THE AGEING OF BRUISES IN LAMBS

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ABSTRACT

Bruising in lambs processed for human consumption is a significant economic problem. A reduction in the prevalence of bruises could be achieved relatively efficiently if their important places of occurrence could be identified by ageing these lesions with respect to the known times of occurrence of events of possible aetiological significance. To this end efforts were made to age experimental bruises in lambs by objectively assessing semi-quantitative histopathological data using a mathematical model based on Bayes' theorem of inverse probabilities, by enzyme histochemical and isoelectric focussing studies and by measurement of muscle pH.

The Bayesian method for objective histopathological ageing was developed and tested on data representing 178 bruises. It was successful in identifying bruises as either 1-20 hours or more than 24 hours old. The 'accuracy' with which a bruise of known age could be identified as such depended on the nature and number of tissue samples studied. The degree of 'confidence' with which an individual bruise of unknown age could be aged, however, depended both on the 'accuracy' of the method and on the relative number of bruises estimated to belong to each of the two age categories considered. In general a degree of 'confidence' of 80-90% can be expected in practice, and in this respect the performance of the Bayesian method is superior to that achieved by purely subjective means. A pilot survey involving 107 bruises collected from an export meat works established both the practical value of the objective ageing method and its superiority over alternative epidemiological approaches to the problem of utilising data pertaining to trucking times and holding times in meat works yards. Of the bruises studied, 60% were estimated to have been inflicted within the works, and 40% prior to arrival.

Enzyme studies on bruises aged 4-144 hours old revealed no detectable relationships between observed changes in either histochemical or isoenzyme activities and bruise ages. In light of contradictory published results pertaining to other types of wounds, this lack of success was thought to reflect the relatively mild nature of the tissue reaction in bruises.

Statistically significant relationships could not be demonstrated between absolute or relative muscle pH and the ages of bruises from 4-48 hours old.

A newly recognised condition of 'subcutaneous haemorrhagic speckling' in the carcase adipose tissue of young lambs processed for human consumption was investigated. From histopathological and epidemiological evidence, the primary cause of the lesions was shown to be electrical stunning. However, secondary aetiological factors were proposed as having influenced the prevalence and severity of lesions. Attempts to elucidate the pathogenesis of 'speckling' with the intention of formulating a rational approach to its prevention were unsuccessful.

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TABLE OF CONTENTS

LIST OF TABLES

TABLE

TABLE

TABLE

TABLE

LIST OF FIGURES

INTRODUCTION

Bruising in livestock is an important source of economic loss to the New Zealand meat industry. During the 1978/79 killing season the problem in sheep was estimated to have cost $1.3 million (Millar and Glover, 1980) and during the 1976/77 season bruising in cattle cost approximately $2 million (Marshall, 1977). Losses of $22.3 million and $45 million per annum have been recorded in Australia and the United States of America respectively (Rich, 1973; Anon, 1975).

Epidemiological investigations in Australia have shown the prevalence of bruising to be affected by both animal associated and extrinsic factors (Anon, 1972; Meischke *et al.*, 1974). The former included sex, temperament, age and the presence or absence of horns, whilst the latter included stockyard design, animal handling techniques and methods of transport. Transport associated factors such as truck design, distance travelled, number of stops and driver skills were found to be particularly important in Queensland, where animals are frequently hauled over long distances (Wythes, 1981). The prevalence of bruising in New Zealand has likewise been shown to be affected by such factors as stock handling techniques, yard design, trucking distances, wool length and the number of times animals are washed prior to slaughter (Marshall, 1977; Petersen, 1978). Epidemiological studies have thus highlighted a range of possible factors in the aetiology of bruising. However, the data collected has been insufficient to allow the relative importance of each of these factors to be ascertained. If bruises could be aged in relation to known times of occurrence of events of specific interest, the various aetiological factors could be defined and ranked in order of importance with a degree of certainty previously unachievable.

The most established method of ageing wounds for forensic purposes is to match their gross and histopathological characteristics with those in similar wounds of known ages (Walcher, 1930; Raekallio, 1977). Nevertheless, many of the criteria used for wound ageing in humans have been based on relatively few and widely varying observations.

From experiments on laboratory animals (Raekallio, 1961; Hirvonen, 1968a; Ojala, 1968) it appears that the two most important sources of variation when ageing bruises are differences between observers and between the species of animals studied (Raekallio, 1973). In this study the reaction to bruising injury was therefore characterised using lesions inflicted on sheep, since this was the species of practical interest. Those features judged as being of potential value for ageing purposes were used to age bruises objectively using a mathematical model based on Bayes' theorem of inverse probabilities as adapted for use in computerised medical diagnosis (Bailey, 1968). Once developed, the model was tested in a pilot survey carried out under normal operating conditions in an export meat works.

Wounds can also be aged according to their histochemically and electrophoretically determined enzyme profiles. This approach is of particular interest in that these profiles appear to change most dramatically during the early stages of wound healing when routine histopathological changes are most ambiguous (Raekallio, 1977). Histochemically demonstrated enzyme activities and isoenzyme patterns were therefore determined in bruises of known ages, and the results interpreted in relation to the duration of injury.

Local tissue pH changes are known to occur during inflammation (Walter and Israel, 1974). An attempt was therefore made to establish a correlation between the pH and the age of bruised muscle. This aspect of wound ageing has not been reported previously.

The final part of this thesis details an investigation into a previously unrecognised haemorrhagic lesion affecting the adipose tissue of the carcases of lambs slaughtered in an export meat works. The lesions were described, their aetiology defined and attempts made to elucidate their pathogenesis.

CHAPTER I

REVIEW - THE AGEING OF WOUNDS

There is a relative paucity of information relating specifically to the ageing of bruises, so the following review pertains to the ageing of wounds in general. It is divided into four main sections. In the first section, factors that may affect the time-course of the tissue reaction to injury are described, and the implications of failing to consider these factors when extrapolating from the literature are noted. In the second section the gross and chemical changes characteristic of bruises are described, and in the third and fourth sections respectively histopathological and enzymic changes in wounded tissues are discussed.

I. FACTORS AFFECTING THE TIME-COURSE OF THE TISSUE REACTION TO INJURY

Tissue reactions to injury encompass stages of degeneration, necrosis, repair and regeneration. Certain chemical or morphological features are associated with each of these stages and after determining the times after injury at which they occur it is possible to use them to age wounds (Walcher, 1930; Raekallio, 1977). Unfortunately this method of ageing cannot be used without reservation, since although the sequences of change following injury are constant (Fishback and Fishback, 1932; Godman, 1957; Price *et al.*, 1964a; Ordman and Gillman, 1966; Veress *et al.*, 1966; Hurley, 1972; Pullar, 1973; Sciuba *et al.*, 1978), both their magnitudes and time-courses may be affected by such variables as the type of injury, its severity, the tissue affected and the species of animal involved. The influence of each of these factors, along with the effects of contributory variables such as the age of the animal, previous injury and differences in interpretation of observations must therefore be considered when reviewing published observations of the temporal changes in wounds.

1. The effect of type and severity of injury on the time-course of the ensuing tissue reaction

The characteristics of an inflammatory response may vary according to the type and severity of injury inflicted (Hurley, 1972). There has been little direct comment on the effect of type of injury on the time-course of the ensuing tissue reaction, but Hurley and Edwards (1969) attributed observed differences in times of onset of vascular permeability in burns as compared to crush injuries to the type of injury as well as its severity. Hirvonen (1968b), on the other hand, noted that the time-course of the inflammatory reaction in adipose tissue was independent of the type of injury.

A positive relationship generally exists between the severity of injury and both the degree of the ensuing tissue reaction (Millar, 1934; Godman, 1957; Simpson, 1965; Ojala, 1968; Hurley and Edwards, 1969; Steele and Wilhelm, 1970) and its rate of progression (Millar, 1934; Veress, 1966; Hurley *et al.*, 1967; Hirvonen, 1968a, Hurley and Edwards, 1969). If possible the severity of injury should therefore be taken into account when studying reactive tissue changes in wounds.

2. The effects of species of animal and type of tissue affected on the time-course of the tissue reaction to injury

The relationship between the rate of healing and the species of animal affected cannot be clearly defined because of a paucity of published experimental evidence. Both Hamdy *et al.* (1957a) and McCausland and Dougherty (1978) have observed similar rates of healing between bruises in different species of livestock. Bruises in poultry, however, are said to heal between two and three times faster than equivalent lesions in mammals (Hamdy *et al.*, 1961a). Reactions to a variety of types of injury appear to proceed faster in rodents than in man (Fatteh, 1966a; Berg and Ebel, 1969; Ojala *et al.*, 1969; Pullar, 1973), although Raekallio (1965) has recorded similar reaction rates to incision injuries in humans and guinea pigs. On balance,

the probability exists of there being significant differences between the reaction rates to injury in different species of animals, and for this reason a need for caution has been recognised when extrapolating results from laboratory animals to man for forensic purposes (Fatteh, 1966a; Pullar, 1973; Raekallio, 1977).

It is generally agreed that reactions to injury proceed faster in adipose tissue than in muscle and faster in muscle than in skin (Ordman and Gillman, 1966; Hirvonen, 1968a; Hirvonen and Ojala, 1968; Ojala *et al.*, 1969; Robertson and Hodge, 1972; Pullar, 1973; Sciuba *et al.*, 1978), notwithstanding the fact that McCausland and Dougherty (1978), when studying bruises in lambs and calves, noted no differences between the rates of the reactions in muscle and adipose tissue. The reasons for the relationship between rate of healing and tissue type are open to conjecture, but the degree of tissue vascularity appears to exert an effect (Simpson, 1965; Ordman and Gillman, 1966; Hirvonen and Ojala, 1968).

3. The effects of subject age, multiple wounding and observer variation on the rate of the tissue reaction to injury

As judged by gross appearance, bruises resolve more slowly in older people (Smith, 1965; Simpson, 1979). By measuring tissue bilirubin levels, Hamdy *et al.* (1957c) have demonstrated an apparent decrease in the rate of healing of bruises in older as compared to younger poultry and rabbits. Raekallio (1972), on the other hand, found that the histological reaction to injury was noticeably retarded only in cases of advanced senility with cachexia, and McCausland and Dougherty (1978) expressed the opinion that age was unlikely to be a factor affecting the rate of the histological reaction to bruising in livestock. It appears, therefore, that the age of the animal affected has relatively little effect on the histological reaction to injury.

Raekallio (1965) found that severe and multiple wounding caused an impairment of the local tissue response to injury but he was unable to explain his observations. In contrast, Hamdy *et al.* (1957c, 1961a), by measuring tissue bilirubin levels, demonstrated increased rates of healing in bruises inflicted on previously bruised rabbits or poultry, and these authors claimed to have been able to passively transfer a 'healing factor' present in blood from bruised animals.

Due to the subjective nature of histopathological judgements, times recorded for reactive changes in injured tissue reflect a component of observer variation. This factor should be borne in mind if times of occurrence for the histological features of wound healing are to be estimated using a number of diverse sources.

II. THE GROSS APPEARANCE OF BRUISES

Bruises are wounds characterised by haemorrhage into interstitial spaces (Raekallio, 1973). This haemorrhage usually occurs from vessels damaged by trauma from a blunt object, although incidental bruising may occur in tissues adjacent to incisions and lacerations (Simpson, 1979). Although the severity and dimensions of a bruise depend primarily on the nature of the striking object and its force of impact, its characteristics are also influenced by such factors as tissue vascularity, tissue density, support from adjacent tissues, depth of underlying bone and time elapsed from injury (Smith, 1936; Hamdy *et al.*, 1957a; Simpson, 1979). In its gross appearance therefore, a bruise does not necessarily provide accurate information as to its specific cause (Simpson, 1979).

The colour changes so characteristic of bruises represent accumulations of haemoglobin or its breakdown products (Simpson, 1979). The sequence of change is constant (Hamdy *et al.*, 1957c) but the rate may vary according to severity of haemorrhage (Smith, 1936; Hamdy *et al.*, 1957c), site (Simpson, 1965), vitality of tissue affected (Simpson, 1979), species of animal (Hamdy *et al.*, 1961c; McCausland and Dougherty, 1968) and possibly environmental temperature (Hamdy *et al.*,

1957c). Nevertheless, colour changes are useful indicators of age and Hamdy *et al.* (1957a) have recorded the following sequence in bruised cattle, sheep, pigs and rabbits: dark red up to two days, green-purple at three days, yellow-green at four days, orange at five days, light orange at six days and a return to normal colour at seven days. Caution must be exercised if examining bruises in dead subjects, since the above colours fade rapidly after death and thus become unreliable as a means of estimating age (May and Hamdy, 1966).

III. BLOOD DEGRADATION PRODUCTS AND TISSUE CONDUCTIVITY IN BRUISES

Hamdy *et al.* (1957a) noted that haemoglobin concentrations in cattle bruises increased 15 minutes after trauma and continued to increase for the next 5 days. A return to pre-bruise levels had occurred by day 9, which was 2 days after visual evidence of resolution. Inorganic iron was not released from the extravasated haemoglobin until 2 days after bruising. They also used a sensitive colourimetric technique to measure bilirubin concentrations in normal and bruised muscle. They could not detect bilirubin in normal muscle but demonstrated it in 2 day old bruises. Concentrations increased thereafter with bruise age, peaking at 4 days and returning to pre-bruise levels by day 9. A less sensitive but considerably more simple test was able to detect bilirubin in bruises 60 hours and older (Hamdy *et al.*, 1958, 1961c).

Tissue conductivity, as measured by inserting electrodes into muscle, has been shown to increase in bruises (Hamdy *et al.*, 1957a). The conductivity varied according to tissue fat content and severity of injury, but average values representing several bruises increased 15 minutes after injury, peaked at 40 hours and returned to normal by day 7.

IV. HISTAMINE AND SEROTONIN LEVELS IN WOUNDS

Vasoactive amines are released into tissue spaces early in the pathogenesis of most inflammatory reactions (Walter and Israel, 1974). For this reason Raekallio and Makinen (1966, 1969a, 1969b, 1970) measured free histamine and serotonin concentrations in skin incisions affecting laboratory animals and humans. Histamine concentrations increased significantly within 30 minutes of injury and returned to normal within 60 minutes, whereas serotonin concentrations increased significantly within 15 minutes of injury and returned to normal several hours later. Because both the release and degradation of these compounds are enzyme-regulated processes requiring oxygen, their tissue concentrations did not rise in wounds inflicted *post mortem*. *Ante mortem* concentrations remained stable for at least 5 days after death. Raekallio and Makinen therefore concluded that histamine and serotonin levels were of value for estimating the ages of skin wounds too young to be aged using conventional histological criteria. However, they emphasised the need to measure concentrations of these compounds relative to those in contralateral control tissues, since both serotonin and histamine concentrations exhibit wide individual animal and site variations.

V. THE HISTOPATHOLOGY OF WOUNDS

A. The histopathology of wounds involving skeletal muscle

1. Degenerative changes in muscle fibres following injury:

Following injury, muscle undergoes progressive degeneration, the severity of which is indicated by the histological appearance of affected fibres (Fishback and Fishback, 1932; Veress *et al.*, 1966), but the sequence of which is almost independent of the type of injury (Adams *et al.*, 1967; Carlson, 1973). Muscle fibres are usually described as having undergone either cloudy swelling (cell swelling), hyaline, vacuolar, granular or fatty degeneration, but other criteria such as loss of cross striations, contraction of sarcoplasm and

nuclear degeneration have also been used to indicate the severity of change from normal (Fishback and Fishback, 1932; Veress *et al.*, 1966; Adams *et al.*, 1967). Since the histological appearance of damaged muscle fibres alters with time, the possibility exists of using the type of degenerative change present to estimate the age of wounds involving muscle. However, the morphology of degenerating muscle fibres depends not only on the time elapsed since injury but also on the severity of the insult, since this factor affects both the rate of degeneration (Veress *et al.*, 1966; Hurley and Edwards, 1969; Josza and Reffy, 1978) and the ultimate severity of the degenerative change (Price *et al.*, 1964a; Veress *et al.*, 1966; Adams *et al.*, 1967). The severity of injury is rarely uniform within or between muscle fibres, so several stages of degeneration and necrosis usually co-exist at any one time (Veress *et al.*, 1966; Adams *et al.*, 1967; Benoit and Belt, 1970; McCausland and Dougherty, 1978; Josza and Reffy, 1978; Ali, 1979). Furthermore, degenerative changes induced by the original injury are modified by the effects of delayed ischaemia (Hopkinson and Watts, 1963). For these reasons degenerative changes in muscle are unlikely to be of great value for estimating the ages of wounds. This is substantiated by the wide variation in times reported for the onset and duration of degenerative changes in a variety of wounds, including bruises (McCausland and Dougherty, 1978; Josza and Reffy, 1978), incisions (Ojala, 1968), burns (Hurley *et al.*, 1967), ischaemia (Moore *et al.*, 1956; Scully and Hughs, 1956), cold injury (Price *et al.*, 1964; Veress *et al.*, 1966), chemical injury (Veress *et al.*, 1966; Adams *et al.*, 1967; Benoit and Belt, 1970), crush wounds (Hurley and Edwards, 1969) and bullet wounds (Hopkinson and Watts, 1963).

2. The leucocytic reaction following injury to muscle:

Small numbers of polymorphonuclear and mononuclear leucocytes are released into tissue spaces immediately after injury as components of haemorrhage, but within hours these cells comprise only a small proportion of the interstitial leucocyte population, due to the arrival of the cellular exudate (Ojala, 1968; McCausland and Dougherty, 1978). Times recorded for the arrival and subsequent

changes in the numbers of leucocytes vary, but it is accepted that the first cells to appear in significant numbers are polymorphonuclear neutrophils (Ojala, 1968; Ojala and Lempinen, 1969). These are noted in tissue sections from 30-60 minutes after injury and increase in number over a period of approximately 12-24 hours (Hopkinson and Watts, 1963; Hurley *et al.*, 1967; Hirvonen and Ojala, 1968; Ojala, 1968; Hurley and Edwards, 1969; Ojala *et al.* 1969; McCausland and Dougherty, 1978). Their numbers then decline, but small numbers of neutrophils may persist among interstitial macrophages for at least 3 days after injury (Ojala, 1968). Macrophages arrive in significant numbers later than neutrophils (Raekallio, 1977) but may be observed as early as 1-2 hours after injury. They increase in numbers for 48-72 hours thereafter, during much of which time they are the predominant cell in the exudate (Ojala, 1968; Hall-Craggs, 1974; McCausland and Dougherty, 1978). Absolute numbers of leucocytes vary according to the severity of injury (Hurley and Edwards, 1969) but McCausland and Dougherty (1978) found relative numbers of macrophages and neutrophils a useful indication of age in as much as the macrophage/neutrophil ratios inverted in bruises older than 24 hours. Leucocytes invade and phagocytose damaged muscle tissue but reported times for this event vary widely from 4 hours (Ojala, 1968) to 72 hours (Hall-Craggs, 1974) after injury.

It thus appears that times of arrival of macrophages and neutrophils, their relative numbers and the period of time before which they invade damaged muscle fibres are all factors of potential value for estimating the ages of wounds.

3. Fibroplasia and vascular changes following injury to muscle:
Using light microscopy, regenerative changes in vascular endothelium become visible within 2-3 days of injury to muscle (Le Gros Clark, 1946; Hopkinson and Watts, 1963; Fishbein *et al.*, 1978; McCausland and Dougherty, 1978). Affected endothelial cells become plump and rounded and possess swollen vesicular nuclei showing occasional mitotic figures. Ultrastructural changes precede those visible by light microscopy. Hurley and Edwards (1969) observed electron microscopic evidence of damage to endothelial cells 4 hours after

crush injury, and regenerative changes were detected after 24 hours. By 48 hours regenerative changes were more common, but the ultra-structural characteristics of affected endothelial cells were no different to those observed at 24 hours. Fibroblasts are usually observed in endomysial, fascial and perivascular sites within 2-3 days of injury (Le Gros Clark, 1946; Godman, 1957; Hopkinson and Watts, 1963; Fishbein *et al.*, 1978; McCausland and Dougherty, 1978), but they have also been detected in wounds only 2-4 hours old (Ojala, 1968; Ojala *et al.*, 1969). Over subsequent days they are observed in increasing numbers throughout interstitial spaces and are most numerous in areas of more severe damage (Le Gros Clark, 1946; Ojala, 1968). Fine strands of newly synthesised collagen may be detected in close association with fibroblasts at from 3-7 days following injury (Hopkinson and Watts, 1963; Ojala, 1968; Fishbein *et al.*, 1978).

Fibroplasia may become a prominent feature of incision or crush injuries to muscle (Le Gros Clark, 1946; Ojala, 1968) but it is not usually a prominent feature in bruises (Robertson and Mansfield, 1957).

4. Regenerative changes in muscle fibres following injury:
Muscle fibres begin to regenerate within 2-4 days of injury (Carlson, 1973). The regenerative process is described as being either 'continuous' or 'discontinuous' according to whether new fibres are formed by extension from viable remains of injured fibres or by fusion of individual muscle precursor cells called myoblasts (Ali, 1979). The mode of regeneration depends on the extent to which muscle architecture has been disrupted and hence on both the type and degree of injury. 'Continuous' regeneration occurs if sarcolemmal sheaths have been destroyed, whilst 'discontinuous' regeneration occurs if their integrity has been preserved. Since muscle is rarely damaged uniformly following injury, both types of regeneration are usually found in the same lesion (Ali, 1979).

Myoblasts can be recognised by their basophilic cytoplasm and enlarged oval nuclei with prominent nucleoli, but they are often difficult to distinguish from macrophages by light microscopy. They

are usually observed immediately beneath sarcolemmal basement membranes within 2-3 days of injury, but have been noted after only 2-4 hours following chemical injury where the vascular and supporting architecture of muscle were preserved intact (Benoit and Belt, 1970; Dolwick *et al.*, 1977). Numbers of myoblasts increase rapidly to peak at 3 days, by which time they extend throughout sarcolemmal tubes, coexisting alongside phagocytosing macrophages. After 3 days their numbers decline as they fuse to form multinucleate sacroplasmic straps called sarcoblasts or myotubes (Carlson, 1973; Ali, 1979).

During the 'continuous' mode of regeneration small buds of intensely basophilic sarcoplasm are observed within 3-4 days of injury, projecting from the ends of surviving muscle fibres. These buds subsequently elongate and by the 5th day have become invested with a central chain of immature myonuclei (Millar, 1934; Ali, 1979). In 'continuous' regeneration sarcoblasts thus form by a process of extension.

Regardless of whether they are formed by the processes of extension or fusion, sarcoblasts are characterised at an early stage in their formation as thin straps of relatively basophilic sarcoplasm possessing central chains of immature myonuclei and longitudinal striations (Ali, 1979). They are observed in regenerating muscle from the 3rd day after injury and over the next few days become the predominant regenerative feature (Carlson, 1973). After the 4th day they increase in width (Le Gros Clark, 1946). Cross striations do not become visible by light microscopy until 6-10 days after injury, depending on the rate at which myofilaments aggregate to form myofibrils (Millar, 1934; Godman, 1957; Hall-Craggs, 1974).

As they mature, sarcoblasts become less basophilic and more cross-striated, their nuclei decrease in size and their nucleoli become less prominent. Eventually sarcoblast nuclei migrate to the periphery of the myotube, at which time the sarcoblasts, though thinner than normal muscle fibres, become in other respects morphologically indistinguishable from them (Carlson, 1973). This process takes in excess of 20 days to complete (Benoit and Belt, 1970; Dolwick *et al.*, 1977).

B. The histopathology of wounds involving adipose tissue

Subcutaneous adipose tissue is particularly prone to traumatic injury because of its superficial position and its relative lack of connective tissue support. Moreover, the tissue reaction following injury is generally florid, probably because of the highly vascular nature of this tissue (Roberts *et al.*, 1956; Hirvonen, 1968b). A study of the reaction to injury in adipose tissue is therefore of potential value for wound ageing purposes.

1. Degenerative changes in adipocytes following injury:

Following bruising of the adipose tissue of guinea pigs, Hirvonen (1968b) noted that a proportion of adipocytes immediately ruptured, releasing their lipid contents into tissue spaces. Less severely damaged adipocytes became swollen and rounded, and some of these cells ruptured within 2-4 hours of injury. Hirvonen did not detect degenerative changes in adipose cell nuclei, but Panabokke (1958) observed karyolysis within 2 hours of inducing fat necrosis in rats.

Degenerative changes in the lipid component of fat cells occur soon after injury. Within 1 minute, damaged and normal adipocytes could be clearly distinguished by U/V light, due to the appearance of birefringent crystals in the lipid of damaged cells (Hirvonen, 1968b). These crystals persisted for up to 2 weeks, but had finally disappeared by 4 weeks after injury. With conventional light microscopy and haematoxylin and eosin stain (H+E), injured adipocytes were observed to become foamy and cloudy 2-4 hours after injury, though the time depended to some extent on severity of damage (Hirvonen, 1968b; Flock *et al.*, 1973). The altered appearance was due to crystal formation within the lipid droplet. With special stains, distinct changes in the lipid component of injured adipocytes were detected in wounds only 10 minutes old. Neutral lipid stains changed colour or became less intense and fatty acid crystals could be demonstrated in the centres of lipid droplets. These features became more marked with time. Eventually a proportion of necrotic adipose cells came to stain only for fatty acids, either in liquid or solid form (Ito, 1973).

Saponification has been recorded as an early feature of fat necrosis in rats (Roberts *et al.*, 1956), but soaps are not a prominent component of bovine fat necrosis (Ito *et al.*, 1968).

More chronic degenerative changes include calcification, the formation of cholesterol clefts and the formation of 'oil cysts'. Calcification of necrotic adipocytes and the connective tissue stroma has been noted within 24-48 hours of injury in rats (Panabokke, 1958; Storck and Bjorntorp, 1971). Intense calcification was a common feature of chronic fat necrosis lesions in cattle and pigs (Ito *et al.*, 1968; Ito, 1973). Cholesterol clefts surrounded by histiocytes have been observed in 5 day old lesions of fat necrosis in rats (Cameron and Mallik, 1954). Oil cysts, which are spaces lined by histiocytes or giant cells and containing degrading lipid, are a feature of older lesions (Seneviratne, 1963). They appear to form from 1-2 weeks after injury (Hirvonen, 1968; Panabokke, 1968), and may persist for several months (Cameron and Mallik, 1954) before being replaced by fibrous tissue.

Regeneration is not a sequel to fat necrosis (Cameron and Mallik, 1954; Hirvonen, 1968b; Ito, 1973). Necrotic adipocytes either persist for long periods of time in macrophage-filled spaces, calcify, or become replaced by fibrous tissue (Ribelin and De Eds, 1960).

2. The leucocytic reaction following injury to adipose tissue:
Leucocyte infiltration may be the earliest reactive change seen following damage to adipose tissue (Ojala *et al.*, 1969). A neutrophil exudate has been observed within 30 minutes of injury to this tissue, and the arrival of macrophages has been noted after 60 minutes (Hirvonen, 1968a; Ojala *et al.*, 1969). The neutrophils became absent from 5 day old bruises but macrophages and lymphocytes were present in large numbers for up to 2 months, after which time their numbers declined. Times recorded for giant cell formation vary from 1-5 days after injury (Cameron and Mallik, 1954; Hirvonen, 1968b). They become very numerous in areas of severe damage and persist for in excess of 4 months after injury.

3. Fibroplasia and vascular changes following injury to adipose tissue: Fibroplasia is a prominent feature of chronic fat necrosis (Farr, 1923; Cameron and Mallik, 1954; Ito, 1973). Fibroblasts have been noted in human and guinea pig adipose tissue as early as 2-4 hours after bruising and incision injury (Hirvonen, 1968a, 1968b; Ojala *et al.*, 1969). They were first observed perivascularly and adjacent to connective tissue septa, but after 8 hours were widespread throughout interstitial spaces. Maximum numbers of fibroblasts were noted within 2-3 days of injury, but after 5 days numbers decreased as they matured to fibrocytes (Hirvonen, 1968b). In contrast to the above, where fibroplasia was noted within a few hours of injury, Cameron and Mallik (1954) first observed fibroblasts 48 hours after local freezing injury to rats. Numerous fibroblasts were still present in wounds 2 weeks old.

Mucosubstances have been detected within fibroblasts 1-8 hours after bruising or incision injury to guinea pig adipose tissue (Hirvonen, 1968a, 1968b). These substances were also present extracellularly in wounds more than 8 hours old. Ojala *et al.* (1969), however, were not able to demonstrate mucosubstances in human adipose tissue in wounds up to 4 hours old, which was the latest age studied.

In guinea pig adipose tissue, reticulin fibres were first noted in close association with fibroblasts in 8 hour old wounds (Hirvonen, 1968a, 1968b). By 12-24 hours these fibres had matured into recognisable collagen. By 7 days after freezing, collagen fibres had either formed dense connective tissue bundles replacing ruptured adipocytes, or they existed as light networks around damaged but unruptured adipose cells. Cameron and Mallik (1954) failed to detect reticulin fibres in rats until 5 days after freezing injury. Newly formed collagen was detected after 7 days.

Vascular lesions are not commonly mentioned in descriptions of the histological reactions of adipose tissue to injury. Ito *et al.* (1968) observed hyaline degeneration of blood vessels in acute and chronic porcine fat necrosis, but remarked on the lack of vascular involvement

in bovine lesions. Cameron and Mallik (1954) observed swollen endothelial cells 24 hours after freezing injury to rats. Hirvonen (1968b) observed capillary proliferation in 2-3 day old bruises in guinea pigs, and noted that a rich capillary network persisted for 2-4 weeks after injury.

C. The histopathology of wounds involving skin

When studying the time-course of histological events following injury, forensic pathologists have concentrated mainly on reactions in skin, despite the fact that reactive changes occur faster and are more pronounced in muscle or adipose tissue (Hirvonen, 1968a, 1968b; Robertson and Hodge, 1972). This preoccupation with skin mainly reflects the fact that it is consistently involved in wounds of forensic importance (Simpson, 1979), although Robertson and Hodge (1972) studied abrasions in preference to bruises because they found the reaction sequence more consistent in the former. If the epithelium is excluded from consideration, the histological reaction in skin can be regarded as comprising those changes associated with leucocyte populations, ground substance, connective tissue and blood vessels. Each of these will be considered separately.

1. Degenerative changes in skin following injury:

The morphology and staining properties of mature dermal connective tissue have been observed to alter following incision injury (Ordman and Gillman, 1966; Hirvonen, 1968a; Ojala *et al.*, 1969; Robertson and Hodge, 1972). Within 2-8 hours collagen fibres became swollen and homogeneous. They also became more eosinophilic with H+E stain but lost their affinity for connective tissue stains. The above changes were accentuated during the first 16-24 hours after injury (Hirvonen, 1968a). However, they are not peculiar to *ante mortem* wounds, since Robertson and Hodge (1972) also observed similar features in agonal and *post mortem* wounds left for several hours before processing.

According to Furukava (1959), elastin fibres retracted from lines of incision in *ante mortem* wounds, and thus appeared wavy, whereas in *post mortem* wounds they remained straight. Fatteh (1971) however, was unable to confirm these results, and he considered the light microscopic appearance of elastin fibres of no value in determining whether a wound was inflicted prior to, or after death.

2. The leucocyte reaction following injury to skin:

Leucocyte margination has been observed within 30 minutes of injury and is thus one of the earliest reactive changes affecting skin (Walcher, 1930; Hirvonen, 1968a). It is, however, a difficult criterion to assess (Pullar, 1973) and furthermore, it has been observed in wounds inflicted immediately after death (Raekallio, 1977). Leucocyte margination is therefore of little practical value for wound ageing purposes. The earliest reliable leucocytic response to injury is neutrophil exudation. Usually this commences from 1-8 hours after injury, although delays of up to 24 hours have been recorded (Fatteh, 1966a, 1966b; Raekallio, 1965; Hirvonen, 1968a; Hirvonen and Ojala, 1968; Ojala *et al.*, 1969; Malik, 1970; Robertson and Hodge, 1972). The neutrophils accumulated in interstitial spaces rapidly, and maximum numbers were usually reached within 24 hours of injury. After this, their numbers declined in favour of macrophages (Ross and Benditt, 1961; Fatteh, 1966b; Steele and Wilhelm, 1970). In skin wounds, the macrophage exudate usually arrives approximately 8 hours after injury and becomes a prominent feature by 24 hours. The decline in numbers is slow compared to that of neutrophils, and a vigorous response may be observed for at least 8 days after injury (Robertson and Hodge, 1972). Regardless of actual leucocyte numbers, macrophages tend to be in excess of neutrophils in the exudate of wounds more than 12-24 hours old (Ross and Benditt, 1961; Sciuba *et al.*, 1978).

Eosinophils are not usually a feature of the histological reaction to injury but they have been observed in both incisions and abrasions. Florid eosinophil exudates have been observed in 4-6 hour old abrasions affecting dogs and in 2 day old incisions in pigs (Riddle and Barnhart, 1964; Ordman and Gillman, 1966). Steele and Wilhelm (1970) observed them also in guinea pig incisions, where their

arrival 2-6 hours after injury coincided with that of neutrophils. In contrast to the neutrophils however, the eosinophils aggregated peripheral to areas of damage. Maximum numbers of eosinophils had accumulated within 5-15 hours of injury, but the subsequent decline in population was slow, and eosinophils still persisted at the margin of wounds 50 days old. By this time other leucocytes had disappeared. Eosinophils have also been observed amongst and peripheral to scar tissue in rat incisions (Bassett *et al.*, 1977). Numbers increased rapidly from day 6-12 and remained elevated for in excess of 30 days, at which time the experiment was terminated. These authors suggested that the eosinophils shared a function in the synthesis of sulphated glycosaminoglycans or in the remodelling of collagen.

3. The histochemistry of acid glycosaminoglycans in skin wounds:
Acid glycosaminoglycans, formerly called acid mucopolysaccharides, are components of ground substance produced locally by connective tissue cells. They can be demonstrated histochemically with a variety of stains, of which alcian blue is one of the most specific. The staining properties of acid glycosaminoglycans alter rapidly following injury, probably because of changes in configuration caused by altered local pH and electrolyte concentrations (Gersh and Catchpole, 1960). Loss of staining has been observed in 1 hour old bruises as well as in abrasions, incisions and electrocution wounds (Raekallio, 1961; Nevelos and Gee, 1970). Nevelos and Gee (1970) believed the change was caused by haemorrhage, and cited in evidence the fact that acid glycosaminoglycan staining remained as normal in bloodless wounds such as those caused by strangulation and hanging. The initial phase of decreased acid glycosaminoglycan staining is generally superceded by a period of increased staining, the intensity of which may cause the wound to become clearly delineated from surrounding tissue (Hirvonen, 1968a). This change may occur as early as 2-6 hours after injury (Gersh and Catchpole, 1949), in which case it reflects further structural alterations to pre-existing acid glycosaminoglycan polymers. Usually, however, it occurs later, with the advent of repair, and is due to the synthesis of new material by fibroblasts (Raekallio, 1973). Increased intrafibroblastic and extracellular staining has thus been observed mainly in wounds from 16-72 hours old (Dunphy and Udupa, 1955;

Raekallio, 1961; Hirvonen, 1968a). Subjectively assessed acid glycosaminoglycan levels remained elevated for up to 6 days in rat incisions, after which time there was a sharp decrease, coinciding with the appearance of newly synthesised collagen (Dunphy and Udupa, 1955).

In contrast to the above observations, Pullar (1973) found histochemical staining of acid glycosaminoglycans to be of limited value as a technique for ageing wounds, as subject age and nutritional status were of equal importance in determining the character of the reaction.

4. Fibroplasia and vascular changes following injury to skin:
Fibroblasts are first observed in skin wounds from 16 hours to 3 days after injury (Raekallio, 1961; Ross and Benditt, 1961; Ordman and Gillman, 1966; Hirvonen, 1968a; Raekallio, 1977). They increase rapidly in number and to some extent in size, until approximately day 7, after which time the fibroblast population decreases. Fine argyrophilic reticulin fibrils have been observed in 1-5 day old wounds (Levenson *et al.*, 1965; Robertson and Hodge, 1972; Basset *et al.*, 1977). Initially, these fibrils were closely associated with fibroblasts, but they eventually formed a loose network throughout interstitial spaces. Reticulin had begun to mature into van Gieson positive collagen fibrils by days 4-7 after injury (Raekallio 1961; Ross and Benditt, 1961; Ordman and Gillman, 1966; Robertson and Hodge, 1972; Raekallio, 1977). The amounts of collagen increased rapidly thereafter as the amounts of reticulin decreased. At first the collagen was formed as a loose network of fibrils within the interstitium, but in wounds more than 5 days old maturing fibrils had condensed into coarser, more deeply staining fibres. These in turn condensed further to form compact connective tissue bundles of scar tissue (Ross and Benditt, 1961).

Newly formed elastin is not a prominent feature of healing wounds. Ordman and Gillman (1966) observed a few fine, wavy elastin fibrils in wounds 15 days old, but these had increased little in number or maturity by day 49.

Using light microscopy, proliferating endothelial cells are difficult to distinguish from fibroblasts and adventitial cells (Levensen *et al.*, 1965). It is not until they have formed vascular channels that newly formed endothelial cells are clearly identifiable, and it may be for this reason that endothelial cell proliferation is usually observed later than fibroblasia. Autoradiographic as well as light microscopic evidence of endothelial cell replication has been detected within 32 hours of necrotising thermal injury in rats (Sholley *et al.*, 1977). These changes reached maximum intensity within 48-72 hours, during which time mitotic figures were also detected. More commonly, however, newly formed capillaries have been detected by light microscopy from between 3-8 days after injury (Raekallio, 1961; Ordman and Gillman, 1966; Sholley *et al.*, 1977). Revascularisation of the reactive site becomes obvious after day 12 (Robertson and Hodge, 1972).

VI. ENZYMIC ACTIVITY IN WOUNDS

Quantitative and qualitative changes in tissue enzyme activities occur in wounds. Such changes might be of potential use for the ageing of bruises.

A. Technical considerations in measuring tissue enzyme activities

The two branches of enzyme study in relation to tissue injury are enzyme histochemistry as performed on tissue sections and enzyme assay techniques as performed on tissue homogenates. Many technical disadvantages are associated with enzyme histochemistry (Pearse, 1968), yet despite these the majority of data pertaining to wounds have stemmed from this approach. This largely reflects the advantages of being able to correlate levels of enzymic activity with changes in tissue morphology, thus permitting events to be monitored as they affect individual cells (Dubowitz and Pearse, 1961; Morales and Fine, 1966; Pullar, 1973; Shannon *et al.*, 1974). In so doing an increased degree of sophistication is attained in the interpretation of results.

Biochemical assays of total enzyme activities are inferior in this respect, since they are relatively insensitive to tissue changes as they affect minority cell populations (Lojda and Gutmann, 1976). This is a significant liability considering the complex and subtle nature of tissue reactions in wounds. Improvements in techniques for demonstrating isoenzymes have, however, greatly enhanced the potential usefulness of the purely biochemical approach to wound ageing (Bishop, 1979; Mauro, 1979). Advantages of this method not shared by enzyme histochemistry include an increased sensitivity to change and objective, readily measured and easily interpreted results (Barka, 1961; Dannenberg *et al.*, 1963; Holmes and Masters, 1967; Makinen and Raekallio, 1968a, 1973; Dahle and From, 1971; Bonte, 1978). Within the known limitations of each technique, a good correlation has generally existed between histochemical and biochemical observations (Hachmias and Padykula, 1958; Beckett, 1962; Barron *et al.*, 1966; Meijer, 1972; Max and Wagner, 1979; Radzun *et al.*, 1980). However, since the two techniques yield different types of information it is desirable to employ both when investigating the enzymic activity of wounded tissues (Pearson and Kar, 1966).

B. The nature of enzyme changes in wounds

The majority of reported experiments have demonstrated histochemical and biochemical changes in the enzymic activity of damaged tissues (Pullar, 1973; Mauro, 1979). These changes appear relatively non-specific for a wide variety of pathological conditions (Jasmin, 1966), although variations between different kinds of wounds have occasionally been recorded (Maeir and Zaiman, 1966). It therefore appears reasonable to extrapolate, with caution, between observations based on different types of insults. However, in common with morphological events, variations in the nature and time sequence of enzymic reactions vary according to the tissue (Raekallio and Levonen, 1963) and the species of animal affected (Markert and Hunter, 1959; Raekallio, 1961; Fatteh, 1966a; Holmes and Masters, 1968; Hou-Jensen, 1968; Weller *et al.*, 1973; Bonte and Hermann, 1978; Hancock *et al.*, 1978). Controversy exists over the effect of severity of injury on subsequent

enzymic activity. Brown and Hamdy (1964, 1965a, 1965b) observed significantly increased enzyme activities with increased severity of bruising. Others, however, have found the effects of severity of injury to be insignificant (Shannon *et al.*, 1974). Since the most florid enzymic reactions in wounds occur peripheral to the areas of severe tissue damage (Fatteh, 1966a; Raekallio, 1977), the severity of injury appears likely to influence the degree of the response.

Opinions differ as to the nature, the degree and the significance of enzyme changes in wounds. As a generalisation, enzymic activity decreases during necrosis then gradually increases during regeneration (Smith, 1965; Fine *et al.*, 1966; Raekallio and Makinen, 1967; Ojala, 1968; Wiesmann *et al.*, 1969; Josza, 1974; Di Mauro *et al.*, 1978; Josza *et al.*, 1978). However, both increased (Fatteh, 1966b; Makinen and Raekallio, 1967; Hirvonen, 1968a, 1968b; Hancock *et al.*, 1978; Meijer and Israel, 1979a) and unchanged (Maeir and Zaiman, 1966; McCausland and Dougherty, 1978) enzyme activities have been recorded. The time sequences, and to an extent the nature, sensitivity and degree of response, depends on the individual enzyme studied (Fatteh, 1966a; Fine *et al.*, 1966; Raekallio, 1973). It has therefore been recommended that the enzymic activity of wounds be studied using a battery of enzymes representing several different metabolic pathways (Fine *et al.*, 1966).

C. The time course of enzyme changes in wounds

Opinions are divided as to the usefulness of enzyme studies when ageing wounds. The minority opinion is that enzyme activities merely accompany morphological events such as cell exudation and fibroblast proliferation and are therefore of little practical advantage for ageing purposes (Hodson *et al.*, 1962; Hirvonen, 1968b; Hou-Jensen, 1968). The most convincing argument in this respect comes from the work of Hou-Jensen (1968) who monitored histochemical enzyme activities in the wounds of control and X-irradiated rats. He reported a lack of enzymic activity in the wounds of the irradiated and therefore leukopaenic animals, but observed a vigorous response associated with

the cellular exudate in the controls. On this evidence he attributed the enzymic response in injury solely to the influx of enzymatically active leucocytes. The majority of opinion based on histochemical and biochemical evidence, however, is that enzymic reactions significantly precede the earliest unambiguous response to injury and that they provide a useful tool for wound ageing purposes (Fatteh, 1966a, 1966b; Fine *et al.*, 1966; Makinen and Raekallio, 1967; Hirvonen, 1968a; Raekallio and Makinen, 1967a, 1969a, 1971a, 1971b; Danse and Steenberger-Botterwerg, 1974; Hancock *et al.*, 1978; Wagner *et al.*, 1978; Max and Wagner, 1979). They are especially useful during the earliest stages of the tissue response (Pullar, 1973; Raekallio and Nieminen, 1979).

D. Observations on specific enzyme activities in wounds

Little is known of the mechanisms by which enzymes are activated by injury, and available explanations for their being so are largely speculative (Holmes and Masters, 1967; Di Mauro *et al.*, 1978; Bocking and Riede, 1979; Mauro, 1979; Miranda *et al.*, 1979). However, it is logical to assume that altered tissue enzyme activities reflect changes in the metabolism of damaged cells. Detailed observations on individual enzyme activities are therefore considered below according to the major metabolic functions in which they participate. These are grouped as follows: glycolytic pathway, citric acid cycle, pentose phosphate pathway, hydrolases and miscellaneous. Several enzymes are associated with each of these pathways, but the majority of studies on damaged tissues have concentrated on a few so-called 'marker' enzymes, the activities of which are indicative of the functional states of their appropriate metabolic pathways.

1. The glycolytic pathway:

For wound ageing purposes the most widely investigated glycolytic enzymes are aldolase, glycogen phosphorylase and lactate dehydrogenase.

(a) Aldolase: Histochemical observations pertaining to aldolase must be interpreted with caution because of the solubility of this enzyme and the marked effects that technique can therefore have on visible activity (Beckett, 1962). Histochemical activity has been observed to decrease from between 1-5 days after rupture of skeletal muscle (Josza, 1974). Reversions to foetal isoenzyme patterns have been observed in various neuromuscular and dystrophic diseases (Miranda *et al.*, 1979).

(b) Glycogen phosphorylase: Due to its lability and solubility, the histochemical demonstration of glycogen phosphorylase is affected by technical factors such as the type of fixative employed and the composition of the incubating medium (Takeuchi and Kuriaki, 1955; Ogata, 1968). In addition, this enzyme is readily inactivated during autolysis (Morales and Fine, 1966). Nevertheless, provided tissues are processed expeditiously, and standard methods of demonstration are employed, a good correspondence between biochemically and histochemically measured activities can be achieved (Beckett, 1962).

Glycogen phosphorylase activity is one of the first to alter in wounds. It undergoes a rapid and pronounced decrease possibly within minutes, but almost certainly within 12 hours after injury (Smith, 1965; Fine *et al.*, 1966; Morales and Fine, 1966; Snow, 1973; Josza *et al.*, 1978). Activity does not return until muscle regeneration is almost complete, despite the persistence or earlier reappearance of other glycolytic enzymes (Smith, 1965; Snow, 1973). The lack of glycogen phosphorylase activity in regenerating muscle has been taken to indicate the relative unimportance of anaerobic glycolysis during muscle differentiation (Snow, 1973). This view has been challenged, however, by Wagner *et al.* (1976) who demonstrated normal lactate production in regenerating muscle despite a 60% loss in biochemically demonstrated phosphorylase activity.

Alterations in the number and relative activities of glycogen phosphorylase isoenzymes occur within 2 hours in skin wounds (Bonte, 1978). This appears to reflect a reversion to foetal isoenzyme patterns (Di Mauro *et al.*, 1978).

(c) Lactate dehydrogenase: Normal muscle shows strong lactate dehydrogenase activity, with variations in intensity according to fibre type (Dubowitz and Pearse, 1961; Kar and Pearson, 1963). Following various types of injury including ischaemia, incisions and bruising, there is a progressive decline in both histochemically and biochemically demonstrable lactate dehydrogenase activity (Wiesmann *et al.*, 1969; Wagner *et al.*, 1976). When measured histochemically, this decrease may be preceded by a transient increase in activity, but this is an artefact caused by mitochondrial swelling (Josza, 1974). The change in activity frequently precedes light microscopic evidence of tissue damage and in this respect lactate dehydrogenase is one of the earliest enzymes to alter following injury (Josza, 1974). The speed of the decrease varies according to the nature of the insult, but has generally been observed to commence within 2 hours of injury (Fine *et al.*, 1966; Morales and Fine, 1966; Ojala, 1968; Josza, 1974). Activity then declines progressively and has usually disappeared from degenerating muscle fibres within 72 hours. Lactate dehydrogenase is also one of the first enzymes to regain activity with tissue regeneration, indicating the importance of anaerobic glycolysis during this phase of healing (Ojala, 1968; Snow, 1973). A return of activity has been observed as early as 24 hours in bruises (Josza, 1974), but in most wounds the progressive increase has been observed to commence much later - at least 4 days after injury (Ojala, 1968; Wiesmann *et al.*, 1969; Snow, 1973; Wagner *et al.*, 1976).

Normal muscle possesses five electrophoretically demonstrable lactate dehydrogenase isoenzymes, with a predominance of the LDH 5 cathodal type (Kar and Pearson, 1963; Harris and Hopkinson, 1976). Variation in isoenzyme composition exists between different muscles and between fibre types, but this normal variation is minor in degree compared to the changes that have been observed in abnormal muscle (Dubowitz and

Pearse, 1961; Kar and Pearson, 1963; Pearson and Kar, 1966). During muscle development the isoenzyme composition of normal fibres changes in a reproducible manner from a foetal to a mature pattern, with a predominance of anodal and cathodal bands respectively (Mauro, 1979). A reversion to a foetal pattern of activity has been observed in necrotic and regenerating muscle following anoxia and physical injury (Mauro, 1979; Bonte and Bode, 1981). It has not, however, been possible to establish a relationship between lactate dehydrogenase isoenzyme patterns and the duration or the severity of tissue damage.

2. The citric acid cycle:

When studying injured tissues the enzyme usually selected to represent the citric acid cycle is succinate dehydrogenase. Other enzymes from this pathway, such as malate dehydrogenase and isocitrate dehydrogenase, have proven less useful in this respect (Pearson and Kar, 1966).

(a) Succinate dehydrogenase: Histochemical and biochemical assay data pertaining to succinate dehydrogenase activity are in general agreement (Nachmias and Padykula, 1958). Normal muscle shows a moderate histochemical reaction for the enzyme, with variation according to fibre type (Davies and Gunn, 1972). Activity may also exist in leucocytes, fibroblasts and fibrocytes, depending on the species of animal considered (Ojala, 1968).

When observed, changes in succinate dehydrogenase activity usually occur later than those for other dehydrogenases. A progressive decline to a complete absence of enzyme activity has been reported between 2 hours and 6 days after injury (Raekallio, 1961; Beckett, 1962; Smith, 1965; Fine *et al.*, 1966; Hirvonen, 1968a; Ojala, 1968; Josza, 1974; Bonte and Bode, 1981). This decrease could generally be related to the onset and severity of mitochondrial dissolution (Beckett, 1962). Succinate dehydrogenase activity reappears in regenerating muscle fibres at from 2-14 days after injury. The time for its reappearance appears to depend on the nature of the insult and the species of animal affected (Smith, 1965; Ojala, 1968; Snow, 1973). Leucocytes and fibroblasts demonstrate an increase in succinate dehydrogenase activity in wounds, commencing from 4-12 hours

after injury (Raekallio, 1964; Ojala, 1968). These cells may contribute significantly to the overall enzyme activity in the area of damage (Raekallio, 1964).

3. The pentose phosphate pathway:

The pentose phosphate enzyme pathway synthesises nucleic acids from glucose-6-phosphate and is thus an important metabolic pathway in developing and regenerating tissues (Metzler, 1977). It comprises a sequence of two oxidative enzymes, namely glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, followed by five non-oxidative enzymes. The non-oxidative enzymes demonstrate only a minor increase in regenerating tissues. Both of the oxidative enzymes, on the other hand, are capable of responding rapidly to regenerative stimuli, and appear to be the rate determining steps for the pentose phosphate pathway (Max and Wagner, 1979). Both glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase show similar responses following tissue damage. The former is the most frequently studied.

(a) Glucose-6-phosphate dehydrogenase: Although a high level of glucose-6-phosphate dehydrogenase activity can be detected both histochemically and biochemically in foetal muscle, activity is present only in very low levels in normal adult vertebrate muscle (Ogata and Mori, 1964; Max and Wagner, 1979). Following injury, however, the enzyme is rapidly synthesised. Within 2 hours glucose-6-phosphate dehydrogenase levels may double, and by 24 hours they may have increased ninefold (Snow, 1973; Max and Wagner, 1979). Maximum values coincide with maximum levels of regenerative activity as judged histologically, but the earliest detectable increases in activity occur very soon after injury and may precede histological evidence even of degenerative change (Wagner *et al.*, 1978). Enzyme levels return to normal approximately 14 days after injury (Smith, 1965; Snow, 1973). Extravascular macrophages may acquire glucose-6-phosphate dehydrogenase activity approximately 10 hours after injury to muscle, but this source of enzyme has been shown to be minor in comparison to the muscular component (Max and Wagner, 1979).

4. Hydrolases:

The most commonly studied hydrolases in damaged tissues have been acid phosphatase, alkaline phosphatase, leucine aminopeptidase and non-specific esterase.

(a) Acid phosphatase: Histochemical observations have frequently suggested that acid phosphatase is either absent from, or exists only in trace amounts in skeletal muscle (Maeir and Zaiman, 1966; Ojala, 1968; Shannon *et al.*, 1974). More sophisticated histochemical techniques designed to minimise the loss of acid phosphatase activity through enzyme inhibition or diffusion into the substrate medium have, however, consistently demonstrated moderate amounts of activity in this tissue (Lojda and Gutmann, 1976; Engel, 1979; Ohiyumi and Ohiyumi, 1979). Acid phosphatase is also present in leucocytes, fibroblasts and fibrocytes (Raekallio, 1964; Fatteh, 1966b; Hirvonen, 1968b; Rudolph and Schnable, 1979).

Histochemically detected acid phosphatase activity has generally been shown to increase in damaged muscle fibres (Maeir and Zaiman, 1966; Neerunjun and Dubowitz, 1974; Shannon *et al.*, 1974; Engel, 1979; Meijer and Israel, 1979a), though this has not always been the case (Ojala, 1968; McCausland and Dougherty, 1978). The increase occurs during regeneration and has usually been noted between 2-7 days after injury. The magnitude of the response approximates the severity of muscle damage and is believed to reflect an increase in the number of sarcoplasmic lysosomes (Brown and Hamdy, 1964; Maeir and Zaiman, 1966; Ohiyumi and Ohiyumi, 1979). A return to normal levels occurs approximately 14 days after injury (Shannon *et al.*, 1974).

According to the majority of observations on wounds, the earliest histochemically detectable acid phosphatase response occurred in local interstitial fibroblasts at between 2-6 hours after injury, depending on the species of animal affected (Fatteh, 1966a, 1966b; Ojala, 1968; Malik, 1970; Raekallio, 1977). This increase pre-dated the arrival of the acid phosphatase rich cellular exudate by several hours and was therefore considered a useful means of ageing wounds. Others, however, have failed to substantiate these observations (Hirvonen,

1968a; Hou-Jensen, 1968). The latter two authors have attributed the increases in enzyme activity entirely to leucocytes and have therefore concluded that the histochemical evaluation of acid phosphatase activity offers no advantages over routine histology for wound ageing purposes.

Biochemical assays of acid phosphatase activity in wound homogenates have generally concurred with histochemical observations, although differences have arisen in cases where the cellular exudate has contributed substantially to the overall activity (Brown and Hamdy, 1964; Shannon *et al.*, 1974; Meijer and Israel, 1979b). The limited amount of data available has also suggested the potential value of isoenzyme studies when ageing wounds. Certain electrofocussed acid phosphatase isoenzymes demonstrated an increased activity within 2 hours in skin wounds (Bonte, 1979; Bonte and Bode, 1981), and the relative activities of electrophoretically separated isoenzymes in skeletal muscle have been shown to alter with the onset of necrosis (Meijer and Israel, 1979b). Makinen and Raekallio (1971), on the other hand, failed to detect changes in electrofocussed acid phosphatase isoenzyme patterns in skin wounds.

(b) Alkaline phosphatase: Alkaline phosphatase activity cannot be detected histochemically in normal, necrotic or regenerating muscle fibres (Ogata and Mori, 1963; Ojala, 1968). The activity of this enzyme in fibroblasts may increase from between 3-8 hours after injury, depending on the species of animal affected (Raekallio, 1964; Fatteh, 1966a; Ojala, 1968; Malik, 1970; Hancock *et al.*, 1978). However, because this increase usually coincided with the arrival of an enzyme positive leucocyte exudate, the histochemical evaluation of alkaline phosphatase activity was considered of limited value for wound ageing purposes. McCausland and Dougherty (1978) failed to observe changes in enzyme activity in bruises of different ages.

The total alkaline phosphatase activity of wound homogenates may increase within 6 hours of injury, but the results are difficult to evaluate for ageing purposes because of the variable contribution made by infiltrating leucocytes (Manning and Di Pasquale, 1967). The relative activities of electrofocussed alkaline phosphatase

isoenzymes have been found to differ between skin wounds aged from 2 hours to 5 days old (Makinen and Raekallio, 1973). Isoenzyme studies may therefore prove useful for ageing wounds, especially since this approach enables the contributions of individual tissue components in the overall reaction to be evaluated separately.

(c) Non-specific esterase: Slight to moderate non-specific esterase activity can usually be demonstrated histochemically within normal muscle fibres (Ogata and Mori, 1963; Meijer and Israel, 1979a), although an absence of activity has been recorded (Maeir and Zaiman, 1966; Snow, 1973). The enzyme has usually increased in activity within 7 days of injury, and the degree of the increase has approximately parallelled the severity of injury (Maeir and Zaiman, 1966; Snow, 1973; Meijer and Israel, 1979a). Occasionally, however, unchanged (Morales and Fine, 1966) or decreased (Barron *et al.* 1966; Ojala, 1968) activity has been recorded. Ojala (1968) observed a complete loss of enzyme from muscle fibres within 8 hours of incision injury.

Interstitial fibroblasts have commonly shown increased esterase activity in wounds. The time-course of this response has varied from 10-60 minutes according to the observer and the species of animal affected, but in each case it preceded the arrival of the enzyme rich cellular exudate and was therefore considered useful for wound ageing purposes (Raekallio, 1964; Fatteh, 1966a; Malik, 1970; Hancock *et al.*, 1978; Raekallio and Nieminen, 1979). This conclusion is not, however, unanimous. Neither Hirvonen (1968b) nor Hou-Jensen (1968) observed an increase in fibroblast esterase activity in skin wounds until after leucocytes had arrived, and Ojala (1968) failed to observe any fibroblast enzyme activity in muscle wounds. Esterase activity in leucocytes has been observed in wounds 8 hours and older (Ojala, 1968).

Isoelectric focussing techniques have demonstrated both new esterase isoenzymes and altered relative activities of pre-existing isoenzymes in wounds from 30 minutes to 4 hours old (Jarecki *et al.*, 1970; Bonte, 1978). There was a good correlation of results between wounds of similar ages.

(d) Leucine aminopeptidase: Aminopeptidase activity has not been detected histochemically in the normal striated muscle fibres of those animal species in which wounds have been studied (Ogata and Mori, 1963; Ojala, 1968; Meijer and Israel, 1979a). Foci of activity have occasionally been observed in damaged fibres (Maeir and Zaiman, 1966; Meijer and Israel, 1979a), but this has not always been the case (McCausland and Dougherty, 1978). Moderate enzyme activity exists both in leucocytes and in interstitial fibroblasts (Raekallio, 1964; Fatteh, 1966a; Hou-Jensen, 1968; Rudolph and Schnable, 1979). Fibroblastic aminopeptidase activity has been observed to increase at a variable time after injury, from 2 hours to 7 days depending on the observer, the nature of the injury and the species of animal affected (Raekallio, 1960, 1964; Monis, 1963; Raekallio and Makinen, 1967a; Hirvonen, 1968b; Malik, 1970). Enzyme activity in individual leucocytes has also been observed to increase within 8 hours of injury to muscle (Ojala, 1968).

Makinen and Raekallio (1967, 1968a) measured a progressive increase in total enzyme activity in homogenates of skin wounds aged from 2-24 hours old. After 24 hours, enzyme activities declined. Histochemical observations on tissue samples from the same wounds were in agreement with the enzyme assays. New aminopeptidase isoenzymes and altered relative isoenzyme activities have been observed in skin wounds 30 minutes to 8 hours old (Raekallio and Makinen, 1967a, 1967b, 1969a, 1971a). These changes were not due to extravasated blood or leucocytes, so changes in aminopeptidase isoenzyme patterns were considered potentially useful for wound ageing purposes.

5. Miscellaneous enzymes:

Two enzymes, namely creatine phosphokinase and myosin adenosine triphosphatase have been grouped under the heading of 'miscellaneous enzymes' because they do not belong to any of the enzyme systems formerly considered. Both are present in high concentrations in muscle.

(a) Creatine phosphokinase: Few published observations exist concerning the histochemical activity of creatine phosphokinase in damaged muscle fibres because this enzyme could not be accurately demonstrated in tissue sections prior to the introduction of a 'reactive film' technique by Khan *et al.* (1971a). Josza *et al.* (1978) observed a decrease in creatine phosphokinase activity in muscle fibres that had been ruptured for between 1-5 days.

Creatine phosphokinase activity as measured in muscle homogenates falls during the first 4 days after injury, then rises to reach normal values during the subsequent regenerative period (Wiesmann *et al.*, 1969). A reversion to a foetal isoenzyme pattern may occur during muscle regeneration, followed by a gradual return to normal during the process of maturation (Miranda *et al.*, 1979).

(b) Myosin adenosine triphosphatase: Most normal muscle has a 'checkerboard' staining pattern for adenosine triphosphatase, with individual fibres staining either strongly or weakly according to their metabolic types (Davies and Gunn, 1972; Kaldor and Di Battista, 1975). The enzyme activity in necrotic fibres usually decreases between 2 hours to 3 days after injury, resulting in a uniformly weak staining reaction in all fibres (Raekallio and Levonen, 1963a; Morales and Fine, 1966; Riley, 1973; Josza *et al.*, 1978). Occasionally, however, adenosine triphosphatase activity in necrotic fibres remains unchanged (Neerunjun and Dubowitz, 1974). Early in the course of regeneration, muscle fibres acquire a uniformly strong adenosine triphosphatase reaction with no distinction between fibre types (Smith, 1965; Riley, 1973; Engel, 1979). Variation in staining reappears as the newly formed fibres mature (Neerunjun and Dubowitz, 1974).

Both leucocytes and fibroblasts possess moderate adenosine triphosphatase activity in laboratory animals (Raekallio and Levonen, 1963a; Hirvonen, 1968b; Raekallio and Nieminen, 1979). The fibroblastic activity increased in wounds over a period of 1-24 hours, and the leucocytic activity increased during phagocytosis.

CONCLUSION

The sequential morphological response of injured tissues provides a reliable and well documented means of ageing wounds despite variations in times reported for recognisable histological events to occur. A limitation with the histological or routine histochemical approach, however, is the existence of a so-called 'lag phase' during which time no morphological changes can be detected. The duration of the 'lag phase' varies, but according to Raekallio (1977) the earliest unambiguous morphological events occur from between 4-8 hours after injury. Prior to this time wounds cannot be aged using routine histological techniques.

Damaged tissues also show sequential changes in enzyme activity. The suitability of these for wound ageing purposes is not well established but they have proven of some use in this respect. The most pronounced enzymic changes have occurred within the first few hours of injury, i.e. during the histological 'lag phase', and for this reason the value of enzyme studies has been most apparent when they have been applied to age wounds inflicted close to the time of death.

CHAPTER II

AGEING BRUISES ACCORDING TO THEIR GROSS AND HISTOPATHOLOGICAL CHARACTERISTICS

INTRODUCTION

Wounds and bruises are usually aged by comparing their gross and histopathological characteristics with those in similar lesions of known ages. Such methods are largely subjective and many of the established age criteria in use have been based on limited numbers of widely varying observations (as reviewed in Chapter I). One of the most common sources of variation is species differences in rates of healing. For this reason the time-course of the tissue reaction to bruising injury was studied in sheep, since this was the species of practical interest.

The bruises studied were of known ages and comprised both the 'impact' type as caused by striking or crushing injury, and the 'wool pull' type as caused when sheep are lifted by their wool. Various histopathological changes in these bruises have been described and scored semi-quantitatively according to their degrees of change from normal. This information has then been used to develop a mathematical method for ageing bruises according to histopathological changes. The usefulness of this method in practice has been evaluated in a pilot survey of bruises sampled from an export meat works.

MATERIALS AND METHODS

I. IMPACT BRUISES

1. Experimental animals

Fifty healthy male, male-castrate or female New Zealand Romney and

Perendale lambs were used. They ranged in age from 3-12 months old.

2. Production of bruises

In an attempt to produce a relatively standard impact-type bruise, wool over the surface to be bruised was parted and a 1500g lead weight with a 0.8 cm^2 striking surface was dropped onto the skin through a perforated plastic tube 1.0m in length (Figure 2.1). Up to six bruises, usually of different ages, were produced on each animal. The sites bruised were as follows:

(i) The forelimbs over the lateral aspect of the distal third of the humerus;

(ii) The hindlimbs midway over the lateral aspect of the femur;

(iii) Approximately 5.0cm each side of the dorsal processes of the vertebral column and immediately cranial to a line drawn between the two iliac crests.

In this way a total of 178 bruises aged 1, 2, 4, 8, 12, 16, 20, 24, 30, 36, 48, 60 and 72 hours old were inflicted (Table 2.1). Originally there were to have been five bruises of each age, since equal within-group numbers considerably simplify most statistical analyses. However, inequality of numbers did not present difficulties with the statistical analyses eventually used, so all available data have been included. The extra bruises came from three sources:

(i) Bruises produced whilst perfecting bruising techniques and methods of fixation;

(ii) Partially successful bruises, where haemorrhage was visible grossly in adipose tissue but where muscle appeared not to have been bruised. These cases were processed for histology and the decision whether or not to reject data rested later on the presence or absence of histological evidence of haemorrhage, i.e. of bruising;

(iii) Bruises added later in the experiment when, in order to estimate their ages, it became necessary to re-define the age categories into which bruises were placed as either 1-8, 12-20, 24-36, 48, 60 or 72 hours old.

FIGURE 2.1 : The weight, and plastic tube, along with the hand vice used to inflict impact and wool pull bruises respectively. The wooden ruler is 40cm long.

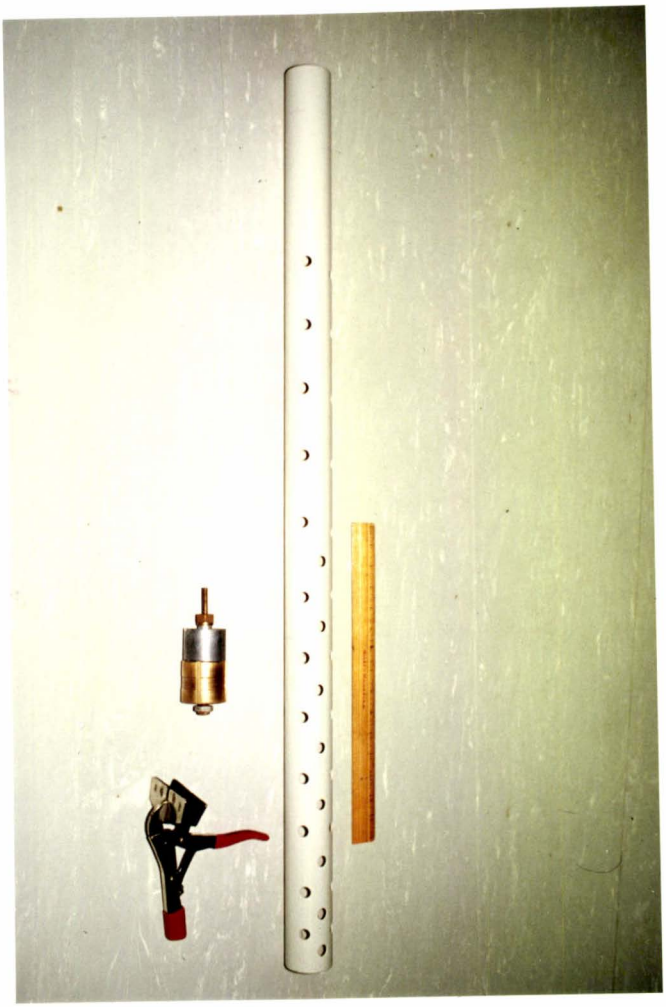


TABLE 2.1 : THE AGES, NUMBERS AND POSITIONS OF IMPACT BRUISES INFLICTED ON EXPERIMENTAL LAMBS.

AGE (hours)	POSITION OF BRUISE ON CARCASE						NUMBER OF BRUISES
	LF	RF	LL	RL	LH	RH	
1	3	1	1	0	1	2	8
2	1	4	0	0	0	3	8
4	1	4	2	1	2	2	12
6	0	4	0	0	0	1	5
8	2	0	1	1	3	2	9
12	0	2	0	2	3	2	9
16	3	1	0	3	4	3	14
20	2	0	0	0	3	2	7
24	1	2	1	1	1	2	8
30	1	2	0	0	3	4	10
36	2	2	0	2	2	4	12
48	5	6	3	1	4	3	22
60	9	3	1	2	8	6	29
72	4	7	2	0	2	10	25
						TOTAL	178

LF, RF - left and right forelimbs
LL, RL - left and right lumbar areas
LH, RH - left and right hind limbs

In addition to the above, two 96 hour, two 120 hour and two 144 hour old bruises were sampled. Data from these lesions were not included in statistical analyses but their histological features were noted.

3. Processing of bruised tissues

At predetermined times after bruising the lambs were killed by a transverse incision through the soft tissues of the neck and an almost simultaneous severence of the spinal cord through the atlanto--occipital joint. After the skin was removed from each animal its

bruises were examined grossly and their colour, consistency, dimensions and severity of haemorrhage were recorded. Three samples approximately 7.0mm thick were taken from most of the bruises, but less than three samples were taken from a few of the smaller lesions. Where possible, both muscle and adipose tissue were included in the same sample. Control tissues obtained from unbruised muscle were treated in the same way, except that only one sample was taken from each site. The fixed tissues were embedded in paraffin and sections cut at approximately 7.0μm thickness. The majority of sections were stained using only haematoxylin and eosin (H+E), van Gieson and Perl's stain for iron. However, a number of sections were examined using the following additional stains:
Giemsa, periodic acid Schiff/alcian blue, von Kossa's stain for calcium and either Mallory's phosphotungstic acid-haemotoxylin (PTAH) or Martius scarlet blue (MSB) stains (Luna, 1968; Lendrum, 1969).

4. Examination and scoring of tissue sections

Tissue sections were examined without prior knowledge of bruise ages and the observations were related to age only after all the bruises had been processed. The salient histopathological features of each section were noted and used retrospectively to compile a description of the sequential tissue changes in bruises with age. In addition, a number of histopathological features associated with degenerative change, inflammation, repair and regeneration in muscle and adipose tissue were scored semi-quantitatively on a scale of 1-5. A score of 1 indicated no change from the normal state as noted in control tissues, and a score of 5 indicated the most severe change from normal. The features thus scored, and an example of the score sheet used are shown in Figure 2.2. Most of these degenerative and reactive changes were studied in H+E stained paraffin sections, but Perl's stain was used to detect haemosiderin, and Giemsa stain was used to identify mast cells and eosinophils. In addition to the semi-quantitative scoring, cell counts were carried out in order to calculate macrophage/neutrophil ratios both in interstitial spaces and within degenerate muscle fibres. These ratios were subsequently

SLIDE NUMBER _____

AGE OF BRUISE _____ (entered retrospectively)

Macrophage/neutrophil ratio in exudate - muscle _____

- adipose tissue _____

HISTOPATHOLOGICAL FEATURE SCORE

	Muscle	Adipose tissue
Physical disruption of tissue	_____	_____
Severity of haemorrhage	_____	_____
Degeneration/necrosis	_____	_____
Regenerative changes in muscle	_____	_____
Exudation of neutrophils	_____	_____
Exudation of macrophages	_____	_____
Exudation of eosinophils	_____	_____
Hypertrophy of endothelial cells	_____	_____
Mitotic figures in endothelial cells	_____	_____
Proliferation of new capillary loops	_____	_____
Fibroplasia	_____	_____
Haemosiderin in macrophages	_____	_____
Calcification	_____	_____
Mast cells: Intact	_____	_____
Degranulated	_____	_____

FIGURE 2.2 : An example of the score sheets used when studying histopathological changes in bruises.

assigned scores from 1-3 according to whether they were less than or equal to 1.00, from 1.01-2.99 or from 3.00 upwards. Lymphocytes were not scored since they appeared to occur in passive association with haemorrhage.

5. Analyses of data and methods used to estimate the ages of bruises

Immediately after they had been scored interpretively, bruises were aged according to experience acquired during the development of the experiment. These subjective estimates of age were later compared with objective estimates based on semi-quantitative data. When analysing data or estimating bruise ages objectively, muscle and adipose tissues were considered separately and data were used only from tissues with histological evidence of haemorrhage. Bruises were grouped into six categories: 1-8, 12-20, 24-36, 48, 60 and 72 hours old. All available data were used to construct contingency tables (e.g. Table 2.2) relating scores from the histopathological features studied to the known ages of bruises. Score categories 4 and 5 were subsequently merged so as to minimise the number of contingency tables cells containing information from less than five tissue sections.

Histopathological parameters likely to be of use for estimating the ages of bruises were selected after chi-square analyses of data in the above tables. These analyses were performed by a Burroughs 6300 computer, using a programme available in the computer package known as 'Statistical Package for the Social Sciences' (Nie *et al.*, 1975). The extent to which scores from the potentially useful features were merely associated with tissue haemorrhage rather than age was then investigated using a further series of chi-square analyses in which severity of haemorrhage was taken into account. Features with significant relationships between scores and ages were used in two mathematical models designed to estimate the ages of bruises. These were: a Bayesian probability model and a multiple linear regression model. Both models were 'developed' using data from one tissue section selected from each of 20 bruises per age category. The best model was then 'tested' using data from the remaining tissue sections.

TABLE 2.2 : EXAMPLE OF A CONTINGENCY TABLE USED FOR CHI-SQUARE ANALYSIS. IN THIS CASE THE RELATIONSHIP BETWEEN BRUISE AGES AND SCORES FOR MACROPHAGE EXUDATION IS INVESTIGATED.

AGE (hours)	SCORE 1	2	3	4	TOTAL NO. SLIDES
1-8	18	42	20	3	83
12-20	3	13	28	14	58
24-36	3	15	24	7	49
48	0	6	9	11	26
60	0	4	9	17	30
72	0	5	8	12	25

Chi-square = 88.84

Degrees of freedom = 15

Level of significance = 0.0001, i.e. there was a statistically significant association between bruise age and the degree of macrophage exudation

(a) The Bayesian probability model: This model is based on Bayes' probability theorem as adapted for use in a mathematical method of medical diagnosis (Bailey, 1965). Bruises had been grouped into one of six age categories (D_{1-6}), and during the semi-quantitative histological study each histopathological feature had been assigned a score (S_x) for each bruise. The Bayesian probability model was 'developed' in three steps, culminating in the application of Bayes' equation. The calculations performed in each step are outlined below.

(i) For each score (1-5) of each histopathological feature, the probability $P(S_x|D_j)$ of that score occurring in bruises representing a particular age category was calculated for each of the six age categories in turn, using data from 20 bruises per age category. These probabilities were calculated as follows:

Probability of a particular score from a particular histopathological feature occurring in a bruise in a given age category = $\frac{\text{No. bruises with that score + 1}}{\text{Tot. No. bruises in age category + No. score categories (i.e. 4)}}$

In mathematical terms - $P(S_x|D_j) = \frac{a_{xjk} + 1}{n_{xj} + z_{(x)}}$

where

- $P(S_x|D_j)$ is the probability of observing score S_x in a bruise from age category D_j
- a_{xjk} is, for a particular histopathological feature (x), the number of bruises with score (k) in age category D_j
- n_{xj} is the total number of bruises scored for histopathological feature (x) in age category D_j
- $z_{(x)}$ is the number of score categories in age category D_j

(ii) The probability $P(S_c|D_j)$ of observing in a bruise any particular combination of scores (S_c) representing several histopathological features was calculated for each age category as the product of the probability of observing each of the scores in the combination,

i.e. $P(S_c|D_j) = P(S_1|D_j).P(S_2|D_j)...P(S_x|D_j)$

The highest probability figure thus calculated indicated the age group in which the combination of scores was most likely to be observed. It was not, however, the actual probability of that set of scores representing a bruise in that age category. This probability figure was calculated in the final step.

(iii) The actual probability $P(D_j|S_c)$, relative to those of the other age categories, that a bruise was of a given age was calculated by applying Bayes' equation to the probabilities of observing the combination of scores in bruises from each age category. The age of the bruise was indicated by the highest probability figure thus calculated.

$$\text{Bayes' equation - } P(D_j|S_c) = \frac{P(S_c|D_j)}{\Sigma^6_{j=1} P(S_c|D_j)}$$

where

- $P(D_j|S_c)$ is the relative probability that a set of scores (S_c) represents a bruise in age category D_j
- $P(S_c|D_j)$ is the probability of observing a set of scores (S_c) in bruises from age category D_j
- $\Sigma P(S_c|D_j)$ is the sum of the above probability values, which have been calculated for each of the six age categories in turn

To illustrate the above, a worked example for one bruise is reproduced in results (Table 2.9).

When developing the model it was necessary to perform all three of the above steps. However, for all subsequent applications it was necessary to perform only steps (ii) and (iii).

(b) Linear regression analysis: In this model the ages of individual bruises were estimated directly by entering scores for the selected histopathological features into a multiple regression equation with age as the dependent variable. The equation had been fitted to the raw data by a computer, using a programme in the computer package known as 'Statistical Package for the Social Sciences' (Nie *et al.*, 1975).

Although the mathematical models were objective and their results therefore repeatable, the scores for the degrees of histopathological change were assigned subjectively. The repeatability of scoring was therefore assessed by re-scoring the 120 sections used for developing the mathematical models. The ages of bruises were then re-estimated by applying the selected mathematical model to the new scores, and the results compared with those obtained using the original data.

II. WOOL PULL BRUISES

1. Production of bruises

Up to four 'wool pull' bruises per animal were inflicted on the dorsal surfaces of 20 of the lambs used in the 'impact' bruise study. Each bruise was produced by pulling vigorously on a clump of wool grasped firmly in a hand-vice (Figure 2.1). In this way 60 lesions ranging in age from 1-72 hours old were created for histopathological study. Muscle and adipose tissue samples were collected and processed as for impact bruises.

2. Analysis of the histopathological data

Histopathological data were interpreted using the Bayesian probability method developed for impact bruises. Lesions were aged as either 1-20 hours or 24-72 hours old.

RESULTS

I. GROSS AND MICROSCOPIC APPEARANCE OF IMPACT BRUISES

1. Gross appearance of bruises

There was little variation in the colour of most bruises from 1-72 hours of age. All were essentially red, although 5/76 of the 48-72 hour old bruises also had yellow-tinged exudates. The exudate was orange-yellow in one of the two 96 hour bruises, green in one of the two 120 hour bruises and orange-red in one of the two 144 hour bruises. These discolourations were most noticeable in adipose tissue. Occasionally, bruised muscle appeared brown and indurated, but this was not an age-dependent observation.

Bruises in the limbs were usually more severe than those in the lumbar area. However, the bruises in each position on the carcase were not of uniform severity and it was not possible to produce a bruise of standard severity by the method used. The haemorrhage in surface fat or fascia often appeared more severe than that in underlying muscle, but the haemorrhage in affected muscle was frequently more extensive than that in the surface tissue and usually extended to a depth of up to 5.0cm. Haemorrhage and exudate were present between fascial planes, sometimes extending beyond the immediate limits of traumatised tissue.

2. The histopathology of bruises

The following histopathological description is based on a study of 178 bruises inflicted by a standard impact force (Table 2.1). Special stains utilised in the study are listed in Table 2.3. Most bruises ranged in age from 1-72 hours old, but in addition there were two each of bruises 96, 108, 120 and 144 hours old. The description is designed to serve as a guide to the interpretive ageing of bruises, but also serves to demonstrate the rationale behind the choice of the histopathological features eventually scored semi-quantitatively.

TABLE 2.3 : A LIST OF THE SPECIAL STAINS USED ON TISSUE SECTIONS FROM IMPACT BRUISES, SHOWING THE NUMBERS OF BRUISES OF EACH AGE STAINED.

	CONTROL	AGE (hours) 1	2	4	6	8	12	16	20	24	30	36	48	60	72	96	120	144	TOTAL
Giemsa	16	11	11	15	7	12	10	12	5	7	6	11	10	8	7	2	3	1	154
PAS/Alc.Bl.	16	7	6	10	4	6	9	7	1	-	4	7	6	8	7	2	2	2	104
van Gieson	16	3	1	9	3	1	3	-	-	-	5	6	3	3	21	2	2	1	79
PTAH	16	3	5	2	1	5	6	6	1	-	-	2	4	5	3	-	-	-	59
von Kossa	16	-	3	2	1	1	1	3	1	3	7	11	5	5	20	-	-	2	81
MSB	16	5	5	5	5	5	5	5	5	-	-	-	-	-	-	-	-	-	56
Perl's iron	16	8	8	12	5	9	9	14	7	8	10	12	22	29	25	2	2	2	200

Microscopic evidence of haemorrhage was always present in grossly damaged tissues and was frequently detected in tissues judged grossly not to have been bruised. The extravasated erythrocytes had usually spread extensively through interstitial spaces and areas of damage, where they remained morphologically intact at least for the six day period of the experiment. Erythrophagocytosis by macrophages was first observed in 8 hour old bruises and became increasingly common with time. The rate of removal of erythrocytes by this means however, did not appear sufficient to cause a decrease in the apparent severity of haemorrhage as observed in older bruises. In addition to erythrocytes, small numbers of neutrophils, lymphocytes and monocytes had also been released from blood vessels during haemorrhage. The presence of a few interstitial leucocytes therefore preceded the arrival of a true cellular exudate.

Degenerative changes were observed in the muscle fibres of most bruises. These changes appeared to be characterised by a progression of histological events ranging from swelling and eosinophilia through to vacuolation, fragmentation and eventual liquefaction of sarcoplasm. However, the morphology of affected fibres varied widely both within and between fibres in the same bruise and did not appear to be closely related to age. Cross striations and myofibrils usually became indistinct soon after the onset of degenerative change but the cross striations occasionally persisted, staining distinctly with PTAH even in some necrotic fragments of muscle. Small PTAH positive granules were arrayed in parallel lines between myofibrils in some necrotic fibres. Some endomysial sheaths in necrotic areas remained intact, whilst others had fragmented.

Necrosis of adipose tissue overlying bruised muscle was noted in approximately 25% of bruises up to 72 hours old. In most cases, necrosis involved individual widely scattered adipocytes, but focal lesions involving several adipocytes were not uncommon. More extensive areas of fat necrosis were rare however, even in steatitis associated with the older bruises. The most common manifestations of necrosis were degenerate fat deposits within the remains of dead fat cells. These deposits varied in extent and morphology between affected cells,

being either slightly eosinophilic or basophilic and either non-granular, granular or crystalline in nature. Leucocytes had infiltrated many areas of necrosis and were the sole indicators of degenerative change in those necrotic cells without an intracellular deposit. Some macrophages within cell spaces possessed large amounts of cytoplasm, and they had, on occasions, fused to form giant cells. Using von Kossa's stain, mild calcification was observed in most areas of fat necrosis but only a few areas were severely calcified. There was no apparent association between the presence of fat necrosis and the ages of bruises, nor was there an association between age and the histological appearance of fat necrosis, which often varied considerably within tissue sections.

Neutrophils considered to be part of a cellular exudate, rather than a component of haemorrhage, were noted in some bruises only 1 hour old. Despite variations between bruises of the same age, numbers of neutrophils increased thereafter until 20 hours, after which time they tended to decrease. Macrophages on the other hand, first appeared in the exudate at 4 hours and their numbers showed a general increase with age. The ratio of macrophages to neutrophils in the exudate varied widely between bruises, but macrophages usually predominated over neutrophils in bruises more than 24 hours old. Interstitial eosinophils were noted in muscle and adipose tissue in 10/138 bruises stained with Giemsa. These bruises varied widely in age being 1, 2, 4, 8, 16, 20, 24, 30, 48 and 72 hours old, so the presence of eosinophils was considered to be coincidental and not related to the age of the lesion. Margination of leucocytes in small blood vessels was observed infrequently despite the fact that exudation of leucocytes was common. Lymphocytes represented a small proportion of interstitial leucocytes and their numbers did not appear to alter with the age of bruises. They were presumed to have arrived passively during haemorrhage.

Leucocytes had infiltrated muscle fibres in many bruises, but the presence or absence of an infiltrate did not in itself correlate well with age. When an infiltrate was observed, its composition approximately reflected that in the exudate, i.e. neutrophils first appeared in the

degenerative muscle fibres of some 1 hour old bruises, increased in numbers for a period of time but became less numerous in older bruises; macrophages first appeared within fibres in 4 hour old bruises and increased in numbers thereafter. The actual numbers of infiltrating macrophages and neutrophils varied widely between both fibres and bruises even of the same age, but macrophages usually predominated over neutrophils after 24 hours (Figures 2.3, 2.4).

A selection of bruises from 1-60 hours old, all bruises aged 72 hours old and the two 144 hour old bruises were stained with von Kossa's stain for calcium (Table 2.3). Calcium deposits in muscle were observed in only 7/62 bruises: one from each of the 6, 30 and 48 hour old bruises and two from each of those that were 72 and 144 hours old. In the bruises up to and including 72 hours old, calcification was mild and affected very few necrotic fibres but it was more severe and extensive in the two 144 hour old bruises.

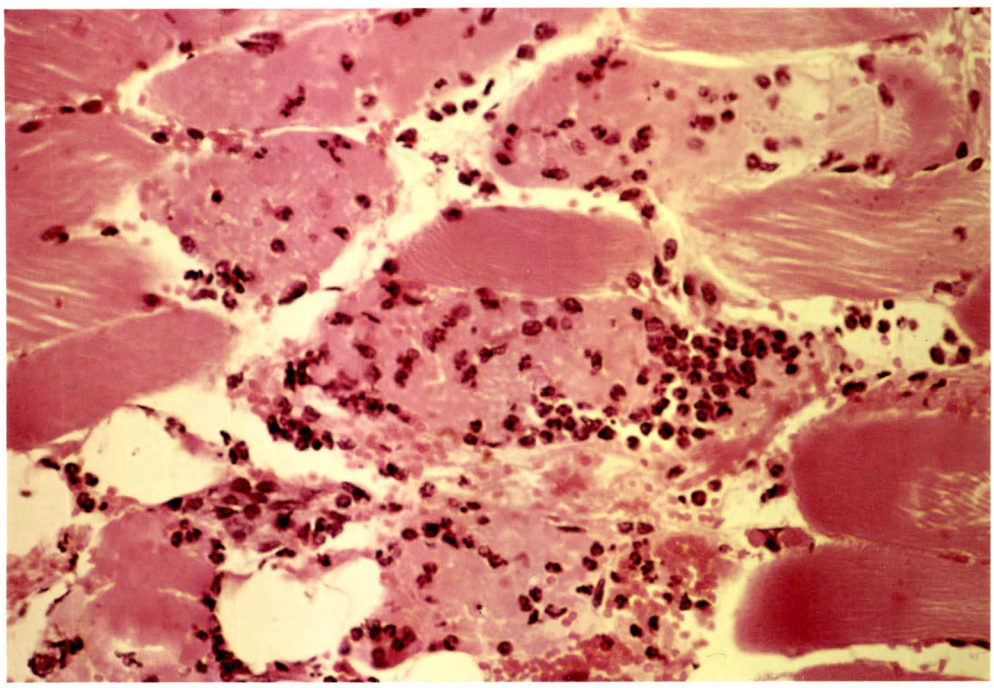
Mast cells were easily recognised using Giemsa stain but few were observed in either muscle or adipose tissue. Degranulation rarely occurred, even in young bruises.

The staining characteristics of fibrin were investigated in 40 bruises aged from 1-72 hours old, using either MSB or PTAH stains (Table 2.3). With MSB, strands of fibrin stained either red, or, when the strands were very fine, blue. Fibrin less than 16 hours old did not stain yellow as suggested by Lendrum *et al.* (1969). With PTAH, fibrin strands in bruises of all ages stained purple.

Muscle regeneration was not detected prior to 36 hours but was observed regularly thereafter. The most common regenerative change was the appearance of myoblasts amongst macrophages infiltrating necrotic fibres (Figure 2.5). A proportion of myoblasts were readily identified by their large, clear oval or slightly irregular nuclei with prominent nucleoli, but their overall resemblance to adjacent macrophages made a reliable distinction between the two cell types difficult by light microscopy. Hence it was not possible to calculate the percentage of bruises more than 36 hours old showing evidence of muscle regeneration.

FIGURE 2.3 : The number of neutrophils in the infiltrate exceeds that of macrophages, as was generally the case in bruises up to 20 hours old. This bruise was 16 hours old.
(Paraffin section, H+E x 260)

FIGURE 2.4 : The number of macrophages in the infiltrate exceeds that of neutrophils, as was generally the case in bruises 24 hours and older. This bruise was 60 hours old.
(Paraffin section, H+E x 260)



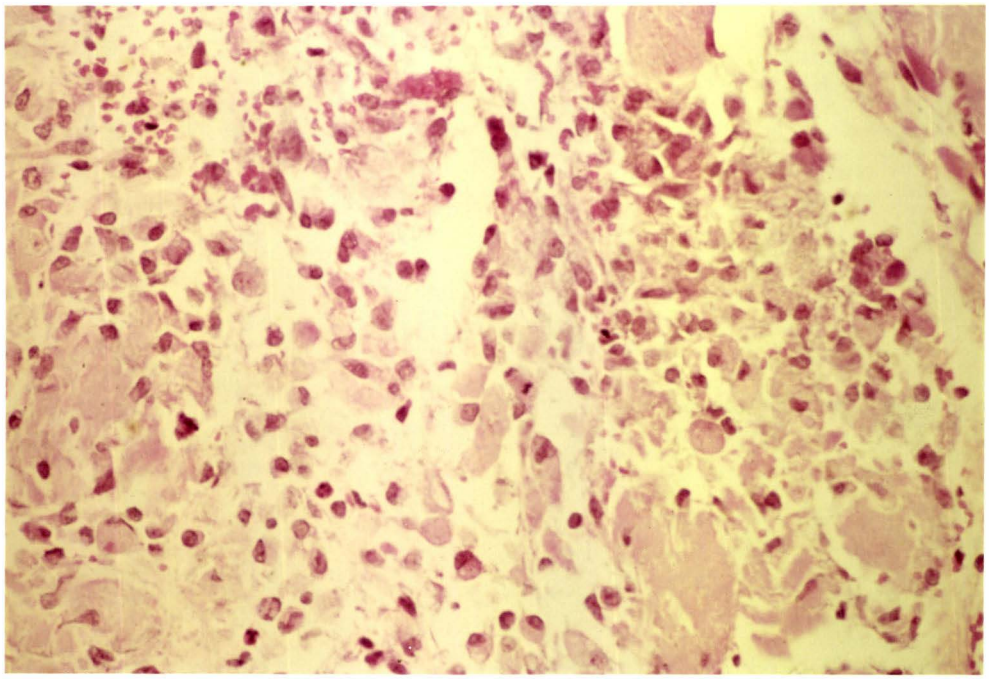
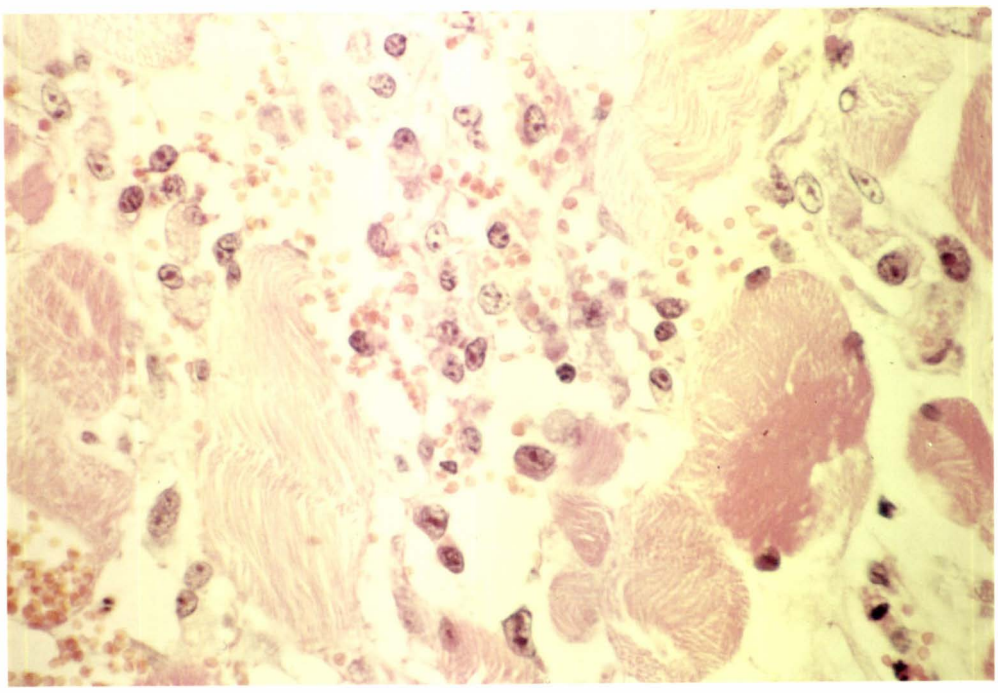
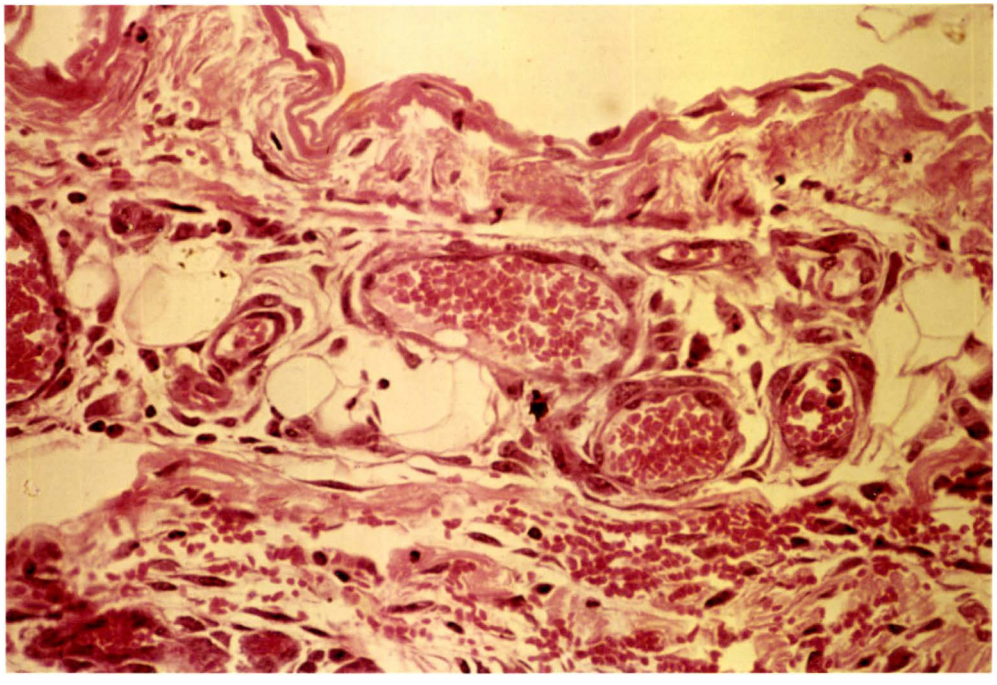


FIGURE 2.5 : Myoblast-like cells indicative of early regenerative change. This bruise was 36 hours old.
(Paraffin section, H+E x 500)

FIGURE 2.6 : Endothelial hypertrophy in a 60 hour old bruise.
(Paraffin section, H+E x 260)





Also present in regenerating muscle, but less commonly observed, were enlarged, clear, elongated myonuclei with prominent nucleoli but no discrete cytoplasm. Most of these nuclei were centrally placed in otherwise apparently normal fibres, though some were observed in necrotic fibres.

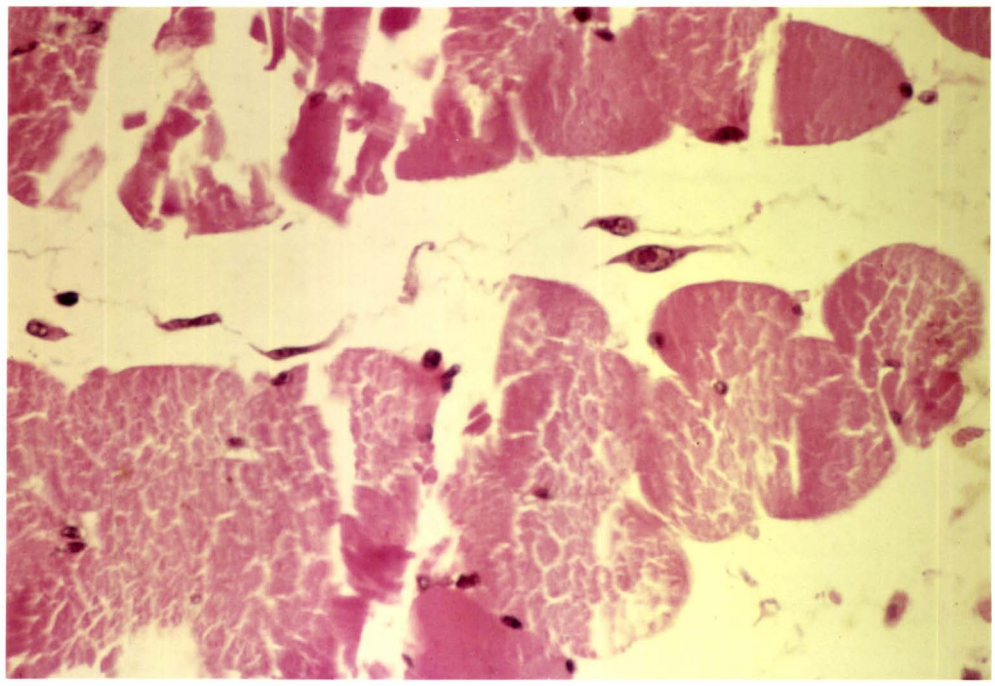
The visible indications of repair in both muscle and adipose tissue were endothelial hypertrophy, proliferation of capillary loops and fibroplasia. Proliferating capillary loops were observed infrequently and only in a few older bruises undergoing severe reactive changes. Endothelial hypertrophy was observed in 7% of bruises less than 36 hours old and in 55% of bruises 36 hours and older. It mainly involved small to medium sized venules, the endothelial cells of which became rounded and contained enlarged nuclei with prominent nucleoli (Figure 2.6).

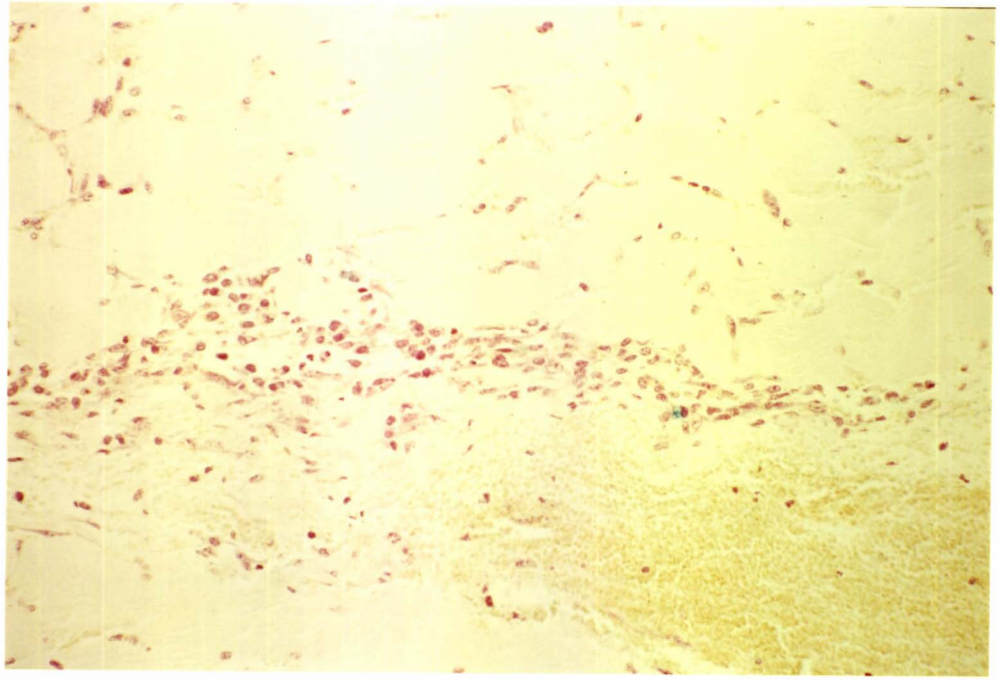
The earliest evidence of fibroplasia was hypertrophy, hyperchromasia and proliferation of fibroblasts in close association with blood vessels (Figure 2.7). This was rarely detected in bruises less than 24 hours old but was noted in approximately 20% of bruises 24 hours and older. Newly formed collagen was not detected using van Gieson stain until after 72 hours, when it appeared as fine red fibrils closely associated with fibroblasts. The fibroplastic reaction was usually mild and most bruises appeared likely to have healed without significant scar formation. However, in a few cases fibroplasia was severe. Haemosiderin was readily detected by Perl's stain as blue intracytoplasmic granules of various sizes within macrophages (Figure 2.8). It was observed in 50% of bruises 48 hours and older but was rarely detected prior to this. Usually only a small proportion of the macrophages present contained haemosiderin.

Acid glycosaminoglycans, as detected by PAS/alcian blue staining, were observed in the interstitial spaces of all of the bruises examined (Table 2.3). However, the amounts detected were too small to allow difference between bruises to be assessed.

FIGURE 2.7 : Hypertrophy of fibroblasts in a 60 hour old bruise.
(Paraffin section, H+E x 500)

FIGURE 2.8 : Haemosiderin positive macrophages in a 72 hour old bruise.
(Paraffin section, Perl's stain, x 160)





II. SELECTION OF HISTOPATHOLOGICAL FEATURES SUITABLE FOR AGEING IMPACT BRUISES MATHEMATICALLY

1. Initial selection of histopathological features

Most of the histopathological features described in the previous section were scored semi-quantitatively on a scale of 1-5 according to the degree of change from normal, with a score of 1 representing the normal state. Macrophage/neutrophil ratios were calculated from cell counts and scored as 1-3 according to whether ratios were <1.00, 1.01-2.99 or >3.00 respectively.

It became apparent that the original experimental design, in which bruises were to be classified as 1, 2, 4, 6, 8, 12, 20, 24, 30, 36, 48, 60 or 72 hours old was too optimistic in anticipating the accuracy with which bruises might be aged. As a consequence, and before the data were analysed, bruises were grouped into the following six age categories: 1-8, 12-20, 24-36, 48, 60 and 72 hours old. This restructuring necessitated the production of extra bruises to provide at least 20 bruises in each age category. Data from all available tissue sections showing evidence of haemorrhage were then used to construct contingency Tables (e.g. Table 2.2) relating scores from individual pathological features to each of the six age categories. The tables thus constructed for muscle contained data from 271 tissue sections representing 145 bruises; those constructed for adipose tissue contained information from 339 tissue sections representing 148 bruises. After inspection of these tables, four histopathological features were excluded from further consideration because too few of their assigned scores were greater than 1, the score assigned to normal tissue. These were: margination of leucocytes, proliferation of capillary loops, degranulation of mast cells and calcification. Muscle regeneration was also excluded from further consideration because of the difficulty in distinguishing between myoblasts and macrophages by light microscopy.

2. Further selection of histopathological features according to results of chi-square analyses on data in contingency tables

In contingency tables for the remaining features, score categories 4 and 5 were merged so as to minimise the number of cells containing information from less than five tissue sections. Data in these tables were then subjected to chi-square analyses to identify those features with significant associations between scores and the known ages of bruises. The results are summarised in Table 2.4. Histopathological features with scores statistically associated with age at the $P<0.05$ rejection level were selected as being of potential use for estimating the ages of bruises. In both muscle and adipose tissue these were: necrosis, neutrophil exudation, macrophage exudation, fibroplasia, endothelial cell hypertrophy, haemosiderin and ratios of macrophages to neutrophils in the inflammatory exudate. In muscle, statistically significant associations also existed between bruise ages and scores for severity of tissue haemorrhage. Scores for severity of disruption of muscle, severity of disruption of adipose tissue and severity of haemorrhage in adipose tissue were not significantly related to age.

TABLE 2.4 : STATISTICAL SIGNIFICANCE OF ASSOCIATIONS BETWEEN SCORES FROM VARIOUS HISTOPATHOLOGICAL FEATURES AND BRUISE AGES. THE RESULTS ARE FROM CHI-SQUARE ANALYSES OF THE RESPECTIVE CONTINGENCY TABLES.

HISTOPATHOLOGICAL FEATURE	SIGNIFICANCE OF TEST	
	Muscle	Adipose Tissue
Physical disruption of tissue	0.279*	0.173*
Severity of haemorrhage	0.001	0.210*
Necrosis	0.001	0.029
Neutrophil exudate	0.001	0.001
Macrophage exudate	0.001	0.001
Fibroplasia	0.001	0.001
Hypertrophy of endothelial cells	0.001	0.001
Haemosiderin in macrophages	0.001	0.001
Macrophage/neutrophil ratio	0.001	0.001

* No significant association between score and age of bruise

3. Relationships between bruise ages and scores for histopathological features whilst controlling for the effects of haemorrhage

The degree of haemorrhage may, in theory, affect the severity of tissue reactions to injury. For this reason possible indirect effects of haemorrhage were examined before deciding on the significance of the relationships between histopathological scores and bruise ages. To this end, contingency tables relating scores and bruise ages were again prepared for both muscle and adipose tissue, but this time each table was constructed so that it contained information only from tissue sections with the same score for haemorrhage. The data in these tables were again subjected to chi-square analyses to determine which histopathological features remained significantly associated with age ($P<0.05$) once the direct effects of haemorrhage had been negated. The results from this series of analyses are presented in Table 2.5 and 2.6 for muscle and adipose tissue respectively. Most of the original conclusions concerning associations between scores and bruise ages were still valid after the effects of haemorrhage were accounted for, but the conclusions pertaining to muscle necrosis and fat necrosis were not confirmed. These features were therefore considered unsuitable for estimating the ages of bruises.

4. Histopathological features eventually chosen for use when estimating the ages of bruises

Following the selection procedures described above, six histopathological features in muscle and adipose tissue were chosen as of potential use for estimating the ages of bruises.

These were:
- The neutrophil exudate
- The macrophage exudate
- Fibroplasia
- Endothelial cell hypertrophy
- Haemosiderin in macrophages
- The macrophage/neutrophil ratio in the exudate

For all of the above features except haemosiderin, scores 4 and 5 were merged to minimise the number of score categories represented by small

numbers of bruises. For haemosiderin, score categories 2-5 were merged, i.e. haemosiderin was scored as being present or not present.

TABLE 2.5 : STATISTICAL SIGNIFICANCE OF ASSOCIATIONS BETWEEN SCORES FOR VARIOUS HISTOPATHOLOGICAL FEATURES IN MUSCLE AND BRUISE AGES, WHILST CONTROLLING FOR THE EFFECTS OF HAEMORRHAGE.

HISTOPATHOLOGICAL FEATURE SCORED 1-5	STATISTICAL SIGNIFICANCE OF TEST FOR EACH DEGREE OF HAEMORRHAGE			
	1 (107)	2 (158)	3 (85)	4 + 5 (20)
Disruption of muscle fibres	0.854	0.157	0.968	0.430
Necrosis of muscle fibres	0.426	0.539	0.009	0.418
Neutrophil exudate	0.676	0.002	0.001	0.082
Macrophage exudate	0.369	0.001	0.003	0.320
Fibroplasia	0.532	0.001	0.001	0.001
Hypertrophy of endothelial cells	0.391	0.003	0.001	0.012
Haemosiderin	0.249	0.001	0.001	0.071
Macrophage/neutrophil ratio	0.664	0.001	0.001	0.004

( ) Number of tissue sections considered

TABLE 2.6 : STATISTICAL SIGNIFICANCE OF ASSOCIATIONS BETWEEN SCORES FOR VARIOUS HISTOPATHOLOGICAL FEATURES IN ADIPOSE TISSUE AND BRUISE AGES, WHILST CONTROLLING FOR THE EFFECTS OF HAEMORRHAGE.

HISTOPATHOLOGICAL FEATURE SCORED 1-5	STATISTICAL SIGNIFICANCE OF TEST FOR EACH DEGREE OF HAEMORRHAGE			
	1 (19)	2 (160)	3 (109)	4 + 5 (70)
Disruption of adipocytes	0.471	0.206	0.363	0.600
Necrosis of adipocytes	0.233	0.061	0.336	0.975
Neutrophil exudate	0.072	0.002	0.001	0.001
Macrophage exudate	0.209	0.001	0.001	0.001
Fibroplasia	0.268	0.001	0.001	0.001
Hypertrophy of endothelial cells	0.062	0.001	0.005	0.013
Haemosiderin	0.184	0.001	0.001	0.001
Macrophage/neutrophil ratio	0.097	0.001	0.001	0.001

( ) Number of tissue sections considered

III. DEVELOPMENT OF A MATHEMATICAL MODEL FOR AGEING IMPACT BRUISES OBJECTIVELY

The histopathological features potentially of use for ageing bruises had been selected as above. It remained to choose a mathematical model with which to objectively evaluate and interpret the semi-quantitative scores for these features. Two different models were considered: A Bayesian probability model (Bailey, 1965) and a multiple linear regression model (Nie *et al.*, 1975). Both models were 'developed' using identical data from 120 tissue sections. The most suitable model was then chosen according to its ability to correctly age the bruises that furnished the developmental data, since this provided an indication of the maximum potential performance of the model.

A. Developing the Bayesian probability model

In developing this model, data were derived from one tissue section from each of 20 bruises per age category studied. Muscle and adipose tissue were considered separately. The probability $P(S_x|D_j)$ of a score occurring in bruises representing a particular age category was calculated for each score from each of the chosen histopathological features for each of the six age categories in turn. These probability figures are presented in Tables 2.7 and 2.8 for muscle and adipose tissue respectively. The probability $P(S_c|D_j)$ of observing a combination of scores representing several histopathological features was calculated for each age category as the product of the probability of observing each of the separate scores in the combination. The highest probability figure thus calculated indicated the age group in which the combination of scores was most likely to be observed. The actual probability $P(D_j|S_c)$, relative to the other age categories, that the combination of scores represented that age category was then calculated using Bayes' equation,

$$\text{i.e. } P(D_j|S_c) = \frac{P(S_c|D_j)}{\sum_{j=1}^{6} P(S_c|D_j)}$$

A worked example for one bruise is shown in Table 2.9.

TABLE 2.7 : PROBABILITIES OF OBSERVING INDIVIDUAL SCORES FOR SELECTED HISTOPATHOLOGICAL FEATURES OF BRUISED MUSCLE, CALCULATED FOR EACH OF SIX AGE CATEGORIES OF BRUISES.

AGE (hours)	NEUTROPHIL EXUDATE				MACROPHAGE EXUDATE				FIBROPLASIA				ENDOTHELIAL HYPERTROPHY				HAEMO-SIDERIN		MACRO/NEUT RATIO		
	1	2	3	4,5	1	2	3	4,5	1	2	3	4,5	1	2	3	4,5	1	2+	1	2	3
1-8	.24	.31	.38	.07	.27	.48	.21	.03	.83	.10	.03	.03	.90	.03	.03	.03	.93	.07	.77	.18	.05
12-20	.04	.25	.54	.17	.04	.38	.38	.21	.71	.21	.04	.04	.88	.04	.04	.04	.91	.09	.67	.19	.14
24-36	.13	.50	.29	.08	.08	.33	.42	.17	.38	.29	.29	.04	.75	.13	.08	.04	.91	.09	.28	.56	.17
48	.33	.50	.13	.04	.04	.29	.25	.42	.04	.21	.38	.38	.38	.13	.29	.21	.61	.36	.07	.53	.40
60	.46	.46	.04	.04	.04	.17	.33	.46	.08	.08	.38	.46	.25	.21	.29	.25	.32	.68	.17	.22	.61
72	.67	.17	.13	.04	.04	.21	.33	.42	.08	.25	.13	.54	.46	.13	.29	.13	.14	.86	.06	.19	.75

TABLE 2.8 : PROBABILITIES OF OBSERVING INDIVIDUAL SCORES FOR SELECTED HISTOPATHOLOGICAL FEATURES OF BRUISED ADIPOSE TISSUE, CALCULATED FOR EACH OF SIX AGE CATEGORIES OF BRUISES.

AGE (hours)	NEUTROPHIL EXUDATE				MACROPHAGE EXUDATE			FIBROPLASIA				ENDOTHELIAL HYPERTROPHY			HAEMOSIDERIN	
	1	2	3	4,5	1,2	3	4,5	1	2	3	4	1	2	3,4	1	2+
1-8	.03	.31	.31	.34	.54	.36	.11	.90	.03	.04	.03	.88	.08	.04	.92	.08
12-20	.04	.29	.42	.21	.35	.52	.13	.67	.25	.08	.04	.86	.14	.05	.76	.24
24-36	.04	.38	.42	.17	.17	.48	.39	.38	.21	.25	.17	.52	.17	.30	.74	.26
48	.42	.38	.17	.04	.26	.44	.30	.13	.21	.33	.33	.52	.33	.14	.50	.50
60	.54	.29	.13	.04	.22	.61	.17	.17	.13	.38	.33	.35	.39	.26	.32	.68
72	.63	.21	.13	.04	.30	.43	.22	.17	.33	.21	.29	.50	.18	.32	.14	.76

TABLE 2.9 : THE CALCULATIONS REQUIRED WHEN USING HISTOPATHOLOGICAL DATA TO AGE BRUISES ARE SHOWN FOR ONE BRUISE IN MUSCLE.

	NEUTROPHIL EXUDATE	MACROPHAGE EXUDATE	FIBROPLASIA	ENDOTHELIAL CELL HYPERTROPHY	HAEMOSIDERIN		
	Score assigned to histopathological feature						
AGE (hours)	2	3	3	1	2	Product of probabilities	Relative probability of bruise being of stated age**
	Probability of observing score above in each age category from Table 2.7						
1-8	.31	.21	.03	.90	.07	.0001	.00
12-20	.25	.38	.04	.88	.09	.0003	.01
24-36	.50	.42	.29	.75	.09	.0041	.14
48	.50	.25	.38	.38	.36	.0065	.22
60	.46	.33	.38	.25	.68	.0098	.33*
72	.17	.33	.13	.46	.86	.0087	.30
				Total for this bruise		0.0295	1.00

* Most likely age for combination of scores

** Calculated using Bayes' formula

1. The performance of the Bayesian probability model using data from muscle:

The term 'accuracy' as used in subsequent sections is defined as the percentage of bruises of known ages correctly aged as such. When only two age categories were considered the above measures became closely analogous to the epidemiological concepts of sensitivity and specificity as used to describe the performance of disease testing procedures (Schwabe *et al.*, 1977). The potential 'accuracy' of the Bayesian probability model was assessed by applying it to data from the 120 bruises from which the probabilities of observing scores were calculated. Separate scores for the macrophage and neutrophil exudates and scores for macrophage/neutrophil ratios were considered in two separate analyses since a theoretical requirement when using Bayes' equation is that the probabilities of observing scores for the parameters in the equation be causally independent of each other. The ages of bruises estimated using scores for the histopathological features in Table 2.9, i.e. separate macrophage and neutrophil scores, are compared to their known ages in Table 2.10. The ages of bruises estimated using the scores for macrophage/neutrophil ratios are compared to their actual ages in Table 2.11. Not all age categories in these tables contain 20 bruises. This is because the probabilities assigned to some combinations of scores tied on two or more age categories, causing the affected bruises to be rejected from consideration. The 'accuracies' achieved by the two applications of the Bayesian probability model are compared for each age group in Table 2.12. From inspection of Tables 2.10, 2.11 and 2.12 the following conclusions were drawn:

(i) The Bayesian probability model as applied to data from muscle was not very 'accurate' in placing bruises into one of six age categories.

(ii) The model was more 'accurate' and less bruises were rejected because of tied results when separate scores for macrophage and neutrophil exudates were used rather than scores assigned to macrophage/neutrophil ratios.

Because of these considerations the use of ratios was discontinued and the number of age categories was reduced.

TABLE 2.10 : THE ESTIMATED AGES OF EXPERIMENTAL BRUISES, AS DETERMINED BY APPLYING THE BAYESIAN PROBABILITY MODEL TO HISTOPATHOLOGICAL DATA FOR MUSCLE, ARE COMPARED WITH THEIR KNOWN AGES. MACROPHAGES AND NEUTROPHILS WERE SCORED SEPARATELY RATHER THAN AS RATIOS.

ACTUAL AGE (hours)	ESTIMATED AGE (hours)						TOTAL NUMBER OF BRUISES
	1-8	12-20	24-36	48	60	72	
1-8	17	2	1	-	-	-	20
12-20	6	13	-	-	-	-	19*
24-36	4	1	11	1	-	1	18*
48	-	-	5	9	3	3	20
60	-	-	1	3	12	4	20
72	-	-	-	2	2	15	19*

* Some bruises rejected because of tied age estimates

TABLE 2.11 : THE ESTIMATED AGES OF EXPERIMENTAL BRUISES, AS DETERMINED BY APPLYING THE BAYESIAN PROBABILITY MODEL TO HISTOPATHOLOGICAL DATA FOR MUSCLE, ARE COMPARED WITH THEIR KNOWN AGES. THIS TIME MACROPHAGES AND NEUTROPHILS ARE SCORED AS RATIOS.

ACTUAL AGE (hours)	ESTIMATED AGE (hours)						TOTAL NUMBER OF BRUISES
	1-8	12-20	24-36	48	60	72	
1-8	13	1	1	-	-	-	15*
12-20	12	4	4	-	-	-	20
24-36	2	1	9	2	-	1	15*
48	-	-	3	6	4	4	17*
60	-	-	1	5	8	5	19*
72	-	-	-	2	4	12	18*

* Some bruises rejected because of tied age estimates

TABLE 2.12 : THE PERCENTAGE 'ACCURACIES' OF THE BAYESIAN PROBABILITY MODEL IN AGEING BRUISES USING HISTOPATHOLOGICAL DATA FROM MUSCLE OR ADIPOSE TISSUE ARE SHOWN FOR THE 120 BRUISES USED TO DEVELOP THE MODEL.

AGE (hours)	ACCURACY		
	MUSCLE		ADIPOSE TISSUE
	separate scores*	ratios**	separate scores*
1-8	85%	87%	80%
12-20	68%	20%	45%
24-36	61%	60%	40%
48	45%	35%	25%
60	60%	42%	50%
72	79%	67%	60%

* Separate scores for the macrophage and neutrophil exudates

** Scores assigned to macrophage/neutrophil ratios

2. The performance of the Bayesian probability model when used to place bruises into one of only two broad age categories:

The effect of reducing the number of age categories on the 'accuracy' of ageing bruises was explored in two ways. In the first, the six age categories in Table 2.10 were simply merged to form two groups representing bruises 1-20 hours and 24-72 hours old. In the second, new probabilities of observing scores for histopathological features were calculated for age groups 1-20 hours and 24-72 hours and Bayes' equation was used to age bruises directly as such. The increases in 'accuracy' thus attained are shown in Table 2.13. It was concluded that the Bayesian probability model was more 'accurate' when used to place bruises into one of two rather than six age categories. However, the greatest overall 'accuracy' in so doing was achieved by first placing bruises into one of six age groups then regrouping them, rather than by placing them directly into one of only two age categories.

TABLE 2.13 : THE PERCENTAGE 'ACCURACIES' OF THE BAYESIAN PROBABILITY MODEL IN AGEING BRUISES INDIRECTLY OR DIRECTLY INTO TWO BROAD AGE CATEGORIES ARE CALCULATED USING HISTOPATHOLOGICAL DATA FROM THE 120 BRUISES USED TO DEVELOP THE MODEL.

AGE (hours)	ACCURACY		
	MUSCLE		ADIPOSE TISSUE
	indirect*	direct	indirect*
1-20	97%	100%	93%
24-72	94%	82%	83%

* Bruises first placed into one of six age categories

3. The performance of the Bayesian probability model using data from adipose tissue:

For adipose tissue, the model was applied to scores equivalent to those used for muscle, but without considering scores for macrophage/neutrophil ratios. Comparisons between estimated and actual ages of bruises are shown in Table 2.14. The 'accuracy' of the model for each age category is presented for comparison with muscle in Table 2.12. The 'accuracy' of the model after bruises had been regrouped into age categories 1-20 hours and 24-72 hours is compared in Table 2.13 with that for muscle.

4. The effects of re-scoring of tissue sections on bruise age estimates:

The repeatability of semi-quantitative histopathological scoring was expected to be less than perfect. Variability was therefore measured by re-scoring the 120 muscle sections used when developing the Bayesian probability model. As indicated in Table 2.15, repeatability for all features other than haemosiderin was relatively poor, although scores for the most part deviated by only ± 1 score unit from the originals.

TABLE 2.14 : THE ESTIMATED AGES OF EXPERIMENTAL BRUISES, AS DETERMINED BY APPLYING THE BAYESIAN PROBABILITY MODEL TO HISTOPATHOLOGICAL DATA FOR ADIPOSE TISSUE, ARE COMPARED WITH THEIR KNOWN AGES.

ACTUAL AGE (hours)	ESTIMATED AGE (hours)						TOTAL NUMBER OF BRUISES
	1-8	12-20	24-36	48	60	72	
1-8	16	4	-	-	-	-	20
12-20	8	9	2	-	1	-	20
24-36	4	4	8	2	2	-	20
48	2	1	4	5	3	5	20
60	2	-	1	1	10	6	20
72	-	1	2	1	4	12	20

TABLE 2.15 : THE PERCENTAGES OF SCORES DUPLICATED AFTER RE-SCORING THE 120 BRUISES USED WHEN DEVELOPING THE BAYESIAN PROBABILITY MODEL.

HISTOPATHOLOGICAL FEATURE	PERCENTAGE OF DUPLICATED SCORES
Neutrophil exudate	59%
Macrophage exudate	57%
Fibroplasia	63%
Endothelial cell hypertrophy	77%
Haemosiderin in macrophages	100%

The new scores were used to re-estimate the ages of bruises into two broad age categories, using the probability figures $P(S_x|D_j)$ calculated from the original data for the six age categories. The results are compared in Table 2.16.

TABLE 2.16 : COMPARISONS BETWEEN THE 'ACCURACIES' OF THE BAYESIAN PROBABILITY MODEL IN AGEING BRUISES USING THE ORIGINAL AND NEW SCORES FOR THE 120 MUSCLE SECTIONS USED TO DEVELOP THE MODEL.

AGE (hours)	ACCURACY	
	Original scores	New scores
1-20	97%	92%
24-72	94%	90%

As can be seen, the 'accuracy' of the model when estimating ages from the new scores was of the same order of magnitude as that achieved using the original scores (Table 2.13). It was concluded that although scores assigned to individual histopathological features frequently differed from the originals, the effects of these variations were not great when sets of scores representing several features were used to estimate the ages of bruises.

B. Developing the multiple regression model

A multiple regression equation was derived for muscle, using the data representing the same 120 bruises used to develop the Bayesian probability model. The equation was fitted by the method of least squares, using a sub-programme available in the computer package known as 'Statistical Package for the Social Sciences' (SPSS).

The equation so derived was -

$$y = 0.58x_1 + 0.71x_2 - 0.07x_3 - 0.64x_4 + 0.49x_5 + 1.11$$

where

- y was the estimated age of the bruise. This took values from 1-6 according to whether the bruise was estimated as being 1-8, 12-20, 24-36, 48, 60 or 72 hours old respectively.

- x_1-x_5 were the semi-quantitative scores (1-4) for fibroplasia, haemosiderin in macrophages, hypertrophy of endothelial cells, exudation of neutrophils and exudation of macrophages respectively.

The above equation was used to estimate individual bruise ages directly from scores for the five selected histopathological features. Its maximum potential 'accuracy' was assessed by applying it to the data from which it had been derived, i.e. that from the 120 bruises. Comparisons between estimated and actual ages of bruises are presented in Table 2.17. The 'accuracy' of the linear regression model is compared to that of the Bayesian probability model in Table 2.18. Examination of this table shows that the 'accuracy' of the regression equation was less than that of the Bayesian model when bruises were partitioned into one of six age categories. As was the case with the Bayesian model however, the 'accuracy' was considerably increased if bruises were subsequently re-classified as 1-20 hours or 24-72 hours old.

It was concluded that the multiple regression equation had no practical advantages over the Bayesian probability model. For this reason, and because of certain theoretical factors discussed later, the Bayesian probability model was selected as the model of choice for further ageing of bruises.

C. Assessment of the performance of the Bayesian probability model when applied to data not previously used to develop the model

The age of each new tissue section of a bruise was estimated by applying the Bayesian probability model to scores for the five previously selected histopathological features. Where a single bruise was represented by more than one section, each section was treated as a separate bruise. Estimated and actual ages of the new sections of bruises are compared in Table 2.19 and 2.20 for muscle and adipose tissue respectively. In Table 2.21 the performance of the Bayesian probability model when applied to the new data is

TABLE 2.17 : THE ESTIMATED AGES OF EXPERIMENTAL BRUISES, AS DETERMINED BY APPLYING THE MULTIPLE REGRESSION MODEL TO HISTOPATHOLOGICAL DATA FOR MUSCLE, ARE COMPARED WITH THEIR KNOWN AGES.

(hours)	ESTIMATED AGE (hours)						TOTAL NUMBER OF BRUISES
	1-8	12-20	24-36	48	60	72	
1-8	8	11	1	-	-	-	20
12-20	11	9	-	-	-	-	20
24-36	2	10	8	-	-	-	20
48	-	2	6	6	6	-	20
60	-	-	2	9	8	1	20
72	-	-	6	2	12	-	20

TABLE 2.18 : THE PERCENTAGE 'ACCURACY' OF THE MULTIPLE LINEAR REGRESSION MODEL IS COMPARED WITH THAT OF THE BAYESIAN PROBABILITY MODEL, USING THE SAME DATA FOR MUSCLE.

AGE (hours)	ACCURACY	
	Multiple regression	Bayesian model
1-8	40%	85%
12-20	45%	68%
24-36	40%	61%
48	30%	45%
60	40%	60%
72	0%	79%
1-20	98%	97%
24-72	83%	94%

TABLE 2.19 : COMPARISONS BETWEEN ESTIMATED AND ACTUAL AGES OF EXPERIMENTAL BRUISES. AGE ESTIMATES WERE MADE BY APPLYING THE BAYESIAN PROBABILITY MODEL TO HISTO-PATHOLOGICAL DATA FROM MUSCLE SECTIONS NOT PREVIOUSLY USED FOR DEVELOPING THE MODEL.

ACTUAL AGE (hours)	ESTIMATED AGE (hours)						TOTAL NUMBER OF BRUISES
	1-8	12-20	24-36	48	60	72	
1-8	31	18	5	-	-	2	56
12-20	10	17	5	-	-	1	33
24-36	10	-	13	5	-	1	29
48	1	-	4	2	3	-	10
60	-	-	-	-	9	1	10
72	-	-	1	3	4	2	10

TABLE 2.20 : COMPARISONS BETWEEN ESTIMATED AND ACTUAL AGES OF EXPERIMENTAL BRUISES. AGE ESTIMATES WERE MADE BY APPLYING THE BAYESIAN PROBABILITY MODEL TO HISTO-PATHOLOGICAL DATA FROM ADIPOSE TISSUE SECTIONS NOT PREVIOUSLY USED FOR DEVELOPING THE MODEL.

ACTUAL AGE (hours)	ESTIMATED AGE (hours)						TOTAL NUMBER OF BRUISES
	1-8	12-20	24-36	48	60	72	
1-8	58	16	2	-	-	-	76
12-20	26	9	4	2	-	1	42
24-36	5	10	25	5	6	-	51
48	4	3	2	3	3	-	15
60	1	1	2	3	7	4	18
72	-	2	2	5	6	2	17

TABLE 2.21 : THE 'ACCURACY' OF THE BAYESIAN PROBABILITY MODEL WHEN APPLIED TO DATA PREVIOUSLY USED FOR ITS DEVELOPMENT IS COMPARED WITH THAT ACHIEVED WITH NEW DATA.

AGE (hours)	ACCURACY			
	MUSCLE		ADIPOSE TISSUE	
	original*	new**	original*	new**
1-20	97%	85%	93%	93%
24-72	94%	81%	83%	75%

* Data used for developing the model

** New data

compared to its maximum performance after it had been applied to the data from which it was developed.

As expected, the model was slightly less 'accurate' when applied to the new data, but this would be a more realistic assessment of its 'accuracy' if it was applied to data from bruises of unknown ages.

1. Investigation into the advantages of considering more than one sample per bruise:

It was logical to assume that bruises would be aged with greater 'accuracy' if more than one sample of muscle or adipose tissue was considered for each bruise, or if individual bruises were aged only when there was concordance between age estimates made from both muscle and adipose tissue. Both of these options were explored.

(a) The effect of duplicate within-bruise sampling on the 'accuracy' of ageing bruises: The possible advantages of duplicate within-bruise sampling were explored using 62 paired muscle samples and 80 paired adipose tissue samples. Previously, each pair of sections had been treated as representing two separate bruises. Individual bruises were aged only when there was agreement between the estimated ages of sample pairs, with muscle and adipose tissue being considered separately.

Concordance of estimated ages occurred in only 72% and 73% of bruises when considering data from muscle and adipose tissue respectively. The 'accuracy' of ageing individual bruises using paired within-bruise samples (Table 2.22) was slightly higher than when bruises were aged on the basis of one sample only (Table 2.21). However, when considering paired samples, 18% of data had to be rejected because of a lack of concordance between age estimates.

TABLE 2.22 : THE 'ACCURACY' OF THE BAYESIAN PROBABILITY MODEL IN AGEING BRUISES WHEN THERE WAS CONCORDANCE BETWEEN PAIRED WITHIN-BRUISE SAMPLES.

AGE (hours)	ACCURACY	
	MUSCLE*	ADIPOSE TISSUE**
1-20	93%	95%
24-72	86%	81%

* 18% of bruises rejected for lack of concordance

** 17% of bruises rejected for lack of concordance

(b) The 'accuracy' of ageing bruises when there was concordance between age estimates from muscle and adipose tissue: Sections from those bruises with data available for both muscle and adipose tissue were aged only if there was agreement between age estimates from the two tissues. A total of 118 tissue sections fulfilled this criterion. With concordance of age between the two tissues, the 'accuracy' of ageing bruises was enhanced considerably (Table 2.23) relative to that when either muscle or adipose tissue were considered separately (Table 2.21). However, this increase in 'accuracy' was gained at the cost of having to reject 13% of muscle and adipose tissue pairs from consideration because of a lack of concordance between age estimates from the two tissues.

TABLE 2.23 : THE 'ACCURACY' OF THE BAYESIAN PROBABILITY MODEL IN AGEING BRUISES WITH CONCORDANCE BETWEEN ESTIMATES MADE FROM MUSCLE AND ADIPOSE TISSUE.

AGE (hours)	ACCURACY*
1-20	96%
24-72	97%

* 13% of sections were rejected for lack of concordance

2. Comparisons between the 'accuracies' of interpretive and Bayesian methods of ageing bruises:

The Bayesian probability model would be of practical value for ageing bruises only if it was more 'accurate' than a pathologist making interpretive assessments of age based on first principles and personal experience. Immediately after scoring tissue sections not used when 'developing' the Bayesian probability model, bruises had been aged interpretively according to histopathological changes in both muscle and adipose tissue. Comparisons between the 'accuracies' of the interpretive and Bayesian methods of ageing bruises are presented in Table 2.24.

TABLE 2.24 : THE 'ACCURACY' OF THE BAYESIAN PROBABILITY MODEL IN AGEING BRUISES IS COMPARED TO THAT OF INTERPRETIVE AGEING.

AGE (hours)	ACCURACY		
	INTERPRETIVE METHOD	BAYESIAN METHOD	
		MUSCLE	ADIPOSE TISSUE
1-20	78%	85%	93%
24-72	72%	81%	75%

The above table shows mathematical ageing to have been more 'accurate' than interpretive ageing.

IV. APPLICATION OF THE BAYESIAN PROBABILITY MODEL TO WOOL PULL BRUISES

1. Gross pathology

It was possible to distinguish grossly between experimental impact and 'wool pull' bruises since haemorrhage in the latter was more diffuse and largely confined to the subcutaneous fascia. Even though the fascial haemorrhage was quite severe in the majority of the 'wool pull' bruises, only mild haemorrhage was usually observed in the underlying adipose tissue. Muscle usually appeared unaffected grossly.

2. Histology

Haemorrhage in the 'wool pull' bruises was usually very mild, with 55% of adipose tissue sections being allotted the lowest haemorrhage score of 2. Muscle was affected in only 15% of cases. The tissue reactions in many of the bruises were slight and therefore relatively uninformative. The data were processed using the Bayesian probability method and the probabilities of observing histopathological scores calculated for impact bruises. The method was 100% 'accurate' in ageing 1-20 hour old bruises but only 57% 'accurate' for bruises 24-72 hours old.

V. THE 'CONFIDENCE' OF AGEING BRUISES OF UNKNOWN AGES BY APPLICATION OF THE BAYESIAN PROBABILITY METHOD

In previous sections the performance of the Bayesian probability model has been described in terms of the 'accuracy' of ageing, which was defined as the percentages of bruises of known ages 1-20 hours or 24-72 hours old that were correctly aged as such. This concept of 'accuracy' takes no cognisance of the number of incorrectly aged bruises from each alternate age category, and as such would be of limited value in measuring the performance of the model after it had been applied to bruises of unknown ages. A better measure of performance

in these situations would be the degree of certainty with which individual bruises had been aged as either 1-20 hours or 24-72 hours old. This is referred to as the 'confidence' of ageing. The method of calculating 'confidence' is outlined after first defining the different concepts of 'accuracy' and 'confidence' mathematically, with reference to Figure 2.9.

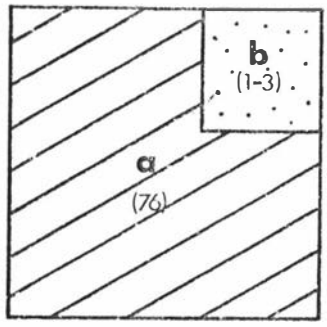
(a) 'Accuracy' of the Bayesian probability model: 'Accuracy' denotes the ability of the model to correctly identify as such, bruises of known ages 1-20 hours and 24-72 hours old respectively, i.e. a/a+b and c/c+d in Figure 2.9. In probability terms 'accuracy' is defined as 'the probability of a bruise being aged by the Bayesian method as 1-20 hours or 24-72 hours old, given that it was actually of that age', i.e. P(E|A), where the symbols stand for the probability (P) of estimated age (E) given (|) actual age (A). The values for 'accuracy' were determined experimentally, using bruises of known ages, and are assumed to remain constant for all future applications of the Bayesian method to bruises of unknown ages.

(b) The 'confidence' of ageing individual bruises: 'Confidence' is defined as the percentage of those bruises estimated to be either 1-20 hours or 24-72 hours old that were actually of that age, i.e. a/a+d and c/c+b in Figure 2.9. In probability terms, it is defined as the probability of a bruise actually being 1-20 hours or 24-72 hours old, given that it was estimated as such by the Bayesian method, i.e. P(A|E). This mathematical definition emphasises the fundamental difference between the concepts of 'accuracy' and 'confidence'.

1. Method of calculating the 'confidence' with which individual bruises have been aged

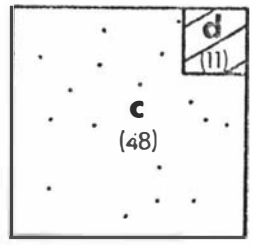
When an applied model is less than 100% 'accurate' a proportion of 1-20 hour and 24-72 hour old bruises will have been aged incorrectly (error terms b and d in Figure 2.9). The magnitude of this error depends on the degree of 'accuracy'. This factor therefore influences the 'confidence' of ageing. 'Accuracy' was found to vary according

Actual age 1-20 hours



n_x = a+b = 89

Actual age 24-72 hours



n_y = c+d = 59

- bruises estimated as 1-20 hours old = a+d

- bruises estimated as 24-72 hours old = c+b

Estimated age (hours)	Actual age (hours) 1-20	24-72	Total
1-20	76	11	87
24-72	13	48	61
	89	59	148

FIGURE 2.9 : A diagrammatic and figurative representation of the performance of the Bayesian probability model in ageing a population of bruises of ages n_x actually 1-20 hours old and n_y actually 24-72 hours old.

to the tissues used for ageing purposes and the number of samples examined per bruise, but in all cases values were less than 100% (Tables 2.21, 2.22, 2.23).

Irrespective of the 'accuracies' of ageing, the larger the number of bruises actually in one of the age categories relative to the other, the less significant the error term (b or d in Figure 2.9) from the alternate age category - and *vice versa*. In order to calculate the 'confidence' of ageing individual bruises it is therefore necessary to take into account the actual number of bruises in each age category. With bruises of unknown ages these numbers cannot be known with certainty but values can be estimated given knowledge of the 'accuracy' of the model and the numbers of bruises apparently of each age.

Because of the above considerations, 'confidence' values must be calculated afresh after each application of the ageing method to groups of bruises of unknown ages. The method of so doing is illustrated below, taking as an example the data previously used to determine the 'accuracy' of the model when applied to data from muscle only (Table 2.21) as presented in Figure 2.9.

In practice the following data from Figure 2.3 would be known or assumed

- The 'accuracy' of the model in identifying 1-20 hour old bruises i.e. $P(E_x|A_x) = 76/89 = \underline{0.85}$
- The 'accuracy' of the model in identifying 24-72 hour old bruises i.e. $P(E_y|A_y) = 48/59 = \underline{0.81}$
- The number of bruises estimated to be 1-20 hours old = 87
- The number of bruises estimated to be 24-72 hours old = 61
- The total number of bruises in the population studied = 148

The 'confidence' of ageing bruises is calculated by applying Bayes' rule to the above data as follows.

The 'confidence' of ageing bruises as 1-20 hours old =

$$P(A_x|E_x) = \frac{P(D_x).P(E_x|A_x)}{P(D_x).P(E_x|A_x) + P(D_y).(1 - P(E_y|A_y))} \quad (1)$$

The 'confidence' of ageing bruises as 24-72 hours old =

$$P(A_y|E_y) = \frac{P(D_y).P(E_y|A_y)}{P(D_y).P(E_y|A_y) + P(D_x).(1 - P(E_x|A_x))} \quad (2)$$

The values of $P(D_x)$ and $P(D_y)$ in the above equations can be estimated from the respective 'accuracies' of the model and the apparent prevalences of bruises of each age (Schwabe *et al.*, 1977).

$$P(D_x) = \frac{\text{apparent prevalence of } x + P(E_y|A_y) - 1}{P(E_y|A_y) + P(E_x|A_x) - 1} \quad (3)$$

$$P(D_y) = 1 - P(D_x) \quad (4)$$

For the example in Figure 1.9

$$P(D_x) = \frac{87/148 + 48/59 - 1}{48/59 + 76/89 - 1}$$

$$= \underline{\underline{0.61}}$$

$$P(D_y) = 1 - 0.61$$

$$= \underline{\underline{0.39}}$$

Therefore -

$$P(A_x|E_x) = \frac{(0.61).(0.85)}{(0.61).(0.85) + (0.39)(1 - 0.81)}$$

$$= \underline{0.88}$$

$$P(A_y|E_y) = \frac{(0.39).(0.81)}{(0.39).(0.81) + (0.61).(1 - 0.85)}$$

$$= \underline{0.78}$$

In this example the percentage 'confidences' with which bruises were aged by the Bayesian probability model were 88% and 78% for 1-20 hour and 24-72 hour old bruises respectively. In other words, there was an 88% likelihood of having correctly aged individual bruises of unknown ages as 1-20 hours old and a 78% likelihood of having correctly aged them as 24-72 hours old.

The profound effect the relative number of bruises actually in each age category has on the 'confidence' of ageing bruises can be demonstrated by applying the above equations (1-4) to figures constructed for a hypothetical situation represented in Table 2.25.

TABLE 2.25 : THE EFFECT VARIATION IN THE RELATIVE NUMBERS OF BRUISES ACTUALLY AGED 1-20 HOURS or 24-72 HOURS OLD HAS ON THE 'CONFIDENCE' OF AGEING INDIVIDUAL BRUISES. THE 'ACCURACY' OF THE MODEL IS ASSUMED CONSTANT AT 0.85 AND 0.81 FOR 1-20 HOUR AND 24-72 HOUR OLD BRUISES RESPECTIVELY.

ACTUAL NUMBER OF BRUISES			'CONFIDENCE' OF AGEING INDIVIDUAL BRUISES	
1-20 hours	24-72 hours	Total	1-20 hours	24-72 hours
150	50	200	93%	65%
100	100	200	82%	84%
50	150	200	61%	95%

The larger the population of one age group relative to the other, the greater the 'confidence' with which bruises at that age will have been aged as such, and *vice versa*.

2. Method of calculating the 'confidence' with which single bruises have been aged

A special situation exists when only a single bruise or a small number of bruises are considered at any one time. On these occasions the real, as opposed to the apparent, age distributions of bruises cannot be calculated with an acceptable degree of certainty. Each bruise must therefore be assumed to have an equal probability of being either 1-20 hours or 24-72 hours old, i.e. $P(D_x) = P(D_y) = \underline{0.50}$. Since both the 'accuracy' of the model and the prevalences of bruises will now be considered as constants, the 'confidence' with which the model operates will also be a constant, able to be calculated in advance for each set of samples that may be used for ageing bruises. Appropriate 'confidence' values thus calculated are listed in Table 2.26, using 'accuracy' figures previously estimated for different tissue sampling regimens (Tables 2.23, 2.24 and 2.25).

TABLE 2.26 : THE 'CONFIDENCE' WITH WHICH SINGLE OR SMALL NUMBERS OF BRUISES CAN BE AGED BY THE BAYESIAN PROBABILITY MODEL, USING 'ACCURACY' FIGURES FROM TABLES 2.21, 2.22 AND 2.23.

DATA CONSIDERED FOR AGEING	'CONFIDENCE' OF AGEING	
	1-20 hours	24-72 hours
Muscle only - one section per bruise	82%	84%
Adipose tissue only - one section per bruise	79%	92%
Muscle and adipose tissue (concordance)	97%	96%
Paired muscle samples "	85%	93%
Paired adipose tissue samples "	93%	94%

Comment: The 'confidence' with which an individual bruise can be aged has been shown to depend on two independent but interacting factors, namely the 'accuracy' of the model and the age distribution of the bruises under study. The 'confidence' with which a bruise can be aged is therefore determined by a set of conditions peculiar to each group of bruises considered. Nevertheless, at all times four general principles will act to enhance the 'confidence' with which the bruises from any one age category will have been aged. These principles are best understood with reference to Figure 2.9.

(i) The greater the 'accuracy' of the model in identifying bruises of a particular age, the greater the 'confidence' with which those bruises will have been aged.

(ii) The greater the 'accuracy' of the model in identifying one particular age group of bruises, the greater the 'confidence' with which bruises in the alternate age category will have been aged.

(iii) The larger the number of bruises in any one age category relative to the other, the greater the 'confidence' with which those bruises will have been aged.

(iv) The larger the number of bruises in any one age category relative to the other, the lower the 'confidence' with which bruises in the alternate age category will have been aged.

VI. PRACTICAL APPLICATION OF THE BAYESIAN AGEING METHOD: THE AGEING OF BRUISES IN A PILOT STUDY AT A MEAT WORKS

INTRODUCTION

A semi-quantitative mathematical method had been developed to age bruises objectively as either 1-20 hours or 24-72 hours old. This was intended for use in determining the places of origin of bruises by comparing their estimated ages with times for events thought likely to contribute to the bruising problem in New Zealand. Although certain expectations and limitations of the method could be deduced from experimental data it remained to assess the usefulness of the technique in practice. To this end a pilot survey was conducted under normal operating conditions in an export meat works. Readily available epidemiological data were collected during the survey in order to compare the traditional epidemiological and objective approaches to a problem of this kind.

MATERIALS AND METHODS

A total of 107 bruises, each of which was severe enough to require trimming under Ministry of Agriculture and Fisheries supervision, were collected from lambs processed under normal working conditions in an export meat works. All such bruises in any particular mob of lambs under consideration were sampled. One tissue sample per bruise was immediately fixed in formalin then processed for histology. Tissue sections were stained with H+E and Perl's iron stains. Bruises were subsequently aged as either 1-20 hours or 24+ hours old by applying the Bayesian probability model to histopathological data from muscle and adipose tissue considered separately.

The following information was recorded for each bruise: gross appearance, farm of origin, number of lambs from that farm, trucking distance and *ante mortem* holding time in meat works yards. In addition

the position of each bruise was classified according to Figure 2.13.

RESULTS

1. Gross characteristics of bruises

Most of the 107 bruises sampled were more severe than those inflicted experimentally but they were otherwise grossly similar. The majority were 'impact' bruises, with only 5 lesions grossly resembling experimental 'wool pull' bruises. It was not possible to age any of the bruises grossly since all of them were red in colour.

2. Histopathological characteristics of bruises

The bruises sampled were histopathologically similar to those inflicted experimentally. Bruising injury affected muscle only in 8%, adipose tissue only in 43% and both muscle and adipose tissue in 49% of the 107 bruises studied.

3. Ageing of bruises using the Bayesian probability method

Bruises were aged objectively as either 1-20 hours or 24+ hours old by applying the Bayesian probability method to the appropriate data. Of the bruises with both muscle and adipose tissue affected, only 67% of the age estimates concurred. The balance were aged according to results from muscle, since the scoring of histopathological changes was considered to be more reliable in this tissue.

From knowledge of the apparent age distributions for each set of data, and assuming the respective 'accuracies' of the method remained as previously determined (Table 2.21, 2.22 and 2.23), 'confidence' in ageing individual bruises was calculated as previously demonstrated. The results are presented in Table 2.27.

TABLE 2.27 : 'CONFIDENCE' LEVELS OF AGEING INDIVIDUAL BRUISES CALCULATED AFTER APPLYING THE BAYESIAN PROBABILITY METHOD TO DATA FROM MUSCLE, ADIPOSE TISSUE OR BOTH.

TISSUE	NUMBER OF BRUISES	ESTIMATED AGE		'CONFIDENCE' LEVELS	
		1-20 HOURS	24+ HOURS	1-20 HOURS	24+ HOURS
Muscle	26	19	7	0.96	0.56
Adipose tissue	46	27	19	0.80	0.90
Muscle and adipose tissue	35	27	8	0.99	0.85
	107				

From the above figures, the Bayesian method appeared potentially useful for ageing bruises observed in lambs processed for export. However, to determine the causes of bruising, the estimated ages of individual bruises must be related to the times of occurrence of events thought to contribute to the bruising problem. In this survey the bruise ages were considered in relation to holding times in meat works yards.

4. Bruise ages considered in relation to holding times in meat works yards

By considering bruise ages in relation to holding times in meat works yards, bruises were divided into two categories: those inflicted in the meat works and those inflicted prior to arrival. The results of these comparisons are presented in Table 2.28.

TABLE 2.28 : BRUISES AGED AS 1-20 HOURS OR 24+ HOURS OLD ARE CLASSIFIED ACCORDING TO THE KNOWN HOLDING TIMES OF LAMBS IN MEAT WORKS YARDS PRIOR TO SLAUGHTER

ESTIMATED AGES	HOLDING TIMES		
	<20 hours	21-23 hours	>24 hours
1-20 hours	(a) 7%	(b) 26%	(c) 36%
24+ hours	(d) 9%	(e) 15%	(f) 7%
Totals	16%	41%	43%

Certain shortcomings in the application of the Bayesian probability method become apparent when age estimates and holding times are considered in relation to each other. These arise because the method can be used to age bruises with an acceptable degree of 'confidence' only as either less than 20 hours or more than 24 hours old. Consequently, the places of origin of bruises cannot be determined in the following circumstances (with reference to Table 2.28):

(i) Both holding times and estimated ages are <20 hours (a);

(ii) Both holding times and estimated ages are >24 hours (f);

(iii) Holding times are from 21-23 hours (b,e).

On the other hand, the method is potentially useful when:

(i) Estimated ages are <20 hours and holding times are >24 hours (c);

(ii) Estimated ages are >24 hours and holding times are <20 hours (d).

The percentage values (a-f) in Table 2.28 depend on the interrelationships between the distribution patterns for bruise ages and holding times, and will therefore vary unpredictably with each survey. In the present survey only 45% (c+d) of bruises could be classified according to whether they occurred within or prior to arrival at the meat works. No firm decision as to places of origin of the remaining 55% of lesions could be made despite the fact that a proportion of these bruises were aged with a 'confidence' level of in excess of 90%. In future surveys this percentage could be reduced

substantially by not considering bruises from lambs held in the meat works for 21-23 hours. Had this policy been followed in this survey the percentage of bruises for which no firm decision could be made would have been reduced to only 24%.

Bruises could only be classified as having occurred within the meat works when holding times were >24 hours and as having occurred prior to arrival at the works when holding times were <20 hours. The proportion of bruises classified according to place of origin was therefore influenced by the distribution of the holding times of the lambs from which bruises were sampled. Since this distribution was uneven in the present survey the estimated ratio of 9:36 (d:c in Table 2.28) for bruises inflicted prior to arrival as compared to within the meat works would not have reflected the actual situation. For valid interpretation the results had to be adjusted for a hypothetical equal ratio of 50:50 for holding times <20 hours and >24 hours respectively. This was done as follows:

(i) Estimated place of origin of bruises with respect to the meat works.

Prior to arrival		Within the works
9	:	36

(ii) Actual ratio of holding times in meat works yards.

<20 hours		>24 hours
16	:	43

(iii) Hypothetical ratio of holding times in meat works yards to which results are to be adjusted.

<20 hours		>24 hours
50	:	50

(iv) Proposed places of occurrence of bruises.

Prior to arrival	Within the works
9 x 50/16 = 28	35 x 50/43 = 42

From the above situation it was proposed that 40% of bruises were inflicted prior to lambs arriving at the works and 60% were inflicted within the works.

5. Epidemiological data

Certain readily available epidemiological data were collected during the survey. These were used to compare the traditional and ageing approaches to determining the origins of bruises.

(a) Between-farm clustering of bruises: The 107 bruises comprising the survey originated from only 37 farms, which suggested a clustering effect whereby bruises predominantly affected lambs from only a minority of properties. This was confirmed by examining the difference between each mob-specific percentage bruising prevalence and the daily average bruising prevalence for the day on which that mob was killed (Figure 2.10). A paired 't' test analysis of the above data demonstrated a significantly increased ($P<0.05$) prevalence of bruising in the affected mobs as compared to the daily averages (Table 2.29). This indicated a tendency for bruises to be unevenly distributed between the mobs of lambs presented for slaughter, i.e. a tendency for bruises to occur in clusters.

TABLE 2.29 : RESULTS OF STUDENT'S PAIRED 't' TEST ASSESSING THE SIGNIFICANCE OF DIFFERENCES BETWEEN MOB-SPECIFIC AND APPROPRIATE DAILY AVERAGE BRUISING PREVALENCES.

Average daily bruising prevalence	0.83
Average bruising prevalence in affected mobs	1.30
Number of paired samples	37
Student's 't' statistic	2.1
Level of significance	<0.05

(b) Trucking distance: Trucking distances were presumed to reflect trucking times. Percentage prevalences of bruising in affected mobs of lambs are plotted against trucking distances in Figure 2.11. Within the limits of the survey the histogram suggests there was no relationship between trucking distance and the prevalence of bruising.

(c) Holding times in meat works yards: For practical and regulatory reasons all animals are rested in export slaughterhouses for a variable period of time before slaughter. Holding times for the lambs in this survey are shown in Figure 2.12, in which percentage prevalences of bruising are plotted against times held in the yards. The range of times for lambs in this survey was from 19-41 hours, but holding times may in fact range from between 11-72 hours. Within the limits of this survey the histogram in Figure 2.12 suggests there is no relationship between holding times and the prevalence of bruising.

(d) Carcase position of bruises: Bruises were classified according to the positions delinated in Figure 2.13. The percentage of bruises in each position and the estimated origins of those bruises that could be so classified are presented in Table 2.30.

TABLE 2.30 : THE CARCASE POSITIONS OF BRUISES ARE COMPARED WITH THEIR ESTIMATED PLACES OF OCCURRENCE, EITHER BEFORE OR AFTER ARRIVAL AT THE MEAT WORKS.

POSITION ON CARCASE	PERCENTAGE OF TOTAL	PLACE OF ORIGIN		
		Within*	Without*	No Decision
Lateral hind leg	33%	14	3	17
Medial hind leg	27%	9	5	15
Rump	7%	2	-	6
Back	6%	1	-	5
Shoulder/thorax	9%	4	1	5
Distal foreleg	13%	6	1	7
Generalised	4%	2	-	4
		38	10	59
		(36%)	(9%)	(55%)

* These figures are not adjusted for equal holding time distributions

From the above table it is apparent that 73% of bruises affected either the fore or hind limbs. In this survey the actual numbers of bruises affecting each carcase position were considered too small to allow

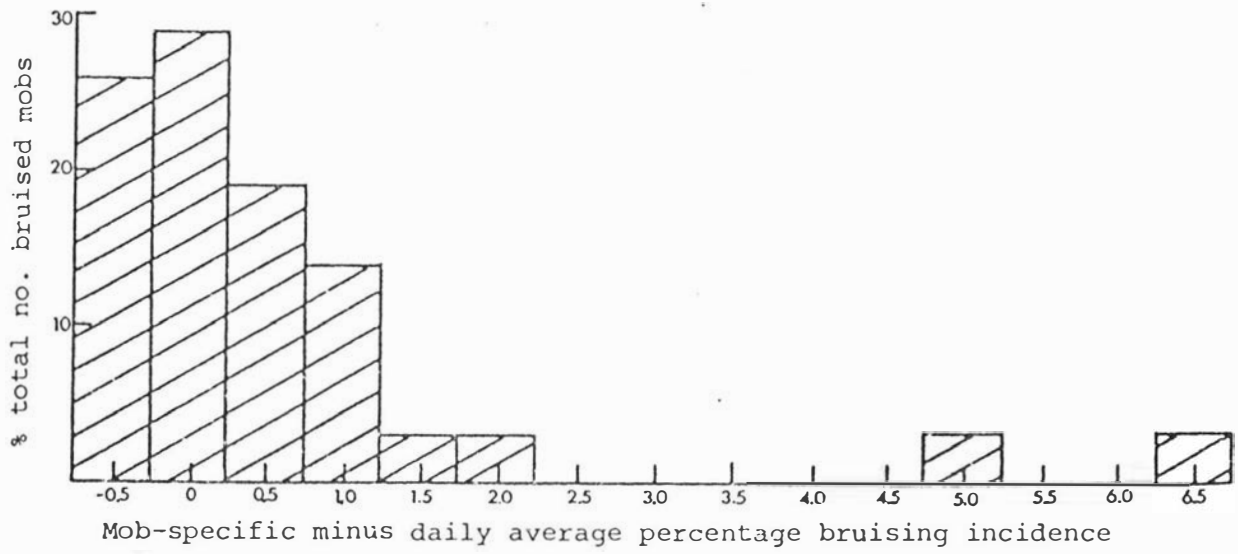


FIGURE 2.10 : Percentages of the total number of bruised mobs plotted against differences between mob-specific and appropriate daily average bruising prevalences. This shows the tendency for bruises to cluster within certain mobs of lambs rather than to occur with even distribution in all mobs slaughtered.

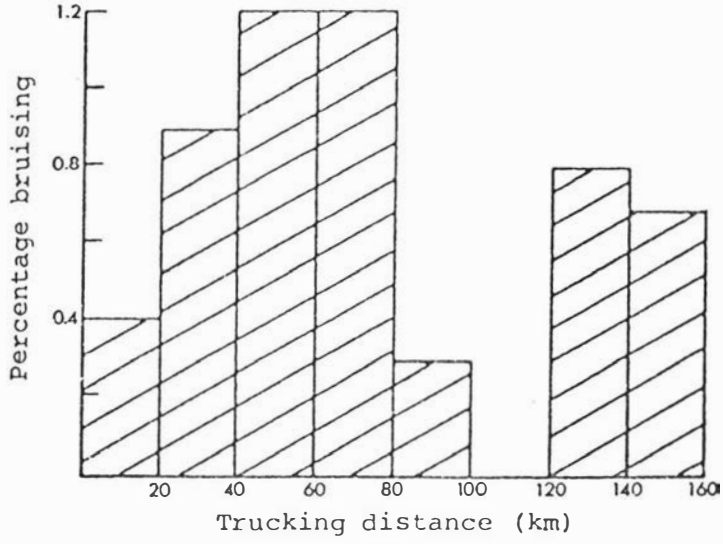


FIGURE 2.11 : Percentage bruising in lambs surveyed plotted against trucking distance to the meat works.

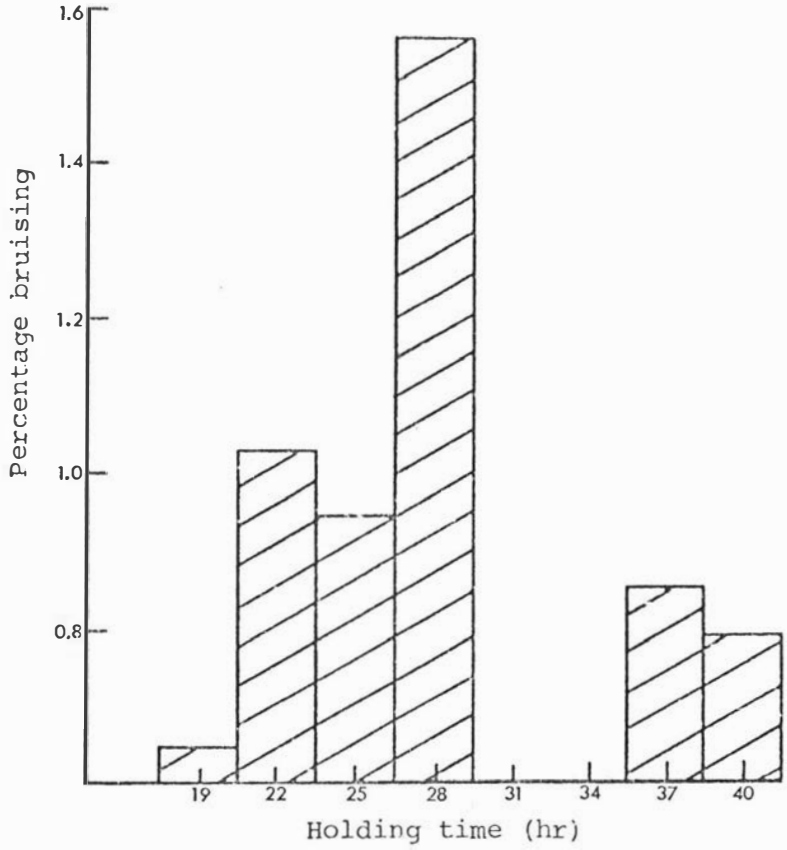


FIGURE 2.12 : Percentage bruising in lambs surveyed plotted against holding time in meat works yards.

1) Lateral hind leg
2) Medial hind leg
3) Rump
4) Back
5) Thorax/shoulder
6) Brisket
7) Distal foreleg
8) Generalised

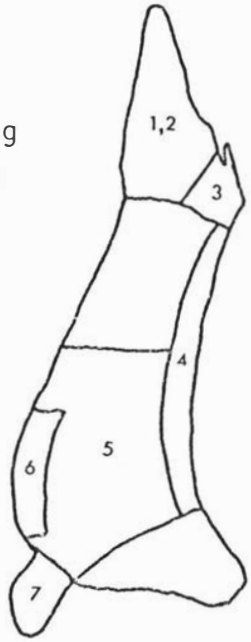


FIGURE 2.13 : Areas into which carcases were divided when classifying bruises according to carcase position.

an accurate adjustment to be made for equal holding time distributions. No comment is therefore made concerning the places of occurrence of bruises affecting specific parts of lamb carcases.

DISCUSSION

I. EXPERIMENTAL ANIMALS

Bruises as they affect lambs, adult sheep and cattle all cause significant economic loss to the New Zealand meat industry (Marshall, 1977; Millar and Glover, 1980). However, for logistical reasons this investigation into the ageing of bruises was restricted to only one class of stock. Lambs were selected as the experimental animal of choice for convenience of handling and because bruising in lambs is a relatively more important source of economic loss than it is in adult sheep (Millar and Glover, 1980). The application of experimental results would therefore be of greater practical significance. The lambs used were healthy male, female or male castrate Perendale or New Zealand Romneys aged from 3-12 months old. It was not considered necessary to control for sex, breed or age since these factors have been shown not to affect histological reactions to injury (Raekallio, 1972; McCausland and Dougherty, 1978).

II. PRODUCTION OF BRUISES

The majority of lambs processed for export in New Zealand meat works are killed within 72 hours of mustering on their farms of origin. It was therefore sufficient to study experimental bruises with a maximum age of only 72 hours. Each animal was bruised six times: once on each limb and twice in the lumbar region. It has been suggested that multiple injury either increases (Hamdy *et al.*, 1957c, 1961a) or decreases (Raekallio, 1965) rates of wound healing, but in both studies the experimental evidence was limited and the results inconclusive. Multiple bruising was therefore considered unlikely to influence tissue reaction rates sufficiently to affect this experiment.

Nevertheless, potential within or between animal variations were minimised by varying both the sites and the ages of bruises inflicted on each animal.

The aim when inflicting bruises was to create lesions similar to those experienced by the meat industry, but of constant severity. To this end all impact bruises were inflicted by dropping a constant weight of 1500g with a striking surface of 0.8cm^2 through a vertical distance of 1.0m. This technique produced bruises similar in severity though less extensive than those seen in practice. Unfortunately it proved impossible to inflict a 'standard bruise' because intrinsic animal factors such as the depth of overlying adipose tissue, the thickness of muscle and the presence of intra or extra-muscular fibrous septae all influenced the final results. Of particular importance was the need to strike muscle directly over bone. Failure to do this resulted in a very mild bruise. The flexibility of the spine made bruising of the lumbar muscles difficult. For this reason lumbar bruising was discontinued and bruises were inflicted only on the limbs.

III. GROSS APPEARANCE OF BRUISES

Most of the experimental bruises were red, although 10% of lesions 48 hours and older showed colour changes similar in sequence to those described in cattle (Hamdy *et al.*, 1957a), i.e. from yellow to green to orange. Occasionally therefore, it is possible to age a bruise by its colour, but since colour changes occur in only a small proportion of bruises this criterion is of limited practical value. All of the 107 bruises sampled in a pilot freezing works survey were red.

IV. HISTOPATHOLOGY OF BRUISES

At first, tissue sections were studied using a battery of stains intended to demonstrate more clearly certain histopathological features of wounds. Most of these stains were progressively discontinued

because they failed to provide useful information, and only H+E and Perl's iron stains were applied to all tissue sections. Expectations and results for special stains are discussed with the histopathological changes they were intended to illustrate.

1. Haemorrhage and fibrin

Extravasated erythrocytes appeared to be removed from bruises primarily by macrophage erythrophagocytosis. This was observed as early as eight hours after injury, but was unsuitable as a criterion of age because its absence was difficult to verify and its extent appeared to reflect both the severity of haemorrhage and macrophage numbers. There was no noticeable depletion of erythrocytes in the older bruises, and since intact red blood cells have been observed in the interstitial spaces of bruises several weeks old (Robertson and Mansfield, 1957), severity of haemorrhage was considered a reliable measure of the severity of bruising irrespective of the duration of injury.

Both the staining characteristics and the morphology of fibrin have been used to age wounds. According to Lendrum *et al.* (1962), recently deposited fibrin stains preferentially with dyes of small molecular size whereas older fibrin stains better with dyes of larger molecular size. Using this principle they developed the Martius scarlet blue (MSB) stain which they claimed would stain fibrin younger than 16 hours old yellow and older fibrin red. Cook (1964) studied fibrin morphology in wounds using Lendrum's acid picro Mallory and Mallory's phosphotungstic acid haematoxylin (PTAH) stains, and claimed to be able to age wounds within broad limits according to the coarseness and general appearance of fibrin strands. In this experiment fibrin was stained with PTAH and MSB stains. In view of the results obtained by Lendrum *et al.* (1962) only bruises from 1-20 hours old were stained with MSB but a wider range of bruises was stained with PTAH (Table 1.3). Neither the age-dependent morphological features described by Cook (1964), nor the yellow staining of fibrin less than 16 hours old noted by Lendrum *et al.* (1962) were observed in our study. These results, which are in accord with those of McCausland and Dougherty (1978), indicate that for bruises neither the morphology nor the staining properties of fibrin are useful indicators of age.

2. Histopathological changes in muscle fibres

Muscle necrosis was observed in 65% of bruises but there was no apparent association between its severity and the age of affected bruises. There appeared to be a morphological sequence of degenerative change in injured fibres, ranging from cell swelling with eosinophilia through to vacuolation, clumping, fragmentation and liquefaction of myotubal contents. However, variation within and between muscle fibres of the same bruise, and between fibres in different bruises of the same age made it difficult to relate the stage of necrosis to age. This was not unexpected, since although muscle fibres do undergo a series of morphological changes following injury (Fishback and Fishback, 1932; Veress *et al.*, 1966; Adams *et al.*, 1967; Carlson, 1973), the rates of change may vary according to the severity of damage (Veress *et al.*, 1966; Josza and Reffy, 1978), and this is rarely uniform even following a standard insult (Benoit and Belt, 1970; Ali, 1979). Furthermore, secondary ischaemic damage may be superimposed on that caused by the initial insult (Hopkinson and Watts, 1973).

Muscle regeneration was not expected to be a prominent feature in the bruises studied because it usually commences 2-4 days after injury (Carlson, 1973). It was observed in only 10% of our bruises 36 hours and older but was not detected earlier than this. When observed, regeneration was therefore a good criterion of age. Regenerative change was mainly characterised by proliferating subsarcolemmal myoblasts ('discontinuous' regeneration). Sarcoplasmic budding ('continuous' regeneration) was also observed, though less frequently. According to Ali (1979), 'discontinuous' regeneration usually follows myonecrosis with sarcolemmal preservation, whereas 'continuous' regeneration predominates when necrosis is accompanied by sarcolemmal disruption. Both types of necrosis occurred in bruises so the apparent predominance of 'discontinuous' regeneration probably reflected the relative ease with which myoblasts, as compared to sarcoplasmic buds, could be detected. Myoblasts can be distinguished by their enlarged oval nuclei with prominent nucleoli, but it is not always possible to differentiate them from macrophages by light microscopy (Carlson, 1973; Reznik, 1973). This inability to confirm the absence of muscle regeneration with confidence by light microscopy

decreased the usefulness of this histopathological feature as a criterion for ageing bruises.

Dystrophic calcification is an early feature of myonecrosis and may in fact be the first light microscopic sign of degenerative change (Oberc and Engel, 1977). A selection of bruises ranging in age from 2-144 hours old were stained with von Kossa's stain (Table 2.3) in order to ascertain the usefulness of calcification as a criterion for ageing bruises. Of the 62 bruises studied, only seven, aged 6, 30, 48, 72 and 144 hours old, showed muscle calcification. Furthermore, in all except the 144 hour old bruises this calcification was very mild and affected only a few necrotic fibres. It was therefore concluded that calcification, though more likely to occur in the muscle of older bruises, was uncommon in lesions up to 72 hours old and was thus of little value for ageing bruises.

3. Fat necrosis

Fat necrosis was not observed in control tissues but it was present in 25% of the experimental bruises. The necrotic foci varied widely in appearance both within and between bruises of the same age and there was no apparent association between their presence and bruise age.

4. The cellular exudates

Two of the more widely applied criteria for ageing wounds are the presence and relative numbers of neutrophils and macrophages in the cellular exudate. Some leucocytes were present in interstitial spaces as components of haemorrhage, but leucocyte numbers in the inflammatory exudates were relatively greater and there was little difficulty in differentiating between active and passive infiltration. This is important in as much as Raekallio (1973) attributed much of the variation in the timing of histological events following injury to differing opinions as to what constituted an active rather than a passive change.

Exudative neutrophils were detected in both 1 hour and 72 hour old bruises so their presence or absence could not be used as a 'cut off' criterion of age. Macrophages, on the other hand, were useful in this respect since they were present only in bruises 4 hours and older. Both the actual and relative numbers of neutrophils and macrophages appeared to alter systematically with age. The extent and composition of the cellular exudate was therefore of use for ageing bruises.

Small to moderate numbers of interstitial eosinophils were observed in 10 out of 20 bruises, aged 1, 2, 4, 8, 16, 20, 24, 30, 48 and 72 hours old. Eosinophils have been observed in acute and chronic inflammation induced by physical trauma (Riddle and Barnhart, 1964; Ordman and Gillman, 1966; Steele and Wilhelm, 1970; Bassett *et al.*, 1977), but in this experiment the sporadic incidence and the wide range of ages over which eosinophils occurred suggested that their presence was incidental.

5. Tissue repair

Fibroplasia was scored according to the reactive morphology of fibroblasts rather than their numbers, since the latter vary according to the severity of injury (Ojala, 1968; Robertson and Hodge, 1972), whereas fibroblast morphology depends on its duration (Ross and Benditt, 1961). Reactive changes were easily discerned by light microscopy even though very few fibroblasts were observed in some bruises. Evidence of fibroplasia was detected in 80% of bruises aged from 24-72 hours old and in 18% of younger bruises. Since only mild changes were observed in bruises less than 24 hours old, this histopathological feature was considered useful for ageing purposes.

With van Gieson stain, newly formed collagen was detected only in bruises more than 72 hours old. These results concur with observations from a variety of wounds involving muscle, adipose tissue and skin, where newly formed collagen has been observed from 3-7 days after injury (Ojala, 1968; Robertson and Hodge, 1972; Raekallio, 1977; Fishbein *et al.*, 1978). On the other hand, newly synthesised collagen

has been detected with van Gieson stain in guinea pig incisions 12-24 hours old (Hirvonen, 1968a, 1968b).

Endothelial hypertrophy was observed in the small blood vessels of 55% of 36-72 hour old bruises and in 7% of bruises less than 36 hours old. These results are in accord with previous observations of wounds affecting muscle, adipose tissue and skin (Hirvonen, 1968b; Sholley *et al.*, 1977; Fishbein *et al.*, 1978; McCausland and Dougherty, 1978). The reactive changes in endothelium were detected approximately 12 hours later than those in fibroblasts. This, together with the fact that the degrees of change were readily assessable, made endothelial hypertrophy a valuable criterion of bruise age. Mitotic figures were uncommon in endothelial cells and because of the mild and diffuse nature of the tissue reaction in bruises, new capillary loops were observed only in a few older and more severe lesions.

6. Haemosiderin

Haemosiderin was detected in 50% of bruises 24 hours or older, and in 3% of bruises less than 24 hours old. Usually it was present in only a few interstitial macrophages, but with Perl's iron stain these cells were easily detected. The presence or absence of haemosiderin was therefore considered a useful criterion for ageing bruises.

7. Mast cells and free histamine

Mast cells are very sensitive to trauma and may degranulate immediately after physical injury, thereby releasing histamine and other vasoactive chemicals into tissue spaces (Cheville, 1976). This has been observed to occur within minutes of applying heat injury to the skin of sheep (Vegad, 1979). Unfortunately, there were too few mast cells in either muscle or adipose tissue to enable the presence of widespread degranulation to be related to age.

Elevations in free histamine concentrations, which reflect the degree of mast cell degranulation, have been shown to occur in skin wounds

aged 10-40 minutes old (Raekallio and Makinen, 1966, 1969a, 1970). These experiments suggested the use of histamine assays for ageing very young bruises. However, a pilot experiment involving three controls and three bruises aged 10, 30 and 60 minutes old (results not included) showed the amounts of free histamine in bruises to be a factor of ten below the sensitivity of the assay technique (Shore *et al.*, 1959) employed. This presumably reflected the small number of mast cells in muscle and adipose tissue. It was considered impracticable to use free histamine concentrations as a means of ageing bruises.

8. Acid glycosaminoglycans

It has been shown, using PAS/alcian blue, Hale's colloidal iron and toluidine blue stains, that acid glycosaminoglycans in bruises and incisions vary in amount according to the age of the wound (Raekallio, 1961; Hirvonen, 1968a; Nevelos and Gee, 1970). Control tissues and a selection of bruises of various ages (Table 2.3) were therefore stained with PAS/alcian blue, which is reputedly one of the most sensitive histochemical stains for acid glycosaminoglycans (Lendrum, 1969). However, the amounts detected in bruises were too small to allow differences to be assessed. Attempts to measure changes using Hale's colloidal iron stain were similarly unsuccessful. This approach to the ageing of bruises was therefore abandoned.

In conclusion, the following histopathological features were considered likely to be of use for ageing bruises: the neutrophil and macrophage exudates, fibroplasia, endothelial hypertrophy, haemosiderin in macrophages, muscle regeneration and possibly muscle necrosis.

V. MATHEMATICAL METHODS AVAILABLE FOR PROCESSING HISTOPATHOLOGICAL DATA

When histopathological data are used to age wounds subjectively there are three potential sources of variation, giving rise to sampling,

observational and interpretational errors respectively. The latter two are responsible for most of the discrepancies between results recorded in the literature (Raekallio, 1977). Observational errors can be reduced by measuring histopathological changes objectively, but the techniques are exacting and for routine application impracticable (Pearse, 1968; Pette *et al.*, 1979). Errors of interpretation can be eliminated by using mathematical models to interpret data objectively (Rogers *et al.*, 1979), and this procedure is relatively straightforward.

Most of the mathematical models available for interpreting biological data are capable of handling ordinal scores (i.e. data ranked subjectively rather than actually measured). In fact, it is often easier to deal with this type of information (Baily, 1965). The histopathological changes in bruises were therefore scored semi-quantitatively on a subjective scale of 1-5. The mathematical models in current use are of two basic types: statistical and logical (Rogers *et al.*, 1979; Sher, 1980). The operational principles of each will be discussed in general terms, and the models of choice then discussed in detail.

1. Logical models

Given a set of data, logical models, also called 'decision tree' models, progress to a diagnosis deterministically by leading the investigator through a series of predetermined decision points which are usually binary (Hirschfeld *et al.*, 1974). These models thus mimic the process of differential diagnosis (Sher, 1980). Their two main advantages are that they do not have to be based on large amounts of data and they can be used to differentiate between a large number of alternatives, many of which may be poorly defined (Altman *et al.*, 1976). For this reason they are popular in psychiatric medicine. The disadvantages with these models are that although they can differentiate between many alternatives, they make a single choice with no indication of the relative likelihood of that particular choice being correct (Hirschfeld *et al.*, 1974).

2. Statistical models

Statistical models operate by mathematically analysing the structure of a reference sample in order to determine how best to categorise a new observation presenting a given combination of characteristics. They are more popular than their 'logical' counterparts, partly because they are relatively simple to apply and understand and partly because the type of data upon which decisions are made often exhibit a high degree of variability, which statistical models are well equipped to handle (Anderson and Boyle, 1968). Three characteristics of statistical models stand in contrast to those of logical models:

(i) The data upon which they are based must be reasonably extensive, since it must be analysed mathematically;

(ii) They are most successful when used to differentiate between relatively few (2-10) clearly defined alternatives. Of great advantage, however, is their ability to calculate the likelihood of each proposed alternative. A measure of certainty is thus attached to each decision. This attribute can be developed into considerations of the relative importance of mistaken decisions, and to provide the option for a 'non-decision' should the degree of risk exceed that stipulated by the operator;

(iii) In general the statistical models are easy to design and apply, especially with the ready availability of computer packages (Anderson and Boyle, 1968; Altman *et al.*, 1976; Rogers *et al.*, 1979; Sher, 1980).

There are four kinds of statistical models, namely Bayesian probability, discriminant function analysis, matching and regression analysis.

(a) Bayesian probability model: The Bayesian probability model is one of the more popular models, being both widely applicable, straightforward and statistically robust (Bailey, 1965). It uses the frequencies with which individual events occur in a population to calculate the probability that a given combination of events will occur. Data

can be subjective, objective or both, and a large number of symptoms may be considered either all at once or in a stepwise manner according to the calculated usefulness of each (Knill-Jones *et al.*, 1973). It is the most common model used to deal with histological data (Rogers *et al.*, 1979).

(b) Discriminant function analysis: Discriminant function analysis was pioneered by Fischer in 1929, and is most commonly used in numerical taxonomy. A series of attributes that characterise an object or alternative are considered as existing in a multidimensional space. They can thus be visualised and described mathematically as clusters, and each cluster, though it may overlap to a variable extent with other clusters, denotes a particular alternative. From considerations of the means and variances of sets of cluster in relation to each other, a new set of attributes can be assigned a score and according to the score's magnitude the set of attributes can be ascribed to a particular alternative (Anderson and Boyle, 1968). In its general approach discriminant function analysis is thus similar to the Bayesian probability model.

(c) Data matching models: Data matching models use the 'nearest neighbour' approach. In their simplest form they match a set of data as closely as possible to sets of data comprising a reference sample. However, considerably more sophisticated variants exist, in which symptom comparisons are weighted according to their diagnostic importance, and final decisions based on composite scores (Rogers *et al.*, 1979). The main drawback with matching techniques is the large reference sample needed to make valid comparisons.

(d) Regression analysis: Given values for one or more independent variables, regression equations can be calculated to estimate the value of a dependent variable. The equations may be linear or non-linear, simple or multiple, and they are fitted by maximising the proportion of the variance of the dependent variable explained by the independent variables of interest (Nie *et al.*, 1975). Regression analysis is popular for several reasons, not the least of which is the fact that the independent variables are weighted according to their usefulness. However, the equations are calculated on the following

assumptions:

(i) The data is measured rather than subjectively assessed;

(ii) The data is bivariate normal in distribution;

(iii) The variance of the dependent variable is the same for every value of the independent variable.

Biological data often fail to fulfil these conditons, but the Pearson correlation coefficient (r) upon which the equations are based is statistically robust and the usefulness of regression analysis has been demonstrated despite theoretical non-compliance of data (Munford, *personal communication*).

The superiority of one type of model has not been unequivocally demonstrated (Rogers *et al.*, 1979). When the appropriate variables have been controlled all models have performed similarly and it has been suggested that the type of data rather than the type of model has the greatest influence on the results. Either the Bayesian probability model or discriminant function analysis were considered theoretically most suitable for the amount and type of data available for ageing bruises. Of these, the former was favoured because of the following two assumptions inherent in applying discriminant analysis:

(i) The data base should be large so that stable discriminant functions can be calculated;

(ii) The distribution of the data should be multivariate normal with equal covariance matrices (Nie *et al.*, 1975).

For the above reasons Anderson and Boyle (1968) recommended the use of the Bayesian model rather than discriminant analysis when dealing with discreet data (such as our semi-quantitative scores).

VI. ESTIMATING THE AGES OF BRUISES MATHEMATICALLY USING SCORES FROM SEVERAL HISTOPATHOLOGICAL PARAMETERS

Two mathematical models, the Bayesian probability model and a multiple linear regression equation, were used to estimate the ages of bruises objectively, using scores from the selected histopathological features listed in results. The Bayesian probability model was chosen for its simplicity, flexibility and suitability for histological data, whereas the multiple linear regression equation was tested because of its widespread use and proven success with biological data (Nie *et al.*, 1975).

It is a basic precept when using mathematical models for biological classification that they first be 'developed' using data from a reference sample, then 'tested' on new data (Oyama and Tatsuoka, 1956; van Woerkom and Brodman, 1961; Anderson and Boyle, 1968; Boyle and Anderson, 1963; Knill-Jones *et al.*, 1973; Sher, 1980). It is impossible to evaluate the performance of a model realistically without so doing, and not to do so may result in overestimating its performance by as much as 20% (Hirschfeld *et al.*, 1974). Nonetheless, a model may be tested back on itself provided the aim is to assess its potential, as opposed to its actual, performance (Rogers *et al.*, 1979).

Available data were therefore divided into two groups: a reference sample for developing the models and assessing their potential performances, and a testing sample for assessing the real worth of the best model. The reference sample consisted of data from one tissue section selected at random from each of 20 bruises per age category, i.e. 120 tissue sections. The remaining data formed the test sample. Data from muscle and adipose tissue were considered separately because both tissues are not always affected in practice, and combining data would eliminate these bruises from consideration.

1. Selection of histopathological features

The histopathological features considered for use when objectively ageing bruises (Figure 2.2) were those conventionally used to age wounds subjectively (Raekallio, 1977). Their scoring on a scale of 1-5 was necessarily imprecise, but the application of Bayes' equation to such data is non-controversial (Colquhoun, 1971). As far as possible the scoring was unbiased, and to this end bruises were scored without knowledge of their ages. Only macrophage/neutrophil ratios were objective measurements, having been calculated from cell counts. Often, when quantitatively scoring histological changes, a grid pattern system is adopted to ensure that tissue sections are studied on a random basis (Bassett *et al.*, 1977). Though desirable, this approach would have been unsatisfactory for bruises because the tissue reactions were frequently mild and confined to small areas within tissue sections. Scoring was therefore based on an evaluation of the entire sections. Scores were assigned according to the degree rather than the extent of change, e.g. scores for leucocytes depended on the density of the cellular exudate rather than on overall numbers of cells. The extent of damage was assumed to reflect primarily the severity of injury rather than its duration, though it was realised that the early stages of reactive change are self propagating (Ryan, 1977) and that for muscle in particular, ischaemic damage may become superimposed on that directly due to the initial insult (Hopkinson and Watts, 1963). Histopathological features with scores significantly affected by the severity of injury were eventually identified statistically by controlling for severity of injury in chi-square tests.

Bruises had to be re-aggregated from fourteen to only six age categories (i.e. 1-8, 12-20, 24-36, 48, 60 and 72 hours) since it became obvious that they could not be aged more accurately than this. At the same time numbers of bruises were increased so that for muscle and adipose tissue considered separately there were data from at least 20 bruises per age category. Extra data acquired whilst perfecting experimental techniques or from enzyme studies were also included in the data base since equality of numbers was not a prerequisite for the methods of analysis eventually used.

Histopathological features were selected according to the degree and validity of associations between their scores and bruise ages. The selection process was effected by carrying out two sets of chi-square analyses on data from tissues with histological evidence of haemorrhage. Tissues without haemorrhage were excluded from analysis because haemorrhage was considered the fundamental criterion of a bruise.

When features of potential use for estimating bruise ages were selected, all valid data were used to construct contingency tables relating scores and bruise ages. All data had to be used, without regard to possible bias from multiple sampling, in order to fulfil the chi-square analysis requirement that not more than 20% of table cells should contain less than five data units each (Nie *et al.*, 1975). Even so, it was necessary to combine score categories 2-5 for haemosiderin and categories 4 and 5 for the other variables. The significance levels of chi-square tests performed on those variables not already excluded because of insufficient data are shown in Table 2.4. Scores for haemorrhage and tissue disruption were included as controls since they were expected not to be associated with age. The significance levels calculated for haemorrhage were of particular interest because both the magnitude and possibly the time-course of a tissue reaction may be affected by the severity of injury (Steele and Wilhelm, 1970), and scores for haemorrhage were presumed to reflect this factor. Also, extravasated blood can itself induce a tissue reaction indistinguishable from that in bruises. This had been shown by a small experiment (results not recorded) whereby autologous blood had been injected into lambs and tissues examined 16, 48, 120 and 144 hours later. It had to be assumed therefore, that haemorrhage would itself account for part of the tissue reaction in bruises. For this reason it was disturbing to find a significant association between age and scores for the haemorrhage in muscle, since it raised the possibility that the significant associations between age and the remaining muscle parameters were spurious: merely reflecting the relationship between muscle haemorrhage and age. Three things had to be considered in evaluating this possibility:

(i) With large samples, statistical significance does not necessarily imply a substantive relationship (Finch, 1978);

(ii) The interpretation of statistical results must always be with reference to the knowledge of the investigator, i.e. statistical significance suggests but does not dictate a conclusion (Colquhoun, 1971);

(iii) There was no reason to assume a real relationship between muscle haemorrhage and age in the absence of a significant parallel relationship between haemorrhage in adipose tissue and age ($P<0.21$).

Nevertheless, it was considered necessary to construct a second set of contingency tables relating scores to bruise ages, this time controlling for the direct effects of haemorrhage (Tables 2.5 and 2.6). In this way it was shown that except for muscle and fat necrosis, the relationships between bruise ages and scores for the features in Table 2.4 were valid despite the confounding influence of haemorrhage.

2. Developing the Bayesian probability model

The Bayesian probability model was developed by calculating the frequencies with which scores for fibroplasia, endothelial swelling, haemosiderin and either the macrophage and neutrophil exudates or macrophage/neutrophil ratios occurred in the reference sample. These frequencies, and Bayes' equation, were then used to test the model back on itself in order to empirically tailor it to its best performance, i.e. select the best combination of histopathological features and the most efficient score categories to use, and the best age categories into which to classify bruises. The developing process is discussed progressively under subheadings: (a) data base, (b) selection of indicants used for estimating ages, (c) age classification.

(a) Data base: The choice of 20 bruises per age category for the reference sample was to some extent arbitrary and was partly dictated by experimental practicability. A large reference sample is desirable since frequencies of observation can be calculated more reliably (Finch, 1978). However, Bailey (1965) developed a successful Bayesian probability model using a reference sample of less than 100 cases to distinguish between 12 disease categories, and Altman *et al.* (1976) found that increasing the reference sample for his model resulted in only a 1% improvement in performance. Ledley (1965) has shown that conditional probabilities calculated on 20 or more observations are stable. Therefore 20 bruises per age category was considered an adequate reference sample for our model.

(b) Selection of indicants used for age estimation: The variables used in Bayes' equation should, in theory be statistically independent of each other, but with biological data this requirement is rarely met (Hirschfeld *et al.*, 1974). Statistical non-independence can be adjusted for mathematically, but the computations are such that unless a large reference sample is available the results are unreliable for predictive purposes. With the reference samples usually available it is therefore recommended that the assumption of independence be disregarded unless it is strongly violated (Anderson and Boyle, 1968; Colquhoun, 1971). Not to do so may be counterproductive (van Woerkom and Bradman, 1961). Fryback (1978) has shown that for small samples the performance of the Bayesian model is 'maximised' by using a small number of the most informative variables and ignoring statistical interdependence.

Five histopathological variables were therefore used when estimating the ages of bruises. Their statistical independence was not formally evaluated and the only precaution in this respect was not to include scores for macrophage/neutrophil ratios in the same equation as those for the macrophage and neutrophil exudates considered separately.

The term $P(D_j)$ in Bayes' equation is the probability of sampling a bruise of a particular age by chance. These probabilities could have been calculated for both the reference and test samples, but since they

cannot be calculated in practice they were assumed equal. This approach is valid and not likely to decrease the performance of the model appreciably (Bailey, 1965; Altman *et al.*, 1976).

(c) Age classification and the potential performance of the model: In order to maximise the performance of a model, the alternatives between which it must distinguish should be clearly defined (Rogers *et al.*, 1979). For this reason bruises were aggregated into discreet rather than continuous age categories, i.e. 1-8, 12-20 hours etc. In this way data were more clearly polarised, thus enhancing their predictive worths. In so doing, however, bruises of between-category ages were artificially forced into one or other adjacent age categories. This was a source of error, but errors in ageing bruises at interfaces are unavoidable and the prime concern with the Bayesian model was to have clearly defined score/age relationships.

Using Bayes' equation, the probability that a combination of scores represented a bruise of a given age (i.e. $P(D_j|S_c)$) was calculated for each age category in turn, and the age of the bruise was taken as being the category assigned the highest probability value. This probability was not a measure of the 'accuracy' of the model; it was a measure of how 'sure' the model was of itself when ageing the bruise (Hirschfeld, 1974).

The 'accuracy' of the model could have been increased by ageing only those bruises in which $P(D_j|S_c)$ was greater than a predetermined arbitrary figure (Collen *et al.*, 1964; Anderson and Boyle, 1968; Knill-Jones *et al.*, 1973). However, this would have meant rejecting some bruises from consideration and for our model it was empirically determined that the loss of information was out of proportion to the gains in 'accuracy'. All bruises were therefore aged, regardless of the magnitude of the probability assigned to the age category of choice.

The model demonstrated only a limited ability to assign bruises to one of six age categories (Table 2.12). The six age categories were therefore re-aggregated into two, i.e. 1-20 hours and 24-72 hours old, and the bruises placed into one of these according to the magnitudes of

the combined probabilities for each group. Combining probabilities in this way is theoretically valid (Knill-Jones *et al.*, 1973). The model was also redesigned so that bruises were aged directly as either 1-20 hours or 24-72 hours old. However, greater 'accuracy' was obtained if bruises were first placed into one of six age categories then re-aggregated, so this was the method adopted. When using separate scores for macrophage and neutrophil exudates 3% of bruises could not be aged because their age probabilities were equal. With macrophage/neutrophil ratios the equivalent figure was 12%, and since the use of ratios did not enhance the performance of the model (Table 2.12) their use was discontinued.

3. Scoring variation

Histopathological features were scored subjectively, and as such, scoring variation was expected. For this reason muscle for the 120 bruises comprising the reference sample were re-scored. As is evident from Table 2.15 the repeatability of scoring was poor. The new scores usually deviated by ± 1 from the originals. However, when bruises were aged using combinations of the new scores the 'accuracy' of the model changed by only 5%. It was therefore concluded that although individual scores frequently differed from the originals, the net effects of scoring variation were slight when sets of scores were used, and the 'accuracy' of the model was not limited by scoring error.

4. Developing the multiple linear regression model

A linear multiple regression equation was fitted to the same data used to develop the Bayesian probability model. The equation derived was then tested back on itself in order to assess its maximum potential 'accuracy'. Theoretically the type of data was inadequate for regression analysis, but in practice this model has been shown to work despite violations of statistical assumptions. The Bayesian probability model was compared with regression analysis because the technique is relatively simple to apply and is often successful. Of

the other statistical models available for comparison, discriminant analysis was unsuitable for the type and amount of data available, and matching models require large amounts of data.

The 'accuracies' of the regression equation and Bayesian models are compared in Table 2.18. When used to place bruises into one of six age categories, regression analysis was markedly inferior to the Bayesian model. When used to age bruises as either 1-20 hours or 24-72 hours old, however, its performance was comparable. Nevertheless, the Bayesian model was considered the model of choice, mainly because of its theoretical suitability for the type and amount of data available. Violations of the theoretical assumptions made in deriving the regression equation would render this approach less reliable with small samples of new data.

5. Assessing the realistic 'accuracy' of the Bayesian probability model

In all, there were 271 muscle sections representing 145 bruises, and 339 adipose tissue sections representing 148 bruises. For both muscle and adipose tissue considered separately, one tissue section from each of 120 bruises had been used to 'develop' the Bayesian probability model. It is a fundamental principle that a mathematical model be tested on data other than that used to develop it (Rogers *et al.*, 1979), and the remaining tissue sections formed a convenient pool of such data. For muscle there were 151 sections representing 79 bruises, of which 62 had at least two slides per bruise; for adipose tissue there were 219 tissue sections representing 117 bruises, of which 80 had at least two slides per bruise. This test sample was acquired mainly by multiple sampling rather than the production of extra bruises both for logistical reasons and to provide information about the effects of within-bruise variation on the accuracy of ageing. The validity of this approach rests on whether or not within-bruise score variations were of similar magnitude to score variations between bruises of the same age. A series of Kruskal-Wallis analyses of score variances (Siegel, 1956) had shown that in a statistical sense

samples belonged to one of only two bruise populations: either 1-20 hours or 24-72 hours old (results not recorded). It was therefore theoretically acceptable to consider each tissue section as independent and to pool data regardless of multiple sampling. The realistic 'accuracy' of the Bayesian probability model so assessed is compared in Table 2.21 to its maximum potential 'accuracy'. The decreased performance with the test data was expected because statistical models usually perform best when applied to the reference data to which they have been tailored (Fleiss *et al.*, 1972). Nevertheless, at 85% and 81% for muscle, and 92% and 75% for adipose tissue, the 'accuracy' of the model in ageing 1-20 hour and 24-72 hour old bruises was as good as that reported for other histopathological data (Rogers *et al.*, 1979). Inspection of Tables 2.10, 2.11, 2.19 and 2.20 show that the greatest sources of error, both when developing and testing the model, were in ageing 24-36 hour old bruises, i.e. those near the age category interface.

The possible advantages of duplicate within-bruise sampling were investigated by examining bruise age estimates from 61 paired muscle only samples, 80 paired adipose tissue only samples and 118 paired muscle and adipose samples. The respective 'accuracies' of the Bayesian probability model operating under these conditions are shown in Tables 2.22 and 2.23. Small increases in the 'accuracy' of ageing were gained by duplicate sampling of either muscle or adipose tissue considered separately, but in so doing 18% of bruises had to be rejected from consideration because of a lack of concordance between age estimates. A greater increase in 'accuracy' was achieved by considering muscle and adipose tissue together, but in doing so it was still necessary to reject 13% of the tissue pairs because of a lack of concordance. Bruises were thus aged with greatest 'accuracy' by the Bayesian probability method when age estimates were based on concordances between results from more than one tissue sample. Such gains in 'accuracy' were, however, offset by the necessity of rejecting a proportion of bruises from consideration because of a lack of concordance between age estimates from appropriate sample pairs. Whether or not the extra work entailed in processing more than one sample per bruise would be justified would depend on the purpose for

which bruises were to be aged.

For the meat industry the main concern would be to examine as many bruises as possible to establish population trends. In this situation gains through increasing the 'accuracy' of ageing by considering more than one sample per bruise would be offset by the logistical need, in so doing, to limit the number of bruises considered in any one study. On the other hand, when ageing an individual bruise for forensic purposes, or when investigating a specific bruising problem within the meat industry, it would be important to age each bruise as 'accurately' as possible. In such cases the extra workload would be justified and more than one sample should be considered from each bruise.

6. Validation of the Bayesian probability model

The Bayesian probability model would be of little practical value if its anticipated 'accuracy' was less than that achieved after ageing bruises interpretively. A comparison of results after mathematical and interpretive ageing is therefore presented in Table 2.24. Mathematical ageing was more 'accurate' than, and therefore superior to, interpretive ageing. This is particularly true bearing in mind the fact that the performance of the Bayesian probability model could have been increased still further, albeit at the cost of having to discard data, by considering only those age estimates with probability values $P(D_j|S_c)$ for age estimates exceeding predetermined arbitrary values.

7. The 'confidence' of ageing bruises of unknown ages by application of the Bayesian probability model

The performance of a mathematical model as applied to biological data can be assessed in four ways, each of which is expressed in terms of the table below (Ransohoff and Feinstein, 1978).

ESTIMATED AGE OF BRUISE	ACTUAL AGE OF BRUISE		TOTAL
	1-20 hours	24-72 hours	
1-20 hours	a	b	a+b
24-72 hours	c	d	c+d
	a+c	b+d	N

(a) 'Accuracy' in detecting 1-20 hour old bruises $= \frac{a}{a+c}$

(b) 'Accuracy' in detecting 24-72 hour old bruises $= \frac{d}{b+d}$

(c) 'Confidence' of ageing 1-20 hour old bruises $= \frac{a}{a+b}$

(d) 'Confidence' of ageing 24-72 hour old bruises $= \frac{d}{c+d}$

In practice performance is usually expressed as percentages of correct to attempted diagnoses, i.e. a/a+b and d/c+d (Rogers *et al.*, 1979). These terms have been referred to as the 'confidence' of ageing in this study. 'Confidence' values vary according to the relative numbers of bruises actually in each age category (a+c, b+d), but fortunately its values can be calculated from knowledge of the 'accuracy' of the mathematical model and the apparent number of bruises of each age (a+b, c+d). It is therefore possible to estimate the degree of certainty with which individual bruises collected in a survey have been aged. This information is useful when determining the origins of bruises by comparing their estimated ages to times of occurrence of events of interest such as trucking, washing etc.

VII. PRACTICAL APPLICATION OF THE BAYESIAN AGEING METHOD TO BRUISES IN A PILOT MEAT WORKS STUDY

The pilot meat works survey was undertaken in order to assess the practical usefulness of the Bayesian probability method for ageing bruises. To this end the bruises were aged in relation to holding times in meat works yards, thus classifying them as having been inflicted either after or prior to arrival at the works. Additional epidemiological data were collected during the survey in order to permit a comparison between the ageing and more traditional inferential approaches to a problem of this kind.

The survey was conducted under normal working conditions in an export meat works. Only bruises severe enough to require trimming under Ministry of Agriculture and Fisheries supervision were collected, since it was these lesions that caused carcase downgrading.

The 'confidence' with which individual bruises can be aged histologically depends both on the tissue considered (i.e. muscle or adipose tissue or both) and the age distribution of the bruises collected during the survey. The latter factor is subject to chance but a degree of control can be exercised over the former. In order to maximise the 'confidence' of ageing, both muscle and adipose tissue were considered. Nevertheless, 67% of bruises had to be aged according to one set of data only, due either to a lack of concordance between age estimates or because only one tissue was affected. Despite this, 94% of bruises were aged with a 'confidence' level in excess of 80%, which suggested the potential usefulness of the Bayesian ageing method in practice.

The value of the method must ultimately be measured in terms of its ability to age bruises in relation to times of occurrence of predetermined events of interest. In this survey bruises were considered in relation to holding times in meat works yards. In so doing, inherent deficiencies in the ageing approach became apparent. These arose from the limitation of the Bayesian method in being able

to age bruises only as either 1-20 hours or 24+ hours old. One of six situations may arise after bruises have been aged as such in relation to holding times (Table 2.28), and of these only two permit bruises to be classified according to place of origin. A varying proportion of the data processed for each future survey would therefore become redundant. Fortunately the amount of data thus wasted can be reduced by excluding from consideration those bruises collected from animals with holding times from 21-23 hours. In this way the efficiency of the ageing method in terms of the input required in order to draw valid conclusions can be raised to an acceptable level. A further problem arose from the fact that bruises could only be classified as having occurred within the meat works when holding times were >24 hours and as having occurred prior to arrival at the works when holding times were <20 hours. The proportion of bruises classified according to the place of origin was therefore influenced by the distribution of holding times of the lambs from which the bruises were sampled. It is possible, as was done in this survey, to adjust the results retrospectively from knowledge of the distribution of holding times. In future surveys, however, it would be more appropriate to ensure an equal distribution of holding times represented before processing the data.

Additional epidemiological data were collected in order to evaluate the usefulness of the Bayesian ageing method in relation to that of formerly available investigative techniques. The between-farm clustering of bruises (Table 2.29) strongly suggested the action of sporadic and therefore potentially identifiable incidents affecting individual mobs of lambs, but with no indication as to their nature. Had bruises been inflicted predominantly either prior to or after arrival at the meat works, it was reasonable to expect a relationship to be manifest between the prevalence of bruising and either trucking distances or holding times respectively. Such relationships were not observed. This may indicate that the majority of bruises were inflicted at a single point in time, e.g. whilst loading or unloading, during washing, immediately prior to slaughter etc. On the other hand, it may reflect the small sample size of the survey, thereby exposing a relative insensitivity in the inferential as compared to

the more direct ageing approach to this problem.

A total of 73% of bruises affected either the fore or the hind limbs of lamb carcases. From their distribution, extent and severity, these bruises appeared to have been inflicted by a prolonged insult such as the trapping of limbs between two surfaces. If so, the distinctive nature of this type of insult and the characteristic positions of the resulting bruises would provide a useful additonal means of investigating their places of origin without the need to first determine their ages. This aspect of the problem was not specifically explored.

The pilot survey demonstrated the practicability of the Bayesian ageing method in determining the likely places of origin of bruises observed in lambs processed for export. Within limits imposed by its ability to age bruises only as either 1-20 hours or 24+ hours old, the potential worth of the method in this respect appeared to exceed that of formerly available epidemiological techniques, which suffer from being primarily inferential in nature and thus dependent on larger numbers of observations than are required by the ageing approach. The most serious drawbacks with the latter are the variations in the levels of 'confidence' with which individual bruises can be aged and in the proportions of data made redundant after processing. Even with these factors operating to decrease its efficiency, however, the ageing method is likely to prove useful in practice.

VIII. WOOL PULL BRUISES

Experimental wool pull bruises aged from 1-72 hours old were inflicted by grasping a tuft of wool firmly in a hand-vice and vigorously shaking the animal. The severity of this insult exceeded that usually experienced in practice but the lesions so produced were nevertheless mild.

Grossly, the wool pull bruises could be distinguished from impact bruises by their superficial nature and 'flared' appearance. From available evidence though, the sequence and timing of the tissue reactions in bruises was not expected to alter significantly according to the type of injury *per se* (Hirvonen, 1968b). The Bayesian probability model developed for impact bruises was therefore applied to data from the wool pull bruises. The 'accuracy' of ageing 1-20 hour old bruises was 100% but it was only 57% for 24-72 hour old bruises. This poor performance appeared to reflect the lack of assurance with which very mild histopathological changes could be scored, and since wool pull bruises are characterised by mild reactions, histological ageing was deemed unsuitable for this type of lesion. Fortunately, wool pull bruises are becoming less common in lambs processed for slaughter, as was substantiated by the fact that only 5/107 bruises in the previously considered survey were suggestive of this type of lesion. The inability to age the bruises mathematically was therefore of little practical concern.

CHAPTER III

ENZYMIC ACTIVITY IN BRUISED MUSCLE

MATERIALS AND METHODS

The histochemical and isoenzymic activities of the following ten enzymes were examined in bruised muscle: acid phosphatase, aldolase, alkaline phosphatase, aminopeptidase, creatine kinase, glucose-6-phosphate dehydrogenase, lactate dehydrogenase, myosin adenosine triphosphatase, non-specific esterase and succinate dehydrogenase. For logistical reasons the investigations were organised into two separate experiments, identical in format but each involving only five enzymes. In these experiments one impact bruise was inflicted, in the manner previously described, on each of the limbs of four lambs. Each lamb was bruised 4, 24, 48 and 144 hours before death. The experiment was so designed that each limb was represented by a bruise of a different age. Immediately after slaughter by exanguination, the bruises were excised, assigned code numbers and frozen in liquid nitrogen. Part of each bruise was then removed to a cryostat for sectioning. The remaining tissues were stored at -70^{o}C until the completion of the enzyme histochemistry part of the experiment, after which time they were processed for isoenzyme studies.

1. Enzyme histochemistry

Eleven frozen sections of 10μm thickness were cut from each bruise. Five of these were used for demonstrating the activity of the five enzymes under investigation, five were used as the appropriate controls and the remaining section was stained with H+E. The treatment of the sections depended on the enzyme studied. Details for each enzyme arranged alphabetically are presented in appendix. Relevant light microscopic features associated with each enzyme were scored subjectively for each bruise as -, +, ++ or +++ according to the degree of

enzyme activity. Features of particular interest were photographed.

2. Isoenzyme studies

(a) Preparation of tissues: The frozen tissues were thawed and visible adipose tissue removed. The bruised muscle was then weighed, cut up finely with scissors, placed in a 1:5 w/v solution of 0.1% Triton X-100 in distilled water and homogenised in an ice bath for 3 minutes, using a Sorvall Omnimixer. Thereafter, the enzyme solutions were maintained at a temperature of $4^{o}C$ or lower. The homogenates were first centrifuged for 30 minutes x 4,000g in order to precipitate the coarse sediment, then re-centrifuged for 30 minutes x 15,000g. The final supernatant was dialysed for 24 hours in 100 volumes of 1% glycine, centrifuged for 30 minutes x 15,000g then freeze dried. The lyophilised cell extracts were reconstituted with distilled water to one tenth of their pre-freeze drying volume, thus effecting a 2-fold concentration of tissue enzyme. The reconstituted solutions were centrifuged for 5 minutes x 3,000g then dispensed as 200µl volumes into plastic vials for storage at $-20^{o}C$ until required. Heart, liver and small intestines, which served as control tissues, were prepared in the same way as bruised muscle. A pooled sample of 20ml of blood per animal had been collected and mixed with a glucose/citrate anticoagulant. This blood was centrifuged for 10 minutes x 3,000g in order to separate the plasma, buffy coat and erythrocytes. The plasma and erythrocyte fractions were freeze/thawed three times, dialysed as for muscle and freeze dried. The plasma was reconstituted to one tenth of its pre-freeze drying volume with 0.1% Triton X-100 in distilled water, but the erythrocyte fraction could only be reconstituted to its original volume. The buffy coat cells were washed three times in 0.9% phosphate buffered saline pH 7.4 (PBS) and resuspended to approximately $600x10^{9}$ cells/litre. They were then sonicated for 30 seconds, dialysed as for muscle, freeze dried and reconstituted to one twentieth their pre-freeze dried volume in 0.1% Triton X-100. The leucocytic enzymes were thus concentrated to approximately 1,000 times their levels in whole blood.

(b) Isoelectric focussing: Isoenzymes were separated by isoelectric focussing in polyacrylamide gels, using a 30 watt constant power generator (LKB* 1203), a multiphore horizontal electrophoresis unit with cooling plate and electrode attachments (LKB 2117-301) and Ampholine PAGplates, either pH 3.5-9.5 (LKB 1804-101) or pH 4.0-6.5 (LKB 1804-102). Immediately prior to each run, the required number of samples were thawed and absorbed onto squares of blotting paper which were placed on the gel. For pH 3.5-9.5 gels the anodal and cathodal buffers were 1.0M phosphoric acid and 1.0M sodium hydroxide respectively; for the pH 4.0-6.5 gels the corresponding buffers were 0.1M glutamic acid/0.5M phosphoric acid and 0.1M β-alanine. A constant power of 25 watt was applied to each gel for a period of 1.5 hours for the pH 3.5-9.5 gels and 2.5 hours for the pH 4.0-6.5 gels. The squares of blotting paper were removed 30 minutes after application of the current. At the completion of the run the edges of the gels to which the electrodes had been applied were trimmed off and the isoenzyme patterns for each enzyme stained by the appropriate chromogenic technique as specified in appendix. After the gels had been incubated for the required period in substrate medium they were fixed and preserved by immersion for 30 minutes in an aqueous solution of 8% glacial acetic acid/10% glycerol/25% ethanol. This fixative denatured the enzymes, thereby halting the chromogenic reaction and preventing further diffusion of the isoenzyme bands. Each gel was photographed and, if necessary for analysis, scanned with a 'Quick Scan Flur Vis' integrating densitometer (Helena Laboratories**). The scanned gels were overlayed with plastic for storage.

Creatine kinase activity could not be demonstrated with sufficient clarity in polyacrylamide gels. Its activity was therefore demonstrated after electrophoresis in 'Titan III' cellulose acetate plates (Helena Laboratories). Six 0.25μl samples were placed on each of three plates

* LKB-Producter AB, Box 305, S-161 26 Sweden.

** Helena Laboratories, P.O.Box 752, Beaumont, Texas.

resting on electrode wicks standing in 0.06M sodium barbitol/0.06% mercaptoethanol buffer pH 8.6. Electrophoresis was conducted at 250 volts for 90 minutes. Enzyme activity was visualised using the chromogenic method outlined in appendix.

The zymograms of each of the enzymes studied were evaluated visually and, for complex patterns, with the aid of densitometric tracings.

RESULTS

I. ENZYMES OF THE GLYCOLYTIC PATHWAY

The histochemical activities and isoenzyme compositions of two glycolytic enzymes, aldolase and lactate dehydrogenase, were demonstrated and the results evaluated as previously described.

1. Aldolase

(a) Enzyme histochemistry: A summary of visually assessed enzyme activities in bruised muscle is presented in Table 3.1. Normal muscle fibres stained positively for aldolase, with an expected variation in intensity according to fibre type (Figure 3.1). The enzyme is associated with mitochondria and the punctate staining of fibres reflected this fact. There was both an absence of staining and enhanced staining in different necrotic muscle fibres (Figure 3.2). The enhanced staining was interpreted as mitochondrial rather than cytoplasmic. No association was detected between the intensity of staining, the severity of necrosis or the age of the bruise.

The leucocytes associated with areas of frank haemorrhage, and which therefore had presumably arrived passively, stained negatively for aldolase. A number of neutrophils and macrophages comprising the cellular exudate stained positively, but neither the proportion of leucocytes showing aldolase activity, nor the intensity of the reaction

in individual cells were related to bruise ages.

The majority of bruises showed moderately positive aldolase activity within connective tissue septal fibroblasts (Figure 3.3). This staining was enhanced in the two 144 hour old bruises in which coarse septa occurred (Fig. 3.4).

From consideration of the data summarised in Table 3.1, it was concluded that the histochemical activity of aldolase was an unsatisfactory means of ageing bruises, at least within the age range studied.

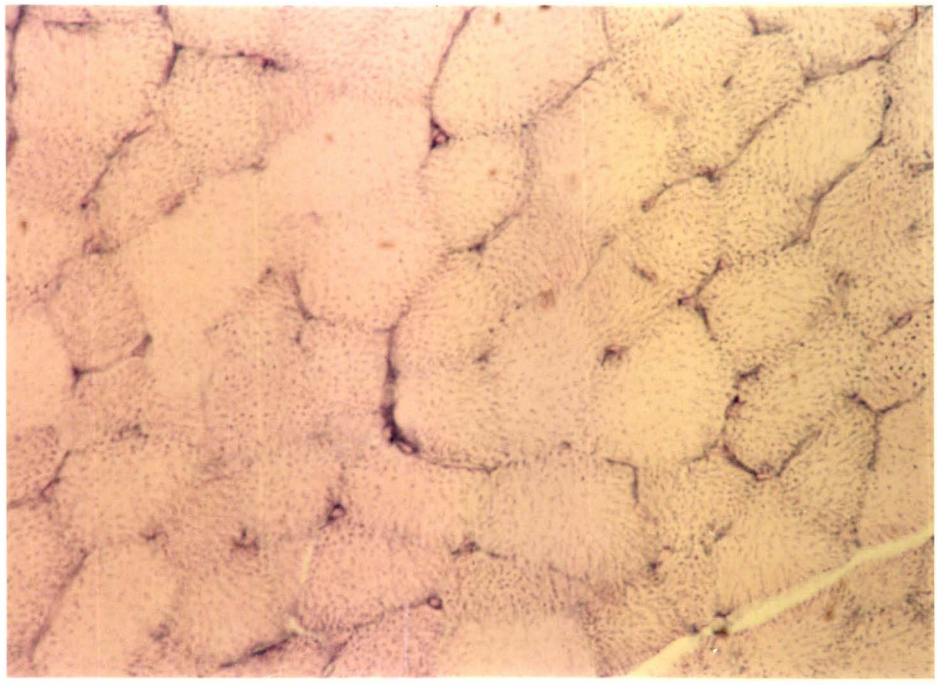
(b) Isoenzyme studies: Electrofocussed aldolase isoenzyme activities are shown as purple bands in Figure 3.5. In eleven of the homogenates of bruised muscle only one aldolase enzyme band was demonstrated, each with the same isoelectric point (pI). An additional band was focussed in each of the remaining five bruise samples (B24, B48, D4, D24, D48). This additional band could not be related to common factors either of anatomical position or of bruise age. Aldolase isoenzyme patterns as demonstrated by isoelectric focussing in polyacrylamide gels therefore proved of no value as a means of ageing bruises. Plasma showed strong aldolase activity resolved into four distinct bands. Activity was very mild in erythrocytes and remained undetected in the purified leucocyte sample. The uncomplicated nature of the aldolase zymogram obviated the need for a densitometer scan.

2. Lactate dehydrogenase

(a) Enzyme histochemistry: A summary of visually assessed enzyme activities in bruised muscle is presented in Table 3.2. Apparently normal muscle fibres demonstrated faintly positive diffuse as well as strongly positive punctate lactate dehydrogenase activities, with an expected variation in intensity of the latter according to fibre type (Figure 3.6). Diffuse staining was absent from all necrotic fibres, but punctate lactate dehydrogenase staining was variable. In some necrotic fibres it was enhanced, but usually it was decreased or absent (Figure 3.7). In general the extent of the decrease was related

FIGURE 3.1 : Bruise 4 hours old. Variation in aldolase staining according to fibre type in apparently normal muscle.
x 320

FIGURE 3.2 : Bruise 48 hours old. Decreased and increased aldolase staining in necrotic muscle fibres.
x 320



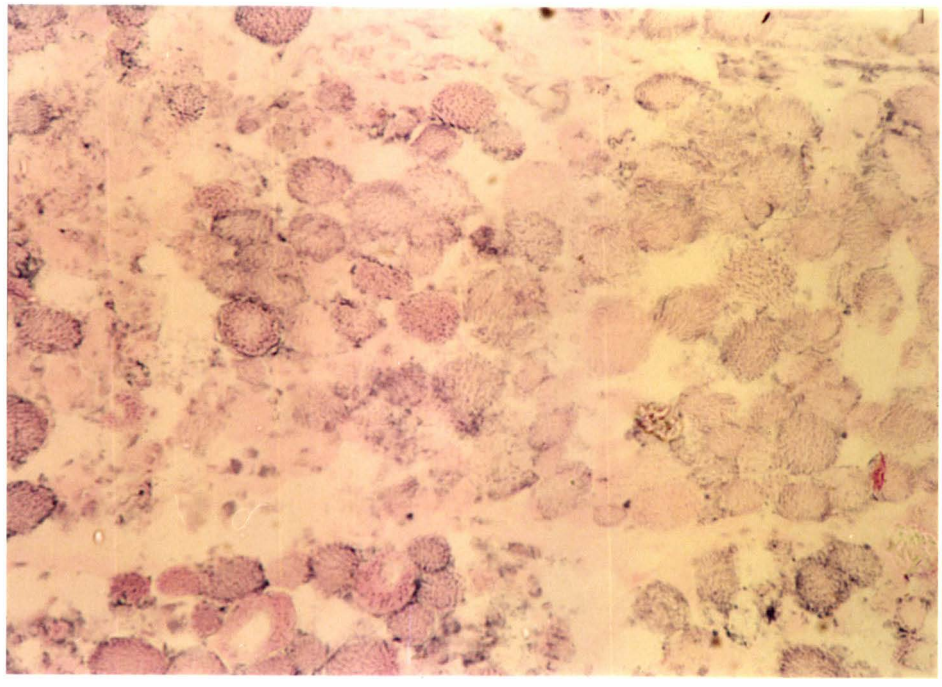
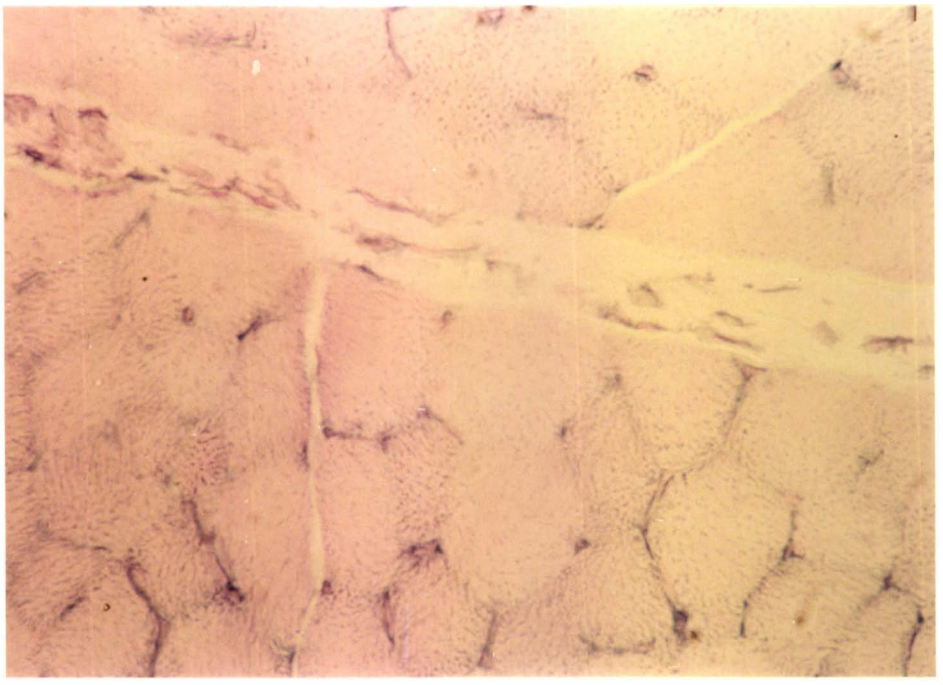


TABLE 3.1 : SUMMARY OF THE VISUALLY ASSESSED DEGREES OF ACTIVITY OF HISTOCHEMICALLY DEMONSTRATED ALDOLASE IN VARIOUS TISSUE COMPONENTS OF BRUISED MUSCLE.

AGE (hours)	SEVERITY OF INJURY			MUSCLE FIBRES		MUSCLE INFILTRATE		INTERSTITIAL EXUDATE		c.t. SEPTA
	haem.	necro.	wbc	normal	necrotic	neut.	mononuc.	neut.	mononuc.	
4	+	-	+	+	+,++	n.s.	n.s.	n.s.	-,+	+
4	+++	+++	++	n.s.	-,+	n.s.	n.s.	n.s.	-,+	n.s.
4	+	-	-	+	+	n.s.	n.s.	n.s.	+	+
4	+	-	+	+	+	n.s.	n.s.	+	+	+
24	+++	+++	+++	+	-,+	+	+	-,+	-,+	+
24	+++	+++	+++	n.s.	+	+	+	-	+	-
24	++	+++	+++	+	-,+,++	+	+	-,+	-,+	+
24	++	+	++	+	-,+	n.s.	n.s.	-	-	+
48	++	+++	+++	n.s.	-,+,++	n.s.	+	+	+	+
48	++	-	+	+	-,+	-	-	-	-	+
48	+++	+++	+++	+	-,+,++	+	+	n.s.	+	+
48	+++	+++	+++	+	-,+,++	+	+	+	+	+
144	+++	+++	++	n.s.	-,+	n.s.	-,+	n.s.	-,+	n.s.
144	+	-	-	+	+,++	n.s.	n.s.	n.s.	+	-,+,++
144	+++	++	+++	n.s.	+,++	n.s.	-,+	n.s.	+	++
144	+	-	++	+	+	+	+	-,+	-,+	n.s.

FIGURE 3.3 : Bruise 144 hours old. Moderately positive aldolase staining of fibroblasts in connective tissue septum.
x 320

FIGURE 3.4 : Bruise 144 hours old. Enhanced aldolase staining of fibroblasts in connective tissue septum.
x 320



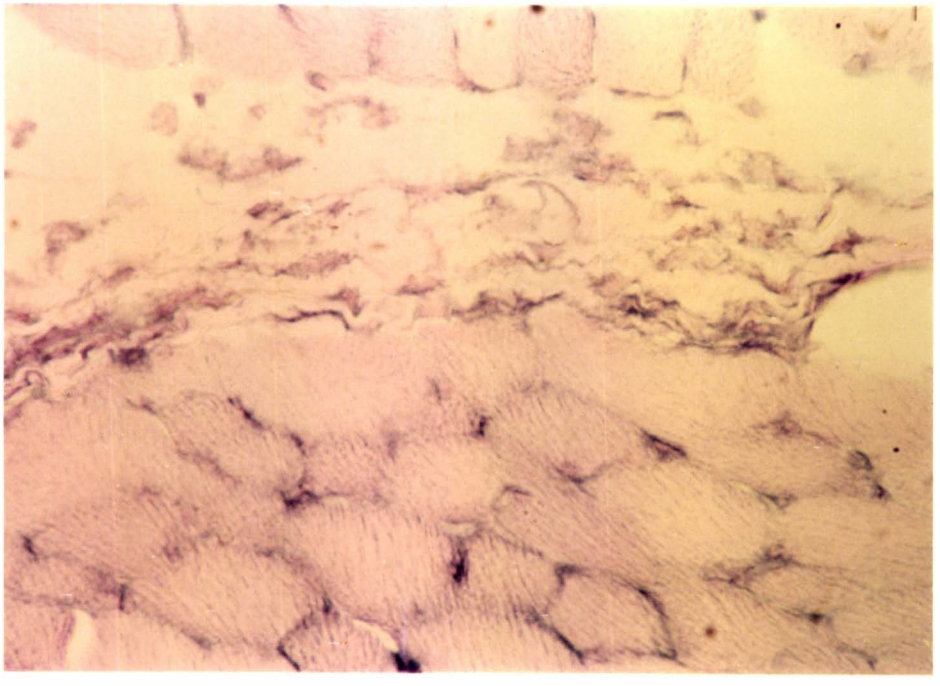
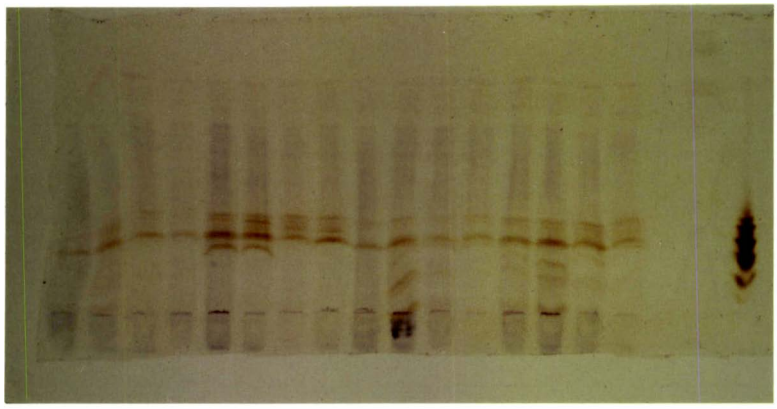


FIGURE 3.5 : Aldolase zymogram of bruised muscle, leucocytes, plasma and erythrocytes after isoelectric focussing at 25 watt constant power for 1.5 hours in PAG with ampholine buffer range pH 3.5-9.5. The gel was incubated in aqueous substrate medium for 30 minutes at 37^{o}C.

FIGURE 3.6 : Bruise 4 hours old. Variation in lactate dehydrogenase staining according to fibre type in apparently normal muscle.
x 320



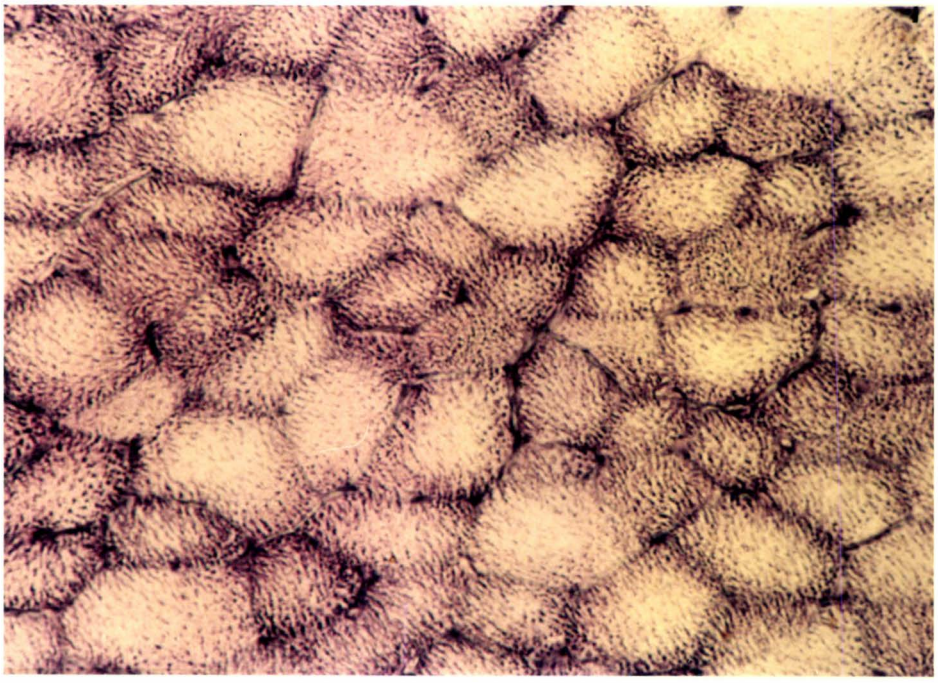
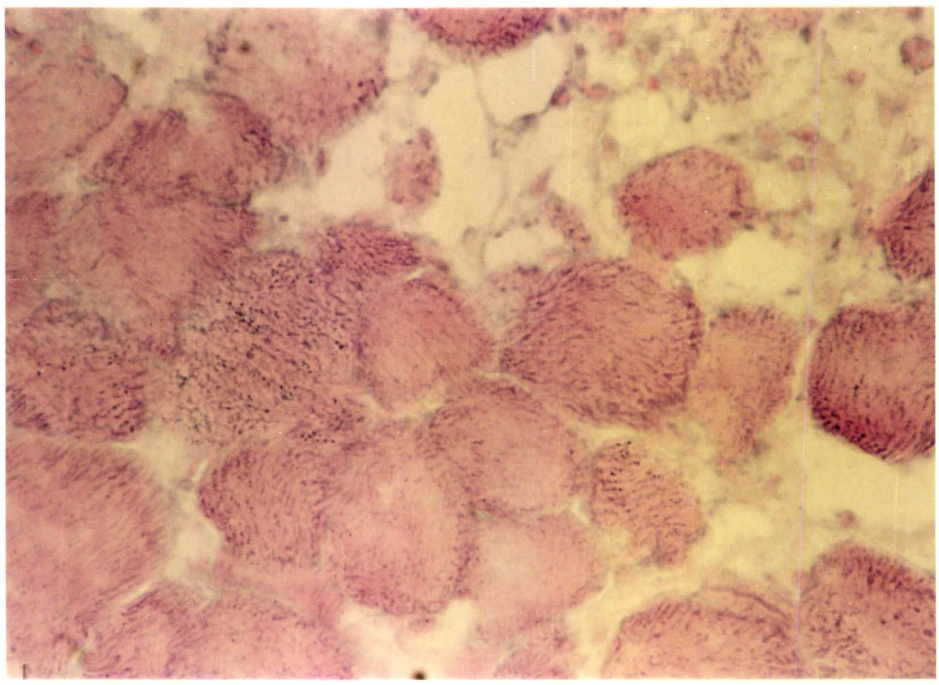


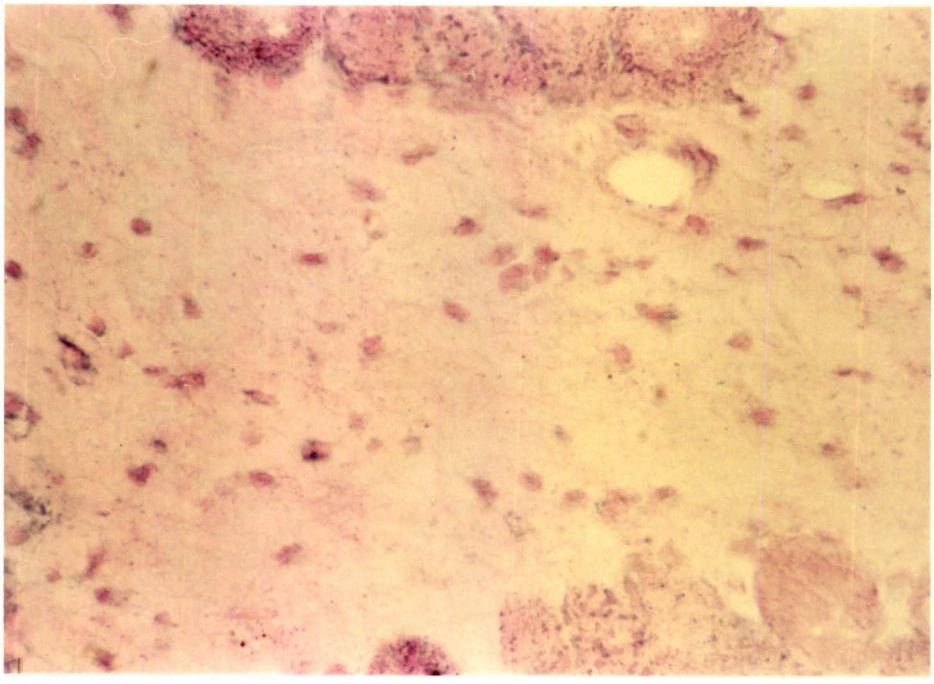
TABLE 3.2 : SUMMARY OF THE VISUALLY ASSESSED DEGREES OF ACTIVITY OF HISTOCHEMICALLY DEMONSTRATED LACTATE DEHYDROGENASE IN VARIOUS TISSUE COMPONENTS OF BRUISED MUSCLE.

AGE (hours)	SEVERITY OF INJURY			MUSCLE FIBRES		MUSCLE INFILTRATE		INTERSTITIAL EXUDATE		c.t. SEPTA
	haem.	necro.	wbc	normal	necrotic	neut.	mononuc.	neut.	mononuc.	
4	+	-	+	+,++	+	n.s.	n.s.	+	+	+,++
4	+++	+++	++	n.s.	+	n.s.	n.s.	+	+	+
4	+	-	-	+,++	-,++	n.s.	n.s.	n.s.	n.s.	+
4	+	-	+	+,++	+,++	n.s.	n.s.	+	+	++
24	+++	+++	+++	+	-,+	+	+	n.s.	++	+
24	+++	+++	+++	+,++	-,+,++	+	+	+	+	+,++
24	++	+++	+++	+,++	-,+	++	++	+	+	+,++
24	++	+	++	n.s.	-,+,++	+	+	+	+	+
48	++	+++	+++	n.s.	+,++	++	++	++	++	++,+++
48	++	-	+	+,++	-,+	+	+	++	++	+,++
48	+++	+++	+++	n.s.	-,+	++	++	++,+++	++,+++	++,+++
48	+++	+++	+++	n.s.	-,+,++	+,++,+++	+,++,+++	+++	+++	+,++
144	+++	+++	++	+,++	+,++	++	++	n.s.	++	n.s.
144	+	-	-	+,++	n.s.	n.s.	n.s.	+	+	++
144	+++	++	+++	+,++	+,++	++	++	n.s.	n.s.	n.s.
144	+	-	++	n.s.	+,++	+	+	n.s.	+	n.s.

FIGURE 3.7 : Bruise 24 hours old. Decreased lactate dehydrogenase staining in necrotic muscle fibres.
x 320

FIGURE 3.8 : Bruise 24 hours old. Variably positive lactate dehydrogenase staining in the leucocyte exudate.
x 320





to the severity of necrosis, but punctate staining occasionally persisted even in necrotic fibres undergoing dissolution. Altered staining in damaged fibres was unrelated to the factor of bruise age. On no occasion was altered lactate dehydrogenase activity observed in morphologically normal fibres.

The majority of leucocytes showed a degree of positive activity for lactate dehydrogenase (Figure 3.8). Increased enzyme activity was frequently observed in the cellular exudate (Figure 3.9), but this was not clearly related to bruise age.

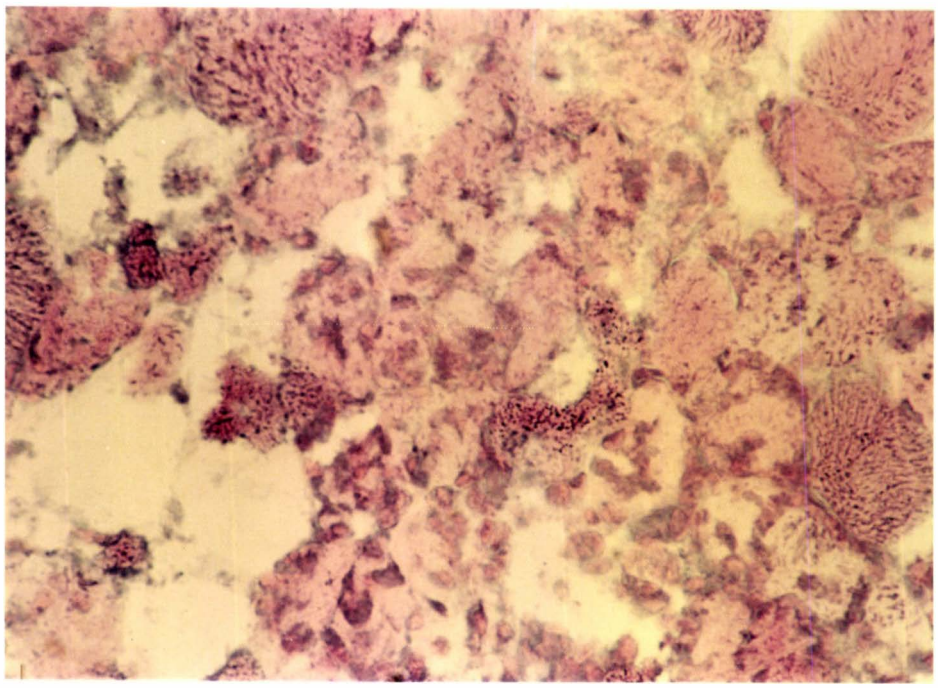
Both moderately positive (Figure 3.10) and strongly positive (Figure 3.11) staining was observed in connective tissue septal fibroblasts. The degree of staining was not, however, related to the age of the bruise.

(b) Isoenzyme studies: Electrofocussed lactate dehydrogenase isoenzyme activities are shown as purple bands on Figure 3.12. A large number of isoenzyme bands were resolved from each homogenate of bruised muscle and it was therefore necessary to analyse the zymogram with the aid of a densitometer. The densitometric scan of each bruise sample could be interpreted in terms of the 25 bands shown on the representative tracing in Figure 3.13. There was an overall similarity between the scans of the bruises but each one varied in detail. These variations could not be related to common factors of anatomical position, bruise age or the animal affected. Lactate dehydrogenase isoenzyme patterns as demonstrated by isoelectric focussing therefore proved of no value as a means of ageing bruises.

The plasma and erythrocyte samples both showed lactate dehydrogenase activity but the positions and staining intensities of the respective isoenzyme bands were such as not to interfere with the interpretation of those representing muscle. Enzyme activity was not detected in the purified leucocyte sample.

FIGURE 3.9 : Bruise 24 hours old. Increased lactate dehydrogenase staining of intramuscular leucocytes.
x 320

FIGURE 3.10 : Bruise 24 hours old. Moderately positive lactate dehydrogenase staining of fibroblasts in connective tissue septum.
x 500



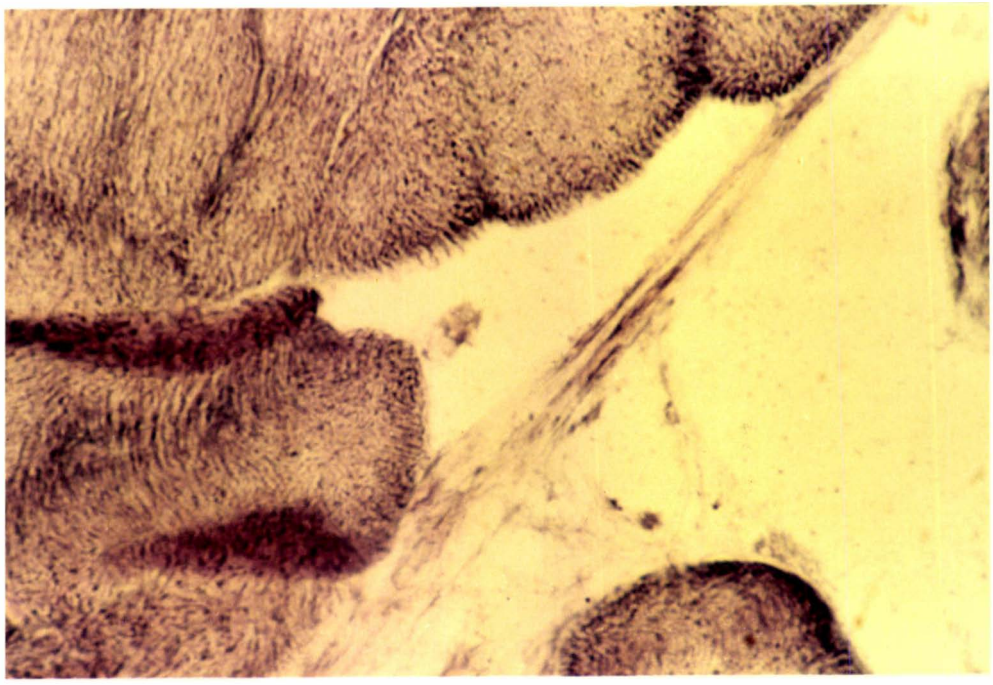
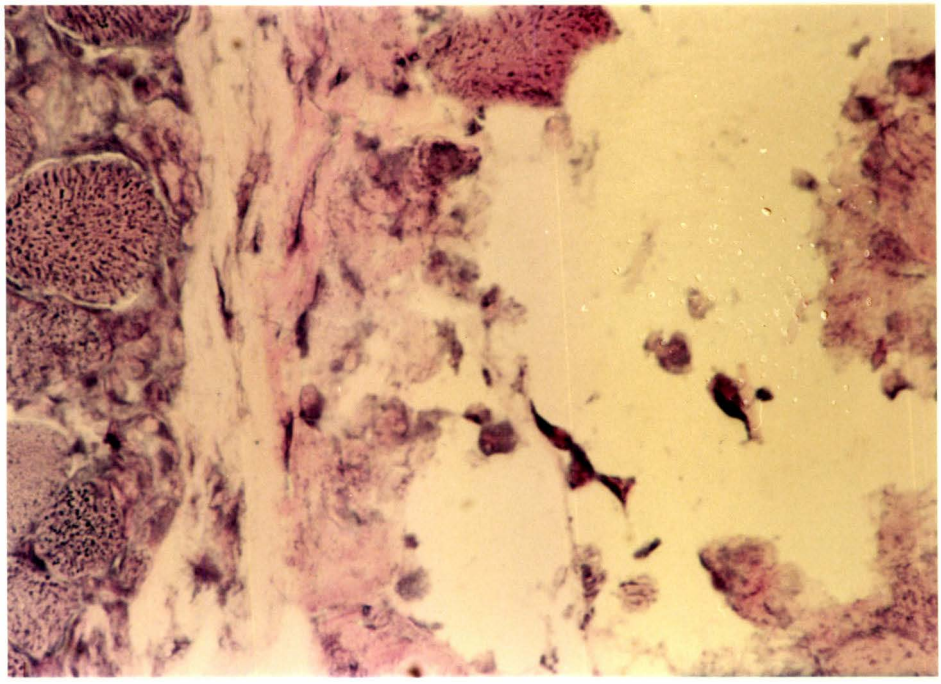


FIGURE 3.11 : Bruise 48 hours old. Strongly positive lactate dehydrogenase staining of fibroblasts in connective tissue septum.
x 320

FIGURE 3.12 : Lactate dehydrogenase zymogram of bruised muscle, leucocytes, plasma and erythrocytes after isoelectric focussing at 25 watt constant power for 1.5 hours in PAG with ampholine buffer range pH 3.5-9.5. The gel was incubated in aqueous substrate medium for 30 minutes at $37^{o}C$.



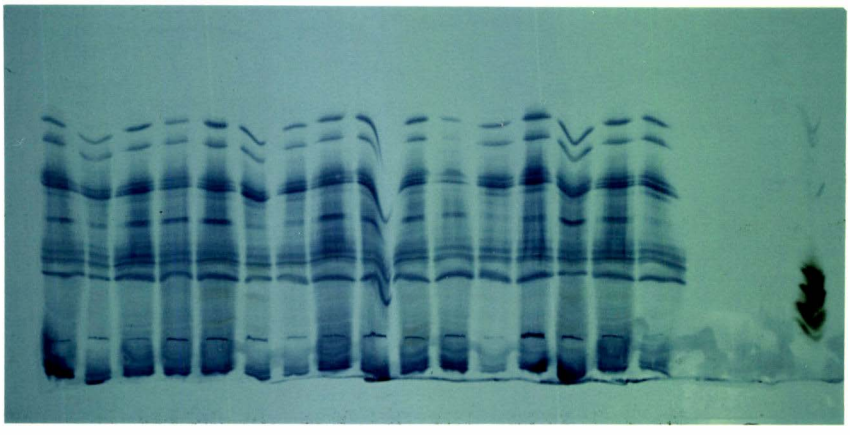
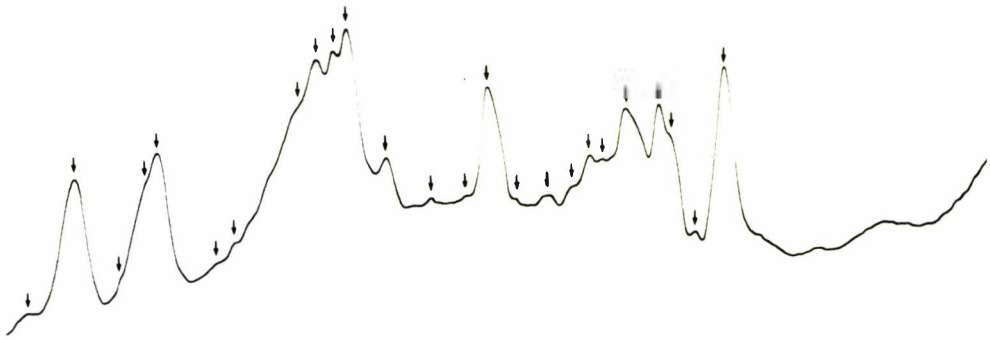
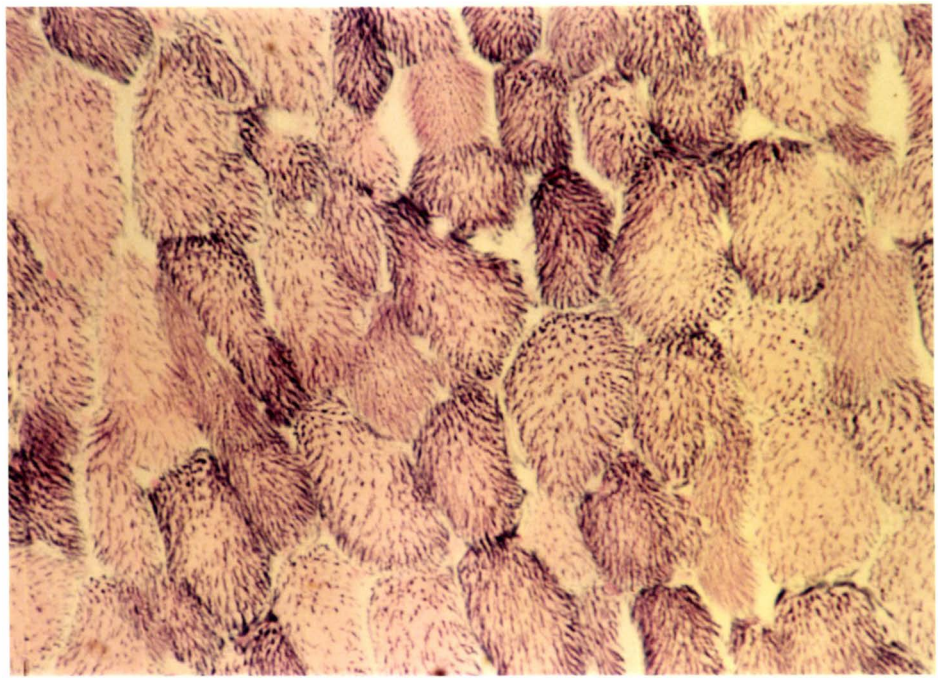


FIGURE 3.13 : A representative densitometer scan of electrofocussed lactate dehydrogenase isoenzymes obtained from bruised muscle, showing the 25 bands common to each sample of bruised muscle. This particular bruise was 4 hours old. The gel had ampholine buffer range pH 3.5-9.5.

FIGURE 3.14 : Bruise 4 hours old. Variation in succinate dehydrogenase staining according to fibre type in apparently normal muscle.
x 320





II. ENZYMES OF THE CITRIC ACID CYCLE

The only citric acid cycle enzyme studied was succinate dehydrogenase.

1. Succinate dehydrogenase

(a) Enzyme histochemistry: A summary of visually assessed enzyme activities in bruised muscle is presented in Table 3.3. Apparently normal muscle fibres demonstrated faintly positive punctate staining for succinate dehydrogenase, with an expected variation in intensity according to fibre type (Figure 3.14). Degenerative change was usually accompanied by a decrease or loss in enzyme activity (Figure 3.15), although enhanced activity was occasionally observed (Figure 3.16). The intensity of staining could not be related either to the severity of necrosis or the age of the bruise.

Interstitial leucocytes lacked succinate dehydrogenase activity and intramuscular leucocytes stained positively in only three of the sixteen bruises examined. Leucocyte succinate dehydrogenase activity could not therefore be related to age.

Connective tissue septal fibroblasts failed to stain for succinate dehydrogenase.

(b) Isoenzyme studies: Electrofocussed succinate dehydrogenase isoenzyme activities are shown as purple bands in Figure 3.17. A variable number of up to six poorly defined isoenzyme bands were resolved from homogenates of bruised muscle. Variations in isoenzyme patterns were not attributable to anatomical position or bruise age. Succinate dehydrogenase zymograms as demonstrated by isoelectric focussing therefore proved of no value as a means of ageing bruises.

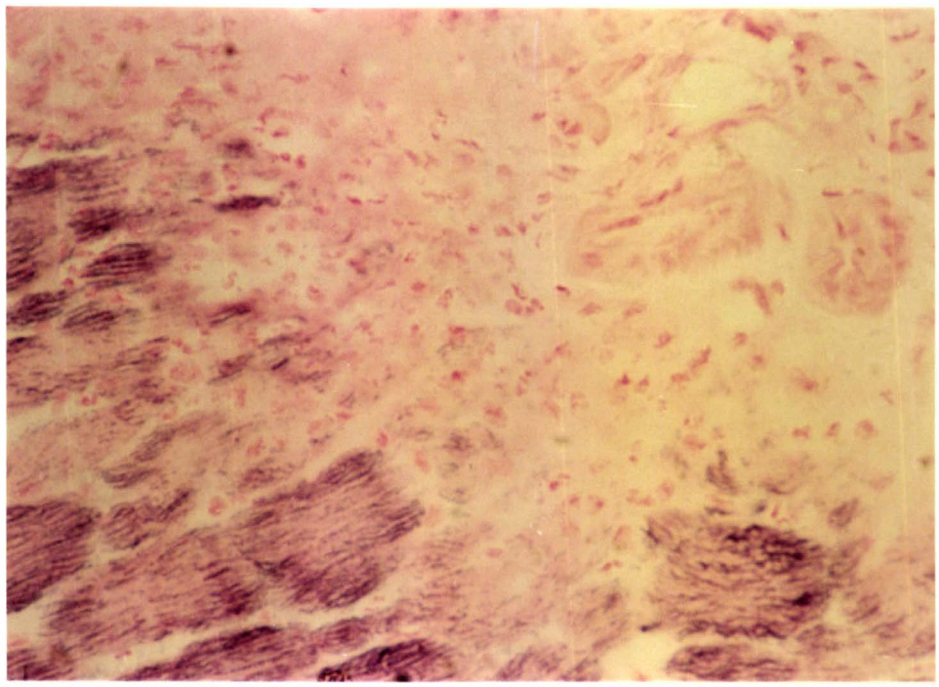
Both the plasma and erythrocyte samples showed moderate succinate dehydrogenase activity differentiated into several isoenzyme bands. No activity was detected in the purified leucocyte sample.

TABLE 3.3 : SUMMARY OF THE VISUALLY ASSESSED DEGREES OF ACTIVITY OF HISTOCHEMICALLY DEMONSTRATED SUCCINATE DEHYDROGENASE IN VARIOUS TISSUE COMPONENTS OF BRUISED MUSCLE.

AGE (hours)	SEVERITY OF INJURY			MUSCLE FIBRES		MUSCLE INFILTRATE		INTERSTITIAL EXUDATE		c.t. SEPTA
	haem.	necro.	wbc	normal	necrotic	neut.	mononuc.	neut.	mononuc.	
4	+	-	+	+,++	+	n.s.	n.s.	-	-	-
4	+++	+++	++	n.s.	-,+	n.s.	n.s.	-	-	n.s.
4	+	-	-	n.s.	+,++	n.s.	n.s.	n.s.	n.s.	-
4	+	-	+	+	+,++	n.s.	n.s.	-	-	-
24	+++	+++	+++	-,+	-,+,++	++	++	-	-	-
24	+++	+++	+++	+,++	+,++	-	-,+	-	-	n.s.
24	++	+++	+++	-,+	-,+	-	-	-	-	-
24	++	+	++	+	-,+,++	-	-	-	-	-
48	++	+++	+++	n.s.	-,+,++	-	-	-	-	-
48	++	-	+	-,+	-,+,++	-	-	-	-	-
48	+++	+++	+++	+,++	-,+,++	-	-	-	-	-
48	+++	+++	+++	+	-,+	-	-	-	-	-
144	+++	+++	++	n.s.	++	-	-	-	-	n.s.
144	+	-	-	+,++	n.s.	n.s.	n.s.	-	-	-
144	+++	++	+++	n.s.	-,+,++	-	-,+	-	-	-
144	+	-	++	+	+,++,+++	-	-	-	-	-

FIGURE 3.15 : Bruise 48 hours old. Decreased succinate dehydrogenase activity in necrotic muscle fibres.
x 320

FIGURE 3.16 : Bruise 144 hours old. Enhanced succinate dehydrogenase staining in degenerative muscle fibre.
x 320



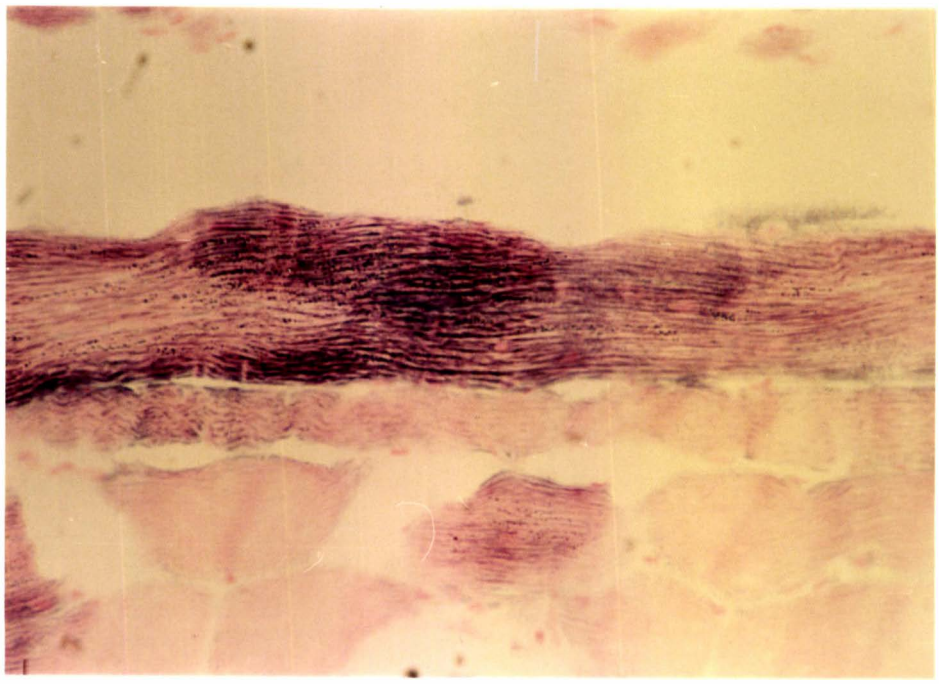
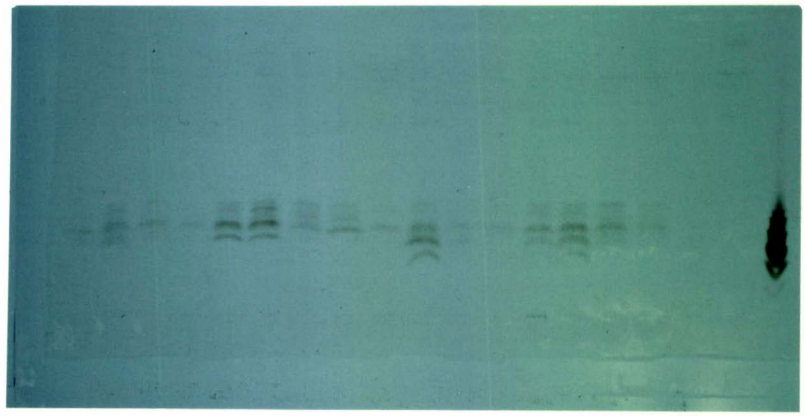
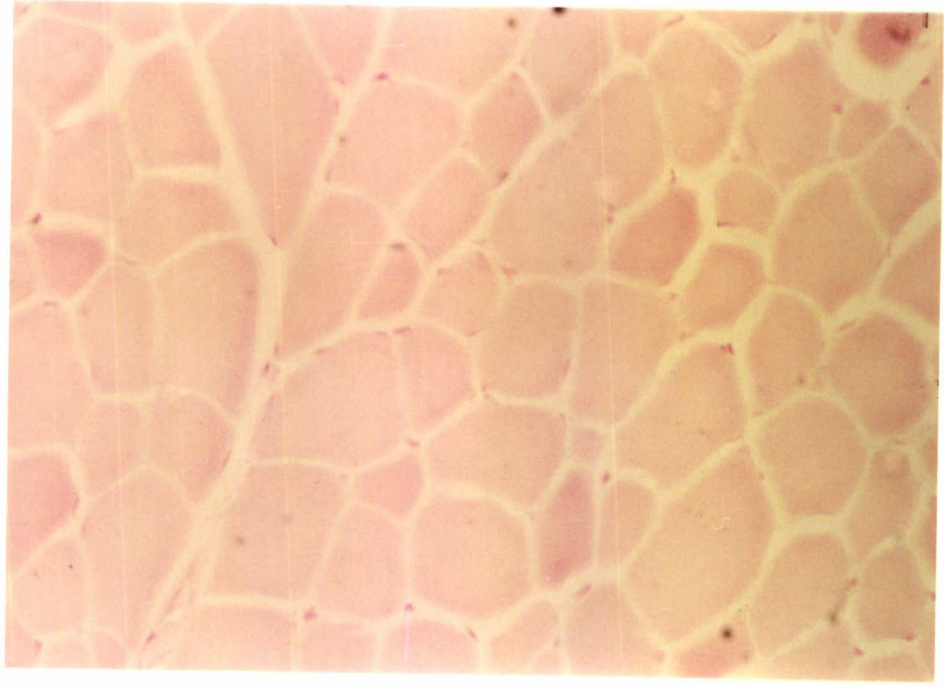


FIGURE 3.17 : Succinate dehydrogenase zymogram of bruised muscle, leucocytes, plasma and erythrocytes after isoelectric focussing at 25 watt constant power for 1.5 hours in PAG with ampholine buffer range pH 3.5-9.5. The gel was incubated in aqueous substrate medium for 30 minutes at 37^{o}C.

FIGURE 3.18 : Bruise 4 hours old. Absence of glucose-6-phosphate dehydrogenase activity in apparently normal muscle fibres. x 320





III. ENZYMES OF THE PENTOSE PHOSPHATE PATHWAY

The only pentose phosphate pathway enzyme studied was glucose-6-phosphate dehydrogenase.

1. Glucose-6-phosphate dehydrogenase

(a) Enzyme histochemistry: A summary of visually assessed enzyme activities in bruised muscle is presented in Table 3.4. Apparently normal muscle fibres showed no glucose-6-phosphate dehydrogenase activity (Figure 3.18). The majority of fibres undergoing degenerative change in bruises of all ages, however, showed a variable degree of punctate staining (Figure 3.19). The intensity of this reaction could not be related either to the severity of necrosis or to the age of the bruise.

Exudative leucocytes in the 4 hour old bruises lacked glucose-6-phosphate dehydrogenase activity but a positive reaction was observed in a proportion of the interstitial leucocytes in most of the 24 hour and older bruises. The activation of glucose-6-phosphate dehydrogenase in leucocytes therefore appeared to be time related and therefore potentially of use for ageing bruises. Neither the intensity of the reaction nor the relative number of interstitial leucocytes affected was related to age. Leucocytes infiltrating muscle fibres invariably stained moderately or strongly positive for glucose-6-phosphate dehydrogenase (Figure 3.19).

Fibroblasts within connective tissue septa failed to demonstrate glucose-6-phosphate activity in 4 hour old bruises but a proportion of these cells showed a faintly positive reaction in most of the 24 hour and older bruises (Figure 3.20). The enzymic reaction in fibroblasts therefore appeared of potential use for bruise ageing purposes.

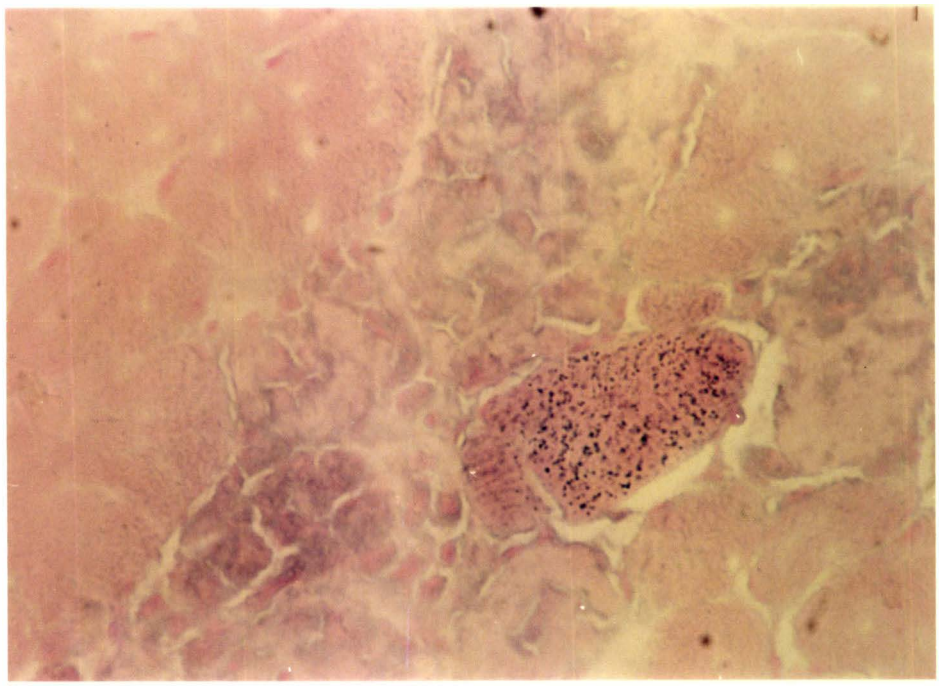
(b) Isoenzyme studies: Electrofocussed glucose-6-phosphate dehydrogenase isoenzyme activities are shown as purple bands in Figure 3.21. Only two bruises, aged 4 hour and 24 hour old and each

TABLE 3.4 : SUMMARY OF THE VISUALLY ASSESSED DEGREES OF ACTIVITY OF HISTOCHEMICALLY DEMONSTRATED GLUCOSE-6-PHOSPHATE DEHYDROGENASE IN VARIOUS TISSUE COMPONENTS OF BRUISED MUSCLE.

AGE (hours)	SEVERITY OF INJURY			MUSCLE FIBRES		MUSCLE INFILTRATE		INTERSTITIAL EXUDATE		c.t. SEPTA
	haem.	necro.	wbc	normal	necrotic	neut.	mononuc.	neut.	mononuc.	
4	+	-	+	-	+	n.s.	n.s.	-	-	-
4	+++	+++	++	n.s.	-	n.s.	n.s.	-	-	-
4	+	-	-	-	+	n.s.	n.s.	-	-	-
4	+	-	+	-	+	n.s.	n.s.	-	-	-
24	+++	+++	+++	-,+	-,+	++	+	-	-,+	+
24	+++	+++	+++	n.s.	-,+	++	++	-	-,+	-
24	++	+++	+++	-,+	-,+,++	++	++	-,+	-,+	-,+
24	++	+	++	-	-,+	++	++	-,+	-,+	-,+
48	++	+++	+++	-	-,+,++	++	++	-	-	-,+
48	++	-	+	-	-,+	n.s.	++	-,+	-,+	-,+
48	+++	+++	+++	-	-,+,++	++	++	+	+	+
48	+++	+++	+++	-	-,+,++	++	++	-,+	+	+,++
144	+++	+++	++	-	-,++,+++	++	++	-	-,+,++	+,++
144	+	-	-	-	n.s.	n.s.	n.s.	n.s.	-	-
144	+++	++	+++	-	-,+,++	n.s.	++	-,+	-,+	+
144	+	-	++	-	-,+,++	+	n.s.	+	+	+

FIGURE 3.19 : Bruise 24 hours old. Punctate glucose-6-phosphate dehydrogenase activity in degenerative muscle fibre. Intramuscular leucocytes show a moderately positive reaction.
x 320

FIGURE 3.20 : Bruise 48 hours old. Faintly positive glucose-6-phosphate dehydrogenase staining of fibroblasts in connective tissue septum.
x 500



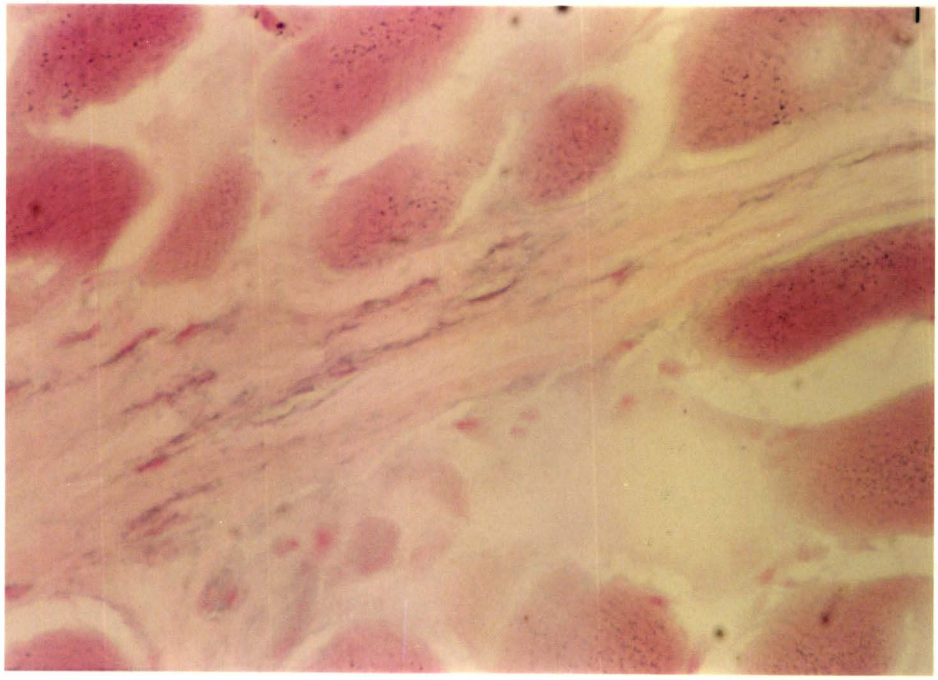
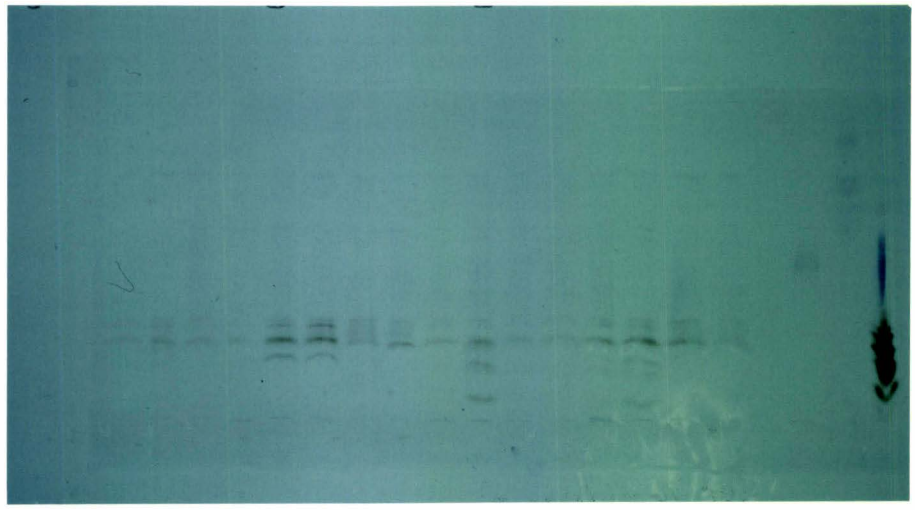
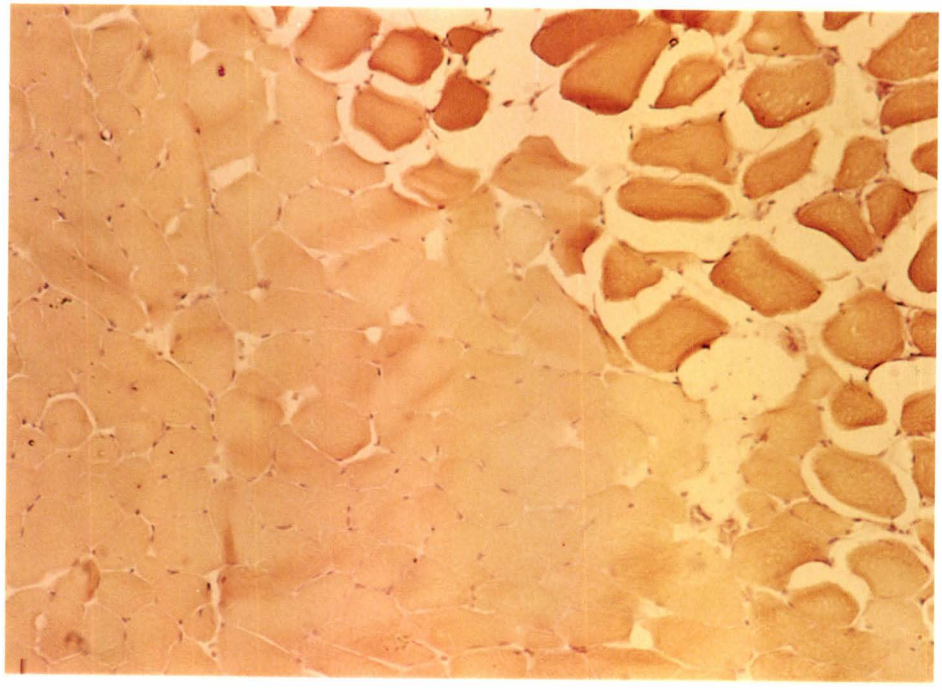


FIGURE 3.21 : Glucose-6-phosphate dehydrogenase zymogram of bruised muscle, leucocytes, plasma and erythrocytes after isoelectric focussing at 25 watt constant power for 1.5 hours in PAG with ampholine buffer range pH 3.5-9.5. The gel was incubated in aqueous substrate medium for 30 minutes at 37^{o}C.

FIGURE 3.22 : Bruise 4 hours old. Absence of acid phosphatase activity in apparently normal and degenerative muscle fibres.
x 125





from the same animal, showed enzyme activity. In both cases there was one faintly stained band with the same pI. Glucose-6-phosphate dehydrogenase isoenzyme patterns as demonstrated by isoelectric focussing therefore proved of no value as a means of ageing bruises.

Plasma showed moderate glucose-6-phosphate dehydrogenase activity resolved into five distinct bands. A moderate degree of enzyme activity was also observed in erythrocytes but this was poorly resolved into isoenzyme fractions. No activity was detected in the purified leucocyte sample.

IV. HYDROLYTIC ENZYMES

Four hydrolytic enzymes were studied. These were acid phosphatase, alkaline phosphatase, non-specific esterase and leucine aminopeptidase.

1. Acid phosphatase

(a) Enzyme histochemistry: A summary of subjectively assessed enzyme activities in bruised muscle is presented in Table 3.5. Neither apparently normal nor necrotic muscle fibres showed acid phosphatase activity (Figures 3.22 and 3.23). Necrotic fibres occasionally appeared to stain positively for enzyme but this was due to densely infiltrating macrophages (Figure 3.24).

Neutrophils failed to stain for acid phosphatase. Macrophages showed a variable degree of activity, from negative to strongly positive (Figures 3.23 and 3.25). However, neither the intensity of staining nor the proportion of macrophages affected could be related to the age of the bruise.

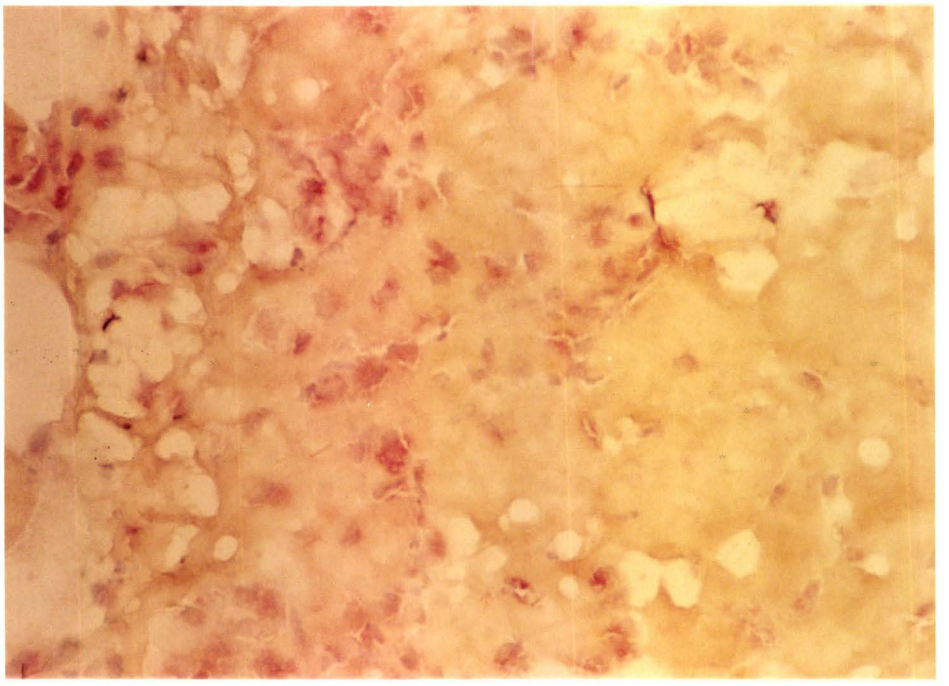
In the majority of bruises, fibroblasts in connective tissue septa failed to stain for acid phosphatase. In five bruises, however, a proportion of these cells showed a positive reaction (Figure 3.26). Neither the intensity of this reaction nor the relative numbers of connective

TABLE 3.5 : SUMMARY OF THE VISUALLY ASSESSED DEGREES OF ACTIVITY OF HISTOCHEMICALLY DEMONSTRATED ACID PHOSPHATASE IN VARIOUS TISSUE COMPONENTS OF BRUISED MUSCLE.

AGE (hours)	SEVERITY OF INJURY			MUSCLE FIBRES		MUSCLE INFILTRATE		INTERSTITIAL EXUDATE		c.t. SEPTA
	haem.	necro.	wbc	normal	necrotic	neut.	mononuc.	neut.	mononuc.	
4	+	+	+	-	-	n.s.	-,+	-	-,+	-
4	+	+	+	-	-	-	-	-	-,+	n.s.
4	+	+	+	-	-	n.s.	-,+,++	-	-,+,++	-
4	+	+	+	-	-	n.s.	n.s.	-	-	-
24	-	-	-	-	n.s.	n.s.	-	n.s.	n.s.	-
24	+	-	+	-	-	n.s.	+,++	-	-,+,++	-,+,++
24	+	+	+	-	-	n.s.	n.s.	n.s.	-,+	-
24	++	++	++	-	-	-	-,+	-	-,+	-
48	++	+++	+++	-	-	-	-,+,++	-	-,+	-
48	++	+++	+++	-	-	n.s.	-,+,++	n.s.	-,+,++	n.s.
48	+	+	+	-	-	-	-,+	-	-,+	-,+
48	+	+	+	-	-	-	-,+,++,+++	-	-,+,++	-,++
144	-	-	-	-	n.s.	n.s.	n.s.	n.s.	-,+	-
144	+	-	+	-	-	n.s.	+++	n.s.	-,+,++	-
144	+	+	+	-	-	n.s.	-	n.s.	-,+,++	-,+
144	++	+++	+++	-	-	-	-,+,++	-	-,+,++	-,+,++

FIGURE 3.23 : Bruise 48 hours old. Absence of acid phosphatase activity in necrotic muscle fibres. Intramuscular macrophages show a positive reaction. x 320

FIGURE 3.24 : Bruise 24 hours old. Apparently positive acid phosphatase staining of a necrotic muscle fibre, actually due to infiltrating macrophages. x 320



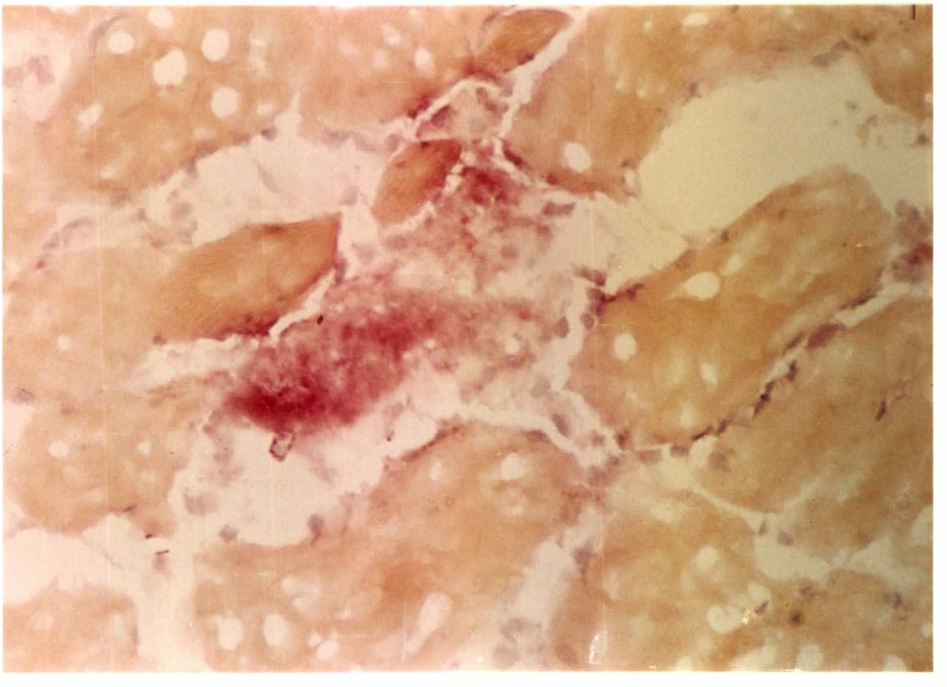
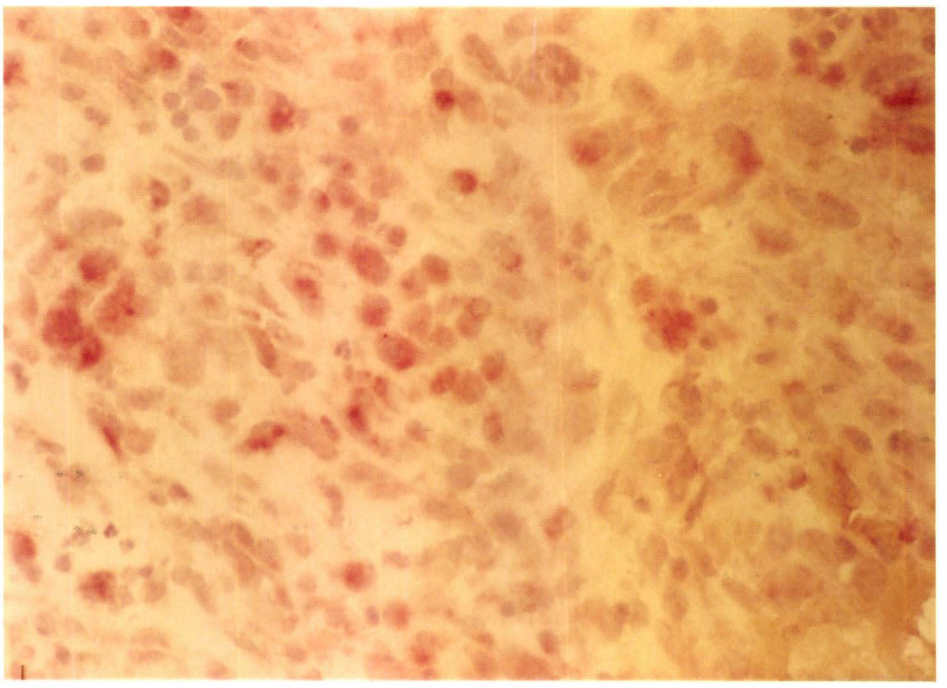
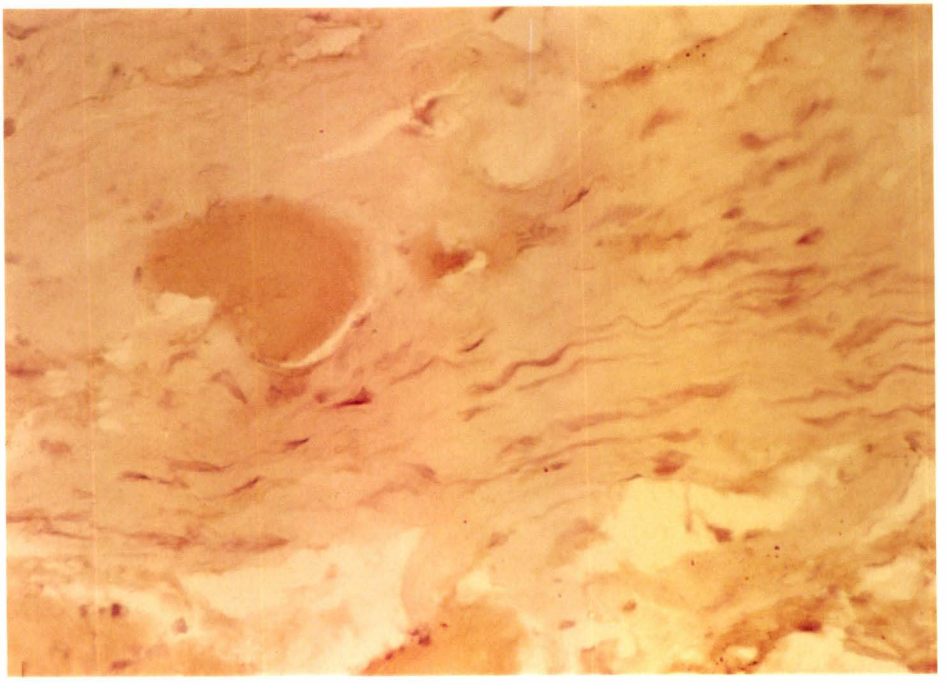


FIGURE 3.25 : Bruise 144 hours old. Absence of acid phosphatase staining in interstitial polymorphs, and a variably positive reaction in macrophages. x 320

FIGURE 3.26 : Bruise 48 hours old. Acid phosphatase staining of fibroblasts in connective tissue septum. x 320





tissue cells affected could be related to bruise age. Nonetheless, the fact that four of the affected bruises were aged 48 hours or older and the remaining one was 24 hours old implies that fibroblastic acid phosphatase activity may occur only in bruises 24 hours or older.

(b) Isoenzyme studies: Electrofocussed acid phosphatase isoenzyme activities are shown as red bands in Figure 3.27. The control liver sample demonstrated moderate acid phosphatase activity poorly resolved into several isoenzyme bands. The isoenzyme bands resolved from homogenates of bruised muscle were poorly focussed and very mild in activity. The results were therefore analysed with the aid of a densitometer. Each densitometer scan could be interpreted in terms of the peaks in the representative scan presented in Figure 3.28. Considerable differences existed between bruise samples but these could not be related to common factors of anatomical position, bruise age or the animal affected. Acid phosphatase isoenzyme patterns as demonstrated by isoelectric focussing therefore proved of no value as a means of ageing bruises. Plasma, erythrocyte and leucocyte fractions were not examined for acid phosphatase activity.

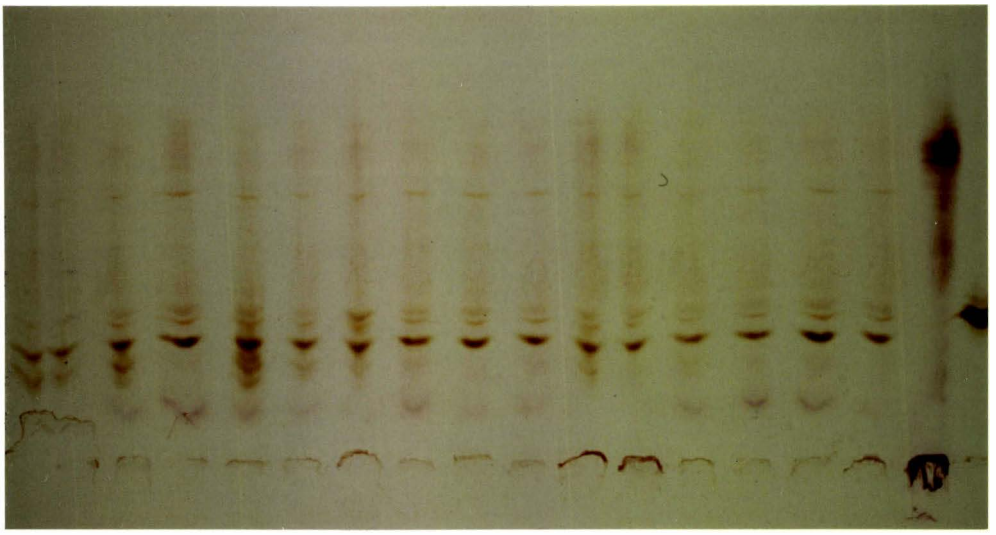
2. Alkaline phosphatase

(a) Enzyme histochemistry: A summary of subjectively assessed enzyme activities in bruised muscle is presented in Table 3.6. Neither apparently normal nor necrotic muscle fibres showed alkaline phosphatase activity. Necrotic fibres occasionally appeared to stain positively but this was due to densely infiltrating macrophages (Figure 3.29). Capillary endothelium between muscle fibres stained strongly positive. This activity was maintained in areas of necrosis (Figure 3.30).

Neutrophils showed moderate or strong alkaline phosphatase activity (Figure 3.30). Macrophages showed a varying degree of activity ranging from negative to strongly positive (Figure 3.31). Neither the intensity of leucocyte staining nor the proportion of cells affected could be related to the age of the bruise.

FIGURE 3.27 : Acid phosphatase zymogram of bruised muscle, liver and erythrocytes after isoelectric focussing at 25 watt constant power for 1.5 hours in PAG with ampholine buffer range pH 3.5-9.5. The gel was incubated in aqueous substrate medium for 30 minutes at 37^{o}C.

FIGURE 3.28 : A representative densitometer scan of electrofocussed acid phosphatase isoenzymes obtained from homogenates of bruised muscle, showing the four isoenzyme bands represented in all the muscle samples. This particular bruise was 144 hours old. The unmarked peaks represent haemoglobin or unidentified protein.



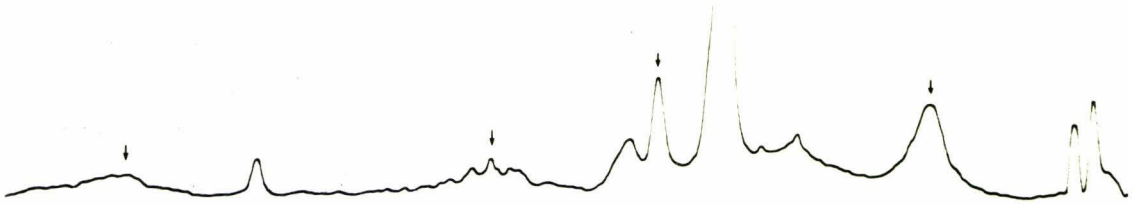
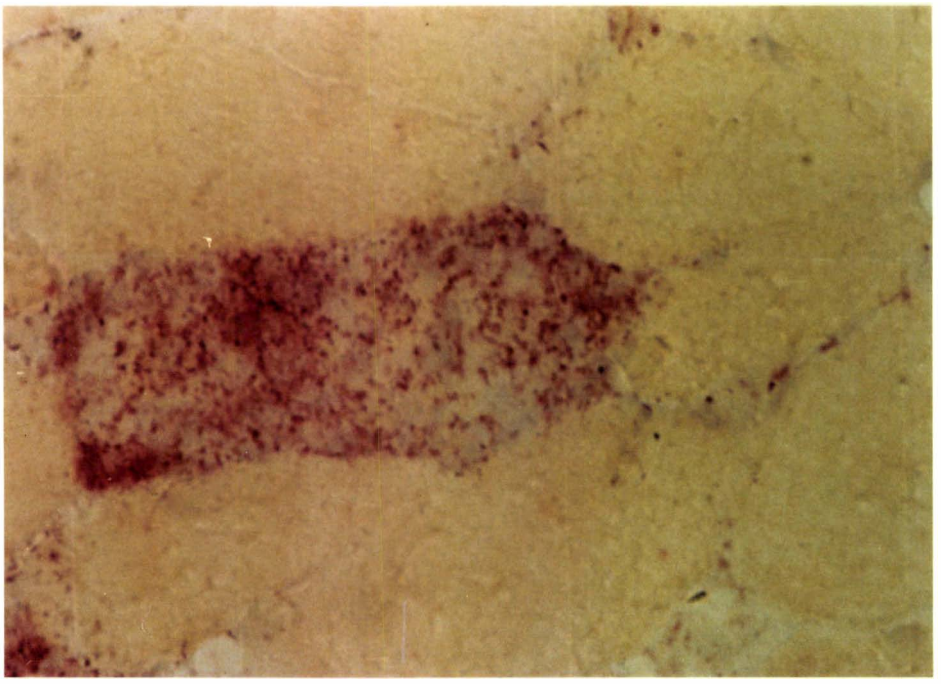


TABLE 3.6 : SUMMARY OF THE VISUALLY ASSESSED DEGREES OF ACTIVITY OF HISTOCHEMICALLY DEMONSTRATED ALKALINE PHOSPHATASE IN VARIOUS TISSUE COMPONENTS OF BRUISED MUSCLE.

AGE (hours)	SEVERITY OF INJURY			MUSCLE FIBRES		MUSCLE INFILTRATE		INTERSTITIAL EXUDATE		c.t. SEPTA
	haem.	necro.	wbc	normal	necrotic	neut.	mononuc.	neut.	mononuc.	
4	+	+	+	-	-	n.s.	-	+,++	-,+,++	-,++
4	+	+	+	-	-	n.s.	n.s.	+,++	-,+,++	n.s.
4	+	+	+	-	-	n.s.	-,+	n.s.	-,+	-
4	+	+	+	-	-	n.s.	n.s.	+,++	-,+,++	-,+
24	-	-	-	-	n.s.	n.s.	n.s.	n.s.	++	-
24	+	-	+	-	-	n.s.	n.s.	n.s.	-,+	-
24	+	+	+	-	-	n.s.	++	n.s.	+	-
24	++	++	++	-	-	+	+,++,+++	+,++	-,+,++	n.s.
48	++	+++	+++	-	-	n.s.	-,+,++,+++	+	-,+,++,+++	-,++
48	++	+++	+++	-	-	+	-,+,++	+	-,+,++,+++	n.s.
48	+	+	+	-	-	+	-,+,++	++	-,+,++	-
48	+	+	+	-	-	++	++	++	++	+,++
144	-	-	-	-	n.s.	n.s.	-	n.s.	-,+	-
144	+	-	+	-	-	n.s.	n.s.	n.s.	-	-
144	+	+	+	-	-	n.s.	-,+	n.s.	-	-
144	++	+++	+++	-	-	n.s.	-	n.s.	-,+	-

FIGURE 3.29 : Bruise 24 hours old. Apparently positive alkaline phosphatase staining of a necrotic muscle fibre, actually due to infiltrating macrophages.
x 500

FIGURE 3.30 : Bruise 24 hours old. Strongly positive alkaline phosphatase staining of capillary endothelium.
Neutrophils are also strongly positive.
x 125



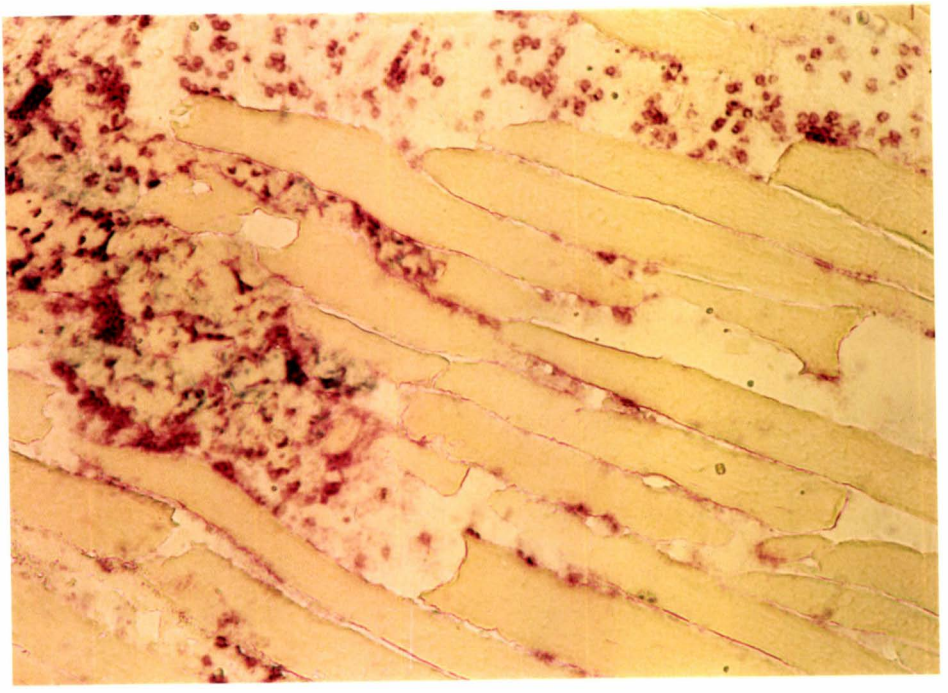
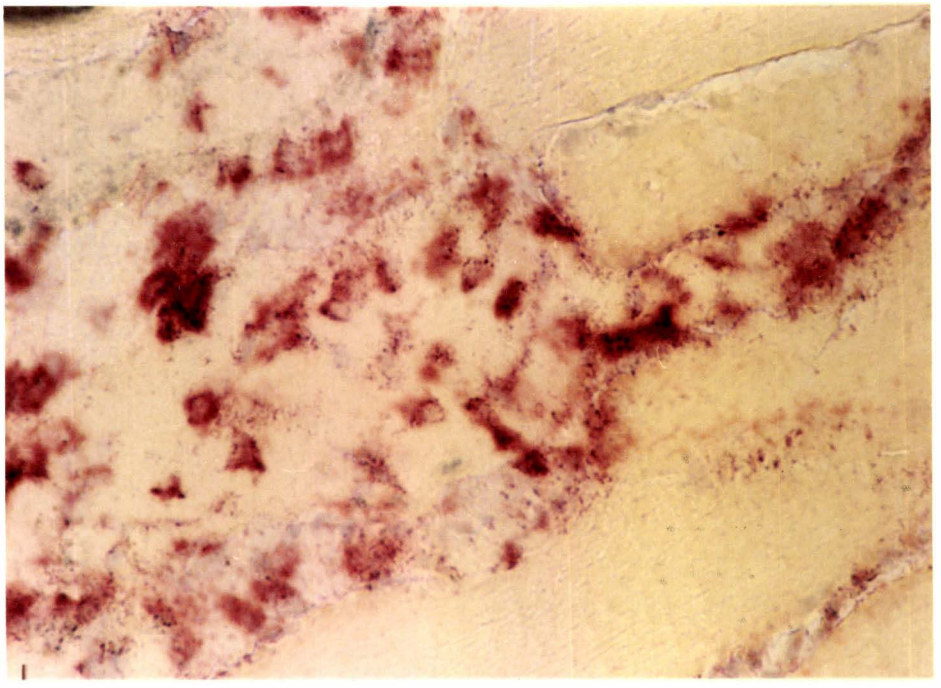
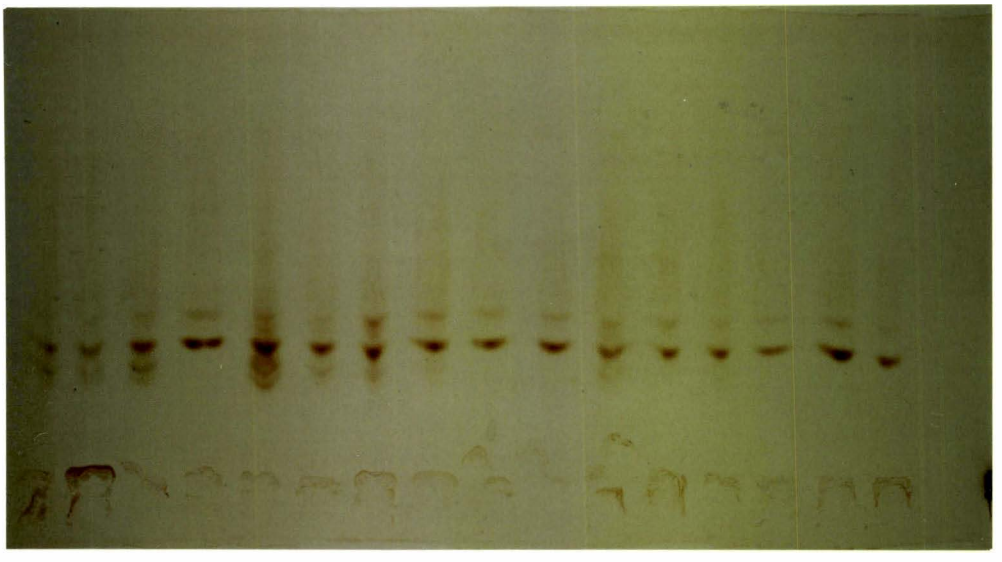


FIGURE 3.31 : Bruise 24 hours old. Variable alkaline phosphatase staining of infiltrating macrophages.
x 500

FIGURE 3.32 : Alkaline phosphatase zymogram of bruised muscle, small intestine and liver after isoelectric focussing at 25 watt constant power for 1.5 hours in PAG with ampholine buffer range pH 3.5-9.5. The gel was incubated in aqueous substrate medium for 30 minutes at $37^{o}C$.





In the majority of bruises, fibroblasts in connective tissue septa failed to stain for alkaline phosphatase. However, moderately positive staining was observed in a proportion of connective tissue cells in four bruises. Since two of these were 4 hours and two were 48 hours old, this positive staining reaction could not be related to age.

(b) Isoenzyme studies: Electrofocussed alkaline phosphatase isoenzymes showed as red bands (Figure 3.32). The control small intestine sample demonstrated faint enzyme activity but no alkaline phosphatase staining was detected in the homogenates of bruised muscle. Alkaline phosphatase isoenzyme patterns as demonstrated by isoelectric focussing therefore proved of no value as a means of ageing bruises. Plasma, erythrocyte and leucocyte fractions were not examined for alkaline phosphatase activity.

3. Leucine aminopeptidase

(a) Enzyme histochemistry: A summary of visually assessed enzyme activities in bruised muscle is presented in Table 3.7. Apparently normal muscle and the majority of those fibres undergoing degenerative change showed no leucine aminopeptidase activity. In eight bruises, moderate enzyme activity was detected in one or several isolated necrotic muscle fibres, but this activity was not related to age and appeared in most cases to be caused by infiltrating macrophages (Figure 3.33). Occasionally, however, positive staining occurred in the absence of a visible cellular exudate (Figure 3.34).

Neutrophils failed to stain for leucine aminopeptidase. Macrophages showed a variable degree of activity, ranging from negative to strongly positive (Figure 3.33). Neither the intensity of staining nor the proportion of cells affected could be related to the age of the bruise.

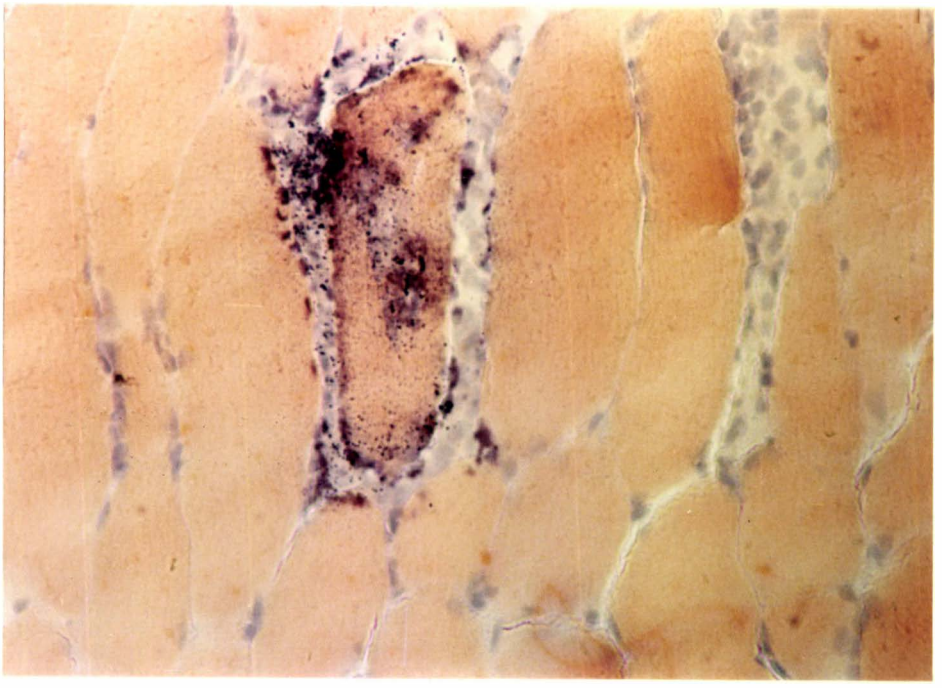
Fibroblasts in the connective tissue septa of most of the bruises failed to stain for aminopeptidase activity. A moderate degree of activity was observed in two of the 144 hour old bruises (Figure 3.35).

TABLE 3.7 : SUMMARY OF THE VISUALLY ASSESSED DEGREES OF ACTIVITY OF HISTOCHEMICALLY DEMONSTRATED LEUCINE AMINOPEPTIDASE IN VARIOUS TISSUE COMPONENTS OF BRUISED MUSCLE.

AGE (hours)	SEVERITY OF INJURY			MUSCLE FIBRES		MUSCLE INFILTRATE		INTERSTITIAL EXUDATE		c.t. SEPTA
	haem.	necro.	wbc	normal	necrotic	neut.	mononuc.	neut.	mononuc.	
4	+	+	+	-	-	n.s.	n.s.	-	+	-
4	+	+	+	-	-,+	n.s.	n.s.	n.s.	+,++	-
4	+	+	+	-	-,++	n.s.	n.s.	-	-,+,++,+++	-
4	+	+	+	-	-,++	n.s.	n.s.	-	+,++	-
24	-	-	-	-	n.s.	n.s.	n.s.	n.s.	-	-
24	+	-	+	-	-	n.s.	+	n.s.	-	-
24	+	+	+	-	-,+++	n.s.	++	n.s.	-,+	-
24	++	++	++	-	-,++	-	-	-	-,+,++	-
48	++	+++	+++	-	-	n.s.	-	-	-	-
48	++	+++	+++	-	-	-	-	n.s.	-,++	-
48	+	+	+	-	-	n.s.	-	-	-,+	-
48	+	+	+	-	-	n.s.	-	-	-,+	-
144	-	-	-	-	n.s.	n.s.	n.s.	n.s.	-,+	-
144	+	-	+	-	-,++	n.s.	++	n.s.	-,+,++	-
144	+	+	+	-	-	n.s.	n.s.	n.s.	-,+,++	-,+
144	++	+++	+++	-	-,++	n.s.	-,+	n.s.	-,+,++,+++	-,++

FIGURE 3.33 : Bruise 144 hours old. Apparently positive leucine aminopeptidase staining of a necrotic muscle fibre, actually due to infiltrating macrophages.
x 320

FIGURE 3.34 : Bruise 4 hours old. Positive leucine aminopeptidase staining in the absence of infiltrating macrophages.
x 320



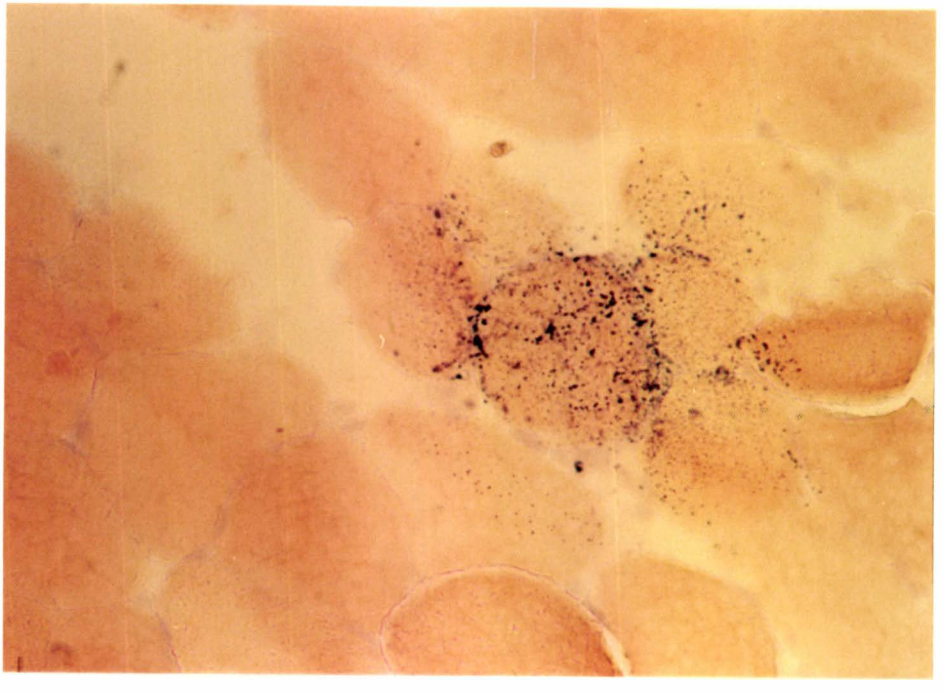
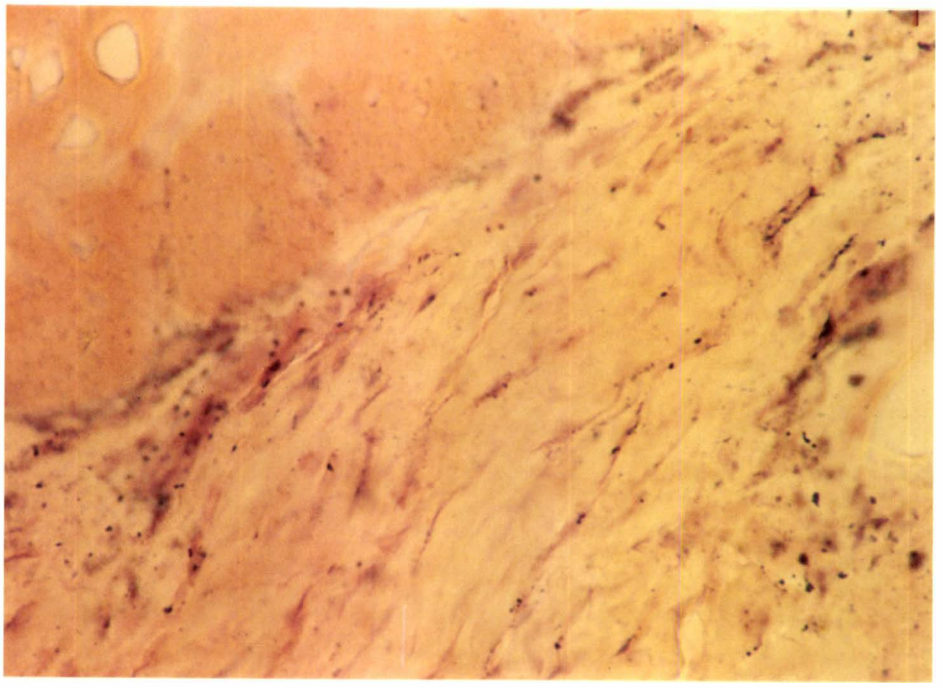
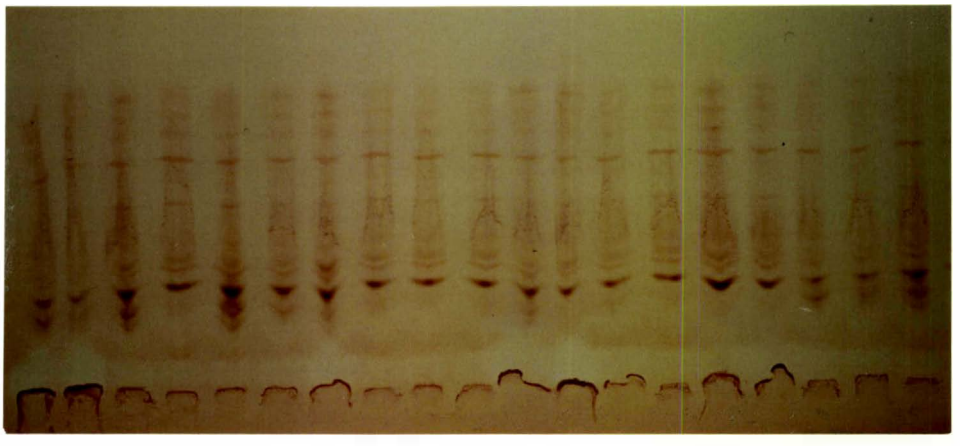


FIGURE 3.35 : Bruise 144 hours old. Moderate leucine aminopeptidase staining of fibroblasts in connective tissue septum.
x 500

FIGURE 3.36 : Leucine aminopeptidase zymogram of bruised muscle homogenates after isoelectric focussing at 25 watt constant power for 1.5 hours in PAG with ampholine buffer range pH 3.5-9.5. The gel was incubated in aqueous substrate medium for 30 minutes at $37^{o}C$.





(b) Isoenzyme studies: Electrofocussed leucine aminopeptidase isoenzyme activities are shown as faint black bands in Figure 3.36. One, or occasionally two, isoenzymes were resolved from the homogenates of bruised muscle. Neither the number, the position nor the intensity of the bands could be related to common factors of anatomical position, bruise age or the animal affected. Leucine aminopeptidase isoenzyme patterns as demonstrated by isoelectric focussing therefore proved of no value as a means of ageing bruises. Plasma, erythrocyte and leucocyte fractions were not examined for leucine aminopeptidase activity.

4. Non-specific esterase

(a) Enzyme histochemistry: A summary of visually assessed enzyme activities in bruised muscle is presented in Table 3.8. Normal muscle fibres stained either slightly positive or negative for esterase activity (Figure 3.37). Degenerative fibres showed either unchanged or variably increased enzyme activities (Figure 3.38). The intensity of the reaction could not be related either to the severity of necrosis or to the age of the bruise.

Esterase activity in leucocytes ranged widely from negative to strongly positive. Neither the intensity of the leucocytic reaction nor the proportion of cells affected could be related to the age of the bruise.

Fibroblasts within connective tissue septa showed a variably degree of activity, ranging from negative to moderately positive (Figure 3.39). Neither the intensity of this reaction nor the relative number of cells affected could be related to the age of the bruise.

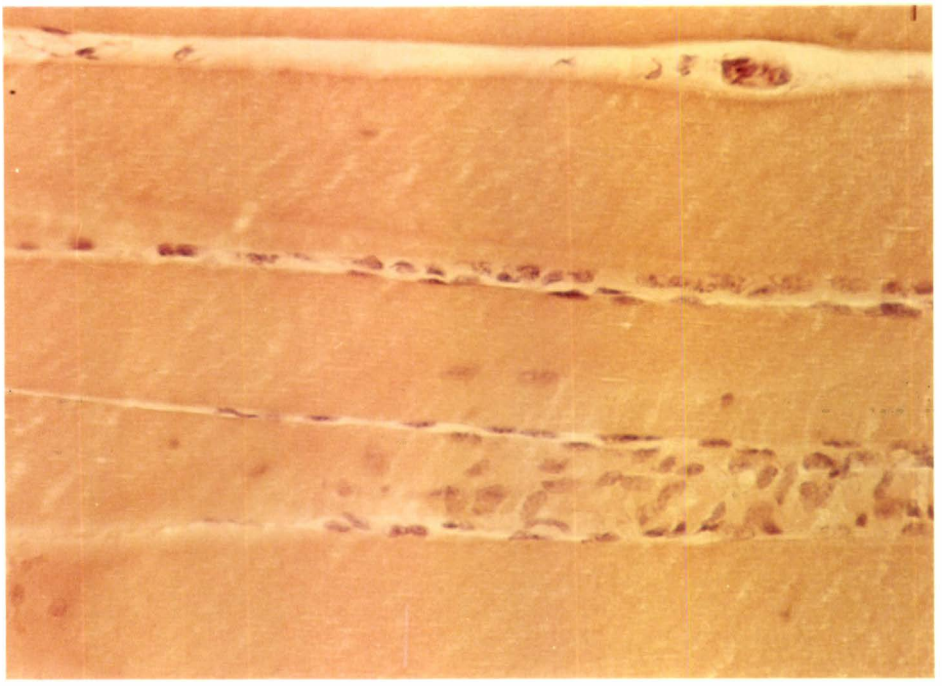
(b) Isoenzyme studies: Electrofocussed non-specific esterase isoenzymes as resolved in ampholine gels of pH 3.5-9.5 are shown as brown bands in Figure 3.40. A large number of isoenzymes were focussed in close apposition to one another and within a pH range of approximately 4.0-7.0. To facilitate analysis, esterase isoenzymes were therefore separated using an ampholine gel of pH 4.5-6.5. The

TABLE 3.8 : SUMMARY OF THE VISUALLY ASSESSED DEGREES OF ACTIVITY OF HISTOCHEMICALLY DEMONSTRATED NON-SPECIFIC ESTERASE IN VARIOUS TISSUE COMPONENTS OF BRUISED MUSCLE.

AGE (hours)	SEVERITY OF INJURY			MUSCLE FIBRES		MUSCLE INFILTRATE		INTERSTITIAL EXUDATE		c.t. SEPTA
	haem.	necro.	wbc	normal	necrotic	neut.	mononuc.	neut.	mononuc.	
4	+	+	+	-,+	-,+,++	n.s.	n.s.	+	-,+,++	-
4	+	+	+	-,+	-,+,++,+++	n.s.	n.s.	-,+	-,+,++	n.s.
4	+	+	+	-,+	-,+,++,+++	n.s.	n.s.	-	-,+,++	-,+
4	+	+	+	-,+	-,+,++,+++	n.s.	n.s.	+	-,+,++	+
24	-	-	-	-,+	n.s.	n.s.	n.s.	n.s.	-,+,++	-,+
24	+	-	+	-,+	-,+,++,+++	n.s.	-,+	n.s.	-,+,++	-,+,++
24	+	+	+	-,+	-,+,++,+++	n.s.	n.s.	n.s.	-,+,++	-,+,++
24	++	++	++	-,+	-,+,++,+++	n.s.	+,++,+++	-,+	-,+,++	-,+
48	++	+++	+++	-,+	-,+,++	n.s.	++,+++	-,+	-,+,++	-,+++
48	++	+++	+++	-,+	-,+,++,+++	n.s.	++,+++	n.s.	-,+,++	-,+
48	+	+	+	-,+	-,+,++,+++	n.s.	+,++	-	-,+,++,+++	-,+
48	+	+	+	-,+	-,+,++,+++	-,+	-,+,++	-,+	-,+	-,+,++
144	-	-	-	-,+	n.s.	n.s.	n.s.	n.s.	n.s.	-,+
144	+	-	+	-,+	-,+,++,+++	n.s.	-,++	n.s.	-,+,++	-
144	+	+	+	-,+	-,+,++,+++	n.s.	n.s.	n.s.	-,+,++	-,+,++
144	++	+++	+++	-,+	-,+,++,+++	n.s.	-,+,++,+++	n.s.	-,+,++,+++	-,+,++

FIGURE 3.37 : Bruise 4 hours old. Negative or slightly positive non-specific esterase staining of apparently normal muscle fibres.
x 320

FIGURE 3.38 : Bruise 24 hours old. Unchanged and increased non-specific esterase staining in necrotic muscle fibres.
x 320



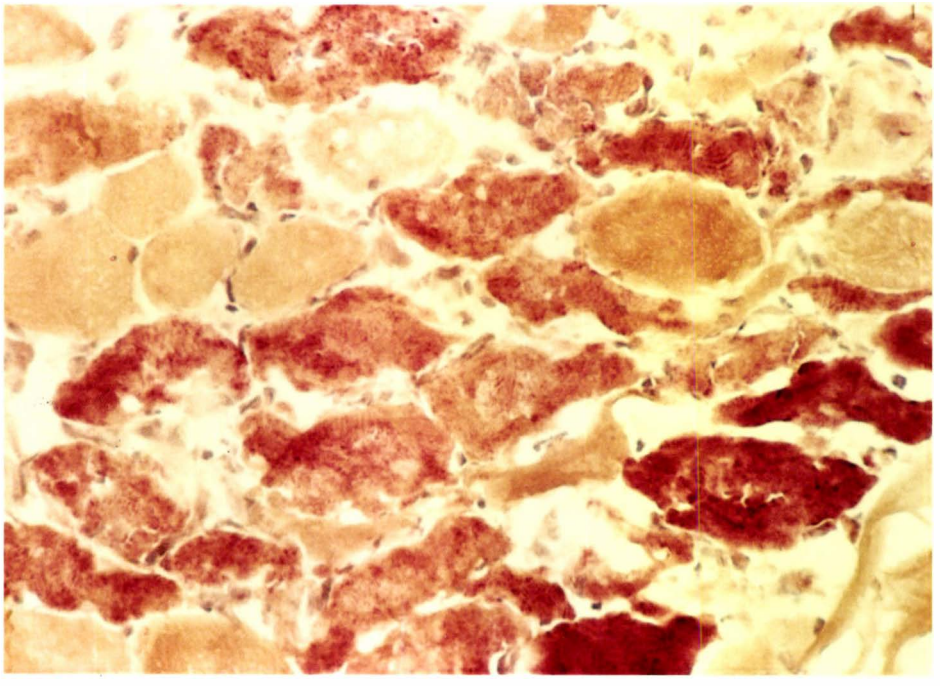
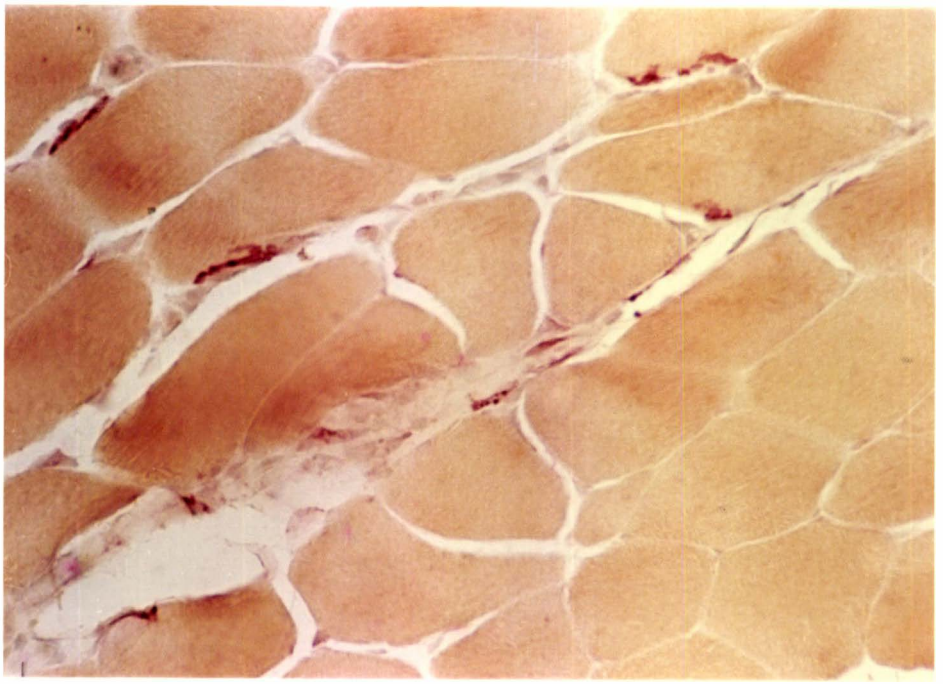
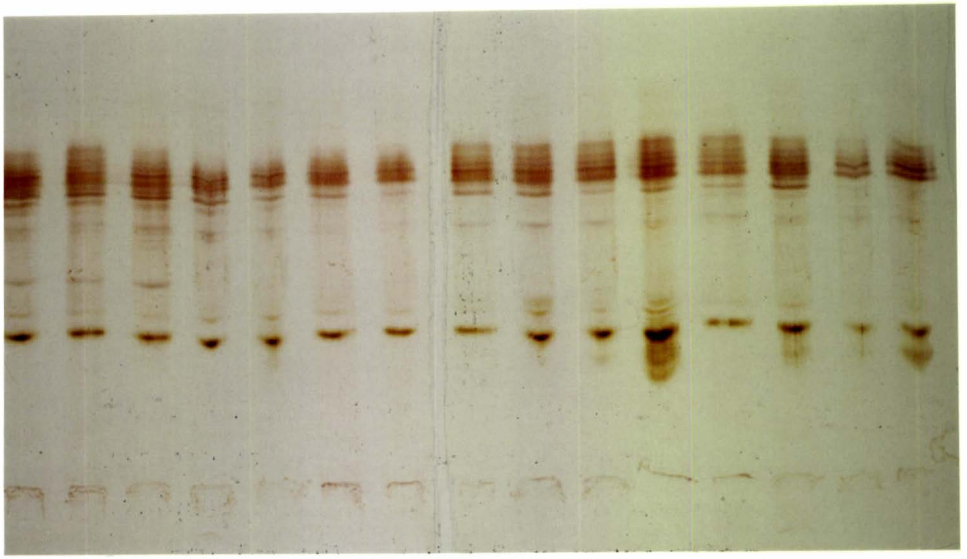


FIGURE 3.39 : Bruise 48 hours old. Strongly positive non-specific esterase staining in interstitial fibroblasts.
x 320

FIGURE 3.40 : Esterase zymogram of bruised muscle after isoelectric focussing at 25 watts constant power for 1.5 hours in PAG with ampholine buffer range pH 3.5-9.5. The gel was incubated in aqueous substrate medium for 15 minutes at $25^{o}C$.





resulting zymogram is presented in Figure 3.41. The resolution of non-specific esterase isoenzymes was clearly superior in the pH 4.5-6.5 gel. The results were analysed with the aid of a densitometer. The scan of each bruise could be interpreted in terms of the 17 bands indicated in the representative tracing in Figure 3.42. Differences existed between the scans of each bruise sample but these could not be related to common factors of anatomical position, bruise age or the animal affected. Non-specific esterase isoenzyme patterns as demonstrated by isoelectric focussing therefore proved of no value as a means of ageing bruises.

V. MISCELLANEOUS ENZYMES

Two enzymes, creatine phosphokinase and myosin adenosine triphosphatase are considered below under the heading of 'miscellaneous enzymes'.

1. Creatine phosphokinase

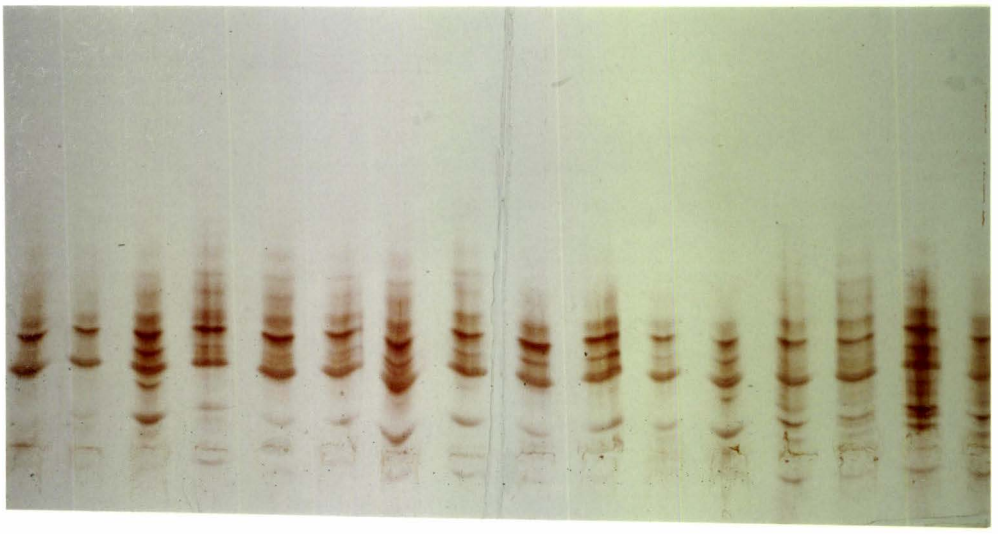
(a) Enzyme histochemistry: A summary of visually assessed enzyme activities in bruised muscle is presented in Table 3.9. Normal muscle fibres showed moderate diffuse and strong punctate creatine phosphokinase activity (Figure 3.43). In many necrotic fibres this activity persisted unchanged (Figure 3.44), but in others there was a decrease in both types of staining (Figure 3.45). There was no apparent relationship between the nature or intensity of the histochemical reaction and either the severity of necrosis or the age of the bruise.

Leucocytes demonstrated strong creatine phosphokinase activity (Figure 3.46). Fibroblasts in connective tissue septa likewise showed a strongly positive reaction in bruises of all ages (Figure 3.47).

(b) Isoenzyme studies: Creatine phosphokinase activity could not be detected in electrofocussed polyacrylamide gels incubated in aqueous substrate medium because the enzyme diffused out of the gel and reacted with the tetrazolium dye in solution. The substrate was therefore

FIGURE 3.41 : Esterase zymogram of bruised muscle after isoelectric focussing at 25 watts constant power for 2.5 hours in PAG with ampholine buffer range pH 4.5-6.5. The gel was incubated in aqueous substrate medium for 15 minutes at 25^{o}C.

FIGURE 3.42 : A representative densitometer scan of electro-focussed non-specific esterase isoenzymes obtained from bruised muscle, showing the 17 peaks represented in all the muscle samples. This particular bruise was 24 hours old. The gel had an ampholine buffer range of pH 4.5-6.5.



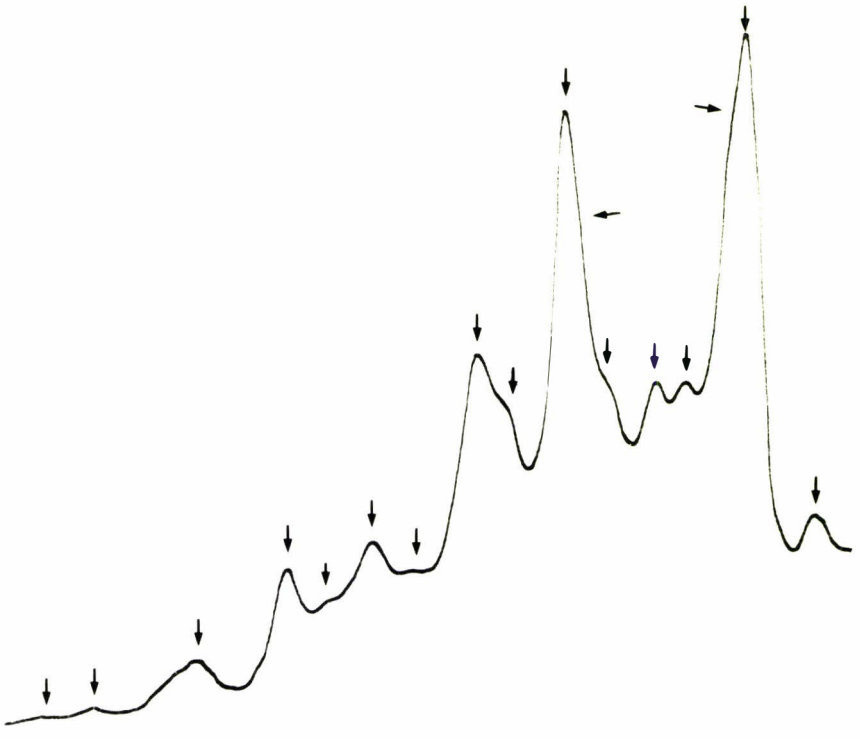
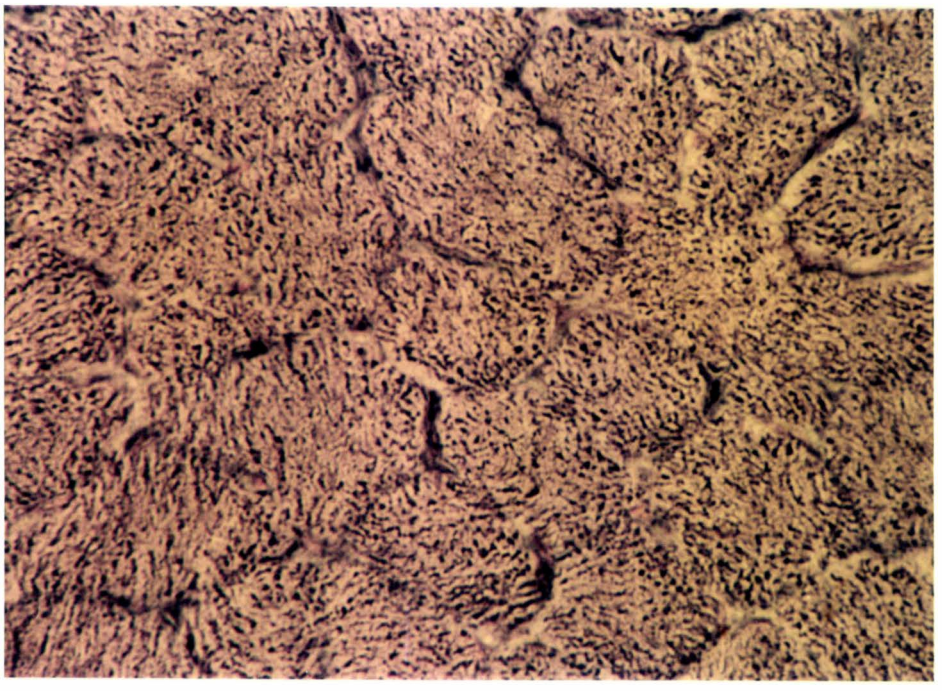


TABLE 3.9 : SUMMARY OF THE VISUALLY ASSESSED DEGREES OF ACTIVITY OF HISTOCHEMICALLY DEMONSTRATED CREATINE PHOSPHOKINASE IN VARIOUS TISSUE COMPONENTS OF BRUISED MUSCLE.

AGE (hours)	SEVERITY OF INJURY			MUSCLE FIBRES		MUSCLE INFILTRATE		INTERSTITIAL EXUDATE		c.t. SEPTA
	haem.	necro.	wbc	normal	necrotic	neut.	mononuc.	neut.	mononuc.	
4	+	-	+	++	++	n.s.	n.s.	+++	++,+++	+,++
4	+++	+++	++	n.s.	+,++	++	++	+++	+++	++
4	+	-	-	++	+++	n.s.	n.s.	+++	+++	+++
4	+	-	+	++	++	n.s.	n.s.	++	++	++
24	+++	+++	+++	++	++	++	++	+++	+++	++
24	+++	+++	+++	n.s.	+,++	++	++	++	+++	++
24	++	+++	+++	++	+,++	++	++	++	++	++
24	++	+	++	++	+	++	++	+++	+++	++
48	++	+++	+++	n.s.	++,+++	++	++	+++	+++	+++
48	++	-	+	++	++	++	++	++	++	+++
48	+++	+++	+++	++	+++	++	++	+++	+++	+++
48	+++	+++	+++	++	++	++	++	++	++	+++
144	+++	+++	++	++	+	+++	+++	+++	+++	n.s.
144	+	-	-	++	n.s.	n.s.	n.s.	n.s.	n.s.	+++
144	+++	++	+++	++	+,++	++	++	+++	+++	++
144	+	-	++	++	++	n.s.	n.s.	+++	+++	+++

FIGURE 3.43 : Bruise 144 hours old. Moderate diffuse and strong punctate creatine phosphokinase staining in apparently normal muscle fibres.
x 500

FIGURE 3.44 : Bruise 24 hours old. Unchanged creatine phosphokinase staining in severely necrotic muscle fibres.
x 320



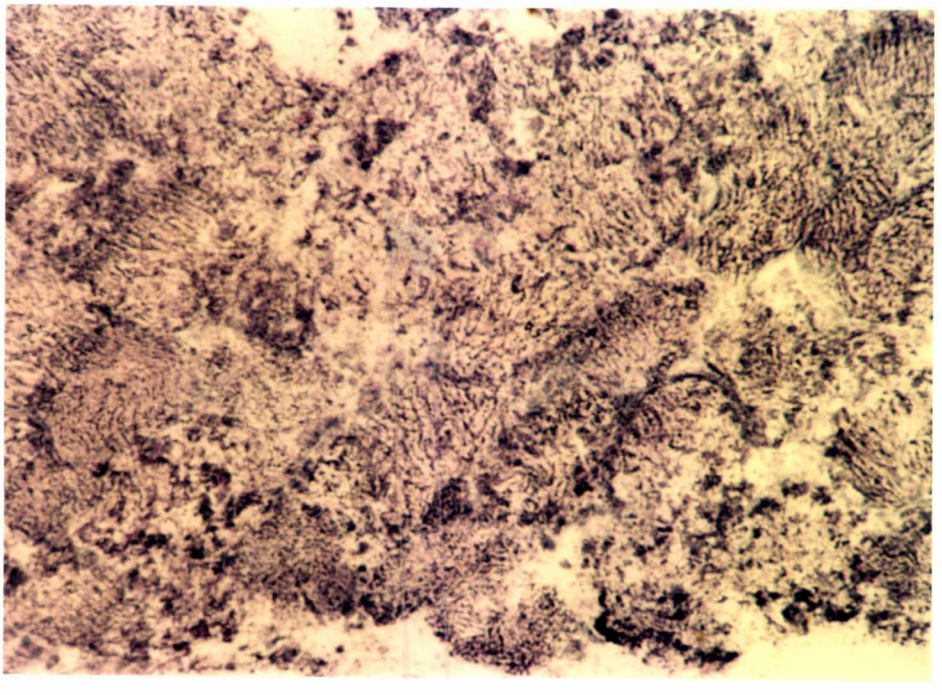
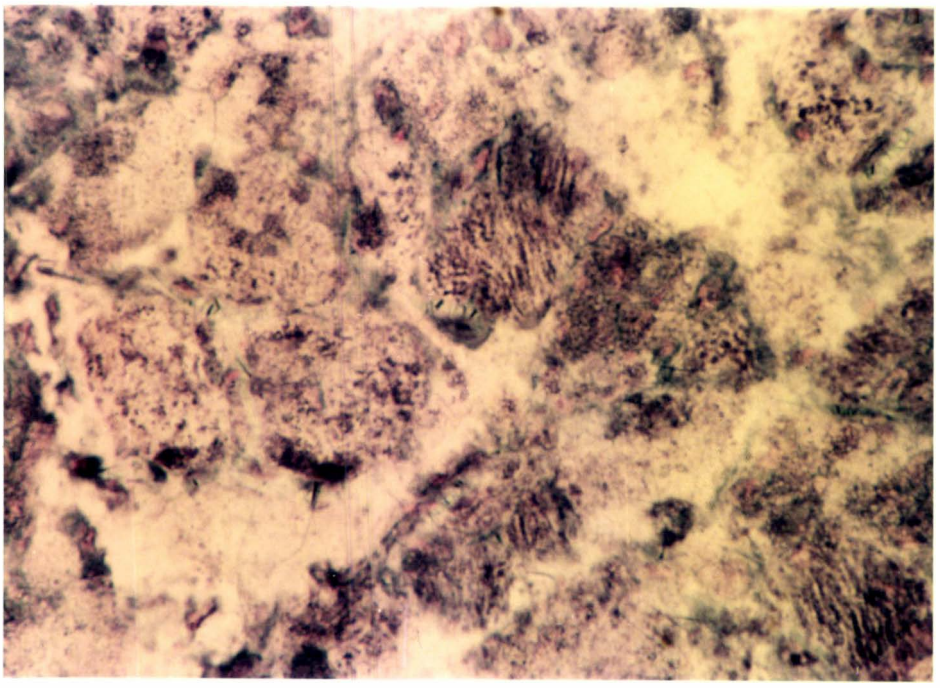


FIGURE 3.45 : Bruise 48 hours old. Decreased creatine phosphokinase staining in necrotic muscle fibres.
x 320

FIGURE 3.46 : Bruise 144 hours old. Strongly positive creatine phosphokinase activity in the leucocytic exudate. The necrotic fibres show decreased activity.
x 320



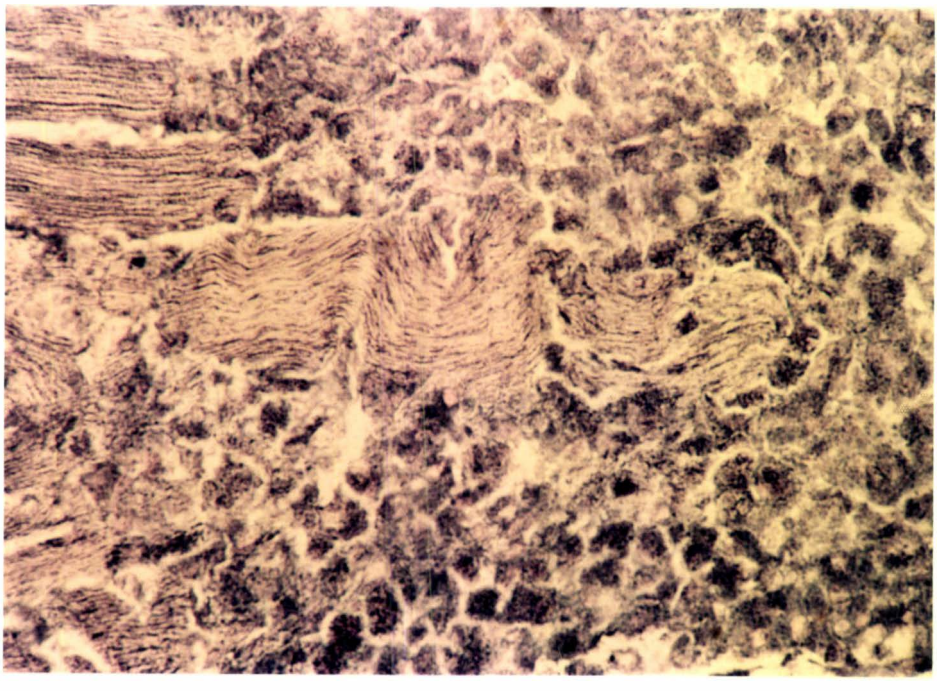
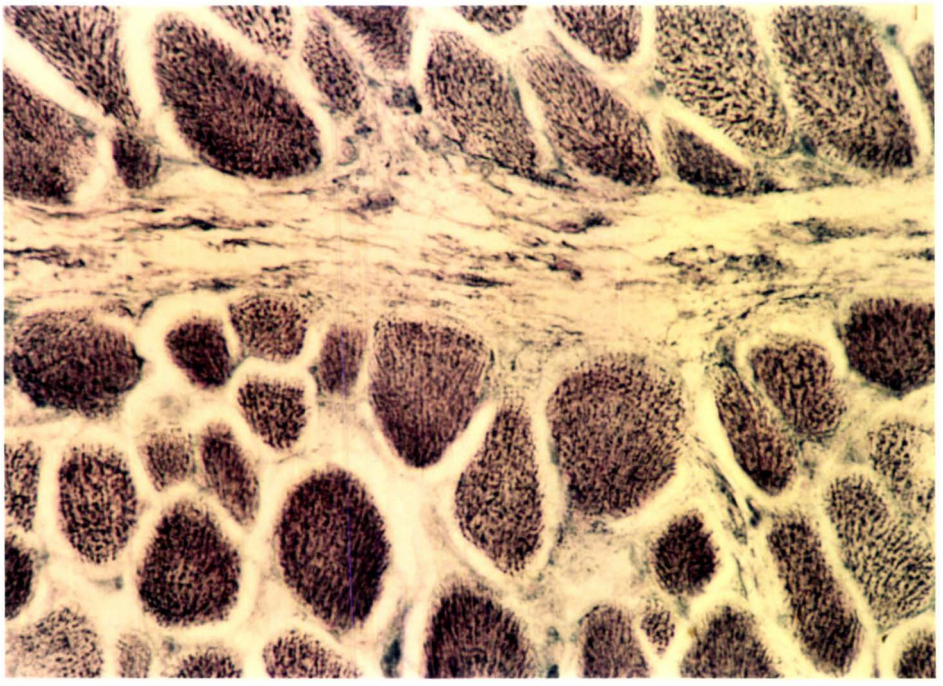
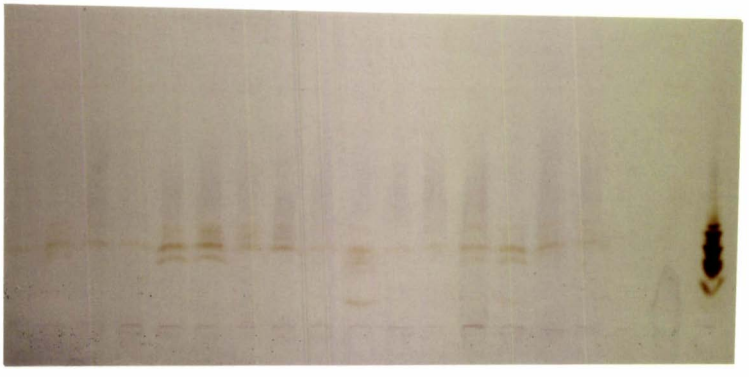


FIGURE 3.47 : Bruise 48 hours old. Strongly positive creatine phosphokinase staining of fibroblasts in connective tissue septum. x 320

FIGURE 3.48 : Creatine phosphokinase zymogram of bruised muscle, leucocytes, plasma and erythrocytes after isoelectric focussing at 25 watt constant power for 1.5 hours in PAG with ampholine buffer range pH 3.5-9.5. The substrate was incorporated in a 2% agarose gel which was applied to the electrofocussed gel for 30 minutes at $37^{O}C$.





incorporated into a 2% agarose gel which was applied directly to the electrofocussed gel. The resulting zymogram is shown in Figure 3.48. Creatine phosphokinase activity was detected in bruise homogenates and plasma. However, diffusion remained a problem, as was indicated by residual staining of the substrate gel. Isoenzyme patterns were therefore investigated after electrophoresis in cellulose acetate plates (Figure 3.49). There was only one isoenzyme per bruise, situated in a position characteristic of that for normal mature striated muscle. Differences existed between the intensity of creatine phosphokinase activities between bruises, but these could not be related to common factors of anatomical position, bruise age or the animal affected. Creatine phosphokinase isoenzyme patterns, as demonstrated both by isoelectric focussing in polyacrylamide gels and by electrophoresis in cellulose acetate plates, therefore proved of no value as a means of ageing bruises.

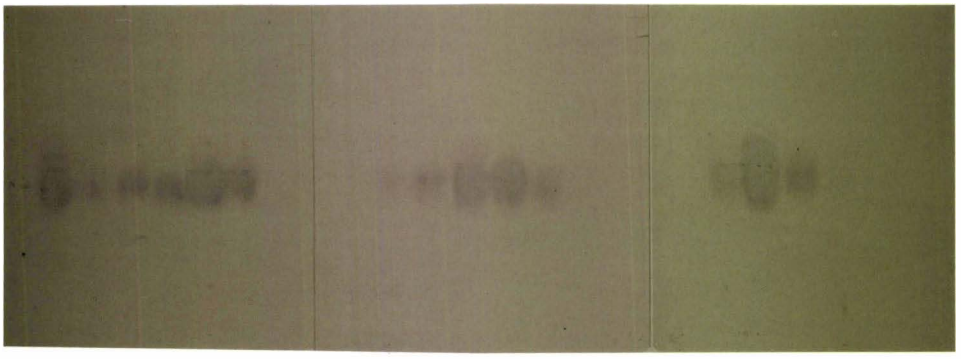
2. Myosine adenosine triphosphatase

(a) Enzyme histochemistry: The normal expected variation in adenosine triphosphatase activity according to fibre type (Figure 3.50) was maintained even in advanced stages of myonecrosis (Figure 3.51). Enzyme activity in leucocytes and fibroblasts within connective tissue septa was very mild and therefore difficult to evaluate (Figure 3.52) but there were no apparent differences between the staining characteristics of these cells in bruises of different ages.

(b) Isoenzyme studies: No attempt was made to produce an adenosine triphosphatase zymogram since preliminary experiments had revealed technical difficulties in so doing.

FIGURE 3.49 : Creatine phosphokinase zymogram of bruised muscle, leucocytes and erythrocytes after electrophoresis at 250 volts for 90 minutes in cellulose acetate plates. The substrate was applied to a second cellulose acetate plate as a 0.5% noble agar gel. This plate was firmly applied to the first and the pair incubated for 20 minutes at 37°C.

FIGURE 3.50 : Bruise 48 hours old. Variation in adenosine triphosphatase staining in normal muscle fibres according to fibre type in apparently normal muscle.
x 50



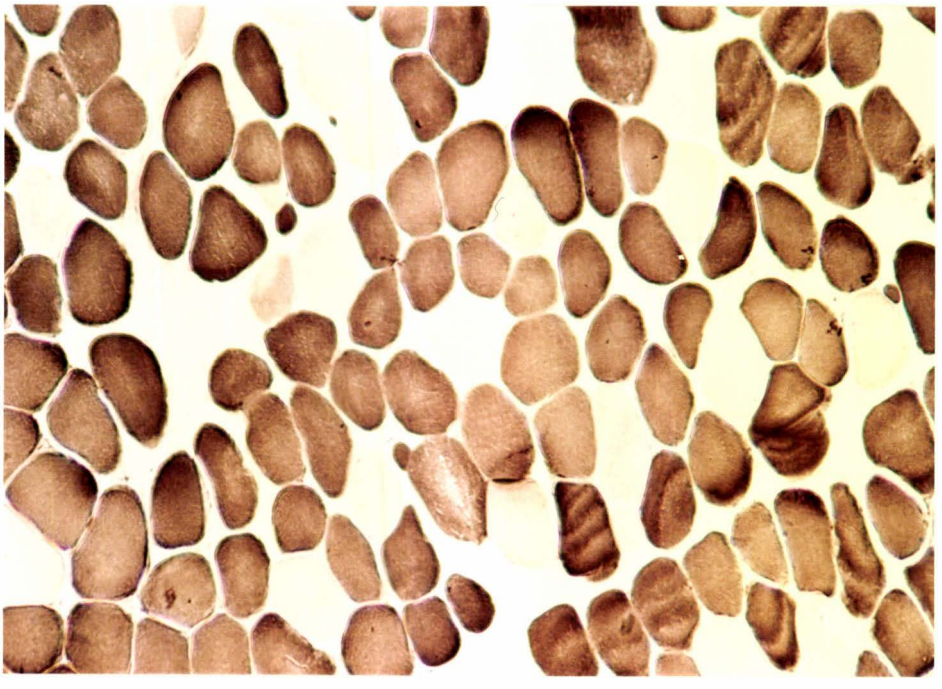
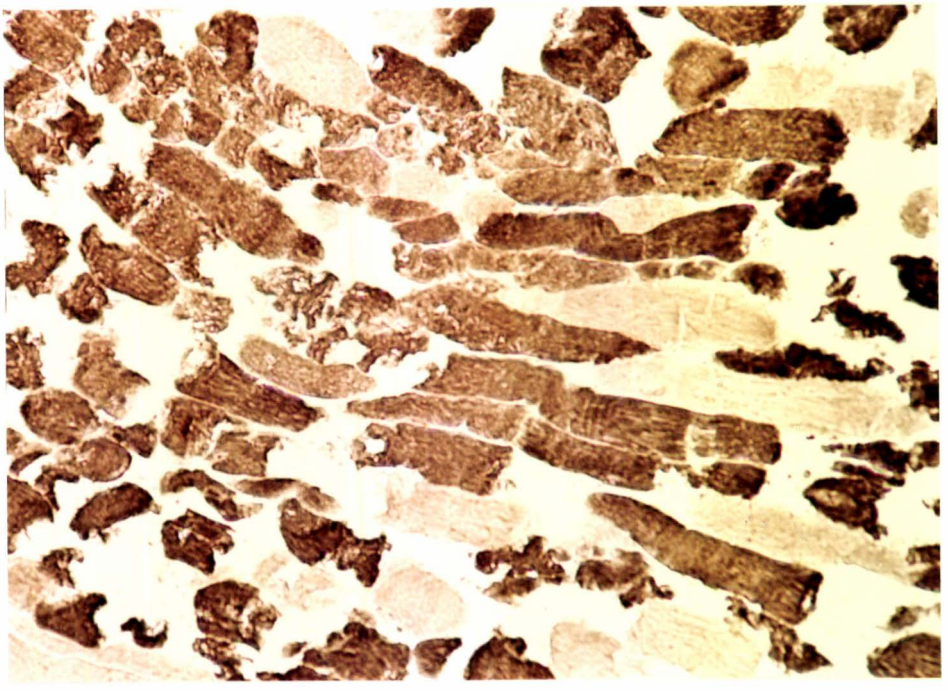
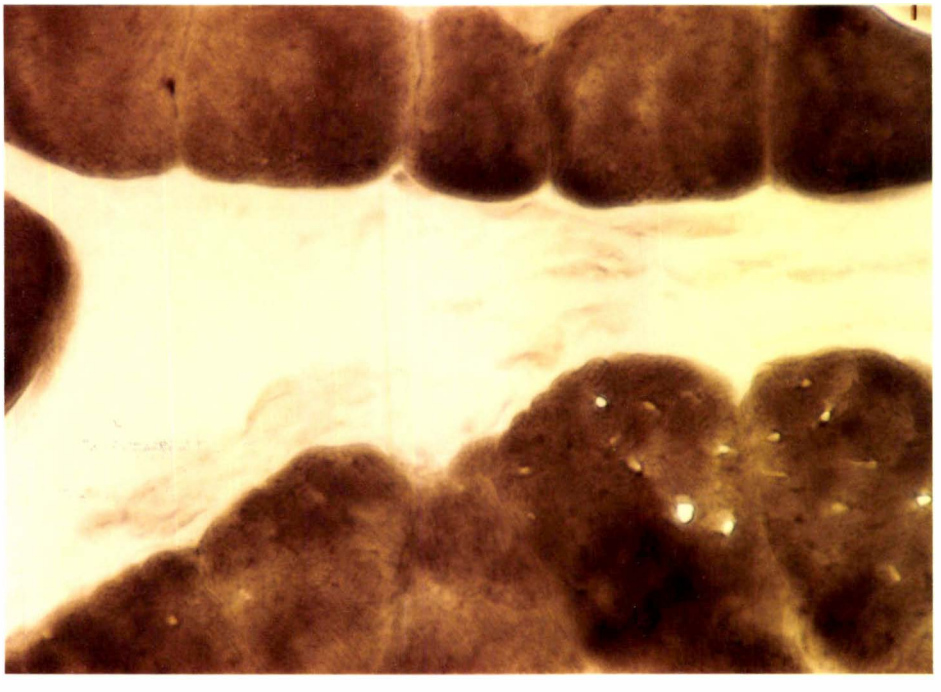


FIGURE 3.51 : Bruise 24 hours old. Variation in adenosine triphosphatase staining according to fibre type is maintained in areas of muscle necrosis. x 125

FIGURE 3.52 : Bruise 24 hours old. Very mild and therefore equivocal adenosine triphosphatase staining of fibroblasts in connective tissue septum. x 500





DISCUSSION

The activities of 10 enzymes were examined in bruises, and in some cases leucocytes, plasma and erythrocyte preparations. The latter samples were included in case age-dependent changes occurred in the bruises, whereupon it would have been desirable to have identified the tissue component to which this activity was attributable. Both histochemical and isoelectric focussing techniques were utilised because separate advantages and disadvantages are inherent in each (Dahl and From, 1971; Pullar, 1973; Bocking and Riede, 1979). The enzymes studied were those with previously recorded evidence indicating a potential usefulness for wound ageing purposes. The experimental bruises studied were aged from 4-144 hours old, thereby encompassing a range of tissue reactions from early degenerative change through to repair and regeneration. Only bruised muscle was studied because adipose tissue has a non-specific affinity for the majority of the chromogenic dyes used for detecting enzymes.

Enzyme histochemistry

A number of observations have attested to the usefulness of enzyme histochemistry for ageing wounds by virtue of sequential changes in tissue enzyme activities that occur in response to injury. The recorded times of change vary due to factors such as the observer, the tissue involved and the species of animal affected, but the approximate order of altered enzymic activity relates to glycogen phosphorylase, non-specific esterase, adenosine triphosphatase, leucine aminopeptidase, acid phosphatase, alkaline phosphatase and various dehydrogenases (Raekallio, 1973). The earliest enzymic changes have usually occurred in fibroblasts (Raekallio and Nieminen, 1979) and these have preceded other light microscopic responses to injury. Enzyme changes in muscle have usually occurred later, concurrently with visible signs of necrosis and regeneration of damaged fibres (Hirvonen and Ojala, 1968).

In our studies, variation in enzyme staining was frequently observed in apparently normal muscle fibres. This was expected and can be

explained by the existence of two main types of muscle fibres with different enzyme profiles according to whether they possess a predominantly oxidative or glycolytic metabolism (Meijer, 1970). Degenerative muscle fibres generally showed decreased histochemical activity for aldolase, creatine phosphokinase, lactate dehydrogenase, succinate dehydrogenase and non-specific esterase, and increased activity for leucine aminopeptidase and glucose-6-phosphate dehydrogenase. Acid and alkaline phosphatase activities remained unchanged in damaged fibres. Leucocytes and connective tissue fibroblasts occasionally showed increased enzyme activities. However, patterns of change failed to emerge in any of these tissues and no relationship could be established between enzyme activities and bruise ages.

In light of the body of evidence supporting the concept that histochemical enzyme activities show time-related changes in wounds (Raekallio, 1977), it is difficult to explain the lack of an observed relationship for the bruises in this experiment. The procedures to which muscle was subjected when demonstrating the enzymes included freezing, fixation, sectioning and staining. Each of these can exert a degree of adverse effect on enzyme activity sufficient to affect the results of an experiment of this nature (Pearse, 1968). Care was therefore taken to process the bruises studied using only reliable histochemical techniques of proven sensitivity, and unless more recent developments dictated otherwise, preference was given to methods previously used successfully for wound ageing purposes. For this reason the lack of observed relationships between histochemical enzyme activities and bruise ages appears unlikely to reflect technical inadequacies in the experiment.

It is thought more likely that our failure to demonstrate age related changes in enzyme activities was associated with the relatively mild inflammatory and reparative reactions in the bruises studies, as compared to the more intensive reactions in incision and thermal injuries (Fatteh, 1966a; Fine *et al.*, 1966; Makinen and Raekallio, 1967; Hirvonen, 1968a; Raekallio and Makinen, 1969a, 1971a; Wagner *et al.*, 1978; Max and Wagner, 1979). However, against this is the fact that we did observe non-temporal related changes in fibroblasts,

the cell type showing the most significant changes in the other studies, and the fact that Josza (1974) has recorded age related histochemical enzyme changes in bruises. It is unfortunate that in the reports mentioned above the evidence is largely descriptive and there is insufficient documentation of quantitative or semi-quantitative data to allow independent assessments of these experiments in the light of our results. Our conclusions are in accord with those of Hodson (1962), Hirvonen, (1968a), Hou-Jensen (1968) and McCausland and Dougherty (1978) who expressed their beliefs that histochemical enzyme reactions following injury provide no advantage over conventional histological methods for ageing wounds.

Isoenzyme studies

A number of experiments have suggested the worth of isoenzyme studies for wound ageing purposes, but these have lacked the scale and methodical quality of corresponding enzyme histochemical investigations.

The various techniques for separating isoenzymes include protein precipitation, chromatography, gel filtration, electrophoresis and isoelectric focussing. Of these, isoelectric focussing, in which proteins are separated according to their isoelectric points, is one of the most sensitive methods and has the added advantage of technical simplicity (Latner, 1973; Metzler, 1977). Where possible, the isoenzyme patterns of bruised muscle homogenates were therefore analysed after isoelectric focussing in polyacrylamide gels. Measures were taken to increase the intensity of enzyme staining and to minimise artefacts such as skewing of bands and protein streaking. These included extraction of enzyme in 0.1% Triton X-100, physical removal of particulate adipose tissue, concentration of enzyme by freeze-drying and dialysis of supernatants against 1% glycine. These procedures were based on methods previously employed in the investigation of wounds and had been shown in preliminary experiments not to fundamentally alter zymogram patterns.

For most of the enzymes, few or no isoenzyme bands were resolved from

the muscle homogenates. Only non-specific esterase and lactate dehydrogenase were separated into a large number of bands. In no case could a relationship be detected between either the position or the relative intensities of isoenzyme bands and common factors of anatomical position, bruise age or the animal affected.

The lack of an association between the ages of bruises and isoenzyme patterns places the results of this experiment in apparent conflict with the majority of recorded observations of isoenzyme patterns in both skin and muscle wounds (Jarecki *et al.*, 1970; Raekallio and Makinen, 1971b; Makinen and Raekallio, 1973; Bonte, 1978; Meijer and Israel, 1979b; Miranda *et al.*, 1979; Bonte and Bode, 1981). Undoubtedly some enzyme activity was lost during the preparation of the zymograms. However, the methods of extraction and visualisation of isoenzymes were closely based on, and in most cases identical to, those used in previous experiments purporting to have demonstrated altered isoenzyme patterns in damaged tissues. Critical losses of enzyme activity were therefore unlikely to have occurred. Furthermore, comparisons between histochemical and biochemical observations revealed an overall correlation between these two sets of results, suggesting that the zymogram results accurately reflected the true enzyme status of bruised muscle. Also significant was the fact that even those enzymes with strong activity, e.g. esterase and lactate dehydrogenase, showed no association between isoenzyme patterns and bruise ages. The apparent conflict between the results of this experiment and other recorded observations is unlikely, therefore, to reflect technical inadequacies in the methods used for demonstrating isoenzymes in bruises.

Published conclusions concerning isoenzyme patterns during the early stages of wound healing have been based on results for incision wounds in skin. Our inability to reproduce these results in experimental bruises may therefore reflect differences in the relative intensities of the fibroblastic response in the two types of wounds. In this respect the situation regarding isoenzyme studies is similar to that previously considered for enzyme histochemistry. A regression to foetal isoenzyme patterns has been demonstrated in older wounds affect-

ing muscle (Wagner *et al.*, 1978; Miranda *et al.*, 1979), but the severity of injury was such that muscle regeneration had become a prominent feature of the tissue reactions concerned. This was not true of the experimental bruises in this study since regeneration failed to become a prominent feature of the reaction even 144 hours after injury. The absence of altered enzyme patterns characteristic of regenerating muscle in older wounds (Mauro, 1979) was therefore not surprising.

The published experiments from which conclusions regarding time-related changes in isoenzyme patterns have been based were designed primarily to investigate differences between *ante mortem* and *post mortem* wounds, with wounds of various ages being considered mainly in this context. In some respects existing conclusions concerning *ante mortem* wounds of various ages are therefore open to question. In particular there has been insufficient evaluation of the magnitude of within-age as compared to between-age sample variations. The proposed temporal progressions in isoenzyme patterns of wounds may therefore have merely reflected sample variations that existed independently of age considerations. Such was the case in our experiment. Only Bonte (1978) has demonstrated good correlations between zymograms of wounds of the same ages, but he failed to identify the source of his wounds. Had all wounds of a particular age in his experiment been derived from one animal, a reasonable degree of within-age correlation might have been expected, since for unknown reasons even control tissues from distant sites in the same animal may acquire similar isoenzyme patterns to the site of injury (Makinen and Raekallio, 1973).

It was concluded that under the conditions of this experiment neither histochemical nor isoenzyme studies proved of value for differentiating between bruises aged 4, 24, 48 and 144 hours old. However, by virtue of the appearance of previously absent leucine aminopeptidase and glucose-6-phosphatase activity in degenerative muscle fibres within 4 hours of injury, stains for these enzymes may be of limited use in ageing bruises less than 4 hours old.

CHAPTER IV

pH CHANGES IN BRUISED MUSCLE

INTRODUCTION

Tissues undergoing inflammation frequently become more acidic than normal due to a build up of lactic acid as an end product of anaerobic glycolysis, chiefly by infiltrating leucocytes (Ryan, 1977). Changes in pH could therefore be expected to occur in bruised muscle. These anticipated changes were to be related to age in an attempt to discover an alternative approach to the ageing of bruises other than by histology or enzyme studies.

MATERIALS AND METHODS

I. TECHNIQUE FOR MEASUREMENT OF MUSCLE pH

A New Zealand Romney lamb was killed by a transverse incision severing the soft tissues of the neck and the spinal cord at the level of the atlanto-occipital joint. Two x 2.0g samples of muscle were taken from the *Biceps femoris* or the *Triceps brachii* of each leg immediately after death. One sample from each site was stored for 1 hour *post mortem* in a small capped vial then homogenised in 20ml 5mM sodium iodoacetate for 3 minutes in a 'Colworth Stomacher'. This prevented further production of lactic acid by anaerobic glycolysis. The remaining samples were overlayered with paraffin oil to exclude atmospheric oxygen, maintained at 27^{o}C for 24 hours then homogenised as above (Petersen, 1982). Each homogenate stood in atmospheric conditions at room temperature for 8 hours, during which time sequential pH measurements were made.

II. MEASUREMENT OF BRUISE AND CONTROL MUSCLE pH VALUES

1. Experimental animals

A total of 25 Suffolk-cross lambs approximately 6 months of age and of mixed sex were divided into five groups of five lambs. Each group was kept in a pen and given hay and water *ad libitum* for six days, then deprived of food and water for 24 hours prior to slaughter.

2. Bruising of animals

The right fore and hind limbs were bruised once each at the same sites and by the same impact method described in previous chapters. All the lambs in any one group were bruised at one time so as to produce five groups of ten bruises, aged either 4, 16, 24, 36 or 48 hours old. The left fore and hind limbs remained unbruised.

3. Slaughter of animals and processing of muscle samples

Each lamb was killed by exanguination as described, and two x 2.0g samples of (a) bruised muscle, (b) control muscle adjacent to bruises and (c) control muscles from equivalent contra-lateral sites to the above were taken as soon as possible after death. These samples were processed for pH measurement as described previously, except the first samples were homogenised 3 hours *post mortem* and pH values measured 4 hours after this, whilst pH values of the 24 hours *post mortem* samples were measured only 30 minutes after they had been homogenised.

RESULTS

I. TECHNIQUE FOR MEASUREMENT OF MUSCLE pH

Homogenate pH values of the samples processed 1 hour *post mortem* were measured 10 minutes, 20 minutes, 1, 1.5, 2, 3, 4 and 8 hours after standing in atmospheric conditions at room temperature. Those of the samples processed 24 hours *post mortem* were measured 10 minutes, 15 minutes, 1.5 and 7 hours after standing under the same conditions. The results are plotted in Figures 4.1 and 4.2 respectively. Homogenate pH values of the 1 hour *post mortem* samples rose sharply over the first 2 hours but stabilised after 3 hours, whilst those of the 24 hour *post mortem* samples had stabilised within 15 minutes. The rise in pH was thought to be due to equilibration between the partial pressures of sample and atmospheric carbon dioxide. It was therefore concluded that the pH of samples taken close to time of death should be measured at least 3 hours after homogenisation in iodoacetate, whereas a delay of only 30 minutes was sufficient for samples processed after 24 hours.

II. MEASUREMENT OF BRUISE AND CONTROL MUSCLE pH VALUES

1. Bruise pH values

The pH of bruises of various ages was measured in samples taken 3 hours and 24 hours after death. These values are presented graphically in Figures 4.3 and 4.4. Inspection of these figures revealed an overlap between the pH values of bruises of each age. Student's 't' tests showed the hind limb samples to be significantly lower ($P < 0.05$) than those of equivalent forelimb samples. The presence or absence of a relationship between bruise pH and bruise ages was examined further by performing analysis of variance tests on these data. In neither case was there a statistically significant relationship between bruise pH and age.

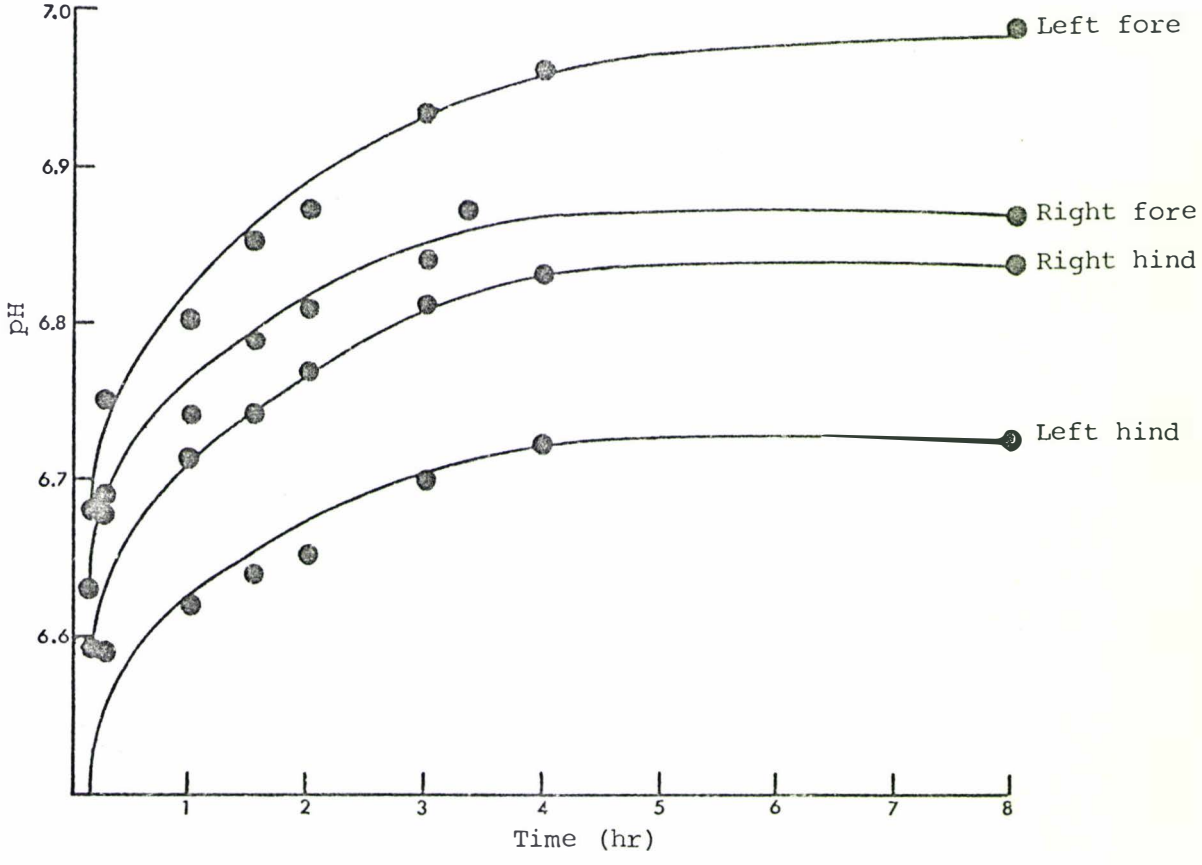


FIGURE 4.1 : Sequential pH measurements of normal muscle sampled 1 hour *post mortem* and homogenised in 5mM sodium iodoacetate.

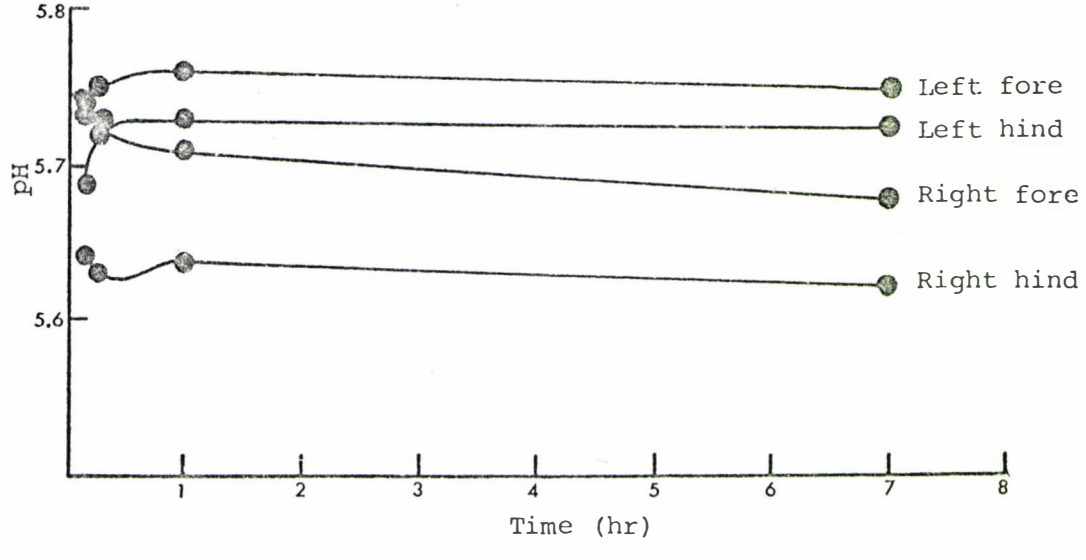


FIGURE 4.2 : Sequential pH measurements of normal muscle sampled 24 hours *post mortem* and homogenised in 5mM sodium iodoacetate.

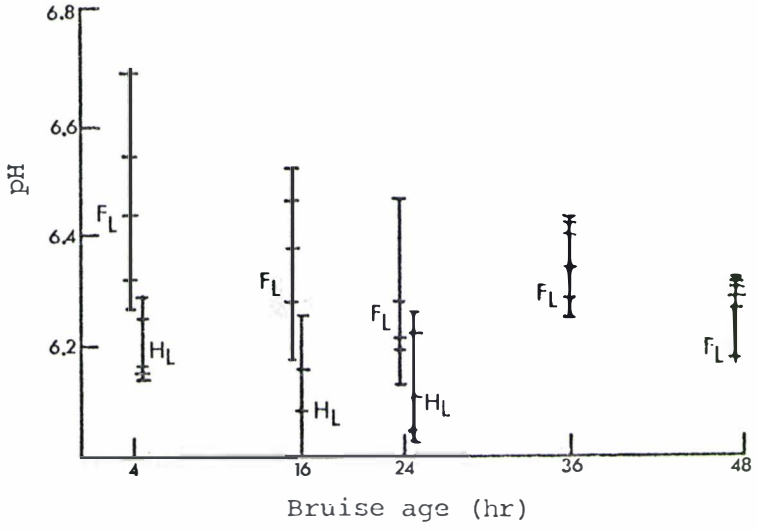


FIGURE 4.3 : Ranges of fore and hind limb pH values in 3 hour *post mortem* samples of bruises of different ages. Hind limb values are offset to the right for clarity.

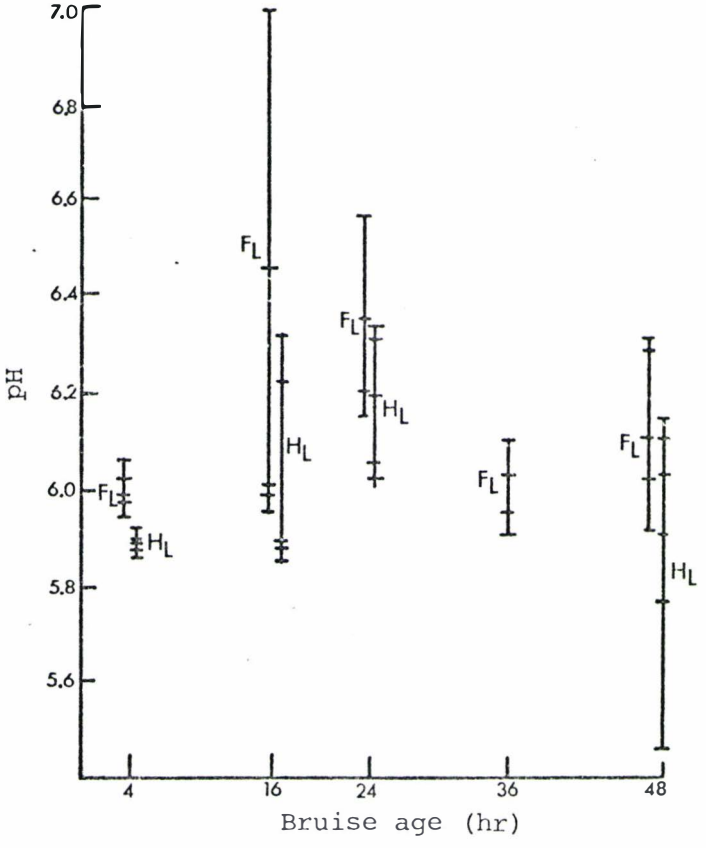


FIGURE 4.4 : Ranges of fore and hind limb pH values in 24 hour *post mortem* samples of bruises of different ages. Hind limb values are offset to the right for clarity.

2. Bruise pH values relative to those of appropriate controls

The differences in Figures 4.3 and 4.4 between the fore and hind limb bruise pH values probably reflected underlying differences in normal pH between muscles. Normal muscles may in fact differ both within and between muscles of the same animal, and between muscles of different animals (Petersen, *personal communication*). For this reason the above bruise pH values were examined in relation to those of appropriate controls, using the latter as estimates of pre-bruise pH.

(a) Selection of appropriate control muscle samples: The following three control tissues were selected for study:

(i) Normal tissue from within bruised muscle = C1;

(ii) Normal tissue from a similar position to the above in unbruised contralateral muscle = C2;

(iii) Normal tissue from a similar position to the bruise but from contralateral muscle = C3.

Pre-bruise pH values can be estimated using either a contralateral or ipsilateral control muscle sample. The differences between contralateral (C1-C2) and ipsilateral (C2-C3) normal pH values were therefore examined using Student's paired 't' test to see which pair of values were the most similar. Data for 3 hour and 24 hour *post mortem* samples are summarised in Table 4.1. There were no significant differences between within-muscle controls, nor, with one exception, between contralateral controls. The exception was with 3 hour *post mortem* foreleg contralateral controls.

From these results it was concluded that as estimates of pre-bruise pH, either within-muscle or between contralateral muscle controls were acceptable, but that within-muscle controls were marginally superior. In practice, within-muscle sampling would also be more acceptable to the meat industry.

TABLE 4.1 : RESULTS OF STUDENT'S PAIRED t TESTS EXAMINING LEVELS OF SIGNIFICANCE OF pH DIFFERENCES BETWEEN CONTRALATERAL (C1-C2) AND ALSO BETWEEN WITHIN-MUSCLE (C2-C3) CONTROLS.

Sample time	LEVEL OF SIGNIFICANCE OF pH DIFFERENCES	
	Between contralateral sides (C1-C2)	Within-muscle (C2-C3)
3 hours		
Fore limb	0.05	n.s.
Hind limb	n.s.	n.s.
24 hours		
Fore limb	n.s.	n.s.
Hind limb	n.s.	n.s.

(b) Bruise pH values relative to those of within-muscle controls: The significance of differences between bruise and within-muscle control pH values were examined using Student's paired 't' test. The results which are summarised in Table 4.2, showed that with one exception bruise pH values were significantly higher than those in controls, but only by an average of 0.2 of a pH unit. The exception was with 3 hour *post mortem* hind limb bruise samples, which were not significantly different in pH from controls.

Differences in pH between bruises and within-muscle control samples are illustrated graphically in Figures 4.5 and 4.6 for 3 hour and 24 hour *post mortem* samples respectively. Inspection of these figures revealed an overlap between the pH differences of bruises of each age but no apparent differences between the values relating to fore and hind limb samples. Analyses of variance on these data showed pH differences between bruises and controls to be unrelated to the age of the bruise.

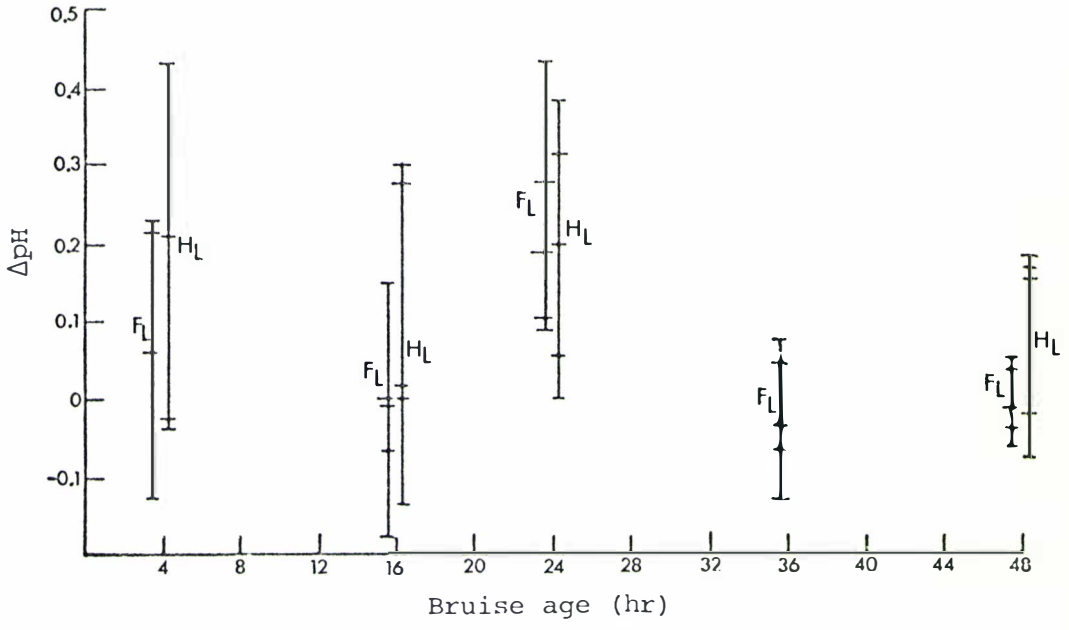


FIGURE 4.5 : Ranges of bruise/control pH differences for 3 hour *post mortem* fore and hind limb muscle samples of bruises of different ages. Hind limb values are offset to the right for clarity.

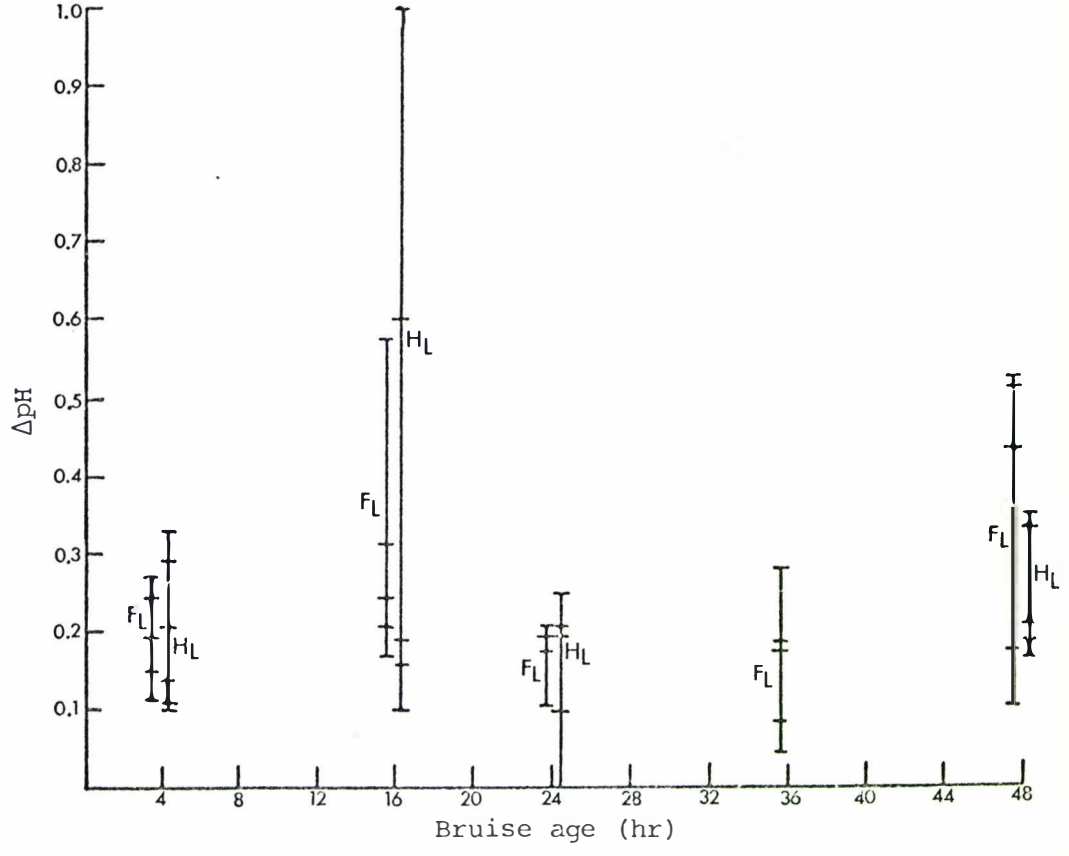


FIGURE 4.6 : Ranges of bruise/control pH differences for 24 hour *post mortem* fore and hind limb muscle samples of bruises of different ages. Hind limb values are offset to the right for clarity.

TABLE 4.2 : AVERAGE MUSCLE pH OF BRUISES AND CONTROLS, ALONG WITH RESULTS OF STUDENT'S t TESTS ON pH DIFFERENCES BETWEEN BRUISE/CONTROL SAMPLE PAIRS.

	FORE LIMB		HIND LIMB	
	3 hour samples	24 hour samples	3 hour samples	24 hour samples
Bruise pH	6.34	6.14	6.11	5.98
Control pH	6.27	5.92	6.14	5.69
Difference in pH	0.07	0.22	-0.03	0.29
Significance of difference	$P<0.05$	$P<0.001$	n.s.	$P<0.01$

DISCUSSION

Localised tissue pH changes occur in areas of inflammation (Walter and Israel, 1972). At first there may be a transient basic phase due to plasma exudation (Cheville, 1976) but the predominant reaction is acidic and reflects the accumulation of lactic acid as an end product of anaerobic glycolysis by invading leucocytes and partially ischaemic tissues (Ryan, 1977). The tissue reaction to bruising injury is one of inflammation, repair and regeneration, so changes in pH were expected to occur in bruised muscle. By relating these anticipated changes to the ages of experimental bruises, an attempt was made to develop an alternative approach to the ageing of these lesions other than by histology or enzyme studies.

The bruises studied ranged in age from 4-48 hours old so as to encompass a spectrum of tissue changes from degeneration through to early regeneration and repair. Extraneous factors such as stress and planes of nutrition affect glycogen levels in muscle, so the *ante mortem* treatment of lambs was standardised as far as was practicable under the experimental conditions. All of the lambs in any one group were bruised at one time in order to equalise the degrees of pre-bruise stress.

Muscle pH was measured using a standard technique in which tissue samples were first homogenised in a 5mM sodium iodoacetate solution to prevent further production of lactic acid by anaerobic glycolysis. Samples were taken 3 hours and 24 hours after death. The 24 hour samples were to provide an indirect measure of the amounts of and availability of stored glycogen in bruised muscles, whereas the 3 hour samples were to provide as close an estimate of *ante mortem* bruise pH as was practicable under the conditions of the experiment. It was recognised that the pH of 3 hour *post mortem* samples would be somewhat different to those of bruised muscle at the time of slaughter because of continuing anaerobic glycolysis during the intervening period. However, this experiment was undertaken primarily to determine the practical value of pH measurements as a means of ageing bruises, and similar *post mortem* delays would be unavoidable under normal operating conditions in a meat works.

The upward drifts in pH measured in the iodoacetate homogenates of 3 hour *post mortem* samples continued for 3 hours, by which time a change of approximately 0.3 pH units had occurred. From their direction and time-course, the observed drifts in pH were probably due to equilibration between the partial pressures of sample homogenate and atmospheric carbon dioxide. Similar changes occur when fresh blood is equilibrated with air (Greenway, *personal communication*).

Absolute pH values varied widely between bruises of the same age, and an analysis of variance on these data showed there was no significant relationship between muscle pH and bruise age. There were, however, differences between fore and hind limb bruise pH's, with those of the hind limbs being consistently lower than equivalent forelimb bruises. For this reason pH changes were investigated relative to controls of normal muscle, using the latter as estimates of pre-bruise pH. Most of the bruises were slightly but significantly ($P<0.05$) less acidic than control tissues. Since the differences were more pronounced in the 24 hour *post mortem* samples this presumably reflected an *ante mortem* depletion of glycogen stores in bruised muscle. An analysis of variance showed there was no statistically significant relationship between paired bruise/control pH differences and the ages of bruises.

In conclusion, it was not possible to demonstrate statistically significant relationships between bruise ages and either absolute or relative muscle pH values.

CHAPTER V

PETECHIAL HAEMORRHAGE IN THE CARCASE FAT OF SLAUGHTERED LAMBS

INTRODUCTION

At the beginning of the 1977/78 killing season a number of meat works experienced a previously unreported problem of petechial haemorrhages affecting the carcase fat of young lambs processed for export (Frazerhurst *et al.*, 1978). In subsequent seasons these lesions, which became known as 'subcutaneous haemorrhagic speckling', became a nation-wide problem of considerable economic significance (Gilbert *et al.*, 1979). They affected mainly young lambs and occurred with an average prevalence of approximately 5% during the first few weeks of each killing season.

The emergence of 'speckling' as a recognised entity coincided with the introduction of electrical stunning and associated slaughtering techniques designed to fulfil new statutory requirements for the humane and hygienic slaughter of sheep. An analogy was immediately drawn, therefore, between 'speckling' and 'blood splash' haemorrhages, the latter of which are known to be associated with electrical stunning (Frazerhurst, 1978). However, for poorly understood reasons, 'blood splash' had ceased to exist as a significant problem subsequent to the 1976/77 killing season (Frazerhurst *et al.*, 1978). Furthermore, 'subcutaneous haemorrhagic speckling' proved to be grossly and microscopically distinct from 'blood splash', and preliminary investigations had dissuaded against the aetiological role of electrical stunning (Frazerhurst *et al.*, 1978). No published descriptions of similar lesions could be located. It therefore became necessary to investigate the aetiology and pathogenesis of 'speckling'.

The following investigations were conducted at one meat works during the first two seasons in which 'speckling' occurred. The lesions were described, their primary cause established and attempts were made to elucidate their pathogenesis.

MATERIALS AND METHODS

A. Histology

For routine light microscopy, tissues were fixed in 10% formol saline, blocked in paraffin, sectioned, then stained with haematoxylin and eosin (H+E), Lendrum's acid picro-Mallory and Perl's iron stains. Epoxy-resin embedded sections were also prepared from affected adipose tissue that had been fixed for 24 hours in a mixture of 3% glutaraldehyde and 2% formaldehyde in 0.1M phosphate buffer pH 7.2, and post-fixed for 4 hours in 1% osmium tetroxide in 0.1M phosphate buffer pH 7.2. For light microscopy, 1.0µm sections were stained on an 80°C hotplate with 1% toluidine blue in phosphate buffer pH 7.4 for 40 seconds and counterstained with 4% basic fuchsin for 20 seconds. Sections for electron microscopy were stained with uranyl acetate and lead citrate.

B. Immunohistochemistry

Ten control and ten affected tissues were examined for antibody complexes using an immunoperoxidase technique (Petts and Roitt, 1971; Brandtzaeg, 1974). Tissue blocks were fixed for 24 hours in 95% ethanol at 4°C, dehydrated for 24 hours in absolute ethanol, cleared overnight in xylene, brought to room temperature slowly in a xylene bath then vacuum embedded in low melting point (52°C) wax. The embedded tissues were stored at 4°C prior to sectioning. Paraffin sections were cut at approximately 10µm, floated on water briefly at 40°C then dried for 30 minutes at 37°C. Prior to use, these sections were deparaffinised by two immersions in cold xylene followed by two 20 second immersions in cold 95% ethanol. Endogenous tissue peroxidase activity was inhibited by immersing the sections for 30 minutes in a 0.5% solution of hydrogen peroxide in methanol, after which they were washed for 30 minutes in three changes of phosphate buffered saline (PBS). Affected and control tissues were incubated for 30 minutes in rabbit anti-sheep gammaglobulin of the following dilutions:

1/5, 1/10, 1/20, 1/40. The incubated sections were then washed for 30 minutes in three changes of PBS and re-incubated for 30 minutes with peroxidase conjugated goat anti-rabbit IgG. Unbound conjugated antibody was washed out by immersing the sections for 15 minutes in three changes of PBS. Finally, the bound peroxidase conjugates were labelled by incubating the sections for 15 minutes in a freshly made solution of 0.025% 3'3 diaminobenzidine tetra HCl (DAB) and 0.01% hydrogen peroxide in 0.05M tris-sucrose buffer pH 7.6. The labelled sections were washed in tap water, counterstained in weak haematoxylin, dehydrated and mounted in D.P.X. mountant.

C. Epidemiological studies

1. Statistical analysis of population factors associated with petechiation in carcase fat: A series of retrospective statistical analyses relating the prevalence of petechiation to a series of lamb population factors was carried out on data collected between November 1977 and February 1978. Information was collected from 275 mobs of between 50 and 666 lambs, and the prevalence of carcases showing petechiation was compared with a number of factors. These were: farm of origin, 'clumping' of affected carcases within mobs, mean carcase weight and wool yield, transport company and driver, transport time (0-6 hours), holding time before slaughter (16-48 hours), washing (0, 1 or 2 times), the chain on which the mob was slaughtered and the prevalence of conventional bruising. Each of these factors was treated as an independent variable or a covariable in computer-assisted statistical analyses, with the arcsin transformation of the proportion of affected carcases as the dependent variable (Nie *et al.*, 1975). The term 'mob of lambs' defines a group of lambs that remained identifiable from their farm of origin to the point at which the carcases were graded. Petechiation was recorded as present or absent by company graders.

2. Split-line trial to determine whether lesions occurred at or prior to slaughter: A mob of 317 lambs that had been on the freezing works premises for 20 hours was split into two groups of 160 and 157 lambs respectively. The lambs in group A were slaughtered immediately; those in group B were driven to the point of slaughter, then held a further 20 hours before being killed and processed over the same chain. For both groups the prevalence of petechiation was recorded, and samples were taken for light microscopy.

3. Factors associated with the process of slaughter: Assuming the lesions occurred close to the time of death, the following trial was designed to identify the cause of petechiation in adipose tissue, i.e. whether due to the methods of restraint, stunning or slaughter. Lesions were recorded by one person, who also scored them on a scale from 1-4 according to severity. Lambs were either restrained in a moving 'crush' conveyor or walked up a ramp, and unless otherwise indicated they were stunned by a 1.25 amp, 400 volt, 50 Hertz current applied for 4 seconds with a Thornton Mk II* head-to-back stunner. In 'gash-cutting', a knife was used to sever the soft tissues and major blood vessels of the neck, and the spinal cord was cut at the atlanto-occipital joint. In 'pig-sticking', a knife was thrust through the thoracic inlet in such a way as to sever the anterior vena cava and the bicarotid trunk. Mobs of lambs were selected from farms with a previously high prevalence of lesions. Individual mobs were assigned randomly to one of four groups designated A to D (Table 5.3). Each mob was then subdivided into four, or for those in group A, six approximately equal treatment groups. Every alternate treatment group was slaughtered using the procedure under investigation, and the remainder were killed as controls, using the routine method of slaughter, i.e. electrical stunning whilst in the 'crush' conveyor, followed by exanguination by 'pig-sticking'. The subdivisions in group A were designed to study the effects of electrical stunning, those in group B to study the effects of 'pig-sticking' and those in group C to study the effect of the conveyor. Group D was designed to compare the

* Thornton Engineering Group Ltd, P.O.Box 223, Auckland.

traditional method of 'gash-cutting' without stunning or restraint in a crush conveyor with the current method of slaughter. Three treatments were necessary within group A because for humane reasons lambs could not be subjected to 'pig-sticking' without first being stunned, and the first treatment served to establish the presence of lesions in the mobs within that group under normal working conditions.

D. Pathogenesis of petechiation in adipose tissue

1. Measurement of post-stunning systemic blood pressure: One femoral artery in each of five lambs was cannulated under sodium pentobarbitone general anaesthesia, and the animals were allowed to recover overnight. Three lambs were then electrically stunned using a Thornton Mk II head-to-back handpiece coupled to a Thornton control box delivering a current of 0.5-0.9 amp at 400 volts and 50 Hertz. The current application time was 4 seconds for two of these lambs and 10 seconds for the third. The remaining two lambs were stunned using a Paralec* head-only stunner and the Thornton control box, delivering a 0.7-0.9 amp, 400 volt, 50 Hertz current for 10 seconds duration. One of these lambs was allowed to recover, then re-stunned with a further 10 second application of the Paralec handpiece. In all cases systemic blood pressures were monitored by a Bell and Howell type PI pressure transducer (CFC Division, Pasadena, California 91109, USA) attached to the femoral cannula, and recorded on a Devices 8 Channel Recorder (Devices Sales Ltd, Hertfordshire, UK). Electrocardiograms were recorded using a Model M2 Pen Recorder (Devices Instrument Company) using four needle electrodes: two placed immediately cranial to the shoulder joints, one in the abdominal wall immediately cranial to the left stifle and a reference electrode placed mid-way between the right iliac crest and the right ischiatic tuberosity (Smith, 1978).

* McKenzie and Holland (N.Z.) Ltd, P.O.Box 35097, Naenae.

2. Attempts to produce petechial haemorrhages in inguinal fat:

Attempts were made to produce petechial haemorrhages by electrically stimulating the nerves innervating the inguinal adipose tissue overlying the pubic symphysis in three female lambs in good body condition. These animals remained under sodium pentobarbitone general anaesthesia throughout the experiment. The procedures outlined in Table 5.1 were applied after first isolating the left and right genito-femoral nerves of each lamb as they emerged from the external inguinal hiatus along with the pudendal artery and vein (Rosell, 1966; Habel, 1970). An oscilloscope was used to monitor the electrical stimulus applied to the nerves, and histology was used to retrospectively identify the stimulated tissue. Samples of adipose tissue were fixed in 10% formol saline and processed for routine light microscopy.

TABLE 5.1 : OUTLINE OF PROCEDURES APPLIED DURING ATTEMPTS TO PRODUCE PETECHIAL HAEMORRHAGES BY THE DIRECT ELECTRICAL STIMULATION OF NERVES INNERVATING INGUINAL FAT.

EXPERIMENTAL PROCEDURE		
Lamb A	Lamb B	Lamb C
*Stimulate right genito-femoral nerve	*Stimulate right genito-femoral nerve	**Stimulate right genito-femoral nerve
Sample inguinal fat right of midline	Sample inguinal fat right of midline	Sample inguinal fat right of midline
Intravenous histamine 0.4mg/kg	'Physostigmine' nerve blocker, I/V, 1mg/kg	Histamine 0.4mg/kg and 'Physostigmine' nerve blocker, I/V 1mg/kg
*Stimulate left genito-femoral nerve	*Stimulate left genito-femoral nerve	**Stimulate left genito-femoral nerve
Sample inguinal fat left of midline	Sample inguinal fat left of midline	Sample inguinal fat left of midline

* 4 seconds with 50 Hertz, 10V rectified square wave of 5m seconds duration.

** 4 seconds with 50 Hertz, 10V alternating sinusoidal wave.

RESULTS

A. Gross pathology

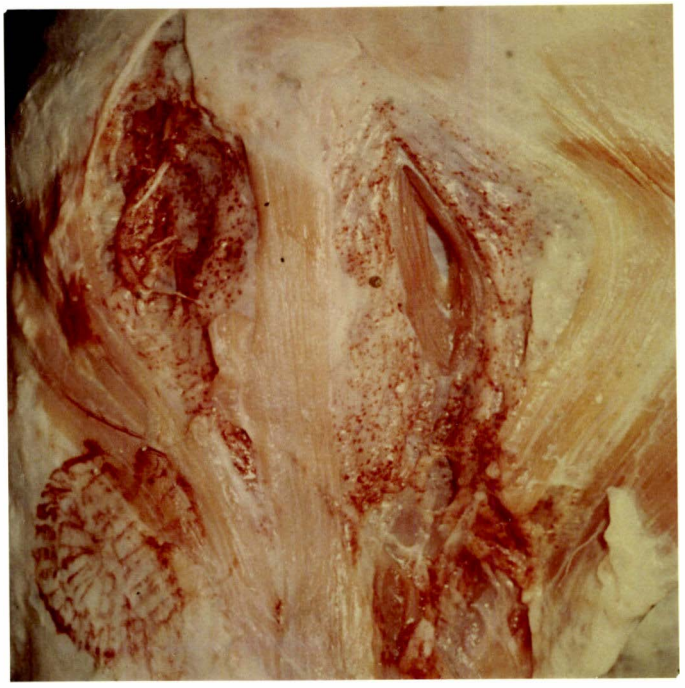
Petechiation of carcase adipose tissue was observed only in lambs. The lesions were multiple, discrete petechial haemorrhages, 0.5-3.0mm in diameter and occurring in adipose tissue or its fascia but not in muscle (Figure 5.1). Their distribution was approximately bilaterally symmetrical. Although they could be found in almost any area of external adipose tissue they were most constant and severe in the lumbar and flank areas. If lesions were not visible superficially, incision of the *Cutaneous trunci* muscle frequently exposed them in underlying fat. The inguinal region and cranial aspect of the shoulder were other commonly affected areas, and very mild petechiation was occasionally observed in adipose tissue beneath the parietal pleura. No lesions were observed in the pelts, on the sclera, or in the conjunctival or oral mucosae. Petechiated lungs were commonly found in both affected and unaffected carcases but the remaining viscera were grossly unaffected.

B. Histopathology

The haemorrhages were discrete and confined to the interstitial spaces of adipose tissue or associated fascia (Figure 5.2). Points of apparent capillary rupture were occasionally detected in epoxy-resin sections but no vasular degenerative changes were observed. A characteristic feature of the lesions was a small central fibrin and platelet thrombus (Figure 5.3). This was visible in most areas of haemorrhage but was absent in some because of the plane of section. Few leucocytes were distributed throughout the haemorrhage, but frequently up to 100 neutrophils and mononuclear leucocytes could be observed adjacent to the thrombi. The size and appearance of the mononuclear cells suggested they were lymphocytes, an opinion verified by light and electron microscopy on epoxy-resin embedded sections. Inorganic iron was not detected by Perl's stain for iron.

FIGURE 5.1 : Petechial haemorrhages occurring in adipose tissue but not in muscle.

FIGURE 5.2 : Discrete haemorrhage in adipose tissue and its associated fascia.
(Paraffin section, H+E x 65)



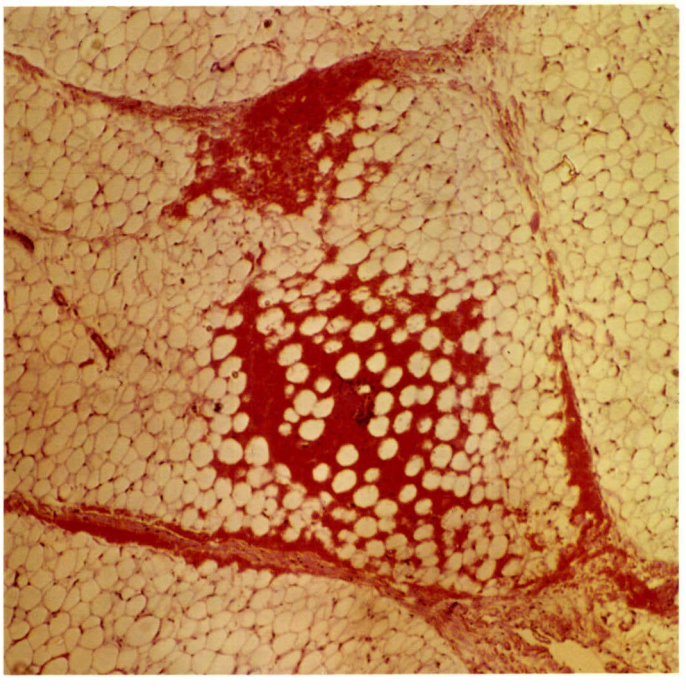
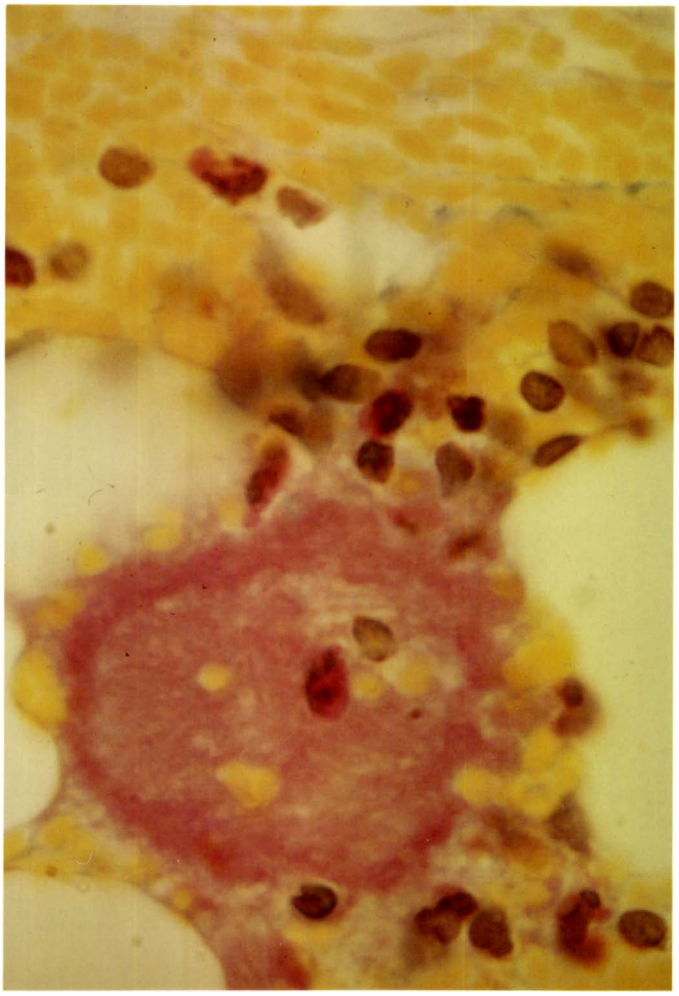


FIGURE 5.3 : A capillary thrombus with closely associated leucocytes.
(Paraffin section, picro-Mallory x 1250)



Similar thrombi with a few closely associated leucocytes were observed in some sections of retropleural adipose tissue. Some sections of petechiated lung also showed focal haemorrhages with thrombi and closely associated white blood cells. These lesions were similar to those seen in adipose tissue.

C. Immunohistochemistry

The immunoperoxidase technique used for antibody detection failed to reveal antibody complexes in adipose tissue affected by 'speckling'.

D. Epidemiological studies

1. Statistical analysis of population factors associated with petechial haemorrhage: Data relating population factors to the prevalence of petechial haemorrhage were compiled by export company personnel. Not all mobs were affected, but on average, petechial haemorrhage was observed in 5% of slaughtered lambs, with an incidence of up to 15% in some cases. The week by week incidence of petechiation in carcase fat, as recorded by company graders, was expressed as a percentage of carcases produced. The results have been compared in Figure 5.4 with equivalent figures for conventional bruising. There was no statistically significant correlation between the two types of lesions. Data on petechiation were not plotted after 7th January 1978 because of the very low prevalence of the condition after this time.

Statistical analyses were performed on data collected from 275 mobs of between 50 and 666 lambs. The prevalence of petechiation in carcase fat could not be significantly related to any of the following factors: mean carcase weight or wool yield, transport company or driver, transport time (0-6 hours), holding time in establishment yards (16-48 hours), number of times lambs were washed (0, 1 or 2 times) or the chain over which lambs were slaughtered. Although it was a common subjective impression, there was no statistical evidence that petechiated carcases

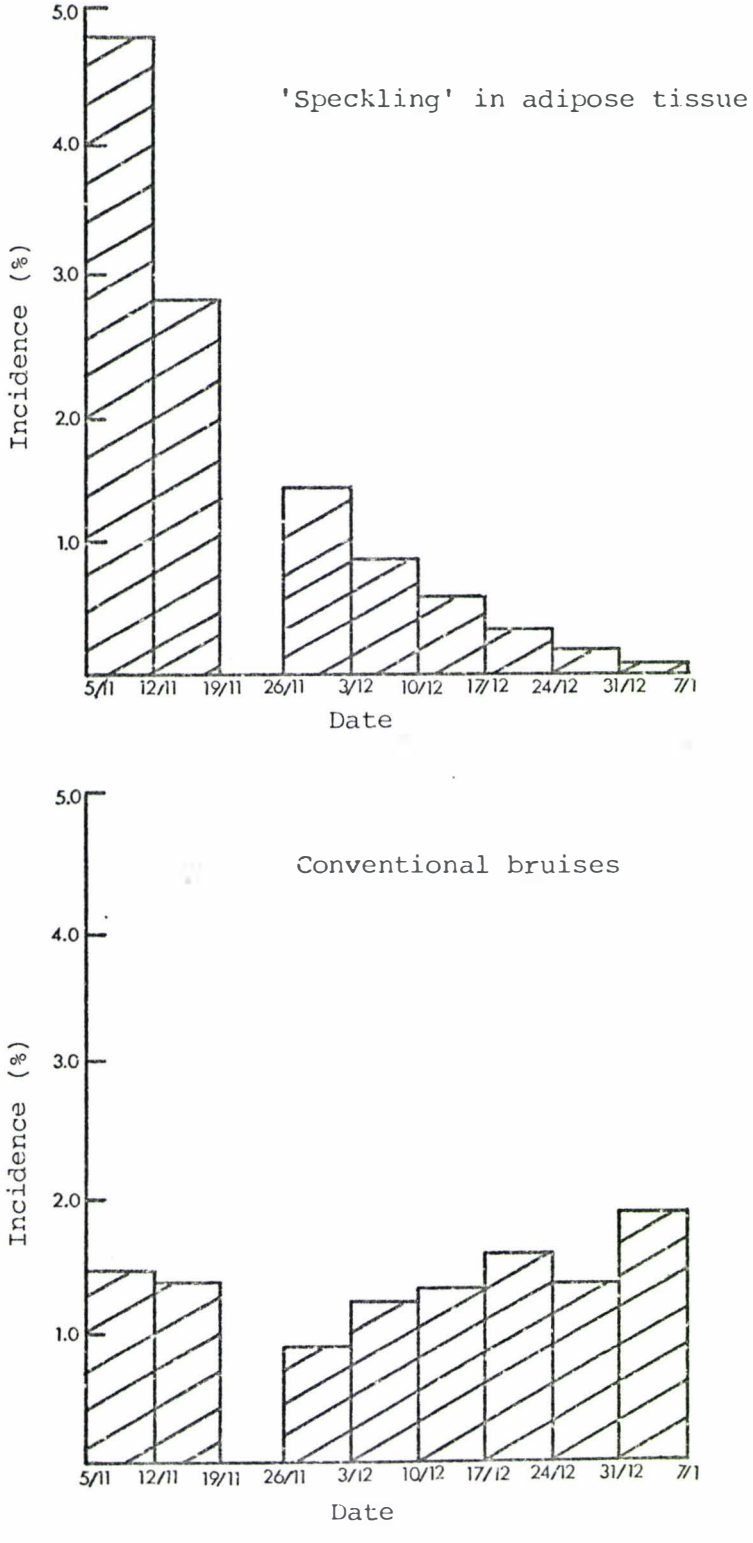


FIGURE 5.4 : The weekly incidence of petechiation in carcase adipose tissue in comparison with that of conventional bruising.

occurred in 'clumps' within affected mobs. There were, however, significant differences ($P<0.01$) in prevalences between mobs killed on the same chain in any one day. Prevalences of petechiation of carcase fat were investigated in lambs from 10 farms that had sent for slaughter at least two drafts of lambs on at least two occasions. Petechiation was not related to the farm of origin.

2. Split-line trial to determine whether lesions occurred at or prior to slaughter: A total of 317 lambs that had been on the premises for 20 hours were split into two groups. The lambs in group A were slaughtered immediately but those in group B were held a further 20 hours, then slaughtered over the same chain. The respective group prevalences of petechiation were recorded, and samples from eight lambs per group were collected for histology. The results of this trial are summarised in Table 5.2.

TABLE 5.2 : THE PREVALENCES OF PETECHIATION IN THE CARCASE FAT OF TWO GROUPS OF LAMBS FORMED FROM A SINGLE MOB. THE LAMBS IN GROUP B WERE KILLED 20 HOURS AFTER THOSE IN GROUP A.

GROUP	NORMAL	PETECHIATED	TOTAL
A	95	65	160
B	106	51	157

A chi-square analysis showed no significant difference between the prevalence of petechiation in the two groups. Histologically, the petechial haemorrhages were similar in both groups of lambs.

3. Experiment to isolate factors associated with the process of slaughter: The prevalence of petechiation relative to methods of slaughter is shown in Table 5.3. There were significant differences ($P<0.001$), as measured by chi-square analysis, between treatments within groups A and D but not between treatments within groups B and C. The table thus shows a clear association between electrical stunning and petechiation. Though the data are not shown, there was no significant differences between treatments with regard to severity of carcase lesions as measured on a scale of 1-4.

TABLE 5.3 : EXPERIMENT TO ISOLATE FACTORS ASSOCIATED WITH THE PROCESS OF SLAUGHTER.

GROUP	TREATMENTS			NUMBER OF LAMBS			
	crush conveyor	electrical stunning	method of exsanguination	normal	petechiation	total	
A	*yes	yes	pig stick	128	79	207	= 77.85
	yes	yes	gash cut	142	62	204	d.f.2
	yes	no	gash cut	194	5	199	P<0.001
				464	146	610	
B	*yes	yes	pig stick	19	67	86	= 2.37
	yes	yes	gash cut	28	58	86	d.f.1
				47	125	172	N.S.
C	*yes	yes	pig stick	183	44	227	= 2.40
	bypassed	yes	pig stick	197	32	229	d.f.1
				380	76	456	N.S.
D	*yes	yes	pig stick	71	39	110	= 42.37
	bypassed	no	gash cut	97	0	97	d.f.1
				168	39	207	P<0.001

* routine method of slaughter.

E. The pathogenesis of petechiation in adipose tissue

1. Measurement of post-stunning systemic blood pressure: Three lambs were electrically stunned using a Thornton Mk II head-to-back handpiece. Two of these received a 0.5-0.9 amp, 400 volt, 50 Hertz current of 4 seconds duration and the third received a similar current applied for 10 seconds. Another two lambs were stunned using a Paralec head-only handpiece delivering a 0.7-0.9 amp, 400 volt, 50 Hertz current of 10 seconds duration. One of these lambs was allowed to recover, then re-stunned with a further 10 second application of the Paralec handpiece. Post-stunning electrocardiograms (ECG) and systemic blood pressures were monitored as previously described.

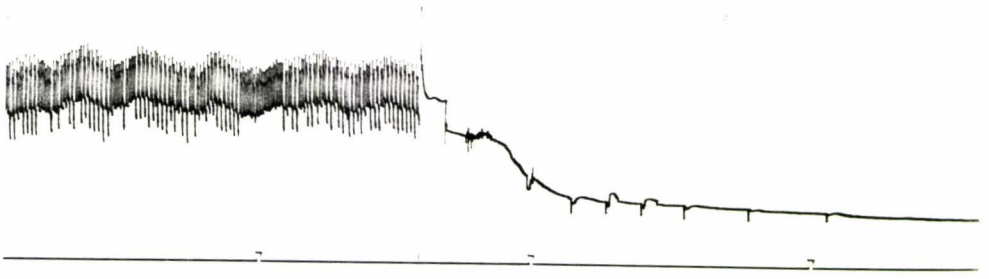
The Thornton Mk II head-to-back handpiece immediately induced an ultimately fatal cardiac fibrillation in each of the three lambs to which it was applied. The pre-stun arterial blood pressures of each animal were 150/80, 140/90 and 120/80 mm Hg respectively. As the current was applied there was a transient increase in arterial pressure of approximately 60mm Hg above normal (Figure 5.5). The timing of this pressure pulse corresponded to the onset of tonic skeletal muscle spasm caused by the electric current. Stunning was followed by a rapid progressive decrease in arterial pressure.

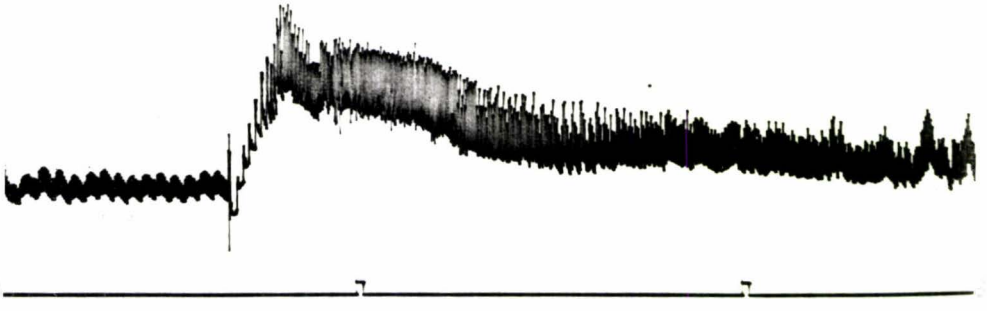
The electrocardiographs of the two lambs stunned with the Paralec head-only handpiece remained normal, and the animal allowed to recover had done so within five minutes. The pre-stun systemic blood pressures were 120/70mm Hg in both lambs. Upon application of the current there was a momentary fluctuation in arterial pressure similar in duration and intensity to that described for head-to-back stunning. The post-stun arterial pressure then increased in a stepwise manner to reach maximum values of 350/230 and 360/250 mm Hg respectively within 7 seconds of stunning (Figure 5.6). Following this, the arterial pressures declined gradually to plateau at 160/80mm Hg 90 seconds after stunning, at which time pressure recordings were discontinued.

None of the lambs showed petechial haemorrhages in adipose tissue.

FIGURE 5.5 : Changes in arterial pressure following electrical stunning using a 'Thornton Mk II' head-to-back current applicator.

FIGURE 5.6 : Changes in arterial pressure following electrical stunning using a 'Paralec' head-only current applicator.





2. Attempts to produce petechial haemorrhages in inguinal fat:
Attempts were made to produce petechial haemorrhages by electrically stimulating the nerves innervating the inguinal adipose tissue of three female lambs in good body condition. The procedures applied in each case have been outlined in Table 5.1. At no time were gross or microscopic haemorrhages observed in the inguinal fat.

DISCUSSION

From the beginning of the 1977/78 killing season it was clear that the lesions subsequently to be called 'subcutaneous haemorrhagic speckling' (Anon, 1978) were grossly and histologically distinct from both 'blood splash' and bruising, which were the only two haemorrhagic lesions previously recognised in apparently healthy lambs slaughtered for export (Gilbert *et al.*, 1979). Grossly, 'speckling' was characterised by its punctate nature, its restriction to carcase adipose tissue or associated fascia and its anatomical distribution in this tissue. When first encountered, the lesions were approximately bilaterally symmetrical and widely distributed, though they were most severe over the loins and often could not be detected unless the surface fascia of the carcase was incised (Frazerhurst *et al* ., 1978). In subsequent seasons, however, their distribution changed subtly, with a tendency to concentrate more around the hindquarters, which was an area not previously involved during the initial outbreak (Frazerhurst *et al.*, 1978; Gilbert *et al.*, 1979). Haemorrhages due to bruising were comparatively easy to distinguish from those of 'speckling' since they were not punctate, were usually visible on the surface of the carcase, involved the underside of the pelt and frequently involved skeletal muscle (Frazerhurst *et al.*, 1978). Electrically induced 'blood splash' haemorrhages were infrequently observed but they are usually ecchymotic rather than petechial in nature and do not involve adipose tissue (Warrington, 1974; Kirton *et al.*, 1978). Blood splashed lungs, however, were commonly observed and were grossly similar to 'speckling'. Histologically, 'speckling' in adipose tissue was distinct from either bruising or blood splash, since only the former lesions were characterised by discrete interstitial haemorrhages with small central thrombi closely surrounded by moderate numbers of leucocytes.

In the early stages of the investigation considerable reliance was placed on histopathological and retrospective epidemiological data, since without firm reasons for doing so disruptive trials could not be justified in an export meat works under full production. Epidemiological evidence showed the prevalence of 'speckling' to be associated with the age of the lambs, but it was independent of such pre-slaughter variables as farm of origin, trucking company, trucking times, holding times in meat works yards, number of times lambs were washed and the prevalence of conventional bruising. This suggested that the lesions occurred close to the time of death. The presence in paraffin sections of moderate numbers of leucocytes aggregated around a central thrombus had originally been interpreted as evidence of an inflammatory reaction of at least 30 minutes duration. This opinion was modified once light microscopy on semi-thin epoxy resin sections had shown the leucocytes to be comprised of approximately equal numbers of neutrophils and lymphocytes. Since lymphocytes are not normally involved in acute inflammation (Cheville, 1976), and since the polymorph/lymphocyte ratio was approximately that of normal ovine blood, the leucocytes were finally considered to have arrived passively during haemorrhage. The aggregation of leucocytes around a central thrombus was explained by proposing a sieving action by intact, closely apposed adipocytes allowing a free passage only to erythrocytes.

That 'speckling' did in fact occur close to the time of slaughter was proven by the trial in which histologically indistinguishable lesions had been observed with similar prevalences in the two groups of a split mob of lambs, the second group of which had been detained for an additional 20 hours before being slaughtered in an identical manner to the first group. Had 'speckling' existed before the mob was split, i.e. before slaughter, the lesions in the two groups of lambs would have been histologically dissimilar. This presupposition was based on previously acquired experimental evidence following the subcutaneous injection of autologous whole blood into sheep.

1. The primary aetiology of 'speckling'

Prior to 1976, all sheep in New Zealand meat works had been walked from the holding yards to the point of slaughter, tipped onto their sides and exanguinated by a transverse 'gash cut' that almost simultaneously severed the ventral soft tissues of the neck and the spinal cord at the level of the atlanto-occipital joint. From the 1976/77 killing season on, new measures were introduced whereby sheep were transported to the point of slaughter in a moving V-shaped conveyor, electrically stunned whilst in the conveyor, then ejected forwards for exanguination. In this case exanguination was effected by severing the major blood vessels of the neck whilst leaving the spinal cord and oesophagus intact. 'Speckling' had been shown to occur close to the time of death. Its likely cause was therefore considered to be one or a combination of the above three newly introduced slaughtering procedures. In this regard the initial restriction of the problem to only a few meat works could be explained in terms of the wide variations in equipment and slaughtering techniques adopted throughout the country at that time. Each factor is considered below.

(a) Method of exsanguination: Petechial haemorrhages in adipose tissue are commonly observed following all types of rapidly induced anoxic or hypoxic death (Polson, 1965). Their pathogenesis is poorly understood but they are believed to arise when poorly supported capillaries rupture under the combined effects of hypoxic endothelial damage and agonal fluctuations in blood pressure (Gordon, 1975). The newly introduced method of exsanguination by 'pig sticking' was considered a potential cause of 'speckling' because the animals so bled underwent violent agonal respiratory efforts not previously observed to the same extent after 'gash cutting', wherein the central connections of the phrenic nerves were severed by cutting the spinal cord. However, a trial conducted by Frazerhurst *et al.* (1978) failed to implicate the method of exsanguination as the cause of the condition.

(b) The restraining conveyor: From histological evidence, Frazerhurst *et al.* (1978) deduced that 'subcutaneous haemorrhagic speckling' was caused by shear forces which probably resulted from electrically induced muscular spasms in lambs firmly restrained in narrowly set V-shaped 'crush' conveyors. They cited as support the fact that grossly similar lesions could be inflicted manually by grasping the wool of immediately post-stunned lambs and applying a rapid tangential shearing force to that segment of skin. They also observed a decreased prevalence of 'speckling' once narrowly set conveyors had been opened so as to restrain lambs less firmly. Using the same manual technique of Frazerhurst *et al.* (1978), we were able to create petechial haemorrhages grossly similar to those of 'speckling'. However, these lesions lacked the characteristic central thrombi with closely associated leucocytes, and were thus histologically distinct from true 'speckling'. Also, petechial haemorrhages had occasionally affected inguinal and subpleural adipose tissue, where it was difficult to envisage the appropriate shear forces acting. Muscular spasms in restrained electrically stunned lambs were therefore considered unlikely to be the primary cause of 'speckling', although a contributory effect on their prevalence and severity could not be excluded.

(c) Electrical stunning: Despite early opinions to the contrary, the balance of evidence implicated electrical stunning as the cause of 'speckling'. This was confirmed in a trial designed to isolate the respective contributions of electrical stunning, the conveyor and the method of exsanguination (Table 5.3), and in which only the former influenced the prevalence of lesions. The primary aetiological role of electrical stunning subsequently became well established (Blackmore and Petersen, 1981; Anon., 1982). Nevertheless, the prevalence of 'speckling' appeared also to be influenced by undefined secondary factors. Only in this way could the relatively low average prevalence of 'speckling', its seasonal distribution pattern and its variation in prevalence between different mobs of lambs be explained (Petersen and Wright, 1978; Gilbert *et al.*, 1979).

2. Secondary factors in the aetiology of 'speckling'

A variety of secondary factors were proposed as possibly influencing the prevalence of 'speckling'. These could be conveniently classified as animal associated, environmental or works associated. However, their prospective roles were difficult to investigate under the conditions in which meat works operate, and to date the significance of each remains undefined. Only a few of the more substantiated possibilities are considered below.

The predominance of lesions in young lambs, with the occasional involvement of older sheep (Gilbert *et al.*, 1979) may have reflected an effect of maturity on capillary support and thereby on the propensity of these vessels to rupture under haemodynamic stress (Tocantins, 1947; Walter and Israel, 1974). However, since only eight meat works experienced a 'speckling' problem during the 1977/78 killing season (Frazerhurst *et al.*, 1978), additional factors must have been operating. The initial restriction of 'speckling' to a few widely scattered meat works implicated environmental factors in the aetiology of the lesions. The demonstration of a correlation between the pasture content of coumarinogenic plants and the prevalence of 'blood splash' (Restall, 1980), and evidence of a direct effect of certain coumarin analogues on capillary fragility (Nalbandian *et al.*, 1965; Khan *et al.*, 1971b), supported this concept. However, Petersen and Pauli (1983) failed to detect a statistically significant association between one-stage prothrombin times and the prevalence of 'speckling'. The sudden widespread occurrence of 'speckling' subsequent to the 1977/78 killing season also became evidence against the potential role of environmental factors, implicating instead the electrical stunning process itself as the most important variable besides age influencing the prevalence of 'speckling'. Current strength and application time affect the prevalence of lesions, and these factors were shown to vary widely between meat works (Gilbert *et al.*, 1979; Anon., 1982). However, no attempt was made to evaluate their significance. Petersen and Wright (1978) attributed the widespread emergence of 'speckling' to the general adoption of the 'Thornton Mk II' head-to-back current applicator, and they believed their lesions to have been caused by local field

effects generated by its mid-thoracic electrode. That the distribution of 'subcutaneous haemorrhagic speckling' is influenced by the position of the electrodes and thus by local field density pattern has since been demonstrated convincingly (Blackmore and Petersen, 1981; Anon., 1982). Nevertheless, from our observations generalised lesions do occur following head-only stunning and they are usually most severe over the loins, which is an area at considerable distance from the point of application of the current.

Despite its established primary cause, the secondary aetiological factors influencing the prevalence of 'subcutaneous haemorrhagic speckling' presented a complex picture. An alternative approach was therefore adopted, whereby attempts were made to elucidate the pathogenesis of the lesions with the intention of formulating a rational approach to their prevention.

3. The pathogenesis of 'speckling'

From histological and epidemiological evidence, 'subcutaneous haemorrhagic speckling' appeared to have arisen from capillaries ruptured by undefined forces induced by electrical stunning. In this respect they resembled 'blood splash' lesions. 'Blood splash' is a poorly understood phenomenon, but a central feature in its pathogenesis appears to be a sustained increase in post-stunning arterial blood pressure (Warrington, 1974; Kirton *et al.*, 1978). Thus it may occur with a high incidence following head-only stunning, which causes a dramatic increase in arterial blood pressure (Figure 5.6), but it rarely occurs following head-to-back stunning (Frazerhurst, 1978) after which there is a rapid fall in arterial pressure (Figure 5.5). The relationship between blood pressure and 'speckling' is less straightforward than that for 'blood splash' since the former occurs with approximately equal prevalence following both types of stun (Gilbert *et al.*, 1979). Nevertheless, an increase in systemic blood pressure may still be a vital factor in its pathogenesis since an immediate momentary post-stun increase in arterial pressure appears to occur in both cases. This pressure pulse, which is probably caused by an intense spasm of

intercostal and diaphragmatic muscles may be sufficient to rupture the capillaries of adipose tissue (Gilbert and Devine, 1982). Possibly of greater significance is the demonstration by Petersen and Carr (personal communication) of a sustained ten-fold increase in central venous pressure following head-to-back stunning and a two-fold increase following head-only stunning. They estimated that post stun changes in arterial and venous blood pressures would combine to cause up to a four-fold increase in intra-capillary pressure, and they suggest this as a possible cause of capillary rupture. Against this, however, is the fact that for purely haemodynamic reasons, capillaries are resistant to damage caused by increases in systemic blood pressure acting alone (Rowley, 1964). Rowley failed to detect ruptures or even increased permeability after subjecting the capillaries of subcutaneous adipose tissue in rats to ten times their normal hydrostatic pressures. As an alternative hypothesis, 'subcutaneous haemorrhagic speckling' may have resulted from rapid electrically induced local fluctuations in intra-capillary blood pressures, possibly with concurrent electrically induced increases in capillary permeability. Such changes are known to occur following direct or indirect electrical stimulation of the vasculature of subcutaneous adipose tissue (Ballard, 1978).

Within seconds of electrically stimulating either the sympathetic trunk or the peripheral nerve innervating the inguinal adipose tissue of dogs, there is vasoconstriction with an increased ratio of pre/post capillary resistance and a considerable fall in capillary pressure (Rosell, 1966; Ngai *et al.*, 1966; Oberg and Rosell, 1967). Upon cessation of stimulation, pre- and post- capillary vessels undergo an immediate, prolonged maximal vasodilation which causes intracapillary pressures to rise above normal (Ngai *et al.*, 1966). For poorly defined reasons the actual and relative magntiude of the above changes vary widely between individuals of the same species, between different capillary beds and between capillaries of the same bed (Ballard, 1978). However, in all cases they appear to be mediated via adrenergic sympathetic nerves, with no involvement of the central nervous system (Ngai *et al.*, 1966; Ballard, 1978; Bevan, 1979). In addition to fluctuations in capillary pressure, electrical stimulation causes an increase in capillary permeability similar in order of magnitude to that induced by histamine (Fredholm

et al., 1970). The mechanism of this action is not understood but the response appears to be unique to adipose tissue (Ballard, 1978).

The above evidence supports the proposed hypothesis that 'speckling' in adipose tissue resulted from localised electrically induced increases in capillary pressure and permeability. Attempts were therefore made to reproduce 'speckling' by electrically stimulating the right or left *genito femoral* nerves as they innervated the right and left sides respectively of the inguinal fat of three young lambs. Unilateral nervous stimulation alone failed to produce lesions. The contralateral nerve of each animal was therefore stimulated whilst attempts were made to enhance particular vascular reactions considered likely to predispose to capillary rupture. With the first lamb, the contralateral genital nerve was stimulated after the intravenous injection of physostigmine, which in dogs enhances the vasodilation of pre-capillary sphincters but has minimal post capillary effects (Ngai *et al.*, 1966). This was expected to increase the presumed post-stimulation increase in capillary pressure. With the second lamb, the contralateral nerve was stimulated after intravenous injection of histamine, since in dogs this causes a greatly increased capillary permeability (Fredholm *et al.*, 1970). The contralateral nerve of the third lamb was stimulated after the injection of both physostigmine and histamine. Adrenaline has been shown not to alter the prevalence of 'speckling' occurring in practice (Anon., 1982), and intravenous noradrenaline has been shown to exactly mimic the constrictor effects of sympathetic nervous stimulation (Fredholm *et al.*, 1970). Sympathomimetics were not therefore injected prior to nervous stimulation. All attempts to reproduce 'speckling' experimentally were unsuccessful. This failure may have been caused either by unfavourable animal-associated factors, which is not unlikely since the lesions occur in practice with an average prevalence of only 5%, or it may have reflected an inadequacy in the proposed hypothesis as to the pathogenesis of the lesions.

To date speckling remains a problem in most New Zealand meat works. Worthwhile reductions in its severity and prevalence have been achieved by changing the field characteristics of the electric current used to stun sheep (Blackmore and Petersen, 1981; Anon., 1982), but the problem

is unlikely to be eliminated until the pathogenesis of the lesions has been further elucidated.

SUMMARY AND CONCLUSIONS

I. THE AGEING OF BRUISES

Bruises in carcases processed for human consumption cause substantial economic losses to the meat industry both here and overseas. Knowledge of the ages of bruises in relation to known times of specific events of interest would provide a useful means of investigating the aetiology of these lesions with the aim of lowering their prevalence. For this reason attempts were made to age experimentally inflicted bruises from histopathological observations, enzyme studies and pH measurements.

1. Histopathology

Published observations pertaining to the histopathology of a variety of wounds including bruises have suggested the potential usefulness of this approach for ageing purposes.

Experimental bruises of various ages from 1-72 hours old were examined and a number of histopathological features in both muscle and adipose tissue scored semi-quantitatively according to their degrees of change from normal. After relating scores to ages, the following features were selected as being of use for ageing purposes: neutrophil and macrophage exudates, fibroplasia, endothelial hypertrophy and the present of haemosiderin in interstitial macrophages. In order to eliminate errors associated with the subjective interpretation of histopathological data, combinations of scores for the above features were interpreted objectively using a mathematical model based on Bayes' theorem of inverse probabilities.

It had been intended to age bruises as either 1-8, 12-20, 24-36, 48, 60 or 72 hours old. Unfortunately, even when performing at its maximum capability the Bayesian model was unable to do this with a satisfactory degree of success. However, it was sufficiently accurate to be of potential worth if used to age bruises as either 1-20 hours or over 24 hours old. The 'confidence' with which individual bruises could be so aged depended on the type and number of tissues examined per bruise (Tables 2.21, 2.22, 2.23), and because of the respective error terms, also on the relative number of bruises estimated to be of each age. As a general estimate, bruises could be aged with an 80-90% degree of certainty, which was in excess of the maximum of 78% achieved after the subjective interpretation of equivalent data.

The practical usefulness of the Bayesian ageing method was examined by applying it in a pilot study to 107 bruises collected from an export meat works. By relating the estimated ages of these bruises to holding times of lambs in meat works yards, it was estimated that 60% of these lesions were inflicted within the works and 40% without. Due to the constraints in being able to age bruises only within the broad limits of 1-20 hours and 24+ hours, a substantial proportion (45%) of the information could not be used in the above estimates (Table 2.30). In future surveys, however, the amount of data made redundant could be reduced substantially by a judicious selection of the range of holding times of the lambs from which bruises were collected.

In the above survey a comparison of the ageing and more traditional epidemiological approaches to determining the places of occurrence of bruises suggested the superiority of the former method, at least when applied to small numbers of bruises. The Bayesian method for ageing bruises was therefore considered to be of practical value for determining the places of occurrence of these lesions.

The ability of the histopathological approach in being able to accurately place bruises into one of only two broad age categories appeared primarily to reflect the combined effects of biological variation in the severity and time-course of the observed tissue reaction to this type of injury, and to variations in the semi-

quantitative scoring of the appropriate histopathological changes. The Bayesian method itself was entirely objective in its evaluation of data according to the relative probability of observing combinations of scores in bruises of various ages. The distinction between bruises less than 20 hours and greater than 24 hours old rested mainly on the onset of repair and the presence of haemosiderin in the latter, with modifications according to the degrees of the macrophage and neutrophil exudates. Increasing the data base upon which the model was based would therefore have done little to enhance its sensitivity in distinguishing between bruises of various ages, though it may have enhanced the 'accuracy' with which bruises were aged as either 1-20 hours or more than 24 hours old.

2. Enzyme studies

Although opinions are not unanimous in this respect, most published evidence has suggested the usefulness of enzyme studies for the ageing of wounds, especially during the first few hours after injury. The activities of ten enzymes representing the major metabolic pathways in striated muscle were therefore examined by applying established enzyme histochemical and isoenzyme electrofocussing techniques to experimental bruises aged 4-144 hours old.

Altered histochemical enzyme activities and electrofocussed isoenzyme profiles were demonstrated in bruises. However, in no case could relationships be established between these changes and bruise ages. In this respect our findings were at variance with many published observations pertaining to wounds. Because established techniques were employed in this experiment, these discrepancies were considered unlikely to have reflected technical inadequacies in experimental methodology. The most likely explanation lay with the nature of the tissue reaction in the bruises studied, since this was relatively mild in comparison with that in the incision wounds upon which the majority of observations have been centred.

3. pH of bruised muscle

Localised pH changes of a predominantly acidic nature are known to occur in areas of inflammation, but there appears to be no published information relating tissue pH to the duration of injury. For this reason muscle pH values were measured in bruises 4, 16, 24, 36 and 48 hours old in order to investigate a possible alternative method for the ageing of these lesions other than by histology or enzyme studies.

Bruise and control muscle pH's were measured using a reliable and sensitive technique involving the homogenisation of tissues in a sodium iodoacetate solution of sufficient concentration to prevent further production of lactic acid by *post mortem* anaerobic glycolysis. Samples were taken 3 hours and 24 hours after death. The 24 hour samples provided an indirect measure of the amounts or availability of stored glycogen in bruised muscles, whereas the 3 hour samples provided as close an estimate of *ante mortem* bruise pH as was practicable under the conditions of the experiment. It was recognised that the pH of 3 hour *post mortem* samples would be somewhat different to those of bruised muscle at the time of slaughter because of continuing anaerobic glycolysis during the intervening period. However, the pH experiment was undertaken primarily to investigate the practical value of the pH approach to the ageing of bruises, and since similar *post mortem* delays would be unavoidable in the meat works, this situation was accepted.

Absolute pH varied widely between bruises of the same age, and an analysis of variance test showed there was no significant relationship between muscle pH and age. Hind limb bruise pH's were, however, significantly lower than the equivalent forelimb values. For this reason pH changes were investigated relative to controls of normal muscle, the latter being estimates of pre-bruise pH. Most of the bruises were slightly, though significantly ($P<0.05$), less acidic than control tissues. Since the differences were more pronounced in 24 hour *post mortem* samples this presumably reflected an *ante mortem* depletion of glycogen stores in bruised muscle. An analysis of variance showed there was no significant relationship between bruise/control pH differences and age.

II. SUBCUTANEOUS HAEMORRHAGIC SPECKLING

At the beginning of the 1977/78 killing season a previously unreported condition subsequently to be known as 'subcutaneous haemorrhagic speckling' affected an average of 5% of young lambs slaughtered in eight widely scattered New Zealand export meat works. In following seasons the majority of works in the country were affected. The pathological characteristics, aetiology and pathogenesis of these lesions were investigated.

Grossly, 'speckling' manifested as multiple petechial haemorrhages widely scattered throughout carcase adipose tissue, though with a tendency to be more severe over the loins and hindquarters. In this respect they were clearly distinguishable from the haemorrhages of 'blood splash' and conventional bruising. Microscopically the lesions were characterised by central thrombi surrounded by moderate numbers of closely associated leucocytes. From histological and epidemiological evidence, 'speckling' was shown to have occurred close to the time of death. Subsequent trials established the primary cause of the lesions to be electrical stunning. However, because 'speckling' exhibited a pronounced seasonal distribution pattern and affected only 5% of mainly very young lambs, a variety of secondary aetiological factors were proposed as influencing the prevalence and severity of lesions. In the meat works situation, however, it was not practicable to conduct trials of the scale needed to investigate the roles of these secondary factors. As an alternative approach, attempts were made to determine the pathogenesis of 'speckling' with the intention of formulating a rational approach to its prevention.

From the distribution and histological appearance of 'subcutaneous haemorrhagic speckling', the lesions appeared to have arisen from capillaries that had ruptured under haemodynamic stress. Measurements of systemic blood pressure suggested that capillary rupture was unrelated to post-stunning changes in arterial pressure. 'Speckling' was therefore proposed to have resulted from localised electrically induced increases in capillary pressure and permeability. Attempts were therefore made to reproduce the lesions by direct stimulation of

the nerves innervating the inguinal adipose tissue of three young lambs, both prior to and after the injection of various vasoactive chemicals which published evidence had suggested would affect capillary permeability. These attempts were unsuccessful.

To date, 'speckling' remains a problem in most New Zealand meat works. Worthwhile reductions in its severity and prevalence have been achieved by changing the field characteristics of the electric current used to stun sheep, but the problem is unlikely to be controlled until the pathogenesis of the lesions has been further elucidated.

APPENDIX

MATERIALS AND METHODS USED IN THE ENZYME STUDIES ON BRUISED MUSCLE

ACID PHOSPHATASE

A. Histochemistry

Reference: Pearse (1968).

Preparation of tissue sections

Fresh frozen sections cut at approx. 10μm.
Fix in 10% neutral buffered formalin for 15 minutes at 4°C.
Wash in distilled water.
Air dry.

Incubating solutions

1) Pararosanilin stock solution

pararosanilin	=	1.0g
distilled water	=	20ml
conc. HCl	=	5ml

Heat gently and filter

2) Michaelis veronal acetate buffer pH 5.0

sodium barbiturate	=	2.94g
$NaCH_3COOH\ 3H_2O$	=	1.94g
distilled water	=	100ml

3) Substrate medium

α naphthyl phosphate*	=	10mg (OMIT FROM CONTROLS)
pararosanilin stock	=	6.4ml
$NaNO_2$	=	256mg
veronal acetate buffer	=	40ml
distilled water	=	80ml

Adjust to pH 6.0 with 1 N NaOH.

Add distilled water to final volume = 160ml

Technique

Filter the substrate medium into Coplin jars containing slides.

Incubate for 60 minutes at 25°C.

Rinse in distilled water for 2 minutes.

Counterstain with Mayer's haemalum for 2 minutes.

Wash for 15 minutes.

Dehydrate with alcohol followed by xylene.

Mount in D.P.X.

B. Polyacrylamide gel isoelectrofocussing

Reference: Harris and Hopkinson (1976).

Incubating solutions

1) 0.2M acetate buffer pH 5.0

Solution A

0.2M acetic acid	=	11.4ml/litre

Solution B

0.2M anhydr. sod. acetate	=	16.4g/litre

148ml A + 352ml B + 500ml H_2O = 1000ml buffer pH 5.0

* All substrates, enzymes, coenzymes, tetrazolium dyes and diazo dyes were obtained from Sigma Chemical Co., St Louis, Missouri, U.S.A.

2) Substrate medium

α naphthyl phosphate	=	100mg
Fast Blue B	=	100mg
acetate buffer	=	100ml

Technique

Wash gel for 5 minutes in acetate buffer at 4°C.
Incubate gel in substrate medium for 30 minutes at 25°C.
Immerse in trichloroacetic acid/glycerol fixing solution for 30 minutes.
Photograph and scan gel.
Cover with plastic for storage.

ALDOLASE

A. Histochemistry

References: Pearse (1968); Pette *et al.* (1979).

Preparation of tissue sections

Fresh frozen sections cut at approx. 10μm.
Fix in acetone for 2 minutes at 2°C.
Air dry.

Incubating solutions

1) 0.05M arsenate-HCl buffer pH 7.6

2) Substrate medium - Due to the solubility of the enzyme, the substrate medium is made up as a series of 1.5% agarose gels formed between two cover slips spaced by matchsticks. One of each of the cover slips is removed and the gels placed over the tissue sections.

Solution A

0.05M arsenate-HCl buffer pH 7.6	=	50ml
agarose	=	1.5g

Solution B

sodium fructose 1,6,diphosphate	=	406mg (OMIT FROM CONTROLS)
NAD	=	12mg
NBT	=	25mg
arsenate-HCl buffer	=	50ml

A + B = 100ml 1.5% agarose substrate gel

Technique

Apply substrate gels firmly.
Incubate for 30 minutes at 37°C.
Remove gels and wash slides in water.
Fix sections in 10% formalin for 1 hour.
Wash in water.
Immerse in acetone for 10 minutes to dissolve monoformazans.
Air dry.
Counterstain with 1% safranin for 20 seconds.
Dehydrate in alcohols followed by xylene.
Mount in D.P.X.

B. Polyacrylamide gel electrofocussing

Reference: Harris and Hopkinson (1976).

Incubating solutions

1) 0.1M Tris-HCl pH 8.0

2) Substrate medium

Fructose 1,6 diphosphate	=	600mg
G3PD (1220 I.U./ml)	=	198µl
MTT	=	45mg
PMS	=	15mg
NAD	=	120mg
$Na_2 AsO_3$	=	90mg
Tris-HCl buffer	=	150ml

Technique

Wash gel for 5 minutes in Tris-HCl buffer at 40°C
Incubate gel in substrate medium for 30 minutes at 25°C in darkness.
Immerse in fixing solution for 30 minutes.
Photograph and scan.
Cover with plastic for storage.

ALKALINE PHOSPHATASE

A. Histochemistry

Reference: Pearse (1968)

Preparation of tissue sections

Fresh frozen sections cut at approx. 10µm.
Fix in 10% neutral buffered formol saline for 15 minutes at 4°C.
Wash in distilled water.
Air dry.

Incubating solutions

1) 0.1M Tris-HCl buffer pH 10.0

2) Substrate medium

α naphthyl phosphate	=	100mg (OMIT FROM CONTROLS)
Fast Red Tr	=	100mg
Tris-HCl buffer	=	100ml

Technique

Filter the substrate medium into Coplin jars containing slides.
Incubate for 60 minutes at 25°C.
Wash for 1-3 minutes.
Counterstain with Mayer's haemalum for 2 minutes.
Wash for 15 minutes.
Mount in glycerine jelly.

B. Polyacrylamide gel electrofocussing

References: Angellis *et al.* (1966); Harris and Hopkinson (1976).

Incubating solutions

1) 1.0M Tris-HCl buffer pH 9.7

Tris	=	121.1g/litre
$MgSO_4 \cdot 7H_2O$	=	123mg/litre
$ZnSO_4 \cdot 7H_2O$	=	14.4mg/litre

2) Substrate medium

α naphthyl phosphate	=	100mg
Fast Blue B	=	100mg
$MgSO_4 \cdot 7H_2O$	=	123mg
$ZnSO_4 \cdot 7H_2O$	=	14.4mg
Tris-HCl buffer	=	100ml

Technique

Wash gel for 5 minutes in buffer at 4^oC.
Incubate gel in substrate medium for 90 minutes at 37^oC.
Immerse gel in fixing solution for 30 minutes.
Photograph gel.
Cover with plastic for storage.

CREATINE KINASE

A. Histochemistry

References: Khan (1971, 1973); Pette *et al.* (1979).

Preparation of tissue sections

Fresh frozen sections cut at approx. 10μm.

Air dry.

Immerse in acetone for 12 minutes at -70°C (inhibits respiratory enzymes).

Immerse in 70% ethanol for 8 minutes at -20°C (inhibits ATP-ase).

Wash thoroughly.

Air dry.

Incubating solutions

1) 0.1M glycylglycine buffer pH 6.9

glycylglycine	=	13.2g/l
magnesium acetate	=	4.3g/l
glucose	=	3.6g/l

2) Substrate medium - Due to the solubility of the enzyme, the substrate medium is made up as a series of 1.5% agarose gels formed between two cover slips spaced by matchsticks. One of each of the cover slips is removed and the gels placed over the tissue sections.

Solution A

0.1M glycylglycine buffer 6.9	=	50ml
agarose	=	1.5g

Solution B

phosphocreatine	=	330mg (OMIT FROM CONTROLS)
ADP	=	130mg
AMP	=	26mg (inhibits myokinase)
NADP	=	66mg
hexokinase	=	82.5I.U.
G6PD	=	130I.U.
PMS	=	33mg
NBT	=	33mg
glycylglycine buffer	=	50ml

50ml A + 50ml B = 100ml 1.5% agarose substrate gel

Techique

Apply substrate gels firmly.
Incubate 10 minutes at 25^{o}C in darkness.
Remove gels.
Wash sections.
Immerse in acetone for 10 minutes to dissolve monoformazans.
Counterstain with 1% safranin for 20 seconds.
Dehydrate in alcohol followed by xylene.
Mount in D.P.X.

B. Polyacrylamide gel electrofocussing

Reference: Shaw and Prasad (1970).

Incubating solutions

1) 0.1M Tris-HCl buffer pH 7.0

2) Substrate medium

creatine phosphate	=	400mg
ADP	=	210mg
NADP	=	36mg
MTT	=	36mg
PMS	=	4.5mg
glucose	=	270mg
G6PD	=	75I.U.
hexokinase	=	135I.U.
$MgCl_2$	=	300mg
Tris-HCl buffer	=	150ml

Technique

Wash gel in Tris-HCl buffer for 5 minutes at 4^{o}C.
Enzyme activities were demonstrated using two separate techniques, A and B.

Method A: Incubate gel in substrate medium for 15 minutes at 37^{o}C in darkness.
Immerse gel in fixing solution for 30 minutes.
Photograph and scan gel.
Cover with plastic for storage.

Method B: Cast the substrate medium into a 2% agarose gel plate and apply firmly to the enzyme gel.
Incubate for 30 minutes at 37°C.
Remove enzyme gel and treat as for Method A.

C. Cellulose acetate electrophoresis

References: Shaw and Prasad (1970); Turner *et al.* (1974).

Incubating solutions

1) Electrode buffer

0.06M sodium barbitol	= 12.4g/l
0.06% mercaptoethanol	= 600µl/l

2) Substrate buffer

0.1M Tris-HCl pH 7.2

3) Substrate medium - Due to the solubility of the enzyme the substrate medium is made up as a 0.5% agar gel.

phosphocreatine	= 731mg
hexokinase	= 2.3mg
G6PD	= 130I.U.
glucose	= 150mg
AMP	= 35mg
ADP	= 80mg
NADP	= 50mg
MTT	= 25mg
PMS	= 2.5mg
$MgCl_2 \cdot 7H_2O$	= 150mg
Tris-HCl buffer	= 100ml
noble agar	= 0.5g

Technique

Pre-soak 'Titan III' cellulose acetate plates in the electrode buffer for 20 minutes, then apply four 0.25µl samples of enzyme solution to each plate.

Electrophorese at 250 volts for 90 minutes.

Apply 1.0ml of substrate gel to an unused cellulose acetate

plate that has been pre-soaked in substrate buffer for 20 minutes, blotted and air dried.

Allow the substrate to soak in for 1 minute, blot the plate and apply it firmly to the enzyme plate.

Incubate for 20 minutes at 37°C in darkness.

Fix and clear plate as follows:

5% acetic acid for 10 minutes

methanol for 2 minutes

clearing solution for 5 minutes

Air dry plate and photograph.

ESTERASE

A. Histochemistry

Reference: Pearse (1968).

Preparation of tissue sections

Fresh frozen sections cut at approx. 10µm.

Fix in 10% neutral buffered formalin for 15 minutes at 4°C.

Wash in distilled water.

Air dry.

Incubating solutions

1) 0.2M phosphate buffer pH 7.4

Solution A

$Na_2HPO_4 \cdot 12H_2O$ = 7.164g/100ml

Solution B

$NaH_2PO_4 \cdot 2H_2O$ = 3.121g/100ml

40.5ml A + 9.5ml B = 50ml buffer pH 7.4

2) Hexazotized pararosanilin solution

Solution A

pararosanilin hydrochloride	= 1g
distilled water	= 20ml
conc. HCl	= 5ml

Warm gently, cool, filter, store in dark.

Solution B

Freshly prepared 4% sodium nitrite.

Immediately prior to use, mix equal volumes of A and B, adjust to pH 5.0.

3) Substrate medium

α naphthyl acetate	=	50mg (OMIT FROM CONTROLS)
phosphate buffer	=	75ml
water	=	25ml
hexazotized pararosanilin	=	8ml

Technique

Incubate in substrate medium for 20 minutes at 25°C.
Wash briefly in water.
Counterstain in Mayer's haemalum for 5 minutes.
Wash for 30 minutes.
Dehydrate in alcohols followed by xylene.
Mount in D.P.X.

B. Polyacrylamide gel electrofocussing

Reference: Jarecki *et al.* (1970).

Incubating solutions

1) 0.1M Tris-HCl buffer pH 7.4

2) Substrate medium

α naphthyl phosphate	=	40mg
Fast Red Tr	=	100mg
Tris-HCl buffer	=	100ml

Technique

Wash gel for 5 minutes in Tris-HCl buffer at 4°C.
Incubate gel in substrate medium for 15 minutes at 25°C.
Immerse gel in fixing solution for 30 minutes.
Photograph and scan.
Cover with plastic for storage.

GLUCOSE-6-PHOSPHATE DEHYDROGENASE

A. Histochemistry

References: Pearse (1968); Pette and Wimmer (1979).

Preparation of tissue sections

Fresh frozen sections cut at approx. 10μm.
Fix in acetone for 2 minutes at 2°C.
Air dry.

Incubating solutions

1) 0.05M glycylglycine-HCl buffer pH 7.6

glycylglycine free base	=	6.6g/l
adjust pH with HCl		

2) Substrate medium - Due to the solubility of the enzyme, the substrate medium is made up as a series of 1.5% agarose gels formed between two cover slips spaced by matchsticks. One of each of the cover slips is removed and the gels placed over the tissue sections.

Solution A

0.05M glycylglycine-HCl buffer pH 7.6	=	50ml
agarose	=	1.5g

Solution B

glucose-6-phosphate	=	673mg (OMIT FROM CONTROLS)
NADP	=	149mg
NBT	=	123mg
KCN	=	6.5mg
PMS	=	6.1mg
EDTA	=	187mg
0.05M glycylglycine-HCl buffer pH 7.6	=	50ml

A + B = 100ml 1.5% agarose substrate gel

Technique

Apply substrate gels firmly

Incubate 15 minutes at 37°C in darkness.

Remove gels and wash slides in water.

Immerse in acetone for 10 minutes to dissolve monoformazans.

Immerse in 10% formalin for 5 minutes.

Counterstain with 1% safranin for 20 seconds.

Dehydrate in alcohols followed by xylene.

Mount in D.P.X.

B. Polyacrylamide gel electrofocussing

References: Harris and Hopkinson (1976); Vergnes (1979).

Incubating solutions

1) 0.2M Tris-HCl pH 8.0

2) Substrate medium

glucose-6-phosphate	= 300mg
NADP	= 100mg
MTT	= 12mg
PMS	= 12mg
$MgCl_2.H_2O$	= 400mg
0.2M Tris-HCl pH 8.0	= 150ml

Technique

Wash gel for 5 minutes in Tris-HCl buffer at 4°C.

Incubate gel in substrate solution for 30 minutes at 37°C in darkness.

Immerse gel in fixing solution for 30 minutes.

Photograph and scan.

Cover with plastic for storage.

LACTATE DEHYDROGENASE

A. Histochemistry

References: Fahimi (1964); Pette *et al.* (1979).

Preparation of tissue sections

Fresh frozen sections cut at approx. 10μm.
Fix in acetone for 2 minutes at 2°C.
Air dry.

Incubating solutions

1) 0.2M Tris-HCl buffer pH 7.4

2) Substrate medium - Due to the solubility of the enzyme, the substrate medium is made up as a series of 1.5% agarose gels formed between two cover slips spaced by matchsticks. One of each of the cover slips is removed and the gels placed over the tissue sections.

Solution A

0.2M Tris-HCl buffer pH 7.4	=	50ml
agarose	=	1.5g

Solution B

Lithium lactate	=	1.15g (OMIT FROM CONTROLS)
EDTA	=	186.6mg
NBT	=	123mg
PMS	=	6mg
NAD	=	10mg
KCN	=	6.5mg
Tris-HCl buffer	=	50ml

A + B = 100ml 1.5% agarose substrate gel

Technique

Apply substrate gels firmly.
Incubate 15 minutes at 37°C in darkness.
Remove gels and wash slides in water.
Immerse in acetone for 10 minutes to dissolve monoformazans.

Dry and counterstain with 1% safranin for 1-2 minutes.
Dehydrate in alcohols followed by xylene.
Mount in D.P.X.
Store in the dark.

B. Polyacrylamide gel electrofocussing

Reference: Chua *et al.* (1978).

Incubating solutions

1) 0.05M Tris/glycine buffer pH 8.4

Tris	=	6g/l
glycine	=	28.8g/l

2) Substrate medium

Lithium lactate	=	600mg
β-NAD	=	40mg
NBT	=	20mg
PMS	=	1mg
water	=	16ml
Tris/glycine buffer	=	90ml

Technique

Wash gel for 5 minutes in Tris/glycine buffer at $4^{o}C$.
Incubate gel in substrate solution for 30 minutes at $25^{o}C$ in darkness.
Immerse gel in fixing solution for 30 minutes.
Photograph and scan.
Cover with plastic for storage.

LEUCINE AMINOPEPTIDASE

A. Histochemistry

References: Burstone and Cook (1956); Shaw and Cook (1979).

Preparation of tissue sections

Fresh frozen sections cut at approx. 10μm.
Fix in 10% neutral buffered formalin for 15 minutes at 4°C.
Wash in distilled water.
Air dry for 2 hours.

Incubating solutions

1) 0.2M Tris-HCl buffer pH 7.1

2) Substrate medium

ℓ-leucyl β-naphthylamide	= 35mg (OMIT FROM CONTROLS)
Fast Black K	= 105mg
Tris-HCl buffer	= 35ml
water	= 140ml

Technique

Filter the substrate medium into Coplin jars containing slides.
Incubate for 60 minutes at 25°C.
Wash for 2 minutes.
Counterstain with Mayer's haemalum for 3 minutes.
Wash for 15 minutes.
Mount in glycerine jelly.

B. Polyacrylamide gel electrofocussing

Reference: Shaw and Prasad (1970).

Incubating solutions

1) 0.2M Tris-maleate buffer pH 6.0

Solution A

Tris	= 24.2g/l
maleic acid	= 23.2g/l

Solution B

0.2M NaOH = 8g/l

250ml A + 130ml B + 620ml H_2O = 1000ml buffer pH 6.0

2) Substrate medium

ℓ-leucyl β-naphthylamide	= 40mg
Black K	= 100mg
Tris-maleate buffer	- 100ml

Technique

Filter substrate medium

Wash gel in buffer for 5 minutes.

Incubate gel in substrate medium for 10 minutes at 37°C.

Immerse gel in fixing solution for 30 minutes.

Photograph and scan gel.

Cover with plastic for storage.

MYOSIN ADENOSINE TRIPHOSPHATASE

A. Histochemistry

Reference: Davies and Gunn (1972)

Preparation of tissue sections

Fresh frozen sections cut at approx. 10μm.

Fix in cacodylate buffered formaldehyde pH 7.0 for 2 minutes.

Wash in distilled water.

Air dry.

Incubating solutions

1) Cacodylate buffered formaldehyde pH 7.0

sodium cacodylate.$3H_2O$	= 0.14g
0.2M HCl	= 6.3ml
40% formaldehyde	= 20ml
distilled water	= 200ml

2) Substrate medium

ATP (disodium salt)	= 90mg
$CaCl_2.6H_2O$	= 120mg
Tris	= 1.45g
distilled water	= 45ml

Adjust to pH 9.5 with 0.1N HCl

Add distilled water to volume = 60ml

Technique

Incubate in substrate medium for 20 minutes at 37°C.
Wash in two changes of distilled water.
Immerse in 2% cobalt chloride for 3 minutes.
Wash in two changes of distilled water.
Immerse in 1% ammonium sulphide for 30 seconds.
Wash.
Mount in glycerine jelly.

B. Polyacrylamide gel electrofocussing

Reference: Davies and Gunn (1972)

Incubating solutions

As for histochemistry.

Technique

Wash gel briefly in distilled water.
Immerse in cacodylate buffered formalin for 3 minutes
Dip in distilled water.
Incubate in substrate medium for 15 minutes at 25°C.
Wash twice in distilled water.
Immerse in 2% cobalt chloride for 5 minutes.
Wash for 1 hour with several changes of distilled water at 4°C.
Immerse in 1% ammonium sulphide for 2 minutes.
Wash.
Immerse gel in fixing solution for 30 minutes.
Photograph.
Cover with plastic for storage.

SUCCINATE DEHYDROGENASE

A. Histochemistry

Reference: Pearse (1968).

Preparation of tissue sections

Fresh frozen sections cut at approx. 10μm.
Fix in acetone for 2 minutes at 2°C.
Air dry.

Incubating solutions

1) 0.1M phosphate buffer pH 7.6

Solution A

KH_2PO_4 = 9g/l

Solution B

Na_2HPO_4 = 9.4g/l

10ml A + 90ml B = 100ml buffer pH 7.6

2) Substrate medium

sodium succinate	=	1.35g (OMIT FROM CONTROLS)
NBT	=	50mg
distilled water	=	75ml
phosphate buffer	=	25ml

Technique

Incubate in substrate medium for 50 minutes at 37°C.
Wash.
Fix in 10% neutral buffered formalin for 10 minutes.
Wash.
Air dry.
Immerse in acetone for 10 minutes to remove monoformazans.
Air dry.
Counterstain with 1% safranin for 20 seconds.
Dehydrate in alcohols followed by xylene
Mount in D.P.X.

B. Polyacrylamide gel electrofocussing

Incubating solutions

1) 0.1M Tris-HCl pH 7.4

2) Substrate medium

sodium succinate	=	675mg
MTT	=	40mg
$MgCl_2.6H_2O$	=	10mg
$CoCl_2.6H_2O$	=	60mg
sodium azide	=	60mg (inhibits respiratory chain)
NaCN	=	49mg (inhibits respiratory enzymes)
Tris-HCl buffer	=	100ml

Technique

Wash gel for 5 minutes in Tris-HCl buffer at 4^{o}C.

Incubate gel in substrate medium for 30 minutes at 25^{o}C.

Immerse gel in fixing solution for 30 minutes.

Photograph and scan.

Cover with plastic for storage.

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