th	20.5	10.0	
15th	23.0	10.5	
16th	22.5	9.5	
17th	24.0	9.0	

Results : Control cell preparations made prior to tooth burial showed the cells of the pulp to be intact. Two teeth retrieved from the surface and subsurface daily, revealed that many pulp cells remained intact until the 3rd day (July 14th), on the 4th day cell loss was apparent, and by the fifth day few cells remained. At 1 week (July 17th) a few cells still remained. The remaining 2 teeth were retrieved and evaluated at 3 months (September 17th) by which time the pulp chambers were devoid of soft tissue.

LYTTON

Summer Subsurface and Surface Deposition of Extracted Pig Teeth

Experiment 5, (Catalogue T.U.).

Period of Experiment : 4 weeks (August 29th to September 25th), retrieval at 1 and 4 weeks (September 4th, September 25th).

Location : Lytton.

Elevation : 1,087 metres above sea level.

Burial Depth : 30 cm.

Sample Size : 12 anterior teeth (subsurface), 12 anterior teeth (surface).

Soil Temperature at Burial Depth : 26C (at burial), 26C (at 1 week), 13C (at 4 weeks).

Soil pH : 7 (at burial), 7 (at 1 week), 8.5 (at 4 weeks).

Soil Type : Sandy

For the climate recorded at Lytton (by AES Climate Services) for the experimental period see Experiment 2 data.

Results: Control cell preparations made prior to tooth burial showed the cells of the pulp to be intact. Five subsurface teeth recovered at 1 week (September 4th) revealed dry papery pulp tissue, red in colour, adhering to the walls of the pulp chamber. Putrefactive degeneration was noted but some cells were still intact. Five subsurface teeth recovered at 4 weeks (September 25th) revealed damp papery acellular tissue adhering to the chamber walls. Rhabditiform worms, part of the normal soil fauna, were also isolated from the chamber. Four teeth recovered

at 1 week from the ground surface showed dry papery pulp tissue, red in colour, clinging to the chamber walls. Many cells were still intact. Three surface exposed teeth recovered at 1 month, revealed wet acellular pulps.

Again it should be noted that the first week was one of high temperatures and no rainfall. The pig pulps appear to have preserved better than the human pulps (in Experiment 2). The pig remains were taken at the time of slaughter, kept on ice overnight, and transported the next day to the site for disposal. The human specimens,however, were stored frozen by dental surgeons for 1 week prior to burial. Perhaps frozen storage and thawing of the pulps rendered the cells more fragile at the time of site disposal.

VANCOUVER

Winter Subsurface and Surface Deposition of Extracted Pig Teeth

Experiment 6, (Catalogue D.W.).

Period of Experiment : Burial 2 months (October 10th 1987 to January 24th 1988), retrieval at 1 week (October 17th) and 2 months (December 20th). Surface 1 week (October 5th to October 12th 1988), retrieval daily.

Location : Vancouver.

Elevation : less than 150 metres above sea level.

Burial Depth : 30 cm.

Sample Size : 15 anterior teeth (buried), 17 anterior teeth (surface).

Soil Temperature at Burial Depth : 19C (at burial), 15C (at 1 week), 4C (at 2 months).

Soil pH : 5.6 (at burial), 5.6 (at 1 week), 5 (at 2 months).

Soil Type : Humic.

Climate Recorded for the Experimental Period (about 10 km from the site) at Vancouver International Airport, by AES Climate Services.

(1 Week Burial).

	Temperature (centigrade)		Rainfall (mm)
	Maximum	Minimum	
October 10th	17.2	3.4	
11th	17.4	4.3	Trace
12th	15.6	3.8	0.2
13th	13.9	4.5	

October 14th	13.5	6.9
15th	13.5	5.4
16th	13.7	5.0
17th	12.6	7.1

Results : Control cell preparations made prior to deposition showed the cells of the pulp to be intact. Five subsurface teeth retrieved at 1 week revealed wet acellular pulps, 5 subsurface teeth retrieved at 2 months gave the same result. The climate data for this period was from Vancouver International Airport, located at the edge of the sea. Usually the airport temperature readings are a few degrees colder than those recorded at Burnaby Metrotown, located nearer to the burial sites. However, Burnaby Metrotown was not recording during the relevant period.

Climate Recorded at Burnaby Metrotown (by AES Climate Services) for the 1 Week Period of Tooth Exposure

	Temperature (centigrade)		Rainfall (mm)
	Maximum	Minimum	
October 5th	18.5	12.5	
6th	17.0	8.0	
7th	18.5	10.5	
8th	16.5	8.5	
9th	19.0	5.5	
10th	23.5	7.0	
11th	14.0	8.5	
12th	17.0	12.5	

Results: Control cell preparations made prior to surface deposition showed the cells of the pulp to be intact. Tissue from this part of Experiment 6 was paraffin embedded and sectioned and stained with haematoxylin and eosin. Two or 3 pulps were retrieved at daily intervals (October 6th to October 12th). Cellular disintegration was apparent by the 4th day, and although remnants of cells were observed at 1 week, few nuclei still remained intact and their nuclear granules appeared atypical.

VANCOUVER

Summer Subsurface and Surface Deposition of Unextracted Pig Teeth

Experiment 7, (Catalogue Q.R.I.).

Period of Experiment : 2 weeks subsurface (July 11th to July 17th 1988), retrieved at 1 week, and 2 weeks (July 24th).

Surface specimens 5 days (July 11th to July 16th), retrieved at day 1 (July 11th), day 2 (July 12th),day 3 (July 13th), and day 5 (July 15th).

Location : Vancouver.

Elevation : less than 150 metres.

Burial Depth: 30 cm.

Sample Size : 6 jaws subsurface, 4 jaws surface.

Soil Temperature at Burial Depth: 11C (at burial), 13C (at 1 week).

Soil pH : 6 (at burial), 6 (at 1 week).

Soil Type : Humic.

Climate Recorded for the Experimental Period (about 3 km from the site)at Burnaby Metrotown by AES Climate Services.

	Temperature (centigrade)		Rainfall
	Maximum	Minimum	
July 11th	15.0	11.5	4.7
12th	14.5	11.0	6.6
13th	19.5	11.5	
14th	20.5	10.0	

15th	23.0	10.5
July 16th	22.5	9.5
17th	24.0	9.0
18th	29.0	11.0
19th	32.5	13.5
20th	31.0	15.5
21st	25.0	13.0
22nd	23.0	10.0
23rd	26.0	11.0
24th	28.0	11.0

Results: Control cell preparations made prior to jaw deposition showed the cells of the pulp to be intact. Two subsurface jaws (3 teeth from each jaw), evaluated at 1 week (July 17th), showed many cells still intact. Some pulps showed many intact fibroblasts and white blood cells, in others the fibroblasts had mostly degenerated leaving white blood cells still intact. Of 2 jaws retrieved (3 teeth from each jaw) at 2 weeks (July 24th), the pulp cells showed putrefactive deterioration. Very few cells still remained. Four surface deposited jaws (3 teeth removed at each time interval) showed many intact pulp cells up to 3 days, at 5 days few cells remained intact.

At 1 month (June 19th) the remaining surface jaws were re-examined. The jaws were becoming dry, some soft tissue still remained on the mandible surface, adipocere could be seen under the dry brown skin. Fly puparia were noted on the mandibular ramus. Extraction of molars was now possible (soft tissue loss

makes them loose in the sockets). Fly larvae were found inhabiting the pulp cavities. No pulp tissue remained in 3 molars examined, but some pulp tissue still remained in 2 anterior teeth.

LYTTON

Summer Subsurface and Surface Deposition of Unextracted Pig Teeth

Experiment 8, (Catalogue T.U.).

Period of Experiment : 4 weeks (August 29th to September 25th), retrieval at 1 and 4 weeks (September 4th and September 25th).

Location : Lytton.

Elevation : 1087 metres above sea level.

Burial Depth : 30 cm.

Sample Size : 8 jaws subsurface, 9 jaws surface.

Soil Temperature at Burial Depth: 26C (at burial),26C (at 1 week), 13C (at 4 weeks).

Soil pH : 7 (at burial), 7 (at one week), 8.5 (at four weeks).

Soil Type : Sandy.

For the climate recorded at Lytton (by AES Climate Services) for the experimental period see Experiment 2 data.

Results : Control cell preparations made prior to jaw deposition showed the cells of the pulp to be intact. From 2 buried pig mandibles retrieved at 1 week (September 4th), 7 extracted teeth revealed red wet noxious pulp tissue which was acellular.At 1 month (September 25th), 2 jaws from which 3 anterior and 2 molars were extracted, revealed acellular white papery tissue adhering to the walls of the pulp chambers. The jaws exposed to the ground surface were disturbed by animals, in spite of elaborate precautions taken to secure them (a chain link mesh held in place with large tent pegs driven into the ground,with the mesh further

secured by heavy rocks placed around the perimeter). At 1 week, 5 jaws were missing, and on the remaining 3, the mandibular condyles had been chewed. Many animal tracks were readily visible on the ground surface in the vicinity.The surface tissue on the mandibles was brown and dry making it difficult to dislodge the teeth.In the 5 teeth extracted at 1 week, very little pulp tissue remained.This tissue was dry, white, and papery in appearance. No cells remained intact. Five teeth extracted at 4 weeks presented the same result.

VANCOUVER

Winter Subsurface and Surface Deposition of Unextracted Pig Teeth

Experiment 9 (Catalogue D.X.).

Period of Experiment : Burial 2 month (October 10th to December 20th 1987), retrieval at 1 week (October 17th) and 2 months (December 20th). Surface deposition 9 days (November 6th to November 14th 1988), retrieval daily.

Location : Vancouver.

Elevation : less than 150 metres.

Burial Depth : 30 cm.

Sample Size : 1 pig's head subsurface, 7 jaws surface.

Soil Temperature at Burial Depth: 19C (at burial), 15C (at 1 week), 4C (at 2 months).

Soil pH : 5.6 (at burial), 5.6 (at 1 week), 5 (at 2 months).

Soil Type : Humic.

For the subsurface material the climate was recorded at Vancouver International Airport (by AES Climate Services). See Experiment 6 for the data.

Climate Recorded at Burnaby Metrotown for the Experimental Period (about 3km from the site) by AES Climate Services.

	Temperature (centigrade)		Rainfall(mm)
	Maximum	Minimum	
November 6th	11.5	6.0	0.3
7th	11.0	3.0	10.7

8th	6.5	4.0	11.5
November 9th	9.5	5.0	12.2
10th	8.0	5.5	5.9
11th	8.0	1.0	22.2
12th	11.5	3.0	0.3
13th	12.0	4.5	
14th	6.5	0.0	1.4

Results: Control cell preparations made prior to deposition showed the cells of the pulp to be intact. After 1 week burial 5 pulps from extracted anterior teeth were wet and acellular. From 5 anterior teeth retrieved from the head at 2 months, the same result was obtained. Samples of pulps from surface deposited pig jaws (3 anterior teeth per time interval), were paraffin sectioned to analyse the putrefactive process. At day 1 the cells were all intact with no signs of necrotic change. At day 3 some nuclear atrophy was observed and some margination of chromatin (aggregation of chromatin at the nuclear periphery) was noted. By the 6th day most of the tissue contained sheets of ghost cells (nuclear outline only, no chromatin structure remaining); in a few areas nuclear chromatin was still apparent, although even here many nuclei were atrophied. By the 9th day most nuclei had undergone autolysis and appeared as debris.

VANCOUVER

Daily Accounting of Human Pulp Cell Decomposition

Experiment 10 (Catalogue Super)

Period of Experiment: Burial and Exposure 7 days (May 24th-May 31st 1989) retrieval daily.

Location: Vancouver.

Elevation: less than 150 metres.

Burial Depth: 30 cm.

Sample Size: 2 male and 2 female pulps per retrieval.

Soil Temperature at Burial Depth: 11C at burial, 12C at 7 days.

Soil pH: 6 at burial, 6 at 7 days.

Soil Type: Humic.

Climate Recorded for the Experimental Period (about 3 km from the site) at Burnaby Metrotown by AES Climate Services

	Temperature (centigrade)		Rainfall (mm)
	Maximum	Minimum	
May 24th	12.0	6.0	0.4
25th	17.0	8.5	4.7
26th	10.5	8.0	28.2
27th	12.0	7.5	1.0
28th	14.5	7.5	
29th	16.5	7.0	
30th	17.5	11.0	
31st	21.5	10.5	

Results : Chapter 5, Section A, p. 86/91.