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OXYTALAN FIBRES IN MARSUPIAL

PERIODONTAL LIGAMENTS

A project report submitted in partial fulfilment
for the degree of Master of Dental Surgery.

by

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SUMMARY

The arrangement and distribution of oxytalan fibres in Australian marsupials has not previously been reported. A wombat, three wallabies, one possum and eight marsupial mice of two species were examined histologically for the presence and distribution of oxytalan fibres within the mandibular molar and incisor periodontiums. Anatomic observations permitted comparison of the marsupials with different eutherian orders of Rodentia, Ungulate-like herbivores and Carnivora. Furthermore, evidence was obtained which indicated anatomic adaptations of the masticatory apparatus of each marsupial to a particular diet.

The marsupial periodontiums contained large numbers of oxytalan staining fibres. The wombat was the only marsupial studied which consistently demonstrated elastic and oxytalan fibres within the periodontal ligament. All marsupials had elastic fibres located in the corium of the supra-alveolar region of the periodontium. Comparative differences in the number, size and specific distribution of oxytalan fibres were noted and related to masticatory function. There was a basic oxytalan fibre pattern which recurred in all the marsupials studied. The oxytalan fibre system formed a continuous network of fine, branching fibres which completely invested each tooth in the periodontal ligament and supra-alveolar regions. The oxytalan system continued to link adjoining teeth transseptally. However, the mandibular incisors of the wombat, wallaby and possum had no apparent transseptal communication. There were two distinct forms of oxytalan fibres which were distributed as -

(1) fine fibres which emerged from the cementum to form a complex, branching network around, and between the teeth, and

(2) thicker, ribbon-like fibre tracts restricted from the

cervical to the apical third regions of all periodontal

ligaments, except in the wombat.

Attachment of oxytalan fibres to alveolar bone was not substantiated.

Oxytalan fibres appeared to have an intimate association

with the periodontal vasculature. Frequently, the oxytalan fibres

accompanied blood vessels into the alveolar bone. A three-dimensional

reconstruction technique has been developed to study the course of

oxytalan fibres within the periodontal ligament.

The staining reactions of fifty five dye substances were

tested under varying conditions for their ability to reveal oxytalan

fibres in the marsupial periodontal ligament. The pH values,

spectrophotometric absorption patterns, and the fluorescent properties

of the dye solutions were analysed.

The enzyme digestions and staining reactions employed in this

study provided support for the view that a mucopolysaccharide matrix

constitutes the stainable fraction of the oxytalan fibres demonstrated

histologically. It is hypothesized that the marsupial oxytalan fibres

may differ from their eutherian counterparts by the presence of more

available aldehyde and carboxyl groups within, or upon, the staining

moiety of oxytalan fibres. However, the staining mechanisms are

complex and may involve physical and/or chemical phenomena which are

also influenced by many variables.

On the basis of their morphology and distribution, oxytalan

fibres have been demonstrated in the marsupials with the use of many

more dyes than have been previously reported in the literature for

other species.

SIGNED STATEMENT

This project report is submitted in partial fulfilment of the
requirements of the Degree of Master of Dental Surgery in The University
of Adelaide.

This report contains no material which has been accepted for the
award of any other degree or diploma in any University. To the best
of my knowledge and belief, it contains no material previously
published or written by another person except when due reference is
made in the text of the report.

WAYNE J. SAMPSON.

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INTRODUCTION:

Since their discovery by Fullmer in 1958, oxytalan fibres
have been described in histologic, comparative, histochemical and
electron microscopic investigations upon many animals and under
conditions of health and disease. Fullmer has established criteria
which define oxytalan fibres as distinct entities from collagen,
reticulin and elastic fibres. However, he has also reported certain
similarities between oxytalan and elastic fibres.

Between 1958 and 1966, Fullmer published a series of articles
in which he described the distribution of oxytalan at various anatomic
sites and concluded that the presence of oxytalan seemed to be
proportional to the degree of stress to which the tissues were subjected.
He described oxytalan fibres in the periodontal ligaments of man and
other animals including the dog, sheep, deer, cattle, swine, guinea
pig, rat, mouse, monkey and rabbit. Different animals have been
investigated by other authors but information is lacking regarding
the presence and distribution of oxytalan in marsupials.

Numerous hypotheses have been offered to explain the
functional role of oxytalan fibres in the periodontal ligament.
Furthermore, the chemistry and structure of oxytalan is incompletely
understood despite histochemical and histo-morphological investigations
utilizing both light and electron microscopy.

Fullmer has stated that oxytalan fibres are a normal
component of the periodontal ligament. He has hypothesized that
oxytalan fibres represent an immature or modified form of elastic
tissue which is functionally related to stress but does not mature

to conventional elastic tissue under normal conditions. The staining
moiety of the oxytalan fibre has been attributed to a mucopolysaccharide
matrix.

AIMS OF THE INVESTIGATION:

The objectives of the present study are –

1. To evaluate Fullmer's hypotheses concerning oxytalan fibres, using marsupial material.

2. To examine anatomic features of the mandible and dentition of selected Australian marsupials.

3. To examine the periodontiums of the marsupials employing histologic procedures.

4. To establish whether oxytalan fibres are present in the marsupial periodontal ligaments.

5. To compare the marsupial oxytalan fibres with Fullmer's criteria for oxytalan.

6. To study the distribution of these fibres.

7. To evaluate the findings on a comparative basis with previously reported studies.

8. To further examine the staining properties of oxytalan fibres.

CHAPTER 1.   LITERATURE REVIEW: OXYTALAN.

1.1. Discovery:

        While examining formalin-fixed human periodontal ligaments,
Fullmer (1958) added an initial oxidative step with peracetic acid and
revealed aldehyde fuchsin positive fibres where only white collagen
had been previously observed. These new fibres were not classical
elastic fibres as they required oxidation before they could be
demonstrated and, even then, they stained with only three of the five
recognized elastic fibre stains (i.e. aldehyde fuchsin, Taenzer-Unna
orcein and Weigert's resorcin fuchsin). Furthermore, these new fibres
resisted elastase digestion and had a distinctive distribution in the
periodontium. The regions in which Fullmer was able to demonstrate
the new fibres were areas traditionally associated with stress so that
he initially thought they might be a specialized form of collagen which
formed as a response to functional demands. The fibres were not
considered to be pro-collagen or aged collagen as they could not be
seen in granulation tissue or skin.

        In the following paper, Fullmer and Lillie (1958) formally
named this new fibre "oxytalan". The name was derived from the Greek,
meaning "acid-enduring", in cognisance of the necessity for an oxidative
step before the fibres could be demonstrated histologically. The
authors endeavoured to establish certain criteria whereby oxytalan might
be recognized as a fibre unique and distinct from collagen, reticulin
or elastic fibres. Oxytalan was shown to have similarities, but also
significant differences, when compared with elastic fibres (Chapter
2.8).

1.2. Distribution:

1.2.(a) Man.

Fullmer (1958) demonstrated oxytalan fibres in the perio-
dontal ligaments of developing and erupted teeth, tendons, ligaments,
adventitia of blood vessels, epineurium, perineurium and the connective
tissue sheath around hair follicles. In the periodontal ligament the
oxytalan fibres were not birefringent like collagen, varied in cross-
section from 3μ to less than 0.5μ and on occasions reached 2 millimetres
in length. Some fibres were branched and, overall, the orientation
varied in different parts of the periodontal ligament. Oxytalan fibres
were embedded in cementum, associated with blood vessels, thought to
be attached to bone, and were seen in greatest numbers apically and at
the level of the cemento-enamel junction. However, it was not possible
to trace a single fibre that extended from the tooth to the bone.
Some fibres followed the same course as the principal collagen bundles,
some were oblique to the collagen bundles whilst a few, unattached,
ribbon-like fibres ran apico-occlusally as in the guinea pig perio-
dontium. Rannie (1963) found oxytalan fibres to be thick and perpendic-
ular to the occlusal plane at the apex, and fine and parallel to that
plane at the cemento-enamel junction. In the gingival region, some
oxytalan fibres followed the free gingival collagen bundles without
always reaching the epithelial basement membrane (Fullmer 1966).
Other fibres of oxytalan tended to follow the transseptal collagen
bundles but few, if any, joined across the alveolar crest. Some fibres
circumscribed the tooth. Fullmer (1960a, 1966) noted that the greatest
density of oxytalan fibres was in the gingival region. Those fibres at
the apex formed a complex network and those in the middle third of the
periodontal ligament were the finest and least numerous.

Fullmer (1959a) was unable to demonstrate oxytalan in the
human foetus prior to four months gestation. He found the fibres in

the oral mucosa adjacent and peripheral to the outer enamel epithelium and occlusal to the developing tooth. Oxytalan fibres were inserted into cementum as it was laid down.

Very fine oxytalan fibres were also disclosed in embryonic human pulp tissue (Fullmer 1959b) by means of the peracetic acid-orcein technique rather than the peracetic acid-aldehyde fuchsin method which obscured the fibres due to the large amount of stainable mucopoly-saccharide present. Some fine oxytalan fibres were also found in umbilical cords but few were seen in the pulp tissue of fully developed teeth. Oxytalan was only found in reparative tissue if the injured tissue originally possessed it e.g. in granulation tissue from the oral mucosa but not from the skin.

In general, most authors seemed to agree with Fullmer regarding the distribution of oxytalan in normal healthy human periodontal tissues (Kohl and Zander 1962, Rannie 1963, Löe and Nuki 1964). Fullmer, in articles published in 1960a, 1962, 1963, 1965 and 1966, repeated his statements that oxytalan was a normal component of the periodontal ligament. However, it is interesting to note that Fullmer did not specifically mention oxytalan fibre attachment to bone in his 1961 and 1962 articles, thus differing from his earlier state-ments concerning oxytalan attachment to alveolar bone and, particularly, the crestal bone. Parker (1972) and Edwards (1968a) did not find oxytalan fibres inserting into bone. Sims (1973) refuted bone attach-ment although the fibres were seen to accompany blood vessels adjacent to, and penetrating, the alveolar walls.

Hasegawa (1960) studied "normal" dermal-epidermal junctions adjacent to certain dermatologic pathoses and found considerable numbers of aldehyde fuchsin positive fibres. This seemed to contradict Fullmer (1958) who did not find oxytalan in the skin.

Tedeschi and Sommers (1962) suggested that Hasegawa's

findings were incorrect, because the tissue studied was most unlikely to be normal as it was probably stretched and stressed by the nearby lesions. Furthermore, oxytalan was not demonstrable in sections of dermis, granulation tissue or the fibrous stroma of viscera.

Löe and Nuki (1964) supported Fullmer's statements that oxytalan was a component of normal tissue and added that the fibres nearest the bone were finer and more branched than those near the cementum.

Goggins (1966) examined the periodontal ligaments of deciduous and developing teeth and found a general similarity in the oxytalan distribution. However, there were significant differences from Fullmer's descriptions for the adult human dentition -

(i)   there were many more ribbon-like fibres running in an apico-occlusal direction and extending into the middle third region of the deciduous periodontal ligaments,

(ii)   there were fewer oxytalan fibres embedded in the cementum of deciduous teeth,

(iii)   a definite network of fine fibres could be seen close to the deciduous cementum but not attached to it. (It is of interest to note that Shackleford (1971a) observed a similar array of collagen with the scanning electron microscope and which he called the "Indifferent Fiber Plexus"), and

(iv)   there were larger and more numerous oxytalan fibres in the bifurcation areas of permanent teeth.

Oxytalan was also found running parallel to the dental follicle of the developing primary and secondary teeth.

Miake (1970) found elastic fibres embedded into the cementum of deciduous periodontal ligaments, especially where resorption was advanced or coronal caries was extensive. The distribution of these

fibres was very similar to that of oxytalan but the sections, both decalcified and non-decalcified, were not pre-oxidized yet still showed positive for elastic fibres in about 50% of the cases examined. Fullmer (1958, 1960a, 1963 and 1965) acknowledged the presence of an occasional elastic fibre in human periodontal ligaments but said it was a rare finding (Fullmer 1966) except in the pathologic condition of scleroderma (Fullmer and Witte 1962).

Oxytalan fibres have been described as being thickest and most numerous in the transseptal region of rotated teeth (Boese 1969, Edwards 1968a, 1970).

Boese (1969) believed oxytalan crossed the alveolar crest to the adjoining tooth but this was denied by Edwards (1968a, 1968b, 1970) who also dismissed the idea of an alveolar attachment. Edwards (1968a) also found flat, ribbon-like oxytalan fibres in the middle third of the periodontal ligament. These fibres were perpendicular to the principal collagen fibres and were not apico-occlusally aligned as described by Goggins (1966).

Roche (1972) examined the gingival col but could find no regular pattern of oxytalan fibres. There was great variation in density and orientation from specimen to specimen as the oxytalan fibres weaved and intertwined with the gingival collagen bundles.

1.2.(b) Animals.

The presence and distribution of oxytalan has been investigated in a wide variety of animals. In general, the arrangement of the fibres followed a similar plan to that in man, although significant differences were noted.

Fullmer (1958) found and examined oxytalan fibres in the Achilles tendons and patellar ligaments of monkeys, mice, rats, guinea pigs, and a turkey. Fullmer (1959b) noticed small, fine oxytalan

fibres in a rooster's comb. Fullmer (1960a, 1966) published the results of comparative studies on the periodontal tissues of mice, rats, guinea pigs, a rabbit, deer, dog, sheep, swine, a monkey and also some human embryos. He found that the dog, swine, deer and cattle had elastic fibres of similar morphology to the transseptal oxytalan fibres of man. The elastic fibres co-existed with oxytalan which was found in the middle and apical thirds of the periodontal ligaments of the same animals. This suggested a possible relationship between oxytalan and elastic fibres, as will be described in Chapter 2.8 of this report. Fullmer also noted that part of the same fibre would stain like an elastic fibre and the other part like oxytalan (e.g. cattle, swine and deer). The proportion of elastic fibres to oxytalan varied according to (1) the tooth examined, (2) the species of animal, and (3) the region of periodontal ligament observed. For example, in the rabbit and guinea pig, the proportion of elastic to oxytalan fibres varied greatly in the areas between the molars of the same animal. He found oxytalan, which was morphologically indisting-uishable from elastic fibres, co-existing with elastic fibres in the gingival regions of some animals. Frequently, oxytalan fibres were seen to pass to the epithelial basement membrane and ramify below the epithelial cells. The thickness of the fibres, the degree of branching, and the numbers of fibres varied among the animals studied.

Rannie (1963) examined the periodontal tissues of monkeys and rats. It was stated that the apical oxytalan fibres were strong and perpendicular to the occlusal plane while at the cemento-enamel junction they were fine and parallel to the occlusal plane. Oxytalan fibres attached to both cementum and bone.

Carmichael (1968) studied laboratory mice and found that the majority of oxytalan fibres ran between cementum and the periodontal blood vessels. The main oxytalan orientation was oblique across the

periodontal ligament although some fibres were perpendicular to the direction of the collagen bundles. Another group of fibres was observed to be located toward the mesial end of the incisor periodontal ligament where they were closely related to the blood vessels and also proceeded toward the gingiva. No elastic fibres were found and there was reported to be no attachment of oxytalan fibres to bone.

Shackleford (1971b) said that armadillos, which have poorly functional teeth, possessed oxytalan fibres running parallel to the long axis of their teeth. He noted the similarity to the distribution of oxytalan fibres in human deciduous teeth, rat incisors and the continuously erupting guinea pig molars.

Beynon (1967) examined oxytalan fibres in developing mouse molars and found them –

    (i)   inserting into the earliest formed cementum at the level of the break-up of Hertwig's epithelial root sheath,

    (ii)   lining the pulpal surface of the epithelial diaphragm running between the pre-odontoblasts and into the basement membrane, and

    (iii)   passing between the odontoblasts in the coronal pulp to become obscured in the pre-dentine.

Hyaluronidase digestion was positive, indicating the acid mucopoly-saccharide nature of the stainable portion of the fibres.

Soule (1967) studied oxytalan in the periodontal ligaments of the caiman and the alligator and found small numbers of oxytalan fibres attached to the primary cementum and extending a short distance across the ligament between the collagen bundles. The fineness and sparcity of the oxytalan fibres was explained in terms of the short functional life of these teeth. Both elastic and oxytalan fibres were

present in the periodontal ligament.

In a study of the Trigger fish, Soule (1969) found very few oxytalan fibres in the periodontal ligament. Those present were mainly located in the cervical region. No elastic fibres were found.

Duthy (1972) was able to demonstrate both oxytalan and elastic fibres in the temporomandibular joint of the rabbit.

Hurst (1972) described the oxytalan distribution in the Rhesus monkey periodontal ligament. The author found long, thick fibres which were greatest in number adjacent to the cementum and ran parallel to the tooth root. Smaller fibres were observed to lie in a horizontal direction in the transseptal region. No elastic fibres were seen in the periodontal tissues.

1.3. Functional Considerations:

In all articles since 1958, Fullmer consistently emphasised that oxytalan was found as a component of normal tissues subjected to mechanical stress and might serve the function of assisting those tissues to resist damage from overstretching. The oxytalan fibres parallel and intertwined with the collagen bundles were thought to strengthen them (Fullmer 1958, 1962, 1965 and 1966).

Rannie (1963) suggested that the fibres running perpendicular to the collagen bundles might provide a system which would counter disruptive forces on the periodontium. He further postulated that the anchorage of oxytalan fibres in cementum and bone could prevent ischaemia produced by obliteration of the vascular channels when the tissues were overstretched. Rannie also noted that the number of fibres increased as the functional demand increased.

Parker (1972) said that the numbers of oxytalan fibres, particularly in the transseptal region, were such that any function of oxytalan would be secondary to that of collagen. However, he also said

that

> *the fact that they are found consistently in areas of*
> *stress might indicate that they are produced by the body*
> *as a safeguard against abnormal forces causing separation*
> *and destruction of tissues.*

Oxytalan fibres have a low turn-over rate and high stability (Fullmer 1961, 1963). The biochemical stability, and cemental and gingival attachments of oxytalan fibres, could play an important part in the relapse of orthodontically rotated teeth (Boese 1969, Edwards 1968a, 1968b, 1970) and the reopening of extraction spaces (Edwards 1971). This was said to occur because the transseptal fibres stretched the gingiva on application of torsional forces and oxytalan did not readily remodel to relieve the stretching. Boese (1969), Edwards (1968a, 1968b, 1970 and 1971) also noted a significant increase in size and number of oxytalan fibres during derotation. Brain (1969) noted the importance of the supra-alveolar fibre system in the relapse of orthodontically rotated teeth.

On the basis of morphological, histochemical and both light and electron microscopic examination of oxytalan, Fullmer (1959b, 1960a, 1962, 1965, 1966) concluded that it represented a specially modified form of elastic fibre or an immature elastic fibre which did not mature to become a classical elastic fibre. Fullmer believed that there were sufficient differences to warrant the classification of oxytalan as a separate fibre type. Fullmer (1965) concluded that oxytalan fulfilled the role that elastic fibres would; namely, to prevent overstretching of tissues by nature of its elastic recoil when stretched. Roche (1972) agreed that oxytalan had elastic properties.

Fullmer (1962) had suggested that the observed connection of many oxytalan fibres to the periodontal blood vessels might imply that the fibres mechanically suspend the vessels in the periodontal ligament.

Carmichael (1968) developed Fullmer's concept of a suspensory function and postulated that oxytalan fibres might act to maintain the patency of the blood vessels when the tooth was under occlusal loading. As corroborative evidence, he cited the work of Castelli and Dempster (1965) who found that the periodontal vessels of monkey incisors unexpectedly failed to collapse when measured tensions were applied to the teeth.

However, Sims (1973) attributed a different function to the fibres by nature of their attachment to the periodontal vessels. Citing Bien (1966) he hypothesised that oxytalan fibres could perform a more sophisticated regulatory function rather than behave as a purely supportive or suspensory system for the periodontal vasculature.

Löe and Nuki (1964) attributed a neural function rather than a mechanical function to oxytalan fibres. This viewpoint was based upon the finding that the observed oxytalan distribution appeared very similar to the nerve distribution shown by silver staining techniques.

Goggins (1966) disagreed strongly with Löe and Nuki's concept and said that although the oxytalan distribution was super-ficially similar to the nerve distribution shown by Bernick (1957), there were more differences than similarities. For example, Bernick (1959) did not find any nerve fibres in developing rat periodontal ligaments, whereas Goggins (1966) noted an abundance of demonstrable oxytalan around developing human teeth. Trott (1962) said that the collagen fibre organization and orientation in the periodontal ligament of the rat was due to functional stimuli created by the eruptive forces - no reference was made to oxytalan.

1.4. Non-Pathologic changes:

Fullmer (1959a) observed the presence of oxytalan in areas where functional stresses were generated - e.g. periodontal ligaments

and muscle tendons. As a result of these stresses, the fibres were
reported to be increased in size and number. For example, around
teeth serving as bridge abutments. The number of fibres and the
actual thickness of the individual fibres increased with age and the
degree of stress. Other fibre changes were minimal between the ages
of 25 and 80 years (Fullmer 1962). Fullmer also observed that oxytalan
was only formed in reparative tissues where it was present before
injury (Fullmer 1959b, 1963, 1965).

1.5. Pathologic Changes:

Fullmer (1959a) stated that oxytalan was a component of
normal tissues which were subjected to functional stresses. Fullmer
(1963) noted that the number and size of oxytalan fibres increased in
healthy tissues with function. In certain disease states the size,
number and distribution of the fibres altered.

Oxytalan was demonstrated in dental granulomas and radicular
cysts (Fullmer 1960b), the stroma of ameloblastomas and an
ameloblastic fibroma (Fisher and Fullmer 1962), dilantin induced
gingival hyperplasia (Baratieri 1967a, 1967b), fibro-osseous jaw
lesions (Hamner and Fullmer 1966), sclerosing haemangiomas (Tedeschi
and Sommers 1961), dermal fibromas and giant cell tumors (Tedeschi and
Sommers 1962).

Fullmer (1961) studied periodontal disease in human tissue
and found great variation in the degree of fibre and bone loss which
was independent of the degree of sulcular involvement. Collagen seemed
to suffer destruction before oxytalan (Fullmer 1963) and this impression
was supported by the work of Kanouse (1966) with ascorbic acid deficient
guinea pigs. Fullmer (1961) noted that with repair of the periodontal
tissues, the collagen and oxytalan fibres seemed to be poorly
orientated for function. The degree of periodontal destruction

appeared to depend upon the age of the individual and the duration of the disease process.

Fullmer and Witte (1962) found that both collagen and oxytalan fibres increased in numbers in the periodontium of individuals suffering from scleroderma. In areas of collagen sclerosis, elastic fibres could be demonstrated. This implied that oxytalan might undergo maturation to become elastic fibres in this pathologic condition thus supporting the concept of the relationship between oxytalan and immature elastic tissue.

Hurst (1972) experimented with the reimplantation of extracted teeth in the Rhesus monkey. He found oxytalan to have completely regenerated its original distribution and appearance in the periodontal ligament 2 weeks after reimplantation. However, the teeth were splinted, and as ankylosis occurred from diminished function, he found the oxytalan in the periodontal ligament decreased in size, number of fibres, and staining intensity. The transseptal fibre system did not show a decrease in the number of oxytalan fibres, but did indicate that they diminished in size with reduced function.

1.6. Electronmicroscopy:

Fullmer and Carmichael (1966) examined rat periodontal ligaments and reported that the oxytalan fibres consisted of bundles containing filaments approximately $150^{O}A$ in diameter and an inter-filamentous amorphous substance of similar diameter. No periodicity of the fibrils was seen.

Greenlee, Ross and Hartman (1966) remarked upon the striking similarity between the developing elastic fibres which they had described in the rat flexor tendon and the oxytalan fibres described by Fullmer and Carmichael (1966).

Griffin and Harris (1967) found fibres in human periodontal

ligaments which were very similar to those seen by Fullmer and
Carmichael (1966) except that the diameters were smaller (75 - 150°A).
Beaded elements with diameters of 75 - 175°A were also seen. Those
beaded fibres had an associated amorphous component. The
investigators further noted that the fibrils branched and formed a
part of larger fibres (1250 - 3500°A diameter) which were said to
correspond with the oxytalan fibres demonstrated by light microscopic
techniques. Low (1962) described microfibrils of 100°A diameter which
aggregated around basement membranes and elastic fibres. He
considered the microfibrils to be separate extra-cellular entities.
Ayer (1964) suggested that these microfibrils might aggregate to form
elastic fibres. Griffin and Harris (1967) suggested that the micro-
fibrillar structures actually formed oxytalan.

Harris and Griffin (1967) used normal, peracetic oxidized,
and peracetic acid-β-glucuronidase digested human periodontal tissue.
These workers found that the microfibrillar substance behaved
similarly to oxytalan with regard to staining, loss of the amorphous
substance and reaction to enzyme digestion. They equated the
amorphous ground substance associated with the microfibrils to the
mucopolysaccharide portion of the oxytalan fibre.

Hall et al. (1955) concluded that the electronmicroscopic
picture of elastic fibres was unresolved when they reviewed the
available literature. All they could determine was that branching,
non-banded fibrils could be seen held together by a dense amorphous
substance. They wondered whether the fibrils might be of a
collagenous nature and hypothesized that either elastic fibres might
be produced from the breakdown products of collagen degeneration, or
both collagen and elastic fibre precursors might originate from the
same cell type.

Ayer (1964) believed the amorphous matrix to be no more than

an artifact produced by poor resolution of closely applied fibrils.
He also observed the presence of beaded fibrils with "knobs" which
possibly represented retracted, disconnected side branches of elastic
fibres.

Bodley and Wood (1972) examined rat tracheas and described
elastic fibres comprising a central amorphous material surrounded by
peripherally arranged microfibrils of 160°A diameter. The central
amorphous material was thought to provide the classical recoil
properties and staining peculiarities of elastic fibres.

Selvig (1968) examined human periodontal tissues and found
non-banded fibrils interspersed with banded collagen fibrils. These
non-banded fibrils were similar to those called oxytalan by Fullmer
and Carmichael (1966) and Harris and Griffin (1967). Selvig disputed
this designation on the basis that both non-banded and banded sections
could be discerned within the same fibril, and because he found an
inverse relationship between the banded and non-banded fibrils. He
concluded that the non-banded fibrils were in fact degenerating
collagen since they occurred in greatest numbers where the collagen
turnover rate was highest. Maximum turnover occurred in areas near
the cementum and bone at the root apex and alveolar crest, as shown
by the autoradiographic studies of Anderson (1967), Carniero and
Fave de Moraes (1965) and Baumrind and Buck (1970). Selvig also found
many banded and non-banded fibrils around teeth with chronic
periodontal involvement.

Beynon (1967) supported Fullmer and Carmichael (1966) with
regard to the non-striation of oxytalan fibrils. He also remarked
upon the great similarity between the histologically observed
distribution of oxytalan in developing mouse molars and that of the
fibrils observed by Selvig (1963) in an electron microscopic study
of Hertwig's epithelial rooth sheath and the formation of early

cementum and dentine.

Shackleford (1971a, 1971b) was unable to demonstrate oxytalan with the scanning electron microscope.

1.7. Formation and Development:

Fullmer (1959b) discovered that oxytalan fibres could not be demonstrated in human foetuses until the fourth month of gestation. The oxytalan staining elements were first found in the developing dental pulp.

Fullmer (1959a) reported the presence of oxytalan fibres in six month human foetuses at two locations in the oral mucosa -

(i) adjacent and peripheral to the outer enamel epithelium, and

(ii) occlusal to the developing tooth.

The fibres in (i) eventually were included in the forming periodontal ligament and those in (ii) became the gingival fibres. Oxytalan seemed to first develop as a mass of mucopolysaccaride between the collagen bundles. As further development occurred, fibres could be seen to be laid down in the ground substance.

It is of interest to note that Varadi (1970) studied elastic fibres in embryonic skin and aorta with the electron microscope and showed that the microfibrillar portion developed before the amorphous material. As development continued the amorphous material increased in proportion to the fibrillar component which showed a decrease.

According to Fullmer (1959a), the oxytalan fibres in the periodontal ligament became embedded in cementum as it was deposited upon the forming root.

An increase in size and number of oxytalan fibres was observed to occur in response to functional demands and stresses (Fullmer 1959a, Rannie 1963, Fullmer 1963, 1965, Edwards 1968a). Very little change could be observed as age increased from 25 to 80 years (Fullmer 1962).

Pre-elastic and oxytalan fibres were found to be histologically indistinguishable (Fullmer 1960a). Therefore, it was hypothesized that the stainable mucopolysaccharide portion of oxytalan did not mature to that of classical elastic fibres except in disease states, such as scleroderma, which involved a general over-maturation of the connective tissues.

Hamner and Fullmer (1966) discovered oxytalan in fibro-osseous jaw lesions but concluded that, because oxytalan and pre-elastic fibres were indistinguishable, no definitive deduction could be made concerning the possible odontologic origin of such lesions.

Kanouse (1966) believed oxytalan to be a form of precursor collagen. Selvig (1968) considered oxytalan to be degenerating collagen, while Fullmer (1958) originally thought the fibres might represent a stress modified form of collagen.

The concept that collagen and elastic fibres possess some similarities in their compositional sub-units is supported by a considerable number of investigations which have demonstrated the apparent transmutability between them (e.g. Hall et al. 1955, Banga, Balo and Szabo 1956, Fullmer and Lillie 1957).

1.8.   Histochemistry:

1.8.(a)   Demonstration of oxytalan.

Originally, Fullmer (1958) used Gomori's aldehyde fuchsin after oxidation of the tissue sections by the peracetic acid method of Greenspan. This staining technique could not distinguish between oxytalan and pre-elastic fibres in the developing ligamentum nuchae of cattle and the periosteum of human foetuses (Fullmer 1960a).

Fullmer and Lillie (1958) found oxytalan positive reactions with aldehyde fuchsin, Taenzer-Unna orcein and Weigert's resorcin fuchsin, but not with the other accepted elastic tissue stains

(orcinol-new fuchsin, Verhoeff's iron haematoxylin, azure A or Hale's colloidal iron stain). A slight reaction to the periodic acid – Schiff technique occurred in rodents but only demonstrated elastic fibres. Enzyme digestions with elastase, testicular hyaluronidase, β-glucuronidase and lysozyme were included to distinguish between elastic fibres and oxytalan.

Fullmer (1959b) used Taenzer-Unna orcein with peracetic acid oxidation to demonstrate fine oxytalan fibres in developing human pulps. Orcein was said to be more specific for the fibre portion of oxytalan. Aldehyde fuchsin seemed to be less discriminatory and caused heavy staining which tended to mask the fibres present. Orcein was said to be useful for defining oxytalan in areas rich with acid mucopolysaccharide.

Rannie (1963) substituted the oxidant potassium monoper-sulphate instead of peracetic acid which was sometimes unpredictable and often caused considerable tissue damage.

However, performic acid, bromine or acid permanganate could also be used as oxidants (Fullmer 1965).

An acid-alcohol differentiation step was added by Löe and Nuki (1964) to improve the quality of the staining procedure by removing excessive back ground staining artifacts.

Rannie (1963) was able to demonstrate oxytalan with dyes of the quinone-imine group (celestine blue, toluidine blue and methylene blue).

Horobin and James (1970) investigated the staining of elastic fibres with direct blue 152 and also provided a large list of other dyes which would stain elastic tissue (Table 13, Appendix I, p.210.). The authors were unable to demonstrate oxytalan fibres.

Fullmer and Lillie (1958) found that oxytalan could only be demonstrated after oxidation of the tissues and then with fewer dyes

than would successfully stain elastic fibres (Table 1. Chapter 3. p. 53.). They also found that the intensity of the elastic fibre staining reaction was increased after oxidation.

1.8.(b) Mechanisms of staining.

1.8.(b)(i) aldehyde fuchsin

Bangle (1954) theorized that the reactivity of Gomoris' aldehyde fuchsin was due to azomethines (Schiff bases) and that these reacted with three basic tissue groups –

(i)   specific proteins (e.g. β-cell granules of the pancreas),

(ii)   specific mucopolysaccharides,

(iii)   aldehydes.

The author noted similarities between some staining properties and the spectral absorptive spectra of aldehyde fuchsin, crystal violet and methyl violet 2 B.

Landing et al. (1956) stated that aldehyde fuchsin stained structures in the posterior pituitary following pre-oxidation which liberated strongly acidic groupings from cystine or cysteine.

Fullmer and Lillie (1958) proposed that the stainable fraction of oxytalan and elastic fibres was mucopolysaccharide and that the binding between protein and mucopolysaccharide was different for oxytalan. They also said that the staining reactions of different dyes varied and that probably different groupings were involved. For example, the active groups could exist in a reduced form, thus explaining the requirement of oxidation before oxytalan staining occurs. The authors found that interspersion of aldehyde blockade, deamination or acetylation following peracetic oxidation failed to prevent aldehyde fuchsin staining of oxytalan or elastic fibres. Methylation before or after oxidation prevented staining and alkali treatment (saponification) failed to restore it. They also

investigated the possible binding sites but discounted participation of S-S or $O-SO_4$ groups. The likely nature of the bond was concluded to be more strongly covalent than ionic. The $O-SO_4$ group was discounted due to the failure of alcian blue or azure A to stain oxytalan fibres. Nile blue, oil red O and lipid extraction tests failed to reveal a lipid component in oxytalan.

Spicer and Meyer (1960) believed aldehyde fuchsin strongly stained sulphated mucins. Non-sulphated and sialic acid containing acid mucopolysaccharides reacted weakly.

Sumner (1965) found that, following oxidation, staining was probably due to ionic linking with acidic products of cystine. Without oxidation, elastic fibres still stained but the mechanism was probably not ionic, although the mechanism was of an undetermined nature.

Ortman et al. (1966) said the active dye molecule was para-rosaniline which was not very stable and could account for the loss of staining properties with solution aging.

Mander et al. (1968) agreed with Bangle (1954) that the active species was the Schiff's base. They hypothesized that the possible staining reaction involved oxidation of α-hydroxyamine residues in oxytalan to imines which then tautomerized to enamines. It was thought that these enamine groupings then acted as the nucleophilic species in the staining reaction. They attributed the loss of staining activity of aldehyde fuchsin to the formation of hexa-N-alkyl fuchsin after several weeks aging.

Lillie (1969b) found basic fuchsin to have a triplet composition –

(i)   pararosaniline,

(ii)   rosaniline, and

(iii)   magenta II.

Pearse (1968) said aldehyde fuchsin was a triphenyl methane

type dye.

1.8.(b)(ii) orcein

Fullmer (1960a) found that orcein would not stain the internal
elastic-like membrane of venous and lymphatic vessels without pre-
oxidation with peracetic acid. This result was considered to be
analogous to pre-elastic fibre staining. Orcein was noted to be more
selective than aldehyde fuchsin, staining finer oxytalan fibres and
less mucopolysaccharide, thereby facilitating the identification of
such fibres in developing pulp (Fullmer 1959b) and in mucous connective
tissues (Fullmer 1960a).

Fullmer and Lillie (1956) concluded that elastic fibre
staining was not prevented by measures which blocked carboxyl, hydroxyl,
amine or aldehyde groups but noted that orcein stained collagen well
when those polar groups were blocked. After deamination and acetyla-
tion, orcein would not stain, thus indicating possible involvement of
quinine or amyl hydroxyl groupings.

Fullmer (1960a) observed enhancement of the orcein staining
reaction in acid solution and following oxidation of the tissue
sections.

Roman et al. (1967) demonstrated well defined, coarse, and
fine elastic fibres by use of an orcein-haematoxylin-iodized ferric
chloride staining procedure. The ferric chloride appeared to intensify
the orcein reaction.

Lillie et al. (1968) claimed to heighten elastic fibre
staining with acid orcein-iron and acid orcein-copper solutions.

Weiss (1954) calculated the heat of reaction between orcein
and elastic fibres and found it to be within the range characteristic
of hydrogen bonds (-6000 to -6500 calories/mole).

Goldstein (1962) believed that the binding force was not

ionic as the staining reaction was not inhibited in highly concentrated solutions of sodium chloride but was prevented in highly concentrated solutions of urea (which has a high affinity for hydrogen bonds).

Friedberg and Goldstein (1969) attributed orcein selectivity for elastic fibres to -

(i) the presence of a relatively large number of dye-binding sites per unit volume of elastic fibre. The binding probably occurred by some non-ionic mechanism.

(ii) the relatively non-polar nature of elastic fibres which repelled cationic dye particles to a lesser extent than tissue components carrying a positive charge at low pH.

(iii) the low permeability of elastic fibres, such that once dying was achieved, alcoholic extraction was resisted.

Lillie (1969a) investigated some 50 dyes in order to clarify the mechanism of orcein staining. He concluded that the elastic fibre staining components of orcein belonged to amino-orceins rather than hydroxy-orceins or amino-orceimines.

Lillie (1969b) stated that orcein could be manufactured from orcinol. This is of interest because orcinol-new fuchsin failed to stain oxytalan whereas orcein did. There were also some 14 different fractions comprising the compound orcein and all of those fractions had different properties which complicated the staining mechanism.

1.8.(b)(iii) resorcin fushsin

Puchtler et al. (1961) concluded that resorcin fuchsin probably bound to polysaccharide esters and did not depend upon the presence of protein. Many materials (agar, glycogen, collagen, basement membranes, and reticulin) could be induced to stain after acetylation,

sulphation and phosphorylation procedures which introduced ester

groups.   The authors thought it was likely that a phenolic hydroxyl

group of the resorcinol moiety of the dye was free to become reactive.

1.8.(b)(iv)   other dyes

Fullmer and Lillie (1958) failed to demonstrate oxytalan

with nile blue, oil red O, azure A, alcian blue, Hale's colloidal iron,

P.A.S., Verhoff's iron haematoxylin or orcinol-new fuchsin.

Rannie (1963) was able to demonstrate oxytalan fibres with

methylene blue, toluidine blue and celestin blue dyes which are of

the Quinone-imine type.

Methylene blue (a tetramethyl thionin), toluidine blue

(related to thionin and methylene blue) and celestin blue (an Oxazin)

were dyes which stained acid mucins (Lillie 1969b).

Horobin and James (1970) found that there was no relation-

ship between the dye class, formal charges, or the presence of

hydrogen binding groups in the staining reactions of elastic fibres.

They found, however, that staining was definitely associated with the

presence of 5 or more aromatic rings in the dye molecule.   This

suggested that dye binding to elastic fibres might be by Van der Waal's

forces for both the direct dyes and the standard elastic fibre stains

(Table 13. Appendix I, p.210.).   The authors used direct blue 152 and

proposed a method for its manufacture and use.   They did not find

oxytalan fibres in Zenker fixed periodontal tissues.

1.8.(c)   Reliability of histochemical procedures.

Fullmer and Lillie (1957) found that acetylation or

benzoylation of formalin fixed collagen induced stainability with the

elastic fibre stains, except aldehyde fuchsin, but blocked the standard

reactions for collagen, such as Van Gieson, Masson's Trichrome and

Mallory's aniline blue methods. Deamination induced aldehyde fuchsin
staining of collagen.

Fullmer (1960a) noted that the nature of dye binding must
differ according to the dye type, the fibre type and the different
reactive groupings on the same fibre. For example, orcein had a
different mechanism of reaction than did aldehyde fuchsin or resorcin
fuchsin. This created a degree of uncertainty about the identity of
substances demonstrated by histochemical methods alone. Pearse (1968)
and Lillie (1969b) found that the method of tissue preservation or
fixation could alter the biochemistry of the tissues. Variability
was said to exist at many stages between preparation, staining and
final observation and interpretation of the tissues.

Puchtler et al. (1961) demonstrated that the staining
conditions of resorcin fuchsin could be modified to produce positive
reactions for collagen, glycogen, basement membranes and reticulin.
They said that no information could be deduced concerning the protein
moiety and that the specificity of the dye could be seriously
challenged.

Rannie (1963) said that even though orcein was more specific
for elastic fibres than aldehyde fuchsin, the dye also stained keratin
after oxidation. Consequently, there was inadequate evidence to relate
oxytalan to elastica as the orcein reaction might just as correctly be
assumed to be displaying keratin.

Aldehyde fuchsin stained mucopolysaccharide (Bangle 1954,
Fullmer and Lillie 1958) but indicated nothing about the protein
portion. The stainable fraction of oxytalan, but not elastic fibres,
was removed by β-glucuronidase (Fullmer 1966), hyaluronidase and
lysozyme (Fullmer and Lillie 1958) while the fibrous portion remained,
in an unstained condition.

Fullmer (1965) observed that collagen, precollagenous

reticulin and stromal reticulin of spleen and lymph nodes did not
stain with aldehyde fuchsin under conditions for oxytalan, but
sarcolema and basement membranes around kidney tubular epithelial cells
did stain.   Therefore, aldehyde fuchsin was not specific for elastic
tissue or oxytalan (Goggins 1966). Löe and Nuki (1964) used the
observed stainability of the motor end plate as support for their
contention that oxytalan could be nervous tissue.

The fact that pre-elastic fibres could not be distinguished
from oxytalan supported the doubts regarding the specificity of
elastic fibre stains (Fullmer 1960a, 1965).

Lev and Spicer (1964) demonstrated how easily staining
reactions can be misleading. Normally, alcian blue at pH 1 will react
with $-SO_4$ groupings and at pH 2.5 will stain both $-SO_4$ and $-COOH$
groups. However, if the sections are rinsed in water before mounting
the $-SO_4$ specificity is lost because the water raises the pH to the
level for $-COOH$ reactivity as well. Similarly, Carlo (1964) suggested
alcian yellow would show sulphate half esters and alcian blue would
reveal the carboxyl groupings. However, Sorvari and Nänto (1971)
seriously doubted the reliability of such a method as a useful
laboratory procedure. Alcian blue even stained nuclear chromatin
after β-glucuronidase liberated, or produced, a nuclear polysaccharide
complex (Salthouse 1961).

Zugibe (1970) showed that normally periodic acid – Schiff
negative acid mucopolysaccharides could be induced to react positively
after prolonged exposure to periodic acid (4 – 16 hours).

Banga, Balo and Szabo (1956) were able to produce a meta-
collagen by (1) thermohydral contraction, (2) chemical contraction –
relaxation, and (3) extraction by chemicals (for example, phosphate
buffers). Collagen treated in this manner behaved physically and
chemically like elastic tissue. Similarly, Hall et al. (1955) were

able to produce "elastin" from collagen treated with alkaline borate buffers and sodium metaperiodate at pH 5.

Ballantyne (1968) further illustrated the relative non-specificity of histochemical determinations when he was able to demonstrate elastic fibres reacting positively with the naphthoic acid hydrazide technique to give a false positive result for monoamine oxidase.

CHAPTER 2.   THE FIBROUS CONNECTIVE TISSUES.

Collagen

Reticulin

Elastic Fibres

Oxytalan

2.1. Explanatory Notes:

An understanding of the characteristics of the connective tissue fibres enhances the description of oxytalan as a separate fibre type. The distinction and recognition of any one of the fibres relies upon the establishment of adequate differences in physical and chemical properties. However, despite the differences which do exist, each connective tissue fibre type has certain similarities with one or more of the other fibres with the result that it may be broadly related to another fibre type. For example, Fullmer (1960a) hypothesized that oxytalan represented an immature or modified form of elastic fibre. This conclusion was reached by observation of certain differences and similarities of oxytalan to pre-elastic and mature elastic fibres. The differences were sufficient to warrant the naming of oxytalan as a separate connective tissue element. Banga et al. (1956) and Hall et al. (1955) observed certain similarities between collagen and elastic fibres, but their differences in physical and chemical behaviour are such that they are recognized as separate fibre types.

A comprehensive literature review of all the connective tissue fibres would be disproportionate for the present study which mainly focuses on the distribution of one fibre, oxytalan. However, it is felt that a discussion involving oxytalan necessitates an

appreciation of its relationship to the other connective tissue fibres.

Consequently, short summaries are presented for each fibre with an attempt to relate them to each other by listing the relevant similarities and differences in their properties.

The major references are Ramachandran (1963), Lowther (1963), Elden (1968), Pearse (1968), Ham (1969) and Melcher and Bowen (1969). All necessary minor references are contained therein unless specifically indicated.

As a broad generalization, the connective tissues comprise both fibrous and amorphous (ground substance) moieties. The amorphous material is difficult to analyse because a complex number of physical and chemical inter-reactions are produced between the various tissue components. For example, the physical property of viscosity shows much variability, ranging from fluid to gel-like depending on the body location and function to be performed. The ground substance is composed of proteins, carbohydrates (acidic and neutral, some of which are sulphated), lipids, interstitial fluid, plasma proteins, electrolytes, hormones, vitamins, enzymes, substances for anabolism and products of catabolism. The classification of the carbohydrate-protein compounds is difficult and has been confused. Barrett (1971) offered the following division of mucosubstances (carbohydrate-protein complexes) –

(1) GLYCOPROTEINS	(2) PROTEOGLYCANS
often branched	linear
No simple sequence of sugar residues	Alternating sequence of sugars
Sialic acid and fucose present	Absent
Uronic acid and sulphate normally absent	Present
Small molecules	Large molecules

The mucopolysaccharides fall into the proteoglycan category. Acid mucopolysaccharides can have simple organic acid side groups (-COOH, as occur in hyaluronic acid and chondroitin) or sulphuric acid groups (-O-SO$_4$, as occur in chondroitin sulphates). Glycoproteins (e.g. mucus) are characterized by the presence of N-acetyl-D-glucosamine, N-acetyl-D-galactosamine and sialic acid.

Connective tissues are found in areas of the body which undergo physiological deformation and as the age and functional demand change, then the relationship of fibrous to amorphous components alters. For example, as age increases, the hydration of the tissues decreases and the proportion of fibrous to amorphous components increases. Furthermore, increasing amounts of mucopolysaccharide relative to collagen are found in tendons, ligaments and cartilage, respectively.

2.2. Collagen:

Collagen is the most plentiful of the connective tissue fibres and is found in both vertebrates and invertebrates (excluding protozoa). In mammals, it comprises 25-35% of the total body nitrogen and is found in skin, tendon, bone, artery walls, fascia surrounding muscles,and in the periodontium. In fishes, it is found in fins, skin, scales and the swim bladders.

The structure and composition of collagen does not vary greatly in different areas of the same animal, except perhaps the size of the fibres, but may do so between species.

In histological sections, collagen is usually described as a coarse, non-branching fibre which is birefringent when unstained but stains characteristically with a number of dye techniques (e.g. Van Gieson, Mallory's aniline blue, Masson's trichrome). It is also distinguished by means of a characteristic wide angle X-ray diffraction pattern which provides information regarding the organisa-

tion of the collagen structure at the molecular level. When examined with the electron microscope, collagen is readily identified by its banded structure ($640^{O}A$ periodicity) which stains more intensely with phosphotungstic acid. Collagen is composed of a triple helix of three intertwined polypeptide chains each of which again has a helical configuration for its backbone.

Collagen exists in three physical states —

(1) fibrous (as in tendons and ligaments),

(2) crystalline molecules (tropocollagen), and

(3) random coils (gelatin).

From native (naturally occurring) collagen, two subunits can be produced, i.e. (a) insoluble collagen, and (b) soluble collagen. The proportion of soluble to insoluble collagen changes as the animal ages, with a predominance of the insoluble form being present in aged collagen tissue. Tropocollagen is the soluble fraction which is derived by treating native collagen at $0^{O}C$ in buffers or dilute acetic acid at pH 3.5 (i.e. neutral salt or acid extraction). Tropocollagen is believed to be the basic building block of collagen as it is triple stranded like collagen, has a similar amino acid content, and can be aggregated back into the form of native fibrils. If tropocollagen is treated with heat ($40^{O}C$), potassium thiocyanate, urea or lithium bromide at room temperature, then further fractions are formed. These fractions are of undetermined nature but may represent dispersion of the three chains of the triple helical structure or intra-helical fractionation. By electron microscopic observation, tropocollagen is $2,800^{O}A$ in length and $14^{O}A$ wide. The aggregation of tropocollagen to reform native collagen depends on (1) pH (greater than 6.5 is needed), (2) ionic strength and valency of the ions concerned, (3) temperature and, (4) purity of the tropocollagen mixture.

The arrangement of the tropocollagen molecules determines

the form of the collagen fibrils seen with the electron microscope. The banded appearance of collagen is due to the manner in which the tropocollagen molecules overlap each other in the composite fibril. The collagen types are -

(1)   FLS (fibrous long spacing). This is seen as fibrils with a periodicity of 2,000 - 3,000°A and is formed by the action of certain inducing agents (usually polymers of high negative charge, such as serum glycoprotein and chondroitin sulphate).

(2)   SLS (segment long spacing). This form occurs in short segments (about 2,500 - 3,000°A) and is obtained from phosphate extracts (by addition of R.N.A., D.N.A., heparin, chondroitin sulphate before dialyzing against citrate buffers) or by A.T.P. precipitation from acid solutions.

(3)   Native collagen. This is the fibril seen under the electron microscope with the characteristic 640°A periodicity due to the overlap of the tropocollagen molecules by approximately one quarter of their length. The light and dark bands are probably a feature of the side groups in the molecules. There are more overlapping tropocollagen molecules in the light regions than the dark.

Due to the lessened content of soluble tropocollagen and possible cross-linking effects, mature collagen is not readily dissolved by dilute acids, alkalis, or proteolytic enzymes such as trypsin or chymotrypsin, unless the fibres are denatured by pre-treatment with (1) 6M urea or (2) heating. Collagen is broken down by collagenase. The amount of water bound to collagen at saturation

is 100 - 120% of its dry weight. On heating above the shrinkage
temperature (67°C), thermohydral contraction occurs and collagen fibres
shrink to about one third of their original length, lose their
characteristic X-ray diffraction pattern, become more susceptible to
trypsin digestion, and lose their birefringence. On cooling, neither
the original length of the fibre nor its X-ray pattern are restored.
However, stretching the fibre can at least partly restore some of the
physical properties. On excessive heating, collagen breaks down into
gelatin. Collagen fibres cannot be stretched more than 10% of their
resting length without fracture.

Biochemically, collagen is unique from the other connective
tissue fibres in the proportions of its amino acid residues. Glycine
comprises one third of the total and about 20% is imino acids (proline
and hydroxyproline). The hydroxylysine content is also higher than in
other proteins while aromatic and sulphur containing amino acids are
low (tyrosine, methionine). Collagen comprises approximately 50% non-
polar amino acid residues. Banga and Balo (1960), Lazarow and Spiedel
(1964), and Bouteille and Pease (1969) have demonstrated the presence
of a carbohydrate, or mucopolysaccharide, intimately associated with
collagen. However, the carbohydrate composition of collagen seems to
vary with the origin and mode of preparation. The vertebrate collagens
and gelatins have a lower hexose content (0.5 - 1.3% dry weight) than
invertebrates (3 - 14%). Collagen contains a minimal amount of acetal
phospholipids (these are the substances responsible for the positive
reaction with fuchsin-sulphurous acid in untreated control sections
for the Feulgin and periodic acid-Schiff techniques).

The majority of authors share the opinion that collagen is
formed by the fibroblast. Precursor substances are secreted from the
cell and collagen fibres form extracellularly. Striated fibrils have
been found in very close relationship to the cellular basement

membranes but conclusive evidence of fibrils being present within the cell protoplasm is lacking. The fibroblast also secretes mucopoly-saccharides (e.g. hyaluronic acid and chondroitin sulphate) and it is thought that these amorphous substances form a gel outside the fibro-blast and aid collagen fibrillogenesis by -

(1) restricting the rapid diffusion of secreted tropocollagen such that

(2) the rate of formation of nucleation sites for new fibrils is enhanced due to the composition of the mucopolysaccharides.

The orientation of the newly formed fibre is determined by the surface of the fibroblast cell and the growth of the fibre is analogous to crystallization of soluble collagen on to preformed fibrils as the motile fibroblast moves away.

As the age of the animal increases, the rate of collagen synthesis decreases and consequently the proportion of soluble collagen diminishes and the stability of the fibres increases (except in uterine involution). Soluble collagen is easily catabolized which is a feature of a lack of cross-linkages between the peptide chains. Changes in collagen are seen in lathyrism where an abnormal collagen is produced, in vitamin C deficiencies where new collagen formation is impeded, and in a variety of disease states involving aberrations in collagen anabolism or catabolism.

Immature collagen can be non-banded or have an atypical periodicity (e.g. $200^{o}A$) which changes to $640^{o}A$ with maturation. Many invertebrates have a non-banded form of collagen.

Various treatments can falsify the histological identifica-tion of collagen as it can be made to represent elastic fibres (Banga, Balo and Szabo 1956, Fullmer and Lillie 1957, and Hall et al. 1955).

Characteristic features which distinguish collagen from other proteins are –

(1) Histochemistry (under normal conditions) e.g. birefringence and staining reactions.

(2) The amino acid content (one third glycine, 20% imino acids plus different quantities of other residues). Proline and hydroxyproline are characteristically high.

(3) A typical wide angle X-ray diffraction pattern with $3^{\circ}A$ meridional arc and $12^{\circ}A$ equatorial spots.

(4) The banded structure seen with the electron microscope.

(5) The peak corresponding to the NH....O hydrogen bond stretching frequency in the collagen infra-red absorption spectrum is at $3,330 \text{ cm}^{-1}$, having shifted by 30 cm^{-1} from the $3,300 \text{ cm}^{-1}$ peak of other proteins.

(6) In solution, collagen has a negative specific rotation of about -350° which drops to -120° on heating above the transition temperature.

(7) On heating, shrinkage occurs and the X-ray diffraction pattern becomes amorphous.

2.3. Reticulin:

There has been some controversy in the past concerning the actual nature of these fibres, with three views having been expressed –

(1) collagen and reticulin are one and the same.

(2) they are the same chemically, but differ physically.

(3) they differ both physically and chemically.

Furthermore, there has been a divergence in the use of the term "reticulin" to describe the structures seen histologically. Most authors are imprecise with the terminology but Pearse (1968, p.214) cited Puchtler (1964) who believed that "reticulin" should describe

the interfibrillar substance and "reticulum" or "reticular fibres"
should be reserved for the structures seen histologically. Melcher
and Bowen (1969) concluded that reticulin should not be referred to
as "reticular fibres" because electron microscopic studies indicated
that the fibrous appearance of reticulin at the light microscope
level of mangification was an illusion. Their studies showed that at
electron microscope levels of magnification, reticulin appeared as an
amorphous substance containing fibrils. This material filled the
continuum between cells and around bundles of collagen fibres with
the result that it was seen as a branching network at light micro-
scopic levels of magnification. This distribution of reticulin, in
conjunction with the histologic methods used to demonstrate it, creates
the appearance of fibres. Therefore, in accordance with Melcher and
Bowen the term "reticulin" will be adopted for descriptive purposes.

Reticulin is usually described as fine, branching fibres
which are associated with the renal basement membrane of kidney cortex
and lymphoid tissue. Reticulin is also found in the dermal papillae
subjacent to gingival and skin epithelium.

Unlike collagen, reticulin is isotropic in paraffin sections.
The staining reactions of reticulin and collagen differ. Reticulin
stains black with silver impregnation, magenta with the periodic acid-
Schiff reaction, red with sulphation metachromasia and pink, or
unstained, with Van Gieson. Collagen, by contrast, stains yellow-
brown, faint pink, blue, and red respectively. Using conventional
histology and light microscopy, reticulin presents as a branching,
fibrous network. When examined with the electron microscope, the
reticulin structures appear as a membranous, feltwork of interlacing
fibrils of very small diameter scattered throughout an abundant
amorphous matrix. The fibrils have a $640^{\circ}A$ periodicity. Reticulin
has been described as filling the continuum between cells and collagen

bundles. Reticulin has the same X-ray diffraction pattern as collagen.

Similar to collagen, reticulin is digested by collagenase and is not dissolved by trypsin. On boiling, reticulin does not revert to gelatin nor does it seem to show the same thermohydral properties as collagen.

The composition of reticulin varies from organ to organ, with age and under different physiological conditions. The amino and imino acid content is similar to collagen except for apparent deficiencies in proline and hydroxyproline, and the raised hydroxylysine content in renal reticulin. There is a high percentage of the lipid, myristic acid, (10 - 11%) and carbohydrate (4.2%) firmly bound to the protein moiety.

The origin of both collagen and reticulin is probably the fibroblast. It has been postulated in the literature that reticulin may represent an immature form of collagen. Reticulin is distinguished from other fibre types by histological staining techniques and by the detection of minor differences in composition, structure and distribution (c.f. elastic fibres and oxytalan). Orcein does not demonstrate reticulin in the spleen and lymph nodes but does show oxytalan in developing human pulp tissue, after four months gestation (Fullmer 1959b).

2.4. A Comparison Between Collagen and Reticulin:

2.4.(a) Similarities.

    (i)   Some authors believe collagen and reticulin to be one and the same with reticulin representing an immature form of collagen (Pearse 1968).

    (ii)   both are attacked by collagenase but are resistant to trypsin digestion.

    (iii)   both are possibly formed by the fibroblast.

(iv)   reticulin has fibrils of $640^{\circ}A$ periodicity, which is the same as collagen, when viewed with the electron microscope.

(v)   they both have the same X-ray diffraction pattern.

(vi)   the biochemical differences between collagen and reticulin are small (particularly the protein moiety).

(vii)   when collagen is heated it becomes isotropic, which is a characteristic of reticulin.

2.4.(b) Differences.

(i)   Some authors consider that collagen and reticulin differ physically and/or chemically (Pearse 1968). Others believe that reticulin does not even exist!

(ii)   Histologically, collagen is coarse and non-branching, whereas reticulin appears to be fine and branching.

(iii)   The histological staining reactions differ. For example, reticulin is argyrophilic and reacts positively with the periodic acid-Schiff technique whereas collagen does not.

(iv)   Collagen is birefringent whereas reticulin is isotropic in paraffin sections.

(v)   Although reticulin has a basically similar amino and imino acid content to collagen, there are deficiencies in proline and hydroxyproline. The hydroxylysine content is increased and there is more lipid and carbohydrate contained in reticulin.

(vi)   Unlike collagen, reticulin does not decompose to gelatin when boiled.

(vii)   Reticulin has an ultrastructure of amorphous matrix containing irregularly arranged fibrils. This leads to a membranous structure as distinct from collagen,

which forms a more ordered, fibrous system.

(viii)   The fibres are found in different anatomical areas.
         For instance, reticulin is found in the stroma of lymph
         nodes, spleen and kidney, while collagen is generally
         found in denser structures such as ligaments and tendons.
         This relationship seems to indicate an association with
         the degree of mechanical deformation which is to be
         withstood.

2.5.   Elastic Fibres:

Elastic tissue, which includes the protein and amorphous
polysaccharide components, is usually found in association with
collagen and occurs in the intima of blood vessels, tendons, skin,
lung, and forms a large proportion of the walls of major arteries
such as the aorta. Elastic fibres have been observed within the
periodontal ligaments of several animals (e.g. swine, deer and cattle,
Fullmer 1960a) but are rarely found in human periodontal ligaments.

Compared with collagen and reticulin, elastic fibres are
highly refractile and composed of long, narrow, branching fibres (less
than 1μ to a few microns thick when compared with collagen fibres
which range from 1μ to 12μ in diameter).

Elastic fibres can be demonstrated histologically by a large
variety and number of stains (Table 13, p.210, Table 14, p.212,
Appendix I). The best known and accepted stains being aldehyde fuchsin,
orcein, resorcin fuchsin, orcinol-new fuchsin and Verhoeff's iron
haematoxylin. The specificity of these dyes is not particularly high
for the protein moiety of the elastic fibre as it seems that the muco-
polysaccharide matrix is most frequently stained. Various chemical
pretreatments, such as acetylation, benzoylation or deamination, can
induce collagen to react with the elastic tissue stains mentioned

above (Fullmer and Lillie 1957). Elastic fibres react well with phenol
and naphthol dyes (Fullmer 1965, Melcher and Bowen 1969). The binding
of the dyes is not due to ionic bonds (Goldstein 1962) because of the
high percentage of non-polar residues present in the elastic tissue.

Horobin and James (1970) believed the high content of non-
polar groups would favour dye binding by short range Van der Waal's
forces. At the present time, the mechanism(s) of elastic tissue
staining excites much controversy and remains unresolved.

Electron microscopic studies indicate that elastic fibres
are composed morphologically of two parts:

(1)   a central region composed of an amorphous material,

(2)   peripherally arranged microfibrils.

The microfibrils are approximately 150°A in diameter, non-
banded, but occasionally show beading which Ayer (1964) suggested might
represent elastically recoiled, discontinued side branches. Ayer
(1964), however, believed the appearance of the amorphous material was
merely an artefact of poor resolution. Many researchers express
conviction that the amorphous material does in fact exist (Greenlee,
Ross and Hartman 1966, Ross and Bornstein 1969). Bodley and Wood
(1972) attributed the classical recoil properties and selective stain-
ing properties of elastic tissue to the central amorphous material.

Ramachandran (1963) suggested that elastic protofibrils had
a triple helical structure similar to collagen, but existed in a shrunken
state even at room temperature. Bodley (1969) noted that the elastic
microfibrils had a periodicity of density every 160°A. He postulated
that the periodic density was due to the microfibril being composed of
a single strand (protein?) which was less than 20°A wide and arranged
in a helix with a slope of 26°.

Elastic fibres vary in morphology and staining behaviour
depending on the site and animal examined (Hall and Gardner 1955).

The fibres are almost insoluble in organic and inorganic solvents, and have a low iso-electric point at pH 6.0 compared with collagen at pH 10.0. The term, elastic fibre, refers to the considerable extensibility possible before fracture. The refractive index is low (1.47 to 1.54), the X-ray diffraction pattern is similar to that of thermohydrally shrunken collagen, and elastic fibres display yellow fluorescence under ultra violet light (Pearse 1968). Despite the stable, cross-linked composition of elastic fibres, Partridge et al. (1955) could solubilize them by partial hydrolysis with 0.25M oxalic acid at 100°C. This treatment produced two fractions, α (molecular weight 60-84,000) and β (molecular weight 5,500), when increasing concentrations of ammonium sulphate were added.

Elastic fibres are digested by elastase but the action of the enzyme is unsure and may only attack the chromotrophic mucopolysaccharide substance. However, Partridge et al. (1955) believed the elastase enzyme was proteolytic. Hall and Gardner thought elastase had a dual composition. Loeven (1963) agreed that elastase was proteolytic but was unsure whether it should be called elastomucase or elastolipoproteinase. Bodley and Wood (1972) examined the effects of enzyme attack on elastic tissue at electron microscopic magnification and found that the amorphous material was more affected than the microfibrillar moiety. They concluded, however, that embedding the tissues in Epon may have influenced the observed results. Unlike collagen, elastic tissue is resistant to collagenase. Thomas and Partridge (1960) wrote that the elastolytic failure of proteolytic enzymes like trypsin and chymotrypsin was due to

(1) failure to penetrate the crosslinked structure of elastic fibres, or

(2) very restricted peptide bond specificity of the enzymes.

Biochemically, elastic tissue, when compared to collagen, contains less arginine, histidine, lysine, very little hydroxyproline (Partridge et al. 1955) and no tryptophan. The content of leucine, valine, alanine, and tyrosine residues is higher. The content of glycine and proline is similar to that of collagen. Thomas, Elsden and Partridge (1963) located the amino acids desmosine and isodesmosine to which they attributed the cross-linkage and high stability of the elastic fibre. Ross and Bornstein (1969) showed that the microfibrils are protein which is rich in polar, hydroxy-, and sulphur-, containing amino acids and which possess less glycine, valine, proline and alanine than the amorphous material. The amorphous portion is rich in glycine, valine, proline and alanine.

Immature elastic tissue (pre-elastic fibres) cannot be histologically demonstrated in humans until the fourth month of gestation and then only when the tissues are pre-oxidized (as for the demonstration of oxytalan fibres - Fullmer 1965). Quite likely, the precursors of pre-elastic fibres, which eventually mature into elastic fibres, are secreted from the fibroblast together with collagen pre-cursors. There is some debate whether endothelial cells can form elastic fibres, and the other connective tissue elements, in developing arteries. Fyfe et al. (1968) have shown islets of a homogeneous material located close to developing smooth muscle cells or endothelial cells. Fine, electron-dense fibrils, which they called pro-elastin, aggregated in the homogenous material and polymerized after excretion from the formative cells. Fyfe and his co-workers also demonstrated dark areas on the secreting cells which possibly secreted pro-elastin and may also have represented the site of manufacture of the smooth muscle myofilaments. This finding could perhaps explain the close association between smooth muscle cells and elastic membranes in arteries. Varadi (1970) showed that the microfibrils are the first

component of elastic fibres to develop in the human foetus. As
development progresses, the proportion of amorphous material to micro-
fibrils alters so that the microfibrils eventually comprise the lesser
quantity. Also, the amino acid desmosine is at its highest level during
embryonic life.

As the age of the individual animal increases, the stability
and yellow pigmentation of the elastic fibres also increase, as
indicated by the slow turnover rate of $\alpha - C^{14}$ (Pearse 1968). As age
increases, the elastic fibres split longitudinally, then fragment and
finally form granules. These physical changes occur concommitantly with
alterations in the amino acid content. For example, glutamic and
aspartic acids increase. The calcium content may also increase up to
14%. Elastic fibres are highly extensible and one of their functions
seems to be involved with maintaining tissue tonus. The loss of elastic
properties with advancing age is associated with a loss in elasticity,
as exemplified by the wrinkling of skin in old age. The term elacin
has been associated with degenerating elastic tissue.

Gawlik (1965), quoted by Pearse (1968, p.230.), described
elastic-like fibres which he named "elaunin", from the Greek meaning
"I stretch". These fibres stained with orcein, aldehyde fuchsin,
resorcin fuchsin and cresyl violet but not Verhoeff's iron haematoxylin
or orcinol-new fuchsin. They differed from oxytalan in that pre-
oxidation of the tissues was not required to demonstrate them and this
property was attributed to a variation in the mucopolysaccharide matrices.
The nature of elaunin fibres is open to conjecture (Pearse 1968).

2.6. A Comparison Between Collagen and Elastic Fibres:

2.6.(a) Similarities.

(1) Collagen and elastic fibres occur together in areas
subjected to mechanical stress - for example, ligaments

and tendons.

(2) Collagen will stain with elastic tissue stains under certain conditions. For instance, acetylation and benzoylation of collagen induces its reactivity to orcein, Weigert's resorcin fuchsin, orcinol-new fuchsin and Verhoeff's iron haematoxylin. Deamination induces aldehyde fuchsin staining (Fullmer and Lillie 1957).

(3) Banga et al. (1956) were able to produce a metacollagen which behaved quite similarly to elastic fibres with regard to elasticity, refractivity, liability to elastase digestion, electron microscopic appearance and resistance to acid attack.

(4) The glycine and proline contents of elastic tissue and collagen are similar (Rudall, 1968, p.132).

(5) Collagen and elastic fibres share similar basic subunits with different sheath mucoids, amino acid arrangement and structural organization (Banga et al. 1956).

(6) Elastic tissue has an X-ray diffraction pattern similar to that of thermohydrally shrunken collagen.

(7) According to Ramachandran (1963), elastic tissue protofibrils have a triple helical structure like the tropocollagen molecule.

(8) Collagen and elastic fibres are believed to originate from the fibroblast.

(9) Immature collagen can be non-banded. Degenerating collagen has also been thought to be non-banded (Selvig 1968).

(10) Treatment of collagen with alkaline borate buffers or sodium metaperiodate can produce an electron microscopic picture like that for elastic fibres (Hall et al. 1955).

2.6.(b) Differences.

(1) Elastic tissue is found in arteries and areas where the resilience of the tissues depends upon the extensibility and elastic recovery of elastic fibres. In the dermis, for example, degeneration of elastic fibres with age results in loss of skin tone and wrinkling.

(2) Elastic fibres are more extensible than collagen and reticulin which fracture when stretched more than ten per cent of their resting length.

(3) Elastic fibres are refractile and not birefringent like collagen.

(4) Elastic fibres are branching and thinner than collagen (1μ compared with 12μ).

(5) Under normal histological conditions, elastic fibres and collagen react selectively with different dyes. For example, elastic tissue reacts with phenolic dyes but collagen stains poorly.

(6) Elastic fibres are composed of 90% non-polar amino acid groupings whereas collagen has only 50%. Therefore, collagen has more ionized, reactive sidegroups.

(7) Elastic tissue has less arginine, histidine, lysine, hydroxyproline and tryptophan, but more leucine, valine and tyrosine than collagen.

(8) Elastic fibres are dissolved by elastase but not by collagenase. This is the reverse reaction of collagen to these enzymes.

(9) Elastic microfibrils are usually reported to be non-striated when viewed under the electron microscope.

(10) Elastic fibres are rarely found in human periodontal ligaments, whereas collagen is the major fibrous component.

(11) Collagen displays the characteristic of thermohydral

contraction on heating whereas elastic fibres do not.

(12) Elastic fibres do not swell in acid or dissolve in

0.01M acetic acid.

2.7. Oxytalan:

The study of oxytalan is restricted because the fibres exist in such small quantities that accurate biochemical analysis and physicochemical testing have not been very rewarding. Therefore, present information regarding oxytalan is based upon histological observations with the light and electron microscopes. Histochemical procedures have been employed but knowledge of oxytalan remains more qualitative than quantitative. The function of oxytalan is an enigma (Ten Cate, in Melcher and Bowen 1969, p.78).

Oxytalan fibres are generally found in areas of stress where elastic fibres can not be normally demonstrated. Although they bear certain similarities to elastic fibres, sufficient differences exist to justify the classification of oxytalan as a distinct connective tissue component.

2.8. A Comparison Between Elastic and Oxytalan Fibres:

2.8.(a) Similarities (from Fullmer 1965).

(1) Some animals, e.g. cattle, have elastic fibres in their

periodontal ligaments with a distribution similar to

the oxytalan fibres in man.

(2) Cattle, dogs, swine and deer have oxytalan fibres at the

apical and middle third regions but elastic fibres at

the cervical third of the periodontal ligament. Elastic

fibres have, at times, been seen closely associated with

oxytalan in the transseptal areas.

(3)   Some parts of the same fibre will stain for both oxytalan and elastic fibres.

(4)   When oxidized, oxytalan and elastic fibres are digested by elastase. The rate of elastic tissue digestion increases after oxidation.

(5)   Following oxidation, three of the five standard elastic tissue stains will demonstrate oxytalan.

(6)   On rare occasions, elastic fibres can be found in human periodontal ligaments with a distribution analogous to that of oxytalan.

(7)   The present staining techniques can not distinguish between oxytalan and pre-elastic fibres.

(8)   Some oxytalan fibres seem to develop into genuine elastic fibres in the pathological condition of scleroderma (which involves an overmaturation of connective tissues). This finding lends support to the concept that oxytalan represents an immature form of elastic fibre.

(9)   Many fine extensions of elastic fibres from the dermis into the epithelial basement membrane of normal human skin can be stained with the peracetic-aldehyde fuchsin technique, but not the normal staining techniques for elastica.

(10) The stainable portion of both elastic tissue and oxytalan seems to reside predominantly in the associated mucopolysaccharide matrix.

(11) The electron microscopic interpretations of both elastic and oxytalan fibres are very similar. Both seem to be composed of non-banded filaments associated with an amorphous matrix.

(12) Oxytalan and elastic fibres seem to be biochemically stable as they are resistant to destruction, e.g. in periodontal

disease and ascorbic acid deficiencies, oxytalan is retained for longer periods than is collagen.

(13) Rannie (1963) noted that oxytalan and elastic tissue stain with some basic dyes of the Quinone-imine group including celestin blue, methylene blue and toluidine blue.

Observation of such similarities in the periodontal ligaments of man and animals led Fullmer (1965) to suggest that oxytalan represented - *the elastic-like fibres produced by connective tissue cells at this anatomic site in lieu of elastic fibres.*

Fullmer (1960a) stated that -

The data from investigations of oxytalan fibres are consistent with the concept that they represent either abortive or modified efforts toward the production of an elastic-like product by connective tissue cells in certain structures. They are present in connective tissue structures that require little or no elasticity. In these sites, such as periodontal membranes and tendons, it is plausable to assume that the development of characteristic elastic tissue is either not required, or may actually be detrimental to function, and therefore an altered or incomplete elastic structure is formed.

2.8.(b) Differences.

(1) Oxytalan is found in areas where genuine elastic fibres are not normally observed, e.g. in healthy periodontal ligaments of man.

(2) The tissues must be pre-oxidized before oxytalan can be demonstrated. However, this procedure also enhances the intensity of elastic tissue staining.

(3) With age, elastic fibres degenerate in a manner which is observable whereas oxytalan seems to alter very little between the ages of 25 to 80 years (Fullmer 1962).

(4) Even after oxidation, oxytalan can only be demonstrated with a smaller number of histological dyes than those which react positively with elastic tissue.

(5) Although pre-elastic and oxytalan fibres are initially
indistinguishable, and may even share a common origin,
elastic fibres undergo biochemical changes (maturation).
This is illustrated by the greater diversity of histo-
chemical reactions which occur with the larger number of
elastic positive stains.

(6) Oxytalan is susceptible to alkali treatment whereas elastic
fibres are more resistant.

(7) Elastase will not digest oxytalan until the tissues have
been pre-oxidized.

(8) Hyaluronidase, β-glucuronidase and lysozyme reactions
prevent oxytalan, but not elastic fibres, staining after
pre-oxidation.

(9) There may be more mucopolysaccharide associated with oxytalan
than with elastic fibres.

The relationship between oxytalan and elastic fibres is
similar to that which exists between collagen and reticulin. Melcher
and Eastoe (in Melcher and Bowen, p.212, 1969) stated that -

> *Persistance of immature elastic fibres in mature*
> *connective tissues has a possible analogue to*
> *reticulin which, ...,*
> *may be an immature precursor of collagen fibres*
> *persisting in mature connective tissues.*

CHAPTER 3.   <u>CRITERIA FOR CALLING A FIBRE OXYTALAN</u>.

This section is based upon the investigations of Fullmer and Lillie (1958) and Fullmer (1960a), the results of which are summarized in Table 1, p. 53. and Table 2, p. 54.

Fullmer realised the difficulty in distinguishing between pre-elastic tissue and oxytalan. Nevertheless, he established the following criteria for their identification.

3.1. <u>Pre-elastic Fibres</u>:

This term should be applied during embryogenesis or during postnatal life to those developing elastic tissue fibres which fail to stain with selective elastic tissue stains, but may stain with aldehyde fuchsin or orcein after oxidation.

> *The term pre-elastic should be restricted to either those fibres which can be expected to mature eventually into elastic fibres or are extensions of recognizable elastic fibres. On this basis, the anatomic site of the fibre will be the most useful single criterion to the distinction between oxytalan and pre-elastic fibres.*
>
> Fullmer (1960a).

3.2. <u>Oxytalan Fibres</u>:

If the elastic-like tissue in an adult structure is –

(a)   only demonstrated after oxidation,

(b)   stainable with only three of the five standard elastic tissue stains (aldehyde fuchsin, orcein and Weigert's resorcin fuchsin), and then only after oxidation,

(c)   resistant to elastase digestion,

(d)   not stained by peracetic-aldehyde fuchsin or paracetic-orcein techniques when treated with testicular hyaluronidase, β-glucuronidase or lysozyme at the stage between oxidative and staining steps,

then the fibres are concluded to be oxytalan (Fullmer 1960a). Thus

oxytalan is distinguished by its distribution and staining peculiarities.

TABLE 1:

Staining reactions of oxytalan and elastic fibres from Fullmer and
Lillie 1958.

REACTIONS	OXYTALAN FIBRES	ELASTIC FIBRES
Direct		
The 5 elastin stains	-	-
azure A pH4	-	-
Hale	-	-
Schiff	-	pink*
Peracetic Oxidized		
orcein	brown**	brown
aldehyde fuchsin	purple	purple
resorcin fuchsin	purple**	purple
Verhoeff's stain	-	black
orcinol-new fuchsin	-	purple
azure A pH4	-	-
Hale	-	-
Schiff	-	pink*

* The elastic fibres in rodents but not in man give a direct
  Schiff reaction.

** Some fibres are reactive; others are not.

TABLE 2:

Effect of enzymatic hydrolysis on staining reactions of formalin fixed oxytalan and elastic fibres with aldehyde fuchsin from Fullmer and Lillie 1958.

ENZYMATIC REACTIONS	OXYTALAN[1] FIBRES	ELASTIC[2] FIBRES
undigested	purple	purple
elastase	purple	dissolved
peracetic–elastase	dissolved	dissolved
testicular hyaluronidase	purple	purple
peracetic–hyaluronidase	unstained[3]	purple
lysozyme[4]	purple	purple
peracetic–lysozyme	unstained	purple

1   Stained by the peracetic-aldehyde fuchsin sequence.

2   Stained by aldehyde fuchsin.

3   Here the fibres slowly lose their stainability in serially exposed preparations, meanwhile unstained portions of fibres adjacent to stained portions are still visible.

4   Fresh frozen sections of human gingiva and tendon were used with lysozyme. Formalin and alcohol fixed oxytalan and elastic fibres were resistant to lysozyme.

CHAPTER 4.   MATERIALS AND METHODS.

4.1. Materials:

The animals used for this study were -

(1) Three Tammar wallabies (Macropus eugenii) obtained from the
     Zoology Department, University of Adelaide,

(2) One hairy nosed wombat (Lasiorhinus latifrons) from the
     Department of Oral Pathology and Oral Surgery, University of
     Adelaide,

(3) One brush tailed possum (Trichosurus vulpecula) from the
     Central Animal House, Waite Agricultural Research Institute,

(4) Eight marsupial mice comprising two different genera,

     (a) five Dunnarts (Sminthopsis crassicaudata), and

     (b) three Mardos (Antechinus flavipes).

     These specimens were provided by the Institute of Medical and
     Veterinary Science, field station, Gilles Plains.

     More detailed descriptive information about the animals is
contained in Appendix II. The animal heads were preserved in 10%
neutral buffered formalin prior to examination.

4.2. Methods:

In outline, the methodology involved -

(1) A gross anatomical study of the dentition and jaw morphology
     to illustrate features related to function. The temporo-
     mandibular articulation was not studied in detail.

(2) Dye techniques were employed to histologically demonstrate
     the presence and distribution of oxytalan fibres in the
     periodontal tissues of the marsupials selected for study.

(3) Limited investigation was conducted into aspects of the

histochemistry of oxytalan staining.

(4)   A technique for three-dimensional reconstruction of
histologic tissue sections has been developed.

4.2.(a)   Anatomy.

Photographic records of the marsupial mandibles were made
before they were treated for histological examination.   Diagrams were
prepared from X-ray and photographic records of the jaws.

4.2.(b)   Histologic preparation.

4.2.(b)(i)   fixation

As soon as the animals were killed their heads were placed
in 10% neutral buffered formalin solution (Appendix III).   The mandibles
were carefully dissected to allow collation of gross anatomic data and
then returned to fresh solutions of 10% neutral buffered formalin
solution until processed further.

4.2.(b)(ii)   decalcification

All mandibles and teeth studied were decalcified in 40%
formic/formate solution (Appendix III) except one wallaby mandible
which was demineralized with R.D.O. (Du Page Kinetic Laboratories Inc.)
and used for a pilot study into three-dimensional reconstruction of
histologic sections.   To facilitate penetration of the decalcification
solutions into bone, the mandibles were divided through the symphysis
and distal to the last molar tooth.   Periodic X-ray examination revealed
the progress of demineralization (Fig.1.).   The small, delicate
marsupial mice completed decalcification in $2\frac{1}{2}$ to 3 days.   The possum
and wallaby mandibles, because of their larger size and increased bone-
thickness, were further sectioned into incisor and molar segments
(Diag. 1.)   To enhance the decalcifying process, the solutions were
agitated by passing air through the specimen bottles which were
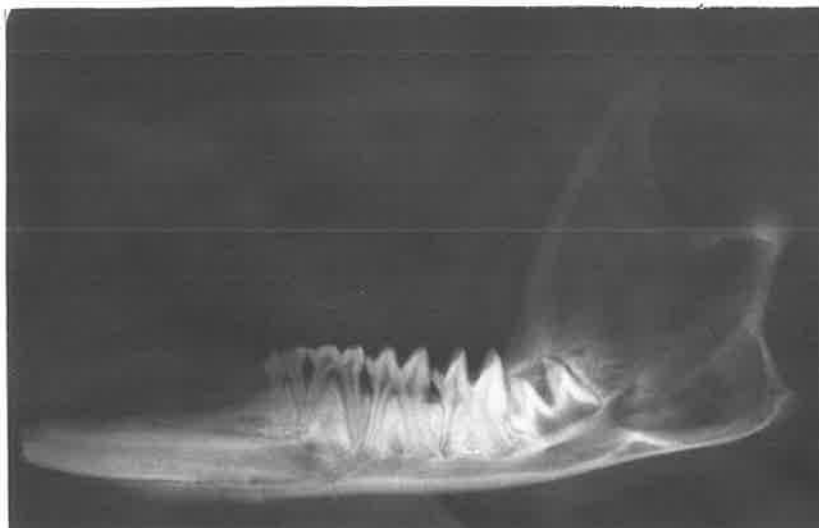
Fig. 1. Radiographs of wallaby mandibles –
(a) before decalcification, (b) after 7 days, and
(c) after 17 days. When radiopacity is no longer evident
the tissues are decalcified.
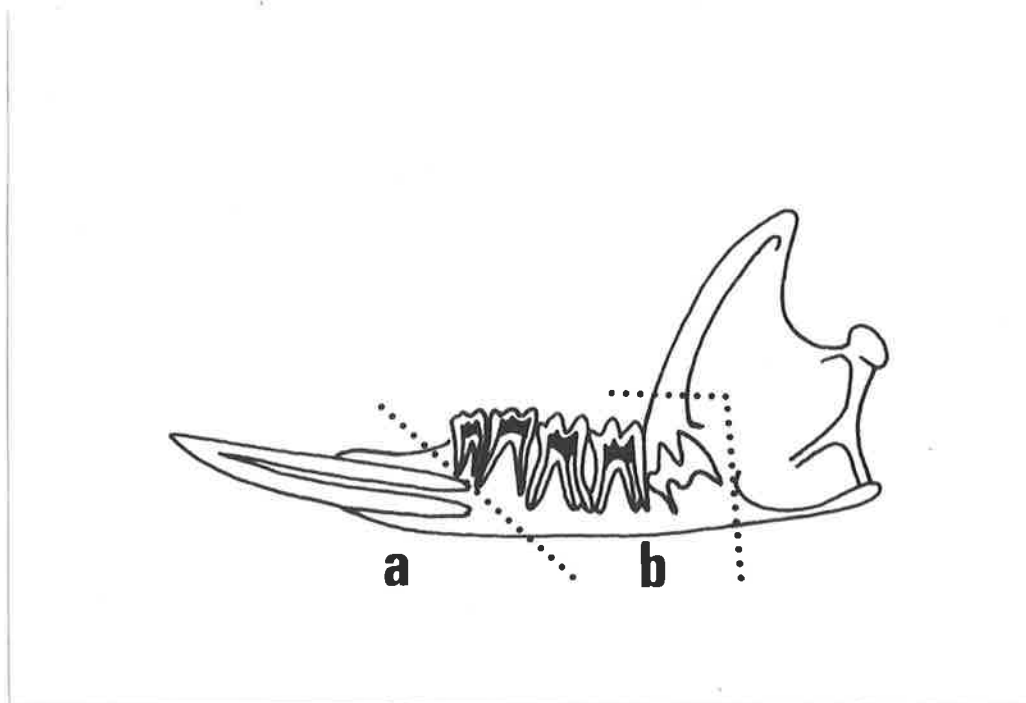
Diag. 1. Division sites of the wallaby mandible into incisor (a) and molar (b) segments prior to the double embedding process.
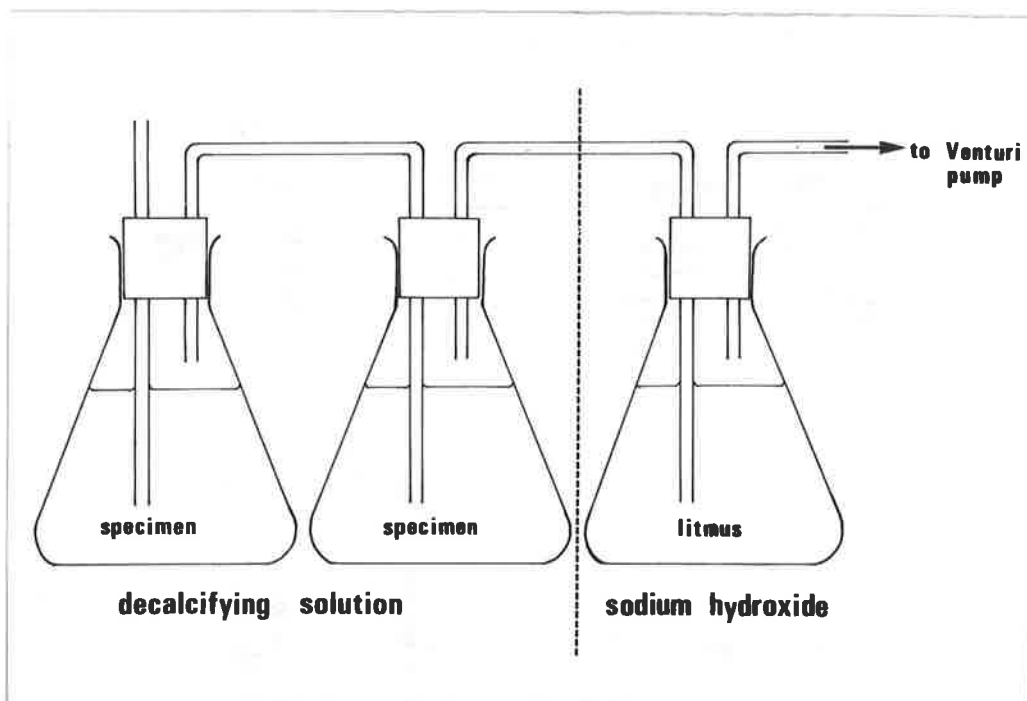
Diag. 2. Apparatus used to speed the decalcification process. The decalcifying solution is 40% formic/formate.

connected to a Venturi pump (Diag. 2.). A sodium hydroxide bottle was used to neutralize any formic acid fumes and the pH was monitored to assure non-acidity. The wallaby material showed radiographic decalcification at eighteen days while the possum required twenty-four days. Wombat demineralization needed seventy-seven days because of the large teeth and immense, solid, bony buttresses supporting the dentition. Initial division of the jaw was performed with a vice and hacksaw to produce molar and incisor segments of approximately one cubic inch. A period of decalcification was continued until further sectioning with a scalpel could be achieved. In this manner manageable portions of the mandibular incisor and molars were obtained for horizontal, sagittal and coronal sectioning.

The wallaby mandible in R.D.O. solution was decalcified in eleven hours.

4.2.(b)(iii) neutralization

When decalcification was complete, the specimens were placed in 5% sodium sulphate (Na_2SO_4) for from 8-36 hours depending upon the length of time the tissues were kept in the formic/formate solution. The wallaby mandible was merely washed in distilled water since R.D.O. does not require neutralization.

4.2.(b)(iv) processing

The specimens were prepared for paraffin wax embedding by the normal Double Embedding Technique (Appendix III). Because of their size, the portions of possum and wallaby mandibles required 4-6 days in celloidin and the wombat material 7 days.

4.2.(b)(v) paraffin embedding

The specimens were infiltrated with paraffin wax (Appendix III) and then vacuumed in clean paraffin wax at 56°C for a minimum of

fifteen minutes prior to blocking. Each specimen was marked on the lingual aspect to facilitate orientation of the blocks for serial sectioning. With physical size being the determinant, the tissue pieces were then blocked in either plastic or metal moulds using the "Tissue-Tek II Tissue Embedding Center" machine.

4.2.(b)(vi) sectioning

The blocks were orientated so that the tissues could be cut in -

(1) coronal,

(2) sagittal, and

(3) horizontal planes.

The marsupial mouse material was serially sectioned at 10μ on an ERMA rotary microtome. However, the large size of the possum, wombat and wallaby material necessitated the cutting of individual 10μ sections on an M.S.E. sledge microtome. Each section was then flattened on to clean glass microscope slides using the hot plate and warm air oven technique.

A total of 4,857 sections were mounted on to glass slides - wallaby (1,459), possum (220), wombat (503), S. crassicaudata (1,513) and A. flavipes (1,162). Additional sections were prepared for the three-dimensional study described below (Chapter 4.3.).

4.2.(c) Staining.

4.2.(c)(i)

For the specific purpose of visualizing the micro-anatomic features of the marsupial dentitions, representative sections were selected for each tooth segment (molar and incisor) and each plane of tissue sectioning for all animals. These sections were then stained with the haematoxylin and eosin technique (Appendix IV).

4.2.(c)(ii)

For the demonstration of oxytalan fibres, sections were pre-oxidized with potassium monopersulphate (Oxone) and then stained with aldehyde fuchsin - Halmi, orcein-light green, or Weigert's resorcin fuchsin-Halmi techniques. For every oxidized section an unoxidized, adjacent, control section was stained under identical conditions. The staining techniques are contained in Appendix IV. Two of every ten slides (one oxidized, the other unoxidized) were stained with aldehyde fuchsin-Halmi. Two in twenty slides were treated with orcein-light green or resorcin fuchsin-Halmi. This method provided a comprehensive scan of the material with further slides needing to be stained only when an area of particular interest demanded special investigation. Therefore, adequate sections were available for further experimentation. The results of the staining procedures were examined by light microscopy and the oxytalan fibre distribution recorded by a coded system (Appendix V, Table 15, p.229.) which simplified analysis of each slide and tabulation of the results.

4.2.(c)(iii)

To determine the distribution of other tissue elements associated with oxytalan fibres, the following staining procedures (Appendix IV) were used -

Elastic fibres: Verhoeff's iron haematoxylin, orcinol - new fuchsin, orcein, resorcin fuchsin, aldehyde fuchsin, and the iron-orcein techniques of Roman et al. (1967) and Lillie et al. (1968).

Collagen: Van Gieson's stain, Pollack's Trichrome, Halmi, Mallory's Trichrome, and eosin (plus haematoxylin).

Reticulin:	silver impregnation (Naoumenko and Feigin 1974).
Nerve fibres:	the Hirano-Zimmerman, Bielschowsky and Bodian techniques.
Mucopoly-saccharides:	periodic acid-Schiff and the Hale colloidal iron methods.
Keratin and pre-keratin:	Ayoub-Shklar method.

4.2.(d) Enzyme digestions.

For each enzyme experiment, a group of four serial wallaby sections was used according to the following procedure.

Slide 1: (OXONE) - (ENZYME + BUFFER) - (ALDEHYDE FUCHSIN)

Slide 2: (OXONE) - (BUFFER) - (ALDEHYDE FUCHSIN)

Slide 3: (ENZYME + BUFFER) - (OXONE) - (ALDEHYDE FUCHSIN)

Slide 4: (BUFFER) - (OXONE) - (ALDEHYDE FUCHSIN)

Every effort was made to maintain constancy of the conditions for oxidation, enzyme digestion and staining steps so that the only variables were the order in which the steps were performed and the absence of enzyme activity in slides 2 and 4.

The enzymes used were: β-glucuronidase, elastase, collagenase, pepsin, hyaluronidase, bromelain, and neuraminidase.

Preparations of the enzyme solutions are contained in Appendix VI.

To minimise observer bias in the interpretation of the results, three independent persons were asked to assess any changes in the stainability, numbers and appearance of oxytalan, elastic and collagen fibres. The observers did not know the type of enzyme used or the method of treatment because slides 1, 2, 3, and 4 of each group were randomly rearranged and coded.

4.2.(e)   Investigation of dye properties.

4.2.(e)(i)   dye solutions

To further investigate staining characteristics of oxytalan fibres, 55 dye substances were applied to both oxidized and non-oxidized control sections. Many of the dyes were available from laboratory stocks. Additional dye substances were chosen to provide a broad coverage of the various dye classes. The wallaby incisor was selected as the initial experimental material to screen all the different dye solutions to eliminate variation in staining behaviour which might be caused by biochemical tissue differences between the different marsupials. The dyes tested are listed in Table 10, p.147.   Staining solutions of the dyes were prepared as follows –

A: Solutions and techniques used by other authors for any particular dye were followed whenever possible. The different dyes were used irrespective of whether they were originally intended to identify various tissue components other than oxytalan and elastic fibres. For example, collagen, elastic fibres, carbohydrates, lipids or mucopolysaccharides, etc.

B: The dyes were also tested as 1%, or saturated solutions, in deionized distilled water.

C: 0.05%, 0.5%, 1%, or saturated solutions of the dyes in 0.1N hydrochloric acid were also employed.

D: Alcohol soluble dyes were prepared in 70% or 95% ethyl alcohol to concentrations ranging from 0.1% to 3%, with the concentrations being determined at random, but guided by the solubility.

E: 0.1% dye solutions in a 50/50 mixture of 2-methoxymethanol and Universal buffer (Tris (hydroxymethyl) aminomethane)

pH 9.0 were prepared and tested.

Although the sections were stained for varying times, they were checked at 5, 10, 20, 30, 40 minutes, 1 hour, 2 hours, 6 hours, 24 hours and, in the case of one particular solution of the dye celestin blue B, up to 4 days. Sections were also examined at different stages of manipulation for the presence of oxytalan and elastic fibres or for any changes in the staining reaction at the stages of (1) dye application, (2) water washing, and (3) alcohol dehydration. Acetone and methanol dehydration were also assessed. Therefore, sections were either mounted in dye solution, water, glycerin, or synthetic resin.

The staining solutions and techniques which revealed oxytalan-like fibres in the wallaby periodontal membrane were repeated, under identical conditions, for the wombat, possum and marsupial mice. The staining solutions and procedures found to be useful are contained in Appendix VII.

The dyes and staining solutions were further analysed by pH measurements, spectrophotometric absorption patterns, light field, dark field, phase, and ultraviolet microscopic evaluation of the staining reactions.

An additional 16 dyes ordered from Germany (Chroma Co.) were not received for testing.

4.2.(e)(ii) pH analysis

pH measurements were made at $20^{o}C$ with an E.I.L. Direct Reading pH meter (model 23A) which was periodically tested for accuracy with B.D.H. Buffer tablet solutions at pH 4.0, 7.0 and 9.0.

Recordings were made of the pH of the working stain solutions and also of a 0.5% aqueous solution of each dye substance.

4.2.(e)(iii) spectrophotometry

Tracings of the spectral absorption patterns of all dye

solutions were recorded by a Unicam SP 800 ultra violet spectrophotometer (deuterium lamp). The machine automatically compared samples of the dye solution with samples of the dye solvents and compensated so that the tracings produced were a feature of the dye substance itself. Continuous tracings of the spectral absorptance patterns of the dyes were obtained over the range of 200 mμ to 700 mμ. The results were qualitative when comparing different dyes, but were quantitative when comparing the absorptive peaks for a single dye over the complete ultra violet and visible spectral range.

4.2.(f) Microscopy.

4.2.(f)(i) light microscope

Care was always taken to compare oxidized sections with the corresponding non-oxidized control sections when examining slides for the presence and distribution of oxytalan fibres. Serial sections were used to ensure that the evaluation of oxidized and control sections would enable a more accurate interpretation of the findings. Each stained slide was examined with an Olympus microscope, model EH. The findings were recorded using the symbols shown in Table 15, Appendix V. Sections which displayed features of interest were photographed and line diagrams were drawn as composites of the slides examined.

4.2.(f)(ii) phase contrast

An Ortholux microscope (Leitz) was converted for Heine phase contrast. Stained and unstained sections were examined for information regarding oxytalan fibres in the marsupial periodontal ligaments.

4.2.(f)(iii) dark field

A dry dark field condenser was attached to the Ortholux microscope which then permitted dark field examination of those sections studied by phase contrast.

4.2.(f)(iv) ultra violet microscopy

Stained and unstained deparaffinized sections were examined
for ultra violet fluorescence with a Zeiss Universal ultra violet
microscope. The microscope system had a super pressure HBO 200 W
mercury light source, BG 12 exciter filter and barrier filters ranging
from 410 mμ to 650 mμ.

All dyes used in this study were examined. Particular
attention was given to those sections treated with the fluorescent
dyes fluorescein, rhodamine B, lissamine rhodamine RB 200 - phloxine
rhodamine and procion brilliant yellow M 4 R solutions.

A dark field condenser was used to enhance any fluorescent
effects. Both oxidized and non-oxidized sections were examined for
oxytalan fibre fluorescence.

4.2.(g) Methylation and saponification tests for carboxyl groups.

Slides were selected and prepared for the methylation
blockading technique described by Culling (1974) - Appendix IX. The
test slides were manipulated so that the results of the methylation
could be assessed before, and after oxidation in 10% Oxone for 90
minutes. The sections were divided into equally treated pairs and one
was stained with aldehyde fuchsin and the other with alcian blue
(pH 2.5). Control, oxidized but not methylated, sections were also
stained with aldehyde fuchsin and alcian blue (pH 2.5).

The effects of saponification (Appendix IX) following either
oxidation only, methylation only, or methylation plus oxidation were
assessed by aldehyde fuchsin and alcian blue (pH 2.5) staining of
equally treated slide pairs. The effects of methylation followed by
saponification and then by oxidation were also noted.

Methylation times were 4 hours and 24 hours at 60°C.
Saponification was for 30 minutes.

4.2.(h)   Photography.

4.2.(h)(i)

Macroscopic photographs illustrating anatomic features of the marsupial mandibles were taken with a Minolta SLT 101 reflex camera fitted with a bellows extension, a P. and K. (UR 65Z) ring flash unit and Braun F 700 power pack.

4.2.(h)(ii)

Photomicrographs were taken in both black and white, and colour. Colour slides were produced with an Ortholux microscope fitted with an Orthomat 35 mm. attachment and Leitz automatic exposure system. The film used was Kodachrome professional II $(3,400^{O}K)$ and Ektachrome ER tungsten $(3,200^{O}K)$. The advantage of using Ektachrome was that the exposed film could be immediately developed with a Kodak E.4. colour processing kit and assessed without delay.

Black and white photographs were taken with an Aristophot attachment to the Ortholux microscope. This permitted the use of 5" x 4" Ilford FP 4 cut sheet film.

Black and white negatives were produced from colour slides with a Honeywell Repronar 400A copier.

4.2.(h)(iii)

Line drawings were copied from acetate sheets on to Microfilm with the Leitz Reprovit II camera system.

Films were processed and prints produced in accordance with the manufacturers' instructions.

4.3.   Three-Dimensional Reconstruction:

4.3.(a)   Technique.

This experiment was devised to provide a method whereby histologic sections might be photographed and accurately superimposed

to build a laminated, three-dimensional composite of a chosen field.

One formalin-fixed wallaby mandible was bisected through the symphysis and the left half firmly clamped in a vice. The jaw was stabilized to prevent vibration and movement while four, spaced, parallel holes were drilled through the incisor with a 0.5 mm. Spirec dental bur held in an engineering drill mount. The vice could move only in the horizontal plane and the drill only in the vertical plane, ensuring parallelism of the drilled holes.

The incisor segment was then resected and decalcified in R.D.O. solution (Chapter 4.2.(b)(ii)). The tissue was paraffin blocked following double embedding procedures. Serial sections were cut at 10μ with an M.S.E. sledge microtome and each section was placed on clean glass slides. Eighty serial sections were obtained and numbered in order of production from the tissue block.

To compensate for rotational and translation changes in the placement of the sections upon the glass slides, a mathematical technique involving a rotation and translation of axes was used (Chapter 4.3.(b)). From the mathematical formulae it is possible to calculate the location of any desired point. This is done by measuring the X and Y co-ordinates of the centre of at least two of the drilled reference holes in slide 1. A microscope stage which measures accurately in the X and Y directions is needed. A point is then chosen in the area of the periodontal ligament which is intended for study and the X and Y co-ordinate values recorded. From the next serial slide (slide 2), the X and Y co-ordinates are recorded for the corresponding two reference holes measured in slide 1. These co-ordinates from slides 1 and 2 are adequate to calculate the co-ordinates of the point, in the periodontal ligament of slide 2, which was selected for study from slide 1 (equations 5 and 6 in

Chapter 4.3.(b)). This point is visualized by setting the microscope stage to the calculated values. Therefore, if the same microscopic magnification has been used for the selected area in slides 1 and 2, the calculated point will define the corresponding area in slide 2 to that seen originally in slide 1.

A single point is not sensitive to a rotational vector and the area seen microscopically after calculation of the co-ordinates will still incorporate the angular difference between slides 1 and 2. This factor has been mathematically compensated by calculating the value in degrees of the angular change (equations 2 and 3 in Chapter 4.3.(b)). Thus the rotational difference between slides 1 and 2 can be eliminated by either (1) rotating the microscope stage, or (2) rotating the camera by the calculated number of degrees.

It is imperative that slide 1 be related to slide 2 and then slide 2 to slide 3, etc. and not slide 1 to slide 2, then slide 1 to slide 3 etc. This is important because the 10μ thickness of each tissue section reduces the accuracy of the superimposition if the plane of sectioning is not absolutely perpendicular to the long axis of the centres of the dilled reference holes. Thus slide 1 is used to calculate the values defining the desired area in slide 2. Once the values of slide 2 are obtained they are used to calculate the same area for slide 3, etc.

To reduce the calculation time required to produce the coordinates and angular changes of the chosen point in the periodontal ligament, the X and Y values of the reference holes in all slides plus the coordinate values of the selected field from slide 1 are measured, tabulated, and fed into a data processing computer. A program has been developed for the computer to calculate the coordinates and angles for all slides and present the results in table form. It is then a simple

procedure to read off the X and Y coordinates for the desired area,
set the values on the microscope stage, rotate the camera, and take
the photograph. It is emphasized that the photographs must be taken in
sequential order (slide 1, slide 2, slide 3, ...) because the angular
change is only related to the immediately preceding slide. For this
procedure, an Ortholux microscope fitted with a rotatable Aristophot
camera system was used. The 5" x 4" negatives were traced on to 5" x 4"
clear perspex sheets. The perspex tracings were easily superimposed and
stacked to represent a reconstruction of the sectioned tissue in three-
dimensions. Two-dimensional composite tracings of the course of oxytalan
fibres could also be produced, if required.

4.3.(b) <u>Calculations</u>.

    Although four holes were drilled in the test tooth, the
translational and rotational changes between two compared slides can
be computed from as few as two reference holes. The extra holes were
drilled as a means of checking the accuracy of the computations.

    From the calibrated microscope stage, the coordinates of the
reference holes are immediately known. For convenience, the reference
holes in slide 1 will be nominated as A and B with X, Y coordinates
(a_1, a_2) and (b_1, b_2). For slide 2, these references become A´ and B´
with X, Y coordinates $(a_1´, a_2´)$ and $(b_1´, b_2´)$. The desired point in
the periodontal ligament is known in slide 1 but unknown in slide 2.
Let the point be C with coordinates (c_1, c_2) in slide 1 and C´ with
coordinates $(c_1´, c_2´)$ in slide 2 (Diag. 3(a)).

<u>Rotation of axes</u> –

    From diagram 3(b) it is obvious that the position of the section
differs in both rotational and translational vectors. Because the edges
of the glass slides represent the X and Y axes utilised by the micro-
scope stage, then one needs to know how these axes change when the
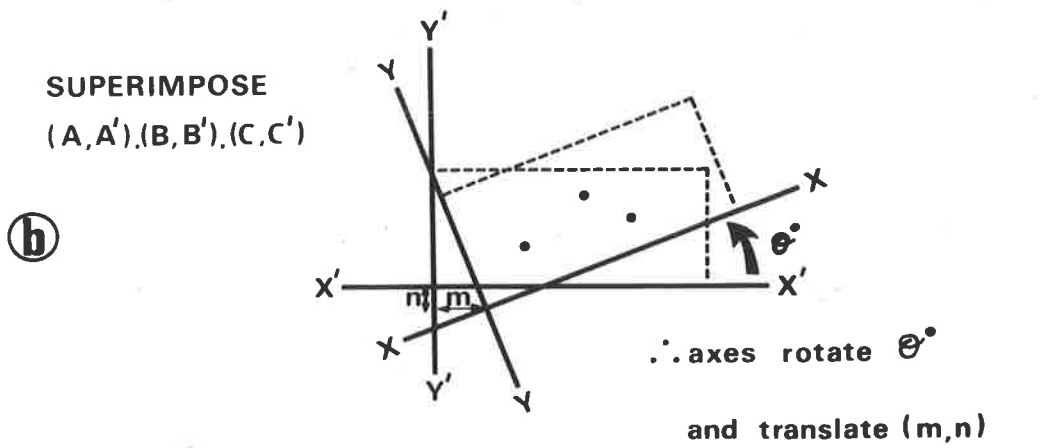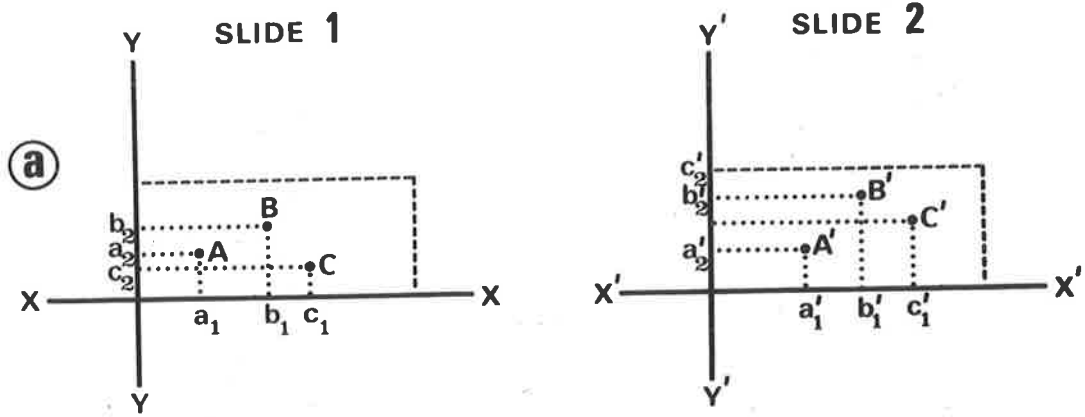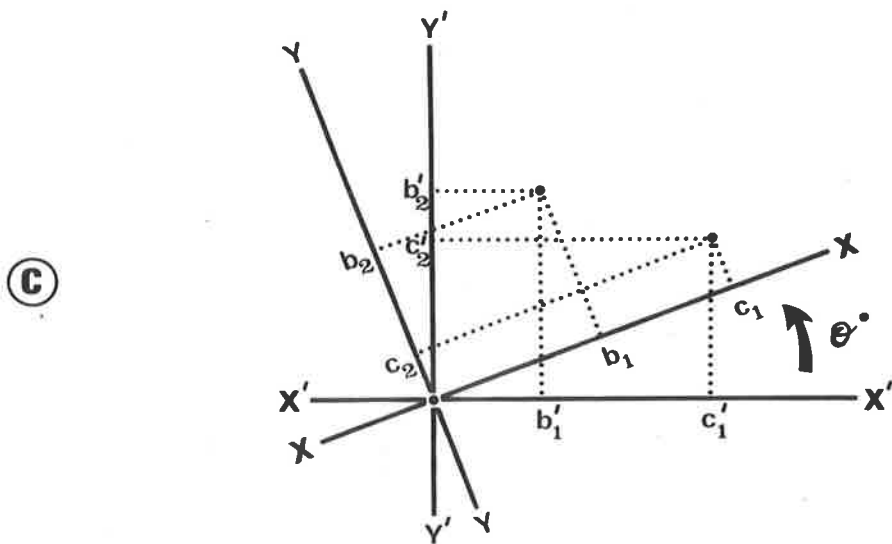
diagram. 3.

sections are superimposed over the reference holes A, A´ and B, B´
(and of course C, C´)(Diag. 3(b)). Axes parallel to the sides of the
glass slides will effectively rotate θ° and translate vertically (n
units) and horizontally (m units).

value of θ° –

Knowing (by measurement) the values of (a_1, a_2), $(a_1´, a_2´)$ and
(b_1, b_2), $(b_1´, b_2´)$ then θ° is determined by expressing $a_1´$ in terms of
a_1 and a_2; $a_2´$ in terms of a_1 and a_2; $b_1´$ in terms of b_1 and b_2; $b_2´$
in terms of b_1 and b_2; and then solving for θ. For simplification of
calculation, the axes are shown meeting at A and equations produced
for B (Diag. 3(c)). As indicated in diagrams 4(a) and 4(b) the following
expressions are obtained:

$$b_1´ = b_1 \cos \theta - b_2 \sin \theta$$

$$b_2´ = b_2 \cos \theta + b_1 \sin \theta$$

Similar equations apply to $c_1´$ and $c_2´$.

In reality, translation of the axes (m, n) occurs because the
centre of rotation is not through A, but at a point distant which has
moved m units of length in the horizontal dimension and n units
vertically when slide 2 is compared to slide 1 (Diag. 3(b)). The
equations then become –

$$a_1´ = (a_1 \cos \theta - a_2 \sin \theta) + m$$

$$a_2´ = (a_2 \cos \theta + a_1 \sin \theta) + n$$

$$b_1´ = (b_1 \cos \theta - b_2 \sin \theta) + m$$

$$b_2´ = (b_2 \cos \theta + b_1 \sin \theta) + n$$

Before $c_1´$ and $c_2´$ can be determined, the values of θ, m, and n are
needed. Let $\cos \theta = \alpha$ and $\sin \theta = \beta$, then substitution produces 4
equations with 4 unknowns.

(a)

b_2

$b_2\sin\theta$

b_1

θ

b_1'

$b_2\sin\theta$

$b_1\cos\theta$

$$\therefore\ b_1' = b_1\cos\theta - b_2\sin\theta$$

(b)

$\sin\theta(b_1 - b_2\tan\theta)$

$b_1 - b_2\tan\theta$

$b_2\tan\theta$

$\dfrac{b_2}{\cos\theta}$

b_2'

b_2

θ

b_1
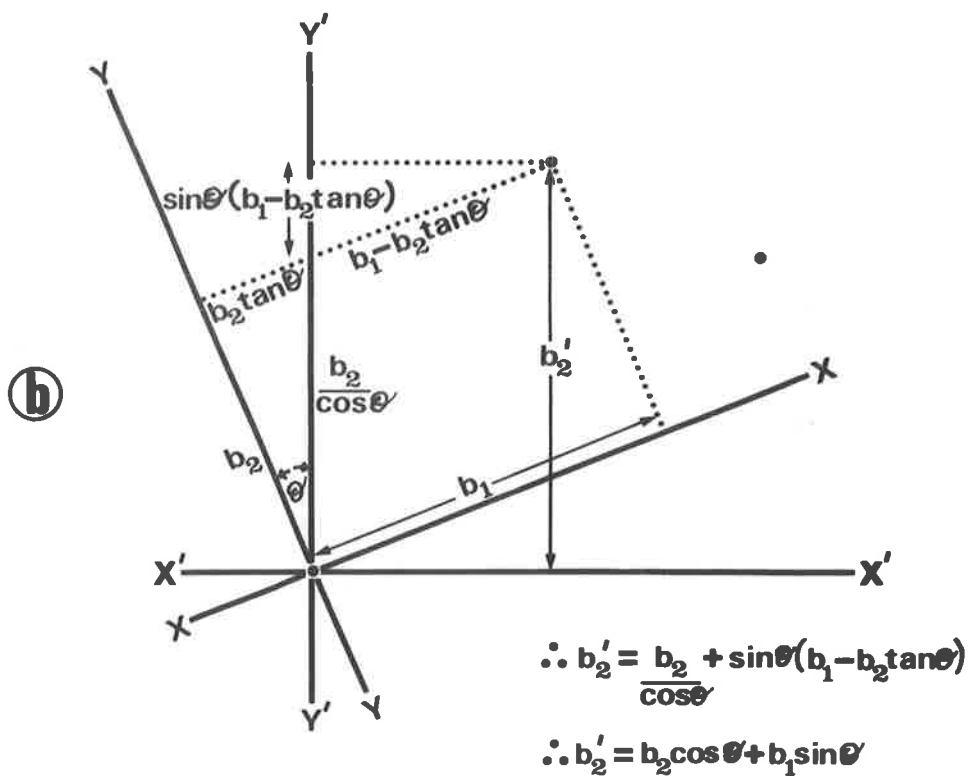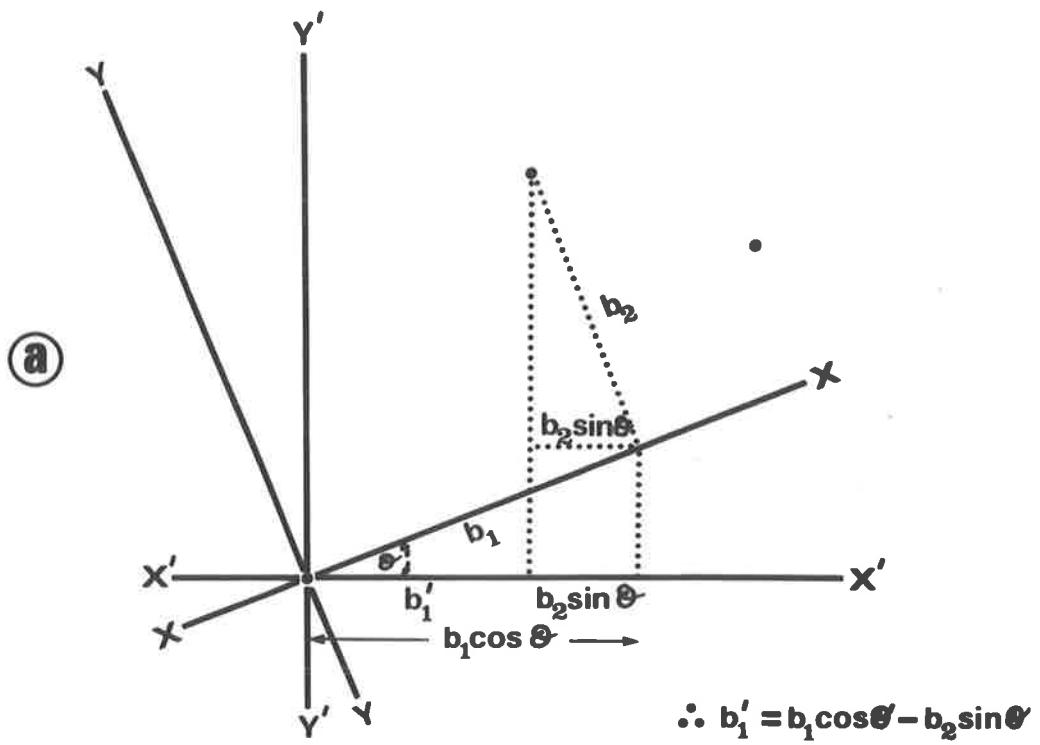
$$\therefore\ b_2' = \frac{b_2}{\cos\theta} + \sin\theta(b_1 - b_2\tan\theta)$$

$$\therefore\ b_2' = b_2\cos\theta + b_1\sin\theta$$

diagram. 4.

equation (1)

$$a_1' = (a_1\alpha - a_2\beta) + m$$

$$a_2' = (a_2\alpha + a_1\beta) + n$$

$$b_1' = (b_1\alpha - b_2\beta) + m$$

$$b_2' = (b_2\alpha + b_1\beta) + n$$

Solving in terms of a_1, a_2, b_1, and b_2 produces the following equations.

equation (2)

$$\alpha = \frac{(a_1-b_1)(a_1'-b_1') + (a_2'-b_2')(a_2-b_2)}{(a_2-b_2)^2 + (a_1-b_1)^2} = \cos\theta$$

equation (3)

$$\beta = \frac{(a_1-b_1)(a_2'-b_2') - (a_1'-b_1')(a_2-b_2)}{(a_2-b_2)^2 + (a_1-b_1)^2} = \sin\theta$$

Since $a_1' = (a_1\alpha-a_2\beta) + m$; and $a_2' = (a_2\alpha+a_1\beta) + n$

equation (4)

$$\text{Then, } m = a_1' - (a_1\alpha-a_2\beta); \text{ and } n = a_2' - (a_2\alpha+a_1\beta)$$

So far, equations (2), (3) and (4) have defined the rotational and translational changes between slide 1 and slide 2. From measured values of (a_1,a_2), (a_1',a_2'), (b_1,b_2) and (b_1',b_2') the values of θ^0, m, and n can be quantified. However, the value of (c_1,c_2) is known but (c_1',c_2') is not.

Calculation of (c_1',c_2') –

From equation (1)

$$c_1' = (c_1\alpha-c_2\beta) + m; \quad c_2' = (c_2\alpha+c_1\beta) + n$$

$$\therefore c_1' = (c_1\alpha-c_2\beta) + (a_1'-a_1\alpha+a_2\beta)$$

$$\therefore c_1' = a_1'+\alpha(c_1-a_1) - \beta(c_2-a_2)$$

$$\therefore\ c_1' = a_1' + (c_1-a_1)\ \frac{(a_1-b_1)(a_1'-b_1') + (a_2-b_2)(a_2'-b_2')}{(a_1-b_1)^2 + (a_2-b_2)^2}$$

$$-(c_2-a_2)\ \frac{(a_1-b_1)(a_2'-b_2') - (a_1'-b_1')(a_2-b_2)}{(a_1-b_1)^2 + (a_2-b_2)^2}$$

$$\therefore\ c_1' = a_1' + (a_1'-b_1')\ \frac{(c_1-a_1)(a_1-b_1) + (c_2-a_2)(a_2-b_2)}{(a_1-b_1)^2 + (a_2-b_2)^2}$$

$$+(a_2'-b_2')\ \frac{(a_2-b_2)(c_1-a_1) - (c_2-a_2)(a_1-b_1)}{(a_1-b_1)^2 + (a_2-b_2)^2}$$

Since a_1, b_1, and c_1; and a_2, b_2, and c_2 are measured from slide 1,
then the expression of those values becomes a constant.

i.e. let
$$F_1 = \frac{(c_1-a_1)(a_1-b_1) + (c_2-a_2)(a_2-b_2)}{(a_1-b_1)^2 + (a_2-b_2)^2}$$

and let
$$F_2 = \frac{(a_2-b_2)(c_1-a_1) - (c_2-a_2)(a_1-b_1)}{(a_1-b_1)^2 + (a_2-b_2)^2}$$

equation (5)

Then $c_1' = a_1' + (a_1'-b_1')\ F_1 + (a_2'-b_2')\ F_2$

Similarly, $c_2' = (c_2\alpha + c_1\beta) + n$

$\therefore\ c_2' = (c_2\alpha + c_1\beta) + (a_2' - a_2\alpha - a_1\beta)$

$\therefore\ c_2' = a_2' + \alpha(c_2-a_2) + \beta(c_1-a_1)$

which simplifies, as above, to -

equation (6)

$c_2' = a_2' - F_2\ (a_1'-b_1') + F_1(a_2'-b_2')$

Computer -

A fortran program, using the above formulae was fed into the
University of Adelaide's C.D.C. 6400 computer with instructions to
present, in tabulated format, the measured data (a_1, a_2, b_1, b_2,

c_1, c_2, a_1', a_2', b_1', b_2') and the calculated values of c_1' and c_2', α, β, F_1 and F_2. Special attention was paid to ensure that the values of c_1' and c_2' resulted from comparison with only the calculated values of the immediately preceding slide. The last set of values calculated always form the basis for calculation of the next succeeding slide in the series.

CHAPTER 5.   FINDINGS.

5.1. Gross Anatomy:

5.1.(a) Mandible.

The mandibular shapes of the different experimental animals are illustrated in Figures 2(a),2(b),3(a),3(b),4(a),4(b),5(a),5(b). The overall physical dimensions decreased in magnitude from the large, bulky wombat down to the small,delicate mandible of Sminthopsis crassicaudata. When viewed from the occlusal aspect, the mandibles all resembled a "V" shape.

Of particular interest were the buccal and lingual flanges located in the area of the gonial angle. These cup-like extensions of bone housed the masseter and medial pterygoid muscles, respectively. The flanges were large and highly developed in the wombat, smaller in the wallaby and possum, and persisted in the marsupial mice as very much finer structures. The marsupial mice possessed a long, delicate, spur-like lingual extension of bone which was directed postero-medially from the gonial angle. Careful attention was paid to the relative sizes of the masticatory musculature at dissection. The impression was gained that the larger and seemingly more powerful muscles were associated with correspondingly large buccal and lingual flanges (muscle attachment areas). For all animals the buccal flange was well developed when compared with the overall mandibular dimensions.

The marsupial coronoid process provided attachment for the temporalis muscle and in all animals appeared as a solid structure pointing supero-distally (Figs.2(a),3(a),4(a),5(a), Diag.5.). However, the possum had a coronoid process which was broader sagittally, and shorter vertically, when compared with the corresponding structures of the other animals.
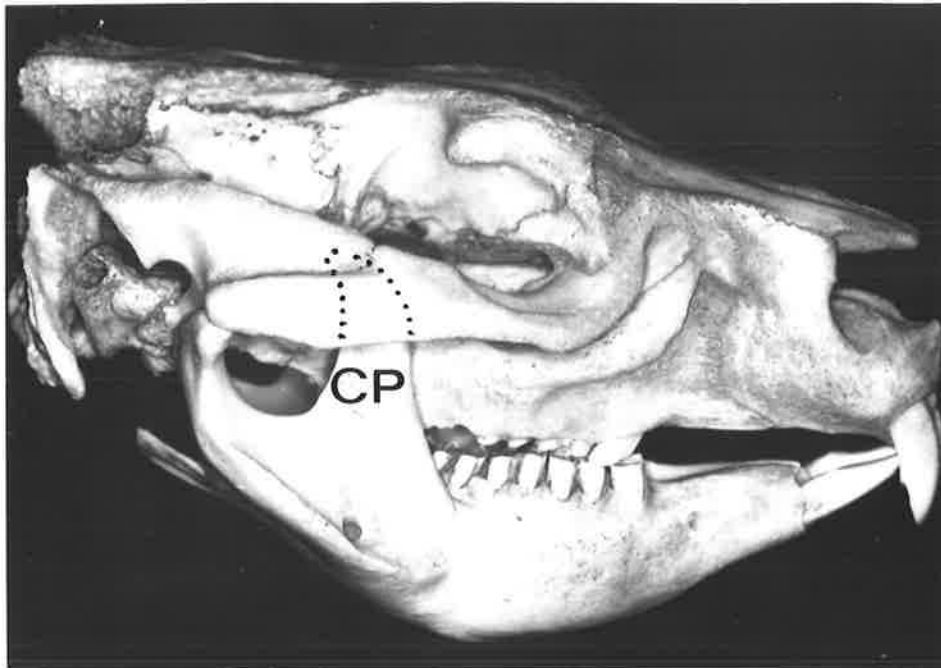
Fig. 2(a). Wombat skull and mandible. The coronoid process (CP) has
            been obscured by the zygomatic process, but is indicated
            in dotted outline. The incisors are chisel-shaped through
            attrition.

Fig. 2(b).

Occlusal view of the wombat
mandible. The buccal (BF)
and lingual (LF) flanges
are large. The condylar
head (CH) is cylindrical
medio-laterally. From
this view the mandible
presents a "V" shape.
The buccal tooth segments
do not conform with the
general "V" shape of the
mandible.
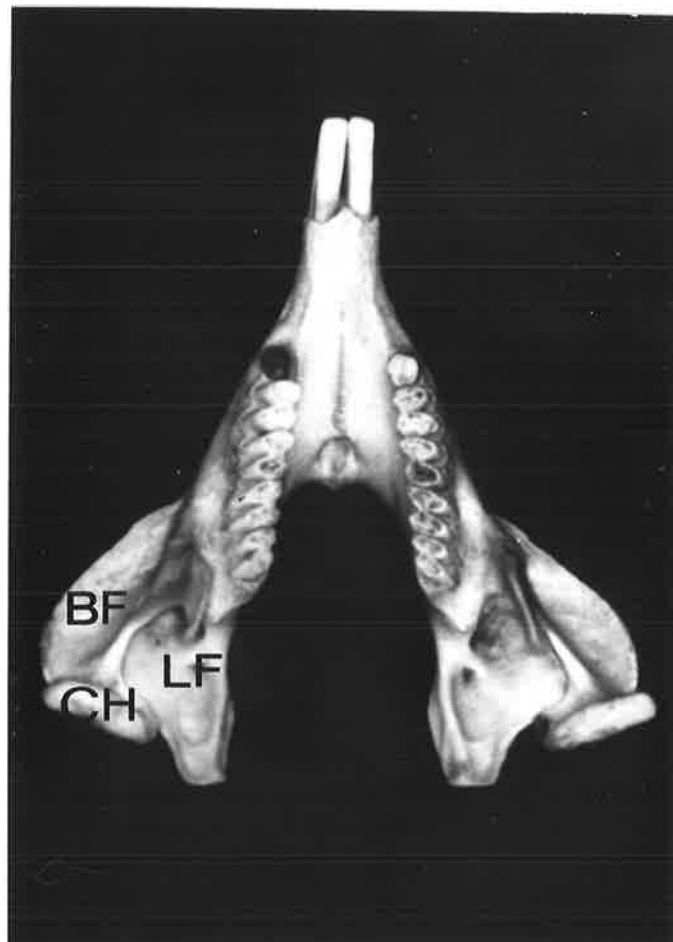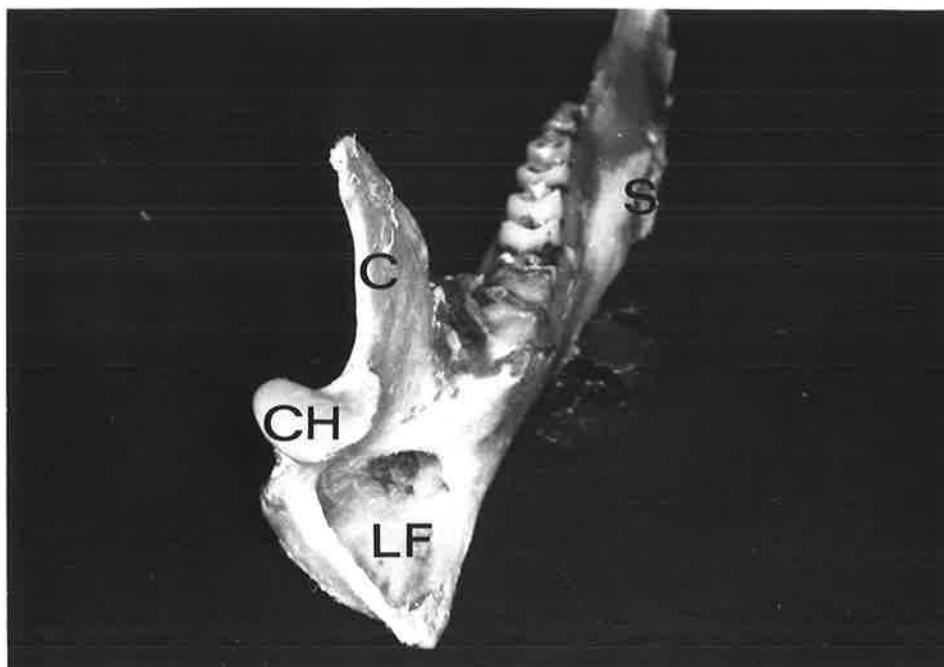
Fig. 3(a). Left side of the wallaby mandible, viewed from the
            postero-lingual aspect. The coronoid process (CP) and
            the lingual flange (LF) are well developed. The condylar
            head (CH) is a flattened, plate-shape. The symphysis (s)
            is cartilagenous.

Fig. 3(b).

Occlusal view of the
wallaby mandible,
illustrating the "V" shape,
buccal (BF) and lingual
(LF) flanges, and the
procumbent incisors.
Enamel (E) can be seen as
a thin, white margin on
the distal aspects of the
mandibular incisors. The
molar tooth segments do
not entirely conform to the
"V" shape of the mandible.
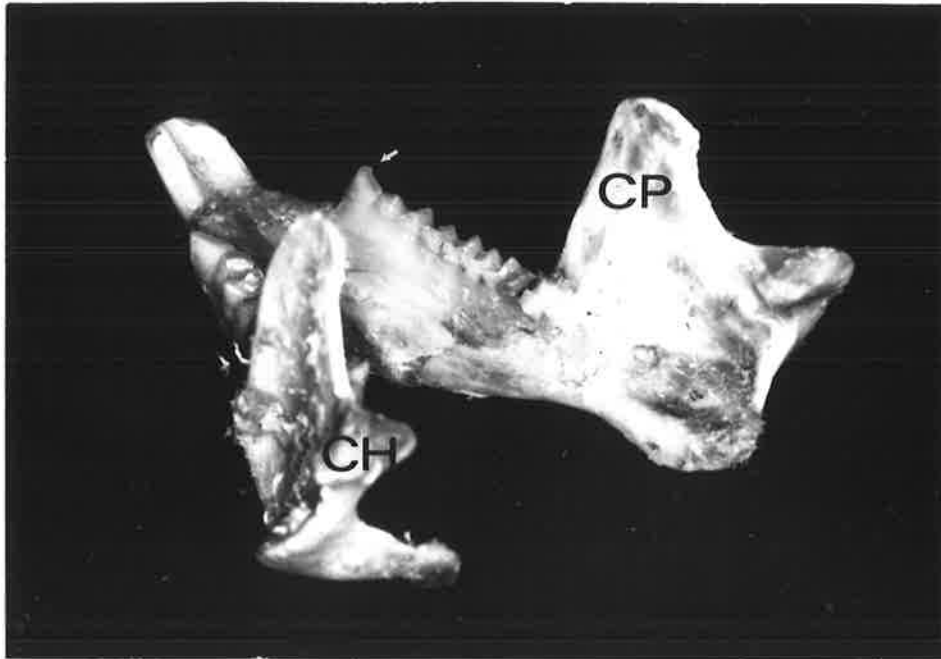
Fig. 4(a).   Possum mandible viewed from the postero-lingual aspect.
The coronoid process (CP) is broader and flatter than the
coronoid processes of the wombat and wallaby. The first
tooth in the buccal segment has a pointed, carnassial form
(arrow). The condylar head (CH) is flattened and plate-
shaped like that of the wallaby.

Fig. 4(b).

Occlusal view of the
possum mandible, showing
the "V" shape. The
carnassial form of the
first tooth in the buccal
segment is shown (arrows).
The buccal flange (BF)
houses the masseter muscle
attachment and the lingual
flange (LF) contains the
attachment of the medial
pterygoid muscle. The
molar tooth segments do not
entirely conform with the
"V" shape of the mandible.
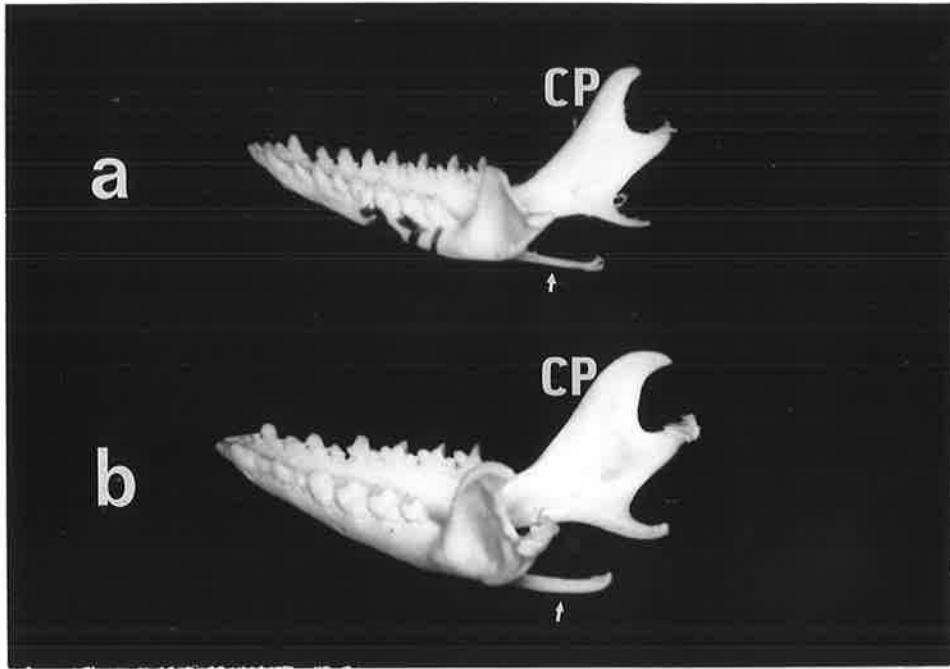
Fig. 5(a). Mandibles of Sminthopsis crassicaudata (A) and
Antechinus flavipes (B) viewed from the postero-buccal
aspect. The coronoid process (CP) is long and curved.
There are long, spur-like lingual extensions (arrows).
Unlike the wombat, wallaby or possum, there are no
edentulous regions separating the molar and incisor
segments.

Fig. 5(b).

Occlusal view of the skull
and mandible of
S. crassicaudata (A) and
A. flavipes (B). The
mandibles are "V" shaped
and the dental arches
conform to that same
pattern. The lingual spurs
are well illustrated
(arrows). The condylar
heads (CH) cylindrical
medio-laterally and fit
snugly into the glenoid
fossae (GF).
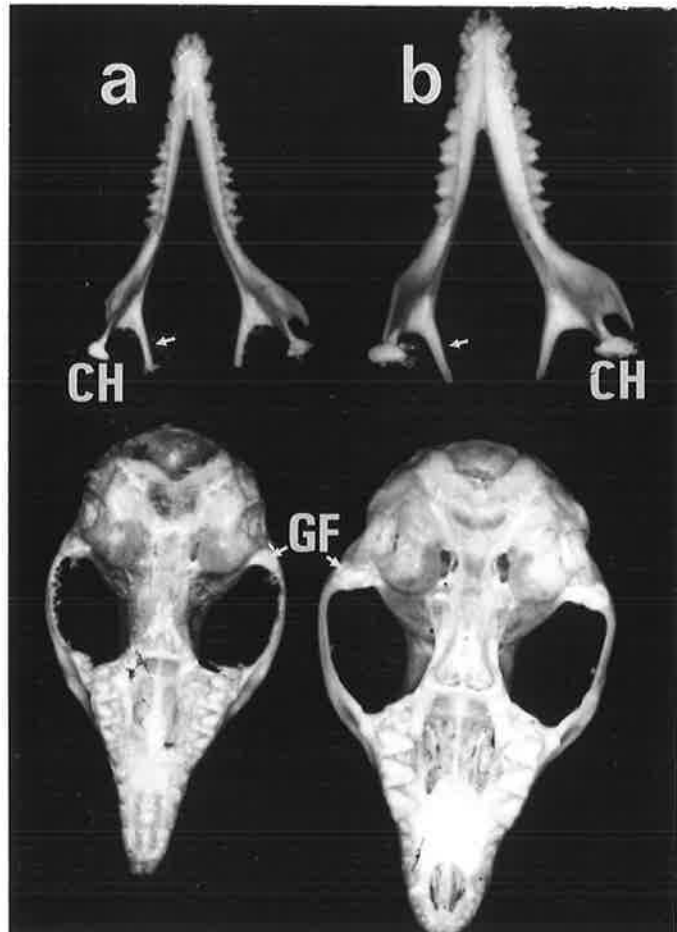
Jaw sizes and dental formulae.

WOMBAT $I_1^1 C_0^0 Pm M_5^5$

WALLABY $I_1^3 C_0^0 Pm M_5^5$

POSSUM $I_1^3 C Pm M_5^5$

A. FLAVIPES $I_3^4 C_1^1 Pm_3^3 M_4^4$ S. CRASSICAUDATA

135MM

72 MM

64 MM

21MM

16 MM
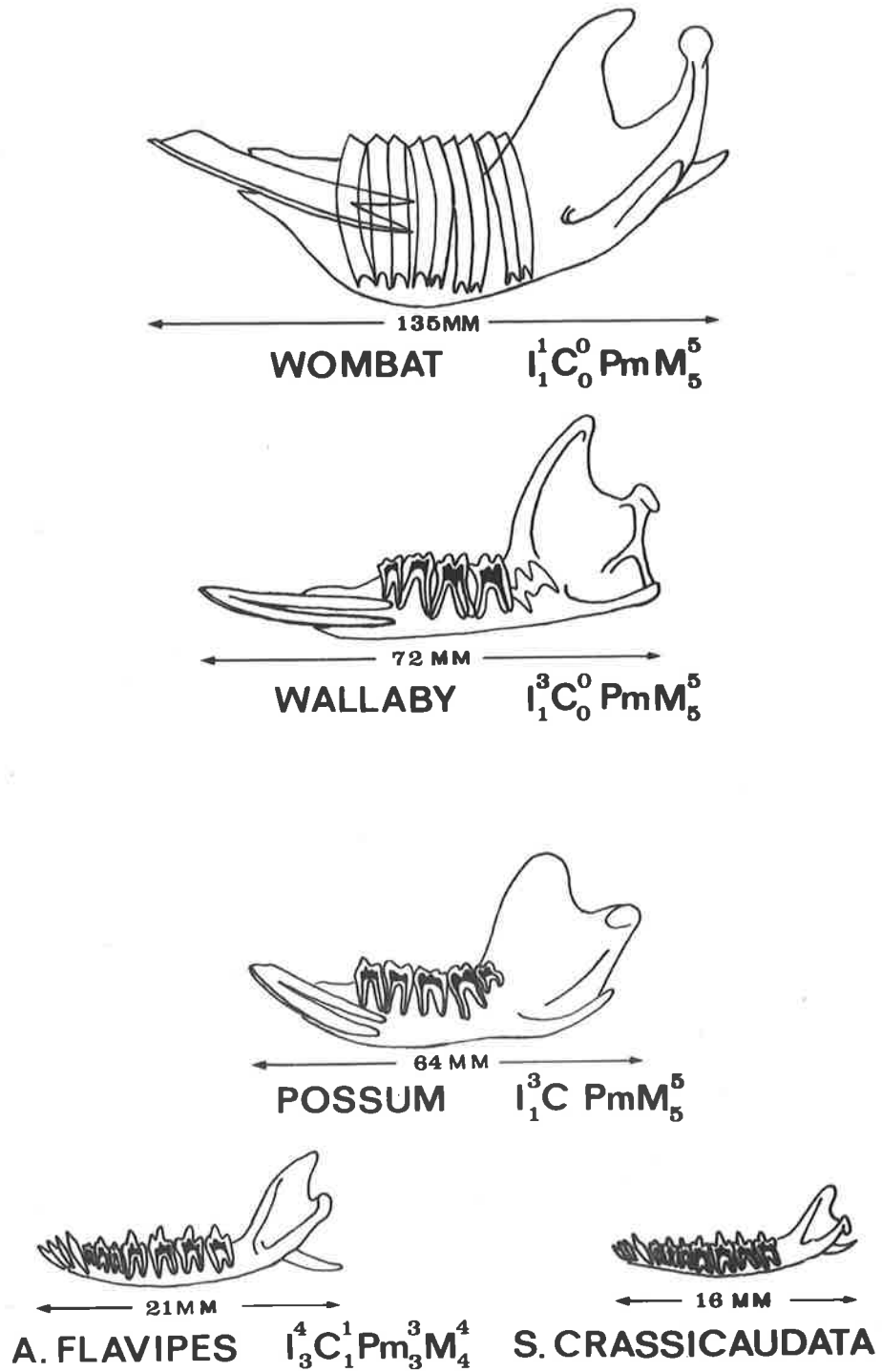
diagram. 5.

The condylar processes of the wombat and marsupial mice were longer than those of the wallaby and possum (Figs.2(a),3(a),4(a),5(a), Diag.5.). The morphology of the condylar heads also varied. The wallaby and possum displayed flattened, almost plate-like condylar articular surfaces (Figs.3(a),4(a)). The wombat had a more rounded condylar surface which was medio-laterally cylindrical (Figs.2(a),2(b)). The glenoid fossa areas of the wallaby and possum skulls were ill-defined with very low articular eminances, indicating a potential for considerable freedom in articulation. The marsupial mice, however, had rounded condylar heads which fitted snugly into definite fossae (Figs.5(a),5(b)). The wombat condyle articulated with a convex area which was more representative of an articular tubercle as there was no glenoid fossa.

5.1.(b) Dentition.

The wombat, wallaby and possum were classified as Diprotodonts because of the presence of two very large and procumbent mandibular incisors. The marsupial mice possessed more than two lower incisors and were consequently classified as Polyprotodonts. (Diag.5.).

5.1.(b)(i) wombat

The dental formula was $I_1^1 \ C_o^o \ (PmM)_5^5$. The mandibular incisors were huge, tusk-like structures which possessed cementum covered enamel on the infero-distal aspect. With attrition, a knife edge of enamel was produced. Both maxillary and mandibular incisors had lingual wear facets which resembled chisel edges and extended inciso-gingivally. Such a pattern could only result from a gnawing, rodent-like incisor mastication.

The molars were very long, curved, cylindrical structures which had cementum enveloped enamel on the buccal aspects. With attrition, sharp ridges formed on the bucco-occlusal aspects. All,

except the first molar (or premolar) occurred as paired cylinders (Diag.5.). There was no definite crown/root division.

When viewed antero-posteriorly, the occlusal plane was concave and the lateral surfaces of the condylar heads were located on a predominantly convex imaginary arc (Diag.6.). However, the medial aspect of the condylar surface, described a concave curvature.

5.1.(b)(ii)   wallaby

The dental formula was $I_1^3 \, C_o^o \, (PmM)_5^5$ (Diag.5.). There was difficulty in determining how many of the buccal teeth might be pre-molars. In young animals, there were seven teeth of which two were replaced by the most mesially erupting tooth of the adult dentition (Diag.7(a),(b)). Probably, at least one of the shed teeth was deciduous while the other may have represented a premolar.

Enamel covered the entire crown of the molars while the procumbent mandibular incisors only had enamel on the infero-distal aspect. Unlike the wombat, cementum did not completely encompass the enamel and there was a definite crown/root demarcation. Occlusion and attrition produced a sharp labio-incisal edge of enamel. However, the wear facets on the maxillary and mandibular incisors suggested a milling, and shearing masticatory function.

When viewed antero-posteriorly, the occlusal surfaces of the molar segments were located upon a convex curve. The plane of the condylar heads was concave (Diag.6.).

5.1.(b)(iii)   possum

The dental formula was $I_1^3 \, C_o \, (PmM)_5^5$. There was uncertainty regarding the nature of the small maxillary canine which could have been a permanent, deciduous, or even supernumary tooth.

The most mesial molar in the buccal segment differed from the wallaby molars, and the second and third molars, in that it had a

Arcs drawn through the condylar and occlusal surfaces of mandibles viewed antero-posteriorly.

wombat

A.flavipes
S.crassicaudata
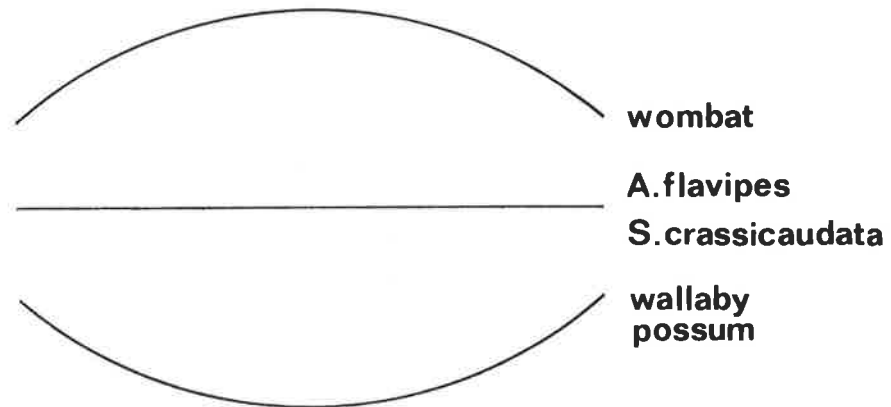
wallaby
possum

CONDYLAR HEAD PLANE

OCCLUSAL PLANE

possum
wallaby

A.flavipes
S.crassicaudata

wombat
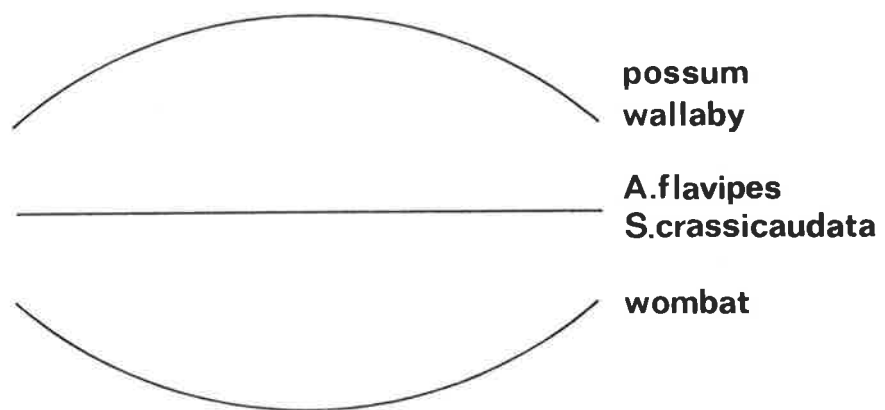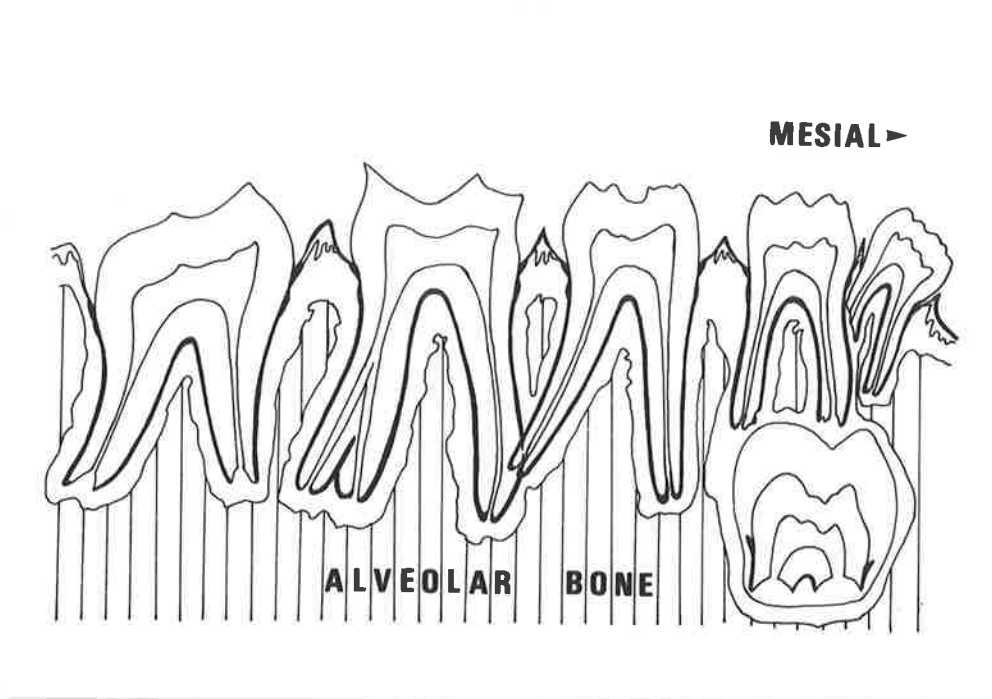
diagram.6.

Diag. 7(a). When the most mesial tooth in the wallaby buccal
segment erupts, it displaces two other teeth. The most
distal molar is not shown. The enamel has not been shown
in this diagram which is a composite of numerous
histological sections.
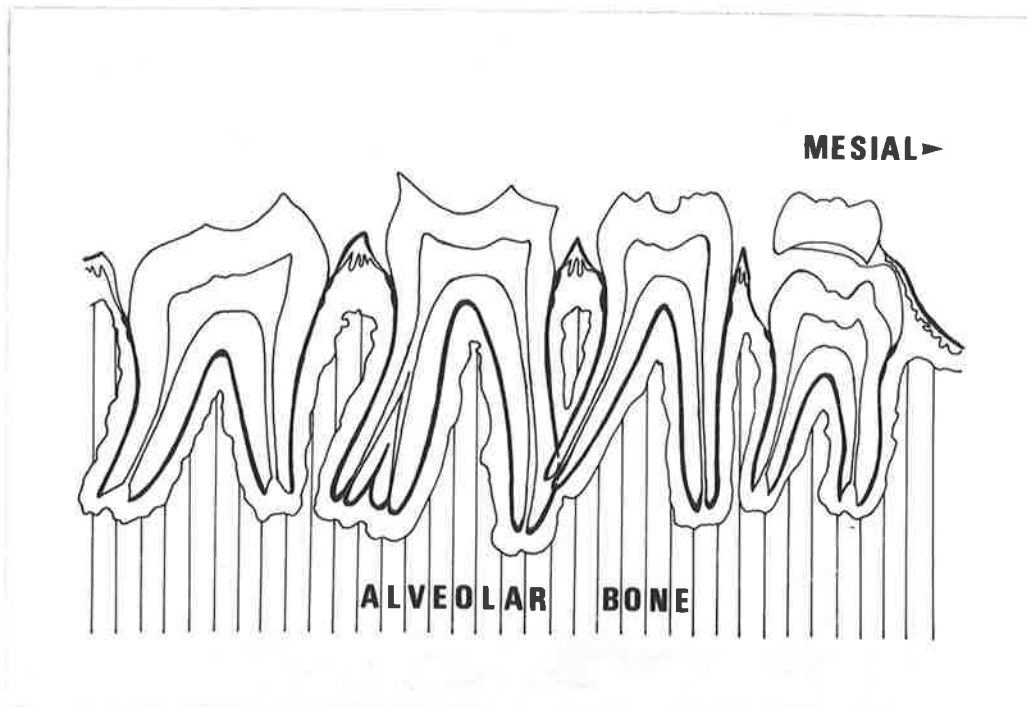
Diag. 7(b). Showing a further stage in the eruption process.
The enamel has been lost during decalcification.

large pointed cusp which was carnassial in form (Figs.4(a),4(b)). The most distal of the molars was a rather small tooth (Diag.5.). The molar crowns were completely covered by enamel and the molars had a definite crown/root structure.

The mandibular incisors were procumbent, but more curved and upright than the wallaby incisors. However, there was enamel on the infero-distal aspects of the possum incisors which produced a sharp cutting edge with attrition.

When viewed antero-posteriorly, the occlusal plane was convex and the condylar head plane was concave (Diag.6.).

5.1.(b)(iv) marsupial mice

Antechinus flavipes and Sminthopsis crassicaudata differed significantly from each other only with regard to their relative sizes and have therefore been considered together.

The dental formula was $I_3^4 \, C_1^1 \, Pm_3^3 M_4^4$. Each molar and premolar had many, sharp pointed cusps. There was no edentulous area separating the incisor and molar segments as occurred in the wallaby, wombat and possum. Enamel completely covered the crowns. The teeth had a definite root form (Diag.5.).

Viewed antero-posteriorly, the occlusal and condylar planes were predominantly flat (Diag.6.).

When viewed from the occlusal aspect, the left and right molar segments were almost parallel to each other in the wombat, wallaby and possum. In the marsupial mice the molar segments conformed to the "V"-shape of the mandible (Figs.2,3,4,5).

5.2. Histology of the Marsupial Mandibular Dentition:

5.2.(a) Dental histologic observations.

5.2.(a)(i) wombat

The molars, except the first, comprised long, curved cylinders

of tubular dentine fused into pairs which continuously erupted from persistent pulps (Diags.5,8.). The pulp chambers rapidly diminished from the forming root ends and extended occlusally a little further than half the tooth length. Enamel abutted dentine over the entire buccal surface and extended interproximally for about one third to one half of the tooth width. Cellular cementum covered the entire outer surface of the tooth, including the enamel. With attrition, the occlusal surface, therefore, became composed of dentine, enamel and cementum.

The incisor also arose from a persistent pulp. As with the molars, the incisor pulp chamber rapidly narrowed from a wide, open apex (Diag.5.). The dentine was tubular adjacent to the pulp chamber, but soon became sclerotic. Enamel covered the entire inferior and distal aspects of the incisor. Surrounding the entire incisor surface, including enamel, was a thick layer of cellular cementum.

The persisting pulp appeared to be a combination of retained enamel organ and proliferating Hertwig's epithelial root sheath. Ameloblasts, which formed the enamel, extended occlusally for a short distance from the open apex. Away from the apex, the columnar ameloblasts became more cuboidal and eventually merged into the cemento-blasts. Consequently, cementum was deposited on top of the enamel (Diag.8.). The tooth surface not covered by enamel was laid down by the continuing action of Hertwig's epithelial root sheath.

periodontal ligament.

The periodontal ligament mainly comprised dense bundles of collagen fibres which were obliquely orientated from the cementum and attached to the alveolar bone at a more occlusal level. However, the fibre orientation was not always oblique since areas of circumferential and vertically directed fibre bundles were frequently observed. This might have been suggestive of an

wombat molar

lingual

buccal
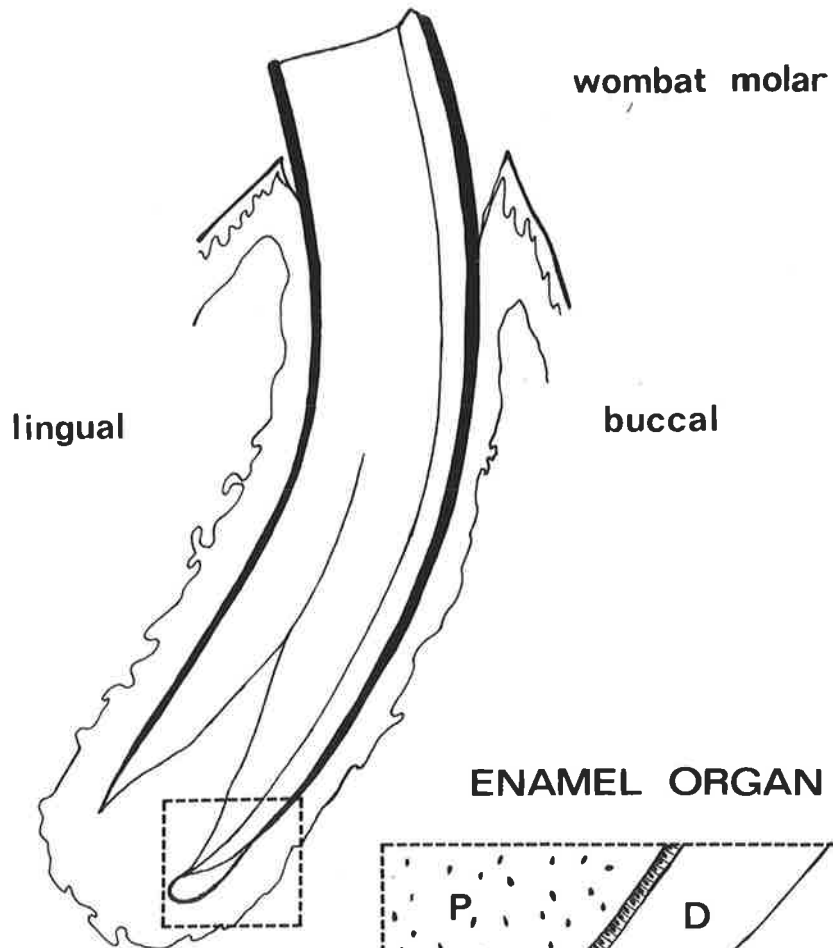
ENAMEL ORGAN
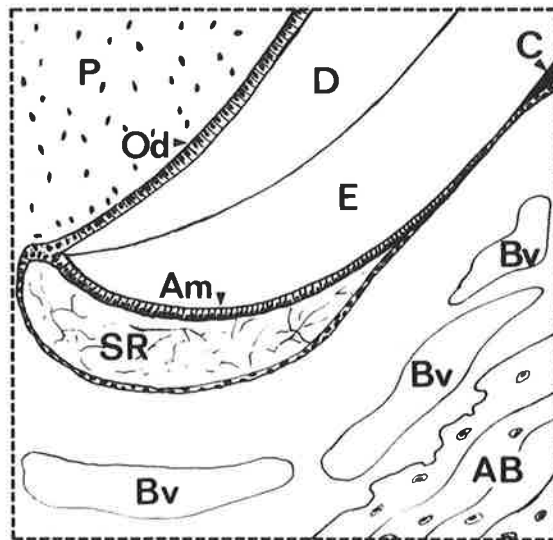
AB= alveolar bone
Bv= blood vessel
C= cementum
D= dentine
E= enamel
Am= ameloblasts
Od= odontoblasts
P= pulp
SR= stellate reticulum

diagram. 8.

intermediate plexus. The periodontal ligament was richly supplied
with thin-walled blood vessels. The sizes of the blood vessels
progressively increased from their positions near the cementum to
their location adjacent to the alveolar wall where they became very
large. The alveolar wall was very irregular and fenestrated from
the blood vessels passing into, and leaving, the ligament space. An
occasional artery could be seen within the periodontal ligament.
The blood vessels appeared to be smaller near the root apex than
opposite the middle third of the periodontal ligament. Furthermore,
the blood vessels appeared smaller and less numerous on the mesial
and distal aspects than the buccal and lingual sides of the tooth.

supra-alveolar region.

The collagen fibre system was not clearly divided into dento-
alveolar, circular, transseptal and dento-gingival groups, but
seemed to predominantly comprise circular and dento-gingival groups.

The level of the gingival attachment was situated occlusal to
the alveolar crest. The gingival sulcus was shallow, non-keratinized
and without rete peg formations. The gingival crest was lightly
keratinized, blunted in contour, and had shallow rete peg extensions
(Diags.8, and 11, p.108).

The supra-alveolar vasculature comprised a complex lattice-work
of blood vessels which were much smaller in diameter than those
present in the periodontal ligament.

5.2.(a)(ii) wallaby

Unlike the wombat, the wallaby possessed molars and incisors
which were not derived from persistent pulps and possessed a definite
root system. Enamel covered the entire molar crown but only the
inferior and distal aspects of the incisor crown. Cellular cementum
covered the root surfaces of all teeth and the superior and mesial

aspects of the incisor crown. The cellularity and thickness of the cementum decreased from the apex toward the occlusal surface. Where contact was made with enamel, the cementum overlapped the enamel for a short distance (Diags.7,9.).

The dentine appeared to be normal, tubular dentine. However, the pulp chamber was large and extended for much of the tooth length. The incisor pulp was highly vascular, with many large vessels running parallel to the long axis of the tooth. The molars contained less vascular pulp tissue but conversely displayed greater cellularity within a loose connective tissue stroma.

periodontal ligament.

The principal collagen bundles were attached to cementum and alveolar bone with no definite evidence of an intermediate plexus. The collagen fibre orientation was primarily oblique with the bone attachment being more occlusal than the cemental attachment. Many large endothelial-walled blood vessels existed throughout the periodontal ligament, but the largest of these vessels were in the middle third region of the periodontal ligament. At the incisor apex the vessels appeared to be smaller and to run parallel to the tooth surface. The molars did not display apico-occlusally aligned apical blood vessels but had numerous, large vessels located adjacent to the alveolar wall. An occasional artery could be demonstrated near the apex. Frequent entry and exit of blood vessels through the alveolar bone created an irregular, sieve-like appearance to the socket wall.

supra-alveolar region.

The gingival attachment was located above the level of the alveolar crest in all instances, except the inferior and distal aspects of the mandibular incisor where the sulcus extended far below the alveolar crest (Diag.9.).
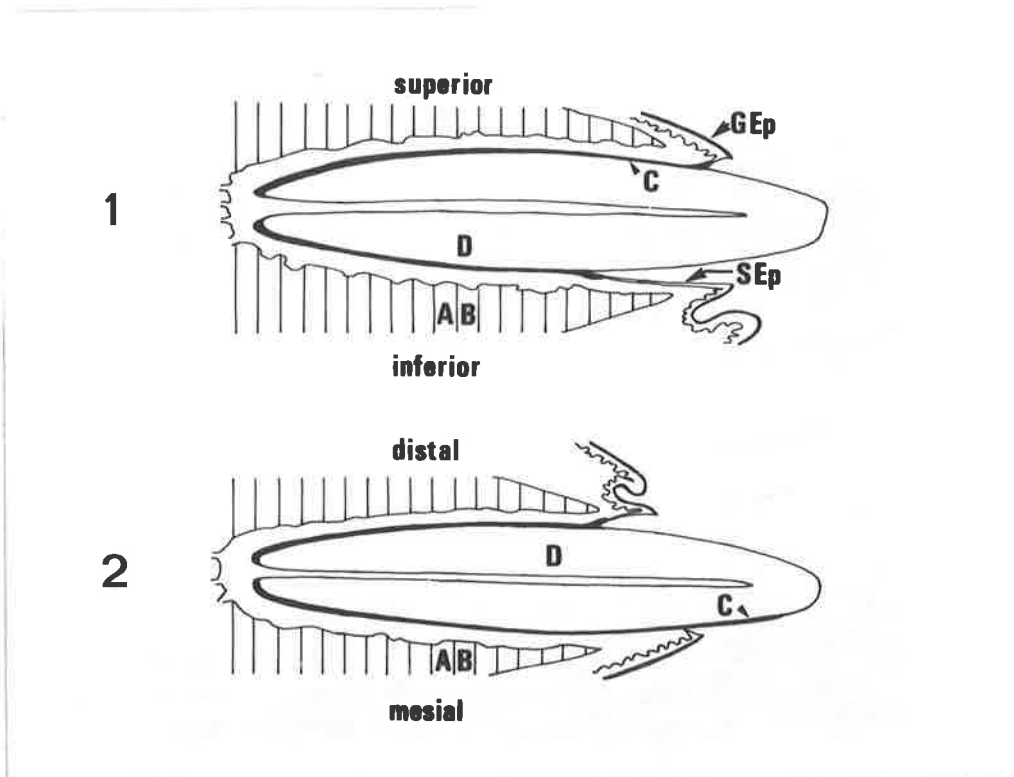
Diag. 9. Wallaby mandibular incisor sectioned in the sagittal
plane (1) and the horizontal plane (2), showing general
form and level of gingival attachment. Cementum (C)
overlaps the enamel, except on the mesial aspect of the
tooth. The enamel has been lost during processing.
Alveolar bone (AB). Dentine (D). Gingival epithelium (GEp).
Sulcular epithelium (SEp).

The collagen fibre system comprised dento-alveolar, circular, dento-gingival (free and attached gingiva) and transseptal groups around the teeth. However, the incisor appeared to lack the transseptal system of fibres.

There were many small blood vessels which frequently anastomosed in this area. The incisor displayed a particularly well-defined plexus of vessels which commenced at the cemento-enamel junction and extended toward the gingival crest, subjacent to the gingival sulcus.

The gingival sulcus was lined with non-keratinized epithelium but had no rete pegs. The gingival crest was more triangular than the wombat's interdental papillae and was keratinized with rete peg extensions into the dermis (Diags.7,9, and 12. p.121).

5.2.(a)(iii) possum

The possum dentition was histologically similar to that of the wallaby, having teeth with definite root and crown divisions which were not derived from persistent pulps. The cementum and enamel distribution was almost identical to the wallaby and there was a slight cemental overlap of enamel at the cemento-enamel junction. The dentine was normal, tubular dentine and the molar pulp tissue comprised a cellular, loose connective tissue stroma. The incisor pulp chamber was extensive and highly vascular with many large blood vessels.

periodontal ligament.

The only significant differences from the wallaby description concerned the narrower width of the periodontal ligament and the decreased incidence of extremely large-volume blood vessels in the possum. Proportionally, however, there were more large, thin-walled vessels in the middle third region adjacent to the alveolar bone than in the apical third areas. Similar to the wallaby, the possum incisor periodontal ligament contained a network of apico-occlusally

orientated vessels near the root apex.

supra-alveolar region.

As in the wallaby, the possum had the gingival attachment at a level occlusal to the alveolar bone crest, except on the inferior and distal aspects of the mandibular incisors. The gingival sulcus was lined with non-keratinized epithelium which was two to three cells thick and was without rete pegs. The gingival crest was of a similar triangular shape to the wallaby, keratinized, and had rete pegs.

The blood vessels formed a complex network at the level of the cemento-enamel junction. The plexus continued toward the gingival crest subjacent to the gingival sulcus.

The collagen fibres were arranged in dento-alveolar, circular, dento-gingival and transseptal groups, except where there were no transseptal fibres between the mandibular incisors.

5.2.(a)(iv) marsupial mice.

The only relevant difference between Sminthopsis crassicaudata and Antechinus flavipes was that the size of the latter exceeded the former.

All teeth, of both animals, had distinct root and crown portions which were not derived from persistent pulps. The dentine was tubular and enamel covered the crowns of all teeth. However, the cellular cementum covering the root surfaces did not overlap the enamel at the cemento-enamel junction and thus differed from the wallaby and the possum. Moreover, just below the cemento-enamel junction, there were large cemental bulges on the mesial and distal surfaces of the molars and premolars. Furthermore, there was a large accumulation of cellular cementum (hypercementosis) on the apical third of the roots (Diag.10.). The pulp chambers were moderately large and contained a

molar   incisor
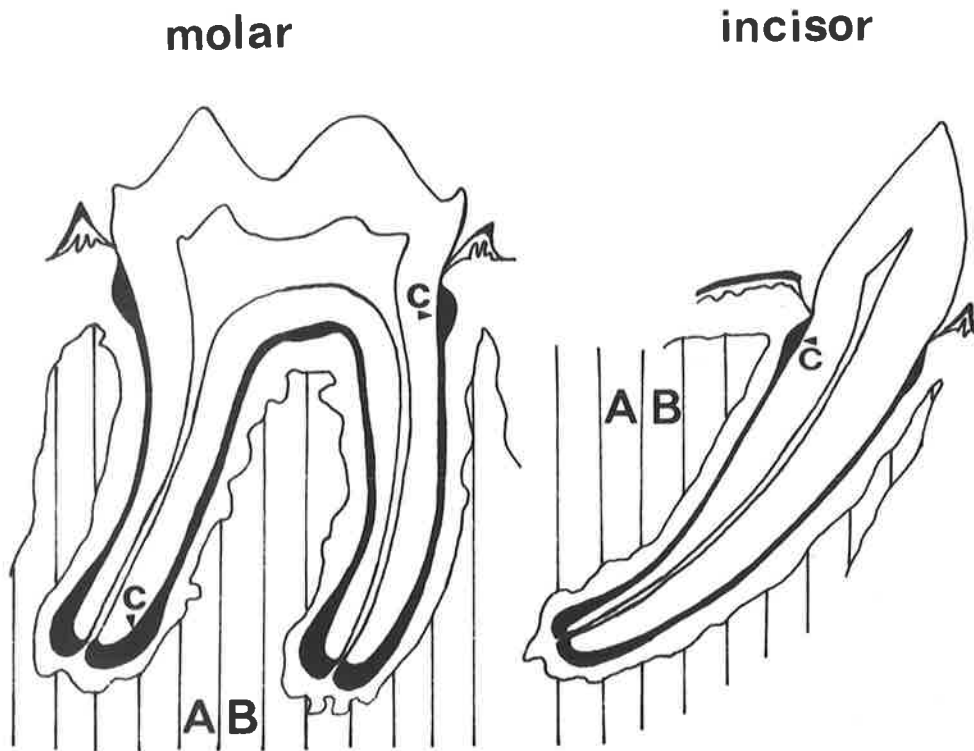
A.flavipes and S.crassicaudata

AB = alveolar bone
C = cementum

diagram.10.

vascular, cellular stroma of loose connective tissue.

<u>periodontal ligament.</u>

The teeth were firmly anchored to alveolar bone by dense bundles of collagen fibres arising from cementum and travelling obliquely to attach into alveolar bone at a more occlusal level. No intermediate plexus was apparent.

The vascularity was not particularly marked, but in relation to the size of the teeth, the vessels were quite large. Generally, the vessel sizes increased toward the tooth apex, as occurred in the other experimental animals. The largest vessels were located very close to the alveolar socket wall. Consequently, the socket wall was frequently perforated by numerous blood vessels, particularly at the apical end where the dental nerve and artery entered the periodontal ligament.

<u>supra-alveolar region.</u>

The gingival sulcus was lined with non-keratinized epithelium, and attached at the cemento-enamel junction. The gingival crest was spear-shaped (Diags.10, and 13. p.132.), keratinized, and had extensive rete peg formations.

There was a plexus of small, branching blood vessels at the level of the cemento-enamel junction. This plexus extended transseptally and on to the buccal and lingual aspects of the teeth as far as the free gingival crest.

The collagen fibre system involved dento-alveolar, dento-gingival, circular and transseptal groups. As with the other experimental animals, some of the circular fibres could represent bucco-lingually orientated intrapapillary fibres and would be present in all the experimental animals.

5.2.(b) <u>Distribution of the Connective Tissues.</u>

5.2.(b)(i)   <u>nerve fibres</u>

Fibres could be seen branching from the inferior dental nerve trunk and passing into the periodontal ligaments, primarily at the root apex area. The nerve fibres passed into the pulps of the teeth and also branched out into the periodontal ligament, travelling toward the occlusal. Nerve fibres could also be found in the corium and ramifying below the gingival epithelium. The nerve fibres ran a tortuous course through the periodontal ligament (Fig.6.) but branched more frequently at the apical foramen and in the supra-alveolar regions. Some nerve endings could be seen within the basal cell layers of the gingival epithelium with the predominant pattern comprising circular formations (Fig.7.).

5.2.(b)(ii)   <u>collagen</u>

Collagen was the major fibrous component in the periodontium of all the experimental animals. The principal and supra-alveolar fibre bundles were well demonstrated by the relevant staining techniques.

5.2.(b)(iii)   <u>reticulin</u>

An occasional fibre staining for reticulin could be seen randomly arranged within the periodontal ligaments of the wallaby, possum and marsupial mice. The wombat displayed greater numbers of reticulin fibres scattered throughout the periodontal ligament. All animals displayed concentrations of reticulin adjacent to the basement membrane of the gingival epithelium (Fig.8.). Further fibres were found around acini of the submandibular gland, muscle fibre bundles, in nerve sheaths, within the media of arteries, and throughout the gingival corium as a spidery network.

5.2.(b)(iv)   <u>mucopolysaccharides</u>

The presence of acid mucopolysaccharides in the periodontal

Fig. 6.

Possum periodontal
ligament showing a nerve
fibre (N). Fibroblast
nuclei stain black.
(Bodian's stain. x400)
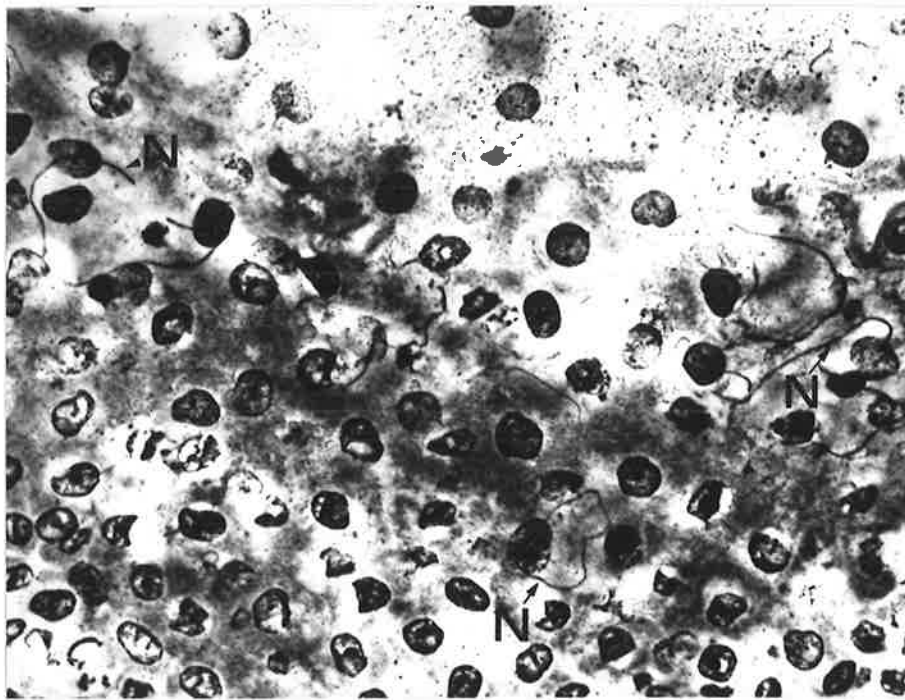
Fig. 7. Horizontal section through the basal layer of the
gingival epithelium of Antechinus flavipes. Nerve
fibres (N) are arranged in circular formations.
(Bodian's stain. x400).
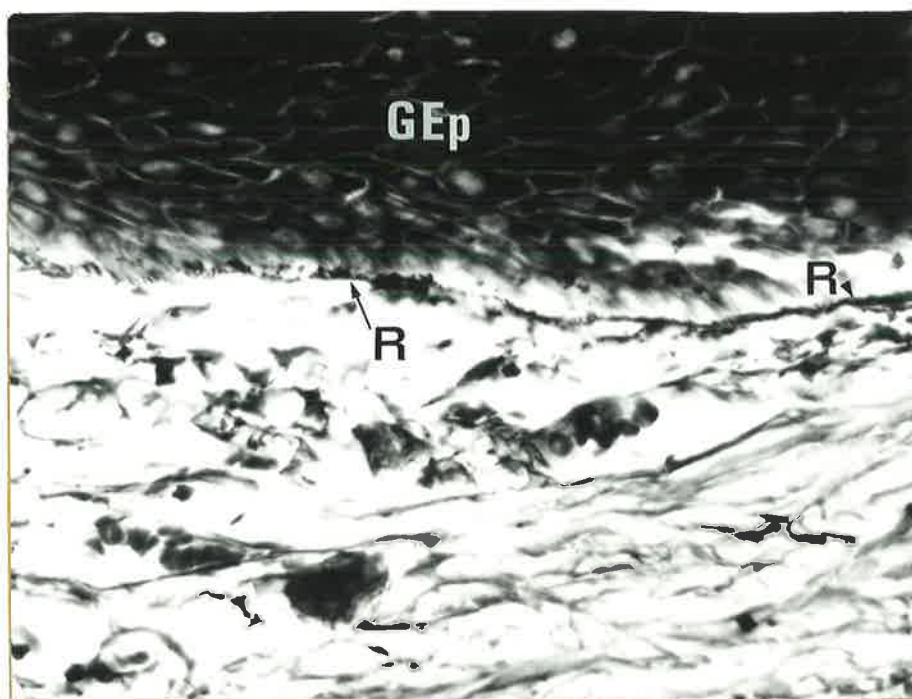
Fig. 8. Reticulin (R) located adjacent to the basement membrane
of the gingival epithelium (GEp) in the wallaby.
(Naoumenko and Feigin reticulin stain. x400).

Fig. 9. Oxytalan-like structures (arrows) in the wallaby
periodontal ligament.
(periodic acid-Schiff reaction. x250).

ligament was not substantiated by the Hale colloidal iron technique in
either control or oxidized tissue sections. However, after oxidation,
a slight and diffuse positive reaction was noted for material which
morphologically resembled elastic fibres (Table 3. p.104.). The
periodic acid-Schiff (P.A.S.) method produced a weakly positive reaction
in the periodontal ligaments of the wombat and wallaby (Fig.9.). It
could not be stated whether the reactive material was neutral muco-
polysaccharide or an indication of aldehyde groups. However, the
observed structures strongly resembled oxytalan fibres (Chapter 5.2.
(b)(vi)). Little difference could be detected between control or
oxidized sections.

5.2.(b)(v) elastic fibres

Positive reactions for elastic fibres were obtained using the
five standard elastic stains (aldehyde fuchsin, orcein, resorcin
fuchsin, orcinol-new fuchsin and Verhoeff's iron haematoxylin) in
unoxidized sections. The iron-orcein techniques of Roman et al. (1967)
and Lillie et al. (1968) produced excellent definition of individual
elastic fibres.

As determined by the staining reactions, elastic fibres were
consistently found in the arterial intima (Fig.10.), in the corium of
the vestibular mucosa and gingival crest (Fig.11.), and to varying
degrees within the periodontal ligament. Elastic fibres were found
close to the epithelial basement membrane of the gingival crest and
sulcus.

The wombat was the only experimental animal which consistently
contained elastic fibres within the periodontal ligament. The fibres
were orientated apico-occlusally and were closely associated with large
blood vessels adjacent to the alveolar bone. The fibre numbers decreased
toward the apex and very few were found in areas of the periodontal

Fig. 10.   Elastic fibres in the intima, media and adventitia of the
            inferior dental artery of the wombat.   The elastic fibres
            are stained purple.   (aldehyde fuchsin-Halmi. x250).
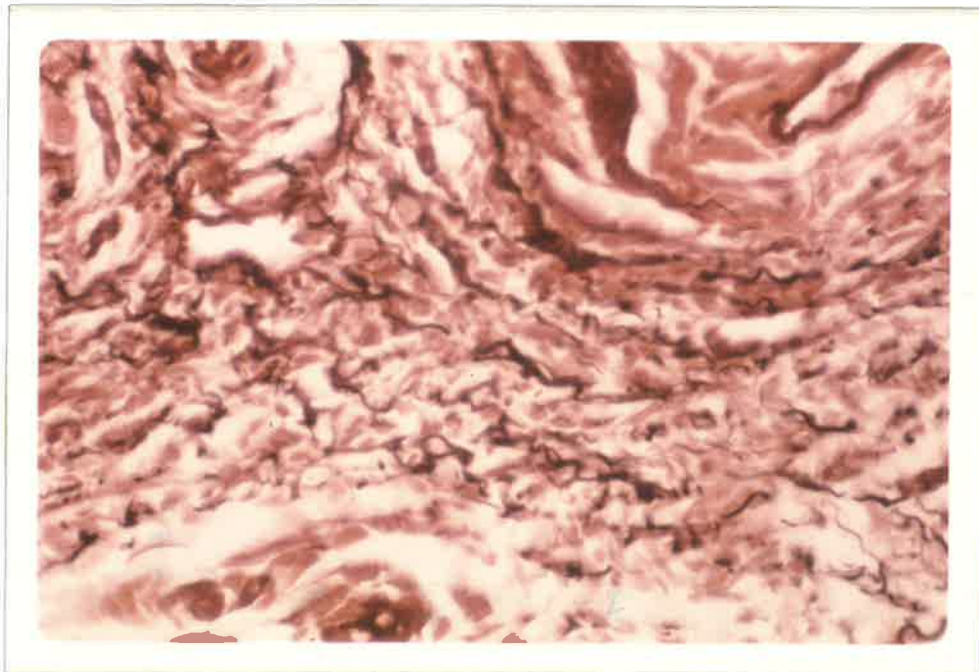
Fig. 11.   Elastic fibres in the corium of the wallaby gingival crest.
            The elastic fibres stain black.   (iron-orcein staining
            technique of Roman et al. (1967). x250).

ligament adjacent to the enamel bearing tooth surfaces i.e. the disto-inferior aspect of the incisor and buccal aspect of the molars (Table 5. p.111.).

The wallaby and possum infrequently revealed elastic fibres within the periodontal ligament and, when present, they were found in small numbers near the cemento-enamel junction (Table 6. p.119. and Table 7. p.124.). The possum had reduced numbers of elastic fibres in the corium of the gingival sulcus and crest when compared with the wombat and wallaby.

The marsupial mice appeared to be devoid of elastic fibres within their periodontal ligaments with only small amounts being present on the buccal and lingual aspects of the free and attached gingiva (Table 8. p.130.).

After potassium monopersulphate (Oxone) oxidation the intensity of the elastic fibre staining reaction markedly increased.

5.2.(b)(vi) oxytalan fibres

Tissue sections which had not been pre-oxidized, reacted positively for elastic fibres with the standard elastic fibre stains. With the exception of the wombat, few elastic fibres were present within the periodontal ligament. Following a period of pre-oxidation with 10% potassium monopersulphate, previously non-reactive oxytalan fibres were found to stain with aldehyde fuchsin (Fig.12, Fig.13), orcein, resorcin fuchsin and orcinol-new fuchsin. The reactions are summarized in Table 3, p.104. It was interesting to note that oxytalan stained brown with iron-orcein whereas elastic fibres stained an intense black (Table 4. p.104.).

The difference between control and oxidized sections was illustrated (Fig. 14, and Fig.15)) where the arterial internal elastic lamina stained in both sections, but oxytalan fibres were seen only in
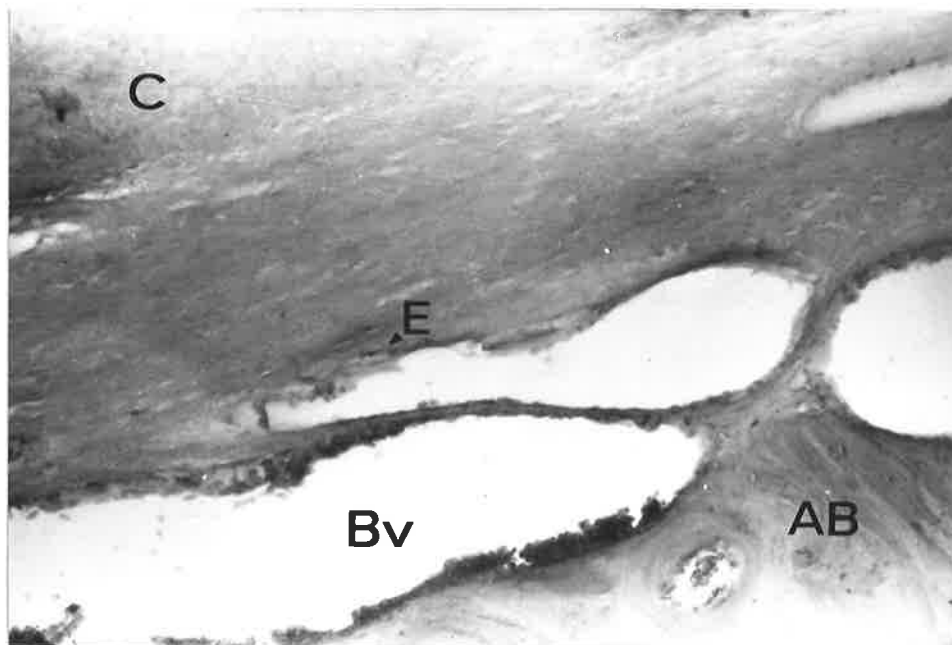
Fig. 12. Wombat periodontal ligament. Cementum (C). Blood vessels
(Bv). Alveolar bone (AB). Elastic fibres (E).
(aldehyde fuchsin-Halmi. x100).
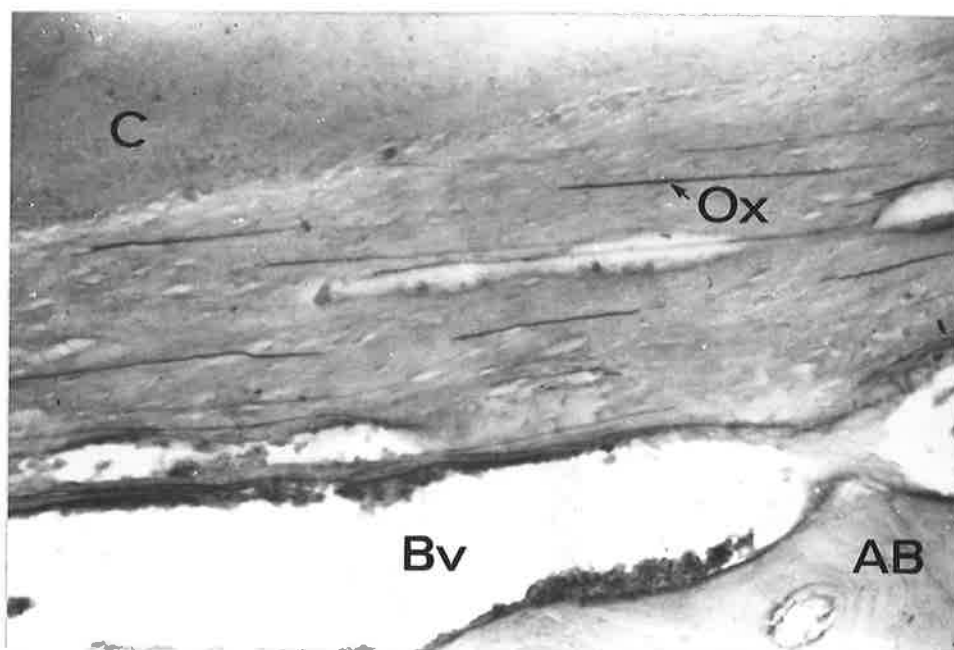
Fig. 13. Adjacent section to Fig. 12. Oxytalan fibres (Ox) are
visible in the wombat periodontal ligament following
oxidation with Oxone. Cementum (C). Blood vessels (Bv).
Alveolar bone (AB). (Oxone-aldehyde fuchsin-Halmi. x100).

TABLE 3:

Composite findings of the staining reactions of the marsupials studied
and the results of Fullmer and Lillie (1958).

REACTIONS	OXYTALAN FIBRES	ELASTIC FIBRES
Direct (non-oxidized)		
The 5 elastic fibre stains	−	+
Azure A	−	+
Hale	−	(+) *
Schiff	pink *	pink **
Oxidized		
orcein	brown	brown
aldehyde fuchsin	purple	purple
resorcin fuchsin	purple-black	purple-black
orcinol-new fuchsin	purple-maroon *	purple-maroon
Verhoeff's iron haematoxylin	−	black
Azure A	pale blue *	light blue *
Hale	−	blue *
Schiff	pink *	pink **

* Fullmer and Lillie described negative results with these dyes
for their material.

** Fullmer and Lillie acknowledged a slight P.A.S. positive
reaction in elastic tissues of rodents.

TABLE 4:

Iron-orcein reactions (Roman et al. 1967; Lillie et al. 1968).

Direct (non-oxidized)		
Iron-orcein	−	black
Oxidized		
Iron-orcein	brown	black

Fig. 14.   Photomicrograph of the apical third region of the wombat
molar periodontal ligament.   The internal elastic lamina
of the small artery has stained purple.
(aldehyde fuchsin-Halmi. x250).
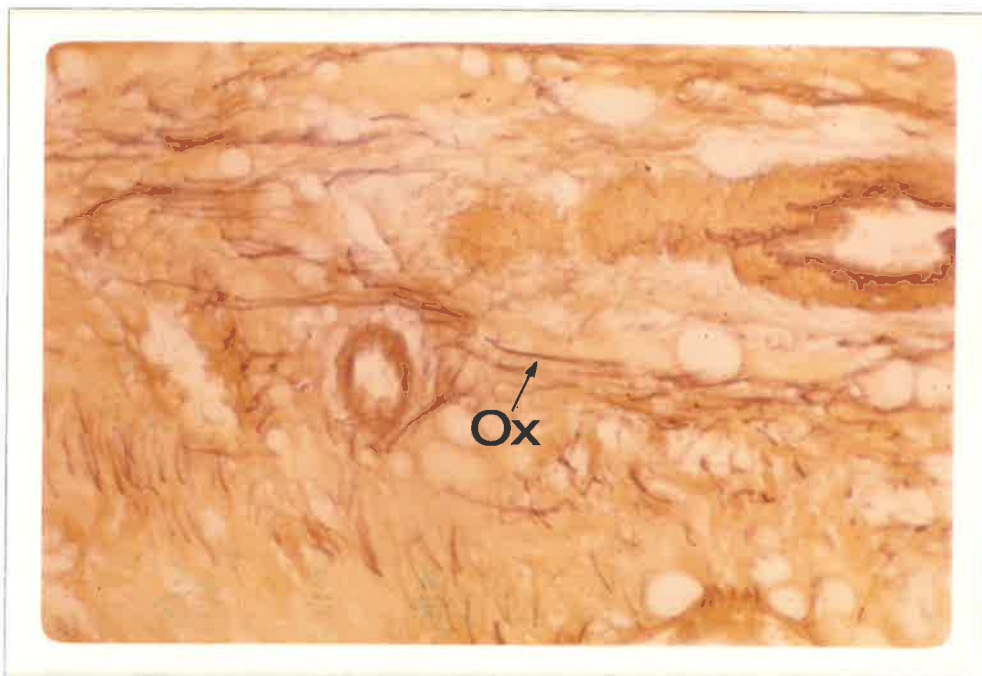
Fig. 15.   Adjacent section to Fig. 14.   Oxytalan fibres (Ox) can be
seen in large numbers in areas where no fibres were
apparent before oxidation of the tissues.
(Oxone-aldehyde fuchsin-Halmi. x250).

the oxidized section.

The fibres referred to as oxytalan fibres in this present study complied with the criteria established by Fullmer and contained in Chapter 3 of this report.

5.2.(c) Regional Distribution of Oxytalan Fibres.

5.2.(c)(i) wombat

periodontal ligament.

Oxytalan fibres were embedded in cementum and could be traced through the entire thickness of the cemental layer. The fibres varied in diameter and could be observed to branch within the cementum (Fig.16.). It seemed that the number and size of oxytalan fibres embedded in the cementum increased from the apical end of the molar and incisor teeth towards the occlusal surface. Fibres were fewer and of smaller diameter in the cementum overlying the enamel surfaces on the disto-inferior aspect of the incisor and the buccal aspect of the molars.

The oxytalan fibres emerged into the periodontal ligament oblique to the cemental surface and predominantly perpendicular to the principal collagen fibres. However, some of the finer oxytalan fibres intermingled with, and occasionally followed, the collagen fibres for a short distance across the periodontal ligament. These fine fibres frequently branched and rejoined to form a complex oxytalan meshwork, particularly adjacent the cemental surface.

The predominant pattern of the oxytalan fibres was to cascade toward the apex of the tooth. These fibres also branched but seemed to become thicker in diameter as they extended across the periodontal ligament toward the alveolar bone. The oblique, parabolic path rapidly straightened toward the alveolar bone. This resulted in long, thick oxytalan fibres being found in large numbers and orientated apico-occlusally (Fig.17. Diag.11. p.108.).

Fig. 16.

Oxytalan fibres (Ox)
often branch within the
cementum (C). Sagittal
section of wombat molar.
(Oxone-aldehyde fuchsin-
Halmi. x250).
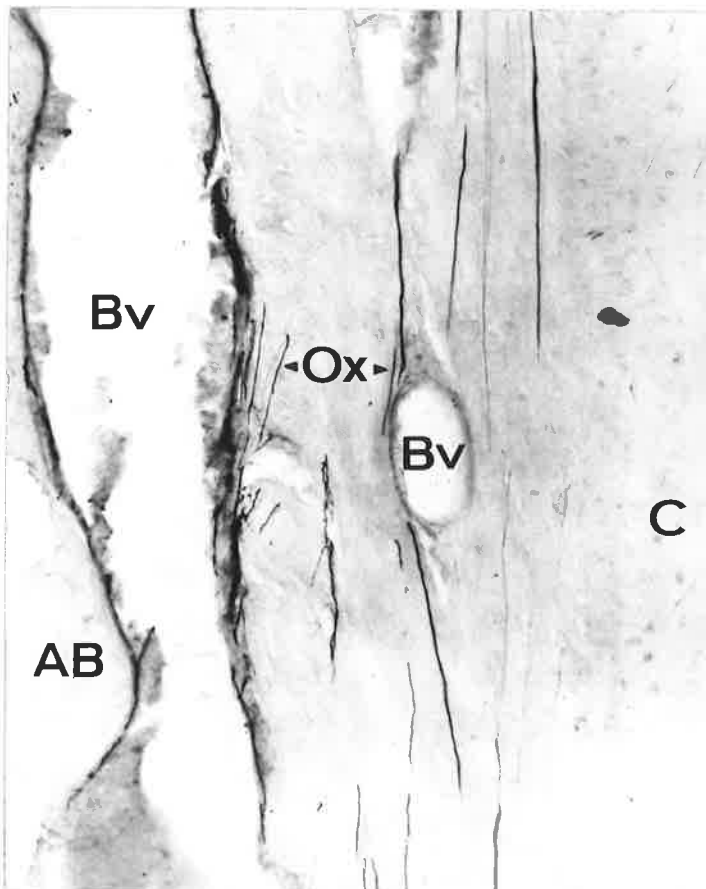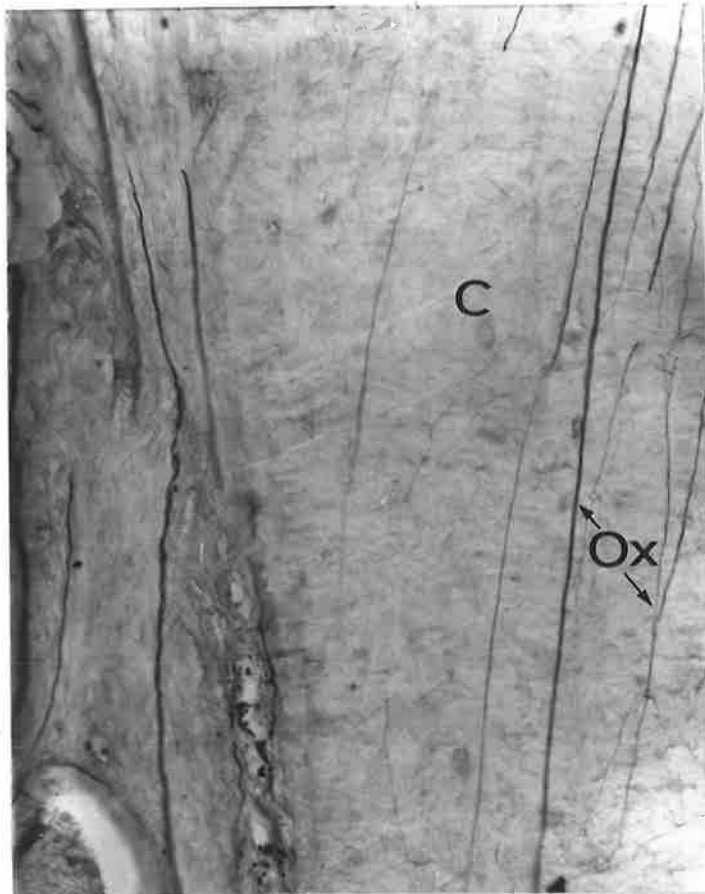
Fig. 17.

Sagittal section of wombat
molar. Oxytalan fibres
(Ox) are orientated apico-
occlusally and are
frequently observed to
have a close relationship
to the periodontal blood
vessels (Bv). Cementum (c).
Alveolar bone (AB).
(Oxone-aldehyde fuchsin-
Halmi. x100).

wombat molars
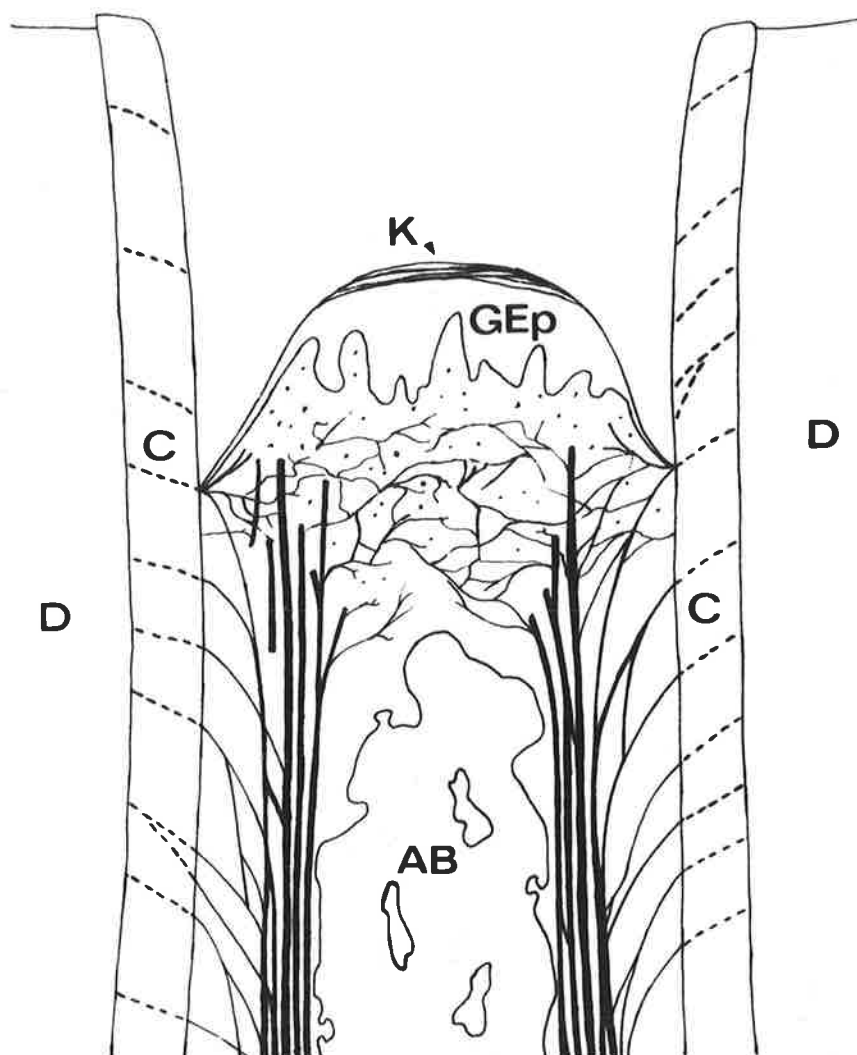
K = keratin
GEp = gingival
      epithelium
C = cementum
D = dentine
AB = alveolar bone

Oxytalan fibre distribution
-in mesio-distal section

diagram. 11.

The oxytalan fibres were situated very close to the blood vessels in the periodontal ligament and were intimately associated with the large blood vessels abutting the alveolar socket wall. Occasionally, widely separated blood vessels appeared to be linked together by oxytalan fibres (Fig.17, and Fig.18.). Oxytalan fibres could be seen following blood vessels into the alveolar bone, but no convincing evidence for fibre attachment into bone was found.

Proportionally fewer of the thick variety of oxytalan fibres were found on the disto-inferior surface of the incisor and buccal aspects of the molars. This distribution was similar to the elastic fibres. Furthermore, the numbers and sizes of the oxytalan fibres tended to gradually increase from the cervical third to the apical third levels of the periodontal ligament (Table 5, p.111.).

supra-alveolar region.

As the oxytalan fibres emerged from cementum above the level of the alveolar crest, they either curved apically into the periodontal ligament to join the long, thick fibres or continued with an orientation similar to the collagen fibre system. There were more fine, branching oxytalan fibres which interweaved with the supra-crestal collagen bundles compared with those in the periodontal ligament. Thus a fine network of oxytalan extended transseptally between adjoining teeth and passed bucally and lingually through the crestal corium toward the attached gingiva. Oxytalan fibres could be seen in a dense network of fine fibres near the basement membrane of the sulcular and free gingival epithelium. Numerous fibres appeared to run circumferentially around the teeth.

By contrast to the fibre proportions in the periodontal ligament, there appeared to be more oxytalan fibres in the buccal than lingual supra-alveolar regions (Table 5, p.111.).

Numerous, apico-occlusally aligned, thick fibres could be
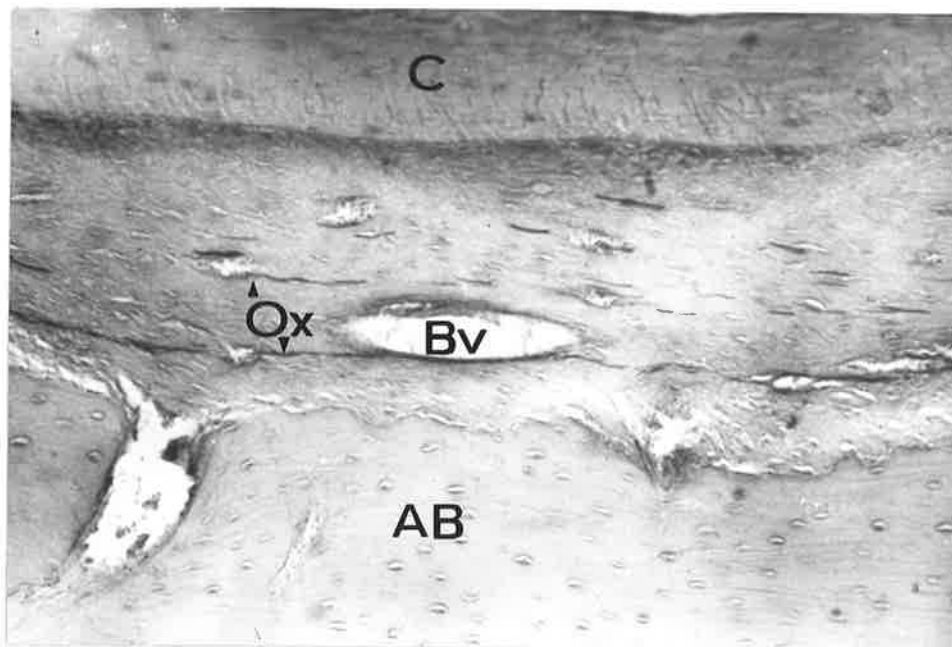
Fig. 18.   Buccal aspect of the wombat molar periodontal ligament.
Oxytalan fibres (Ox) appear to link blood vessels (Bv).
Cementum (C).   Alveolar bone (AB).   (Oxone-aldehyde
fuchsin-Halmi. x100).

Fig. 19.

Immature pulp tissue at
the apex of a continuously
erupting wombat molar.
Oxytalan fibres (Ox)
appear as short, wavy
structures against a
background of diffusely
staining amorphous
material. (Oxone-
aldehyde fuchsin-Halmi.
x250).
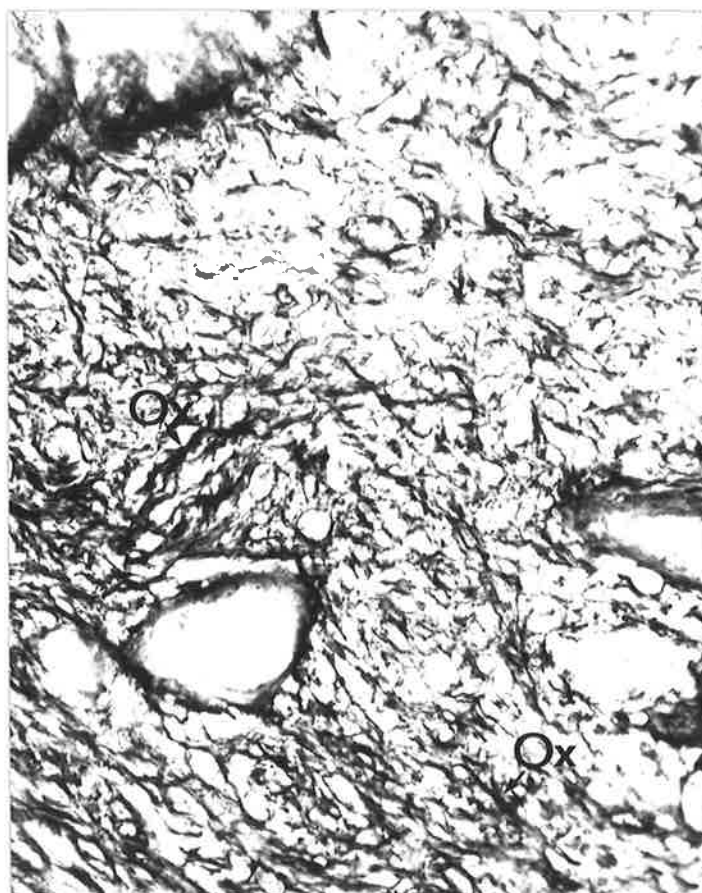
TABLE 5:

Oxytalan and elastic fibre distribution in the wombat (aldehyde
fuchsin staining)

	OXYTALAN	ELASTIC FIBRES
Wombat molars –		
(1) periodontal ligament		
mesial (C/3, M/3, A/3.)	++ , ++ , ++ .	+ , + , (+) .
distal (C/3, M/3, A/3.)	++ , ++ , ++ .	+ , 0 , (+) .
buccal (C/3, M/3, A/3.)	+ , ++ , ++ .	0 , 0 , (+) .
lingual (C/3, M/3, A/3.)	++ , +++, +++.	+ , + , + .
apex	+ some fibres pass under the apex and around the enamel organ.	0
bifurcation*	+	0
(2) supra-alveolar region		
crest (Buccal, lingual,	+++, + ,	0 , 0 ,
mesial, distal)	++ , ++ .	(+) , (+) .
sulcus (Buccal, lingual,	++ , + ,	0 , 0 ,
mesial, distal)	+ , + .	(+) , (+) .
transseptal (Mesial, distal)	++ , ++ .	(+) , (+) .
(3) pulp**	+	0
(4) circumdental fibres	+	0

TABLE 5:   (continued)

	OXYTALAN	ELASTIC FIBRES
Wombat incisor –		
(1) periodontal ligament		
superior (C/3, M/3, A/3.)	N.S.,N.S., +++.	N.S.,N.S., ++ .
inferior (C/3, M/3, A/3.)	N.S.,N.S., +++.	N.S.,N.S., + .
distal (C/3, M/3, A/3.)	N.S.,N.S., ++ .	N.S.,N.S., 0 .
mesial (C/3, M/3, A/3.)	N.S.,N.S., +++.	N.S.,N.S., ++ .
(2) apex	++ fine network	0
(3) pulp**	+	0

N.S. – not studied in detail.

 * = not a true bifurcation, rather an area of cemental fusion.

 ** = only in the immature pulp tissue.

Note: refer to Appendix V for explanation of the symbols.

seen above the level of the alveolar crest and extending almost to the level of the gingival attachment (Diag.11.). These oxytalan fibres appeared to represent a continuation of the thick fibre system of the periodontal ligament which could have been drawn into the supra-alveolar region by the continuing process of tooth eruption. Oxytalan fibres could be seen still embedded in cementum occlusal to the gingival attachment.

apex.

Oxytalan fibres continued around the apices of the molars and incisors to effectively link the fibre systems on the buccal, lingual, mesial, and distal aspects of the periodontal ligament. The fibres passing around the apex formed a fine, branching network which appeared to be very closely related to the walls of blood vessels and the basement membrane of the ameloblastic cells of the persisting enamel organ. The large, apico-occlusally directed oxytalan fibres extended almost to the apex where they rapidly dissipated and reduced to a fine network. The oxytalan fibres appeared to be more poorly organized on the buccal aspect of the molar apical region.

pulp.

The apical end of the molars and incisors was wide and flaring and contained a high proportion of vascular mesenchymal tissue. In oxidized sections only, this formative pulp stained positively and diffusely for mucopolysaccharide. Small, coiled oxytalan fibres were also present (Fig.19.). However, evidence of oxytalan fibres and mucopolysaccharide was lacking when examining the coronal, more mature, pulp tissue.

5.2.(c)(ii) wallaby

periodontal ligament.

The oxytalan fibres were embedded in cementum where they

occasionally branched and then passed obliquely into the periodontal ligament. Similar to the wombat, some oxytalan fibres travelled short distances intertwined with the principal collagen fibre bundles. The oxytalan fibres branched and formed a plexus which was most evident near the cemental surface (Fig.20.). However, most oxytalan fibres appeared to cascade toward the apex, becoming more perpendicular to the collagen fibres until many of them became orientated in an apico-occlusal direction. The number and thickness of oxytalan fibres emerging from the cementum increased towards the apex. Within the periodontal ligament space, the oxytalan fibres frequently branched and intercommunicated, with the result that thick fibres could be seen against a background of fine, meshed fibres (Fig.21.). The thick, ribbon-like oxytalan fibres ran predominantly in an apico-occlusal direction. The close relationship between the fibres and periodontal blood vessels was demonstrated by fibres which appeared to pass across the periodontal membrane and aim directly for a particular vessel (Fig.22.). Oxytalan fibres could also be seen accompanying blood vessels into alveolar bone (Fig.23.) but, similar to the wombat, there was not sufficient evidence to indicate fibre attachment to bone.

The thicker variety of oxytalan fibres seemed to branch less frequently than the finer types. If the tissue sections were not examined serially, the impression was often gained that the thick, ribbon-like fibres were orientated in an apico-occlusal direction without cemental attachment. When examined serially, it became apparent that the thick fibres were formed as the result of an aggregation of thinner fibres of cemental origin (e.g. Fig.24.).

Generally, the number of fibres, particularly the thick type, increased toward the apex but there were proportionally fewer oxytalan fibres on the distal aspect of the incisor than the superior, inferior and mesial sides. Conversely, the wallaby molars had comparatively

Fig. 20.

Gradations in oxytalan
fibre size within the
wallaby periodontal
ligament. The oxytalan
fibres emerging from the
cementum (C) are of lesser
diameter than the thicker,
ribbon-like oxytalan
fibres (arrows).
(Oxone-aldehyde fuchsin-
Halmi. x250).
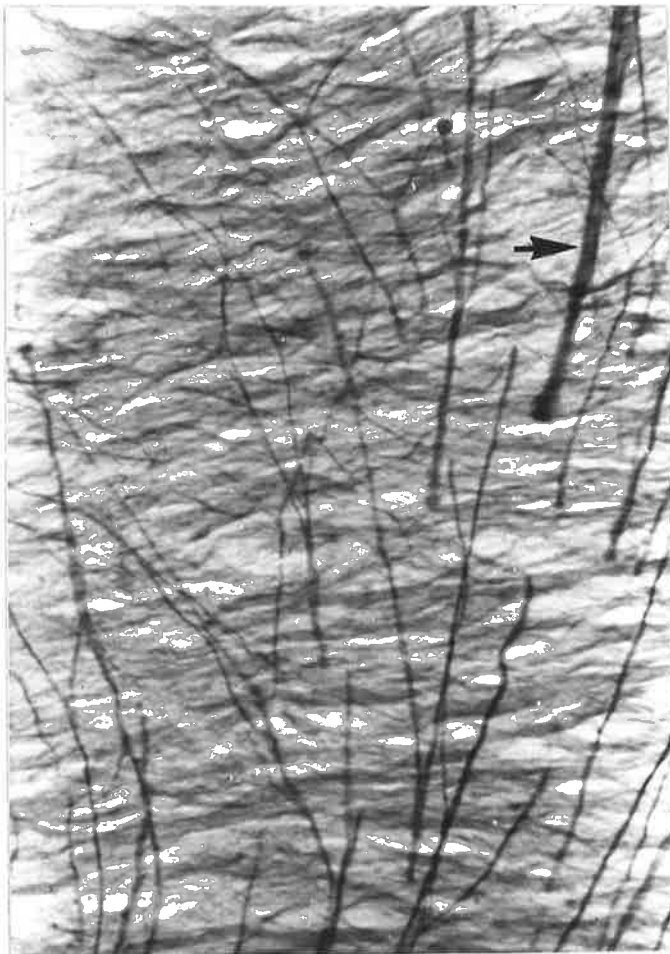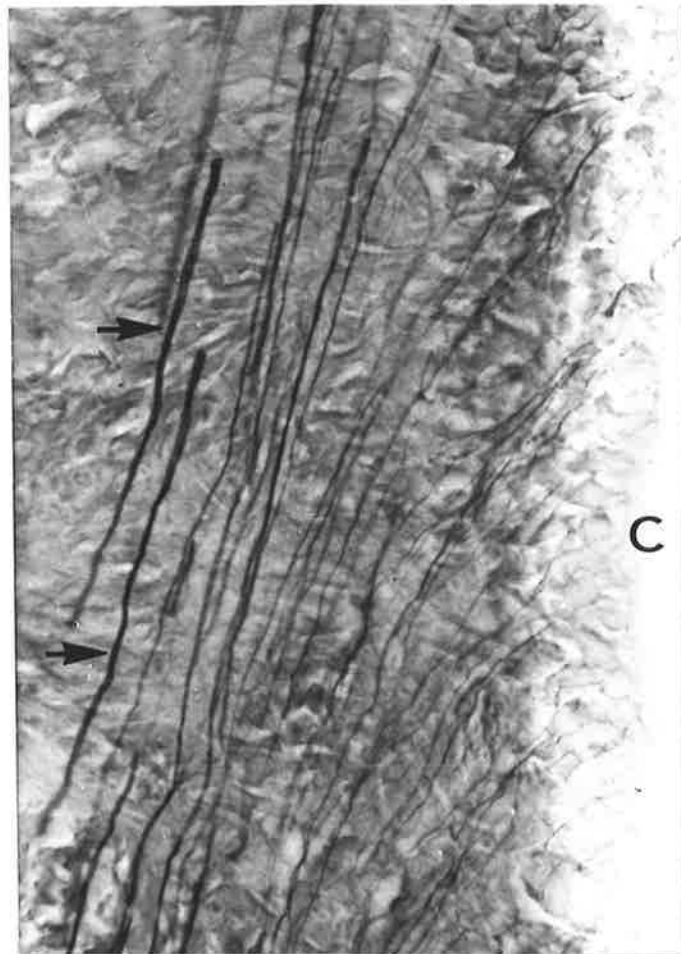
Fig. 21.

Wallaby molar periodontal
ligament. Fine branching,
oxytalan fibres form a
network among thicker
diameter oxytalan fibres
(arrow). (Oxone-aldehyde
fuchsin-Halmi. x400).

Fig. 22.

Photomicrograph of an
oxytalan fibre (Ox)
adjacent to a large blood
vessel (Bv) at the middle
third level of the wallaby
incisor periodontal
ligament. Sagittal
section. (Oxone-aldehyde
fuchsin-Halmi. x250).
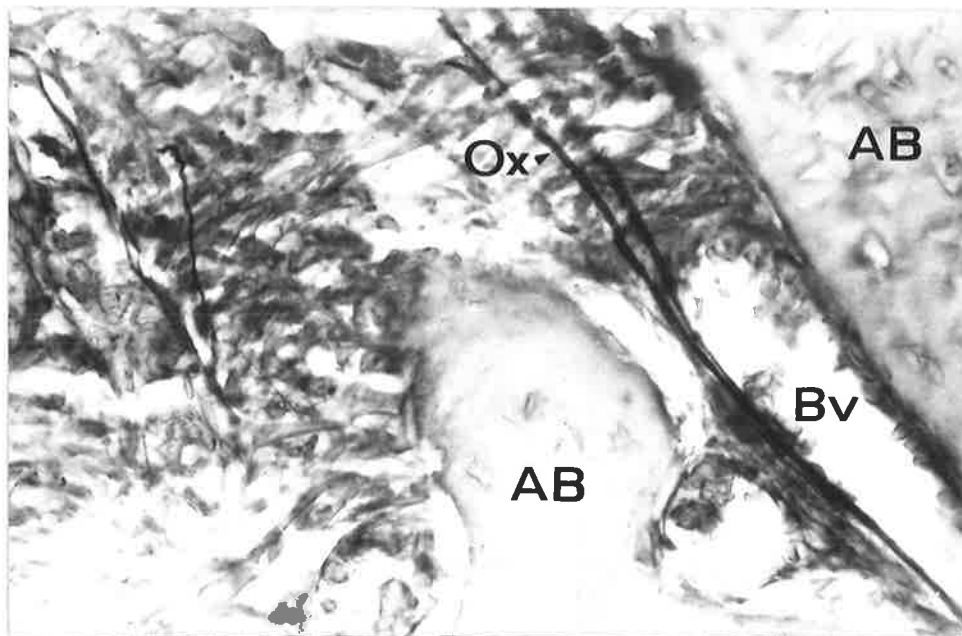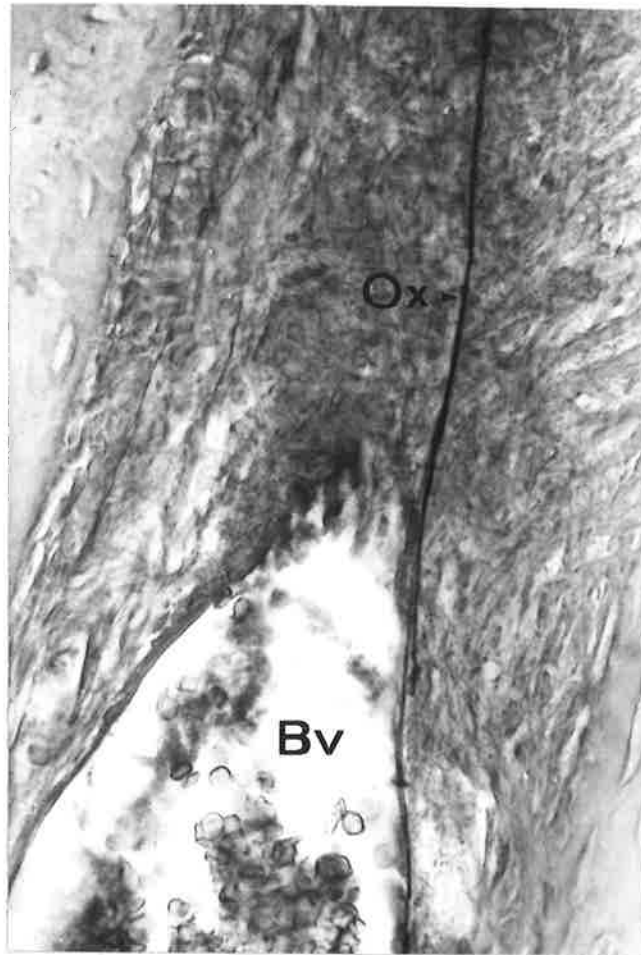
Fig. 23. Oxytalan fibres (Ox) following a blood vessel (Bv) into
the alveolar bone (AB). Sagittal section at the middle
third level of the wallaby molar periodontal ligament.
(Oxone-aldehyde fuchsin-Halmi. x250).

Fig. 24.

Sagittal section of wallaby incisor periodontal ligament at the middle third level. Oxytalan fibres (Ox) curve from the cementum (C), frequently branch, and join thicker aggregates of oxytalan fibres - oxytalan tracts (OxT). (Oxone-aldehyde fuchsin-Halmi. x250).
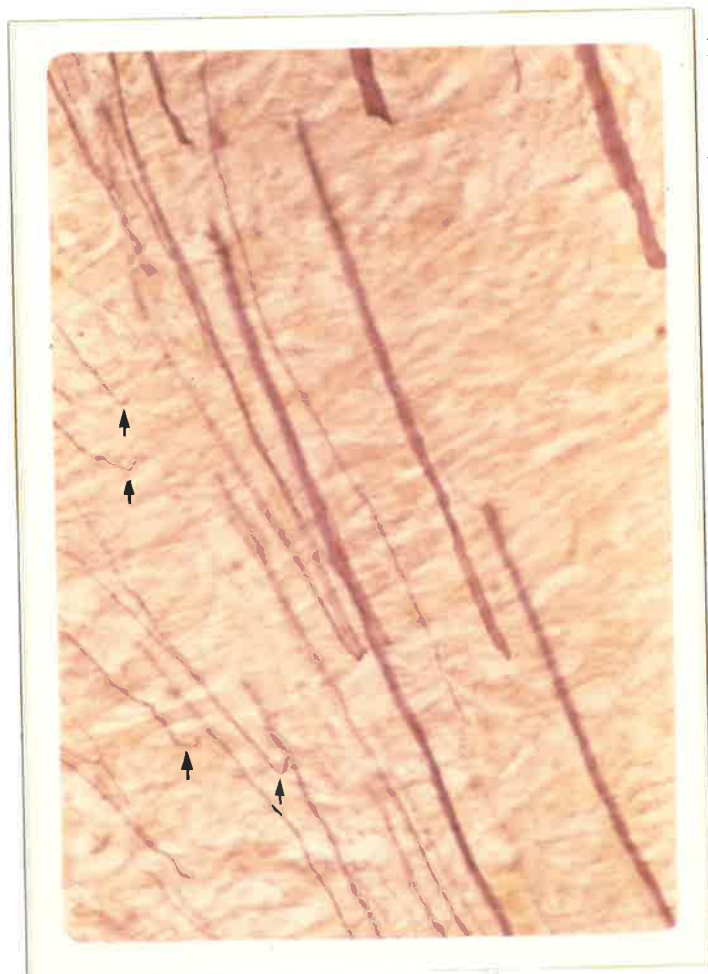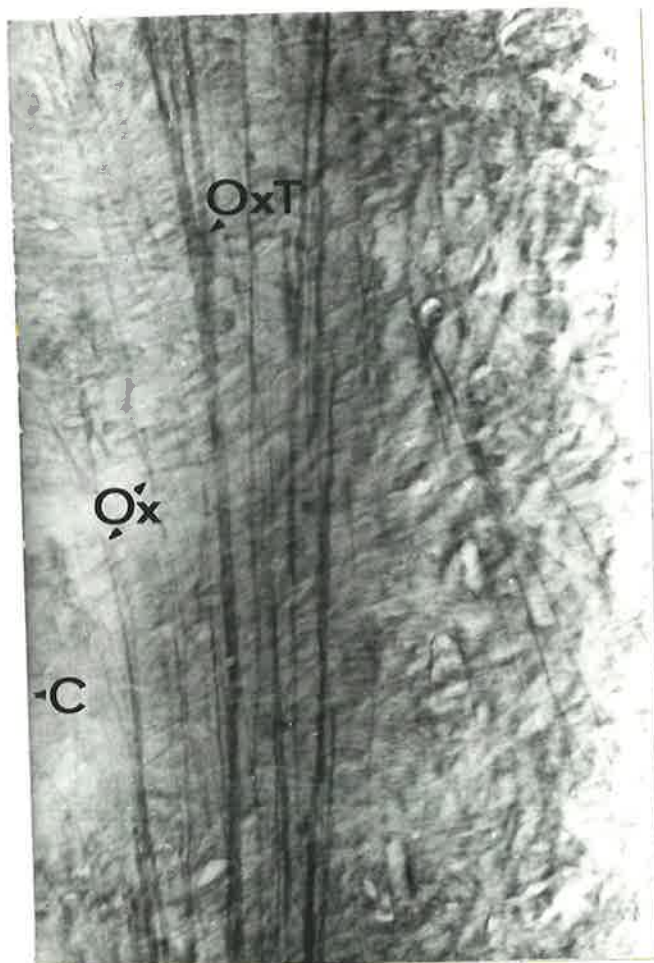
Fig. 25.

Oxytalan fibres and oxytalan fibre tracts in the wallaby molar periodontal ligament. Several fibres appear to have elastically recoiled (arrows) following preparation of the section. (Oxone-aldehyde fuchsin. x400).

more oxytalan fibres on the distal aspect of the periodontal ligament
(Table 6, p.119.).

It was interesting to note that in numerous sections
oxytalan fibres appeared corrugated and with their cut ends curled over
(Fig.25.). The impression was obtained that these fibres had recoiled
from a stretched state when sectioned on the microtome.

supra-alveolar region.

Oxytalan fibres could be seen arising from cementum at the
level of the cemento-enamel junction, frequently branching and follow-
ing courses similar to the dento-gingival, transseptal and circular
collagen fibre bundles (Diag.12.). Small diameter oxytalan fibres
were found close to the basement membrane of the gingival and sulcular
epithelium and in these regions they mingled with elastic fibres.

No obvious transseptal oxytalan fibres existed between the
mandibular incisors. Oxytalan fibres could be traced in the molar
supra-alveolar tissues from the mesial tooth to the distal aspect of
the most distal molar. It was, therefore, a continuous system of
supra-alveolar communication.

An important observation was the absence of thick, ribbon-
like oxytalan fibres above the level of the alveolar crest of bone.

apex.

The fibre pattern changed at the apex where the thick fibres
broke up into progressively finer, branching fibres to eventually form
a filamentous network (Fig.26.). This fibre network, which was
associated with a mucopolysaccharide-like material, passed beneath
the apex to unite the oxytalan fibre systems on the mesial, distal,
buccal, and lingual aspects of the periodontal ligament.

bifurcation.

Only a small number of fine, branching fibres of cemental
origin could be seen.

TABLE 6:

Distribution of oxytalan fibres in the wallaby (aldehyde fuchsin staining technique).

	OXYTALAN	ELASTIC FIBRES
Wallaby molars-		
(1) periodontal ligament		
mesial (C/3, M/3, A/3.)	+ , ++ , +++.	(+), 0 , 0 .
distal (C/3, M/3, A/3.)	++ , +++, +++.	+ , (+), 0 .
buccal (C/3, M/3, A/3.)	+ , ++ , ++ .	(+), 0 , 0 .
lingual (C/3, M/3, A/3.)	+ , ++ , ++ .	(+), 0 , 0 .
apex	++	0
bifurcation	+	0
(2) supra-alveolar region		
crest	+	++
sulcus	+	+
transseptal	++	(+)
(3) pulp**	(+)	0
(4) follicle of developing tooth	0	0
(5) periodontal ligament of developing tooth	(+)	0
(6) erupting teeth		
mesial (C/3, M/3, A/3.)	++ , +++, +++.	0 , 0 , 0 .
distal (C/3, M/3, A/3.)	++ , +++, +++.	0 , 0 , 0 .
bifurcation	+	0
apex	++	0
above C.E.J.	+	0

** = few oxytalan fibres are present.

TABLE 6:   (continued)

	OXYTALAN	ELASTIC FIBRES
Wallaby incisor -		
(1) periodontal ligament		
superior (C/3, M/3, A/3.)	++ , ++ , +++.	+ , (+), 0 .
inferior (C/3, M/3, A/3.)	++ , ++ , +++.	+ , (+), 0 .
mesial (C/3, M/3, A/3.)	++ , ++ , ++ .	+ , 0 , 0 .
distal (C/3, M/3, A/3.)	+ , ++ , ++ .	(+), 0 , 0 .
apex	++	0
(2) supra-alveolar region		
crest	+	++
sulcus	+	+
(3) pulp**	(+)	0

** = few oxytalan fibres are present.

Note: refer to Appendix V for explanation of the symbols.

wallaby molars
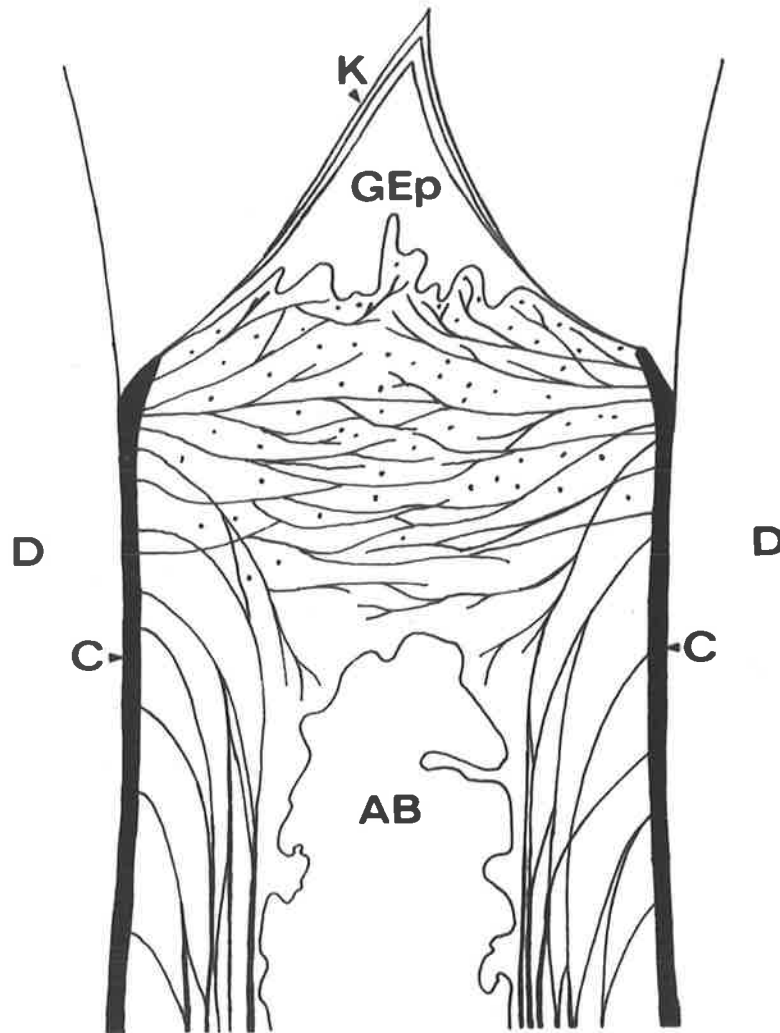
Oxytalan fibre distribution

-in mesio-distal section

K = keratin
GEp = gingival
        epithelium
C = cementum
D = dentine
AB = alveolar bone

diagram. 12.
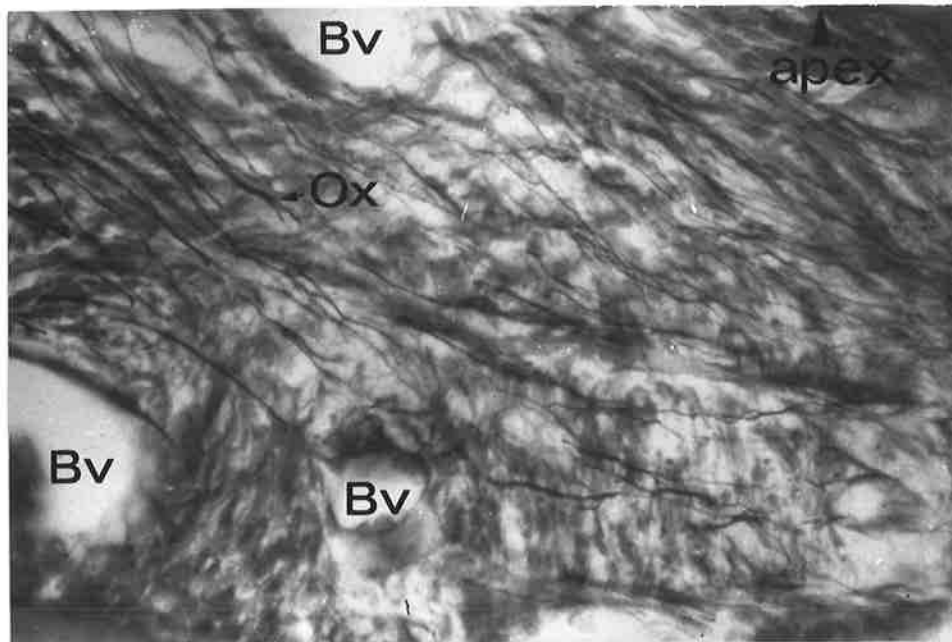
Fig. 26.   Network of fine, branching oxytalan fibres (Ox) passing
           beneath the apex of a wallaby molar. At the apical
           foramen of the tooth, there is a large amount of
           diffusely staining amorphous material. Blood vessels (Bv).
           (Oxone-aldehyde fuchsin. x250).

Fig. 27.

Fine, branching oxytalan
fibres and thick, oxytalan
tracts (OxT) in the
periodontal ligament of an
erupting wallaby molar.
Cementum (C). The finer
oxytalan fibres form what
appears to be a plexus
adjacent to the cemental
surface. (Oxone-aldehyde
fuchsin-Halmi, x250).
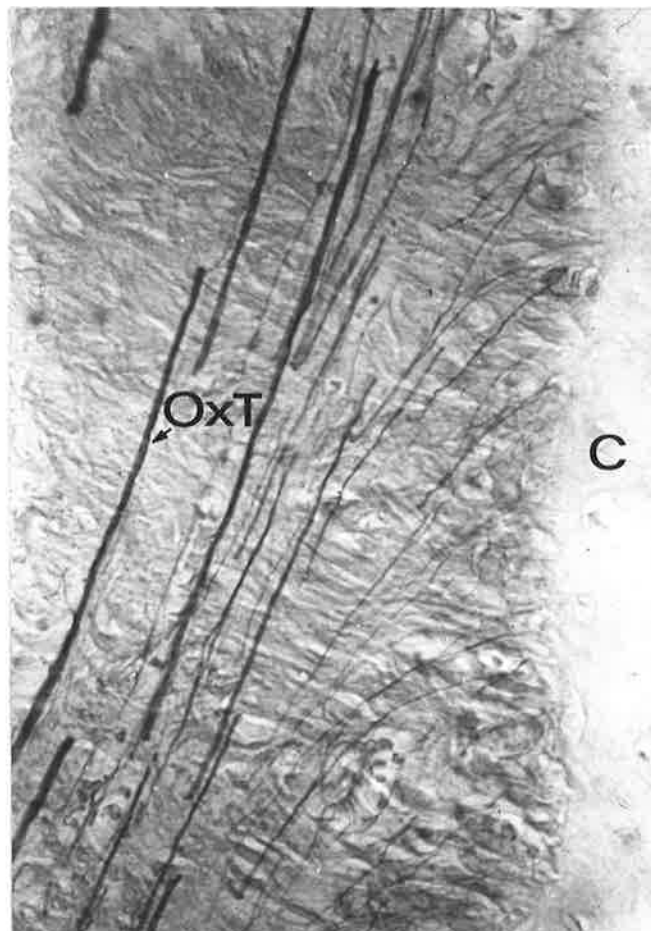
<u>pulp</u>.

Occasionally, an oxytalan-like fibre appeared to be present in the mature pulp tissue.

<u>erupting tooth</u>.

The periodontal ligament contained many oxytalan fibres of cemental origin which cascaded in large numbers toward the apex. There were far fewer oxytalan fibres at the level of the cemento-enamel junction and extending transseptally, than occurred in fully erupted teeth. Within the periodontal ligament there were many ribbon-like fibres which were orientated apico-occlusally but did not appear to branch as frequently as corresponding oxytalan fibres around fully erupted teeth. Numerous fine fibres of cemental insertion were also present (Fig.27.).

Unlike the oxytalan fibre system of fully erupted teeth, the proportions of fibres on the mesial, distal, buccal, and lingual aspects of the periodontal ligament were approximately equal (Table 6.).

5.2.(c)(iii)   <u>possum</u>

The oxytalan fibre distribution was generally similar to the system already described for the wallaby, but with certain modifications.

(1) Fibre proportions - As shown in Table 7, p.124. the inferior and mesial aspects of the incisor periodontal ligament showed a gradual increase in the number of oxytalan fibres from the cervical to apical regions. However, the superior and distal aspects displayed more fibres of varying sizes in the cervical rather than the apical region. The molars showed marginally greater fibre concentrations on the mesial and buccal aspects of the periodontal ligament whereas the wallaby had the greatest oxytalan fibre concentration on the distal aspects of its molars. Both animals had the same oxytalan fibre pattern of cemental attachment, a

TABLE 7:

Distribution of oxytalan fibres in the possum (aldehyde fuchsin staining technique).

	OXYTALAN	ELASTIC FIBRES
Possum molars -		
(1) periodontal ligament		
mesial (C/3, M/3, A/3.)	+ , ++ , ++ .	0 , 0 , 0 .
distal (C/3, M/3, A/3.)	+ , + , ++ .	0 , 0 , 0 .
buccal (C/3, M/3, A/3.)	+ , ++ , ++ .	0 , 0 , 0 .
lingual (C/3, M/3, A/3.)	+ , + , ++ .	0 , 0 , 0 .
apex	+	0
bifurcation	+	0
(2) supra-alveolar region		
crest	+	0
sulcus	+	0
transseptal	(+)	0
(3) pulp**	+	0
(4) circumdental fibres	(+)	0

TABLE 7:   (continued)

	OXYTALAN	ELASTIC FIBRES

Possum incisor -

(1) periodontal ligament

superior (C/3, M/3, A/3.)	+++, + , ++ .	(+), 0 , 0 .
inferior (C/3, M/3, A/3.)	+ , (+), ++ .	0 , 0 , 0 .
mesial (C/3, M/3, A/3.)	+++, + , + .	(+), 0 , 0 .
distal (C/3, M/3, A/3.)	+ , + , + .	0 , 0 , 0 .
apex	++	0

(2) supra-alveolar region

crest	+	+
sulcus	+	0
transseptal	0	0
circumdental fibres	(+)	0

(3) pulp**	+	0

** = small, coiled oxytalan fibres in the immature pulp.

Note: refer to Appendix V for explanation of the symbols.

cascade appearance in the periodontal ligament, and a close
relationship to the blood vessels.

(2) Fibre dimensions - When compared to the wallaby, the possum had
fewer oxytalan fibres of the thick, branching variety. Only a
few large, apico-occlusally aligned fibres were seen in the apical
third regions of the periodontal ligament. The oxytalan fibre
system was continuous around the apex and in the supra-alveolar
regions as a network of very fine, branching fibres.

No definite evidence for oxytalan fibre attachment to alveolar
bone was observed. Small, delicate oxytalan fibres were found in
the incisor apical pulp tissue amidst a moderately dense staining
background of mucopolysaccharide (Fig.28.).

5.2.(c)(iv) marsupial mice

There were no significant differences between the oxytalan
fibre patterns of S. Crassicaudata and A. flavipes.

periodontal ligament.

From their cemental attachment, the oxytalan fibres swept
out into the periodontal ligament and passed apically (Fig.29.). The
oxytalan fibres were much smaller in diameter than those in the wombat,
wallaby, or possum. Apart from that difference, the basic fibre pattern
was quite similar to the other experimental animals. Fine, branching
fibres could be seen nearer the cementum than the alveolar bone. Some
oxytalan fibres intertwined with the principal collagen bundles, but
the majority were oblique to the collagen system. Larger, less
frequently branching oxytalan fibres were seen further toward the
alveolar bone side of the periodontal ligament. The oxytalan fibres
appeared closely related to the periodontal blood vessels (Fig.30.).
Large fibres could be observed to divide and send branches directly
toward a blood vessel.

No definite attachment of oxytalan fibres into alveolar bone

Fig. 28.

Sagittal section of possum incisor apex. Oxytalan fibres (Ox) can be seen passing beneath the apex. Small, delicate oxytalan fibres (Ox) can be found within the immature apical pulp tissue with diffusely staining amorphous material. (Oxone-aldehyde fuchsin. x250).
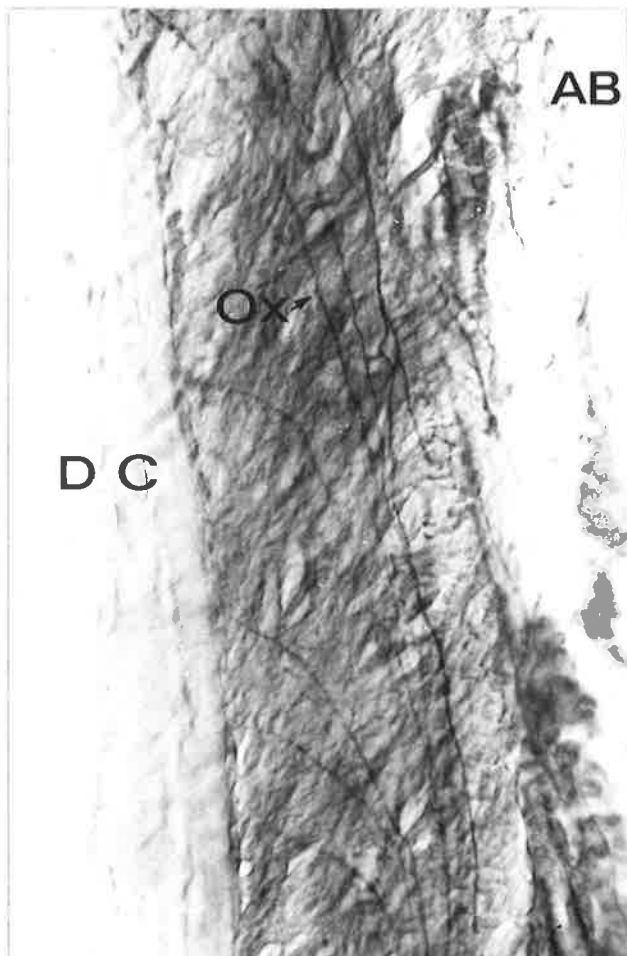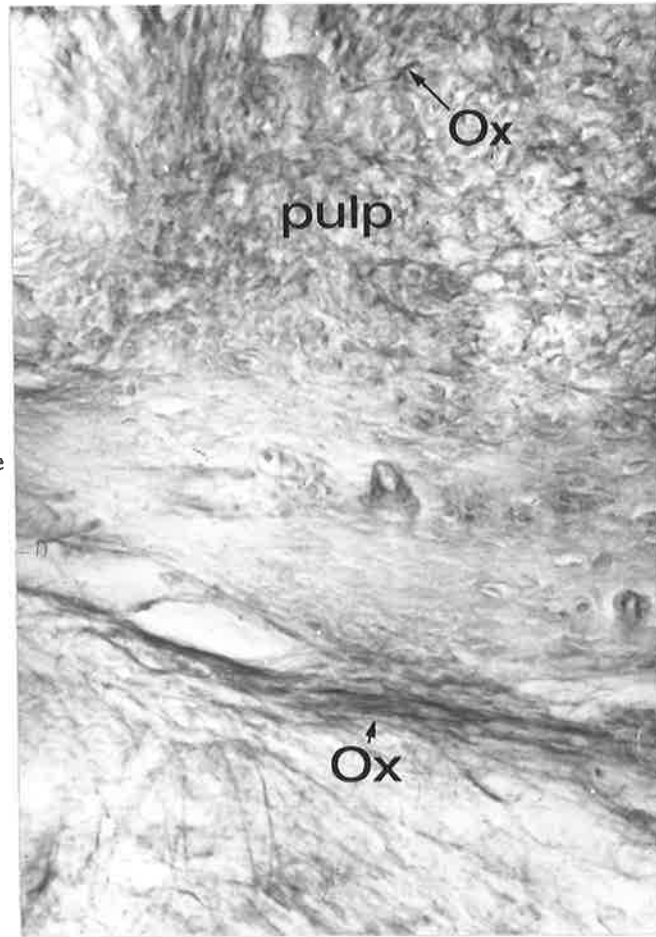
Fig. 29.

Sagittal section of a molar from A. flavipes. Oxytalan fibres (Ox) emerge from the cementum (C) and cascade into the periodontal ligament. Alveolar bone (AB). Dentine (D). (Oxone-aldehyde fuchsin-light green. x400).
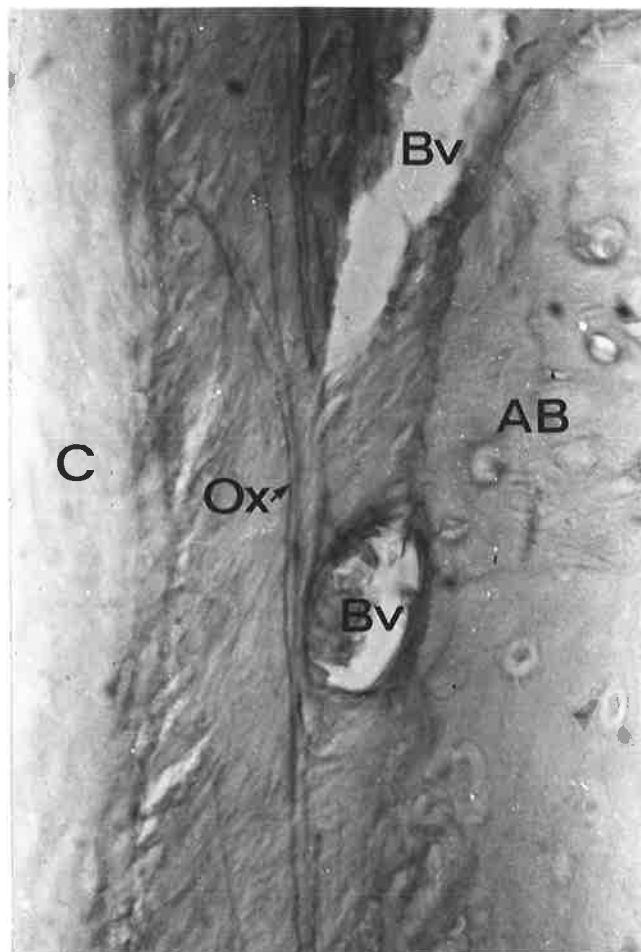
Fig. 30. Sagittal section of a molar from S. crassicaudata.
Oxytalan fibres (Ox) are closely associated with the
periodontal blood vessels (Bv). Cementum (C).
Alveolar bone (AB). (Oxone-aldehyde fuchsin. x400).

could be verified.

The total number of oxytalan fibres and the frequency of large, ribbon-like, apico-occlusally directed fibres increased from the cervical to the apical region of the periodontal ligament (Table 8, p.130.). There appeared to be more of both the fine, branching fibres and the thicker, less branching oxytalan fibres located on the mesial and buccal aspects than the distal or lingual aspects of the periodontal ligament.

supra-alveolar region.

Oxytalan fibres of minute diameter arose from cementum and, intertwining with the collagen fibre bundles, passed transseptally toward the gingival crest and into the corium of the buccal and lingual attached gingiva. A plexus of fine, branching oxytalan fibres could be seen near the basement membranes of the sulcular and gingival crest epithelium. Some oxytalan fibres appeared to travel bucco-lingually, particularly interproximally in the region of the intra-papillary collagen fibres. Contrary to the other animals studied, the marsupial mice occasionally showed evidence of an arcade of oxytalan fibres which passed over the interdental alveolar crest and connected the periodontal ligaments of adjacent teeth (Diag.13.).

apex.

A meshwork of extremely fine, small-diameter oxytalan fibres passed completely around the root apices. No oxytalan fibres could be seen entering the apical foramen.

pulp.

Definite oxytalan fibres were not seen with great frequency within the pulp tissue.

5.2.(d)   Summary of the arrangement of oxytalan fibres.

Except for the specific differences mentioned above, the oxytalan fibre system was quite similar for all of the animals examined.

TABLE 8:

Distribution of oxytalan fibres in the marsupial mice (aldehyde fuschin staining technique).

	OXYTALAN	ELASTIC FIBRES
S. crassicaudata –		
(1) periodontal ligament		
mesial (C/3, M/3, A/3.)	+ , + , + .	0 , 0 , 0 .
distal (C/3, M/3, A/3.)	(+), + , + .	0 , 0 , 0 .
buccal (C/3, M/3, A/3.)	+ , + , + .	0 , 0 , 0 .
lingual (C/3, M/3, A/3.)	+ , + , + .	0 , 0 , 0 .
apex	+	0
bifurcation	(+)	0
(2) supra-alveolar region		
crest	(+)	(+)
sulcus	(+)	0
transseptal	(+)	0
(3) pulp	0	0

TABLE 8:   (continued)

	OXYTALAN	ELASTIC FIBRES
A. flavipes –		
(1) periodontal ligament		
mesial (C/3, M/3, A/3.)	++ , ++ , ++ .	0 , 0 , 0 .
distal (C/3, M/3, A/3.)	+ , + , ++ .	0 , 0 , 0 .
buccal (C/3, M/3, A/3.)	++ , ++ , ++ .	0 , 0 , 0 .
lingual (C/3, M/3, A/3.)	+ , ++ , + .	0 , 0 , 0 .
apex	+	0
bifurcation	(+)	0
(2) supra-alveolar region		
crest	+	(+)
sulcus	(+)	0
transseptal	+	0
(3) pulp	0	0

Note: refer to Appendix V for explanation of the symbols.

marsupial mouse molars
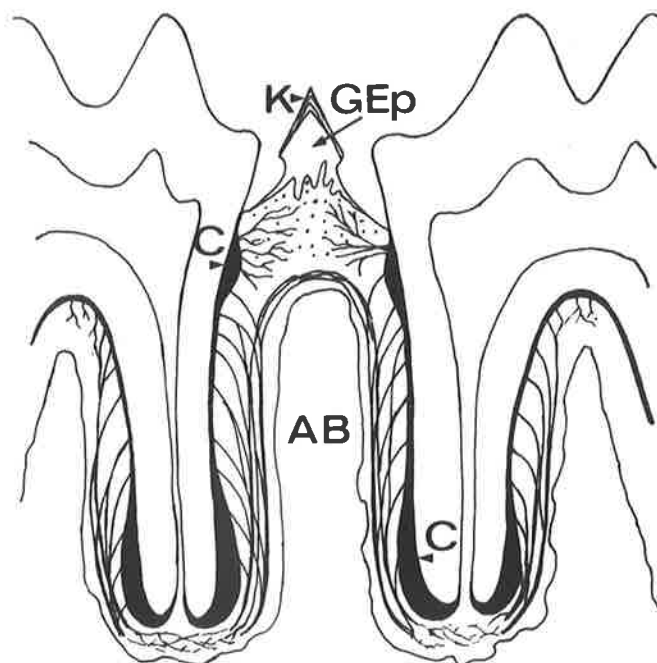
Oxytalan fibre distribution

-in mesio-distal section

K = keratin
GEp = gingival
         epithelium
C = cementum
AB = alveolar
        bone

diagram. 13.

The common features of

(1)   cemental attachment,

(2)   distinctive and unique appearance in the periodontium,

(3)   close relationship with periodontal blood vessels, and

(4)   continuity of the oxytalan fibres as a definite system
      around and between groups of teeth,

were consistently observed. The oxytalan fibre system appeared to
comprise two fibre types. Firstly, fine, branching fibres in the
supra-alveolar regions and periodontal ligament. Secondly, the
appearance of thick, ribbon-like fibres confined to the periodontal
ligament in all animals except the wombat. Differences did exist
regarding numbers and sizes of oxytalan fibres on a regional and species
basis. However, the general pattern was as follows:-

5.2.(d)(i)   cemental attachment.

        Oxytalan fibres could be traced for the entire thickness of
the cemental layer (Fig.31.). The fibres occasionally branched within
the cementum but on emergence from the cemental surface, they frequently
branched and divided. Generally, the number and diameter of the
emerging fibres increased toward the root apex.

5.2.(d)(ii)   periodontal ligament.

        Once the oxytalan fibres emerged from cementum, they usually
curved out into the periodontal ligament and arced inferiorly toward
the root apex. The oxytalan fibres branched at differing distances
from the cemental surface and either ramified with the plexus of fine,
branching oxytalan fibres found near the cementum (Fig.32.) or
contributed to the thicker fibre aggregates as they passed apically.
However, some small-diameter oxytalan fibres intermingled and travelled
parallel with the principal collagen bundles for varying distances.
Attachment of oxytalan fibres to alveolar bone was not evident. All

Fig. 31.

Photomicrograph showing
oxytalan fibres (Ox)
embedded in cementum (C)
and emerging into the
periodontal ligament.
Sagittal section of a
wallaby molar.
(Oxone-aldehyde fuchsin-
Halmi. x250).
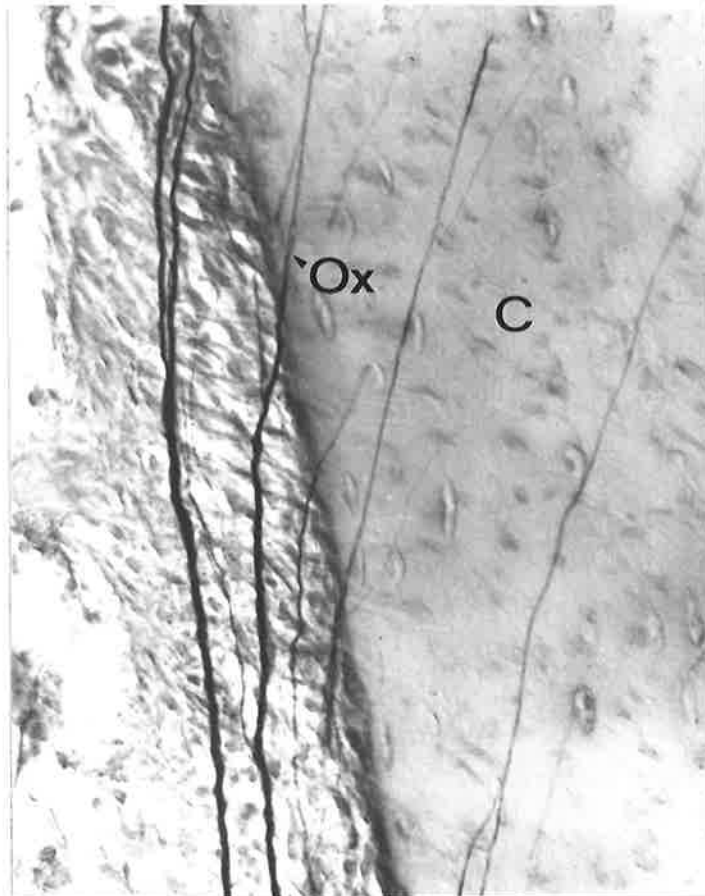
Fig. 32. Fine meshwork of branching oxytalan fibres near the
         cemental surface of a wallaby molar. The cementum (C)
         lies near the top left hand corner of the photograph.
         (Oxone-aldehyde fuchsin. x400).

animals used in this study possessed thick, ribbon-like oxytalan fibres
which were aligned apico-occlusally (Figs.33,34.). High power (x1000)
magnification of these thick "fibres" indicated that they were the
result of aggregation and coalescence of a number of individual,
smaller diameter fibres (Fig.35.).

5.2.(d)(iii) supra-alveolar region.

Usually, the oxytalan fibres were fine, branching structures
which intertwined with, and seemed to follow, the free and attached
collagen fibre bundles. No thick oxytalan fibre types were found in
the supra-alveolar regions of the wallaby, possum or marsupial mice.
However, thick fibres were seen in this area of the wombat periodontium,
probably as the result of elevation and then disconnection of the
oxytalan fibres as the tooth continuously erupted.

5.2.(d)(iv) apex.

The oxytalan fibre system continued around the root apices as
a fine, branching network (Diag.14.).

5.2.(d)(v) pulp.

Where the root apices were still forming and the pulp tissue
was relatively undifferentiated, oxytalan fibres could be found in
association with large amounts of mucopolysaccharide staining material
dispersed through the pulp tissue. Definite oxytalan fibres could not
be seen within the more mature pulp tissues.

5.2.(d)(vi) blood vessels.

It was regularly observed that oxytalan fibres seemed to bear
a very close relationship with the periodontal blood vessels (Figs.17,
18,22,23,30.). Oxytalan fibres could be seen by-passing blood vessels
(Fig.36.), sending branches toward them (Figs.37,38.), and appearing
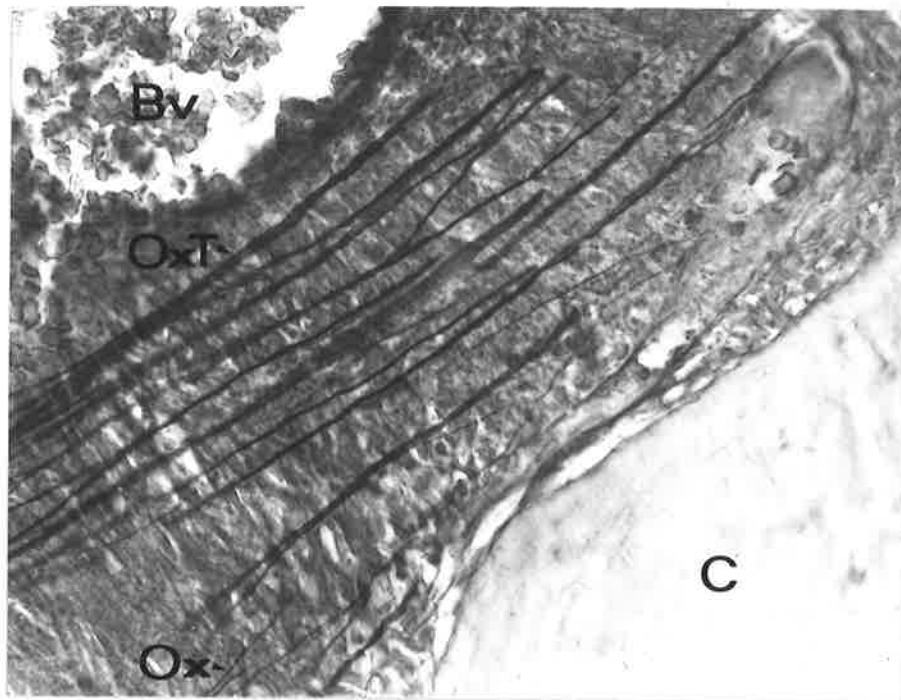
Fig. 33. Oxytalan fibres (Ox) and fibre tracts (OxT) on the distal
aspect in the apical third of a wallaby molar periodontal
ligament. The oxytalan structures frequently branch.
Cementum (C). Blood vessel (Bv). Sagittal section.
(Oxone-aldehyde fuchsin. x250).
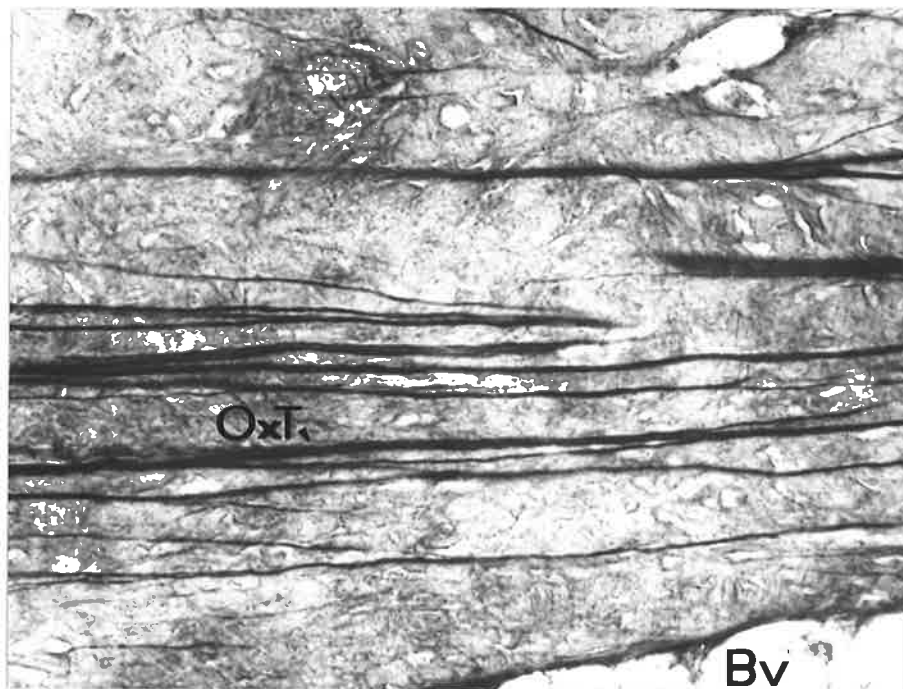
Fig. 34. Thick, branching oxytalan fibre tracts (OxT). The tracts
(or groups of oxytalan fibres) appear to intercommunicate.
Periodontal blood vessel (Bv). Sagittal section of the
apical third region of a wallaby incisor. (Oxone-aldehyde
fuchsin. x400).
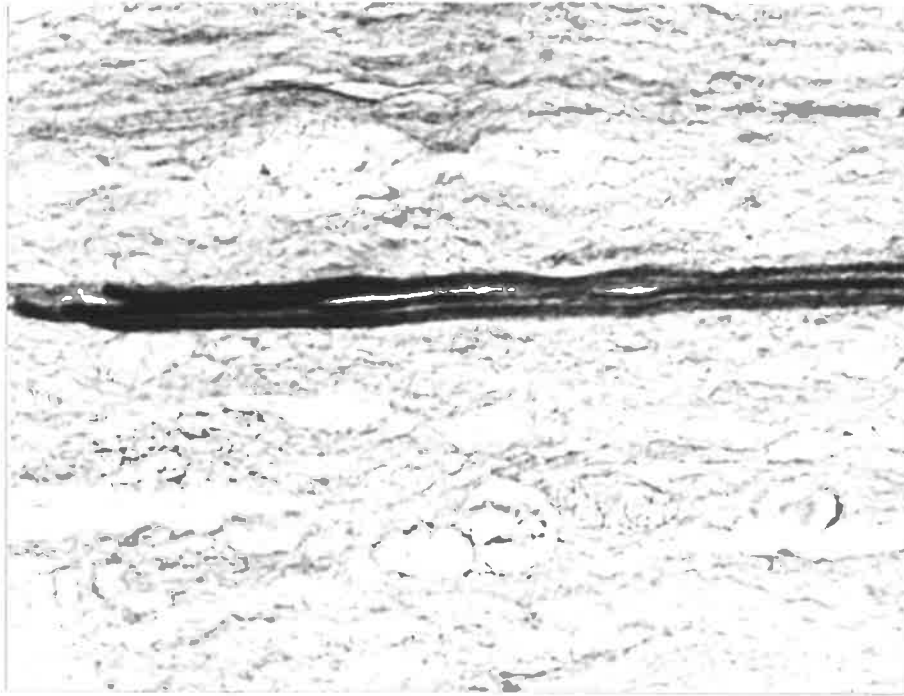
Fig. 35. Oil immersion photomicrograph of a thick oxytalan fibre
in the incisor periodontal ligament of the wombat. At
lower magnification, this bundle of fibres appears as a
single, ribbon-like fibre. The curled ends of the fibres
indicates elastic recoil. (Oxone-aldehyde fuchsin. x1000).
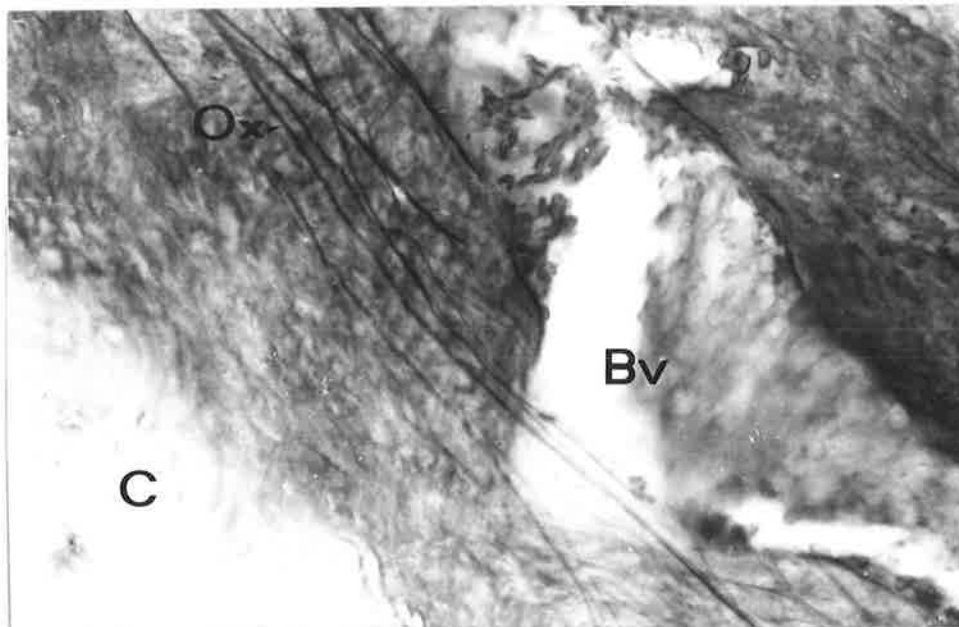
Fig. 36. Oxytalan fibres (Ox) closely associated with a blood
vessel (Bv) in the wallaby periodontal ligament. Some
fibres bypass the vessel. Cementum (C). Sagittal section
at the middle third level of the incisor. (Oxone-aldehyde
fuchsin-Halmi. x250).

Fig. 37.

Sagittal section of a
wombat molar periodontal
ligament. Oxytalan
fibres are located very
close to the walls of the
blood vessels (Bv).
(Oxone-aldehyde fuchsin.
x250).
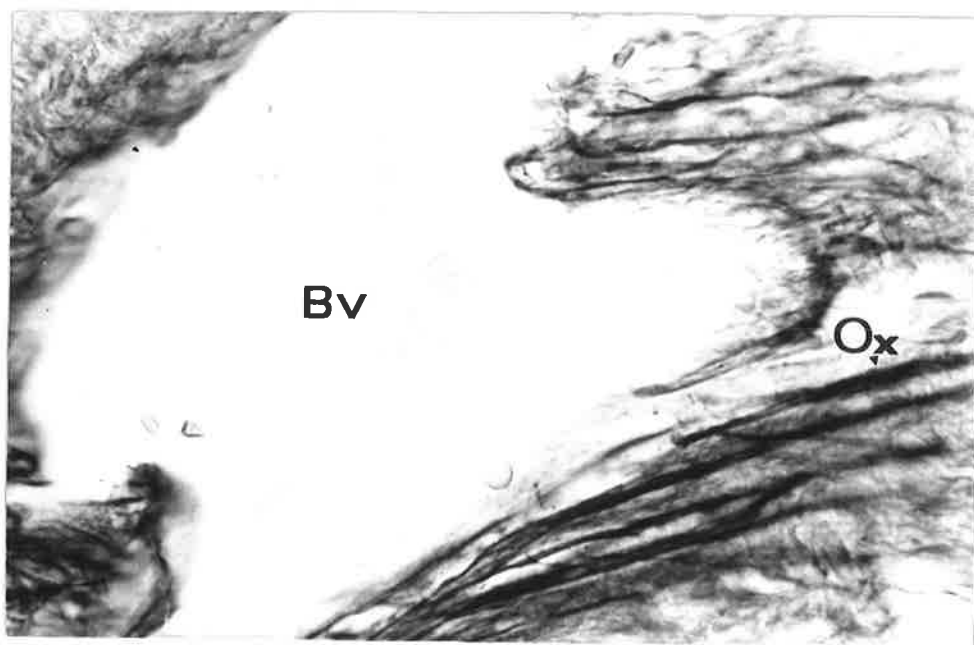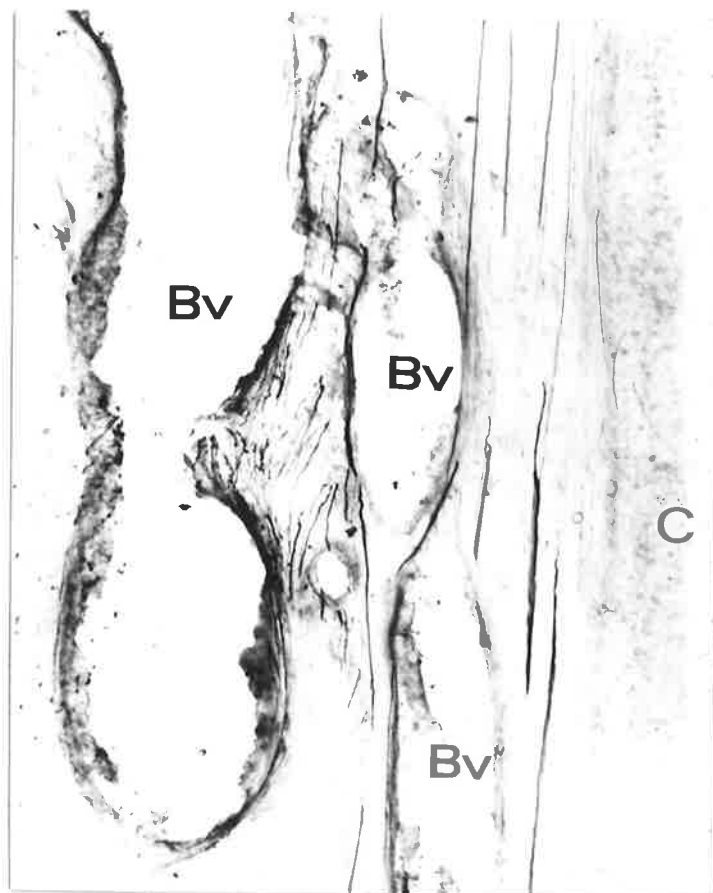
Fig. 38. Oxytalan fibres (Ox) closely associated with the wall of
a blood vessel (Bv) in the wallaby incisor periodontal
ligament. The fibres are branches from larger diameter
oxytalan fibres not seen in this view. (Oxone-aldehyde
fuchsin. x400).
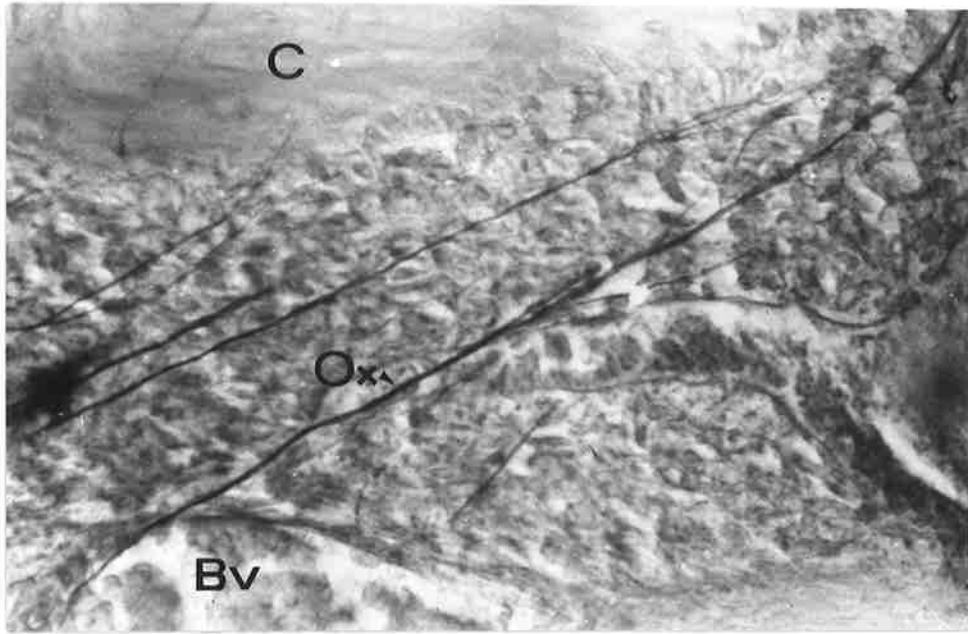
Fig. 39. Sagittal section at the apical third level of a wallaby
periodontal ligament. Oxytalan fibres (Ox) emerge from
cementum (C) and can be seen heading directly toward a
blood vessel (Bv). (Oxone-aldehyde fuchsin. x250).
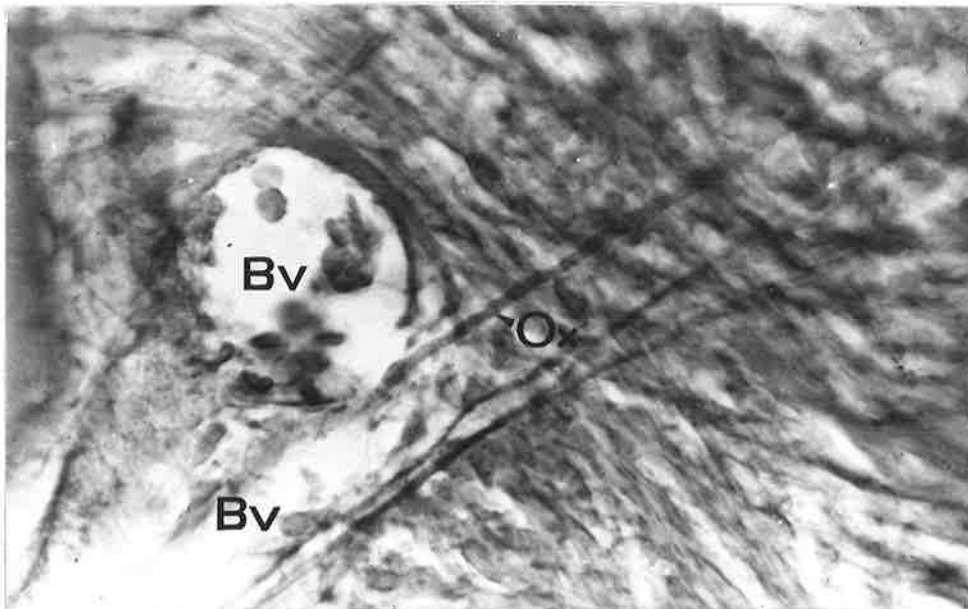
Fig. 40. Oxytalan fibres (Ox) orientated toward blood vessels (Bv).
Sagittal section of the distal aspect of a wallaby molar
at the middle third level. (Oxone-resorcin fuchsin. x400).
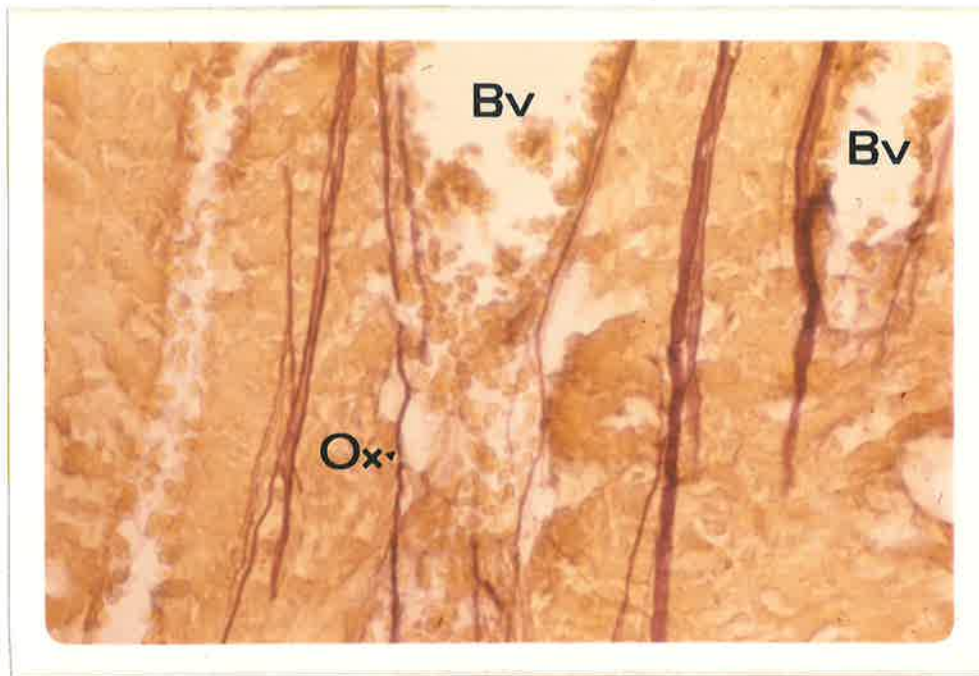
Fig. 41.   Sagittal section of wombat periodontal ligament
illustrating the usual finding of a close relationship
between the oxytalan fibres (Ox) and the blood vessels
(Bv).   (Oxone-aldehyde fuchsin-Halmi. x250).
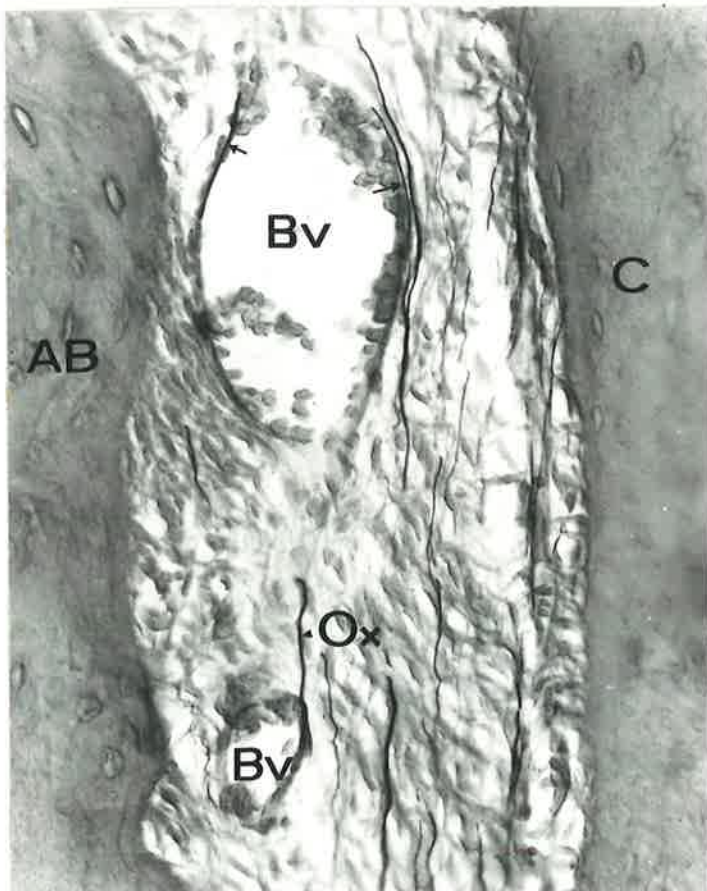
Fig. 42.

Sagittal section of a
wallaby periodontal ligament.
Oxytalan fibres (Ox) are
closely applied to the walls
of the periodontal blood
vessels (Bv).   Branches of
the oxytalan fibres appear
to become an integral part
of the vessel perimeter
(arrows).   Cementum (C).
Alveolar bone (AB).
(Oxone-aldehyde fuchsin.
x250).
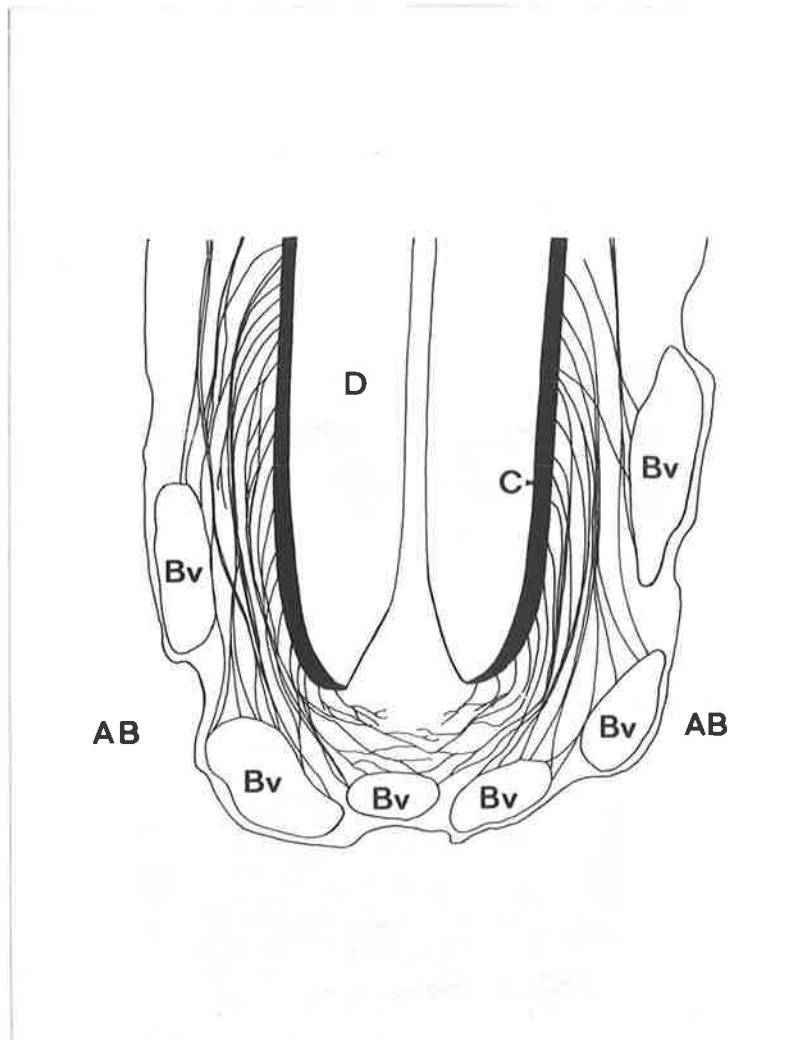
Diag. 14. Schematic representation of oxytalan fibre
distribution at the apex of incisor and molar teeth.
The large, ribbon-like oxytalan fibre tracts break
down and contribute to the plexus of very fine
diameter fibres passing around the apex. The oxytalan
fibres are closely associated with the walls of blood
vessels (Bv). In oxidized sections, there are
considerable amounts of mucopolysaccharide-staining
material in the region of the apical foramen.
Alveolar bone (AB). Dentine (D). Cementum (C).

to travel directly to a particular vessel (Figs.39,40.). Oxytalan

fibres appeared to be associated with the walls of the blood vessels

(Figs.41,42.) although the relationship to the fine structure of the

wall could not be ascertained by normal histologic methods. Realization

of this fact provided the stimulus for an attempt to reconstruct the

three-dimensional course of individual oxytalan fibres through the

periodontal ligament.

Oxytalan fibres could be observed accompanying blood vessels

into alveolar bone (Fig.23.) but no direct attachment to bone was

found. Oxytalan fibres were only located close to the alveolar bone

when blood vessels approximated the alveolar wall. At the root apex,

where the large oxytalan fibres broke up into a delicate, branching

network passing beneath the apex, the fibres were intimately associated

with the blood vessels (Diag.14.).

5.3. Enzyme Reactions:

The independent examiners recorded remarkably similar

observations which have been compiled as a composite finding in Table 9,

p.143. The results were not quantitative but qualitatively indicated

enzyme activity by the histologic appearance of the test tissue sections.

The staining reactions of oxytalan, elastic and collagen fibres were

used as criteria of the enzyme activity.

5.3.(a) Oxytalan.

The enzymes β-glucuronidase, bromelain, elastase, collagenase

and neuraminidase definitely decreased or eliminated aldehyde fuchsin

staining for oxytalan fibres but only when preceded by ninety minutes

of oxidation with Oxone. The enzyme pepsin produced a slight, but

observable, reduction in the oxytalan staining properties when applied

either before or after the oxidation stage. Hyaluronidase produced an

TABLE 9:

Effects of enzyme digestions as determined by staining with aldehyde fuchsin.

ENZYME	OXYTALAN	ELASTIC FIBRES	COLLAGEN
(1) β-GLUCURONIDASE			
Oxone-β-GLUCURONIDASE	much reduced	normal	normal
Oxone-control	normal	normal	normal
β-GLUCURONIDASE-Oxone	normal	normal	normal
control-Oxone	normal	normal	normal
(2) COLLAGENASE			
Oxone-COLLAGENASE	moderate reduction	normal	slightly reduced
Oxone-control	normal	normal	normal
COLLAGENASE-Oxone	normal	normal	normal
Control-Oxone	normal	normal	normal
(3) PEPSIN			
Oxone-PEPSIN	slight reduction	normal	moderate reduction
Oxone-control	normal	normal	normal
PEPSIN-Oxone	slight reduction	normal	moderate reduction
control-Oxone	normal	normal	normal
(4) HYALURONIDASE			
Oxone-HYALURONIDASE	normal*	normal	normal
Oxone-control	normal	normal	normal
HYALURONIDASE-Oxone	normal	normal	normal
Control-Oxone	normal	normal	normal

    * a very slight reduction at 3 hours incubation.

(5) BROMELAIN			
Oxone-BROMELAIN	eliminated	eliminated	normal
Oxone-control	normal	normal	normal
BROMELAIN-Oxone	normal	normal	normal
control-Oxone	normal	normal	normal

TABLE 9:   (continued)

ENZYME	OXYTALAN	ELASTIC FIBRES	COLLAGEN
(6) NEURAMINIDASE			
Oxone–NEURAMINIDASE	slight reduction	normal	normal
Oxone–control	normal	normal	normal
NEURAMINIDASE–Oxone	normal	normal	normal
Control–Oxone	normal	normal	normal
(7) ELASTASE			
Oxone–ELASTASE	absent	greatly reduced	normal
Oxone–buffer	normal	normal	normal
ELASTASE–Oxone	normal	reduced	normal
Buffer–Oxone	normal	normal	normal

N.B.   (1) In all elastase treated sections, the epithelium was destroyed, with the exception of the keratinized surface layer. Of particular interest was the finding of a similar surface layer remnant where the crevicular epithelium was removed – this could possibly represent either a keratinized sulcus or dental cuticle.

(2) Only formalin-fixed tissue was used.

(3) Collagen disruption was determined by morphologic appearance (e.g. vacuolation and subjective assessment of fibre density).

indefinite effect after three hours incubation of the pre-oxidized tissues.

Normal, typical oxytalan staining occurred for all control (buffer minus enzyme) sections.

5.3.(b) Elastic Fibres.

Elastase reduced aldehyde fuchsin staining of elastic tissue whether applied before or after the oxidation stage. However, the reduction in staining was greater when oxidation preceded the enzyme application. Bromelain was the other enzyme which was noted to affect elastic fibre staining. Bromelain completely eliminated all elastic tissue staining following pre-oxidation.

Normal staining reactions occurred for all control sections.

5.3.(c) Collagen.

The enzyme effects were subjectively assessed on the basis of morphologic appearance (e.g. disruption, vacuolization, disintegration), and fibre density in the periodontal ligaments of the tissue sections studied. Pepsin produced observable disruption of collagen fibres both before and after oxidation. Collagenase appeared to slightly disrupt the collagen system in the wallaby sections when the tissues were pre-oxidized.

All other enzymes and control solutions produced negligable effects upon collagen.

5.3.(d) Other tissues.

In all elastase treated sections, the epithelium was totally destroyed with exception of the stratum corneum. Of particular interest was the finding of a remnant similar to the stratum corneum in the gingival sulcus where the sulcular epithelium was removed. This remnant might indicate the presence of a lightly keratinized sulcus or the

presence of a dental cuticle.

5.4. Investigation of Dye Properties:

5.4.(a) Dyes.

The 55 dye substances investigated are listed in Table 10,
p.147.   Included is a classification of the dye types (chemical family)
and the acidic or basic behaviour of the dye chromophore (from Lillie
1969b). All oxytalan-positive dyes, except chlorazol black E and
orcein, are basic dyes.

As outlined in Chapter 4.2.(e)(i), the dyes were made into
staining solutions and applied to serial oxidized and non-oxidized
control tissue sections. From this mass screening, the dyes were
separated into those which stained oxytalan-like and elastic-like
fibres (Table 11, p.149.). It was considered that the only useful dyes
were those which positively reacted with structures having morphologic
and geographic similarities to the oxytalan (and elastic) fibres
demonstrated by aldehyde fuchsin, orcein, and the resorcin fuchsin
techniques.

Table 12, p.150. summarizes some of the conditions under
which the sections were manipulated to observe the results of the stain-
ing reactions. In particular, oxytalan staining was reduced, or lost,
in alcohol for dyes such as astra blue, azure A, celestin blue B, cresyl
fast violet (and acetate), crystal violet, methyl violet 6B, neutral
red, nile blue, thionin and toluidine blue. This observation might
indicate the possible degrees of specificity and/or the type of bonding
between the dyes and the chromophilic moieties of the oxytalan and
elastic fibres.

Furthermore, relative differences existed between the
different dyes regarding their ability to clearly define oxytalan-like
fibres (Table 11, p.149.). For example, aldehyde fuchsin produced

TABLE 10:

List of dyes investigated.

DYE	TYPE	
Acid fuchsin	TRIAMINOTRIPHENYLMETHANE	acidic
Alcian blue 8GX	PTHALOCYANINE	basic
Alcian green 2GX	PTHALOCYANINE	basic
Alcian yellow GX	PTHALOCYANINE	basic
Amido black 10B	DISAZO	acidic
Aniline blue W.S.	TRIAMINOTRIPHENYLMETHANE	acidic
Anthracene blue W.R.	HYDROXYANTHRAQUINONE	acidic
Astra blue	TRIARYLMETHANE	basic
Azure A	QUINONE-IMINE (THIAZIN)	basic
Basic fuchsin	TRIAMINOTRIPHENYLMETHANE	basic
Benzo fast pink 2BL (direct red 75)	DISAZO	acidic
Biebrich scarlet	DISAZO	acidic
Bismarck brown Y	DISAZO	basic
Celestin blue B	QUINONE-IMINE (OXAZIN)	basic
Chlorazol black E	TRISAZO	acidic
Congo red	DISAZO	acidic
Cresyl fast violet	QUINONE-IMINE (OXAZIN)	basic
Cresyl fast violet acetate	QUINONE-IMINE (OXAZIN)	basic
Crystal violet	TRIAMINOTRIPHENYLMETHANE	basic
Eosin	HYDROXY-XANTHENE (FLUORONE)	acidic
Evan's blue	DISAZO	acidic
Fast blue RR	DIAZONIUM	—
Fast green FCF	DIAMINOTRIPHENYLMETHANE	acidic
Fluorescein	HYDROXYXANTHENE	acidic
Giemsa	MIXTURE (QUINONE-IMINE & FLUORONE)	acidic
Haematoxylin	NATURAL	basic
Light green SF	DIAMINOTRIPHENYLMETHANE	acidic
Lissamine Rhodamine RB200 – phloxine Rhodamine	AMINOXANTHENE + HYDROXYXANTHENE	acidic
Luxol fast blue G	DISAZO (DIARYLGUANIDINE SALT)	basic

TABLE 10: (continued)

DYE	TYPE	
Luxol fast blue MBS	SULPHONATED COPPER PTHALOCYANINE (DIARYLGUANIDINE)	basic
Luxol fast blue ARN	DISAZO(DIARYLGUANIDINE SALT OF SULPHATED AZO)	basic (?)
Metanil yellow	MONOAZO	acidic
Methylene blue (polychrome)	QUINONE-IMINE (THIAZIN)	basic
Methyl blue	TRIAMINOTRIPHENYLMETHANE	acidic
Methyl green	TRIAMINOTRIPHENYLMETHANE	basic
Methyl orange	MONOAZO	weak acid
Methyl red	MONOAZO	weak acid
Methyl violet 6B	TRIAMINOTRIPHENYLMETHANE	basic
Neutral red	QUINONE-IMINE (AZIN)	weak base
Nile blue	QUINONE-IMINE (OXAZIN)	basic
Nitro blue (tetrazolium)	TETRAZOLIUM	–
Oil red O	DISAZO	weak acid
Orcein	NATURAL DYE	weak acid
Orcinol-new fuchsin	NATURAL + ARYLMETHANE	weak acid + strong base
Ponceau 2R	MONOAZO	acidic
Pontamine sky blue 6BX	DISAZO	acidic
Procion yellow M4R	DICHLOROTRIAZINYL	basic
Resorcin fuchsin	ARYLMETHANE	basic
Rhodamine B	XANTHENE (RHODAMINE)	basic
Ruthenium red	MINERAL PIGMENT	–
Sirius red F3BA	POLYAZO	acidic
Sirius supra blue FGL-CF	DISAZO	acidic
Sudan black B	DISAZO	basic
Thionin	QUINONE-IMINE (THIAZIN)	strongly basic
Toluidine blue	QUINONE-IMINE (THIAZIN)	basic dye

N.B. The staining results of this study apply to marsupial
material and one batch of each dye. Some variability
in the staining quality may exist between different
batches of the same dye type.

TABLE 11:

List of dyes found to react positively with oxytalan-like structures.

DYE	WALLABY	WOMBAT	POSSUM	M. MICE both species
Alcian blue 8GX (0.1N HCl)	++	+	(+)	+
Alcian blue 8GX (3% CH$_3$COOH)	++	++	+	++
Alcian green 2GX	+	+	(+)	(+)
Alcian yellow GX	+	+	(+)	(+)
Astra blue	+	+	+	0
Azure A	+	++	+	0
Basic fuchsin (aldehyde fuchsin)	+++	+++	++	++
Basic fuchsin (P.A.S.)	(+)	(+)	0	0
Bismarck brown Y	++	+++	++	++
Celestin blue	+	+	(+)	0
Chlorazol black E	(+)	+	0	0
Cresyl fast violet (0.1N HCl)	+	++	+	0
C.F.V. (0.6% +CH$_3$COOH)	++	++	++	+
C.F.V. acetate (0.1N HCl)	+	+	+	0
Crystal violet	+	+	+	(+)
Methyl violet 6B	+	++	+	+
Neutral red	++	++	++	+
Nile blue	+	++	+	+
Orcein	++	++	+	+
Orcinol-new fuchsin	++	++	+	+
Resorcin fuchsin	+	+	(+)	(+)
Sudan black B	++	++	+	++
Thionin	++	+	+	(+)
Toluidine blue	++	++	++	+

Symbols.

+++ = very good to excellent oxytalan staining.

++ = good.

+ = visible but not all fibres stained.

(+) = weak

0 = no definite fibres revealed.

TABLE 12:

Oxytalan positive dyes, pH values, and manipulations of some dye solutions.

DYE	pH(aqueous)	pH(solutions)	MOUNTANT
Alcian blue 8GX	3.5		
(1) in 0.1N HCL		1.1	Xam (no water rinse)
(2) in 3% CH₃COOH		2.5	Xam (water rinse)
Alcian green 2GX	3.8	1.5	Xam (no water rinse)
Alcian yellow GX	3.9	1.4	Xam (no water rinse)
* Astra blue	3.5	1.7	water or dye solution
* Azure A	4.7	1.6	water or dye solution
Basic fuchsin	6.0		
(1) aldehyde fuchsin		1.4	Xam
(2) P.A.S.		1.4	Xam
Bismarck brown Y	3.4	1.5	Xam
* Celestin blue B	3.3	1.6	water
Chlorazol black E	9.3	8.4	Xam
Cresyl fast violet	4.9		
* (1) in 0.1N HCL		1.2	dye solution
(2) in 0.6% CH₃COOH		3.7	Xam
* Cresyl fast violet acetate	5.0	1.1	dye solution
* Crystal violet	5.3	2.0	dye solution
* Methyl violet 6B	4.6	1.5	water or dye solution
* Neutral red	4.2	4.0	water or dye solution
* Nile blue	4.0	1.8	dye solution
Orcein	7.0	1.6	Xam
Orcinol-new fuchsin	5.5	3.1	Xam (no water rinse)
Resorcin fuchsin	3.9	1.5	Xam
Sudan black B	5.1	1.3	Xam (rapid dehydration)
* Thionin	3.5	1.6	dye solution
* Toluidine blue	4.0	3.7	water or dye solution

* The staining of oxytalan-like fibres rapidly faded when these particular dye solutions were used and the sections were subsequently dehydrated in ethanol. Furthermore, the staining results were not preserved when acetone or methanol were substituted for ethanol. However, these staining solutions did not contain acetic acid - the resistance to ethanol extraction was markedly increased in an acetic acid preparation.

noticeably more diffuse staining of greater numbers of oxytalan fibres than did orcein. When orcein and aldehyde fuchsin staining reactions were compared, the corresponding orcein-positive oxytalan fibres appeared finer and more precisely defined. Resorcin fuchsin and orcinol- new fuchsin appeared to stain fewer fibres more diffusely than either orcein or aldehyde fuchsin. Of the remaining dyes, bismarck brown Y and sudan black B consistently appeared to stain the thick, and fine branching varieties of oxytalan-like fibres with excellent clarity. By contrast, chlorazol black E reacted poorly with oxytalan-like fibres. However, the structures which were stained by chlorazol black E corresponded morphologically to the fine oxytalan fibre types, but these were in far fewer numbers than those displayed by orcein.

Comparison of the staining reactions for the wombat, wallaby, possum and marsupial mice revealed species differences for the same dye and the same staining technique (Table 11, p.149.). The wombat and wallaby produced greatly improved staining reactions for oxytalan-like structures compared with either the possum or the marsupial mice.

The method of preparation of the dye solution appeared to alter the staining reaction. For example, alcian blue as a 0.5% solution in 3% acetic acid produced less diffuse staining of oxytalan-like fibres than alcian blue as a 1% solution in 0.1N hydrochloric acid. Likewise, cresyl fast violet as a 0.6% solution in a 0.25% acetic acid solution created a better defined, more intense, and more resistant oxytalan-like reaction to ethanol extraction than a 0.05% solution of the same dye in 0.1N hydrochloric acid. Furthermore, celestin blue B stained very well in acetic acid solution but stained very poorly in hydrochloric acid solution. The trend for all dyes was to stain better as the concentration of the dye solutions increased until the stage was reached where the background staining became too

intense for useful demonstration of oxytalan or elastic-like fibres. Similarly, the addition of small concentrations of glacial acetic acid to the dye solutions appeared to clarify the staining reaction and increase its permanence.

Another important variable appeared to be the length of application of the dye solution. An increase in the staining time increased the intensity of the staining reaction until a stage was reached when insignificant changes occurred. However, it was apparent that the equilibrium point between time and stain intensity, varied with the type of dye, the composition, and the concentration of the dye solution. For example, orcein required a longer staining time than did aldehyde fuchsin. The most suitable staining time for a particular dye solution was determined by trial and error with routine checks to decide when intensity and contrast of the stain reaction was optimal.

Furthermore, the length of oxidation before application of the dye solution appeared to influence the intensity of the staining reaction. For example, oxytalan fibres in the marsupial mouse periodontal ligament reacted more positively with orcein following two hours of Oxone pre-oxidation compared with one hour of pre-oxidation. The temperature of the staining solution probably influenced the rate of the staining reaction. For example, orcein appeared to produce better staining intensity after 45-60 minutes incubation at 50°C.

For all dyes, the staining reaction for elastic fibres was more precise and of far greater intensity following a period of pre-oxidation with Oxone. However, aldehyde fuchsin staining of oxytalan and elastic fibres was lost if the sections were re-oxidized following the initial oxidation and staining stages.

Excepting sirius red F3BA, ponceau 2R, congo red, luxol fast

blue G (Salthouse 1965), acid fuchsin and Verhoeff's iron haematoxylin, all dyes which stained for elastic fibres also stained for oxytalan-like fibres.

Generally, when oxytalan fibres were compared with elastic fibres, the staining reaction was noticeably more diffuse around the elastic fibres. The elastic fibres appeared more ragged in outline than the oxytalan fibres which had clearly defined perimeters. Both elastic and oxytalan fibres appeared to be composed of an envelope of amorphous staining material surrounding a more densely stained central core. The more diffuse the reaction of a particular dye solution appeared, the less defined the central core became. This staining variability may imply a low specificity of the dye for the central core.

The periodic acid-Schiff technique produced a weak positive reaction in the periodontal ligaments of the wombat and wallaby. The observed structures were faint, rose pink in colour, and strongly resembled oxytalan fibres in appearance and distribution. Oxidation with Oxone was not essential. The P.A.S. reaction occurred in areas of the periodontal ligament where elastic fibres could not be demonstrated by conventional techniques.

Some differences between oxytalan and elastic fibres might be implied by the finding that oxytalan fibres did not pick up the black deposits produced in elastic fibres by the iron-orcein techniques. Furthermore, elastic fibres stained a darker shade of blue than oxytalan fibres when azure A was used.

Appendix VII contains the methods of preparation of the solutions and the staining results for the dyes which reacted positively with oxytalan-like fibres.

5.4.(b) pH Analysis.

The pH values varied for each dye according to the nature

of the solvent. No absolute relationship seemed to exist between pH

and the staining of oxytalan fibres although there did appear to be a

trend favouring pH values on the acid side of neutrality. Perhaps by

coincidence all but one of the staining solutions which proved positive

for oxytalan were within the range of pH 1.1 to pH 4.0. The exception

was chlorazol black E (pH 8.4). However, many of the solutions which

did not stain oxytalan fibres were also within the pH range mentioned

above, particularly those dye solutions prepared as 1% in 0.1N hydro-

chloric acid. Therefore, the pH of the staining solution could be

quite easily misleading.

In aqueous solution, 6 of the 21 oxytalan-positive dyes

(19%) had values greater than pH 5.0. A mean value of pH 4.7 was

obtained for aqueous solutions of oxytalan-positive dyes whilst a mean

value of pH 6.0 resulted from aqueous solutions of the oxytalan-

negative dyes tested.

Although of questionable significance, an average value of

pH 2.3 was obtained for all the oxytalan-positive staining solutions.

Of the dye solutions made from the oxytalan-negative dyes, an average

value of pH 3.6 was obtained.

Table 12, p.150. illustrates the oxytalan-positive dyes,

their pH in aqueous solution, and the pH values of a representative

sample of some of the staining solutions made from them.

5.4.(c)   Spectrophotometric analysis.

Spectral absorption curves were obtained for all dyes and

all staining solutions. Considerable variation existed between the

different dyes regarding the number, magnitude and wavelength of the

absorptive peaks (maxima). For example, fluorescein had one sharp peak

(446-448mμ) in the visible range whereas chlorazol black E had a very

broad absorption over the entire visible range (375-750mμ) (Diag.15.).

diagram. 15.

diagram. 16.

Legend:
- ——— ORCEIN
- ——▲ ORCINOL–NEW FUCHSIN
- ······ ALDEHYDE FUCHSIN
- ——■□ RESORCIN FUCHSIN

Y-axis: ABSORBANCE (2·0, 1·8, 1·6, 1·4, 1·2, 1·0, 0·8, 0·6, 0·4, 0·2, 0·0)

X-axis: WAVELENGTH (mµ) (200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500, 525, 550, 575, 600, 625, 650, 675, 700)
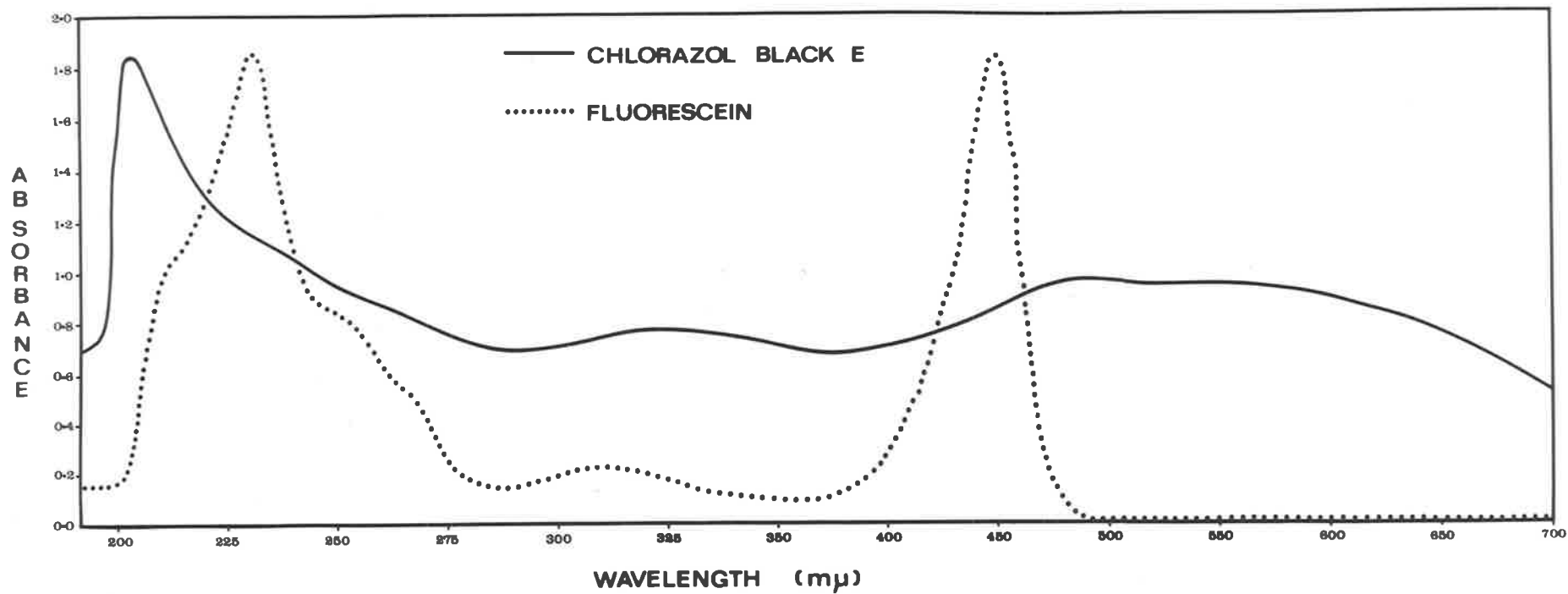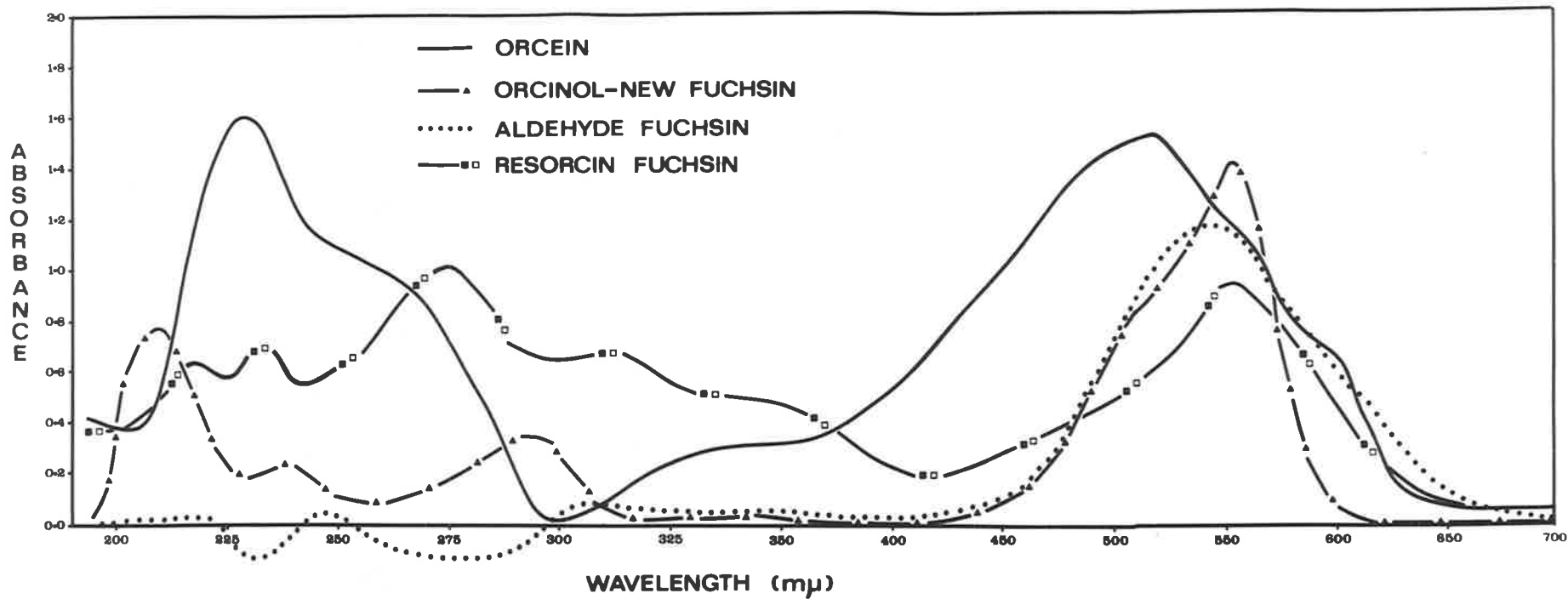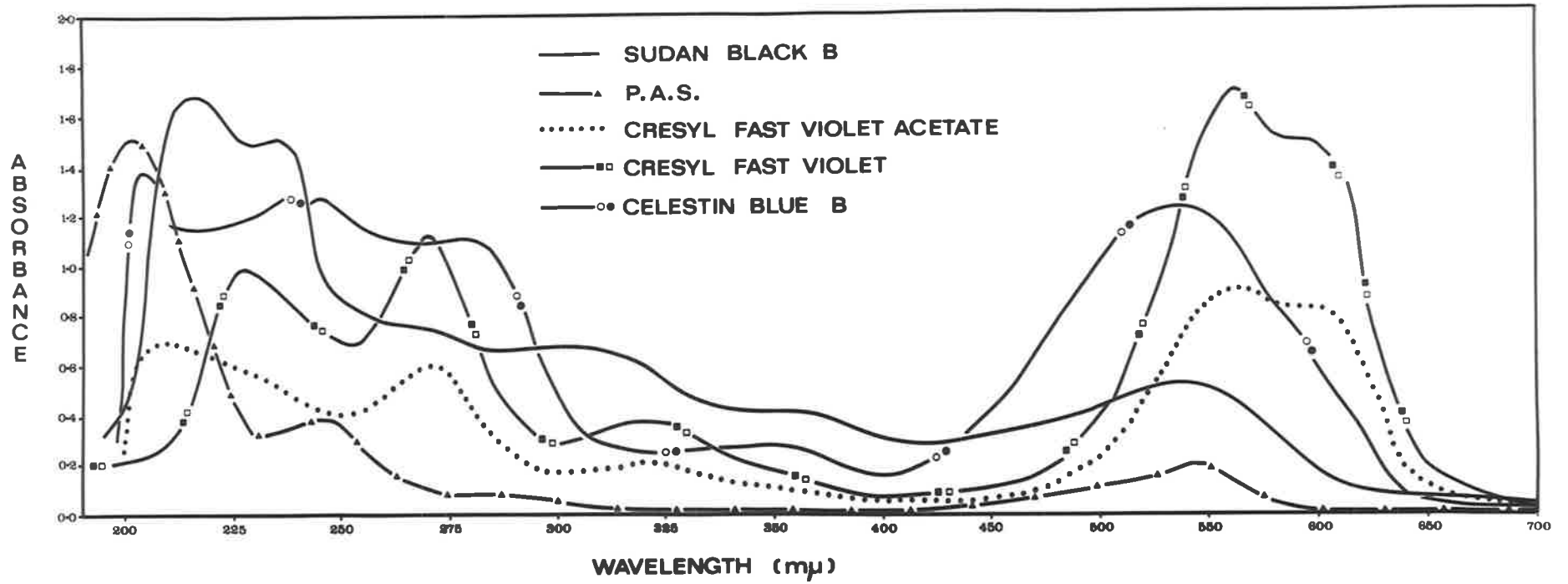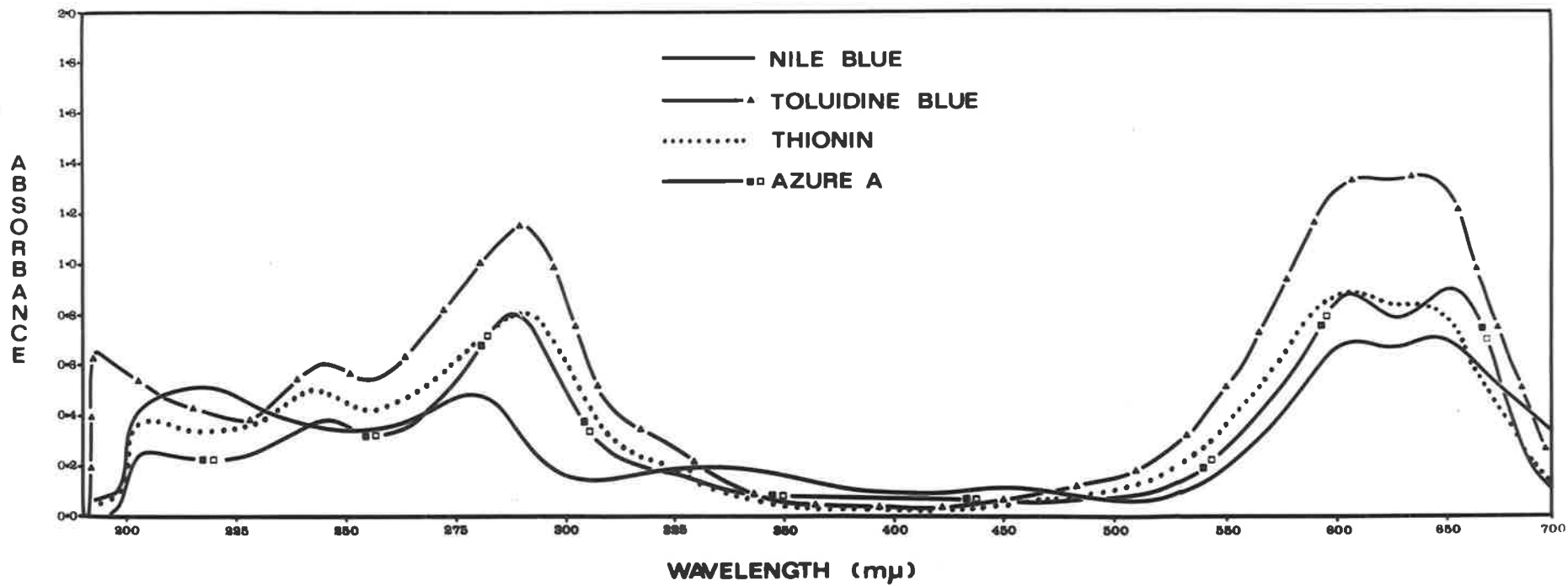
diagram. 17.
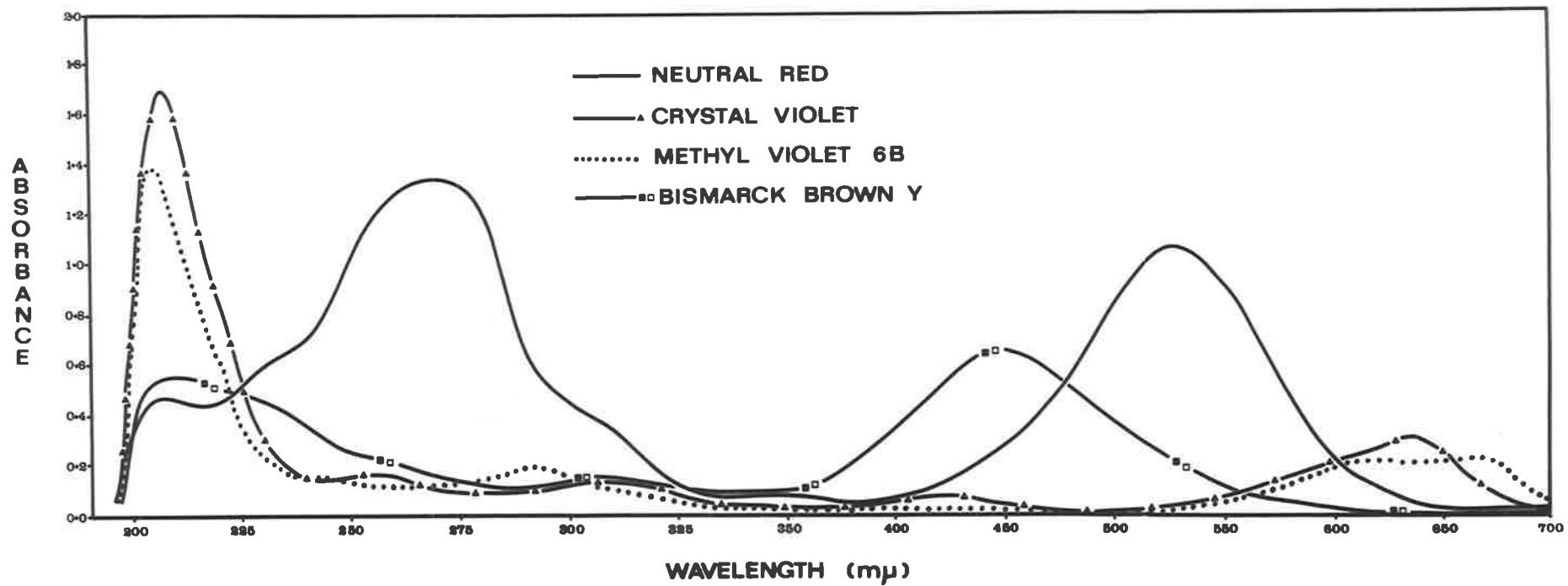
diagram. 18.

diagram. 19.
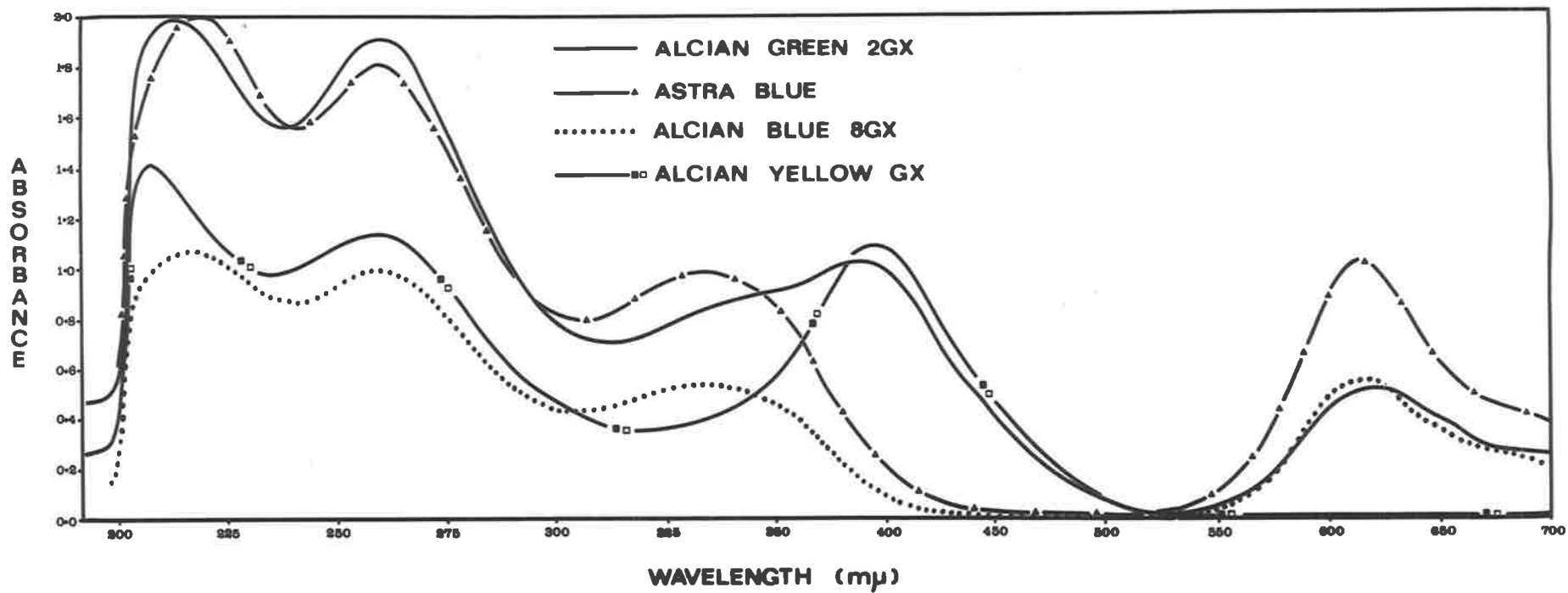
diagram. 20.

The absorption patterns of representative examples of the dye solutions which stained positively for oxytalan-like fibres are shown in Diags. 15,16,17,18,19,20.

Changes in dye concentration produced increases in the absorptance values and emphasized small peaks. However, for the purpose of this study, the concentrations were adjusted for each individual solution to fit all absorption peaks on to a continuous graph over the ultra violet and visible wavelengths.

For any one dye, it was found that variation in the type of solvent (e.g. water, alcohol or dilute acid etc.) could produce a wavelength shift of the absorptive maxima. Variation in the degree and direction of the shift occurred with the different dye solvents.

The values of the absorption peaks of representative solutions of all dye substances used in this study are included in Table 16, p.240. of Appendix VIII.

The collective distribution of the absorptive maxima for the oxytalan-negative dyes are shown in Bar Graph 1, p.162. and for the oxytalan-positive dyes in Bar Graph 2, p.163. Only distinct and separate peaks have been considered and their maximal wavelengths recorded.

(a) Oxytalan-negative dyes: A total of 146 peaks were measured. Of these, 51 occurred within the visible spectrum (35%) and the remaining 95 were within the ultra violet range. The total number of peaks in the visible spectrum represented 54% of the total number of peaks occurring in the ultra violet.

(b) Oxytalan-positive dyes: The total number of peaks measured was 89, of which 37 occurred in the visible spectrum (42%). The remaining 52 peaks were in the ultra violet range. The total number of visible peaks represented 71% of the total number of ultra violet absorption maxima.
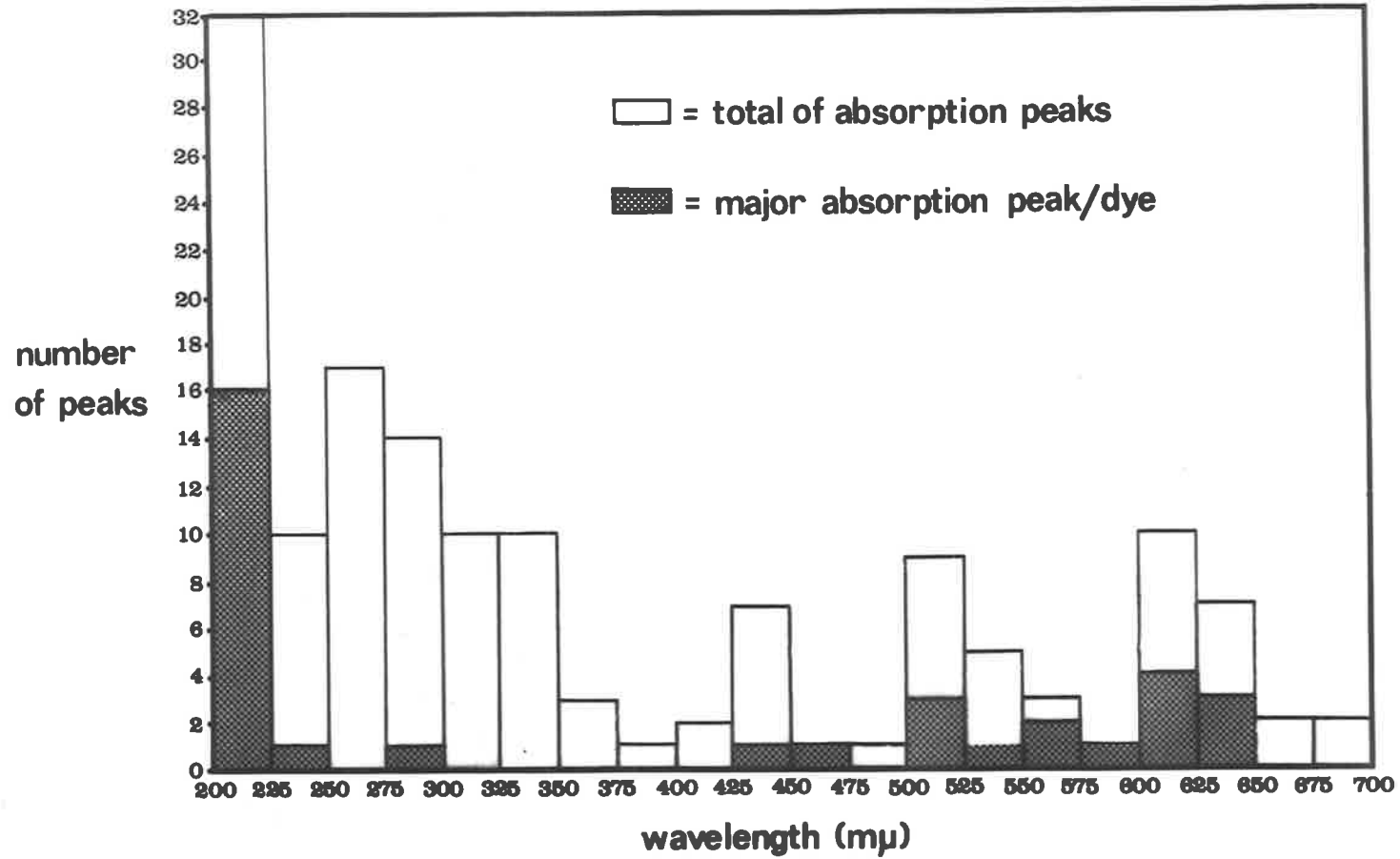
BAR GRAPH. 1.

DYES POSITIVE FOR OXYTALAN
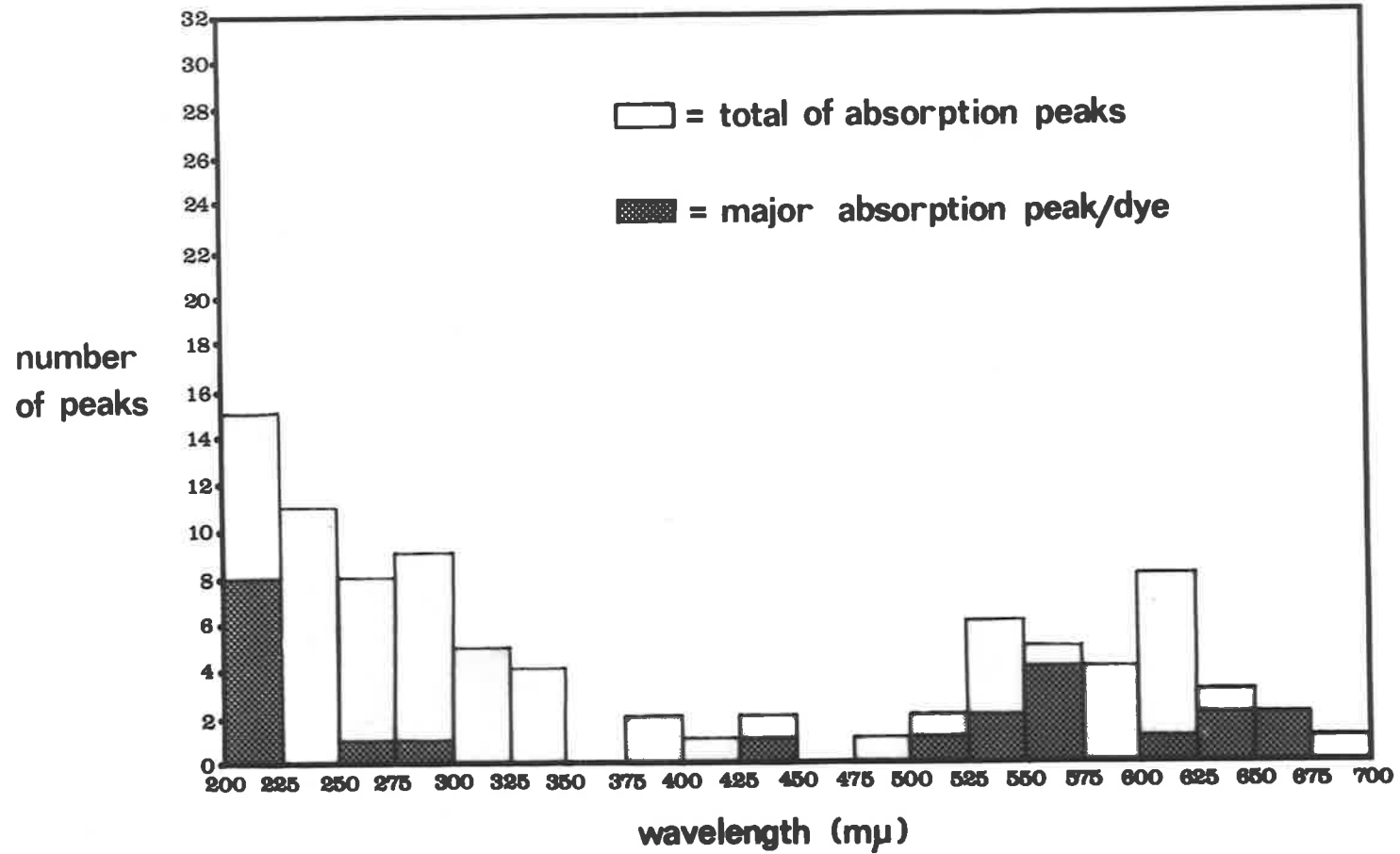
BAR GRAPH.2.

The absorptive patterns of the dye solutions were examined and the peak displaying the greatest absorptance for any one pattern was recorded and designated the major absorptive peak. For the 34 oxytalan-negative dyes measured, 16 of the major absorptive peaks occurred within the visible spectrum (i.e. 47%). The 23 oxytalan-positive dyes (including one measurment of the periodic acid-Schiff solution) indicated 13 major absorptive peaks in the visible spectrum (i.e. 59%).

Similarities in the spectral curves were detected for -

(1) thionin, azure A, nile blue and toluidine blue.

(2) methyl violet 6B and crystal violet.

(3) alcian blue 8GX, alcian green 2GX, alcian yellow GX and astra blue.

(4) cresyl fast violet and cresyl fast violet acetate.

5.4.(d) <u>Methylation and saponification tests for carboxyl groups.</u>

(i) Methylation and saponification had little noticeable effect upon oxytalan stainability with aldehyde fuchsin. Following 24 hours of methylation, oxytalan, and elastic fibre, staining reactions were insignificantly affected. The stage of interspersion of oxidation had no apparent effect.

(ii) Alcian blue (pH 2.5) staining of oxytalan fibres was eliminated when oxidation preceded methylation and was greatly reduced when oxidation followed methylation. Saponification restored the oxytalan staining reaction with alcian blue (pH 2.5).

5.5. <u>Microscopy:</u>

5.5.(a) <u>Phase contrast.</u>

No additional information regarding the staining mechanism(s) of oxytalan fibres could be ascertained. The oxytalan fibres in stained

sections were clearly visible but contrast effects from background structures reduced any advantages the technique might offer over normal light microscopy.

In unstained sections, structures were seen within the periodontal ligament which had an orientation very similar to the structures seen with aldehyde fuchsin.

5.5.(b) Dark field.

For transmitted white light, the dark field condenser offered little advantage. However, for ultra violet microscopy, the dark field technique intensified tissue fluorescence and was a great advantage.

5.5.(c) Fluorescence.

All dyes used in this study, and their staining solutions, were tested for fluorescence of oxytalan and elastic fibres under ultra violet illumination. The techniques employed failed to reveal definite fluorescing oxytalan-like structures in the formalin-fixed periodontal ligaments of any of the animals studied.

Elastic fibres and the internal elastic lamina of many arteries fluoresced with several of the dyes used. Yellow-green fluorescence occurred with nitro blue tetrazolium, alcian blue 8GX (faint) and anthracene blue WR. Yellow-green auto-fluorescence of elastic tissue also occurred in unstained, deparaffinized sections. After oxidation with Oxone, the elastic tissue fluorescence appeared to increase but no oxytalan fluorescence was apparent. Red elastic intimal fluorescence occurred with congo red (faint), Evan's blue and sirius red F3BA. Yellow fluorescence of elastic tissue occurred with the dyes procion yellow M4R, celestin blue B and lissamine rhodamine RB200-phloxine rhodamine. Celestin blue B exhibited the brightest,

most clearly defined fluorescence of elastic fibres and the arterial intima.

Fluorescein and procion yellow M4R produced excellent, but non-specific tissue fluorescence. Other dyes which fluoresced for different tissue elements such as nuclei, bone, muscle and red blood cells, did not show convincing evidence of oxytalan or elastic fibre fluorescence in either oxidized or control sections.

5.6. Three-Dimensional Reconstruction:

Following the methodology described in Chapter 4.3(a) and 4.3(b) of this study, an area was selected from slide 1 of the specially prepared serial sections to test the accuracy of the system. The computer program was developed into a workable form which read the input data and printed out the required coordinates and the angular changes between successive slides into a tabulated format. The X and Y coordinates of the reference holes were measured and the coordinate values of the test area computed. However, when superimposition and reconstruction of the test area was attempted it became obvious that inaccuracies of a small, but significant level were present in the technique. At x100 magnification a superimposition error of approximately 6% was present. The major problem concerned the inaccessability to a microscope stage which could measure with sufficient accuracy in the X and Y directions. The provided microscope stage could only produce coordinates which were reasonably accurate at 0.1mm. The success of superimposition would depend largely upon the accuracy of the initial measurements and it was felt that a stage which could measure accurately to at least 0.01mm. would be essential if the mechanical errors were to be reduced to an acceptable level.

There are several other possible sources of error which should also be considered.

(1) <u>Tissue distortion during processing</u>. Luna (1968), Lillie (1969b), and Pearse (1972) have stated that during paraffin embedding, tissue shrinkage of up to 30% may occur. However, celloidin embedding does reduce the degree of tissue shrinkage and possible distortion of the tissue components.

(2) <u>Inaccuracy in placing reference holes</u>. Any wobble in the drill will reduce the precision and ease of locating the centres of the holes and hence reduce the calculation accuracy for the test area. The tissues must also be firmly held so that the drilled holes remain perfectly parallel.

(3) <u>Sectioning</u>. The sections <u>must</u> be serially cut and the thinner the tissue slices the better and more accurate will be the superimposition result. Any change of the plane of the tissue sectioning will also produce inaccuracies.

(4) <u>Staining</u>. If a section comes loose, or tears, folds may occur and alterations in the relationship of the tissue components could result.

CHAPTER 6.   DISCUSSION.

6.1. Animals:

The marsupials studied show considerable variation and dietary adaptation of their masticatory apparatus, a finding which is shared by many authors including Wood-Jones (1924), Scott and Symons (1964, 1967), and Noble (1973). The wombat has continuously erupting molar and incisor teeth which closely resemble the rodent dentition. However, the incisors wear obliquely but do not form the extreme chisel shape commonly found in eutherian rodents. The temporo-mandibular articulation also differs from the usual antero-posteriorly orientated glenoid groove of the rodents. The wombat mandibular condyle articulates with a tubercle-like ridge which could allow both antero-posterior and lateral jaw excursions during mastication. This finding agrees with Hiiemae (1967) and infers a specialization of the rodent-like incisors for gnawing and the molar segments for grinding the exclusively vegetarian diet of the wombat.

The wallaby has powerful medial pterygoid and masseter muscles and a flattened temporo-mandibular articulation which would be well-suited to considerable lateral masticatory excursions. A very similar articulation is found in ungulate-like herbivores (Scott and Symons 1964). The molar segments have occlusal surfaces comprising low transverse ridges which would be most suitable for a grinding mastication common to herbivores (Noble 1973). The mandibular incisors do not develop from persistent pulps as Scott and Symons (1964, 1967) reported for the closely related kangaroo species. However, the enamel distribution ensures that with attrition a sharp incisor ridge is maintained for efficient cropping of grass. It is of interest to note other reports which state that the tooth numbers in the buccal segment

constantly decrease with age. According to Tomes (1923), Widdowson (1946), and Scott and Symons (1964, 1967), this process commences with displacement of two teeth by the erupting first buccal tooth in the adult dentition. The process continues with progressive loss of the most anterior teeth in the buccal segment as they move mesially. Occasionally, only one molar will remain in the buccal segment of old animals. This phenomenon of progressive tooth loss with mesial movement forms a means of assessing the kangaroo's age (Callaby 1968).

The possum temporo-mandibular articulation and dentition are essentially similar to the wallaby and hence qualifies as a herbivorous marsupial with an ungulate type of masticatory apparatus. However, the following differences exist. The first tooth in the mandibular buccal segment has an enlarged, carnassial cusp form resembling a carnivorous adaptation. The broad, shortened coronoid process could allow greater potential for lateral masticatory excursions to avoid interferences with the carnassial tooth. In fact, the possum does partake of a mixed diet of leaves, flowers, seeds, fruits, insects and has been known to consume newly hatched birds (Walker 1964).

The marsupial mice possess many of the features found in carnivorous animals. The temporo-mandibular articulation tends towards a ball and socket joint and the many sharp cusped teeth are well adapted for puncturing, grasping and tearing the prey. However, the cusp form is similar to the insectivore dentition described by Scott and Symons (1964). Both A. flavipes and S. crassicaudata feed upon insects, small lizards and other mice (Walker 1964).

From his comparative studies, Atkinson (1965) noted certain characteristic features of the occlusal and condylar head planes when the mandibles were viewed from an antero-posterior aspect. He observed that the occlusal planes were concave in rodents, convex in

herbivores (e.g. antelopes and kangaroos), and flat in carnivores. The condylar head curvatures were convex for rodents, concave for herbivores and flat for carnivores. The present study confirms Atkinson's findings (Diag. 6, p. 85 .).

It is believed that the wombat masticatory apparatus indicates certain rodent-like characteristics, whereas the wallaby and possum are essentially herbivorous (ungulate-like), and the marsupial mice display numerous carnivorous and insectivorous features (Diag. 21.). In all animals, however, the buccal and lingual flanges are very obvious at the mandibular gonial angle region, a finding which Scott and Symons (1964, 1967) say is characteristic of marsupials. However, static anatomical studies of jaw and dental morphology do not necessarily describe the complexities of mastication. For example, Noble (1973) cited the findings of other workers who theorized that functional twisting of the mandible could occur about its long axis with the cartilagenous symphysis as pivot.

6.2. Oxytalan Fibres:

The fibres seen within the periodontal ligaments of all the experimental animals comply with the oxytalan criteria established by Fullmer and Lillie (1958) and Fullmer (1960a).

6.2.(a) Distribution.

The finding of abundant oxytalan fibres in all marsupials supports Fullmer's statements (1959a, 1960a, 1963) that oxytalan is a component of normal, healthy periodontal tissues. The animals were captured live from their natural environment and were consequently assumed to be free of any diseases likely to affect the results of this study.

Within the limits of the periodontal ligament, the oxytalan

Marsupial classifications.

RODENT-LIKE

WOMBAT $\quad I_1^1 C_0^0 Pm M_5^5$

WALLABY $\quad I_1^3 C_0^0 Pm M_5^5$

UNGULATE
-LIKE

POSSUM $\quad I_1^3 C \, Pm M_5^5$

DIPROTODONTIA

POLYPROTODONTIA

CARNIVORE-INSECTIVORE

A. FLAVIPES $\quad I_3^4 C_1^1 Pm_3^3 M_4^4$

S. CRASSICAUDATA

$I_3^4 C_1^1 Pm_3^3 M_4^4$
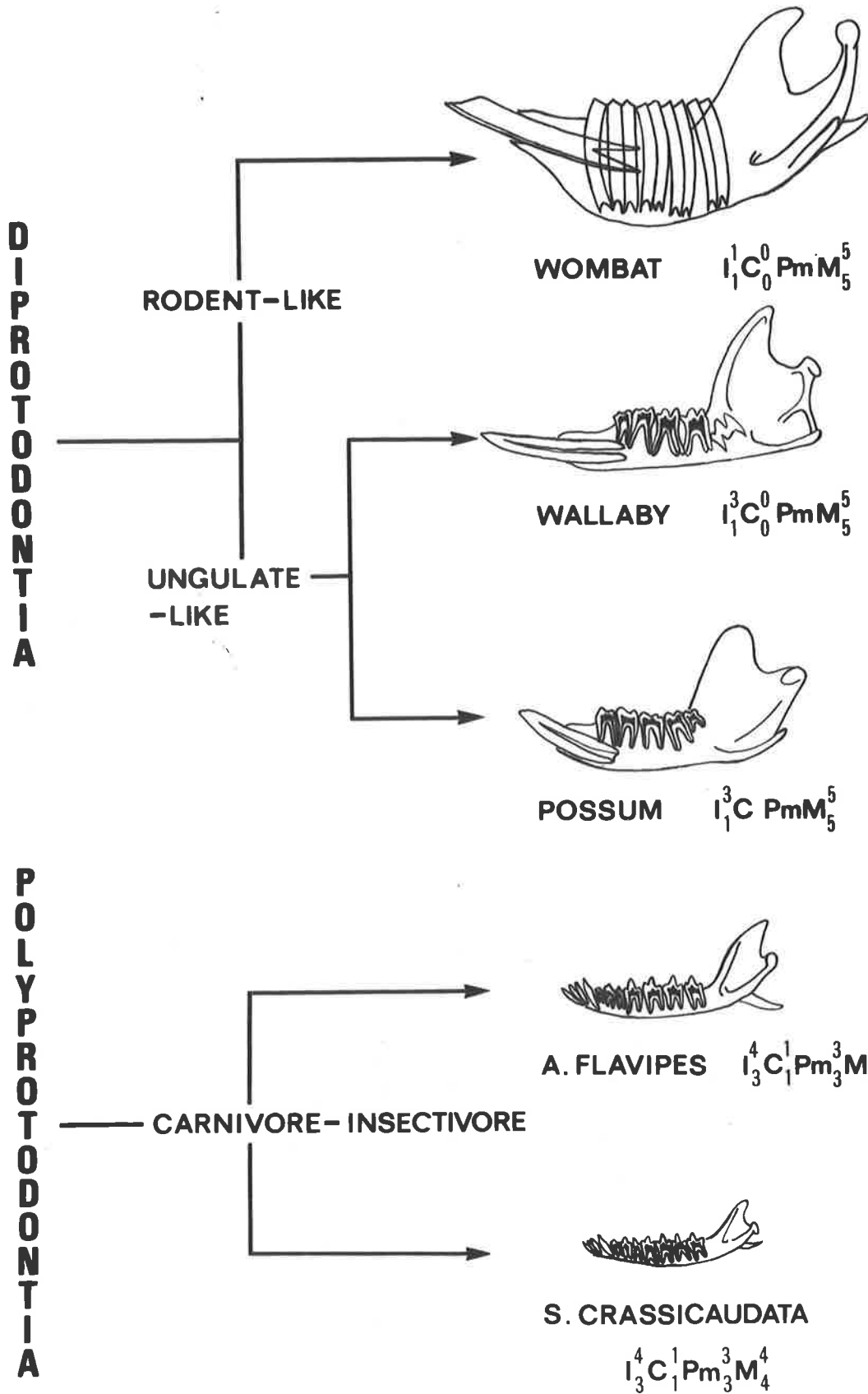
diagram. 21.

fibres occur as fine, intricate, branching structures and also as
thick, ribbon-like cords (which agrees with Fullmer 1958, 1965, 1966,
Rannie 1963, Goggins 1966, Edwards 1968a). Comparatively, the two
oxytalan fibre types vary from animal to animal in number, size, and
extent at different levels and aspects of the tooth root. For example,
the wombat displays fewer of the fine, branching fibres and has a far
greater predominance of long, thick, apico-occlusally directed fibres
than the marsupial mice. The marsupial periodontal ligaments contain
more numerous and larger oxytalan fibres at the apical third than at
the middle third level. This finding accords with Fullmer (1958, 1966).
However, complete concurrence with Fullmer's description of greater
oxytalan fibre concentrations at the gingival level is not justified.
By definition, the periodontal ligament ends at the level of the
alveolar crest and structures above that level become the supra-alveolar
system. From the present study, the supra-alveolar oxytalan system
comprises small, branching fibres which are not as numerous as those
found at the apical third of the periodontal ligament. Furthermore,
thick oxytalan fibres are only seen in the supra-alveolar region of the
wombat (Diag. 11, p.108.) and these are probably a result of
continuous tooth eruption which elevates the long, ribbon-like fibres
which then separate from their cemental attachment.

Roche (1972) could find no definite pattern of oxytalan
distribution in the supra-alveolar region. Although such an impression
can be gained by examination of a few sections, the overall findings of
the present study more closely agree with Fullmer (1958), Rannie (1963)
and Kohl and Zander (1962). Usually the oxytalan fibres intertwine and
follow the course of the dento-gingival, circular, and transseptal
collagen fibres. At the level of the cemento-enamel junction, or
subjacent to the gingival attachment in the wombat, the oxytalan fibres

either pass with the supra-alveolar collagen system or cascade apically

into the periodontal ligament where they run oblique, and then

perpendicular to the principal collagen bundles. Diagrams 11 (p.108),

12 (p.121.), and 13 (p.132.) summarise the basic supra-alveolar

oxytalan systems of the wombat, wallaby, possum and marsupial mice.

The incisors of all experimental animals, excepting the marsupial mice,

do not have a well organized transseptal collagen fibre system. Instead,

these animals have small numbers of poorly organized, fine, branching

oxytalan fibres. The wombat molars also have a poorly developed

transseptal fibre system which is probably a feature of the continual

eruption processes.

The branching oxytalan fibres which pass towards the gingival

crest do not appear to contact the epithelial basement membrane as

described by Fullmer (1960a) and Hasegawa (1960), although they do

come into close proximity with it. However, nerve fibres have been

found within the stratum basale of the gingival crest epithelium.

The oxytalan fibres which turn down into the periodontal

ligament frequently branch and travel oblique to the principal

collagen fibre bundles. These oxytalan fibres supplement the fine

network which extends from the cementum across the greater width of

the periodontal ligament, and contributes to the thicker fibre

aggregates called tracts by Sims (1973).

Goggins (1966) reported that there was a fine plexus of

oxytalan fibres adjacent, but unattached, to the cementum of

deciduous teeth. In the wallaby, such a plexus can be seen, but it

is attached to the cementum.

Within the periodontal ligaments of the experimental animals,

the number and size of the oxytalan fibres emerging from cementum,

and the number and size of the oxytalan tracts, appear to increase

toward the apex. However, at the apex, the large fibres disperse

and the oxytalan system continues completely around the apex as a very

fine complex of branching fibres. This finding agrees with Fullmer

(1958).

Fullmer (1958) and Edwards (1968a) did not report a transseptal

communication between the oxytalan systems of adjacent teeth. Although

the number and form of the communicating links differ between the

animals studied, there does appear to be a definite, continuous system

between and around the teeth as reported by Boese (1969) and Sims (1973).

The oxytalan system appears to be continuous from the supra-alveolar

region of the most mesial member of a group of teeth to the corres-

ponding distal aspect of the most distal tooth. The oxytalan fibres

in the supra-alveolar areas are of the fine and branching variety.

These connect with the periodontal ligament system of similar fine,

branching fibres and thicker, apico-occusually directed fibres. These

ribbon-like oxytalan fibres are usually found only between the cervical

and apical third regions of the periodontal ligament.

Fullmer (1958, 1966), Goggins (1966) and Edwards (1968a)

believed the thick, apico-occlusally aligned oxytalan fibres had no

attachment within the periodontal ligament. Such an impression is

readily formed when individual sections are examined. Observation of

many serial sections indicates that these thick "fibres" are aggregates

of smaller fibres of cemental origin. A schematic reconstruction of

the possible mechanism for formation of the large fibres (or more

correctly fibre tracts) is represented in Diag. 22. The concept is

presented in its simplest terms where the fibres are shown to join

but do not branch. Actually, the oxytalan fibres frequently branch

and consequently create a much more complicated structural arrangement.

Figures 20, 24, 27, 29 lend support to the model for

Schematic representation of oxytalan fibre tract formation.
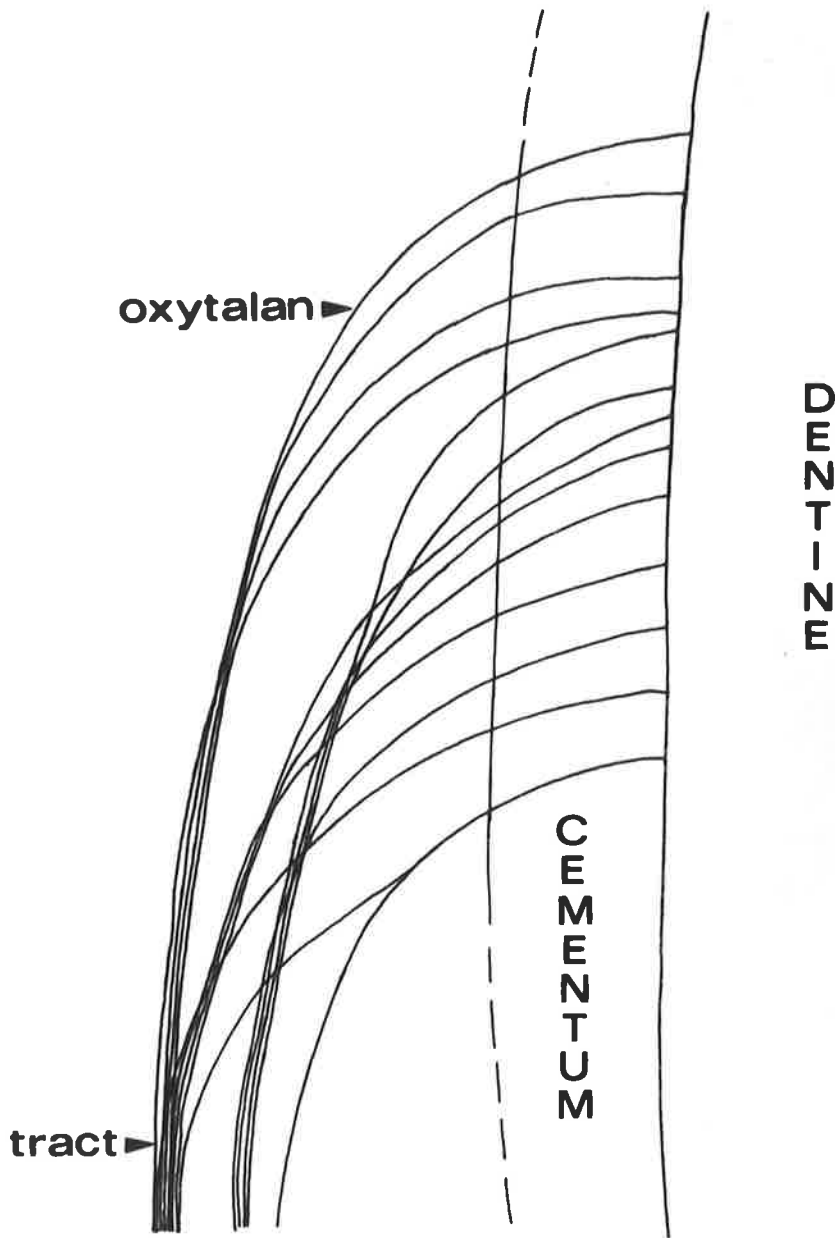
diagram. 22.

oxytalan fibre tract formation. Figure 35 is a x1,000 magnification showing fibres running parallel to each other in a neat bundle, which at lower power magnification appears as a single, ribbon-like oxytalan fibre.

In addition to the readily observable large oxytalan fibre tracts, there is also a background of much finer fibres which branch to form a complex network, particularly around the apex. This network could be an oxytalan analogue to the indifferent fibre plexus of collagen described by Shackleford (1971a).

6.2.(b) Attachments.

The present study concurs with other authors regarding the embedding of oxytalan fibres into cementum. Fibres can be traced as far as the dento-cemental junction. This finding supports Fullmer (1966) and Beynon (1967) who noted that oxytalan was laid down into the forming cementum from the periodontal ligament side of Hertwig's epithelial root sheath. The cemental anchorage of oxytalan fibres is strong. Evidence for this finding is the persistence of oxytalan fibres within the cemental layer of the continuously erupting wombat molar occlusal to the gingival attachment.

No proof has been found to suggest that oxytalan fibres insert into alveolar bone as was stated by Fullmer (1958) and Rannie (1963). Oxytalan fibres can, however, be frequently observed to accompany blood vessels into the alveolar bone. Furthermore, oxytalan tracts and fibres can be seen in close approximation to the alveolar socket margin when the periodontal blood vessels are peripheral and adjacent to the bone, as in the wombat and wallaby. In some sections it does appear that the oxytalan fibres are actually inserting into bone. However, close and careful examination of serial sections indicates that the seemingly attached fibres are in a different plane

and really overlie the bone surface. This fact can be verified by altering the microscope depth of focus through the tissue thickness. Consequently, the appearance of oxytalan inserting into bone could be an illusion which is emphasised when sections are cut obliquely through the periodontal ligament. These findings are in agreement with Carmichael (1968) and Sims (1973) who could find no evidence for bony insertion of oxytalan fibres.

Many fine oxytalan fibres have been found in close association with the periodontal vessels. The larger fibre tracts often pass close to, or send branches toward, the vessel walls. These findings are in agreement with the observations of Fullmer (1962), Carmichael (1968) and Sims (1973). Light microscope observations suggest that oxytalan fibres are attached to the vessel wall. Absolute proof of this relationship may be provided through in vivo, scanning electron microscopic, or three-dimensional reconstruction experiments.

6.2.(c)   Three-dimensional reconstruction.

A method has been devised for a three-dimensional study of the components of the periodontal ligament including oxytalan fibre attachment to the vessel walls. However, mechanical inaccuracy of the equipment available, and necessary revisions of the histological preparations have prevented completion of this aspect of the project.

Conceivably, the technique could be developed and applied to studies including −

(1) embryology,

(2) anatomy, and the

(3) histology of different tissues.

6.2.(d) Erupting teeth.

Goggins (1966) and Fullmer (1959a) found oxytalan fibres running parallel with the outer enamel epithelium of unerupted human teeth. In the wallaby, no similar relationship has been observed. A small number of randomly orientated oxytalan fibres are dispersed throughout the periodontal ligament of the unerupted wallaby teeth. This finding contrasts with Goggins (1966), who described abundant oxytalan fibres in developing human periodontal ligaments.

A well developed oxytalan fibre system comprising many fine fibres and thick fibre tracts is evident by the time the wallaby molars are due to erupt into the oral cavity. The presence of few oxytalan fibres above the level of the cemento-enamel junction indicates that the supra-alveolar system might develop after the tooth fully erupts, comes into function, and is subjected to masticatory stresses.

6.2.(e) Functional considerations.

Many theories regarding the possible functions of oxytalan have been reported. These include -

(1) a mechanical supportive and strengthening component to the collagen fibres (Fullmer 1958, Parker 1972, Rannie 1963).

(2) a counter to extrusive or disruptive forces on the periodontium by the implied elastic nature of the oxytalan fibres (Hurst 1972, Roche 1972). In particular, the relapse of orthodontically rotated teeth has been attributed to the elasticity of dento-gingival oxytalan fibres in the supra-alveolar region (Boese 1969, Edwards 1968a, 1968b, 1970). Edwards (1971) believed oxytalan fibre proliferation and tissue bunching contributed to reopening of extraction spaces. Parker (1972) considered that the action of oxytalan was secondary to collagen because there were many more

collagen fibres present in relation to oxytalan.

(3) a vascular suspensory role due to oxytalan attachment to blood vessels (Fullmer 1962).

(4) a mechanical means of maintaining vascular patency under compressive forces acting on the periodontal ligament (Carmichael 1968).

(5) a regulatory mechanism, sensory or otherwise, which functions under various states of tension during oscillation of the tooth with mastication (Sims 1973).

The present study was not designed to offer suggestions regarding the dynamic function of oxytalan fibres. However, from histological observations, there is no reason to discount a theory describing a working relationship with the periodontal blood vessels.

Fullmer (1959a, 1962, 1963), Rannie (1963), Edwards (1968a, 1968b, 1970, 1971) and Boese (1969) observed increases in the number and size of oxytalan fibres in response to stress, particularly tensile stretching forces. Undoubtedly, masticatory forces generated by the different marsupials studied are variable in strength, duration and complexity of directional delivery to the periodontium. Each animal displayed variation in the numbers and sizes of the oxytalan fibres and fibre tracts in the supra-alveolar, cervical third, middle third and apical thirds of the periodontal ligament. Moreover, the fibre distribution varied between mesial, distal, buccal and lingual aspects of the teeth. These findings are consistent with the concept that variation in oxytalan morphology and numbers occurs as a response to variation in the delivery of masticatory forces to different locations in the periodontium. An observation of interest was the decrease in number and size of oxytalan fibres and fibre tracts from the wombat to the tiny marsupial mice. This finding is consistent with the possible

degrees of masticatory stress which each animal is capable of producing. For example, the wombat grinds its food with powerful lateral movements while the marsupial mice chew with a snapping, vertical motion. In accordance with lateral movements being predominant in the wombat, fewer oxytalan fibres are found on the buccal aspects of the molars. Possibly, the tensile stresses would be less on the buccal aspects.

However, the question should be asked why oxytalan fibres are found in the dental pulp where tensile forces would be expected to be minimal. Fullmer (1959b) and Beynon (1967) described the presence of oxytalan fibres in developing, but not adult, pulp tissue. The presence of short, wavy oxytalan fibres in the immature apical pulp tissue of the continuously erupting wombat teeth and at the incomplete apices of the wallaby and possum, agrees with the above authors. Moreover, no definite oxytalan fibres are found in the more mature pulp tissue located further occlusally. Densely staining amorphous material, which has the appearance of mucopolysaccharide, is found associated with the oxytalan in the immature pulp and is only visible following pre-oxidation. The amorphous material has staining qualities very similar to oxytalan and may well have an embryological significance since Fullmer (1959a) described oxytalan fibres condensing from masses of diffuse mucopolysaccharide.

It could be asked why oxytalan fibres are found in the periodontal ligaments of erupting teeth which have yet to enter the oral cavity. Perhaps the forces of eruption which help organize the principal collagen fibres (Trott 1962) also play some role in establishing oxytalan fibres in the periodontal ligament and developing pulp.

The field concerning the possible function of oxytalan fibres remains unresolved.

6.2.(f)   Relationship to elastic fibres.

Fullmer (1960a, 1966) and Soule (1967, 1969) have described the presence of elastic fibres within the periodontal ligaments of numerous animals. The consistent finding of elastic fibres in the periodontal ligament of the wombat and the less frequent observation of elastic fibres near the cemento-enamel junction of the wallaby and possum corresponds with some other species. Indeed, the presence of elastic fibres with a similar distribution to oxytalan in different animals, and the observation of fibres which stained for both oxytalan and elastic fibres within the same fibre, partly formed the basis for Fullmer's (1960a) hypothesis that oxytalan was a modified form of elastic fibre.

However, it is impossible to histologically distinguish between pre-elastin and oxytalan (Fullmer 1960a) and it could be said that the co-existence of elastic fibres with oxytalan fibres could indicate that the oxytalan observed in the wombat, wallaby and possum might represent immature elastic tissue or pre-elastin. The animals were all adult. Consequently, the possibility of oxytalan being pre-elastin can be eliminated by definition (Chapter 3.1.). Many authors have shown that the structure and biochemical behaviour of elastic tissues varies from animal to animal and from site to site within the same animal. This information illustrates how cautious one must be to avoid forming premature conclusions. This present study has not provided sufficient evidence to modify Fullmer's (1960a, 1965) statements that oxytalan might represent a modified form of elastic tissue.

In addition to the similarities and differences between oxytalan and elastic fibres previously reported (Chapter 2.8.), the present study offers the following information.

6.2.(f)(i)   Similarities.

(1)   All dyes tested, which indicated a positive reaction for
      oxytalan, also displayed elastic fibres.

(2)   Numerous oxytalan fibres appeared to have recoiled subsequent
      to sectioning i.e. the oxytalan fibres showed evidence of
      elastic properties.

(3)   Pre-oxidation of the sections not only revealed oxytalan
      but also appreciably improved the quality and intensity of
      elastic fibre staining (this agreed with Fullmer and Lillie
      1958).

(4)   Bromelain removed all elastic and oxytalan fibre staining
      by aldehyde fuchsin but only after pre-oxidation. Thomas
      and Partridge (1960) attributed elastolytic and proteolytic
      properties to bromelain.

(5)   Elastase reduced oxytalan stainability with aldehyde fuchsin
      only if the tissues were oxidised prior to enzyme application.
      This finding agreed with Fullmer and Lillie (1958).

(6)   Four of the classical elastic tissue stains (aldehyde fuchsin,
      orcein, resorcin fuchsin and orcinol-new fuchsin) demonstrated
      oxytalan fibres in the marsupial periodontal ligaments. How-
      ever, it must be noted that Fullmer and Lillie (1958) did not
      find orcinol-new fuchsin to positively react with oxytalan
      in their experimental material.

(7)   Faint, but positive reactions with periodic acid-Schiff
      (P.A.S.) were seen in areas of the marsupial periodontal
      ligaments where only oxytalan fibres were consistently
      found. Fullmer and Lillie (1958) have noted slight P.A.S.
      reactivity of some rodent elastic fibres.

(8)   As the numbers and size of oxytalan fibres decreased from

the wombat through to the marsupial mice, the numbers of
elastic fibres found within the periodontal ligaments also
diminished.

6.2.(f)(ii) Differences.

(1) A number of dyes stain for elastic fibres but not oxytalan
(i.e. Verhoeff's iron haematoxylin, Hale's colloidal iron,
ponceau 2R, sirius red F3BA, congo red, acid fuchsin and
luxol fast blue G). Salthouse (1965) noted elastic fibre
staining with luxol fast blue G.

(2) For any one stain technique, the elastic fibres appeared to be
larger, coarser and associated with more variably staining
amorphous material. The oxytalan structures seemed to have
an amorphous component with more distinct limits.

(3) Many more oxytalan than elastic fibres were present in the
periodontal ligament.

(4) The fact that pre-oxidation was required before oxytalan
fibres could be demonstrated, indicates some tangible difference
in the staining moiety.

(5) The iron-orcein staining techniques of Roman et al.(1967)
and Lillie et al.(1968), indicate a further difference
because elastic fibres stained an intense black and oxytalan
fibres retained only the purple-brown colour of orcein. This
difference poses the question as to whether oxytalan fibres
lack the iron conjugating factors thought to be present in
elastic fibres.

(6) Furthermore, no effect upon the aldehyde fuchsin stainability
of elastic fibres could be detected following β-glucuronidase,
collagenase, pepsin, neuraminidase and possibly hyaluronidase
digestions. Oxytalan stainability was affected only when the

enzyme digestion followed a period of pre-oxidation. These findings agree with Fullmer and Lillie (1958) and Fullmer (1960a).

6.2.(g) Relationship to other fibres.

On the basis of morphology and distribution, the marsupial oxytalan fibres are unlikely to represent nerve tissue as was suggested by Löe and Nuki (1964). Moreover, from this present study, it is not possible to evaluate the ideas of Selvig (1968) and Kanouse (1966) who likened oxytalan to degenerating or immature collagen fibres. However, on the basis of morphology and staining characterisitcs, dyes which reacted with oxytalan did not react well, if at all, with collagen under normal staining conditions.

6.2.(h) Staining reactions.

It has been said by Fullmer and Lillie (1958) and Fullmer (1960a, 1965) that the stainable portion of oxytalan is a mucopoly-saccharide matrix and not necessarily the protein moiety. Puchtler et al.(1961) questioned the specificity of the elastic fibre stain resorcin fuchsin on the basis that keratin, glycogen, basement membranes, reticulin and even collagen could be induced to react positively after certain pretreatments. Histochemically, the assumed specificity of the elastic tissue dyes such as aldehyde fuchsin, orcein and resorcin fuchsin can be altered dramatically by methylation, acetylation, benzoylation or other blockade steps (Fullmer and Lillie 1957, Fullmer 1960a). The work of Banga et al.(1956) and Hall et al.(1955) further illustrated the complexities involved in analysing biologic structures. A review of the questionable specificity of certain histochemical staining reactions is contained in Chapter 1.8.(c) of this report. However, the fact that oxytalan fibres do not stain with every known

chemical dye at least indicates some degree of selectivity, be it physical and/or chemical in nature. The identification of the reactions responsible for the histological demonstration of oxytalan fibres is extremely difficult. Before the actual staining step is reached, the tissues have been insulted with preservative(s), decalcification, dehydration, paraffin embedding, sectioning, deparaffinizing and hydration. All these procedures affect the chemical and physical properties of the tissues (Pearse 1968, Barka and Anderson 1963) and even variation in the rates and times of exposure to those treatments will produce more diverse results. In addition to these problems, the conglomeration of tissue proteins, electrolytes, enzymes, hormones, and the products of anabolism and catabolism which all vary from animal to animal, and from site to site within that animal at any one particular time in its life cycle, further complicate the staining reactions. Study of the biochemistry of oxytalan fibres is hampered by the fact that their size and arrangement has not permitted micro-assays.

Considering the differences between the various dye solutions and the variety of possible physical and/or chemical binding sites in the tissues, it would be optimistic to expect determination of more than a few reaction trends. Whatever the chemical reactions of the staining processes may be, this investigation confirms that there are differences in the mechanisms for aldehyde fuchsin, orcein and resorcin fuchsin dyes as reported by Fullmer (1960a). Orcein-positive fibres appear to be more discrete and seem to have less associated amorphous material than corresponding fibres stained by aldehyde fuchsin. Resorcin fuchsin seems to stain even fewer fibres, and then mostly those which are demonstrated as thick fibres (or oxytalan tracts) by the other dyes. Fullmer and Lillie (1958) believed orcinol-new fuchsin displayed "a high degree of specificity....for elastic fibres"

because it did not stain oxytalan or any other tissue excepting the
enamel matrix of unerupted, decalcified teeth. However, the marsupial
material indicates orcinol-new fuchsin staining of oxytalan fibres in
the periodontal ligament. Verhoeff's iron haematoxylin would seem to
be more specific for elastic fibres than the other elastic tissue stains.

6.2.(h)(i) oxidation.

Fullmer and Lillie (1958) and Pearse (1968) have mentioned
that peracetic and performic acids are known to split disulphide bonds
(S-S) to form sulphonic and, perhaps, other acids. Although Fullmer and
Lillie (1958) discounted the presence of S-S bonds within oxytalan
fibres and the formation of sulphonic acid, it is interesting to
conjecture upon the formation of carboxyl groupings by oxidation.
Theoretically, aldehydes can be formed by oxidation of primary alcohols
(ketones from secondary alcohols) and both alcohols and aldehydes are
easily oxidized to carboxylic acids, particularly in acidic aqueous
solutions (Stranks et al. 1965). Potassium monopersulphate (Oxone) is
an oxidant which is strongly acidic (pH 1.5) in a 10% aqueous solution.
Fullmer and Lillie (1958) stated that oxytalan may differ from elastic
tissue by possessing stainable groups in a more reduced form. One
aspect of oxidation might be to increase the proportion of carboxyl
groups available for stain interaction. In fact, Fullmer (1960a) has
noted an enhancement of elastic fibre staining following a period of
oxidation. Furthermore, Landing et al. (1956) believed aldehyde
fuchsin reacted with strongly acid groups produced from oxidation of
tissues containing sulphydryl or disulphide groups (cystine or cystein).

6.2.(h)(ii) reactive groups.

From the results of numerous chemical tests of oxytalan
staining in mammalian tissues, Fullmer and Lillie (1958) discarded the

possibility of formation of $-O-SO_4$ groups by oxidation, partly because oxytalan staining did not occur with the Hale, azure A or alcian blue methods. However, this present study has reported positive staining of structures morphologically identical with oxytalan fibres with azure A and alcian blue. According to Schultz-Haudt et al. (1964), Carlo (1964), and Spicer and Meyer (1960), alcian blue will stain sulphated mucopolysaccharides. However, Lev and Spicer (1964) explained that specificity for sulphated mucopolysaccharides only occurred at very low pH levels and that carboxyl groups took up alcian blue at pH values greater than 2.5. Thus, the results of the present study would indicate the presence of both sulphated and carboxyl groups in marsupial oxytalan structures because positive results were seen at pH 1 and greater than pH 2.5. However, the intensity and clarity of staining with alcian blue was improved at pH 2.5 and with the addition of acetic acid which would indicate a possible predominance of carboxyl groups being present. Nevertheless, the animal material used in this study differs from that used by other workers. Furthermore, Sorvari and Nänto (1971) actually questioned the specificity of alcian dyes and disputed the ability of such dyes to accurately distinguish staining groups as a laboratory technique.

Bangle (1954) believed aldehyde fuchsin stained specific proteins, specific mucopolysaccharides, and aldehydes. In particular, evidence for aldehyde fuchsin staining sulphated mucopolysaccharides was provided by Spicer and Meyer (1960), and Melcher (1967) as cited in Melcher and Bowen (1969) p.211. As tentative support for those findings it has been observed in the present study that the pH of aldehyde fuchsin (pH 1.4) is within the range reported by others to favour sulphated mucopolysaccharide staining. In fact, the quality and specificity of aldehyde fuchsin staining seems to improve the lower the pH becomes,

further indicating the presence of sulphated groupings. Lillie (1969b)
stated that only sulphuric or sulphonic residues take up basic dyes at
pH values less than or equal to pH 1.0.

Fullmer and Lillie (1958) concluded that the stainable portion
of oxytalan was a mucopolysaccharide. This is supported by the present
finding of oxytalan-like staining with alcian blue (pH 1), toluidine
blue, celestin blue, cresyl fast violet and cresyl fast violet acetate
which stain acid or sulphated mucins (Pearse 1968, Lillie 1969b). It
should be noted that Rannie (1963) reported oxytalan staining with
methylene blue, toluidine blue and celestin blue. However, Hale's
colloidal iron technique for acid mucopolysaccharides does not reveal
oxytalan fibres and this finding would seem to indicate the absence of
acid mucopolysaccharide. According to Culling (1974), Braden (1955),
and Thonard and Scherp (1962), Hale's technique is neither reliable nor
specific for acid mucopolysaccharide.

Fullmer and Lillie (1958) also explored the possibility of
reactive lipid groups within oxytalan fibres, but could find no positive
staining with the oil red O or nile blue techniques for lipid. The
present study has revealed structures morphologically identical to
oxytalan which react with nile blue and sudan black B techniques (both
are used as lipid stains - Luna 1968). Consequently, one could say that
lipids are present. However, caution is advisable considering the lack
of absolute specificity displayed by most dye substances (Chapter 1.8.
(c)). For example, cresyl fast violet has been used in various techniques
to display nuclei, plasma, amyloid, mucin, mast cell granules, white
blood cells, tumourous tissues and for bulk staining of nerve tissues.
Therefore, all that can be said is that similar reactive groups may be
located somewhere within, or upon, the complex structures histologically
recognized as lipid or oxytalan fibres. The hypothesis of an increase

in effective carboxyl groupings in marsupial oxytalan fibres, as presented in this report, is supported by positive reactions from sudan black B and nile blue because lipids do contain high proportions of carboxyl groups (Culling 1974). Therefore, the marsupial material used in this study could differ from the eutherian material of Fullmer and Lillie (1958) either by the presence of reactive lipids, or by an overall increase in the available carboxyl groups.

The possibility that aldehyde groups are present in marsupial oxytalan fibres could be inferred from the positive reactions with periodic acid-Schiff techniques before oxidation. Lillie (1969b) referred to the reaction between aldehydes and the Schiff base. Bangle (1954) said that aldehyde fuchsin reacted via the formation of a Schiff's base which would then react with aldehydes. Zugibe (1970) said that Schiff staining occurred via aldehydes produced from periodic acid oxidation. Furthermore, glycogen, neutral mucopolysaccharides, glycoprotein with neuraminic acid residues, some unsaturated lipids, formaldehyde residues and serine were periodic acid-Schiff positive. However, Zugibe also found that acid mucopolysaccharides could produce a positive reaction after prolonged oxidation (4-16 hours). According to Culling (1974), the chemical basis of the periodic acid-Schiff reaction is that periodic acid will cleave the carbon-carbon bond where those carbon atoms have adjacent hydroxyl (-OH) groups (1:2 glycol groups), or adjacent hydroxyl and amino ($-NH_2$) groups (1:2 amino, hydroxy groups). Hotchkiss (1948), cited in Culling (1974, p.263), stated that any substance which satisfies the following criteria will give a positive result with the periodic acid-Schiff reaction.

(1) The substance must contain the 1:2 glycol grouping, or the equivalent amino or alkyl-amino derivative, or the oxidation product CHOH-CO.

(2)   It must not diffuse away in the course of fixation.

(3)   It must give an oxidation product which is not diffusible.

(4)   Sufficient concentration must be present to give a detectable
      final colour.

Providing the periodate oxidation is short, as it was in the
present study, then reasonable confidence can be expressed that periodic
acid-Schiff positivity is not due to acid mucopolysaccharide staining.
Therefore, marsupial oxytalan fibres probably have a different
composition of the staining moiety compared with eutherian animals which
are periodic acid-Schiff negative.

This present study can not discount the possibility that the
amorphous material around individual oxytalan and elastic fibres, and in
the developing pulp tissue, is the staining moiety. However, orcein,
bismarck brown Y and chlorazol black E appear to stain for finer diameter
oxytalan elements. Undoubtedly, the staining reactions differ among
the dyes and probably the reactive tissue and dye groupings also vary.
However, it seems possible that the amorphous material may be a muco-
polysaccharide whose acidity is increased by oxidation.

6.2.(h)(iii) dyes.

All the dyes, with the exception of chlorazol black E and
orcein, which this study found to react positively with oxytalan
structures, are basic dyes. The pH values of the aqueous dye solutions,
and particularly the staining dye solutions, do not reflect the acid or
base nature of the dye. This is because the commercial dyes are
obtained as salts and consequently the pH of the solution can be more
influenced by the potassium, sodium, chloride, sulphate, acetate ions
etc. than the chromophore (colour bearing radicle)(Lillie 1969b). By
definition, an acid dye carries a negative charge (anion) and basic dye
carries an overall positive charge (cation). The complexity of

structure exhibited by the dyes used in this study can be appreciated from Diags. 23, 24, 25, 26. The majority of oxytalan-positive dyes have either amine ($-NH_2$) groups or available nitrogen present in the molecular structure which would explain some of the basic properties of the dye. However, a basic dye can be easily made acidic by the addition of a strong acid grouping. For example, basic fuchsin (which will demonstrate oxytalan when it is prepared as aldehyde fuchsin and resorcin fuchsin) can be made into acid fuchsin (which will not stain oxytalan and poorly stains elastic fibres) by the process of sulphonation (addition of $-SO_3$ groups).

Theoretically, a basic dye (nett positive charge) should react with a negatively charged tissue component (e.g. carboxyl radicles, or sulphate groups etc.). Mander et al.(1968) believed that α-hydroxyamine residues were oxidized to -imines by periodate and then tautomerized to enamines within the oxytalan structure. However, it is most likely that the operative reactive groups vary with the type of dye, and the available tissue components. Accordingly, oxytalan stainability could be due to a number of different groups present within, or upon, the structure. These different groups do not necessarily have to function at the same time but could vary according to the chemical dye composition, pH of the dye solution, presence of ionic contaminants in the dye solution, shape of the dye molecule (stoichiometry), temperature of the reaction environment, and the method of dye application (e.g. concentration and time). Even the density and permeability of the tissues will affect the rate and intensity of dye interaction (Friedberg and Goldstein 1969, Lillie 1969b). Furthermore, the staining fraction of oxytalan is more likely to be mucopolysaccharide than protein. A further complication which could be considered is the occurrence of impurities and inconsistencies in the composition of

ALCIAN BLUE

$C_{32}H_{12}N_8Cu + 4X$
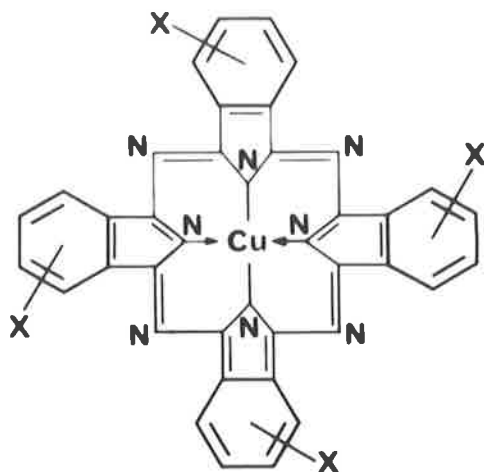
(X = an onium group)

M.Wt. $\simeq$ 1381·588

ALCIAN GREEN and ALCIAN YELLOW are similar
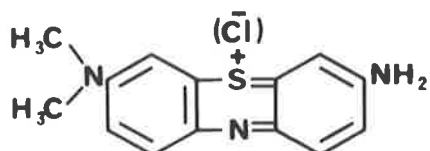
AZURE A $C_{14}H_{14}N_3SCl$

M.Wt. 291·813

PARAROSANILIN

$C_{19}H_{18}N_3Cl$
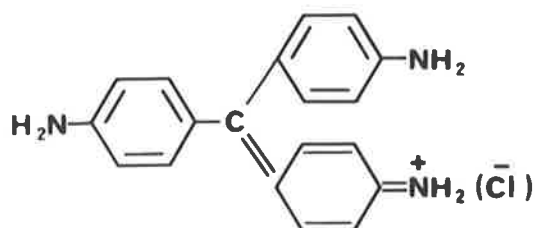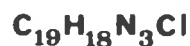
M.Wt. 323·834

ROSANILIN

$C_{20}H_{20}N_3Cl$
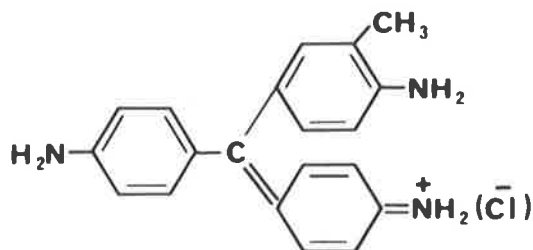
M.Wt. 337·861

MAGENTA II

$C_{21}H_{22}N_3Cl$
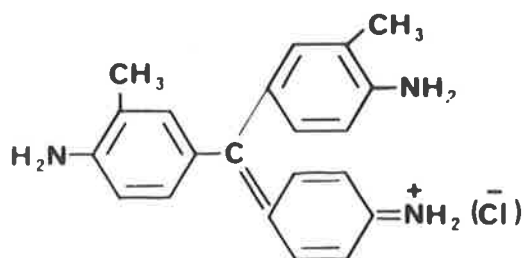
M.Wt. 351·888

BASIC FUCHSIN

diagram. 23.
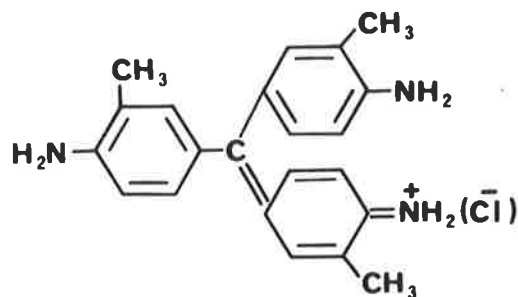
NEW FUCHSIN

(MAGENTA III)

$C_{22}H_{24}N_3Cl$

M.Wt. 365·915
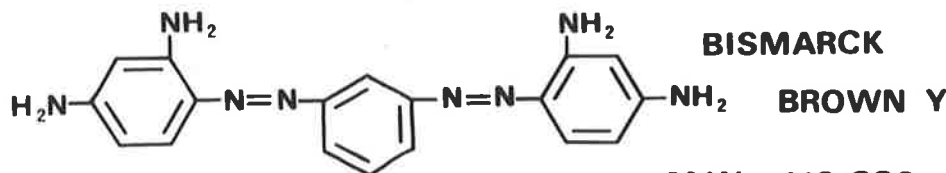
BISMARCK BROWN Y

$C_{18}H_{20}N_8$ M.Wt. 419·336
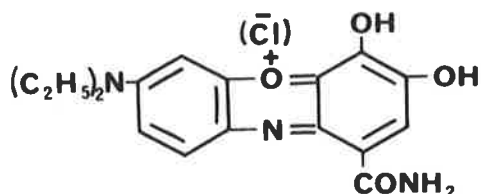
CELESTIN BLUE B

$C_{17}H_{18}N_3O_4Cl$

M.Wt. 363·812
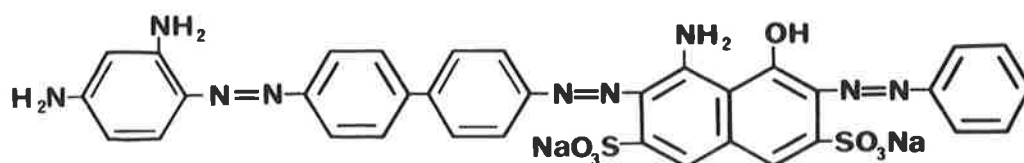
CHLORAZOL BLACK E $C_{34}H_{25}N_9O_7S_2Na_2$

M.Wt. 781·760
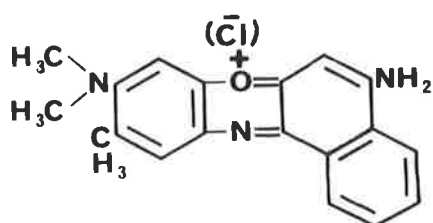
CRESYL FAST VIOLET

$C_{19}H_{18}N_3OCl$

M.Wt. 339·834
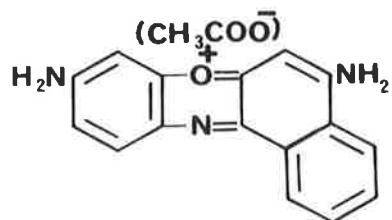
CRESYL VIOLET ACETATE

$C_{18}H_{15}N_3O_3$

M.Wt. 321·342

diagram. 24.

CRYSTAL VIOLET

$C_{25}H_{30}N_3Cl$

M.Wt. 407·996

METHYL VIOLET 6B

$C_{24}H_{28}N_3Cl$

M.Wt. 393·969

NEUTRAL RED

$C_{15}H_{17}N_4Cl$

M.Wt. 288·790

NILE BLUE

$C_{20}H_{20}N_3OCl$

M.Wt. 353·861

ORCEIN

variations of R_1, R_2, R_3, produce 14 different orcein fractions
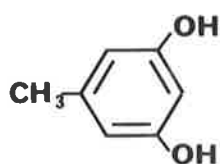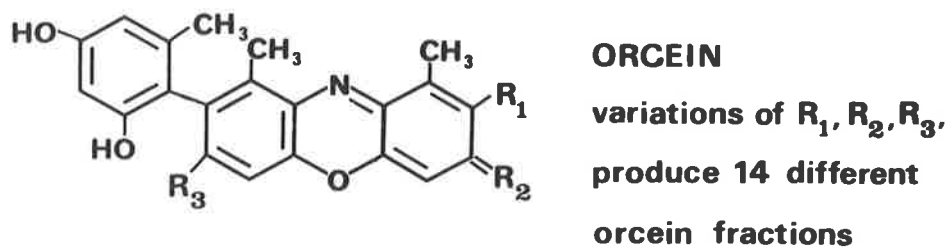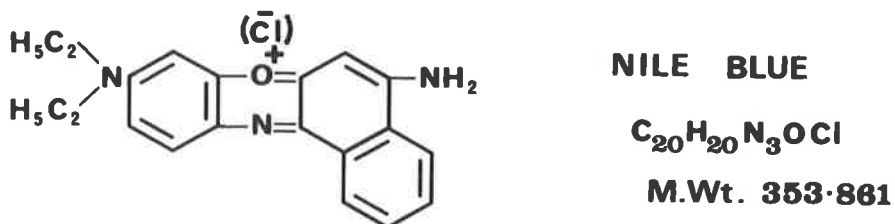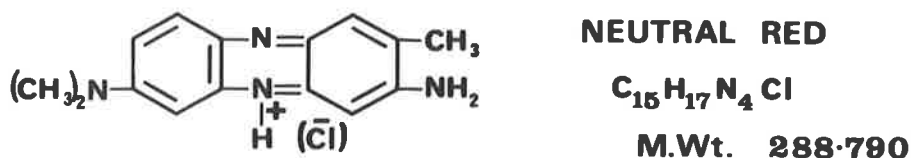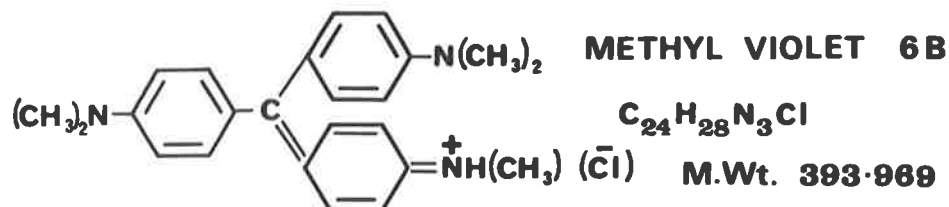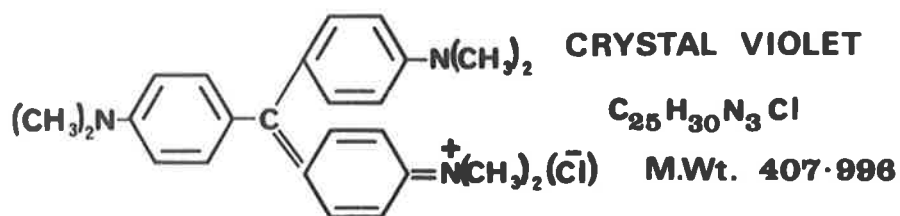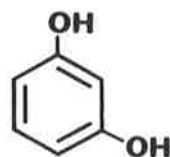
ORCINOL

RESORCINOL

diagram.25.

SUDAN   BLACK   B $C_{29}H_{24}N_6$

M. Wt. 456·559

THIONIN

$C_{12}H_{10}N_3SCl$
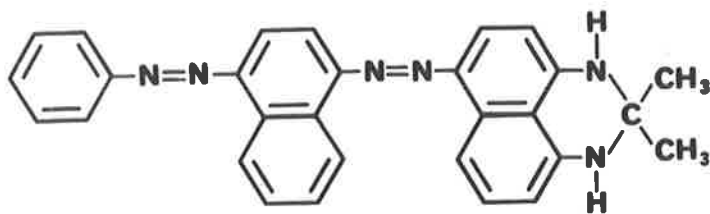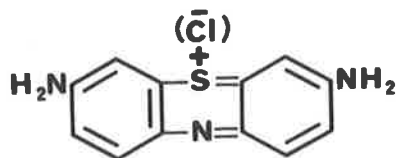
M.Wt. 263·759

TOLUIDINE   BLUE

$C_{15}H_{16}N_3SCl$
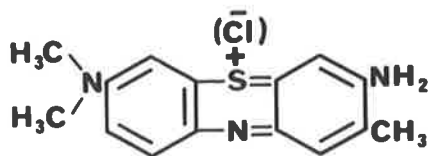
M.Wt. 305·840

diagram. 26.

the commercially available dyes.

No correlation between oxytalan-positive staining and the dye type has been revealed by this present study. Individual dyes belonging to the pthalocyanine, triarylmethane, quinone-imine, arylmethane, disazo and trisazo dye classes, and the naturally occurring orcein complexes do not show a predictable reactivity with oxytalan structures in the marsupials studied. Horobin and James (1970) found no association between dye class, formal charge, or the presence of hydrogen bonding groups and elastic fibre staining. These authors did find an association between elastic fibre staining and the presence of five or more aromatic rings in the dye structure. As can be seen from Diagrams 23, 24, 25, 26, not all of the dyes have five or more aromatic rings in the pure state. However, that is not to say that further interactions may not take place in solution to effectively produce more complicated, multi-ringed structures.

According to Lillie (1969b), the majority of dyes, particularly the water soluble ones, are salts. For example, sizeable numbers of the basic dyes are cationic with anions of chloride or acetate. Considering the ionic equilibrium dissociation constant, the addition of hydrochloric acid or acetic acid to the aqueous dye solutions would tend to reduce the dissociation of the chloride or acetate dye salt, respectively, as well as reducing the pH of the staining solution. The advantage of lowering the dye solubility might be to reduce the rate of dye removal from the stained tissues during preparation of the sections for mounting and microscopic examination. In fact, it would appear that the addition of acetic acid to alcian blue 8GX and cresyl fast violet does increase the stability of the staining results over those solutions where hydrochloric acid is the additive. However, such an observation could also be attributed to the differences in pH of the

staining solutions. Lillie (1969b) stated that only sulphuric or sulphonic residues take up basic dyes at pH values less than or equal to pH 1.0, phosphoric acid residues commence uptake at approximately pH 1.5, and carboxyl residues operate at about pH 3.0. Whichever group does take up the dye will be determined by the hydrogen ion concentration of the stain solution i.e. the pH of the stain solution should be greater than the pKa of the tissue for an acid-base reaction to occur. Therefore, the finding of improved staining with the addition of acetic acid would support the hypothesis that marsupials contain more -COOH groups after oxidation because acetic acid would lower the pH to pH 2.5-3.0 where carboxyl dissociation is favoured.

Fullmer and Lillie (1958) found that oxytalan stainability with aldehyde fuchsin was lost after 16 hours of methylation and was not restored by saponification. Theoretically, methylation procedures will desulphate the tissues as well as blocking carboxyl groups and with saponification the carboxyls, but not the sulphates, are restored (Culling, 1974). This present study offers support for the hypothesis that marsupial oxytalan fibres might contain reactive carboxyl groups following oxidation. Staining of oxytalan fibres with alcian blue (pH 2.5) was lost after methylation and restored by saponification, which indicates carboxyl groups are present. However, oxytalan fibre staining with aldehyde fuchsin was not significantly affected by methylation or saponification, indicating that aldehyde fuchsin does not depend on carboxyl or sulphate groups for its reactivity. More extensive study of the effects of different blockade techniques upon the staining reactions of all dyes upon the marsupial material could provide further information.

Pearse (1972) indicated that basic dyes can react with acidic groups such as carboxyl, sulphate, and phosphate by:

(1)   <u>salt linkage</u>

$$R_T - \overset{\overset{\text{O}}{\|}}{C} -OH + Rd^+Cl^- \rightarrow R_T - \overset{\overset{\text{O}}{\|}}{C} - O^- \; Rd^+ + HCl$$
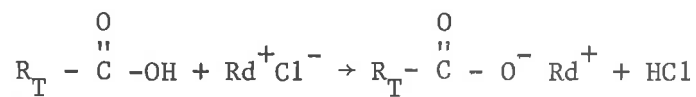
(R_T = tissue radicle)

(Rd = dye radicle.)

or   (2)   <u>condensation reaction</u>

$$R_T - \overset{\overset{\text{O}}{\|}}{C} -OH + H_2N-Rd \rightarrow R_T-\overset{\overset{\text{O H}}{\| \;\;|}}{C} -N-Rd + H_2O$$
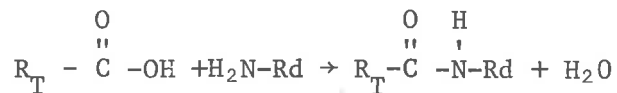
The condensation type of reaction is said to be more resistant
to dehydration and mounting than is the salt linkage. Consequently,
the improved dye stability observed when acetic acid is added to some of
the dye solutions may imply not only a lowering of the dye solubility
but also an increased incidence of condensation type reactivity.
However, Horobin and James (1970) discounted the hydrogen bonding and
suggested Van der Waal's forces as the most likely reaction involved in
dye binding to elastic tissue, principally because of the highly non-
polar nature of elastic fibres. Most authors seem to follow the
probability that dye binding to elastic fibres occurs by a non-ionic
process (Goldstein (1962), Weiss (1954), Sumner (1965), Lillie (1969a),
Horobin and James (1970)). If Van der Waal's forces are involved,
then the number of polarizable aromatic dye rings as well as the
stoichiometric considerations of dye size and shape, and binding site
shape and accessibility become very important. The issue regarding
oxytalan fibre dye binding reactions is unresolved and undoubtably
complex and different for each dye type. Dye reactions can be both
physical and/or chemical in nature (Lillie 1969b). Quite possibly, the
diversity of reactions observed in this study reflect both physical
and chemical interactions. For example, differential solubility plays
an important part in the staining reaction of sudan black B which is
more soluble in lipid than alcohol and therefore resists alcohol

extraction. Furthermore, the effect of alcohol on stained sections has not been satisfactorily determined (Culling 1974) and is another perplexing area of the staining reaction yet to be explained.

Metachromasia depends on the ability of tissue elements, called chromotropes, to produce marked changes in the absorption spectrum of certain basic dyes with which they are being stained. Principal chromotropes in animals are chondroitin sulphate, heparin, hyaluronate and sometimes nucleic acids. It is interesting to note that Taylor (1961) found thionin, azure A, methylene blue, toluidine blue, cresyl fast violet and nile blue A to be metachromatic dyes. With the exception of methylene blue, all those dyes produced positive oxytalan staining reactions in the marsupial material used for this present study. Furthermore, alcohol dehydration of the stained tissues results in loss of metachromasia, (Lillie 1969b) a phenomenon which has been observed in this study.

6.2.(i) Spectrophotometry.

Although some spectrophotometric differences have been observed in this present study between those dye solutions which stain and fail to stain oxytalan fibres, it is hazardous to reach many con- clusions. Firstly, the wavelength of the absorptive peaks is an indication of colour of the dye solution and the degree of absorption is related to the concentration of dye present. Secondly, the absorption pattern will change with the solvent used and the number and type of additives also in solution. Therefore, care is needed to interpret the results of spectral analysis. The number of peaks is characteristic for a particular dye, but these can be confused by impurities and tautomeric forms of the dye (Lillie 1969b). However, the spectrophotometric tracings (Diags. 17, 18, 19, 20) do show similarities between (1) Cresyl fast violet and cresyl fast violet

acetate; (2) thionin, azure A, nile blue and toluidine blue;
(3) methyl violet and crystal violet; (4) alcian blue 8GX, alcian
green 2GX, alcian yellow GX and astra blue;

6.2.(j) <u>Fluorescence studies</u>.

Very little useful information was gained from these
experiments as they were designed purely to scan for any dye technique
which might offer a more efficient means of examining oxytalan fibres
by ultra violet fluorescent microscopy. The tissues in the present
study were formalin-fixed and, as a suggestion for further work in this
field, perhaps the use of freeze dried tissue might offer different
results.

6.2.(k) <u>Enzymes</u>.

Following pre-oxidation, oxytalan stainability with aldehyde
fuchsin was reduced or eliminated by all enzymes used in this study,
with the possible exception of hyaluronidase. However, the questionable
staining reduction observed in this study after 3 hours incubation of
the tissue sections with hyaluronidase can be reconciled with the fact
that Fullmer and Lillie (1958) incubated their material for 24-72 hours.
Fullmer (1960a, 1965) concluded that β-glucuronidase, and hyaluronidase,
removed mucopolysaccharide from the structure histologically called
oxytalan. That finding is supported by the present study. The
collagenase and pepsin reduction of oxytalan staining was mildly
surprising and might indicate some subunit similarity between
collagen and oxytalan, or enzyme non-specificity. Pepsin appears to
reduce oxytalan staining independently of tissue pre-oxidation. The
reaction of pepsin and collagenase might be explained by a relative
nonspecificity due to the presence of a carbohydrate or muco-
polysaccharide associated with collagen (Lazarow and Spiedel, 1964;
Bouteille and Pease, 1969; and Bloom and Fawcett, 1968) which could

be similar to the mucopolysaccharide said to be associated with oxytalan
(Fullmer 1960a). However, pepsin has been previously found to have
elastolytic properties (Pearse, 1972). Bromelain and elastase were the
only two enzymes tested and found to have an effect on oxytalan and
elastic fibre staining. Bodley and Wood (1972) studied enzyme hydrolysis
effects upon the ultrastructure of elastic fibres and found elastase
and pepsin attacked the amorphous material whilst collagenase and
hyaluronidase had little observable effect.

The results of this present study seem to support Fullmer's
concept that the stainable fraction of oxytalan is mucopolysaccharide.
However, it must be remembered that variation in interpreting the
results of enzyme digestions can be created by many factors such as pH,
temperature, tissue inhibitors or activators, specificity of the
enzyme, and physical structure of the enzyme and/or substrate (Pearse,
1972; Thomas and Partridge, 1960). Furthermore, the elastase
digestion of the gingival epithelium, excluding the stratum corneum,
agrees with similar findings by Fullmer and Lillie (1958). This may
suggest either a similarity of substrate between elastic fibres,
epithelial cells and intercellular cementing substance, or a non-
specificity due to the inherent nature of the enzyme or the presence
of an impurity similar to a protease as suggested by Fullmer and Lillie
(1958). Furthermore, following elastase digestion, a thin tissue
remnant persists in the gingival sulcus. This remnant appears similar
to the gingival stratum corneum and may either represent a lightly
keratinized sulcus or a dental cuticle which was described by Cran
(1972).

The results of the enzyme reactions used in this study
indicate differences and similarities between oxytalan and elastic
fibres. These comparisons probably reflect the nature of the staining

components of elastic and oxytalan fibres to aldehyde fuchsin. More
detailed investigation of the substrate reactions of the enzymes would
be required before more definitive information regarding the composition
of elastic and oxytalan fibres could be obtained.

CHAPTER 7.   CONCLUSIONS.

1. The marsupials studied displayed anatomical differences between
    their masticatory apparatuses. The wombat, wallaby and possum are
    Diprotodonts and the marsupial mice (Antechinus flavipes and
    Sminthopsis crassicaudata) are Polyprotodonts. Morphologically,
    the wombat dentition and mandibular articulation could be
    classified as rodent-like. The wallaby and possum could be
    categorized as herbivores, but the possum demonstrated some
    carnivorous dental features. The marsupial mice indicated
    carnivorous and insectivorous morphologic features. The various
    animals showed species differences and adaptation to suit their
    natural diets.

2. The wombat was the only animal studied which had a dentition entirely
    derived from persistent dental pulps. The periodontal ligament
    and supra-alveolar collagen fibre systems indicated adaptation to
    continuous tooth eruption.

3. Oxytalan fibres were found in large numbers within the marsupial
    periodontiums. This finding supports Fullmer's hypothesis that
    oxytalan fibres are a normal component of the periodontal ligament.

4. The wombat consistently contained elastic fibres within its
    periodontal ligament whilst the wallaby and possum revealed only an
    occasional elastic fibre at the level of the cemento-enamel
    junction. The marsupial mice had no elastic fibres within the
    periodontal ligament, but like all the other animals, did have
    elastic staining fibres in the supra-alveolar corium. Similarly,
    the number and size of the oxytalan fibres decreased progressively
    from the wombat, wallaby, and possum to the marsupial mice. This

observation may lend support to Fullmer's hypotheses regarding a
relationship between oxytalan and elastic fibres, and an increase
in oxytalan fibre number and size with masticatory demand.

5. The oxytalan fibres formed a continuous system extending from the
    supra-alveolar corium to the apex of all teeth. Furthermore, the
    system continued around the apex and transseptally linked adjoining
    teeth.

6. An abundant network of fine, frequently branching fibres of cemental
    origin comprised much of the oxytalan fibre system.

    (i)   Above the level of the alveolar crest, the oxytalan fibres
            predominantly followed and intertwined with the prevailing
            collagen fibre system (i.e. transseptal, circular and
            dento-gingival).

    (ii)   Within the periodontal ligament, commencing at the
            cemento-enamel junction or subjacent to the gingival
            attachment of the wombat, the oxytalan fibres cascaded
            apically and oblique to the principal collagen fibres.

    (iii)   The oxytalan fibres, after emerging from the cellular
            cementum, increased in thickness as they traversed the
            periodontal ligament.

    (iv)   The oxytalan system continued around the root apex as
            a network of very fine branching fibres.

7. In addition to the branching oxytalan fibre system there were long
    ribbon-like aggregates, or tracts, of clearly defined oxytalan
    fibres which were orientated perpendicular to the collagen bundles.
    These structures extended from the cervical to apical third regions
    of the wallaby, possum, and marsupial mouse periodontal ligaments.
    The thick fibre tracts did not extend around the root apex and were

only found above the level of the alveolar crest in the wombat.

8. The large, ribbon-like "fibres" were found nearer the alveolar bone
    than the cementum. Their arrangement strongly suggested that they
    were the result of a coalescence of finer, branching oxytalan fibres
    of cemental origin.

9. The oxytalan fibre distribution varied in specific location within
    the periodontiums of the animals studied. However, the most
    numerous and largest oxytalan fibres occurred in the apical third
    region of the periodontal ligament, and not at the coronal third as
    was stated by Fullmer (1958).

10. Small, wavy oxytalan-positive structures were seen in the immature
    pulp tissue of the continuously erupting wombat teeth and the
    incomplete apices of the wallaby and possum. This observation is
    in agreement with Fullmer's (1959b) findings in the human pulp.
    Associated with these fibres was a background of diffuse amorphous
    material, probably mucopolysaccharide, which stained with oxytalan-
    revealing stains following oxidation. No definite oxytalan fibres
    were found within the mature pulp tissue.

11. The large numbers, and the distribution of oxytalan fibres within
    the marsupial periodontal ligament are consistent with Fullmer's
    hypothesis that oxytalan fibres are related to the degree of stress
    to be resisted by the tissues. Specifically, the oxytalan fibre
    size and concentration varied in direct proportion to the assumed
    masticatory stresses generated by the diet and chewing patterns of
    the different animals.

12. Contrary to Fullmer's observations, the oxytalan fibres found in
    this study did not attach to alveolar bone. However, oxytalan
    fibres could be seen more closely approximating the alveolar

surface when blood vessels abutted or entered the alveolar bone.

13. A close morphologic association has been observed between the periodontal blood vessel wall and oxytalan fibres. Evidence for actual attachment was strongly suggested. However, the histologic techniques employed did not clarify the precise relationship. A three-dimensional reconstruction of the tissues and an electron microscopic investigation is indicated.

14. A method for three-dimensional superimposition of several histologic sections was developed but remains incomplete due to equipment limitations. Further work is suggested in this field.

15. Oxytalan fibres approximated but did not appear to attach to the basement membranes of the gingival epithelium or to the ameloblasts of the retained enamel organ in the wombat.

16. Oxytalan fibres and nerve fibres did not resemble each other with regard to morphology, distribution, and histological staining properties. Nor did oxytalan fibres correlate with reticulin.

17. Adequate similarities in histologic staining reactions existed between oxytalan and elastic fibres to support Fullmer's hypothesis that oxytalan might represent an immature or modified form of elastic fibre. However, differences existed which support another of Fullmer's conclusions - namely, that oxytalan is a distinct connective tissue entity. Considering the chemical and physical complexities of the tissues and the delicacy and uncertainty of many of the dye staining interactions, it is readily understandable why no clear cut distinctions exist between oxytalan and elastic fibres.

18. The fibres described as oxytalan in the present study fulfilled the majority of Fullmer's criteria for defining oxytalan as a distinct

connective tissue fibre. The exceptions were the observed
positive reactivity with orcinol-new fuchsin and faint staining
with periodic acid-Schiff techniques. For marsupial tissues,
Verhoeff's iron haematoxylin would appear to be the most specific
of the elastic fibre stains.

19. Of the 55 dye substances tested for oxytalan reactivity, 22 were
found to demonstrate oxytalan fibres with varying degrees of
specificity, intensity, and permanence. Bismarck brown Y, sudan
black B, alcian blue 8GX, and cresyl fast violet produced very
good staining of elastic and oxytalan fibres. Aldehyde fuchsin
and orcein produced excellent results. All oxytalan-positive dyes
were basic dyes, except chlorazol black E.

20. Pre-oxidation of the marsupial tissues enhanced elastic fibre
staining as well as revealing oxytalan fibres in the periodontium.
Pre-oxidation could conceivably increase the proportion of aldehyde
and carboxyl groups from alcohol residues present in either the
mucopolysaccharide or protein components of oxytalan fibres.

21. The addition of small amounts of acetic acid to many of the dye
solutions appeared to improve the resistance of the oxytalan
staining to alcohol dehydration. Perhaps acetic acid increased
the potential for covalent bonding between the dyes and reactive
tissue groups, or merely influenced the pH, or reaction equilibrium
to favour tissue staining.

22. No relationship between dye class and oxytalan staining was
apparent. However, the pH of the staining solution did appear to
be an important factor in the staining reaction.

23. The precise nature of the type of bond and reactive groups
involved in oxytalan staining are unsure and possibly quite

different for each dye and each dye solution. However, Fullmer's contention that the stainable fraction of oxytalan is a mucopolysaccharide was supported by appearance, enzyme digestions, and the dye staining reactions of the marsupial oxytalan fibres.

24. The possibility that marsupial oxytalan structures differ bio-chemically from the eutherian material reported by other workers, was substantiated by the large number and the variety of dyes found to react positively for marsupial oxytalan. The possibility that marsupials, like rodents, have increased proportions of stainable aldehyde groups in elastic and oxytalan fibres was supported by reactivity with the periodic acid-Schiff technique before and after tissue oxidation.

25. Methylation and saponification experiments indicated the presence of carboxyl groups in the marsupial oxytalan fibres.

26. Most authors conclude oxytalan and elastic fibre staining to be non-ionic in nature. The methods employed in the present study did not disprove the concept of covalent bonding and did lend some support to the possibility that both physical and chemical factors could be involved with the dye/tissue interactions.

27. Further investigations should be conducted to improve the quality of some of the staining techniques. However, on the basis of morphology, oxytalan staining has been observed in the marsupials examined, with the following dyes -

        alcian blue 8GX, alcian yellow GX, alcian green 2GX,

        astra blue, azure A, bismarck brown Y, celestin blue B,

        chlorazol black E, cresyl fast violet, cresyl fast violet

        acetate, crystal violet, methyl violet 6B, neutral red,

        nile blue, sudan black B, thionin, toluidine blue,

orcinol-new fuchsin, and in the wombat and wallaby the periodic acid-Schiff technique.

28. The staining results obtained for marsupial material should be repeated using eutherian material.

CHAPTER 8.   APPENDICES.

8.1.   APPENDIX I: Lists of elastic fibre dyes.

8.1.(a)   TABLE 13:

Dyes found to stain elastic fibres by Horobin and James (1970).

Dye	Class	Charge per molecule	Number of aromatic rings	Number of hydrogen bonding groups
Orcein	oxazole	1+	e.g.5	e.g.4
Weigert's resorcin fuchsin	triphenyl-methane	n+	>3	?
Aldehyde fuchsin	triphenyl-methane	n+	>3	?
Verhoeff's iron haematoxylin	metal complex	0	4	4
PTA haematoxylin	metal complex	n-	4	4
Chlorantine Fast Red	disazo	2-	5	1
Chlorazol Black E	trisazo	2-	6	4
Congo Red	disazo	2-	6	2
Mucicarmine	metal complex	n+	6	?
Aniline Blue	triphenyl-methane	3-	5-6	2
Amido Black 10B	disazo	2-	4	2
Azocarmine G	azine	1-	6	1
Biebrich Scarlet	disazo	2-	4	1
Bismarck Brown	disazo	2+	3	2
Chicago Blue 6B	disazo	4-	6	4
Chlorantine Fast Green BLL	complex	4-	9	5
Direct Blue 10	disazo	2-	6	6
Direct Blue 15	disazo	4-	6	6
Direct Blue 152	disazo	2-	6	4

TABLE 13 (continued)

Dye	Class	Charge per molecule	Number of aromatic rings	Number of hydrogen bonding groups
Direct Violet 37	disazo	2-	6	6
Durazol Blue 8G150	phthalo-cyanine	3-	8	0
Durazol Paper Blue 10G	phthalo-cyanine	3-	8	0
Elastin Purple FP*	oxazole	2+	5	5
Evan's Blue	disazo	4-	6	4
Isamine Blue	triphenyl-methane	2-	7	4
Luxol Fast Blue B	phthalo-cyanine	n-	8	0
Luxol Fast Blue G	trisazo	4-	8	1
Magdala Red	azine	1+	7	1
Methyl Blue	triphenyl-methane	3-	6	2
Monsol Fast Blue GS	phthalo-cyanine	3-	8	0
Naphthol Black B	disazo	4-	6	1
Primuline	thiazole	1-	5	1
Resorcin Blue*	oxyzole	1+	5	5
Saffron	polyene	2-	0	0
Sudan Black B	disazo	0	5	2
Thioflavin S	thiazole	n-	5	2
Trypan Blue	disazo	4-	6	4
Victoria Blue B*	triphenyl-methane	1+	5	1
Victoria Blue 4R*	triphenyl-methane	1+	5	0
Vital Red	disazo	3-	6	2

* (see Lillie 1969a)

8.1(b) TABLE 14:

A list of dyes which have been successful in demonstrating elastic fibres (from Pearse, 1968).

Dye introduced by	Dye used	
Unna (1886)	0.5% dahlia in alcoholic HNO_3.	
Koppen (1889)	5% crystal violet in 5% phenol.	
Tänzer (1891)	1% orcein in acid alcohol (HCL) pH 1.3.	
Pranter (1902)	1% orcein in acid alcohol (HNO_3) pH 0.9.	
Weigert (1898)	0.25% resorcin fuchsin in acid alcohol.	
Harris (1902)	Mucicarmine with acid alcohol differentiation.	acid staining
Michaelis (1901)	Resorcin and other phenols with fuchsin, safranin, methyl violet etc.	
Verhoeff (1908)	Iron Haematoxylin.	
Matsuura (1925)	Phosphotungstic acid-congo Red.	
Petry (1952-3)	Phosphomolybdic acid-haematoxylin.	
Gomori (1950)	Aldehyde fuchsin.	
Klüver and Barrera (1953)	Alcohol Luxol Fast Blue.	neutral staining
Herxheimer (1886)	Lithium carbonate-haematoxylin.	
Salthouse (1944)	Ammoniacal Chlorazol Black.	
Witke (1951)	Alkaline Primulin (fluorescence).	alkaline staining
Lautsch et al. (1953)	Alcoholic Chlorazol Black - Benzazurin.	

8.2. APPENDIX II: The animals studied

The following are brief summaries of the zoologic features of
marsupials used in the present study.

8.2.(a) The Hairy Nosed Wombat - (Lasiorhinus latifrons) from Ride
(1970).

i. Description:-

a. Body size - ranges in size up to 1.25 metres in
length and 27 kilograms in weight.

b. Features - has the largest, most pointed ears of
all species of wombat. Body hair is fine and
silky. The nose is covered with soft, short hair.

c. Behaviour - a powerful, but docile, burrowing
animal which is not gregarious. Usually has one
offspring in summer. The young are independent
after about 4 months.

ii. Habitat Distribution:-

A plains dweller which is physiologically well
adapted to desert areas. e.g. Nullabor, Murray Valley,
Eyre and Yorke Peninsulas.

iii. Diet:-

Entirely vegetarian (herbivorous)

8.2.(b) The Tammar, or Dama Wallaby - (Macropus eugenii)
From Marlow (1962) and Wood-Jones (1924-1925).
Until recently this animal was called Thylogale eugenii and
was first described by Desmarest (1817). This animal is a
member of the subclass Metatheria (Marsupiala) and the
family Macropodidae, of which there are 17 genera and 52
species including kangaroos, "rat" kangaroos, wallabies and

wallaroos.

i.   Description:-

    a. Body size - it is the smallest species of wallaby. Head-body length is 60 cm and tail length is 40 cm.

    b. Features - the snout is naked.

    c. Colour - the head and body are grizzled grey, the belly is grey-white, the shoulders are rufous and there is a faint, dark dorsal stripe. The tail is grey with a dark tip.

ii.   Habitat Distribution:-

This is mainly restricted to the southern portions of Australia, including the tip of Eyre Peninsula, St. Peter's Island, Nuyt's Archipelago, Kangaroo Island, Wallaby Island, Houtmann's Abrohls and the south-west corner of Western Australia from Geraldton to Hopetown. It lives mainly in sclerophyll forest and well timbered areas.

iii.   Diet:- herbivorous.

8.2.(c)   The Brush Tailed Possum - (Trichosaurus vulpecula)

From Walker (1964). There are two species (1) T. caninus and (2) T. vulpecula.

i.   Description:-

    a. Body size - head to body length varies from 30 cm to 60 cm. The tail is about 25 cm to 35 cm in length.

    b. Features - tail is furry, prehensile and naked on the inner surface.

    c. Colour - variable over a wide range including grey, black and white, cream, with males sometimes having

a reddish tinge over the shoulders.

    d. Behaviour - a powerful, vicious fighter. Prolific
breeder all year round. Young leave the pouch at
4 months and ride on the mother's back till about
6 months old.

ii. <u>Habitat Distribution</u>:-

Usually found in forest areas, but may be found in
treeless areas, in caves, and other animal burrows.
Also found in semi-arid areas of Central Australia
near water courses and eucalypts. Frequently found
in cities, parks and roofs of houses.

iii. <u>Diet</u>:-

Mainly herbivorous (shoots, leaves, flowers, fruits).
Also eats seeds, insects and even kills young birds.

8.2.(d) <u>The Mardo, which is also known as the Yellow Footed Antechinus,
the Broad Footed Marsupial Mouse, or the Yellow Footed
Marsupial Mouse</u> - (Antechinus flavipes).

From Walker (1964) Subclass (Metatheria), family (Dasyuridae).

First described by McLeay (1841). One of about 10 species.

i. <u>Description</u>:-

    a. Body size - head to body about 10 cm to 15 cm.
Tail length about 10 cm to 15 cm.

    b. Features - short, dense, coarse fur. Short,
broad feet. Tail is slender. Nocturnal, poorly
developed pouch, partly arboreal and hence has
well developed foot pad striations.

    c. Colour - varies from pale, pinkish fawn, to grey,
to copper-brown. Belly is creamy to white and
upper surfaces of feet are off-white to yellow.

ii.   Habitat Distribution:-

Mainly in thinly wooded country, around sandstone
outcrops and in open forests of Australia, Tasmania
and New Guinea.

iii.   Diet:-

Carnivorous. Preys on other mice and eats nectar.

8.2.(e)   The Dunnart, which is also known as the Narrow Footed
Marsupial Mouse, or the Pouched Mouse - (Sminthopsis
crassicaudata). From Walker (1964). First described by
Thomas (1887). One of about about 10 recognised species.

i.   Description:-

a.   Body size - head to body length is about 7 cm to
12 cm and the tail length is approximately 5 cm
to 7 cm.

b.   Features - soft, fine, but dense fur. Tail is
stumpy at the base. The pouch is better developed
than in some other marsupial mice. Nocturnal,
hops on its hind legs at top speed but has a
peculiar quadrupedal ramble at slow speeds. The
tail is held in a distinctive upward curve. The
feet are slender and the foot pads striated or
granular.

c.   Colour - back and sides are buff to grey with
greyish-white to white belly. There is a dark
median facial stripe.

ii.   Habitat distribution:-

Ranges from dry, sandy deserts to humid forests in
Australia, Tasmania, Southern New Guinea and the
Aru Islands.

iii.   <u>Diet</u>:-

Mainly insectivorous but sometimes eats lizards and

smaller mice.

8.3. APPENDIX III: Histologic preparation of the specimens.

8.3.(a) The buffered neutral formalin solution was made to the

following formula -

37-40% formalin	100ml.
distilled water	90ml.
sodium dihydrogen phosphate	4.0gm.
disodium hydrogen orthophosphate	6.5gm.

8.3.(b) Decalcifying Solution (40% formic/formate) -

sodium formate	7.0gm.
distilled water	100ml.
formic acid	40ml.

8.3.(c) The tissues were processed at 37°C using the Double Embedding

Technique. After neutralization in 5% sodium sulphate, the

decalcified sections were treated according to the following

stages:

1.	70% ethanol	overnight
2.	80% ethanol	one hour
3.	90% ethanol	one hour
4.	95% ethanol	one hour
5.	absolute ethanol	one hour (minimum)
6.	absolute ethanol	one hour (minimum)
7.	absolute ethanol	one hour (minimum)
8.	one part absolute ethanol one part methyl salicylate	} one hour (minimum)
9.	methyl salicylate plus 0.5% celloidin	} 2 days
10.	methyl salicylate plus 1% celloidin	} 2 days (minimum)

8.3.(d)   Paraffin Embedding for blocking:-

1. Two-thirds methyl salicylate
                                      } one hour (minimum)
    One-third paraffin wax

2. One-half methyl salicylate
                                      } one hour
    One-half paraffin wax

3. One-third methyl salicylate
                                      } one hour
    Two thirds paraffin wax

4. Paraffin wax (first change)   two hours

5. Paraffin wax (second change)   two hours

6. Paraffin wax (third change)   two hours (minimum for

                                         larger tissues).

These specimens were then vacuumed in clean paraffin wax at
56°C for 15-60 minutes prior to blocking.

8.4. APPENDIX IV: Conventional staining techniques.

8.4.(a)   HAEMATOXYLIN and EOSIN:

The haematoxylin solution was made to the formulation of Harris (Luna, 1968: p.34).

Eosin Y (G.T. Gurr) was made to a 1% aqueous solution.

1. Deparaffinize and hydrate to distilled water.

2. Harris' haematoxylin (15 minutes).

3. Wash in tap water (10-15 minutes).

4. Eosin (30 seconds or till desired counterstain).

5. Dehydrate 95% ethanol, absolute ethanol (2 changes).

6. Clear in xylol and mount with Xam.

8.4.(b)   ALDEHYDE FUCHSIN: (modified from Fullmer and Lillie, 1958).

Preparation: Add 1.0ml of concentrated hydrochloric acid and 1.0ml of paraldehyde (U.S.P.) to 100 ml of 0.5% solution of basic fuchsin (G.T.Gurr) in 60-70% ethanol. Allow to stand at least 24 hours before using.

1. Deparaffinize and bring the sections to water.

2. Oxidize in 10% aqueous solution of Oxone (Du Ponts' monopersulphate compound) for 90 minutes.*

3. Wash in running water (for 2 minutes).

4. Stain oxidized and control sections with Gomori's aldehyde fuchsin (8 minutes).

5. Rinse in 2 changes of 95% ethanol and leave in a third change for 5 minutes.

6. Rinse in acid/alcohol for 30 seconds**

   70% ethanol.....................100ml.

   concentrated hydrochloric acid.....4ml.

7. Rinse in running water (2 minutes).

8. Counterstain in the following solution modified by

Halmi for 5-10 seconds***

        distilled water .... 100ml.

        light green SF .... 0.2gm.

        orange G .... 1.0gm.

        phosphotungstic acid ..... 0.5gm.

        glacial acetic acid ...... 1.0ml.

9. Rinse briefly in 0.2% glacial acetic acid in 95%

ethanol.

10. Dehydrate in ethanol, clear xylol and mount in Xam.

Modifications:

* Fullmer and Lillie originally used Greenspan's peracetic

acid for 10-30 minutes. Rannie (1963) substituted 10%

Oxone for 90 minutes.

** Fullmer and Lillie used 70% ethanol. Löe and Nuki used

acid-alcohol.

*** Fullmer and Lillie originally counterstained with Halmi

for 15 seconds.

Staining reactions:

Elastic fibres, oxytalan, mucins....deep purple.

Other tissues take up the green and yellow of the counterstain.

8.4(c) ORCEIN: (modified from Fullmer 1959b).

Preparation: To 1.0gm of natural orcein (G.T. GURR) in 100ml

of 70% ethanol, add 1.0ml of concentrated hydrochloric acid.

Incubate for 1 hour at 60°C. Then filter the solution.

1. Deparaffinize and bring sections to water.*

2. Oxidize in 10% Oxone for 90 minutes (or longer).**

3. Wash in running water (2 minutes).

4. Stain oxidized and control sections in orcein for 30-60 minutes at 50°C.***

5. Differentiate in 70% ethanol for 5 minutes (3 changes).

6. Sections to water.

7. Counterstain with modified Halmi (1 second) or light green (10 seconds).

8. Rinse briefly in 0.2% glacial acetic acid in 95% ethanol.

9. Dehydrate, clear and mount in Xam.

Modifications:

* Fullmer originally brought the sections to absolute ethanol.

** Originally 10-30 minutes of peracetic acid.

*** Fullmer initially stained with orcein for 15 minutes at 37°C.

Preparation of light green:

Make a solution of 0.2gm of light green SF (G.T. Gurr) in 200ml of 95% ethanol.

Staining reactions:

Elastic and oxytalan fibres..........brown-brownish purple.

Keratin, dentinal tubules..........brown.

Bone, dentine..........light green.

Collagen and epithelial cells..........bluish green.

8.4.(d) RESORCIN FUCHSIN: (modified from Weigert's technique contained in Luna, 1968: p.80).

Preparation: 1. Stock solution,

basic fuchsin....................2.0gm.

resorcinol.......................4.0gm.

distilled water..................200ml.

Mix the ingredients in a porcelain dish, bring to the boil for one minute, then add 2ml of 29% ferric chloride solution.

Cool, filter and leave the precipitate to dry on the filter paper. Return the filter paper to the porcelain dish, which should be dry but still contains whatever part of the precipitate remains adherent to it. Add 200ml of 95% alcohol and heat very carefully, dissolving all precipitate from the filter paper before discarding it. Add 4.0ml of hydrochloric acid. The solution keeps for several months.

2. working solution,

stock solution...................10.0ml.

70% alcohol.....................100ml.

concentrated hydrochloric acid...2.0ml.

1. Deparaffinize and hydrate to distilled water.

2. Oxidize in 10% Oxone for 90 minutes.

3. Stain oxidized and control sections in resorcin fuchsin working solution for 2 hours or more.*

4. Rinse in 95% ethanol.

5. Wash in tap water.

6. Counterstain with Halmi for 1 second or light green for 10-15 seconds.

7. Rinse with 0.2% acetic acid in 95% ethanol.**

8. Dehydrate, clear and mount in Xam.

Modifications:

* Luna suggested only 30 minutes staining time.

** Luna used only 95% ethanol.

Staining reactions:

Elastic and oxytalan fibres..........purple-black.

8.4.(e) ORCINOL-NEW FUCHSIN: (from Fullmer, 1956)

Preparation: Add 2.0gm of new fuchsin (Magenta III C.I. 42520)

and 4.0gm of orcinol to 200 cc of distilled water, boil for 5

minutes and add 25cc of Liquor Ferri Chloride (U.S.P. IX) and

boil for 5 minutes or more (or use 15.5gm of $FeCl_3.6H_2O$ and

water to make 25cc if U.S.P. unavailable). Cool, collect the

precipitate on filter paper and dissolve it in 100cc of 95%

ethanol. If orcinol-new fuchsin is commercially available,

use 2.0gm of dye in 100cc of 95% ethanol as the staining

solution.

1. Deparaffinize and bring sections to absolute ethanol.

2. Oxidize in 10% Oxone for 90 minutes.

3. Stain oxidized and control sections in orcinol-new

    fuchsin for 45 minutes at $37^{o}C$.

4. Wash in 3 changes of 70% ethanol.

5. Dehydrate, clear and mount in Xam.

Modification:

* Fullmer originally stained for 15 minutes.

Staining reactions:

Elastic fibres and keratin..........dark purple-pink.

Oxytalan fibres..........dark purple-maroon.

8.4.(f) VERHOEFF'S IRON HAEMATOXYLIN: (after Verhoeff)

The preparation and staining technique are contained in

Culling (1974): pages 421-422.

Staining results:

Elastic fibres and nuclei..........black to blue-black.

Cytoplasm and muscle...............yellow.

Collagen...........................red.

8.4.(g)   <u>IRON-ORCEIN</u>:

Preparation and staining procedures were closely followed

according to the techniques of

      (1) Roman, Perkins, Perkins and Dolnick (1967), and

      (2) Lillie, Gutierrez, Madden and Henderson (1968).

<u>Staining reactions</u>:

Elastic fibres (both coarse and fine)..........purple-black.

8.4.(h)   <u>VAN GIESON COLLAGEN STAIN</u>:

The dye preparation and staining technique followed the

standard method described in Luna (1968): p.76.

<u>Staining reactions</u>:

Collagen....................red.

Muscle and keratin.........yellow.

Nuclei....................blue to black.

8.4.(i)   <u>POLLACK'S TRICHROME FOR CONNECTIVE TISSUES</u>:

Preparation and usage are described in Luna (1968): pages

116-117.

<u>Staining results</u>:

Muscle and elastic fibres..........red.

Collagen, cartilage, bone.........green.

Keratin...........................orange-red.

8.4.(j)   <u>MALLORY'S METHOD FOR COLLAGEN</u>:

Preparation and staining procedure were followed according to

Luna (1968): pages 75-76.

<u>Staining results</u>:

Collagen, reticulin, and basophil granules.......deep blue.

Cartilage, mucin, amyloid.........................lighter blue.

Fibrin...red.

Red blood cells..........orange-vermillion.

Nuclei.................blue-purple.

8.4.(k)   SILVER STAIN FOR RETICULIN:

The procedure described by Naoumenko and Feigin (1974) was

closely followed with one modification.*

*   the tissues were toned in gold chloride (0.5% aqueous)

for 2 minutes.

Staining results:

Reticulin.............black.

Collagen..............light grey.

Bone, dentine.........brown.

8.4.(1)   METHODS FOR NERVE FIBRES:

The techniques and preparations for (1) Bielschowsky,

(2) Hirano-Zimmerman, and (3) Bodian, were followed as

described in Luna (1968): pages 193-194, 198-199 and 195-196

respectively.

Staining results:

Nerve fibres..........black.

Background............varied with the technique from blue to

purple.

8.4.(m)   PERIODIC ACID-SCHIFF REACTION:

The technique and method were followed as described in Luna

(1968): pages 159-160 with modifications.*

*   the haematoxylin and light green staining steps were

omitted.

Staining results:

P.A.S. positive material including carbohydrates, reticulin

and basement membranes ....rose to purplish red.

Elastic fibres and oxytalan in marsupials.....pale pink.

8.4.(n)   HALE'S COLLOIDAL IRON:

Preparation: stock colloidal iron solution -

To 250 cc of boiling distilled water add 4.4cc of 29% $FeCl_3$

(U.S.P.XVI) and stir. When the solution has turned dark red,

remove from the flame and allow to cool. The water must be

kept on the boil during addition of $FeCl_3$ otherwise the

formation of colloidal (hydrous) Fe_2O_3 will be incomplete.

This stock is stable for several months. Allow to cool and

dialyze for 24 hours against three changes of distilled water

5-10 times the volume of the iron solution. Filter.

working solution:

glacial acetic acid............ 5cc.

distilled water................15cc.

stock colloidal iron...........20cc.

1. Deparaffinize sections to distilled water.

2. Rinse briefly in 12% acetic acid (30 seconds).

3. Treat test section(s) for 60 minutes in a freshly made
    working solution of colloidal iron. Do not place control
    section(s) in colloidal iron.

4. Rinse sections for 3 minutes in each of 4 changes of 12%
    acetic acid.

5. Treat test section plus control in fresh solution of 50:50
    2% hydrochloric acid and 2% potassium ferrocyanide for 20
    minutes at room temperature.

6. Wash in running water for 5 minutes.

7. Counterstain as desired (e.g. Harris' Haematoxylin or
    P.A.S.).

8. Dehydrate clear and mount.

Staining reactions:

Acid mucopolysaccharides..........bright blue.

The reaction demonstrates acid mucins well, but is not as

specific as alcian blue.

8.4.(o)   KERATIN AND PRE-KERATIN:

The technique of Ayoub-Shklar was followed as described by

Luna (1968): pages 82-83.

Staining reactions:

Keratin and pre-keratin..........brilliant red.

Epithelial cells................poorly stained.

8.5. APPENDIX V:

TABLE 15:

Code used to systematise the recording of histologic sections for the presence of oxytalan fibres.

FIBRES	DISTRIBUTION
density	none $\quad$ indefinite $\quad$ sparse $\quad$ moderate $\quad$ dense
	0 $\qquad$ (+) $\qquad$ + $\qquad$ ++ $\qquad$ +++
attachment	free-end $\quad$ bone $\quad$ blood vessel $\quad$ cementum
	− $\qquad$ B $\qquad$ Bv $\qquad$ C
size	fine $\quad$ fine-branched $\quad$ thick $\quad$ thick-branched
	F $\qquad$ Fb $\qquad$ T $\qquad$ Tb
location	apical$\frac{1}{3}$ $\quad$ middle$\frac{1}{3}$ $\quad$ cervical$\frac{1}{3}$ $\quad$ gingival $\quad$ transseptal
	A/3 $\qquad$ M/3 $\qquad$ C/3 $\qquad$ G $\qquad$ Ts
	bifurcation $\quad$ pulp
	Bf $\qquad$ P
aspect	mesial $\quad$ distal $\quad$ buccal $\quad$ lingual
	M $\qquad$ D $\qquad$ Bu $\qquad$ Li
direction to the main collagen groups	oblique $\quad$ parallel $\quad$ apico-occlusal $\quad$ circular
	Ob $\qquad$ Pa $\qquad$ A-O $\qquad$ Ci

8.6. APPENDIX VI: Enzyme digestions.

8.6.(a) β-Glucuronidase:

Enzyme: from bovine liver type B-I (Sigma Chemical Company).
Activity is 530,000 Fishman units/gram.

Preparation: 15gm per 50cc of 0.1M acetate buffer (pH 4.5)
incubated for 48 hours at 37°C.

The acetate buffer was prepared from tables contained in Barka
and Anderson (1963).

8.6.(b) Elastase:

Enzyme: from hog pancreas, type III, chromatographically
purified. Lyophilized, water soluble powder (Sigma Chemical
Company). Activity is approximately 60 units/milligram.

Preparation: .01% solution of elastase in Tris buffer
(pH 8.8) and incubated for 60 minutes at 37°C.

The Tris(hydroxymethyl)aminomethane − "Universal Buffer" − was
obtained from the Sigma Chemical Company and made to pH 8.8
according to buffer tables in Barka and Anderson (1963).

8.6.(c) Collagenase:

Enzyme: extracted from Clostridium histolyticum, type I
practical grade (Sigma Chemical Company). Activity − 5 mg will
solubilize 50mg of collagen at pH 7.5 in 20 hours at 37°C.

Preparation: 1mg collagenase/cc of 0.9% sodium chloride and
incubated in humid conditions at 50°C for 45−60 minutes.

8.6.(d) Pepsin:

Enzyme: 1:2,500 powder, PKg free (B.D.H. Chemical Company).

Preparation: 2mg pepsin/cc of 0.02 N hydrochloric acid at
pH 1.6 and incubated for 2−3 hours at 37°C.

8.6.(e)   Hyaluronidase:

Enzyme: from Ovine testes, type II (Sigma Chemical Company).
Activity of 550NF units/mg.

Preparation: 1mg hyaluronidase/cc of 0.85% saline solution and
incubated for 3 hours at 37°C.

For effect upon oxytalan stainability, incubation should
continue longer (e.g. 24 hours).

8.6.(f)   Neuraminidase:

Enzyme: from Clostridium perfringens, type V (Sigma Chemical
Company). 1 unit will liberate 1.0 μ mole of N-acetyl
neuraminic acid/minute at pH 5 and 37°C.

Preparation: 500 units/cc neuraminidase diluted with an equal
volume of 0.1 M acetate buffer, containing approximately 1%
NaCl and 0.1% CaCl$_2$ at pH 5.5 for 16-24 hours at 39-41°C.
(Pearse 1972).

The acetate buffer was made according to tables in Barka and
Anderson (1963).

8.6.(g)   Bromelain:

Enzyme: from pineapple, practical grade II (Sigma Chemical
Company). Activity is 1800 units/mg.

Preparation: 0.5% bromelain in 0.9% sodium chloride solution
(pH 6.0) and incubated for 3 hours at 37°C.

8.7. APPENDIX VII: Additional oxytalan positive dye solutions.

8.7.(a) Alcian Blue:

Preparation: (a) alcian blue pH 1.0 (from Luna, 1968: p.164).

1% alcian blue 8GX (G.T. Gurr) 0.1N hydrochloric acid.

1. Deparaffinize and hydrate to distilled water.

2. Oxidized and control sections are stained for 30 minutes.

3. Blot the sections dry with filter paper - no water rinse.

4. dehydrate in ethanol, clear and mount in Xam.

Staining reactions:

This method provides a more selective staining of sulphated

muco-substances which stain dark blue.

Elastic fibres, oxytalan..........bright blue.

Preparation: (b) alcian blue pH 2.5 (modified from Luna, 1968:

p.163). 1% alcian blue 8GX in 3% acetic acid solution. Adjust

pH to 2.5.

1. Deparaffinize and hydrate to distilled water.

2. Stain oxidized and control sections in alcian blue solution

    (40 minutes).

3. Wash in water.

4. Dehydrate ethanol, clear xylol and mount in Xam.

Staining reactions:

Acidic sulphated muco-substances, hyaluronic acid and sialo-

mucins are dark blue.

Elastic fibres, oxytalan..........dark blue (more specific than

alcian blue pH 1.0).

8.7.(b) Alcian Green:

Preparation: as for alcian blue pH 1.0. 1% alcian green 2GX

(G.T. Gurr) in 0.1N hydrochloric acid.

Staining reactions:

Elastic fibres, oxytalan.........apple green.

Dye specificity is improved when made in a 3% acetic acid
solution.

8.7.(c) Alcian Yellow:

Preparation: as for alcian blue pH 1.0. 1% alcian yellow GX
(G.T. Gurr) in 0.1N hydrochloric acid.

Staining reactions:

Elastic fibres, oxytalan..........pale yellow-gold.

Again the dye specificity can be improved when used as a 3%
acetic acid solution.

8.7.(d) Astra Blue:

Preparation: 1% astra blue (G.T. Gurr) in 0.1N hydrochloric
acid.

1. Deparaffinize sections and hydrate to water.

2. Astra blue solution for 40-45 minutes.

3. Mount in dye or water and examine as soon as possible.

Staining reactions:

Elastic fibres, oxytalan..........turquoise blue.

Dye specificity can be improved by addition of acetic acid.

8.7.(e) Azure A:

Preparation: 1% azure A (Chroma Co.) in 0.1N hydrochloric acid.

1. Deparaffinize and hydrate to water.

2. Stain oxidized and control sections with azure A solution
    for 40-45 minutes.

3. Rinse in distilled water.

4. Mount in distilled water (coverslip, blot around edges,
    and seal with fingernail polish or Xam).

5. Examine sections as soon as possible as dye fades.

Staining reactions: (Metachromatic).

Elastic fibres.........light blue.

Oxytalan...............lighter blue.

Keratin................purple-blue.

8.7.(f) Bismarck Brown Y:

Preparation: 1% bismarck brown Y (Chroma Co.) in 0.1N hydro-

chloric acid.

1. Deparaffinize and hydrate to water.

2. Stain oxidized and control sections in bismarck brown

    solution for 40 minutes.

3. Wash in water.

4. Quickly dehydrate in ethanol, clear and mount in Xam.

Staining reactions:

Elastic fibres, oxytalan.........chocolate brown.

This is a very good stain for oxytalan.

8.7.(g) Celestin Blue B:

Preparation: A. 1% celestin blue B (Chroma Co.) in 0.1N

                   hydrochloric acid.

               B. 1% celestin blue B in 3% acetic acid.

1. Deparaffinize and hydrate to water.

2. Stain oxidized and control sections for 30 minutes or as

    long as it takes to see fibres.

3. Quickly blue in distilled water and coverslip the section

    (Solution A).

4. Wash in water, dehydrate in ethanol, clear and mount Xam.

    (Solution B).

Staining reactions:

Elastic fibres, oxytalan.........grey (A), violet-blue (B).

Dentine.........violet-blue (B). Bone..........blue (B).

This is not a very satisfactory technique with solution A.

However, metachromasia and excellent oxytalan staining occur

with solution B.

8.7.(h) Chlorazol Black E:

Preparation: 3% aqueous chlorazol black E (Chroma Co.) plus

4ml of 1N hydrochloric acid/50 ml of solution.

1. Deparaffinize and hydrate to water.

2. Stain oxidized and control sections with chlorazol black E

    solution for 45 minutes.

3. Wash in distilled water.

4. Dehydrate in ethanol, clear and mount.

Staining reaction:

Elastic fibres, oxytalan.........black.

Only fine oxytalan structures are seen which geographically

correspond to the thick oxytalan tracts seen with aldehyde fuchsin.

8.7.(i) Cresyl Fast Violet:

Preparation: A. .05% cresyl fast violet (G.T. Gurr) in

                  0.1N hydrochloric acid.

              B. 0.6% cresyl fast violet (G.T. Gurr) in 0.25%

                  acetic acid solution.

1. Deparaffinize and hydrate to water.

2. Stain oxidized and control sections in either solution (A)

    for 30 minutes or in solution (B) for 20 minutes.

3. Those sections stained in solution (A) should be cover-

    slipped and mounted in dye solution.

4. Those sections stained in solution (B) are rinsed in water,

    dehydrated quickly in ethanol, cleared in xylol and mounted

    in Xam.

Staining reaction:

Elastic fibres, oxytalan.........violet-purple.

The addition of acetic acid in solution (B) seems to improve the intensity of staining as well as increasing resistance to ethanol extraction.

8.7.(j) Cresyl Fast Violet Acetate

Preparation, staining method and staining results are virtually identical to those of solution (A) of cresyl fast violet. Solution (B) gives better results than solution (A) of cresyl fast violet acetate.

8.7.(k) Crystal Violet:

Preparation: 1% crystal violet (G.T. Gurr) in 0.1N hydrochloric acid.

1. Deparaffinize and hydrate sections to water.

2. Stain oxidized and control sections in crystal violet for 30 minutes.

3. Sections are coverslipped in dye solution.

Staining reaction:

Elastic fibres, oxytalan.........maroon-purple.

Epithelium......................dark purple.

Addition of acetic acid to a less concentrated solution will improve staining and ease of observation of the tissues.

8.7.(1) Methyl Violet:

Preparation: 1% methyl violet 6B (B.D.H.) in 0.1N hydrochloric acid.

1. Deparaffinize and hydrate to water.

2. Stain oxidized and control sections with methyl violet solution for 20 minutes.

3. Sections can be either mounted in dye solution or else

quickly dehydrated in ethanol, cleared, and mounted in Xam.

However, dye is rapidly lost in ethanol, acetone or methanol.

Staining reaction:

Elastic fibres, oxytalan..........blue-purple.

8.7.(m) Neutral Red:

Preparation: 1% neutral red (G.T. Gurr) in 0.1N hydrochloric
acid. Staining technique is the same as methyl violet and neutral
red is also lost in ethanol very easily.

Staining reaction:

Elastic fibres, oxytalan..........red-magenta.

When used in the neutral red-fast green technique of Monroe and
Frommer (1967), oxytalan and elastic fibre staining becomes
patchy and indistinct.

8.7.(n) Nile Blue:

Preparation: .05% nile blue (G.T. Gurr) in 1% sulphuric acid
(from Pearse, 1972 and modified from Lillie, 1956).

1. Deparaffinize sections and hydrate to water.

2. Stain oxidized and control sections in nile blue solution
    for 30 minutes.

3. Rinse in water or coverslip directly in dye solution.

4. Coverslip in water and seal.

Staining reaction:

Elastic fibres, oxytalan..........navy blue.

Bone, dentine...................mauve-purple.

Slightly metachromatic. Dye staining of oxytalan is easily lost
in ethanol.

8.7.(o) Sudan Black B:

Preparation: 2% sudan black B (G.T. Gurr) in 95% ethanol plus

lcc of concentrated hydrochloric acid/50ml of solution.

1. Deparaffinize and hydrate sections to water.

2. Stain oxidized and control sections in sudan black solution
   for 15-30 minutes.

3. Rapidly dehydrate in ethanol, clear, and mount in Xam.

Staining reaction:

Elastic fibres, oxytalan.........dark brown, black.

8.7.(p) Thionin:

Preparation: 1% thionin (B.D.H.) in 0.1N hydrochloric acid.

1. Deparaffinize and hydrate to water.

2. Stain oxidized and control sections in thionin solution
   for 30 minutes.

3. Wash briefly in water and coverslip. The sections should
   be observed as soon as possible.

Staining reactions:

Elastic fibres, oxytalan.........purple.

Collagen, fibroblasts............blue.

This is not a very satisfactory technique and it might be better
to use thionin similarly to toluidine blue.

8.7.(q) Toluidine Blue:

Preparation: 0.1% toluidine blue (G.T. Gurr) in distilled
water (from Luna, 1968: p.162).

1. Deparaffinize and hydrate the sections to water.

2. Stain oxidized and control sections with toluidine blue
   solution for 20 minutes.

3. Rinse in distilled water.

4. Coverslip in distilled water and seal around the edges with
   fingernail polish or Xam. The sections should be examined
   as soon as possible.

Staining reactions:

Acid mucopolysaccharides..........pink.

Elastic fibres, oxytalan..........pink-purple.

8.8. APPENDIX VIII:

8.8.(a) TABLE 16:

Spectrophotometric analysis of representative staining
solutions for the dyes used in this study.

DYE	WAVELENGTH OF ABSORPTIVE PEAKS
Acid fuchsin	(210-212), (547-550), (289-293), (251-253).
Alcian blue 8GX	(213-217), (256-259), (608-616), (330-335).
Alcian green 2GX	(209-212), (257-259), (384-386), (616-624).
Alcian yellow GX	(205-208), (256-259), (390-394).
Amido black 10B	(620-622), (602-606), (203), (319-323), (396-400), (440-448).
Aniline blue WS	(608-616), (204-206), (319-321), (283-285), possible peaks (640), (652), (520-530).
Anthracene blue WR	(204-207), perhaps (475-485) long, flat curve.
Astra blue	(216-218), (257-258), (610), (330-333).
Azure A	(652-654), (606), (288), (244).
Basic fuchsin	
(i) aldehyde fuchsin	(542-546), (247-249).
(ii) P.A.S.	(201-203), (243-245), (540-544), (284-287).
Benzo fast pink 2BL	(203-205), (231-234), (526-532), (612-616), (670).
Biebrich scarlet	(204-206), (226-229), (506-509), (352-356), (272-276).
Bismarck brown Y	(444-448), (206-211).
Celestin blue B	(536-540), (246-248), (279-281), (204-206), (345-355).
Chlorazol black E	(202-203), (500-600) continuous absorption over the visible spectrum, (320-325).
Congo red	(202-204), (500-504), (340-345), (335-337).

TABLE 16: (continued)

DYE	WAVELENGTH OF ABSORPTIVE PEAKS
Cresyl fast violet	(560-564), (592-596), (270), (226-229), (317-320).
Cresyl fast violet acetate	(558-564), (582-586), (208-212), (269-271), (316-319).
Crystal violet	(206-207), (630-636), (253-258), (309-314), (416-426).
Eosin	(519-522), (196-200), (253-256), (300-303), (341-344).
Evan's blue	(610-616), (204-206), (320-325).
Fast blue RR	(205-208), (219-224), (293-298), (340-355), (425-435).
Fast Green FCF	(634-636), (206-208), (308-311), (407-411), (251-256).
Fluorescein	(446-448), (228-230), (306-311).
Giemsa	(642-646), (288-290), (241-243), (610-614).
Haematoxylin	(208-212), (288-290), (442-448).
Light green SF	(636-640), (212-213), (260-263), (318-322), (440-446).
Lissamine rhodamine RB200-phloxine rhodamine	(564-568), (197-199), (217-221), (255-258), (281-284), (307-309), (349-352).
Luxol fast blue G	(212-216), (680-684), (640-646), (263-268), (346-352).
Luxol fast blue ARN	(206-207), (225), (510-520), (600-610)
Luxol fast blue MBS	(214-218), (280-283), (248-255), (632-642), (678-682).
Metanil yellow	(205-207), (418-426), (273-276).
Methylene blue	(284-286), (604-608), (228-232).

TABLE 16: (continued)

DYE	WAVELENGTH OF ABSORPTIVE PEAKS
Methyl blue	(205-206), (614-616), (552-554), (518-520), (321-323), (284-286).
Methyl green	(220-221), (259-260), (638-642).
Methyl orange	(510-514), (203-206), (274-277), (315-320).
Methyl red	(518-522), (203-205), (215-220), (284-288).
Methyl violet 6B	(204-205), (662-668), (620-624), (292-294).
Neutral red	(268-271), (528-532), (231-234), (204-209), (301-305).
Nile blue	(642-646), (604-608), (215-217), (278-279), (330-336), (432-442).
Nitro blue (tetrazolium)	(203-205), (258-262), (435-445) diminutive.
Oil red O	(225-227), (251-253), (271-273), (510-514), (330-335), (285-287).
Orcein	(514-520), (228-230), (594-598), (492-496).
Orcinol-new fuchsin	(556-558), (208-212), (293-295), (233-235).
Ponceau 2R	(218-220), (207-210), (245-250), (504-508), (269-271), (327-332).
Pontamine sky blue	(618-624), (208-212), (324-327).
Procion yellow M4R	(200-204), (262-264), (430-434).
Resorcin fuchsin	(554-556).
Rhodamine B	(556-559), (190), (252-255), (354).
Ruthenium red	(466-468), (332-336), (528-534), (201-203).
Sirius red F3BA	(530-532), (232-235), (280-285).
Sirius supra blue FGL-CF	(596-600), (295-298), (260-264).
Sudan black B	(213-217), (234-236), (299-303), (536-544).

TABLE 16: (continued)

DYE	WAVELENGTH OF ABSORPTIVE PEAKS
Thionin	(602-610), (687-692), (289-291), (242-244).
Toluidine blue	(636-642), (606-610), (289-291), (239-241).
Verhoeff's iron haematoxylin	(217-220), (664-674), (540-546), (277-282).

N.B. The spectral absorptive peaks have been listed in diminishing order according to the absorptive values for any one dye. The visible spectrum ranges from about 375 mμ to approximately 750 mμ and the ultra violet recordings commenced at 200 mμ up to 375 mμ. It is most important to realize that the spectro-photometric peaks will shift considerably depending upon the dye solvent. The values listed here represent some of the staining solutions used in this study and consequently, may differ from values derived from standard test solutions.

8.9 APPENDIX IX: Methylation and saponification tests for carboxyl

groups.

8.9.(a) Carboxyl Blockade:

from Culling (1974): p.241.

(1) bring sections to water.

(2) treat with 0.1 N HCl in absolute methanol at $37^{o}C$ for

4-48 hours (mild methylation) or at $60^{o}C$ for 4-24 hours

(normal methylation).

(3) rinse in alcohol.

(4) stain as required (e.g. aldehyde fuchsin or alcian blue).

8.9.(b) Saponification:

from Culling (1974): p.292.

(1) sections to 70% ethanol.

(2) treat with 0.5% KOH in 70% ethanol for 30 minutes at room

temperature.

(3) rinse carefully in 70% ethanol.

(4) wash in slowly running tap water for 10 minutes.

(5) stain as required (e.g. aldehyde fuchsin or alcian blue).

CHAPTER 9.   BIBLIOGRAPHY.

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